Advance Access Publication 30 June 2007

eCAM 2009;6(1)77–83
doi:10.1093/ecam/nem068

Original Article

Acupuncture on the Blood Flow of Various Organs Measured Simultaneously by Colored Microspheres in Rats

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We examined how acupuncture affected the blood flow of muscle, kidney, stomach, small intestine, brain, lung, heart, spleen and liver. Wistar rats anesthetized with urethane ($n=27$) were allocated into the control ($n=10$), ST-7 (Hsia-Kuan, $n=10$) and LI-4 (Hoku, $n=7$) groups. To measure organ blood flow, colored microspheres (CMS) were injected through a catheter positioned in the left ventricle and blood samples were drawn from the femoral artery. Yellow CMS ($3.6–4.2\times10^5$) and blue CMS ($6.0–6.9\times10^5$) were injected at intervals of about 30 min. An acupuncture needle (ϕ 340 μm) was inserted into the left ST-7 point (left masseter muscle) or the right LI-4 point after the first sampling and left for about 30 min (10 twists at 1 Hz, 2-min intervals). The mean blood flow of nine organs varied widely from 4.03 to 0.20 (ml/min/g). Acupuncture to the ST-7 produced significant changes of the blood flow (percentage change from baseline) in the muscle, kidney, brain and heart ($P<0.05$, versus control), but those of LI-4 were not significant. The blood flow of the left masseter muscle after acupuncture to ST-7 (left masseter muscle) tended to increase ($P=0.08$). Changes in blood pressure during the experimental periods were almost similar among these three groups. Acupuncture stimulation increases the blood flow of several organs by modulating the central circulatory systems, and the effects differed with sites of stimulation.

Keywords: acupuncture – colored microsphere – organ blood flow – rat

Introduction

It is known that acupuncture stimulation affects blood flow, and there are some studies for skin (1), muscle (2) and brain (3). We examined whether acupuncture not only stimulates a local area but also the blood flow of other organs, thus clarifying how acupuncture stimulates an organism. Since it is necessary to examine the effect on organ blood flow according to different areas of stimulation, the blood flow of various organs was measured in anesthetized rats using colored microspheres that can quantitatively measure multiple organ blood flow. We examined how acupuncture stimulation of the regions (Hsia-Kuan or Hoku) influenced the blood flow of various organs. Although microsphere measurement for regional blood flow has radiolabeled microspheres (4–6,7–17,18) and colored microspheres (19–28), we used the colored ones in this experiment. There are two techniques for colored microsphere measurement. One technique can calculate blood flow by counting the total number of microspheres in each sample (19–23) and the other by extracting colored dye from the microsphere s (24–28). We used the latter technique in our experiment.

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Methods

Preparation

After 24 h without food, Male Wistar rats ($n = 27$, body weight: 250–420 g, free water intake) were anesthetized with intra-peritoneal injection (1.2 g/kg) of urethane. After tracheotomy, a cannula was inserted and a respirator artificially regulated breathing (respiratory frequency: 90 cycles/min, tidal air: 10 ml/kg, SN-480-7, Shinano, Japan). Pancuronium bromide (2 mg/kg) was administrated from a catheter that was placed in the jugular vein of rats. In addition, CO_2 concentration in the expiration was monitored (1H26, NEC) and maintained at about 3%. A second catheter (PE-50) was positioned in the right femoral artery to monitor blood pressure. The blood pressure and heart rate were recorded on a thermal array recorder (RTA-1200, Nihon Kohden). A third catheter (PE-10) was inserted into left ventricular via the right carotid artery for the colored microsphere injection. And, a fourth catheter (PE-50) was positioned in the left femoral artery for withdrawal of blood samples by a syringe pump at a rate of 0.84 ml/min (Model210, KD Scientific Inc. USA). The rectal temperature was monitored using a thermistor and maintained about 37.5°C by means of a heating pad (MK-900, Muromachi Kikai Co.). In this experiment, yellow and blue microspheres (15 ± 0.2 μm, Dye-Track Triton Technology Inc. USA) were used to measure organ blood flow.

The experiment was divided into three groups. First group comprised the controls ($n = 10$, no stimulation). Second group was stimulated with acupuncture for 30 min at left ST-7 ($n = 10$, ST-7 group). The third group was stimulated by acupuncture for 30 min at right LI-4 ($n = 7$, LI-4 group). In the ST-7 group or LI-4 group, acupuncture was inserted into the left ST-7 (Hsia-Kuan, masseter muscle) or right LI-4 (Hoku) point (LI-4 is located on the dorsum of the hand, between the first and second finger) in the forelimb. Acupuncture stimulation was given using rotation (the needle was rotated right and left at 1 Hz) and was repeated 10 times at intervals of 2 min.

The position of the left ventricle catheter was confirmed by autopsy at the end of experiment.

Measurement of Blood Flow by Colored Microspheres

The infusion of colored microspheres started at least 60 min after surgery and confirmation of stabilized blood pressure and heartbeats. The microspheres were stirred with a test tube mixer (NS-80, Iuchiseieidou) for 5 min before infusion. The reference blood was drawn from 10 s before the microsphere infusion, and continued for 75 s. The microsphere (yellow or blue) infusion (20 s) was started 10 s after beginning to draw blood. Saline (0.5 ml) was then infused for 30 s to flush the microspheres in the catheter. In all experiments, yellow microspheres (0.12–0.14 ml, 360 000–420 000 microspheres) were injected first and blue (0.2–0.23 ml, 600 000–690 000 microspheres) ones second. After yellow injection, additional fluid was not replaced except by injection of blue. The injection of blue microspheres started 30 min after the first blood sampling was finished in the control group. In ST-7 or LI-4 group, acupuncture stimulation was applied after the first sampling. About 30 min after inserting the acupuncture needle, blue micro spheres were injected.

After the second blood sampling, cardiac arrest was achieved in the rats with potassium chloride. After the main large blood vessels were bound with a ligature, tissue samples that measure organ blood flow were excised and weighed. In the control, ST-7 and LI-4 group both masseter (right and left) and trapezius (right and left), or only masseter, kidney (right and left), stomach, small intestine, brain (divided into two samples, one including cerebrum and the other including the cerebellum), lung, heart, spleen and liver were excised.

Each tissue sample was put in a test tube, adding 4 M KOH containing 2% Tween 80 (16 M KOH containing 20% Tween 80 was added to the blood sample). Both tissue and blood samples were put at 60°C for 4 h in the dryer (FC-410, Toyo Seisakujyo). These samples were stirred with a test tube mixer per 1 h. Afterwards, tissue or blood samples were filtered under reduced pressure through a polyester filter to collect the microspheres. The filter and DMF (dimethylformamide) were put in the micro test tubes to dissolve dye from the microspheres in the solution after the filters dried. Then the filter was removed from the tube, and the solution was centrifuged (5 min, 4000 g and 3 min, 2000 g), and absorbance of the supernatant fluid was measured by a spectrophotometer (UV-1600, Shimadzu Seisakusho LTD.).

Figure 1 shows examples of the absorbance by the above processing. Since the peak of absorbance in yellow microspheres appears at 448 nm wavelength and blue appears at 672 nm (24,27), we measured 448 nm for yellow microspheres in the blood sample of and 672 nm for blue microspheres. Tissue samples containing both microspheres were measured at wavelength absorbencies of 448 nm and 672 nm. Tissue samples with no absorbency peak were deleted from our data.

Calculation of Organ Blood Flow

Organ blood flow was calculated using the equation below:

$$Qm = (Am \times Qr)/Ar$$

Qm shows blood flow of the tissues (ml/min/g). Qr shows the withdrawal rate of the blood samples. Am shows the

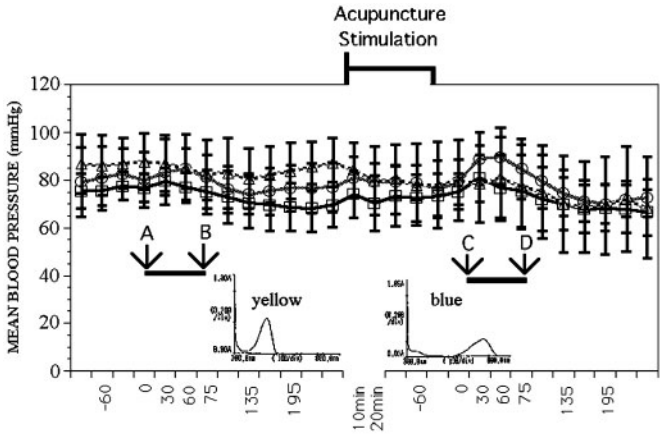

Figure 1. Variation of mean blood pressure in the control group, ST-7 group, LI-4 group (mean ± SD), and the absorbance of the reference blood samples (yellow and blue). It shows temporal variation of mean blood pressure in control group (blank square) ($n = 10$), ST-7 group (blank circle) ($n = 10$), LI-4 group (blank triangle) ($n = 7$). The number of horizontal respectively means time (seconds or minutes) of the first or second reference blood sample withdrawal. (A) Start of the first reference blood sample withdrawal, (B) end of the first reference blood sample withdrawal, (C) start of the second reference blood sample withdrawal, (D) end of the second reference blood sample withdrawal. The crossbar in the figure means the period that the reference blood sample was withdrawn. The absorbance in the figure indicated yellow (448 nm) and blue (672 nm) in the each reference blood sample.

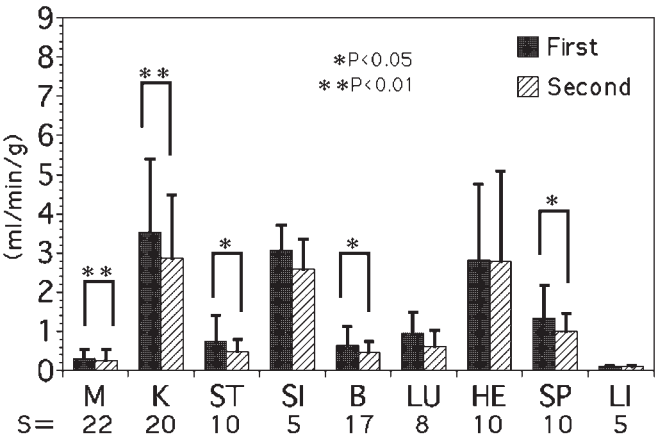

Figure 2. Organ blood flow in the control group (mean ± SD). It shows blood flow of the first and second organ blood flow in the control group (M, muscle; K, kidney; ST, stomach; SI, small intestine; B, brain; LU, lung; HE, heart; SP, spleen and LI, liver). The black bar graph expresses the first organ blood flow. The slant bar graph expresses the second organ blood flow. S means the number of tissue sample. $^{*}P < 0.05$,$^{**}P < 0.01$.

absorbance (AU) of the microspheres per 1 g. Ar shows the absorbance (AU) of all microspheres in the blood samples.

Statistical Analysis

Data were expressed as the mean ± SD. The percentage changes of blood flow were expressed as 100% the first value of blood flow, and the percentage was showed by box and whisker plot. Wilcoxon signed rank test, Mann–Whitney U-Test, One-way or Two-way ANOVA was used for data analysis. Differences of $P < 0.05$ were considered statistically significant.

Results

Time Courses of Mean Blood Pressure During the Experiment in Control, ST-7 and LI-4 Groups

Figure 1 shows the time course of mean blood pressure (mmHg) in the control, ST-7 and LI-4 group. The mean blood pressures before the first withdrawal in the control, ST-7 and LI-4 were 76.0 ± 6.0, 80.7 ± 12.7 and 86.4 ± 11.7. Although blood pressure of the control group tended to be low, there was no significant difference among the three groups ($P = 0.15$). One-way ANOVA was applied to this analysis. The temporal changes of blood pressure were also similar among three groups, and no significant differences ($F(2,14) = 1.94$, $P = 0.17$) and interaction ($P = 0.69$) among three groups. On the other hand, heart rate (beats/min) before the first withdrawal of the control group, ST-7 and LI-4 were 383.7 ± 25.3, 424.6 ± 40.3 and 427.4 ± 27.6.There was no significant difference [$F(2,14) = 3.02$, $P = 0.07$] and interaction ($P = 0.45$) among the three groups (data not shown). Two-way ANOVA was applied to these analyses.

Organ Blood Flow in the Control Group

The second measurements of organ blood flow were slightly lower than those of the first in every organ, with significant differences in the muscle, kidney, stomach, brain and spleen (Fig. 2). Wilcoxon signed rank test was applied to these analyses and the mean variations (ml/min/g) of first and second organ blood flow in each organ were as follows; kidney: −0.65; small intestine: −0.49; lung: −0.33; spleen: −0.32; stomach: −0.26; brain: −0.17; muscle: −0.05; heart: −0.03 and liver: −0.02.

The first and second blood flow of the left masseter muscle in the control group were 0.35 ± 0.24, 0.36 ± 0.45, respectively ($P = 0.35$, no figure), and right masseter of the control group were 0.16 ± 0.18 and 0.12 ± 0.10, and there was no significant difference ($P = 0.34$, no figure).

Change of Organ Blood Flow in the ST-7 Group

Figure 3 shows the first and second organ blood flows in the ST-7 group. Though the second blood flow was slightly higher than the first blood flow in the muscle, stomach, small intestine, brain and heart, there was

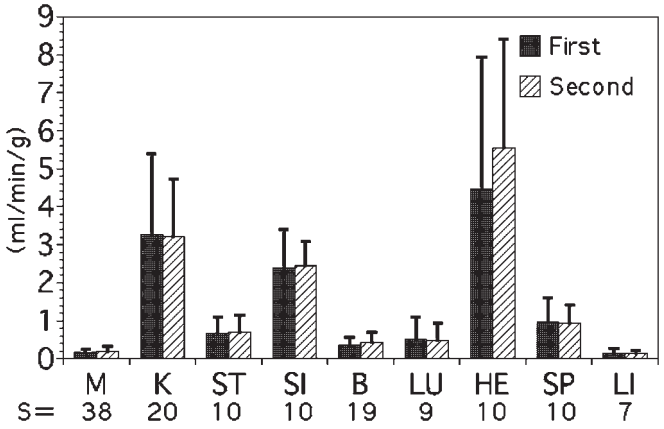

Figure 3. Organ blood flow in the ST-7 group (mean ± SD). It shows blood flow of the first and second organ blood flow in the ST-7 group. S means the number of tissue sample.

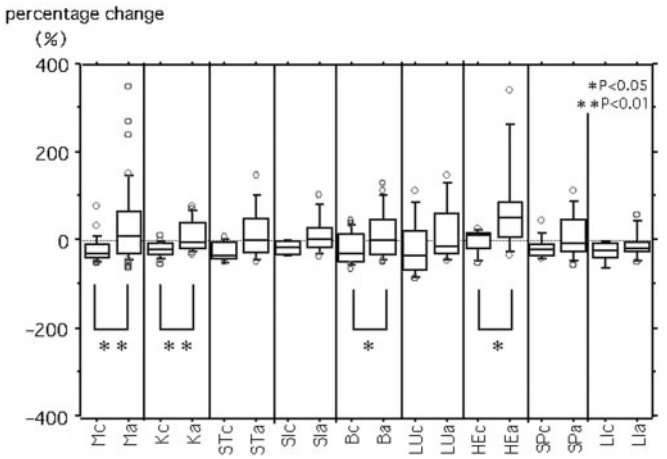

Figure 4. Percentage change of organ blood flow in the control group and ST-7 group. It shows box and whisker plot of percentage change of organ blood flow in the control group and ST-7 group (c, control group; a, acupuncture group; M, muscle; K, kidney; ST, stomach; SI, small intestine, B, brain; LU, lung; HE, heart; SP, spleen and LI, liver). The values are shown the lower extreme, 10 and 25%, median, 75 and 90% and the upper extreme. $*P<0.05$,$**P<0.01$.

no significant difference between the first and second blood flows in any organ. Wilcoxon signed rank test was applied to these analyses. The mean variation (ml/min/g) of each organ blood flow was: heart: +1.07; brain: +0.07; small intestine: +0.05; muscle: +0.04; stomach: +0.02; liver: −0.02; lung: −0.03; spleen: −0.03 and kidney: −0.04.

The first and second blood flows of the left masseter on the side receiving acupuncture stimulation were: 0.13 ± 0.06, 0.23 ± 0.19 (data not shown). There was no significant difference between the first and second values, but we did observe a trend to increase ($P = 0.08$, Wilcoxon signed rank test). Meanwhile, the first and second blood flows of the right masseter were 0.10 ± 0.05, 0.12 ± 0.08, showing no change ($P = 0.40$, data not shown).

A box and whisker plot of the percentage change (%) of organ blood flow in the control group and ST-7 shows significant differences in muscle, kidney, brain and heart, applying a Mann–Whitney U–test to the analyses (Fig. 4). Mean percentage change (%) of the blood flow in each organ of the ST-7 group were: heart: +76.0; muscle: +34.6; brain: +18.1; lung: +17.2; stomach: +15.3; small intestine: +14.6; kidney: +11.8; spleen: −9.2 and liver: −9.2.

Mean percentage change of the left masseter stimulated by acupuncture in the ST-7 group was +57.2. The value of the right masseter in the ST-7 group that was not stimulated was +28.9. The control group values were: left masseter: −10.8; right masseter: −11.3. While the blood flow rate decreased in both masseters of the control group, the blood flow rate of the left masseter of the ST-7 group had increased more than the right of the same group. However, this difference between the left and right masseters of the ST-7 group was not statistically significant. (Mann–Whitney U-test).

Organ Blood Flow Change in the LI-4 Group

Figure 5 shows the first and second organ blood flow measurements of the LI-4 group. Though the second blood flow increased slightly more than the first in the brain, lung and heart, there was no significant difference. There was a significant decrease in the muscle. Wilcoxon signed rank test was applied to these analyses. Mean variation (ml/min/g) of blood flow of each organ blood flow was: heart: +2.15; lung: +0.16; brain: +0.11; liver: 0.00; stomach: −0.01; muscle: −0.05; small intestine: −0.18; spleen: −0.54 and kidney: −0.90. Figure 6 shows the percentage change (%) of the control group and LI-4 group by box and whisker plot. There was no significant difference between the control group and LI-4 group. Mann–Whitney U-test was applied to these analyses. Mean percentage change (%) of organ blood flow in LI-4 group were described subsequently; lung: +46.3; heart: +34.7; brain: +11.0; liver: +7.4; small intestine: −1.1; stomach: −10.8; kidney: −16.9; muscle: −30.0 and spleen: −33.3.

Discussion

The colored microsphere technique used in this experiment has various advantages for organ blood flow measurement. It can measure blood flow of multiple organs simultaneously. In principle, microspheres are trapped at the peripheral capillary, and when infusion volume increases, the measurement accuracy will rise (18). However, disturbances may occur in the rat's circulation. Kobayashi *et al.* (22) described that a bolus injection of less than one million colored microspheres caused no significant hemodynamic disturbances in rats,

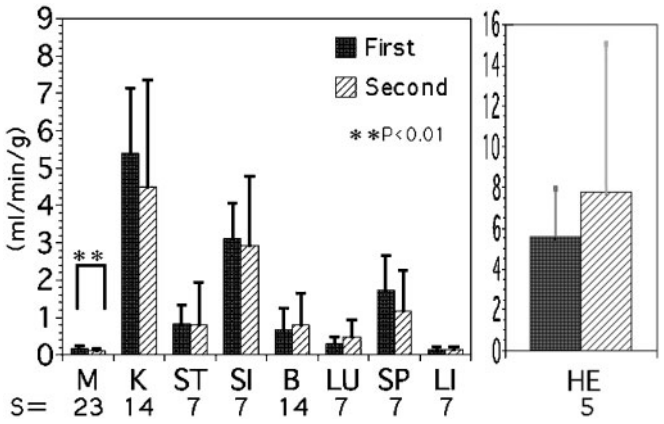

Figure 5. Organ blood flow in the LI-4 group (mean ± SD). It shows blood flow of the first and second organ blood flow in the LI-4 group. $^{**}P < 0.01$.

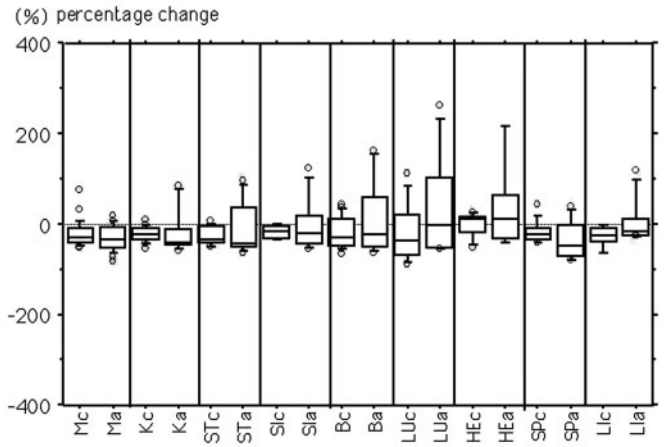

Figure 6. Percentage change of organ blood flow in the control group and LI-4 group. It shows box and whisker plot of percentage change of organ blood flow in the control group and LI-4 group.

and 500 000 colored microspheres were repeatedly injected up to four times (cumulative dose of 2 000 000 colored microspheres) without producing any adverse hemodynamic effects. Generally, blood vessels have the following diameters: capillaries are 1–8 μm, arterioles are 20–30 μm and arteriovenous anastomoses are 2–150 μm. Therefore, if 15 μm microspheres are used, a measurement of true capillaries or nutrient blood flow will result (22), and many researchers use 15 μm microspheres (6,7–17,21,22,24–28,29–32). In our experiment, we used 15 μm colored microspheres with a density of 1.0–1.09 g/ml and the colored microspheres were suspended in distilled water. In this experiment, infusion volume of blue microspheres increased more than yellow microspheres, because blue needs a greater number of microspheres than yellow to obtain the same absorbance from colored dye extraction of blue and yellow microspheres (3). Some values of mean organ blood flow values (ml/min/g) in rats are reported by measuring microspheres.

The mean organ blood flow of other investigations (ml/min/g) was also measured by microspheres in rats. A comparison of the mean values of our experimental results with conventional results is as follows: muscle: 0.06–1.27 (0.20); kidney: 3.8–10.13 (3.91); stomach: 0.5–2.38(0.74); small intestine: 1.7–4.63(2.77); brain: 0.46–1.66(0.54); lung: 0.46–2.59(0.60); heart: 3.11–6.17(4.03); spleen: 1.15–3.49(1.30) and liver: 0.14–0.4(0.14) (6,7,9–11,22,25,27,31,32). The values in parentheses show our results.

Although blood flow has a different value for each per organ, our results corresponded with those of past reports. There was a significant decrease in muscle, kidney, stomach, brain and spleen in the control group (Fig. 2) and the blood flow of other organs also tended to decrease. Blood pressure decreased significantly from the time of the first blood sampling and after the second. Though we considered that the infusion volume of microspheres in this experiment would not cause significant change in blood flow, these results were beyond expectation. Though the reason is unclear, it may have been caused by microspheres being caught in capillaries. In results of blood flow measurement using colored microspheres and Laser Doppler in the small intestines of anesthetized rats, Wahlberg *et al.* (14) described that blood flow and blood pressure decreased when the second infusion of 120 000–140 000 microspheres (cumulative dose of 240 000–280 000 microspheres) was done. They suggested that microspheres trapped in capillaries were the cause. From this evidence, we consider that a low volume of microsphere infusion is needed to achieve stable organ blood flow.

There was a significant increase of blood flow in the muscle, kidney, brain and heart between the control group and ST-7 group (Fig. 4). These results suggest that acupuncture stimulation affected blood flow of multiple organs. Lee (33) reported that acupuncture stimulation by twirling at GO-26 (located at midpoint of the philtrum) for 30 min caused significant increase in stroke volume and cardiac output, and a significant decrease in total peripheral resistance in anesthetized dogs. Meanwhile, mean blood pressure and heart rate did not change significantly. In our experiment, we found no significant temporal change of mean blood pressure and heart rate between the control and ST-7 groups (Fig. 1), when acupuncture was performed at the trigeminal area (close to the area that Lee stimulated in the ST-7 group). Perhaps that acupuncture might cause an increase in stroke volume and decrease in total peripheral resistance. As a result, although mean blood pressure did not change significantly, it is still possible that blood flow of each organ was affected.

In the case of acupuncture to the left masseter, muscle blood flow of the left masseter tended to increase more

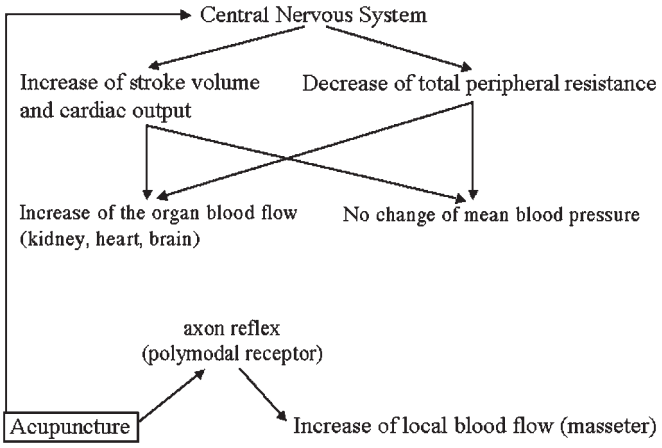

Figure 7. Hypothesis of acupuncture on the blood flow of various organs.

than the right masseter ($P = 0.08$). This phenomenon suggests that acupuncture increased muscle blood flow by some peripheral mechanism. Robert Porszasz *et al.* (34) reported antidromic vasodilatation in the striated muscle of rats, and there is a research paper suggesting that the axon reflex in the skeletal muscle may occur with acupuncture (35). From the above, we considered that peripheral mechanisms such as antidromic vasodilation and axon reflex may contribute to the increase of blood flow of the area stimulated by acupuncture. However, acupuncture not only increases blood flow of the stimulated area, but also simultaneously increased multiple organ blood flow including brain in comparison with the control group, suggesting that organ blood flow in the whole body in being influenced through the central nervous system (CNS) without change in blood pressure. It is also possible that polymodal receptors take part as peripheral receptors of acupuncture stimulation. Polymodal receptors are distributed throughout the body and react to mechanical, nociceptive and chemical stimulation. Polymodal receptors fully react by acupuncture stimulation in this experiment (36,37). Polymodal receptors also cause axon reflex in the skin (36, 37). For these reasons, we consider that polymodal receptors are the peripheral receptors of acupuncture stimulation. Below, we describe our hypothesis for the mechanism of acupuncture stimulation on blood flow of various organs (Fig. 7). Acupuncture stimulation increases blood flow of the stimulated area by causing axon reflex via polymodal receptors and increased stroke volume and cardiac output, as well as a decrease in total peripheral resistance via CNS at the same time.

Furthermore, to examine organ blood flow with a different stimulation point, LI-4 was selected, because LI-4 is often used clinically in acupuncture treatment. Our results differed from those of the ST-7 group in that there was no significant difference between the control and LI-4 groups (Fig. 6). This suggests that organ blood flow is affected differently by stimulating different points. We considered that the regulation mechanism of the automatic nerve system is different, because ST-7 (trigeminal nerve area) and LI-4 (spinal nerve area) have different innervations.

Adachi *et al.* (38) reported that noxious mechanical stimulation of the skin on the hind paw, forepaw or face caused a significant increase in cerebral blood flow in anesthetized rats, whereas noxious mechanical stimulation of the back failed to produce any significant changes in cortical blood flow. They speculated that differences in magnitude of changes in cortical blood flow and blood pressure elicited by similar stimulation of different cutaneous areas may be related to differences in conduction of impulses and/or in density of innervations of different skin areas. In addition, Noguchi *et al.* (2) reported that electro-acupuncture stimulation of a hind paw produced an increase in skeletal muscle blood flow in muscle biceps femoral's of hind limbs accompanied by an increase in systemic arterial blood pressure in anesthetized rats. Ohsawa *et al.* (39) reported that acupuncture like stimulation (the needle was twisted right and left) of an area corresponding to Tzu-San-Li point in humans induced a decrease in mean arterial pressure which was accompanied by a decrease in renal sympathetic nerve activity in anesthetized rats, and it is also known that electrical acupuncture stimulation of Tzu-San-Li in anesthetized rats produce depressor of mean arterial pressure (40). Inoue *et al.* (41) reported that manual acupuncture stimulation of 10 mm lateral to the L6 vertebra spinous process in anesthetized rats (the needle was rotated) did not produce consistent changes in sciatic nerve blood flow, with increased and decreased blood flow as well as no change in observed blood flow. Sugiyama reported that manual acupuncture (the needle was rotated) of the Tsu-San-Li point in human produced an increase in muscle sympathetic nerve activity (42). All these reports suggest that different methods or regions of acupuncture stimulation influence blood pressure and autonomic nerves differently.

While conventional acupuncture research on the circulatory system has targeted individual organs or tissue blood flow, our method of using colored microsphere may be the first time that we have been able to grasp changes in multiple organ blood flow by acupuncture at the same time. It is our hope that these results have great significance for future acupuncture research.

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Received September 22, 2006; accepted April 12, 2007