Effects of long-term ethanol consumption on adhesion molecules in liver

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Hepatic fibrosis is an outcome of many chronic liver diseases, such as viral and autoimmune hepatitis, and of alcohol consumption and biliary obstruction¹. Hepatic injury, whether subclinical or overt, is a perturbation of normal liver homeostasis, with the extracellular release of free radicals (i.e. “oxidant stress”), intracellular constituents and/or cytokines and signalling molecules². Many processes related to the consumption or breakdown of alcohol that contribute to alcohol-induced liver disease are mediated by cytokines, which are produced and secreted by cells throughout the body³. 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 mechanisms³. Ethanol-associated endotoxaemia and subsequent release of inflammatory mediators⁴, such as γ-interferon (IFN-γ), pro-inflammatory cytokine tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), as well as vascular endothelial growth factor (VEGF)-A⁵,⁶, may cause hepatocyte injury via oxyradical-dependent or -independent mechanisms⁴, which may aggravate to fibrosis and elevate pro-angiogenic factors⁵,⁶. TNF-α constitutes a major factor in the development of alcohol-induced liver injury⁷. TNF-α makes a moderate contribution to the alanine transaminase (ALT) elevation, necroinflammation, apoptosis and a small contribution to the fatty liver⁸. TNF-α and other cytokines may be associated with releasing TGF-β1 that is believed to be involved in hepatic fibrosis⁹. Vascular endothelial growth factor (VEGF), a glycosylated peptide with multiple isoforms potently induces endothelial proliferation¹⁰, increases vascular permeability¹¹ and may be associated with angiogenesis due to long-term ethanol consumption⁵,⁶.

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 liver¹². The chronobiology of the events related to liver regeneration starts with a release of VEGF by the hepatocytes¹³. Fibrogenesis in human ethanol injury is due to the activity of stellate cells, Kupffer cells, and to a lesser extent, to endothelial cells¹⁴. VEGF induces adhesion molecules on endothelial cells during inflammation¹⁵. Endothelial adhesion molecules play an important role in the pathogenesis of several diseases¹⁶.
Therefore, in this study we focused on examining
the expression of adhesion molecules such as
intercellular adhesion molecule-1 (ICAM-1), vascular
cell adhesion molecule-1 (VCAM-1), platelet
endothelial cell adhesion molecule-1 (PECAM-1) in
the setting of chronic alcohol induced liver damage of
male albino Wistar strain rats (16-18 weeks-old, 200-
220 g) in a time dependent manner.

**Materials and Methods**

**Chemicals**—Ethanol from Bengal Chemicals, Kol-
kata; percoll from Amersham Pharmacia Biotech;
FITC-conjugated goat anti-mouse IgG secondary an-
tibody from Bangalore Genei; purified mouse anti-rat
CD106 (VCAM-1; Cat. 559165), anti-rat CD31 (PE-
CAM-1; Cat. 555025), anti-rat CD54 (ICAM-1; Cat.
554967) monoclonal antibodies and other cytokine
kits from BD Biosciences (Becton, Dickinson &
Company; USA) were used. All other chemicals were
purchased from Sisco Research Laboratory, India.

**Animal selection**—Male albino Wistar strain rats
(30, 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 to standard diet and
water *ad libitum*. The animals were weighed daily and
their 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
as it was found tolerable and simultaneously caused
maximum liver damage in male Wistar rats in
previous dose-dependent study.

The rats were divided into the following 5 groups
of 6 each:

- **Group I:** Control rats—the rats were fed normal
diet and water.
- **Group II:** Rats were treated with 1.6 g ethanol/kg body
weight/day for 4 weeks.
- **Group III:** Rats were treated with 1.6 g ethanol/kg body
weight/day for 12 weeks.
- **Group IV:** Rats were treated with 1.6 g ethanol/kg body
weight/day for 24 weeks.
- **Group V:** Rats were treated with 1.6 g ethanol/kg body
weight/day for 36 weeks.

Absolute ethanol (20 ml) was diluted with 80 ml
distilled water to get desired concentration (i.e. 20%
v/v). Freshly prepared dilute ethanol was fed
intragastrically at a dose of 1 ml/100 g body weight.
The Animal Ethics Committee of the Institution
approved the procedures in accordance with the
CPCSEA guideline.

**Experimental procedure**—The rats were sacrificed
after over-night fasting at the end of experimental
period, by applying intraperitoneal Na-pentobarbital
(Nembutal, 60 mg/kg body weight) (euthanasia).
The liver tissues were collected, cleaned with ice-cold
saline, blotted dry, and immediately transferred to ice
chamber.

Liver was homogenized in (10% w/v) 0.25 M
sucrose solution, diluted with 0.9% saline, and these
diluted samples were used for estimation of tissue
protein and transaminase (aspartate transaminase,
AST, EC. 2.6.1.1; alanine transaminase, ALT, EC.
2.6.1.2) activities.

**Flow cytometry analysis**—The tissues were
homogenized and then passed through a 40 µm nylon
cell strainer (Becton Dickinson). The suspension was
centrifuged at 400 g for 10 min at room temperature.
Pellet was resuspended in 4 ml 30% percoll, and
overlaid on the top of a gradient containing 3.5 ml of
70% percoll solution, prepared by dilution in Hank’s
balanced salt solution (HBSS). The gradient was
centrifuged at 500 g for 20 min at room temperature.
Approximately 5 ml cells were collected from the 37
to 70% interface and washed with HBSS containing
10% fetal Bovine serum (FBS).

The cells thus obtained were diluted to 10^6 cells
/100 µl of phosphate buffered saline (PBS; pH 7.4)
containing 0.5% bovine serum albumin (BSA), and
were incubated with 5 µg mouse monoclonal anti-rat
adhesion molecule (VCAM-1 or ICAM-1 or PECAM-
1) for 1 h at 4°C. Then cells were washed with
antibody binding buffer (ABB) containing PBS (pH
7.4), 0.5% BSA and 0.1% sodium azide (NaN3) thrice,
and subsequently were stained with FITC-
conjugated goat anti-mouse IgG secondary antibody
(0.5 mg/ml ABB) at 4°C for 1 h. After washing thrice,
cells were analyzed by flow cytometry (FACScan,
Becton Dickinson Inc.). For each sample 30000 cells
were analyzed (DIVA software, Becton Dickinson
Inc). Results are expressed as the corrected mean
fluorescence intensity (MFI).

**Statistical analysis**—All data were analyzed using
the statistical package SPSS (version 11.0, SPSS
Inc., Chicago, IL) and the results were expressed
as mean ± SD. The sources of variation for
multiple comparisons were assessed by the analysis
of variance (ANOVA), followed by post-hoc
test. The difference were considered significant at
P<0.05.
Results
A time-dependent effect of ethanol on protein content and transaminases activities of rat liver homogenates is presented in Table 1. Protein level decreased significantly after 12th week of ethanol exposure, compared to the control group. Activities of liver specific enzymes such as AST and ALT increased significantly after 4 weeks of ethanol exposure (Table 1).

Time dependent expression of adhesion molecules in liver tissues of ethanol exposed animals were compared with control animals in this study. Flow cytometric analysis revealed that ICAM-1, VCAM-1 and PECAM-1 expression in liver tissues of rats were induced with duration of ethanol exposure (Table 2; Figs 1-3). ICAM-1 and PECAM-1 significantly expressed after 4 weeks of ethanol exposure, whereas VCAM-1 expressed significantly after 12 weeks of ethanol exposure compared to the control group (Table 2).

Discussion
Protein deficiency is often associated with liver disease, due to decreased dietary intake or deficiencies in digestion and absorption24. Decreased protein level and increased activities of liver marker enzymes in response to the chronic ethanol exposure, compared to the control group (Table 1), indicated that these animals suffered from liver damage in a time-dependent manner. Ethanol inhibits the secretion of protein from the liver25.

Chronic ethanol consumption for 6 weeks enhanced gut ischemia-reperfusion (I/R)-induced hepatic microvascular dysfunction and hepatocellular injury in the pericentral region and terminal hepatic venules via an enhanced hepatic expression of ICAM-1 in male Wistar rats26. In the present study liver cells of ethanol-treated rats showed elevated expression of ICAM-1 with duration of exposure (Table 2, Fig. 1). Chronic alcoholics exhibited significantly higher serum levels of endothelial adhesion molecules than abstainers and moderate drinkers in a clinical study16. An upregulation of ICAM-1 and VCAM-1 was also detected in liver biopsies obtained from patients with alcoholic hepatitis and cirrhosis16. Elevation of expression of ~110-kDa transmembrane VCAM-1 was also noted in the liver cells of ethanol-treated rats in a time dependent manner in the present study (Table 2, Fig. 2). Upregulation of ICAM-1 expression may be related with liver fibrogenesis37. The overexpression of ICAM-1 in alcohol-induced liver injury28, most likely involves TNF-α29,30. Expression of VCAM-1 on endothelial cells is also induced by inflammatory cytokines, and in certain pathologic conditions31.

Table 1—Effect of ethanol on protein content and activities of aspartate (AST; EC. 2.6.1.1) and alanine (ALT; EC. 2.6.1.2) transaminase in liver homogenate of rats for different time period

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>4 weeks</th>
<th>12 weeks</th>
<th>24 weeks</th>
<th>36 weeks</th>
<th>F value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/100 mg tissue)</td>
<td>23.3 ± 0.38</td>
<td>21.8 ± 0.38</td>
<td>19.68 ± 0.61</td>
<td>18.65 ± 0.95</td>
<td>18.4 ± 1.06</td>
<td>22.172</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST (U/100 mg tissue)</td>
<td>20.5 ± 1.26</td>
<td>62.5 ± 3.28</td>
<td>108.67 ± 6.5</td>
<td>149 ± 8.12</td>
<td>165.1 ± 8.1</td>
<td>122.778</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALT (U/100 mg tissue)</td>
<td>17.3 ± 0.71</td>
<td>53.3 ± 3.6</td>
<td>99.17 ± 4.92</td>
<td>161.3 ± 7.96</td>
<td>181.3 ± 7.72</td>
<td>149.096</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

P values: a<0.001, b<0.01 compared to control group; c<0.01 compared to 4 weeks ethanol treated group; d<0.001, e<0.01 compared to 12 weeks ethanol treated group.

Table 2—Induction of intercellular adhesion molecule-1(ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) [in percent (%) of total cell population] in liver homogenate of ethanol exposed rats for different time period

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>4 weeks</th>
<th>12 weeks</th>
<th>24 weeks</th>
<th>36 weeks</th>
<th>F value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM</td>
<td>11.4 ± 0.29</td>
<td>13.8 ± 0.64b</td>
<td>17.8 ± 0.53c</td>
<td>19.2 ± 0.75cd</td>
<td>23.8 ± 2.44</td>
<td>95.931</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VCAM</td>
<td>11.1 ± 0.26</td>
<td>11.8 ± 0.49</td>
<td>17.1 ± 0.45ac</td>
<td>18.2 ± 0.72</td>
<td>22.6 ± 1.98</td>
<td>137.199</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PECAM</td>
<td>6.7 ± 0.23</td>
<td>10.5 ± 0.33a</td>
<td>12.1 ± 0.39ad</td>
<td>15.1 ± 0.48</td>
<td>16.38 ± 1.61</td>
<td>140.589</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

P values: a<0.001, b<0.01 compared to control group; c<0.001, d<0.05 compared to 4 weeks ethanol treated group; e<0.01, f<0.01 compared to 12 weeks ethanol treated group.
Fig. 1 – ICAM-1 expression in liver tissues for (a) control group of rats; and (b) 4 weeks, (c) 12 weeks, (d) 24 weeks and (e) 36 weeks of ethanol exposed rats
Fig. 2– VCAM-1 expression in liver tissues for (a) control group of rats; and (b) 4 weeks, (c) 12 weeks, (d) 24 weeks and (e) 36 weeks of ethanol exposed rats.
PECAM-1 is expressed on the surface of EC and leukocytes, and plays an important role in endothelial-leukocyte and endothelial-endothelial cell-cell interactions through the regulation of cell adhesive and migratory mechanisms\textsuperscript{32}. The attachment of PECAM-1 on the cell surface can activate mitogen-activated protein kinase (MAPK) [originally called extracellular signal-regulated kinases (ERKs)] and small GTPases, impacting both cadherin-mediated cell-cell and integrin-mediated cell-matrix interactions\textsuperscript{32}. PECAM-1 may support the effective signaling required for wound healing endothelial migration\textsuperscript{33}. CD31-mediated endothelial cell-cell interactions are involved in angiogenesis\textsuperscript{34}. Elevated PECAM-1 expression in the liver cells of ethanol-treated rats in this study (Table 2, Fig. 3), may participates in maintenance of adherens junction integrity and permeability, organization of the

Fig. 3– PECAM-1 expression in liver tissues for (a) control group of rats; and (b) 4 weeks, (c) 12 weeks, (d) 24 weeks and (e) 36 weeks of ethanol exposed rats
intermediate filament cytoskeleton, regulation of catenin localization and transcriptional activities, and control of apoptotic events.\textsuperscript{35}

In conclusion, adhesion molecules may be associated with the initiation of hepatic injury during alcohol intoxication.

Acknowledgement

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References

8 Ji C, Deng Q & Kaplowitz N, Role of TNF-alpha in ethanol-induced hyperhomocysteinemia and murine alcoholic liver injury, Hepatology, 40 (2004) 442.
18 Das S K & Vasudevan D M, Protective effects of silymarin, a milk thistle (Silybum marianum) derivative on ethanol-induced oxidative stress in liver, Indian J Biochem Biophys, 43 (2006) 306.

