Prophylactic effect of *Withania somnifera* on inflammation in a non-autoimmune prone murine model of lupus

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ABSTRACT: The immunosuppressive properties of an aqueous suspension of *Withania somnifera* (WS) root powder were investigated in a pristane induced female Balb/c model of a systemic lupus erythematosus (SLE) like disease. The course of disease is initiated by peritoneal inflammation caused by pristane which results in development of SLE like symptoms, *i.e.* autoantibody production, proteinuria, and nephritis within a period of five to six months. The model of SLE was established by injecting 0.5 mL of pristane intraperitoneally into female Balb/c mice (12-18 weeks old). WS root powder (500 mg and 1,000 mg per kg body weight) was administered orally from one month prior to disease induction and for the following 6 months. Parameters of inflammation like nitric oxide (NO), Interleukin 6 and tumour necrosis factor-α and reactive oxygen species (ROS) in serum and/or ascitic fluid were measured. Prophylactic administration of WS root powder (500 mg and 1,000 mg per kg body weight) potently inhibits the pro-inflammatory cytokines, NO, and ROS in the ascitic fluid as well as in serum. Therefore, our results indicate a preventive effect of WS root powder on the mouse model of lupus.

Keywords: Systemic lupus erythematosus, *Withania somnifera*, pristane, Balb/c, prophylactic effect

1. Introduction

*Withania somnifera* (WS, Ashwagandha) (L.) Dunal (Solanaceae) is one of the most precious medicinal herbs in Ayurveda. It is generally regarded as health food and is consumed as a general tonic for rejuvenation, vitality, enhancing longevity, endurance and prevention of diseases. Therefore, for thousands of years, WS has been used to treat a wide range of conditions, for example, dyspepsia, emaciation, syphilis, anxiety, insomnia, nervous disorders, gynaecological disorders, musculoskeletal disorders, infections, asthma, male and female infertility *etc.* (1,2). The roots of WS are believed to be most potent for therapeutic purposes (3).

Studies have been conducted to investigate the immunomodulatory properties of WS. Singh *et al.* (4) have reported *in vitro* inhibition of nuclear factor-kappa B (NF-kB) and activator protein transcription factors (which play important roles in stimulating many genes involved in the inflammatory response) using a crude ethanol extract of WS (4). Suppressive effects of WS root powder on gouty arthritis and experimentally induced arthritis have also been reported (5-8). Withaferin A, a chemical constituent of WS was found to inhibit NF-kB in a cellular model of cystic fibrosis inflammation (9). Withanolide sulphoxide, another active constituent from WS roots also inhibits NF-kB along with cyclooxygenase 2 (COX-2) enzyme, which is selectively induced by proinflammatory cytokines at the site of inflammation, to restrain tumor cell progression (10).

Systemic lupus erythematosus (SLE) is an autoimmune disorder with inflammation as a main hallmark along with production of autoantibodies. Treatment is symptomatic and non-steroidal anti-inflammatory drugs are given for the initial stages of the disease. Pertaining to side effects of these drugs, herbal product(s) having the same properties would be beneficial to patients. Anti-inflammatory properties of WS root powder have been investigated in animal models of arthritis (11-14) in order to authenticate its use as a treatment drug. In a clinical study, WS in herbal formulation proved to be effective against rheumatoid arthritis, proposing its role in this autoimmune disorder (15). In order to study the prophylactic properties of WS in lupus, a model in Balb/c mice was generated by intraperitoneal injection of pristane (2,6,10,14-tetramethylpentadecane). This hydrocarbon oil induces a
strong immune response in the peritoneum of female Balb/c mice which leads to development of SLE like symptoms such as autoantibody production, arthritis, proteinuria, and glomerulonephritis (16). In view of several reports on anti-inflammatory properties of WS monitoring the prophylactic effect of this candidate drug (WS root powder) on the Balb/c model of SLE-like disease appears to be important. The present study was therefore carried out to understand this effect.

2. Materials and Methods

2.1. Animal groups

Eight female Balb/c mice (obtained from Central Animal House facility, Panjab University, Chandigarh, India) were used in the following groups: PT group, mice given 0.5 mL of pristane intraperitoneally; WST1000 group, PT mice given WS at 1,000 mg/kg body weight one month prior to pristane treatment; WST500 group, PT mice given WS at 500 mg/kg body weight one month prior to pristane treatment; and PBST group, mice given 0.5 mL sterile PBS. The present study was cleared by the institutional ethical committee, Panjab University, Chandigarh, India.

2.2. Induction of SLE-like disease (PT)

Female Balb/c mice (2-3 months old) were acclimatized for one month in a conventional animal house facility (Central Animal House Facility, Panjab University, Chandigarh, India). The mice were injected with 0.5 mL of pristane (Sigma-Aldrich, St Louis, MO, USA) intraperitoneally (17). After six months, sera and ascitic fluid were collected for further studies.

2.3. Drug regimen

Commercially available WS root powder (Dabur India Limited, Ghaziabad, India) and its aqueous suspension in 2% gum acacia was orally given at different dose levels (500 and 1,000 mg/kg body weight) one month prior to pristane injection and was continued for 6 months.

2.4. Preparation of sample for analysis

Peritoneum of mice from each group was aspirated with 1 mL of PBS. The fluid was collected and centrifuged at 1,000 × g for 10 min. The supernatant was used for detection of nitric oxide (NO), interleukin 6 (IL-6), and tumour necrosis factor-α (TNF-α). Cells in the pellet were used for measurement of reactive oxygen species (ROS).

2.5. Reactive intermediates

Parameters such as NO and ROS were assessed in ascitic fluid/serum of all the above mentioned groups at the end of the study. NO levels were assessed by measuring nitrite levels in the ascitic fluid and serum using Griess reagent (0.1% naphthylenediamine-HCl/1% sulfanilamide in 5% phosphoric acid; 1:1, v/v) (18). The pink color produced by nitrite (the stable end product of NO metabolism) with Griess reagent was read at 540 nm.

ROS generation was analysed in intraperitoneal macrophages by using dichlorofluorocein diacetate (DCFH-DA, Sigma-Aldrich) (19). Ten μL of 1 μM DCFH-DA was added to the suspension of 1 × 10^6 cells in 2 mL PBS and the mixture was incubated at 37ºC for 30 min in the dark. The suspension was pelleted by centrifuging (4ºC) at 1,000 × g for 10 min. After washing three times with PBS, the suspension was kept on ice until flow cytometric detection (BD Biosciences, San Jose, CA, USA). Data acquired was analyzed using the CELLQuest program.

2.6. Cytokine analysis

Cytokines (IL-6 and TNF-α) were measured in ascitic fluid as well as serum using ELISA kits (GEN-PROBE, Diaclone, France) according to manufacturer’s instructions. Briefly, wells of the microtiter strips coated with monoclonal antibodies specific for mIL-6 and mTNF-α were incubated with samples and biotinylated polyclonal antibodies specific for mIL-6 and mTNF-α simultaneously. Streptavidin-horseradish peroxidase and 3,3',5,5'-tetramethylbenzidene were used as the chromogen. Absorbance was measured at 450 nm as the primary wavelength and optionally at 620 nm as a reference wavelength. Data analysis was carried out using a standard curve for both the cytokines.

2.7. Autoantibody screening

Autoantibodies were detected in serum by indirect fluorescence using a kit (The Binding Site, Birmingham, UK). In brief, samples (diluted 1/20) were incubated with the substrate slides containing mouse liver, kidney, and stomach sections. After washing, fluoresceinisothiocyanate (FITC)-labelled sheep anti-mouse IgG (H + L) conjugate was added. After washing again, slides were viewed with a fluorescent microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany).

2.8. Histopathology

Kidney, spleen, liver, and lung tissues were fixed in 10% formalin and paraffin sections were stained with hematoxylin and eosin (H&E) dye before viewing them under a light microscope (Eclipse 80i; Nikon, Tokyo, Japan).
3. Results

3.1. Inflammatory response caused by pristane in the peritoneum of PT mice

Intraperitoneal injection of pristane resulted in an inflammatory response and this lead to formation of lipogranulomas. Figure 1b clearly shows serosal membranes full of lipogranulomas. Effusion of fluid in the peritoneal cavity (ascites) resulted in abdominal swelling. A significant elevation ($p < 0.0001$) of NO was observed in ascitic fluid of the PT group (34.7 ± 8.5 μM) as compared to the PBST group (1.36 ± 0.15 μM) (Figure 2a). IL-6 levels were also raised in PT (1,560 ± 401 pg/mL) as compared to PBST groups (0.65 ± 0.08 pg/mL) ($p < 0.0001$) (Figure 3a). A similar result was detected when TNF-α levels were analyzed in PT (30.2 ± 9.0 pg/mL) as compared to PBST groups (0.8 ± 0.1 pg/mL) ($p < 0.0001$) (Figure 3b). ROS levels (expressed as mean fluorescence value) were also significantly increased ($p < 0.0001$) in PT (648 ± 101) as compared to PBST groups (79.0 ± 7.8) (Figure 4).

3.2. Inflammatory response caused by pristane in the serum of PT mice

Serum nitrite levels were significantly raised ($p < 0.0002$) in the PT group (12.5 ± 4.8 μM) compared to the PBST group (2.03 ± 0.19 μM) (Figure 2b). Similar results were found when IL-6 (68.2 ± 25.5 pg/mL in PT group as compared to 8.76 ± 2.34 pg/mL in PBST group; $p < 0.0001$) and TNF-α (24.2 ± 11.0 pg/mL in PT group as compared to 6.36 ± 1.87 pg/mL in PBST group; $p < 0.0005$) levels were detected (Figures 3c and 3d). Animals from the PT group showed presence of autoantibodies in serum (Figure 5b) as compared to the PBST group (Figure 5a). Figure 5b demonstrates peripheral and diffused staining of the nuclei of mouse kidney tubular cells depicting autoantibodies against ds-DNA and histones.

3.3. Anti-inflammatory effect of WS treatment in peritoneal cavity

After seven months treatment the peritoneal cavity of groups WST1000 and WST500 was found to be clear with little or no lipogranulomas (Figures 1c and 1d), however, 62.5% of the animals still showed some small round calcified material in the gut. Ascitic fluid was virtually absent in both the WST1000 and WST500 groups. A significant reduction in peritoneal nitrite levels of the WST1000 group (2.40 ± 1.04 μM) and the WST500 group (2.35 ± 1.02 μM) (Figure 2a) compared to the PT group ($p < 0.0001$) were observed. A similar trend was observed when IL-6 and TNF-α levels were checked in WST1000 (86.9 ± 29.6 pg/mL and 7.87 ± 2.86 pg/mL, respectively) and WST500 (80.8 ± 30.4 pg/mL and 9.76 ± 3.32 pg/mL, respectively) groups compared to the PT group ($p < 0.0001$) (Figures 3a and 3b). A significant reduction in ROS levels was observed.

Figure 1. Lipogranulomas on serosal membrane separating gut organs from lungs and heart. (a) PBST, (b) PT, (c) WST1000, (d) WST500.

Figure 2. Nitrite levels of ascitic fluid (a) and serum (b) in PBST, PT, WST1000, and WST500. Values are expressed as mean ± S.D. ($n = 8$). * Significant difference from PBST. Significant difference from PT group.
Figure 5. ROS levels in the peritoneal macrophages of various animal groups. (a-d) Flow cytometric profiles for PBST (a), PT (b), WST1000 (c), and WST500 (d). (e) Graphical representation of mean fluorescence obtained using DCFH-DA stained peritoneal macrophages. Values are expressed as mean ± S.D. (n = 8). * Significant difference from PBST. # Significant difference from PT group.
in both WST1000 (125 ± 54) and WST500 (118 ± 35) groups when compared to the PT group (648 ± 101) ($p < 0.0001$) (Figures 4c and 4d). Nitrite, IL-6, TNF-α, and ROS levels were still significantly elevated in WST1000 and WST500 groups as compared to the PBST group ($p < 0.05$).

3.4. Anti-inflammatory effect of WS in serum

Serum nitrite level was reduced significantly in WST1000 (7.46 ± 2.40 μM) and WST500 (8.04 ± 2.23 μM) groups as compared to the PT group ($p < 0.02$ and $p < 0.03$, respectively) (Figure 2b). IL-6 and TNF-α levels were also decreased significantly ($p < 0.05$) in both groups WST1000 (34.1 ± 10.9 pg/mL and 7.87 ± 2.86 pg/mL, respectively) and WST500 (38.5 ± 11.7 pg/mL and 9.76 ± 3.32 pg/mL, respectively) as compared to the PT group (Figures 3c and 3d). However, autoantibodies were still detected in both WST1000 and WST500 groups (Figures 5c and 5d, respectively). As compared to the PBST group, the levels of nitrites, IL-6, and TNF-α levels were significantly elevated in WST1000 and WST500 groups ($p < 0.05$).

3.5. Effect of WS on histopathological alterations caused due to pristane

Histology of kidney, spleen, liver, and lung was found to be altered in PT mice. The treatment groups (WST1000 and WST 500) showed a radical effect on the pristane induced histopathologic alterations. Figure 6 demonstrates histomicrographs of the above tissues in PT, WST1000 and WST 500 groups. Kidneys of the PT group showed focal inflammation in the interstitium (Figure 6a). The inflammatory infiltrate is shown with an arrow. Figure 6b shows normal kidney pathology as observed in WST1000 and WST500 groups. The spleen of PT group animals depicts reactive enlargement of follicles in the lower half of the spleen (marked with arrows; Figure 6c) while normal histology of spleen was observed in WST1000 and WST500 groups (Figure 6d). Figure 6e shows one of the necrotic areas in liver of PT mice depicting inflammatory cells along with oil droplets. Surrounding the necrotic area, Kupffer cell (KC) hyperplasia along with reactive enlargement of liver nuclei (NC) was observed (as shown with arrows). Figure 6f depicts normal liver without any focus of inflammation as found in WST1000 and WST500 groups. Figure 6g illustrates a micrograph of lung with infiltration of mononuclear cells which are predominantly plasma cells in the PT group. Figure 6h shows normal histology of lung as observed in WST1000 and WST500 groups.

4. Discussion

Anti inflammatory properties of WS root powder have been widely investigated in animal models (5-7, 11-14), however its role in SLE still needs attention. The data observed in the present study revealed anti-inflammatory activity of the WS root powder in the mice model of this complex autoimmune disorder.

When introduced in peritoneum pristane induces a strong immune response leading to formation of lipogranulomas which remain adherent to the serosal surface of the peritoneal cavity. Interestingly, after seven months of oral WS root powder treatment, the lipogranulomas were reduced or disappeared from the peritoneal cavity of mice at both dose levels. As suggested by Shaheen et al. (20), the macrophages in the lipogranuloma are the likely source of several cytokines that are critical for the development of autoimmunity in the pristane induced lupus model (particularly IL-6). It has been reported earlier that IL-6 deficient Balb/c mice when injected with pristane could not develop plasmacytomas (21), highlighting its significant role in disease development. Pro-inflammatory cytokines such as IL-6 and TNF-α were analyzed as they are known to be responsible for the production of autoantibodies in lupus (22). IL-6 plays a significant role not only in antibody generation (23), but also in triggering helper and cytotoxic T cells (24).
The synergic effects of IL-6 and TNF-α further worsen the situation by elevating inflammation, far from the site of action. The current study depicts this picture in the PT group of animals. The WS1000 and WS500 treated groups however showed a significant lowering of the above mentioned cytokines. It has therefore been suggested that any molecule/drug than can suppress or block these cytokines can be used as a means of therapy.

In the present study, WS root powder in both 500 as well as 1,000 mg/kg body weight doses could bring about reduction in the levels of IL-6 as well as TNF-α whereas untreated the PT group continued to exhibit increased IL-6 and TNF-α. This observation suggests that reduction of these pro-inflammatory cytokines points towards the anti-inflammatory effect brought about by WS mediating inhibition of cytokine synthesis. The WS root powder treatment led to impassive autoantibody production in the PT model of lupus. This is supported by work carried out by Rasool and Varalakshmi (6) on experimentally induced (adjuvant induced arthritis) inflammation in vitro and in vivo where complement activity, lymphocyte proliferation and delayed type hypersensitivity responses were suppressed but humoral antibody response was unaltered. Other than autoantibody production, renal pathology is another hallmark of the pristane induced lupus model. WS, in the present study has clearly shown its protective effect on renal pathology as well as on histopathology of different tissues such as spleen, liver, and lung. In line with these studies, our report also deals with the role of WS root powder on NO and ROS levels. The overproduction of NO, an important signalling molecule involved in many physiological processes, has been speculated to cause abnormal lymphocyte function contributing to pathogenesis of autoimmunity (26,27). During chronic inflammation there is sustained production of ROS which is speculated to be involved in the pathogenesis of many autoimmune diseases (28). Elevated NO levels in the ascitic fluid and ROS production by peritoneal macrophages in the PT group were found to be decreased in WS1000 and WS500 groups. Sumantran et al. (29) using an explant model of in vitro cartilage damage study on patients with osteoarthritis have also shown marked reduction in NO release when treated with WS root extract. Ascitic fluid formation and abdominal swelling were also markedly reduced in both treatment groups, further confirming its action against inflammation caused by pristane. Anti-inflammatory properties of WS root powder have been widely investigated in animal models of arthritis. Clinical trials using herbal formulation of WS proved to be effective for osteoarthritis (30). Results in the present study suggest that one month pretreatment of animals with WS root powder prior to induction of the disease and subsequent treatment for another six months, had inhibitory effects on peritoneal inflammation and the resultant elevation of inflammatory parameters in serum of this model of autoimmune disorders.

Concluding the current work, pristane induced lupus depicts a model of SLE like disease in mice where NO, cytokines and ROS levels are disturbed. We have studied the prophylactic effect of WS root powder on the above mentioned model and found it to be effective against inflammatory response caused by pristane. W. somnifera was also found to be effective against the renal pathology occurring in this model. Since, autoantibody production was unaltered; we cannot articulate that W. somnifera root powder at 500 and 1,000 mg/kg body weight was able to hinder the progression of disease completely. Future studies at the molecular level are necessary to understand the biochemical processes going on in the presence or absence of this natural product.

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