Twirling reinforcing-reducing manipulation — central mechanism underlying antihypertensive effect on spontaneous hypertension in rats

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Abstract

OBJECTIVE: To investigate antihypertensive effect in rats in order to confirm that twirling reinforcing-reducing manipulation (TRRM) might be the central mechanism underlying the action.

METHODS: In the study, "F-2-fluoro-deoxy-D-glucose positron emission tomography ("F-FDG-PET) was employed. Fifty-six spontaneous hypertensive rats (SHRs) were randomly divided into a model group, a single-needle acupuncture (SNA) group, a twirling reinforcing group (SNA + TRF) and a twirling reducing (SNA + TRD) group. Fourteen Wistar rats were assigned to the control group. The acupuncture intervention at Taichong (LR 3) acupoint was administered once daily in the SNA, SNA + TRF and SNA + TRD groups for 14 days, with 1 d interval between the two weeks. The blood pressure (BP) of all rats was measured repeatedly and "F-FDG-PET scans were conducted on the 14th day. PET images were processed with Statistical Parametric Mapping 8.0.

RESULTS: After the intervention, systolic BP showed a significant decrease in the SNA, SNA+TRF and SNA + TRD versus the model groups (all P < 0.01) and in the SNA + TRF and SNA + TRD versus the SNA groups (both P < 0.01), with the SNA+TRD group exhibited the best antihypertensive effect (P < 0.01). The key brain regions activated by TRRM were mainly concentrated in the cerebellum, hippocampus, hypothalamus, medulla oblongata, insular cortex, midbrain, thalamus and visual cortex.

CONCLUSION: TRRM could significantly lower the BP of SHRs by improving the cerebral glucose metabolism of the activated key brain regions and the underlying central mechanism may be related to the central rennin-angiotensin system and neuro-transmission.

Keywords: Twirling reinforcing reducing method; Hypertension; Blood pressure; Positron-Emission tomography; Central mechanism

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INTRODUCTION

Hypertension, a major risk factor for cerebrovascular disorders affecting human health, is characterized by systolic blood pressure (BP) of ≥140 mm Hg or diastolic BP of ≥90 mm Hg. It has been estimated that the number of adults with hypertension will increase to 1.5 billion worldwide in 2025, resulting in an increasingly serious worldwide public health challenge. Essential hypertension (EH), which accounts for 95% of all hypertensive cases, is generally considered a paradigmatic multi-factorial disease that is determined by a combination of genetic factors, environmental stimuli and their interaction. In recent years, increasing evidence of central nervous system (CNS) involvement has been considered either a cause or consequence of EH. Clinically, EH has been involved in cerebral neuronal damage and abnormalities of the cerebral vascular structure, ultimately results in cognitive decline, vascular dementia, cerebral haemorrhage and other cerebrovascular disorder, placing a heavy medical and financial burden on families and society. Hence, the prevention and treatment of early hypertension has become the primary problem to be solved.

Acupuncture provides an alternative treatment approach to hypertension and has been a critical constituent of Traditional Chinese Medicine (TCM) for the past 2500 years. Data from clinical investigations and laboratory animals have provided compelling evidence that acupuncture has become an effective treatment for EH. Twirling reinforcing-reducing manipulation (TRRM), regarded as a critical factor in acupuncture effect, is one of the most commonly used acupuncture manipulations in clinical practice. This type of manipulation originates from Huang Di Nei Jing and was established according to the therapeutic principles of “treating exuberance with drainage while treating deficiency with supplementation” in TCM. Previous studies have shown that different RRTM have different antihypertensive effect both in human and SHRs experiments. Moreover, the application of correct RRTM, according to deficiency or excess of disease, is a prerequisite for curative effect. Despite increasing use and experimental study of TRRM, the central mechanism underlying its antihypertensive effect are still not well understood.

Positron emission tomography (PET), a non-invasive cerebral functional imaging technique that enables the activity of cellular metabolism to be observed, has made it possible to intuitively investigate the effect of different acupuncture manipulations in the treatment of EH. Recently, 18F-2-fluoro-deoxy-D-glucose (18F-FDG) has been employed to detect cerebral rates of glucose metabolism which is considered to be an imaging biomarker with good sensitivity, and by far it is the most widely available and applied clinical PET tracer. In this study we aimed to identify the key brain regions from the perspective of altered cerebral glucose metabolism and to reveal the underlying mechanism of TRRM for treating hypertension for the first time.

MATERIALS AND METHODS

Animals

Fifty-six 9-week-old male SHRs weighing 200-230 g and fourteen age-matched male Wistar rats were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd., (Beijing, China) under licence number SCXK (Beijing) 200223. All rats were housed with 5 individuals per clean cage, with free access to water and food, under conditions of controlled illumination (12:12 h light/dark cycle, lights on 08:00-20:00), humidity (50%-60%), and temperature (18-22 °C). All experimental procedures were conducted in accordance with the World Health Organization’s International Guiding Principles for Biomedical Research Involving Animals and were approved by the Animal Care and Use Committee of Beijing University of Chinese Medicine, Beijing, China (permit No. BUCM-3-2016090301-3003). We made all efforts to minimize animal suffering and sacrifice in this experiment process.

After 1 week of acclimatization, Forty SHRs (systolic BP ≥ 140 mm Hg) were randomly divided into the following 4 groups (n = 14/group) by means of random number table: a model group, a single-needle acupuncture (SNA) group, a twirling reinforcing manipulation (SNA + TRF) group and a twirling reducing manipulation (SNA + TRD) group. Fourteen Wistar rats were assigned to the control group.

Acupuncture intervention

All the interventions were performed by a single skilled acupuncturist at 14:00. Each rat was loosely immobilized headfirst in a specially designed black cloth restrainer such that the anterior portion of the rat’s body was firmly in the restrainer with the hind legs exposed. The Taichong (LR 3) point (Taichong, located in the dorsum of the foot, in the depression anterior to the junction of the first and second metatarsals) and sterile disposable stainless steel needles (13 mm length, 0.18 mm diameter; Zhongyan Taihe, Beijing Zhongyan Taihe Medical Instrument Co., Ltd., Beijing, China) were used in the treatment.

In the SNA group, the needles were perpendicularly inserted into the bilateral Taichong (LR 3) to a depth of approximately 2 mm by the right hand, followed by retention for 20 min. In the SNA + TRF group, the needles were perpendicularly inserted into the bilateral Taichong (LR 3) to a depth of approximately 2 mm by the right hand, and then the reinforcing twirling manipulation was performed with a range of ±360° at a frequency of 60 times/min for 3 min, during which the forward thumb force was exerted with heavy strength, while the backward thumb force was exerted...
with light strength, followed by retention for 17 min. In the SNA + TRD group, all acupuncture treatments were the same as in the SNA + TRF group except that forward thumb force was exerted with light strength, while the backward thumb force was exerted with heavy strength. The rats in the control and model groups were untreated but handled and fixed for 20 min in the same restrainer as the first three groups. All the interventions were conducted once daily for 14 d, with 1 d interval between the two weeks.

**Measurement of blood pressure**

The systolic BP of the caudal artery was measured by two experienced technicians between 8:00 and 12:00 at a controlled temperature of (20 ± 2) °C. Each rat was gently placed in a restrainer and its tail was fixed using the rat-tail fixing facility. The ventral portion of each rat was placed on the heat pad, while the BP measurement cuffs were put in place. Once a batch of 3 rats was in place, calm rats were preheated at 36 °C for 10 min, after which their systolic BP was measured with a non-invasive BP instrument (BP-6, Chengdu Thai Union Biological Instrument Co., Ltd., Chengdu, China) and recorded by the pulse recording sensor facility while the rats were quiet and conscious. To ensure the accuracy and reproducibility of the measurements, the rats were trained to acclimate to the rat-tail fixing facility and the measurement environment at the same time once daily for 1 week prior to the experiment. During the experiments, three trials, spaced 5 min apart, were conducted to measure and record the BP. The data resulting from the 3 trials were averaged. BP was recorded one day before the intervention and on the 3rd, 8th, 13th day.

**18F-FDG-PET imaging**

All rats were sent to the PET Experimental Centre of the Institute of High Energy Physics of the Chinese Academy of Sciences before 8:00 on the 14th day. Cerebral images were acquired using PET after 24 h of fasting. The experimental procedures was as follows: (a) Blood glucose was measured in samples from tail vein bleeds via the glucose oxidase method (One Touch Ultra Glucose Kit, Johnson (China) Medical Equipment Co., Ltd., Shanghai, China). (b) The rats were allowed to rest for 20 min in a dark and quiet room. (c) A tracer (fluodeoxyglucose [18F]), 18F-FDG, synthesised with Mini Tracer accelerator, 123-185 MBq) was injected after 40 min via the tail vein for imaging, which continued for the next 10 min post-injection: the SNA + TRF and the SNA + SNA + TRD groups were accordingly subjected to the RRTM intervention for 2 min before and after injection, respectively, while the SNA group was subjected to direct needle acupuncture 2 min before the injection. After a approximately 20-min retention, the rats were allowed free movement within a small box for 15 min. The rats in the model and the control groups were free to move in a small box for 35 min after the injection. Then, all of the rats were anaesthetized with 1.5% isoflurane in 100% oxygen for 5 min. (d) PET was performed using a primate PET scanner (Eplus-260, micro-PET system, Institute of High Energy Physics, Chinese Academy of Sciences, Beijing, China) covering the whole brain and neck, in which LYSO-based detector blocks with a 23 cm transaxial field of view (FOV) and a 6.4 cm axial FOV were used. The emission projection data were acquired in list mode format and were Fourier rebinned into two-dimensional (2D) sinograms. Images were then reconstructed using the 2D OSEM algorithm (4 iterations and 24 subsets), resulting in a 0.5 mm × 0.5 mm × 1.0 mm 3-voxel size for a 380 × 380 × 63 image volume. The PET images were corrected for detector efficiency, deadtime, decay, photon scatter and attenuation. All scans were saved in Analyze 7.5 format.

**Statistical analysis and image processing**

The data of systolic BP m, expressed as the means ± standard deviation ( x ± s ), were analysed using SPSS 17.0 (SPSS Inc., Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago, IL, USA) and. At each time point, comparisons of systolic BP among the five groups were analysed via two-way analysis of variance (ANOVA), followed by Tukey’s post hoc test. Statistical significance was considered only when P < 0.05. The pre-processing and data analysis of the PET images were performed using spmratIHEP3 based on SPM 8.0 (Welcome Department of Cognitive Neurology, London, UK), which comprised an FDG-PET rat brain template and atlas in Paxinos & Watson space. First, the body tissues and background in individual images were manually removed using MRicr, and the origin of the image was repositioned at D3V (dorsal 3rd ventricle), which corresponded to the standard FDG-PET template in Paxinos space. Then, the datasets were analysed in spmrat IHEP automatically as described below. (a) The individual images of the rat brain were spatially normalized into Paxinos & Watson space, which involved scaling up the voxel size in the Analyze header by a factor of 4, registering to a FDG-PET template, subsequently removing extracranial tissues via the intracranial image, and shearing the matrix to cut off the background. (b) The normalized images were smoothed with a Gaussian kernel of 2 × 2 × 4 mm3 full wave at half maximum. Then, the pre-processed images were analysed based on the framework of the general linear model (GLM). A two-sample t-test was performed to identify the difference in FDG signals between groups, in which proportional scaling and intensity normalization was applied to account for global confounds. Finally, the brain regions with significant FDG changes were yielded based on a voxel-level height threshold of P < 0.01 (uncorrected) and a cluster size of no less than 50 voxels.
RESULTS

Changes in blood pressure

As shown in Figure 1, one day before intervention, systolic BP was significantly higher in the model, SNA, SNA + TRF and SNA + TRD groups than in the control group (all P < 0.01) and remained at a higher level on the 3rd to 13th days (all P < 0.01), suggesting that the BP of SHRs maintained a steady state of hypertension during the course of the study. Moreover, there were no significant differences between the first four groups at baseline (all P > 0.05). Compared with the model group, systolic BP was significantly decreased in the SNA+TRF and SNA+TRD groups on the 3rd day (both P < 0.01) and in the SNA, SNA+TRF and SNA+TRD groups on the 8th and 13th day (all P < 0.01). Compared with the SNA group, systolic BP showed a significant decrease in the SNA + TRF and SNA + TRD groups on the 8th (both P < 0.01) and 13th (both P < 0.01) days. Interestingly, compared with the SNA + TRF group, the SNA + TRD group exhibited better antihypertensive effect on the 8th (P < 0.05) and 13th (P < 0.01) days.

Changes in cerebral glucose metabolism in different brain regions

Tables 1-2 show the specific brain regions in which a significant elevation of cerebral glucose metabolism was observed in the control and the SNA versus the model groups, and in the SNA+TRF and the SNA + TRD versus the SNA groups, respectively. Significant hypometabolism was reflected by the maximum t value (Max T) in each cluster. The location is indicated by the peak coordinates of the point of the maximum effect in Paxinos and Watson space, in which the X-axis is negative to the left of the midline and positive to the right; the Y-axis is positive ventrally and negative dorsally; and the Z-axis is positive in the direction of the olfactory bulb relative to the bregma and negative in the direction of the cerebellum (Figure 2).

As shown in Table 1, compared with the control group, we observed that significantly decreased glucose metabolism mainly in the brain regions of the thalamus, cerebellum, striatum and visual cortex on the 14th day in the model group (Figure 2A). Higher rates of glucose metabolism in the cerebellum, midbrain, and hippocampus were found in the SNA versus the model groups (Figure 2B).

As shown in Table 2, compared with the SNA group, the SNA+TRF group exhibited increased glucose metabolism in the cerebellum, hippocampus, midbrain, thalamus, visual cortex, sensory cortex and motor cortex (Figure 2C), while the SNA + TRD group exhibited higher levels of glucose metabolism mainly in the

Table 1 Brain regions activated in the control, model and SNA groups

<table>
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<tr>
<th>Anatomical structural</th>
<th>Max_T</th>
<th>Peak coordinates (mm)</th>
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<td>X</td>
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<td>Control versus model groups</td>
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<tr>
<td>Dorsal thalamus — lateral nucleus</td>
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<td>Cerebellum — 9th cerebellar lobule</td>
<td>9.90</td>
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<td>Cerebellum — 6th cerebellar lobule</td>
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<td>Striatum</td>
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<td>Visual cortex</td>
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<td>4</td>
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<tr>
<td>SNA versus model groups</td>
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<tr>
<td>Cerebellum — simple lobule A</td>
<td>9.49</td>
<td>3</td>
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<tr>
<td>Midbrain — Lateral periaqueductal gray</td>
<td>6.14</td>
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<tr>
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<td>-1</td>
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<td>-2</td>
</tr>
<tr>
<td>Hippocampus — postsubiculum</td>
<td>4.32</td>
<td>4</td>
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</tbody>
</table>

Notes: control group (n = 14): untreated Wistar rats; model group (n = 14): untreated SHRs; SNA group (n = 14): SHRs that received single needle acupuncture at Taichong (LR 3). SHR: spontaneous hypertensive rat; SNA: single needle acupuncture.
hypotheses, medulla oblongata, cerebellum, hippocampus and insular cortex of the brain (Figure 2D). The SNA + TRD group also showed increased glucose metabolism compared with that of the SNA + TRF group in the hypothalamus, striatum and insular cortex (Figure 2E).

**DISCUSSION**

In this study needle alone Taichong (LR 3) could significantly lower the systolic BP of SHRs and hypertensive patients.\(^{14,15}\) Moreover, in the present study, the antihypertensive effect was consistent with the previous experiments.\(^{14,15}\) Therefore, we set up only five groups in the present study: the control, model, SNA, SNA + TRF and SNA + TRD groups, without a non-LR3 acupuncture group.

We investigated the antihypertensive effect of the SNA, SNA + RFT, SNA + RDT at Taichong (LR3) in SHRs. Compared with the model group, systolic BP was significantly decreased in the SNA, SNA + TRF and SNA + TRD groups on the 8th and 13th day, indicating that needle Taichong (LR 3) could significantly lower the systolic BP of SHRs from the 8th day. Compared with the SNA group, systolic BP showed a significant decrease in the SNA + TRF and SNA + TRD groups on the 8th and 13th days, suggesting that the effect of acupuncture with TRRM were better than those of single-needle acupuncture in lowering the blood pressure of SHRs, and it also began to stabilize on the 8th day. Additionally, compared with SNA + RFT, the SNA + TRD group exhibited better antihypertensive effect from the 8th days. Therefore, we concluded that antihypertensive effect of the three acupuncture groups means that SNA + TRD > SNA + TRF > SNA in the short-term. We also found that the key brain regions activated by TRRM were mainly concentrated in the cerebellum, hippocampus, hypothalamus, medulla oblongata, insular cortex, midbrain, thalamus and visual cortex. The key brain regions play crucial roles in the pathogenesis of hypertension and could, to some extent, help to elu-
The cerebellum and hippocampus, notably, showed significantly higher glucose metabolism in the SNA + RFT versus the SNA groups and in the SNA + RDT versus the SNA group. The cerebellum is involved in the pathogenesis of hypertension. The RAS is composed of two arms: the pressor arm, including Ang II/ACE (angiotensin-converting enzyme)/AT1Rs, and the depressor arm, represented by Ang-(1-7) [angiotensin-(1-7)]/ACE2/Mas receptors. All of the components of the RAS are present in the brain, including the PVN and RVLM. Studies have shown that, in the PVN, activated RAS can contribute to the Ang II-induced hypertensive response, which can be attenuated via superoxide dismutase 1 (SOD1) overexpression in the PVN of SHRs. Thus, the neurotransmission of PVN-RVLM pathway is critically involved in the control of the blood pressure. The PVN-RVLM pathway is also the location of the rennin-angiotensin system (RAS), which is involved in the pathogenesis of hypertension. The RAS is composed of two arms: the pressor arm, including Ang II/ACE (angiotensin-converting enzyme)/AT1Rs, and the depressor arm, represented by Ang-(1-7) [angiotensin-(1-7)]/ACE2/Mas receptors. All of the components of the RAS are present in the brain, including the PVN and RVLM. Studies have shown that, in the PVN, activated RAS can contribute to the Ang II-induced hypertensive response, which can be attenuated via the overexpression of ACE2. Its underlying central mechanism may account for the shift of the RAS towards the antihypertensive axis (ACE2/Ang-(1-7)/Mas). We therefore speculate that the increased glucose metabolic activity observed in the PVN and RVLM may be related to the symptoms of blood pressure regulation by the sympathetic nervous system and the RAS.

In addition, the RAS also exists in the dorsal thalamus, striatum, and midbrain, involved in the central pathogenesis of hypertension. It has been demonstrated that Ang II receptor binding capacity in the thalamus, striatum, hypothalamus and ACE activity are significantly elevated in the midbrain of SHRs with sodium intake. Besides, it has also been shown that cerebral blood flow (CBF) in the thalamus, cerebellum, and parietal cortex is significantly decreased, while the calculated cerebrovascular resistance is increased in SHRs. Thus, CBF may be another factor that could be involved in the antihypertensive effect of TRRM.
However, the central regulation of blood pressure is a complicated process that involves several cerebral regions, rather than only one region. It has been demonstrated that, in essential hypertensive patients, the hypothalamus-related brain network exhibited increased functional connectivity with the cerebellum, limbic system, thalamus, medulla, frontal lobes, and brainstem after short-term acupuncture treatment, indicating that acupuncture may regulate the cardiovascular system through a complicated brain network involving the cortical level, hypothalamus, and brainstem. There are several limitations of this study. First, we evaluated changes in rats’ BP according to a single index, the systolic BP of the tail artery. Multiple indicators should be used in future studies to obtain a more objective evaluation. Second, we were unable to directly explain the relationship between the cerebral metabolic activity activated by TRRM and changes in brain regions that pathologically define EH. In the future, we will examine these regions independently using specific PET tracers and will hopefully provide a better understanding of the antihypertensive effect of TRRM on hypertension.

In conclusion, our study demonstrated that RTTM could significantly lower the BP of SHRs by improving cerebral glucose metabolism in the key brain regions including cerebellum, hippocampus, hypothalamus, medulla oblongata, insular cortex, midbrain, thalamus, and visual cortex. Several possible hypotheses regarding this link were put forth and we supposed that the underlying central mechanism may be mostly related to the activated central RAS and neurotransmission. Further investigations regarding the central mechanism behind the antihypertensive effect of RRTM are warranted.

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