CHAPTER 7

PROTECTIVE EFFECTS OF INDIKANTHA GHRITHA ON CYCLOPHOSPHAMIDE INDUCED TOXICITY
7.1. INTRODUCTION

Despite many advances in the treatment of cancer in recent decades, its long-term therapeutic outcome remains poor. As an important method to treat cancer, chemotherapy is widely used in clinic. However, the chemo-therapy has many toxic and side effects. The life quality of patients who receive chemotherapy treatment is poor because of the side effects of drugs. At the same time, immunosuppression of chemotherapy can accelerate the recrudescence and metastasis of tumors. (Artym J, et al. 2005) (Philips FS, 1961). Improving the curative effect and mitigating the toxic effect of chemotherapy have become the important task of present clinical researches. At the same time, more and more efforts have been made in searching natural materials and foods as a complementary means for the treatment of cancer.

While screening the immunomodulatory activity, most of the studies employ agents like cisplatin, cyclophosphamide (CTX), or corticosteroids in order to induce the immunosuppression in the experimental animals. CTX is an immunosuppressive agent used in the long-term treatment of arthritis (Chou RC et al, 2006), systemic lupus erythematosus (Zhong S et al, 2006), scleroderma (Martinez FJ and McCune WJ, 2006), glomerulonephritis (Mok CC, 2006), interstitial pneumonia (Murota H et al, 2006), hepatitis (Vajro P et al, 2006), multiple sclerosis (Gladstone DE et al, 2006) and other chronic inflammatory diseases. CTX causes depletion of lymphoid tissues, effectively decreasing the ability of the host to raise an adequate specific immune response. The major side effect of anticancer drugs, e.g. cyclophosphamide, is the non-specific cytostatic action on normal healthy cells, especially those with high proliferating capacity like the hematopoietic cells. The extensive death of the immune cells results in leukopenia which severely weakens the immune system of cancer patients and therefore greatly increases the chance of disseminated infections which could be fatal. As a result, drug-free period is always clinically necessary in cancer patients receiving chemotherapy, so as to allow their immune systems to restore function (Colvin M and Chabner BA, 1990).

Currently, chemoprevention strategies using natural products are very attractive and have earned serious consideration as a potential means of management of cancer. Scientists and medical professionals have shown increased interest in this
field, as they recognize the true health benefits of natural remedies. It is also very important to note that suitable chemo preventive natural agents should have little or no toxicity, a high efficacy, to be orally administrable, to have a known mechanism of action and of low cost. To improve the clinical application of these drugs, it is very important to know whether they are able to promote restoration of damaged immune functions. In this study, a cyclophosphamide-treated mouse model is proposed as an approximation for immunomodulation studies in immunocompromised individuals.

Cyclophosphamide (CTX) is one of the most popular alkylating anticancer drugs in spite of its toxic side effects including immunotoxicity, hematotoxicity, mutagenicity (Hill DL, 1975). Cyclophosphamide is not a reactive compound, but it undergoes activation in the body. The initial activation reaction is carried out by cytochrome P450 mediated microsomal oxidation in the liver to produce 4-hydroxy cyclophosphamide, which is in spontaneous equilibrium with the tautomer, aldophosphamide. This equilibrium mixture diffuses from the hepatocyte into the plasma and is distributed throughout the body. Since 4-hydroxy cyclophosphamide is relatively nonpolar, it enters target cells readily by diffusion (Russo JE et al, 1989). The characteristic toxicities of the alkylating agents are hematopoietic and gastrointestinal. In general, the clinical dose-limiting toxicity for alkylating agents is hematopoietic toxicity, particularly suppression of granulocytes. The degree and duration of granulocyte depression after cyclophosphamide administration can be reduced by the concomitant use of hematopoietic inducing drugs (Harada T, et al, 2006). Damage to the gastrointestinal tract is a toxicity that frequently occurs with high-dose regimens. Mucositis, stomatitis, esophagitis, and diarrhoea occur with high doses of alkylating agents (Hui MK, 2006). Pulmonary damage in the form of interstitial pneumonitis and fibrosis has been associated with almost all of the alkylating antitumor drugs (Patel AR, et al, 1976). Although the exact mechanism of the pulmonary toxicity is not known, it is presumably due to direct toxicity of the alkylating agents to pulmonary epithelial cells. The typical presentation of this toxicity is the onset of a nonproductive cough and dyspnea, which may progress to tachypnea and cyanosis and even to severe pulmonary insufficiency and death ((Radin AE, 1970). Cyclophosphamide produce bladder toxicity, which is not seen
with other alkylating agents. This toxicity is a hemorrhagic cystitis, which may progress to massive hemorrhage (Philips FS, 1961). The toxicity has been demonstrated to be due to metabolites of these drugs, which are excreted into the urine. The metabolite principally responsible for this toxicity is acrolein. The majority of a dose of cyclophosphamide (<70%) is excreted in the urine as the inactive metabolite carboxyphosphamide. Renal function does not significantly affect the toxicity of cyclophosphamide, most likely because spontaneous decomposition, and not renal excretion, determines the clearance of the principal active metabolites (Cox PJ, 1979).

The use of alternative therapy in cancer patients has become increasingly popular, although this approach has not been scientifically tested and has the potential to interact with conventional treatment. Plants have the capacity to synthesize a diverse array of chemicals, and understanding how phytochemicals function in plants may further our understanding of the mechanisms by which they benefit humans.

To improve the clinical application of Indukantha ghritha, it is very important to know whether they are able to promote restoration of damaged immune functions. In this report, a cyclophosphamide-treated mouse model is proposed as an approximation for immunomodulation studies in immunocompromised individuals. Any of these actions would extend the therapeutic application of CTX in cancer patients in which Indukantha Ghritha could be used together with the cytotoxic agent in cancer therapeutic regimen. However, the protective effect of IG on CTX-induced immunosuppression was undefined. In the present study, we investigated whether IG could protect the bone marrow of CTX in mice. We also profiled the changes of the expression of cytokines in response to the damage by CTX and protection by IG. The present study was undertaken to assess the protective effects of IG in mice treated with CTX.
7.2. MATERIALS AND METHODS

7.2.1. Treatment protocol
Preliminary studies revealed that the effective therapeutic dose of IG was 250mg/kg orally for 14 days, at which no mortality could be seen up to 28 days. Hence, the experimental group was divided into three groups (n=8). Group 1 was the normal untreated control received the plain ghee as vehicle while groups 2 received cyclophosphamide at a total dose of 250 mg/kg intraperitoneal injections scheduled at day 1 (150 mg/kg) and day 4 (100 mg/kg). Group 3 mice were administered with 250mg/kg of IG for 14 days along the same dose schedule of CTX as prescribed earlier. Blood samples were collected at various intervals of time and mice were sacrificed on day 15. The immunosuppression, characterizing its effects on body weight, white blood cells, including neutrophils, lymphocytes and monocytes, bone marrow cellularity, lymphocyte proliferative response, immunophenotyping of lymphocytes, cytokine evaluation etc were determined. The tissues were collected for histological assessments.

7.2.2. Body weight monitoring.
The body weight of mice was monitored from day 0 and then every 3 days until day 21 after CTX injection.

7.2.3. Effect of IG on hematological parameters during cyclophosphamide treatment
As described previously in the chapter 3

7.2.4. Effect of IG on Bone marrow cellularity during cyclophosphamide treatment
As described previously in the chapter 3

7.2.5. Determination of effect of IG Lymphocyte proliferative response
As described previously in the chapter 3

7.2.6. Immunophenotypic study.
As described previously in the chapter 3

7.2.7. Determination of Phagocytic activity
As described previously in the chapter 3

7.2.8. Western blotting
As described previously in the chapter 5
7.2.9. Cytokine analysis
As described previously in the chapter 4

7.2.10. Histological Study.
As described previously in the chapter 2

7.2.11. Statistical analysis

The results were expressed as the mean ± S.D. The differences between the control and the treatment in these experiments were evaluated for statistical significance by using student “t” test. A p value of ≤0.05 was considered significant.

7.3. RESULTS

The administration of IG restored the immune response in mice to normal levels, suggesting that IG negates the inhibitory action of CTX treated mice.

7.3.1. Effect of IG on the body weight of CTX-injected mice

In order to evaluate the effect of IG on the body weight of mice, we first monitored the body weight of CTX-injected mice treated with vehicle control and Indukantha ghritha. The mean weight of mice prior to CTX injection was 21.61±0.73g. Figure 7.1 showed a significant decrease in the body weight of control mice on days 3 to 14 after CTX injection. After administration with Indukantha ghritha a significant increase in the body weights of CTX+IG treated group occurred compared to the CTX treated mice. In contrast, control mice showed no significant change in body weight throughout the experiment.

![Graph showing changes in body weight after CTX treatment in IG administered mice.](image-url)
7.3.2. Effects of Indukantha Ghritha on the recovery from cyclophosphamide-induced leucopenia.

The aim of this study was to evaluate the efficacy of a simplified 250 mg/kg regimen of CTX for murine immunosupression, and characterizing its effects on white blood cells, including neutrophils, lymphocytes and monocytes, and its impact in animal models of tumor. Administration of cyclophosphamide has significantly lowered the levels of total WBC (2060 ± 440) as compared to control group (9100 ±570) in blood. Recovery of WBC count started on day 7 in IG administered CTX treated group and returned back to the normal level on day 14. However, the rate of recovery of WBC number in mice treated with IG 250 mg/kg was significantly increased. IG also caused substantial restoration of the percentage of the lymphocyte population in circulating blood (from 29.87 to 75.12 %). The initial number of neutrophils in normal mice was 12.62 ± 2.50%. Figure 7.2C illustrates how CTX (250 mg/kg) induced severe neutropenia by day 4 and the absolute neutrophils count remained below this value throughout days 3 and 7 for all animals. By day 7, mice had 4.37±1.76% neutrophils, and on IG treatment at day 14 they all had returned gradually to normal. Lymphocyte and monocyte counts also declined on CTX treatment, and started to recover after day 14 on IG treatment. Indukantha Ghritha was found to be effective in protecting cyclophosphamide-induced myelosuppression as evidenced by increasing the levels of total WBC count significantly (p< 0.01).

7.3.3. Effect of IG on Bone marrow cellularity during cyclophosphamide treatment

Hematopoietic elements in the marrow mice contained erythroid, granulocytic, lymphoid, and megakaryocytic cells. In contrast, CTX treated mice showed a marked decrease in cellularity. IG increased the number of hematopoietic cells in the bone marrow of CTX treatment mice. There was a decline (6.28±1.24 x10^6cells/femur) in bone marrow hematopoietic cells of CTX group, compared with control animals (15.93±0.36 x10^6cells/femur). In contrast, there was substantial increase (13.93±1.46 x10^6cells/femur) in the number of bone marrow cells of Indukantha Ghritha administered cyclophosphamide treated group (Figure 7.3). Thus
Figure 7.2. Restoration of (A) total WBC, (B) lymphocyte and (C) neutrophil count by Indukantha Ghritha in CTX treated mice. * or ** denotes significant difference from control group with p < 0.05 or P < 0.01 respectively.
Figure 7.3. Effect of orally administered Indukantha Ghritha on Bone marrow cellularity in mice treated with CTX. IG = Indukantha Ghritha, CTX = Cyclophosphamide. * denotes significant difference from control group with p ≤ 0.01.

Figure 7.4. Effect of IG on lymphocyte proliferative response in CTX treated mice. IG = Indukantha Ghritha, CTX = Cyclophosphamide. * denotes significant difference from control group with p ≤ 0.01.
in vivo treatment with IG significantly reverses bone marrow hypocellularity in immunocomprised animals.

7.3.4. Determination of effect of IG Lymphocyte proliferative response

Cyclophosphamide is a potent alkylating agent which inhibits lymphocyte proliferation, leading to repression of the function of B and T lymphocytes as well as to a reduction of their numbers. Administration of IG restores impaired lymphocyte proliferative response in mice treated with CTX. The figure 7.4 given below showing the lymphocyte proliferative response points out that IG administration is capable of reverting the cytopenic effect by increased hematopoiesis to nearly the normal levels.

7.3.5. The immune inhibitory effect of cyclophosphamide on Balb/c mice

The CD4+ and CD8+ lymphocytes in Balb/c mice treated with cyclophosphamide were significantly lower than those in normal ones (10.87±2.23% vs 26.12±1.64%, P<0.01, and 8.37±1.84% vs 14.87±1.24%, P<0.01), which showed that the cellular immune function was significantly inhibited. It was demonstrated that IG strongly elevated the pool of CD3+ T cells in CTX-immunocompromised mice. In conclusion, we showed for the first time that IG, given orally to CTX-immunosuppressed mice, could reconstitute a T-cell mediated immune response by renewal of the T cell pool. Indeed CTX reduces the number of B lymphocytes (CD19) to mount an appropriate antibody response. IG possessed immunopotentiating activities, being effective in restoring cyclophosphamide (CTX) induced immunosuppression such as depressed lymphocyte proliferation, production of white blood cells. Both humoral and cellular immune function may be inhibited to a varying degree by CTX.

7.3.6. Phagocytic activity

CTX also induces immunological function disturbances such as impaired phagocytic capability and reduces phagocytic activity of macrophages. IG influenced macrophage response as it significantly increased the phagocytic index of mice (figure 7.6). The increase in ingested yeast particles reflects the enhancement of phagocytic function of mononuclear macrophage and non-specific immunity.
Phagocytosis by macrophages is important against the smaller parasites and more rapid clearance of parasite from blood.

Figure 7.5. Effect of IG on immunophenotypic analysis of lymphocyte subsets in CTX treated mice using FACS analysis. IG = Indukantha Ghritha, CTX = Cyclophosphamide. * denotes significant difference from control group with \( p \leq 0.01 \).

Figure 7.6. Phagocytic activity of peritoneal macrophages induced by IG in mice treated with CTX treatment. IG = Indukantha Ghritha, CTX = Cyclophosphamide. * denotes significant difference from control group with \( p \leq 0.01 \).
6.3.7. Western blotting

To determine whether IG alters downstream TCR-signal-transduction-dependent events during CTX treatment. As shown in figure 7.8 western blot analysis revealed that IG treatment apparently induced the expression of TCR signal proteins in CTX treated mice. Results implicated the reduction by CTX and normalization by IG of T cell signal proteins like TCR, CD3, PKC, ZAP-70 when compared with control animals. CTX is the non-cytostatic drug that acts non-specifically on both tumor cells and normal healthy cells with high proliferating capacity like immune cells. Strikingly, as shown in Figure 7.9, TCR-mediated signal transduction pathway was inhibited by CTX. In IG-treated CTX group, up-regulated TCR, CD3, PKC, ZAP-70 expression, as indicated by intense bands compared with CTX treated animals. We demonstrated that the magnitude of protein expression was strongly diminished by CTX action, was reconstituted by IG.

6.3.8. Histological Study.

Histological analysis was performed on cross sections of intestine, bone marrow, urinary bladder and lungs after staining with hematoxylin and eosin as shown in Fig. 7.10. Cross sections from cyclophosphamide treated mice and IG administered CTX treated mice were compared with control mice. The control mice had normal histology in the GI tract. In contrast, the CTX treated mouse had severe inflammation and crypt abscesses were prevalent in ileum. These histological features of a mixed inflammatory cell infiltrate, mucin depletion, and occasional crypt distortion. To learn whether restoration of hematopoietic cells in the bone marrow altered the lineage of hematopoietic cells, H&E-staining of bone marrow were performed after drug treatment. Effects on the proximal femoral epiphyses were evaluated histomorphometrically at day 14 after the experimental period. Results showed a significant reduction in bone marrow cellularity of the cyclophosphamide-treated femora compared to the controls. Cell differentiation throughout the growth plates was clearly disturbed, involving nesting of cells, loss of polarity, as seen by areas of excessive hyalinization. The gastrointestinal tract is a major component of the human immune system with a total lymphoid mass which is comparable with bone
marrow. The Peyer's patches are the principal sites of interaction among luminal antigens and lymphocytes, while the scattered lymphocytes in the lamina propria and epithelium are the effector cells that mediate immune response. The gut is also a site of synthesis and release of a specialised form of immunoglobulin A (secretory IgA) which is resistant to digestion. These immunological mechanisms are important because the gut has a huge surface area which interacts with the numerous potentially noxious agents including micro-organisms and dietary antigens. The intestinal tract is also one of the most metabolically active tissues in the body, with mucosal renewal taking place every three to five days, it is not surprising therefore that the gut is often the target organ for pathological processes in the immunosuppressed patients. The deleterious effects of immunosuppression on the integral functioning of the gut are assuming greater importance now that the use of potent long term immunosuppression has become widespread. Histology of CTX treated mice showed gut mucosal erosions, bleeding, and viscous perforation. The use of IG along with CTX was found to be effective in restoring mucosal integrity of intestine. H & E stained section of urinary bladder in control mice showed normal histology with intact urothelium, blood vessels with thin walls, no edema or infiltrate present, two smooth muscle layer without alterations. The CTX treated mice showed extensive urothelial damage, urothelium was absent from many regions of the bladder, marked inflammatory infiltrate with abundant lymphocytes, and polymorphonuclear white blood cells, marked edema separating the smooth muscle layers, and marked congestion. In IG administered CTX treated mice showed considerable improvement in comparison with histological findings of CTX treated group with urothelium present, mild to moderate vascular congestion, mild edema, and few white blood cells infiltrates.

7.3.9. Cytokine analysis in CTX treated mice.
ELISA method was performed to analyze the levels of IFN-\(\gamma\), IL-4, IL-12 and GM-CSF cytokines in serum of IG administered CTX treated mice. The levels of all the cytokines in CTX treated mice significantly decreased when compared with those in healthy control mice. As shown in figure 7.8 the concentration of IFN-\(\gamma\), IL-12, GM-CSF and IL-4 significantly increased by IG administration in CTX treated mice.
Figure 7.7. Restoration of phagocytic activity of macrophages by IG in CTX treated mice.
Figure 7.8. Reconstitution of cytokines after IG administration in CTX treated mice. IG = Indukantha Ghritha, CTX=Cyclophosphamide. * denotes significant difference from control group with p ≤ 0.01.

Figure 7.9. Restoration of T cell signal proteins in CTX treated mice after IG administration.
Figure 7.10. Representative H & E stained section of Bone arrow, Intestine, Urinary bladder, lungs of control (untreated), CTX alone and IG along with CTX treated animals.
Figure 7.11. Flow cytometric analysis of lymphocyte subsets in control, CTX alone and IG administered CTX treated mice.
These results indicating that these cytokines act synergistically to induce hematopoiesis in mice.

7.4. DISCUSSION

The present findings suggest that IG has immunostimulatory effect which can accelerate the recovery from leukopenia induced by CTX. This protection includes preservation of body weight, and a marked reduction in the histopathological findings of urinary bladder, bone marrow, intestine, lungs etc. Oral administration of IG at the dose of 250mg/kg daily significantly promoted the recovery rate of immune system in mice in a 14 day treatment. It also significantly increased the number of WBC and bone marrow cellularity. Chemotherapeutic drugs exert its cytotoxic effect via transfer of its alkyl groups to DNA, leading to cell cycle arrest and apoptosis (Ahmed AR and Hombal SM, 1984). Patients under chemotherapeutic regimen are often subject to leukopenia which greatly increases the chance of opportunistic infections. As a result drug-free period is routinely introduced during regimen to prevent any or even fatal infections. Such a long drug-free period is indeed undesirable because it allows the restoration of tumor tissue into active proliferating stage, as vascular endothelial cells can proliferate again and tumor will be nourished by supply of nutrients and oxygen (Aviles A et al, 1996). However, in mice treatment with IG 250mg/kg was sufficient enough to fully restore their normal immune response. IG could increase the rate of lymphocyte proliferation in vitro, phagocytic activity of peritoneal macrophages, T cell signal proteins etc. Oral administration of IG, a classical prescription of IG, was shown to protect mice against cyclophosphamide induced inhibition of IFN-γ, IL-12, GM-CSF, IL-4. All of these results are consistent with the present findings that IG is a tonic to hematopoietic system.

Blood profile analysis revealed elevation of leukocytosis by IG in CTX-treated mice. Furthermore, IG showed an increase in the cellularity of bone marrow isolated from CTX-treated mice (from 6.28 to 13.9x10^6 cells/femur). However, cyclophosphamide would also adversely affect the repairing capacity and the defensive mechanism of the immune system that have been damaged during chemotherapy. In this regard, IG was shown to be beneficial to cancer patients because it normalized immunologic parameters. This also promoted the defensive
mechanism and also the repairing capacity of the tissues which has been damaged by CTX administration. Since bacterial growth in lungs was enhanced in leukopenic mice, therefore, lungs could be a more susceptible target than blood for pathogens in cyclophosphamide-treated patients. The present investigation has revealed that cyclophosphamide at 250mg/kg dose, produced significant impairment of humoral as well as cell-mediated immune responses and inhibited phagocytosis. It is suggested that IG may prove to be useful as a component of combination therapy in cancer patients under CTX treatment regimen.