Indian J. Applied & Pure Bio. Vol. 37(3), 821-827 (2022). **ISSN: 0970-2091**
A web of Science Journal

Effect of *Withania somnifera* (L.) Dunal extract on Acetylcholinesterase (AChE) Activity in stressed rat brain: A Histochemical study

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Abstract

Any physical or emotional pressure or the body's reaction to an undue challenge or demand is defined as stress. Stress can affect all the organ systems of the body especially, the nervous system. The hippocampus, which is under the influence of the Hypothalamo-Pituitary-Adrenal (HPA) axis, is vulnerable to stress reactions. In the present study, we studied the effect of immobilization stress and the effect of *Withania somnifera* (L.) Dunal on acetylcholinesterase (AChE) activity in adult female Swiss albino rats. In stressed animals, AChE activity was weak and in drug treated animals, intense reactivity was observed in all the cell layers of the hippocampus. AChE activity restored in stressed animals after the drug treatment. Another interesting finding is that stress differentially affects the hippocampus; it affects different regions and cell layers differently. The study thus demonstrates the antistress neuroprotective effects of *Withania somnifera*.

Stress influences every physiological activity of the body and adversely affect the normal state of a subject[3]. Neurotransmitters and hormones, specifically associated with the hypothalamo-pituitary-adrenal axis and free radicals are inherent to stress-induced effects[10]. Catecholamines such as dopamine, norepinephrine, and epinephrine have been extensively studied in stress[14]. Studies are available, which highlight the importance of cholinergic mechanism in stress. Acetylcholine (ACh) is affected by immobilization stress[8]. Effect of stress on hippocampal cholinergic system varies with the strain of rats[9]. Cholinergic neurotransmitters play a crucial role in learning and memory. Cholinergic antagonists impair the learning and memory and reduce the release of acetylcholine in hippocampal neurons[6]. It is difficult to measure and localize the acetylcholine directly therefore either Choline acetyltransferase (ChAT) or AChE enzymes are studied.

Stress activates hypothalamus and hippocampal subregion of brain and can induce a decrease in AChE content, this decrease is differential as observed in the dorsal hippocampus only[7]. Das *et al*.,[5] compared effect of the different stressors on AChE activity, chronic unpredictable stress reduces enzyme levels more than chronic predictable stress and affects the learning and memory process in a similar way.

Many plant species are reported for their neuroprotective properties. Schimidt *et al*.,[17] investigated the neuroprotective role of green tea in cafeteria diet fed animals, associated with a model of β-Amyloid (Aβ) injection that induces cognitive impairments related to Alzheimer disease (AD). Green tea reduces oxidative stress and improves antioxidant status in the hippocampus. Green tea preserves short and long-term memories in a rat model of AD-like disease. Jain *et al*.,[11] and Shukla *et al*.,[19] studied the neuroprotective effects of *Withania somnifera* (L.) Dunal on stressed adult female swiss albino rats both at light and electron microscopy level. Speers *et al*.,[20] demonstrated that *Withania somnifera* root and leaf extracts exhibit anti-stress and anti-anxiety activity in animals and humans, it is also helpful in improving the symptoms of depression and insomnia. Besides this several other plants, *Semecarpus anacardium*[2], *Asparagus racemosus*[16,22] from our lab are reported to possess neuroprotective properties. *Withania somnifera* is well reported for its neuroprotective and cognitive enhancing properties. The present investigation was conducted to assess whether the cognition-enhancing effects, in stressed female rats, of *Withania somnifera* are because of neurochemical alterations of cholinergic transmitter systems. Therefore, histochemistry to analyse AChE activity was performed in hipppocampus of adult female rats.

Present study was carried out on adult female Swiss albino rats (B.W 60±5gms) age one and half years. Rats selected for experimental purpose were completely disease and infection free. Animals were kept in plastic cages in controlled laboratory conditions (12 hrs light and dark cycle at 27±2^{O}C) for two weeks after receiving them from supplier. After two weeks rats were divided into experimental (E) and control group (C). All the animals were provided rat chow and water *ad libitum* during the experiment. All experiments and protocols were approved by IAEC [Reg. No. 973/ac/06/CPCSEA].

Control group treatment :

Age match rats were (N=5) kept in pathogen free environment in an isolated room. Room was locked for 24 hrs. This was necessary to avoid any stressful situation. After opening the room rats were decapitated immediately and brain was dissected out and fixed.

Experimental groups :

In this group animals were divided into three subgroups E1, E2 and E3.

E1: Rats were exposed to 14 hours of immobilization stress for 30 days.

E2: Ashwagandha root extract administered for 30 days.

E3: Rats given daily stress and dose of root extract for 30 days.

Stress protocol :

Animals of group E1 and E3 were subjected to immobilization stress. Rats were kept in tightly fitted ventilated plastic boxes for 14 hours daily for 30 days.

Drug and Dose schedule :

Withania somnifera root powder extract is available commercially as STRESSCOM CAPSULE (Dabur India Limited). Each capsule contains hydroalcoholic extract of ashwagandha root. Dabur has standardized extract for withanolids and withanoles. Soya lecitins, bee wax and arachis oil is used as a base for medicate and packed in a soft capsule. Extract from the capsule was dissolved in distilled water and a single daily dosage of 80 mg/kg of body weight was given orally using a feeding tube, experimental Groups E2 and E3 received drug treatment between 10.00 to 12.00 am. Treatment continued for 30 days; on 31st day rats were sacrificed.

Histochemical Studies :

15µm thick cryostat sections passing through hippocampal subregion were processed for histochemical localization of Acetylcholinestrase using Karnovsky and Roots[12] method.

The distribution of Acetylcholinestrase enzyme in hippocampal sub region (CA1-Dg) of control and experimental rats was studied. Identification of hippocampal sub region was based on stereotaxic rat brain atlas by Paxinos and Watson[15].

Serial sections of the forebrain were processed for histochemical demonstration of enzyme activity. The site of enzyme activity was determined by presence of brownish deposit on reaction site. The reaction intensity is an indication of the quantity of enzyme present and distribution corresponds to the distribution of acetylcholine. A distinct pattern of staining was observed in the hippocampal sub region; different intensities of histochemical reaction were observed in an area specific manner. On the basis of intensity of colour reaction, the reactivity of AChE in the hippocampus is classified into intense, moderate and weak (Table-1).

In CA1 hippocampal area intense reactivity was observed in hippocampal pyramidal cells of control and drug treated group. In stress treated animals AChE reactivity was very weak or absent. In control group AChE reactivity was moderate in oriens (Or), intense in pyramidal (Py) and absent in stratum radiatum (Rad) and lacunosum moleculare (Lmol) layer of CA1 subregion of hippocampus (Fig 1.1). In group E1 (Fig 1.2) reactivity was absent (-) in oriens (Or), pyramidal (Py), stratum radiatum (rad) and lacunosum molecuare (Lmol) layers. In group E2 (Fig 1.3) moderate reactivity was observed in Oriens (Or) while very light reactivity (+) was observed in stratum radiatum, lacunosum cell layer. The pyramidal cell layer of CA1 region contains a very dark reactivity (+++). In group E3 (Fig 1.4) moderate reactivity (++) was observed in stratum Oriens (Or) while no reactivity (-) was observed in radiatum and Lacunosum molecular cell layer. The pyramidal cell layer of drug treated animals showed moderate reactivity (+++).

Pyramidal neurons in CA2 subregion of control rats exhibited intense reactivity this

was followed by Stratum Oriens (Or), Stratum radiatum (Rad) and Lacunosum molecular cell layer (L mol) with moderate, weak and no reactivity (Fig 1.1). Stressed animals exhibited weak reactivity in all the cell layers (Fig 1.2). Group E2 animals which were treated with extract only exhibited historeactivity at par with control animals, strong reaction was observed in pyramidal neurons rest all cell layers demonstrated weak or no reaction (Fig 1.3). Stressed animals treated with Withania extract i.e group E3 animals showed commensuration of AChE reactivity, moderate activity was observed in pyramidal and Stratum Oriens (Or) neurons (Fig 1.4).

Distinct changes were observed in CA3 subregion (Fig 1.1-1.4) after the stress treatment. Pyramidal neurons were found to be affected most, intense reactivity was observed in Control and Drug treated animals, whereas the reactivity was weak in stressed animals and moderate in group E3 animals. Rest all other cell layers followed this pattern of reactivity.

In control group and E2 group the reactivity was intense (Fig 1.1 and 1.3). In group E1, stress treated, reactivity was absent (Fig 1.2). Group E3 the reaction was comparatively weak to control group (Fig 1.4).

In control group (Fig 1.1) reaction of AChE was moderate while in stress treated group (Fig 1.2) there was no reaction found in Dg area. Group E2 (Fig 1.3,) dose treated group reaction intensity was very moderate and AChE positive fibbers were also visible. In group E3 (Fig 1.4) stressed animals simultaneously treatment with drug reactivity was less intense as compared to control.

Table-1. Histochemical localization of AChE Activity in hippocampus layer of the rat following groups treated after immobilization stress and herbal extract of *Withania somnifera* for 30 days

Hippocampal Sub Area	CA1				CA2				CA3				CA4				DG	
Cell Layers → / Experimental Groups ↓	Or	Py	Rad	LMol	Or	Py	Rad	LMol	Or	Py	Rad	LMol	Or	Py	Rad	LMol	Mol	GrDg
C	++	+++	+	-	++	+++	+	-	+	+++	+	-	++	+++	++	-	-	++
E1	-	-	-	-	+	+	+	+	+	+	+	-	-	++	-	-	+	+
E2	++	+++	+	+	+	+++	+	-	-	+++	-	-	-	+++	-	-	-	++
E3	++	+++	-	_	++	++	+	_	+	++	+	-	+	++	++	-	_	+

Cell layers of Hippocampus: Or: Oriens, Py : pyramidal cell layer, Rad:Stratum Radium, LMol: Molecular lacunosum, Mol: Molecular layer of DG, GrDg: Granular layer of DG

Reaction Intensity: - = no Reactivity, + = Light Reactivity, ++ = Moderate Reactivity, +++ = Intense Reactivity

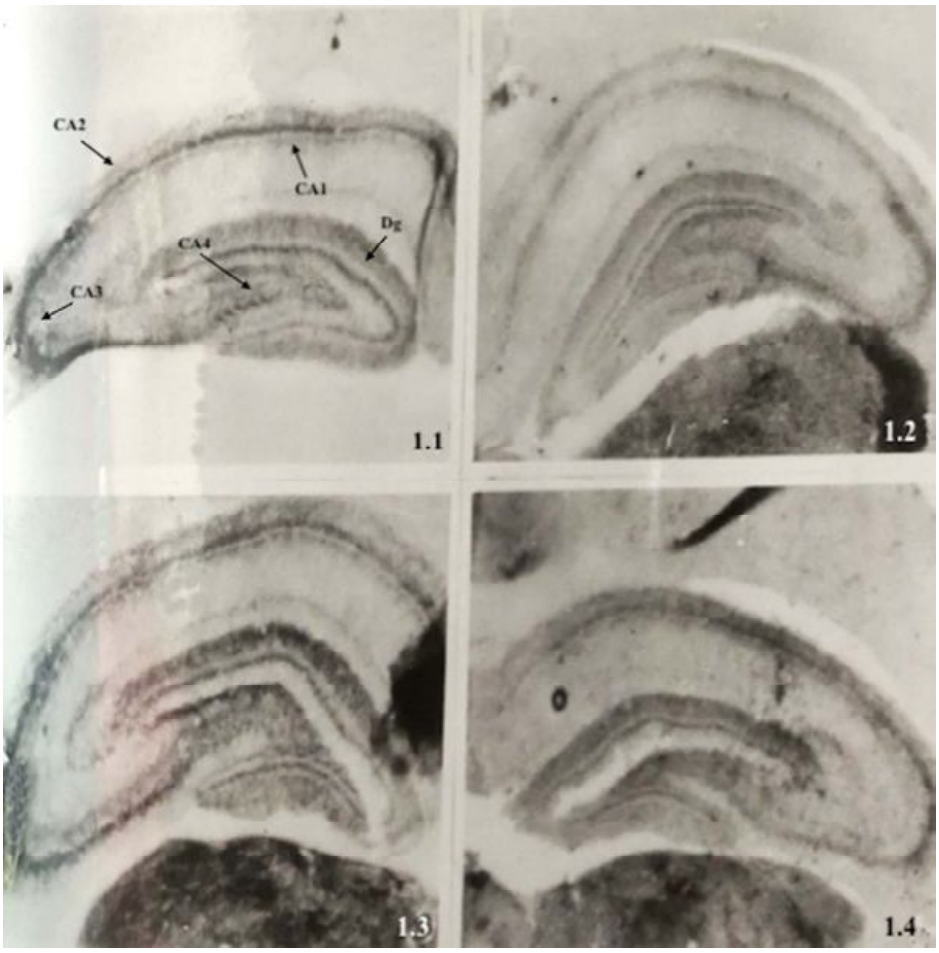

Figure 1: Photomicrographs depicting AChE histochemical reaction in Dorsal Hippocampus of Control and Experimental animals. (1.1) Control animals; (1.2) Stressed animals; (1.3) Only *Withania Somnifera* extract treated animals and (1.4) Stress and *Withania Somnifera* extract treated animals (X 4).

The results of the present investigation demonstrated the AChE reactivity in the stress (14 hrs immobilization) treated group was very less or negligible. In drug-treated (*Withania somnifera*) group, the reactivity was intense; in animals treated with stress and drug the AChE, reactivity was moderate, exhibiting a positive trend. Common to all groups, the stratum Oriens (Or) and molecular layer of Dg shows AChE positive Reactions. Stratum radiatum (Rad) shows very less reactivity in all groups; no reactivity was observed in lacunosum molecular layer (L mol). Interestingly there was no reactivity or negligible reaction was observed in all six layers of stress treated group. The maximum reactivity in all the layer was observed in the drug-treated group and is comparable to control group animals. Among

all the six-cell layers pyramidal cell layer exhibited historeactivity across all the groups. It was followed by stratum Oriens, Stratum radiatum and lacunosum molecular. In all experimental groups, the maximum reactivity was observed in CA3 in relation to various sub-areas of hippocampus and CA1, CA4, DG and CA2 followed this. These observations are in concurrence with the previously reported results where the regional difference in enzyme reactivity is reported.

There is no uniform pattern is reported for cholinergic neurotransmitters in the rat brain, which varies with strain and type of stressors *i.e.* increase, decrease or unaffected[7]. Among all the stressors, whether unpredictable or predictable, immobilization stress is considered as the most severe stress. As Hippocampus is under the influence of the HPA axis, it is suggested as an integral part of the adaptive response to various kind of stressors. Das *et al*.,[4] demonstrated that chronic immobilization stress may lower the AChE.

Findings of the present study indicate that stress in rodents is associated with extensive atrophy of a population of hippocampal neurons that moderate the functions of hippocampal neurons. In the present study, chronic immobilization stress decreased the AChE activity probably because of less availability of acetylcholine. Alzheimer's disease is also marked by the deficit in cholinergic neurotransmitters, as happens during stress, characterized by low choline acetyltransferase (ChAT), enzyme helpful in the synthesis of acetylcholine, levels[21].

Withania extract treatment increased the expression of AChE activity probably because of better availability of acetylcholine in the hippocampal subarea. Withania root extract has the potential to increase the expression of ChAT, enzyme helpful in the synthesis of acetylcholine[1]. Ashwagandha has neuroprotective action by acting on AChE and can be enhanced by binding with M1 receptor (Muscarinic acetylcholine receptors)[18]. Withanolide, a steroidal lactone, probably stimulates signalling pathway similar to steroids, especially estradiol, which exhibited neuroprotective action and memory (spatial and working) consolidation via cholinergic mechanisms[13].

The present study proposes neuroprotective properties of *Withania somnifera* owing to cholinergic alterations. *Withania somnifera* treated group significantly increased in AChE activity in stress treated animals, ultimately increased acetylcholine content. An increase in acetylcholine can be attributed to its neuroprotective effects and consequentially to the cognition-enhancing and memory-improving effects. It is quite possible that Withania extract might have delayed the degeneration process and still a possibility of returning to normal neurotransmitter homeostasis.

Present study exhibited restoration of AChE after *Withania somnifera* extract treatment on stressed animals. Withania can be an effective antistress agent and able to minimize the stress induced changes in brain specially in hippocampus. We do not claim but suggest that *Withania* can be explored for the treatment of a chronic age-related neurodegenerative condition such as Parkinson's and Alzheimer's disease.

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