Effects of Boric Acid on Invasion, Migration, Proliferation, Apoptosis, Cell Cycle and miRNAs in Medullary Thyroid Cancer Cells

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Abstract

Background: Medullary thyroid cancer (MTC), which makes up 5-10% of all thyroid cancers, is a highly aggressive and chemotherapeutically resistant cancer originating from parafollicular C cells of the thyroid. In recent years, testing of new biological, chemical or natural compounds as an alternative to existing agents used in cancer treatment has been a popular research topic. One of these compounds is boric acid originating from the element boron, and studies have shown that it has anti-carcinogenic, anti-osteoporotic and anti-oxidant effects.

Purpose: The aim of this study is to determine the therapeutic effects of boric acid on cell proliferation, invasion, migration, colony formation, cell cycle and apoptosis mechanisms in TT medullary thyroid cancer cell line under in vitro conditions.

Methodology: The effects of boric acid on cell viability was determined by XTT assay. Total RNA was isolated by Trizol reagent. Gene and miRNA expressions were analyzed by RT-PCR. Effects of boric acid on apoptosis were analyzed by TUNEL assay and genotoxicity was performed by comet assay. Effects of boric acid on cell invasion, colony formation and cell migration were detected by matrigel-chamber, colony formation assay and wound healing assay, respectively.

Results: In order to determine the effect of boric acid on cell viability and 50% lethal dose, the XTT method was employed and IC50 dose was found to be 35 μM at 48th hour. A real-time PCR test was used to investigate changes in cell cycle and apoptosis related genes and proteins and identified miRNAs under the addition of boric acid. In addition, *miR-21* was significantly reduced in medullary thyroid cancer associated with cancer aggressiveness and poor prognosis. Using the Tunnel test, the apoptosis rate in the dose group cells were found as 14%. Matrigel invasion test showed a 30.8% decrease in invasion in the dose group and colony formation test decreased 67.9% in the dose group. Using the wound healing assay, it has been found that migration was reduced, and the Comet assay has shown that DNA fractures increased after treatment with boric acid.

Conclusion: In conclusion, the findings of this study have shown that boric acid can be used as a potential anticancer agent in medullary thyroid cancer and other cancers caused by similar mechanisms.

Research Highlights

* To introduce a potential new chemical for the treatments of thyroid cancer

* Boric acid accelerates and increases the anti-cancer effect.

* Boric acid shows an cytotoxic effect by inhibition of thyroid cancer cell line's viability.

* Boric acid also affects the invasive and colony formation capabilities of cell line.

Introduction

Medullary thyroid cancer is a rare type of thyroid cancer that produces calcitonin hormone and originates from parafollicular C cells of neural crest origin. It accounts for 5–10% of all thyroid cancer types [1]. The mechanism of medullary thyroid cancer is defined within the activation of *RET*. Sporadic medullary carcinoma (sMTC) consist of 75% cases of medullary carcinoma. The remaining 25% cases are familial medullary carcinoma (hMTC) which is specified with RET oncogene [2].

Genetic diagnosis in hMTC provides an opportunity to provide a possible cure with total prophylactic thyroidectomy. However, this is different when most sMTC are diagnosed, and metastases are present to lymph nodes and distant organs, particularly bone, liver and lung. Today, a surgical protocol consisting of total thyroidectomy and lymph node dissection is applied in both types. The effectiveness of systemic chemotherapy is low, and the side effect profile is high, and tyrosine kinase inhibitors had limited effects in advanced metastatic MTC [3, 4].

RET oncogene mutations reveal some level of explanatory mechanisms in explaining MTC tumorigenesis, but these are not sufficient. The role of *RET* in MTC progression is still poorly illuminated and *RET* has been shown to cooperate with different signal transduction pathways, especially in the metastatic context [5].

In this context, in recent years, studies on MicroRNAs and genes related to cell cycle and apoptosis have been very much to reveal progression, tumor development and similar in MTC and other types of cancer [6–13]

When the studies conducted and the prognosis of the disease are evaluated, studies regarding effective and new class agents should be performed. New treatment strategies are required for MTC.

Boron as a non-metal element does not exist in the form of a pure element in nature. Borax, boric acid, colemanite, kernite, ulexite and borate are different forms of boron structures. Absorbed boron is found mostly as Boric acid in the human’s systems.

Previous in vivo and in vitro studies showed that boron has a wide variety of biological activities such as anti-osteoporotic [14], antioxidant [15, 16], a regulator of the immune system [17, 18], mineral and vitamin metabolism [19]. and steroid hormone metabolism [20, 21]. The anti-cancer effect of boric acid demonstrated in many studies. These effects are also existing in the prostate [22–25], cervix [26, 27], lung [28], breast cancer [29, 30], and in malign melanoma [31].

The aim of this study is to evaluate the anticancer effect of boric acid on TT medullary thyroid cancer cells in vitro and try to understand molecular mechanism of boric acid activity. For this purpose, the effects of boric acid on cell cycle control and apoptosis genes, migration, invasion, proliferation and some of miRNA's connected with medullary thyroid cancer were evaluated.

Material And Methods

Cell culture:

Thyroid medullary carcinoma TT cell line (ATCC, CRL 1803™) and human thyroid fibroblast (HTF; ScienCell Cat No: 3730) cell lines were used in this study. Both cell lines were cultured under suitable conditions at 37°C in 5% CO_2 and cells were grown in Dulbecco Modified Eagle Medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Capricorn Scientific), 20 units/ml penicillin and 20 µg/mL streptomycin, 0,1 mM amino acid solution (Biological Industries) and 1mM sodium pyruvate (Biological Industries). Different concentrations (10µM, 20µM, 35µM, 50µM, 75µM, 100µM, 200µM, 500µM) of boric acid (Etimaden) were applied to the cells in a time and dose-dependent manner.

Cell Proliferation Xtt Assay

Effects of boric acid on cell proliferation in TT medullary thyroid carcinoma and Human thyroid fibroblast were detected by XTT (2,3-bis(2-methoxy-4 nitro-5- sulfophenyl)- 2H-tetrazolium-5-carboxanilide) assay according to manufacturer procedure (Cell Proliferation Assay with XTT Reagent-Cell Proliferation Kit ; Biotium cat no : 30007). TT and HTF cells were seeded into 96-well plates at a concentration of 1×10^4 cells per well. After 24 hours of incubation, the cells were treated with 10 µM, 20 µM, 35 µM, 50 µM, 75 µM, 100 µM, 200 µM, 500 µM concentrations of boric acid during 24, 48 and 72h. Dose concentration range was selected by using references in the literature as Hacıoğlu et al 2109 and Barranco WT and Eckert CD 2004 [23, 32]. Untreated cells were used as control cells. After the incubation period, XTT mixture added and then formazan formation was determined spectrophotometrically at 450 nm (reference wavelength 630 nm) by a microplate reader (Biotek). Viability (%) was calculated using the background- corrected absorbance as follows:

Viability (%) = Absorbance of experiment well / Absorbance of control well × 100

IC_{50} doses of boric acid on TT cells were evaluated by GraphPad Prism 8 computer programme. IC50 dose was used as dose group in other studies such as invasion, migration, TUNEL, Real-Time PCR and comet assay.

Rna Isolation, Cdna Synthesis, And Real-time Pcr (Rt-pcr):

RNA isolation, cDNA synthesis, and Real-time PCR (RT-PCR):
Total RNA from control and dose group of TT medullary thyroid carcinoma cells were isolated by Trizol Reagent (Invirogen, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystem, USA), according to the manufacturer’s protocol.

CCND1 (cyclin D1), CCND2 (cyclin D2), cyclin-dependent kinase (CDK)2, CDK4, CDK6, retinoblastoma (RB), p21, p16, caspase-3, caspase-9, caspase-8, caspase-10, B- cell lymphoma 2 (Bcl- 2), B- cell lymphoma-extra-large (Bcl-xl), BH3 interacting domain death agonist (BID), death receptor 4 (DR4), death receptor 5 (DR5), Fas- associated protein with death domain (FADD), FAS, tumor necrosis factor (TNF), tumor necrosis factor receptor type 1- associated DEATH domain protein (TRADD), apoptotic protease activating factor 1 (APAF-1), Bax, p53 upregulated modulator of apoptosis (PUMA), NOXA genes were used for cell cycle, and cell apoptosis pathway expression analysis in this study and expression profiles were calculated using the beta-actin (house-keeping gene) as the reference. Real-time PCR tests were performed by according to the SYBR Green qPCR Master Mix (Applied Biosystem, USA) protocol. RT-PCR assay was performed using gene-specific primers. The sequences of primers were given in Supplementary Materials (Supplementary Table 1).

miRNA expression change was also determined by using RT-PCR. miRNA cDNA synthesis kit (abm) was used for cDNA synthesis and subsequently relative quantification of *hsa-miR-21-5p*, *hsa-miR-224-5p*, *hsa-miR-200a-3p*, *hsa-miR-130a-3p*, and *hsa-miR-146a-5p* was analyzed by RT-PCR according to evagreen (abm) master mix (abm) protocol. miRNAs expressions were normalized to U6 as the human endogenous control.

Tunel Assay:

The apoptotic effects of boric acid in TT medullary thyroid cancer cells were assessed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. The control and dose group of TT cells were fixed with 4% (w/v) paraformaldehyde, and the cell apoptosis was analyzed using a commercial kit (Tunel In Situ Cell Detection Kit, AAT bioquest) according to the manufacturer's protocol. TT cells were stained with Hoechst dye and then observed under a fluorescence microscope (Olympus Inc., Tokyo, Japan). The total cells and TUNEL-positive cells were counted in 10 randomly selected fields in the fluorescence microscope. The results were expressed as a percentage of TUNEL-positive cells defining the ratio of apoptotic cells to the total cells.

Matrigel-invasion Assay:

Analyzing the number of invasive chambers were performed using Matrigel invasion chambers (Thermo scientific LOT: 159467) following the manufacturer's instructions. Briefly, 2 x 10^5 cells with serum-free DMEM were plated onto the upper chambers of Matrigel-coated filter inserts, and serum-containing DMEM medium (500 µL) was added to the lower chambers as a chemoattractant. After incubation at 37°C for 24h, filter inserts were removed from the wells. A cotton-tipped swab removed the non-invasive cells on the upper surface of the filter. The cells that invaded the lower surface of the filter were fixed with methanol for 10 minutes (min) and stained with crystal violet. Using an inverted microscope, the cells that invaded in the lower surface of the filter were counted. The assays were performed in triplicate. The percentage of invasion was computed using the following equation:

Invasion (%) = The number of cells in matrigel matrix basement membrane / The number of cells in control membrane X 100

Colony Formation Assay

Colony formation assay was performed to examine the colony formation of TT cells treated with boric acid. Cells at the exponential growth phase were digested with trypsin and counted using the trypan blue dye exclusion test. Then, the cells were suspended in a DMEM medium containing 10% fetal bovine serum. The seeding of the cells into a six-well plate was done at a density of 10^3 cells/well. The medium was changed every three days for 2 to 3 weeks. When macroscopically visible colonies appeared in the culture dish, colonies were fixed in methanol for 10 minutes and stained with crystal violet. The morphology of colonies was observed under a microscope, and the numbers of colonies were counted.

Wound-healing Migration Assay

Anti-migratory effects of boric acid on TT medullary thyroid cancer cells were determined by wound-healing assay. The control and dose group TT cells were seeded in 60×15 mm style cell culture dishes at 10^6 cells per well and allowed to adhere overnight at 37°C with 5 % CO_2. After the growing cell layers had reached about 90% confluence, a scratch was made via sterile 200-µL plastic pipette tip. The cells were washed with 2 ml serum-free DMEM to remove all detached cells. Cells were further incubated for 24 hours with an IC50 dose of boric acid. Then evaluated the closure at 24 hours using a light microscope. Images of the TT cell proliferation were taken. The assays were performed in triplicate.

Comet Assay

Effects of boric acid on DNA damage and genotoxic situations in TT medullary thyroid cancer cells were evaluated. Slides were prepared by covering with high-melting agarose (HMA). Control and dose group cells were dissolved in PBS after tripsinization and cell suspension was mix with low-melting agarose in the microcentrifugation tube. All slides were immersed in freshly prepared cold lysing solution [2.5M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris (pH 10) and 10% DMSO] and incubated at 75 minutes. 4°C). Following incubation, the slides were placed in an electrophoresis buffer [0.3 M NaOH, 1mMNa2EDTA; pH 13] for 20 min at 4°C. Electrophoresis process were performed at (25 V (300 mA, approx. 0.74 V/cm)) for 35 min at 4°C. Then, the slides were washed three times with dH20, and incubated in neutralization buffer [0.4 M Tris; pH 7.5] for 5 minutes each. The slides were incubated in methanol for 5 minutes at − 20°C to fix the cells to the slides. After the staining of slides with ethidium bromide, cells were evaluated by fluorescence a microscope (Nikon) with the comet assay IV Version 4.3.2 forthe Basler FireWire programme. Head length (µm), tail length (µm), head density (%), tail density (%) and tail moment parameters were measured.

Statistical analysis

Real-time PCR data were evaluated by the ΔΔCt method for the statistical analysis of the findings and quantified with a computer program. The comparison of the groups was performed with the “Volcano Plot” analysis, from “RT^2 Profiles PCR Array Data Analysis”, which is assessed statistically using the “student *t*-test.” IBM SPSS Version 21 (SPSS Inc., Chicago, IL, USA) analysis program was used to perform the parametric and nonparametric analysis of dose and control groups. $P < 0.05$ was considered to indicate statistical significance.

Results

Anti-Proliferative effects of boric acid in TT medullary thyroid cancer

The anti-proliferative activity of boric acid in TT medullary thyroid cancer cells was determined by XTT assay. Live cells reduced tetrazolium to orange colored formazan. In this way, the effect of boric acid on the TT cell line was investigated depending on time and dose and the IC50 dose of boric acid was found to be 35μM in the 48th hour (Fig. 1).

Mrna Expression Of Genes And Mirna By Real-time Pcr

cDNAs, which are synthesis from total RNA from control and dose group cells were performed, and Real Time PCR was used to detect the expression of genes and proteins which are missioned in apoptosis pathways and cell cycles and some of miRNAs, which are related with medullary thyroid cancer. It was observed that boric acid in TT cell line caused a significant increase in the apoptosis-related gene expression of *caspase-3*, *caspase-9*, *Bax, NOXA*, *APAF-1* which are inducing apoptosis and a significant decrease in the apoptosis related protein expression of *bcl-2* and *bcl-xl* which are negative regulator of apoptosis (Table 1). Furthermore, there were statistically significant changes and decreases among the genes related to the cell cycle, they were *cyclin D1*, *CDK4* (Cyclin dependent kinase 4), *CDK6* (Cyclin-dependent kinase 6) and *cyclin E* (Table 1). There were statistically significant decreases among the miRNAs related to the MTC, they were *miRNA-21* and *mi-RNA-224* (Table 1).

Apoptotic effects of boric acid in the TT cell line.

TUNEL test was used to check the data about apoptosis. In the light of the data obtained, the percentage of apoptotic cells was compared between the dose and the control group. In the control group, the rate of apoptotic cells was determined as 4%, and in the dose group in which IC50 (35 μM) dose of boric acid was added, the rate of apoptotic cells was determined as 14% (Fig. 2A and Fig. 2B).

Anti-invasive effect of boric acid in the TT cell line.

Matrigel invasion chamber assay was used to detect of effect boric acid on invasion in the TT cell line. It observed that boric acid inhibited the invasion of dose groups, compared with the control groups (Fig. 3A). According to the results, the invasion capacities of the control cells were 63.22 ± 3.5%, while the percentage of invasion was 32.44 ± 2.5% in the dose group administered boric acid (Fig. 3B) (p = 0,046).

Effects of boric acid on colony formation of the TT cell line.

In order to understand the effect of boric acid on colony formation, colonies larger than 0.1 cm were evaluated. While the average colony in the control group cells was 412, the number of colonies was determined as 134 in the dose group cells treated with boric acid (35 μM). This significant decrease was expressed graphically after the counting process, and it was found that the number of colonies was suppressed by 67.9% in TT cells with boric acid exposure (Fig. 4A). The colony images of the cells were evaluated, it was remarkable that the number of colonies between the control group colony number and the boric acid administered dose group was significantly different, and that there was significant decrease in the number of colonies of the TT cell line after boric acid (Fig. 4B).

Effects of boric acid on migration in the TT cell line.

Wound healing migration assay was used for detecting of the effect of boric acid on migration in the TT cell line. Cells in the control and dose groups were photographed at 0 and 24 hours (Fig. 5). As a result of the experiment, it has been shown that boric acid inhibits cell migration more than the control group.

Detection of DNA damage caused by boric acid in the TT cell line.

Comet assay was used for observed to DNA damage of the TT cell line caused by boric acid. Damage in DNA was assessed by measuring the head and tail length, density and tail moment, which occurred after the migration of the DNA, whose supercoil structure was impaired, towards the anode in gel electrophoresis. The resulting values are listed in Table 2. In Fig. 6, there is the appearance of a comet made up of solid and damaged DNA.

Discussion

Today, in addition to standard chemotherapy, many new agents are expected to be used in the treatment of cancer in the future. One of these new agents is Boron element and Boron origin compounds. In this study, we aimed to determine the anti-cancer properties of boric acid by examining the effects of boric acid on medullary thyroid cancer cells in vitro such as changes of migration, invasion, cell proliferation and expression of cell cycle and apoptosis genes and miRNAs. In the literature, no studies examining the effects of boric acid directly on medullary thyroid cancer cells, so similar experiments in studies examining the effects of boric acid on other cancer cell lines were evaluated for mutual comparison [6].

The cytotoxic effects of boric acid at various doses and 24, 48 and 72 hours were investigated and the amount causing death in 50% of TT cells which mean IC50 (LD50) dose was determined as 35 μm at

48th hours. This IC50 dose was used for ongoing tests in dose groups for evaluate to change of variables compare the control groups.

In this study, we compare to evaluated expression of genes and proteins which are related apoptotic pathway and cell cycle regulation in control and dose treatment group of TT cell lines for understanding that anti-cancer effects of boric acid in TT cells. In addition, we evaluated expression of some of miRNAs which are related to MTC.

The caspase-3 as a member of cysteine-aspartic acid protease family cooperating with caspase-8 and caspase-9 activates with both extrinsic (death ligand) and intrinsic (mitochondrial) pathways in the apoptotic cells [33].

In addition, *Caspase-9* is an initiator critical to the intrinsic apoptotic pathway found in many tissues. *Caspase-9* activates *caspase − 3, 6* and *7*, continuing the apoptosis [10, 34]. In an in-vitro study by Barranco and Eckhert in 2004, it was shown that activity and expression of *caspase-3* did not change after treatment with boric acid in DU-145 cells [23]. Scorei et al. determined that calcium fructoborate and boric acid inhibits proliferation of breast cancer cells and they showed that calcium fructoborate acid increases expression and activity of *caspase-3*, but they didn't detect any increase of *caspase-3* in cells which treated with boric acid [35]. However, Hacioglu et al. showed a significant dose-depended increase in expression of *caspase-3* in DU-145 cells [32]. Furthermore, Meacham et al. showed that boric acid induces apoptosis in both prostate and breast cell lines and they confirmed this with a caspase-3 assay. According to our results, we observed a significant increase in expression of *caspase-3* and *caspase-9.*

Bcl-xl, *Bcl-2*, and *Bax* are members of the *Bcl-2* gene family. While *Bcl-xl* and *Bcl-2* act as anti-apoptotic protein, preventing the release of pro-apoptotic factors such as cytochrome c, *Bax* plays an active role in the release of cytochrome-c and pro-apoptotic factors from mitochondria [36]. Hinze et al. showed that *Bcl-2* was strongly expressed, and *Bcl-xl* was moderately expressed in medullar thyroid cancer. [37]. Hacioglu et.al detected an increase in expression of *Bax* in DU-145 cells which treated with boric acid [32]. In a study examining the effects of borax, a salt of boric acid, on hepatocellular cancer cells, apoptosis was found to be induced in cells after borax, and it was demonstrated that *Bax* increased and *Bcl-2* decreased [38]. A significant increase was determined in the mRNA expression of *Bax* and a decrease in the mRNA expression of *Bcl-2* and *Bcl-xl* in the boric acid treatment group cells compared with the control group cells.

NOXA gene is a pro-apoptotic member of the *Bcl-2* protein family. The expression of

NOXA is regulated by *p53* which is a tumor suppressor gene [39]. Yarmand et

al., demonstrated that *RET* proto-oncogene was found to negatively regulate the expression of *NOXA*, which are pro-apoptotic genes via activated transcription factor 4 (ATF4) [40]. *Apoptotic protease activating factor 1 (APAF-1)* is involved in the transmission of the death signal in the apoptotic pathway. APAF-1 combines with cytochrome c released from mitochondria to form apoptosome complex and this

complex stimulates the caspase 9, leading to apoptosis of the cell [41]. According to our results, were observed a significant increase *NOXA* and *APAF-1* in boric acid treated cells. When these results and other studies on similar subjects are evaluated, it can be said that boric acid induces apoptosis in TT cells by increasing the expression of apoptotic genes and decreasing the expression of anti-apoptotic genes.

Furthermore, boric acid and its apoptotic effects on TT cells were confirmed by TUNEL assay. According to the results, apoptotic cell percentage were compared between the dose group and the control group. In the control group, the rate of apoptotic cells was determined as 4%, and in the dose group in which IC50 (35 µM) dose of boric acid was added, the rate of apoptotic cells was determined as 14%.

The transfer of genes in one cell to another occurs by cell division. Therefore, such an important event is controlled by many control mechanisms. Cyclin-dependent kinase (CDK) s and cyclins are essential components for cell control points. For the continuation of the cell cycle, CDKs should be transformed into active kinases by forming complexes with cyclins. *Cyclin C* with *CDK3* in G0 phase, *cyclin D* and *E* with *CDK 4* and *CDK 6* in G1 phase, *cyclin A* and *E* with *CDK 2* in S phase, *cyclin A* with *CDK 1* and *CDK 2* in G2 phase and *cyclin B* with *CDK 1* in M phase are known to play a role in cell cycle control [42].

In the study of investigating the expression of *cyclin D1*, in primary thyroid cancers, used tumor tissues of 35 thyroid cancer cases, 4 of which were medullary thyroid cancer, and the rate of *cyclin D1* positive cells in medullary thyroid cancer cells was found to be 27.4% and ıt has been suggested that positivity may be related to the aggressiveness of cancer [12].

In similar studies by Drosten et al. and Watanebe et al. on the carcinogenesis of medullar thyroid cancer, *cyclin D1* was found to be highly expressed at the mRNA level in cells expressing oncogenic RET [43, 44]. There are studies that have been proven to play a role in the pathogenesis of cyclins and cyclin dependent kinases in cancers other than MTC [45, 46, 47, 48]. Furthermore, there are several studies that different substances prevent cell proliferation by inhibiting the *cyclin D1* and *CDK4* and *CDK6* levels in MTC at the cell cycle control points [49–52,].

In our study, it was found a significant decrease the expression of *cyclin D1*, *cyclin E*, *CDK4* and *CDK 6* in dose groups compared to the control groups. No studies have been found in the literature where boric acid and MTC are used together and the expressions of cell control point genes are investigated. In the study of cellular changes in DU-145 prostate cancer cells after the addition of boric acid by Barranco and Echert, they determined that *cyclin A*, *B1*, *E*, *D1* levels associated with prostate cancer aggressiveness and this was evaluated as anti-proliferative effect of boric acid outside of apoptosis. [53]. Considering that cyclins and CDKs play a proliferative role in cells, suppression of cyclins (*cyclin D1*, *E*) and *CDK4* and *CDK6* after exposure to boric acid may stop cell proliferation by arresting the cells at the G1-S control point.

Recently, gene regulation roles of microRNAs (miRNAs) in development mechanism of MTC have been studied extensively. Multiple miRNA disorders with potential prognostic, therapeutic and predictive effects in MTC have been discovered. In the study conducted by

Pennelli et al. examining the *PDCD4 (Proglamated cell death 4)* / *miR-21* pathway in medullary thyroid cancer revealed that the increased expression of *miR-21* had a consistent relationship with the development of MTC and high *miR-21* values were associated with high calcitonin levels, lymph node metastasis, advanced disease and resistant disease [54]. In another study Y.H.Chu et al. demonstrated over-expression of *micro RNA 21* in MTC cells. In the same study, there was a significant decrease in cell proliferation and invasion after removal of *miR-21* and *MALAT-1* in MTC cell culture [7]. In another study containing 42 MTC cases conducted by Y.H.Chu et al. on over-expression of *micro RNA 21* in MTC cells, *miR-21* was found significantly higher in MTC cells than normal thyroid tissue. In the same study, there was a significant decrease in cell proliferation and invasion after removal of *miR-21* and *MALAT-1* in MTC cell culture [7]. In the study involving 34 sMTC, 6 hMTC and 2 C cell hyperplasia, which examined miRNA profiles in family and sporadic medullary thyroid cancers by Mian et al., *miR-21* was found to be increased 4.2 times in diseased tissue compared to normal thyroid tissue [55]. Recent research has revealed that *miR-21* supports cell proliferation by suppressing tumor suppressor genes such as *PTEN*, *RECK*, *PDCD4*, and *TPM1*[56, 57]. In our study, in the TT cell line treated with boric acid (35 µM), *miR-21* was found to be reduced 4.3 times compared to the control group. With the decrease in *miR-21*, the elimination of pressure on tumor suppressor genes can be thought to induce apoptosis. In the same study, we found *miR-224* 8.9 times reduced compared to the control group. In studies on *miR-224* in MTC, the increase of *miR-224* has been associated with the absence of lymph node metastasis, low-stage disease and good prognosis [55, 58]. However, in studies conducted on different types of cancer such as breast cancer, cervical cancer and lung cancer, the increase of *miR-224* expression has been associated with poor prognosis, cancer aggressiveness and advanced disease [59–62]. Although studies on MTC show that *miR-224* is associated with good prognosis and low-stage disease, in our study, it was found that *miR-224* expression decreased in TT cells with boric acid added. As a result of tests performed during the study, if the boric acid is thought to decrease proliferation and induce apoptosis in TT cells, studies involving MTC and *miR-224* related mostly patient and healthy control groups are needed.

In order to strengthen our study, the matrigel invasion test was performed and the invasion capacities of the dose group and the control group were compared. The mean invasion percentage was 63.22 ± 3.5% in the control group cells, and 32.44 ± 2.5% in the dose group cells. It was determined that the invasion decreased by 30.7% after the addition of boric acid. In the study conducted by Barranco and Eckhert and evaluating the effects of boric acid on DU-145 prostate cancer, the effects of boric acid on invasion capacity were investigated using the matrigel invasion test. DU-145 cells were exposed to boric acid for 8 days at 250 µM and 1000 µM doses, and it was determined that the invasion decreased by 82% and 97%, respectively. In the same study, it was found that in the culture of the boric acid treated cell, although the general actin concentrations did not decrease, the F-actin around the cell decreased at a dose of 1000 µM boric acid [53] In addition, the effects of boric acid on migration with wound healing test were evaluated within the scope of our study and dose group and control group were photographed at 0 and 24 hours. As a result of the experiment, boric acid has been shown to inhibit cell migration compared to the control group. In our study, no experiment was conducted on the mechanism of action of suppressing the invasion and migration of boric acid in TT cells. However, given the studies that actions have been found

to be associated with invasion and migration in malignant cells, it can be considered that the effect of boric acid on invasion and migration in TT cells by reducing the amount of F-actin in around the cells [63].

In our study, the effects of boric acid on colony formation were also investigated. According to the data obtained, while the average colony in the control group cells was 412, the number of colonies was determined as 134 in the dose group cells treated with boric acid (35 μM). As a result, it was found that the number of colonies was suppressed by 67.9% in TT cells after boric acid exposure.

The comet assay test was used to detect the damage done by boric acid on DNA. According to the test results, it was determined that the head length and density decreased, the tail length and density increased, and the tail moment increased in the dose group where boric acid was applied compared to the control group. This result shows that boric acid induces cell death by increasing DNA damage in TT cells.

Conclusion

In conclusion, carcinogenesis is a very complex process considering cell cycle control points, the functioning of genes and proteins involved in apoptosis, enzymatic reactions. This study has a power in order to demonstrate of ant anti-carcinogenic effect of boric acid in multiple aspects in cell culture of MTC. In our study, it was determined that boric acid reduces proliferation, stops the cell cycle and induces apoptosis, reduces invasion and colony formation in TT medullary thyroid cancer cells. When all the results are evaluated, the idea arises, that boric acid can be used as a possible therapeutic agent in both medullary thyroid cancer and maybe other types of cancer. It is important in this respect to accelerate and expand in vitro studies and to start in vivo studies using experimental animals.

Abbreviations

Medullary thyroid cancer : MTC

Sporadic medullary carsinoma: (sMTC)

familial medullary carsinoma:(hMTC)

Thyroid medullary carcinoma: TT cell line

TUNEL :Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Human thyroid fibroblast: HTF

XTT: 2,3-bis(2-methoxy-4 nitro-5- sulfophenyl- 2H-tetrazolium-5-carboxanilide)

Complementary DNA :cDNA

B- cell lymphoma 2 : Bcl- 2

B- cell lymphoma- extra-large: Bcl-xl

BH3 interacting domain death agonist: BID

Death receptor 4 :DR4

Death receptor 5 :DR5

Fas- associated protein: FAS

FAS death domain :FADD

Tumor necrosis factor: TNF

Tumor necrosis factor receptor type 1- associated DEATH domain protein :TRADD

Apoptotic protease activating factor 1 :APAF-1

Proglamated cell death 4p53 :PDCD4

Declarations

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Tables

Table 1:

The mRNA expression changes of cell cycle and apoptosis genes, and miRNAs mRNA expression in TT cancer cells treated with boric acid compared with the control group cells. Data were obtained by RT-PCR assay via ΔΔCt method in RT2 Profiles PCR Array Data Analysis online program.

Apoptosis Related Genes		
Gene Names	Fold Regulation	p value
Caspase-3	2.3613	0.015[a]
Caspase-9	1.3332	0.046[a]
BAX	4.2143	0.024[a]
BCL2L	-2.1713	0.038[a]
BCLXL	-2.1624	0.002[a]
NOXA	3.0652	0.022[a]
APAF1	7.8504	0.030[a]
Cell Cycle Related Genes		
Gene Names	Fold Regulation	p value
Cyclin D1	-1.4746	0.042[a]
CDK4	-9.8864	0.002[a]
CDK6	2.0665	0.03[a]
Cyclin E	-9.7741	0.049[a]
Apoptosis Related miRNAs		
miRNAs Names	Fold Regulation	p value
hsa-miR-21-5p	-4.3648	0.02[a]
hsa-miR-224-5p	-8.9801	0.004[a]

BAX: BCL2 Associated X, Bcl-2: B-cell lymphoma 2, BCL2L: B-cell lymphoma-extra large, NOXA: Phorbol-12-myristate-13-acetate-induced protein 1, APAF1: Apoptotic Peptidase Activating Factor 1, CDK4: Cyclin Dependent Kinase 4, CDK6: Cyclin Dependent Kinase 6, [a] $p < 0.05$ statistically significant.

Table 2:

Comet Assay - Genotoxicity Test Results

	Control Group	Dose Group	P Value
Head lenght (µm)	48,72 ± 10,29	44,31 ± 9,22	0,024
Tail lenght (µm)	38,15 ± 12,5	44,68 ± 11,48	0,002
Head density	84,04 ± 11 (%)	66,92 ± 16,86 (%)	< 0,001
Tail density	15,96 ± 11 (%)	33,07 ± 16,86 (%)	< 0,001
Tail moment	3,2 ± 2,78	7,4 ± 4,38	< 0,001

Figures

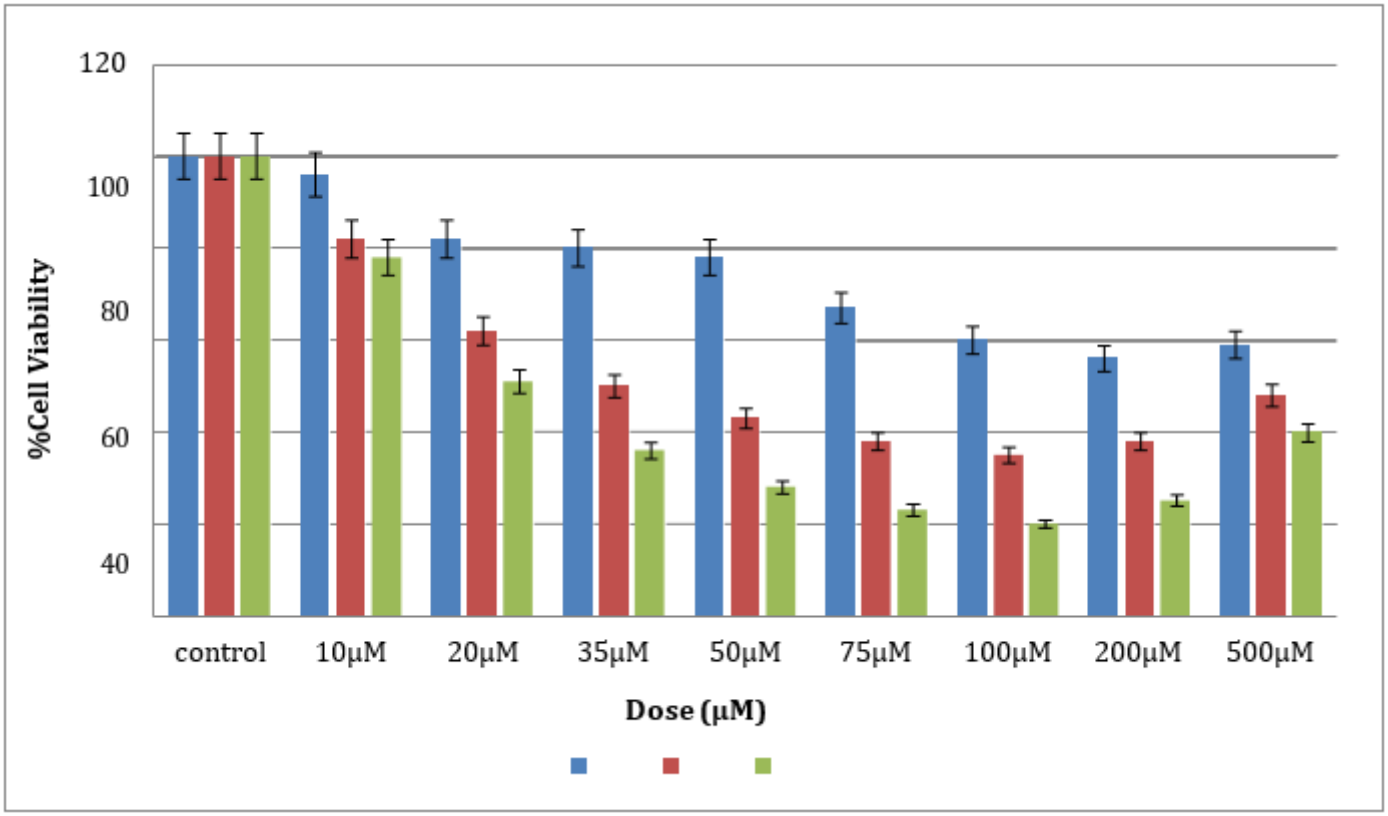


Figure 1

Effect of Boric acid on the viability of TT cell line at different time and doses. The cells were treated with boric acid at different concentrations and time intervals and their proliferation was assessed by XTT assay. Data are the average results of three independent experiments. *IC50 dose of boric acid in TT thyroid cancer cells was detected 35µM at 48th hour.

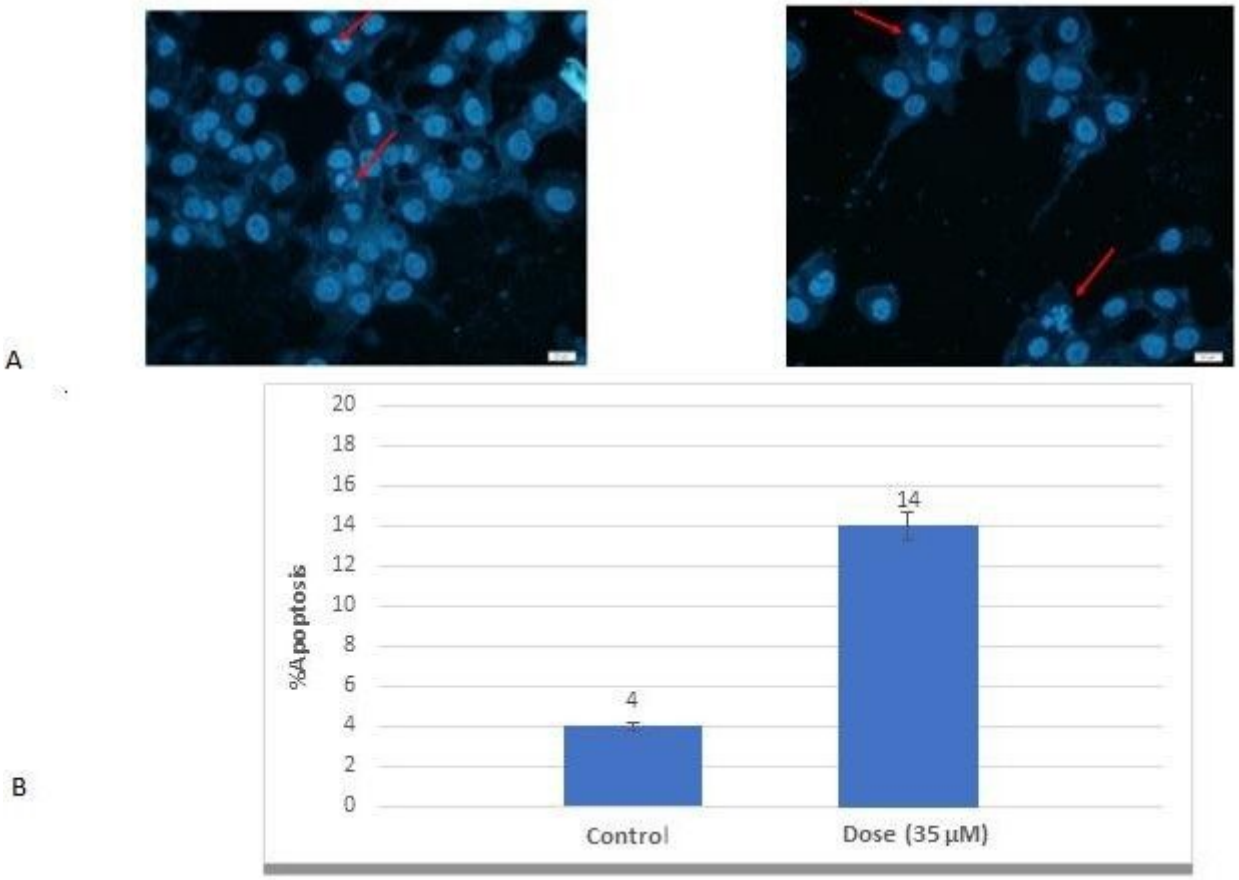
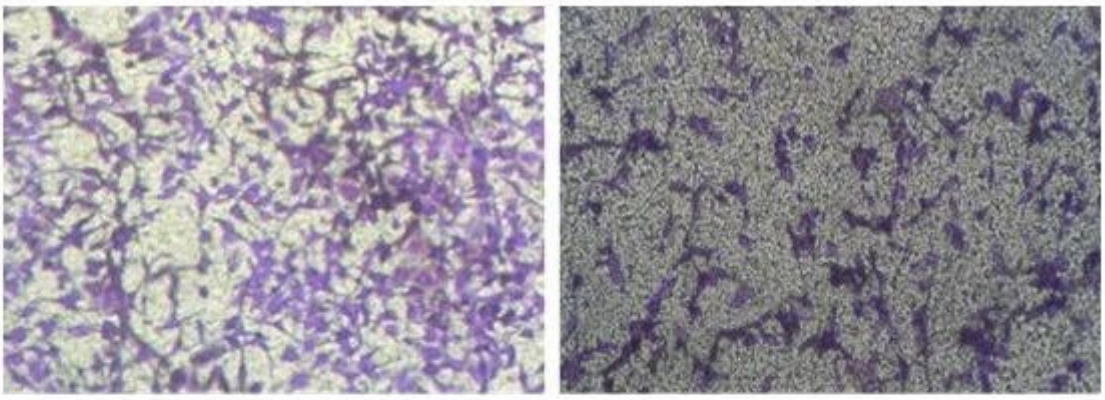


Figure 2

(A) Fluorescence microscopy images at 20x magnification of dose group cells after hoechst staining. The red arrows show the apoptotic cells, (B) Average apoptotic cell percentages in the control and dose groups.

A



B

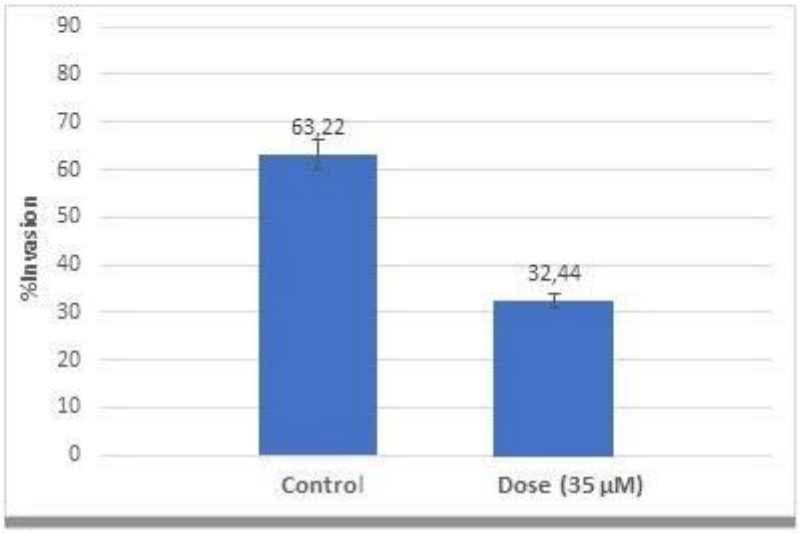


Figure 3

(A) Crystal viole stained images of invaded cells in control and boric acid treated cells (Magnification: 40x). (B) % Invasion Rate in TT cells with boric acid compared to control group. (Data were expressed as mean ± SD, n = 10, (p=0,046).

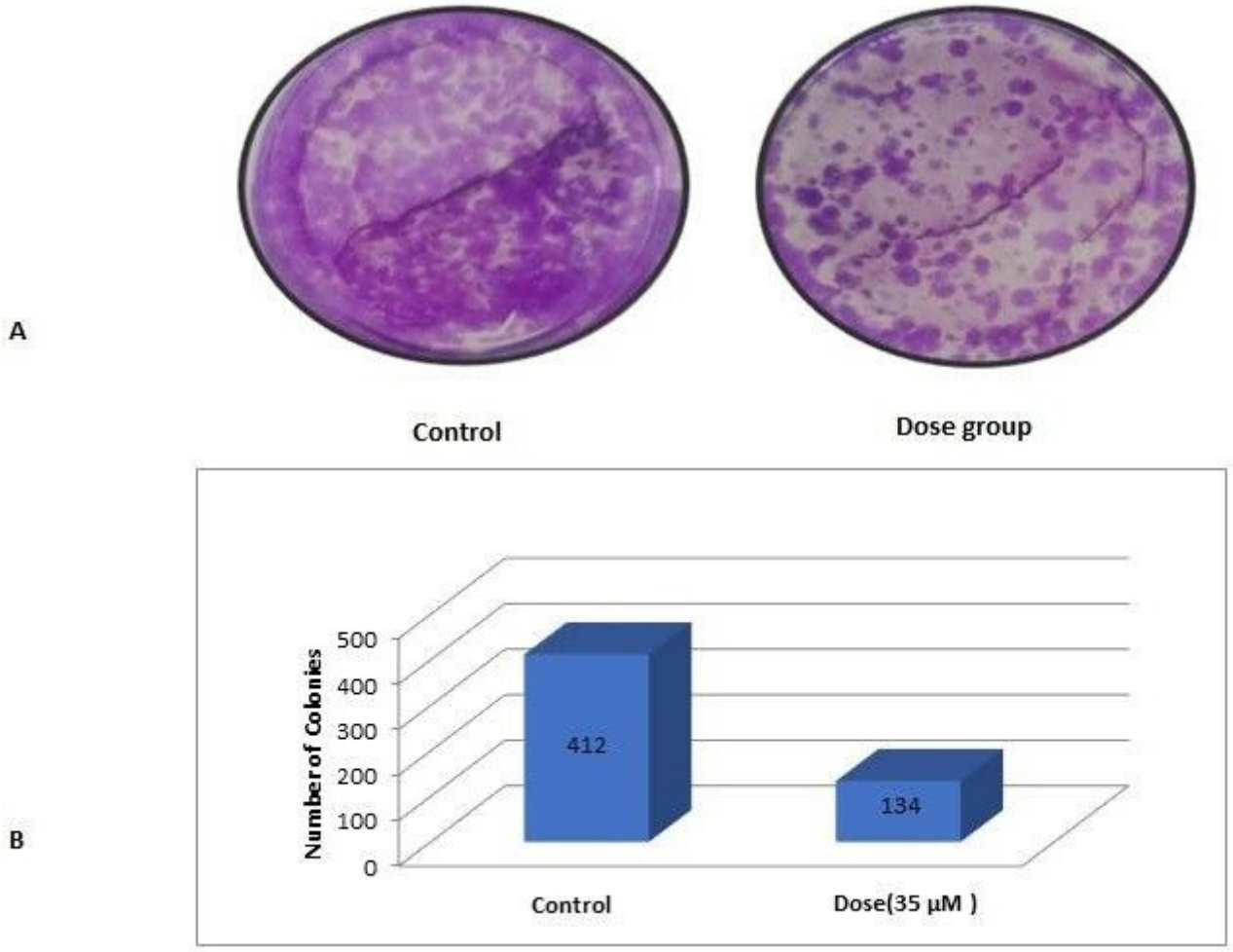


Figure 4

(A) TT cells Control and Dose group colony image (B) Average colony counts in the control and dose groups.

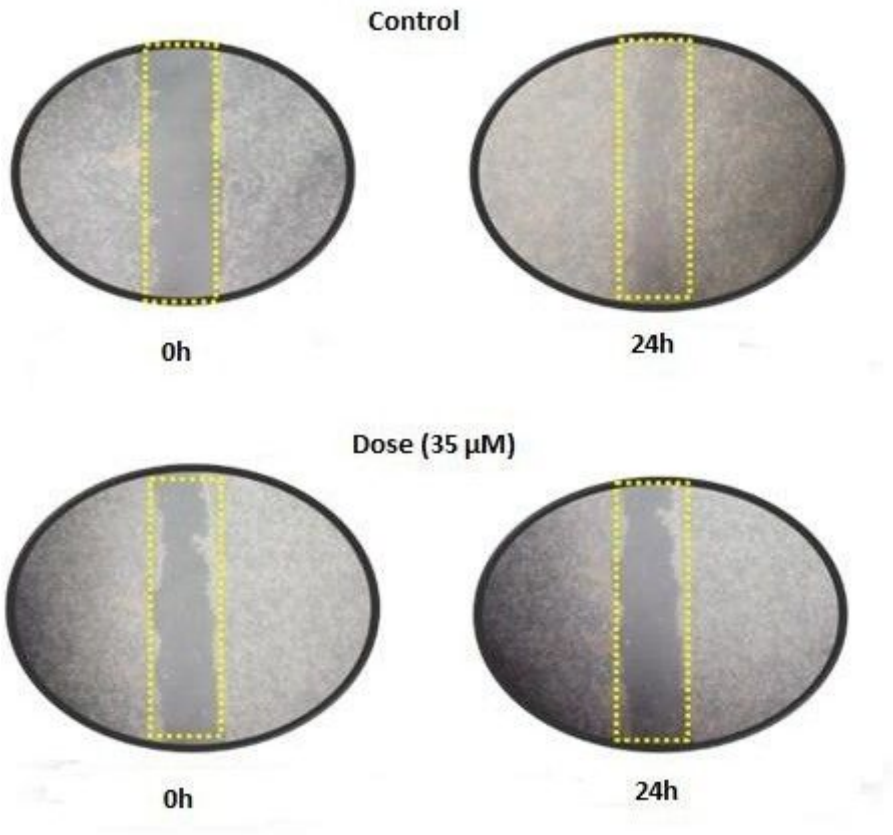


Figure 5

Wound healing assay results showed that boric acid reduced cell migration. Control and dose (35 µM BA) images at 0, 16, and 24 h were given.

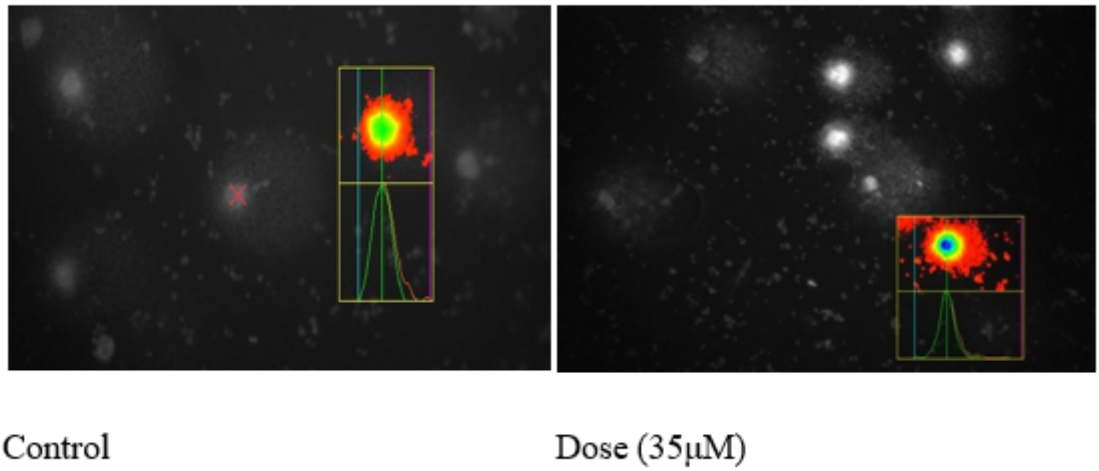


Figure 6

Comet images of the control and dose group were given. Comet assay: DNA damage in TT cells after 48 h exposure to boric acid at 35 µM dose: Head length, head intensity, tail length, tail intensity, tail moment, tail migration demonstrative images of genotoxicity.