Research Paper

Protective effects of Withania somnifera extract in SOD1\textsuperscript{G93A} mouse model of amyotrophic lateral sclerosis

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A B S T R A C T

Withania somnifera (WS; commonly known as Ashwagandha or Indian ginseng) is a medicinal plant whose extracts have been in use for centuries in various regions of the world as a rejuvenator. There is now a growing body of evidence documenting neuroprotective functions of the plant extracts or its purified compounds in several models of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS). Based on the extract’s beneficial effect in a mouse model of ALS with TDP-43 proteinopathy, the current study was designed to test its efficacy in another model of familial ALS. Our results show that administration of WS extracts by gavage to mice expressing G93A mutant form of superoxide dismutase (SOD1) resulted in increased longevity, improved motor performance and increased number of motor neurons in lumbar spinal cord. The WS treatment caused substantial reduction in levels of misfolded SOD1 whereas it enhanced expression of cellular chaperons in spinal cord of SOD1\textsuperscript{G93A} mice. WS markedly reduced glial activation and prevented phosphorylation of nuclear factor kappaB (NF-κB). The overall immunomodulatory effect of WS was further evidenced by changes in expression of multiple cytokines/chemokines. WS also served as an autophagy inducer which may be beneficial at early stages of the disease. These results suggest that WS extracts might constitute promising therapeutics for treatment of ALS with involvement of misfolded SOD1.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is usually an adult onset fatal neurodegenerative disorder characterized by selective motor neuron death. Most cases of ALS are sporadic in nature wherein a definite causative factor or inheritance pattern cannot be determined. However, in approximately 10% of all ALS cases, the disease can be inherited (familial ALS or fALS). Mutations in the gene encoding Cu–Zn superoxide dismutase (or SOD1) were the first to be discovered as causative of ALS (Rosen et al., 1993). Currently, > 160 mutations in SOD1 have been identified in fALS patients, most which arise from missense point mutations, and account for approximately 20% of all fALS cases (Bunton-Stasyshyn et al., 2015) and 2–7% of sporadic cases (http://alsod.iop.kcl.ac.uk/Overview?gene_id=SOD1). SOD1 encodes for an anti-oxidant enzyme whose main function is to catalyze the transformation reaction of toxic superoxide radicals to oxygen and hydrogen peroxide. This enzyme activity is compromised in human ALS cases arising from almost all known mutations in SOD1 (Saccon et al., 2013). Yet, this ‘loss of function’ does not cause the development of the disease (Borchelt et al., 1995; Borchelt et al., 1994). Evidence suggests that mutant SOD1 proteins are prone to misfold and to form aggregates, particularly in lower motor neurons, resulting in gain of toxic functions such as endoplasmic reticulum stress, mitochondrial dysfunction and axonal transport impairment (Ajroud-Driss and Siddique, 2015).

Withania somnifera is a perennial herb belonging to the family Solanaceae, whose extracts have been in use for centuries in Indian, Chinese and Arabic traditional medicines. Withania somnifera extracts (root, leaf or fruit) have been reported to possess anti-inflammatory, antitumor, anti-stress, antioxidant, immunomodulatory, hemopoietic, and rejuvenating properties (Mishra et al., 2000). Apart from the extracts, Withaferin A, a steroidal lactone present in extracts of the plant, has also been extensively investigated as an anti-cancer drug. In models of neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and traumatic brain injury and cerebral ischemia, Withania somnifera extracts have been reported to be broadly neuroprotective (Bhatnagar et al., 2009; Kuboyama et al., 2014). It has also been used as an adjunctive therapy to improve cognitive function in people suffering from bipolar disorder with positive outcomes.
2.3. Analysis of clinical symptoms

Animal Care. the Care and Use of Experimental Animals of the Canadian Council protocols. Experiments were carried out in accordance with the Guide to We did not observe any significant difference between survival of male and female mice, irrespective of treatment. The Animal Care Ethics Committee of Université Laval approved all in vivo experimental protocols. Experiments were carried out in accordance with the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

2.3. Analysis of clinical symptoms

The onset of disease was defined as the time when mice started to exhibit a decline of body weight after reaching a peak. End stage was defined as the loss of righting reflex (the age when the animal could not right itself within 30s when placed on its side). Scoring on the basis of hind-limb reflexes was used to monitor the progression of disease in SOD1<sup>G93A</sup> mice, as previously described (Urushitani et al., 2006). Briefly, mice were scored using a 3-point system where non-symptomatic animal (hind limbs extending to form an angle of 120 degrees) was assigned a score of 3 to end stage animal (loss of reflex with hind limbs paws held close to the body and inability to walk) being assigned a score of 0. The reflex scoring was done by animal facility technicians who were blinded for the treatment paradigm but had experience in grading SOD1<sup>G93A</sup> mice paralysis. All other experiments were not conducted blind to treatment group.

2.4. Motor performance test

To test motor coordination, treated and un-treated mice were trained to run on an accelerating rotarod at 3-rpm speed with 0.25-rpm/s acceleration. Mice were subjected to three trials per session every week and the longest latency to fall from the rotating rod was recorded. To assess hindlimb strength the mice were placed on a wire grill which was then turned over gently, allowing the animal to hang from the grill. The time elapsed before the hindlimbs of the mice lets go of the grill was recorded with a cutoff time set at 90 s.

2.5. Immunohistochemistry

Spinal cords collected post sacrifice were sectioned and subjected to immunofluorescent staining by methods previously described (Dutta et al., 2017a). Details of antibodies used are provided in Table 1. Spinal cord sections were stained with cresyl violet to visualize Nissl positive neurons. Briefly, sections were de-fatted by immersing them in 1:1 ethanol-chloroform mixture followed by rehydration through graded alcohol to distilled water. Sections were then stained with warm 0.1% cresyl violet followed by multiple washes with distilled water. The sections were finally differentiated and dehydrated by passing through graded alcohol, cleared in xylene and mounted with DPX. Dissected dorsal root ganglia (DRG) were post fixed in 3% glutaraldehyde for a period of 48 h, washed in PBS, treated with 1% osmium tetroxide for 2 h, and dehydrated through increasing concentrations of ethanol. Prior to embedding in Epon resin, DRG were further dissected to ensure that all ventral root (VR) axons would be sampled at 3 mm from the DRG cell body. Semi-thin cross sections were stained with Toluidine Blue, rinsed, and cover slipped. Slides were visualized under Zeiss Apotome or Leica DM5000B microscope.

2.6. Immunoprecipitation for misfolded SOD1

Spinal cords from sacrificed mice were homogenized in TNG-T lysis buffer (50 mM Tris–HCl pH: 7.4; 100 mM NaCl; 10% glycerol; 1% Triton X), sonicated and centrifuged for 20 min at 9000 g at 4°C. Immunoprecipitation was performed using protein A/G-coated paramagnetic beads (Dynabeads; Invitrogen, Carlsbad, CA, USA) according to previously published protocol (Gros-Louis et al., 2010). Briefly, the beads were first coated with monoclonal anti-misfolded SOD1 antibody (clone B8H10; 1.0 μg of antibody per 40 μL of beads) for 2 h at room temperature. Following washes to remove unbound antibodies, the beads were incubated with volumes of spinal cord lysates corresponding to 300 μg of total protein, at 4°C, overnight. On the following day, the beads washed, protein was eluted and separated on a 14% SDS-polyacrylamide gel and then transferred electrophotographically to PVDF membrane. After blocking with 5% non-fat milk, membranes were incubated with polyclonal primary antibody against SOD1 (see Table 1). Immunodetection was performed with a goat anti-rabbit-horseradish peroxidase labeled secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and visualized on X-ray film.
Table 1

List of antibodies used for western blots (WB) and immunofluorescence (IF).

| Antibody against | Dilution for WB | Dilution for IF | Company |
|------------------|-----------------|----------------|---------|
| Actin            | 1:15000         |                | Millipore, CA, USA |
| Atg-5            | 1:1000          |                | Novus Biologicals; CO, USA |
| Beclin-1         | 1:1000          |                | Novus Biologicals; CO, USA |
| CD68             | 1:1000          |                | Serotec, CA, USA |
| Cox-2            | 1:1000          |                | Cell Signaling Technologies, CA, USA |
| ChAT             | 1:2500          | 1:250          | Millipore, CA, USA |
| GAPDH            | 1:2000          | 1:300          | Santa Cruz, CA, USA |
| GFAP             |                 |                | Cell Signaling Technologies, CA, USA |
| HSP-27           | 1:1000          | 1:1000         | Cell Signaling Technologies, CA, USA |
| HSP-60           | 1:1000          | 1:1000         | Cell Signaling Technologies, CA, USA |
| HSP-70           | 1:1000          | 1:1000         | Cell Signaling Technologies, CA, USA |
| Iba-1            |                 | 1:500          | Wako Chemicals, VA, USA |
| Phospho-IkBα     | 1:1000          | 1:1000         | Cell Signaling Technologies, CA, USA |
| LC3-II           | 1:2500          |                | Novus Biologicals, CO, USA |
| NeuN             | 1:1000          | 1:1000         | Cell Signaling Technologies, CA, USA |
| NFκB             |                 | 1:500          | Santa Cruz, CA, USA |
| Phospho-NFκB (Ser 536) | 1:1000        |                | Cell Signaling Technologies, CA, USA |
| SOD1 mAb (misfolded; clone BBH10) | 1:250          |                | Generated from hybridoma in the lab. |
| SOD1 pAb         | 1:500           |                | Enzo Life Sciences; NY, USA. |

*For immune-precipitation

Fig. 1. WS treatment improved survival and motor performance of SOD1<sup>G93A</sup> mice.

(a) Kaplan-Meier curves plotted with the mouse survival data showed that WS extract increased the median life span of SOD1<sup>G93A</sup> mice by 7.5 days. Statistical significance was assessed by Mantel-Cox log rank test; Chi square 4.016, \( p < .05 \). \( n = 16 \) in either groups. Median survival in WS = 166.5 days; in Veh = 159 days.

(b) Motor performance of the mice was assessed by the accelerating rotarod test (speed 3 rpm; acceleration 0.2/s; cut-off 300 s.). The maximum latency to fall from the rod was plotted. Data shows a clear increase in performance in WS-treated mice which significantly differed from that of Veh-treated mice between the age of 90 and 130 days. (c) Loss of body weight is considered as a major indicator of initiation of disease. Change in body weight of WS- and Veh-treated SOD1<sup>G93A</sup> mice is expressed as % of peak body weight. \( N = 16 \) for both groups of transgenic mice and 3 for wild type. (d) Mean age at which peak body weight was achieved was plotted. In WS-treated group the peak body was attained at 110.5 ± 3.021 days of age whereas in Veh-treated group it was attained at 115.8 ± 3.042 days of age. The difference in means was not statistically significant. (e) Latency to fall from a grid was assessed to test for hindlimb strength with a maximum cut off time set at 90 s. The data shows that there was a significant difference among the two treatment groups between 119 and 126 days of age. (f) Hindlimb reflex scoring also showed significant difference between 120 and 140 days of age. For b, c, e and f the differences in mean between the WS and Veh-treated groups were assessed by \( t \)-test with Welch's correction at each time point; *\( p < .5 \); **\( p < .01 \).
(Biomax MR1; #8701302; Kodak, Rochester, NY, USA) following exposure to enzyme chemiluminescent reagent (Thermo Fischer, IL, USA).

2.7. Immunoblot analysis

Immunoblot analysis was performed from samples containing equal amount of protein (quantified by Bio-Rad Protein assay; Bio-Rad, CA, USA) by methods previously described (Dutta et al., 2017a). Details of antibodies used are given in Table 1.

2.8. Cytokine array

The cytokine expression profiles from spinal cord extracts of WS or Veh-treated mice were performed with mouse cytokine antibody array kit (Raybio C-series Mouse Inflammation Antibody Array C1; Cat#AAM-INF-1-8; RayBiotech, Norcross, GA, USA) as previously described (Patel et al., 2015). Briefly, spinal cord lysates containing 300 μg of total protein were incubated with the array membranes overnight at 4°C. After washing with the buffers provided with the kit, membranes were incubated with biotin-conjugated antibodies overnight. Signal detection was performed according to the RayBiotech protocol, by exposing membranes to X-ray film (Biomax MR1; #8701302; Kodak, Rochester, NY, USA), and the obtained results were analyzed using Protein array analyzer macros in ImageJ software (version 1.51j8). Data were normalized to positive controls present in the array, quantified in arbitrary units and expressed as fold change in WS-treated group over corresponding values in Veh-treated group.

2.9. Statistical analysis

Data were analyzed using Graphpad Prism 5.0 software with significance level set at p < .05 unless otherwise mentioned. Difference between 2 groups was analyzed by unpaired t-test with Welch’s correction.

3. Results

3.1. WS treatment ameliorated motor performance and slightly increased longevity of SOD1G93A mice

Treatment of SOD1G93A mice (n = 16 per group) with WS extract starting at 50 days of age resulted in a median life span of 166.5 days as compared to 159 days in Veh-treated mice (an increase of 7.5 days) (Fig. 1a). The two survival curves were compared using Mantel-Cox log rank test and were found to differ significantly (p < .05). Along with increased survival, WS treatment also ameliorated motor performance of the transgenic mice. When assessed on an accelerating rotarod starting at 70 days of age, mice from either group displayed an initial increase in latency to fall from the rod even though performance of Veh-treated transgenic mice lagged from that of WS-treated mice. Between 90 days and 135 days of age there was a significant improvement of motor performance in the WS-treated SOD1G93A mice when compared to Veh-treated SOD1G93A mice. For mice of both groups there was a peak performance at around 120 days which was followed by a rapid decline. In contrast, age-matched wild type mice treated similarly with WS extract or Veh exhibited a gradual improvement in motor performance which peak at about 150 days of age (Fig. 1b). Loss of body weight is also considered as an important predictor of the time of initiation of the disease. The body weights of mice of each group were monitored and data were expressed as percent of peak body weight (Fig. 1c). No significant changes were observed in the body weights of the transgenic mice treated either with WS extract or Veh during almost the entire life span, except near the endpoint (150 days and beyond). Age-matched wild type controls of both treatment groups, on the other hand, showed a constant increase in body weight throughout the duration of the experiment (Fig. 1c). Also, the mean age at which SOD1G93A mice from either treatment groups attained peak body weights were not significantly different (WS-treated 110.5 ± 3.021 days; Veh-treated 115.8 ± 3.042 days; Fig. 1d). Grid- hang test performed to assess hindlimb strength showed that there was no significant difference in the latency to fall from the grid between the two groups until the age of 112 days. Beyond this age, the performance decreased more rapidly in mice of the Veh-treated group than in the WS-treated group. However, beyond 130 days, no difference was observed again (Fig. 1e). Reflex scoring was done based on the position of the hindlimbs with respect to the body with non-symptomatic mice scored as 3 and end stage mice scored as 0. Analysis showed that WS-treated SOD1G93A mice had significantly better scores between the age of 120 and 140 days in comparison to Veh-treated mice (Fig. 1f).

3.2. Treatment with WS extract reduced levels of misfolded SOD1 species and upregulated heat shock proteins

Protein aggregates containing misfolded SOD1 in motor neurons of transgenic SOD1G93A mice as well as human iALS cases constitute a feature of the disease pathology (Wood et al., 2003). In the SOD1G93A mouse, the misfolded SOD1 protein can be detected very early in age. Here, we have analyzed the levels of misfolded SOD1 in the spinal cord of mice at 125 days of age. Immunofluorescent staining of spinal cord sections of mice with an antibody (B8H10) specific for misfolded SOD1 revealed less neuronal staining for misfolded SOD1 in WS-treated group as compared to Veh- treated group (Fig. 2a). The overall B8H10 signal intensities from multiple sections in either treatment group were calculated using ImageJ software and the results clearly show a significant reduction in WS-treated group (Fig. 2b). Misfolded SOD1 species were also immunoprecipitated from spinal cord lysates using antibody B8H10 followed by immunodetection with polyclonal anti-SOD1 antibody which recognizes all forms of SOD1. Our results show that in spinal cord of WS-treated mice, misfolded SOD1 species were detected at significantly reduced levels (approximately 2.4-fold less) as compared to age-matched vehicle controls (Fig. 2c and d).

Heat shock proteins are molecular chaperons that among other functions, guide misfolded or unfolded proteins to revert to their functional conformation (Hohfeld et al., 2001). When compared to age-matched vehicle control mice, WS-treated SOD1G93A mice increased levels of Hsp-70, Hsp-60 and Hsp-27 by approximately 3.3 folds, 3.5 folds and 1.4 folds, respectively. The upregulation of heat shock proteins could be due to activation of HSF-1 which is characterized by shift in the bands on immunoblots, as it becomes phosphorylated. The monomeric HSF-1 protein is usually visualized on SDS-PAGE at about 65–75 kDa region but the phosphorylated bands are usually detected at higher levels. Our results show that even though HSF-1 was phosphorylated to some degree in Veh-treated SOD1G93A mice, WS treatment enhanced the effect which possibly led to higher expression of the heat shock proteins (Fig. 2 e and f).

3.3. WS extract conferred neuroprotection in SOD1G93A mice

As mentioned earlier, ALS is characterized by a marked loss of spinal motor neurons which could be as high as 70% in SOD1G93A mice (Julien and Kriz, 2006). To assess the number of motor neurons in spinal cords of the SOD1G93A mice, tissue sections were immunostained with the neuronal nuclear marker NeuN and motor neuron-specific marker choline acetyl transferase (ChAT). A co-staining with ChAT confirmed the identity of double positive cells as motor neurons. Motor neurons display significant heterogeneity with respect to their size, with alpha motor neurons ranging from 250 to 900 μm² (Friese et al., 2009; Misawa et al., 2012; Pasetto et al., 2017). Thus, for this study’s purpose, we have considered a minimal cut-off soma area ≥ 250 μm² for motor neurons. Immunostaining spinal cord sections of WS-treated SOD1G93A mice showed that at 125 days of age, NeuN positive large motor neurons were also strongly immune-reactive for ChAT, indicating
functional motor units. In contrast, the same type of cells in age-matched Veh-treated mice was faintly immune-reactive to ChAT (Fig. 3a-d and S1). Multiple lumbar spinal cord sections from either treatment group were stained for Nissl bodies with cresyl violet and their images were analyzed for motor neurons based on soma area using ImageJ software in the ventral horn gray matter. Results show that WS treated mice possessed more motor neurons (Mean = 12.67 ± 1.257) as compared to Veh-treated mice (Mean = 8.444 ± 0.9296) in the ventral horn of spinal cords at 125 days of age (Fig. 3e and f). Moreover, ventral roots from L4 level were sectioned and stained with Toluidine blue followed by counting of axons with diameter ≥ 4 μm which generally are alpha-motor axons (Sabado et al., 2014). It was observed that ventral roots from WS-treated mice contained a significantly higher number of motor axons as compared to Veh-treated mice (154.2 ± 4.87 vs 123.9 ± 4.626) thus providing further evidence of neuroprotective effect of WS extract (Fig. 3g-h, and S2). However, some degree of degenerating features such as axonal swelling and abnormal accumulations of organelle were present in either group. In the dorsal roots, there was not much indication of loss of sensory axons (Fig. 3i and S2).

3.4. WS treatment reduces inflammation in the spinal cord of SOD1G93A mice

Astrocytes and microglia were visualized in in spinal cord sections by immunostaining for their respective marker, the intermediate filament protein GFAP and the ionic calcium binding adapter protein 1 (Iba-1). WS treatment attenuated the reactive phenotypes of astrocytes in spinal cords of SOD1G93A mice at 125 days of age as determined by less hypertrophic cell bodies and a significant decrease in the GFAP signal intensity when compared to Veh-treated mice (Fig. 4a,b). Even though the microglial Iba-1 signal intensity was not affected by WA treatment (Fig. 4c,d), the levels of TLR2 and CD68 in spinal cords of WS-treated SOD1G93A mice significantly decreased, indicating a shift towards anti-inflammatory polarity. This was further supported by the decreased level of cyclooxygenase (COX)-2, an enzyme which also contributes to inflammation (Fig. 4g and h). WS extracts can exert anti-inflammatory effects by a multitude of mechanisms, chief among which is its inhibitory effect on p65 NF-κB activation. Evidence in support of the significance of this protein in ALS pathology has been accumulating over last few years. In the present study we observed that WS treatment significantly inhibited the phosphorylation of p65 NF-κB thereby...
preventing its activation (Fig. 4g and i).

3.5. Differential effect of WS extract on cytokine and chemokine production in the spinal cord of SOD1G93A mice

Cytokines and chemokine levels in the CNS are greatly altered in ALS (Martinez et al., 2017). For the current study we decide to use a membrane-based array from which out of 40 cytokines/chemokines that could be detected. Twenty-one cyto/chemokines were found to be significantly altered by WS treatment in SOD1G93A mice (12 upregulated and 9 downregulated). The cyto/chemokine concentrations were assessed from the spot intensities (Fig. 5a) which were then converted
to a heat-map using the Protein array analyzer macros in ImageJ thereby generating numerical values (Fig. 5b). These were then normalized with intensities of positive controls present on the membrane. Table 2 shows the per cent increase or decrease in cytokine levels in WS-treated SOD1G93A mice as compared to Veh-treated SOD1G93A mice. A comparison between mean values of each modulated cytokine is represented in Fig. 5c and d.

3.6. WS promoted autophagy in SOD1G93A mice

Autophagy is a major pathway for elimination of aggregated proteins and damaged organelles and thus plays a crucial role in cellular homeostasis in ALS. Withaferin A present in WS extract is reportedly an autophagy inducer (Hahm and Singh, 2013). In spinal motor neurons (classified on the basis of soma area) of WS-treated mice, we

Fig. 3. Neuroprotective effects of WS extract.
(a-b) NeuN and ChAT co-staining was done for identification of motor neurons in spinal cord sections from WS- and Veh-treated mice. Pictures are represented as single channel grayscale images for both NeuN and ChAT. Magnification 10×, scale = 100 μm. Selected region from ventral horn, as depicted by shadowed region on the 10× images were magnified 300% using Adobe photoshop CS5 and also represented as single channel grayscale images. (c, d) Results show that motor neurons in WS-treated spinal cords are strongly immunoreactive for ChAT in 125-day old SOD1G93A mice. In comparison, motor neurons in age-matched Veh-treated mice were faintly immunoreactive for ChAT indicating degeneration associated loss of function. The colored single channel and merged images of these representative sections are shown in supplementary fig. 1. (e) Spinal cord sections from either treatment groups were stained for Nissl and imaged at 20× magnification. Scale = 100 μm. (f) The number of motor neurons present in ventral horn each section was counted using ImageJ with minimum cut-off area set for 250 μm². Data were compiled from 3 mice per group with at least 6 sections per mouse. Statistical significance was tested with unpaired t-test with Welch's correction; *p < .05. (g, h) Morphometric analysis of motor axons was performed from toluidine blue-stained cross-sections of L4 ventral root after imaging at 20× magnification (g-upper panel). Motor axons were defined as those with diameter ≥ 4 μm and counted using ImageJ and Adobe photoshop CS5 software. Data shows presence of significantly higher number of motor axons in WS-treated mice. Data were gathered from 3 animals with a minimum of 6 sections per mouse. Statistical significance was derived using unpaired t-test with Welch's correction; ***p < .001.

Fig. 4. WS treatment attenuated glial activation and NF-κB phosphorylation.
GFAP and Iba-1 staining was done on spinal cord sections of both WS- and Veh-treated SOD1G93A mice to visualize astrocytic and microglial phenotypes, respectively. WS treatment markedly attenuated astrocytic activation in spinal cord in comparison to Veh-treated (a-b) and the overall GFAP signal intensity was significantly reduced (c). WS treatment also resulted in phenotypic alteration in Iba-1 positive microglia (d-e) even though the overall Iba-1 signal intensity did not differ significantly between the groups (f). GFAP and Iba-1 intensities were calculated using ImageJ. Data were compiled from 3 animals per group with at least 6 sections per mouse. Magnification = 40×; Scale = 50 μm. Statistical significance was calculated by unpaired t-test with Welch's correction; ***p < .001. Immunoblotting revealed reduction in levels of microglial markers Tlr2 and CD68 and inflammatory marker COX-2 (g-h). Data are representative of 3 animals per group. NF-κB phosphorylation was also found to be significantly decreased in WS-treated mice spinal cord (g-i). *p < .05, **p < .01 as estimated by unpaired t-test with Welch's correction.
observed prominent p62 positive autophagic granules (Fig. 6a-d) which were not observed in Veh-treated animals. In Veh-treated animals the signal was more diffused in the neuronal cytoplasm with presence of much smaller granules (Fig. 6e-h). We further examined the levels of other proteins associated with autophagy and found that p62 levels were significantly reduced. However, levels of Beclin-1, a key regulator of autophagy initiation, and free ATG5 or ATG5 bound with ATG12, even though moderately elevated in WS group, were not significantly different from that of Veh. This could be indicating that active autophagic processes irrespective of the treatment were being maintained. Although LC3-I levels were not much different between the treatment and control groups, LC3-II was found to be expressed at significantly higher level in WS-treated group (Fig. 6i-j). As the amount of LC3-II can be directly correlated with the number of autophagosomes (Mizushima and Yoshimori, 2007), the combined results suggest that WS treatment promoted autophagy in the SOD1G93A mice.

4. Discussion

ALS is characterized by a progressive irreversible loss of motor neurons and currently there is no cure for the disease. The only two approved drugs currently used to treat patients are Riluzol and Edaravone/Radicava which attenuates glutamate-mediated excitotoxicity and reduces oxidative stress, respectively (Miller et al., 2003; Takei et al., 2017). It is now recognized that ALS is a multifactorial disease encompassing a network of cellular pathways (Eisen, 1995; Kiernan et al., 2011). Thus, a combinatorial approach to therapy could be more effective than a single drug targeting selective pathways.

Withania somnifera is a medicinal plant whose extracts has been used for centuries, more as a rejuvenator, rather than targeted against any specific disease. However, based on its inherent broad spectrum anti-inflammatory properties, multiple studies have reported beneficial effects in different models of neurodegeneration. Withaferin A is the most studied component of the plant extract and is a potent anti-inflammatory compound. Nevertheless, since the whole plant extract consists of several other bioactive compounds, it is possible that the net therapeutic effect could arise from a concomitant or synergistic action.
of multiple molecules. Withaferin A has been previously reported to confer neuroprotection in two different mice models of fALS (Patel et al., 2015) but it could be toxic at high dose, and data concerning chronic treatment in mammalian models are not clear about potential side effects (Vaishnavi et al., 2012). In comparison, WS extracts are better tolerated at high dosages in animal models and humans with little or no side effects (Dutta et al., 2017b).

The WS treatment described here led to an increase in the life-span of SOD1<sup>G93A</sup> mice which was similar to that reported previously with Withaferin A (Patel et al., 2015), along with moderate improvement in clinical features. The WS-treated SOD1<sup>G93A</sup> mice exhibited a significantly better motor performance from the onset stage of the disease when compared to control mice. At 125 days of age, there was higher number of spinal motor neurons and of ventral root axons. WS extracts contain at least three known withanolides, apart from Withaferin A, which reportedly possess potent anti-acetylcholinesterase activity (Choudhary et al., 2004). Moreover, the spinal motor neurons of WS-treated SOD1<sup>G93A</sup> mice exhibited at 125 days of age a stronger ChAT immunoreactivity than Veh-treated SOD1<sup>G93A</sup> mice (Fig. 3c, d; S1). So, the WS treatment can contribute to maintain positivity for ChAT which is known to be reduced in ALS (Oda et al., 1995).

A most compelling evidence of neuroprotective effect of WS came
from the reduced levels of misfolded SOD1 species detected in the spinal cord of WS-treated SOD1<sup>G93A</sup> mice at 125 days of age. Two possible mechanisms may explain this reduction in levels of misfolded SOD1 species. Firstly, oxidative stress is known to contribute to ALS pathology and is one of the causes responsible for SOD1 misfolding (Rakhit et al., 2002). WS extracts are known to reduce oxidative stress in a wide variety of neuronal and non-neuronal cells (Anwer et al., 2012; Durg et al., 2015; Khan et al., 2015). Secondly, WS extracts could prevent SOD1 misfolding by inducing production of molecular chaperones that help refolding the misfolded protein or to maintain their native conformation. Heat shock proteins (Hsps) constitute a group of chaperones whose activities regarding protein structure homeostasis in ALS have been studied in detail over the years (Kalmar et al., 2014). The effects of WS extracts or its component Withaferin A on induction of Hsps are controversial. Several studies based on the use of cultured cell lines reported that WS extract or withaferin A reduce Hsps70 and 90 activities (Kataria et al., 2012; Kumar et al., 2014; Yu et al., 2010) whereas others reported that they induce the activities of Hsps70, 32 and 27 (Grogan et al., 2013; Khan et al., 2012). In our previous study, WS treatment also upregulated the levels of Hsps70 and 27/25. Interestingly, Hsps60 was also found to be significantly upregulated. Hsps60 is a mitochondrial chaperone and its role in ALS is not well elucidated. Its basal level in SOD1<sup>G93A</sup> model does not significantly differ from that of SOD1<sup>WT</sup> mice (Li et al., 2010). In TDP-43 and FUS mouse models of ALS, the TDP-43 and FUS proteins have been reported to abnormally interact with Hsps60 to induce mitochondrial damage (Khâlil and Lievens, 2017). Its upregulation could be beneficial for maintaining motor neuron mitochondrial functions, which are altered by misfolded SOD1 (Vande Velde et al., 2011).

Apart from direct neuroprotective effects, WS can modulate inflammation in the spinal cord to minimize neuronal damage. As revealed by immunofluorescent staining of spinal cord sections at 125 days of age, astrocytes exhibited a marked reduction in reactive phenotype. Moreover, WS-treated SOD1<sup>G93A</sup> mice had significantly reduced TLR2 and CD-68 expression indicating reduced microgliosis and anti-inflammation phenotype. The classical concept of M1/M2 polarity of microglia is now under scrutiny as under pathological conditions the canonical markers of M1/M2 polarization phenotypes are found to be co-expressed by single microglial cells (Mathys et al., 2017; Ransohoff, 2016). Thus, even though CD68 levels were found to be lower in WS-treated mice, it would not be accurate to infer the absence of M1 microglia in spinal cord sections. CD68 labels the lysosome and is therefore commonly considered a marker of activated phagocytic microglia, even though some expression can also be detected in resting microglia (Bodea et al., 2014; Chistiakov et al., 2016; Hopperton et al., 2017). Thus, CD68 can be detected in microglial autophagic process at the final step when an autophagosome fuses with a lysosome to form autophagolysosome, at which step the engulfed cargo is degraded. In the current context, a reduced level of CD68 is possibly indicative of non-phagocytic microglia and/or inhibited autophagolysosome formation or clearance. However, in alpha motor neurons, an autophagic mechanism seems to be active (Fig.6).

The role of NF-κB in ALS pathogenesis has been under investigation in recent years. Phosphorylation plays a critical role in the activation of NF-κB. NF-κB activation leads to alteration of expression of several genes associated with inflammation (Christian et al., 2016) and several studies based on pharmacologically or constitutively suppressed NF-κB activation in ALS models have shown beneficial outcomes (Dutta et al., 2017a; Frakes et al., 2014; Hu et al., 2018). Here, in the SOD1<sup>G93A</sup> model, activation of p65 NF-κB by phosphorylation was found to be inhibited by WS extract which could have diverse impacts. Within the nervous system, p65-NF-κB is expressed in all glial cells as well as in neurons (Kalschmidt and Kalschmidt, 2009) and we have previously reported that WS treatment is effective in reducing P65 activity both in neurons and microglia (Dutta et al., 2017a). Studies by Frakes et al. (2014) suggest that NF-κB occurs predominantly in microglia in SOD1<sup>G93A</sup> mice and that microglial NF-κB inhibition with a super repressor inhibitor of NF-κB (IκBα-SR) substantially delayed disease progression. On the other hand, NF-κB inhibition in astrocytes did not confer neuroprotection. So, it is expected that the protective effects of WS in SOD1<sup>G93A</sup> mice resulted from the attenuation of NF-κB signaling at least in part in microglia.

A common role of NF-κB is to act as transcription factor for the synthesis of cytokines and chemokines that can affect multiple cellular functions. Due to WS treatment a total of 21 cytokines and chemokines were found to be significantly deregulated in spinal cord of early-symptomatic mice. Chemotactic cytokines, including MCP-1, Eotaxin-1 (CCL11), Eotaxin-2 (CCL24), Fractalkine (CX3CL1) and lymphotactin (XCL1) were found to be expressed at higher levels upon WS treatment and these are responsible for attracting a wide array of peripheral cells such as monocytes, lymphocytes, eosinophils, T cells and basophils. Among these, MCP-1 expression is reportedly increased in ALS (Kuhle et al., 2009) and further increase due to WS treatment could help recruitment of infiltrating cells of monocyte lineage, to fight the disease (Schwartz and Baruch, 2014; Zondler et al., 2016). However, the significance of this chemotraction in the context of spinal cord is debatable as the infiltration, even though demonstrated in peripheral nerves (Chiu et al., 2009) may not be a contributing factor to overall disease pathology in ALS (Peake et al., 2017). It is to be noted that levels of two other chemotactic chemokine CX3CL1 and RANTES, whose levels are elevated in plasma as well as in cerebrospinal fluid (CSF) of ALS patients (Rentzos et al., 2007), were downregulated by WS treatment. Another interesting change was an upregulation of the adipo-kine leptin following WS treatment. Increased leptin levels have recently been reported to be associated with longer survival of ALS patients in a population-based case-control study (Nagel et al., 2017). Among the downregulated proteins in WS-treated group, apart from the chemotactic cyto/chemokines, the greatest change observed was in the level of interferon gamma (IFN-γ), a proinflammatory cytokine whose levels have been reported to be increased in ALS by multiple studies (Babu et al., 2008; Liu et al., 2015). Moreover, IFN-γ induces motor neuron death by a non-cell autonomous mechanism (Ambosser et al., 2012). WS treatment also led to the significant reduction of interleukin (IL)-1 beta, IL-3, IL-4, IL-6 and IL-12p70 levels. IL-1 beta is upregulated in ALS patients and is known to accelerate disease progression in fALS mouse models with misfolded SOD1 (Meissner et al., 2010). Likewise, higher IL-6 levels have also been reported in ALS patients and in animal models. However, decreasing IL-6 in SOD1<sup>G93A</sup> model did not confer protection (Han et al., 2016). WS-mediated decrease in IL-3 levels is likely beneficial as high levels of IL-3 indirectly led to motor neuron degeneration and ALS-like symptoms in animal models (Chavany et al., 1998). The role of IL-4 in ALS is unclear. IL-4 is usually involved in differentiation of Th0 cells to Th2, which could further secrete IL-4 themselves to regulate immune function. In SOD1<sup>G93A</sup> mice, it has been reported that Tregs suppressed microglial activation by secreting IL-4 (Zhao et al., 2012). Thus, a WS-mediated decrease in IL-4 levels could indicate either a suppression of Th0 cell differentiation or a stimulation of microglial activation. IL-12 is a 70-kDa heterodimeric cytokine composed of two disulfide-bound subunits designated p35 (35 kDa) and p40 (40 kDa). IL-12p40 is thought to be a natural antagonist of IL-12p70, which is biologically active, at least in part by competitive binding to the IL-12 receptors. However, IL-12p40 independently can act as chemoattractant for macrophage (Cooper and Khdrer, 2007). Among other cells, microglia are known to be reactive to IL-12p70 and can produce IFN-γ as a response. In fact, microglia can produce IL-12p70 too in a positive feedback loop (Kawankochi et al., 2006; Taucuik et al., 2001). Thus WS-mediated increase in IL-12p40 and decrease in the active form IL-12p70 indicates anti-inflammatory effect
but may also promote chemotherapeutics.

Autophagy is a critical cellular homeostatic mechanism with a role in clearance of misfolded or aggregated proteins. As autophagy induction has been reported in cancer cells with withaferin A, we examined if WS extract affected the autophagic pathway in the SOD1G93A model. Microscopy revealed the presence of prominent large autophagosomes in motor neurons of lumbar spinal cord from WS-treated SOD1G93A mice. In comparison, motor neurons from Veh-treated mice exhibited very few and much smaller granular structures. In spinal cord motor neurons of SOD1G93A mice the transcription factor EB (TFEB) and one its key targets, becn1, were reported to be downregulated (Chen et al., 2015). Becn1 encodes Beclin-1, which is a key regulator of autophagy initiation. Thus, a down-regulation of becn1 suggests a reduced autophagy function in motor neurons of SOD1G93A mice. In our study WS treatment resulted in an upregulation of LC3-II proteins and a downregulation of p62. Increased LC3-II levels, as mentioned above, were symptomatic, autophagosome markers in spinal cord motor neurons of SOD1G93A mice the transcription factor EB (TFEB) and one its key targets, becn1, were reported to be downregulated (Chen et al., 2015). Becn1 encodes Beclin-1, which is a key regulator of autophagy initiation. Thus, a down-regulation of becn1 suggests a reduced autophagy function in motor neurons of SOD1G93A mice. In our study WS treatment resulted in an upregulation of LC3-II proteins and a downregulation of p62. Increased LC3-II levels, as mentioned above, were observed upon autophagy induction. Thus p62 levels can be used as a marker to study autophagic flux (Bjorkoy et al., 2009). Thus our study indicates that WS may confer protection in part by promoting autophagy. A recent study reported inhibition of autophagy in motor neurons of SOD1G93A mice resulted in acceleration of disease onset (Hsueh et al., 2015). Thus, autophagy activation early on could be beneficial as it would delay disease onset but at later stages, this could be detrimental for the animals. In the present study, WS administration was initiated when animals were 50 days old, at which time its pro-autophagic effect should be protective. At about 125 days, when animals were symptomatic, autophagosomes markers in spinal cord of WS-treated animals were still elevated and results indicated an ongoing active autophagic process. Since our treatment was continued till the end stage, there is a possibility that the pro-autophagic effect might have counteracted other disease ameliorating properties of WS, thereby limiting survival increase to a modest 7.5 days.

In summary, based on its effect on multiple disease processes, WS extract is a promising therapeutic candidate for both sporadic and familial ALS but the time course of treatment needs to be carefully adapted which necessitates further studies. Furthermore, the effects of individual components of WS extract needs to be evaluated in context of this disease model. A prior study with withaferin A revealed potent anti-inflammatory effect of this compound with a life span increase on SOD1G93A mice comparable to WS extract reported here in the same mouse model. It should be pointed out that WS extract contains only minute quantity of withaferin A. Hence, it is likely that other components in WS may also have beneficial effects in ALS pathogenesis. Moreover, semi-synthetic withaferin A analogs are currently being evaluated by our group as potential therapeutics for ALS.

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Appendix A supplementary data

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