Anti-Inflammatory Effect of L-carvone on Lipopolysaccharide-Induced Acute Lung Injury

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Abstract

Acute lung injury is among the most serious conditions that affect the lung which is characterized by an exacerbation of inflammatory response that can result from a severe lung infection. The enantiomers of carvone (L and D) are found in various plants species each with special pharmacological effects; where, the levo (L)-carvone is chiral monoterpenoid ketone present in the essential oils of dill, caraway, and spearmint and possess many pharmacological properties like antioxidant, anti-inflammatory, antimicrobial, anti-diabetic, and anticonvulsant effects. In a previous study, L-carvone inhibited mucositis induced by irinotecan. This study is the first to evaluate the lung anti-inflammatory protective effects, and potential mechanism of action of L-carvone in acute lung injury induced by lipopolysaccharide by measuring the gene expression level of inflammatory mediators (tumor necrosis factor-alpha, cyclooxygenase-2 and nuclear factor-kappa of activated B cells) by conducting real-time quantitative polymerase chain reaction test.

Fifty adult mice were allocated into 5 Groups as follows: - control group (mice received normal saline, Group I). Mice in the -induction group received (lipopolysaccharide 10mg/kg/day intraperitoneally, Group II) and were euthanized 2 hours later. Group III (Vehicle group, Mice received corn oil 0.1 ml + lipopolysaccharide 10 mg/kg). Mice in the -treatment groups received either [(50mg/kg/day), Group IV] or [(100mg/kg/day), Group V] of oral L-carvone for 5 consecutive days before lipopolysaccharide injection.

Pretreatment with L-carvone (50mg/kg/day) (Group IV) markedly attenuated pro-inflammatory cytokines as observed by significant (P<0.05) reduction in mRNA expressions of tumor necrosis factor-alpha (7.56 ±1.195 vs 29.20±4.9) and cyclooxygenase 2 (5.72±0.329 vs.10.58 ±0.777) in mice’s lung tissue compared to those in the induction group, non-treated mice (lipopolysaccharide model group) (Group II). Increasing the dose of L-carvone to 100mg/kg/day (Group V) also resulted in a significant reduction in mRNA expressions of tumor necrosis factor-alpha (7.84±1.4 vs 29.20±4.9) and cyclooxygenase2 (4.589± 0.946 vs 10.58±1.641) compared to that expression in the induction group, non-treated mice (lipopolysaccharide model group) (Group II); however, the attenuating effect is dose-independent. Furthermore, the results revealed that nuclear factor-kappa of activated B cells mRNA gene expression was significantly lowered by L-carvone 50mg/kg/day (Group IV) (5.01±0.826 vs 11.88±1.227) and 100mg/kg /day (Group V) (6.81±1.362 vs 11.88±1.227) compared to induction group, non-treated mice (lipopolysaccharide model, Group II).

Conclusion This study clearly revealed that L-carvone exerted anti-inflammatory and lung-protective effects on lipopolysaccharide-induced acute lung injury. The observed effects were dose-independent and resulted from hampering of the NF-κB signaling pathway.

Keywords: Acute lung injury, Lipopolysaccharide, TNF-α, COX2, L-carvone.

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الخلاصة

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Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), is a serious inflammatory condition affecting the lung with a high percentage of morbidity and mortality (1). The ALI is caused by neutrophil leakage, production of a huge amount of pro-inflammatory cytokines, injury to airway epithelium and alveolar endothelium which lead to pulmonary fluid accumulation, and the impairment in gases exchange.

Lipopolysaccharide (LPS) is a component of the gram-negative bacterial cell wall, which is a glycolipid that consists of many disaccharide units and polar lipid head group and can be employed to create an ALI model in the mice lung which mimicked its characteristic and pathological features in the human (2,3). Previous studies have demonstrated that LPS could cause ALI by activating the toll-like receptor 4 (TLR4) signaling pathway, which controls the transcription of pro-inflammatory cytokines, such as interleukin β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). These cytokines are responsible for the activation of the innate immune system which results in injury in the lung tissue (4-6).

Carvones are chiral monoterpeneoid ketones (3-Isopropenyl-2-methyl-2-cyclohexenone) that are abundant in many plants including caraway, angelica, and spearmint. In recent years it attracts attention as alternative medicine. It is present in nature in two enantiomers (+, D) mainly present in caraway Carum carvi and (-, L) carvone mainly present in Mentha spicata. Many studies evaluate pharmacological effects like anti-inflammatory, antioxidant effects, antibacterial, anti-diabetic, anticonvulsant, and antinociceptive effects. According to the previously mentioned effects of l-carvone, the anti-inflammatory and possible lung protective effect in ALI induced by LPS was investigated in an animal model in this study (7-10).

The anti-inflammatory effect of L-carvone was previously reported through the inhibition of myeloperoxidase activity (MPO), reduction of prostaglandin E2 (PGE2), interleukin-1β (IL-1β), tumor necrosis factor (TNF-α), nitric oxide (NO'), inducible nitric oxide synthase (iNOS) expression, cyclooxygenase E2 (COX2), an increase in glutathione levels (GSH) (10).

Moreover, L-carvone was able to produce a protective effect on irinotecan-induced intestinal mucusitis in mice by reducing pro-inflammatory cytokines TNF-α production and diarrheal score (11).

Materials and Methods

Chemicals and kit

Levo-carvone (l-carvone) was obtained from Sigma Aldrich/USA, LPS O55:B5 was purchased from sigma Aldrich/ Germany, NF-κB, GAPDH, COX 2, and TNF α Primers were purchased from Macrogen / South Korea, RNA extraction kit was purchased from Dong Sheng Biotech/China, Trans Start® Green qPCR Supermix was purchased from Transgen Biotech/ China, Easy Script® One-Step gDNA removal and cDNA synthesis.

Animal selection

Fifty (50) Albino male mice weighing (20-30) grams were divided into five groups were brought from and maintained in the Animal Facility of the College of Pharmacy, University of Baghdad, under conditions of the controlled temperature, humidity, and light periodicity (12-hour light/dark cycle). Mice had free access to a standard diet and water during the experimental period.

Experimental protocol

This study was approved by the Scientific and Ethical Committees of the College of the Pharmacy/University of Baghdad. Mice employed in this study were randomly-divided into five groups of ten mice each, as follows:
Group I. Mice received 0.1 ml intraperitoneal injection of normal saline for five constitutive days and were considered as the normal group (negative control group).

Group II (lipopolysaccharide model group). Mice received a single dose of intraperitoneal LPS in dose (10 mg/kg) and were euthanized after two hours. This group served as the acute lung injury model group (induction group) \(^{(12)}\).

Group III (Corn oil + LPS). Mice received corn oil (0.1 ml) orally for five consecutive days. On day 5, mice received lipopolysaccharide (10 mg/kg) and were euthanized after 2 hrs. The purpose of using corn oil + LPS was to verify if the corn oil has an anti-inflammatory effect or not (as corn oil is used to dissolve L-carvone).

Group IV (L-carvone treatment). Mice received L-carvone solution in dose (50 mg/kg/day) orally for five consecutive days \(^{(13)}\). On day 5, mice received lipopolysaccharide (10 mg/kg) and were euthanized after 2 hrs.

Group V (L-carvone treatment). Mice received L-carvone solution (100 mg/kg/day) orally for five consecutive days \(^{(13)}\). On day 5, mice received lipopolysaccharide (10 mg/kg) and were euthanized after 2 hrs. Administration of L-carvone is done by oral gavage at 8:00 AM daily from day 1 through day 5. Euthanization is done by diethyl ether followed by cervical dislocation.

Determination of lung tissue gene expression of TNF-α, NF-κB, and COX2

The right lung was isolated and 50-100 mg of it was placed in a tube containing 1 ml of TRIzol (RL solution) and frozen for later use. Analysis and calculation of gene expression levels of TNF-α, NF-κB, COX2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), depend on mRNA concentration after converting it to complementary DNA. The process includes total RNA extraction and purification using (DongSheng Biotech /China), complementary DNA (cDNA) synthesis using random primers (Transgen Biotech / China), The SYBR green PCR Master Mix (TaqDNA polymerase, SYBER Green1, dNTPs, PCR enhancer and stabilizer) was used for real-time PCR analysis. The cycle time values of the interested genes were first normalized with GAPDH/ Housekeeping gene which is a reference gene used to achieve accurate normalization for the RT-qPCR of the same sample. The changes in the mRNA expression in all the groups were calculated by the comparative method of \(2^{-\Delta\Delta Ct}}\). The primer pairs of the expected products were as follows (forward and reverse, respectively): NF-κB \((5'\, AAGCAACAGGCAGGACATG-3' and 5'\, AGCACATCTTCATTCCAC-3')\), TNF-α \((5'\, TACCCACGGTACCTGACAAAC \, 3' \, and 5'\, ACAACCTACCACTCATGC-3')\), COX2 \((5'\, GCTGACCCAGGGACGAAATTC-3' \, and 5'\, CACC ATAGATCCAGTGCGG-3')\) and GAPDH \((5'\, CGGGTTCTTATAATACG \, 3' \, and 5'\, CAAATACGGGCAATCGGTC-3')\) primers were purchased from Macrogen / South Korea, real-time PCR was performed using Corbett research RT-qPCR device according to the manufacturer instructions \(^{(14,15)}\).

Statistical analysis

Data presented as mean± standard error of the mean (SEM). Statistical Package for the Social Sciences (SPSS, version 25) was used for data analysis and student T-test was used. One-way ANOVA and Tukey used for comparison among groups were \(P<0.05\) considered significant.

Results

Effect of L-carvone on TNF-α mRNA level

Analysis of the data in the Table (1) revealed that, the level of TNF-α mRNA is significantly elevated \((P<0.05)\) in the LPS model (Group II) compared to corresponding level in the normal control (Group I) \((29.20±4.9 \, \text{vs.} \, 1.141±0.31)\). Furthermore, administration of L-carvone in dose 50 mg/kg/day (Group IV, L-carvone treatment group) significantly \((P<0.05)\) attenuated the TNF-α mRNA level in lung tissue \((29.20±4.9 \, \text{vs.} \, 7.56±1.195)\) compared to those levels in the non-treated mice (Group II, LPS model group) (Figure 1). Similarly, increasing the dose of L-carvone to 100 mg/kg/day (Group V, L-carvone treatment group) also resulted in significant \((P<0.05)\) attenuation of the TNF-α mRNA level \((29.20±4.9 \, \text{vs.} \, 7.84±1.40)\) compared to non-treated mice (Group II, LPS model group). In addition, data of Table 1 and Figure 1 pointed out that there is a non-significant \((P>0.05)\) difference between the (Group III, corn oil + LPS) and that in (Group II, LPS model group) in terms of TNF-α mRNA level which revealed that corn oil used as a vehicle in the study has no effect on the TNF-α mRNA gene expression level (which prove corn oil had no anti-inflammatory effect). Further, data analysis reveals that there is a non-significant \((P>0.05)\) difference in the TNF-α mRNA expression level between (Group IV) mice treated with 50 mg/kg/day and (Group V) mice treated with 100 mg/kg/day L-carvone. Table 1 and Figure 1.
Table 1. Effect of L-carvone on TNF-α, COX2, and NF-κB mRNA gene expression levels.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TNF-α gene expression level (Mean ± SEM)</th>
<th>COX2 gene expression level (Mean ± SEM)</th>
<th>NF-κB gene expression level (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G I: Negative Control (Normal saline treated group)</td>
<td>1.1418±0.318204</td>
<td>1.1113±0.294431</td>
<td>1.1571±0.369087</td>
</tr>
<tr>
<td>G II: LPS group (ALI model group)</td>
<td>29.20±4.767602</td>
<td>10.58±0.777646</td>
<td>11.88±0.575529</td>
</tr>
<tr>
<td>G III: (Corn oil + LPS group)</td>
<td>28.81±4.501522</td>
<td>10.78±2.751145</td>
<td>11.31±0.684981</td>
</tr>
<tr>
<td>G IV: L-carvone treatment group 50mg/kg/day</td>
<td>7.83±0.399415</td>
<td>5.72±0.329006</td>
<td>6.81±0.719718</td>
</tr>
<tr>
<td>G V: L-carvone treatment group 100mg/kg/day</td>
<td>7.56±1.15581</td>
<td>4.59±0.946045</td>
<td>5.00±0.800742</td>
</tr>
</tbody>
</table>

#: Significant (P<0.05) differences compared to the normal (negative) control Group I mice.
a: Non-significant (P>0.05) differences compared to the LPS model Group II mice.
*: Significant (P<0.05) compared to the LPS model Group II mice.
b: Non-significant (P>0.05) differences between Groups IV and V mice.

Effect of L-carvone on COX2 mRNA level

Results of Table (1) showed that the COX2 mRNA gene expression level was significantly-elevated (P<0.05) in LPS model group (Group II) compared to the corresponding gene expression level in normal control group (10.58±0.777 vs. 1.1± 0.29); and the administration of L-carvone in a dose of 50mg/kg/day (Group IV) significantly (P<0.05) attenuated the COX2 mRNA gene expression level in lung tissue (10.58±0.777 vs. 5.72±0.329) compared to non-treated animals (Group II, LPS model group) (Figure 2). Moreover, doubling the dose of L-carvone to 100mg/Kg/day (Group V, L-carvone treatment) also resulted in significant (P<0.05) attenuation of COX2 mRNA level (10.58±0.777 vs. 4.59± 0.946) compared to such level in non-treated mice (LPS model group). In addition, data of Table (1) and Figure (2) pointed out that there was a non-significant (P>0.05) difference in the COX2 mRNA expression level between the (Group III, corn oil + LPS) and non-treated animals (Group II, LPS model group), which can indicate that the utilization of corn oil as a vehicle in the study has no effect on the COX2 mRNA gene expression level (in other word, corn oil had no anti-inflammatory effect).

In addition, data analysis reveals that there is a non-significant (P>0.05) difference in the COX2 mRNA expression level between (Group IV) mice treated with 50mg/kg/day and (Group V) mice treated with 100mg/kg/day L-carvone. Table (1) and Figure (2).
RT-qPCR analysis of COX2 mRNA expression level in lung tissue of mice treated with L-carvone 50 mg/kg or 100 mg/Kg/day for 5 days and euthanized 2 hrs after LPS administration (n=10 mice in each group). Data represent mean ±SEM. 
# : Significant (P<0.05) differences compared to the normal (negative) control Group I mice. a: non-significant difference (P>0.05) (Group III, Corn oil+ LPS) compared to the LPS model Group II mice. 
*: Significant difference (P<0.05) of L-carvone Groups (IV and V) each compared to the lipopolysaccharide (LPS) model Group (II) mice. 
b: Non-significant (P>0.05) difference between Groups IV and V mice.

Effect of L-carvone on NF-κB mRNA level:
Results shown in Table (1) revealed that levels of NF-κB mRNA gene expression were significantly-elevated (P<0.05) in the LPS model group compared to the corresponding gene expression level in the normal control group (11.88±1.227 vs. 1.15 ± 0.369), while in the mice’s group received L-carvone 50mg/kg/day (Group IV) showed that there was significant (P<0.05) down-regulation of NF-κB mRNA gene level (11.88±1.227 vs. 6.81±1.362) compared to non-treated mice (LPS model group) (Figure 3). Furthermore, administration of L-carvone 100mg/kg/day (Group V) to mice also resulted in a significant (P<0.05) reduction in NF-κB mRNA gene expression level (11.88±1.227 vs. 5.01±0.826) compared to such level in non-treated mice (LPS model, Group II). In addition, data pointed out in Table (1) and Figure (3) there were non-significant (P>0.05) differences in NF-κB mRNA gene expression level between the corn oil group (corn oil + LPS Group III) and LPS model group which can indicate that corn oil used as a vehicle in the study has no effect on the NF-κB mRNA gene expression level. 

Also, results of Table (1) and Figure (3) revealed that there was a non-significant difference (P>0.05) in the NF-κB mRNA gene expression level between the treatment groups with L-carvone 50mg/kg/day (Group IV) and 100mg/kg/day (Group V).

Discussion
Acute lung injury (ALI) is a serious inflammatory condition with a high mortality rate in the intensive care unit around the world. It is caused by many factors which affect the lung either directly or indirectly (16, 17). One of the causes of ALI is the lipopolysaccharide (LPS), which is a component of the gram-negative bacterial cell wall. In the present study, the expression of pro-inflammatory mediators TNF-α, COX2, and NF-κB were significantly-elevated in mice intraperitoneally-injected with 10mg/kg LPS to induce ALI (Group II mice); results of this study are consistent with those of others (18-21).

Pretreatment with L-carvone at doses of 50mg/kg/day or 100mg/kg/day for 5 days (Groups IV and Group V), respectively each decreased the gene expression levels of TNF-α, COX2, and NF-κB in lung tissue of treated mice and each compared to those expression levels in LPS model (ALI, Group II) but the effects are not significant (P>0.05) (dose-independent effect). Table 1 and Figures (1, 2, and 3).
Levo (L)-carvone can reduce the mRNA gene expression levels of TNF-α, COX2, and NF-κB, which indicates the anti-inflammatory effect of L-carvone that has been supported by other studies (22-24); furthermore, it has recently been reported that L-carvone can inhibit the elevated TNF-α level and diarrhea score in intestinal mucositis induced by irinotecan (11). Results of many in vitro studies have shown that the anti-inflammatory effect of L-carvone is related to the suppression of TNF-α-induced neutrophil adherence; moreover, studies showed that L-carvone inhibited TNF-α production by macrophage cell line RAW264.7 stimulated with LPS, which further proved the anti-inflammatory effect of L-carvone also in this study; moreover, L-carvone inhibited nitric oxide (NO) and interleukin-1β (IL-1β), interleukin-1α (IL-1α), and NF-κB which are critical inflammatory mediators associated with severe acute and chronic inflammatory diseases (22-24).

Furthermore, a study of others revealed that the inflammatory cytokines TNF-α, IL-1β, IL-6, COX2 and NF-κB play critical roles in the development of ALI; thus, inhibiting these cytokines is of significant concern in attenuating ALI (23). Tumor necrosis factor-α (TNF-α) is a crucial endogenous mediator that is mostly-produced by monocytes and macrophages; it can activate the inflammatory response, and plays a major role in the regulation of the inflammatory process in the lung, and causes damage to the cells of vascular endothelium (24).

Furthermore, the COX2 enzyme is involved in converting arachidonic acid (AA) to inflammatory prostanoids, which are involved in the development of early and late phase endotoxemia; moreover such enzyme is a critical component in the inflammatory response downstream of the NF-κB signaling pathway; where, it can be stimulated by several pro-inflammatory factors such as IL-6 and TNF-α, oxidative stress (OS), and growth factors (23, 24).

Macrophages, dendritic cells, and neutrophils are the major component of the innate immune system involved in inflammation; furthermore, these cells express pattern recognition receptor (PRRs) that detect various microbial components, which is called (pathogen-associated molecular patterns) (29).

Moreover, Vidyà MK et al. (2018) reported that LPS activated the PRRs receptor (Toll-like receptor 4), which is a classical pathway that initiates intracellular inflammatory signal transduction which stimulates macrophages to produce pro-inflammatory cytokines production (30), and it is essential for inflammatory M1 macrophage polarization and inflammatory cytokines production (31). Also, its activation resulted in the activation of NF-κB through myeloid differentiation 88 dependent (MyD88 dependent) and MyD88 independent pathway (32); furthermore, the NF-κB is a crucial transcription factor of M1 macrophages and is required for the induction of a large number of inflammatory genes, such as TNF-α, IL-1β, IL-6, and COX-2 (29).

Moreover, OS can contribute to the pathogenesis of ALI by the activation of transcription factor NF-κB and the downstream pro-inflammatory cytokines (33). Levo (L)-carvone showed an antioxidant effect in a previous study by reducing the superoxide-free radical ($O_2^\cdot-$); and, the antioxidant treatment in oxidant-induced lung injury has been widely observed to suppress NF-κB activation and the outspread neutrophilic lung inflammation (34). In the current study, the lung-protective effects of L-carvone could either be due to its direct inhibition of NF-κB signaling pathway or indirect effect by inhibition of OS/ NF-κB pathway which has been shown in Table (1) and Figure (3).

Furthermore, L-carvone was able to exert a protective effect in ALI induced by LPS by hampering NF-κB activation and its downstream pro-inflammatory cytokines (TNF-α, COX2) production; the results of the present study are consistent with the results of other, which revealed that downregulation of NF-κB resulted in ameliorating lung injury in mice (35); in addition, L-carvone induced the production of the anti-inflammatory factor interleukin-10 (IL-10) which further proves its anti-inflammatory effect (23).

**Conclusion**

Based on the observed effects, L-carvone (in dose-independent effect) has lung-protective and anti-inflammatory effects on LPS-induce ALI; and its anti-inflammatory effect is ensured by hampering NF-κB gene expression and its downstream pro-inflammatory cytokines TNF-α and COX2, thus protecting the lungs from acute inflammatory damage.

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