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Research Article

Mucuna Seed Extract Treatment Alleviates SDS-Induced Oxidative Stress and Neuronal Damage in Carp Brain

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ABSTRACT

The neurotoxic effects of a synthetic detergent sodium dodecyl sulphate (SDS) on brain function in freshwater carp *Cirrhinus cirrhosus* and the efficacy of methanol extract of *Mucuna pruriens* seeds for alleviating such effects were demonstrated. Fish (n=36, 2 replicates) were exposed to SDS (2.53 mg/l) for 0 (control), 15 and 30 days. After 30-days treatment, methanol extract of *Mucuna* seed was injected for continuous seven days and sampling was done on each alternate odd days. Levels of different enzymatic and non-enzymatic antioxidants, Na^+-K^+-ATPase, acetylcholinesterase; monoamine oxidase; nitric oxide, cortisol, estradiol-17β (in females) and testosterone (in males) were measured in *Cirrhinus* brain tissue and serum. 30-days treatment with SDS caused significant decrease in glutathione, catalase, superoxide dismutase and estradiol-17β, while glutathione S-transferase, glutathione reductase, malondialdehyde and cortisol level increased significantly ($P<0.05$). Administration of *Mucuna* extract (15.5 mg/kg body weight) was found to restore the neurological activity and reduce stress in a time-dependent manner as the biochemical and neurological parameters in fish after 7-day extract administration showed no significant difference ($P>0.05$) compared to those in control without SDS treatment.

Keywords: *Mucuna pruriens*, SDS, Stress, Neural damage, *Cirrhinus cirrhosis.*

INTRODUCTION

Various agricultural and industrial wastes enter aquatic environment and may induce physiological changes in fish. Some of these pollutants may directly enhance reactive oxygen species (ROS) formation whereas others act indirectly by binding with cellular thiols and reducing antioxidant potential[1]. A wide spectrum of inter-site differences in oxidative damage have been observed in fish from polluted compared to non-polluted areas[2]. Hence, an integrated approach with the appreciation of balance between prooxidant manifestations and antioxidant defence in biological systems needs to be a control point to assess toxic effects under stressful environmental conditions. Neurons are relatively sensitive to reactive oxygen species (ROS) and many neurodegenerative disorders have been linked to damage caused by ROS[3]. Detergents present in urban or rural sewage may reduce dissolved oxygen, alter pH and salinity of receiving freshwater bodies, thereby affecting oxygen consumption ability of fishes[4]. However any potential impact of these synthetic detergents on central nervous system and the mechanism of such detergent-induced impairment of nervous system function in fish remains unclear[5]. Many plant extracts are reported to possess antioxidant activity and act as a potent scavenger of superoxide radical[6]. The Indian medicinal herb, *Mucuna pruriens* has been reported to possess anti-depressant potency, libido enhancing effect, antispasmodic, antipyretic, anti-inflammatory activity, and has been used to manage Parkinson's disease[7]. Indian major carps are susceptible to exposure to various pollutants including synthetic detergents. In this study, *Cirrhinus cirrhosus*, an economically important Indian major carp was treated with a synthetic detergent, sodium dodecyl sulphate (SDS), to elucidate the neurotoxic effects of any detergent surfactant in a fish model. Subsequently, the antioxidant and neuroprotective efficacies, if any, of *Mucuna* seed extract were evaluated for considering the use of the plant extract as source of new drugs against neurotoxicity. The aim of the study was to measure functional responses in reproductive and stress physiology as well as documenting brain functions and biochemical responses during exposure to pollutants in aquatic environment.

MATERIALS AND METHODS

Collection of fish

Healthy *Cirrhinus* (100±3.8 gm) were collected from the fish farm of West Bengal Government, oxygen packed and transported to the laboratory. In the laboratory, the fish was kept in 45 l aerated aquaria maintaining the physicochemical parameters such as water temperature ($T=27\pm2^0C$), pH (7.2-7.5) and dissolved oxygen (5-5.5 mg/l) at appropriate values.

Experimental design

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Fish were divided into 2 groups (n=36, 2 replicates). One group was exposed to SDS at the concentration of 2.53 mg/l, while the control group was not exposed to SDS. This sub-lethal concentration (1/10th of the 96 h LC50) for exposure was determined based on the 96 h LC50 value (25.3 mg/l) of SDS for the concerned fish. SDS powder was weighed and thoroughly mixed with tap water. The exposure solution was renewed completely each day in order to ensure constant concentrations. After 15 and 30 days of SDS-treatment, brain tissue and serum samples were collected from 6 fish each from both control and the treatment groups. Remaining fish from SDS-treatment groups (30 days) were injected intraperitoneally with *Mucuna* extract (15.5 mg/kg body weight) for seven consecutive days and brain tissue and serum were collected from 6 fish each after 1, 3, 5 and 7 days of *Mucuna* treatment. All samples were stored in -20˚C for biochemical analysis.

Preparation of Mucuna extract

Mucuna seeds were procured from local plant market, washed in sterile distilled water, air-dried and powdered. Powdered plant materials (250 gm) were extracted with 500 ml methanol in a Soxhlet apparatus and the extracts were evaporated to dryness under pressure at 45ºC using rotary evaporator and stored under nitrogen at -20ºC in amber glass bottle.

Measurement of enzymatic and non-enzymatic antioxidants in brain tissue

Brain tissues were homogenized with Tris buffered saline (10 mM Tris–HCl, 0.1 mM EDTA-2Na, 10 mM sucrose, 0.8% NaCl, pH 7.4), centrifuged and supernatant was used to measure levels of different enzymatic and non-enzymatic antioxidants such as malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidise (GPx), glutathione S-transferase (GST) according to standard methods described earlier[8]. In brief, the level of MDA equivalents was measured in brain by thiobarbituric acid reactive substances (TBARS) assay with minor modifications. SOD activity was measured following a spectrophotometric method based on assessment of $O_2^{\cdot-}$-mediated nitro blue tetrazolium reduction by an aerobic mixture of NADH and PMS. For measurement of CAT activity, absorbance was monitored at 240 nm up to 90 s at 15 s intervals. The assay was validated by treating the tissue homogenates with sodium azide, a known inhibitor of CAT activity[9]. For GPx activity, absorbance was measured at 492 nm against blank (100 µl extra OPD solution instead of sample). The activity of GR was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm, in a reaction mixture containing 950 µl of 0.15 mM NADPH, 0.5 mM glutathione, and 3 mM $MgCl_2$ in 50 mM Tris (pH 7.5) and 50 µl extract. The GST activity was spectrophotometrically assessed using glutathione (GSH, 2.4 mM/l) and 1-chloro-2, 4-dinitrobenzen (CDNB, 1 mM/l) as substrate. Protein concentrations in the supernatants were determined using Bradford's procedure.

Measurement of hormones in the serum

Serum 17-β estradiol (E_2) (in females) and testosterone (in males) were measured using Enzyme Immunoassay kit (Biocheck Inc, 837 Cowan Road, Burlingame, Ca 94010) and in house prepared standards in buffered matrix following manufacturer's protocol. The absorbance of the yellow colour inside the solution of the wells was measured at 450 nm with Tecan-Spectra automatic Microplate reader. The intra- and interassay coefficient of variation of this assay were 2.8% and 6.4% respectively. Serum cortisol was measured by photometric method using Pars Azmoon kits following manufacturer's protocol. The intra- and interassay coefficient of variation of this assay were 2.9% and 6.4% respectively.

The tissue sample from each fish which were used for testosterone (T) measurement using Enzyme Immunoassay kit (Biocheck Inc, 837 Cowan Road, Burlingame, Ca 94010) and in house prepared testosterone standard in buffered matrix. The absorbancy of the yellow colour inside the solution of the wells was measured at 405 nm with Tecan-Spectra automatic Microplate reader. The intra- and interassay coefficient of variations of this assay were 2.6% and 5.4% respectively.

Measurement of activity of brain enzymes

Na+-K+-ATPase activity, expressed as µmol Pi liberated/mg protein/h in the brain was measured by liberating PO_4 from a hydrolysis reaction with ATPase[10]. AChE activity in brain homogenates was determined with an AChE kit according to standard method[11]. The activity of 1 U of AChE was defined as the number of hydrolyzed micromoles of acetylthiocholine iodide per min per microgram of protein. The activity of MAO was determined using a detection kit, which assessed the production of benzyl aldehyde from the reaction of MAO and its specific substrate, aniline hydrochloride[12].

Statistical analysis

Two separate principal component analysis (PCA) for all the antioxidant enzymes with two hormones and brain enzymes were done to understand the ordination. First, interspecific patterns of stress were examined using a PCA on the correlation matrix of standardized enzymes and hormones. This analysis allows comparison of stress physiology, brain enzymes and serum hormones within a multivariate morphological space and identification of patterns of correlation among physiological variables. Eigenvalues of each component of the PCA were considered to interpret results. All data were expressed as Mean±SD and analyzed by one-way ANOVA. Where F values indicated significance, the means were compared by a *post hoc* multiple range test taking $p < 0.05$ as the threshold.

RESULTS

Activities of GST and GRD were observed to significantly ($P<0.05$) increase with the increasing duration of the SDS-treatment, while SOD and CAT activity showed a sharp decrease in their expression along with time. No significant change ($P>0.05$) in the activity of GPx was monitored in any of the SDS-treatment groups compared to that in control. Administration of

Table 1: Effect of *Mucuna* on antioxidant parameters in the SDS treated *Cirrhinus.*

INDICES	TEST GROUPS						
	Control	S1	S2	M1	M3	M5	M7
SOD (U/mg protein)	19.83±0.95^{a}	14.6±0.45^{b}	13.1±0.4^{c}	14.2±0.7^{b}	14.9±0.6^{b}	16.36±0.55^{b}	18.03±1.31^{a}
CAT (U/mg protein)	18.73±0.65^{a}	16.26±0.45^{b}	14.16±0.25^{c}	14.5±0.36^{c}	14.9±0.2^{c}	16.23±0.45^{b}	17.96±0.80^{a}
GPx (U/mg protein)	13.36±0.25^{a}	12.76±0.06^{a}	12.23±0.15^{a}	11.9±0.2^{a}	12.13±0.55^{a}	12.63±0.31^{a}	12.86±0.25^{a}
GST (U/mg protein)	26.26±6.74^{c}	34.83±1.51^{a}	36.73±1.06^{a}	36.96±1.46^{a}	35.16±0.31^{a}	34.6±0.96^{a}	32.6±1.15^{b}
GR (U/mg protein)	9.43±0.35^{c}	12.83±0.75^{a}	12.76±0.15^{a}	12.46±0.80^{a}	11.63±0.75^{a}	10.63±0.55^{b}	9.73±0.41^{c}
GSH (nmol/mg protein)	54.46±2.25^{a}	47.46±1.65^{b}	44.36±1.15^{c}	43.8±1.1^{c}	45.16±0.95^{c}	48.36±0.85^{b}	52.1±0.8^{a}
MDA (nmol/mg protein)	7.43±0.25^{c}	9.733±0.15^{b}	11.5±0.26^{a}	11.16±0.25^{a}	10.36±0.38^{a}	9.1±0.3^{b}	8.43±0.21^{c}

Notes: SOD- Super Oxide Dismutase, CAT – Catalase, GPx – Glutathione Peroxidase, GST - Glutathione S-transferase, GR – Glutathione reductase, GSH – Reduced Glutathione (nmol/mg protein), MDA - Malondialdehyde; S1 and S2 - 15 and 30 days of SDS treatment; M1, M3, M5 and M7 – Days (1, 3, 5 and 7) of observation after *Mucuna* treatment. Data are means ± SD; (n = 6). Different alphabets within same row indicate significant variation in mean value.

Table 2: Effect of *Mucuna* on neurological and serum parameters in the SDS treated *Cirrhinus.*

INDICES	TEST GROUPS						
	Control	S1	S2	M1	M3	M5	M7
MAO (U/mg protein)	13.67±0.25^{a}	10.36±0.13^{b}	9.27±0.06^{c}	9.09±0.07^{c}	9.25±0.07^{c}	10.85±0.07^{b}	12.97±0.09^{a}
NO (U/mg protein)	1.98±0.05^{a}	1.11±0.05^{c}	0.79±0.06^{d}	0.79±0.03^{d}	0.87±0.41^{d}	1.07±0.07^{c}	1.66±0.06^{b}
AchE (U/mg protein)	7.94±0.42^{a}	5.21±0.16^{c}	4.14±0.06^{d}	4.11±0.06^{d}	4.93±0.05^{c}	6.14±0.17^{b}	7.42±0.28^{a}
Na+-K+ATPase (U/mg protein)	1.86±0.09^{a}	1.05±0.08^{c}	0.90±0.05^{d}	0.88±0.02^{d}	1.02±0.09^{c}	1.46±0.08^{b}	1.78±0.07^{a}
Testosterone (ng/ml)	2.03±0.14^{a}	1.6±0.1^{a}	1.56±0.07^{a}	1.66±0.15^{a}	1.73±0.11^{a}	1.76±0.12^{a}	1.83±0.11^{a}
17β-Estradiol (ng/ml)	3.06±0.15^{a}	2.3±0.1^{b}	2.03±0.12^{b}	1.86±0.05^{c}	2.23±0.31^{b}	2.7±0.1^{a}	2.8±0.1^{a}
Cortisol (ng/ml)	1.03±0.12^{d}	1.66±0.15^{b}	1.9±0.2^{a}	1.96±0.2^{a}	1.93±0.3^{a}	1.63±0.1^{b}	1.4±0.11^{c}

MAO –Mono amine oxidase, NO – Nitric oxide, AchE- Acetyl cholinesterase. The abbreviations used for representing different fish groups are the same as in Table 1. Data are means ± SD; (n = 6). Different alphabets within same row indicate significant variation in mean value.

Mucuna extract significantly re-established (P<0.05) the levels of the enzyme activity and such significant response was observed after 5 days of *Mucuna* extract treatment (Table 1). GSH level decreased significantly even at 15 days SDS-treatment group compared to that in control. MDA levels were observed to increase with duration of the SDS-treatment. Administration of *Mucuna* extract significantly restored (P<0.05) the levels of the GSH and lipid peroxidation and the response was noted after 5 days of treatment (Table 1). The activity of AChE, MAO and NO in the brain was significantly inhibited in the SDS-administration groups (P<0.05), and the level of inhibition was duration dependent, as activity was significantly reduced after 30 days of treatment than that after 15 days treatment. Administration of *Mucuna* extract significantly restored (P<0.05) the levels of the enzyme activity though the duration of treatment required for the restoration varied for different enzymes. The activity of MAO and AchE was observed to increase significantly (P<0.05) after 5 days of *Mucuna* treatment whereas, NO activity was observed to be significantly affected (P<0.05) after 7 days of administration (Table 2). There was a decrease in the activity of $Na^{+}K^{+}$ATPase enzyme in both the SDS-treatment duration categories than that of the control fish. However, no significant variation (P>0.05) was observed for Na^{+} K^{+} ATPase activity between 15-days and 30-days SDS-treatment groups. Levels of Na^{+} K^{+} ATPase activity started to increase significantly (P<0.05) after 5 days of *Mucuna* injection though highest activity was observed after 7

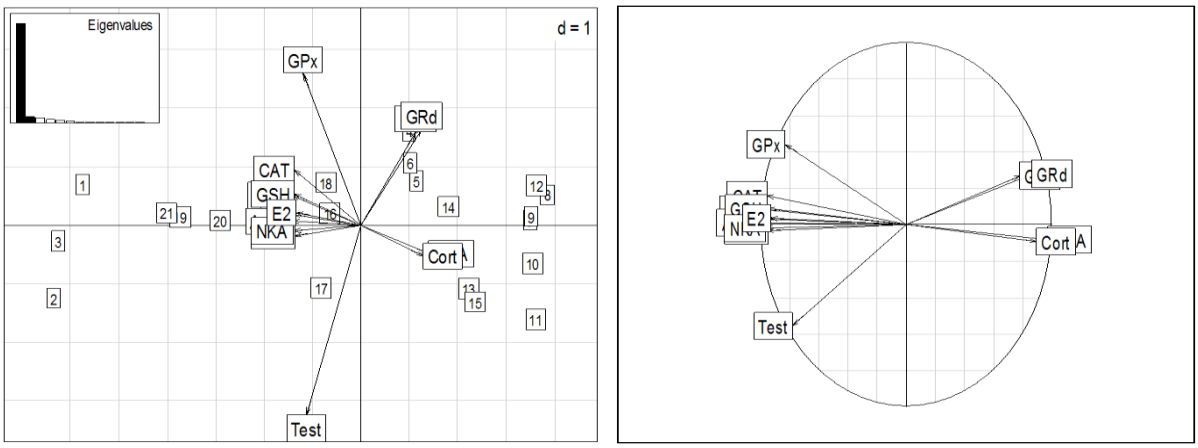

Figure1: Ordination diagram of PCA of SDS concentrations, exposure time and all parameters measured in the fish brains after chronic exposure to SDS.

days of treatment (Table 2). Cortisol level increased, while 17β-estradiol level decreased significantly ($P<0.05$) with duration of the SDS-treatment. No significant decrease ($P>0.05$) in testosterone level was observed after SDS treatment. Injection with *Mucuna* extract significantly reinstated ($P<0.05$) the levels of the 17β-estradiol after 5 days of treatment though no such effect was noted in testosterone profiles. Levels of cortisol were found to decrease after 7 days of *Mucuna* treatment (Table 2).

DISCUSSION

Oxidative stress is the ultimate manifestation of a multi-step pathway, culminating in an imbalance between pro-oxidant and antioxidant defence mechanisms due to the depletion of antioxidants, or the excessive accumulation of ROS, or both, which leads to tissue damage[13]. It has been demonstrated that exposure to various contaminants like SDS could produce ROS which cause various organ lesions[14]. MDA, a lipid peroxidation indicator, was used to evaluate the oxidative stress. The duration dependent significant increase in MDA level indicated the generation of oxidative stress in fish exposed to SDS. Due to the inhibitory effects on oxy-radical formation, the SOD-CAT system provides the first line of defence against oxygen toxicity[15]. Decrease in the activity of SOD-CAT system was most likely a response to toxicant stress, and failed to neutralize the impact of increased ROS generation[16]. SOD and CAT activities in fish tissues were strongly inhibited with prolonged duration of exposure, which could be due to the flux of superoxide radicals, resulting in increase in cellular H_2O_2[17]. GR plays an important role in cellular antioxidant protection and adjustment processes of metabolic pathways, and GPx catalyzes the reduction of H_2O_2 and lipid peroxide[3]. Together they constitute an important line of defence against oxidative stress induced by such type of xenobiotics. In the present study, GR and GST were found to be stimulated at higher SDS treatment duration, suggesting that the SDS-induced accumulation of ROS has interfered with the antioxidant enzymes and they tried to neutralize the effect of increased ROS generation in the brain of exposed fish. A significant negative correlation was observed between the antioxidant enzymes and MDA (Figure 1, Table 3) suggesting severe oxidative stress by SDS. It might be inferred that the possible mechanisms of SDS-induced oxidative stress might be a higher oxygen consumption rate to increase metabolic rate to detoxify SDS toxicity eventually leading to higher ROS production. AChE plays a crucial role in synaptic transmission at cholinergic synapses by controlling the action of acetylcholine, whereas MAO also plays a vital role in the metabolism of different neurotransmitters[18]. The inhibition of AChE and MAO activities under SDS stress might occur due to the altered affinity for free-SH groups and consequential inhibition of their function[19]. NO plays an important role in cell signalling, neurotransmission, cell-protection and regulatory effects in various cells at a physiological concentration[20]. Therefore, alterations in NO production may be a causal factor in the development of neurotoxicity. Similar to some previous reports[21], here also SDS was found to decrease NO production in the brains of exposed fish, while increasing ROS levels. ROS was reported to contribute to the induction of neurodegenerative disorders[22]. The present study supports the view that SDS exposure might hamper the neurological function in fish brain, associated with oxidative stress. The inhibition of Na^+-K^+-ATPase activity produces membrane depolarization, leading to the suppression of neuronal and excitatory transmission. A significant negative correlation of Na^+/K^+-ATPase activity with MDA, but a strong positive correlation with the neurological parameters was observed (Figure 1, Table 3). The possible reasons may be related to the special physiological characteristics in fish brain. Brain tissue, as the centre of the nervous system in all vertebrate, contains large amounts of polyunsaturated fatty acids, which are particularly vulnerable to free radical attacks. It was reported that tributyltin exposure caused brain damage in kelpfish, associated with increasing of ROS stress and decreasing of Na^+/K^+-ATPase activity[17]. Similarly, here, the SDS-induced inhibition of Na^+-K^+-ATPase after long-term exposure most likely disturbed the Na-K pump, which

Table 3: Principal component loadings of PCA with neurological and serum parameters in the SDS treated *Cirrhinus.*

	Comp1	Comp2
MAO	-0.9794226	0.08593054
NO	-0.9729949	0.03201235
AchE	-0.9863789	0.01120752
MDA	0.9691811	-0.08285455
SOD	-0.9509695	-0.03109691
CAT	-0.9567638	0.16204503
GPx	-0.8304068	0.44332395
GST	0.7797093	0.27062062
GRd	0.8635483	0.28045743
GSH	-0.9551003	0.09051793
NKA	-0.9646339	-0.01675676
Test	-0.7814060	-0.55427282
E2	-0.9301656	0.03711670
Cort	0.8941092	-0.09083890

might be responsible for the SDS neurotoxicity. All the stress parameters play crucial role in a concert to maintain the pattern of stress hormone but it is not related to any specific enzymes of the system. A significant negative correlation (Figure 1, Table 3) was observed between cortisol and reproductive steroids. The decline in this female sex steroid pattern was evident throughout the study. However, production of testosterone was not much affected. Therefore, it might be postulated that this carp could not withstand stress caused by SDS toxicity and it would further affect its reproductive efficiency, mainly in females. However, the hypothesis needs further experimental proof to be established. Administration of *Mucuna* seed extract to SDS-treated fish resulted in a significant ($P<0.05$) decrease in MDA levels, GST and GR activities; and increase in activities of SOD, CAT and neurological parameters regulating the brain function compared to those in fish which were exposed to SDS for 30 days but not to *Mucuna* extract. These observations indicate that with its antioxidant property, the plant extract may play a crucial role in maintaining the neurological parameters in the brain and protect the tissue from possible neurotoxic damage. The antioxidant and neuroprotective activity of the extracts may be attributed to the presence of specific phytochemicals such as flavonoids, terpenoids and tannins[23].

CONCLUSION

With prolonged exposure, SDS-induced stress led to functional damage in the brains of the fish, by accumulation of oxidative substances, inhibition of antioxidant defences and the neurological system in the brain and Na^+-K^+-ATPase activity as well. *Mucuna* extract treatment exhibited prominent antioxidant and neuroprotective activity and strongly inhibited lipid peroxidation in fish exposed to SDS. Thus, the plant might act as a possible natural source of antioxidants and could be useful in therapy of free radical pathologies. However, further study will be necessary to correlate this observation with other aquatic organisms and higher vertebrates, and for clarifying the extent of pharmacological activity of *M. Puriens*.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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