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Long-term ethanol consumption initiates atherosclerosis in rat aorta through inflammatory stress and endothelial dysfunction

Alireza Shirpoor [a,*], Siamak Salami [b], Mohammad Hassan Khadem-Ansari [c], Behnam Heshmatian [a], Behrouz Ilkhanizadeh [d]

[a] Department of Physiology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[b] Department of Clinical Biochemistry, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Departments of Biochemistry and Nutrition, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[d] Department of Pathology, Faculty of Medicine Urmia, University of Medical Sciences, Urmia, Iran

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ABSTRACT

Controversy exists on whether alcohol has a direct cardioprotective effect or it provokes atherosclerosis, so the present study sought to assess the effect of chronic consumption of ethanol on the markers of endothelial function, vessel rigidity, and atherosclerosis in the aorta of rat. Male Wistar rats were selected randomly and exposed to ethanol (4.5 g/kg of 20% w/v solution in saline) once per day for 6 weeks. Blood pressure, hemodynamic parameters, foam cell formation, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, endothelial-leukocyte adhesion molecule-1, and high-sensitivity C-reactive protein (CRP) were assessed in ethanol treated rats and compared with either sham or control rats. The results revealed a concurrent significant increase of adhesion molecules, CRP levels, systolic, diastolic, pulse, and dicrotic pressures as well as enhanced formation of foam cell in ethanol-treated rats. These findings implicate that long-term ethanol exposure provokes atherogenic and hemodynamic changes via significant induction of proinflammatory response, augmenting of cell adhesion molecules, stiffness in rat aorta wall and induction of foam cell formation.

1. Introduction

Alcohol as an old drug and popular beverage has attracted human interest several centuries ago. However scientific debate on the risks and benefits of alcohol started from that time and continues today (Kloner and Rezkalla, 2007). Although moderate alcohol consumption has been reported to confer a protective effect for specific diseases, current social patterns of alcohol consumption is a considerable global public health burden as reported in UK, US and Switzerland (Balakrishnan et al., 2009; Baumberg, 2006; Rehm and Monteiro, 2005; Rehm et al., 2007). Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality in developed countries and atherosclerosis is the important underlying pathology of them (Libby et al., 2002). Controversy exists on whether alcohol has a direct cardioprotective effect or not. Studies from some laboratories do not support the concept that alcohol has a direct cardioprotective effect, but cardioprotective effect of moderate alcohol consumption was also reported. It is clearly shown that binge drinking is a major cause of atherogenic changes such as hyperlipidemia, vasoconstriction, increased clotting activity, and a lower threshold for ventricular fibrillation (Costanzo et al., 2010a, 2010b). On the contrary, association of the light to moderate alcohol consumption with reduced cardiovascular mortality and incidence of ischemic stroke was reported (Kohsaka et al., 2011). Not more than thirty years ago, atherosclerosis was believed as result of lipid accumulation in the arterial wall, but better understanding of the atheroma plaque formation has led to a new concept of atherosclerosis as a chronic low grade inflammatory disease of the arterial endothelial cells (Hansson, 2005; Libby et al., 2002; Liuzzo, 2001). Endothelial cells play a crucial role in regulation of oxidative stress, permeability of vasculature to plasma constituents, platelets aggregation, thrombosis, vascular tone and blood pressure through controlling the release of several vasoactive substances including nitric oxide (NO) (Callow, 2002; Chatterjee and Catravas, 2008; Russo et al., 2002). It has recently reported that chronic alcohol ingestion is associated with increased aortic inflammation, oxidative endothelial injury and a decrease in the aortic endothelial NO-generating system giving rise to loss of vascular relaxation response and hypertension in rats (Husain et al., 2011). Another indicator of endothelial dysfunction and an important step in atherogenesis is the expression of the adhesion molecules on the surface of the vascular endothelium in response to the lesions (Fries et al., 1993). Several of these, namely, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial-leukocyte adhesion molecule-1 (E-selectin), have been identified on endothelial cells

* Corresponding author. Tel.: +98 441 2770988; fax: +98 441 2780801.
E-mail address: ashirpoor@yahoo.com (A. Shirpoor).

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(Bevilacqua et al., 1989) or on cells in human atherosclerotic lesions (Poston et al., 1992) and eventually lead to monocyte adhesion to the vascular wall and trans-endothelial migration. The later phenomenon triggers the formation of foam cells and vascular cell cytotoxicity. The adherence of monocytes is a key step in this process, and it occurs as a result of the up-regulation of adhesion molecules on both the endothelium and the leukocytes during the development and progression of atherosclerosis (Ross, 1999). It has been shown that moderate wine consumption, especially red wine, has beneficial anti-inflammatory effects on the endothelial activation which is possibly because of its polyphenol content (Sacanella et al., 2007), but the effect of chronic alcohol consumption on the foam cell formation was not investigated.

The observed ambiguity in the pro- or anti-inflammatory effects of ethanol on pathology of the atherosclerosis implicates a more complex cross talk between ethanol and cellular signal transducers than previously expected. Therefore, the effect of chronic ethanol consumption on the general and endothelial related inflammatory markers, blood pressure, and selected hemodynamic parameters, and foam cell formation are investigated in the current study using improved methodologies such as carotid arterial cannulation and waveform analyses of hemodynamic parameters.

2. Materials and methods

2.1. Animals and study design

All procedures utilizing rats were performed according to 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, National Medical and Health Service. Twenty-four 6-month-old male Wistar rats with initial body mass of approximately 275 ± 20 g were used in this study. The rats were divided into three groups: sham (SHR), control (CR), and ethanol-treated rats (ETR). ETR group received 4.5 g/kg of ethanol (Merck KGaA, Darmstadt, Germany) solution in saline (20% w/v) intragastrically by gavage once per day for 6 weeks. Control group was treated with vehicle only (normal sterile saline). To rule out any effect of the intragastrical gavage on the measured parameters, same numbers of the rats without any intervention were assigned as sham. Foods were supplied ad libitum to all groups throughout the experiments. Rats were anesthetized by ethyl carbamate (urethane) (1 g/kg, IP) and depth of anesthesia was assessed by pinching a hind paw and monitoring of the blood pressure and heart rate. In case of withdrawal reflex and/or variation of cardiovascular parameters, a supplementary dose of urethane was given (0.1–0.2 g/kg-IP).

2.2. Arterial blood pressure monitoring

Arterial blood pressure was directly recorded from carotid. Briefly, the rats were anesthetized with urethane (1 g/kg, IP) and were placed on a temperature-regulated table in supine position. A polyethylene catheter was inserted into the left carotid artery for blood pressure measurement. The catheter was filled with normal saline and heparin (75 IU/ml). The catheter was connected to a pressure transducer and physiograph (NARCO bio-system-USA). Blood pressure was measured after a 15-minute stabilization period. The pressure catheter was calibrated prior to each experiment. All data were recorded and stored digitally. Data were analyzed using Powerlab software (ADInstruments, Australia).

2.3. Blood samples

Blood samples were collected directly from carotid artery, mixed with ethylene diamine tetra acetic acid (EDTA) as an anticoagulant and centrifuged at $1000 \times g$ for 10 min within 30 min of collection. The plasma samples were aliquoted and stored at −80 °C without repeated freeze–thaw cycles.

2.4. Tissue harvesting and processing

The thoracic cavity and abdomen of animals were opened and aortas were dissected from the root to the abdominal descending part. Adventitial tissue and fat was removed from the excised aorta and the aortic arch was fixed in buffered formalin and embedded in paraffin after standard dehydration steps.

2.5. Biochemical assays

2.5.1. Soluble ICAM-1

Plasma level of rat soluble ICAM-1 (or CD54) was measured using a Rat sICAM-1 (CD54) immunoassay kit (RIC100, R&D systems, USA). This assay employs the quantitative sandwich enzyme immunoassay technique with a specific polyclonal antibody for rat sICAM-1 molecule conjugated to horseradish peroxidase. All steps were done according to the protocol provided by the manufacturer. Optical density of each well was determined within 30 min, using a microplate reader set to 450 nm. Wavelength correction was set to 540 nm. The mean minimum detectable dose (MDD) of rat sICAM-1 was 2.0 pg/mL.

2.5.2. Soluble VCAM-1

Plasma level of rat sVCAM-1 was measured using a rat sVCAM-1 ELISA kit (E0547r, USCN Life Science Inc., China). This assay employs a quantitative sandwich enzyme immunoassay technique using a specific antibody for sVCAM-1. All steps were followed as described by the manufacturer. Optical density of each well was determined within 30 min, using a microplate reader set to 450 nm. The detection range and sensitivity of rat sVCAM-1 were 0.78–50 ng/mL and 0.195 ng/mL, respectively.

2.5.3. Soluble endothelium selectin

Rat soluble endothelium-Selectin (sE-Selectin), an early mediator of leukocyte-endothelial adhesion, is expressed on activated endothelium and elevated serum levels were reported in a variety of inflammatory conditions. Plasma level of rat sE-Selectin was measured using a rat sE-Selectin ELISA Kit (E0549r, USCN Life Science Inc., China). This assay employs a quantitative sandwich enzyme immunoassay technique using a specific biotin-conjugated polyclonal antibody specific for sE-selectin without reported cross reactivity. The assay was performed as described by the manufacturer. Optical density of each well was determined within 30 min, using a microplate reader set to 450 nm. The detection range and sensitivity of the rat sE-selectin kit were 78–5000 pg/mL and 19.5 pg/mL respectively.

2.5.4. C-reactive protein

CRP is a member of the pentaxins, which synthesize in the liver and normal serum concentrations of which are very low (less than 10 mg/L). 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 MININEPHTM according to the protocol provided by the manufacturer (ZK044.L.R, The Binding Site Ltd, Birmingham, U.K.). The approximate measuring range and sensitivity were 3.51–12 mg/L and 0.44 mg/L, respectively.

2.5.5. CD68 staining

Tissues were fixed in buffered formalin and embedded in paraffin after standard dehydration steps. Tissue sections (4 μm thick) from formalin-fixed paraffin-embedded aorta were deparaffinized by immersing in xylene, rehydrated by gradual ethanol passage and washed in Tris buffer. For assessment of activated macrophages as an indicator of atherosclerotic process, the sections were stained for

CD68 immunohistochemically. Anti-CD68, clone KP1, was used to stain the slides after appropriate Ag retrieval step, and optimal results were achieved by the EnVision™ visualization system (Dako Denmark A/S, Danmark). Hematoxylin was used as counterstain. Appropriate negative controls were included in assessment. All slides were inspected by two expert pathologists independently.

2.6. Statistical analysis

Data were expressed as mean ± SD. To compare treated group with control group, the data for biochemical and physiological parameters were analyzed statistically using one-way analysis of variance (ANOVA) followed by Tukey's multiple range test using SPSS V.17 for windows (SPSS Inc, USA). The 0.05 level of probability was used for statistical significance.

3. Results

3.1. Blood pressure parameters

As shown in Table 1, systolic, diastolic, mean arterial, and dicrotic pressures or reflected pressure in ETR was significantly higher than sham and control groups ($p<0.001$). There was no significant change in pulse pressure among the groups. Heart rate in ETR was significantly higher than in the sham and control groups ($p<0.037$). No significant difference was found between the control and the sham groups ($p<0.9$). Fig. 1 shows the digital wave form of blood pressure among the different groups. Rats in ETR group had reduced diastolic time compared with the control and sham groups ($p<0.05$). There was no significant change in systolic time value among the different groups ($p<0.9$). Crest time (CT), the time interval from the foot point of the wave to the first peak, showed a significant increase in ETR compared with the sham and control groups ($p<0.001$). Significant differences in crest time ratio (CT/cycle time) were not found among the different groups ($p<.1$). The interval between first and reflected peaks (T_{DVP}) was significantly longer in ETR than that in the sham and control groups ($p<0.05$). There were no significant differences in CT + T_{DVP} value among the different groups ($p<0.9$).

3.2. CRP

Fig. 2 presents the levels of CRP and adhesion molecules in the different groups.

The level of CRP in ETR (17.36 ± 1) was significantly higher than those in SHR (3.38 ± .24) and CR (3.75 ± .2) ($p<.005$). No significant differences were found between SHR and CR ($p<0.9$) (Fig. 2A).

Table 1
Blood pressure parameters in three groups of rats.

	SHR	CR	ETR
Heart rate	390 ± 2.37	384 ± 8	431 ± 15.6*
SBP (mm Hg)	78.5 ± 3.25	75.24 ± 3	109.5 ± 5.5*
DBP (mm Hg)	62.3 ± 3.5	57.8 ± 1.69	95 ± 6.1*
M .A.P (mm Hg)	67.69 ± 2.9	63.36 ± 2	99.7 ± 5.9*
P.P. (mm Hg)	12.99 ± 2.5	17.81 ± 3.6	14.5 ± 4.5*
P. dicrotic (mm Hg)	74.86 ± 3.18	72.5 ± 2.8	105.69 ± 5.88*
Systolic time	0.0606 ± 0.002	0.0609 ± 0.001	0.0601 ± 0.002
Diastolic time	0.0942 ± 0.0015	0.106 ± 0.0053	0.0786 ± 0.004*
Crest-time	0.046 ± 0.0017	0.0498 ± 0.00045	0.0387 ± 0.006*
Crest-Tc	0.303 ± 0.0073	0.284 ± 0.0081	0.2635 ± 0.016*
T_{DVP}	0.0212 ± 0.00145	0.0163 ± 0.0003	0.035 ± 0.0612*
Crest-time + T_{DVP}	0.069 ± 0.00163	0.0667 ± 0.00068	0.0695 ± 0.00519*

Values are the means ± SEM.
Systolic blood pressure (SBP), diastolic blood pressure (DBP), Mean arterial blood pressure (MAP), and pressure of dicrotic (P. dicrotic).
* $p<.05$ compared with sham and control groups.

3.3. Cell adhesion molecules

As shown in Figure. 2B, plasma levels of sICAM in SHR, CR, and ETR were 82.5 ± 3.99, 81.28 ± 6.3, and 680 ± 63.6, respectively. Therefore, level of sICAM in ETR was drastically higher than those in SHR and CR ($p<.001$), but no significant differences were detected between the SHR and CR ($p<0.1$).

The plasma levels of sVCAM in ETR, CR, and SHR were 35.93 ± 6.9, 14.41 ± 1.25, and 13.85 ± .12, respectively. A significant difference was found between ETR and either SHR or CR, but no significant difference was found between SHR and CR ($p>0.5$) (Fig. 2C).

Regarding E-selectin, its plasma concentrations in SHR, CR, and ETR were 60 ± 7.9, 70.8 ± 4.75, and 124 ± 7.1, respectively. A significant increase in plasma E-selectin amount was observed in ETR

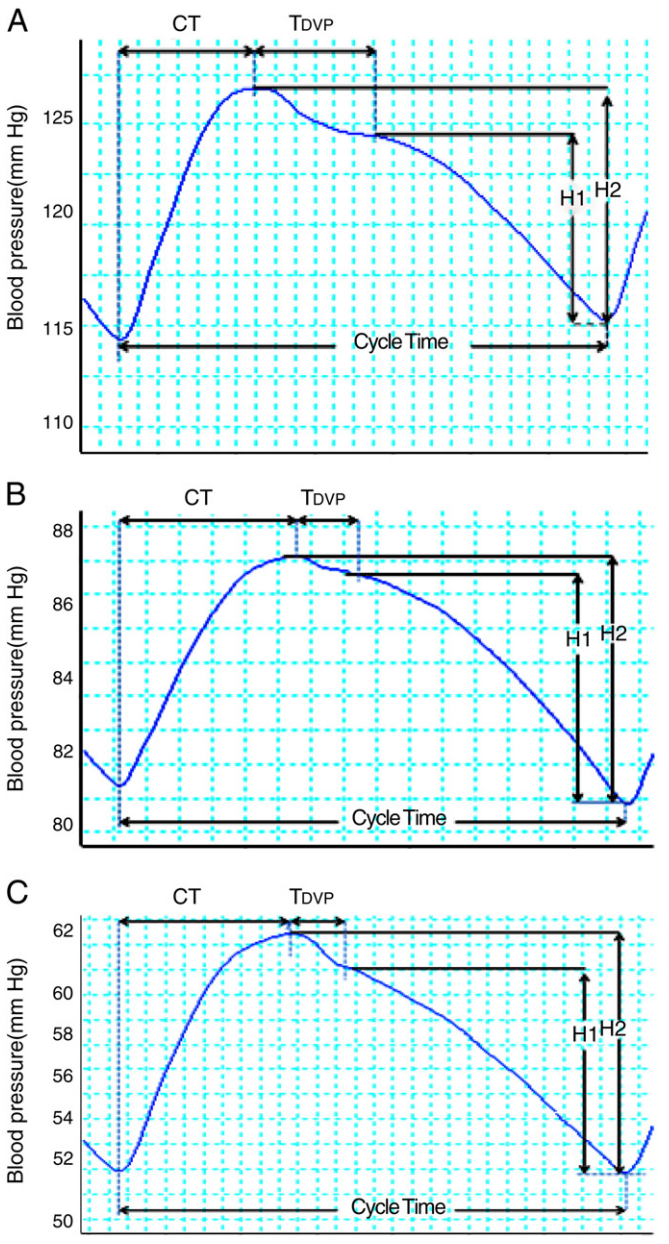

Fig. 1. Waveforms recorded from ethanol-treated (A), control (B), and sham (C) rats. CT (crest time) = the time interval from the foot point of wave to the first peak in milliseconds. TDVP = time difference between first and second peak in milliseconds. H2 = amplitude of first peak. H1 = amplitude of second peak. DVP is abbreviation of digital volume pulse.

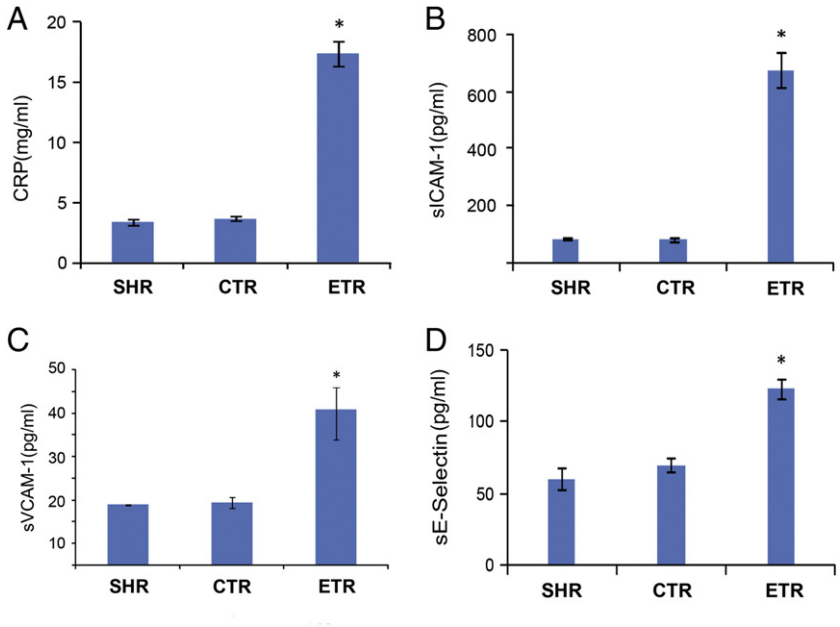

Fig. 2. Plasma level of CRP (A), sICAM-1 (B), sVCAM-1 (C) and E-selectin (D) in different groups. Values are the means ± SEM, *$p<0.05$ compared with sham and control groups.

compared with SHR and CR ($p<0.001$), but there was no significant difference between SHR and CR ($p<0.5$) (Fig. 2D).

3.4. Atherosclerotic changes of aorta

The presence of foam cells and the formation of atherosclerotic plaques were evaluated in hematoxylin–eosin-stained slides from all groups, but positive results were only found in ETR. Thus, a significant difference was found between ETR and the other groups ($p<0.001$).

Presence of plaque macrophages was investigated by CD68 staining and incidence of activated $CD68^+$ macrophages in ETR was significantly higher than other groups ($p<0.001$) (Fig. 3).

4. Discussion

It has been recently reported that chronic ethanol consumption induces a significant elevation in blood pressure which is related to increased aortic inflammation, elevated angiotensin II levels, induction of NADPH oxidase causing endothelial injury, reduction of superoxide dismutase activity, down-regulation of endothelial NO generating system and impaired vascular relaxation in rats (Husain et al., 2011). In spite of remarkable above mentioned observations, the relation of the chronic ethanol consumption with induction and progression of the atherosclerosis was remained unclear. So, the main objective of the current study was to evaluate the impact of long-term consumption of ethanol on initiation of proinflammatory response, induction of endothelial dysfunction with subsequent vascular and hemodynamic changes, and induction of atherosclerosis. Hence, multiple markers of inflammation (CRP), markers of endothelium dysfunction (sVCAM, sICAM, E-selectin), immunohistochemical markers of foam cell formation and hemodynamic parameters, were assessed in ETR and control rats. ETR have received oral dose of 4.5 g/kg ethanol (20%W/V) for 6 weeks, which is approximately equal to 4 standard alcoholic drinks per day in humans and could be considered as heavy dose but not binge drinking (Husain et al., 2011). The blood alcohol levels of ETR, control and sham rats were 241 ± 12, 1.7 ± 0.65 and 1.4 ± 0.87 mg/dL, respectively. It proves that the plasma level of ethanol in treated rats is higher than allowed alcohol levels (100 mg/dL) for drivers. The results revealed that, ethanol consumption in rat causes a massive increase in blood pressure. The variables related to blood pressure are reliable reflectors of in vivo hemodynamic data and artery wall properties. Ethanol-related elevated blood pressure and atherosclerosis in human and animal models have been reported in some previous studies (Fuchs, 2005; Shaish et al., 1997), but for the first time, to the best of our knowledge, we took advantage of the digital counter analysis of blood pressure wave to assess ethanol induced blood pressure parameter changes using carotid arterial cannulation. Thus, even with aggressiveness of the method, the results are more reliable and informative

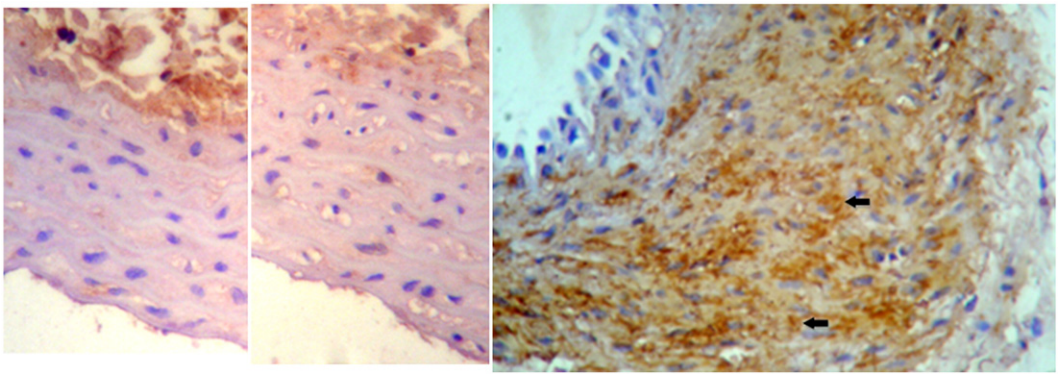

Fig. 3. Representative micrographs of aorta tissue stained with hematoxylin–eosin and CD68 from sham rats (A), control (B), and ethanol-treated (C). Original magnification ×400.

than the results obtained by tail-cuff methods (Ferrari et al., 1990; Fritz and Rinaldi, 2008; Ibrahim et al., 2006). Other than significant increase in systolic, diastolic, and mean arterial pressures, we also found a significant increase in dicrotic or reflected pressure. It has been reported that dicrotic notch height elevation is evidence of diminished NO production, and an altered NO synthesis triggers consequent changes in the physical and geometrical properties of vessels (Alastruey et al., 2009), so the results of current study supports the findings of the other study reporting ethanol induced down regulation of NO generating system (Husain et al., 2011). NO has an important vasodilatation effect on vessel diameters, tone, resistance, and compliance of vasculature (Chatterjee and Catravas, 2008; Russo et al., 2002). We had previously shown that ethanol consumption leads to an elevated level of plasma homocysteine (Shirpoor et al., 2009), and it has been shown that homocysteine impairs the nitric oxide synthase (NOS) pathway (Stuhlinger et al., 2001). The observed concurrent increase of general and dicrotic notch pressures might be combined effects of an increased level of adhesive molecules and impairment of NO synthesis. Positive association between ethanol consumption and hypertension in human subjects has also been reported recently (Wakabayashi, 2011).

We found that ethanol consumption evokes a significant increase in plasma level of CRP. It is an inflammatory factor that is used as a sensitive marker of acute inflammatory response. It is well established that CRP promotes the activities and expressions of multiple factors like as IL-6 and adhesion molecules that are implicated in atherogenesis, and it also facilitates the uptake of low-density lipoprotein by macrophage (Nava et al., 1995; Pasceri et al., 2001; Zwaka et al., 2001). It has been reported that CRP or its related actions initiate nitric oxide depletion and elevated expression of IL-6, IL-8, and vascular adhesive molecules (Zhong et al., 2006). In addition, it is capable of stimulating migration and proliferation of smooth muscle cells in the vessels (Wang et al., 2003). It also appears that CRP as inflammatory markers is in correlation with the extent of plaque formation as revealed by MRI (Taniguchi et al., 2004).

In addition, significant rise of plasma levels of sVCAM, sICAM, and E-selectin was found in ETR compared with the control rats. ICAM-1 and VCAM-1 are adhesive molecules that facilitate the attachment of white blood cells to the endothelium and consequent transendothelial migration (Clausell et al., 1999). The release of these molecules in plasma can be considered a marker of atherosclerosis and endothelial dysfunction (Bau et al., 2007). In patients with angina without flow-limiting lesions, elevated levels of these markers can be assumed as indicators of endothelial dysfunction (Clausell et al., 1999). E-selectin is an adhesive molecule that mediates the initial rolling, arrest, and attachment of leukocytes to the endothelium, whereas ICAM-1 and VCAM-1 mediate firmer leukocyte adhesion to the endothelium (Clausell et al., 1999). Drastically increased level of ICAM-1 which observed in current study might be considered as an early marker of atherosclerosis due to long-term ethanol consumption. Association of serum level of ICAM-1 and early stages of atherosclerosis development as well as with inflammation has reported previously (Hulthe et al., 2002; Kitagawa et al., 2002). In contrast, down regulation of cell adhesion molecules after consumption of moderate level of wines was reported but cardioprotective effect of alcoholic beverages was attributed to their polyphenol content (Sacanella et al., 2007).

Finally, the current study is the first report revealing the in vivo effect of chronic ethanol consumption on the differentiation of immigrating monocytes to $CD68^{+}$ macrophages in the atherosclerotic plaque in rats. So, we found that foam cells and plaque formation in the aorta wall in the ethanol treated rats were significantly higher than controls. The important role of macrophages in the development of atherosclerotic lesions, in cholesterol accumulation, and as sources of secreted enzymes and growth factors were clearly demonstrated (Ghosh et al., 2010; Jessup et al., 2002). Elevated CRP and concurrent overproduction of adhesive molecules possibly promote the development of atherosclerosis. Up-regulation of adhesion molecules and the binding of monocytes to the endothelium are early changes preceding the appearance of atherosclerotic lesions leading to inflammatory cell infiltration of the plaque and progression of the atherosclerotic process (Costacou et al., 2005). It has been also suggested that an increased expression of VCAM-1 and ICAM-1 is associated with an increased intimal leukocyte accumulation, and a large quantity of adhesion molecules were detected in arterial sites that are prone to the development of atherosclerotic lesions (Cybulsky et al., 2004; Takahashi et al., 2002). A modest association between alcohol consumption and carotid plaques was reported in men only, but no significant relationship with intima–media thickness (IMT) and carotid plaques was found in women (Zureik et al., 2004).

Instead of different methodological approaches, an interesting convergence exists between the findings of current study and recently reported results, which support pro-inflammatory effect of chronic ethanol consumption in the rat (Husain et al., 2011). Whereas their study was focused on the mechanism of the ethanol induced injuries in the aorta, the progression of injures to atherosclerosis was not investigated and our findings provide not only supportive evidences for the steps that they studied but also with different and somehow better methodology like as carotid arterial cannulation instead of tail cuff method, also shed light on the mechanism of ethanol induced atherosclerosis. On the other hand, a meta-analysis of eight publications revealed that in patients with cardiovascular disease, light to moderate alcohol consumption (5 to 25 g/day) was significantly associated with a lower incidence of cardiovascular and all-cause mortality (Costanzo et al., 2010a). Causal impact of average volume of alcohol consumption, i.e. dosing effect, on the reported outcomes was explained recently (Di Minno et al., 2011). In addition, methodological issues, drinking patterns, beverage type or gender differences should be considered in the interpretation of the current controversial and somehow odd figure of association between ethanol consumption and risk of cardiovascular disease (Brinton, 2010; da Luz and Coimbra, 2004; Fuchs et al., 2001; Kauhanen et al., 1999; Kloner and Rezkalla, 2007). It has also been suggested that alcoholic beverages with medium-level polyphenol content such as cava or with high-level polyphenol content such as red wine induces further reductions of inflammatory markers of atherosclerosis in relation to the alcoholic beverages with negligible levels of polyphenols, such as gin. Hence, some of the atheroprotective effect of alcoholic beverages could be partially mediated by the anti-inflammatory activity of polyphenolic content (Avellone et al., 2006; Chaves et al., 2009; Estruch et al., 2011; Sacanella et al., 2007; Shen et al., 2006; Vazquez-Agell et al., 2007).

In conclusion, elevation of CRP, augmentation of adhesive molecules, increase in blood pressure with disturbed hemodynamic parameters and development of the foam cells due to differentiation of monocytes to macrophages in the long-term ethanol treated rats are strong evidences of a proinflammatory response to the consumed ethanol. Therefore, the reported cardioprotective effects of alcohol in human subjects might be interpreted cautiously and further studies are needed to quantify the safe dose of alcohol with minimum side effects.

Conflict of interest

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, our work.

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