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Acupuncture in a rat model of asthma

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TITLE:

Acupuncture in a Rat Model of Asthma

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KEYWORDS:

Acupuncture, Asthma, High platform, Rat fixation, Whole-body plethysmography

ABSTRACT:

A high platform can fix rats without restriction and completely expose the acupoints on the back during acupuncture manipulation. This article describes methods for the fabrication of the high platform, establishes a rat model of asthma and measures changes in respiratory function using a noninvasive and real-time whole-body plethysmography (WBP) system.

INTRODUCTION:

Asthma is a chronic disease characterized by bronchoconstriction and airway hyper-responsiveness[1]. Approximately 339.4 million people worldwide are diagnosed with asthma[2]. The prevalence of asthma is increasing globally, with 100 million new cases expected in the next decade[3]. Inhaled corticosteroids and long-acting β_2-agonists do not effectively control the symptoms of all patients[4]. Therefore, the use of alternative therapies such as acupuncture to treat asthma has been receiving increased attention[5]. An effective method for treating asthma is to needle GV14 (Dazhui), bilateral BL12 (Fengmen) and bilateral BL13 (Feishu)[6]. Acupuncture significantly reduces the levels of secretory immunoglobulin A (SIgA) in the saliva

and nasal secretions of patients with allergic asthma and reduces peripheral blood eosinophil counts with a total effective rate of 85%[6]. Acupuncture at BL13 and ST 36 (Zusanli) in mice with chronic asthma decreases the level of IL-17 by 40% and decreases the smooth muscle thickness by 33% compared with that of the ovalbumin (OVA) group, and thus relieving inflammation symptoms[7,8]. However, the mechanism of acupuncture treatment for asthma is not completely clear.

Animal models are an important tool for asthma research[9] because the animal model can be used for the continuous measurement of respiratory function to assess the effectiveness of the asthma treatment[10]. Meanwhile, studies of the pathophysiology of asthma require test samples, including samples from the trachea, lung and bronchoalveolar lavage to verify changes in the levels of key factors[11]. Rat models of asthma are commonly used to assess asthma pathophysiology because they produce long-lasting airway responses and show immediate and late-phase airway responses[12]. However, due to repeated experimental stimulation, the rats often become irritable. Therefore, a suitable method to fix rats is needed. Commonly used methods for rat fixation include anesthesia and binding[13]. Although the anesthesia method provides better exposure of the acupoints, it may affect nerve conduction and ultimately affect the experimental results[14]. The binding method does not induce the same physiological effects as anesthesia, but restrained rats will experience skeletal muscle tension. Also, the needle cannot easily reach the designated position[15]. Here, we introduce a high platform that can be used to fix rats without restricting them and fully expose the acupoints on the back. The rats can be placed on the high platform during the administration of acupuncture. The high platform is able to accommodate the rat only while it is standing, and as it is placed a certain distance from the ground, the rats will not move because of a fear of heights[16].

This article describes in detail the fabrication of the high platform, the establishment of a rat model of asthma, the operation of needling a rat on the high platform and the measurement of respiratory function using a whole-body plethysmography (WBP) system.

PROTOCOL:

All animal experiments were reviewed and approved by the Committee on the Ethics of Animal Experiments of Shanghai University of Traditional Chinese Medicine (Ethical clearance number PZSHUTCM190308020).

1. Fabrication of the high platform

1.1. Set the length and width of the supporting device to 86 cm x 17 cm (**Figure 1**).

1.2. Fix the connecting device vertically to the supporting device. The connecting device should be approximately 30 cm long to prevent the rat from running to the top surface of the high platform and have a width of 14 cm.

1.3. Fix the horizontal pedal (5 cm x 5 cm) to the connecting device. The suspended height at the bottom of the horizontal pedal should be greater than or equal to 50 cm.

NOTE: Depending on the number of experimental rats, multiple horizontal pedals can be used, with one rat placed on each pedal.

2. Establishment of a rat model of asthma

2.1. Sensitizing the rats with OVA

2.1.1. Prepare the sensitization solution on day 0. Prepare each 1 mL of the sensitization solution with 1 mg of OVA and 10 mg of aluminum hydroxide, with normal saline as the solvent.

2.1.2. Intraperitoneally inject each rat with 1 mL of the sensitization solution on day 0. Draw the sensitizing solution with a suitable syringe for later use.

2.1.3. Grab the tail and lift up the rat, place it on a rough surface, and gently pull the tail back. Grasp the skin of both ears and neck with the left thumb and index finger while tightly holding the back skin with the remaining three fingers and palms (be careful to refrain from applying excess force that will suffocate the rat).

2.1.4. With the right hand, perform an intraperitoneal injection in the rat with the prepared syringe with 1 mL of the sensitization solution.

2.2. Challenging rats with OVA

2.2.1. Select a box with a lid (the length, width and height are 39 cm x 28 cm x 23 cm), and drill a small hole in the side. Connect the sealed box, nebulizing cup and the compression nebulizer with a rubber tube (**Supplementary Figure 1**).

NOTE: The nebulized particle size of the compressed nebulizer is 1 - 5 μm, the nebulization rate is greater than 0.2 mL/min, and the ultimate pressure is 205 KPa.

2.2.2. On the 14th day after sensitization, open the lid of the homemade sealed box and place the rat in the box (the box can accommodate 5 rats), and then put the sealed box in a biosafety cabinet.

2.2.3. Prepare the challenge solution. Thoroughly mix 300 mg of OVA with 30 mL of normal saline.

2.2.4. Turn on the ultrasonic nebulizer and add 30 mL of the challenge solution into the nebulizing cup. Adjust the dosage of challenge solution and the number of rats according to the volume of the homemade sealed box.

2.2.5. Repeat nebulization several times (once a day) or adjust the procedure according to the severity of asthma symptoms.

3. Acupuncture treatment

3.1. Select acupoints, including GV14, bilateral BL12 and bilateral BL13, according to the theory of traditional Chinese medicine for treating asthma (**Supplementary Figure 2**). Administer manual acupuncture on day 2 and perform once every other day for a total of 7 session (**Figure 2**)**.**

3.1.1. Acupuncture the GV14 located between the seventh cervical vertebra and the first thoracic vertebra (the hollow of the scapula), on the midline of the back (**Figure 3**).

3.1.2. Acupuncture the BL13 located below the third thoracic vertebra, on both sides of the intercostal spaces (lower edge of the scapula).

3.1.3. Acupuncture the BL12 located below the second thoracic vertebra, on both sides of the intercostal spaces (below the scapula, position identified according to BL13).

3.2. Grasp the rats and place them on the high platform.

3.3. Hold the disposable stainless-steel needle (0.3 mm c 13.0 mm, **Supplementary Figure 3**) handle with the thumb and index finger of the right hand, and press the back of the rat with the palm of the right hand.

3.4. Touch the depression in the middle of the upper edge (GV14) or the lower edge of the scapula (BL12 and BL13), and then gently prick the skin of the rat with the needle tip to locate the acupoints.

3.5. Quickly insert the needle after determining the specific location of the acupoint and rapidly remove the right hand.

3.6. Evenly twisted the needles approximately 360° at a rate of 60 times/min for 10 - 20 s, manipulate them every 5 min, and then withdraw them after 20 - 30 min (**Supplementary Figure 4**).

4. The Penh measurement

4.1. Prepare methacholine: Place 100 mg of methacholine into a 5 mL centrifuge tube and add PBS to a volume of 2 mL (50 mg/mL methacholine). Then, dilute the 50 mg/mL methacholine solution to 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL and 3.125 mg/mL.

4.2. Instrument inspection

4.2.1. Turn on the signal amplifier (the aerosol distribution system and the rodent bias flow supply system are all turned off, and the nebulization connector is closed).

4.2.2. Check the channels: Click **Calibrate**, return the coarse adjustment baseline to 0 and switch to the direct current (DC) position. Fine-tune the baseline to 0 and inject 5 mL of gas with a syringe. Then, switch to the alternating current (AC) position.

4.2.3. After checking the channels, ensure that the error value is less than 0.5%.

4.3. Setting parameters

4.3.1. Turn on the aerosol distribution system and the rodent bias flow supply system. Adjust the flow rate to 4.5 mL according to the liquid level on the mercury ball.

4.3.2. Click **Create Study Options** and select **Dose Response Study**, select **Rat** of species and click **Next**.

4.3.3. Select **Simple Dose List**, set the **Number of Doses** to **6** and click **Next**.

4.3.4. Select the **Penh** and **Area** parameters: Set the animal adaptation time to 5 min, the nebulization time to 2 min, the response time to 3 min, and the recovery time to 2 min.

4.3.5. Sequentially add the following solutions to the nebulizer (200 µL per rat): methacholine (0 mg/mL, 3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL and 50 mg/mL). Real-time changes in Penh can be observed (**Figure 4**).

4.3.6. After all 6 solutions with different concentrations have been reacted, click **File** and **End Session** to complete the experiment.

REPRESENTATIVE RESULTS:

With acupuncture at the GV14, bilateral BL12 and bilateral BL13 points, the rat maintains a prone position. The rat can twist its head only to the outside and not the inside because one direction is blocked with a wooden board (**Figure 5**).

As the methacholine concentration increased, Penh shows an increasing trend and the growth rate gradually increases. At concentrations of 3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL and 50 mg/mL, Penh increased by 18.40% ± 10.70%, 27.55% ± 26.30%, 88.10% ± 45.63%, 427.60% ± 172.92% and 882.27% ± 121.97%, respectively, showing an effect that increases with increasing dose (**Figure 6**).

Figure 1. Fabrication of the high platform. The high platform consists of a supporting device, three connecting devices and three pedals. The length and width of the supporting device are 86 cm x 17 cm, the length and width of the connecting device are 30 cm x 14 cm, the length and width of the pedal are 5 cm x 5 cm. Each connecting device is separated by 20 cm.

Figure 2. Protocol for establishing a rat model of asthma. Sensitization is performed on day 0, and acupuncture is performed every other day on the high platform from days 2 to 14. After challenge, the change in Penh is measured.

Figure 3. Anatomical positions of GV14 (Dazhui), BL12 (Fengmen), and BL13 (Feishu) on the rats. (**A**) Anatomical dissection of the back of a rat showing the scapula, first rib, second rib, third rib, and second thoracic vertebra (T2). (**B**) The sagittal plane of the ribs and thoracic vertebra. (**C**) The cross section of the scapula. (**D**) GV14: Located in the hollow of the scapula, midline of the back; BL13: Located at the lower edge of the scapula; BL12: Located below the scapula, positioned according to BL13.

Figure 4. Real-time changes in respiratory function. The blue curve shows real-time changes in Penh. The green curve shows the total change in the tidal volume (TVb) over a certain period. The purple curve shows the total change in Penh over a certain period.

Figure 5. Acupuncture of GV14 (Dazhui), BL12 (Fengmen), and BL13 (Feishu) in rats on the high platform. (**A**) Top view of the positions of GV14, BL12 and BL13 in rats. (**B**) Lateral view of an acupunctured rat on a high platform. The rat is placed on the platform and maintained in the prone position during acupuncture.

Figure 6. Measurement of Penh. WBP is used to measure the Penh of the normal rat. As the concentration of methacholine increases, the Penh also gradually increases. The data are presented as the mean ± SEM (n = 3).

Supplementary Figure 1. The nebulizer specifications. The nebulized particle size of the compressed nebulizer is 1 - 5 μm, the nebulization rate is greater than 0.2 mL/min, and the ultimate pressure is 205 kPa.

Supplementary Figure 2. The schematic positions of GV14 (Dazhui), BL12 (Fengmen), and BL13 (Feishu) in rat.

Supplementary Figure 3. Needle specifications. The length of the needle is 13 mm and the diameter is 0.3 mm.

Supplementary Figure 4. The schematic of acupuncture manipulation. After inserting the needle, evenly twisted the needles approximately 360° in different directions.

DISCUSSION

The GV14, bilateral BL12 and bilateral BL13 acupoints used in this study are located on the back, and thus the high platform is suitable for fixing rats. In other studies, an adequate fixation method should be selected according to the position of the acupoints[15]. For acupoints on the abdomen, the rat can be placed downward in a black cone (similar to a pastry bag used by bakers)[17]. A chain is attached to the restraint cone for fixation, and the hind leg of the rat

is also fixed. In this way, the front of the animal's body is firmly fixed in the cone, while the abdomen and the back are exposed. For acupoints on the leg, the rat can be placed vertically in a special transparent bucket, leaving the tail and hind legs naturally outside the bucket[18]. The transparent barrel covers the body of the rat, but it is convenient for acupuncture on the leg.

Several critical steps should be considered when performing these methods. For acupuncture manipulation, the harder the palm of the right hand presses the rat back, the easier it is to insert the needle. For respiratory function measurement, the WBP system measures changes in the Penh that reflect changes in pulmonary resistance of rats in a noninvasive manner and minimizes effects of psychological stress[19,20]. The effect of the external environment on rats should be minimized. The rat should be placed in the testing room and then covered with a plastic bag to reduce the irritation caused by the external environment.

The limitation of this experiment is that the needle may fall off if the rat swings about during the acupuncture. Therefore, needling must be manipulated every 5 min to adjust the depth. In addition, the high platform is suitable for rats, but not for smaller animals, such as mice.

In summary, we provide a detailed description of method for the fabrication of a high platform for fixing rats without restriction during acupuncture. The high platform can completely expose the acupoints on the back of the rat. The use of the high platform and noninvasive respiratory function measurements may facilitate the analysis of acupuncture as a treatment for asthma. Studying the therapeutic mechanism of acupuncture on the back may bring new treatments to respiratory diseases such as asthma and chronic obstructive pulmonary disease.

DISCLOSURES

The authors have nothing to disclose.

ACKNOWLEDGMENTS

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Figure 1

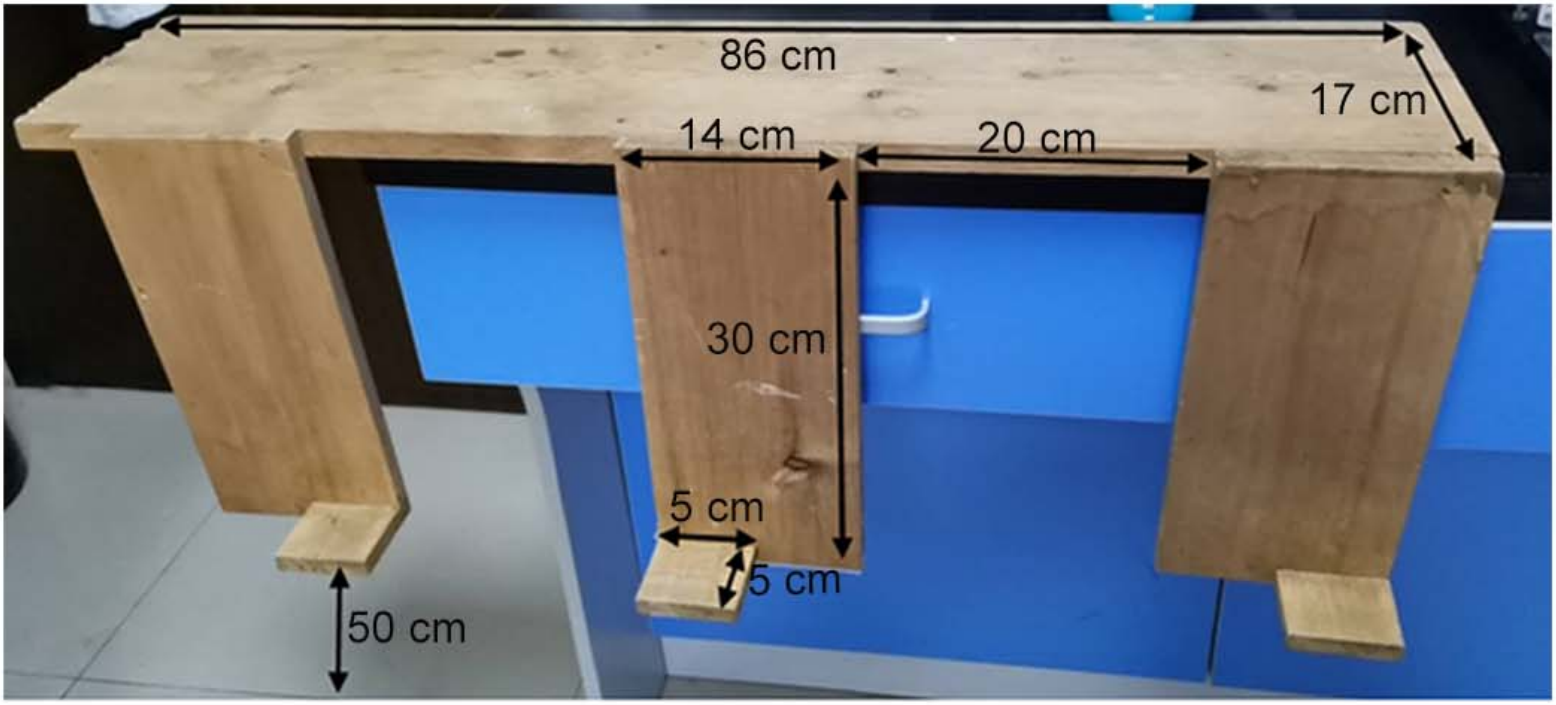

Figure 2

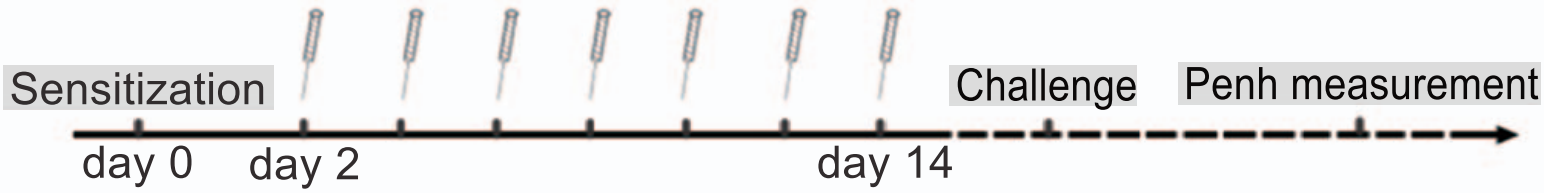

Figure 3

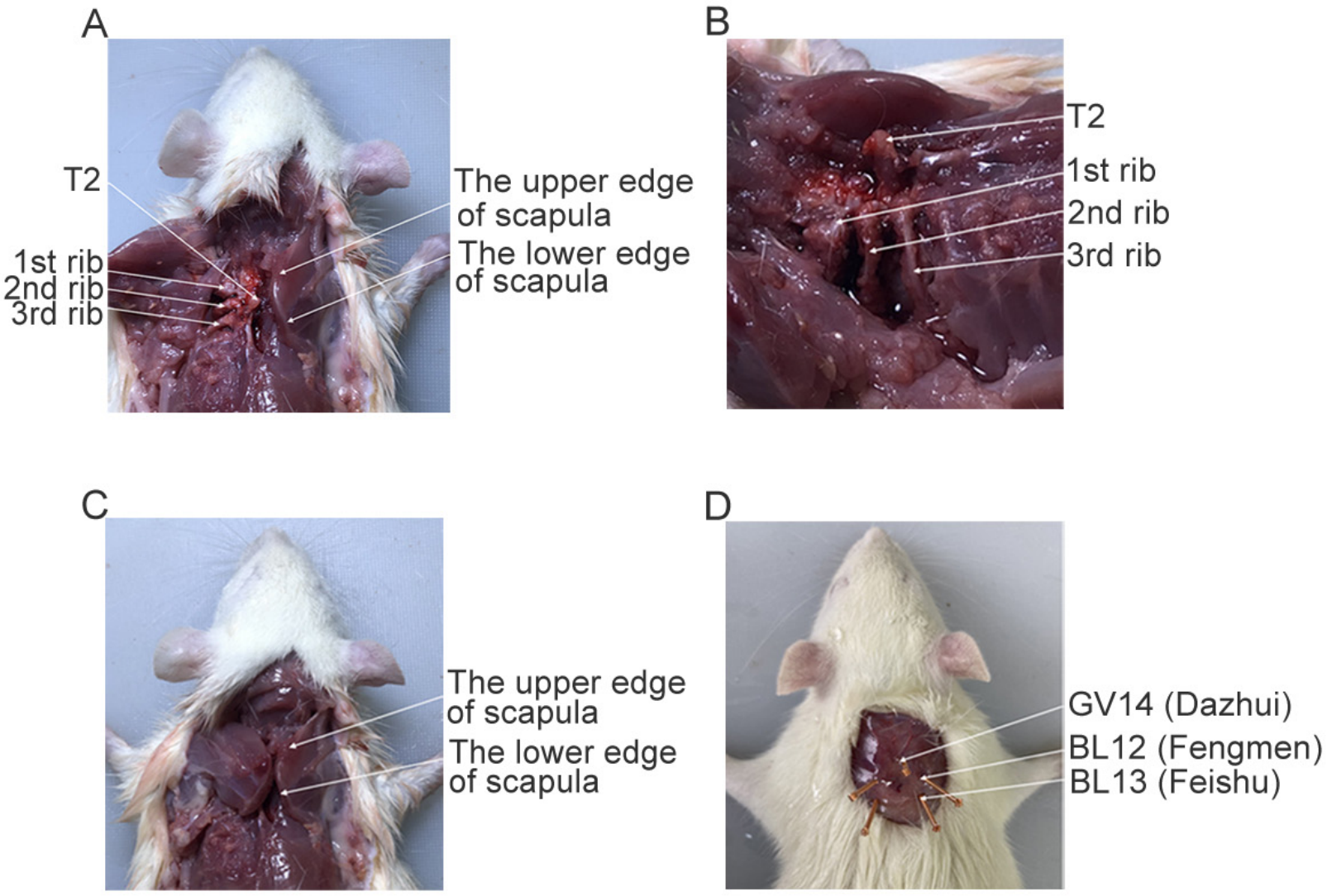

Figure 4

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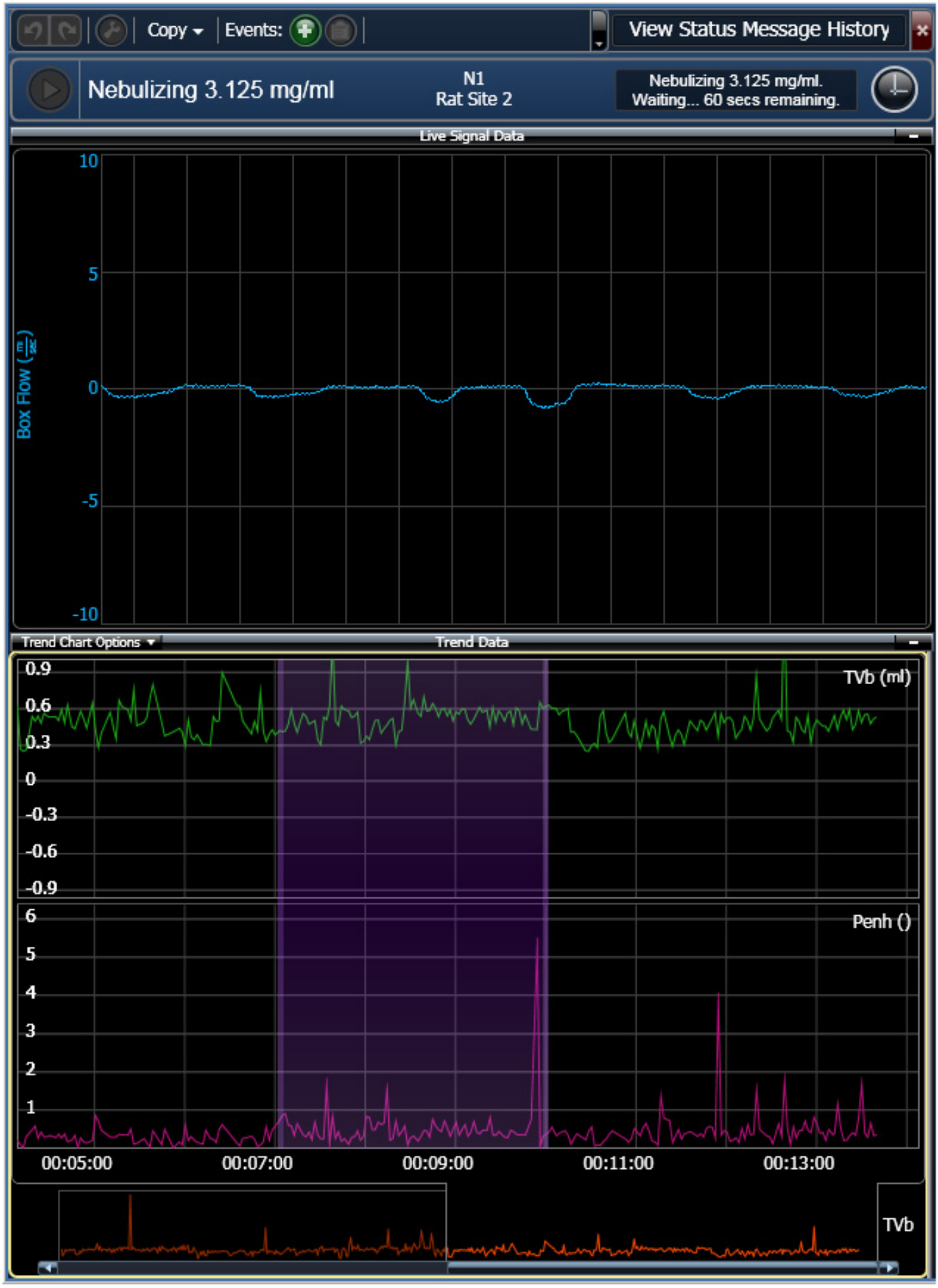


Figure 5

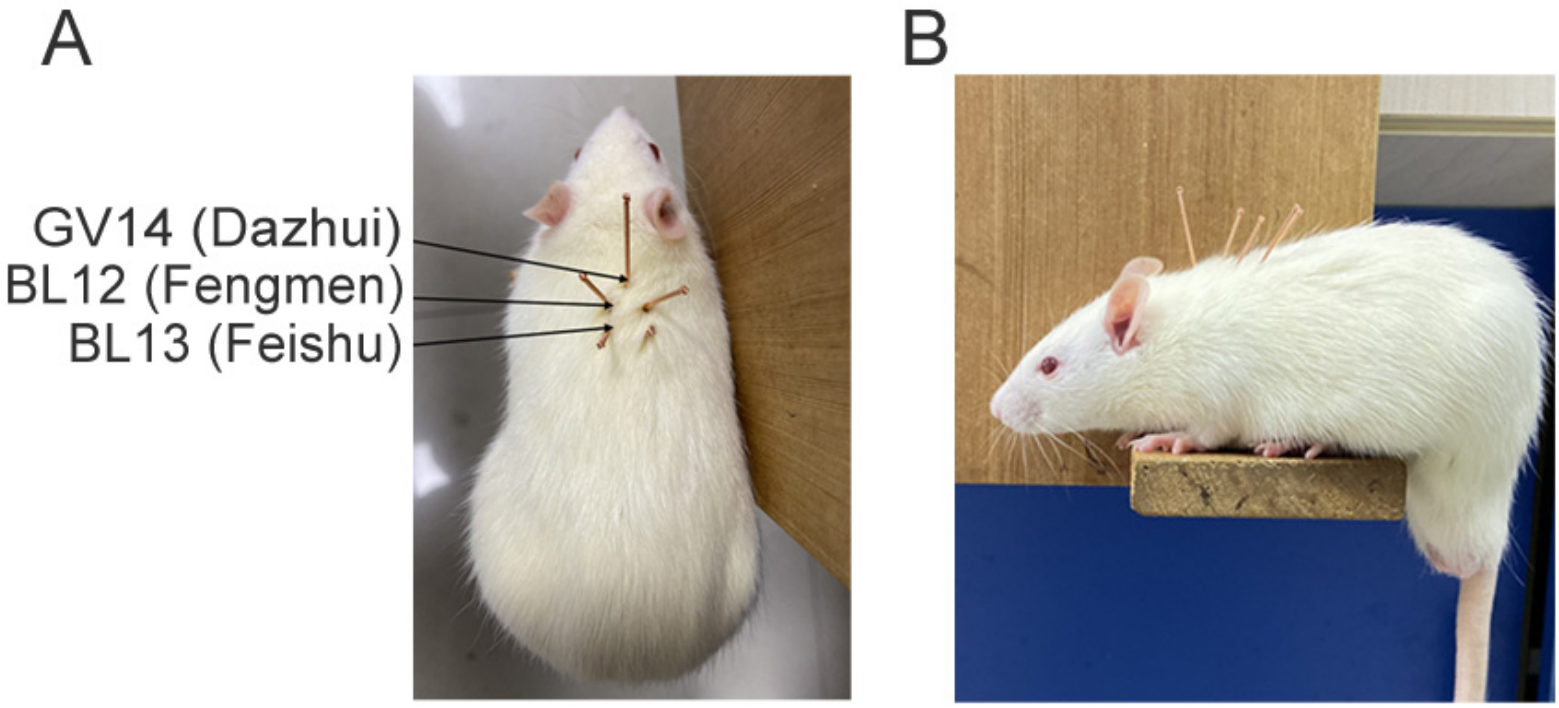
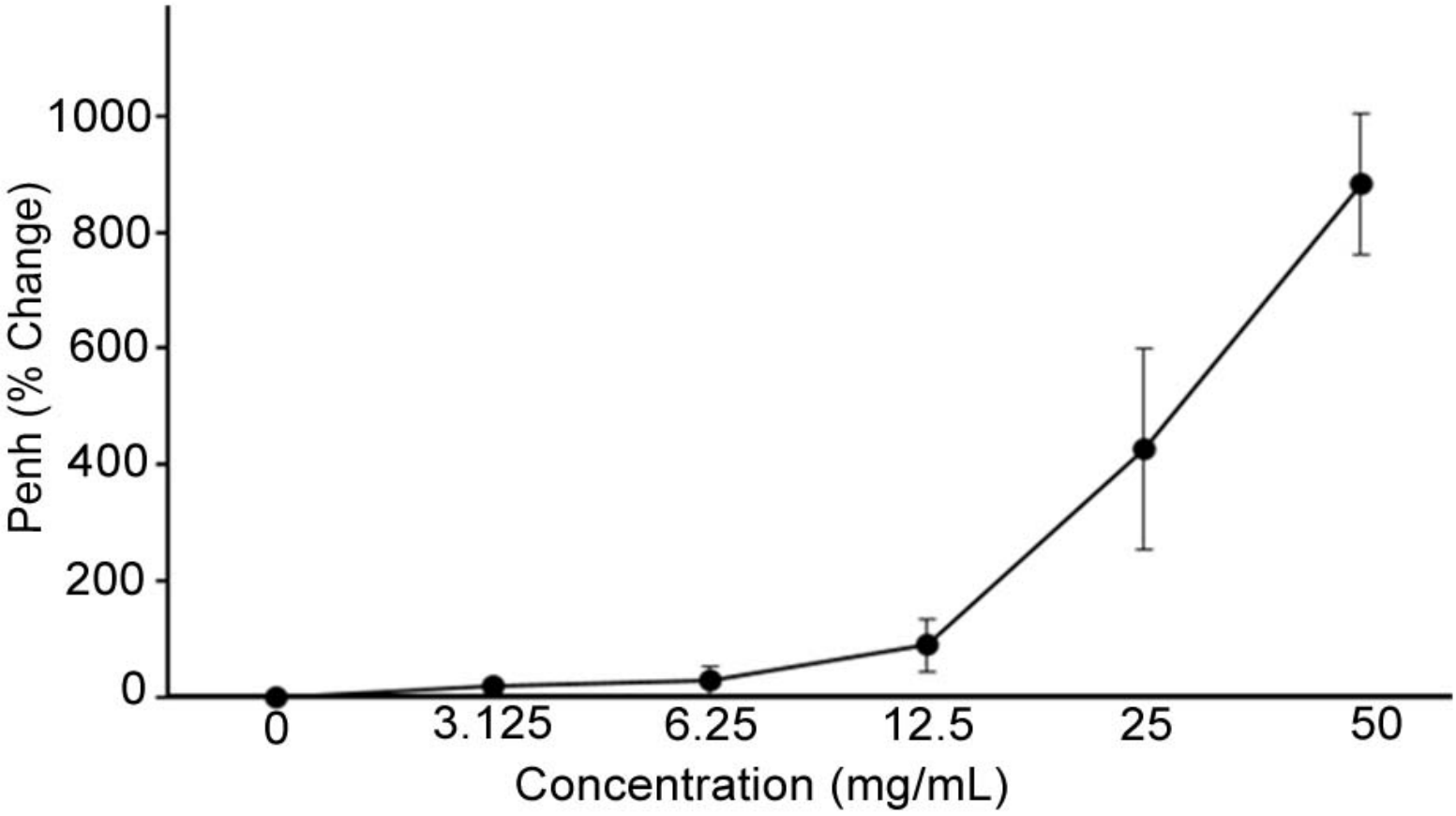


Figure 6



Name of Material/Equipment	Company	Catalog Number
0.3 mm x 13.0 mm acupuncture needle	China Suzhou Medical Device Factory	
$Al(OH)_3$	American Thermo	77161
Compression nebulizer	Jiangsu Lude Medical Electronics Co., Ltd.	NB-212C
Fine Pointe V2.0	American Buxco	
Laboratory gas drying unit	American Drierite	26800
Methacholine	American Sigma	MKCF6054
Ovalbumin	American Sigma	A5503-25G
Phosphate Buffer solution (1x)	Gelifesciences	SH30256.01
Seal box	IRIS Corporation of Japan	
Whole-body plethysmography respiratory function measurement system	American Buxco	

Editorial Comments:

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Reply: Thank you for your suggestion. The manuscript has been re-revised by native speakers of American Journal Experts to avoid any spelling or grammatical errors (The verification page is https://secure.aje.com/certificate/verify and the verification code is 1E76-DA24-1D96-2A45-B473).

Abstracts: Please provide a summary/short abstract that states the goal of the work in 50 words.

Reply: Abstract has been adjusted within 50 words and now read: A high platform can fix rats without restriction and completely expose the acupoints on the back during acupuncture manipulation. This article describes methods for the fabrication of the high platform, establishes a rat model of asthma and measures changes in respiratory function using a noninvasive and real-time whole-body plethysmography (WBP) system.

Protocol Language: Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

1) Some examples NOT in the imperative: 1.1-1.5, 2.1.1-2.1.2, etc.

Reply: These sentences have been modified in the imperative tense.

1) The section of 1.1 now read:

   Set the length and width of the supporting device to 86 cm × 17 cm (Figure 1).

2) The section of 1.2 now read:

   Fix the connecting device vertically to the supporting device. The connecting device should be approximately 30 cm long to prevent the rat from running to the top surface of the high platform and have a width of 14 cm.

3) The section of 1.3 now read:

   Fix the horizontal pedal (5 cm × 5 cm) to the connecting device, and the suspended height at the bottom of the horizontal pedal should be greater than or equal to 50 cm.

4) The section of 1.4 now read:

   Note: Depending on the number of experimental rats, multiple horizontal pedals can be used, with one rat placed on each pedal.

5) The section of 2.2.1 now read:

   Select a box with a lid (the length, width and height are 39 cm × 28 cm × 23 cm), and drill a small hole in the side. Connect the sealed box, nebulizing cup and the compression nebulizer with a rubber tube. The nebulized particle size of the compressed nebulizer is 1 - 5 μm, the nebulization rate is greater than 0.2 mL/min, and the ultimate pressure is 205 KPa.

6) The section of 2.2.2 now read:

On the 14th day after sensitization, open the lid of the homemade sealed box and place the rat in the box (the box can accommodate 5 rats), and then put the sealed box in a biosafety cabinet.

Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Reply: Thank you for your suggestion. The specific details have been added to the protocol steps and now read:

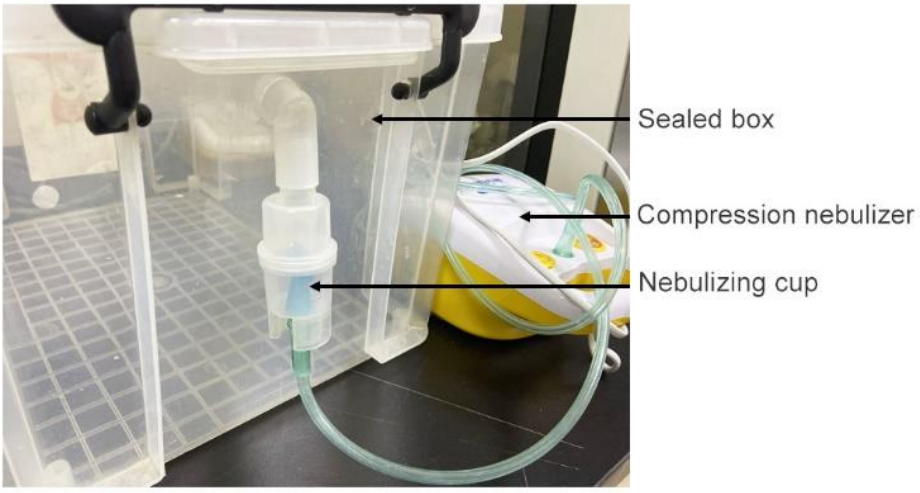
4.2.2 Check the channels: click “calibrate”.

4.3.2 Click “create study options” and select “dose response study”, select “rat” of species.

4.3.3 Select “simple dose list”, set the “number of does” to “6” and click “next”.

1) 2.2.3: Mention nebulizer specifications and describe how it was adapted for small animals.

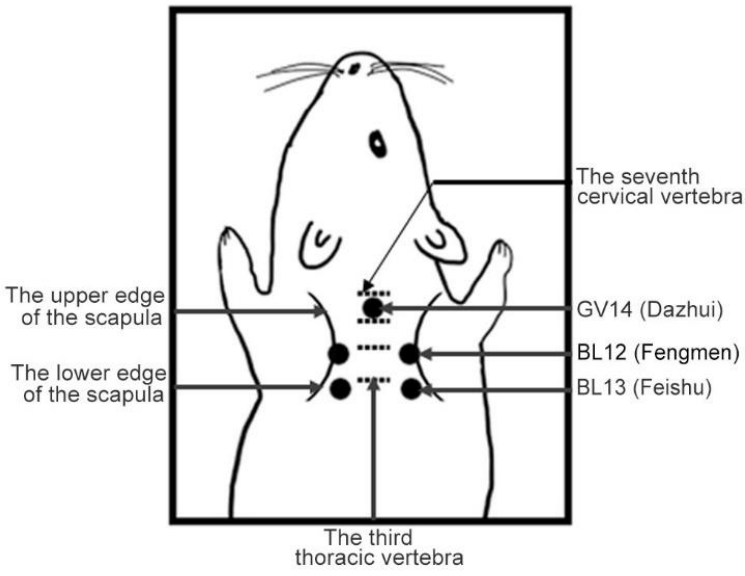
Reply: The nebulized particle size of the compressed nebulizer is 1-5 μm, the nebulization rate is greater than 0.2 mL/min, and the ultimate pressure is 205 KPa. The nebulizer specifications have been showed in supplementary figure 1.



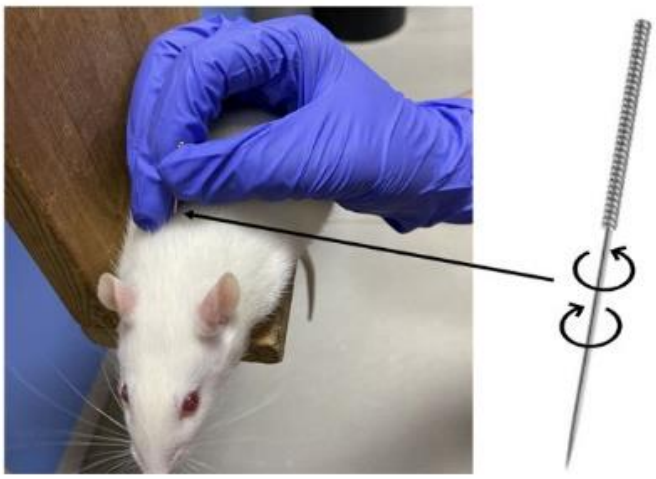
For the detail of the nebulizer can be adapted for small animals: The nebulizing device includes a sealed box, open the lid of the self-made sealed box and place the rat in the sealed box. The corresponding changes have been highlighted in page 6 line 11-15.

2) 3.1: Provide a schematic showing these points. This can be a supplementary figure. How exactly is acupuncture performed? Mention needle specifications.

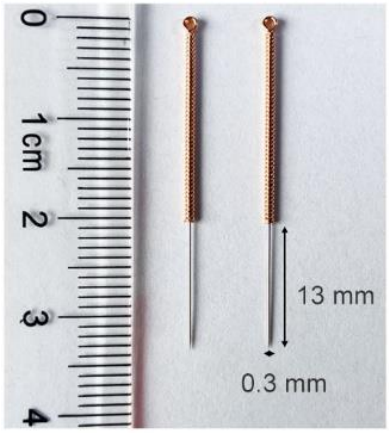
1) **Reply:** The schematic of these points has been showed in supplementary figure 2.



2) Acupuncture is performed as follow: Evenly twisted the needles approximately 360° to the left and the right at a rate of 60 times/min for 10 - 20 s, manipulate them every 5 min, and then withdraw them after 20 - 30 min. The schematic has been showed in supplementary figure 4.



3) The needle is disposable stainless-steel needle (0.3 mm × 13.0 mm) and the specifications have been showed in supplementary figure 3.



Results: Remove the subheadings.

Reply: The subheadings in the results have been removed.

Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Reply: The discussion has covered the above in detail and in paragraph form.

1) Modifications and troubleshooting:

   The needle may fall off if the rat swings about during the acupuncture. Therefore, needling must be manipulated every 5 min to adjust the depth.

2) Limitations of the technique:

   The limitations of the technique is that the high platform is suitable for rats, but not for smaller animals, such as mice.

3) Significance with respect to existing methods:

   We provide detailed methods for the fabrication of a high platforms for fixing rats without restriction during acupuncture. The high platform can well expose the acupoints on the back of the rat.

4) Future applications:

   Use of the high platform and noninvasive respiratory function measurement can facilitate the study of acupuncture in treating asthma. Studying the therapeutic mechanism of acupuncture on the back may bring new treatments to respiratory diseases such as asthma and chronic obstructive pulmonary disease.

5) Critical steps within the protocol:

Several critical steps should be considered when performing these methods. For acupuncture manipulation, the harder the palm of the right hand presses the rat back, the easier it is to insert the needle. For respiratory function measurement, the WBP system measures changes in the Penh that reflect changes in pulmonary resistance in rats in a noninvasive manner and minimizes effects of psychological stress[1,2]. The effect of the external environment on rats should be minimized. The rat should be placed in the testing room and then covered with a plastic bag to reduce the irritation caused by the external environment.

References: Please spell out journal names.

Reply: The journal names in references have been spell out.

Table of Materials: Please sort in alphabetical order.

Reply: The table of materials have been sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

1. Please show and give ethical approval for this research (ethical clearance)

Reply: Ethical approval has been given in page 5 line 16-18 and now read: All animal experiments were reviewed and approved by the Committee on the Ethics of Animal Experiments of Shanghai University of Traditional Chinese Medicine (Ethical clearance number PZSHUTCM190308020).

2. Please rotate right 90^{o} picture number 1, 2, 3 and 5

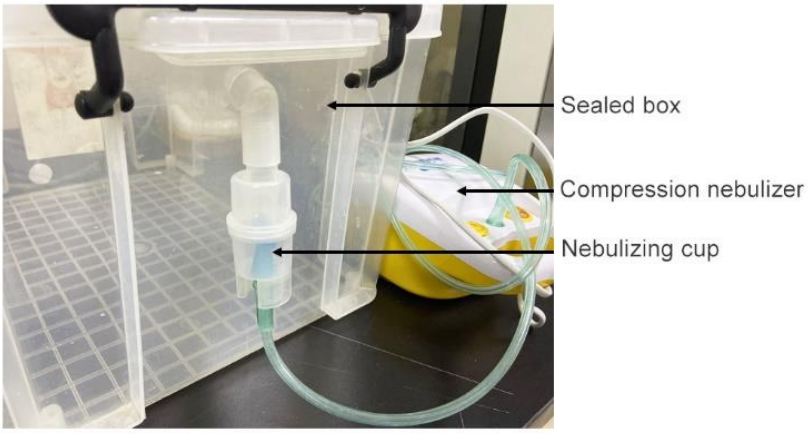
Reply: The pictures (figure number 1, 2, 3 and 5) have been rotated right 90^{o}.

3. In line 107 = what size of sealed box do you use?

Reply: The length, width and height of sealed box are 39 cm × 28 cm × 23 cm. The information of box size have been added in page 6 line 11.

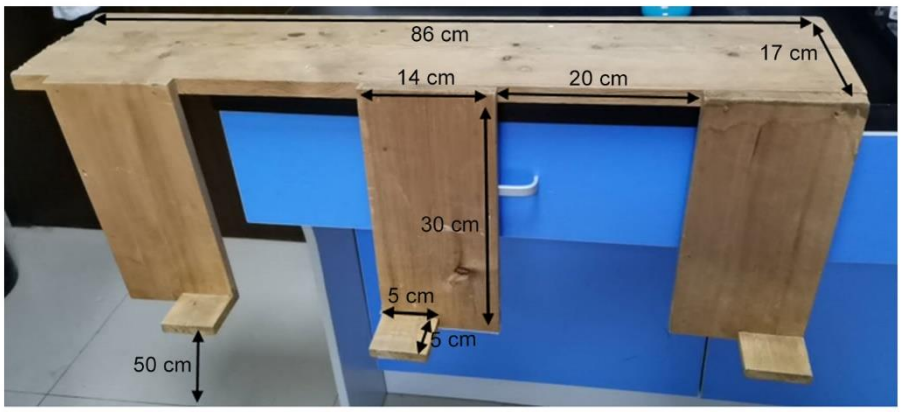
4. In line 111 = How to connect sealed box with the nebulizer?

Reply: Sealed box has a small hole in it to connect an ultrasonic atomizer with a rubber tube. The corresponding information have been added in page 6 line 11-15 and the specifications have been showed in supplementary figure 1.



5. In line 201 = what is the height of pedal from the ground? You should add the details in this sub or you can attach in figure number 1.

Reply: The height of pedal from the ground is 50 cm and have been attached in figure 1 and new figure 1 is now as follow:



6. In your Introduction/Discussion you should include other recent preclinical research on the effects of acupuncture in mouse model of chronic asthma - please see/cite

- Nurwati I, Purwanto B, Mudigdo A, Saputra K, Prasetyo DH, Muthmainah M. Improvement in inflammation and airway remodelling after acupuncture at BL13 and ST36 in a mouse model of chronic asthma. Acupunct Med. 2019;37(4):228-36.

- Nurwati I, Purwanto B, Mudigdo A, Saputra K, Sutrisno TC. Reduction of interleukin-17 level by acupuncture at Feishu (Bl 13) is strengthened by acupuncture at Zusanli (ST 36) in a mouse model of chronic asthma: An experimental study. Med Acupunct. 2015;27(4):278-82.

Reply: Thank you for your suggestion. The above two references have been cited as reference 7 and 8, the corresponding changes have been highlighted in page 3 line 12-14 and now read: Acupuncture at BL13 and ST 36 (Zusanli) in mice with chronic asthma decreases the level of IL-17 by 40% and decreases the smooth muscle thickness by 33% compared with that of the ovalbumin (OVA) group, and thus relieving inflammation symptoms[3,4].

Reviewer #2:

Manuscript Summary:

The manuscript described a detailed method for acupuncture manipulation in rat model of asthma. Major Concerns:

1. The authors should perform acupuncture on the rat model of asthma. However, the panel figure 6 only described the parameters from the asthmatic rat, the control rat and the asthmatic rat received acupuncture treatment should be included and compared using statistics methods.

Reply: Thank you for your suggestion. The experimental results of respiratory function have been showed in our previous article[5]. We highlight corresponding methods here according to the demand of JoVE.

Minor Concerns:

please further check grammars throughout the manuscript.

Reply: Thank you for the suggestion. The grammars have been re-revised by American Journal Experts (The verification page is https://secure.aje.com/certificate/verify and the verification code is 1E76-DA24-1D96-2A45-B473).

Can authors discuss how sham acupuncture can be used and performed in this type of animal study?

Reply: Different kinds of sham acupuncture are widely applied in randomized controlled trials (RCT) to investigate if acupuncture has therapeutic effects beyond the psychological effects in the clinic[6]. However, the pathologic-specific and non-specific

physiological effects of acupuncture are usually needed to be examined in animal study[7,8]. In order to highlight the pathologic-specific effect of acupuncture, it is critical to evaluate and know the physiological background effect of acupuncture (not psychological effect) in animal[7,8]. Therefore, acupuncture in normal rats has often been selected as control group in this type of animal study[5,9-11].

Reviewer #3:

Manuscript Summary:

The MS by Zhou et al. described the work to establish a rat model for acupuncture treatment of asthma. The study design and the goal of this work have sufficient scientific merit, and it would interest the general readers in this field. Minor Concerns:

1. The idea behind the high-platform is to immobilize rats for fear of heights. However, the fear of heights can cause anxiety, stress, and discomfort. Moreover, it has been reported that stress exacerbates clinical symptoms in patients with asthma (Brain, Behavior, and Immunity 21(8), pp. 993-999). So, it may be necessary to consider the effect of fear response itself on asthma in the experiment.

Reply: Thank you for the suggestion. According to the reference "Stress and inflammation in exacerbations of asthma. Brain Behav Immun. 2007; 21(8): 993-999", stress links to immune system and the directionality can vary depending on the type of stressor. From our investigation, the rats keep quiet and breathe smoothly on the high platform, showing that the high platform is an acceptable environment. In addition, rats in each group are placed on the high platform to unify the operating background.

Meanwhile, there are a total of 7 times of acupuncture in high platform, and repeated operations minimize the stress response.

2. The authors described that it is necessary to perform needles every 5 min to adjust the depth and enhance the acupuncture effect with this high-platform system. I am wondering whether repeated acupoints' stimulation under high-platform will also cause allergic or stress reactions in the rat.

Reply: Thank you for your suggestion. Adjust the depth every 5 minutes is to prevent the needle from falling, the movements are gentle and rat remains quiet. So it will not cause allergic or stress reactions in the rat.

References

1 McFadden, E. R., Jr., Luparello, T., Lyons, H. A., Bleecker, E. The mechanism of action of suggestion in the induction of acute asthma attacks. *Psychosomatic medicine.* **31** (2), 134-143 (1969).

2 Savov, J. D. *et al.* Ozone-induced acute pulmonary injury in inbred mouse strains. *American journal of respiratory cell and molecular biology.* **31** (1), 69-77 (2004).

3 Nurwati, I. *et al*. Reduction of interleukin-17 level by acupuncture at Feishu (BL 13) is strengthened by acupuncture at Zusanli (ST 36) in a mouse model of chronic asthma: An experimental study. *Medical acupuncture*. **27**(4), 278-282 (2015).

4 Nurwati, I. *et al.* Improvement in inflammation and airway remodelling after acupuncture at BL13 and ST36 in a mouse model of chronic asthma. *Acupuncture in medicine.* **37** (4), 228-236 (2019).

5 Zhou, D. D. *et al.* Metallothionein-2 is associated with the amelioration of asthmatic pulmonary function by acupuncture through protein phosphorylation. *Biomedicine & pharmacotherapy.* **123**, 109785 (2020).

6 Jiang, Y. L. *et al.* Assessments of different kinds of sham acupuncture applied in randomized controlled trials. *Journal of acupuncture and tuina science.* **9** (4), 199-203 (2011).

7 Yin, L. M. *et al.* Effects of acupuncture on the gene expression profile of lung tissue from normal rats. *Molecular medicine reports.* **6** (2), 345-360 (2012).

8 Xu, Y. D. *et al.* Non-specific physiological background effects of acupuncture revealed by proteomic analysis in normal rats. *BMC complementary and alternative medicine.* **14**, 375 (2014).

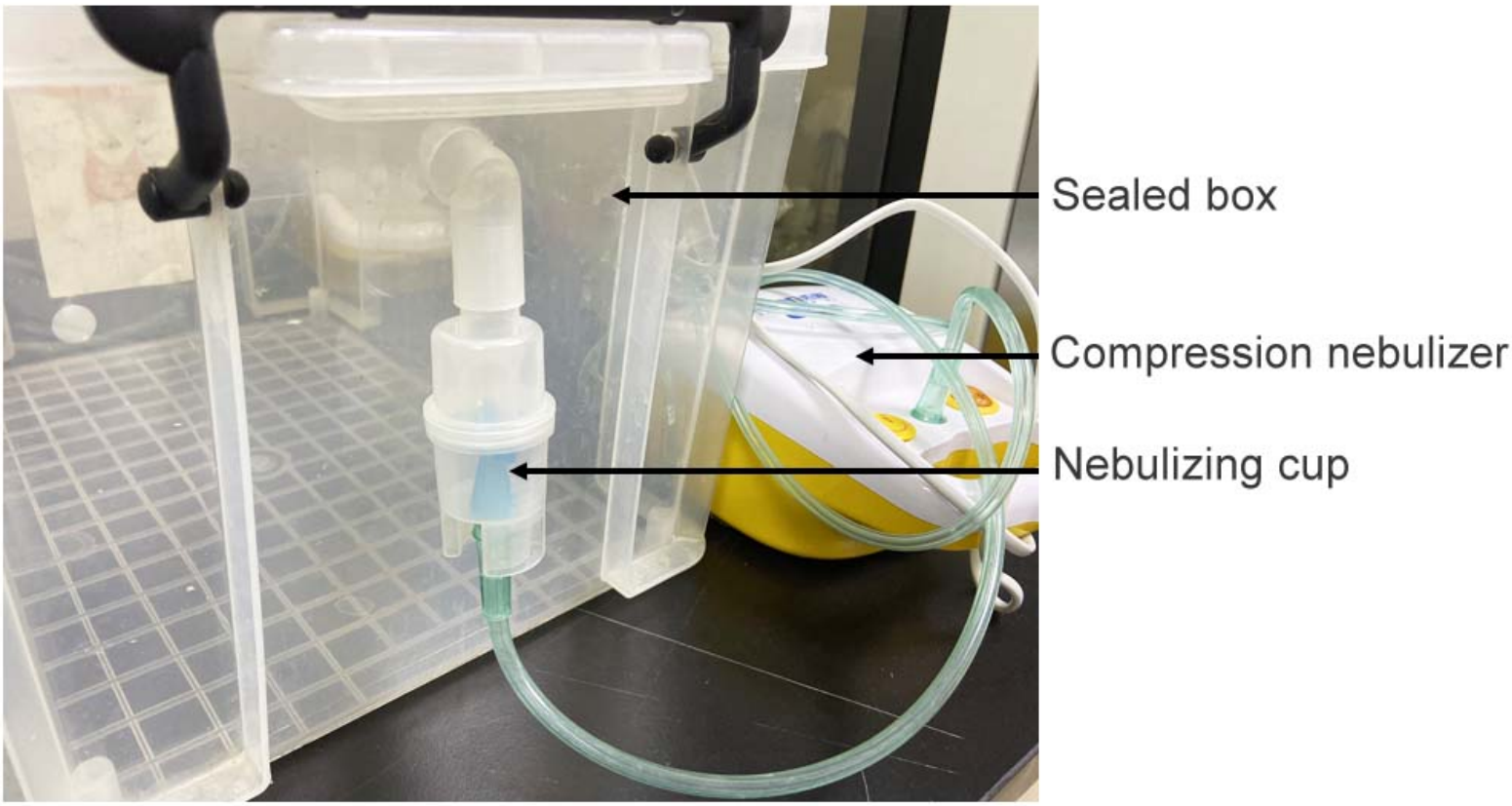
9 Yin, L. M. *et al.* Use of serial analysis of gene expression to reveal the specific regulation of gene expression profile in asthmatic rats treated by acupuncture. *Journal of biomedical science.* **16** (1), 46 (2009).

10 Xu, Y. D. *et al.* Proteomic analysis reveals the deregulation of inflammation-related proteins in acupuncture-treated rats with asthma onset. *Evidence-based complementary and alternative medicine.* **2012**, 850514 (2012).

11 Yin, L. M., Yang, Y. Q., Xu, Y. D., Wang, Y., Zhang, Q. H. Gene expression profile of acupuncture in treating asthma and the anti-asthmatic effects of differently expressed gene S100A9. *Respirology.* **18** (Suppl.1), 13 (2013).

Supplemental figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);supplementary figure 1.eps



Supplemental figure 2

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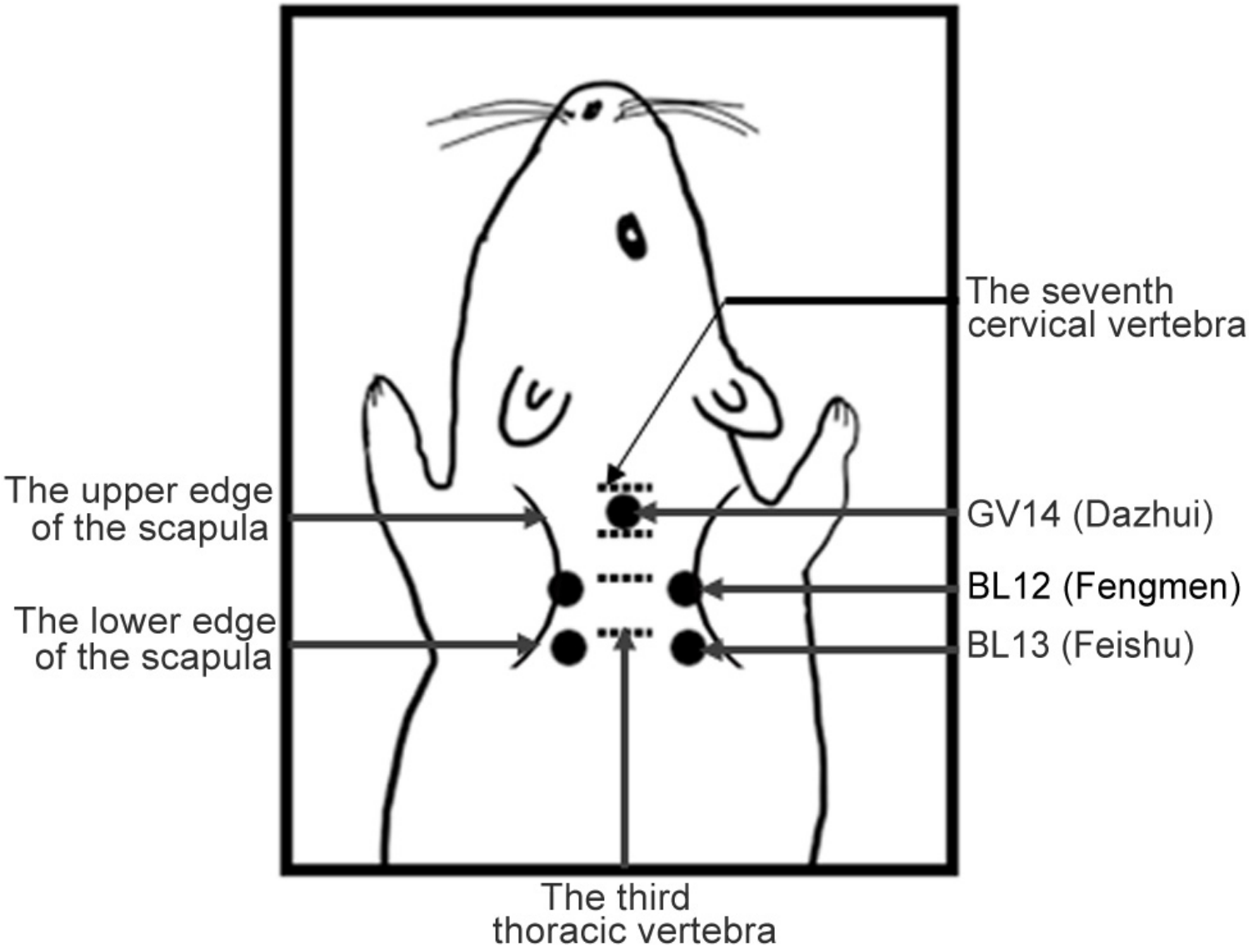
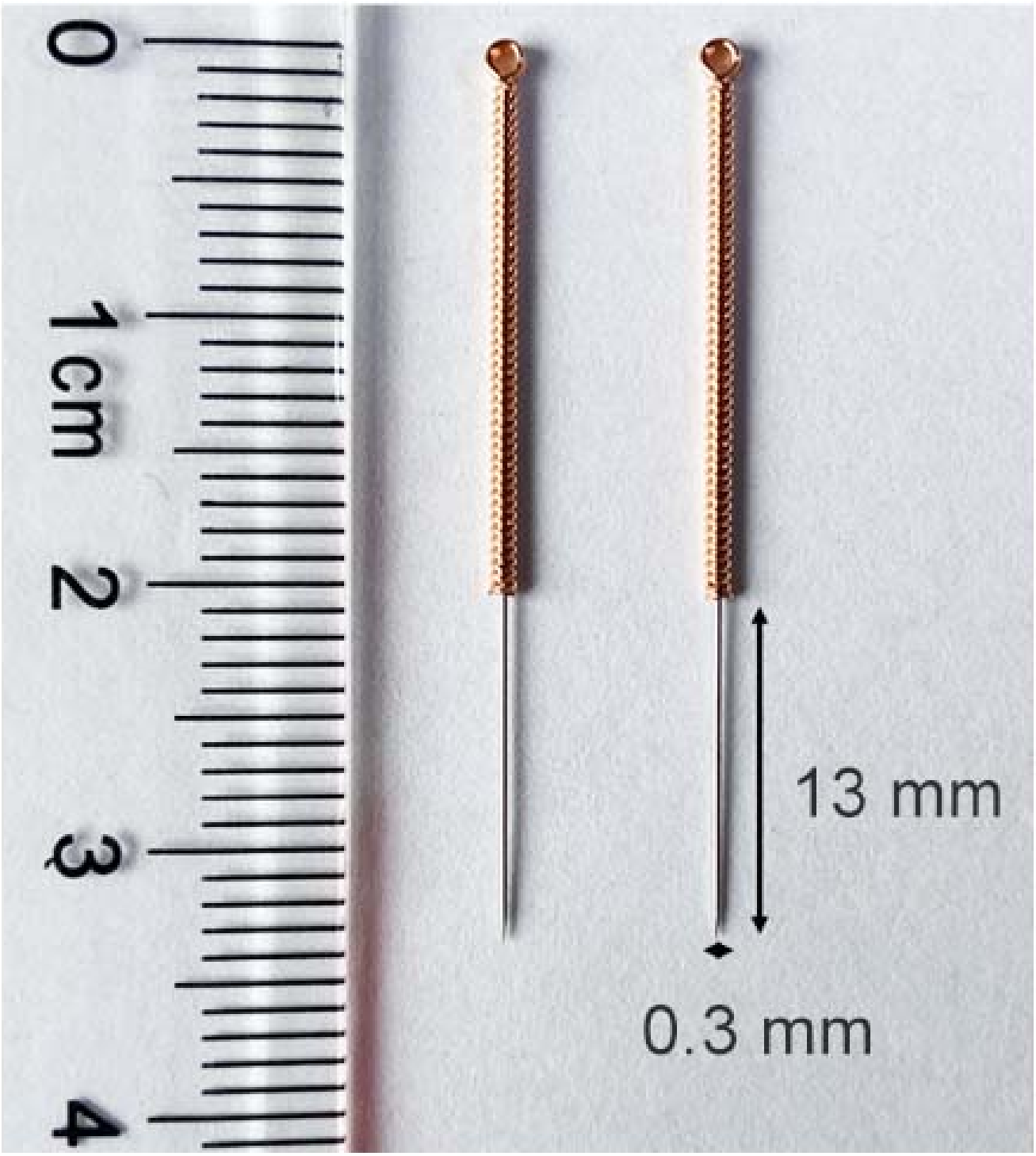


Supplemental figure 3

Click here to access/download;Supplemental File (Figures, Permissions, etc.);supplementary figure 3.eps



Supplemental figures 4

Click here to access/download;Supplemental File (Figures, Permissions, etc.);supplementary figure 4.eps