Opium may affect coronary artery disease by inducing inflammation but not through the expression of CD9, CD36, and CD68

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ABSTRACT

The molecular mechanisms of opium with regard to coronary artery disease (CAD) have not yet been determined. The aim of the present study was to evaluate the effect of opium on the expression of scavenger receptors including CD36, CD68, and CD9 tetraspanin in monocytes and the plasma levels of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), malondialdehyde (MDA), and nitric oxide metabolites (NO_x) in patients with CAD with and without opium addiction. This case–control study was conducted in three groups: (1) opium-addicted patients with CAD (CAD+OA, n=30); (2) patients with CAD with no opium addiction (CAD, n=30); and (3) individuals without CAD and opium addiction as the control group (Ctrl, n=17). Protein and messenger RNA (mRNA) levels of CD9, CD36, and CD68 were evaluated by flow cytometry and reverse transcription-quantitative PCR methods, respectively. Consumption of atorvastatin, aspirin, and glyceryl trinitrate was found to be higher in the CAD groups compared with the control group. The plasma level of TNF-α was significantly higher in the CAD+OA group than in the CAD and Ctrl groups (p=0.001 and p=0.005, respectively). MDA levels significantly increased in the CAD and CAD+OA groups in comparison with the Ctrl group (p=0.010 and p=0.002, respectively). No significant differences were found in CD9, CD36, CD68, IFN-γ, and NO_x between the three groups. The findings demonstrated that opium did not have a significant effect on the expression of CD36, CD68, and CD9 at the gene and protein levels, but it might be involved in the development of CAD by inducing inflammation through other mechanisms.

Significance of this study

What is already known about this subject?

- The molecular mechanism of opium with regard to coronary artery disease (CAD) has not yet been determined.
- Opium-induced oxidation may alter the expression of the scavenger receptors and their regulatory factors.
- Oxidative stress may affect nitric oxide production, which has contradictory roles in relation to cardiovascular diseases.

What are the new findings?

- The findings showed that the plasma level of tumor necrosis factor alpha rose significantly in opium-addicted patients with CAD in comparison with the CAD and control groups.
- It was found that opium did not affect the expression of CD9, CD36, and CD68 receptors in patients with CAD.
- The results also demonstrated that opium did not affect the plasma levels of malondialdehyde, interferon gamma, and nitric oxide metabolites in patients with CAD.

How might these results change the focus of research or clinical practice?

- Further large-scale studies are needed to reveal the exact mechanisms and effects of opium and routine medications on the expression of CD9, CD36, and CD68 receptors in patients with CAD.

INTRODUCTION

Coronary artery disease (CAD) is considered one of the main causes of death around the globe.[1] One of the known factors that can lead to CAD is atherosclerosis,[2 3] which is caused by chronic inflammation and plaque formation in medium-sized to large-sized arteries.[4] Various risk factors are involved in the development of atherosclerotic plaques, including arterial hypertension, smoking, lifestyle, high-fat diet, physical inactivity, and diabetes.[5–7] Moreover, studies have shown that opium consumption may have destructive effects on atherosclerotic plaque formation.[8 9] Another factor that plays a role in plaque formation is oxidized low-density lipoprotein (ox-LDL).[4] Macrophages take up ox-LDL via scavenger receptors, such as CD68 and CD36. It has been demonstrated

that either CD36 or CD68 plays a critical role in the pathogenesis of CAD and its complications such as atherosclerosis.[10] Scavenger receptors act through various signaling pathways and perform their function via interactions with other receptors, such as integrins, Toll-like receptors, and tetraspanins.[10 11] CD9, which is a member of the tetraspanin family, is expressed on various cells, such as platelets and macrophages. It has been shown that this important molecule can interfere with the functions of the scavenger receptors on macrophages, and the genetic deletion of CD9 leads to a decrease in CD36 signaling in response to ox-LDL and a reduction in foam cell generation.[12] Thus, it is hypothesized that CD9 may affect the pathogenesis of atherosclerosis and CAD in a scavenger receptor-dependent manner. However, the altered expression of these receptors, including scavenger receptors and CD9, by direct or indirect risk factors, may change monocyte functions.

Opium, as a narcotic drug, may interfere with CAD.[13] The molecular mechanism of action of this substance with regard to this disease has not yet been determined. In some societies such as in Asian communities, the traditional belief among people is that opium might have a positive effect on cardiovascular health and diabetes, hypertension, and dyslipidemia.[14] Studies have shown that using opium has a direct correlation with incidence of cardiovascular diseases (CVDs) and increases the risk of inflammation and oxidative stress, which both contribute to the development of atherosclerotic plaques.[13] For example, the expression of CD36 in macrophages was enhanced as a result of oxidative stress, thereby increasing the absorption of ox-LDL.[15] Therefore, the oxidation induced by opium may alter the expression of scavenger receptors and their regulatory factors. Moreover, oxidative stress affects lipids and leads to the generation of secondary compounds, such as malondialdehyde (MDA), as a result of the peroxidation of polyunsaturated fatty acids.[16 17] In addition, oxidative stress may also affect nitric oxide (NO) production, which has contradictory roles in relation to CVD.[18]

The present study aimed to evaluate the expression of CD9, CD36, and CD68 on the monocytes of patients with CAD with and without opium addiction at both the messenger RNA (mRNA) and protein levels. Moreover, the plasma levels of tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), as proinflammatory cytokines, and MDA and nitric oxide metabolites (NO_x), as oxidative stress markers, were compared between the studied groups.

MATERIALS AND METHODS

Subjects

As a case–control study, the present research was carried out on a total of 77 individuals with suspected CAD who were considered candidates for coronary angiography and were referred to Shafa Hospital of Kerman University of Medical Sciences, Kerman, Iran (from July 2017 to February 2018). Coronary angiography was performed on all the subjects. Some of the subjects in the research were under treatment with drugs such as atorvastatin, glyceryl trinitrate, aspirin, clopidogrel, bisoprolol, valsartan, metoprolol, captopril, carvedilol, isosorbide, and losartan. The inclusion criteria were as follows: men with symptoms of ischemic heart disease and those diagnosed with CAD by coronary angiography. The exclusion criteria included patients with a history of cancer, diabetes, autoimmune and respiratory diseases, cerebral infarction, and use of alcohol, cigarettes, methadone, and other opiates, such as morphine, heroin, and similar narcotics. All opium-addicted patients used opium more than 500 mg daily for at least 1 year before sample collection. Subjects were sorted into three groups: (1) severe CAD (stenosis >50%) and opium-addicted (CAD+OA, n=30); (2) severe CAD (stenosis >50%) with no opium addiction (CAD, n=30); and (3) normal coronary arteries with no opium addiction as the control group (Ctrl, n=17).

Samples and data collection

Demographic information was collected through interviews with the subjects. Body mass index was computed as the ratio of weight to the square of height. About 10 mL of blood were drawn from subjects after overnight fasting (collected before breakfast at 08:00) and poured into two separate tubes containing EDTA. One tube was centrifuged (10 min at 3000 revolutions per minute (RPM)) and plasma was separated and then kept at −70°C for measurement of biochemical parameters, TNF-α, IFN-γ, NO_x, and MDA. The other tube was used for monocyte separation, evaluation of cell surface CD36, CD68, and CD9, and extraction of total mRNA as described in the following sections. Based on a previous article[19] and using the following formula, the minimum sample size for the comparison of IFN-γ between the study groups, considering α=0.05, β=0.2, d=48.89, and effect size (ES)=0.983, was calculated to be 18 subjects in each group.

$$n = \frac{Z^2 p(1-p)}{d^2}$$

Biochemistry factors

The plasma levels of fasting plasma glucose, total cholesterol (TC), triglycerides, high-density lipoprotein cholesterol (HDL-c), creatinine, and urea were determined using standard kits (Pars Azmoon, Tehran, Iran) and an autoanalyzer (Roche Hitachi 911) in a standard laboratory context. Additionally, low-density lipoprotein cholesterol (LDL-c) was calculated using the Friedewald equation. Plasma sodium and potassium levels were measured via an ion-selective electrode.[20]

Separation of monocytes

First, isolation of peripheral blood mononuclear cells (PBMCs) was performed from anticoagulated blood by density gradient centrifugation using Ficoll histopaque. Subsequently, PBMCs were incubated with anti-human CD14 magnetic particles (catalog number: 557769; BD Biosciences, USA) at room temperature for 50 min, and then the monocytes were isolated by the cell separation magnet (catalog number: 552311; BD Biosciences).

Analysis of CD9, CD36, and CD68 mRNA expression

To evaluate mRNA expression, reverse transcription-quantitative PCR (RT-qPCR) was performed. Briefly, RNA was extracted from monocytes by total RNA extraction solution (catalog number: K-3090; Bioneer, Daejeon, South Korea) and purified RNA was qualitatively and quantitatively

assessed using a NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific, Wilmington, USA). In addition, complementary DNA (cDNA) was synthesized via the reverse transcription system by the cDNA Synthesis Kit (catalog number: 6110A; Takara Bio, California, USA). The primers used for amplification of CD9, CD36, and CD68 are listed in online supplemental table S1. Beta-actin was used as the housekeeping gene. Moreover, RT-qPCR was performed using RealQ Plus Master Mix Green (catalog number: 4324402; Amplicon, Odense, Denmark) in a real-time PCR machine (Mic, Australia) according to the following program: the *CD9* gene was initially denatured for 15 min at 95°C, followed by 35 cycles of denaturation (20 s) at 95°C, annealing (20 s) at 69°C, extension (20 s) at 72°C, along with a final extension at 72°C for 5 min. RT-qPCR for *CD36*, *CD68*, and beta-actin was performed as follows: initial denaturation for 15 min at 95°C, followed by 40 cycles of denaturation (30 s) at 95°C, annealing (60 s), extension (35 s) at 60°C, and the final extension for 5 min at 72°C. The expressions of these molecules were computed using the $2^{-\Delta\Delta CT}$ formula.[21]

Flow cytometry analysis

To detect CD9, CD36, and CD68 on monocytes, PBMCs were fixed with a fixation solution (catalog number: 420801; BioLegend, San Diego, USA) and incubated at room temperature in darkness for 20 min. They were then centrifuged and the diluted intracellular staining permeabilization wash buffer (catalog number: 421002; BioLegend) was added to the cellular deposition and centrifuged (the process was repeated twice). After centrifugation, the cells were washed with phosphate-buffered saline (PBS). PBMCs were stained using the antibodies listed in online supplemental table S2. Anti-human CD14 was used to identify monocytes. Approximately 10,000 stained cells were evaluated using a flow cytometry instrument (catalog number: 342976; BD FACSCalibur, USA).

Measuring cytokine levels

Plasma levels of IFN-γ and TNF-α were evaluated using commercially available ELISA kits (Karmania Pars Gene Company, Kerman, Iran) according to the manufacturer's instruction.

MDA measurement

One of the substances measured as a lipid peroxidation index is MDA. The measurement of this compound was conducted by the thiobarbituric acid (TBA) assay protocol. In this method, in the presence of the trichloroacetic acid-TBA-hydrochloric acid reagent, MDA reacts with TBA and produces a pink color whose final absorbance was measured at 535 nm.[22 23]

Evaluation of NO_x

Due to the short half-life of NO in the blood, its stable metabolites (NO_x), nitrite (NO_2^-), and nitrate (NO_3^-) were detected by the Griess method.[24 25] First, using $ZnSO_4$ and in the presence of 0.3 M NaOH, plasma deproteinization was performed. Then vanadium (III) chloride ($VaCl_3$) (for converting nitrate into nitrite) and the Griess reagent (2% sulfanilamide in 5% phosphoric acid and 0.1% NEDD (N-(1-naphthyl) ethylenediamine dihydrochloride) in deionized water) were added to the deproteinized plasma and incubated at 37°C for 30 min. Finally, the optical density was measured at 540 nm.[26]

Table 1 Demographic and biochemical parameters of subjects in the three groups

Variables	CAD+OA (n=30)	CAD (n=30)	Ctrl (n=17)	P value
Age (years)	56.13±1.4	53.13±1.7	49.88±1.9	0.028*
BMI (kg/m^2)	27.43±2.0	26.72±0.6	23.85±1.1	0.311
FPG (mg/dL)	99.00±4.8	98.96±3.7	105.9±4.5	0.222
TG (mg/dL)	130.82±14.9	120.5±8.9	128.8±11.6	0.759
TC (mg/dL)	141.00±6.9	152.66±5.8	180.05±9.6	0.003*
HDL-c (mg/dL)	35.37±1.0	40.20±1.4	48.11±2.7	<0.001*
LDL-c (mg/dL)	79.62±6.0	88.36±4.9	106.69±7.8	0.009*
Sodium (mmol/L)	138.14±0.4	137.51±0.4	138.71±0.9	0.338
Potassium (mmol/L)	4.15±0.0	4.08±0.0	4.06±0.1	0.746
Cr (mg/dL)	1.07±0.0	1.09±0.0	1.04±0.0	0.666
Urea (mg/dL)	33.59±1.9	34.34±1.3	34.91±2.8	0.906
Hyperlipidemia (yes)	21 (70)	19 (63.33)	7 (41.17)	0.154
Hypertension (yes)	11 (36.66)	16 (53.33)	4 (23.52)	0.205

Continuous and categorical variables are presented as mean±SEM and number (%), respectively.
*Significant difference was set at p<0.05.
BMI, body mass index; CAD, coronary artery disease; CAD+OA, coronary artery disease and opium addiction; Cr, creatinine; Ctrl, control; FPG, fasting plasma glucose; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

Statistical analysis

Continuous and categorical variables are presented as mean and SEM or number and percentage. In addition, one-way analysis of variance (ANOVA) and χ^2 test were used to compare the variables between the study groups. One-way ANOVA followed by the Bonferroni test was applied for pairwise comparison of log(y) between the study groups using the logarithm function as the transforming method for normalizing the response variables. All analyses were performed in SPSS V.24, and the significance level was set at 0.05.

RESULTS

Demographic analysis

Table 1 presents the subjects' demographic and clinical characteristics. There were significant differences in age, TC, HDL-c, and LDL-c between the three groups (p=0.028, p=0.003, p<0.001, and p=0.009, respectively). However, no significant differences were observed with regard to other variables (p>0.05 for all comparisons).

Comparison of drug usage

Comparisons of drug consumption between the three studied groups are depicted in online supplemental table S3. Significant differences were found in the consumption of aspirin (p=0.015), glyceryl trinitrate (p=0.006), atorvastatin (p=0.008), and clopidogrel (p=0.012) between the three studied groups before coronary angiography. However, the analysis did not reveal any significant differences in the consumption of other drugs between the three studied groups (p>0.05 for all comparisons).

Table 2 Pairwise comparison using one-way ANOVA followed by Bonferroni test

Response	Comparison	MD±SE	95% CI†	P value
TNF-α	CAD+OA vs Ctrl	3.228±1.425	1.350 to 7.714	0.005*
	CAD+OA vs CAD	2.504±1.266	1.402 to 4.468	0.001*
	CAD vs Ctrl	1.289±1.363	0.600 to 2.768	>0.999
IFN-γ	CAD+OA vs Ctrl	1.514±1.212	0.944 to 2.430	0.104
	CAD+OA vs CAD	1.254±1.126	0.936 to 1.680	0.185
	CAD vs Ctrl	1.207±1.196	0.777 to 1.876	0.888
MDA	CAD+OA vs Ctrl	1.865±1.189	1.219 to 2.852	0.002*
	CAD+OA vs CAD	1.182±1.120	0.894 to 1.565	0.437
	CAD vs Ctrl	1.576±1.162	1.091 to 2.277	0.010*
NO_x	CAD+OA vs Ctrl	1.070±1.038	0.977 to 1.172	0.207
	CAD+OA vs CAD	0.997±1.025	0.938 to 1.059	>0.999
	CAD vs Ctrl	1.075±1.034	0.991 to 1.165	0.100

*Significant difference was considered at p<0.05.
†Back-transformed coefficients using the exponential function.
ANOVA, analysis of variance; CAD, coronary artery disease; CAD+OA, coronary artery disease and opium addiction; Ctrl, control; IFN-γ, interferon gamma; MD, mean difference; MDA, malondialdehyde; NO_x, nitric oxide metabolites; TNF-α, tumor necrosis factor alpha.

Plasma levels of TNF-α, IFN-γ, MDA, and NO_x

Comparisons of the mean plasma levels of TNF-α, IFN-γ, MDA, and NO_x between the groups are presented in table 2. The plasma level of TNF-α was significantly higher in the CAD +OA group than in the CAD and Ctrl groups (p=0.001 and p=0.005, respectively). However, no significant differences were observed between patients with CAD and the Ctrl group (p>0.05 for both comparisons). MDA plasma levels significantly increased in the CAD and CAD +OA groups in comparison with the Ctrl group (p=0.010 and p=0.002, respectively). However, no significant difference was observed between the CAD +OA group and the CAD group (p>0.05 for both comparisons). In addition, there was no significant difference regarding the mean level of IFN-γ and NO_x between the studied groups (p>0.05 for both comparisons).

Gene expression of cluster of differentiation (CD) markers

Figure 1 and table 3 demonstrate the comparison of the mean levels of CD9, CD36, and CD68 mRNA expression in the CAD +OA, CAD, and Ctrl groups. The analysis did not reveal any significant difference in CD9, CD36, and CD68 mRNA expression in two-by-two comparisons between the study groups (p>0.05 for all comparisons).

Protein level of CD markers

Figure 2 and table 4 show the comparison of the mean levels of CD9, CD36, and CD68 proteins in the CAD +OA, CAD, and Ctrl groups. No significant differences were observed in terms of CD9, CD36, and CD68 proteins between the three study groups (p>0.05 for all comparisons).

DISCUSSION

The findings of the current study indicated that the plasma level of TNF-α rose significantly in the CAD +OA group in comparison with the CAD and Ctrl groups. However, the results indicated that opium did not affect the expression of CD9, CD36, and CD68 receptors and the plasma levels of MDA, IFN-γ, and NO_x in patients with CAD.

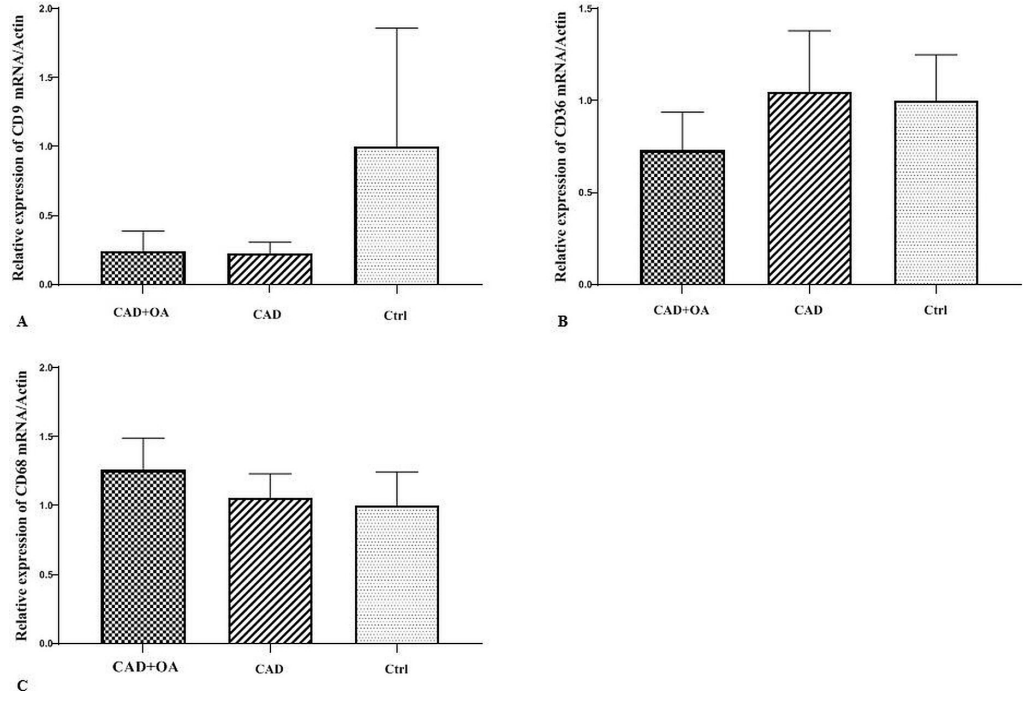

Figure 1 CD9, CD36, and CD68 mRNA expression levels in the CAD+OA, CAD, and Ctrl groups. (A) No statistically significant difference was found in the CD9 mRNA expression level between the three study groups (p>0.05 for all comparisons). (B) No significant difference was found in the CD36 mRNA expression level between the three study groups. (C) No significant difference was found in the CD68 mRNA expression level between the three study groups (p>0.05 for all comparisons). CAD, coronary artery disease; CAD+OA, coronary artery disease and opium addiction; Ctrl, control.

Table 3 Comparison of CD9, CD36, and CD68 mRNA expression between the CAD+OA, CAD, and Ctrl groups

	CAD +OA		CAD		Ctrl			
	Mean	SD	Mean	SD	Mean	SD	P1	P1
CD9	0.24	0.64	0.23	0.23	1.00	3.21	0.959	0.553
CD36	0.73	1.06	1.05	1.74	1.00	0.97	0.127	0.439
CD68	1.26	1.18	1.06	0.93	1.00	0.94	0.659	0.919

P1: CAD+OA vs. Ctrl.
P2: CAD vs. Ctrl.
CAD, coronary artery disease; CAD+OA, coronary artery disease and opium addiction; Ctrl, control.

We found that consumption of atorvastatin, clopidogrel, aspirin, and glyceryl trinitrate was significantly higher in the CAD+OA and CAD groups in comparison with the control group. Given the anti-inflammatory effect of these drugs, the plasma levels of TNF-α were not significantly different in patients with CAD compared with the control group. However, in the CAD+OA group, the plasma levels were significantly higher than in the CAD and control groups. By affecting endothelial cells, triggering growth factors and chemoattractants, and interfering with the thrombotic process, TNF-α assists in the development of CAD.[27] With respect to the effects of opium on TNF-α, Yarahmadzehi *et al*[28] revealed that this cytokine was increased in the brain tissue of opium-treated rats. A study conducted by Zhao *et al*[29] indicated that atorvastatin reduced the serum levels of TNF-α in hypercholesterolemic rabbits compared with controls. In another research conducted by McFarland *et al*,[30] it was demonstrated that statins could decrease TNF-α secretion from the Tohoku Hospital Pediatrics-1 (THP-1) cell line. Furthermore, Al-Bahrani *et al*[31] found that aspirin and clopidogrel decreased the production of TNF-α in the leukocytes of patients who had acute stroke. On the other hand, it has been hypothesized that opium may modulate the production of cytokines by affecting the immune system cells.[17] Although reports about the effects of opium on the immune system are limited, there are numerous studies on the impact of opium derivatives, namely morphine, on the immune system, showing that morphine can induce oxidative stress.[32] Sawaya *et al*[33] indicated that morphine enhanced the expression of TNF-α in U937, astrocytes, and microglia, and such an increase may be mediated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. Moreover, Peng *et al*[34] reported that mice treated with morphine exhibited an enhanced mRNA expression of TNF-α. Therefore, prior studies confirm our hypothesis that opium may be involved in the development of CAD through its effect on the immune system and increasing TNF-α, which is a proinflammatory cytokine. Although the plasma levels of IFN-γ, a proinflammatory cytokine, were higher in the CAD+OA group than the other two groups, the difference between the three groups was not significant. Because IFN-γ is found in high amounts in the atherosclerotic plaque areas,[35–37] and since our subjects received anti-inflammatory drugs, the level of this cytokine in the CAD groups was not significantly different from

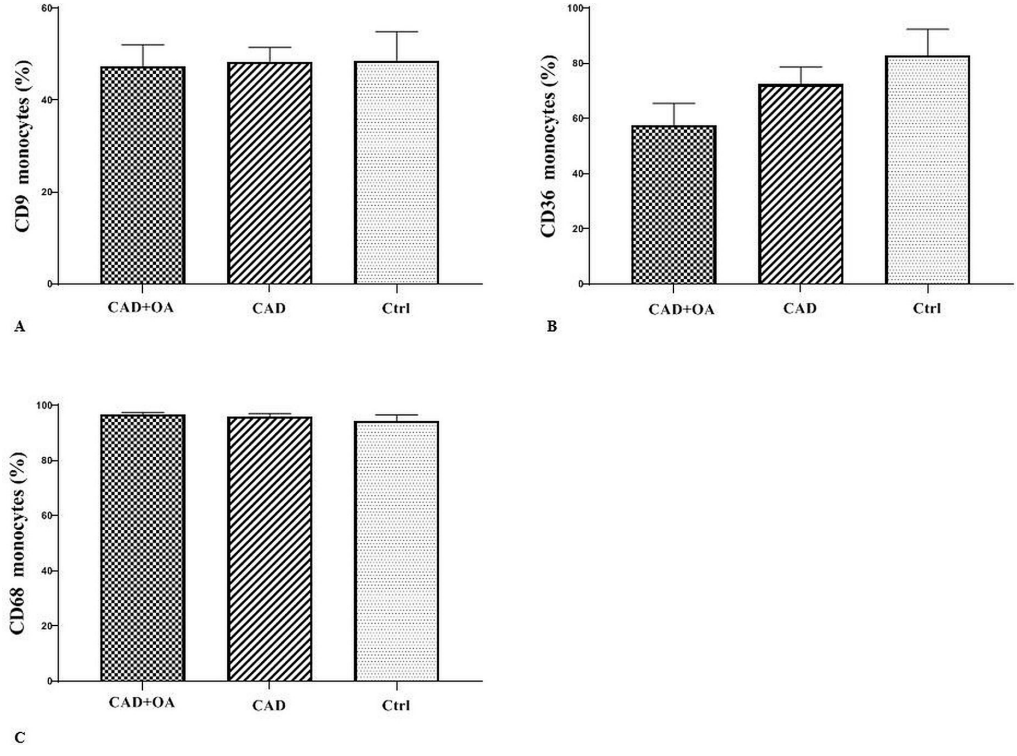

Figure 2 CD9, CD36, and CD68 protein levels in the CAD+OA, CAD, and Ctrl groups. (A) There was no significant difference in the CD9 protein level between the three study groups (p>0.05 for all comparisons). (B) No significant difference was found in the CD36 protein level between the three study groups (p>0.05 for all comparisons). (C) There was no significant difference in the CD68 protein level between the three groups (p>0.05 for all comparisons). CAD, coronary artery disease; CAD+OA, coronary artery disease and opium addiction; Ctrl, control.

Table 4 Comparison of CD9, CD36, and CD68 protein levels between the CAD+OA, CAD, and Ctrl groups

	CAD+OA		CAD		Ctrl				
	Mean	SD	Mean	SD	Mean	SD	P1	P2	P3
CD9	47.33	22.17	48.20	16.91	48.61	19.95	>0.999	0.406	>0.999
CD36	57.59	37.12	72.38	32.67	82.86	29.97	>0.999	0.169	0.658
CD68	96.70	3.19	95.89	5.66	94.24	7.16	>0.999	>0.999	>0.999

P1: CAD+OA vs. CAD.
P2: CAD+OA vs. Ctrl.
P3: CAD vs. Ctrl.
CAD, coronary artery disease; CAD+OA, coronary artery disease and opium addiction; Ctrl, control.

that in the control group. Enayati *et al*[38] showed that the expression of IFN-γ in PBMCs of patients with CAD was not significantly different from that of the control group.

The crucial roles played by the scavenger receptors and the upregulation of the molecules in patients with CAD have been documented previously.[39 40] However, it was observed that mRNA or protein levels of CD36 and CD68, as the scavenger receptors of macrophages, and CD9, as a family member of tetraspanins, did not alter in the studied groups. Thus, according to the present results, it seems that treatment of both CAD and CAD +OA patients with atorvastatin, clopidogrel, aspirin, and glyceryl trinitrate regulated the expression of scavenger receptors independent of opium. Previous in vivo studies have also shown that inflammation increases the expression of CD68 as a scavenger receptor.[41] Since CAD +OA patients and patients with CAD without opium addiction were taking aspirin, this drug reduced inflammation,[42] and consequently the expression of CD68 did not change in monocytes. The findings of a study performed on the human macrophage THP-1 cell line treated with opium were consistent with our results, showing that no significant differences were found in the expression of CD36, CD68, and CD9 at the gene and protein levels.[43] As described previously, CD9 is a family member of tetraspanins that is capable of altering the function of scavenger receptors.[44] In a study on mice, it was shown that CD9 is associated with CD36 at the macrophage level and contributes to the uptake of ox-LDL via CD36. Moreover, in mice lacking the *CD9* gene, lipid accumulation in macrophages was decreased and consequently reduced foam cell formation.[44] Furthermore, it has been shown that CD9 expression is reduced in macrophages due to the effect of lipopolysaccharides (LPS), as inflammatory promoter molecules, and a decrease in the amount of tetraspanin may contribute to the progression of inflammatory diseases. In addition, statins were shown to decrease lung inflammation in mice by increasing macrophage CD9 expression.[45] Therefore, due to the contradictory results, the precise role and function of CD9 in relation to CAD and opium require further investigation.

Given the fact that oxidative stress is involved in CVD development, it can be concluded that oxidative stress is the main cause of MDA production.[46] The current study demonstrated that MDA serum levels significantly increased in CAD or CAD +OA patients; hence, it may be deduced that oxidative stress affects CAD and CAD +OA patients. In an in vivo study, the serum MDA level was higher in rats that received opium than in the control group.[47] Some studies examined MDA levels in patients with CAD and normal individuals. It was shown that the level of MDA in subjects with CAD was nearly twofold higher.[48 49] Thus, our results confirmed the impact of both CAD and addiction to opium on the MDA levels. However, there were no significant differences between CAD and CAD +OA patients, implying that opium addiction does not have synergistic effects with CAD to increase the levels of MDA.

It is believed that NO may have different effects on the cardiovascular system, depending on the isoform that is produced. Studies have shown that reactive oxygen species can react with NO to produce peroxynitrite ($ONOO^-$), thereby reducing NO levels and producing another free radical to cause further damage to cellular components.[50] NO has been implicated in the maintenance of tissue perfusion, protection against LPS-derived toxic lipids, and maintenance of red blood cells in septicemia.[51] In general, it can be said that NO has a dual role in the body, and in addition to its physiological function it can play a pathological role. In a study carried out on opium addicts, the circulating NO level was higher in addicts than in the control group, but the difference was not significant.[52] In another study on subjects with CAD, the mean plasma NO levels were lower than those in the control group.[53] In the study by Kalantarian *et al*,[54] there was no significant difference between serum NO levels in subjects with CAD and the control group. In contrast to those studies, in another investigation, NO was found to be higher in patients with CAD than in the control group.[55] In the present study, opium did not affect the NO_x plasma levels. The NO level was higher in the CAD+OA group than in the Ctrl group even though there was no statistically significant difference. It is assumed that different diets, medications, blood pressure, and the presence or absence of other factors which are not included are effective in oxidative stress and the NO level may be the possible cause of these discrepancies.[56]

Various studies have shown that opium has different and contradictory effects (as a potential risk/protective/impartial factor) on CVD. A population-based study by Sadeghian *et al*[57] revealed that using opium was a risk factor for CAD, excluding cigarette smokers, and they reported the same general findings. However, a study carried out on 460 patients with myocardial infarction showed that the inpatient mortality rate was lower in opium users. Even though this difference was not significant, among patients with anterior wall infarction, the mortality rate was significantly higher in non-opium users.[58] On the other hand, a study conducted by Sharafi *et al*[59] assessing 1-year major adverse cardiac events in 1545 patients did not show a significant difference between patients with and without opium use

disorder. Similarly, Roohafza *et al*[60] claimed that the initial death rate was similar between opium users and non-users.

A number of limitations must be considered when interpreting the present results. These limitations include the fact that it was difficult and time-consuming to select the Ctrl group subjects out of all the candidates for coronary angiography without any criticism of coronary vessel stenosis, as well as to choose the CAD +OA group out of patients who were addicted to opium but did not consume cigarettes. Nevertheless, the current research has several advantages as well. In addition to these novel findings, this investigation evaluated the association between opium addiction, excluding cigarette use, and CAD development and routine medications. Furthermore, in the present study, CAD diagnosis was carried out by coronary angiogram in all the subjects, whereas some other studies identified CAD by an ECG or a thallium scan.

CONCLUSIONS

The findings showed that opium might increase inflammation in the body by increasing the plasma levels of TNF-α, a proinflammatory cytokine, and interfering with anti-inflammatory drugs, and thus opium may be involved in the development of CVD. In addition, the findings revealed that opium did not show a significant effect on the expression of CD36, CD68, and CD9 at the gene and protein levels, which can be due to the consumption of antiplatelets and statins. However, it may be involved in the development of CAD by inducing inflammation through other pathways. Nevertheless, further large-scale studies are needed to reveal the exact mechanism and effects of opium and routine medications on the expression of these genes.

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