Journal of Chemical Neuroanatomy 80 (2017) 1–10

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Mucuna pruriens reduces inducible nitric oxide synthase expression in Parkinsonian mice model

Satyndra Kumar Yadav[1], Sachchida Nand Rai[1], Surya Pratap Singh[*]

Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi 221005, India

ARTICLE INFO

Article history:
Received 12 July 2016
Received in revised form 29 October 2016
Accepted 30 November 2016
Available online 2 December 2016

Keywords:
Nitric oxide
Intraperitoneally
Immunoreactivity
Lipid peroxidation
Dopaminergic neurons

ABSTRACT

Parkinson's disease is one of the most common neurodegenerative disease found in aged peoples. Plentiful studies are being conducted to find a suitable and effective cure for this disease giving special impetus on use of herbal plants. The study aimed at investigating the effect of ethanolic extract of *Mucuna pruriens* (Mp) on level of nitric oxide (NO) in paraquat (PQ) induced Parkinson's disease (PD) mouse model and its subsequent contribution to lipid peroxidation. Twenty four Swiss albino mice were divided into three groups; Control, PQ and PQ + Mp. PQ doses were given intraperitoneally, twice in a week and oral dose of ethanolic extract of Mp seed was given for 9 weeks. Nitrite content and lipid peroxidation was measured in all treated groups along with respective controls. RNA was isolated from the nigrostriatal tissue of control and the treated mice and was reverse transcribed into cDNA. PCR was performed to amplify iNOS mRNA and western blot analysis was performed to check its protein level. We had also perfused the mice in all treated group and performed Tyrosine hydroxylase (TH) and iNOS immunoreactivity in substantia nigra region of mice brain.

PQ-treatment increased nitrite content, expression of iNOS and lipid peroxidation compared to respective controls. Mp treatment resulted in a significant attenuation of iNOS expression, nitrite content and lipid peroxidation demonstrating that it reduces nitric oxide in PQ-induced Parkinson's disease. Interestingly; we also observed that mRNA, protein expression and immunoreactivity of iNOS was significantly decreased after Mp treatment and TH immunoreactivity was significantly improved after the treatment of Mp. Our results demonstrated that Mp protects the dopaminergic neurons from the NO injury in substantia nigra.

1. Introduction

Parkinson's disease (PD) is the second most common chronic neurodegenerative movement disorder, characterized by the progressive degeneration of the dopaminergic neurons in the substantia nigra pars compacta region of the midbrain and subsequently depletion of dopamine in the striatum (Miller, 2009; Yadav et al., 2012). The degeneration of dopaminergic neurons leads to various motor complications like tremor, rigidity, bradykinesia and abnormal postural reflexes (Fahn, 2003). Deterioration of the neuronal connection occurs between two brain regions vital for normal motor function i.e., the substantia nigra (SN) and the striatum and it is a major pathological hallmarks of PD (Wooten, 1997), this is manifested via the depleted dopamine influx to the striatum originating from the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta. An another important pathological hallmark of PD is the presence of cytosolic filamentous inclusions known as Lewy bodies (LBs) and Lewy neurites (Lewy, 1912; Forno, 1996) in some surviving nigral dopaminergic neurons. PD is a heterogeneous disease likely to be caused by numerous etiological factors, interconnected with PD (Orth and Tabrizi, 2003), including ageing, environmental toxins (pesticides, heavy metals, etc.) and genetic factors. People involved in agricultural practices, such as farming, living in rural environment and drinking well water are at high risk of PD (Uversky, 2004). Paraquat, a bipyridyl and cationic non-selective herbicide, causes PD after sustained exposure (Yadav et al., 2012). Paraquat appeared as a risk factor, since it is structurally similar to 1-methyl-4-phenylpyridine (MPP^+), an active metabolite of MPTP (Widdowson et al., 1996). Paraquat

* Corresponding author.
E-mail addresses: satyndra_yadav@yahoo.co.in (S.K. Yadav), raibiochem@gmail.com (S.N. Rai), suryasinghbhu16@gmail.com, singh35@bhu.ac.in (S.P. Singh).
[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.jchemneu.2016.11.009
0891-0618/

readily crosses the blood brain barrier (BBB) through neutral amino acid transporters (McCormack and DiMonte, 2003) and dopamine transporter (DAT) and it causes neurotoxicity by redox cycling. It subsequently induces free radical generation by inhibiting the mitochondrial complex I and leading to neurodegeneration of dopaminergic neurons (Yadav et al., 2012). In PD pathogenesis oxidative stress plays major role, the main cause of oxidative stress is the impairment of mitochondrial electron transport chain (ETC) which leads to the production of superoxide anions. Super oxide anion gets converted into hydroxyl radical, hydrogen peroxide (H_2O_2) and peroxy radicals. Superoxide gets converted into peroxynitrite and nitro-tyrosyl radicals in the presence of nitric oxide (NO) (Betarbet et al., 2005; Mariani et al., 2005). PQ is able to elicit some decrease in striatal dopamine nerve terminal density and to induce neurobehavioral syndrome characterized by reduced ambulatory activity (Brooks et al., 1999).

In the Indian Ayurvedic medical system, the dried endocarp powder of the *Mucuna pruriens* (Mp) bean is used to treat Parkinson's disease since ages (Manyam, 1990; Mariani et al., 2005). Ayurvedic practitioners and review of the literature points out that PD patients treated with Mp do not develop bradykinesia. Mp has been shown to contain natural levodopa (LD) (Vaidya et al., 1978; Mahajani et al., 1996; Modi et al., 2008), which is an active constituent in ethanolic extract of Mp seed. Different preparations of Mp (from the seeds) are used for the management of numerous free radical-mediated diseases such as ageing, rheumatoid arthritis, diabetes, atherosclerosis, male infertility and nervous disorders. Mp contains many micro-nutrients including amino acids, Zn, Se, carbohydrates (Prakash et al., 2001; Ghosal et al., 1971; Mehta and Majumdar, 1994; Panikkar et al., 1987) and various plant alkaloids (Rakshit and Majumdar, 1956; Ghosal et al., 1971). After analysis of Mp seed extract by the help of HPTLC, it was found that percentage of L-DOPA is 5.6 ± 0.43 (Modi et al., 2008).

Earlier studies assured that Mp is a significant source of natural anti-oxidant (Kumar et al., 2010; Tripathi and Upadhyay, 2001), thus, it is possible that Mp may act through a mechanism of free radical removal in the management of the Parkinson's disease.

Nitric oxide (NO) is a potent vasodilator and a significant secondary mediator of several biological functions which quickly combines with superoxide to form peroxynitrite, a potent and versatile oxidant, which can attack a wide range of biological targets (Beckman, 1996; Tripathi and Upadhyay, 2001). Even though NO plays a significant role in neurotransmitter release, neurotransmitter re-uptake and regulation of gene expression, its excessive production leads to neurotoxicity (Dawson and Dawson, 1998). NO mediates neurotoxicity by excitotoxicity, DNA damage or post-translational modification of proteins (Zhang et al., 2006). Increase in oxidative stress and nitric oxide synthase (NOS)-mediated nitric oxide production are well known during microglial activation (Liberatore et al., 1999). As compared with saline treated controls we have reported that PQ treated mice showed increased lipid peroxidation activity in the striatum. For this, we studied three parameters; first changes in motor functions by rotarod test, second oxidative stress occurring in nigrostriatal region by lipid and nitrite biochemical estimation and third expression of mRNA and protein of iNOS in the substantia nigra of mice brain by RT-PCR, immunohistochemistry and western blotting performed on Parkinsonian mice.

2. Material and Methods

2.1. Animals and preparation of plant extract

In our study, we used male Swiss albino mice weighing 25 ± 5 g. All experimental procedures were directed according to the National Guidelines on the Proper Care and Use of Animals in Laboratory Research and were approved by the Institutional Ethical Committee. Mice were kept in a controlled temperature of $22 \pm 3\,^{\circ}C$ on a 12 h: 12 h light-dark cycle. Food pellets and water were made available to these animals ad libitium (Chouhan et al., 2013). Mp seed powder was purchased from the Ayurveda pharmacy, Institute of Medical Science, Banaras Hindu University, Varanasi, India. We prepared ethanolic extract of Mp seed powder by soaking 500 g of the powdered material in 1000 mL of ethanol overnight and afterwards extracting in a soxhlet apparatus by using the continuous hot extraction method at $60\,^{\circ}C$ for 35 hrs. After this we concentrated the extract under reduced pressure and stored at $4\,^{\circ}C$. The amount of plant extract was expressed in terms of dry weight (Eze et al., 2011).

2.2. Chemicals

Acetic acid, disodium hydrogen phosphate, potassium chloride and sodium dihydrogen phosphate were procured from Sisco Research Laboratories (SRL; Mumbai, India). Streptavidin-peroxidase, normal goat serum and the DAB system were procured from Bangalore Genei Pvt. India Ltd, Bangalore, India. Folin Ciocalteau reagent, hydrogen peroxide (H_2O_2) and potassium-dichromate were purchased from Merck (Darmstadt, Germany). Primary antibodies for inducible iNOS were procured from Santa Cruz, Biotechnology (Santa Cruz, CA, USA) and the primers were purchased from IDT.

2.3. Treatment of the mice

24 Mice were divided into three groups with eight animals in each group in one set of experiment and we performed two sets of experiments everytime. The first group was treated with saline (i.p.) and used as controls. In second group, it was injected (i.p.) by PQ (10 mg/kg body weight) twice in a week for 9 weeks to induce PD. The third group was injected (i.p.) with PQ (10 mg/kg body weight) twice in a week and additionally treated with Mp seed extract (100 mg/kg body weight), which was given orally for 7 days prior to PQ treatment and 9 weeks after (Rajasankar et al., 2007).

2.4. Behavioural Studies

Behavioral studies were performed using rotarod. In rotarod test, animals were trained for 3 consecutive days before the day of final treatment at a fixed speed (5 rpm) for 5 min. The time after which the animals fell down was determined and the maximal observation time was 5 min. The experimental readings were taken 24 h after the last treatment in all animals groups. At least 4 experimental readings were recorded and the results were averaged to obtain a single value for each animal (Manna et al., 2006).

2.5. Decapitation and dissection of brains

After behavioural test, mice were sacrificed by cervical dislocation to make sure minimal pain (Prakash et al. 2001, 2013; Surendran, 2007). The brain were removed, micro dissected on a glass plate over ice and the nigrostriatal area was collected and stored at $-80\,^{\circ}C$ until further used for biochemical assays, PCR and western blot. Rest of the mice was perfused for performing the immunohistochemistry.

Half of the animals (4) in each group were perfused for immunohistochemistry and other half were used for biochemical estimations. From the second set of experiment, half of the animals (4) were used for PCR experiments and the other half were used for western blotting and each experiment was repeated 8 times.