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# Inhibition by Moxa Smoke of NO Production and iNOS Expression in Mouse Macrophage-like Cells Raw 264.7

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**Abstract.** *The biological activities of Moxa, used as moxibustion, have not been well documented. We investigated the effect of Moxa smoke on nitric oxide (NO) production by mouse macrophage-like Raw 264.7 cells. Moxa smoke failed to stimulate the Raw 264.7 cells to produce detectable amounts of NO, but rather inhibited the NO production by lipopolysaccharide (LPS)-activated Raw 264.7 cells. The 50% inhibitory concentration ($IC_{50}$) of NO production by Moxa smoke (0.16%) was one order lower than the 50% cytotoxic concentration ($CC_{50}$) (4.67%). Western blot and RT-PCR analyses demonstrated that a slightly higher concentration of Moxa is required to reduce the iNOS expression at protein and mRNA levels ($IC_{50}$=0.99 and 2.03%, respectively). The inhibition of NO production by Moxa smoke is, thus, probably due to both the inhibition of iNOS expression and radical-scavenging activity. The present data suggest the possible anti-inflammatory effect of Moxa smoke.*

Moxa is a dried cotton-like material from the leaf of yomogi (*Artemisia capillaris*), and used as moxibustion. Extracts of yomogi have shown various biological activities such as antioxidant (1), apoptosis-inducing (2) and anti-inflammatory activity (3). On the other hand, no detailed study of the biological activity of Moxa has been reported. We found that a hot-water extract of Moxa has higher radical intensity (*in vitro*), radical scavenging activity (*in vitro*) and antimicrobial activity (*in vivo*) than the highly cytotoxic ethanol extract of Moxa (4). The cytotoxic substances have been partially purified from the $CH_2Cl_2$-extract of Moxa by three cycles of silica gel column chromatography (5). The active fractions (which contained two peaks on HPLC separation) showed higher cytotoxicity against five human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, melanoma A-375, promyelocytic leukemia HL-60, T-cell leukemia MT-4) than three normal human cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) (5). On the other hand, Moxa smoke showed oxidation potential (measured by nitric oxide (NO) monitor), produced carbon radical (measured by ESR spectroscopy), modified the conformation and/or activity of Mn-superoxide dismutase (MnSOD) (measured by activity staining after polyacrylamide gel electrophoresis), and induced internucleosomal DNA fragmentation in HL-60 cells (measured by agarose gel electrophoresis), but not in other cell lines (HSC-2 and human mammary carcinoma MCF-7) (6). Addition of *N*-acetyl-l-cysteine, a popular antioxidant, significantly reduced the cytotoxic activity, oxidation potential and carbon radical intensity of Moxa smoke (6), suggesting its pro-oxidant action. We have recently found that Moxa smoke has some tumor-specific cytotoxicity, by comparing its cytotoxic activity against human oral carcinoma cells and normal oral cells (7). In the present study, we investigated whether Moxa smoke modifies the function of macrophage, by measuring NO production by unstimulated and lipopolysaccharide (LPS)-stimulated mouse macrophage-like Raw 264.7 cells. Since the NO concentration is determined by the intracellular concentration of inducible NO synthase (iNOS), the enzyme activity of iNOS and the quenching of NO radical by radical scavengers present in the assay systems, we also investigated whether Moxa smoke affects the expression of iNOS protein and mRNA by Western blot and RT-PCR analyses, respectively.

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*Key Words:* Moxa smoke, LPS, mouse macrophage, iNOS expression, NO.

## Materials and Methods

*Materials.* The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA, USA); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT),

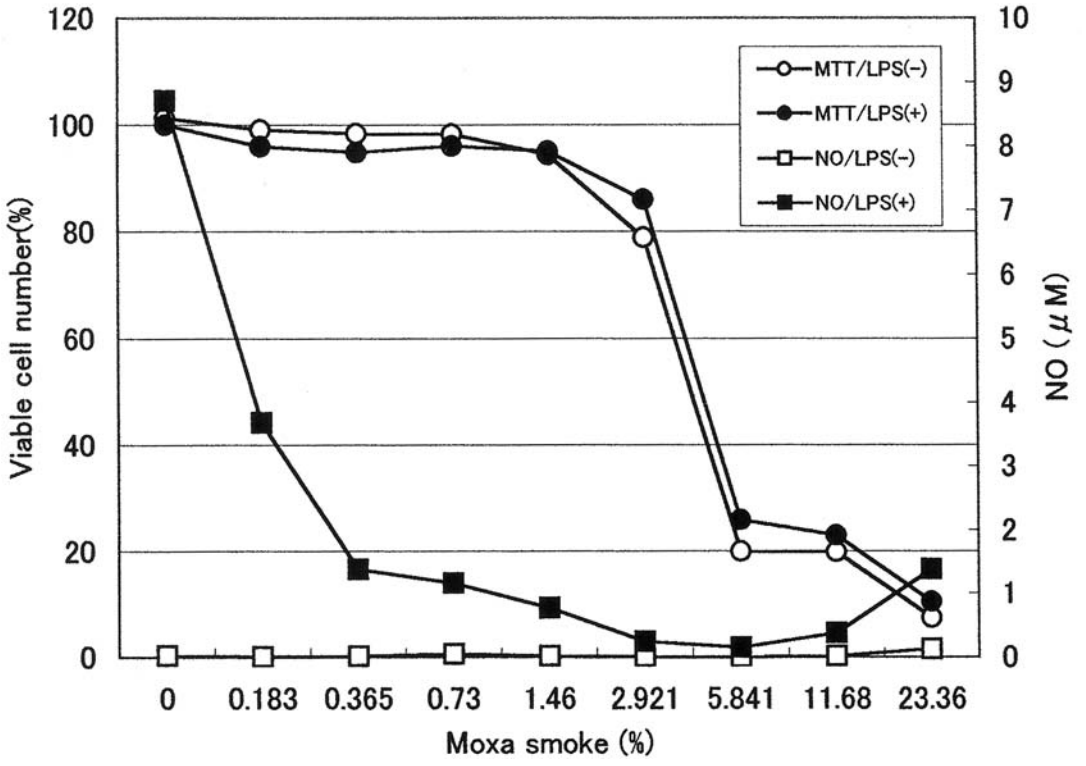


Figure 1. *Moxa smoke inhibited the NO production by LPS-stimulated Raw 264.7 cells. Near-confluent Raw 264.7 cells were incubated for 24 hours with the indicated concentrations of Moxa smoke, in the absence (○,□) or presence (●,■) of 100 ng/mL LPS in phenol red-free DMEM supplemented with 10% FBS, and then the viable cell number (○,●) and extracellular concentration of NO (□,■) were determined by MTT method and Griess method, respectively.*

phenylmethylsulfonyl fluoride (PMSF), dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind, Osaka, Japan); LPS from *E. coli* (serotype 0111:B4) (List Biological Laboratories, Inc).

*Preparation of Moxa smoke.* Moxa smoke was collected *via* capillary tube into phosphate-buffer saline without $Ca^{2+}$ and $Mg^{2+}$ (PBS) (-)(pH7.4) under reduced pressure, with an aspirator. The yield of Moxa smoke from the starting material was 1.0-2.7%, assuming that $1A_{260}$=0.2 mg/mL (4).

*Cell culture.* Mouse macrophage-like Raw 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% $CO_2$ atmosphere.

*Assay for cytotoxic activity.* Raw 264.7 cells were inoculated at 5 ~ 6 x $10^4$ cells/well in 96-microwell (Becton Dickison Labware NJ, USA), unless otherwise stated. After 24 hours, the medium was removed by suction with an aspirator, and replaced with 0.1mL of fresh medium containing various concentrations of Moxa smoke without or with 100 ng/mL LPS. The cells were incubated for another 24 hours, and the relative viable cell number was then determined by MTT method. In brief, the cells were washed once with PBS(-), replaced with fresh culture medium containing 0.2 mg/mL MTT and incubated for 30 minutes. The cells were lysed with 0.1 mL of DMSO, and the absorbance at 540 nm of the cell lysate was determined, using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). The absorbance at 540 nm of control cells was usually in the range of 0.40 to 0.90. The 50% cytotoxic concentration ($CC_{50}$) was determined from the dose-response curve.

*Assay for NO concentration.* Near confluent Raw 264.7 cells were incubated for 24 hours with each test sample in phenol red-free DMEM supplemented with 10% FBS and the NO production by Raw 264.7 cells was quantified by Greiss reagent, using the standard curve of $NO_2^-$. To eliminate the interaction between the sample and Greiss reagent, we also measured the NO concentration in the culture medium without the cells, and subtracted it from that with the cells. The concentration which caused a 50% inhibition ($IC_{50}$) for the LPS-stimulated NO production was determined from the dose-response curve. The efficacy of inhibition of NO production was estimated by the selectivity index SI, which was calculated by the following equation: $SI = CC_{50}/IC_{50}$

*Western blotting.* The cell pellets were lysed with 100 μL of lysis buffer (10 mM Tris-HCl, pH7.6, 1%Triton X-100, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF) for 10 minutes in ice water, and then incubated for 50 minutes at 4˚C with RT-5 ROTATOR (Titec, Sitama, Japan). The cell lysates were centrifuged at 16,000 xg for 20 minutes at 4˚C to remove the insoluble materials and the

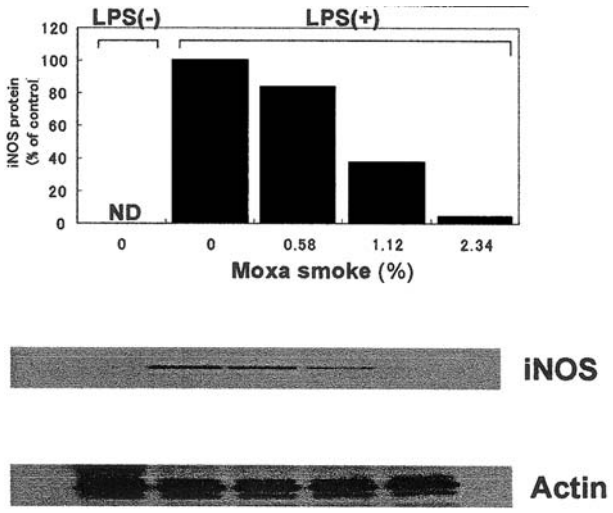


Figure 2. *Effect of Moxa smoke on the intracellular concentration of iNOS protein in Raw 264.7 cells. Raw 264.7 cells were incubated for 24 hours with the indicated concentrations of Moxa smoke in the absence or presence of 100 ng/mL LPS. The iNOS protein expression was quantified by Western blot analysis and densitometry. The intracellular iNOS protein was expressed as the ratio to that of actin. The iNOS protein concentration without LPS was below the detection limit, and therefore omitted.*

supernatant was collected. The protein concentrations of the supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA,USA). Cell lysate (equivalent to 10 μg protein) was mixed with 2 x sodium dodecyl sulfate (SDS)-sample buffer (0.1M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue, 1.2% 2-mercaptoethanol), boiled for 10 minutes, and applied to the SDS-7% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in PBS(-) saline plus 0.05% Tween 20 for 90 minutes and incubated with anti-iNOS antibody (1:1000, Santa Cruz Biotechnology, Delaware, CA, USA) for 90 minutes at room temperature or overnight at 4˚C, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 hour at room temperature. Immunoblots were detected by Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA), and analyzed on a power Macintosh 7600/120 using the public domains NIH Image program.

*Assay for mRNA expression*. Total RNA was isolated by ISOGEN RNA extraction reagent (Nippon Gene, Tokyo, Japan). A reverse transcriptase reaction (RT) was performed with 1.0 μL of total RNA, using the Rever Tra Ace (Toyobo Co., Ltd), using oligo $(dT)_{20}$ primer. Single strand cDNA obtained by RT reaction was amplified, using the KOD plus (Toyobo Co., Ltd), using iNOS and β-actin specific primers, according to the protocol. The RT-PCR products were applied to 2% agarose gel electrophoresis and the ethidium bromide-stained gel was then photographed under UV light.

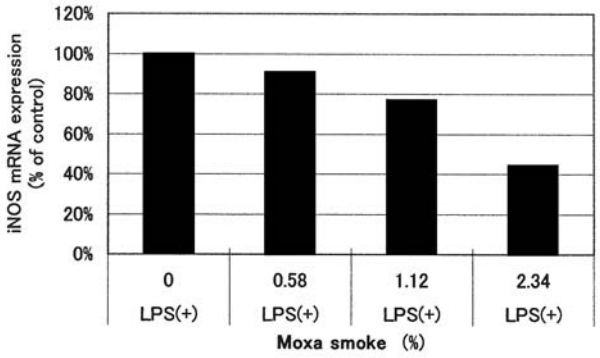


Figure 3. *Effect of Moxa smoke on LPS-stimulated iNOS mRNA expression. Raw 264.7 cells were incubated for 24 hours with the indicated concentrations of Moxa smoke in the absence or presence of 100 ng/mL LPS. RNA was then isolated, and the RT-PCR product was applied to agarose gel electrophoresis and quantified by densitometry. The expression of iNOS mRNA was expressed as the ratio to that of β-actin mRNA. The iNOS mRNA expression without LPS was below the detection limit, and therefore omitted.*

## Results

*Inhibition of NO production.* Moxa smoke dose-dependently reduced the viable cell number of Raw 264.7 cells ($CC_{50}$=4.67%, calculated to be 199.8 μg/mL, based on the absorbance spectrum) (Figure 1). Moxa smoke alone did not stimulate Raw 264.7 cells to produce any detectable amount of NO, but it inhibited the NO production by LPS-stimulated Raw 264.7 cells, even at noncytotoxic concentration ($IC_{50}$=0.16%), yielding the SI viable of 29.5 (Figure 1).

*iNOS expression.* We next investigated the possibility that this compound may reduce the intracellular concentration of iNOS. Western blot analysis showed that unstimulated Raw 264.7 cells expressed only a background level of iNOS protein, but, upon stimulation with LPS, they began to express detectable amounts of iNOS protein. This LPS-induced iNOS expression was dose-dependently inhibited by Moxa smoke. The inhibitory effect of Moxa smoke was detected above 0.58% (25 μg/mL) (Figure 2). At 2.34% (100 μg/mL) of Moxa smoke, the intracellular concentration of iNOS declined nearly to the baseline level. From the dose-response curve, the $IC_{50}$ for iNOS protein expression was calculated to be 0.99%, six times higher than the $IC_{50}$ for NO production (0.16%).

RT-PCR analysis demonstrated that treatment with 0.58% (25 μg/mL) Moxa smoke resulted in a slight decline in iNOS mRNA expression. At 2.34% (100 μg/mL) of Moxa smoke, iNOS mRNA expression was less than 50% of the control level. The $IC_{50}$ was calculated to be 2.03%, about thirteen times higher than the $IC_{50}$ for NO production (Figure 3).

## Discussion

NO is produced from l-arginine by NOS in the presence of NADPH, and displays diverse biological activities such as vasodilation, inhibition of endothelial leukocyte adhesion and regulation of energy metabolism (8). We have previously reported that LPS stimulated Raw 264.7 cells to produce tumor necrosis factor (TNF), NO, citrulline and asparagines (9, 10). The present study demonstrated, for the first time, that Moxa smoke reduced the extracellular NO concentration in LPS-activated Raw 264.7 cells, and therefore may inhibit the biological action of the NO radical. The inhibitory activity of Moxa smoke was accompanied by the decline of both iNOS protein and mRNA expression. However, the $IC_{50}$ for the NO production (0.16%) was much smaller than that for iNOS protein (0.99%) and mRNA expression (2.03%). This suggest that Moxa smoke inhibits the NO production by a combination of its inhibition of iNOS expression and other mechanisms such as NO scavenging activity. We found that Moxa (3.7%) reduced the radical intensity of NO (produced from NOC-7) by 50% (7).

The possibility that Moxa smoke might interfere with the activation pathway such as NF-Î B triggered by LPS in Raw 264.7 cells remains to be investigated (11). Since Moxa smoke inhibits the LPS-stimulated translation of iNOS protein more efficiently than transcription of iNOS mRNA, it may affect the protein stability and modification. We have recently found that Moxa smoke reduced the intracellular concentration of $PGE_2$ elevated by LPS in Raw 264.7 cells, and this effect was diminished by addition of arachidonic acid (Matsumoto *et al.* in preparation). These data, taken together with the present study, suggest the possible anti-inflammatory effect of Moxa smoke. At the time of inflammation, a huge amount of NO is produced *via* activation of iNOS independently of $Ca^{2+}$, and reacts with active oxygens to generate tissue-damaging peroxynitrites and further aggravate the respiratory inflammation. Since Moxa smoke inhibits the NO generation by activated macrophages, this gaseous material may be useful for the treatment of asthma.

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