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Influence of Electroacupuncture Stimulation on Nitric Monoxide Production in Vascular Endothelial Cells in Rats

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Abstract. *Background/Aim: In Chinese medicine, blood stasis termed as 'Oketsu' means 'preceding state' or 'symptomatic of sickness'. Traditional Chinese medicine may improve blood flow by vasodilation or blood clotting inhibition. Although acupuncture influences the blood circulatory system, its underlying mechanisms remain unclear. Herein we evaluated changes in NO, as reflected by changes in* NO_2^-*, platelet aggregation, oxidative stress and endocrine responses after acupuncture stimulation in rats. Materials and Methods: Acupuncture stimulation was administered to rats randomly divided into five groups: control,* N^G*-nitro-L-arginine methyl ester hydrochloride (L-NAME) injection, restraint stress (RS), restraint plus acupuncture stimulation (RA), and restraint plus acupuncture with L-NAME (RLA). Results: Compared to those in the RS group, levels of* NO_2^-*, endothelial nitric oxide synthase (NOS) protein and its mRNA significantly increased and those of hydroperoxide and soluble P-selectin significantly decreased in the RA group. Conclusion: Acupuncture stimulation regulates vascular endothelium NOS function and affects vascular resistance and blood characteristics through NO. Additionally, NO produced may modulate excessive reactive oxygen development and blood platelet activation.*

Physiological blood circulation involves the transportation of oxygen and materials, and maintains organic homeostasis. Two major factors contribute to blood flow: blood pressure and blood hydrodynamic characteristics (1). The relationship between control of vascular resistance and blood hydrodynamic characteristics is important; however, it remains unclear.

In Chinese medicine, blood stasis is called '*Oketsu*', which signifies 'the preceding state' or 'symptomatic of sickness' (2). *Oketsu* is the syndrome found in *e.g.* menoxenia and sprain. Improving *Oketsu* naturally alleviates symptoms and disease (2). *Oketsu* is regarded as 'the reduction of blood flow' and is studied from the perspective of blood fluidity and vascular resistance (3, 4). Some studies indicate that traditional herbal medicines improve blood flow by vasodilation or inhibiting blood coagulation (5, 6). Acupuncture dilates blood vessels and lowers blood pressure by an autonomic nervous reflex (7). We reported that acupuncture stimulation improves blood fluidity and inhibits platelet aggregation, and β-blocker inhibits these acupuncture functions in animal experiments with rats (8). Furthermore, we showed that acupuncture stimulation reduced the blood catecholamine levels (8). Thus, it is evident that acupuncture stimulation affects the circulatory system as well as blood characteristics. In other words, these facts indicate that blood characteristics and blood circulatory system impact one another. However, the underlying mechanism that changes blood characteristics has not been understood.

In the present study, we recorded changes in nitric monoxide, a vasodilative factor, and blood characteristic, platelet aggregation, oxidative stress and endocrine changes after applying acupuncture stimuli to rats.

Materials and Methods

Experimental animals. Specific pathogen-free 8-week-old male Wistar rats were purchased from Japan Bio-Supply Center (Tokyo, Japan) and maintained at 25±2˚C, humidity 55±5%, with a 12-h light–dark cycle in our animal facilities. The rats were randomly divided into five groups of six animals each: Control, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME; Dojindo Laboratories, Kumamoto, Japan) injection, restraint stress (RS), restraint plus acupuncture stimulation (RA), and restraint plus L-NAME with acupuncture (RLA). Furthermore, none of the animals were given a chow diet or water during experiments (6 hours). This study was approved by the Ethics Committee for Animal Experiments of Showa University (04104).

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Key Words: Electroacupuncture, nitric monoxide, endothelial nitric oxide synthase, hydroperoxide, soluble platelet selectin.

Stimulation and reagents. We used the restraint stress method to investigate the mechanism of acupuncture stimulus. Based on preliminary research, we hypothesized that various stressors change blood characteristics (9). Restraining of rats in a rectangular acrylic box for 6 h was used as the stressor. Acupuncture stimuli were applied for 1 h after 5 h of restriction, as reported previously (10). The acupuncture needle used was 0.20 ×40 mm (Seirin Co., Shizuoka, Japan). Acupoints were pricked to apply the needle equivalent to the human locus: ZuSanli (ST36), on the outside of the *crus superior*, where the effect on blood fluidity was confirmed (9) and is generally known to improve *Oketsu* (11). The control group did not receive acupuncture stimulation. Acupuncture was administered at a depth of 5 mm and stimulated electrically (3-5 V, 30-200 μA, rectangular and biphasic) at a frequency of 1 Hz to permit the muscle to shrink slightly. An Ohm Palser LFP-4000A (Zen Iryoki Co., Fukuoka, Japan) was used as the acupuncture stimulus device. The LFP-4000A has four output lines that can stimulate eight points simultaneously. Furthermore, it can be useful for electroacupuncture and transcutaneous electrical nerve stimulation.

L-NAME [20 mg/kg; Dojindo Laboratories] was injected into the abdominal cavity for the L-NAME and RLA groups after 4 hours of restraining to inhibit the function of endothelial nitric oxide synthase (eNOS) wherever nitric monoxide (NO) production is enzymatic (12).

Blood sampling and serum preparation. The blood sample was obtained from the inferior vena cava of the experimental rat anesthetized by abdominal injection of a combination anesthetic (M/M/B) which was prepared with 0.15 mg/kg of medetomidine (Nippon Zenyaku Kogyo Co.,Ltd., Fukushima, Japan), 2.0 mg/kg of midazolam (Sandoz K.K., Tokyo, Japan), and 2.5 mg/kg of butorphanol (Meiji Seika Pharma Co., Ltd., Tokyo, Japan). The animals were euthanatized by cervical dislocation after blood sampling promptly. After blood samples keep to clot for 2 h at room temperature, the serum was provided by centrifuging for 20 min at 2,000 × *g* and store samples at –20˚C.

Detection of serum NO_2^- *level*. As a metabolic product of NO, NO_2^- was measured as a surrogate for NO because the half-life of NO is very short (3-6 s). The amount of nitrite/nitrate in the serum produced by vascular endothelial cells was measured using an NO_2/NO_3 Assay Kit-FX (NK08; Dojindo Laboratories), according to the manufacturer's instructions. After the serum was centrifuged with a centrifugal filter (Amicon Ultra-0.5 Centrifugal Filters' Merck Millipore Ltd., Tullagreen, Ireland) for albumin removal, 80 μl serum was transferred to an empty 96-well plate, 2,3-diaminonaphthalene reagent included in the kit were added to each well, and the plate was incubated for 15 min at room temperature. Fluorescence intensity was measured using a fluorescence plate reader (Twinkle LB970; Berthold Japan, Tokyo, Japan), with excitation and emission wavelengths of 355 and 450 nm.

Immunohistological staining for eNOS detection on blood vessels. The thoracic aorta was washed several times with saline to remove unwanted materials (*e.g.* blood and connective tissues), fixed in 4% paraformaldehyde–phosphate-buffered saline (PBS), followed by fixing in 5%, 15% and 30% sucrose–PBS; specimens were then embedded in Tissue-Tek (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and cut into 10-μm sections.

A primary antibody for eNOS (rabbit IgG, diluted 1:100; ab87750; Abcam Co., Ltd., Tokyo, Japan) was applied to the fixed cryosections and left overnight at 4˚C. To demonstrate endothelial integrity, a primary antibody for CD31 [mouse IgG, diluted 1:100; ab64543; Abcam Co., Ltd.] was applied to fixed cryosections and incubated overnight at 4˚C. The sections were then washed with PBS and incubated for 1 hour with fluorescein isothiocyanate (FITC; goat anti-rabbit IgG, dilution 1:500; ab6717; Abcam Co., Ltd) for eNOS and Texas Red (goat anti-mouse IgG, dilution 1:500; ab6787; Abcam Co., Ltd;) for cluster of differentiation 31 (CD31). After rinsing in PBS, sections were coverslipped using VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA). Digital images were obtained using a confocal laser scanning microscope (A1si; Nikon Co., Ltd., Tokyo, Japan), and fluorescence was evaluated as above on at least four aortic sections per animal with NIT-Elements ver3.22 Analysis (Nikon Co., Ltd.).

PCR primers and reagent kits. Reagents used for mRNA isolation (TaqMan Gene Expression Cells-to-Ct™) and real-time reverse transcription-polymerase chain reaction (RT-PCR; TaqMan Gene Expression Assays) were purchased from Applied Biosystems (Foster City, CA, USA). Assays were conducted according to the manufacturer's instructions (13). For real-time RT-PCR comparison of gene expression, we selected eNOS (*NOS3*: TaqMan Gene Expression Assays; Assay ID: Rn02132634_s1). The 18S ribosomal RNA (*Rn18s*: TaqMan Gene Expression Assays; Assay ID: Rn03928990_g1) was used as a housekeeping gene to normalize for RNA loading.

mRNA isolation and quantitative RT-PCR. Total RNA was isolated from vascular endothelial cells, obtained as previously described by McKenzie *et al*. (14), using 50 μl Lysis Solution (P/N4383583). Each sample of total RNA was subjected to RT using 20× RT Enzyme Mix (P/N 4383585) and 2× RT Buffer (P/N43833586) with a T100 Thermal Cycler (Bio-Rad Co., Hercules, CA, USA). After the RT reaction, the cDNA templates were amplified by PCR using TaqMan Gene Expression Assays, PCR primers and RT Master Mix (P/N 4369016). Predesigned and validated gene-specific TaqMan Gene Expression Assays (13, 15) from Applied Biosystems were duplicated for quantitative RT-PCR, according to the manufacturer's protocol. PCR assays were conducted as follows: 10-min denaturation at 95˚C, 40 cycles of 15 s denaturation at 95˚C and 1 min annealing and extension at 60˚C. Samples were analyzed using an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems) (15, 16). Relative quantification (RQ) studies (17) were prepared from collected data [threshold cycle numbers (*Ct*)] with ABI Prism 7900HT Sequence-Detection System software 2.3 (Applied Biosystems).

Detection of serum soluble (s)P-selectin. Serum sP-selectin levels were measured with commercially available ELISA test kits (KT-28051: Kamiya biomedical Co., Seattle, WA, USA), with a minimum detectable level of 0.057 ng/ml.

Reactive oxygen metabolite test for oxidative stress detection. The oxidative stress level was measured using the Reactive Oxygen Metabolites test (d-ROMs test; Wismerll Co., Ltd. Tokyo, Japan). Briefly, the color reaction when the sample serum was mixed with chromogen was measured using the Free Radical Elective Evaluator (Wismerll Co., Ltd.). This test measures the hydroperoxide level to reflect production of peroxide (18).

Statistical analysis. Data are expressed as means±standard deviations. All assays were repeated three times to ensure reproducibility. Statistical significance between the controlled and experimental groups was analyzed by one-way analysis of variance followed by the Scheffe test. A probability (*p*) value of less than 0.05 was considered statistically significant.

Results

Serum NO_2^- detection. We examined whether acupuncture stimulation affected serum level of nitrous acid ion (NO_2^-) using the NO_2/NO_3 Assay Kit-FX (Figure 1). Serum NO_2^- levels in the RS and L-NAME injection groups were significantly decreased compared to those in the control group ($p<0.05$). The NO_2^- level in the RA group was significantly increased compared to that in the RS group ($p<0.05$). However, there was no detectable increase in the NO_2^- level in the RLA group.

Vascular eNOS expression. We examined whether acupuncture stimulation affected the release of vascular eNOS. Figure 2 shows a vascular immunology chromatic image. The expression of vascular eNOS in the RS and L-NAME injection groups was reduced compared to that in the control group, while that in the acupuncture stimulation group was increased compared to that in the RS group. Furthermore, the intensity of eNOS in vascular endothelial cells was measured (Figure 3). eNOS fluorescence intensity in the RS and L-NAME injection groups was significantly lower compared to that in the control group ($p<0.05$). The fluorescence intensity of eNOS in the RA group was significantly increased compared with that in the RS group ($p<0.05$). However, an increase in eNOS fluorescence intensity was not detected in the RLA group.

eNOS mRNA in vascular endothelial cells. We further examined whether acupuncture stimulation affected the expression of eNOS mRNA in endothelial cells using real-time RT-PCR (Figure 4). The expression of eNOS mRNA in the RS and L-NAME injection groups was significantly decreased compared with that in the control group ($p<0.05$), whereas it was significantly increased in the RA group compared with that in the RS group ($p<0.05$). However, the NO_2^- level did not increase in the RLA group.

Serum oxidative stress level detection. The serum oxidative stress level was examined to determine the effects of acupuncture stimulation (Figure 5) by the d-ROM value, which reflects hydroperoxide level and peroxide production. The d-ROM value in the RS and L-NAME injection groups significantly increased compared to that in the control group, whereas it significantly decreased in the RA group compared with that in the RS group (both $p<0.05$). However, no decrease in d-ROM value was observed in the RLA group.

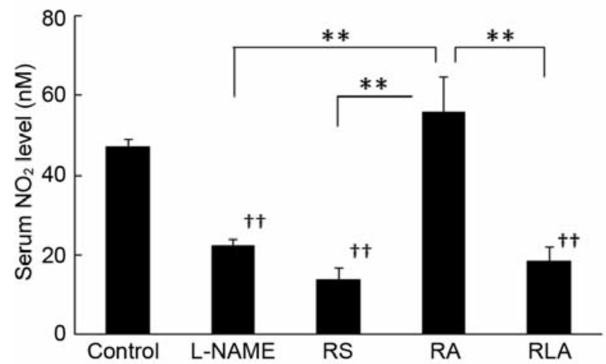

Figure 1. *Serum NO_2^- level as a surrogate for NO (n=6 in each group). Data are the mean±standard deviation. RS: Restraint stress group, RA: restraint + acupuncture stimulation group, RLA: restraint + N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) + acupuncture group. Differences statistically significant at: **p<0.01; ††p<0.01 vs. control.*

Serum sP-selectin level detection. ELISA was used to determine whether acupuncture stimulation affected serum sP-selectin (Figure 6). The sP-selectin level in the RS and L-NAME injection groups significantly increased compared with that in the control group but significantly decreased in the RA group compared with that in the RS group (both $p<0.05$). However, a decrease in the sP-selectin level was not detected in the RLA group.

Discussion

Nitric monoxide has a role in various physiological homeostatic mechanisms. NO secreted by vascular endothelial cells increases cGMP, generated following guanylate cyclase activity in vascular endothelium and vascular smooth muscle. cGMP relaxes vascular smooth muscle. Thus, NO in vascular endothelial cells maintains smooth microcirculation. Disorders of blood circulation can cause pain and inflammation. Acupuncture has been used to improve disorders of blood circulation for a long time. However, there exists no evidence that acupuncture stimulation affects blood vessel function and blood characteristics. Therefore, the present study was conducted to determine whether acupuncture stimulation affects NO production of a vascular endothelial origin.

Firstly, the level of NO_2^- as a surrogate for NO was measured. Serum NO_2^- levels were found to decrease in the RS group but increased in the RA group. NO is secreted by vascular endothelial cells, macrophages and nerve cells. It is generally thought that NO secreted within blood in real time (not accompanied with inflammation) is mainly of aortic vascular endothelial origin (19). Therefore, in the next experiment, we examined whether acupuncture stimulation

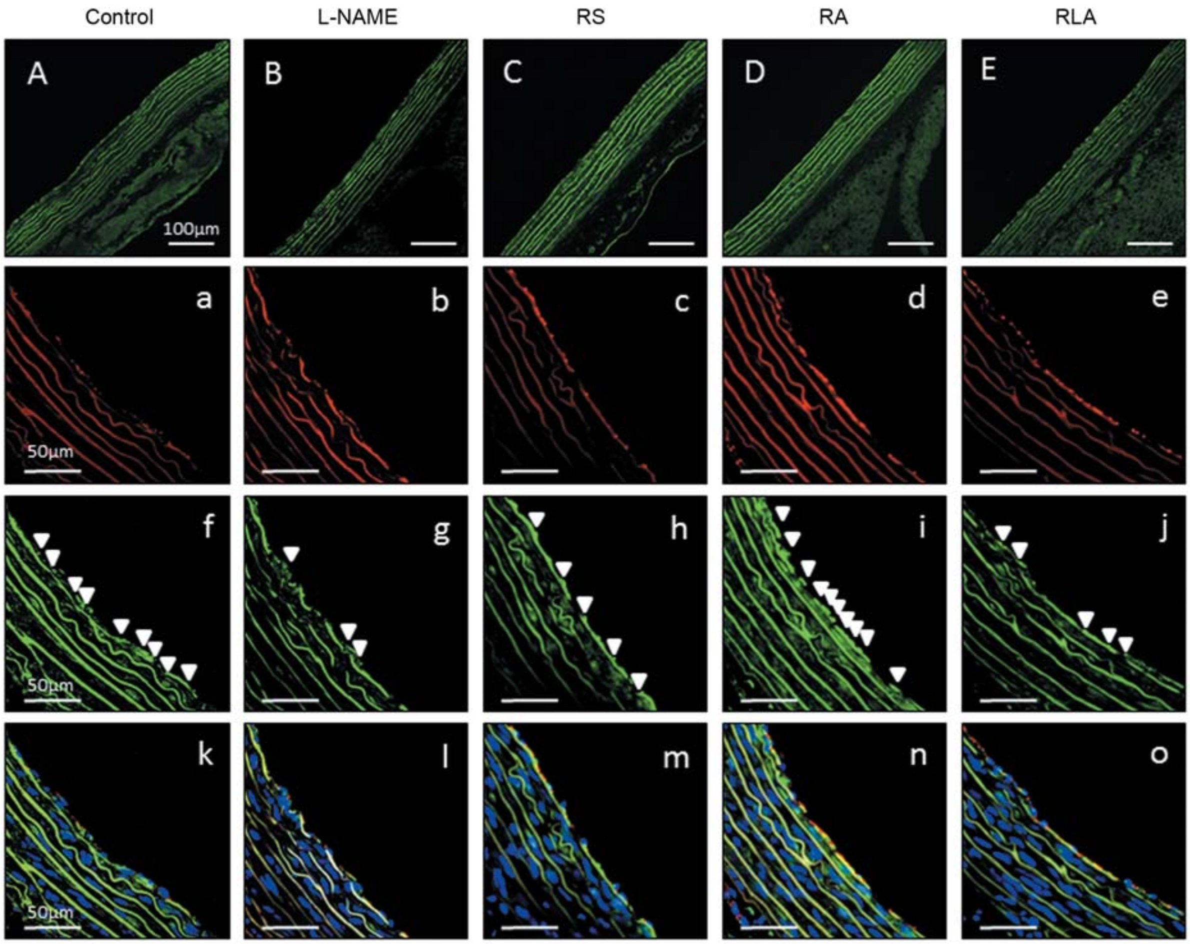

Figure 2. *Image of vascular immunohistological staining. Digital images were obtained using a confocal laser scanning microscope. A-E: Vascular endothelial cells which were dyed by antibody to cluster of differentiation 31 (CD31) (×200 magnification). Green indicates reaction with antibody to endothelial nitric oxide synthase (eNOS). f-j: Chromatic image using eNOS antibody. Arrowheads show where eNOS developed in unison with a vascular endothelial cell. k-o: Merging of images shown in a-e and f and g. Blue shows the nucleus stained by 4',6-diamidino-2-phenylindole. a-o: ×600 magnification. L-NAME: N^G-nitro-L-arginine methyl ester hydrochloride group, RS: Restraint stress group, RA: restraint + acupuncture stimulation group, RLA: restraint + L-NAME + acupuncture group.*

affected eNOS production. Animal blood vessels were used for observation of eNOS expression in vascular endothelial cells using fluorescence immunohistological staining and eNOS mRNA in vascular endothelial cells using a real-time RT-PCR method.

On immunohistological examination, eNOS was more frequently expressed in vascular endothelial cells in the RA group than in the RS group. Furthermore, eNOS mRNA was more frequently expressed in vascular endothelial cells in the RA group compared to that in the RS group, that was comparable to the pattern observed for eNOS protein. Additionally, administration of L-NAME (an NO-producing antienzyme) inhibited NO secretion due to acupuncture stimulation.

The eNOS secretion of the vascular endothelial cell membrane is activated by mechanical stimulus (shearing stress) against a vascular endothelial cell and blood-vessel agents (vascular endothelium growth factor, acetylcholine and bradykinin) (20, 21). NO is generated during oxidation of L-arginine by the function of the enzyme (NOS) and co-enzyme (tetrahydrobiopterin: BH4). Inactivation of NOS, and deficiency of L-arginine and BH4 inhibit NO generation. In particular, inactivation of NOS induces the of excessive reactive oxygen species (22, 23). Therefore, in the third experiment, we examined the serum oxidative stress level in this model. The oxidative stress level (d-ROM value) was higher in the low-NO-level groups (i.e. RS group, L-NAME group and RLA group) than in the high-NO-level group

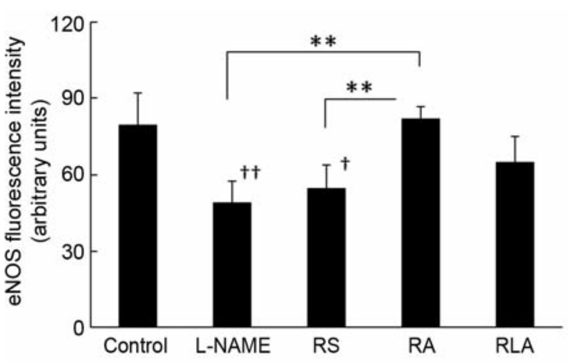

Figure 3. *Endothelial nitric oxide synthase (eNOS) fluorescence intensity (n=6 in each group). Fluorescence was evaluated on at least four aortic sections per animal. Data are the mean±standard deviation. RS: RS: Restraint stress group, RA: restraint + acupuncture stimulation group, RLA: restraint +* N^G*-nitro-L-arginine methyl ester hydrochloride (L-NAME) + acupuncture group. Differences statistically significant at: **p<0.01 vs. RA; †p<0.05, ††p<0.01 vs. control.*

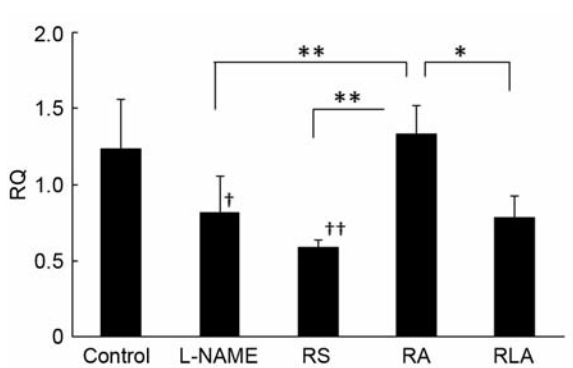

Figure 4. *Relative quantity (RQ) of endothelial nitric oxide synthase (eNOS) mRNA expression in vascular endothelial cells (n=6 in each group). Data are the mean±standard deviation. L-NAME:* N^G*-nitro-L-arginine methyl ester hydrochloride group, RS: Restraint stress group, RA: restraint + acupuncture stimulation group, RLA: restraint + L-NAME + acupuncture group. Differences statistically significant at: *p<0.05, **p<0.01; †p<0.05, ††p<0.01 vs. control.*

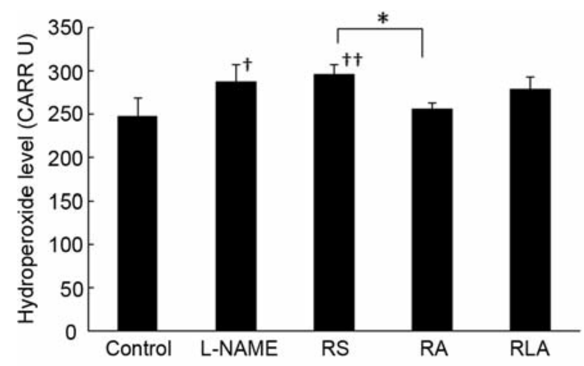

Figure 5. *Serum oxidative stress level detection with reactive oxygen metabolites test (n=6 in each group). 1 CARR U is equivalent to 0.08 mg* H_2O_2*/dl. Data are the mean±standard deviation. L-NAME:* N^G*-nitro-L-arginine methyl ester hydrochloride group, RS: Restraint stress group, RA: restraint + acupuncture stimulation group, RLA: restraint + L-NAME + acupuncture group. Differences statistically significant at: *p<0.05; †p<0.05, ††p<0.01 vs. control.*

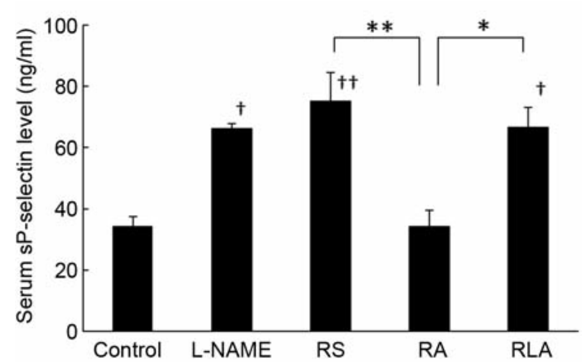

Figure 6. *Serum soluble P-selectin (sP-selectin) detection with enzyme-linked immunosorbent assay (n=6 in each group). Data are the mean±standard deviation. L-NAME:* N^G*-nitro-L-arginine methyl ester hydrochloride group, RS: Restraint stress group, RA: restraint + acupuncture stimulation group, RLA: restraint + L-NAME + acupuncture group. Differences statistically significant at: *p<0.05, **p<0.01; †p<0.05, ††p<0.01 vs. control.*

(control group and RS group). The results showed restraint stress may inhibit the scavenger function of NO. Thus, results suggest that an increase in NO secretion by acupuncture stimulation inhibits the production of reactive oxygen species.

Nitric monoxide has two facets of function: vascular damage and vascular protection. Thus, NO at an optimum level (low concentration) acts to protect blood vessels and at excessive levels (high concentration) acts as a negative factor that can injure blood vessels (22, 23). NO was transferred from vascular endothelial cells to platelets, and it reduced platelet activation following cGMP activity in platelets (24, 25). Therefore, in the final experiment, platelet activity was examined in serum samples. sP-selectin levels were found to be lower in the RA group compared to those of the RS group. Since the reduction of sP-selectin levels by acupuncture was also inhibited by L-NAME, NO may be just one of the factors inhibiting sP-selectin.

Vascular mechanical stress in the physiological range affects shear stress, whereas hard mechanical stress causes excessive stretching of vascular smooth muscles (20). Therefore, moderate shear stress induces secretion of NO and prostaglandin from vascular endothelial cells and induces vasodilation and protection of the vascular endothelium (20). Excessive stress damages the vascular endothelial cell and increases release of several cytokines and chemokines, with the resultant disorder of blood flow causing increases in the level of reactive oxygen species (20, 26). Our results suggest that restraint stress increases blood shear stress through decreased blood vessel flexibility and that acupuncture stimulation may improve these functions.

We previously reported that acupuncture stimulation improved blood catecholamine levels, which were reduced by restraint stress. Furthermore, there is a possibility that catecholamine affected NO dynamics in this experimental study. We expect to examine the mechanism, including sympathomimetic action, of acupuncture stimulation in the regulation of NO secretion from the vascular endothelium in future research.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding the publication of this article.

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