AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Immunomodulatory Effect of *Withania somnifera* (Ashwagandha) on Cyclophosphamide Induced Toxicity in Rats

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ABSTRACT

Cyclophosphamide is well known anticancer drug used for the treatment of several types of cancers. In combination with other drugs cyclophosphamide is prescribed to treat breast cancer, leukemia and ovarian cancer. But cyclophosphamide reduces the production of blood cells from the bone marrow. In the present investigation to combat the toxicity of cyclophosphamide, aqueous extract of immunomodulator plant like Ashwagandha was studied against toxicity of cyclophosphamide. After administration of cyclophosphamide @ 250 mg/kg b.w. (tablet) orally by gastric intubation method to rats, marked reduction in total count of WBC, ALC and Platelets were observed and slightly reduction in RBC was observed on day 4th. When Ashwagandha (300mg/kg b.w.) administered five days prior to cyclophosphamide administration and continued for ten days then significant increase in total count of WBC, ALC and Platelets were observed after treatment but there is no significant statistical difference in the RBC count was seen in all groups during the period of the study. Thus findings of present investigation showed that therapeutic potency of Ashwagandha ameliorate the toxicity produced during cancer chemotherapy by mitigating the bone marrow depression.

Keywords: Cyclophosphamide, Ashwagandha, Rats, Red Blood Cell, White Blood Cell, Platelets and Absolute Lymphocyte Count.

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Received 25 May 2015, Accepted 06 June 2015

Please cite this article as: Mohammad A *et al*., Immunomodulatory Effect of of *Withania somnifera* (Ashwagandha) on Cyclophosphamide Induced Toxicity in Rats. American Journal of PharmTech Research 2015.

INTRODUCTION

Cancer treatment requires surgery, chemotherapy, and radiotherapy. Chemotherapy is the primary treatment to control malignant disease, acts by killing the malignant cells (that divides rapidly, one of the main properties of cancer cell). It means chemotherapy also harms the cells that divide rapidly under normal circumstances in bone marrow. Immunosuppression is the major drawback in chemotherapy due to its toxic side effects like myelosuppression, mucosal ulceration and alopecia etc. Cyclophosphamide is a drug that is used primarily for treating several types of cancer. In order to work, cyclophosphamide first is converted by the liver into two chemicals, acrolein and phosphoramide. Acrolein and phosphoramide are the active compounds, and they slow the growth of cancer cells by interfering with the actions of deoxyribonucleic acid (DNA) within the cancerous cells. Unfortunately, normal cells also are affected, and this results in serious side effects. In addition to slowing the growth of cancerous cells, cyclophosphamide also suppresses the immune system and is referred to as immunosuppressive. The main use of cyclophosphamide is with other chemotherapy agents in the treatment of lymphomas, some forms of brain cancer, and leukemia[1] and some solid tumors[2]**.** Since cyclophosphamide itself a immunosuppressant drug and cancer patients also suffer from immunity hence it reduces the production of platelets, number of red blood cells and number of white blood cells. Cancer chemotherapy drugs are immunosuppressant's, cytotoxic, and exert variety of side effects. Botanical based immunomodulators are often employed as supportive or adjuvant therapy to overcome the undesired effects of cytotoxic chemotherapeutic agents and to restore normal health. Cytoprotection means protection of cell from noxious chemicals or other stimuli or Enhancing the ability of cell to resist injury[3]. Cytoprotective agents will reduce or prevent these toxicities, the agents should ideally be selective for normal cell versus cancer cells, these are effective in reducing or preventing toxicity should have no negative impact on anticancer therapy and have minimal adverse effect[4]. In the present investigation attempt was made to evaluate aqueous root extract of ashwagandha is used as a cytoprotective action if any, to mitigate the toxicity being produced by cyclophosphamide.

MATERIALS AND METHOD

Animals

In the present investigation, experiments were performed on 12-14 weeks old healthy charles foster rats. For the optimal growth and development, the rats were kept in ideal condition under a well regulated light and dark (12h:12h) schedule at 23±1°C in the animal house, Mahavir cancer

Institute & Research Centre, patna, India (CPCSEA Regd. No. 1129/bc/07/CPCSEA, dated 13/02/2008) and the experiment was duly approved by the IAEC. Animals were given food and water *ad libitum*.

Cyclophosphamide:

Cyclophosphamide drug in tablet form was procured from pharmacy of Mahavir cancer institute.

Ashwagandha:

Dry root of *W. somnifera* (Ashwagandha) were purchased from Haridwar Medicinal Store, Haridwar, Uttrakahand, India. The identity of the medicinal plant was confirmed by Dr. Ramakant Pandey (Botanist), Department of Biochemistry, Patna University, Patna, Bihar, India. Preparation of aqueous root extract: 10g of root powder was dissolved in 100ml of distilled water in a conical flask and boiled at 100·C in water bath for 6 hrs and then filtered through Whatmann no.1 filter paper .The filtrate was then stored at room temperature for further study.

Methodology

Study Design:

Eighteen rats were used in the study and were grouped into three groups. Group A: 6 untreated rats kept as control and served with equal volume of distilled water by gavage method. Group B: rats treated with cyclophosphamide @ 250 mg/kg b.w. Group C: Ashwagandha (300mg/kg b.w.) administered five days prior to cyclophosphamide administration and continued for ten days. This cycle was continued at interval of fifteen days. Blood extracted from control and treated group of rats on day 4^{th} after cyclophosphamide administration. In third group Ashwagandha (300mg/kg b.w.) administered five days prior to cyclophosphamide administration and blood taken on 9^{th} day (5 days prior to cyclophosphamide and 4^{th} day after cyclophosphamide) for total count of WBC, RBC and Platelets.

Collection of Blood

The blood from the control and treated rats were obtained from heart puncture. Rats were anaesthetized for this purpose. Collection of blood from heart puncture is one of the most effective methods, which causes least stress to the animal. The blood was collected in EDTA vaccutainer tube for haematological (WBC, RBC and Platelets) study. White blood cell count (WBC) - A 1:20 dilution of blood was made by adding 10 µl of blood to 200 µl of wbc diluting fluid in a plastic tube. After tightly corking the tube the suspension was well mixed by rotation. The improved Neubauer counting chamber was loaded with the diluted blood by means of pasteur pipette. The loaded counting chamber was allowed for two minutes for cells to settle, after which the preparation was viewed under the microscope 10 mm objective. The cells were counted in the 4

large corner squares of the counting chamber. The calculation of total white blood cells was made using the formula N x 2.5 x 20.

Red blood cell count (RBC):

A 1:200 dilution of blood was made by adding 10µl of blood to 2000 µl of wbc diluting fluid in a plastic tube. A clean dry improved Neubauer counting chamber with cover slip already in position was loaded with diluted blood using pasteur pipette. The chamber was left undisturbed for 2 minutes for the cells to settle. The cells were counted under the microscope using 40 mm objective. Cells were counted in 80 small squares in the central ruled area of the counting chamber. The calculation of Red blood cells was made using the formula Nx50 x 200.

Platelets Count:

Thin film of blood smear was made and stained by Leishmann's stain. Observation was made at 100 x magnification. Number of thrombocytes observed at five fields and after averaging of five fields, calculated value was multiplied by 20,000. (N x 20,000).

Absolute Lymphocyte Count:

Absolute lymphocyte count was made by multiplying the total number of WBC with percentage of lymphocyte. (ALC = Total no. of wbc x % of lymphocyte)

Statistical analysis

Data were analyzed with statistical software (Graphpad Prism 5) and values were expressed as Mean ± SEM. And differences between the groups were statistically analyzed by one-way analysis of variance (ANOVA) using the Dunnett's test.

RESULTS AND DISCUSSION

Analysis of Haematological Parameters

Table 1: remin

Parameter	Control (n = 6) (I)	Cyclophosphamide Treated (n = 6) (II)	Ashwagandha + Cyclophosphamide Treated (n = 6) (III)
RBC(10^6/ cumm)	4.093 ± 0.1116	3.717 ± 0.1763	3.790 ± 0.0327
WBC (cumm)	7300 ± 434.4	4183 ± 279.8	6350 ± 354.7
ALC (lymphs/mm3)	5165 ± 352.8	2958 ± 227.3	4634 ± 281.2
PLT (cumm)	433167 ± 19857	276167 ± 18934	321833 ± 9460

Values are expressed as Mean ± SEM, one way ANOVA followed by Dunnet's Test, Treated groups are compared with control group. RBC = Red Blood cells, WBC = White Blood Cells, ALC = Absolute Lymphocyte Count , PLT = Platelets.

There was significant statistical difference ($p < 0.001$) was observed in the WBC of cyclophosphamide treated group with compare to control. A significant increase however was seen

in the *Ashwagandha* treated group of WBC. There was also significant statistical difference ($p < 0.001$) were observed in the Absolute Lymphocyte Count (ALC), & Platelets (PLT), white blood with compare to control. A marked significant statistical increase ($p < 0.001$) was observed in the ALC, WBC, & PLT during the period of the study in the *Ashwagandha* treated group except RBC count. There was no significant statistical difference ($p>0.05$) in the RBC count was seen in all groups during the period of the study. Most of the synthetic chemotherapeutic agents available today are immunosuppressant's, cytotoxic, and exert variety of side effects that are particularly evident in cancer chemotherapy. Botanical based immunomodulators are often employed as supportive or adjuvant therapy to overcome the undesired effects of cytotoxic chemotherapeutic agents and to restore normal health. Thus in the present study the role of *Withania somnifera* as immunomodulator has been studied against cyclophosphamide toxicity. The root extract of *Withania somnifera* has been shown to have health promoting effects such as anti-stress, anti-arthiritic, anti-inflammatory, analgesic, anti-pyretic, anti-oxidant and immunomodulatory properties [5,6,7,8,9,10]. Root extract of WS was tested for immunomodulatory effects in three myelosuppression models in mice: cyclophosphamide, azathioprin, or prednisolone[11]. Significant increases in hemoglobin concentration, red blood cell count, white blood cell count, platelet count, and body weight were observed in WS-treated mice compared to untreated control mice. Similar study was also observed in my investigation that ashwagandha showed marked increases in the WBC and platelets count after bone marrow suppression induced by cyclophosphamide. The authors also reported significant increases in hemolytic antibody responses toward human erythrocytes which indicated immunostimulatory activity. The effect of WS was also studied on the functions of macrophages obtained from mice treated with the carcinogen ochratoxin A (OTA). OTA treatment of mice for 17 weeks significantly decreased the chemotactic activity of the macrophages. Interleukin-1 (IL- 1) and tumor necrosis factor alpha (TNF-α) production was also markedly decreased[12]. In different study with the aqueous suspension of WS root powder was investigated for their *in vivo* and *in vitro* immunomodulatory properties. WS showed potent inhibitory activity towards the complement system, mitogen induced lymphocyte proliferation and delayed-type hypersensitivity reaction. In a study of Gautam et al[13]. Immunopotentiation on oral feeding of standardized aqueous extract of WS was evaluated in laboratory animals immunized with DPT (Diphtheria, Pertussis, Tetanus) vaccine. Treatment of immunized animals with test material for 15 days resulted in significant increase of antibody titers to B. pertussis. Immunized animals (treated and untreated) were challenged with B. pertussis 18,323 strain and the animals were observed for 14 days. Treated animals showed significant increase in antibody titers as

compared to untreated animals after challenge. Immunoprotection against intracerebral challenge of live B. pertussis cells was evaluated based on degree of sickness, paralysis and subsequent death. Reduced mortality accompanied with overall improved health status was observed in treated animals after intracerebral challenge of B. pertussisindicating development of protective immune response. In another study, WS also stimulated immunological activity in Balb/c mice. Treatment with five doses of WS was found to enhance the total WBC count on 10th day. Bone marrow cellularity as well as alpha-esterase positive cell number also increased significantly. Treatment with WS along with the antigen (SRBC) produced an enhancement in the circulating antibody titre and the number of plaque forming cells (PFC) in the spleen. Maximum number of PFC (985 PFC/10(6) spleen cells) was obtained on the fourth day. WS inhibited delayed type hypersensitivity reaction in mice (Mantoux test). Administration of WS also showed an enhancement in phagocytic activity of peritoneal macrophages when compared to control in mice. These results confirm the immunomodulatory activity of WS extract in indigenous medicine[14]. The actions of WS on the immune system are subtler than simply suppressing the immune/ inflammatory response. WS modulates the immune response, increasing the expression of T-helper 1 (Th1) cytokines, as well as CD4 and CD8 counts, and natural killer (NK) cell activity[15,16,17]. In my investigation significant increase in ALC (Absolute Lymphocyte Count) count after ashwagandha treatment supports above study as ALC is the predictor of CD4 and CD8 count.

CONCLUSION

Cyclophosphamide is a good anti cancer drug and being used in variety cancer cases but its toxicity causes myelosuppresion mucosal ulceration and alopecia etc. The present investigation was aimed to combat the toxicity of cyclophosphamide through aqueous extract of immunomodulator plant like Ashwagandha as a adjuvant therapy. Ashwagandha (300mg/kg b.w.) administered five days prior to cyclophosphamide administration and continued for ten days then significant increase in total count of WBC, ALC and Platelets were observed after treatment but there is no significant statistical difference in the RBC count was seen in all groups during the period of the study. Thus findings of present investigation showed that therapeutic potency of Ashwagandha ameliorate the toxicity produced during cancer chemotherapy by mitigating the bone marrow depression.

ACKNOWLEDGEMENT

The authors are thankful to Mahavir Cancer Institute and Research Centre for providing the infrastructural facility for compilation of research work.

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