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YEDITEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHYSIOLOGY

INVESTIGATION OF THE EFFECT OF HEXAGONAL BORON NITRIDES (hBNs) ON MOUSE HIPPOCAMPAL CELL LINE

MASTER OF SCIENCE THESIS

İREM ÇULHA

SUPERVISOR

Prof. Dr. Bayram YILMAZ

İSTANBUL, 2018

THESIS APPROVAL FORM

Institute : Yeditepe University Institute of Health Sciences

Programme : Physiology Master's Programme

Title of the Thesis : Investigation of the effect of hexagonal boron nitrides on mouse hippocampal cell line

Owner of the Thesis : İrem Çulha

Examination Date : June 21, 2018

This study have been approved as a Master Thesis in regard to content and quality by the Jury.

	Title, Name-Surname (Institution)	(Signature)
Chair of the Jury:	Prof. Dr. Bayram Yılmaz	
Supervisor:	Prof. Dr. Bayram Yılmaz	
Member/Examiner:	Prof. Dr. Ertuğrul Kılıç	
Member/Examiner:	Associate Prof. Ali Özhan Aytekin	

APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..22.06.18..... and numbered .2018/11-09

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

21.06.2018

İrem Çulha

ACKNOWLEDGEMENTS

There are a number of individuals who I would like to acknowledge and thank for their contributions on for the completion of this thesis. I am thankful for my supervisor Prof. Dr. Bayram Yılmaz who has given the opportunity to complete my work in Physiology Department and his patient guidance. Beside my advisor, I would like to thank the rest of my thesis committee; Prof. Dr. Ertuğrul Kılıç from Medipol University and Assoc. Prof. Ali Özhan Aytekin for their insightful comments and encouragement, but also for the contributions which lead me to widen my research from various perspectives. My sincere thanks also goes to Prof. Dr. Işıl Kurnaz form Gebze Technical University for kindly sharing the cell line used in this thesis.

With all the support they gave me and how they patiently guided me, I give my sincere thanks to Melis Emanet and Özlem Şen, who helped me with the experiments and also performed the synthesis of the nanomaterials used (hBNs) for this thesis. I extend gratitude to my parents and my fiance for their love and support during my journey.

Finally, I would especially like to thank my father, Prof. Dr. Mustafa Çulha for not only supporting me as a father, but for being the best co-supervisor and pushing me to the limits I never even knew I had.

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LIST OF SYMBOLS AND ABBREVIATIONS

AD	Alzeihmer's disease
BA	Boric Acid
BBB	Blood brain barrier
BN	Boron Nitride
BNNT	Boron Nitride Nanotubes
CNS	Central Nervous System
CuO	Copper (II) oxide
CuO NPs	Copper (II) oxide nanoparticles
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DOX	Doxorubicin
ETP	Etoposide
FBS	Fetal Bovine Serum
GO	Graphene Oxide
hBNs	Hexagonal boron nitrides
MWCNTs	Multi-walled Carbon Nanotubes
N2A	Neuro2a
NP	Nanoparticles
PD	Parkinson's disease
PI	Propodium Iodide
PSA	Penicillin Streptomycin Ampicillin
ROS	Reactive Oxygen Species
SD	Standard Deviation
SiNPs	Silica Nanoparticles

SPI	Spinal Cord Injury
TiO_2	Titanium Dioxide

ABSTRACT

Culha, I. (2018). Investigation of the effect of hexagonal boron nitrides (hBNs) on Mouse hippocampal cell line. Yeditepe University, Institute of Health Sciences, Department of Physiology, Msc Thesis, Istanbul.

It is a challenge to treat a neurological disease and brain cancer not only due to poor understanding of their molecular basis but also delivery of therapeutic drugs. In this thesis, it is aimed to understand the potential of hBNs as a therapetic agent to relieve cellular stress. First, the cytotoxicity of hBNs and their possible degradation product, boric acid (BA), on Embryonic Mouse Hippocampal Cell Line (mHippoE-14) was assessed in the concentration range of 10-1000 μg/mL for both materials for 24 h and 72 h exposure. hBNs and BA showed concentration and time dependent cytotoxicity while hBNs were showing less cytotoxic behavior. Further; cell death mechanism, reactive oxygen species (ROS) generation, cell cycle, and apoptotic body formation were investigated using hBNs and BA concentrations with 4.4, 22, and 44 μg/mL boron content. hBNs drove the cells to apoptosis with the lowest concentration at 24 h exposure and with the increased exposure time the higher concentrations caused apoptosis as well. For BA, an increase in necrosis was observed at lowest two concentrations with 72 h of exposure. BA drove cells to apoptosis starting from the lowest concentration with both exposure times. Both hBNs and BA caused increased ROS production with the increased concentration and exposure times but the increase was more significant with BA exposure. 24 h exposure of hBNs and BA had no significant effect on cell cycle while 72 h exposure caused significant changes on cell cycle, especially on G0-G1 phase. Apoptotic bodies were not observed. The study was further expanded by exposing the cells to doxorubicin (DOX) to cause a stress on cells, and then were treated with hBNs and BA concentrations containing 4.4, 22 and 44 μg/mL boron and were exposed for 24 h and 72 h. It was found that both hBNs and BA increased cell viability when exposed for 72 h. This study suggests that hBNs and BA have very low toxicity and could be used as therapeutic agents at low concentrations to decrease oxidative stress caused by various drugs used for treatment of neurological diseases and brain cancer. However, since hBNs slowly degrade in biological medium, they can be used as a controlled releasing agent as compared to ionic BA in addition to their nanocarrier feature.

Key words: neuron, cytotoxicity, hexagonal boron nitride, boric acid, doxorubicin

ÖZET

Culha, I. (2018). Hekzagonal bor nitrürlerin (hBNs) fare hipokampal hücre hattı üzerine etkilerinin araştırılması. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Fizyoloji Anabilim Dalı, Master Tezi, İstanbul.

Nörolojik rahatsızlıklarının tedavisi moleküler temellerinin anlaşılmasının güçlüğü ve tedavi edici ajanların hedef bölgeye taşınımından dolayı zordur. Tezin amacı hBN'nin terapatik ajan olarak hücreler üzerindeki stresi azaltmak için kullanılabilecek potansiyeli olduğunu araştırmaktır. hBN ve bozunum ürünü olduğu düşünülen borik asidin (BA) embriyonik fare hipokampal hücre hattı (mHippoE-14) üzerine sitotoksisiteleri 10-1000 μg/mL derişim aralığında 24 s ve 72 s maruz bırakılarak araştırılmıştır. Deneyler sonucunda hBN ve BA'in zaman ve derişime bağlı olarak sitotoksisite yarattığı, hBN'lerin ise BA'e göre daha az sitotoksiteye neden olduğu saptanmıştır. Hücre ölümü mekanizması, reaktif oksijen türleri (ROS) oluşumu, hücre döngüsü analizi ve apoptotik cisim oluşumu araştırılması yapıştırmıştır, bu deneyler için 4.4, 22, ve 44 μg/mL bor içeren hBN ve BA derişimleri kullanılmıştır. 24 s sonunda düşük derişimli hBN'lerin artan maruz bırakılma süresi ve derişimlerde de apoptoza götürdüğü, BA'in ise 72 s maruz bırakılma sonrasında en düşük iki derişimde nekroza götürdüğü saptanmıştır. BA'in en düşük derişimden itibaren apoptoza götürdüğü görülmüştür. BA derişim miktarı ve maruz bırakılma süresinin artışıyla beraber ROS oluşumunu arttırdığı saptanmıştır. Hücre döngüsü üzerine 24 s maruziyetinin anlamlı bir etkisi olmadığı, 72 s maruziyetin ise hücre döngüsünde G0-G1 fazı üzerine etkisi daha fazla olduğu saptanmıştır. hBN ve BA'in 24 s ve 72 s maruziyetinin apoptotik cisim oluşumuna neden olmadığı görülmüştür. Hücereler üzerinde stres yaratmak amacıyla doksorubisine (DOX) maruz bırakılmıştır, daha sonrasında 4.4, 22, ve 44 μg/mL bor içeren hBN ve BA derişimleri ile 24 ve 72 s muamele edilmiştir ve sitotoksisitelerine bakılmıştır. hBN ve BA'in 72 s sonunda hücre canlılığını arttırdığı gözlemlenmiştir. Bu çalışma hBN ve BA'in düşük derişimlerde nörolojik hastalıklarda ve beyin kanserinde kullanılan ilaçların yarattığı oksidatif stresi azaltabilmek için tedavi amaçlı ajan olarak kullanılabileceği görülmüştür. hBN'nin biyolojik besiyerinde yavaş bozunuma uğraması nedeniyle, iyonik BA'e göre kontrollü salınım için daha uygun olabileceği saptanmıştır.

Anahtar kelimeler: sinir hücreleri, sitotoksisite, hekzagonal boron nitrür, borik asit, doksorubisin

1. INTRODUCTION and PURPOSE

The brains basic unit, the neuron, is the key part of the central nervous system (CNS). The anatomical structure of the neuron contains a cell body, an axon which is the cell bodies longer extension and dendrites which transfers signals from the apex of the neuron toward the cell body, axon terminal and then finally to other neurons or effector organs. During signal transmission, if the nerve is damaged then this interruption leads to problems in signaling and these functional disturbances lead to neurological disorders (1). Neurological disorders can also be caused by infection, trauma, degenerative disorders or cancer. Disease progression prevention can not be adequate enough in most circumstances in current neurological disease treatments due to the difficulties of transporting therapeutic drugs or agents used for imaging across the blood-brain barrier (BBB) into the nervous system and accomplishing targeted delivery to proper regions of the brain (2). To overcome these difficulties for effective treatment, novel carriers and tools are needed. The concept of nanomedicine has been applied widely to clarify these various challenges in medicine, which include being used to overcome barriers mentioned above and improving delivery of biologically active molecules to the nervous system (3). One of the limitations for drug delivery improvement is the risk of potential secondary toxicities, which has caused extensive research in therapeutic agent and biomarker discovery for treatment and bioimaging. If the research is successful, nervous system disorders could be discovered and treated with more efficient and a variety of options for practical use in the medical field (4).

The most common type of neurological disorders are neurodegenerative disorders that include Alzeihmer's disease (AD) and Parkison's disease (PD) which show prevalence among other types of neurodegenerative disorders (5). Mitochondria dysfunction, oxydative stress level increase, protein aggregation, excitotoxicity, iron accumulation and inflammatory changes are some of the common mechanisms of neurodegeneration (6). Another pathology seen in the brain is tumors in specific regions. The most common and aggressive primary brain tumor in humans, glioblastoma multiforme (GBM) has no other treatment options other than chemotherapy, surgical removal and radiotherapy (7). The biggest obstacle of achieving optimal recovery from cancer drug treatments is the blood-

BBB which restrains small-molecule drugs that are trying to access the brain tumors (8). To improve GBM treatment, it is important to deliver anti-cancer drugs to the tumor, and to discover different delivery methods in which drugs can penetrate the BBB to access the tumor as well (9). Another challenge is that as the drug enters the cancerous or diseased site, it also causes damage to non-cancer cells and has severe side effects on different organs as well. Drugs used for cancer, such as doxorubicin (DOX) cause multi-organ injury and this is partially because of the oxidative damage it causes (10, 11).

The hippocampus is the region of the human brain lying deep in the medial temporal lobes which has a major role in memory and learning (12). It forms posterior part of the limbic system and is concerned with also hunger, mood, pleasure and apetites. The hippocampus and its adjacent temporal and parietal lobe stuctures are called the hipocampal formation, which have several and indirect connections with portions of the cerebral cortex (13). The hippocampus is the structure that is affected the earliest and the most severely in several neurological disorders such as AD and epilepsy (14). Hippocampus is also one of the unique regions in brain where the neurogenesis continues even in adult life (15). This region also has an important role in emotional behaviour (16), regulation of hypothalamic functions (17) and spatial navigation (18).

Nanotechnology refers to the study of physicochemical properties of materials in the size of 1 to 100 nm and the construction of novel tools and approaches from nanomaterials for their use in a wide range of applications including in medicine (19). There are different types of nanomaterials that vary depending on the purpose of usage. The main reason use of nanomaterials in medicine are for delivery, targeting and controlled release. For optimal use of nanomaterials, they should meet certain requirements which include biocompatibility, stability and low toxicity via itself and degradation products. These properties are largely related to the nanomaterials chemical structure. Thus, the structure of a nanocarrier contains a material or polymeric structure that has low toxicity. Amoung many nanomaterials boron-based ones, namely boron nitride nanotubes (BNNTs) and hexagonal boron nitrides (hBNs) are recently gained attention due to their low toxicity

and high stability as nanocarriers and positive effect on cell viability possibly through their degradation products (20).

Although these novel nanomaterials are attempted to be used as nanocarriers, there is not much known about their affect through their degradation products. In a recent report, it is claimed that hBNs can suppress the growth of prostatate cancer in mouse. Although the nature of the degradation products is not known, it is proposed that it could be boric acid (BA) (21). In another study performed with cancerous HeLa cells and healthy HUVEC cells, BNNTs attached to DOX showed an increase in cancer cell death (22). Based on these prelimiminary reports, with their low toxicity and high biocompatibility in addition to their nanocarrier potential, their positive effect on cell viability shoud be investigated.

DOX has highly potent cytotoxicity to induce Human Proximal Tubule Cells HK-2 cells apoptosis by significantly changing caspase activities (23). A study has shown that madecassoside has protective effects against DOX-induced nephrotoxicity *in vivo* studies and and *in vitro* studies which Human Proximal Tubule Cells HK-2 and mice models were used (24).

Since hBNs are hydrophobic in nature, they may easily be able to pass the BBB and deliver their cargo. Once it makes its way into the brain by passing the BBB, it may not only deliver their cargo but also influence the healthy cells exposed to toxic drug molecules in a positive way releasing the stress generated by the drug molecules. In this thesis, the hBNs and thier possible degredation product, BA, were first evaluated for thier cytotoxicty using Embryonic Mouse Hippocampal Cell Line (mHippoE-14) using several molecular tests. Then, the influence of hBNs and BA on these healthy cells' viability were evaluated by exposing the cells to DOX, a cancer drug, to cause stress to the cells. For this, first the cells were exposed to a DOX concentration, which was high enough to cause a cellular stress and low enough to not kill all cells, then the cells were exposed to hBNs and BA. It was important to use a hippocampal cell line to see the effect of hBNs and BA for possible treatment developments for several disorders due to dysfunctions in the hippocampus.

2. LITERATURE REVIEW

As many as 1.5 billion people worldwide were reported to suffer from CNS-related diseases (25). CNS diseases such as stroke, spinal cord injury (SPI), and brain tumors have devastating outcomes and have expensive treatments and therapy. CNS treatment is currently insufficient due to the BBB, which strictly regulates the entry of foreign substances including drugs used for treatment. In many instances the drug entry into the CNS is very limited, thus can not reach its optimal potential treatment effect. This has caused the development of multiple drug delivery strategies including the use of nanoparticles for enhancing the delivery (26).

The small size of the nanomaterails cause their properties to diverge from their significantly large form. In addition to their very large surface area and increased reactivity, their interaction with electromagnetic radiation, electrical and thermal conductivity, physical strength and colloidal behaviour changes as the size gets smaller. The idea of making novel tools and materials by modifying the size of bulk materials resulted in a massive research effort starting from late 1990s. As the idea evolved, it was realized that the molecular machinary at celllular level operates at the nanometer scale as well. Combining the biomachinary with the idea of nanotechnology resulted in the birth of nanobiotechnology, which aimed to interfere with the cellular machniary using nanomaterials and nanomachines for disease treatment, detection and diagnosis. Since nanobiotehnology is a broder definition, later the use of nanomaterials and nanotechnology concept in medicine is named as “nanomedicine” (27).

Figure 1 compares the sizes of an eukaryotic cell and its components to nanomaterials. The average size of an eukaryotic cell is given as 10 μm while size of a mitochondrion is about 1 μm. The diameter of a SWCNT ranges from 1 to 5 nm while a single stranded DNA is about 1.5 nm. For example, the size of hBNs used in this thesis is about 50 nm.

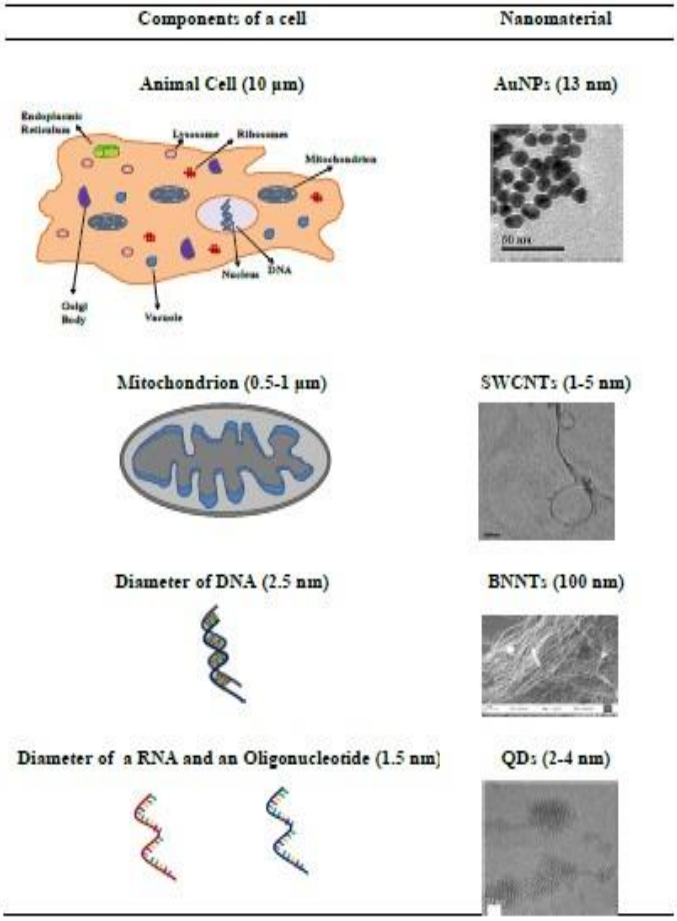

Figure 1. The size comparison of components of a cell and selected nanomaterials (28).

The advent of nanomaterials has also provided extraordinary opportunities for biomedical applications. Scientists have also studied the effects of nanomaterials used for targeted therapy, drug delivery and imaging techniques for developing treatments of CNS-related diseases. Nanomaterials have biologically stable properties, which make them optimal for targeted therapy (29). The main focus of nanomedicine is to discover novel nano-systems for biomedical applications and methods using numerus nanoparticles as mentioned above (30). As mentioned earlier, nanomedicine involves the use of nano-scale materials in medicine, which their properties differ from those of the same material with larger scaled size. The human body consists of many biological mechanisms that occur at the nano-scale, thus nanoparticles may potentially cross the natural barriers in the human body and enter different sites therefore interact with biomolecules in the blood or directly

with cells, tissues and organs. This easy entry may be an advantage for drug delivery and imaging techniques. However, this uncontrolled distribution of nanosized objects can cause serious problems. Thus, nanoparticles planned to utilize in nanomedicine studies, as with all medical devices or drugs, must go through toxicity assessment, broad characterization, and clinical trials before their potential is fully understood for the patients benefit (31).

There are numerous nanoparticles and nanostructures studied for drug delivery and targetting in the literature. Examples of these are gold (AuNPs) and silver nanoparticles (AgNPs) (32), magnetic iron oxide nanoparticles (Fe_2O_3) (33), carbon based nanomaterials (Graphene, SWCNTs, etc) (34, 35, 36) and polymeric nanoparticles (37). The AuNPs are widely investigated not only for delivery and targeting but also for photothermal threapy due to their plasmonic properties and low toxicity (38, 39). Fe_2O_3 nanoparticles are already used in MRI imaging as contrast agent due to thier low toxicity. Carbon based nanomaterials are also widely exploited due to thier low toxicity, electrical conductivity and physical strength. Among these mentioned nanomaterials, GO has attaracted great attention due to its physical properties and low toxicity (40). Since it is not possible to cover all nanomaterials used for delivery here due to its very broad applications in medicine and out of the scope of this thesis, only a few relevant examples will be mentioned.

The unique features of inorganic and polymeric nanomaterials are being widely applied in commercial products and the industrial field, though the potential adverse effects of nanomaterails in humans have not been completely clarified yet (41). In a study, the influence of multi-walled carbon nanotubes (MWCNTs) before and after functionalization, GO and hybrid coating deposited on a titanium surface on the nerve cell responses were investigated and this study showed that GO showed positive effect on cell viability, neurite outgrowth and neural cell morphology and significantly improved the properties of the other nanocomposite coatings (42). Two types of titanium dioxide (TiO_2) nanoparticles have been used for neurotoxic evaluation on human SH-SY5Y neuronal cells. The results of this study showed that both types of TiO_2 showed similar effects and did not reduce cell viability but were effectively internalized by the cells and induced alterations in the cell cycle which was dose-dependent apoptosis, and genotoxicity (43). In another study, the cellular uptake of silica nanoparticles (SiNPs) in

mouse neuro2a (N2a) and human SK-N-SH neuroblastoma cells were treated with 10.0 g/mL of 15-nm SiNPs for 24 h by transmission electron microscopy. It was found that SiNPs significantly decreased cell viability, caused cellular apoptosis, and caused an increase in levels of intracellular reactive oxygen species (ROS) in a dose-dependent way in both cell lines (41). A different study investigated the mechanism of CuO nanoparticle (CuONPs)-induced neurotoxicity and neuroprotective effect of crocetin on HT22 hippocampal cells. Exposure of CuONps in presence and absence of 5 μM crocetin both increased cell death after 24 h, enhanced apoptosis and decreased ROS generation (44).

hBNs were first discovered in 1842, yet their stable forms/phases were obtained after almost 100 years (45). Boron and nitrogen atoms of hBNs are connected through a honeycomb-shaped covalent bonding. The two-dimensinal (2D) layers of hBNs are stuck on each other through weak van der Waals interactions (46) and create hBN films (47) or spheric structures (48). In a recent study, it was shown that hBNs and could be used as a drug delivery agent by loading with doxorubicin and folate for cancer studies (22). Having outstanding physical and chemical characteristics, hBNs bioavailability and its cell based positive effect should be studied in more detail for their use in medicine. It was seen that hBNs were not toxic up to 100 μg/mL up to 48 h in biocompatibility assays of HEK-293T and CHO cells (49). In an other study it was shown that up to 40 μg/mL hBNs did not effect cell growth in IAR-6-1 neoplastic cells (20). These studies that were performed on the *in vitro* level have indicated that hBNs use in medical and biomedical fields are promising. A recent study showed that their degredation product could possibly be BA and it decreased cancer cell viability, this was shown by using it against prostate cancer cells (21). It is believed that these materials effect cells via their nano-size and degradation products, but there are no studies indicating that BA is the degradation product in biological environment.

The most commonly reported post treatment effect of cancer treatment is mild cognitive impairment due to neurotoxicity caused by cancer treatment is referred to as ‘’chemobrain’’ (50). Anti-neoplastic drugs such as cisplatin, doxorubicin, 5-fluorouracil and methotrexate are known to cause cognitive dysfunction during and after cancer treatment (51, 52). Research has shown that these anti-neoplastic drugs may cause damage on neuronal axons, microglia and oligodendrocytes (51, 53).

DOX is the main compound of the anthracycline antibiotic which is used for treatment of various cancers. The most serious side effect of this class of compounds is cardiotoxicity (54). DOX in the presence of molecular oxygen is a chemotherapeutic drug which causes reactive oxygen species (ROS) production (55). Properties of DOX cause DNA damage, interfere with transcription, mitotoxicity, formation of free radicals and lipid oxidation (56). As it is known that DOX crosses the BBB poorly, in adequate doses it can penetrate the brain and cause cytotoxic effects that lead to thinning of the brain cortex and reduce brain connectivity by causing abnormal activity and damage to synapses, and neurites in neurons (57). The oxidative stress caused by DOX is believed to be caused by ROS generated in the mitochondria. A recent study showed that oxidative stress caused by doxorubicin contributing to neurotoxicity is not only caused by ROS generation in the mitochondria but in the peroxisomes as well (56).

The aim of this study was to investigate the effect of hBNs and their believed degradation product (BA) on healthy hippocampal cells. First their cytotoxicity was assesed through a number of molecular tests and thier influence on cell viability of healthy cell exposed to a cancer drug, DOX, to generate oxidative stress, was investigated. The aim of this was to show whether boron containing hBNs and BA had any influence on cell viability by decreasing cellular stress.

3. MATERIALS and METHODS

3.1. Synthesis of hBNs

The synthesis of hBNs was reported somewhere else (58). Briefly, 2 g of boric acid were suspended in 3 mL of 4.1 M ammonia solution. This mixture was transferred onto a silicon carbide boat and dried on a hot plate adjusted to 100°C for approximately 20 min. Then, this silicon carbide boat was placed in a Protherm Furnace PTF 14/50/450 and heated until 1300°C with a heating rate of 10°C/min under ammonia gas flow for 2 h. Following the heating, the silicon carbide boat was removed from furnace at around 550°C and hBNs were scratched from the surface of the silicon carbide boat with the help of spatula and stored under room conditions.

3.2. Cell culture

Embryonic Mouse Hippocampal Cell Line mHippoE-14 was utilized to assess the cytotoxicity of hBNs and it is believed degradation product BA. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 1% penicillin streptomycin ampicillin (PSA) antibiotics and 10% Fetal Bovine Serum (FBS). The cells were incubated in water-jacketed incubator in a 5% CO_2, 95% air atmosphere at 37 °C.

3.3. WST-8 assay for assesing cell viability

Cells were seeded into 96-well plates at a density of $5x10^4$ cells/well and after 24 h incubation the concentrations 10, 20, 50, 100, 200, 300, 500, 750 and 1000 μg/mL of hBNs and BA containing culture mediums were added. After 24 h and 72 h of incubation the hBNs and BA containing mediums in 96-well plates, they were removed and washed three times in PBS therefore were replaced with fresh culture medium containing WST-8 reagent with 1:10 ratio and incubated for a further 1 h. The cell survivability upon exposure to varying concentrations of hBNs and BA, was calculated as the ratio of absorbance readings (450 nm) yielded by the treated and untreated (negative control) wells, after correction for blank absorbance reading of the reaction mixture incubated without cells at 37 °C for 1 h.

The same concentration range of 10, 20, 50, 100, 200, 300, 500, 750 and 1000 μg/mL was used for both hBNs and BA to assess the cytotoxicity range and to study their effects on the hippocampal cell line. After the cell viability assay, the boron concentrations of hBNs and BA were calculated to be equal.

3.4. Cell Death Mechanism Assessment

To determine the percentage of apoptotic cells, the Annexin-V and propidium iodide (PI) staining was carried out using an Annexin-V FITC apoptosis detection kit (Sigma, Germany). The cells were seeded in 24-well plates at a density of 25×10^3 cells/ well and were incubated for 24 h. 10% dimethyl sulfoxide (DMSO) was used as positive control for apoptosis, 0.025% Triton X-100 was used as positive control for necrosis and non-treated cells were used as negative control. After treating the cells with 4.4, 22 and 44 μg/mL boron including hBNs and BA for 24 h and 72 h, the detached and attached cells were collected and washed with PBS. Subsequently, the cells were re-suspended in solution containing 200 μl of 1× binding buffer, 0.5 μl of Annexin-V FITC and 0.1 μl of PI per sample, 200 μL Annexin V solution was added first and after incubation in the dark for 10 min at 37 °C 200 μL PI solution was added and then they were incubated in the dark for 15 min at 37 °C. After incubation, the BectonDickinson FACS Calibur flow cytometry system (BectonDickinson, USA) was used to quantify the number of viable, apoptotic and necrotic cells. The data were analyzed by FACS Comp software.

3.5. Reactive oxygen species (ROS) detection assay

The ROS detection test was applied to the hBNs and BA exposed mHippo E-14 cells. The cells were seeded in 24-well plates at a density of 25×10^3 cells/well and incubated for 24 h at 37 °C. The following day the media were removed, and the cells were treated with buffer containing 20 μM ROS detection reagent (2' ,7' dichlorofluorescindiacetate, DCFDA) and incubated for 45 min at 37 °C in the dark. After incubation, the ROS detection reagent was removed and the concentrations 4.4, 22 and 44 μg/mL boron including hBNs and BA containing media were added and incubated for 24 h and 72 h at 37 °C. The DCFDA is transformed to a non-fluorescent compound by deacetilation after diffusion into the cells. Finally, the cells were analyzed with a flow cytometer.

3.6. Cell cycle analysis

Approximately 25 x 10^3 cells/well were plated in 24-well plates and allowed to adhere to the bottom of the plates overnight. The cells were then treated with 4.4, 22 and 44 μg/mL boron including hBNs and BA dissolved in media for 24 h and 72 h. 600 μL of 10^{-5} M colchicine was used as positive control. After the treatment, cells were collected and fixed overnight using 70% ethanol and incubated at -20 °C. The following day they were pelleted and re-suspended in 0.5% BSA in PBS, 50 μg/mL RNAse in PBS and 0.1 mL PI from 1M stock for each sample. The samples were analyzed using flow cytometry.

3.7. Detection of apoptotic bodies using confocal microscopy

For 24 h analysis, 15 x 10^4 cells per well were seeded, and for 72 h analysis 5 x 10^4 cells per well were seeded in 6-well plates and were incubated for 24 h at 37°C. The following day the cells were treated with 4.4, 22 and 44 μg/mL boron including hBNs and BA for 24 h and 72 h. 10% DMSO was used as positive control and non-treated cells were used as negative control. After exposure to hBNs and BA, cells were fixed with 4% paraformaldehyde desolved in PBS for 20 min and were washed three times with PBS. Then the cells were treated with 1 mg/mL of sodium borohydride and incubated for 5 min in room temperature. After washing three times again with PBS, the cells were stained with DAPI dye 5 μg/mL per well, incubated in room temperature in dark then was washed again three times. Each coverslip was inverted onto a drop of ProlongTM antifade (Molecular Probes) placed on a glass slide and was held overnight in the dark before observation with a Nikon TE2000 microscope attached to a C1 confocal unit.

3.8. WST-8 assay for hBN-doxorubicin and BA-doxorubicin

Cells were seeded into 96-well plates at a density of 5x10^4 cells per well and after 24 h incubation 4 μM Doxorubicin was added to cause stress and was incubated for 4 h, then the 4.4, 22 and 44 μg/mL boron including hBNs and BA were added. After 24 h and 72 h of incubation the hBNs and BA containing medium in 96-well plates was washed three times in PBS and was replaced with fresh culture medium containing WST-8 reagent with 1:10 ratio and incubated for a further 1 h. The cell survivability upon exposure to varying

levels of oxidative stress and varying concentrations of hBN and BA, was calculated as the ratio of absorbance readings (450 nm) yielded by the treated and untreated (negative control) wells, after correction for blank absorbance reading of the reaction mixture incubated without cells for the same duration (1 h) at 37 °C.

3.9. Statistical Analysis

All data were expressed as mean ± standard deviation (SD) and to determine if each two sample datasets were different significantly when compared by Student's *t*-test. A value of $p<0.05$ were considered as statisticaly significant.

4. RESULTS

4.1. hBNs synthesis

The synthesized hBNs were characterized with spectroscopic and imaging techniques. Figure 2. A shows the schematic presentation of two layers of hBNs. As seen, boron and nitrogen atoms are connected through a hexagonal network. As mentioned earlier, layers of hBNs are held together through weak van der Vaals interactions and naturally several layers are stuck together to form thicker layers with round and flat shape as seen in the TEM image in Figure 2 B. The average size of hBNs is about 50 nm in diameter with an almost round shape. The FT-IR spectrum presented on Figure 2 C confirms the molecular structure of hBNs. Since hBNs are quite hydrophobic and thier dispersion in aqueous media requires longer sonication times, it is necesseary to demonstrate they are satisfactorily dispersed in water and cell culture medium. Figure 2.D shows DLS spectra of hBNs demonstrating size distribution in water and cell culture medium. The average size of hBNs in water and cell culture medium is found to be 78.82 nm and 190.10 nm, respectively. Note that the size of hBNs is larger due to solvation of hBNs by water molecules, whic is called hydrodynamic size. When hBNs are added into cell culture medium, thier size get larger since proteins and other small molecules are adsorbed onto hBNs. It is also possible that hBNs can adhere together forming larger aggregates in cell culture medium.

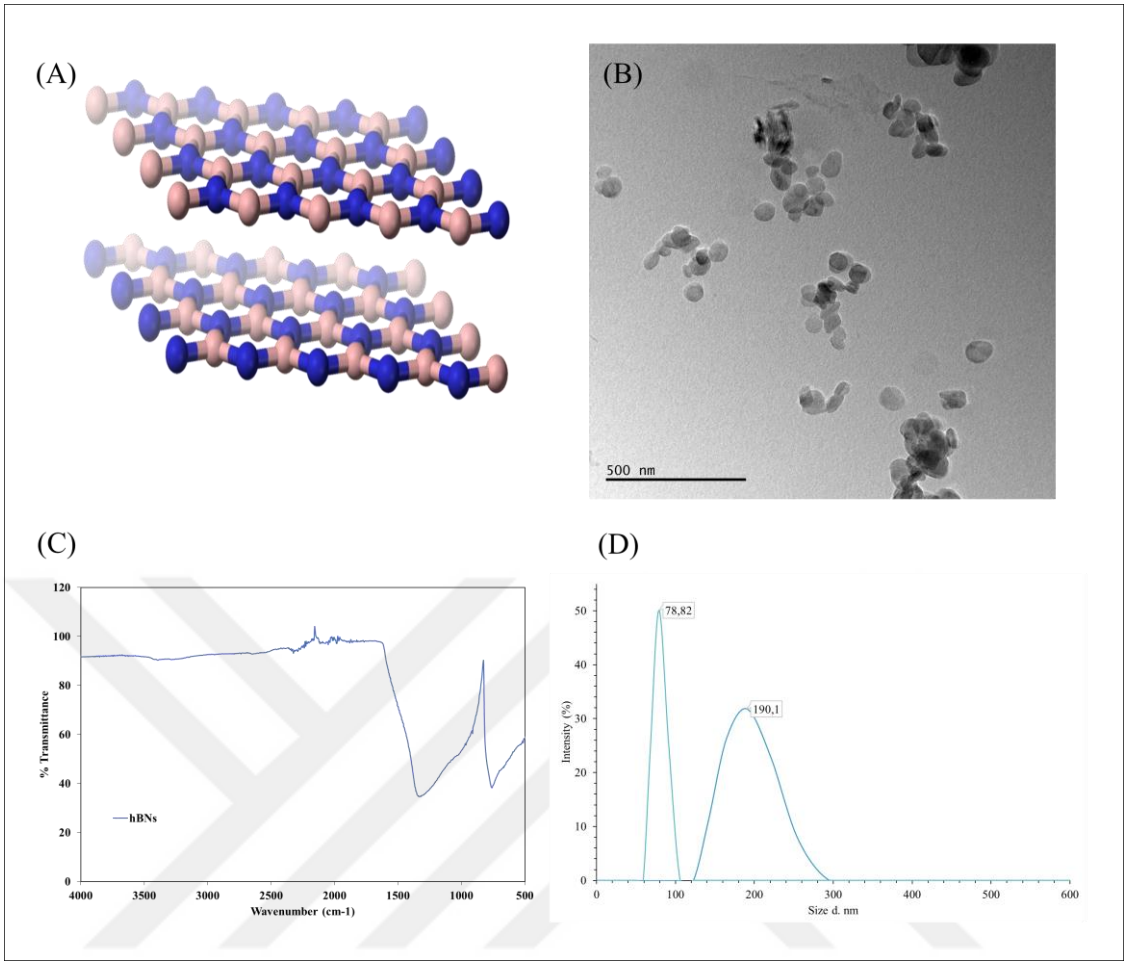

Figure 2. Schematic representation of hBN (A), TEM image (B), FT-IR spectrum (C) and DLS spectra (D) in water (78.82 nm) and cell culture media (190.10) (58).

4.2. Viability Tests/Cytotoxicity Studies for hBN and BA

In oder to evaluate the cytotoxic behavior of hBNs and BA, a concentration rage of 10-1000 µg/mL of both was used. Figure 3 shows the cell viability assessment with WST-8 assay after 24 h and 72 h of hBNs and BA exposure. Each concentration of both hBNs and BA is repeated four times. In order to realize possible errors, all concentrations of both hBNs and BA are repeated two times in separate weeks. The concentration range of 10-1000 µg/mL indicates that hBNs are less toxic than the concentration range of BA, for both 24 h and 72 h of exposure. As the concentration is increased, the viability of the cells increased between the concentrations 10-200 µg/mL, then started to decrease the viability

of cells on the first day (24 h). A dose and time dependent cytotoxicity of both hBNs and BA was observed. The two week average was calculated, and then analyzed using Student's *t*-test. The 24 h average of two experiments showed that only 20 μg/mL of hBNs showed significant increase in cell viability ($p<0.05$). Other concentrations of hBNs showed unsignificant decrease in cell viability. The experiments showed that all concentrations of BA caused an unsignificant decrease in cell viability.

For 72 h exposore, there was a significant increase in cell viability in concentrations of 10, 20, 50, 100 and 200 μg/mL of hBNS ($p<0.05$). An increase in cell viability is also seen in 300 μg/mL and decrease in 500 and 750 μg/mL but it is not statisticaly significant. The concentration of 1000 μg/mL has caused a significant decrease in cell viability. For BA, the statistical analysis have proved that 10 and 20 μg/mL have caused a significant increase in cell viability. It also showed that there was an unsignificant increase of viability at the concentration 50 μg/mL and a significant decrease in viability after 100 μg/mL. The SD of hBNs concentrations including 500, 750 and 1000 μg/mL are very high, the reason of the large standard deviation is because of the high concentration and because of the hBNs not being able to disperse smoothly/equally in DMEM due to their chemical features.

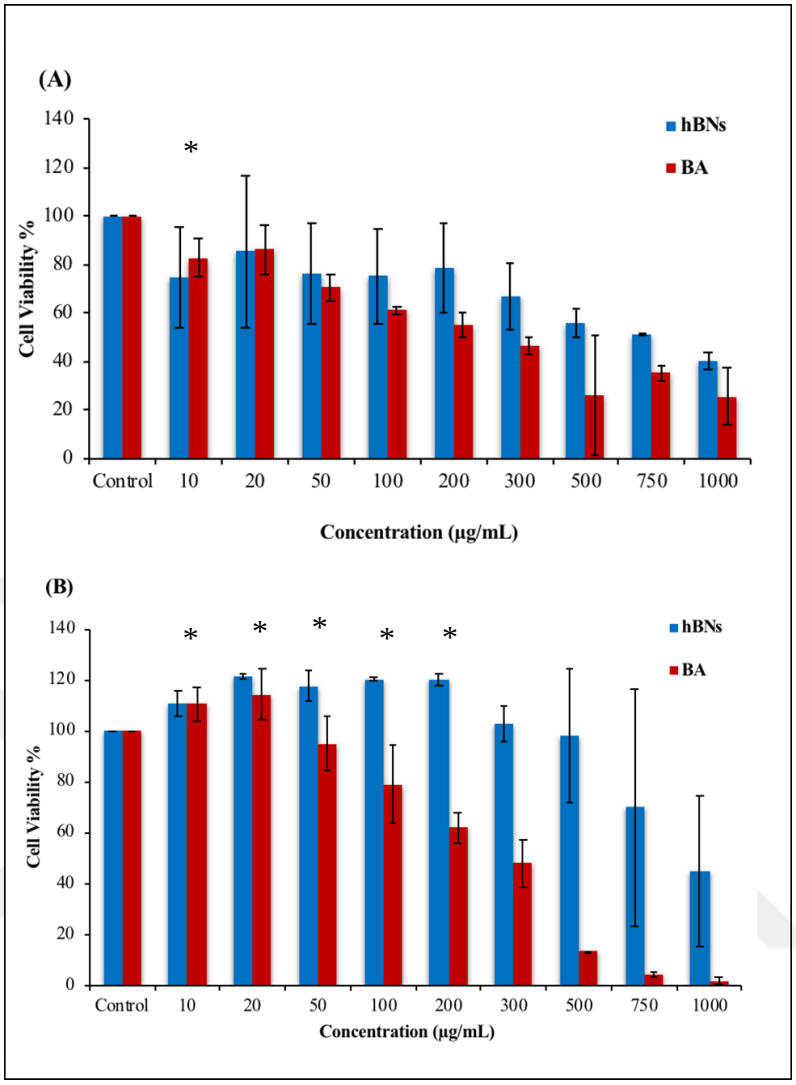

Figure 3. Cell viability after 24 h (A) and 72 h (B) of hHBs and BA exposure. The results are the average of two repeats. * Different from control $p<0.05$, statistically analysed by Student's *t*-test.

Although an increase cell viability with the exposure of both hBNs and BA was not observed with 24 h exposure, a clear increase in cell viability, especially with hBNs exposure, over 100% up to 200 µg/mL, was observed. This suggested that the concentration of the tested agents should have been lower than 200 µg/mL. Thus, three concentrations, 10 µg/mL. 50 and 100 µg/mL, were decided to use in further studies. However, instead of using the regaent concentrations, the boron concentrations in hBNs and BA were decided to use considering the fact that boron played a role in increased cell viability. Since hBNs concentrations decided to use were 10 µg/mL. 50 and 100 µg/mL

and the boron content in hBNs for these concentrations were calculated as 4.4, 22.0 and 44.0 μg/mL, the BA concentration was adjusted to this boron content by multiplying its concentration by 2.5, which is the ratio of boron conent of hBNs and BA. Further experiments were performed using the boron conents as concentrations.

4.3. Cell Death Mechanism

Figure 4 shows cell death mechanism assessment of mHippo cells with the exposure of hBNs (A, C) and BA (B, D) for 24 h and 72 h. At 24 h exposure, hBNs containing 4.4 and 22 μg/mL boron content did not have a significant decreasing effect on number of live cells. All concentrations of hBNs had no significant effect on necrosis and early apoptosis. While a significant increase in late apoptosis at 4.4 μg/mL was observed, no significant effect on late apoptotic cells with 22 and 44 μg/mL was observed. All BA concentrations exposure of 24 h showed no significant effect on live cell numbers and necrosis. With 22 μg/mL boron content of BA, a significant increase of early apoptotic cells was observed. With the 4.4 and 22 μg/mL concentrations, a significant increase in late apoptotic cells was seen.

With 72 h exposure, all hBNs concentrations including 4.4 μg/mL, 22 μg/mL and 44 μg/mL boron had no significant effect on necrosis. A significant increase in late apoptotic cells in all the concentrations of hBNs was seen. While a decrease of live cells with 4.4 μg/mL was observed, an increase with 22 μg/mL and again a decrease with 44 μg/mL was observed. On the other hand, a significant increase in early apoptosis with 44 μg/mL were seen. At the end of 72 h exposure, 4.4 μg/mL boron including BA caused a significant decrease in live cell numbers and significant increase in necrotic/necrosis cells. The decrease in necrosis in concentratrions 22 and 44 μg/mL are not significant. The decrease in early apoptotic cells in 4.4 μg/mL was significant while the decrease in 22 and 44 μg/mL boron content including BA was not significant.

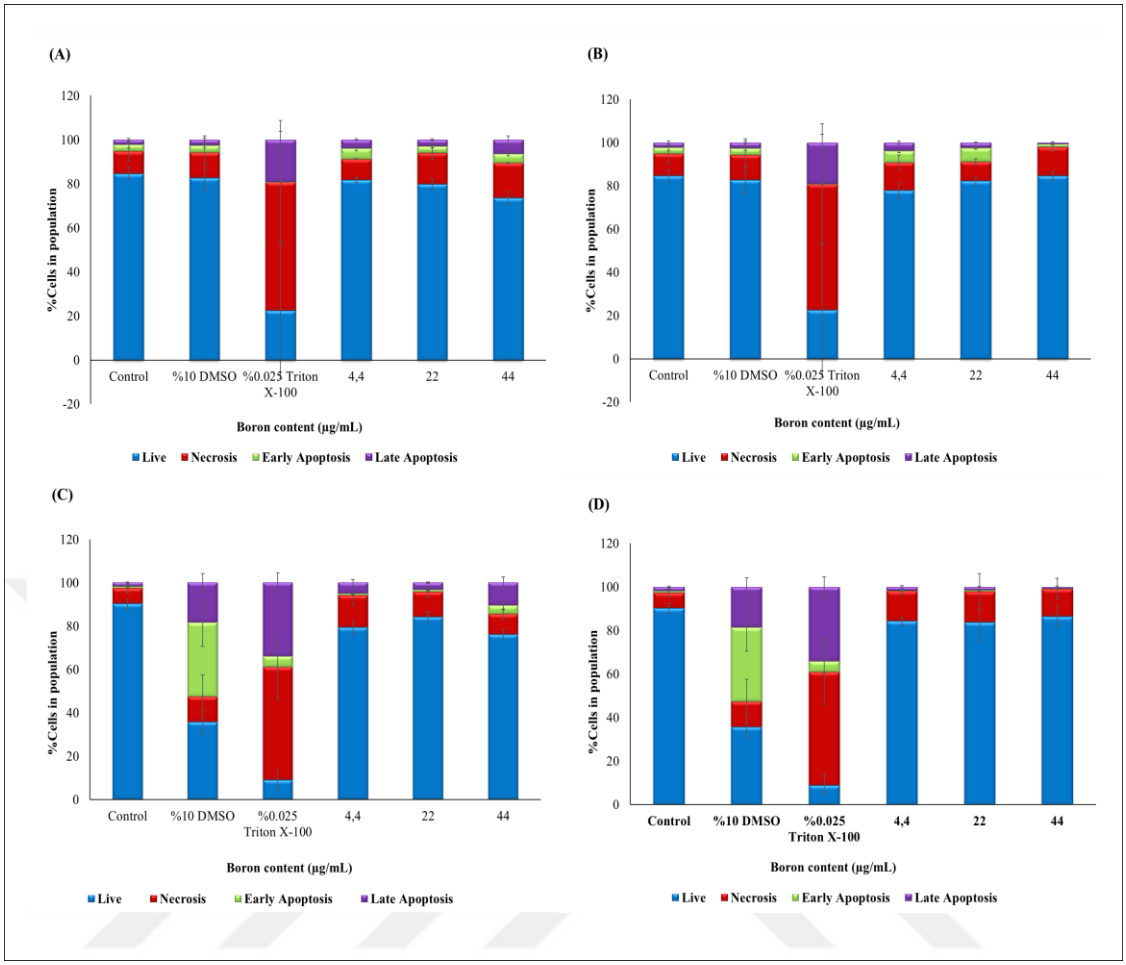

Figure 4. Cell death mechanism detection of hBNs (A) and BA (B) for 24 h, hBNs (C) and (BA) for 72 h treated mHippoE-14 cells.

4.4. ROS Analysis

24 h exposure of hBNs containing 44 µg/mL boron caused a significant increase in ROS production while no significant change in ROS production in all other concentrations was observed. The increase in ROS production in BA containing 4.4 µg/mL boron was not significant.

72 h exposure caused a significant increase with hBNs containing 44 µg/mL boron. For BA, a significant increase in ROS production with BA including 4.4 µg/mL boron was observed while the higher concentrations, 22 and 44, µg/mL caused lower ROS generation.

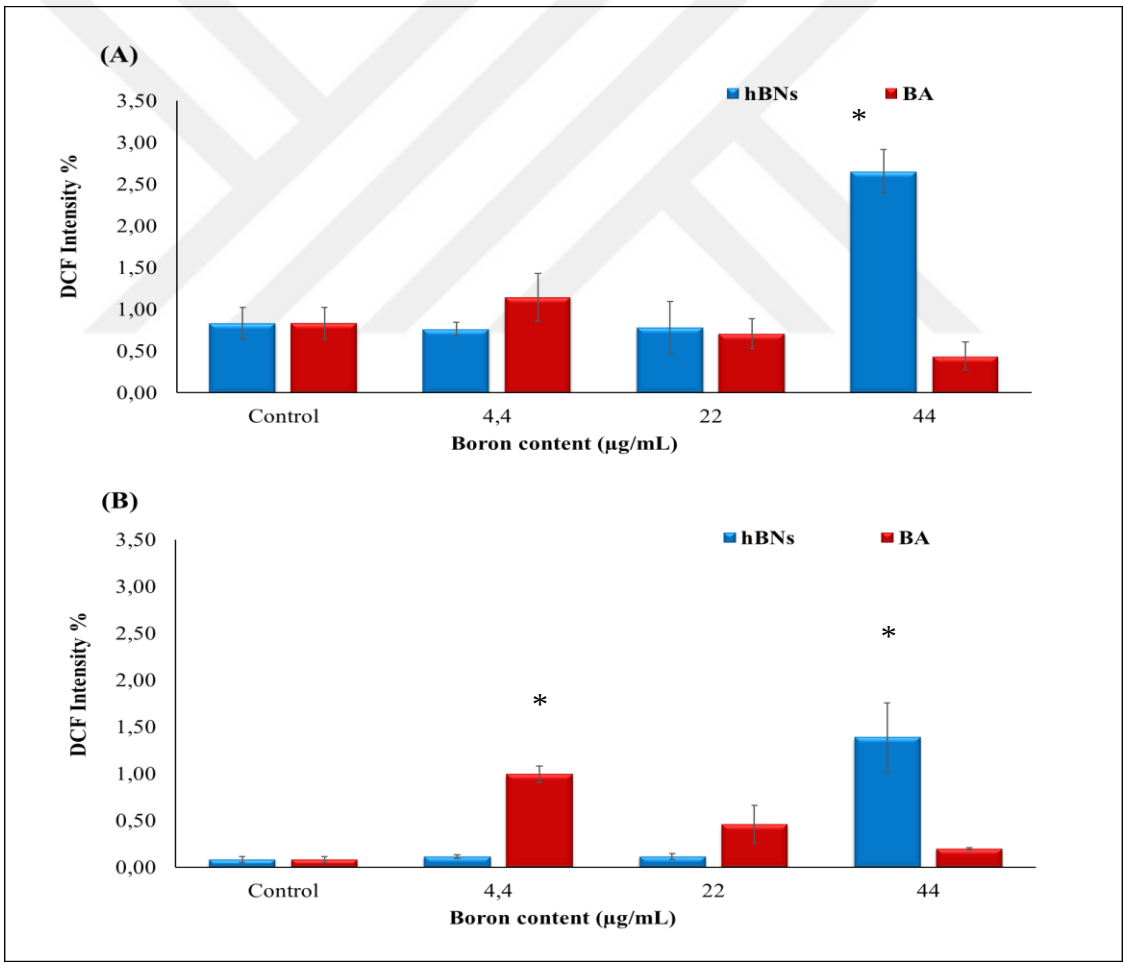

Figure 5. ROS analysis of (A) 24 h and (B) 72 h of hBNs and BA treated mHippoE-14 cells. * Different from control $p<0.05$, statistically analysed by Student's *t*-test.

4.5. Cell Cycle Analysis

There was no significant difference in cell cycle for any concentrations when exposed to hBNs and BA of exposure for 24h.

72h exposure of 22 µg/mL boron containing hBNs significantly increased G0-G1 phase, decreased the G2-M phase and had no significant effect on S phase. Other concentrations of hBNs had no significant effect on cell cycle. 4.4 and 22 µg/mL boron containing BA caused a significant increase at G0-G1 phase, and a unsignificant decrease in 44 µg/ml. A significant decrease was also seen in S phase of 22 and 44 µg/mL exposure. 44 µg/mL caused a significant increase in G2-M phase while the increases seen in 4.4 and 22 µg/mL were not significant.

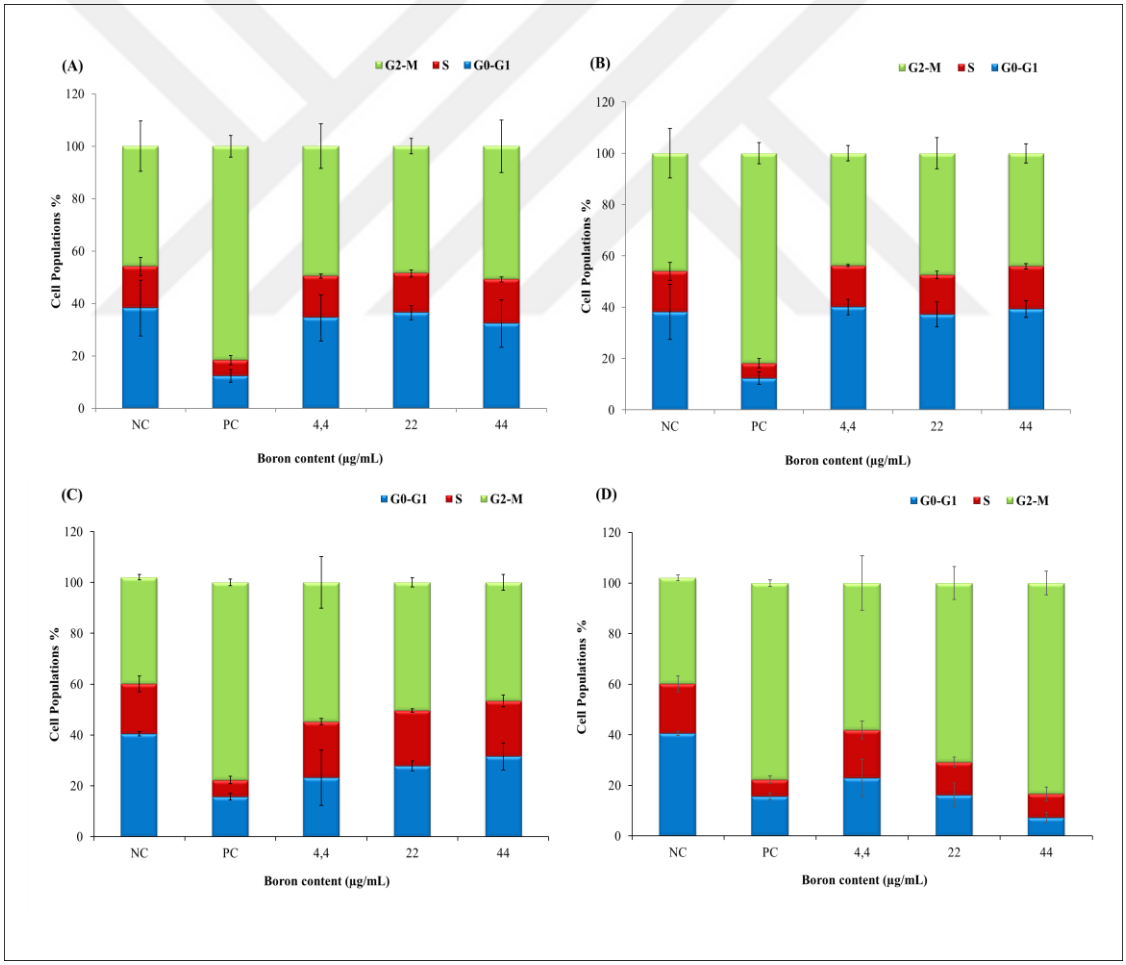

Figure 6. Cell cycle analysis of hBNs (A) and BA (B) for 24 h, hBNs (C) and (BA) for 72 h treated mHippoE-14 cells.

4.6. Detection of apoptotic bodies

24 h and 72 h exposure of hBNs and BA containing 4.4, 22 and 44 µg/mL boron concentrations showed no significant effect on formation of apoptotic bodies. Figures 7.1, 7.2, 7.3 and 7.4 show images taken with confocal microscopy. DAPI was used to stain genomic DNA and all images were acquired at 63x magnification.

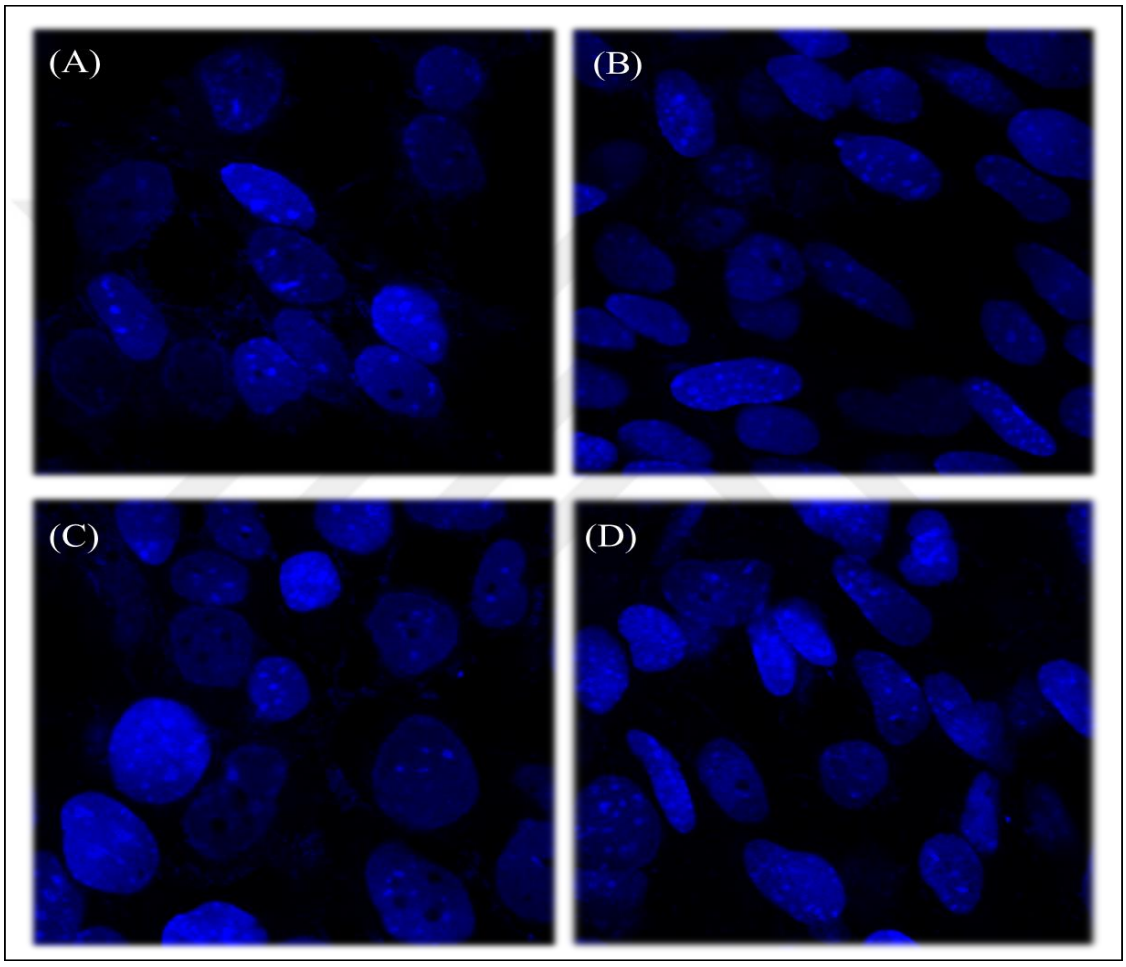

Figure 7.1. Detection of apoptotic bodies of hBNs (A) control, (B) 4.4 µg/mL boron, (C) 22 µg/mL boron and (D) 44 µg/mL boron exposure of 24 h. The images were taken with confocal microscopy by 63x magnification.

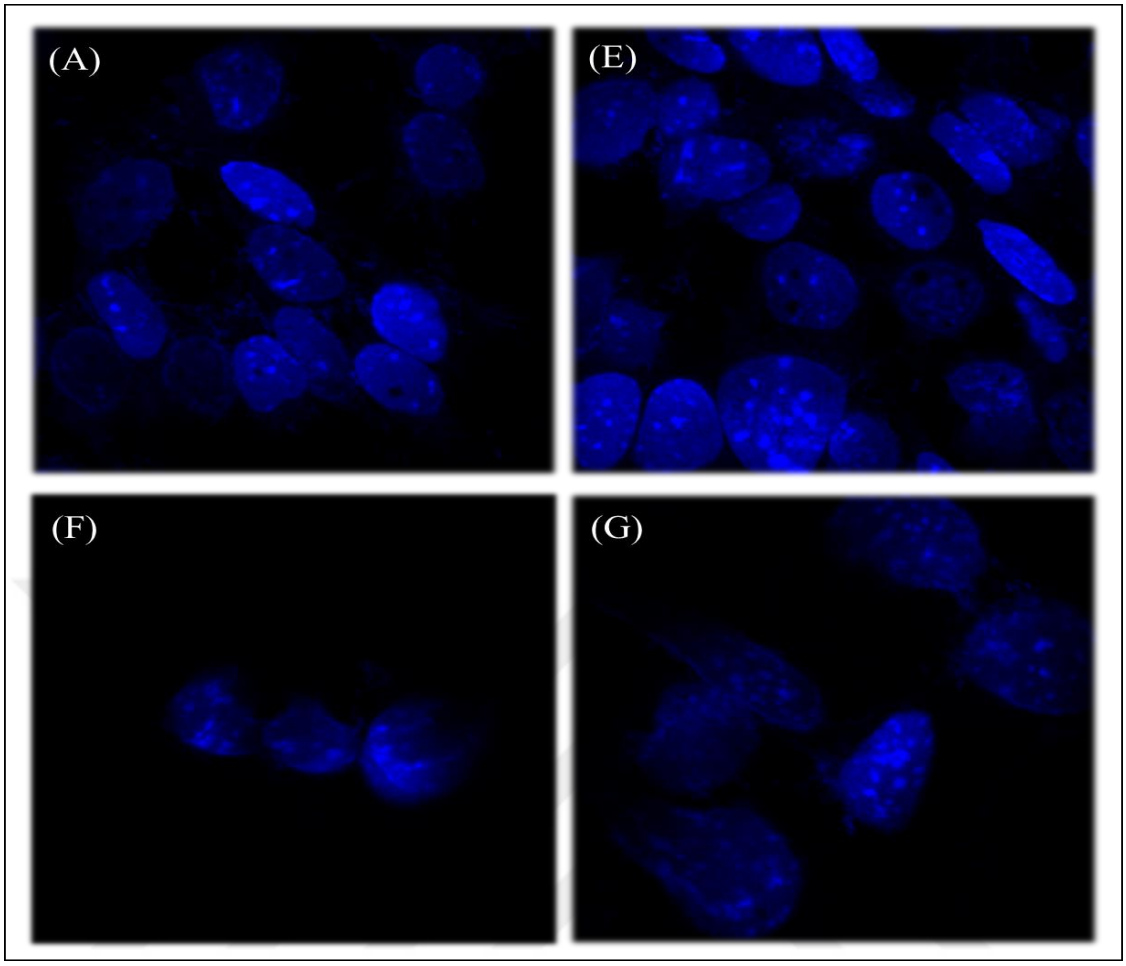

Figure 7.2. Detection of apoptotic bodies of BA (A) control, (E) 4.4 μg/mL boron, (F) 22 μg/mL boron and (G) 44 μg/mL boron exposure of 24 h. The images were taken with confocal microscopy by 63x magnification.

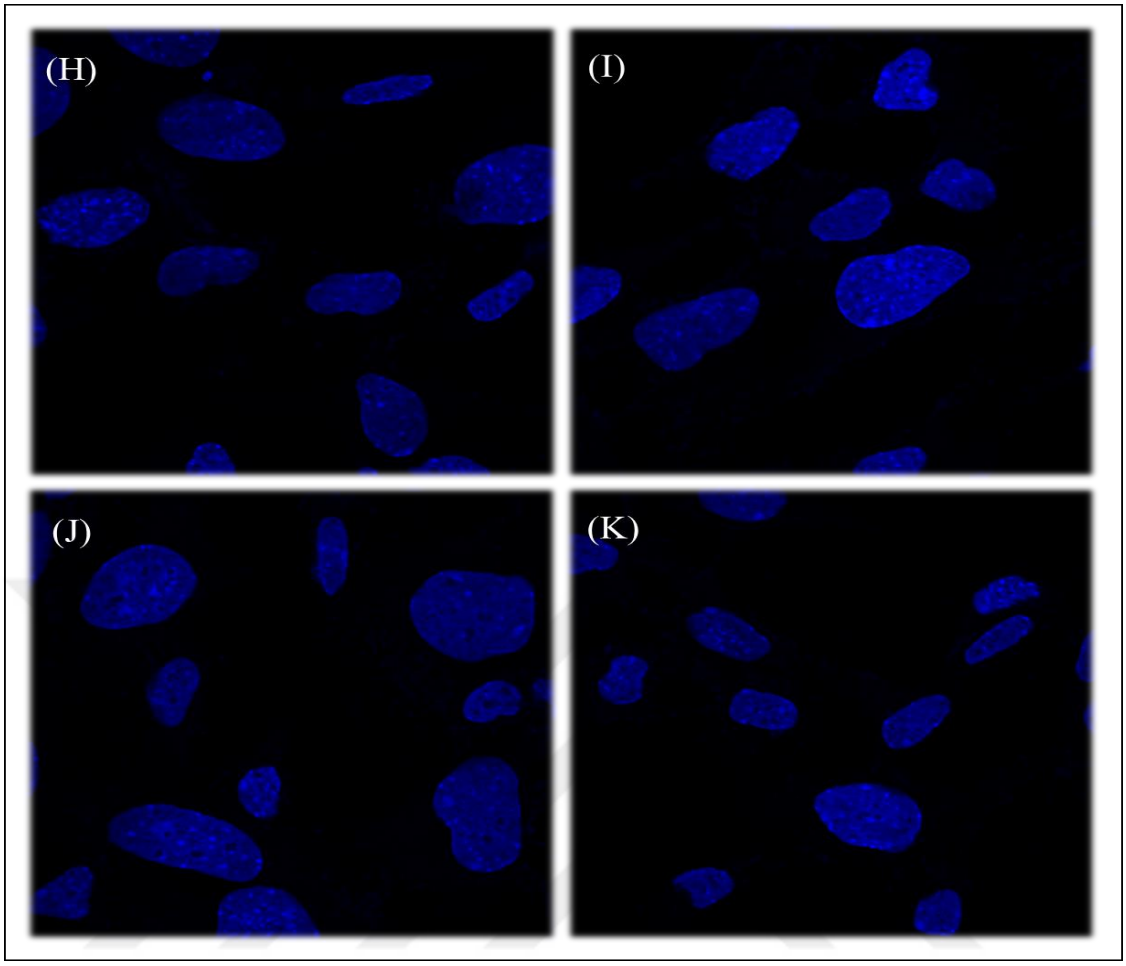

Figure 7.3. Detection of apoptotic bodies of hBNs (H) control, (I) 4.4 µg/mL boron, (J) 22 µg/mL boron and (K) 44 µg/mL boron exposure of 72 h. The images were taken with confocal microscopy by 63x magnification.

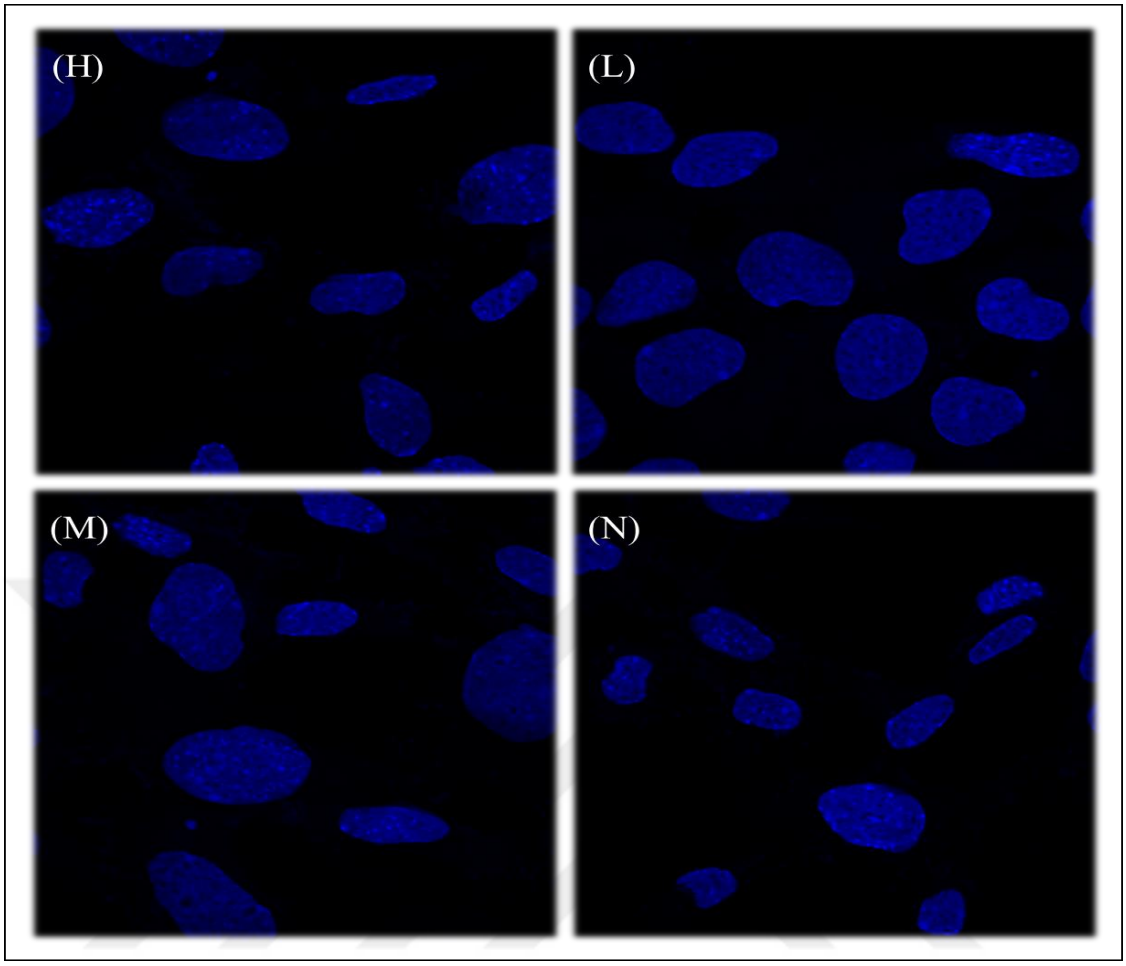

Figure 7.4. Detection of apoptotic bodies of BA (H) control, (L) 4.4 µg/mL boron, (M) 22 µg/mL boron and (N) 44 µg/mL boron exposure of 72 h. The images were taken with confocal microscopy by 63x magnification.

4.7. Investigation of the effect of hBNs and BA on DOX exposed cells

Figure 8 shows the viability of the cells exposed to 4 µM DOX for 5 h and then exposure of these cells to hBNs and BA. After the DOX treatment, the cells exposed to hBNs and BA for 24 h showed almost no significant affect on cell viability. However, when the cells were exposed to both hBNs and BA for 72 h, a significant increase in cell viability was seen for concentrations of both materials.

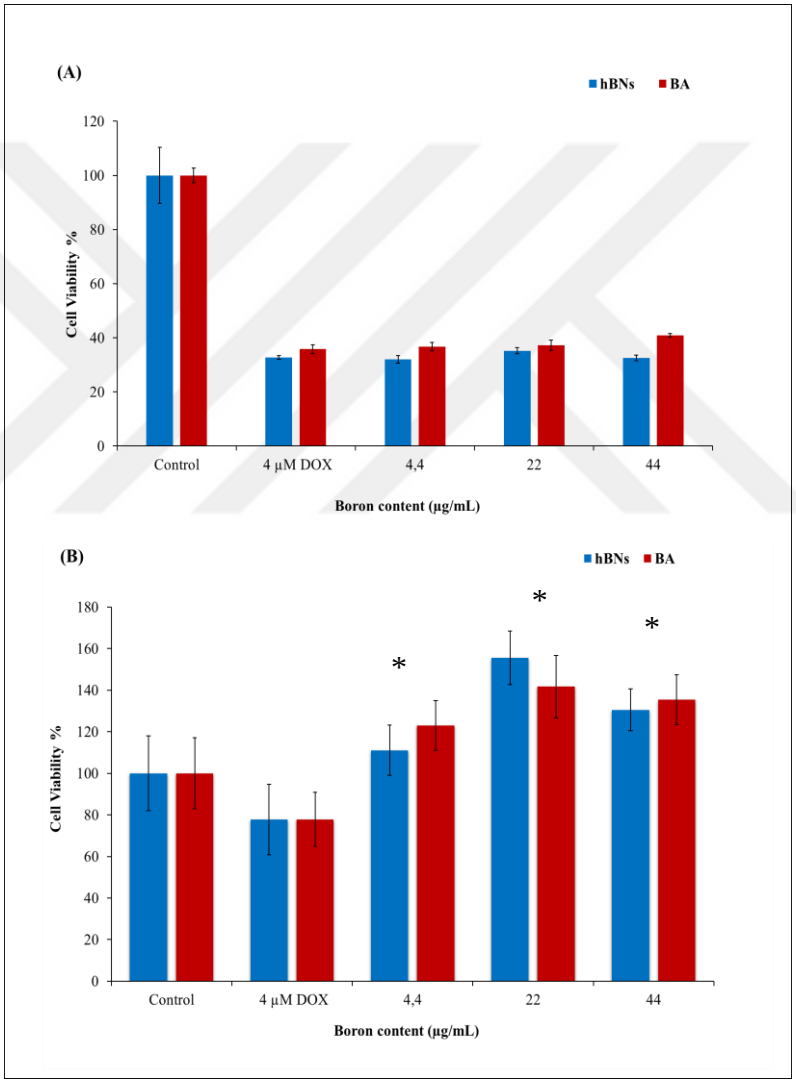

Figure 8. Cell viability of DOX exposed mHippo cells 24 h (A) and 72 h (B) exposure of hBNs and BA. * Different from control $p<0.05$, statistically analysed by Student's *t*-test.

5. DISCUSSION AND CONCLUSION

Boron nitride (BN) has atracted attention and is widely used in varoius fileds due to its unique properties including biocompatibility, excellent mechanical properties, and good thermal conductivity (59). A study has showed that an anticancer targeted drug delivery system based on folate-conjugated BN nanospheres (BBNS) was developed and cytotoxicity was tested on HeLa cells, then they were loaded with DOX and resulted with strong potential for drug targeted cancer therapy (60).

The hBNs and BA had varying cytotoxic effect on mHippo cells at 24 h and 72 h exposure times. A time and dose dependent toxicity of both hBN and BA was clear. When compared, the hBNs showed lesser cytotoxic effect on cells than BA for all concentrations (10-1000 μg/mL) for 24 and 72 h. For 24h exposure, the cell viability at the concentration 10 μg/mL hBNs significantly increased but never reached the cell viability level of the control group. The concentrations including 300 μg/mL and the higher ones caused a significant decrease in cell viability. For 72 h exposure of hBNs and BA, an increased cell viability compared to 24 h exposure was observed. The reason for the increased cell viability after 72 h of hBNs exposure can be attributed to the increased release of boron degredation products into the cell culture and cells with increased incubation time. As the hBNs are uptaken through endocytosis into the cells, endosomes carrying hBNs fuse with lysosomes, which has acidic content. This process might help to increase the degradation of hBNs in the cells.

There are two different mechanisms of cell death, apoptosis and necrosis (61). Apoptosis is a controlled cell death mechanism. In necrotic cell death, the cell swells, plasma membrane loses its integrity, and the cells lose its intracellular contents. This process is irreversible and only starts when the cells die (62). PI was used with Annexin-V to determine if cells were viable, necrotic, or apoptotic. PI does not stain live or early apoptotic cells, it only stains late apoptotic and necrotic cells. All experiments were done with exposure of hBNs and BA for 24 h and 72 h. It was observed that the live cell numbers were not significantly affected by hBNs concentrations including 4.4 and 22 μg/mL boron at 24 h exposure. The experiment has showed that the concentrations of

hBNs had no significant ($p<0,05$) effect on early apoptosis, yet 4.4 μg/mL boron containing concentrations caused a significant increase in late apoptosis but generally had no significant effect on necrosis. 24 h exposure of BA concentrations showed no significant effect on live cell population and did not cause necrosis. 22 μg/mL boron containing BA caused the cells to be significantly apoptotic. 72 h exposure caused a decrease in live cells in 4.4 μg/mL boron including hBNs while increased the live cell population in 22 μg/mL and again decreased in 44 μg/mL. As concentration of hBNs increases, a gradual increase can be seen in late apoptosis with 72 h exposure. 72 h exposure of BA containing 4.4 μg/mL boron, on the other hand decreased live cell population significantly and lead to necrosis. BA containing 44 μg/mL boron caused significant late apoptosis on the cells. The reason for the decrease in live cells and the increase of late apoptosis of cells exposed to hBNs could be due to the stress caused by being incubated for 72 h when compared to the cell line which needs passage almost every 48 h. As the incubation time increases, the medium volume decreases and more cells come into contact with each other and cause more stress.

Detection of apoptotic bodies were detected by staining the nucleus of the hippocampal cells with DAPI dye and were then studied with confocal microscopy. All experiments were done with exposure of hBNs and BA for 24 h and 72 h. The confocal images were used to detect whether boron concentrations of 4.4, 22 and 44 μg/mL of hBNs and BA have caused nucleus damage of the mHippo cells. The images showed that there no DNA damage occured in all concentrations of hBNs and BA. The cytotoxicity assay WST-8 also showed that the concentrations used for following experiments; 10, 50 and 100 μg/mL, have had no toxic effect and the DNA fragmantation experiment was validated with this experiment. A previous study was carried out to investigate boric acids protective effect on wound healing and DNA damage in human epithelial cell line and the study stated that BA had reduced DNA damage caused by agents like irinotecan (CPT11), DOX and etoposide (ETP) and had improved wound healing as well (63).

It is known that cancer cells are metabolically active and are under increased oxidative stress, which is likely associated with dysfunction of metabolic regulation and uncontrolled cell proliferation and thus caused ROS generation. All appototic body

detection experiments were done with exposure of hBNs and BA for 24 h and 72 h. The ROS detection experiments show that there is a significant increase in ROS production at hBNs concentration including 44 μg/mL boron, and BA does not have any significant effect on ROS production with 24 h exposure. 72 h exposure of hBNs only caused significant increase in ROS production in only concentration of hBNs containing 44 μg/mL boron. As for BA, it only caused significant ROS production increase in the boron concentration of 4.4 μg/mL and caused significant decrease in 22 μg/mL of boron. As ROS production increased, the confocal microscopy study showed no DNA damage. The reason of this could be because of not only the mytochondria being able to generate ROS, there is also another organell that can generate ROS which is the peroxisome. (64). Peroxisomes oxidize amino acids and fats, and they synthesize lipids, and also catabolize ROS produced during oxidative reactions (65).

Cell cycle analysis characterizes cells in different cell cycle phases and is used to determine the cellular response to biological stimulations and drugs. The cell cycle assay is based on measuring the DNA content in the cell population, thus it is important to use this assay to strengthen the previous experiments that have showed there is significantly no DNA damage ($p<0.05$). There was no significant difference ($p<0,05$) in cell cycle for any concentrations when exposed to hBNs and BA with 24 h exposure. The S phase is were the cell undergoes DNA replication, the hBNs concentrations did not significantly affect this phase therefore did not cause an arrest at S phase and did not cause any disruption in DNA replication with the exposure for 24 h. hBNs did not cause any significant changes of the G0-G1 and G2-M phases as well with 24 h exposure. 72 h exposure of hBNs caused a significant increase in G0-G1 phase and a significant decrease in G2-M phase at 22 μg/mL containing boron concentration of hBNs. BA also caused a significant increase in G0-G1 at 4.4 and 22 μg/mL boron containing concentrations. The S phase significantly increased with and 44 μg/mL boron containing BA concentrations, which showed that cell cycle arrest occured.

The first WST-8 experiments were performed to understand the toxic concentration range and the optimal concentrations were choosed to better explain the effect of hBNs and BA on the cell line which was 10, 50 and 100 μg/mL. The aim of the study was to analyze whether hBNs and BA could significantly decrease the stress caused by theraupetic agents such as DOX used in this study. DOX, is a chemotherapeutic agent that produces ROS reactive superoxide anion by redox cycling of the quinone moteity in the presence of molecular oxygen (66). DOX is a fluorescent neurotoxin, which can be transported in the reverse direction in the brain, and has the feature of killing neurons in the brain (67). As DOX is used for cancer treatment, the potential of killing neurons is a tremendous side effect that causes comparably worse outcomes (68). In this study, hippocampal cells were used to determine whether hBNs and BA could possibly decrease the damaging effect of drugs that have potential effects of damaging healthy cells or tissues such as DOX. To determine this, the cells were exposed to 4 μM DOX for 5 hours, just enough time to cause stress on the cells. After this exposure, the hBNs and BA were added at increasing concentrations and the cells incubated for both 24 h and 72 h. As it is seen in Figure 8A, the group only treated with 4 μM DOX has significantly decreased cell viability to compared to the control group, but when exposed to hBNs and BA concentrations containing equal amounts of boron, the cell viability significantly incrased ($p<0.05$) compared to the group only exposed to 4 μM DOX. As it did not reach the control groups cell viability level, it still showed a significant increase in cell viability at 24 h exposure. 72 h exposure of hBNs and BA showed a significantly higher increasement in cell viability when compared to 24 h exposure. The cell viability increase seen in 72 h exposure is similar to the cell viability of the the non-treated group. The results showed that as incubation time increased, hBNs and BA concentrations caused cell viability to significantly increase more, even back to the normal level seen in the control group and even higher. This proves our aim of the study which was to be able to use hBNs and BA as therapeutic materials to be exposed with, before or after exposure of anticancer-drugs such as doxorubicin which have prolonged toxic effects mostly after anti-cancer treatments.

In conclusion, this study reveals that both hBNs and BA are not cytotoxic at concentrations lower than 50 μg/mL and hBNs show lesser cytotoxicity than BA. When a low concentration of hBNs is used there is not almost detectable change in ROS production, cell cycle, and DNA demage. Although both hBNs and BA helped to increase the cell viability after exposure to DOX to cause a reasonable stress, the slow degredation of hBNs offer the controlled release feature for its possiblee use for not only treatment of neurological disorders but also relieve the stress caused by the treatment. The results of this study suggest that hBNs should be further investigated by expanding to other neuron cells and animal tests.

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APPENDIX 1. Curriculum Vitae

Personal Informations

Name	İrem	**Surname**	Çulha
Place of Birth	Adana	**Date of Birth**	12.06.1994
Nationality	Turkish	**TR ID Number**	12007318866
E-mail	culhairem@gmail.com	**Phone number**	05343215459

Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Doctorate			
Master			
University	Physiotherapy and Rehabilitation	Yeditepe University Faculty of Health Sciences	2016
High school		İstek Uluğbey Science High School	2012

[#] All the grades must be listed if there is more than one (KPDS, ÜDS, TOEFL; EELTS vs),

Languages	**Grades ([#]))**
YDS	75
IELTS	8

Work Experience (Sort from present to past)

Position	**Institute**	**Duration (Year - Year)**
		-
		-

Computer Skills

Program	**Level**
Microsoft Word, Excel and Powerpoint	Good

***Excellent , good, average or basic**

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

Articles published in other journals

Proceedings presented in international scientific meetings and published in proceedings book.

Journals in the proceedings book of the refereed conference / symposium

Others (Projects / Certificates / Rewards)
