Int.J.Curr.Microbiol.App.Sci (2016) *5*(4): 713-719

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 4 (2016) pp. 713-719**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.504.082

Antigenotoxic Effect of *Withania somnifera* (Ashwagandha) Extract Against DNA Damage Induced by Hydrogen Peroxide in Cultured Human Peripheral Blood Lymphocytes

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A B S T R A C T

Hydrogen peroxide is well known to produce reactive oxygen species which cause damage to nucleic acids, proteins and disintegrate plasma membrane. It is also responsible for aging and neoplasmic progressions. The present study investigated the antigenotoxic effect of *Withania somnifera* extract against DNA damage induced by hydrogen peroxide in cultured human peripheral blood lymphocytes (PBL) using sister chromatid exchange (SCE) assay as a biomarker of genotoxicity. Heparinised fresh blood from healthy individuals was treated with hydrogen peroxide in presence of *Withania somnifera* extract in order to observe its antigenotoxic potential. A significant ($p<0.05$) dose dependent increase in frequency of SCEs was observed as the concentration of hydrogen peroxide increases. However, *Withania somnifera* (35 µg/ml and 70 µg/ml) in presence of 20 µM of hydrogen peroxide had significantly reduced ($p<0.05$) the frequencies of SCEs as compared to PBL exposed to hydrogen peroxide only. Hence, our findings suggest that *Withania somnifera* can protect against DNA damage induced by hydrogen peroxide in cultured human PBL.

Keywords

Hydrogen peroxide, *Withania somnifera*, Sister Chromatid Exchanges, DNA damage, Peripheral blood lymphocytes.

Article Info

Accepted:
22 March 2016
Available Online:
10 April 2016

Introduction

Asian continent has rich diversity of plants with great medicinal value needed to be explored for safer and effective treatment. Ayurvedic medicinal system includes a number of plants that can be used against genotoxicity. These can be considered as naturally occurring chemo preventive drugs that are safer and cost effective synthetic antioxidants. *Withania somnifera* (WS) commonly known as "Ashwagandha" is well known and extensively used traditional Ayurvedic medicine. The root smells like horse ("Ashwa") that is why it is called Ashwagandha. It is also referred as Indian ginseng, used in various musculoskeletal conditions such as arthritis, rheumatism and

also used for disease prevention in athletes as well as during pregnancy (Bone *et al*., 1996). The active ingredients of Ashwagandha include alkaloids (isopelletierine, anaferine, cuscohygrine, anahygrine, etc.), steroidal lactones (withanolides, withaferins) and saponins. (Mishra *et al*., 2000). The root extracts of Ashwagandha has been studied widely and shown anti-oxidant, anti-arthritic, anti-inflammatory, anti-stress, analgesic, anti-pyretic, and immunological properties (Davis and Kuttan, 2002, Umadevi *et al*., 2012, Gupta and Singh, 2014).

Oxidative stress involves production of reactive oxygen species (ROS) which cause damage to nucleic acids, proteins and disintegrate plasma membrane. It is also responsible for aging, neoplasmic progressions and several chronic neurogenerative diseases (Daroui *et al*., 2004). Hydrogen peroxide (H_2O_2) is well known for generating reactive oxygen species (ROS) that are responsible for DNA damage (Marilia de arruda *et al*., 1990).

Sister chromatid exchanges (SCEs) involve interchanges of DNA replication products between sister chromatids at apparently homologous loci, suggested to represent homologous recombination repair of DNA double strand breaks (Sonoda *et al*., 1999). They are analysed from 2nd division metaphases using a staining method based on sister chromatid differentiation by bromodeoxyuridine labelling. They occur as a normal event during cell cycle but due to stress conditions their frequency increases which indicated the extent of DNA damage.

Keeping in mind adverse effects of H_2O_2, the present study was focused on protective effect of Ashwagandha against H_2O_2 induced genotoxic damage in cultured human peripheral blood lymphocytes (PBL) using sister chromatid exchange assay as a biomarker of genotoxicity.

Materials and Methods

Preparation of Extract

Withania somnifera (Ashwagandha) extract was prepared from commercially available tablets. Each tablet contained1 mg of Ashwagandha. The stock solution (1mg/ml) was prepared in DMSO, sterile filtered (0.22µm) and stored at -20ºC until use.

Subjects and Sampling

5 ml venous blood was taken from healthy individuals in vacutainer tubes containing sodium heparin as an anticoagulant for lymphocyte culture set up. A consent form and questionnaire was also got filled by the donors for their health status history and consent. All the individuals participated in the study were men of age group 18 to 30 years and were bonafide resident of Haryana state, North India (Aryan race). The protocol was duly approved by human ethical committee of Kurukshetra University.

Human Lymphocyte Culture

Short term PBL cultures were set up using earlier studied technique of Moorhead *et al*. 1960 with minor modifications. Cultures were set up in duplicate by adding (0.4 ml) whole heparinized blood into 5 ml of RPMI 1640 culture medium (Himedia) containing L-glutamine (1%), fetal calf serum (20%) (Himedia), penicillin (100 UI/ml) and streptomycin (100 µg/ml) solution (Himedia), and phytohaemagglutinin (2%) (Bangalore genei). The cultures were incubated in 5 % CO_2 at 37 °C for 72 hours.

Sister Chromatid Exchange Assay

For SCE analysis, 5-bromo-2-deoxyuridine (Sigma) was added after 24 hours of

incubation in final concentration of 10 µg/ml of culture. H_2O_2 was added at the beginning of culture in concentrations ranging from 10 to 30 µM. Out of these concentrations, maximum genotoxic dose of H_2O_2 *i.e.* 20 µM was chosen to check the ameliorative effect of Ashwagandha.

To check ameliorative potential of Ashwagandha against H_2O_2, cultures were set up separately having various combinations of Ashwagandha and H_2O_2. In one set up, heparinised fresh blood was treated with 20 µM of H_2O_2 along with 35 µg/ml of Ashwagandha while in other 70 µg/ml concentrations of Ashwagandha was added against 20 µM concentration of H_2O_2.

Blood was also treated with Ashwagandha alone to check its genotoxic effects, if any. Blood without H_2O_2 and Ashwagandha served as a control while blood having Dimethylsulphoxide (DMSO) was taken as negative control. The cultures were then incubated for 72 hours at 37 °C and 5 % CO_2. Colchicine (Sigma) was added 45 minutes (min) prior to the harvesting in final concentration of 0.2 µg/ml.

The cells were harvested by centrifugation, and then treated with hypotonic solution (0.075 M KCl) and fixed in methanol: acetic acid (3:1). From a suspension of fixed cells, slides were prepared by the air drying method, and stained with Hoechst 33258 (Sigma) and 4% Giemsa stain (Himedia) solution following the method of Perry and Wolff (Perry and Wolff, 1974). For calculating the frequency of SCE per cell, 50 metaphase plates were analyzed.

Statistical Analysis

All treatments were performed in duplicates and results were expressed as means ± S.E. The student t test was used for calculating the statistical significance using SPSS 16.0.

Results and Discussion

Sister Chromatid Exchange (SCE) Analysis

In present study, SCE assay was carried out in Human PBL for evaluation of anti-mutagenic potential of Ashwagandha against H_2O_2. Treatment of Human PBL with H_2O_2 had proved its genotoxic nature as there was dose dependent increase in frequency of SCEs was observed due to change in concentration of H_2O_2 from 10 µM to 30µM (Table 1 and figure 1).

Ashwagandha had shown ameliorative effect in dose dependent manner against H_2O_2 as there was significant reduction ($P<0.05$) in frequency of SCEs ($P<0.05$) as compared to the PBL treated with H_2O_2 only (Table 2 and figure 2). Ashwagandha was also analyzed for any genotoxic effect in absence of H_2O_2 and no significant increase in SCEs was found with the doses of Ashwagandha used *i.e.* 35 and 70µg/ml, as compared to untreated PBL (Table 3).

Table.1 Mutagenic Effect of Hydrogen Peroxide

Hydrogen peroxide (µM)	Metaphase Scored	SCE/Cell ±SE
Untreated	50	2.93±0.33
10	50	3.95±0.32
15	50	4.60±0.29
20	50	7.33±±0.70[a]
30	50	No Differentiation

[a]$P<0.05$ (Significant as compared to untreated)

Table.2 Antigenotoxic Effect of Ashwagandha against H_2O_2 Induced DNA Damage

Hydrogen peroxide + Ashwagandha (µM + µg/ml)	Metaphase Scored	SCE/Cell ±SE
Untreated	50	2.93±0.33
20 + 00	50	7.33±0.70
20 + 35	50	5.13±0.48[b]
20 + 70	50	4.06±0.57[b]
DMSO (20 µl)	50	2.57±0.19

[b]P<0.05 (Significant as compared to Hydrogen peroxide treatment)

Table.3 Non-mutagenic Effect of Ashwagandha

Ashwagandha Treatment (µg/ml)	Metaphase Scored	SCE/Cell ±SE
Untreated	50	2.93±0.33
35	50	3.01±0.11[c]
70	50	3.06±0.23[c]

[c]P>0.05(Non-significant as compared to untreated)

Figure.1 Induction of SCE in Cultured Human PBL by H_2O_2

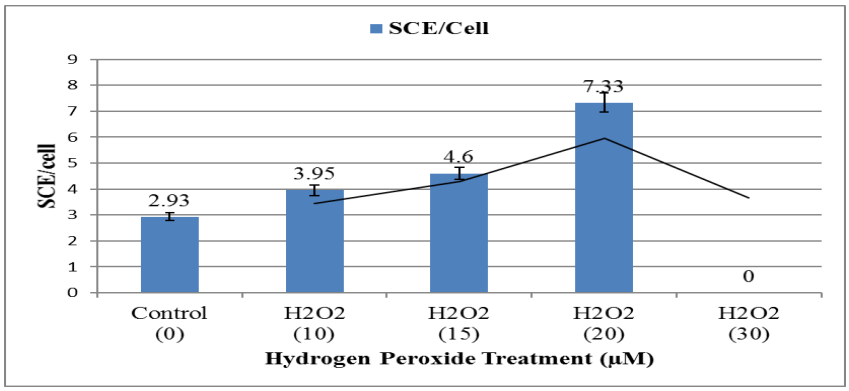
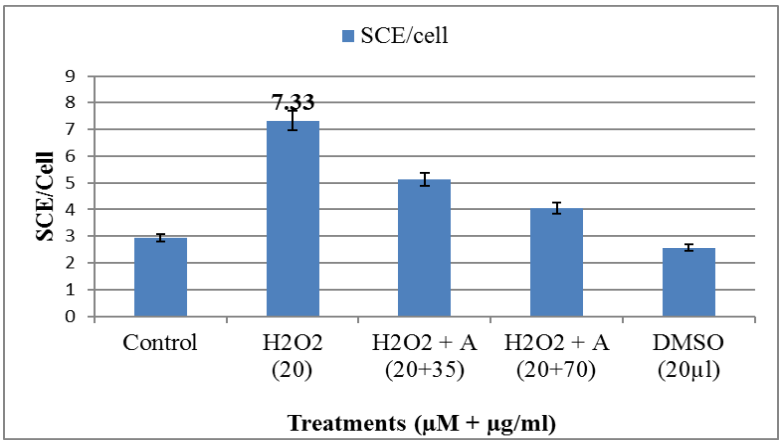


Figure.2 Reduction in SCE Frequency by Ashwagandha against H_2O_2 Treated Cultured Human PBL



(Control: Untreated, H_2O_2: Hydrogen Peroxide, A: Ashwagandha, DMSO: Dimethyl Sulphoxide)

Hence, on the basis of above findings, we suggest that hydrogen peroxide is toxic to human lymphocytes while Ashwagandha can ameliorate the DNA damage induced by hydrogen peroxide in cultured human PBL.

Genotoxicity assessment provides a tool to analyses individual susceptibility to certain chemicals used in routine. Genotoxicity found to have a direct relationship with cancer risk (Hagmar *et al.,* 1998). In present study, we found that Ashwagandha had ameliorative effect against the H_2O_2 induced genotoxicity. We observed that the PBL exposed to 10-30 µM of H_2O_2 had shown dose dependent increase in SCE frequency. Similar to our findings, several reports are available in literature supporting the genotoxicity of H_2O_2. Salvia *et al*. (1999) had found that H_2O_2 (10^{-4} M) had significantly increased the chromosomal aberrations as well as SCE in cultured PBL. Genotoxic potential of H_2O_2 was also studied by Siddique *et al*. (2009a, 2009b). They observed that 50, 100 and 200 µM of H_2O_2 had significantly increased the lipid peroxidation, SCEs and micronuclei frequency in dose dependent manner. Similarly, Beevi *et al*. (2010) also reported that H_2O_2 induced the DNA damage in human lymphocytes. They incubated the lymphocytes with series of concentrations (0-500µM) of H_2O_2 for 10 minutes and observed that treatment with H_2O_2 had reduced the cell viability in dose dependent manner. They observed apparent DNA damage at concentrations above 50 µM and maximum cell death as well as DNA damage was observed at 200 µM concentration of H_2O_2.

The results of the present study support the ameliorative role of Ashwagandha against H_2O_2. Similar to our findings, protective effects of Ashwagandha root extract against hydrogen peroxide and β-amyloid (1-42)-induced cytotoxicity in differentiated PC12 cells (Cell line derived from rat adrenal medulla) was observed by Kumar *et al.* (2010) using MTT assay. Their results suggest that pretreatments of differentiated PC12 cells with aqueous root extract of Ashwagandha had significantly protected the differentiated PC12 cells against both H_2O_2 and β-amyloid (1-42) induced cytotoxicity. Similarly, Bernhardt *et al*. (2011) also observed the antigenotoxic effect of ashwagandha against DNA damage induced by commercial grade malathion (CGM) in mice leucocytes using comet assay. They observed that leucocytes treated with ashwagandha had less damaging index (DI) and percentage of lesioned cells as compared to CGM treated leucocytes.

Application of doxorubicin (Dox) for the treatment of cancer is restricted due to its severe side effects. Fong *et al*. (2012) observed that combination of Dox with ashwagandha had significantly minimized the ill effects of Dox. Treatment of various epithelial ovarian cancer cell lines (A2780, A2780/CP70 and CaOV3) with combination of ashwagandha and Dox showed a time and dose-dependent synergistic effect on inhibition of cell proliferation and induction of cell death, thus reducing the dosage requirement of Dox. Ashwagandha leaf extract was also found to protect the albino mice against toxicity induced by lead nitrate (Kumar *et al*. 2014). They observed that pre-treatment of C6 (glioma cell line) cells with 0.1% Ashwagandha extract had significantly protect the cells against the toxicity caused by 25 μM to 400 μM of lead nitrate. They also analysed the protective effect of ashwagandha under *in vivo* conditions in young male albino rats and observed that Ashwagandha also provided a significant protection to lipid peroxidation (LPx) levels, catalase, and superoxide dismutase (SOD) in brain tissue as well as peripheral organs,

liver and kidney, suggesting its ability to act as a free radical scavenger protecting cells against toxic insult.

Present study has shown an increase in DNA damage in dose dependent manner when the PBL were treated with hydrogen peroxide. However, Ashwagandha had significantly reduced the DNA damage induced by H_2O_2 suggested the use of indigenous herbs, shrubs to develop safer medicine.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgement

This work was proposed and guided by Dr. Anita Yadav. The work was carried out in Animal Biotechnology Laboratory, Department of Biotechnology, Kurukshetra University, Kurukshetra (Haryana), India. We are thankful to all blood donors for their useful contribution in our research.

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How to cite this article:

Neeraj Kumar, Anita Yadav, Ranjan Gupta and Neeraj Aggarwal. 2016. Antigenotoxic Effect of *Withania somnifera* (Ashwagandha) Extract Against DNA Damage Induced by Hydrogen Peroxide in Cultured Human Peripheral Blood Lymphocytes. *Int.J.Curr.Microbiol.App.Sci.*5(4): 713-719. doi: http://dx.doi.org/10.20546/ijcmas.2016.504.082