Chapter 2

Nitric Oxide in Acupuncture Mechanism

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1. Introduction

In Japan and other parts of East Asia, acupuncture is an important component of traditional medicine, where it is widely used for pain relief and other healing effects for a variety of disorders [1]. However, studies in the United States and European countries that investigated the effectiveness of acupuncture have been inconclusive or equivocal [2], and the mechanism of acupuncture is not fully elucidated [3]. Recent findings suggest specific neural pathways that transmit acupuncture stimulation to distant body areas via the central nervous system, which may support traditional meridian theory [4]. In addition to such a possible central mechanism, a peripheral mechanism has also been suggested to be active in acupuncture treatment, as several studies have noted increases in skin temperature and muscular blood perfusion at acupuncture sites [5-8].

Nitric oxide (NO) is a key regulator of vascular tone and blood flow in local circulation, the importance of which has been well documented in various *in vitro* and *in vivo* studies [9, 10]. There have been several findings indicating that the level of NO can vary according to pathophysiological events and metabolic alteration. Considering the effects of acupuncture on local perfusion, an involvement of NO in the acupuncture mechanism can be speculated.

NO typically functions in a very limited area, while its half-life is short and the amount produced is small. In addition, food or water intake, daily activity or time of measurement, and the activities of various resident bacteria can greatly affect the amount of NO-related metabolites such as nitrite and nitrate, thus interfering with determination of actual NO level [11-13]. Therefore, it is quite difficult to demonstrate true changes in local NO concentration coupled with physiological or pathological effects in individuals or at the organ level. Furthermore, it is highly controversial whether acupuncture used in animals and humans is exactly the same in regard to physiological and anatomical considerations, and findings obtained only in animal studies may be of limited significance [3]. Thus, to demonstrate the

involvement of NO in the acupuncture mechanism, evidence obtained with human subjects is absolutely necessary.

Our laboratory has been investigating traditional medicine from the standpoint of Western medicine and basic science, and has also extensively studied reactive oxygen species (ROS) and NO metabolism in various physiological and pathological conditions [9, 10]. Through these studies, we have found strong evidence that changes in NO level are coupled with biological functions in human subjects [12,13]. In this chapter, such results demonstrating the functions NO in the human body will be briefly shown, followed by findings showing that acupuncture has a significant impact on the level of NO at the same magnitude as shown in those results [14], which may validate the notion that NO has an important function in the mechanism of acupuncture.

2. NO in human respiratory system

2.1. Study design

To clarify the effect of NO on pulmonary gas exchange, the relationship between exhaled NO and arterial blood gases was studied in healthy human subjects.

2.2. Methods

Exhaled NO and arterial blood gases were analyzed in 23 healthy non-smokers without airway inflammation (20 males, 3 females; 41±4 [mean ± SD] years old) in a special clean room, in which the concentration of NO was less than 4 parts per billion (ppb). In order to minimize environmental external NO, the subjects remained in the room for at least 1 hour before measurement were performed. Previous studies have reported that exhaled NO was increased in asthmatic patients and others with airway inflammation, and may possibly reflect the inflammatory status of the airway [15, 16]. Therefore, subjects with upper or lower respiratory inflammation were strictly excluded from the present study. In addition, measurements were performed at the same time each day after more than 10 hours of fasting to eliminate the effects of external NO metabolites from diet. These careful procedures were considered important to determine the exact concentration of exhaled NO.

After 1 hour in the special clean room, blood samples were anaerobically obtained from the radial artery of each subject, then analyzed for arterial blood gases (pH, PaO_2, $PaCO_2$) with a blood gas analyzer (Model 288; Chiron, MA). Next, exhaled NO was measured using a chemiluminescence NO analyzer CML-500 (Shimizu, Kyoto, Japan). Each subject was instructed to perform a slow vital capacity maneuver over 30-40 seconds into unobstructed tubing while wearing nose clips. Air samples were continuously collected through a side arm as the plateau concentration at the end of expiration.

2.3. Results

Analysis of the exhaled air samples revealed that the concentrations of exhaled NO ranged from 13 to 53 ppb. Values for PaO_2, $PaCO_2$, and pH in the blood samples were 88.9±2.0 mmHg, 40.2±0.8 mmHg, and 7.40±0.01, respectively. Among these examined parameters, arterial PaO_2 was significantly correlated with the concentration of exhaled NO (Figure 1). However, no significant correlation was found between that concentration and pH or $PaCO_2$.

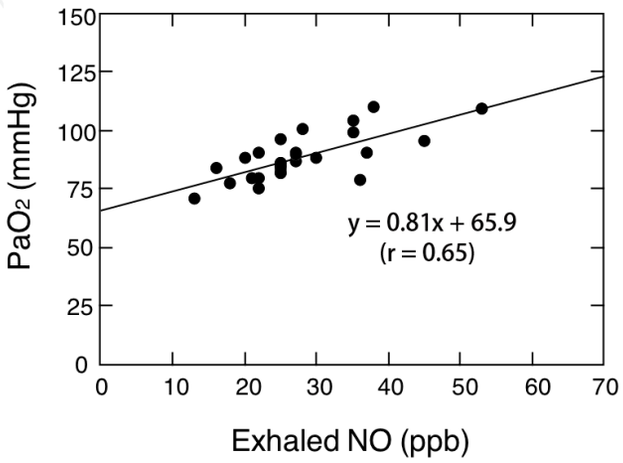

Figure 1. Relationship between PaO_2 and exhaled NO. Exhaled air samples were collected from 23 healthy subjects using a slow vital capacity maneuver. PaO_2 was significantly correlated with the concentration of exhaled NO (r = 0.65).

2.4. Interpretation

Our findings indicate that exhaled airway NO is involved in arterial oxygenation in the respiratory system. This is not surprising when considering the potent effects of NO on vascular and/or airway smooth muscular tone, which may control ventilation-to-perfusion matching in the respiratory system. Theoretically, NO concentrations in respiratory gas exchange are not equal throughout the respiratory zone, as they are dependent on the amount of inspirable NO generated in the upper airway in addition to intrinsic NO generated in the lower respiratory zone. Thus, a well-ventilated zone, which requires much perfusion, could receive much more NO than a poorly ventilated one. In this manner, airway NO actively controls gas exchange in the respiratory system, even though its concentration is very low.

3. NO in human circulatory system; adverse effects of cigarette smoking

3.1. Study design

Cigarette smoke contains superoxide and other reactive oxygen species (ROS) [17-19], thus it has been speculated that some of the adverse effects of smoking may be caused by oxidative

damage to endothelial cells, resulting in NO shortage. To investigate this possibility, changes in plasma level of NO and other antioxidants in healthy subjects with a cigarette smoking habit after smoking a single cigarette were compared between those divided into real smoking and sham smoking groups.

3.2. Methods

Changes in plasma concentrations of nitrate plus nitrite, used as an index of NO formation in the circulation, as well as changes in plasma concentrations of major antioxidants (ascorbic acid, cysteine, methionine, uric acid) in healthy young smokers after smoking a single cigarette were measured. Subject number, age, height, weight, and smoking history in the smoking group were 20, 28±4 (mean ± SD) years old, 169±8 cm, 61±7 kg, and 6±3 pack-years (number of cigarettes in packs smoked daily times number of years), respectively, while those in the sham smoking group were 15, 27±3 years old, 170±8 cm, 61±8 kg, and 6±2 pack-years, respectively, which were not significantly different.

Both food and water contain substantial amounts of nitrate, thus it is especially important to control those factors before attempting to determine endogenous plasma NO formation in humans [11, 20]. We limited and standardized the food and water intake by the subjects prior to the experiment, in which all were asked to not eat for 10 hours, and not drink or smoke for 6 hours prior to the experiment. Measurements were performed at the same time each day while the subjects were at rest. Blood was sampled at the following intervals: just prior to smoking a cigarette, 5 minutes after smoking that cigarette, and again 60 minutes later.

3.3. Results

The plasma concentration of nitrate plus nitrite was significantly decreased by 3.4±1.1 μM in the smoking group after smoking a single cigarette as compared with the sham smoking group (23.9±1.1 μM in the smoking group vs. 27.3±1.2 μM in the sham smoking group) (Figure 2). The concentrations of antioxidants (ascorbic acid, cysteine, methionine, uric acid) were also significantly decreased. After 60 minutes, all parameters returned to pre-experimental levels. In the sham smoking group subjects, no significant changes were noted.

3.4. Interpretation

These results show that smoking a single cigarette decreases the plasma concentration of nitrate plus nitrite, as well as ascorbic acid and other antioxidants. We previously demonstrated that cigarette smoke contains superoxide and a large number of other ROS [17-19]. Inhalation of these ROS by smoking increases oxidative stress in smokers, and induces a reduction in NO and antioxidant levels. Since an adequate level of NO is essential for sufficient coronary circulation, such transient changes contribute to coronary vasoconstriction, which is routinely observed after smoking [21,22].

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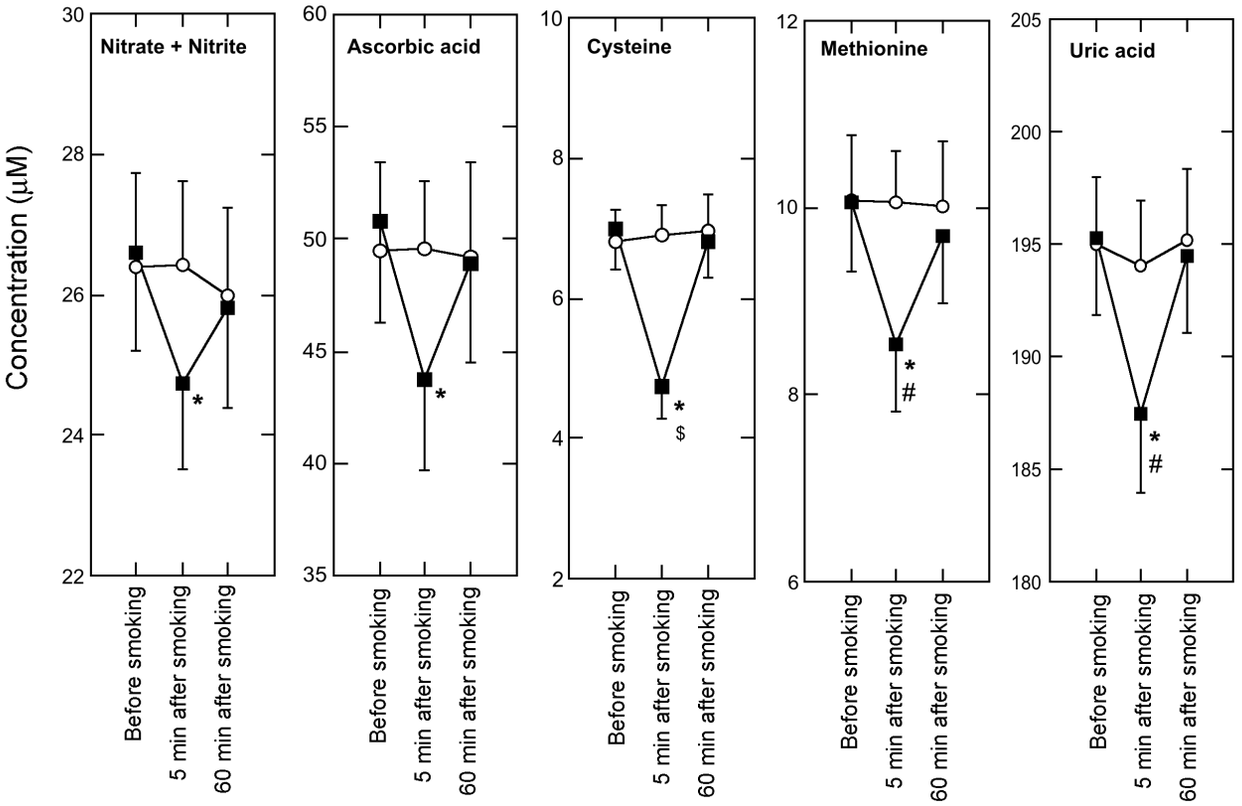

Figure 2. Changes in plasma concentrations (mean ± SD) of nitrate and nitrite, ascorbic acid, cysteine, methionine, and uric acid in smokers at 5 and 60 minutes after smoking a single (closed square) or sham (open circle) cigarette. * $P< 0.01$ *vs.* values before smoking, $ $P< 0.01$ *vs.* corresponding values in control group, # $P< 0.05$ *vs.* corresponding values in control group.

4. Change in concentration of NO required for physiological effects

It is difficult to compare NO concentrations in the respiratory system by measuring exhaled breath (13-53 ppb range) with those in the circulatory system by measuring plasma (23.9-27.3 μM range). However, it is important to note that units of ppb are considered to present a more precise scale than units of μM. Thus, based on our results, it seems certain that NO functions in the human body in a range less than μM depending on the environmental condition.

5. NO in acupuncture treatment in humans; effects on local circulation

5.1. Study design

NO is a key regulator of local circulation, and the development and persistence of pain can be affected by changes in circulation. Thus, we speculated that acupuncture can regulate NO

levels. We studied the effects of acupuncture on local NO levels and circulation in a randomized, double blind, crossover study with healthy human subjects.

5.2. Methods

Procedures for acupuncture

Twenty subjects (28±5 years old [mean±SD], height 169±6 cm, weight 65±7 kg) with no knowledge of or experience with acupuncture therapy were randomly assigned to initially undergo either real or sham acupuncture treatment using computer-generated random numbers. All acupuncture treatments were performed at the same time of day, and none of the subjects ate for 10 or drank for 6 hours prior to treatment, the same as in the study of cigarette smoking. For the real acupuncture group, the subjects were blindfolded and received acupuncture at the Li4, P6, L6, and H5 acupoints in a forearm by a well-trained acupuncturist (Figure 3). In Japan, these acupoints are considered to be effective for relieving muscle and joint pain in the forearm and hand. Each acupoint was manually stimulated twice for 1 minute each by finely twisting the needle, according to standard acupuncture technique, then the needle was left *in situ* for 10 minutes. 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 with the true acupuncture. One week after the first treatment (real or sham), the same subjects underwent the opposite treatment (real or sham acupuncture) performed in the same manner as described above.

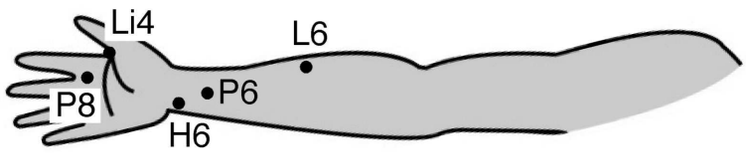

Figure 3. Acupoints used in the study.

Measurements of change in palmar circulation in acupunctured forearm

Before, and 5 and 60 minutes after acupuncture, subcutaneous circulation in the palm on the side of the treated forearm was investigated using a laser tissue blood flow meter (FLO-N1, Omegawave, Tokyo, Japan) by an investigator who did not know whether the subjects had undergone real or sham acupuncture [23]. The laser tissue blood flow meter allows measurement of microvascular blood flow in tissue at approximately 1-4 mm deep below the probe. The flow probe was placed at the center of the palm and allowed to stabilize, then the average reading over a period of 5 minutes was obtained.

Measurements of NO formation in acupunctured forearm

Following the measurement of microcirculation, 2 ml of whole blood from an axillary vein was drawn from the subject and collected into heparinized tubes. NO generation in blood was determined using an HPLC (high performance liquid chromatography) method by

measuring the plasma concentrations of nitrate and nitrite [12], and confirmed by an ESR (electron spin resonance) method that directly measured NO signals [24], as described below.

a. HPLC method

Plasma was isolated by centrifugation of the collected blood samples (5 minutes at 750 x *g*, 4˚C) and deproteinized by 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 using a reverse-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 Griess reagent in a reaction coil and change in absorbance was monitored at 540 nm. The flow rate of the mobile phase, which consisted of 10% methanol containing 0.15 M $BaCl_2$-NH_4Cl and 0.5 g/l EDTA-4Na, was 0.33 ml/minute. Griess reagent was delivered at a rate of 0.1 ml/minute.

b. ESR method

The remainder of the blood sample (400 μl) was quickly transferred to ESR tubes (4 mm inner diameter) and frozen with liquid nitrogen, then analyzed by an ESR method at 110 K using a JES-RE1X spectrometer (JOEL, Tokyo) with 100 kHz field modulation. ESR analysis was conducted with a microwave power of 8 mW at a frequency of 9.099 GHz, 325±50 mT field, 3-minute sweep time, 0.125-mT modulation amplitude, and 0.1-second time constant. As a positive control, fresh non-treated blood was incubated for 10 minutes with NOC7 ([1-hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-3-aminopropyl]-3-methyl-1-triazene), which chemically generates NO with a half-life of 5 minutes.

Chemicals

Reagents for HPLC analysis were obtained from EICOM (Kyoto, Japan) and NOC7 was obtained from Dojin Co. (Kumamoto, Japan). Other reagents used were of analytical grade.

5.3. Results

Palmar circulation in acupunctured forearm

Prior to receiving acupuncture, microvascular blood flow in the palm of the forearm that underwent real acupuncture did not differ from that in the forearm that underwent sham acupuncture (Figure 4-A). At 5 and 60 minutes after acupuncture, microvascular blood flow in the palm of the sham acupunctured forearm was unchanged, while that in the real acupunctured side was significantly increased as compared with both the pre-acupuncture and sham values.

NO formation in acupunctured forearm: HPCL study

Prior to receiving acupuncture, there were no differences in regard to plasma concentrations of nitrate plus nitrite between the real and sham acupuncture groups (Figure 4-B). After acupuncture as well, no significant changes in plasma concentrations of nitrate plus nitrite were noted in either the non-treated forearm in subjects who underwent real acupuncture or

those that underwent sham treatment. However, that concentration in the acupunctured forearm was significantly increased as compared with both the pre-acupuncture value and values obtained for the opposite non-treated and sham-acupunctured forearms at 5 and 60 minutes after real acupuncture treatment. Plasma concentration of nitrate plus nitrite was 24.6±6.1 μM in the acupunctured forearm before acupuncture, 27.5±6.5 (mean±SD) μM at 5 minutes after acupuncture, and 27.1±6.6 μM at 60 minutes. Thus, the amount of increase above the pre-acupuncture value was 2.8±1.5 μM after 5 and 2.5±1.4 μM after 60 minutes.

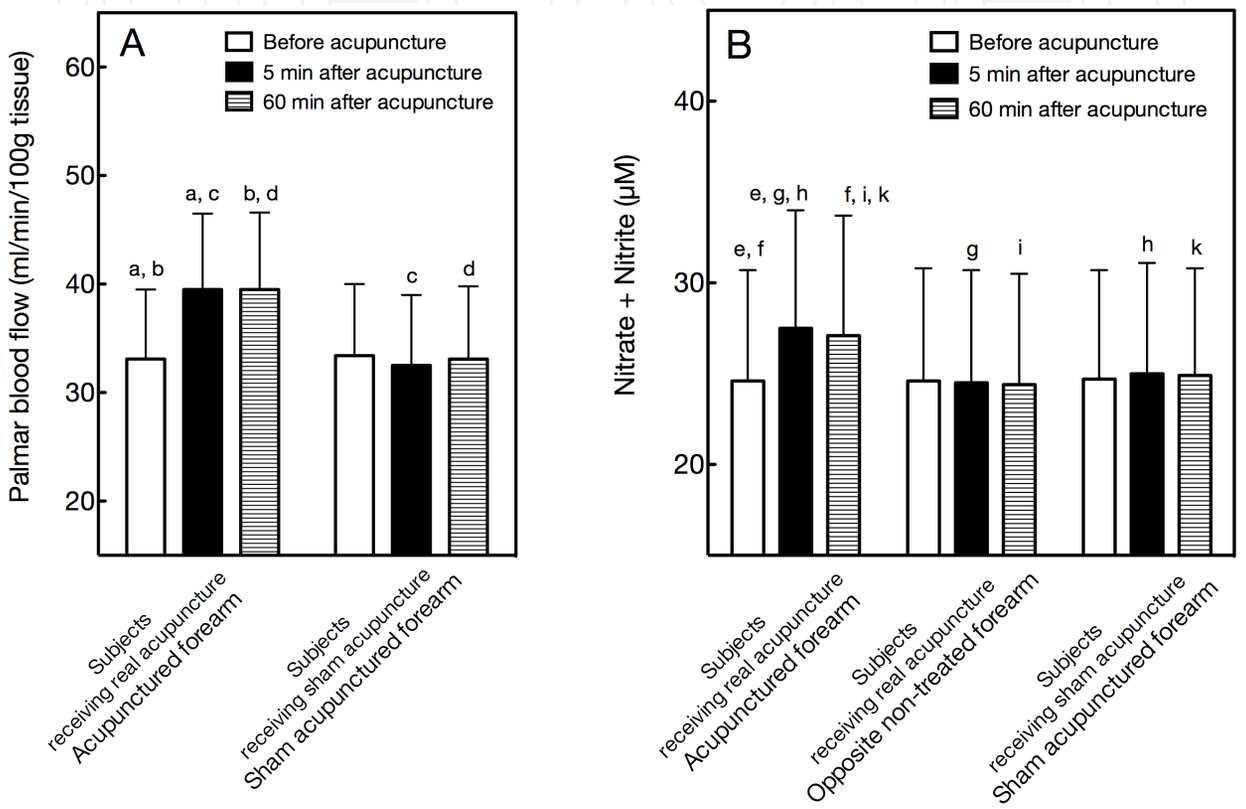

Figure 4. Changes in blood flow (mean±SD) in palmer subcutaneous tissue (A), and plasma concentrations of nitrate and nitrite in samples from an axillary vein (B) in subjects who received real or sham acupuncture in a forearm. Alphabetic characters of a, b, c, d, e, f, g, h, i, and k indicate a significant difference ($p < 0.05$) between values.

NO formation: ESR study

The ESR spectrum attributed to Cu^{2+} of ceruloplasmin was observed in blood samples obtained before acupuncture (Figure 5-A). At 5 minutes after acupuncture, a small peak with a *g* value of 2.0 developed and was superimposed on the Cu^{2+}-ceruloplasmin adduct spectrum [24] in blood samples obtained from the acupunctured forearm (arrows in Figure 5-B), but not in blood samples from the non-treated and sham forearms (data not shown). Although the ESR signal observed was small, it was assigned to the NO-hemoglobin adduct spectrum with reference to the typical ESR spectrum of NO-hemoglobin generated by incubation of NOC7, a short-lived potent NO releaser, with a fresh non-treated blood sample, as shown in Figure 5-C and D.

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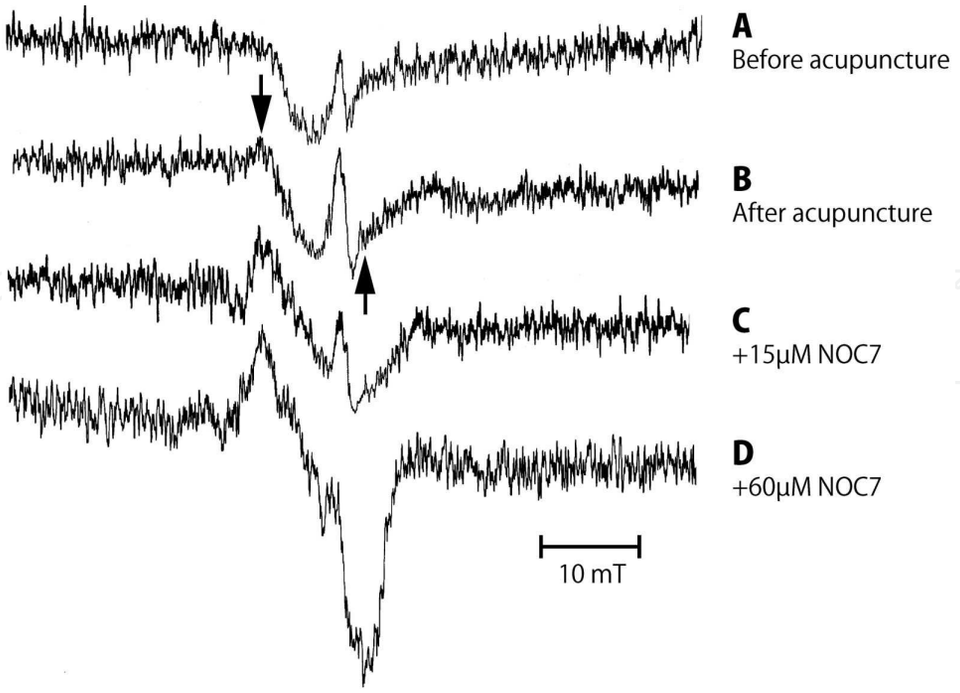

Figure 5. ESR spectra of blood samples from acupunctured forearm. Prior to acupuncture (A), 5 minutes after acupuncture (B), and 10 minutes after incubation of fresh non-treated blood with 15 μM (C) and 60 μM (D) of NOC7 as a positive control. Arrows in B indicate the NO-hemoglobin adduct with a *g* value of 2.0 superimposed on the originally generated Cu^{2+}-ceruloplasmin adduct. Detailed ESR settings are described in the text.

Correlation between NO formation and palmar circulation

In the real acupunctured forearm, the amount of increase in microvascular blood flow in the palm above the pre-acupuncture value was significantly correlated with that in plasma concentration of nitrate plus nitrite, with a regression line of y = 2.3x and correlation coefficient (r) of 0.79 (Figure 6). In the sham forearm, no correlation was found between the amount of increase in microvascular blood flow in the palm and that in plasma concentration of nitrate plus nitrite.

5.4. Discussion and interpretation

We found that acupuncture increased the plasma concentration of nitrate plus nitrite in treated regions, coupled with an increase in blood flow. Although measurements of NO itself were not performed, previous studies have shown that concentrations of nitrate and nitrite, end-products of NO metabolism, are reliable indicators of NO formation *in vivo* [25].

It should be noted that blood in the axillary vein of the acupunctured forearm contained nitrate plus nitrite derived from locally generated NO in the forearm itself, in addition to that basally generated throughout the body. Thus, to confirm that basal NO generation did not affect the change in concentration of nitrate plus nitrite in blood from the acupunctured forearm, that in blood from the opposite non-treated forearm was also measured, which showed no significant change. This finding indicates that background basal NO level did

not change in the present subjects. Thus, the increased NO level in the acupunctured forearm was primarily from changes that occurred in the forearm itself.

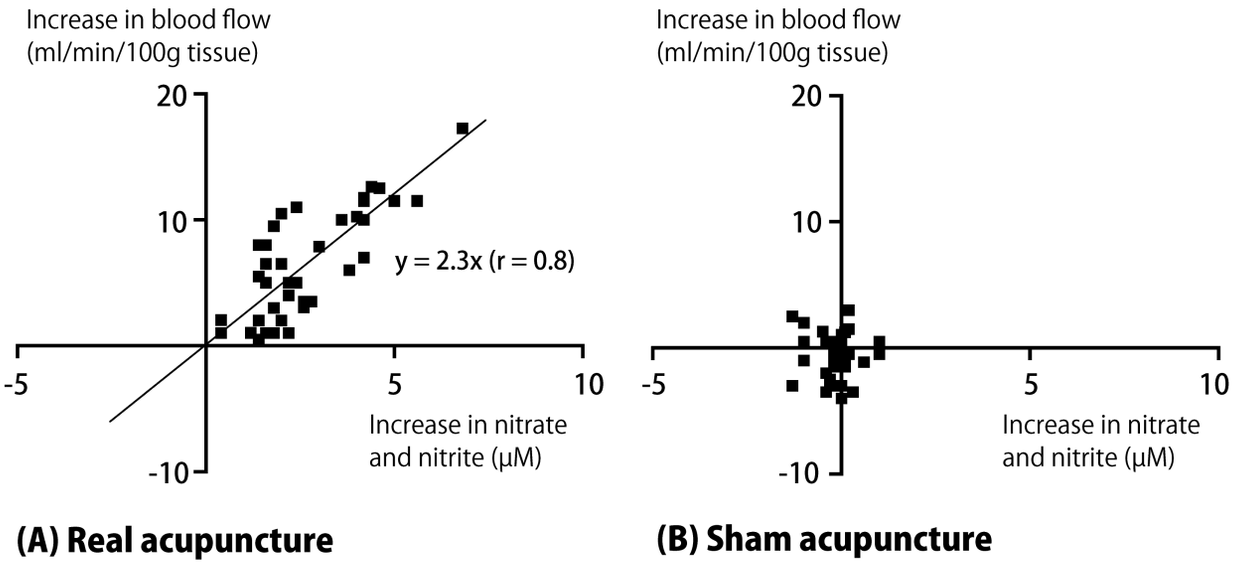

Figure 6. Relationship between amount of increase in palmar blood flow above the pre-acupuncture value, and that in plasma concentration of nitrate plus nitrite in real acupunctured (A) and sham-acupunctured (B) forearms. There was a significant correlation after real acupuncture, with a regression line of y = 2.3x and correlation coefficient (r) of 0.79, but not after sham acupuncture.

An ESR method was used to detect and confirm NO generation in the forearm treated with acupuncture. An advantage of ESR is that it can directly and specifically detect NO even in biological samples, whereas it is not highly sensitive and does not provide quantitative findings [26]. Because the affinity of hemoglobin for NO is very high, 300,000 times greater than that for oxygen molecules, NO generated in blood rapidly reacts with hemoglobin to form an NO-hemoglobin adduct, which is detected as a characteristic ESR spectrum and interpreted as evidence of NO generation [24]. Thus, even a small ESR peak for NO-hemoglobin, as seen in the present study, is strong and reliable evidence for NO generation in the forearm from which blood samples were collected. To the best of our knowledge, this is the first study to demonstrate that manual acupuncture treatment increases plasma NO in acupunctured regions in human subjects.

The increase in palmar microvascular blood flow seen in the acupunctured forearm side indicates that acupuncture controls regional circulation, and agrees well with previous studies that reported increases in skin temperature and muscular blood flow in regions subjected to acupuncture [5-8]. The significant correlation between the amounts of increase in blood flow and NO level in the acupunctured forearm indicates that NO-dependent mechanisms are involved in regulation of circulation by acupuncture. Regional ischemia is an important factor in development and prolongation of certain types of pain, and improvement in blood flow washes out pain-generating metabolic products [27, 28]. Thus, the regulatory effect on re-

gional circulation through control of NO level partly accounts for the biological mechanism of the effects of acupuncture on pain.

6. NO a key molecule in acupuncture

The findings of NO dynamics in human subjects described in the previous sections 2 and 3 indicate that changes in NO concentration in μM amounts have a strong influence on vital body functions. Therefore, the magnitude of NO generated by acupuncture shown in the present study is of great biological significance. Furthermore, coupled physiological effects were also observed, as expected. We consider it reasonable to conclude that NO plays a key role in the effects of acupuncture. A recent animal study showed that acupuncture increased muscle blood flow to some degree, even in denervated hindlimb muscles, indicating that a local regulation mechanism may have a dominant role in acupuncture [29]. When considering the importance of NO as a biological molecule for basic vital functions [9, 10], its local regulation is a compelling candidate for an acupuncture-related molecular mechanism.

7. Further advancements in studies of NO metabolism related to acupuncture

Based on an understanding of the importance of NO generation in acupuncture, the question regarding which mechanism is involved becomes important. In addition to the present findings, several studies have reported higher levels of generated NO on the skin surface at meridians and acupoints in resting or acupuncture treated human subjects [30-32]. Furthermore, an increase in NO generation at certain acupoints during electrical acupuncture treatment at distant acupoints belonging to the same meridian has been shown using dermal micro-dialysis [33].

Some resident bacteria could produce NO-related metabolites, thus it is possible that the bacterial organisms in the area of the skin contribute to these NO phenomena [20, 31]. On the other hand, it should be noted that many acupoints and meridians are located close to blood vessels and peripheral neural fibers, which might express nitric oxide synthase (NOS) enzymes with various stimuli. In fact, enhancement of the expression of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) by acupuncture was reported [34, 35], though in animal studies. Together with other studies, we suggest that the acupoint and meridian NOS system function to regulate the level of NO in acupunctured individuals with the aid of surrounding blood vessels and local neural fibers. These should be confirmed in future studies.

8. Conclusion

Acupuncture elevates the level of local NO in treated regions, thereby increasing local blood flow. The importance of local perfusion in patients suffering from pain suggests that these effects contribute to pain relief by acupuncture. Our findings may be useful for elucidation of the complex mechanisms underlying the clinical effects and mechanism of acupuncture.

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