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ATTENUATION OF ENDOTOXIN-INDUCED OXIDATIVE STRESS AND MULTIPLE ORGAN INJURY BY 3,4-METHYLENEDIOXYPHENOL IN RATS

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Running title: Sesamol on endotoxin-induced multiple organ injury

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ABSTRACT

Endotoxin is a potent inducer of lipid peroxidation, which is associated with the development of endotoxemia. 3,4-Methylenedioxyphenol (sesamol) is one of the sesame oil lignans with a high anti-lipid-peroxidation effect. Whether sesamol can attenuate endotoxin-induced lipid peroxidation and multiple organ injury is unknown. After a dose response for sesamol in endotoxin-challenged rats was established, experiments were conducted to assess its effects on hydroxyl radical, peroxynitrite, and superoxide anion counts, activities of superoxide dismutase, catalase, and glutathione peroxidase, as well as the productions of nitric oxide and the expression of inducible nitric oxide synthase. Additionally, the effects of sesamol on endotoxin-induced hepatic and renal injuries were assessed. Sesamol (a) dose-dependently reduced serum lipid peroxidation in endotoxin-challenged rats; (b) decreased hydroxyl radical and peroxynitrite, but not superoxide anion counts; (c) increased the activities of superoxide dismutase, catalase, and glutathione peroxidase in endotoxin-treated rats; (d) reduced nitric oxide production and inducible nitric oxide synthase expression; and (e) attenuated hepatic and renal injuries induced by endotoxin in rats. We concluded that sesamol might protect against organ injury by decreasing nitric-oxide-associated lipid peroxidation in endotoxemic rats.

Keywords: sesamol; endotoxin; lipid peroxidation; nitric oxide; rats
INTRODUCTION

In past decades, sesame oil has been regarded as a daily nutritional supplement to increase cell resistance to lipid peroxidation (1). Recently, it has been suggested that sesame oil increases hepatic detoxification of chemicals, reduces the incidence of chemically induced mammary tumors, and protects against oxidative stress (2). We previously reported (3-6) that sesame oil potently attenuates oxidative-stress-associated multiple organ injury triggered by endotoxin in rats, but its anti-oxidative component is not well clarified (2).

3,4-Methylenedioxyphenol (sesamol) (Fig. 1) is one of the sesame oil lignans with a high anti-lipid peroxidation effect (1). Sesamol has potent inhibitory effects on the lipid peroxidation of liposomes induced by Fe^{2+}, on the lipid peroxidation of rat liver microsomes induced by CCl_{4} or NADPH, and on the lipid peroxidation of mitochondria induced by ascorbate/Fe^{2+} (7). To manage the critical situation in endotoxemia, sesamol might be more beneficial than sesame oil. First, water-soluble sesamol can easily be prepared and administered to mitigate liver- and kidney-damaging oxidative stress. Second, during sepsis, impaired gastric mucosa, and acid secretion may decrease the digestion and absorption of drugs (8, 9). However, the effects of sesamol on endotoxin-induced lipid peroxidation and organ injury have never been investigated.

Endotoxin induces lipid peroxidation, which plays an important role in the pathogenesis of multiple organ injury during endotoxemia (10, 11). Reactive oxygen intermediates (ROI), including singlet oxygen, hydrogen peroxide, and radicals such as superoxide anion and hydroxyl radical (12), are important mediators of cellular injury and play a putative role in endotoxin-associated oxidative stress (13-15). In addition, oxidative stress is associated with the alterations of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (16, 17). In general, superoxide anion is catalyzed into hydrogen peroxide and water by SOD and CAT; both are considered the crucial enzymes of the anti-oxidative defense
system (18, 19). GPx prevents ROI from interacting with critical cellular constituents, such as the phospholipids of biomembranes, nucleic acids, and proteins (20-23). Additionally, an endotoxin challenge activates inducible NO synthase (iNOS), which results in the overproduction of nitric oxide (NO). NO is the main source of hydroxyl radical and peroxynitrite, both of which are the important mediators of lipid peroxidation (LPO) and NO-mediated cell injury in endotoxemia (24, 25). The effect of sesamol on endotoxin-induced lipid peroxidation and multiple organ injury are still unknown. The aim of this study was to assess the effects of sesamol on lipid peroxidation and multiple organ injury in endotoxin-challenged rats.

**MATERIALS AND METHODS**

**Materials**

Endotoxin lipopolysaccharide (LPS) derived from *E. coli*, serotype 055:B5 and sesamol were obtained from Sigma-Aldrich (St. Louis, MO).

**Animals**

The animal care and experimental protocols were in accord with nationally approved guidelines. Thirty-six male SPF Wistar rats weighing 200-250 g were obtained from and housed in our institution's laboratory animal center. Rats were housed individually in a room with central air conditioning (25°C, 70% humidity) and a 12-h light:dark cycle. They were allowed free access to tap water and a pelleted rodent diet (Richmond Standard; PMI Feeds, Inc., St. Louis, MO).

In a dose-response study, rats were given sesamol subcutaneously (s.c.) ranging from 1 to 30 mg/kg (n = 6/group) just before an intraperitoneal (i.p.) LPS (5 mg/kg) injection; serum lipid peroxidation was assessed 6 h later. In other studies, rats were divided into four groups of six. Saline-group rats were given saline (1 mL/kg, s.c.) alone; SM-group rats were given
sesamol (10 mg/kg, s.c.) alone; LPS-group rats were challenged with LPS (5 mg/kg, i.p.) dissolved in saline (2); and LSM-group rats were given sesamol (10 mg/kg, s.c.) just before an injection (i.p.) of LPS. Parameters were measured 6 h after each treatment.

To confirm the effect of sesamol on endotoxin-induced lipid peroxidation, superoxide anion, peroxynitrite, hydroxyl radical, SOD, CAT, and GPx were also assessed. To examine the role of NO in the effect of sesamol on lipid peroxidation, serum nitrite level and iNOS protein expression were determined. Additionally, hepatic and renal functions were evaluated by determining serum aspartate aminotransferase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine levels. All rats were also given a histological examination.

**Blood collection**

Blood samples were collected from the femoral veins of rats under ethylether anesthesia. Blood was drawn into serum separation tubes, allowed to clot for 30 min at room temperature, and then centrifuged at 1000 × g for 10 min at 4°C.

**Measurement of serum lipid peroxidation levels**

Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Whole blood (500 μL) was centrifuged at 2500 × g for 10 min at 4°C. Supernatant (200 μL) was taken for lipid peroxidation measurement using a commercial assay kit (Lipid Peroxidase Assay Kit; Merck Biosciences GmbH, Darmstadt, Germany), and the spectrophotometer was read at 586 nm (26).

**Superoxide anion, hydroxyl radical, and peroxynitrite counts**

The ongoing production of oxygen radicals—superoxide anion, peroxynitrite, and hydroxyl radical—in blood isolated from endotoxin-treated rats was measured using a high-performance chemiluminescence (CL) analyzer (model CLA-2100; Tohoku Electronic
Industrial Co. Ltd., Rifu, Japan). Briefly, 400 μL of a whole blood sample was mixed with 200 μL of PBS in a stainless dish, and then the background CL count was read for 60 sec. One hundred microliters of lucigenin, indoxyl βD-glucuronide, or luminol (17 mM dissolved in PBS, for determination of superoxide anion, hydroxyl radical, and peroxynitrite, respectively) was injected into the machine, and CL was counted for another 1200 sec at 10-sec intervals. The data were analyzed using Chemiluminescence Analyzer Data Acquisition Software (CLA-DAS) (Tohoku Electronic Industrial Co.) (27).

**Measurement of SOD activity in lysed blood cells**

Blood samples were collected in tubes containing EDTA as an anticoagulant. Whole blood (500 μL) was centrifuged at 2500 × g for 10 min at 4°C. Pellets were resuspended in 4 packed-cell volumes of ice-cold water. The stored frozen red blood cells were then homogenized and centrifuged at 2500 × g for 10 min at 4°C. SOD activity was measured (Superoxide Dismutase Assay Kit; Calbiochem-Novabiochem International, Inc., La Jolla, CA) in 40 μL of the supernatant, and the spectrophotometer was read at 525 nm.

**Measurement of CAT activity in lysed blood cells**

Blood samples were collected in tubes containing the anticoagulant heparin. Five hundred microliters of whole blood was centrifuged at 2500 × g for 10 min at 4°C. The plasma supernatant was discarded and the pellet washed 3 times in ice-cold 0.9% NaCl. The erythrocyte pellet was resuspended in 4 volumes of ice-cold de-ionized water and kept on ice for 10 min. Thirty microliters of diluted sample was used to assess CAT activity (Catalase Assay Kit; Calbiochem-Novabiochem), and the spectrophotometer was read at 520 nm.

**Measurement of GPx activity in lysed blood cells**

Briefly, whole blood sample (100 μL) was centrifuged (2500 × g, 10 min) and washed 3 times in ice-cold 0.9% NaCl; 1 mL of ice-cold de-ionized water was then added. One hundred
microliters of hemolysate was mixed with 1 mL of Drabkin's solution (0.77 KCN and 0.6 mM K$_3$[Fe(CN)$_6$] in 1 mM potassium dihydrogen phosphate, pH 7.0). Five hundred microliters of transformation solution (4.5 mM KCN and 0.45 mM K$_3$[Fe(CN)$_6$] adjusted with 0.25 M potassium dihydrogen phosphate to pH 7.0) was added to 1 mL of hemolysate. Five hundred microliters of hemolysate was transferred to a cuvette containing 100 µL of GSH, NADPH, GSSG reductase, and buffer. Measurement started upon adding 100 µL of H$_2$O$_2$. The absorbance differences during 2 min were measured at 366 nm using a spectrophotometer (24).

**Measurement of serum nitrite concentration**

Briefly, the concentration of nitrite in serum was measured following the Griess reaction by incubating 100 µL of sample with 100 µL of Griess reagent (Sigma) at room temperature for 20 min. The absorbance was measured at 550 nm using a spectrophotometer (29). Nitrite concentration was calculated by comparing it with standard solutions of known sodium nitrite concentrations.

**Rat leukocyte isolation**

We isolated rat leukocytes from whole blood using erythrocyte lysis buffer. The leukocyte pellet was homogenized in 200 µL of ice-cold lysis buffer containing 20 mM Hepes (pH 7.2), 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin. We centrifuged this solution at 12,000 rpm for 30 min and then determined the protein concentration in the supernatant using protein assay dye (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard (6).

**Western blotting**

We loaded 50 µg of protein on 8% or 10% SDS-PAGE, and then transferred it to nitrocellulose sheets (NEN Life Science Products, Inc, Boston, MA) in a transfer apparatus
(Bio-Rad) run at 1.2 A for 3 h. After we blocked the blots in 5% non-fat skim milk in TBST, we incubated the blots with primary iNOS polyclonal antibody (dilution 1:1000) (BD Biosciences, San Diego, CA) against target protein in 5% non-fat skim milk, and then with anti-rabbit IgG conjugated with alkaline phosphatase (dilution 1:3000) (Jackson ImmunoResearch Laboratories, Inc., Philadelphia, PA). Immunoblots were developed using BCIP/NBT solution (Kirkegaard & Perry Laboratories, Inc., Baltimore, MD).

**Blood biochemistry study**

Four marker enzymes were used as biochemical indicators of hepatic and renal dysfunction. Hepatic dysfunction and injury were assessed by measuring rises in serum levels of AST and ALT. Renal dysfunction and injury were assessed by measuring rises in serum levels of BUN and creatinine. Serum samples were spotted to slides (Vitros DT; Johnson & Johnson Co., Rochester, NY) and evaluated for all the above indicators using a blood biochemical analyzer (Ektachem DTSCII; Eastman Kodak, Rochester, NY).

**Histological evaluation of organ injury**

Hepatic and renal injury was further assessed by histological examination. Briefly, organ tissues were fixed in 4% formaldehyde buffered with a phosphate solution (0.1 M, pH 7.4) at room temperature. Organ fragments were washed in phosphate buffer, dehydrated in graded concentrations of ethanol, and then embedded in paraffin. From each tissue, 4-μm-thin sections were obtained and stained with hematoxylin and eosin to evaluate hepatic and renal morphology (30). Quantified histological changes versus viable regions of the liver lobule and cortical tubules in every animal were examined using a light microscope. Hepatic and renal cell swelling and necrosis were evaluated qualitatively using five grades: (0) normal; (I) areas of cell swelling and necrosis involving < 25% of hepatocytes or cortical tubules; (II) similar changes involving > 25% but < 50% of hepatocytes or cortical tubules; (III) similar changes involving > 50% but < 75% of hepatocytes or cortical tubules; (IV) similar changes involving >
75% of hepatocytes or cortical tubules. The mean score of all fields was taken as the hepatic and renal injury scores of these sections (31, 32).

**Statistical analysis**

Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) and then the Tukey Honestly Significant Difference (HSD) method was used to make pairwise comparisons between the treatments. Statistical significance was set at \( P < 0.05 \).

**RESULTS**

*Dose response of sesamol in endotoxin-induced lipid peroxidation in rats*

In the present study, we generated a dose-response curve (range, 1-30 mg/kg) against lipid peroxidation in LPS-treated rats. LPS significantly increased serum lipid peroxidation compared with the saline group. It showed that 10 and 30 mg/kg of sesamol produced the maximal inhibition of lipid peroxidation (90-95% inhibition) (Fig. 2). Thus, we chose 10 mg/kg of sesamol, the minimal effective dose, for this study.

*Effects of sesamol on oxidative stress in endotoxin-treated rats*

To further examine the effects of sesamol on endotoxin-induced lipid peroxidation, we ran hydroxyl radical and superoxide anion counts and analyzed the activities of oxidative-stress-associated enzymes—SOD, CAT, and GPx. LPS increased the hydroxyl radical and peroxynitrite counts, but not the superoxide anion counts. Sesamol significantly reduced hydroxyl radical (Fig. 3A) and peroxynitrite (Fig. 3B) generation, but it did not affect the superoxide anion level (Fig. 3C). In addition, LPS potently decreased SOD, CAT, and GPx (all \( P < 0.05 \)) (Figs. 4A-4C). Sesamol significantly reversed all the changes induced by LPS (\( P < 0.05 \)). No significant difference was found between the SM group and the Saline group for the mentioned tested parameters.
Effects of sesamol on serum nitrite production and leukocyte iNOS expression

To further examine the involvement of nitric oxide in sesamol-associated protection, serum nitrite production and leukocyte iNOS expression levels were determined. LPS markedly increased serum nitrite production. Sesamol reduced LPS-induced nitrite production. No significant difference was found between the Saline and SM groups (Fig. 5A). In addition, LPS markedly increased leukocyte iNOS expression, but sesamol decreased LPS-induced iNOS expression (Fig. 5B).

Effect of sesamol on LPS-induced hepatic and renal injuries in endotoxin-treated rats

To assess the protective effects of sesamol on LPS-induced hepatic and renal dysfunction, we determined the levels of AST, ALT, BUN, and creatinine. LPS significantly increased the levels of AST ($P < 0.0001$) (Fig. 6A), ALT ($P < 0.0001$) (Fig. 6B), BUN ($P < 0.0001$) (Fig. 6C), and creatinine ($P = 0.007$) (Fig. 6D). In the LSM group, sesamol significantly attenuated all of them (all $P < 0.05$). Microscopic examination of livers and kidneys from rats in the LPS group showed cell swelling and coagulative necrosis. Tissue from the LSM group showed marked attenuation in LPS-initiated morphological changes (Fig. 6E). In addition, sesamol significantly decreased LPS-increased hepatic and renal histological scores (Fig. 6F).

DISCUSSION

We demonstrated that a single dose of sesamol, a sesame oil lignan, ameliorated lipid peroxidation and multiple organ injury induced by endotoxin in rats. Sesamol suppressed lipid peroxidation and diminished the generation of NO, peroxynitrite, and hydroxyl radical in endotoxin-challenged rats. We suggest that inhibition of iNOS expression might be involved in sesamol-associated anti-oxidation in endotoxemia in rats.

While hydroxyl radical, peroxynitrite, and superoxide anion are causes of
endotoxin-induced lipid peroxidation, hydroxyl radical and peroxynitrite may be potent contributors and major causes of endotoxin-induced oxidative stress (33). LPS significantly decreased the activities of SOD and CAT, but it did not alter the superoxide anion level 6 h after it had been injected. In contrast, superoxide anion counts increased 12 h after LPS injection without affecting the activities of SOD or CAT (5), which may have neutralized superoxide anion, thus preventing an increase during the first 6 h (16). Further, sesamol potently reduced LPS-induced lipid peroxidation and the generation of hydroxyl radical and peroxynitrite. Consequently, sesamol might reduce oxidative stress by inhibiting the generation of hydroxyl radical and peroxynitrite. In addition, sesamol maintained the normal activities of SOD, CAT, and GPx, all of which might reduce both free radicals and lipid peroxidation. This provided a further confirmation of the anti-lipid-peroxidation effect of sesamol in endotoxin-treated rats.

Sesamol may inhibit hydroxyl radical- or peroxynitrite-initiated lipid peroxidation, or both, by reducing NO production in LPS-treated rats. Sesamol significantly decreased NO production and iNOS protein expression; this might be attributed to the attenuation of LPS-induced oxidative stress and its resultant organ protection. NO is a highly and spontaneously chemically reactive free radical. It reacts with superoxide anion to generate peroxynitrite and hydroxyl radical (34), both of which are important mediators of oxidative stress and NO-mediated cell injury in many pathological states (24, 25). Sesamol might attenuate LPS-induced oxidative stress and LPS-associated organ injury by reducing the NO-mediated hydroxyl-radical generation in rats.

Sesame oil used as a therapeutic agent protects rats from experimental sepsis (4). Although various lipids, such as olive oil and fish oil, inhibit the inflammatory response induced by LPS (35-37), most of them are used as nutritional supplements, which decreases their relevance in managing inflammatory diseases such as endotoxemia and septic shock.
The suppression of iNOS expression may be involved in the reduction of NO and subsequent lipid peroxidation in sesamol-associated protection against LPS-induced endotoxemia. iNOS, responsible for the over-production of NO, is highly expressed after LPS-induced activation of phagocytes, such as neutrophils (38) and macrophages (39). Nuclear transcription factor NF-kappaB and pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, play the crucial role in regulating iNOS expression and NO production (40-42). Therefore, we hypothesize that sesamol inhibits LPS-induced iNOS expression by affecting NF-kappaB- and pro-inflammatory cytokine-associated signal transduction; however, more investigations of the mechanisms underlying sesamol-exerted iNOS inhibition are needed. We conclude that the reduction of nitric-oxide-associated oxygen free radical generation might be associated with the protection of sesamol against endotoxin-induced lipid peroxidation and organ injury in rats.
ACKNOWLEDGMENTS

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FIGURE LEGENDS

**Figure 1.** Structure of 3,4-methylenedioxyphenol.

**Figure 2.** Dose response of sesamol in endotoxin-induced lipid peroxidation in rats. Rats were given saline (1 mL/kg) or sesamol (1-30 mg/kg) just before a lipopolysaccharide (5 mg/kg) injection. The gray bar represents the lipid peroxidation level of rats without any treatment for comparison. Values are mean ± standard deviation (SD) (n = 6/group). Significant differences of measurement traits were analyzed using one-way ANOVA. The differences between those treatments with different letters are statistically significant (P < 0.05).

**Figure 3.** Effects of sesamol on ongoing production of free radicals in blood from endotoxin-treated rats. Rats were given intraperitoneal saline (1 mL/kg) (Saline group), sesamol (10 mg/kg) (SM group), LPS (5 mg/kg) (LPS group), or LPS (5 mg/kg) plus sesamol (10 mg/kg) (LSM group). The production of hydroxyl radical (A), peroxynitrite (B), and superoxide anion (C) was assessed 6 h after treatment. Data are expressed as mean ± SD (n = 6/group). Significant differences of measurement traits were analyzed using one-way ANOVA. The differences between those treatments with different letters are statistically significant (P < 0.05).

**Figure 4.** Effect of sesamol on the activity of defense enzymes in lysed blood cells in endotoxin-treated rats. Rats were given intraperitoneal saline (1 mL/kg) (Saline group), sesamol (10 mg/kg) (SM group), LPS (5 mg/kg) (LPS group), or LPS (5 mg/kg) plus sesamol (10 mg/kg) (LSM group). The activities of superoxide dismutase (SOD) (A), catalase (CAT) (B), and glutathione peroxidase (GPx) (C) were assessed 6 h after treatment. Data are expressed as mean ± SD (n = 6/group). Significant differences of measurement traits were analyzed using one-way ANOVA. The differences between those treatments with different
letters are statistically significant ($P < 0.05$).

**Figure 5.** Effects of sesamol on serum nitrite production and leukocyte iNOS protein expression in endotoxin-treated rats. Rats were given intraperitoneal saline (1 mL/kg) (Saline group), sesamol (10 mg/kg) (SM group), LPS (5 mg/kg) (LPS group), or LPS (5 mg/kg) plus sesamol (10 mg/kg) (LSM group). Serum nitrite (A) and leukocyte iNOS expression (B) were determined 6 h after treatment. Data are means ± SD ($n = 6$/group). Significant differences of measurement traits were analyzed using one-way ANOVA. The differences between those treatments with different letters are statistically significant ($P < 0.05$).

**Figure 6.** Sesamol attenuated hepatic and renal injuries in LPS-treated rats. (A-D) Rats were given intraperitoneal saline (1 mL/kg) (Saline group), sesamol (10 mg/kg) (SM group), LPS (5 mg/kg) (LPS group), or LPS (5 mg/kg) plus sesamol (10 mg/kg) (LSM group). Data are expressed as mean ± standard deviation ($n = 6$/group). Significant differences in measurement traits were analyzed using one-way ANOVA. Differences between treatments with different letters are statistically significant ($P < 0.05$). (E) Livers and kidneys were from Saline, LPS, and LSM groups, respectively. The arrows point out the cell degeneration and necrotic area (Hematoxylin and eosin stain; magnification = ×100). (F) Hepatic and renal histological score. Data are expressed as mean ± standard deviation ($n = 6$). Significant differences in measurement traits were analyzed using one-way ANOVA. Differences between treatments with different letters are statistically significant ($P < 0.05$).
Figure 1

3,4-methylenedioxyphenol
Figure 2

![Bar chart showing LPO (μM MDA) levels at different doses (mg/kg). The doses are 0, 1, 3, 10, and 30, with corresponding LPO levels indicated by 'a', 'b', and 'ab'.](image-url)
Figure 3
Figure 4

(A) SOD (U/mL)

(B) CAT (x10^4 U/mL)

(C) GPx (x10^{-1} ΔAbs)

Saline, SM, LPS, LSM
Figure 5

(A) and (B) show the results of nitrite levels and protein expression under different conditions. The graphs indicate significant differences in nitrite levels and protein expression between the groups.

(A) Bar graph showing nitrite levels (μM) for Saline, SM, LPS, and LSM. The bars are labeled with letters indicating statistical significance.

(B) Western blot analysis showing protein expression for iNOS and G3PDH under the same conditions. The blots display the intensity of protein expression in each group.
Figure 6
Figure 6