Full Length Research Paper

Neuroprotective response of the hippocampus region of the brain to *Withania somnifera* and *Asparagus racemosus* root extract: An *in vitro* study

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Neuroprotective effects of two medicinally important herbs *Withania somnifera* (WS) and *Asparagus racemosus* (AR) were investigated in primary hippocampal neuron cell culture. The root extracts of WS and AR were obtained by Soxhlet extraction using distilled water as solvent. The 7 to 8 days old hippocampal cells in culture were treated with100 µM Glutamate (Glu) for 10 min at room temperature in Hank's balanced saline solution (HBSS). One hour (1 h) after exposure to Glu, cells were treated with root extract of WS, AR or WS + AR [10 µg/ml dissolved in dimethyl sulfoxide (DMSO)], respectively. Results of the study suggested that percent cell viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay] was more significant in groups treated with Glu + WS and Glu + WS + AR, when compared to group treated with Glu + AR. More than two-fold increase in free Ca^{2+} was observed in culture in presence of Glu, but after treatment with WS, this increase was significantly reduced ($P < 0.01$). No significant change was observed in presence of AR. A significant decrease in lactic dehydrogenase (LDH) was observed after WS, AR as well as WS + AR, when compared with Glu treated group. There was significant ($P < 0.01$) decrease in malondialdehyde (MDA) in presence of WS or AR. Reactive oxygen species (ROS) level was significantly inhibited in both Glu + WS and Glu + AR treated group, compared to Glu and Glu + WS + AR treated group. Significant elevation of reduced glutathione (GSH) and glutathione disulfide (GSSG) was also obtained after WS and AR treatment. A marked increase in nerve growth factor (NGF) and pro-brain derived growth factor (pro-BDNF) expression was observed after AR and WS + AR treatment; whereas no observable change was observed in WS treated group. It was therefore, concluded that both plants in combination (WS + AR) have more effective role in neuroprotection. Study also showed neurotrophic factor modulatory activity of these plants. Present study suggest that WS + AR in combination could have alternative therapeutic potency for treatment of diseases associated with neuron cell loss.

Key words: *Withania somnifera*, *Asparagus racemosus*, hippocampus, glutamate, *in vitro*, neuroprotection.

INTRODUCTION

Hippocampal neurons are most vulnerable to damaging effects of various stressors. Cell loss in hippocampus during normal aging and age related disorders have been reported and is linked to deficit in endogenous antioxidant (Jing et al., 2008). Degeneration of cell bodies in machinery (Manchair et al., 1990; Christian, 1998; Contestabile, 2001; Cantuti et al., 2003; Halliwel, 2006); hippocampus results in loss of memory and learning

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activities and also cognitive behavior. Hippocampal neurons are more susceptible due to presence of high concentration of glucocorticoid receptors, which get activated due to altered glucocorticoid homeostasis in aging and chronic stress and in turn render hippocampal neurons vulnerable to oxidative stress (Christian et al., 1997). Recent reports have shown that natural products of plant origin can reduce oxidative stress and prevent neuronal cell death (Bhatnagar et al., 2000; Shukla et al., 2000; Bastianetto et al., 2000; Kim et al., 2001; Suk et al., 2002). Protective effects of various plants extracts have been shown in culture and animal models of Alzheimer's disease (AD) (Thimmappa et al., 2005) and Parkinson's disease (PD) (Sankar et al., 2009). The mechanisms underlying neuroprotective effects of herbal extracts may be as diverse as the plants and could be associated with free radical scavenging action or by potentiating the endogenous antioxidant defence system or modulation of neurotransmitters and growth factor expression. Thus, in present investigation, *in vitro* studies were carried out with an aim to study protective effects of *Withania somnifera* (WS) and *Asparagus racemosus* (AR) root extracts in hippocampal neuronal culture model.

MATERIALS AND METHODS

Plant collection, identification and extract preparation

Fresh roots of WS and AR were collected from botanical garden of the Unic College of Science, M. L. Sukhadia University, Udaipur. Plant was identified by Prof. K. G. Ramawat, Head of Department of Botany, M. L. Sukhadia University, Udaipur, India and a voucher specimen was deposited at the Department of Botany, M. L. Sukhadia University, Udaipur, India. Roots were air-dried at room temperature for 3 weeks. The crushing of the roots was done in laboratory using pestle and mortar, after which it was ground into powder. About 200 g of powdered roots was weighed and placed in conical flask and 1 L of distilled water was added thereafter. The mixture was then shaken and allowed to stand for 30 min, after which it was boiled for 1 h, cooled and shaken vigorously, before filtration through Whatman No.1 filter paper. The filtrate was concentrated in rotator evaporator and stored at 4°C until used and yield was 7.75% (w/w)

Animals

Male adult Wistar rats (BW 150 g) were obtained from National Institute of Nutrition, Hyderabad. After receiving from supplier, rats were placed for 7 days in an isolated room at controlled conditions of temperature (22 ± 2°C), humidity and light (12 to 12 h night and dark cycle) to minimize the effects of change of place. Rats were kept for breeding as per norms of Centre for prevention and control of use of experimental animals (CPCSEA) India. All experiments were cleared by Institutional Ethical Committee (IAEC). Laboratory is registered with CPCSEA.

Primary hippocampal cell culture

New born (2 h) rat pups were used for primary culture. Pups were decapitated; hippocampi were removed quickly and placed in a sterile Petri dish with the culture medium. The tissue was then mechanically triturated through an 18-guage needle attached to a 5 ml syringe. Single cell suspension was seeded in 96-well plate coated with 0.01% poly-L-lysine, at a density of 10^6 cells per well. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was used to culture the cells. Ampicillin and streptomycin were added to medium. Cells were cultured in presence of 5% CO_2 at 37°C and 100% humidity for 8 to 10 days before using them for further experimentation.

Experimental design

Seven (7) to 8 days old hippocampal cell culture was used in study. Culture wells were divided into control (Group I) and four experimental groups (group II to V). Control group cells were treated with phosphate-buffered saline (PBS) only. Group II culture was treated with Glutamate (Glu) only. Cells of groups III, IV and V were treated with WS, AR and WS + AR plant extracts [10 µg/ml dissolved in dimethyl sulfoxide (DMSO)], respectively. After 24 h of the exposure with plant extracts, cultured cells were exposed to 100 µM Glu for 10 min at room temperature in Hank's balanced saline solution (HBSS) (Hansen et al., 1989). Immediately after Glu exposure, culture was washed twice with HBSS and replaced with fresh culture medium. Culture was returned to the incubator for 24 h prior to various analyses.

Cell viability assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to check the cell viability was carried out by the protocol of Hansen et al. (1989). Reactive oxygen species (ROS) level was measured spectrofluorimetrically as described by Myhre et al. (2003). Intracellular Ca^{2+} was measured in cultured neurons using calcium sensitive dye Fluo-3 AM as described by Mattson et al. (1993). Lactic dehydrogenase (LDH) assay was done by the protocol of Koh and Choi (1982). Reduced glutathione (GSH) and oxidized form, glutathione disulfide (GSSG) were measured as described by Hissin and Hilf (1976). Malondialdehyde (MDA), end products of lipid peroxidation was measured spectrophotometrically by Utley et al. (1976).

Immunocytochemistry

Cultured hippocampal cells grew on cover slips in 24-well plate were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and incubated with blocking buffer (10% goat serum and 0.1% triton X-100 in PBS, pH 7.4). Primary antibody (Sigma) for nerve growth factor (NGF) and pro-brain derived growth factor (pro-BDNF) (rabbit polyclonal, Sigma) diluted 1:1000 was added to the culture and cells were incubated overnight at 4°C. Cells were washed with PBS and then incubated with secondary antibody (biotinylated goat anti-rabbit, Vector laboratories, USA) for 1 h at room temperature and after washing with PBS, staining was done using Vectastain ABC kit (Avidin-Biotin Complex Kit, Vector laboratories, USA). Colour was developed in diamino benzidine hydrochloride (DAB). Sections were dehydrated and mounted in DPX. Photomicrographs were taken on Olympus BX51 Fluorescence microscope.

Statistics analysis

Results are expressed as the mean ± SD. The data were analyzed by one way analysis of variance (ANOVA) using PRISM (Graphpad Software Inc.).

1A
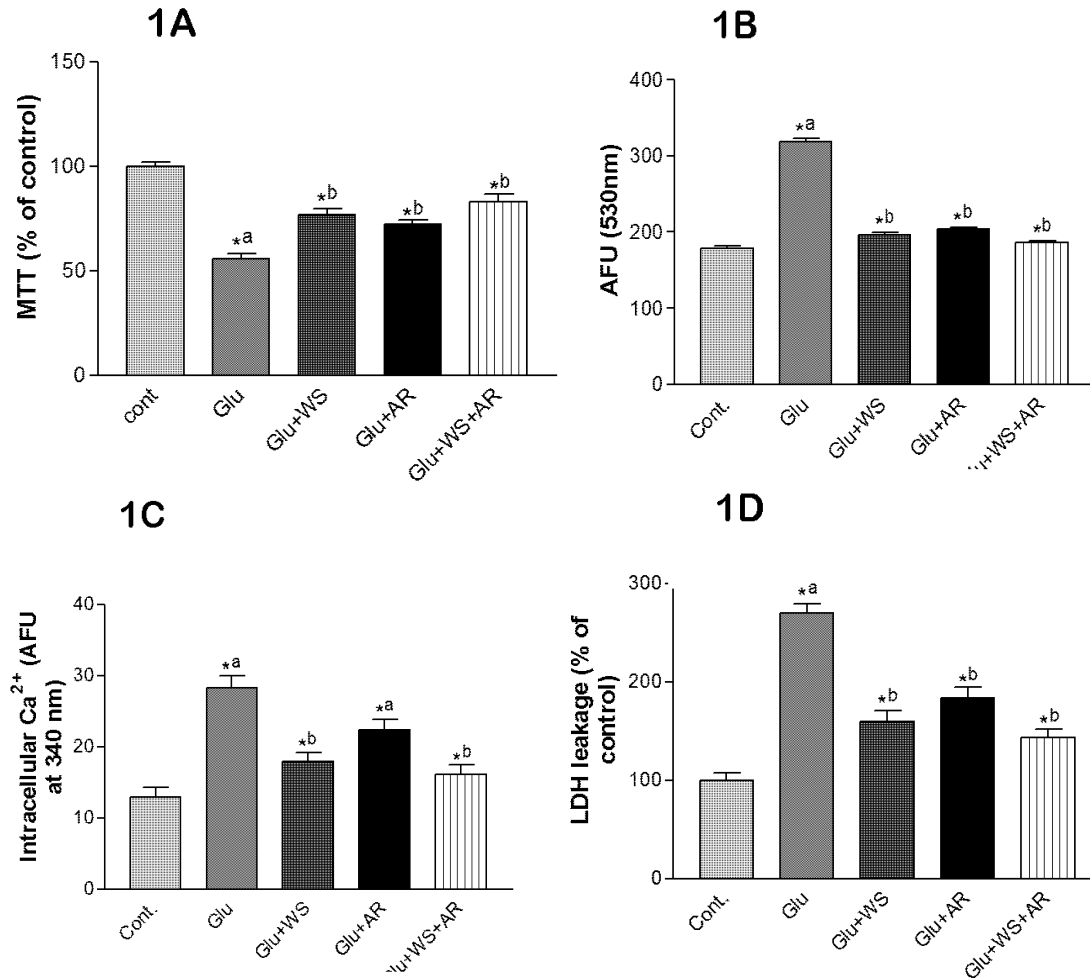

1B

1C

1D

Figure 1. Effects of WS, AR and WS + AR treatment on hippocampal cells in primary culture exposed to Glu. A, Cell viability assay showing decline in MTT reduction after Glu treatment was nearly 45% (p < 0.001). MTT reduction increased significantly (p < 0.001) in presence of WS, AR and WS + AR extracts in Glu treated group; B, AFU measurement in Glu treated group showing nearly 1.5-fold increases in ROS level. This increase was inhibited significantly (p > 0.05) in presence of WS, AR as well as in WS + AR; C, two fold increase in free Ca2+ concentration was observed after Glu treatment. This increase in Ca2+ was inhibited significantly (p < 0.01) in the presence of WS as well as WS + AR. Effects were more significant in WS and WS + AR group as compared to AR alone (p > 0.05); D, LDH showed 150% (p < 0.01) increase after Glu treatment as compared to control. A significantly (P < 0.01) decreased LDH level was observed after WS, AR or WS + AR treatment; E, GSH level declined significantly (p < 0.01) after Glu treatment as compared to control group. Significant (p<0.01) elevation in GSH level was observed in all the three groups that is, treated with WS and AR. GSH increase more significant (p < 0.001) WS + AR group; F, GSSG level increased significantly (p < 0.01) after Glu treatment as compared to control group. Significant (p < 0.01) decrease in GSSG level was observed in all the three groups that is, treated with WS, AR and WS + AR; G, MDA level was increased more than two fold in Glu treated group as compared to control group (p < 0.001). Presence of WS or AR as well as WS + AR extract significantly (P < 0.001) prevented increase in MDA.

RESULTS

Cell viability assay

Decline in MTT reduction after Glu treatment was nearly 45% (p < 0.001) as compared to control group (Figure 1A). MTT reduction increased significantly in presence of WS, AR and WS + AR extracts in Glu treated group. Effects of WS and WS + AR were more significant (p <

0.001) in WS and WS + AR group than in AR group.

Reactive oxygen species (ROS)

Arbitrary fluorescence units (AFU) measurement in Glu treated group showed nearly 1.5-fold increases in ROS level as compared to control. This increase was inhibited significantly (p > 0.05) in presence of WS, AR as well as
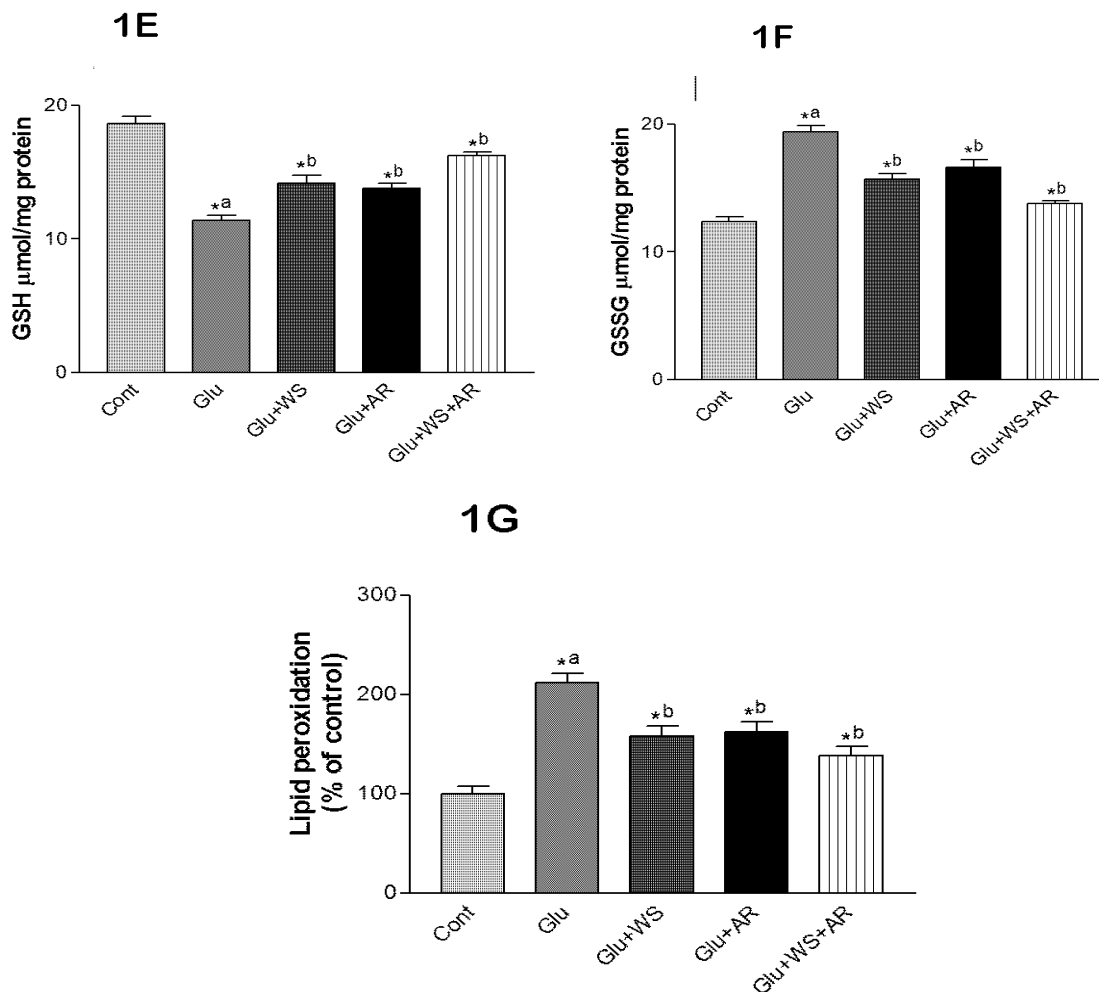
1E

1F

1G

Figure 1. Contd.

in WS + AR (Figure 1B).

Free Ca⁺⁺

Two-fold increase in free Ca^{2+} concentration (Figure 1C) was observed in Glu treatment. This increase in Ca^{2+} was inhibited significantly ($p < 0.01$) in the presence of WS as well as WS + AR. Effects were more significant in WS and WS + AR group as compared to AR alone ($p > 0.05$).

Lactic dehydrogenase (LDH) assay

LDH showed 150% ($p < 0.01$) increase after Glu treatment as compared to control. A significantly ($P < 0.01$) decreased LDH level was observed after WS, AR or WS + AR treatment (Figure 1D).

Reduced glutathione (GSH) assay

GSH level declined significantly ($p < 0.01$) after Glu

treatment as compared to control group. Significant ($p < 0.01$) elevation in GSH level was observed in all the three groups that is, treated with WS and AR. GSH increase was more significant ($p < 0.001$) in WS + AR group (Figure 1E).

Glutathione disulfide (GSSG) assay

Conversely, GSSG level increased significantly ($p < 0.01$) after Glu treatment as compared to control group. Significant ($p < 0.01$) decrease in GSSG level was observed in all the three groups that is, treated with WS, AR and WS + AR (Figure 1F).

Malondialdehyde (MDA)

MDA level was increased more than two-fold in Glu treated group as compared to control group ($p < 0.001$). Presence of WS or AR as well as WS + AR extract significantly ($P < 0.001$) prevented increase in MDA
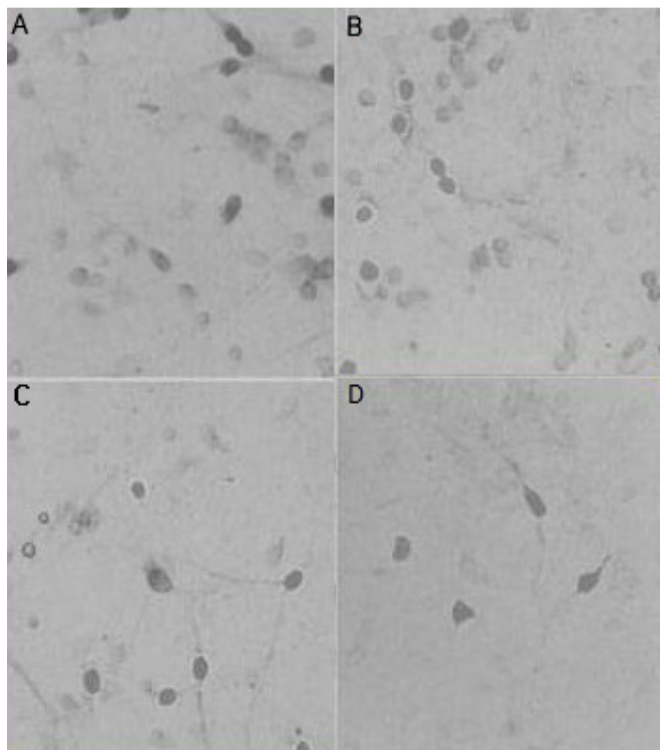
Figure 2. Immunocytochemical localization of NGF and BDNF in cultured cells. A, Control group showing significant pro-BDNF activity in cytoplasm of neuron cell bodies; B, a non-significant reduction in the expression after Glu treatment; C, a significant increase in pro-BDNF immunoreactivity was observed after AR; D, a significant increase in pro-BDNF immunoreactivity was observed after WS + AR treatment.

(Figure 1G).

Immunocytochemistry

Immunocytochemical localization of NGF and proBDNF in cultured cells, showed a non significant reduction in the expression after Glu treatment but a significant increase in ProBDNF immunoreactivity was observed after AR and WS + AR treatment (Figures 2A to D).

DISCUSSION

Researchers are in search of a substance which could not only effectively quench the free radicals but also have anti or pro cholinergic, anti-amyloid and anti-inflammatory properties for the treatment of neurodegenerative disorders. Several reports have shown these properties in two medicinally important plants WS and AR (Bastianetto et al., 2000; Mishra et al., 2000; Thimmappa et al., 2005; Bhatnagar et al. 2005; Sankar et al., 2009; Bhatnagar et al., 2009). In present study, we studied the effects of AR and WS extracts individually and in

combination on Glu induced neuronal degeneration in primary culture of hippocampal cell. Changes observed in MTT reduction, Free Ca^{++}, LDH level, GSH, GSSG, ROS and MDA after WS, AR or WS + AR treatment to Glu treated cells showed protective effects of these extracts. A significant decline in MTT reduction after Glu treatment and significant MTT reduction increase in presence of WS + AR extracts compared to WS or AR in Glu treated group showed that percentage cell viability increased after the treatment of both the plant extracts. Glu exerts its neurotoxic effects through activation of N-methyl-d-aspartate (NMDA) and N-methyl-p-aspartate (AMPA) receptors (Pereira and Oliveiro, 2000; Calabrese et al., 2007). Over activation of these receptors, it triggers an excessive entry of Ca^{2+}, initiating a series of cytoplasmic and nuclear processes that promote neuronal cell death. An increased entry of Ca^{2+} into the mitochondria is believed to enhance the mitochondrial electron transport, increasing the production of ROS such as $^{.}O_2^{-}$. Rise in Ca^{2+} also activate neuronal nitric oxide synthase (nNOS) and stimulate nitric oxide (NO) production that in turn reacts with peroxide to produce peroxynitrite, which is a strong oxidant and cause neuronal damage. We have recently also shown that WS extract protects against Glu (Bhatnagar et al., 2009). In this study, two-fold increase in free Ca^{2+} concentration was observed after Glu treatment. This increase in Ca^{2+} was inhibited significantly (p < 0.01) in the presence of plant extracts. Effects were more significant in WS and WS + AR group as compared to AR alone. These observations further substantiate the results of cell viability assay. LDH is membrane damage marker. During peroxidation, levels of both LDH and MDA significantly change. In present study, LDH and MDA are decreased significantly after treatment with WS or AR as well as WS + AR. Low GSH, and high GSSG and ROS are indicative of increased oxidative stress. In our study, GSH level declined significantly after Glu treatment. Conversely, GSSG level increased significantly after Glu treatment. Changes in GSH and GSSG were reversed after treatment with plant extracts. Similarly, AFU also showed nearly 1.5-fold increases in ROS level in Glu treated group. This increase was also inhibited significantly in presence of WS, AR as well as in WS + AR.

Immunocytochemical localization of NGF and BDNF in cultured cells was attempted first time after treatment with WS or AR extract. Neurotrophic factors are endogenous molecules that play crucial role in the maintenance, survival and differentiation of various neuronal populations in brain (Larsson et al., 1999; Griesbach et al., 2002). Results showed a non-significant reduction in the expression after Glu treatment but a significant increase in NGF and pro-BDNF expression was observed after AR and WS + AR treatment particularly. The mechanism underlying the observed changes in NTF expression might be that plant extract directly enhance the expression of NTF. Earlier, *in vitro* and *in vivo* studies

have shown that NTFs can attenuate neuronal injury initiated by excitotoxic damage, ischemia and trauma (Griesbach et al., 2002; Tsuzynski, 2000). NTFs such as NGF, BDNF and glial cell line-derived neurotrophic factor (GDNF) have been shown to promote cell survival by preventing acute damage mediated by EEAs, ROS and Ca^{2+} influx (Tsuzynski, 2000; Tsuzynski, 2007). Aggravated death of hippocampal neurons in global forebrain ischemia was shown after decreased BDNF activity (Larsson et al., 1999). In rats, intracerebroventricular or intraparenchymal infusion of NGF significantly prevented the massive cell loss or when cells were genetically transduced to over express NGF (Tsuzynski, 2000).

In conclusion, our results clearly suggest neuroprotective effects of both WS and AR. Interestingly, WS + AR in combination showed high free radical scavenging as well as neurotrohin modulatory property. Taken all the evidences together, it seems that WS and AR have a therapeutic potency in diseases associated with neuron cell loss.

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REFERENCES

Bastianetto S, Ramassamy C, Dore S, Christen Y, Poirier J, Quirion R (2000). The Ginkgo biloba extract (EGb 761) protects hippocampal neurons against cell death induced by beta-amyloid. Eur. J. Neurosci. 12:1882-1890.

Bhatnagar M, Sharma D, Salvi M (2009). Neuroprotective Effects of Withania somnifera Dunal.: A Possible Mechanism. Neurochem. Res. 34:1975-1983.

Bhatnagar M, Shukla SD, Jain S, Mundra A (2000). Cytoprotective effects of Shankpushpi and E. asenoides preparation on hippocampal cells in mice. Ind. Drugs 37:280-285.

Bhatnagar M, Sissodia SS, Bhatnagar R (2005). Antiulcer and antioxidant activity of Asparagus racemosus WILLED and Withania somnifera DUNAL in rats. Ann. Y Acad. Sci. 1056:261-278.

Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stell AMG (2007). Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nat. Rev. Neurosci. 8:766-775.

Cantuti GI, Schukkit-Hale B, Joseph JA (2003). Neurobehavioral aspects of antioxidants in aging. Int. J. Dev. Neurosci. 8:67-381.

Christian B (1998). Effects of glucocorticoids on oxidative stress-induced hippocampal cell death: implications for the pathogenesis of Alzheimer's disease. Exp. Gerontol. 33:689-696.

Christian B, Frank L, Thorsten T, Martina W, Thomas S, Florian H (1997). Glucocorticoid Enhance Oxidative Stress-Induced Cell Death in Hippocampal Neurons in Vitro. Endocrinol. 138:101-106.

Contestabile A (2001). Oxidative stresses in neurodegeneration: Mechanisms and therapeutic perspective. Curr. Top. Med. Chem. 1:553-568.

Griesbach GS, Hovda DA, Molteni R, Gomez-Pinilla F (2002). Alterations in BDNF and synapsin I within the occipital cortex and hippocampus after mild traumatic brain injury in the developing rat : reflections of injury induced neuroplasticity. J. Neurotrauma 19:803-814.

Halliwel B (2006). Oxidative stress and neurodegeneration. Where are we now? J. Neurochem. 97:1634-1658.

Hansen MB, Nielsen SE, Berg K (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119:203-210.

Hissin PJ, Hilf R (1976). A flluorimetric method for determination of oxidized and reduced glutathione in tissue. Anal. Biochem. 74:214-226.

Jing B, Jing Bo, Liu HJ, Lai C, Zhang XL, J-An L (2008). Protective effects of Catalpol against H2O2 induced oxidative stress in astrocyte primary culture. Neurosci. Lett. 442:224-227.

Kim YO, Leem K, Park J, Lee P, Ahn DK, Lee BC, Park HK, Suk K, Kim SY, Kim H (2001). Cytoprotective effect of Scutellaria baicalensis in CA1 hippocampal neurons of rats after global cerebral ischemia. J. Ethnopharmacol. 77:183-188.

Koh JY, Choi DW (1982). Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay, J. Neurosci. Meth. 20:83-90.

Larsson E, Nanobashvili A, Kokaia Z, Lindvall O (1999). Evidence for neuroprotective effects of endogenous brain-derived neurotrophic factor after global forebrain ischemia in rats. J. Cereb. Blood Flow. Metab. 19:1220-1228.

Manchair E, Shashi KS, Mayur DB (1990). Oxidative stress and antioxidant therapy in Parkinson's disease. Neurobiol. 48:1-19.

Mattson MP, Tomaselli KJ, Rydel RE (1993). Calcium destabilizing and neurodegenerative effects of aggregated beta-amyloid peptid are attenuated by basic FGF. Brain Res. 621:35-49.

Mishra LC, Singh BB, Dagenais S (2000). Scientific basis for the therapeutic use of Withania somnifera (Ashwagandha): a review. Altern. Med. Rev. 5:334-346.

Myhre O, Andersen JA, Aarnes H, Fonnum F (2003). Evaluation of the probes 2',7'-dichlorofluorescin diacetate , luminol and lucigenin as indicators of reactive species formation. Biochem. Pharmacol. 65:1575-1582.

Pereira CF, Oliveiro CR (2000). Oxidative glutamate toxicity involves mitochondrial dysfunction and operturbation of intracellular Ca++ homeostasis. Neurosci. Res. 37: 227-236.

Sankar SR, Manivassagam T, Surendran S (2009). Ashwagandha leaf extract: a potential agent in treating oxidative damage and physiological abnormalities seen in mouse model of Parkinson's disease. Neurosci. Lett. 45:411-419.

Shukla SD, Jain S, Sharma K, Bhatnagar M (2000). Stress induced neurodegenration and protective effects on Semecarpus anacardium Linn: and Withania somnifera Dunn in hippocampus of albino mice: an ultrastructural study. Ind. J. Exp. Biol. 38:1007-1013.

Suk KJ, Kim SY, Leem K, Kim YO, Park SY, Hur J, Baek J, Lee KJ, Zheng HZ, Kim H (2002). Neuroprotection by methanol extract of Uncaria rhynchophylla against global cerebral ischemia in rats. Life Sci. 70:2467-2480.

Thimmappa S, Anekonda P, Reddy H (2005). Can herbs provide a new generation of drugs for treating Alzheimer's disease? Brain Res. Rev. 50:361-376.

Tsuzynski MH (2000). Intraparenchymal NGF infusions rescue degenerating cholinergic neurons. Cell transplant 9:629-636.

Tsuzynski MH(2007). Nerve growth factor gene therapy in Alzheimer's disease. Alzheimer's dis. Assoc. Disord. 21:179-189.

Utley HG, Bergheim F, Hochstein P (1976). Effects of sulfydrl reagents on peroxidation of microsomes, Arch. Biochem. Biophy. 118:29-32.