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Original Article

Ethanol induces oxidative stress and apoptosis in human umbilical vein endothelial cells

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Abstract: We investigated the cytotoxicity of ethanol on human umbilical vein endothelial cell (HUVEC). Human umbilical vein endothelial cells were treated with ethanol (50, 100, or 200 mM) for 24 hours. Cell proliferation was determined by a methyl thiazolyl tetrazolium (MTT) assay. In addition, cell supernatants were collected to determine levels of malondialdehyde (by a thiobarbituric acid kit), superoxide dismutase activity (by a xanthine oxidase kit), nitric oxide release (by a nitrate reductase kit), and expression and release of soluble ICAM-1 (by an ELISA assay). In addition, apoptosis was determined using an Annexin V/PI kit and flow cytometry. High concentrations of ethanol inhibited HUVEC proliferation, increase levels of malondialdehyde, decrease superoxide dismutase activity, and inhibited secretion of nitric oxide. Human umbilical vein endothelial cells treated with ethanol also exhibited up-regulated expression or release of ICAM-1. Furthermore, ethanol-treated HUVECs exhibited a dose-dependent increase in apoptosis. Ethanol can damage endothelial cells by promoting oxidative stress and inflammatory responses, leading to apoptosis.

Keywords: Ethanol, HUVEC, oxidative stress, ICAM-1, apoptosis

Introduction

Alcohol is one of the most important drinks in daily life. The alcohol drinking popular is increasing recently and it has been reported that there are over five hundred million people drinking alcohol in China. Previous study showed that there are 84.1% male and 29.3% female drinking alcohol in China. However, it has been noticed that 65% of alcohol drink is harmful due to the over concentration. It is reported that every year over 2 million people die from bad living habit including excessive drinking [1]. It is discovered that alcohol users have more chance to gain cardiovascular disease and have higher mortality [2].

Recent study shows that low dosage of alcohol usage might repress coronary disease instead of promoting the disease. However over dosage of alcohol could induce coronary disease, myocardiosis and arrhythmia, etc. World Health Organization (WHO) has issued the alcohol taken limitation that male could not take over 20 g alcohol per day and 10 g for female. The alcohol taken limitation in China is: <20 g/day is regarded as mild, 20-25 g/day is moderate and >50 g/day is severe. If take 38% alcohol drink as an example, Chinese drinkers take almost 41.04/g alcohol each day, almost 2 times as WHO limitation and 3 times as health drinking limitation (15 g) in China.

It has been studied that drinking and angiocardiopathy are "U" type related [3]. When low dosage of alcohol is used, the coronary disease is inhibited; however the high dosage of alcohol might induce angiocardiopathy, even leading to death. Low dosage alcohol could inhibit angiocardiopathy might due to oxidative stress, NO release, reduction expression of adhesion molecule and increase of HDL-C induced by alcohol. Wollny et al. [4] group has revealed that when treated the rat with ethanol, red wine and white wine, the ethanol could repress thrombus formation. However excessive alcohol usage could induce myocardial injury and thus causing myocardial disease. In early stage of alcohol

induced myocardial injury, cardiac muscle cell number is decreasing and more collagen is formed. In late stage of alcohol induced myocardial injury. Large number of cardiac muscle cells are dead and fibrillation region are formed in cardiac muscles. Alcohol might induce cardiac muscle cell dysfunction, such as apoptosis, contractile protein degradation, mitochondria and ER stress, etc. Shahryari et al. [5] group showed that after alcohol treatment, hamsters' cholesterol, glycerin triaurate and LDL-C have been increased dramatically.

In our study, we used different concentration of ethanol to treat human umbilical vein endothelial cells and measured the cell number increasing, MDA, SOD and NO release amount. We detected the ICAM-1 amount released to apoptotic cell culture medium and discussed the mechanism of alcohol induced human umbilical vein endothelial cells apoptosis.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC China Center for Type Culture Collection Luojia Shan, Wuhan, Hubei) were cultured in DMEM medium supplemented with 10% fetal bovine serum, then placed into an incubator at 37°C under a humidified 5% CO_2/95% atmosphere. After cells reached confluence, they were passaged at 1:2 or 1:3. Prior to experiments, HUVECs were digested in buffer containing 0.25% trypsin and 0.2% EDTA and plated. The health of endothelial cells was judge by their morphology under an inverted microscope.

Experimental group

Endothelial cells were treated with different concentrations of ethanol (0, 50, 100, and 200 mM) for 24 hours. Each treatment was repeated 6 times.

Detection of HUVECs proliferation

Cell proliferation was measured by the MTT assay (Sigma Company, USA). After endothelial cells were exposed to ethanol for 24 hours, 20 μL thiazolyl tetrazolium (5 g/L) per well was added and cells were incubated for another 4 hours at 37°C. Next, 150 μL dimethyl sulphoxide (DMSO) was added into each cell well for 10 min to dissolve the accumulated formazan. The number of live cells was determined by the absorbance at 490 nm on a microplate reader (BioTek Company, USA).

Malondialdehyde, superoxide dismutase, and nitric oxide released

Following ethanol treatment, 100 μL of the supernatant was collected from each well. The malondialdehyde (MDA Nanjing Institute of Biological Engineering Zhongyang Road, 258-27#, Nanjing, Jiangsu) level was detected using a thiobarbituric acid detection kit. Superoxide dismutase (SOD Nanjing Institute of Biological Engineering Zhongyang Road, 258-27#, Nanjing, Jiangsu) activity was measured by a xanthine oxidase kit, and nitric oxide (NO Nanjing Institute of Biological Engineering Zhongyang Road, 258-27#, Nanjing, Jiangsu) levels were determined using a nitrate reductase kit. All experiments were performed according to the manufacturer's instructions.

Determination of soluble ICAM-1

Soluble ICAM-1 (ADL Company, USA) levels in supernatants were detected by an ELISA assay (ELISA instrument BioTek Company, USA). First, 50 μL of standards and specimens were added into appropriate wells. A solution conjugated with biotin (10 μL) and reagent labeled with enzyme (100 μL) was added into the required wells. After incubated at 37°C for 60 minutes, each well was washed 5 times with distilled water. Substrates A (50 μL) and B (50 μL) were added and incubated at 37°C for 15 minutes, then the stop solution (50 μL) was added. Absorbance of each well was detected at 450 nm.

Apoptosis measurement

Cell apoptosis was measured with an Annexin V and propidium iodide (PI) kit (BD Company, 2350 Qume Drive, San Jose, CA 95131-1807, USA) followed by flow cytometry. Human epithelial cells were collected after ethanol treatment and diluted to 5×10^5-10×10^5 cells/ml. Cells were washed twice with PBS, and re-suspended in 200 μL binding buffer containing 10 μL FITC-conjugated anti-Annexin V monoclonal antibody and 5 μL PI. Cells were incubated at room temperature in the dark for 15 minutes. Apoptosis was immediately quantified by flow cytometry (BD Company, 2350 Qume Drive, San Jose, CA 95131-1807, USA). In the dot

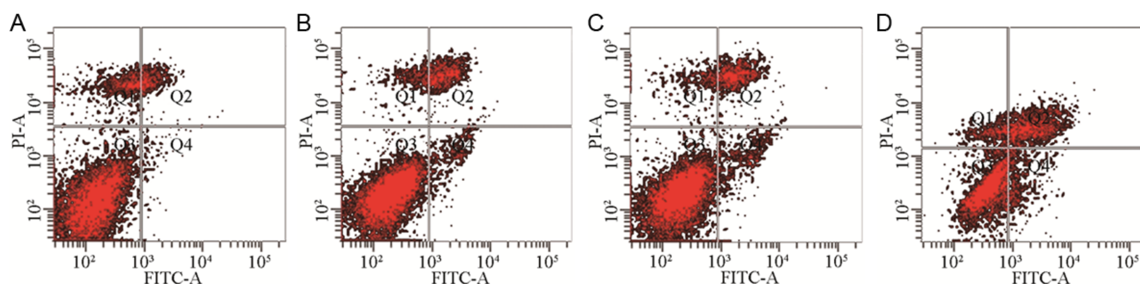

Figure 1. Flow cytometric analysis of cell apoptosis.

Table 1. Effect of ethanol on human umbilical vein endothelial cell viability and levels of MDA, SOD, NO, and ICAM-1 in the supernatant

Groups	N	MTT (OD)	MDA (nmol/mL)	SOD (U/mL)	NO (μmol/L)	ICAM-1 (ng/mL)
Normal	8	0.529±0.021	0.652±0.047	46.86±5.19	94.02±5.51	4.971±0.139
50 mM ethanol	8	0.446±0.048*	0.949±0.193	40.67±4.79*	85.56±1.92*	5.893±0.549#
100 mM ethanol	8	0.392±0.034*	1.567±0.204*	35.38±4.93*	77.72±5.92*	6.494±0.756*
200 mM ethanol	8	0.332±0.026*	1.966±0.301*	31.03±3.67*	75.54±2.59*	7.383±0.415*

Note: *indicates $P<0.01$ compared to the control group; #indicates $P<0.05$ compared to the control group. Abbreviation: MDA, malondialdehyde; SOD, superoxide dismutase; NO, nitric oxide.

plots (**Figure 1**), quadrant Q1 represents dead cells, quadrant Q2 represents late apoptotic cells, quadrant Q3 represents normal cells, and quadrant Q4 represents early apoptotic cells.

Statistical analysis

Data are expressed as means ± standard deviation. Comparisons among all groups were analyzed by one way ANOVA. Comparisons between individual treatment groups were analyzed by post-hoc SNK tests. A probability (*P*) value <0.05 was considered statistically significant.

Results

HUVECs proliferation

After endothelial cells were cultured in medium containing 50, 100, or 200 mM ethanol for 24 hours, cell viability, as revealed by the MTT assay, decreased significantly compared to the control group ($P<0.01$). The decrease in cell number was dose-dependent, and there were significant differences between all of the different dose groups ($P<0.05$, **Table 1**).

MDA and SOD activity

After HUVECs were treated with 50, 100, or 200 mM ethanol for 24 hours, concentrations of malondialdehyde (MDA) and superoxide dismutase activity (SOD) were determined in culture supernatants. Although there was no significant difference between the 50 mM ethanol group and the control group, concentrations of malondialdehyde following 100 mM ethanol treatment (1.567 nmol/ml±0.20 nmol/mL) and 200 mM ethanol treatment (1.966 nmol/ml±0.301 nmol/mL) were significantly higher than the control group ($P<0.01$). In contrast, SOD activity decreased significantly in all three ethanol treatment groups compared to the control group ($P<0.01$). Levels of superoxide dismutase in 50, 100, and 200 mM ethanol groups were 40.67 U/ml±4.79 U/ml, 35.38 U/ml±4.93 U/ml, and 31.03 U/ml±3.67 U/ml, respectively. Thus, malondialdehyde levels increased and superoxide dismutase activities decreased with increasing ethanol concentrations, and there were significant differences between most dose groups ($P<0.05$, **Table 1**). These results strongly suggest that ethanol treatment induced oxidative stress in HUVECs.

NO release

After HUVECs were treated with different doses of ethanol for 24 hours, nitric oxide levels in all ethanol treatment groups were significantly decreased compared to the control group ($P<0.01$). There was no significant difference between the 100 mM ethanol group and the

Table 2. Effect of ethanol on apoptosis

Groups	N	Early apoptosis rate (%)	Total apoptosis rate (%)
Control		0.87±0.16	3.13±1.31
50 mM ethanol	8	4.32±0.62*	11.3±1.62*
100 mM ethanol	8	7.35±1.72*	12.17±2.32*
200 mM ethanol	8	8.57±1.15*	15.06±3.15*

Note: *indicates $P<0.01$ compared with control group.

200 mM ethanol group, but there were significant differences between the control and the 50 mM group and between the two high dose groups and the other two treatment group ($P<0.05$, **Table 1**). Therefore, ethanol dose was inversely correlated with nitric oxide release into cell supernatants.

Soluble ICAM-1

Soluble ICAM-1 protein levels were significantly increased in all ethanol-treated groups compared with the control group ($P<0.01$, **Table 1**) and increased with higher concentrations of ethanol. There was no significant difference between the 50 mM ethanol group and the 100 mM ethanol group, but all other treatment groups were significantly different from each other.

Apoptosis of HUVECs

Apoptosis of endothelial cell was quantified after ethanol treatment using double labeling with FITC-anti-Annexin V mAb and PI and subsequent flow cytometry (**Figure 1**). The early apoptosis rates and total apoptosis rates in all ethanol treatment groups were significantly higher than the control group ($P<0.01$, **Table 2**). The early apoptosis rate was 4.32%±0.62% following 50 mM ethanol, 7.35%±1.72% at 100 mM, and 8.57%±1.15% at 200 mM, while the total apoptosis rates in each ethanol treatment group were 11.3%±1.62%, 12.17%±2.32%, and 15.06%±3.15%, respectively. There was no significant difference in total apoptosis rate between the 50 mM ethanol group and the 100 mM ethanol group, but the other groups showed significant differences between each other ($P<0.05$).

Discussion

Cardiovascular disease had become a major threat to human health. Studies have shown that excessive drinking leads to hypertension, alcoholic cardiomyopathy, cardiac arrhythmia, stroke, liver cirrhosis, pancreatitis, and various types of cancer [6-8]. Heavy alcohol consumption was associated with increased mortality from stroke, particularly hemorrhagic stroke, and cardiovascular disease in men [9]. Excessive drinking led to an increased incidence of cardiovascular disease, possibly by inhibiting myocardial energy metabolism, inhibiting lipoprotein lipase, chronic excitation of the sympathetic nervous system, and by increasing the incidence of hypertension [10-15]. In contrast, moderate drinking was shown to be healthy, and could reduce the incidence and mortality of cardiovascular disease. Our study found that high concentrations of ethanol could reduce superoxide dismutase activity and nitric oxide release, while increasing lipid peroxidation (malondialdehyde accumulation) and ICAM-1 release from endothelial cells. Ethanol promoted apoptosis of endothelial cells in a dose-dependent manner over 50-200 mM, possibly by oxidative stress.

Mitochondrial enzymes reduce the yellow thiazolyl tetrazolium to insoluble blue-purple formazan crystals. Dead and dying have minimal reductase activity, so formazan is not produced. Therefore, the generation of formazan crystals is proportional to cell number within a certain range of cells. This study found cell numbers (as indicated by absorbent values in ethanol-treated HUVEC cultures) were significantly reduced compared to the untreated control group, indicating that the proliferation and viability of HUVECs was decreased by ethanol.

Malondialdehyde is one of the end products of lipid peroxidation, so determination of malondialdehyde reflects the extent of oxidative stress. Superoxide dismutase is a major cellular antioxidant enzyme that can remove oxygen free radicals. Indeed, superoxide dismutase activity is closely linked to oxidative damage by free radicals. Determination of superoxide dismutase can reflect the endogenous protective capacity of cells against oxidative damage, while malondialdehyde levels reflect the severity of attack by free radicals. Oxidative stress leads to dysfunction of cardiovascular cells and is the central cause of atherosclerosis (AS) [16-18]. A recent study found that chronic alcohol consumption could increase lipid peroxidation and induce endothelial nitric oxide synthase

expression in the aorta [19]. Our study confirmed that ethanol can reduce superoxide dismutase activity and increase malondialdehyde levels in endothelial cell culture supernatants. Thus, one of the main modes of ethanol-induced endothelial cell injury is through oxidative stress.

Nitric oxide is a major intercellular signaling molecule between different types of cardiovascular cells. It is mainly secreted by vascular endothelial cells and diffuses rapidly through the membranes of the surround smooth muscle cells. This intercellular diffusion of nitric oxide relaxes vascular smooth muscles and so dilates blood vessels. In addition, nitric oxide reduced the expression of inflammatory factors and inhibited activation and aggregation of platelets [20-22]. Production of nitric oxide is a sign of endothelial cell function, while dysfunctional endothelial cells usually show reduced NO secretion. Our study also demonstrated that endothelial cells stimulated with high concentrations of ethanol had a reduced ability to secrete nitric oxide. Reduced vasodilation concomitant with reduced NO release could lead to hypertension during ethanol exposure.

Intercellular adhesion molecule-1 is a member of the immunoglobulin super-family (IgSF) that mediates the infiltration of inflammatory cells into both normal and inflamed tissues. Thus, increased expression of ICAM-1 is closely related to the occurrence and development of inflammation. High serum levels of soluble ICAM-1 are a sign of inflammation and can damage endothelial cells [23]. This study demonstrated that ethanol could mediate pro-inflammatory and pro-AS effects by promoting expression and release of ICAM-1 from endothelial cells.

Apoptosis is a form programmed death that is stimulated by a variety of physiological and pathological factors. Vessels free of atherosclerosis showed no significant apoptosis, while the apoptosis rate was about 4 times higher in vessels with atherosclerosis. Abnormal endothelial apoptosis is an important cause of atherosclerosis at both the early formation stage and the late development stage [24]. Thus, abnormal apoptosis of endothelial cells might be an initial step in the pathogenesis of AS [25]. The present study demonstrated that ethanol can induce apoptosis in endothelial cells. While the molecular mechanisms of ethanol cytotoxicity remained to be elucidated, the simultaneous demonstration of lipid peroxidation and reduced SOD activity suggests a reduced antioxidant capacity, followed by oxidation of cellular components (oxidative stress), leading to eventual (mitochondrial-dependent) apoptosis.

In summary, high concentrations of ethanol were toxic to endothelial cells. The molecular mechanisms of ethanol-induced cytotoxicity might be related to oxidative stress, inflammation, and the promotion of cell apoptosis.

Disclosure of conflict of interest

None.

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