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# Influence of Acupuncture on Expression of Mitochondrial Fusion and Fission Mediators in Rat Liver

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

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## ABSTRACT

**Background:** We aimed to elucidate the mechanism of change in mitochondrial fusion- and fission-related mediators for maintaining cell function through the effects of acupuncture treatment and apply findings to a liver disease model. We focused on the optic atrophy-1 (*OPA1*) and fission protein 1 (*Fis1*) genes of rat liver cells.

**Methods:** Sprague Dawley rats were divided into a control group (no treatment) and LR2, LR3, LR4 and LR8 groups (acupuncture treatment to those points). Acupuncture was performed on each point for 10 minutes once daily for 4 days. Changes in the mRNA expression of Peroxisome proliferator-activated receptor-gamma coactivator 1-α (*PGC-1α*) and *Fis1* were observed *via* quantitative real-time polymerase chain reaction (qRT-PCR); changes in *OPA1,* mitofusin-2 (*MFN2*), mitofusin-1 (*MFN1*), dynamin-related protein 1 (*DRP1*) and adenosine monophosphate-activated protein kinase (*AMPK*) proteins were observed through western blotting and endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) expressions were observed through immunohistochemistry.

**Result:** *OPA1* decreased in the LR3 and LR8 groups and *Fis1* increased in the LR2 and LR4 groups. *AMPK* and *PGC-1α* decreased significantly in all acupuncture groups. eNOS and nNOS expression reduced in all acupuncture groups. Therefore, acupuncture can regulate mitochondrial fusion/fission by influencing the following mediators: *AMPK, PGC-1α, OPA1, Fis1*, eNOS and nNOS.

**Key words:** Acupuncture, *AMPK*, eNOS, *Fis1,* nNOS, *OPA1*, *PGC-1α*.

## INTRODUCTION

Acupuncture is a method which has been used in various diseases by selecting specific acupoints. Especially it is an effective treatment for liver diseases related to mitochondrial function, including various types of obesities (Cho *et al.*, 2009; Shu *et al.*, 2020; Meng *et al.*, 2019) and chronic inflammation (Lim *et al.*, 2020; Ma *et al.*, 2020; Li *et al.*, 2019; Wang *et al.*, 2013; Liu *et al.*, 2015). Mitochondria-mediated signaling pathways play an important role in hepatocellular survival and problems associated with this pathway are known to be a major cause of hepatocellular damage. Therefore, observing changes in gene expression related to mitochondrial function *in vivo* can help in the treatment of liver diseases (Grattagliano *et al.*, 2011).

To explore the mechanisms of treatment effects in the pathological model of acupuncture, it is necessary to observe mitochondrial changes, a key component of cell function in normal animal models. In this study, the mechanism of acupuncture related to mitochondrial fusion and fission gene changes was investigated, whereupon we examined the gene expression related to the pathways of *AMPK* and *PGC-1α* in liver cells, eNOS and nNOS.

In this study, we focused on several mRNAs and proteins related to fission and fusion to observe the effects of acupuncture on mitochondrial fission and fusion in the livers of rats. Therefore, we first observed the mRNAs of *PGC-1α* and *Fis1* and we tried to explore experimental evidence that acupuncture regulates the mediators involved in mitochondrial fission and fusion by observing and reaffirming protein factors related to mitochondrial fission and fusion, such as *AMPK*.

[1]Department of Biomedical Science and Engineering, Gwangju Institute of Science and Technology, Bukgu, Gwangju-61005, Republic of Korea.
[2]Department of Korean Medicine, Dongshin University, Naju, Jeollanamdo-58245, Republic of Korea.
[3]Department of Health Administration, Dongshin University, Naju, Jeollanamdo-58245, Republic of Korea.
[#]These authors contributed equally to this work.

**Corresponding Author:** Daehwan Youn, Dongshin University, Naju, Jeollanamdo-58245, Republic of Korea.
Email: human22@dsu.ac.kr

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## MATERIALS AND METHODS

Twenty seven-week-old Sprague-Dawley male rats (Samtaco, Korea) weighing 260 g were used for animal testing in the experiment. This research was conducted from 2019 to 2020 at the laboratory of Acupoint and Meridian, Korean Medicine School of Dongshin University, Republic of Korea. All animal care and experimental protocols were

approved by the College Animal Management and Use Commission of Dongshin University (Approval numbers, DSU-2019-05-01 and DSU-2020-02-01). The rats spent 1 week adjusting to a temperature of 23°C±1°C and humidity of 60%±5% in a temperature- and humidity-controlled chamber. During the adjustment period, the rats freely fed upon sufficient amounts of pellets and water.

The rats were randomly divided into five groups: the control group (n=4), which received no treatment and the LR2 (n=4), LR3 (n=4), LR4 (n=4) and LR8 groups (n=4), which received acupuncture at each of the LR2, LR3, LR4 and LR8 acupoints. All the rats were kept under isoflurane (Hana Pharm, Korea) anesthesia. They were punctured 4 times in total for 4 days (1 per day). The LR2 acupoint is located at the border between the first and the second toe. the LR3 acupoint is located between the first and second metatarsal bones and the LR4 acupoint is located in the anterior of the medial malleolus, in the depression medial to the tibialis anterior tendon. The LR8 acupoint is located in the medial depression of the tendons of the semitendinosus of the semimembranosus muscles semimembranosus muscles (World Health Organization, 2008).

All rats were sacrificed and post-mortem examination was performed immediately. Liver tissues were dissected out, washed in saline and stored at -80°C until analysis. Total RNA (1 µg) was extracted from liver tissues (50 mg) using trizol isolation reagent (Invitrogen, USA). RNA concentration was quantified using Qubit 4 (Invitrogen, USA). RNA (1 µg) was reverse transcribed into cDNA using a cDNA synthesis Master Mix (Legene, USA). Real-time PCR reactions were performed on the CFX Connect Real-Time PCR Detection System (Bio Rad, USA) using SB-Green qPCR Master Mix (Legene, USA). *PGC-1α* and *Fis1* sequences are shown in Table 1. The thermal cycling conditions for the genes were as follows: denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 10 sec, annealing at 60°C for 15 sec and extension at 60°C for 30 sec. Melting curve analysis was performed at 95°C for 10 sec and 65°C for 5 sec. Results are expressed as the fold changes calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Liu *et al*., 2020). Liver tissues (50 mg) were lysed using protein extraction solution (iNtronbio, Korea) and quantified using a bicinchoninic acid assay kit (Pierce, USA). The following proteins were visualized (Table 2). To visualize proteins, the western blotting method was performed overnight at 4°C and using peroxidase-conjugated AffiniPure goat anti-rabbit IgG (1:10000; Jackson Immuno research, USA) for 1 hr at 25°C. Band intensity was quantified using the Amersham Imager 600 (GE Life Sciences, USA).

Liver tissues were fixed in 10% formalin solution. The formalin-fixed paraffin-embedded tissue sections were deparaffinized, rehydrated in an ethanol series and subjected to epitope retrieval (Sayed *et al*., 2021). For eNOS and nNOS immunostaining, the sections were incubated with a primary antibody (anti-eNOS antibody: Invitrogen, USA, 1:100; anti-nNOS antibody: Invitrogen, USA, 1:8) overnight at 4°C. The sections were then washed 3 times with 0.01 M PBS and treated for 15 min with HRP-conjugated goat anti-rabbit IgG (Vectastain ABC Kit: Vector Labs, USA) at room temperature. The tissues were washed 3 times with 0.01 M PBS and incubated with HRP-conjugated streptavidin (Vectastain ABC Kit: Vector Labs, USA) for 15 min at room temperature. The sections were then washed 3 times with 0.01 M PBS and stained with DAB (Vector Labs, USA). Immunoreactivity was examined using Celleste software (Invitrogen, USA) (Zong *et al*., 2019).

GraphPad Prism 8.4.1 software (San Diego, California, USA) was used for computation and statistical analysis. All results were expressed as mean±SD and were tested for distribution (Dunnett's multiple comparisons test, P-value). In addition, $p<0.05$ indicated statistical significance.

## RESULTS AND DISCUSSION

Fig 1 shows that there was a significant decrease in *OPA1*, a mitochondrial fusion gene, in LR3 and LR8 groups. A significant increase was observed in *Fis1*, a mitochondrial fission gene, in LR2 and LR4 groups. A significant decrease was observed in *AMPK* and *PGC-1α* expressions, one of the mechanisms controlling mitochondrial fusion and fission, in all acupuncture groups. Mitochondria are very important organelle required to produce energy in the form of ATP through the process of cellular respiration. Therefore, the balanced adjustment of mitochondrial forms is important for the continuous supply of energy in cells, which is achieved through the mitochondria fusion and fission processes in the quantitative and qualitative adjustments of various regulatory proteins.

*OPA1* plays an important role in maintaining the mitochondrial crista structure and reduces damage to mitochondrial DNA (mtDNA), proteins and lipids (Youle and Van Der Bliek, 2012). Additionally, while *OPA1* can regulate proliferating cells, *OPA1* knock-down inhibits mitochondrial fusion. A previous study reports that this inhibition is used as a therapeutic mechanism to debilitate the growth of cancer cells *in vitro* (Li *et al*., 2020). In the present study, the possibility of controlling activation of fusion in mitochondrial hepatocytes was observed through

**Table 1:** The nucleotide primer sequences.

| Genes | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---|---|---|
| *PGC-1 α* | GGCACAGTCAAGGCTGAGAATG | ATGGTGGTGAAGACGCCAGTA |
| *Fis1* | TTTGAATACGCCTGGTGCCT | TACCTTTGGGCAACAGCTCC |
| *GAPDH* | GGCACAGTCAAGGCTGAGAATG | ATGGTGGTGAAGACGCCAGTA |

Abbreviations: *PGC-1α,* Peroxisome proliferator-activated receptor-gamma coactivator 1-*α*; *Fis1,* fission protein 1.

**Table 2:** Protein information.

| Protein | Dilution | Company |
|---|---|---|
| *OPA* | 1 : 300 | Abcam, UK |
| *MFN2* | 1 : 500 | Invitrogen, USA |
| *MFN1* | 1 : 500 | Invitrogen, USA |
| *DRP1* | 1 : 1000 | Abcam, UK |
| *AMPK* | 1 : 1000 | Cell Signaling Technology, USA |
| *β-Actin* | 1 : 1000 | Invitrogen, USA |

Abbreviations: *OPA*: Optic atrophy 1; *MFN2*: Mitofusion 2; *MFN1*: Mitofusion 1; *DRP1*: Dynamin-related protein; *AMPK*: Adenosine monophosphate-activated protein kinase.

reduced expression of *OPA1* through acupuncture of LR3 and LR8 acupoints.

During cell-growth inhibition, the mitochondrial fission gene *Fis1* present in the outer mitochondrial membrane is a protein that acts as a receptor for *DRP1*. This means that a greater amount of *DRP1* is moved around the mitochondrial membrane *via DRP1* phosphorylation. Therefore, mitochondrial fission is promoted as a result of binding with mitochondria fission factor and *Fis1* protein (Youle and Van Der Bliek, 2012). In this study, we observed that acupuncture on acupoints LR2 and LR4 increases only *Fis1* and contributes to mitochondrial fission activation

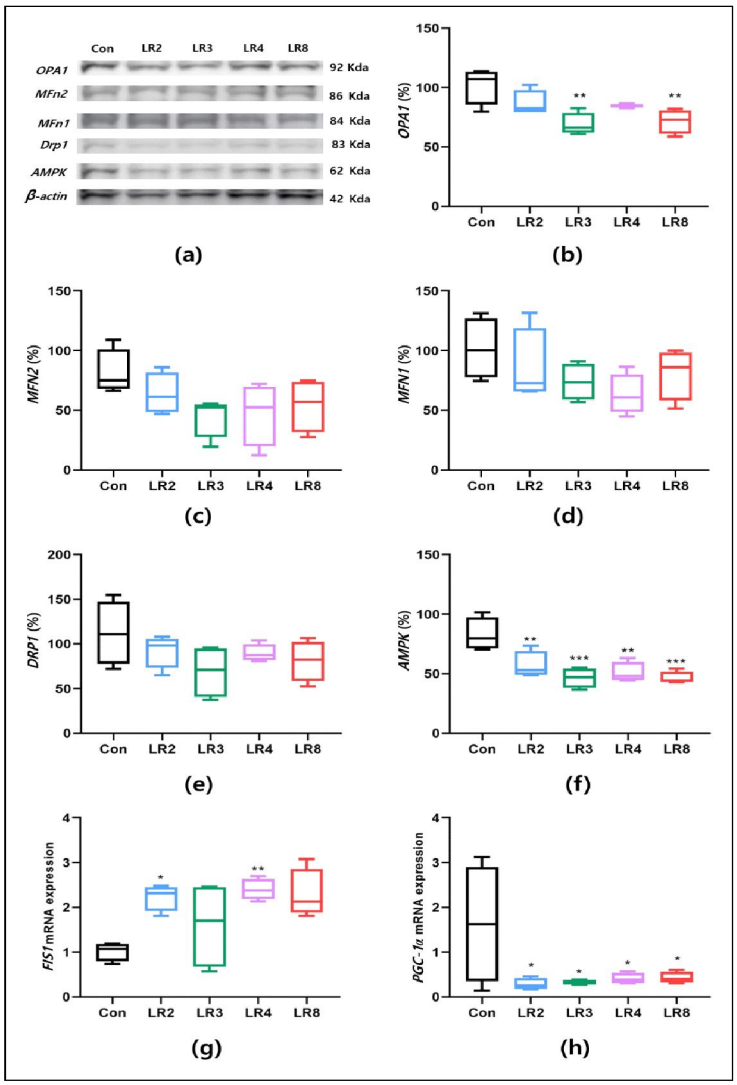


**Fig 1:** Acupuncture-related changes in the gene expressions of *OPA1, MFN2, MFN1, DRP1, AMPK, Fis1* and *PGC-1α* in rat livers. Protein expressions of *OPA1* (b), *MFN2* (c), *MFN1* (d), *DRP1* (e) and *AMPK* (f) detected with western blotting. mRNA levels of *Fis1* (g) and *PGC-1α* (h) were analyzed by qRT-PCR. Con: Control group with no treatment, LR2, LR3, LR4 and LR8: Treatment groups that received acupuncture on those respective acupoints. Data are presented as mean; SEM.
*p<0.05; **p<0.01; ***p<0.001 compared with the Con group.

independently of *DRP1*. This occurs because acupuncture of acupoints LR2, LR3, LR4 and LR8 affects different gene factors, but these factors are involved in activation of mitochondria function.

Several studies have reported that decrease in *OPA1* and increase in *Fis1* induce abnormal cell function (Li *et al*., 2020; Suzuki *et al*., 2003; Bi *et al*., 2019) and that acupuncture can be used for treatment of hyperactive diseases such as liver disease through inhibition of mitochondrial fusion and promoting fission (Hernández-Alvarez and Zorzano, 2021; Senft and Ronai, 2016). These studies provide important context in interpreting the results from the present study.

Immunohistochemical analysis revealed that the expression of eNOS and nNOS, which are important regulatory mechanisms involved in mitochondrial biogenesis, decreased in all acupuncture groups (Fig 2). NO-mediated regulation of mitochondrial fusion and fission is diffused between the cytoplasm and mitochondria and interaction with other molecules or proteins signal physiological stimuli or promote cell growth (Nisoli *et al*., 2003; Barsoum *et al*., 2006). NO is produced *via* NO

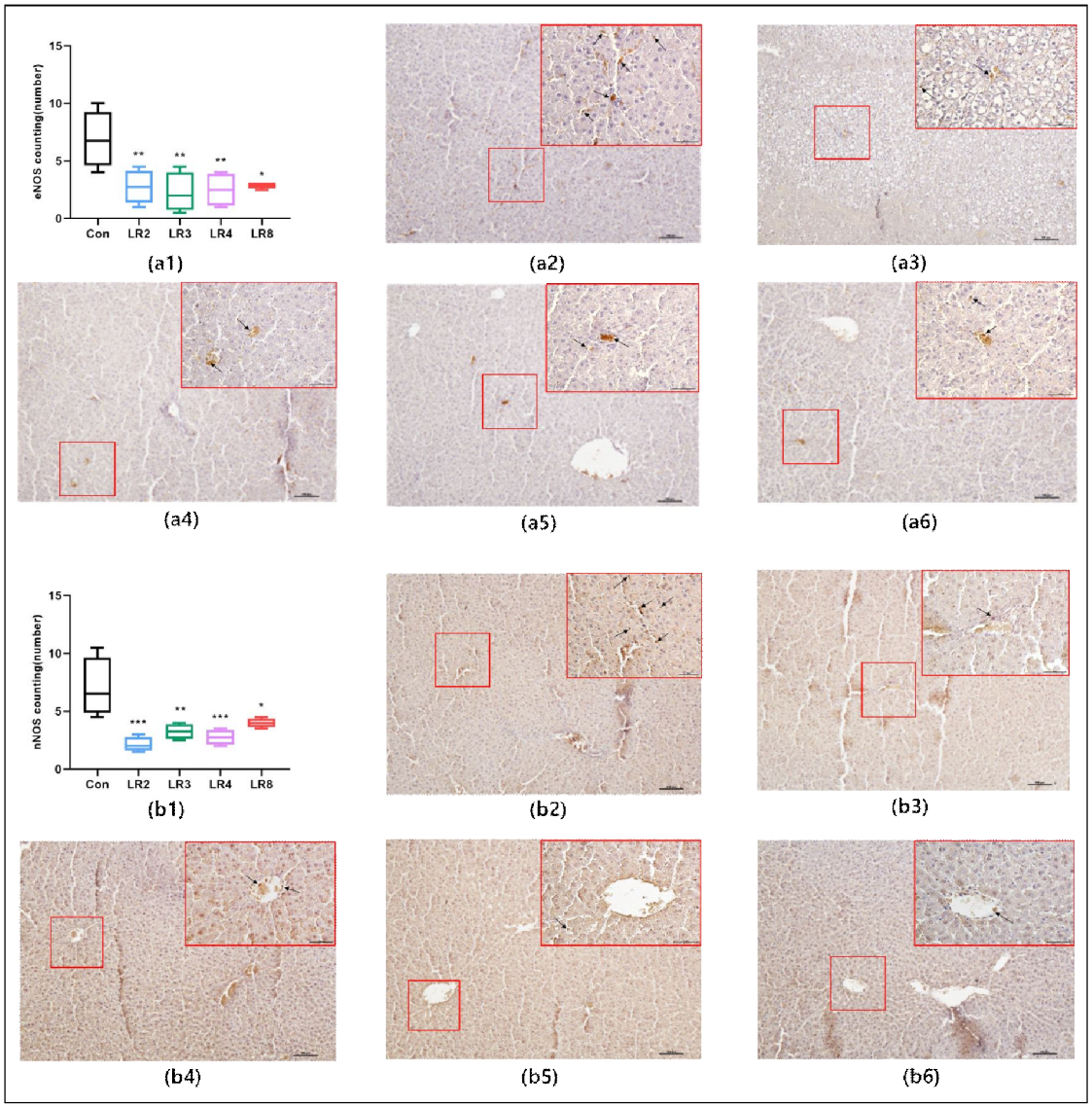


**Fig 2:** Immunohistological microphotographs (×10,×40 magnification) of liver tissues observing the effect of acupuncture on mitochondrial eNOS and nNOS expression.

a1: con, a2, a3, a4, a5 and a6: Photographs of eNOS expression in the LR2, LR3, LR4 and LR8 groups that received acupuncture treatment on those acupoints, respectively. b1: con, b2, b3, b4, b5 and b6: Photographs of nNOS expression in the LR2, LR3, LR4 and LR8 groups that received acupuncture treatment on those acupoints, respectively.

Data are presented as mean; SEM. *p<0.05; **p<0.01; ***p<0.001 compared with the Con group. Scale bars μ=50 μm; v=100 μm.

synthases (NOS) of the three main isoforms eNOS, nNOS and inducible NOS (iNOS) (Tengan *et al.*, 2012). In this study, the expression of both eNOS and nNOS decreases in rats subjected to acupuncture on the LR2, LR3, LR4 and LR8 acupoints. Our results also demonstrate the possibility that acupuncture can control mitochondrial fusion and fission functions through the NOS mechanism. These results are consistent with a study which reported that mitochondrial biosynthesis, fusion and fission are all affected by NOS suppression (Miller *et al.*, 2013).

Inactive *AMPK* reduces the activity of the *PGC-1α* pathway, thereby decreasing mitochondrial biosynthesis signals and reducing the mitochondrial content of the cell. It also promotes the decrease of *OPA1* and the increase of *Fis1* levels owing to the decrease in *PGC-1α* activity (Yu and Yang, 2010; Singh *et al.*, 2016). The molecular mechanisms of NO-dependent regulation of *PGC-1α* and mitochondrial biosynthesis are related to *AMPK*. In addition, much cellular energy stresses such as exercise, starvation, or mitochondrial dysfunction that increase AMP by decreasing ATP enhance *AMPK* activity and promote mitochondrial biosynthesis. In contrast, *AMPK*, when down-regulated, suppresses abnormal cell proliferation (Steinberg and Kemp, 2009; Cui *et al.*, 2018). In particular, in mice subjected to eNOS knock-down, musculoskeletal mitochondrial biosynthesis was reduced and cGMP induction of *PGC-1α, AMPK* and mitochondrial biosynthesis was disrupted (Tedesco *et al.*, 2010; Chen *et al.*, 2010).

Our research confirmed the decrease in *OPA1*, eNOS and nNOS expression and the increase in *Fis1* expression due to acupuncture treatment LR2, LR3, LR4 and LR8 acupoints and these changes were found to be caused by changes in *AMPK/PGC-1* signaling pathway. We suggest that acupuncture can influence mitochondrial fusion and fission pathways by triggering mediators of *AMPK, PGC-1α, OPA1* and *Fis1* in the rat liver of non-disease conditions. Additional in-depth studies in disease model applications and in the morphological observation of mitochondria will provide an opportunity to apply this research to the treatment of hepatocellular diseases through the regulation of mitochondrial fusion and fission factors.

## CONCLUSION

In this study, the effect of acupuncture on the fusion and fission of mitochondria in liver cells in normal rats was observed. Our results indicate that acupuncture on points LR3 and LR8 reduced *OPA1* expression, thereby controlling activation or activity of mitochondrial fusion. Furthermore, LR2 and LR4 acupuncture increased *Fis1* expression and activated mitochondrial fission. These acupuncture effects were observed to be caused by the reduction of eNOS, nNOS, *AMPK* and *PGC-1α*.

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**Conflict of interest:** None.

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