

## Effect of mouse recombinant leptin on ethanol elicited damage in the mouse hepatocellular carcinoma cell lines

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ORIGINAL RESEARCH ARTICLE

### ABSTRACT

Obesity is associated with hepatocellular carcinoma (HCC). Leptin, an anti-obesity hormone exerts potent modulatory properties both in vivo and in vitro. We have previously shown the reduction of lipotoxicity with leptin in vivo. The aim of this study was to evaluate the effect of leptin on ethanol induced fibrogenesis and apoptosis in mouse hepatocellular carcinoma (HCC) cell lines. Mouse HCC cell lines were treated for 48 h with and without ethanol (500 mM) and leptin (31.2 nM), subsequently analyzed for cell proliferation, flow cytometry, biochemical and molecular studies. Ethanol exposure significantly reduced the cell viability as evidenced by 3-(4,5 dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay ( $P < 0.05$ ). Moreover, ethanol treated cells significantly lowered DNA synthesis as evidenced by thymidine incorporation ( $P < 0.05$ ) and increased DNA fragmentation. Ethanol incubation also significantly increased the % of apoptotic cells ( $P < 0.05$ ). These results were compared with that of untreated control cell lines. Leptin co-treatment with ethanol significantly enhanced ( $P < 0.05$ ) cell viability and DNA synthesis, whereas significantly ( $P < 0.05$ ) decreased apoptotic cells and DNA ladder formation. In addition, ethanol exposure significantly ( $P < 0.05$ ) increased cytokine (TNF $\alpha$ ), reactive oxygen species (ROS), TBARS and mRNA expressions of caspase-3, procollagen type I, MMP 2, MMP 9 and TIMP-1 compared to untreated control mouse HCC cell lines. Leptin co-administration significantly ( $P < 0.05$ ) down regulated the above indices when compared to ethanol alone exposed mouse HCC cell lines. Furthermore, ethanol exposure significantly ( $P < 0.05$ ) lowered antioxidant enzymes activities. Leptin co-administration along with ethanol significantly ( $P < 0.05$ ) improved antioxidant enzymes activities. Thus, our experimental data provide evidence that leptin treatment to ethanol exposed mouse HCC cell lines results in attenuating fibrogenesis and apoptosis, thereby warranting further population based mechanistic studies.

### KEYWORDS

apoptosis; antioxidant; caspase-3; ethanol; hepatocellular carcinoma; leptin

## 1. INTRODUCTION

Hepatocellular carcinoma (HCC), a lethal tumor in the liver, represents 3<sup>rd</sup> leading cause of cancer related deaths worldwide (Forner et al., 2012; Galati et al., 2016). Chronic heavy alcohol consumption led to fatty liver, steatohepatitis and progression to fibrosis and/or cirrhosis and eventually ends with HCC (Lobo et al., 2016). Hepatic fibrosis is characterized by increased accumulation of collagens, primarily type I collagen

and other extracellular matrix (ECM) components in the hepatocytes (Arriazu et al., 2013; Robert et al., 2016). Under normal physiological conditions, these ECM proteins are continuously remodelled by the family of enzymes known as matrix metalloproteinases (MMPs) leading to a controlled deposition of ECM components (Arriazu et al., 2013). Tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMPs that participate in controlling the local activities of MMPs in the liver (Kossakowska et al., 1998; Ramachandran and Iredale 2012). The

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<b>Nomenclature</b>	
ALD	Alcoholic liver disease
CAT	Catalase
GSH	Reduced glutathione
HCC	Hepatocellular carcinoma
MMPs	Matrix metalloproteinases
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TIMP	Tissue inhibitor of metalloproteinases

alteration of balance between MMP and TIMP can lead to excessive deposition of ECM proteins resulting in severe fibrosis (McCrudden and Iredale 2000). Furthermore, numerous other factors are also involved in the development of alcoholic liver disease (ALD), which includes oxidative stress and inflammation (Balasubramanian et al., 2007a; Leung and Nieto 2013). In this context, cell death particularly apoptosis also plays a significant role in the progression of alcohol-induced liver disease to cirrhosis (Lieber 2004; Feldstein and Gores 2005; Balasubramanian et al., 2007a). However, the molecular mechanisms of alcohol induced apoptosis remain unidentified.

Leptin, the product of the obese gene (Zhang et al., 1994), is well known as a hormone with anti-obesity properties, controlling food intake in conditions of energy excess. Indeed, the role of leptin in ethanol-induced HCC remains obscure. However, several published studies have indicated that circulating leptin levels were increased in cirrhosis and obesity (McCullough et al., 1998; Shimizu et al., 1998). A previous study has shown that serum leptin levels are elevated in patients with alcohol-induced cirrhosis (Henriksen et al., 1999), which cannot be explained simply by the correlation with body mass index. In addition, hepatic stellate cells (HSCs) have been shown to produce leptin when they are transdifferentiated in vitro (Potter et al., 1998). These observations suggest a possible role for leptin in the hepatic fibrogenesis. As distinct from the above, we have reported a protective role of leptin against ethanol-induced hypercholesterolemia in mice (Balasubramanian and Nalini 2003). Studies have also demonstrated that leptin exerts proliferative and antiapoptotic activities in a variety of cell types, including human hepatocarcinoma cells (Balasubramanian et al., 2007a) and vascular endothelial cells (Artwohl et al.,

2002). Furthermore, our previous in vitro study shows the evidence that co-administration of leptin along with ethanol decreased oxidative stress and apoptosis (Balasubramanian et al., 2007a). It is unclear whether leptin has any potential effect on preventing hepatic cell fibrosis and apoptosis and thus, leptin warrants further detailed evaluation of its mechanism of action at the molecular and cellular levels. In this study, our aim was to explore the effect of leptin on ethanol-induced fibrosis, inflammation and apoptosis in mouse HCC cell lines.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

Mouse recombinant leptin was purchased from Sigma Chemical Co. (St Louis, MO, USA). Ethanol was obtained from E.Merck, Darmstadt, Germany. All other chemicals used were of analytical grade and the organic solvents were distilled before use.

### 2.2. In vitro model and cell culture conditions

Mouse hepatoma cell line (BNL 1ME A.7R.1), was obtained from the National Centre for Cell Science, Department of Biotechnology, Pune, India. Cells were routinely grown in monolayer culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/mL) and streptomycin (100 mg/mL). Cells were grown at 37 °C in disposable 25 mL plastic bottles, in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The culture medium was changed every other day and the cells were usually split 1:3 when they reached confluence. After treatment with or without leptin (31.2 nM), ethanol (500 mM) and leptin+ethanol, the cells were incubated for 48 h in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C.

Group 1 cells were treated with vehicle and served as control

Group 2 cells were treated with 31.2 nM leptin

Group 3 cells were treated with 500 mM ethanol

Group 4 cells were treated with 31.2 nM leptin and 500 mM ethanol

After treatment, the cells were incubated for 48 h in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. At the end of 48 h incubation, cell viability was estimated by MTT assay for all the treated cell lines. The above optimal dose fixed for both leptin and ethanol, primarily

based on our earlier study (Balasubramaniyan et al., 2007b).

### 2.3. [<sup>3</sup>H] Thymidine uptake assay for cell proliferation

The mouse HCC cell lines were cultured in a 96 well plate for three days at 4x10<sup>5</sup> cells/well. Cells were pulsed with 1μCi of [<sup>3</sup>H] methyl thymidine (BRIT-Mumbai, India) for 48 h and then harvested onto a membrane filter using a filter-mate harvester (Packard Instruments). The incorporation of <sup>3</sup>H-thymidine was used as a measure of DNA synthesis using liquid scintillation counter (Packard Instruments). Assays were performed in quadruplicate.

### 2.4. Apoptosis visualization by laddering

Apoptotic DNA ladder was visualized by isolating genomic DNA as described by Park et al (Park and Patek 1998). Briefly, 4x10<sup>6</sup> mouse HCC cells seeded in six-well plates were lysed in 30 μL of TE lysis buffer (10mM Tris HCl, pH 7.5, 1 mM EDTA, and 0.25% Triton X-100) and resuspended by gentle vortexing and incubating at 37 °C for 20 min. Lysis was followed by ribonuclease A treatment and deproteinization by proteinase K at 37 °C. The lysate was centrifuged at high speed and the supernatants were removed to a fresh tube. 6X DNA loading dye was added to the DNA and analyzed on 1.8% agarose gel in TBE buffer (54 g Tris base, 27.5 g of boric acid and 20 mL of 0.5 M EDTA pH 8.3). The gel was stained with ethidium bromide to visualize DNA fragmentation.

### 2.5. Apoptosis and cell cycle analysis by propidium iodide staining

Cells were harvested, washed with 1X PBS (137 mM NaCl, 2.7 mM KCl, 15 mM KH<sub>2</sub>PO<sub>4</sub> and 9.6 mM Na<sub>2</sub>HPO<sub>4</sub>) and fixed in 70% chilled ethanol at 4 °C for 10 min. After hydrolyzing with 5 mg/mL of ribonuclease A for 20 min and staining with 50 μg/mL of propidium iodide (PI), the DNA content was determined on the FL-2 A channel with a flow cytometer (FACS Vantage, BD) equipped with a 488 nm argon laser. The data were subjected to cell cycle analysis using modfit software. The number of cells in sub-G<sub>0</sub> phase was expressed as the percentage of total events (10,000 cells).

### 2.6. mRNA expression studies

Total RNA was isolated from mouse hepatoma cell

lines using TRIZOL (Sigma Chemical Co, St. Louis, MO) according to the manufacturer's instructions and cDNA synthesis using the thermoscript RT-PCR system (Invitrogen Life Technologies). The cDNA then used as template for PCR amplification of mouse α1 (I) procollagen, MMP-2, MMP-9, TIMP-1 and caspase-3 using gene specific primers. The primer sets used were from Integrated DNA Technologies, Inc. Coralville, IA) (Table 1).

Each sample was amplified for mouse b-actin to ensure equal cDNA input. The PCR conditions for 35 cycles of amplification were as follows: 94 °C (1 min.), corresponding annealing temperature (1 min.), 72 °C (1 min.) (Thermoscript TMRT-PCR system; Invitrogen Life Technologies, Carlsbad, CA, USA) along with the final extension of 10 min. at 72 °C. RT-PCR products were resolved on 1.5 % agarose gels followed by staining with ethidium bromide.

**Table 1.** Primer used for PCR.

Name of the gene	Primer sequence	Annealing temperature °C
α1 (I) Procollagen	F: 5'-GAG CCG AGA GTA CTG GAT CG-3' R: 5'-TGC TGT AGG TGA AGC GAC TG-3'	55
Caspase-3	F: 5'-TGT CAT CTC GCT CTG GTA CG-3' R: 5'-AAA TGA CCC CTT CAT CAC CA-3'	53
MMP-2	F: 5'-CAG GCT CTT CTC CTT TCA CAA C-3' R: 5'-AAG CCA CGG CTT GGT TTT CCT C-3'	54
MMP-9	F: 5'-TGG GCT ACG TGA CCT ATG ACA T-3' R: 5'-GCC CAG CCC ACC TCC ACT CCT C-3'	56
TIMP-1	F: 5'-ACC CAC AGA CGG CCT TCT GCA ATT C-3' R: 5'-GGC TAT CTG GGA CCG CAG GGA CTG C-3'	62
β-actin	F: 5'-TGG AAT CCT GTG GCA TCC A-3' R: 5'-TAA CAG TCC GCC TAG AAG CA-3'	57

### 2.7. Measurement of cytokine

The concentration of TNF-α in cell free culture supernatants was detected by standard two-site sandwich ELISA as described in the BD Pharmingen (San Diego, CA) manual.

## 2.8. Measurement of oxidative stress and antioxidant enzymes

ROS were determined by spectrofluorometer with 2',7'-dichloro dihydro fluorescein diacetate (Wang and Joseph, 1999). Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al. (1979). Reduced glutathione (GSH) was determined by Ellman method as described previously (Ellman 1959). Superoxide dismutase (SOD, E.C.1.15.1.1) and catalase (CAT, E.C.1.11.1.6) activities were assayed by the methods of Sinha (1972) and Kakkar (1984).

## 2.9. Statistical analysis

Data were analysed by t test-Mann Whitney U test, or one way ANOVA for Newman-Keuls multiple comparisons;  $p < 0.05$  was considered statistically significant. Results are presented as mean  $\pm$  SEM using GraphPad Prism 5.0.

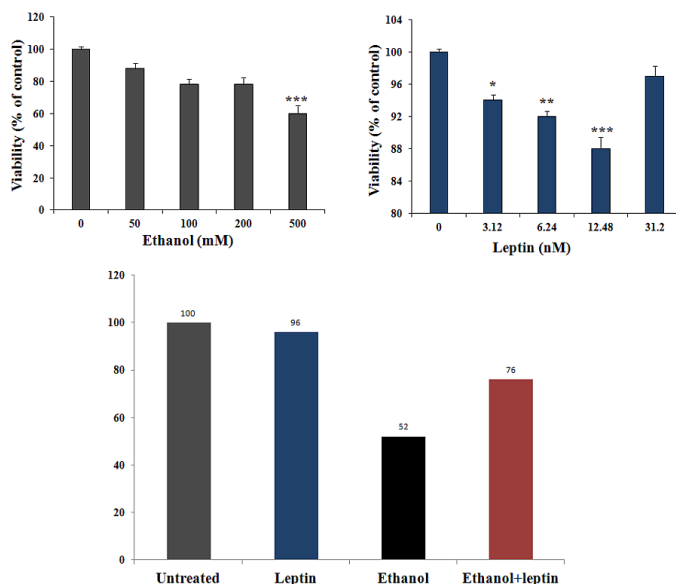
# 3. RESULTS

## 3.1. Effect of leptin and ethanol on cytotoxicity

The average values of cells grown in the regular medium were considered as 100% viable. The toxicity of ethanol was increased with different doses to the mouse HCC cells. Significant toxicity could be observed even at ethanol concentration as low as 50 mM, with further increase in the toxicity as the ethanol concentration was elevated (Figure 1a). Ethanol concentration as high as 500 mM had a maximum toxic effect on the mouse HCC cells (60%). Furthermore, we have assessed the cytotoxic response of leptin to mouse HCC cells. No significant toxicity could be observed at a dose of 31.2 nM leptin, which showed 97% cell viability in the culture medium and the doses of 3.12, 6.24 and 12.48 nM leptin showed 94, 92 and 88 % viability, respectively (Figure 1b).

Figure 1c shows the effect of leptin on ethanol-elicited toxicity as assessed by MTT assay. Ethanol incubated mouse HCC cells showed 52% viability, and leptin treatment together with ethanol showed 76% cell viability. Leptin alone treated cells had no such a significant cell death as observed by 96% cell viability. Essentially, similar results were obtained after morphological visualization of the cells under the light microscope. Most of the cells lost normal morphology

when treated with ethanol for 48 h, whereas leptin-treated cells retained their shape and structure (Data not shown).



**Figure 1.** Mouse HCC cell lines viability was assessed by MTT. In concentration–response study, cells were treated with 0, 50, 100, 200 and 500mM ethanol for 48 h (top left image). In concentration–response study, cells were treated with 0, 3.12, 6.24, 12.48 and 31.2 nM leptin for 48 h (top right image). Effect of leptin (31.2 nM) and ethanol (500 mM) on cell survival as determined by MTT assay (bottom image). Data are expressed as mean  $\pm$  standard error of mean (SEM). Note: \* $p < 0.05$  compared to untreated mouse HCC cell lines;  $^{\$}p < 0.05$  compared to alcohol exposed mouse HCC cell lines.

## 3.2. Effect of leptin on ethanol induced apoptosis

a) Thymidine incorporation: DNA synthesis was significantly decreased on ethanol-exposed cell lines compared to untreated cell lines ( $p < 0.0001$ ). Co-administration of leptin with ethanol significant increased the DNA synthesis ( $p < 0.05$ ) when compared to ethanol alone-incubated cell lines. Leptin alone incubated cell lines showed no such significant changes of DNA synthesis when compared to untreated cell lines (Fig 2a).

b) DNA ladders are believed to be important biochemical markers of apoptosis. Ethanol incubation to mouse HCC cells showed a clear DNA ladder formation compared to untreated cells, whereas no DNA ladder was seen in either leptin alone or leptin+ethanol incubated cell

lines (Figure 2b).

c) DNA analysis by flow cytometry: When compared to untreated mouse HCC cell lines (Figure 2c1), ethanol treatment significantly ( $p < 0.05$ ) elevated percentage of apoptotic cells (Figure 2c3). Leptin co-treatment along with ethanol (Figure 2c4) significantly ( $p < 0.05$ ) decreased the percentage of apoptotic cells when compared to ethanol alone-incubated cells. Leptin alone treatment (Figure 2c3) showed insignificant increase of apoptotic cells when compared to untreated cells.

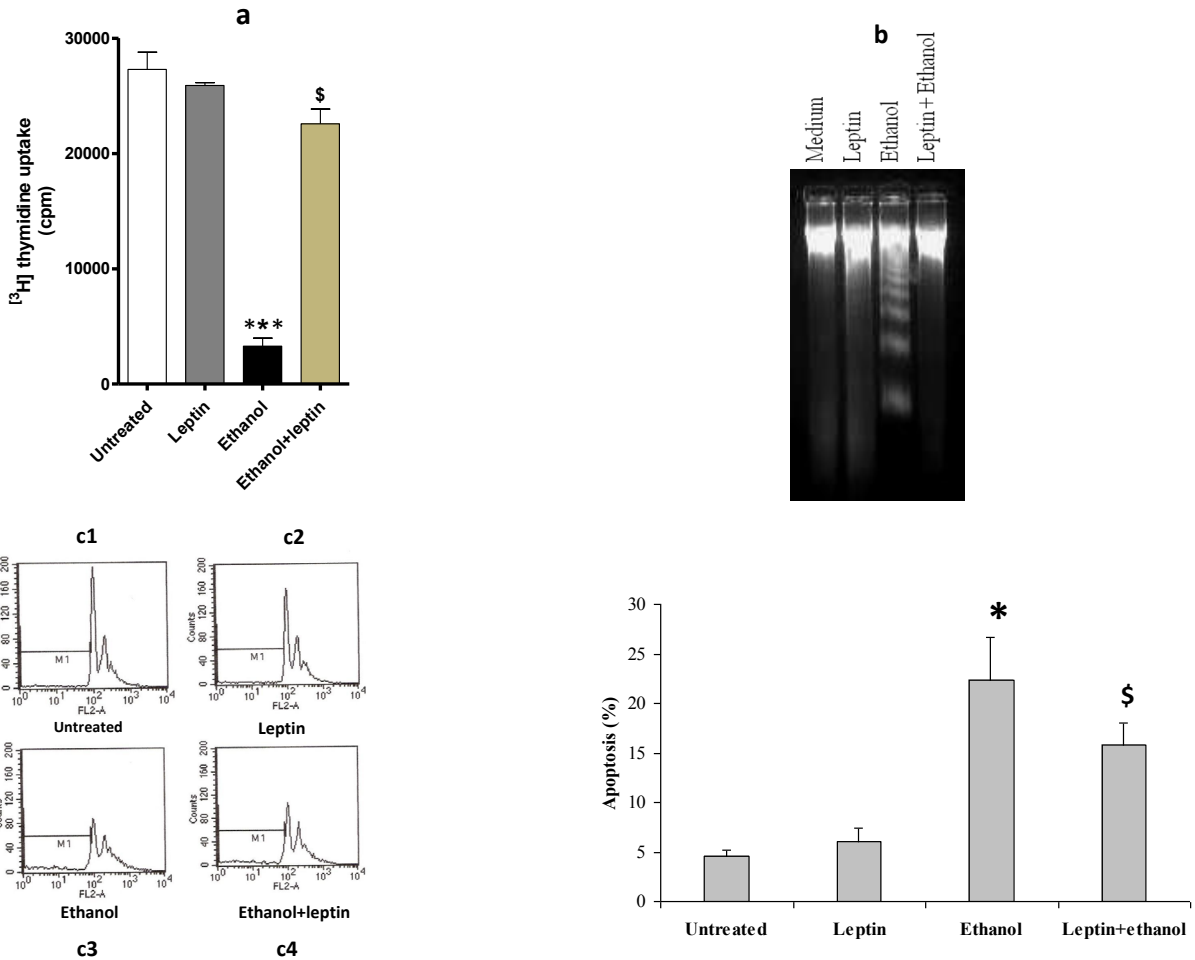
### 3.3. Effect of leptin on ethanol-induced caspase-3 mRNA expression

When compared to untreated mouse HCC cell lines, ethanol incubation significantly ( $p < 0.05$ ) elevated caspase 3 mRNA expression (Figure 3a). Leptin

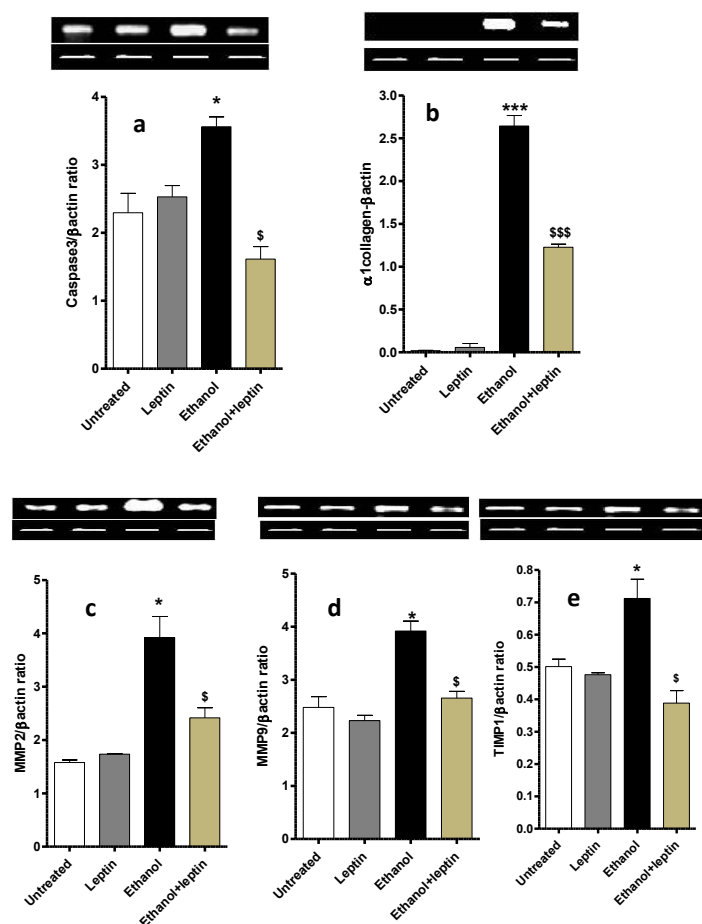
co-administration along with ethanol significantly ( $p < 0.05$ ) decreased caspase 3 mRNA expression compared to ethanol alone-incubated cell lines. Leptin per se showed no such significant alteration of caspase 3 mRNA expression compared to untreated medium.

### 3.4. Effect of leptin on ethanol-induced $\alpha 1$ (I) procollagen mRNA expression

When compared to untreated mouse HCC cell lines, ethanol incubation significantly ( $p < 0.0001$ ) elevated  $\alpha 1$  (I) procollagen mRNA expression (Figure 3b). Leptin co-administration along with ethanol significantly ( $p < 0.0001$ ) decreased  $\alpha 1$  (I) procollagen mRNA expression compared to ethanol alone-incubated cell lines. Leptin per se showed insignificant increase of  $\alpha 1$  (I) procollagen3 mRNA expression compared to untreated medium.



**Figure 2.** (a) Effect of leptin on ethanol induced cell proliferation as assessed by [<sup>3</sup>H] thymidine assay; (b) Effect of leptin on ethanol induced DNA fragmentation in mouse HCC cell lines and (c) Effect of leptin on ethanol induced cell cycle and the induction of apoptosis in mouse HCC cells. Data are expressed as mean  $\pm$  standard error of mean (SEM). Note: \* $p < 0.05$  compared to untreated mouse HCC cell lines; § $p < 0.05$  compared to alcohol exposed mouse HCC cell lines.



**Figure 3.** Effect of leptin on (a) ethanol-induced caspase-3 mRNA expressions by RT-PCR in mouse HCC cell lines, (b) ethanol-induced procollagen  $\alpha 1$  (I) mRNA expressions by RT-PCR in mouse HCC cell lines, (c) ethanol-induced MMP 2 mRNA expressions by RT-PCR in mouse HCC cell lines, (d) ethanol-induced MMP 9 mRNA expressions by RT-PCR in mouse HCC cell lines and (e) ethanol-induced TIMP 1 mRNA expressions by RT-PCR in mouse HCC cell lines. Data are expressed as mean  $\pm$  standard error of mean (SEM). Note: \* $p < 0.05$  compared to untreated mouse HCC cell lines; \$ $p < 0.05$  compared to alcohol exposed mouse HCC cell lines, \*\*\* $p < 0.0001$  compared to untreated mouse HCC cell lines, and \$\$\$ $p < 0.0001$  compared to alcohol exposed mouse HCC cell lines.

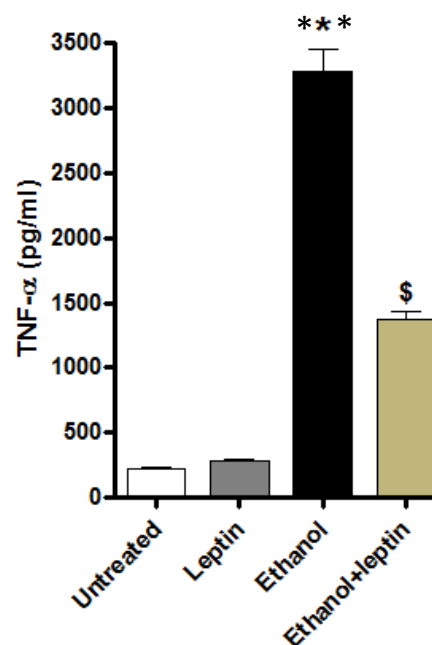
### 3.5. Effect of leptin on ethanol induced MMPs 2&9 and TIMP 1 mRNA expressions

When compared to untreated mouse HCC cell lines, ethanol incubation significantly ( $p < 0.05$ ) elevated MMPs 2&9 mRNA expression (Figures 3c and d). Leptin co-administration along with ethanol significantly ( $p < 0.05$ ) decreased MMPs 2&9 mRNA expression compared to ethanol alone-incubated cell lines. Leptin

per se showed no such significant alteration of MMPs 2&9 mRNA expression when compared to untreated medium. Furthermore, TIMP 1 mRNA expression also significantly ( $p < 0.05$ ) elevated following ethanol incubation to mouse HCC cell lines when compared to untreated medium (Figure 3e). Co-administration of leptin along with ethanol significantly ( $p < 0.05$ ) decreased TIMP 1 mRNA expression.

### 3.6. Effect of leptin on ethanol-induced TNF $\alpha$ production

Ethanol incubation significantly ( $p < 0.0001$ ) increased TNF  $\alpha$  concentration in mouse HCC cell lines when compared to untreated HCC cell lines (Figure 4). Co-administration of leptin along with ethanol significantly decreased TNF  $\alpha$  concentration compared to ethanol per se treated cell lines.



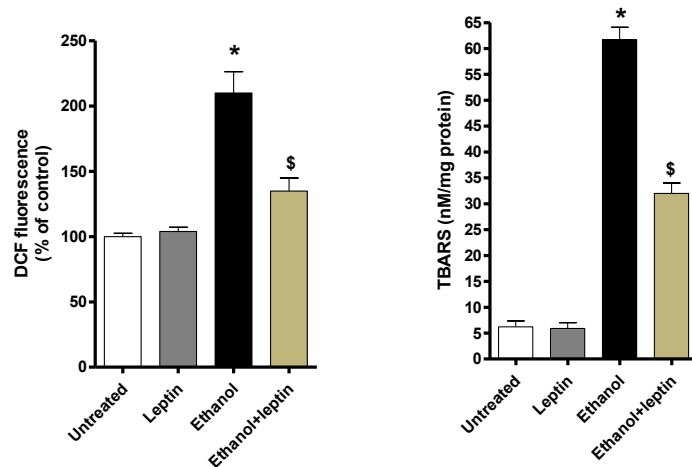
**Figure 4.** Effect of leptin on ethanol-induced TNF $\alpha$  levels in mouse HCC cell lines. Data are expressed as mean  $\pm$  standard error of mean (SEM). Note: \*\*\* $p < 0.0001$  compared to untreated mouse HCC cell lines; \$ $p < 0.05$  compared to alcohol exposed mouse HCC cell lines.

### 3.7. Effect of leptin on ethanol-induced oxidative stress

When compared to untreated cell lines, ethanol exposure significantly ( $p < 0.05$ ) elevated ROS production (Figure 5a). Leptin co-administration along



with ethanol significantly ( $p < 0.05$ ) decreased ROS formation compared to ethanol per se exposed cell lines. We also observed significantly ( $p < 0.05$ ) elevated levels of TBARS (Figure 5b), a marker of peroxidation products to ethanol treated cell lines as compared with untreated cells. Leptin co-treatment along with ethanol significantly lowered the TBARS levels when compared to ethanol alone-incubated cell lines. Leptin per se had no effect on alteration of ROS or TBARS in mouse HCC cell lines.



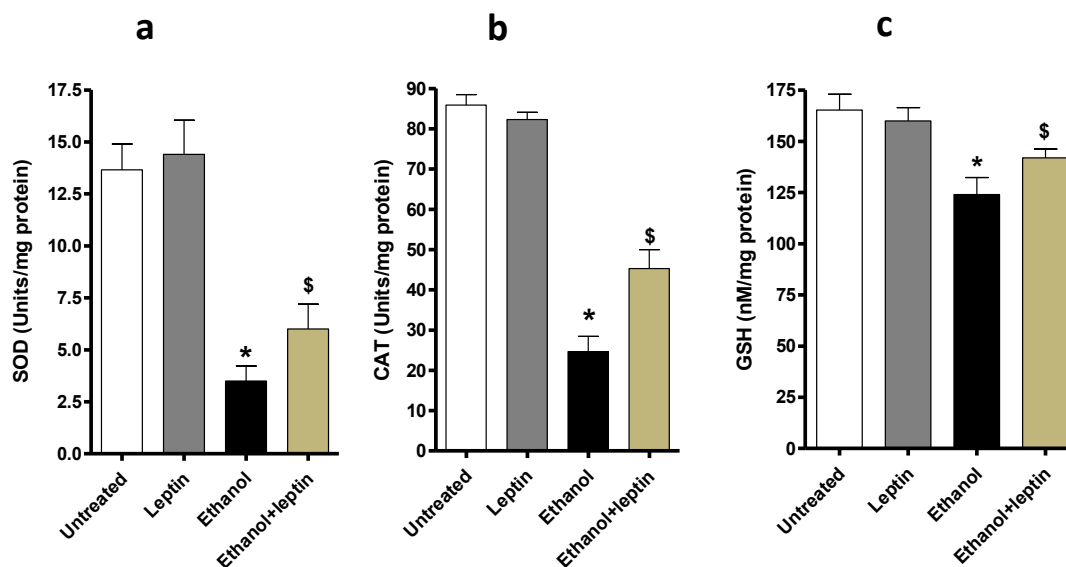
**Figure 5.** Effect of leptin on ethanol induced ROS formation in mouse HCC cell lines (left image) and TBARS levels in mouse HCC cell lines (right image). Data are expressed as mean  $\pm$  standard error of mean (SEM). Note: \* $p < 0.05$  compared to untreated mouse HCC cell lines; \$ $p < 0.05$  compared to alcohol exposed mouse HCC cell lines.

### 3.8. Effect of leptin on ethanol-induced antioxidant enzymes

The activities of SOD (Figure 6a), CAT (Figure 6b) and the concentration of GSH (Figure 6c) were significantly ( $p < 0.05$ ) lowered following ethanol exposure to mouse HCC cell lines compared to untreated medium. Co-treatment of leptin with ethanol significantly ( $p < 0.05$ ) elevated the activities of SOD, CAT and GSH when compared to ethanol alone-incubated cell lines. Leptin per se did not significantly alter the above indices compared to untreated control cell lines.

## 4. DISCUSSION

Use of cultured hepatoma cells have added to our understanding of the molecular mechanisms by which ethanol metabolism damages hepatic cells and eventually causes liver disease. Use of these systems has allowed detailed investigation of the effects of individual metabolic pathways of ethanol metabolism and specific biochemical changes. In this study, we showed that leptin co-treatment along with ethanol to mouse HCC culture resulted in decreased apoptosis, fibrosis, inflammation and oxidative stress whilst increasing the antioxidant enzyme activities. First, we have analysed the proliferative capacity of ethanol and leptin on mouse HCC cell lines by MTT assay, an acceptable cellular assay. Ethanol incubation results in decreased cell proliferation and observed a maximum toxic effect in a concentration-dependent manner.



**Figure 6.** Effect of leptin on ethanol induced (a) SOD activity in mouse HCC cell lines, (b) CAT activity in mouse HCC cell lines and (c) GSH activity in mouse HCC cell lines. Data are expressed as mean  $\pm$  standard error of mean (SEM). Note: \* $p < 0.05$  compared to untreated mouse HCC cell lines; \$ $p < 0.05$  compared to alcohol exposed mouse HCC cell lines.

Leptin administration to ethanol treated cells showed increased cell number even on treatment with the maximum dose of 31.2 nM.

Apoptosis has been shown to occur in both experimental and clinical alcoholic liver disease, but the signalling pathways are yet to be fully understood. The most common biochemical marker of apoptosis is the fragmentation of DNA into internucleosomal fragments caused by endonuclease action (Kajstura et al., 2007). In the current study both DNA fragmentation resulting in lesser DNA content was observed in the mouse HCC cells after ethanol treatment. We also measured apoptosis using FITC-annexin V with PI staining and flow cytometry which significantly increased cell death caused by ethanol in the early sub-G<sub>0</sub> phases of the cell cycle. In this context, previous reports emphasize the role of ethanol on apoptosis death of many cell types (Benedetti et al., 1988; Han et al., 2005).

Central to the process of apoptosis are the caspases, a unique class of proteinases. Activation of caspases, especially caspase-3, results in the cleavage of various target proteins that are critical for apoptosis. Mitochondria, the main target subcellular organelle for damage by ROS, endure permeability transition thereby undergoing a disruption of the mitochondrial membrane potential. Such conditions cause the release of cytochrome c from the mitochondria to the cytosol, triggering cells to undergo apoptosis by activating caspase-3 (Tada-Oikawa et al., 1999). The current study showed a simultaneous increase in the mRNA expression of caspase-3 and also the cellular production of ROS on chronic ethanol supplementation, whereas leptin co-treatment along with ethanol to mouse HCC cell lines increased cell viability, DNA synthesis and decreased DNA fragmentation and cell death in the early sub-G<sub>0</sub> phases of the cell cycle. Collectively these findings represent the protective role of leptin on ethanol-elicited cytotoxicity and apoptosis.

We have also described the possible protective effect of leptin on ethanol-induced toxicity by measuring oxidative stress and the antioxidant status. Ethanol affects the liver more than any other organ, since its metabolism implies generation of free radicals that can damage cell structure and function (Zhong and Lemasters 2004). Our in vivo studies showed increased lipid peroxidation and decreased endogenous antioxidants on chronic ethanol supplementation (data not shown). In the present in vitro experiments, ethanol significantly elevated TBARS and cellular ROS and significantly lowered the levels of the major antioxidants such as SOD, CAT and GSH. Thus, the metabolism of ethanol increases

free radicals production, causing cellular damage as well as alterations in the liver function. In this context, Bailey and Cunningham (Bailey and Cunningham 1998) showed that incubation of rat liver hepatocytes with ethanol was increased dichlorofluorescein fluorescence (DCF), a measure of ROS, largely due to H<sub>2</sub>O<sub>2</sub> production.

Leptin administration along with ethanol decreased TBARS and ROS thereby enhancing cell viability suggesting its protective effect. Moreover, leptin treatment significantly increased GSH concentration, CAT and SOD activities in ethanol-exposed mouse cultured cell lines. The decreased lipid peroxidation and ROS upon leptin treatment might be due to the induction of the hepatic activities of SOD and CAT. Moreover, hyperinsulinemia is known to play a role in the intracellular H<sub>2</sub>O<sub>2</sub> generation (Mukherjee and Lynn 1977). Watson et al. (1999) have shown that insulin resistance in ob/ob mouse, may have contributed to the increased production of H<sub>2</sub>O<sub>2</sub> and therefore a reduction in the antioxidant enzyme activities. Moreover, our in vivo leptin treatment to mice causes a decrease in body weight due to increased fat oxidation resulting in the loss of body fat (Balasubramanian and Nalini, 2006a). This loss in body fat and normalization of insulin levels by leptin (Balasubramanian and Nalini, 2006b) could potentially reverse the oxidative stress and antioxidant deficiency occurring in ethanol-exposed cell lines. More recently Ercan et al. (2007) found that leptin pre-treatment down regulates caspase-3 immunoreactivity and lipid peroxidation in the stomachs of rats exposed to cold-restraint stress.

Finally, we have addressed the possible protective effect of leptin on ethanol-mediated fibrosis by analysing fibrotic markers such as collagen type I and extracellular matrix-degrading enzymes like MMP 2 and 9 and also its inhibitor TIMP-1. Incubation of cells with ethanol stimulates type I procollagen mRNA (Figure 3b) and up-regulates expression of the tissue inhibitor of metalloproteinase (TIMP)-1 (Figure 3e), thus blocking collagen degradation. Our previous observations revealed that leptin significantly inhibits transforming growth factor - $\beta$  (TGF- $\beta$ ) both in vivo and in vitro (Balasubramanian et al., 2007b). TGF- $\beta$  is known to enhance collagen formation through the SMAD signalling pathway, which may be the cause for the negative regulatory role of leptin on the enhancing effect of ethanol on collagen production.

Ethanol exposed cells also up regulate the mRNA expressions of MMP 2 (Figure 3c) and MMP 9 (Figure 3d). MMP 2 is secreted by activated hepatic stellate cells and is increased in the presence of type I



collagen. MMP 9 is secreted by activated kupffer cells mainly involved inflammatory pathway. Plasma levels of MMP 9 have been shown to be increased in patients with hepatocellular carcinoma (Murawaki et al., 2000). Ethanol treatment increased MMP 9 gelatinolytic activity and promoted cell invasion through the up-regulation of MMP-9 gene transcription in HepG2 cells (Chen et al., 2007). Moreover, ethanol stimulates the secretion of matrix metalloproteinases 2 and 9 in MCF-7 human breast cancer cells (Etique et al., 2006). A similar increase in the expression of MMP-9 was also identified in this study on ethanol exposure to mouse HCC cell lines when compared to untreated cells. On the other hand leptin co-treatment along with ethanol resulted in the decreased expression of MMP 2 and 9.

TIMPs are low molecular weight glycoproteins that are specific inhibitors of matrix-degrading enzymes. TIMP-1 overexpression results in the suppression of proliferative and invasive potential of HepG2 cells in vitro (Xia et al., 2006). Our results also showed that TIMP-1 mRNA expression was significantly elevated with ethanol supplementation and this increase was significantly reduced following leptin administration, which therefore appears to confer a dual beneficial action of leptin on inhibiting fibrogenesis (procollagen  $\alpha 1$  (I) expression) and relieving the suppression of extracellular matrix degradation (blocking TIMP-1 mRNA expression). The interaction between MMPs and TIMPs is complex, and, because these molecules act locally as well as having multiple activities including activation of growth factors, affecting cell proliferation, and inhibition of apoptosis, the relationship remains unclear.

In summary, leptin co-treatment along with ethanol to mouse HCC cell lines results in decreased oxidative stress and cytokine response and increased cell viability, DNA synthesis and antioxidant enzyme. Leptin co-treatment together with ethanol also results in a down regulation of profibrogenic markers as well as a marker of apoptosis. Thus, our findings suggest that leptin may have a role against alcohol induced HCC progression in in vitro. Indeed, further molecular studies are needed to confirm a potential role of leptin against HCC.

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