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3-Pyridinylboronic acid normalizes the effects of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure in zebrafish embryos

Fümet Duygu Üstündağ[a], İsmail Ünal[b], Derya Cansız[c], Ünsal Veli Üstündağ[d], Hülya Kara Subaşat[e], A. Ata Alturfan[c], Pınar Mega Tiber[a] and Ebru Emekli-Alturfan[b]

[a]Faculty of Medicine, Department of Biophysics, Marmara University, Istanbul, Turkey; [b]Faculty of Dentistry, Department of Basic Medical Sciences, Marmara University, Istanbul, Turkey; [c]Faculty of Medicine, Department of Biochemistry, Istanbul University-Cerrahpaşa, Istanbul, Turkey; [d]Faculty of Medicine, Medical Biochemistry, Department Medipol University, Istanbul, Turkey; [e]Graduate School of Natural and Applied Sciences, Department of Energy, Mugla Sıtkı Kocman University, Muğla, Turkey

ABSTRACT

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that damages dopaminergic neurons. Zebrafish has been shown to be a suitable model organism to investigate the molecular pathways in the pathogenesis of Parkinson's disease and also for potential therapeutic agent research. Boron has been shown to play an important role in the neural activity of the brain. Boronic acids are used in combinatorial approaches in drug design and discovery. The effect of 3-pyridinylboronic acid which is an important sub-class of heterocyclic boronic acids has not been evaluated in case of MPTP exposure in zebrafish embryos. Accordingly, this study was designed to investigate the effects of 3-pyridinylboronic acid on MPTP exposed zebrafish embryos focusing on the molecular pathways related to neurodegeneration and apoptosis by RT-PCR. Zebrafish embryos were exposed to MPTP (800 μM); MPTP + Low Dose 3-Pyridinylboronic acid (50 μM) (MPTP + LB) and MPTP + High Dose 3-Pyridinylboronic acid (100 μM) (MPTP + HB) in well plates for 72 hours post fertilization. Results of our study showed that MPTP induced a P53 dependent and Bax mediated apoptosis in zebrafish embryos and 3-pyridinylboronic acid restored the locomotor activity and gene expressions related to mitochondrial dysfunction and oxidative stress due to the deleterious effects of MPTP, in a dose-dependent manner.

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1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 3-pyridinylboronic acid; zebrafish embryo; neurodegeneration; apoptosis

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzeimer's disease in the world. The neurodegenerative aspect of PD is associated with the selective loss of different types of neurons (Franco *et al.* 2017). The effective treatment method in PD has not been found yet and the current treatment is for the relief of symptoms.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that induces selective dopaminergic neuron loss in the mammalian midbrain. MPTT exposure leads to characteristic symptoms of PD in different model organisms (Langston *et al.* 1984). MPTP exposure in zebrafish embryos has been shown to damage dopaminergic neurons and decrease dopaminergic cell numbers in the diencephalon (Lam *et al.* 2005). In zebrafish, the dopaminergic system is well characterized both in embryonic and adulthood. For this reason, zebrafish is a suitable model organism to investigate the molecular pathways in the pathogenesis of PD and also for potential therapeutic agent research (Unal and Emekli-Alturfan 2019).

Boron is an essential mineral among the 3 A group elements of the periodic table and does not exist as an element in nature. It is found in compounds with carbon and other elements (Das *et al.* 2013; Kuru *et al.* 2019). Boronic acid is one of the most commonly used boron compounds (Białek *et al.* 2019). Recently, new boron-based compounds have been tested as anticancer, antibacterial, antifungal, antiviral, anticoagulant and antidiabetic agents, while they have been proposed for the treatment of cancer, cardiovascular, central nervous system, lung and metabolic diseases as well as inflammatory processes (Hall 2011; Das *et al.* 2013; Soriano-Ursúa *et al.* 2014; Yahsi *et al.* 2015). Based on these described properties, boron has significant potential in the design of therapeutic agents. It is known that boron has an important role in the neural activity of the brain. In recent studies, it has been reported that boron deprivation causes symptoms such as decreased brain electrical activity, loss of consciousness and psycho-motor activity, decreased movement and skills, and short-term memory weakness (Penland 1994; Białek *et al.* 2019).

Boronic acids are one of the most commonly used boron compounds in organic chemistry as they have a major role in a wide variety of cross-linking reactions (Białek *et al.* 2019; Plescia and Moitessier 2020). In addition to their key roles in crosslinking reactions in supramolecular chemistry, some boronic acid species have been identified for their antimicrobial,

CONTACT Ebru Emekli-Alturfan ebruemekli@yahoo.com Faculty of Dentistry, Department of Basic Medical Sciences, Marmara University, Istanbul, Turkey
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antineoplastic, enzyme inhibitory and serine-protease inhibitory activities (Smoum *et al*. 2012; Fontaine *et al*. 2014). One of the main reasons why boronic acids are included in drug discovery efforts is their approval for treatment by the Federal Drug Administration (Trippier and McGuigana 2010; Plescia and Moitessier 2020).

Substituted pyridines are important components of many drugs and drug candidates (Kadayat *et al*. 2018). Recently, the use of pyridine boronic acids in terms of hydrogen-bound derivatives has attracted attention because of the production of different supramolecular communities based on crystal engineering principles in supramolecular chemistry (Kara *et al*. 2006; Yahsi *et al*. 2015). Pyridinylboronic acids are appropriate for use as subclass of heterocyclic boronic acids in combinatorial approaches in product design and discovery (Liu *et al*. 2013; Fontaine *et al*. 2015). Pyridinium orientation has been shown to determine the mitochondrial uncoupling of the mitochondria-targeted, broad-spectrum anticancer agent F16 (Xu *et al*. 2018). On the other hand, mitochondrial uncoupling has been also suggested to affect neurons by modulating multiple neuroprotective pathways (Geisler *et al*. 2017). Although the beneficial effects of boron in the neural activity of the brain have been shown before, the effect of 3-pyridinylboronic acid has not been evaluated so far in PD. Accordingly, the aim of this study was to investigate the effects of 3-pyridinylboronic acid on MPTP exposed zebrafish embryos focusing on the molecular pathways related to neurodegeneration and apoptosis.

Methods

Chemicals tested

MPTP (CAS no. 23007–85-4) was purchased from Sigma-Aldrich, St Louis, MO, USA. 3-Pyridinylboronic acid (CAS no. 1692–25-7) was purchased from Sigma-Aldrich. They were all analytical grade with the highest purity available. Syntheses and crystallizations were carried out in the air in standard glassware. Complete evaporation of a dilute HCl solution of 3-Pyridinylboronic acid causes the quantitative formation of white crystals of 3-Pyridinylboronic acid. The resulting white crystals were collected, washed with ethanol and dried in the air.

Maintenance of zebrafish

Wild type AB/AB Strain zebrafish were maintained in apparently disease-free conditions. Fish were kept in an aquarium rack system (Zebtec, Tecniplast, Italy) at 27 ± 1 °C under a light/dark cycle of 14/10 h and they were fed with commercial flake fish food complemented with live Artemia twice a day. Reverse osmosis water that contains 0.018 mg L^{-1} Instant Ocean™ salt was used for all of the experiments. After natural spawnings, fertilized embryos were gathered and staged according to their developmental and morphology as described before (Westerfield 1995).

Embryo exposure

Stock 10 mg MPTP solution was prepared by dissolving in 2 mL E3. Embryos were exposed to MPTP (800 μM); MPTP + Low Dose 3-Pyridinylboronic acid (50 μM) (MPTP + LB) and MPTP + High Dose (100 μM) 3-Pyridinylboronic acid (MPTP + HB) in well plates for 72 hours post fertilization (hpf). Embryo medium was used as the blank control. Each exposure group contained three replicate wells having 20 embryos in each for the analyzes of development, mortality and hatching. Each day the exposure solutions were changed with fresh solutions. Each day developmental parameters were monitored under a stereomicroscope (Zeiss Discovery V8, Germany). Hatching rates were also documented every 24 h. The hatching rate is defined as the ratio of hatching embryos to the living embryos in each well. The development indicators including yolk sac, anal pore, pectoral fin, and swim bladder were used for embryo staging as explained before (Westerfield 1995).

Locomotor activity

The locomotor activity of the zebrafish embryos was evaluated as described previously (Goody *et al*. 2012). This was performed by placing a 60 mm Petri dish containing embryo medium on top of the motility wheel which is on the microscope stage. Then, by using an embryo poker tool the zebrafish embryo was positioned in the middle of the motility wheel and the time it took for an embryo to swim a predetermined distance was recorded and the average escape response was calculated.

Reverse transcription (cDNA synthesis) and quantitative Real-Time PCR

At the end of the experiment, RNA was isolated from the embryos in each group. Rneasy Mini Kit and Qiacube (Qiagen, Hilden, Germany) were used according to the instructions of the manufacturer. A single-stranded cDNA was produced from 1 μg of total RNA using RT^2 Profiler PCR Arrays (Qiagen, Hilden, Germany). DNA Master SYBR Green kit (Qiagen, Hilden, Germany) was used to perform RT-PCRs. Beta actin was used as the house keeping gene. Relative levels of transcription were calculated using the ΔΔCT method based on the normalization of the values using the house keeping gene (Livak and Schmittgen 2001). The list of the primers used is given in Table 1.

Statistical analysis

One-way analysis of variance (ANOVA) with *post hoc* Dunn's multiple comparison test was used to analyze the differences between the groups using GraphPad Prism 8. $p < 0.05$ was considered as significant.

Table 1. Forward and reverse primers used in the study.

Fas	Forward primer	5′-GTGACGCTAATGCAAAAATGAAG-3′
	Reverse primer	5′-CGATGTCCTGCAGAGTGGTG-3′
bax	Forward primer	5′-GGCTATTTCAACCAGGGTTCC-3′
	Reverse primer	5′-TGCGAATCACCAATGCTGT-3′
casp3a	Forward primer	5′-ATGAACGGAGACTGTGTG-3′
	Reverse primer	5′-TTAAGGAGTGAAGTACATCTCTTTG-3′
p53	Forward primer	5′-GGGCAATCAGCGAGCAAA-3′
	Reverse primer	5′-ACTGACCTTCCTGAGTCTCCA-3′
park2	Forward primer	5′-GCGAGTGTGTCTGAGCTGAA-3′
	Reverse primer	5′-CACACTGGAACACCAGCACT-3′
pink1	Forward primer	5′-GGCAATGAAGATGATGTGGAAC-3′
	Reverse primer	5′-GGTCGGCAGGACATCAGGA-3′
bdnf	Forward primer	5′-ATAGTAACGAACAGGATGG-3′
	Reverse primer	5′-GCTCAGTCATGGGAGTCC-3′
β actin	Forward primer	5′-AAGCAGGAGTACGATGAGTCTG-3
	Reverse primer	5′-GGTAAACGCTTCTGGAATGAC-3′
lrrk2	Forward primer	5′-CCCTAAACCGCAGAGTATCA-3′
	Reverse primer	5′-ATTCATAGTCCACCGGTCTG-3′
	Forward primer	5′-GGCCGGTAAAAGAGCGTTAG-3′
dj1	Reverse primer	5′-ACCCATGAGTCCTCCACTA-3′

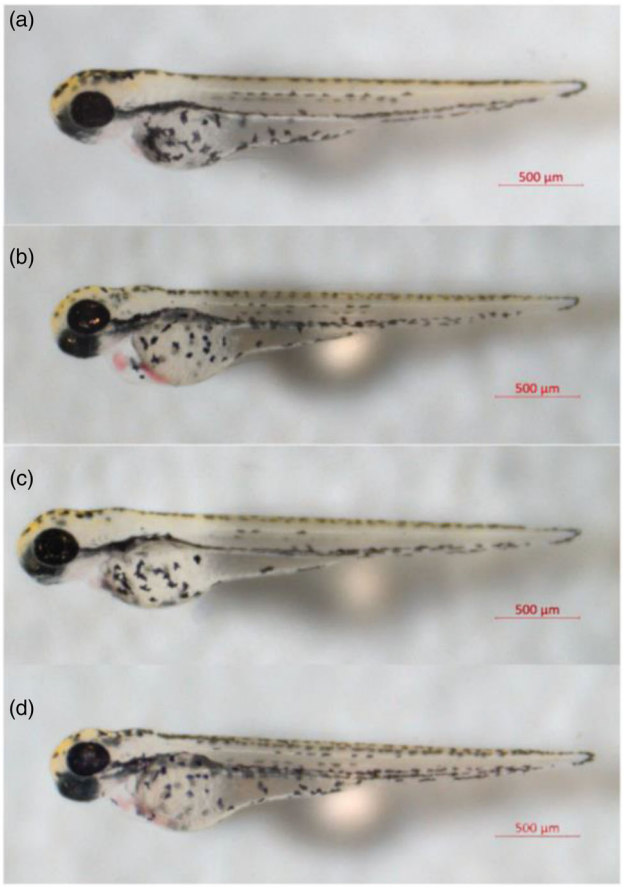

Figure 1. Representative figures of zebrafish embryos at 72 hpf (a) control group with normal growth and development, (b) blood stasis and pericardial edema with MPTP exposure, (c) MPTP + LB exposed embryo (d) MPTP + HB exposed embryo. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; LB: Low dose 3-Pyridinylboronic acid; HB: High dose 3-Pyridinylboronic acid.

Results

Results of hatching rate and morphological analyzes

The images of the zebrafish embryos at 72 hpf are presented in Figure 1. Normal growth and development were observed in the control group (Figure 1(a)) whereas, blood stasis and pericardial edema were observed at 72 hpf in the MPTP group (Figure 1(b)). The hatching rates of the embryos are given in Figure 2. Although statistically not significant, delayed hatching was observed in the MPTP group which slightly increased in the MPTP + HB group (Figure 2).

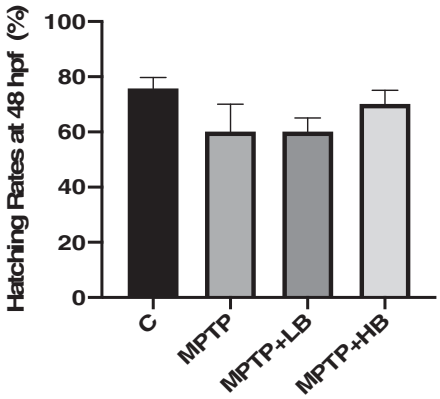

Figure 2. Hatching rates of the embryos at 48 hours post fertilization (hpf). Data are expressed as mean + SD from the three independent experiments. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; LB: Low dose 3-Pyridinylboronic acid; HB: High dose 3-Pyridinylboronic acid; SD: standard deviation.

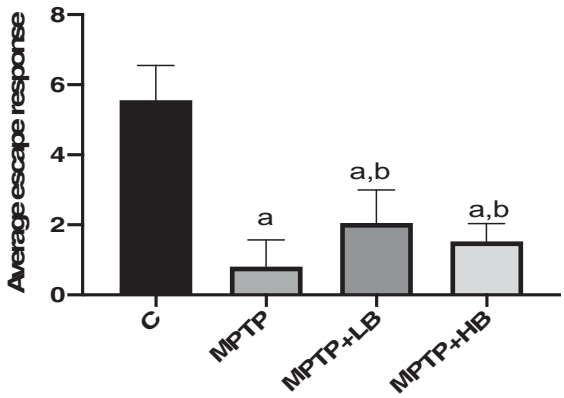

Figure 3. Locomotor activity of the embryos in groups assessed as average escape response. Data are expressed as mean + SD from the three independent experiments. [a] significantly different from the control group, $p < 0.05$; [b] significantly different from the MPTP group, $p < 0.05$. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; LB: Low dose 3-Pyridinylboronic acid; HB: High dose 3-Pyridinylboronic acid; SD: standard deviation.

Results of locomotor activities

The results of the locomotor activities are given in Figure 3. Locomotor activities in all exposure groups decreased significantly when compared with the control group. On the other hand, significant increases were observed both in the MPTP + LB and the MPTP + HB groups when compared with the MPTP group.

Results of gene expression analyses

The mRNA expression levels of *pink1* and *park2* increased significantly in the MPTP group when compared with the Control group. On the other hand, both low and high dose 3-pyridine boronic acid decreased *pink1* and *park2* expressions significantly when compared with the MPTP group (Figure 4).

There was a significant increase in the expression of *lrrk* in the MPTP group compared with the control group. Both low and high dose 3-pyridineboronic acid decreased *lrrk* expressions significantly when compared with the MPTP group (Figure 5). *bdnf* and *dj1* expressions decreased significantly in the MPTP group and low and high dose 3-pyridineboronic acid led to increases in *bdnf* and *dj1* expressions compared with the MPTP group (Figure 5).

Significant increases were observed in *p53* and *casp3a* expressions in the MPTP group, both low and high 3-pyridineboronic acid decreased *p53* and *casp3a* expressions when compared with the MPTP group (Figure 6). *bax* expressions increased significantly in all exposure groups when compared with the Control group. A significant decrease was observed in the MPTP + HB group when compared with MPTP group (Figure 7). *fas* expressions decreased significantly in the MPTP and MPTP + LB groups when compared with the Control group and increased significantly in the MPTP + HB group when compared both with the MPTP and the MPTP + LB groups (Figure 7).

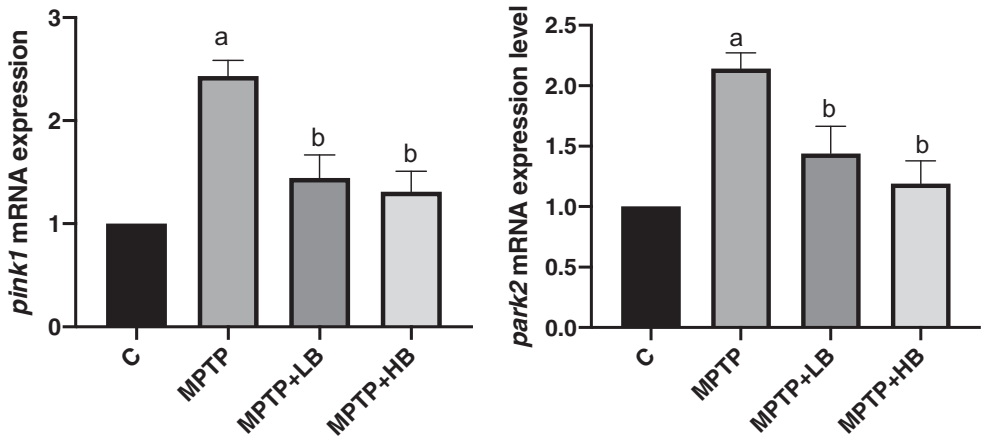

Figure 4. *pink 1* and *park2* expressions of the groups. Data are expressed as mean + SD from the three independent experiments. [a] significantly different from the control group, $p < 0.05$; [b] significantly different from the MPTP group, $p < 0.05$. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; LB: Low dose 3-Pyridinylboronic acid; HB: High dose 3-Pyridinylboronic acid; SD: standard deviation.

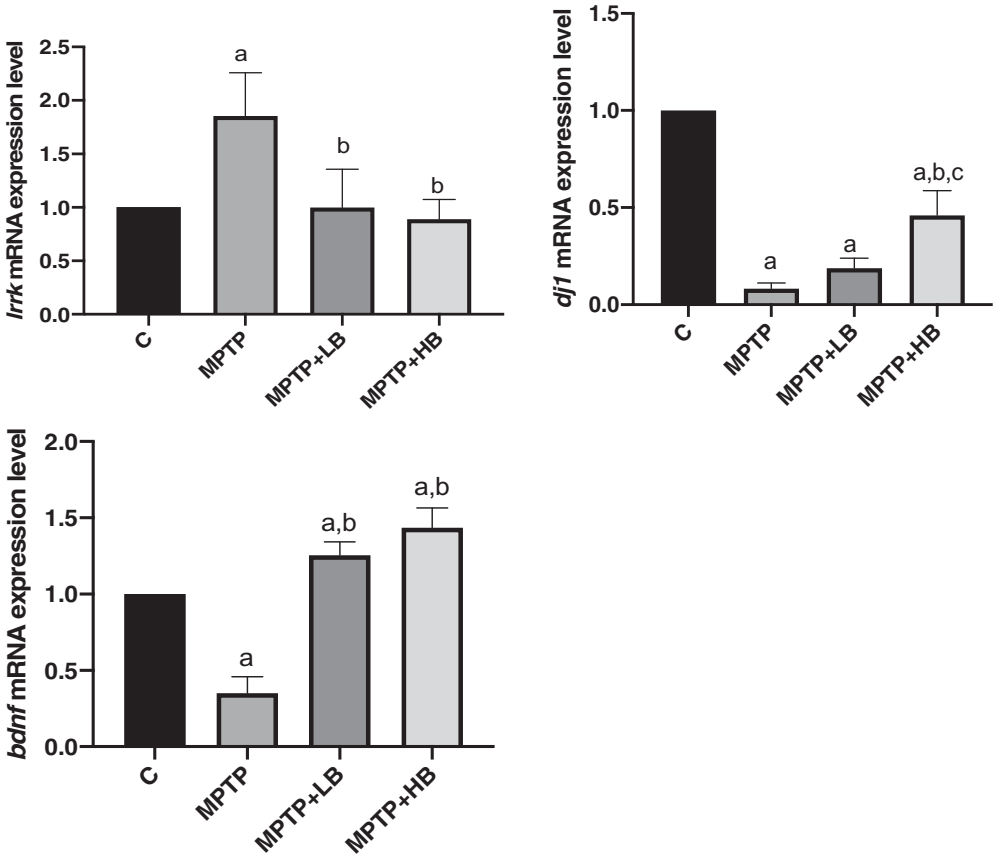

Figure 5. *lrrk*, *dj1* and *bdnf* expressions of the groups. Data are expressed as mean + SD from the three independent experiments. [a] significantly different from the control group, $p < 0.05$; [b] significantly different from the MPTP group, $p < 0.05$; c significantly different from the MPTP + LB group. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; LB: Low dose 3-Pyridinylboronic acid; HB: High dose 3-Pyridinylboronic acid; SD: standard deviation.

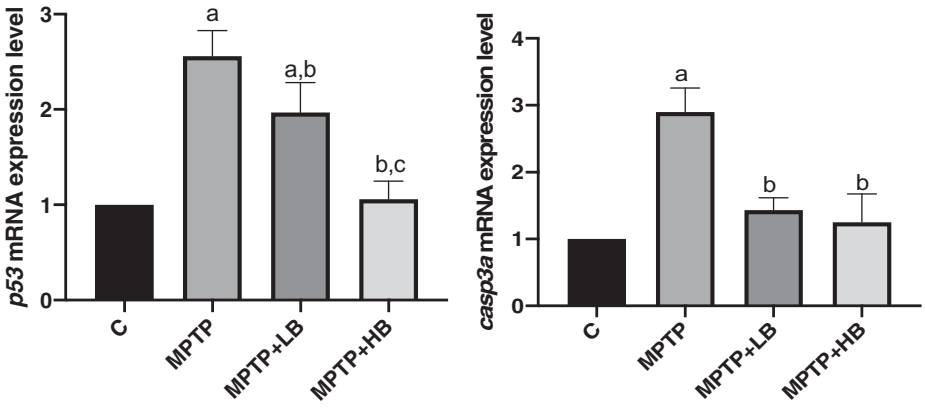

Figure 6. *p53* and *casp3a* expressions of the groups. Data are expressed as mean + SD from the three independent experiments. [a] significantly different from the control group, $p < 0.05$; [b] significantly different from the MPTP group, $p < 0.05$; [c] significantly different from the MPTP + LB group. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; LB: Low dose 3-Pyridinylboronic acid; HB: High dose 3-Pyridinylboronic acid; SD: standard deviation.

Discussion

In this study we evaluated the effects of 3-pyridineboronic acid on MPTP exposed zebrafish embryos focusing on neurodegenerative pathways and apoptosis. 3-pyridineboronic acid improved the impaired locomotor activity and gene expressions related to mitochondrial dysfunction due to MPTP exposure.

MPTP is a neurotoxin shown to induce selective loss of dopaminergic neurons in the mammalian midbrain, leading to characteristic symptoms of PD in different animal models including zebrafish (Unal and Emekli-Alturfan 2019). When MPTP is metabolized by monoamine oxidase-B, 1-Methyl-4-phenylpyridinium (ion) (MPP^+) is formed as the ultimate toxic agent. Zebrafish embryos have been shown to be susceptible to the dopaminergic neurotoxin MPTP (Lam *et al.* 2005). Several mechanisms have been suggested to be effective in the molecular pathways of PD including oxidative stress, inflammation, apoptosis, mitochondrial dysfunction and finally defects in protein degradation (Schmidt and Ferger 2001).

In the present study, MPTP exposure decreased locomotor activity in zebrafish embryos. MPTP exposure has been shown to decrease the number of dopaminergic cells in the diencephalon which was reversed by monoamine oxidase B inhibitor. Similar to the results of our study MPTP exposure led to defects in the swimming responses of zebrafish larvae (Lam *et al.* 2005).

When MPP^+ is taken by the dopaminergic neurons, it inhibits mitochondrial oxidative phosphorylation complex 1, leading to mitochondrial stress. Mitophagy is the process where the damaged mitochondria is targeted for lysosomal degradation. Pink1 and Parkin work coordinately to regulate mitophagy. Defects in Pink1/Parkin regulated mitophagy leads to the accumulation of damaged mitochondria which contributes to PD (Greenamyre *et al.* 2001). In our study, *pink1* and *park2* expressions increased significantly in the MPTP group when compared with the Control group. Increased *pink1* and *park2* expressions may indicate increased mitochondrial stress due to MPTP.

On the other hand, both low and high dose 3-pyridinylboronic acid decreased *pink1* and *park2* expressions. 3-Pyridinylboronic acid has shown a protective effect by reducing mitochondrial stress as evidenced by the normalized *pink* and *park2* expressions.

There are a few number of studies examining the effects of boron-containing compounds against PD. Küçükdoğru *et al.* (2020) investigated the effects of hexagonal boron nitride nanoparticles (hBNs) against the toxicity of MPTP in experimental PD model. Similar to our findings, their results indicated the therapeutic potential of hBNs against MPP + toxicity and they suggested that hBNs can be used as new neuroprotective agent and drug delivery system in PD. On the other hand, 3-thienylboronic acid (3TB) was reported to cause motor disruption and neuronal damage in mice (Farfán-García *et al.* 2016). In another study, tetraphenylboron (TPB) anion increased the inhibitory effects of MPP^+ in isolated mouse liver mitochondria and a greater dopaminergic neurotoxicity was reported in mice receiving the combination of TPB and MPTP (Heikkila *et al.* 1990). Pérez-Rodríguez *et al.* (2017) examined the toxic effects of four boronic acids having a five-membered cycle similar to 3TB. In their study motor disruption was not induced by all boronic acids with five-membered cycle and they suggested that degrees of the motor system disruption depended on the diverse chemico-morphological changes of the compounds (Pérez-Rodríguez *et al.* 2017).

A significant increase was observed in the expression of *lrrk* in the MPTP group compared with the control group. Mitochondrial impairment has been shown to activate LRRK2 before the presence of neurodegeneration (Di Maio *et al.* 2018). Accordingly Di Maio (2018) showed that rotenone treatment and α-synuclein induced reactive oxygen species (ROS) activated LRRK2. Both low and high dose 3-pyridinylboronic acid decreased *lrrk* expressions significantly which may be due to decreased ROS formation. Boron has been shown to stimulate antioxidant enzymes, in particular the enzymes related to the ascorbate cycle (Pasa *et al.* 2016). Boron treatment has been shown to improve the arsenic-induced changes in the oxidant-antioxidant system

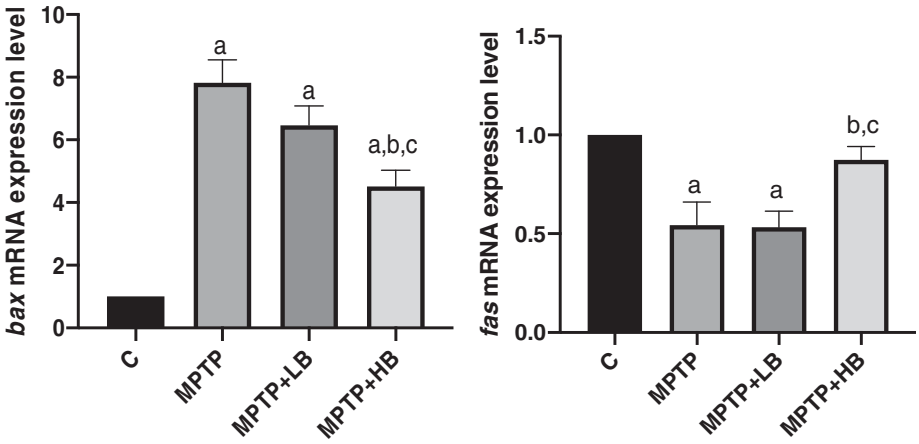

Figure 7. *bax* and *fas* expressions of the groups. Data are expressed as mean + SD from the three independent experiments. [a] significantly different from the control group, $p < 0.05$; [b] significantly different from the MPTP group, $p < 0.05$; [c] significantly different from the MPTP + LB group. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; LB: Low dose 3-Pyridinylboronic acid; HB: High dose 3-Pyridinylboronic acid; SD: standard deviation.

parameters and ameliorated the increase in DNA damage and proinflammatory cytokine gene expressions (Ince *et al.* 2019).

3-pyridineboronic acid increased the expressions of *bdnf* and *dj1* which were decreased in the MPTP group. BDNF is a neurotrophic factor, and it is important for the neuronal development, protection, survival and synaptic plasticity (Tang *et al.* 2016). DJ-1 acts as an oxidative stress sensor to lower synuclein accumulation in PD models (Lee *et al.* 2017). Based on our results, 3-pyridinylboronic acid may be suggested to lower ROS formation by the stimulation of antioxidant enzymes leading to a decrease in *lrrk* and increase in *bdnf* and *dj1* expressions. Similar to our results appropriate supplementation of boric acid (160 mg/L) has been shown to promote ostrich chicks' brain development by promoting BDNF expression and reducing cell apoptosis. It was previously reported that oxidative stress induced by MPTP leads to apoptosis through the activation of BCL-2 family proteins and pro-apoptotic BAX (Yang *et al.* 1997; Crompton 2000). Apoptosis of dopaminergic neurons due to caspase activation has been shown to be involved in PD pathogenesis. Caspase-3 is both vital for the development of the normal brain and for the typical progress of apoptosis. It is known as the executioner of downstream apoptosis (Porter and Jänicke 1999; Yamada *et al.* 2010). MPTP has been shown to activate caspase-3, 8, 9, and 11 and apoptosis in the substantia nigra of MPTP-treated rats (Hartmann *et al.* 2001; Turmel 2001; Viswanath *et al.* 2001; Yamada *et al.* 2010).

In accordance with these reports in our study, significant increases were observed in *p53, casp3a* and *bax* expressions in the MPTP group. P53 is an important transcriptional activator as it acts like a sensor of cellular stress in conditions including DNA damage and oxidative stress. Normally when the cell is not stressed, P53 protein concentration is very low due to its degradation by E3 ubiquitin ligase. When P53 is activated in response to stress stimuli it regulates the transcription of many genes to control different cellular processes. Accordingly P53 controls apoptosis to induce cell death through transcriptional activation of both the death receptor and many downstream target genes, including BAX (Aubrey*et al.* 2018). BAX expression is upregulated by P53, and BAX is involved in p53-mediated apoptosis (Chipuk *et al.* 2004).

In the present study, both low and high 3-pyridinylboronic acid decreased *p53* and *casp3a* expressions when compared with the MPTP group. On the other hand, *bax* expression decreased only in the high 3-pyridinylboronic acid exposed MPTP group. In accordance with our findings, Routray and Ali (2019) reported that boron inhibits apoptosis through the stabilization of the structure of mitochondrial membrane that inhibits cytochrome c release from the mitochondria.

Apoptosis is initiated by intrinsic or extrinsic pathways. The death receptors such as Fas and TNFR are involved in the extrinsic apoptotic pathway. The intrinsic pathway triggers apoptosis in response to internal stimuli including stress and DNA damage. Bax and Bcl-2 group of molecules modulate the intrinsic pathway (van Loo 2002). Fas receptor (CD95, TNF receptor superfamily member 6) is a death receptor that is localized on the surface of many cells. Fas activates a signal transduction pathway leading to apoptosis (Nagata and Golstein 1995). P53 regulates apoptosis through the transport of Fas from cytoplasmic stores. Therefore, the overexpression of p53 increases the expression of Fas in cell surface (Bennett *et al.* 1998). However, in our study, decreased *fas* expression was observed in the MPTP group.

Conclusion

Based on the results of our study MPTP may be suggested to induce a P53 dependent and Bax mediated apoptosis in zebrafish embryos rather than the extrinsic apoptotic pathway as *fas* expression was not activated. Moreover our study is first to show that 3-pyridinylboronic acid had a positive effect by restoring the altered gene expressions related to mitochondrial dysfunction and increased oxidative stress due to the deleterious effects of MPTP, in a dose-dependent manner. Given the rapid increase in the use of boronic acid

compounds in drug design and discovery, we believe the results of our study would contribute to the current knowledge on the biological properties of 3-pyridinylboronic.

Limitations of the study

In this study oxidant and antioxidant parameters were not measured which can be considered as a limitation. Oxidant-antioxidant analyses could provide supporting data for the mitochondrial dysfunction as evidenced by gene expressions. Also lack of prior research on the biological activities of 3-pyridinylboronic limited the discussion of previous data on the subject.

Disclosure statement

The authors report no conflicts of interest.

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