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Effects of Bromelain on Noradrenaline Release During Anxiety-Like Behaviour Following Intra-Medial Forebrain Bundle 6-OHDA Injection in Rat Model of Parkinsonism

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ABSTRACT

Introduction*: Parkinson's disease (PD) is characterized by emotional dysfunction which is a typical manifestation of non-motor symptoms. Non-motor symptoms may occur simultaneously with motor impairment in PD. Anxiety is a comorbidity in PD. This study therefore addresses the question of whether prior/post 6-OHDA lesion treatment with bromelain can show an effect on the behavioural signs of parkinsonism and damage to the CNS noradrenergic system in experimental rats.*

Methods*: Open field and despair swim tests were used to assess certain psychomotor functions and emotional reactivity in the neurotoxin injected rats. The level of noradrenaline in certain regions of the brain as well as in the blood was further analyzed by enzyme linked immunosorbent assay.*

Findings*: The results showed that unilateral injection of the neurotoxin into the rats' medial forebrain bundle caused a decrease in the rearing time and a corresponding increase in the time spent grooming. There was a further indication that 6-OHDA injection resulted in the reduction of noradrenaline in the systemic environment. Also, the duration of immobility was increased by 6-OHDA injection but not statistically significant. Pretreatment with bromelain caused a change in the grooming behaviour not consistent with noradrenaline level.*

Conclusion*: This finding suggests that bromelain treatment interferes with the regulation of mood and emotion during the early phase of parkinsonism in the 6-OHDA rat model of PD which was accompanied by changes in noradrenaline level.*

Keywords

6-OHDA, Noradrenergic, Anxiety, Bromelain.

Introduction

Mood disturbances are recognized as common non-motor, psychiatric comorbidities in idiopathic Parkinson's disease (PD) [1,2]. However, anxiety appears to be under-recognized and under-treated in patients with PD due to diagnostic imprecision, symptom overlap with motor and cognitive features of PD as well as under-reporting of symptoms by the patients [3]. Notably, people with PD report relatively higher rates of anxiety when compared to people who have other neurodegenerative disorders [4] which further implies a unique aetiology and neuropathology of mood disorders in PD [5]. It is widely speculated that memory performance is restricted by anxiety, as anxiety is thought to compete with task-relevant processes [6.] Het et al., [7] argued about the significance

of timing in the onset of PD and further emphasized that anxiety is induced prior to impaired memory functions.

Grooming and rearing are complex patterned neuro-behavioural indicators which are often seen as anxiogenic responses in animals [8]. Regulation of these behaviour is clearly centrally controlled and mediated by multiple brain regions as well as by various neuro-mediators, hormones and psychotropic drugs [9,10]. The thalamus was reported to be activated in the context of emotionally arousing environment and produced fear and anxiety-like behaviour in rats [11] and blockade of some receptors in the thalamus has anxiolytic effects [12]. In rats, the paraventricular thalamic nucleus was activated when rats were exposed to foot-shock [13]. Further evidences were provided to explain that the information associated with emotionally charged events was transmitted to the thalamus, which then provided excitatory inputs to the PFC and other areas of the forebrain involved with the emotional states [14,15]. Also, the integrity of the thalamic fibre tract was reported to be compromised in neurodegenerative disorder in humans [16].

Progressive degeneration of dopaminergic neurons is strongly associated with PD, culminating in motor dysfunctions [17]. The neuroanatomical and functional relationship between dopamine (DA) and noradrenaline (NA) have revealed that these central neurotransmitters share a biosynthetic pathway and sympathetic nerve stimulation releasing NA was speculated to compensate for the neuronal loss of DA in PD [18] by binding to DA receptors. In fact, diminished NA signalling was reported to exacerbate PD neuropathology while mechanisms to augment NA tone were further suggested to provide neuroprotection [19]. In the present study, we aimed at investigating the effect of bromelain (an anti-inflammatory agent) on the noradrenergic system and perhaps, its behavioural consequences in the early stage of PD.

Materials and methods Experimental 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 hr light/dark cycle with free access to standard rat chow and water in the Biomedical Resource Centre 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 [20]. 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 to the animals and were monitored until they were confirmed to be fully anaesthetised. Following 30 min after the administration of desipramine, the anaesthetised rats were then 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) using the stereotaxic coordinates (AP – 4.7, ML + 1.6, DV – 8.4) [21]. 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) (6N), pre-surgery bromelain treatment (40 mg/kg i/p; Sigma-Aldrich, USA) for 7 days 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 post-surgery bromelain daily treatment starting from 24hr after surgery for 7days (24Br) (Table 1). Behavioural assessment took place 24hr after the last drug/vehicle injection.

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

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

Behavioural Assessment

On PND 58 before surgery, all the rats were tested for anxiety-like behavior and depression using open field and forced swim tests. The same tests were repeated 24hr after the last post- surgical treatment with bromelain or saline.

Open field test

The open field box is a square open arena (68× 68× 45) cm^3 designed to evaluate neurobehavioural activities in rats [22]. The apparatus was cleaned with 70% alcohol before introducing each rat into the box; this was to remove any olfactory cue which could have been left by the previous rat. The activity of the rat in the open field box was recorded over a period of 10 min commencing at 8:00 hr on the day. Rearing was scored as the duration of time spent with extension of the fore-limbs and placement of the paws on the walls of the box in an exploratory manner, as previously

described [23,24]. Grooming was time spent by each animal using the forelimbs to clean the face and the body. Anxiety was recorded as the number of entries and the duration of time spent in the centre square [25]. Following this test, the rats were returned to their respective cages in the holding room where they remained for 2 hr before being taken for the forced swim test.

Forced Swim test

Each rat was placed inside a clean, open-top plexiglas cylinder (diameter: 15 cm, height: 40 cm) containing 30 cm of water maintained at 25 ± 1°C. The rats were placed in the cylinder for the first time; and allowed to swim vigorously until the activity began to subside and was interspersed with phases of immobility or floating. Immobility reached a plateau and the rats remained motionless. The first session lasting 15 min was conducted without behavioural recording. This was done to acclimatize the rats to the test situation, thereby providing a stable and a high level of immobile behaviour during the subsequent test session. The rats were further allowed to dry in a heated environment (30°C) before being returned to their different home cages. They were again placed in the cylinder 24 hr later and the total duration of immobility was measured during a 6 min test. An animal was judged immobile whenever it remained floating passively in the water in a slightly hunched but upright position, its nose just above the surface of the water.

Animal Sacrifice and tissue collection

A subset of the animals (6 per group) was randomly selected and sacrificed by decapitation 24 hr after the last behavioural test at PND 70. The blood was collected in heparinized tubes and centrifuged for 15 min at 1160 ×g in a 4^{0}C refrigerated centrifuge (HERMLE LABTECH, Germany) after which plasma was pipetted into eppendorff tubes. The brain was also 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 thalamus and pre- frontal cortex (PFC) were dissected out, weighed and placed in eppendorff tubes. Then, both the plasma and the tissues already placed in eppendorffs were further snap-frozen in liquid nitrogen before being stored in a -80ºC bio-freezer until further use.

Determination of NA levels in the plasma, thalamus and PFC

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 noradrenaline concentration was analysed in the plasma, PFC and thalamic tissues using a Sandwich-ELISA method using ELISA kits (Elabscience Biotech., Texas, USA). The NA analysis 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 noradrenaline only in the samples. The PFC and thalamic 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 noradrenaline metabolism. The tissues were sonicated immediately using a sonicator (CML- 4, Fischer, USA) and further centrifuged at 1160 ×g for 10 min at 4ºC. The supernatant was pipetted into new eppendorff tubes. The standard, control and the samples (plasma, PFC and thalamic tissues) were respectively pipetted into each well of the noradrenaline micro-ELISA plate. Biotinylated detection antibody specific for noradrenaline (50 µL) was added to each well and incubated for 1 hr at room temperature. Following this was the addition of avidin conjugated horseradish peroxidase (HRP) (100 µL) to each well and incubated for 1 hr at room temperature. This was then followed by the addition of tetramethylbenzidine (TMB) liquid 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 NA was quantified using a microtitre plate (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 control were analyzed in triplicates. The assay guidelines provided by the manufacturer (Catalogue No: E-EL- R0047) were followed.

Statistical Analysis

All results are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., USA). Data normality was assessed by Kolmogorov- Smirnov test. The behavioural outcomes were compared using two-way repeated measures ANOVA while all other outcomes were compared with control using one-way ANOVA followed by Bonferroni post hoc analysis. Effects were considered statistically significant at p value < 0.05.

Results

Open field test

The time spent rearing in the open field box was analyzed in the four groups viz: pre-surgery saline treated, saline injected with post-surgery saline treatment (NN), pre-surgery saline treatment, 6-OHDA injected with post-surgery saline treatment (6N), pre-surgery bromelain treatment followed by 6-OHDA injection with post-surgery saline treatment (Br6) and pre- surgical saline treatment, 6-OHDA injected with post-surgery bromelain treatment starting from 24 hr after surgery (24Br) (n = 10 rats per group). There was no difference between the group's pre-surgery. The treatment was found to affect the time spent rearing $F(1, 36) = 72.5$ $p < 0.001$: Figure 1. The time spent rearing was decreased in the saline treated, 6-OHDA injected rat (NN post vs. 6N post) $p < 0.01$. Also, time spent grooming was significantly affected by the treatment $F(1, 36) = 20.14$, $p = 0.004$; Figure 2. The time spent on grooming was significantly increased in the saline treated, 6-OHDA injected rats in comparison to the control group (6N post vs. NN post) $p < 0.001$. Pretreatment with bromelain caused a significant reduction in the grooming time compared to the saline treated, 6-OHDA injected rats (Br6 post vs. 6N post), $p < 0.05$.

Forced Swim Test

The period of immobility in the forced swim test was further analysed in the rats of the four groups. There was a significant

effect of the injected neurotoxin as the post-surgery period of immobility was significantly increased compared to the pre-surgery values $F(1, 36) = 26.07$, $p < 0.0001$, Figure 3. The period of immobility was increased by the injection of 6-OHDA but not statistically significant. Also, the period of immobility was further increased as well decreased by bromelain treatment prior and post 6-OHDA injection respectively, but not significant.

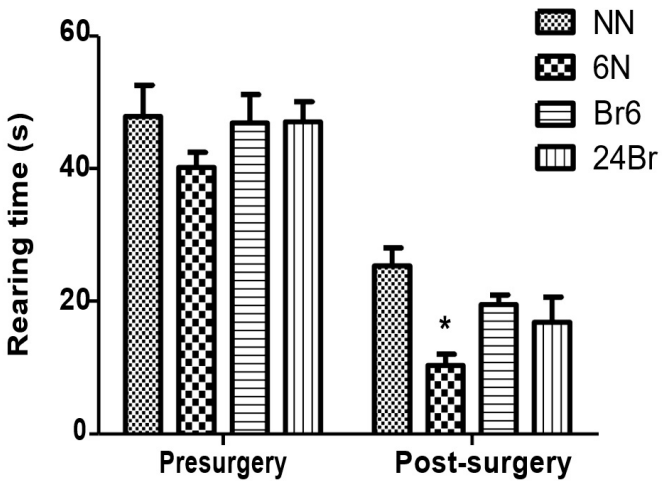

Figure 1: Graph showing the rearing time before (pre) and after (post) 6-OHDA injection in the saline treated rats (NN), 6-OHDA treated rats (6N), presurgery bromelain treated rats (Br6) and post-surgery bromelain treated rats (24Br). Treatment was significant $F(1, 36) = 72.5$, $p < 0.001$. *(NN vs. 6N), $p < 0.01$. n = 10/group.

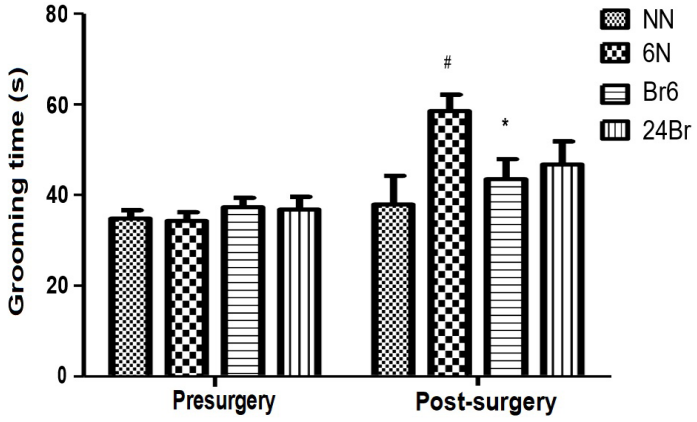

Figure 2: Graph showing the grooming time before (pre) and after (post) 6-OHDA lesion in the saline treated rats (NN), 6-OHDA injected rats (6N), presurgical bromelain treated rats (Br6) and post-surgical bromelain treated rats (24Br). Treatment was significant $F(1, 36 = 20.74$; $p = 0.004$. *(NN vs. 6N), $p < 0.001$; #(6N vs. Br6), $p < 0.05$. n = 10/group.

NA levels in the plasma, thalamus and PFC

The neurotoxin (6-OHDA) caused a significant decrease in the systemic noradrenaline concentration ($F_{(3,20)} = 7.135$; $p = 0.0023$, Figure 4). Post-surgical treatment with bromelain produced a significant increase in the systemic concentration of noradrenaline compared to the saline treated 6-OHDA injected rats (6N vs. 24Br) $p < 0.05$, Figure 4. The thalamic noradrenaline concentration was reduced by 6-OHDA injection, although not statistically significant (Figure 5). Also, bromelain pretreatment reversed the thalamic noradrenaline concentration compared to the 6-OHDA injection, but not significant. Likewise, the bromelain post treatment slightly increased the thalamic noradrenaline levels compared to the 6-OHDA, but not significant (Figure 5). In the PFC, the NA level was decreased by the injection of 6-OHDA compared to saline injection, but not statistically significant. Also, the cortical noradrenaline concentration was increased by bromelain pretreatment compared to 6-OHDA injection but not significant (Figure 6).

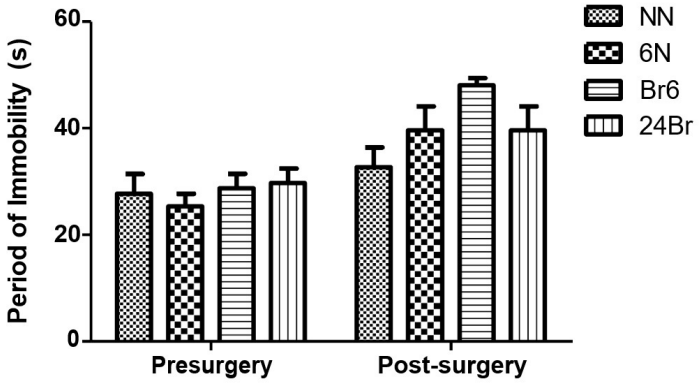

Figure 3: Graph showing the period of immobility. The result shows both pre and post-surgery in the saline treated rats (NN), 6-OHDA injected rats (6N), bromelain pretreated, 6-OHDA injected rats (Br6), 6-OHDA injected, post-surgery bromelain treated rats at 24hr (24Br). (n=10). $F(1,36) = 26.07$, $p < 0.0001$.

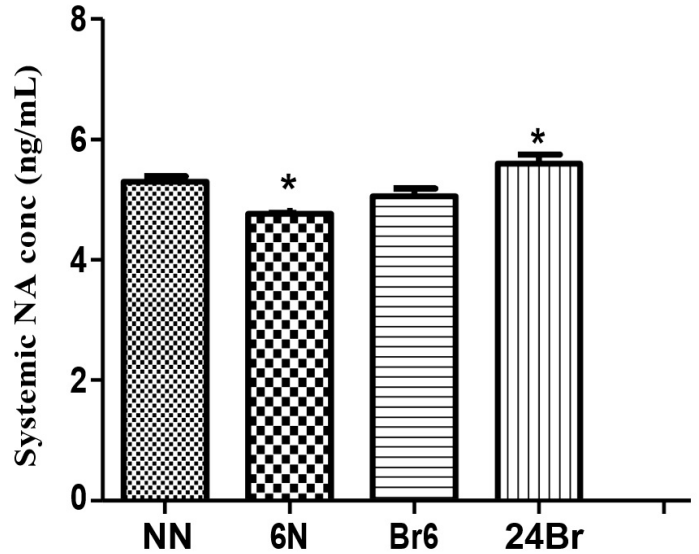

Figure 4: Graph showing the systemic NA concentration in the saline treated rats (NN), 6- OHDA injected rats (6N), bromelain pretreated, 6-OHDA injected rats (Br6) and 6-OHDA injected, post-surgery bromelain treated rats (24Br). $F(3, 20) = 7.135$, $p = 0.0023$. *(NN vs. 6N) $p < 0.05$; *(6N vs. 24Br) $p < 0.05$.

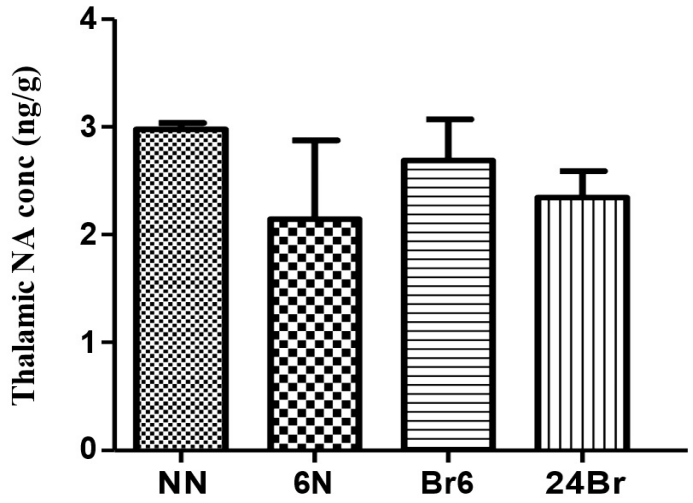

Figure 5: Graph showing the thalamic NA concentration in the saline treated rats (NN), 6- OHDA injected rats (6N), bromelain pretreated, 6-OHDA injected rats (Br6) and 6- OHDA injected, post-surgery bromelain treated (24Br). F(3, 20) = 0.9844, p = 0.422.

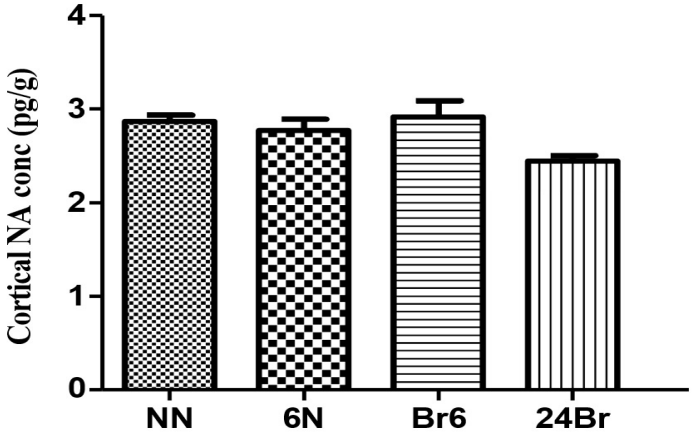

Figure 6: Graph showing the cortical NA concentration in the saline treated rats (NN), 6-OHDA injected rats (6N), bromelain pretreated, 6-OHDA injected rats (Br6) and 6-OHDA injected, post-surgery bromelain treated at 24hrs (24Br). F(3, 20) = 3.738, p = 0.03. n= 6/group.

Discussion

In this study, we investigated the behavioural consequences as well as plasma noradrenaline levels in the early stage of parkinsonism using a 6-OHDA lesioned rat model of PD, and the influence of bromelain treatment on behaviour and the secretion of noradrenaline within the systemic environment and in the brain.

The open field test showed that intra MFB injection of the neurotoxin (6-OHDA) resulted in the development of a state of anxiety in the rat model of PD. We found that the rearing time following the 6-OHDA lesion was significantly shorter while the time spent grooming was increased. This suggests a state of apprehension or fear and emotional distress in the early phase of parkinsonism which could have been incurred by the excitotoxic damage caused by the neurotoxin. These results agreed with previous studies and further confirmed that the state of anxiety is a typical reactionary response to PD [26-28]. The degree of anxiety is conventionally linked with the state of alertness during exploration in a novel environment [29]; therefore, the suppressed rearing is suggested to have resulted from a feeling of apprehension. There is no doubt that the neurotoxin at the relatively high dose used in the present study possesses a strong effect on the noradrenergic innervation of areas of the brain that are concerned with the interplay of sensational feelings [30]. In our study, the period of immobility of the 6-OHDA injected rats in the forced swim test was not different from the control animals. This suggests that depressive-like behaviours may not emanate at the onset of dopaminergic neurodegeneration; however, there is tendency for its occurrence at any time during the disease [31].

In this study, another major observation is a marked reduction of NA in the systemic environment following intra-MFB 6-OHDA injection. This finding is consistent with other studies describing biochemical changes in PD. Also, it is like the findings by Wong et al. [32] that show extensive redundancy in the sympathetic innervation and a decline in the circulating endogenous NA as a result of a high rate of oxidative deamination of NA in the cardiac sympathetic nerve in the early stage of PD before the development of classic symptoms of PD [32]. In this case, there is reason to argue that the reduction of NA within the plasma in PD can be taken as being as a result widespread loss of noradrenergic nerve supply to some visceral organs especially the brain and heart.

Bromelain as reported by Mauer et al. has a wide range effect which is a result of its diverse mechanisms. In our result, post-surgery bromelain treatment was found to increase the concentration of NA within the systemic system. There was a previous suggestion that anxiety in parkinsonism is because of lower NA transporter binding in the LC but with elevated plasma concentration of NA. The elevated systemic NA may further be explained as a result of auto-feedback mechanism by adrenal medulla to maintain micro-homeostasis. Conversely, the kininogen (vasodilator) was known to reduce the reactivity to noradrenergic stimuli. In line with this, the evidence provided by Majima et al. that bromelain depletes plasma levels of kininogen (vasodilator) which helps to understand that bromelain indirectly allows the release of NA in the plasma. Conversely, bromelain pre-treatment alleviated the anxiety by reducing the 6-OHDA-induced increase in time spent engaged in grooming behaviour. It is a known fact that most of the anxiolytic drugs act by modulating gamma aminobutyric acid (GABA) receptors and potentiating GABAergic inhibition by opening calcium channels and causing hyperpolarizastion in neurons that are directly activated by its receptors [33]. In addition, Chaudhary et al. provided evidence that bromelain enhances the release of GABA within the whole brain of neurotoxin intoxicated animals. Consistent with this report, we may then suggest that the reduced grooming activities following bromelain pretreatment of 6-OHDA lesioned rats may be the behavioural output resulting from the balance between the systemic level of GABA and NA. Perhaps, the systemic level

of GABA may have out-weighed the effect of NA which may have resulted in suppression of grooming activities as observed in 6-OHDA lesioned rats pretreated with bromelain.

Conclusion

In conclusion, these findings suggest that anxiety is implicated in the neurodegeneration that occurs at the early stage of the 6-OHDA intra-MFB rat model of parkinsonism. The level of NA is quite affected by the neurotoxin. Also, the release of NA in the systemic environment is caused by bromelain following the 6-OHDA injection. The elevation of NA may partly be responsible for the anxiety-like state at the early stage of parkinsonism. Unlike, pre-surgery treatment with bromelain is perhaps advisable as a treatment for anxiety in the rat model of parkinsonism.

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