

Preventive role of Curcumin against hepatotoxic effects of Methotrexate and Cyclophosphamide

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ABSTRACT

Chemotherapy kills cells that proliferate rapidly. It also causes damage to cells in normal conditions i.e. bone marrow cells, gastrointestinal tract, hair follicle, etc. resulting in very common side effects. The present study described here, we have studied the hepatotoxic effects of two common anti-cancer drugs - Cyclophosphamide and Methotrexate. Methotrexate's acts by checking DNA and RNA synthesis by inhibiting dihydrofolate reductase and thymidylate synthetase which results in toxic side effects. It actively enters cells by a system used by folate thereafter binding and inhibiting the enzyme. This enzyme recycles dihydrofolic acid which is formed during thymidylate synthesis and thereby maintains reduced folate rate. Dihydrofolate reductase reduces folic acid to tetrahydrofolate, an important co-factor in the synthesis of purine nucleotides. In the study discussed here, Swiss Albino mice (*Mus musculus*) were injected with sublethal doses of Cyclophosphamide and Methotrexate with varying dosage and exposure periods. Mice were tested for hepatotoxicity by estimating the common biochemical parameters. The highest toxic dose was treated with Curcumin supplementation, which reduced the toxic effects. The present study will help in better comprehension of side effects of usage of these common chemotherapeutic drugs and measures to prevent these side effects.

KEY WORDS: Methotrexate, Cyclophosphamide, hepatotoxicity, Curcumin.

1. INTRODUCTION

Chemotherapy is the treatment of cancer with an anti-neoplastic drug alone or in combination with other drugs following a specific treatment regimen. It acts by killing cells that divide rapidly. This means that it also causes damage to normal cells i.e. bone marrow cells, gastrointestinal tract, hair follicle, etc resulting in very common side effects. Some drugs show less toxic effects than other, enabling adjustment in treatment regime helping the patients in certain situations. Methotrexate is indicated in the treatment of gestational choriocarcinoma, chorioadenomadesruens and hydatidiform mole. In acute lymphocytic leukemia, Methotrexate is used in the treatment of meningeal leukemia and is used in collaboration with other anti-cancer drugs. Methotrexate is also used the therapy of meningeal leukemia. Methotrexate is also used in collaboration with other anti-cancer drugs in the treatment of terminal or intermediae non-Hodgkin's lymphomas. Methotrexate's anti-tumor activity is due to inhibition of folic acid reductase, which causes inhibition of DNA synthesis and blocking of cell proliferation. A number of physical side-effects are found with application of Methotrexate. They include: dry cough, shortness of breath, diarrhoea, vomiting, white patches or sores inside mouth or on lips, blood in urine or stools, swelling, rapid weight gain, etc.

At the cellular level, Methotrexate treatment inhibits deamination of adenosine and potentiates adenosine-induced vasodilatation. These effects on the adenosine kinetics in humans is responsible for efficacy of Methotrexate. Methotrexate modifies the kinetic pathway of adenosine in live humans (Riksen, 2006). Methotrexate treatment inhibits neutrophil adherence by enhancing adenosine release from fibroblasts since digestion of extracellular adenosine by added adenosine deaminase completely abrogates the implications of Methotrexate on neutrophil adherence without, itself, leading to adherence (Cronstein, 1976).

Cyclophosphamide is used for the treatment of numerous malignant processes and certain autoimmune diseases. The therapeutic process is dependent on prompt control of pathological problems and replacing it with less toxic, alternative drug at the earliest in order to minimize associated morbidity. Regular laboratory examinations are required to monitor renal function, avoid bladder complications, and screen for bone marrow toxicity. Adverse drug reactions from Cyclophosphamide are related to the dose of medicine and include chemotherapy-induced nausea and vomiting, bone marrow suppression, stomach ache, hemorrhagic cystitis, diarrhea, declouring of the skin/nails, alopecia (hair loss) hair thinning, hair colour and texture change, and lethargy.

Other side effects may include bruise, joint pain, less urine, sores in mouth, abnormal decrease in urine, or unusual tiredness or weakness. Neutropenia or lymphoma arising secondary to Cyclophosphamide usage can lead patients to a variety of bacterial, fungal and opportunistic infections. Pulmonary injury is rare, but can have two different expressions: an early, acute pneumonitis and a chronic, progressive fibrosis. Cardiotoxicity is a big concern with oncology patients given higher dose regimens.

Curcumin is the product obtained by solvent extraction of turmeric i.e., the ground rhizomes of *Curcuma longa* L. and later purifying it by crystallization. The anti-oxidant and anti-cancer characters of Curcumin are well known from centuries. Nitric oxide synthase (iNOS) is hugely found in macrophages that provide the 'oxidative burst'

necessary for defense against pathogens. iNOS is induced due to an oxidative environment, and the NO generated out of that can react with superoxide radicals to form peroxynitrite, which is highly toxic to cells.

It has been shown that curcumin down regulates the iNOS activity in macrophages, thus reducing the production of reactive oxygen species (ROS) formed in response to oxidative stress. Additional studies in microglial cells (brain macrophage analogs) demonstrated reduced NO generation and protection of neural cells from oxidative stress following curcumin treatment, thus the spice may be useful in reducing the neuro inflammation associated with degenerative conditions such as Alzheimer's disease.

The antioxidant property of curcumin was reported (Sharma, 1976) in 1976. It acts as a chelator of free oxygen radicals (Subramanian, 1994). It protects haemoglobin from oxidation (Unnikrishnan, 1995). *In vitro*, curcumin can block the production of ROS like superoxide anions, H₂O₂ and nitrite radical formation by activated macrophages, which are important cells involved in inflammation (Joe, 1994). Curcumin also lowers formation of ROS *in vivo* (Joe, 1994).

The present study aims to ascertain the nature of hepatotoxicity caused by Methotrexate and Cyclophosphamide at various doses, so that a dose-response relation can be found. The second phase of the study focussed on use of Curcumin supplementation as a preventive antidote against the toxic effects of Methotrexate and Cyclophosphamide. We hope that the present study will throw some light on preventing the toxic side effects of these chemotherapeutic drugs.

2. MATERIALS AND METHODS

Animal model: Healthy male Swiss Albino mice (*Mus musculus* L.), about 3 to 4 months old, and weighing between 20 and 25 gm were procured from M/s Scientific Concern, Kolkata, India, and reared in animal cages. The mice were randomly divided into different experimental groups with 4 (four) mice per cage for each control and treated series. The animals were maintained under standard conditions of temperature and humidity with a repetitive 12 hrs light/dark cycles. They were fed with normal diet and drinking water *ad libitum*. All animals were acclimatized to the facility for 1 week before the start of the study. The Guide for the Care and Use of Laboratory Animals (Institute of Medical Animal Laboratory Resources, National Academy Press, USA) was followed throughout the experimental duration. The experimental protocol also met the National Guidelines on the Proper Care and Use of Animals in Laboratory Research (Indian Science Academy, New Delhi, India).

Treatment Protocol:

Vehicle control: This group of mice were injected with distilled water @ 1ml/100gm body weight. This group served as the negative control as both Methotrexate and Cyclophosphamide were dissolved in distilled water.

Methotrexate treatment groups: Each treatment group consisted of 4 mice. 3 separate groups were injected with Methotrexate i.p. (Sigma-Aldrich USA) @ 3 mg/kg body weight, 6 mg/kg body weight and 9 mg/kg body weight. Each dose was observed for 7 days. The dose causing the highest toxicity (9 mg/kg body weight) was counter treated with Curcumin supplementation @5 gm/ kg body weight and 10 gm/ kg body weight for 7 days exposure period.

Cyclophosphamide treatment groups: Each treatment group consisted of 4 mice. 3 separate groups were injected with Cyclophosphamide i.p. (Sigma-Aldrich USA) @ 3 mg/kg body weight, 6 mg/kg body weight and 9 mg/kg body weight. Each dose was observed for 7 days. The dose causing the highest toxicity (9 mg/kg body weight) was counter-treated with Curcumin supplementation @5 gm/ kg body weight and 10 gm/ kg body weight.

Methodology: Male Swiss Albino mice (*Mus musculus*) of same age and body weight were treated with above mentioned sublethal doses of Methotrexate and Cyclophosphamide. Mice were sacrificed after required exposure period as per treatment protocol. Liver was dissected out and homogenised in Tissue homogeniser. Homogenates were centrifuged and supernatant was used for enzyme assays. For estimation of specific activity of phosphatase enzymes, the method of Bergmeyer and Brent (1974) was followed with some minor modifications. Absorbance was measured at 440 nm using Spectrophotometer (Labman LMSP V320). Enzyme activity was determined from the standard curve drawn using Paranitrophenol (PNP) as the standard solution. The specific activity was expressed as microgram of PNP produced per mg of protein per minute following the protocol by Lowry (Lowry, 1931).

3. RESULTS

Effect of Cyclophosphamide and Curcumin: It was seen that Cyclophosphamide treatment lowered down the enzyme activities of the two main phosphatase enzymes in the liver – acid and alkaline phosphatase (Fig.1 and Fig.2). Both the enzyme activities were decreased considerably, although the change was not statistically significant at 5% level of significance. The 3rd dose @9 mg/ kg body weight showed maximum toxic effect of Cyclophosphamide. This 3rd dose was chosen for counter-treatment with Curcumin supplementation. Application of curcumin along with Cyclophosphamide was successful in regaining the activity of the alkaline phosphatase enzyme near to the lowest effect, but the result was otherwise in case of acid phosphatase. We hypothesize that more doses of Curcumin may be required to regain its activity back to normal condition.

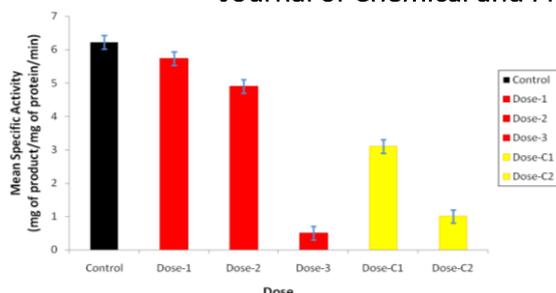


Figure.1. Comparative activity of acid phosphatase

(Mean \pm SD) in response to Cyclophosphamide (Dose-1, 2 and 3) and Curcumin (Dose C-1 and C-2)

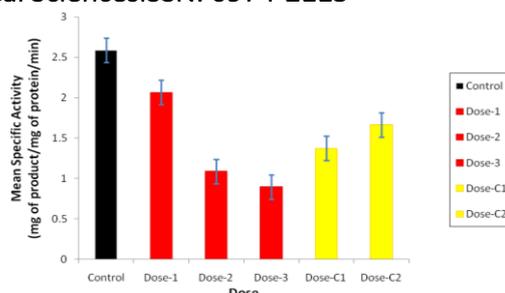


Figure.2. Comparative activity of alkaline phosphatase

Effect of Methotrexate and Curcumin: It was seen that Methotrexate treatment lowered down the enzyme activities of the two main phosphatase enzymes in the liver – acid and alkaline phosphatase (Fig.3 and Fig.4). Both the enzyme activities were decreased considerably, although the change was not statistically significant at 5% level of significance. The 3rd dose @9 mg/ kg body weight showed maximum toxic effect of Methotrexate. This 3rd dose was chosen for counter-treatment with Curcumin supplementation. Co-treatment with curcumin along with Methotrexate was successful in regaining the activity of both the acid and alkaline phosphatase enzymes near to the normal effect, which clearly shows the anti-cytotoxic properties of curcumin.

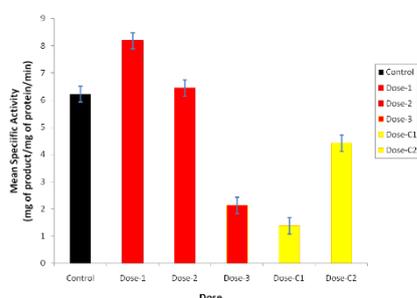


Figure.3. Comparative activity of acid phosphatase

(Mean \pm SD) in response to Methotrexate (Dose-1, 2 and 3) and Curcumin (Dose C-1 and C-2)

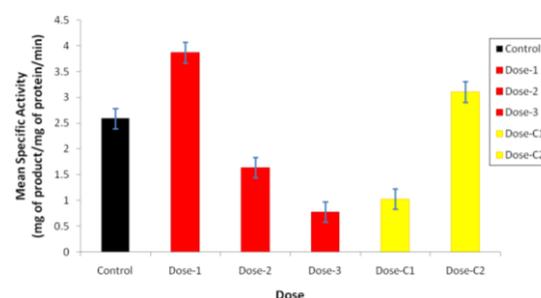


Figure.4. Comparative activity of alkaline phosphatase

DISCUSSION

It was found from the above study that both Methotrexate and cyclophosphamide show hepatotoxic effects at sublethal doses. It also shows that Curcumin supplementation with food can reduce these side effects to a considerable extent. It has been reported that Methotrexate binds promptly to dihydrofolate reductase, checking the reduction of dihydrofolate to its active form, tetrahydrofolic acid. Tetrahydrofolic acid is essential for the transfer of one carbon reactions required for the production of thymidylate, a precursor to DNA, and the purines adenosine and guanosine, which are precursors of both DNA and RNA. In normal doses, Methotrexate is excreted unchanged in the urine. In higher doses, it can be partially metabolized by the liver to 7-hydroxymethotrexate (Leme, 17975). When it is used in high doses during leucovorin rescue, Methotrexate diffuses into both normal and malignant cells. Leucovorin enters normal cells, blocking the effects of Methotrexate. When Methotrexate was used for therapy in children who were suffering from acute leukemia, it can cause hepatic cirrhosis and fibrosis (Colsky, 1955; McIntosh, 1977; Hutter, 1960). Fatty change, focal hepatitis, or portal fibrosis seen in control patients made it difficult to assess its role in hepatotoxicity. Renal excretion is the main route of removal and depends on the dose and route of administration.

At the biochemical level, Cyclophosphamide treatment resulted in the decrease in the activities of acid and alkaline phosphatases in the liver. Phosphatases are enzymes that catalyze the removal of phosphoric acids from different monophosphoric esters, an important reaction in several body processes including neoplastic growth. Similar types of observations have been recorded by other scientists (Hernandez-Zavala, 1998; Mazumder, 1998; Vinitha, 1995) particularly in liver and cardiac tissue. The main implication of measuring alkaline phosphatase is to check the occurrence of mainly liver diseases (ALP-1) or bone diseases (ALP-2). The level decreases mainly as a result of liver, bile duct or gall bladder dysfunction, particularly in adults. The study of alkaline phosphatase levels becomes significant in effectively denoting the alteration of toxicity levels during the period of drug administration (Guha, 2003).

Corroborating these findings with our results, it can be seen that to meet the ever-increasing demand of inorganic phosphate for intracellular utilization that is affected by chronic Methotrexate toxicity, the terminal phosphate groups from phosphate compounds are cleaved by the action of alkaline phosphatase continuously, thereby causing its low activity. The similar effects of Methotrexate can decrease the activities of liver enzymes aspartate amino transferase and alanine amino transferase since both of them are pyridoxal phosphate-linked enzymes. The

other alternate reason may be direct toxicity of Methotrexate on liver cells, since histological studies of hepatic samples from other studies have reported enlargement and fibrosis of varying degrees in the portal regions.

On the other hand, exposure to Cyclophosphamide may alter the binding of protamines to DNA because of increased DNA damage, that may also result in protamine alkylation, because protamines are reported to be very susceptible to alkylation. If this is happening, the end point may be abnormal protamine deposition and blockage of normal disulphide bond formation, thereby preventing proper formation of chromatin and checking the ability of fertilization.

Previous workers investigated that Curcumin could check the DNA damage caused by arsenic as assessed by single cell gel electrophoresis (SCGE) using peripheral blood lymphocytes, from healthy donors. It was observed that DNA injury by arsenic could be significantly reduced by curcumin and the effect was more prominent when the blood cells were pre-incubated with Curcumin prior to arsenic treatment. Arsenic is known to cause DNA damage by generating ROS and increasing lipid peroxidation levels. Curcumin checked the damage by quenching ROS, limiting lipid peroxidation level and increasing the level of detoxification enzymes (phase II) like catalase, superoxide dismutase and glutathione peroxidase. Curcumin also increased the DNA repair activity against arsenic induced toxicity. Curcumin has been used in India for several novel properties since centuries. So it is time tested and has no side effects of application, and hence can be used safely as food supplements in chemotherapy patients. We hypothesize that more doses over longer periods of exposure needs to be tested to standardize the protocol before moving over to clinical trials.

4. CONCLUSION

It can be concluded from the study that both Methotrexate and Cyclophosphamide have hepatotoxic effects when administered for a long duration which is evident from reduced activity of the liver enzymes. This may cause severe adverse effects in chemotherapy patients. So, more care should be taken while administering these as a chemotherapy regime. It was also proved that Curcumin supplementation can reduce these effects to a considerable extent, which is a ray of hope for patients facing such adverse effects.

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