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Antimutagenic activity of Ashwagandha

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Abstract

Objective: To investigate the protective effect of methanolic extract of *Withania somnifera* (Ashwagandha) on Mitomycin C induced damage in mouse bone marrow and antioxidant enzymes in liver. Materials and Methods: Protective effect of methanolic extract of Ashwagandha (250 mg/kg b.w. p.o, for 7 days) was assessed against formation of micronuclei (MN) in polychromatic cells (PCEs) and normochromatic cells (NCEs) induced by Mitomycin C (4 mg/kg b.w., i.p) using mouse bone marrow micronucleus test. The effect of Mitomycin C and Ashwagandha extract on liver enzymes, superoxide desmutase (SOD), and glutathione (GSH) was also evaluated. Results: The frequency of occurrence of micronucleated cells in bone marrow was reduced significantly when methanolic extract of Ashwagandha was administered before Mitomycin C. There was a reversal of decrease in P/N ratio. The level of SOD and GSH in liver was significantly depressed by Mitomycin C treatment and the Ashwagandha treatment enhanced level of these enzymes significantly. Conclusions: These findings suggest that Ashwagandha extract is effective in preventing DNA damage, and one of the mechanisms of action might involve scavenging of active oxygen radicals generated in reactions initiated by the mutagens.

Keywords: Ashwagandha, Antimutagens, Micronucleus test, Mitomycin

1. Introduction

Untoward mutations are associated with a number of serious diseases like cancer, aging, arthritis, cardiovascular diseases and infectious diseases [1]. Many of the pollutants released into the atmosphere and water, the residues of pesticides and toxins present in the foods and drugs, are common agents of mutagenic damage in human population. Application or routine chemoprevention of genetic damage through the use of foods and food additives having antimutagenic property is an ideal means for preventing mutagenic damage [2]. Various plants have been tested for their antimutagenic property, however, still there is a need for more effective and useful antimutagenic phytochemicals.

Withania somnifera Dunal (Solanaceae), commonly known as 'Ashwagandha' is a

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subtropical undershrub used as an ayurvedic herb. It has generated a lot of scientific interest in recent years. Roots of this plant are used in several indigenous drug preparations. The plant is attributed with curative properties against a number of diseases including cancer [3]. It is categorized as an adaptogen, used to promote health and longevity by augmenting defense against diseases, arresting the aging process, revitalizing the body in debilitated conditions, increasing the capability of the body to resist adverse environmental factors and creating a sense of mental well being [4].

The diverse pharmacological activities of this plant are due to its antioxidant activity [5]. It has also been proposed as an adjuvant during radiation therapy [6]. Further studies are needed to explore the clinical potential of this plant as a mutagen scavenger. Hence, in the present study the antimutagenic activity of methanolic extract of Ashwagandha (250 mg/kg b.w) was studied against Mitomycin C-induced clastogenic effects using *in vivo* mouse bone marrow micronucleus test. Mitomycin C is a potent mutagen, inducing clastogenic effects.

This assay is an almost complete system for measuring various genetic mishaps during mutagenesis. An attempt is also made to evaluate the antioxidant activity of the extract in terms of its effect on liver enzyme, superoxide desmutase (SOD) and glutathione (GSH) before and after Mitomycin C treatment.

2. Materials and methods

2.1 Preparation of extract

Ashwagandha roots were collected from Anju Phytochemicals Ltd, Bangalore. The roots were powdered and defatted using petroleum ether. The defatted powder was refluxed with methanol for four hours. The solvent was removed by filtration and the residue was extracted again for two hours. The extracts were then pooled together and evaporated to dryness using a rotary vacuum evaporator.

2.2 Animals

Swiss albino mice of either sex (6-8 weeks old) weighing 25±2 g, bred in our laboratory were used for the study. The animals were housed in polypropylene cages and maintained at controlled temperature (27 ±2ºC) under standard conditions of 12:12 light and dark cycle. They were fed with standard diet (Amrut Laboratory Animal Feed, Pune). Water was supplied *ad libitum*. Ethical clearance for the use of animals was obtained from the Institutional Animal Ethics Committee.

2.3 Micronucleus test

Experimental protocol suggested by Hyashi et al [7] was adopted for micronucleus test. The protocol is in accordance with OECD (Organization of Environmental Carcinogen Detection) and WHO guidelines for mutagenicity studies.

2.3.1 Treatment

The animals were divided into four groups of 18 animals each. Group I served as control. These animals were fed normal diet and were administered 0.2 ml of water by oral route for 7 days. The group II received the challenge as 4 mg/kg b.w of Mitomycin C, by i.p. route. The groups III and IV received methanolic extract of Ashwagandha (250 mg/kg) by oral route for seven consecutive days. After half an hour of the last dose of Ashwagandha, the animals of group IV received the challenge.

2.3.2 Extraction of bone marrow

From each group, 6 animals were sampled and sacrificed by cervical dislocation at 24, 48 and 72 h after the last treatment. The animals were cut open, femur and tibia were removed and the bone marrow was flushed out into a cavity block using bovine serum albumin. A suspension was made and centrifuged. The

supernatant was drawn off and the marrow was used for preparation of smear.

2.3.3 Staining and scoring of slides

The smear was made on clean slides and fixed in absolute methanol. The slides were then stained with May Grunwald's stain, diluted with phosphate buffer (pH 6.8) and Giemsa stain freshly diluted with phosphate buffer for 15 and 10 min respectively. The slides were washed with buffered water and allowed to stand undisturbed for differentiation. They were then dried and scanned for presence of micronuclei in polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs). 1000 PCEs and NCEs per animal were counted to calculate P/N ratio.

2.4 Estimation of antioxidant enzymes in liver

Liver of the animals was collected and cleaned in buffered saline (pH 7.4). 5% liver homogenates were prepared using 0.25 % sucrose in phosphate buffer and centrifuged at 500x g for 10 min.

Estimation of proteins: Standard Folin-Ciocalteu method was used to estimate the amount of proteins [8].

Estimation of enzymes: Amount of superoxide desmutase (SOD) was estimated using the method described by Beauchamp and Fridovich [9]. The amount of glutathione (GSH) was estimated using the method described by Tietze [10].

2.5 Data analysis

The results obtained for different groups at different time intervals were subjected to one way ANOVA. The individual group differences were evaluated using unpaired't' test. Data were considered significant at $p < 0.05$.

3. Results

The results of micronucleus test in terms of P/N ratio and % frequency of MN in PCEs and NCEs are shown in Tables 1, 2 and 3. The effect of Mitomycin C and Ashwagandha treatments on SOD and glutathione are depicted in Figs I and II.

P/N ratio and frequency of MN in PCEs and NCEs in control animals were found to be within the limit stated in the literature [11]. This suggests that the present laboratory conditions have not increased the frequency of spontaneous MN and, hence is considered suitable for the screening of antimutagenic compounds.

One way ANOVA indicated a significant difference in P/N ratio for different groups at all the tested intervals ($p<0.001$; ANOVA). Post hoc analysis indicated that the variation in the P/N ratio was statistically insignificant ($p>0.05$; unpaired't') at all the time intervals, when compared to the respective control group. On the other hand P/N ratio declined significantly with passage of time after the challenge ($p<0.001$; unpaired't'). The Ashwagandha treatment exhibited protective effect on the damage caused by Mitomycin. There was significant reversal of decrease in P/N ratio

Table 1
Effect of Mitomycin C and Ashwagandha on P/N ratio

Treatment	P/N ratio		
	24 h	48 h	72 h
Control	1.01±0.05	1.01±0.05	1.01±0.05
MMC	0.70±0.03***	0.64±0.01***	0.58±0.01***
WS	1.00±0.01	1.00±0.01	1.00±0.01
MMC+WS	0.79±0.03#	0.74±0.05##	0.85±0.02###

Values are mean ± SEM (n=6) Statistics: Unpaired't' test,
* Control *vs.* MMC treated group *** -p<0.001
# MMC *vs.* MMC + WS #-p<0.05, ##-p<0.01, ###-p<0.001
MMC-Mitomycin C WS-Methanolic extract of *W. somnifera*

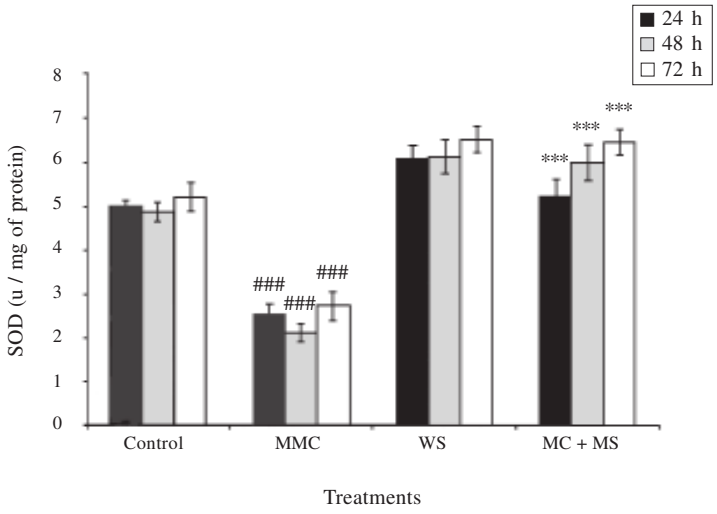

Fig 1. Effect of Ashwagandha and Mitomucin C on SOD

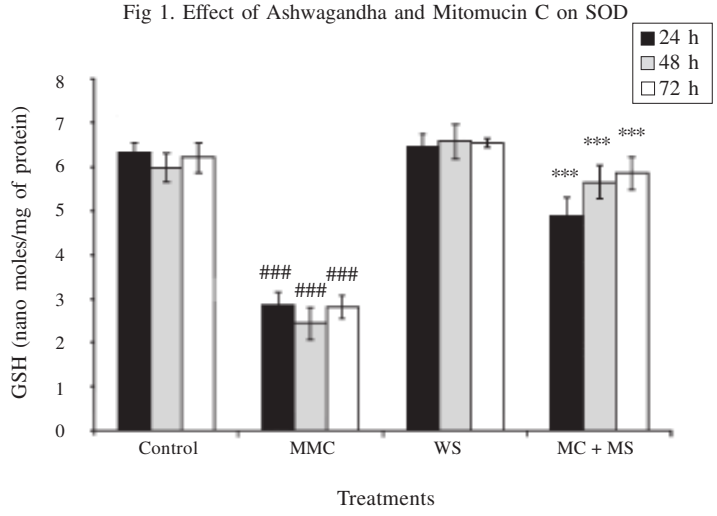

Fig 2. Effect of Ashwagandha and Mitomycin C on GSH

* Control *vs.* MMC treated group *** - p<0.001 # MMC *vs.* MMC + WS
***-p<0.001, MMC-Mitomycin C WS- Methanolic extract of Ashwagandha

(p<0.05; unpaired‘t’) at 24 h, (p<0.01; unpaired‘t’) at 48 h and (p<0.001; unpaired‘t’) at 72 h.

The frequencies of micronuclei in PCEs and NCEs in different groups are shown in Table 2 and 3 respectively. One way ANOVA indicated a significant difference for different groups at all the tested intervals (p<0.001; ANOVA). Application of unpaired’t’ test indicated no significant increase (p>0.05;unpaired‘t’) in the frequencies of MN in PCEs and NCEs in Ashwagandha-treated test animals when compared to control groups.

However, there was a significant increase (p<0.001; unpaired ‘t’) in frequency of MN in PCEs and NCEs after the challenge at all the tested intervals when compared to the control group. Ashwagandha treatment caused a significant protection against the damage caused by MMC. The % of miconuclei in PCEs decreased significantly (p<0.001; unpaired‘t’) in Ashwagandha and MMC treated group when compared to challenge treated group after all the three intervals.

However, the protective effect of the Ashwagandha treatment in NCEs was moderately significant (p<0.01; unpaired ‘t’) after 24 and 48 h, and significant (p<0.05; unpaired ‘t’) after 72 h.

One way ANOVA indicated a significant (P<0.001; ANOVA) difference in the level of SOD and GSH in different groups at all tested intervals. There was a significant (p<0.001; unpaired ‘t’) increase in the level of SOD and GSH in animals treated with Ashwagandha extract compared to control group, which may be attributed to its antioxidant property. MMC caused a significant decrease (p<0.001; unpaired ‘t’) in the antioxidant enzymes when

Table 2
Effect of Mitomycin C and Ashwagandha on frequency of micronuclei in PCEs after 24, 48 and 72 h.

Treatment	% of Micronuclei in PCE		
	24 h	48 h	72 h
Control	0.39±0.04	0.39±0.04	0.39±0.04
MMC	3.74±0.20***	4.29±0.28***	4.00±0.31***
WS	0.43±0.02	0.45±0.01	0.48±0.06
MMC+WS	1.70±0.21###	1.40±0.11###	1.34±0.22###

Values are mean ± SEM (n=6) Statistics: Unpaired't' test,
* Control *vs.* MMC treated group *** - p<0.001
# MMC *vs.* MMC + WS ### - p<0.001
MMC-Mitomycin C WS-Methanolic extract of *W. somnifera.*

Table 3
Effect of Mitomycin C and Ashwagandha on frequency of micronuclei in NCEs after 24, 48 and 72 h.

Treatment	% of Micronuclei in NCE		
	24 h	48 h	72 h
Control	0.20±0.02	0.22±0.02	0.20±0.02
MMC	1.10±0.11***	0.94±0.04***	0.78±0.12***
WS	0.23±0.01	0.33±0.02	0.23±0.02
MMC+WS	0.86±0.21##	0.62±0.22##	0.52±0.21#

Values are mean ± SEM (n=6) Statistics: Unpaired't' test,
* Control *vs.* MMC treated group *** - p<0.001
# MMC *vs.* MMC + WS #- p<0.05, ## - p<0.01
MMC-Mitomycin C WS-Methanolic extract of *W. somnifera*

compared to control group. However, the decrease was significantly reversed (p<0.001; unpaired 't') by Ashwagandha treatment.

4. Discussion

Micronuclei are chromatid/chromosome fragments that are left behind after expulsion of the main nucleus during maturation of erythroblasts to erythrocytes in the bone marrow. These represent the consequence of DNA damage caused by externally administered substances. The P/N ratio gives an index of the mitotic activity [12].

In the present study, Mitomycin C caused a significant DNA damage measured in terms of increase in frequency of MN in both PCEs and NCEs and decrease in P/N ratio of all the tested intervals, suggesting the suppression of the proliferative activity of the bone marrow. Mitomycin C is a cell cycle non-specific alkylating agent.

It is considered to be one of the most toxic drugs. It impairs DNA replication by cross-linking base pairs, particularly the G-C moiety of DNA duplex. Oxidative DNA damage has been implicated as a factor playing a role in mutagenesis and carcinogenesis.

In the present study, the level of SOD and GSH was significantly depressed (p<0.001) by Mitomycin C treatment. Hence the DNA damage by Mitomycin C may be due to depletion of antioxidant enzymes.

The Ashwagandha treatment exhibited protective effect on the damage caused by Mitomycin. Ashwagandha *per se* lacks any mutagenic potential at the dose tested and there was a significant reversal of decrease in P/N ratio and increase in frequencies of micronuclei in both PCEs and NCEs after all the three intervals. Ashwagandha is a well-known antioxidant and is reported to cause an increase in antioxidant enzymes in liver and brain of treated animals [13, 14].

In the present study also, it caused a significant increase in level of SOD and GSH in liver of treated animals. Decrease in level of SOD and GSH induced by Mitomycin C treatment was

reversed significantly by Ashwagandha treatment. These findings suggest that Ashwagandha extract is effective in preventing DNA damage, and one of the mechanisms of action might involve scavenging of active oxygen radicals, generated in reactions initiated by the mutagen.

The study hence indicates that Ashwagandha could prove to be a good natural source of a potent and relatively safe antimutagenic agent. Hence daily intake of Ashwagandha extract may provide protection against the deleterious implications of environmental or endogenous mutagens.

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