

## Effects of Chronic Ethanol and Vitamin C Administration on Production of Tumor Necrosis Factor- $\alpha$ and Interleukin-6 in Rats

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Chronic alcoholism complicated by alcoholic liver disease (ALD) is characterized by activation of inflammatory responses. Alcohol intake increases gut permeability allowing substances such as lipopolysaccharides (LPS) which are strong inducers of proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) to enter the circulation. Vitamin C is an antioxidant with many cellular activities seemed to protect cells against alcohol-induced peroxidation. In present study, serum levels of TNF- $\alpha$  and IL-6 were measured by ELISA method in four groups of albino rats, each group consists of 10 rats. Group (I) was untreated group (control), group (II) was treated with ethanol, group (III) was treated with ascorbic acid and group (IV) was treated with ethanol + ascorbic acid. Results revealed that both TNF- $\alpha$  and IL-6 serum levels were very highly significantly increased in group (II) and (IV) than control group (I) ( $P < 0.001$ ). Group (III) showed significantly ( $P < 0.001$ ) decreased TNF- $\alpha$  serum level than group (II) and (IV) while it showed significantly ( $P < 0.001$ ) increased IL-6 serum level than control group (I) and also significantly decreased IL-6 serum level than group (IV). Serum IL-6 level was significantly ( $P < 0.01$ ) decreased in group (III) than (II). These results indicate that serum levels of the proinflammatory cytokines TNF- $\alpha$  and IL-6 may serve as predictive biomarkers for progression of ALD. In addition, using TNF- $\alpha$  neutralizing agent (or its antagonist)/or IL-6 as an anti-apoptotic factor could be useful as a treatment strategy of ALD.

Chronic ethanol consumption causes serious alteration of immune system including changes in chemotaxis of granulocytes, lymphocyte response, T-cell number and natural killer cell (NK) activity. In addition, it could be a major factor underlying the increased incidence of certain forms of cancer (Lee et al., 1994). Liver is the most severely affected organ by alcoholism since over 80% of ingested alcohol is metabolized in the liver without feedback mechanism (Zima et al., 2001). Chronic ethanol intoxication has been shown to enhance generation of reactive oxygen species, mainly superoxide radical and hydrogen peroxide (Kukielka et al., 1994 and Lindros, 1995).

Ethanol ( $C_2H_5OH$ ) is a small water soluble molecule that is absorbed rapidly and completely from the gastrointestinal tract. Its vapor can be readily absorbed in the lungs.

Over 90% of alcohol consumed is oxidized in the liver, the rest is excreted through the lungs and in the urine (Lee et al., 1994). Mitochondrial oxidative stress has been accepted to play an important role in the pathogenesis and progression of alcoholic liver injury (Nordmann et al., 1992 and Fernandez-Checa et al., 1993).

Ascorbic acid (Vitamin C) is considered to be the most important antioxidant in extracellular fluids (Stocker et al., 1991 and Sies et al., 1995). It has also many cellular activities of an antioxidant nature as well (Moser et al., 1991). Vitamin C has been shown to efficiently scavenge superoxide hydrogen peroxide, hypochlorite, the hydroxyl radical, peroxy radicals and  $O_2$  (Nishikimi, 1975 and Kwon et al., 1988). Ascorbic acid can also act to protect membranes against peroxidation by enhancing the activity of

tocopherol by maintaining tocopherol in the reduced action form (Etsuo et al., 1995).

Chronic alcoholism complicated by alcoholic liver disease (ALD) is characterized by activation of the inflammatory responses mediated mainly by inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) secreted by different cell types including, liver cells: hepatocytes, endothelial cells, Kupffer cells and stellate cells (Neuman, 2003). Persistent cytokine secretion in the liver resulting in chronic inflammation leads to conditions such as hepatitis, fibrosis, and cirrhosis. Increased cytokine levels and activities correlated with the clinical hepatitis (Andus et al., 1995 and Hill et al., 1992).

TNF- $\alpha$  is a multifunctional pro-inflammatory cytokine that plays a major role in the acute phase responses (Beuter et al., 1987 and Le et al., 1987). TNF- $\alpha$  is called "Cachectin" for its role in inducing tissue wasting in cachectic states (Haslett, 1998 and Beuter et al., 1987). TNF is produced by activated monocytes / macrophages, neutrophils, T and B lymphocytes, mast cells, basophiles, eosinophils, NK cells and some tumor cells (Charon, 1998) TNF- $\alpha$  induction is one of the earliest events in hepatic inflammation, triggering a cascade of other cytokines that co-operate to kill hepatocytes, recruit inflammatory cells and initiate a wound healing response that includes fibrogenesis (Diehl, 2000).

Interleukin-6 (IL-6) is a proinflammatory cytokine that acts on a variety of cells. It is synergizing with IL-1 and TNF- $\alpha$  to co-stimulate T cells; inducing the acute phase response in liver cells and hypothalamic fever center; enhancing B-cell proliferation, differentiation and immunoglobulin production; and supports growth of transformed hepatocytes (Oppenheim et al., 1997). IL-6 is produced by activated T and B lymphocytes, monocytes, endothelial cells, epithelial cells, and fibroblasts. Its expression

is induced by TNF- $\alpha$ , IL-1 and platelet-derived growth factor (Oppenheim et al., 1997). In patients with alcoholic cirrhosis, IL-6 production is significantly increased (Deviere et al., 1989 and Laso et al., 1997). It also has potential hepatic anti-apoptotic role and plays an important part in liver regeneration and repair from injury (Cressman et al., 1996).

IL-6 together with TNF- $\alpha$  were found to be correlated with mortality in alcoholic hepatitis (AH) (Khorus et al., 1991 and Hill et al., 1992). However, the role of both TNF- $\alpha$  and IL-6 during chronic liver injuries and fibrogenesis in alcoholic hepatitis remains to be clarified. Thus, the present study aimed to investigate the effect of administration of alcohol and vitamin C on the production of the pro-inflammatory cytokines; TNF- $\alpha$  and IL-6 in rats.

## Material and Methods

Forty healthy male albino rats weighing about 110-130 gram each, were used in this study. They were acclimatized for it at least one week prior to experiments. During this period, the rats were housed in cages with two animals each at random. Animals were given the regular laboratory standard diet and water ad libitum. Rats were divided into 4 groups each consists of 10 rats. Group I served as control group. Group II rats treated with ethanol (0.3 ml absolute ethanol/ 100g body weight/day), group III rates treated with ascorbic acid (0.2g/100g body weight/day) and group IV rats treated with ethanol and ascorbic acid (0.3 ml absolute ethanol/100g body weight + 0.2 g ascorbic acid/100 g body weight /day). (El-Sokkary et al., 1999 and Suresh et al., 2000).

Preparation of experimental animal (rats): Ascorbic acid (AA) and ethanol were given orally by gastric incubation daily for a period of 30 days. AA was freshly dissolved in distilled water whereas absolute ethanol was diluted with distilled water in the ratio of 1:1 (v/v) so that each rat received 0.6 ml/100 g body weight/day of the diluted ethanol. At the end of treatment, rats were exsanguinated by cardiac puncture under light anaesthesia, then sacrificed by decapitation.

### Methods

The blood samples of all studied groups were left to clot and then centrifuged at 3000 rpm for 10 minutes. Sera

were separated and kept at  $-80^{\circ}\text{C}$  to be used for determination of TNF- $\alpha$  and IL-6 serum levels.

- Determination of TNF- $\alpha$  and IL-6 serum levels

Rat TNF- $\alpha$  (or IL-6) concentration (pg/ml) was determined using a quantitative sandwich-type enzyme immunoassay technique according to manufacturer's instructions (ELISA Kit-Bender Med Systems. Austria).

A monoclonal antibody specific for rat TNF- $\alpha$  (or IL-6) had been coated onto wells of a micro-titer plate (microwell strips), then washed twice with washing buffer. Diluted samples and serial dilutions of TNF- $\alpha$  (or IL-6) standard were pipetted into the microwell strips. Standard concentrations ranged from 39-2500 pg/ml for TNF- $\alpha$  and from 31-2000 pg/ml for IL-6. Biotin-conjugate antibody were added to all wells, the plate was covered and incubated at room temperature ( $18-25^{\circ}\text{C}$ ) for 2 hours on a microplate shaker (set at 200 rpm.). The microwell strips were washed 4 times, then diluted Streptavidin-HRP were added, incubated at room temperature for 1 hour on the microplate shaker at 200 rpm. The microwell strips were washed 4 times and TMB substrate solution was pipetted into wells, then the plate wrapped to avoid exposure to light and incubated for 15

minute. A colour was produced in proportion to the amount of TNF- $\alpha$  (or IL-6) present in the sample. The enzyme reaction was stopped by adding stopping solution (1M phosphoric acid). The absorbance of colour was measured using a spectrophotometer at 450 nm. TNF- $\alpha$  (or IL-6) concentration (pg/ml) of each sample was determined from the standard curve plotted using values of standard concentrations of each cytokine and their corresponding colour absorbance readings.

#### Statistical analysis

Statistical analysis was performed in this study using the SPSS-package (release 3, SPSS Inc. Chicago III) running on MCROVAX 3500. A difference was considered significant at  $P < 0.05$ , highly significant at  $P < 0.01$  and very highly significant at  $P < 0.001$ . The correlation was considered significant when  $P < 0.05$ .

## Results

Results revealed that all groups of rats had a significant increase ( $P < 0.001$ ) in body weight at the end of the experiment than initial weights (Table 1).

Table 1. Statistical analyses of body weight changes (g) in control, ethanol, ascorbic acid and ethanol + ascorbic acid treated groups of rats.

	Control group (n=10)	Ethanol group (n=10)	Ascorbic acid group (n=10)	Ethanol + Ascorbic acid group (n=10)
			Initial weights of rats	
Mean $\pm$ S.E.M.	109.5 $\pm$ 3.20	119 $\pm$ 0.67	110 $\pm$ 2.89	114 $\pm$ 2.67
			Final weights of rats	
Mean $\pm$ S.E.M.	197.6 $\pm$ 10.43	168.4 $\pm$ 8.05	191.5 $\pm$ 8.27	168.9 $\pm$ 8.20
Mean difference	88.1	49.4	81.5	54.9
$\pm$ S.E.M.	11.78	7.59	9.6	8.8
*P<	0.001	0.001	0.001	0.001
	( $\uparrow$ 80%)	( $\uparrow$ 42%)	( $\uparrow$ 74%)	( $\uparrow$ 47%)

P values significantly different from initial weight

\*P< 0.05 was considered significant

%; Changes in body weight from the initial value.

The final weights were significantly lower ( $P < 0.05$ ) in ethanol and ethanol + ascorbic acid treated groups as compared to the

corresponding values in control group (Table 2).

Table 2. Statistical analyses of final body weights (g) in control, ethanol, ascorbic acid and ethanol + ascorbic acid treated groups.

	Control group (n=10)	Ethanol group (n=10)	Ascorbic acid group (n=10)	Ethanol + Ascorbic acid group (n=10)
Final weight (g) Mean $\pm$ S.E.M.	197.6 $\pm$ 10.43	168.4 $\pm$ 8.05	191.5 $\pm$ 8.27	168.9 $\pm$ 8.20
*P		0.024 (14.8% $\downarrow$ )	0.627	0.027 (14.5% $\downarrow$ )
P1			0.071	0.968
P2				0.078

P: Values as compared with control group.

P1: Values as compared with ethanol group.

P2: Values as compared with ascorbic acid group.

\*P < 0.05 was considered significant.

%; Changes of body weight as compared to control.

Table (3) represents range, mean values  $\pm$  S.E.M. of TNF- $\alpha$  serum levels and the statistical analysis of these results. The serum TNF- $\alpha$  level was very highly significantly increased (P < 0.001) in both groups of rats treated with ethanol (61.30 $\pm$ 3.81) and ethanol + ascorbic acid (53.65 $\pm$ 3.38) treated groups as compared to its corresponding value (36.15 $\pm$ 2.68) in control group. The serum TNF- $\alpha$  level did not show any significant

difference between the ascorbic acid (34.90 $\pm$ 2.42) treated group and the controls.

The serum TNF- $\alpha$  level was significantly decreased (P < 0.001) in the ascorbic acid treated group as compared to that of ethanol treated group. The TNF- $\alpha$  serum level in ethanol + ascorbic acid treated group was significantly increased (P < 0.001) than that of ascorbic acid treated group while it did not show any significant difference when compared to that of ethanol treated one.

Table 3. Statistical analyses of serum TNF- $\alpha$  (pg/ml) in control, ethanol, ascorbic acid and ethanol + ascorbic acid treated groups.

	Control group (n=10)	Ethanol group (n=10)	Ascorbic acid group (n=10)	Ethanol + Ascorbic acid group (n=10)
Range	23.6-49.7	43.6-76.8	24.6-45.4	32.5-70.6
Mean	36.15	61.30	34.90	53.65
$\pm$ S.E.M.	2.68	3.81	2.42	3.38
*P		0.001	N.S	0.001
*P1			0.001	N.S
*P2				0.001

P: Values as compared with control group.

P1: Values as compared with ethanol group.

P2: Values as compared with ascorbic acid group.

\*P < 0.05 was considered significant

Table (4) shows the range, mean values  $\pm$  S.E.M. of IL-6 serum levels and the statistical analyses of these results. The serum IL-6 level was significantly increased ( $P < 0.001$ ) in ethanol, ascorbic acid and ethanol + ascorbic acid treated groups than that of controls. It was significantly decreased ( $P < 0.01$ ) in the ascorbic acid treated group than ethanol

treated one. The IL-6 serum level did not show any significant difference between ethanol + ascorbic acid treated group and ethanol treated one. The serum IL-6 level was significantly increased ( $P < 0.001$ ) in the ethanol + ascorbic acid treated group than ascorbic acid treated one.

Table 4. Statistical analyses of serum IL-6 (pg/ml) in control, ethanol, ascorbic acid and ethanol + ascorbic acid treated groups.

	Control group (n=10)	Ethanol group (n=10)	Ascorbic acid group (n=10)	Ethanol + Ascorbic acid group (n=10)
Range	18.17-39.83	41.50-79.81	34.83-59.90	49.82-83.18
Mean	26.50	60.66	47.43	65.25
$\pm$ S.E.M.	1.79	4.07	2.75	3.49
*P		0.000	0.001	0.001
P1			0.01	N.S
P2				0.001

P: Values as compared with control group.

P1: Values as compared with ethanol group.

P2: Values as compared with ascorbic acid group.

\* $P < 0.05$  was considered significant.

## Discussion

Alcoholic liver disease is a major cause of alcohol-related morbidity and mortality (Maun et al., 2003). Alcoholic hepatitis is associated with severe alterations of immunoinflammatory response characterized by imbalanced ratio of Th1/Th2 cytokine production and infiltration of both neutrophils and T lymphocytes (Tsukamoto et al., 2001). Alcohol intake increases gut permeability allowing substances such as lipopolysaccharides (LPS), which are a strong inducers of cytokines such as IL-1, TNF- $\alpha$  and IL-6 to enter the circulation (Bjarnason et al., 1989 and Bode et al., 1987). Ethanol also impairs the function of the hepatic reticuloendothelial system, which may result in increased plasma endotoxin concentration (Bode et al., 1987). This may explain the elevated serum levels of both TNF- $\alpha$  and IL-6

in both ethanol and ethanol + ascorbic acid treated groups than that of control in present study. This result agreed with the findings that TNF- $\alpha$  and IL-6 serum levels are correlated with disease severity in alcoholic hepatitis (Hill et al., 1992 and Bird et al., 1990). The elevated levels of cytokines and chemokines during chronic alcoholic consumption are likely to contribute to the pathogenesis of alcoholic liver disease in humans (McClain et al., 1986 and Hill et al., 1994) and animal models (Bautista, 1995 and Bautista, 1997). This result is also in accordance with that of Lancaster (1995) who demonstrated that pro-inflammatory cytokines including TNF- $\alpha$  and IL-6 increase as a result of alcohol induced cellular damage and this causes an increase in nitric oxide (NO) production by stimulating inducible NOS (iNOS). Hink et al. (2001) have claimed that H<sub>2</sub>O<sub>2</sub> activity stimulates

iNOS expression. In this case, Uzun and his associates (2005) assumed that the increase in NO concentration and the contribution of NO to cellular damage could be secondary. This result is also in agreement with the finding of Dai et al. (2003) who demonstrated that in ethanol-fed rats, the level of TNF- $\alpha$  was significantly higher than that in control group (Dai et al., 2003). Similar results were obtained in mice (Hong et al. 2002) and in human studies (Daniluk et al., 2000 and Tilg et al., 2003). In this aspect, TNF- $\alpha$  as a pro-inflammatory cytokine is believed to exert vascular effects by increasing vascular permeability and causing vasodilatation thought to be mediated through nitric oxide (NO) dependant pathways (Moncada et al., 1993). TNF- $\alpha$  has been reported to be elevated in alcoholic liver diseases, in particular, alcoholic hepatitis (AH) (McClain et al., 1999). Our results also may denote the causative role of TNF- $\alpha$  in the pathogenesis of alcoholic hepatitis which is supported by animal model studies (McClain, 1991, McClain et al., 1993 and Arnon et al., 1995). This role has been investigated by McClain et al. (1989) and Mathurin et al. (2000). They suggested that, in the liver, endotoxin interacts primarily with Kupffer cells leading to secretion of TNF- $\alpha$  which interacts with receptors on both Kupffer cells and hepatocytes, and production of other inflammatory cytokines such as IL-1, IL-6, and IL-8 which are correlated with the clinical course of AH. This chain of events is important for the development and progression of alcoholic liver disease. In addition, in ethanol fed rats, increased TNF- $\alpha$  production by CD<sub>4</sub><sup>+</sup> cells enhances liver injury and promotes hepatocyte apoptosis (Czaja et al., 1995; Pastorino et al., 2000 and Neuman 2003). TNF- $\alpha$  was found to be responsible for the observed clinical aspects of AH such as anorexia, fever, wasting, hypoalbuminemia and neutrophilia (Bazzoni et al., 1996).

In the present study, the elevated serum levels of TNF- $\alpha$  (as a cachectin and apoptotic inducing factor) in ethanol and ethanol + ascorbic acid treated group of rats seemed to be the mechanism by which ethanol administration caused lower percentage of weight gain and the lower net weight of these two groups. Baniluk et al. (2001) and Hanck et al. (2000) suggested that TNF- $\alpha$  is a reliable diagnostic parameter for evaluating the severity of liver damage and qualification of patients for liver transplantation. (Mc Clain et al., 1999) Moreover, gene expression of TNF- $\alpha$  in peripheral blood mononuclear cells (PBMCs) in patients with alcoholic cirrhosis is stage-dependent and increases with severity of cirrhosis (Hanck et al., 2000).

Our results are also in accordance with the findings of Ono et al. (2004) who found that IL-6 level was increased in chronically ethanol fed rats compared to the controls. Similar results were also obtained in murine (Shea-Donohue et al., 2001 and Natsume et al., 1999) and humans (McClain et al. 2004). IL-6 concentration correlated with hyper gamma globulinemia in patients with decompensated cirrhosis (Daniluk et al., 2001). Petero et al. (2000) suggested that IL-6 might be involved in fibrotic changes, partly by modulating intrahepatic expression of other cytokines such as IL-1.

The significantly increased serum level of IL-6 in both ascorbic acid and ethanol + ascorbic acid treated groups of rats in the present study suggested that the protective role of ascorbic acid may be performed by increasing the production of IL-6 which is able to establish and maintain an adequate level of FLICE-inhibitory protein (FLTP) and downstream anti-apoptotic factors (Kovalovich et al., 2001).

It has been established that IL-6 has an important role in liver regeneration and repair from injury (Cressman et al., 1996). These facts provide evidence for the important role of IL-6 in reducing chronic liver injury and

fibrosis (Hink et al., 2001). The increased level of IL-6 caused by ascorbic acid is considered to be one of the protective actions of ascorbic acid. Ascorbic acid is a non-enzyme antioxidant. Antioxidants are the main defense mechanisms evolved in the body against the damaging effects of free radicals (Cheesman et al., 1993).

The pathogenesis of alcohol induced liver disease seemed to involve oxidative tissue injury (Niemela et al., 1995) as well as imbalance of oxidant/antioxidant towards auto-oxidation either by acting as a pro-oxidant or by reducing the antioxidant level or by both mechanisms (Diluzio et al., 1967).

In conclusion, the higher serum levels of TNF- $\alpha$  and IL-6 in ethanol treated groups of rats and their lower levels in ascorbic acid treated group in present study indicated that these two pro-inflammatory cytokines could be used as predictive markers for alcoholic liver disease. The use of IL-6 as a protective / anti-apoptotic factor or TNF- $\alpha$  antagonist could be helpful for treatment strategy that needs further investigations.

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