CHAPTER-4

Blood lymphocyte CYP2E1: A biomarker to predict early stage alcoholic liver cirrhosis
4.1 INTRODUCTION

Studies in preceding chapter have shown that polymorphism in genes responsible for ethanol metabolism and detoxification such as CYP2E1, GSTs and MnSOD modifies the susceptibility to alcoholic liver cirrhosis. However, in contrast, to measuring genetic polymorphism and correlating them with other determinants of risk, another approach has been to measure interindividual variation in the expression of CYPs, involved in xenobiotic metabolism (Hayashi et al., 1994; Willey et al., 1997; Raucy et al., 1999). In recent years there has been great interest in developing assays that can be used as biomarkers of the extent and persistence of effects caused by exposure to toxic agents. Lymphocytes have obvious advantages for use in the development of non-invasive assays to screen human population for toxicant exposure and the applicability of these cell types as indicative of toxicant exposure has been well documented (Lucier et al., 1987; Harris, 1989). Lymphocytes have been shown to express several members of CYP gene family, whose protein products are involved in the oxidative metabolism of wide variety of drugs and chemicals (Raucy et al., 1997; Dey et al., 2001, 2002). Recent studies from our laboratory have demonstrated the expression of xenobiotic metabolizing CYPs in freshly isolated unstimulated rat blood lymphocytes (Dey et al., 2001, 2002, 2005).

Studies have shown that freshly isolated PBLs of human and laboratory animal express measurable levels of CYP2E1 mRNA and protein (Song et al., 1990; Raucy et al., 1997, 1999; Dey et al., 2002, 2005; Haufroid et al., 2003). Dey et al. (2002, 2005), earlier characterized CYP2E1 activity in freshly isolated and uncultured rat blood lymphocytes and reported significant increase in CYP2E1 dependent NDMA-d activity.
and lipid peroxidation in freshly prepared lymphocytes isolated from ethanol pretreated rats. The increase in NDMA-d activity in rat blood lymphocytes was further found to be associated with increase in mRNA and protein expression of CYP2E1 (Dey et al., 2002, 2005). RT-PCR studies showed that as observed in liver, acute treatment of ethanol resulted in increase in the mRNA expression of CYP2E1 in freshly isolated rat blood lymphocytes. Western blotting studies using polyclonal antibody raised against rat liver CYP2E1 demonstrated significant immunoreactivity, comigrating with the liver isoenzymes, in freshly isolated control rat blood lymphocytes. Thus these studies demonstrating similarities of the blood lymphocyte CYP2E1 with the liver enzyme suggest that lymphocyte CYP2E1 levels in freshly isolated rat blood lymphocytes could be used to monitor tissue enzyme levels (Dey et al., 2005).

Since expression of CYP2E1 is altered by many factors affecting chemical metabolism and toxicity, monitoring of CYP2E1 levels in individuals, who are regular alcohol users or are exposed to environmental chemicals or carry certain pathophysiological changes which result in induction of CYP2E1, could help in identifying individuals who are at risk. The candidate in the present study therefore aimed to develop peripheral blood lymphocyte CYP2E1 expression as a possible biomarker of alcoholic cirrhosis by investigating mRNA and protein expression of CYP2E1 and associated catalytic activity in freshly prepared blood lymphocytes isolated from non-alcoholic healthy controls, non-alcoholic cirrhotic and alcoholic cirrhotic patients.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

N-nitrosodimethylamine (NDMA), NADPH, bovine serum albumin (BSA) and Histopaque 1077 were procured from Sigma-Aldrich, St. Louis, MI, U.S.A. Polyclonal rabbit anti-human CYP2E1 antibodies was procured from Chemicon, U.S.A. Oligo (dT)20, RNAse out, dNTP mix, Reverse Transcriptase (RT), Taq DNA polymerase and all other reagent for PCR were procured from Fermentas, CANADA. All other chemicals used were of highest purity commercially available and procured either from E. Merck, India or Sisco Research Laboratories Pvt. Ltd., India.
4.2.2 Study group

The study group consisted of eight patients suffering from alcoholic liver cirrhosis (non-cholestatic) and equal number of patients suffering from non-alcoholic liver cirrhosis visiting the OPD facility of Gastroenterology Department of Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, India was included in the study. Eight healthy non-alcoholic individuals were also enrolled in the study and served as controls. Patients with alcoholic liver cirrhosis were diagnosed on the basis of their liver biopsy. All the alcoholic cirrhosis patients were negative for serum antinuclear and anti-mitochondrial antibodies and negative for antibodies to the hepatitis C virus. The hepatitis B surface antigen was also negative in all the eight alcoholic cirrhotic patients. Alcoholic cirrhotic patients were actively drinking alcohol prior to inclusion in the study. Patients with non-alcoholic liver cirrhosis were also diagnosed on the basis of their liver biopsy and out of the eight non-alcoholic cirrhotic patients three were positive for hepatitis B surface antigen and five were positive for antibodies against hepatitis C virus. Non-alcoholic control group did not showed any evidence of liver disease as judged by physical examination and normal liver function test. It was ensured that the alcoholic and non-alcoholic cirrhotic patients and non-alcoholic controls belonged to the same ethnicity (Indo-European community) of North India. Table 4.1 summarizes the main characteristics of the individuals enrolled in the study.

The protocol for research work was approved by the human ethics committee of Indian Institute of Toxicology Research (IITR), Lucknow and it conforms to the provisions of the declaration of Helsinki in 1995. Informed consent was obtained from the study subjects for inclusion in the study and before the collection of blood samples it was ensured that the subject anonymity was preserved. All study subjects completed a questionnaire covering medical, residential and occupational history. The questionnaire also included details such as frequency of alcohol intake. Subjects who consumed less than 10g/day of alcohol were classified as non-alcoholics. Approximately 4 ml of blood was collected from each subject. RNA was isolated from 0.5ml of whole blood while 3ml of blood was used for preparing lymphocytes. Lymphocytes were used for enzyme estimation and ELISA studies while RT-PCR and semi-quantitative RCR studies were carried out with total RNA isolated from whole blood. Though correlation has been reported for CYP2E1 mRNA and protein in blood lymphocytes of alcoholic and non-
Table 4.1: Main characteristics of study group

<table>
<thead>
<tr>
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<th>Non-alcoholic controls</th>
<th>Non-alcoholic cirrhosis</th>
<th>Alcoholic cirrhosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sample</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Mean age</td>
<td>39 ± 9.2</td>
<td>42 ± 7.6</td>
<td>45 ± 9.8</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>---</td>
<td>---</td>
<td>135 ± 12</td>
</tr>
<tr>
<td>(mean ± SD) gm/day</td>
<td>---</td>
<td>---</td>
<td>14 ± 3.6</td>
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<tr>
<td>Duration (Years)</td>
<td>---</td>
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<td></td>
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<tr>
<td>Histology</td>
<td>---</td>
<td>Non-cholestatic</td>
<td>Non-cholestatic</td>
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<tr>
<td></td>
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<td>early stage cirrhosis</td>
<td>early stage cirrhosis</td>
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alcoholics (Raucy et al., 1999), CYP2E1 is known to be regulated at various levels such as pre-transcription, transcription, pre-translation, translation and post-translation (Ronis et al., 1996). Therefore in the present study, CYP2E1 mRNA, protein and activity of NDMA-\(d\), a marker of CYP2E1 catalyzed reaction, were investigated in freshly isolated blood lymphocytes. ELISA was preferred over western immunoblotting as it required lesser amount of lymphocyte protein when compared to immunoblotting studies.

### 4.2.3 RNA isolation

Total RNA was extracted from whole blood by RiboPure-blood kit (Ambion USA) according to manufacturer protocol. 500 \(\mu\)l of anti-coagulated blood was centrifuged at 13,200rpm and pellet was lysed with 800\(\mu\)l of lysing solution and 50\(\mu\)l sodium acetate. 500\(\mu\)l of acid phenol: chloroform was added to separate the aqueous and organic phase. 600\(\mu\)l of 100% ethanol was added to the aqueous phase and contents were transferred to the filter cartridge assembly supplied with kit. After centrifugation at 13,200 rpm the RNA was washed with wash solution 1 and wash solution 2/3 (supplied with kit). RNA was eluted with suitable amount of elution solution. Eluted RNA was used for cDNA synthesis.

### 4.2.4 Semi-Quantitative RT-PCR analysis

Total RNA isolated from whole blood of healthy controls and patients suffering from alcoholic and non-alcoholic cirrhotic patients were analyzed for CYP2E1 mRNA by RT-PCR analysis. cDNA was prepared from total RNA with Reverse Transcriptase (RT) enzyme (1.5U/\(\mu\)l) in a reaction mixture containing cDNA synthesis buffer, 0.01M DTT, 4U/\(\mu\)l RNAse out, and 2mM dNTPs and incubating them at 50\(^{\circ}\)C for 60min. 2\(\mu\)l of this RT product was used for subsequent PCR reactions. The reaction mixture for PCR of CYP2E1 in 25 \(\mu\)l contained 10X PCR buffer, 1.5 mM MgCl\(_2\), 0.2 mM dNTP mix, 0.5 \(\mu\)M of each CYP2E1 primers, 2 \(\mu\)l of cDNA and 1 \(\mu\)l Taq enzyme. The details of the sequence of primers used for CYP2E1 have been described earlier (Hakkola et al., 1994). PCR was carried out in Gene Amp PCR system 9700 of Applied Biosystem using 35 cycles of denaturation at 94\(^{\circ}\)C for 5 min, annealing of primers at 60\(^{\circ}\)C for 1min, extension at 72\(^{\circ}\)C for 1.5 min and final extension at 72\(^{\circ}\)C for 10 min. Prior to amplification of CYP2E1, normalization was carried out with \(\beta\)-actin, the housekeeping
gene. The PCR reaction for β-actin contains 10X PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP mix, 0.5 μM of each β-actin primers (Hakkola et al., 1994), 2 μl of cDNA and 1 μl Taq enzyme. PCR was carried out using 35 cycles of denaturation at 94°C for 5 min, annealing of primers at 59°C for 1 min, extension at 72°C for 1.5 min and final extension at 72°C for 10 min. PCR products (365bp for CYP2E1 and 541bp for β-actin) were analyzed by electrophoresis in 2.5% and 1.8% agarose gel respectively stained with ethidium bromide in VERSA DOC Imaging system, Model 1000 (Biorad, USA). Densitometric analysis of the bands was carried out using Quantity One Quantitation Software version 4.3.1 (Biorad, U.S.A).

4.2.5 Quantitative Real Time PCR (RT-PCR) Analysis

For Quantitative PCR, cDNA was synthesized by High-Capacity cDNA Reverse Transcription Kit provided by Applied Biosystems. For RT, the reaction mixture in 20μl contained 10μl 2X master mix (2X RT buffer (2μl), 0.8 mM dNTP Mix (0.8μl), 2X Random Primers (2μl), RNase Inhibitor (1μl), 1μl MultiScribe Reverse Transcriptase and 3.2μl Nuclease-free H₂O) and an equal volume of diluted RNA sample. RT reaction was carried out in a thermal cycler consisting of 1 cycle of 25°C for 10 min, 37°C for 120 min and 85°C for 5 sec followed by placing the reaction vial at 4°C. The PCR reaction mixture for CYP2E1 and β-actin in 20μl contained 1X TaqMan Universal PCR Master Mix provided by Applied Biosystems, 0.5μM of each primer, 0.2μM of probe, 2μl cDNA and Nuclease-free H₂O. TaqMan assays for each gene target were performed in triplicate on cDNA samples in 96-well optical plates on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). PCR parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min.

4.2.6 Lymphocyte isolation

Lymphocytes were isolated from the blood by the method of Boyum (Boyum, 1968) with slight modifications. In brief, 4.0 ml of whole blood was diluted with 4.0 ml of phosphate buffered saline (PBS), pH 7.4, and carefully layered over 2.0 ml of Histopaque 1077. After centrifugation at 400 x g for 30 min at room temperature, the upper layer was discarded and the opaque interface containing mononuclear cells was transferred into a clean centrifuge tube. After repeated washing of the lymphocytes with
PBS and recentrifugation at 250 x g, the resulting pellet was resuspended in 0.5 ml of PBS and used for enzyme estimations.

4.2.7 Protein estimation

Protein content of the samples was estimated by the method of Lowry et al. (1951). Suitable volume of protein sample was diluted to 1.0 ml with distilled water and to 1.0 ml aliquot, 5.0 ml of alkaline copper reagent (2% copper sulphate, 2% sodium potassium tartarate, 2% sodium carbonate in 0.2N sodium hydroxide) was added. The solution was kept for 10 minutes at room temperature. The final color was developed by the addition of 0.5 ml of Folin Ciocalteau phenol reagent (1N). The intensity of the color developed 30 minutes later was measured at 660 nm in a spectrophotometer. The amount of protein in the sample was determined by comparing it with the standard protein curve of BSA.

4.2.8 Enzyme assay

NDMA-d activity was assayed by the method of Dey et al. (2002). The assay mixture contained a suitable amount of lymphocytes, 70.0 mM Tris-HCl, pH 7.4, 10.0 mM semicarbazide, 14.0 mM MgCl2, 215.0 mM KCl, 1.0 mM NADPH and 4.0 mM NDMA in 1.0 ml final volume. The reaction mixture was incubated at 37°C for 30 minutes and the reaction was stopped by the addition of 1.5ml of 12.5% TCA solution. After centrifugation at 2000 rpm for 10 minutes, 2 ml of the supernatant was mixed with an equal amount of Nash reagent. The tubes were then incubated at 70°C for 20 min and the HCHO formed was measured at 415 nm (Nash, 1953).

4.2.9 Enzyme-linked immunosorbent assay (ELISA)

Polyvinyl chloride 96-well plates (Falcon) were coated with poly L-lysine and washed twice with double distilled water. Protein were then bound on 96-well plates overnight at 4°C with sequentially increasing concentrations (from 0.001µg to 100µg) in phosphate buffered saline (PBS, 0.1 M phosphate buffer, 150 mM NaCl, pH 7.2). The binding step(s) was followed by a blocking step by incubating of the plates with 4% BSA, 5% sucrose and 0.05% sodium azide (NaN₃) in PBS for 2 hours at room temperature. The plates were then washed twice for 15 min with wash buffer (0.05% Tween 20 in PBS). Primary rabbit anti-human CYP2E1 antibodies and secondary
alkaline phosphatase (AP) conjugated anti-rabbit IgG antibodies were diluted appropriately with 20 mM Tris (pH 7.5), 2% BSA, 0.05% Tween 20, 150 mM NaCl. Plates were incubated with primary antibody for 1 h at ambient temperature, followed by two wash-steps. Incubation with secondary antibodies was also carried out for 1 h at ambient temperature. Two 15-min wash steps were done in the buffer described above, and a final 15-min step was done in PBS without additives. The bound AP conjugate was detected by adding 100μl of p-nitrophenyl phosphate (PNPP). The alkaline phosphatase reaction was stopped after 20 min by the addition of 50μl of 3 M NaOH. Optical densities at 405 nm were measured using an ELISA plate reader. The assay was performed in triplicates.

4.2.10 Statistical analysis

Students 't' test was employed to calculate the statistical significance between non-alcoholic control and cirrhotic patients. p<0.05 was considered to be significant when compared with the non-alcoholic controls.

4.3 RESULTS

Total RNA was isolated from blood of eight non-alcoholic controls and equal number of sex matched alcoholic and non-alcoholic liver cirrhotic patients, CYP2E1 mRNA were quantified by RT-PCR and Real Time PCR, as described under Materials and Methods, using a set of forward and reverse primers that ensured a specific CYP2E1 cDNA amplification. In order to improve the accuracy and precision of the correlation studies, mRNA expression of the β-actin, a house keeping gene, was also studied and the abundance of CYP2E1 mRNA was normalized against the house keeping gene.

4.3.1 Semi-Quantitative PCR analysis

PCR amplification of the RT product obtained from the RNA isolated from non-alcoholic controls, with the primer pairs of β-actin, resulted in the formation of PCR products of expected band size of 541bp. As evident from figure 4.1A almost equal intensity of the PCR product was obtained from the PCR amplification of the RT product isolated from RNA extract from blood lymphocytes of non-alcoholic control or non-
alcoholic cirrhotic or alcoholic cirrhotic patients (Figure 4.1 A and 4.1B). Results of PCR amplification of RT products obtained from RNA isolated from the whole blood of non-alcoholic controls or non-alcoholic cirrhotic or alcoholic cirrhotic patients, with primers specific for CYP2E1 are shown in figure 4.2A. As evident from the figure, PCR product of expected band size of 365 bp were formed with the RNA isolated from the blood of non-alcoholic controls or non-alcoholic cirrhotic or alcoholic cirrhotic patients. Densitometric analysis of the PCR products revealed that the intensity of the bands was higher in the PCR products obtained from blood lymphocytes of patients suffering from alcoholic liver cirrhosis when compared to non-alcoholic controls or non-alcoholic cirrhotic patients (Figure 4.2A). As evident from figure 4.2B, mRNA expression of CYP2E1 in alcoholic cirrhotic patients was found to be 1.9 and 1.6-folds higher than non-alcoholic controls and non-alcoholic cirrhotic patient respectively which was found to be statistically significant. No significant change was however, observed in the mRNA expression of CYP2E1 in non-alcoholic cirrhotic patients when compared with non-alcoholic controls (Figure 4.2A).

4.3.2 Quantitative Real-Time PCR (RT-PCR) Analysis

To validate the data of semi-quantitative PCR, RT-PCR was also carried out to study the mRNA expression of CYP2E1 in lymphocytes of non-alcoholic control, non-alcoholic cirrhotic and alcoholic cirrhotic patients. The mRNA expression of housekeeping gene (β-actin) was used as an endogenous control. The expression of β-actin was found to be uniform throughout all the samples (control and patients) analyzed confirming the integrity of RNA used in assays. RT-PCR clearly demonstrated that the mRNA expression of CYP2E1 in alcoholic cirrhotic patients increased 2-fold in blood lymphocytes when compared to the non-alcoholic controls. This increase in CYP2E1 mRNA expression in the alcoholic cirrhotic patient was found to be statistically significant (Table 4.2). A 1.4-fold higher induction was also observed in the mRNA expression of CYP2E1 blood lymphocytes isolated from non-alcoholic cirrhotic patients when compared with non-alcoholic controls which was found to be statistically significant (Table 4.2).
Figure 4.1: (A) Ethidium Bromide-stained agarose showing mRNA expression of β-actin in blood lymphocytes of non-alcoholic control, non-alcoholic cirrhotic patients (NACP) and alcoholic cirrhotic patients (ACP). Lane 1 contains -mRNA, lane 2, 3, 4 contains RT-PCR. Lane 5 contains 100bp DNA ladder. (B) Densitometric analysis of β-actin in blood lymphocytes of non-alcoholic control, NACP and ACP.
Figure 4.2: (A) Ethidium Bromide-stained agarose showing mRNA expression of CYP2E1 in blood lymphocytes of non-alcoholic control, non-alcoholic cirrhotic patients (NACP) and alcoholic cirrhotic patients (ACP). Lane 1 contains mRNA, lane 2, 3, 4 contains RT-PCR. Lane 5 contains 100bp DNA ladder. (B) Mean optical density of CYP2E1 in blood lymphocytes of non-alcoholic control, NACP and ACP.

Mean optical density = optical density of CYP2E1 / optical density of β-actin.

*p < 0.005 when compared with non-alcoholic control.
Table 4.2: Fold induction of CYP2E1 by Real Time PCR analysis in blood lymphocytes of non-alcoholic cirrhotic patients and alcoholic cirrhotic patients when compared with non-alcoholic controls

<table>
<thead>
<tr>
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<th>Fold Induction</th>
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<tbody>
<tr>
<td>Non-alcoholic cirrhosis (NACP)</td>
<td>1.41* ± 0.06 (n=8)</td>
</tr>
<tr>
<td>Alcoholic cirrhosis (ACP)</td>
<td>1.96* ± 0.09 (n=8)</td>
</tr>
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</table>

All the values are mean ± S.E. of eight NACP & ACP.
* p<0.001 when compared with non-alcoholic controls.
Figure in parenthesis indicate number of NACP & ACP.
4.3.3 N-nitrosodimethylamine demethylase (NDMA-d) activity

The catalytic activity of CYP2E1 dependent NDMA-d in blood lymphocytes of healthy non-alcoholic controls and patients suffering non-alcoholic and alcoholic liver cirrhosis is summarized in Table 4.3. As evident from the table the specific activity of CYP2E1 in the peripheral blood lymphocytes of alcoholic cirrhotic patients was found to be significantly higher (1.3 fold) when compared with non-alcoholic controls or non-alcoholic cirrhotic patients. In contrast no significant change in the NDMA-d activity was observed in the blood lymphocytes of non-alcoholic cirrhotic patients when compared with non-alcoholic controls (Table 4.3).

4.3.4 Enzyme-linked immunosorbent assay (ELISA)

Figure 4.3 summarizes the CYP2E1 protein activity using ELISA at different protein concentration in non-alcoholic controls, non-alcoholic cirrhotic patients and alcoholic cirrhotic patients. Consistent with the mRNA expression studies, the results from ELISA also indicate that CYP2E1 protein was expressed in blood lymphocytes of non-alcoholic controls, non-alcoholic cirrhotic and alcoholic cirrhotic patients. As evident from figure 4.3, the level of CYP2E1 protein in blood lymphocytes of all the three groups gradually increased with the increase in protein concentration. Maximum activity was found at 100 µg of protein concentration and the protein expression in blood lymphocytes of alcoholic cirrhotic patients was found to be higher when compared to non-alcoholic controls or non-alcoholic cirrhotic patients (Figure 4.3). As evident from the figure, the CYP2E1 protein activity in the peripheral blood lymphocytes of alcoholic cirrhotic patients was found to be 1.25-fold higher than non-alcoholic controls. However, no statistically significant change was observed in CYP2E1 protein in the blood lymphocytes of non-alcoholic cirrhotic patients when compared with non-alcoholic controls (Figure 4.3). The CYP2E1 protein expression in blood lymphocytes of alcoholic cirrhotic patients was found to be increased (1.15-fold) when compared with non-alcoholic cirrhotic patients which however, was not found to be statistically significant (Figure 4.3).
Table 4.3: CYP2E1 dependent N-nitrosodimethylamine demethylase (NDMA-d) activity in blood lymphocytes of non-alcoholic controls, non-alcoholic cirrhotic patients and alcoholic cirrhotic patients

<table>
<thead>
<tr>
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<th>Specific Activity</th>
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<tbody>
<tr>
<td><strong>Non-alcoholic Control</strong></td>
<td>1.78 ± 0.026 (n=8)</td>
</tr>
<tr>
<td><strong>Non-alcoholic cirrhosis (NACP)</strong></td>
<td>1.90 ± 0.038 (n=8)</td>
</tr>
<tr>
<td><strong>Alcoholic cirrhosis (ACP)</strong></td>
<td>2.36* ± 0.123 (n=8)</td>
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</table>

Values are expressed in n moles HCHO/min/mg protein.
All the values are mean ± S.E. of eight control and patients.
* p<0.001 when compared with control and non-alcoholic cirrhotic patients.
Figure in parenthesis indicate number of non-alcoholic controls and patients.
Figure 4.3: CYP2E1 protein activity using ELISA at various protein concentration in blood lymphocytes of non-alcoholic controls, non-alcoholic cirrhotic patients (NACP) and alcoholic cirrhotic patients (ACP).
4.4 DISCUSSION

Consistent with earlier reports (Song et al., 1990; Raucy et al., 1997, 1999; Haufrroid et al., 2003, 2005), the present study has provided evidence for the mRNA expression of CYP2E1 in freshly isolated peripheral blood lymphocytes of healthy individuals. Though CYP2E1 is primarily a hepatic enzyme, studies have shown that CYP2E1 mRNA is expressed in peripheral blood lymphocytes of human (Song et al., 1990) and laboratory animals (Soh et al., 1996; Dey et al., 2002, 2005). The expression of CYP2E1 in these uncultured and unstimulated peripheral blood lymphocytes is regulated in a manner similar to that observed in the liver (Song et al., 1990; Raucy et al., 1997, 1999). Our study using ELISA has further showed that CYP2E1 protein was expressed in control blood lymphocytes isolated from healthy individuals. As mRNA and protein were elevated concordantly in the peripheral blood lymphocytes following exposure to ethanol (Raucy et al., 1999) and western immunoblotting required larger amounts of blood sample, ELISA was used to study protein expression instead of blotting. Earlier studies using western immunoblotting have indentified the expression of CYP2E1 in blood lymphocytes isolated from humans (Song et al., 1990; Raucy et al., 1997). Western immunoblotting with freshly prepared peripheral blood lymphocytes isolated from experimental animals also revealed constitutive as well as inducible expression of CYP2E1 protein (Raucy et al., 1995; Dey et al., 2002, 2005).

CYP2E1 mRNA and protein expressed in blood lymphocytes was further found to be catalytically active as reflected by the present study indicating significant activity of NDMA-d in freshly prepared peripheral blood lymphocytes isolated from healthy individuals. Previous studies have also shown that freshly prepared blood lymphocytes were found to catalyse the demethylation of N-nitrosodimethyamine (NDMA) and NADPH dependent lipid peroxidation in Wistar rats. Similarities were observed in the NADPH dependent lipid peroxidation and NDMA-d activity in the blood lymphocytes with liver microsomes further suggesting that CYP2E1 lymphocytes levels in freshly isolated peripheral blood lymphocytes could be used to monitor tissue enzyme levels (Dey et al., 2002, 2005). Using chlorzoxazone as a substrate to monitor CYP2E1 catalyzed reactions, Raucy et al. (1997, 1999) reported that freshly isolated peripheral blood lymphocytes catalyze the oxidation of chlorzoxazone in healthy individuals. Raucy
et al. (1999) have further reported that ethnicity does not play a major role in the constitutive expression of CYP2E1 in the lymphocytes as the levels of CYP2E1 mRNA and protein were found to be similar among the healthy Whites, Novajo and Mexican-American ethnic groups.

A significant increase in the CYP2E1 mRNA expression in freshly prepared peripheral blood lymphocytes isolated from alcoholic liver cirrhotic patients when compared to the non-alcoholic controls or non-alcoholic cirrhotic patients is in agreement with the earlier studies (Yano et al., 2001; Jones et al., 2002) indicating that CYP2E1 expression increases significantly in patients suffering from alcoholic liver disease. Chronic ethanol consumption is known to increase the expression of CYP2E1 in liver which increases the formation of reactive oxygen species leading to the liver damage (Lieber, 1991, 1998). Yano et al., (2001) reported that CYP2E1 mRNA levels of mononuclear cells in alcoholic healthy controls increased almost 100-times compared with non-alcoholic healthy controls. Interestingly, when these subjects abstained from drinking, this increase in CYP2E1 mRNA decreased to the levels observed in non-alcoholic healthy subjects by 4-days of abstinence (Yano et al., 2001). Further, CYP2E1 mRNA levels in patients with alcoholic liver disease were found to be almost similar to that observed in alcoholic healthy subjects. However, progression of the disease to hepatic fibrosis and cirrhosis did not resulted in any significant difference in CYP2E1 mRNA levels suggesting that CYP2E1mRNA in mononuclear cells may not be influenced by the progression of disease (Yano et al., 2001). Studies in laboratory animals have also shown that the expression of CYP2E1 mRNA and protein in the lymphocytes is influenced by the same factors that affect the concentration of hepatic enzymes. CYP2E1 induction by either ethanol or fasting occurred both in liver and lymphocytes in a parallel manner (Soh et al., 1996; Raucy et al., 1997, 1999; Dey et al., 2002, 2005).

That the increased mRNA expression of CYP2E1 in blood lymphocytes of alcoholic cirrhotic patients leads to increased protein expression and associated enzyme activity was demonstrated by an increase in the CYP2E1 immunoreactive protein and NDMA-d activity in the blood lymphocytes of alcoholic liver cirrhotic patients when compared with non-alcoholic controls or non-alcoholic cirrhotic patients. Raucy et al., (1997) also reported a 2-fold increase in the CYP2E1 immunoreactive protein in
alcoholics when compared with non-alcoholic controls. This increase in lymphocyte CYP2E1 content in alcoholic subjects was associated with nearly 2-fold increase in chlorzoxazone clearance rate and a 2-fold decrease in the AUC for chlorzoxazone. Dey et al. (2002, 2005) who characterized CYP2E1 activity in freshly isolated and uncultured rat blood lymphocytes also reported significant increase in CYP2E1 dependent NDMA-d activity and lipid peroxidation in freshly prepared lymphocytes isolated from ethanol pretreated rats. The increase in NDMA-d activity in rat blood lymphocytes was further found to be associated with increase in mRNA and protein expression of CYP2E1 (Dey et al., 2002, 2005). The increase in CYP2E1 protein expression and associated NDMA-d activity observed in the blood lymphocytes of alcoholic cirrhotic patients in present study is in agreement with earlier reports demonstrating a higher chlorzoxazone oxidation in patients with alcoholic liver disease when compared with alcoholics without clinical and biochemical signs of liver disease suggesting the role of CYP2E1 in the development of alcoholic liver disease (Dupont et al., 1998). Dilger et al. (1997) also showed a 3-fold increase in chlorzoxazone oxidation in actively drinking patients with alcoholic liver disease when compared with abstaining patients with the disease. Elevated CYP2E1 activity as measured by chlorzoxazone metabolism was found to rapidly decline during withdrawal of alcohol. The contribution of CYP2E1 inducibility as a risk factor for the development of alcoholic liver disease has also been shown in experimental studies using chronic ethanol-fed rats, in which the modulation of CYP2E1 by treatments with diallylsulfide or phenylethylisothiocyanate, specific inhibitors of CYP2E1, reduced the extent of alcohol induced liver damage (Albano et al., 1996).

A significant increase observed in the CYP2E1 mRNA expression in freshly isolated peripheral blood lymphocytes of non-alcoholic liver cirrhotic patients when compared to the non-alcoholic controls could be attributed to the hepatitis C virus infection. CYP2E1 activation has been proposed to be one potential factor responsible for the progression of fibrosis in chronic hepatitis C disease. In hepatitis C virus (HCV) infected patients, the presence of hepatic steatosis has been associated with the increased expression of CYP2E1 mRNA in the liver (Gochee et al., 2003). In contrast, Haufroid et al. (2005) did not observed induction in CYP2E1 activity either in liver or blood lymphocytes of chronic HCV patients, mainly without cirrhosis, suggesting absence of relationship between CYP2E1 induction and patients with early stages of hepatitis C.
infection. However, as almost none of patients included in the study exhibited histological fibrosis, Haufroid et al. (2005) suggested that that the development of cirrhosis in chronic HCV patients may be associated with an increase in CYP2E1 activity.

Interestingly, our data indicating increase in CYP2E1 mRNA and protein expression and NDMA-d activity in the freshly isolated peripheral blood lymphocytes of alcoholic liver cirrhotic patients when compared with the non-alcoholic controls is in contrast to the earlier reports indicating a decrease in hepatic CYP2E1 activity in cirrhotic patients (George et al., 1995). CYP2E1 was found to be reduced in microsomes of cirrhotic livers from patients with cholestasis, but not in other non-cholestatic cirrhotic livers. The reasons for this inconsistency could be due to the fact that our study involved the actively drinking individuals with non-cholestatic early stage of alcoholic liver cirrhosis, whereas the study of George et al. (1995) included the non-drinking patients suffering from cholestatic end stage of cirrhosis. Cholestatic cirrhosis has been shown to lead to the accumulation of bile acids in the liver which induces cellular oncotic necrosis and apoptosis leading to cell death (Spivey et al., 1993). It is however not clear whether this oncotic necrosis and apoptosis leads to reduced CYP2E1 activity or whether necrosis and apoptosis is a consequence of altered CYP2E1 activity. Dilger et al. (1997) have also reported continuously decreasing CYP2E1 activity in non-drinking or abstaining patients with progressively severe manifestations of alcoholic liver disease. Using bile duct ligation and carbon tetrachloride (CCl4)-induced models of cirrhosis, Bastien et al. (2000) also reported that the CYP content and the individual CYP isoenzymes are differentially altered by cirrhosis in the rats. In rats with mild cirrhosis, CYP contents and the activity of CYP1A2, 2C and 3A isoenzymes were comparable with the controls while an increase in the activity of CYP2E1 was observed. In rats with more severe form of cirrhosis the contents of all the four CYPs were found to be reduced (Bastien et al., 2000).

In conclusion, the results of the present study have shown the CYP2E1 mRNA is expressed in freshly prepared uncultured human blood lymphocytes. Significant increase in the CYP2E1 mRNA and protein expression and associated NDMA-d activity in the freshly prepared blood lymphocytes isolated from non-cholestatic alcoholic liver cirrhotic patients when compared to non-alcoholic controls and non-alcoholic cirrhotic patients have shown that blood lymphocytes CYP2E1 could possibly be used to predict alcohol
induced toxicity as reported in experimental animals (Raucy et al., 1995; Dey et al 2002, 2005). Further this increase in CYP2E1 mRNA expression and protein activity in freshly prepared blood lymphocytes of alcoholic liver cirrhotic patients with early stage of alcoholic cirrhosis have indicated suitability of using blood CYP2E1 mRNA expression profile as a biomarker to detect the hepatic level of CYP2E1.