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Original Research Article

Cytoprotective effects of rosmarinic acid against cigarette smoke extract-induced cytotoxicity in H9C2 cardiomyocytes are mediated through modulation of the *NLRP3*/*NF-κB* inflammatory signaling pathway

Narges Atefipour[1,2], Mahin Dianat[1,3,*], Zahra Mansouri[1], Fereshteh Nejaddehbashi[1], Fatemeh Dianat[4]

[1]*Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[2]*Medicinal Plant Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.*
[3]*Persian Gulf Physiology Research Center, Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.*
[4]*Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*

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**** Corresponding Author:***
Tel: +989163110437
Fax: +986133337370
dianat@ajums.ac.ir

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Abstract

Objective: Cigarette smoke contains toxic substances that cause oxidative stress and damage to cardiac tissues. Mitochondrial dysfunction and inflammasome activation contribute to smoking-related cardiovascular diseases. This study investigated the preventive effects of rosmarinic acid (RA) on oxidative stress and inflammation induced by cigarette smoke extract (CSE).

Materials and Methods: H9C2 cells were divided into seven groups: Negative control: Phosphate buffered saline (100 µM), Positive control: H_2O_2 (100 µM), CSE: (10%), CSE+RA (5, 10, and 25 µM) and RA 25 (25 µM). Cell viability, oxidative stress (Superoxide dismutase, Glutathione peroxidase, and Catalase), lipid peroxidation, and pro-inflammatory and anti-inflammatory factors (IL-1β, IL-18, and IL-10), and gene expressions (*NF-κB* and *NLRP3*) were evaluated.

Results: CSE reduced antioxidant defenses, increased IL-1β and IL-18, and decreased IL-10. Additionally, pro-inflammatory genes *NF-κB* and *NLRP3* were significantly expressed. This condition was associated with a decrease in cell viability. RA demonstrated a significant protective effect against the detrimental impacts of CSE on H9C2 cells.

Conclusion: Exposure of cardiac cells to CSE induces cytotoxicity through increased oxidative stress and activation of inflammatory pathways. RA, as a natural antioxidant, not only alleviates oxidative damage but also exhibits anti-inflammatory and cytoprotective effects. These benefits may arise from its ability to modulate inflammatory mediators and enhance cellular resilience against stress.

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Introduction

As reported by the World Health Organization (WHO), cardiovascular diseases (CVDs) represent the foremost cause of mortality worldwide, claiming approximately 17.9 million lives annually (Organization 2021). Epidemiological studies have shown that smoking is a significant risk factor for CVDs, along with lung cancer and emphysema. Many studies have shown a link between oxidative stress and the pathogenesis of most chronic diseases caused by tobacco smoke (Das et al. 2012; Tsai et al. 2020). Cigarette smoke (CS) is a complex mixture of approximately 4,000 chemicals, including free and long-lived radicals. Studies have shown that oxidative stress caused by free radicals significantly contributes to cardiac and vascular endothelial cell damage and can lead to pathological cardiovascular events (Nagler et al. 2020).

Oxidative stress occurs when there is an imbalance between free radical production and the body's antioxidant defenses, leading to damage to crucial biomolecules including lipids, proteins, and nucleic acids. The body counters oxidative stress with various enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). SOD converts superoxide radicals into hydrogen peroxide, which CAT then breaks down into oxygen and water, thus reducing oxidative damage (Jomova et al. 2024; Owumi et al. 2020). Reactive oxygen species (ROS) can interact with different biomolecules, particularly lipids, proteins, and nucleic acids, and can cause lipid peroxidation, resulting in harmful by-products like malondialdehyde (MDA) which is a marker for increased oxidative stress (do Nascimento et al. 2023; Lismont et al. 2019).

Inflammasomes are intracellular receptors in the cytosol that detect microbial pathogens and signals from cellular stress, leading to inflammatory responses. Upon formation, inflammasomes activate the protease caspase-1 which promotes the release of pro-inflammatory cytokines like interleukin-1β (IL-1β) and interleukin-18 (IL-18), attracting immune cells such as macrophages to the affected tissues (Chang 2023). The NLR family pyrin domain containing 3 (*NLRP3*) inflammasome specifically plays a significant role in infectious diseases by activating these inflammatory responses. Its activation is upregulated by the transcription factor nuclear factor kappa-B (*NF-κB*) in response to cytokines and microbial factors. Triggers like adenosine triphosphate (ATP) and viral RNA can stimulate *NLRP3*, often through potassium ion efflux, leading to the conversion of pro-IL-1β and pro-IL-18 into their active forms, thus initiating a robust inflammatory response (Dianat et al. 2025; Péladeau and Sandhu 2021).

Rosmarinic acid (RA) is a phenolic compound belonging to the *Boraginaceae* species and *Lamiaceae* subfamily. Owing to its potent antioxidant properties, RA has garnered considerable attention in scientific research. In regions such as Japan, India, and Southern Europe, RA is traditionally employed to address a variety of health conditions including digestive disorders, cardiovascular diseases, and diabetes. In addition to its antioxidant effects, RA exhibits anti-inflammatory, anti-tumor, antibacterial, and antiviral properties. Studies have demonstrated the protective effects of RA in the contexts of liver and lung fibrosis, as well as in the management of cardiovascular diseases (Atefipour et al. 2024b; Kim et al. 2005; Rahbardar et al. 2022).

The H9C2 cell line is widely used in cardiovascular research because it retains many characteristic features of adult cardiomyocytes including relevant signaling pathways and metabolic functions, and has been extensively utilized in studies of oxidative stress and drug-induced cardiotoxicity, and for evaluation of cardioprotective compounds (Watkins et al. 2011).

Despite the established link between smoking and cardiovascular damage, the precise molecular mechanisms involving the *NLRP3* inflammasome and *NF-κB* signaling in cigarette smoke extract (CSE)-induced cardiomyocyte injury are not fully defined. Moreover, there is a lack of evidence regarding the efficacy of natural anti-inflammatory agents, such as RA, in targeting this specific inflammatory cascade in cardiac cells.

To address these gaps, the present study aimed to: (1) definitively characterize the activation of the *NLRP3*/*NF-κB* pathway in H9C2 cardiomyocytes following CSE exposure, and (2) evaluate the therapeutic potential of RA in alleviating CSE-induced cytotoxicity and inflammation by modulating this pathway.

Materials and Methods

The protocol was approved by the Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (grant No. CMRC-0232), and the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1402.460).

Cell treatments and preparation of cell lysates

Embryonic BD1X rat heart tissue cell line (H9C2) was obtained from the Pasteur Institute of Iran (IPI). Then, the cells were divided into seven independent treatment groups (n=3):

Negative control group: Phosphate buffered saline (100 µM; 24 hr).

Positive control group: H_2O_2 as a well-known oxidant (100 µM; 24 hr).

CSE group: CSE 10% (24 hr) (Panayiotidis et al. 2004)

CSE+RA 5: CSE 10% + 5 µM RA (24 hr) (Ghaffari et al. 2014)

CSE+RA 10: CSE 10% + 10 µM RA (24 hr) (Ghaffari et al. 2014)

CSE+RA 25: CSE 10% + 25 µM RA (24 hr) (Ghaffari et al. 2014)

RA: 25 µM RA (24 hr).

In this study, the H9C2 cell line was grown in T25 flasks at a density of 5×10^5 cells/ flask in Dulbecco's Modified Eagle Medium (DMEM) (Bio-Idea Co., Iran), supplemented with 15% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated at 37°C in a humidified incubator with 5% CO_2. Following this, the cells were divided into various treatment groups. The positive control group received an exposure to 100 µM H_2O_2 (Sigma-Aldrich Co., USA). Experimental groups were treated with CSE 10% and various concentrations of RA (5, 10, and 25 µM) (Sigma-Aldrich Co., USA). Cell lysates and culture supernatants were subsequently collected from each group for further analysis.

Preparation of CSE

For this purpose, Winston Red Cigarettes (R.J. Reynolds Tobacco Company, USA), which contain 1 mg of nicotine and 14 mg of tar, were utilized. The filter was removed from the cigarettes, and the smoke was generated by a peristaltic pump set at a low speed such that approximately 60 mm of the cigarette was burned over 15 min, and then bubbled into a flask containing 25 mL of culture medium.Subsequently, the pH of the medium was adjusted to approximately 7.4. A syringe equipped with a 0.2 µm pore diameter filter was employed to eliminate bacteria and larger particles from the smoke extract. Following this, specific amounts of streptomycin and penicillin were added. The resulting culture medium was considered to contain 100% CSE which was then diluted to the desired concentration of 10% (Panayiotidis et al. 2004).

Cell viability

A colorimetric MTT assay was conducted to assess the viability of H9C2 cells and the cytotoxic effects of CSE. To

identify the optimal dosage of RA, H9C2 cells were plated in 96-well plates at a density of 5×10^3 cells/well and subsequently treated with CSE and varying concentrations of RA + CSE. Following a 24-hr incubation period, 30 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well, and the cells were further incubated at 37°C for an additional four hours. After incubation, the medium was removed, and the resulting formazan crystals were dissolved in 150 µl of dimethyl sulfoxide (DMSO). The absorbance was measured at a wavelength of 570 nm using an ELISA reader (ELx808 Absorbance Microplate Reader, ELISA Technologies Inc., USA). Cell survival was quantified using the formula: (Optical density test / Optical density control) × 100 (Benov 2021; Dianat et al. 2025).

Analysis of oxidative stress

Lipid peroxidation was assessed by measuring MDA levels using the thiobarbituric acid reactive substances (TBARS) assay, as described by De Leon and Borges (De Leon and Borges 2020). Briefly, the samples reacted with thiobarbituric acid (TBA). The absorbance of the resulting pink chromogen was measured at 532 nm. The concentration of MDA was calculated using a standard curve prepared from known concentrations of MDA. The xanthine/xanthine oxidase system was used to measure SOD activity according to the method explained by Cheung et al. (Cheung et al. 2008). The measurement was done at a wavelength of 340 nm. The enzymatic function of GPx was assessed using the methods described by Paglia and Valentine (Paglia and Valentine 1967), and the absorbance was measured at a wavelength of 340 nm. CAT activity was determined by measuring the breakdown of H_2O_2 at a wavelength of 240 nm, following the procedures outlined by Aebi (Aebi 1974).

ELISA

The concentrations of IL-1β, IL-18, and IL-10 in cell culture supernatants were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (SunLong Biotech, China). The assays were performed according to the manufacturer's instructions using a sandwich ELISA protocol. Briefly, 50 µl of standards or undiluted samples was added to the antibody-pre-coated microplate wells. After incubation for 30 min at 37°C, the plates were washed five times. Then, 50 µl of horseradish peroxidase (HRP)-conjugated detection antibody was added to each well and incubated for another 30 min at 37°C. Following a second wash step, 50 µl of chromogen substrate solutions A and B was added to each well and incubated for 15 min at 37°C in the dark. The reaction was stopped by adding 50 µl of stop solution, and the optical density was immediately measured at 450 nm using a microplate reader. A standard curve was generated for each assay using the provided standards, and cytokine concentrations in samples were determined by interpolation from the standard curve. All samples were normalized to total protein concentration, determined using a protein assay.

Gene expression

The real-time PCR method was used to investigate the effect of CSE and RA on the expression levels of *NF-κB* and *NLRP3* in H9C2 cells. Total RNA was extracted from cell lysates using a commercial RNA extraction kit. cDNA was synthesized from the extracted RNA using a Quantitate Reverse Transcriptase kit. Real-time PCR reactions were performed in a final volume of 20 µl, containing 2 µl of cDNA, 1 µmol/L of each forward and reverse primer (Table 1), and 10 µl of Real-Time PCR Master Mix. The remaining volume was adjusted with RNase-free water. The PCR amplification was carried out under the following cycling conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15

sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. The specificity of the amplification was confirmed by melt curve analysis. The relative expression of each target gene was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the expression level of GAPDH.

Statistical analysis

The data were analyzed using GraphPad Prism 8.0. The normality of all data sets was confirmed using the Shapiro-Wilk test. No outliers were identified or removed, and there were no missing data. Therefore, the results, expressed as mean ± standard deviation (SD), were subjected to one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. A $p<0.05$ was considered statistically significant. The number of independent samples (n) in each group was 3.

Table 1. The specific primers

Gene name	Forward primer sequence	Reverse primer sequence	Product size (bp)
GAPDH	AGTTCAACGGCACAGTCAAG	TACTCAGCACCAGCATCACC	119
NF-kB	TCAACATGGCAGACGACGAT	TTGAAGGTATGGGCCATCTGT	134
NLRP3	TGAGCTCCAACCATTCTCTGAC	TTCACCAACCCCAGCTTCTG	127

Results

Effect of RA treatment on cell viability

The viability of H9C2 cells was determined using the MTT assay, which measures metabolic activity as an indicator of cell viability. The results were normalized to the control group (set as 100% viability) and expressed as percentage of viability. Exposure to H_2O_2 and CSE significantly reduced cell viability (39.0 ± 2.8% and 53.0 ± 3.2%, respectively) compared to the control group ($p<0.001$). Treatment with RA at 5, 10, and 25 µM significantly attenuated the CSE-induced reduction in viability in a dose-dependent manner, restoring viability to 64.0 ± 2.9%, 81.0 ± 3.1%, and 89.67 ± 2.7%, respectively ($p<0.05$- $p<0.001$) (Figure 1).

Effect of RA treatment on oxidative stress factors

This study found that treatment with H_2O_2, a well-known oxidizing agent, and CSE significantly elevated the levels of MDA, a key marker of lipid peroxidation, when compared to the control group ($p<0.001$). Additionally, H_2O_2 and CSE exposure led to a notable decrease in the activities of CAT, SOD, and GPx ($p<0.001$). Importantly, when various concentrations of RA were introduced to cells treated with CSE, the results indicated a marked enhancement in the cells ability to counteract free radicals compared to the CSE group ($p<0.001$), as illustrated in Figure 2 (A-D).

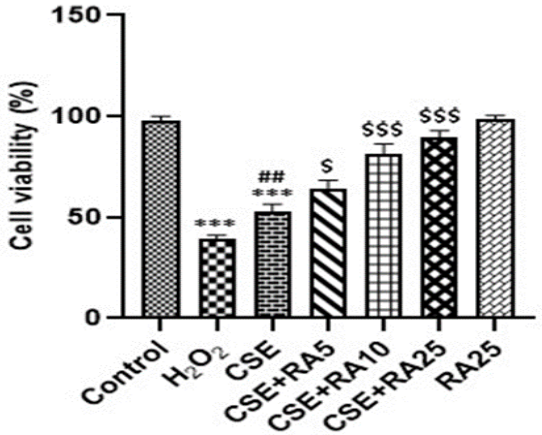

Figure 1. Effect of RA on cell viability using MTT assay in all groups including: Negative control, Positive control (H_2O_2), CSE (10%), CSE+RA5 (CSE 10%+ RA 5 µM), CSE+RA10 (CSE 10% + RA 10 µM), CSE+RA25 (CSE 10%+ RA 25 µM), RA25 (RA 25 µM). Results are expressed as mean ± SD (n=3). One-way ANOVA was used followed by Tukey's test. $^{***}p<0.001$ vs. control group. $^{\#\#}p<0.01$ vs. H_2O_2 group. $^{\$}p<0.05$ and $^{\$\$\$}p<0.001$ vs. CSE group.

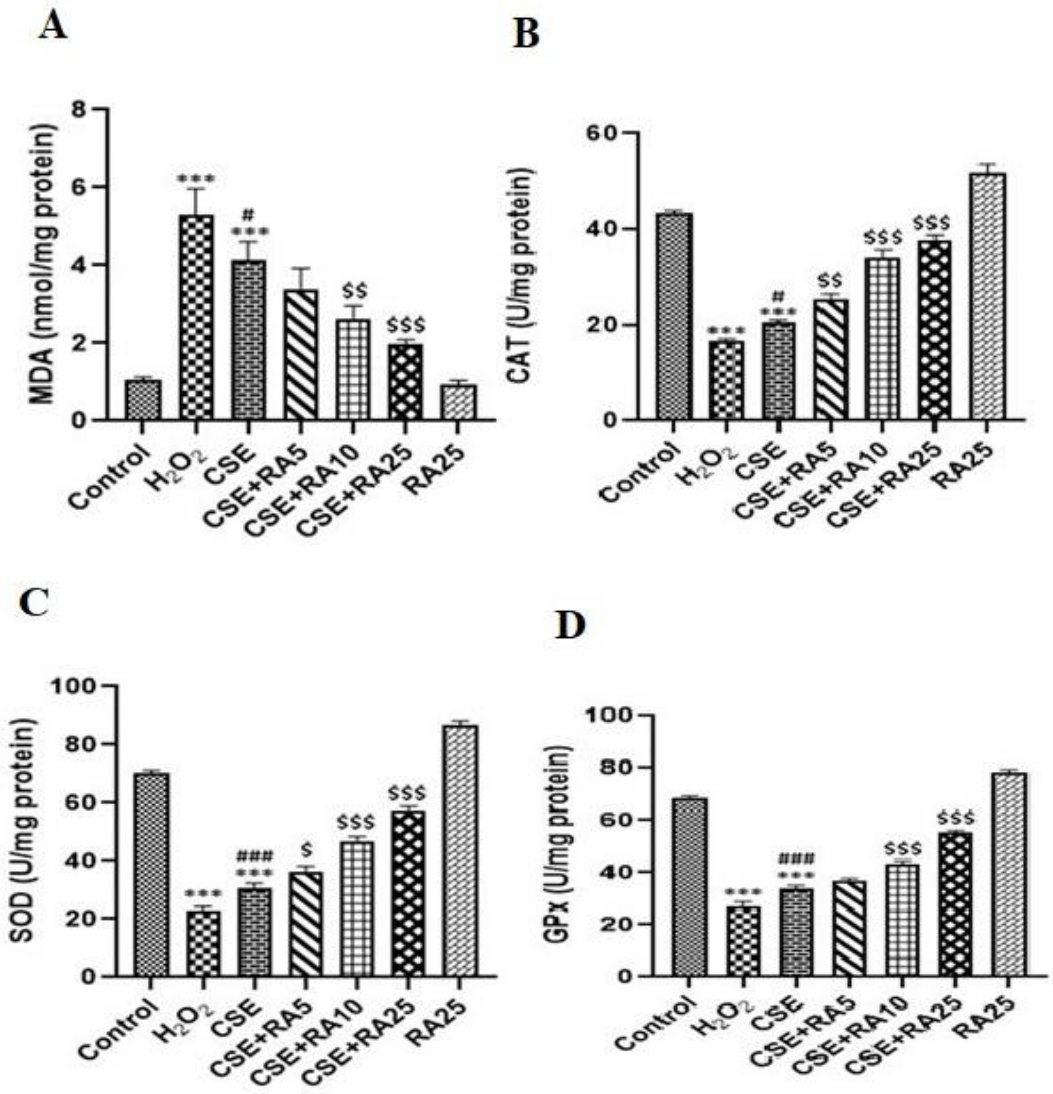

Figure 2. Effect of RA on A: MDA, B: CAT, C: SOD, and D: GPx in all groups including: Negative control, Positive control (H_2O_2), CSE (10%), CSE+RA5 (CSE 10%+ RA 5 µM), CSE+RA10 (CSE 10%+ RA 10 µM), CSE+RA25 (CSE 10%+ RA 25 µM), RA25 (RA 25 µM). Results are expressed as mean ± SD (n=3). One-way ANOVA was used followed by Tukey's test. $^{***}p<0.001$ vs. Control group. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. H_2O_2 group. $^{\$}p<0.05$, $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ vs. CSE group.

Effect of RA treatment on inflammatory cytokines

IL-1β, IL-18, and IL-10 levels were assessed to evaluate variations in inflammatory cytokines. Following exposure to H_2O_2 and CSE, the levels of IL-1β and IL-18 exhibited a significant increase ($p<0.001$), while the level of IL-10 experienced a notable decrease ($p<0.001$) compared to the control group. However, treatment with RA significantly reduced the levels of IL-1β and IL-18 ($p<0.001$), while it increased the level of IL-10 ($p<0.001$) compared to the CSE group, as illustrated in Figure 3 (A-C).

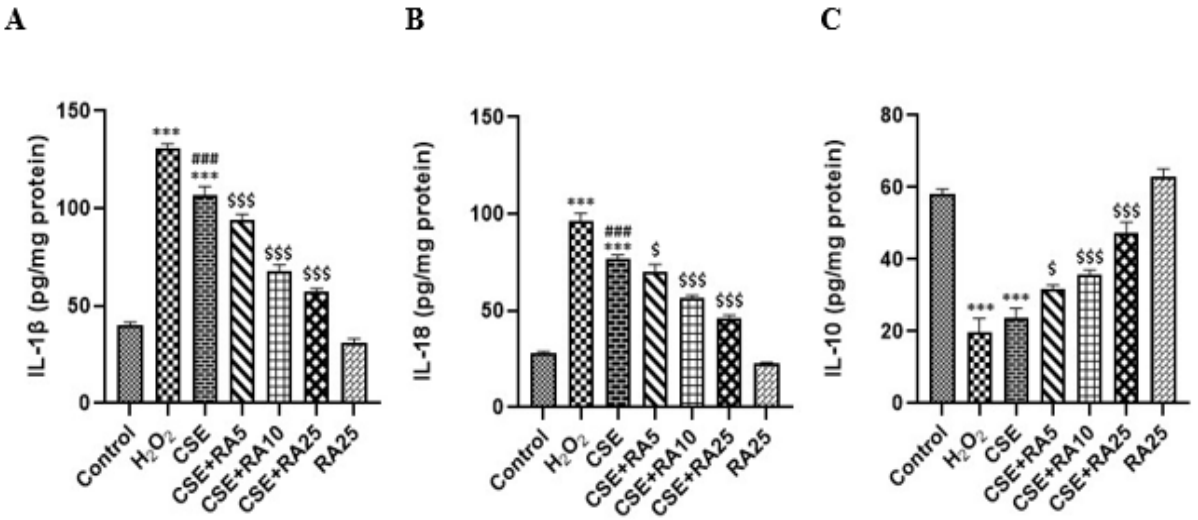

Figure 3. Effect of RA on A: IL-1β, B: IL-18, and C: IL-10 in all groups including: Negative control, Positive control (H_2O_2), CSE (10%), CSE+RA5 (CSE 10%+ RA 5 µM), CSE+RA10 (CSE 10%+ RA 10 µM), CSE+RA25 (CSE 10%+ RA 25 µM), RA25 (RA 25 µM). Results are expressed as mean ± SD (n=3). One-way ANOVA was used followed by Tukey's test. $^{***}p<0.001$ vs. control group. $^{###}$ $p<0.001$ vs. H_2O_2 group. $^{\$}$ $p<0.05$ and $^{\$\$\$}$ $p<0.001$ vs. CSE group.

Effect of RA treatment on inflammatory biomarker gene expression

To elucidate the mechanism by which, RA inhibits inflammation, the gene expressions of *NLRP3* and *NF-κB* were evaluated using real-time PCR. This study found that exposure to both H_2O_2 and CSE significantly elevated the expression of *NLRP3* and *NF-κB* genes compared to the control group ($p<0.001$). Treatment with increasing concentrations of RA (5, 10, and 25 µM) significantly reduced the expression of these inflammatory genes in a dose-dependent manner. Notably, the reduction was most pronounced at the 25 µM concentration which was significantly more effective than lower concentrations in suppressing *NLRP3* and *NF-κB* gene expression compared to the CSE group, as indicated in Figure 4 (A, B).

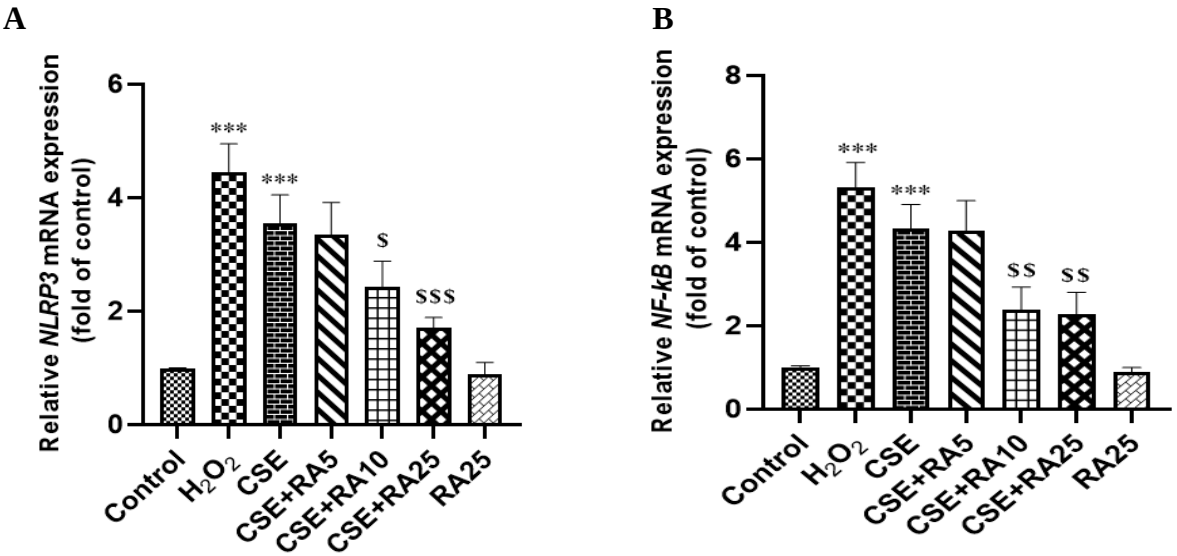

Figure 4. Effect of RA on A: *NLRP3* and B: *NF-κB* mRNA expression in all groups including: Negative control, Positive control (H_2O_2), CSE (10%), CSE+RA5 (CSE 10%+ RA 5 µM), CSE+RA10 (CSE 10%+ RA 10 µM), CSE+RA25 (CSE 10%+ RA 25 µM), RA25 (RA 25 µM). Results are expressed as mean ± SD (n=3). One-way ANOVA was used followed by Tukey's test. $^{***}p<0.001$ vs. control group. $^{\$}$ $p<0.05$, $^{\$\$}$ $p<0.01$ and $^{\$\$\$}$ $p<0.001$ vs. CSE group.

Discussion

Cigarette smoke is a major risk factor for cardiovascular diseases, largely through the induction of oxidative stress and inflammation (Rudrapal et al. 2022). Our study demonstrates that RA confers a protective effect against CSE-induced damage in H9C2 cardiomyocytes by enhancing antioxidant defenses and suppressing the *NF-κB*/*NLRP3* inflammasome axis.

Our findings confirm the cytotoxic effects of CSE on H9C2 cells, consistent with previous research showing that exposure to constituents of cigarette smoke can induce oxidative stress, subsequently causing cellular damage and dysfunction (Xue et al. 2023). Treatment with RA, particularly at 25 µM, significantly restored cell viability. The potent cytoprotective effect at 25 µM likely represents the peak of RA bioactive benefits within our experimental model.

A principal mechanism by which, RA confers protection is through the attenuation of oxidative stress, a pivotal contributor to numerous pathological and physiological processes, including inflammation. This phenomenon is characterized by an imbalance between the production of ROS and the capacity of antioxidant defense systems. Key antioxidant enzymes such as SOD, CAT, and GPx play a vital role in protecting cells from oxidative damage by scavenging ROS and maintaining redox homeostasis (Ferraz et al. 2024). We found that RA (specially at a concentration of 25 µM) significantly reversed the CSE-induced depletion of key antioxidant enzymes (CAT, SOD, and GPx) and reduced lipid peroxidation, as evidenced by decreased MDA levels. This suggests that RA not only scavenges ROS, but also bolsters the endogenous antioxidant system. Consequently, these findings underscore the potent antioxidant properties of RA in safeguarding cardiomyocytes against oxidative damage.

The transition from oxidative stress to inflammation is a critical step in CSE-induced pathology. Our data provide a clear molecular connection: CSE-induced oxidative stress led to the activation of the *NF-κB* and *NLRP3* inflammasome pathways, resulting in a pro-inflammatory cytokine profile (elevated IL-1β and IL-18, and suppressed IL-10). The observed reduction in IL-10 was particularly significant, as this anti-inflammatory cytokine is a known potent suppressor of *NF-κB* signaling (Li et al. 2021a; Li et al. 2021b; Stijlemans et al. 2022). Our results indicate that RA treatment effectively suppressed the CSE-induced upregulation of both *NF-κB* and *NLRP3* gene expression. This coordinated inhibition is crucial. *NF-κB* is a primary transcription factor for *NLRP3* and pro-IL-1β, serving as the priming signal for inflammasome activation. The activation signal is often provided by ROS themselves (Li et al. 2021b). By reducing ROS, RA can disrupt both the priming and activation steps of the *NLRP3* inflammasome. This mechanistic insight is supported by Guan et al. who showed that targeting the *NF-κB*/*NLRP3* axis alleviates inflammation (Guan et al. 2022b). Therefore, we propose that the reduction in oxidative stress leads to decreased *NF-κB* activation which in turn, reduces *NLRP3* expression and the subsequent maturation of IL-1β and IL-18. The concurrent elevation of IL-10 by RA may further reinforce this effect by creating an anti-inflammatory feedback loop.

The protective effects of RA observed in our study are consistent with a growing body of evidence. For instance, previous research reported that RA has cardioprotective effects by inhibiting *NF-κB* expression (Atefipour et al. 2024a; Guan et al. 2022a). Similarly, another study on a different cell type showed RA ability to suppress *NLRP3* inflammasome assembly (Yao et al. 2019). Our study consolidates these findings in a CSE-exposed cardiomyocyte model, providing a more

comprehensive picture of its cardioprotective potential.

This study acknowledges several key limitations. Firstly, the use of the H9C2 rat cell line may limit the direct applicability of the findings to human cardiomyocytes. Secondly, the research focused on gene expression (*NF-κB* and *NLRP3*) and lacked protein-level analysis, as well as molecular evidence for *Nrf2* pathway involvement.

To address these, future research should:

Use animal models for greater physiological relevance.

Employ protein-level techniques (e.g. Western blot) to investigate *NF-κB* and *Nrf2* pathways directly.

Conduct pharmacokinetic studies to see if effective RA concentrations can be achieved in living organisms.

These approaches could significantly deepen our understanding of the underlying mechanisms and therapeutic potentials involved.

In conclusion, our study provides compelling evidence that RA protects H9c2 cardiomyocytes from CSE-induced damage. The mechanism involves a concerted action: enhancing the cellular antioxidant system to combat oxidative stress and concurrently suppressing the pro-inflammatory *NF-κB*/*NLRP3* signaling cascade. While further investigation is needed to fully elucidate the upstream signaling and in vivo applicability, RA emerges as a promising therapeutic candidate for mitigating smoking-associated cardiotoxicity.

Acknowledgment

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Conflicts of interest

All authors declare no conflicts of interest.

Funding

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Ethical Considerations

All ethical considerations related to the research protocol were observed in accordance with the Declaration of Helsinki.

Code of Ethics

The protocol was approved by the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1402.460).

Authors' Contributions

Dianat M and Atefipour N were responsible for the design of this project. Dianat M and Atefipour N designed the experiments. Atefipour N, Mansouri Z, and Dianat F collected and processed the data. Atefipour N and Nejaddehbashi F analyzed and interpreted. Atefipour N and Mansouri Z wrote the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Abbreviations

CSE: Cigarette smoke extract. CVD: Cardiovascular diseases. COPD: Chronic obstructive pulmonary disease. CAT: Catalase. DMSO: Dimethylsulfoxide. ELISA: Enzyme-linked immunosorbent assay. GPx: Glutathione peroxidase. IL-1β: Interleukin-1β. IL-18: Interleukin-18. IL-10: Interleukin-10. MI: Myocardial infarction. MDA: Malondialdehyde. NLRP3: NLR family pyrin domain containing 3. NF-κB: Nuclear factor kappa-B. OD: optical density. PBS: Phosphate-buffered saline. RA: Rosmarinic acid. ROS: Reactive oxygen species. SOD: Superoxide dismutase

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