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Association of Cell Viability in Huntington Chorea Rat Models and the Neuroprotective Role of Withania Somnifera in Public Health

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Abstract

Huntington's disorder (HD) is a genetic, fatal neurodegenerative disorder that causes abnormal, involuntary jerky movements, postures and defects in cognition, mood and behaviour. Huntington's disease involves with degeneration of neurons in basal nuclei and so has no cure like other neurodegenerative disorders and the affected person struggles in life for 15-20 years. The basal nuclei are involved in the inhibition of unwanted motor activities and so responsible for fine motor movements. Loss of these neurons will inflict on the quality of life of the person. Neurodegenerative disorders are almost a compulsory evil when a person is ageing. As nerve cell division and regeneration are impossible, we planned to analyze the possibility of protecting the neurons already in action by pretreating them with Withania somnifera, a traditional herbal plant, well known for its neuroprotective role. We selected 4 groups of experimental rat models, treated prior with the crude extract of Withania somnifera and the active principle withanolide A. On analysis, large lesion and dead tissue mass were found in the brain samples without pretreatment. Wherein brain samples with pretreatment, the lesion was minimalized and so was the number of dead cells. By analyzing the actual cell death, we analyzed the protective role of the plant extract and the active principle we employed. As neurodegeneration is a sure process in age and regeneration is a question unanswered, prevention or delaying nerve cell death is a need in the present community health care that was achieved by this study.

Keywords: *Basal Ganglia, Huntington's Rat Model and Neurotransmitter, Striatum, Withania Somnifera, Withanolide A.*

Introduction

HD is a fatal autosomal dominant disorder commonly affecting middle-aged people with movement and behaviour [1, 2], but may affect any age group. As a neurodegenerative disorder, it is not reversible and people affected by it must depend on the caregiver for the remaining days of life. As it affects the basal ganglia [3], the person affected will express abnormal jerky dance-like movement [4], and postures along with mood swings and memory deficit. This will invariably affect the quality of life of a person and lead to other clinical complications and even suicide [5].

Research says the global prevalence of HD is around 2.7 per 100,000 people with a high occurrence in Australia, Europe and North America (3-7 per 100,000) [6] and 1.75 per 100,000 among the Indian immigrant population in the UK. [7]. No recent evidence to prove the prevalence of HD among the Indian Population but it is been stated, likely to be 3-7 per 100,000 in like European population. Being a heavily populated country the impact of this rate is heavy on India than any other

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country in the world [8]. Considering the worst-case scenario that there is no cure for HD and is a genetic transmittable disorder, it is undeniable to find an alternative to HD like prevention to improve the health care of the people affected and is very much needed in a heavily populated country like India.

According to World Health Organization (WHO), 80 percent of people around the world are using herbs for some healthcare aspects. Jain (2001) [9], studied the neuroprotective effects of Withania somnifera stress com capsules (Dabur Ltd., India) on immobilization stress-induced female adult Swiss rats. Based on the histological observations he concluded that medication with root extract of Withania somnifera largely reduced the degenerating nerve cells in the CA2 and CA3 regions of the hippocampus. Withania somnifera otherwise known as ashwagandha, poison gooseberry, winter cherry or Indian ginseng is a plant of the nightshade family, also called as "Queen of Ayurveda" and is a very important plant in Ayurveda, the Indian traditional medicine [10]. According to Dhalla *et al*., (1961) [11], Ashwagandha induces regeneration of axons, dentrites and synopses. According to Suganya *et al*., (2010) [12], the Withanolide A is an important secondary metabolite in the dry root of Withania somnifera, which has potent anti-tumor and antioxidant properties. According to Parihar and Hemnani (2004) [13], oxidative stress and excitotoxicity are the basics for nerve cell death and Ashwagandha Withanolides were found to have the property of nerve cell regeneration and synapse formation in laboratory experiments (*withania somnifera*.com).

We have formulated this present study, which is a pioneer in research to explore the protective role of Ashwagandha and its Withanolide A in preventing nerve cell death on experimentally induced Huntington's rat models and so to conclude its role in preventing HD on the offspring of HD patients or at least delaying neurodegeneration to provide a little push in health care and quality life for people affected by HD.

Materials and Methods

Animals

We used SD male rats of 200–250gm for this study, strictly maintained in compliance with institutional ethical guidance. The experiment was fully approved by (IAEC/XIII/11/CLBMCP/2008-09) CPCSEA.

Animal groups

A basic study was conducted by surgically creating experimental Huntington's chorea rat models using kainic acid excitotoxin [14]. Then using the biochemical parameters (analyzed within 24 hours of lesion (Table 1), the working effective dosage of the selected drug was found. Based on the biochemical parameters the effective dosage was confirmed as WS 125 mg for ethanolic extract of withania somnifera and WD 100µg for withanolide A.

Table 1. Groups of Animals used for Biochemical Study for this Present Study 4 Groups of Animals were Selected with 6 Animals in Each Group (Table 2), Based on the Basic Study of Biochemical Parameters

S.No	Animal groups	Lesion surgery	Pre and post treatments of WS	Pre and post treatments of WD
1	SC	NOT	NOT	NOT
2	Lesion control-LC	Yes	NOT	NOT
3	WS 100 mg	YES	YES	NOT
4	WS 125 mg	YES	YES	NOT
5	WS 150 mg	YES	YES	NOT
6	WD 5 µg	YES	NOT	YES

7	WD 50 µg	YES	NOT	YES
8	WD 100 µg	YES	NOT	YES

Table 2. Animal Groups Used for Cell Viability Analysis

S. No	Groups of animals	Lesion	Pre and post treatments of WS	Pre and post treatments of WD
1	Sham (SC)			
2	Lesioncontrol (LC)	YES	NOT	NOT
3	WS 125mg (WS 125)	YES	YE	NOT
4	WA100µg(WD100)	YES	NOT	YES

Materials

The plant Withania somnifera was authenticated by the Plant Anatomy Research Centre, National Institute of Herbal Science, Chennai. A phytochemical analysis was also performed to confirm the presence of sugar, steroids, terpenoids and proteins in the ethanolic extract. The total ash was found to be 6.29% W/W.

Ethanolic extract of *Withania somnifera* was prepared by soxhletion following Elayaraja et al., 2010 [15]. A course powder of Withania somnifera was taken in a simple and placed in a condenser. Ethanol was added to the powder and exhalation was carried out for 10 to 12hrs. Later the solvent will be collected in turbovial, preconcentrated and dried using turbovap. We can get 1.25 to 1.5 gms of ethanolic extract of Withania somnifera from 100 g root powder.

The active principle Withanolide A was purchased from Fluka – Sigma USA and the excitatory neurotoxin Kainic acid was purchased form Caymen chemicals – USA, dissolved in 0.9% NaCl [16].

For this study live cell exclusion technology was adopted and was done by trypan blue staining.

Trypan blue is a basic stain that penetrates the compromised cell membrane [17]. Because Trypan blue only stains the dead cells and leave the live cells, by using this technique we easily quantify the amount of live cells and also can find out the effects of drugs in preventing the neuronal damage.

Procedure

The procedure, live cell exclusion technology was done on the 3rd day of experimental lesion surgery for Huntington's chorea model. All the animals have undergone a lesion surgery on a stereotaxic frame by injecting 0.5 microliters of kainic acid into the rat striatum except SC who received 0.5 microliters of normal saline instead of kainic acid.

On the day of surgery, the animals were taken under anesthesia and placed in the stereotaxic frame. The previous suture was removed with care following SOP. Then the animals were injected with 2 µl of 1% trypan blue in saline at the same coordinates of experimental lesion surgery [18] with care by using a Hamilton syringe. The scalp was re-sutured following SOP.

Animals were sacrificed after 24 hours, ran with normal saline and perfused in 4% formaldehyde in saline. The brains of the animals were removed, processed by routine

paraffin technique, cut and counterstained with eosin for non-Trypan blue-stained cells.

Results

The histology of SC group brain samples showed a small mass of dead tissue stained with eosin, without cell structure. The peripheral region of the dead tissue mass was surrounded with dead cells stained with trypan blue with intact cell structure (Figures 1, 5, 9).

The histology of the LC group shown (Figures 2, 6), a very large area of lesion with dead tissue mass when compared with SC animals and was surrounded by dead cells stained by trypan blue vital staining. In WS125 brain samples (Figures 3, 7), the dead tissue mass was less and also the cells stained with trypan blue that shown the protective nature of the drug employed. But for WD100 animals the dead tissue mass and the surrounding dead cells were minimal which clearly proved that the drug protected the striatal neurons from degeneration (Figures 4, 8, 10).

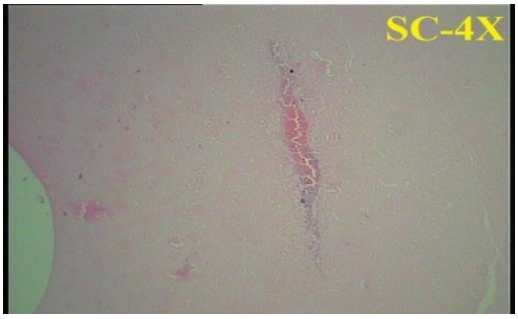

Figure 1. Histology of SC Rat Striatum Showing the Size of Lesion Stained with Trypan Blue and Eosin in 4X

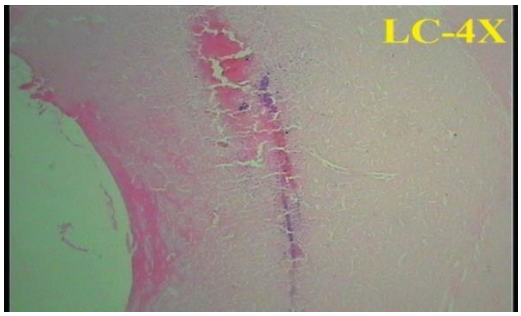

Figure 2. Histology of LC Rat Striatum Showing the Size of Lesion Stained with Trypan Blue and Eosin in 4X

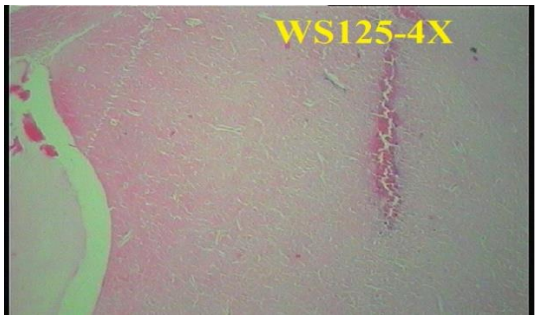

Figure 3. Histology of WS125 Rat Striatum Showing the Size of Lesion Stained with Trypan Blue and Eosin in 4X

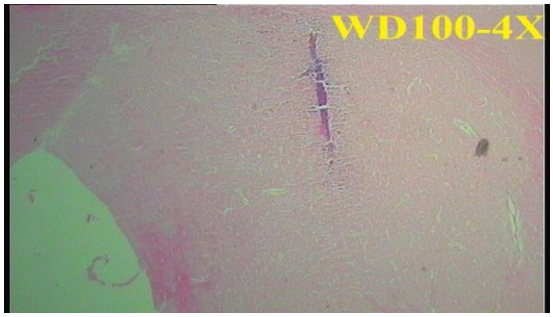

Figure 4. Histology of WD100 Rat Striatum Showing the Size of Lesion Stained with Trypan Blue and Eosin in 4X

Figures 1, 2, 3, 4 – Comparison of Lesion Between Histology of SC, LC, WS125 and WD100 Rat Striatum Stained with Trypan Blue and Eosin in 4X

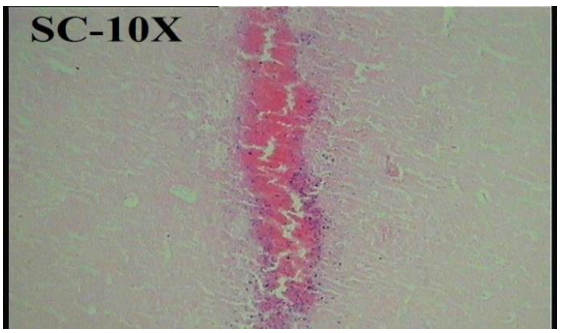

Figure 5. Showing the Level of Trypan Blue Stained Dead Cells Spreading Around the Lesion in SC Group Striatum Under 10X

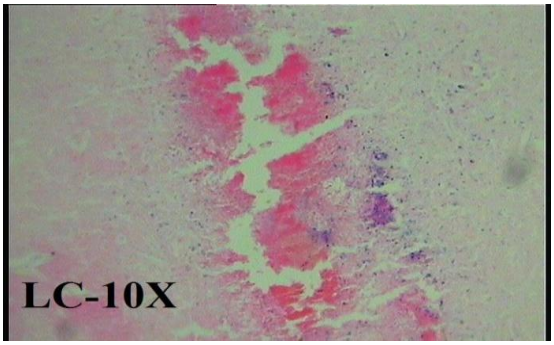

Figure 6. Showing the Level of Trypan Blue Stained Dead Cells Spreading Around the Lesion in LC Group Striatum Under 10X

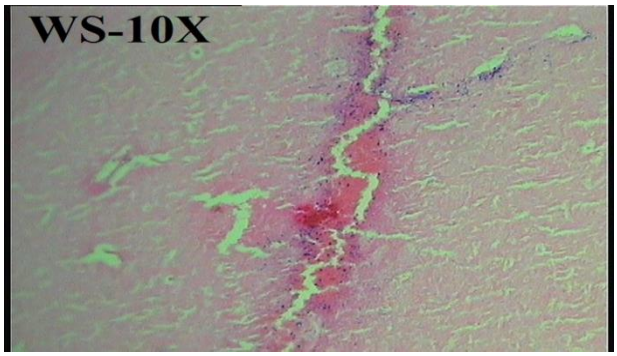

Figure 7. Showing the Level of Trypan Blue Stained Dead Cells Spreading Around the Lesion in WS Group Striatum Under 10X

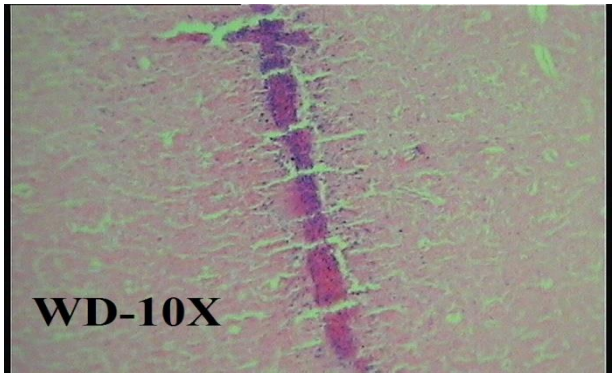

Figure 8. Showing the Level of Trypan Blue Stained Dead Cells Spreading Around the Lesion in WD Group Striatum Under 10X

Figures 5, 6, 7, 8 – Comparison Between SC, LC, WS125 and WD100 Rat Striatum on Trypan Blue Stained Dead Cells Spreading Around the Lesion in 10X

Photographs Were also Taken (Figures 9,10) in Higher Magnification to Differentiate Between Dead Cells and Dead Tissue Mass Stained by Trypan Blue and Eosin.

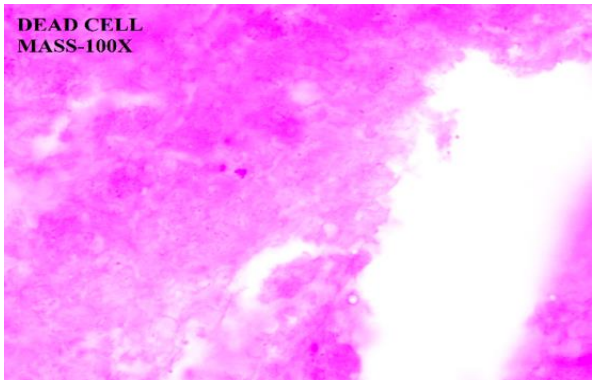

Figure 9. Showing the Dead Tissue Mass in LC under 100X, Stained with Trypan Blue and Eosin

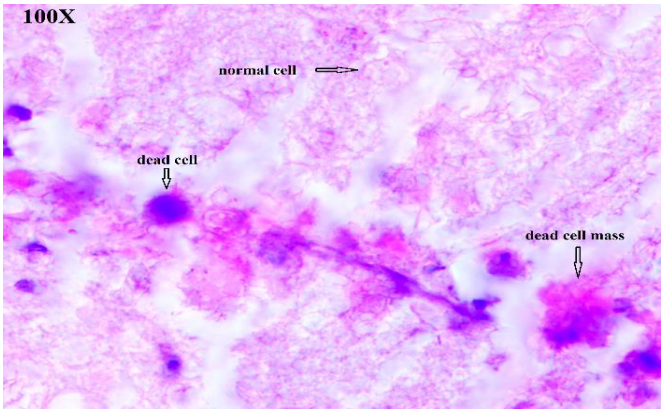

Figure 10. Showing the Difference Between the Dead Cells, dead Tissue Mass and Normal Cells in 100X, Stained with Trypan Blue and Eosin

Sections were also Photographed with h & e Staining to Compare the Level of Lesion within Groups and to Support Trypan Blue Staining Technique (Fig. 11, 12, 13, 14)

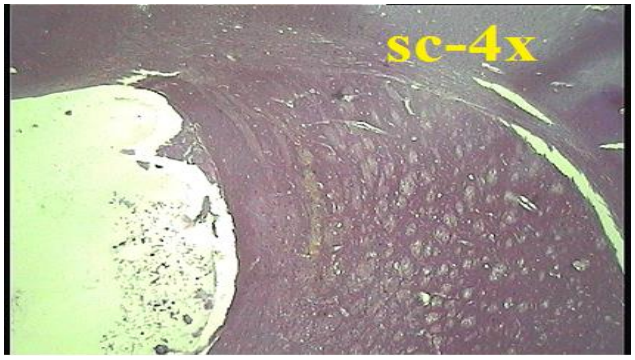

Figure 11. Histology of SC Rat Striatum Stained with H & E in 4X

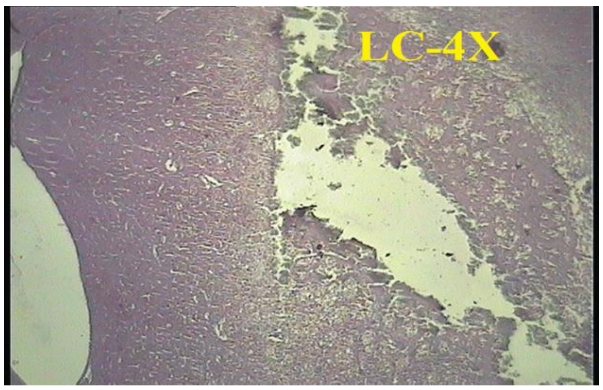

Figure 12. Histology of LC Rat Striatum Stained with H & E in 4X

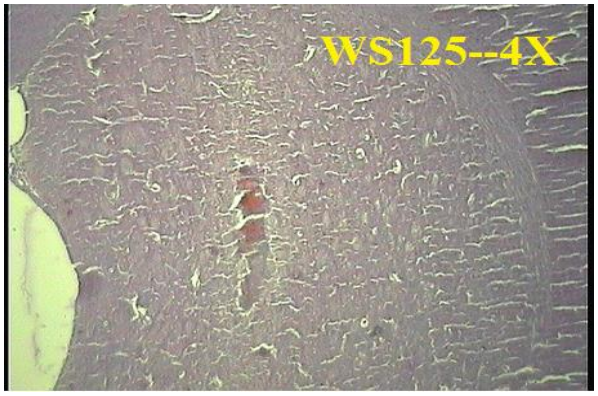

Figure 13. Histology of WS125 Rat Striatum Stained with H & E in 4X

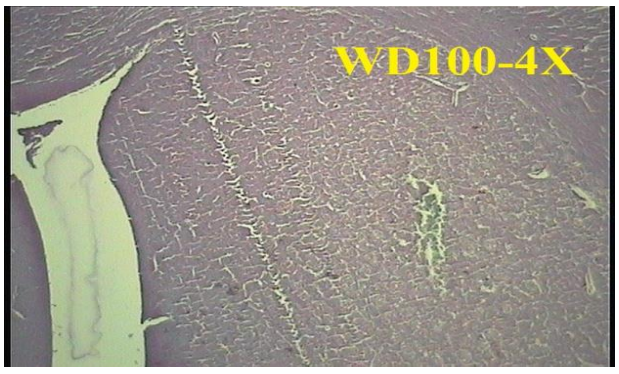

Figure 14. Histology of WD100 Rat Striatum Stained with H & E in 4X

Figures 11, 12, 13, 14 - Showing the Comparison Between the Histology of SC, LC, WS125 and WD100 Rat Striatum Stained with H & E in 4X

Discussion

Animal studies are a must in health care because it is not possible to find several vaccines and drugs to fight diseases like HIV, Alzheimer and Hepatitis, without animal research and they yield more or less equal results like humans do.

In this present study, a model of HD was made in rats that prior fed with both crude extract and the active principle to be analyzed the withanolide A, to analyze the neuroprotective role of the plant in animals and so in human health care. To analyze the neuroprotective role of the plant extract, the striatal histology of the animal model was analyzed with Trypan blue staining. Trypan blue is a stain that works under the principle of live cell exclusion technology and will stain the intact proteins in a cell. As trypan blue is a negatively charged dye it cannot pass through the negatively charged live cell membrane but can cross through the compromised cell membrane and stain the proteins inside the cells Jessop *et al.,* 2018 [19]. Crowley *et al.,* (2016) [20], analyzed cell death using trypan blue with the help of a light microscope and confirmed only dead cells with intracellular proteins are taking trypan blue stain. Though it is a stain for dead cells, will not stain the dead cells with denatured proteins and so the dead cell mass. This technique was used in this present study to compare the spread of dead cells and dead tissue mass between the four groups SC, LC, WS125 and WD100 (Fig. 1, 2, 3, 4, 5, 6, 7, 8) and so to analyze the level of protection the plant has given to the nerve cell of the animal to be correlated with human health care.

The striatum of LC animals showed a large mass of dead tissue stained with eosin covered with trypan blue stained dead cells (Fig. 2, 6). As the striatum of LC group animals was not protected with pre-treatment, the nerve cells could not be able to withstand the action of excitotoxin which leads to damage, not only to the cell membrane, but also to the protein inside the cell and so the area around the lesion was left with large dead tissue mass that will be stained only by eosin and a few dead cells with intact protein stained with trypan blue. The striatum of SC animals shown only small dead tissue mass covered with trypan blue stained dead cells (Fig. 1, 4). Though this group animals undergone dummy surgery with normal saline, the needle prick region shown a small dead tissue mass covered with dead cells. The damages to the neurons done by the needle prick is responsible for this dead cells and tissue mass (Fig. 9, 10). The striatal histology of WS125 also shown dead tissue mass and dead cells but the size of the dead tissue mass was very small and present around only the needle prick area and covered with dead cells that clearly proves the protective nature of the extract given. But in case with WD100, we have seen only a small mass of dead tissue covered with trypan blue stained dead cells (Fig. 3, 4, 7, 8), that is a clear indication of prior protection of striatum by the drug employed. As an steroidal component, Withanolide-A hopefully can pass the cell membrane and can protect the cell well and so the actual spread of lesion was not much with drug treated (Fig. 5, 6, 7, 8, 11, 12, 13, 14) animal group.

Neurodegenerative disorders, including HD are chronic permanent disorders of life that affect the quantity and quality of a person affected. As of now we can only treat the symptoms of certain diseases by cannot cure them. This present study suggest a plan to care the root cause of the array of disorders the nerve cell damage and death. If we can prevent the damage and death of the nerve cell of human as like the animals used in the experiment, it is a great boon to mankind in health care to at least delay the occurrence of the disease or its adverse effect and so improve the quality of life of the affected person in society more simply without much effort and money, which is very essential in a highly populated country like India.

Conclusion

Withania somnifera is a very old herb of India and one of the constituents of our traditional nervine tonic to boost the activities of the brain and memory. It has no side effects and easily available in all parts of the world. It is even available along with some daily consuming supplements like tea powder, chocolate to the community.

Herewith we can suggest, people with any neurodegenerative disorders, including HD can give a try of these commercially available products for their health care and this technique, trypan blue live cell exclusion staining can be used to access the actual dead cells in any organ undergoing degeneration. The usage of withania somnifera is not only restricted to people with neurodegenerative disorders but the offspring of the patients and even common people can consume it on a daily basis with other supplements like tea to have a healthy life, as neurodegeneration is a part and parcel of all human in old age and can affect anyone at any time to keep a quality life in the community.

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Institutional Review Board Statement

Approved - (IAEC/XIII/11/CLBMCP/2008-09) CPCSEA.

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Conflicts of Interest

The authors declare no conflicts of interest.

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