<Research Article>

Effects of electrical acupuncture stimulation on the exercise endurance and muscle glycogen concentration of the *Tibialis Anterior* muscle of mice

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Summary To enhance endurance exercise performance, it is essential to maintain skeletal muscle glycogen levels prior to exercise and blood glucose concentrations during exercise. Although electroacupuncture stimulation has been suggested to affect glucose metabolism, its effects on exercise capacity and related metabolism remain unclear. Therefore, in this study, we aimed to investigate the effects of electroacupuncture stimulation of the tibialis anterior muscle and these metabolic parameters. Electroacupuncture stimulation was administered to both tibialis anterior muscles of male C57BL/6J mice at 1 Hz and 0.02 mA for 20 min. Following this stimulation, the mice performed running exercise at 25 m/min, and we measured the time to exhaustion. Subsequently, indices of glucose and lipid metabolism were measured in tissues. We found that the running time was significantly prolonged in the electroacupuncture group. Furthermore, glycogen concentrations in the tibialis anterior muscles prior to exercise were higher and the decrease in blood glucose concentration post-exercise was attenuated in the electroacupuncture group. However, no significant effects of electroacupuncture stimulation were found on free fatty acid concentrations. These findings suggest that electroacupuncture stimulation enhances glucose metabolism in skeletal muscle and improves exercise tolerance. Further research is warranted to elucidate the mechanisms underlying these effects.

Key words: Electroacupuncture, Exercise, Skeletal muscle, Glycogen, Metabolism

1. Introduction

Exercise involves the activation of energy metabolism in association with skeletal muscle contraction, which has health-promoting effects. The primary energy sources for exercise are carbohydrates, fats, and proteins, and the utilization rates of these energy substrates are influenced by the intensity and duration of exercise[1]. Low-intensity, short-duration activity is

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principally associated with free fatty acid oxidation, whereas high-intensity, long-duration exercise is predominantly associated with the utilization of glucose derived from skeletal muscle and liver glycogen[2].

Glucose, a primary energy source, is obtained through dietary intake and endogenous production in the liver and kidneys[3]. Specifically, the liver is integral to the energy supply during exercise and regulation of blood glucose levels, primarily through the generation of glucose via glycogenolysis and gluconeogenesis[4]. The main precursors for gluconeogenesis include amino acids (such as alanine), lactate derived from skeletal muscle and the bloodstream, and also glycerol released by adipose tissue[1,5]. These non-carbohydrate substrates are converted to glucose by rate-limiting enzymes including phosphoenolpyruvate carboxykinase 1 (PCK1), glucose-6-phosphatase catalytic subunit 1 (G6PC1), and fructose bisphosphatase 1 (FBP1)[6] (Fig. 1). Deficiencies in or hyperactivity of these enzymes can disrupt glucose metabolism, thereby impairing blood glucose regulation[5]. In contrast, because skeletal muscle glycogen is consumed during physical activity, it is crucial to continuously supply blood glucose to skeletal muscles to sustain exercise. This process is facilitated by solute carrier family 2 (facilitated glucose transporter) member 4 (GLUT4), a glucose transporter protein that mediates the uptake of glucose into skeletal muscle[7]. Mice which lack GLUT4 exhibit reduced glucose uptake in muscle tissue and demonstrate shortened endurance capacity during exercise[8]. These findings underscore the importance of blood glucose homeostasis in supporting sustained physical activity[9].

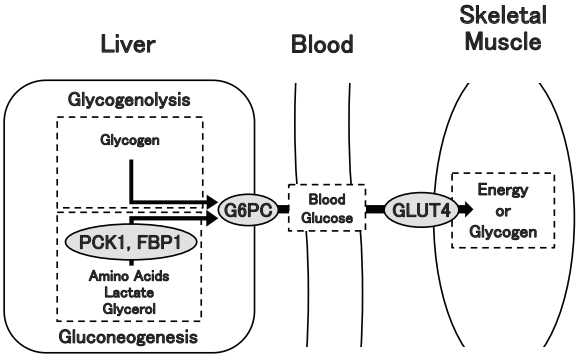

Fig. 1. Schematic representation of glucose metabolism during exercise. The liver provides energy to skeletal muscles and regulates blood glucose levels by producing glucose through glycogenolysis and gluconeogenesis. FBP1, fructose bisphosphatase 1 ; GLUT4, glucose transpoter 4 ; G6PC1, glucose-6-phosphatase catalytic subunit 1 ; PCK1, phosphoenolpyruvate carboxykinase 1.

Acupuncture, a traditional therapeutic practice that originated in China around 100 BCE, has been demonstrated to be an efficacious therapy for various conditions, including lower back and post-operative pain, positioning it as a viable alternative medicine option[10-12]. In clinical settings, electroacupuncture (EA), which integrates acupuncture with electrotherapy, is frequently employed[13.] EA induces muscle contraction *via* direct electrical stimulation and influences glucose and lipid metabolism. Previous studies have shown improvements in blood glucose concentrations in rodent models of depression and type 2 diabetes; lower serum triglycerides, total cholesterol, and free fatty acid concentrations in models of both diabetes and non-alcoholic fatty liver disease; and an improvement in glucose tolerance and lower free fatty acid concentrations in healthy rats[14-17]. Furthermore, it has been shown that EA can mimic the physiologic effects of exercise, particularly in functional rehabilitation[18,19].

Given the potential beneficial effects of EA on glucose and lipid metabolism and exercise tolerance, few studies have investigated its effects in healthy mice. Therefore, in the present study, we aimed to characterize the effects of EA during running exercise and its relationship with glucose and lipid metabolism before and after exercise.

2. Materials and Methods

Animals

All the animal experiments were conducted in accordance with the guidelines of the Tsukuba University Animal Resource Center (approval number 23-460). Male C57BL/6J mice were obtained from CLEA Japan (CLEA Japan, Inc., Tokyo, Japan), Inc. and acclimatized in a specific pathogen-free environment with *ad libitum* access to food and water for a duration of 2 weeks. Following this adaptation period, mice aged between 8 and 15 weeks were randomly assigned to two groups: a control (CON) group and

an EA group.

EA stimulation

EA was administered to the mice in the EA group while under isoflurane inhalation anesthesia using a Narcobit-E apparatus (Natsume Seisakusho, Tokyo, Japan). Sterilized stainless steel acupuncture needles (Seirin, Shizuoka, Japan) with a diameter of 0.20 mm and a length of 30 mm were inserted into the tibialis anterior muscles of both hind limbs to a depth of approximately 3 mm. The acupuncture needles were subsequently connected to an Ohm Pulsar LFP-2000e device (Zen Iryoki, Fukuoka, Japan), and low-frequency electrical stimulation was delivered at a frequency of 1 Hz and an amplitude of 0.02 mA for 20 min. The mice in the CON group were maintained in a resting state for 20 min under anesthesia.

Exercise tolerance testing

Exercise tolerance testing was performed following EA stimulation using a TMS-M4 treadmill (Melquest, Toyama, Japan). The treadmill was configured with a 0° incline, and the frequency of the electrical impulses delivered in the shock grid area at the base was set to 1 Hz. Prior to the exercise tolerance assessment, the mice were acclimatized to the treadmill by running for 10 min on 2 consecutive days. On the third day, the mice rested, and on the fourth day, EA stimulation was applied for 20 min, as previously described. Six hours post-EA stimulation, the mice underwent a warm-up for 12 min, commencing at a speed of 5 m/min and progressively increasing by 5 m/min every 3 min. Continuous running was then performed at a speed of 25 m/min, and the duration of running until exhaustion was recorded (n=7–8 per group). Exhaustion was recorded when the mice remained on the electrical shock grid for 5 consecutive seconds. Mice who ran for <1 h were excluded from the analysis.

Biochemical parameter analysis

For the analysis of biochemical parameters, mice were prepared according to the protocols for exercise tolerance testing and then underwent 1 h of running. Following this exercise, the mice were euthanized by cervical dislocation, and blood samples were collected from the abdominal vena cava of each mouse (n=7–8 per group). The samples were centrifuged to obtain plasma samples, which were subsequently stored at −80°C until analyzed. The plasma glucose concentrations were measured using a LabAssay™ Glucose kit (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) and the free fatty acid concentrations were measured using a LabAssay™ NEFA kit (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). The lactate concentrations were determined using a Lactate Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan), and the tissue glycogen levels were measured using a Glycogen Assay Kit (Abcam, Cambridge, UK), in accordance with the manufacturers' protocols.

cDNA synthesis and quantitative PCR

Liver tissue samples from the mice were homogenized in RNAiso plus (Takara Bio, Shiga, Japan), and RNA was extracted according to the manufacturer's protocol. cDNA was synthesized from 500 ng of RNA using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. The resulting cDNA was diluted 10-fold with MilliQ water and subsequently used for quantitative PCR (qPCR). qPCR primers were designed using Primer-BLAST (NCBI) (Table 1). The qPCR was conducted using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio, Shiga, Japan) in a 10 μL volume for each reaction. Relative gene expression was assessed using the ΔΔCt method, with *18S rRNA* serving as the reference gene for normalization.

Table 1 Primer sequences used in this study

Target gene	Primer sequences (5' to 3')		Amplicon size (bp)
Pck1	Forward	GCAGTGAGGAAGTTCGTGGA	164
	Reverse	GTGAGAGCCAGCCAACAGT	
G6pc1	Forward	TGAGACCGGACCAGGAAGTC	195
	Reverse	GCAAGGTAGATCCGGGACAG	
Fbp1	Forward	GCATCGCACAGCTCTATGGT	122
	Reverse	ACAGGTAGCGTAGGACGACT	
18srRNA	Forward	CGCCGCTAGAGGTGAAATTC	101
	Reverse	CGAACCTCCGACTTTCGTTCT	

Western blotting

RIPA buffer supplemented with Protease Inhibitor

Cocktail Set III, DMSO Solution, Animal-derived-free (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and Phos STOP (Roche, Basel, Switzerland) was used in order to prepare the protein extraction solution. The tibialis anterior muscle was homogenized using BioMacher II (Nippi, Tokyo, Japan). The lysate was centrifuged at 17,900 × g for 20 min and the supernatant was collected. The total protein concentration in the supernatant was determined using the TaKaRa BCA Protein Assay Kit (Takara Bio, Shiga, Japan).

Equal amounts of protein (10 μg per lane) were loaded onto Mini-PROTEAN TGX Gels (Bio-Rad, CA, US) for separation using SDS-PAGE. After electrophoresis, proteins were transferred to a PVDF membrane using the Trans-Blot Turbo RTA Transfer Kit, PVDF (Bio-Rad, CA, US) and the Trans-Blot Turbo Transfer System (Bio-Rad, CA, US) under the following conditions: 2.5 A, 25 V, for 8 min.

Following the transfer, the PVDF membrane was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) and incubated with shaking for 30 min at room temperature. The membrane was then incubated with a primary antibody against GLUT4 (Cell Signaling Technology, #2213, MA, US) at a 1:1000 dilution in Tris-Buffered Saline Tween-20 (TBS-T) solution for 2 h at room temperature. After three washes with TBS-T, the membrane was incubated with HRP-conjugated anti-mouse IgG (Cell Signaling Technology, #7076, MA, US) at a 1:3000 dilution for 30 min at room temperature. GAPDH (Cell Signaling Technology, #5174, MA, US) was used as a loading control, with HRP-conjugated anti-rabbit IgG (Cell Signaling Technology, #7074, MA, US) as the secondary antibody.

Chemiluminescent detection was performed using EzWestLumi Plus (ATTO, Tokyo, Japan) and the protein bands were visualized using FUSION-FX7. EDGE imaging system (Vilber Lourmat, Marne-la-Vallée, France). Signal intensities of the GLUT4 bands were quantified and normalized to those of GAPDH as a loading control.

Statistical analysis

Statistical analyses were conducted using Prism version 9.5.1 (GraphPad Software, CA, US). The Mann–Whitney U test was used to compare two groups and one-way ANOVA was used for comparisons among multiple groups. A p-value of < 0.05 was considered to indicate a statistically significant difference.

3. Results

Effect of EA stimulation on exercise tolerance

The effect of EA on endurance was evaluated using treadmill running to exhaustion following EA stimulation. The mean running time from the initiation of exercise to exhaustion was recorded, and the CON group exhibited a mean running time of 90 min, whereas the EA group demonstrated a significantly longer mean running time of 138 min (Fig. 2).

Effects of EA stimulation on glucose and lipid metabolism

We assessed the effects of EA stimulation on the glucose and lipid metabolism associated with exercise following 1 h of running exercise post-EA stimulation.

The mean plasma glucose concentration of the mice prior to exercise was 176.1 mg/dL for the CON group and 177.1 mg/dL for the EA group, and after running, the concentrations were 111.0 mg/dL for the CON group and 137.2 mg/dL for the EA group. There were no significant differences between the two groups, before or after exercise (Fig. 3A). With respect to the effects of EA stimulation, the mean plasma glucose concentration significantly decreased in the CON group post-exercise, but the reduction in the EA group was significantly attenuated.

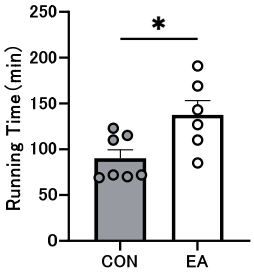

Fig. 2. Effect of EA stimulation on the exercise tolerance of the mice. Data are expressed as mean ± SEM (n=6–7). CON, control group; EA, electroacupuncture group. *$P < 0.05$ vs. CON group (Mann–Whitney U test).

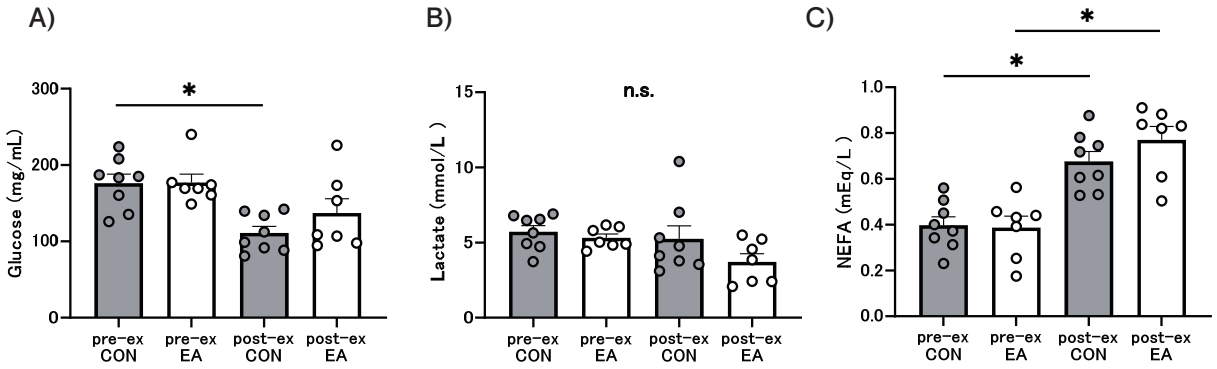

Fig. 3. Effect of EA stimulation on glucose and lipid metabolism. A) Plasma glucose concentration. B) Plasma lactate concentration. C) Plasma free fatty acid concentration. Data are expressed as mean ± SEM (n=7–8). CON, control group; EA, electroacupuncture group; ex, running exercise. n.s., not significant, *$P < 0.05$ vs. pre-ex CON group (1-way ANOVA).

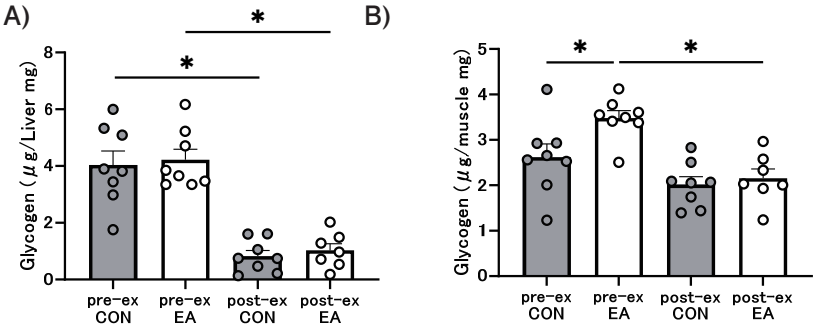

Fig. 4. Effect of EA stimulation on glycogen storage. A) Glycogen concentrations of the liver. B) Glycogen concentrations of the tibialis anterior muscle. Data are expressed as mean ± SEM (n=7–8). CON, control group; EA, electroacupuncture group; ex, running exercise. *$P < 0.05$ vs. pre-ex CON group (1-way ANOVA).

The mean plasma lactate concentrations before running were 5.7 mmol/L for the CON group and 5.3 mmol/L for the EA group, and following exercise, these values were 5.3 mmol/L for the CON group and 3.7 mmol/L for the EA group. No significant differences were found between the groups under running conditions (Fig. 3B), and no significant effect of EA stimulation was observed.

The mean plasma free fatty acid concentrations prior to running were 0.4 mEq/L for the CON group and 0.4 mEq/L for the EA group, and after running, the concentrations increased to 0.7 mEq/L for the CON group and 0.8 mEq/L for the EA group. Both groups exhibited a significant increase in free fatty acid concentration during exercise (Fig. 3C), but there was no significant effect of EA stimulation.

The mean liver glycogen concentrations prior to running were 4.0 μg/mg for the CON group and 4.2 μg/mg for the EA group, and following running, the glycogen concentrations were 0.8 μg/mg for the CON group and 1.0 μg/mg for the EA group (Fig. 4A). Thus, liver glycogen concentrations in both of the groups significantly decreased during exercise; however, there were no significant differences seen in the mean liver glycogen concentrations in the CON or EA groups before or after running. In contrast, the mean glycogen concentrations in the tibialis anterior muscle prior to running were 2.6 μg/mg for the CON group and 3.5 μg/mg for the EA group, and after exercise, these values were 2.0 μg/mg for the CON group and 2.1 μg/mg for the EA group (Fig. 4B). Glycogen concentration before exercise was significantly higher in the EA group than in the CON group. There was no significant difference in the mean glycogen concentration in the tibialis anterior muscle before and after running in the CON group; however, there was a significant decrease in the EA group during exercise.

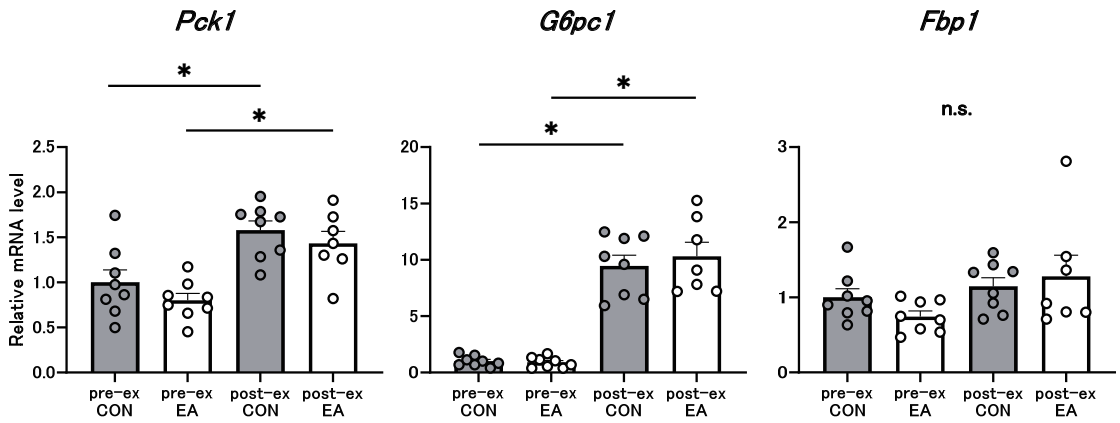

Fig. 5. Effect of EA stimulation on gluconeogenic gene expression. The reference gene used was *18S*. Data are expressed as mean ± SEM (n=7–8). CON, control group; EA, electroacupuncture group; ex, running exercise. n.s., not significant, $*P < 0.05$ vs. pre-ex CON group (1-way ANOVA).

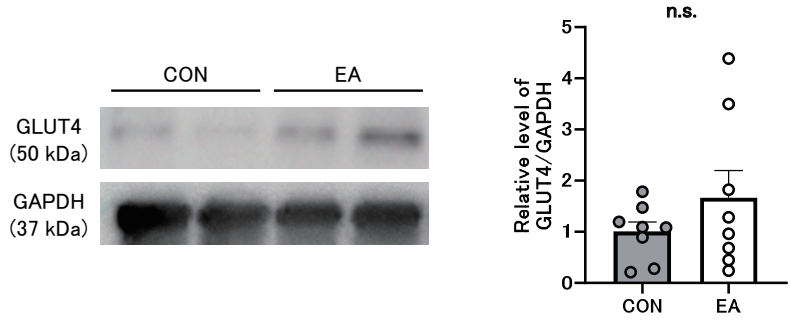

Fig. 6. Effect of EA stimulation on the relative level of GLUT4 in the tibialis anterior muscle. The protein levels of GLUT4 in the anterior tibialis muscle following EA stimulation were determined by Western blotting. GAPDH was used as the loading control. Data are expressed as mean ± SEM (n=8). CON, control group; EA, electroacupuncture group. n.s., not significant (Mann–Whitney U test).

Effect of EA stimulation on the mRNA levels of enzymes involved in glucose release

To evaluate the effect of EA stimulation prior to running on glucose production during exercise, we analyzed the relative mRNA levels of *Pck1, G6pc1*, and *Fbp1* by qPCR (Fig. 5). During running, the relative mRNA levels of *Pck1* increased significantly (by 1.6-fold) in the CON group, whereas the EA group demonstrated a significant increase of 1.4-fold. The CON group exhibited a significant 9.5-fold increase in *G6pc1* mRNA levels during running, whereas the EA group exhibited a significant 10.3-fold increase. However, there were no significant differences in relative mRNA levels of *Fbp1* between the EA and CON groups.

Effect of EA stimulation on blood glucose uptake

To assess the effect of EA stimulation on glucose uptake in the tibialis anterior muscle, we measured the relative amount of GLUT4 by Western blotting before running. The results showed a 1.7-fold increase in GLUT4 expression in the EA group compared to that in the control group, although this difference was found to be not statistically significant (Fig. 6).

4. Discussion

In the present study, we investigated the effects of EA stimulation on running endurance in mice. We found that EA stimulation significantly extended the running time of the mice and increased the glycogen concentrations of their tibialis anterior muscles.

Endurance exercise is characterized by changes in energy substrate utilization that depend on the intensity and duration of exercise[1]. At low exercise intensity, there is an increase in the utilization of circulating free

fatty acids, whereas during high-intensity and prolonged exercise, there is an increase in utilization of blood glucose, leading to a decrease in blood glucose concentration[1,20,21]. Additionally, lactic acid produced as a byproduct of energy metabolism accumulates to a greater extent during high-intensity exercise[22]. In the present study, the CON group exhibited a decrease in blood glucose concentration and an increase in circulating free fatty acid concentration during running, with no significant change in lactic acid concentration. These findings suggest that the intensity of the running exercise was moderate.

In the EA group, the reduction in blood glucose levels after running was attenuated. Because EA stimulation has been suggested to influence glucose metabolism, in this study we focused on glycogen as a relevant factor. In the CON group, liver glycogen concentration decreased significantly after running; however, no change in glycogen concentration was observed in the anterior tibialis muscle. It has been previously reported that exercise induces an increase in the mRNA levels of enzymes involved in glucose release, and similar results were obtained in this study, supporting the notion that blood glucose levels during exercise are sustained by enhanced glucose release from the liver[23]. However, no significant differences were observed in the mRNA levels of the enzymes related to glucose release before and after running in the EA group. These findings suggest that EA stimulation did not exert a significant effect on glucose release from the liver.

In contrast, the glycogen concentration in the tibialis anterior muscle of the EA group increased significantly prior to running exercise, although no effect was observed on the amount of GLUT4, the transporter responsible for glucose uptake in the skeletal muscle. Previous studies have reported that EA stimulation increases GLUT4 levels; however, these findings contrast with those of the present study, which analyzed healthy mice, as prior studies primarily involved repeated EA stimulation in animal models with pathological conditions or those subjected to resistance training[24-26]. To date, no reports have indicated that EA stimulation increases the skeletal muscle glycogen concentration in wild-type mice, making this study the first to provide new insights into the effects of EA stimulation on healthy organisms. In previous in vitro studies, the electrical stimulation of muscle cells was shown to induce the translocation of GLUT4 to the sarcolemma, thereby increasing glucose uptake[27-30]. Based on these findings, EA stimulation may have initially increased GLUT4 levels in the tibialis anterior muscle. However, this effect may have diminished 6 h after EA stimulation prior to running exercise.

In the present study, the EA stimulation group exhibited increased glycogen concentration in the tibialis anterior muscle. Because the glycogen concentration in the tibialis anterior muscle was not measured immediately following EA stimulation, further studies are needed to investigate the temporal relationship between skeletal muscle glycogen concentration and GLUT4 levels after EA stimulation.

In this study, the glycogen concentration in the tibialis anterior muscle was found to be higher in the EA group. However, as the glycogen concentration immediately following acupuncture stimulation was not measured, the relationship between the time course of skeletal muscle glycogen concentration after acupuncture stimulation and GLUT4 levels remains unclear. Additionally, given that the tibialis anterior muscle is part of the ankle extensor group, it is unclear whether the increase in glycogen concentration in this muscle directly contributes to the enhancement of running performance. However, these findings warrant further investigation in future studies.

In summary, EA stimulation of the tibialis anterior muscle prior to running exercise prolonged the exercise duration and attenuated the decrease in blood glucose levels. Furthermore, the glycogen concentration in the tibialis anterior muscle increased following EA stimulation before running. These findings suggest that EA stimulation may influence glycogen content in skeletal muscles, thereby contributing to the extension of running performance and maintenance of blood glucose levels. Given that enhancing skeletal muscle glycogen storage is crucial for improving exercise performance, EA stimulation may offer a potential approach for carbohydrate loading in sports[31].

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Conflicts of interests

All authors declare that there is no conflict of interest regarding the publication of this article.

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