ABSTRACT

*Withania somnifera* (Solanaceae) is reported to have various biological activities including antioxidant, antitumor, antistress, anti-inflammatory, immunomodulatory, hematopoetic, anti-ageing, amylolytic, anti-depressive rejuvenating properties. Considering the antioxidant properties of WS, the aim of this study was to access the efficacy of WS in reducing lead-induced changes in mice brain. Animal exposed to lead nitrate showed significant decrease in brain SOD, CAT, GSH, GST and total protein. This was accompanied by simultaneous increase in the TBARS level. These influences of lead were prevented partially by concurrent daily administration of WS root extract. Histological examination of brain also revealed patho-physiological changes in lead nitrate exposed group and treatment with WS improved neuro histopathology.

The results thus led us to conclude that administration of WS significantly protects against lead-induced neurotoxicity. Our data suggests that WS contains active ingredients that can counteract the deleterious effects of lead nitrate.

Keywords: *Withania somnifera*, Oxidative stress, Lead nitrate, Histology, Brain

INTRODUCTION

Lead poisoning is a potential factor in brain damage, mental impairment and severe behavioral problems, as well as anemia, kidney insufficiency, neuromuscular weakness, and coma.\(^1\) Increasing concern has been expressed about the rapidly increasing level of chemicals in the environment, particularly lead, which has well known hazardous effects.\(^2\) Lead is an environment pollutant and metabolic poison with a variety of toxic effects, among which is its adverse influence on renal, hepatic and reproductive system.\(^3\) Its exposure mainly occurs through the respiratory and gastrointestinal systems. Several antioxidant molecules such as glutathione (GSH) and glutathione disulphide (GSSG) and antioxidant enzymes such as SOD, CAT, glutathione peroxidase (GPx), and glutathione reductase (GR) are the most common parameters used to evaluate lead induced oxidative damage.\(^4,5\)

A limited amount of data suggest that the biochemical and molecular mechanism of Pb toxicity involve the induction of oxidative stress in target cells, partially via activation of reactive oxygen species (ROS), followed by DNA damage and apoptosis.\(^6\) Nevertheless, indirect support for the involvement of reactive radicals in Pb toxicity has come from the studies demonstrating beneficial effects of antioxidants on Pb induced toxicity in various tissues, including kidney, brain, liver, sperm and blood cells.\(^5,6\)

*Withania somnifera* (WS) commonly known as Ashwagandha, Indian Ginseng, and Winter cherry belonging to family Solanaceae, is an important herb in Ayurvedic and indigenous medical systems for centuries in India.\(^4\) The traditional use of Ashwagandha was to increase vital fluids, muscle fat, blood, and lymph and cell production. It helps to counteract chronic fatigue, weakness, dehydration, bone weakness, loose teeth, thirst, impotency, premature aging emaciation, debility, convalescence and muscle tension.\(^7,8\)

Historically, the plant has been used as an antioxidant, adaptive and more recently to treat ulcers, bacterial infection, venom toxins and senile dementia. The roots are the main portion of the plant used therapeutically. The phytocchemicals present in WS are well known potent free radical scavengers and it has also been reported that the root extract of *Withania somnifera* tends to reverse the changes in lipid peroxidation and damage to cells.\(^9\)

Despite the fact that Ashwagandha has myriad medicinal properties, very few reports on its use in metal detoxification are available. Hence, there is a strong demand for its use in metal detoxification especially lead elimination from tissues. With this perspective in mind and the above mentioned properties of Ashwagandha, the present study was carried out to investigate the effect of hydro melathanolic root extract of *Withania somnifera* on some neurological parameters in Swiss albino mice subjected to lead nitrate.

MATERIALS AND METHODS

Chemicals

Lead nitrate was purchased from Central Drug House (India). All other chemicals used in the study were of analytical reagent grade and obtained from Sisco Research Laboratories, SD fine chemicals, HIMEDIA and Central Drug House (India), Qualigens (India/Germany).

Experimental plant

*Withania somnifera* roots were collected from Pharmacological garden of Banasthali University, Banasthali, India. The plant was identified with the help of available literature and authenticated by Botanist of Krishi Vigyan Kendra, Banasthali University, Banasthali, Tonk district.

Preparation of hydromethanolic WS root extract

The dried and powered WS roots (50g) were extracted successively with 80% methanol and 20% H₂O in a soxhlet extractor for 48 h at 60°C. After extraction, the solvent was evaporated to dryness at 40°C by using a rotary evaporator and the yield was 5g/kg was stored at 4°C. It was dissolved in distilled water whereas needed for experiment.\(^10\)

Experimental animal

Male Swiss albino mice (*Mus musculus* L) weighing approximately 15–30 g (2-2.5 months) were obtained from Haryana Agricultural University Hisar (India) for experimental purpose. The Animal Ethics Committee of Banasthali University, Banasthali, India has approved experimental protocol. They were housed in polypropylene cages in an air-conditioned room with temperature maintained at 25°C±3°C, relative humidity of 50%± 5% and 12h alternating light and dark cycles. The mice were provided with a nutritionally adequate chow diet (Hindustan lever Limited, India) and drinking water *ad libitum* throughout the study.
**Experimental design**

In the present study 36 male Swiss albino mice weighing 25-30g (3-4 months old) were used for brain biochemical parameters. For this six groups with six mice in each group were taken and treated by oral gavage once daily as follows:

Group-1: received 1ml distilled water; served as control.

Group-2: received lead nitrate (20 mg/kg body weight/day) dissolved in distilled water

Group-3 and 4: received hydromethanolic WS root extract at a dose of 200 & 500 mg/kg body weight/per day, respectively.

Group-5 and 6: received lead nitrate at a dose of 20 mg/ kg body weight/per day along with a dose of hydromethanolic WS root extract at a dose of 200 and 500 mg/kg body weight/per day, respectively.

The dose for lead nitrate and plant was decided on the basis of published reports.11,12

**Brain oxidative stress parameters**

After 42 days of treatment the mice were fasted overnight and then sacrificed under light ether anesthesia. Brain lobules were dissected out, washed immediately with ice-cold saline to remove blood, and the wet weight was noted and then stored at -80°C for various biochemical assays, and histological studies. Half of each brain was processed for biochemical analysis and the other half was used for Histopathological/histological examination.

**Biochemical analysis**

Organ (brain) was sliced into pieces and homogenized with a blender in ice-cold 0.1 M sodium phosphate buffer (pH-7.4) at 1-4°C to give 10% homogenate (w/v). The homogenate was centrifuged at 10,000 rpm for 15-20 min at 4°C twice to get enzyme traction. The resulting supernatant was separated and used for various biochemical estimations. For biochemical assays, brain was dissected out, cleaned, washed and used to determine. Lipid peroxidation (LPO),15 Super oxide dismutase (SOD),16 Catalase (CAT),17 Glutathione-S-Transferase (GST),18 Reduced Glutathione (GSH),19 and total Protein content,20 in various groups of mice.

**Histological examination**

Histological analysis of brain was done according to the method of McManus Mowry.21 Brain fragments removed from the mice were fixed in Bovins solution, dehydrated in an ethanol series, and embedded in paraffin wax for histological procedure. Brain was cut to obtain representative section of all brain lobules.

**Statistical analysis**

The data was analyzed using the statistical package for social science program (S.P.S.S.11). The results were expressed as Mean ± S.E.M (standard error of mean) and % of change- Level of significance between groups were set at P <0.05. For comparison between different experimental groups, one way analysis of variation (ANOVA) was used followed by post hoc Tukey’s test.

**RESULTS**

**Histological features of brain**

The brain histology of control mice showed well developed neurons. No vascular damage or haemorrhages were observed (Figure 1). The brain of lead treated mice revealed necrosis of tissue, vacuolization and pyknosis of nuclei. Cells were bigger in size with large vascular spaces around them (Figure 2). The Group III and IV (Withania somnifera root 200mg/kg body weight and 500 mg/kg body weight) showed no histological differences when compared with control group I (Figure 3 and 4). The combined treatment with Withania somnifera 200 mg/kg body weight and 500 mg/kg body weight along with lead nitrate resulted in some improvement but vacuolization still persist. However in the high dose, better recovery was noticed and no sign of damage was seen (Figure 5 and 6).

**Biochemical parameters**

Table 1 demonstrate the effect of lead nitrate and WS root extract either alone or in combination on lipid peroxidation (LPO), activity of antioxidant enzymes and non enzymatic antioxidant level, in control and experimental group of animals. The brain LPO level was significantly increased in lead treated mice than that of the control group (P<0.001). Administration of Withania somnifera root extract alone to mice had no significant effect on the lipid per-oxidation level as compared to untreated mice. However, treatment with hydro methanolic extract of Withania somnifera root extract along with lead caused significant reduction (P<0.001 for low and high dose) in LPO level when compared with lead nitrate group.

**Table 1: Effect of lead nitrate and withania somnifera root extract either alone or in combination on some brain biochemical variables**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>LPO (nmole MDA/g fresh wet tissue)</th>
<th>SOD (Units/mg protein)</th>
<th>CAT (umol.H₂O₂/min/mg protein)</th>
<th>GST (nmol CDNB formed/min/mg/ protein)</th>
<th>GSH (nmol GSH/gm tissue)</th>
<th>Protein (mg/g fresh wt of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>Untreated</td>
<td>90.78±2.22</td>
<td>5.26±0.30</td>
<td>41.78±2.57</td>
<td>183.85±2.10</td>
<td>231.96±2.51</td>
<td>32.35±1.14</td>
</tr>
<tr>
<td>2. LN</td>
<td>20</td>
<td>187.88±1.41</td>
<td>2.83±0.15</td>
<td>17.03±0.55</td>
<td>107.27±2.41</td>
<td>110.93±1.16</td>
<td>14.01±0.23</td>
</tr>
<tr>
<td>3. WS</td>
<td>200</td>
<td>90.91±2.25</td>
<td>5.32±0.22</td>
<td>43.97±3.06</td>
<td>197.93±3.12</td>
<td>261.34±16.30</td>
<td>22.39±0.95</td>
</tr>
<tr>
<td>4. WS</td>
<td>500</td>
<td>89.44±1.39</td>
<td>6.02±0.38</td>
<td>48.13±2.03</td>
<td>193.10±3.63</td>
<td>254.35±16.10</td>
<td>28.53±1.08</td>
</tr>
<tr>
<td>5. LN+ WS</td>
<td>20+200</td>
<td>91.22±2.09</td>
<td>5.21±0.19</td>
<td>39.19±2.52</td>
<td>170.81±2.59</td>
<td>230.02±2.57</td>
<td>24.32±1.09</td>
</tr>
<tr>
<td>6. LN+ WS</td>
<td>20+500</td>
<td>87.8±1.5</td>
<td>5.11±0.26</td>
<td>42.89±2.69</td>
<td>176.85±1.38</td>
<td>257.91±15.29</td>
<td>25.54±1.70</td>
</tr>
</tbody>
</table>

Values are Means±S.E.M; n = 6; *P<0.001 compared to normal animals; #P<0.02 compared to untreated animals; P<0.001 compared to lead exposed animals; LN: Lead nitrate

**Activity of antioxidant enzymes**

Activity of antioxidant enzymes (SOD and CAT) were significantly declined in lead treated mice as compared to control mice (P<0.001). Treatment with Withania somnifera root extract at both dose had no significant but moderate effect on antioxidant enzymes (SOD and CAT) as compared to untreated animals. Co-administration of Withania somnifera root extract with lead provided protection to SOD and CAT enzymes in groups V and VI (P<0.001 for low and high dose).

Activity of GST concentration was significantly reduced in lead treated mice than that of control group (P<0.001). However, treatment with plant root extract (low and high dose), augmented

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the GST level when compared with normal group. When *Withania somnifera* root extract was given to animals along with lead offered some protection to mice by enhancing the GST levels.

GSH concentration was significantly diminished (P<0.001) in lead group II, compared to control group in brain tissue of mice. An insignificant increase (P>0.05 for low and high dose) in the activity of non-enzymatic antioxidant GSH was observed in plant treated groups as compared to normal animals. Administration of plant root extract along with lead to animals improved GSH content compared to lead group (P<0.001 for low and high dose).

The mean value of total protein was significantly decreased by lead intake when compared with control (P<0.001). Low & high dose of plant root extract changed the total protein content in brain tissue of mice (group III & IV) as compared to untreated mice. On administration of plant root extract along with lead in both groups V and VI increased the total protein content as compared to lead group II (P<0.001 for low and high dose). It was exciting to observe that Co-current administration of plant root extract with lead nitrate offered significant protection to almost all the brain biochemical parameters.
DISCUSSION

The histology of the brain was altered by lead nitrate treatment for 42 days. Lead ions bind with –SH group in biomembranes and damage them via lipid peroxidation. Heavy metals labilize lysosomal membranes inhibit protein synthesis affects structure and synthesis of RNA and DNA and disturbs structure and function of mitochondrial membrane. In this way inorganic lead might induce cellular damage in brain. On the other hand, the ingestion of *Withania somnifera* root extract along with lead nitrate was found to revert the adverse effect of lead nitrate by improving the histological picture of brain. This might be due to presence of withanolides in the roots. Therefore *Withania* can be suggested to play an important role in alleviating lead induced toxic effects in the brain of mice.

Lead causes oxidative stress by inducing the generation of reactive oxygen species and by reducing the antioxidant cell defence systems by depleting glutathione, by inhibiting sulphhydril-dependent enzymes, by interfering with some essential metals needed for antioxidant enzyme activities, and/or by increasing cell susceptibility to oxidative attack by altering the membrane integrity and fatty acid composition. Consequently, the resulting impaired oxidant/antioxidant balance can be partially responsible for the effects of lead.

Lipid peroxidation, a basic cellular deteriorative change, is one of the primary effects induced by oxidative stress and occurs readily in the tissues due to presence of membrane rich in polyunsaturated highly oxidizable fatty acids. Several non-enzymatic antioxidant molecules (Reduced glutathione and glutathione disulphide) and antioxidant enzymes (SOD, CAT, glutathione peroxidase, and glutathione reductase) are the most common parameters used to evaluate lead induced oxidative damage. CAT and SOD are metalloproteins and accomplish their antioxidant functions by enzymatically detoxifying the peroxides (OH, H₂O₂) and superoxide anion. CAT decomposes H₂O₂ to H₂O and O₂ whereas superoxide dismutase dismutates superoxide into H₂O₂ and needs copper and zinc for its activity.

Results of the current investigation also indicate a significant alteration in the peroxidative process following lead nitrate exposure. The increase in LPO level and decrease in the endogenous antioxidant enzymes (SOD, CAT, and GST) and nonenzymatic antioxidants (GSH and protein content) were observed in the present study. The results obtained are in consistent with our previous report as well as others reports. The interesting finding is that the Aswagandha extract was able to scavenge the oxidative damage produced due to lead nitrate toxicity as evidence by decreased lipid peroxidative process and increased antioxidant status of the body. The source of proxidant during lead induced oxidative stress is not known, it is suggested that autooxidation of excessively accumulated amino levulinic acid due to inhibition of amino levulinic acid dehydratase, may result in formation of highly reactive cytotoxic compounds like oxidative free radicals like superoxide and hydrogen peroxide. The most abundant oxidative free radicals generated in living cells are superoxide anions and derivatives, particularly the highly reactive and damaging hydroxyl radical which induces peroxidation of cell membrane lipids. Glibananada and Hussain observed that the improper balance between reactive oxygen metabolites and antioxidant defence results in “oxidative stress”. Participation of iron in Fenton reaction in vivo, leading to production of iron in fenton reaction in vivo, leading to production of more reactive hydroxyl radicals from superoxide radicals and H₂O₂ results in increased lipid per oxidation. This might be one of the reasons for significant attention in LPO and significant changes in the activity of antioxidant enzymes, observed in the present study.

However, a few studies shows that superoxide radicals can also inhibit the catalase (CAT) activity and the increase H₂O₂ levels resulting from CAT inhibition could finally inhibit the SOD activity. CAT activity in tissues (liver, kidney and brain) of lead treated mice showed a dip compared to the control group. This might be due to the inhibitory action of lead on CAT.

Reduced Glutathione plays a pivotal role in the protection of cells against oxidative stress. It can act as a non-enzymatic antioxidant by direct interaction of the SH group with ROS, or it can be involved in the enzymatic detoxification reactions for ROS as a cofactor. GSH concentration in the present study suggests the utilization of glutathione by glutathione peroxidase. Glibananada and Hussain found that the GPX catalyses the oxidation of GSH to GSSG. This oxidation reaction occurs at the expense of (H₂O₂). Direct coupling of lead to GSH, which results in the formation of a GSH-lead complex that is subsequently excreted in the bile, has been demonstrated in vivo. When the activity of ALAD is impeded, an effect of lead exposure which has been confirmed experimentally by several authors, the amount of δ-ALA increase. Since δ-ALA itself is known to be a potent inducer of lipid peroxidation (LPO) and ROI formation both in vivo and in vitro, its accumulation may facilitate the depletion of GSH from lead burdened cells.

The decrease in GST activity after the exposure to Pb in the present study could be caused by Pb-induced changes in the enzyme structure as well as by the lack or insufficient amount of GSH, being a substrate for this enzyme.

In the present study, decrease in protein level was also noticed. Lead binds to plasmatic protein, where it causes alterations in high number of enzymes. Administration of *Withania somnifera* root extract along had slight effect on LPO, SOD, CAT, GST and GSH activity but no effect of plant extract was seen on protein content. However, treatment with plant root extract in two different doses (200 and 500 mg/kg body weight) along with lead decreased the lipid peroxidation in brain as compared with lead nitrate treated animals, thus indicating protective role of this plant extract in lead nitrate intoxication. Moreover elevated levels of the antioxidant enzymes (SOD, CAT and GST) and non-enzymatic potential (GSH) further support the antioxidant role of the root extract.

The hydromethanolic root extract of *Withania somnifera* contain several active ingredients. These active ingredients are well known potent free radical scavengers and it has also been reported that the root extract of *Withania somnifera* tends to reverse the changes in lipid peroxidation and damage to cells. Mechanism by which the *Withania somnifera* extract exerts a neuroprotective effect could be attributed to (i) presence of natural antioxidants, (ii) its free radical scavenging and antioxidant properties. The exact underlying mechanism is still unclear and further research is needed. From the present study it is evident that *Withania somnifera* root extract is capable in treating/preventing lead toxicity to some extent. Thus, it appears likely that these directory supplements could be beneficial for population in endemic areas against lead toxicity.

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