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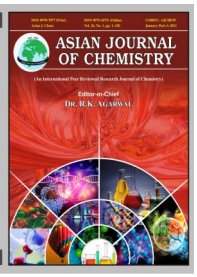

Subduing the Hepatotoxic and Urotoxic Effects of Cyclophosphamide using Natural Antioxidants

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Cyclophosphamide is a commercially popular chemotherapeutic drug and is used in the treatment of malignant and non-malignant forms of cancer. Action of hepatic microsomal enzymes on cyclophosphamide metabolizes it into phosphoramide mustard and acrolein. These are toxic and are majorly found to have adverse effects on the kidney and liver causing symptoms like dypsia, fibrosis, hemorrhagic cystitis and necropsis. Acrolein-one of the metabolites was found to interact with majority of biomolecules like DNA, RNA and proteins that may lead to cellular apoptosis. Cyclophosphamide metabolism was also known to induce the production of reactive oxygen species leading to total cellular damage, which causes various acute and chronic diseases. Herbal plants are found to be rich in antioxidant and have anticancerous and tumor reducing properties. Present study aimed in achieving modulatory action of the poly-herbal combination BC and vitamin E on cyclophosphamide induced hepato- and uro-toxicity. Wistar rats (male and female) were treated orally with the 100 mg/kg of the BC and 50 mg/kg of Vit-E for whole of the study (30 days). 200 mg/kg of cyclophosphamide was administered in 2 split doses through *i.p.* on day 20 and 21. Serum was used to analyze the clinical chemistry parameters such as total protein, albumin, bilirubin (total and direct), urea, creatinine and enzymes like AST and ALT. Parameters that were significantly altered due to cyclophosphamide administration were modulated during treatment using BC + Vit-E (BCE). By these preliminary studies, it has been hypothesized that the effects of cyclophosphamide were treated to a great extent and therapeutic effect of cyclophosphamide has been sensitized by BCE.

Keywords: Hepatotoxic, Urotoxic, Cyclophosphamide, Antioxidants.

INTRODUCTION

Cyclophosphamide (CP) commercially popular as endoxan, cytoxan, neosar, procytox, revimmune is an effective alkylating chemotherapeutic drug, which has been commercially popular for treatment of malignant and non-malignant forms of cancer. Generally cyclophosphamide is well absorbed into the system after administration, having bioavailability greater than 75 %. 5-25 % of the administered dose remains as unchanged drug, which is excreted in urine after an elimination half-life of 3 to 12 h[1]. Cyclophosphamide is principally found to bio-transform in the liver, by a mixed function microsomal oxidase system where it forms active alkylating metabolites like 4-hydroxy-cyclophosphamide and phosphoramide mustard[2]. These metabolites, especially 4-hydroxy-cyclophosphamide is thought to cross-link to tumor cell DNA, thereby interfering with the growth of rapidly proliferating malignant cells[3]. It is eliminated primarily in the urine and is found to contain several toxic and non-toxic metabolites. Adverse reactions of this drug include nausea and vomiting often followed by stomach ache, diarrhea, mouth sores, darkening of the skin/nails, hair loss or thinning of hair, lethargy or unusual tiredness accompanied by joint pain and weakness. It is also known to cause easy bruising/bleeding and slow-healing existing wounds and also lowers the body's ability to fight an infection[4]. Frequent complications include events like gross and microscopic hematuria, unusual decrease in the amount of urine, hemorrhagic cystitis[5,6]. It also known to cause temporary (rarely permanent) sterility[7]. Cyclophosphamide is itself carcinogenic, potentially causing transitional cell carcinoma of the bladder as a long term complication[4]. Another serious potential side effect may include acute myeloid leukemia, referred to as secondary AML, which occurs due to the treatment of primary tumors[8]. Studies determine that cyclophosphamide induction causes deleterious effect on testicular activities along with testicular oxidative stress[7,9]. Acrolein-one of the metabolites is found to interact with majority of biomolecules like DNA, RNA and proteins that may lead to cellular apoptosis[10]. Cyclophosphamide metabolism is also known to induce the production of reactive oxygen species which causes total cellular damage, further leading to various acute and chronic diseases[11].

Enhancing the body's defense system may be effective in counteracting the oxidative stress and related problems; classes of allopathic drugs are available for the same, but various side effects and high cost of these drugs have forced us to search for an alternative .The Indian System of Medicine is known to

provides a viable alternative that counteracts these symptoms and also scores over the side effects and the cost factor of the allopathic counterpart. Recent years have seen renewed interest in treatment of diseases using herbal drugs[12]. Certain plant derivatives are known to have antioxidant and immunomodulatory properties. They also show positive effects on liver, kidney and other parts of gastro intestinal system, cardio vascular system and nervous system. Since ancient times various plants and their derivatives have played a vital role in treatment of various diseases, hence these have been used in folk medicines and traditional healing systems around the world[13]. Poly-herbal drug formulations made using plant based pharmacological agents that may be found to exert synergistic, antagonistic, agonistic, potentiative actions by virtue of diverse active principles present in them. Their combination working together in a dynamic way is often intends to produce maximum therapeutic efficiency with minimum side effects[14-16]. Herbal drugs are generally found to be nontoxic and even World Health Organization recommends the evaluation of the effectiveness of plants as medicine in condition where we lack safe modern drugs.

BC is an indigenously developed combination of 9 different plants which include *Eclipta alba*, *Withania somnifera*, *Tinospora cardifolia*, *Piper nigrum*, *Terminelia chebula*, *Terminelia ballerica*, *Emblica officinalis*, *Andrographis paniculata*, *Phylanthus amarus* in different proportions. These plant extracts are classified in Ayurveda as "RASAYANA" which are involved in improving defense mechanism of the body[13,17]. These classes of drugs rejuvenate the body by providing energy and antioxidants. They are also known to provide longevity of life. Table-1 shows that all plants in the combination have been individually studied for their protective action on kidney, liver, urinary bladder, pancreas, cardio vascular system and nervous system. They are found to reduce inflammation caused by toxicity of drug metabolism and also counteract increased free radical oxidation and act as potential aphrodisiac[18,19]. Hence may act as medication for temporary and permanent sterility caused by induction of cyclophosphamide. Vitamin E is a well known naturally existing antioxidant present in lipid fractions[16]. It is found in a variety of forms and in many kinds of food. Only 20-60 % of vitamin E is absorbed from dietary sources and as the dose increases, vitamin E fraction absorption decreases. Vitamin E is composed of two homologous series, tocopherol with saturated side chain and tocotrienols with unsaturated side chain. Plasma and tissues predominantly contain D-α-tocopherol which is a form of vitamin-E and is an important structural component of the cell membrane. Vitamin E principally acts as a free radical scavenger in lipid peroxidation thereby protecting cell membrane from free radical quenching in the early stages. (±) D-α-tocopherol succinates have protective effects of on testicular dysfunctions caused by induction of cyclophosphamide[20]. Thereby suggesting that cyclophosphamide treatment at its clinical dose is associated with anti-gonadal activities as well as induction of oxidative stress in gonad that can be ameliorated significantly by co-administration of (±) D-α-tocopherol[21]. The exact mechanism of its antioxidant action is not yet completely understood[16]. Recent study have shown that a combination of antioxidants consisting of vitamin C, vitamin E and N-acetylcysteine provided protection against heart damage induced by chemotherapy without reducing the particular drug's effectiveness.

The drug on the whole is anticipated to exhibit antioxidant, antiinflammatory, immunomodulatory and rejuvenative effects on various organs like liver, pancreas, gastrointestinal organs, heart, kidney and renal pathways. Studies have explained the effectiveness of vitamins E administered parallel with chemotherapy in several kinds of tumor models[22]. Several investigators have offered the seemingly paradoxical conclusion about use of antioxidants together with free radical-generating compounds may be a useful strategy in the treatment of solid tumours[22]. The present study aimed in achieving synergistic action of the antioxidative and immunomodulatory activities of both the polyherbal combination BC and vitamin E (BCE) for subduing cyclophosphamide induced hepato- and uro-toxicity. As cyclophosphamide is a widely used drug for treatment of malignant and non-malignant tumours, the result of this study, if favourable, would envisage and serve as a potential remedy to reduce such toxicity.

EXPERIMENTAL

Cyclophosphamide was purchased from GLS Pharmaceuticals, New Delhi, India. (±) D-α-Tocopherol: Analytical grade, purchased from Sigma Aldrich, Bangalore, India. Poly herbal drug BC: An indigenously developed combination of 9 different plants.

Animal model: Albino Wistar rats of both sexes (200 ± 20 g) procured from SASTRA University, Thanjavur, India were used for the study. Animals were fed with commercially available standard rat pellet feed. The feed and water were provided ad *libitum*. The rats were housed under conditions of controlled temperature (25 ± 2 °C) and were acclimatized

TABLE-1
MEDICINAL PROPERTIES OF INDIVIDUAL PLANTS IN THE COMBINATION HAVE BEEN STUDIED PREVIOUSLY INDICATED THE FOLLOWING PROPERTIES[31]

Plant	Properties
Eclipta alba	Antioxidant[21], immunomodulatory activities[23], hepatoprotective activities[24]
Withania somnifera	Antioxidant[25], immunomodulatory activities[26], hepatoprotective[27], uroprotective activities[28], potential rejuvenator[29]
Tinospora cardifolia	Antioxidant[30], immunomodulatory activities and hepatoprotective[31], uroprotective activities[32]
Piper nigrum	Antioxidant and hepatoprotective activities[33], radical scavenging[34], immunomodulatory activities[35]
Terminalia chebula	Antioxidant and radical scavenging[36], immunomodulatory activities and hepatoprotective activities[37]
Terminalia bellerica	Antioxidant and free radical scavenging [38], immunomodulatory activities and antidiabetic[39]
Emblica officinalis	Antioxidant and radical scavenging[40], hepatoprotective activities[41]
Andrographis paniculata	Antioxidant and hepatoprotective[42], immunomodulatory activities and anti cancer[43], uroprotective activities[14]
Phyllanthus amarus	Antioxidant[44], hepatoprotective[45], anti-inflammatory[46]

to 12:12 h light dark cycles. The animal experiments were conducted according to guidelines of Institutional Animal Ethics Committee (Approval no. 187/SASTRA/IAEC/RPP). The rats were divided into 4 groups of 8 animals each. Group I served as the controls. Group II animals received a dose of 200 mg/kg b.w, of cyclophosphamide dissolved in saline, intraperitoneal, in 2 split doses of 100 mg/kg b.w, delivered on consecutive days (20th and 21st day). Group III animals received BCE orally as 100 mg/kg b.w BC and 50 mg/kg b.w vit-E on all the days of the experimental period (30 days). Group IV animals were co-treated with BCE (as in group iii) for all the days of the experimental period (30 days) and cyclophosphamide (as in group ii) delivered on consecutive days (20th and 21st day). At the end of experimental period (31st day), all the animals were sacrificed by cervical decapitation. Prior to sacrifice, blood was collected from orbital plexus bleeding and serum was obtained to perform biochemical assays.

Biochemical analysis: The serum was analyzed in an automated A15 biochemistry analyzer, Biosystems Inc., Spain using the Biosystems auto-analyzer kits. Collected serum was used to analyze biochemical parameters like total proteins, albumin, bilirubin (total and direct), aspartate transaminase (AST), alanine transaminase (ALT), urea and creatinine were done as per the manufacturer's instructions.

Statistical analysis: Results were expressed as mean ± standard deviation for 6 animals in each group. Differences between groups were assessed by the one-way analysis of variance (ANOVA) and post hoc testing was performed using the least significance difference (LSD) test. A $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Cyclophosphamide administration was found to induce many biochemical changes that were indications of damage to the organs. Table-2 and 3 summarize the results of total protein, albumin, total bilirubin, direct bilirubin, urea and creatinine. Significantly decreased levels of total proteins and albumin were observed in diseased group (cyclophosphamide) of animals than control. During the treatment with BCE, the protein levels in the serum were found to normalize in female rats. In BCE alone administered groups, moderate increase in the levels of total proteins and albumin were observed and was significant in female total protein levels. In previous studies, no significant alterations were found in the biochemical parameters in BC alone administered group of rats (unpublished data). An alteration in the levels of total and direct bilirubin was observed and was significant in male BCE group. Similarly, urea and creatinine showed significant increase in the cyclophosphamide treated group in both male and female rats. These levels were found to reduce in BCE and treatment group and the reduction was more significant in males than females.

Fig. 1 and 2 shows the activities of marker enzymes ALT and AST, respectively that were slightly decreased in the cyclophosphamide treated groups when compared to the control group. Interestingly, these levels were increased in treatment group.

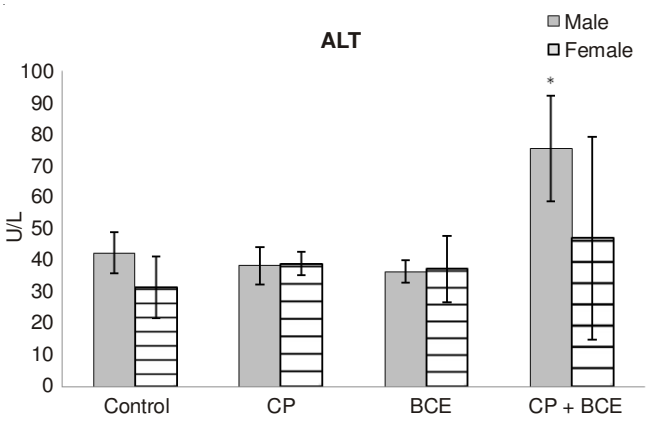

Fig. 1. Effect of cyclophosphamide and BCE on the activities of ALT in serum. Results are given as mean ± S.D. for 6 rats. Comparisons: cyclophosphamide (CP) with control, BCE with control and BCE + cyclophosphamide with cyclophosphamide. *Statistically significant ($p < 0.05$)

TABLE-2
LEVELS OF CLINICAL CHEMISTRY PARAMETERS IN EXPERIMENTAL MALE RATS

GROUPS	CONTROL	CP	BCE	CP + BCE
TOTAL PROTEIN	6.48 ± 0.5	5.51 ± 0.34*	6.61 ± 0.36	5.24 ± 0.20
ALBUMIN	2.95 ± 0.19	2.47 ± 0.36*	3.025 ± 0.24	2.19 ± 0.30
BILIRUBIN TOTAL	0.43 ± 0.15	0.44 ± 0.32	0.68 ± 0.15*	0.33 ± 0.03
BILIRUBIN DIRECT	0.11 ± 0.06	0.19 ± 0.14	0.27 ± 0.04*	0.20 ± 0.21
UREA	28.33 ± 2.52	31.33 ± 2.08*	27.25 ± 4.79	28.67 ± 9.50
CREATININE	0.89 ± 0.00	0.93 ± 0.04*	0.84 ± 0.04*	0.83 ± 0.04*

Results were expressed as mean ± S.D. for 6 rats. Units-Total protein and Albumin: g/dl; Urea, Creatinine, Total and Direct bilirubin: mg/dl; Comparisons: CP with control, BCE with control and CP + BCE with CP. *Statistically significant ($p < 0.05$)

TABLE-3
LEVELS OF CLINICAL CHEMISTRY PARAMETERS IN EXPERIMENTAL FEMALE RATS

GROUPS	CONTROL	CP	BCE	CP + BCE
TOTAL PROTEIN	6.23 ± 1.01	5.67 ± 0.12	7.27 ± 0.43*	6.36 ± 0.08*
ALBUMIN	3.03 ± 0.55	2.36 ± 0.42*	3.37 ± 0.26	2.77 ± 0.19*
BILIRUBIN TOTAL	0.63 ± 0.27	0.64 ± 0.11	0.87 ± 0.14	0.65 ± 0.11
BILIRUBIN DIRECT	0.31 ± 0.04	0.27 ± 0.04	0.40 ± 0.21	0.29 ± 0.02
UREA	24.25 ± 10.21	38.3 ± 5.51*	32.50 ± 7.23	27.33 ±13.05*
CREATININE	0.92 ± 0.22	0.90 ± 0.06	0.91 ± 0.01	0.93 ± 0.06

Results were expressed as mean ± S.D. for 6 rats. Units-Total protein and Albumin: g/dl; Urea, Creatinine, Total and Direct bilirubin: mg/dl; Comparisons: CP with control, BCE with control and CP + BCE with CP. *Statistically significant ($p < 0.05$).

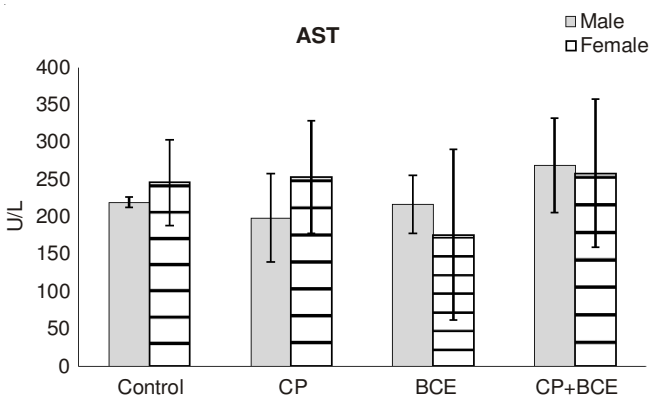

Fig. 2. Effect of cyclophosphamide and BCE on the activities of AST in serum. Results are given as mean ± S.D. for 6 rats. Comparisons: cyclophosphamide with control, BCE with control and BCE + cyclophosphamide with cyclophosphamide. *Statistically significant ($p < 0.05$)

Hepatic dysfunction was the most common toxicity reported in patients treated with cyclophosphamide and total body irradiation. Hepatic tissues were the primary sites for the metabolism and microsomal activation of the drugs, leading to the formation of toxic metabolite which causes damage to the kidney and liver tissues. The pathological state where there seems to be an increased production and/or ineffective scavenging of reactive oxygen species may play a crucial role in determining tissue injury finally leading to multi organ toxicity[47]. High doses of cyclophosphamide can cause death within 10 days of its administration[48], whereas administration of intermittent massive doses of cyclophosphamide has been found effective in chemotherapy[7]. In the present study, cyclophosphamide was found to induce damage to liver and kidney tissues where cyclophosphamide is metabolized[10]. Albumin is an important marker for the assessment of liver functions[49]. Albumin and globulins contribute together for the levels of serum total proteins. Decrease in the levels of total protein in serum could be partly due to immune-suppressive effect of cyclophosphamide and partly due to decrease in albumin level[50]. These abnormalities were normalized during the treatment with BCE. Significantly, increase in the level of total protein in female rats during BCE alone administration depicts the general well-being effect of BCE. Interestingly, significant decrease in level of creatinine and significant increase in total and direct bilirubin in male rats in BCE alone group has no evidence of toxicity at biochemical and marker enzyme levels. Conversely, urea and creatinine levels that were elevated in cyclophosphamide toxicity were normalized during treatment with BCE.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were the important markers for the liver tissue damage. Due to the predominant cytosolic location of ALT, it can be rapidly released in blood following irreversible hepatocellular damage or necrosis[51] and also reversible damage through cytoplasmic blebbing[52,53]. This could be the reason for the elevated levels of ALT in the treatment group of the present study. Near normal level of AST in the treatment group could be due to the shorter circulating half-life of AST than ALT[54] that got released together into the blood. These could be the rationale for the observations of altered levels of ALT and AST in the present study and indicates a possible potentiating effect on cyclophosphamide-induced therapeutic efficiency *in vivo*. Further studies on the potentiating role of the drug in cyclophosphamide-induced toxicities and related problems are warranted.

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