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PROTECTIVE EFFECT OF NAC ON BUSULFAN INDUCED CLASTOGENESIS IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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ABSTRACT

In accordance with the previous studies N-acetyl cysteine (NAC) has been shown to be protective against a wide range of toxic compounds. As an antioxidant it has the ability to reduce the DNA damage. While the busulfan (BUS) has also been used for clinical purposes for long time but its high concentration has shown to be carcinogenic. The aim of this study is to analyse the protective effect of NAC against busulfan induced clastogenecity through chromosomal aberration (CA) analysis on human peripheral blood lymphocytes. Busulfan which is used as prior treatment for bone marrow transplantation for long time has been reported to have carcinogenic effects. Thus the potentiality of NAC against busulfan has been investigated in following seven groups: Group 1 – normal control, group 2 – Busulfan 2µg/ml, group 3 – Busulfan 4µg/ml, group 4 – 1 mM NAC + 2µg/ml BUS, group 5 – 1 mM NAC + 4µg/ml BUS, group 6 – 100 µg/ml Vit C+ 2µg/ml BUS, group 7 - 100 µg/ml Vit C+ 4µg/ml BUS. In this study group 3 showed higher frequency of CA when compared to group 1 and group 2 and thus showing clastogenecity effect. The post treatment of NAC on busulfan treated cells showed lower rate of CA and its effect has been compared with the standard Vitamin C. In this study NAC showed a protective effect on busulfan induced genotoxicity by determining the analysis of CA.

INTRODUCTION

Major source of DNA damages leading to mutation and cancer are the reactions of DNA with Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). This causes oxidative and nitrosative stress in human cells, which leads to DNA damage[1]. A significant chromosomal instability could be a risk factor for cancer[2]. Clastogenesis is characterized by the chromosomal aberration which is caused due to some mutations at genetic level. Most theories of the mechanisms of chromosomal aberrations involve the concepts of clastogens directly acting on DNA to produce strand breakage. Cellular targets affected by oxidative stress include DNA, phospholipids, proteins, and carbohydrates on the cell membrane[3]. Oxidized and injured DNA has the potential to induce genetic mutation. Antioxidants neutralize free radicals as the natural by-product of normal cell processes. Free radicals are molecules with incomplete electron shells which make them more chemically reactive than those with complete electron shells. Exposure to various environmental factors, including tobacco smoke and radiation, can also lead to free radical formation. In humans, the most common form of free radicals is oxygen. When an oxygen molecule (O_2) becomes electrically charged or "radicalized" it tries to steal electrons from other molecules, causing damage to the DNA and other molecules. Over time, such damage may become irreversible and lead to disease including cancer. Antioxidants are often described as "mopping up" free radicals, meaning they neutralize the electrical charge and prevent the free radical from taking electrons from other molecules[4]. Some of the antioxidants are betacarotene, Vitamin C, Vitamin E, Selenium, Lipoic acid, Poly MVA and Bioflavanoids.

Busulfan is a cancer drug, which was used as chemotherapeutic drug for the treatment of chronic myeloid leukemia (CML). It is an alkylating agent which is used clinically in bone marrow transplantation, chronic lymphocytic leukemia. Although it was used in medical purpose it has been reported to possess certain side effects like pulmonary fibrosis, hyperpigmentation, seizures, hepatic veno-occlusive disease (VOD),alteration of immune function and carcinogenic effect[5]. The N-AcetylCysteine (NAC) is the amino acid L-Cysteine plus an acetyl (-CO-CH_3) group attached to the amino (NH_2) group and it's a powerful antioxidant. It readily enters cells and is hydrolyzed to cysteine. N-Acetyl Cysteine was used as cancer-chemoprotective agent against lung tumours in mice[6]. NAC was reported to have the ability to function like nucleophile and as a precursor of cysteine and reduced glutathione. Hence, our present study is to evaluate the potential effects of NAC against busulfan induced clastogenecity.

MATERIAL AND METHODS

Chemicals

NAC and BUS from Sigma, RPMI-1640 and Fetal calf serum from Invitrogen, phosphate buffered saline, Phytohaemagglutinin (PHA), colchicine, potassium chloride, Tryphan blue, Giemsa stain from Himedia. All other chemicals are analytical grade.

Dose fixation

Concentration of drugs was chosen as per previous studies. The highest non toxic level of NAC on the growth of human lymphocytes culture has been reported as 10mM[7]. BUS suppresses the mitotic activity of human lymphocytes in PHA culture. Increasing BUS concentration, decrease in the number of mitoses. For appropriate culturing of lymphocytes, 5µg/mL has been observed as the highest concentration of BUS.

Experimental design

To 0.5 ml of the lymphocyte, 5ml culture medium (RPMI-1640) supplemented with NaHCO3 (7.5% (w/v)), 20% FCS, 200mM-glutamine, penicillin 100 units/ml and streptomycin100µg/ml were added in a 15ml screw cap flat bottom vials. PHA-M (0.2 ml) was added to the culture to initiate cell division. Cells were incubated at 37°C in humidified 5% CO2 atmosphere.

The following samples were considered for the experiment

Group 1 – Normal control

Group 2 – Busulfan 2µg/ml

Group 3 – Busulfan 4µg/ml

Group 4 – 1 mM NAC + 2µg/ml BUS

Group 5 – 1 mM NAC + 4µg/ml BUS

Group 6 – 100 µg/ml Vit C+ 2µg/ml BUS

Group 7 - 100 µg/ml Vit C+ 4µg/ml BUS

All the pretreatment hours was calculated from the time of colchicine addition to the samples. After treatment, cells were cultured for another 24 hours. Then, cells were arrested in metaphase by the addition of colchicine (0.2ml of 0.01%) and incubated for final 2hr 30min.

Chromosomal aberration test

The chromosomal aberration assay was carried out using conventional techniques prescribed by[8]. Human blood samples were obtained by venipuncture from non-smoking volunteer, aging from 21 to 37 years with their signed informed consent. The study was approved by the university ethical committee. Heparinized total blood (0.5mL) was added to 4.5ml medium

containing RPMI 1640, 20% inactivated fetal bovine serum, antibiotics (penicillin and streptomycin) and stimulated with 2% phytohemagglutin (PHA) and incubated for 48 h at 37°C. Human peripheral blood lymphocytes were incubated for 24 h before NAC and/or busulfan were simultaneously added to the culture medium, and left until harvest. An untreated control culture was established as well. Cultures were harvested 48 h after the treatments. Colchicine was added to the cultures at a final concentration of 0.4% µg/ml of the culture medium 21/2 hrs prior to harvesting. After 72 hr incubation, cells were carefully transferred to clean centrifuge tube and centrifuged at 1200rpm for 10 min and the pellet was washed twice with ice cold PBS (pH 7.2). To the pellet prewarmed hypotonic solution (0.075M KCL) was added and incubated at 30mins for 37ºC. After incubation, it was centrifuged at 1000rpm for 5min, the pellet was suspended in ice cold Carnoy's fixative (methanol: acetic acid in the ratio of 3:1) and incubated for an hour at 37°C. After the incubation, it was again centrifuged at same condition and the pellet was suspended in few drops of fresh fixative and a slide has been prepared.

Slide preparation

The cell suspension was dropped on to clean, chilled microscopic slides and dried gently over a hot plate. The slides were stained with 4% giemsa for 10mins after two or three days.

Scoring of slides

Randomly about 50 well spread metaphases from each sample was counted. Numerical and structural aberrations were recorded.

Analysis of chromosomal damage

In order to check whether NAC mediate any protective effect against BUS induced DNA damage, lymphocyte culture treated with BUS alone, NAC alone, simultaneous treatment of BUS and NAC and preincubation of NAC at 6 and 12 hr prior to BUS treatment were established. Chromosomal structural aberrations were scored in giemsa stained chromosomal preparation.

RESULTS AND DISCUSSION

The protective effect of NAC on busulfan induced DNA damage was investigated and to compared with vitamin-C. The metaphase analysis of human blood lymphocytes revealed number of chromosomal aberrations in human peripheral blood lymphocytes treated with busulfan. In this study NAC of 2µg/ml and 4µg/ml has been used to analyze its potential activity of against busulfan. The results showed that rate of chromosomal aberration shows very high on cells treated with busulfan alone and the cells that are treated with busulfan. However, NAC showed significantly decreased rate of chromosomal aberration.

One therapeutic effect of busulfan may be depends on the generating ROS (Reactive Oxygen Species) between non-target cells are also damaged in this process. The development of chemoprotection is not only for raising the effectiveness of cancer treatment but also for studying the underlying mechanisms of anticancer agents induced cytotoxicity. Thiol supplementation to maintain tissue redox balance has been studied by various researches. One of the mechanisms of protection is free radical scavenging and it is based on the supposition that free radicals formed from the hydrolysis of water. It is known that GSH plays a crucial role in the detoxification of drug or radiation induced oxygen free radicals. NAC, an aminothiol and synthetic precursor of intracellular cysteine and GSH, has been used for many years as a mucolytic drug. The potential of NAC on Busulfan induced clastogenecity was assessed at different concentrations employing human peripheral blood lymphocytes through CA analysis. Lymphocytes are routinely used in human bio-monitoring to assess the toxic and cytoprotective effects of diet on both DNA damage and repair. Conventional techniques for measuring chromosomal changes require proliferating cells so that chromosomes can be seen at mitosis. Hence in this study its effect showed an increased rate of chromosomal aberration in human peripheral blood lymphocytes and such carcinogenic effect of busulfan are suppressed with N-Acetyl Cysteine. Treatment with NAC showed significantly decreased rate of chromosomal aberration. In conclusion NAC on study showed protective effect against busulfan induced genotoxicity by determining the fragments of chromosomal aberrations. However further studies are needed to explore its mechanisms.

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