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RESEARCH ARTICLE

Withanolide D of Ashwagandha improves Apoptosis in the Bone Marrow of Leukemic Murine Model

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ABSTRACT

Background: Leukemia is one of the most occurring haematological pathologies in the world which develops due to the impairment in the hematopoietic machinery. Cellular death via apoptosis is severely impaired in this pathophysiological condition and leads to the progression of the disease.

Objective: We have tried to unearth the efficacy of Withanolide D, a steroidal lactone derived from *Withania somnifera* or Ashwagandha on some of the apoptotic machinery components, i.e. TERT, BCL2 and PUMA in the experimental leukemic mice.

Materials and Methods: LD₅₀ and EC₅₀ values of Withanolide D were estimated. Three groups of animals were taken for experimental purpose i.e. Group I = Leukemic, (L); Group II = Control, (C); Group III = Leukemic treated with Withanolide D, (L + WD). Group III received Withanolide D via oral route and other two groups received equal volume of distilled water. Various cytological, immunofluorescence and flow cytometric studies were taken into consideration post administration.

Result: Leukemic group showed increased cellular proliferation and decreased cellular death as compared to control. Post Withanolide D administration TERT, BCL2 and PUMA expression started to shift towards normal status. This shift in the expressional values of increased apoptosis rate and decreased cellular proliferation revealed by cytological, immunofluorescence studies and flow cytometric investigations.

Conclusion: As Withanolide D decreased the suppression of apoptosis and impaired the progression of the disease, so, we can conclude that Withanolide D of Ashwagandha may hold a promise towards a new therapeutic strategy in leukemia.

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ABBREVIATIONS

PUMA: p53 Upregulated Modulator of Apoptosis; BCL2: B-Cell Lymphoma 2; TERT: Telomerase Reverse Transcriptase; HPLC: High Performance Liquid Chromatography; ENU: N-Ethyl-N-Nitrosourea; WD: Withanolide D; LD50: Lethal Dose 50; EC50: Effective Concentration 50; MFI: Mean Fluorescence Intensity

INTRODUCTION

Deregulated haematopoiesis is the basis of Leukaemia where hematopoietic machinery gives rise to abnormal cell lineages, the hallmark of leukemic pathophysiology [1-4]. In the battle against this dreadful disease many strategies, therapeutic regimen, were formulated and implemented which bears various side effects and other complications so we turned towards Mother Nature in search of more effective, natural alternatives.

Withania somnifera or Ashwagandha is one of the most ancient and widely used herbs in traditional Indian Ayurvedic medicine mainly found in Indian subcontinent

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along with many parts of south Asia [5,6]. In Ayurvedic system this plant is used in various diseases and disorders. It is extensively studied for its biologically active constituents and contains several pharmacologically important steroidal lactones i.e. Withanolides, Glycowithanolides, Withaferins etc. Various studies on Withanolides portrayed their effect on inhibition of metastasis, angiogenesis, cell cycle arrest, cytotoxicity, apoptosis, cytoskeletal destabilization etc [7-9].

In leukemic pathophysiology suppression of apoptotic mechanism leads to progression of the disease. BCL2 or B-cell lymphoma 2 is ubiquitously expressed in all leukocytes. This protein has a pivotal role in cellular survival, cellular proliferation and activation. In leukemic condition BCL2 plays a very important role in the survival and progression of the disease by suppressing the drug induced apoptosis [10-16].

On the other hand PUMA (p53 Upregulated Modulator of Apoptosis), a member of the BH3-only protein family is widely known as potent mediator of apoptosis. A wide variety of stimuli, such as infection, toxins, cytokine withdrawal, deregulated oncogene expression, growth factor etc induces PUMA mediated apoptosis [17-20].

Another key molecule is TERT (Telomerase Reverse Transcriptase), whose expression increases in apoptosis resistant cells. Various cancer cells have been noted with high level of telomerase activity that helped in the maintenance of immortal state and decreased apoptosis rate [21-23].

In our present study we tried to unveil the efficacy of Withanolide D on the apoptotic machinery particularly focusing on PUMA, BCL2 and TERT in experimental leukemic mice which may open up a new avenue in the therapeutic modality of leukemia.

MATERIALS AND METHODS

Disease induction

Three groups of animals were taken into consideration for experimental purposes.: Group-I: Litter pups of 10-14 days weighing 4-5 gms were given intra-peritoneal injection of ENU, a potent carcinogen, at a dose of 80 mg/kg of body weight for developing leukemia (N = 60) by 6-8 months time period as confirmed by peripheral blood hemogram [24]. Group-II: Control group of mice (N = 18) received equal volume of saline in similar condition. Group III: Post development of Leukaemia these animals received Withanolide D (N = 30) by oral route for 30 days.

HPLC

20 grams of Ashwagandha powder was dissolved in 100ml of distilled water over night at 4 degree Celsius, followed by filtration under sterile condition. Filtrate was treated as 100%. This filtrate was filtered again through

0.22micron filter membrane and used for High Performance Liquid chromatography. Withanolide D (WD) was separated and collected from Ashwagandha whole extract.

Estimation of LD₅₀ and administration of Withanolide D

To determine the LD₅₀ value isolated Withanolide D was administered *via* oral route in the leukemic mice at a dose of 50, 60, 70, 75 and 80 mg/kg of bodyweight. It was found that at the dosimetry of 75 mg/kg of bodyweight 50 percent of all the experimental animals died.

To denote EC₅₀ value half of LD₅₀ value was taken into consideration. Withanolide D was administered at a dose of 27.5, 37.5 and 47.5 mg/kg of bodyweight. 50% of experimental animals showed change in their physiological, haematological and cytological parameters at a dose 37.5 mg/kg bodyweight.

So, Withanolide D was administered *via* oral route for 30days at a dose of 37.5 mg/kg of bodyweight.

Dye exclusion method

Bone marrow cells were flushed out from the femurs of experimental animals. Single cells were prepared from the extracted marrows portions. Single cell suspension was divided into two parts, one part was used for flow cytometric analysis and other for the dye exclusion method. Single cells were incubated with 0.04% Trypan blue (Sigma, USA) stain at 1:1 ratio for 10 minutes at room temperature. Post incubation cells were charged into hemocytometer (Rohem, India) for evaluation.

Immunocytochemistry

We have performed immunocytochemical studies on PFA-fixed bone marrow cells, using monoclonal antibody against PUMA, BCL2, TERT (Cell Signaling, USA) tagged with Alexa 488 (Invitrogen, USA) and DAPI to stain nucleus (Sigma, USA) for evaluating the expression pattern with and without the treatment.

Flow cytometry

Cells from the control and treated groups were fixed at room temperature in 1.5% PFA and pelleted. Fixed cells were permeabilised by resuspension with vortexing in 500 µl of chilled 90% methanol and incubated at 4°C for 15 min to 20 min. Thereafter, cells were washed twice in FACS fluid (PBS containing 1% BSA) and were divided into different sorting tubes with 1.5 x 10⁶ cells per 100 µl of fresh FACS fluid. 2 µl, anti-PUMA antibody and anti-BCL2 antibody (Cell Signaling, USA) were then added into respective sorting tubes and incubated for 30 min at 37°C. This was followed by the addition of secondary antibody Alexa 488 (Invitrogen, USA) to each primary antibody containing tubes and incubated further for 30 min at 37°C. Finally cells were washed by centrifugation and resuspended in FACS fluid

for acquisition and analysis by BD FACS Calibur (Becton-Dickinson, USA) using CellQuest Pro software (v9.1 Becton-Dickinson, USA).

Statistical evaluation

Intergroup comparison was evaluated by student's t test and for multiple comparisons one way Analysis of Variance (ANOVA) was used. All data are presented as mean $\pm$ standard deviation. Each experiment was performed three times for statistical significance. Significance was defined as $p < 0.01$.

RESULTS

Dosimetry and estimation of LD₅₀ and EC₅₀ values

Aqueous solution of Withanolide D was administered in experimental leukemic animal groups in order to estimate the LD₅₀ value. LD₅₀ refers to a dose at which 50% of all test subjects die. Previous studies have estimated LD₅₀ value of Withanolide D in normal Swiss albino mice but LD₅₀ value of Withanolide D in leukemic mice was unavailable. Withanolide D was administered at a dosimetry of 50 mg/kg, 60 mg/kg, 70 mg/kg, and 80 mg/kg of bodyweight via oral route. 100% mortality rate was noted at 80mg/kg of bodyweight dose and at 70 mg/kg of bodyweight 33.33% mortality rate was observed. A fraction between 70 and 80 mg/kg bodyweight, i.e. 75 mg/kg bodyweight was administered and 50% of all test animals died (Table 1).

Similarly effective concentration or EC₅₀ value was evaluated. Experimental animals were divided into 3 groups and they received an aqueous solution of Withanolide D via oral route. Three different doses of Withanolide D i.e. 27.5 mg/kg, 37.5 mg/kg and 47.5 mg/kg of bodyweight was administered. At a dose of 37.5 mg/kg of bodyweight Withanolide D showed maximum effect in 50% of the experimental animals (Table 2).

Summarily, at a dose of 75 mg/kg of bodyweight, Withanolide D showed severe toxicity in leukemic mice and 50 percent of animals died. The estimated Effective

concentration of Withanolide D was 37.5 mg/kg of bodyweight.

Assessment of cellular death by dye exclusion method

Post Withanolide D administration bone marrow cells were assessed for the cellular death ratio. In Control(C) group (Group II) percentage of live cell was $91 \pm 0.85\%$ and dead cell was $9 \pm 1.04\%$. On the other hand leukemic (L) group (Group I) showed increased ($p = 0.01$) survivability with a percentage of $97.78 \pm 1.12\%$ and decreased mortality, $2.22 \pm 0.95\%$. Post WD administration the percentages of live cells have decreased to $94.15 \pm 1.33\%$ and percentage dead cells increased to $5.85 \pm 0.63\%$ in group III (L+WD). Cytochemical and immunocytochemical methods revealed augmented cellular death in the treated group compared to the leukemic group (Figure 2 a-h).

Withanolide D increases pro apoptotic activity

PUMA has been widely related to pro-apoptotic function. In our study expressional level of PUMA decreased significantly in the leukemic group (MFI 138.09 ± 1.32) as compared to control (MFI 297.16 ± 2.01). Another key component is BCL2 and its expression level in the leukemic group notably increased (MFI 323.66 ± 1.36) as compared to the control group (MFI 184.95 ± 2.42). Simultaneous decline in the expressional level of PUMA and increase in BCL2 expression clearly indicated towards deregulation in apoptotic machinery and survival of the abnormal cells.

On the other hand when the leukemic animals were treated with Withanolide D the expression levels of PUMA (MFI 229.66 ± 2.11) was markedly increased & BCL2 (MFI 265.33 ± 1.98) significantly decreased ($p = 0.01$) than that of their leukemic counterpart. Administration of Withanolide D released the suppression on the apoptotic machinery and with this release rate of apoptosis increased. Increased apoptosis rate not only hindered expansion of abnormal cells but also helped bone marrow micro environment to overcome the catastrophe caused by leukemia (Figure 1 a-h).

Table 1: LD₅₀ value of Withanolide D.

Withanolide D mg/kg	Dead	Survived	Total	Rate	% Mortality	% Survival
80	4	0	6	6/0	100	0
75	3	3	6	3/3	50	50
70	2	4	6	2/4	33.33	66.66
60	1	5	6	1/5	16.66	83.33
50	0	6	6	0/6	0	100

Table 2: EC₅₀ value of Withanolide D.

Withanolide D mg/kg	Total	Activity observed	Rate	%
27.5	6	1	1/6	16.67
37.5	6	3	3/6	50
47.5	6	6	6/0	100

Figure 1 Expressional pattern of PUMA and BCL2.

a. Histogram plot describing PUMA expression among all the three groups. b. Upper row: immunofluorescence pattern of PUMA in all the three groups. Bottom row: 3D plot of the expression pattern of PUMA. c. Histogram plot describing BCL2 expression among all the three groups. d. Upper row: immunofluorescence pattern of BCL2 in all the three groups. Bottom row: 3D plot of the expression pattern of BCL2. e. Bar graph depicting expression pattern of PUMA in all the three experimental groups. f. Bar graph depicting expression pattern of BCL2 in all the three experimental groups. g. Comparison between PUMA and BCL2 expression in Control, Leukemic and Leukemic administered with Withanolide D. h. Heatmap of expressional comparison between PUMA and BCL2.

Expressional change of TERT post Withanolide D administration

In our immunochemical studies it was noted that the expressional value of TERT in leukemic condition was increased than that of control. In concurrence to our findings

other studies have showed augmented level of TERT in most tumor cells. Increased TERT levels are often associated with the proliferative capacity of somatic cells and in leukaemic condition this proliferative phenomenon helped in the progression of abnormal leukemic cells by supporting the anti apoptotic function.

Figure 2 Assessment of cellular viability and death.

a. Bar graph depicting comparison between living and dead cells in Control, Leukemic and Leukemic administered with Withanolide D. b. Heat map depicting live and dead cells among all the three experimental groups. c. Graph describing dead cell numbers in all the three groups. d. Graph describing live cell numbers in all the three groups. e. Graph portraying variation in dead cell numbers in all the three groups. f. Graph portraying variation in live cell numbers in all the three groups. g. Percentage of dead cells in all the three groups. h. Percentage of live cells in all the three groups.

Post Withanolide D administration expression of TERT shifted towards normal value and triggered the apoptosis machinery as decreased telomerase activity has been directly associated with increased vulnerability to apoptosis (Figure 4a).

DISCUSSION

Fine balance between cell proliferation and death maintains the healthy environment in an organism and any imbalance leads to abnormality and ultimately to diseased

Figure 3 Graphical representation of Withanolide D mediated modulation of apoptotic pathway.

a. Withanolide D decreased TERT and BCL2 expression and simultaneously increased PUMA expression. Augmented PUMA initiated and activated apoptotic machinery which was aided by decreased TERT and BCL2 expression. Combined action of aforementioned molecules eliminated abnormal cells, halted disease progression.

b. Graphical representation of Withanolide D mediated activation of apoptosis in leukemic bone marrow cells.

condition. In our leukemic model the balance between cellular proliferation and death was severely hindered by abnormal cellular proliferation and decreased cellular death or apoptosis. Apoptosis is an intricately tuned mechanism comprises of many key components of extrinsic and intrinsic pathway and in our experimental endeavour we have taken

three representative key components TERT, BCL2 and PUMA regarding the associated particular anti-apoptotic and pro-apoptotic functionality. In leukemic model augmented levels of TERT jammed the apoptotic machinery and helped in the survival of the abnormal cells. Increased abnormal cells led to progression of the disease. Not only increase in the anti

apoptotic and proliferative mechanism helped leukaemic progression but also pro apoptotic system is involved. PUMA is a very potent activator of apoptosis. Augmented TERT and BCL2 levels and decreased PUMA expression tipped the apoptotic system off balance. As a result abnormal cells were proliferated in large numbers. Withanolide D, when administered into the leukemic system started to interact with the key components, i.e., TERT, BCL2 and PUMA. Expressional decline of TERT and BCL2 indicated increased

vulnerability to apoptosis in the abnormal hematopoietic cells. Simultaneously increased PUMA expression also set the apoptotic system in motion. The two way pressure on the apoptotic machinery lead to cellular death. Increased death rate and decline in the proliferative action significantly decreased the disease progression (Figure 3).

As Withanolide D showed promising result, so, we can conclude that Withanolide D of Ashwagandha may hold a

Figure 4 TERT expression and HPLC isolation of Withanolide D.

a. TERT expression in bone marrow cells. Upper row depicts DAPI stained bone marrow cells of Control, Leukemic and Leukemic administered with Withanolide D. Middle row depicts Alexa 488 stained bone marrow cells of Control, Leukemic and Leukemic administered with Withanolide D. Bottom row depicts composite image of DAPI + Alexa 488 stained bone marrow cells of Control, Leukemic and Leukemic administered with Withanolide D. b. Isolation of WD from Ashwagandha via HPLC method. c. HPLC graph depicting peak of Withanolide D(WD) with structural image of WD.

promise to a cheaper, safer and easily available alternative for the therapeutic management of leukemia.

LIMITATIONS OF THE STUDY

There are many signalling molecules involved in the intrinsic and extrinsic pathway of apoptotic machinery in Leukaemia which could have given us a more elaborate view of the scenario but we have taken some representative key molecules only, for the initial study, to progress surely in future, in a broader aspect.

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