Vasoprotective effect of vitamin E: Rescue of ethanol-induced atherosclerosis and inflammatory stress in rat vascular wall

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ABSTRACT

Chronic ethanol consumption increases the incidence of cardiovascular disease. The mechanisms underlying ethanol-induced susceptibility to cardiovascular disease continue to be defined. This study examines the hypothesis that chronic ethanol consumption plausibly induces vascular wall abnormalities via inflammatory reactions. In addition, it intends to find out whether vitamin E inhibits the abnormalities induced by ethanol in rats' vascular wall. Twenty four male Wistar rats were divided into three groups (n = 8): Control ©, ethanol (E), and vitamin E treated ethanol (VETE) group. After 6 weeks, the aortic and coronary wall changes, vascular endothelial growth factor (VEGF), alpha-1 glycoprotein and haptoglobin amounts in plasma, C-reactive protein levels(CRP), as well as the amount of aortic IL-6 were evaluated. The results revealed the elevation of polymorphonuclear (PMN) leukocyte in the vascular wall, disorganization of endothelium with ballooning of cells, proliferation of vasa-vasorum with an increase in the IL-6, CRP, as well as a decrease in VEGF and an increase in alpha-1 glycoprotein and haptoglobin in the ethanol group compared to the control group. Significant amelioration of aortic and coronary wall changes, along with the restoration of elevated level of IL6, CRP, and the decreased level of VEGF compared to that of the controls were found in vitamin E-treated animals. These findings strongly support the idea that heavy and chronic ethanol consumption initiates atherosclerosis by inflammatory stress, and that these effects can be alleviated by vitamin E as an anti-inflammatory agent.

1. Introduction

Alcohol consumption and problems engendered by it have been known in human societies since the beginning of the recorded history. Although moderate alcohol consumption has been reported to achieve a protective effect in the case of specific diseases, current social patterns of alcohol consumption have imposed a great burden on global public health; as reported in UK, US and Switzerland [1,2]. Chronic alcohol consumption is now recognized as a predisposing factor that increases the incidence of cardiovascular disease through mechanisms that continue to be defined [3]. Recent studies on the rat model of chronic ethanol ingestion demonstrated the ability of ethanol to enhance susceptibility to experimental cardiovascular injury by causing a variety of defects such as inflammation in vascular walls, dysfunction in endothelial cells, and rise in blood pressure [3,4]. Although the precise cellular and biochemical causes of ethanol-induced susceptibility to cardiovascular injury have

not yet been defined, considerable evidence indicates that ethanol-induced oxidative and inflammatory stress play a critical role in ethanol susceptibility to cardiovascular injury, and many ethanol-induced adverse effects can be prevented or attenuated by antioxidants [5].

The relationship between ethanol consumption and inflammatory reactions is biphasic and complex. It has been reported that in most cases, acute ethanol administration prevents inflammatory responses associated with resistance to infection [6,7]. Contradictorily, it has also been found that long-term ethanol consumption is associated with elevated circulating levels of the proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin(IL)-1 and IL-6 [8,9]. We have recently demonstrated that long term pure ethanol administration once a day for six weeks, induces the development of pro-inflammatory and pro-atherosclerotic processes; as shown by an increase in blood pressure, serum levels of C-reactive protein (CRP), intercellular adhesion molecule-1, endothelial leukocyte adhesion molecule-1 (E-selectin) and the appearance of infiltrated monocytes in the aorta, as well as foam-cell formation in early plaques, suggesting a potential implication for ethanol itself to possibly induce atherosclerosis via inflammation processes [4]. Many experimental studies have provided strong evidence supporting that inflammation is a key regulatory process linked to several risk

factors for atherosclerosis and its complications along altered arterial biology [10–12]. Inflammatory nature of atherosclerosis on the one hand and long-term ethanol ingestion-induced inflammatory reactions on the other hand, tempted us to re-examine the hypothesis indicating that chronic ethanol consumption plays an important role in the development of atherosclerosis and its consequential adverse effect on vascular system via inflammation. In addition, the possible ameliorative effect of vitamin E on ethanol-induced histopathological alterations and inflammatory responses were investigated.

2. Materials and methods

All experiments and procedures were performed in accordance with the Principles of Laboratory Animal Care (NIH publication no.85-23, revised 1985) as well as the specific rules provided by the Animal Care and Use Committee and National Medical and Health Service. Twenty-four male Wistar rats (6 months old, 240 ± 10 g) were assigned to three equal sized groups, that is, a control (C), ethanol (Etoh), and vitamin E-treated (VETE) group. Ethanol-treated rats received 4.5 g/kg BW ethanol (Merck KGaA, Darmstadt, Germany) solution in normal saline (20%w/v) intragastrically by gavage once a day for 6 weeks. According to preliminary studies, rats in the VETE group received a non-toxic dose of 300 mg of vitamin E (Merck GmbH, Germany) intragastrically by gavage in addition to their regular daily diet and same ethanol. The control group was treated with vehicle only (normal saline). Food and water were supplied ad libitum to all groups throughout the experiment. After 6 weeks, all rats were anesthetized using ethyl carbamate (urethane) (1 g/kg), intraperitoneally, and anesthesia was assessed through pinching a hind paw. After weighing, the thoracic cavity was opened and blood sample was collected directly from the heart by syringe, then it was mixed with ethylene diamine tetra acetic acid (EDTA), as an anticoagulant and was then centrifuged at 4000 $\times g$ for 20 min within 30 min of collection. The plasma was then separated and stored at -80 °C without repeated freeze–thaw cycles. After blood sampling, abdomen of each animal was opened and the aorta and heart were dissected from the root to the abdominal descending part. Adventitial tissues and fat were removed from the excised tissues. Next, they were freed from blood clots, weighed, and then the tissues were fixed in buffered formalin and were embedded in paraffin after taking standard dehydration steps.

Some sections of the aorta were washed with ice-cold physiological saline solution, then dried and processed for biochemical measurements. Using Ultra Turrax (T10B, IKA, Germany) homogenizer in an ice-cold extraction buffer (10% wt/vol) containing a 50 mM phosphate buffer (pH 7.4), the tissues were homogenized and then centrifuged at 10,000 $\times g$ for 20 min at 4 °C in a refrigerated centrifuge. The supernatant sample was collected and stored at -80 °C until the time of analysis, without a repeated freeze–thaw cycle.

2.1. Biochemical assay

2.1.1. C-reactive protein

CRP is a member of the pentaxins which is synthesized in the liver and its normal serum concentration is very low. Its concentration increases rapidly following inflammation, so it is probably the most useful and reliable indicator of the acute-phase response. Serum level of CRP was measured by nephelometric methods using MININEPH™ according to the protocol provided by the manufacturer (ZK044.L.R, The Binding Site Ltd, Birmingham, U.K.). The approximate measuring range was 3.51–12 mg/l at a sample dilution of 1/40. Furthermore, the sensitivity limit was 0.44 mg/l when using a 1/5 sample dilution.

2.1.2. IL-6

The concentration of rats' aortic IL-6 was measured by a standard sandwich enzyme-linked immunosorbent assay kit (EK0412, Boster Biological Technology Ltd, USA). All steps were taken as they were described in the protocol provided by the manufacturer. The optical densities were read at 450 nm and the concentration of the samples was calculated from the standard curve as pg/ml IL-6. The minimum detectable dose (lower limit of detection) and the detection level of rats IL-6 were typically less than 5 pg/ml and 62.5–4000 pg/ml, respectively.

2.1.3. Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) or vasculotropin, is a homodimeric 34–42 kDa, heparin-binding glycoprotein with potent angiogenic, mitogenic and vascular permeability-enhancing activities specific to endothelial cells. Rat vascular endothelial cell growth factor ELISA kit (csb-e04757r, CUSABIO, China) was used to measure VEGF concentration in the homogenates. It is a sandwich enzyme immunoassay for the in vitro quantitative measurement of rat VEGF with detection range of 1.56–100 pg/ml. The minimum detectable dose (lower limit of detection) of rat VEGF was typically less than 0.39 pg/ml. All steps were followed according to the protocol provided by the manufacturer and the plate was read at 450 nm. Standard curve and concentration of VEGF in samples were calculated by the software provided by the manufacturer (Curve Expert1.3).

2.2. Capillary zone electrophoresis (CZE)

Plasma proteins were analyzed with capillary zone electrophoresis (Capillary, Sebia, France) making use of a high resolution buffer kit (Sebia, Cedex, France). In evaluation of the formal serum protein electrophoresis, it was found that it is reproducible, rapid, and reliable serum electrophoresis in clinical chemistry which makes it possible to fracture and diagnose proteins [13]. Rat serum proteins were separated based on their size to charge ratio in the interior of a small capillary filled with an electrolyte, and each fraction was directly documented and quantified using corresponding absorbance at 200 nm. Electrophoretic fractions were identified and named by taking advantage of standard patterns of serum protein electropherogram. Fractions of haptoglobin and alpha-1acid glycoprotein were regarded as acute phase proteins, α-1 antitrypsin and α-2 macroglobulin as protease inhibitors, and β-1 globulin as an elastase inhibitor.

2.3. Histopathologicalogical examinations

5 μm-thick sections of formalin fixed paraffin embedded (FFPE) aorta and heart tissues were used for histopathological staining. The general structure of the vascular wall and infiltration of polymorphonuclear leukocyte were examined using Harris' Hematoxylin and Eosin (H&E) Staining Protocols. To determine the glycogen content of aorta and heart tissues, the slides were subjected to the periodic acid-Schiff (PAS) stain procedure, according to the kit instructions.

2.4. PCNA staining

Tissue sections (4 μm thick) from the formalin-fixed paraffin-embedded heart were deparaffinized by first immersing them in xylene, then rehydrating them by gradual ethanol passage, and finally washing them in Tris buffer. Monoclonal anti-PCNA antibody (Dako Denmark A/S, Denmark) was used to stain the slides after the appropriate Ag retrieval step. Optimal results were achieved with the EnVision™ visualization system (Dako Denmark A/S, Denmark). Hematoxylin was used as a counterstain. Appropriate negative controls were included in the assessment. Moreover, all slides were independently inspected by two expert pathologists.

PCNA-positive indices were considered as indicators of muscle cell proliferation.

Scoring was performed in the following fashion.

To assess the percentage of PCNA-positive indices, all cells contained in each cross section of coronary artery were scored. The criteria for quality scoring of PCNA-positive indices were as follows: normal, PCNA-positive indices present in less than 5% of muscle cells; mild, PCNA-positive indices present in less than 25% of muscle cells; mild to moderate, PCNA-positive indices present in 25% to 50% of muscle cells; moderate to severe, PCNA-positive indices present in 50% to 75% of muscle cells; severe, PCNA-positive indices present in 75% to 100% of muscle cells. The sections were inspected under light microscope and photomicrographs were taken.

2.5. Statistical analysis

Data are expressed as means ± SEM. The statistical evaluation was performed through analysis of variance and performing Student's t test. A p value of 0.05 or less was considered as the level of significance.

3. Results

Chronic ethanol ingestion increased inflammatory proteins in plasma and aorta tissues.

We have previously reported that chronic ethanol ingestion in rats leads to inflammatory stress in the aorta characterized by elevations of adhesion molecules and foam cell formation in aortic walls [4]. To further characterize potential mechanisms of inflammatory stress in the vascular wall by chronic ethanol ingestion, acute phase proteins were measured in the current study. As shown in Table 1, chronic ethanol ingestion significantly increased IL-6 production in rat aorta compared to the control group (p < 0.005), an effect attenuated by vitamin E treatment. The CRP levels in plasma obtained from the ethanol group were significantly higher than that in the control group (p < 0.05); however, these levels diminished significantly in VETE group compared to the ethanol group (p < 0.05), (Table 1). Alpha-1 glycoprotein and haptoglobin levels were both significantly higher in the ethanol treated animals when compared with those in the control group (p < 0.05). Vitamin E treatment along ethanol, alleviated alpha-1 glycoprotein and haptoglobin levels compared to ethanol group, but those levels were still significantly higher than that in the control group (p < 0.05) (Table 1 and Fig. 1). Although it was not one of the goals of the current study to measure the amount of these proteins, it is worth noting that no significant difference was observed among different groups in terms of α-1 antitrypsin and α-2 macroglobulin as protease inhibitors, and β-1 globulin as an elastase inhibitor level (Table 1, Fig. 1).

3.1. Vascular structure

Histologic sections stained with hematoxylin and eosin (H&E) showed differences between control, ethanol, and VETE vessel structure

Table 1
Effect of vitamin E on ethanol-induced changes in plasma serine protease levels, IL-6, CRP, and VEGE.

	Control	Ethanol	Ethanol + Vit E
IL-6	190 ± 5.4	394 ± 12.4*	185 ± 12.49
CRP (mg/dl)	2.05 ± 0.2	5.71 ± 0.37*	3.52 ± 0.2**
VEGF	4.85 ± 0.17	2.61 ± 0.14*	6.12 ± 0.32**
Alph-1 antitrypsin (g/dl)	1.2 ± 0.03	1.1 ± 0.05	1.2 ± 0.03
Beta-1 (g/dl)	0.3 ± 0.03	0.36 ± 0.02	0.3 ± 0.02
Alpha 1 glycoprotein acid (g/dl)	0.044 ± 0.001	0.054 ± 0.002*	0.051 ± 0.001
Beta-2 (g/dl)	1.8 ± 0.11	1.7 ± 0.05	1.3 ± 0.05**
Alpha 2 macroglobulin (g/dl)	0.3 ± 0.02	0.31 ± 0.03	0.23 ± 0.02
Albumin (g/dl)	2 ± 0.05	2.2 ± 0.07*	2.5 ± 0.05**
Haptoglobin (g/dl)	0.19 ± 0.006	0.3 ± 0.03*	0.27 ± 0.009**

Data are expressed as means ± SEM.
* p < 0.05 compared to the respective control values.
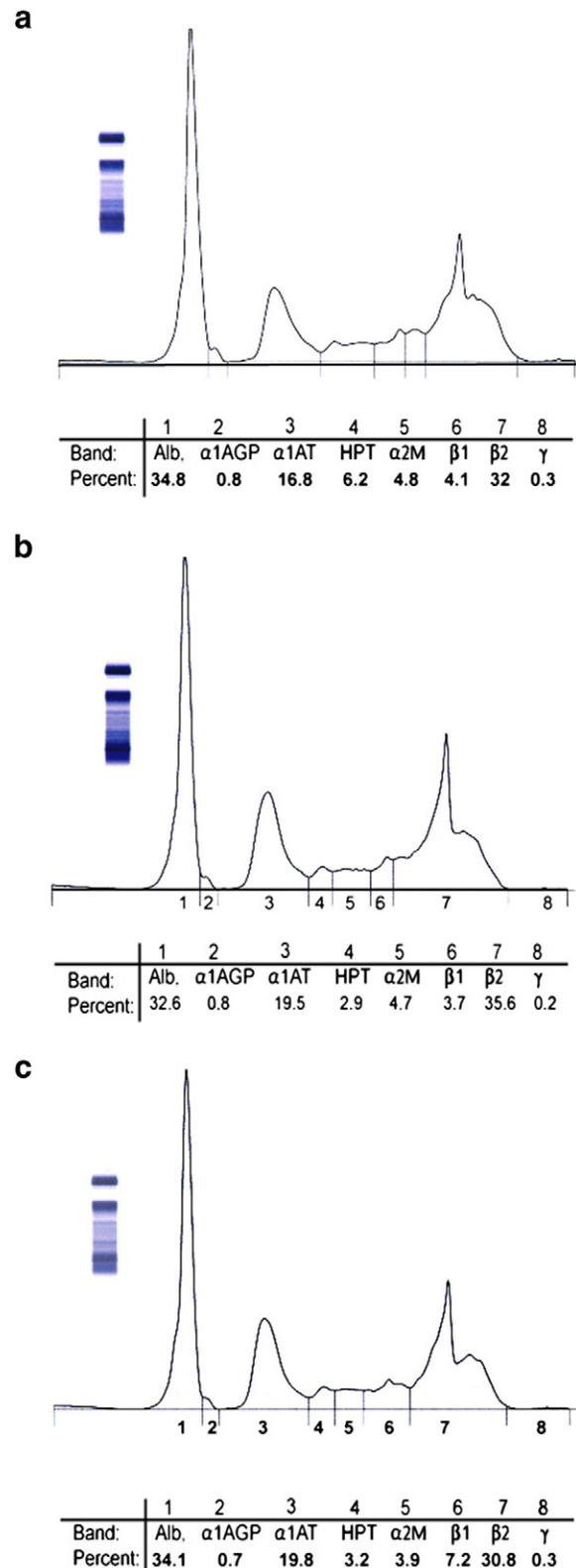** p < 0.05 compared to the respective value of control and ethanol + vitamin E groups.

Band:	1 Alb.	2 α1AGP	3 α1AT	4 HPT	5 α2M	6 β1	7 β2	8 γ
Percent:	34.8	0.8	16.8	6.2	4.8	4.1	32	0.3

Band:	1 Alb.	2 α1AGP	3 α1AT	4 HPT	5 α2M	6 β1	7 β2	8 γ
Percent:	32.6	0.8	19.5	2.9	4.7	3.7	35.6	0.2

Band:	1 Alb.	2 α1AGP	3 α1AT	4 HPT	5 α2M	6 β1	7 β2	8 γ
Percent:	34.1	0.7	19.8	3.2	3.9	7.2	30.8	0.3

Fig. 1. Capillary zone electropherogram of plasma proteins in control (a), ethanol (b), and vitamin E treated ethanolic groups. In comparison to control group, significant increase in alpha 1 glycoprotein and haptoglobin as an acute phase inflammatory proteins were found in ethanol group (p < 0.05). Vitamin E treatment along ethanol alleviated alpha-1 glycoprotein and haptoglobin levels compared ethanol group, but those levels are still significantly higher than control group (p < 0.05). Alb = albumin, α1AGP = alpha-1acid glycoprotein, α1AT = alpha-1 antitrypsin, HPT = haptoglobin, α2M = alpha-2 macroglobulin, β1 = beta-1, β2 = beta-2, γ = gamma.
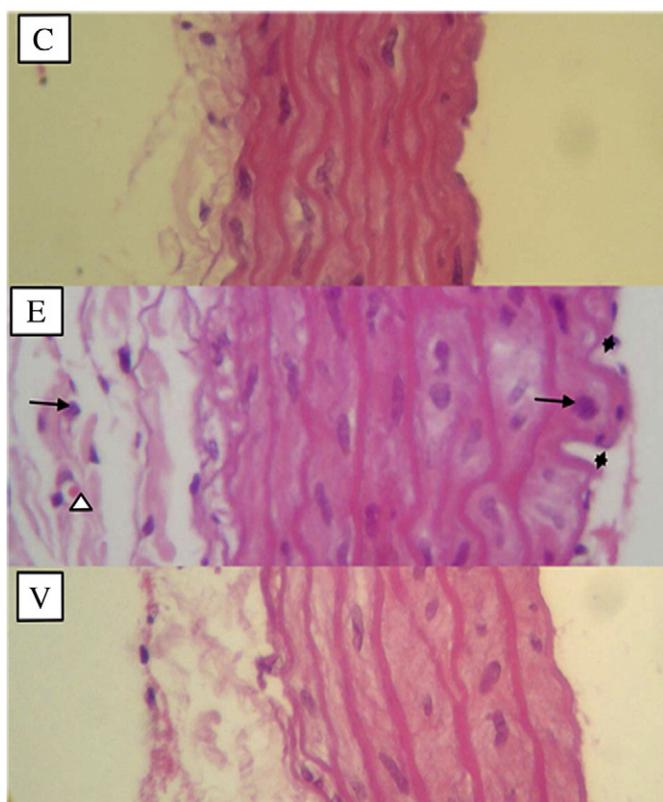
Fig. 2. Histopathology of aorta tissue in control, ethanol rats, and ethanol + vitamin rats (hematoxylin–eosin, original magnification ×400). (C) Control aorta tissue with normal vascular cell. (E) Increased aorta wall thickness, PMN accumulation along activated and disorganized arrangement cells of endothelial layer in ethanol group. (V) Vitamin treatment along ethanol alleviated vascular structural changes induced by ethanol. (✦) endothelial disarrangement, (➔) PMN, (△) glycogen droplet.

Fig. 4. Severe vasa-vasorum proliferation along infiltrated PMN and mononuclear lymphocytes in aorta wall of ethanol group (magnification ×400).

Furthermore, in ethanol animals, coronary endothelial cells showed activated histology as semidisorganized arrangement and ballooned nuclei. There was no significant difference in terms of vessel tissue structure between the VETE and control group.

Severe vasa-vasorum proliferation along infiltrated PMN and mononuclear lymphocytes was also present in aortic walls of the ethanol group. The thickness of the aortic media wall was statistically greater in the ethanol rats compared to the control animals ($p < 0.005$), while it remained unchanged in the VETE rats compared to the control rats.

3.2. Vascular smooth muscle cell proliferation

The ratio of proliferated cells (PCNA-positive indices) in the coronary of the control, ethanol, and VETE rats is shown in Fig. 5. The PCNA-positive indices (as indicators of proliferation) were increased (30%, mild to moderate) in the ethanol-treated group compared to the indices in the control group. The ratio of proliferated cells (PCNA-positive indices) in the coronary of the control and VETE groups was under 5% and was considered as having normal status.

in the form of endothelium disturbance presented as endothelial cell prominency, focal PMN infiltration, as well as vasa vasorum proliferation (Figs. 2, 3 and 4). In ethanol treated animals, focal PMN infiltration was observed in all parts of the aorta and coronary artery in comparison to the controls. In addition, prominent PMN marginalization was seen in coronary vessels of the ethanol group compared to the control group.
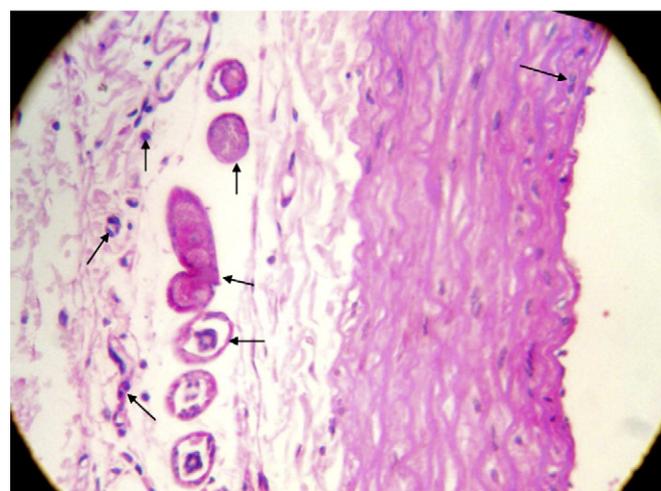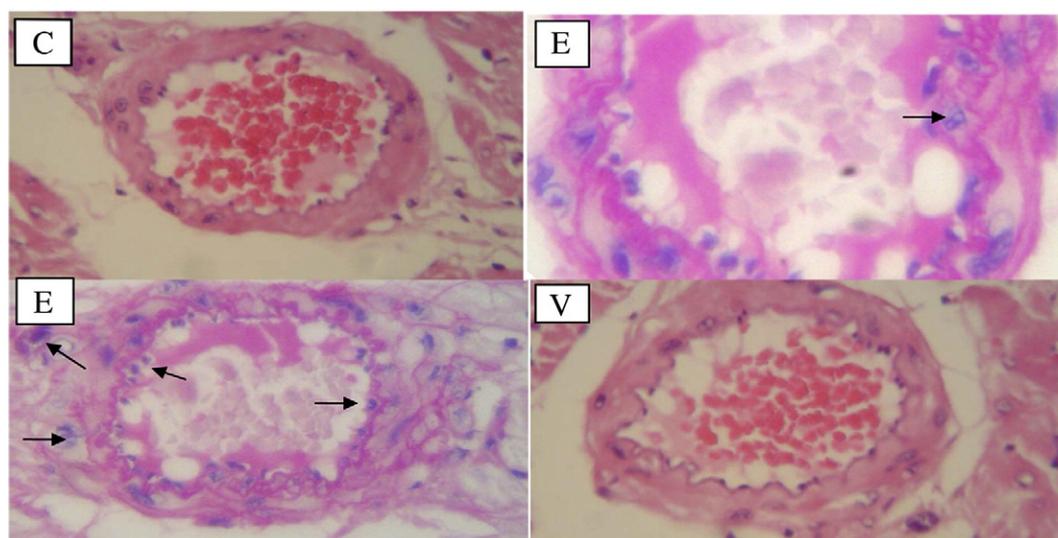

Fig. 3. Morphological changes in the coronary of different group (magnification ×400).Arrows indicated PMN accumulation, marginalization and migration in coronary wall of samples from ethanol group.
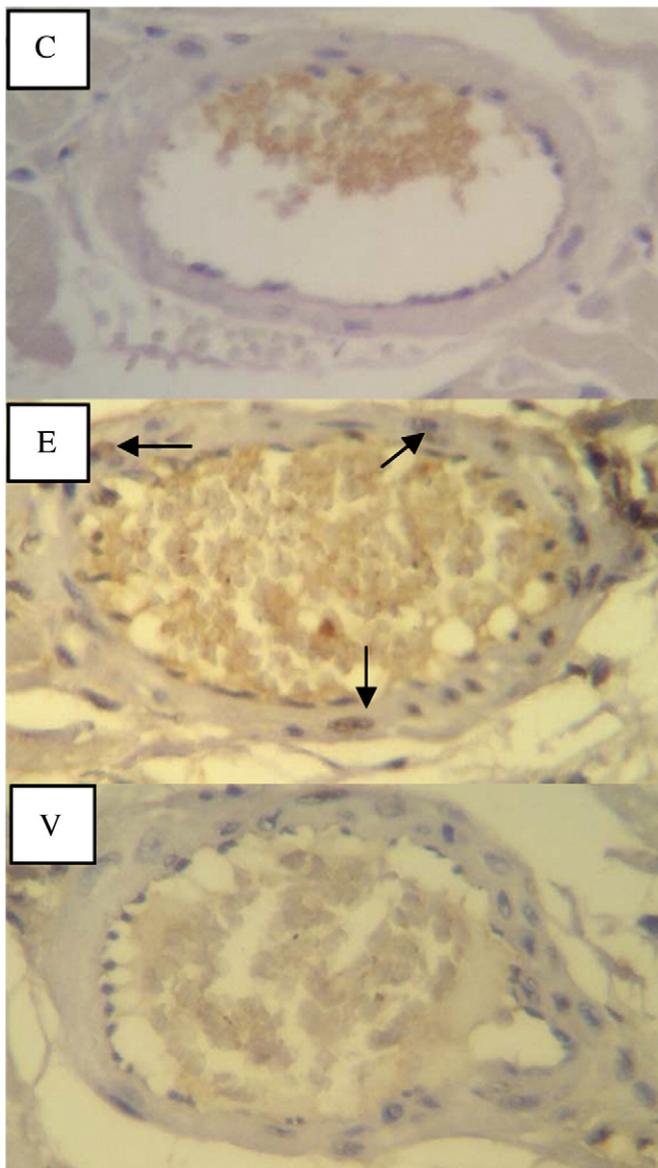
Fig. 5. Result of immunohistochemical staining in different experimental groups with PCNA positive cell expression in coronary wall (magnification ×400). Arrows indicated PCNA positive index or proliferated nucleus from ethanol group coronary sample. C: control, E: ethanol, V: vitamin E treated along ethanol group.

4. Discussion

After the consumption of ethanol, an increase in the levels of different inflammatory markers such as IL-6, CRP, alpha 1 glycoprotein and haptoglobin with a parallel decrease in VEGF was observed. Histopathologic changes such as endothelium disturbance, PMN accumulation, cell enlargement, as well as vasa-vasorum proliferation in the aorta, and mild to moderate coronary smooth muscle cell proliferation were also found. All these factors favor atherosclerosis. Significant amelioration of vascular structure changes, as well as restoration of inflammatory marker levels were found in vitamin E-treated rats as opposed to the control rats. In addition, the amount of VEGF was significantly increased in the vitamin E-treated group to meet that of the control and ethanol groups. It has long been documented that immune-competent cells are involved in every step in the atherosclerosis. The first considerable vascular wall lesion consisted of macrophage-derived foam cells containing lipid droplets. In the later stages of atherosclerosis the monocytes/macrophages and activated T-lymphocytes as well as eosinophils are accumulated in the atherosclerotic lesions [14,15].

In the current study, as assessed by light microscopy, it was found that rats in the ethanol group have gone through alteration in the structure of their coronary and aortic walls including an increase in the thickness of their vessel walls, an elevated number of PMNs with marginalization and migration in all their aortic and coronary layers, an increase in their coronary VSMS proliferation, endothelial activation, and cell arrangement disorganization. Adhesion of monocytes to the endothelium is the earliest event in vascular inflammation in atherogenesis, which is followed by the infiltration and differentiation of monocytes into macrophages. This key phase is mediated by an interaction between monocytes and the molecules expressed on the surface of endothelial cells [4,16]. A recent publication of our group pointed out that chronic ethanol consumption increased cell adhesion molecules (CAMs) in aorta of rats [4].

CAMs are essential in initiation of atherosclerosis because they facilitate the immigration of leukocytes into vessel walls [17]. Infiltrated leukocytes and their resulted PMN development are known to play a significant role in inflammation due to their ability in carrying out a series of effector mechanisms of innate immunity [18]. PMNs mobilize cytoplasmic granules and secretory vesicles which contain a variety of membrane-bound receptors for endothelial adhesion molecules; extracellular matrix proteins; and soluble mediators such as protease, different interleukins, neutrophil elastase, and ROS [19,20]. Signals from PMNs cause the release of secretory organelle contents and secreted effectors arouse inflammatory responses [19]. In addition, PMNs generate lipid mediator of leukotriene B4, a substance that not only stimulates the generation of ROS, but also constitutes a chemotactic factor for neutrophils and other leukocytes, increases endothelial adhesiveness, and augments vascular permeability by promoting the release of HBP/CAP37/azurocidin [21]. Moreover, PMNs induce production of several cytokines that have a proinflammatory role in promoting systemic inflammatory responses and recruiting inflammatory cells locally [22,23]. Accordingly, infiltration of leukocytes into vascular tissues may predispose development of atherosclerosis via contribution of inflammatory responses in vascular walls. A recent study by Zernecke et al. demonstrated the functional role of PMNs in the formation of murine atherosclerotic lesions [24].

The results of our study showed mild to moderate VSMCs proliferation in coronary of the rats in the ethanol group. The hallmark in the progression of atherosclerosis is the proliferation and migration of VSMC into the vascular damage area; therefore, contributing to the thickening of the vascular wall. Although VSMC proliferation and migration have been identified to be of potential significance in the pathogenesis of atherosclerosis [25], the mechanism underlying cell proliferation in the coronary wall of ethanol group is not fully known. Previous studies suggested that during atherosclerosis, VSMC locomotion and growth are preceded and followed by inflammatory responses [26,27]. Here we have shown that concentration of CRP, IL-6, haptoglobin and alpha 1 glycoprotein is significantly higher in the ethanol group compared to the control group. Clinical studies have identified inflammation markers of CRP and IL-6 as well known cardiac risk factors [28,29]. These proteins are found to be associated with an increased risk of myocardial infarction and stroke. In addition, IL-6 has been identified as an independent risk factor for coronary artery diseases [28,30]. The association between alcohol consumption and the inflammatory markers in one hand and between alcohol consumption and cardiovascular diseases on other hand is complex. Results from a number of studies have demonstrated that consumption of a moderate amount of ethanol suppresses inflammatory responses and reduces incidences of atherosclerosis [31–33]. The favorable effect of moderate ethanol consumption may result from its effect on acute phase proteins which are among the most stable and predictive inflammatory markers concerning cardiovascular diseases [34]. Epidemiological studies have suggested that moderate ethanol intake can have a lowering effect on the CRP [31,32]. Results from a number of previous studies and data presented in the current study indicate that heavy ethanol consumption

increases inflammatory cytokines and plays a key role in the development of atherosclerosis and its clinical manifestations [8,9]. Our results showed a significant increase in the acute phase proteins including IL-6, CRP, haptoglobin, and alpha 1 glycoprotein acid amounts, with parallel VSMC proliferation and PMN accumulation in the artery wall of the ethanol group.

Another interesting result of the current study is the proliferation of vasa-vasorum surrounded by an abundance of infiltrated leukocyte in adventitia of aorta in the ethanol group. Although a limited number of studies have shown ethanol-induced alterations in some measures of inflammatory stress in the aorta, such as foam cell formation and elevation of cytokines, this is the first in vivo study demonstrating that ethanol consumption encourages proliferated vasa-vasorum formation along vessel wall thickness and infiltrated leukocyte in the wall of aorta. Angiogenesis is the formation of new blood vessels from a pre-existing vascular network, and is a significant incident in physiological situations such as embryonic development and in pathological conditions such as rheumatoid arthritis, tumor progression and atherosclerosis [35,36]. Angiogenesis is a documented property of the atherogenic development in artery diseases which is aroused frequently following activation and proliferation of a dense network of vessels in the adventitia adjacent to a plaque [37]. Angiogenic vasa-vasorum has been diagnosed in complicated regions of human aortic lesions through immune-staining experiments [38]. These angiogenic neovessels are most often localized in inflammatory/macrophages infiltrated regions of vessels [39]. Interestingly, results of the current study showed that the vasa-vasorum region in the aortic wall is full of infiltrated leukocyte along the increased aortic walls, and this result serves as a strong evidence for the initiation of an atherosclerosis process induced by ethanol in aortic walls. We also observed that chronic ethanol consumption significantly reduced the amount of VEGF in aortic walls obtained from the ethanol group. VEGF is a mitogen that aids vascular endothelial cell proliferation and angiogenesis. Furthermore, neovascularization has been shown to be significant in atherosclerotic plaque development [40]. In contrast to the result of this study, previous studies have demonstrated that ethanol consumption increases VEGF in coronary arteries [41,42]. Such an inconsistency in the reported literature may be due to differences in the strain of the animals used, duration of the experiments, or dose and kind of alcohol consumption. Moreover, ethanol consumption increases expression of different angiogenic growth factors such as transformation of growth factor-β_1 (TGF- β_1), basic fibroblast growth factor (bFGF), Flt-1, and Flk-1 in addition to VEGF [41]. Although it has not been fully discussed in the present report, it could be assumed that in addition to causing a reduction in VEGF, prolonged consumption of ethanol may initiate angiogenesis via other angiogenic factors. However, it is necessary to further examine various effects of ethanol on different angiogenic growth factors and angiogenesis in rats using different alcohol consumption levels ranging from light to very heavy. In addition, this study is significant in that its results provide a theoretical basis for contradictory outcomes and may, therefore, encourage further experimentation.

The second issue addressed in this study was the rescue effect of vitamin E on vascular wall changes and inflammation induced by chronic ethanol administration, since recent works consider vitamin E administration as having positive features and protective characteristics [5,43]. In the current study, we found that alcohol exposure related increases in inflammatory cytokines and decreases in VEGF could be restored to their normal levels through vitamin E treatment. In addition, we observed that VSMC proliferation and structural changes were also alleviated in vitamin E treated group compared to the control group. It has been demonstrated in previous reports that vitamin E has preventive effects against atherosclerosis, in addition to a number of other diseases [44,45]. The protective effect of vitamin E is caused by its antioxidant and anti-inflammatory properties. Mechanistically, vitamin E reduces the process of LDL oxidation which results in endothelial damage and foam-cell formation from macrophages and VSMC [46]. Vitamin E's free radical scavenging ability permits it to eliminate lipid

soluble free radicals, alongside the network of cellular antioxidant defenses, consisting of catalases, SOD and other enzymes, as well as small organic molecules such as L-ascorbic acid [45]. It has also been shown that vitamin E has intrinsic anti-inflammatory effects and suppresses pro-inflammatory cytokine expressions such as TNF-α, COX-2, and IL-6 in chronic ethanol consumed rat pancreas, and inhibits expression of VCAM-1 and ICAM-1 in endothelial cells and also reduces CRP expressions in healthy volunteers and in the diabetics [43,47,48]. Besides having antioxidant and anti-inflammatory properties, as strongly supported by the results of some studies, vitamin E may prevent the development of atherosclerosis by its ability to inhibit cell proliferation in VSMC via reduction of PKC activity and activation of protein phosphatase 2A (PP2A) [49,50]. A growing body of evidence supports PKC activation role in the development of atherosclerosis [51,52]. In this case, vitamin E inactivated PKC in VSMC by de-phosphorylating it, which is mediated by activation of PP2A [50]. This unique ability of vitamin E as an antioxidant, gives virtue to it for prevention of LDL oxidation and inhibition of hyaluronic acid depolymerization, by inhibiting the cellular production of superoxide, via inhibition of NADPH oxidase [53,54]. Drawing from the results of the current study and the previous ones, we suggest that ethanol induced inflammatory cytokines may trigger atherogenic signaling pathways and contribute to the proliferation of VSMCs and atherosclerosis. In conclusion, the results of this study can assist us in defining the chronic ethanol-dependent alterations in vascular structures and cellular functions. At the structural level, we determined that with chronic high dose ethanol consumption, aorta wall becomes thicker with proliferated vasa-vasorum surrounded by PMN, and VSMC proliferation is slightly increased in coronary artery. In addition, the elevation of systemic and vascular biomarkers of inflammation showed a more significant and stronger association between ethanol induced vascular structure changes via inflammatory stress than a number of traditional risk factors. Furthermore, vitamin E alleviated structural changes and restored inflammatory cytokines in the experimental group compared to the control group. However, any one of these observations contributes to the development of ethanol-induced adverse effect on vascular wall and will keep the field of ethanol vascular research busy for a very long time. In addition, incremental value of information regarding risk stratification based on a modified definition of the ethanol-induced adverse effect must be established and validated in future large scale prospective studies.

Acknowledgment

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