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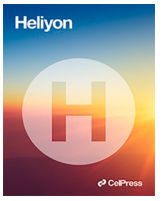

Research article

Treadmill training impacts the skeletal muscle molecular clock after ischemia stroke in rats

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ABSTRACT

Objective: Stroke is frequently associated with muscle mass loss. Treadmill training is considered the most effective treatment for sarcopenia. Circadian rhythms are closely related to exercise and have been extensively studied. The skeletal muscle has its molecular clock genes. Exercise may regulate skeletal muscle clock genes. This study evaluated the effects of early treadmill training on the skeletal muscle molecular clock machinery in rats with stroke and determined the relationship of these changes with exercise-induced improvements in skeletal muscle health.

Materials and methods: Overall, 168 Sprague-Dawley rats were included in this study. We established an ischemic stroke rat model of sarcopenia. Finally, 144 rats were randomly allocated to four groups (36 per group): normal, sham, middle cerebral artery occlusion, and training. Neurological scores, rotating rod test, body weight, muscle circumference, wet weight, and hematoxylin-eosin staining were assessed. Twenty-four rats were used for transcriptome sequencing. Gene and protein expressions of skeletal muscles, such as brain muscle arnt-like 1, period 1, and period 2, were measured by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assays.

Results: Neurological function scores and rotating rod test results improved after treadmill training. Nine differentially expressed genes were identified by comparing the sham group with the hemiplegic side of the model group. Seventeen differentially expressed genes were identified between the hemiplegic and non-hemiplegic sides. BMAL1, PER1, and PER2 mRNA levels increased on both sides after treadmill training. BMAL1 expression increased, and PER1 expression decreased on both sides, whereas PER2 expression decreased on the hemiplegic side but increased on the non-hemiplegic side.

Conclusion: Treadmill training can mitigate muscle loss and regulate skeletal muscle clock gene expression following ischemic stroke. Exercise affects the hemiplegic side and has a positive regulatory effect on the non-hemiplegic side.

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1. Introduction

Since its introduction [1], research on sarcopenia has increased [2–4]. According to the 2018 consensus of the European Working Group on Sarcopenia in Older Persons [3], the definition of sarcopenia has been updated and confirmed as a muscular disease. The diagnostic code was obtained from the International Classification of Diseases-10. Sarcopenia is an ongoing systemic disease of the skeletal muscle. It is a clinical syndrome characterized by reduced muscular strength and mass. Sarcopenia can be classified as primary or secondary. Aged-induced sarcopenia, known as primary sarcopenia, occurs in approximately 15% of older adults [5] and increases the risk of falls, fractures, and disabilities in older adults. The reported incidence rates of secondary sarcopenia in cardiovascular, diabetic, and respiratory diseases are 31.4, 31.1, and 26.8% [6], respectively. Systemic muscle mass loss and functional decline may occur following a stroke. Secondary or stroke-related sarcopenia [7–9], can affect a patient's quality of life and clinical outcomes. A recent systematic review has revealed that the incidence of secondary sarcopenia in patients with stroke can be high at 47% [10]. Stroke is a leading disabling disease worldwide [11], and its annual incidence in China is increasing. In 2017, approximately 1.96 million people died of stroke, and the annual medical cost of stroke was approximately 25 billion yuan [12]. As the number of patients surviving a stroke increases, the effects of muscular dystrophy after stroke have gradually become a focus [13,14].

Physical disability in older adults lowers their quality of life and often results in psychological stress. Unfortunately, little attention has been paid to aging individuals with disabilities or functional limitations. Old age is associated with an increased use of healthcare services and health costs. Improving functional disability reduces the need for support, hospitalization, and costs in older adults [15].

Limb paralysis after a stroke is a multifactorial clinical condition that may lead to muscle denervation, limb alienation, and catabolic activation. It can cause phenotypic changes in skeletal muscle fibers and wasting. Some clinical studies have demonstrated that muscle mass in hemiplegia decreases within 4 h of stroke [16]. Limbs without paralysis are generally regarded to have the same size and strength as healthy muscles. However, muscular weakness may occur in non-hemiplegic limbs within 1 week of stroke [17]. The loss of muscle mass was greater 3 weeks after stroke, and approximately 24% of the paralytic limb muscle volume decreased between 6 and 12 months after stroke [18]. A retrospective study has demonstrated that exercise in patients with stroke can reverse the decline in muscular mass and strength of hemiplegic and non-hemiplegic limbs. Strength training may also improve walking speed and prognosis [9].

Skeletal muscle loss is a major health concern. The skeletal muscle is integral to physical activity, is related to respiratory function [19], and has been identified as the largest endocrine organ that releases myokines that influence metabolism and health [20]. Although the skeletal muscle is important for maintaining health, most individuals experience skeletal muscle loss due to aging and various diseases. Previous studies have suggested that Alzheimer's disease (AD) causes muscle atrophy, associated with myonuclear reduction in rats with AD [21]. Several studies have concluded that muscle atrophy is one of the most common symptoms of aging [22]. Increasing muscle volume may be difficult in older patients in the acute and chronic stroke phases. A previous study has also reported that the myonuclear content in rodents decreases in response to hindlimb suspension, denervation, and immobilization, with lower satellite cell (SC) content in response to hindlimb suspension and immobilization. These findings suggest that the myonuclear and SC contents in humans and rodents are not maintained indefinitely and may be reduced with skeletal muscle atrophy [23]. Previous studies have indicated muscle mass decreases in patients admitted to the intensive care unit [24] and those with aneurysmal subarachnoid hemorrhage during the acute phase [25]. Another study reported that muscle mass changes were not observed 1 year after stroke onset [26]. Therefore, older patients who have experienced a stroke may be unable to regain skeletal muscle mass in the acute and chronic stroke phases without rehabilitation or training. Considering rehabilitation or training, even older people may have increased skeletal muscle mass.

Acute and subacute muscle atrophy may be more relevant for recovery, and prevention or reversal of muscle atrophy at the beginning of the post-attack phase may be especially important. Rehabilitation medicine can improve limb function in patients with stroke and minimize the impact of disease factors on their quality of life. Skeletal muscle is a dynamic and highly plastic tissue capable of remodeling its properties in response to various stimuli such as chronic diseases and exercise training [27,28]. Exercise is the most effective treatment for patients with sarcopenia [29]. Exercise intervention is considered one of the main strategies for combating sarcopenia-related injuries, and the 2018 European Consensus states that exercise is the first-line treatment for sarcopenia. Moreover, exercise has anabolic effects on muscles, reduces age-related oxidative damage and chronic inflammatory responses, increases autophagy, and improves mitochondrial function, actin profiles, insulin-like growth factor signaling pathways, and insulin sensitivity [30]. Muscle strength and aerobic fitness are factors that maintain performance capacity [31]. Currently, the recognized exercise methods include resistance and aerobic exercises.

Resistance exercise [32] can significantly improve the protein synthesis rate in skeletal muscles, an effective strategy for low-anabolic muscles. Studies by Snijders et al. [33] have demonstrated that long-term resistance exercise intervention after 24 weeks can prevent muscle mass loss in both men and women and improve muscle strength with age. Engardt et al. [34] have reported that physical exercise was associated with reduced muscular atrophy and significant improvements in motor function, including knee extension and flexion movements, in all the hemiplegic muscles. Particularly, regarding bone metabolism, Amato et al. [35] suggested that could be an influence of resistance training on bone turnover, positive for osteocalcin (formation marker). However, this increased formation activity, also evidenced by an increase in calcium. Therefore, the resistance training for people with Parkinson's seems to affect bone metabolism. Home-based resistance training could be a strategy to support drug therapy to prevent and reduce BMD loss associated with PD, maintain healthy body composition, and improve functional physical performance. The most similar exercise type used by both mice and humans is aerobic exercise, which includes treadmill exercise and wheel-running in mice. Treadmill exercise has simple, easily modifiable parameters. The most significant advantage for treadmill exercise is that the exercise intensity can be precisely controlled to better investigate the effects induced by different intensities [36]. Aerobic exercise, as a repeated exercise

involving a large number of large muscle groups, can increase energy production by mitochondria and capillary density of skeletal muscle, thus promoting oxygen consumption and cellular homeostasis, and delaying skeletal muscle atrophy [37]. Moreover, due to its convenience, operability, and diverse forms, aerobic exercise is highly beneficial for delaying the aging progression of the aging population in an active and healthy aging mode. It has been foreseen to become an important and feasible strategy for the long-term prevention and mitigation of aging induced skeletal muscle atrophy [38]. Aerobic exercise has the potential to minimize the effects of muscle atrophy by enhancing motor unit recruitment and favoring the development of high oxidative muscle fibers. Treadmill training reduces muscle loss and increases protein synthesis, myogenic fiber count, and fiber cross-section. This leads to an improvement in muscle mass and strength [39] and an increase in exercise ability and survival [40–46].

In 2017, scientists in the United States won the Nobel Prize for discovering biological clock genes and their underlying regulatory mechanisms of action. Since then, research on biorhythms has attracted considerable attention from researchers in sports medicine and exercise physiology. The biological clock is an invisible "clock" in the body that regulates the physiological and biochemical behavior of the body. It is a universal molecular oscillator that controls and regulates many behavioral and physiological phenomena [47]. The mammalian biological clock is a molecular clock network that includes the central circadian clock located in the suprachiasmatic nucleus of the hypothalamus [48] and peripheral biological clocks in peripheral tissues and organs, such as the skeletal muscles, heart, and kidneys [49]. The basic molecular clock mechanism is a self-regulated transcriptional feedback loop composing Brain and Muscle Arnt-like 1 (BMAL1), Circadian Locomotor Output Cycles Kaput (CLOCK), cryptochrome 1 (CRY1), cryptochrome 2 (CRY2), period 1 (PER1), and period 2 (PER2). The circadian rhythm is most closely linked to exercise and is the most widely studied rhythm, which may affect exercise ability and performance. Moreover, exercise as a signal input can affect circadian rhythms [50]. Consequently, a close interaction has been proposed among skeletal muscles, the circadian clock system, and exercise [51].

Almost all aspects of mammalian physiology change relative to the time of day. Many of these variations are directly or indirectly driven by the circadian clock, an evolutionarily conserved time-keeping mechanism present in virtually all cells of the body [52]. Consistent with the circadian modulation of muscle physiology, hundreds of transcripts in skeletal muscles oscillate within a 24 h in humans and mice [53,54]. Moreover, insulin sensitivity, mitochondrial respiration, and glucose and lipid-related metabolites follow similar patterns in the muscle tissue [55–57]. Similarly, daily variations in resistance and endurance exercise peak performance have been reported during the normal active phase in humans [58] and rodents [59] but not in all trials [58,60]. The robustness and timing of such performance peaks may be highly variable, depending on several parameters, including chronotype, time from awakening, muscle and liver glycogen levels, nutritional status, and temperature [61,62]. Training studies at different times of the day suffer from confounding factors, such as light-mediated inhibition of voluntary locomotion in animals or restricted analysis of reporter gene-based approaches. Therefore, whether the putative enhanced effects of timed exercise training on health parameters in both clinical and preclinical contexts [63,64] depend on the zeitgeber properties of the exercise remains unclear.

Currently, research on the circadian rhythms of skeletal muscles is mainly focused on rodent models. Rodent experiments are typically conducted during the day, which is the sleep period of rodents. However, circadian rhythm and exercise ability change over time. One study examined the difference in the exercise capacity of mice between two time points within their active phase: 2 h after lights-off and 2 h before lights-on (named Early and Late, respectively) [59]. They reported that running durations at 55% and 45% of the maximal aerobic capacity were longer at later times. In our experimental design, we ensured that the duration of the exercise training and time of each sample were as consistent as possible. Therefore, in future experiments, we will consider designing different times of the day to observe changes in circadian clock genes.

Increased expression of the circadian rhythmic proteins BMAL1, PER1, and CLOCK is associated with cerebral infarction size, brain swelling, neurological deficits, neuronal survival, and post-ischemic apoptosis [65]. Studies on rodent skeletal muscle have reported that PER2 mRNA levels increase approximately 1 h after acute aerobic exercise [66]. Furthermore, PER2 knockout mice exhibit a reduced ability to exercise at low and moderate levels during their later active phases [59]. These data provide additional evidence for the relationship between molecular clocks and athletic capability. However, this topic warrants further investigation.

The skeletal muscle is the main functional organ involved in post-stroke disability, and exercise is the main method to improve sarcopenia associated with stroke. The probable mechanism is that the regulatory effect of exercise on the molecular clock alters the skeletal muscle rhythm and promotes the metabolism and function of skeletal muscles. Exercise as a time signal for the skeletal muscle clock may be an effective tool for resetting the clock in patients with stroke-related sarcopenia.

In this study, a rat model of right central brain artery occlusion was established using a wire embolism. A sarcopenia pattern was successfully implemented in rats with middle cerebral artery occlusion (MCAO). We performed transcriptome sequencing of hemiplegic and non-hemiplegic skeletal muscles. We determined early changes in body weight and gastrocnemius muscle mass on the hemiplegic and non-hemiplegic sides of rats following stroke. The treadmill training intervention was also administered. The morphology of the gastrocnemius muscle fibers was observed by hematoxylin-eosin (H&E) staining. Changes in BMAL1, PER1, and PER2 expressions in the hemiplegic and non-hemiplegic gastrocnemius muscles were detected by quantitative real-time polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Animals

We acquired 168 healthy male Sprague-Dawley rats (weight, 280–300 g) from the Kunming Laboratory Animal Center (Kunming, China). All the rats were housed at 25 °C ± 2 °C on a 12-h reverse light/dark cycle in separate cages (five rats per cage) and had free access to food and water. All animal protocols were approved by the Animal Ethics Committee of Kunming Medical University

(KMMU20220901).

2.2. Grouping and experimental design

After 1 week of adaptation, 144 rats were randomly divided into four groups: the normal group, which rats that did not receive surgery and treatment; the sham group, which rats were exposed blood vessels according to the MCAO surgical method [67] but not ligated; the MCAO group, which rats that only received MCAO surgery; the training group, which rats received MCAO surgery and were given treadmill training. The observation points were set before molding and 10 and 21 days after molding. Overall, 24 rats were used for transcriptome sequencing. A flowchart of the experimental design is presented in Fig. 1.

2.3. Construction of a cerebral ischemia/reperfusion injury model

We established a cerebral ischemia/reperfusion injury model of right MCAO caused by a thread embolism following previously reported guidelines [67]. The rats were anesthetized using an anesthesia machine and mounted on an operating table. Neck hair was shaved and disinfected. An incision measuring approximately 1.5 cm was made in the middle of the neck. The right common carotid artery (CCA) was carefully isolated from the internal carotid artery (ICA) and external carotid artery (ECA). To avoid damaging the vagus nerve, the proximal ends of the CCA and ICA were carefully clamped, and two surgical knots were tied 5 mm away from the bifurcation of the ECA. An incision was made from the middle of the two surgical knots, the plug line was inserted, the direction of the plug line was adjusted, and the plug line was gently pushed toward the intracranial direction, as visually observed in the ICA. The pushing was continued and stopped when slight resistance was encountered. The insertion depth of the plug was generally 1.8–2 cm (starting point of the ECA and ICA bifurcation), and the tip of the plug passed through the beginning of the middle cerebral artery. After entering the smaller anterior cerebral artery, the knot was tied tightly, the excess thread was trimmed, the skin was stitched, and the rat was returned to its cage. The plugs were carefully removed after 2 h. In the sham group, the CCA, CIA, and right ECA were exposed but not ligated.

The success of this model was indicated by the presence of hemiplegia, which was more severe in the ischemic contralateral limb after recovery from anesthesia. Neurological function was scored 24 h after MCAO using the 5-points scoring standard of Longa et al. [67] as follows: 0, no signs of nerve injury; 1, the contralateral forepaw cannot be fully extended; 2, autonomic movement, the body to the hemiplegic side of the circle; 3, during voluntary movement, the body falls to the opposite side; and 4, loss of consciousness, inability to walk spontaneously. Signs of neurological impairment were scored after the rats were awake, and the model was successfully prepared if the score was 1. Rats with scores of 1–3 were included in this study.

2.4. Treadmill training protocol

The training group completed treadmill training on day 3 posy-surgery. The rats first underwent 2 days of adaptive exercise; on day 1 and 2, the treadmill slope was 0°, the exercise speed was 10 m/min, and the exercise time was 20 min. On day 3, the treadmill slope was 0°, the speed was increased to 15 m/min, the exercise time was 30 min, and the exercise frequency was 5 days/week. The rats were subjected to daily treadmill training between 10 a.m. and 2 p.m.

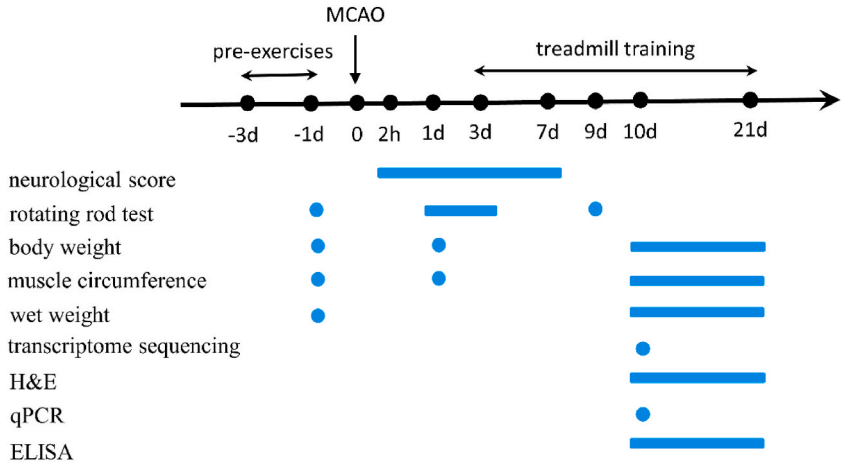

Fig. 1. Experimental design. Rats were subjected to middle cerebral artery occlusion (MCAO) after 3 days of pre-running training. MCAO rats underwent treadmill training beginning on day 3 and continuing until day 21. During the intervention, we measured neurological scores, rotating rod test, body weight, muscle circumference, and wet weight. After sacrifice, muscle tissues were obtained for laboratory testing, including transcriptome sequencing, quantitative real-time polymerase chain reaction, hematoxylin–eosin staining, and enzyme-linked immunosorbent assay.

2.5. Rotating rod test

Motor function in the MCAO and training groups was assessed using the rotating rod test. The assessment time points were preoperatively and 24, 72 h, and 9 days postoperatively. The rats were trained on a rotating rod that was gradually accelerated from 0 to 40 rpm within 200 s. We recorded the length of time that the rats remained on the rod. The test interval for each rat was 10 min, and the mean residence time was calculated for three consecutive periods.

2.6. Skeletal muscle tissues preparation

Hemiplegic and non-hemiplegic gastrocnemius muscle tissues were obtained 10 and 21 days after MCAO modeling and then preserved. The skeletal muscles of the rats were sampled daily between 10 a.m. and 4 p.m. The gastrocnemius muscle was divided into three parts: two for qPCR and ELISA and one for H&E staining. The isolated bilateral gastrocnemius muscle samples were quickly cut along the direction of the muscle filament, placed in 2.5% glutaraldehyde and 4% paraformaldehyde solutions, and stored in liquid nitrogen. Thereafter, the samples were stored in a refrigerator at −80 °C for further experiment. For H&E staining, the skeletal muscle was divided into two 2 mm slices, placed in a fixing solution of 4% paraformaldehyde, and stored in a refrigerator at 4 °C. Paraffin sections (6 μm thick) were cut depending on the direction of the muscle fiber sections for H&E staining.

2.7. Measurement of calf muscle circumference and wet weight of the gastrocnemius muscle

We straightened the calf circumference of each rat, formed a circle around the widest part of the leg circumference with a rope, marked it with a ruler, and measured the length of the marked rope. After anesthesia, the gastrocnemius muscle on both sides was quickly and completely peeled off, the residual liquid on the surface was removed, and the wet weight of the gastrocnemius muscle on the hemiplegic and non-hemiplegic sides was measured using an electronic balance.

2.8. H&E staining of the skeletal muscle

The muscle specimens were fixed in 4% paraformaldehyde and sequentially dehydrated in a gradient ethanol solution until bottled. Paraffin sections were cut along the cross-section of the muscle fibers at 6 μm thickness. Before staining, xylene dewaxing, gradient ethanol rehydration, hematoxylin dyeing for 2–3 min, and rinsing with tap water were performed. The nucleating effect of hematoxylin was observed under a microscope. If the nucleus color was too dark, 1% hydrochloric acid ethanol solution was used for color separation for a few seconds, rinsed fully with tap water, stained with eosin dye solution for 1 min, rinsed with tap water, dehydrated with a series of gradient ethanol solutions, and sealed with transparent xylene and neutral resin. Eight sections were selected from each group for observation under an optical microscope (200×), and four fields were randomly selected for each section to be photographed. Cross-sectional area (CSA) is the main parameter to assess muscle regeneration capability. MyoView is an efficient and accurate software for measuring CSAs [27]. In our study, we assessed the structural characteristics and CSA of the gastrocnemius muscle fibers in each group.

2.9. Transcriptomic methods

Total RNA was extracted from the hemiplegic and non-hemiplegic muscles of 12 rats in the sham and MCAO groups using the TRIzol reagent. RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was assessed using the RNA Nano 6000 Assay Kit on an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). One microgram RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using the Hieff NGS Ultima Dual-mode mRNA Library Prep Kit for Illumina (Yeasen Biotechnology Co., Ltd., Shanghai, China) following the manufacturer's recommendations, and index codes were added to the attribute sequences for each sample. mRNA was purified from total RNA using poly T oligo-attached magnetic beads. First-strand and second-strand cDNA were synthesized. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activity. After adenylation of 3′ ends of DNA fragments, the NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization. Library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, MA, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. PCR was performed using the Phusion High-Fidelity DNA polymerase, Universal PCR primers, and the Index (X) Primer. Finally, the PCR products were purified (AMPure XP system), and library quality was assessed using an Agilent Bioanalyzer 2100 system.

2.10. Differential expression analysis

For samples with biological replicates, a differential expression analysis of the two conditions/groups was performed using DESeq2. DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on a negative binomial distribution. The resulting P values were adjusted using Benjamini and Hochberg's approach to control for the false discovery rate. Genes with an adjusted P-value $<$ 0.01 and Fold Change$\geq$2 found by DESeq2 were assigned as differentially expressed. For samples without biological replicates, a differential expression analysis of the two samples was performed using EdgeR. The false discovery rate (FDR) $<$ 0.01 and fold change$\geq$2 were set as the threshold for significantly differential expression.

2.11. Detecting BMAL1, PER1, and PER2 mRNA expressions via qPCR

Total genomic RNA was extracted using an easy-to-isolate RNA reagent (CAT #R701; Vazyme) after crushing gastrocnemius muscles in liquid nitrogen. cDNA synthesis was performed using HiScript III RT SuperMix for qPCR (gDNA wiper) (CAT#R323; Vazyme). qPCR was used to quantitatively detect the mRNA expression of associated genes. The primer names and sequences are listed in Table 1 qPCR was performed using the Taq Pro Universal SYBR qPCR Master Mix (CAT#Q712; Vazyme) according to the manufacturer's instructions. The reaction procedure of qPCR is as follows: pre-denaturation for 5 min at 95 °C. cDNA was amplified in 50 cycles at 95 °C for 15 s and 60 °C for 30 s. After the thermal cycle, the solution curve was measured at 75 °C for 5 s and 95 °C for 5 s. A dissociation curve was used to determine the specificity of the amplified product and formation of primer dimers. The relative expression levels were normalized and calculated by $2^{-\triangle\triangle}$CT. All qPCR experiments were repeated at least thrice, and the results are expressed as mean ± standard deviation.

2.12. ELISA for detecting the expression of clock genes in the gastrocnemius muscle

We added 50 μl of standard product at a different concentration to the standard well, 50 μl of sample to be measured was added to the sample well, and none was added to the blank well. In addition to the virgin pore, 100 μl of horseradish peroxidase was added to each pore in the standard pore and sample and incubated for 60 min. After repeated washing five times, the 50 μl substrate A and 50 μl substrate B were added to each well and incubated at 37 °C for 15 min away from light. Finally, a 50 μl termination solution was added to each well, and the optical density (OD) was measured at a wavelength of 450 nm in 15 min. We used the OD value of the measured standard product as the horizontal coordinate and the concentration value of the standard product as the vertical coordinate, drew a standard curve, obtained a linear regression equation, substituted the OD value of the sample into the equation, and calculated the sample concentration.

2.13. Statistical analysis

Data are expressed as mean ± standard error of the mean. The GraphPad Prism Software (version 8.0; GraphPad Software Inc., San Diego, California, USA) was used for statistical analysis of all data and generating the charts. After verifying that all data were normally distributed, a one-way analysis of variance was performed to analyze the neurological scores, qPCR, ELISA, body weight, calf muscle circumference, and gastrocnemius wet weight. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Neuro function scores for assessing the success of brain ischemia–reperfusion models in rats

Neurological function scores in the MCAO and training groups were greater than 0, 2, and 24 h after modeling, indicating signs of neurological impairment. This suggested that the rat cerebral ischemia–reperfusion model was successfully developed. Neurological function scores were measured again at 3 and 7 days after modeling, and neurological function scores were significantly lower at these two-time points than at 2 h after modeling (MCAO, 3d, $P < 0.01$; 7d, $P < 0.001$; Training, 3d, $P < 0.0001$; 7d, $P < 0.001$; Fig. 2).

3.2. Rotating rod test for assessing rat motor function after modelling

The rotating rod test was performed on the rats in the MCAO and training groups before 24, 72 h after, and 9 days after modeling. The rotational speed and test time were monitored to assess motor function. The results revealed that the rotation speed of the training group was slower than that of the MCAO group 72 h after molding ($P < 0.05$; Fig. 3-A). The training group had a shorter test time than the MCAO group ($P < 0.05$; Fig. 3-B). Nine days after modeling, the rotational speed and test time of the formation group were not significantly different from those of the MCAO group. However, the rotation speed was faster, and the test time was longer than that in the MCAO group ($P > 0.05$; Fig. 3). These results indicate that treadmill training could increase the rotational speed in the rod test, extend the test duration, and improve motor function.

3.3. Acute weight loss and muscular loss in experimental rats following cerebral ischemic injury

Weight loss was observed in the MCAO and training groups at 1, 10, and 21 days after MCAO and was significantly different from

Table 1
Primers sequence for quantitative real-time PCR.

Gene Target	Forward	Reverse
BMAL1	5′-TGTTGGCCAGAGTGAATGCT-3′	5′- CTGGCCTGGAACTTGCTACA-3′
PER1	5′-TTGACACCTCTTCTGTGGCG-3′	5′-ACACATAGCAGGGGGTTTCG-3′
PER2	5′-CTGCCACCTCAGACTCACTG-3′	5′- TCACTCTCCTCCGTGTCTGT-3′

BMAL1: Brain and Muscle ARNT-Like 1; PER1: Period Circadian Regulator 1; PER2: Period Circadian Regulator 2.

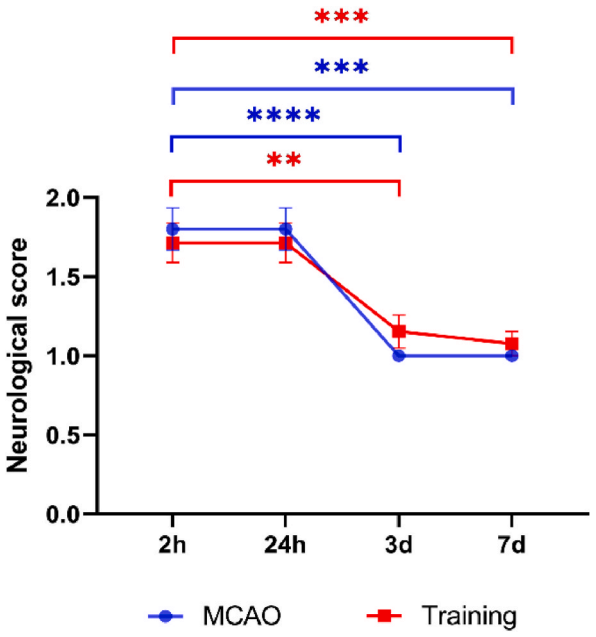

Fig. 2. Neurological scores on 2 h, 24 h, 3 days, and 7 days after MCAO. Two-way ANOVA. Data are expressed as mean ± SEM. **P < 0.01, ***P < 0.001, ****P < 0.0001.

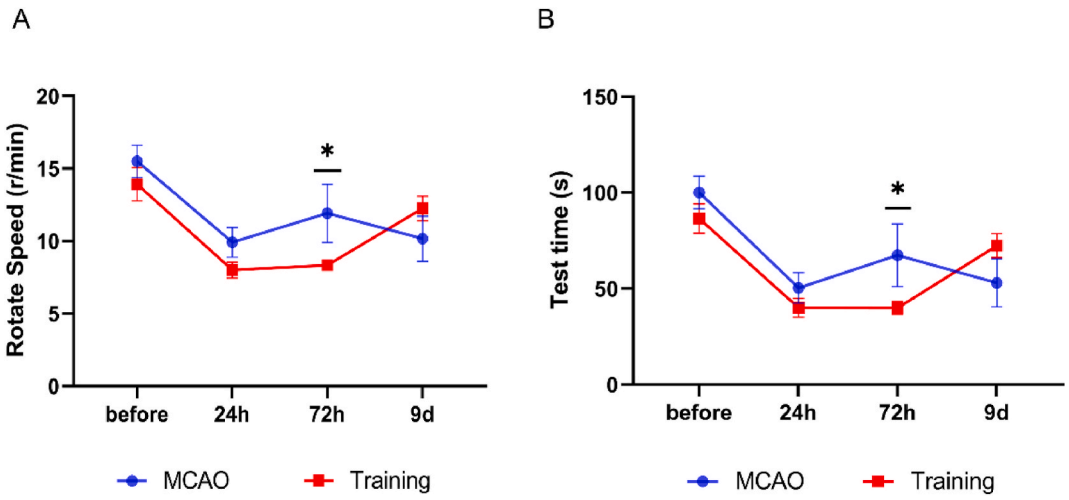

Fig. 3. Rotating rod test in the MCAO and training groups. A. Rotate speed of the MCAO and training group. B. Test time of the MCAO and training group. Two-way ANOVA. Data are expressed as mean ± SEM. *P < 0.05.

that in the sham group (Fig. 4-A). During the observation period, the weights of the two groups decreased significantly on day 10 (P < 0.001; Fig. 4-A) and recovered on day 21. Although no significant difference was observed between the training and MCAO groups, a significant difference between the training groups was noted on days 21 and 10 (P < 0.01; Fig. 4-B).

Similarly, muscle tissue loss and wet weight of the gastrocnemius were reduced in both the MCAO and training groups 10 days after MCAO. Compared with the control and sham groups, the calf muscle circumference and wet weight of the gastrocnemius on the hemiplegic and non-hemiplegic sides of rats in the two groups were significantly reduced (muscle circumference, P < 0.0001; Fig. 5-A, and -B; wet weight of gastrocnemius muscle, P < 0.001; Fig. 6-A). However, the muscle circumference and wet weight of the gastrocnemius did not differ significantly between the hemiplegic and non-hemiplegic sides in the MCAO and training groups after early treadmill training intervention (muscle circumference, P > 0.05; Fig. 5-C and -D; wet weight, P > 0.05; Fig. 6-B and -C).

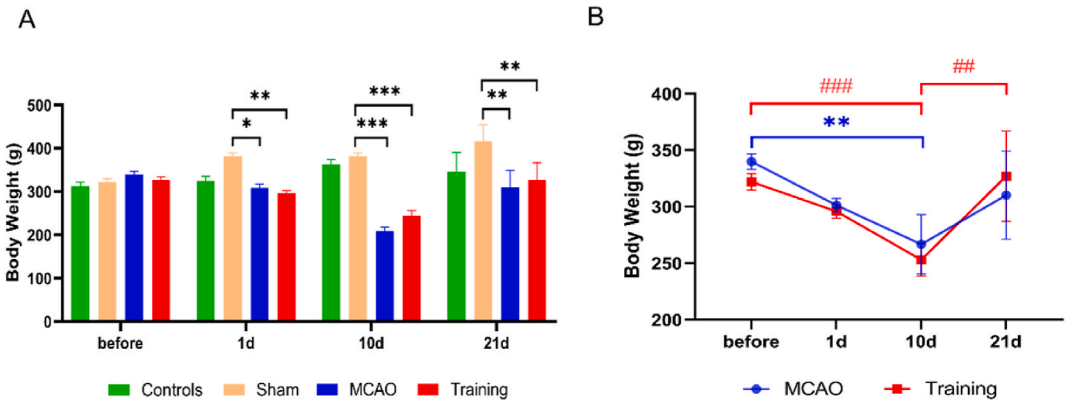

Fig. 4. Body weight were reduced after MCAO. (B, *MCAO group; #Training group). Two-way ANOVA. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ## <0.01, ### <0.001.

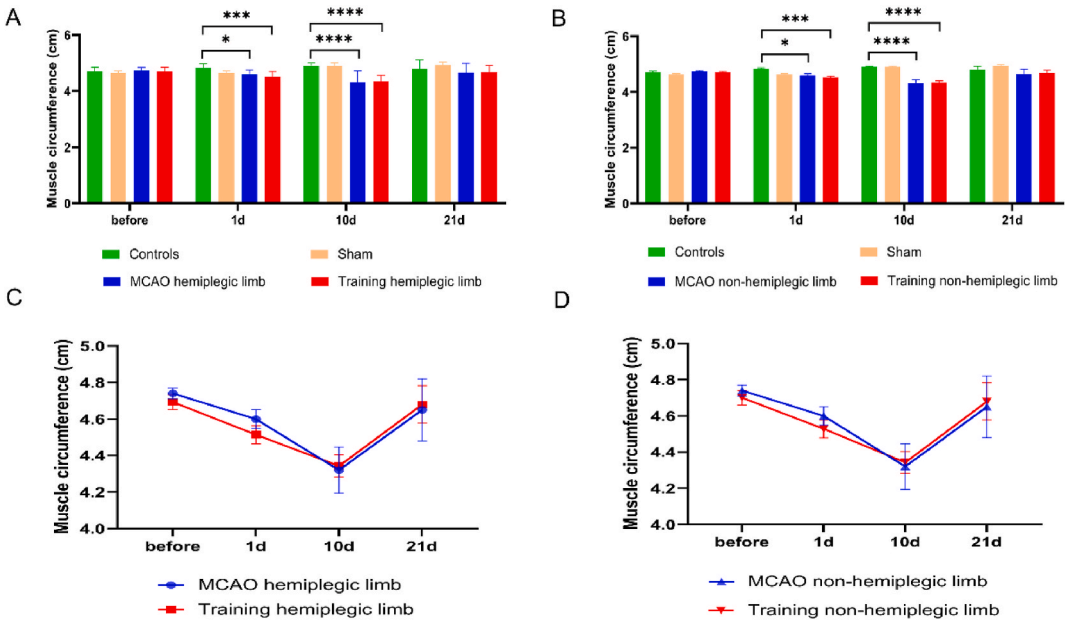

Fig. 5. Muscle tissues of the gastrocnemius were reduced. A, and C. Muscle tissues loss of the hemiplegic side of MCAO and training group. B, and D. Muscle tissues loss of the non-hemiplegic side of MCAO and training group. Two-way ANOVA. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

3.4. H&E staining was performed on the gastrocnemius muscle to observe the morphological changes and inflammatory response of muscle fibers

Ten days after MCAO, the H&E staining indicated that the muscle fibers on the hemiplegic side in the MCAO group were severely atrophied with different sizes, necrotic muscle fibers, slight hyperplasia of connective tissue, nuclear migration, and slight inflammatory cell infiltration. The sizes of the muscle fibers on the hemiplegic side in the training group differed, and some muscle fibers atrophied and became smaller. However, 21 days after MCAO, muscle fibers in the MCAO and training groups were much more ordered and uniform in size than those in the control group (Fig. 7).

After the treadmill training period, we assessed muscle fibers in both whole muscle cross-sections and isolated myofibers. The average muscle CSA in response to MCAO and resistance training is presented in Fig. 8(A–G). CSA of single muscle fiber decreased significantly in the gastrocnemius skeletal muscle fibers of MCAO rats on the hemiplegic side on day 10 compared to the control group (P < 0.01, Fig. 8-B). CSA of whole muscle fiber decreased in the gastrocnemius skeletal muscle fibers of MCAO rats on the hemiplegic side on day 21 compared to the control group (P < 0.05, Fig. 8-C). Additionally, on the hemiplegic side on day 21 compared to day 10 of the training group, the CSA of whole muscle fiber increased with statistical differences (P < 0.05, Fig. 8-G). Although no significant difference was observed between 10 and 21 days in CSA of single muscle fibers of the training group. (P > 0.05, Fig. 8-H).

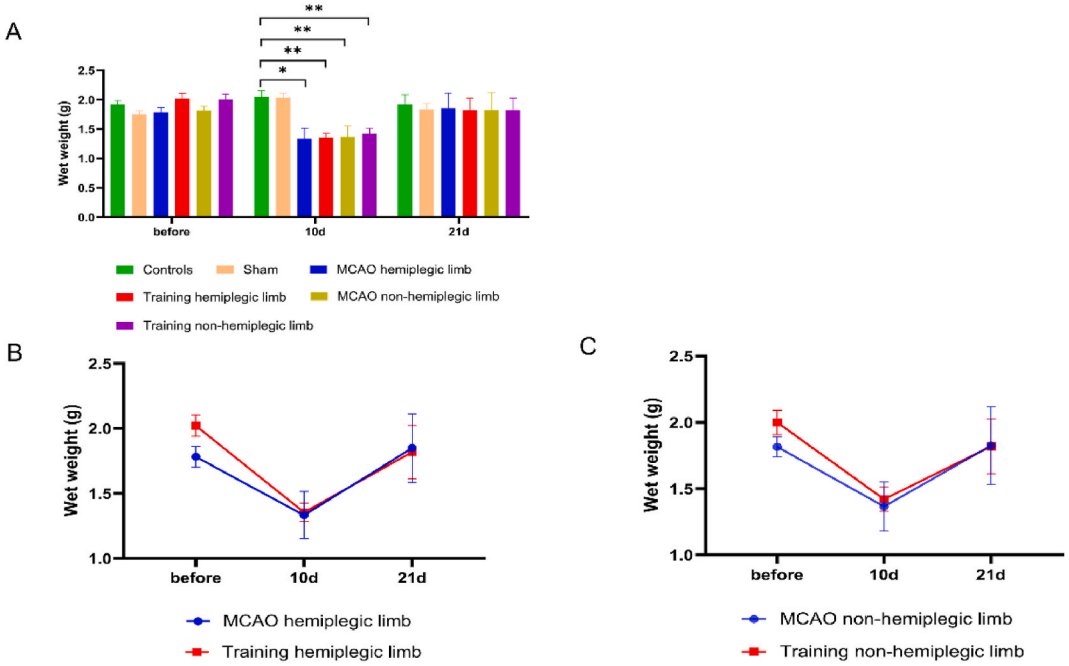

Fig. 6. Wet weight of the gastrocnemius were reduced after MCAO. B. Wet weight of the hemiplegic side of MCAO and training group. C. Wet weight of the non-hemiplegic side of MCAO and training group. Two-way ANOVA. Data are expressed as mean ± SEM. ***P < 0.001.

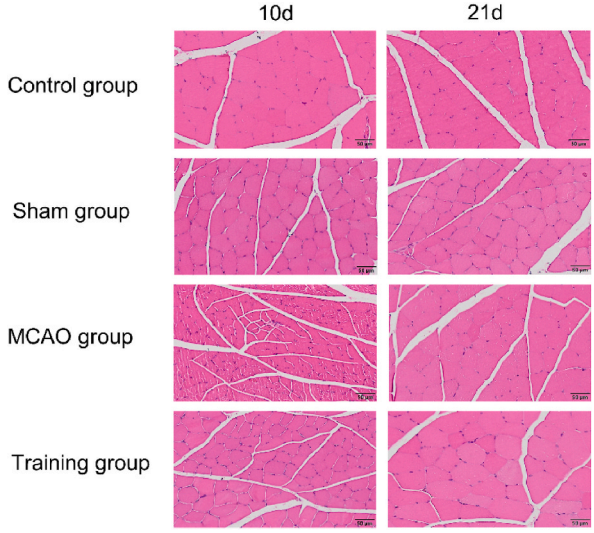

Fig. 7. H&E staining showed sections of gastrocnemius in the hemiplegic side on days 10 and 21 (×200, scale bar = 50 μm). On 10 days, the muscle fibers were atrophied and varied in size.

3.5. *Differentially expressed genes*

By comparing the sham group with the hemiplegic side of the model group (model A/sham group), nine differentially expressed genes were identified, four upregulated and five downregulated (Fig. 9A–B). PER1 and PER2 expressions were upregulated, whereas BMAL1 expression was downregulated.

By comparing the sham group with the non-hemiplegic side of the model group (model UA/sham group), 17 differentially expressed genes were identified, 10 upregulated and seven downregulated (Fig. 10A–B). PER1 and PER2 expressions were upregulated, whereas BMAL1 expression was downregulated.

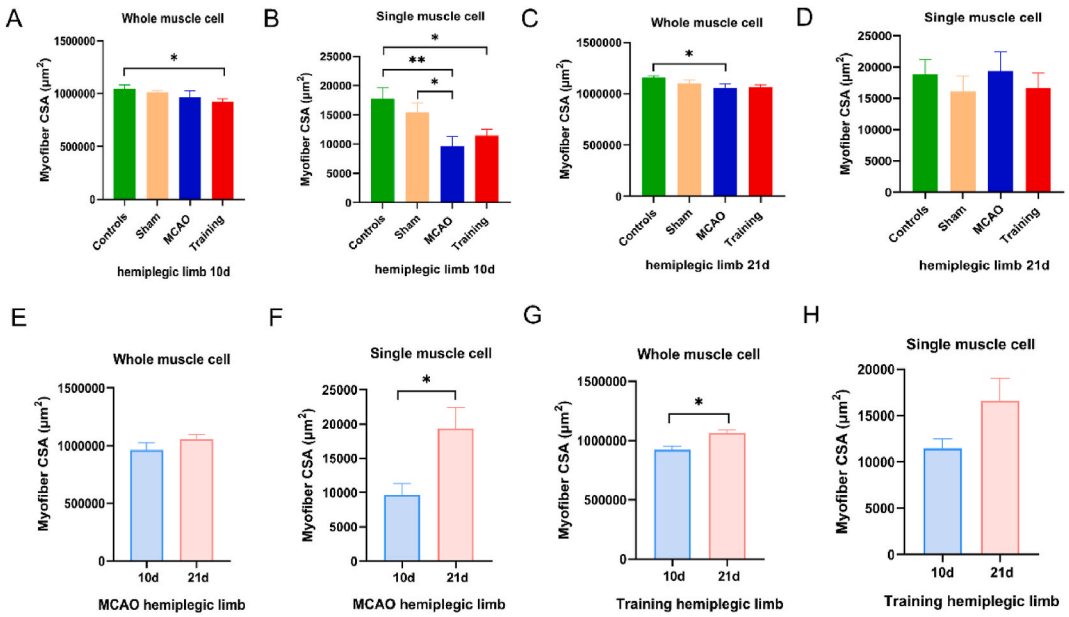

Fig. 8. Statistical results of cross section area of gastrocnemius muscle fibers stained by H&E. The cross-sectional area of muscle fibers was assessed on 10 and 21 days. A-D, One-way ANOVA; E-H, *t*-test. Data are expressed as mean ± SEM. **P < 0.01, *P < 0.05.

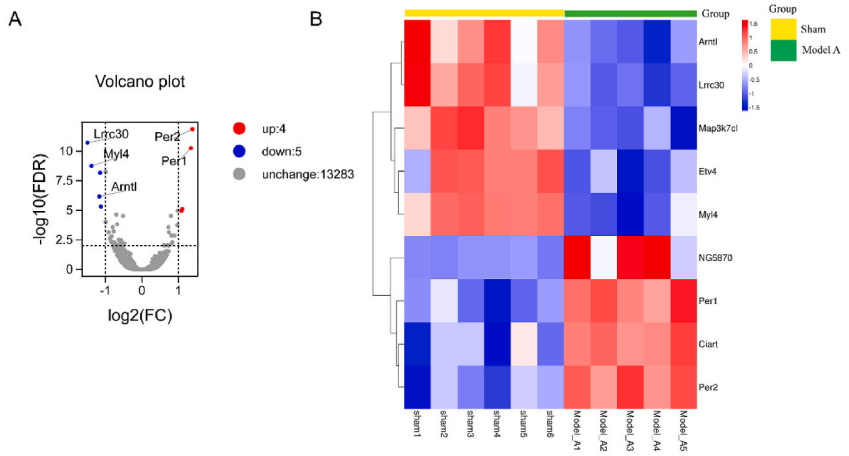

Fig. 9. Differentially expressed genes. A. Volcano plot of the hemiplegic side of model/sham group. B. Heatmap of top 9 genes.

3.6. Treadmill training increased the mRNA levels of the clock genes of the skeletal muscles

We measured the mRNA levels of the three clock genes 10 days after MCAO. We observed a decrease in BMAL1 mRNA levels and an increase in PER1 and PER2 mRNA levels in the gastrocnemius muscle on the hemiplegic side in the MCAO group. No significant differences were observed between the control and sham groups (P > 0.05; Fig. 11-A, -C, and -E). In the training group, the BMAL1, PER1, and PER2 mRNA levels increased after treadmill training. The mRNA levels of PER1 and PER2 in the training group were significantly different from those in the control group (PER1, P < 0.05; PER2, P < 0.05; Fig. 11-C and -E).

The mRNA levels of BMAL1, PER1, and PER2 in the non-hemiplegic gastrocnemius muscle were higher in the MCAO group than in the control and sham groups, and no significant differences were observed between the MCAO and control groups (Fig. 11-B, -D, and-F). In addition, the mRNA levels of PER1 and PER2 in the training group significantly increased (PER1, P < 0.01; PER2, P < 0.001; Fig. 11-D and -F).

3.7. Treadmill training affects the expressions of skeletal muscle clock genes

Using ELISA, we detected the expressions of three clock genes (BMAL1, PER1, and PER2) 10 and 21 days after MCAO in the

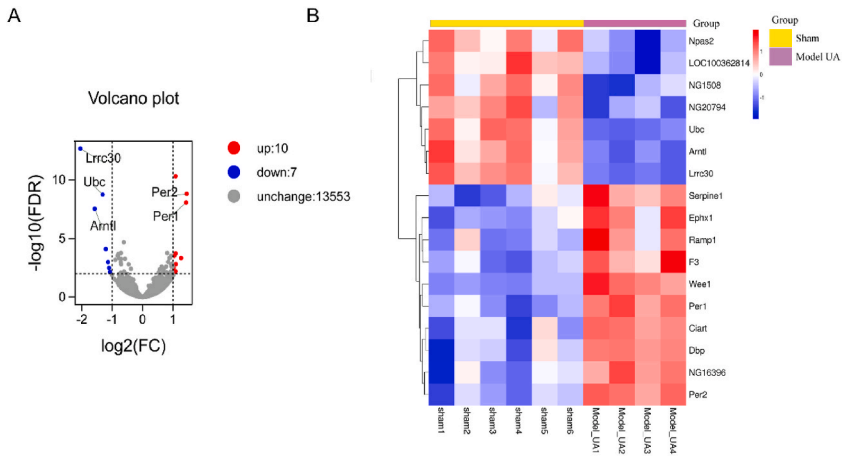

Fig. 10. Differentially expressed genes. A. Volcano plot of the non-hemiplegic side of model/sham group. B. Heatmap of top 17 genes.

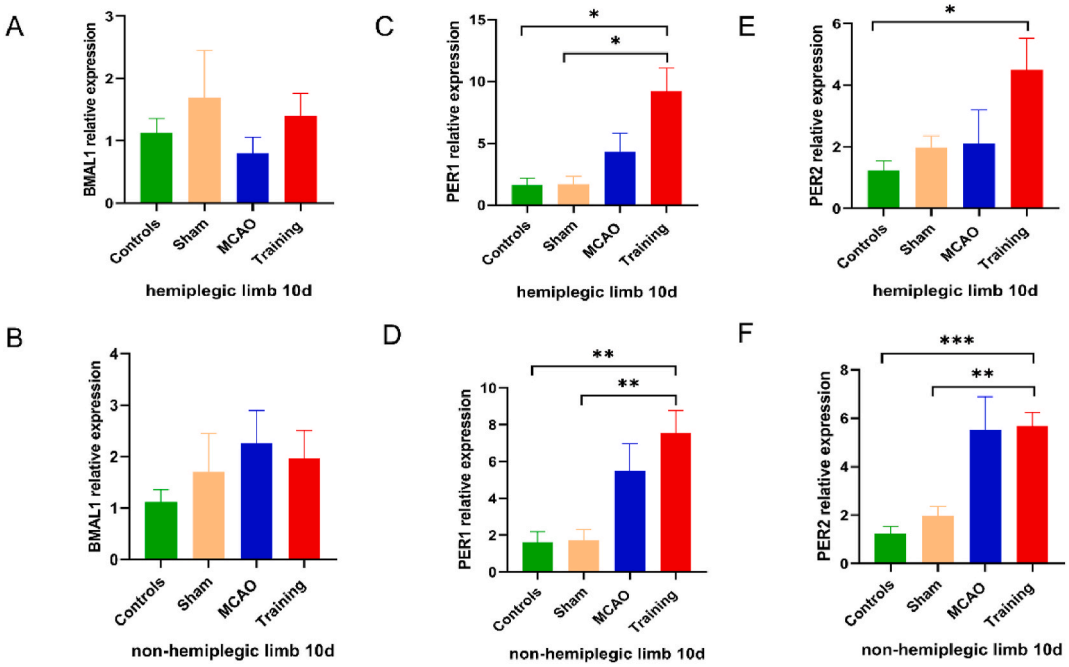

Fig. 11. The mRNA levels of BMAL1, PER1, and PER2 in the hemiplegic (A, C, and E) and non-hemiplegic (B, D, and F) gastrocnemius muscles on 10 days. One-way ANOVA. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01.

hemiplegic and non-hemiplegic gastrocnemius muscles.

3.7.1. Treadmill training increased BMAL1 expression on both the hemiplegic and non-hemiplegic gastrocnemius muscles

The level of the BMAL1 expression on the hemiplegic side increased in the training group. However, no significant difference was observed between the training and MCAO groups ($P > 0.05$; Fig. 12-A and -B). No significant difference in BMAL1 expression was observed between the two groups at 10 and 21 days after MCAO ($P > 0.05$; Fig. 12-C and -D). Ten days after MCAO, BMAL1 expression on the non-hemiplegic side of the training group significantly decreased compared to the control and sham groups ($P < 0.05$; Fig. 12-E). We also observed that BMAL1 expression in both the MCAO and training groups significantly increased at 21 days (Fig. 12-F) compared to that at 10 days (MCAO group, $P < 0.05$; Fig. 12-G; training group, $P < 0.001$; Fig. 12-H).

3.7.2. Treadmill training decreased PER1 expression on both the hemiplegic and non-hemiplegic gastrocnemius muscles

The PER1 expression on 10 and 21 days following MCAO are presented in Fig. 13(A–H). Ten days after MCAO, the PER1 expression on the hemiplegic side significantly decreased in the training group compared with that in the control group ($P < 0.05$; Fig. 13-A).

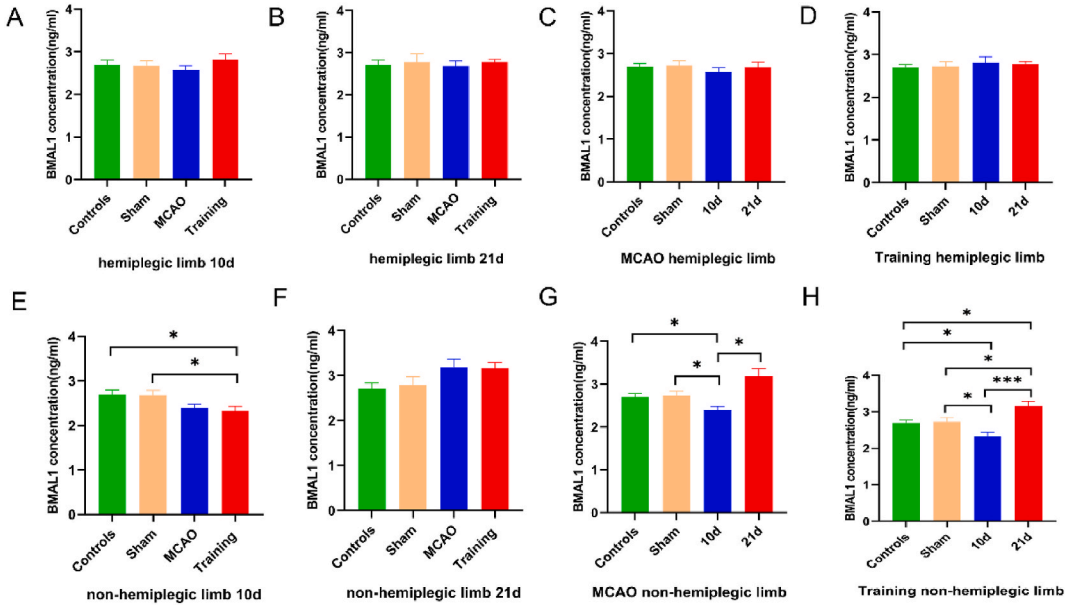

Fig. 12. The BMAL1 expression on 10 and 21 days following MCAO in the hemiplegic (A, B, C, and D) and non-hemiplegic (E, F, G, and H) gastrocnemius muscles. One-way ANOVA. Data are expressed as mean ± SEM. *P < 0.05, ***P < 0.001.

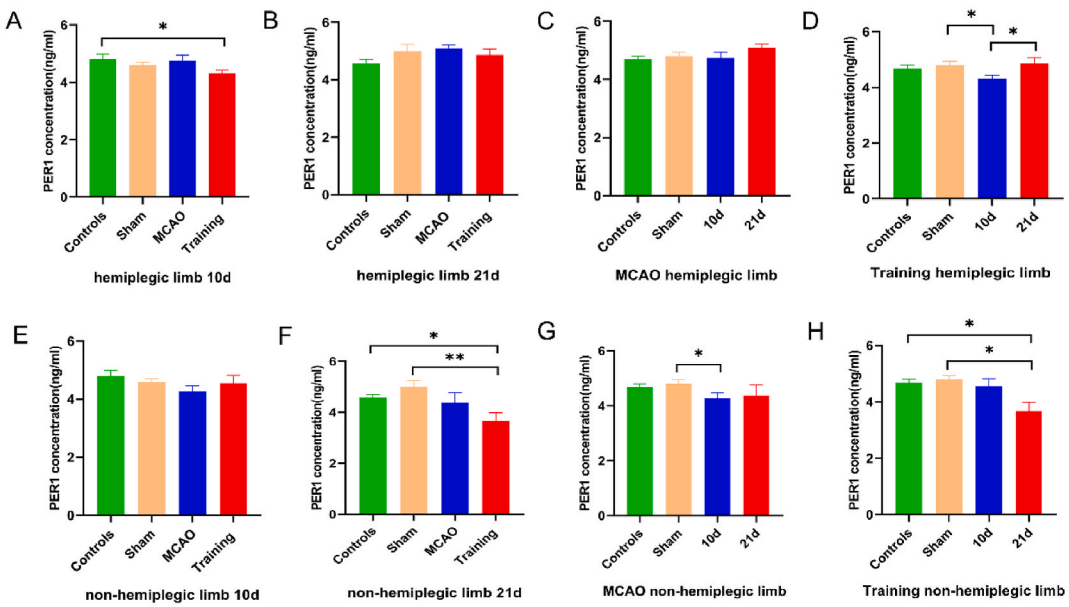

Fig. 13. The PER1 expression on 10 and 21 days following MCAO in the hemiplegic (A, B, C, and D) and non-hemiplegic (E, F, G, and H) gastrocnemius muscles. One-way ANOVA. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01.

PER1 expression also significantly increased on the hemiplegic side of the training group compared with that in the 10-day group (P < 0.05; Fig. 13-D). However, PER1 expression on the non-hemiplegic side decreased 21 days after MCAO in the training group. Significant differences were observed between the control and sham groups (control group, P < 0.05; sham group, P < 0.01; Fig. 13-F).

3.7.3. Treadmill training decreased PER2 expression on the hemiplegic side and increased PER2 expression on the non-hemiplegic side

On the hemiplegic side of the gastrocnemius, PER2 expression in the MCAO group on days 10 and 21 was higher than that in the training group (day 10, P < 0.05; Fig. 14-A; day 21, P > 0.05; Fig. 14-B). PER2 expression at day 21 was higher than that at day 10, although no significant difference was observed between the two groups (P > 0.05; Fig. 14-C and -D). On the non-hemiplegic side of the gastrocnemius, the PER2 expression in the training group was higher than that in the MCAO group (10 days, P < 0.05; Fig. 14-E. 21

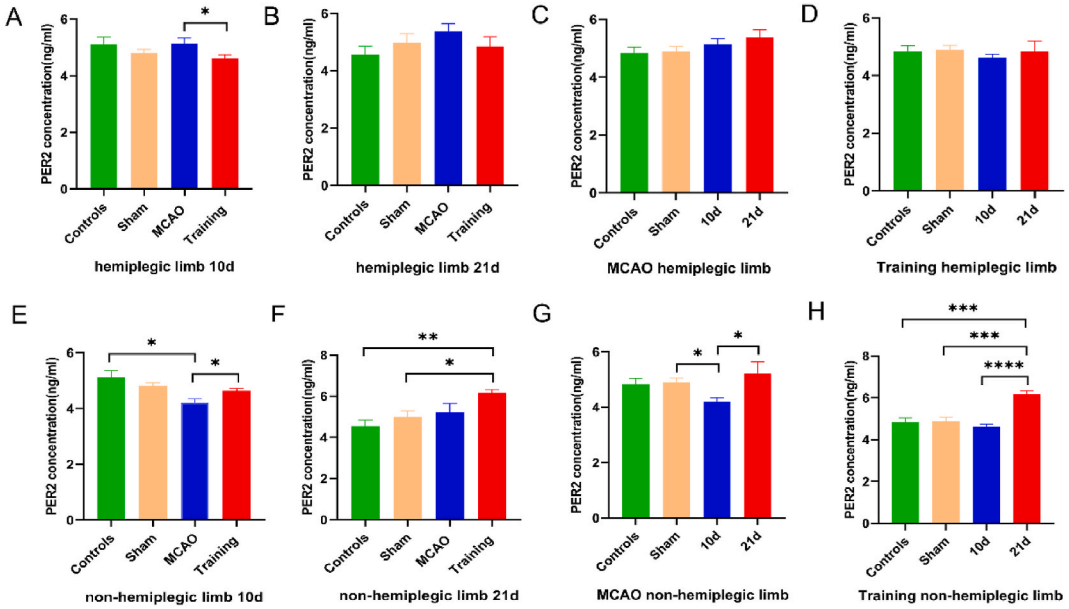

Fig. 14. The PER2 expression on 10 and 21 days following MCAO in the hemiplegic (A, B, C, and D) and non-hemiplegic (E, F, G, and H) gastrocnemius muscles. One-way ANOVA. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

days, vs. control group, P < 0.01; vs. sham group, P < 0.05; Fig. 14-F). Moreover, the PER2 expression in the two groups at 21 days was significantly different from that at 10 days (MCAO group, P < 0.05; Fig. 14-G; training group, P < 0.0001; Fig. 14-H).

4. Discussion

The current study examined the effects of ischemic stroke and endurance training on the skeletal muscle and whether they alter the expression of clock genes in the skeletal muscle. Three novel results were obtained and discussed in more detail as follows. First, ischemic stroke causes a decrease in muscle circumference, wet weight of the gastrocnemius muscle, and cross-sectional area of muscle fibers in both hemiplegic and non-hemiplegic limbs, thereby causing muscle atrophy. Endurance training can improve post-stroke muscle atrophy. Second, stroke interferes with the expression of skeletal muscle clock genes on both the hemiplegic and non-hemiplegic sides. Lastly, endurance training increased BMAL1 expression and decreased PER1 expression on both the hemiplegic and non-hemiplegic sides. Interestingly, endurance training reduced PER2 expression on the hemiplegic side and increased it on the non-hemiplegic side.

Our results are consistent with those of previous studies demonstrating that stroke causes muscle atrophy, which is associated with decreased muscle fibers and motor function in MCAO rats. Although the exact mechanism of muscle atrophy after stroke is unknown, adaptive changes in the muscle tissue structure of the hemiplegic limb can be observed within 4 h of cerebral infarction [68]. The non-hemiplegic limb also exhibits muscle weakness within 1 week after the stroke [69]. In this context, we were interested in determining whether the muscle tissue of the non-hemiplegic limb also changes after stroke. We observed significant reductions in muscle circumference and wet weight of the gastrocnemius on both the hemiplegic and non-hemiplegic sides 10 days after MCAO, which updated our previous understanding. Studies have demonstrated that in the initial stage of stroke, the muscle volume of the hemiplegic lower extremity decreases by 20–24% compared with the non-hemiplegic side, and the intermuscular fat increases by 17–25% compared with the non-hemiplegic side [70]. From 3 weeks to 6 months after the stroke, the non-hemiplegic leg exhibits a similar reduction in muscle mass and increased in intermuscular fat to the hemiplegic leg [71]. In our study, we observed severe atrophy of muscle fibers in the gastrocnemius muscle on the hemiplegic side 10 days after MCAO, with varying muscle fiber sizes. Thus, substantial changes in muscle fibers may have already occurred during the early stages of stroke. Our results also confirmed that endurance training can effectively increase muscle circumference and gastrocnemius wet weight, making muscle fibers more orderly and uniform in size, indicating that endurance training improves muscle fiber structure. Previous studies have demonstrated that after stroke, the dynamic balance between pro-inflammatory and anti-inflammatory factors is disrupted, and inflammatory cytokines can be detected on both the hemiplegic and non-hemiplegic muscle tissues [72]. These findings suggest the involvement of inflammatory cytokines in development of stroke-related sarcopenia. Exercise training can destroy the vicious cycle of chronic inflammation through various anti-inflammatory factors secreted by the skeletal muscle [73]. Nevertheless, the relationship between muscle inflammatory response and exercise training after a stroke requires further study.

Changes in skeletal muscle clock genes were evaluated to understand the relationship between stroke and skeletal muscle rhythm, and the molecular clock mechanism by which exercise regulates skeletal muscle rhythm. Our results revealed that stroke reduced BMAL1 expression and slightly increased PER1 and PER2 expressions in the gastrocnemius muscle on the hemiplegic side. Thus, stroke

interferes with circadian rhythm expression in skeletal muscles. As a key factor in muscle synthesis, BMAL1 positively regulates skeletal muscle cell proliferation and differentiation. PER1 and PER2 are the core components of the molecular clock and work in concert with BMAL1 [74] to negatively regulate BMAL1. Studies have reported that 40-week-old mice with a deletion of the BMAL1 gene lose nearly half of their muscle mass compared with normal mice and have a reduced lifespan. This is attributed to the loss of BMAL1, which leads to a reduction in muscle fiber diameter and a change in muscle fiber type in mice [75,76]. Loss of muscle activity caused by denervation reduces BMAL1 and PER1 expression levels in skeletal muscles, resulting in significant muscle atrophy [77–79]. Our study also revealed that exercise training increased BMAL1 expression levels and decreased PER1 and PER2 expression levels in the hemiplegic gastrocnemius muscle. Some studies have demonstrated that exercise can alter the expression of molecular clocks in mammalian skeletal muscles [80]. PER2 is associated with exercise endurance and athletic ability [81]. Another study, using muscle biopsy, has reported that after resistance exercise, the expression of BMAL1, CRY1, and PER2 in the lower extremities on the exercising side was upregulated compared with that on the non-exercising side. Resistance exercise may indirectly regulate skeletal muscle rhythm and synchronize it through repeated muscle contractions [82]. Moreover, alterations in peripheral skeletal muscle clock genes directly affect skeletal muscle activity. Exercise can change the skeletal muscle rhythm by regulating the expression of the skeletal muscle molecular clock, thus affecting skeletal muscle metabolism and function. Surprisingly, we observed different results in non-hemiplegic skeletal muscles in that stroke reduced BMAL1, PER1, and PER2 expression levels in non-hemiplegic gastrocnemius muscles. This suggests that changes in the expression of these three clock genes are consistent in non-hemiplegic skeletal muscles during the early stages of stroke. The BMAL1 and PER2 expression levels in the non-hemiplegic gastrocnemius muscle increased, whereas those of PER1 decreased. This suggests that stroke also interferes with non-hemiplegic skeletal muscle clock factors, which has not been reported in the domestic and foreign literature. Our results also revealed that early exercise training resulted in lower BMAL1 expression and higher PER1 and PER2 expression in the non-hemiplegic gastrocnemius muscle. With the extension of the exercise training time, the expressions of BMAL1 and PER2 in the non-hemiplegic gastrocnemius muscle increased, whereas that of PER1 decreased. Hence, exercise promoting PER2 expression in the non-hemiplegic gastrocnemius muscle and inhibiting PER1 expression is a very interesting phenomenon. Further, long-term aerobic exercise resulted in changes in PER2 expression. This further confirms that PER2 is closely related to exercise endurance and athletic ability. However, the negative regulatory effect of exercise training on PER1 remains unclear, and further studies are needed. Studies have reported that PER2-impaired mice do not exhibit changes in muscle contraction ability, but demonstrate reduced running endurance and exercise ability [82]. Rodent skeletal muscle studies revealed that PER2 mRNA increases approximately 1 h after acute aerobic exercise [59]. These studies suggest that both acute and chronic treadmill training can affect PER2. Although exercise increases PER2 expression, PER2 affects motor ability in animal models [59]. Therefore, PER2 expression may be associated with exercise.

Exercise is the most effective treatment for patients with sarcopenia [30]. A recent retrospective study has demonstrated that exercise in patients with stroke can reverse the decline in muscle strength, whereas strength training can improve walking speed and prognosis [9]. According to the results of our experiment, exercise had little effect on core clock genes in the hemiplegic gastrocnemius muscle. However, the effect on central clock genes in the non-hemiplegic gastrocnemius muscle was significant. This suggests that after cerebral ischemic stroke, the central conduction pathway of the contralateral limb is interrupted owing to the interruption of the upper motor neuron transmission pathway, leading to a temporary interruption of the peripheral skeletal muscle clock genes. However, because the central conducting pathway of the ipsilateral limb is not interrupted, the circadian rhythm genes of the ipsilateral limb skeletal muscle are more sensitive. Therefore, early exercise intervention can improve the core clock genes of hemiplegic skeletal muscle after cerebral ischemic stroke and the core clock genes of non-hemiplegic skeletal muscle.

This study had some limitations. First, we did not examine metabolic markers of skeletal muscle atrophy to determine an association between reduced muscle strength and stroke occurrence. Second, we did not assess the molecular signaling pathways associated with skeletal muscle atrophy. Future studies should focus on signaling pathways to elucidate skeletal muscle plasticity in stroke-associated sarcopenia. Finally, we did not assess the characteristics of the brain tissue and clock gene expression in the brain tissue of stroke animals.

In conclusion, stroke leads to muscle atrophy that disrupts the expression of skeletal muscle rhythms and clock factors. Endurance training regulates disturbed skeletal muscle clocks, helping improve metabolic health. Endurance training may be an effective treatment for stroke-related sarcopenia. However, whether the molecular clock of skeletal muscle is affected by long-term training and how it is related to exercise-induced metabolic improvements are unclear. Further studies are required to elucidate the underlying mechanisms. The relationship between central clock genes and athletic ability warrants further investigation. Studies on the relationship between circadian rhythms and stroke-related sarcopenia, or the regulation of circadian rhythms to treat stroke-related sarcopenia, may emerge.

5. Limitations

Currently, investigations on the circadian rhythms of skeletal muscles have mainly focused on rodent models. By comparing the gene expression phase of the central clock, the results revealed that the circadian profile of nocturnal activity and daytime sleep differed from that of humans by 8–10 h. Rodent experiments are often performed during the day (rodent sleep period). Whether these results should be applied to daytime human physiological activity (human activity period) is unknown. Consequently, the results of rodent experiments should be designed and considered at different time points to extend them to humans. Therefore, the use of rodents to investigate human circadian rhythms is limited. In the future, primary muscle cell culture will be a powerful tool for analyzing skeletal muscle rhythms.

6. Conclusions

Moderate-intensity aerobic exercise in the early stages of stroke can regulate the skeletal muscle clock genes, which influencing exercise performance. In addition, moderate-intensity aerobic exercise in the early stages of stroke had a small effect on the hemiplegic skeletal muscle clock genes and a significant positive effect on the non-hemiplegic skeletal muscle clock genes.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Mai Li: Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yong Yin:** Supervision, Project administration, Conceptualization. **Dongdong Qin:** Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

MCAO	Middle Cerebral Artery Occlusion Model
H&E	Hematoxylin-eosin staining
qPCR	Quantitative real-time polymerase chain reaction
ELISA	enzyme linked immunosorbent assay
BMAL1	Brain and Muscle ARNT-Like 1
CLOCK	Circadian Locomotor Output Cycles Kaput
PER1	Period Circadian Regulator 1
PER2	Period Circadian Regulator 2
CRY	Cytochrome
EWGSOP2	European Working Group on Sarcopenia in Older People
SCN	suprachiasmatic nucleus
SD	Sprague-Dawley
CCA	Common carotid artery
ICA	Internal carotid artery
ECA	External carotid artery
MCA	Middle cerebral artery
ACA	Anterior cerebral artery
HRP	Horseradish Peroxidase
OD	Optical Density
AD	Alzheimer's disease
SC	Satellite cell
CSA	Cross-sectional area
FDR	False discovery rate

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