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ORIGINAL ARTICLE

Effects of bromelain on motor responses following intra-medial forebrain bundle 6-OHDA injection in rat model of parkinsonism

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Abstract

Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta. The conventional therapeutic measures which include the widely used L-DOPA therapy, are inefficient especially when dopamine loss is severe, and the physical symptoms are full blown. Since neuroinflammation is a core feature of PD, this raised the question of whether early treatment with an anti-inflammatory agent may provide a more efficient intervention for PD. In this study, we investigated the effect of bromelain (an anti-inflammatory drug) on motor responses and dopamine levels in a parkinsonian rat model. Male Sprague-Dawley rats were lesioned stereotaxically with the neurotoxin 6-OHDA. The anti-inflammatory agent, bromelain (40 mg/kg i.p) was used to treat a subset of the rats prior to or 24 h post 6-OHDA lesion. Locomotor activity was assessed after 6-OHDA injection, using the cylinder and step tests. The cortical and striatal concentrations of dopamine were also measured. 6-OHDA injection resulted in marked motor impairment which was prevented by pretreatment with bromelain prior to the lesion. Also, the injection of 6-OHDA into the medial forebrain bundle resulted in a significant reduction in dopamine concentration in the striatum and PFC. Bromelain treatment did not alter the suppression of cortical and striatal dopamine levels. Pre-treatment with bromelain reduced the motor dysfunction in the parkinsonian rat model of PD. The efficacy of treatment with bromelain does not appear to be via preservation of the dopaminergic system. The efficacy of bromelain in 6-OHDA injected rats still remains unclear.

Keywords Parkinson's disease · 6-OHDA · Dopamine · Bromelain

Introduction

Dopamine (DA) is a catecholamine neurotransmitter widely distributed in the central nervous system and some peripheral areas within the systemic environment (Mccabe 2017; Rodríguez-Nogales et al. 2016). In the brain, DA is involved in the control of movement, emotions, cognition, reward and memory (Lud Cadet et al. 2010). Neurodegenerative disorders such as Parkinson's disease (PD) are related to decreased DA transmission (Bhat et al. 2015). The loss of DA in PD causes an imbalance in the initiation and execution of movements specifically through hyper-activation of the indirect (inhibitory) pathway which is primarily associated with D_2-like dopamine receptor activity (Espinoza et al. 2015). DA within the prefrontal cortex (PFC) is reported to be crucial for mediating cognitive control processes that permit optimal performance during complex cognitive tasks (Robbins and Cools 2014). However, it is vulnerable to disruption and this could result in maladaptive behavioural functions (Crone and Dahl 2012). The nigrostriatal source of DA in the brain is intimately involved in the disturbance in motor functions as the substantia nigra pars compacta (SNpc) sends its projection to the dorsal striatum and further regulates spontaneous motor activity in conjunction with cortical circuitry (Feyder et al. 2011; Leisman et al. 2014).

Despite numerous studies aimed at elucidating the neurodegenerative processes involved in PD, the aetiology remains obscure and PD patients continue to suffer severe adverse effects with current therapies for the disease (Farzanehfar 2018; Fifel and Videnovic 2018). L-DOPA loses its efficacy over time, especially when the motor symptoms of the disease are severe (Bezard et al. 2001; Rascol et al. 2003). For

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instance, neurocognitive side effects are becoming more evident with deep brain stimulation of the sub-thalamic nucleus because of the spread of stimulation by the electrode to the surrounding structures (Benabid et al. 2009). Also, disappointing clinical outcomes and severe dyskinesia were reported for neural transplantation which has been used as an alternative therapy for the disease (Stocchi et al. 2002; Björklund et al. 2003).

Neuroinflammation is a possible mechanism in PD (Wang et al. 2015). Some reports have further implicated sustained inflammatory responses, glial cell activation and T- cell infiltration as common features of PD both in humans diagnosed with the disease and in animal models and that they play vital roles in the degeneration of DA neurons (Hirsch et al. 2012; Lv et al. 2015). Higher levels of pro-inflammatory mediators including Tumour necrosis factor (TNF-α), interleukin-1β (IL-1β) and interferon-gamma (IFN-γ) were reported in the midbrain of a patient with PD which was attributed to the presence of reactive microglia in the SN pars compacta (Mcgeer et al. 1988). In addition, a large group of pro-inflammatory cytokines were reported to be up-regulated with PD diagnosis and this was suggested to contribute to acceleration of nigral DA neuron degeneration (Mccoy et al. 2006; Ferrari et al. 2006). In animal model of PD, molecules such as α-synuclein, ATP and metalloproteinase-3 (MMP-3) were released from the degenerating DA neurons and the neuroinflammatory responses in the parkinsonian brain were amplified as the symptoms of the disease became obvious (Saijo et al. 2009; Zhang et al. 2005).

Bromelain is an anti-inflammatory cysteine protease derived from pineapple stem that acts through down-regulation of plasma kininogen (Gaspani et al. 2002), inhibition of prostaglandin (PG) E_2 expression, degradation of advanced glycation end product receptors and regulation of angiogenic biomarkers (Stopper et al. 2003) as well as antioxidant action upstream in the COX-pathway (Santanam et al. 2013). Beneficial effects of bromelain have been suggested in a variety of inflammatory diseases and animal models of inflammation. These include immunologically mediated arteriosclerosis in rat aortic allograft (Tochi et al. 2008), and an experimental allergic encephalomyelitis model for human autoimmune disease, multiple sclerosis (Darshan and Doreswamy 2004). Also, a study reported a significant improvement in acute knee pain after one month of treatment with bromelain (Walker et al. 2002). Evidence obtained in-vitro supports a neuroprotective effect in microglia (Abbasi Habashi et al. 2012). However, there is no information on the efficacy of bromelain in the treatment of neurodegenerative diseases such as PD. Therefore, in this study, we aimed to investigate the effect of bromelain treatment on spontaneous motor activity and dopamine levels in a rat model of PD and whether it has potential as an early phase adjunct treatment for the disease.

Materials and methods

Animals and surgery

All animal experiments were performed according to the NIH guidelines for the care and use of laboratory animals and were approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (AREC/019/016D). Male Sprague-Dawley rats were housed under a 12 h light/dark cycle with free access to standard rat chow and water in the Biomedical Resource Unit of the University of KwaZulu-Natal. At PND 51, the animals were divided into two major groups viz. pre-surgically treated rats with daily injections of bromelain (40 mg/kg i/p; Sigma-Aldrich, USA), ($n = 10$) and saline treated animals (10 ml/kg i/p; Adcock- Ingram, SA) ($n = 30$) for 7 days. The dose of bromelain was based on previous experiments (Onken et al. 2008). At PND 60, the animals were injected with desipramine (15 mg/kg i/p; Sigma, Munich, Germany) a norepinephrine reuptake blocker which serves to prevent 6-OHDA uptake by noradrenergic neurons. The rats were deeply anaesthetized with ketamine (90 mg/kg/i.p; Bayer Pty Ltd., SA) and xylazine (5 mg/kg/i.p; Intervet Pty Ltd., SA) was administered to stabilize systemic arterial pressure. The drugs were administered as a concoction and the animals were monitored until they were confirmed to be fully anaesthetized. Following 30 min after the administration of desipramine, the anaesthetized rats were positioned on a stereotaxic frame (Kopf Instruments, Tujunga, USA). The neurotoxin 6-OHDA (10 μg) dissolved in normal saline (4 μL) containing 0.2% ascorbic acid (Sigma, St. Louis, MO, USA) was injected into the left medial forebrain bundle (MFB), the preferred site of lesion because it avoids the confounding effect of direct mechanical damage to the striatum or substantia nigra, using the stereotaxic coordinates AP – 4.7, ML + 1.6, DV – 8.4 (Paxinos and Watson 1998; Howells et al. 2005). Control animals were injected with normal saline (4 μL). Following surgery, the animals were placed on a heating pad until recovery. This was followed by an injection of temgesic (0.05 mg/kg/s.c; Reckitt Benckiser Ltd., UK) for pain relief. The animals were further sub-divided into four groups viz.: pre-surgery saline treatment (10 mL/kg i.p, daily for 7 days) followed by intra-MFB saline injection, with post-surgery saline treatment (10 mL/kg i.p, daily for 7 days; Adcock-Ingram, South Africa) (**NN**), pre-surgery saline treatment (10 mL/kg i.p, daily for 7 days) followed by intra-MFB 6-OHDA injection, with post-surgery saline treatment (10 mL/kg i.p, daily for 7 days) (**6 N**), pre-surgery bromelain treatment (40 mg/kg i.p, daily for 7 days; Sigma-Aldrich, USA) followed by intra-MFB 6-OHDA injection with post-surgery saline treatment (10 mL/kg i.p, daily for 7 days) (**Br6**) and pre-surgery saline treatment (10 mL/kg i.p, daily for 7 days) followed by intra-MFB 6-OHDA injection, with daily post-surgery bromelain treatment starting from 24 h after

Table 1 Treatment schedule of the four groups: pre-surgery saline treated, saline injected, with saline post-surgery treated rats (**NN**); pre-surgery saline treated, 6-OHDA injected, with saline post-surgery treated rats (**6 N**); bromelain pre-surgery daily treatment for 7 days followed by 6-OHDA injection, with saline post-surgery treated rats (**Br6**); and pre-surgery saline treated, 6-OHDA injected, with post-surgery bromelain daily treatment starting from 24 h after surgery for 7 days (**24 Br**). n = 10 rats per group

Groups	Pre-surgical bromelain Treatment	6-OHDA (10 μg/4 μL) Lesion	Post-surgical bromelain Treatment
NN	–	–	–
6 N	–	+	–
Br 6	+	+	–
24 Br	–	+	+

surgery for 7 days (**24Br**). Behavioural assessment took place after the last drug/vehicle injection (Table 1).

Behavioural assessment

On PND 59, the day before surgery, all the rats were tested for forelimb use asymmetry using the cylinder and step tests. The same tests were repeated after the last post-surgical treatment with bromelain or saline. After each test, the cylinder and the floor were cleaned with 70% alcohol.

Cylinder test

The cylinder is an open-top transparent plexiglas cylinder (diameter: 20 cm, height: 30 cm) designed to evaluate locomotor asymmetry in rodent models of central nervous system (CNS) disorders (Schallert and Woodlee 2005). The animals were taken to the experimental room 1 h prior to the experiment. Each rat was placed in the plexiglas cylinder in the room and the behaviour was video recorded for 5 min/session. The number of times that the animal made independent use of forelimbs for support, weight shifting movements along the wall and landing after vertical exploration was assessed (Tillerson et al. 2001; Mpofana et al. 2014). The % use of impaired forelimb was determined using $\{\frac{(Right+{}^1/_2 Both)}{Righti+Left+Both}\} \times 100\%$.

Step test

The Step test involves the measurement of the impaired limb's step length (Schallert et al., 2000). The rat was held in a position such that the head and the untested forelimb of the rat were gently oriented forward by using the thumb and index finger, so as to minimize head turning (Woodlee et al. 2008; Mpofana et al. 2014). While held in this position, stepping movements were assessed for each forelimb along a ruler placed on the table and the length of the step taken was recorded. Three steps were taken for each forelimb and the mean was recorded as the length of step taken by each forelimb.

Animal sacrifice and tissue collection

A subset of the animals (6 per group) was randomly selected and sacrificed by decapitation 12 h following the last behavioural test (step test) procedure. The brain was removed immediately after decapitation and placed in a frozen 0.9% saline slush so as to suppress the degradation of the brain structures during dissection. The striatum and prefrontal cortex (PFC) were dissected, weighed, and placed in eppendorff tubes. Then, the tissues were snap-frozen in liquid nitrogen before being stored in a − 80 °C bio-freezer until further use. The remaining animals (4 per group) were also decapitated. The brains were removed and stored in the −80 °C bio-freezer for further research purposes.

Determination of dopamine levels in the PFC and striatum

In order to support our behavioural results and establish a neurochemical basis for the difference in behaviour caused by the injected neurotoxin and drug treatment, the dopamine concentration was analysed in both the PFC and striatal tissues using Sandwich-ELISA kits (Elabscience Biotech., Texas, USA). The dopamine assay protocol consisted of an extraction procedure that was followed by quantification. Both steps were conducted on the same day. The micro ELISA plate provided with the kit was pre-coated with specific antibody that recognized dopamine only in the samples. The PFC and striatal tissues were removed from the bio-freezer and EDTA-HCL buffer (4 mL/1 mg of tissue) was added to each tube containing the tissue to minimize the metabolism to 2–3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The tissues were sonicated immediately using an ultrasonic tissue disrupter (CML-4, Fischer, USA) and centrifuged at 1160×g for 10 min at 4 °C. The supernatant was pipetted into new eppendorff tubes. The standard, control and the samples were respectively pipetted into each well of the dopamine micro ELISA plate. Biotinylated detection antibody specific for dopamine (100 μL) was added to each well and incubated for 1 h at room temperature. Following this was the addition of conjugate (100 μL) to each well and incubation

for ½ hr. at room temperature. This was then followed by the addition of substrate (90 μL) to each well. The plate was then incubated at room temperature for 15 min. Following incubation, stop solution (50 μL) made of sulphuric acid was added. The absorbance of dopamine was quantified using a microtitre plate reader (SPECTROstar Nano, BMG LABTECH GmbH, Ortenberg, Germany) at a wavelength of 450 ± 2 nm within 10 min as per the manufacturer's protocol. All samples, standard and controls were analysed in triplicate. The assay guidelines provided by the manufacturer (Catalogue No: E-EL-R0046) were followed.

Statistical analysis

Data are displayed as group averages ± SEM. Statistical analysis was performed using Graph Pad Prism 5 (Graph Pad Software Inc., USA). The behavioural outcomes were compared using two-way repeated measures ANOVA followed by Bonferroni post hoc tests. One-way ANOVA followed by Bonferroni post hoc tests was used to analyse tissue dopamine concentrations. Effects were considered statistically significant at p value <0.05.

Results

Cylinder test

The percentage use of the impaired forelimb was analysed in rats of the four groups viz.: saline pre-surgical treatment, saline injected with saline post-surgical treatment (NN), saline pre-surgical treatment, 6-OHDA injected with saline post-surgical treatment (6 N), bromelain pre-surgical treatment followed by 6-OHDA injection with saline post-surgical treatment (Br6) and saline pre-surgical treatment, 6-OHDA injected with post-surgical bromelain treatment at 24 h (24Br) ($n = 10$ rats per group). There was a significant effect of the injected neurotoxin as the post-surgery percentage use of impaired forelimbs was significantly decreased compared to the pre-surgery values ($F(1, 36) = 41.34$, $p < 0.0001$, Fig. 1).

Step test

A significant effect of the neurotoxin was observed in the average step length which increased post lesion $F(1, 36) = 26.08$, $p < 0.0001$. There were no significant differences between groups pre-surgery. In the post-surgery groups, the average step length was significantly increased by treatment ($F(3, 36) = 5.30$, $p = 0.0039$), with 6-OHDA injection compared to saline injection ***(NN post vs. 6 N post, $p < 0.001$). Bromelain pre-treatment reduced the 6-OHDA-induced increase in the average step length *(6 N vs. Br6, $p < 0.05$; Fig. 2).

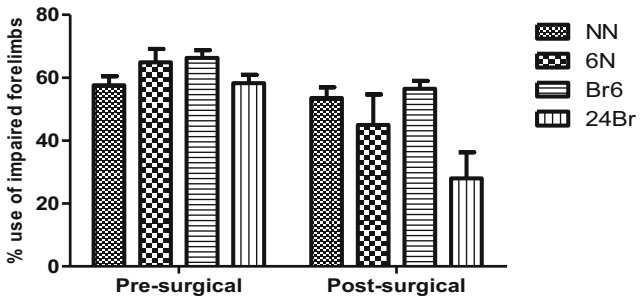

Fig. 1 Graph showing the % use of impaired forelimbs before (pre) and after (post) 6-OHDA injection in the saline treated rats (NN), saline treated, 6-OHDA injected rats (6 N), bromelain pretreated, 6-OHDA injected rats (Br6) and 6-OHDA injected, post-surgery bromelain treated rats (24 Br). n = 10/group

Dopamine levels in PFC and striatum

The striatal concentration of dopamine in the pre-surgery saline treated, 6-OHDA injected, with saline post-surgery treated rats (6 N) was significantly reduced compared to the control group (6 N vs. NN, $p < 0.05$, Fig. 3). The concentration of striatal dopamine in the bromelain treated groups (Br6 and 24Br) was not statistically different from the 6-OHDA injected, saline treated group (6 N vs. Br6, $p > 0.05$, Fig. 3) and (6 N vs. 24Br, $p > 0.05$, Fig. 3).

The cortical concentration of dopamine in the pre-surgery saline treated, 6-OHDA injected with saline post surgical treatment rats (6 N) was also significantly reduced compared to the control (6 N post vs. NN post, $p < 0.05$, Fig. 4). However, the cortical concentration of dopamine in the bromelain treated groups was not statistically different from the

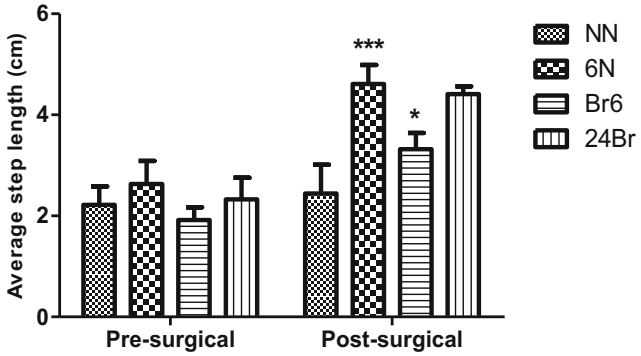

Fig. 2 Graph showing the averge step length before (pre) and after (post) 6-OHDA lesion in the saline treated rats (NN), saline treated, 6-OHDA injected rats (6 N), bromelain pretreated, 6-OHDA injected rats (Br6) and 6-OHDA injected, post-surgery bromelain treated rats (24 Br). Two-way repeated measures ANOVA showed the effect of both 6-OHDA and bromelain. 6-OHDA significantly increased the average step length $F(1, 36) = 26.08$, $p < 0.0001$. ***(NN post vs. 6 N post) $p < 0.001$. Bromelain pretreatment significantly reduced the average step length *(6 N post vs. Br6 post) $p < 0.05$. $n = 10$/group

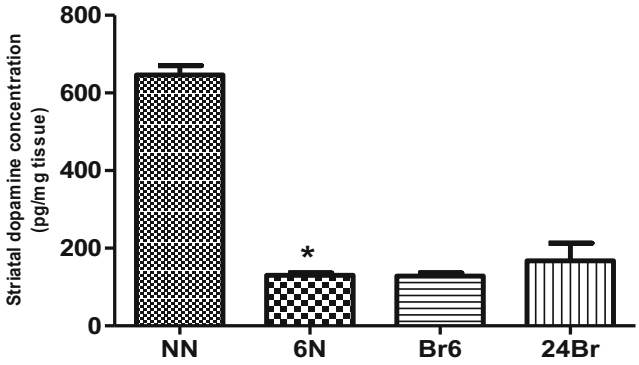

Fig. 3 Graph showing striatal dopamine concentration in the saline treated rats (NN), saline treated, 6-OHDA injected rats (6 N), bromelain pretreated, 6-OHDA injected rats (Br6) and 6-OHDA injected, post-surgery bromelain treated rats (24 Br) $F(3, 18) = 82.79$, $p < 0.0001$. *(NN vs. 6 N) $p < 0.05$

pre-surgery saline treated, 6-OHDA injected and with saline post surgical treatment group (6 N).

Discussion

We investigated the role of bromelain treatment on motor impairment in the cylinder test and the initiation of stepping movements by the contralateral paw, as well as on the dopamine concentration in both the PFC and striatum following a unilateral injection of 6-OHDA in the medial forebrain bundle.

In our study, a battery of behavioural tests was employed in order to assess the extent of motor impairment, each evaluating different aspects of motor function. Accurate detection of the motor impairment depends on the behavioural test employed. The behavioural incompetence directly relates to the damage of the nigrostriatal tract and begins to manifest

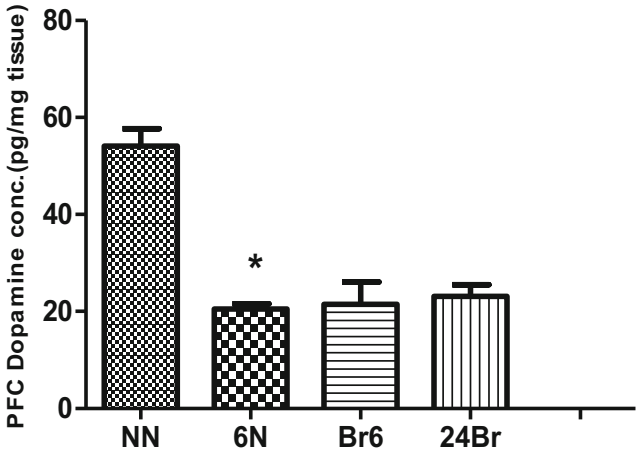

Fig. 4 Graph showing the PFC dopamine concentration in the saline treated rats (**NN**), saline treated, 6-OHDA injected rats (**6 N**), bromelain pre-treated, 6-OHDA injected rats (**Br6**) and 6-OHDA injected, post-surgery bromelain treated rats (**24Br**). $F(3, 20) = 25.70$, $p = 0.0001$. *(NN vs. 6 N), $p < 0.05$

when the severity of the lesion reaches a critical threshold around 60–80% loss of nigral dopamine cell bodies and their axons (Cheng et al. 2010). Each successful forepaw movement is linked with the propriospinal neurons located within the supra-spinal descending pathway while the brain stem and spinal cord are largely responsible for the regulation of exploratory movement in the cylinder test (Weidner et al. 2001; Piecharka et al. 2005). Our result showed that the motor impairment as a result of the 6-OHDA injection is a reflection of the extent of damage incurred by the neurotoxin in the neural circuitry which is projected to the limbs. This observation is supported by the extent of DA loss in both the striatum and PFC. The depletion of DA in the striatum and PFC was reported using similar lesion and coordinates (Robbins et al. 1990). This further suggests that the impairment of forelimb function following neurotoxin injection may not depend solely upon the striatal loss of DA but may also be a result of the loss of DA within the PFC. Also, the role of dopamine activity in motivation is attributed to mesolimbic DA system (IKEMOTO and PANKSEPP 1999). This therefore implies that the motivational behaviour to initiate stepping movement is abated due to the impairment of the PFC dopaminergic pathways.

The marginal limb-use improvement in the presurgical bromelain treated rats as shown by the step test prompted further investigation to determine whether dopamine concentration in the striatum and PFC complimented the behavioural results. The observed limb impairment with the 6-OHDA injection correlated with the neurochemical alterations at the striatal level. We clearly detected the loss of DA 7 days after 6-OHDA injection in comparison with saline injection and the determination of striatal DA concentration suggested that almost 20% still remained after 7 days of 6-OHDA injection. This observation supported that the loss of DA was attributed to the retrograde degeneration of nerve fibres through the production of reactive oxygen species by the 6-OHDA (Robertson and Robertson 1989; Yuan et al. 2005). The DA level in the PFC was similarly decreased by 6-OHDA injection into the MFB and this pattern of dopamine concentration following 6-OHDA intra-MFB lesion as seen in this experiment further supported the marked impairment in the accuracy and speed of response with disrupted discrimination performance as observed in lesioned rats (Robbins et al. 1990). The reduced PFC level of DA emphasizes the successful establishment of the PD model in rats and that the motor deficit induced by intra-MFB administration of 6-OHDA in rats may not be solely related to time dependent alteration in dopamine levels within the striatum but also in close association with dopamine depletion within the PFC. Also, the DA loss in the PFC after a period of 7 days suggests a severe effect of 6-OHDA lesion and a tendency to affect other neural networks that are concerned with the regulation of motor execution and movement inhibition.

In our study, pre-surgery bromelain treatment resulted in a significant decrease in the average step length compared to saline treatment prior to the 6-OHDA injection. This shows that early treatment with bromelain conferred behavioural protection. However, the prevention of behavioural impairment is unlikely to be attributed to a dopaminergic mechanism because the concentration of dopamine in the PFC and striatum of bromelain treated rats was not different from the saline treated, 6-OHDA injected rats. This is further explained in agreement with some previous reports that aside dopamine loss in PD, quite a number of factors like oxidative damage, mitochondrial dysfunction and neuroinflammation play pertinent roles in PD (Sherer et al. 2002; Li et al. 2004; Biesmans et al. 2013). However, there is no experimental evidence that motor complication in parkinsonism is entirely mediated by striatal depletion. There is possibility that bromelain may not exert its effect on the rats' behaviour by restoring dopamine level but rather by facilitating compensatory mechanisms such as inflammatory suppression in disease conditions. This is in agreement with a study where bromelain was demonstrated to reduce the secretion of granulocyte-macrophage colony stimulating factor in rats with disease that cohort with PD (Onken et al. 2008). In addition, bromelain was shown to alleviate inflammation through inhibition of cytokines/chemokines and further removed a number of cell surface molecule attractants that are vital for leukocytes migration in animals (Fitzhugh et al. 2008). The efficacy of bromelain on these factors appeared to be dependent upon its proteolytic activity. Therefore, the results of this study show that bromelain may not be able to protect against 6-OHDA induced dopaminergic neurodegeneration or striatal nerve terminal loss. Therefore, it is arguable that the drug may have acted as an anti-inflammatory agent and may have provided some protection against motor dysfunction via a different pathway. The improvement on the average step length by bromelain pre-treatment in our result technically suggests that the drug may have acted via a different mechanism aside dopamine restoration. There is tendency that bromelain acts through series of molecular events in pathological condition. This is in agreement with certain reports that bromelain significantly reduced the concentration of IL-13 and CD8+ T cells in the unhealthy animals and increased phosphorylation of Akt and FOXO3A to prevent mitochondrial dysfunction as well as protein degradation (Juhasz et al. 2008; Secor et al. 2008). These mechanisms were pointed out to be initiated before the onset of the physical symptoms of dopaminergic neural degeneration. With respect to our results in this study, we suggest that the efficacy of early treatment with bromelain may be more pronounced on the molecular mechanisms rather than the direct effect on dopamine levels in the brain.

Conclusion

In summary, we demonstrated impaired function of the affected forelimb in the 6-OHDA model of PD in rats and an improvement by pre-treatment with bromelain which did not appear to depend on preservation of dopamine levels in the striatum or PFC. Reduction in the impairment of forelimb function was observed in bromelain pre-treated rats independent of the 6-OHDA induced loss of dopamine neurons. We provided evidence to support the reduced level of dopamine in PFC and striatum as the neurochemical basis for motor dysfunction in Parkinson's disease. Despite early onset of treatment with bromelain, there was no improvement of 6-OHDA-induced dopamine loss. Regardless of that, early treatment with bromelain might represent a protective option to restore motor function in patients with Parkinson's disease via molecular events independent of dopamine loss in Parkinson's disease.

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