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RESEARCH ARTICLE

Effects of long term ethanol consumption mediated oxidative stress on neovessel generation in liver

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Abstract
Angiogenesis, the growth of new blood vessels, is essential during tissue repair. Though most molecular mechanisms of angiogenesis are common to the liver and other organs, there was no report available whether alcoholic liver disease also causes angiogenesis. In this study, we examined the effects of long term ethanol (1.6 g/kg body weight/day) consumption on angiogenic responses in the liver of male Wistar strain albino rats (16–18 weeks old, weighing 200–220 g) up to 36 weeks. Chronic ethanol consumption was associated with not only elevated oxidative stress, and altered cytokines expression, but also developed large von Willebrand factor, fibrosis and activation of matrix metalloproteinases. Moreover, vascular endothelial growth factor-receptor 2 (VEGF-R2, fetal liver kinase 1: Flk-1/KDR) expression and neovessel generation in the rat liver were noted after 36 weeks of ethanol consumption. Thus our study provides novel evidence that long-term ethanol consumption is associated with angiogenesis through delicate and coordinated action of a variety of mediators.

Keywords: Ethanol, matrix metalloproteinases, oxidative stress, vascular endothelial growth factor-receptor, von Willebrand factor

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Introduction

Hepatic injury, whether subclinical or overt, is a perturbation of normal liver homeostasis, with the extracellular release of free radicals, intracellular constituents and/or cytokines and signalling molecules (Friedman, 2000). Ethanol-associated endotoxaemia and subsequent release of inflammatory mediators, such as pro-inflammatory cytokine tumor necrosis factor-α (TNF-α), as well as transforming growth factor-β1 (TGF-β1) and vascular endothelial growth factor (VEGF) (Das et al. 2009a, 2009b), may cause hepatic injury via oxyradical-dependent or -independent mechanisms (Fernandez-Checha et al. 1997).

Tissue repair plays a critical role in determining the final outcome of toxicity, i.e., recovery from injury or progression of injury, leading to liver failure and death. It also encompasses regeneration of hepatic extracellular matrix and angiogenesis, the processes necessary to completely restore the structure and function of the liver (Mehendale, 2005). Liver regeneration is a complex physiological response to hepatic injury. The remnant organ initiates a series of reactions to promote cell growth and to restore the functional liver mass (Fausto, 2000). Though most molecular mechanisms of angiogenesis are common to the liver and other organs, there are potential differences (McCuskey and Reilly, 1993; Medina et al. 2004). Hypoxia is one of the pathogenic mechanisms contributing to liver damage secondary to acute and chronic ethanol consumption (Arteel et al. 1997). In a rat experimental model of alcoholic liver disease, hypoxia is evidenced (Bardag-Gorce et al. 2002). Local low levels of oxygen have been postulated not only contribute to liver damage, but also induce several factors involved in angiogenesis (Bardag-Gorce et al. 2002). However, available conflicting reports raised concern, whether alcohol induced liver damage also causes angiogenesis (Nanji et al. 1996; Radek et al. 2008; Sarphie et al. 1997). In fact, hepatic angiogenesis has been observed in the context of different inflammatory, fibrotic, and ischemic conditions (Carmeliet, 2003).

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Therefore, in this study, we examined the role of chronic ethanol-induced oxidative stress and immunological alterations on angiogenic responses in the liver.

Materials and methods

Chemicals – Ethanol from Bengal Chemicals, Kolkata; thiobarbituric acid from Loba Chemie, India; 4-aminophenazone, 3,5-dichloro-2-hydroxybenzenesulfonic acid (3,5-DCHBS), glutamate dehydrogenase and yeast glutathione reductase (GR) from Sigma Chemical Co., St. Louis, USA; immunohistochemical kits (vWF, VEGF-Flk) from Abcam Inc, MA, USA; transforming growth factor (TGF)-β1 ELISA kit from Bender Medsystems, Austria; and other cytokine kits from BD Biosciences (Becton, Dickinson & Company, USA), Complete Mini protease inhibitor cocktail tablets from Roche Diagnostics, Indianapolis, IN, USA were used. All other chemicals were purchased from Sisco Research Laboratory, Mumbai, India unless otherwise stated.

Animal Selection – Twenty four male albino Wistar strain rats (16–18 weeks-old, 200–220 g) were housed in plastic cages inside a well-ventilated room. The room was maintained under standard husbandry condition. All animals had free access of standard diet (Das and Vasudevan, 2006a, 2006b) and water *ad libitum*. The animals were weighed daily and its general condition was recorded including their daily intake of liquid. A dose of 1.6 g ethanol/kg body weight/day was selected for administration to animals based on our previous dose-dependent study in male Wistar rats (Das and Vasudevan, 2005). The rats were randomly divided into three ethanol treatment groups along with appropriate control. Ethanol was diluted with distilled water to get desired concentration and administered intragastrically for 4, 12 or 36 weeks. Control rats were fed isocaloric glucose solution instead of ethanol (1.6 g/kg body wt) per day. The Animal Ethics Committee of the Institution approved the procedures in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA-India) guideline.

Experimental procedures– The rats were sacrificed after over-night fast at the end of each experimental schedule by administration of intraperitoneal Na-pentobarbital (Nembutal, 60 mg/kg body weight) (euthanasia) (Fortunato et al. 2007). The liver tissues were collected, cleaned with ice-cold saline, blotted dry, and immediately preserved for further analysis.

Biochemical analysis– Frozen liver tissues were homogenized in 100 mg tissue/mL cold lysis buffer (20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton ×-100) and 1 tablet of Complete Mini protease inhibitor cocktail tablets/10 mL, and were used for estimation of tissue protein (Lowry et al. 1951), reduced glutathione (GSH) (Beutler et al. 1963) and thiobarbituric acid reactive substance (TBARS) (Sinnhuber et al. 1958) levels; and the activities of glutathione peroxidase (GPx) (Paglia and Valentine, 1967), glutathione reductase (GR) (Pinto and Bartley, 1969), glutathione-s-transferase (GST) (Habig et al. 1974), catalase (Das and Vasudevan, 2006a) and superoxide dismutase (SOD) (Paoletti et al. 1986). Cytokines [IL-1β, IL-10, TNF-α and TGF-β1] were estimated using Sandwich ELISA, according to manufacturer's instruction.

Immunohistochemistry – Briefly, four micrometer paraffin-embedded liver sections were deparaffined in xylene for 20 min, absolute alcohol for 10 min, methanol: H_2O_2 (300 mL: 3 mL) for 30 min and rehydrated in graded alcohol (90%, 70% and 30%). The tissues were then immersed in PBS and in 5% BSA, followed by primary antibody for 1 h. After washing again in PBS, secondary antibody was added for 1 h. The slides were then washed in PBS and incubated in PBS-DAB- H_2O_2 (300 mL: 300 mg: 0.6 mL) for 30 min. The slides were further washed in running tap water and placed in haematoxylin, washed again and dehydrated with graded alcohol and kept in xylene overnight. After draining off excess xylene, mounted on DPX, viewed under microscope, digitized and scanned using standard imaging program.

Multiwell zymogram (Total matrix metalloproteinase activity) – 100 μL tissue homogenates were placed in 24-well containing plate and incubated at 37°C for 30 mins for enzyme activation. Zymo gel [15 mg gelatin dissolved in 3.75 mL of Tris buffer, pH 8.8; 3.75 mL acrylamide-bisacryamide (30 g%: 0.3 g%); 7.125 mL double distilled water; 150 μL 10% ammonium persulphate (freshly prepared) and 15 μL TEMED] was added and allowed to settle for 1 h. The gels were then placed in 6-well containing plates with zymo buffer (calcium chloride buffer, pH 7.5; consisting of 3.03 g Tris–HCl and 0.36 mg $CaCl_2$ in 500 mL double distilled water) and incubated overnight. After removing the zymo buffer, the gels were stained with Coomasie brilliant blue for 3-4 h and destained (Ambili et al. 1998).

Histopathological Examination – Liver tissues were fixed in formalin, routinely processed and embedded in paraffin. 4 μm thick sections were stained with hematoxylin and eosin to assess morphological changes under microscope.

Statistical analysis – All data were analyzed using the statistical package SPSS (version 11.0, SPSS Inc., Chicago, IL). Results were expressed as mean ± SD (standard deviation). The sources of variation for multiple comparisons were assessed by the analysis of variance (ANOVA), followed by Post-Hoc test with Bonferroni's and Tukey's multiple comparisons test. The difference were considered significant at $P < 0.05$.

Results

A time-dependent effect on oxidative stress related parameters in ethanol exposed rat liver homogenates up to 36 weeks are presented in Table 1. While reduced glutathione (GSH) content and activities of GPx, GR and

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Table 1. Effect of ethanol on reduced glutathione (GSH) and thiobarbituric acid reactive substance (TBARS) contents, and activities of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione s-transferase (GST), catalase and superoxide dismutase (SOD) in liver homogenate of rats for different time period.

Parameters	Control	4 weeks	12 weeks	36 weeks
TBARS (μmole MDA formed/ min/100 mg tissue)	0.651 ± 0.009	0.858 ± 0.012	1.075 ± 0.041[a]	1.426 ± 0.084[a,d,g]
GSH (nmoles/mg protein)	67.93 ± 1.19	51.52 ± 1.46[a]	40.9 ± 1.25[a,e]	33.57 ± 1.83[a,d,h]
GSSG (nmoles/mg protein)	5.2 ± 0.09	5.76 ± 0.12[c]	6.23 ± 0.1[a]	6.98 ± 0.09[a,d,g]
Redox ratio (GSSG/GSH)	0.076 ± 0.002	0.112 ± 0.005	0.153 ± 0.007[a]	0.211 ± 0.014[a,d,h]
GPx (nmole NADPH breakdown/ min/mg protein)	82.17 ± 1.25	66.5 ± 1.26[b]	56.33 ± 1.98[a,f]	45.83 ± 3.19[a,d,i]
GR (nmole NADPH breakdown/min/mg protein)	54.67 ± 0.88	37.67 ± 1.76[a]	25.83 ± 1.25[a,d]	20 ± 1.06[a,d,i]
GST (μmole CDNB conjugate formed/mg protein/min)	6.88 ± 0.09	11.16 ± 0.37[a]	10.95 ± 0.3[a]	9.63 ± 0.2[a,e,i]
Catalase (μmole H_2O_2 decomposed/min/mg protein)	39.48 ± 0.59	34.65 ± 0.8[a]	32.1 ± 0.63[a]	25.48 ± 0.67[a,d,g]
SOD (U/mg protein)	6.26 ± 0.06	9.44 ± 0.1[a]	8.7 ± 0.18[a,e]	5.9 ± 0.05[d,g]

Values are mean ± SD of 6 rats in each group.
p Values: [a]< 0.001, [b]<0.01, [c]<0.05 compared to control group and; [d]<0.001, [e]<0.01, [f]<0.05 compared to 4 weeks ethanol treated group; [g]<0.001, [h]<0.01, [i]<0.05 compared to 12 weeks ethanol treated grouped group.

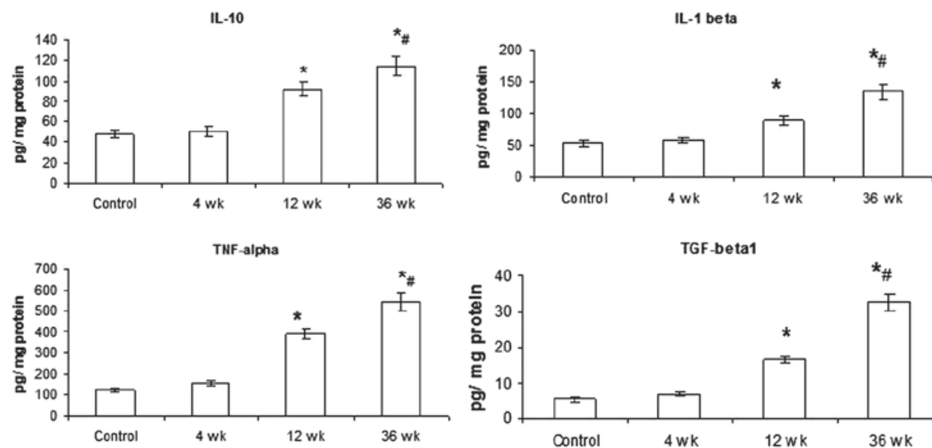

Figure 1. Time dependent effects of ethanol on different cytokines expression in rat liver tissues. Values given are average of 3 experiments ± SD. * indicates $p < 0.05$ compared to the control group and 4 wk ethanol treated group; # indicates $p < 0.05$ compared to the 12 wk ethanol treated group.

Catalase decreased significantly after 4 weeks of ethanol exposure (Table 1), TBARS level increased significantly after 12 weeks of ethanol exposure (Table 1), compared to the control group. Interestingly, though GST and SOD activities increased significantly after 4 weeks of ethanol exposure compared to the control group (Table 1), these activities decreased later with duration of ethanol exposure (Table 1). Ethanol treatment significantly increased liver cytokines [IL-1β, IL-10, TNF-α and TGF-β1] activities after 12 weeks of exposure (Figure 1).

Figure 2 shows increased large von Willebrand factor (vWF) in rat liver tissues due to ethanol (1.6 g/ kg body weight/ day) exposure for longer duration (Figure 2a and b). Chronic ethanol consumption activated expression of VEGF-receptor-2 (VEGFR2; fetal liver kinase 1; Flk-1/ KDR) with duration of exposure (Figure 3a and 3b). Histopathological examination of ethanol treatment for 4 (Figure 4b) or 12 weeks (Figure 4c) showed generation of fibrosis as evidenced by pale pink to bluish color in hematoxylin and eosin staining, and further long-term exposure (36 weeks) caused neovessels generation (Figure 4d). These changes were accompanied with elevated total matrix metalloproteinase activity (Figure 5).

Discussion

Ethanol-induced liver injury is characterized by increased formation of reactive oxygen species (ROS). GSH is a major non-protein thiol in living organisms, which plays a key role in coordinating the body's antioxidant defense processes. Depletion of GSH due to ethanol exposure in this study (Table 1) renders the cell more susceptible to oxidative stress (Videla et al. 1984). Elevated lipid peroxidation or TBARS (Table 1) mediated by free radicals is considered to be a primary mechanism of cell membrane destruction and cellular damage (Plaa and Witschi, 1976). Alterations in GPx, GR and GST activities, followed by thiol depletion in this study are important factors sustaining a pathogenic role for oxidative stress (Das and Vasudevan, 2005).

SOD catalytically dismutes superoxide radical anion ($O_2^{\cdot-}$) to H_2O_2, while catalase and GPx render H_2O_2 harmless within cells by converting it into water and oxygen (Gabbita et al. 2000). Initial increase in SOD activity may probably be an adaptive response towards oxidative stress (Kono and Fridovich, 1982). But, long term ethanol exposure diminished its activity. Decreased

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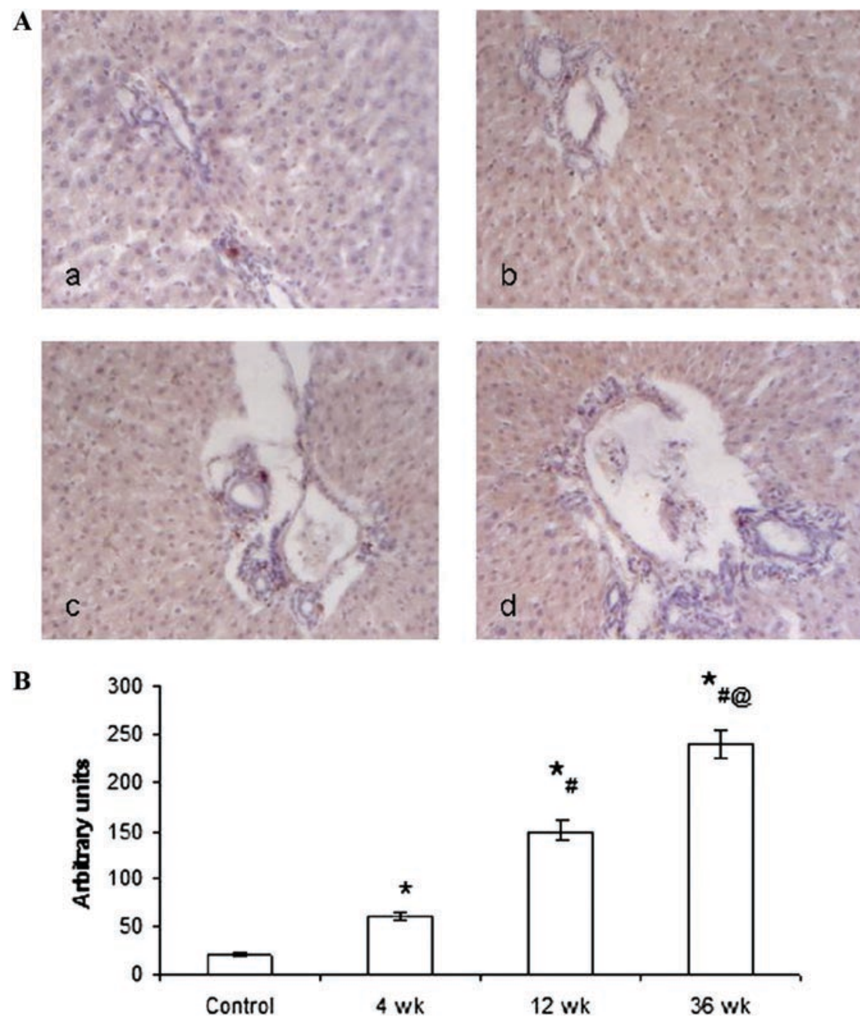

Figure 2. a – Expression of von Willebrand Factor in liver tissues for (a) control group of rats; and (b) 4 weeks, (c) 12 weeks, and (d) 36 weeks of ethanol exposed rats. b – The scanning image analysis of von Willebrand factor expression in rat liver tissue with duration of ethanol exposure. Values given are average of 3 experiments ± SD. *p* Values: *<0.05 compared to the control group, #<0.05 compared to the 4 weeks ethanol treated group, and @<0.05 compared to the 12 weeks ethanol treated group.

catalase activity (Table 1) might be due to loss of NADPH or generation of superoxide or increased activity of lipid peroxidation or combination of all (Das and Vasudevan, 2005). Generation of reactive oxygen species, such as super oxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) due to ethanol consumption are involved in the signaling pathways mediating stress and growth responses, including angiogenesis (Lelkes et al. 1998).

Cytokines are multi-functional proteins that play a critical role in cellular communication, activation, inflammation, cell death, cell proliferation, and migration as well as healing mechanisms (Crews et al. 2006). Though anti-inflammatory and immunosuppressive activities of IL-10 may limit alcohol-induced liver damage (Hill et al. 2002), its activity in the liver enhanced after 12 weeks of ethanol exposure and persisted (Figure 1) in agreement with other study (Qin et al. 2008). Long-term ethanol consumption also increased in pro-inflammatory cytokines TNF-α and IL-1β in the liver. TNF-α, a major factor in the development of alcohol-induced liver injury, contributes to necroinflammation, apoptosis and fatty liver (Das et al. 2010). In addition, TNF-α and other cytokines may be associated with releasing TGF-β1, which is believed to be involved in hepatic fibrosis (Das et al. 2010). IL-1β also has direct action on hepatic fibrogenesis (Zhang et al. 2006). These alterations in immune status may lead to impairment of host defenses against infections.

The role of von Willebrand Factor (vWF), a large multimeric carrier glycoprotein of factor VIII (an essential cofactor in coagulation), in liver injury and repair is unknown. Although many hemostatic changes in liver disease promote bleeding, compensatory mechanisms also are found, including high levels of the platelet adhesive protein vWF (Lisman et al. 2006). vWF promotes platelet adhesion to the damaged vessel wall (Mazurier et al. 1998), particularly at the high shear rates encountered in small blood vessels. In our study, large vWF were observed in the rat livers due to prolonged ethanol consumption (Figure 2a and b). In another study, severe

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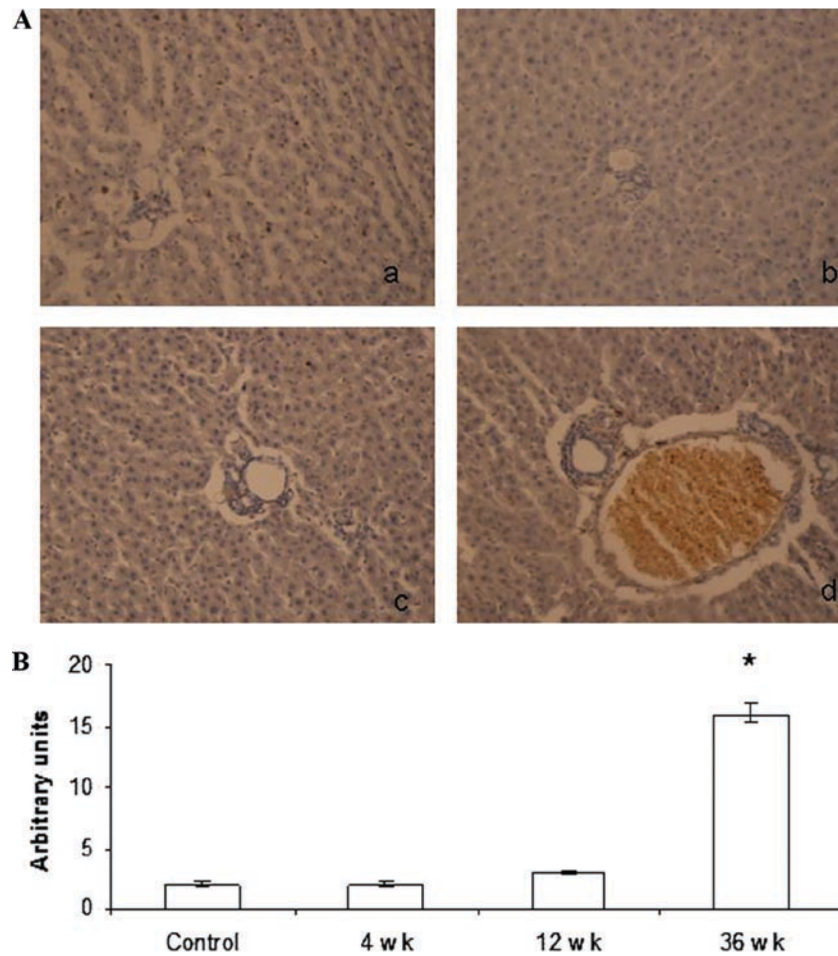

Figure 3. a – Effects of ethanol exposure on VEGF-Flk expression of in rat liver tissues for (a) control group; and (b) 4 weeks, (c) 12 weeks, and (d) 36 weeks of treatment. b – The scanning image analysis of VEGF-Flk expression in rat liver tissue with duration of ethanol exposure. Values given are average of 3 experiments ± SD.* indicates $p < 0.05$ compared to the control group.

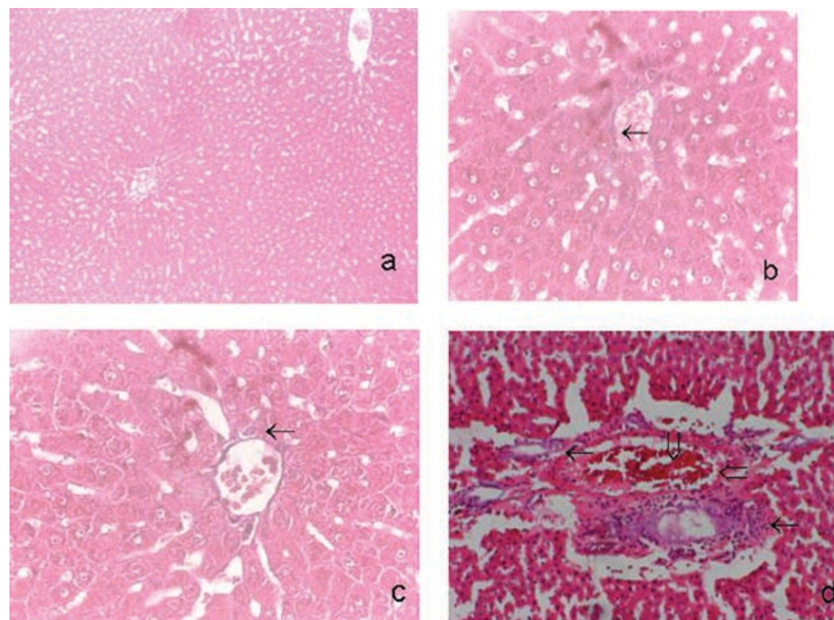

Figure 4. Histopathological examination of rat liver tissues for (a) control group; and (b) 4 weeks, (c) 12 weeks, and (d) 36 weeks of ethanol (1.6 g/kg body wt/day) treatment. Pale pink to bluish colour shown by arrows (→) indicated appearance of fibrosis; and ⇒ indicated neovessels generation after 36 wks of ethanol exposure.

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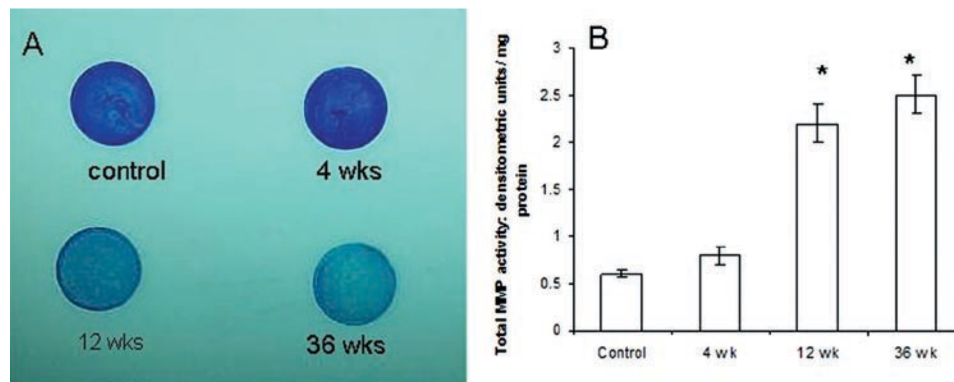

Figure 5. Changes in total matrix metalloproteinases activity on rat liver due to ethanol (1.6 g/kg body weight/day) exposure for different time period. (A) Extracts of liver tissue samples (100 µL) from control and ethanol exposed rats of different time interval (up to 36 weeks) were copolymerised with acrylamide-bisacryamide containing gelatin in Tris buffer (pH 8.8). After polymerization, the gels were then incubated in substrate buffer, stained with Coomasie brilliant blue and destained with methanol-acetic acid-water. (B) Activity measured by densitometric analysis. Values given are average of 3 experiments ± SD. * indicates $p < 0.05$ compared to the control group.

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alcoholic hepatitis was found associated with an increase in large vWF multimer that caused microcirculatory disturbance and multiorgan failure (Matsuyama et al. 2007). High concentrations of vWF may indicate the presence of microangiopathy (Lisman et al. 2006). Elevated levels of vWF contribute to the induction of primary hemostasis despite reduced functional properties of the molecule. This phenomenon might compensate for defects in platelet number and function in liver diseases (Lisman et al. 2006). Studies suggest that the vWF is deposited within the liver sinusoids early after liver damage. The factor is only partially produced locally during the acute phase of the disease, but is overproduced in chronic disease states (Baruch et al. 2004). vWF elevation paralleled the onset of angiogenesis and was considered an early indicator of endothelial activation (Choi et al. 2009).

Vascular endothelial growth factor (VEGF) plays a crucial role in virtually all pathological situations in which angiogenesis occur (Yano et al. 2006). VEGF has been shown to be highly protective for liver cells (Moriga et al. 2000; Taniguchi et al. 2001). In endothelial cells, H_2O_2 stimulates cell migration, proliferation (Pearlman et al. 1995) and was found to induce VEGF mRNA (Shih et al. 1999). The chronobiology of the events related to liver regeneration starts with a release of VEGF by the hepatocytes (Maharaj et al. 2006). The VEGF interacts with three subtypes of VEGF receptors on the cellular membrane known as VEGFR-1 (fms-like tyrosine kinase 1: Flt-1), VEGFR-2 (fetal liver kinase 1: Flk-1/KDR), and VEGFR-3 (Flt-4) to activate signaling pathways (Namiecinska et al. 2005). VEGF-A and B participates in the regulation of normal (physiological) and pathological angiogenesis (Das and Vasudevan, 2007). The VEGF-A binds to both VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), while the VEGF-B binds to VEGFR-1 (Flt-1) only (Iyer et al. 2006). One study suggested that progression of angiogenesis may be mediated by upregulation of VEGF and Flt-1, especially under the influence of ethanol (Tan et al. 2007). Our previous studies reported that chronic ethanol consumption was associated with elevation of VEGF-A level (Das et al. 2009a, 2009b). In this study, VEGF-Flk1 expression was observed in rat livers after 36 weeks of ethanol treatment (Figure 3). In resting liver, VEGFR2 expression is limited to endothelial cells of the large hepatic vessels. During liver regeneration, VEGFR2 expression predominantly increases on endothelial cells of large vessels and to a lesser extent on sinusoidal endothelial cells often in close proximity to large vessels. Additionally hepatic stellate cells have been found to express VEGFR2 *in vitro* (Dufour and Clavien, 2010). It is suggested that VEGF and Flk-1/KDR are involved in proliferation, migration, and tubule-like structure of endothelial cells (Choi et al. 2009).

Histopathological analysis revealed appearance of fibrosis in liver tissues of rats after 4 weeks of ethanol treatment (Figure 4b), and its intensity increased with further exposure up to 12 weeks of ethanol treatment (Figure 4c). we exceptionally conducted this study for such a long period. Fibrotic tissues offer resistance to blood flow and to the delivery of oxygen, and become hypoxic (Garcia-Monzon et al. 1995). Local low levels of oxygen have been postulated not only to contribute to liver damage, but also to induce several factors involved in angiogenesis (Bardag-Gorce et al. 2002). Thus chronic hepatic damage is associated with liver fibrosis that may lead to the pathological vessel growth (Vogten et al. 2004), and we are providing first ever evidence that longer term (36 weeks) ethanol consumption resulted neovessels generation in Figure 4d.

Fibrogenesis in human ethanol injury is due to the activity of stellate cells, Kupffer cells, and to a lesser extent to endothelial cells (Chedid et al. 2004). Fibrosis or scarring, in the liver is characterized by excessive extracellular matrix (ECM) deposition, resulting from both increased ECM protein synthesis and decreased degradation (Cohen and Nagy, 2011). Angiogenesis involves remodeling the basement membrane and interstitial

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extracellular matrix (ECM) using proteases produced by the endothelial cells (ECs) and other adjacent cells (Norrby, 1997). The ECM directs cellular differentiation, migration, proliferation, and fibrogenic activation or deactivation (Schuppan et al. 2001). Specialized proteinases, including matrix metalloproteinases (MMPs) and others, degrade the basement membrane and the ECM to allow subsequent EC migration and proliferation (van Hinsbergh et al. 2006). In this study the gelatin degrading MMP activities expressed in liver homogenate due to ethanol exposure (Figure 5). These proteases switch on neovascularization by activation, liberation, and modification of angiogenic growth factors and degradation of the endothelial and interstitial matrix (van Hinsbergh et al. 2006).

Our study thus provides first ever novel evidence that long-term (36 weeks) ethanol (1.6 g/kg body wt/ day) consumption is associated with severe oxidative stress and immunological alterations that triggers angiogenesis through delicate and coordinated action of a variety of mediators. However, the pathophysiology of neovessel generation involves complex interactions between different cell types in the liver. Therefore, further understanding of the complex impact of ethanol on different cell types, particularly, in the context of fibrosis and angiogenesis are needed, in order to facilitate the development of therapeutic interventions for patients with alcoholic liver disease.

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Declaration of interest

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