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## Effects of Cigarette Smoke and Opium on the Expression of CD9, CD36, and CD68 at mRNA and Protein Levels in Human Macrophage Cell Line THP-1

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### ABSTRACT

Cigarette smoking and opium use are risk factors for coronary artery disease (CAD). It has been known that scavenger receptors such as CD36 and CD68 play critical roles in the pathogenesis of CAD. CD9, as a member of the tetraspanin, has been shown to interact with scavenger receptors. The aim of this study was to investigate the effects of these risk factors on expression levels of CD9, CD36, and CD68 on the THP-1 cell line.

The THP-1 cell line treated with cigarette smoke extract (CSE) and opium, both individually and combinatory, in 24 h incubation. The protein and mRNA levels of CD9, CD36, and CD68 were evaluated by flow cytometry and quantitative reverse transcription-Polymerase Chain Reaction (qRT-PCR) techniques, respectively.

CD36 and CD68 mRNA and protein expression levels were significantly increased in the cells treated with cigarette smoke extract compared to the control ( $p < 0.001$  in mRNA expression levels and  $p = 0.016$  and  $p = 0.012$  in protein expression levels, respectively). The CSE increased the level of CD9 protein expression compared to the control group ( $p = 0.041$ ) on the human macrophage cell line THP-1. No significant differences were observed in the CD9, CD36, and CD68 gene expression and at the protein levels between opium-treated THP-1 cells and controls.

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In conclusion, cigarettes by increasing the levels of CD36, CD68, and CD9 can be a risk factor in the development of many inflammatory diseases, including cardiovascular diseases, chronic obstructive pulmonary disease (COPD) and lung carcinoma.

**Keywords:** Cigarette; CD9; CD36; CD68; Coronary artery disease; Opium

## INTRODUCTION

Cigarette smoking contributes to around 90% cases of lung cancers worldwide and represents a major risk factor for the development of several diseases in both men and women including, chronic obstructive pulmonary disease (COPD), asthma, myocardial infarction (MI) and fatal coronary artery disease (CAD).<sup>1</sup>

Cigarette smoke-induced oxidative stress activates the endothelium as well as macrophages and platelets by the induction of adhesion molecules expression including intracellular adhesion and vascular cell adhesion molecules. Endothelial cells release inflammatory and pro-atherogenic cytokines. After adhesion and transendothelial migration, macrophages take up oxidized lipids produced by oxidative modification through smoke-increased reactive oxygen species (ROS) production. Scavenger receptor-mediated uptake of the oxidized lipids, which leads to induction of foam cells formation within the artery wall, and subsequent death of foam cells induces the release of these lipids and the formation of lipid-rich artery plaques.<sup>2,3</sup>

In addition to the prevalence of cigarette smoking worldwide, in some Asian and Middle Eastern countries, opium ingestion or inhalation has traditionally been considered as a way to lower blood glucose and lipids medicine and to prevent many disorders like heart diseases.<sup>4,5</sup> There are studies that have not shown any association between opium consumption and atherosclerosis<sup>6,7</sup> but the result of some studies indicates that opium consumption is a significant risk factor for CAD.<sup>8,9</sup>

In vitro and ex vivo studies on mononuclear cells showed that cigarette smoke increased the expression of numerous markers, such as scavenger receptors (CD36, CD68), pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF- $\alpha$ ), and endothelial adhesion molecules.<sup>10-12</sup> CD36 is a type 2 scavenger receptor with multiple functions. CD36 binding to oxidized-LDL (ox-LDL) triggers signaling cascades that are required for macrophage foam cell

formation.<sup>10-12</sup> CD68, macrosialin, is a highly glycosylated membrane protein, which is widely expressed on mononuclear phagocytes.<sup>13</sup> CD68 expression appeared to correlate with macrophage activation.<sup>14-16</sup> CD68 was found to predominately localize to late endosomal and lysosomal compartments with a modest level of cell surface expression. Its ability to bind ox-LDL and phosphatidylserine is cited as evidence that CD68 served as a scavenger receptor.<sup>17,18</sup>

CD9, as a member of the tetraspanin family, is expressed in the various cells, such as platelets and macrophages.<sup>19</sup> The previous study showed that CD9 and CD36 co-associate on the macrophage surface, suggesting that CD36 may be part of the tetraspanin web.<sup>20</sup> Loss of this association by genetic deletion of CD9 led to a modest but statistically significant decrement in CD36 mediated signaling in response to ox-LDL and a concomitant modest decrease in lipid accumulation and foam cell formation.<sup>19,20</sup> Thus, CD9 expression may be associated with the expression of scavenger receptors like CD36.

To the best of our knowledge, no study has been reported to evaluate the effects of cigarette smoke extract (CES) and opium on expression levels of CD9, CD36, and CD68. On the other hand, since most opium addicts are cigarette smoker and due to the information presented here, the aim of this project was to evaluate the alone and in combination effects of CSE and opium on the expression levels of CD9, CD36 and CD68 at both mRNA and protein levels on human macrophage cell line THP-1 in an in vitro study.

## MATERIALS AND METHODS

### Preparation of CSE

The mainstream smoke from camel cigarettes was drawn into 35 mL of pre-warmed Phosphate-buffered saline (PBS) by a vacuum. Each cigarette was lit for 5 min, and five cigarettes were used per 35 ml of PBS to generate a CSE-PBS solution<sup>21-23</sup> and this solution was considered as the concentration 100% CSE. The pH of

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CSE was adjusted to 7.2-7.4, and the solution was sterile-filtered through a 0.22- $\mu$ m filter. The CSE was diluted with medium and used immediately for treatment.

### Preparation of Opium Solution

Opium was received from the anti-narcotics Police Department of Kerman (Iran). Gas chromatography-mass spectrometry (GC-MS) analysis showed that the opium was made of more than 30% alkaloids of different types (i.e. 16% morphine, 5.5% codeine, 4.4% thebaine, and 3.2% papaverine), non-alkaloid organic compounds and water. To create a stock solution (2.86 g/mL), based on our previous study,<sup>24</sup> 150 mg powdered opium was dissolved in 1ml PBS. Afterward, the supernatant was obtained using centrifugation (1200 rpm, 10 minutes) and filtered through the 0.22- $\mu$ m filter. 10  $\mu$ L from opium stock added to 10 ml PBS, 2.86 $\times$ 10<sup>-5</sup> g/mL RPMI solution was prepared. The computations were done based on our previous study<sup>24</sup> and 2.86 $\times$ 10<sup>-4</sup> g/mL opium concentration in our previous study led to significant induction of apoptosis in certain cell lines (i.e. AA8, N2a, WEHI).

### Cell Culture, Treatment and Viability Assessment

The human macrophage cell line THP-1 (was obtained from national cell bank, Pasteur Institute of Iran, Tehran, Iran) was grown in RPMI-1640 (BioSera, France) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco-Thermo Fisher Scientific, Waltham, MA, USA), 1% Penicillin-Streptomycin (Sigma-Aldrich, Saint Luis, MO, USA) in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. For toxicity evaluation of CSE and opium, MTT assay was performed.<sup>25</sup> Different

concentrations of CSE and opium solutions were prepared and cells were incubated for 24 h (Figure.1). The concentration of 0.625% from CSE and 3.5 $\times$ 10<sup>-6</sup> g/mL opium concentration, both individually and combinatory, were selected. The human macrophage cell line THP-1 was incubated in a CO<sub>2</sub> incubator at 37°C for 24 h.

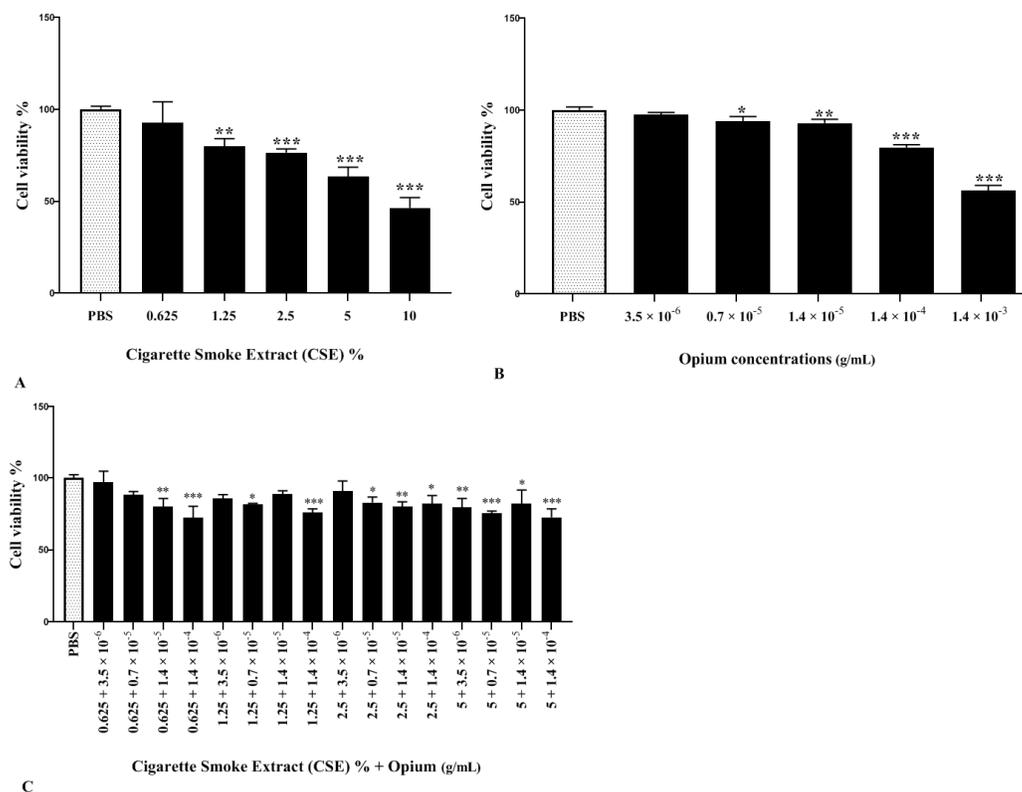
### Relative Expression of CD9, CD36, and CD68 by qRT-PCR

After treatments of THP-1 cells with CSE and opium, the separation of cells was done by Trypsin and total RNA was extracted using RNX (SinaClon, Iran) and cDNA was constructed using Takara cDNA Synthesis Kit for qRT-PCR (Japan). The amounts of RNAs purified from cells qualitatively and quantitatively were determined using Nanodrop spectrophotometer (ND-1000, ThermoFisher Scientific, Wilmington, USA). cDNA was synthesized by the cDNA Synthesis kit (Catalog Number: 6110A, Takara Bio, California, USA). The primers used for this study are listed in Table 1.

qRT-PCR was performed,<sup>26</sup> using RealQ Plus Master Mix Green (Ampliqon, Odense, Denmark) in Real-Time PCR machine (Mic, Australia) using the following program: for CD9 gene, initially denaturation 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 and final extension at 72°C for 5 min. Real-time PCR for CD36, CD68, and beta-actin were 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

**Table 1. Primers used for quantitative reverse transcription-Polymerase Chain Reaction (qRT-PCR)**

Gene		Primers
CD9	Forward	5'- TCATGATGCTGGTGGGCTTC-3'
	Reverse	5'- GGAATATCCCCAGATGGCCG-3'
CD36	Forward	5'- ATGTGCAAAATCCACAGGAAGTG-3'
	Reverse	5'- GCCACAGCCAGATTGAGAAC-3'
CD68	Forward	5'- CTACATGGCGGTGGAGTACAA-3'
	Reverse	5'- GAATGATGCTCGAGTTGCTGC-3'
Beta-Actin	Forward	5'- TGGCACCCAGCACAATGAA-3'
	Reverse	5'- CTAAGTCATAGTCCGCTAGAAGCA-3'



**Figure 1.** The percentage of cell viability after 24 h incubation with (A) cigarette smoke extract (CSE), (B) opium concentrations and (C) combination of CSE and opium vs Phosphate-buffered saline (PBS) solution as control. The difference between the percentage of cell lines viability is significant ( $p < 0.001$ ). \*  $p$ -value  $< 0.05$  in compare with PBS, \*\*  $p$ -value  $< 0.01$  in compare with PBS, \*\*\*  $p$ -value  $< 0.001$  in compare with PBS.

(35 s) at 60°C and final extension for 5 min at 72°C. The expressions of these molecules were computed using the  $2^{-\Delta\Delta CT}$  formula.

#### CD9, CD36, CD68 Protein Quantification by Flow Cytometry

Flow cytometry was performed using FACS Calibur cytometers (BD Biosciences, USA) running CellQuest (BD Biosciences, USA) acquisition and analysis software. To evaluate CD36, CD68, and CD9 expression, treated cells were isolated from flask by Trypsin and stained using the following antibodies: (1) PerCP-Cy<sup>TM</sup>5.5 Mouse anti-Human CD9 (clone: M-L13, isotype: Mouse BALB/c IgG1,  $\kappa$ , BD Biosciences, USA) and PerCP-Cy<sup>TM</sup>5.5 Mouse IgG1  $\kappa$  Isotype Control (clone: MOPC 21, isotype: Mouse IgG1,  $\kappa$ , BioLegend, USA), (2) FITC Mouse Anti-Human CD36

(clone: CB38, isotype: Mouse IgM,  $\kappa$ , BD Biosciences, USA) and FITC-conjugated isotype-matched negative control (clone: G155-228, isotype: Mouse BALB/c IgM,  $\kappa$ , BD Biosciences, USA), (3) PE Mouse Anti-Human CD68 Set (clone: Y1/82A, isotype: Mouse IgG2b,  $\kappa$ , BD Biosciences, USA), and PE Mouse IgG2b  $\kappa$  Isotype Control (clone: 27-35, isotype: Mouse IgG2b,  $\kappa$ , BD Biosciences, USA), and (5) APC anti-human CD14 Antibody (clone: M5E2, isotype: Mouse IgG2a,  $\kappa$ , BioLegend, USA) and appropriate APC Mouse IgG2a,  $\kappa$  Isotype Ctrl Antibody (clone: MOPC-173, isotype: Mouse IgG2a,  $\kappa$ , BioLegend, USA) to determine the monocytes.

#### Data Analysis

Data analysis was performed using GraphPad Prism software (version 8). The data were presented as mean

and standard deviation (SD). The differences among groups were evaluated using a one-way analysis of variance (ANOVA)/Kruskal-Wallis with posthoc Tukey. In this study,  $p$ -values less than 0.05 were considered to be statistically significant.

## RESULTS

### Cell Viability

Figure 1 depicts the viability of THP-1 cells treated with (A) CSE, (B) opium concentrations and (C) combination of CSE and opium vs. PBS solution as a control. As a result, 0.625% CSE concentration and  $3.5 \times 10^{-6}$  g/mL opium concentration, both individually and combinatory, were selected for the treatment of cells. However, no significant differences were found in the cell viability in the mentioned concentrations in comparison to the control group.

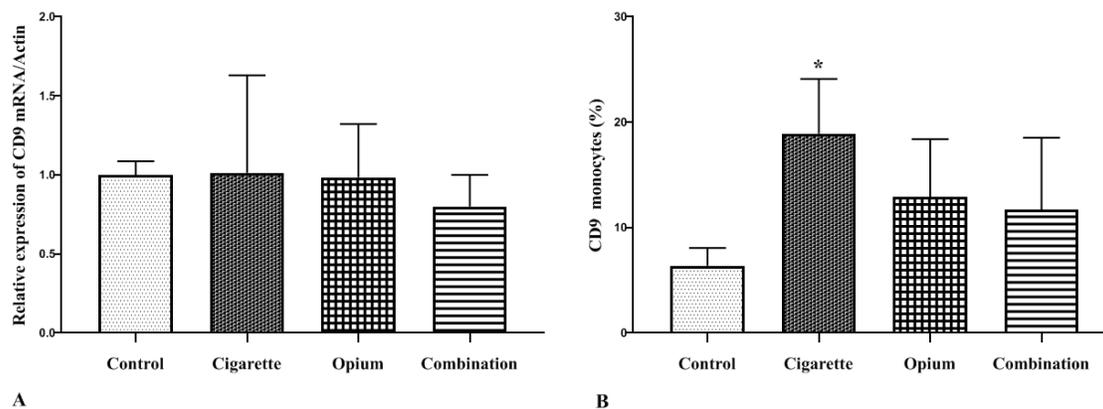
### CD9 Gene Expression and Protein Levels

Figure 2 shows the comparison of mean levels of CD9 protein and mRNA expression among treated THP-1 cells with CSE, opium, and the combination of CSE and opium. The analysis showed no significant difference between cells treated with opium and the control cells with respect to CD9 mRNA expression and at the protein levels ( $p > 0.999$  and  $p = 0.418$ , respectively). No significant differences were found

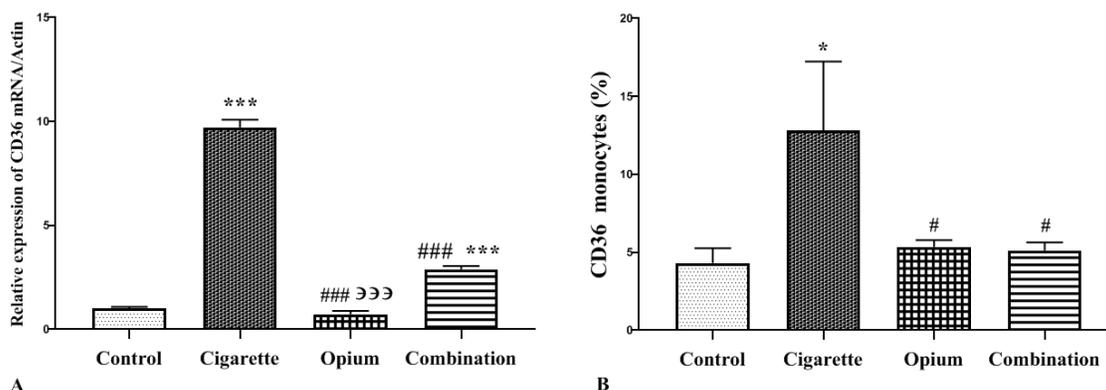
in the mean levels of CD9 mRNA expression in monocytes treated with the CSE ( $p > 0.999$ ), while the protein level of CD9 was found higher than control cells ( $p = 0.041$ ). Regarding the treatment of monocytes with opium and CSE simultaneously, no significant differences were found both in the CD9 mRNA expression and at the protein levels ( $p = 0.912$  and  $p = 0.647$ , respectively).

### CD36 Gene Expression and Protein Levels

CD36 gene expression of THP-1 cells at the protein levels among the study groups are provided in Figure 3. CSE, both individually and in combination with opium, significantly up-regulated CD36 gene expression compared to the control ( $p < 0.001$ ), but, at the protein level, only significant difference was found in monocytes treated with CSE in comparison with the control, opium and combination groups ( $p = 0.016$ ,  $p = 0.031$ , and  $p = 0.049$ , respectively). No significant differences were observed in the CD36 gene expression and at the protein levels between opium-treated THP-1 cells and controls ( $p = 0.470$  and  $p = 0.965$ , respectively). Regarding the gene expression levels of CD36, the pairwise comparison between groups showed that the combination treatment (CSE+opium) led to a further increase in the expression of mRNA of CD36 when compared with opium and control groups ( $p < 0.001$ ).



**Figure 2.** CD9 gene expression and protein levels among treated cells with cigarette smoke extract (CSE), opium, and combination of CSE and opium. (A) There were no significant differences in the CD9 gene expression among treated cells ( $p = 0.734$ ). (B) The analysis did not show any significant differences in CD9 protein levels between treated cells ( $p = 0.058$ ). \*  $p$ -value  $< 0.05$  in comparison to the control group.

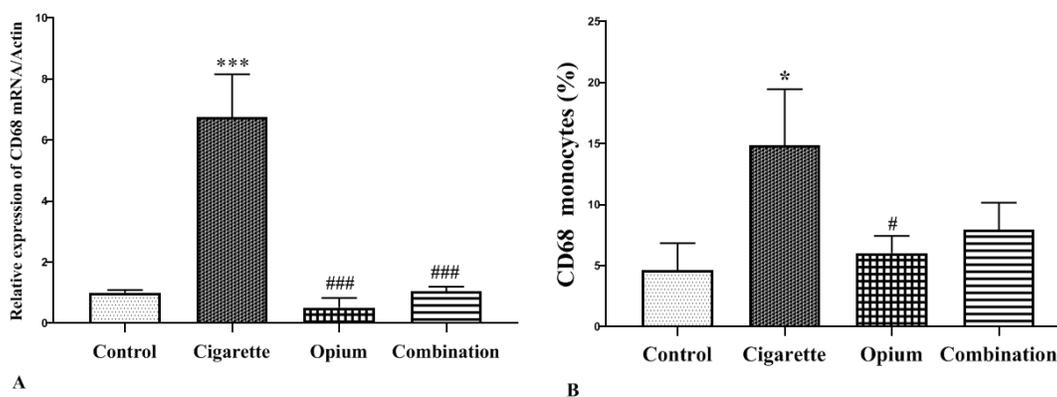


**Figure 3. CD36 gene expression and protein levels among treated cells with cigarette smoke extract (CSE), opium and combination of CSE and opium. (A) There were significant differences in CD36 gene expression among treated cells ( $p < 0.001$ ). (B) There were significant differences in CD36 protein levels among treated cells ( $p = 0.011$ ). \*  $p$ -value  $< 0.05$  in compare with control group, \*\*\*  $p$ -value  $< 0.001$  in compare with control group, #  $p$ -value  $< 0.05$  in compare with cigarette group, ###  $p$ -value  $< 0.001$  in compare with cigarette group, ###  $p$ -value  $< 0.001$  in compare with combination group.**

#### CD68 Gene Expression and Protein Levels

Figure 4 depicts the comparison of mean levels of CD68 mRNA expression and the amount of protein among THP-1 cells treated with CSE, opium, and the combination of CSE and opium. CD68 mRNA expression levels were significantly increased in monocytes treated with CSE in comparison with control, a combination of opium and CSE and opium groups ( $p < 0.001$ ). Moreover, the CSE, individually,

increased the expression of CD68 protein levels compared with opium and control groups ( $p = 0.027$  and  $p = 0.012$ , respectively). However, no significant differences were found in the levels of CD68 mRNA expression and amount of protein in cells treated with opium, both separately and combined, compared control group. ( $p = 0.845$  and  $p = 0.999$  in mRNA expression levels and  $p = 0.947$  and  $p = 0.677$  in protein expression levels, respectively).



**Figure 4. CD68 gene expression and protein levels among treated cells with cigarette smoke extract (CSE), opium, and combination of CSE and opium. (A) There were significant differences in CD68 gene expression among treated cells ( $p < 0.001$ ). (B) There were significant differences in CD68 protein levels among treated cells ( $P = 0.011$ ). \*  $p$ -value  $< 0.05$  in compare with control group, \*\*\*  $p$ -value  $< 0.001$  in compare with control group, #  $p$ -value  $< 0.05$  in compare with cigarette group, ###  $p$ -value  $< 0.001$  in compare with cigarette group.**

## DISCUSSION

In this study, we investigated the effect of cigarette and opium, both individually and combinatory protocol, on the mRNA expression and protein levels of CD9, CD36, and CD68 in human macrophage cell line THP-1. The CSE, not opium, increased the expression of the scavenger receptors, CD36 and CD68, at the gene and protein levels. Also, CD9 protein expression was increased by the CSE in THP-1 cells.

One of the main causes of morbidity and mortality, through CAD, in the world is cigarette smoking.<sup>27</sup> Numerous researches have revealed that cigarette smoking is related to inflammation and oxidative stress.<sup>28</sup> Cigarette comprises more than 4000 known components. These include nicotine, ammonia, polyphenols, carbon monoxide, nitrogen oxides, and etc. In addition to cigarette smoking, the studies have shown that the use of opium has a direct correlation with cardiovascular disease, which can increase the risk of inflammation and oxidative stress, both of which contribute to the development of atherosclerotic plaques.<sup>29</sup> Opium addiction is common in many Asian countries. One of the reasons for the high prevalence of the opium consumption is the traditional rumor among people that may have a positive effect on cardiovascular health, diabetes, high blood pressure, dyslipidemia and etc.<sup>29</sup> To the best of the authors' knowledge, no research has been performed to evaluate the effects of simultaneous exposure of CSE and opium on expression levels of CD9, CD36, and CD68 yet. The results of the current study revealed that cigarettes significantly up-regulated the expression of CD36 at the gene and protein levels. The macrophages were taking-up the ox-LDL via scavenger receptors, such as

CD68 and CD36. It has been documented that either CD36 or CD68 plays a critical role in the pathogenesis of CAD and its complications such as atherosclerosis.<sup>30</sup> LDL is converted to ox-LDL through oxidative stress, and ox-LDL stimulated gene expression of CD36 in macrophage.<sup>31,32</sup> Therefore, one of the inducers CD36 expression is oxidative stress. It has been reported that cigarette by increasing the pro-oxidant and decreasing anti-oxidant factors raises the production of ROS and oxidative stress.<sup>33</sup> Investigations have revealed that nicotine, as one of the main cigarette components, increases CD36 by binding to nicotinic receptors at the

cells surface of human THP1 macrophages and peripheral blood mononuclear cells (PBMCs).<sup>34</sup> Also, nicotine significantly increases ROS, and these reactive species up-regulate CD36 expression in THP1 macrophages.<sup>34</sup> It has been indicated that morphine and heroin, as opium derivatives, induce systemic oxidative stress,<sup>29</sup> but opium contains more than 20 alkaloids and more than 70 components<sup>35</sup> and these compounds may have agonistic or antagonistic effects on morphine in relation to oxidative stress. Like oxidative stress, cigarettes activates an immunologic response that is concomitant with increased levels of inflammatory markers, such as white blood cell count and C-reactive protein.<sup>36-38</sup> Results obtained from a study on mice treated with lipopolysaccharide (LPS), as an inducer of inflammation, showed that CD36 expression up-regulated in macrophage.<sup>39</sup> Hashimoto et al also showed that LPS enhances expression of CD36 in murine bone marrow macrophages.<sup>40</sup> On the other hand, studies have been shown that CD36 plays an important role in the activation of pro-inflammatory pathways and generation of oxidative stress.<sup>41,42</sup> Therefore, it can be hypothesized that one of the pathways of inducing inflammation and oxidative stress by cigarettes may be through CD36. In the present study, in contrast to cigarette, either mRNA or protein levels of CD36 and CD68 in cells treated with opium was not altered in comparison to the control group. Unlike the cigarette, there are controversial results in relation to opium and inflammation. For example, Asadikaram et al shown the secretion of transforming growth factor-beta (TGF- $\beta$ ), that plays either anti-inflammatory through an enhanced number of T regulatory lymphocytes, or pro-inflammatory through the expansion of T helper 17 cells (Th17) lineage, reduced in Jurkat cells treated with opium.<sup>43</sup> However, Ayatollahi-Mousavi et al revealed that the plasma levels of TGF- $\beta$  increased in opium addicts.<sup>44</sup> Moreover, another study by Peng et al revealed that morphine increased the mRNA expression of TNF- $\alpha$ , pro-inflammatory cytokine, in mice compared to controls.<sup>45</sup> Martucci et al indicated that Interleukin-1  $\beta$  (IL-1 $\beta$ ), as an anti-inflammatory cytokine, reduced in the morphine-treated mice.<sup>46</sup>

The present consequences displayed that cigarette, but not opium increased CD68 gene expression and the protein levels. Similar to CD36, *In vivo* investigations have been reported that inflammatory stimuli lead to the up-regulation of CD68, as a marker of activated

macrophages, on the macrophages<sup>47</sup> and as the scavenger receptors of macrophages, is involved in harvesting ox-LDL and thus effect on atherogenic foam cell formation.<sup>48</sup> John et al in a study conducted on female C57BL/6 mice exposed to CSE showed that CD68 mRNA expression elevated in lung tissue.<sup>49</sup> In contrast to the present findings, Luo C et al showed that high Uric acid, as a natural free radical scavenger, through oxidative stress, disrupted CD68 expression in THP-1 cells.<sup>50</sup> Therefore it may be concluded that inflammation, but not oxidative stress, increased the expression of CD68.

Numerous studies have shown that there are several signaling pathways for scavenger receptors and their performance by interacting with other receptors such as tetraspanins.<sup>30,51</sup> The current results showed that CD9 increased in protein levels on the THP-1 cells treated with the CSE in comparison to controls. It has been revealed that macrophage CD9 is downregulated by LPS<sup>52</sup> and reduction in the levels of this tetraspanin may be involved in the progression of inflammatory diseases.<sup>53</sup> In agreement with this hypothesis, Jin and et al presented that statins execute anti-inflammatory effects through upregulating macrophage CD9.<sup>53</sup> However, the exact functions of CD9 are controversy and there are some reports, which proved the pro-inflammatory roles played by CD9. For instance, it was revealed that the expression of CD9 in the rats treated with morphine group was more than the control group.<sup>54</sup> Huang and et al also showed that CD9 expression has a positive association with CD36 at mice macrophage and is involved in gathering ox-LDL through CD36 and also lipidic accumulation in the *cd9* null macrophages is impaired, which leads to a reduction in the development of foam cell.<sup>20</sup> Thus, it appears that CD9 functions may be dependent on several factors that may act in a network manner. Although due to funding limitation the present study suffered from some limitations, such as; treatment of human macrophage cell line THP-1 was not performed at different times and different concentrations of opium and CSE, and also, with the main components of opium (morphine, papaverine, codeine, and noscapine) and cigarette (nicotine, ammonia, polyphenols, carbon monoxide, nitrogen oxides). However, our study revealed an important mechanism for cigarette induced disorders. In conclusion, CSE increased the expression of CD36 and CD68 at the gene and protein levels and CD9 expression at the protein level. Therefore SCE

may play an important role in the pathogenesis and development of many inflammatory diseases, including cardiovascular diseases, COPD and lung carcinoma through up expression of CD36, CD68, and CD9. Furthermore, our results revealed that opium has not shown significant effect on the expression of CD36, CD68 and CD9 at the gene and protein levels at least at the time and dose which design in the present study, hence the role of opium in development of inflammatory diseases such as cardiovascular diseases in human (which reported by some researchers) are mediated by other mechanisms.

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