Antioxidant effect of indirect moxibustion on healthy subjects: a pilot study

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OBJECTIVE: Moxibustion has long been thought to promote vital energy or immunity through clinical experience. This study aimed to investigate the clinical effects of indirect moxibustion on antioxidant and immunomodulation in a normal population.

METHODS: Twenty-five healthy volunteers with no objective or subjective disorders were recruited. The participants were treated with indirect moxibustion on acupoints Guanyuan (CV 4) and Shenque (CV 8) three times per week for 4 weeks. The serum levels of reactive oxygen species (ROS) and malondialdehyde (MDA), the total antioxidant capacity (TAC), the activities of catalase and superoxide dismutase (SOD), and the total glutathione content were determined before and after the 12th moxibustion. Lymphocyte subpopulations and 42 cytokines in the peripheral blood were analyzed using flow cytometry and antibody array, respectively.

RESULTS: Compared with the initial time point, the serum levels of ROS and MDA were significantly lowered by moxibustion, while TAC was increased ($P<0.01$ for all). A significant increase was observed in catalase activity ($P<0.05$), but not in SOD or total glutathione. There were no significant changes in lymphocyte subpopulations or cytokines in the peripheral blood. Fifteen of 25 participants reported at least one symptom in which they felt subjective improvement after moxibustion.

CONCLUSION: Indirect moxibustion on acupoints CV 4 and CV 8 improved the antioxidant defense system, which may be a mechanism explaining the clinical effects of moxibustion.

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Key words: Moxibustion; Point CV 4 (Guanyuan); Point CV 8 (Shenque); Antioxidants; Pilot projects

INTRODUCTION

Moxibustion, one of the main therapies in traditional Oriental medicine, involves the creation of thermal and chemical stimulants by burning moxa on certain acupoints of the body. Moxibustion has been practiced especially to treat chronic illness conditions and prevent disease because it has long been thought to promote vital energy and immune function through clinical experience.

Many recent studies have proven the scientific evidence supporting the efficacy of moxibustion for diverse disorders such as stroke sequelae, ulcerative colitis, and menopausal hot flashes. A few studies elucidated the mechanisms of moxibustion in human and animal models. They involved thermal action, sympathetic vibration at the skin surface, and immunomodulation. However, there is lack of scientific basis explaining the
mechanistic action of a wide range of moxibustion applications.

Oxidative stress is considered to be an important element in the pathogenesis of various conditions including inflammation, cancer, and aging, and the antioxidant effects of many therapies and remedies have been well demonstrated.\textsuperscript{11,12} Some reports have revealed the antioxidant property of acupuncture treatment.\textsuperscript{13-15}

Based on traditional theory and clinical applications, moxibustion is also anticipated to induce antioxidative activity in the body. However, no clinical investigations of a connection between moxibustion and antioxidant effects have been performed.

Therefore, we investigated whether chronic indirect moxibustion at two acupoints (Guanyuan, CV 4 and Shenque, CV 8) affects oxidative stress and antioxidant capacity in healthy adult volunteers.

**METHODS**

**Subjects**
Adults considered to be healthy by self-judgment were recruited, and volunteers showing abnormalities on radiological or hematological examinations were excluded. A physician and radiologist performed these examinations at Daejeon University Hospital. We also excluded night workers, alcohol drinkers, smokers, medication users, and overweight individuals (body mass index of >30). Of those recruited, 25 subjects (5 men and 20 women) were included (median age, 47 years; range, 20-65 years). Informed consent was obtained from each subject, and the ethical committee of Daejeon University Hospital approved the study protocol (authorization number: DJOMC-33-1).

**Study design and moxibustion treatment**
All moxibustion treatments in every subject were performed by a licensed doctor throughout the trial. Moxibustion was performed on subjects at two acupoints on the Conception Vessel Meridian: Guanyuan (CV 4, located at 3 cun below the center of the umbilicus) and Shenque (CV 8, located at the center of the umbilicus) three times per week for 4 weeks. One moxa corn (3.5 g of wormwood fiber) was burned on the top of salt inside bamboo; diameter, 30 mm; length, 40 mm; KyeGoo Inc., Incheon, Korea) was burned for 30 min per treatment while the volunteers were lying on their backs (Figure 1).

To examine the effects of indirect moxibustion on the status of the antioxidant defense system, lymphocyte subpopulations and cytokines were evaluated in blood (serum) samples collected on the day before the first moxibustion treatment and 1 hour after the final moxibustion under conditions of an 8-h fast.

**Chemical materials**

1,1,3,3-tetraethoxypropane (TEP), N,N-diethyl-para-

**Figure 1** Moxibustion treatment and moxa. Moxibustion was performed at acupoints CV 4 and CV 8 three times per week for 4 weeks (A). One moxa comprised 3.5 g of wormwood fiber inside a bamboo with a salt base (B).

phenyldiamine (DEPPD), ferrous sulfate, trichloroaceticacid(TCA), 5,5-dihioibis-(2-nitrobenzoicacid)(DTNB), potassium phosphate, reduced glutathione, myoglobin, 2,2¢-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), glutathione reductase, and the reduced form of β-nicotinamide adenine dinucleotide phosphate (β-NADPH) were purchased from Sigma (St. Louis, MO). Thiobarbituric acid (TBA) was obtained from Lancaster Co. (Lancashire, England). Hydrogen peroxide was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). 1-butanol was purchased from J.T. Baker (Mexico City, Mexico).

**Total reactive oxygen species**
The total reactive oxygen species (ROS) level in serum was determined according to the method of Hayashi.\textsuperscript{16} Briefly, hydrogen peroxide was used to generate a calibration curve by standard methods. DEPPD solution and ferrous sulfate solution were prepared beforehand. A total of 5 mL of standard solution or serum was added to 140 mL of 0.1 M sodium acetate buffer (pH 4.8) in each well of 96-well plates and incubated at 37°C for 5 min. A total of 100 mL of DEPPD and ferrous mixture solution were added to each well, and the ROS level was determined at 505 nm using a spectrophotometer.

**Lipid peroxide as malondialdehyde**
Lipid peroxide levels in the serum were determined using thiobarbituric acid-reactive substances (TBARS) as described by Kamal.\textsuperscript{17} The concentration of TBARS was expressed as malondialdehyde (MDA) mM in serum. Briefly, 250 mL of serum or standard solution was added to 2.5 mL of 20% TCA. It was mixed with 1 mL of 0.67% TBA and heated at 100°C for 30 min, followed by cooling on ice and vigorous vortexing with 4 mL of n-butanol. After centrifugation at 3000 × g for 20 min, the upper organic layer absorbance was measured at 535 nm with a spectrophotometer and compared with the TEP standard curve.

**Total antioxidant capacity**
The total antioxidant capacity (TAC) was determined according to the method of Kambsyashi.\textsuperscript{18} A total of 90 mL
of 10 mM phosphate-buffered saline (pH 7.2), 50 mL of myoglobin solution (45 mM), 20 mL of 3 mM ABTS solution, 20 mL of diluted serum sample, and trolox were added to a 96-well microplate and mixed well at 25°C for 3 min. Next, 20 mL of H2O2 was added to each well and incubated for 5 min. The absorbance was read using a plate reader at 600 nm (Molecular Device Corp., USA). The TAC was expressed as trolox equivalent antioxidant capacity (TEAC).

Catalase, superoxide dismutase, and total glutathione
Catalase activity in the serum was determined using the method of Beers and Sizer. Briefly, 100 mL of diluted serum or standard solution was mixed with 2.9 mL of substrate solution (0.0036% [w/w] hydrogen peroxide in 50 mM potassium phosphate), followed by measurement of absorbance at 240 nm after 5 min. Superoxide dismutase (SOD) activity in the serum was determined using an SOD assay kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s protocol. Bovine erythrocyte SOD (Sigma) was used as a standard.

The total glutathione content was determined according to the method of Evans. Briefly, 50 mL of diluted serum (in 10 mM PBS, pH 7.2) or glutathione standard was combined with 80 mL of DTNB/NADPH mixture (10 mL of 4 mM DTNB and 70 mL of 0.3 mM NADPH) in a 96-well microplate. A total of 20 mL (0.06 U) of glutathione reductase solution was added to each well, and the absorbance was measured using a plate reader at 405 nm (Molecular Devices, Sunnyvale, CA).

Lymphocyte subpopulations
Complete blood counts including differential white blood cell counts were analyzed using an auto hemato-analyzer (Celltack-α, Nihon, Japan). A lymphocyte subclass analysis of cytotoxic T cells, helper T cells, B cells, and natural T cells was performed using flow cytometry (BD FACSCaliber, BD Biosciences, USA) with a Multitest IMK Kit (BD Biosciences, Canada) according to the manufacturer’s protocol.

Cytokine antibody array
The expression of 42 serum cytokines was examined using the Human Cytokine Antibody Array III (Ray Biotech, Inc., USA), which contained antibodies against ENA-78, GCSF, GM-CSF, GRO, GRO-α, I-309, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, p40, p70, IL-13, IL-15, IFN-γ, MCP-1, MCP-2, MCP-3, MCSP, MDC, MIG, MIP-1β, RANTES, SCF, SDF-1, TARC, TGF-β1, TNF-α, TNF-β, EDF, IGF-1, angiogenin, oncostatin M, thrombopoietin, VEGF, PDGF BB, and leptin. Images were captured with an image analyzer (Bio-Rad Laboratories, Segrate, Italy) and analyzed using Quantity One software (Bio-Rad Laboratories).

RESULTS
Effects of moxibustion on serum ROS, MDA, and TAC levels
The mean concentration of serum ROS was (136 ± 51) units at the initial time point of moxibustion and significantly decreased to 101±35 units after 4 weeks of moxibustion (P<0.01) (Figure 2A). The serum MDA level also significantly decreased from (6±2) to (4±2) mM after the moxibustion trial (P<0.01) (Figure 2B). Meanwhile, the TAC, expressed as TEAC, significantly increased by about 30% [from (335 ± 74) to (401 ± 33) units] with moxibustion (P<0.01) (Figure 2C).

Change in catalase and SOD activities and total glutathione content in serum
The serum catalase activity was significantly increased with 4 weeks of moxibustion from 41 ± 17 to 58 ± 34 units (P<0.05) (Figure 2D). However, no significant change was observed in SOD activity (from 3.2±0.4 to 3.4±0.4 units) or total glutathione content (from 63±17 to 58±16 mM) (Figure 2E, F).

Analysis of lymphocyte subpopulations and serum cytokine profiles
Lymphocyte subpopulations, including cytotoxic T cells, helper T cells, B cells, and natural killer cells, were analyzed. No significant changes were observed in their relative proportions or absolute cell numbers between peripheral blood samples before and after the moxibustion trial (Figure 3A, B).

In addition, the semi-quantitative analysis of 42 cytokines showed no significant changes between serum samples taken before and after the moxibustion trial (Figure 3C, D).

Improvement in subjective symptoms by self-reporting
Twelve of 20 female participants reported at least one subjective symptom or physical feeling in which they felt improvement after moxibustion. The improved symptoms included constipation (five subjects), menstruation or menstrual pain (three subjects), digestion or appetite (four subjects), fatigue (three subjects), and

Self-reporting of changes in subjective symptoms
A doctor performed medical examinations in the form of a questionnaire or handwritten case report form regarding specific symptoms before and after the clinical study. All participants were instructed to report any subjective feelings or changes in symptoms, including adverse effects.

Statistical analysis
Statistical comparisons of all biological parameters between before and after moxibustion were analyzed with a paired t-test. Statistical significance was fixed at P<0.05.
A skin condition (one subject). One of five male participants reported that his hands and feet became warmer after the trial. One woman subject complained of mild itching and rubefaction of the skin around the navel.

DISCUSSION

Like acupuncture, moxibustion has been a major therapeutic in traditional Oriental medicine. Numerous studies on the clinical applications of acupuncture and their corresponding mechanisms have been performed, but there are very few studies on moxibustion. Moxibustion is generally thought to be preferable as a preventive therapeutic compared with acupuncture. Therefore, we investigated the effect of moxibustion at CV 4 and CV 8 on the status of the oxidative stress condition and immune-related parameters in healthy volunteers. These two acupoints (CV 4, CV 8) have been used to enhance vital energy in both patients with pathological disorders and healthy patients.

We observed that indirect moxibustion at these acupoints lowered the serum concentration of total ROS (Figure 2A). ROS are normally produced during cellular metabolism, but they are also a major contributor of many diseases through damage of cell structures by DNA alteration and oxidation of proteins and lipids. MDA is a quantitative marker of lipid peroxidation by ROS, and the serum level of MDA is also significantly reduced by indirect moxibustion (Figure 2B). These results were in accordance with the increased TAC (Figure 2C). Oxidative stressors are normally eliminated by various antioxidant mechanisms, including free radical scavengers, catalase, SOD, and the glutathione oxidation/reduction system.

Our results presented significantly increased serum levels of catalase activity (Figure 2-D) and no changes in SOD activity or GSH contents (Figure 2E, F).

Several have presented antioxidant action as an important mechanism of acupuncture in clinical and animal experiments. Previous findings confirmed the elevation in SOD and depletion of MDA in a global brain ischemia rat model. Some clinical studies reported the anti-oxidative effects of moxibustion in aged people. Other studies have reported its effect on hypertriglyceridemic or hypertensive patients. However,
er, those studies differ from our current study with respect to the experimental model, acupoints, and type of moxibustion or protocol. Our results suggest that moxibustion treatment at acupoints CV 4 and CV 8 has an antioxidative capacity as evidenced by the significantly decreased ROS and MDA concentrations and the increased TAC level and catalase activity after moxibustion. These results could be indicative of reduced susceptibility to oxidative damage, which is possibly relevant to the preventive effect of moxibustion therapy. We examined whether repeated indirect moxibustion influences immunological parameters, especially in lymphocyte subpopulations and serum cytokines. The acupoints CV 4 and CV 8 treated in this study are traditionally believed to help immune function, and two animal studies showed immune-modulating effects such as changes in serum IL-12 levels and NK cell activities.\textsuperscript{22,23} However, we observed no significant changes in either analysis of lymphocyte subpopulations or 42 cytokines in the peripheral blood (Figure 3). For more accurate quantification of important cytokine candidates, including interferon-gamma and IL-12, we performed analyses using individual ELISA kits, but no changes were observed (data not shown). In contrast to our results, one group observed increased helper T cells in healthy women and increased cytotoxic T cells in women with systemic lupus erythematosus after indirect moxibustion on Zusanli (ST 36) and Sanyinjiao (SP 6) for 1 week.\textsuperscript{34} These differences might have been caused by dissimilarities in experiment designs, such as rodent versus human bodies, pathologic and physiological states, locations of moxibustion, or direct versus indirect moxibustion.

We did not quantify the improvements in symptoms because all volunteers were basically healthy. Thirteen participants reported that they felt subjectively better at the end of the trial, which might have been at least partially linked to the antioxidative action of moxibustion. Our study includes a key limitation in interpretation of the results because this study was not based on a randomized controlled trial. It is very difficult to blind subjects using sham moxibustion treatment.\textsuperscript{35,36} We must consider the possibility of biased results in a single-arm study. The present study results in healthy subjects may reinforce the application of moxibustion for the management of human ailments. Taken together, we can conclude that repeated indirect moxibustion on CV 4 and CV 8 has antioxidative stress activity in healthy subjects. Further studies are needed to confirm this finding in larger-scale trials with a control group.

Figure 3 Lymphocyte subpopulations and human cytokine array
Blood was collected before the trial and after the last moxibustion. A lymphocyte subclass analysis was performed using the FACS system (A, B). A total of 42 cytokines in serum were analyzed using the Human Cytokine Antibody Array III (C, D). The cytokines are listed in the Subjects and Methods section.
REFERENCES


