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Neuroprotective effects of bee venom acupuncture therapy against rotenone-induced oxidative stress and apoptosis

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ABSTRACT

Parkinson's disease (PD), the most common neurodegenerative movement disorder, is characterized by dopaminergic neurodegeneration, mitochondrial impairment, and oxidative stress. Exposure of animals to rotenone induces a range of responses characteristic of PD, including reactive oxygen species production and dopaminergic cell death. Although L-dopa is the drug of choice for improving core symptoms of PD, it is associated with involuntary movements. The current study was directed to evaluate the neuroprotective effect of bee venom acupuncture therapy (BVA) against rotenone-induced oxidative stress, neuroinflammation, and apoptosis in PD mouse model. Forty male Swiss mice were divided into four groups: (1) received saline solution orally and served as normal control, (2) received rotenone (1.5 mg/kg, s.c. every other day for 6 doses), (3) received rotenone concomitantly with L-dopa (25 mg/kg, daily, p.o. for 6 days), and finally (4) received rotenone concomitantly with BVA (0.02 ml once every 3 days for two weeks). Rotenone-treated mice showed impairment in locomotor behavior and a significant reduction in brain dopamine, serotonin, norepinephrine, GSH levels, and paraoxonase activity, whereas a significant increase was observed in brain malondialdehyde, tumor necrosis factor-α, interleukin-β levels besides DNA damage, and over-expression of caspase-3, Bax, and Bcl-2 genes. Significant improvement of the aforementioned parameters was demonstrated after BVA compared to L-dopa therapy. In conclusion, bee venom normalized all the neuroinflammatory and apoptotic markers and restored brain neurochemistry after rotenone injury. Therefore, BVA is a promising neuroprotective therapy for PD.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease affecting middle aged and elderly people (Braak et al., 1995). It is characterized by loss of dopaminergic neurons of the substantia nigra (SN) and depletion of dopamine in the striatum (STR) that results in the typical symptoms of parkinsonism, including resting tremor, rigidity, and bradykinesia (Fahn and Przedborski, 2005). Neuroinflammation plays a critical role in the pathogenesis of PD (Hirsch et al., 2003). The primary mediators of neuroinflammation are activated microglia, which are innate immune cells of the central nervous system (CNS) found in and around the degenerating dopamine neurons (Block and Hong, 2005; Kreutzberg, 1996; McGeer and McGeer, 2008). Microglia are dramatically activated in response to neuronal damage and produce several potentially neurotoxic substances, including reactive oxygen species (ROS) and/or proinflammatory cytokines (Block et al., 2007).

Rotenone, a commonly used natural insecticide or fish poison, is highly lipophilic and readily passes to the brain (Talpade et al., 2000), where dopaminergic neurons are very susceptible to the induced mitochondrial complex I inhibition (Betarbet et al., 2006; Sherer et al., 2002). Chronic exposure of rats to rotenone recapitulates key features of parkinsonism, including selective loss of dopaminergic neurons and locomotor deficits (Deng et al., 2010). Effective experimental PD models should present dopaminergic degeneration, cytoplasmic inclusions, and motor malfunction (Sherer et al., 2002). According to our study and other previous reports, rotenone reproduced many features of PD, including systemic mitochondrial impairment, oxidative damage, nigrostriatal dopaminergic degeneration, L-dopa responsiveness, and cytoplasmic inclusions (Cannon et al., 2009; Mao et al., 2007; Sherer et al., 2002).

The crucial role of the neuroinflammatory process in PD suggests that inhibition of the microglial reaction might be a therapeutic avenue for reducing neuronal degeneration. There are no proven neuroprotective therapies for PD, and only symptomatic treatments are available. These include drugs therapy, such as L-dopa and dopamine agonists, MAO-B and COMT inhibitors, surgery, and physiotherapy (Hunot and Hirsch, 2003). L-dopa is the drug of choice in PD due to its ability to initially improve core symptoms by

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increasing basal ganglia dopamine activity. However, after 5 years of therapy, 50% of patients experience motor response complications, and the benefit from each dose becomes weaker ("wearing off" fluctuations), more unpredictable ("on–off" fluctuations), and associated with involuntary movements (dyskinesias). In addition, patients continue to suffer from fluctuations in motor function that are inherent to the disease itself (Verhagen Metman, 2002).

In the light of significant limitations of conventional therapy, complementary and alternative therapies represent an attractive strategy. Previous studies have suggested that bee venom (BV) results in antinociceptive and anti-inflammatory effects on arthritis, neuralgia, and chronic inflammation in humans (Lee et al., 2005) and in an animal model of inflammatory disease (Baek et al., 2006). Therefore, BV has been widely used in oriental medicine to treat some immune-related diseases, such as rheumatoid arthritis in humans and experimental animals (Kwon et al., 2002; Son et al., 2007). Recently, clinical trials have suggested that BV might be beneficial in the treatment of neurodegenerative diseases of the CNS, including PD (Cho et al., 2012; Mirshafiey, 2007). Several studies have shown that BV produced a marked suppression of leukocyte migration in the inflammatory-mediated animal models (Kwon et al., 2003) and a significant inhibition of microglia or macrophage activation (Han et al., 2007; Moon et al., 2007; Park et al., 2007). In addition, BV acupuncture (BVA) has been shown to protect dopaminergic neurons effectively against MPTP-induced toxicity due to inhibition of microglial activation in mice (Doo et al., 2010; Kim et al., 2011). Moreover, BV has a direct neuroprotective effect on SH-SY5Y human neuroblastoma cells from MPP^+-induced apoptotic cell death (Doo et al., 2012). However, such therapeutic uses are not supported by convincing evidence to date, and the underlying mechanism of BV amelioration of neurodegenerative diseases of the CNS, such as PD, remains to be elucidated. Our focus was to evaluate the neuroprotective role of BVA therapy against rotenone-induced oxidative stress, neuroinflammation, and apoptosis in mouse model of PD.

2. Materials and methods

2.1. Animals

Swiss male albino mice 20–22 g of body weight (age: 5–6 weeks) were obtained from animal house colony of the National Research Centre (NRC, Cairo, Egypt). Mice were housed under standardized conditions with free access to standard laboratory food and water. Animal procedures were performed in accordance with the Ethics Committee of the NRC and followed the recommendations of the National institutes of health guide for care and use of laboratory animals (Publication No. 85-23, revised 1985).

2.2. Experimental design

Animals were divided into four groups (10 mice each). Group (1) received saline solution orally and served as normal control. Group (2) received rotenone (Sigma–Aldrich Chem. Co, MA, USA), 1.5 mg/kg, s.c., dissolved in DMSO every other day at 6 doses for induction of parkinsonian behavior (Gawad et al., 2004). Group (3) received rotenone concomitantly with L-dopa (Sinemet® tab (carbidopa/L-dopa, 25/250), Merk & Co. Inc., Whitehouse Station, NJ, USA), 25 mg/kg, daily, p.o., for 6 days (De Leonibus et al., 2009). Group (4) received rotenone concomitantly with BVA (Sigma–Aldrich Chem. Co, USA) with 0.02 ml (1:2000 w/v) to acupoint GB34 bilaterally once every 3 days for 2 weeks (Doo et al., 2010).

2.3. Neurobehavioral measures

At the end of the experimental period, all mice were screened for motor behavioral impairment using the wire hanging test and the cylinder test.

2.3.1. Wire hanging test

Neuromuscular strength was evaluated through the wire hang test, where mouse was placed with its forelimbs on a wire mounted horizontally of 20 cm length, 50 cm above the surface. Latency time to fall was recorded. 30 seconds cut off time was taken. Soft padding was placed on the landing area to avoid injury of the mice (Sanberg et al., 1996).

2.3.2. Cylinder test

Cylinder test uses rearing frequency to assess the locomotor activity. Spontaneous movement was measured by placing the animal in a small transparent cylinder (height, 15.5 cm; diameter, 12.7 cm) for 5 min. The number of rears was recorded after each treatment. A rear was counted when an animal made a vertical movement with both forelimbs removed from the ground. This test has been successfully used previously to assess behavioral deficits in the rats receiving subcutaneous or intravenous rotenone (Fleming et al., 2004).

2.4. Biochemical analysis

Mice were killed by decapitation under ether anesthesia, brains were excised, a part of the harvested brains was kept in 10% formol-saline for histopathological investigation, while the other part was washed with ice-cold saline (0.9%), weighed, and stored at –80 °C for further biochemical and molecular analyses. The brain was homogenized with 0.1 M phosphate buffer saline at pH 7.4, to give a final concentration of 10% w/v for the biochemical assays.

2.4.1. Oxidative stress markers

Lipid peroxidation was estimated by measuring thiobarbituric acid reactive substances (TBARS) in brain samples according to the method of Placer et al. (1966) and the results were expressed as nmole malondialdehyde (MDA)/g wet tissue. In addition, brain glutathione (GSH) was evaluated by the method of Ellman (1959) and expressed as μmol/g wet tissue.

2.4.2. Brain paraoxonase -1 (PON1) activity

The determination of PON1 activity was carried out in accordance with the method of Gatica et al. (2006). This assay involves the hydrolysis of phenylacetate (substrate) by PON1/arylesterase activity releasing phenol. The phenol formed after the addition of a 40-fold diluted homogenate sample was spectrophotometrically measured at 217 nm. Blanks were included to correct the spontaneous hydrolysis of phenylacetate. The activity of PON1 was expressed in K unit/g wet tissue. One unit was defined as the enzyme quantity that disintegrates 1 nmol phenylacetate per minute.

2.4.3. Brain cytokine levels

Brain TNF-α and IL-1β were determined by enzyme linked immunosorbent assay (ELISA) following the methods of Kitaura et al. (2004) and Tamaoki et al. (1999), respectively, using commercial ELISA kits (Invitrogen Corporation Camarillo, California,USA) and microtiter plate reader (Fisher Biotech, Germany). An aliquot of sample or calibrator containing the antigen to be quantified is allowed to bind with a solid phase antibody. After washing, enzyme labeled antibody is added to form a sandwich complex of solid phase Ab-Ag-Ab enzyme. Excess (unbound) antibody is then washed away, then enzyme substrate is added. The enzyme catalytically

converts the substrate to product, the amount of which is proportional to the quantity of antigen in the sample.

2.4.4. Brain monoamines neurotransmitters

Determination of brain serotonin, dopamine, and norepinephrine contents was carried out using high performance liquid chromatography (HPLC) system, Agilent technologies 1100 series equipped with a quaternary pump (Quat pump, G131A model). Separation was achieved on ODS-reversed phase column (C18, 25 × 0.46 cm, i.d. 5 μm). The mobile phase consisted of potassium phosphate buffer/methanol (97:3, v) and was delivered at a flow rate 1 ml/min. UV detection was performed at 270 nm and the injection volume was 20 μl. The concentration of the neurotransmitters was determined by external standard method using peak areas. Serial dilutions of standards were injected and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentration in samples was obtained from the curve (Abdel-Salam et al., 2011).

2.5. Determination of caspase-3 activity

Activation of caspase-3 that occurred during the apoptotic process in mouse brain was assessed according to the user's manual for the caspase-3 assay kit. The supernatant obtained by a centrifugation of lysed cells was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (Acetyl-Asp-Glu-Val-Asp p-nitroanilide) and incubated for 90 min at 37 °C. Absorbance was measured with a microplate reader at a test wavelength of 405 nm.

2.5.1. DNA fragmentation analysis

Apoptotic DNA fragmentation was qualitatively analyzed by detecting the laddering pattern of nuclear DNA as described (Lu et al., 2002). Briefly, brain tissues were homogenized, washed in PBS, and lysed in 0.5 ml of DNA extraction buffer (50 mM Tris–HCl, 10 mM EDTA, 0.5%Triton, and 100 μg/ml proteinase K, pH 8.0) for overnight at 37 °C. The lysate was then incubated with 100 μg/ml DNase-free RNase for 2 h at 37 °C, followed by three extractions of an equal volume of phenol/chloroform (1:1, v) and a subsequent re-extraction with chloroform by centrifuging at 15,000 rpm for 5 min at 4 °C. The extracted DNA was precipitated in two volumes of ice-cold 100% ethanol with 1/10 volume of 3 M sodium acetate, pH 5.2 at –20 °C for 1h, followed by centrifuging at 15,000 rpm for 15 min at 4 °C. After washing with 70% ethanol, the DNA pellet was air-dried and dissolved in 10 mM Tris–HCl/L mM EDTA, pH 8.0. The DNA was then electrophoresed on 1.5% agarose gel and stained with ethidium bromide in Tris/acetate/EDTA (TAE) buffer (pH 8.5, 2 mM EDTA, and 40 mM Tris–acetate). A 100-bp DNA ladder (Invitrogen, USA) was included as a molecular size marker and DNA fragments were visualized and photographed by exposing the gels to ultraviolet transillumination.

2.5.2. Expression analysis of apoptosis related genes (Caspase-3, Bcl-2, and Bax)

2.5.2.1. Isolation of total RNA. Total RNA was isolated from brain tissues of treated mice by the standard TRIzol® Reagent extraction method (cat#15596-026, Invitrogen, Germany). Briefly, tissue samples were homogenized in 1 ml of TRIzol® Reagent per 50 mg of the tissue. Afterwards, the homogenized sample was incubated for 15 minutes at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol® Reagent was added. Then the samples were vortexed vigorously for 15 seconds and incubated at room temperature for 3 minutes. The samples were centrifuged for no more than 12,000× *g* for 15 minutes at 4 °C. Following centrifugation, the mixture was separated into lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per 1 ml of TRIzol® Reagent used for the initial homogenization. Afterwards, the samples were incubated at 15 to 30 °C for 10 minutes and centrifuged at 12,000× *g* for 10 minutes at 4 °C. The RNA was precipitated which was often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at no more than 7500× *g* for 5 minutes at 4 °C. The supernatant was removed and RNA pellet was air-dried for 10 minutes. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip. Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

2.5.2.2. Reverse transcription (RT) reaction. The complete Poly(A) + RNA isolated from brain tissues was reverse transcribed into cDNA in a total volume of 20 μl using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5 μg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM MgCl2, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50 μM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M-MuLV reverse transcriptase. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards the reaction tubes containing RT preparations were flash cooled in an ice chamber until being used for DNA amplification through quantitative real time-polymerase chain reaction (qRT-PCR).

2.5.2.3. Quantitative real time-polymerase chain reaction (qRT-PCR). PCR reactions were set up in 25 μL reaction mixtures containing 12.5 μL 1 × SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 μL 0.2 μM sense primer, 0.5 μL 0.2 μM antisense primer, 6.5 μL distilled water, and 5 μL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0 °C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 sub-steps: (a) at 95.0 °C for 15 sec; (b) at 55.0 °C for 30 sec; and (c) at 72.0 °C for 30 sec. The third step consisted of 71 cycles which started at 60.0 °C and then increased about 0.5 °C every 10 sec up to 95.0 °C. At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers. Each experiment included a distilled water control.

The quantitative values of RT-PCR (qRT-PCR) of Bax (Bax-F: 5′-CGA GCT GAT CAG AAC CAT CA-3′, Bax-R: 5′-CTC AGC CCA TCT TCT TCC AG-3′, NCBI: NM-017059.2); Bcl2 (Bcl2-F: 5′-CTC AGT CAT CCA CAG GGC GA-3′, Bcl2-R: 5′-AGA GGG GCT ACG AGT GGG AT-3′, Khalil and Booles, 2011); caspase 3 (Casp3-F: 5′-GGA CCT GTG GAC CTG AAA AA-3′, Casp3-R: 5′-GCA TGC CAT ATC ATC GTC AG-3′, NCBI: NM-012922.2) genes were normalized on the bases of β-actin (β -actin-F: 5′-CAC GTG GGC CGC TCT AGG CAC CAA-3′, β-actin-R: 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′, Khalil and Booles, 2011) expression. At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

2.6. Calculation of gene expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae (Bio-Rad Laboratories Inc, 2006):

$$Ef = 10\text{-}1/slope$$

$$Efficiency\,(\%) = (Ef - 1) \times 100$$

The relative quantification of the target to the reference was determined by using the ΔCT method if E for the target (Caspase 3, Bcl_2, and Bax) and the reference primers (β-Actin) are the same (Bio-Rad Laboratories Inc, 2006):

$$Ratio(reference/target\ gene) = Ef\ C_T(reference) - C_T(target)$$

2.7. Statistical analysis

Behavioral and biochemical results were assessed using one way-analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons. GraphPad Prism, 5.00 for windows software (CA, USA) was used to calculate these results. Molecular data were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System (SAS, Version 9.1, Statsoft Inc., Tulsa, USA) followed by Duncan's multiple rank-test to assess significant differences between groups. Data were expressed as mean ± S.E.M. Statistical significance is marked as following: The highest mean is given the letter (a), the following mean not statistically different from the first is given (ab). If it is significantly different it is given the letter (b). All values not containing the same letter are statistically different. All statements of significance were based on probability of $p < 0.05$.

3. Results

3.1. Neurobehavioral measures

3.1.1. Wire hanging test

The present study showed that rotenone-treated mice had a shorter latency period in wire hanging test as compared to the control group. Co-administration of L-dopa to rotenone-treated mice succeeded in normalizing the latency period as compared to the control group. BVA of rotenone-treated mice showed an increased muscular strength similar to L-dopa-treated group (Fig. 1a).

Table 1
Effect of bee venom acupuncture (BVA) or L-dopa treatment on brain MDA level, paroxonase activity, and GSH level in rotenone-induced Parkinson's disease in mice.

Groups	Parameters			
	Control	Rotenone	Rotenone + L-Dopa	Rotenone + BVA
MDA Nmol/g wet tissue	183.5 ± 13.1^{cd}	326.1 ± 19.3^{a}	200.6 ± 18.1^{b}	172.4 ± 17.5^{d}
Paraoxonase KU/g wet tissue	35.6 ± 13.1^{a}	12.8 ± 6.3^{c}	25.6 ± 5.1^{b}	32.4 ± 10.3^{a}
GSH μg/g wet tissue	450.7 ± 20.1^{b}	226.9 ± 10.3^{c}	440.7 ± 9.3^{b}	478.6 ± 17.5^{a}

Each value represents the mean ±SE (n = 10). Means followed by the same alphabetical letters are not significantly different at $p < 0.05$.

3.1.2. Cylinder test

The present results showed that rotenone-treated mice had a low rearing frequency in cylinder test as compared to the control group. Co-administration of rotenone with either L-dopa or BVA succeeded in normalizing the rearing frequency as compared to the control group (Fig. 1b).

3.2. Oxidative stress markers

The relevant data showed a significant increase in brain MDA levels (77.7%) after rotenone induced PD as compared to the control group. Concomitant use of rotenone with L-dopa showed a significant improvement in brain MDA levels (39%), while concomitant use of rotenone with BVA succeeded in normalizing MDA levels as compared to rotenone-induced PD group.

Brain PON1 activity and GSH levels were significantly lowered (64.1%, 49.7%, respectively) in rotenone-induced PD group as compared to the control group. Concomitant L-dopa treatment of rotenone-treated mice improved PON1 (100%) and succeeded in normalizing GSH levels, while BVA concomitant use restored both PON1 activity and GSH level in comparison with rotenone-induced PD group (Table 1).

3.3. Brain cytokine levels

The present results showed significant elevation in brain TNF-α, and IL-1β (172.8%, 167% respectively) in rotenone-treated group versus the control group. Mice that received rotenone with L-dopa treatment revealed significant improvement in both TNF-α (50.5%)

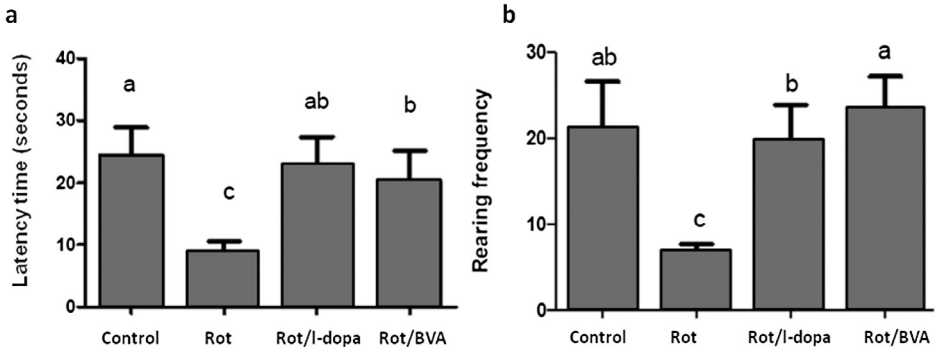

Fig. 1. Effect of bee venom acupuncture (BVA) on behavioral activity in rotenone-treated mice. The severity of motor dysfunction in rotenone-treated mice was quantified using wire hanging test of neuromuscular strength (a). Mice were placed by forelimbs on a wire mounted horizontally 50 cm above the surface and latency time to fall was recorded with maximum time of 30 seconds. Rearing frequency (in cylinder test) was used to examine spontaneous activity (b), which was retained by BVA. Values are mean ± SEM (n = 6/group). Statistical analysis was carried out by one-way ANOVA followed by Tukey–Kramer multiple comparison test. Columns followed by the same alphabetical letters are not significantly different at $p > 0.05$.

Table 2
Effect of bee venom acupuncture (BVA) or L-dopa treatment on brain inflammatory cytokine markers in rotenone-induced Parkinson's disease in mice.

Groups	Parameters			
	Control	Rotenone	Rotenone + L-Dopa	Rotenone + BVA
TNF-α Pg/g wet tissue	29.8 ± 3.6[d]	81.3 ± 7.2[a]	200.6 ± 18.1[b]	31.5 ± 4.2[cd]
IL-1β Pg/g wet tissue	59.3 ± 13.5[d]	158.4 ± 35.3[a]	85.7 ± 12.3[b]	70.1 ± 8.3[cd]

Each value represents the mean ±SE (n = 10). Means followed by the same alphabetical letters are not significantly different at $p < 0.05$.

Table 3
Effect of bee venom acupuncture (BVA) or L-dopa treatment on brain monoamine levels in rotenone-induced Parkinson's disease in mice.

Groups	Parameters			
	Control	Rotenone	Rotenone + L-Dopa	Rotenone + BVA
Dopamine μg/g wet tissue	4.1 ± 0.8[a]	1.03 ± 0.1[c]	3.9 ± 1.3[a]	2.9 ± 0.9[b]
Serotonin μg/g wet tissue	3.3 ± 0.3[b]	0.9 ± 0.02[c]	4.3 ± 1.1[a]	4.1 ± 1.2[ab]
Noradrenaline μg/g wet tissue	1.5 ± 0.03[c]	0.5 ± 0.04[d]	1.9 ± 0.2[a]	1.4 ± 0.9[bc]

Each value represents the mean ±SE (n = 10). Means followed by the same alphabetical letters are not significantly different at $p < 0.05$.

and IL-1β (45.9%), while BVA reinstated both brain TNF-α and IL-1β (Table 2).

3.4. Brain monoamine neurotransmitters

As shown in Table 3, rotenone-induced PD mice showed a significant decline in brain dopamine, serotonin, and noradrenaline (75%, 72.8%, and 66.7%, respectively) versus control group. Normalization of brain dopamine was achieved by concomitant use of L-dopa, while a superior effect was observed on both brain serotonin, and noradrenaline in comparison with rotenone-induced PD group. Profound enhancement of brain dopamine (181.5%) was observed after treatment with BVA of rotenone-injected mice, whereas normalization of both serotonin and noradrenaline was achieved when compared to rotenone-induced PD group.

3.5. Caspase-3 activity

The results of the present study revealed that rotenone induced a significant elevation in caspase-3 activity in brains of mice (271.4%) as compared to the control animals. However, treatment with L-dopa significantly reduced the level of caspase-3 activity (153.6%) when compared to rotenone-treated mice. Moreover, treatment with BVA succeeded in normalizing caspase-3 activity in comparison with the control mice (Fig. 2a).

3.6. Expression analysis of Caspase-3, Bcl-2, and Bax genes

Expression of apoptosis related genes (Caspase-3, Bcl-2, and Bax) in brain tissues of mice exposed to rotenone alone or in combination with either L-dopa or BVA is summarized in Fig. 2b–d. There was a significant increase in mRNA levels of caspase-3, Bcl-2 and Bax genes in brain tissues of mice exposed to rotenone as compared to control group (Fig. 2b–d). However, treatment with L-dopa and BVA significantly decreased the expression levels of these genes as compared to rotenone treated group. While the expression levels of caspase-3 and Bax genes in L-dopa treated group were still significantly higher than those of control group (Fig. 2b, d), treatment

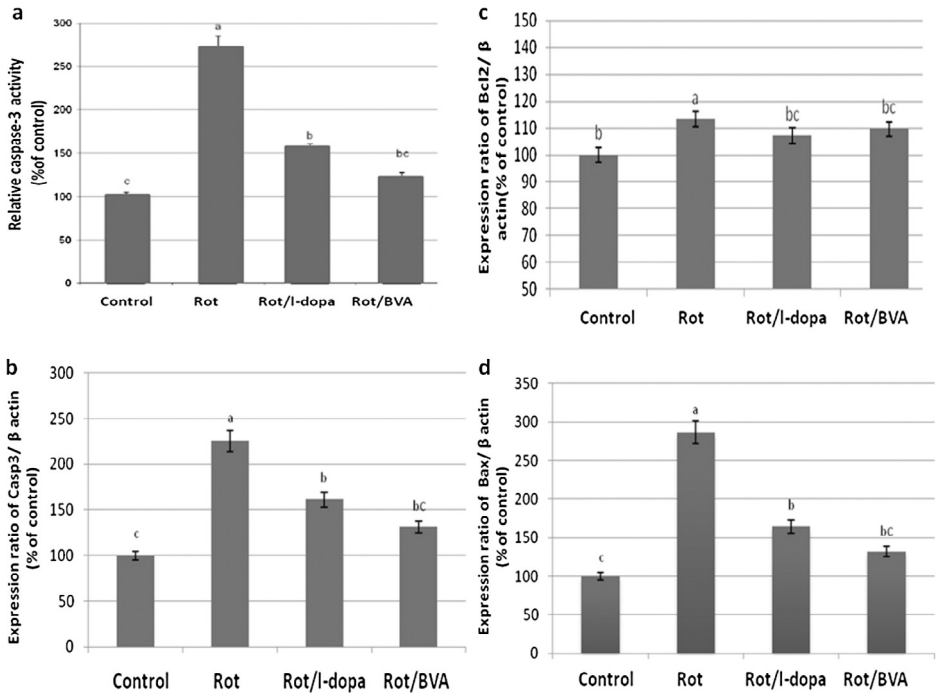

Fig. 2. Bee venom acupuncture (BVA) reduced the caspase-3 activation and the expression of apoptosis genes (Bax, Bcl2) in comparison to L-dopa in brain of rotenone (Rot)-treated mice. (a): casp-3 activity, (b): casp-3 gene expression, (c) Bcl-2 gene expression, and (d) Bax gene expression. Caspase-3 activity was determined using caspase-3 colorimetric assay kit according to the manufacturer protocol. Expression levels were normalized to β-actin expression levels and expressed relative to saline-treated mice. Values represented as percentage of control animals, (n = 8/group). Statistical analysis was performed using General Liner Models (GLM) procedure and followed by Duncan's multiple-rank test. Columns followed by the same alphabetical letters are not significantly different at $p < 0.05$.

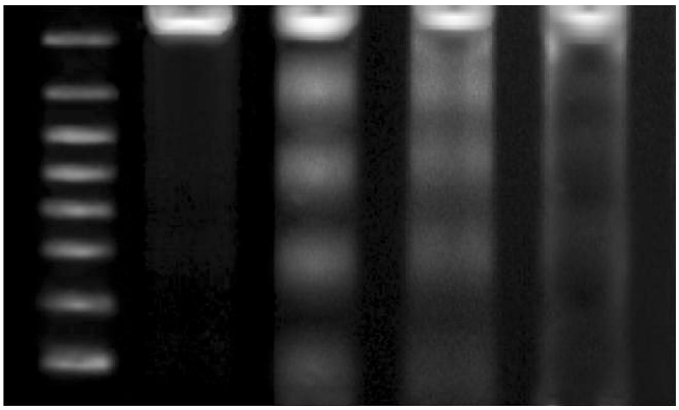

Fig. 3. DNA fragmentation detected with agarose gel of DNA extracted from brain tissues and analyzed by DNA gel electrophoresis laddering assay. Lane 1 represents DNA ladder. Lane 2 represents control samples, Lane 3 shows DNA fragmentation in brain tissues following rotenone exposure, Lane 4 shows DNA fragmentation in brain tissues following L-dopa treatment of rotenone-treated mice, and Lane 5 shows DNA fragmentation in brain tissues following BVA treatment of rotenone-exposed mice.

with BVA significantly decreased the expression levels of caspase-3 and Bax genes to normal levels.

3.7. DNA fragmentation

Rotenone treatment increased the DNA damage as compared to control group. However, treatment with L-dopa showed a significant reduction in DNA damage versus mice treated with rotenone alone. In addition, BVA treatment succeeded to inhibit DNA damage more effectively than L-dopa as compared to control group (Fig. 3).

4. Discussion

The current findings revealed enhanced motor activity by BVA treatment of rotenone-induced PD model in mice, resembled in wire hanging and cylinder tests, corroborated with restored brain dopamine content. Furthermore, inflammation and oxidative damage caused by rotenone were antagonized by BVA, which was reflected in the apoptotic markers in a comparable effect to L-dopa treatment. Motor functions were not enhanced by acupuncture alone in previous reports (Cristian et al., 2005; Shulman et al., 2002). Motor restoring effect of BVA can be explained by restored monoamine levels in brain. Dopamine is a major neurotransmitter in motor functions (Zhou et al., 2008). Rotenone primarily causes dopaminergic neuronal loss, which is responsible for the parkinsonian motor symptoms observed in rotenone experimental models (Cenci and Lundblad, 2005). Serretti et al. (2006) mentioned that dysfunction in biogenic amines plays a role in PD, which is noticed in the current study where dopamine, serotonin, and noradrenaline were depleted in the brain after rotenone administration and reversed by BVA and L-dopa treatment. Such an effect is likely to be a direct action of BV where acupuncture was not proven to increase dopamine level in the brain (Huang et al., 2010). BV was reported to maintain the survival of tyrosine-hydroxylase containing neurons in SN of MPTP-treated mice (Kim et al., 2011). Moreover, BVA induced an increase in noradrenaline level, which is related to enhanced motor functions (Thomas and Palmiter, 1997), in a direct relationship to the increased dopamine level, the main precursor for its synthesis.

Furthermore, neuronal degeneration in PD is mediated through inflammatory reactions and pro-inflammatory cytokines (Gao et al., 2003). In accordance, IL-1β and TNF-α are elevated in brain of PD patients (Mogi et al., 1994; Nagatsu et al., 2000). In the present study, BVA restored the rotenone-induced elevation in TNF-α and IL-1β, reinforcing the anti-inflammatory effect of BVA that can be a plausible mechanism by which BVA attenuates brain neurodegeneration, hence indirectly raises the DA level. BV directly inhibits leukocyte migration and generation of TNF-α, IL-1, and ROS in inflammatory conditions (Lee et al., 2008; Nam et al., 2003; Park et al., 2007).

BV is a rich mixture of peptides, such as apamin and melittin, enzymes, biologically active amines, and nonpeptide components (Matysiak et al., 2011). Apamin, through acting on Ca^{2+}-activated K^+ (SK) channels, reserved dopaminergic neurons in MPTP/probenecid-intoxicated mice (Alvarez-Fischer et al., 2013) and elevated dopamine level in prefrontal cortex of rats (Steketee and Kalivas, 1990). Mellitin, the major component of BV, inhibits the activity of Na/K-ATPase pump (Yang and Carrasquer, 1997) that adds protection to dopaminergic neurons in vitro (Salthun-Lassalle et al., 2004). Moreover, melittin suppresses inflammation by inhibiting the enzyme phospholipase A (Saini et al., 1997), which causes tissue degradation and, thus, dysfunction (Mihelich and Schevitz, 1999). Additionally, Yang et al. (2011) have reported improved motor activity and decreased neuroinflammation in mice model of amyotropic lateral sclerosis after melittin treatment. Based on these previous observations, the current protective effect of BVA can be justified. However, Alvarez-Fischer et al. (2013) reported that BV produces neuroprotection independently from the anti-inflammatory properties. Indeed, oxidative damage plays a central role in the pathogenesis of PD as proven in human and animal studies (Sian et al., 1994). Inflammatory processes produce ROS that initiate oxidative damage (Miller et al., 2009). The high content of dopamine and lipids in dopaminergic cells augment their oxidative damage (Milusheva et al., 2010). Rotenone being a mitochondrial complex I inhibitor imposes free radical damage (Radad et al., 2006). In the current experiment, this insecticide generated a depletion of GSH, in addition to a reduced PON1 activity in brain corroborated with increased lipid peroxidation products, supporting previous reports (Gawad et al., 2004; Zaitone et al., 2012). PON1 enzyme protects membranes against lipid peroxidation being associated with the high density lipoprotein (HDL) (Rosenblat and Aviram, 2009), and was found to be reduced in PD (Ikeda et al., 2011). This unequivocal oxidative state of brain indicates reduction of the cellular antioxidant capacity leading to cell disruption (Drukarch and van Muiswinkel, 2000), mimicking the situation in PD brain (Ilic et al., 1999). In the present work, BVA showed powerful antioxidant effect manifested in reduced lipid peroxides besides normalized GSH level and PON1 activity; hence the neuroprotective effect presented by BVA in mice could be through the restoration of the antioxidant pool of the brain tissue, thus preventing neuronal injury. Such an effect is also described in the study of Choi et al. (2009), where BVA protected dopamine neurons from oxidative damage in MPTP-intoxicated mice. In subsequent events, BV decreased the expression level of Bax gene induced by rotenone treatment and hence suppressed the apoptotic pathway, which was further demonstrated by alleviated DNA fragmentation and suppressed caspase-3 activation induced by rotenone. Bcl-2 and Bax play an important role in the mitochondria-dependent apoptotic pathway (Doo et al., 2012). Induction of apoptosis factors would generate activation of the caspase cascade (Miller et al., 1997). The BVA proved anti-apoptotic activity might have a role in preserving the dopaminergic cells and preventing the neurodegenerative process detected in this study, where a decrease in Bax/Bcl2 ratio associated with decreased caspase-3 were reported. Apamin, the BV protein, prevents apoptosis indirectly by blocking SK channels on dopamine neurons (Salthun-Lassalle et al., 2004). In addition, BV was reported to directly inhibit apoptosis in SH-SY5Y human neuroblastoma cells (Doo et al., 2012). Furthermore, previous studies reported direct neuroprotective effect of BV on MPP^+ or MPTP/probenecid-induced

apoptotic cell death (Alvarez-Fischer et al., 2013; Doo et al., 2012) supporting the current observation.

In conclusion, the present results confirm that BVA therapy has a neuroprotective effect on rotenone-induced neurodegeneration that is mediated through inhibition of neuroinflammation, oxidative stress, and apoptosis. From these findings, we suggest BVA therapy as a new approach in PD treatment or as an adjuvant therapy in preference to its individual components, which act synergistically.

Conflicts of interest

All authors declare no conflicts of interest.

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