Stress induced neuron degeneration and protective effects of *Semecarpus anacardium* Linn. and *Withania somnifera* Dunn. in hippocampus of albino rats: An ultrastructural study

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Effects of herbal formulations were studied on hippocampal neuron cell bodies. Study was carried out in adult Swiss albino rats. Experimental rats (E) were divided into three groups. Group E1 rats were given immobilization stress for 14 hr/day for 30 days. Rats in E2 and E3 group were given daily single dose (40 mg/kg/body wt.) of alcoholic extract of *S. anacardium* and *W. somnifera*. After 1 hr giving the plant extract, the rats were subjected to stress. Treatment continued for 14 hr for 30 days. Control rats were kept in complete nonstress condition. Ultrastructural characteristics of neuron cell bodies in hippocampal sublayer (CA1-CA4 and Dg) was studied in rats of E1, E2 and E3 groups and compared with control. Results of the present study demonstrated, that both CA2 and Dg, 85% of neuron cell bodies exhibited degenerating characteristics, (which includes karyorrhexis, membrane blebbing, chromatin condensation, chromatin fragmentation and intracellular spacing). Interestingly, after the treatment with *S. anacardium* cells demonstrating degenerating characteristics was significantly reduced (80%) as compared to treatment with *W. somnifera*. Study suggests that probably both the herbal drugs have cytoprotective properties.

Prolonged stress immobilization, heat, cold or swimming types are associated with neuron cell degeneration or neuron loss in hippocampal and other brain areas. Selye¹, Sapolsky et al.² and Sharma et al.³ have demonstrated stress related loss of pyramidal or granule cells in hippocampus and cerebral cortex area of rat brain. Neuronal degeneration in hippocampal sub regions of brain has been also reported by Olanow et al.⁴ in various neuropathological disorders i.e. Parkinson's, Alzheimer's, Amyotrophic lateral sclerosis etc. Sapolsky et al.⁵ demonstrated that during stressful situation, glucocorticoids are secreted which enhances the effects of neurotoxic insults. High glucocorticoid secretion accelerate age related loss of pyramidal neurons in hippocampus.⁶ Sloviter et al.⁷ demonstrated that chronic removal of glucocorticoids by adrenalectomy causes extensive damage to granule cells in dentate gyrus but prevent loss of pyramidal neuron cell bodies.

Recently antistress adaptogenic effects⁸ and cytoprotective effects⁹ in certain herbal preparations have been demonstrated. Sugaya et al.¹⁰ demonstrated neuroprotective action of TJ-960 (a herbal formulation) on pyramidal cells in CA1 of rats given cobalt stress.

Most of these reports available in the literature are biochemical or light microscopy histological studies. Ultrastructural evidences on antistress or cytoprotective effects of herbal drug on neuron cell bodies are lacking.

Hippocampus is known as seat of learning and memory, damage to hippocampus affects both the processes. It will be interesting to investigate that various drugs, which are used in memory enhancement and as adaptogens, in Ayurvedic system of medicine, might also, produce neuroprotective effects on the hippocampal neuron cell bodies.

In view of the above, present investigation was carried out to elucidate the ultrastructural changes in degenerating hippocampal neuron cell bodies of stressed rats and in hippocampus of rats, treated with extract of *Semecarpus anacardium* Linn. and *Withania somnifera* Dunn. Ultrastructural characteristics of neuron cell bodies in both the experimental groups and control group are presented.

**Materials and Methods**

*Animals—Thirty five adult Swiss albino rats used in the present study, (Body wt. 70 to 85 g) were...*
purchased from the J.L.N. Veterinary College, Mhow. After obtaining them from the supplier, rats were housed in polyurethane cages to acclimatize them to laboratory conditions. All the rats were given access to food and water, *ad libitum* and were maintained on 12:12 hr light/dark cycles at 27°C±2°C. All the animals were kept under these conditions for two weeks. Prior to the start of experiment, rats were divided into control and experimental groups.

**Control group**—Rats (N=5) were kept in pathogen free environment in isolated room. Room was locked for 24 hr. This was necessary to avoid stressful situation even of handling. After opening the room, rats were decapitated immediately. Tissues were dissected and fixed in 2.5% gluteraldehyde, prepared in Phosphate buffer (pH 7.2).

**Experimental group**—Rats (N=30) were divided into three experimental groups:
- E1: - Rats (N=10) were given 14 hr immobilization stress daily for 30 days.
- E2: - Rats (N=10) were given 14 hr immobilization stress for 30 days and simultaneously treated with *S. anacardium* extract (40 mg/kg/bdy wt).
- E3: - Rats (N=10) were subjected to same stress protocol for 30 days and were given *W. somnifera* extract simultaneously. (40 mg/kg/bdy wt.)

**Stress protocol**—All the experimental animals were subjected to immobilization stress and for this, rats were kept in tightly fitted ventilated plastic boxes for specific time period i.e. 14 hr daily for 30 days. Colonic/Rectal body temperature was measured immediately before and after the stress and other hyperactivities were also recorded for stress determination e.g. prostration, salivation etc.

**Drug preparation**—The nuts of *S. anacardium* were procured from the local supplier. Cut and dried nuts were purified by absorption method, keeping them in contact with brick powder for at least 10-15 days. After purification the nuts were packed in high quality of filter paper and successive solvent extract (chloroform, chloroform methanol, ethyl alcohol) was prepared by continuous extraction method in soxhlet extractor. After drying, the extract was dissolved in butterfat (ghee).

*W. somnifera*—root powder extract is available commercially as stresscom capsules (Dabur India Ltd.). Capsule consists of hydroalcoholic extract of ashwagandha root. This extract was standardized for withanolids and withnoles. Soya lecithin, bees wax and arachis oil was used as a base for medicament and packed in soft gelatin capsules. Root powder extract was dissolved in distilled water and given orally.

**Dose schedule**—All the groups received drug treatment between 10:00 A.M. to 12:00 Noon. Single daily dose of 40 mg/kg of body weight was given orally using feeding tube. Treatment was continued till 30 days and on 31st day rats were sacrificed.

**Tissue preparation**—Rats were sacrificed by decapitation, brains were dissected out. Hippocampus was separated under stereomicroscope and fixed in 2.5% gluteraldehyde at 4°C for 16 hr. After fixation the tissues were washed in 0.2 M phosphate buffer pH 7.2 at 4°C. After washing tissues were taken to the E.M. facility at Department of Anatomy, AIIMS, New Delhi. Tissues were rinsed again in 0.2 M phosphate buffer pH 7.2 at 4°C and postfixed in 1% osmium tetroxide for 100-120 min at 4°C.

**E.M. Procedure**—Later, tissues were rinsed for three times in 0.2 M phosphate buffer for 10-15 min. Subsequently tissues were dehydrated in acetone and cleared in toluene. Infiltration was carried out in 1:3, 1:1 and 3:1 ratio of embedding media and toluene for 1 hr each, respectively. Tissues were then infiltrated in pure embedding media for 1 hr. Then polymerization carried out at 50°C in pre set incubator for 14 hr. Tissues were then left for 24-28 hr at 60°C. Embedding media used for the preparation consisted of Araldite CY212, DDSA (Dodecynyl succinic anhydride), DMP-30 (Triimethyl aminomethyl phenol) and dibutyl phthalate in ratio of 10: 10: 0.4: 1. Blocks were trimmed and sectioned at the thickness of 0.5μm and stained with toluidine blue for light microscopy. Ultra thin sections of 60-90nm were cut on Richert ultra cut microtome (TM - 60) and picked on copper grids (100 mesh size). Ultra thin sections were stained with uranyl acetate and lead citrate. Sections were examined and photographed under PHILIPS CM - 100 electron microscope at an accelerating voltage of 80 KV (E.M. facility, AIIMS).

**Cell counting**—Cell counting was done in 10 photographs, taken from 5 different grids, of each group, at the magnification of 870X.

**Results**

Ultrastructural characteristics of pyramidal and granule cell bodies of hippocampal sublayers in control and experimental rats were studied.

**Control group** (Figs 1,4,7&10)—Stratum Pyramidal (Pyramidal cell layer, Py) the principal layer
Fig. 1—Control, demonstrating pyramidal cells of CA2 region, note the normal cytomorphology, with large nucleus. 870X; Fig. 2—E1. Demonstrating degenerating pyramidal cell bodies (D), with intracellular spaces (IS). 870X.; Fig. 3—E3, Rats given hydroalcoholic extract of W.somnifera. Note the large cell bodies with distinct nucleus and cytoplasm. 870X.; Fig. 4—Pyramidal cells of CA2 region of Control rat, demonstrating normal morphology with distinct nucleus (n), less electron dense cytoplasm, endoplasmic reticulum (E), and mitochondria (M) 2600X.; Fig. 5—E1. Degenerating pyramidal cell bodies (D) after stress treatment, exhibiting fragmented nuclei (n), swelled mitochondria (M) and electron dense cytoplasm. 2600X.; Fig. 6—E3, Rats given hydroalcoholic extract of W.somnifera, hippocampal cell bodies of CA2 region showing large nucleus (n), with less electron dense cytoplasm and distinct cell organelles. 2600X.
Fig. 7—Heart of 9 days old chick embryo (x 7). A: normal heart; B: abnormal large heart; Fig. 8—E1, Demonstrating degenerating granule cell bodies (D), with intracellular space (IS). 870X.; Fig. 9—E2, given alcoholic extract of S. anacardium. Note the large cell bodies with distinct nucleus and cytoplasm. 870X.; Fig. 10—Granular cells of Dg region of Control rat, demonstrating normal morphology with distinct nucleus (n), less electron dense cytoplasm, endoplasmic reticulum (E), and mitochondria (M). 2600X.; Fig. 11—E1, Degenerating Granular cell bodies (D) after stress treatment, exhibiting fragmented nuclei (n), swelled mitochondria (M) and electron dense cytoplasm. 2600X.; Fig. 12—E3, Rats given alcoholic extract of S. anacardium, hippocampal cell bodies of Dg region showing large nucleus (n), with less electron dense cytoplasm and distinct cell organelles. 2600X.
of ammon's horn (CA1-CA4) in hippocampus of the control rats exhibited ultrastructural characteristics of a typical neuron, which includes lightly electron dense cytoplasm, rich with mitochondria (M), large clusters of endoplasmic reticulum (E), free ribosome (R), which constitutes the nissl material. Nuclear chromatin (n) was for the most part uniformly dispersed with only limited condensation at the periphery and the cell membrane was intact.

The neuron cell bodies in dentate gyrus (Dg) also exhibited typical neuronal ultrastructural features with notable aggregation of large clusters of granular endoplasmic reticulum (E). The nuclei of these cells appeared oval or round in section with occasional shallow indentation. The chromatin was uniformly dispersed throughout the nucleus. Around the nucleus there was narrow rim of the cytoplasm containing the usual cellular organelles. The vesicle and cisternae of golgi apparatus (G) were widely distributed around the nucleus and in the dendrites. Mitochondria (M) were found uniformly distributed throughout the cytoplasm.

Group E1 (Figs 2, 5, 8 & 11)—30 days exposure to chronic stress demonstrated significant number of (85%) pyramidal cells (CA2) and granule cells (Dg) degenerated (D) in the hippocampal sub layers. Both (pyramidal and granular) cells were condensed and separated from the neighbouring cells, as intercellular spaces were increased. Nuclear chromatin (n) was found to be fragmented (Karyorrhexis) and nucleolus was disintegrated in the form of the cloud of coarse osmiophilic granules. Complete internal folds were apparent on the nuclear membrane. The cell transiently adopted a deeply convoluted outline.

Cells, which were in presumably more advanced stage of degeneration, showed cytoplasmic vacuolization. Electron dense cytoplasm consisted of few small-condensed mitochondria. The matrix of mitochondria became electron lucent and displayed focal flocculent densities. In CA3 and CA4 very few cells were found in degenerated state.

Group E2 (Figs 9 & 12)—Cell counting data showed that after the treatments with S. anacardium extract the number of degenerating cell bodies were significantly (80%) reduced (Table 1). Interestingly neuron cell bodies in granule cell layer of Dg were found more protected as compared to pyramidal cell layer.

Group E3 (Figs 3 & 6)—In animals treated with W. somnifera, neuron cell bodies in hippocampal sub regions in particular pyramidal cell layer of ammon’s horn (CA2) and granule cells (Dg) demonstrated neuroprotective characteristics. Cell counting data showed that 60% of cell bodies exhibited ultrastructural characteristics of typical neuron as

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<th>Group</th>
<th>Adjuvants used (Amount)</th>
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<tr>
<td>I</td>
<td>FCA/FIA</td>
<td>FIA</td>
</tr>
<tr>
<td>II</td>
<td>Alugel only</td>
<td>Alugel</td>
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<td>III</td>
<td>SPLPS + Alugel</td>
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<td>IV</td>
<td>Porin + Alugel</td>
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<td>V</td>
<td>SPLPS + Porin in Alugel</td>
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Fig. 13—Granule cells of Dentate gyrus (Dg) after W. somnifera treatment; Fig. 14—Pyramidal cells of CA2 region after the S. anacardium treatment.
Discussion

Present study provides the first ultrastructural evidence of neuroprotective properties in S. anacardium and W. somnifera. Though results demonstrate that the S. anacardium have more cytoprotective effects as compared to W. somnifera.

In present study long-term immobilization stress produced significant neuron cell degeneration in both pyramidal (CA2) and granule cells (Dg) of hippocampal subregions. Light microscopic studies, conducted in our laboratory showed presence of significant number of dark cell bodies in both the regions. Ultrastructural characteristics of these cells studied in the present report showed condensation of nuclear chromatin, nucleolus disintegration, and reduction in nuclear size, compaction of various cytoplasmic organelles, mitochondria, golgi body and dilation of endoplasmic reticulum. At low magnification cells appeared as they were separated from each other, as intracellular spaces were apparent, highly electron dense cytoplasm was found accumulated at the periphery, and the membrane blebbing was observed.

After treatment with the extract of W. somnifera or S.anacardium, number of degenerating cell bodies (dark cells) in pyramidal (CA2) and granule cell layer (Dg) were significantly reduced. It was interesting to note that S.anacardium exhibited more cytoprotective effects on dentate gyrus neuron cell bodies, while W.somnifera on pyramidal neuron cell bodies of CA2 region. In both cases most of the neuron cell bodies in pyramidal cell layer and granule cell layer demonstrated ultrastructural characteristics of a typical healthy neuron cell body. Which includes electron dense cytoplasm rich with mitochondria, rough endoplasmic reticulum, and free ribosomes, nuclear chromatin was uniformly dispersed with limited condensation at nuclear membrane margin. Finally, cell membrane appeared to be intact.

Neuroprotective activity of both the plants S. anacardicum and W. somnifera observed in this study is strictly a ultrastructural morphological description and it is not possible to elucidate the mechanism of degeneration and possibly the reversal.

The most likely biochemical explanation for degeneration of cell bodies associated with immobilization stress, is increase in corticosteroid level. Stress itself produce cellular oxidation due to generation of free radicals. As hippocampal cells are significant target tissues for glucocorticoids, it is assumed that free radicals might have exacerbated the glucocorticoid toxicity, which possibly lead to neuronal degeneration.

Both the drugs W. somnifera and S. anacardium were found effective in reducing the glucocorticoid level after the stress treatment and simultaneously both the drugs have shown significant free radical scavenging activity in stress treated animals (Personal communication). Here, it may be hypothesized that glucocorticoid modulating and free radical scavenging activity of these drugs go hand in hand, in preventing the stress induced neuronal loss.

The ultrastructural characteristics observed in degenerating cells suggests close similarities with apoptotic characteristics shown earlier. Nitatori et al. demonstrated degenerated cells in CA1 pyramidal cell layer of hippocampus after the transient ischemia and exhibited apoptotic characteristics.

Cytoprotective properties of herbal formulation have been reported in literature but the cellular evidences are lacking. Sugaya et al. demonstrated the cytoprotective action of T.J. 960 (a herbal formulation) against cobalt toxicity on hippocampal cell bodies.

In conclusion, the present study demonstrates that chronic immobilization stress elicits the cellular damage in hippocampus and this brain damage after stress treatment may lead to permanent neurological deficits. In addition to this, present study also demonstrates that both indigenously available herbal drugs have neuroprotective and antistress activity. Possibly plant products may be useful in controlling stress-related elements in human beings.

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References