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RESEARCH ARTICLE

p-Cymene Modulate Oxidative Stress and Inflammation in Murine Macrophages: Potential Implication in Atherosclerosis

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Abstract: ***Background*:** p-Cymene (p-CYM) is a common chemical used in air fresheners.

***Objective*:** The study was designed to investigate the molecular effect of p-CYM on macrophages.

***Materials and Methods*:** Macrophages (RAW 264.7) were treated with p-CYM (50 uM/L, 150 uM/L and 250 uM/L) for 6 hours, and 24 hours). Gene involved in inflammation, such as the Tumor Necrosis Factor-alpha (TNF-α), and the Monocyte Chemoattractant Protein-1 (MCP-1) and other genes known for their antioxidant activity such as the Paraoxonase 1 (PON-1) were analyzed.

***Results*:** Cells treated with p-CYM have shown 30% up-regulation of MCP-1 after 24 hour of exposure; and also a differential up-regulation of TNF-α. However, treatment with p-CYM has resulted in a considerable (37%) dose-dependent downregulation of PON-1 after 24 hours of exposure. PON-1 is known for its antioxidant properties protecting High-Density Lipoproteins (HDL) from oxidation.

Conclusion: Our findings demonstrate that exposure to p-CYM over time promotes oxidative stress by downregulating antioxidants genes as shown in PON-1 and also stimulates inflammation, a key process during the initiation and progression of atherosclerosis.

Keywords: Atherosclerosis, inflammation, oxidative stress, p-Cymene, macrophages, High-Density Lipoproteins (HDL).

1. INTRODUCTION

Atherosclerosis is a chronic inflammatory disorder of the large and medium-sized arteries characterized by asymmetric focal thickening of the inner layer of the artery, the intima. Atherosclerosis is a major cause of stroke, coronary artery disease (CAD), and peripheral arterial disease, which are responsible for the most cardiovascular morbidity and in most developed countries [1, 2]. Studies have shown that oxidative stress and inflammation play a significant role in the pathogenesis atherosclerosis. Macrophages migrated from blood circulation, dominate the early manifestation of atherosclerotic lesions by releasing pro-inflammatory cytokines, chemokine, oxygen and nitrogen radicals during the oxidative modification of low-density lipoprotein (LDL) [2, 3] in injured intima. Several risk factors promote inflammation and contribute to the progression of atherosclerosis including hypercholesterolemia, hypertension, infection, cigarette smoking [4], and air pollution.

It has been known that air pollution constitutes an important cardiovascular risk factor [5]. Airborne particulate matters from outdoor air pollution or indoor cooking oil and smoking are correlated to increased cardiopulmonary deaths [6]. In a study, Suwa *et al*. [7], found that exposure to air particular material <10μm (PM_{10}) to hyperlipidemia rabbits for four weeks accelerates atherosclerotic lesions and elevates the vulnerability of plaque rupture. The authors also found that alveolar macrophages phagocytized PM_{10} was stored in the lung and could activate Tumor Necrosis Factor-α (TNF-α) and some other cytokines such as interleukin-1β, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF). This leads to the up-regulation of the production of Monocyte Chemoattractant Protein-1 (MCP-1) to endothelial cells and induces the accumulation of macrophages and T-lymphocytes in atherosclerotic lesions. Another study performed on mice observed that long-term exposure to particular materials with a concentration of 2.5μm ($PM_{2.5}$) results in pulmonary inflammation and generation systemic Reactive Oxygen Species (ROS), chemokine, and cytokines to the systemic circulation [8].

p-CYM (4-Isopropyltoluene) is a chemical utilized in air freshener's products. It is classified as an alkylbenzene related to monoterpenes and it consists of benzene ring *para*-substituted with an isopropyl group and methyl group [9-12]. p-CYM is a naturally occurring aromatic and Volatile Organic Compound (VOC) that found in most plants, fruits and

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herbs such as orange juice, carrots, thyme, and oregano [13, 14]. It is widely used in pharmaceutical industries and for the production of fungicides, pesticides, as flavoring agent [15]. Studies demonstrate p-CYM has an anti-nociceptive effect and antimicrobial function against Gram-positive bacterium *Staphylococcus aureus* and Gram-negative *Escherichia coli* [16, 17]. Furthermore, a recent study demonstrates that p-CYM has an anti-immune-modulatory role by regulating macrophages inflammatory mediators TNF-α, IL-1β, IL-6, and IL-10 productions and blocking nuclear factor-κβ (NF-κβ) and mitogen-activated protein kinase (MAPK) signaling pathway [3, 18, 19]. However, recent research performed on mice models revealed that sub-chronic exposure to a low dose of VOC has a toxic impact on the lung and cause inflammation, oxidative stress, and immune modulation [20, 21]. Acute exposure of rats through gavage with a range of 620-10700 mg/kg bw of p-cymene and studied for clinical signs and mortality have shown survival at doses of 620 to 2100 mg/kg/ body weight, however rats died at dosages above 3200 mg/kg body weight [22]. Rats were also acutely exposed to atmospheres saturated with 9.7 mg /L of p-cymene for a period of 5 hours. Signs reported during the first 30 minutes were those typical of irritation, excitement, pawing at the eyes and nose, increased blinking, squinting, and eye closure [22]. There is no data on human exposure and toxicity from p-CYM.

The purpose of this research study is to investigate the toxic effect of prolonged exposure of p-CYM on murine macrophages inflammatory cytokines, chemokine.

2. MATERIALS AND METHODS

2.1. Materials

p-Cymene (99%) was purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). RPMI Medium 1640(1X) mediums, FBS (Fetal Bovine Serum), and Pen Strep (Penicillin Streptomycin) were all purchased from Life Technologies (Carlsbad, CA). The murine macrophages were RAW 264.7 and purchased from ATCC (Manassas, VA). TRIzol Reagent was purchased from ThermoFisher Scientific (Waltham, MA). All the Gene expression reagents and Western Blotting reagents were obtained from Bio-Rad (Hercules, CA).

2.2. Cell Culture and Treatment

The murine macrophages RAW 264.7 cells were cultivated in RPMI 1640 Medium supplemented with 10% (v/v) FBS and 1% (v/v) Pen Strep. Cells grown to late log phase were treated with p-CYM (50 uM, 150 uM, 250 uM in serum-free growth medium for 6 hours, and 24 hours). The dosages were selected and calculated based on the lowest overall possible tolerability from animal studies, given the consideration that the exposure to molecule from the air freshener is extremely low [22]. Ethanol control was used with the same amount of ethanol to dissolve p-CYM. The negative control group was cultured with only serum-free growth medium, along with the same incubation and duration. Protein and RNA were isolated immediately after each incubation period using NP-40 Lysis buffer and Trizol reagent according to the protocols provided by the manufacturer. Proteinase inhibitors were present throughout the protein isolation procedure. Both protein and RNA samples were stored at -80°C freezer until analysis.

2.3. Protein and RNA Quantification

Qubit Fluorometric Quantitation was used during the quantification procedure. Qubit RNA BR assay Kit was used for RNA quantification. The Qubit Protein Assay Kit was used for protein quantification.

2.4. Quantitative Real-time PCR (qPCR)

Complementary DNA (cDNA) was generated from 400ng of the total RNA and 100ng was taken for qPCR. cDNA synthesis and qPCRs were performed with Universal Probes Supermix according to the manufacturer protocol. qPCR was done in sealed 96-well plates with iScript Reverse Transcription Supermix and SsoFast EvaGreen Supermix on an RTPCR MyiQTM2 system (Bio-Rad; Hercules, CA). Threshold cycle (CT) was determined by Bio-Rad iQ5 software. The melting curve and efficiency level were evaluated for all primer pairs. The level of gene expression was calculated using the Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH) as an internal control gene. The mRNA gene expression values were calculated using $2^{-\Delta C_T}$ method.

2.5. Western Blot Analysis

Protein was precipitated and diluted with PBS. After adding 0.01%(w/v) Bromphenol Blue, 5 ug of the total protein from cell lysate of each group, samplese were incubated in 100°C water-bath for 5 min to denature the protein samples. Equal amounts of protein were added to and separated on SDS/4-20% polyacrylamide gels and transferred to Polyvinylidene Difluoride (PVDF) membranes. TNF-α, PON-1 and β-Actin antibodies were used to detect relevant proteins. The antibodies were diluted 1:1000, 1:2000 and 1:25000 respectively. The secondary horseradish peroxidase-conjugated anti-rabbit IgG was diluted 1:5000, 1:10000 separately. The protein bands were detected by the luminol-based Enhanced Chemiluminescence (ECL) HRP substrate. All the membranes were imaged and analyzed by Biospectrum AC imaging system and Visionworks Software (UVP).

2.6. Statistical Analysis

Individual conditions were assayed in triplicate. All data presented as means of replicate tubes. The difference between means was determined using a Student's T test. A $p<0.05$ is accepted as statistically significant. Statistical analyses were performed using Microsoft Excel.

3. RESULTS AND DISCUSSION

Each group of murine macrophages RAW 264.7 cells experiment had more than 90% confluence, which provided a very good source for RNA and protein isolation. There was no significant reduction in the amount of the cells for each group as a result of the treatments. Additional experiments

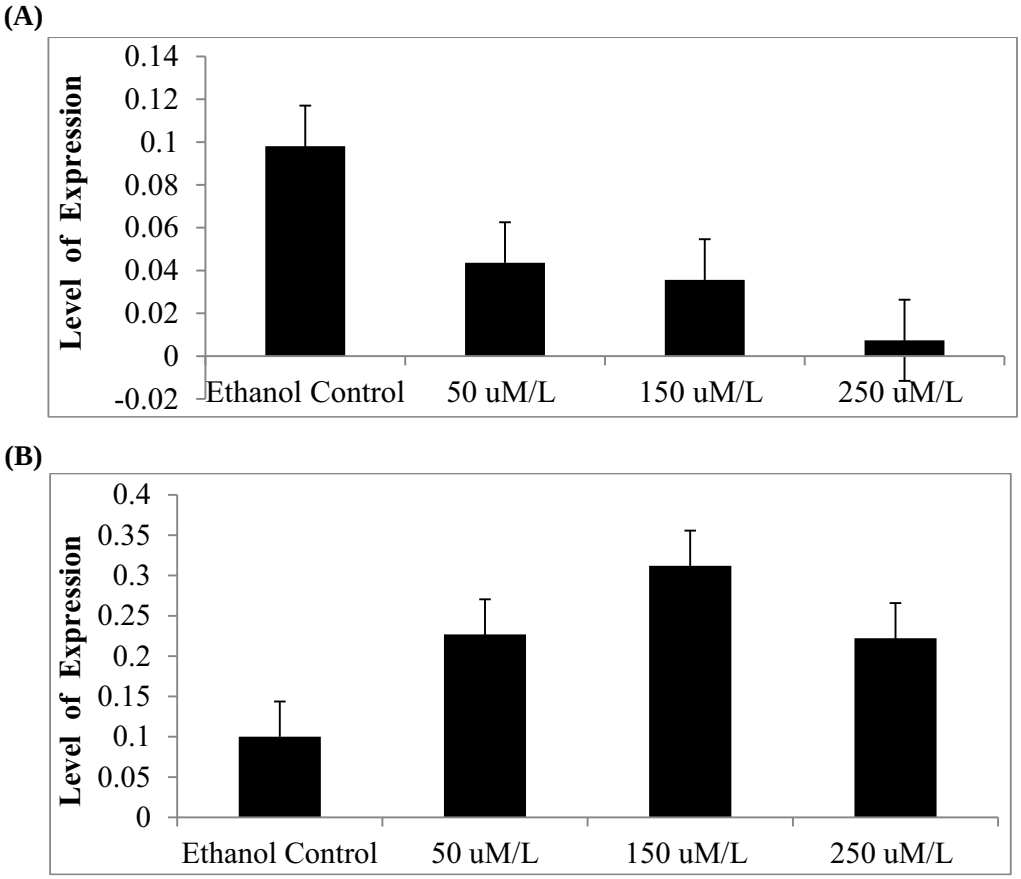

Fig. (1). (A) Macrophage levels of MCP-1 gene expression after 6 hours of treatments. The data shows dose dependent acute decreases in MCP-1 gene expression levels. **(B)** Macrophage levels of MCP-1 gene expression increased after 24 hours of treatments.

were conducted for the cell viability, plasma membrane damage, blebbing and apoptosis after exposure to the different tested p-Cymene concentrations. However, the cells seem to tolerate the exposure to these concentrations without any physiological or morphological changes observed. To our knowledge, there is no available data or study on human p-Cymene exposure and toxicity.

3.1. Gene Expression

The outcome of the macrophage treatment with p-CYM showed that acute treatment results in anti-inflammatory responses as shown by the dose-response decreases of the TNF-α, and MCP-1 gene expression. On the other hand, the treatment has shown the induction of oxidative stress that resulted in slight decreases in PON-1 gene expression.

Monocyte chemoattractant protein-1 (MCP-1, also known as chemokine CCL2) is an important chemokine mediator of inflammation. They are small heparin-binding proteins that constitute a large family of peptides (60–100 amino acids) structurally related to cytokines. MCP-1 is known to regulate cell trafficking. Six hours of exposure to p-CYM have resulted in dose-dependent decreases in MCP-1 levels (Fig. **1a**). These results indicate that p-CYM has acute anti-inflammatory action. Prolonged exposure to p-CYM resulted in substantial increases in MCP-1 expression (Fig. **1b**). MCP-1 levels have shown a p-CYM concentration dose-dependent like increases during a 24 hours treatment. However, interestingly, the 250uM p-CYM concentration showed a lower MCP-1 expression compared to the other treatments *i.e.*, 50uM and 150uM p-CYM. This indicates that chronic exposure to p-CYM induces an increase in MCP-1. The highest levels of MCP-1 expression were attained by the 150uM p-CYM group for all hours of collection. Nevertheless, it is evident that p-CYM clearly modulates the MCP-1 gene expression. Increased MCP-1 gene expression is reported in atherosclerosis progression and relevant metabolic studies [23-25]. MCP-1 upregulation with p-CYM shows a pro-atherosclerotic effect for this molecule.

The trend seeing in MCP-1 responses to p-CYM treatment was repeated with the TNF-α gene expression (Fig. **2a**) confirming the acute effect of p-CYM on inflammation. The effect was dose-dependent. The gene expression data was confirmed by the western blot results (Fig. **2b**). The levels of TNF-α increased with chronic treatment of the macrophages with p-CYM. After 24 hours, the levels of TNF-α gene expression rebounded in a similar to a trend to what was seeing in MCP-1 gene expression (Fig. **2c**), this confirmed with Western blot data (Fig. **2d**). The increases in TNF-α occurred gradually with the low dose that initially had higher increases with the acute treatment returned more prominently. This data confirm that p-CYM has an acute anti-inflammation effect; however, the prolonged exposure results in

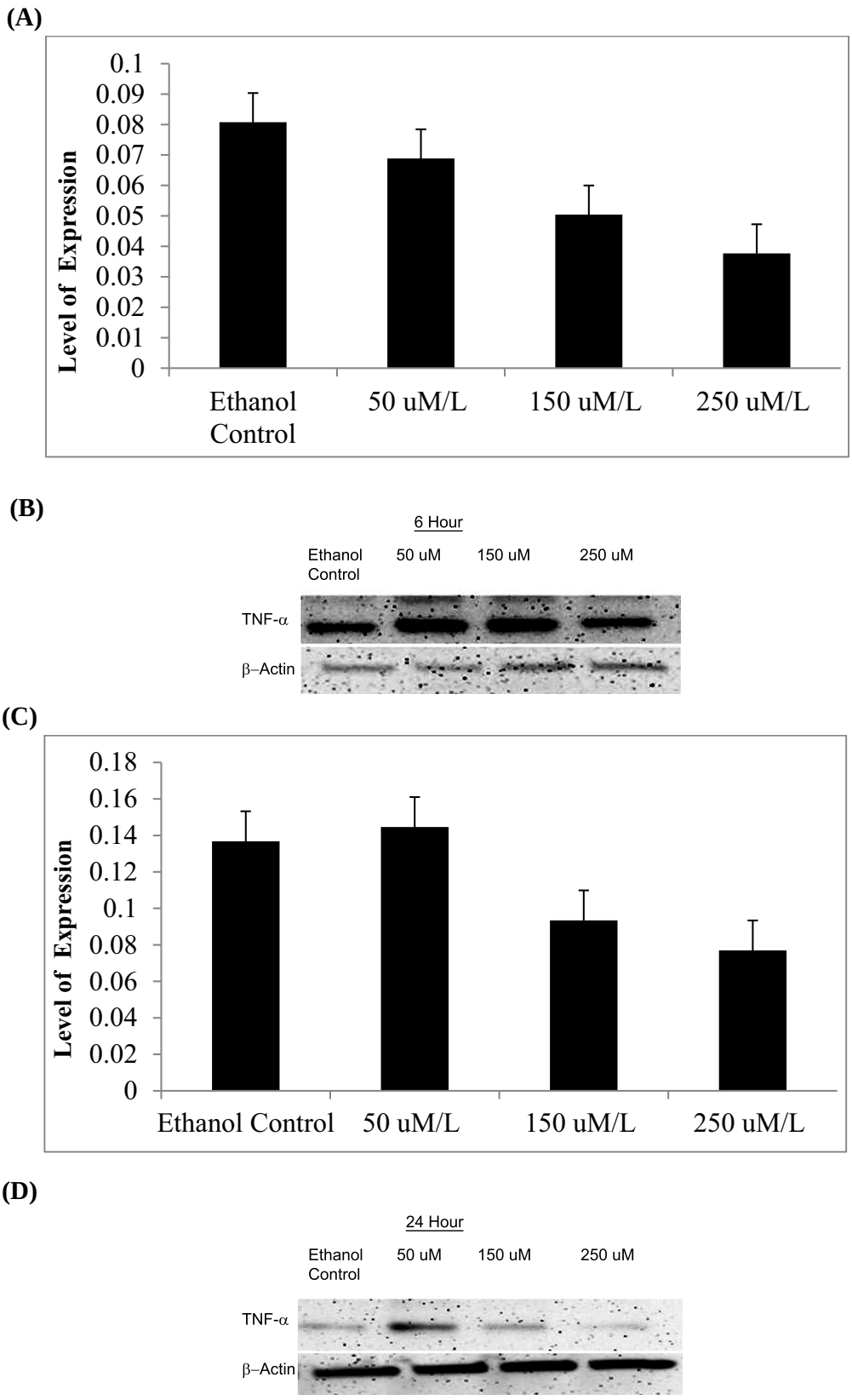

Fig. (2). (**A**) Macrophage levels of TNF-α gene expression acutely decreased after 6 hours of treatment in dose dependent responses. (**B**) The Western blot data confirm the dose dependent decreases in TNF-α gene expression. (**C**) Macrophage levels of TNF-α gene expression shows gradual increases in TNF-α after 24 hours of treatments. (**D**) Western blot data shows the gradual increases TNF-α after 24 hours of treatment.

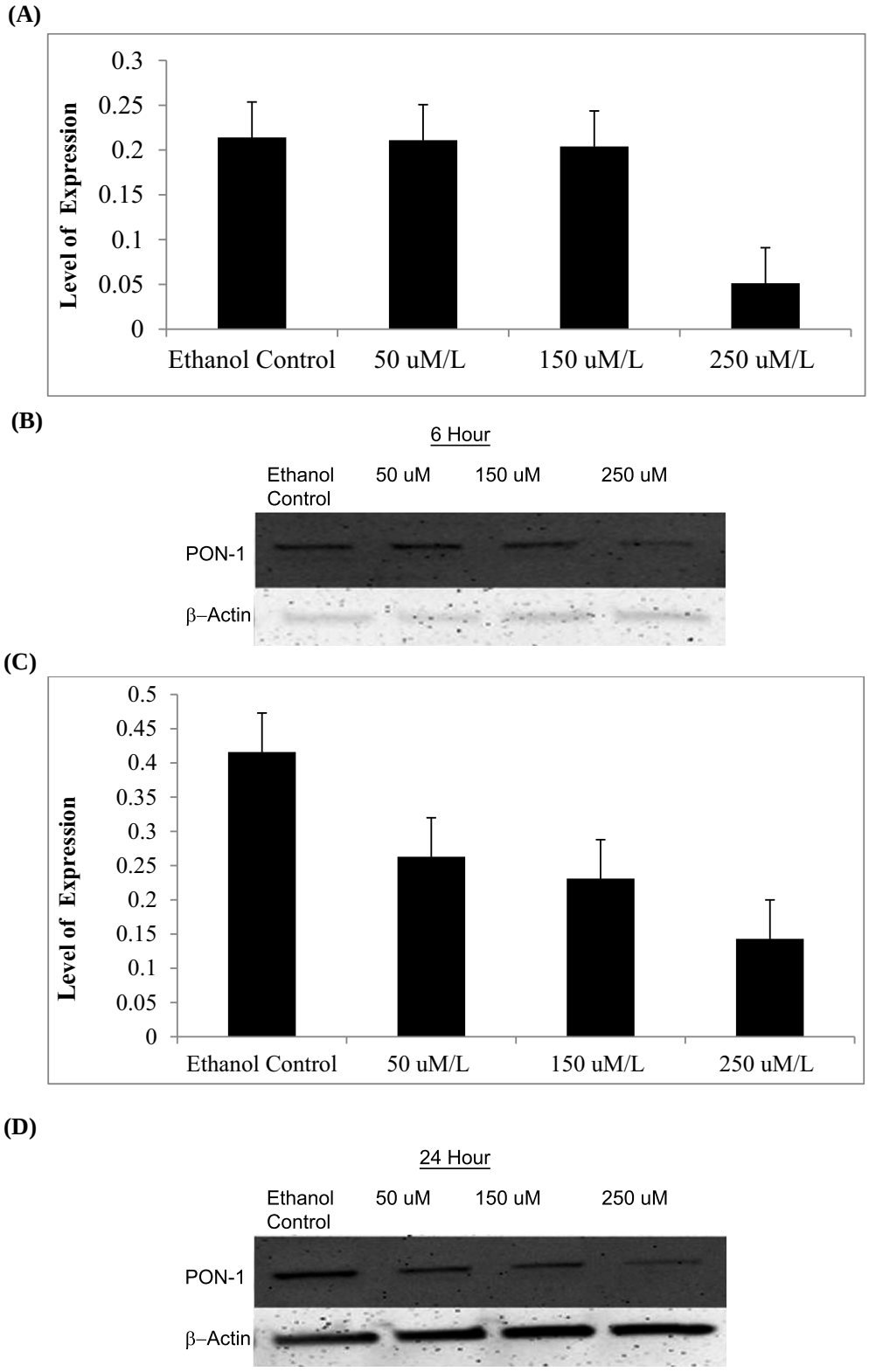

Fig. (3). (**A**) Macrophage levels of PON1 gene expression shows insignificant decrease in PON1 levels with 50 and 150 uM/L after 6 hours of treatments, however considerable decreases in PON1 levels with p-cymene treatment. (**B**) Western blot data confirms the responses seeing in the level of gene expression in Fig. **3a**. (**C**) Macrophage levels of PON1 gene expression continued to decrease after 24 hours in dose dependent responses. (**D**) Western Blot Analysis**:** 5 µg of total protein from cell lysates of each group was loaded and separated on SDS/4-20% polyacrylamide gels. TNF-α, PON-1 and β-Actin were detected separately at ~26 kDa, ~80 kDa (dimer) and ~42 kDa.

increased inflammation. Association of increased TNF-α gene expression and plasma levels in with atherosclerosis and cardiovascular disease is well documented [26-29]. TNF-α is an acute phase reaction gene that is secreted mainly by macrophages in response to inflammation-inducing stimuli. The primary role of TNF-α is in the regulatory immune cells. Its increases in this study were due to the pro-inflammatory effect of p-CYM. Overall, the inflammation-inducing effect of p-CYM was clearly shown by both genes MCP-1 and TNF-α gene expressions as a result of chronic exposure.

p-Cymene not only induces inflammation but also has a pro-oxidant effect as shown by the PON-1 responses. The acute and chronic treatments of macrophages with p-CYM have resulted in decreased PON-1 gene expression (Fig. **3a-d**). The effects of the treatment were dose-dependent. The down regulations of PON-1 gene expression may have been a result of inhibitory effects of p-CYM on the PON-1 gene and protein. The reduction in protein expression may also be a result of protein inhibition and degradation. The pro-oxidant overwhelming impact of the p-CYM molecule may have affected PON-1 activity that resulted in doses dependent diminishing effects to the protein. PON-1 is a hydrolytic enzyme with a wide range of substrates, and capability to protect against lipid oxidation, there is enormous in vitro and *in vivo* data to demonstrate the beneficial effects of PON1 in atherosclerosis [30]. The exact antioxidant mechanism of PON1 is not known however its antioxidant potential is directly linked to its HDL protective role. PON1 binds to HDL through the interaction of hydrophobic N-terminus to phospholipids, and through PON1– ApoA interaction [30]. It is also reported that PON1 directly suppresses macrophage pro-inflammatory responses [31]. p-CYM degradation or inhibition of PON1 associated with its atherogenic properties.

In summary, the results of these experiments meritoriously demonstrated p-CYM's effect on macrophages as an inflammation-inducing substance. p-CYM not only modulates the expression of inflammatory cytokines, but also effectively inhibits the antioxidant activity in macrophages over the 24-hour period, as shown by a decreased PON1 activity. These characteristics indicate possible atherosclerosis pathogenesis of p-CYM. p-CYM is commonly used as part of some air fresher, which makes it a potential inducer of atherosclerosis. Additional studies are needed to evaluate the effects of p-CYM in presence of other pro-inflammatory modulators and other chemicals commonly used in household formulations. Animal studies can provide further verification of our results to affirm the possible atheorpathogenesis role of p-CYM.

CONCLUSION

Our findings demonstrate that exposure to p-CYM over time promotes oxidative stress by downregulating antioxidants genes as shown in PON-1 and also stimulates inflammation responsible for the initiation and progression of atherosclerosis.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The murine macrophages, RAW 264.7 and purchased from ATCC (Manassas, VA).

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data used to support the findings of this study are included in the article.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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