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HESPERIDIN A BIOFLAVONOID MODULATES THE EXPRESSION LEVELS OF SNCA AND PARKIN IN 6-HYDROXYDOPAMINE INDUCED NEUROTOXICITY IN RATS

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ABSTRACT

Parkinson disease (PD) is one of the neurodegenerative disease and oxidative stress plays a vital role in its causation. The present study was carried out to evaluate the role of hesperidin in the expression of SNCA, LRRK2, Parkin and PINK1 during 6 hydroxydopamine induced Parkinson rat model. Animals were divided into 5 groups: GroupI served as normal. GroupII was induced with 6-hydroxydopamine (8µg/2µl in 0.1% ascorbic acid-saline). Group III: 6 hydroxydopamine + 50mg/kg b.w hesperidin. GroupIV: 6-hydroxydopamine + 50mg/kg b.w hesperidin+100mg/kg b.w of L-Dopa. GroupV: 6-hydroxydopamine+100mg/kg b.w L-Dopa. The mRNA and protein expression of SNCA, LRRK2, Parkin and PINK1 was evaluated. Hesperidin 50mg/kg b.w and L-Dopa 100mg/kg b.w treated rats showed better results. It may be attributed that treatment with hesperidin and L-Dopa in combination suppress the expression level of SNCA and LRRK2, while it enhanced the expression level of parkin and PINK1. By western blot analysis, group IV treated animals showed suppressed protein levels of SNCA and LRRK2 genes while elevation in the protein level of Parkin and PINKI was noticed. The findings of these studies show that hesperidin can ameliorate 6 hydroxydopamine induced degeneration of dopaminergic neurons during Parkinson disease.

Key words: Parkinson disease, 6-OHDA, Hesperidin, L-Dopa, SNCA, LRRK2, Parkin, PINK1.

INTRODUCTION

Neurodegenerative disorders are a group of devastating disorders of the central nervous system, in which progressive loss of structure and function of neurons, including neuronal death is observed. With the progress of research, many similarities are appearing that ultimately relate these diseases to one another on a subcellular level. Recent studies demonstrate that neuroinflammation and oxidative stress are two hallmarks of various neurodegenerative disorders[1-2]. Parkinson disease (PD) is a neurodegenerative movement disorder characterized by resting tremor, bradykinesia, stiffness of movement and postural instability. These movement problems are largely a consequence of substantial loss in the substantia nigra pars compacta and concomitant loss of dopamine (DA) neurotransmitter, and the deposition of protein within the brain as intracellular inclusions called Lewy bodies [3].

Hesperidin, a bioflavonoid is an abundant and inexpensive by-product of citrus family. A deficiency of this substance in the diet has been linked with abnormal capillary leakiness as well as pain in the extremities causing aches, weakness and night leg cramps. *In-vitro* investigation has demonstrated that the antioxidant properties of flavonoids are linked with their capability for scavenging free radicals, chelating metals and inhibiting the activity of oxidases. They play a special role in protecting brain. *In-vitro* antioxidant property of hesperidin has been proven that hesperidin is capable of scavenging free radicals more efficiently as the concentration increases and it may be suggested that hesperidin has great importance as therapeutic agent in preventing (or) slowing the progress of aging and age associated oxidative stress related degenerative disease [4].

Familial PD has two forms; autosomal dominant heredity (ADPD) and autosomal recessive heredity (ARPD). ADPD has been observed to be caused by mutations in SNCA and LRRK2. ARPD is caused by homozygous (or) compound heterozygous mutations in parkin and PINK1.

Alpha synuclein is a protein of 140 amino acids expressed abundantely in the brain. Alpha synuclein is also the main component of pathogenic Lewy bodies and Lewy neuritis. Mutations of the alpha synuclein gene have been linked to PD, alpha synuclein antibody detects the alpha isoform of synuclein [5]. SNCA may be involved in the regulation of dopamine release and transport. It is expressed principally in brain but is also expressed in low concentrations in all tissues except in liver. It is concentrated in presynaptic nerve terminals. Genetic alterations of SNCA resulting in aberrant polymerization with fibrils, are associated with several neurodegenerative diseases (synucleinopathies). SNCA fibrillar aggregates represent the major non A-beta component of Alzheimer disease amyloid plaque and a major component of Lewy body inclusions. They are also found within Lewy body (LB) like intraneuronal inclusions. Familial forms of the disease usually begin at earlier ages and are associated with atypical clinical features. Defects in SNCA are the cause of dementia Lewy bodies (DLB). Hallmark lesions of neurodegenerative synucleinopathies contain alpha-synuclein that is modified by nitration of tyrosine residues and possibly by dityrosine cross-linking to generate stable oligomers that are ubiquitinated. The predominant conjugate is the diubiquitinated form.

Leucine-rich repeat kinase 2 (LRRK2) contains amino-terminal leucine-rich repeats (LRR), as Ras-like small GTP binding protein-like (ROC) domain, a MLK protein kinase domain and carboxy-terminal WD40 repeat. At least 20 LRRK2 mutations have been linked to PD, the G2019S mutation being the most prevalent [6]. The G2019S mutation causes increased LRRK2 kinase activity, which induces a progressive reduction in neurite length, leading to progressive neurite loss and decreased neuronal survival [7]. The MLK inhibitor CEP-1347 is explored in PD clinical trials indicating that LRRK2 may have value as therapeutic target for treatment of PD [8]. LRRK2 antibody detects endogenous levels of total LRRK2 protein.

Parkin is a protein of 465 amino acids with an amino-terminal ubiquitin domain and a carboxy-terminal RING-box [9]. In the case of autosomal recessive juvenile parkinsonism (AR-JP) deletions have been found in the gene on chromosome 6 encoding the protein parkin [10]. Parkin antibody detects endogenous levels of total parkin protein.

PINK1, PTEN induced putative kinase1, is a mitochondrial serine/threonine kinase involved in the normal function and integrity of mitochondria, as well as a reduction of cytochrome C release from mitochondria . PINK1 phosphorylates parkin and promotes its translocation to mitochondria. Mutations of PINK1 are associated with loss of protective function, mitochondrial dysfunction, aggregation of Alpha-synuclein, as well as proteasome dysfunction [11-13].

So in the present study the impact of hesperidin on the level of SNCA, LRRK2, parkin and PINK1 was investigated.

MATERIALS AND METHODS

6-OHDA, ascorbic acid, Hesperidin and Apomorphine were purchased from sigma Aldrich. All other chemicals used were of analytical grade.

Experimental Animals: Adult male wistar rats (145-150g) were used for the study. Animals were purchased from Tamilnadu Veterinary and Animal Science University, Madhavaram, Chennai and were housed under controlled temperature provided with food and water ad libitum. The protocol was approved by the institutional animal ethics committee of the Saveetha University, Chennai. (IAEC NO: SU/BRULAC/RD/008/2013).

Experimental protocol: The animals were divided into 5 groups, each containing 6 animals.

Group I: Animals were treated normally and served as control.

Group II: Rats were infused with 6-hydroxydopamine (8µg/2µl in 0.1% ascorbic acid-saline) in right striatum once and were maintained for the development of Parkinson's disease for 45 days.

Group III: Rats were infused with 6-hydroxydopamine (8µg/2µl in 0.1% ascorbic acid-saline) in right striatum once and maintained for the development of Parkinson's disease for 21 days. On 22nd day, Hesperidin (50 mg/kg b.w) dissolved in distilled water were given for next 24 days.

Group IV: Rats were infused with 6-hydroxydopamine (8µg/2µl in 0.1% ascorbic acid-saline) in right striatum once and were maintained for the development of Parkinson's disease for 21 days. On 22nd day, Hesperidin (50mg/kg b.w) and L-Dopa (100mg/kg b.w) dissolved in distilled water were given for next 24 days.

Group V: Rats were infused with 6-hydroxydopamine (8µg/2µl in 0.1% ascorbic acid-saline) in right striatum for once and were maintained for the development of Parkinson's disease for 21

days. On 22nd day (L-DOPA 100mg/kg b.w) was given for next 24days.

6-OHDA induced lesions: All animals in the experimental groups were anaesthetized with Ketamine (100mg/kg b.w) (i.p) and Xylazine (10 mg/kg b.w) (SC). Each animal was mounted on a stereotaxic apparatus, skin overlying the skull was cut to expose it, and the co-ordinates for the striatum were measured accurately (anterio-posterior 0.5 mm, lateral 2.5 mm, dorso-ventral from dura) with the tooth bar set at 0 mm [14]. Thereafter, all animals in experimental groups were lesioned by injecting (8µg/2µl in 0.1% ascorbic acid-saline) in the right striatum, while the Group I served as a control. The injections were made manually, with the help of a Hamilton syringe, through the burr holes made on the skull surface in experimental groups. The injection rate was 1µl/min and the needle was kept in place for an additional 1 min before being slowly retracted.

Post-operative care: Recovery from anaesthesia took approximately 4-5hrs. The animals were kept in a well-ventilated room at 25±3^{0}C in individual cages until they gained full consciousness; they were then housed together in groups of 6 animals per cage. Food, water mixed with 1ml of Ibuprofen was kept inside the cages for two days, allowing animals easy access, without physical tr
auma due to overhead injury. Animals were then treated with normal food, water and the bedding of the cages were changed twice per week, as usual.

Collections of Brain Samples: The animals were sacrificed and striatal portion of the brain was separated and homogenised separately in ice-cold phosphate buffer (P^H 7.5) at a concentration of 15% (W/V). This would release soluble protein leaving only membrane and non-vascular matter in a sediment form. Homogenised samples were then centrifuged at 5000rpm for 10min. Aliquot was taken for expression studies. Tissue homogenate was stored at -20°C until use for further analyses.

RT-PCR (Reverse transcription): The experimental groups were analysed for the expression of SNCA, LRRK2, Parkin and PINK1 by RT-PCR. β-actin gene was used as control. Total RNA was extracted from brain striatum tissue of all groups using Trizol reagent (SIGMA, USA). After homogenization of brain striatum tissue samples (100mg), proteins were extracted with chloroform and total RNA was precipitated with isopropanol. The precipitated RNA was washed with 75% ethanol and resuspended in 50µl of DEPC-treated water. Finally the DNA free RNA was prepared prior to RT-PCR using DNase I, RNase-free kit. Reverse transcription was carried out to obtain cDNA using two step RT-PCR kit (QIAGEN Germany).
The primers used for SNCA, LRRK2, Parkin and PINK1 were based on the rat sequences and it is shown in **Table 1.** Samples were processed following the manufacturer's directions. The reaction mixture was loaded in a PCR thermal cycler for 35 cyclic reactions. The PCR products were separated using agarose gel electrophoresis visualized and documented using Quantity One Software (BIO RAD, USA) [15].

PROTEIN EXPRESSION: The brain striatum samples were homogenised in 10 volumes of 20mM Tris HCL (P^H 7.4) containing 5mM EDTA and 10mM β-mercaptoethanol using a homogenizer. The homogenates were centrifuged at 10,000 rpm for 30 min, 4°C and the recovered supernatants were used. The proteins were separated by 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis. SDS-PAGE was done as per the standard procedures [16].
After electrophoresis, the proteins were transferred to nitrocellulose membrane, blocked overnight with 5% non-fat dried milk in PBS-T at 2-8°C, reacted with rabbit monoclonal primary antibodies (anti SNCA, anti LRRK2, anti Parkin and anti PINK1 IgG antibody, 1:1000 dilution) and incubated overnight at 4°C and washed. Anti-rabbit secondary antibody was used for SNCA, LRRK2 and parkin, whereas anti-mouse secondary antibody was used for PINK1. The membrane was then washed three times with TBST, incubated further with alkaline phosphatase conjugated with anti-rabbit secondary antibody at room temperature for 2 hours, and then washed three times with TBST. After reaction with horseradish peroxidase conjugated with rabbit secondary antibody, the immune complexes were visualized by using the chemiluminescence ECL PLUS detection reagents following the manufacturer's procedure (Amersham Bioscience). Antibody to anti SNCA, anti LRRK2, anti Parkin and anti PINK1 were purchased from Santa cruz biotechnology.

RESULTS

RT-PCR: Figure1 shows mRNA expression of genes like SNCA, LRRK2, Parkin and PINK1. An upregulated expression of SNCA and LRRK2 was observed in brain striatal tissue of rats with 6-OHDA induced Parkinson disease (Group II) than in control rats (Group I). Interestingly Hesperidin treated rats (Group III) showed mild reduction of m-RNA expression patterns in SNCA and LRRK2 whereas in Hesperidin and L-Dopa treated rats (Group IV)

showed significant downregulation in SNCA and LRRK2. Rats treated with standard L-Dopa (Group V) also showed mild reduction in m-RNA expression patterns in SNCA and LRRK2. A downregulated expression of Parkin and PINK1 was observed in striatal tissue of rats with 6-OHDA induced Parkinson disease (Group II) than in control rats (Group I). Interestingly Hesperidin treated rats (Group III) showed mild upregulation of m-RNA expression patterns in parkin and PINK1 whereas in Hesperidin and L-Dopa treated rats (Group IV) showed significant upregulation in Parkin and PINK1. Rats treated with standard L-Dopa (Group V) also showed mild upregulation in m-RNA expression patterns in Parkin and PINK1.

Western blot: Figure2 shows protein expression in brain striatal tissue of experimental animals and was confirmed by immunoblotting. A higher level of expression in SNCA and LRRK2 was observed in rats with Parkinson induced animal (Group II) than in control animals (Group I). Hesperidin treated rats (Group III) showed mild downregulation in SNCA and LRRK2. Hesperidin and L-Dopa treated rats (Group IV) showed significant downregulation in SNCA and LRRK2, whereas standard L-Dopa rats showed mild downregulation in SNCA and LRRK2 respectively. A low level of expression in parkin and PINK1 was observed in striatal tissue of rats with Parkinson induced animal (Group II) than in control animals (Group I). Hesperidin treated rats (Group III) showed mild upregulation in Parkin and PINK1. Hesperidin and L-Dopa (Group IV) treated rats showed significant upregulation in parkin and PINK1, whereas standard L-Dopa rats showed mild upregulation in parkin and PINK1 respectively.

DISCUSSION

Lifestyle factors and dietary habits have also been shown to influence the risk of developing PD. Epidemiological data showing that consumption of coffee and non-steroidal anti-inflammatory drugs reduces the risk of PD are intriguing [17-18]. A high caloric intake have been investigated as potential risk factor of PD. This was confirmed by a study showing that eating foods high in animal fat is associated with increased risk of PD [19].

L-Dopa is dopamine precursor that has long been considered to be the gold standard drug for treatment of PD. L-dopa can improve motor function daily activities and quality of life in PD patients, whereas other non-motor symptoms such as postural instability, freezing, mood and sleep disorders, autonomic dysfunction and dementia do not respond to this drug. Sadly, chronic treatment with L-dopa is also associated with some motor complications, motor fluctuation and dyskinesia and hence there is an urgent need to find new drugs to treat this disease. Dopamine agonists can directly stimulate the postsynaptic receptors in the striatum. This category of drugs include two basic groups ergot derivative (bromocriptine) and non-ergot dopamine agonist (pramipexole). Side effects of dopamine agonists include hallucination, sleepiness during daytime, and compulsive disorders [20].

Recently, mutations in LRRK2 have been shown to cause a disease that resembles parkinson's disease in having both neuronal loss and protein inclusion pathology [21]. LRRK2/dardarin mutations are important in understanding the relationships between sporadic parkinson's disease and genetic forms for several reasons. Firstly, most mutations cause clinically typical parkinson's disease and in some cases in which pathology has been reported, alpha-synuclein positive Lewy bodies are seen [22].

Parkin is the target in individual affected by autosomal recessive juvenile parkinsonism (AR-JP) [23]. Functional analysis has shown parkin activity to be that of an E3 ubiquitin ligase, the specific involvement of which in protein degradation was observed [24].

PINK1 encodes a mitochondrial kinase, linking genetic data with previous suggestions that mitochondrial dysfunction may be important in the pathogenesis of parkinson's disease [25]. Hesperidin was found to possess a strong antioxidant potential in invitro condition [26]. It is also shown that hesperidin shows better results in behavioural activities in 6-OHDA induced parkinson model [27].

We evaluated the anti-parkinson effect of the hesperidin by analysing the gene expression of SNCA, LRRK2, parkin and PINK1. The RT-PCR analysis showed the upregulation of SNCA and LRRK2 and downregulation of parkin and PINK1 in striatal portion of Parkinson disease, while hesperidin (50mg/kg b.w) in combination with L-Dopa (100mg/kg b.w) treated animals showed downregulation of SNCA and LRRK2 and upregulation of parkin and PINK1 of mRNA expression patterns.

Our study clearly indicate that hesperidin and L-dopa in combination significantly downregulated the gene expression of SNCA and LRRK2 while it upregulated parkin and PINK1. Thus, by western blot analysis also, it was noted that overall treatment of experimental rats with (Hesperidin 50mg/kg b.w)+L-Dopa (100mg/kg b.w) suppress the expression of SNCA, LRRK2 and triggers the expression of parkin

and PINK1 in 6-OHDA induced parkinson animal model and therefore synergistically act as neuroprotective drug.

CONCLUSION

The present study highlights that hesperidin in combination with L-Dopa would alter the expression levels of genes which are dominant and recessive in status of neurotoxicated animals. The findings of this study show that hesperidin (50mg/kg b.w) in combination with L-Dopa (100mg/kg b.w) can suppress the expression of SNCA and LRRK2, whereas enhance the expression of parkin and PINK1 respectively and thereby ameliorate 6-OHDA induced degeneration of dopaminergic neurons in Parkinson disease.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare in relation to this article.

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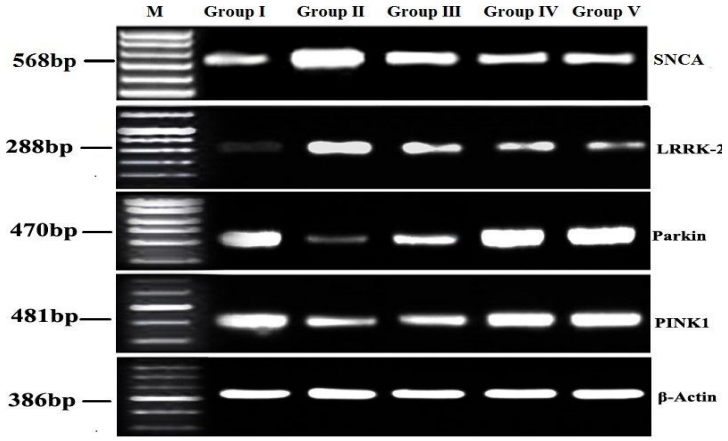

Figure 1: mRNA expression of SNCA, LRRK2, Parkin and PINK1

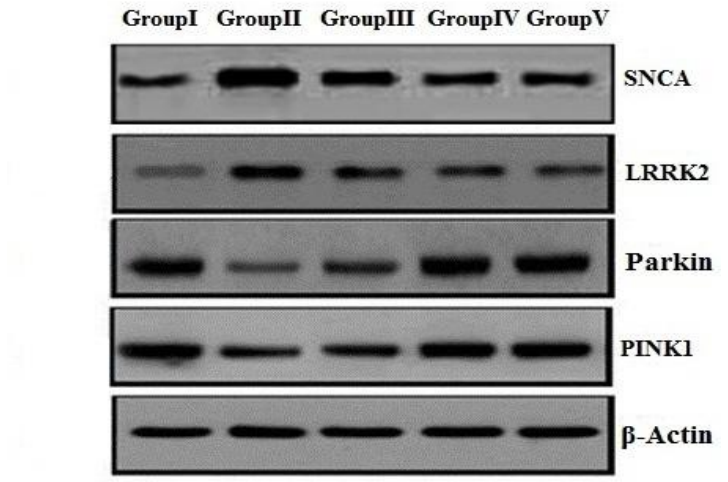

Figure 2: Protein expression of SNCA, LRRK2, Parkin and PINK1

Table 1: Sequences of Primers for RT PCR assay.

Gene	Primer Forward	Primer Reverse
SNCA	5′-CATGGATGTATTCATGAAAGG-3′	5′-GAGTGTAGGGTTAATGTTCC-3′
LRRK2	5′-TGGGTTGGTCACTTCTGTGC-3′	5′CATTGGCTGGAAATGAGTGC-3′
Parkin	5′CCAAACCGGATGAGTGGTGAGTGC3′	5′ACACGGCAGGGAGTAGCCAAGTTG3′
PINK1	5′CTTATAGGAAAGGGCCCGGATGTCG3′	5′GATGATGTTAGGGTGTGGGGCAAGC3′
β-actin	5′GTAGACAAAATGGTGAAGGTCGGTG3′	5′CTCGCTCCTGGAAGATGGTGATGGG3′

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