Neuroprotective Effects of *Mucuna pruriens* Against Stress-Induced Oxidative Damage

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Abstract

Stress is known to alter the cellular homeostasis by oxidative damage in brain which in turn involves in various forms of neurotoxicity including neuronal death. The seed powder of the leguminous plant, *Mucuna pruriens* (MP) has long been used in traditional Ayurvedic Indian medicine for various neurological diseases including Parkinsonism. The neuroprotective and neurorestorative effect of *MP* was attributed to its antioxidant activity. In the present study the antioxidant potential of *MP* was evaluated in stressed rat brains. Adult male and female rats were stressed (immobilization for 6 hours/day during 21 days) and also treated with *Mucuna pruriens.* Oxidative stress was assessed by measuring the extent of lipid peroxidation and reduced Glutathione activities and also totals antioxidant activity in rat brain. Immobilization stress caused an alteration in oxidative stress markers with a significance increase in brain lipid peroxidation and depletion of reduced glutathione and total antioxidant activities in brain homogenate of stressed rats. *MP* has significantly minimized the oxidative stress effects by significant decrease in lipid peroxidation level and also an increase in reduced glutathione level as well as total antioxidant activities. Results of the present study indicated that *MP* has antioxidant potential against stress-induced oxidative damage in rat brain.

J Physiol Biomed Sci. 2011; 24(2): 28-33.

Keywords: *stress, oxidative stress, antioxidant, Mucuna pruriens*

In the last few years, several reports indicated that long lasting stress affects synaptic plasticity, dendritic morphology and neurogenesis in animals[1] and induces both clinical & anatomical features of neurotoxic damage in humans.[2,3] Stress induced neuronal damage were also reported.[4,5] There are many reports suggesting the possible mechanisms like altered hypothalamo-pituitary-adrenal axis, an increased brain corticosterone level, altered neurotransmitters and enhanced free radical generation causing oxidative damage in nervous tissue.

Stressful conditions leads to the formation of excessive free radicals which are a major internal threat to cellular homeostasis. Restraint as a stress model combines both emotional and physical components of stress in addition to producing robust increases in the production of reactive oxygen species and consequent oxidative damage, with a concomitant decline in *in vivo* antioxidant defenses.[6,7] Oxidative damage is an established outcome of restraint stress, which has been suggested to induce many damaging processes contributing to the pathology of stress-induced neurotoxic effects. Of all the organs in the body, the central nervous system takes more than its share of oxidative abuse.[8,9] The brain is particularly vulnerable to oxidative stress due to its high rate of oxygen consumption and elevated content in iron and polyunsaturated fatty acids.[10] Fortunately neurons are endowed with defense and repair mechanisms, capable to override the oxidative stress associated damage.[11] However under pathological conditions, an over production of reactive oxygen species may occur, overcoming the capacity of the antioxidant systems and resulting in sustained neuronal oxidative insults.[12-14] From all these reports it is evident that stress can alter cellular homeostasis by oxidative damage in brain which in turn involves in various forms of neurotoxicity including neuronal death. Immobilization stress in mice has been shown to cause neuronal death in the cerebral cortex by apoptosis, which was effectively prevented by antioxidant pretreatment with an associated decrease in reactive oxygen species population.[15] The antioxidant defense system is mainly represented by antioxidant enzymes, synthesized antioxidant and dietary antioxidant molecules. Various synthetic antioxidants have been used for neurodegenerative diseases; but naturally occurring antioxidants have been reported to possess a broad spectrum of biological, pharmacological and therapeutic activities against free radicals and oxidative stress.[16] Synthetic antioxidants used in oils and fatty foods are suspected to be carcinogenic. Therefore, the importance of search for natural antioxidants has greatly increased in the recent years.[17]

MP (also known as "the cowhage" or "velvet" bean) is an annual twining herb with trifoliate leaves, purple flowers and turgid S-shaped pods covered with hairs. The plant belongs to the family Leguminosae. It is found in bushes and hedges at damp places, ravines and scrub jungles throughout the plains of India and in other parts of the

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tropics including Central and South America. It is cultivated for its pods as vegetable and young leaves as fodder. The pods are anthelmintic and seeds exhibit anti-inflammatory and aphrodisiac property.[18] In India seeds are used as a tonic and for male vitality in traditional medicine.[19] Seeds from *MP* (Atmagupta) have been described as a useful therapeutic agent in various dieseases of the human nervous and reproductive system.[20] It is also used for the management of several free radical-mediated diseases, such as rheumatoid arthritis, diabetes, atherosclerosis, male infertility and nervous disorders, in the Ayurvedic system of medicine.[21] Ayurvedic texts describe Kampavata, a nervous malady bearing similarities to Parkinson's syndrome, responding to *MP*.[22] Levodopa (L-dopa) was first isolated from the seeds of MP in 1937[23] and when the value of L-dopa for the treatment of Parkinson's disease became known, scientific interest in plants rich in L-dopa was revived. *MP* seed in addition to L-dopa contains several identified and other unidentified compounds.[24] A study by Dhanashekaran et al.,[25] suggests that the neuroprotective and neurorestorative effect of *MP* is due to its antioxidant activity. With these findings there is reason to believe that *MP* by its antioxidant or other unknown mechanism could reverse the stress-induced neurotoxic effects in all age groups. Hence in the present study we would like to evaluate the neuroprotective effect of *MP* on stress induced oxidative damage in rat brain.

Materials and Methods

Animals

Male and female Wistar rats (200-225 g) bred in house are used in the present study. Animals were maintained under controlled conditions of light (10 h light : 14 h dark), temperature (22 ± 3°C), and humidity (approximately 50 ± 10 %). All rats were maintained on the standard rat food and water *ad libitum*. The experiment was approved by the institutional animal ethics committee. Breeding and maintenance of the animals were done as per the guidelines of Government of India for use of Laboratory animals (Government of India notifies the rules for breeding and conducting animal experiments, proposed in the gazette of India Dec 15, 1998: which was reproduced in Indian Journal of Pharmacol. 1999; 31: 92-5).

MP treatment schedule and animal groups

Although no studies have yet appeared regarding proper dosage of *MP* for animals and humans, Zandu Pharmaceutical works (Mumbai, India) along with Manyam et al.[20] have taken to use a dosage of 2.5 g/kg/day and 5.0 g/kg/day of their *MP* supplement formulation. *MP* treatment (oral) was started one week prior to immobilization stress. *MP* (Kapikacchu choorna – An Ayurvedic name for MP, in which it is available to the patients in the powder form containing only *MP*) in the powder form was obtained from SDM Ayurvedha College, Udupi, India (where it was used for patients) and it was dissolved with distilled water (5 g in 30 ml). A constant volume of 5 ml having different amount of *MP* (as calculated from body weight) was administered orally using infant feeding tubes at every morning. The MP treatment in Group 5 and 6 was started a week before stressing procedure. Eight rats were used in each group (four males and four female rats in each group).

Group 1: Rats – Control group received distilled water
Group 2: Rats received 21 days restraint stress (6 h daily)
Group 3: Rats received 2.5 g/kg body weight dose of MP for 4 weeks
Group 4: Rats received 5 g/kg body weight dose of MP for 4 weeks
Group 5: Rats received 21 days stress (6 h daily) + MP (2.5 g/kg body weight dose) for 4 weeks
Group 6: Rats received 21 days stress (6 h daily) + MP (5.0 g/kg body weight dose) for 4 weeks

Stressing procedure

Four month old male and female Wistar rats were assigned to a daily restraint stress for 21 days (6 hours daily from 9 am to 3 pm) in a wire mesh restrainer.[26] The wire mesh restrainer has a wooden base and stainless steel wire mesh restrainer hinged to the base. A pad lock and latch will help to secure the rat in the restrainer. The restrainer with dimensions of 11 cm (L) x 8 cm (B) x 8 cm (H) will be used to stress. This type of restrainer will only restrict the animal movement without any pain, discomfort or suffocation.

Antioxidant studies

Rats were sacrificed by decapitation at the end of *MP* treatment in groups 3 to 6 and at the end of the restraint stress in group 2. The whole brain was removed and rinsed with ice-cold 0.1 M phosphate buffer (pH 7.4) 10 times (w/v). The homogenate was immediately centrifuged at 10,000'g for 15 min and aliquots of supernatant was separated and used for antioxidant studies.

Measurement of lipid peroxidation The quantitative estimation of malondialdehyde (MDA), a measure of lipid peroxidation in the whole brain homogenate was performed according to the method by Gayathri et al.[27] One ml of supernatant was precipitated with 2.5 ml of ice cold tricholoroacetic acid (TCA). The mixture was centrifuged at 3000g for 10 min. To 2 ml of this supernatant, 0.67 % of thiobarbituric acid (TBA) was added and kept in boiling water bath for 10 min and cooled it. The pink chromogen developed was read immediately at 532 nm using a Systronic-117 UV-Visible spectrophotometer. Thiobarbituric acid-reactive substances (TBARS) concentration was calculated using molar extinction coefficient of chromophore (1.56×10^5 mol/l^{-1} cm^{-1}) and the value was expressed in nmol/g protein.

Estimation of reduced glutathione Reduced glutathione (GSH) was estimated according to the method described by Ellman et al.[28] and later by Jyothi D'souza et al.[29] One ml of supernatant was precipitated with one ml of 4% sulphosalicylic acid and cold digested at 4ºC for one hour. The samples were centrifuged at 1,200g for 15 min at 4ºC. To one ml of this supernatant, 2.7 ml of phosphate buffer (0.1 mol/l, pH 8) and 0.2 ml of 5,5' dithio-bis(2-nitro-benzoic acid, DTNB) were added. The yellow colour that

developed was read immediately at 412 nm using a Systonic-117 UV-visible spectrophotometer. Results are calculated using the molar extinction coefficient of chromophore (1.36 x 10^4 mol/l^{-1} cm^{-1}) and expressed µg/g tissue.

Estimation of total antioxidants The total antioxidants concentration was estimated according to the method described by Koracevic, et al.[30] Each sample (A_1) had its own control (A_0, sample blank), in which 2 mmol/l Fe-EDTA mixture, 10 mmol/l H_2O_2 and 10 mmol/l sodium benzoate were added after 20 % acetic acid. For each series of analysis a negative control (K_1 and K_0) was prepared, containing the same reagents as A_1 or A_0, except that brain homogenate was replaced with 0.1 M sodium phosphate buffer, pH 7.4. Standard containing 1 mmol/l uric acid (UA_1 and UA_0) are used for calibration.

Content pipetted into each tube (in milliliters) is shown in the following table:

	A_1	A_0	K_1	K_0	UA_1	UA_0
Homogenate	0.01	0.01	-	-	-	-
Uric acid	-	-	-	-	0.01	0.01
Buffer	0.49	0.49	0.50	0.50	0.49	0.49
Na-benzoate	0.50	0.50	0.50	0.50	0.50	0.50
Acetic acid	-	1.00	-	1.00	-	1.00
Fe-EDTA	0.20	0.20	0.20	0.20	0.20	0.20
H_2O_2	0.20	0.20	0.20	0.20	0.20	0.20

These were incubated for 60 minutes at 37ºC, then the followings were added:

Acetic acid	1.00	-	1.00	-	1.00	-
TBA	1.00	1.00	1.00	1.00	1.00	1.00

The mixture was incubated for 10 minutes at 100ºC (in a boiling water bath), and then cooled in an ice bath. Absorbance was measured at 532 nm against deionised water. The total antioxidants (TAO) level in whole brain was expressed as µmol/g protein.

Statistical analysis

The data were expressed as mean ± SD. The significance of differences among the groups were assessed using one way analysis of variance (ANOVA) test followed by Bonferroni's multiple comparison test. *P* values < 0.05 were considered as significant. Comparison of data between male and female group was assessed by Mann-Whitney U test.

Results

No gender differences were observed in any measures and therefore, data from males and females were collapsed into one group.

Effect of *MP* on brain lipid peroxidation levels in stressed rats

Immobilization stress has caused a decline in antioxidant defense as indicated by a significant ($P < 0.001$) rise in brain MDA levels in stressed rats, when compared with control or rats treated with different doses of *MP* without stress. Stressed rats with *MP* treatment (at both doses) had shown significantly ($P < 0.001$) reduced MDA levels, when compared with rats received stress without MP treatment (Figure 1). There were no dose dependent effects of *MP*.

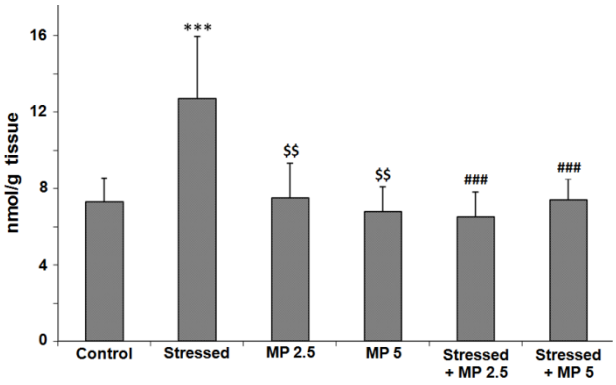

Figure 1 Malondialdehyde (MDA) levels in whole brain homogenate of various groups of rats, expressed as nmol/g tissue. Values are mean ±SD. ***, $P < 0.001$ comparing stressed rats with control; $$, $P < 0.001$ comparing MP treatment (2.5 and 5 g/kg body weight) with stressed rats; ###, $P < 0.001$ comparing stressed rats and rats received both stress and *MP* treatment; F = 9.582.

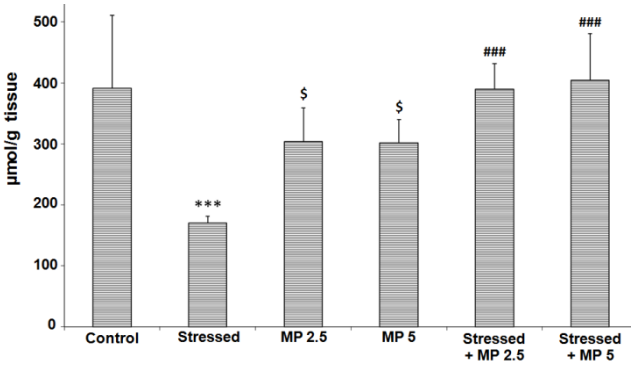

Figure 2 Reduced glutathione levels in whole brain homogenate of various groups of rats, expressed as µg/g tissue. Values are mean ±SD. ***, $P < 0.001$ comparing stressed rats with control; $, $P < 0.05$ comparing *MP* treatment (2.5 and 5 g/kg body weight) with stressed rats; ###, $P < 0.001$ comparing stressed rats and rats received both stress and *MP* treatment; F = 10.183. The largest value in the data set was used for normalization.

Effect of *MP* on brain reduced glutathione levels in stressed rats

A highly significant ($P < 0.001$) fall in the level of brain reduced glutathione was observed in stressed rats when compared to control group. This fall in reduced glutathione level was also significant ($P < 0.05$) when stressed rats were compared with rats treated with *MP* (at both doses). Stressed rats when treated with *MP* (at both doses), showed a significant ($P < 0.001$) elevation in the level of brain reduced glutathione when compared with stressed rats who have not received *MP* treatment (Figure 2). There were no dose dependent effects of *MP*.

Effect of *MP* on total brain antioxidant activity in stressed rats

We observed a significant ($P < 0.001$) reduction in total antioxidant activity in the brains of stressed rats when compared to control rats. The total antioxidant activity of was also significantly less ($P < 0.05$) in stressed rats when

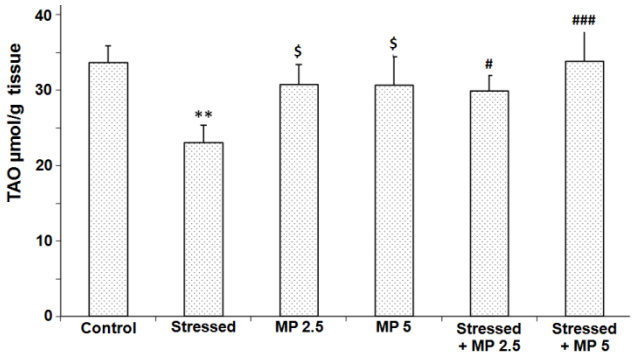

Figure 3 Total antioxidant (TAO) activities in whole brain homogenate of various groups of rats, expressed as TAO µmol/g tissue. Values are mean ±SD. ***, $P < 0.001$ comparing stressed rats with control; $, $P < 0.05$ comparing *MP* treatment (2.5 and 5 g/kg body weight) with stressed rats; #, $P < 0.05$ and ###, $P < 0.001$ comparing stressed rats and rats received both stress and *MP* treatment; F = 7.340.

compared with rats treated with both doses of *MP*. Stressed rats with *MP* treatment at 5 g/kg dose has shown a highly significant increase in ($P < 0.001$) total antioxidant activity when compared with rats received only stress. The *MP* treatment for stressed rats at 2.5 g/kg dose has shown a moderately significant ($P < 0.05$) rise in total antioxidant activity (Figure 3).

Discussion

Reactive oxygen species generated by severe stress (restraint stress) significantly compromises the inbuilt antioxidant system of animals.[6,7] The results of the present study clearly demonstrates that restraint stress results in oxidative damage to rat brain, as evidenced by significant rise in brain malondialdehyde (MDA – an end product of lipid peroxidation) levels, decreased brain reduced glutathione level and also significant reduction in total antioxidant activities. There are similar reports claiming oxidative damage in nervous tissue after restraint stress. Repeated restraint stress induces oxidative damage in rat hippocampus.[31] Immobilization stress resulted in reduced glutathione level and lipid peroxidation in peripheral tissues of rats.[32] It is also known to cause oxidative damage to lipid, protein and DNA in the brain of rats.[33]

The consequences of oxidative stress to nervous tissue is many, as brain is particularly vulnerable to oxidative stress due to its high rate of oxygen consumption. Oxidative stress induces many damaging processes in stress disorders such as mitochondrial dysfunction, dysregulation of calcium homeostasis,[34] disruption of energy pathway,[35] damage to neuronal precursors, impairment of neurogenesis,[36] induction of signaling events in apoptotic cell death.[37] Oxidative stress ultimately leads to morphological changes and finally neuronal atrophy/death.[38,39] Recent *invitro* studies on the underlying mechanisms of stress induced neuronal damage have demonstrated that corticosterone released from the adrenal cortex during stress either induces the formation of reactive oxygen species[40] or decreases antioxidant enzyme activity, resulting in increased neurotoxicity in cortical cultures.[41] Oxidative stress is thought to contribute to the development of a wide range of diseases including Alzheimer's disease, Parkinson's disease and neurodegeneration in motor neuron diseases. The brain is uniquely vulnerable to oxidative injury, due to high metabolic rate and high levels of polyunsaturated lipids, the target of lipid peroxidation. As a result, antioxidants are commonly used as medication to treat various forms of brain injury.[42]

In the present study *MP*, a natural antioxidant at both doses has significantly reduced brain MDA levels and elevated brain reduced glutathione level in stressed rats, as compared with stressed rats which did not receive pretreatment of *MP*. An increased total antioxidant activity with *MP* treatment in stressed rats also demonstrates the antioxidant effects of *MP*. In an earlier study, *MP* cotyledon powder inhibited hydrogen peroxide-induced lipid peroxidation in the rat brain. It also scavenged reactive oxygen species generated through t-butyl hydroperoxide and also scavenged free radicals in rat brain.[25] Alcoholic extract of the seeds of *MP* has an antilipid peroxidation property, which is mediated through the removal of superoxide and hydroxyl radicals.[43] Methanolic extract of *MP* showed a significant antioxidant effects in *in vitro* model.[44] Another study by Rajeshwar et al.,[45] shows strong antioxidant activities of *MP* and it was attributed to its ability to inhibit DPPH and hydroxyl radical, nitric oxide and superoxide anion scavenging, hydrogen peroxide scavenging activities. However in an *in vivo* study on rats, alcohol extract of seeds of *MP* has not affected the level of reduced glutathione content and superoxide dismutase activity in the liver. In the present study *MP* clearly demonstrated antioxidant effect against restraint stress.

Lipid peroxidation is a process whereby free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process followed by a free radical chain reaction mechanism. It often affects polyunsaturated fatty acids (in brain), because they contain multiple double bonds in between which lie methylene-CH2-groups that possess especially reactive hydrogens. These continue reactions will be damaging the cell membrane, which consists mainly of lipids. In addition, end products of lipid peroxidation may be mutagenic and carcinogenic. For instance, the end product malondialdehyde reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts to them. In the present study *MP* has significantly reduced brain MDA level.

Glutathione is a tripeptide containing glutamic acid, cysteine and glycine and is widely present in animals and plants. It primarily acts by reducing inactive disulfide linkages of enzymes to active sulhydryl group, while the sulfhydryl group of glutathione becomes oxidized.

Thus, glutathione plays an important role in defense against membrane peroxidation and also by reducing hydrogen peroxide with glutathione peroxidase. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems as well as reacting directly with oxidants. In the present study *MP* has significantly

elevated brain reduced glutathione level in stressed rats. A study by Misra and Wagner[19] on identifying the active components from the seed of *MP*, revealed about 19 types of aminoacid apart from L-Dopa, which was the major constituent. The major amino acids identified were β-alanine/taurine/L-argininene, glutamic acid and glutathione. Hence the antioxidant properties can be attributed to presence of glutamic acid in *MP* .In the same study, it was also observed that propanol extract of *MP* which had least amount of L-Dopa shown significant neuroprotective activity, suggesting that whole extract of *MP* seeds could be superior, instead of selective components. Dhanasekaran et al[25] claim that, presence of polyphenols and glutathione may have the direct impact on the antioxidant activities of *MP*. In analyzing the major constituents of *MP*, Tripathi et al[21] claims presence of tryptamine and 5-hydroxy-tryptamine and another study by Sidhuraju et al.,[46] claims no alkaloids in *MP*. The possible role of serotonin present in the MP in neuroprotective efficacy is yet to be tested.

Restraint as a stress model combines both emotional and physical components of stress. Exposure to chronic restraint stress in rats, and psychological stress in humans, is implicated in the pathophysiology of mood and anxiety disorders.[47] Free radical damage by reactive oxygen species has been suggested to play a critical role in the patho-physiology of neurodegenerative diseases, neuropsychiatric disorders and stress-induced depressions in humans.[39,48,49] Restraint stress is known to cause mitochondrial dysfunction due to overproduction of nitric oxide in brain.[50] Such damage to mitochondrial electron transport chain has been suggested to be an important factor in the pathogenesis of a range of neurodegenerative disorders.[51] Oxidative stress is one of the major toxic mechanisms for the neurodegeneration of dopaminergic neurons in the substantia nigra that leads to Parkinson's disease.[52] Atrophy and dysfunction of the human hippocampus and prefrontal cortex is a feature of aging in some individuals, and this dysfunction predicts later dementia. Stressful conditions are believed to be the one of the cause. From all these data, it is evident that the effects of stress-induced oxidative damage in nervous tissue are of many folds. Though the precise mechanism by which stress-induced brain damage is still a matter of debate, it is evident that long term stress can results in various form of neurotoxicity which is also due to oxidative damage. Since oxidative damage in nervous tissue plays a major role in inducing neurotoxicity, antioxidant therapy in stressed conditions becomes an avenue for tackling stress-induced neurotoxicity.

In view of developing cost effective remedy to stress-induced neurotoxicity in poor countries, the utility of the plant product as therapeutic agent is in vogue. The natural antioxidant, MP selected in the present study was in use in this part of the world since decades without much research. The results of this study will have impact in delivery of better health care which is less cumbersome, more functional and would be within the reach of common man from the economy aspect as well. Hence this study is very relevant in delivering the health care at the doorstep of the common man in the current scenario of stressful life style.

Conflict of interest

None to declare.

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