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RESEARCH ARTICLE

The Effects of Manual Acupuncture on Mitochondrial Fusion and Fission Gene Expression in Rat Spleen

Yu-Mi Lee[1,†], Dong-Hee Choi[2,†], Jeong-Hye Park[2], Min-Woo Cheon[3], Jae Gwan Kim[1], Jeong-Sang Kim[2], Taejin Choi[4], Hye-Ran Kim[2], Daehwan Youn[2,*]

[1]Department of Biomedical Science and Engineering, Gwangju Institute of Science and Technology, Gwangju, Korea
[2]Department of Korean Medicine, Dongshin University, Naju, Korea
[3]Department of Health Administration, Dongshin University, Naju, Korea
[4]DongHaeng Convalescent Hospital, Gwangju, Korea

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Correspondence to
Daehwan Youn
Department of Korean Medicine, Dongshin University, Naju, Korea
E-mail human22@dsu.ac.kr
†Yu-Mi Lee and Dong-Hee Choi contributed equally to the writing of this article.

Background: A significant amount of research has been conducted to establish the validity of acupuncture, and it has been demonstrated through animal disease model studies that acupuncture influences mitochondrial changes. However, to more accurately examine the mechanisms of acupuncture treatment effectiveness in pathological models, it is crucial to investigate changes in disease-free animals. Among various hypotheses regarding the effects of acupuncture on the body, we focused on the result that acupuncture stimulation is related to mitochondria.
Objectives: We examined the effects of acupuncture mitochondrial fission and fusion-related mediators in disease-free Sprague Dawley (SD) rats' spleen meridian acupoints.
Methods: SD rats were divided into control, SP1, SP2, SP3, SP5, and SP9 acupuncture groups. Acupuncture was performed at each point for 10 minutes daily for four days. Peroxisome proliferator-activated receptor-gamma coactivator 1-α (*PGC-1α*) and fission protein 1 (*Fis1*) levels were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR), while dynamin-related protein 1 (*DRP1*), optic atrophy-1 (*OPA1*), mitofusin-1 (*MFN1*), and mitofusin-2 (*MFN2*) levels were assessed via western blotting. Mitochondria protein concentrations and NADH dehydrogenase activity in spleen tissues were measured using enzyme-linked immunosorbent assay (ELISA).
Results: PGC-1α expression decreased in the SP1 ($p < 0.01$), SP5 ($p < 0.05$), and SP9 ($p < 0.05$) groups, while *Fis1* expression increased in the SP1 ($p < 0.01$), SP5 ($p < 0.01$), and SP9 ($p < 0.05$) groups. *DRP1*, *OPA1*, *MFN1*, and *MFN2* levels exhibited no significant changes. Mitochondrial protein concentrations decreased in the SP2 ($p < 0.01$), SP3 ($p < 0.01$), SP5 ($p < 0.01$), and SP9 ($p < 0.01$) groups, while NADH dehydrogenase activity decreased in the SP2 ($p < 0.05$) and SP9 ($p < 0.05$) groups.
Conclusion: Acupuncture at the SP9 acupoint influenced the mitochondrial fission pathway by modulating *PGC-1α* and *Fis1* mediators in the rat spleen under non-disease conditions.

Keywords: Acupuncture, Mitochondrial fission, Spleen, *PGC-1α*, *Fis1*, NADH dehydrogenase

INTRODUCTION

Mitochondria play a critical role in cellular homeostasis by generating the majority of energy required for cellular functions, while also undergoing continuous fusion and fission events [1]. These dynamic processes of mitochondrial fission and fusion contribute to the regulation of mitochondrial morphology and function; however, impaired or imbalanced processes are linked to various diseases, including cancer and spleen-related disorders [2]. The spleen, a secondary lymphoid organ, is responsible for capturing and eliminating pathogens through innate and adaptive immunity. Previous research has demonstrated that alterations in specific genes are associated with the preservation of mitochondrial function in splenocytes [3].

Various approaches have been developed to explore the functional activity of diseases and mitochondria. Among these, acupuncture is a well-known method that stimulates

specific acupoints along meridians, which connect the skin surface and internal organs, and is being investigated for its potential effects on mitochondrial changes [4]. Recent studies have revealed that acupuncture may play a significant role in the prevention and treatment of AD by modulating energy metabolism and mitochondrial dynamics [5]. Other research has reported that electro-acupuncture at the SP9 acupoint increased mean blood flow and perfusion in the rat spleen [6], while additional studies have shown that acupuncture can reduce liver damage by regulating genetic factors related to mitochondrial energy metabolism [7]. In Parkinson's disease research, mice treated with acupuncture exhibited significant recovery in terms of reduced lipid ratios and improved mitochondrial structure [8]. These recent investigations highlight the substantial impact of acupuncture on the recovery from various diseases by modulating and influencing mitochondrial function.

Although the spleen is less prone to diseases compared to other organs, there are limited acupuncture studies focusing on splenic meridians for spleen-related disorders. Nonetheless, numerous studies have reported the effects of acupuncture on spleen meridians and the importance of the spleen. One study examining the spleen meridian revealed that SP6 acupressure could reduce anxiety and the need for painkillers in pain management [9]. Chou et al. [6] reported that the average blood flow and perfusion of the spleen increased during SP9 stimulation, while no such increase was observed in the liver. These findings provide scientific evidence supporting the organ-specific effects of acupuncture according to traditional medicine principles.

Despite the growing body of research on the relationship between acupuncture and mitochondria, as well as between acupuncture and the spleen, there is a scarcity of studies examining the connection between acupuncture, the spleen, and mitochondria. The effects of acupuncture-mediated mitochondrial fission and fusion in splenocytes remain to be elucidated. Additionally, it is crucial to observe mitochondrial changes in disease-free animal models and investigate the underlying mechanisms of therapeutic effects in pathological models. Consequently, this study aims to explore the mechanisms of acupuncture in relation to alterations in mitochondrial fission and fusion factors in the disease-free rat spleen.

MATERIALS AND METHODS

1. Animal subjects

Seven-week-old male Sprague Dawley (SD) rats (Samtaco, Osan, Korea), weighing 260 g, were acclimated for one week in a temperature- and humidity-controlled chamber (23 ± 1℃, 60 ± 5% humidity) prior to the experiment. During this period, the rats had ad libitum access to food and water. All animal care and experimental protocols were approved by the Dongshin University College Animal Management and Use Commission (Approval numbers: DSU-2018-02-02, DSU-2019-05-01).

2. Group assignment and acupuncture stimulation

Rats were randomly assigned to one of six groups: a control group (n = 4), which received no treatment, and SP1 (n = 4), SP2 (n = 4), SP3 (n = 4), SP5 (n = 4), and SP9 groups (n = 4), which underwent acupuncture at SP1, SP2, SP3, SP5, and SP9 acupoints, respectively. Acupuncture was administered once daily for four days under 2% isoflurane anesthesia (Hana Pharm, Hwaseong, Korea). The control group received anesthesia only, with no acupuncture stimulation. Manual stimulation involved 27 clockwise torsions over 10 minutes; acupoint locations are detailed in Table 1 and Fig. 1 [10].

3. Tissue collection

One day after the completion of acupuncture treatment, rats were euthanized, and spleen tissues were harvested during post-mortem examinations. Tissues were rinsed in saline and stored at –80℃.

Table 1. Location of acupoints

Acupoint	Method of location of acupoints
SP1	On the great toe, medial to the distal phalanx, 0.1 F-cun proximal-medial to the medial corner of the toenail, at the intersection of the vertical line of the medial border and horizontal line of the base of the toenail.
SP2	On the great toe, in the depression distal to the first metatarsophalangeal joint, at the border between the red and white flesh.
SP3	On the medial aspect of the foot, in the depression proximal to the first metatarsophalangeal joint, at the border between the red and white flesh.
SP5	On the medial aspect of the foot, anteroinferior to the medial malleolus, in the depression midway between the tuberosity of the navicular bone and the prominence of the medial malleolus.
SP9	On the tibial aspect of the leg, in the depression between the inferior border of the medial condyle of the tibia and the medial border of the tibia.

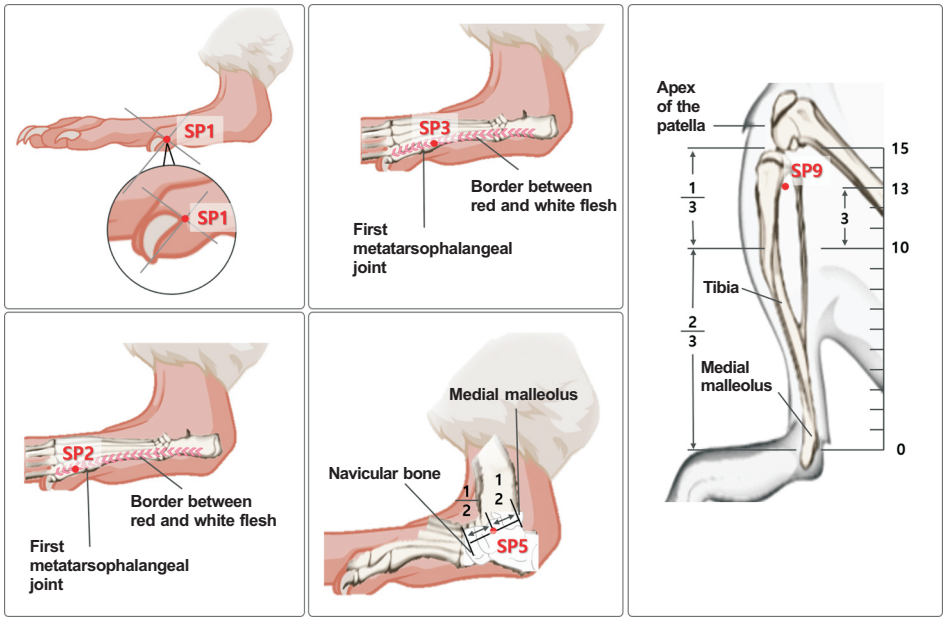

Fig. 1. Illustration of SP1, SP2, SP3, SP5, and SP9 acupoints located at rat with reference anatomical structures.

4. Mitochondrial protein concentrations and NADH dehydrogenase activity

Mitochondrial protein concentrations and NADH dehydrogenase activity in spleen tissues were assessed using a mitochondria isolation kit (Thermo Fisher, Rockford, USA) and an NADH dehydrogenase (complex I) enzyme activity microplate assay kit (Abcam, Cambridge, UK), following the manufacturers' instructions.

Table 2. Primer sequences

Target gene	Primer sequence
GAPDH	F: 5′-GGC ACA GTC AAG GCT GAG AAT G-3′ R: 5′-ATG GTG GTG AAG ACG CCA GTA-3′
PGC-1α	F: 5′-GGC ACA GTC AAG GCT GAG AAT G-3′ R: 5′-ATG GTG GTG AAG ACG CCA GTA-3′
Fis1	F: 5′-TTT GAA TAC GCC TGG TGC CT-3′ R: 5′-TAC CTT TGG GCA ACA GCT CC-3′

5. RNA isolation and qRT-PCR

Total RNA (1 μg) was extracted from 50 mg of spleen tissue using 800 μl of Trizol isolation reagent (Thermo Fisher Scientific, Waltham, USA). RNA concentration was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA). RNA was then reverse-transcribed into cDNA using a cDNA synthesis Master Mix (LeGene Biosciences, San Diego, USA). Real-time PCR reactions were conducted on the CFX Connect Real-Time PCR Detection System (Bio Rad, Hercules, USA) using SB-Green qPCR Master Mix (LeGene Biosciences, San Diego, USA). *PGC-1α* and *Fis1* sequences are shown in Table 2.

Thermal cycling parameters were as follows: 95℃ for 2 minutes, followed by 40 cycles at 95℃ for 10 seconds, annealing at 60℃ for 15 seconds, extension at 60℃ for 30 seconds, and a melting curve analysis performed at 95℃ for 10 seconds and 65℃ for 5 seconds. Results were expressed as fold change and calculated using the comparative $2^{-\Delta\Delta CT}$ method [11].

6. Western blotting

Spleen tissues (50 mg) were lysed in 600 μl protein extraction solution (IntronBio, Sungnam, Korea), and protein concentrations were determined using a bicinchoninic acid assay kit (Thermo Fisher). Proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked with 5% skim milk-TBST for 1 hour at room temperature and incubated with primary antibodies: *Drp1* (1:1000, Abcam, Cambridge, UK), *OPA1* (1:300, Abcam, Cambridge, UK), *MFN1* (1:500, Thermo Fisher Scientific, Waltham, USA), *MFN2* (1:500, Thermo Fisher Scientific, Waltham, USA), and *β-actin* (1:1000, Thermo Fisher Scientific, Waltham, USA) at 4℃ overnight. The following day, membranes were incubated with a per oxidase-conjugated affinipure goat anti-rabbit IgG antibody (1:10000, Jackson Immuno Research, West Grove, USA) for 1 hour at 25℃. Band intensity was quantified using the Amersham Imager 600 (GE Life Sciences, Piscataway, USA).

7. Statistical analysis

Data were presented as mean ± standard deviation and analyzed using GraphPad Prism software (GraphPad Software version 8.4.1, San Diego, USA). Statistical significance was assessed using one-way ANOVA, followed by the post

hoc Dunnett test. Experimental group data were compared with control group data at $\alpha = 0.05$ ($p < 0.05$) and $\alpha = 0.01$ ($p < 0.01$) significance levels.

RESULTS

1. *PGC-1α* and *Fis1* gene expression

Relative to the control group, a significant reduction in *PGC-1α* expression levels was observed in spleen tissue of the SP1 ($p < 0.01$), SP5 ($p < 0.05$), and SP9 ($p < 0.05$) groups. Conversely, *Fis1* expression levels exhibited a significant increase in the SP1 ($p < 0.01$), SP5 ($p < 0.01$), and SP9 ($p < 0.05$) groups in comparison to the control group (Fig. 2).

2. *DRP1*, *OPA1*, *MFN1*, and *MFN2* protein expression

No significant alterations in the levels of *DRP1*, *OPA1*, *MFN1*, and *MFN2* were detected among any of the groups compared to the control group (Fig. 3).

3. Mitochondrial protein concentrations

In comparison to the control group, mitochondrial protein concentrations within spleen tissue demonstrated a significant decrease in the SP2 ($p < 0.01$), SP3 ($p < 0.01$), SP5 ($p < 0.01$), and SP9 ($p < 0.01$) groups (Fig. 4).

4. NADH dehydrogenase activity

Relative to the control group, the activity of NADH dehydrogenase in spleen tissue was diminished in the SP2 ($p < 0.05$) and SP9 ($p < 0.05$) groups (Fig. 5).

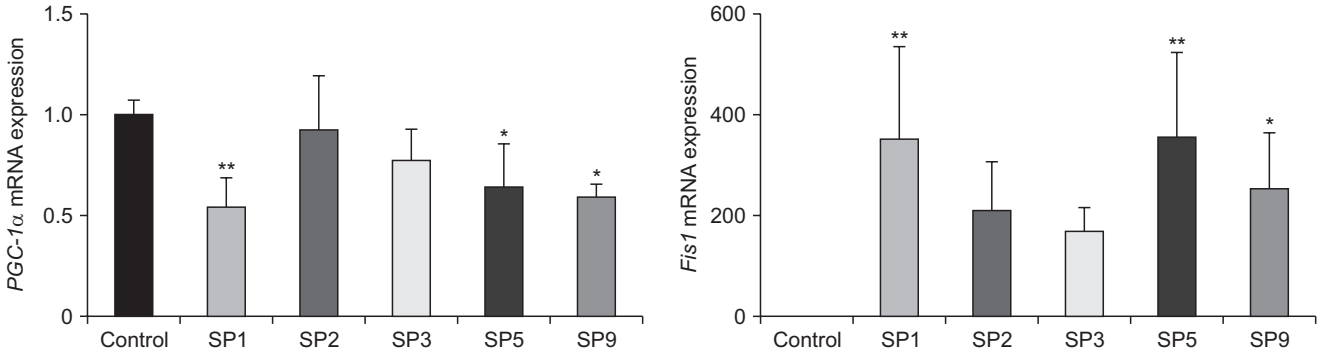

Fig. 2. Effect of acupuncture at acupoints on *PGC-1α* and *Fis1* in rat spleen. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$ vs. Control group.

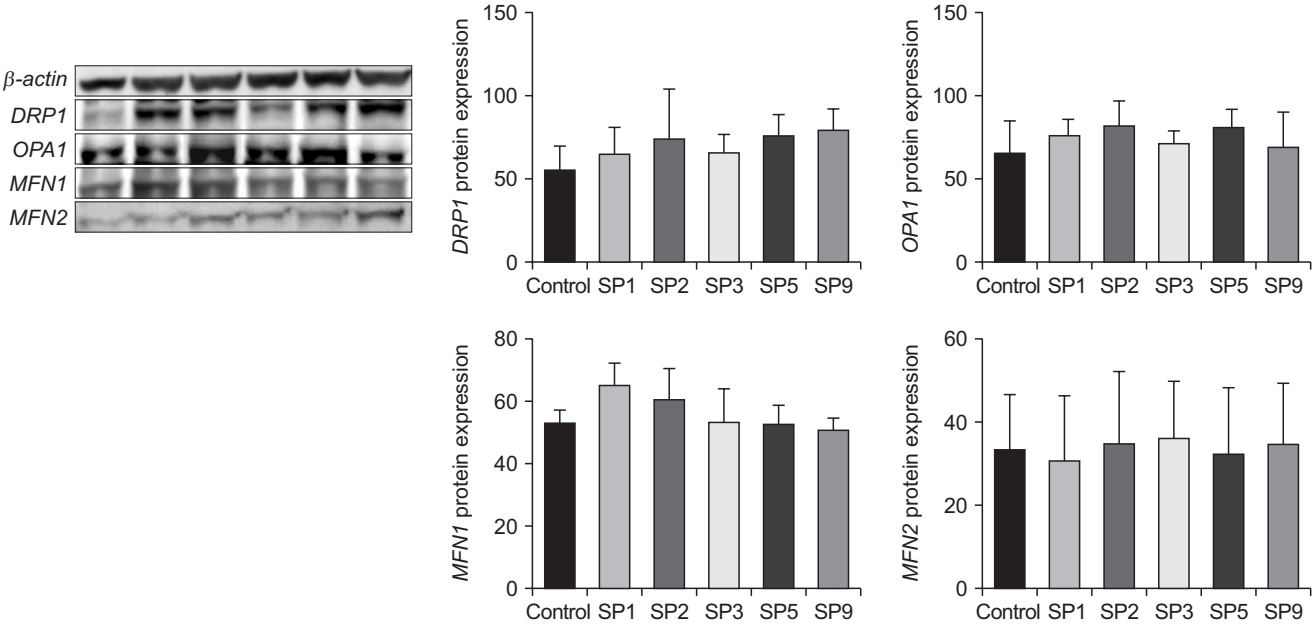

Fig. 3. Effect of acupuncture at acupoints on *DRP1*, *OPA1*, *MFN1*, and *MFN2* in rat spleen. Data are presented as mean ± SD.

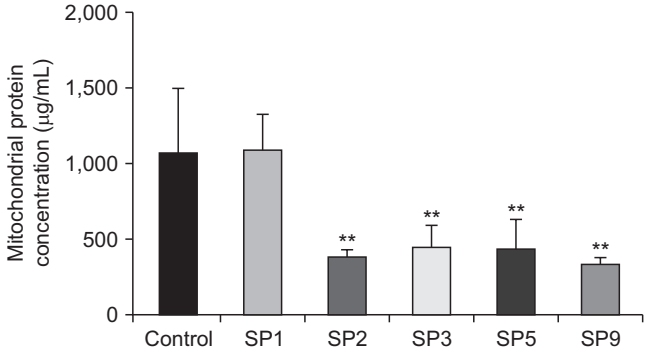

Fig. 4. Effect of acupuncture at acupoints on mitochondrial protein concentration in rat spleen. Data are presented as mean ± SD. $^{**}p < 0.01$ vs. Control group.

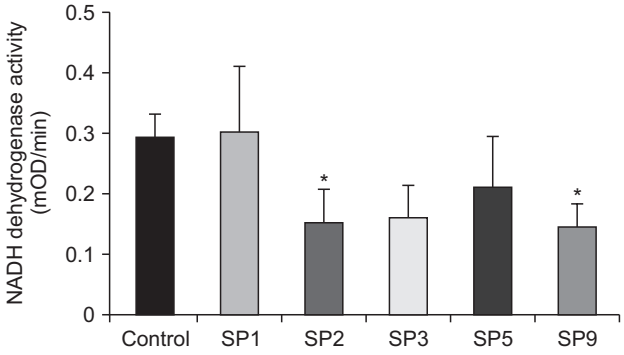

Fig. 5. Effect of acupuncture at acupoints on NADH dehydrogenase activity in rat spleen. Data are presented as mean ± SD. $^{*}p < 0.05$ vs. Control group.

DISCUSSION

The mitochondrion is a fundamental organelle that supplies ATP energy for cellular respiration [12]. Maintaining a balanced mitochondrial output is essential for the smooth supply of energy to cells, which is achieved via protein-mediated mitochondrial fission and fusion pathways, crucial for sustaining normal cell functions [13]. Key proteins regulating this balance include fission factors *Fis1* and *DRP1*, and fusion proteins *OPA1*, *MFN1*, and *MFN2*, which bind to inner and outer mitochondrial membranes (IMM and OMM, respectively) [14]. Additionally, *PGC-1α* is a central regulator of mitochondrial biogenesis among the many genes facilitating mitochondria-related fission and fusion, activating mitochondrial biogenesis continuously during damaged mitochondria recovery phases to provide healthy organelles [15].

Our examination of mitochondrial fission and fusion processes in the spleen during acupuncture stimulation of spleen meridians revealed that *PGC-1α* levels decreased in the SP1, SP5, and SP9 groups compared to the control group. *Fis1* levels significantly increased in the SP1, SP5, and SP9 groups compared to the control group, while *DRP1*, *OPA1*, *MFN1*, and *MFN2* levels remained unchanged. Although *Fis1* and *DRP1* data did not exhibit similar trends, it is generally accepted that *Fis1* interacts with *DRP1* and may act as its receptor [16]. In mammals, Fis1 is not specifically required for *DRP1*, as *DRP1* or dynamin 2 (*Dyn2*) have been implicated in independent mitochondrial fission [17]. This observation suggests that acupuncture may contribute to mitochondrial fission independently of *DRP1*.

Mitochondria are critical organelles since they generate ATP and regulate calcium responses by discarding damaged mitochondria through fission and regenerating mitochondria via fusion. However, mitochondrial numbers and concentrations play crucial roles in maintaining homeostatic functions, as organelle dysfunction and defects are associated with various diseases, neurological abnormalities, and abnormal metabolic states [18]. Mitochondrial dysfunction primarily affects the electron transport chain. NADH dehydrogenase, the first enzyme in this chain, represents the entry point for electrons into the respiratory chain and plays central roles in cell metabolism and energy generation. In contrast, NADH dehydrogenase defects can cause changes in tissues with high metabolic rates [19]. In our study, mitochondrial concentrations significantly decreased in the SP2, SP3, SP5, and SP9 groups, and NADH dehydrogenase activity also showed significant decreases in the SP2 and SP9 groups. These observations may have resulted from decreased *PGC-1α* and increased *Fis1* levels. In particular, acupuncture at the SP9 acupoint reduced mitochondrial concentrations and NADH dehydrogenase activity by acting on *PGC-1α* and *Fis1*.

In Korean medicine, the spleen is considered to be involved in ingestion and digestion, governs the blood, and provides nutrients to the whole body. Previous research has experimentally demonstrated the effects of acupuncture on the spleen, such as Sun et al.'s report [20] that acupuncture of the five Shu acupoints in the spleen meridian can lower blood uric acid levels by promoting uric acid excretion and increasing urine volume. Our study's results suggest that acupuncture on SP9 is related to mitochondrial activation in the spleen as one of its regulatory mechanisms, as also reported by Sun et al. [20]. Furthermore, the results indicate that SP9 is an acupoint that can affect organs under normal physiological conditions, as stated in Chang's study [21].

Based on our data, we observed the expression of genetic factors related to mitochondrial fission upon acupuncture in disease-free rat spleens. If additional in-depth studies on disease models and mitochondrial morphology are conducted, cell diseases could be treated by regulating mitochondrial fission factors through acupuncture.

CONCLUSIONS

In this study, we investigated the impact of acupuncture on mitochondrial fission and fusion processes within the spleen of healthy rats. Our findings revealed that acupuncture in all experimental groups (SP1, SP2, SP3, SP5, and SP9) predominantly enhanced factors associated with fission rather than fusion. Specifically, acupuncture at the SP9 point resulted in the inhibition of mitochondrial protein concentrations and NADH dehydrogenase activity by modulating the activity of *PGC-1α* and *Fis1*.

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AUTHORS' CONTRIBUTIONS

Yu-Mi Lee: Writing- Original draft preparation, Validation. Dong-Hee Choi: Writing- Methodology, Verification, and experiment. Jeong-Hye Park: Experiment. Min-Woo Cheon: Reviewing and Editing. Jae Gwan Kim: Reviewing and Editing. Jeong-Sang Kim: Reviewing and Editing. Taejin Choi: Conceptualization. Hye-Ran Kim: Conceptualization. Daehwan Youn: Writing, Supervision, Conceptualization.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Yu-Mi Lee, https://orcid.org/0000-0001-9190-2357
Dong-Hee Choi, https://orcid.org/0000-0002-2418-9560
Jeong-Hye Park, https://orcid.org/0000-0002-6384-3908
Min-Woo Cheon, https://orcid.org/0000-0002-0979-4316
Jae Gwan Kim, https://orcid.org/0000-0002-1010-7712
Jeong-Sang Kim, https://orcid.org/0000-0003-3984-6049
Taejin Choi, https://orcid.org/0000-0002-9911-9567
Hye-Ran Kim, https://orcid.org/0000-0002-0658-3345
Daehwan Youn, https://orcid.org/0000-0002-5522-6723

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