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Research Article

IMMUNOHISTOLOGICAL STUDY ON BASAL NUCLEI TREATED WITH ETHANOLIC EXTRACT OF WITHANIA SOMNIFERA AND WITHANOLIDE A IN HUNTINGTON'S RAT MODEL

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A B S T R A C T

Neurodegenerative disorders include a wide array of nervous system related disabilities like Alzheimer's, Parkinson's and Huntington's disease, and are closely knitted with age. With the present scenario they are incurable and progress negatively that makes the management of the person a nightmare.

Free radicals always play big roles in nerve cell degeneration. The good old herb Withania somnifera (Queen of Ayurveda) was widely proved for its free radical scavenging ability and neuroprotection for ages in India. As prevention is better than cure, this herb drawn our attention towards a study in neuroprotection in experimental rat models.

We used 4 animal groups for this basic study. They were CO, LC, WS125 and WA100. We created a stereotaxic Huntington's rat model by inducing kainic acid bilaterally into the striatum. The rats were treated with drugs ten days prior and after lesion surgery to analyze the preventive and regenerative ability of the drugs as few studies claimed regeneration even in distinctive regions of adult rat brain. On tenth day we collected the brain samples, immunostained them and counterstained with hematoxylin to give a good contrast. The results were amazing, shown both neuroprotection and new cell formation in striatum.

INTRODUCTION

The nervous system is the controlling center of our body and the neurons and glia are forming the building blocks of it. Normally neurons don't reproduce or replace themselves so when they are damaged or die they cannot be replaced by the body. Degeneration of neurons is the prime cause of any neurodegenerative disorder and is a normal process after 40 years. Regeneration of neurons in the central nervous system is always a controversial topic as the neurons are generally considered as non-regenerative.

Neurodegenerative disorders are the most common disorders of old age and affect millions of people worldwide. As there is no cure for this disorder and is mainly involving with day today activities in old age, it is a boon to mankind if we find a way to regenerate the lost neurons or to prevent or slowdown the degeneration of neurons.

Degenerative nerve diseases are life-threatening and managing dementia is breathtaking. Huntington's disease (HD) is one such neurodegenerative disorders that lead to progressive cell death mainly in the striatum, a part of the basal ganglia. At present there is no cure for HD, and full-time care is required in later stages of this disease (Pollard, 2003).

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As the disease advances, uncoordinated, jerky body movements become more apparent, along with a decline in mental abilities.

Withania somnifera also known as Ashwagandha is called as the queen of ayurveda, has long been used in the traditional Ayurvedic system of medicine to enhance memory and improve cognition (Choudhary, 2017). Prakash *et al* (2013) evaluated the neuroprotective effect of *Withania somnifera* root extract on Parkinsonian mice and proved its protective role. The active principle of *Withania somnifera* the Withanolide A was well known for its regenerative properties (http://withaniasomnifera.com). Dhalla *et al* (1961) registered the ability of *Withania somnifera* in regeneration of axons and dentrites. Dongre *et al* (2015) conducted a pilot study on adult females and concluded that Withania somnifera improved the body's physical and psychological condition. Kumar *et al* (2017) studied the active principles of Withania somnifera and concluded that the phytochemicals confers neuroprotection by selective inhibition of neuronal nitric oxide synthase.

As neurodegeneration characterized by progressive degeneration of the structure and functions of the central nervous system it will create social and financial burdens to millions of people worldwide and rise dramatically with age (Gendelman *et al,* 2015). At present there is no cure for such disorders that stimulated us to supplement some herb to play the role as neuro-protector in a regular fashion. As the herb *Withania somnifera* and its active principle withanolide A

were in talk with neuroprotection and nerve cell regeneration we decided to employ the extract of *Withania somnifera* and its active principle withanolide A in neuroprotection of striatum in a rat model and analyzed the results in the light of Immunostaining.

MATERIALS AND METHODS

Equipments

1. Stereotaxic frame – INCO-AMBALA.
2. Microtome - INCO-AMBALA.
3. Dental micromotor hand drill
4. Coplin jars
5. Vectastain ABC elite kit from VECTORS LAB - Japan

Ethanolic Extract of Withania Somnifera:

The ethanolic extract of *Withania Somnifera* was prepared by soxhletion method following Elayaraja *et al* (2010) and the IP dosage was adjusted to 1ml for each animal.

Withanolide A

Withanolide A was purchased from Sigma FLUKA-USA. The I.P. dosage of this drug was prepared by dissolving the drug in a few drops of methanol (SIGMA-ALDRICH) and then in normal saline and the volume was adjusted to 1ml for each animal (Nakatonmi *et al,* 2002).

Animals

Adult male Sprague Dawly rats (200–250gm) were housed under standard laboratory conditions and maintained in compliance with strict institutional guidelines. The room environment was maintained at 20º ± 2º; alternating 12 h light–dark cycle with food and water *ad libitum*. The total experimental design was approved by the Institutional Animal Ethical Committee. Maximum effort was taken to minimize the unwanted stress to the animals and to reduce the number of animal to be used for this study.

For this study we divided the animals into 4 groups with 6 animals each (Table-1). Group 1 had control animals. Group 2 had lesion control animals. Group 3 had WS 125mg drug treated animals with lesion. Group 4 had WD100µg drug treated animals with lesion. The drug group animals were given the specific volume of drugs for 10 days prior to lesion surgery, on the day of surgery and till the end of the study.

Table 1 Animal Groups

Animal groups	Control (CO)	Lesion control (LC)	WS 125	WA100
Lesion surgery	NO	YES	YES, WS 125mg treatment before and after surgery	YES, WA100µg treatment before and after surgery

Table showing the animal groups and drugs used in this study
WS 125mg - Withania somnifera ethanolic extract 125mg/kg body wt.
WA100µg - Withanolide A 100µg/kg body wt.

Lesion surgery was performed with the aid of stereotaxic frame by injecting 0.5µl of kainic acid into the striatum of animals bilaterally.

Analysis of neuroprotection and new cell formation in basal ganglia

Aim: To stain the newly formed cells in striatum and to analyze the area of lesion.

Methodology: BrdU Immunostaining by Vectastain ABC elite kit- counterstained with hematoxylin.

5-Bromo Deoxy Uridine (BrdU) administration

Principle: BrdU is a uridine derivative and a structural analog of thymidine, is a common reagent used for cell proliferation assays to detect the newly formed cells. It can be incorporated into DNA during the synthesis-phase of the cell cycle as a substitute for thymine, thereby serving as a marker for proliferation. Cells marked by BrdU incorporation may be detected by multiple detection methods using enzyme-linked anti-BrdU antibodies.

BrdU incorporation procedure

BrdU was dissolved in saline. From that 1ml was given to the animals intraperitoneal at a concentration of 50mg/kg body weight / day, on 3rd, 5th, 7th and 9th days after lesion surgery.

On 10th day we deeply anaesthetized all animals with double dose of Pentothal sodium, ran intracardially with 0.9% NaCl and perfused with Somogyi fluid.

Then we collected the brain samples, sliced and incubated 1-3hrs at room temperature in Somogyi fluid, rinsed and kept in 0.1M PBS at pH7.3 for 1 hr.

Processing and Embedding

We followed standard processing and embedding techniques and dried the sections at 37˚C overnight.

Immunostaining reagents

A number of buffers can be used in VECTASTAIN elite ABC system. One of the most common is 1X PBS (10 mM sodium phosphate, pH 7.5, 0.9% saline). The VECTASTAIN working solutions are

1. Blocking serum
2. Primary Antibody Dilution Buffer
3. Biotinylated antibody
4. VECTASTAIN elite ABC reagent
5. Diaminobenzidine Tetrahydrochloride (DAB)

Immunostaining procedure

For staining procedure we used humidifying chambers, coupling jars and staining boxes. The procedure follows

1. Deparaffinized the sections using xylene
2. Sections were hydrated in graded alcohol
3. Rinsed for 5 min in tap water
4. Incubated 30 min in 0.3% h2o2 in water
5. Washed in buffer for 5 min
6. Incubated 20 min in normal blocking serum- blotted the excess serum
7. Incubated 1hr in primary antibody diluted in buffer
8. Washed 5 min in buffer
9. Incubated 30 min in biotinylated secondary antibody
10. Again washed 5 min in buffer
11. Incubated 30 min in VECTASTAIN ABC elite reagent
12. Washed 5 min in buffer

13. Incubated in DAB for 2-10 min
14. Rinsed in tap water
15. Cleared and mounted with DPX and then viewed under the light microscope

RESULTS

We produced an equivalent animal model of Huntington's Chorea, a neurodegenerative disorder by this study as the lesion inducing agent was an excitatory neurotoxin. The histology of lesion control group animals showed a very larger lesion on 10th day and the lesion was significantly larger when compared with other group animals (figure-1).

Figure showing the histology of Lesion Control rat striatum stained with Immunostaining

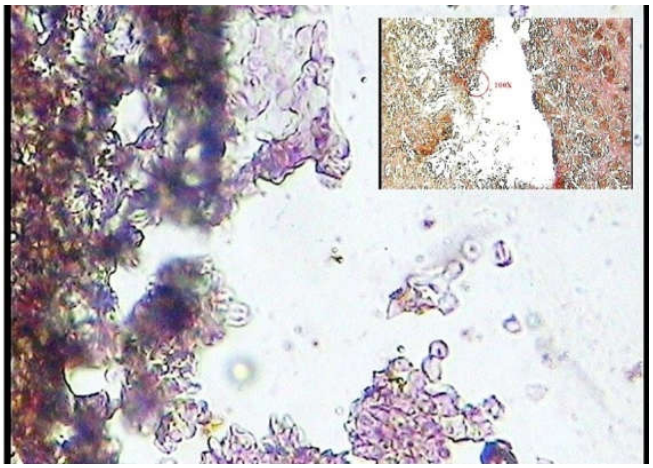

Figure1 VECTASTAIN ABC elite kit counterstained with Hematoxylin

*Main figure -100X magnification
*inside figure – 10X magnification

The striatum of WS125 group animals showed a significantly smaller lesion with larger number of newly formed brown colored cells started covering the area of lesion **(figure-2,3)**.

Figure showing the histology of WS125 rat striatum on 10th day after lesion, stained with Immunostaining in 10X

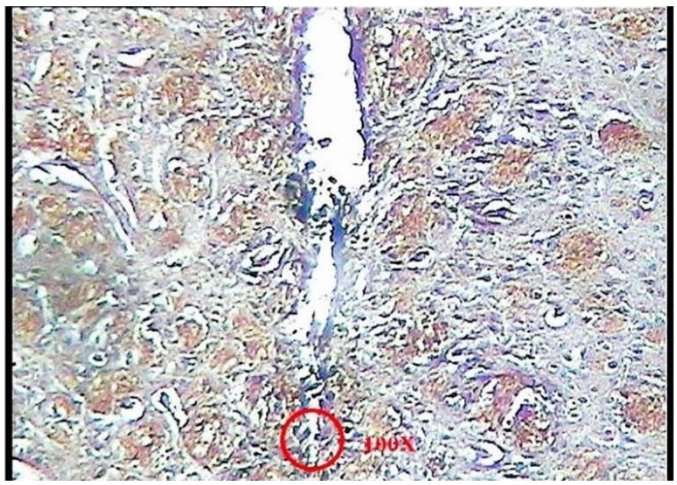

Figure 2 VECTASTAIN ABC elite kit counterstained with Hematoxylin.

*10X magnification

Figure showing the histology of WS125 rat striatum on 10th day after lesion, stained with Immunostaining in 100X

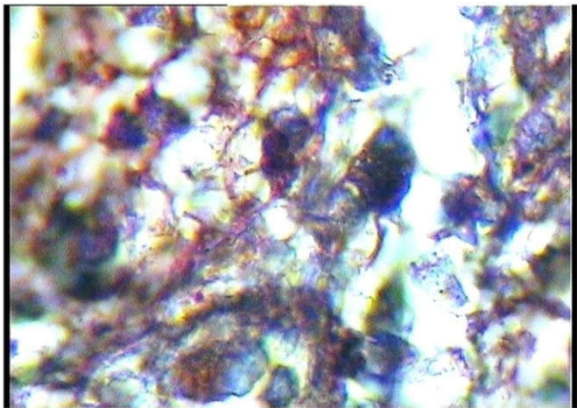

Figure 3 VECTASTAIN ABC elite kit counterstained with Hematoxylin.

***100X magnification**

The histology of animal groupWD100 showed a very small lesion with large number of newly formed brown cells that covered the lesion almost and formed connections with the nearby neurons **(figure-4,5)**. The above said results shown both the drugs were very good in neuroprotection and stimulation to form new cell that can be used well against neurodegeneration.

Figure showing the histology of WD100 rat striatum on 10th day after lesion, stained with Immunostaining in 10X

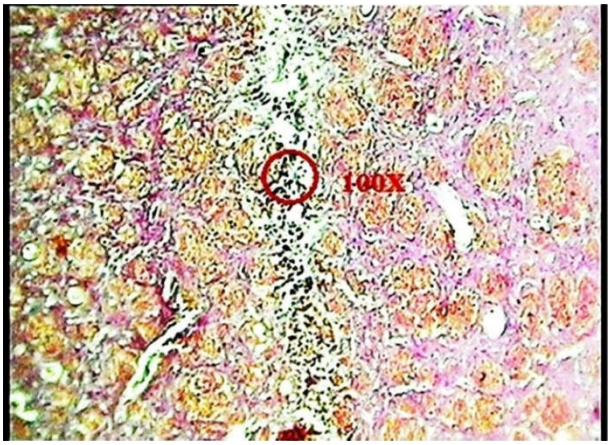

Figure 4 VECTASTAIN ABC elite kit counterstained with Hematoxylin.

***10X magnification**

Figure showing the histology of WD100 rat striatum on 10th day after lesion, stained with Immunostaining in 100X

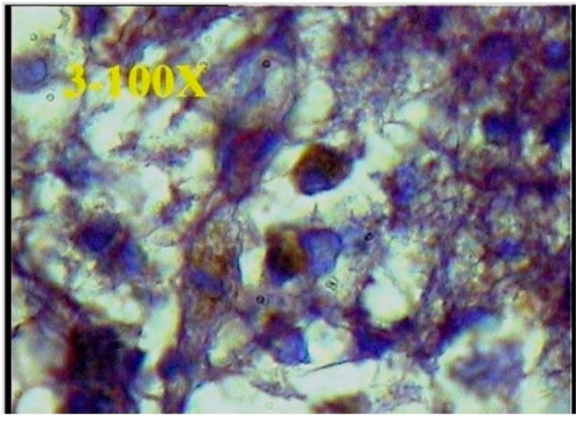

Figure 5 VECTASTAIN ABC elite kit counterstained with Hematoxylin.

*100X magnification

Some studies showed the regeneration of nerve cells in the hippocampal region and the subventricular zone (Nakatomi *et al*, 2002; Sani *et al,* 2015) even in adult human. According to Nakaguchi *et al* (2011) new neurons are continuously generated by endogenous neural stem cells in the subventricular zone (SVZ) of the adult mammalian brain, including the human brain and some of these new neurons migrate to the injured striatum and differentiate into mature neurons, such new neurons may be able to replace degenerated neurons and improve or repair neurological deficits.

In this present study normal histological pattern was observed with CO animals. In LC group the lesion was very much larger, in higher magnification cell debris and degenerating neurons were still found inside the lesion. The brain samples of WS125 showed a good amount of brown colored new cell with fibers. In WD100 striatum samples the newly generated cells almost covered the lesion area, formed fibers and established connections with the nearby cells. There were new cells in the lesion area, large enough to compare with the neurons present in the other areas.

DISCUSSION

In LC samples newly generated cells and fibers were not found in the region of lesion. Histology of WS125 samples showed only small lesion in the striatum with a good number of newly generated cells on the site of lesion which was confirmed by immunostaining. The samples belonging to WD100 showed only a very small lesion almost closed on 10^{th} day by the newly generated cells that also was confirmed by immunostaining. We also analyzed the margins of lesion belongs to WS125 and WD100 in higher magnification and found they were totally different from the lesion margins of LC samples. Only cell debris and degenerating nerve cells were found along the lesion margins of LC samples, in higher magnification on 10^{th} day. Fewer number of glial cells were found but the lesion was still persisting as it was vast. But in both the drug groups the cells were still viable along the margin of lesion and the histo-morphology also looking very much normal. This clearly stated both the drugs WS125 and WD100 were very good in neuroprotection and stimulated the formation of new cells.

The newly formed brown colored cells were carefully analyzed in the light of literature to separate them into nerve cells and glial cells (Ludwig and Bhimji, 2015). A good number of new cells formed in the area of lesion were large with large nucleus with large amount of cytoplasm and with multipolar appearance (Zhang and Fenderson, 2015). They were much different from glia that looked small with heterochromatin granules in nucleus and with less cytoplasm.

From the above said results it is understandable that both the herb *Withania somnifera* and its active principle Withanolide A are very good in protection and even stimulated the striatum to produce new cells. Withanolides as per theory occupies the receptor sites in the cell membrane and prevent the attachment of deleterious components and protect the cells (Tiwari *et al,* 2014), and the results were seen through the above immunostained sections. As like all the other researchers we do have controversial ideas regarding regeneration of nerve cells (Naegele, 2018), but the results what we got made us think about. Else we can put it in this manner, new cells may be generated in critical conditions under the stimulation of certain agents but they fail to make proper connections with the particular tract leads to its failure. As prevention is always better than cure, we can definitely utilize this herb and its active principle along with our daily food or drink like tea, in minimal quantity as a supplement to protect our nervous system for a healthy living.

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