CHAPTER 6 PHASE III STUDIES

IN VITRO STUDIES ON THE EFFECT OF FA AND/OR CISPLATIN ON NORMAL HUMAN LYMPHOCYTES

6.1. INTRODUCTION

From the phase I and phase II studies it was clear that ferulic acid sensitized cancer cells for cisplatin chemotherapy by increasing the cytotoxic activity. Being a dietary polyphenol, it can be cytotoxic towards cancer cells but should it not be to cytotoxic to the normal cells. So, the purpose behind the phase III study is to find the effect of FA in normal cells. It was also aimed to find whether FA is protective towards normal cells or not.

In the phase I and phase II studies the prooxidant activity of FA was considered to be an important factor for its chemosensitizing potential. Moreover, the increased cytotoxicity of cisplatin in FA and cisplatin treated cancer cells showed that FA was one of the reasons. Whereas Sudheer et al. (2007) suggested the protective action of FA in lymphocytes. Hence, it is worth to study the cytotoxicity and protective effect of FA in chemotherapy.

Lymphocytes were used in many studies to find the protective action of a phytochemical on normal cells (Srinivasan et al 2005; Sudheer et al. 2007). Lymphocytes are a type of white blood cells generated by the immune system to defend the body against cancerous cells, pathogens and foreign matter. Lymphocytes circulate in blood and lymph fluid and are found in body tissues including the spleen, thymus, bone marrow, lymph nodes, tonsils, and liver. Lymphocytes provide a means for immunity against antigens. This is accomplished through two types of immune responses: humoral immunity and cell mediated immunity. Humoral immunity focuses on identifying
antigens prior to cell infection, while cell mediated immunity focuses on the active destruction of infected or cancerous cells.

The dose of a chemotherapeutic drug such as cisplatin that is necessary to overcome even a small increase in cellular resistance can result in severe cytotoxicity in normal cells. Therefore, it is urgent to explore novel approaches to reduce drug dosage, minimize side effects, enhance the efficacy of therapy, and promote the application of cisplatin in cancer therapy. Cisplatin-DNA crosslinks cause cytotoxic lesions in tumors and in other normal dividing cells. Phytochemicals found in herbs has been reported to possess antioxidant properties, which protects normal cells from oxidative stress (Van Acker et al. 2000). Various research reports have proved polyphenols protect normal cells during chemotherapy (Thresimma et al. 1996; Greggi Auntes et al. 2001).

Phase I studies revealed that FA showed chemosensitizing action on cervical cancer cells for cisplatin chemotherapy. It was difficult to explore and confirm the particular efficacy of any phytochemical by testing it against a single type of cancer cell line alone. So, the phase II study was designed to explore the Chemosensitizing effects of FA and/or cisplatin on Dalton’s lymphoma cell lines in vitro.
6.2. MATERIALS AND METHODS

6.2.1. Chemicals

Ferulic acid (FA), cisplatin, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT), trypan blue, Ficoll - histopaque and lymphocyte culture chemicals such as heat inactivated fetal bovine serum (FBS), RPMI-1640, L-glutamine, penicillin-streptomycin, trypsin were purchased from Sigma Chemicals Co., St. Louis, USA. All other chemicals and solvents of analytical grade were obtained from S.D. fine chemicals, Mumbai, Fisher Inorganic and Aromatic Limited, Chennai and Central Drug House (P) Ltd, New Delhi, India.

6.2.2. Human lymphocyte culture

Lymphocytes from normal human blood from healthy individuals were isolated using Ficoll–histopaque (Boyum 1968). Blood was diluted 1:1 with phosphate buffered saline (PBS) and layered onto Histopaque with the ratio of blood and PBS: Histopaque maintained at 4:3. The blood was centrifuged at 1340 rpm for 35 minutes at room temperature. The lymphocyte layer was removed and washed twice in PBS at 1200 rpm for 10 minutes each and then washed with RPMI-1640 media and approximately 1x10^6 cells were layered in the media.

6.2.3. Effect of ferulic acid on the viability of human lymphocytes

The effect of FA on the viability of normal human lymphocytes was determined by trypan blue viability test adopted from Gupta (2002).

Principle

Trypan blue is a cationic chromosomal dye that intercalates with DNA. Loss of viability results in the nuclear uptake of the dye, giving characteristic blue colour to the nucleus. In a viable cell, uptake of the dye is prevented by
the presence of the intact plasma membrane. From the colour appearance of the cell, the viability can be calculated.

**Materials**

1. Trypan blue dye : 0.4%
2. Light microscope
3. Haemocytometer and cover slip

**Procedure**

To isolated lymphocytes, different concentrations of FA were added. Then few drops of trypan blue stain (0.4%) were added and the viable cells against dead cells (total 200 cells) were recorded after 5 minutes using haemocytometer under a microscope.

The percentage of viable cells was calculated using the formula,

\[
\% \text{ Viability} = \frac{\text{Total number of viable cells}}{\text{Total number of viable and non-viable cells}} \times 100
\]

**6.2.4. Study Design**

Isolated lymphocytes were divided into 4 groups as follows:

**Group 1**: Normal lymphocytes

**Group 2**: Normal + FA (10 µg/mL)

**Group 3**: Normal + Cisplatin (5 µg/mL)

**Group 4**: Normal + FA pretreatment (10 µg/mL) 1 hour before Cisplatin treatment (5 µg/mL)

Group 1 served as normal lymphocyte control group. Group 2 lymphocytes were treated with FA (10 µg/mL) alone. Group 3 was treated
with 5 μg/mL of cisplatin alone. 1 hour prior to cisplatin (5 μg/mL) treatment, 10 μg/mL of ferulic acid (FA) was added to the normal lymphocytes on group 4.

6.2.5. Effect of FA on cisplatin treated lymphocytes

The protective effect of FA on lymphocytes treated with cisplatin was estimated through the proliferation activity of cell populations by MTT assay (Mosmann 1983).

Principle

MTT assay is based on the detection of mitochondrial dehydrogenase activity in living cells. In MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay, the yellow tetrazolium salt is metabolized by NAD-dependent dehydrogenase) to form a dark blue formazan product.

Reagents

1. 0.5 g/L MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide)

2. Dimethyl sulfoxide (DMSO)

Procedure

Yellow colour MTT is converted to the purple formazan product only by metabolically active mitochondria and the absorbance is directly proportional to the number of viable cells. MTT solution (0.5 g/L) was added to each lymphocyte culture well after treatment with FA and cisplatin for 24 hours. After MTT addition, the colour was allowed to develop for additional 4 hour incubation. An equal volume of DMSO was added to stop the reaction and to solubilize the blue crystals. Samples were transferred into culture plates and the absorbance was measured at 490 nm in microplate reader.
6.2.6. Statistical analysis

All values were expressed as means ± SD. The data were statically analyzed using one-way analysis of variance (ANOVA) and the significant difference among treatment groups were evaluated by Duncan’s Multiple Range Test (DMRT). The results were considered statistically significant at p<0.05. All statistical analysis were made using SPSS 17.0 (Statistical package for social sciences) software package (SPSS, Tokyo, Japan).
6.3. RESULTS

The effects of FA and cisplatin in normal cells were tested in human lymphocytes. Since FA was cytotoxic towards cancer cells, its cytotoxicity towards normal cells was also checked. Moreover, cisplatin was known to be cytotoxic to normal cells. The effect of FA on normal cells during the cisplatin therapy was also studied.

Figure 6.1. shows the effect of the plant polyphenol, ferulic acid (FA) in normal human lymphocytes on percentage cell viability. It was found that, when lymphocytes were treated with FA in a concentration range of 2 µg – 20 µg, there was no decrease in the % cell viability of lymphocytes. From 2 to 20 µg the cell viability was maintained almost 100%. This showed that FA was not cytotoxic towards normal cells.
Data are presented as the means ± SD; n=6
Lymphocytes were treated with FA in a concentration range of 2-20 µg

Figure 6.1. Effect of FA on cell viability of normal human lymphocytes
Data are presented as the means ± SD; n=6
Lymphocytes were treated with 0.5% DMSO, FA

**Figure 6.2. Protective effect of FA on cisplatin treatment in normal human lymphocytes**

Figure 6.2 shows the effect of FA on cisplatin treatment in normal human lymphocytes. Treatment of cisplatin (5 µg/ml) significantly decreased the cell viability when compared to normal cells. At the same time, when lymphocytes were treated with FA (10 µg/ml), no decrease in cell viability was seen. In FA pretreated lymphocytes treated further with cisplatin, the cell viability was found to be increased significantly when compared to cisplatin alone treated lymphocytes.
6.4. DISCUSSION

The suitable method adopted for studying cytogenetic effects induced by a suspected agent in human beings is the micro culturing of human peripheral blood lymphocytes.

The anticancer agent cisplatin can also act as a potential human carcinogen through its DNA binding effect on cells (Greene 1992). It forms intrastrand and interstrand cross links in the DNA molecule and appears to correlate well with the cytotoxicity of the drug. The tumour cells amass an overburden of mutations which lead eventually to the cell's death (Gilman et al. 1985). The cytotoxic action of cisplatin in human lymphocytes through its has already been reported by several authors like Adler and El-Tarrass (1989); Ohe et al. (1990); Osanto et al. (1991); Krishnaswamy and Dewey (1993); Choudhury et al. (2000); Jin and Ikushima (2004) and Somisetty and Devi (2011). Cisplatin was found to be cytotoxic towards normal cells (figure 6.2). Cisplatin exerts cytotoxic effect by binding its highly reactive hydrated platinum complex to the DNA of cells (Erickson et al. 1981; Roberts et al. 1988). Much effort has been put into reducing the cytotoxic side effects of Cisplatin by administration of polyphenols as modulating agents.

In the present study, polyphenol FA did not show any cytotoxic action over normal lymphocytes. But on the other hand, FA was found to be cytotoxic against cancer cells (results from the Phases I and II of the present study). This showed that, FA was cytotoxic only towards cancer cells but not normal cells i.e., selective protection over normal cells. This differential effect of FA on normal and cancer cells might be due to different redox environment present in normal and cancer cells (Raffoul et al. 2007). The results of the present study are in accordance with the previous studies which suggested that FA is protective over normal cells. Lin et al (2010) showed that FA did not show any toxic effects in normal human umbilical vein endothelial cells.
(HUVECs). Bandugula et al. (2012) also reported that FA did not show cytotoxicity and DNA damage on human lymphocytes.

Moreover in the present study, the cytotoxicity induced by cisplatin was reduced by the treatment of FA. This may be due to the protective effect of FA on normal cells. This protective effect is eliminated when FA was absent, indicating that FA has to be taken up by the cells to exert its protective effect. In a study, Prasad et al. (2006) showed the protective effect of FA on radiation induced damage in normal human lymphocytes. In a study on nicotine–induced DNA damage and cellular changes in rat peripheral blood lymphocytes, FA protected the normal cells against damage (Sudheer et al. 2007). In a similar study by Prasad et al. (2007), FA protected the normal lymphocytes against UV-B induced cell damage.

6.5. CONCLUSION

The results of the present study showed that, FA does not show any toxic effects on normal cells and also protected normal cells from cisplatin induced cytotoxicity.