Synergistic Effects of Glutathione and Vitamin E on ROS Mediated Ethanol Toxicity in Isolated Rat Hepatocytes

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ABSTRACT
In this study, the potential synergistic relationship between reduced glutathione and vitamin E on ethanol toxicity in isolated rat hepatocytes were examined. It was observed that treatment of ethanol (5-25 mM) caused decrease in cell viability but in presence of Rotenone, cell viability decreased to a greater extent. Inhibitory concentration (IC50) was found to be 20 mM of ethanol treated with rotenone (10 μM) for 2 h. Treatment of ethanol with rotenone (20 mM±10 μM) caused increase in lipid peroxidation, increase of lactate dehydrogenase and decrease in reduced glutathione content. Reduced glutathione and α-tocopherol, alone and in combination, were added to hepatocyte suspension in an attempt to protect cells against alcohol induced cell damage. Pretreatment with reduced glutathione and vitamin E independently, increased the cell viability to 78.97 and 70.42%, respectively, but a combination of reduced glutathione and vitamin E increased cell viability to 92.12%. Vitamin E reduced lipid peroxidation by 41.32% but in combination with reduced glutathione decreased lipid peroxidation by 61.15%. The results suggest an interaction between GSH and Vitamin E in protecting ethanol induced hepatocytes damage.

Key words: Ethanol, rotenone, hepatotoxicity, antioxidants, oxidative stress

INTRODUCTION
Hepatic dysfunction due to overconsumption of alcohol has widely increased in the last few years (Hao et al., 2004; Caetano et al., 2009; Kypri et al., 2009). Hepatic dysfunction in chronic alcoholics is mainly due to the generation of excessive amount of Reactive Oxygen Species (ROS), resulting in the detrimental effects on the cellular antioxidant defense system (Adachi and Ishii, 2002; Nagy, 2004) as well as enhancement of the lipid peroxidation (LPC) (Sun et al., 2001) and mitochondrial dysfunction (Cunningham et al., 1999). Ethanol metabolism alters the cytosolic and mitochondrial redox states [NADH/NAD+] and it may exacerbate mitochondrial ROS production by increasing the flow of electron down the respiratory chain. Thus chronic ethanol exposure may accelerate an oxidative mechanism directly and indirectly which eventually causes cell death and extensive tissue damage (Higuchi et al., 2001; Adachi et al., 2004; Li et al., 2004). Damage to the liver can be successfully prevented or controlled by supplementation with antioxidants (Turkdogan et al., 2001; Bansal et al., 2005).
Rotenone is known to be a potent inhibitor of complex I of the mitochondrial respiratory chain in all cell types, by inhibiting the function of mitochondrial NADH dehydrogenase activity (Sherer et al., 2003; Molina-Jimenez et al., 2004) therefore leading to a decrease in aerobic metabolism and development of a lactic acidosis. This inhibition of the mitochondrial respiratory chain leads to an increase in the production of hydrogen peroxide and superoxide (O$_2^*$) and oxygen radical species (Sakka et al., 2003; Molina-Jimenez et al., 2004). Rotenone is effectively broken down by the liver to produce less toxic and excretable metabolites. Approximately 20% of the oral dose (and probably most of the absorbed dose) is excreted within 24 h (Ray, 1991); around 80% as water-soluble products with the remainder as hydroxylated rotenoids (Pukami et al., 1996). In the present study non-enzymatic antioxidants such as GSH (reduced glutathione) and Vitamin E (Vit E) were employed as defense against oxygen free radicals. The purpose of present study was to demonstrate that chronic ethanol exposure increased ROS generation in hepatocytes, which may result in decrease in cell viability and increase in lipid peroxidation. For this, isolated rat hepatocytes were incubated with ethanol and rotenone, a complex I inhibitor of electron transport chain. Rotenone is a natural toxin produced by several tropical plants and is used in solution as a pesticide and insecticide.

MATERIALS AND METHODS

**Animals:** Male albino Wistar rats weighing 150±20 g were purchased from Central Drug Research Institute (CDRI), Lucknow, India for the study and kept in standard temperature, moisture, diet and water condition. Approval of the protocol and animal use were obtained from the institutional ethical committee. This study sponsored by CSTUP was initiated in July 2008 and came to certain conclusion by December 2010.

**Chemicals:** Rotenone (EC no: 650-005-00-2; CAS no: 83-79-4), HEPES, collagen, collagenase (Type IV, from *Clostridium histolyticum*), ethylene glycol-bis-tetra acetic acid (EGTA), LDH assay kit, total Glutathione assay kit, heparin, methylthiozoletetrazolium (MTT), FBS, RPMI media were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals and reagents used were purchased from Himedia, BDH, Merck and Qualigenes Ltd. HPLC grade quartz double distilled water was employed throughout the studies.

**Primary cell-culture:** Hepatocytes were isolated from liver of overnight fasted rat after subjecting it to two-stage collagenase perfusion with HEPES buffer (Seglen, 1976). Cell viability was checked by trypan blue dye exclusion test within an hour of cell isolation. Only preparations with cell viability greater than 95% were used for subsequent experiments. Hepatocytes were maintained in RPMI-1640 media supplemented with heat-inactivated 10% fetal bovine serum (FBS) and 1% of 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin-B at 37°C in a 5% CO$_2$-95% air incubator (Thermo-forma) with controlled humidity. The cells were seeded at a density of 1.0×10$^4$ cells in 0.1% collagen pre-coated 96-well plate, and used for the ethanol exposure experiments after being cultured overnight.

**Experimental design:** The experiments were designed into two stages as a) concentration-response experiments and b) protective experiments. The concentration-response experiments were designed to determine the effects of different concentration of ethanol alone and with rotenone on cell viability. The first series of experiments were performed by incubating hepatocytes with different concentration of ethanol ranging from 5 mM to 25 mM alone and with rotenone (10 µM),
measured the cell viability in terms of MTT reduction after 2 h incubation. Rotenone was prepared as a 10 mM solution in Dimethylsulfoxide (DMSO), filtered through 0.22 μM filter and used for subsequent treatment. Hepatocytes treated (control samples) with an equivalent vehicle concentration i.e 0.15% (v/v) had no effect on cell viability. The second series of experiments were performed by pre-incubating the hepatocytes with reduced GSH or Vitamin E, alone and in combination, to study the possible protection of the hepatocytes against Lipid Peroxidation (LPO) and glutathione depletion.

Based on result obtained from concentration-response experiments, a single concentration of ethanol with rotenone (20 mM±10 μM) was chosen for these experiments. Hepatocytes were pre-incubated with either GSH (8 mM), Vitamin E (4 mM) or in combination of both GSH (8 mM) and Vitamin E (4 mM) for a period of 10 min prior to the addition of Rotenone (10 μM) and ethanol (20 mM) showed complete protection from cellular toxicity. RPMI-1640 media was used in control cell suspension.

**MTT assay:** Cell viability was determined by a colorimetric MTT assay, as described by Mosmann (1983).

**MDA determination:** In this assay the evaluation of end product malondialdehyde (MDA) formed due to membrane lipid peroxidation was measured. Briefly, in 10 μL lysate, 50 μL of 50 mM phosphate buffer, 10 μL of 1 mM butylated hydroxy toluene (BHT), 75 μL of 1.3% Thiobarbituric Acid (TBA) was added in distilled water. The lipids were isolated by precipitating them with 50 μL of 50% trichloroacetic acid (TCA). The mixture was then incubated at 60°C for 40 min and then kept on ice for 15 min. Reaction was stopped by adding 10 μL of 20% Sodium Dodecyl Sulphate (SDS). This assay measures the amount of pink coloured MDA-TBA adduct at 530 nm and to account for the interference of phytochemicals it is also read at 600 nm (Wallin et al., 1993).

**GSH content:** GSH content was measured using standardized kit from Sigma Chemical Co., USA. Assays were performed in 96-well microplates by the method of Griffith (1980) and change in absorbance monitored at 410 nm.

**LDH activity-based cytotoxicity assay:** LDH (lactate dehydrogenase) was measured using standardized kit from Sigma Chemical Co., USA. LDH activity was measured both in floating dead cells and viable adherent cells. The floating cells were collected from culture medium by centrifugation (240xg) at 4°C for 5 min and LDH content from the pellet was used as an index of apoptotic cell death (LDHp). The LDH released in the culture medium (LDHe; extracellular LDH) was used as an index of necrotic cell death and LDH present in the adherent viable cells as intracellular LDH (LDHi). The percentage of apoptotic and necrotic cell death was calculated as follows:

\[
\text{% Apoptosis} = \frac{\text{LDHp}}{\text{LDHp} + \text{LDHi} + \text{LDHe}} \times 100
\]

\[
\text{% Necrosis} = \frac{\text{LDHe}}{\text{LDHp} + \text{LDHi} + \text{LDHe}} \times 100
\]

**Preparation of reagents:** Perfusate solutions, washing media, and incubation media were prepared using deionized water. Rotenone was prepared as a 10 mM solution in dimethylsulfoxide.
Control samples were incubated with an identical concentration of DMSO (0.35% v/v) to replicate incubation condition with Rotenone. DMSO had no effect on ROS generation and cell viability.

Reduced GSH was prepared in RPMI-1640 media, pH 7.4 and Vitamin E (DL-α-tocopherol) was dissolved in absolute ethanol immediately before use. The final ethanol concentration in the hepatocytes incubations was less than 0.01%.

**Statistical analysis:** All the data are expressed as Mean±Standard Deviation (SD). When comparing the groups the data were analyzed by one-way variance model (ANOVA) using statistical software SPSS 17 version. The statistical significance are shown as (**p<0.01) and (*)p<0.001) for denotation.

**RESULTS**

**Hepatocytes cell viability:** The cell viability results from hepatocytes suspension incubated with varying concentration of ethanol (5-25 mM) in absence and presence of rotenone (10 μM) showed decrease in cell viability both in ethanol and ethanol plus rotenone treated cells. However, in ethanol plus rotenone treated cells, cell viability decreased more rapidly. These results suggest that ethanol stimulate hepatocytes ROS production and mild decrease in cell viability but in presence of rotenone (complex I inhibitor) ROS generation increased two fold and decreased the cell viability in dose dependant manner of Ethanol (Fig. 1). Inhibitory concentration (IC<sub>50</sub>) was determined and was found to be 20 mM of ethanol treated with rotenone. This concentration of ethanol with rotenone (20 mM±10 μM) was used for further experiments.

**Effect of GSH and Vitamin E on cellular toxicity:** The protective effects of GSH, Vitamin E (Vit E) and combination of GSH with Vitamin E on the viability of rat hepatocytes showed preventive effects, when determined with MTT cytotoxicity assay. The pretreatment of cells with GSH (8 mM), Vit E (4 mM) and GSH (8 mM) plus VitE (4 mM) increased the cell viability significantly. Pretreatment independently with GSH (8 mM) and Vit E (4 mM) increased the cell viability to 78.97 and 70.42%, respectively, but combination of GSH and VitE increased cell viability to 92.12% (Fig. 2). Ethanol was a common treatment in all the cases.

**LDH activity**

**Correlation with necrosis and apoptosis of primary hepatocytes:** Cell death through oxidative stress may be accomplished by two distinct mechanisms, necrosis or apoptosis. To further

![Fig. 1: Effect of ethanol alone (5-25 mM) and with rotenone (10 μM) on the viability of cultured primary rat hepatocytes (5-25 mM). Values are mean±S.D of 5 determinations in each case. Significantly different from control cells (*p<0.001)
Fig. 2: Protective effect of GSH, Vitamin E and GSH+Vitamin E on toxicity. Treatment was carried out in different combinations of Ethanol, Rotenone, GSH, Vitamin E and a comparison was drawn between these groups. Values are Mean±SD of 5 determinants in each case *p<0.001. EtOH: Ethanol, R: Rotenone, GSH: Reduced glutathione, Vit E: Vitamin E

Fig. 3: EtOH+R treated group was compared with control group, EtOH+R+GSH and EtOH+R+Vit E treated groups were compared with EtOH+R treated groups and EtOH+R+GSH+Vit E treated group was compared with EtOH+R+GSH and EtOH+R+Vit E. Values are Mean±SD of 5 determinants in each case. *p<0.001, **p<0.01 when compared EtOH+R+GSH+Vit E treated group with EtOH+R+GSH EtOH: Ethanol, R: Rotenone, GSH: Reduced glutathione, Vit E: Vitamin E.

characterized the possible mechanism involved in ethanol and rotenone treated cell death and efficacy of GSH, Vitamin E, GSH plus Vitamin E as protective agents, the ratio of necrosis and apoptosis in primary hepatocytes was analyzed using LDH activity-based assay. Intracellular LDH release was evaluated as a result of breakdown of plasma membrane and alteration of its permeability. The cells were exposed to ethanol and rotenone for 2 h, LDH leakage was taken as the cell death indicator. Figure 3 shows LDH leakage increased significantly in the presence of ethanol and rotenone (20 mM±10 μM) with 53.23% (p<0.001) apoptotic and 28.14% (p<0.001)
Table 1: Effect of GSH and Vit E on cell GSH content and MDA level

<table>
<thead>
<tr>
<th>Condition</th>
<th>GSH level (2-nitro-5-thiobenzoic acid formation/min/10^6 cells)</th>
<th>Lipid peroxidation (nM MDA formation/10^6 Treatment cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>213±0.68</td>
<td>0.31±0.05</td>
</tr>
<tr>
<td>EtOH-R</td>
<td>98±4.61*</td>
<td>1.21±0.15*</td>
</tr>
<tr>
<td>EtOH-R+GSH</td>
<td>168±2.32*</td>
<td>0.86±0.10*</td>
</tr>
<tr>
<td>EtOH-R+Vit E</td>
<td>190±3.24*</td>
<td>0.71±0.08*</td>
</tr>
<tr>
<td>EtOH-R+GSH+Vit E</td>
<td>191±2.95*</td>
<td>0.47±0.02*</td>
</tr>
</tbody>
</table>

Values are Mean±SD of 5 determinants in each case *p<0.001. EtOH: Ethanol, R: Rotenone, GSH: Reduced glutathione, Vit E: Vitamin E.

necrotic cells indicating apoptosis as a predominant mechanism responsible for cell death. Cells pre-incubated with GSH, Vit E showed decrease in apoptotic by 15.34%, 19.77% (p<0.001) as well as necrotic cells by 14.01 and 17.08% (p<0.001), respectively whereas pre-incubation with GSH plus Vit E decreased the number of apoptotic cells by 10.56% (p<0.01) and necrotic 7.41% (p<0.001) (Fig. 3).

Incubation with ethanol plus Rotenone increased the MDA formation significantly as 1.21±0.15 nM MDA formation per 1.0×10^4 cells. In hepatocytes pretreated with GSH, Vit E and combination of GSH plus Vit E, the peroxidative decomposition of phospholipids was reduced to 0.86±0.10, 0.71±0.08 and 0.47±0.02 nM MDA formation per 1.0×10^4 cells, respectively (p<0.001) (Table 1).

DISCUSSION

The present study investigated the protective effects of GSH, Vitamin E and combined effect of GSH plus Vitamin E on ROS mediated ethanol toxicity. Ethanol is oxidized by alcohol dehydrogenase enzyme present in the liver which causes redox imbalance in the hepatic cell [NADH/NAD^+]. NADH generated during ethanol oxidation is transported to the mitochondria through the malate aspartate shuttle. Thus, ethanol oxidation increases the availability of oxidizable NADH to the mitochondrial electron transport chain. Mitochondrial electron transport chain generates superoxide as an unavoidable by-product and primary ROS at two complexes, complex I and complex III (Cadenas and Davies, 2000; Turrens, 2003; Brand et al., 2004). The complex I (NADH-ubiquinone reductase) may generate superoxide (O_2^-) during the re-oxidation of the flavin mononucleotide (FMN) present within the enzyme complex (Turrens and Boveris, 1980). Thus, Rotenone used in the present study, inhibitor of complex I blocks the transfer of an electron from the iron-sulfur center N-2 to ubiquinone (Li et al., 2003) increased superoxide (O_2^-) production from the FMN semiquinone radical during ethanol oxidation (Bailey et al., 1999). It was also evident that increased ROS production causes decrease in cell viability and increased LPO. Studies have demonstrated that ethanol-induced liver injury is related to excessive generation of free radicals and consequently oxidative stress (Morel and Barouki, 1999; Koch et al., 2000; Jordao et al., 2004) and thereby responsible for cell death. LPO plays an important role in raising oxidative stress for injured liver (Slater, 1984) which is determined indirectly by evaluating the increase in MDA levels (Draper and Hadley, 1990) and decrease in GSH levels (Guerr and Grisolia, 1980). In the current study, we observed a higher level of MDA and a lower level of GSH in the isolated rat hepatocytes treated with ethanol plus rotenone (Table 1). The pretreatment with vitamin E and GSH attenuated the ethanol and rotenone elicited increase in cytotoxicity (Fig. 3). GSH is a hydrophilic tripeptide and is critical to glutathione redox cycling and enzyme
regulation. GSH reacts directly with (O₂•), hydroxyl radical (OH•) and organic free radicals and is critical for the detoxification processes of xenobiotic metabolism (Yu, 1994; Marks et al., 1996). Liver is the main producer of GSH and it is mostly concentrated in the liver. Experimental GSH depletion can trigger suicide of the cell by a process known as apoptosis (Slater et al., 1995; Duke et al., 1996). The results strongly suggest that hepatocytes treated with ethanol and Rotenone caused GSH depletion which results in decrease in cell viability and increased cellular apoptosis. However, pretreatment of cells with GSH alone showed increase in cell viability and decrease in apoptotic cell (Fig. 2, 3). Vitamin E, the major lipid soluble antioxidant present in all membrane, acts as a powerful terminator of lipid peroxidation (Machlin, 1980). It has been shown that the level of MDA increases whereas superoxide dismutase and GPx decreases in stored blood and therefore supplementation with antioxidants and vitamins is needs prior to blood transfusion (Marjani et al., 2007). The present study showed that pretreatment with Vit E causes the decrease in lipid peroxidation (Table 1), which is main cause of oxidative stress of ethanol exposure. The result of the cell viability assay, LDH leakage assay and lipid peroxidation assay suggests that synergistic action of GSH and vitamin E is more protective than GSH or Vitamin E alone treatment (Fig. 2, 3, Table 1). Many researchers have suggested that GSH was effective at reducing and regenerating Vitamin E to its active antioxidant state. Leedle and Aust (1990) hold the view of vitamin E regeneration by GSH which by contrast is not supported by others. Our study supports Leedle and Aust (1990) as was evident from our experiments that synergistic treatment confers better protection against ethanol induced cellular toxicity. Oxidative stress is the most known intermediary pathway initiating liver disease in diabetics and alpha tocopherol is recommended as an adjuvant therapy (Abdel-Hamid et al., 2008). Certain plants like Urtica pilulifera possess natural antioxidant properties, increases the proteins concentration and reduces lipid in lipemic liver, reduces phospholipid composition and can thus be used as food supplement or in pharmaceutical industry (Mahmoud, 2006; Mahmoud et al., 2006). Similarly some other plants like Solanum surattense leaf extract possesses antihyperglycemic, antilipidperoxidative, and hepatoprotective antioxidant potential (Serdev et al., 2007). Thus to combat oxidative stress comprehensively, plant sources with potent antioxidant potential could provide wonderful therapy.

CONCLUSION
The results from the present study indicates that hepatic dysfunction in chronic alcoholics may be primarily due to the generation of excessive reactive oxygen species. Hepatocytes treated with ethanol and rotenone causes GSH depletion, reduced cell viability and increased apoptosis. However, supplementation with GSH and Vitamin E together confers better and enhanced protection than with GSH or Vitamin E alone. The results are indicative of possible hepatoprotective therapy in alcohol induced liver dysfunction.

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REFERENCES


