Published online in http://ijam.co.in

ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 15 (2), 2024; 420-425

The effect of an Indian traditional Ayurvedic formulation (Rasa-sindoor) on Parkinson disease mouse model

Research Article

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Abstract

Ayurveda is a traditional medication system in India. Experimental validation of ayurvedic formulations to combat with progressive, non-curable diseases like cancer, neurodegenerative disorders and knowing their mode of action are of current interest. The present study was aimed to explore the effect of dietary supplement of Rasa-sindoor (RS), an organo-metallic derivative of mercury, on drug (MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced mouse model of Parkinson disease (PD). For this, mice were divided into three groups: (1) vehicle control (Normal saline treated), (2) Treated 1 (MPTP treated: 15mg/kg body weight, twice a day in 2 hours interval for 2 days, intraperitoneal injection) and (3) Treated 2 (MPTP: same as Treated 1 and Rasa Sindoor: 1gm/kg RS twice a day for 4 days). Total cellular RNA was extracted from sacrificed mice brain tissue and Immunohistochemistry (IHC) was performed for Tyrosine Hydroxylase (TH) in 100 μ section of mid-brain region to check dopaminergic neurodegeneration. IHC revealed reduced TH activity in Treated 1 and Treated 2 as compared to vehicle control while Treated 2 showed greater TH activity than that of Treated 1. This preliminary observation was followed by a microarray based gene expression analysis, which results differential expression of a new set of genes involving neurogenesis, growth cone formation and axon guidance, activation of latent precursor cells etc.; validated by semi-quantitative real time PCR. Our result suggests that RS is neuroprotective and its mode of action may be through prevention of cell apoptosis and activation of latent precursor cells and cell growth.

Keywords: Ayurveda, Rasa-sindoor, MPTP, Neuroprotective, Immunohistochemistry, Micro-array.

Introduction

Parkinson disease (PD, OMIM #168600) is a common motor disorder characterized pathophysiologically by midbrain dopaminergic neurodegeneration accompanied by the appearance of intraneuronal inclusions enriched with α-synuclein, the Lewy bodies (1). Though there are ever increasing list of proteins and pathways those are known to be associated with PD, the disease etiology still remains elusive. The disease can be caused by genetic predisposition, oxidative stress, abnormal protein processing, mitochondrial dysfunction, neuro-inflammation, environmental exposure and so on. The main challenge concerning a better therapeutic approach to the treatment and prevention of PD is the enigma of its underlying causes. Primarily the disease symptom develops due to insufficient dopamine, whatever the cause behind. Dopamine metabolism and mitochondrial dysfunction predominantly cause generation of oxidative stress which plays the central role in disease pathogenesis. So the only therapeutic approach to combat with PD is administration of dopamine precursor L-DOPA which alleviates PD symptoms, though chronic administration of L-DOPA often causes motor and psychiatric side effects (2).

To get insight into the PD pathogenesis different experimental models are being developed to date especially those produced using neurotoxins. One such very commonly used neurotoxin is MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). Being hydrophobic in nature MPTP easily crosses the blood-brain barrier and enters into the glial cell where it is converted into MPDP with the help of enzyme Mono-amine oxidase B (MAO-B) and finally forms MPP^+ which being an analogue of dopamine enters to the dopaminergic neuron through the DAT (Dopamine transporter) receptor. MPP^+ is the main toxic metabolite of MPTP, which in turn produces Super-oxide radical, inactivates tyrosine hydroxylase (TH), cause DNA Damage and ultimately degeneration of dopaminergic neurons (3).

Ayurveda is practiced in India from 5000 to 3000 BC and still continued. Not only in India, also in other neighboring Asian countries, Ayurveda serves as an alternative medicine system (4). The basic concept of Ayurveda includes three bioentities: *vata*, *pitta* and *kapha* representing the psychomotor activities, digestive and metabolic activities and the growth aspects respectively. The neurological disorders are considered in Ayurveda as an imbalance of *vata*, especially the

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disorder mentioned as *kampavata* bears the resemblance of clinical symptoms of 'shaking palsy' described by doctor James Parkinson in 1817. Formulations derived from body parts of a few important medicinal plants (Aswagandha: root of *Withania somnifera*, Atmagupta: seed of *Mucuna prureins*, Bala: root of *Sida cordifolia* etc.) have already been described to be significantly effective for improving disease conditions in PD (5, 6). Ayurveda medicines such as Suvarna Bhasma which has potential to treat neurological diseases has been shown to prevent drug induced PD in zebrafish (7).

Metal based formulations, such as red sulfide of mercury, known as rasasindura/ rasasindoor/ rasasinduram/ sindur or sindoor in ancient Indian literature was used extensively in various diseases. Physico-chemcal characterization of it reveals its function of protease inhibitor along with mild anti-oxidant activity (8). The formulation used in present study is an organo-metallic derivative of mercury, called Rasa-sindoor (RS), prepared from processed and purified mercury and sulphur mixed with aloe-vera juice in 1:1:0.7 ratios (9). It is used generally for improving immune system, rejuvenation, in cardiac diseases, inflammatory conditions etc. RS is also being used for tuberculosis, bleeding disorders etc. for centuries. The dietary supplement of RS, on neurodegeneration in *Drosophila* fly models of Huntington and Alzheimer's diseases have been examined to cause suppression of degeneration of photoreceptor neurons along with rescue of premature death of flies (10). It has also been found to improve the age-associated memory deficits in mice (11). In this context, the objective of the present study is to explore the effect of RS in neurodegeneration model, i.e. MPTP-induced mouse model of PD, using high throughput gene expression array.

Materials and methods

Animals

8-10 week old male PARKS Mice weighing 25-30 gm were used for this study. They were housed in constant temperature and humidity with 12 h dark and light cycle supplied with sufficient food pellet and water. All procedures were in accordance with the National Guidelines of the proper care and use of animals in laboratory research.

Experimental set up

Mice were grouped into three sets for experiment. The first group of mice received normal saline, kept as vehicle control (CONDITION 1). The second group of mice (Treated 1) were treated with MPTP (intraperitoneal injection), i.e., 15 mg/ kg body weight twice a day at two hour intervals for two days (CONDITION 2). The rest group of mice (Treated 2) received MPTP as in Treated 1; along with this they were fed RS (1 gm/kg) with the help of feeding syringe twice a day for four days (CONDITION 3). All the mice were sacrificed two days after the last MPTP injection, i.e. on day 4. The experiment was repeated twice.

Immunohistochemistry for Tyrosine Hydroxylase (TH) immunoreactivity

The whole brain was dissected and fixed in 10% neutral buffered saline (NBF) for 2 days. On day 3 it was kept under running water for 4-5 hours for removing fixative. The mid-brain region (Atlas mouse brain bregma -1 to -5) was taken for sectioning. The SNpc region lies in mouse brain bregma (-2.54) to (-3.48). 100 µ thick tissue sectioning was performed in vibratome under chilled Phosphate Buffered Saline (PBS). Approximately ten sections of 100 µ thickness were obtained from the particular region of interest which was mounted in PBS in 24 well plate. After that sections were incubated in 0.01% H_2O_2 for 45 min in dark. Blocking was performed in normal goat serum (1:100) for 1 hour. Sections were incubated in mouse monoclonal anti-Tyrosine Hydroxylase [ab49640, abcam] primary antibody (1:100) overnight at 4°C. Further procedures were followed as directed by manufacturer's protocol of Avidin-Bioton Complex (ABC- VECTASTATIN Universal kit-PK-6200). After incubating in peroxidase substrate solution for development of desired stain intensity, sections were transferred into PBS and then on glass slides. Next day after completely drying, sections were mounted on Dibutyl Phathalate Xylene (DPX) and images were obtained using bright field microscope at 4X and 10X magnification. TH positive cells were counted as described in earlier report (12).

Extraction of RNA

Mid-brain region was taken (cerebellum and anterior part were removed) and homogenized with glass homogenizer using TRI-reagent (Sigma- T9424). Total cellular RNA was extracted following the instruction manual. RNA integrity was checked by 1.5% agarose gel electrophoresis.

DNaseI treatment

Trace contamination of genomic DNA (if any) was removed by DNase I digestion following standard protocol. The purified RNA was quantified in spectrophotometer.

Gene expression array

Microarray based gene expression analysis was performed using GeneChip® Mouse Gene 2.0 ST Array (Affymatrix). A total of six arrays for three experimental conditions in duplicate were used for this study. The experimental conditions are (1) vehicle control (Normal saline treated), (2) Treated 1 (MPTP treated: 15mg/kg body weight, twice a day in 2 hours interval for 2 days, intraperitoneal injection) and (3) Treated 2 (MPTP: same as Treated 1 and Rasa Sindoor: 1gm/kg RS twice a day for 4 days). Data was analyzed using the software Expression console and Transcriptome Analysis Console (Affymatrix). Differential Expression Analysis at Gene Level and Exon Level were performed. The threshold level for relative fold change was set at ±2 and the condition1, i.e. normal saline treated vehicle control, was taken for

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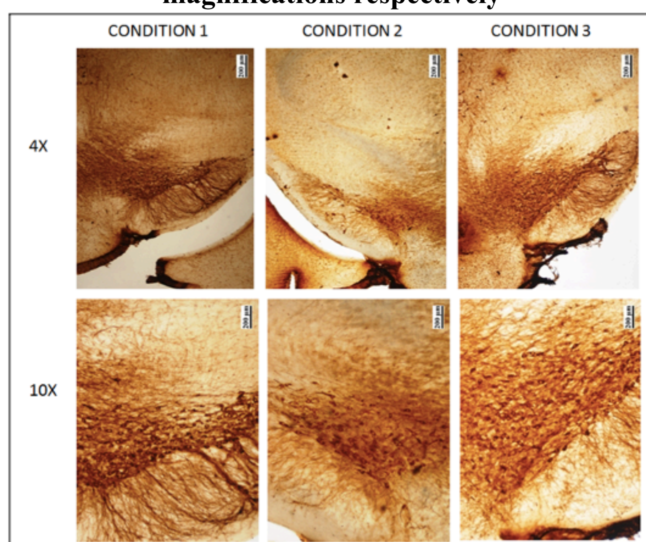
normalization. *p*-values of less than 0.05 were considered significant.

Reverse transcription and Semi-quantitative real time PCR

For further validation of expression array data, SYBR Green based semi-quantitative real time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher K0221) followed by cDNA synthesis using reverse transcription kit (Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit) following instruction manual.

Fig 1: The representative images of Tyrosine Hydroxylase immunostaining of substantia nigra-pars compacta region of mice mid brain of all three experimental conditions obtained at 4X and 10X magnifications respectively



Results

IHC was performed first to confirm the effect of MPTP and MPTP+RS. The primary antibody used for IHC was against the enzyme Tyrosine hydroxylase (TH), an enzyme which is involved in catecholamine biosynthesis and an important marker of dopaminergic neurons. The TH positive cells of approximately ten sections (100µ) from SNpc region [mouse brain bregma (-2.54) to (-3.48)] were considered for counting. The average cell count as found from three repetitive experiments for each condition was compared. This results in significant recovery of dopaminergic neurons in CONDITION 3 as compared to CONDITION 2 [*p* value= 0.0015; i.e., <0.01]. Thus, our results demonstrate a significant reduction of TH immunoreactivity in the SN-PC of MPTP treated mice, compared to control in which the fibers of dopaminergic neurons are observed prominently after immunostaining. A significant restoration in number of TH-positive cells was also observed in mice group co-treated with MPTP and RS. The representative images of all three conditions obtained at 4X and 10X magnifications respectively were depicted in **[Fig: 1].** Based on this initial indication of neuro-protective effect of RS, we performed gene expression analysis of these three experimental conditions using microarray [GeneChip® Mouse Gene 2.0 ST Array] in duplicate i.e. biological replicate. *In vivo* gene expression profiling in SNpc of three group of mice results expression of a new set of genes those are mainly involved in neurogenesis, growth cone formation and axon guidance, activation of latent precursor cells etc. 23 genes were found to be differentially regulated, 4 top ranked were taken for validation by qPCR [**Table: 1**].

Table 1: List of differentially expressed genes identified by microarray and validated by qPCR

Name of the gene	Transcript cluster ID	Condition 2 vs. 1 (Fold change & *p*-value)	Condition 3 vs. 2 (Fold change & *p*-value)	Condition 3 vs. 1 (Fold change & *p*-value)
Plxnb3	17535644	down (-3.3) & (0.009)	retained	n/c
Gh	17270829	n/c	up (17.92) & (0.04)	up (17.65) & (0.04)
Prl	17286107	n/c	up (10.67) & (0.006)	up (11.2) & (0.007)
Th	17244737	down (-2.4) & (0.005)	retained	n/c

Plexin B3 (*PlxnB3*)

It is a member of Plexin family. These are characterized by sema domain, consisting of a highly conserved variant form of the seven-blade beta-propeller fold, hence known as semaphorin receptors (13). During the development of the nervous system, neurons respond to either attractive or repulsive guidance signals to navigate to their final targets (14). As per previous data, plexins appear to be mainly involved in the repulsive activities of semaphorins on neuronal cells. It is also evident that Plexin B3 stimulates neurite outgrowth and interacts with Rin, a neuron specific small GTPase which is involved in downstream signaling of Plexin B3. Thus Plexin B3 positively influences neuronal morphogenesis in semaphorin-independent signaling mechanisms (15). In the present study we have found down regulation of this gene expression in MPTP treated mice group as compared to control, which was restored in MPTP treated RS fed mice group.

Table 2: Primer sequences and PCR conditions for Semi-quantitative real time PCR

mRNA	Primer sequence (5'-3')	Annealing (ºC)
Growth Hormone (*Gh*)	F: TGACCGTCAGCCTGCTCT	60º
	R: GCAGCCTGGGCATTCTGAAT	
Prolactin (*Prl*)	F: GGTGACTGCCAGACTTCTC	60º
	R: AGTGGGGCAGTCATTGATGA	
PlexinB3 (*PlxnB3*)	F: CAGGCCTTTAATGATGTGCGA	60º
	R: AGTTCTGGGTTGAGATGCCA	
Tyrosine Hydroxylase (*Th*)	F: GCAGAGTCTCATCGAGGAT	60º
	R: CTCGAAGCGCACAAAGTACT	
Gapdh	F: GGGTGGAGCCAAAAGGGTC	60º
	R: GGAGTTGCTGTTGAAGTCGCA	

Growth hormone (*Gh*)

The expression of growth hormone/insulin-like growth factor 1 (GH/IGF-1) has been found to be upregulated in the third group of mice co-treated with MPTP and RS. It is involved in brain growth, development, myelination and effect the proliferation of new neurons, endothelial cells astrocytes and oligodendrocytes (16). It is also evident from the previous report that GH therapy induces cell genesis in adult brain. Over expression of such a neuroprotective gene in RS fed mice indicates the neuroprotective effect of RS on PD mouse model as well.

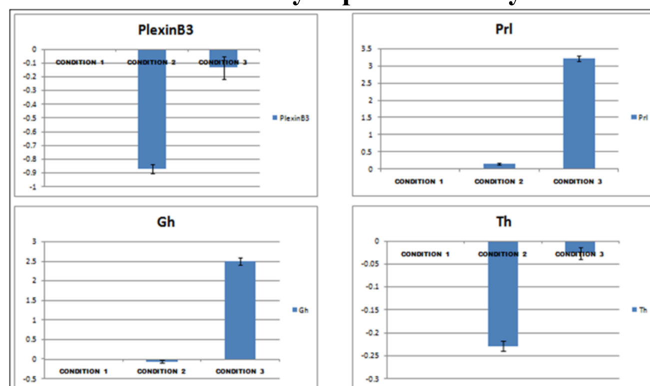
Prolactin (*Prl*)

The over expression of Prl has also been found exclusively in RS fed MPTP treated mouse. It has a stimulatory effect on brain and functions in neuroendocrine feedback loop. The feedback effect of Prl to anterior periventricular dopaminergic (DA) neurons is to increase the expression of TH and activate DA synthesis (17). Thus Prl exerts neurotrophic effects in cell differentiation, maintenance and axon guidance.
The differential expression of all these genes (PlxnB3, Gh and Prl) identified by expression array along with Th were further cross-validated by qPCR **[Fig: 2]**. The primers used for this have been listed in **Table: 2**.

Discussion

MPTP, an inhibitor of complex I of the mitochondrial respiratory chain was initially identified as a cause of inducing Parkinsonian syndrome in human drug addicts (18). Since then it is being used for developing PD model *in vivo* as well as *in vitro*. It has therefore, long been established that systemic administration of MPTP to primates and rodents replicates most of the clinical symptoms as well as the major biochemical and pathological hallmarks of PD. Sub-acute dose of MPTP i.e. 15mg/kg body weight, twice a day in 2 hours interval for 2 days had successfully induced PD in mice of PARKS strain as clearly observed by TH immunoreactivity in our study. It is the most distinctive test for observing DA neurons since TH is the key regulatory enzyme in dopamine biosynthesis. We observed a significant reduction in TH immunoreactivity in MPTP treated mice compared to normal saline treated which was significantly restored in RS fed MPTP treated mice. This was the key observation of the present study. Based on this the further experiments were performed.

Fig 2: Bar diagrams based on qPCR result of the differentially expressed genes (*PlxnB3, Gh, Prl, Th*) identified by expression array



In ayurveda neurological disorders are included in *vata.* However, RS is known to be therapeutically effective in diseases due to *kapha*. This formulation is also being used for wide varieties of disorders like *Rajyayakshma* (Tuberculosis), *Prameha* (Diabetes), *Rakta-pitta* (Bleeding disorders), Pandu (Anaemia) etc (19, 20). It is a well known rejuvenator. The testing for toxicity of RS revealed that there were no mortality, no significant changes in renal function test and no drug related morphological changes in histo-pathological examination, no behavioural changes in albino rat, administered orally with different doses (a maximum of 100 mg/kg) of RS for 28 days (21). Toxicological study based on the effect of RS and one of its intermediate (kajjali) on NIH3T3 cell line and zebrafish larvae has shown to cause no toxicity or morphological changes (22). The present study was inspired by the initial observation of the effect of dietary supplement of RS, on neurodegeneration in fly models of Huntington and Alzheimer's diseases by Dwivedi *et al.*, 2012 (10). They had reported RS to cause suppression of degeneration of photoreceptor neurons along with rescue of premature death of fly models. The present study is first to explore differential gene expression caused by dietary supplementation of RS on PD mouse model. From the microscopic observation in IHC study against TH immunoreactivity and DA neuron count **[Fig 1]**, it is clear that TH immunoreactivity has been

improved in RS supplemented PD mouse. The genes identified by high throughput cDNA microarray were a few (23 genes were found to be differentially regulated; data not shown) but significant. Such as, PLXN B3 is a highly potent stimulator of neurite outgrowth. As per gene ontology (GO) classification it belongs to semaphorin receptor family and involved in several biological processes like cell differentiation and proliferation, response to stimulus, cell signaling etc. It positively regulates axonogenesis by axon extension, guidance, its regeneration and also collateral sprouting. Down-regulation of this gene after MPTP treatment was recovered significantly in MPTP treated mice those were supplemented with RS. The other two genes exclusively identified to be up-regulated in MPTP treated RS fed mice, can be implemented as the effect of RS itself. These are growth hormone (Gh) and Prolactin (Prl). The anterior pituitary hormone prolactin is a growth regulator for many tissues including that of nervous system. It plays a role in cell survival by suppressing apoptosis. It can also specifically trigger the DA neuron proliferation by TH over expression and can activate a pool of latent precursor cells in the adult mouse hippocampus (23). However, on the other hand, the GH is involved in a wide spectrum of important biological processes including cell proliferation, differentiation, metabolism, signaling, system development and many others. It also regulates adult neurogenesis by activation of neural stem/ progenitor cells in dentate gyrus and sub-ventricular zone (24). The proliferating glia-like cells promote formation of transiently amplifying cells which in turn generate neuroblasts. Following this cells migrate towards olfactory bulb where they differentiate to different subtypes of interneurons (25). The majority of them are known to become GABAergic granule neurons with a small percentage of dopaminergic neurons as well.

In our study over-expression of these hormones after dietary supplement of RS in PD mouse model clearly supports its role in prevention of neuro-degeneration and promotion of neurogenesis as well. However, finding the exact mode of action of dietary supplement of RS will require introduction of a suitable tracer molecule which may help to identify the target pathway/s involved in the whole process. The present study thus provides convincing evidences to conclude that, RS could be used as an effective neurogenesis stimulating factor to combat with degenerative neurological disorders like PD.

Acknowledgement

We acknowledge Head of the Dept., Dept. of Zoology, BHU for providing the facility of availing animal house, Prof. SC Lakhotia for providing RS, Prof. Rajiva Raman for providing GeneChip® Mouse Gene 2.0 ST Arrays, Coordinator of Interdisciplinary School of Life Sciences (ISLS), BHU for microarrry and real-time PCR instrumentation facility. Indian Council of Medical Research (ICMR) is acknowledged for providing fellowship to T. Halder.

Conflict of interest

The authors have no conflict of interest to disclose.

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