Benefits of Betanin in Rotenone-Induced Parkinson Mice

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Research Article

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Abstract

The present study aimed to investigate betanin's neuroprotective effect in mice with rotenone-induced Parkinson-like motor dysfunction and neurodegeneration. Forty male ICR mice were divided into 4 groups: Sham-veh, Rot-veh, Rot-Bet100 and Rot-Bet200. Rotenone (Rot) at 2.5 mg/kg/48 h was subcutaneous injected, and betanin (Bet) at 100 and 200 mg/kg/48 h were given alternately with the Rot injections in Rot-Bet groups for 6 weeks. Motor dysfunctions were evaluated weekly using hanging wire and rotarod tests. Malondialdehyde (MDA), reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), neuronal degeneration in the motor cortex (MC), striatum (Str) and substantia nigra par compacta (SNc) were evaluated. The immunohistochemical densities of tyrosine hydroxylase (TH) in Str and in SNc were also measured. We found that rotenone significantly decreased the time to fall in a hanging wire test after the 4^{th} week and after the rotarod test at the 6^{th} week ($p<0.05$). The percentage of neuronal degeneration in MC, Str and SNc ($p<0.05$) significantly increased, and the TH density in Str and in SNc ($p<0.05$) significantly decreased. Betanin at 100 and 200 mg/kg significantly prevented MC, Str and SNc neuronal degeneration ($p<0.05$) and prevented the decrease of TH density in Str and in SNc ($p<0.05$). These findings appeared concurrently with improved effects on the time to fall in hanging wire and rotarod tests ($p<0.05$). Treatment with betanin significantly prevented increased MDA levels and boosted GSH, CAT and SOD activities ($p<0.05$). Betanin exhibits neuroprotective effects against rotenone-induced Parkinson in mice regarding both motor dysfunction and neurodegeneration. Betanin's neurohealth benefit relates to its powerful antioxidative property. Therefore, betanin use in neurodegenerative disease therapy is interesting to study.

Introduction

Parkinson disease (PD) is clinically incurable and still needs knowledge of distinct pathological mechanisms. An ideal PD animal model used in pathomechanism studies has been developed in genetic and neurotoxic models (Zeng et al. 2018). Rotenone is one major neurotoxin used to reproduce PD pathology in animal models. It caused damage specific to the nigrostriatal pathway, especially the dopaminergic neurons in the substantia nigra par compacta (SNc) (Terron et al. 2018). It also caused damage to various brain areas, including the cortex, hippocampus, striatum, substantia nigra, medulla and cerebellum (Abdel-Salam O 2014). Because it is highly hydrophobic, rotenone can easily cross the blood brain barrier (BBB). Once it reaches the inside of a neuron, its alteration effect on oxidative mechanism can cause neurodegeneration. This includes the selective inhibition of mitochondria complex I, resulting in mitochondrial dysfunction and rising reactive oxygen species (ROS), especially in dopaminergic neurons (Terron et al. 2018). PD's distinct pathomechanism is not fully understood, but oxidative stress is one proposed major pathological mechanism related to PD (Inden et al. 2011). Therefore, rotenone could help oxidative stress in reproduced PD pathology. In addition to selective neurodegeneration, rotenone could induce some motor deficits that resemble PD symptoms, such as reduced muscle strength and loss of coordination and balance (Rahimmi et al. 2015). Moreover, a report indicated rotenone-induced striatum damage with the alteration of dopaminergic function (Crutchfield

and Dluzen 2006). One pathological hallmark of PD is the loss of dopaminergic neurons in substantia nigra (Davie 2008), and the projecting area of the neural circuit (e.g., the striatum and motor cortex) was also affected. Therefore, rotenone is suitable for inducing Parkinson-like neurodegeneration and motor dysfunction in animals.

Oxidative stress plays a major role in neurodegenerative disease, and antioxidative substances are part of therapeutic intervention. Betanin, a powerful antioxidant, has been used as natural red food colorant that can prevent lipid oxidation in meats. It has various health benefits, including inhibition effects on low-density lipoprotein (LDL), inducible nitric oxide synthase (iNOS), cyclooxygenase (COX) and lysosome protease activities (Ahmadi et al. 2020; Allegra et al. 2007; Esatbeyoglu et al. 2015; Indumathi et al. 2018). The powerful antioxidation depends on how it affects the erythroid 2-related factor 2 antioxidant response element (Nrf2-ARE), which activates the mRNA and protein expression of antioxidative enzymes, glutathione S-transferase, heamoxygenase-1 and NAD(P)H quinone dehydrogenase 1 (Krajka-Kuzniak et al. 2013). An abrogate effect on the lipid peroxidation process of reducing the malondialdehyde (MDA) level and the myeloperoxidase (MPO) activity has been reported (Tural et al. 2020). Betanin's anti-cancer effect as an angiogenesis inhibitor and apoptotic inducer via caspase 3, 7 and 9 activations in human lung cancer cell lines was also reported (Zhang et al. 2013). Betanin's ameliorative effect on oxidative stress-induced apoptotic death in exposed PC 12 cells 6-OHDA (in vitro PD model) has recently appeared with precise SAPK/JNK and PI3 K partial inhibition (Hadipour et al. 2020). Our recent study indicates betanin's neuroprotective effect against trimethyltin-induce neurodegeneration in mice, which involves various antioxidative properties (Thong-Asa et al. 2020). A multifunctional molecule with powerful antioxidative properties, betanin is interesting as therapeutic intervention for neurodegenerative diseases. To elucidate betanin's protective effect on neuronal pathology and behavioral correlation, the present study investigates betanin's beneficial effect against rotenone-induced neurodegeneration and motor dysfunctions in mice.

Materials And Methods

Chemicals and reagents

Betanin, rotenone and other analytic chemicals and reagents were purchased from Chemical Express Co., Ltd., Merck, Millipore, Germany and Agilent, USA.

Animals

Forty male ICR mice, 8 weeks old and weighing 30-50 grams, were purchased from the National Laboratory Animal Center (NLAC) at Mahidol University, Salaya, Nakorn Pathom. They were housed in a room with controlled humidity (55%) and temperature (25 ^{o}C). They had free access to standard food (No. 082G) and reverse osmosis (RO) water.

Experimental protocol

The experimental protocol was approved by the Animal Ethics Committee in the Faculty of Science at Kasetsart University (ID#ACKU63-SCI-002). Mice were divided to 4 groups: Sham-veh, Rot-veh, Rot-Bet100 and Rot-Bet200. Mice in the Rot groups received subcutaneous injections of rotenone at 2.5 mg/kg/48h (Rahimmi et al. 2015). Betanin at 100 and 200 mg/kg was dissolved into normal saline (veh was also use as a vehicle) and given via intra-gastric gavage to Bet groups every 48h alternately with rotenone injections. All treatments were given continuously for 6 weeks.

Hanging wire test

A four-limbs hanging test was used to evaluate muscle strength and balance. A cage lid was used as a hanging grid and positioned 25 cm above soft bedding to protect the mouse when it falls. Each mouse was placed on the grid. When it grabbed the grid with four paws, the grid was inverted and the hanging time began. Each mouse had 120 sec maximum with two more tries (three tries total), and the time to fall (sec) was recorded. All mice received training (for base line) before starting the experiment and were tested once a week for 6 weeks (Aartsma-Rus and van Putten 2014).

Rotarod test

We used a rotarod test to evaluate fore and hind limb motor coordination and balance impairments (Aartsma-Rus and van Putten 2014). Before the rotenone injection, mice were briefly trained in the rotarod at 11–15 rpm until all mice reach a stable performance (for baseline). A weekly rotarod test was delivered until the end of the experiment. For the test, each mouse was placed on a rotating tube at a steady speed of 5 rpm. The speed was increased from 5 to 15 rpm in 15 sec, and this speed was maintained for at least 180 sec, with two more tries. The running time was collected and expressed as time to fall (sec) for each mouse.

Biochemical analysis

After behavioral tests, mice were euthanized with 180 mg/kg sodium pentothal. They were quickly decapitated and the fresh brains were collected for biochemical analyses. The brains were washed in cold normal saline and homogenized in a 10% w/v phosphate buffer saline (PBS). Half of the homogenate was separated for a malondialdehyde (MDA) assay, and the rest was further centrifuged (10,000g, 4 ^{o}C). Supernatant was collected for reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) assessments (Sakamula and Thong-Asa 2018).

Histological analysis

Brains were processed and embedded in paraffin blocks. Five-micrometer sections were created serially using microtomes. The motor cortex (MC) and striatum (Str) sections were collected at bregma -0.34 mm, and SNc sections were collected at bregma -3.52 mm (Paxinos and Franklin 2008). Five sections with 125 μM space intervals were collected from each mouse and stained with 0.1% cresyl violet (Thong-Asa et al. 2020). Brain sections were deparaffined and rehydrated via serial changes of xylene and 100%, 95%,

80% and 70% ethanol. They were dipped in distilled water before staining with 0.1% cresyl violet for 30 sec. They were dehydrated and cleared in reverse via serial changes of ethanol, xylene and finished by sealing with cover glasses. The brain areas of interest (e.g., MC, Str and SNc) were captured via 3 non-overlapping images in each hemisphere. At 100x magnification, two investigators counted viable and degenerating cells in a blind fashion using NIH image J. The data were interpreted as the percentage of degeneration using formula % degeneration = 100 x [degenerating/[viable + degenerating] (Somrednganand Thong-Asa 2018).

Tyrosine hydroxylase (TH) immunohistochemistry

Brains were fixed in cold 4% paraformaldehyde for 24 h (4 ^{o}C) and then transferred to PBS containing 20% sucrose. When the brains sunk, they were frozen as 20-µm thick sections with cryostat. Five brain sections covering the Str and SNc area were selected from each mouse with a minimum 100-µm space interval. Brain sections were rinsed in PBS 3x5 min then incubated in 3% H_2O_2/10% methanol 5-10 min at room temperature (RT). They were washed in PBS for two 5-min intervals and in PBS-T (0.1% Triton X-100 in PBS) for two 5-min intervals and then in the blocking solution (PBS containing 5% goat serum) for 60 min at RT. After a brief rinse in PBS, they were covered by anti-tyrosine hydroxylase (AB152) diluted at 1:500 in carrier solutions (PBS-T containing 2% goat serum). Brain sections were incubated at RT for 3 h before being transferred to a 4 ^{o}C environment for a 24 h incubation. The next day, brain sections were rinsed in PBS-T for three 5-min intervals and incubated with biotinylated goat anti-rabbit antibodies diluted at 1:200 (PBS-T containing 2% goat serum) for 60 min at RT. They were washed in PBS-T for 10 min, washed in PBS for two 10-min intervals and then incubated in an ABC solution for 60 min. They were washed in PBS for two 5-min intervals and covered with diaminobenzide for 15 min. After washing in PBS for two 5-min intervals, brain sections were mounted onto cover glasses.

Three non-overlapping images of Str and SNc were capture in each hemisphere. At 100x magnification, TH density was analyzed using NIH Image J and represented as the % TH density related to the % of control (Javed et al. 2016).

Statistical analysis

Animal weights, latency to falls in hanging wire and rotarod tests, biochemical data and the % of neuron degeneration in MC, Str, SNc and TH optical density related to % of control were analyzed via a one-way variance analysis, followed by a Fisher's PLSD post hoc test. Statistical significance was accepted for p-values under 0.05.

Results

Body weights and mortality rate

To verify the unspecific effects of rotenone that may reduce body weight, we measured the mice's weight from baseline till the experiment's end. Mice with rotenone injections showed gradual weight loss with no

statistical significance (p>0.05, Fig. 1a). None of the Sham-veh or Rot-Bet100 mice died during the experimental period. The mortality rate of Rot groups stayed below 20%. Two Rot-veh mice died in the 2nd and 3rd weeks, and 1 Rot-Bet200 mouse died in the 4th week of the experiment.

Motor dysfunctions

Behavioral tests indicated that motor coordination, balance and strength gradually decreased in rotenone-treated mice. Fig. 1b indicated early signs of muscle strength and balance impairment for Rot-veh mice in the hanging wire test from the 4th to 6th weeks (p=0.0016, 0.0077 and 0.025, respectively) compared to Sham-veh mice. The motor coordination and balance in rotarod mice gradually decreased, with a significant difference only in the experiment's 6th week (p=0.0042, Fig. 1c). Treatment with betanin at 100 and 200 mg/kg significantly prevented the decline of muscle strength in hanging wire tests from the 4th to 6th weeks (Rot-Bet100 compared to Rot-veh, p=0.0063, 0.0052 and 0.0071; Rot-Bet100 compared to Rot-veh, p=0.0399, 0.0471 and 0.0046, respectively; Fig. 1b). Betanin also prevented the decline of motor coordination in rotarod at the 6th week (Rot-Bet100 compared to Rot-veh, p=0.0105; Rot-Bet200 compared to Rot-veh, p = 0.0037; Fig. 1c).

Brain oxidative status

Rotenone-induced lipid peroxidation was indicated by significantly increased MDA levels (p=0.0041, Fig. 2a). It significantly decreased CAT and SOD activities (p=0.0037 and 0.0012, respectively, Fig. 2b, 2c) but not the GSH level (p>0.05, Fig. 2d) when comparing Rot-veh to Sham-veh. Betanin at 100 and 200 mg/kg significantly decreased MDA levels (p=0.0013 and 0.0057, respectively; Fig. 2a) and significantly increased CAT activity (p=0.0427 and 0.0255, respectively, Fig. 2b) and SOD activity (p = 0.0035 and 0.0168, respectively, Fig. 2c) when comparing Rot-Bet100 and Rot-Bet200 to Rot-veh. Rotenone did not change GSH levels, but treatments with betanin significantly increased GSH levels at both 100 and 200 mg/kg (p=0.0089 and 0.0127, respectively; Fig. 2d).

Brain histology

We demonstrated significant neuronal degeneration in SNc, Str and MC induced via rotenone injection. The % of degeneration of SNc, Str and MC significantly increased in Rot-veh compared to Sham-veh (p=0.0006, <0.0001 and <0.0001, respectively). Betanin treatment significantly reduced the % of degeneration of SNc, Str and MC when comparing Rot-Bet100 (p=0.0044, <0.0001 and <0.0001, respectively) and Rot-Bet200 (p=0.0400, <0.0001 and 0.0003, respectively) to Rot-veh (Fig. 3-5).

TH density

Tyrosine hydroxylase immunological staining density appeared in SNc and in Str, revealing that rotenone significantly reduced the TH staining density in SNc and in Str (p<0.001 and <0.0001, respectively; Fig. 6-7) compared to Sham-veh. Rotenone injections at 6 weeks decreased TH density about 38–40% in both regions. Betanin treatment reduced TH density by about 7–12% in Str and by about 20–22% in SNc. In

SNc, betanin significantly prevented TH density reduction when comparing Rot-Bet100 ($p = 0.0041$) and Rot-Bet200 ($p=0.0026$) to Rot-veh (Fig. 6). It also significantly differs from Sham-veh (Rot-Bet100, $p= 0.0010$ and Rot-Bet200, $p=0.0034$; Fig. 6). In Str, both betanin doses significantly prevented a decrease of TH density when comparing Rot-Bet100 ($p=0.0003$) and Rot-Bet200 ($p=0.0001$) to Rot-veh (Fig. 7).

Discussion

The present study demonstrated betanin's neurohealth benefit in ameliorating rotenone-induced neurodegeneration concurrently with Parkinson's-like symptoms in mice. Using 2.5 mg/kg/48 h of rotenone for 6 weeks to reproduce Parkinson's in animal models clearly induced some Parkinson's symptoms in ICR mice. Rotenone significantly reduced the muscle strength, motor coordination and balance concurrently, with significant neuronal degeneration in SNc, Str and MC and with reduced TH density in SNc and Str. Moreover, rotenone significantly changed the brain oxidative status, significantly increased MDA levels and reduced CAT and SOD activities. These results confirm rotenone's involvement via the oxidative stress neurodegenerative pathomechanism.

The PD pathomechanism not fully understood, but it correlated to the nigrostriatal pathway (Cannon et al. 2009). One major pathogenesis was the rising ROS, which contributed to oxidative damage. Attacking cell macromolecules caused the alteration of mitochondrial function and neuroinflammation, so ROS subsequently led to neuronal damage (Guo et al. 2018). Rotenone's alteration effect on oxidative mechanisms, resulting in neurodegeneration, was relevant to PD regarding the oxidative pathomechanism. It induced mitochondrial dysfunction, including the inhibition of complex I in the mitochondrial respiratory chain, which led to ATP depletion and ROS leakage. Via the rising ROS, various cascades were activated and led to apoptosis, necroptosis and necrosis neuronal death (Callizot et al. 2019). Rotenone-induced neuroinflammation occurs via the increase of TNFα, IL-1β, IL-6 (Javed et al. 2016). It also induced neuronal oxidation by increasing MDA and NO levels, alternated with decreased antioxidants, such as CAT, SOD and GSH. (Hasan et al. 2020). When using rotenone-induced PD in rodents (a low or high dose), the frequency of rotenone exposure led to differences in timelines and in behavioral and neurological deficits in rats and mice (Richter et al. 2007; Zhang et al. 2017). Rotenone caused weight loss and high mortality rates for doses up to 2.5 mg/kg via daily injection (Zhang et al. 2017). Rahimmi and colleagues used 2.5 mg/kg/48 h in rats and confirmed no unspecific effects on body weight. After the benefit of reproducing the motor and neurological deficits within 3 weeks (Rahimmi et al. 2015), we delivered rotenone at 2.5 mg/kg/48 h to ICR mice and found benefits in the reduction of weight change and mortality rates. Motor deficits appeared from 4 to 6 weeks, depending on the tests. Muscle weakness represented via hanging wire tests appeared in the 4^{th} week, and decreased motor coordination in the rotarod appeared during the 6^{th} week of rotenone injection. The expression of motor deficits in rotarod appear later when compared to a rat model with the same frequency and dose of rotenone exposure. The difference of animal species in rotenone endurance and behavioral test intensity must be considered (Aartsma-Rus and van Putten 2014).

The behavioral deficits associated with brain tissue oxidation were indicated via the significant increase of MDA levels and the decrease of CAT and SOD in the present study. In addition, we found significantly increased neuronal degeneration and reduced TH density in SNc, Str. These results confirmed rotenone-induced motor and neurological deficits, including oxidative stress and neuronal damage. The degeneration of neuronal cell appeared specifically on the nigrostriatal structure in SNc dopaminergic neurons and in Str neurons. We also found neurodegeneration beyond the nigrostriatal pathway (e.g., in the MC) that involve motor dysfunction. This resembles the previous report that rotenone's effect is not limited to the nigrostriatal pathway but involves various brain areas, including the cortex, hippocampus, striatum, substantia nigra, medulla and cerebellum (Abdel-Salam O 2014). This may contribute to behavioral deficits as well.

The present study indicated betanin's neuroprotective effect against rotenone-induced neuronal degeneration and motor dysfunctions. We found betanin's ameliorative effect associates with antioxidative properties. Its preventive effect against oxidative stress was indicated by a significant reduction of MDA levels and the boosting effect on CAT, SOD and GSH. This relates to the previous study indicating betanin's abrogate effect on the lipid peroxidation process via both the MDA level and via MPO activity (Tural et al. 2020). It also relates to betanin's effect on mRNA and on the protein expression of antioxidative enzymes (Krajka-Kuzniak et al. 2013). The protection against oxidative stress-induced apoptotic death in PC 12 cells 6-OHDA exposed an association with SAPK/JNK, and partial PI3 K inhibition was also recently reported (Hadipour et al. 2020). We indicated betanin's protective effect against neurodegeneration in nigrostriatal structures such as SNc and Str, along with dopaminergic neuron preservation in these two areas. In addition to the nigrostriatal structure, the MC neurons damaged by rotenone were also protected. This confirmed rotenone's effect was not limited to nigrostriatal pathway but included other brain areas (Abdel-Salam O 2014). We also confirmed betanin's neuroprotective effect against neuronal damage in other brain areas. Our recent report supports this by indicating betanin's neuroprotective effect against trimethyltin-induced neurodegeneration in mice, and the pathomechanism correlated with oxidation as well (Thong-Asa et al. 2020).

Conclusion

In summary, the present study indicates betanin's neuroprotective effect against rotenone-induced neurodegeneration and motor dysfunctions in mice with PD-like symptoms. Betanin neuroprotection involves potential antioxidative properties. Therefore, betanin use in neurodegenerative disease therapy is interesting to study.

Declarations

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Data availability

Available upon request.

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Contributions

Wachiryah Thong-asa conceived and designed research, analyzed data and wrote the manuscript. Sujira Jedsadavitayakol and Suchawalee Jutarattananon conducted experiments. All authors read and approved the manuscript for publication.

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Compliance with ethical standards

Ethics declarations

Ethics approval

“All applicable international and national guidelines for the care and use of animals were followed”.

Consent to participate

All authors agree to participate.

Consent for publication

All authors agree to publish.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

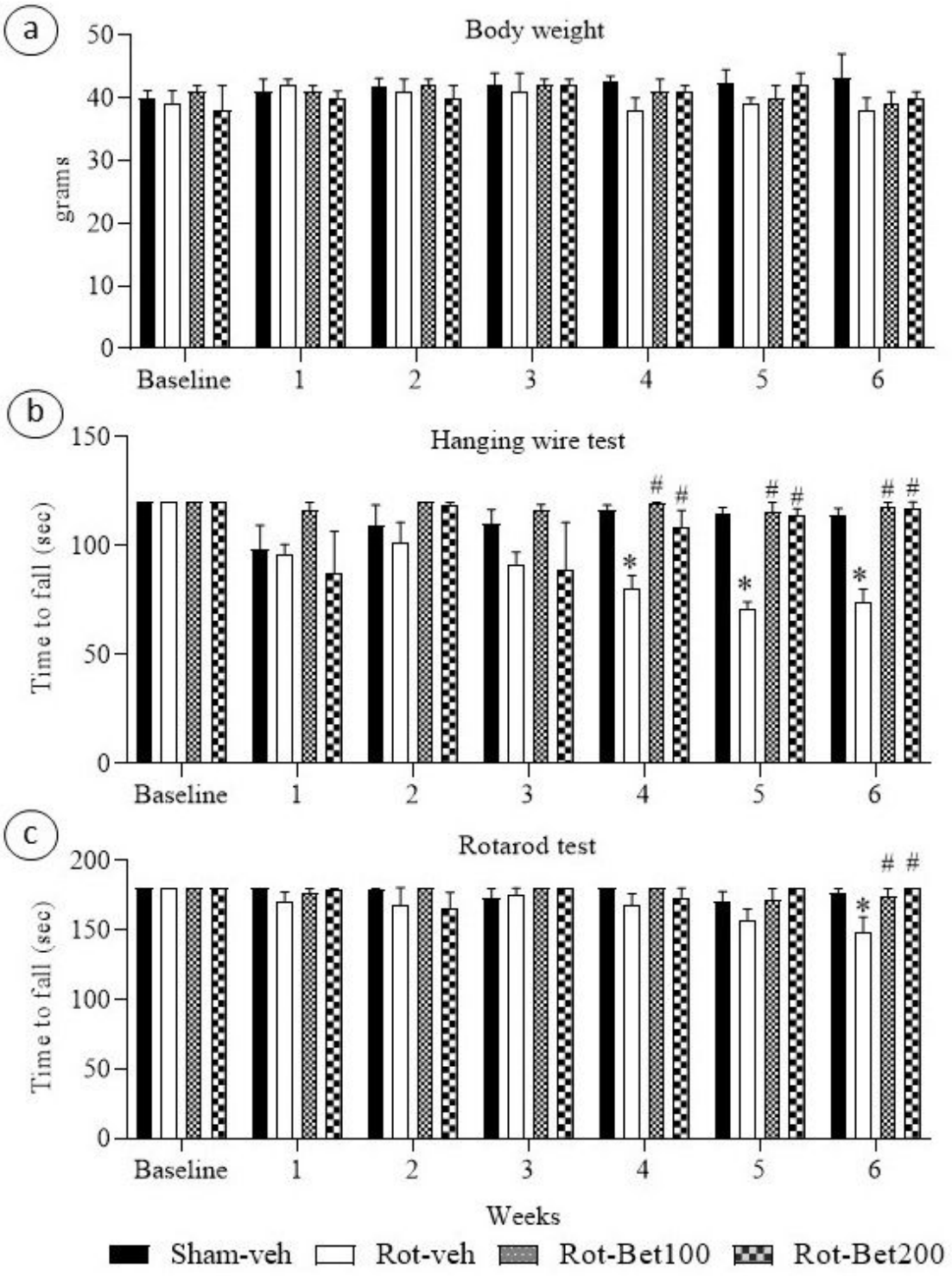


Figure 1

Body weight measurements and behavioral evaluations. Body weights (a), muscle strength indicated by the time to fall in hanging wire tests (b), motor coordination and balance indicated by the time to fall in rotarod tests (c). *indicates a significant difference compared to Sham-veh; #indicates a significant difference compared to Rot-veh.

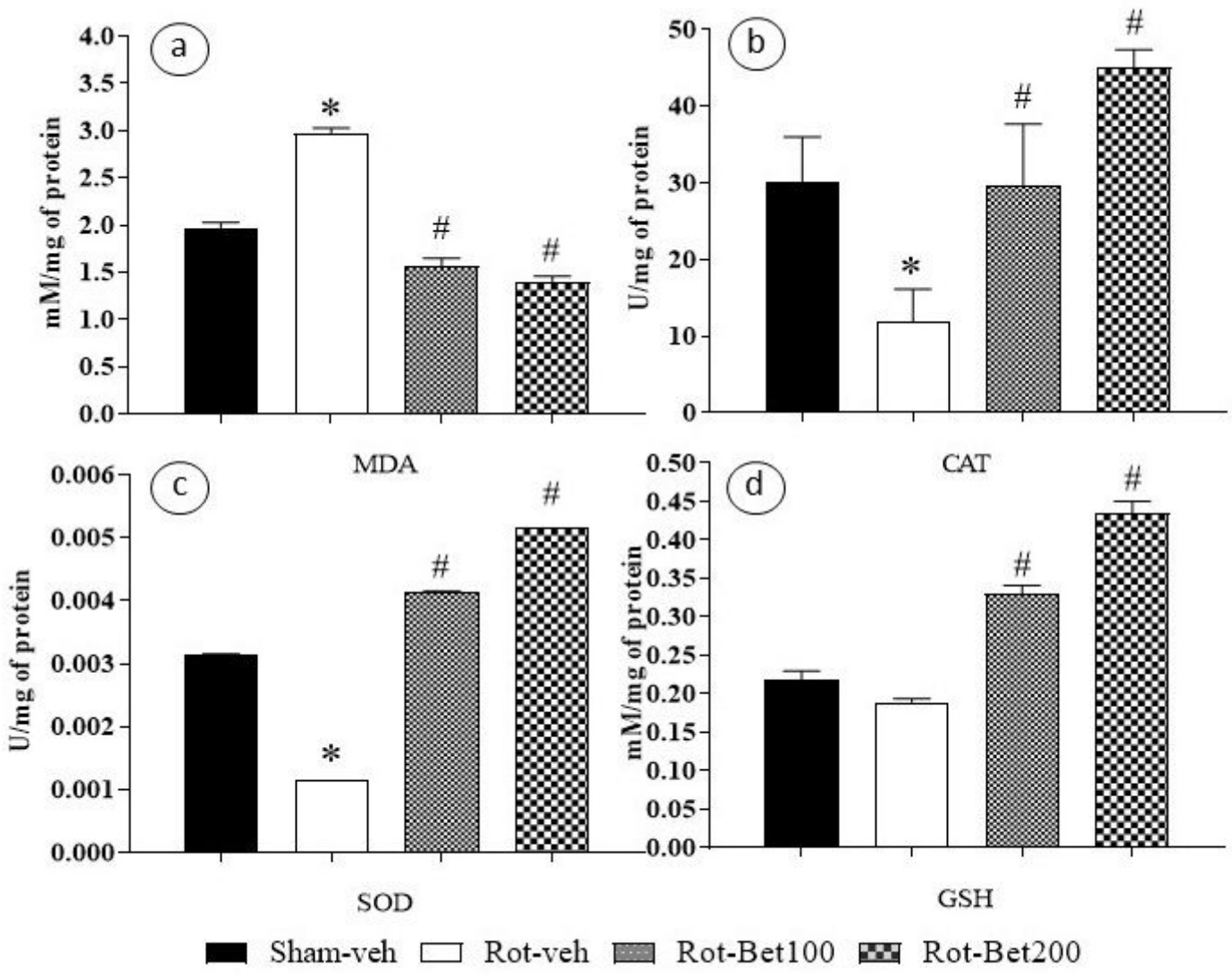
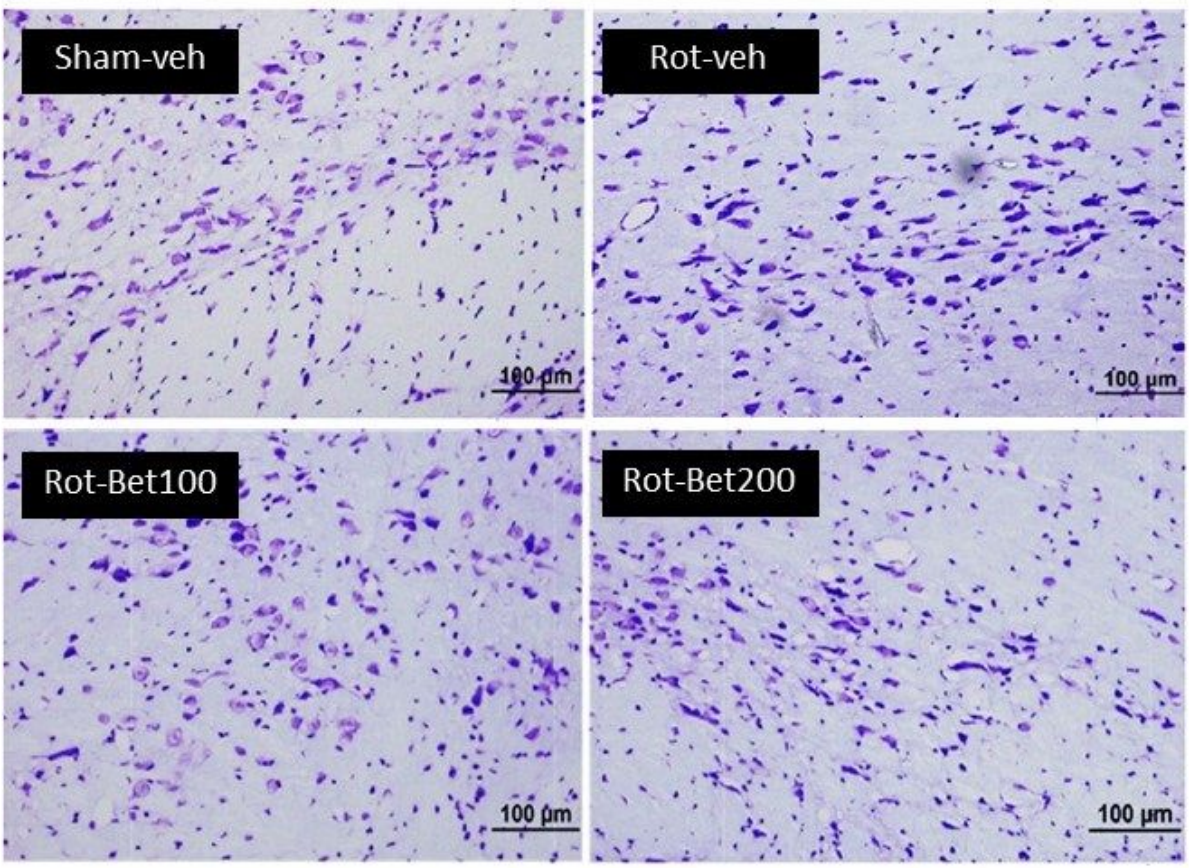


Figure 2

Biochemical evaluations. MDA (a), CAT (b), SOD (c) and GSH (d). *indicates a significant difference compared to Sham-veh; #indicates a significant difference compared to Rot-veh.



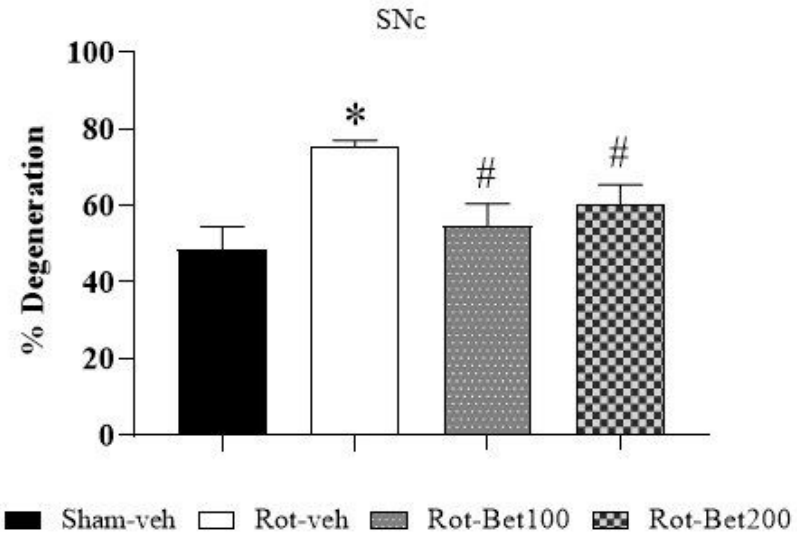


Figure 3

Photomicrographs of SNc at 100x magnification, including staining with 0.1% cresyl violet for Sham-veh, Rot-veh, Rot-Bet100 and Rot-Bet200, respectively, and with a 100-μm scale bar. Histograms show the % of neuronal degeneration in SNc. *indicates a significant difference compared to Sham-veh; #indicates a significant difference compared to Rot-veh.

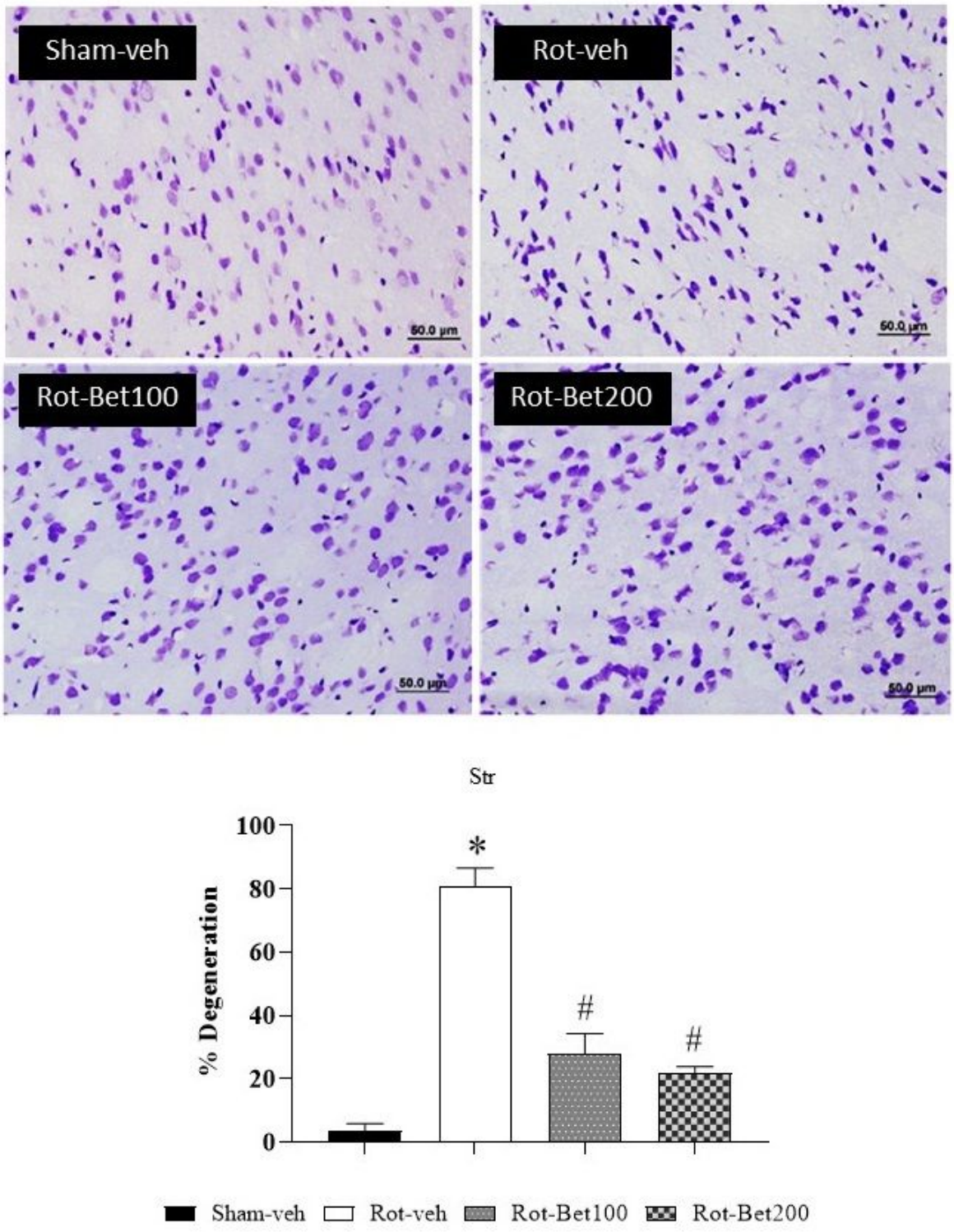


Figure 4

Photomicrographs of Str at 100x magnification, including staining with 0.1% cresyl violet for Sham-veh, Rot-veh, Rot-Bet100 and Rot-Bet200, respectively, and with a 50-µm scale bar. Histograms show the % of neuronal degeneration Str. *indicates a significant difference compared to Sham-veh; #indicates a significant difference compared to Rot-veh.

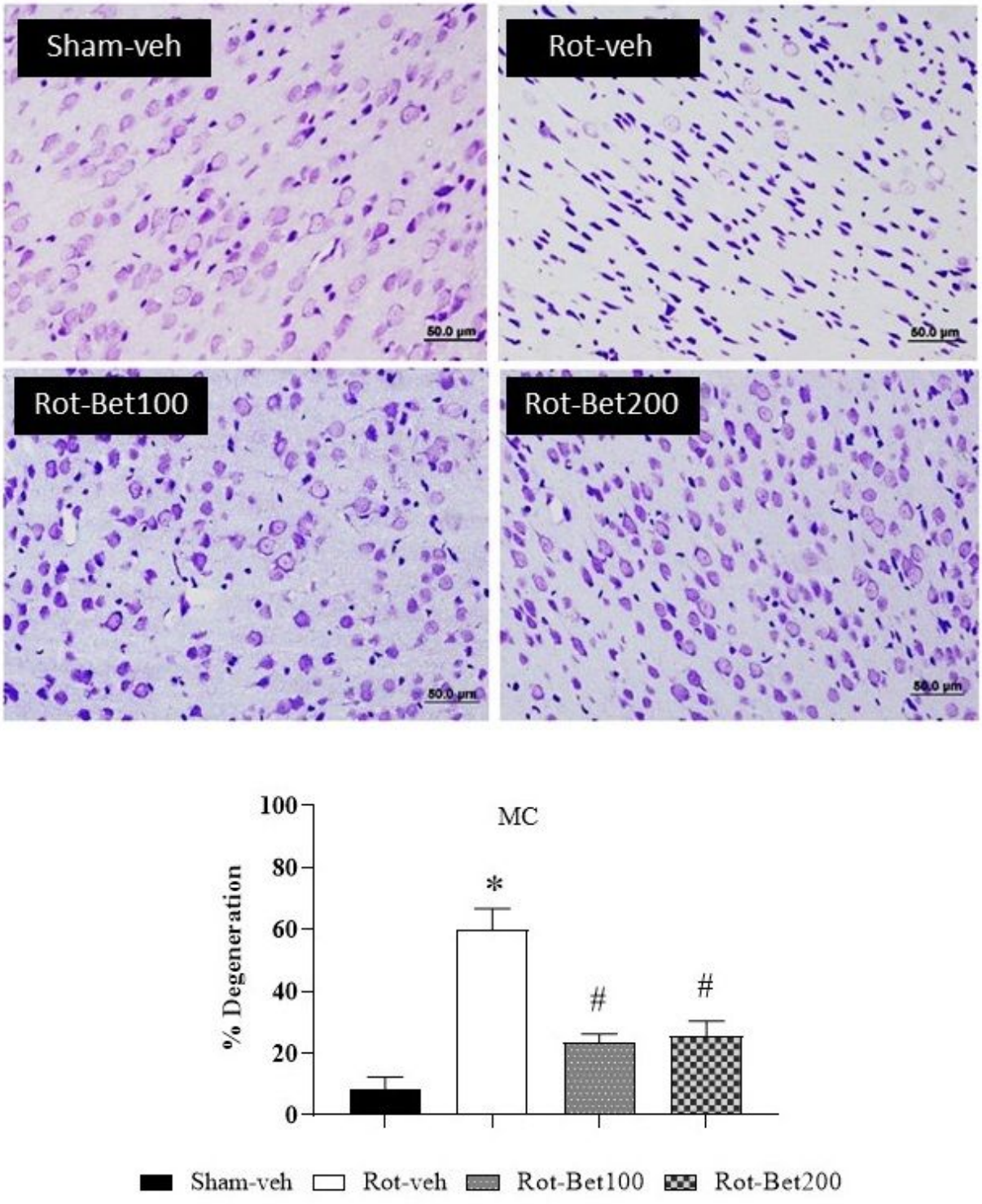


Figure 5

Photomicrographs of MC at 100x magnification, including staining with 0.1% cresyl violet for Sham-veh, Rot-veh, Rot-Bet100 and Rot-Bet200, respectively, and with a 50-μm scale bar. Histograms show the % of neuronal degeneration in MC. *indicates a significant difference compared to Sham-veh; #indicates a significant difference compared to Rot-veh.

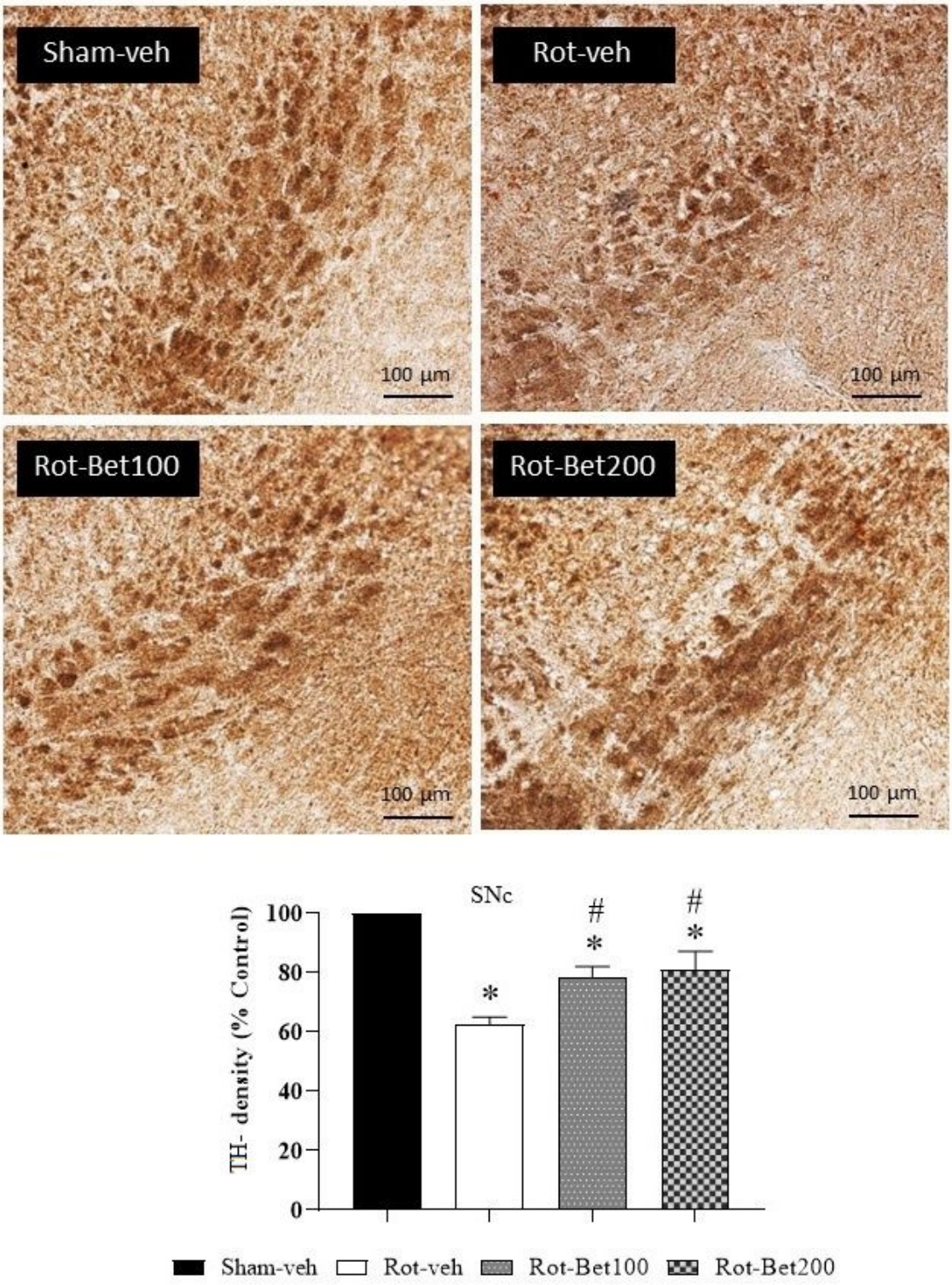


Figure 6

TH immunohistochemistry in SNc. Photomicrographs of TH staining in SNc captured at 100x magnification of Sham-veh, Rot-veh, Rot-Be100 and Rot-Bet200, respectively, and with a 100-µm scale bar. A histogram shows TH density in SNc related to the % of control (j). *indicates a significant difference compared to Sham-veh; #indicates a significant difference compared to Rot-veh.

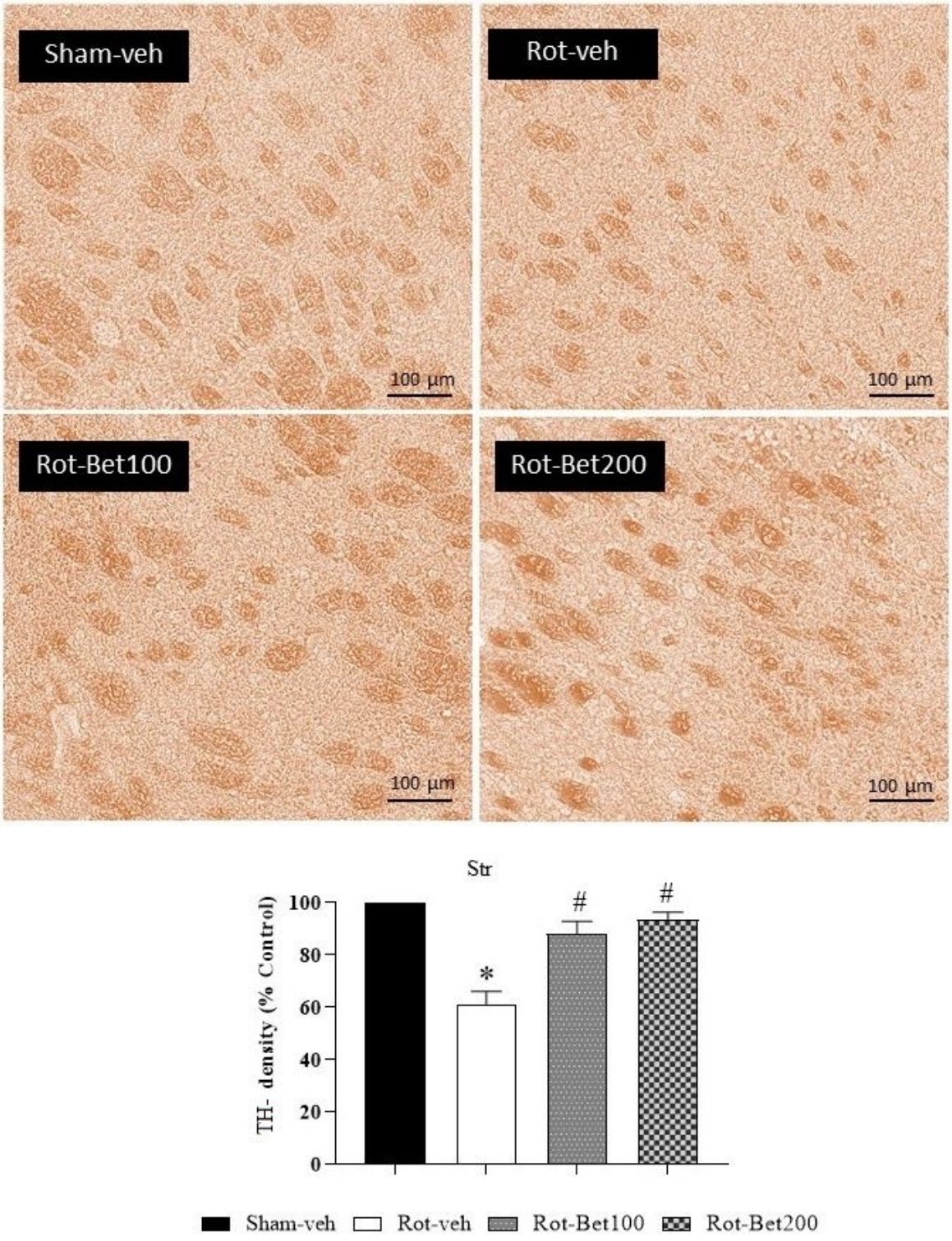


Figure 7

TH immunohistochemistry in Str. Photomicrographs of TH staining in Str were captured at 100x magnification of Sham-veh, Rot-veh, Rot-Be100 and Rot-Bet200, respectively, and with a 100-µm scale bar. A histogram shows TH density in Str related to the % of control. *indicates a significant difference compared to Sham-veh; #indicates a significant difference compared to Rot-veh.