

An Experimental Study of Acupuncture to Rat Model of Obesity: Involvement of SIRT1 Signaling Pathway

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ABSTRACT

Obesity is a global health challenge. While manual acupuncture has been recognized for its potential to reduce body weight, the efficacy of different manual acupuncture techniques in obesity treatment remains to be elucidated. This study aims to investigate the differential effects of various manual acupuncture methods in an obese rat model. We categorized rats into five groups: a control group, a model group, a conventional acupuncture group, a high-frequency twisting group, and a low-frequency twisting group. All groups, except the control, were fed a high-fat diet for eight weeks. The conventional acupuncture group underwent standard acupuncture treatment, whereas the high-frequency and low-frequency twisting groups received conventional acupuncture with respective frequency modulations. All interventions spanned four weeks. Acupuncture treatments were found to facilitate a reduction in body weight. The Lee's index was significantly lower in the acupuncture-treated groups. These interventions also rectified the disrupted lipid profiles in rat serum. Enzyme-linked immunosorbent assays (ELISA) revealed that acupuncture treatments substantially reduced pro-inflammatory cytokine levels in the liver. Moreover, liver morphological alterations and the presence of lipid droplets were notably ameliorated following acupuncture treatments. Comparative analysis indicated superior efficacy in the high-frequency and low-frequency twisting groups over conventional acupuncture, with the high-frequency twisting group demonstrating the most pronounced effects. Additionally, variations in the SIRT1/SREBP-1c/PPAR γ signaling pathway were observed, contributing to the differential impacts on obesity. This study demonstrates that acupuncture treatments exert a significant inhibitory effect on obesity. Among the various acupuncture modalities assessed, the high-frequency twisting technique emerged as the most effective, showing superior results in body weight reduction and other related indices. These findings suggest that the integration of high-frequency twisting with conventional acupuncture may represent an optimal approach for the treatment of obesity.

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Authors' Contribution

MP and BA conceived and designed this experiment. MP and PS did the experiments, collected and analyzed the data and drafted the manuscript. PZ and BA provided positive suggestions and amended the final manuscript. BA revised and improved the language of the final version of the manuscript.

Key words

Acupuncture, Reinforcing-reduction by entwisting, Obesity, SIRT1 signaling pathway

INTRODUCTION

Obesity has emerged as a global health crisis today, intricately associated with a multitude of severe conditions, including metabolic syndrome, diabetes, cardiovascular disease, renal failure (Sui *et al.*, 2012), and even polycystic ovary syndrome. Epidemiologically,

obesity is defined as a BMI of 30 kg/m² or higher (Caballero, 2019).

Its prevalence spans across various age groups, from childhood (Arenz *et al.*, 2004) and preschool years (Lanigan *et al.*, 2010) to adolescence (Sommer and Twig, 2018), adulthood, and the elderly population (Kalish, 2016). It has become an increasing epidemic (Jiménez-Cruz *et al.*, 2004), leading to poor quality of life. Except for excessive high fat food intake, the environment also contributes to the occurrence of obesity (Koch *et al.*, 2021). Reduction of body weights can lead to a healthier body and has been shown to improve quality of life in obese persons undergoing a variety of treatments. For example, 5–10% body weight reduction can decrease the risk of diabetes by 58% for obese subjects (Knowler *et al.*, 2002).

Acupuncture, a traditional complementary medicine, has been applied to a diverse array of diseases, such as

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asthma (Brinkhaus *et al.*, 2017), functional constipation (Lee *et al.*, 2018), and insomnia (Garland *et al.*, 2019).

Serving as a non-pharmacologic intervention, acupuncture has been effective in relieving pain, reducing stress (Schroeder *et al.*, 2017), and facilitating cognitive improvement (Du *et al.*, 2020).

An important application of acupuncture is body weight control. Some clinical trials demonstrated that acupuncture helps body weight reduction (Abdi *et al.*, 2012; Kim *et al.*, 2021). Furthermore, a systematic review and meta-analysis led to the conclusion that acupuncture is effective for simple obesity compared with placebo (Zhong *et al.*, 2020). An over-review of current systematic reviews still suggested larger and more solid evidence in the future in this field (Chen *et al.*, 2022). However, Electroacupuncture and manual acupuncture, two common clinical modalities, involve various needle operations such as piercing, lifting, inserting, and twisting at acupuncture points. This study compares these operations in an obesity animal model and explores their intrinsic mechanisms.

MATERIALS AND METHODS

Reagents and chemical

The primary antibodies for SIRT1 and others were obtained from Abcam. Lipid profile assay kits were obtained from Nanjing Jiancheng (Nanjing Jiancheng, Jiangsu, China). Inflammatory cytokines and other apolipoproteins were obtained from Beyotime (Beyotime, Nanjing, Jiangsu, China). Other chemicals were of analytic grade.

Animals, modeling and treatments

Animal study approval was obtained from the Laboratory Animal Ethics Committee of the Affiliated Hospital of Nanjing University of Chinese Medicine (Lab Animal Grant number: 2022DW-23-02). Wistar rats at 6 weeks with half females and males were provided by SPF (Beijing) Biotechnology Co. Ltd. Rats were randomly assigned into two groups at first (Week 0): the control group (n=8, Control) and obesity model group (n=32). Rats in the control group were fed with a standard rodent diet provided by Research Diets Corporation (D12450J, 3.8 kcal/g BW; 10% fat, 70% carbohydrates, and 20% protein). Meanwhile, rats in obesity model group were fed with a specific high-fat diet provided by Research Diets Corporation (D12492, 5.5 kcal/g BW; 46.5% fat, 38.5% carbohydrates, and 15% protein) (Zecchin *et al.*, 2007; Shu *et al.*, 2020).

After 8 weeks of feeding (Week 8), rats in the model group were assigned into 4 groups (n=8): model group (Model), conventional acupuncture group (conventional acupuncture group), high-frequency twisting group (high

frequency acupuncture group) and low frequency twisting group (low frequency acupuncture group).

In the control group, there were no administrations and treatments. In the model group, there was also no administration and treatment. The other groups were subjected to acupuncture treatment. The rats were fixed on the small animal operation table, and routine disinfection with a 0.25mm × 25mm sterile acupuncture needle was used to pierce into the acupuncture point directly by 3mm at Zhongwan (RN12), Zusanli (ST36), Qihai (RN06), Tianshu (ST25) and Sanyinjiao (SP6) in the normal acupuncture group. The locations of acupuncture points were followed by previously established criteria (Yin *et al.*, 2008). Meanwhile, the Zusanli (ST36), Tianshu (ST25), Sanyinjiao (SP6) were treated on both sides. In the high-frequency twisting group, rats were given 120 twists per minute beyond the above-mentioned pierce, and the low-frequency group was given 60 twists per minute. Twisting was composed of first rotating the needle handle clockwise by 360 degrees and then rotating it counterclockwise by 360 degrees per time. Each acupoint was treated for 2 min each time, and the needle was kept for 20 min each time once every other day, 10 times a course of treatment, for a total of 1 course of treatment.

General observation

The body weights and body lengths of rats in groups were recorded. The Lee's index method is a golden criterion for obesity evaluation using a previously mentioned formula:

$[LI = \text{body weight (g)}^{1/3} * 1000 / \text{body length (cm)}]$ (Wu *et al.*, 2014; Zakaria *et al.*, 2021). The general situation of rats was also observed.

Biochemical analysis of serum

After 4 weeks of acupuncture treatment (week 12), rats were fasted for 10 h and then executed after anaesthesia using 2% pentobarbital sodium. Blood was collected and centrifuged at 3000 rpm, 2664 g, 4°C for 10 min. The levels of fasting blood glucose (FBS), total cholesterol (TC), triglycerides (TG), and high-density lipoprotein-cholesterol (HDL-C), adiponectin (ADP), fast insulin (FINS), and free fatty acid (FFA) in the serum of rats were measured using an automatic immune analyzer (AU5800, Roche Diagnostic Systems, Mannheim, Germany). Serum low-density lipoprotein (LDL) was measured using the formula in previously reported (Friedewald *et al.*, 1972).

Enzyme linked immunosorbent assay (ELISA) for cytokines in liver

The contents of liver inflammatory cytokines (tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6))

were tested by ELISA (Muniroh *et al.*, 2021). The liver tissue was harvested, and the operation procedures were followed by manual instruction. The standard curve was pre-determined using a microplate reader (PT-3502, Ponetov, Beijing, China) at 455 nm. The optical density values were tested 3 times and averages were adopted.

Histological observation

Rat liver was fixed using 4% paraformaldehyde and embedded in paraffin (Qiu *et al.*, 2020). Then rat livers were cut into 4 μ m sections and stained with hematoxylin eosin (HE). The morphological changes were observed under a light microscope. Some other parts of rat liver were harvested on ice and subjected to liquid nitrogen rapid freezing. Livers were cut into 4 μ m sections, and lipid contents were visualized as red stained areas in or above liver cells (Karunakaran *et al.*, 2021).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using TRIZOL according to the manufacturer's kits and previously reported (Yang *et al.*, 2019). Concisely, the concentration and purity of the RNA in the rat liver were determined. cDNA was synthesized for RT-PCR amplification according to the instructions in the First Strand cDNA Synthesis Kit (Invitrogen). The sequences of the primers used for the RT-PCR assay are ***. The qPCR was performed using the following conditions: 30s at 95 °C for denaturation, 5 s at 95 °C for annealing, and 40 s at 60 °C for extension by an Automatic PCR instrument (Z480, Roche, Swiss).

Western blot analysis

To further validate the role of the SIRT1 signaling pathway, the western blot was employed (Zhu *et al.*, 2021). Part of the mouse lung was harvested on ice, and the total protein was extracted. After bicinchoninic acid determination, proper protein was separated on gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Consequently, a blocking solution was employed to block the membranes, and the PVDF membranes were incubated with the respective primary antibodies overnight. On the second day, secondary antibodies were co-incubated for 2 h and the chemiluminescence signals were detected.

Statistical analysis

The data was expressed as the mean and standard deviation (SD). The body weight and Lee index were compared by repeated measurements using a one-way ANOVA. Statistical significance was detected using SPSS software (version 27.0, SPSS, Chicago, IL, USA) and set

as $P < 0.05$. The other analysis was performed by one-way ANOVA, followed by Least Significant Difference (LSD) or Dunnett-T (DT). Results were presented by Graph Pad Prism 6.0 (GraphPad Software Inc., Diego, CA, USA).

RESULTS

Acupuncture alleviates the body weights increase

At week 0 as shown in Figure 1A, there were no statistical differences in body weights between all groups. At week 8 (Fig. 1B), the body weights in the model and other groups increased significantly compared with the control group. After acupuncture treatment, body weights in 3 acupuncture treatment groups decreased compared with the model group at week 12 (Fig. 1C). Among them, the lowest body weights were found in the high-frequency twist group.

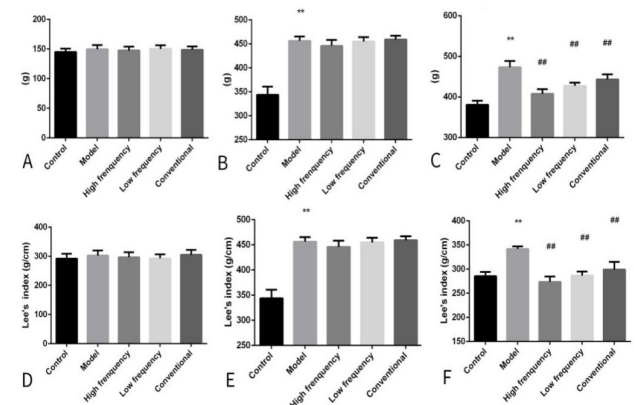


Fig. 1. The body weights and Lee index of rats. (A) Body weights before study (0th week), model establishment (8th week), and after last acupuncture (12th week). (B) Lee index at (0th week), model establishment (8th week), and after last acupuncture (12th week). Statistical differences among the model group and other groups were considered significant at the level of * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$. Meanwhile, differences among the normal acupuncture group and other acupuncture groups were considered at # $P < 0.05$, ## $P < 0.01$ or ### $P < 0.001$.

There were no statistical differences among Lee's indexes between groups in Week 0, as shown in Figure 1D. At Week 8 (Fig. 1E), the Lee's index in the model and other groups increased compared with the control group. After acupuncture treatment, Lee's indexes in acupuncture groups decreased compared with the model group at Week 12 (Fig. 1F). Among them, the lowest Lee's index was founded in the high frequency twist acupuncture group.

Acupuncture alleviated the disrupted lipid profiles

As shown in Figure 2A, FBS levels in the rat serum

of control group were maintained at relatively low levels. However, FBS levels increased significantly in the model compared with control group due to high fat diet feeding. Acupuncture treatment reduced the levels of FBS in 3 acupuncture groups. Among them, the greatest reduction was found in high frequency twist acupuncture group. Similar tendencies were founded in other lipid profiles, for example TC levels (Fig. 2B), TG levels (Fig. 2C), LDL-C levels (Fig. 2D), insulin levels (Fig. 2F), FFA (Fig. 2G). An opposite tendency was found in the (Fig. 2E) levels due to HDL-C is a protective factor in obesity.

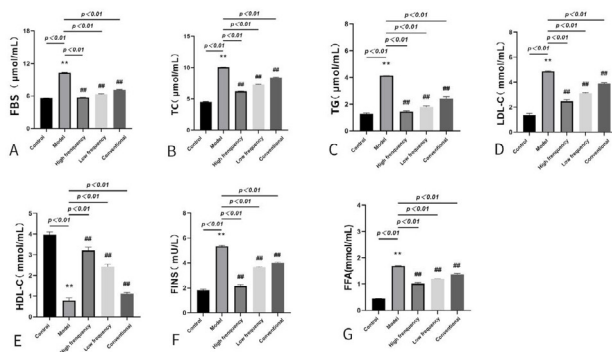


Fig. 2. The FBS levels (A), TC levels (B), TG levels (C), LDL-C levels (D), ADP levels (E), insulin levels (F), FFA (G) levels, and HDL-C levels (H) in the serum of rats in groups. Statistical differences among the model group and other groups were considered significant at the level of $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$. Meanwhile, differences among the normal acupuncture group and other acupuncture groups were considered at $\#P < 0.05$, $\#\#P < 0.01$ or $\#\#\#P < 0.001$.

Acupuncture improved the liver morphology

As shown in Figure 3A, hepatocytes in the control group ranged in alignment and showed clear hepatic lobule structures. However, hepatocytes in model group showed the characteristics of steatosis and hepatocellular ballooning. The hepatocytes and steatosis were swelling and enlarged. The hepatic cords arranged disorderly while inflammatory cells increased. Most importantly, different sizes of lipid droplets existed widely in the hepatocytes. After acupuncture treatments, the lipid droplets decreased in acupuncture groups. An analogous trend of augmented lipid accumulation, manifested as red droplets, was observed in the model group (refer to Fig. 3B). Notably, acupuncture interventions ameliorated liver morphological alterations, particularly the lipid accumulation within the liver.

Acupuncture attenuated the increase of cytokines

The rise of inflammatory cytokines is another

characteristic of obesity. The pro-inflammatory cytokines containing TNF- α (Fig. 3C), IL-6 (Fig. 3D), levels were maintained at relatively low levels in the control group. IL-6 and TNF- α levels were found to be increased in the model group compared with the control group. By contrast, acupuncture treatments attenuated the increase of IL-6 and TNF- α levels. In addition, the levels of TNF- α and IL-6 in the 2 twist groups were lower than in the conventional acupuncture group.

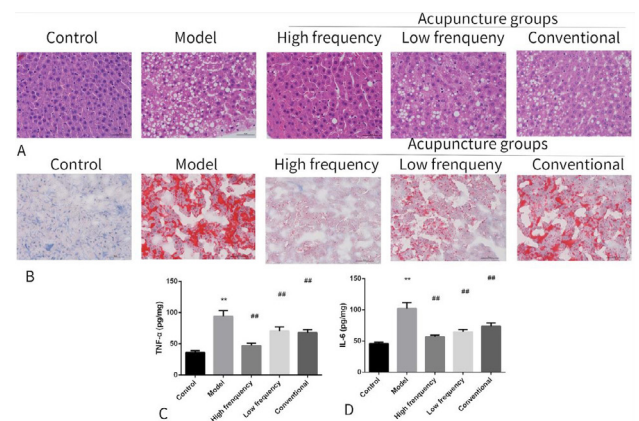


Fig. 3. The morphometry observation of rat liver. Representative HE staining of the liver in rats (A). Magnification= $\times 200$. The IL-6 levels (B), TNF- α levels (C), Apo-A1 levels (D) and Apo-B levels (E) in the liver of rats within groups. Statistical differences among the model group and other groups were considered significant at the level of $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$. Meanwhile, differences between the normal acupuncture group and other acupuncture groups were considered at $\#P < 0.05$, $\#\#P < 0.01$ or $\#\#\#P < 0.001$.

Acupuncture's intrinsic mechanism

In this study, PCR results demonstrated that the high fat diet administration down-regulated the expression levels of SIRT1 and up-regulated the downstream SREBP-1c and peroxisome proliferator-activated receptor gamma (PPAR γ) gene activation in model group, as shown in Figure 4A. However, acupuncture administration up-regulated expression levels of SIRT1 while down-regulating the SREBP-1c and PPAR γ genes compared with the model group. In addition, the expression levels of SIRT1, SREBP-1c and PPAR γ in 2 twist groups were higher than in the conventional acupuncture group. Among them, the highest SIRT1 levels were found in the high-frequency twist acupuncture group.

The western blot results showed a similar tendency. The levels of SREBP-1c and PPAR γ proteins were enhanced in the model group as shown in Figure 4D. However, acupuncture administration down-regulated

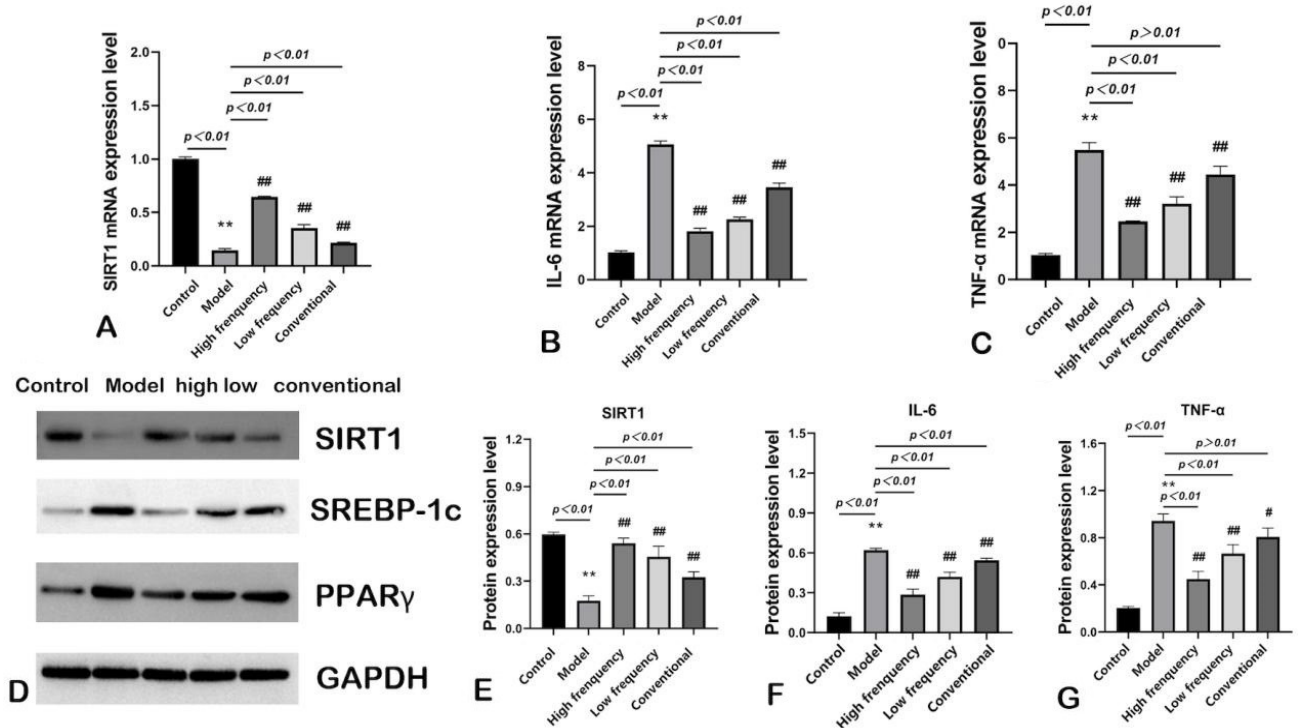


Fig. 4. Mechanism validation of acupuncture in a rat model of obesity. (A) The gene of the PCR results. (B) Representative western blot images and quantification of western blot results. Statistical differences among the model group and other groups were considered significant at the level of $*P<0.05$, $**P<0.01$ or $***P<0.001$. Meanwhile difference between the normal acupuncture group and other acupuncture groups was considered at $\#P<0.05$, $\##P<0.01$ or $\###P<0.001$.

the expression levels of SREBP-1c and PPAR γ proteins compared with model group. In addition, the expression levels of SIRT1, in 2 twist groups were higher than in the normal acupuncture group. Among them, the highest SIRT1 levels were found in the high-frequency twist group.

DISCUSSION

This study explored the differences between several types of manual operations in acupuncture. Interestingly, it was found that the high-frequency twisting group exhibited the highest efficacy among all acupuncture treatments. The expression levels within the SIRT1/SREBP-1c/PPAR γ signaling pathway were identified as playing pivotal roles in mediating the differences observed between the acupuncture treatments. The SIRT1 signaling pathway plays a pivotal role in the efficacy of acupuncture in treating obesity. The sirtuins family, comprising seven members (SIRT1-7), has garnered significant attention, with sirtuin 1 (SIRT1) being the most extensively studied in recent decades (Herranz and Serrano, 2010).

They regulate many cellular and physiological

processes, such as maintaining energy under circumvent conditions (Ren *et al.*, 2020). Predominantly located in the nucleus, SIRT1 exerts protective roles in the liver. For instance, mice deficient in SIRT1 exhibited severe hepatic steatosis (Xu *et al.*, 2010), and a marked reduction in SIRT1 expression was observed in patients with severe obesity (Costa *et al.*, 2010). Furthermore, SIRT1 is known to regulate several important downstream genes and proteins in fatty liver disease and obesity (Schug and Li, 2011; You *et al.*, 2015). Firstly, a reduction in SIRT1 *in vivo* leads to macrophage recruitment and the release of cytokines, including IL-6 and TNF- α (Gillum *et al.*, 2011).

Secondly, SIRT1 activation promotes mitochondrial biogenesis and, in conjunction with p AMPK, inhibits PPAR γ /SREBP-1c-induced fatty acid synthase (FAS) activity (Chyau *et al.*, 2020). PPAR γ , a ligand-activated transcription factor, is regarded as the “master” regulator of adipogenesis (Perrini *et al.*, 2020). SREBP-1 is also a crucial regulator of sterol and fatty acid synthesis, while PPAR regulates fatty acid storage and glucose metabolism (Tontonoz *et al.*, 1994). The high-frequency twist acupuncture achieved the most effect among 3 acupuncture groups. The twisting acupuncture manipulation is a specific

manipulation (Han *et al.*, 2015), which exerts a needle-tissue interaction force and may enhance the interaction of neurons and tissue inside the body. In general, it was recognized as a clinical application to reinforce the Qi, and stimulate the body (Feng *et al.*, 2016). Some twirling and rotating acupunctures at specific frequencies can promote blood flow perfusion at acupoints (Xu *et al.*, 2019). Notably, higher frequencies have been associated with optimal acupuncture effects in ischemic stroke models in rats (Zhang *et al.*, 2013). In this study, high-frequency twist acupuncture emerged as the most effective technique in the rat model of obesity.

This study has some strengths and limitations. The main strength is the comparison of differences between acupuncture treatments in the obesity model, which has been little explored in the past. The main limitation is the lack of SIRT agonists or inhibitors. However, we tried this in the preliminary experiment, but the SIRT agonist or inhibitor was not stable in the animal, different from the cell study.

CONCLUSION

In summary, the present study has elucidated that acupuncture treatments contribute to the reduction of body weight. Among the various acupuncture treatment groups, the high-frequency twisting group demonstrated the greatest effectiveness in terms of body weight reduction and other related indices. This study suggested that conventional acupuncture plus high-frequency twisting was most suitable for obesity treatment.

DECLARATIONS

Funding

The study received no external funding.

Ethical statement and IRB approval

The animal study was reviewed and approved by the Animal Ethics Committee in Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (2022DW-23-02).

Data availability statement

The raw data supporting the conclusions of this article had been uploaded in the data in supplementary.1.

Statement of conflict of interest

The authors have declared no conflict of interest.

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