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The *In vivo* Biochemical and Oxidative Changes by Ethanol and Opium Consumption in Syrian Hamsters

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Abstract

Daily consumption of opium and alcohol can make people have many health problems, including coronary artery disease diseases (CAD) which has been found to be the most common cause death in opium addicts. The aim of this study was to investigate the effect of simultaneous consumption of alcohol and opium on the lipid profile and oxidative stress in Syrian golden hamsters. Twenty-four male golden Syrian hamsters were randomly divided into four treatment groups (n=6): 1-control (received normal chow), 2-opium (received 40 mg/kg of opium two times per day), 3-alcohol (received 6.0 g/kg of 30% ethanol two times per day), 4-combination group (received a combination of the above mentioned doses of opium and ethanol). After one month of treatment, hamsters were sacrificed and blood samples were collected. Lipid levels and atherogenic index were markedly increased in the combination group compared with the controls ($p < 0.001$). Serum alanine aminotransferase (ALT) ($p < 0.05$), aspartate aminotransferase (AST) ($p < 0.05$), and gamma-glutamyl transferase (GGT) ($p < 0.01$), were significantly increased in alcohol-treated group compared with the control animals. The increase in ALT ($p < 0.01$) and GGT ($p < 0.001$) levels were more significant in the combination group when compared with the controls. The plasma concentration of malondialdehyde (MDA) was markedly increased in the ethanol ($p < 0.01$), opium ($p < 0.01$) and combination groups ($p < 0.001$) compared with the controls. Glutathione (GSH), nitric oxide (NO) and catalase (CAT) levels as well as superoxide dismutase activity were markedly reduced in the ethanol ($p < 0.05$), opium ($p < 0.05$), and combination groups ($p < 0.001$) compared with the control group. Results of this study clearly showed that opium and ethanol are capable to provoke the oxidative stress when administered alone or in combination. Moreover, combination opium and alcohol increased total cholesterol, LDL-C, TG, VLDL-C, atherogenic index and non-HDL-C levels.

Keywords: ethanol, opium, superoxid dimutase, malondialdehyde, nitric oxide, cholesterol

1. Introduction

Atherosclerosis, the leading cause of mortality in developed countries is a multifactorial disease that is related to numerous risk factors, including diabetes, obesity, dyslipidemia and hypertension. Many diseases, including atherosclerosis, develop as a result of imbalance between generating and scavenging of free radicals, a condition called oxidative stress (Meagher & Rader, 2001).

Although abuse of opium significantly reduced recently compared to other drug, in some Asian and middle eastern societies, opium is still the major drug of abuse. Traditional beliefs with no scientific basis may result in

harmful behavior. In Iran for instance some people, particularly the elderly, use opium believing it will lower blood sugar and lipids and prevents atherosclerosis. This might be because morphine administration was traditionally used for treatment of acute myocardial infarction (AMI) (Mohammadi, 2009). Howevcer, Masoomi el al. in a 20-year prospective study have shown that coronary artery diseases (CAD) were the most common reason of death in opium addicts in Iran. Moreover, results of some other studies have shown that a standardized mortality ratio for CAD is higher in opium addicts when compared with the non-addicts (Masoomi, 2010).

Some people consume opium and alcohol simultaneously and this type of dependence is an important health problem (Mohammadi, 2009). The aim of the present study was first, to assess the effect of consumption of ethanol or opium alone on lipid profile, lipid peroxidation, nitric oxide level, antioxidant activity, and liver enzymes, and second, to investigate whether simultaneous consumption of ethanol and opium results in a synergistic or antagonizing effect.

2. Method

2.1 Study Desing

Twenty-four male golden Syrian hamsters were used in this study. Animals were kept on a 12h light/12h dark cycle in an ambient temperature of 22 ± 1 °C. After acclimatizing for one week, animals were randomly divided into 4 treatment groups (n = 6) as follows: 1-control group (fed on normal chow), 2-addicted group (received 40 mg/kg body weight/day), 3-alcohol group (received 30% ethanol (vol/vol) at the dose of 6.0 g/kg body weight/day), and 4-combination group (received 40 mg/kg body weight/day of + 6.0 g/kg body weight/day of ethanol).

The opium group received opium as follows: a 10 mg/ml suspension of opium (Court of Justice, Kerman, Iran) in hot water was prepared, and after cooling at room temperature was administered by intragastric injection two times per day, starting with 5 mg/day within a period of 8 days the dose was increased to 40 mg per day and kept throughout the rest of the experiment. Hamsters were monitored daily and body weights were recorded every 48 hours. Hamsters were sacrificed 24 hours after the final treatment, and blood samples were collected from their hearts (Mohammadi, 2009; Husain, 2005; Mohammadi, 2012). This study was approved by the ethics committee of the Kerman University of Medical Sciences (Kerman, Iran, 2009).

2.2 Plasma Biochemical Measurements

The plasma levels of total cholesterol, triglyceride, HDL-C, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT) were measured using commercial kits (Pars Azmoon Co., Ltd., Tehran, Iran) and a Hitachi 902 (Japan) autoanalyser. The Atherogenic Index (AI) was calculated using the Rosenfeld equation and LDL-C level was calculated using the Freidwald equation. LDL-C: HDL-C ratio was also calculated (Abbasi-Oshaghi, 2012).

2.3 Antioxidant Assays

Activity of serum super oxide dismutase (SOD) was determined by the Misra and Fridovich method (Misra HP, 1972). Absorbance of the samples was measured at 480 nm for 4 min, and the activity of enzyme was expressed as the quantity of enzyme which inhibited the epinephrine oxidation by 50 percent, this is equivalent to one unit. Activity of CAT was measured using the Aebi method (1984). Absorbance was measured at 240 nm for 60 s in a UV-spectrophotometer (UV/VIS, PG Instrumental, America). Reduced glutathione (GSH) was assayed by the method of Beutler et al. (1963). The absorbance of colored product was read instantly at 412 nm in a spectrophotometer (Husain, 2005; Beutler, 1963; Husain, 2005).

2.4 Assay of Lipid Peroxidation

This assay was performed by measuring malondialdehyde (MDA) levels as described by Ohkawa et al. Briefly, 100 µl of plasma was added to 50 µl of 8.1% SDS, vortexed and incubated for 10 min at room temperature. Equal volumes (375 µl each) of 20% acetic acid (pH 3.5) and thiobarbituric acid (0.6%) were added and after one hour in the boiling water, samples were allowed to cool at room temperature. Then 1.25 ml of butanol: pyridine (15:1) was added to the samples, vortexed and centrifuged at 1000 rpm for 5 min. The absorbance of organic pink layer (750 µl) was measured at 532 nm. 1, 1, 3, 3-tetraethoxypropane was used as a standard (Husain, 2005).

2.5 Assay of Plasma Nitric Oxide Levels

The level of plasma nitric oxide was measured by Gries' reaction. In this reaction, acidified NO_2 produces a nitrosating agent that reacts with sulfanic acid and produces diazonium ion. In the second step, diazonium ion coupled with N^- (1-naphthyl) ethylenediamine. Absorbance of the product, chromophoric azo-derivative, was

measured at 520 nm (UV/VIS, PG Instrumental, America) (Husain, 2005; Vasdev, 2006; Guan, 2011; Hassan, 2013).

2.6 Statistical Analysis

Data were analyzed by ANOVA and expressed as mean ± SEM, and considered significant if $p < 0.05$. Analyses were completed with the statistical package SPSS (version 16, SPSS, Inc).

3. Results

Table 1 shows TC, LDL-C, HDL-C, TG and VLDL-C, in different groups. Serum total cholesterol levels were significantly increased in the ethanol ($p < 0.05$) and combination ($p < 0.01$) groups, while it showed a non-significant decrease in the opium group. Serum TG and VLDL-C significantly increased in ethanol ($p < 0.01$) and combination ($p < 0.001$) groups, however the increase in the opium group was not significant. LDL-C markedly increased in combination group ($p < 0.001$). Levels of HDL-C significantly increased only in ethanol group ($p < 0.05$). Table 2 shows the atherogenic index (AI), LDL/HDL ratio and non-HDL-C. These parameters markedly increased in combination groups compared with the controls ($p < 0.001$).

Serum ALT ($p < 0.05$), AST ($p < 0.05$), and GGT ($p < 0.01$), were significantly increased in the alcohol group compared with control animals. Serum ALT ($p < 0.01$) and GGT ($p < 0.001$) increases were more significant in combination group compared with control animals (Table 3).

SOD activity was markedly reduced in opium ($p < 0.05$) and alcohol ($p < 0.05$) as well as in combination groups ($p < 0.001$) (Figure 1). GSH and catalase levels were also markedly reduced in ethanol ($p < 0.05$) and opium ($p < 0.05$) groups compared with controls (Figure 2 and Figure 3 respectively). The reduction was more significant in the combination group ($p < 0.001$). The plasma concentration of MDA markedly increased in ethanol ($p < 0.01$) and an opium ($p < 0.01$) groups compared with controls and was more significant in combination group ($p < 0.001$) (Figure 4). NO level was significantly reduced in ethanol ($p < 0.05$) and opium ($p < 0.05$) groups compared with controls (Figure 5).

Table 1. Effects of alcohol and opium on lipid profiles

Groups/parameters	TC(mg/dl)	TG(mg/dl)	HDL-C(mg/dl)	VLDL-C(mg/dl)	LDL-C(mg/dl)
Control	78. 2 ± 5.1	88.4 ± 4.4	34.4 ± 3.4	17. 1 ± 2.1	27.2 ± 4.0
Ethanol	128. 4 ± 7.0^{a}	187 .2 ± 6.3^{b}	71.8 ± 5.5^{a}	37.7 ± 3.2^{b}	22.7 ± 2.3
Opium	71.1 ± 4.6	101. 2 ± 7.0	24.1 ± 2.0	22.5 ± 3.0	49.7 ± 4.7
ETHo/Opioum	135.4 ± 6.2^{b}	230. 2 ± 9.2^{c}	39 .5 ± 3.2	66. 3 ± 5.5^{b}	121.6 ± 6.5^{b}

Results are expressed as mean ± SEM. $^{a}p < 0.05$ compared to control. $^{b}p < 0.01$ compared to control.$^{c}p < 0.001$ compared to control. TC: Total cholesterol, TG: Triglyceride, LDL-C: Low-density lipoprotein cholesterol, HDL-C: High-density lipoprotein cholesterol, VLDL-C: Very low-density lipoprotein cholesterol.

Table 2. Effects of alcohol and opium on atherogenic index (AI), LDL/HDL ratio and non-HDL-C

	AI	LDL/HDL ratio	non-HDL-C
Control	1.27 ± 0.15	0.79 ± 0.13	43.56 ± 4.61
Ethanol	0.79 ± 0.05^{a}	0.32 ± 0.12^{b}	56.64 ± 6.17
Opium	1.95 ± 0.2 1^{a}	2.04 ± 0.17^{c}	47.1 ± 5.52
Ethanol + Opium	2.4 2 ± 0.34^{c}	3.07 ± 0.24^{c}	95.95 ± 7.48^{c}

Results are expressed as mean ± SEM. $^{a}p < 0.05$ compared to control. $^{b}p < 0.01$ compared to control.$^{c}p < 0.001$ compared to control.

Table 3. Effects of alcohol and opium on serum alanineaminotransferase (ALT), aspartate aminotransferase (AST) and serum Gammaglutamyl transferase (GGT) in U/L

	AST	ALT	GGT
Control	45.65 ± 5.20	43.73 ± 4.10	36.51 ± 4.26
Ethanol	65.69 ± 5.35^{a}	62.45 ± 5.31^{a}	59.24 ± 6.14^{b}
Opium	50.23 ± 6.01	45.55 ± 5.24	44.30 ± 5.62
Ethanol + Opium	72.19 ± 6.83^{a}	75.98 ± 6.80^{b}	69.15 ± 7.11^{c}

Data are expressed as mean ± SEM. $^{a}p < 0.05$ compared to control. $^{b}p < 0.01$ compared to control.$^{c}p < 0.001$ compared to control.

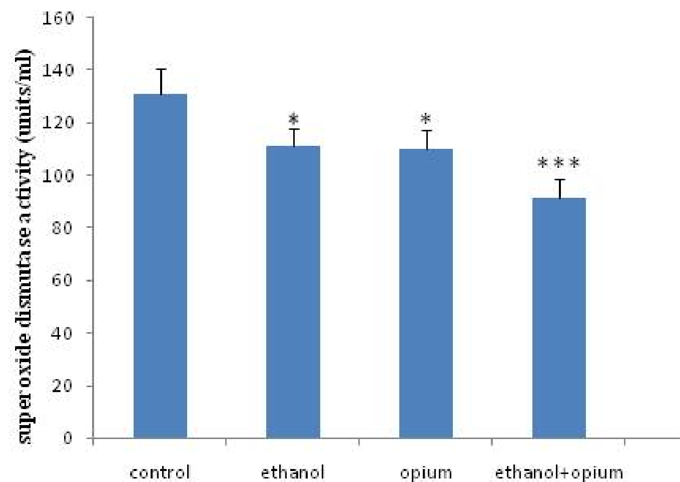

Figure 1. Effect of alcohol and opium on super oxide dismutase (SOD) activitiy. Data are expressed as mean ± SEM. $^{a}p < 0.05$ compared to control. $^{b}p < 0.01$ compared to control.$^{c}p < 0.001$ compared to control

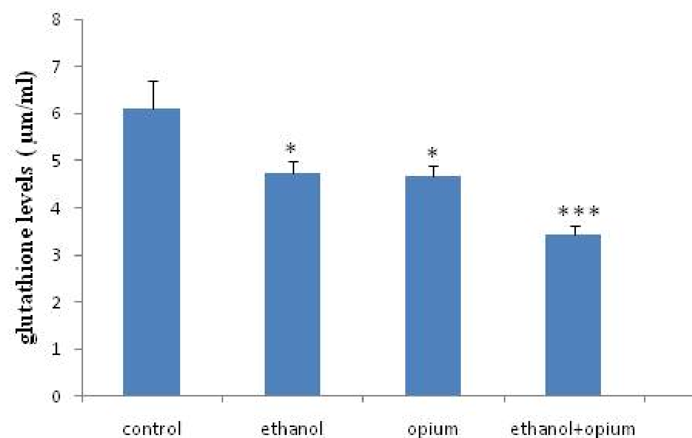

Figure 2. Effect of alcohol and opium on glutathione (GSH)

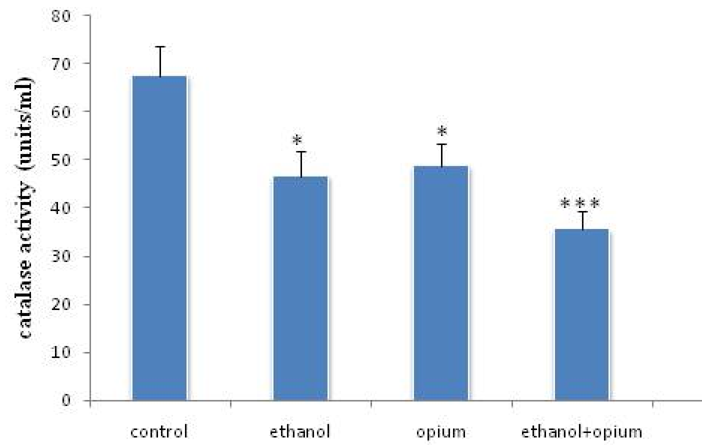

Figure 3. Effect of alcohol and opium on catalase activitiy

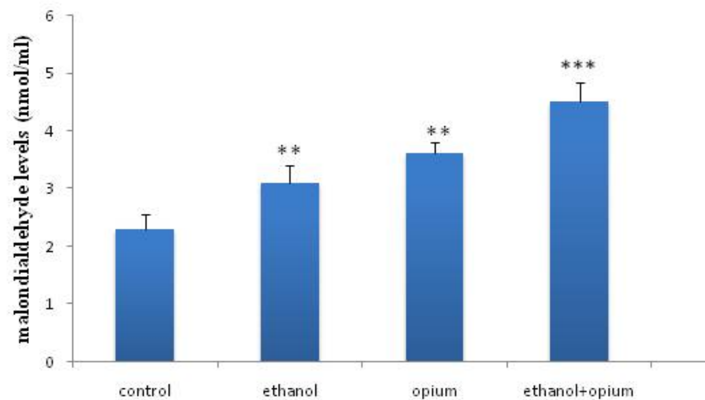

Figure 4. Effect of alcohol and opium on malondialdehyde (MDA)

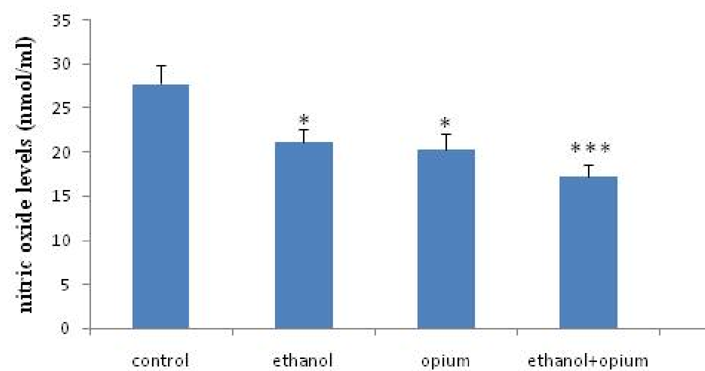

Figure 5. Effect of alcohol and opium on nitric oxide (NO) activitiy

4. Discussion

Atherosclerosis is one of the main causes of mortality in the world, and dyslipidemia has long been related to atherosclerosis. Therefore, correction of abnormal lipid profiles is recognized as a means to prevent

cardiovascular events. It is well known that elevated HDL-C levels have a protective effect on cardiovascular disease, while elevation of LDL-C levels is related with a higher atherosclerosis risk (Vasdev, 2006). In this study LDL-C significantly increased in combination group (121.59 ± 6.5 vs 27.24 ± 4.0). Ordinary consumption of ethanol may be associated with a rise in lipoprotein synthesis which including of reduction of HDL-C degradation and higher hepatic LDL-C metabolism. In this study HDL-C significantly increased in ethanol group (71.78 ± 5.5 vs 34.44 ± 3.4). Retention of LDL or the oxidative modification of LDL is the beginning step in atherosclerosis. Steinberg et al. suggested that oxidation modifys LDL, thus increasing atherogenicity of this particle. The presence of oxidized lipids in the atherosclerotic lesions has been recognized for many years. Results of many studies have showed that oxidized LDL can provoke migration of smooth muscle cell, provoke apoptosis in endothelial cells, macrophages and smooth muscle cells (Schwenke, 1998). Oxidized LDL can also provoke formation of foam cells. These features make this particle very atherogenic (Lonn, 2012).

In the period of postprandial ethanol is also responsible for an additional rise in plasma TG levels through inhibition of free fatty acid oxidation. An increase in plasma free fatty acids and TG levels are associated with a reduction in vasodilation of endothelial and insulin resistance. In this experiment serum TG was markedly increased in ethanol (187 .18 ± 6.3 vs 88.45 ± 4.4) and combination groups (230.17 ± 9.2 vs 88.45 ± 4.4) when compared with the control group. VLDL-C was also markedly increased in ethanol (37.72 ± 3.2 vs 17.13 ± 2.1) and combination groups (66. 33 ± 5.5 vs 17. 13 ± 2.1). Changes in TC, TG and LDL-C levels in opium treated animals were not significant.

Non-HDL cholesterol is a novel risk factor for atherosclerosis, which calculated by subtracting HDL-C from the total cholesterol. Quantity of LDL-C within the lipoprotein particle varies in different individuals, so the measurement of serum LDL-C does not actually reflect the number of particles and consequently a true estimate of cardiovascular risk (Indumati, 2011). Measurment of apolipoprotein-B is a more accurate method for finding the quantity of atherogenic lipoprotein particles in the plasma of patients. In our experiment, non-HDL-C significantly increased in the combination group (95.95 ± 7.48 vs 95.95 ± 7.48). Multivariable examinations in the Strong Heart Study showed that non-HDL-C is a strong predictor of cardiovascular disease (CVD). Other recent studies have also reported that non-HDL-C and apolipoprotein B are more powerful predictors of CVD than LDL-C alone. Also, many experiments have proved a strong relationship between non-HDL-C and apolipoprotein B. The apolipoprotein B assay is not routinely possible while, calculation of non-HDL-C is very easy and simply available for laboratories as well as is an inexpensive. In addition, many studies have shown that the ratio of LDL-C/HDL-C is an exceptional predictor of CHD risk (Indumati, 2011). In this study LDL-C/HDL-C ratio was significantly increased in opium (2.04 ± 0.17 vs 0.79 ± 0.13) and combination groups (3.07 ± 0.24 vs 0.79 ± 0.13) compared to the controls.

Measurements of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (GGT) are generally used as indicators of liver injury. Injured hepatocytes have altered transport function and permeability of plasma membrane, leading to enzymes leakage. This leakage in turn leads to an increase in plasma ALT, AST and GGT levels. Serum ALT is an enzyme mostly produced in hepatic cells; we monitored these enzymes to assess the extent of hepatocellular damage. In this experiment not only ALT (40%), but also AST (44%), GGT (62%) were increased in the alcohol group, the increase in these parameters was more significant in the combination group (64%, 58%, 89% respectively) compared with the controls (Mohammadi, 2012).

Antioxidants contain a mixture of phytochemicals which can be grouped collectively according to their function. A variety of intrinsic antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase and peroxidase are present in organisms which protect them from oxidative stress. Antioxidants are crucial for maintaining health and normal cellular activity. They are capable of deactivating and stabilizing free radicals before they attack cellular components (Sumanth, 2006). Recent evidence shows that oxidative stress and lipid peroxidation are involved in the pathogenesis of atherosclerosis. Lipid peroxidation is one of the initial events happening during in vitro oxidation of LDL, this process is provoked by many oxidants in vivo, and also by major cellular components of the lesions of atherosclerosis (Pratico, 2001). In the vessel wall atherogenic lipids especially oxLDL are responsible for a wide range of cellular dysfunctions (Napoli & Ignarro, 2001).

The high levels of MDA are known as a positive indicator for lipid peroxidation. Compared to controls, in ethanol, opium and combination of ethanol and opium treated animals, MDA was significantly increased (30.12%, 56.52%, and 95.65% respectively).

Catalase is a heme-containing protein mainly found in peroxisomes, that converts H_2O_2 to H_2O with at a high rate. Many studies have suggested that H_2O_2 is involved in the pathogenesis of atherosclerosis by inducing lipid

peroxidation. Under oxidative stress conditions, activity of catalase is decreased. Studies have shown that oxidative stress impairs endothelial cell function (Madhavan, 2010). In ethanol, opium and combination of ethanol and opium treated animals activity of this enzyme was significantly reduced when compared with controls (31.11%, 27.85%, and 47.40% respectively).

Superoxide anion (O_2^-) reacts with polyunsaturated fatty acids to produce reactive and toxic aldehyde metabolites, such as malondialdehyde, which is one of the end products of lipid peroxidation. SOD is a metalloenzyme which and the major defense against O_2^-, catalyzing dismutation of this free radical to H_2O_2 and O_2^-. Therefore SOD and catalase keep cells from toxicity of oxidants by catalyzing the dismutation of O_2^- to H_2O_2 and the decomposition of H_2O_2 to water and oxygen. There was a significant reduction in SOD in alcohol and opium treated groups (14.26% and 16.24% respectively), and there was more reduction I in the SOD activity in the combination group (24.86%) when compared with the control group.

Within the vessels, nitric oxide (NO) inhibits interaction of circulating elements with the vessel wall. For instance nitric oxide NO inhibits platelet aggregation as well as infiltration and adherence of monocytes. It is obvious that dysfunction of endothelium is a primary event in atherogenesis and coronary heart disease (CHD). Inactivation of the NO synthase (NOS) is probably one of the initial events in atherogenesis. Reactive oxygen species (ROS) promote endothelial NOS (eNOS) disconnection consequently leading to decrease NO production. A reduction in NOS activity and/or synthesis may contribute to the beginning and progress of atherosclerosis (Napoli & Ignarro, 2001).

Disorders in NOS dependent pathway may happen by a number of mechanisms such as decrease in NOS affinity for L-arginine, lipoprotein-induced modifications in signal transduction, increased production of superoxide anion followed by degradation of NO, and/or elevated levels of circulating antagonists. Thus, assay of NO activity and/or synthesis may be useful in planning for atherosclerosis treatment and related diseases (Napoli & Ignarro, 2001). This experiment provided data showing that high doses of ethanol and opium significantly reduce NO (24.10% and 26.13% respectively) compared with the control. Co-administration of opium and ethanol more significantly reduced NO (38.12 %). The decrease in NO production observed in atherosclerosis process may be due to increased superoxide production by a dysfunctional endothelium and formation of peroxynitrite. Moreover, ROS directly react with NO and peroxynitrite. This reactive nitrogen species that restricted bioavailability of NO, and is a powerful and cyto-toxic oxidants which may reason injure to vascular tissue (Vasdev, 2006). Pacher et al. (2005) showed that Peroxynitrite is involved in the CVD pathogenesis and diabetes complication such as retinopathy, nephropathy, cardiomiopathy and neuropathy (Pacher, 2005). McKim et al. (2003) reported that the peroxynitrite production is a mediating factor in liver injury of alcoholism (McKim, 2003).

In this study GSH was markedly reduced in opium (22.54%) an alcohol (23.52%) groups and the reduction was almost doubled in the combination group (43.95%). Production of reactive oxygen intermediates in the process of ethanol and opium metabolism leads to lipid peroxidation and oxidation of glutathione (GSH), depletion of reduced glutathione in the liver is in turn responsible for ethanol toxic effects. Glutathione antioxidant system has an important role in cellular protection against reactive free radicals. GSH acts as a free radical scavenger and has an important role in the repairmen of damages caused by free radicals (Madhavan, 2010).

Results of this investigation clearly showed that opium and ethanol are capable to provoke the oxidative stress when administered alone or in combination. Opium and alcohol also harmfully increased total cholesterol, LDL-C, TG, VLDL-C, atherogenic index and non-HDL-C in animals. High levels of alcohol consumption could contribute to increased LDL oxidation and reduced antioxidant activity.

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