

Acupuncture Enhances Generation of Nitric Oxide and Increases Local Circulation

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Although it is widely used, the mechanisms and effects of acupuncture on pain are not completely understood. Recently, increased nitric oxide (NO) synthase activity has been found in meridians and acupoints. Because NO is a key regulator of local circulation, and because change in circulation can affect the development and persistence of pain, we propose that acupuncture might regulate NO levels. We studied the effects of acupuncture on local NO levels and circulation in a randomized, double-blind, crossover study with 20 volunteers, each of whom underwent one session each of real and noninvasive sham acupuncture in a single hand and forearm with a 1-wk interval between treatments. NO concentration in the plasma from the acupunctured arm was significantly increased by 2.8 ± 1.5 $\mu\text{mol/L}$ at 5 min and 2.5 ± 1.4 $\mu\text{mol/L}$ at 60 min after acupuncture. Blood flow in palmar subcutaneous tissue of the acupunctured arm also increased, and this correlated with the NO increase. These changes were not observed in noninvasive sham-acupunctured hands and forearms. In conclusion, acupuncture increases the NO level in treated regions and thereby increases local circulation. These regulatory effects might contribute to pain relief provided by acupuncture.

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Although the mechanism of acupuncture is not fully understood, it is an important component of traditional medicine in Asia (1), where it is widely used for pain relief in many disorders. However, studies in the United States and European countries investigating the effectiveness of acupuncture have been inconclusive or equivocal (2). There is evidence that intraoperative acupuncture, which is considered to produce potent analgesic effects, fails to reduce the need for anesthetics (3). The uncertainties regarding the analgesic effect of acupuncture indicate that its effectiveness is mild or limited. Alternatively, it could be considered that the action of acupuncture against pain is mediated through indirect pathways. Based on this understanding, the documented phenomenon that acupuncture changes local skin temperature (4,5) seems important. The alteration of skin temperature suggests changes in local circulation that may induce an effect on the development and persistence of pain (6,7). Thus, it is not unreasonable to expect that

acupuncture may change local circulation as a possible therapeutic action. However, there is no detailed investigation of such a possibility.

Nitric oxide (NO) is a key regulator of local vascular tone and blood flow (8,9). We have found that the local level of NO varies according to various pathophysiological events and metabolic alteration. Oxygen tension and subsequent generation of reactive oxygen species play crucial roles in determining the local NO level (10). The activation of neutrophils and macrophages also affects the NO concentration in the surrounding tissue (8,9). Cigarette smoking reduces the NO level in the circulation (11). Artificial ventilation also affects exhaled NO levels (10). In the case of acupuncture, Ma (12) demonstrated that NO synthase (NOS) expression is higher in meridian skin regions, including acupoints. Li et al. (13) reported an increase in NO content in blood after warm needling. These findings suggest that acupunctural stimulation could be a modulator of *in vivo* NO levels. Considering this together with the possibility of an acupuncture effect on local circulation as discussed above, we propose that acupuncture might influence circulation in the treated area through regulation of NO levels.

In the present study, we sought to validate this hypothesis by investigating formation of NO and the local change in circulation in acupuncture-treated areas.

METHODS

After receiving approval from the Institutional Ethics Committee of Osaka City University Medical School, the effects of acupuncture on local NO formation and

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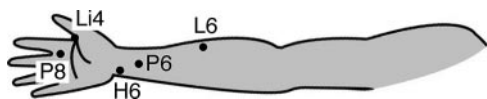


Figure 1. Acupoints used in the study.

circulation in treated areas were examined in healthy Japanese volunteers in a randomized, double-blind, crossover fashion. Operators of the laser tissue-blood flow meter, high performance liquid chromatograph (HPLC), and electron spin resonance (ESR) spectrometer and assessors of the results obtained were blinded to subject treatment. All subjects provided informed consent. Acupuncture was performed at acupoints Li4, P6, P8, L6, and H5. Local NO formation was studied by analyzing the concentration of nitrate and nitrite in plasma obtained from the treated area and confirmed by examining NO-hemoglobin formation in the same samples, whereas local circulation was studied using a laser tissue-blood flow meter. A preliminary study demonstrated that the standard deviation of the pre- to postacupuncture difference in concentration of nitrate plus nitrite is $<2.5 \mu\text{mol/L}$. Under these conditions, a statistical power analysis indicated that a minimum sample size of 17 is required for detecting a difference of $2 \mu\text{mol/L}$ in concentration of nitrate plus nitrite with a power of 0.9 and a significance level of 0.05 in paired data analysis. Therefore, 20 subjects were investigated in this study.

The subjects, who had no advanced knowledge of or experience with acupuncture therapy, were randomly assigned to initially undergo either real or noninvasive sham acupuncture. All subjects were asked not to eat or drink for 10 and 6 h, respectively, before acupuncture. The real acupuncture-assigned subjects were blindfolded and received acupuncture at the Li4, P6, P8, L6, and H5 acupoints in one hand and forearm by a well-trained acupuncturist (Fig. 1). In Japan, these acupoints are considered to be effective treatment for muscle and joint pain in the hand and forearm. A stainless steel acupuncture needle (0.20 mm in diameter and 40 mm in length) without any special coating (Seirin Kasei CO, Shizuoka, Japan) was inserted to a depth of approximately 5–8 mm into each acupoint, which was manually stimulated by delicately twisting the needles according to standard technique for 2 min or until a DeQi sensation (special needle sensation of acupuncture) was achieved, and needles were left *in situ* for 10 min. Noninvasive sham acupuncture subjects were similarly blindfolded, and the same true acupoints were tapped with an empty plastic needle tube. Palpation of the surface of the skin was induced with a blunt adherent instrument for the same period as for the real acupuncture. A week after the first acupuncture treatment (real or noninvasive sham), subjects underwent the opposite treatment depending on their initial assignment.

To evaluate the adequacy of the control treatments, the subjects were questioned regarding their expectations of the treatment (14,15). After both real and

noninvasive sham acupuncture procedures, the subjects completed a questionnaire using a 5-point credibility scale, which included “not at all,” “a little,” “moderately,” “quite a lot,” and “extremely” in ascending order as possible answers (Table 1).

Before acupuncture, 5 and 60 min after the removal of acupuncture needles, subcutaneous circulation of the palm on the side of the treated arm was investigated using a laser tissue-blood flow meter (FLO-N1; Omega-wave, Tokyo, Japan) (16). This laser tissue-blood flow meter allows the measurement of microvascular blood flow in tissue to a depth of approximately 1–4 mm below the probe. The two flow probes were placed in about the center of the palm and, after 2 min of stabilization, an average reading of each over 5 min obtained. The average value given by the two probes was used as an index of palmar circulation.

At the same time as the measurement of circulation, 2 mL of whole blood from each of the axillary veins was drawn from each subject into heparinized tubes. NO generation in the blood was then determined using an HPLC method (11) and confirmed using an ESR method (17), as described below in detail.

In the HPLC method, plasma was isolated by centrifugation of collected blood samples (5 min at $750 \times g$ at 4°C) and deproteinized by the addition of an equal volume of methanol. The samples were then applied to an HPLC system (ENO-20; EICOM, Kyoto, Japan) to determine nitrate and nitrite concentrations. Nitrate and nitrite were separated in 0.15 M $\text{BaCl}_2\text{-NH}_4\text{Cl}$ and 0.5 g/L of EDTA-4Na containing 10% methanol at 0.33 mL/min using a reversed-phase column (NO-PAK), after which nitrate was reduced to nitrite in a reduction column packed with copperized cadmium (NO-RED) at 35°C . The nitrite was then mixed with a Griess reagent (delivered at a rate of 0.1 mL/min) in a reaction coil and absorbance monitored at 540 nm.

In the ESR method, blood samples (400 μL) were transferred without delay to an ESR tube (4-mm inner diameter), frozen in liquid nitrogen, and then analyzed at 110 K using a JES-RE1 \times spectrometer (JOEL, Tokyo, Japan) at 100-kHz field modulation. ESR analysis was conducted at 8 mW with a frequency of 9.099 GHz, $325 \pm 50\text{-mT}$ field, 3-min sweep time, 0.125-mT modulation amplitude, and a time constant of 0.1 s. As a positive control, fresh untreated blood was incubated for 10 min with NOC7 ([1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-3-aminopropyl]-3-methyl-1-triazeno), which chemically generates NO with a half-life of 5 min.

HPLC reagents were obtained from EICOM, and NOC7 was obtained from Dojin (Kumamoto, Japan). Other reagents were of analytical grade.

All results are expressed as mean values \pm SD. Statistical evaluation of NO concentration and palmar blood flow was performed using repeated-measures analysis of variance with Student-Newman-Keuls multiple comparisons test using InStat 3 for Macintosh (GraphPad Software, San Diego, CA). Analysis of

Table 1. Subject Responses to Credibility Questionnaire

(a) How confident do you feel that this treatment can alleviate your arm and hand complaint?

Noninvasive sham acupuncture	Not at all	A little	Moderately	Quite a lot	Extremely	Total
Real acupuncture						
Not at all	0	0	0	0	0	0
A little	0	1	4	0	0	5
Moderately	1	4	0	2	0	7
Quite a lot	0	2	3	2	0	7
Extremely	0	1	0	0	0	1
Total	1	8	7	4	0	20*

(b) How confident would you be in recommending this treatment to a friend who suffers from an arm and hand complaint?

Noninvasive sham acupuncture	Not at all	A little	Moderately	Quite a lot	Extremely	Total
Real acupuncture						
Not at all	0	0	0	0	0	0
A little	1	4	2	0	0	7
Moderately	0	2	4	1	0	7
Quite a lot	0	3	2	1	0	6
Extremely	0	0	0	0	0	0
Total	1	9	8	2	0	20†

(c) How logical does this treatment seem to you?

Noninvasive sham acupuncture	Not at all	A little	Moderately	Quite a lot	Extremely	Total
Real acupuncture						
Not at all	0	0	0	0	0	0
A little	0	2	3	1	0	6
Moderately	1	5	0	1	0	7
Quite a lot	0	1	4	1	0	6
Extremely	0	0	1	0	0	1
Total	1	8	8	3	0	20‡

(d) How successful do you think this treatment would be in alleviating other complaints?

Noninvasive sham acupuncture	Not at all	A little	Moderately	Quite a lot	Extremely	Total
Real acupuncture						
Not at all	0	0	0	0	0	0
A little	0	2	3	0	0	5
Moderately	1	5	1	1	0	8
Quite a lot	0	0	4	2	0	6
Extremely	0	0	0	1	0	1
Total	1	7	8	4	0	20§

Assessment of the credibility of treatment was performed using the McNemar test

* $P = 0.94$; † $P = 0.93$; ‡ $P = 0.93$; § $P = 0.93$.

regression and correlation was also performed using Instat. Power analysis was performed using StatMate 2 for Macintosh (GraphPad Software). McNemar test was performed for credibility assessment using *R* (<http://www.r-project.org>). For all statistical evaluations, differences were considered significant at $P < 0.05$.

RESULTS

The mean subject age was 28 ± 5 yr, height 169 ± 6 cm, and weight 65 ± 7 kg. No adverse effects such as

discomfort, dizziness, or subcutaneous bleeding caused by acupuncture were observed in any subject. DeQi sensation was obtained in each acupoint only after real acupuncture.

Noninvasive sham-acupuncture subjects tended to consider that the treatment was less effective. However, no significant difference was found (Table 1). Thus, we considered that subjects were equally confident in the potential benefit of the treatment whether they received real or noninvasive sham acupuncture.

Table 2. Plasma Nitrate and Nitrite Concentrations and Palmar Blood Flow

	Before acupuncture	After acupuncture	
		5 min	60 min
Plasma nitrate and nitrite ($\mu\text{mol/L}$)			
<i>Subjects receiving real acupuncture</i>			
Acupunctured forearm	24.6 \pm 6.1(a)(b)	27.5 \pm 6.5(a)(c)(d)	27.1 \pm 6.6 (b)(e)(f)
Opposite nontreated forearm	24.6 \pm 6.2	24.5 \pm 6.2(c)	24.4 \pm 6.1 (e)
<i>Subjects receiving noninvasive sham acupuncture</i>			
Noninvasive sham acupunctured forearm	24.7 \pm 6.0	25.0 \pm 6.1(d)	24.9 \pm 5.9 (f)
Palmar blood flow ($\text{mL} \cdot \text{min}^{-1} \cdot 100\text{g}$ of tissue)			
Acupunctured forearm	33.1 \pm 6.4(g)(h)	39.5 \pm 7.0(g)(i)	39.5 \pm 7.1 (h)(j)
Noninvasive sham-acupunctured forearm	33.4 \pm 6.6	32.5 \pm 6.5(i)	33.1 \pm 6.7 (j)

Changes in plasma concentrations of nitrate and nitrite from the axillary vein of the arm in which the subject received real or noninvasive sham acupuncture and coupled changes in blood flow in palmar subcutaneous tissue. (a)(b)(c)(d)(e)(f)(g)(h)(i)(j) indicate the significant difference with $P < 0.05$ between two values

Before acupuncture, palmar blood flow in the real acupunctured arm did not differ from that in the noninvasive sham-acupunctured arm (Table 2). At 5 and 60 min after acupuncture, palmar blood flow in the noninvasive sham-acupunctured arm was unchanged, whereas that in the real acupunctured arm was significantly increased compared with both the preacupuncture value and that in noninvasive sham-acupunctured arm.

Before acupuncture, there were no differences in plasma concentrations of nitrate plus nitrite between subjects undergoing real and noninvasive sham acupuncture (Table 2). After acupuncture, no significant changes in plasma concentration of nitrate plus nitrite were noted in either the nontreated arm in the subjects undergoing real acupuncture or the noninvasive sham-acupunctured arm. However, plasma concentrations of nitrate plus nitrite in the real acupunctured arm were significantly increased compared with both the preacupuncture value and those obtained from the opposite, nontreated arm and noninvasive sham-acupunctured arm at 5 and 60 min after acupuncture. The increase to more than the preacupuncture value was $2.8 \pm 1.5 \mu\text{mol/L}$ after 5 min and $2.5 \pm 1.4 \mu\text{mol/L}$ after 60 min. Our study of plasma NO (11), as well as another clinical study (18), showed that changes in systemic plasma NO levels in amounts similar to those found in the present study were able to alter coronary blood flow, indicating that the increases in local plasma NO level in the present study were clinically relevant changes.

ESR spectrum attributed to the Cu^{2+} of ceruloplasmin (17) was observed for the blood samples obtained before acupuncture (Fig. 2A). At 5 min after acupuncture, a small peak with a g value of 2.0 developed, superimposed on the Cu^{2+} -ceruloplasmin spectrum in blood samples obtained from the real acupunctured arm, as shown by the arrows in Figure 2B, but not in the blood samples from the nontreated arm and noninvasive sham-acupunctured arm (data not shown). Although the ESR signal observed was small, it could be assigned to the NO-hemoglobin adduct spectrum (17) by reference

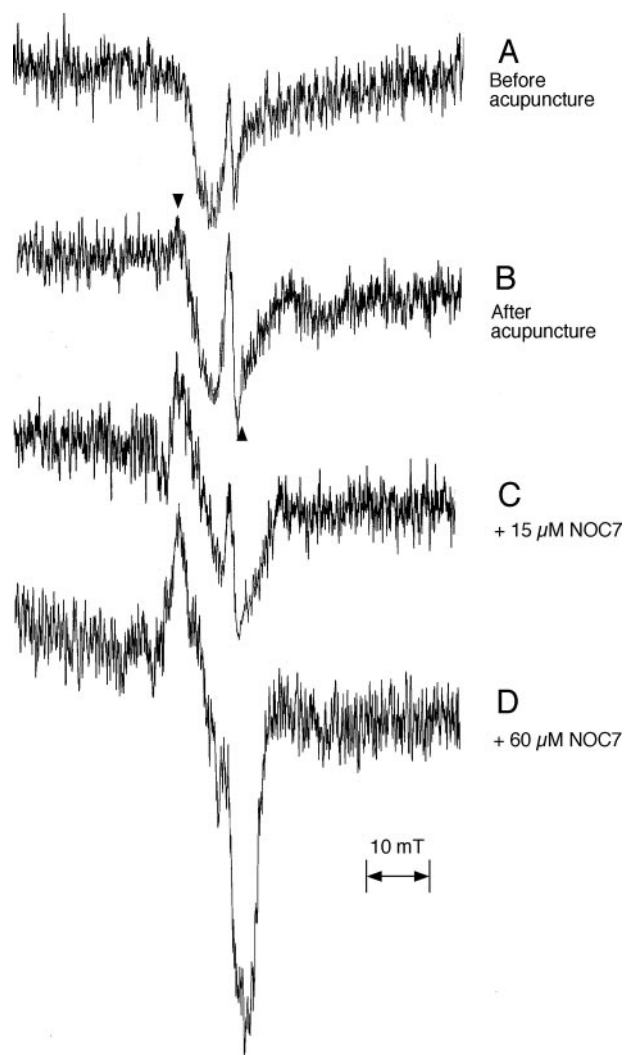


Figure 2. ESR spectra of blood samples from the acupunctured hand and forearm before acupuncture (A), 5 min after acupuncture (B), and electron spin resonance (ESR) spectra of fresh nontreated blood incubated with $15 \mu\text{mol/L}$ (C) and $60 \mu\text{mol/L}$ (D) of NOC7 for 10 min as positive controls. Arrows in (B) indicate the NO-hemoglobin adduct with g value of 2.0 superimposed on the originally generated Cu^{2+} -ceruloplasmin adduct. ESR settings are described in the text.

to the typical ESR spectrum of NO-hemoglobin generated by the incubation of NOC7, a short-lived potent NO releaser, with fresh nontreated blood sample, as shown in Figure 2, C and D.

In the real acupuncture arm, the increase in blood flow in the palm above the preacupuncture value was significantly correlated with the plasma concentration of nitrate plus nitrite with a regression line of $y = 2.3x$ and the correlation coefficient (r) of 0.79 (Fig. 3). In the noninvasive sham-acupunctured arm, no correlation was found between the increase in blood flow in the palm and the plasma concentration of nitrate plus nitrite.

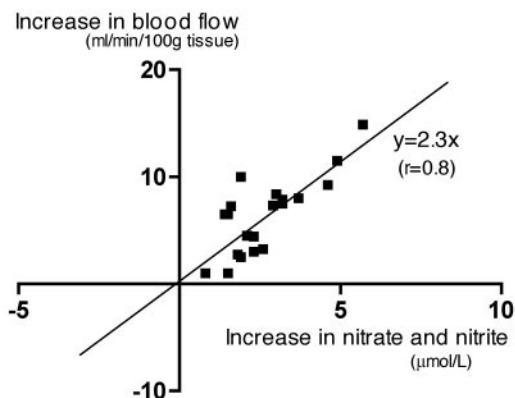
DISCUSSION

We found that acupuncture increased the plasma concentrations of nitrate and nitrite in the treated regions, coupled with an increase in blood flow. Although measurement of NO itself was not performed, previous studies showed that the concentrations of nitrate plus nitrite, which are end-products of metabolism of NO, are reliable indicators of NO formation *in vivo* (11). However, the concentrations of these end-products are easily affected by food or water intake, daily activities, and time of blood sampling (11). Therefore, we limited and standardized food and water intake by our subjects before acupuncture and collected blood samples at the same time of day.

The blood in the axillary vein on the side of acupunctured arm contained nitrate and nitrite derived from the NO locally generated in the hand and forearm, in addition to the basally generated NO throughout the body. Thus, to confirm that basal NO generation did not affect the concentration of nitrate plus nitrite in blood from the acupunctured arm, concentrations of nitrate plus nitrite in blood from the opposite, nontreated arm were measured, and these showed no significant changes. This finding indicates that there was no change in background basal NO level. Thus, the increase in NO level measured in blood from the acupunctured arm resulted from the increase in NO generation in the hand and forearm.

The ESR method used to detect and confirm NO generation in acupunctured hands and forearms has the advantage that it can directly and specifically detect NO, even in biological samples; however, it is neither very sensitive nor quantitative (19). Because the affinity of hemoglobin for NO is very high, NO generated in blood rapidly reacts with hemoglobin to form an NO-hemoglobin adduct (17). This adduct is detected by its characteristic ESR spectrum, which is interpreted as evidence of NO generation. Even a small ESR peak for NO-hemoglobin, as obtained in the present study, is strong evidence for the generation of NO in the arm from which the blood sample is collected. To the best of our knowledge, this is the first study to demonstrate that acupuncture immediately increases the NO level in the treated area in humans.

(A) Real acupuncture



(B) Noninvasive sham acupuncture

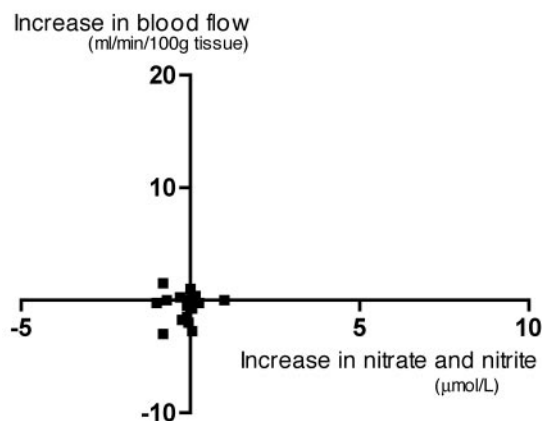


Figure 3. Relationship between the increase in palmar blood flow above the preacupuncture value and the plasma concentration of nitrate plus nitrite in acupunctured hand and forearm (A) and sham-acupunctured hand and forearm (B). The data points represent average values for 5 and 60 min of data after acupuncture treatment. There is a significant correlation after real acupuncture, with a regression line of $y = 2.3x$ and correlation coefficient (r) of 0.79, but not after sham acupuncture.

The increase in palmar blood flow in the acupunctured arm indicates that acupuncture increases circulation in treated regions, which is consistent with a previous study reporting an increase in skin temperature in regions subjected to acupuncture (4). The significant correlation between the increase in blood flow and the increase in NO level in the acupunctured arm indicates that NO-dependent mechanisms are involved there. Because ischemia is an important pathogenic factor in the development and persistence of certain types of pain (6,7), the increase in local circulation as a result of the increase in NO level could contribute to the therapeutic mechanism of acupuncture on pain.

The present findings regarding NO provide further evidence of a mechanism for the effect of acupuncture.

NO has been recognized as a nontraditional intracellular and intercellular messenger (20). Particularly, evidence has been accumulated for the involvement of NO in nociceptive processing. NO inhibits substance P release from spinal cord synaptosomes (21). It functions in the suppression or development of hyperalgesia and allodynia mediated in the spinal cord (22,23). Local application of drugs generating small concentrations of NO, such as SNAP or isosorbide, reduce incision pain through activation of guanylate cyclase (24). It is now considered that NO is involved in the transmission and modulation of nociceptive information throughout the nervous system (25). The same might be true for NO generated in acupuncture. An increase in the local concentration of NO might also exert analgesic effects through the regulation of peripheral pain transduction pathways. In some studies, systemic or distal effects in acupuncture, such as acupunctural anesthesia, cardiac, and antiemetic effects are shown (1). These characteristic effects are believed to be mediated by the central nervous system and opioid peptides (26). We have found that NO does not usually function alone but cooperatively through cross-talk with various active messenger molecules (8,9). This supersystem has been shown to exist throughout the body and it regulates important vital functions, including energy metabolism and circulation (8,9). Advances in the knowledge of this supersystem allow us to assume that NO locally generated in acupuncture could also serve such a supersystem and, through it, communicate with the entire body, which may contribute to the possible systemic or distal effects of acupuncture.

The present findings raise the question of how acupuncture enhances NO generation. Higher NOS activity at meridians and acupoints has been found in rats (12), suggesting that a meridian NOS system works to regulate the NO level in acupunctured subjects. Involvement of sympathetic tone provides another possible explanation for the mechanism of NO generation. The possibility that acupuncture might change sympathetic activity has been suggested (27). Because change in catecholamine status sometimes affects NOS activity in the endothelium (28), sequential reaction from change in sympathetic activity to NOS activation via fluctuation of local catecholamine concentration might be responsible for acupuncture-induced NO generation. Interpretation of the significance of acupuncture-induced NO generation and circulatory change for relieving pain raises the question as to whether subjects, who as good responders generate more NO or have a larger increase in local circulation, experience greater analgesic effects. However, the therapeutic effect of acupuncture on pain is still uncertain (2). The equivocal results in studies of acupuncture on pain indicate that the analgesic effect of acupuncture might be quite weak, making it difficult to validate the clinical significance of NO generated in

acupuncture. These unresolved problems provide objectives for future study.

Because ideal blinding of subjects is difficult in the study of acupuncture, evaluation of blinding success is important. One method is to evaluate the difference in psychological impact between real and sham acupuncture with four specific questions (15). We used this method of assessment. Accordingly, both real and noninvasive sham acupuncture were perceived as similarly credible.

It is also important to ascertain what kind of effect would be demonstrated, for this determines the choice of control (15). Some studies show that sham acupuncture by insertion of an acupuncture needle at an irrelevant site, away from true classical acupoints and meridians, has analgesic effects and even therapeutic benefits (14,29). These findings indicate the importance of needle stimulation as a key factor inducing acupuncture's effects rather than the position of acupuncture needling. Thus, the present study focused on the effect of needle stimulation on the formation of NO and local circulation at real acupoints with controls of noninvasive sham acupuncture at the same acupoints (15). It then remains to be determined how the specificity of real acupoints affect acupuncture, for which purpose penetrating acupuncture at off-site or off-meridian points should be applied as controls (15). However, such penetrating sham acupuncture has the potential for analgesic effects (14,29), indicating the possibility that penetrating sham itself might enhance NO formation and local circulation. Based on this assumption, it is reasonably supposed that the difference between real acupuncture and sham acupuncture with needle penetration in their effect on NO formation and local circulation could be in proportion to their possible analgesic effect. Thus, as we study the specificity of an acupuncture point on the change in NO formation and local circulation, it is important that specificity should be evaluated with reference to analgesic potential. However, because the effectiveness of acupuncture-induced analgesia is still uncertain (2), it seems inadvisable to make a rigorous study of this issue at the present state of our knowledge of acupuncture and research methodology. This unresolved problem is another important objective for future study.

In conclusion, acupuncture increases NO levels in treated regions, thereby increasing local circulation. The importance of local circulation, and NO itself, in pain suggests that these effects might contribute to pain relief obtained with acupuncture. Although numerous questions remain concerning acupuncture, the present findings could help us discover the complex mechanisms underlying the effects of acupuncture.

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