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Research Report

Acetylcholine does not participate in the increase in local muscle blood flow following manual acupuncture in rats

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Abstract

[Objective] The purpose of this study was to investigate the participation of acetylcholine (ACh) in an increase in muscle blood flow (MBF) by manual acupuncture (MA).

[Materials and methods] The experimental animal was the male Sprague-Dawley rat (270-350 g, n = 54). They were pretreated with the muscarinic cholinergic receptor antagonist (atropine; 0.2, 2, and 20 mg/kg IP) or saline as a control. ^{51}Cr-labelled microspheres were used to measure the MBF in the right hindlimb muscles, including the tibialis anterior (TA), extensor digitorum longus (EDL), soleus (Sol), plantaris (Pla), and gastrocnemius (Gas). MA, by using the sparrow pecking technique, was simultaneously applied to the right TA and EDL for 1 minute. The arterial blood pressure was recorded via a catheter inserted into the right common carotid artery.

[Results] MA significantly increased the MBF of both the TA and EDL in both saline-pretreated and atropine-pretreated rats. Atropine did not influence the increase in local MBF by MA at either concentration. There were no significant differences in the mean arterial blood pressure (MAP) before, during, or after MA.

[Discussion and Conclusion] These findings suggest that ACh are not responsible for the increase in local MBF by MA. The further research is necessary to clarify the mechanism.

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Key words: *manual acupuncture, muscle blood flow, radiolabelled microspheres, muscarinic cholinergic receptors*

Ⅰ. Introduction

Acupuncture has been applied to treat various musculoskeletal disorders for pain relief, muscle relaxation, and circulation improvement. The analgesic effect of acupuncture has been considered to be induced by diffuse noxious inhibitory control (DNIC), the descending pain inhibitory system, and the segmental inhibitory system, which is based on the Gate control theory[1)]. The improvement in circulation contributes to pain relief by washing out algesic substances and waste products.

In our previous studies using radiolabelled microspheres for the measurement of muscle blood flow (MBF), we showed that manual acupuncture (MA) with the Japanese traditional needle manipulation technique locally increased MBF without changing the arterial blood pressure[2,3)]. This increase was observed after acute and chronic denervation. These findings indicate that some local vasodilators would be mainly responsible for the increase.

As for the mechanism behind the circulation improvement caused by MA and electroacupuncture (EA), calcitonin gene related peptide (CGRP) released by axon reflex[4)], elevation in blood pressure caused by somato-autonomic reflex[5)], and local generation of nitric oxide (NO)[6)] are assumed to be involved. In addition, ACh

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released by somato-autonomic reflex[7] and axon reflex[8] are proposed. ACh has been well known to be a vasodilator in animal and human skeletal muscle[9], although there is no histological evidence of sympathetic cholinergic vasodilator nerves (SCVNs) in rats[10].

Thus, we investigated the effects of the muscarinic cholinergic receptor antagonist, atropine, on the increase in local MBF by MA using radiolabelled microspheres in rats. The blood pressure was recorded during the experiment.

II. Materials and methods

1. Experimental Animal

Male Sprague-Dawley rats (270-350 g, n = 54, Japan SLC, Inc, Shizuoka, Japan) anesthetized with urethane (1.2 g/kg ip) were used. They were housed in an air-conditioned room under a 12:12 light-and-dark cycle with free access to water and a standard rodent diet. This study was approved by the ethical committee of Meiji University of Integrative Medicine (No. 17-44).

2. Acupuncture stimulation

The acupuncture stimulation was the same as our previous study[2,3]. In brief, the acupuncture used the sparrow pecking technique (SP) consisting of 30 pecking times of 1 minute to the depth of 15-18 mm using a stainless-steel acupuncture needle (0.20 × 30 mm, Seirin Inc., Shizuoka, Japan). The stimulus point was in the tibialis anterior muscle (TA) at a 7-8 mm point below the knee. An acupuncture needle was inserted toward the ankle joint and penetrated both the TA and the extensor digitorum longus (EDL).

3. Antagonist

Atropine sulphate, a muscarinic cholinergic receptor antagonist (Nacalai Tesque Inc., Kyoto, Japan), was used at 3 concentrations, 0.2, 2, and 20 mg/kg IP.

4. Experimental groups

Table 1 describes the experimental conditions and groups. The abbreviations are as follows. 'Atro' and 'Saline' are the rat groups pretreated by atropine and saline, respectively. 'Atro 0.2', 'Atro 2', and 'Atro 20' describe pretreatment with 0.2, 2, and 20 mg/kg IP of atropine, respectively. 'Acp' and 'Cnt' describe acupunctured and non-acupunctured (control) rats, respectively.

5. Muscle blood flow measurement and protocol

Figure 1 showed the schema of MBF measurement and the protocol. ^{51}Cr-labelled microspheres (15 micrometer in diameter) dissolved in saline with 10% Tween 80 (PerkinElmer Japan Co Ltd, Japan) were used for the measurement of MBF as in our previous study[2,3]. The measurement was done according to the following procedure: (1) the reference sample blood (RSB) was withdrawn from the left femoral artery using a pull pump (0.66 ml/min); (2) 10 seconds later, the microsphere solution was injected into the aortic arch by push pump (0.66 ml/min) through a catheter inserted into the right common carotid artery and the remaining microspheres in the catheter were continuously flushed with saline until 120 seconds after the injection start; (3) the rat was killed with an overdose of urethane; (4) the right

Table 1. Mean arterial blood pressure in the saline-pretreated and atropine-pretreated rats with or without manual acupuncture

Experimental groups	Number of rats	Pretreatment	Manual acupuncture (MA)	Mean arterial blood pressure (mmHg)			
				Before MA	During MA	After MA	
				-1-0 min	0-1 min	0-1 min	1-2 min
Saline_Cnt	6	saline	-	88 ± 2	88 ± 4	88 ± 4	90 ± 4
Saline_Acp	6		sparrow pecking	80 ± 4	74 ± 4	76 ± 4	82 ± 4
Atro 0.2_Cnt	7	0.2 mg/kg IP of atropine	-	80 ± 4	80 ± 4	80 ± 4	80 ± 4
Atro 0.2_Acp	7		sparrow pecking	72 ± 6	70 ± 4	74 ± 4	76 ± 6
Atro 2_Cnt	7	2 mg/kg IP of atropine	-	68 ± 4	70 ± 4	68 ± 4	68 ± 4
Atro 2_Acp	7		sparrow pecking	74 ± 2	72 ± 2	74 ± 2	74 ± 2
Atro 20_Cnt	7	20 mg/kg IP of atropine	-	68 ± 4	70 ± 4	70 ± 4	70 ± 4
Atro 20_Acp	7		sparrow pecking	74 ± 4	72 ± 4	76 ± 4	76 ± 4

There were no significant differences in the values or temporal patterns of the mean arterial blood pressure (MAP) between the acupunctured (Acp) and the non-acupunctured (Cnt) groups for each concentration. There were no significant differences in the MAP before, during, or after manual acupuncture (MA). Data were expressed as mean ± standard error.

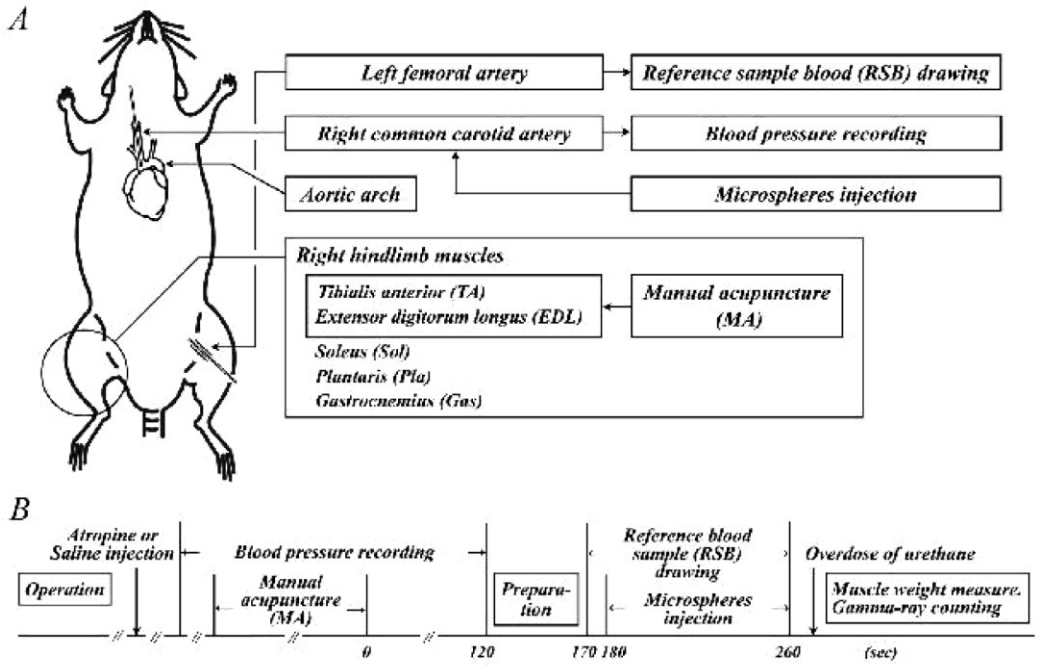

Figure 1. Muscle blood flow measurement and protocol
Figure 1A shows the operation for muscle blood flow (MBF) measurement with ^{51}Cr-labelled microspheres and the target muscles. Figure 1B shows the protocol of atropine injection and the MBF measurement. Refer to the text for the details.

hindlimb muscles, which contained the TA, EDL, the soleus (Sol), the plantaris (Pla), and the gastrocnemius (Gas), were excised and weighed; (5) gamma-radiation levels in the RSB and muscles were counted by gamma counter (Auto Well Gamma System ARS-600, ALOKA Co. Ltd., Japan); and (6) the MBF was calculated using the following equation:

$$MBF=100\times Rm\times V/(Rb\times W)\ (ml/min/100)$$

where Rm and Rb are gamma-radiation doses (cpm) of muscle and RSB, respectively; V is the pull pump speed for drawing blood; and W is the muscle weight (g). The microspheres were injected at 3 minutes after acupuncture stimulation.

6. Blood pressure

The arterial blood pressure was recorded via a catheter inserted into the right common carotid artery before, during, and after MA. This catheter was also used to inject the microspheres. Thus, the recording was stopped 1 minute before the microsphere injection. MAP was calculated every 1 minute after the measurement.

7. Statistics

GraphPad Prism 5 for Windows (GraphPad Software Inc., USA) was used for the statistical analysis. A paired t-test was used to compare MBF data between 2 groups. One-way ANOVA test was used to compare values between multiple groups. A repeated measures two-way ANOVA test was used to compare MAP data between the Acp and Cnt groups at each concentration. If there was a significant difference, the Bonferroni's Multiple Comparison test was subsequently applied to the data. A repeated measures one-way ANOVA test was used to analyse data from each group. If there was a significant difference, the Dunnett's Multiple Comparison test was subsequently applied to the data. The significance level (P) was set at <0.05. All data were expressed as mean ± standard error.

III. Results

Figure 2 shows the MBF of the Saline and Atro groups. MA significantly increased the MBF of both the TA and EDL, which were penetrated by needles. In the Saline, Atro 0.2, Atro 2, and Atro 20 groups, the *P*-values were <0.01, 0.01, 0.05, and 0.001 in the TA, and <0.05, 0.05, 0.05, and 0.01 in the EDL, respectively. There were no significant differences between the Saline_Acp, Atro 0.2_Acp, Atro 2_Acp, and Atro 20_Acp groups, or between the Saline_Cnt, Atro 0.2_Cnt, Atro 2_Cnt, and Atro 20_Cnt groups for each muscle.

Table 1 shows the MAP data. There were no significant differences in the values or temporal patterns between the Cnt and Acp groups for each concentration. There were no significant differences between the values recorded before, during, or after MA.

IV. Discussion

Some hypotheses have been suggested for the mechanism underlying the increase in MBF caused by MA[4,6-8] or EA[5]. Two of these hypotheses involve ACh. One hypothesis is that the increase in MBF is caused by ACh released from SCVNs via the somato-autonomic reflex[7]. The other hypothesis is that the increase in MBF is induced by ACh released from SCVNs via the axon reflex[8]. These hypotheses were derived from the results of some experiments performed using guinea pigs.

It has been known that SCVNs are present in skeletal muscles of cats and dogs[11]. On the other hand, there is no histological evidence of SCVNs in rats[10], mice[10], and humans[12]. However, many studies have reported that topical application of ACh dilates the arterioles of rat skeletal muscles[13-15] and that vasodilation induced by physical or mental stress is attenuated by atropine in human skeletal muscles. These findings suggest the existence of muscarinic cholinergic receptors in rat and human skeletal muscles[16]. Neurotransmitter substances containing ACh are reported to be released from vascular endothelial cells in humans[17]. Joyner et al.[12] supported this report and proposed that local cholinergic mechanisms are responsible for sympathetic vasodilation in human skeletal muscles. Such a mechanism may exist in rats as well as in humans; however, the ACh source is still unclear. We performed our experiments taking these points into consideration. In this study as well as in our previous studies[2,3], we found that MA significantly increased the local MBF. However, atropine did not inhibit the increase in MBF at any concentration. These

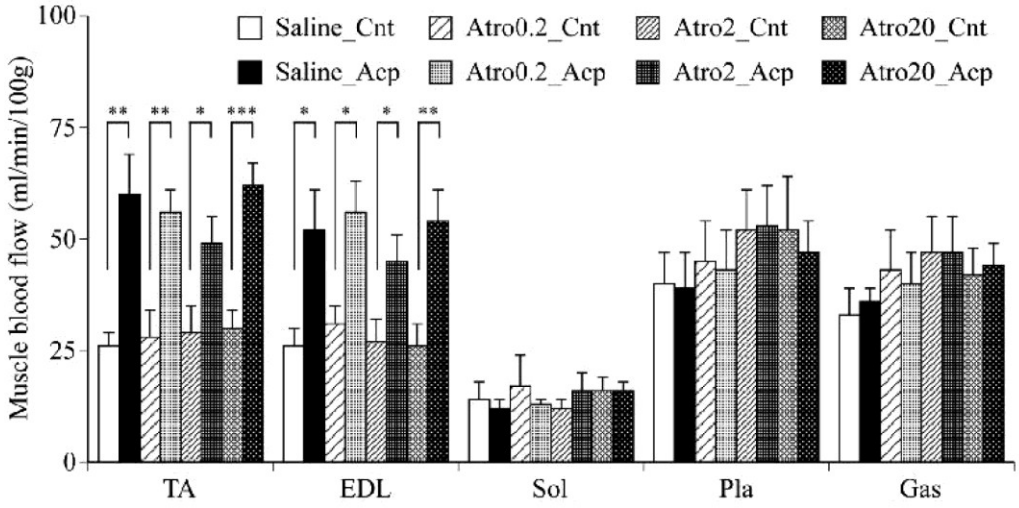

Figure 2. Muscle blood flow in the saline-pretreated and atropine-pretreated rats with or without manual acupuncture
Manual acupuncture (MA) significantly increased muscle blood flow (MBF) of the tibial anterior (TA) and the extensor digitorum longus (EDL), which were penetrated by acupuncture needle. Atropine did not influence any increase in MBF at any concentration. Refer to Table 1 about the experimental condition of each group. Data were expressed as mean ± standard error. ***p <0.001, **p <0.01, and *p <0.05.

findings suggest that ACh is not responsible for the increase in MBF.

Noguchi et al.[5] showed that an elevation in blood pressure caused by EA indirectly increased the MBF in rats. However, there were no significant differences between the values obtained before and after MA in this study, as well as in the data from our previous studies. Thus, it was suggested that arterial blood pressure would not be responsible for the increase in local MBF by MA.

From our findings, it is suggested that the increase in MBF would be mainly caused by local vasodilators. One of the more promising local vasodilators that may fulfil this role is the endothelium-derived relaxation factor nitric oxide (NO). NO is continuously released from the vascular endothelium following various stimulations and dilates muscle vessels against vasoconstriction by acting on the sympathetic adrenergic vasoconstrictor nerves[18]. Tsuchiya et al.[6] suggested its participation in the increase in MBF by MA in humans. The other candidate is CGRP via an axon reflex. Sato et al.[4] reported the possibility of the axon reflex causing vasodilation in skeletal muscle in rats. However, there is no direct evidence yet confirming that CGRP participates in an increased local MBF following MA. In addition, the participation of inflammation-related substances, e.g., prostaglandin[19], adenosine[20], and adenosine triphosphate[20], is expected, because MA causes microdamage of muscle tissue. However, the details are still unclear. Thus, further studies are needed to clarify the mechanism.

V. Conclusion

To further understand the mechanism behind the improvement of muscle circulation following acupuncture, we investigated the effect of atropine on the increase in MBF by MA in rats. Atropine did not influence any increase in MBF at any concentration. These findings suggest that ACh is not responsible for the increase in local MBF by MA.

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References

1)Kawakita K, Shinbara H, Imai K, Fukuda F, Yano T, Kuriyama K. How do acupuncture and moxibustion act? Focusing on the progress in Japanese acupuncture research. J Pharmacol Sci. 2006; 100(5): 443-59.
2)Shinbara H, Okubo M, Sumiya E, Fukuda F, Yano T, Kitade T. Effects of manual acupuncture with sparrow pecking on muscle blood flow of normal and denervated hindlimb in rats. Acupunct Med. 2008; 26(3): 149-59.
3)Shinbara H, Okubo M, Kimura K, Mizunuma K, Sumiya E, Kitade T. Effects of manual acupuncture with the Japanese traditional needle manipulation technique on skeletal muscle blood flow and arterial blood pressure in rats - a comparison of the techniques. JAM. 2011; in press.
4)Sato A, Sato Y, Shimura M et al. Calcitonin gene-related peptide produces skeletal muscle vasodilation following antidromic stimulation of unmyelinated afferents in the dorsal root in rats. Neurosci Lett. 2000; 283(2): 137-40.
5)Noguchi E, Ohsawa H, Kobayashi S, Shimura M, Uchida S, Sato Y. The effect of electro-acupuncture stimulation on the muscle blood flow of the hindlimb in anesthetized rats. J Auton Nerv Syst 1999; 75(2-3): 78-86.
6)Tsuchiya M, Sato EF, Inoue M, Asada A. Acupuncture enhances generation of nitric oxide and increases local circulation. Anesth Analg. 2007; 104(2): 301-7.
7)Kusumoto S, Sato M, Takeshige C. [Experimental research of acupuncture's effect on local pain V: The reflex center of acupuncture needling effect of perivertebral muscle on the recovery from reduced twitch in the gastrocnemius muslce after tetanic stimulation]. J Showa Med Ass. 1985; 45: 279-85.
8)Kuwasawa J, Sato M, Takeshige C. [Experimental research of acupuncture's effect on local pain (V)]. J Showa Med Assoc. 1987; 47(1): 81-8.
9)Costanzo LS. Physiology. 3 rd ed. Philadelphia (PA): Elsevier; 2006. pp. 56-64.
10)Guidry G, Landis SC. Absence of cholinergic sympathetic innervation from limb muscle vasculature in rats and mice. Auton Neurosci. 2000; 82(3): 97-108.
11)Ernsberger U, Rohrer H. Development of the cholinergic neurotransmitter phenotype in postganglionic sympathetic neurons. Cell Tissue Res. 1999; 297(3): 339-61.
12)Joyner MJ, Dietz NM. Sympathetic vasodilation in human muscle. Acta Physiol Scand. 2003; 177(3): 329-36.

13)Anderson GL, Acland RD, Siemionow M, McCabe SJ. Vascular isolation of the rat cremaster muscle. Microvasc Res. 1988; 36(1): 56-63.
14)Kaley G, Rodenburg JM, Messina EJ, Wolin MS. Endothelium-associated vasodilators in rat skeletal muscle microcirculation. Am J Physiol. 1989; 256(3 Pt 2): H 720-5.
15)Ohlstein EH, Vickery L, Sauermelch C, Willette RN. Vasodilation induced by endothelin: role of EDRF and prostanoids in rat hindquarters. Am J Physiol. 1990; 259 (6 Pt 2): H 1835-41.
16)Dietz NM, Rivera JM, Eggener SE, Fix RT, Warner DO, Joyner MJ. Nitric oxide contributes to the rise in forearm blood flow during mental stress in humans. J Physiol. 1994; 480 (Pt 2): 361-8.
17)Milner P, Kirkpatrick KA, Ralevic V, Toothill V, Pearson J, Burnstock G. Endothelial cells cultured from human umbilical vein release ATP, substance P and acetylcholine in response to increased flow. Proc Biol Sci. 1990; 241(1302): 245-8.
18)Martin CM, Beltran-Del-Rio A, Albrecht A, Lorenz RR, Joyner MJ. Local cholinergic mechanisms mediate nitric oxide-dependent flow-induced vasorelaxation in vitro. Am J Physiol. 1996; 270(2 Pt 2): H 442-6.
19)Williams TJ. Prostaglandin E 2, prostaglandin I 2 and the vascular changes of inflammation. Br J Pharmacol. 1979; 65(3): 517-24.
20)Gangarosa LP, Hubbard WC, Hung CT, Park NH. Relative vasodilator potency of ATP, ADP, AMP and adenosine (Ado) in the cat hind limb. Arch Int Pharmacodyn Ther. 1979; 238(1): 114-23.