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Withania somnifera Extract Protects Model Neurons from *In Vitro* Traumatic Injury

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Abstract

Withania somnifera has been used in traditional medicine for a variety of neural disorders. Recently, chronic neurodegenerative conditions have been shown to benefit from treatment with this extract. To evaluate the action of this extract on traumatically injured neurons, the efficacy of *W. somnifera* root extract as a neuroprotective agent was examined in cultured model neurons exposed to an *in vitro* injury system designed to mimic mild traumatic brain injury (TBI). Neuronal health was evaluated by staining with annexin V (an early, apoptotic feature) and monitoring released lactate dehydrogenase activity (a terminal cell loss parameter). Potential mechanisms underlying the observed neuroprotection were examined. Additionally, morphological changes were monitored following injury and treatment. Although no differences were found in the expression of the antioxidant transcription factor nuclear factor erythroid 2-like 2 (Nrf2) or other Nrf2-related downstream components, significant changes were seen in apoptotic signaling. Treatment with the extract resulted in an increased length of neurites projecting from the neuronal cell body after injury. *W. somnifera* extract treatment also resulted in reduced cell death in the model neuron TBI system. The cell death factor Bax was involved (its expression was reduced 2-fold by the treatment) and injury-induced reduction in neurite lengths and numbers was reversed by the treatment. This all indicates that *W. somnifera* root extract was neuroprotective and could have therapeutic potential to target factors involved in secondary injury and long-term sequelae of mild TBI.

Keywords

Ayurveda, *Withania somnifera*, neuroprotection, neurites, SH-SY5Y, traumatic brain injury

Introduction

Traumatic brain injury (TBI) affects 0.5% of the world population each year.[1,2] The majority of these injuries are categorized as mild; nonetheless, even the mild injuries can result in systemic problems, such as memory loss, as a consequence of neuronal loss and connectivity changes. Most of the neurons are not lost immediately but rather days following the injury. The torsional component of human TBI can contribute to axonal injury, and connectivity changes may play an important role in cognitive deficits.[3,4] These neurons are degraded not from the primary injury but from the resulting secondary injury where signaling cascades are activated, and several biochemical changes take place that contribute to substantial functional loss. If the signaling events that occur following the injury are improved by treatment, the damage to neurons and neuronal connections may be reduced, preserving neuronal capacity. One method to preserve neurons is through the increase of antioxidant activity.[5–7] The root of the plant *Withania somnifera* (L.) Dunal (Indian ginseng, with the common name ashwagandha) of family *Solanaceae* is used in Ayurvedic treatment, which increases antioxidant properties.[8–11] Historical use of this plant has been reviewed,[12] and traditional uses over millennia have included treatment of neurological problems.[13–15] *W. somnifera* has been used to treat several

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disorders, including neurodegeneration,[16–18] with no significant side effects.[19,20] In stroke models, extracts from this plant improved motor function[21] and reduced mortality and DNA fragmentation in the brain.[22] The *W. somnifera* root extract has been neuroprotective in hypoxia[23] and model Parkinson disease[24] in rodents. Healthy patients who were given 250 mg of root and leaf extract twice daily displayed improved cognitive performance.[25] The primary chemical components from this plant are withanolides with withaferin A, the most notable of these compounds.[15] Withaferin A has been shown to inhibit transcription factors such as stimulatory protein 1 and nuclear factor kappa-light-chain-enhancer of activated B cells.[26,27]

We sought to determine whether *W. somnifera* could influence neuronal health parameters after mild TBI *in vitro*. In this study, we used *W. somnifera* root extract to treat cultured model neurons that were traumatically injured. SH-SY5Y human neuroblastoma cells have proven to be a useful model for testing neuroprotective treatments including plant extracts.[28,29] The injury process results in a loss of both neurons and neuronal processes.[30] Pretreatment with extract reduced the neuronal loss and increased neuronal process length compared to injured controls. In an effort to determine the mechanism of protection, we explored several potential targets. Based on our previous findings with traumatic injury models,[5,6] we examined transcription factor nuclear factor erythroid 2-like 2 (Nrf2), heat shock protein 70 (HSP70), and related signaling. We did observe significant changes in postinjury cell survival and neurites and a significant reduction in bcl-2-like protein 4 (Bax), suggesting that the extract may reduce apoptotic signaling after injury.

Materials and Methods

Preparation of Extract

Powdered root of *W. somnifera* (L.) Dunal (current name in www.theplantlist.org), obtained from Arya Vaidya Sala, Kottakkal (Kerala, India), was extracted using the chloroform–methanol procedure as described by Sehgal et al.[31] The liquid chromatography-mass spectrometry (LC-MS) chemical fingerprint has been reported and is available in Supplementary Figure 1 in the study by Sehgal et al.[31] The resulting paste was suspended in dimethyl sulfoxide to a final concentration of 2.8%. The suspension was freshly prepared before each experiment and concentrations ranging from 4 to 100 μg/mL were tested in cultures.

Cell Culture

Human neuroblastoma SH-SY5Y cells were obtained from ATCC (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 0.25 U/mL penicillin (Sigma-Aldrich, St Louis, MO, USA), and 50 μg/mL streptomycin (Sigma-Aldrich). Cells were grown at 37 °C in a humidified 5% CO_2 chamber. Media was refreshed every 2 to 3 days when cells reached 85% confluence. At treatment, media was removed and refreshed with maintenance media + *W. somnifera* extract (Fig. 1). Concentrations of the extract tested ranged from 4 to 100 μg/mL. Vehicle-treated cells received the same volume and concentration of dimethyl sulfoxide but no extract.

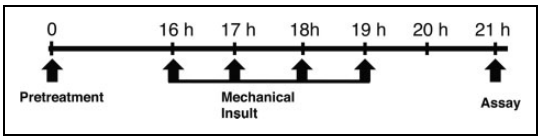

Figure 1. Time line that cultured neurons were exposed to treatment, *in vitro* injury, and assays. SH-SY5Y model neurons were plated on a Flexcell plate with a specialized silastic membrane. Cells were treated 24 h after plating. Sixteen hours posttreatment, 4 injuries consisting of a nitrogen gas pulse of 35.3 psi with a peak injury pressure of 6.0 were delivered to the cultured neurons once every hour. Assays were performed 2 h following the final injury.

In Vitro Traumatic Injury

Injury was performed as optimized and described previously.[31] This injury mimics the torsional strain that is common in traumatic brain injuries.[32–34] In short, undifferentiated SH-SY5Y cells were cultured on 6-well BioFlex plates coated with collagen type I (Flexcell International, Hillsborough, NC) that are designed with a silastic membrane for flexibility. A regulated nitrogen gas pulse at 35.3 psi for 99 ms from a cell injury controller II (Virginia Commonwealth University, Richmond, VA)[35] expanded the membrane inducing a biaxial stretch in the model neurons. Sham injured cells resided in identical conditions in adjacent wells but did not receive the pressure pulse. This injury was performed once per hour for a total of 4 injuries. As reported previously,[5,30] SH-SY5Y cells are more resistant to the biaxial stretch and this degree of pulse exposure amounts to a mild injury. Assays were performed 2 h following the final injury.

Staining for Cell Death

At 2 h postinjury, cells were stained with a mixture that included annexin V conjugated to Alexa-Fluor 488 (1:50 dilution from stock; A13201, Invitrogen), and 5 μg/mL Hoechst 33342 (Invitrogen). The stain mixture, in annexin binding buffer, was added directly to the 250 μL media in each well. Cells were incubated in the dark for 15 min and 5 images per well were taken on a Leica DMI4000B (Wetzlar, Germany) fluorescence microscope at 10× magnification. ImageJ software (National Institutes of Health, Bethesda, MD) was used to count Hoechst-positive and annexin-positive cells. Hoechst was used to determine the total number of cells, and percentage of annexin V–positive cells was determined from approximately 600 total cells per well.

Table 1. Primers Used for mRNA Determinations by qRT-PCR.

Accession Number	Gene	Sense Primer 5′-3′	Antisense Primer 5′-3′	Product Length
NM_002046	GAPDH	CCACTCCTCCACCTTTGAC	ACCCTGTTGCTGTAGCCA	102 bp
NM_005345	HSPA1A	TACTCCGACAACCAACCCG	TGTCTTTCGTCATGGCCCTC	66 bp
NM_145803	TRAF6	TGACAACTGTGCTGCATCAATGGC	GGGCTGTAGGGCAGTCTAGATCA	148 bp

Note: mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

Immunocytochemistry

Nrf2 localization was determined by immunostaining SH-SY5Y cells with 1:100 anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were captured with a Fluoview 1000 confocal microscope (Olympus, Pittsburgh, PA, USA) at 40× magnification as z stacks (settings: sequential, 15% 405 nm laser, 9% 635 nm laser, 10 µs/pixel). Nuclear versus cytoplasmic Nrf2 immunoreactivity was determined with ImageJ. After nuclear autothresholding (intermodes setting), the particle analysis routine (settings: size 20 to 400, circularity 0.1 to 1) was used to determine nuclear counts and generate masks of the nuclear regions of interest. The intensity of the Nrf2 staining in the nuclear regions was measured in the corresponding Cy5 channel. Cytoplasmic regions were then identified, with the nuclear regions excluded, by default autothresholding in the Cy5 channel followed by particle analysis (settings: size 20 to 4,000, circularity 0 to 1), and then the intensities for these cytoplasmic regions were recorded.

Lactate Dehydrogenase Activity

Cell viability was quantified by measuring the release of cytosolic lactate dehydrogenase (LDH) at 2 h postinjury. This assay was performed according to the manufacturer's instructions (Sigma-Aldrich). Briefly, 80 µL of media was taken from the SH-SY5Y culture medium and mixed with 160 µL LDH assay mixture prepared per the manufacturer's instructions. Following the 20-min dark incubation period, the reaction was halted by adding 1 N HCl and sample absorbance was measured on a BioTek plate reader (Winooski, VT, USA) at 490 nm. The background absorbance was measured at 690 nm and subtracted from the readings at 490 nm. Cytotoxicity percentage was calculated as $(A490_{Sample} - A490_{Media}/A490_{Lysed} - A490_{Media}) \times 100\%$, where $A490_{Sample}$ = test culture, $A490_{Media}$ = only media without any cells, and $A490_{Lysed}$ = cells killed with 1% Triton X-100.

Quantitative Reverse Transcription Polymerase Chain Reaction

At 2 h postinjury, RNA was isolated from cells using RNeasy columns (Qiagen, Valencia, CA, USA). RNA was quantified using Nanodrop 2000 (Thermo Fisher Scientific, Rockford, IL, USA) and was amplified using Quantitect SYBR RT-PCR kit (Qiagen), according to the manufacturer's recommendations. The reaction was initiated at 95 °C for 10 min, then denatured at 95 °C for 15 s, annealed at 60 °C for 30 s, extended at 72 °C for 30 s for 40 cycles, and scanned for fluorescent signals in a Bio-Rad Opticon (MJ Research, Hercules, CA, USA). Primer pairs, synthesized by Integrated DNA Technologies (Coralville, IA, USA), were taken from published examples or designed using Primer3: human glyceraldehyde 3-phosphate dehydrogenase (GAPDH),[36] heat shock 70-kDa protein (HSPA1A),[37] nuclear factor (erythroid-derived 2)-like 2 (NFE2L2),[38] and p53 (see Table 1).

Western Immunoblots

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher, Houston, TX, USA) containing 50 mM Tris–HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, and 1% Triton X, plus Halt protease and phosphatase inhibitor (Thermo Fisher). Protein was quantified using Pierce BCA protein assay (Thermo Scientific) and read on a BioTek microplate reader at 570 nm. Protein was denatured by heating at 95 °C for 5 min and separated on a gradient SDS polyacrylamide gel electrophoresis gel (Thermo Fisher). The proteins were transferred onto a nitrocellulose membrane (LI-COR #926-31092, Lincoln, NE, USA), blocked using Odyssey blocking buffer (LI-COR #927-40000), incubated overnight with antibodies as follows, and visualized using a LI-COR Odyssey fluorescence scanner. The primary antibodies were Nrf2 (SC-722) 1:200 rabbit polyclonal (Santa Cruz Biotechnologies), GAPDH (MAB374) 1:1,000 mouse monoclonal (Chemicon Int., Temecula, CA, USA), HSP70 (SC 33575) 1:1,000 rabbit polyclonal (Santa Cruz Biotechnologies), tumor necrosis factor receptor–associated factor 6 (TRAF6; 04-451) 1:1,000 rabbit monoclonal (Chemicon), and HMOX-1 (SC10789) 1:100 rabbit polyclonal (Santa Cruz Biotechnologies), as shown in Table 2.

Protein Antibody Arrays

Total protein was extracted from cells using RIPA buffer as described above. Protein was quantified using Pierce BCA protein assay (Pierce; Thermo Scientific) and read on a BioTek microplate reader at 570 nm. Apoptotic pathway proteins were evaluated with an apoptosis phospho antibody array (PAP247) using an antibody array assay kit (KAS02) from Full Moon Biosystems (Sunnyvale, CA, USA), as

Table 2. Antibodies Used for Protein Expression Analysis.

Antigen	Company	Species	Product Number
Nrf2	Santa Cruz	Rabbit/polyclonal	SC-722
GAPDH	Chemicon	Mouse/monoclonal	MAB374
HSP70	Santa Cruz	Rabbit/polyclonal	SC33575
HMOX-1	Santa Cruz	Rabbit/polyclonal	SC10789
TRAF6	Millipore	Rabbit/monoclonal	04-451
Bax	Cell Signaling	Rabbit/monoclonal	5023
HSP90	Cell Signaling	Rabbit/monoclonal	4877
Cy5	Chemicon	Donkey antimouse	AP182SMI
IR dye 800	Li-Cor	Goat antirabbit	926-32211
IR dye 680	Li-Cor	Goat antimouse	926-68070

Note: Nrf2, nuclear factor erythroid 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSP70, Heat shock protein 70; HMOX-1, heme oxygenase 1; TRAF6, tumor necrosis factor receptorassociated factor 6; Bax, BCL2 associated X protein; HSP90, Heat shock protein 90; Cy5, cyanine 5.

directed by the manufacturer. Briefly, lysate was purified to remove unwanted detergents/buffers from the protein and dissolved in labeling buffer provided. Protein was biotinylated and stored overnight at −80 °C. Antibody arrays were blocked using solution provided in the assay kit for 45 min with agitation and then rinsed thoroughly with H_2O. Biotin-labeled proteins were coupled to microarrays at room temperature for 2 h in coupling solution provided by the manufacturer and rinsed thoroughly with H_2O, incubated in a Cy3-streptavidin (Sigma-Aldrich) solution for 20 min at room temperature, rinsed thoroughly, and finally read with an Agilent DNA (Santa Clara, CA, USA) microarray scanner using a photomultiplier tube (40% setting) at the Moffitt Cancer Center (Tampa, FL, USA). The threshold for the use of a data point was set to 1.5 times background. The mean of each spot was normalized to the local background of the spot to provide a signal mean for each spot. The 6 spot replicates for each antibody were averaged. The biological replicates ($n = 3$) were averaged to obtain a group mean signal that was then normalized to the sham vehicle group to achieve a fold change for expression of each antibody spotted on the array in order to compare differences in expression between groups. Analysis of the phosphorylation state was performed in a similar manner, but the mean signals were compared to the relative total protein antibody and presented as a ratio of phosphorylated/total protein. Any areas that were damaged by scratches, water drops, or shading were designated invalid and not analyzed in any manner.

Measurement of Neurite Length

SH-SY5Y cells were fixed and stained with crystal violet (Sigma-Aldrich), phalloidin conjugated to Alexa-Fluor 488 (Invitrogen), or unstained and imaged under relief contrast conditions on an Olympus CKX41 microscope or Olympus IX81/Fluoview 1000 laser scanning confocal microscope. Images collected after staining with phalloidin (conjugated to Alexa-Fluor 488) and visualized on an Olympus IX81/Fluoview 1000 laser scanning confocal were consistent with neurons visualized by crystal violet staining or relief contrast captured on an Olympus CKX41 microscope. For quantification of neurite length/cell, after crystal violet staining, 10 fields per well were captured with a 4× objective, no contrast, for 6 wells per group. The fields, each 1,400 × 1,050 μm, were preselected in a 2 × 5 array centered in each well, with each field center 3 mm from the center of adjacent fields. Processes were traced and measured automatically with the Neurite Tracer[39] macros for ImageJ. Separate experiments were performed and samples were collected under relief contrast (10× objective, 560 × 420 μm fields) for determination of maximum process lengths and percentages of cells with processes by manual tracing (avoiding overlapping cells), assisted and measured with the NeuronJ plugin for ImageJ.

Statistical Analysis

All data are represented as mean ± standard deviation and analyzed by either unpaired Student t test or analysis of variance + Tukey-Kramer multiple comparison posttest using GraphPad (La Jolla, CA, USA) InStat3 software. A P value of <0.05 was deemed statistically significant.

Results

Pretreatment with extract protected SH-SY5Y model neurons from *in vitro* mechanical injury. Neurons were pretreated 16 h prior to injury with 0, 4, 20, or 100 μg/mL of *W. somnifera* root prepared by sequential methanol–chloroform extractions. Neurons were subjected to 4 traumatic injuries 1 h apart (Fig. 1). Two hours following the final injury, neurons were assayed for survival using annexin V-Alexa 488 and Hoechst stains. There was a significant, 3.6-fold, increase in staining for injured cells compared to uninjured controls exposed only to vehicle. At 4 μg/mL of extract, the injury produced only a 1.9-fold increase in annexin staining, and at 20 μg/mL, there was no increase in annexin-positive cells produced by the injury. The most effective concentration of the *W. somnifera* extract, in terms of reduced annexin staining after injury, was 20 μg/mL (Fig. 2). Toxic effects, based on the annexin V assay, were seen at 100 μg/mL, where there was a 2.3-fold increase in annexin V–positive cells in the uninjured, treated sample. The concentration of 20 μg/mL was used in all successive experiments.

Additionally, neuronal survival was assayed by measuring LDH release into the media from lysed cells 2 h following the final injury. There was significantly more LDH release into the media for cultured neurons exposed to injury compared to sham controls (Fig. 3). Treatment with extract in the injured cells was sufficient to significantly reduce this level of LDH by 20% compared to injured controls (Fig. 3).

To determine whether treatment with *W. somnifera* activated Nrf2 antioxidant signaling, we measured messenger RNA (mRNA) expression of Nrf2 downstream factor HSP70 (Fig. 4a). We complemented these studies by measuring total protein expression of Nrf2 and downstream factors HSP70 and heme oxygenase 1 (HMOX1; Fig. 4a). We also

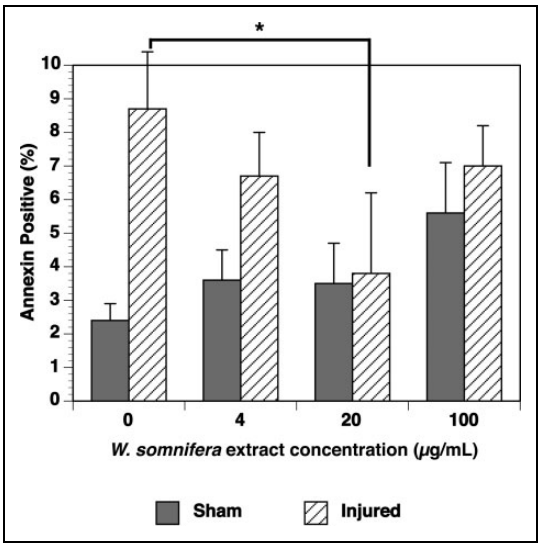

Figure 2. *Withania somnifera* root extract 20 μg/mL prevents SH-SY5Y death. *W. somnifera* root extract at a final concentration of 4, 20, and 100 μg/mL was added to the cultured neurons for 16 h prior to injury. Two hours after the final injury, cells were stained with annexin-V to determine apoptosis (*$P < 0.0007$).

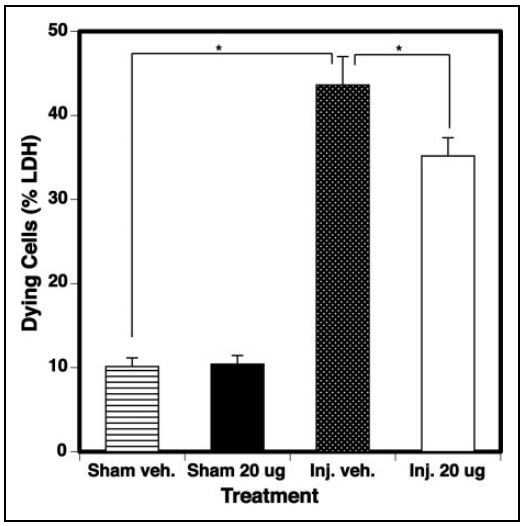

Figure 3. Viability of SH-SY5Y cells improves in the presence of *Withania somnifera* root extract. Following *in vitro* injury, lactate dehydrogenase (LDH) was measured in the media of culture neurons. Pretreatment with 20 μg/mL of *W. somnifera* root extract was sufficient to protect model neurons from cell death (*$P < 0.001$). veh., vehicle, Inj., injury.

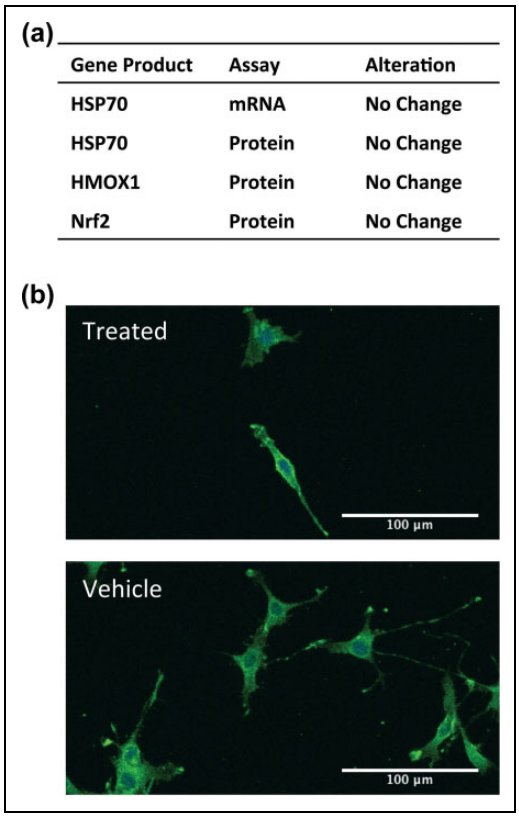

Figure 4. Nuclear factor erythroid 2-like 2 (Nrf2) antioxidant signaling was unchanged following treatment with *Withania somnifera* extract. Heat shock protein 70 (HSP70) messenger RNA (mRNA) was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and showed no significant increase following treatment. Total protein from Nrf2, HSP70, and heme oxygenase 1 (HMOX1) was measured by Western blot (a). Immunocytochemistry for Nrf2 expression following treatment did not indicate activation-associated translocation to the nucleus (b).

monitored Nrf2 activation by measuring nuclear and cytoplasmic protein by immunoblot expression and found no change in expression in either compartment. We saw no difference in HSP70 mRNA or protein for Nrf2, HSP70, or HMOX1. Additionally, to confirm that there was no activation of Nrf2 antioxidant signaling, we performed immunocytochemistry to determine whether there was an increase in Nrf2 nuclear expression following treatment (Fig. 4b). Over 300 cells were measured and there was no difference in Nrf2 immunoreactivity in the cytoplasmic or the nuclear compartments. Nine different fields of cells from the vehicle and extract-treated samples were examined and the Nrf2 immunoreactivity was measured as fluorescent intensity in the nuclear region, identified by DAPI staining, and the cytoplasmic region.

We also examined the expression of other signaling factors that are important for neuronal survival, such as c-Jun and TRAF6. No difference in c-Jun protein expression was observed between any of the groups (data not shown). Similarly, we observed no difference in protein expression of TRAF6, which is known to increase following head injury in vivo[40] (data not shown).

To determine whether treatment altered apoptotic signaling, we monitored some key signaling pathways using the phospho-apoptosis antibody array from Full Moon Biosciences encompassing 247 proteins (Fig. 5a). We saw a significant 51% increase in Bax signaling following injury, which was 37% reduced following treatment with the *W. somnifera* extract. Additionally, we saw a small but significant increase (12%) in 14-3-3 zeta/delta following injury

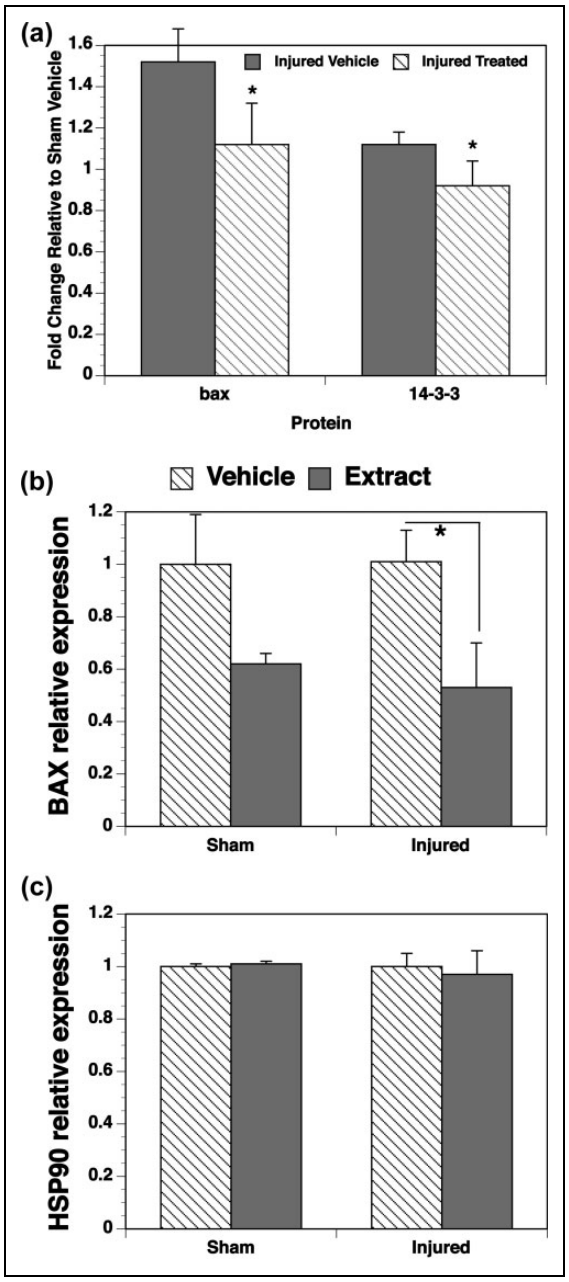

Figure 5. A protein array was used to examine any change in factors involved in apoptotic signaling. Proteins were collected 2 h following the final injury. N = 3 biological replicates were used. Several proteins showed a trend with injury and treatment, however, the only proteins that were significantly changed were Bax (*$P < 0.05$) and 14-3-3, by antibody array analysis (a). With Western immunoblots (b and c), we observed a significant decrease in Bax expression following injury in the presence of treatment compared to vehicle alone (*$P < 0.05$; B). We did not observe any change in heat shock protein 90 (HSP90; c).

and a decrease following treatment (23%). Although we did not achieve significant differences in expression with most of the proteins on the array, and the majority were undetectable in these cells, we observed a strong trend where pro-apoptotic genes such as DAXX increased expression following injury as well as chaperone protein HSP90. These levels were restored to control levels following treatment with *W. somnifera* extract (data not shown). We performed Western blots for Bax and HSP90 to confirm our protein array results. We did observe a significant decrease in Bax following *W. somnifera* extract treatment (Fig. 5b) but no difference in HSP90 (Fig. 5c).

To determine whether the treatment had a functional effect, other than promoting cell survival following injury, we measured neuronal processes as an aspect of the insult that shares features with torsional, axonal injury. *In vitro* traumatic injury resulted in rounded cells with reduced number and length of neuronal processes. When *W. somnifera* extract was present, after injury, we found increases in the average process and in the maximum process length (Fig. 6a-d), and there was a trend with the extract increasing the overall number of cells with processes (Fig. 6e). The injured neurons treated with extract displayed neurites 70% longer than untreated controls (Fig. 6d).

Discussion

Traditional medicines may provide a useful source of therapeutics for modern medicine or at the least help to identify therapeutic targets. We have shown that in cultured neurons, treatment with 20 μg/mL of *W. somnifera* extract can enhance neuronal survival following an *in vitro* injury that mimics TBI and this involves Bax downregulation at the mRNA and protein levels. The decrease in annexin V staining suggests that there is a reduction in early apoptosis produced by the treatment. The concentration of the extract appeared to be very important for neuron survival, as our initial studies indicated that 20 μg/mL was optimal to observe protection and that 100 μg/mL may have been cytotoxic.

To confirm that fewer cells were dying when treated with *W. somnifera* extract, we measured LDH release into the media by cultured neurons. As anticipated, we found an increase in the enzyme release following injury and a reduction in that increase when model neurons were treated with extract.

It has been shown previously that *W. somnifera* extract has antioxidant properties.[8–11] To determine whether treatment increases certain aspects of antioxidant signaling in our model system, we measured a regulator of phase II antioxidants, Nrf2. We did not observe activation of the Nrf2 antioxidant pathway or downstream HSP70 or HMOX1. While p53 expression increased in the injured samples, compared to controls, as seen previously with *in vitro* injuries, we did not observe any treatment effect.

These experiments represent a first step to elucidate the mechanism by which this treatment is working to enhance neuronal health after injury. We observed a decrease in apoptosis with treatment as identified by a reduction in annexin V staining. Upon further examination of apoptotic signaling, we found a decrease in Bax expression, indicating

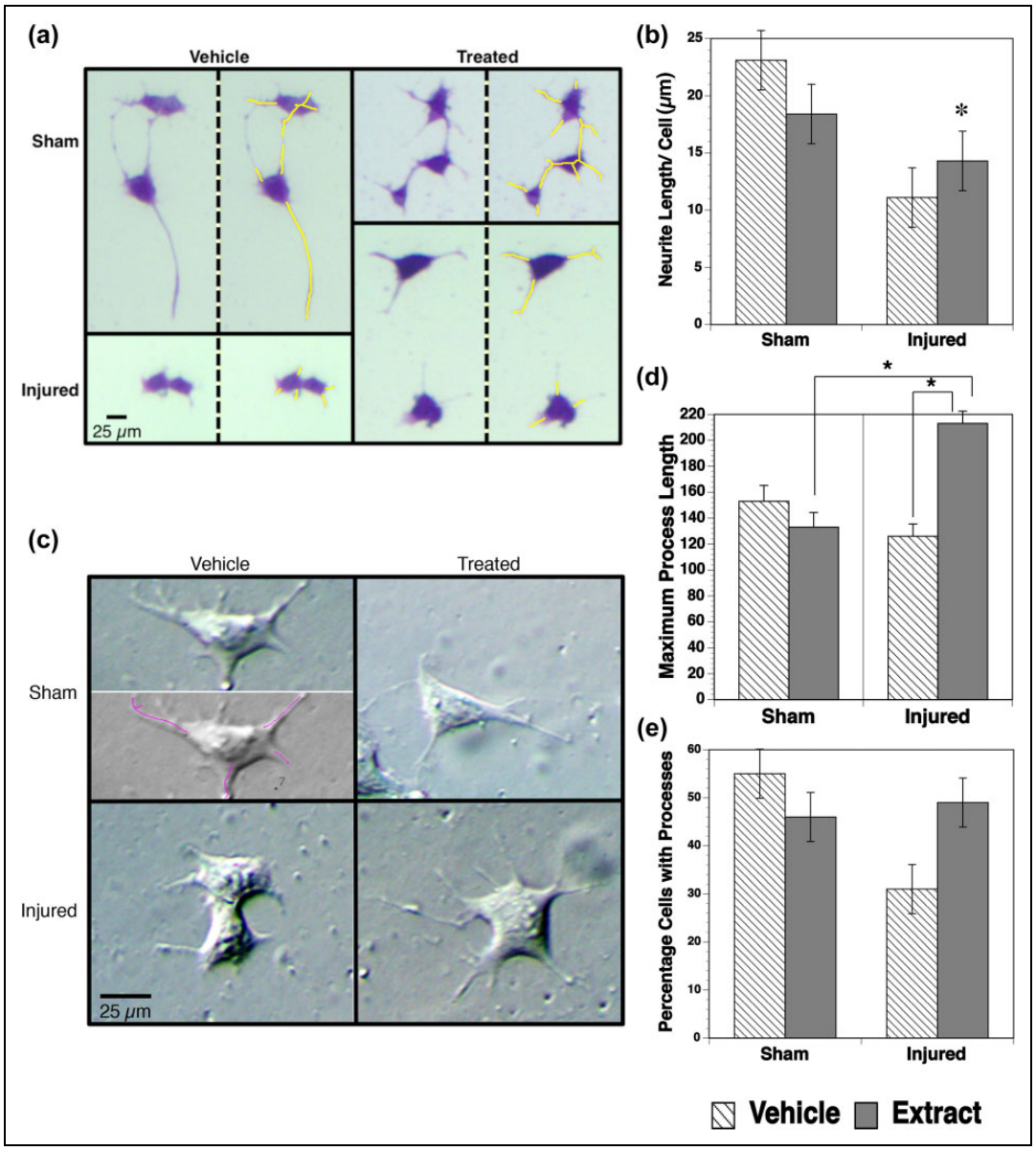

Figure 6. Treatment with *Withania somnifera* root extract increased the length of neurites. Two hours postinjury, cells were stained with crystal violet and phase contrast images were taken (a). Automated tracing of neurites using neurite tracer showed that in the presence of *W. somnifera*, neurites were significantly longer (26,709 total cells were measured, $P < 0.0001$; b). In other experiments, relief contrast images were analyzed using a semi-automated method, which allowed analysis of additional parameters (c). After tracing 350 cells, semi-automated analysis indicated that postinjury, the maximum process length was significantly increased (*$P < 0.0001$) by the treatment (d), while the percentage of cells with processes did show a strong trend with treatment, and it was not significantly different ($P < 0.11$; e).

that the extract can contribute to neuroprotection by reducing an apoptotic initiation pathway. Although we did not observe an increase in Nrf2 antioxidant signaling following *W. somnifera* treatment in this model system, it may be different in an in vivo system where other cell populations, such as astrocytes and microglia, are present and may provide enhanced protection.

Extract from *W. somnifera* contains compounds that promote neurite growth in mice.[41,42] Furthermore, treatment with extract from the root improves cognition[43] and improves Alzheimer pathology in mice.[31] This traditional medicine has been used for centuries to treat anxiety and mental disorders, but the mechanism(s) involved in its efficacy is unknown. A number of bioactive components of *W. somnifera* fruit extract can cross the blood–brain barrier.[44]

The *in vitro* mechanical injury mimics the torsional forces that are observed with head injury. It is well established that mild head injuries with rotational effects have axonal shearing. This diffuse axonal injury may significantly contribute to the functional losses that are observed in patients. The

measurements of neurites following injury demonstrates that not only are more model neurons surviving the injury, but those cells treated with extract display more neurite projections as well as significantly longer neurites compared to injured untreated cells. The loss of these processes is analogous to diffuse axonal injury from a mild traumatic injury, where the rapid stretch of the neuronal axon damages the cytoskeleton and causes increased inflammation resulting in destruction of the axon and consequently the neuronal connectivity. This increase in neurites in model neurons suggests that the treatment has the potential to improve neuronal connectivity and indicates a need for further testing.

Conclusions

W. somnifera is an attractive treatment as it can be delivered orally, and clinical trials have observed no side effects.[19,20,31] Our model system delineates part of the mechanism of *W. somnifera* injury protection and indicates that this treatment may preserve both the number of neurons and the components of neuronal connectivity. Our findings suggest that this may be a valuable treatment for neuronal health following mild traumatic injury and continued research in an in vivo model is warranted.

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Ethical Approval

This study was approved by our institutional review board.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Disclaimer

The contents do not represent the views of the Department of Veterans Affairs or the United States Government.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Bryan-Hancock C, Harrison J. The global burden of traumatic brain injury: preliminary results from the Global Burden of Disease Project. Inj Prev. 2010;16(Suppl 1):A17.
2. Gardner RC, Yaffe K. Epidemiology of mild traumatic brain injury and neurodegenerative disease. Mol Cell Neurosci. 2015;66(Pt B):75–80.
3. Stern RA, Riley DO, Daneshvar DH, Nowinski CJ, Cantu RC, McKee AC. Long-term consequences of repetitive brain trauma: chronic traumatic encephalopathy. PM R. 2011;3(10 Suppl 2):S460–S467.
4. Mac Donald CL, Johnson AM, Cooper D, Nelson EC, Werner NJ, Shimony JS, Snyder AZ, Raichle ME, Witherow JR, Fang R, et al. Detection of blast-related traumatic brain injury in U.S. military personnel. N Engl J Med. 2011;364(22): 2091–2100.
5. Hatic H, Kane MJ, Saykally JN, Citron BA. Modulation of transcription factor Nrf2 in an in vitro model of traumatic brain injury. J Neurotrauma. 2012;29(6):1188–1196.
6. Saykally JN, Rachmany L, Hatic H, Shaer A, Rubovitch V, Pick CG, Citron BA. The nuclear factor erythroid 2-like 2 activator, tert-butylhydroquinone, improves cognitive performance in mice after mild traumatic brain injury. Neuroscience. 2012;223:305–314.
7. Hall ED, Vaishnav RA, Mustafa AG. Antioxidant therapies for traumatic brain injury. Neurotherapeutics. 2010;7(1):51–61.
8. Scartezzini P, Speroni E. Review on some plants of Indian traditional medicine with antioxidant activity. J Ethnopharmacol. 2000;71(1–2):23–43.
9. Mishra LC, Singh BB, Dagenais S. Scientific basis for the therapeutic use of *Withania somnifera* (ashwagandha): a review. Altern Med Rev. 2000;5(4):334–346.
10. Singh RH. Exploring larger evidence-base for contemporary Ayurveda. Int J Ayurveda Res. 2010;1(2):65–66.
11. Alam N, Hossain M, Mottalib MA, Sulaiman SA, Gan SH, Khalil MI. Methanolic extracts of *Withania somnifera* leaves, fruits and roots possess antioxidant properties and antibacterial activities. BMC Complement Altern Med. 2012;12:175.
12. Gupta GL, Rana AC. *Withania somnifera* (ashwagandha): a review. Pharmacognosy Rev. 2007;1(1):129–136.
13. Durg S, Dhadde SB, Vandal R, Shivakumar BS, Charan CS. *Withania somnifera* (ashwagandha) in neurobehavioural disorders induced by brain oxidative stress in rodents: a systematic review and meta-analysis. J Pharm Pharmacol. 2015;67(7): 879–899.
14. Kuboyama T, Tohda C, Komatsu K. Effects of ashwagandha (roots of *Withania somnifera*) on neurodegenerative diseases. Biol Pharm Bull. 2014;37(6):892–897.
15. Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazon J. Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. Molecules. 2009;14(7):2373–2393.
16. Ven Murthy MR, Ranjekar PK, Ramassamy C, Deshpande M. Scientific basis for the use of Indian ayurvedic medicinal plants in the treatment of neurodegenerative disorders: ashwagandha. Cent Nerv Syst Agents Med Chem. 2010;10(3):238–246.

17. Singh RH, Narsimhamurthy K, Singh G. Neuronutrient impact of Ayurvedic Rasayana therapy in brain aging. Biogerontology. 2008;9(6):369–374.
18. Kulkarni SK, Dhir A. *Withania somnifera*: an Indian ginseng. Prog Neuropsychopharmacol Biol Psychiatry. 2008;32(5): 1093–1105.
19. Cooley K, Szczurko O, Perri D, Mills EJ, Bernhardt B, Zhou Q, Seely D. Naturopathic care for anxiety: a randomized controlled trial ISRCTN78958974. PLoS One. 2009;4(8):e6628.
20. Chopra A, Lavin P, Patwardhan B, Chitre D. A 32-week randomized, placebo-controlled clinical evaluation of RA-11, an Ayurvedic drug, on osteoarthritis of the knees. J Clin Rheumatol. 2004;10(5):236–245.
21. Chaudhary G, Sharma U, Jagannathan NR, Gupta YK. Evaluation of *Withania somnifera* in a middle cerebral artery occlusion model of stroke in rats. Clin Exp Pharmacol Physiol. 2003; 30(5–6):399–404.
22. Adams JD Jr, Yang J, Mishra LC, Singh BB. Effects of ashwagandha in a rat model of stroke. Altern Ther Health Med. 2002;8(5):18–19.
23. Baitharu I, Jain V, Deep SN, Hota KB, Hota SK, Prasad D, Ilavazhagan G. *Withania somnifera* root extract ameliorates hypobaric hypoxia induced memory impairment in rats. J Ethnopharmacol. 2013;145(2):431–441.
24. RajaSankar S, Manivasagam T, Sankar V, Prakash S, Muthusamy R, Krishnamurti A, Surendran S. *Withania somnifera* root extract improves catecholamines and physiological abnormalities seen in a Parkinson's disease model mouse. J Ethnopharmacol. 2009;125(3):369–373.
25. Pingali U, Pilli R, Fatima N. Effect of standardized aqueous extract of *Withania somnifera* on tests of cognitive and psychomotor performance in healthy human participants. Pharmacognosy Res. 2014;6(1):12–18.
26. Prabhakaran Y, Dinakaran SK, Macharala SP, Ghosh S, Karanam SR, Kanthasamy N, Avasarala H. Molecular docking studies of withanolides against Cox-2 enzyme. Pak J Pharm Sci. 2012;25(3):595–598.
27. Mohan R, Hammers HJ, Bargagna-Mohan P, Zhan XH, Herbstritt CJ, Ruiz A, Zhang L, Hanson AD, Conner BP, Rougas J, et al. Withaferin A is a potent inhibitor of angiogenesis. Angiogenesis. 2004;7(2):115–122.
28. Friedemann T, Otto B, Klatschke K, Schumacher U, Tao Y, Leung AK, Efferth T, Schroder S. Coptis chinensis Franch. exhibits neuroprotective properties against oxidative stress in human neuroblastoma cells. J Ethnopharmacol. 2014;155(1): 607–615.
29. Hu S, Han R, Mak S, Han Y. Protection against 1-methyl-4-phenylpyridinium ion (MPP+)-induced apoptosis by water extract of ginseng (Panax ginseng C.A. Meyer) in SH-SY5Y cells. J Ethnopharmacol. 2011;135(1):34–42.
30. Kane MJ, Hatic H, Delic V, Dennis JS, Butler CL, Saykally JN, Citron BA. Modeling the pathobiology of repetitive traumatic brain injury in immortalized neuronal cell lines. Brain Res. 2011;1425:123–131.
31. Sehgal N, Gupta A, Valli RK, Joshi SD, Mills JT, Hamel E, Khanna P, Jain SC, Thakur SS, Ravindranath V. *Withania somnifera* reverses Alzheimer's disease pathology by enhancing low-density lipoprotein receptor-related protein in liver. Proc Natl Acad Sci U S A. 2012;109(9):3510–3515.
32. Arundine M, Aarts M, Lau A, Tymianski M. Vulnerability of central neurons to secondary insults after in vitro mechanical stretch. J Neurosci. 2004;24(37):8106–8123.
33. Lau A, Arundine M, Sun HS, Jones M, Tymianski M. Inhibition of caspase-mediated apoptosis by peroxynitrite in traumatic brain injury. J Neurosci. 2006;26(45):11540–11553.
34. Weber JT, Rzigalinski BA, Ellis EF. Traumatic injury of cortical neurons causes changes in intracellular calcium stores and capacitative calcium influx. J Biol Chem. 2001;276(3): 1800–1807.
35. Ellis EF, McKinney JS, Willoughby KA, Liang S, Povlishock JT. A new model for rapid stretch-induced injury of cells in culture: characterization of the model using astrocytes. J Neurotrauma. 1995;12(3):325–339.
36. Zhang Y, Ba Y, Liu C, Sun G, Ding L, Gao S, Hao J, Yu Z, Zhang J, Zen K, et al. PGC-1alpha induces apoptosis in human epithelial ovarian cancer cells through a PPARgamma-dependent pathway. Cell Res. 2007;17(4):363–373.
37. Brooks AR, Lelkes PI, Rubanyi GM. Gene expression profiling of human aortic endothelial cells exposed to disturbed flow and steady laminar flow. Physiol Genomics. 2002;9(1):27–41.
38. Du Y, Villeneuve NF, Wang XJ, Sun Z, Chen W, Li J, Lou H, Wong PK, Zhang DD. Oridonin confers protection against arsenic-induced toxicity through activation of the Nrf2-mediated defensive response. Environ Health Perspect. 2008; 116(9):1154–1161.
39. Pool M, Thiemann J, Bar-Or A, Fournier AE. NeuriteTracer: a novel ImageJ plugin for automated quantification of neurite outgrowth. J Neurosci Methods. 2008;168(1):134–139.
40. Chen J, Wu X, Shao B, Zhao W, Shi W, Zhang S, Ni L, Shen A. Increased expression of TNF receptor-associated factor 6 after rat traumatic brain injury. Cell Mol Neurobiol. 2011;31(2): 269–275.
41. Kuboyama T, Tohda C, Komatsu K. Neuritic regeneration and synaptic reconstruction induced by withanolide A. Br J Pharmacol. 2005;144(7):961–971.
42. Kuboyama T, Tohda C, Komatsu K. Withanoside IV and its active metabolite, sominone, attenuate Abeta(25-35)-induced neurodegeneration. Eur J Neurosci. 2006;23(6):1417–1426.
43. Jarrard LE. On the role of the hippocampus in learning and memory in the rat. Behav Neural Biol. 1993;60(1):9–26.
44. Vareed SK, Bauer AK, Nair KM, Liu Y, Jayaprakasam B, Nair MG. Blood-brain barrier permeability of bioactive withanamides present in *Withania somnifera* fruit extract. Phytother Res. 2014;28(8):1260–1264.