PROPHYLACTIC VERSUS THERAPEUTIC EFFECT OF *WITHANIA SOMNIFERA* ON PRISTANE INDUCED PERITONEAL INFLAMMATION IN FEMALE BALB/C MICE

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Summary

Many pharmacological studies have authenticated the use of *Withania somnifera* (Ashwagandha) as a multipurpose medicinal agent. Multi-herbal formulations containing ashwagandha have been prescribed for treating musculoskeletal conditions like arthritis and rheumatism. The effectiveness of ashwagandha in these conditions may be due in part to its anti-inflammatory properties. Preventive and curative effect of *Withania somnifera* on pristane induced SLE has been reported by our lab (data under communication). In the present report, preventive and curative effect of aqueous suspension of *Withania somnifera* root powder on pristane induced inflammation in peritoneum of female Balb/c mice was compared to ensure the better treatment. *Withania somnifera* (1000 mg/500 mg per kg body weight) was given orally one month before and after the pristane injection in peritoneum to investigate preventive and curative effect respectively. Prophylactic treatment was found to be more effective than curative treatment. Our results therefore suggest possible preventive measure for people predisposed to SLE.

Key words: preventive, curative, *Withania somnifera,* pristane induced SLE, inflammation in peritoneum, female Balb/c mice

Introduction

Withania somnifera L. Dunal (Solanaceae), commonly known as ashwagandha in India is one of the most valuable medicinal herbs used for the treatment of various ailments. It has been used since ancient times without any toxic effects (1). Many pharmacological studies have authenticated its use as a multipurpose medicinal agent (2). Multi-herbal formulations containing ashwagandha have been prescribed for treating musculoskeletal conditions like arthritis and rheumatism (3). The effectiveness of ashwagandha in these conditions may be due in part to its anti-inflammatory properties. Many studies using different inflammatory models have shown preventive as well as curative effect of WS (2).

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by development of antinuclear antibodies and inflammation. There are several spontaneous and induced animal models which have contributed to the understanding of pathogenesis of SLE. Pristane (2,6,10,14-Tetramethylpentadecane) is a naturally occurring hydrocarbon oil which when injected into the peritoneal cavity of different strains of mice or rats induces SLE-like symptoms (4). The Pristane-induced female Balb/c model of SLE-like disease has facilitated the understanding of pathogenesis of environmentally induced lupus due to its close resemblance to human SLE.

The Intraperitoneal administration of pristane induces a strong immune response in the peritoneum which includes production of pro-inflammtory cytokines (chiefly IL-6) responsible for lipogranuloma/plasmacytomas formation. The formation of lipogranuloma is strongly associated with production of autoantibodies (5). The pristane induced inflammatory immune response hence, play part in development of SLE-like disease. We have reported both preventive and curative effect of WS on female Balb/c model of lupus (papers under communication). In the present study, based on our results, preventive and curative effect of WS on the pristane induced peritoneal inflammation has been compared to see the effectiveness of treatment.

In the present study, both prophylactic and curative effect of WS on pristane induced peritoneal inflammation in female Balb/c mice was investigated.

Materials and Methods

Mice

Female Balb/c mice were purchased from the central animal house, Panjab University, Chandigarh and were acclimatized in animal house condition for one month. Each group contained eight mice. Water and palette diet were given *ad libitum.*

Animal model of SLE

Mice (3-4 months old) were given single injection of 0.5 ml pristane intraperitoneally for the induction of SLE-like disease. Control mice were injected with 0.5ml of sterile phosphate buffer saline (PBS) intraperitoneally. Six months after the pristane treatment, ascitic/peritoneal fluid from mice was collected for estimation of nitric oxide (NO), IL-6 and TNF-α in fluid and ROS detection in cells.

Dose preparation: Aqueous suspension of WS pure root powder (Dabur India Limited) in 2% gum acacia was prepared at different dose levels (1000 and 500 mg/kg body weight).

Dose schedule

For prophylactic effect

WS root powder suspension in 2% gum acacia was given orally one month prior to pristane injection and was continued for 6 months.

For therapeutic effect

WS root powder suspension in 2% gum acacia was given orally (daily) from one month after pristane injection and continued till 5 months.

Reactive intermediates

Reactive intermediate such as ROS and NO were measured in macrophages/ascitic fluid/serum of all the above stated groups at the end of the study.

NO estimation: Nitric oxide levels were assessed by measuring nitrite levels (the stable end product of NO metabolism) in the ascitic fluid and serum by Griess reaction (6).

ROS estimation: ROS generation was estimated in ascitic fluid macrophages by using Dichlorofluorocein diacetate (DCFH-DA,10 μM, Sigma-aldrich) (7).

Cytokine analysis

Cytokines such as IL-6 and TNF-α were estimated in ascitic fluid as well as serum by using ELISA kits (GEN-PROBE diaclone, France).

Statistics

Data was expressed as mean ± S.D. Statistical analysis was performed by using one-way ANOVA followed by the least significant difference (LSD) test for multiple comparison.

Results

Lipogranuloma

Fig 1 illustrates lipogranulomas adherent to mesothelial lining. Fig 1a shows clear mesothelium PT mice whereas Fig 1b shows mesothelial lining full of lipogranulomas observed in PT group. WS treatment at 1000mg prophylactically (WST1000P) showed remarkable inhibitory effect on lipogranuloma as evident by clear mesothelium (Fig 1 c). Notable decrease in lipogranuloma formation was found in WST500P group (Fig 1 d). Similarly, WST1000C group also showed decreased extent of lipogranulomas on the mesothelium lining (Fig 1 e). In WST500C group lipogranuloma formation was still high (Fig 1 f).

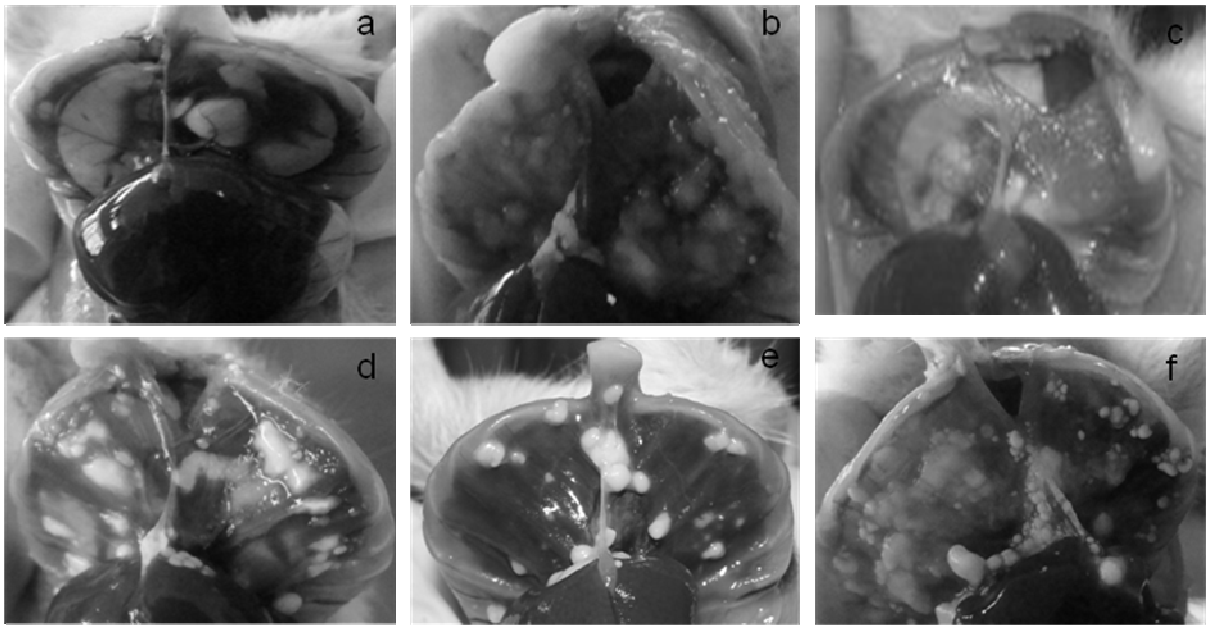

Fig 1: Photomicrograph of lipogranulomas attached to mesothelium. (a) PBST (b) PT (c) WST1000P (d) WST500P (e) WST1000C (f) WST500C.

Reactive intermediates

NO levels which were raised ($p<0.0001$) in ascitic fluid of PT group were remarkably reduced ($p<0.0001$) in ascitic fluid of all WS treated groups (Table1).

Table 1: preventive and curative effect of WS on NO levels (µmoles/L)

Treatment	Dose (mg/kg)	Group name	NO Levels (µmoles/L)
phosphate buffer saline treated		PBST	1.36±0.15
Pristane treated		PT	34.7±8.48*
WS treated preventive	1000	WST1000P	2.4±1.04#
WS treated preventive	500	WST500P	2.35±1.02#
WS treated curative	1000	WST1000C	2.67±0.86#
WS treated curative	500	WST500C	3.46±0.98#

Values are expressed as mean ± SD. (n=8). *Significant difference from control group. #Significant difference from pristane induced SLE

As depicted in Table 2 ROS levels were significantly ($p<0.0001$) raised in pristane induced SLE group as compared to control group. ROS levels in WS preventive group at both dose levels (WST1000P and WST500P) were significantly ($p<0.0001$) reduced as compared to pristane induced SLE group. Similarly, levels of ROS in WS curative groups i.e. WST1000C and WST500C were also decreased significantly ($p<0.0001$ and $p<0.001$ respectively) as compared to pristane induced SLE group. ROS levels in WST1000P and WST500P were found to be non-significantly different from the levels in control. However, levels of ROS in WS curative group at both dose levels were still significantly ($p<0.012$ and $p<0.0001$ respectively) higher than PBST group. ROS levels in WST500C group were significantly higher than in WST1000P, WST500P as well as WST1000C ($p<0.0001$). No significant difference in means was observed among WST1000P, WST500P and WST1000C.

Table 2: Preventive and curative effect of *Withania somnifera* on ROS levels measured as mean fluorescence value (MFV)

Treatment	Dose (mg/kg)	Group name	ROS levels (mean fluorescence value)
phosphate buffer saline treated		PBST	79 ± 7.65
Pristane treated		PT	648 ± 100.4*
WS treated preventive	1000	WST1000P	125.38 ± 54$^{\#}$
WS treated preventive	500	WST500P	188 ± 34.63$^{\#}$
WS treated curative	1000	WST1000C	195.3 ± 64.06$^{*\#}$
WS treated curative	500	WST500C	497 ± 151.4$^{*\#\$@!}$

Values are expressed as mean ± SD. (n=8). *Significant difference from PBST $^{\#}$Significant difference from PT, $^{\$}$ Significant difference from WST1000P, $^{@}$ Significant difference from WST500P, $^{!}$ Significant difference from WST1000C.

Pro-inflammatory cytokines

Remarkable increase ($p<0.0001$) in IL-6 levels were detected in pristane induced SLE group. The highly significant ($p<0.0001$) decrease in the levels of IL-6 was observed in all the WS treated groups (Table 3). However, the IL-6 levels were still higher in all WS treated groups as compared to control group ($p<0.003$, $p<0.005$, $p<0.0001$, $p<0.0001$ respectively). IL-6 levels in WST1000P were significantly lower than those in WST1000C and WST500C groups ($p<0.024$, $p<0.0001$ respectively) and non-significantly different from levels in WST500P. In WST500C levels were significantly higher than WST1000C group ($p<0.0001$).

Similarly, TNF-α levels were also decreased remarkably ($p<0.0001$) in all the WS treated groups; however, the levels were still significantly higher as compared to control with following p values: $p<0.016$, $p<0.003$, $p<0.0001$, $p<0.0001$ respectively Table 3. TNF-α levels in WST1000P and WST500P were significantly lower than those in WST500C ($p<0.003$, $p<0.017$ respectively) and non-significantly different from those in WST1000C. Levels of TNF-α were non-significantly different in WST1000C and WST500C groups.

Fig 3: Preventive and curative effect of WS on IL-6 and TNF-α level in ascitic fluid

Treatment	Dose (mg/kg)	Group name	IL-6 (pg/ml)	TNF-α (pg/ml)
phosphate buffer saline treated		PBST	0.65 ± 0.08	0.8 ± 0.067
Pristane treated		PT	1560.87±400.57*	30.18± 8.96*
WS treated preventive	1000	WST1000P	86.86 ± 29.56$^{\#*}$	7.87 ± 2.86$^{\#*}$
WS treated preventive	500	WST500P	80.78 ± 30.42$^{\#*}$	9.76 ± 3.32$^{\#*}$
WS treated curative	1000	WST1000C	150.65±57.65$^{\#*\$@}$	12.56 ± 5.8$^{\#*}$
WS treated curative	500	WST500C	289 ± 89.18$^{\#*\$@}$	16.54±6.34$^{\#*\$@}$

Values are expressed as mean ± SD. (n=8). *Significant difference from PBST group. $^{\#}$Significant difference from PT $^{\$}$ Significant difference from WST1000P $^{@}$ Significant difference from WST500P.

Discussion

Balb/c model of pristane induced SLE-like disease has significantly contributed to the understanding of human disease (8). Single intraperitoneal injection of 0.5 ml of pristane induces huge inflammatory response in the peritoneum including formation of lipogranulomas, elevation of proinflammtory cytokines such as IL-6 and TNF-α along with reactive intermediates such as nitric oxide (NO) and reactive oxygen species (ROS). The data observed in this study shows immense inhibitory effect of WS on the peritoneal inflammation induced by pristane.

Interestingly, prophylactic treatment of WS at 1000mg/kg exhibited strong inhibitory effect on lipogranuloma formation, clearly suggesting strong anti-inflammatory action at this dose against pristane induced inflammation in peritoneum. A study conducted by Hindawi et al (1992) found prophylactic effect of aerial parts of WS on granuloma induced by subcutaneous implantation of cotton-pellet in rats (9). The anti-granuloma activity was attributed to high content of biologically active steroids mainly withaferin A. Though, withaferin A is present in both roots and leaves of WS it is more concentrated in leaves (10). Apparently, the high content of Withaferin A in 1000 mg/kg root powder as compared to 500mg/kg root powder might be responsible for the resulted anti-lipogranuloma effect in the present study.

Development of reactive intermediates is an essential part of innate immune response. NO have been reported to be overproduced in lupus patients (11). NO levels have been found to be elevated in ascitic fluid of the pristane induced model of SLE. Studies have shown the inhibitory effect of WS on NO production (12, 13, 14). In the present study, WS potently reduced NO production prophylactically as well as curatively at both doses (1000mg and 500mg) depicting its strong inhibitory action against NO producing cells.

Increased ROS production by peritoneal macrophages in response to pristane has been reported by our lab. Recently, the inhibitory effect of WS on ROS production has been revealed by Shukla et al (2011) (15). In contrast, there are couple of reports depicting ROS inducing properties of WS and its withanolides (16, 17). In the present study, prophylactic effect of WS on ROS production at both doses was greater than the curative effect. The curative effect of WS at 500mg/kg was found to be least effective.

IL-6 levels have been associated with lipogranuloma formation and autoantibody production in pristane induced model of SLE (18, 19). In an *in vitro* study conducted by Singh et al (2007), WS was reported to inhibit NF-*κ*B and AP-1 transcription factors (TF) along with pro-inflammatory cytokines. Since, these TFs stimulate the production of these cytokines, inhibition of NF-*κ*B and AP-1 TF was thought to be responsible for suppression of pro-inflammatory cytokines. In the present study WS potently inhibited IL-6 levels prophylactic treatment being more effective than curative treatment. Similarly, TNF-α level were also found to be reduced in all the WS treated groups. Preventive (1000mg/kg and 500mg/kg) as well as curative effects (1000mg/kg) were found to be similar except curative effect at 500mg/kg.

WS possess both immunostimulatory as well as immunosuppressive properties. These contradictory actions of WS should be attributed to its adaptogenic property. An adaptogen possess an extensive range of regulatory activity but manifests its action only against the actual challenge to the system. It is amphoteric and can operate to control significant physiological processes, increasing or decreasing as needed. Therefore it was the amphoteric

nature of WS which is responsible for reduced inflammatory response in peritoneum induced by pristane. Since, SLE is characterized by hyper-immune response initiated by production of autoantibodies; intake of WS may help in reducing the fiery immune response.

Though both preventive and curative effects have been found to be effective, preventive effect was greater than the curative effect and also 1000mg/kg b. wt was the most effective dose. Our results therefore suggest possible preventive measure for people predisposed to SLE. Since our study has also shown potent therapeutic effects, clinical studies on SLE patients using WS alone or in polyherbal formulations would be intriguing.

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Conflict of interest

We declare that we have no conflict of interest.

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Abbreviations

DCFH-DA	Dichlorofluorocein diacetate
IL-6	Interleukin 6
LSD	least significant difference
NFκB	Nuclear factor kappa B
NO	Nitric oxide
PBS	Phosphate buffer saline
ROS	Reactive Oxygen Species
SD	Standard Deviation
SLE	Systemic lupus erythematosus
TNF-α	Tumour necrosis factor alpha
WS	*Withania somnifera*