

Does Ethanol Play a Pro-Oxidant Role during Oxidative Stress in the Liver?

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Received 29 May 2015; accepted 2 August 2015; published 5 August 2015

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Abstract

Oxidative stress has been implicated in the pathophysiology of liver injury during xenobiotic and alcohol metabolism, ischemia/reperfusion injury. In this study we examined if ethanol acted as a pro-oxidant making cells become more sensitive to tert-butylhydroperoxide (tBH) killing. Cell viability was determined in a rat hepatoma cell line (FTO2B) and rat primary hepatocytes in culture in the presence or absence of ethanol pretreatment. To elucidate the contribution of labile iron, deferoxamine (DF, an iron chelator) or lipid free radicals, N,N-diphenyl-p-phenylenediamine (DPPD, a lipid scavenger) were added to the ethanol tBH co-treatment. The levels of glutathione (GSH) and glutathione disulfide (GSSG) in the hepatocytes were also measured. Ethanol treatment (both pretreatment and co-treatment during the 3-hr tBH exposure) increased cell killing dramatically in both FTO2B cells and primary rat hepatocytes. Both DF and DPPD decreased ethanol-enhanced tBH cell killing in hepatocytes. These results demonstrated that co-treatment of FTO2B cells and primary rat hepatocytes with ethanol and tBH increased cell killing. The GSH level was dramatically reduced while GSSG level rose. Both DFP and DPPD reversed or protected the cells from this insult, indicating that ethanol was a pro-oxidant.

Keywords

Ethanol, Liver, Hepatocytes, FTO2B Cells, t-Butyl Hydroperoxide

1. Introduction

One of the major targets of continued alcohol abuse is the liver, which is also the major site of its metabolism. The cause of liver damage due to ethanol appears to be multifactorial, involving enzyme induction, toxic metabolites, glutathione depletion and alterations to the labile iron pool. Oxidative stress has also been implicated in

the pathophysiology of this liver injury and is similar to that seen during xenobiotic metabolism, ischemia/reperfusion injury, and activated phagocytic cell attack [1]. Drug hepatotoxicity, allograft dysfunction following liver transplantation, and liver injury by endotoxin mediated activation of Kupffer cells have also all been associated with oxidative stress [2] [3]. Molecular mechanisms by which oxidants trigger the cascade leading to cell death are, however, not fully understood. Normally, liver cells contain high levels of low-molecular-weight antioxidants and antioxidant enzymes, including vitamin E, reduced glutathione, ascorbic acid, superoxide dismutase and catalase to protect the cells from reactive oxygen species (ROS) and other free radicals. However, in oxidative injury, these antioxidant defense mechanisms become overwhelmed, leading to oxidative stress and ultimately to cell death via either necrotic or apoptotic pathways.

Free radicals and reactive oxygen species (ROS) are continuously produced *in vivo*. These hyper-reactive moieties include the superoxide anion, the hydroxyl radicals, hydrogen peroxide singlet oxygen and a variety of reactive nitrogen species (RNS) besides subsequent downstream free radicals formed by them (including the 1-hydroxyethyl radical) and lipid peroxidation products. Cells are known to produce ROS either as a defense mechanism against invading pathogens, or as a byproduct of enzymatic activity [4]. A major source of ROS is from the mitochondrial electron transport chain. ROS may be produced by other mechanisms. NADH (nicotinic adenine dinucleotide, reduced) oxidase, present in phagocytic cells, is activated by activation of the immune/inflammatory system, and produces large quantities of superoxide anion and hydrogen peroxide. Activated neutrophils also release myeloperoxidase from granules, converting hydrogen peroxide and chloride ions to hypochlorous acid, a powerful oxidizing and chlorinating agent that can react with numerous biological targets to cause cellular injury [5] [6]. Cells have basal level of ROS production that is normally countered by the cellular anti-oxidant defense system. However, disturbances to this homeostatic system that cause an increase in ROS generation, a cellular redox shift, or decrease in the anti-oxidant defense systems lead to diseases resulting from the cellular and tissue injury. It has been suggested that this is one of the mechanisms involved in type 2 diabetes [7] and has also been implicated in the pathogenesis of atherosclerosis and hypertension [8] [9]. While ethanol is known to affect almost every biochemical system in the liver, ethanol abuse is associated with considerable oxidative stress [10]-[12]. There are varieties of mechanisms by which ethanol can affect free radical formation, the liver and other organs of the body, and cause alcoholic liver disease (ALD). These pathways also include the generation of hydroxyethanol free radicals. Some workers have stated that the signaling pathways affected by direct or indirect alcohol exposure range from oxidative stress mechanisms, metabolism related effects, inflammation and apoptosis [13]. Some groups have focused on CYP2E1, part of the microsomal ethanol oxidation system (MEOS), which is inducible by continued alcohol intake. This system has been shown to readily generate ROS, particularly hydrogen peroxide, superoxide and hydroxyl radicals, in the presence of ethanol [14] [15]. It has been shown that alcohol increases lipid peroxidation and also modifies proteins. For instance, many investigators have shown that administration of antioxidants, or agents that reduce the levels of free iron, can prevent or treat the deleterious effect of alcohol [12] [16] [17]. Oxidative stress that occurs in the liver affects not only the resident liver cells but also circulating immune cells such as dendritic cells, neutrophils, and bone marrow derived stem cells that migrate to the liver, cause inflammatory responses, and propagate alcoholic liver injury [13].

In this study we have investigated the direct effect of ethanol by exposing rat hepatocytes in primary culture to ethanol with or without the addition of the organic peroxide, *tertiary*-butyl hydroperoxide (tBH), to simulate oxidative stress. Comparison has also been made to a rat hepatoma cell line which metabolizes ethanol by the alcohol dehydrogenase pathway, and possesses low constitutive levels of CYP2E1. Exposure of isolated hepatocytes to organic peroxides, such as *tertiary*-butyl hydroperoxide, results in rapid oxidation of cellular lipids, and a rapid loss of cell viability [18]-[20]. The aim of this study is therefore to examine if ethanol acts as a pro-oxidant, causing increased sensitivity of hepatocytes to tBH, and by extension to predisposition to liver disease.

2. Experimental Methods

2.1. Cell Culture

Two types of liver cell were used in these experiments: a cultured rat hepatoma cell line, FTO2B, and freshly isolated rat hepatocytes in primary culture.

Hepatocytes were isolated from 175 gm male Sprague Dawley rats (Harlan, Indianapolis, IN) using a modification of the *in situ* collagenase perfusion technique [3] [21]. The cells thus obtained were purified further by

Percoll gradient centrifugation to remove non parenchymal cells. The cells were cultured overnight at 37°C in an atmosphere of 5% CO₂ 95% air in Williams medium E containing 1 µM insulin, 2 mM glutamine, 10⁵ U/l penicillin, 100 mg/l streptomycin, and 10% low endotoxin fetal calf serum (heat inactivated) in 12-well plates, coated with type I collagen, at a density of 200,000 cells per well. The cells were treated as indicated in the text for 3 hr. at 37°C. At the end of treatment the cell viability was determined by assaying for lactate dehydrogenase (LDH) in the culture supernatant and in the lysed cells. LDH levels were measured spectrophotometrically using a commercially available kit (Sigma-Aldrich, St. Louis, MO).

2.2. Cell Culture of FTO2B Cells

The rat hepatoma cell line, FTO2B was cultured at 37°C in 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1) supplemented with 10% bovine serum, 2 mM glutamine, 50 I.U./ml penicillin-streptomycin, and 0.2% sodium bicarbonate. FTO2B cells (2×10^5 /ml) were plated in 12 well plates and allowed to adhere overnight prior to an experiment. Following this pre-incubation, the cells were treated with graded doses of tBH and incubated for 3 hours. Cell viability was determined by assaying the culture supernatant for lactate dehydrogenase (LDH). Lysis of the cells with Triton X-100 was used as a measure of total or 100% killing.

2.3. Treatment

Initially, the dose response curves were determined following a 3 hour incubation of tBH with hepatocytes or FTO2B cells. Subsequently to these determinations, sub lethal doses of t-BH (<LD₅₀) were used throughout the study. After overnight equilibration, FTO2B cells or hepatocytes were incubated in the presence or absence of 100 mM ethanol for a further 24 hr; after which they were challenged with a sub lethal dose of tBH for three hours. In separate experiments, cells with or without ethanol pretreatment were incubated with 200 µM tBH in the presence or absence of 4 µM DPPD, or 20 mM desferrioxamine for 3 hr. At the end of treatment, cell viability was determined by assaying the culture supernatant for lactate dehydrogenase

2.4. Measurement of Glutathione in Hepatocytes

In order to examine the mechanism of action of ethanol in the presence of tBH, this study was extended to measure the levels of glutathione and GSSG in the primary rat hepatocytes. The cells were pretreated with or without ethanol for 24 hr. followed by 30 min. incubation with 200 µM tBH with or without ethanol. Glutathione was assayed by a standard spectrophotometric method [8] [22].

2.5. Materials

Penicillin-streptomycin and glutamine were purchased from Cellgro (Herndon, VA, USA). Culture media was from Gibco Laboratories (Rockville, MD, USA). N,N'-diphenyl-1,4-phenylene diamine (DPPD) was purchased from Aldrich (Milwaukee, WI, USA). Reduced glutathione (GSH) and oxidized glutathione (GSSG) were purchased from Borhringer (Indianapolis, IN).

3. Statistical Analysis

All the results are expressed as mean ± SEM values. The Tukey t-test was applied to data from the FTO2B cells. Values were considered significant at $p < 0.05$.

4. Results

As may be seen in **Figure 1** there was a graded dose response curve of tBH killing of hepatocytes. Doses between 0 and 200 µM tBH had no effect on the viability of the hepatocytes whereas 300 µM produced approximately 50% cell killing. Higher doses of this peroxide resulted in almost complete death of the cultured liver cells. The rat liver cell line (FTO2B cells) were more resistant to killing by tBH; a 50% loss of viability was not observed until the concentration had been raised to between 600 and 800 µM tBH (the data not shown, but the percentage killing may be seen in **Figure 3(a)**). The addition of 100 mM ethanol to 200 µM tBH, which by itself produced little change in cell viability, resulted in a dramatic loss of cell viability after 30 - 60 minutes incubation, as seen in **Figure 2**. This did not occur when the cells were incubated with 100 mM ethanol in the absence

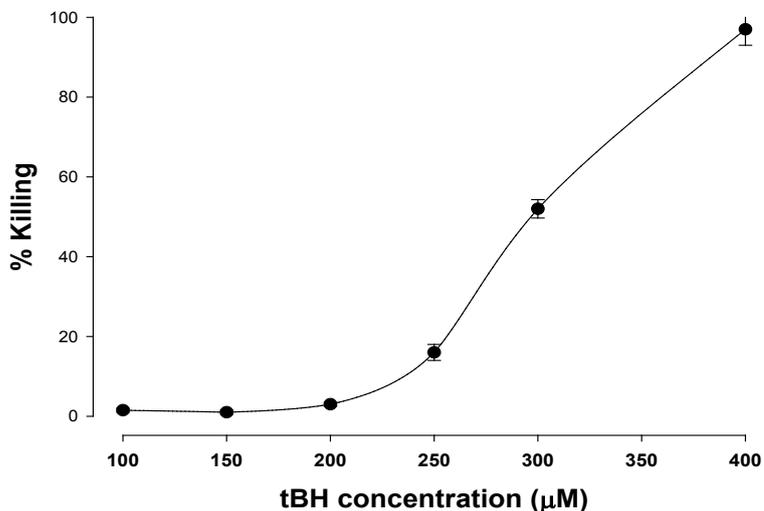


Figure 1. Dose response curve for the killing of rat hepatocytes in primary culture by *tertiary*-butyl hydroperoxide (tBH). Freshly isolated hepatocytes were cultured on collagen overnight and the media changed prior to experimentation. The cells were then treated with various concentrations of tBH, dissolved in the medium and incubated at 37°C for 180 minutes. Cell killing was assessed as the percentage of LDH leakage in this time compared to the values for hepatocytes lysed with triton X-100.

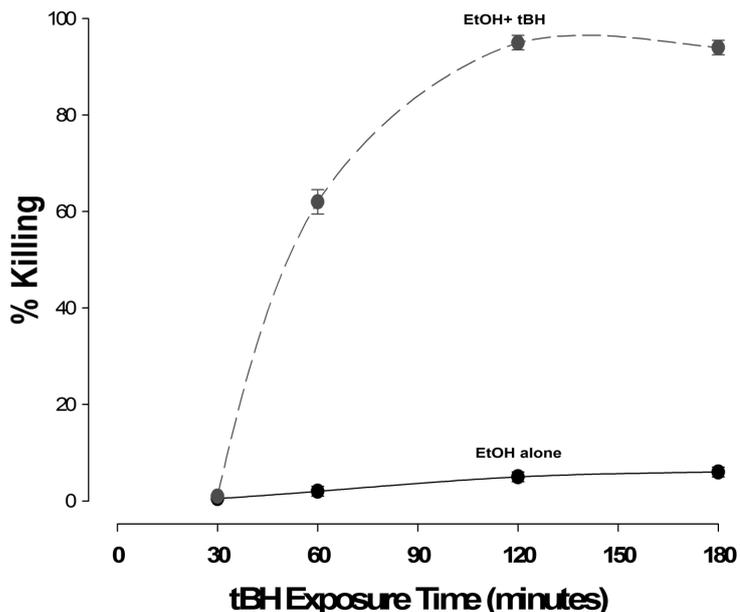


Figure 2. Time curves for the effect of 200 µM *tertiary*-butyl hydroperoxide (tBH) on cultured rat hepatocytes in the presence or absence of 100 mM ethanol. Freshly isolated rat hepatocytes were cultured overnight and the media changed prior to experimentation. At time zero, 200 µM tBH was added, together with media alone (solid line) or media containing 100 mM ethanol (dashed line). At present time intervals, medium was aspirated and the LDH and cellular protein content quantitated. The values were then expressed in terms of cultured cells lysed by the addition of triton X-100.

of tBH (the curves for ethanol were very similar to those observed for low doses of tBH, *i.e.* ≤200 µM—data not shown).

In order to ascertain the contribution of ethanol and its metabolism on the cytotoxic effects of tBH, cells were incubated overnight (24 hours) in the presence or absence of 100 mM ethanol and then for 3 hours with two different concentrations of tBH, again in the presence or absence of ethanol. To compensate for difference engendered by ethanol alone, cells cultured in ethanol for 24 hours were also assayed in media without ethanol when tBH was added and vice versa. As may be seen in **Figure 3(a)**, 600 μM tBH resulted in the killing of only 10% of the FTO2B cells, whereas 800 μM killed almost 60%. Culturing FTO2B cells in ethanol alone resulted in approximately four-fold increase in killing by tBH, at the lower concentration and complete killing of all the cells at the higher concentration. No significant difference was observed between the ethanol/ethanol + tBH groups and media/ethanol + tBH groups suggesting that it was the presence of ethanol together with tBH that was responsible for the cytotoxic activity, rather than the long-term effects of ethanol alone. Similar results were also seen with the cells cultured in the medium without ethanol. When a similar system was employed with the rat hepatocytes in primary culture, lower concentrations of tBH were used. 200 μM tBH produced similar killing in medium alone (ca 10%). However, 300 μM tBH resulted in approximately 70% killing, suggesting that these cells were more sensitive to oxidative stress than the FTO2B cells. Apart from this difference in sensitivity, the cells responded similarly to the FTO2B cells in terms of the presence or absence of ethanol together with tBH, as may be seen in **Figure 3(b)**.

In order to test whether free/labile iron was associated with the cellular killing, such as its utilization in the Fenton/Haber-Weiss reactions to produce hydroxyl radicals or donate free electrons to free radical-mediated reactions, cells were incubated with tBH in the presence or absence of 20 mM desferrioxamine. In the experiments shown in **Figure 4(a)**, desferrioxamine reduced cell killing by tBH in hepatocytes to virtually zero. In every other case, cytotoxic activity was reduced to or below control values. Similar results were obtained with FTO2B cells, as may be seen in **Figure 4(b)**.

Since tBH, a known generator of oxidative stress seems to be involved in lipid oxidation apoptotic/necrotic pathways of cell death, experiments were carried out in the presence or absence of an antioxidant, N,N'-diphenyl-phenylene diamine (DPPD). As may be seen in **Figure 5(a)** and **Figure 5(b)**, the addition of 200 μM DPPD resulted in effective prevention of cell killing, suggesting that lipid peroxidation is involved in the cytotoxic activity of tBH and ethanol.

To ascertain whether tBH altered the oxidative status of the cells, glutathione was also measured in cultured hepatocytes. The level of GSH was high in control cells (media alone: 2750 ng/well) and ethanol-treated cells (ethanol alone: 2500 ng/well) while oxidized glutathione levels were low (200 for media and 100 ng/well for ethanol-treated cells). However, in the presence of tBH and ethanol, the level of GSSG increased (1750 ng/well) while GSH level decreased (400 ng/well), as may be seen in **Figure 6**. These results suggest that glutathione levels can be maintained in the presence of a single oxidant stressor, such as tBH, but not in the presence of an additional factor, such as the presence of ethanol.

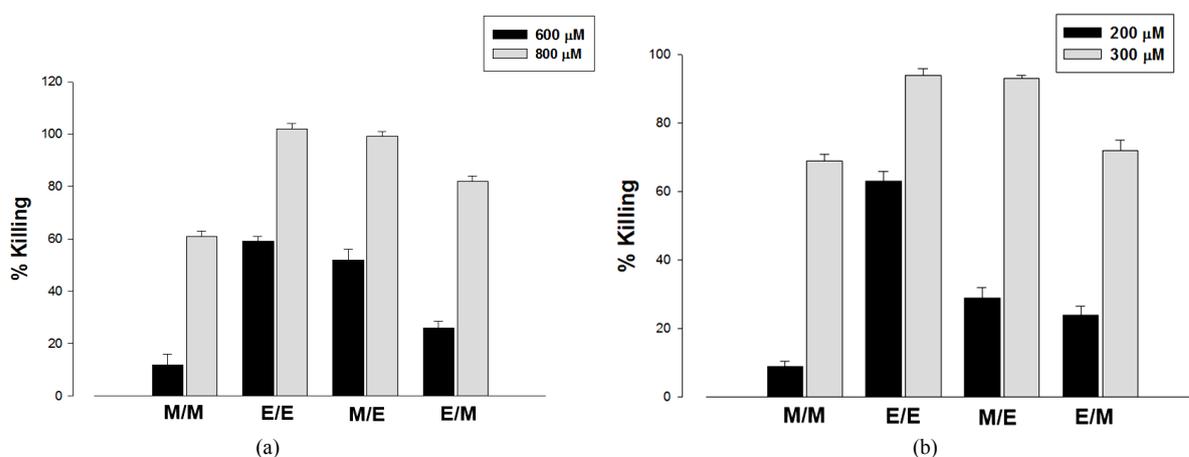


Figure 3. Effects of ethanol on cell killing by *tertiary*-butyl hydroperoxide (tBH). (a) FTO2B cells: 600 μM tBH—black bars; 800 μM tBH—grey bars; (b) Rat hepatocytes in primary culture: 200 μM tBH—black bars; 300 μM tBH—grey bars. M/M = 24-hr culture in media alone followed by 3-hr incubation in media and tBH. E/E = 24-hr culture in ethanol (100 mM) alone followed by 3-hr incubation in ethanol and tBH. M/E = 24-hr culture in media alone followed by 3-hr incubation in ethanol (100 mM) and tBH. E/M = 24-hr culture in ethanol (100 mM) alone followed by 3-hr incubation in media and tBH.

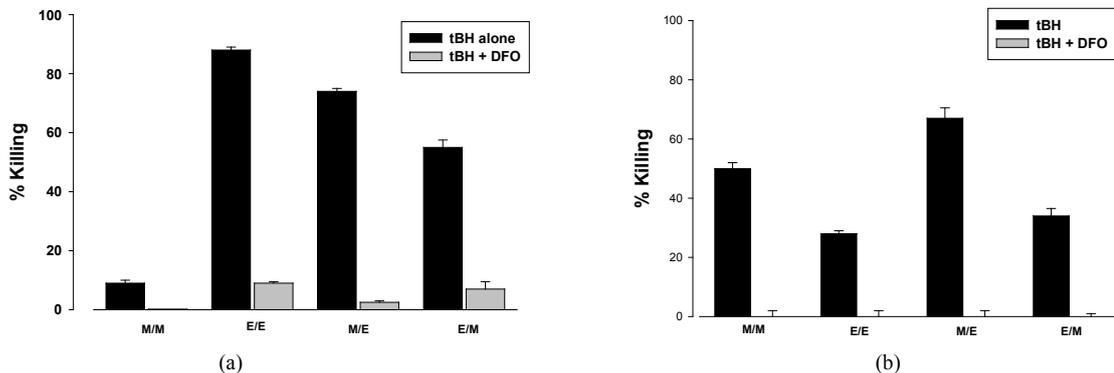


Figure 4. Effects of the addition of desferrioxamine (DFO) on the cell killing by *tertiary*-butyl hydroperoxide (tBH). (a) FTO2B cells: 600 μM tBH—black bars; 800 μM tBH—grey bars; (b) Rat hepatocytes in primary culture: 200 μM tBH—black bars; 300 μM tBH—grey bars. M/M = 24-hr culture in media alone followed by 3-hr incubation in media and tBH +/- DFO. E/E = 24-hr culture in ethanol followed by 3-hr incubation in ethanol and tBH +/- DFO. M/E = 24 hr culture in media followed by 3-hr incubation in ethanol and tBH +/- DFO. E/M = 24-hr culture in ethanol followed by 3-hr incubation in media +/- DFO.

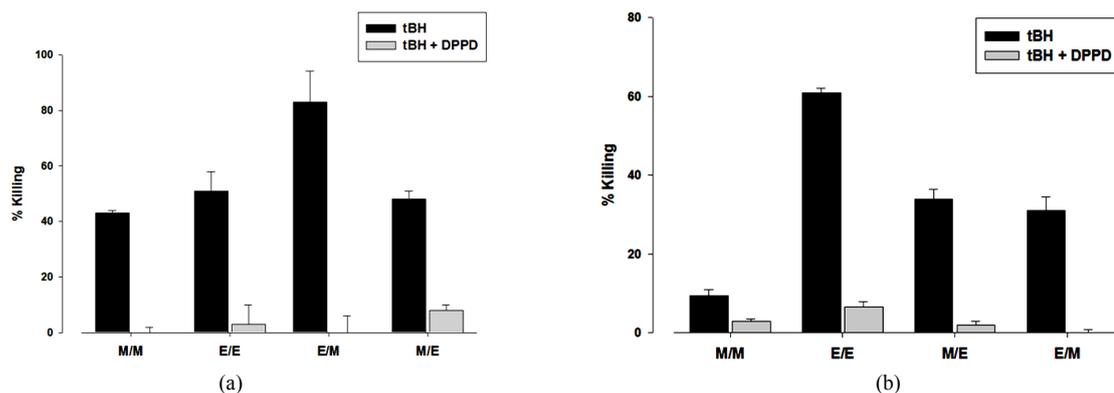


Figure 5. Effects of the addition of N,N-diphenyl-p-phenylenediamine (DPPD) on the cell killing by *tertiary*-butyl hydroperoxide (tBH). (a) FTO2B cells: 600 μM tBH—black bars; 800 μM tBH—grey bars; (b) Rat hepatocytes in primary culture: M/M = 24-hr culture in media alone followed by 3-hr incubation in media and tBH +/- DPPD. E/E = 24-hr culture in ethanol followed by 3-hr incubation in ethanol and tBH +/- DPPD. M/E = 24-hr culture in media followed by 3-hr incubation in ethanol and tBH +/- DPPD.

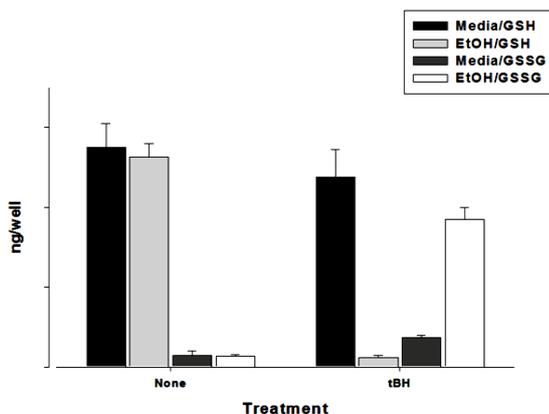


Figure 6. Effects of 200 μM tBH exposure on GSH and GSSG levels in cultured hepatocytes. Rat hepatocytes were cultured in the presence of medium containing 100 mM ethanol or medium alone for 24 hours at 37°C. The medium was then removed and replaced with medium containing 100 mM ethanol or medium alone and tBH and incubated for a further 180 minutes. GSH and GSSG levels were then determined as described in the methods section.

5. Discussion

Response to alcohol has been shown as a factor contributing to immense health hazard. Habitual consumption of alcohol produces a spectrum of hepatic pathology, ranging from simple steatosis (fatty liver) on one extreme, to cirrhosis on the opposite end of the spectrum. Steatohepatitis, a liver disease characterized by hepatic steatosis, inflammation, and increased hepatocyte death, is usually an intermediate stage between simple fatty liver and cirrhosis. Steatosis is a very common result of chronic alcohol ingestion, occurring in many, if not most, human beings and experimental animals that consume alcohol daily. In contrast, cirrhosis is a relatively rare outcome of chronic alcohol ingestion. Cell damage sets in gradually before the whole liver is engulfed. Our study showed no significant cell killing by LDH assay with the 24 hr ethanol pretreatment controls in both hepatocytes and FTO2B cells. *Tert*-butyl hydroperoxide is a toxin that has been frequently employed as a model to study the mechanism of irreversible cell injury resulting from an acute oxidative stress [23]. We found that neither 0.2 mM of tBH nor 100 mM Ethanol alone was significantly toxic to the cells. However, when hepatocytes were treated at the same time with Ethanol and 0.2 mM tBH, cell killing increased dramatically. In effect, the presence of Ethanol converted the 0.2mM tBH from being without effect to a toxic cell insult. This was also observed when Ethanol was only added to tBH without 24 hr Ethanol pretreatment. Both deferoxamine (DF, an iron chelator) and N,N-diphenyl-p-phenylenediamine (DPPD, a lipid radical scavenger) were able to reverse or protect the cells from this insult. In other words, the inclusion of DPPD in the incubation medium prevented cell death. Some researchers have shown that at a concentration of 250 μ M *tert*-butyl hydroperoxide, α -tocopherol-deficient hepatocytes incubated with DPPD were completely protected against cell killing, whereas cells not incubated with DPPD were not [24]. Exposure of isolated rat hepatocytes to allyl alcohol, diethyl maleate and bromoisovalerylurea induce lipid peroxidation, depletion of free protein thiols to about 50% of the control value and cell death [25]. Other studies have also shown that membrane stability may play an important part in anti-oxidant protections, since taurine has been shown to be protective against tBH administration [26].

Glutathione is a critical antioxidant. Our study indicates that its level is normal in the dose for Ethanol and tBH used separately. However, when hepatocytes were exposed to both Ethanol and tBH, the level of GSH became dramatically reduced while GSSG level rose. Other workers have shown that mitochondrial thiols undergo oxidative modification in rats chronically fed alcohol for at least one month [27] and this mitochondrial oxidative stress may also be one of the longer-term damaging consequences of alcohol intoxication. This mitochondrial dysfunction may also lead to increased free radical production following disruption of the electron chain. There has been a controversy whether alcohol liver disease is due to malnutrition or direct hepatotoxicity of ethanol. Some researchers have shown that alcohol liver disease develops as a consequence of priming and sensitizing mechanisms rendered by cross-interactions of primary mechanistic factors and secondary risk factors. The critical role of hepatic macrophages has been highlighted as a priming mechanism and glutathione depletion in hepatocyte mitochondria is considered the most important sensitizing mechanism. Methionine metabolism is also considered as another contributing factor [28]. It has been shown previously that in isolated hepatocytes, *tert*-butyl hydroperoxide is metabolized by the glutathione peroxidase-glutathione reductase enzyme system present in the cytosolic and mitochondrial compartments. Metabolism of *t*-BH may result in a decrease in both the glutathione (GSH)/glutathione disulfide (GSSG) and NADPH/NADP⁺ redox ratios, and in a diminished intracellular concentration of exchangeable Ca²⁺ depending on the concentration of *t*-BH. Some workers described a biphasic action of nitric oxide in its effects on oxidative killing of isolated cells [29]. They observed that low concentrations of nitric oxide protect against oxidative killing, while higher doses enhance killing and these two effects occur by distinct mechanisms.

There is little doubt that the labile iron pool is also involved in this oxidative stress. As well as experimental cell culture, iron accumulation is often increased in patients with alcohol-related liver disease and that this is related to an increase in hepatic transferrin receptors irrespective of non-transferrin-bound plasma iron [30]-[32]. The effects of excess iron may be far-reaching and include oxidative stress-related perturbations of the endoplasmic reticulum [33]. Evidence also suggests that P450 2E1 may be one of the co-factors causing free radical production from this compartment [20] [34]. Other radicals, such as carbon-centered radicals (1-hydroxyethyl), produced by CYP450-Fe-oxycomplexes and hydroxyl radicals from the Fenton-Haber-Weiss reaction may also increase toxicity, as shown by increases in F₂-isoprostanes [35].

6. Conclusion

The data presented above indicate that tBH-ethanol cell killing is associated with peroxidation of cellular lipids.

The ability of DFP and DPPD to prevent tBH-ethanol cell killing may relate to their antioxidant action. In summary, the data observed in this work suggest that the killing of hepatocytes by tert-butylhydroperoxide and ethanol can be attributed to the peroxidation of cellular phospholipids and that free iron is probably involved in the mechanistic pathway. Liver disease is the most common medical complication of alcohol abuse; an estimated 15% - 30% of chronic heavy drinkers eventually develop severe liver disease. Alcohol fatty liver is a reversible condition that may progress to alcoholic hepatitis and eventually to cirrhosis and liver failure [36]. Preventing excessive alcohol consumption is a major part of the solution to alcohol problem.

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