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ORIGINAL PAPER

3-Pyridinylboronic Acid Ameliorates Rotenone-Induced Oxidative Stress Through Nrf2 Target Genes in Zebrafish Embryos

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Abstract

Parkinson's disease (PD) is one of the most common forms of neurodegenerative diseases and research on potential therapeutic agents for PD continues. Rotenone is a neurotoxin that can pass the blood–brain barrier and is used to generate PD models in experimental animals. Boron is a microelement necessary for neural activity in the brain. Antioxidant, non-cytotoxic, anti-genotoxic, anti-carcinogenic effects of boric acid, the salt compound of boron has been reported before. Boronic acids have been approved for treatment by FDA and are included in drug discovery studies and pyridine boronic acids are a subclass of heterocyclic boronic acids used in drug design and discovery as substituted pyridines based on crystal engineering principles. The aim of our study was to determine the effect of 3-pyridinylboronic acid in rotenone-exposed zebrafish embryos, focusing on oxidant-antioxidant parameters and gene expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2) target genes *gclm*, *gclc*, *hmox1a*, *nqo1*, and PD related genes, brain-derived neurotrophic factor, *dj1,* and *tnfα.* Zebrafish embryos were exposed to Rotenone (10 µg/l); Low Dose 3-Pyridinylboronic acid (100 µM); High Dose 3-Pyridinylboronic acid (200 µM); Rotenone + Low Dose-3-Pyridinylboronic acid (10 µg/l + 100 µM); Rotenone + High Dose-3-Pyridinylboronic acid (10 µg/l + 200 µM) in well plates for 96 h post-fertilization (hpf). Our study showed for the first time that 3-pyridinylboronic acid, as a novel sub-class of the heterocyclic boronic acid compound, improved locomotor activities, ameliorated oxidant-antioxidant status by decreasing LPO and NO levels, and normalized the expressions of *bdnf*, *dj1*, *tnf*α and Nrf2 target genes *hmox1a* and *nqo1* in rotenone exposed zebrafish embryos. On the other hand, it caused the deterioration of the oxidant-antioxidant balance in the control group through increased lipid peroxidation, nitric oxide levels, and decreased antioxidant enzymes. We believe that these results should be interpreted in the context of the dose-toxicity and benefit-harm relationship of the effects of 3-pyridinylboronic.

Keywords Parkinson's disease · Rotenone · 3-Pyridinylboronic acid · Zebrafish embryos

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Introduction

Boron is used by plants and humans as a microelement and in nature boron can be found as boron salts. Boric acid, a salt compound of boron, has been reported to be a metal chelator and has antioxidant, non-cytotoxic, anti-genotoxic, anti-carcinogenic effects [1–5]. In brain, boron is necessary for neural activity and boron deficiency leads to defects in brain electrical activity, movement as well as loss of consciousness and psycho-motor activity, and short-term memory weakness [6, 7].

In organic chemistry, boronic acids are used as chemical building blocks and in the Suzuki coupling reactions, they are used as intermediates. Boronic acids have been approved for treatment by FDA and are included in drug discovery studies [8]. In recent years pyridine boronic acids have been used as a subclass of heterocyclic boronic acids in drug design and discovery as substituted pyridines based on crystal engineering principles [9, 10].

Parkinson's disease (PD) continues to be one of the most common neurodegenerative diseases in the world, and research on potential therapeutic agents for PD and neurotoxic agents to be used to generate PD model continues. In PD, oxidative stress plays a key role in the destruction of dopaminergic neurons. Physiologic redox potential maintenance in neurons is disrupted, interfering with various biological processes and ultimately causing cell death. Mitochondrial dysfunction and neuroinflammatory cells are among the known sources and pathways for the formation of reactive oxygen species (ROS) [11] DJ-1 is one of the PD-causing gene products that affects mitochondrial function, resulting in increased ROS production and vulnerability to oxidative stress. Nuclear factor erythroid-2-related factor 2 (Nrf2) is a transcription factor inducing the expression of various cytoprotective and antioxidant enzymes to maintain a possible neuroprotective strategy in PD through the attenuation of oxidative stress [12].

Despite the obvious distinctions between fish and mammals, zebrafish and humans share genomic and physiological homology. Most of the organs, including the nervous system, have physiological and anatomical similarity [13]. The chemistry, cellular populations, and basic anatomical structure of the zebrafish and human nervous systems are all evolutionarily conserved. In the last 20 years, several brain diseases models including PD, have been generated in zebrafish at various stages of development as embryonic, larval, and young adult. Early developmental stages of zebrafish allow researchers to investigate the function of individual genes during system development and maturity. On the other hand, when extrapolating the phenotypes observed to chronic and late-onset conditions, caution must be taken as the adult brain of zebrafish is more complex [13, 14].

There is no study in the literature on the effects of boronic acid compounds on oxidative stress associated with the development of mitochondrial dysfunction, which is effective in the pathophysiology of PD. Therefore, in our study by using zebrafish embryo as a model organism, we wanted to determine the effects of 3-pyridinylboronic acid on the neurotoxin-induced oxidative stress at the molecular level.

Rotenone and 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) are the most commonly used neurotoxins to induce experimental PD models through dopaminergic neurodegeneration [15]. Both MPTP and rotenone inhibit mitochondrial complex I, however, MPP^+, the active metabolite of MPTP, is reported to have a weak inhibitory effect on mitochondrial complex I [16, 17]. We have previously reported that MPTP exposed zebrafish embryos exhibited apoptosis which was dependent on P53 and mediated by Bax and in this model 3-pyridinylboronic acid ameliorated the locomotor activity and normalized the gene expressions that were impaired related to mitochondrial dysfunction [18]. In our present study, we aimed to further determine the effect of 3-pyridinylboronic acid in zebrafish embryos using rotenone as a different neurotoxin that can pass both the blood–brain barrier and the plasma membrane, focusing on oxidant-antioxidant parameters and Nuclear factor erythroid 2-related factor 2 (Nrf2) target genes.

Methods

Chemicals Tested

Rotenone (CAS no: 83–79-4) and dimethyl sulfoxide (DMSO) (CAS no: 67–68-5) were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Rotenone dissolved in 0.1% DMSO. They were all analytical grade with the highest purity available.

3-Pyridinylboronic acid (CAS no.1692–25–7) was obtained from Sigma-Aldrich. Syntheses and recrystallizations were made in air using standard glassware. The complete evaporation of a dilute HCl solution of 3-Pyridinylboronic acid led to the quantitative formation of white 3-Pyridinylboronic acid crystals. After collecting these white crystals they were washed in 95% ice-cold ethanol and they were dried in air [18, 19].

Maintenance of Zebrafish

Zebrafish (AB/AB Strain) were housed in an aquarium rack system (Zebtec, Tecniplast, Italy) in disease-free condition and at 27 ± 1 °C under a light/dark cycle of 14/10 h. Fish were fed commercial flake fish food complemented with live Artemia twice a day. System water pH is between 6.9 and 7.2. Reverse osmosis water that contains 0.018 mg/L

Instant Ocean™ salt was used for all experiments. After natural spawnings, fertilized embryos were gathered and staged according to their developmental and morphology as described before [20].

Embryo Exposure and Determination of Locomotor Activity

Zebrafish embryos were exposed to Rotenone (10 µg/l); 3-Pyridinylboronic acid Low Dose (100 µM); 3-Pyridinylboronic acid High Dose (200 µM); Rotenone + 3-Pyridinylboronic acid Low Dose (10 µg/l + 100 µM); Rotenone + 3-Pyridinylboronic acid High Dose (10 µg/l + 200 µM) in well plates for 96 h post-fertilization (hpf). The blank control was the embryo medium. The workflow of sample preparation and experimental procedures used in the study is given in Fig. 1. Sufficient number of healthy and freshly fertilized eggs for each concentration and control treatment are required for the toxicity studies with zebrafish embryos to maintain reliable dose–response relationships of the tested compounds. In our study, totally six biological replicates of pools of zebrafish embryos (50 embryos/pool) for each group were prepared to attain a sufficient amount for RNA pool (50 embryos/pool; three biological replicates for each group) and for the biochemical analyses (50 embryos/pool; three biological replicates for each group). In the rotenone treated groups a total of ten embryos died which were replaced to maintain sufficient amount for the analyses.

Each day the exposure solutions were changed with fresh solutions. Each day developmental parameters were viewed using a stereomicroscope (Zeiss Discovery V8, Germany), malformations were captured and documented. 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 [20].

Determinations of locomotor activity, gene expression, biochemical and statistical analyses were performed by different investigators blinded to the treatments. The locomotor activity of the zebrafish embryos at 96 hpf ($n = 50$, each group) was evaluated in a blinded manner as described previously [21]. 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.

At the end of exposure period, zebrafish embryos at 96 hpf were immobilized by submersion in ice water for 10 min and then in sodium hypochlorite (6.15%) for 5 min to ensure death.

Reverse Transcription (cDNA Synthesis) and Quantitative Real-time PCR

For RNA isolation from each group, three biological replicates were prepared as pools of zebrafish embryos. Each replicate consisted of 50 embryos. 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 RT2 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 housekeeping gene. Relative levels of transcription were calculated in a blinded manner, using the ΔΔCT method based on the normalization of the values using the housekeeping gene [22]. The list of the primers used are given in Table 1.

Oxidant-antioxidant Parameters

To attain a sufficient amount for the biochemical analyses, three biological replicates were prepared as pools of

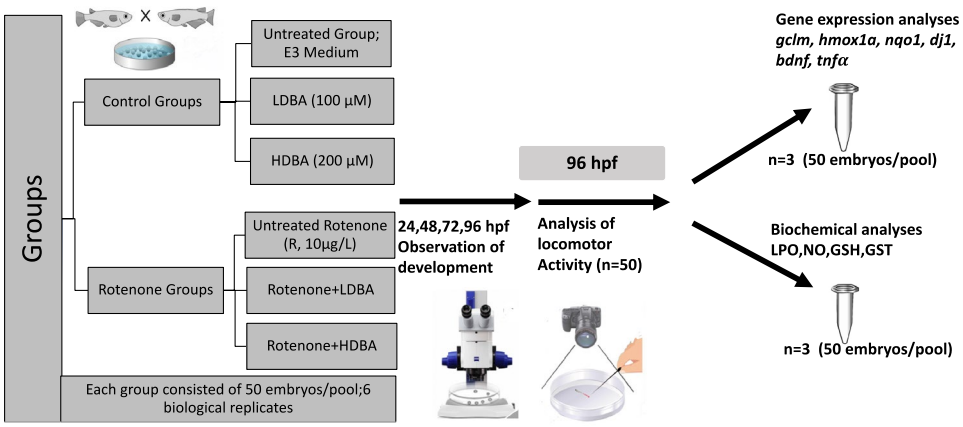

Fig. 1 Workflow of sample preparation and experimental procedures used in the study. *C* control, *R* rotenone, *LCBA* low concentration 3-Pyridinylboronic acid, *HCBA* high concentration 3-Pyridinylboronic acid, *R* + *LCBA* rotenone + low concentration 3-Pyridinylboronic acid; *R* + *HCBA* rotenone + high concentration 3-Pyridinylboronic acid

Table 1 Forward and reverse primers used in the study

	Primerler (forward/reverse)
gclm	5′-TCCAGATCTCACTGCATTCG-3′ 5′-ATGCCTCTGCTCTTGACGAT-3′
gclc	5′-CGCTGCACAATCCTCAACTA-3′ 5′-CGATCAGTTCCGGACATTTT-3′
hmox1a	5′-CCACGTCAGAGCTGAAAACA-3′ 5′-CGAAGAAGTGCTCCAAGTCC-3′
nqo1	5′-CTCAAGGATTTGCCTTCAGC-3′ 5′-CGCAGCACTCCATTCTGTAA-3′
β aktin	5′AAGCAGGAGTACGATGAGTCTG-3′ 5′-GGTAAACGCTTCTGGAATGAC-3′
bdnf	5′-ATAGTAACGAACAGGATGG-3′ 5′-GCTCAGTCATGGGAGTCC-3′
dj1	5′-GGCCGGTAAAAGAGCGTTAG-3′ 5′-ACCCATGAGTCCTCCACTA-3′
tnfα	5′-GCTGGATCTTCAAAGTCGGGTGTA-3′ 5′-TGTGAGTCTCAGCACACTTCCATC-3′

zebrafish embryos. For each pool, zebrafish embryos were homogenized in 1 ml PBS and centrifuged briefly. The supernatant was used for the determination of biochemical parameters. All biochemical analyses were performed and results were calculated by different investigators blinded to the treatments.

Total Protein Determination

Total protein level was determined by the method of Lowry. In this method, proteins are first reacted with copper ions in an alkaline medium. It is then reduced with phosphomolybdic—phosphotungstic acid reagent (foline reagent). The intensity of the blue color, which is directly proportional to the protein concentration, is measured with a spectrophotometer at 500 nm. The results of the parameters are expressed per protein. [23].

Lipid Peroxidation (LPO) Determination

The method of Yagi was used to the absorbance of the pinkish color formed as a result of the reaction between malondialdehyde (MDA), a product of LPO, and thiobarbituric acid (TBA) is evaluated spectrophotometrically. The extinction coefficient of 1.56_105 M^{-1}/cm was used and LPO was expressed in terms of MDA equivalents as nmol MDA/mg protein [24].

Nitric Oxide Determination

The levels of Nitric oxide (NO) were evaluated by using the method of Miranda. In this method, nitrate is reduced to nitrite by vanadium (III) chloride. Then using an acidic media, nitrite formed, and sulfonyl amide reacted with N-(1-Naphtyl) ethylenediamine dihydrochloride and complex diazonium compound was produced. After the measurement of the color complex at 540 nm with a spectrophotometer, the results were calculated and presented as nmol NO/mg protein. [25].

GSH Determination

The colored product formed as a result of the reaction of Elmann reagent, 5–5′ dithiobis 1–2 nitro benzoic acid (DTNB) and sulfhydryl groups is evaluated spectrophotometrically [26].

Glutathione-S-transferase (GST) Determination

The determination of Glutathione-S-transferase activity was performed through the spectrophotometric determination at 340 nm of the absorbance of the product formed by the conjugation of GSH and 1-chloro-2,4-dinitro-benzene (CDNB) [27].

Statistical Analyses

All experiments using zebrafish embryos were performed and replicated three times with 50 embryos per trial. The minimum embryos required to produce statistically significant results was estimated using power analysis-G*Power 3.1 (https://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/). Using the significance level ($\alpha = 0.05$), power ($\beta = 0.8$), and the effect size ($f = 0{,}4$ representing a large effect) it was found that a minimum of 50 embryos were needed per experimental group to obtain a statistically significant sample size. Outliers were detected with the Grubbs test and were excluded. The effects of rotenone and 3-Pyridinylboronic acid on zebrafish embryos were analyzed using the Shapiro–Wilk test for data normality. Statistical significance for the factors rotenone treatment and 3-Pyridinylboronic acid treatments were determined using a Two-Way ANOVA with the Sidak multiple comparison test, in a blinded manner. Statistical analysis was performed using GraphPad Prism 9. Results were given as mean ± standard deviation and $p < 0.05$ was considered significant.

Results

Results of Developmental Analyses

The representative images of the zebrafish embryos are presented in Fig. 2A. Head malformation, pericardial edema, hemorrhage, spinal curvature, and dorsal curvature were observed in the rotenone exposed zebrafish embryos at 48, 72, and 96 hpf. At 72 hpf pericardial edema was observed in

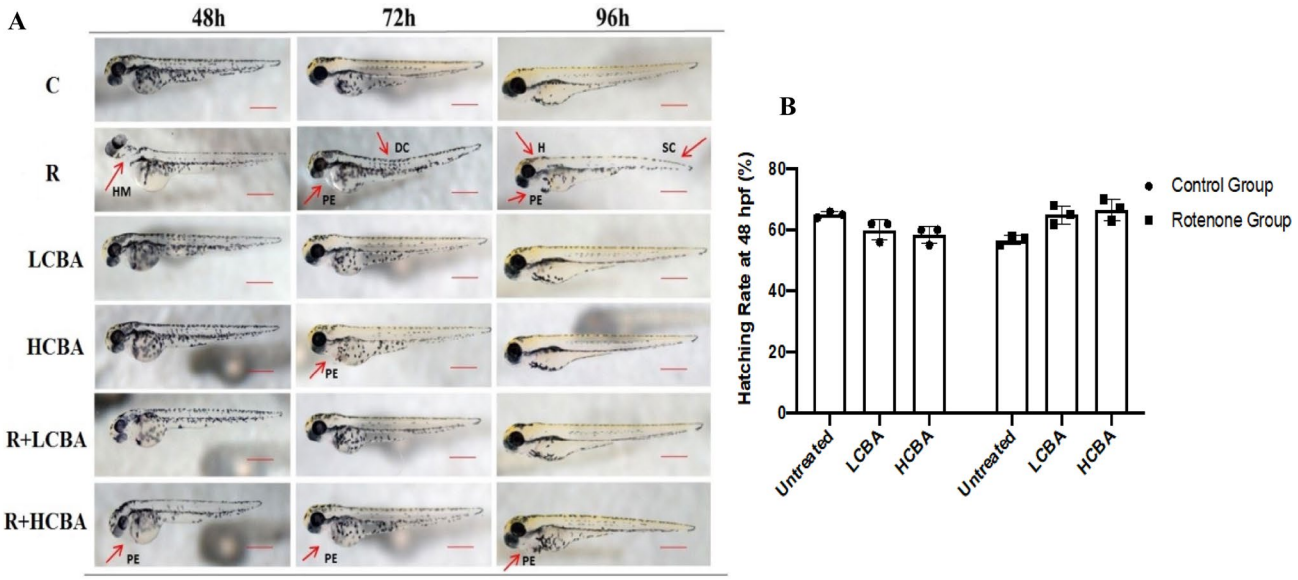

Fig. 2 **A** Representative images of the zebrafish embryos in the groups. *HM* head malformation, *PE* pericardial edema, *H* hemorrhage, *SC* spinal curvature, *DC* dorsal curvature **B** Hatching rates of the groups. Data are expressed as mean ± SD from the three independent experiments ($n = 3$, three biological replicates for each group, 50 embryos/pool). *C* control, *R* rotenone, *LCBA* low concentration 3-Pyridinylboronic acid, *HCBA* high concentration 3-Pyridinylboronic acid, R + LCBA: Rotenone + Low concentration 3-Pyridinylboronic acid, *R* + *HCBA* rotenone + high concentration 3-Pyridinylboronic acid, *SD* standard deviation

the high 3-Pyridinylboronic acid treated embryo. Although not statistically significant, there was a trend for lower hatching rates in 3-pyridinylboronic acid-treated control groups when compared to the Control group. A trend was also seen towards higher hatching rates in 3-pyridinylboronic acid-treated rotenone groups when compared to the Rotenone group (Fig. 2B).

Results of Locomotor Activity

Two-way ANOVA revealed that there was a statistically significant interaction between the effects of rotenone and 3-pyridinylboronic acid treatments ($F = 36.18$, $p < 0.0001$). Simple main effects analysis showed that both rotenone and 3-pyridinylboronic treatments had statistically significant effect on locomotor activities ($F = 270.6$, $p < 0.0001$ and $F = 85.97$, $p < 0.0001$ respectively). Locomotor activity of the groups was determined as average escape response which decreased significantly in the rotenone group compared to the control group ($p < 0.0001$). Only the high 3-pyridinylboronic acid treatment increased locomotor activity significantly in the control groups ($p < 0.01$). In the rotenone groups, both low and high 3-pyridinylboronic acid treatments increased locomotor activities significantly ($p < 0.0001$). Moreover, the locomotor activity of the high 3-pyridinylboronic acid-treated rotenone group was significantly higher than the low high 3-pyridinylboronic acid-treated rotenone group ($p < 0.01$) (Fig. 3).

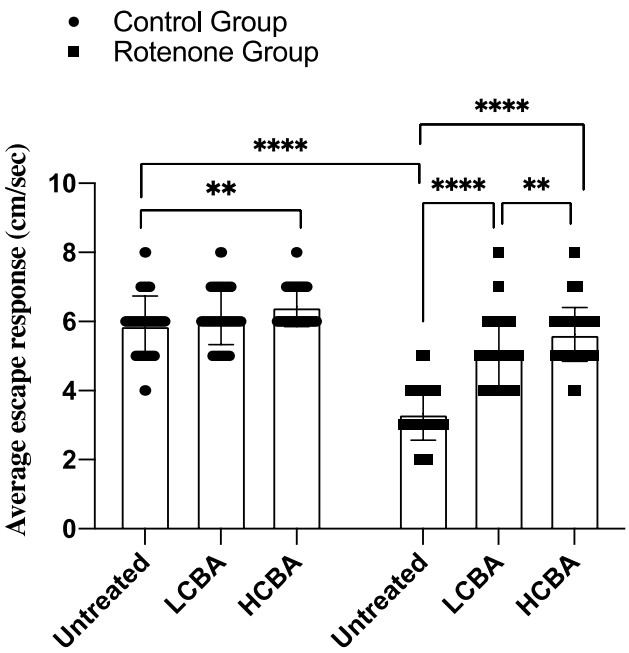

Fig. 3 Locomotor activities of the groups. Data are expressed as mean ± SD ($n = 50$ embryos). $^{**}p < 0.01$; $^{****}p < 0.0001$; *C* control, *R* rotenone; *LCBA* low concentration 3-Pyridinylboronic acid, *HCBA* high concentration 3-Pyridinylboronic acid, *R* + *LCBA* rotenone + low concentration 3-Pyridinylboronic acid, *R* + *HCBA* rotenone + high concentration 3-Pyridinylboronic acid, *SD* standard deviation

Results of Oxidant-Antioxidant Analyses

Results of LPO Analysis

The oxidant-antioxidant status of the 3-Pyridinylboronic acid and rotenone exposed zebrafish embryos were evaluated by the LPO, NO, GSH, and GST assays. For LPO analysis, there was a statistically significant interaction between the effects of rotenone and 3-pyridinylboronic acid treatments ($F=49$, $p<0.0001$). Simple main effects analysis showed that both rotenone and 3-pyridinylboronic treatments had statistically significant effect on LPO levels ($F=16$, $p<0.001$ and $F=38$, $p<0.0001$ respectively). LPO levels of the Rotenone group increased significantly when compared to the Control group ($p<0.0001$). LPO levels increased significantly in the HCBA group when compared to the LCBA group ($p<0.05$). In the rotenone-treated groups, both 3-Pyridinylboronic acid concentrations decreased LPO levels significantly ($p<0.0001$) (Fig. 4A).

Results of NO Analysis

There was a statistically significant interaction between the effects of rotenone and 3-pyridinylboronic acid treatments ($F=18$, $p<0.001$). Rotenone and 3-pyridinylboronic treatments did not have a statistically significant effect on NO levels ($F=0.4$, $p>0.05$ and $F=0.1$, $p>0.05$ respectively). NO levels of the Rotenone group increased significantly when compared to the Control group ($p<0.05$). In the control group, high 3-pyridinylboronic acid treatment increased NO levels significantly ($p<0.0001$). However, in the rotenone group high 3-pyridinylboronic acid treatment led to a significant decrease in NO levels ($p<0.05$) (Fig. 4B).

Results of GSH Analysis

There was a statistically significant interaction between the effects of rotenone and 3-pyridinylboronic acid treatments ($F=23$, $p<0.0001$). Simple main effects analysis showed that 3-pyridinylboronic treatment had a statistically

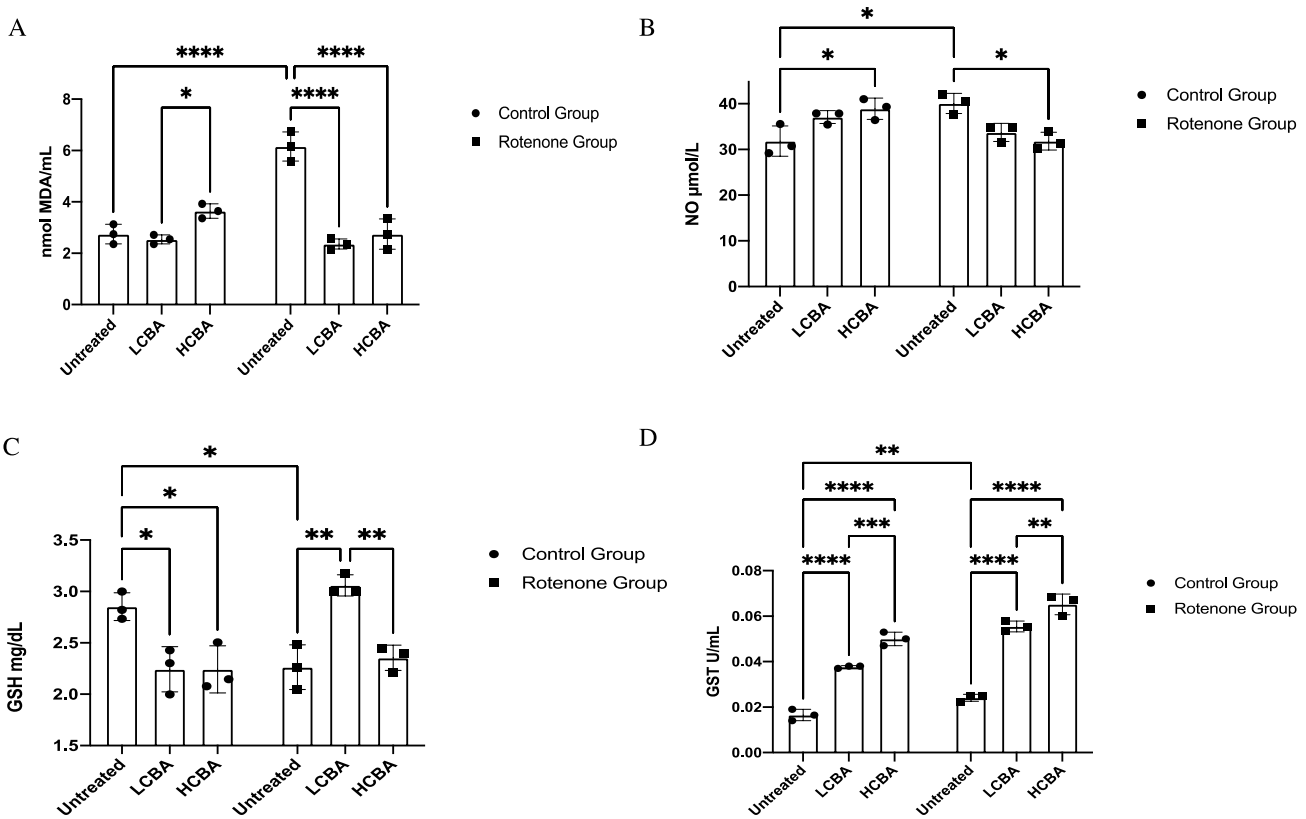

Fig. 4 **A** Malondialdehyde (MDA) levels as an index of lipid peroxidation (LPO); **B** Nitric Oxide (NO) levels; **C** Glutathione (GSH) levels; **D** Glutathione S-Transferase activities of the groups. Data are expressed as mean ± SD from the three independent experiments ($n=3$, three biological replicates for each group, 50 embryos/pool). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$. *C* control, *R* rotenone, *LCBA* low concentration 3-Pyridinylboronic acid, *HCBA* high concentration 3-Pyridinylboronic acid, *R*+*LCBA* rotenone + low concentration 3-Pyridinylboronic acid, *R*+*HCBA* rotenone + high concentration 3-Pyridinylboronic acid, *SD* standard deviation

significant effect on GSH levels (F = 6.2, $p < 0.05$) but rotenone treatment did not have a statistically significant effect on GSH levels (F = 1.8, $p > 0.05$). GSH levels decreased significantly in the R, LCBA, and HCBA groups when compared with the Control group ($p < 0.05$). In the Rotenone groups, only low 3-Pyridinylboronic acid treatment increased GSH levels significantly ($p < 0.01$) and GSH levels of the Low 3-Pyridinylboronic acid treated-Rotenone group was significantly higher than High 3-Pyridinylboronic acid-treated Rotenone group ($p < 0.01$) (Fig. 4C).

Results of GST Analysis

A statistically significant interaction was observed between the effects of rotenone and 3-pyridinylboronic acid treatments (F = 5.6, $p < 0.05$). Both rotenone and 3-pyridinylboronic treatments had statistically significant effect on GST levels (F = 111, $p < 0.0001$ and F = 299, $p < 0.0001$ respectively). GST activities increased significantly in the Rotenone, LCBA, HCBA groups when compared with the Control group ($p < 0.01$; $p < 0.0001$; $p < 0.0001$). When compared with the Rotenone group, GST activities increased in both 3-Pyridinylboronic acid-treated groups ($p < 0.001$). GST activity of the High 3-Pyridinylboronic acid-treated Rotenone group was significantly higher than the Low 3-Pyridinylboronic acid-treated Rotenone group ($p < 0.01$)) (Fig. 4D).

Results of Gene Expression Analyses

Results of gclm Expressions

For *gclm* expressions, the interaction between the effects of rotenone and 3-pyridinylboronic acid treatments was statistically significant (F = 25, $p < 0.0001$). Simple main effects analysis showed that both rotenone and 3-pyridinylboronic treatments had statistically significant effect on *gclm* expressions (F = 28, $p < 0.001$ and F = 7.9, $p < 0.01$ respectively). *gclm* expressions decreased significantly in the Rotenone, and in both Low and High Pyridinylboronic acid-treated control groups ($p < 0.0001$; $p < 0.01$; $p < 0.001$ respectively). In the Rotenone groups only low 3-Pyridinylboronic acid

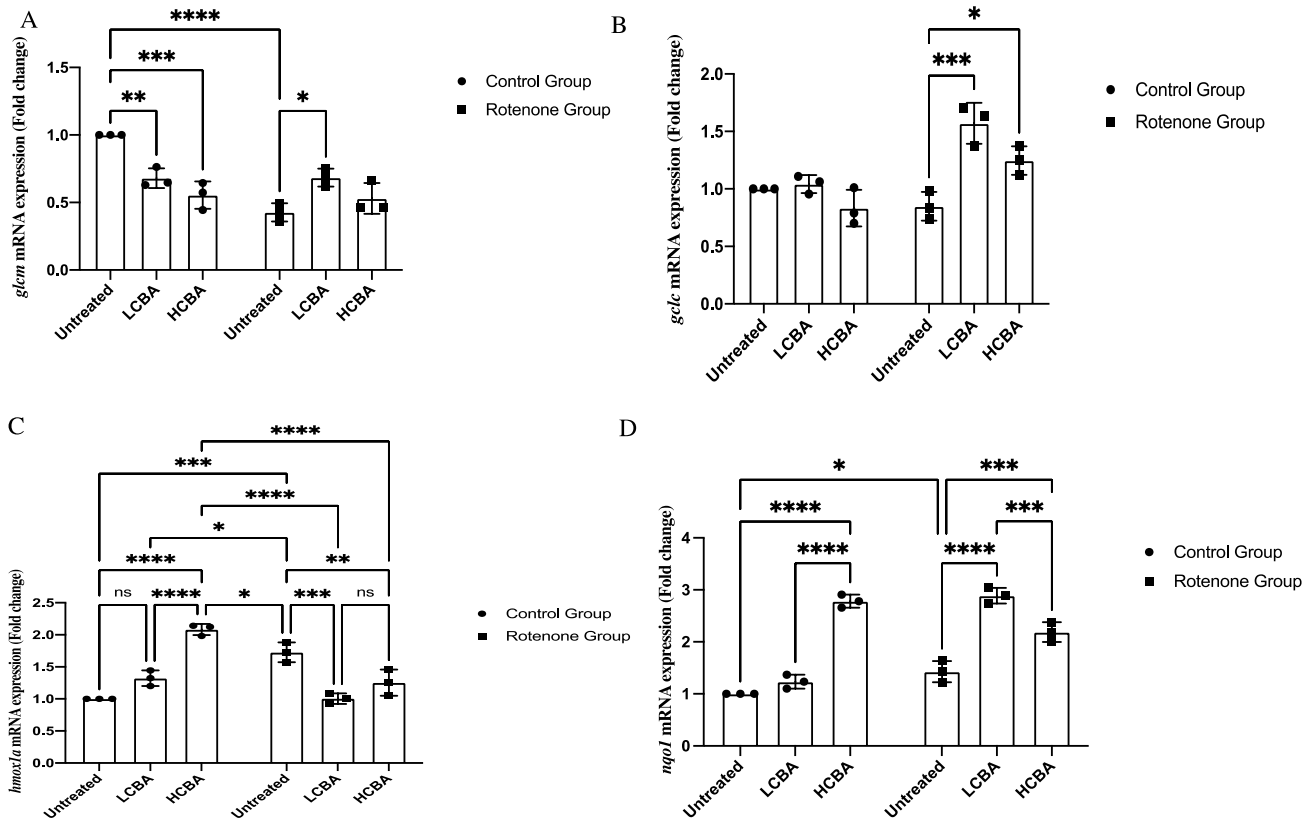

Fig. 5 Bar graph presentation of the fold change of **A** *gclm* **B** *gclc* **C** *hmox1a* **D** *nqo1* transcripts quantified by RT-PCR. All RT-PCR results are normalized to β-actin, the housekeeping gene, and expressed as change from their respective controls. The average values were obtained from three experiments ($n = 3$, three biological replicates for each group, 50 embryos/pool). Data presented are mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. *C* Control, *R* rotenone, *LCBA* low concentration 3-Pyridinylboronic acid, *HCBA* high concentration 3-Pyridinylboronic acid, *R + LCBA* rotenone + low concentration 3-Pyridinylboronic acid, *R + HCBA* rotenone + high concentration 3-Pyridinylboronic acid, *SD* standard deviation

treatment increased *gclm* expression significantly ($p < 0.05$) (Fig. 5A).

Results of gclc Expressions

There was a statistically significant interaction between the effects of rotenone and 3-pyridinylboronic acid treatments ($F = 12$, $p < 0.01$). Both rotenone and 3-pyridinylboronic treatments had statistically significant effect on *gclc* expressions ($F = 19$, $p < 0.001$ and $F = 14$, $p < 0.001$ respectively). In the rotenone groups, both low and high 3-Pyridinylboronic acid treatments increased *gclc* expressions significantly ($p < 0.001$ and $p < 0.05$ respectively) (Fig. 5B).

Results of hmox1a Expressions

A statistically significant interaction was observed between the effects of rotenone and 3-pyridinylboronic acid treatments ($F = 59$, $p < 0.0001$). Rotenone and 3-pyridinylboronic treatments had statistically significant effect on *hmox1a* expressions ($F = 5.6$, $p < 0.05$ and $F = 24$, $p < 0.0001$ respectively). *hmox1a* expressions increased significantly in both Low and High 3-Pyridinylboronic acid-treated control groups ($p < 0.05$ and $p < 0.0001$ respectively) and *hmox1a* expressions of the High 3-Pyridinylboronic acid-treated control group was significantly higher than the Low 3-Pyridinylboronic acid-treated control group ($p < 0.0001$). When compared with the Control group *hmox1a* expression increased in the Rotenone group ($p < 0.001$) and both low and high 3-Pyridinylboronic acid treatments decreased *hmox1a* expressions in Rotenone groups (Fig. 5C).

Results of nqo1 Expressions

For *nqo1* expressions, the interaction between the effects of rotenone and 3-pyridinylboronic acid treatments was statistically significant ($F = 85$, $p < 0.0001$). Both rotenone and 3-pyridinylboronic treatments had statistically significant effect on *nqo1* expressions ($F = 49$, $p < 0.0001$ and $F = 113$, $p < 0.0001$ respectively). *nqo1* expressions increased significantly in the High 3-Pyridinylboronic acid treated control group ($p < 0.0001$) and the High 3-Pyridinylboronic acid-treated control group had significantly higher *nqo1* expressions compared to the Low 3-Pyridinylboronic acid-treated group ($p < 0.0001$). *nqo1* expressions of the Rotenone group was significantly higher than the Control group ($p < 0.05$) and both low and high 3-Pyridinylboronic acid treatments increased *nqo1* expressions in the rotenone groups ($p < 0.0001$ and $p < 0.001$ respectively). *nqo1* expressions of the Low 3-Pyridinylboronic acid-treated Rotenone group were significantly higher than the High 3-Pyridinylboronic acid-treated Rotenone group ($p < 0.001$) (Fig. 5D).

Results of bdnf Expressions

There was a statistically significant interaction between the effects of rotenone and 3-pyridinylboronic acid treatments ($F = 37$, $p < 0.0001$). Simple main effects analysis showed that both rotenone and 3-pyridinylboronic treatments had statistically significant effect on *bdnf* expressions ($F = 118$, $p < 0.0001$ and $F = 292$, $p < 0.0001$ respectively).

bdnf expressions increased in both low and high 3-Pyridinylboronic acid-treated control groups ($p < 0.0001$ and $p < 0.001$ respectively) and the Low 3-Pyridinylboronic acid-treated control group had significantly higher *bdnf* expressions than the High 3-Pyridinylboronic acid-treated control group ($p < 0.0001$). Rotenone treatment led to significantly decreased *bdnf* expressions when compared to the Control group ($p < 0.05$). In the rotenone groups, both low and high 3-Pyridinylboronic acid treatments increased *bdnf* expressions significantly ($p < 0.0001$) and the Low 3-Pyridinylboronic acid-treated rotenone group had significantly higher *bdnf* expressions than the High 3-Pyridinylboronic acid-treated rotenone group ($p < 0.05$) (Fig. 6A).

Results of dj1 Expressions

For *dj1* expressions, the interaction between the effects of rotenone and 3-pyridinylboronic acid treatments was statistically significant ($F = 14$, $p < 0.001$). Rotenone and 3-pyridinylboronic treatments had statistically significant effect on *dj1* expressions ($F = 262$, $p < 0.0001$ and $F = 81$, $p < 0.0001$ respectively). *dj1* expressions increased significantly in the Low 3-Pyridinylboronic acid-treated control group ($p < 0.0001$) and the Low 3-Pyridinylboronic acid-treated control group had significantly higher *dj1* expressions than the High 3-Pyridinylboronic acid-treated control group ($p < 0.0001$). *dj1* expressions decreased significantly in the Rotenone group compared to the Control group ($p < 0.0001$). *dj1* expressions increased significantly in the Low 3-Pyridinylboronic acid-treated rotenone group ($p < 0.001$) and this group had significantly higher *dj1* expressions compared to the Low 3-Pyridinylboronic acid-treated rotenone group ($p < 0.05$) (Fig. 6B).

Results of tnfα Expressions

A statistically significant interaction was observed between the effects of rotenone and 3-pyridinylboronic acid treatments ($F = 65$, $p < 0.0001$). Simple main effects analysis showed that both rotenone and 3-pyridinylboronic treatments had statistically significant effect on *tnf*α expressions ($F = 44$, $p < 0.0001$ and $F = 43$, $p < 0.0001$ respectively).

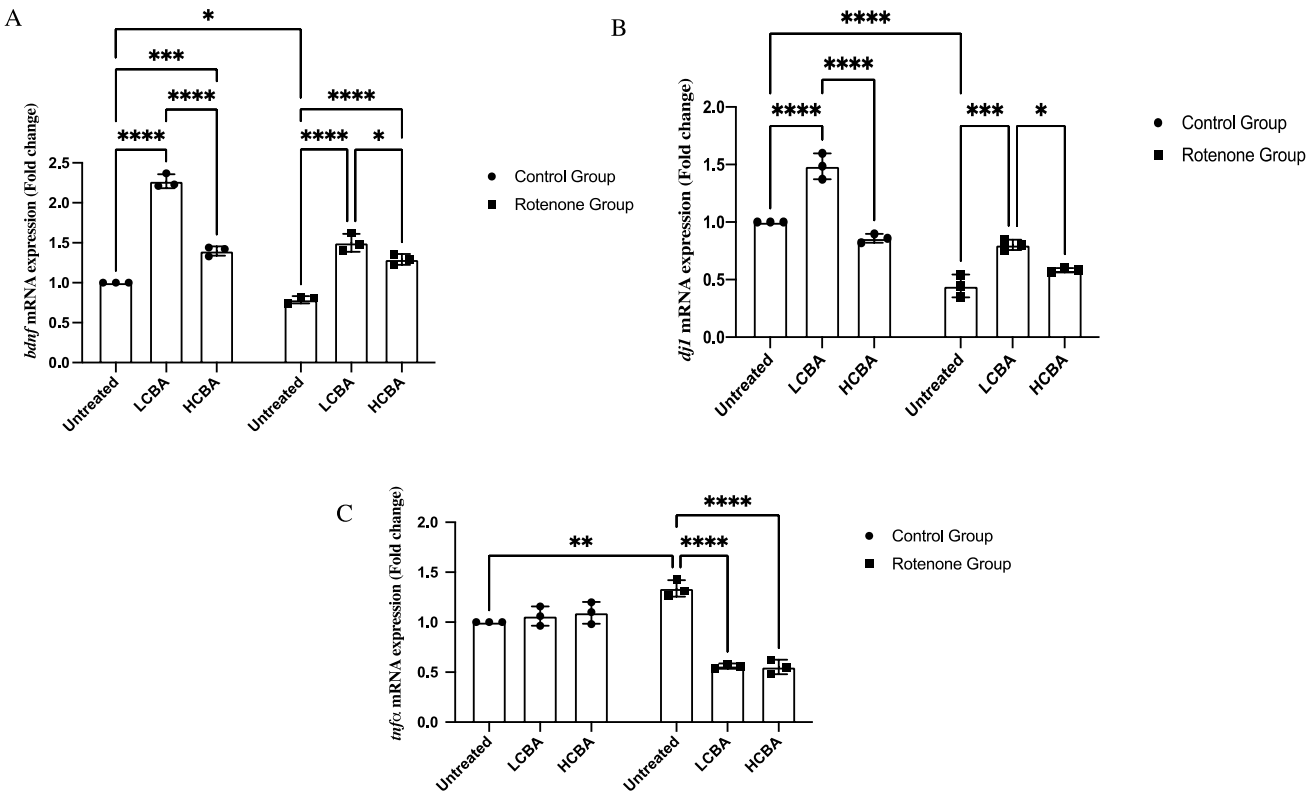

Fig. 6 Bar graph presentation of the fold change of **A** *bdnf* **B** *dj1* **C** *tnf*α transcripts quantified by RT-PCR. All RT-PCR results are normalized to β-actin, the house keeping gene and expressed as change from their respective controls.The average values were obtained from three experiments ($n=3$, three biological replicates for each group, 50 embryos/pool). Data presented are mean ± SD. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$; *C* control, *R* rotenone; *LCBA* low concentration 3-Pyridinylboronic acid; *HCBA* high concentration 3-Pyridinylboronic acid, *R* + *LCBA* rotenone + low concentration 3-Pyridinylboronic acid, *R* + *HCBA* rotenone + high concentration 3-Pyridinylboronic acid, *SD* standard deviation

When compared to the Control group, *tnf*α expressions increased significantly in the Rotenone group ($p<0.01$). On the other hand, both low and high 3-Pyridinylboronic treatments decreased *tnf*α expressions in the rotenone groups ($p<0.001$) (Fig. 6C).

Discussion

In this study, 3-Pyridinylboronic acid improved locomotor activities, ameliorated oxidant-antioxidant status by decreasing LPO and NO levels, and normalized the expressions of *bdnf*, *dj1*, *tnf*α and Nrf2 target genes *hmox1a* and *nqo1* in rotenone exposed zebrafish embryos.

In our previous study, which was the only study on the biological activity of 3-pyridinylboronic acid, 50 and 100 µM 3-pyridinylboronic acid ameliorated the gene expressions related to mitochondrial dysfunction due to MPTP exposure [18]. Nrf2 is a transcription factor and the major mediator that resolves inflammation and after being stabilized following oxidative stress, Nrf2 activates the expression of antioxidants and cytoprotective genes (*hmox1a, nqo1*, *gclm,* and *gclc*) to induce an anti-inflammatory response for healing [28]. Different from our previous study where we showed that MPTP exposure led to a P53 dependent and Bax mediated apoptosis, here in this study we report that rotenone exposure disrupted oxidant-antioxidant status as evidenced by increased LPO, NO levels GST activities, decreased GSH levels, *gclc* expression, and activated adaptive response to oxidative stress through Nrf2 target genes *hmox1a* and *nqo1*.

Rotenone and MPTP are neurotoxins, that have been shown to cause degeneration of dopaminergic neurons, are used to generate experimental PD model. Although MPP^+, inhibits basal respiration similar to rotenone, it induces weaker cytotoxicity. This might be due to an additional effect of rotenone causing cytotoxicity independent of the inhibition of basal respiration. Accordingly it has been reported that MPP^+ interacted through the respiratory chain in a different manner while inhibiting basal cellular respiration, MPP^+ unimpaired electron transport chain but inhibited ATP synthase or related proteins [16]. MPP^+ has also been suggested to act as a cation, was distributed and interacted depending on the mitochondrial membrane potential

[29]. Rotenone is an insecticide and fish poison and cellular respiration inhibition by rotenone lead to a compensatory glycolysis induction, and bioenergetic reserve capacity loss, which in turn activates apoptotic cascade and causes bioenergetic dysfunction and cell death [29]. Here in this study, we showed that rotenone exposure led to decreased *dj1* expressions in zebrafish embryos. In the case of oxidative modification DJ-1 was suggested to act as a cellular redox homeostasis sensor and transportation of DJ-1 to the mitochondria suggested mitochondria as the main localization of DJ-1's neuroprotective activity [30]. As DJ-1 was shown to protect cells from reactive oxygen species, the inhibitory effect of rotenone on *dj1* expression may explain the disrupted oxidant-antioxidant balance.

In our study, we applied higher concentrations of 3-Pyridinylboronic acid as 100 µM and 200 µM and in this model 3-pyridinylboronic acid ameliorated the locomotor activity and normalized oxidant-antioxidant status and gene expressions related to oxidative stress in rotenone exposed zebrafish embryos. LPO is an index of lipid peroxidation and in the High Concentration 3-Pyridinylboronic acid-treated Control group although statistically not significant, there was a trend for an increase in LPO levels compared to the Control group. Moreover, LPO levels in the High 3-Pyridinylboronic acid treated group was significantly higher than the Low 3-Pyridinylboronic acid-treated group. On the other hand, both concentrations of 3-Pyridinylboronic acid normalized the increased LPO levels in the rotenone treated groups. A similar situation was also seen in NO levels. Although the high concentration of 3-Pyridinylboronic acid increased NO levels in the control group, it decreased NO levels in the rotenone exposed group. According to these results, when the increased LPO, NO and decreased GSH values in the 3-Pyridinylboronic acid-treated control group were taken into account, it can be suggested that when 3-Pyridinylboronic acid is given alone, it can shift the oxidant-antioxidant balance towards oxidant damage. On the other hand, in the case of rotenone toxicity, it can be argued that 3-Pyridinylboronic acid affects the oxidant-antioxidant balance in a way to prevent oxidant damage. This argument can be supported by the increased glutathione S-transferase (GSTs) activities in both groups where 3-Pyridinylboronic acid is given alone or in combination with rotenone.

GSTs catalyze the conjugation of glutathione with electrophilic compounds, primarily produced from exogenous xenobiotics by biotransformation [31]. The antioxidant responsive element (ARE) is a cis-acting regulatory element of a number of genes encoding phase II detoxification enzymes and antioxidant proteins [32]. The ARE regulates the transcriptional activation of downstream genes NAD(P)H:quinone oxidoreductase (NQO1) and GSTs [33]. In our study, similar to GST, *nqo1* expression increased in both groups where 3-Pyridinylboronic acid is given alone or in combination with rotenone. As a phase II enzyme, NQO1 catalyzes the reduction of quinone compounds, which prevents the generation of ROS and, protects cells from oxidative damage [34]. Accordingly, increased *nqo1* expressions in the 3-Pyridinylboronic acid-treated control and rotenone groups may indicate the activation of a defense mechanism against increased ROS in these groups.

Hmox1 functions as an adaptive mechanism to protect cells from oxidative damage during stress [35]. Therefore, the increased *hmox* expressions in both 3-Pyridinylboronic acid-treated control groups can be suggested to be related to the increased LPO and NO levels observed in these groups. On the other hand, decreased *hmox* expressions in the 3-Pyridinylboronic acid-treated rotenone groups were in line with the decreased oxidative stress and increased antioxidant defense systems, GST and GSH.

The rate-limiting step in the formation of the cellular antioxidant GSH is catalyzed by glutamate cysteine ligase (GCL). GCL consists of a catalytic subunit (GCLC) and a modifier subunit (GCLM) as two proteins that are coded separately. A variety of cellular stimuli including oxidative stress controls the transcription of both GCLC and GLCM [36]. In our study, in parallel to the decrease in GSH levels 3-Pyridinylboronic acid treatment also decreased *gclm* expressions in the control group and the reduction was more pronounced at the higher concentration. On the other hand, increased *gclm* expressions in the low concentration 3-Pyridinylboronic acid-treated rotenone group can be suggested to be a consequence of the significantly increased oxidative stress indicator lipid peroxidation.

GCLC-deficient mice have been shown to be embryonic lethal, while GCLM-deficient mice were viable and fertile having low GSH levels in their organs and plasma [37]. In our study, while 3-Pyridinylboronic acid treatment did not change *gclc* expressions in the control group, it increased *gclc* expressions significantly in the rotenone group. Increased *gclc* expressions in the rotenone group may be due to a response to oxidative stress observed due to rotenone. On the other hand, the fact that 3-Pyridinylboronic acid treatment decreased *gclm* expressions in the control group but did not change *gclc* expressions may be due to the slight increase in LPO that did not change *gclc* expression in this group.

Boric acid has been shown to alter oxidative metabolism in animals but there are conflicting results in the literature on whether it induces oxidative stress or antioxidant capacity [3, 38, 39]. Decreased antioxidant enzymes have been suggested as the main toxic effect of increasing concentrations of boron compounds [3]. Boron has been suggested to act by affecting the production or utilization of S-adenosylmethionine which is a physiologic precursor of thiols and sulfurated compounds [40]. In previous studies, the effect of boric acid on glutathione reductase enzyme activity was shown to be related to selenium [41, 42]. On the other hand, as boric acid at high doses also decreased the activities of antioxidant enzymes that

are not selenium-dependent, selenium may not be suggested as the only the way that boric acid suppresses antioxidant enzymes. The amount of cAMP is related to the activity of antioxidant enzymes and, at low doses, boron compounds have been suggested to improve activities of antioxidant enzymes through the induction of cAMP accumulation [3].

In our study, although 3-pyridinylboronic acid ameliorated rotenone-induced oxidative stress through Nrf2 target genes, and improved locomotor activity, it caused the deterioration of the oxidant-antioxidant balance in the control group. Gulsoy et al., evaluated the genotoxicity of boron compounds, boric acid (BA) and borax (BX), on zebrafish and reported that DNA damage was highest at 96 h and 24 h for all BX and BA concentrations in peripheral [43]. Boric acid was also shown to cause developmental toxicity in rat, mouse, and rabbit. The toxicity and deficiency of boric acid vary in different animal species and rabbits were found to be most sensitive to the boric acid-induced prenatal mortality [44]. As fish are very sensitive to the alterations in their environment and they play important roles in the evaluation of the potential risks associated with contamination in the aquatic environment of compounds.

In accordance with the previous studies reporting that boron decreased inflammatory biomarkers, we found decreased *tnf*α expression and NO levels in both low and high 3-pyridinylboronic acid-treated rotenone groups. Boron supplementation significantly decreased CRP and TNF-α levels in healthy male volunteers [45].

Although the molecular mechanisms of PD are still not clear, oxidative stress and mitochondrial dysfunction have been suggested to be effective in disease progression [46]. Boron regulates brain activation and boron deprivation results in the decreased electrical activity of brain in both humans and animals [47]. Deprivation of boron caused reduced motor speed, attention, and short-term memory in humans [48]. In our study, both high and low doses of 3-pyridinylboronic acid improved locomotor activity which was decreased in rotenone exposed embryos. BDNF, is suggested to be a potential therapeutic target of PD as a critical regulator of neurodevelopment, central and peripheral nervous systems [49]. Beneficial effects of 3-pyridinylboronic acid were also observed on *bdnf* and *dj-1* as evidenced by their increased expressions. As conclusion although 3-pyridinylboronic acid, as a novel sub-class of the heterocyclic boronic acid compound, ameliorated the rotenone-induced oxidative stress through Nrf2 target genes, and improved locomotor activity in zebrafish embryos, it caused the deterioration of the oxidant-antioxidant balance in the control group. We believe that these results should be interpreted in the context of the dose-toxicity and benefit-harm relationship of the effects of 3-pyridinylboronic.

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Data Availability Data will be available on reasonable request.

Declarations

Conflict of Interest The authors report no conflicts of interest.

Ethical Approval As the zebrafish embryos used were no older than 5 days old, no ethical approval was required for the protocols applied as stated by the Council of Europe (1986), Directive 86/609/EEC.

Consent to Participate All the authors have agreed for authorship, read and approved the manuscript, and given consent to participate.

Consent for Publication All the authors have agreed for authorship, read and approved the manuscript, and given consent for publication.

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