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Effects of Acupuncture on the mRNA Expression of *NF-κB p65/TNF-α* Activated by Fat Accumulation in High-fat Diet-induced Nonalcoholic Fatty Liver Disease Rats

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ABSTRACT

Background: We studied the effects of manual acupuncture (MA) and warm acupuncture (WA) on early-stage nonalcoholic steatohepatitis in relation to fat accumulation by observing the mRNA expression of nuclear factor-κB (*NF-κB*) p65 and tumor necrosis factor-α (*TNF-α*) in a rat model with nonalcoholic fatty liver disease (NAFLD) induced by high-fat diet.

Methods: The experiment was performed using Sprague Dawley rats randomly divided into control (CON), NAFLD, NAFLD + MA and NAFLD + WA groups. Acupuncture was performed on the SP9 + BL18 acupoint from each group for 5 min, twice a week for 8 weeks. Total cholesterol (TC) was measured in the serum and triglyceride (TG) and resistin were measured in the hepatic tissue. In addition, hematoxylin and eosin stain, Masson's trichrome stain, Oil red O stain and perilipin immunohistochemistry were used for histological analysis. Finally, quantitative polymerase chain reactions were performed to observe the changes in *TLR4*, *NF-κB p65* and *TNF-α*.

Result: Resitin and *TLR4* decreased in the NAFLD + WA group. The NAFLD + MA group exhibited reduced levels of TC and TG, which mitigated *NF-κB p65* and *TNF-α* mRNA downregulation through downregulation of the *TLR4*.

Key words: Acupuncture, High-fat diet, NAFLD, *NF-κB p65*, *TLR4*, *TNF-α*.

INTRODUCTION

Acupuncture is a widely used treatment in traditional medicine to relieve or prevent symptoms of pathophysiological conditions and is used to promote health or to treat diseases through diverse techniques. In particular, acupuncture is applied to inhibit the progression of nonalcoholic fatty liver disease (NAFLD) because of its mechanisms involving promotion of lipid metabolism of liver cells and inflammation suppression (Li and Fang, 2022).

NAFLD is a disease where triglyceride accumulates excessively in the liver cells through chronic and persistent accumulation of free fatty acid, which triggers the production of inflammatory cytokines, leading to steatohepatitis and cirrhosis (Engin, 2017; Peiseler *et al*., 2022). Recent studies suggest that, in NAFLD, the regulation of inflammatory factors such as nuclear factor-κB(*NF-κB) p65* and tumor necrosis factor-α (*TNF-α*) plays a crucial role in the progression to early-stage nonalcoholic steatohepatitis. Romics *et al*. (2004) reported that fatty liver is highly sensitive to inflammation activation, particularly involving the activation of *NF-κB p65* and *TNF-α*. Additionally, Kakino *et al.* (2018) and Zhao *et al.* (2018) found that inhibiting these factors can improve NAFLD, indicating that they are key factors in reducing the risk of cirrhosis caused by NAFLD.

In recent years, a study on the effects of acupuncture stimulation on lipid metabolism and regulation of inflammatory action in fatty liver disease reported that electroacupuncture at GB26 reduced inflammatory reactions, such as *TNF-α* and interleukin 6, caused by high-fat diet in rats of the NAFLD model and led to an improvement in dyslipidemia and alterations in steatosis (Wang *et al*., 2023). Most studies on acupuncture have been conducted to investigate the effect of reducing inflammation due to imbalanced lipid metabolism by analyzing lipid metabolism-related effects and specific cytokines (Jie *et al*., 2018); however, there are not enough data on the mechanism of toll-like receptor (*TLR*)*4/NF-κB p65*, which is one of the pathways inducing inflammation related to *TNF-α* due to NAFLD.

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Therefore, this study investigated the mRNA expression of *NF-κB p65* and *TNF-α*, which regulate the development of steatohepatitis in relation to fat accumulation, in a high-fat diet-induced NAFLD rat model using manual acupuncture (MA) and warm acupuncture (WA).

MATERIALS AND METHODS

Five-week-old white Sprague Dawley (SD) rats (140-160 g, Samtaco, Korea) were housed in an experimental environment with constant temperature and humidity of 23°C±1°C and 60%±5%. This research was conducted from 2022 to 2023 at the laboratory of Acupoint and Meridian, Korean Medicine School of Dongshin University, Republic of Korea. This study was approved by the Institutional Animal Care and Use Committee of Dongshin University and conducted in accordance with the ethical guidelines (Approval No.: DSU-2022-01-03 and DSU-2023-01-02). Twenty SD male laboratory animals were allowed to acclimatize for 7 days and were divided into a nontreated control group (CON, n = 5) and an experimental group (n = 15) for which NAFLD was induced. The experimental group was supplied with a 45% high-fat diet (Research diet, USA) for 20 weeks, which was prepared by adding 10% fructose (Samchun, Korea) to drinking water. They were separated into the NAFLD group (NAFLD, n = 5), the MA treatment group (NAFLD + MA, n = 5) and the WA treatment group (NAFLD + WA, n = 5) at week 12 of inducing NAFLD and received treatment for 8 weeks. MA was performed at the SP9 and BL18 acupoints for 5 min and WA was performed at the SP9 and BL18 acupoints by applying moxibustion to the needle springs for 5 min. Each acupoint location was based on the locations according to international standards (World Health Organization, 2008). NAFLD + MA and NAFLD + WA were treated for 8 weeks for a total of 16 treatments. The body weight of the experimental animals was measured every 2 weeks and the liver weight was measured after the experiment. Blood was collected to separate the serum and liver at the end of treatment and biochemistry measurements and tissue observations were conducted, respectively. Serum level of total cholesterol (TC) was measured using a Dri-Chem Clinical Chemistry Analyzer (Fujifilm, Japan). Triglyceride (TG) and resistin in the hepatic tissues were measured using a microplate-based spectrophotometer (Biochrom, UK) using an Enzyme-Linked Immunosorbent kit (Biomatik, USA). The hepatic tissue was fixed in 10%-buffered formaldehyde and cut into 6 ìm sections (Sayed *et al*., 2021). Subsequently, hematoxylin and eosin (Sigma, USA) and Masson's trichrome stain (ScyTek Laboratories, USA) were performed according to the standard protocol (Lee *et al*., 2022). Oil red O staining was performed using an Oil red O stain kit (Statlab, USA) after embedding the tissue sample with frozen section compound (Leica, USA) and cutting it into 10 µm sections. Perilipin immunohistochemistry was performed by culturing the sample with perilipin antibody (1:300, Thermo Fisher Scientific, USA) and then counterstaining with hematoxylin. All stained tissues were analyzed using light microscopy (Nikon, Japan). Total RNA was extracted from hepatic tissues (50 mg) using an 800-µL TRIzol isolation reagent (Thermo Fisher Scientific, USA). mRNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). mRNA was then reverse transcribed into cDNA using a cDNA synthesis Master Mix (LeGene Biosciences, USA). Real-time PCR was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) using SB-Green qPCR Master Mix (LeGene Biosciences, USA). The sequences of *TLR4*, *NF-κB p65* and *TNF-α* are shown in Table 1. Results were expressed as fold change and were calculated using the comparative $2^{-\Delta\Delta CT}$ method (Liu *et al*., 2020).

Data are presented as mean ± standard deviation using GraphPad Prism (version 8.4.1, USA). Statistical significance was determined using a nonparametric one-way analysis of variance test, followed by Dunn's multiple comparisons post-hoc test. The experimental group data were compared with control group data at $\alpha = 0.05$ ($p<0.05$) and $\alpha = 0.01$ ($p<0.01$) levels.

RESULTS AND DISCUSSION

Body and liver weight

Fig 1 (A and B) shows that, as observed in Fan et al. (2005), lipidation of the liver began after 8 weeks on a high-fat diet, progressing to steatohepatitis with pericellular fibrosis by week 12. Rats on the high-fat diet also showed a significantly greater increase in body weight compared to those on a general diet after 10 weeks, along with a higher liver weight due to lipidation. The NAFLD + MA and NAFLD + WA groups started showing a significant decrease in body weight after 12 weeks compared with that of the NAFLD groups and the difference increased further until the completion of the experiment. Moreover, the liver weight decreased significantly with changes in body weight. This indicates that, as in Meng *et al*. (2019) study, acupuncture can significantly affect the increase in liver weight because of lipid accumulation in the liver caused by a high-fat diet.

Table 1: Primer sequences of target gene.

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′
TLR4	F: 5′-GCC TTG AAT CCA GAT GAA AC-3′
	R: 5′-CTG TGA GGT CGT TGA GGT TAG-3′
NF-κB p65	F: 5′-TCC CCT GTA CGA TAG TCG GCT C-3′
	R: 5′-GAG CGT TGC TTT GGA TCA AGG-3′
TNF-α	F: 5′-GCC AAT GGC ATG GAT CTC AA-3′
	R: 5′-CCC TTG AAG AGA ACC TGG GA-3′

Abbreviations: *GAPDH*- Glyceraldehyde 3-phosphate dehydrogenase; *TLR4*- Toll-like receptor 4; *NF-κB p65*- Nuclear factor-κB p65; *TNF-α*- Tumor necrosis factor-α.

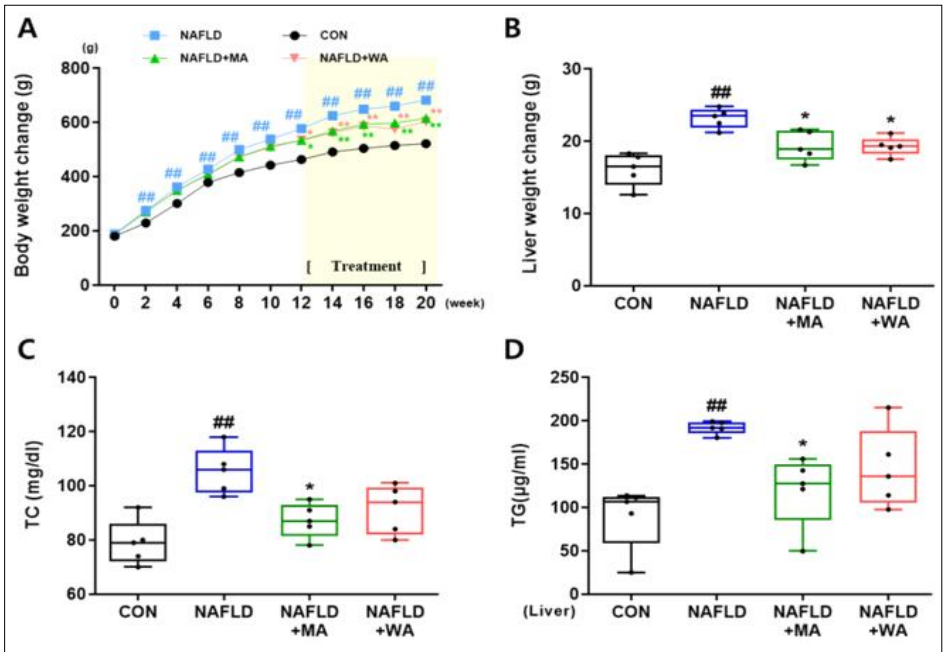
TC and TG

NAFLD due to a high-fat diet is characterized by TG accumulation in the cytoplasm of hepatocytes and high levels of TG accumulation lead to a fatty liver, which causes an increase in inflammatory cytokines or tissue necrosis and fibrosis. The damaged liver leads to a release of TC and TG into the bloodstream, increasing these levels. These serum biochemical markers are used in specific and sensitive evaluations of liver damage in preclinical studies (Palekar *et al*., 2006; Wilkins *et al*., 2013).

As depicted in Fig 1 (C and D), a high-fat diet increased the levels of TC in the serum and TG in the hepatic tissue. The NAFLD + MA group had significantly lower TC levels in the serum and lower TG levels in the hepatic tissue than the NAFLD group. Similarly, the body and liver weights in the NAFLD+MA group reflect the relieving and regulating effects of acupuncture in the stages of lipid accumulation. These findings are consistent with those reported by Wang *et al*. (2019) and Zhang *et al*. (2020), which demonstrated that MA also reduced the elevated TC levels in the liver caused by a high-fat diet.

Histology

NAFLD is defined as the state in which over 5%-10% of the liver cells indicate visible steatosis with no other cause of liver disease (Bondini *et al*., 2007). In the hematoxylin and eosin stain, the NAFLD group showed many accumulated lipid droplets, infiltration and liver lobule structure disorder in multiple locations, whereas acupuncture groups showed a remarkable improvement in lipid droplets and infiltration Fig 2(A). Furthermore, in Masson's trichrome stain, the NAFLD group showed a high rate of lipid droplets compared with that of the control group and fibrosis by infiltration, whereas the acupuncture groups showed a decrease in the area of lipid droplets Fig 2(B). In the Oil red O stain, the control group showed the blue-stained nuclei of liver cells without lipid deposition, whereas the NAFLD group showed diffused lipid droplets stained in red and the nuclei appeared compressed compared with normal cells because of the spread of lipidized cells and the acupuncture groups showed relatively little lipid droplets and more nuclei Fig 2(C). We tracked three indicators, including the symptoms of lipid accumulation in the liver. The histological findings of the liver in this study indicate that, as demonstrated in the research by Alshehri *et al*. (2024), the damaged liver exhibits a general expansion of infiltration and fibrosis compared to normal liver. Importantly, the liver in the adequately treated group showed a significant visual improvement in tissue damage alleviation, reinforcing the effectiveness of the treatment. Finally, perilipin is a protein located on the surface of lipid droplets in adipocytes and steroid-producing cells that are involved in lipid storage by reducing lipid degradation in a dephosphorylated state (Kimmel and Sztalryd, 2016). Our experiment also revealed that perilipin was locally expressed around lipid droplets in the hepatic tissue of the NAFLD model compared with that of the control group, whereas in the acupuncture groups, the expression of perilipin reduced remarkably Fig 2(D).



(A) Differences in Body and (B) Liver weight, (C) Serum TC and (D) Detection of TG in the hepatic tissue by ELISA. Data are presented as mean ± standard deviation. ## p<0.01 compared with the CON group. *p<0.05; **p<0.01 compared with the NAFLD group.

Fig 1: Changes in weight of body and liver, TC and TG.

According to the results of histological analysis in our study, the hepatic tissues and cells in the NAFLD group were lapidated compared with those in the control group and fibrosis due to lipidation of normal cells progressed simultaneously with the occurrence of structural collapse, whereas the acupuncture groups were involved in reducing lipidation-caused fibrosis through structural normalization of tissues by reducing TG level in the tissue and lipidation of liver cells. These results correspond to the above results of the reduced liver weight and lipid-related factors.

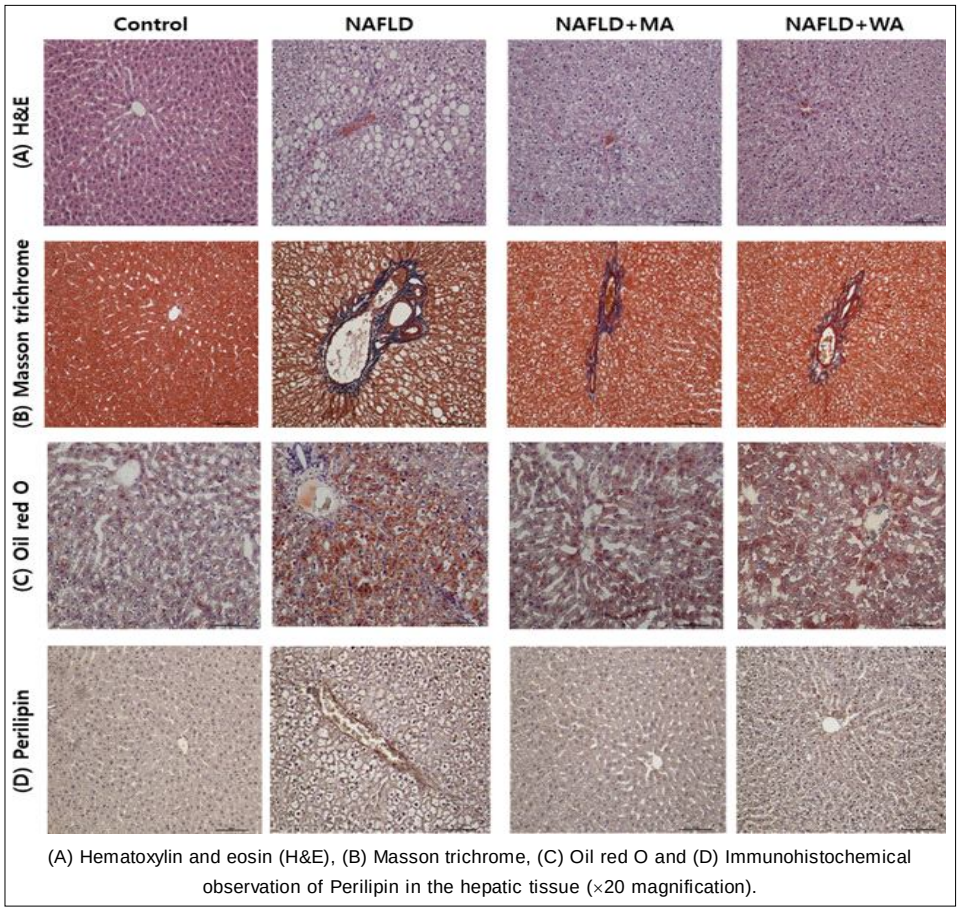
Resistin

As a recent therapeutic experimental approach, Ma *et al*. (2020) reported that acupuncture improved inflammatory cytokines such as *TNF-α* by inhibiting the *NF-κB* signaling pathway and lipid metabolism in an NAFLD model. In this study, we further examined how the effect of acupuncture on fat accumulation induced by a high-fat diet shown above could affect *NF-κB p65/TNF-α*, which can induce inflammation in the liver. Adipokines secreted by the fat tissues are classified into proinflammatory and anti-inflammatory and the proinflammatory adipokines, such as resistin, promote the development and progression of NAFLD as well as cause inflammation (Pang and Lee, 2006). Resistin, the inflammatory cell-activating hormone, is known to be mediated by *NF-κB* to increase its production through *TNF-α* stimulation, inducing inflammation in various cells (Wen *et al*., 2021; Chávez-Tapia *et al*., 2014).

In our study, resistin showed a significant decrease only in the NAFLD + WA group compared with that in the NAFLD group Fig 3(A). These findings suggest that resistin, which activates inflammatory factors that may manifest as fatty deposition in hepatic tissue, can be regulated by WA but is not related to MA.

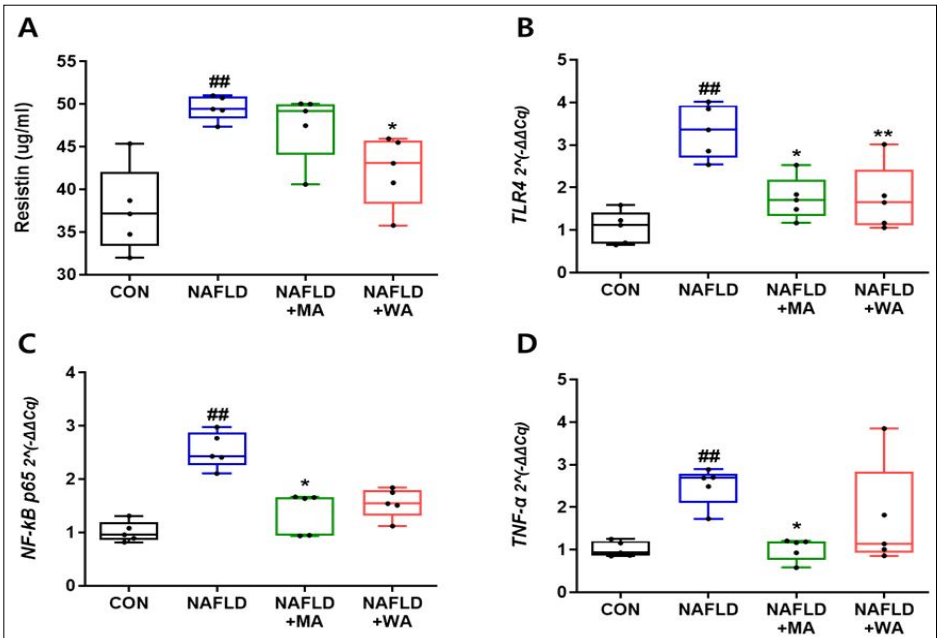
mRNA expression

TLR4 produces various proinflammatory, antiviral and antibacterial cytokines when it is activated by lipidation of the liver (Sung *et al*., 2016). *TLR4* is normally expressed in diverse types of cells, such as hepatocytes, monocytes, Kupffer cells and stellate cells (Broad *et al*., 2007; Sung *et al*., 2016). Triggering the *TLR4* pathway leads to the activation of inflammatory cascades, producing various proinflammatory cytokines, including nuclear translocation of *NF-κB* and *TNF-α*, which causes inflammation and cell death (Long *et al*., 2023). Li and Fang (2022) reported the inflammation-regulating action of acupuncture through inhibition of the *TLR4* signaling pathway and the *NF-κB* pathway in mastocytes as well.



(A) Hematoxylin and eosin (H&E), (B) Masson trichrome, (C) Oil red O and (D) Immunohistochemical observation of Perilipin in the hepatic tissue (×20 magnification).

Fig 2: Histological evaluation of hepatic tissue.



(A) Detection of resistin in the hepatic tissue through ELISA, The mRNA expression of (B) *TLR4,* (C) *NF-kB p65 and* (D) *TNF-α*. Data are presented as mean ± standard deviation. ## p<0.01 compared with the CON group. *p<0.05; **p<0.01 compared with the NAFLD group.

Fig 3: Resistin and mRNA expression in the hepatic tissues.

Our study found that the pro-inflammatory factors *TLR4* significantly decreased in both NAFLD + MA and NAFLD + WA groups Fig 3(B), whereas *NF-κB p65* and *TNF-α* significantly decreased only in the NAFLD + MA group Fig 3 (C and D). These results suggest that WA may have a limited effect on the activation of resistin and *TLR4* mRNA, which are inflammation-related factors potentially triggered by fat accumulation in liver tissue resulting from a high-fat diet. Conversely, MA may facilitate the repair of liver tissue damage associated with fat accumulation by activating *TLR4* mRNA, which subsequently regulates *NF-κB p65* and *TNF-α* mRNA.

CONCLUSION

We evaluated the effects of acupuncture treatment using a model of high-fat diet-induced NAFLD for 8 weeks. MA showed reduced adipocyte accumulation due to TC and TG and it inhibited *NF-κB p65*/*TNF-α* mRNA by regulating *TLR4* mRNA. These findings suggest that MA may have the potential to be used as a treatment to reduce the risk of early-stage nonalcoholic steatohepatitis, which may be caused by NAFLD.

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Disclaimers

The views and conclusions expressed in this paper are those of the authors and do not necessarily reflect the views of their affiliated institutions. The raw data are available from the corresponding author upon request.

Informed consent

The animal study protocol was approved by the Dongshin University Animal Ethics Review Committee and conducted according to ethical guidelines (approval numbers: DSU-2022-01-03 and DSU-2023-01-02).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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