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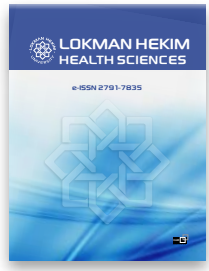

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ORIGINAL ARTICLE

Neuroprotective Potential of Boric Acid in a Rotenone-induced *in Vitro* Parkinson's Disease Model

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Abstract

Introduction: Parkinson's disease (PD) is characterized by progressive dopaminergic neurodegeneration and pathological α-synuclein aggregation. Mitochondrial dysfunction and oxidative stress are central to PD pathogenesis, and mitochondrial complex I inhibitors such as rotenone are widely used to model PD-related neurotoxicity in vitro. Boric acid (BA), a boron-containing compound with reported antioxidant and neuroprotective properties, has not been fully explored in PD-related apoptotic mechanisms. Therefore, this study aimed to investigate the neuroprotective and anti-apoptotic effects of BA in a rotenone-induced in vitro PD model using SH-SY5Y human neuroblastoma cells.

Methods: SH-SY5Y cells were exposed to 50 µM rotenone to induce PD-like neurotoxicity and co-treated with BA at concentrations ranging from 100 to 800 µM. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis-related molecular changes were evaluated by real-time quantitative polymerase chain reaction and Western blot analyses, focusing on key regulators of mitochondrial apoptosis, including BAX (B-cell lymphoma 2 [BCL-2]-associated X protein), BCL-2, and cleaved caspase-3.

Results: Rotenone treatment significantly reduced cell viability compared to control cells. Co-treatment with 200 µM BA markedly restored cell viability (**p<0.01), indicating a strong neuroprotective effect. Molecular analyses demonstrated that BA treatment significantly decreased the BAX/BCL-2 ratio in rotenone-treated cells (p=0.0295), suggesting an attenuation of mitochondria-mediated apoptotic signaling.

Discussion and Conclusion: These findings demonstrate that BA exerts a significant neuroprotective effect against rotenone-induced cytotoxicity by enhancing cell survival and modulating apoptosis-related pathways. BA may represent a promising candidate for mitigating PD-associated neuronal damage, warranting further mechanistic and *in vivo* investigations.

Keywords: Cancer molecular biology; Cell biology; Drug analysis; Molecular biology; neurodegenerative diseases

Parkinson's disease (PD) is one of the most common neurodegenerative conditions, characterized by motor symptoms such as tremors, stiffness, and bradykinesia.[1] The development of dopaminergic neuronal death in PD is a complex process involving oxidative stress, mitochondrial dysfunction, and impairment of mitochondrial complex I

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activity. Mitochondrial malfunction, arising from genetic susceptibility or environmental exposure, leads to excessive production of reactive oxygen species (ROS), thereby promoting programmed cell death in dopaminergic neurons.[2,3] The defining pathological features of PD include progressive degeneration of dopamine-producing neurons and intracellular accumulation of Lewy bodies, primarily composed of α-synuclein, which is closely associated with mitochondrial dysfunction and increased neuronal vulnerability.[4] Collectively, these pathological alterations converge on the activation of apoptotic signaling pathways, ultimately promoting dopaminergic neuronal loss.[5]

Rotenone, a naturally generated chemical found in different plant species, successfully inhibits the activity of mitochondrial complex I, resulting in an accumulation of ROS and neuroinflammation.[6] Prolonged exposure to rotenone can result in neurodegeneration and the development of PD-like symptoms by impairing the OXPHOS system and causing mitochondrial dysfunction.[7] Rotenone, 1-methyl-4-phenylpyridinium, isoquinoline, and tetrahydroisoquinoline, which are complex I inhibitors, have been reported to induce apoptosis in SH-SY5Y cell lines.[8–10] The neurotoxic effect of rotenone in PD is largely attributed to excessive free radical production and oxidative stress.

BA, generally referred to as orthoboric acid (H_3BO_3), is a boron compound with distinctive characteristics and versatile applications in diverse domains. It is widely used in chemistry and medicine due to its versatile characteristics.[11] Boron is crucial for maintaining optimal bodily health and facilitating several biological functions. Boric acid (BA) has demonstrated encouraging neuroprotective properties in numerous trials.[12] Research has demonstrated that BA can inhibit lipid peroxidation and oxidative stress induced by traumatic brain injury, leading to improved catalase (CAT) activity and reduced malondialdehyde levels.[13] Furthermore, BA has shown promising effects in PD research. Studies have demonstrated that BA exhibits dose-dependent antioxidant activity, protecting the dopaminergic system in experimental PD models.[14]

Since some antioxidants can reduce rotenone-induced toxicity, scavenging and preventing the formation of free radicals may be useful as a therapeutic target in PD.[15] Recent evidence indicates that BA exerts neuroprotective effects through several complementary mechanisms. BA forms complexes with hydroxyl-containing biomolecules, influencing membrane stability, calcium signaling, and redox balance.[16,17] It has been shown to act as an antioxidant by reducing ROS generation and enhancing endogenous antioxidant defenses, such as CAT activity.[18,19] In addition, BA supports calcium homeostasis and stabilizes mitochondrial function, preventing excessive ROS formation and apoptosis mechanisms critically impaired during PD pathology.[20,21] Therefore, BA may counteract rotenone-induced mitochondrial dysfunction, oxidative stress, and apoptosis, indicating its potential as a neuroprotective compound in PD models.

Considering the potential therapeutic benefits of BA, this study involved the development of a PD model by treating SH-SY5Y cells with rotenone. Our objective was to mitigate the resultant toxicity using BA and to explore the underlying apoptotic mechanisms responsible for this reduction.

Materials and Methods

This study was carried out in the Cell Culture and Molecular Biology Laboratory of the Department of Medical Biology, Dokuz Eylül University Faculty of Medicine. SH-SY5Y cells were cultured and treated with 50 μM rotenone to establish an *in vitro* PD model, followed by co-treatment with 200 μM BA to evaluate its neuroprotective potential. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and apoptosis-related gene and protein expression levels (BAX, B-cell lymphoma 2 [BCL-2], cleaved caspase-3) were analysed using real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot techniques.

The study was designed to investigate the neuroprotective effects of BA on an *in vitro* rotenone-induced PD model using the SH-SY5Y cell line.

Cell Culture and Reagents

In this experiment, SH-SY5Y cells of human neuroblastoma origin were cultured in DMEM/ F12 medium containing 10% fetal bovine serum, 1% Penicillin/Streptomycin, and L-Glutamine. The cultures were maintained at 37°C with 5% CO_2 in a controlled humidified incubator. Cells were seeded at a density of 5×10^6 cells per T-25 flask and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity Assay

To evaluate cellular cytotoxicity, the MTT assay was performed utilizing the Cell Proliferation Kit I supplied by Roche (catalog number 11465007001), in accordance with standardized procedures. Cells from the SH-SY5Y line were distributed into 96-well plates at a concentration of 1×10^4 cells per well, followed by overnight incubation to facilitate adherence and stabilization.

Rotenone was freshly dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was adjusted to 0.5% (v/v) in all groups, including the control. BA was dissolved in serum-free medium and applied to the cells at final concentrations of 800 µM, 400 µM, 200 µM, and 100 µM. Subsequently, the cells were treated with these concentrations of BA in combination with 50 µM rotenone to evaluate its potential in mitigating the induced toxicity. After 24 h of treatment, 10 µL of MTT solution was added and incubated for 4 h at 37°C in a 5% CO_2 incubator. Following this, 200 µL of solubilization solution was added to each well, and the plates were incubated overnight at 37°C in an incubator with 5% CO_2. The absorbance values at 570 nm were measured for each well using a microplate reader (Thermo Scientific Multiskan Go).[22]

RNA Isolation and cDNA Synthesis

SH-SY5Y cells were cultured in T25 flasks and incubated overnight at 37°C with 5% CO_2. After incubation, the cells were treated with 50 µM rotenone, 200 µM BA, or a combination of 50 µM rotenone + 200 µM BA. After 24 h of treatment, total RNA was extracted using TRIzol™ reagent (Thermo Fisher Scientific, catalog number 15596018) following the manufacturer's recommended protocol. RNA concentration was measured with a microplate reader (Thermo Scientific Multiskan Go). cDNA was synthesized from the isolated RNA using a High-Capacity cDNA Reverse Transcription Kit (New England Biolabs, Inc., catalog number 6300L), in accordance with the supplier's instructions.

RT-qPCR Analysis

Amplifications were performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, cat. no. 1725121) on the LightCycler® 480 real-time PCR system (Roche) with primers listed in Table 1.

The amplification was initiated at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. Transcription levels were normalized to GAPDH Ct values. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as previously described.[23]

Western Blot

SH-SY5Y cells were cultured in T25 flasks and maintained overnight in a humidified incubator at 37°C with 5% CO_2. The cultured cells were then treated with 50 µM rotenone, 200 µM BA, or a combination of 50 µM rotenone + 200 µM BA and incubated for 24 h. After incubation, the cells were harvested, and the pellets were lysed using RIPA lysis buffer supplemented with protease inhibitor. Protein concentration was quantified using the BCA Protein Assay Kit (Thermo Scientific, A55864), and absorbance was measured with a Multiskan Go microplate reader (Thermo Scientific). Approximately 20 µg of protein was loaded onto a 10–12% SDS-PAGE gel, electrophoresed, and transferred to a polyvinylidene fluoride membrane. Membranes were blocked with 5% non-fat milk prepared in PBST (1 × PBS with 0.05% Tween-20) for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against BAX, BCL-2, cleaved caspase-3, and β-actin (Proteintech), diluted in 3% non-fat milk in PBST. After washing, membranes were incubated with HRP-conjugated secondary antibodies diluted 1:2000 in PBST for 1 h at room temperature. Protein bands were visualized using an Enhanced Chemiluminescence detection kit (Thermo Fisher Scientific) and imaged using the FUSION Solo X Chemi Imaging System (VILBER).[24]

Table 1. Designed primer sequences

GAPDH	Forward	5′-GTCTCCTCTGACTTCAACAGCG-3′
	Reverse	5′-ACCACCCTGTTGCTGTAGCCAA-3′
BAX	Forward	5′-AAGAAGCTGAGCGAGT-3′
	Reverse	5′-GCCCATGATGGTTCTG-3′
BCL-2	Forward	5′-TCAGGCTGCTTGGGATAAAGATG-3′
	Reverse	5′-GGCTTCTGGAGGACATTTGGA-3′
CYC-C	Forward	5′-TGGGTGATGTTGAGA AAG G -3'
	Reverse	5'- TTTGTTCCAGGGATGTAC T -3'

CYC-C: Cytochrome c; BCL-2: B-cell lymphoma 2.

Statistical Analysis

Normality of the data was assessed using the Shapiro–Wilk test, and the data were found to be normally distributed. Accordingly, parametric statistical tests were applied where appropriate. Two-group comparisons, including control versus rotenone comparisons performed to validate the *in vitro* PD model, were analysed using the independent samples t-test. For experiments involving more than two groups or multiple concentrations, statistical analysis was performed using the Kruskal–Wallis test followed by non-parametric post hoc multiple comparisons (Dunn's test) with correction for multiple testing. MTT assay analyses were conducted using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). A $p<0.05$ was considered statistically significant. In all figures, statistical significance is indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$. All experiments were performed in triplicate (n=3).

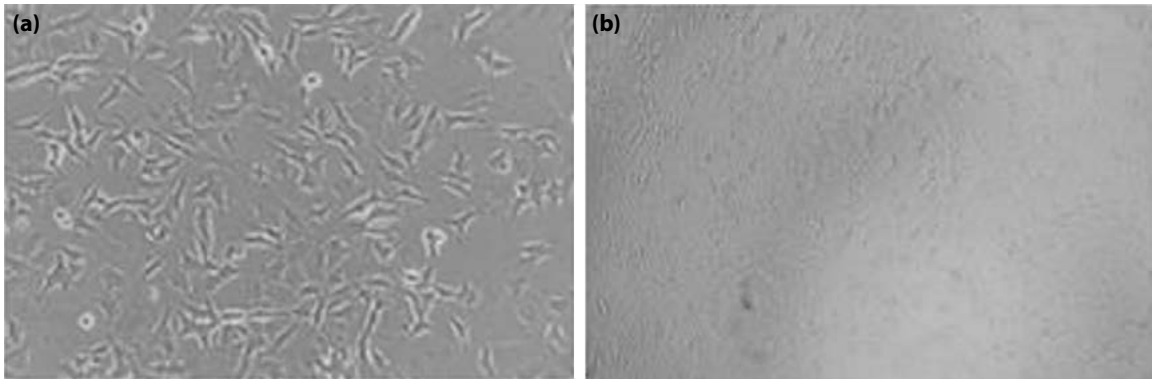

Figure 1. Microscopic images of SH-SY5Y cells. **(a)** Untreated control cells showing normal morphology and proliferation. **(b)** Cells after treatment with 50 µM rotenone (× 5 magnification).

Results

MTT Assay Results Indicating the Neuroprotective Effect of BA on Rotenone-Induced Toxicity in SH-SY5Y Cells

We performed an MTT assay on the SH-SY5Y cell line to evaluate the neuroprotective effects of BA in a rotenone-induced PD model. Representative microscopic images of untreated control and 50 µM rotenone-treated SH-SY5Y cells are shown in Figure 1.

The effects of BA alone on cell viability were evaluated using the MTT assay. Kruskal–Wallis analysis revealed a significant overall difference among the control and BA-treated groups (****$p<0.0001$). However, Dunn's multiple comparisons test showed that none of the tested BA concentrations (100–800 µM) resulted in a statistically significant change in cell viability compared with the control group (Fig. 2a). Although this increase did not reach statistical significance after correction for multiple comparisons, treatment with 200 µM BA was associated with a marked increase in mean cell viability, reaching 125.308% relative to the control group.

Cell viability decreased dramatically to 58.867% compared to the control group (****$p<0.0001$) when 50 µM rotenone was used to induce Parkinson's-like toxicity in SH-SY5Y cells. However, co-treatment with BA attenuated this toxic effect and improved cell viability. The combination of 200 µM BA with rotenone significantly increased cell viability to 137.79%, demonstrating a strong neuroprotective effect compared to the rotenone group (**$p<0.01$). In addition, BA doses of 100 µM, 400 µM, and 800 µM also exhibited a protective effect by increasing cell viability in comparison to rotenone alone, whereas the greatest protective effect was observed with 200 µM BA (Fig. 2b).

RT-qPCR Results Indicating Boric Acid's Neuroprotective Effect

In this study, the selected dose for further analysis was determined to be 50 µM rotenone combined with 200 µM BA. The RT-qPCR results revealed significant findings in the mRNA expression levels related to apoptotic pathways.

The combination of 50 µM rotenone and 200 µM BA reduced the BAX/BCL-2 ratio by 0.54-fold compared with rotenone alone ($p=0.0287$), indicating reduced apoptosis. This reduction in the BAX/BCL-2 ratio indicates a protective effect against apoptosis (Fig. 3a).

The levels of cytochrome c (CYC-C) expression exhibited distinct differences across the various treatment groups. Treatment with BA alone resulted in a marked decrease in CYC-C expression when compared to the rotenone group ($p=0.0018$). Moreover, treatment of rotenone alone led to a significant upregulation of CYC-C expression compared to the control group ($p=0.0006$). The combination of BA and rotenone resulted in a further increase in CYC-C expression compared with rotenone alone; however, this difference was not statistically significant ($p=0.1458$) (Fig. 3b).

Western Blot Results Indicating Apoptotic Modulation by Boric Acid

Western blot analysis showed that BA modulated the BAX/BCL-2 ratio in rotenone-treated cells. In the co-treatment group, the BAX/BCL-2 ratio was significantly decreased compared with the rotenone group ($p=0.0295$), supporting an anti-apoptotic and neuroprotective effect of BA (Fig. 4a).

Cleaved caspase-3 levels decreased to 0.8 in the BA alone-treated group compared with the rotenone group ($p=0.0030$), indicating reduced apoptotic activity. In the BA + rotenone group, cleaved caspase-3 levels

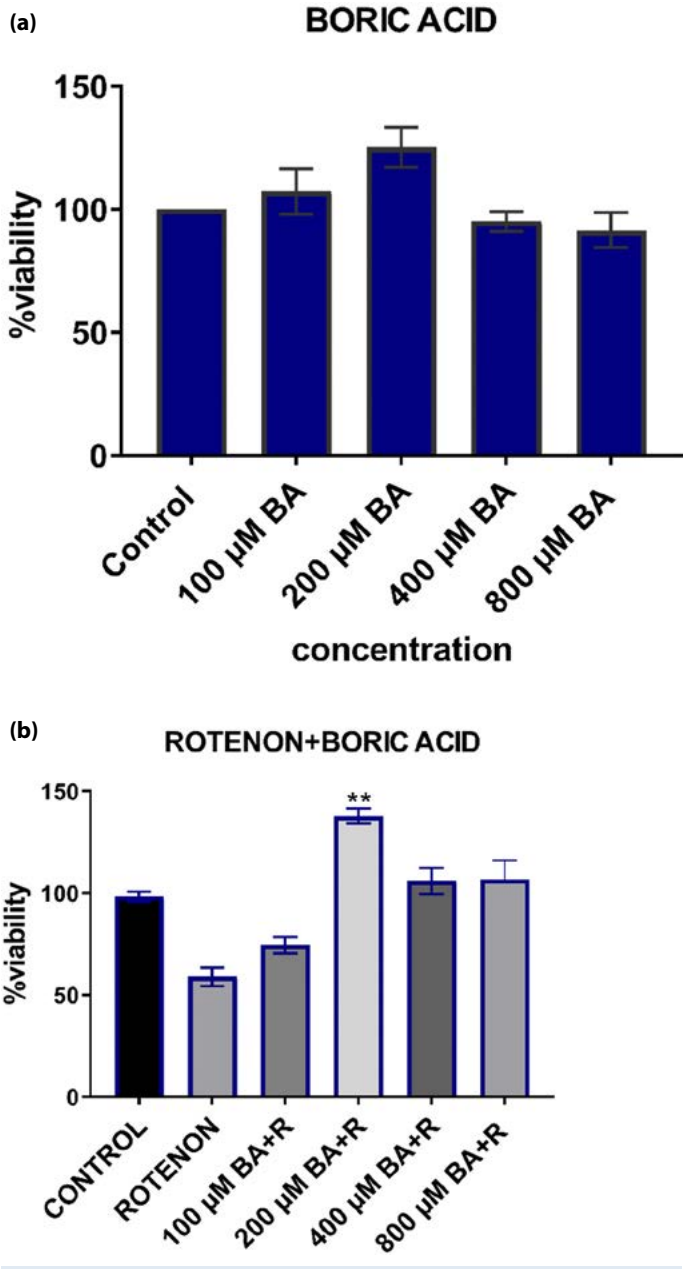

Figure 2. Cytotoxic effects of boric acid in rotenone-induced SH-SY5Y cells. **(a)** MTT assay results of boric acid (100, 200, 400, and 800 µM) on SH-SY5Y cells after 24 h of treatment. **(b)** MTT assay results of 50 µM rotenone and boric acid combinations. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's non-parametric post hoc multiple comparisons with correction for multiple testing. In panel **(a)**, comparisons were made with the control group, whereas in panel **(b)**, comparisons were made with the rotenone-treated group. Data are presented as a percentage of the control group. Experiments were repeated 3 times.

*: p<0.05 versus control; **: p<0.01 versus rotenone.

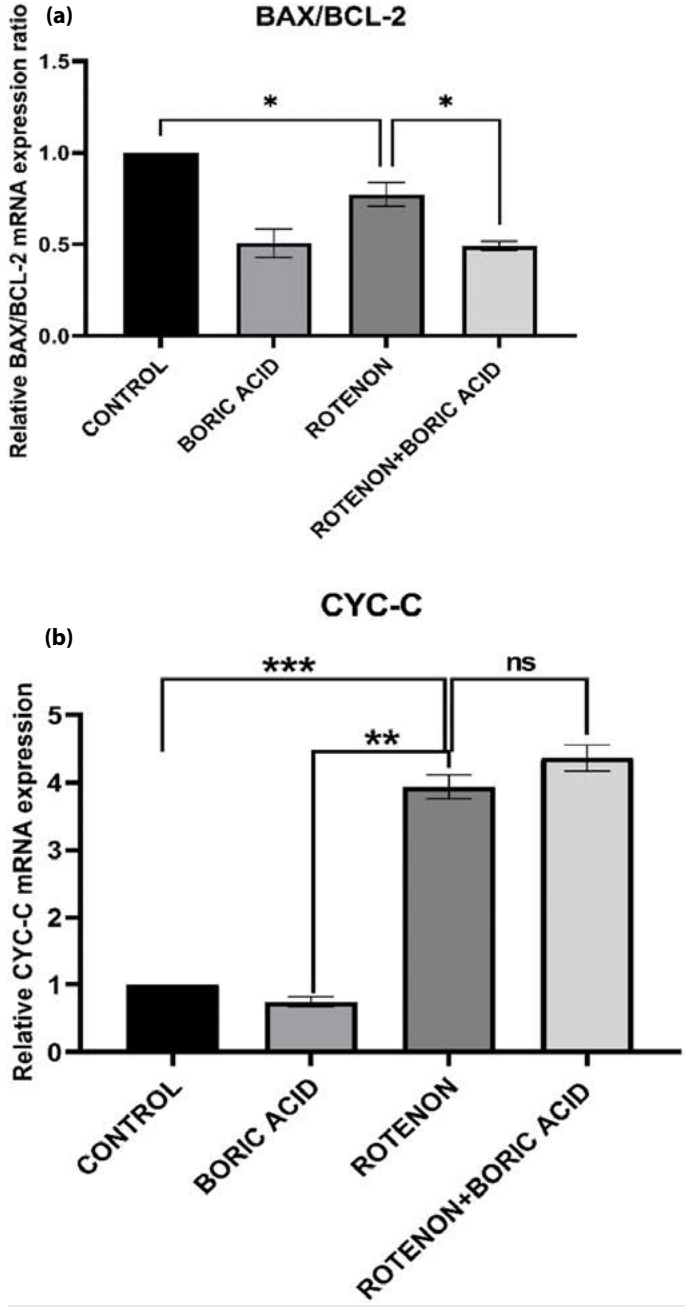

Figure 3. Effects of boric acid on apoptotic gene expression in rotenone-induced SH-SY5Y cells. Relative mRNA expression levels of BAX and B-cell lymphoma 2 (BCL-2), along with the corresponding **(a)** BAX/BCL-2 ratio and **(b)** cytochrome c expression levels. Gene expression levels were determined by real-time quantitative polymerase chain reaction and calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to GAPDH. Experiments were repeated 3 times.

Statistical significance is indicated as follows: *: p<0.05 versus control; and **: p<0.01; ***: p<0.001 versus rotenone-treated group.

were lower than in the rotenone group; however, this reduction did not reach statistical significance (p=0.1084) (Fig. 4b), suggesting only a partial attenuation of rotenone-induced apoptosis.

Discussion

The present study demonstrates that BA exerts a significant neuroprotective effect against rotenone-induced toxicity in SH-SY5Y cells, primarily through modulation of oxidative

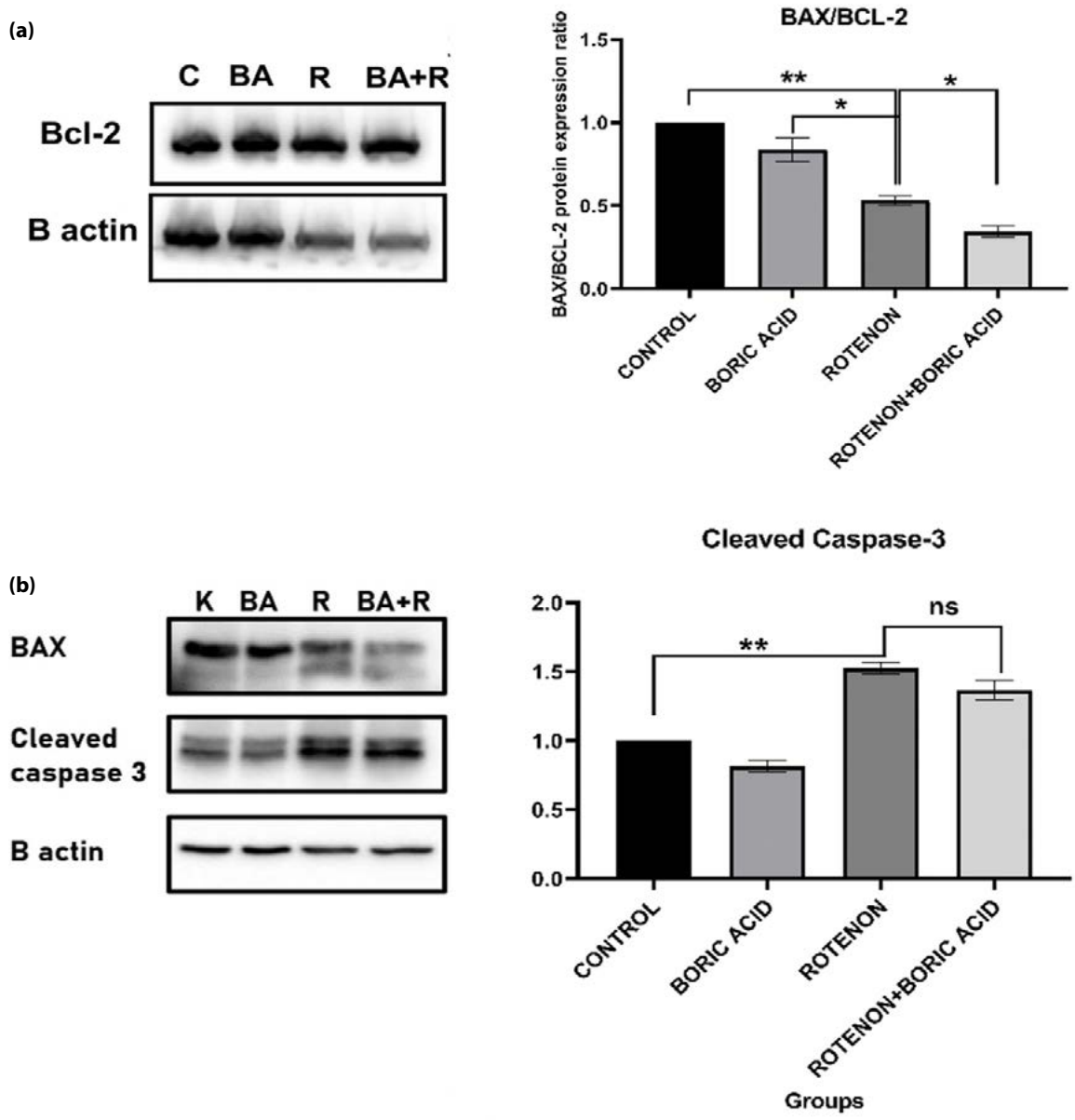

Figure 4. Effects of boric acid on apoptotic protein expression in rotenone-induced SH-SY5Y cells. Relative protein expression levels of BAX and B-cell lymphoma 2 (BCL-2), along with the corresponding **(a)** BAX/BCL-2 ratio, and **(b)** cleaved caspase-3 in SH-SY5Y cells. The intensity of BCL-2 and BAX bands was quantified and shown as relative expression levels after normalization by β-actin. Densitometry tracing was used to illustrate the quantitative differences, positioned next to the figure for the SH-SY5Y cell line.

Statistical significance is indicated as follows: **: p<0.01 versus control; and *: p<0.05; **: p<0.01 versus rotenone-treated group.

stress and mitochondrial apoptotic pathways. Given that mitochondrial dysfunction and excessive ROS generation are central drivers of PD pathogenesis, the ability of BA to enhance cellular antioxidant capacity represents a mechanistically relevant protective strategy. Previous studies have shown that BA increases the activity of key antioxidant enzymes, including superoxide dismutase, CAT, and glutathione peroxidase, thereby limiting oxidative damage and preserving mitochondrial integrity.[12–14] In line with these reports, our findings demonstrate a marked improvement in cell viability following BA treatment, supporting its role in maintaining redox homeostasis under neurotoxic conditions.[12–14]

The MTT cytotoxicity assay demonstrated that 50 μM rotenone significantly reduced cell viability in SH-SY5Y cells (58.87%), confirming its well-established neurotoxic effect. Co-treatment with BA significantly improved cell viability and attenuated rotenone-induced cytotoxicity, with the most pronounced protective effect observed at the 200 μM BA concentration (Fig. 2b). These findings

are consistent with previous studies investigating antioxidant-based neuroprotection in similar *in vitro* PD models. For example, Polyphyllin VI (PPVI) has been reported to increase cell viability from 49.91% under 20 μM rotenone exposure to 81.75% following treatment.[25] Similarly, rosmarinic acid has been shown to restore cell viability by approximately 80% in SH-SY5Y cells treated with 50 μM rotenone, where this concentration induced nearly a 50% reduction in viability, closely aligning with our observations.[26] Notably, in our study, BA treatment increased cell viability to 137.79% relative to the control, indicating a comparatively stronger protective effect than those reported for other antioxidant compounds. This suggests that BA may exert a more pronounced cytoprotective effect in rotenone-induced neurotoxicity, potentially through more effective modulation of oxidative stress and mitochondrial dysfunction.

At the molecular level, apoptosis regulation through the BAX/BCL-2 axis represents a central mechanism governing mitochondria-dependent cell death. The balance between pro-apoptotic BAX and anti-apoptotic BCL-2 is widely recognized as a critical determinant of neuronal survival.[27] In the present study, treatment with 200 μM BA in combination with 50 μM rotenone significantly reduced the BAX/BCL-2 ratio at both mRNA and protein levels, with RT-qPCR analysis demonstrating a 0.54-fold decrease compared to the rotenone-only group (Fig. 3a). This reduction was further confirmed at the protein level by Western blot analysis (Fig. 4a). Notably, analysis of individual protein expression revealed that this effect was driven by both downregulation of BAX and upregulation of BCL-2, indicating a coordinated shift toward an anti-apoptotic state. These findings are consistent with previous studies reporting that neuroprotective agents mitigate mitochondrial apoptosis by modulating the BAX/BCL-2 equilibrium in favor of cell survival. In addition to upstream apoptotic regulators, activation of caspases represents a key hallmark of apoptosis, with cleaved caspase-3 acting as a critical executioner of programmed cell death.[28] In our study, Western blot analysis of cleaved caspase-3 provided further insight into the apoptotic mechanisms. BA treatment alone reduced cleaved caspase-3 levels to 0.8 relative to the rotenone group, indicating a decrease in apoptotic activity ($p=0.0030$). When BA was applied in combination with rotenone, the rotenone-induced elevation in cleaved caspase-3 levels was attenuated; however, this reduction did not reach statistical significance ($p=0.1084$) (Fig. 4b). This partial modulation suggests that BA predominantly affects early-stage mitochondrial apoptotic signaling rather than completely inhibiting downstream caspase activation. Similar observations have been reported in studies where antioxidant or neuroprotective interventions attenuated upstream apoptotic pathways without fully suppressing execution-phase markers, indicating a staged regulation of apoptosis.[25,26,29]

In addition to cytotoxicity and apoptotic markers, validation of the PD model in this study is supported by molecular features consistent with PD pathology. Rotenone is well known to induce α-synuclein phosphorylation and aggregation through mitochondrial complex I inhibition and increased oxidative stress.[6] Although α-synuclein expression was not directly quantified, the observed decrease in cell viability together with increased BAX/BCL-2 ratio and cleaved caspase-3 levels indicates activation of mitochondrial-dependent apoptosis, a hallmark of PD. These findings are in line with previous studies demonstrating that rotenone exposure induces mitochondrial dysfunction, ROS accumulation, and α-synuclein-related pathology in SH-SY5Y cells,[25–29] thereby supporting the validity of our *in vitro* PD model.

Previous studies have shown that various compounds can modulate rotenone-induced toxicity through distinct molecular pathways.[25,28,29] For instance, PPVI has been reported to exert neuroprotective effects through signaling pathways involving FOXO3α, CREB1, and DJ-1,[25] while rosmarinic acid significantly reduced apoptosis in rotenone-treated SH-SY5Y cells as demonstrated by Annexin V analysis.[26] Similarly, Growth Differentiation Factor 15 has been shown to protect against rotenone-induced neurotoxicity by suppressing mitochondrial-mediated apoptotic pathways.[29] In this context, our findings extend the current literature by demonstrating that BA also exerts neuroprotective effects in a rotenone-induced PD model, particularly through modulation of apoptosis-related pathways.

Consistent with previous studies investigating antioxidant-based interventions in PD models, BA demonstrated dose-dependent neuroprotective effects, protecting dopaminergic neurons and reducing oxidative damage in experimental systems.[30] These findings support the broader concept that antioxidants can mitigate rotenone-induced neurotoxicity by targeting oxidative stress and mitochondrial dysfunction. In agreement with this, our results indicate that BA enhances cell viability and modulates apoptotic signaling, suggesting that its neuroprotective effect is mediated through regulation of the oxidant–antioxidant balance and mitochondrial integrity.

Overall, the findings of this study support the growing body of evidence that antioxidant compounds can provide neuroprotection in PD models. However, while BA demonstrated significant neuroprotective effects by modulating apoptotic signaling, these findings require further validation *in vivo* systems. Moreover, since apoptosis is regulated by multiple interconnected pathways, the use of a limited panel of apoptosis-related markers may restrict the mechanistic interpretation of the findings. Future studies incorporating additional apoptotic and mitochondrial markers are necessary to more comprehensively elucidate the molecular mechanisms underlying the neuroprotective effects of BA and to confirm its translational potential.

Conclusion

This study provides evidence that BA can mitigate rotenone-induced neurotoxicity in SH-SY5Y cells by reducing apoptotic activity and enhancing cell viability. While BA demonstrates significant potential as a neuroprotective agent, further research is needed to fully understand its mechanisms of action and to optimize its therapeutic application in PD.

Ethics Committee Approval: The SH-SY5Y human neuroblastoma cell line used in this study is commercially available and does not involve human subjects or animal experiments; therefore, no ethical approval was required.

Informed Consent: Commercial materials were used in the study, not require inform consent.

Conflict of Interest: None declared.

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