THE EFFECT OF BORIC ACID ON STEROID BIOSYNTHESIS PATHWAY IN HEPG2 CELL LINE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ZEYNEP EDA AKYILDIZ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
MOLECULAR BIOLOGY AND GENETICS

FEBRUARY 2022

Approval of the thesis:

THE EFFECT OF BORIC ACID ON STEROID BIOSYNTHESIS PATHWAY IN HEPG2 CELL LINE

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ABSTRACT

THE EFFECT OF BORIC ACID ON STEROID BIOSYNTHESIS PATHWAY IN HEPG2 CELL LINE

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February 2022, 58 pages

Boron is the basic element of borax mineral reserves which a significant amount is found in Turkey. Elemental boron which has wide use in industrial areas recently started to be used in the development of new products in the pharmaceutical industry. However, despite the widespread use in industry and increasing use in health sciences, the molecular mechanism of its effect is still unknown.

Previous microarray studies performed in our laboratory and the analysis of the results have shown that boric acid has an inhibitory effect on the steroid metabolism pathway. According to the analysis, the expressions of several genes in the pathway were found to be changed significantly with boric acid treatment at IC50 concentration.

In this study, in order to confirm the microarray results and to search for the possible role of boric acid in cholesterol synthesis, the gene and protein expressions of some enzymes in this pathway were investigated in the HepG2 cell line. The genes were selected according to their expressions and their roles in the pathway. Expressions of genes which are coding for 7- dehydrocholesterol reductase (DHCR7), Methylsterol Monooxygenase 1 (MSMO1), Emopamil Binding Protein (sterol isomerase) (EBP), Farnesyl-diphosphate Farnesyltransferase 1 (FDFT1), Lanosterol

Synthase (2,3-oxidosqualene-lanosterol cyclase) (LSS), Sterol-C5-desaturase (SC5D), and Squalene Epoxidase (SQLE) were examined at IC10, IC25, and IC50 boric acid concentrations. No significant change was observed in MSMO1 gene expression at all three concentrations of boric acid. On the other hand, significant changes were seen in all DHCR7, SQLE, EBP, and LSS gene expressions. In LSS, whose protein levels were also examined, a significant decrease was observed with IC10 treatment, while no significant increase or decrease was observed in either IC50 or IC25 concentrations. However, in SQLE protein expression a significant decrease was observed in all treatment groups. Contrary to our expectations, the expression of the 7-dehydrocholesterol reductase (DHCR7) gene, which catalyzes the final step in the cholesterol pathway increased significantly at IC25 treated cells.

In light of the data obtained, a more detailed study of the steroid pathway enzymes at the level of both protein and gene expression should be done in order to understand the action mechanism and the possible use of boric acid as a drug in cholesterol metabolism.

Keywords: Boric acid, 7-dehydrocholesterol reductase, steroid metabolism, HepG2 cell line, cholesterol metabolism

ÖZ

BORİK ASİDİN HEPG2 HÜCRE HATTINDA STEROİD BİYOSENTEZ YOLAĞI ÜZERİNE ETKİSİ

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Yüksek Lisans, Moleküler Biyoloji ve Genetik
Tez Yöneticisi: Prof. Dr. N. Tülin Güray

Şubat 2022, 58 sayfa

Bor, Türkiye'de önemli bir orana sahip olan boraks maden rezervlerinin en temel elementidir. Endüstriyel alanda çok geniş kullanıma sahip olan bor, son zamanlarda ilaç sektöründe yeni ürünlerin geliştirilmesinde kullanılmaya başlanmıştır. Endüstriyel alandaki bu geniş kullanımının yanında, sağlık ve ilaç endüstrisinde de kullanımı artmaktadır ancak etkisinin moleküler mekanizmaları hala bilinmemektedir.

Daha önce laboratuvarımızda yürütülen mikrodizin çalışmaları ve sonuçlarının analizleri sonucunda borik asidin, steroid metabolizması üzerinde inhibitör etkisi olduğunu gösterilmiştir. Bu sonuçlara göre yolaktaki bazı genlerin ifadeleri IC50 konsantrasyondaki borik asit muamelesi ile önemli oranda değişmektedir.

Bu tez kapsamında mikrodizin sonuçlarının ve borik asidin steroid metabolizmasındaki olası rolünün doğrulanması amacı ile bazı enzimlerin, HepG2 hücre hattında gen ve protein ekspresyonları araştırılmıştır. Genler, ifadelerine ve yolaktaki rollerine göre seçildi. 7-dehidrokolesterol redüktaz (DHCR7), Metilsterol Monooksijenaz 1 (MSMO1), Emopamil Bağlayıcı Protein (sterol izomeraz) (EBP), Farnesil-difosfat Farnesiltransferaz 1 (FDFT1), Lanosterol Sentaz (2,3-

oksidoskualen)-lanosterol siklaz) (LSS), Sterol-C5-desatüraz (SC5D) ve Skualen Epoksidaz'ı (SQLE) kodlayan genler, IC10, IC25 ve IC50 borik asit konsantrasyonlarında incelenmiştir. Her üç borik asit konsantrasyonunda da MSMO1 gen ekspresyonunda önemli bir değişim gözlenmedi. Öte yandan, DHCR7, SQLE, EBP ve LSS gen ifadelerinde önemli değişiklikler görüldü. Protein seviyeleri de incelenen LSS'de, IC10 tedavisi ile önemli bir düşüş gözlenirken, IC50 veya IC25 konsantrasyonlarında önemli bir artış veya azalma gözlenmedi. SQLE proteininin ekspresyonunda ise tüm tedavi gruplarında önemli bir düşüş gözlendi. Beklentilerimizin aksine, kolesterol yolundaki son basamağı katalize eden 7-dehidrokolesterol redüktaz (DHCR7) geninin ekspresyonu IC25 borik asit konsantrasyonunda önemli ölçüde artmıştır.

Elde edilen veriler ışığında borik asidin kolestrol metabolizmasında etki mekanizmasını ve ilaç olarak olası kullanımını anlamak için hem protein hem de gen ekspresyonu düzeyinde steroid yolu enzimlerinin daha detaylı bir çalışması yapılmalıdır.

Anahtar Kelimeler: Borik asit, 7-dehidrokolesterol redüktaz, steroid metabolizması, HepG2 hücre hattı, kolesterol metabolizması

Dedicated to my father

ACKNOWLEDGMENTS

First and foremost, I would like to thank Prof. Dr. N. Tülin Güray for her endless and valuable guidance, support, kindness, motivation, and trust throughout my study. She always supported, motivated, and encouraged me when I felt desperate. Her great effort made this thesis possible. It was a big honor to work with her.

I also thank the members of the thesis committee Prof. Dr. Orhan Adalı and Prof. Dr. Özlem Yıldırım for their valuable time, suggestions, assistance, and contributions as members of my thesis jury.

I would like to express my sincere appreciation to Merve Akkulak and Özlem Durukan for helping me in every step throughout my experiments and every desperate moment I experienced. Without their inspiring advice and encouragement, I would not be able to complete this thesis.

I would also like to thank Başak Ezgi Saraç for her guidance and valuable pieces of advice for my experiments when I just started my master's degree.

My sincere gratitude goes to my best friends for their endless support, motivation, and friendship. I would like to give special thanks to Tolgahan Sezen for his valuable friendship. He was always there for me every moment. I would also thank my dear friend, Deniz Ak for making me feel so lucky to have her in my life. My deepest appreciation goes to Pınar Diken for her priceless friendship. I would also thank Ömer Rençbereli for his endless kindness, and motivation.

I cannot express my feelings in words towards Tolga Gündoğdu. I am grateful for his support, motivation, friendship, and love. I appreciate him for being always with me, both in my most depressed and happiest moments.

My deepest thanks and gratitude go to my family, especially my mother, A. Leyla Akyıldız, who is the strongest woman I have ever known. I would also thank my sisters, Esra Akyıldız, Seda Baykara, my brother-in-law, Murat Baykara, for being

there for me all the time and for their endless love and support in all stages of my life. I would also like to thank Mustafa Emir Baykara for being more like a brother than a nephew and for his support and motivation.

There is no way I can appreciate my aunt, Prof. Dr. F. Hülya Özcan Önder for her valuable time, guidance, support, kindness, and motivation. I have always admired her the most in my life.

I would like to dedicate this thesis to the beloved memory of my father. If I were not his daughter, I would not be who I am now. Although I miss him so much, I know he always is there for me, and he is always in my heart.

I also wish to thank all in the Biological Sciences Department of METU. This study is partially funded by BAP METU GAP-108-2020-10093.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

ATCC	American Type Culture Collection
BA	Boric Acid
DF	Dilution Factor
DHCR7	7-dehydrocholesterol reductase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EBP	Emopamil binding protein (sterol isomerase)
FBS	Fetal Bovine Serum
FDFT1	Farnesyl-diphosphate farnesyltransferase 1
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
HepG2	Liver Hepatocellular Carcinoma Cells
LSS	Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)
MSMO1	Methylsterol monooxygenase 1
PBS	Phosphate-Buffered Saline
qRT-PCR	Quantitative Real-Time PCR
SC5D	Sterol-C5-desaturase
SQLE	Squalene epoxidase
XTT	2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

CHAPTER 1

INTRODUCTION

1.1 Borax, Boron and Boric Acid

Borax is a boron compound and a salt of boric acid (Wei et al., 2016) which was known to inhibit the growth of tumor cells. The molecular formula of borax is $Na_2B_4O_7.10H_2O$, and the molecular weight is 381.4 g/mol (National Center for Biotechnology Information, 2022). This salt affects 26 different enzymes that are found in plants, animals, cell-culture, and refined chemical reaction systems by acting on the enzyme directly, by binding to cofactors (e.g., NAD) or substrates or by unknown mechanisms (Hunt, 1998).

Boron, on the other hand, is in the 3A group in the periodic table with a molecular weight of 5 and is the lightest element of this group. It is a semimetal, and its symbol is "B". Boron is found in different concentrations in structures such as rocks, seawater, or freshwater. The United States, the Mediterranean, or Kazakhstan regions are very rich soils in boron (Woods, 1994). This element is very important for the world, and Turkey has a considerable amount of boron. Boron, which is widely used in industrial areas such as glass, ceramics, fire extinguishers, trade, energy, and agricultural products, is also used in the development of many new products. In addition to the extensive use of boron in the industry, the use of boron elements in the health and pharmaceutical industries is increasing day by day (BOREN, 2017).

Boric Acid is a hydrate of boric oxide and has mild antiseptic and antifungal properties. It is used to treat yeast infections and herpes. Although the exact toxicity level or status is not known; it is generally cytotoxic to all cells at different doses. Its

chemical formula is H_3BO_3, and its molecular weight is 61.83 g/mol (National Center for Biotechnology Information, 2022).

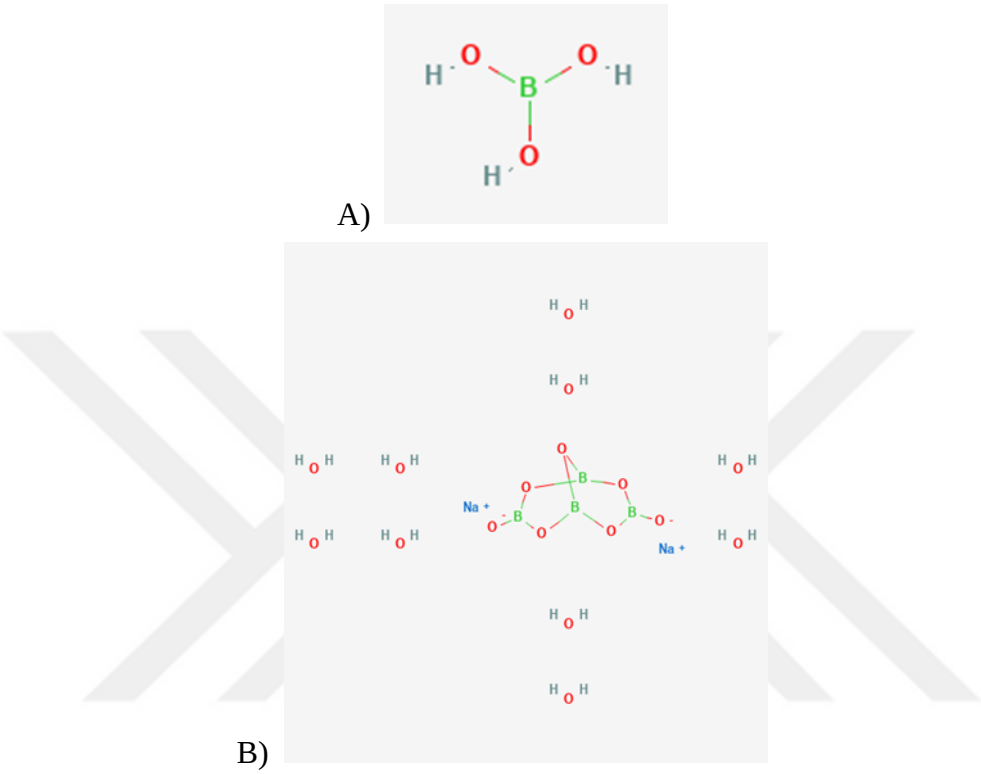


Figure 1.1 A) The 2D structure of boric acid. B) The 2D structure of borax (National Center for Biotechnology Information, 2022).

1.2 Boric Acid and Its Use in Health

Different studies have shown that boron, which is naturally found in several different forms, can be used for the treatment of many types of cancer such as liver, breast, and prostate cancers, all of which are among the most common cancer types (Wang et al., 2012). These studies have promising results for the use of boron in future.

The drug, known as Velcade, a well-known example in the pharmaceutical industry containing the active ingredient bortezomib, is used in the treatment of multiple

myeloma and mantle cell lymphoma. At the same time, the effects of this active ingredient on different types of cancer are also being investigated (Chen et al., 2011). Another example of the use of boron in the health sector is "Boron Neutron Absorption Therapy". This treatment is a promising method for tumors in the head and neck region, which are difficult to treat due to the blood-brain barrier. The rationale of the treatment is to destroy the tumor tissue with minimal damage to other healthy tissues around the tumor (Moss, 2014; Sabuncuoğlu et al., 2006).

1.3 Boric Acid and Liver

The liver might be particularly sensitive to boron intake with ample evidence suggesting a relation between boron and liver function, although the underlying molecular processes remain largely unknown.

There are findings that boric acid reduces lipid peroxidation and oxidative stress while performing various physiological effects in the liver. In addition, it is known through studies that the boric acid-serine complex strongly inhibits the gamma-glutamyl transpeptidase found in the cell membrane (London et al., 2002).

The enzyme gamma-glutamyl transferase, which is known to be inhibited by the boric acid-serine complex, hydrolyzes extracellular glutathione (GSH) and releases the amino acids necessary for the production of intracellular glutathione to be taken into the cell. In HepG2 cells, which are known to express the gamma-glutamyl transferase, it has been shown by studies that the activity of this enzyme is effective in initiating the intracellular lipid peroxidation process and in the formation of cancer, and it has also been found that the serine-boric acid complex is effective in suppressing the lipid peroxidation process (Paolicchi et al., 1997).

This enzyme is also a biomarker used to diagnose liver damage caused by chronic liver diseases, and it has been associated with insulin resistance and liver damage (Petta et al., 2012).

Whether the lipid regulating effects of boric acid and its lipid metabolism regulating effects in the liver is due to the reduction of gamma-glutamyl transferase inhibition in lipid peroxidation is an issue that needs to be investigated. In a study with mice, a newly synthesized boron compound resulted in a decrease in liver and blood lipid levels, a decrease in fasting blood glucose levels, and a reduction in weight gain in a mouse model fed a high-energy diet. In cell experiments, it has been shown that the boron-containing compound inhibits the activity of the SREBP transcription factor (Sterol regulatory-element binding proteins) by binding to the Mediator complex. SREBP is an important transcription factor in regulating lipid homeostasis. In the aforementioned study, the expression of target genes of SREBP decreases as a result of the decrease in the efficiency of SREBP by boron compounds, and as a result, a decrease in lipid biosynthesis is observed (Zhao et al., 2014).

Regulating effects of boric acid on blood were studied in lipid levels of canine (Basoglu et al., 2000), cattle (Basoglu, et al., 2017), rabbit (Basoglu et al., 2010), chicken (Sizmaz, 2014), and rat (Naghii & Samman, 1996). An animal study with rabbits is interesting in terms of examining the effects of boric acid on liver fat metabolism in addition to the blood lipid profile. In this study, it was found that boric acid prevented fatty liver caused by a high-energy diet; it has also been observed to improve the lipid profile in serum. Although the mechanism of the preventive effect of boron on hepatic steatosis is not fully known, it has been suggested that boron reduces oxidative stress associated with lipid peroxidation and affects the serum lipid profile in a healthy way, based on metabolic measurements (Basoglu et al., 2010; Basoglu et al., 2011). The antioxidant property of boric acid is also known from previous studies. Studies have shown that boric acid enhances its antioxidant property when combined with paclitaxel (Turkez, et al., 2010), aflatoxin (Turkez & Geyikoglu, 2010), titanium dioxide (Turkez, 2008), vanadium tetroxide (Geyikoglu & Turkez, 2008), and with lead as well. It is thought that chemicals such as cadmium (Ustundag et al., 2014), arsenic, bismuth, and mercury (Turkez et al., 2012) reduce the genotoxic effect. Another study also showed that boron prevents carbon-

tetrachloride hepatotoxicity by activating the antioxidant defense system (Ince et al., 2012).

1.4 Steroid Metabolism in Human

1.4.1 The Structure of Cholesterol

The molecular formula of cholesterol is $C_{27}H_{46}O$ and its molecular weight is 386.7 g/mol (NCBI, 2022).

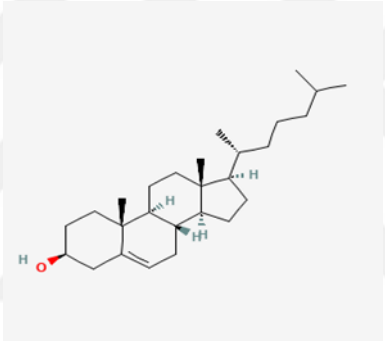


Figure 1.2 The 2D molecular structure of cholesterol (NCBI, 2022).

Cholesterol is largely hydrophobic, similar to other sterols and is synthesized by all cells in mammals and is predominantly localized in cell membranes. In general, it is responsible for interacting with neighboring lipids to regulate the rigidity, selective permeability, and fluidity of the cell membrane bilayer structure. In addition, some transmembrane proteins use cholesterol to bind multiple transmembrane proteins to help maintain or change their structure (Luo, 2020).

1.4.2 Biosynthesis and Uptake of Cholesterol

Cholesterol metabolism is the pathway that includes cholesterol biosynthesis, uptake, efflux, conversion, and esterification. Cholesterol intracellular traffic must be strictly controlled (Xu et al., 2020). Cholesterol synthesis takes place in the cytoplasm and is a very costly process when evaluated in terms of energy. To summarize, Acetyl Co-A molecules are combined to form a 30-carbon compound, but cholesterol is 27-carbon. For this reason, three carbon atoms must be removed (Kumari, 2018). In Figure 1.3, the basic synthesis pathway of cholesterol is given.

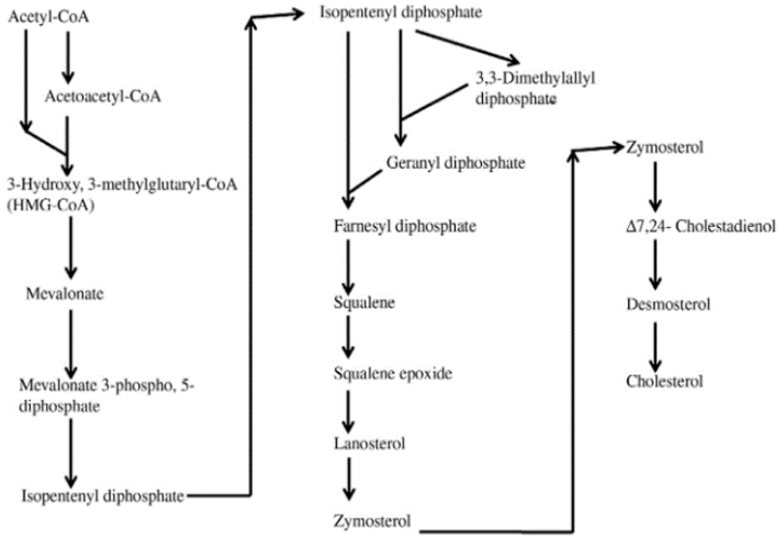


Figure 1.3 The cholesterol synthesis pathway (Kumari, 2018).

There are 2 important enzymes which function in the enzymatic reactions that take place during the synthesis of cholesterol. The first one is 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (HMGCR), which reduces HMG-CoA to MVA, and the latter is squalene epoxidase (SQLE), which catalyzes the oxidation of squalene to 2,3-epoxysqualene.

As shown in Figure 1.4, the two key enzymes 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and squalene epoxidase (SQLE) function in the complex

pathway which convert acetyl-CoA to cholesterol through 30 enzymatic reactions. At the same time rather than constantly making new cholesterol within the cell, most cells can take low-density lipoprotein (LDL) cholesterol from plasma into the cell via endocytosis, where LDLR is used as a mediator. Cholesterol molecule taken into the cell from LDL is then transferred to the endosome and from there to the lysosome. In the final step, it reaches its destination with the help of the NPC2/NPC1 complex and some sterol transfer proteins. If the cholesterol ratio is high, the cell tries to maintain homeostasis by following multiple pathways. One of these ways is to convert cholesterol to cholesteryl ester (CE) by sterol O-acyltransferase (SOAT) and be stored in lipid droplets. In another pathway, it is catalyzed to produce oxysterols, bile acids, and steroid hormones. Excess cholesterol taken into the cell is secreted out of the cell via ABCA1/ABCG1, and the level of cholesterol inside the cell is preserved (Xu et al., 2020).

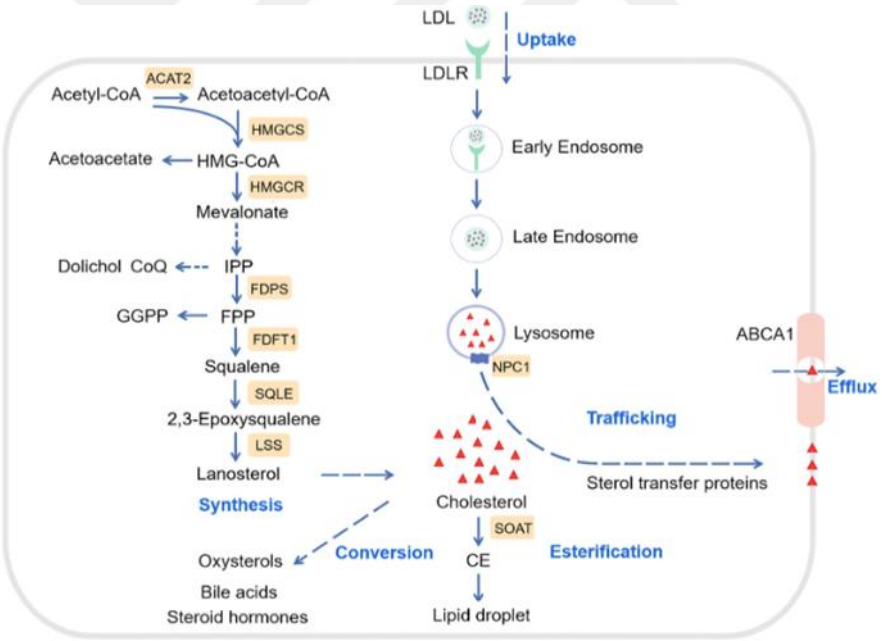


Figure 1.4 The biosynthesis and uptake of cholesterol into a cell. ACAT2: AcetylCoenzyme A Acetyltransferase 2, HMGCS: Hydroxymethylglutaryl-CoA Synthase, HMGCR: 3-Hydroxy-3-Methylglutaryl-CoA Reductase, FDPS: Farnesyl Diphosphate Synthase, FDFT1: Farnesyl-diphosphate farnesyltransferase 1, SQLE: Squalene Epoxidase, LSS: Lanosterol Synthase, NPC1: NPC Intracellular Cholesterol Transporter 1, SOAT: Sterol O-Acyltransferase 1 (Xu et al., 2020)

The Figure 1.5 shows the cholesterol biosynthesis with the enzymes which were investigated in this study.

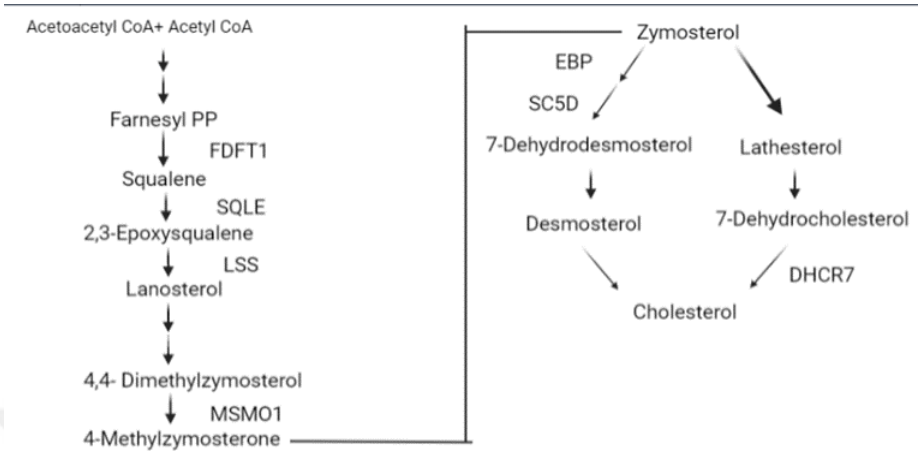


Figure 1.5 Cholesterol synthesis. 7-dehydrocholesterol Reductase (DHCR7), Emopamil Binding Protein (sterol isomerase) (EBP), Farnesyl-diphosphate Farnesyltransferase 1 (FDFT1), Lanosterol Synthase (2,3-oxidosqualene-lanosterol cyclase) (LSS), (Methylsterol) Monooxygenase 1 (MSMO1), Sterol-C5-desaturase (SC5D), Squalene Epoxidase (SQLE).

1.4.3 Cardiovascular Diseases and Cholesterol

The physiological and pathological importance of cholesterol in terms of human health is undeniably high. There is a strong correlation between susceptibility to atherosclerosis or cardiovascular diseases and cholesterol levels (Luo et al., 2022). Atherosclerosis is a vascular disease, caused by the uptake of cholesterol deposited in the arterial walls by macrophages. This accumulation is caused by the low-oxidized low-density lipoproteins oxLDL type of cholesterol. After macrophages take in cholesterol, they turn into a structure called foam cells, and these foam cells can clog the artery wall, causing strokes, heart attacks, and peripheral vascular diseases (Sezgin et al., 2017).

1.4.4 Cholesterol and Cancer

Although the relationship between dietary cholesterol and hypercholesterolemia and tumors is more controversial and unclear (Kuzu, 2016), a study conducted in the past year has shown that high cholesterol level in serum enhances the anti-tumor functions of immune cells in mice, thereby reducing the growth of liver tumors (Qin, 2020). However, other studies have confirmed the tumorigenic or growth-promoting effects of intracellular cholesterol. Cholesterol accumulation in cells has been reported to have an effect not only in liver cancer but also in lung cancer, prostate cancer, and bone metastasis (Xu, 2020).

1.5 Aim of the Study

In this study, the effect of boric acid on lipid metabolism is studied in HepG2 cell lines, a commonly used cell line in toxicology studies. Our laboratory previously studied the effect of boric acid on HepG2 cells at the IC50 level by using microarray technology (Tombuloğlu et al., 2020). The results have shown that steroid biosynthesis pathway as a whole was downregulated indicating a possible inhibitory role of boric acid at this concentration. The genes which were chosen from the microarray results were used in this study in order to investigate the genes related to steroid metabolism. The genes whose expressions were found to be altered as a result of IC50 boric acid treatments are 7-dehydrocholesterol Reductase (DHCR7), Emopamil Binding Protein (sterol isomerase) (EBP), Farnesyl-diphosphate Farnesyltransferase 1 (FDFT1), Lanosterol Synthase (2,3-oxidosqualene-lanosterol cyclase) (LSS), (Methylsterol) Monooxygenase 1 (MSMO1), Sterol-C5-desaturase (SC5D), and Squalene Epoxidase (SQLE). So, in this study, HepG2 cells treated with different concentrations of boric acid which were 4,8 mM, 12 mM, 24 mM (IC10, IC25, IC50 concentrations respectively) used in order to investigate the differences in gene expressions and for some genes at the protein level as well. In this way, we

try to show the effects of boric acid on the steroid pathway in HepG2 cells at the molecular level, so that the possible use of boric acid as a drug may be in question.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

All laboratory chemicals used in cell culture and assays were sterilized and cell culture grade. DMEM medium with low glucose, with L-Glutamine, sodium-pyruvate, and 25mM HEPES, 10X Trypsin-EDTA, heat-inactivated fetal bovine serum (FBS), Penicillin-Streptomycin solution (Pen-strep) (Biowest, France); phosphate-buffered saline solution (PBS) without Mg+2 and Ca+2 (Biowest, France; Biological Industries, Israel), %0,4 Trypan Blue Solution (Sigma Aldrich, USA), dimethyl-sulfoxide (DMSO) (Santa Cruz, USA), were used in cell culture experiments.

Liver hepatocellular carcinoma cell line HepG2 was previously purchased from ATCC (American Type Culture Collection).

Cell culture graded Boric Acid (Sigma, USA) was given as a gift from Dr. Serap Kolukısa from BOREN Institute.

In cytotoxicity experiments, XTT Based Cell Proliferation kit (ATCC, USA) was used.

In Real-Time PCR experiments, RNA Isolation Kit, cDNA Reverse Transcription Kit (Thermo Fischer Scientific, USA); FastStart Universal SYBR Green Master Mix (ROX) (Roche Applied Science, Switzerland) were used.

In protein purification and Western Blot experiments, Pierce™ BCA Protein Assay Kit, PVDF Membrane (Thermo Fischer Scientific, USA), scrapers (Corning, USA), Clarity Western ECL Substrate, tetramethyl ethylenediamine (TEMED) (Bio-Rad Laboratories, USA); phenyl methane sulfonyl fluoride (PMSF), bovine serum

albumin (BSA), bromophenol blue, tween 20, RIPA buffer (Serva, Germany); glycine, ß-mercaptoethanol, sodium dodecyl sulfate (SDS), methanol (Sigma Aldrich, USA); ammonium persulfate (APS), Tris, N"-N"-bis-methylene-acrylamide, acrylamide (Sigma, USA); glycerol (Riedel de-Haen Chemical Company, Germany), pre-stained protein ladder (Fermentas, USA) were used.

All laboratory equipment used for cell culture and other experiments were either analytical, molecular, or cell culture graded. T-75 cell culture flasks, 12 well and 96-well cell culture plates, (Sarstedt, Germany); 6 well plates, serological pipettes (Sarstedt, Germany; SPL, Korea), micropipettes (Eppendorf, Germany), polypropylene centrifuge tubes, pipet gun (Isolab, Germany); microtubes (Axygen, USA), sterile-filtered tips (Isolab, Germany; Biopointe Scientific, USA), RT-PCR tubes (QIAGEN, Germany), Cryovials (Grenier-Bio, Germany), Mr. Frosty (Sigma-Aldrich, USA) and BrightLine Hemocytometer (Marienfeld, Germany) were used.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell Culture Conditions and Preparation of Medium

Human hepatocellular carcinoma line, HepG2, was cultured in Dulbecco's Modified Eagle's medium (DMEM) low glucose with L-Glutamine, sodium pyruvate, and 25 mM HEPES, containing %1 penicillin-streptomycin (Pen-Strep) and %10 fetal bovine serum (FBS). HepG2 cell line was incubated at 37°C and 5% CO_2 atmosphere in an incubator with a water jacket (Series 8000 WJ Thermo-Scientific, USA). Laminar flow cabinet MN 090, NUVE was used for all cell culture experiments. The old medium was changed with a fresh medium every day or every 2 days.

2.2.1.2 Cell Thawing

Cryofrosted cells, which were frozen in cryovials, were defrosted gradually. At first, they were kept at 37°C in a water bath for about one or two minutes. These semi-melted cells were mixed with 1 mL of fresh complete growth medium in cryotubes. After pipetting several times, they were transferred to T75 flasks, and an 8 mL medium was added and pipetted. The cells were incubated in 5% CO_2 and 37°C. After 24 hours, the medium was changed with a new medium to get rid of dimethylsulfoxide (DMSO).

2.2.1.3 Cell Treatment

Boric acid was dissolved in a fresh complete medium at room temperature. A stock solution was prepared fresh before every assay procedure, and it was diluted in an appropriate portion according to assays.

2.2.1.4 Cell Passaging

When HepG2 cells were duplicated, and cell confluence reached approximately 80%, the cells were passaged, that is cells were seeded into another culture flask. In general, the cells reached appropriate confluence on the surface of the flask in 2 or 3 days. After reaching confluence, the complete growth medium was discarded, and the cells were rinsed with 5 mL PBS at once. Two mL of 1X pre-warmed at 37º C Trypsin-EDTA was added to detach the cells from the flask's surface and incubated for 2 or 3 minutes in an incubator. Three mL of medium was added for every 1 mL of trypsin to terminate trypsin activity. As a result, 6 mL of medium was added and gently pipetted to mix equally without clumping. Following pipetting, a total volume of 8 mL was divided into 2 equal volumes, and each 4 mL was added into new 2 flasks. Six mL medium was added to each flask to complete the final volume to 10

mL. Finally, the two new flasks were placed into the incubator. All passaging procedures were performed repeatedly every three or four days.

2.2.1.5 Cell Freezing

Before starting the freezing process, the freezing medium was prepared freshly, containing 90% FBS and 10% DMSO, and kept on the ice until it was used. In this procedure, the first step was the removal of the medium and washing the cells with 5 mL PBS. Two mL trypsin was added and put into the incubator under the same conditions in section 2.2.1.4. After waiting 3-4 minutes, a 6 mL medium was added and pipetted into the mix gently, and this mix was transferred further into a 15 mL falcon tube. Then, it was centrifuged at 1000 rpm for 5 minutes. The supernatant was removed, 1 mL of freshly prepared cold freezing medium was added onto the pellet and mixed well with the help of a pipette. The mixture was transferred into the cryotubes for storage. These cryotubes were placed in Mr. Frosty™ Freezing Container and stored in a -80°C freezer (Sanyo, Japan) for short-term storage. Later, all cryovials were transferred into a liquid nitrogen tank for long-term storage.

2.2.1.6 Cell Counting

In cell staining, viable cells cannot be stained with Trypan Blue since they exclude the dye; whereas dead cells are observed as blue dots since their cell membranes are permeable to Trypan Blue. Using this feature, viable cells were counted on a hematocytometer by using Trypan Blue. After harvesting the HepG2 cells, they were transferred to polypropene centrifuge tubes and centrifuged at 1000 rpm to obtain a pellet. Then, the pellet was dissolved in medium and 100 µL was taken from the pellet and it was further mix was mixed well with 100 µL of Trypan Blue Solution to determine cell viability. Each 100 µL was equal to 1 volume. After the preparation of the Trypan Blue staining mix, 10 µL of this final cell suspension containing trypan blue-treated HepG2 cells was applied to the chambers of a coverslip-covered

hemocytometer. This coverslip allowed the suspension to diffuse using capillary action. Living cells were easily observed under a 10X objective light microscope due to their aforementioned properties.

To explain the calculation of viable cells, Equation 2.1 was given. Each chamber has the same volume of suspension, which is 10^{-4} mL due to that, the calculations were done by the average cell count of the counting chambers and multiplying it with 10^4. The counting chambers are shown in Figure 2.1.

Total Cell Number/mL= Average of Cell Count of Two Chambers x DF x 10^4

DF = Dilution Factor

10^4 = Factor that is calculated from the dimension of the hematocytometer.

Equation 2.1: Equation of Total Cell Count

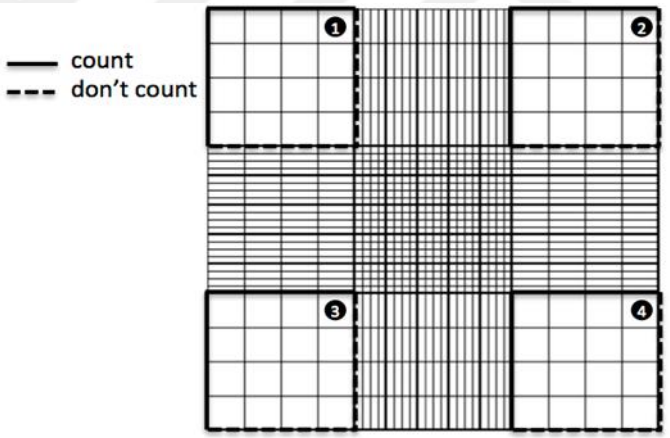


Figure 2.1 Counting Chambers of Hematocytometer

2.2.2 Cytotoxicity Assay

2.2.2.1 XTT Proliferation Assay

In order to evaluation of cytotoxicity of boric acid in HepG2 cell line, XTT (2,3-Bis- (2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) Cell Proliferation Kit was used (Scudiero et al., 1988). The procedure given by the manufacturer was used. XTT is slightly yellow and when it is reduced, its color turns into bright orange, and it is assumed that XTT was excluded from cells because of its negative charges. XTT response is a result of substantially mitochondrial oxidoreductases being transferred to the plasma membrane. Consequently, XTT assay is used to measure the cell viability by measuring the pyridine nucleotide (Berridge et al, 2005; Marshall et al, 1999).

When the cells reached 80-85% confluency in T75 flasks, they were harvested and counted. In a 96 well plate, harvested cells were seeded at a density of 15.000 per well (100 µL/well). After 24 hours, the old medium was discarded, and all wells were rinsed with 50 µL PBS at once. In order to prevent cells from drying, a 100 µL new medium was added quickly. Following the addition of the medium, melted XTT reagents were added according to the manufacturer's instructions onto each well, and the plate was placed into the incubator for 4 hours. After the incubation, the plate was measured in an ELISA reader (Multiskan™ GO- Thermo Scientific, USA). At 630 nm, which is used for non-specific readings, and at 450 nm, the wavelength gives the maximum absorbance for the formed product. Afterward, the values measured at 630 nm were subtracted from the values obtained at 450 nm to exclude non-specific readings in the formula used to find cell viability. According to the formula, the cell viability rate was found as a percentage by making the necessary subtraction from the values of the blank wells without cells, and the percentage of cell viability in the

control group was considered as 100%. The equation is given:

$$\%\text{Cell Viability} = \frac{\text{Avg.OD}_{415}\text{ of treated well (with cell)- Avg.OD}_{415}\text{ of treated well (without cell)}}{\text{Avg.OD}_{415}\text{ of control well (with cell)- Avg.OD}_{415}\text{ of control well (without cell)}} \text{ x } 100$$

In a 96 well plate, wells containing cells were seeded as triplicates, whereas blank wells without cells were seeded as duplicates for each diluted concentration and controls. The example template is given in Figure 2.2.

The XTT assay aimed to find out the IC50 value. This value represents boric acid the concentration that kills 50% of the HepG2 cell population, and it was determined by cytotoxicity curves that were drawn according to equation results.

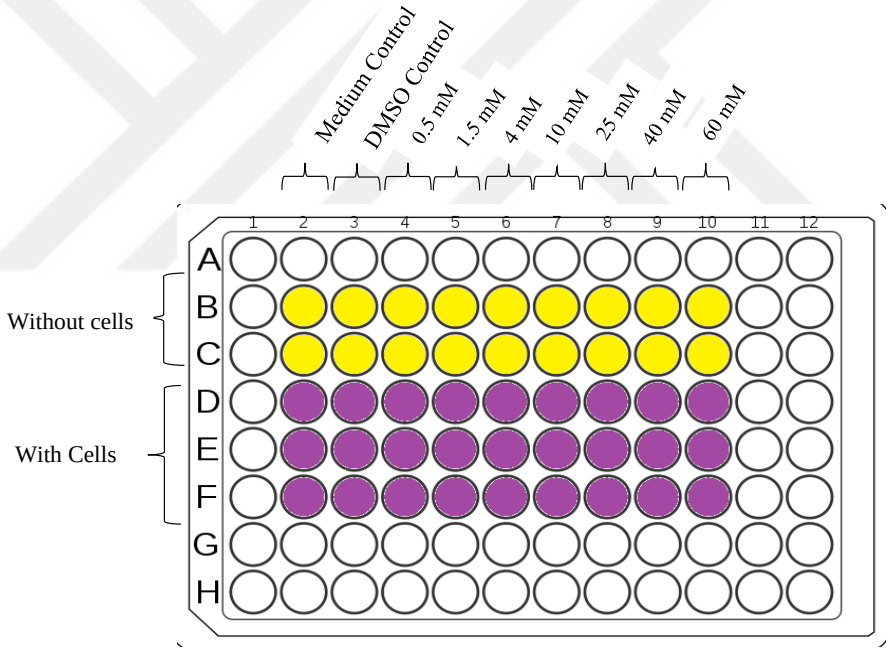


Figure 2.2 An example of treatment pattern of 96 well

2.2.3 Total RNA Isolation

HepG2 cells were seeded in 6 well plates at a density of 10^6 cells/well for IC10, IC25, and IC50 concentrations. In addition, three more wells were used for the control group. One day after seeding, the old medium was replaced with the fresh medium for the control group, the other cells were treated with boric acid at concentrations of IC10, IC25, IC50. After 24 hours of treatment, all wells were rinsed once with PBS for 10 or 15 seconds, and PBS is removed. Then, 1X Trypsin was added into the wells and incubated for 4 minutes in the incubator. After the incubation, the fresh medium was added onto trypsin to inhibit trypsin activation, and the medium-trypsin mix was pipetted well to detach the cells. This cell lysate was transferred to 2 mL centrifuge tubes. In this isolation step, with slight modifications, all procedure was applied according to the manufacturer's instructions. Nanodrop was used to evaluate the RNA purity and determine the concentration of RNA. The purity of RNA was determined by looking at the 260/280 nm ratio. This ratio must be between 1,8 and 2,2 for the purity of RNA. Values other than these values indicate contamination.

For example, above 2,2 specify the contamination of protein or phenol, while below 1,8 remarks contamination of DNA.

2.2.4 cDNA Synthesis

Thermo Fischer Scientific High-Capacity cDNA Reverse Transcription Kit was used to synthesize cDNA.

One μg of total isolated RNA was used. According to the kit's instructions, 10 μL 2X RT Master Mix was prepared first on the ice and pipetted to mix gently, and 10 μL of 2X RT master mix was added into each centrifuge tube. Then, for each concentration and control, 10 μL of RNA samples which were treated with DNase were pipetted and added onto 10 μL of 2X RT master mix. Consequently, 2X RT Master Mix was diluted to 1X. After adding 10 μL master mix, all centrifuge tubes

were pipetted and spun down by microfuge to mix all ingredients well and eliminate any bubbles. The final step was loading the thermal cycler. Thermal cycler conditions are given in Table 2.1.

Table 2.1 Thermal cycler conditions in the cDNA synthesis procedure

Steps	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 minutes	120 minutes	5 minutes	Hold

After thermal cycles completed, all cDNA samples were stored at -20°C for long-term storage.

2.2.5 Quantitative Real-Time PCR (qRT-PCR)

Quantitative Real-Time PCR was used to analyze the effects of boric acid on the HepG2 cell line by using FastStart Universal SYBR Green Master Mix (ROX) and QIAGEN Rotor-Gene (QIAGEN, Germany).

The qRT-PCR reaction mix was prepared with 100 ng cDNA, 5 µM reverse and forward primers, RNase-free distilled water, and SYBR Green Master Mix in a total volume of 20 µl. In addition to controls and treated groups, no template control (NTC) was used for the detection of contamination.

In this study, DHCR7, MSMO1, LSS, EBP, SQLE, FDFT1, and SC5D gene expressions were investigated, and as a housekeeping control gene, RPL13A was used. All primers are given below in Table 2.2.

Table 2.2 The Primer Sequences of Interested Genes

GENE	PRIMER SEQUENCE
RPL13A	F- CCGCCCTACGACAAGAAA R- CAGGGTGGCTGTCACTGC
DHCR7	F- CGGATCGGGAAGTGGTTTGA R- CAGACCCTGCAGCGTGTAAA
MSMO1	F- GTTCCGAGGTTGGAACACCT R- ATAATTCTGAACGGCCGCGA
LSS	F-VCCAGGCCTCCTGATCACTTG R-TCAATGTGCAGGCCCCAG
EBP	F- GGTTTGCAGTGTGTGGGTTC R- GTATCGGCTGTCTCCCTTGG
SQLE	F- GACGGTTACTCTGGTTACTGGG R- GTGGTTTCCAGGGTACCTCC
FDFT1	F- ACTATGTTGCTGGGCTGGTC R- AACAGGCCCATAGAGTTGGC
SC5D	F- GTTTGCAGAGCAGT GGCG R- CAGCAAGAACAGTGCAACAG

2.2.6 Determination of Protein Expression

Total cell protein isolation was performed from a control group and HepG2 cells treated with IC10, IC25, IC50 concentrations. Protein analysis was performed for LSS and SQLE genes whose gene expressions were also checked in Real-time PCR.

2.2.6.1 Protein Isolation

The whole protein isolation procedure was performed by seeding cells into T75 flasks at a density of $3x10^6$. After one day of seeding, 3 plates were treated with the concentration of IC10, IC25, IC50, while 1 plate was chosen for control, and its medium was replaced with a fresh medium. Twenty-four hours after the cells were treated, the old medium was discarded, then all plates were rinsed twice with cold

PBS (4ºC). Following the second washing, 3 mL of cold PBS was added, and the cells were incubated for 5 or 6 minutes and kept on ice during incubation. At the end of the incubation period, cells were scraped with a scraper. The lysates formed in this process were transferred to 2 ml centrifuge tubes, and the tubes were centrifuged at 2500 g for 5 minutes. After discarding supernatant, a commercial RIPA buffer was added on top of the cell pellet, with 1 ml of RIPA buffer per 40 mg pellet and pipetted the mixture to suspend the pellet. In addition to the RIPA buffer, PMSF was added. For increasing the amount of isolated protein, all pellets were sonicated for 1 minute. After sonication, for 15 minutes, pellets were incubated on ice, and every 5 minutes all tubes were vortexed. It was centrifuged at 14,000 x g for 15 minutes and 3 minutes at 17,000 x g to maximally pellet the cell debris. For further analysis, the supernatant was transferred to a new tube and stored at -80ºC for storage.

2.2.6.2 Determination of Protein Concentration

In order to determine the concentration of protein, BCA (Bicinchoninic Acid) method was used. The BSA included in the kit was prepared at concentrations of 0.025, 0.125, 0.25, 0.5, 0.75, 1, 1.5, and 2 mg/mL to be used as a standard.

While preparing the BCA reagent, the A and B solutions in the kit were mixed at a ratio of 50:1. The microplate was kept in dry block heating at 37°C for 30 minutes. The absorbance of the colored products at a wavelength of 562 nm was measured with an ELISA plate reader. The absorbance values of the sample proteins were determined by subtracting the absorbance of the blank sample. Protein concentrations of the samples were calculated using the prepared BSA standards curve.

2.2.6.3 Preparation of Protein Sample

After the determination of protein concentration, for to load proteins onto SDS-PAGE Gel, they were mixed with Sample Dilution Buffer (Appendix). The formula was used to equalize the concentrations of the proteins to be used, obtained from control, and treated cells with IC10, IC25, and IC50 concentrations. By applying the formula, the volume of water needed to be added to obtain the proteins at a 2 mg/mL concentration was found. The sample buffer dilution was then added by calculating the required protein concentration. Afterwards, samples were mixed in 4X volume of sample and sample dilution buffer, and the samples were incubated for 1.5 minutes in a 100ºC heat block.

2.2.6.4 SDS-Page Gel Electrophoresis

The Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) method was used for the separation of proteins (Walker, 2002). Firstly, the gels used in SDS-PAGE Gel Electrophoresis were prepared. They were composed of freshly prepared 4% stacking gel and 10% separating gel (Appendix). After preparing gels, SDS-PAGE Gel Electrophoresis's discontinuous buffer system was used (Laemmli, 1970).

Then, 20 µg of each protein sample and 4 µg from the protein ladder were loaded into the wells as samples and standards, respectively. After loading the samples, the gels were placed in the electrophoresis tank (Mini-Protean, BioRad, USA) and filled with running buffer. This tank system was connected to the power supply (BioRad, USA) and operated at 90 V on the stacking gel and 200 V on the separation gel. The process continued until the bands, which became visible with bromophenol blue in the sample dilution buffer, reached the bottom of the gel.

2.2.6.5 Western Blot Analysis

After separating the proteins according to their molecular weights, the gel was removed from the tank and kept in the transfer buffer to stabilize until the transfer mechanism was set up. While the gel was kept in the transfer buffer, the PVDF membrane was cut evenly with the gel for use in the transfer step. After cutting, the hydrophobic PVDF membrane was kept in 100% methanol for 1 minute in a shaker. After 1 minute, the PVDF membrane taken from the methanol was kept in the transfer buffer for 5 minutes.

Then, a mechanism called a sandwich was prepared for the transfer step. The sandwich mechanism is prepared by placing membrane, gel, and 3 layers of Whatman paper placed on the Whatman papers. In the meantime, extra care was taken in order to avoid bubbles. The prepared transfer sandwich was then placed into the Mini Trans-Blot module (Bio-Rad, USA) and filled with some transfer buffer. The transfer process was carried out by applying a 25 V current for 30 minutes.

After the transfer, the membrane was washed for 15 minutes with TBST (Tris-buffered saline with Tween 20) to remove the transfer buffer. In the blocking stage, the membrane was kept in the blocking solution for one hour to prevent non-specific binding. After 1 hour of blocking, the membrane was incubated overnight at 4˚C on a shaker with primary antibodies prepared in appropriate concentrations with the blocking solutions. Antibodies used in Western blot analysis are given in Table 2.3.

Table 2.3 Antibody concentrations in Western-blot method

Antibody	**Primer Antibody**	**Secondary Antibody**
LSS	Mouse Polyclonal 1:250	HRP-Conjugated anti-mouse 1:4000
SQLE	Mouse Polyclonal 1:250	HRP-Conjugated anti-mouse 1:4000
GAPDH	Rabbit Polyclonal 1:2000	HRP-Conjugated anti-rabbit 1:3000

After waiting overnight for primary antibody binding, the membrane was washed three times for 10 minutes with TBST to remove unbound primary antibodies. The membrane was then incubated with HRP-conjugated secondary antibodies for 1 hour. After the 1 hour of incubation, the membrane was washed three times each with TBST to remove unbound secondary antibodies and prevent non-specific binding. Then, Clarity Western ECL Substrate (Bio-Rad, USA) was used to detect the antibodies bound to the proteins. The 2 solutions in the kit were mixed at a ratio of 1:1 and applied on the membrane in the dark for 5 minutes. Then, the excess ECL mixture on the membrane was removed, and imaging was performed on the membrane imaging device (ChemiDoc MP, Bio-Rad, USA) and the obtained protein band images were analyzed with ImageLab software.

2.3 Statistical Analysis

All experiments were carried out with at least 3 replications. GraphPad Prism version 8.02 was used for statistical analysis, and all results are expressed as mean ± standard deviation (SD), and for all graphs, error bars indicate ± SD. Unpaired, two-tailed student t-test and one-way ANOVA test were used. In these tests, $p<0.05$ was chosen as the level of significance.

CHAPTER 3

RESULTS

The effects of boric acid on the cell cycle and DNA replication were previously confirmed by the cytotoxicity and genotoxicity experiments that were carried out in our laboratory. The previous microarray results also showed that the steroid pathway as a whole was down-regulated (Tombuloglu, 2020). In order to understand the role of boric acid on steroid metabolism, we try to further investigate the expression of some genes which are related to this pathway, including the cholesterol synthesis within the scope of this project.

3.1 Cell Viability Assay by XTT Assay

The cytotoxicity effect of boric acid in HepG2 cells was analyzed by XTT Cell Proliferation Assay. In this assay, cells were seeded in 96-well plates, and they were treated with different concentrations of boric acid for 24 hours. After 24 hours of incubation, plates were measured at 450 and 630 nm by an ELISA plate reader and according to Equation 2.2, cell viability was calculated. The result of this calculation is obtained as IC50, IC25, IC10 values, which are found as 24 mM, 12 mM, and 4,8 mM respectively. IC10, IC25, and IC50 values were calculated using the concentration-dependent cell viability experiments with XTT kit as given in Figure 3.1.

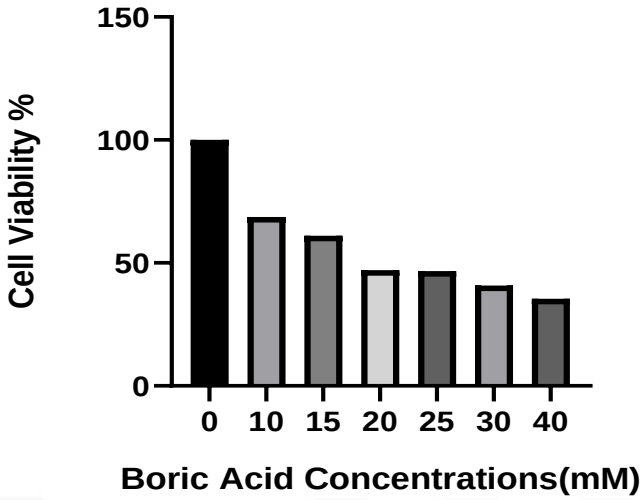


Figure 3.1 % Cell Viability and Concentration Graph

3.2 Gene Expression

The effect of different boric acid concentrations namely IC10, IC25, and IC50 concentrations, on the expressions of some genes which are involved in steroid metabolism was studied in the HepG2 cell line by real-time PCR method.

According to our cytotoxicity experiment results IC10, IC25, IC50 values were calculated and then the HepG2 cells were treated at these concentrations in order to investigate the effect of boric acid on mRNA expression levels of some enzymes of the steroid pathway.

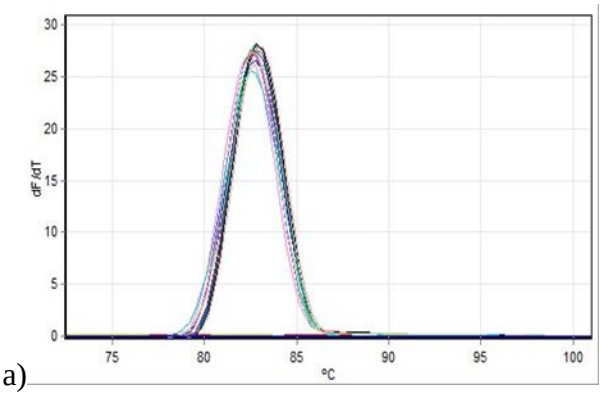
After several trials with GAPDH and beta-actin in qPCR experiments, the RPL13A (ribosomal protein L13a) gene was finally selected to be used as an internal standard due to its high transcriptional stability. Optimization studies were carried out for the expressions of the selected genes and the optimization conditions created by real-time PCR of the DHCR7, LSS, EBP, SQLE, MSMO1, FDFT1, and SC5D genes are given in Table 3.1.

The following qRT-PCR run cycle protocol was used:

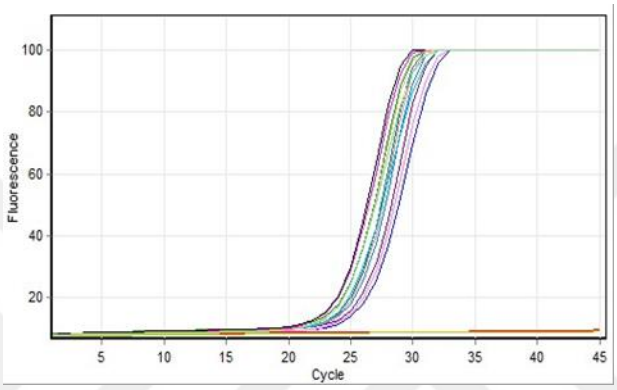
Table 3.1 Genes studied and their optimization conditions. C.R: Cycle Repeat Times

Gene	**1. Step**	**2. Step**	**3. Step**	**4. Step**	**C.R**
DHCR7	95°C-10:00	95°C-00:20	63°C-00:30	72°C 0:20	X44
LSS	95°C-10:00	95°C-00:20	64°C-00:25	72°C 0:20	X44
EBP	95°C-10:00	95°C-00:20	63°C-00:20	72°C 0:20	X44
SQLE	95°C-05:00	95°C-00:20	65°C-00:20	72°C 0:20	X44
MSMO 1	95°C-05:00	95°C-00:20	61°C-00:25	72°C 0:20	X44
FDFT1	95°C-03:00	95°C-00:20	60°C-00:20	72°C 0:20	X44
SC5D	95°C-03:00	95°C-00:20	60°C-00:20	72°C 0:20	X44

The melting curve fluorescent images obtained as a result of the optimization are given in Figure 3.2 and Figure 3.3 below as an example for DHCR7 and RPL13A genes. The rest of the results are given in the Appendix A and B.



a)



b)

Figure 3.2 a) DHCR7 Melt Curve and b) Amplification Curve

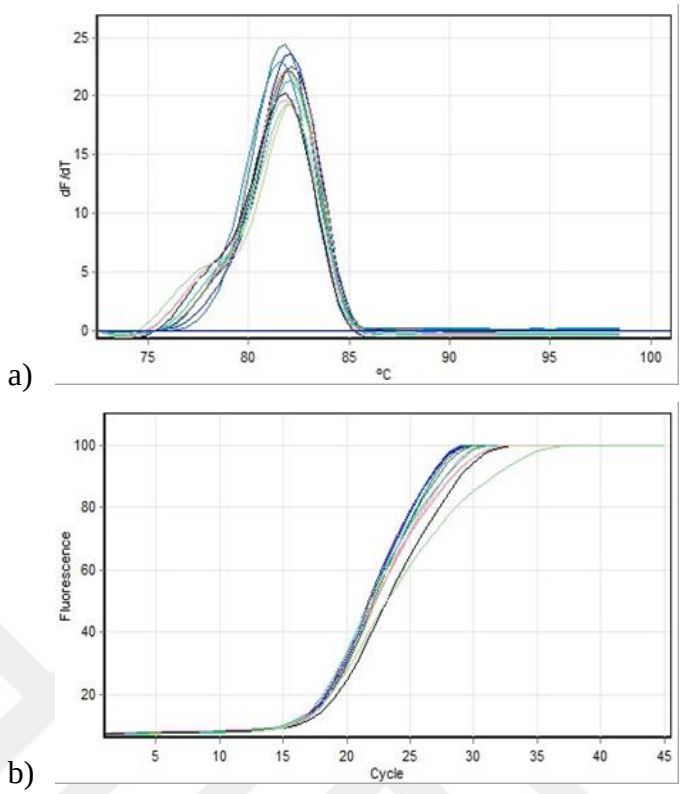


Figure 3.3 a) RPL Melt Curve and b) Amplification Curve

3.2.1 Effects of Boric Acid on mRNA Expression of 7-dehydrocholesterol Reductase (DHCR7)

After treating the cells with boric acid, the expression of the 7-dehydrocholesterol reductase (DHCR7) gene, which catalyzes the final reaction step in the steroid pathway, was found to be increased as can be seen in Figure 3.4; the increase in the cells treated with boric acid at IC25 ratio was even greater. However, as the concentration increased, the expression of the gene started to decrease.

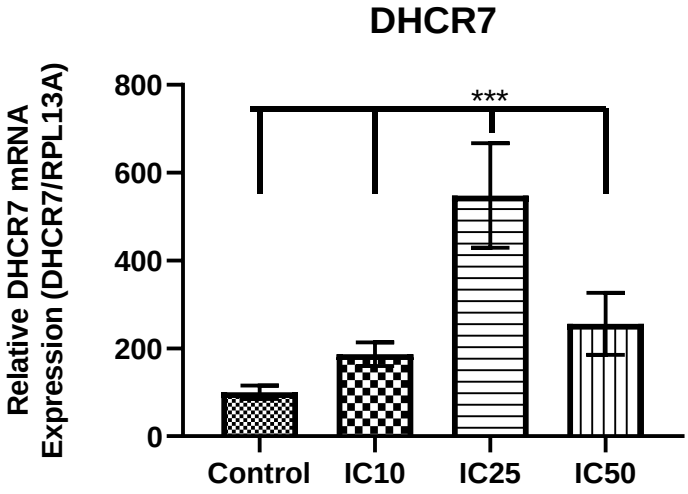


Figure 3.4 Gene expression of DHCR7 in cells treated with three different concentrations of boric acid. Error bars indicate ± SD. *** $p \leq 0.001$

3.2.2 Effects of Boric Acid on mRNA Expression of Squalene Epoxidase (SQLE)

In the SQLE gene analysis, it was observed that the expression of squalene epoxidase (SQLE) was significantly decreased in all cells treated with boric acid; suppression of gene expression was seen depending on boric acid concentrations and the decrease observed was less in cells treated with IC10 (Figure 3.5).

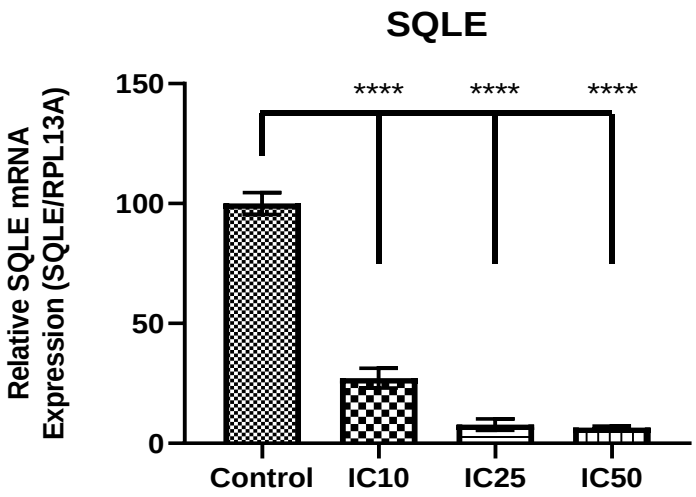


Figure 3.5 Gene expression of SQLE in cells treated with three different concentrations of boric acid. Error bars indicate ± SD. **** $p \leq 0{,}0001$

The expressions for the EBP and LSS genes are given in Figure 3.6 and Figure 3.7, respectively. In the PCR results obtained for these two genes, there was a decrease in expression as seen in the SQLE gene. While a similar decrease was observed in the IC10, IC25, IC50 ratios in the EBP gene, the expression decreased in the LSS gene in cells treated with IC25.

3.2.3 Effects of Boric Acid on mRNA Expression of Emopamil Binding Protein (EBP)

The expressions for the EBP gene are given in Figure 3.6. According to the PCR results obtained for this gene, there was a decrease in expression as seen in the SQLE gene. A similar decrease was observed in all IC10, IC25, and IC50 concentrations of boric acid for the EBP gene.

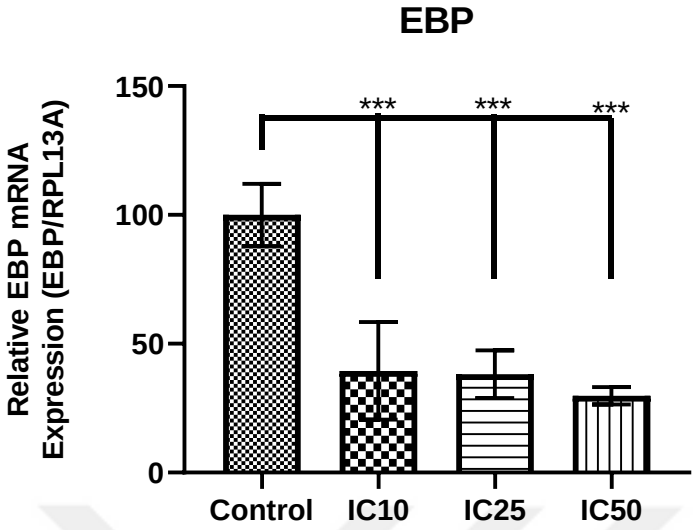


Figure 3.6 Gene expression of EBP in cells treated with three different concentrations of boric acid. Error bars indicate ± SD. *** $p \leq 0{,}001$

3.2.4 Effects of Boric Acid on mRNA Expression of Lanosterol Synthase (LSS)

The expressions for the Lanosterol Synthase (LSS) gene are given in Figure 3.7. According to the PCR results obtained for this gene, there was a decrease initially in expression as seen in both the SQLE and EBP genes, and maximum decrease in LSS gene expression was observed in cells treated with IC25, whereas there is an increase in gene expression in the cells treated with boric acid at IC50 concentration.

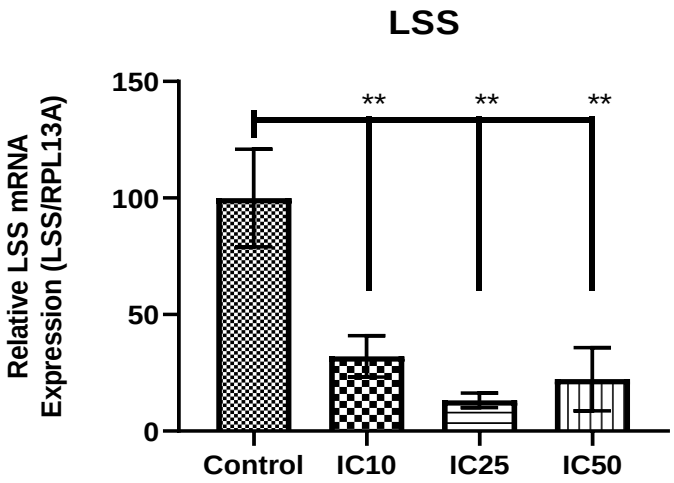


Figure 3.7 Gene expression of the LSS in cells treated with three different concentrations of boric acid. Error bars indicate ± SD. ** $p \leq 0{,}01$.

3.2.5 Effects of Boric Acid on mRNA Expression of Methylsterol Monooxygenase 1 (MSMO1)

MSMO1 gene expressions are given in Figure 3.8. According to PCR results obtained the gene expression was upregulated only at IC25 concentration of boric acid, however, gene expression was suddenly down regulated at IC50 concentration well below the control levels.

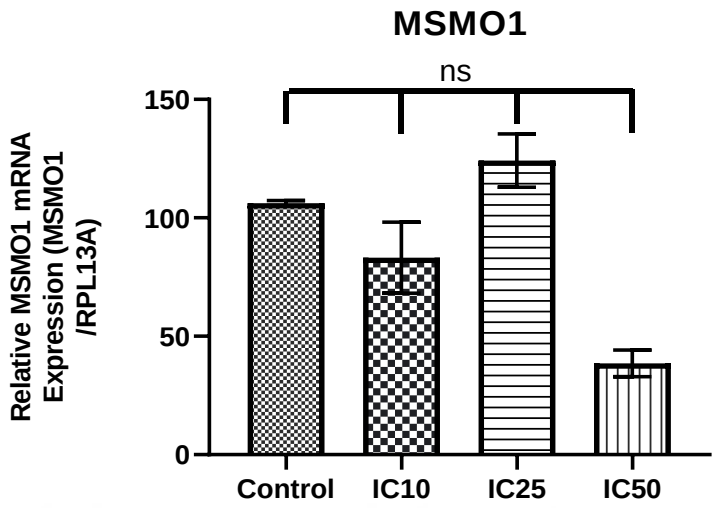


Figure 3.8 Gene expression of the MSMO1 in cells treated with three different concentrations of boric acid. Error bars indicate ± SD.

Although no significant change was observed in the MSMO1 gene, EBP, LSS, and SQLE gene expressions decreased significantly even at IC10 boric acid concentration. SQLE and LSS belong to the Cyclase family, which is nearly always called lyase. They catalyze the reactions to form a cyclic compound. In the cholesterol pathway, LSS converts (S)-2,3-oxidosqualene to lanosterol which is a cyclic molecule (Dean, 1967). SQLE catalyzes the conversion of squalene to 2,3(S)-oxidosqualene in steroid metabolism. SQLE inhibition is targeted for the treatment of hypercholesteremia, cancer, and fungal infections (Padyana et al., 2019). EBP is responsible for one of the last steps of cholesterol biosynthesis, and it converts 8(9)-cholestenol to lathosterol. MSMO1 is mainly localized on endoplasmic reticulum membrane, and it is considered to be responsible for the three-steps in steroid pathway, and its final product is 4,4-dimethyl and 4 alpha-methylsterols.

Among these selected genes, after several trials with different conditions, any threshold cycle (Ct) values couldn’t be obtained for SC5D and FDFT1 throughout 45 cycles. Therefore, their expressions could not be analyzed accurately.

The effect of each enzyme on the pathway can be studied at different levels mainly at the level of gene expression, at the level of protein expression, and through checking post-translational mechanisms mainly by looking at the enzyme activities. In order to confirm the gene expression results, examining the protein expression at different boric acid concentrations will be extremely important in terms of understanding the molecular action mechanisms and the possible use of boric acid on the steroid pathway as an inhibitor.

3.3 Western Blot Analysis

3.3.1 The Effect of Boric Acid on Protein Expression of Lanosterol Synthase (LSS)

The effect of different boric acid concentrations on lanosterol synthase protein expression and the relative protein analysis result is given in Figure 3.9., and the results of Western Blot analysis are given in Figure 3.11. GAPDH was used as the internal reference. The treatment of HepG2 cells with boric acid significantly changed (**$p \leq 0.01$) the relative protein expressions especially at IC10 concentration whereas in IC25, and IC50 treated cells, no significant change was observed compared to control.

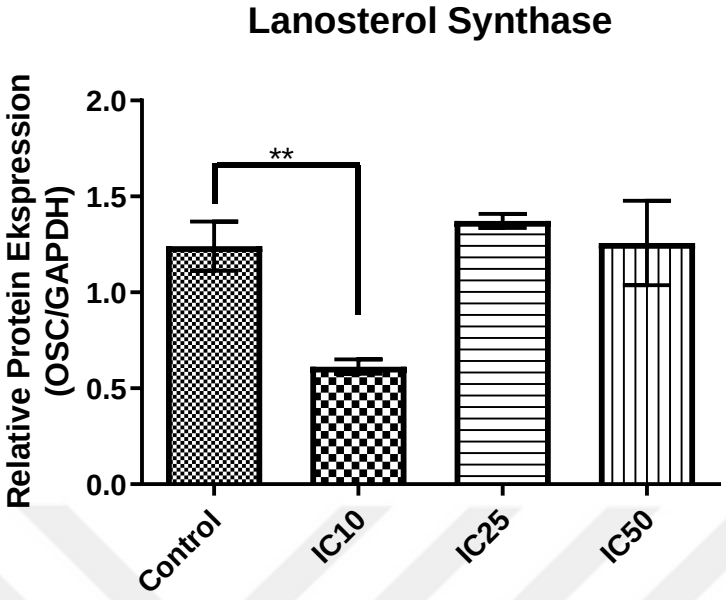


Figure 3.9 The relative protein expressions of lanosterol synthase in control, and boric acid treated HepG2 cells. This data represents three technical replicates. Error bars indicate ± SD. ** $p \leq 0.01$.

3.3.2 The Effect of Boric Acid on Protein Expression of Squalene Epoxidase Synthase (SQLE)

The effect of boric acid on protein expression of squalene epoxidase, the relative protein analysis result is given in Figure 3.10., and the results of Western Blot analysis are given in Figure 3.11. GAPDH was used as an internal reference. As seen from the figure, the treatment of boric acid significantly decreased the relative protein expression in all treated cell groups (*** $p \leq 0{,}001$).

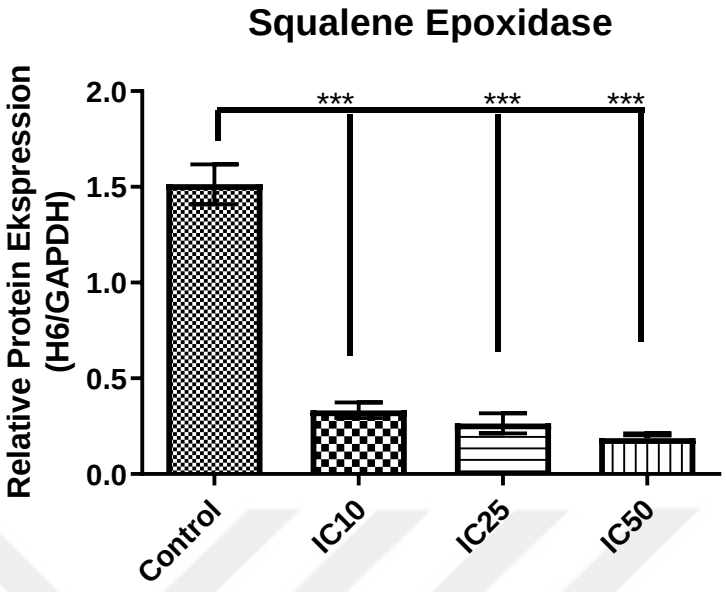


Figure 3.10 The relative protein expressions of squalene epoxidase in control, and boric acid treated HepG2 cells. This data represents three technical replicates. Error bars indicate ± SD. *** $p \leq 0{,}001$

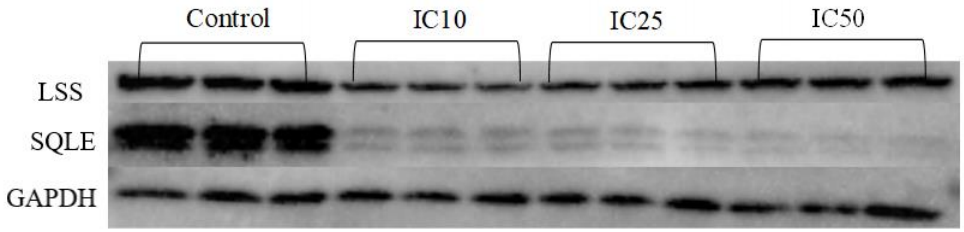


Figure 3.11 Each well contains 20 μg protein. The immunoblot represents the three proteins with molecular weight of 83 kDa for LSS, 55 kDa for SQLE and 37 kDa for GAPDH.

CHAPTER 4

DISCUSSION

Boron, which is part of daily nutrition, has been accepted as an essential chemical element for humans. After consumption, borax and boron-based chemicals are generally reduced to boric acid in the body (Murray, 1998). In living organisms, it is also known that, boric acid forms strong and reversible bonds with biomolecules containing cis-diol groups, and boric acid can interact chemically with molecules containing cis-diols in its structure such as RNA, ATP, NAD, and FAD. Besides, boric acid is known to inhibit serine-proteases by binding to their active sites (Hunt, 1998). Although the biochemical importance and physiological effects of boron and boric acid have been reported in various studies, the number of studies investigating the molecular mechanisms is limited (Donoiu et al., 2018; Hunter, 2009).

As evidenced by Paolicchi (1997), boric acid has various physiological effects on the liver usually by reducing lipid peroxidation and oxidative stress. In this study, he showed that the boric acid-serine complex, which is known as a gamma-glutamyl transferase inhibitor, also suppressed lipid peroxidation in HepG2 cells. Boric acid inhibited enzyme activity indicating that oxidative stress may be an important mechanism at the molecular level and can further have more large-scale effects.

Boric acid is excreted from the human body with a half-life of approximately 21 hours when taken orally or through intravenous injection. Since breaking the B-O bond in its molecular structure requires very high energy, boric acid is removed from the body by being filtered through the kidneys without undergoing any metabolic changes (Murray, 1998).

An increasing number of studies have shown that boron is essential for the completion of the life cycle of some animals and also plays a key role in the replication and development of animal cells. It has also been accepted as an essential

element for animals (Donoiu et al., 2018; Turkez et al., 2012). In addition to these results, it has been shown that, low boron intake may have a negative effect on bone health, brain functions and immune response (Wei, 2016).

As boron is found in significant amounts in plants, soil, and drinking water, living things are exposed to different concentrations of boron in different ways (Murray, 1998; Richold, 1998).

Today, boric acid salts are used in many different areas like industrial and agricultural products, and more recently as health products (BOREN 2017). Examples of these products are laundry detergents, some medicines, cleaning materials, and agricultural fertilizers (Hadrup et al., 2021). During World War I, and before, boric acid and borax were used as food preservatives. Especially during the first World War borax was used in order to preserve and longtime storage of food (Richold, 1998).

In terms of toxicity, it is important to determine the routes of exposure because the level of toxicity is directly related to the route and the level of exposure (Hadrup et al., 2021).

As mentioned before in literature there are limited studies about boron and borax and their health effects. In a study with Borax, it was shown that there was a time- and dose-dependent decrease in the proliferation of HepG2 cells due to the known anti-proliferative effects of borax. Following this finding, the mechanisms underlying this decrease were investigated. Although the inhibitory effect of borax on proliferation was shown, the mechanisms that cause this effect have not been fully explained. By monitoring the changes in mitochondrial membrane potential, it was found that this apoptotic process induced by borax is related with the up-regulation of p53 and Bax genes and the down-regulation of the Bcl-2 gene (Wei, 2016).

Previously our research lab studied the molecular mechanisms underlying the toxicity of boric acid at IC50 concentration in HepG2 cells. After treating the HepG2 cell lines with IC50 concentration of boric acid for 24 hours, whole mRNAs were isolated and microarray experiments were carried out. Among the other results

showing the effect of boric acid on cell cycle, the results have also shown that boric acid down regulated the steroid metabolic pathway (Tombuloglu, 2020). So, in this study we further investigated the effects of boric acid on the steroid pathway in order to understand our previous microarray results. For this purpose, we have selected 7 genes and these genes are: DHCR7 (7-dehydrocholesterol Reductase), EBP (Emopamil Binding Protein (sterol isomerase)), FDFT1 (Farnesyl-diphosphate Farnesyltransferase 1), LSS (Lanosterol Synthase (2,3-oxidosqualene-lanosterol cyclase)), MSMO1 (Methylsterol) Monooxygenase 1), SC5D (Sterol-C5-desaturase) and SQLE (Squalene Epoxidase). In this study, the gene expressions of these 7 genes were measured by qRT-PCR and in addition, we performed Western Blot Analysis in order to determine the protein levels of SQLE (Squalene Epoxidase) and LSS (Lanosterol Synthase (2,3-oxidosqualene-lanosterol cyclase)) genes. At the beginning of the study we have planned to carry out the protein analysis of 7-dehydrocholesterol reductase which is the regulatory enzyme of this pathway but because of pandemic conditions we could not afford to buy its antibody. But to our knowledge, this is the first study in literature related to a relationship between steroid metabolism and boric acid in human.

In this study, the dose-related effect of boric acid on gene expressions of steroid metabolism is investigated. Among the selected genes, after several trials with different conditions, the primers of SC5D and FDFT1 did not give any significant gene expression results (due to primer dimerization, etc) but the conditions of gene expressions were established for the other 5 genes. According to our qRT-PCR results, though there is no significant change in the MSMO1 gene, EBP, LSS, and SQLE gene expressions decreased significantly ($p \leq 0{,}0001$ for SQLE; $p \leq 0{,}001$ for EBP; $p \leq 0{,}01$ for LSS) even at IC10 boric acid concentration. On the other hand, the expression of DHCR-7 increased statistically significantly ($p \leq 0.001$) contrary to our expectations. The gene expression of DHCR7, which is the last enzyme in the path and also the most important regulatory enzyme, was upregulated with boric acid and the results are repeated several times and they are statistically significant. This increase may have been the result of the decrease in the gene expressions of the other

important enzymes in the pathway, in order to compensate the decrease in the rate observed in the entire pathway. Based on previous microarray results, the entire pathway was downregulated. These results we obtained based on the expressions of some genes actually confirmed our previous findings (Tombuloglu et al., 2020). On the other hand, the upregulation observed in 7-dehydrocholesterol reductase gene expression is decreased with increasing the boric acid concentration further to IC50 concentration. Our previous microarray results were also obtained at IC50 concentrations. In order to confirm these results, examining the protein expression and enzyme activities of 7-dehydrocholesterol reductase enzyme at different boric acid concentrations will be extremely important in order to understand the molecular action mechanism of boric acid and using boric acid as a possible steroid pathway inhibitor. Also, there is a present ongoing microarray study in our lab which use IC10 and IC25 concentrations. The results of this study can further clarify the effect of boric acid on the steroid pathway at these concentrations.

According to the results obtained by Western Blot Analysis, the protein level of SQLE was significantly reduced in all boric acid-treated cell groups. For the LSS gene, treatment of HepG2 cells with boric acid significantly decreased their relative protein expression, especially at IC10 concentration, whereas no significant changes were observed in protein levels of cells treated with IC25 and IC50 compared to the control group in which were not treated SQLE and LSS act in succession in the metabolism of steroids, the end product of which is cholesterol. Although there was a significant decrease in the level of the SQLE, there was no significant change in LSS protein expression level in the treated groups other than IC10.

When the qRT-PCR results of the SQLE gene are compared with the Western Blot Analysis results, it is seen that there is a consistency between them, as we expected. There is a significant decrease in both gene expression and protein levels. However, when mRNA expression and protein level were compared in terms of LSS, we encountered opposite results than we expected. When we look at the mRNA expression, although there is a significant decrease in all three treated groups, we see a decrease in the IC25 group meaning that the decrease in the groups with IC10 and

IC50 is close to each other. When the protein levels are considered, no significant change was observed in both IC25 and IC50 groups compared to the control group, while the cells treated with IC10 concentration showed a decrease which is statistically significant. This may be due a regulation at the level of translation.

We have to carry out further studies first related with the gene expression of the DHCR7 in which contrary to our expectations, a statistically significant increase was observed. Future studies are required especially for DHCR7 protein expressions as well. Further examining the results of IC10 and IC25 boric acid treated HepG2 cell microarray analysis together with the transcription factors hopefully will give better understanding of our gene expression results.

CHAPTER 5

CONCLUSION

In this study, for the first time, the effect of boric acid on steroid metabolism was investigated using HepG2 cell line.

The results of our study have shown that boric acid changed the mRNA expressions of DHCR7, LSS, EBP, and SQLE genes. mRNA level of DHCR7 was increased whereas mRNA expressions of LSS, EBP, and SQLE were decreased. In terms of MSMO1, there was no statistically significant change. The treatment of boric acid also changed the protein expressions of both Lanosterol Synthase and Squalene Epoxidase. The protein expression of squalene epoxidase was significantly decreased with boric acid treatment whereas lanosterol synthase protein expression was decreased less with the treatment of IC10 boric acid concentration.

We expected 7-dehydrocholesterol reductase mRNA expression to be downregulated like the other genes. However, the expression increased first with boric acid and decreased at IC50 concentration. This increase can be the result of a compensation in order to prevent the decrease in the rate of whole pathway.

In order to understand these mechanisms better, further experiments are necessary. Unfortunately, we were not able to look at the 7-dehydrocholesterol reductase protein expression as the antibody prices increased drastically above our project budget because of Covid-19 pandemic. Microarray results at IC 10 and IC 25 were obtained in another study performed in our lab. The analysis of the results is still going on. Together with 7-dehydrocholesterol reductase western blot analysis results, they will give more information about the effect of the boric acid on steroid metabolism.

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APPENDICES

A. Real-Time PCR Results For EBP and LSS

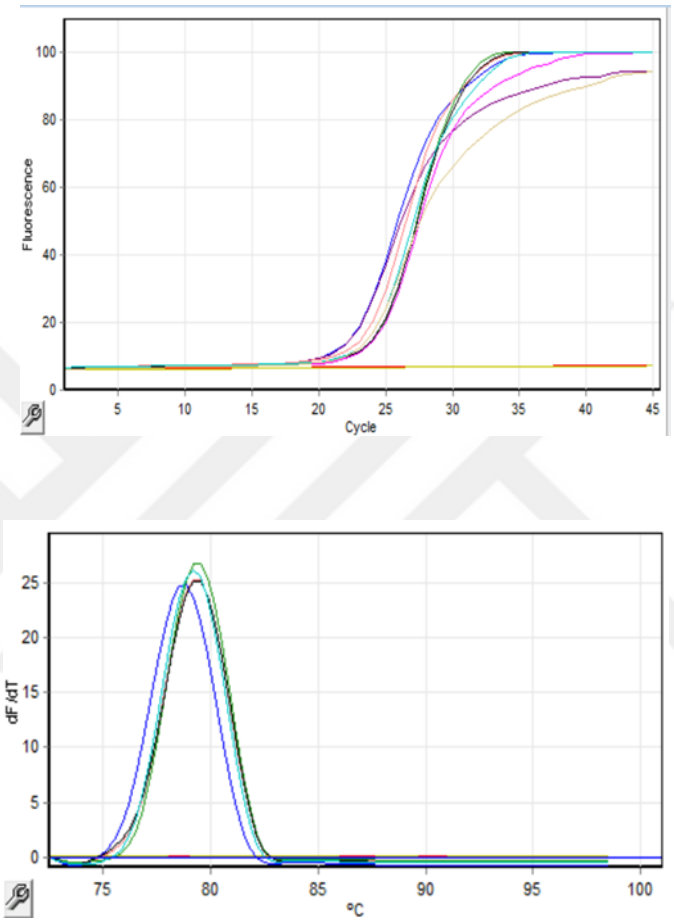


Figure A.1 Amplification and Melt Curves of EBP

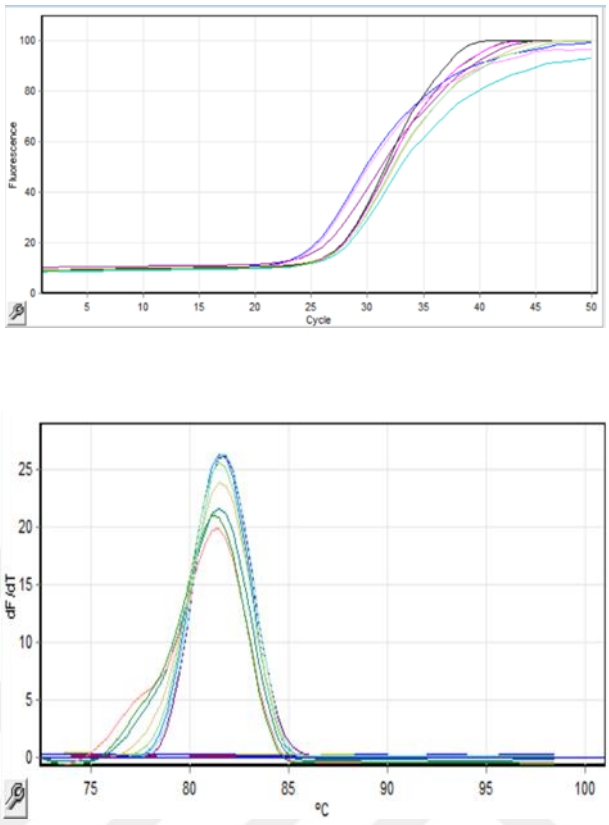


Figure A.2 Amplification and Melt Curves of LSS

B. Solutions and Separating and Stacking Gel Compounds for SDS-PAGE Electrophoresis

Table B.1 Solutions for SDS-PAGE Electrophoresis

Reagents	**Components**
Gel-Solution (Stock)	Acrylamide and N'-N'-Bis-Methylene Acrylamide Dissolving 14.6 g Acrylamide and 0.4 g N'-N'-Bis-Methylene Acrylamide. Total volume is completed to 50 mL with dH_2O.
Separating Buffer (Stock)	1.5 M Tris-HCl, pH: 8.8 Dissolving 18.15 g Tris-base in 50 mL dH_2O. For pH 8.8, it is titrated with 10 M HCl. Total volume is completed to 100 mL with dH_2O.
Stacking Buffer (Stock)	0.5 M Tris HCl, pH: 6.8 Dissolving 6 g Tris-base with dH_2O. For pH 6.8, it is titrated with 10 M HCl. Total volume is completed to 100 mL with dH_2O.
SDS 10% Stock	Dissolving 1 g SDS in dH_2O. Total volume is completed to 10 ml with dH_2O.
APS **(**Ammonium per Sulfate) (Fresh-10%)	For 2 gels, dissolving 25 mg in 250 µL dH_2O.
TEMED	
Sample Dilution Buffer 4X (Stock)	2.5 ml of 1 M pH 6.8 Tris-HCl buffer, and 4 ml Glycerol are prepared. Into this solution, 4 ml glycerol, 0.8 g SDS, 2 mL B-mercaptoethanol, 0.001 g Bromophenol Blue are added. Total volume is completed to 10 ml with dH_2O. After a while, 1 mL mercaptoethanol is added.
Electrophoresis Running Buffer	0.25 M Tris, 1.92 M Glycine (10X Stock is diluted to 1X before using with adding 0.1% SDS. Dissolving 15 g Tris-base in 350 mL dH_2O and adding 72 g glycine. Total volume is completed to 500 ml with dH_2O.

Table B.2 Separating and Stacking Gel Compounds (Required to 2 Gels)

Compounds	**Separating Gel 10%**	**Stacking Gel 4%**
Gel Solution	5 ml	650 µL
dH_2O	6,02 ml	3,05 ml
Separating Buffer	3700 ml	----
Stacking Buffer	----	1,250 ml
10% SDS	150 µL	50 µL
10% APS	75 µL	25 µL
TEMED	15 µL	10 µL
Total Volume	15 ml	5 ml