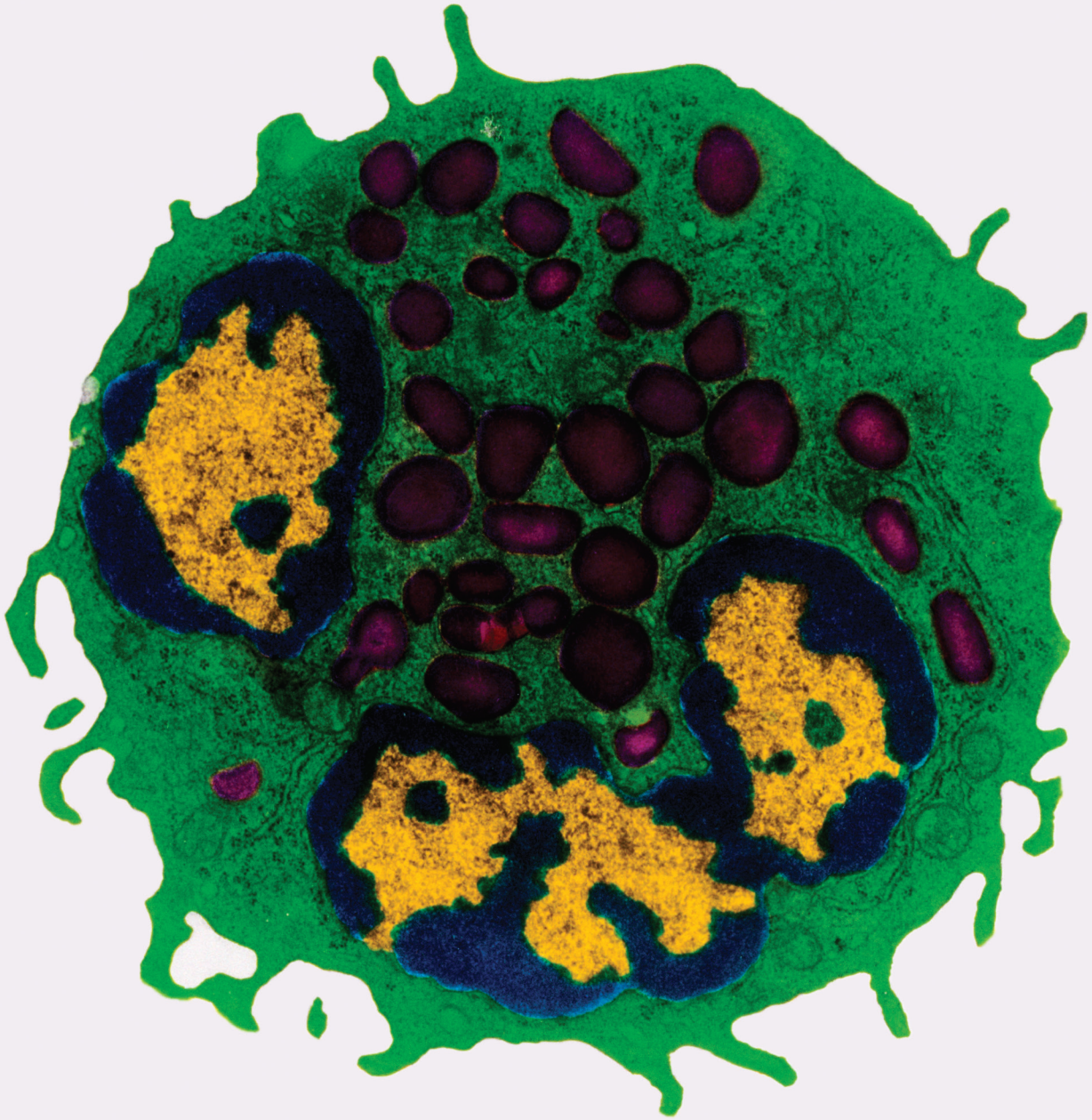
Mediators of Inflammation

The Role of Inflammatory Mediators in Stroke Pathobiology

Lead Guest Editor: Simona Lattanzi
Guest Editors: Mario Di Napoli and Piergiorgio Lochner



The Role of Inflammatory Mediators in Stroke Pathobiology

The Role of Inflammatory Mediators in Stroke Pathobiology

Lead Guest Editor: Simona Lattanzi
Guest Editors: Mario Di Napoli and Piergiorgio Lochner

Contents

Hindawi
Mediators of Inflammation
Volume 2021, Article ID 1849428, 28 pages
https://doi.org/10.1155/2021/1849428

Research Article

High-Intensity Interval Training Improves Physical Function, Prevents Muscle Loss, and Modulates Macrophage-Mediated Inflammation in Skeletal Muscle of Cerebral Ischemic Mice

Lu Luo, Meixi Liu, Hongyu Xie, Yunhui Fan, Jingjun Zhang, Li Liu, Yun Li, Qiqi Zhang, Junfa Wu, Congyu Jiang, and Yi Wu

Department of Rehabilitation Medicine, Huashan Hospital, Fudan University, Shanghai, China

Correspondence should be addressed to Congyu Jiang; lantiono@163.com and Yi Wu; wuyi@fudan.edu.cn

Received 26 September 2021; Accepted 22 October 2021; Published 20 November 2021

Academic Editor: Simona Lattanzi

Although skeletal muscle is the main effector organ largely accounting for disability after stroke, considerably less attention is paid to the secondary abnormalities of stroke-related skeletal muscle loss. It is necessary to explore the mechanism of muscle atrophy after stroke and further develop effective rehabilitation strategy. Here, we evaluated the effects of high-intensity interval (HIIT) versus moderate-intensity aerobic training (MOD) on physical function, muscle mass, and stroke-related gene expression profile of skeletal muscle. After the model of middle cerebral artery occlusion (MCAO) was successfully made, the blood lactate threshold corresponding speed (S_{LT}) and maximum speed (S_{max}) were measured. Different intensity training protocols (MOD < S_{LT}; S_{LT} < HIIT < S_{max}) were carried out for 3 weeks beginning at 7 days after MCAO in the MOD and HIIT groups, respectively. We found that both HIIT and MOD prevented stroke-related gastrocnemius muscle mass loss in MCAO mice. HIIT was more beneficial than MOD for improvements in muscle strength, motor coordination, walking competency, and cardiorespiratory fitness. Furthermore, HIIT was superior to MOD in terms of reducing lipid accumulation, levels of IL-1β and IL-6 in paretic gastrocnemius, and improving peripheral blood CD4+/CD8+ T cell ratio, level of IL-10. Additionally, RNA-seq analysis revealed that the differentially expressed genes among HIIT, MOD, and MCAO groups were highly associated with signaling pathways involved in inflammatory response, more specifically the I-kappaB kinase/NF-kappaB signaling. Following the outcome, we further investigated the infiltrating immune cells abundant in paretic muscles. The results showed that HIIT modulated macrophage activation by downregulating CD86+ (M1 type) macrophages and upregulating CD163+ (M2 type) macrophages via inhibiting the TLR4/MyD88/NFκB signaling pathway and exerting an anti-inflammatory effect in paretic skeletal muscle. It is expected that these data will provide novel insights into the mechanisms and potential targets underlying muscle wasting in stroke.

1. Introduction

Stroke is one of the leading causes of disability worldwide and imposes a tremendous burden on victims, families, and healthcare systems [1]. About 50% of stroke patients have hemiplegia, and 30% of them were unable to walk without assistance [2]. Although the skeletal muscle is the main effector organ largely accounting for disability in stroke patients, most of the researches on motor dysfunction after stroke focus on the concept of neurovascular unit throughout the last two decades [3, 4]. Considerably less attention is paid to the secondary abnormalities of stroke-related skeletal muscle loss or sarcopenia.

Sarcopenia is defined as "progressive and comprehensive syndrome of skeletal muscle loss and strength decline" with increased risk of adverse consequences including higher mortality, quality of life decline, increased rate of falls, and fractures [5]. Sarcopenia is divided into primary sarcopenia caused by aging, and secondary sarcopenia which is activity-related, nutrition-related, or disease-related [6]. Stroke-related sarcopenia is independent of age, showing a rapid muscle mass loss, and significantly bilateral differences in

physical and functional performance [7]. The prevalence of sarcopenia can reach 15% in healthy elderly and 56% in rehabilitated patients. The most widely recognized etiologic factors include denervation, insulin resistance, poor nutritional status, physical inactivity, and inflammation [8].

Despite that sarcopenia contributes to disability and negative outcome after stroke, current clinical practice guideline recommendations fail to adequately address the peripheral muscle adaptations poststroke [9]. Currently, treatment for patients with sarcopenia includes nutritional supplements and hormone-related treatments to improve nutritional status, and weight, which may cause fluid retention, hypogonadism, and orthostatic hypotension [10]. Emerging data suggest that exercise or resistance training is considered the most effective strategy currently available for increasing muscle mass and strength and improving physical function [11, 12]. However, essential training time and sufficient exercise intensity are lacking in most traditional stroke rehabilitation programs [13]. Studies have shown that 76% of time was spent in bed or sitting in the hospitalized stroke, and only 23% of their time are standing or walking [14]. In healthy elderly people, muscle protein synthesis will reduce 30% and muscle mass in lower limbs will reduce 6% after 10 days of bed rest, resulting in a 16% reduction in muscle strength [15]. Well-controlled clinical trials involving the health and patients with stroke have confirmed that high-intensity interval training (HIIT), a new strategy consisting of alternating periods of greater and lower intensity within an exercise session, is more significantly effective and safe than moderate-intensity training for improvements in aerobic capacity, insulin sensitivity, and mitochondrial function [16, 17]. However, the effect of HIIT on physical or muscle mass and composition remains controversial. There is no conclusive evidence to prescribe a specific exercise program in terms of type, intensity, frequency, and duration. Further mechanistic studies are required to develop more effective strategies for preventing or reversing muscle wasting and improving rehabilitation success in patients or animal models with stroke. Therefore, the objective of the present study was to evaluate the effects of high-intensity interval versus moderate-intensity aerobic training on physical function, skeletal muscles, and molecular changes at the genome level in cerebral ischemic mice.

2. Materials and Methods

2.1. Animals. Male C57BL/6 mice (20-25 g), purchased from Shanghai Jihui Laboratory Animal Care Co., Ltd., were initially housed in standard plastic cages (cage size: $26 \times 19 \times 15$ cm) in a temperature-controlled environment ($22 \pm 2^{\circ}$C) with $50 \pm 10\%$ humidity under a 12/12 h light/dark cycle (lights on 7:00 a.m.) with sufficient food and water. All experiment procedures were performed according to the National Institutes of Health guide for the Care and Use of Laboratory Animals. Before use, the mice were allowed to acclimate to laboratory conditions for seven days.

2.2. Middle Cerebral Artery Occlusion Surgery. The middle cerebral artery occlusion (MCAO) model of left middle cerebral artery ischemia for 60 minutes was established after anesthesia by 1% pentobarbital sodium (10mg/kg. ip). The blood flow of the middle cerebral artery was monitored by a laser speckle blood flow meter (RWD Life Science, Shenzhen, China). TTC staining was used to identify the area of infarction. The neurological deficit level of the mice was examined using the modified neurological severity score (mNSS) 24 hours after MCAO surgery. MCAO mice with mNSS more than 6 participated in the experiment (see the Supplementary material) (available here).

After MCAO, mice were excluded according to the following criteria: (1) mNSS less than 6 or overactive ($n = 17$); (2) resisting running on a treadmill ($n = 5$); (3) death during or after the surgery and ineffective arterial occlusion ($n = 12$). Overall, 80 mice were used, and 46 of them were included. Mice were randomly assigned to the sham group ($n = 10$), MCAO group ($n = 12$), MOD group ($n = 12$), and HIIT group ($n = 12$).

2.3. Incremental Test and Exercise Protocols. The protocol was performed with the following adaptations according to the previously published work [18]. Seven days after MCAO, the mice in HIIT and MOD groups were placed on the motor-driven treadmill (Huaibei Zhenghua Biological Instrument Equipment Co. Ltd, China) and warmed up at a rate of 6 m/min for 5 min. Then, accelerate by 3 m/min every 3 minutes until the mouse cannot maintain the applied speed, and the final speed is defined as the maximum speed (S_{max}). Every 20 seconds of acceleration, a small amount of blood (0.2 μL) was collected from the tail vein. The blood lactate concentration (mmol/L) was measured by a portable blood lactate device (Lactate Scout+, EKF Diagnostics, Germany). When the blood lactate concentration measured twice in a row has a significant inflection point or is increased by 1 mmol/L, the blood lactate concentration measured in the previous measurement is regarded as the lactate threshold (LT), and the corresponding treadmill speed is called S_{LT}.

HIIT: the session consists of a 4×4-minute high-intensity treadmill run (S_{LT} + 60-70%($S_{max} - S_{LT}$)), interrupting active recovery (S_{LT}) for 3 minutes between each intensity series. The plan is implemented five times a week for three weeks.

MOD: the speed is fixed at 80-90% S_{LT} to avoid the accumulation of lactate. The project is carried out every day for 3 consecutive weeks. In order to match the total energy expenditure (W) between the groups and only compare the effects of intensity, according to the energy expenditure (exercise + recovery) of the HIIT group, the daily exercise time of the MOD group was adjusted by the following formula:

$$W\,(\mathrm{J/kg \cdot m}) = \mathrm{mass}\,(\mathrm{kg}) \times \mathrm{speed}\,(\mathrm{m/min}) \times \mathrm{time}\,(\mathrm{min}) \times \mathrm{treadmill\ tilt}\,(^{\circ}) \times 9.8. \quad (1)$$

Each program included a 5-minute warm-up (50% S_{LT}) before the formal training. The S_{max} and S_{LT} of the mice were retested every two weeks to adjust the training intensity. The mice in the sham sedentary group were housed in a crowded

cage (cage size: 26 × 19 × 15 cm, 8/per cage) with no access to the treadmill.

2.4. Behavioral Tests. The behavioral tests were performed 2 days before and 3 weeks after exercise intervention. The test sequence was as follows: (1) open field test, (2) grip strength test, (3) rotarod test, (4) cylinder test, (5) ladder rung walking test, and (6) CatWalk XT gait test (see the Supplementary material).

2.5. Pulmonary Function. The pulmonary function of mice was evaluated by a whole-body plethysmograph. Briefly, three days before the test, mice were placed in a sealed box that was connected to transducers and a computer and allowed to acclimate for 15 min every day. On the test day, the mice were placed again in the box to acclimate for 5 min. Afterwards, pulmonary function was recorded and assessed for 5 min, including peak expiratory flow (PEF), peak inspiratory flow (PIF), and minute volume (MV) and tidal volume (TV).

2.6. Body Weight and Muscle Mass. The body weight of the mice was measured at fixed times every 4 days until the end of the experiment, and the body weight growth rate was calculated as follows: growth rate (%) = (current body weight − body weight at the first day)/body weight at the first day × 100%. Paretic gastrocnemius muscle of mice in four groups was isolated, removed, and weighed after being deeply anesthetized.

2.7. Experimental Material Preparation. Mice were deeply anesthetized by 1% pentobarbital sodium (10 mg/kg. ip). For western blotting, RNA sequencing, adenosine triphosphate (ATP)-ase staining, and Oil-Red-O staining, mice in each group were sacrificed and paretic gastrocnemius was quickly removed, placed in Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C for further use. For immunohistochemistry, the mice were transcardially perfused with 50 mL of phosphate-buffered saline (PBS) and then fixed with 50 mL 4% paraformaldehyde (PFA) solution. The paretic gastrocnemius was removed, postfixed for 24 h in the same fixative, and cryoprotected 24 h at 4°C in a 30% sucrose solution. After then, the tissue blocks were embedded in paraffin for further use. Blood sample (300 μL) was collected by enucleating the mouse eyeball for flow cytometric analysis.

2.8. Hematoxylin and Eosin (HE) Staining and Immunohistochemistry. The serial coronal sections in the maximum cross section of paretic gastrocnemius were made to observe the morphology of muscle cells, infiltrating cells by HE staining, and to detect the distribution of CD86 and CD163 proteins by immunohistochemistry (see the Supplementary Materials).

2.9. Adenosine Triphosphate (ATP)-ase Staining and Oil-Red-O Staining. The transverse serial sections were incubated with calcium chloride solution for 5 min and calcium chloride solution for 30 min. Then, the sections were stained with calcium chloride, cobalt nitrate, and ammonium sulphide solutions. Type I muscle fiber is light gray or colorless, and type II muscle fiber is dark gray or black. For Oil-Red-O (ORO) staining to detect lipid deposition, slides were immersed after washing with PBS for 15 min in the ORO working solution and rinsed with deionized water.

2.10. RNA Sequencing and Differentially Expressed Gene Analysis. Total RNA was extracted using the TRIzol reagent according to the manufacturer's protocol. GO enrichment and KEGG pathway enrichment analysis of differentially expressed genes (DEGs) were performed respectively using R based on the hypergeometric distribution. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China) (see the Supplementary Materials).

2.11. Flow Cytometry. To determine the percentage of total leukocytes and lymphocyte subsets, blood samples were stained with various monoclonal antibodies (mAbs) and evaluated by flow cytometry. Cells were analyzed on Becton-Dickinson FACSCalibur flow cytometer using FlowJo software (see the Supplementary Materials).

2.12. Profiling of Cytokines/Chemokines. Cytokines and chemokines in paretic gastrocnemius were measured and quantified using the LEGENDplex™ mouse inflammation panel (BioLegend, 740446) according to the manufacturer's instructions. LEGENDplex™ software was used for analyzing collected data (BioLegend) (see the Supplementary Materials).

2.13. Western Blot Assays. The expression of CD86, CD163, TLR4, MyD88, NFκB, and p-NFκB proteins in the paretic gastrocnemius was detected by western blotting after 3 weeks of training (see the Supplementary Materials).

2.14. Statistical Analysis. Data are expressed as the mean ± standard error (SEM) of at least three independent experiments.

Statistical tests were done on SPSS 23.0 statistical software (SPSS, Chicago, IL, USA) and GraphPad Prism 9.0 (GraphPad Software Inc., USA). Firstly, a normality test was performed. One-way analysis of variance (ANOVA) for multiple comparisons followed by Tukey's post hoc test was performed for the data with normal distribution. The Kruskal–Wallis test was performed for the data with nonnormal distribution. Statistical significance between two groups was determined with unpaired Student's t-test. A probability of 0.05 or less was considered statistically significant.

3. Results

3.1. Establishment of the Animal Model of Cerebral Ischemia Which Induced Skeletal Muscle Atrophy. To determine the change of morphology and function of poststroke skeletal muscle, a cerebral ischemic animal model was established by the MCAO method, which significantly caused ~40% ipsilateral brain infarcts in the lateral striatum and cortex regions shown by TTC staining ($P < 0.01$; Figures 1(c) and 1(d)). The ischemic cerebral blood flow dropped below 30% of the contralateral side during surgery monitored by laser speckle

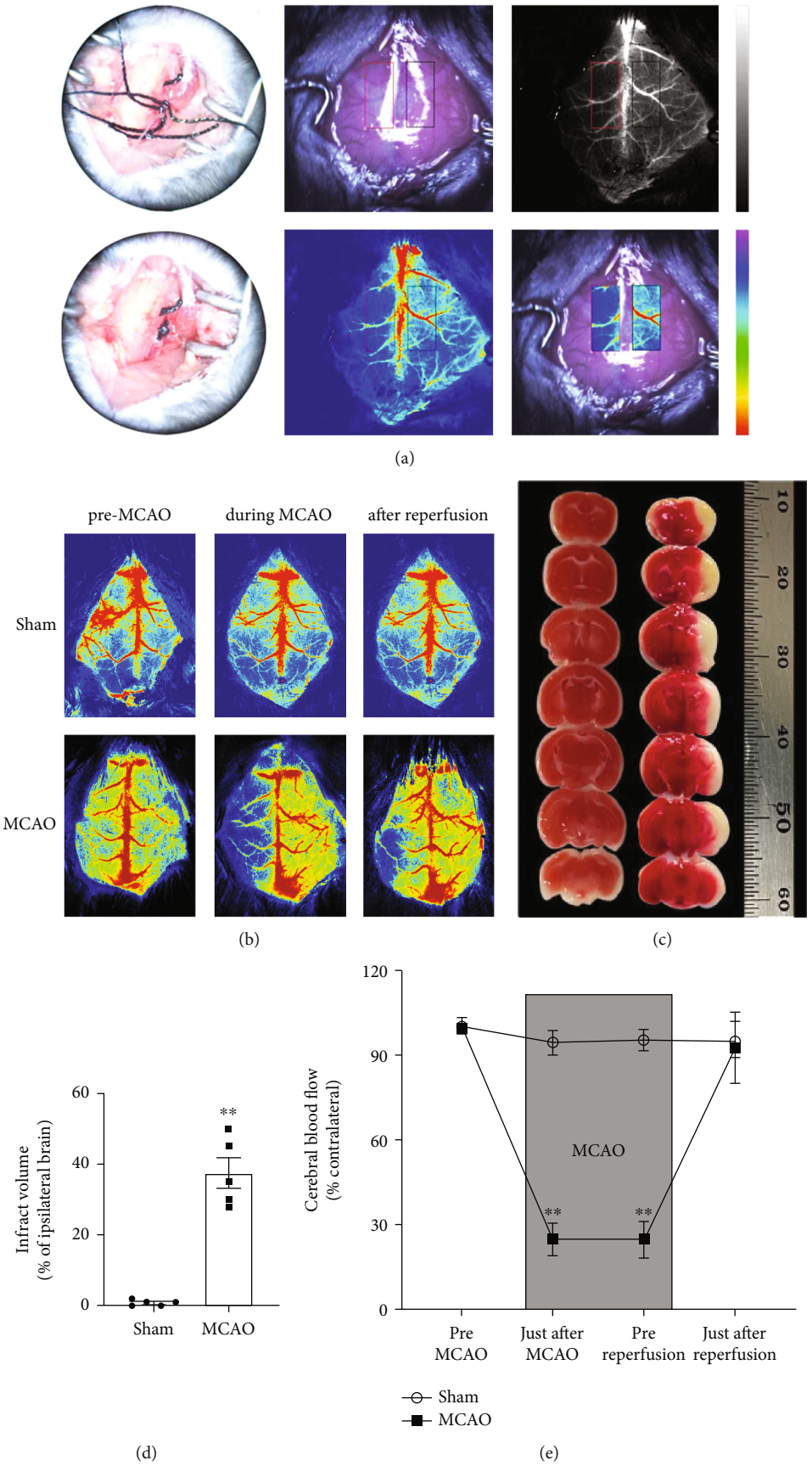


Figure 1: Continued.

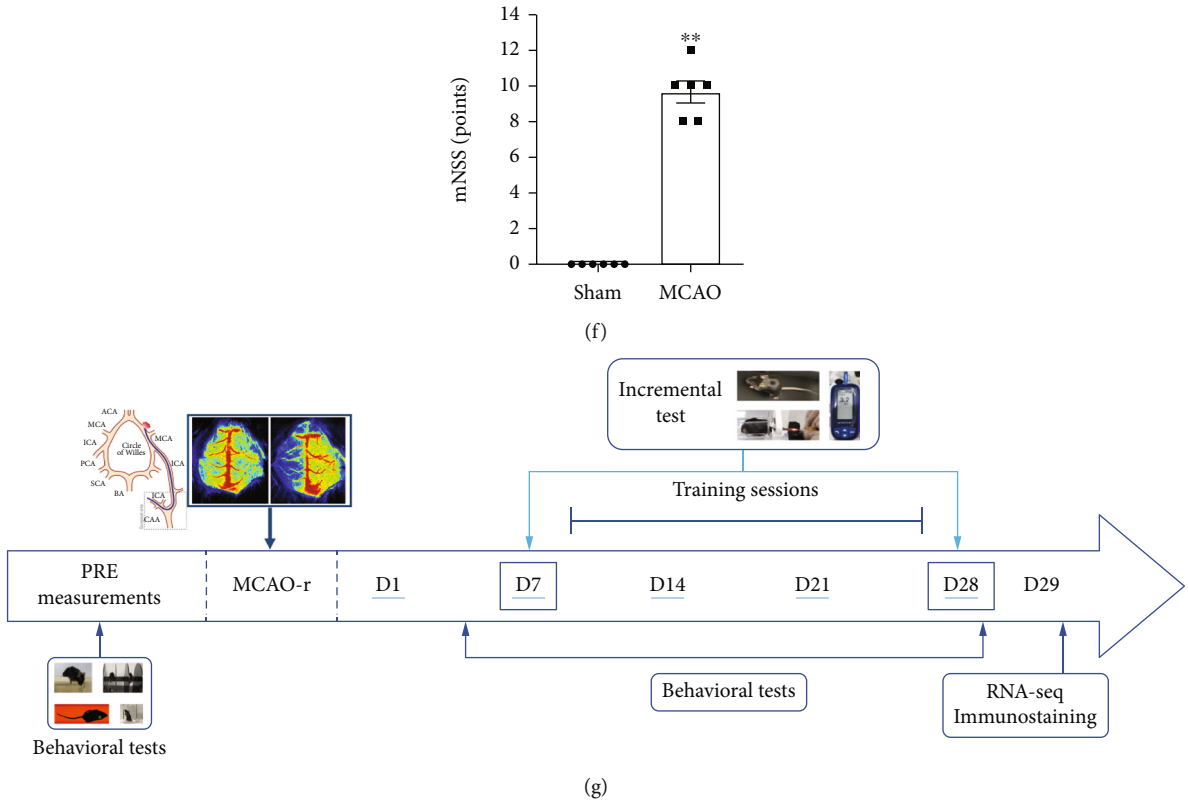


Figure 1: Establishment of the animal model of cerebral ischemia. (a) Middle cerebral artery occlusion (MCAO) was established under cerebral blood flow monitored by laser speckle blood flow meter. (b) Pseudocolor picture of cerebral blood flow before, during, and after MCAO. (c) Triphenyl tetrazolium chloride (TTC) staining showed the infarct size after cerebral ischemia reperfusion. (d) Quantification of cerebral infarct volume of the ipsilateral brain between sham and MCAO groups ($n = 6$). (e) Quantification of cerebral blood flow in the ipsilateral hemisphere normalized to the contralateral hemisphere. (f) Modified neurological severity score (mNSS) of the sham-operated and ischemic mice ($n = 6$). (g) Experimental design of the study. Mice were subjected to MCAO after baseline assessments. Then, animals were randomly subdivided into sham, sedentary MCAO, moderate-intensity aerobic training (MOD), and high-intensity interval training (HIIT) groups 1 week after MCAO. Following 3 weeks of isocaloric training sessions (MOD and HIIT) or sedentarism (sham and MCAO), all groups underwent posttraining assessments. Values are expressed as the mean ± SEM of the mean. $^{*}P < 0.05$ and $^{**}P < 0.01$ as determined by unpaired t-test.

blood flow meter ($P < 0.01$; Figures 1(b) and 1(e)), which severely induced motor deficit as evidenced by higher neurological score ($P < 0.01$; Figure 1(f)) and lower distance moved ($P < 0.05$; Figure 2(j)). Total body weight of ischemic mice was rapidly decreased and significantly lower compared to sham-operated mice. Weight loss peaked at day 4 and recovery of body weight (starting at day 4) remained incomplete and slightly lower compared to the sham group until the end of the study (Figure 2(a)). Muscle mass loss was observed in gastrocnemius muscle of the contralateral leg in the 4th week poststroke. Next, we calculated the ratio of gastrocnemius mass to body weight; the MCAO group also showed a significant decrease in the ratio relative to the sham group ($P < 0.05$; Figures 2(d) and 2(e)).

3.2. Exercise Protocols of High-Intensity Interval Training and Moderate-Intensity Aerobic Training. Different from the moderate-intensity aerobic training recommended by some stroke rehabilitation guidelines [9], we measured the blood lactate threshold (LT) that most MCAO mice could reach during exercise to quantify high-intensity training and low-to-moderate-intensity aerobic training (Figures 3(a) and 3(b)). The intensity below LT is located in the ranges of the moderate intensity (e.g., 40-70% of VO_2 peak). The resting blood lactate concentration of the sedentary MCAO mice was higher than that in the sham group at the 7th day (D7) and was reversed by 3 weeks of HIIT at 28 days (D28) poststroke ($P < 0.05$; Figure 3(c)). HIIT appeared to be more effective to recover aerobic fitness than MOD as indicated by changes in S_{max} and S_{LT}. Before training, S_{LT} and S_{max} in the injured groups were significantly lower compared to those in the sham group ($P < 0.01$; Figure 3(d)). After 3 weeks of treadmill training (D28), S_{LT} and S_{max} increased significantly in the HIIT group ($P < 0.05$; Figure 3(e)). However, no difference was detected between MCAO and MOD groups. In addition, under the same energy expenditure, the total exercise time of the HIIT group was significantly shorter than that of the MOD group, which indicates that HIIT is time efficient and might not be a major obstacle due to its mild exercise tolerance.

3.3. Amelioration of Muscle Wasting and Motor Dysfunction after Exercise Training. Furthermore, we assessed the effect

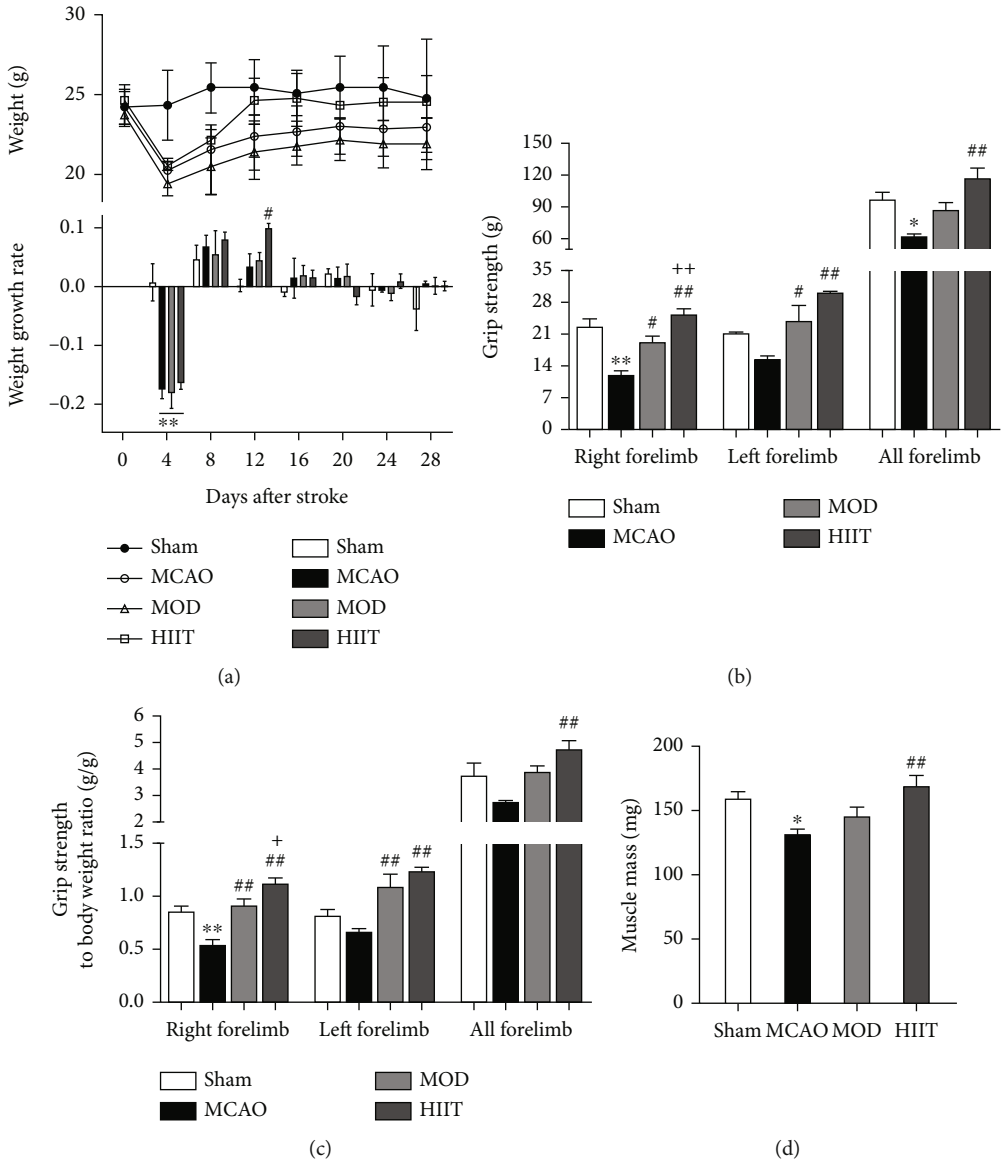


Figure 2: Continued.

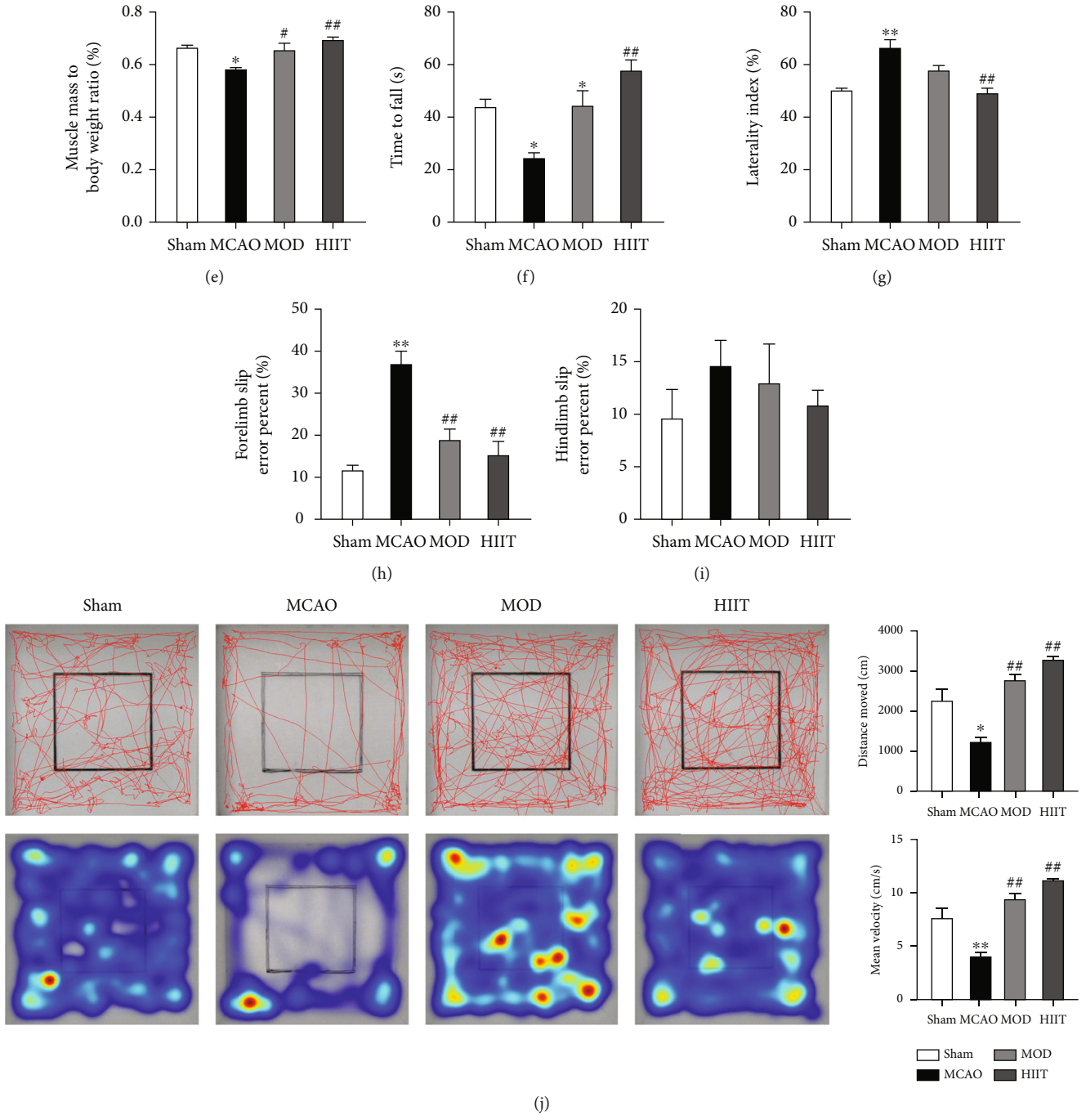


Figure 2: Amelioration of muscle wasting and motor dysfunction after exercise protocols. (a) Changes in mean values of total body weight and weight growth rate (data are expressed as the change with respect to day 0) in each group. (b) The grip strength (g) of bilateral forelimbs measured using a digital force gauge in each group ($n = 5$). (c) The ratio of grip strength and body weight in bilateral forelimbs in each group ($n = 5$). (d) Muscle mass (mg) of paretic gastrocnemius in each group ($n = 6$). (e) The ratio of paretic gastrocnemius mass and body weight in all groups ($n = 6$). (f) Time course of the latency to fall off the rotarod in the rotarod test in each group ($n = 6$). (g) The percentage of laterality in the cylinder test in each group ($n = 6$). (h, i) The percent of forelimb slips and hindlimb slips in the ladder rung walking test in each group ($n = 6$). (j) Motion trajectory and trajectory endpoint heat map of each mouse in the open field test (left); the total distance of motion trajectory (cm) and mean velocity (cm/s) of each mouse (right) ($n = 6$). Values are expressed as the mean ± SEM of the mean. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the MCAO group; $^{+}P < 0.05$ and $^{++}P < 0.01$ compared with the MOD group as determined by one-way ANOVA (Tukey's multiple comparison test) for the data with normal distribution. The letters for no significance were not shown.

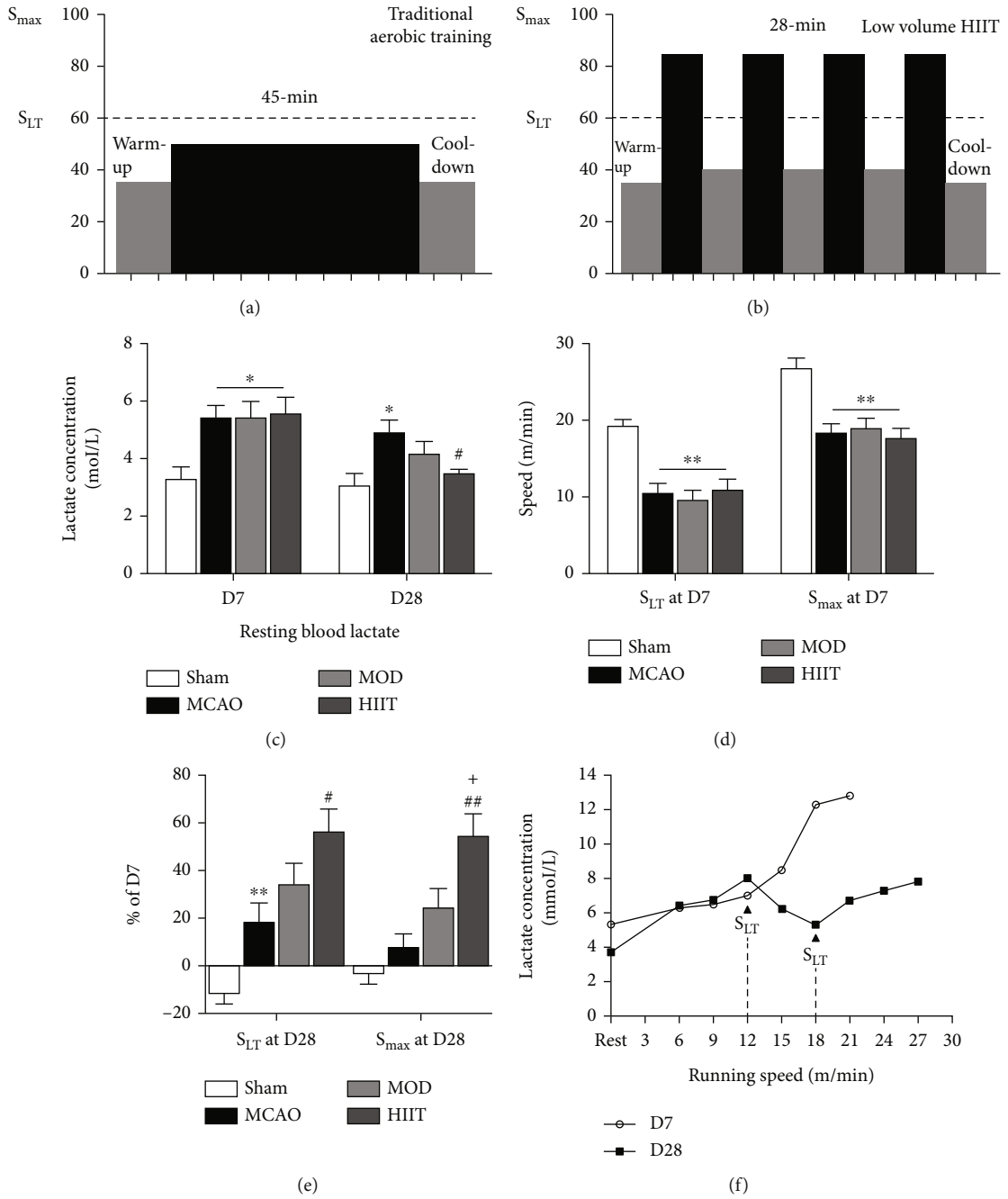


Figure 3: Exercise protocols of high-intensity interval training and moderate-intensity aerobic training. (a, b) Regimens of traditional moderate-intensity aerobic training and low volume high-intensity interval training. (c) Relative percentage (%) of resting blood lactate (mmol/L) at D28 normalized to the D1 ($n = 6$). (d) S_{LT} and S_{max} (m/min) at D7 in each group ($n = 6$). (e) Relative percentage (%) of S_{LT} and S_{max} at D28 normalized to the D1 ($n = 6$). (f) Example of lactatemia kinetic (raw data) before and after HIIT. Arrows indicate the lactate threshold. Values are expressed as the mean ± SEM of the mean. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the MCAO group as determined by one-way ANOVA (Tukey's multiple comparison test) for the data with normal distribution. The letters for no significance were not shown. Abbreviation: S_{LT}: speed associated with the lactate threshold; S_{max}: maximal speed; D7: 7 days after cerebral ischemia; D28: 28 days after cerebral ischemia.

of different exercise protocols on skeletal muscle. Measurement of the muscle weight of paretic gastrocnemius mass and the ratio of gastrocnemius mass to body weight indicated that 3 weeks of HIIT protected against muscle loss induced by cerebral ischemia ($P < 0.01$), whereas MOD did not induce body weight significant changes (Figures 2(a), 2(d), and 2(e)). In the functional tests, the results concerning the muscle strength and motor coordination of mice subjected to MCAO after intervention are shown in Figure 2. In the grip strength test, the MCAO group committed a significant decrease ($P < 0.01$) in the right forelimb and a nonsignificant decrease in the left forelimb when compared

to the sham (Figures 2(b) and 2(c)), evidencing that the MCAO led to the impairment of the contralateral front paws. Similar to the ladder rung walking test, the MCAO group committed more slip errors in the forelimb ($P < 0.01$) but not in the hindlimb (Figures 2(h) and 2(i)). In the rotarod test and cylinder test, a lower time to fall and higher laterality index were observed in the MCAO group in relation to the other groups ($P < 0.05$; Figures 2(f) and 2(g)), suggesting impairment of the muscle strength and motor coordination due to cerebral ischemia. Based on the performance test conducted before and at the end of the training program, both HIIT and MOD significantly improved the forelimb grip strength and reduced the forelimb slip errors ($P < 0.05$; Figures 2(b), 2(c), and 2(h)). In the rotarod test and cylinder test, HIIT, rather than MOD, significantly improved times to fall and reduced laterality index ($P < 0.01$; Figures 2(f) and 2(g)), indicating that HIIT was superior to MOD with regard to improving the motor function after cerebral ischemia. Both HIIT and MOD can significantly improve the low movement distance and movement speed induced by MCAO ($P < 0.01$; Figure 2(j)).

3.4. High-Intensity Interval Training Improved Gait and Pulmonary Functional Parameters. To evaluate the extent of gait impairment after MCAO, we analyzed the run parameters in four groups using the CatWalk automated gait analysis system at 28th day poststroke. The MCAO group displayed obvious but nonsignificant differences from the sham group in period of stand and swing, duty cycle, and mean intensity parameters in the paretic side (Figures 4(b)–4(e)). Furthermore, parameters of duration, average speed, and cadence remained similar between MCAO and sham groups, with minor differences displayed after 3 weeks of HIIT or MOD (Figures 4(f)–4(h)). There was a significant decrease in duty cycle and mean intensity in the right hindlimb after HIIT ($P < 0.05$; Figures 4(d) and 4(e)). From these parameters, it is clear that despite the slight differences observed in walking competency, which might be due to a natural recovery of the animals after cerebral ischemia, HIIT still has the potential to improve certain gait parameters.

The walking energy cost of stroke patients is increased by approximately 1.5- to 2.0-fold that of normal individuals [19]. Therefore, improving cardiorespiratory fitness is an important factor in restoring walking ability. Whole-body barometric plethysmography was used in the present study to noninvasively assess baseline pulmonary function in mice. The results showed that PIF were significantly lower in the MCAO group than those in the sham group ($P < 0.05$; Figure 4(m)). Compared with the MCAO group, PEF and PIF were significantly higher in the HIIT and MOD groups ($P < 0.05$; Figures 4(m) and 4(n)). TV and MV were only significantly higher in the HIIT group ($P < 0.05$; Figures 4(k) and 4(l)), rather than the MOD group.

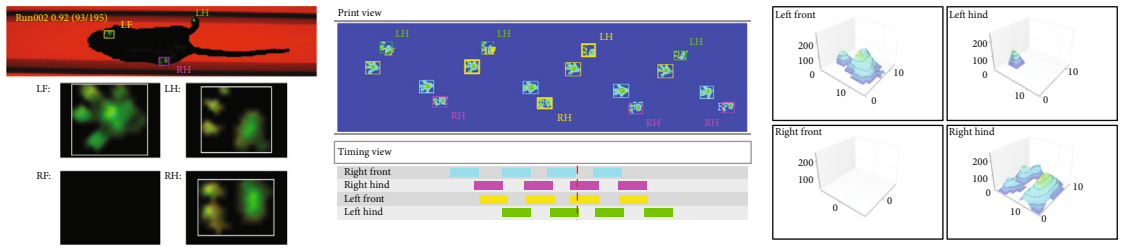
3.5. Effects of Different Exercise Protocols on Paretic Gastrocnemius Muscle Histopathology. Morphology of the muscle was evaluated by HE staining. The normal muscle fibers observed in the sham-operation group were polygonal, arranged together to form muscle bundles. Compared with the sham group, the MCAO group was characterized by small muscle fiber volume, degeneration, uneven fiber distribution, uneven shape, and a significant decrease in average fiber cross-sectional area (CSA) ($P < 0.05$). In addition, compared with sham-operated mice, fibrosis, inflammatory cell infiltration, increased collagen fiber area, and decreased muscle fiber diameter were detected in the paretic gastrocnemius muscle. Three weeks of HIIT, rather than MOD, significantly alleviated stroke-induced reduction in fiber CSA and the infiltrating inflammatory cells ($P < 0.05$). Collectively, the results indicated that HIIT reduced the stroke-induced muscle atrophy by preventing the reduction in cross-sectional area of the muscle fibers of the paretic gastrocnemius muscle (Figures 5(a) and 5(d)).

ORO staining confirmed the massive accumulation of fatty components in the paretic gastrocnemius of the MCAO group ($P < 0.05$), which was reduced dramatically in the HIIT group ($P < 0.05$), instead of the MOD group (Figures 5(b) and 5(e)).

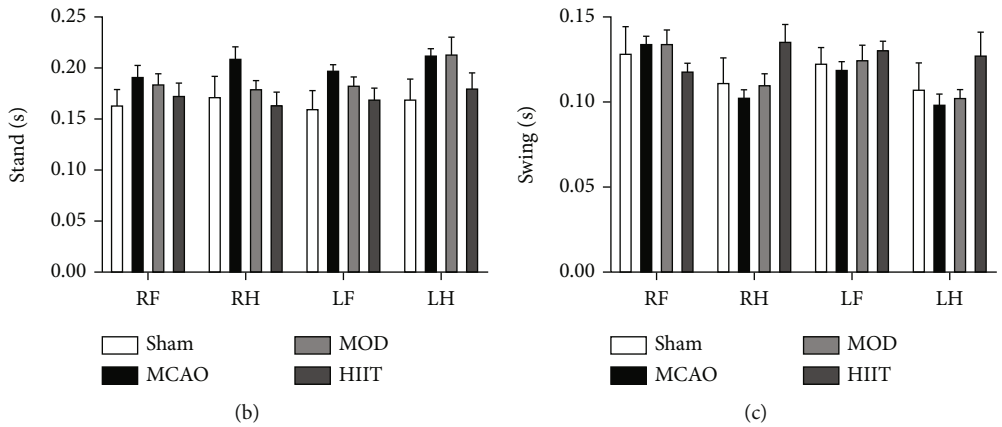
To ascertain whether different exercise protocols produced distinct impacts on fast and slow muscle fibers, we performed ATP-ase staining for slow and fast myosin heavy chain (MyHC) (Figure 5(c)). The proportion of type II fibers increased significantly (with a consequent reduction in type I fibers) in the paretic gastrocnemius muscle in the MCAO group ($P < 0.05$). No significant difference in the proportion of type I and type II fibers was observed between the MCAO and exercise groups. However, the HIIT group presented a greater trend in promoting muscle phenotypic metastasis compared with the MOD group (Figures 5(c), 5(f), and 5(g)).

3.6. Profiling of Differentially Expressed Genes of Skeletal Muscle after Stroke and Exercise Protocols. In order to better explore gene expression changes in skeletal muscle after stroke and investigate molecular origin of the pathophysiological process after different exercise regimens, transcriptome-wide RNA sequencing technology was used followed by further bioinformatics analysis. We found 376 differentially expressed genes with 84 and 292 genes up- and downregulated, respectively, in poststroke muscle, and 269 differentially expressed genes with 162 and 107 genes up- and downregulated, respectively, between HIIT and MCAO groups (Figure 6(b)). These results are visualized via volcano plot and heat map depicting differentially expressed genes (Figures 6(c) and 6(d)). To obtain more detailed gene expression patterns, selected differentially expressed genes associated with GO biological process categorization were shown in the heat map, including "atrophy of muscle," "skeletal muscle fiber type," and "fatty acid oxidation" (Figure 6(a)).

We used GO annotation analysis and KEGG enrichment analysis of differentially expressed genes to characterize their respective biological functions. Our GO annotation analysis was assigned terms in the biological process, cellular component, and molecular function, respectively. Most biological-process-related genes between MCAO and sham groups were annotated with GO terms associated with "transition between fast and slow fiber,"

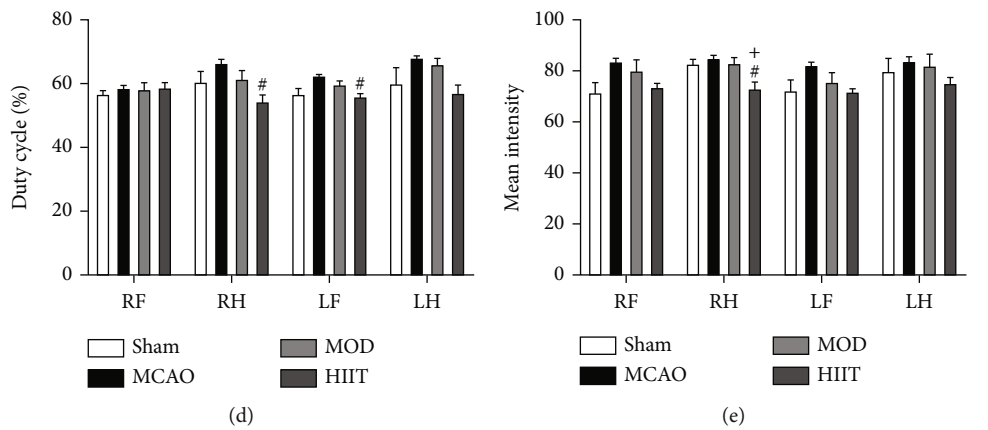


(a)



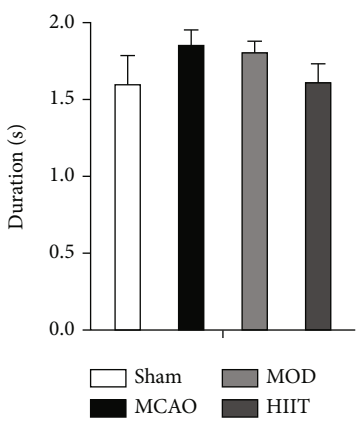
(b)

(c)

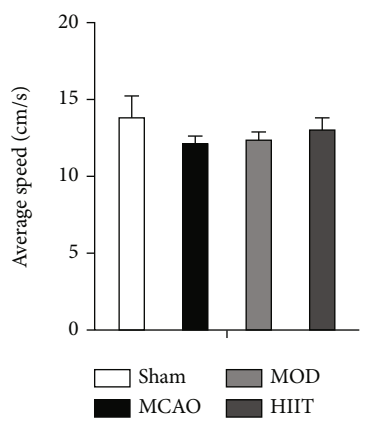


(d)

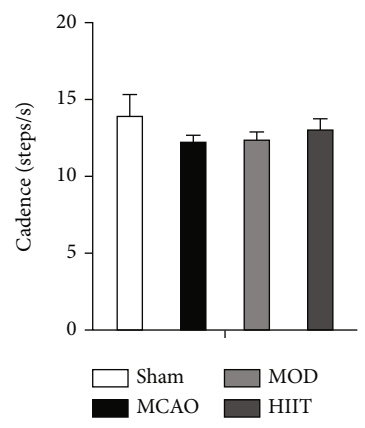
(e)



(f)



(g)



(h)

Figure 4: Continued.

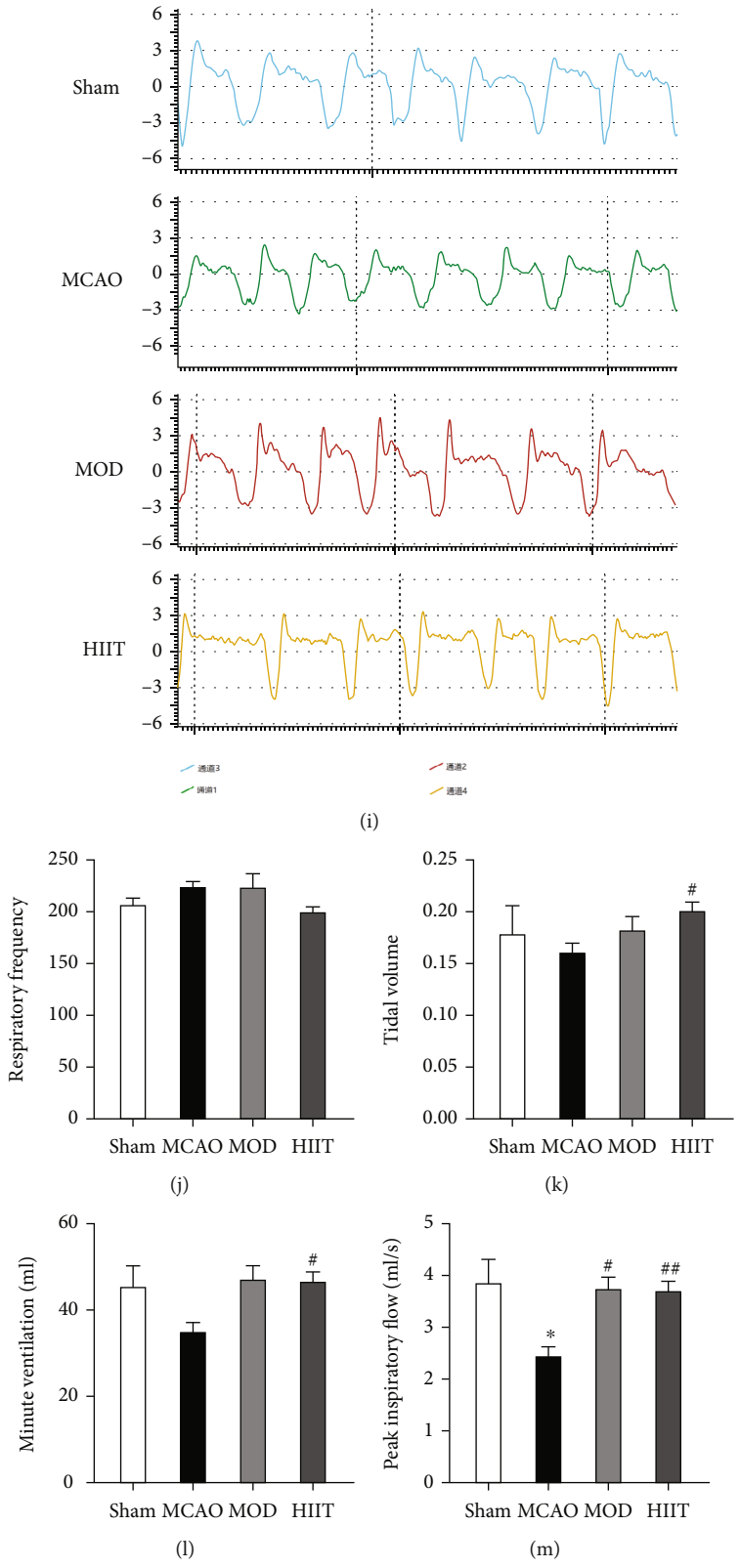


Figure 4: Continued.

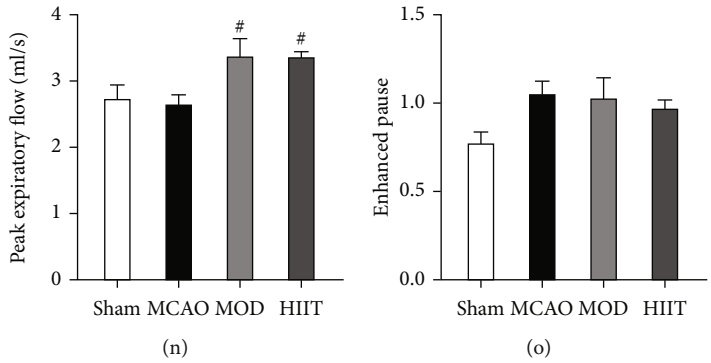


Figure 4: High-intensity interval training improved gait and pulmonary functional parameters. (a) Schematic diagram of labeled footprint and 3D footprint intensity charts. Graph demonstrates statistical differences in the average run characterization parameter in each group, including stand (s) (b), swing (s) (c), duty cycle (%) (d), mean intensity (e), duration (s) (f), average speed (cm/s) (g), and cadence (step/s) (h). (i) The respiration curve illustrates the changes of volume of the plethysmograph induced by the volatility of the mouse's thorax in each group. The graph demonstrates statistical differences in the average pulmonary functional parameter in each group, including respiratory frequency (j), tidal volume (k), minute ventilation (mL) (l), peak inspiratory flow (mL/s) (m), peak expiratory flow (mL/s) (n), and enhanced pause (o). Values are expressed as the mean ± SEM of the mean. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the MCAO group as determined by one-way ANOVA (Tukey's multiple comparison test) for the data with normal distribution. The letters for no significance were not shown. Abbreviation: RF: right forelimb; RH: right hindlimb; LF: left forelimb; LH: left hindlimb.

"skeletal muscle contraction," "positive regulation of protein secretion," and "skeletal muscle cell differentiation" (Figure 7(a)). The most significantly enriched pathways by KEGG enrichment analysis were "immune system," "lipid metabolism," "infectious diseases," "signal transduction," and "transport and catabolism" (Figure 6(e)), indicating that the differentially expressed genes of skeletal muscle after stroke were highly associated with signaling pathways involved in inflammatory response.

Furthermore, most biological-process-related genes between HIIT and MCAO groups were annotated with GO terms associated with "inflammatory response" and "chemokine-mediated signaling pathway" (Figure 7(b)). Most biological-process-related genes between HIIT and MOD groups were annotated with GO terms associated with "regulation of I-kappaB kinase/NF-kappaB signaling" (Figure 7(c)). The results of our GO and KEGG enrichment analysis indicated that HIIT modulated inflammatory response activation and atrophy of muscle induced by stroke might be via the NFκB involved signaling pathway and exerting an anti-inflammatory effect in skeletal muscle.

3.7. Level of Cytokines in Paretic Skeletal Muscle and Lymphocyte Subsets in Peripheral Blood. Thus, we profiled the levels of 13 cytokines, using the bead-based immunoassay LEGENDplex, to explore the local inflammatory status in paretic gastrocnemius muscle. The proinflammatory cytokines are involved in the process of muscle loss, including IL-6 and TNF-α, which were significantly elevated in the MCAO group compared with sham-operated mice ($P < 0.05$). HIIT significantly reduced the levels of IL-1β, IL-6, and elevated IL-10 level compared with the MCAO group ($P < 0.05$). There were no significant differences found in the above cytokines between MOD and MCAO groups (Figure 8(a)).

Flow cytometry analysis results showed that mice subjected to MCAO induced a significant increase in CD8+ T cells and F4/80+CD11b+ cells, the well-known surface marker of mouse macrophages ($P < 0.05$). Although both CD4+ T cell and CD8+ T cell proportions were not significantly altered, HIIT remarkably elevated the ratio of CD4+ and CD8+ T cells ($P < 0.05$). Unlike the HIIT group, MOD failed to significantly affect the proportion of macrophages and T lymphocyte subsets in paretic gastrocnemius (Figures 8(b) and 8(c)).

3.8. HIIT Modulated Macrophage-Mediated Inflammation and Cytotoxic Properties via Inhibiting the TLR4/MyD88/NFκB Signaling Pathway. Since training protected against inflammatory response induced by stroke directly, we examined the effect of HIIT on the infiltrating immune cells in paretic skeletal muscle. Macrophages mediate the recruitment and activation of systemic immune cells and induce cytotoxicity in chronic inflammation. We, therefore, examined the macrophage profile in skeletal muscle poststoke. The gene expression of subsets of macrophages (M1/CD86 and M2/CD163, respectively) is demonstrated in relative expression of β-actin (Figure 9). Western blotting and immunostaining showed that MCAO induced a significant increase in the number of cytotoxic CD86+ (M1 type) macrophages compared to the sham group ($P < 0.05$). No differences were noted in the number of CD163+ (M2 type) macrophages between MCAO and sham groups. We further confirmed that HIIT, instead of the MOD group, significantly decreased the number of CD86+ macrophages and increased the number of CD163+ macrophages in the paretic gastrocnemius muscle compared to the MCAO group ($P < 0.05$; Figure 9).

To further delineate the mechanisms potentially involved in the modulating of macrophage phenotypes after HIIT, we hypothesized that the TLR4/MyD88/NFκB signaling pathway might be a crucial regulator of skeletal muscle

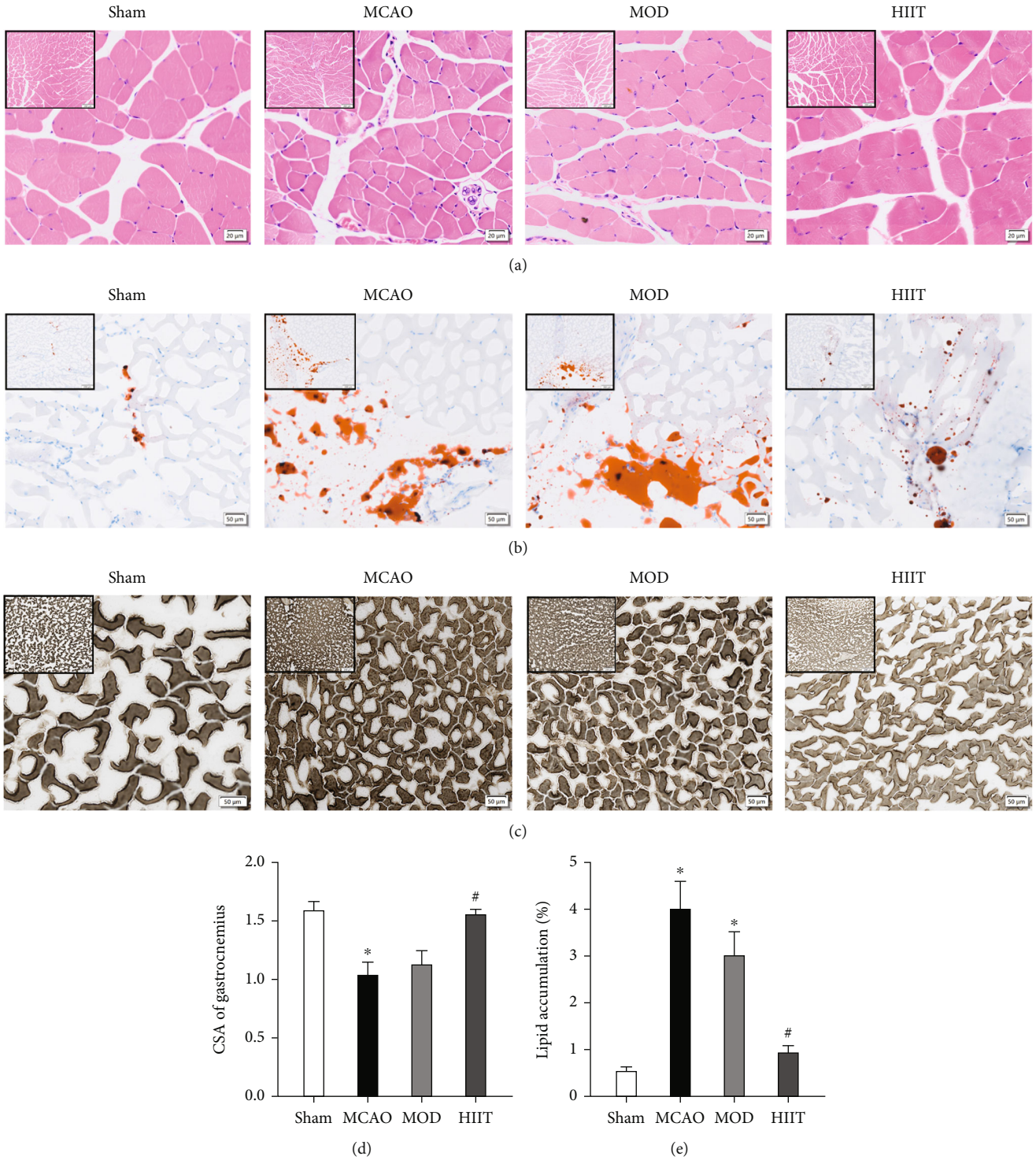


Figure 5: Continued.

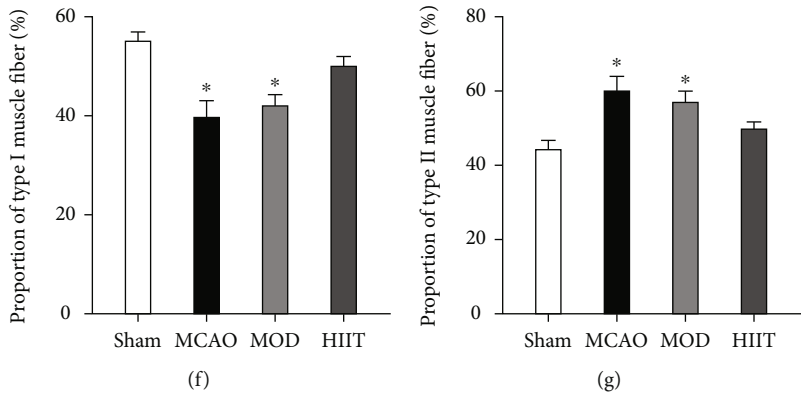


Figure 5: Effects of different exercise protocols on paretic skeletal muscle histopathology. (a) Histological images of HE staining of paretic gastrocnemius muscle tissue in each group (scale bar: 20 μm). (b) Histological images of ORO staining of paretic gastrocnemius muscle tissue in each group (scale bar: 50 μm). (c) Representative gastrocnemius ATP-ase staining in each group (type I fiber: light gray or colorless; type II fiber: dark gray or black) (scale bar: 50 μm). (d) Cross-sectional area (CSA) of paretic gastrocnemius muscle fiber in each group ($n = 5$). (e) Percentage of lipid accumulation in each group ($n = 5$). (f) Proportion of type I muscle fiber in each group. (g) Proportion of type II muscle fiber in each group ($n = 5$). Values are expressed as the mean ± SEM of the mean. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the MCAO group as determined by one-way ANOVA (Tukey's multiple comparison test) for the data with normal distribution. The letters for no significance were not shown.

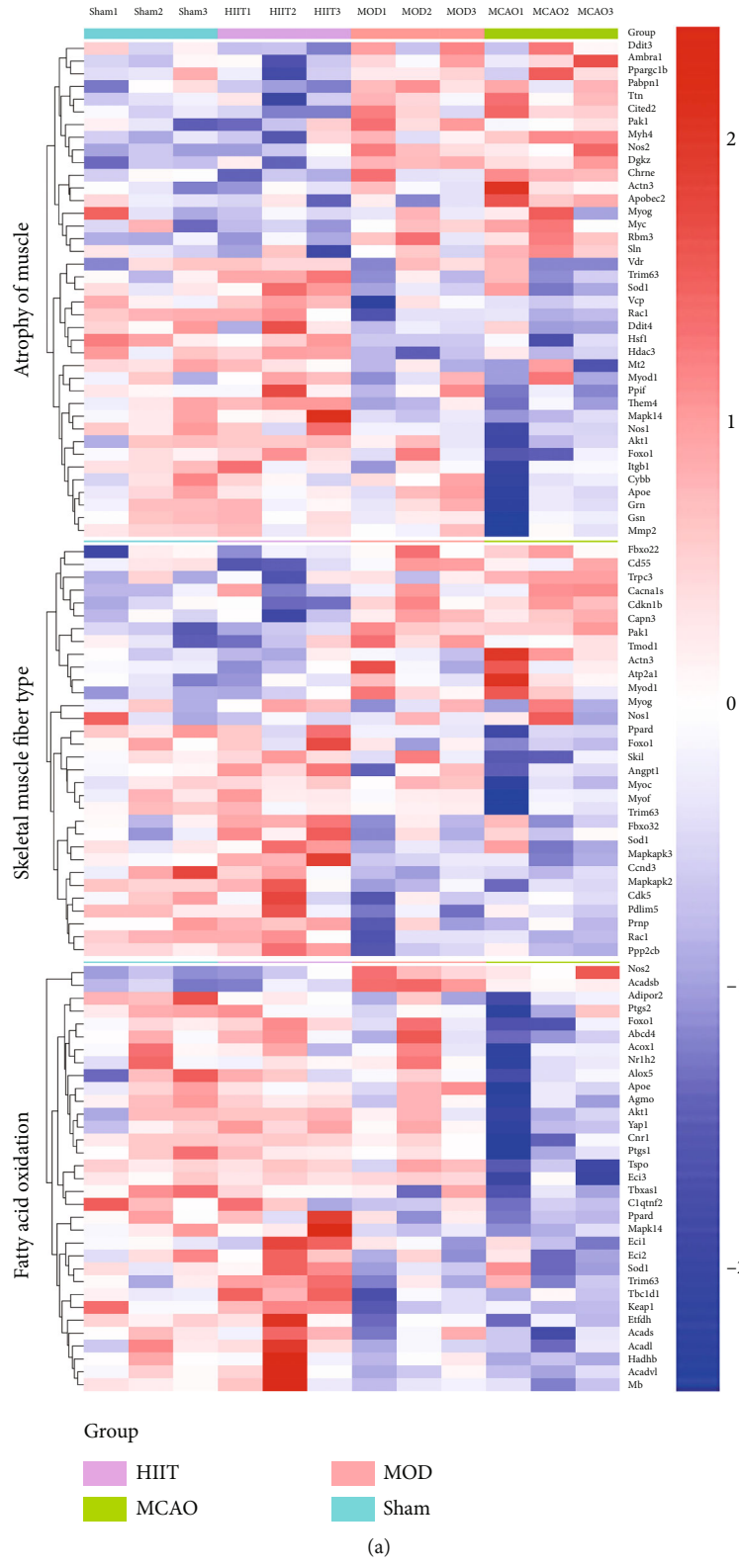
atrophy, as evidenced by the result of GO and KEGG enrichment analysis above. Expression of TLR4, MyD88, NFκB, and p-NFκB was all significantly increased in the paretic gastrocnemius muscle of the MCAO group ($P < 0.05$). Notably, HIIT downregulated the expression of TLR4/MyD88/NFκB signal to control levels ($P < 0.05$; Figures 9(a) and 9(b)), indicating normal reactivity, whereas there were no differences between MOD and MCAO groups.

4. Discussion

This study investigated the effects of high-intensity interval and moderate-intensity aerobic training on muscle mass, strength, physical function (walking competency and cardiorespiratory fitness), and stroke-related gene expression profile of skeletal muscle in a preclinical mouse model of cerebral ischemia. We originally demonstrated that both low volume HIIT and MOD prevented stroke-related skeletal muscle mass loss in mice. HIIT was more beneficial than MOD for improvements in walking competency and cardiorespiratory fitness. Additionally, RNA-seq analysis revealed that the differentially expressed genes between HIIT and sedentary MCAO groups were highly associated with signaling pathways involved in inflammatory response. Following the outcome, we further investigated the infiltrating immune cells abundant in paretic muscles. The results showed that HIIT modulated macrophage activation by stimulating M1-to-M2 polarization via inhibiting the TLR4/MyD88/NFκB signaling pathway, thus exerting an anti-inflammatory effect in paretic skeletal muscle. It is expected that these data will provide novel insights into the mechanisms and potential targets underlying muscle wasting in stroke.

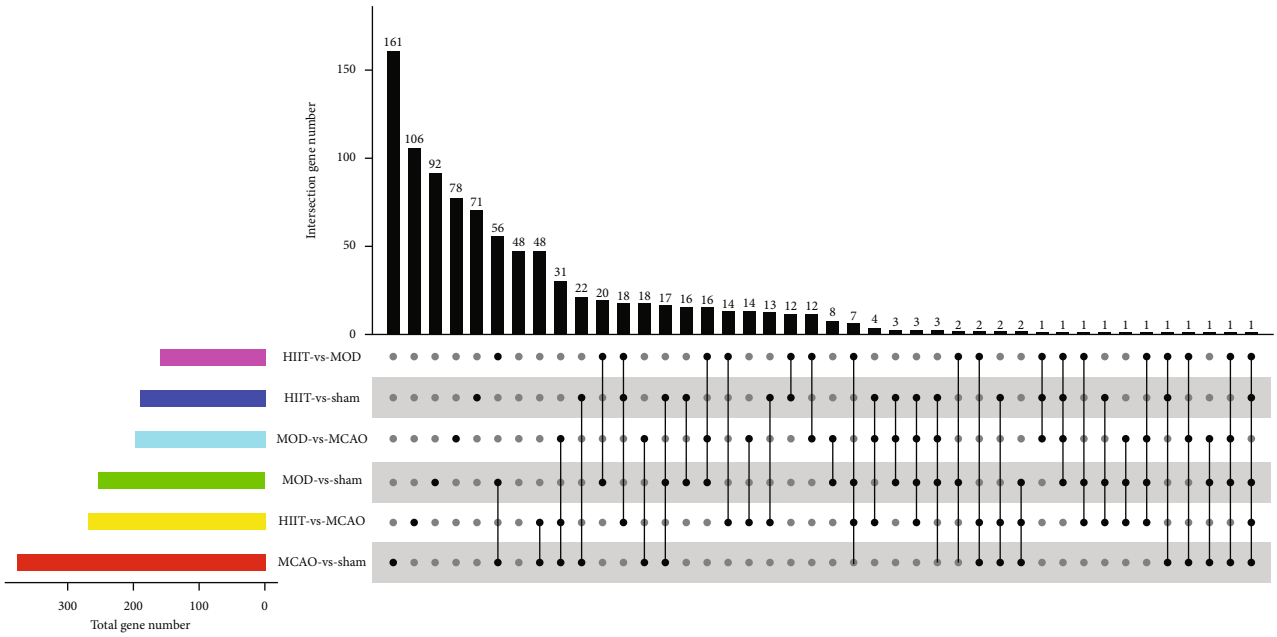
The MCAO model is characterized by high reproducibility and large infarct volumes involving a substantial proportion of the hemisphere (including most of the cortex, striatum, thalamus, hippocampus, and subventricular zone) [20]. Muscle loss or weakness is associated with these motor system impairments due to the nerve fiber degeneration of the motor cortex, striatum, internal capsule, and the descending projection pathways, as well as reduced muscle activation and incoordination [21]. Loss of muscle in the nonparetic limbs is also probable over time as stroke survivors are known to have a sedentary lifestyle [11]. It has been reported that patients with acute stroke were physically active for less than 40 min a day during hospitalization [22]. The open field test in this study also showed that the distance moved and mean velocity of MCAO mice significantly decreased compared with the sham group and the other two exercise groups.

Inactivity and immobilization after stroke are important factors of muscle loss or decreased fiber cross-sectional area as muscle unloading produces a multitude of maladaptive responses, such as insulin resistance, glucose-dependent energy metabolism, and intramuscular lipid disposition [23]. A recent systematic review based on 7 studies involving 1695 stroke patients showed that the prevalence of stroke-related sarcopenia within 1 month was 50% and that at 6 months was 34% [8]. In the present study, the paretic gastrocnemius mass was lower in the MCAO group than that in the sham group until the 4th week postischemia. Next, we calculated the ratio of gastrocnemius mass to body weight. The MCAO group also showed a significant decrease in the ratio relative to the sham group. These results corroborate the previous study predicting that adaptive responses in muscle tissue will be most pronounced in the early phase after stroke [23]. Skeletal muscles make up 40% of the body's mass. It is taken for granted that the loss of muscle mass after stroke is accompanied by weight reduction. A significant decrease in the body weight of animals with cerebral ischemia was observed in the present study as early as 4 days after stroke, which is consistent with those reported by Modo et al. [24], where ischemic animals with cerebral ischemia presented a lower weight gain few days after

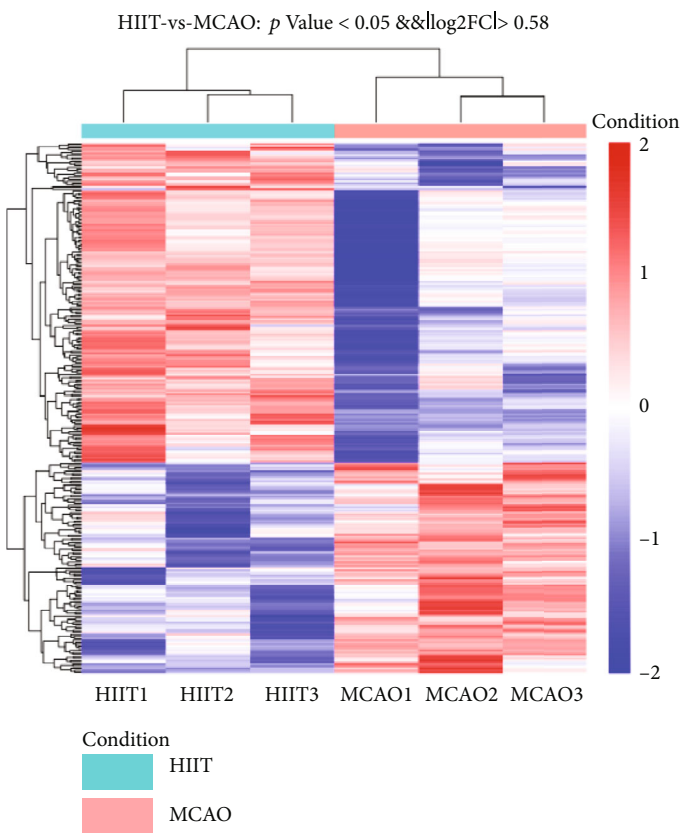


(a)

Figure 6: Continued.

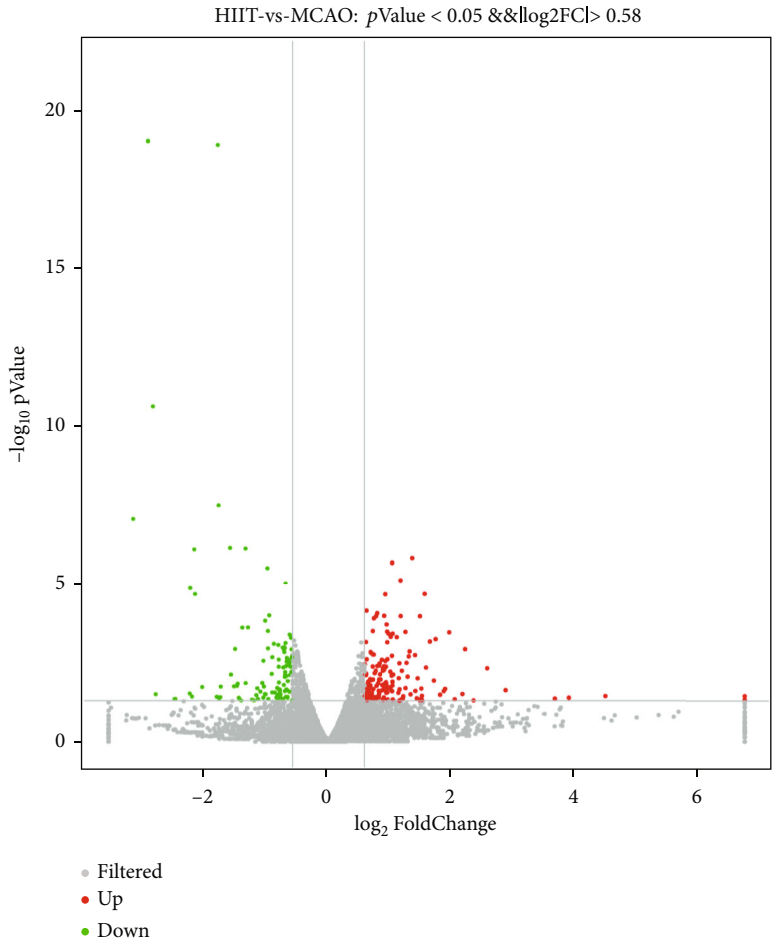


(b)



(c)

Figure 6: Continued.



(d)

Figure 6: Continued.

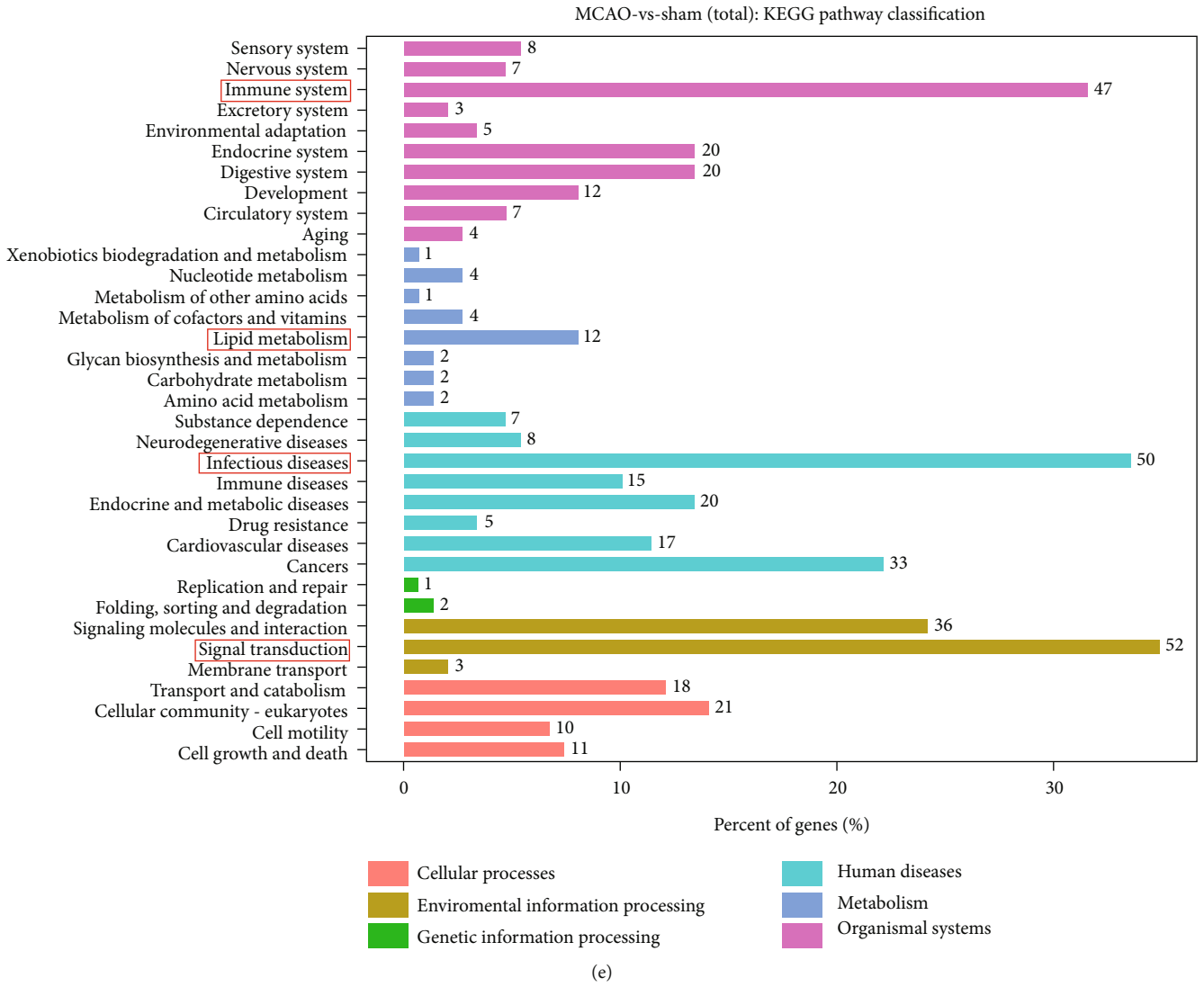
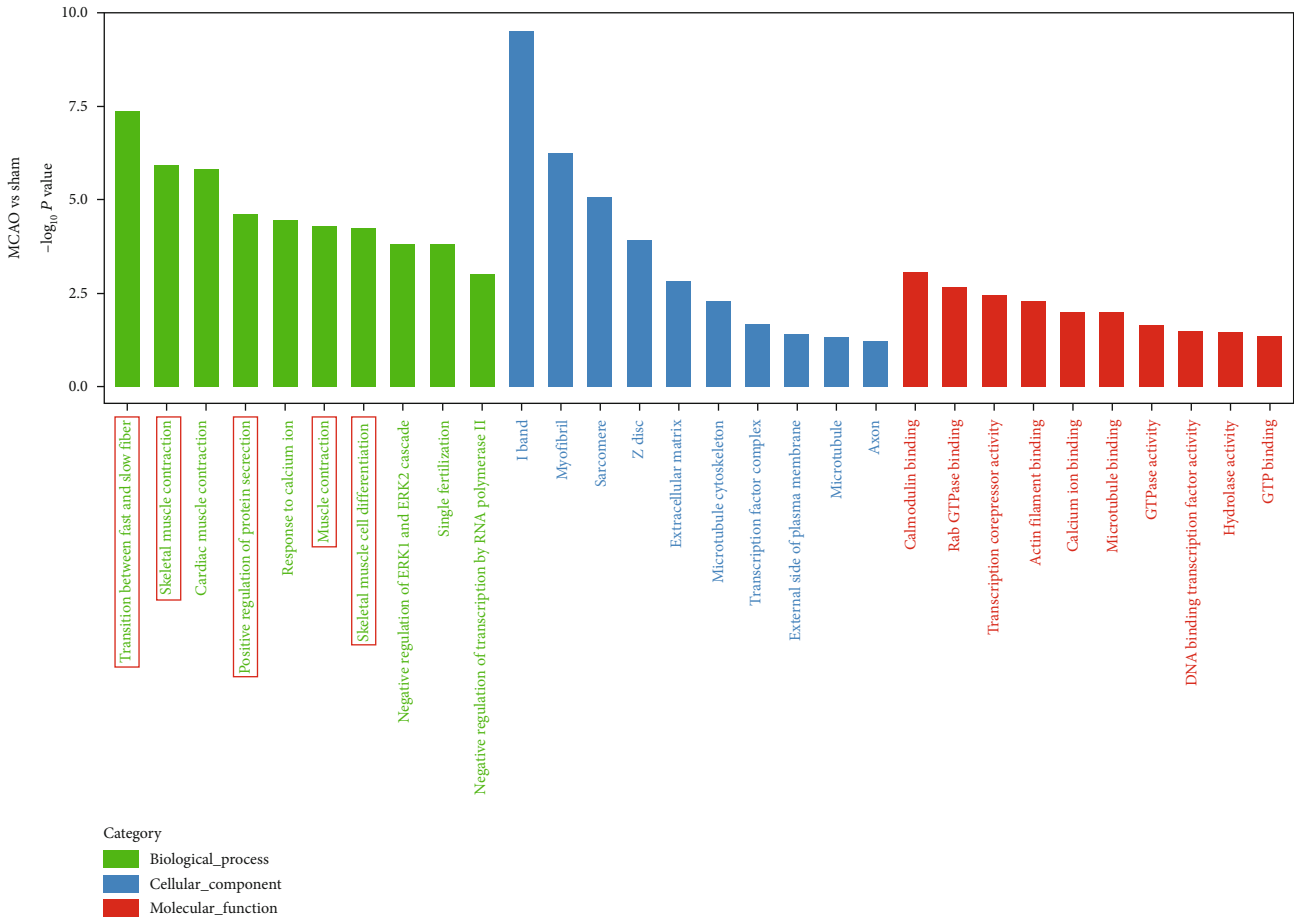


Figure 6: Profiling of differentially expressed genes of skeletal muscle after stroke and exercise protocols. (a) Heat map of differentially expressed genes in each group associated with GO biological process categorization about "atrophy of muscle," "skeletal muscle fiber type," and "fatty acid oxidation." (b) Venn graph illustrates common and unique differentially expressed genes between different comparison groups. (c) Heat map of differentially expressed genes between MCAO and sham groups (dependent group t-test $p < 0.05$ and fold difference ≥ 1.5). Red indicates relatively high expression of protein-coding genes, and blue indicates relatively low expression of protein-coding genes. (d) Volcano map of differentially expressed genes between MCAO and sham groups. Gray represents the genes with insignificant difference. Red and green represent the genes with significant difference. (e) KEGG pathway analysis of differentially expressed genes, including cellular processes, environmental information processing, genetic information processing, human diseases, and metabolism organismal systems. Abbreviation: GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

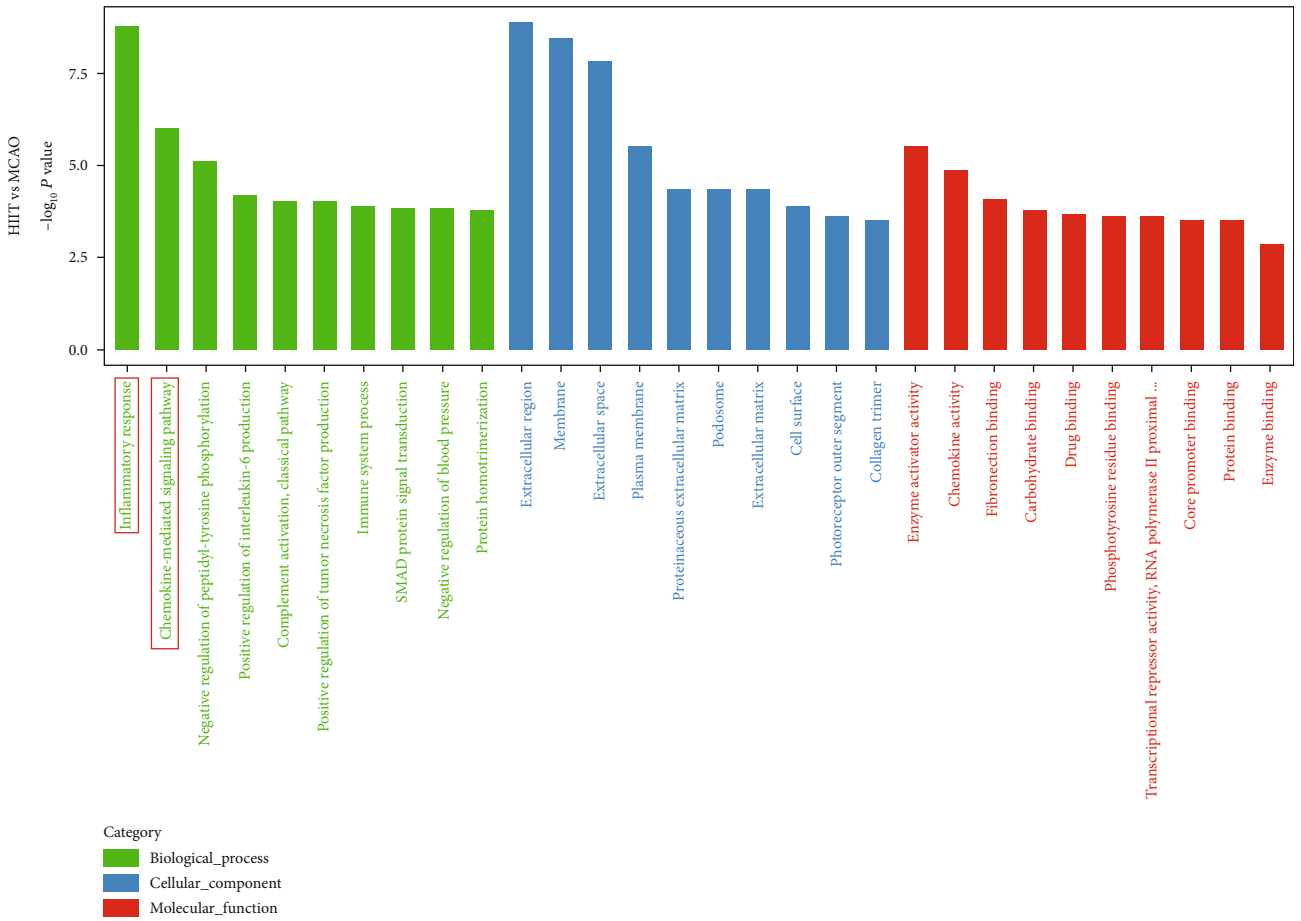
surgery relative to the controls. Compared with the nonexercise group, both 3 weeks of HIIT and MOD significantly reversed the ratio of gastrocnemius mass to body weight. However, HIIT but not MOD significantly improved the weight gain rate at 2nd week after cerebral ischemia and finally contributed significantly to restore paretic gastrocnemius mass after 3 weeks of training.

Recently, there have been suggestions that low muscle strength, rather than low muscle mass, be considered the major determinant of sarcopenia [5]. It is worth noting that the loss of muscle tissue may be replaced by intramuscular lipid configuration, so the actual reduction of functional muscle tissue may be higher than inferred from simple weight assessment. A meta-analysis included 11 trials that report slightly greater fat mass in the paretic arms compared to nonparetic arms in stroke survivors. Although there is no significant increase in whole-body fat mass from 1 to 6 months poststroke, it does increase between 6 and 12 months after the stroke [25]. Herein, our data also indicated that massive lipid droplet accumulation in paretic gastrocnemius was detected by Oil-Red-O staining in the MCAO sedentary group, and it was HIIT but not MOD that significantly reduced the lipid disposition. Skeletal muscle energy flux during contraction is intensity dependent. At the same time, high-intensity exercise was the most efficient exercise regimen regarding depleting glycogen stores,



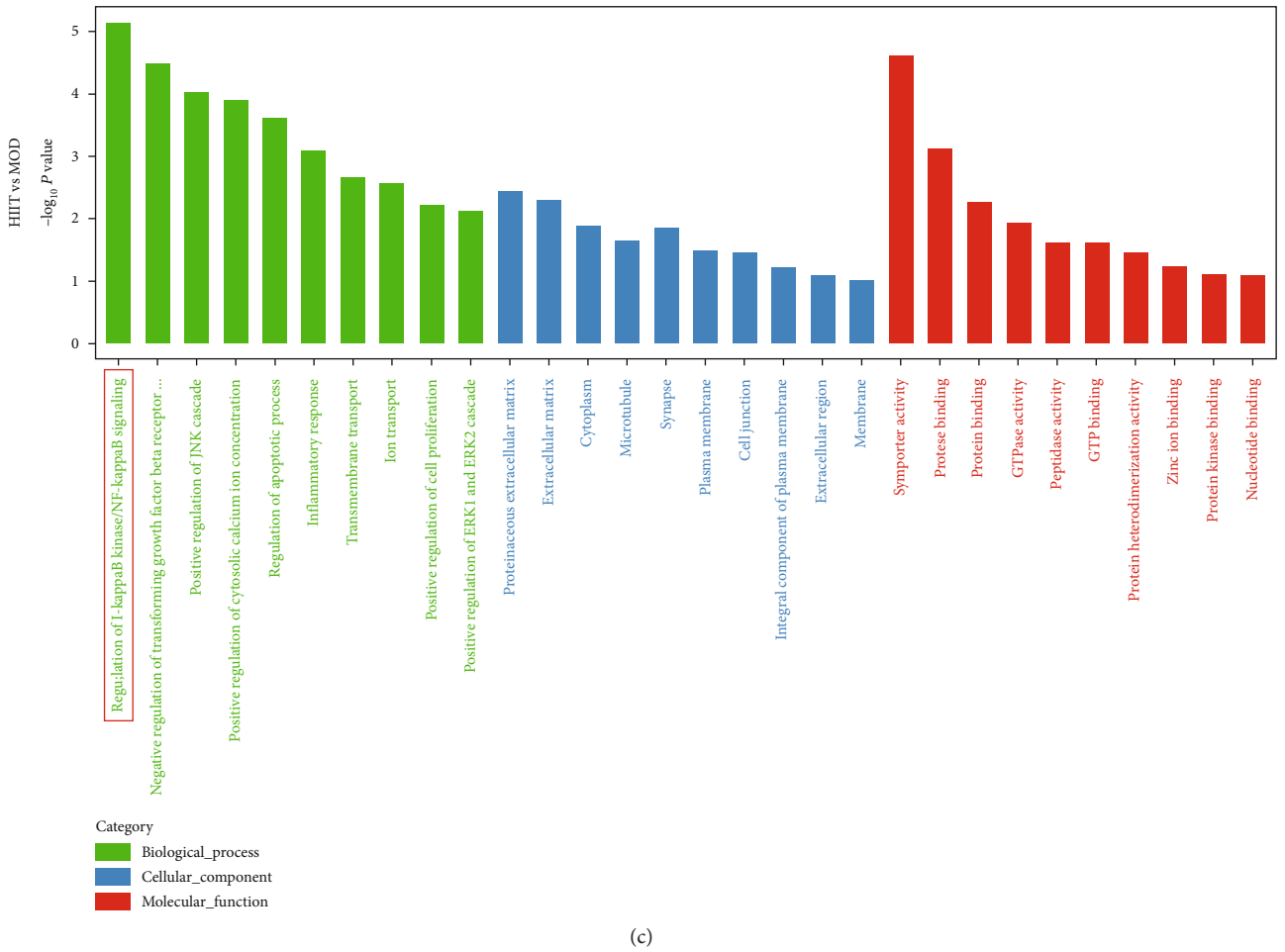
(a)

Figure 7: Continued.



(b)

Figure 7: Continued.



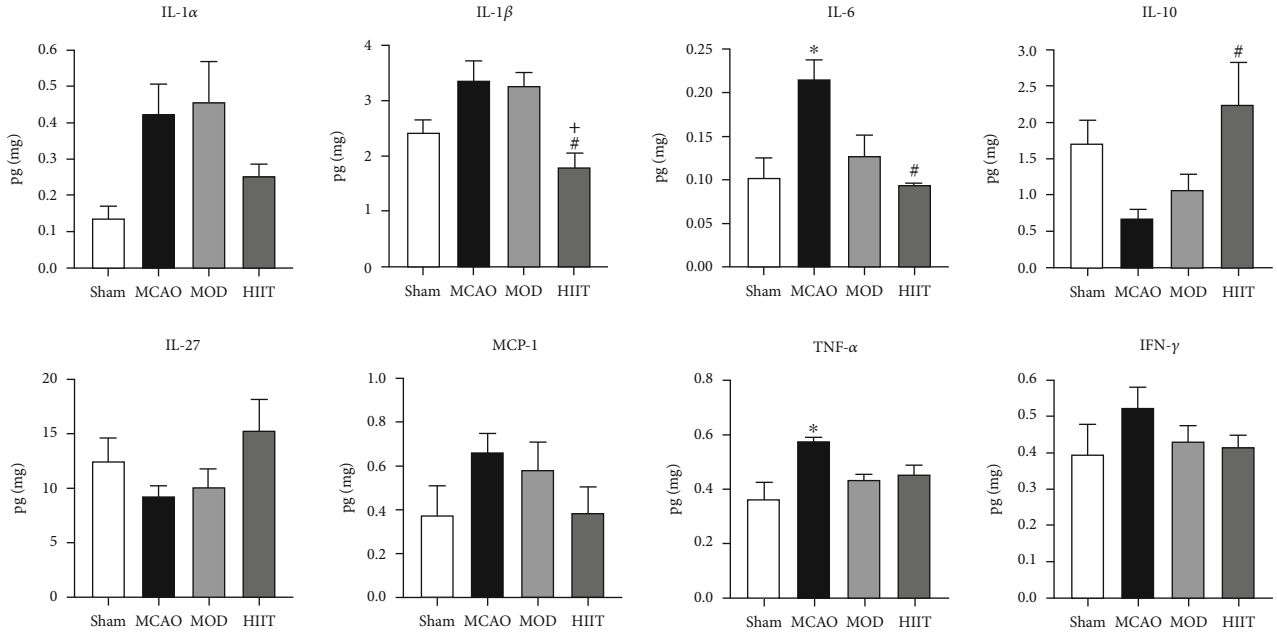
(c)

Figure 7: GO term enrichment analysis of differentially expressed genes, including cellular component, biological process, and molecular function. (a) GO annotation analysis between MCAO and sham groups. (b) GO annotation analysis between HIIT and MCAO groups. (c) GO annotation analysis between HIIT and MOD groups. Abbreviation: GO: Gene Ontology.

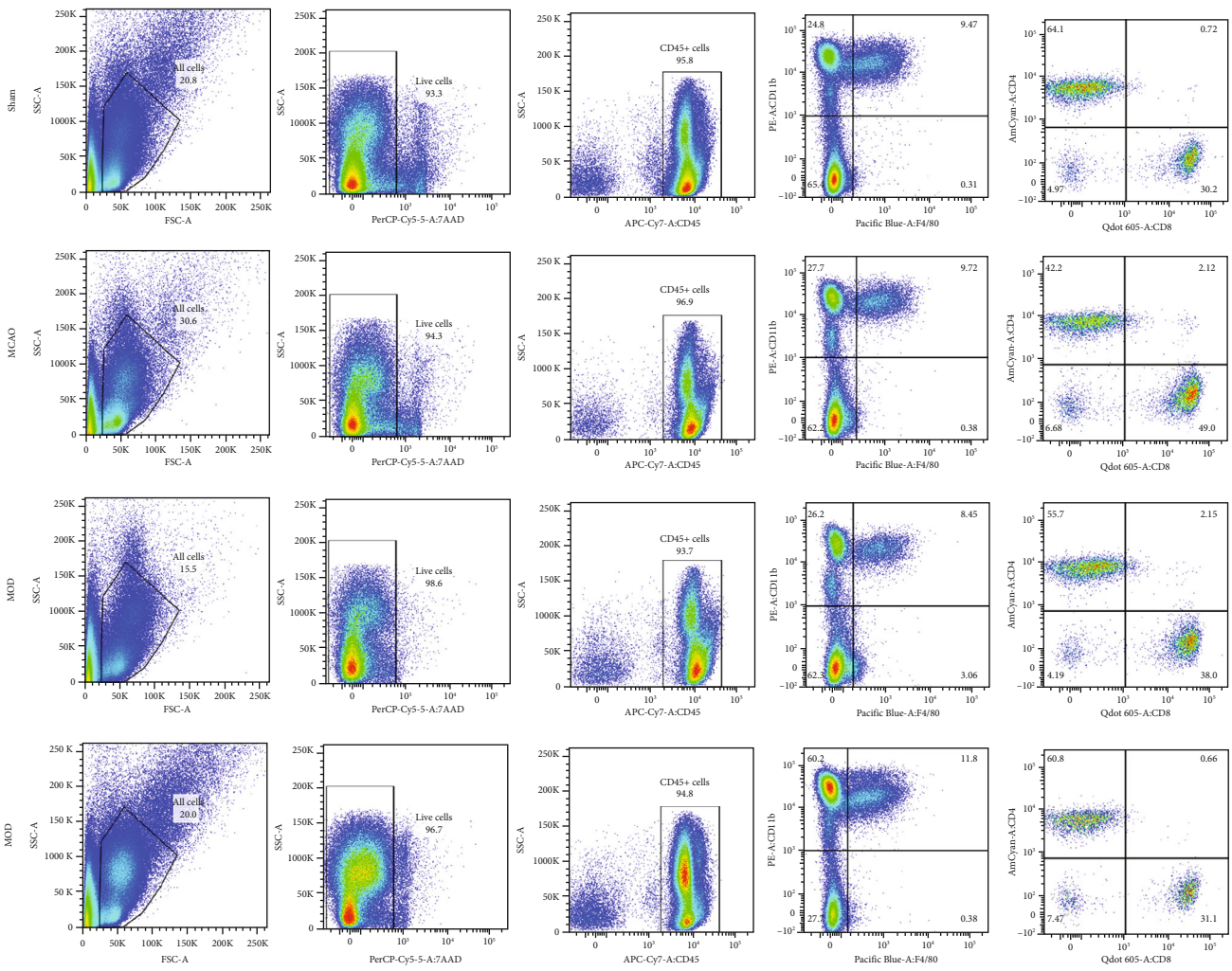
elevating fat oxidation rates, and promoting lipid mobilization and energy expenditure in the postexercise period compared to low- to moderate-intensity exercise [26, 27].

Indeed, skeletal muscle mass deficit more appears to be an independent predictor of poor outcome after stroke. Ohyama et al. [28] revealed that the presence of the skeletal muscle mass defects in over half of patients with acute ischemic stroke, who tended to display worse conditions (i.e., severe neurological impairments and poorer functional outcome) at admission and longer hospital stay. A growing body of evidence supports regular physical exercise as the most effective strategy for improving sarcopenia and physical function [11]. Nevertheless, it is unclear whether the positive effects of exercise interventions can be sustained for an adequate period and maintained at sufficient intensity to prevent incident disabilities [29]. In the present study, a remarkable decrease of forelimb grip strength and latency to fall, as well as a significant increase of laterality index and forelimb slip error percent, was observed in ischemic animals. Similar to muscle mass, both HIIT and MOD reversed the decline in muscle strength of hemiplegic limbs and improved the physical function. In fact, for most people, greater health benefits can be obtained by engaging in intensive or longer physical exercise [30, 31].

Walking performances are important for stroke patients to maintain independent living and participate in family, social activities [32]. Gait abnormalities along with muscle weakness place stroke survivors at a high risk of falls. A recent meta-analysis showed that compared with low-to-moderate-intensity exercise or regular physical activity, high-intensity exercise may be a safe and more effective stimulus to improve the walking ability of stroke patients, with improved walking distance, comfortable gait speed, and stride length [32]. Gait analysis in the present study showed that HIIT can effectively reduce the duty cycle and mean intensity of the right hindlimb. Many stroke patients may be restricted in their daily activities because of their adverse events related to cardiorespiratory fitness, which is considerably low poststroke, with VO_2 peak values ranging from 8 to 22 mL/kg/min, equivalent to 26%~87%, respectively, of that of healthy age- and sex-matched healthy individuals [33]. Cardiorespiratory fitness reflects the ability of circulatory and respiratory systems to supply oxygen for skeletal muscles during moderate- to high-intensity exercise

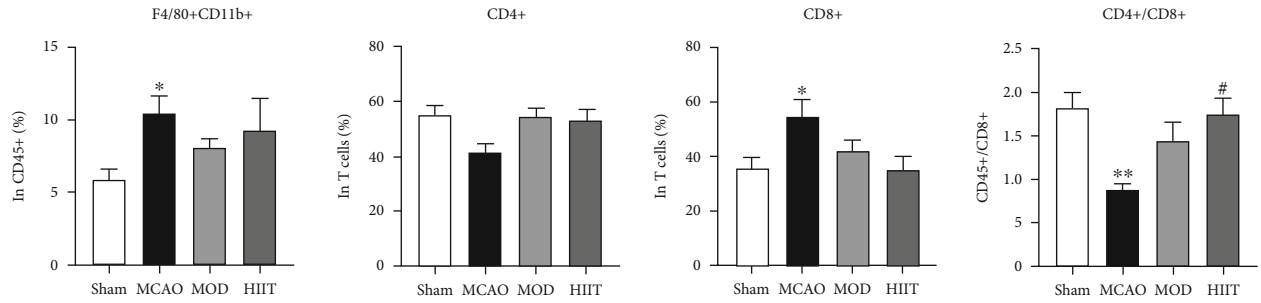


(a)



(b)

Figure 8: Continued.



(c)

Figure 8: Level of cytokines in paretic skeletal muscle and lymphocyte subsets in peripheral blood. (a) The profiles of multiple cytokines in paretic gastrocnemius muscle in each group, including interleukin- (IL-) 1α, IL-1β, IL-6, IL-10, IL-27, monocyte chemoattractant protein (MCP-1), tumor necrosis factor- (TNF-) α, and interferon- (IFN-) γ. (b) Representative fluorescence activated cell sorting (FACS) plots of F4/80+CD11b+ macrophages gated on CD45+ cells, CD4+ cells gated on CD45+CD3+ cells, and CD8+ cells gated on CD45+CD3 +cells in muscle tissue. (c) Bar graph showing the percentage of F4/80+CD11b+ cells in CD45+ cells, CD4+ cells in CD45+CD3+ cells, CD8+ cells in CD45+CD3+ cells, and the ratio of CD4+ and CD8+ cells. Values are expressed as the mean ± SEM of the mean. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the MCAO group; $^{+}P < 0.05$ and $^{++}P < 0.01$ compared with the MOD group as determined by one-way ANOVA (Tukey's multiple comparison test) for the data with normal distribution. The letters for no significance were not shown.

training [34]. Our study reported that HIIT induced a significant increase in tidal volume, minute ventilation, peak inspiratory flow, and peak expiratory flow, while MOD failed.

Human skeletal muscle fibers have great adaptive potential; however, the molecular mechanism of atrophy and phenotypic transition after stroke is not clear [35]. Slow myosin heavy chain (MHC) type I fibers are characterized by a large number of mitochondria, oxidative metabolism, and fatigue resistance. Conversely, fast oxidation glycolysis IIA fiber and glycolysis IIb/X fiber have lower fatigue resistance, and their energy mainly derives from anaerobic glycolysis [36]. In normal aging, muscle fibers shift from fast to slow fibers with more reliance on anaerobic metabolism, resulting in a reduction of muscle strength [37]. In contrast, different from age-related sarcopenia, the characteristic of stroke-related muscle alterations is a slow-to-fast muscle fiber shift, which was a strong predictor of impaired function, such as gait disorders poststroke [7]. In elderly stroke patients, fast type IIx and IIa MHC fibers in paretic vastus lateralis significantly increased compared with nonparetic muscle. The proportion of these fibers is only negatively correlated with the self-selected gait speed of the paralyzed leg [38]. In the present experiment, an exercise protocol of high intensity was applied for continuous 3 weeks, which caused a considerable but nonsignificant increase in the proportion of type I fibers and a decrease in the proportion of type II fibers. Yan et al. [39] reported that sports training may lead to an increase in the proportion of MHC I type fibers in skeletal muscle, but only in the cases of long-term exercise, such as athletes who undergo intense training for years.

In this study, we further used mRNA-seq to determine the effect of different exercise programs on skeletal muscle gene expression of the MCAO mice. Gene ontology analysis implied that signaling pathways involved in inflammatory response might contribute to the protein synthesis and degradation of muscle. According to a different and emerging research and consistent with our results, stroke-related sarcopenia may be associated and even caused by inflammation [40, 41]. A large body of literature showed that inflammatory cytokines activate many molecular pathways involved in skeletal muscle consumption, resulting in the imbalance between protein synthesis and catabolism [42, 43]. Our findings may suggest that the plasma titer of some inflammatory molecules (IL-1β, IL-6, TNF-α, and IL-10), which are important cytokines related to the regulation of Th1 and Th2 implicated in skeletal muscle regeneration through myogenic and myeloid cell activation, could be related to muscle decline and functional impairment [27]. Acute exercise can increase the plasma level of the same proinflammatory cytokines, possibly due to stress response, whereas regular exercise seems to upregulate the anti-inflammatory ability, leading to a decrease in the level of systemic inflammation and circulating inflammatory markers [44, 45]. Extensive studies have driven the discussion about the anti-inflammatory effect of exercise that may be mediated (to some extent) by some activities of myokines released into the blood during long-term contraction [44]. The production and subsequent release of myokines into the circulation seem to be directly related to the duration and intensity of training. This thesis is supported by our results that HIIT determines the decrease of IL-6 and IL-1β levels, which do not reach a continuous level of diminution within low to moderate intensity of training [46].

Among infiltrating immune cells in muscle, macrophages play a central role in the activation and protection of muscle fibers after muscle inflammation and injury [47]. In addition, some studies have found that there are a large number of resident macrophages in the adventitia and fascicular membrane, which control the immune responses in the process of muscle injury [48, 49]. Although the phenotypes of macrophages are heterogeneous in various tissue and environments, there are two phenotypes of activated macrophages, namely, proinflammatory M1 and

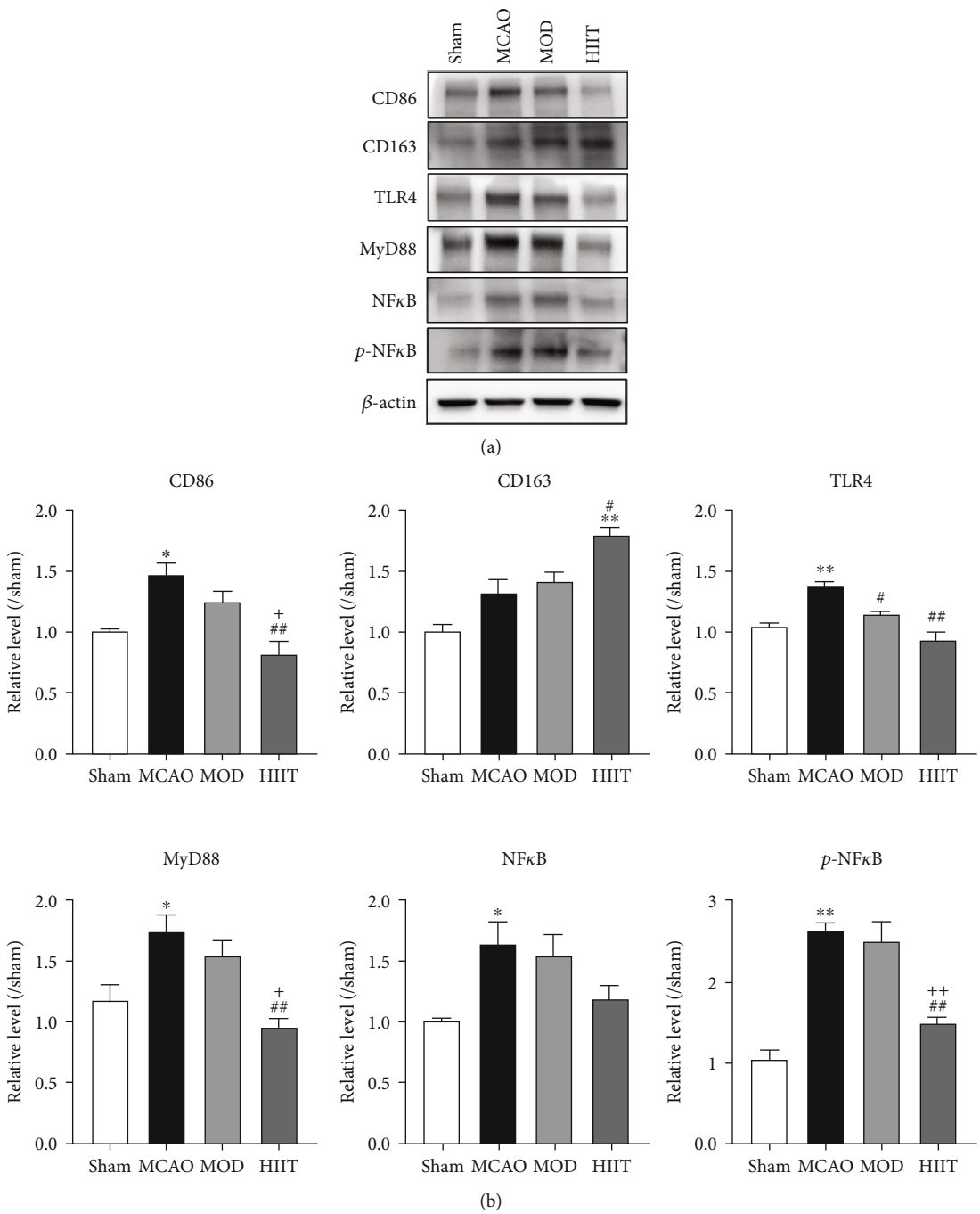


Figure 9: Continued.

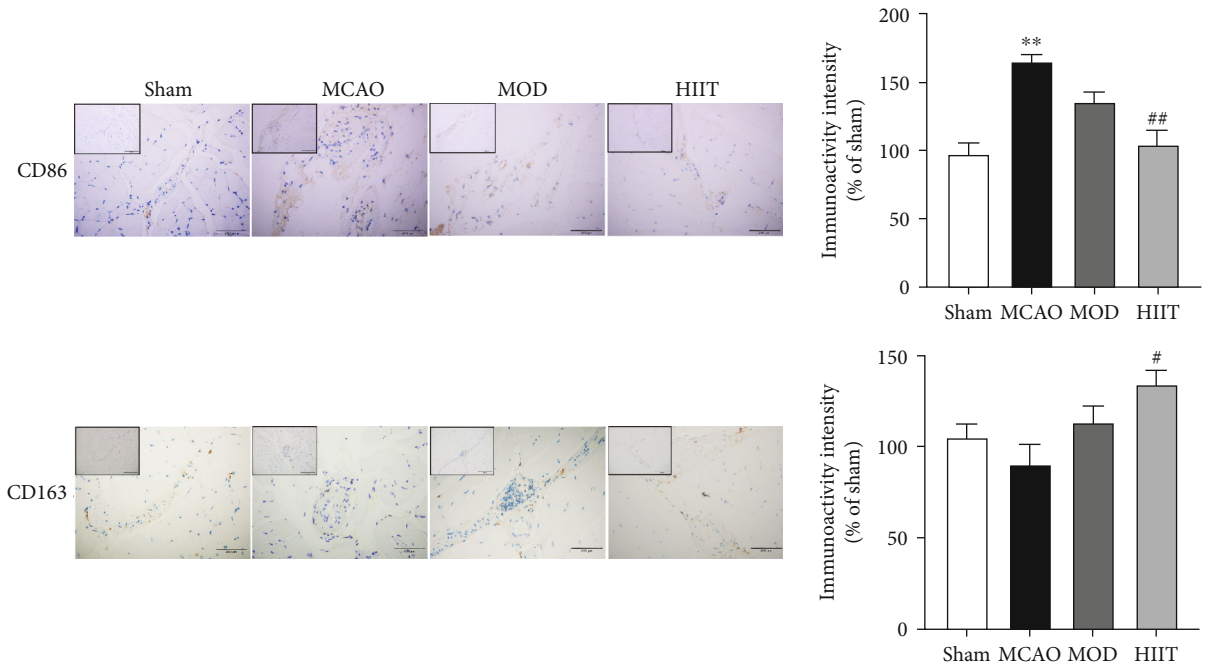


Figure 9: HIIT modulated macrophage-mediated inflammation and cytotoxic properties via inhibiting the TLR4/MyD88/NFκB signaling pathway. (a, b) Representative immunoblots and quantification normalized to sham group condition of CD86, CD163, TLR4, MyD88, NFκB, p-NFκB, and β-actin protein level ($n = 5$). (c) Representative immunostaining and quantification of CD86 and CD163 in paretic gastrocnemius tissues ($n = 5$). Values are expressed as the mean ± SEM of the mean. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the MCAO group; $^{+}P < 0.05$ and $^{++}P < 0.01$ compared with the MOD group as determined by one-way ANOVA (Tukey's multiple comparison test) for the data with normal distribution. The letters for no significance were not shown.

anti-inflammatory M2. M1 (classical activation) macrophages mainly secrete inflammatory cytokines, including IL-1β, IL-6, TNF-α, ROS, NO, and MMP, which in turn promote CD4+/Th1 cells that enter muscle from the blood circulation, consequently causing acceleration of myofiber lysis and protein degradation. The M2 macrophages (alternative activation) abundant during the late phase of tissue repair can release anti-inflammatory cytokines, such as TGF-β, IL-10, and IGF-1, thus prompting the anti-inflammatory effect of CD8+/Th1 cells, which are mainly involved in the phagocytosis and cleaning of the injured site, contributing to myogenesis and tissue repair [50, 51]. In this study, CD86 and CD163 were detected as markers of M1 macrophages and M2 macrophages, respectively. We observed the increase of M1 macrophages and the decrease of M2 macrophages in the paretic gastrocnemius muscle of ischemic mice. Therefore, a definitive understanding of the complex temporally coordinated macrophage roles in stroke-related sarcopenia and the balance of M1 and M2 macrophages is crucial in muscle recovery [10].

Emerging evidence shows that moderate training regulates macrophage activation by stimulating M1 to M2 polarization and playing a global anti-inflammatory role in multiple organs [52, 53]. In skeletal muscle, physical activity stimulates the release of myokines related to M1/M2 ratio regulation (e.g., IL-6, TNF-α, and IL-10), which is involved in skeletal muscle regeneration [54, 55]. The effect of exercise strongly depends on its modality, intensity, and timing. A recent study investigating the direct effects of high-intensity continuous training (HICT) on neuroprotection in the central nervous system (CNS) suggested that HICT protected the CNS against autoimmune neuroinflammation by reducing microglial-derived neurotoxicity, and proinflammatory responses, rather than inducing their shift to M2 phenotype [56]. However, it has also been observed that excessive vigorous exercise still promoted M2 polarization of macrophages in skeletal muscle, and myogenesis increases despite the increase of TNF-α [57]. In our study, results indicated that after 3 weeks of HIIT, rather than MOD, inhibition of M1 phenotype occurred concomitantly with the increase of M2 macrophage marker, and further pathway analyses implicated changes in the regulation of IκB kinase/NFκB signaling between HIIT and MOD groups, which may contribute to modulating M1-to-M2 polarization.

Ample evidence shows that macrophage polarization is mainly regulated by the toll-like receptor (TLR) pathway, which plays a critical role in the nonspecific immune response [58]. TLR4, the earliest receptor protein discovered, binds to corresponding ligands and induces cell activation through myeloid differentiation factor 88- (MyD88-) dependent and MyD88-independent pathways, activating the p65 subunit of downstream NFκB to secrete

inflammatory factors [53]. Our subsequent results also confirmed that HIIT could downregulate the expression of TLR4, MyD88, and phosphorylation of NFκB, thereby inhibiting the M1 type polarization of macrophage and indirectly promoting the M2 type polarization, which is conducive to restore the dynamic balance between the polarization of M1 and M2. That may be a vital mechanism for HIIT to regulate macrophage polarization in both directions, suppress chronic low-grade inflammation of muscles after cerebral ischemia, and promote muscle repair.

In spite of these findings, there are some limitations to be solved in the future. Firstly, the inflammatory response occurs early soon after stroke, which will impede stroke recovery [59]. Moreover, the endogenous recovery in the chronic phase is usually insufficient to significantly improve long-term functional outcomes. The present study failed to explore the role of inflammation markers and cells in predicting stroke outcome in the early stage. In addition, we did not reveal the long-term effects of different exercise regimens on muscle wasting. Future experiments should be dedicated to solve the above problems in clinic setting. Secondly, the global changes of TLR4/MyD88/NFκB signaling are significant in whole muscle tissue. However, it is better to reveal the alteration of TLR4/MyD88/NFκB signaling by isolating infiltrating macrophages using flow sorting.

5. Conclusion

In conclusion, HIIT induces direct beneficial effects on muscle repair and physical function in an experimental model of MCAO compared to MOD. The long-term inflammatory response of muscle after cerebral ischemia serves as a key therapeutic target, and HIIT may regulate macrophage polarization via inhibiting the TLR4/MyD88/NFκB signaling pathway, thereby reducing the cytotoxicity and proinflammatory properties of macrophages. Elucidating the mechanisms underlying the positive effects of exercise training on stroke-related sarcopenia will facilitate the translation of basic research findings to clinical benefits for sedentary patients. Importantly, our study demonstrated the different effects on muscle mass, strength, and gene expression between different exercise regimens in terms of type, intensity, frequency, and duration. Therefore, long-term exercise training and specific exercise program are required to optimally respond to deleterious inflammatory response.

Data Availability

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no competing or financial interests.

Authors' Contributions

Lu Luo and Meixi Liu contributed equally to this work.

Acknowledgments

This study was supported by the Natural Science Foundation of China (NSFC, nos. 81972141 and 81972140) and Shanghai Municipal Key Clinical Specialty (nos. shslczdzk02702).

Supplementary Materials

Supplementary Methods: the concise description of methods and descriptive statistics (Supplemental Table 1, Supplemental Table 2), including behavioral tests, HE staining, immunohistochemistry, RNA sequencing, and differentially expressed gene analysis, profiling of cytokines/chemokines, western blot assays. *(Supplementary Materials)*

References

[1] GBD 2016 Lifetime Risk of Stroke Collaborators, "Global, regional, and country-specific lifetime risks of stroke, 1990 and 2016," *The New England Journal of Medicine*, vol. 379, pp. 2429–2437, 2018.

[2] S. S. Virani, A. Alonso, E. J. Benjamin et al., "Heart disease and stroke statistics-2020 update: a report from the American Heart Association," *Circulation*, vol. 141, pp. e139–e596, 2020.

[3] G. J. Del Zoppo, "Toward the neurovascular Unit A journey in clinical Translation," *Stroke*, vol. 44, no. 1, pp. 263–269, 2013.

[4] Y. Xing and Y. Bai, "A review of exercise-induced neuroplasticity in ischemic stroke: pathology and mechanisms," *Molecular Neurobiology*, vol. 57, no. 10, pp. 4218–4231, 2020.

[5] A. J. Cruz-Jentoft, G. Bahat, J. Bauer et al., "Sarcopenia: revised European consensus on definition and diagnosis," *Age and Ageing*, vol. 48, no. 1, pp. 16–31, 2019.

[6] W. Li, T. Yue, and Y. Liu, "New understanding of the pathogenesis and treatment of stroke-related sarcopenia," *Biomedicine & Pharmacotherapy*, vol. 131, article 110721, 2020.

[7] N. Scherbakov, A. Sandek, and W. Doehner, "Stroke-related sarcopenia: specific characteristics," *Journal of the American Medical Directors Association*, vol. 16, no. 4, pp. 272–276, 2015.

[8] Y. Su, M. Yuki, and M. Otsuki, "Prevalence of stroke-related sarcopenia: a systematic review and meta-analysis," *Journal of Stroke and Cerebrovascular Diseases*, vol. 29, no. 9, article 105092, 2020.

[9] C. J. Winstein, J. Stein, R. Arena et al., "Guidelines for adult stroke rehabilitation and recovery: a guideline for healthcare professionals from the American Heart Association/American Stroke Association," *Stroke*, vol. 47, pp. e98–e169, 2016.

[10] C. Lee, H. Jeong, H. Lee, M. Hong, S. Y. Park, and H. Bae, "Magnolol attenuates cisplatin-induced muscle wasting by M2c macrophage activation," *Frontiers in Immunology*, vol. 11, 2020.

[11] on behalf of the SPRINTT Consortium, E. Marzetti, R. Calvani et al., "Physical activity and exercise as countermeasures to physical frailty and sarcopenia," *Aging Clinical and Experimental Research*, vol. 29, no. 1, pp. 35–42, 2017.

[12] R. T. R. Melo, L. C. M. Damázio, M. C. Lima et al., "Effects of physical exercise on skeletal muscles of rats with cerebral ischemia," *Brazilian Journal of Medical and Biological Research*, vol. 52, no. 12, article e8576, 2019.

[13] S. K. Prajapati, A. Mansfield, W. H. Gage, D. Brooks, and W. E. McIlroy, "Cardiovascular responses associated with daily

walking in subacute stroke," *Stroke Research and Treatment*, vol. 2013, Article ID 612458, 7 pages, 2013.

[14] J. Bernhardt, N. Chitravas, I. L. Meslo, A. G. Thrift, and B. Indredavik, "Not all stroke units are the Same," *Stroke*, vol. 39, no. 7, pp. 2059–2065, 2008.

[15] P. Kortebein, A. Ferrando, J. Lombeida, R. Wolfe, and W. J. Evans, "Effect of 10 days of bed rest on skeletal muscle in healthy older adults," *JAMA*, vol. 297, pp. 1772–1774, 2007.

[16] P. Boyne, K. Dunning, D. Carl et al., "High-intensity interval training and moderate-intensity continuous training in ambulatory chronic stroke: feasibility study," *Physical Therapy*, vol. 96, no. 10, pp. 1533–1544, 2016.

[17] L. Luo, H. Meng, Z. Wang et al., "Effect of high-intensity exercise on cardiorespiratory fitness in stroke survivors: a systematic review and meta-analysis," *Annals of Physical and Rehabilitation Medicine*, vol. 63, no. 1, pp. 59–68, 2020.

[18] L. Luo, C. Li, Y. Deng, Y. Wang, P. Meng, and Q. Wang, "High-Intensity Interval Training on Neuroplasticity, Balance between Brain- Derived Neurotrophic Factor and Precursor Brain-Derived Neurotrophic Factor in Poststroke Depression Rats," *Journal of Stroke and Cerebrovascular Diseases*, vol. 28, no. 3, pp. 672–682, 2019.

[19] I. T. da Cunha Jr., P. A. Lim, H. Qureshy, H. Henson, T. Monga, and E. J. Protas, "Gait outcomes after acute stroke rehabilitation with supported treadmill ambulation training: a randomized controlled pilot study," *Archives of Physical Medicine and Rehabilitation*, vol. 83, no. 9, pp. 1258–1265, 2002.

[20] C. Kleinschnitz, F. Fluri, and M. Schuhmann, "Animal models of ischemic stroke and their application in clinical research," *Drug Design, Development and Therapy*, vol. 9, pp. 3445–3454, 2015.

[21] S. M. Hatem, G. Saussez, M. della Faille et al., "Rehabilitation of motor function after stroke: a multiple systematic review focused on techniques to stimulate upper extremity recovery," *Frontiers in Human Neuroscience*, vol. 10, 2016.

[22] J. Bernhardt, H. Dewey, A. Thrift, and G. Donnan, "Inactive and alone: physical activity within the first 14 days of acute stroke unit care," *Stroke*, vol. 35, pp. 1005–1009, 2004.

[23] N. Scherbakov, S. von Haehling, S. D. Anker, U. Dirnagl, and W. Doehner, "Stroke induced sarcopenia: muscle wasting and disability after stroke," *International Journal of Cardiology*, vol. 170, no. 2, pp. 89–94, 2013.

[24] M. Modo, R. P. Stroemer, E. Tang, T. Veizovic, P. Sowniski, and H. Hodges, "Neurological sequelae and long-term behavioural assessment of rats with transient middle cerebral artery occlusion," *Journal of Neuroscience Methods*, vol. 104, no. 1, pp. 99–109, 2000.

[25] C. English, K. Thoirs, A. Coates, A. Ryan, and J. Bernhardt, "Changes in fat mass in stroke survivors: a systematic review," *International Journal of Stroke*, vol. 7, no. 6, pp. 491–498, 2012.

[26] K. Marcinko, S. R. Sikkema, M. C. Samaan, B. E. Kemp, M. D. Fullerton, and G. R. Steinberg, "High intensity interval training improves liver and adipose tissue insulin sensitivity," *Molecular Metabolism*, vol. 4, no. 12, pp. 903–915, 2015.

[27] Y. Shen, X. Xu, K. Yue, and G. Xu, "Effect of different exercise protocols on metabolic profiles and fatty acid metabolism in skeletal muscle in high-fat diet-fed rats," *Obesity*, vol. 23, no. 5, pp. 1000–1006, 2015.

[28] K. Ohyama, M. Watanabe, Y. Nosaki, T. Hara, K. Iwai, and K. Mokuno, "Correlation between skeletal muscle mass deficit and poor functional outcome in patients with acute ischemic stroke," *Journal of Stroke and Cerebrovascular Diseases*, vol. 29, no. 4, article 104623, 2020.

[29] F. Landi, R. Calvani, M. Cesari et al., "Sarcopenia as the biological substrate of physical frailty," *Clinics in Geriatric Medicine*, vol. 31, no. 3, pp. 367–374, 2015.

[30] A. J. Cruz-Jentoft, F. Landi, S. M. Schneider et al., "Prevalence of and interventions for sarcopenia in ageing adults: a systematic review. Report of the International Sarcopenia Initiative (EWGSOP and IWGS)," *Age and Ageing*, vol. 43, no. 6, pp. 748–759, 2014.

[31] A. M. Martone, F. Lattanzio, A. M. Abbatecola et al., "Treating sarcopenia in older and oldest old," *Current Pharmaceutical Design*, vol. 21, no. 13, pp. 1715–1722, 2015.

[32] L. Luo, S. Zhu, L. Shi, P. Wang, M. Li, and S. Yuan, "High intensity exercise for walking competency in individuals with stroke: a systematic review and meta-analysis," *Journal of Stroke and Cerebrovascular Diseases*, vol. 28, no. 12, p. 104414, 2019.

[33] G. F. Fletcher, G. J. Balady, E. A. Amsterdam et al., "Exercise standards for testing and Training," *Circulation*, vol. 104, no. 14, pp. 1694–1740, 2001.

[34] A. C. Smith, D. H. Saunders, and G. Mead, "Cardiorespiratory fitness after stroke: a systematic review," *International Journal of Stroke*, vol. 7, no. 6, pp. 499–510, 2012.

[35] N. Scherbakov and W. Doehner, "Sarcopenia in stroke-facts and numbers on muscle loss accounting for disability after stroke," *Journal of Cachexia, Sarcopenia and Muscle*, vol. 2, no. 1, pp. 5–8, 2011.

[36] S. Schiaffino, "Muscle fiber type diversity revealed by anti-myosin heavy chain antibodies," *The FEBS Journal*, vol. 285, no. 20, pp. 3688–3694, 2018.

[37] M. Canepari, M. A. Pellegrino, G. D'Antona, and R. Bottinelli, "Single muscle fiber properties in aging and disuse," *Scandinavian Journal of Medicine & Science in Sports*, vol. 20, no. 1, pp. 10–19, 2010.

[38] P. G. De Deyne, C. E. Hafer-Macko, F. M. Ivey, A. S. Ryan, and R. F. Macko, "Muscle molecular phenotype after stroke is associated with gait speed," *Muscle & Nerve*, vol. 30, no. 2, pp. 209–215, 2004.

[39] Z. Yan, M. Okutsu, Y. N. Akhtar, and V. A. Lira, "Regulation of exercise-induced fiber type transformation, mitochondrial biogenesis, and angiogenesis in skeletal muscle," *Journal of Applied Physiology*, vol. 110, pp. 264–274, 2011.

[40] J. M. Argilés, S. Busquets, B. Stemmler, and F. J. López-Soriano, "Cachexia and sarcopenia: mechanisms and potential targets for intervention," *Current Opinion in Pharmacology*, vol. 22, pp. 100–106, 2015.

[41] G. Bano, C. Trevisan, S. Carraro et al., "Inflammation and sarcopenia: A systematic review and _meta_ -analysis," *Maturitas*, vol. 96, pp. 10–15, 2017.

[42] E. Jo, S. R. Lee, B. S. Park, and J. S. Kim, "Potential mechanisms underlying the role of chronic inflammation in age-related muscle wasting," *Aging Clinical and Experimental Research*, vol. 24, pp. 412–422, 2012.

[43] S. L. Budui, A. P. Rossi, and M. Zamboni, "The pathogenetic bases of sarcopenia," *Clinical Cases in Mineral and Bone Metabolism*, vol. 12, pp. 22–26, 2015.

[44] D. Di Raimondo, A. Tuttolomondo, G. Musiari, C. Schimmenti, A. D'Angelo, and A. Pinto, "Are the myokines the mediators of physical activity-induced health benefits?,"

Current Pharmaceutical Design, vol. 22, no. 24, pp. 3622–3647, 2016.

[45] F. Zaldivar, J. Wang-Rodriguez, D. Nemet et al., "Constitutive pro- and anti-inflammatory cytokine and growth factor response to exercise in leukocytes," *Journal of Applied Physiology*, vol. 100, pp. 1124–1133, 2006.

[46] C. P. Fischer, "Interleukin-6 in acute exercise and training: what is the biological relevance?," *Exercise Immunology Review*, vol. 12, pp. 6–33, 2006.

[47] F. De Santa, L. Vitiello, A. Torcinaro, and E. Ferraro, "The role of metabolic remodeling in macrophage polarization and its effect on skeletal muscle regeneration," *Antioxidants & Redox Signaling*, vol. 30, no. 12, pp. 1553–1598, 2019.

[48] B. Chazaud, "Inflammation and skeletal muscle regeneration: leave it to the macrophages!," *Trends in Immunology*, vol. 41, no. 6, pp. 481–492, 2020.

[49] P. T. Reidy, E. E. Dupont-Versteegden, and M. J. Drummond, "Macrophage regulation of muscle regrowth from disuse in aging," *Exercise and Sport Sciences Reviews*, vol. 47, no. 4, pp. 246–250, 2019.

[50] C. Li, M. M. Xu, K. Wang, A. J. Adler, A. T. Vella, and B. Zhou, "Macrophage polarization and meta-inflammation," *Translational Research*, vol. 191, pp. 29–44, 2018.

[51] P. T. Reidy, A. I. McKenzie, Z. S. Mahmassani et al., "Aging impairs mouse skeletal muscle macrophage polarization and muscle-specific abundance during recovery from disuse," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 317, no. 1, pp. E85–e98, 2019.

[52] Z. Han, H. Zhao, Z. Tao et al., "TOPK promotes microglia/macrophage polarization towards M2 phenotype via inhibition of HDAC1 and HDAC2 activity after transient cerebral ischemia," *Aging and Disease*, vol. 9, no. 2, pp. 235–248, 2018.

[53] L. S. Silveira, B. M. M. Antunes, A. L. Minari, R. V. T. dos Santos, J. C. Neto, and F. S. Lira, "Macrophage polarization: implications on metabolic diseases and the role of exercise," *Critical Reviews in Eukaryotic Gene Expression*, vol. 26, no. 2, pp. 115–132, 2016.

[54] E. Ferraro, A. M. Giammarioli, S. Chiandotto, I. Spoletini, and G. Rosano, "Exercise-induced skeletal muscle remodeling and metabolic adaptation: redox signaling and role of autophagy," *Antioxidants & Redox Signaling*, vol. 21, no. 1, pp. 154–176, 2014.

[55] L. A. Perandini, P. Chimin, D. D. S. Lutkemeyer, and N. O. S. Câmara, "Chronic inflammation in skeletal muscle impairs satellite cells function during regeneration: can physical exercise restore the satellite cell niche?," *The FEBS Journal*, vol. 285, no. 11, pp. 1973–1984, 2018.

[56] Y. Zaychik, N. Fainstein, O. Touloumi et al., "High-intensity exercise training protects the brain against autoimmune neuroinflammation: regulation of microglial redox and pro-inflammatory functions," *Frontiers in Cellular Neuroscience*, vol. 15, article 640724, 2021.

[57] A. L. Minari, L. M. Oyama, and R. V. T. dos Santos, "Downhill exercise-induced changes in gene expression related with macrophage polarization and myogenic cells in the triceps long head of rats," *Inflammation*, vol. 38, no. 1, pp. 209–217, 2015.

[58] M. J. Jiménez-Dalmaroni, M. E. Gerswhin, and I. E. Adamopoulos, "The critical role of toll-like receptors - From microbial recognition to autoimmunity: A comprehensive review," *Autoimmunity Reviews*, vol. 15, no. 1, pp. 1–8, 2016.

[59] S. Lattanzi, D. Norata, A. A. Divani et al., "Systemic inflammatory response index and futile recanalization in patients with ischemic stroke undergoing endovascular treatment," *Brain Sciences*, vol. 11, no. 9, 2021.

Hindawi
Mediators of Inflammation
Volume 2021, Article ID 8906561, 16 pages
https://doi.org/10.1155/2021/8906561

Research Article

Cooling and Sterile Inflammation in an Oxygen-Glucose-Deprivation/Reperfusion Injury Model in BV-2 Microglia

Jana Lücht,[1,2] **Nele Rolfs**,[1] **Sylvia J. Wowro**,[3] **Felix Berger**,[1,3] **Katharina R. L. Schmitt**,[1,3] **and Giang Tong**[1]

[1]*German Heart Centre Berlin, Department of Congenital Heart Disease/Pediatric Cardiology, Berlin, Germany*
[2]*Berlin Institute of Health, Berlin, Germany*
[3]*Charité-Universitätsmedizin Berlin, Department of Congenital Heart Disease/Pediatric Cardiology, Berlin, Germany*

Correspondence should be addressed to Katharina R. L. Schmitt; katharina.schmitt@charite.de

Received 2 September 2021; Accepted 2 October 2021; Published 5 November 2021

Academic Editor: Piergiorgio Lochner

Objective. Cold-inducible RNA-binding protein (CIRBP) has been shown to be involved not only in cooling-induced cellular protection but also as a mediator of sterile inflammation, a critical mechanism of the innate immune response in ischemia/reperfusion (I/R) injury. The role of microglia and its activation in cerebral I/R injury warrants further investigation as both detrimental and regenerative properties have been described. Therefore, we investigated the effects of cooling, specifically viability, activation, and release of damage associated molecular patterns (DAMPs) on oxygen glucose deprivation/reperfusion- (OGD/R-) induced injury in murine BV-2 microglial cells. *Methods*. Murine BV-2 microglial cells were exposed to 2 to 6 h OGD (0.2% O_2 in glucose- and serum-free medium) followed by up to 19 h of reperfusion, simulated by restoration of oxygen (21% O_2) and nutrients. Cells were maintained at either normothermia (37°C) or cooled to 33.5°C, 1 h after experimental start. Cultured supernatants were harvested after exposure to OGD for analysis of DAMP secretions, including high-mobility group box 1 (HMGB1), heat shock protein 70 (HSP70), and CIRBP, and cytotoxicity was assessed by lactate dehydrogenase releases after exposure to OGD and reperfusion. Intracellular cold-shock proteins CIRBP and RNA-binding motif 3 (RBM3) as well as caspases 9, 8, and 3 were also analyzed via Western blot analysis. Furthermore, inducible nitric oxide synthase (iNOS), ionized calcium-binding adaptor molecule 1 (Iba1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), interleukin-1α (IL-1α), monocyte chemotactic protein 1 (MCP-1), transforming growth factor β (TGFβ), CIRBP, and RBM3 gene expressions were assessed via reverse transcription polymerase chain reaction, and TNF-α, IL-6, and IL-1β releases into the cultured supernatants were assessed via enzyme-linked immunosorbent assays (ELISA). *Results*. Prolonged exposure to OGD resulted in increased BV-2 necrotic cell death, which was attenuated by cooling. Cooling also significantly induced cold-shock proteins CIRBP and RBM3 gene expressions, with CIRBP expression more rapidly regulated than RBM3 and translatable to significantly increased protein expression. DAMPs including HMGB-1, HSP70, and CIRBP could be detected in cultured supernatants after 6 h of OGD with CIRBP release being significantly attenuated by cooling. Exposure to OGD suppressed cytokine gene expressions of IL-1β, TNF-α, MCP-1, and TGFβ independently of temperature management, whereas cooling led to a significant increase in IL-1α gene expression after 6 h of OGD. In the reperfusion phase, TNF-α and MCP-1 gene expressions were increased, and cooling was associated with significantly lower TGFβ gene expression. Interestingly, cooled Normoxia groups had significant upregulations of microglial activation marker, Iba1, IL-1β, and TNF-α gene expressions. *Conclusion*. BV-2 microglial cells undergo necrotic cell death resulting in DAMP release due to OGD/R-induced injury. Cooling conveyed neuroprotection in OGD/R-injury as observable in increased cell viability as well as induced gene expressions of cold shock proteins. As cooling alone resulted in both upregulation of microglial activation, expression of proinflammatory cytokines, and cold shock protein transcript and protein expression, temperature management might have ambiguous effects in sterile inflammation. However, cooling resulted in a significant decrease of extracellular CIRBP, which has recently been characterized as a novel DAMP and a potent initiator and mediator of inflammation.

1. Introduction

Ischemic brain injury resulting from a deprivation of oxygen and nutrients initiates multiple damage mechanisms including sterile inflammation. Due to necrotic cell death, damage-associated molecular patterns (DAMPs) are released into the extracellular matrix, which can activate microglial cells by binding to respective pattern recognition receptors, e.g., toll-like receptors (TLR) [1]. It has been shown that activation and proliferation of microglial cells in the ischemic region of the brain occur during the first 3 days after cerebral ischemia [2, 3]. As resident immune cells of the brain, microglial cells control the inflammatory process via release of cytokines and matrix metalloproteinases, leading to further recruitment of microglial cells and peripheral leukocyte and monocyte immigration due to a weakened blood brain barrier [4]. In addition to initiating sterile inflammation, microglial cells also clear cellular detritus via phagocytosis and contribute to neuronal regeneration after ischemia/reperfusion (I/R) injury [5]. Several studies have shown that microglial cell activation leads to a detrimental effect in I/R injury [6–8], whilst others demonstrate a neuroprotective effect [9–11]. Therefore, the role of microglial cells in I/R injury warrants further investigation.

Cooling or targeted temperature management (TTM) is an established neuroprotective strategy for I/R injury in the brain routinely applied in neonates after perinatal hypoxic-ischemic encephalopathy and in adults after cardiac arrest [12–15]. A meta-analysis of *in vivo* studies analyzing the effect of hypothermia on focal cerebral ischemia showed that cooling decreased infarct size significantly by 44% and resulted in improved functional outcome. Reduction of infarct size was dependent on time of initiation and degree of cooling. Although cooling to ≤31°C initiated before or at the beginning of ischemia showed the most effective reduction of infarct size, mild cooling to 35°C was also shown to be beneficial [16]. Moreover, a recent meta-analysis focusing on preclinical studies from 2010 to 2015 confirmed the previously described effects of cooling and suggested endovascular cooling as a neuroprotective method even if initiated during ischemia [17]. In clinical trials, intravascular cooling administered by cold saline infusions has been shown to be feasible in patients suffering from ischemic stroke [18, 19]. However, in the intravascular cooling in the treatment of stroke 2 (ICTuS 2) trial, patient recruitment was stopped as thrombectomy, proven to be an efficient treatment for the selected group of patients, and was not included as a treatment. Due to small sample sizes, no statistically significant differences were reported. Yet, patients treated with intravascular cooling presented increased incidence of pneumonia [19]. This is consistent with the results of another randomized multicenter trial analyzing mild cooling (34.0–35.0°C) for 12–24 h, achieved either by intravenous infusion or surface cooling within 6 h of symptom onset to 90 minutes within the start of thrombolysis. Unfortunately, patients' outcomes could not be analyzed due to lack of fundings [20].

Cooling has been shown to be involved in multiple protective mechanisms in the setting of ischemic stroke. Besides attenuating excitotoxicity, calcium influx, oxidative stress, and neuronal apoptosis, there is growing evidence that cooling also reduces the inflammatory response after I/R injury in the brain [21]. Although a well-established protective mechanism lies within the general reduction of metabolism, cold shock proteins such as cold-inducible RNA binding protein (CIRBP) and RNA-binding motif 3 (RBM3) are upregulated under cooling and convey neuroprotection [22–24]. However, extracellular CIRBP has been shown to act as a DAMP and a potent modulator of inflammation. In addition to being released upon necrotic cell death, CIRBP can also be secreted upon, e.g., hypoxia. Administration of recombinant CIRBP resulted both *in vitro* and *in vivo* in increased levels of proinflammatory cytokines and the release of other DAMPs [25]. Moreover, patients suffering from hemorrhagic shock and sepsis showed increased peripheral blood levels of CIRBP, which correlated with poor outcome [25, 26]. Concordantly to other DAMPs such as high-mobility group box 1 (HMGB1), CIRBP binds to the toll-like receptor 4 (TLR4) and myeloid differentiation factor 2 (MD2) complex to initiate inflammation [25]. Furthermore, recent *in vitro* studies show that CIRBP also binds to triggering receptor expressed on myeloid cells-1 (TREM-1) and interleukin 6 receptor (IL-6 R) to activate another pathway of proinflammatory response or promote macrophage endotoxin tolerance [27, 28]. Thus, recent experimental and clinical data underline that extracellular CIRBP is a potent initiator and modulator of inflammation.

The aim of this study is to investigate the effects of cooling initiated during oxygen glucose deprivation (OGD) as a potential neuroprotective strategy in murine BV-2 microglial cells. Therefore, we analyzed the impact of cooling on OGD-induced necrotic cell death, DAMPs release, and cytokines as well as cold-shock protein expressions.

2. Materials and Methods

2.1. Cell Culture. Immortalized murine BV-2 microglial cells [29] were kindly provided by Prof. Ullrich (Zurich, Switzerland). BV-2 cells were cultured as previously described in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% natrium-pyruvate (Biochrom), 10% heat inactivated fetal bovine serum (FBS, Biochrom), and incubated at 37°C, 21% O_2, and 5% CO_2 [30]. Both cultivating media as well as experimental media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Merck Milipore).

2.2. Simulation of Ischemia/Reperfusion Injury. As previously described by our group, deprivation of oxygen and glucose was used to simulate ischemia. Briefly, cells were incubated in glucose/serum-free DMEM at 0.2% O_2 and 5% CO_2 in a CO_2 incubator (Binder, Tuttlingen, Germany). Reperfusion was simulated by restoration of nutrients and incubation at 21% O_2. Control groups were kept in DMEM supplemented with glucose and 10% FBS for the duration of the experiment.

2.3. Time-Temperature Protocol. Prior to experimental start, 500,000 cells were seeded in a 21 cm^2 petri dish (Sarstedt)

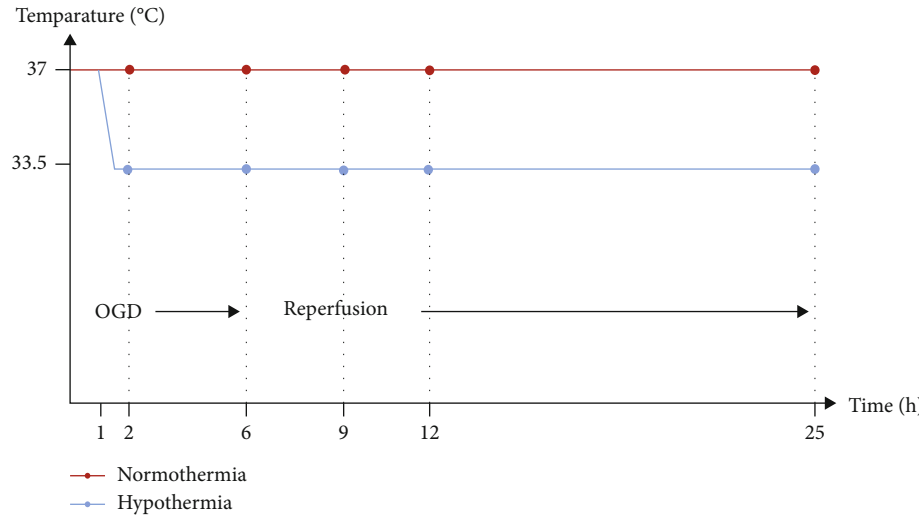


Figure 1: Time-temperature protocol. Cells were exposed to 2 or 6 h of oxygen-glucose deprivation (OGD, 0.2% O_2 in glucose/serum-free medium) followed by up to 19 h of reperfusion (21% O_2 in medium containing glucose and serum) and incubated at 37°C or cooled 1 h after experimental start to 33.5°C. Samples were collected directly after 2 or 6 h of OGD and after 3 h, 6 h, and 19 h of reperfusion, respectively.

and maintained for 24 h. Cells were exposed to OGD and reperfusion according to a time-temperature protocol as illustrated in Figure 1. Experimental groups were exposed to OGD for 2 or 6 h, followed by up to 19 h of reperfusion. Cooling (33.5°C) was initiated after 1 h of OGD and continued until the end of the experiment. Control groups were incubated at 37°C or 33.5°C, 21% O_2, and 5% CO_2 for the duration of the experiment.

2.4. Assessment of Necrotic Cell Death via Lactat Dehydrogenase (LDH) Assay. Necrotic cell death was analyzed via LDH release into cultured supernatants at each experimental timepoints using a colorimetric Cytotoxicity Detection Kit (Roche Diagnostics) according to manufacturer's instructions. The extinction was measured at 490 nm minus 630 nm using a microtiter plate reader (Thermo Fisher Multiskan Ascent). Cytotoxicity is presented as a percentage in relation to maximum LDH content assessed by a lysed normoxic control group as previously described by our research group [30].

2.5. Protein Isolation and Western Blot Analysis. At each experimental time point, cells were harvested, and supernatants were collected for the isolation of intracellular and extracellular proteins, respectively. For intracellular protein analysis, cells were centrifuged at 6,000 x g for 10 minutes, and cell pellets were lysed in radio-immuno precipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (1 : 100, Sigma-Aldrich). Protein concentration was assessed via Pierce Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific). Extracellular proteins were precipitated using a trichloroacetic acid protocol as previously described [30]. Both intra- and extracellular protein samples were incubated with Pierce Lane Marker Reducing Sample Buffer (Thermo Scientific) at 95°C for 5 min and loaded onto a 15% sodium dodecyl sulfate (SDS) polyacrylamide gel for electrophoresis. Afterwards, proteins were transferred onto a polyvinylidene fluoride membrane (PALL Life Sciences) overnight at 30 V using a tank blotting procedure (Bio-Rad Laboratories). Membranes were then blocked for 1 h at room temperature with 5% BSA (Carl Roth) for β-Actin, Caspase 1, Caspase 8, Heat shock protein 70 (Hsp70), and HMGB1 or 5% dry milk (Applied Biosystems) for CIRBP, RBM3, Caspase 3, and Caspase 9 in TBS +0.1% Tween 20. Primary antibodies for β-Actin (1 : 20000, Cell Signaling, Cat#4967), Caspase 1 (1 : 1000, Cell Signaling, Cat#67314), Caspase 8 (1 : 1000, Cell Signaling, Cat#9429), Caspase 9 (1 : 1000, Cell Signaling, Cat#9508), Caspase 3 (1 : 1000, Cell Signaling, Cat#9662), Hsp70 (1 : 1000, Cell Signaling Technology, Cat#4872) HMGB1 (1 : 2000, Chondrex, Cat#7028), CIRBP (1 : 1000, Abclonal, Cat#A6080), and RBM3 (1 : 1000, Proteintech, Cat#14363-1-AP) were diluted in blocking solution and incubated overnight at 4°C. Secondary antibodies (anti-rabbit IgG-HRP, Dianova) were incubated for 1 h at room temperature (1 : 10,000 for CIRBP and HMGB1, otherwise 1 : 20,000). Dura Super Signal West (Thermo Fisher Scientific) was used to visualize protein expression, captured using a ChemiDoc™ Imaging Systems, and Image Lab™ Software (Bio-Rad) was used for densitometry analysis.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). Secreted tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), TNF-α, IL-6, and IL-1β concentrations in cultured supernatants were analyzed at 6 h OGD and 12 h OGD and reperfusion (OGD/R) via ELISA (DuoSet Mouse, R&D Systems) in 96-well plates prepared according to manufacturer's instructions. Briefly, captured antibody-precoated plates were incubated with blocking solution for 1 h at room temperature, supernatants were collected and transferred in duplicates onto a 96-well-plate and incubated overnight at 4°C, and detection antibodies were transferred at a dilution 1 : 60 and incubated for 3 h, followed by light-protected incubation with HRP-conjugated streptavidin for 20 minutes at

Table 1: List of RT-qPCR genes and assay IDs.

Gene	Assay ID
CIRBP	00483336_g1
GAPDH	99999915_g1
Iba1	00479862_g1
IL-1α	00439620_m1
IL-1β	00434228_m1
IL-6	00446190_m1
iNOS (iNOS-2)	00440502_m1
MCP-1 (Ccl-2)	00441242_m1
RBM3	01609819_g1
TGFβ	01178820_m1
TNF-α	00443260_g1

room temperature. Extinction was measured at 450 nm and 540 nm using a microtiter plate reader (Thermo Fisher Multiskan Ascent).

2.7. RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA from BV-2 cells was isolated via acidic phenol/chloroform extraction using RNA Pure™ (Peqlab) followed by DNA digestion using a Turbo DNA-free™ Kit (Ambion) according to manufacturer's instructions. RNA concentration and purity were assessed by spectrophotometric measurements at 260 nm and 280 nm with a Nanodrop 2000 (Nanodrop) and agarose gel electrophoresis. Reverse transcription was performed using 1 μg total RNA via a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a thermal cycler (PTC200, MJ Research) according to manufacturer's instructions. Expression of target genes and GAPDH as reference keeping control was analyzed by real-time qPCR using the TaqMan Gene Expression Assays (summarized in Table 1) and StepOnePlus™ Real-Time PCR System (Applied Biosystems) according to manufacturer's recommendations. We assessed relative quantification of gene expression normalized to glycerinaldehyd-3-phosphat dehydrogenase (GAPDH) as reference gene via the $\Delta\Delta - C_t$ method, and results are depicted as fold changes [31].

2.8. Statistical Analysis. Data was analyzed and illustrated using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA). Groups were compared using one-way analysis of variance (ANOVA) with Tukey posttest. Data from at least 3 independent experiments are presented as mean ± standard deviation (SD), and p values < 0.05 were considered significant.

3. Results

3.1. Necrotic Cell Death. As necrotic cell death due to ischemic brain injury leads to the release of DAMPs and the initiation of sterile inflammation, we compared 2 h versus 6 h duration of OGD in order to establish a time-temperature protocol for induced injury in the BV-2 microglia. Cytotoxicity was assessed via LDH release in the cultured supernatants as illustrated in Figure 2. Exposure to OGD at 37°C for 2 h did not result in significant BV-2 cytotoxicity, whereas 6 h of OGD led to a significant increase in LDH release relative to Normoxia 37°C control. Cooling to 33.5°C initiated after 1 h OGD effectively attenuated BV-2 cell death at the end of the 6 h OGD phase. Restoration of nutrients and oxygen to 21% in the simulated reperfusion phase did not result in further increased cell death. In fact, % cytotoxicity was significantly lower at all investigated reperfusion time points (9, 12, and 25 h) relative to 6 h OGD at 37°C and also not significantly higher than Normoxia 37°C control. Maintenance of cooling at 33.5°C during the reperfusion phase resulted in lower observable cytotoxicity, but did not reach significancy.

3.2. Apoptotic Cell Death. To fully understand the effect of exposure of BV-2 microglia to OGD/R and cooling on programmed apoptotic cell death, we investigated both the intrinsic and extrinsic apoptotic pathways by assessing activation of initiator caspases 9 and caspase 8, as well as their common effector caspase 3 (Figure 3). Activation of caspase 9, the initiator caspase of intrinsic apoptosis, was highly observable after 6 h of OGD in both cooled and normothermic cells but not significant relative to Normoxia 37°C control. Furthermore, activation of caspase 9 steadily decreased after reperfusion (Figure 3(a)). Extrinsic initiator caspase 8 was significantly activated after reperfusion (9 h after experimental start) in cells subjected to cooling and OGD/R relative to Normoxia 37°C control, as well as to Normoxia group cooled to 33.5°C (Figure 3(b)). In consistency with the observable activation of the initiator caspases, effector caspase 3 was also observed to be activated both during OGD and early reperfusion (9 h), though not to significancy and no influence by temperature management was observed (Figure 3(c)).

3.3. Oxidative Stress and Microglial Activity. We analyzed inducible nitric oxide synthase (iNOS) gene expression as an indicator for oxidative stress. iNOS gene expression was significantly increased after 6 h of OGD in both cooled and normothermic groups. iNOS gene expression was significantly higher after 6 h exposure to OGD relative to Normoxia 37°C control and decreased significantly after reperfusion (12 h and 25 h) as compared to 6 h OGD (Figure 4(a)). Cooling had no significant effect on iNOS transcript during exposure to OGD/R. Ionized calcium-binding adaptor molecule 1 (Iba1) is specifically expressed in microglial cells and is commonly used as a marker for microglia activation [32]. Exposure to OGD/R significantly suppressed Iba1 gene expression in comparison to Normoxia 37°C control, and cooling had no observable effect (Figure 4(b)). However, cooling to 33.5°C under normoxic conditions led to significant upregulation of Iba1 transcripts (6 h and 12 h) relative to normothermic Normoxia control as well as OGD/R groups.

3.4. Cold Shock Proteins. A family of cold shock protein has been observed to be induced under hypothermia and other stress conditions. Therefore, we analyzed both gene expression as well as intracellular protein expression of cold shock

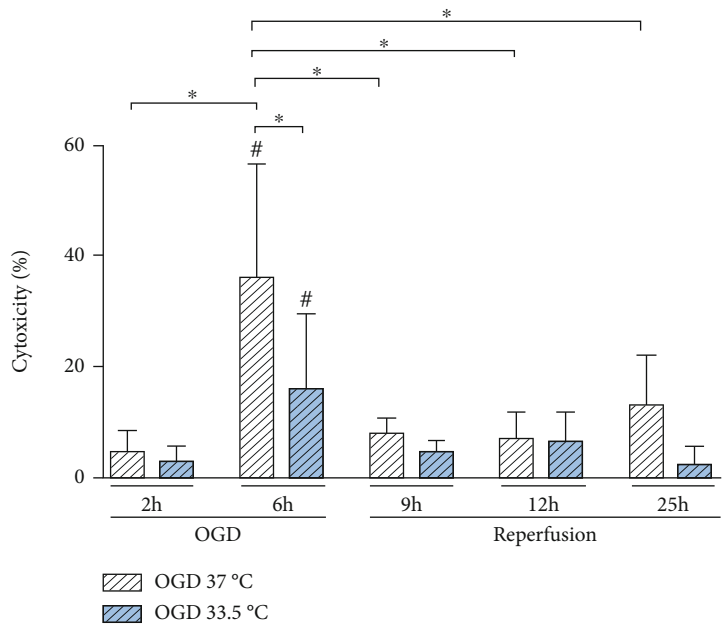


Figure 2: Necrotic cell death measured by LDH release, depicted as % cytotoxicity relative to positive (100%) and Normoxia (0%) controls. Cells were exposed to 2 and 6 h of oxygen-glucose deprivation (OGD, 0.2% O_2 in glucose/serum-free medium) followed up to 19 h of reperfusion (21% O_2 in medium containing glucose and serum) and incubated at 37°C or cooled 1 h after experimental start to 33.5°C. Data from at least 3 individual experiments presented as mean ± SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey posttest; $^{*}p < 0.05$ for group comparison and $^{\#}p < 0.05$ for comparison to Normoxia 37°C were considered significant.

proteins RBM3 and CIRBP. CIRBP transcript was significantly upregulated by cooling in all experimental groups, regardless of exposure to Normoxia or OGD/R, at all observable time points (Figure 5(a)). Cooling-induced RBM3 gene expression kinetics was observably slower than that of CIRBP and reached significancy after 12 h in the Normoxia group and 25 h in the OGD/R group (Figure 5(b)). Moreover, cooling-induced RBM3 expression significantly increased with prolonged duration of cooling, observable by the significantly highest expression at 25 h in both Normoxia and OGD/R conditions. Interestingly, intracellular CIRBP protein expression was not as dynamically induced under cooling and was only significantly higher than Normoxia 37°C control after 25 h cooling under normoxic condition (Figure 6(a)). We observed a tendency towards higher RBM3 protein expression under cooling for both Normoxia and OGD/R treatment conditions, which did not reach significancy (Figure 6(b)).

3.5. Release of DAMPs and Cold-Shock Proteins. Necrotic cell death has been observed to lead to the release of DAMPs, which can initiate the innate inflammatory response. Therefore, we investigated the release of HMGB-1, HSP70, and CIRBP into cultured supernatants after 2 and 6 h of OGD to evaluate the contribution of microglia to DAMPs release. We observed the greatest release of HMGB1 after 2 h exposure to OGD, which did not reach significancy (Figure 7(a)). HSP70 release was significantly higher from cells subjected to OGD for 2 h than Normoxia 37°C control and OGD exposure for 6 h (Figure 7(b)). Neither CIRBP nor RBM3 were detectable in the culture supernatants after 2 h of OGD. However, CIRBP release was significantly higher after 6 h of OGD relative to Normoxia 37°C control and could be attenuated by cooling (Figure 7(c)). RBM3 release showed a higher tendency after 6 h exposure to OGD but did not reach significancy (Figure 7(d)).

3.6. Cytokine Gene Expressions and Releases. In order to assess the inflammatory response in BV-2 microglia, we analyzed a panel of cytokine gene expressions, including TNF-α, IL-6, IL-1β, monocyte chemotactic protein 1 (MCP-1), transforming growth factor β (TGF-β), and interleukin-1α (IL-1α) (Figures 8(a)–8(f), respectively). Interestingly, exposure to 6 h OGD generally resulted in suppressed cytokine expressions with observable significancy in IL-1β and TGF-β expressions (Figures 8(c) and 8(e)). Only IL-6 expression was observed to be unaffected by OGD and even significantly higher expressed relative to Normoxia 37°C control in the 6 h cooled OGD group (Figure 8(b)). Cooling alone significantly induced IL-1β expression under normoxic conditions (Figure 8(c)). Both TNF-α and MCP-1 showed a similar expression pattern to IL-1β with a tendency towards suppression by OGD. However, TNF-α gene expression was significantly induced in all investigated groups after 25 h, and MCP-1 was significantly upregulated in cooled cells relative to Normoxia 37°C control after reperfusion (25 h OGD/R), in contrast to IL-1β, which remained downregulated during reperfusion (Figures 8(a), 8(c), and

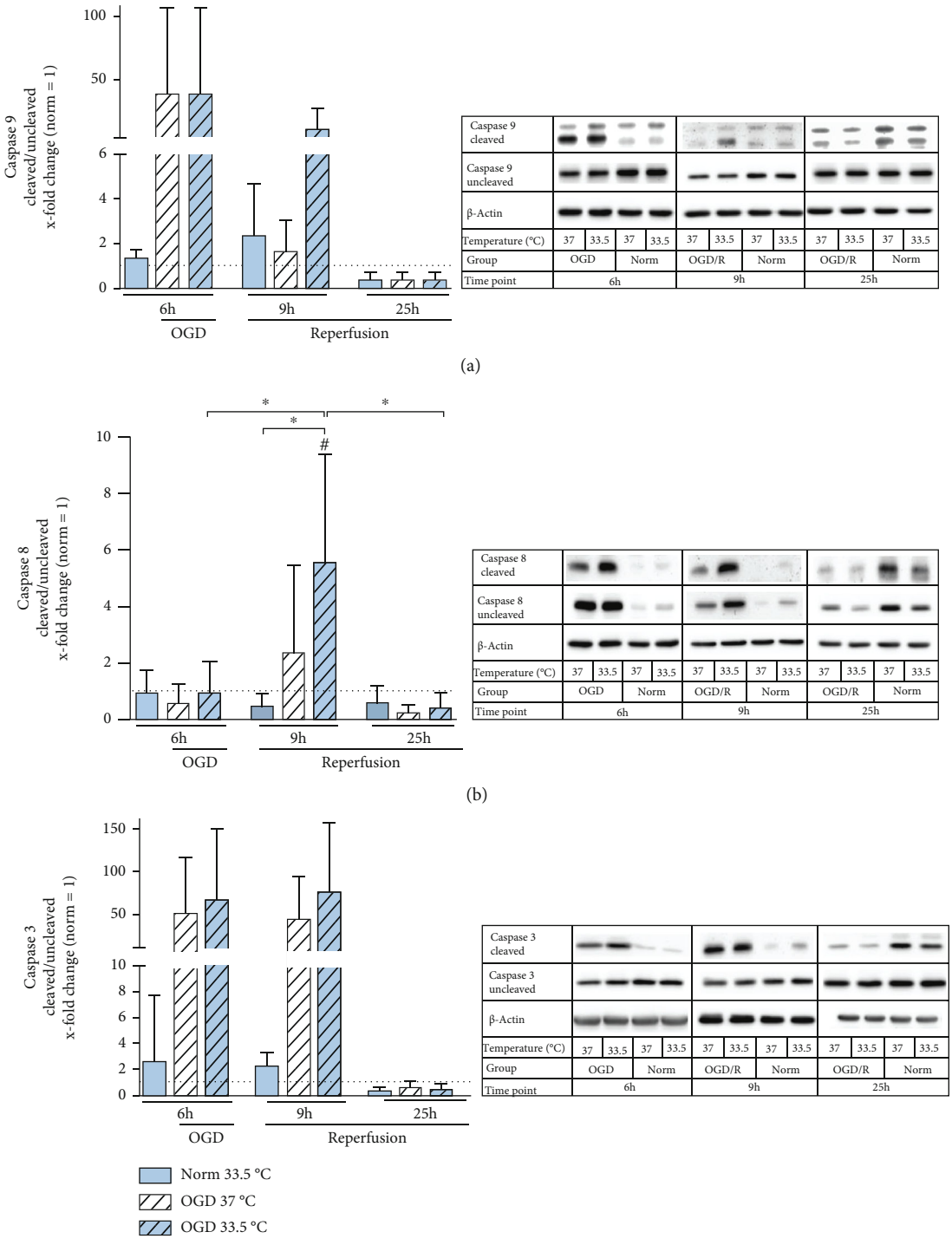


Figure 3: Western blot analysis of apoptotic cell death as assessed by cleavage of (a) caspase 9 as initiator of intrinsic apoptosis, (b) caspase 8 as initiator of extrinsic apoptosis, and (c) caspase 3 as effector caspase presented as x-fold change relative to Normoxia 37°C control and representative immunoblots at respective timepoints. Cells were exposed to 6 h of oxygen-glucose deprivation (OGD, 0.2% O_2 in glucose/serum-free medium) followed by 3 h and 19 h of reperfusion (21% O_2 in medium containing glucose and serum) and incubated at 37°C or cooled 1 h after experimental start to 33.5°C. Data from at least 3 individual experiments are presented as mean ± SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey posttest; $^{*}p < 0.05$ for group comparison and $^{\#}p < 0.05$ for comparison to Normoxia 37°C were considered significant. Panels show representative immunoblots.

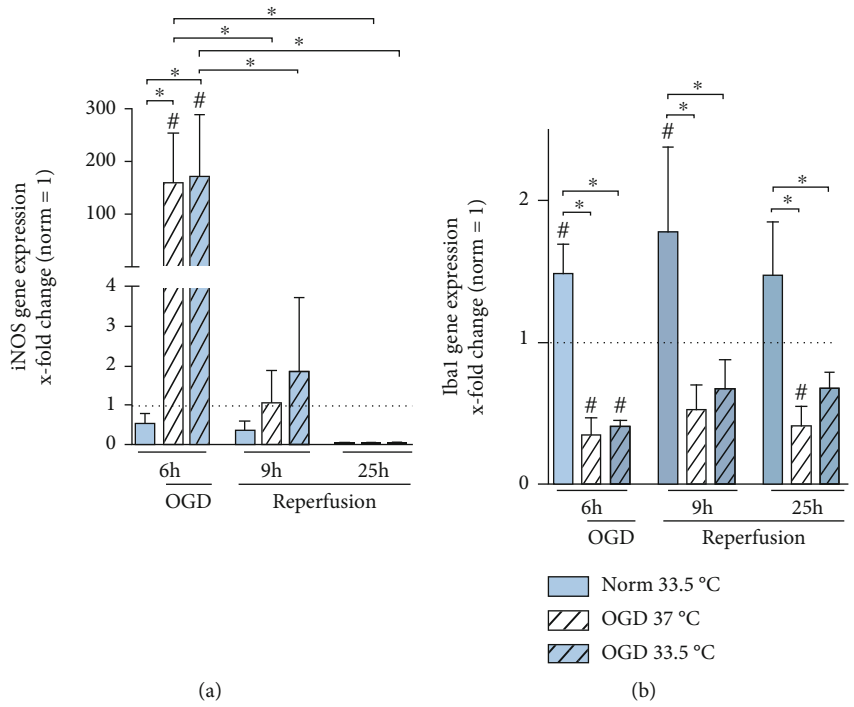


Figure 4: RT-qPCR analyzing (a) iNOS and (b) Iba1 gene expression presented as x-fold change relative to Normoxia 37°C control at respective timepoints. Cells were exposed to 6 h of oxygen-glucose deprivation (OGD, 0.2% O_2 in glucose/serum-free medium) followed by 6 h and 19 h of reperfusion (21% O_2 in medium containing glucose and serum) and incubated at 37°C or cooled 1 h after experimental start to 33.5°C. Data from at least 3 individual experiments are presented as mean ± SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey posttest; $^{*}p < 0.05$ for group comparison and $^{\#}p < 0.05$ for comparison to Normoxia 37°C were considered significant.

8(d)). IL-6 and IL-1α showed a similar expression pattern in the 6 h OGD phase, with IL-6 significantly upregulated relative to Normoxia 37°C control and IL-1α significantly upregulated relative to cooled Normoxia and noncooled OGD-treated groups. Similarly, we did not detect any significant regulation of IL-6 and IL-1α during reperfusion (Figures 8(b) and 8(f)). Furthermore, we analyzed anti-inflammatory TGF-β gene expressions, which was attenuated by OGD and remained suppressed after 12 h in cooled OGD/R group. Interestingly, TGF-β expression recovered after 25 h of OGD/R and was significantly increased in the uncooled cells treated with OGD/R relative to Normoxia 37°C control as well as both cooled Normoxia- and OGD/R-treated groups (Figure 8(e)).

Next, we analyzed the release of proinflammatory cytokines into supernatants via ELISA. BV-2 cell exposure to OGD did not result in a significant increase in TNF-α release (Figure 9). On the contrary, exposure to 6 h of OGD resulted in a significant decrease in TNF-α release relative to the cooled Normoxia-treated cells. Attenuation of TNF-α release by OGD continued into the reperfusion phase (12 h OGD/R) where both cooled and noncooled OGD/R-treated groups were significantly lower relative to both cooled and noncooled Normoxia-treated groups. Additionally, IL-6 and IL-1β were both nondetectable in the cultured supernatants for all experimental groups.

4. Discussion

Sterile inflammation is an important component of I/R injury in the brain where resident immune microglial cells are activated within the first hours after ischemia [2, 3]. Microglia have been shown to contribute to both inflammatory and regenerative responses after I/R injury, thus providing an interesting target for potential neuroprotective therapies [1].

Cooling during and after ischemic brain injury has been shown to influence microglial activation and cytokine release [33–36]. Therefore, the role of microglial activation in ischemic brain injury remains a prevailing research topic. While microglia activation has been observed *in vivo* to be associated with a significant decrease in neurogenesis after focal ischemia and their specific inhibition resulted in increased neurogenesis in the hippocampus, other studies have reported that the number of activated microglia negatively correlate with ischemic damage [2, 8]. Therefore, we investigated the effect of cooling as an established neuroprotective strategy and the role of sterile inflammation on OGD/R-induced injury in murine BV-2 microglial cells.

Our simulated I/R injury model resulted in significant increase in necrotic BV-2 cell death after 6 h exposure to OGD, which could be attenuated by cooling to 33.5°C (Figure 2). Moreover, we also observed increased apoptotic

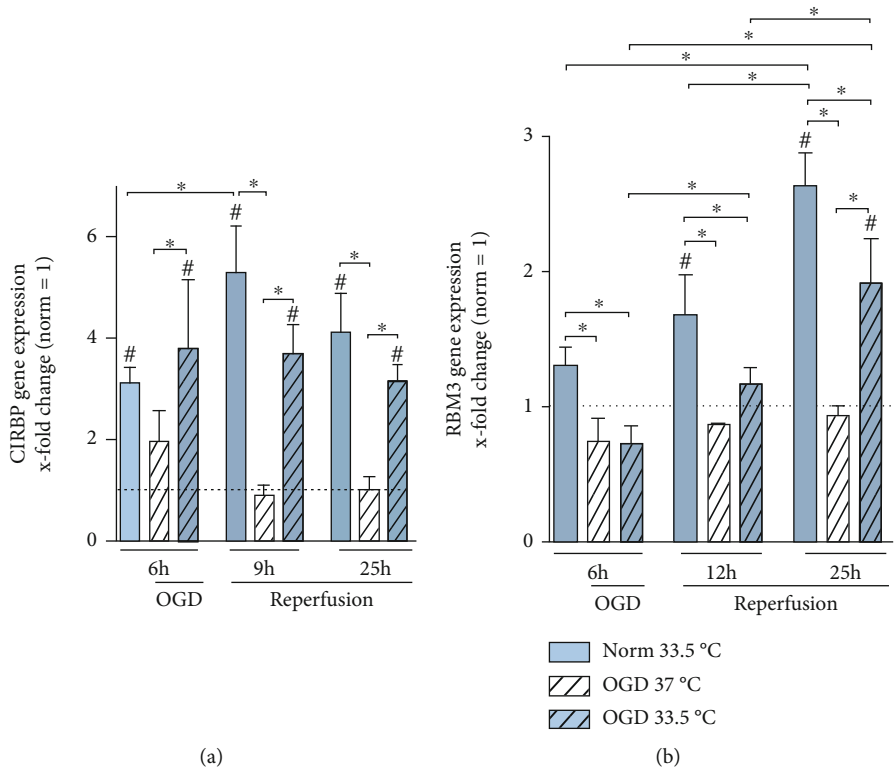


Figure 5: RT-qPCR analyzing cold shock proteins (a) CIRBP and (b) RBM3 gene expression presented as x-fold change relative to Normoxia 37°C control at respective timepoints. Cells were exposed to 6 h of oxygen-glucose deprivation (OGD, 0.2% O_2 in glucose/serum-free medium) followed by 6 h and 19 h of reperfusion (21% O_2 in medium containing glucose and serum) and incubated at 37°C or cooled 1 h after experimental start to 33.5°C. Data from at least 3 individual experiments are presented as mean ± SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey posttest; $^{*}p < 0.05$ for group comparison and $^{\#}p < 0.05$ for comparison to Normoxia 37°C were considered significant.

programmed cell death (Figure 3) as seen in caspase 3 activation in the microglia by exposure to OGD/R. Apoptosis during OGD was primarily via the intrinsic caspase 9 pathway, which was not influenced by temperature management, and via the extrinsic caspase 8 pathway during reperfusion. Interestingly, extrinsic caspase 8 activation was significantly upregulated in the cooled cells as compared to both control groups. We also observed a significant suppression in Iba1 expression in the microglia by exposure to OGD/R. Iba1 is an intracellular protein that is specifically expressed in microglial cells, and its upregulation has been used as a marker for microglial activation [32]. Furthermore, Iba1 has been shown to play an important role in phagocytosis [37, 38] and is upregulated *in vivo* by exposure to ischemia [39]. Selective hypothermia therapy in the brain, however, has been reported to attenuate microglial activation as seen in reduced Iba1 gene expression [40]. Contrary to these findings, Iba1 was significantly upregulated in our cooled Normoxia groups. However, we did observe significant decreases in Iba1 gene expression after 6 h of OGD that could not be restored by cooling (Figure 4(b)). Furthermore, 6 h of exposure to OGD resulted in significantly decreased cell viability, which may also influence the degree of observed microglial activation. Our finding corresponds with a previous *in vivo* study reporting that short durations of ischemia are associated with activation of local microglia, whereas longer duration of ischemia resulted in their degeneration [2].

Moreover, iNOS gene expression was significantly increased from exposure to OGD. Normally, iNOS is undetectable in resting microglia but is upregulated by ischemia, traumatic brain injury, or inflammation, which leads to NO production and oxidative stress [41–43]. iNOS has been shown to be detrimental in ischemic brain injury as experiments with iNOS knockout mice have shown significantly reduced infarct areas and pharmacological inhibition of iNOS results in less ischemic brain damage [44, 45]. Cooling to 33°C has been shown to reduce iNOS expression and NO production in both an *in vivo* model of focal brain ischemia and a neuroinflammatory model induced by lipopolysaccharide (LPS) injection [46]. Furthermore, cooling to 33.5°C has been reported to decrease iNOS gene and protein expression in LPS-stimulated BV-2 cells [47]. However, we did not observe any significant attenuation by cooling of the upregulated iNOS gene expression in the noncooled cells after 6 h of OGD, but we did observe significant decreases in iNOS in both groups after reperfusion (Figure 4(a)).

To further assess the effect of OGD/R on BV-2 microglia activation, we investigated the gene expressions of proinflammatory cytokines (IL-1α, IL-6, TNF-α, and IL-1β),

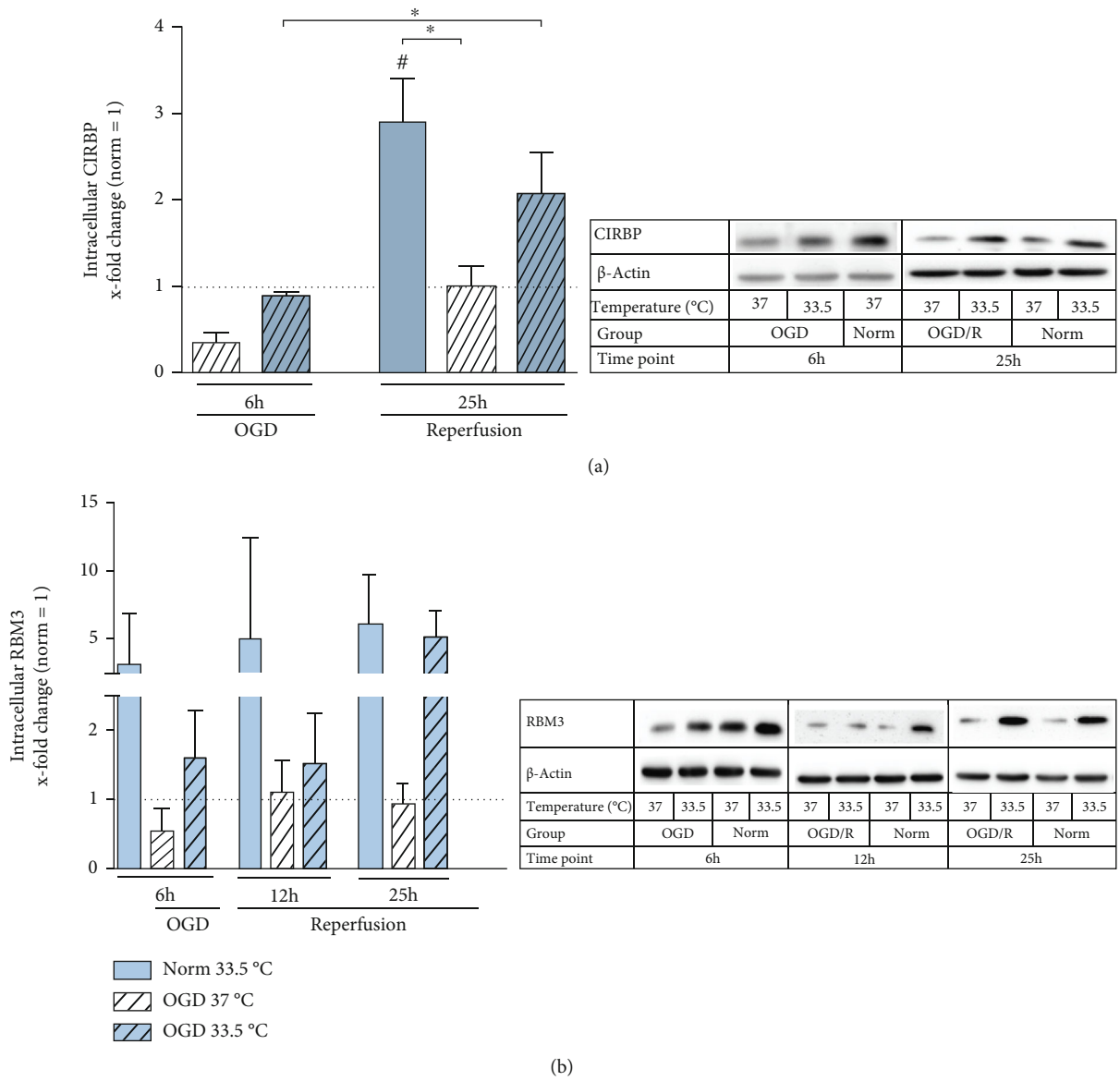
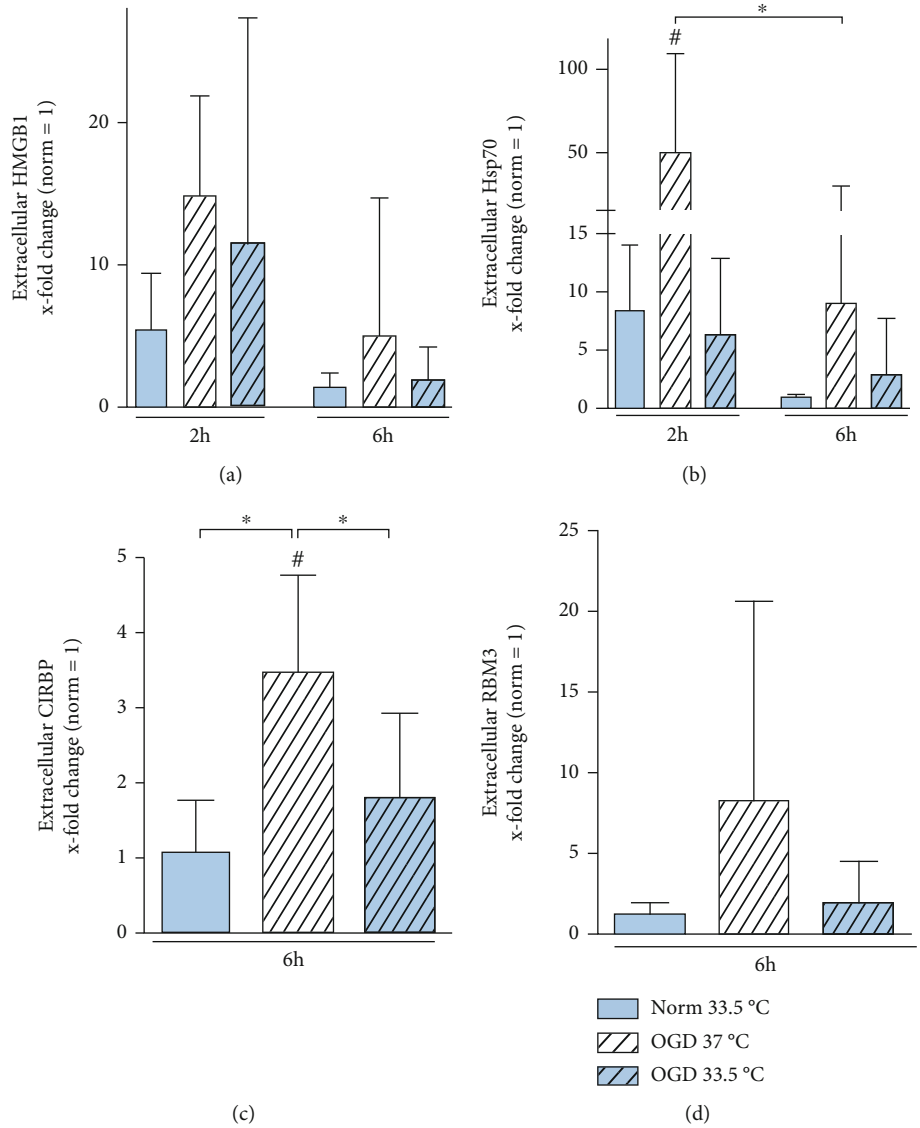


Figure 6: Western blot analysis analyzing intracellular cold shock proteins (a) CIRBP and (b) RBM3 presented as x-fold change relative to Normoxia 37°C control and representative immunoblots at respective timepoints. Cells were exposed to 6 h of oxygen-glucose deprivation (OGD, 0.2% O_2 in glucose/serum-free medium) followed by 6 h and 19 h of reperfusion (21% O_2 in medium containing glucose and serum) and incubated at 37°C or cooled 1 h after experimental start to 33.5°C. Data from at least 3 individual experiments are presented as mean ± SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey posttest; $^{*}p < 0.05$ for group comparison and $^{\#}p < 0.05$ for comparison to Normoxia 37°C were considered significant. Panels show representative immunoblots.

anti-inflammatory cytokine TGFβ, and chemotactic cytokine MCP-1. TNF-α, IL-1β, MCP-1, and TGFβ gene expressions were all significantly suppressed by exposure to OGD in the BV-2 microglia (Figures 8(a) and 8(c)–8(e)). Zhou et al. report an iNOS-dependent upregulation of TNF-α in BV-2 cells subjected to hypoxia [48]. As we detected decreased TNF-α gene expression after 6 h of OGD, no correlation between iNOS and TNF-α regulation could be established in our findings. Previous studies also show that cooling is accompanied by a reduction in TNF-α, IL-6, and IL-1β expressions in the setting of ischemic brain injury and neuroinflammation [33–36]. Seo et al. investigated the influence of cooling initiation time on cytokine expression and reported that TNF-α, IL-1β, iNOS, and IL-6 were all attenuated by cooling independent of time of initiation, but early initiation of cooling was most effective in reducing oxidative stress and transcription of proinflammatory cytokines [36]. Xiong et al. showed that postischemic hypothermia attenuates both TNF-α and IL-6 gene expressions but described a different expression kinetic where TNF-α peaked at 12 and 24 h and IL-6 peaked at 24 and 72 h after ischemia [35]. Since our experimental model investigated up to a



HMGB1								
Hsp70								
CIRBP	Non detectable							
RBM3	Non detectable							
Tempareture (°C)	37	33.5	37	33.5	37	33.5	37	33.5
Group	Norm		OGD		Norm		OGD	
Time point	2h				6h			

(e)

Figure 7: Western blot analysis analyzing extracellular proteins and DAMPs. (a) HMGB1, (b) Hsp70, (c) CIRBP, and (d) RBM3 presented as *x*-fold change relative to Normoxia 37°C control and (e) representative immunoblots at respective timepoints. Cells were exposed to 2 h and 6 h of oxygen-glucose deprivation (OGD, 0.2% O_2 in glucose/serum-free medium) and incubated at 37°C or cooled 1 h after experimental start to 33.5°C. Data from at least 3 individual experiments are presented as mean ± SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey posttest; $^{*}p < 0.05$ for group comparison and $^{\#}p < 0.05$ for comparison to Normoxia 37°C were considered significant. Panels show representative Immunoblots.

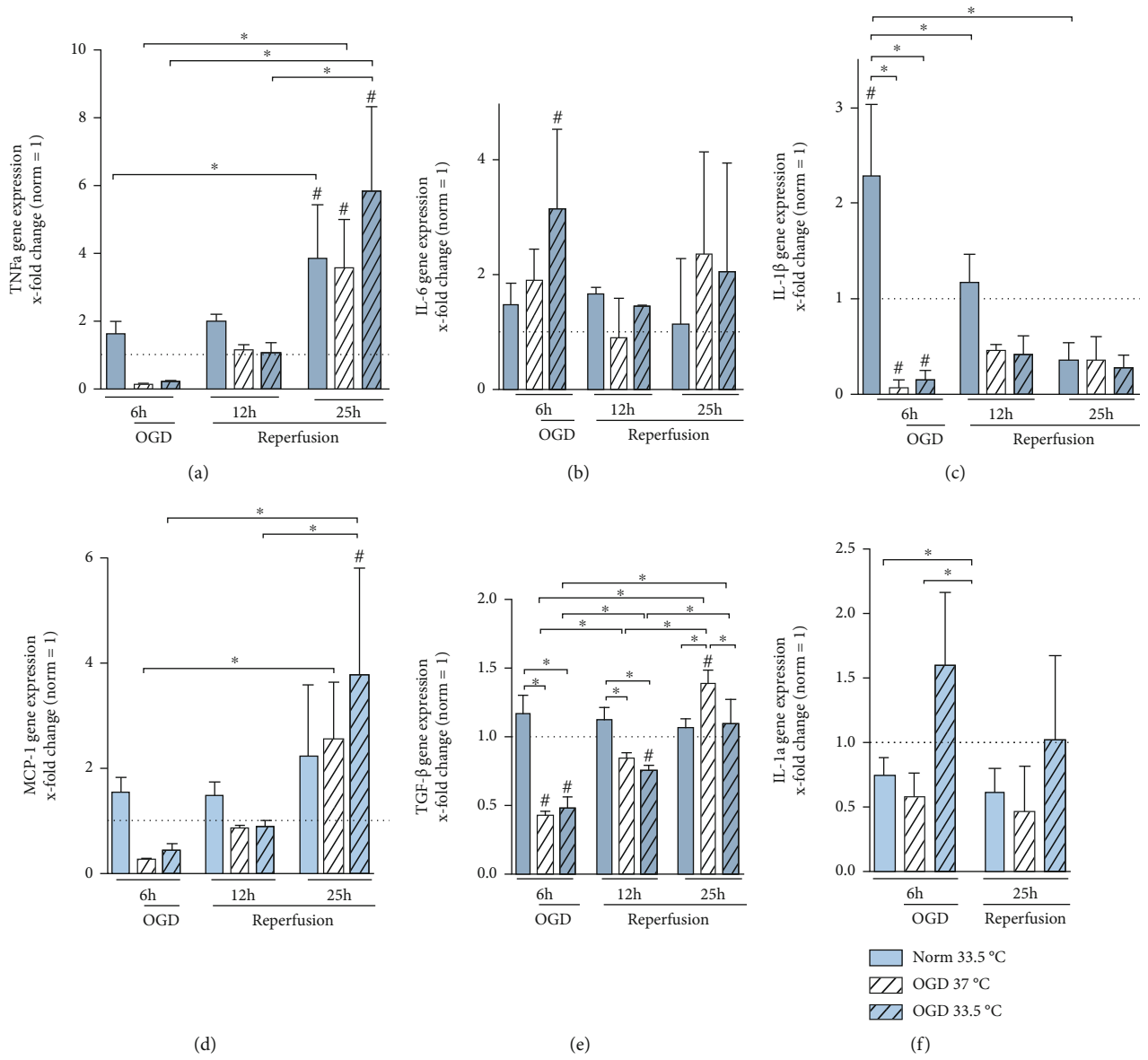


Figure 8: RT-qPCR analyzing cytokine gene expression. (a) TNF-α, (b) IL-6, (c) IL-1β, (d) MCP-1, (e) TGFβ, and (f) IL-1α presented as x -fold change relative to Normoxia 37°C control at respective timepoints. Cells were exposed to 6 h of oxygen-glucose deprivation (OGD, 0.2% O_2 in glucose/serum-free medium) followed by 6 h and 19 h of reperfusion (21% O_2 in medium containing glucose and serum) and incubated at 37°C or cooled 1 h after experimental start to 33.5°C. Data from at least 3 individual experiments are presented as mean ± SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey posttest; $^{*}p < 0.05$ for group comparison and $^{\#}p < 0.05$ for comparison to Normoxia 37°C were considered significant.

maximum of 19 h after reperfusion, a difference in expression kinetics and potential effects of cooling at later timepoints could not assessed.

IL-1α and IL-1β are well-established proinflammatory cytokines that have been shown to convey detrimental effects in I/R injury in the brain [49]. Interestingly, we detected differing gene expression kinetics due to OGD in combination with cooling, where IL-1α was increased and IL-1β was significantly downregulated by cooling after 6 h of OGD (Figures 8(c) and 8(f), respectively). IL-1α has been reported to be released following necrotic cell death, whereupon functioning as a DAMP depending on its subcellular localization. Since we only investigated IL-1α gene expression, its subcellular localization and potential role in as a DAMP in sterile inflammation warrant further investigation.

In contrast to the IL-1 cytokine family, TNF-α and IL-6 have been shown to convey both detrimental and neuroprotective effects [50–52]. In classic IL-6 signaling, binding to the IL-6 membrane bound receptor is considered protective, whereas binding to its soluble receptor is considered proinflammatory [50]. As we only analyzed IL-6 gene expression, no concrete conclusion on potential effects of IL-6 regulation

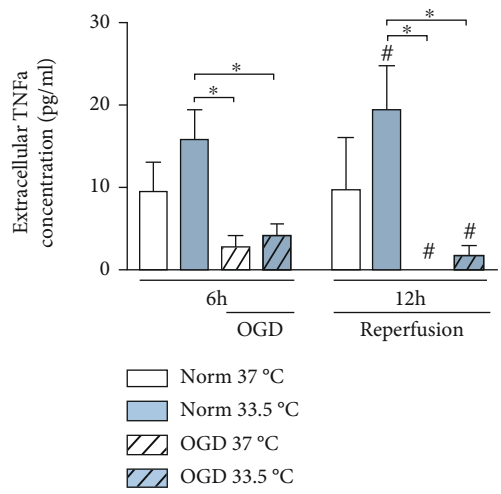


Figure 9: ELISA analysis of TNF-α release presented as absolute concentration. Cells were exposed to 6 h of oxygen-glucose deprivation (OGD, 0.2% O_2 in glucose/serum-free medium) followed by 6 h of reperfusion (21% O2 in medium containing glucose and serum). Cells were incubated at 37°C or cooled 1 h after experimental start to 33.5°C. Data from at least 3 individual experiments presented as mean ± SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey posttest; $^{*}p < 0.05$ for group comparison and $^{\#}p < 0.05$ for comparison to Normoxia 37°C were considered significant.

due to temperature management in OGD/R-injured BV-2 cells can be made. However, IL-6 gene expression in our model of OGD/R-injury in BV-2 microglial cells differs from the other analyzed cytokines as it shows a significant upregulation at 6 h of OGD due to cooling but otherwise no significant regulation due to OGD/R or temperature management (Figure 8(b)).

Additionally, we analyzed secretion levels of TNF-α, IL-6, and IL-1β by ELISAs. IL-6 and IL-1β were below assay detection limits; however, TNF-α secretion was downregulated relative to Normoxia control after exposure to OGD/R and independent of temperature management (Figure 9). Postischemic upregulation of TNF-α protein levels due to cooling has been reported in an *in vivo* model using postischemic cooling to 33°C [34]. We observed an upregulation of TNF-α gene expression due to both cooling alone and after 25 h of OGD/R (Figures 8(a) and 9). Similar to IL-6, TNF-α has also been described as a pleiotropic cytokine, dependent on its binding to either membrane bound or soluble receptor [53]. *In vivo* studies have shown that TNF deficiency results in increased infarction volumes and behavioral dysfunction [51, 52]. Here, we report significant regulations of pro- and anti-inflammatory, as well as pleiotropic cytokines expressions in BV-2 microglia due to exposure to OGD/R and cooling.

Several studies have reported upregulations of CIRBP and RBM3 expressions by hypoxia [54–56]. Liu et al. showed *in vivo* that CIRBP gene expression was significantly upregulated in the cortex after 24 h of ischemia, where cooling as well as the combined treatment of ischemia with cooling resulted in increased CIRBP gene expression 6 h after initiation [55]. Furthermore, Zhou et al. reported upregulation of both gene and protein expressions of CIRBP *in vivo*, as well as in BV-2 cells subjected to 20 h up to 48 h of ischemia/hypoxia [56]. We also observed significant increases in RBM3 and CIRBP gene expressions, as well as their intracellular protein expressions in OGD/R-induced injured BV-2 cells treated with cooling (Figures 5 and 6). Moreover, cooling alone also resulted in significant upregulations of both cold-shock protein gene expressions (Figure 5). As most of the past *in vitro* and *in vivo* studies have analyzed cold shock protein expressions after several hours up to days of ischemia or hypoxia exposure, the lack of observable induced cold shock protein expressions due to OGD exposure alone in our study may be attributed to a shorter duration of OGD.

Interestingly, CIRBP gene expression showed a rapid upregulation after 6 h of OGD at 33.5°C and stayed significantly upregulated through all investigated timepoints, whereas RBM3 was significantly upregulated only after 19 h of reperfusion (OGD/R) at 33.5°C (Figure 5). This is in correlation with our previously reported findings of different expression kinetics between CIRBP and RBM3 in organotypic hippocampal slice cultures (OHSC) treated with moderate hypothermia (33.5°C), where CIRBP gene expression was significantly increased after only 4 h of cooling, while RBM3 showed a delayed regulation with an increase after 24 h of cooling [57], thus confirming the difference in expression kinetics between CIRBP and RBM3 in a BV-2 microglial monoculture. This has also been reported outside of the brain where increased CIRBP transcripts were observed after 24 h of cooling to 25°C, compared to 5 days of cooling required for RBM3 induction in human lung fibroblasts [58]. This difference in the dynamics of cold-shock protein regulation may play an important role in the mechanism of neuroprotection induced by cooling. RBM3 has been shown to reduce neuronal apoptosis in ischemic brain injury [24, 59, 60]. Apoptosis is an active and, therefore, energy-dependent cell death mechanism that primarily contributes to reperfusion-induced injury. Si et al. provide promising data that RBM3 is a key player in the formation of stress granules after OGD-induced injury, a cellular rescue mechanism prohibiting apoptosis [60, 61]. Overexpression of RBM3 in PC12 cells resulted in attenuated apoptotic cell death and increased cell viability, whereas RBM3 knockdown had the opposite effect [60]. Another recent study investigating RBM3 knockout in mice after ischemic brain injury focusing on neural stem/progenitor cells (NSPC) shows that RBM3 plays an important role in neuronal regeneration after ischemic brain injury [59]. In summary, RBM3 has been reported to convey neuroprotection on multimodal levels with studies focusing on intracellular effects. However, homologous cold-shock protein CIRBP is known to have both neuroprotective as well as detrimental effects depending on its location. It has been shown that upregulation of intracellular CIRBP increases cell viability in neural stem cells, which is abolished via small interfering RNA (siRNA) knockdown of CIRBP [23]. Furthermore, overexpression of CIRBP restored cell proliferation in neural stem cells treated with hypoxia, indicating a regulating function of CIRBP in the cell cycle [62].

In our study, cooling alone without OGD/R resulted in an increase in gene expressions of Iba1, cold-shock proteins CIRBP and RBM3, and TNF-α, MCP-1, IL-1β, and TGFβ (Figures 4(b), 5(a), 5(b), 6(a), 6(b), 8(a), and 8(c)–8(e), respectively). Our results indicate that cooling, whilst increasing cell viability and expression of cold shock proteins in a model of OGD/R-induced injury, also activates BV-2 microglial cells and is accompanied by an upregulation of both pro- and anti-inflammatory cytokine gene expressions and the release of TNF-α. Moreover, we have previously reported similar results in BV-2 microglial cells with Iba-1 and MCP-1 gene expressions being upregulated after 24 h of cooling [30]. Furthermore, TNF-α release was increased due to cooling alone in the reperfusion phase 12 h after experimental start (Figure 9). Thus, suggesting that cooling alone induces gene expressions of the microglial activation marker, Iba1, and proinflammatory cytokines. To our knowledge, there are no other studies investigating the potential activation of microglial cells by cooling. Because our study focused predominantly on transcriptional regulation by cooling, further investigation are needed in order to verify the potential proinflammatory effects of temperature management in the absence of OGD/R-induced injury.

To further investigate the role sterile inflammation in the setting of ischemic brain injury, we analyzed DAMP release from BV-2 microglia after OGD-induced injury (Figure 7). Although overexpression of HSP70 has been shown to be neuroprotective, extracellular HSP70 has been characterized as a DAMP that binds to a TLR and activates the inflammatory response [63–65]. High-mobility group 1 (HMGB1) protein is located in the nucleus and can be secreted by both monocytes and macrophages, as well as release after necrotic but not apoptotic cell death [66, 67]. HMGB1 is a well-characterized DAMP in the setting of stroke, where its *in vivo* neutralization results in decreased microglial activation, cytokine and iNOS expressions, and reduced permeability of the blood brain barrier [67, 68]. Clinical studies have shown that elevated HMGB1 serum levels could be detected in patients suffering from myocardial or cerebral ischemia [69]. *In vivo*, ischemic brain injury leads to a decrease of HMGB1 immunoreactive cells in the ischemic cortex and an increase of HMGB1 in serum, which could be attenuated by moderate cooling during ischemia [70].

OGD-induced necrotic microglia cell death resulted in the release of DAMPs, including HMGB1, HSP70, and CIRBP, into the cell cultured media (Figure 7). CIRBP release was significantly attenuated by cooling, but no attenuation in the release of HSP70 and HMGB1 by cooling was observable. However, we could observe a difference in the release dynamics of HMGB1 and HSP70 relative to CIRBP (Figures 7(a)–7(c), respectively). HSP70 and HMGB1 releases were highest after 2 h of OGD and decreased after 6 h, whereas extracellular CIRBP and RBM3 were only detectable after 6 h. Extracellular CIRBP has been identified as a novel DAMP acting as a potent mediator of inflammation, and RBM3 release was also investigated due to its high homology with CIRBP (Figure 7(d)). We observed a significant attenuation of CIRBP release by cooling, but only non-significant reduction in the release of HMGB1, HSP70, and RBM3. The detrimental effect of CIRBP in ischemic brain injury has been described by Zhou et al., as infarct volume could be attenuated by 61% in CIRBP knockdown mice 30 h after middle cerebral artery occlusion. The group also reports a translocation of CIRBP from the nucleus to the cytoplasm upon exposure to hypoxia in BV-2 microglial cells, followed by its eventual release after 20 h to 30 h of hypoxia [56]. In an *in vivo* model of deep hypothermic cardiac arrest, knockdown of CIRBP resulted in decreased cerebral injury and neuronal cell death [71]. Qiang et al. showed that CIRBP is released actively via lysosomal secretion in macrophages treated with hypoxia [25]. Concordantly to previous reports investigating the role of CIRBP in macrophages, CIRBP release appears to be an initiator of proinflammatory cascades involving microglial activation and cytokine release [25, 56, 71] and has been shown to be released earlier than TNF-α in ischemic brain injury models [56, 71]. While treatment of BV-2 cells with recombinant CIRBP induced TNF-α release via NF-κB pathway, this was effectively abolished by CIRBP blockage [56]. It has been reported that incubation with supernatants from BV-2 cells treated with hypoxia induces apoptosis in neurons, which was reproduced by stimulating with recombinant CIRBP and reversed by blocking CIRBP via antiserum or siRNA [56, 71]. However, neurons may be more susceptible to extracellular CIRBP in terms of apoptosis induction. To our knowledge, this is the first report showing that both CIRBP and RBM3 are being released into extracellular matrix in OGD-induced injury BV-2 microglial cells. Whereas extracellular CIRBP promotes sterile inflammation and neuronal cell death, to our knowledge, there are no data on the deleterious effects of extracellular RBM3.

In the present study, we investigated the effects of cooling on BV-2 microglia subjected to OGD/R. In context of analyzing the role of temperature management on sterile inflammation in ischemic brain injury, it is important to note our study limitations. A microglial monoculture was used, so the contributions of other cerebral cells exposed to cooling- and OGD/R-induced injury cannot be assessed. Potential pleiotropic effects of the analyzed cytokines cannot be assessed strictly from the regulated gene transcription data presented. Lastly, our results indicate the passive release of CIRBP from necrotic BV-2 microglia. However, potential active release mechanisms of cold shock proteins, different release kinetics relative to HMGB1 and HSP70, and their role in sterile inflammation remains to be elucidated and warrants further research.

5. Conclusion

Cooling applied during OGD significantly attenuated necrotic cell death in BV-2 microglial cells. Cold-shock proteins RBM3 and CIRBP, both known to convey neuroprotection by increasing cell viability, inhibiting apoptosis, and promoting regenerative mechanisms after ischemic brain injury, were induced by cooling, but show different expression kinetics with a delay for RBM3. Exposure to OGD resulted in significantly higher CIRBP release that could be attenuated by intra-OGD cooling and correlated with

cytotoxicity. As extracellular CIRBP is a potent and novel inductor and mediator of inflammation, our findings further support cooling as a potential neuroprotective strategy against sterile inflammation. Our study focused on and showed the potential protective effects of cooling in OGD/R-injured BV-2 microglial cells, but further investigation on the impact of extracellular cold-shock proteins and the innate immune response are needed.

Data Availability

The raw data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Jana Lücht, Nele Rolfs, Katharina R. L. Schmitt, and Giang Tong contributed equally to this work.

Acknowledgments

This study was financially supported by the Stiftung Kinder-Herz Deutschland gGmbH, Essen, Germany (grant number 2511-3-19-020). Dr. Lücht is a participant in the BIH-Charité Junior Clinician Scientist Program funded by the Charité-Universitätsmedizin Berlin and Berlin Institute of Health.

References

[1] S. C. Zhao, L. S. Ma, Z. H. Chu, H. Xu, W. Q. Wu, and F. Liu, "Regulation of microglial activation in stroke," *Acta Pharmacologica Sinica*, vol. 38, no. 4, pp. 445–458, 2017.

[2] A. Denes, R. Vidyasagar, J. Feng et al., "Proliferating resident microglia after focal cerebral ischaemia in mice," *Journal of Cerebral Blood Flow and Metabolism*, vol. 27, no. 12, pp. 1941–1953, 2007.

[3] M. Lalancette-Hébert, G. Gowing, A. Simard, C. W. Yuan, and J. Kriz, "Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain," *The Journal of Neuroscience*, vol. 27, no. 10, pp. 2596–2605, 2007.

[4] A. R. Patel, R. Ritzel, L. D. McCullough, and F. Liu, "Microglia and ischemic stroke: a double-edged sword," *Int J Physiol Pathophysiol Pharmacol*, vol. 5, no. 2, pp. 73–90, 2013.

[5] C. Herzog, L. Pons Garcia, M. Keatinge et al., "Rapid clearance of cellular debris by microglia limits secondary neuronal cell death after brain injuryin vivo," *Development*, vol. 146, no. 9, 2019.

[6] A. D. Rogove and S. E. Tsirka, "Neurotoxic responses by microglia elicited by excitotoxic injury in the mouse hippocampus," *Current Biology*, vol. 8, no. 1, pp. 19–25, 1998.

[7] D. Giulian, K. Vaca, and M. Corpuz, "Brain glia release factors with opposing actions upon neuronal survival," *The Journal of Neuroscience*, vol. 13, no. 1, pp. 29–37, 1993.

[8] C. T. Ekdahl, J. H. Claasen, S. Bonde, Z. Kokaia, and O. Lindvall, "Inflammation is detrimental for neurogenesis in adult brain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 23, pp. 13632–13637, 2003.

[9] J. Neumann, M. Gunzer, H. O. Gutzeit, O. Ullrich, K. G. Reymann, and K. Dinkel, "Microglia provide neuroprotection after ischemia," *The FASEB Journal*, vol. 20, no. 6, pp. 714–716, 2006.

[10] G. Szalay, B. Martinecz, N. Lénárt et al., "Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke," *Nature Communications*, vol. 7, no. 1, 2016.

[11] Y. Kitamura, D. Yanagisawa, M. Inden et al., "Recovery of focal brain ischemia-induced behavioral dysfunction by intracerebroventricular injection of microglia," *Journal of Pharmacological Sciences*, vol. 97, no. 2, pp. 289–293, 2005.

[12] S. Shankaran, A. Pappas, S. A. McDonald et al., "Childhood outcomes after hypothermia for neonatal encephalopathy," *Obstetrical & Gynecological Survey*, vol. 67, no. 10, pp. 617–619, 2012.

[13] J. P. Nolan, J. Soar, A. Cariou et al., "European Resuscitation Council and European Society of Intensive Care Medicine 2015 guidelines for post-resuscitation care," *Intensive Care Medicine*, vol. 41, no. 12, pp. 2039–2056, 2015.

[14] N. Nielsen, J. Wetterslev, T. Cronberg et al., "Targeted temperature management at 33°C versus 36°C after cardiac arrest," *The New England Journal of Medicine*, vol. 369, no. 23, pp. 2197–2206, 2013.

[15] D. Azzopardi, B. Strohm, N. Marlow et al., "Effects of hypothermia for perinatal asphyxia on childhood outcomes," *The New England Journal of Medicine*, vol. 371, no. 2, pp. 140–149, 2014.

[16] H. B. Van Der Worp, E. S. Sena, G. A. Donnan, D. W. Howells, and M. R. Macleod, "Hypothermia in animal models of acute ischaemic stroke: a systematic review and meta-analysis," *Brain*, vol. 130, no. 12, pp. 3063–3074, 2007.

[17] O. M. Dumitrascu, J. Lamb, and P. D. Lyden, "Still cooling after all these years: meta-analysis of pre-clinical trials of therapeutic hypothermia for acute ischemic stroke," *Journal of Cerebral Blood Flow and Metabolism*, vol. 36, no. 7, pp. 1157–1164, 2016.

[18] P. D. Lyden, R. L. Allgren, K. Ng et al., "Intravascular cooling in the treatment of stroke (ICTuS): early clinical experience," *Journal of Stroke and Cerebrovascular Diseases*, vol. 14, no. 3, pp. 107–114, 2005.

[19] P. Lyden, T. Hemmen, J. Grotta et al., "Results of the ICTuS 2 trial (intravascular cooling in the treatment of stroke 2)," *Stroke*, vol. 47, no. 12, pp. 2888–2895, 2016.

[20] H. B. van der Worp, M. R. Macleod, P. M. W. Bath et al., "Therapeutic hypothermia for acute ischaemic stroke. Results of a European multicentre, randomised, phase III clinical trial," *European Stroke Journal*, vol. 4, no. 3, pp. 254–262, 2019.

[21] K. Kurisu, J. Y. Kim, J. You, and M. A. Yenari, "Therapeutic hypothermia and neuroprotection in acute neurological disease," *Current Medicinal Chemistry*, vol. 26, no. 29, pp. 5430–5455, 2019.

[22] X. Zhu, A. Zelmer, J. P. Kapfhammer, and S. Wellmann, "Cold-inducible RBM3 inhibits PERK phosphorylation through cooperation with NF90 to protect cells from endoplasmic reticulum stress," *The FASEB Journal*, vol. 30, no. 2, pp. 624–634, 2016.

[23] K. Saito, N. Fukuda, T. Matsumoto et al., "Moderate low temperature preserves the stemness of neural stem cells and

suppresses apoptosis of the cells via activation of the cold-inducible RNA binding protein," *Brain Research*, vol. 1358, pp. 20–29, 2010.

[24] S. Chip, A. Zelmer, O. O. Ogunshola et al., "The RNA-binding protein RBM3 is involved in hypothermia induced neuroprotection," *Neurobiology of Disease*, vol. 43, no. 2, pp. 388–396, 2011.

[25] X. Qiang, W. L. Yang, R. Wu et al., "Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis," *Nature Medicine*, vol. 19, no. 11, pp. 1489–1495, 2013.

[26] Y. Zhou, H. Dong, Y. Zhong, J. Huang, J. Lv, and J. Li, "The cold-inducible RNA-binding protein (CIRP) level in peripheral blood predicts sepsis outcome," *PLoS One*, vol. 10, no. 9, article e0137721, 2015.

[27] M. Zhou, M. Aziz, N.-L. Denning, H.-T. Yen, G. Ma, and P. Wang, "Extracellular CIRP induces macrophage endotoxin tolerance through IL-6R–mediated STAT3 activation," *JCI Insight*, vol. 5, no. 5, 2020.

[28] N. L. Denning, M. Aziz, A. Murao et al., "Extracellular CIRP as a novel endogenous TREM-1 ligand to fuel inflammation," *bioRxiv*, vol. 5, no. 5, 2020.

[29] E. Blasi, R. Barluzzi, V. Bocchini, R. Mazzolla, and F. Bistoni, "Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus," *Journal of Neuroimmunology*, vol. 27, no. 2–3, pp. 229–237, 1990.

[30] S. J. Wowro, G. Tong, J. Krech, N. Rolfs, F. Berger, and K. R. L. Schmitt, "Combined cyclosporin A and hypothermia treatment inhibits activation of BV-2 microglia but induces an inflammatory response in an ischemia/reperfusion hippocampal slice culture model," *Frontiers in Cellular Neuroscience*, vol. 13, p. 273, 2019.

[31] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta\_C\_}{}_T$ method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[32] D. Ito, Y. Imai, K. Ohsawa, K. Nakajima, Y. Fukuuchi, and S. Kohsaka, "Microglia-specific localisation of a novel calcium binding protein, Iba1," *Brain Research. Molecular Brain Research*, vol. 57, no. 1, pp. 1–9, 1998.

[33] T. Matsui, H. Kida, T. Iha et al., "Effects of hypothermia on ex vivo microglial production of pro- and anti-inflammatory cytokines and nitric oxide in hypoxic-ischemic brain-injured mice," *Folia Neuropathologica*, vol. 52, no. 2, pp. 151–158, 2014.

[34] A. G. Ceulemans, T. Zgavc, R. Kooijman, S. Hachimi-Idrissi, S. Sarre, and Y. Michotte, "Mild hypothermia causes differential, time-dependent changes in cytokine expression and gliosis following endothelin-1-induced transient focal cerebral ischemia," *Journal of Neuroinflammation*, vol. 8, no. 1, p. 60, 2011.

[35] M. Xiong, Y. Yang, G. Q. Chen, and W. H. Zhou, "Post-ischemic hypothermia for 24 h in P7 rats rescues hippocampal neuron: association with decreased astrocyte activation and inflammatory cytokine expression," *Brain Research Bulletin*, vol. 79, no. 6, pp. 351–357, 2009.

[36] J. W. Seo, J. H. Kim, J. H. Kim et al., "Time-dependent effects of hypothermia on microglial activation and migration," *Journal of Neuroinflammation*, vol. 9, no. 1, pp. 1–22, 2012.

[37] K. Ohsawa, Y. Imai, H. Kanazawa, Y. Sasaki, and S. Kohsaka, "Involvement of Iba1 in membrane ruffling and phagocytosis of macrophages/microglia," *Journal of Cell Science*, vol. 113, no. 17, pp. 3073–3084, 2000.

[38] K. Ohsawa, Y. Imai, Y. Sasaki, and S. Kohsaka, "Microglia/macrophage-specific protein Iba1 binds to fimbrin and enhances its actin-bundling activity," *Journal of Neurochemistry*, vol. 88, no. 4, pp. 844–856, 2004.

[39] D. Ito, K. Tanaka, S. Suzuki, T. Dembo, and Y. Fukuuchi, "Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain," *Stroke*, vol. 32, no. 5, pp. 1208–1215, 2001.

[40] L. Q. Liu, X. R. Liu, J. Y. Zhao et al., "Brain-selective mild hypothermia promotes long-term white matter integrity after ischemic stroke in mice," *CNS Neuroscience & Therapeutics*, vol. 24, no. 12, pp. 1275–1285, 2018.

[41] E. Galea, D. L. Feinstein, and D. J. Reis, "Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 22, pp. 10945–10949, 1992.

[42] C. Iadecola, F. Zhang, S. Xu, R. Casey, and M. E. Ross, "Inducible nitric oxide synthase gene expression in brain following cerebral ischemia," *Journal of Cerebral Blood Flow and Metabolism*, vol. 15, no. 3, pp. 378–384, 1995.

[43] D. Huang, A. Shenoy, J. Cui, W. Huang, and P. K. Liu, "In situ detection of AP sites and DNA strand breaks bearing 3'-phosphate termini in ischemic mouse brain," *The FASEB Journal*, vol. 14, no. 2, pp. 407–417, 2000.

[44] X. Zhao, C. Haensel, E. Araki, M. E. Ross, and C. Iadecola, "Gene-dosing effect and persistence of reduction in ischemic brain injury in mice lacking inducible nitric oxide synthase," *Brain Research*, vol. 872, no. 1–2, pp. 215–218, 2000.

[45] C. Iadecola, F. Zhang, and X. Xu, "Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage," *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, vol. 268, no. 1, pp. R286–R292, 1995.

[46] H. S. Han, Y. Qiao, M. Karabiyikoglu, R. G. Giffard, and M. A. Yenari, "Influence of mild hypothermia on inducible nitric oxide synthase expression and reactive nitrogen production in experimental stroke and inflammation," *The Journal of Neuroscience*, vol. 22, no. 10, pp. 3921–3928, 2002.

[47] T. Kimura, K. Toriuchi, H. Kakita et al., "Hypothermia attenuates neuronal damage via inhibition of microglial activation, including suppression of microglial cytokine production and phagocytosis," *Cellular and Molecular Neurobiology*, vol. 41, no. 3, pp. 459–468, 2021.

[48] M. Zhou, C. M. Wang, W.-L. Yang, and P. Wang, "Microglial CD14 activated by iNOS contributes to neuroinflammation in cerebral ischemia," *Brain Research*, vol. 1506, pp. 105–114, 2013.

[49] H. Boutin, R. A. LeFeuvre, R. Horai, M. Asano, Y. Iwakura, and N. J. Rothwell, "Role of IL-1α and IL-1β in ischemic brain damage," *The Journal of Neuroscience*, vol. 21, no. 15, pp. 5528–5534, 2001.

[50] R. Lissilaa, V. Buatois, G. Magistrelli et al., "Although IL-6 trans-signaling is sufficient to drive local immune responses, classical IL-6 signaling is obligate for the induction of T cell-mediated autoimmunity," *Journal of Immunology*, vol. 185, no. 9, pp. 5512–5521, 2010.

[51] K. L. Lambertsen, B. H. Clausen, A. A. Babcock et al., "Microglia protect neurons against ischemia by synthesis of tumor

necrosis factor," *The Journal of Neuroscience*, vol. 29, no. 5, pp. 1319–1330, 2009.

[52] B. H. Clausen, M. Degn, M. Sivasaravanaparan et al., "Conditional ablation of myeloid TNF increases lesion volume after experimental stroke in mice, possibly via altered ERK1/2 signaling," *Scientific Reports*, vol. 6, no. 1, pp. 1–16, 2016.

[53] P. M. Madsen, B. H. Clausen, M. Degn et al., "Genetic ablation of soluble tumor necrosis factor with preservation of membrane tumor necrosis factor is associated with neuroprotection after focal cerebral ischemia," *Journal of Cerebral Blood Flow and Metabolism*, vol. 36, no. 9, pp. 1553–1569, 2016.

[54] S. Wellmann, C. Bührer, E. Moderegger et al., "Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism," *Journal of Cell Science*, vol. 117, no. 9, pp. 1785–1794, 2004.

[55] A. Liu, Z. Zhang, A. Li, and J. Xue, "Effects of hypothermia and cerebral ischemia on cold-inducible RNA-binding protein mRNA expression in rat brain," *Brain Research*, vol. 1347, no. 51, pp. 104–110, 2010.

[56] M. Zhou, W. L. Yang, Y. Ji, X. Qiang, and P. Wang, "Cold-inducible RNA-binding protein mediates neuroinflammation in cerebral ischemia," *Biochimica et Biophysica Acta, General Subjects*, vol. 1840, no. 7, pp. 2253–2261, 2014.

[57] G. Tong, S. Endersfelder, L. M. Rosenthal et al., "Effects of moderate and deep hypothermia on RNA-binding proteins RBM3 and CIRP expressions in murine hippocampal brain slices," *Brain Research*, vol. 1504, pp. 74–84, 2013.

[58] T. Neutelings, C. A. Lambert, B. V. Nusgens, and A. C. Colige, "Effects of mild cold shock (25°C) followed by warming up at 37°C on the cellular stress response," *PLoS One*, vol. 8, no. 7, article e69687, 2013.

[59] X. Zhu, J. Yan, C. Bregere et al., "RBM3 promotes neurogenesis in a niche-dependent manner via IMP2-IGF2 signaling pathway after hypoxic-ischemic brain injury," *Nature Communications*, vol. 10, no. 1, pp. 1–14, 2019.

[60] W. Si, Z. Li, Z. Huang et al., "RNA binding protein motif 3 inhibits oxygen-glucose deprivation/reoxygenation-induced apoptosis through promoting stress granules formation in PC12 cells and rat primary cortical neurons," *Frontiers in Cellular Neuroscience*, vol. 14, pp. 1–14, 2020.

[61] M. Takahashi, M. Higuchi, H. Matsuki et al., "Stress granules inhibit apoptosis by reducing reactive oxygen species production," *Molecular and Cellular Biology*, vol. 33, no. 4, pp. 815–829, 2013.

[62] Q. Zhang, Y. Z. Wang, W. Zhang et al., "Involvement of cold inducible RNA-binding protein in severe hypoxia-induced growth arrest of neural stem cells in vitro," *Molecular Neurobiology*, vol. 54, no. 3, pp. 2143–2153, 2017.

[63] T. R. Doeppner, F. Nagel, G. P. H. Dietz et al., "TAT-Hsp70-mediated neuroprotection and increased survival of neuronal precursor cells after focal cerebral ischemia in mice," *Journal of Cerebral Blood Flow and Metabolism*, vol. 29, no. 6, pp. 1187–1196, 2009.

[64] L. van der Weerd, M. F. Lythgoe, R. A. Badin et al., "Neuroprotective effects of HSP70 overexpression after cerebral ischaemia–an MRI study," *Experimental Neurology*, vol. 195, no. 1, pp. 257–266, 2005.

[65] J. Y. Kim, J. W. Kim, and M. A. Yenari, "Heat shock protein signaling in brain ischemia and injury," *Neuroscience Letters*, vol. 715, no. November 2019, p. 134642, 2020.

[66] P. Scaffidi, T. Misteli, and M. E. Bianchi, "Release of chromatin protein HMGB1 by necrotic cells triggers inflammation," *Nature*, vol. 418, no. 6894, pp. 191–195, 2002.

[67] K. Liu, S. Mori, H. K. Takahashi et al., "Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats," *The FASEB Journal*, vol. 21, no. 14, pp. 3904–3916, 2007.

[68] J. Zhang, H. K. Takahashi, K. Liu et al., "Anti-high mobility group box-1 monoclonal antibody protects the blood-brain barrier from ischemia-induced disruption in rats," *Stroke*, vol. 42, no. 5, pp. 1420–1428, 2011.

[69] R. S. Goldstein, M. Gallowitsch-Puerta, L. H. Yang et al., "Elevated high-mobility group box 1 levels in patients with cerebral and myocardial ischemia," *Shock*, vol. 25, no. 6, pp. 571–574, 2006.

[70] J. H. Lee, E. J. Yoon, J. Seo et al., "Hypothermia inhibits the propagation of acute ischemic injury by inhibiting HMGB1," *Molecular Brain*, vol. 9, no. 1, pp. 1–10, 2016.

[71] M. Liu, Y. Li, S. Gao et al., "A novel target to reduce microglial inflammation and neuronal damage after deep hypothermic circulatory arrest," *The Journal of Thoracic and Cardiovascular Surgery*, vol. 159, no. 6, pp. 2431–2444.e7, 2020.

Hindawi
Mediators of Inflammation
Volume 2021, Article ID 7104934, 10 pages
https://doi.org/10.1155/2021/7104934

Review Article

The Effect of Statins on C-Reactive Protein in Stroke Patients: A Systematic Review of Clinical Trials

Babak Alikiaii,[1] Zahra Heidari,[2,3] Mohammad Bagherniya,[1,4,5] Gholamreza Askari,[1,4,5] Thozhukat Sathyapalan,[6] and Amirhossein Sahebkar[7,8,9]

[1]*Anesthesia and Critical Care Research Center, Isfahan University of Medical Sciences, Isfahan, Iran*
[2]*Department of Biostatistics and Epidemiology, School of Health, Isfahan University of Medical Sciences, Isfahan, Iran*
[3]*Isfahan Cardiac Rehabilitation Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran*
[4]*Food Security Research Center, Isfahan University of Medical Sciences, Isfahan, Iran*
[5]*Department of Community Nutrition, School of Nutrition and Food Science, Isfahan University of Medical Sciences, Isfahan, Iran*
[6]*Academic Diabetes, Endocrinology and Metabolism, Hull York Medical School, University of Hull, Hull, UK*
[7]*Biotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran*
[8]*Applied Biomedical Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*
[9]*School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran*

Correspondence should be addressed to Amirhossein Sahebkar; amir_saheb2000@yahoo.com

Received 28 May 2021; Accepted 18 August 2021; Published 28 August 2021

Academic Editor: Simona Lattanzi

Background. Statins reportedly have anti-inflammatory effects aside from their lipid-lowering impact. We investigated the effects of statin therapy on the level of C-reactive protein (CRP) or highly sensitive CRP (hs-CRP), a liver-derived marker of systemic inflammation, among stroke patients. *Methods*. An online search was performed in Scopus, PubMed/MEDLINE, ISI Web of Science, and Google Scholar up to November 2020 to recognize clinical trials investigating the effects of statins on the CRP level in stroke patients. *Results*. Overall, nine studies (11 treatment arms) with 1659 participants met the inclusion criteria. Six out of 9 studies (8 out of 11 arms) were categorized as studies with a high-quality methodological approach using the Cochrane Collaboration's tool. Data from 5 treatment arms indicated a significant decrease in CRP concentration, and in one treatment arm, CRP concentration did not suggest any considerable alteration following statin therapy. Moreover, two treatment arms showed a significant reduction in hs-CRP concentration and three treatment arms revealed no significant alteration in hs-CRP concentration following statin therapy. Generally, results were heterogeneous and independent of the type of statin, statin dose, treatment duration, and changes in plasma low-density lipoprotein cholesterol concentration. *Conclusion*. The results suggest that statin therapy could reduce and, therefore, could be considered in these patients as potential anti-inflammatory agents.

1. Introduction

Stroke is a leading cause of severe and long-term disability and is considered the third common cause of human mortality. According to the estimates, annually, 15 million people suffer stroke worldwide, with an annual mortality rate of about 5 million [1]. High blood pressure and atrial fibrillation are the most important risk factors for stroke [2].

Ischemic stroke also referred to as brain ischemia or cerebral ischemia is the most common type of stroke, accounting for 80% of all cases [3]. The leading cause of ischemic stroke is the narrowing of the arteries due to atherosclerosis. However, there are many other causes of cerebral ischemic pathogenesis, including endothelial dysfunction, thrombogenesis, inflammatory and oxidative stress damages, and defects in angiogenesis [4].

Inflammatory damage plays a pivotal role in the pathogenesis of ischemic stroke. The collective contribution of the inflammatory cells in the ischemic tissue usually results in long-standing vascular inflammation and ischemic brain injury [5, 6]. Among various proinflammatory cytokines and mediators, serum C-reactive protein (CRP) is of particular importance. According to several studies, this protein is a neuroinflammation marker and an indicator of treatment efficacy [7]. Furthermore, studies have revealed that CRP could also be potentially used to predict impending atherosclerotic-related diseases, including ischemic stroke and cardiovascular disorders [8].

Statins are inhibitors of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, which have been proved to improve endothelial function, modulate thrombogenesis, and significantly diminish cardiovascular disorders [9–11]. In addition, the preventive and ameliorative effects of statins on myocardial infarctions and stroke have been thought to lower serum cholesterol levels [12]. However, it has been demonstrated that the inhibitory effects of statins on HMG-CoA reductase could result in pleiotropic effects beyond reducing the serum low-density lipoprotein (LDL) and cholesterol [13–20]. In this regard, it has been proved that statins could prevent ischemic stroke through attenuating inflammatory damage [21]. Moreover, various preclinical and clinical studies have reported the beneficial effect of statins on CRP reduction [22–24].

Despite the published clinical trials reporting the effects of statins on the CRP level in stroke patients, the findings of these studies have not been systematically reviewed. Therefore, we aimed to perform a systematic review of published clinical studies assessing the effects of statins on CRP levels in patients with stroke.

2. Material and Methods

This systematic review was designed and reported using the guidelines of the preferred reporting items for systematic reviews and meta-analyses (PRISMA) [25].

2.1. Search Strategy. We performed a conclusive systematic search on medical databases including Scopus, ISI Web of Science, PubMed, and Google Scholar databases from inception up to 12 November 2020 using the following keywords: ("statin therapy" OR "statin" OR "atorvastatin" OR "fluvastatin" OR "lovastatin" OR "pitavastatin" OR "pravastatin" OR "rosuvastatin" OR "simvastatin") AND ("stroke" OR "Brain attack" or "Cerebrovascular accident" OR "CVA" OR "Hemorrhagic stroke" OR "Ischemic stroke") AND ("CRP" OR "hs-CRP" OR "high sensitivity C-reactive protein" OR "C-reactive protein" OR "C-reactive protein") AND ("Intervention Study" OR "Intervention Studies" OR "Controlled trial" OR "Randomized controlled trial" OR "Randomized clinical trial" OR "Non-Randomized Controlled Trials" OR "Clinical Trial" OR "Non-Randomized Controlled Trials" OR "Cross-Over study" OR "Cross-Over trial" OR "Cross Over trial" OR "Cross Over study" OR "Double-Blind Method" OR "Double-Blind" OR "Double-Blind trial" OR "Double-Blind study"). In addition, whenever possible, Medical Subject Headings (MESH) terms were used.

2.2. Study Selection. The title and abstract of all papers, which were found in early search, were independently reviewed by two authors (M.B. and G.A.). Articles that did not meet the inclusion criteria were excluded using a screen form with a hierarchical approach based on the study design, population, exposure, and outcome. To explore additional studies, reference lists of relevant review articles were reviewed. The full text of the eligible citation was reviewed. Any disagreements were discussed and agreed.

2.3. Inclusion Criteria. The search was conducted to identify articles examining the effects of statin therapy on CRP or hs-CRP in stroke patients. In the present systematic review, only original articles following these criteria were included: (1) using clinical trial design, (2) using statins as a drug, (3) conducted on patients with stroke disease as a primary disease, (4) assessing CRP or hs-CRP, and (5) using the English language.

2.4. Exclusion Criteria. Studies were excluded if they (1) were a nonhuman experimental disease, (2) reported duplicate data, and (3) were reviews, letters, editorial articles, study protocol, or case reports.

2.5. Data Extraction. Relevant articles were selected after screening records in the initial search. The following information was extracted from eligible and included articles and reported in Table 1: publication information including first author's last name, publication date, and study location, details of the clinical trial including target population, sample size, gender, the mean of age (years), study design, intervention (treatment), dose, control, duration of treatment, and main results of the studies.

2.6. Quality Assessment. The quality of the included studies was assessed by two independent researchers (M.B.) and (G.A.) using the Cochrane Collaboration's tool [26]. The following vital parts are included in this tool: random sequence generation, allocation concealment, blinding, incomplete outcome data, and selective reporting. Each item was categorized as low/unclear/high risk of bias. Consequently, if a study had more than two items of low risk, it was classified as a study with good quality. If a study had two items of low risk, it was considered a study with acceptable quality, and if a study had less than two items of low risk of bias, it was considered a study with weak quality [26].

3. Results

3.1. Search Results and Study Selection. A total of 5308 studies were acknowledged during the initial search, 5119 references in Scopus, 115 in PubMed, and 74 studies in the Web of Sciences, of which 32 records were duplicated. After reading the title and abstracts, 5222 irrelevant records were omitted. Full texts of these 54 remained articles were reviewed, and according to our inclusion and exclusion criteria, 45 articles were omitted due to the following reasons: review papers ($n = 14$), Chinese language ($n = 9$), working on cardiovascular diseases ($n = 8$), did not report CRP as an outcome ($n = 5$), duplicated data ($n = 4$), retrospective

Table 1: Summarize the studies included in the systematic review (arranged alphabetically by first author's last name).

ID	First author, country, year	Target population	Sample size (intervention, control)	Gender (male/female)	Age (mean ± SD)	Study design	Intervention	Dose	Control	Duration	Main results
1	Antonino Tuttolomondo, Italy, 2016 [28]	Acute ischemic stroke	42 (22/20)	23/19	66.27 ± 19.34	Randomized parallel trial	Atorvastatin	80 mg/day	No treatment	72 h	CRP ↓
2	Antonino Tuttolomondo, Italy, 2016 [28]	Acute ischemic stroke	42 (22/20)	23/19	66.27 ± 19.34	Randomized parallel trial	Atorvastatin	80 mg/day	No treatment	7 days	CRP ↓
3	Christopher Beer, Australia, 2012 [33]	Acute ischemic stroke	40 (20/20)	NM	68.6 ± 13.8	Randomized parallel trial	Atorvastatin	80 mg/day	Placebo	3 days	hs-CRP ↔
4	Christopher Beer, Australia, 2012 [33]	Acute ischemic stroke	38 (17/21)	NM	68.6 ± 13.8	Randomized parallel trial	Atorvastatin	80 mg/day	Placebo	30 days	hs-CRP ↔
5	Antonio Muscari, Italy, 2011 [29]	Ischemic stroke	62 (31/31)	20/42	75.3 ± 11.9	Double-blind, placebo-controlled, parallel group study	Atorvastatin	80 mg/day	Placebo	7 days	CRP ↔
6	Xingyu Chen, China, 2018 [31]	Acute ischemic stroke	117 (60/57)	73/43	61.67 ± 11.67	Preliminary, randomized controlled	Atorvastatin	60 mg/day	Atorvastatin 20 mg/day	7 days	hs-CRP ↓
7	Kazuo Kitagawa, Japan, 2017 [27]	Non-cardiogenic ischemic stroke	1095 (545/550)	755/340	66.2 ± 8.5	Randomized open-label trial	Pravastatin	10 mg/day	No treatment	2 months	hs-CRP ↓
8	Jae-Kwan Cha, South Korea, 2004 [35]	Atherosclerotic ischemic stroke	32 (32/0)	28/4	68.5	Trial	Simvastatin	20 mg/day	No control group	12 weeks	CRP ↓
9	Joan Montaner, Spain, 2008 [32]	Cortical stroke	56 (28/28)	29/27	72.7 ± 12.6	Pilot, double-blind, randomized, multicenter clinical trial	Simvastatin + aspirin or simvastatin + triflusal	Simvastatin 40 mg/day firs week 20 mg/day until day 90 aspirin 300 mg/day or triflusal 900 mg/day and followed with aspirin 300 mg/day or triflusal 600 mg/day until day 90	Placebo + aspirin or placebo + triflusal	90 days	hs-CRP ↔

Table 1: Continued.

ID	First author, country, year	Target population	Sample size (intervention, control)	Gender (male/female)	Age (mean ± SD)	Study design	Intervention	Dose	Control	Duration	Main results
10	A. Vijaya Anand, India, 2009 [34]	Stroke	95 (35/60)	64/31	60.1 ± 7.4	Clinical controlled trial	Atorvastatin	10 mg/day	No treatment	3 months	CRP ↓
11	Guo-jun Cao, China2017 [30]	Cerebral infarction	120 (60/60)	65/55	46.29 ± 7.48	Randomized parallel trial	Clopidogrel (75 mg) + rosuvastatin 10 mg/day	10 mg/day	Clopidogrel (75 mg) + atorvastatin 20 mg/day	6 months	CRP ↓

cohort ($n = 3$), and conference report ($n = 2$). The results of the search are shown in Figure 1. Thus, data extraction was done on nine articles with 11 arms. The characteristics of each selected paper are shown in Table 1.

3.2. Characteristics of the Included Studies. Included studies were published between 2004 and 2018 and were conducted in Japan ($n = 1$) [27], Italy ($n = 2$) [28, 29], China ($n = 2$) [30, 31], and Spain ($n = 1$) [32], and one each from Australia [33], India [34], and South Korea [35]. In total, 1659 participants with a mean age of 46–75 years were allocated to these studies and sample sizes ranged from 32 [35] to 1095 [27]. Eight studies included both men and women, and one study did not report the gender status of participants [33]. The duration of statin intervention ranged between 72 h [28] and six months [30]. Five studies used atorvastatin alone, three at the dose of 80 mg/day [28, 29, 33], one at the dose of 60 mg/day [31], and one at the dose of 10 mg/day [34]. One study used 10 mg/day of pravastatin alone [27], and one study used 20 mg/day of simvastatin alone [35]. Combined therapy was administered in 2 trials. One study used rosuvastatin 10 mg/day plus clopidogrel [30], and in one study, simvastatin, 20 and 40 mg/day, was used in combination with aspirin or triflusal [32]. Finally, five trials measured CRP [28–30, 34, 35] and four trials measured hs-CRP [27, 31–33].

3.3. Main Results. Changes in plasma CRP/hs-CRP concentrations following statin therapy were reported in 11 treatment arms. Data of 5 treatment arms indicated a significant decrease in CRP concentration [28, 30, 34, 35], and one treatment arm CRP concentration did not reveal any significant alteration following statin therapy [29]. Moreover, two treatment arms showed a significant reduction in hs-CRP concentration [27, 31] and the three treatment arms revealed no significant alteration in hs-CRP concentration following statin therapy [32, 33].

When the included studies were arranged according to the type of statin used, CRP concentration in 3 treatment arms was decreased [28, 34], and in one arm, it did not alter after atorvastatin therapy [29]. On the other hand, hs-CRP concentration in one study arm decreased after atorvastatin monotherapy [31]. In 2 treatment arms, alteration in hs-CRP concentration was not significant when single doses of atorvastatin were used [33]. In a different arm, hs-CRP concentration significantly decreased after monotherapy of pravastatin [27]. A significant reduction in CRP concentration was observed after monotherapy of simvastatin in one treatment arm [35], but combination therapy of simvastatin with aspirin or triflusal did not alter the hs-CRP concentration in another study arm [32]. Finally, CRP concentration significantly decreased in one study arm after using rosuvastatin combined with clopidogrel [30].

Overall, statin therapy with lower doses was more effective than higher doses. A significant reduction in CRP/hs-CRP concentrations was observed upon administration of low-dose statin treatments. There was no association between the duration of statin therapy and changes in plasma CRP/hs-CRP concentrations.

3.4. Quality of the Included Studies. As shown in Table 2, 6 out of 9 studies had a high-quality methodological approach [28–33], one study was categorized as a study with acceptable quality [27], and two studies had a weak methodological design [34, 35].

4. Discussion

This review is aimed at systematically and comprehensively evaluating the evidence regarding the effect of statin on CRP in patients with stroke to provide the groundwork for future studies. To our knowledge, this systematic review is the first to assess the association between statin therapy and plasma CRP concentrations among patients with stroke. As described in the results, since the population of statin-treated patients and follow-up durations were heterogeneous across included studies, we did not conduct a meta-analysis. However, the main finding of the current qualitative study is that statin therapy in stroke patients is associated with reducing CRP as an acute-phase reactant and sensitive marker of systemic inflammation. Furthermore, the role of inflammation as a triggering factor for increasing blood viscosity, promoting plaque formation, and accelerating atherosclerosis, through CRP, tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6), as nonspecific markers of the acute stage of the systemic inflammatory response, is well established. Thus, there is a complementary relationship between the level of inflammation and atherosclerotic plaque formation [30, 34, 36].

Although the beneficial effects of statins are mediated predominantly by their lipid-lowering effects, recent evidence from clinical trials suggests that statins have anti-inflammatory effects and their benefits may extend beyond their cholesterol-lowering effects that are important for prognosis and treatment in cardiovascular and stroke events [34, 37, 38]. Statin therapy has suggested protecting vascular events through anti-inflammatory activities reflected by CRP reductions [32, 34]. Growing evidence of CRP-reducing effects of statins indicates that this class of medications reduces the risk of cardiovascular events in patients with coronary artery disease [37, 39–41]. In this regard, Ridker et al. demonstrated that statin therapy resulted in a more significant clinical benefit when CRP levels were high and that statins decreased CRP levels in a manner essentially independent of LDL-C levels [42].

It is well documented that the induction of an inflammatory response plays an essential role in the pathogenesis of brain damage. For example, elevated serum levels of CRP in the inflammatory process of atherosclerosis have been widely considered to increase intimal thickness and plaque rupture, resulting in acute cerebral infarction [30, 31]. This phenomenon implies that the onset and development of atherosclerotic lesions could be modulated by reducing inflammation. In this regard, anti-inflammatory therapies are neuroprotective and preventing neuroinflammation may add a better clinical outcome to ischemic stroke [30, 43].

In addition to their effects in reducing cardiovascular risk, there is increasing evidence that prior or early use of statins may reduce the severity of an acute ischemic stroke

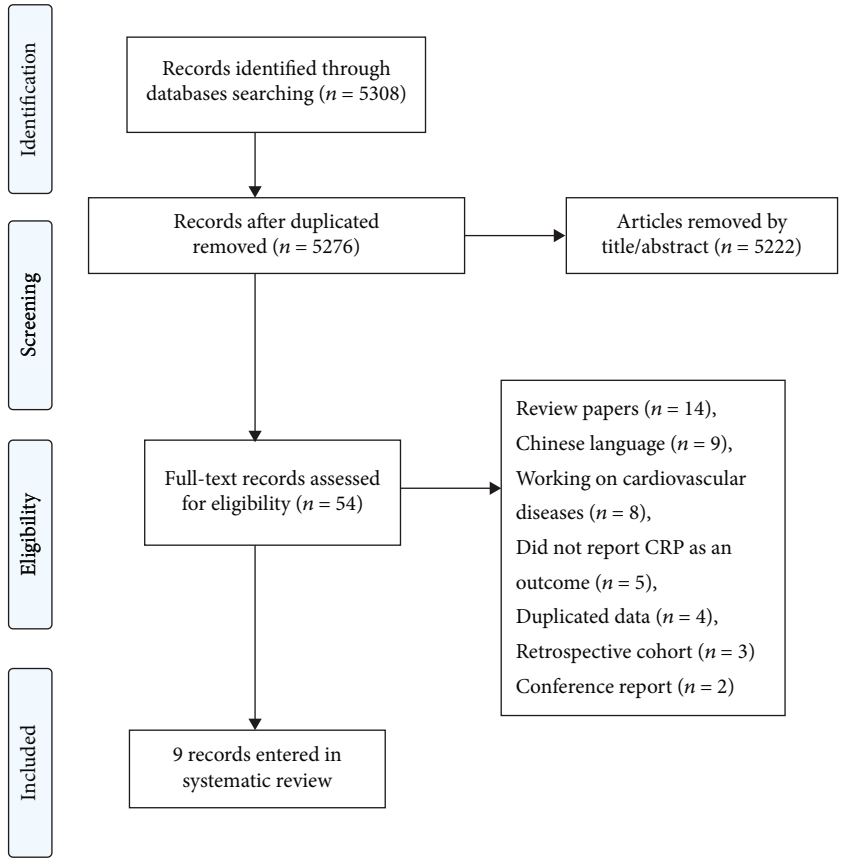


Figure 1: Flow chart of the process of the study selection.

Table 2: Risk of bias assessment for included clinical trials.

First author (publication year)	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other risks of bias
Antonino Tuttolomondo (2016)	L	L	H	H	L	L	U
Christopher Beer (2012)	L	L	L	L	U	L	H
Antonio Muscari,(2011)	L	L	L	L	L	L	U
Xingyu Chen (2018)	L	L	L	L	L	L	L
Kazuo Kitagawa (2017)	L	L	H	H	U	U	H
Jae-Kwan Cha (2004)	H	H	H	H	H	L	U
J. Montaner (2007)	L	L	L	L	L	L	U
Vijaya Anand (2009)	H	H	U	U	U	U	U
Guo-jun Cao (2017)	L	L	U	U	L	L	U

L: low risk of bias; H: high risk of bias; U: unclear risk of bias.

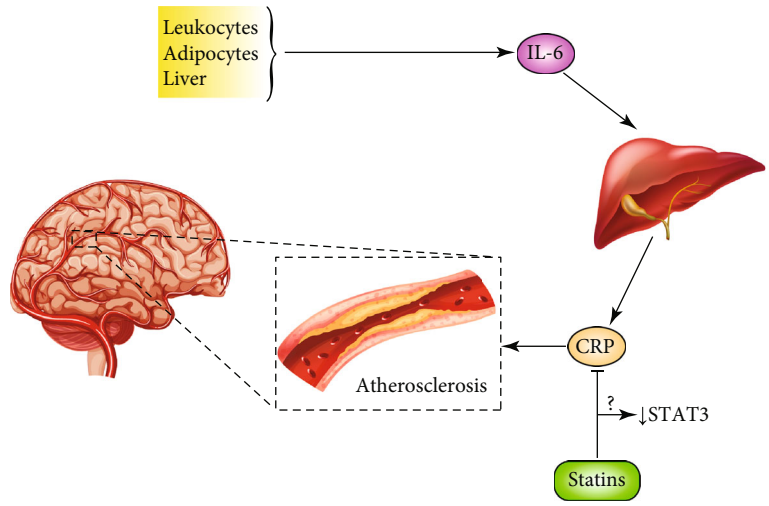


Figure 2: Potential mechanisms leading to statin-induced reduction of CRP release from hepatocytes in stroke. Statins reduce activation of transcription factor STAT3, leading to a decrease of CRP release by hepatocytes. Reduction of the CRP level attenuates inflammatory response and eventually leads to the neuroprotective effects during the stroke.

and improve its outcome [22, 32, 43–46]. It has been shown that statins stabilize atherosclerotic plaque and increase cerebral blood flow [30, 44]. Data from several systematic reviews and meta-analyses support statins' benefit in stroke patients [47–52]. In several trials, statin therapy has been shown to significantly reduce the plasma level of CRP and patients with any stroke history who have low CRP levels after statin therapy have better clinical outcomes than those with higher CRP levels [27, 34, 43, 53, 54].

In the present review, only two studies were designed to directly assess the effects of statins on CRP levels in stroke patients due to their pleiotropic activities [27, 34]. However, the results of these two studies showed a significant reduction in CRP concentrations after statin therapy, confirming the potential anti-inflammatory effects of statins aside from their cholesterol-lowering impact. Moreover, in noncardiogenic ischemic stroke patients, pravastatin decreased the hs-CRP levels and elevated hs-CRP levels were suggested to increase the risk of recurrent stroke and vascular events [27].

In two studies, the effect of statins on the inflammatory markers in stroke patients was evaluated. In a randomized parallel trial, atorvastatin 80 mg/day acutely administered immediately after an atherosclerotic ischemic stroke was found to reduce serum levels of inflammatory markers including CRP, confirming the neuroinflammatory protection of statins [28]. In another trial, atorvastatin and rosuvastatin revealed lipid-lowering and anti-inflammatory effects in stroke patients and reduced CRP levels, although rosuvastatin showed better therapeutic benefits [30]. In addition, in other trials, CRP was also measured after statin usage in stroke patients. These studies showed a decrease in plasma CRP concentration in stroke patients after using statins [31, 35].

In contrast to the above studies, there have also been studies in which statin therapy did not affect the concentrations of CRP in stroke patients. In the study of Montaner and coworkers, assessment of inflammatory markers, including CRP, after using simvastatin did not show any difference in their levels regarding treatment allocation and authors report a nonsignificant increase in mortality and a more significant proportion of infections in the simvastatin group as the primary safety concerns [32]. Moreover, two independent studies demonstrated that administration of atorvastatin 80 mg/day did not affect the concentration of CRP in stroke patients. Muscari and coworkers observed a lack of increase in the CRP level in the atorvastatin group, compared with the significant increase in the placebo group. They conclude that this difference was likely due to the anti-inflammatory effect of atorvastatin [29]. In the second study, atorvastatin prescription for 3 and 30 days did not reduce CRP in stroke patients and did not appear to modify infarct growth substantially [33].

Although some studies did not meet our inclusion criteria, their results concerning statins' effect on CRP levels were interesting. Notably, in two studies, reducing CRP levels following statin therapy was significantly associated with favorable 3-month outcomes and improved patients' survival and readmission rates after acute ischemic stroke [43, 53]. Moreover, in one retrospective study, patients with a history of stroke after prescribing pitavastatin showed a reduced CRP level and potentially limited atherosclerosis in high-risk stroke patients [54]. Furthermore, in the Justification for the Use of Statins in Primary Prevention (JUPITER) trial, a study conducted on healthy subjects without hyperlipidemia and with elevated CRP levels; rosuvastatin 20 mg was administered daily and was shown to decrease both LDL cholesterol and CRP levels, reducing the occurrence of ischemic stroke [55]. In our systematic review, the association between statin therapy and CRP levels did not vary upon the statin type and dose.

The mechanisms by which statins reduce CRP levels in stroke patients are not precisely known. CRP is synthesized mainly by the liver in response to proinflammatory cytokines, particularly IL-6 derived from activated leukocytes, adipose tissue, and in part from the liver [56]. After releasing in the bloodstream, CRP induces upregulation of the vasoconstrictor endothelin-1 and IL-6 by endothelial cells and

increases vascular cell adhesion molecule-1, intercellular adhesion molecule-1, E-selectin, and monocyte chemoattractant protein-1 (MCP-1) and thus enhances leukocyte recruitment in an inflammatory process [38]. As depicted in Figure 2, statins possibly reduce IL-6-induced CRP expression in human hepatocytes. At the transcriptional level, statins act on the geranylgeranyl pathway and decrease activation of the transcription factor STAT3, leading to the attenuation of inflammation and neuroprotection effects after stroke.

Several potential limitations to this study should be noted. First, the total number of studies was limited and the trial population size was relatively small. Second, because studies with almost high heterogeneity were included in this study, estimates from these studies could not be reliably combined to conduct a meta-analysis. Finally, this heterogeneity made us interpret our results cautiously. Differences in the duration of treatment, statin dose, control group treatment, and design of studies were some reasons for potential heterogeneity across studies.

Furthermore, various changes in plasma CRP concentrations are dependent on different pharmacokinetic profiles of statins used. Third, we included only studies published in English and might have missed relevant articles in languages other than English. Finally, if the literature search fails to find all relevant reports, the present study is at risk of bias.

5. Conclusion

Our research showed the beneficial impact of statins in patients after a stroke by reducing CRP levels confirming available evidence regarding the potential benefits of statins as anti-inflammatory agents. These findings could propose that statin treatment should be started in patients with stroke, irrespective of their levels of cholesterol; however, well-designed trials in patients with stroke are needed to precisely examine the CRP-reducing benefits of statin therapy in the future, considering potential differences by dosage, duration of use, study population, and other factors.

Data Availability

There is no raw data associated with this systematic review.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

All authors contributed to the conceptualization, drafting, and final editing of the manuscript. All authors approved the final version for submission.

Acknowledgments

Isfahan University approved this study of Medical Sciences with the grant number 199467 and ethical code IR.MUI.MED.REC.1399.841. There is no financial support for this study.

References

[1] E. S. Donkor, "Stroke in the 21(st) century: a snapshot of the burden, epidemiology, and quality of life," *Stroke Research and Treatment.*, vol. 2018, article 3238165, pp. 1–10, 2018.

[2] R. C. Li, W. D. Xu, Y. L. Lei et al., "The risk of stroke and associated risk factors in a health examination population: a cross-sectional study," *Medicine*, vol. 98, no. 40, article e17218, 2019.

[3] A. K. Boehme, C. Esenwa, and M. S. V. Elkind, "Stroke risk factors, genetics, and prevention," *Circulation Research*, vol. 120, no. 3, pp. 472–495, 2017.

[4] T. D. Musuka, S. B. Wilton, M. Traboulsi, and M. D. Hill, "Diagnosis and management of acute ischemic stroke: speed is critical," *CMAJ*, vol. 187, no. 12, pp. 887–893, 2015.

[5] R. Jin, G. Yang, and G. Li, "Inflammatory mechanisms in ischemic stroke: role of inflammatory cells," *Journal of Leukocyte Biology*, vol. 87, no. 5, pp. 779–789, 2010.

[6] J. Anrather and C. Iadecola, "Inflammation and stroke: an overview," *Neurotherapeutics*, vol. 13, no. 4, pp. 661–670, 2016.

[7] B. Heidari, "C-reactive protein and other markers of inflammation in hemodialysis patients," *Caspian Journal of Internal Medicine.*, vol. 4, no. 1, pp. 611–616, 2013.

[8] A. R. Moon, D. H. Choi, S. Y. Jahng et al., "High-sensitivity C-reactive protein and mean platelet volume as predictive values after percutaneous coronary intervention for long-term clinical outcomes: a comparable and additive study," *Blood Coagulation & Fibrinolysis: An International Journal in Haemostasis and Thrombosis.*, vol. 27, no. 1, pp. 70–76, 2016.

[9] N. K. Kapur and K. Musunuru, "Clinical efficacy and safety of statins in managing cardiovascular risk," *Vascular Health and Risk Management*, vol. Volume 4, no. 2, pp. 341–353, 2008.

[10] X. Han, Y. Zhang, L. Yin et al., "Statin in the treatment of patients with myocardial infarction: a meta-analysis," *Medicine*, vol. 97, no. 12, article e0167e, 2018.

[11] A. Toso, M. Leoncini, and S. De Servi, "Statins and myocardial infarction: from secondary 'prevention' to early 'treatment'," *Journal of Cardiovascular Medicine*, vol. 20, no. 4, pp. 220–222, 2019.

[12] P. Byrne, J. Cullinan, A. Smith, and S. M. Smith, "Statins for the primary prevention of cardiovascular disease: an overview of systematic reviews," *BMJ Open*, vol. 9, no. 4, article e023085, 2019.

[13] A. R. Afshari, H. Mollazadeh, N. C. Henney, T. Jamialahmad, and A. Sahebkar, "Effects of statins on brain tumors: a review," *Seminars in Cancer Biology.*, vol. 73, pp. 116–133, 2021.

[14] A. Bahrami, S. Bo, T. Jamialahmadi, and A. Sahebkar, "Effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors on ageing: molecular mechanisms," *Ageing Research Reviews.*, vol. 58, p. 101024, 2020.

[15] G. Ferretti, T. Bacchetti, and A. Sahebkar, "Effect of statin therapy on paraoxonase-1 status: a systematic review and meta-analysis of 25 clinical trials," *Progress in Lipid Research.*, vol. 60, pp. 50–73, 2015.

[16] H. Mollazadeh, E. Tavana, G. Fanni et al., "Effects of statins on mitochondrial pathways," *Journal of Cachexia, Sarcopenia and Muscle.*, vol. 12, no. 2, pp. 237–251, 2021.

[17] Ž. Reiner, M. Hatamipour, M. Banach et al., "Statins and the Covid-19 main protease: in silico evidence on direct interaction," *Archives of Medical Science.*, vol. 16, no. 3, pp. 490–496, 2020.

[18] A. Sahebkar, C. Serban, D. P. Mikhailidis et al., "Association between statin use and plasma d-dimer levels. a systematic review and meta-analysis of randomised controlled trials," *Thrombosis and Haemostasis.*, vol. 114, no. 3, pp. 546–557, 2015.

[19] A. Sahebkar, C. Serban, S. Ursoniu et al., "The impact of statin therapy on plasma levels of von Willebrand factor antigen," *Thrombosis and Haemostasis.*, vol. 115, no. 3, pp. 520–532, 2016.

[20] C. Serban, A. Sahebkar, S. Ursoniu et al., "A systematic review and meta-analysis of the effect of statins on plasma asymmetric dimethylarginine concentrations," *Scientific Reports*, vol. 5, no. 1, 2015.

[21] J. Zhao, X. Zhang, L. Dong, Y. Wen, and L. Cui, "The many roles of statins in ischemic stroke," *Current Neuropharmacology*, vol. 12, no. 6, pp. 564–574, 2014.

[22] M. Nakamura, T. Fukukawa, K. Kitagawa et al., "Ten-year standardization of lipids and high-sensitivity C-reactive protein in a randomized controlled trial to assess the effects of statins on secondary stroke prevention: Japan statin treatment against recurrent stroke," *Annals of Clinical Biochemistry.*, vol. 55, no. 1, pp. 128–135, 2018.

[23] P. H. Joshi and T. A. Jacobson, "Therapeutic options to further lower C-reactive protein for patients on statin treatment," *Current Atherosclerosis Reports.*, vol. 12, no. 1, pp. 34–42, 2010.

[24] M. A. Albert, E. Danielson, N. Rifai, P. M. Ridker, P. Investigators, and P. Investigators, "Effect of statin therapy on C-reactive protein levels: the pravastatin inflammation/CRP evaluation (PRINCE): a randomized trial and cohort study," *Journal of the American Medical Association*, vol. 286, no. 1, pp. 64–70, 2001.

[25] D. Moher, A. Liberati, J. Tetzlaff, and D. G. Altman, "Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement," *Annals of Internal Medicine.*, vol. 151, no. 4, pp. 264–269, 2009.

[26] J. P. Higgins, "Cochrane handbook for systematic reviews of interventions version 5.0.1," *The Cochrane Collaboration*, 2008, http://www.cochrane-handbook.org.

[27] K. Kitagawa, N. Hosomi, Y. Nagai et al., "Reduction in high-sensitivity C-reactive protein levels in patients with ischemic stroke by statin treatment: Hs-CRP sub-study in J-STARS," *Journal of Atherosclerosis and Thrombosis.*, vol. 24, no. 10, pp. 1039–1047, 2017.

[28] A. Tuttolomondo, D. di Raimondo, R. Pecoraro et al., "Early high-dosage atorvastatin treatment improved serum immune-inflammatory markers and functional outcome in acute ischemic strokes classified as large artery atherosclerotic stroke: a randomized trial," *Medicine*, vol. 95, no. 13, p. e3186, 2016.

[29] A. Muscari, G. M. Puddu, N. Santoro et al., "The atorvastatin during ischemic stroke study: a pilot randomized controlled trial," *Clinical Neuropharmacology*, vol. 34, no. 4, pp. 141–147, 2011.

[30] G. J. Cao, X. F. Zhang, and K. D. Zheng, "Effects of atorvastatin and rosuvastatin on blood lipids, platelet aggregation rate and inflammatory factors in patients with cerebral infarction," *Tropical Journal of Pharmaceutical Research.*, vol. 16, no. 10, pp. 2507–2513, 2017.

[31] X. Chen, X. Zhuang, Z. Peng, H. Yang, L. Chen, and Q. Yang, "Intensive statin therapy for acute ischemic stroke to reduce the number of microemboli: a preliminary, randomized controlled study," *European Neurology*, vol. 80, no. 3-4, pp. 163–170, 2019.

[32] J. Montaner, P. Chacón, J. Krupinski et al., "Simvastatin in the acute phase of ischemic stroke: a safety and efficacy pilot trial," *European Journal of Neurology*, vol. 15, no. 1, pp. 82–90, 2008.

[33] C. Beer, D. Blacker, M. Bynevelt, G. J. Hankey, and I. B. Puddey, "A randomized placebo controlled trial of early treatment of acute ischemic stroke with atorvastatin and irbesartan," *International Journal of Stroke.*, vol. 7, no. 2, pp. 104–111, 2012.

[34] A. V. Anand, M. Chandrasek, S. Kalavathy et al., "The influencing aspects of atorvastatin on C-reactive protein and lipid profile in patients with stroke," *International Journal of Biological Chemistry*, vol. 3, no. 1, pp. 30–34, 2008.

[35] J.-K. Cha, M.-H. Jeong, and J. W. Kim, "Statin reduce the platelet p-selectin expression in atherosclerotic ischemic stroke," *Journal of Thrombosis and Thrombolysis.*, vol. 18, no. 1, pp. 39–42, 2004.

[36] J.-J. Li and X.-J. Chen, "Simvastatin inhibits interleukin-6 release in human monocytes stimulated by C-reactive protein and lipopolysaccharide," *Coronary Artery Disease.*, vol. 14, no. 4, pp. 329–334, 2003.

[37] M. H. Hyun, Y. Lee, B. G. Choi et al., "Roles of achieved levels of low-density lipoprotein cholesterol and high- sensitivity C-reactive protein on cardiovascular outcome in statin therapy," *Cardiovascular Therapeutics.*, vol. 2019, pp. 1–10, 2019.

[38] C. Arnaud, F. Burger, S. Steffens et al., "Statins reduce interleukin-6–induced C-reactive protein in human hepatocytes," *Arteriosclerosis, Thrombosis, and Vascular Biology.*, vol. 25, no. 6, pp. 1231–1236, 2005.

[39] D. O. Kang, Y. Park, J. H. Seo et al., "Time-dependent prognostic effect of high sensitivity C-reactive protein with statin therapy in acute myocardial infarction," *Journal of Cardiology*, vol. 74, no. 1, pp. 74–83, 2019.

[40] B. D. Horne, J. B. Muhlestein, J. F. Carlquist et al., "Statin therapy, lipid levels, C-reactive protein and the survival of patients with angiographically severe coronary artery disease," *Journal of the American College of Cardiology.*, vol. 36, no. 6, pp. 1774–1780, 2000.

[41] P. M. Ridker, C. P. Cannon, D. Morrow et al., "C-reactive protein levels and outcomes after statin therapy," *New England Journal of Medicine.*, vol. 352, no. 1, pp. 20–28, 2005.

[42] P. M. Ridker, N. Rifai, M. Clearfield et al., "Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events," *New England Journal of Medicine.*, vol. 344, no. 26, pp. 1959–1965, 2001.

[43] N.-W. Tsai, L.-H. Lee, C.-R. Huang et al., "The association of statin therapy and high-sensitivity C-reactive protein level for predicting clinical outcome in acute non-cardioembolic ischemic stroke," *Clinica Chimica Acta.*, vol. 413, no. 23-24, pp. 1861–1865, 2012.

[44] S. Yaghi and M. S. Elkind, "Lipid control and beyond: current and future indications for statin therapy in stroke," *Current Treatment Options in Cardiovascular Medicine.*, vol. 18, no. 4, 2016.

[45] F. Colivicchi, A. Bassi, M. Santini, and C. Caltagirone, "Discontinuation of statin therapy and clinical outcome after ischemic stroke," *Stroke*, vol. 38, no. 10, pp. 2652–2657, 2007.

[46] P. S. Chen, C. L. Cheng, Y. H. Kao Yang, and Y. H. Li, "Statin adherence after ischemic stroke or transient ischemic attack is

associated with clinical outcome," *Circulation Journal.*, vol. 80, no. 3, pp. 731–737, 2016.

[47] K.-S. Hong and J. S. Lee, "Statins in acute ischemic stroke: a systematic review," *Journal of Stroke.*, vol. 17, no. 3, pp. 282–301, 2015.

[48] H. Milionis, G. Ntaios, E. Korompoki, K. Vemmos, and P. Michel, "Statin-based therapy for primary and secondary prevention of ischemic stroke: a meta-analysis and critical overview," *International Journal of Stroke.*, vol. 15, no. 4, pp. 377–384, 2020.

[49] B. Christophe, M. Karatela, J. Sanchez, J. Pucci, and E. S. Connolly, "Statin therapy in ischemic stroke models: a meta-analysis," *Translational Stroke Research.*, vol. 11, no. 4, pp. 590–600, 2020.

[50] M.-Y. Eun, J.-M. Jung, K.-H. Choi, and W.-K. Seo, "Statin effects in atrial fibrillation-related stroke: a systematic review and meta-analysis," *Frontiers in Neurology*, vol. 11, 2020.

[51] D. Ní Chróinín, K. Asplund, S. Åsberg et al., "Statin therapy and outcome after ischemic stroke: systematic review and meta-analysis of observational studies and randomized trials," *Stroke*, vol. 44, no. 2, pp. 448–456, 2013.

[52] A. Squizzato, E. Romualdi, F. Dentali, and W. Ageno, "Statins for acute ischemic stroke," *The Cochrane database of systematic reviews*, vol. 8, article Cd007551, 2011.

[53] J. C. Arévalo-Lorido, J. Carretero-Gómez, J. M. Fernández-Recio et al., "Lowering C-reactive protein with statins after an ischemic stroke avoids mortality and readmissions. A prospective cohort study," *Annals of Medicine*, vol. 47, no. 3, pp. 226–232, 2015.

[54] H. Sugimoto, S. Konno, N. Nomoto et al., "The long-term effects of pitavastatin on blood lipids and platelet activation markers in stroke patients: impact of the homocysteine level," *PLoS One*, vol. 9, no. 11, article e113766, 2014.

[55] B. M. Everett, R. J. Glynn, J. G. MacFadyen, and P. M. Ridker, "Rosuvastatin in the prevention of stroke among men and women with elevated levels of C-reactive protein: justification for the use of statins in prevention: an intervention trial evaluating rosuvastatin (JUPITER)," *Circulation*, vol. 121, no. 1, pp. 143–150, 2010.

[56] E. Sucajtys-Szulc, A. Debska-Slizien, B. Rutkowski et al., "Hepatocyte nuclear factors as possible C-reactive protein transcriptional inducer in the liver and white adipose tissue of rats with experimental chronic renal failure," *Molecular and Cellular Biochemistry*, vol. 446, no. 1-2, pp. 11–23, 2018.