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Pretreatment effects of moxibustion on the skin permeation and skin and muscle concentrations of salicylate in rats

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ABSTRACT

The effect of moxibustion on the *in vitro* and *in vivo* skin permeation of salicylate was evaluated in rats. First, the effect of moxibustion pretreatment on the elimination pharmacokinetics of salicylate after *i.v.* injection in rats was determined: no clear difference was observed in the plasma profiles of salicylate (SA) with or without moxibustion pretreatment. However, much higher skin and muscle concentrations of salicylate were observed after its *i.v.* injection. Next, an *in vitro* skin permeation study of SA was performed after moxibustion pretreatment. Moxibustion pretreatment increased the skin permeation of SA, and the extent of the increase in SA skin permeation was related to the strength of moxibustion ignition. More intense treatments produced higher skin permeation. A similar enhancement effect on the skin permeation of SA was observed in *in vivo* studies. Interestingly, the skin/plasma and muscle/plasma ratios of SA were markedly increased by moxibustion pretreatment. These results were due to the induction of enhanced skin permeation and lower clearance into the cutaneous vessels by moxibustion ignition. Combination treatment involving moxibustion and the topical application of drugs such as NSAID may be useful for increasing local pharmaceutical effects by enhancing the drug concentration in the skin and muscle underneath the topical application site.

1. Introduction

Moxibustion therapy affects the blood flow in cutaneous tissues (Tawa et al., 2005) and muscles (Takagi et al., 2007; Matsumoto et al., 2005) as well as the barrier function of the stratum corneum (Cao et al., 2007). It may also have an effect on the local vasodilatory response by increasing the temperature of the skin (Steif et al., 2007). When the body is in a warm environment, the veins and capillaries are more dilated than when it is in a cold environment, and blood flow is higher in warm than in cold environments (Peiffer et al., 2009). Drug permeability through the endothelial membranes of blood vessels and capillaries is also higher in warm than in cold environments (Schimmelb et al., 1964; Nozaki et al., 1980). In addition, the tight junctions in the endothelial membranes of blood vessels and capillaries are opened in inflamed conditions, and so drug molecules can easily permeate across the skin and reach the systemic blood circulation after their topical application to skin (Stewart et al., 1944; Arndt and Lipfert, 1993). The percutaneous absorption kinetics of topically applied drugs depend on the change in temperature induced by moxibustion treatment and the dose of drug applied.

Numerous studies have evaluated the concurrent use of moxibustion with other physical technologies, especially acupuncture (Anastasi and McMahon, 2003; Sugai et al., 2004; Shin et al., 2006). We have reported the change in the *in vitro* skin permeation efficacy of FITC-dextran with a mean molecular weight of 4 kDa (FD-4) induced by a single moxibustion technique (Cao et al., 2007). We also found that the permeation rate and extent of FD-4 permeation through the skin were dependent upon the application technique and type of moxibustion used in an *in vitro* skin permeation study. However, the enhancement effect of drug permeation through skin cannot be verified with only *in vitro* data. *In vivo* data is needed to prove the enhancement effect in addition to *in vitro* data. We have already reported, however, that the drug permeation through excised human skin could be fairly predicted from that through hairless rat skin (Morimoto et al., 1992; Watanabe et al., 2001). Moxibustion treatment might cause skin irritation so that animal experiment instead of human test could be performed to assure the safety.

In the present study, an *in vivo* hairless rat skin permeation study was performed in addition to an *in vitro* study. Salicylic acid (SA) and its sodium salt (SA–Na) were used as model drugs, since the simultaneous use of an NSAID (non-steroidal anti-inflammatory drug) and moxibustion may be useful for treating muscle pain. Skin permeation as well as the skin and muscle concentrations of salicylate was determined to evaluate the effect of moxibustion.

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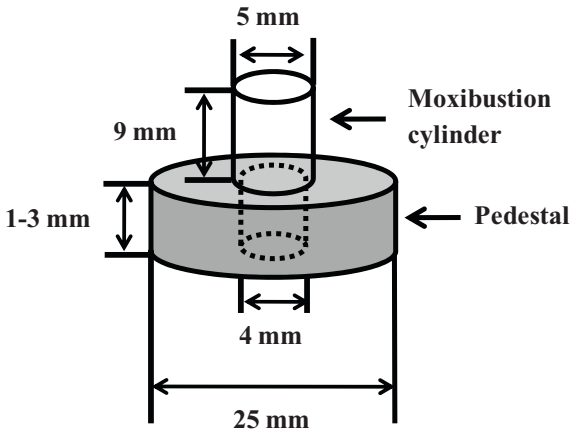

Fig. 1. Schematic view of the moxibustion cylinder and pedestal.

2. Methods

2.1. Materials and animals

SA–Na and Evan's blue dye were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and solvents were of reagent grade and used without further purification.

The SennenQ-off regular Q Ibuki was supplied by Senefa Co. (Nagahama, Shiga, Japan). This moxibustion device has a moxa cylinder (5 mm diameter × 9 mm length) and a pedestal. Only the cylinder was used in the present experiment, and custom pedestals (diameter: 25 mm, thickness: 1–3 mm, hole diameter: 4 mm; Fig. 1) were made for the experiment.

Healthy male hairless rats (WBN/ILA-Ht, body weight: 180–230 g) were purchased from the Life Science Research Center of Josai University (Sakado, Saitama, Japan). All animal feeding procedures and experiments were approved by the Institutional Animal Care and Use Committee of Josai University.

2.2. Measurement of skin temperature

One moxibustion cylinder was applied to the abdomen of each hairless rat (3 mm above the umbilicus on the midline) by fixing the animal on their back under anesthesia, which was induced by the *i.p.* injection of pentobarbital (40 mg/kg). The distance between the cylinder tip and skin surface was set at 1.0, 2.0, or 3.0 mm. The moxibustion cylinder was ignited at 1.0 min and taken off at 6.0 min after application. Thus, the firing period was 5.0 min. The skin surface temperature and subcutaneous tissue temperature were determined according to the method described in our previous paper (Cao et al., 2007).

2.3. In vitro skin permeation experiment

Moxibustion treatment (1 run for 5.0 min or 3 consecutive runs for 15 min) was performed on the abdominal skin of hairless rats (3 mm above the umbilicus along the midline), in the same manner as described above. The distance between the cylinder tip and skin surface was set at 1.0, 2.0, or 3.0 mm. The pretreatment area of the skin was carefully excised, and any underlying fat or muscle tissue was removed 30 min after the first ignition of the moxibustion cylinder. The excised skin was immediately sandwiched between two half-diffusion cells with an effective diffusion area of 0.95 cm^2 and a cell volume of 2.5 mL (Okumura et al., 1989). The stratum corneum side of the skin was treated with 2.5 mL of 3% SA–Na in pH 7.4 phosphate buffered saline (PBS), while the dermis side was treated with the same volume of PBS. Each compartment was magnetically stirred. Aliquots (500 μL) were withdrawn from the dermis side at predetermined time intervals, and fresh PBS was added after each sampling to keep the cell volume constant. The temperature of the whole set was regulated at 32 °C by warm water circulation. The SA concentration of the receiver solution was determined by HPLC.

2.4. In vivo experiments

2.4.1. Intravenous injection experiment

Moxibustion treatment (3 times consecutively for 15 min) was performed on the abdominal skin of hairless rats (3 mm above the umbilicus along the midline), under anesthesia induced by the *i.p.* injection of urethane (1.0 g/kg). The animals were kept on a waterbed kept at 37 °C during the *in vivo* experiment. SA–Na (5 mg/kg) was intravenously injected through the left jugular vein 30 min after the start of the first moxibustion session. Blood sampling was performed periodically from the right jugular vein. The obtained blood sample was centrifuged at 4 °C to obtain plasma. At the end of the experiment (8 h after the injection of SA–Na), the skin and muscle tissues (2.5 cm diameter under the moxibustion site) were excised, and the tissue samples were kept in a freezer until analysis. The control *i.v.* injection of SA–Na was performed without moxibustion. The SA concentrations in plasma and the tissues were determined by HPLC.

2.4.2. Skin application experiment

A glass diffusion cell with an effective diffusion area of 0.95 cm^2 was applied to the skin surface where the moxibustion treatment was carried out. SA–Na solution at a concentration of 20% (1.5 mL) was applied 30 min after the start of the first moxibustion session. Plasma, skin, and muscle were obtained in the same manner as in the *i.v.* injection experiment.

2.4.3. Appearance of i.v. injected Evan's blue

Moxibustion treatment (3 times consecutively for 15 min) was performed on the right abdominal skin of hairless rats, under urethane anesthesia. Then, Evan's blue (50 mg/kg) was intravenously injected, before pictures were taken of the moxibustion-application site as well as the control left abdomen site.

2.5. Salicylate determination methods

The SA concentration was determined by a validated HPLC method with UV detection. The receiver sample (100 μL) in the *in vitro* skin permeation experiments was added to acetonitrile (200 μL) containing an internal standard (propyl *p*-hydroxybenzoate) and centrifuged to obtain the supernatant. Acetonitrile (200 μL) containing the internal standard was added to the plasma sample (100 μL each), which was then centrifuged to obtain the supernatant. The minced skin and muscle samples were added to acetonitrile (5.0 mL) containing the internal standard and physiological saline (4.0 mL) and homogenized using a Polytron (PT3000, Kinematic AG, Switzerland) under iced water. The homogenate was centrifuged at 4 °C. Each supernatant (20 μL) was injected into an HPLC.

The HPLC system (Shimadzu, Kyoto, Japan) comprised a model SLC-10A system controller, an SPD-10A UV detector, an LC-10A pump, and an SIL-$10A_{XL}$ autosampler. The data were acquired and processed with a C-R6A chromatographic integrator (Shimadzu). Chromatographic separation was achieved

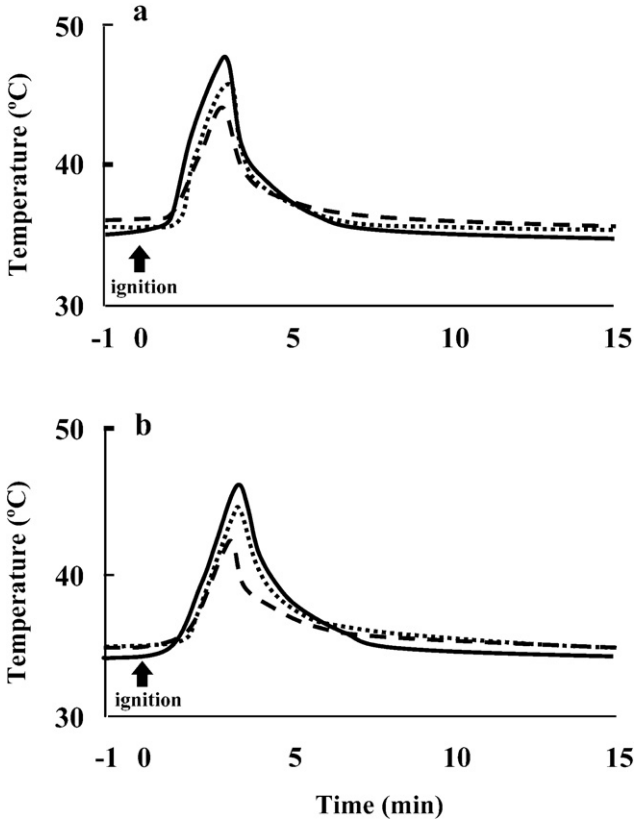

Fig. 2. Effect of moxibustion treatment on the temperature changes at the skin surface (a) and in the subcutis (b) ((—) 1 mm thickness pedestal, (···) 2 mm thickness pedestal, (– – –) 3 mm thickness pedestal). Each line represents the mean value of at least 3 experiments.

using LiChroCART® 250 mm × 4 mm (Merck KGaA, Darmstadt, Germany). The mobile phase was a mixture of methanol and 0.1% phosphate water (55:45). The flow rate of the mobile phase was 0.7 mL/min, and the UV wavelength was set at 225 nm.

3. Results

3.1. Effect of moxibustion on the skin temperature

Fig. 2a and b shows the time courses of the temperature changes at the skin surface and in the subcutaneous tissue, respectively, after one run of moxibustion. In the experiment, a moxibustion cone was applied to the abdomen for 5.0 min. The smoke had disappeared within 1.5 min, but the maximum temperature of the skin surface and subcutaneous tissue was observed at about 2.5 and 3 min after the start of moxibustion, respectively, when the moxibustion cone was still on the abdominal skin. The maximum temperatures at the skin surface and in the subcutaneous tissue produced by moxibustion with the 1-, 2-, and 3-mm pedestals were 47.9, 46.5 and 44.7 °C and 45.6, 43.7 and 42.4 °C, respectively. The highest temperature in the subcutis was lower than that at the skin surface. A short time lag was observed for the change in temperature from the skin surface to the subcutis. These temperatures had returned to the control level (without or before moxibustion) within 10–15 min after the start of the moxibustion. Since the skin permeation experiments were started 30 min after moxibustion ignition, the skin temperature during the permeation experiments was kept at 32 °C using warm water circulation.

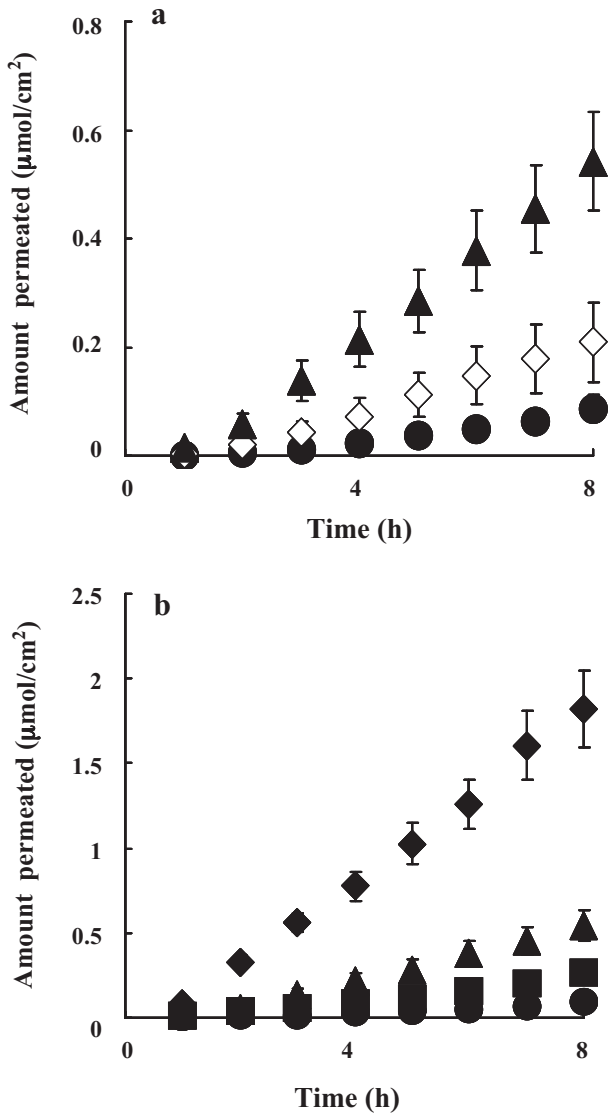

Fig. 3. Effect of the number of moxibustion cylinders (a) and the distance between the cylinder tip and skin surface (b) on the *in vitro* skin permeation of salicylate ((●) control, (◊) 1 run of moxibustion with a 2 mm pedestal, (♦) 3 consecutive runs of moxibustion with a 1 mm pedestal, (▲) 3 consecutive runs of moxibustion with a 2 mm pedestal, (■) 3 consecutive runs of moxibustion with a 3 mm pedestal). Each data point represents the mean ± S.E. (n = 5–7).

3.2. Effect of moxibustion on the in vitro skin permeation of salicylate

Fig. 3 shows the effects of various moxibustion pretreatments on the time course of the cumulative amount of SA that permeated through excised hairless rat skin. Table 1 summarizes the perme-

Table 1
Permeability coefficient of salicylate through excised hairless rat skin.

Type of treatment	Permeability coefficient (cm/s)
Without moxibustion	$(2.33 \pm 0.01) \times 10^{-8}$
1 run of moxibustion with a 2 mm pedestal	$(4.53 \pm 1.47) \times 10^{-8}$
3 consecutive runs of moxibustion with a 1 mm pedestal	$(3.71 \pm 0.01) \times 10^{-7}$
3 consecutive runs of moxibustion with a 2 mm pedestal	$(1.25 \pm 0.01) \times 10^{-7}$
3 consecutive runs of moxibustion with a 3 mm pedestal	$(7.56 \pm 0.01) \times 10^{-8}$

Each value represents the mean ± S.E., n = 5–7.

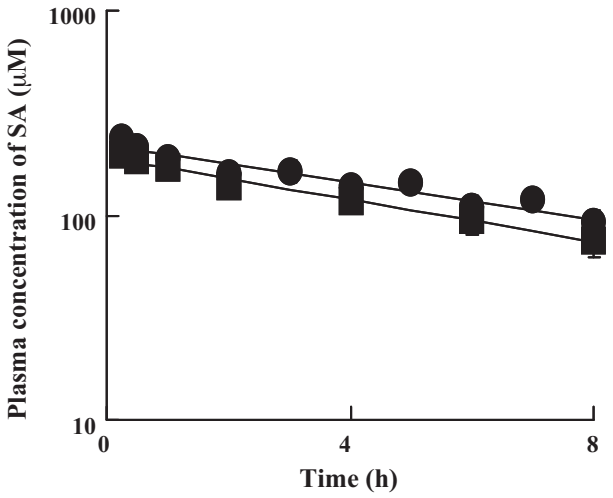

Fig. 4. Effect of moxibustion treatment on the *in vivo* plasma concentration of salicylate following *i.v.* injection ((●) control, (■) 3 consecutive runs of moxibustion with a 1 mm pedestal). Each point represents the mean ± S.E. (n = 3–4).

ability coefficients of SA under each moxibustion treatment (the permeability coefficient can be obtained by dividing the steady-state flux by the SA concentration in the donor solution). The cumulative amount of SA over 8 h was 2.4-, 3.1-, 6.2-, and 21-fold higher than that observed in the control experiment (without moxibustion) during one run of moxibustion with 2-mm pedestal and three consecutive runs of moxibustion pretreatments with 3-, 2-, and 1-mm pedestal, respectively. Increasing the number of moxibustion treatments and decreasing the pedestal thickness markedly increased the skin permeation of SA, which was also the case in *in vitro* studies using FITC-dextran (Cao et al., 2007).

3.3. Effect of moxibustion on the elimination kinetics of salicylate

Before evaluating the effects of moxibustion on the *in vivo* skin permeation of SA, SA–Na (5 mg/kg) was intravenously injected into rats, in order to evaluate the effect of moxibustion on the elimination kinetics of and the skin and muscle disposition to topically applied SA. Fig. 4 shows the time course of the plasma concentration of SA after *i.v.* injection, and Table 2 summarizes the pharmacokinetic parameters obtained with and without moxibustion. Although the saturation kinetics for SA have been reported (Needs and Brooks, 1985), first-order elimination kinetics were observed in the present experiment. No significant difference was

Table 2
Elimination pharmacokinetic parameters of salicylate.

Treatment	k_{el} (h^{-1})	V_d (mL)
Without moxibustion	$(1.83 \pm 0.18) \times 10^{-3}$	31.5 ± 2.0
3 consecutive runs of moxibustion using a 1 mm pedestal	$(2.26 \pm 0.17) \times 10^{-3}$	29.7 ± 2.1

k_{el}, first order elimination rate constant; V_d, volume of distribution.
Each value represents the mean ± S.E. (n = 3).

Table 3
Salicylate amounts in the skin and muscle 4 h after *i.v.* injection.

Treatment	Skin (nmol)	Muscle (nmol)
Without moxibustion	5.42 ± 0.84	8. 68 ± 2.08
3 consecutive runs of moxibustion with a 1 mm pedestal	15.7 ± 5.5*	17.1 ± 1.7*

Each value represents the mean ± S.E. (n = 3).
* $p < 0.05$ Mann–Whitney-U test, compared with the control.

observed in the elimination pharmacokinetics of SA with or without moxibustion, suggesting that moxibustion did not greatly affect the elimination kinetics of SA from the systemic circulation.

Table 3 and Fig. 5 show the amounts of SA in the skin and muscle under the moxibustion site and the skin/plasma and muscle/plasma ratios (s/p and m/p ratio) of SA 4 h after injection, respectively. The plasma amount of SA was calculated from its plasma concentration and volume of distribution. Since a similar plasma concentration range was observed, the same volume of distribution was used for all calculations, in spite of the dose dependent kinetics of SA (Needs and Brooks, 1985). The s/p and m/p ratios under moxibustion were about 4- and 2-fold higher, respectively, compared with those without moxibustion. These results suggest that moxibustion treatment increased the skin and muscle concentrations under the site of moxibustion as well as the skin permeation of the drug. In addition, the effect of moxibustion on the s/p ratio was more marked than that on the m/p ratio.

3.4. Effect of moxibustion on the plasma level (in vivo skin permeation) of salicylate

Fig. 6 shows the time course of the plasma concentration of SA after its topical application. Three-consecutive moxibustion pretreatments using a 1.0 mm pedestal were applied. The plasma concentration increased almost linearly with the passage of time both with and without moxibustion pretreatment. The plasma con-

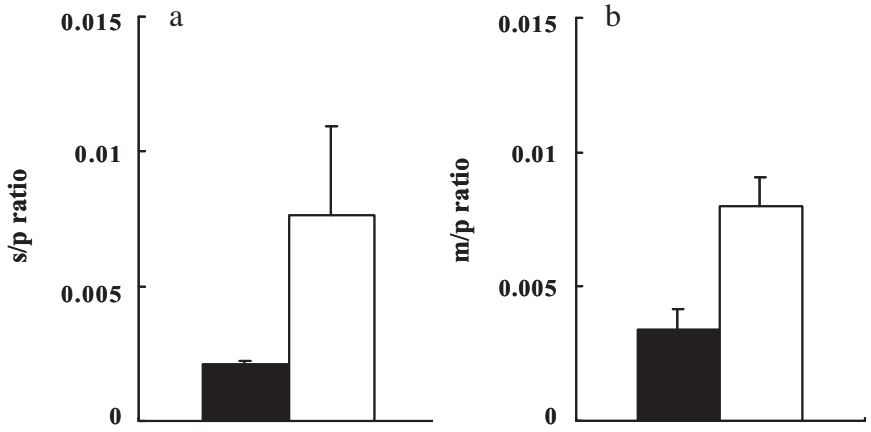

Fig. 5. Effects of moxibustion treatment on the ratio of salicylate in the skin (a) and muscle (b) to the plasma concentration 4 h after *i.v.* injection ((■) control, (□) 3 consecutive runs of moxibustion with a 1 mm pedestal). Each column represents the mean ± S.E. (n = 3).

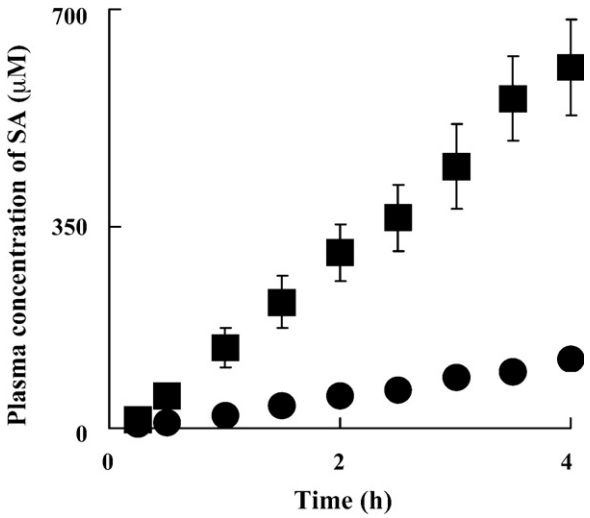

Fig. 6. Effect of moxibustion treatment on the *in vivo* plasma concentration of salicylate following topical application ((●) control, (■) 3 consecutive runs of moxibustion with a 1 mm pedestal). Each point represents the mean ± S.E. ($n = 3$).

centration induced by moxibustion was about 5 times higher than that induced without moxibustion. However, the enhancement of the *in vivo* skin permeation of SA (about 5 times) induced by moxibustion was lower than that the effect seen in the *in vitro* skin permeation experiment (about 21 times, see Fig. 3).

Fig. 7a and b shows the effect of moxibustion on the s/p and m/p ratios of SA, respectively, 4 h after the start of its topical application. Three-consecutive moxibustion pretreatments involving 1.0 mm pedestals were also performed in this study. The s/p and m/p ratios produced by moxibustion were about 3 and 15 times higher than those produced without moxibustion, respectively. Since the plasma concentration itself was increased by moxibustion (see Fig. 6), the skin and muscle concentrations produced by moxibustion were calculated to be about 13 and 81 times higher than those produced without moxibustion (Table 4).

3.5. Effect of moxibustion on the tissue distribution of i.v. injected Evan's blue

The effect of moxibustion treatment was evaluated on the leaching of Evan's blue into the cutaneous and subcutaneous tissues from the systemic circulation in rats. Fig. 8 shows the concentrated Evan's blue dots that formed in the cutaneous and subcutaneous

Table 4
Salicylate amounts in the skin and muscle 4 h after skin application.

Treatment	Skin (nmol)	Muscle (nmol)
Without moxibustion	1.61 ± 0.44	0.06 ± 0.01
3 consecutive runs of moxibustion with a 1 mm pedestal	21.1 ± 1.8*	4.89 ± 0.59*

Each value represents the mean ± S.E. ($n = 3$).
* $p < 0.05$ Mann–Whitney-*U* test, compared with the control.

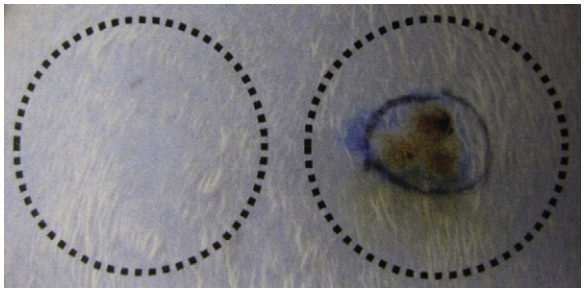

Fig. 8. Effect of moxibustion treatment on the tissue distribution of Evan's blue 25 min after *i.v.* injection. Photograph of the hairless rat abdomen after the intravenous injection of Evan's blue.

tissues 25 min after moxibustion treatment and the *i.v.* injection of Evan's blue. The skin color became blue at the site of moxibustion, suggesting that Evan's blue was easily delivered to the site of moxibustion after intravenous injection. The yellow color in Fig. 8 was due to the moxa resin.

4. Discussion

Although the moxibustion cylinder used in the present experiment was a commercially available model, the moxibustion treatment protocol used in the present study was a little more intense than the treatment protocol that is generally used in humans. For example, the distance between the cylinder tip and skin surface and the thickness of the pedestal are 3 mm or more in the protocols used for general treatment. Therefore, the temperatures of the skin surface and the subcutis found in the present study must be higher than those produced during standard treatment. High temperatures may

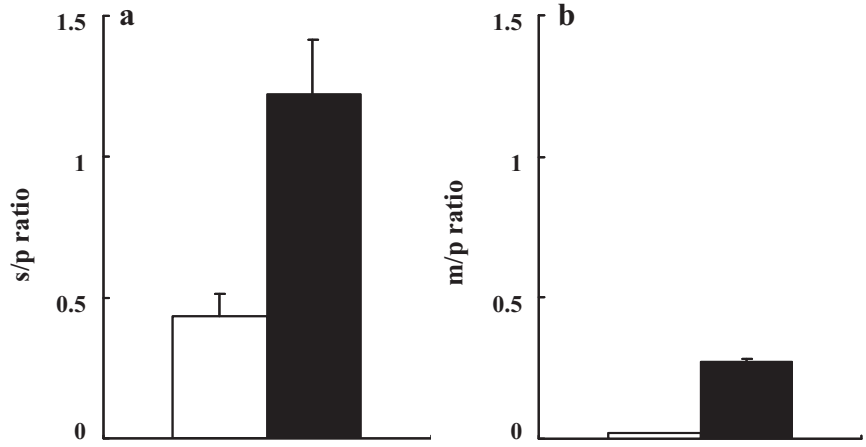

Fig. 7. Effects of moxibustion treatment on the ratio of salicylate in the skin (a) and muscle (b) to the plasma concentration 4 h after topical application ((□) control, (■) 3 consecutive runs of moxibustion with a 1 mm pedestal). Each column represents the mean ± S.E. ($n = 3$).

cause severe inflammation of the skin and subcutaneous tissues (Fig. 2).

SA was used as a model drug in the present study. Thermodynamic energy affects skin permeation of drug. The enhancement of drug permeation through skin after moxibustion treatment, however, was not obtained due to high skin temperature, because the skin temperature showed normal when drug was applied to the skin surface. In addition, the effect of moxibustion on the *in vitro* skin permeability of SA was compared with that of FITC-dextran 4 kDa, which was reported in our previous study (Cao et al., 2007). The enhancement ratio of FITC-dextran permeability; i.e., the permeability of FITC-dextran with/without moxibustion, was higher than that for SA. This was due to the low permeability of the skin to larger compounds in the absence of moxibustion.

Intense moxibustion may cause inflammation at the treatment site. Igarashi et al. (2001) reported that intravenously injected lipid microspheres containing an anti-rheumatoid drug easily leaked from the epithelial membranes into inflammatory foci and that these phenomena were closely related to the high targeting ability of the anti-rheumatoid drug. The inflammation probably expanded into the intercellular region or the tight junctions of cutaneous vessels so drugs in the systemic circulation may easily leach into inflammatory tissues (Tables 3 and 4). This may be the reason for the higher effect of moxibustion on the *in vitro* skin permeation of SA compared with the *in vivo* skin permeation of SA (Figs. 3 and 6). In addition, the *in vivo* skin permeation of SA may inhibit the recovery from inflammation at the site of moxibustion.

An *i.v.* SA injection experiment was performed to determine the volume of distribution of SA and evaluate the effect of moxibustion on the skin and muscle concentrations of SA. The obtained volume of distribution was used to determine the s/p and m/p ratios of SA during moxibustion treatment. These s/p and m/p ratios of SA clearly show the effects of moxibustion on the typical distribution of *i.v.* injected or topically applied SA (Figs. 5 and 7). Some of the components of moxa, such as 1,8-cineole, may be related to the penetration enhancing effect of moxibustion pretreatment. However, increases in the skin and muscle concentrations of SA at the site of moxibustion were observed, even after *i.v.* injection. These phenomena are related to the effects of moxibustion on epithelial membranes in the viable epidermis/dermis and muscle. The present results; i.e., the leakage of Evan's blue from cutaneous and muscle blood capillaries, are similar to the phenomena described by Igarashi et al. (2001) (Fig. 8). In addition, increased drug leakage from cutaneous and muscle blood vessels must be one of the primary reasons for the increases in skin and muscle concentrations of SA induced by moxibustion.

5. Conclusion

The *in vivo* and *in vitro* skin permeation of SA was enhanced by moxibustion pretreatment. Moxibustion pretreatment also increased the skin and muscle concentrations of SA. Moxibustion can be utilized as a method for increasing the skin and muscle concentrations as well as the skin permeation of many kinds of topically applied drugs. After that, we plan to evaluate and find out a safety condition after different for moxibustion treatments in human.

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