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Original Research

Effects of *Ficus Platyphylla*-Induced Hypothermia on Long-term Functional Recovery after Ischaemic Stroke

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Abstract

A stroke is a cerebrovascular disease that results from a blockage in the blood supply to part of the brain or a burst blood vessel in the brain. It is the second leading cause of death worldwide, with an annual mortality rate of about 5.5 million. Antithrombotic therapies have failed to provide a cure for this debilitating cerebrovascular disorder, and hypothermia is gaining interest as a novel strategy for the management of stroke. In this study, we evaluated the effects of *Ficus platyphylla*-induced hypothermia on long-term functional recovery after ischaemic stroke. Histomorphological analysis of the brain demonstrated pathological alterations in the ipsilateral hemisphere of all animals. Animals treated before or immediately after permanent occlusion of the middle cerebral artery (MCAO) had significantly smaller infarct sizes than those given saline. Surgery and treatment did not affect locomotor activity.

There were no significant differences between the groups of mice in terms of parameters associated with situational anxiety, including the number of arm changes and percentile time spent on open arms. There were no significant differences between groups regarding the number of buried marbles and sociability. Surgery and treatment did not affect social recognition, but a significant interaction between surgery and treatment was observed. The time mice remained on the rota rod was relatively similar for all groups tested, with no significant differences related to surgery and treatment, nor was there any surgery/treatment interaction. A learning effect represented by a decrease in exploratory activity was observed irrespective of surgery and treatment, and there was no surgery/treatment interaction. The results suggest that *Ficus platyphylla*-induced hypothermia could be beneficial to long-term functional recovery after ischaemic stroke.

Keywords
Stroke; MCAO; motor functions; anxiety; social recognition memory; hole board

1. Introduction

Stroke is the second leading cause of death worldwide, with an annual mortality rate of about 5.5 million and 50% of survivors being left chronically disabled. Although more young people are affected by stroke in low and middle-income countries [1-3], its incidence is increasing due to the increasingly aging nature of the population. Antithrombotic therapies, including antiplatelet, anticoagulant, or fibrinolytic substances, are recommended for nearly all patients with no contraindications [4]. Pharmacological approaches to treating ischaemic stroke remain limited, suggesting the need for new treatments; hypothermia might be one such new strategy. The metabolic and protective effects of hypothermia have been discussed extensively [5-9]. Preclinical research has shown a therapeutic effect of hypothermia in different pathological conditions such as stroke, traumatic injury, and global ischemia after cardiac arrest [8], with clinical trials producing variable results [7]. A significant disadvantage of systemic cooling is the time needed to attain the target temperature, often outside the 4.5 h therapeutic window [7]. There is growing interest in investigating drug-induced hypothermia as a treatment option for ischaemic stroke. Eight groups of pharmacological compounds that can induce hypothermia have been characterized [10]. These compounds induce hypothermia rapidly without needing specialist equipment, and the procedure can be performed outside a clinical environment.

Recently, we investigated the effect of methanol stem bark extract of *Ficus platyphylla* (Fic) on core body temperature and cerebral ischemia-induced brain damage in mice [11]. The model 'permanent occlusion of the middle cerebral artery' (MCAO) was used, and the brains were observed 24 h after MCAO. The plant extract induced hypothermia for at least 8 hours after intraperitoneal injection [11]. Administration of Fic 1 h before MCAO significantly reduced infarct volume, with no significant effect on infarct volume immediately after MCAO. A higher number of cells and neurons were found in the peri-infarct area in both groups of mice. Fic-induced hypothermia protected the peri-infarct region from synaptophysin reduction and reduced NMDA

receptor 2 immunoreactivity [11]. These results suggest that Fic-induced hypothermia had a protective effect on different levels of ischemia-induced brain damage.

The fact that the number of cells and neurons in the peri-infarct area was higher in Fic-treated mice suggests a positive effect on long-term functional recovery after ischemia-induced brain damage. To test this hypothesis, mice were subjected to behavioral tests to measure their locomotor activity, anxiety, and learning performance 7-17 days after permanent MCAO and Fic treatment.

2. Materials and Methods

2.1 Experimental Animals

Male C57BL/6J mice were purchased from Charles River, Sulzfeld, Germany. All animals were maintained in a temperature and humidity-controlled facility with a 12 h light-dark cycle, with food and water provided ad libitum. Mice were randomly divided into experimental or control groups. All experiments were conducted following European Community regulations and approved by the Saxony-Anhalt Committee on Animal Care (42502-2-1478 UniMD). Every effort was made to minimize the animals' suffering and the number of animals used.

2.2 Extract

The identification, collection, and methanol extraction of *Ficus platyphylla* stem bark and high-performance liquid chromatography analysis of the extract, have been described previously [12, 13]. The quote was dissolved in physiological saline and injected intraperitoneally (ip) at a volume of 10 ml/kg body weight (bw) 1 h prior to MCAO or immediately after MCAO in a dose of 50 mg/kg bw [11]. This dose was selected based on its effect on body temperature [11]. Controls were given the solvent.

2.3 Surgery

MCAO or sham surgery was performed on animals aged 8-11 weeks, as described by Becker et al. 2021. To summarise, mice were anesthetized by ip injection of etomidate (20 mg/kg bw, Hypnomidate®, Janssen-Cilag, Neuss, Germany), and the left middle cerebral artery was exposed. The stem of the middle cerebral artery and both branches were permanently occluded by electrocoagulation. In the sham operations, all procedures were identical except electrocoagulation. During surgery, body temperature was maintained at 37 ± 0.5°C. After MCAO, the skin was closed with tissue adhesive (Histoacryl®, B. Braun Surgical, Rubi, Spain), and the mice received 0.1 mg/kg buprenorphine subcutaneously (sc) for analgesia. Buprenorphine treatment did not alter the infarct volume [14]. They were kept at an ambient temperature of 30°C for 2 h, and their body temperature (37 ± 0.5°C) was monitored continuously. The animals were scored for symptoms of pain 8, 16, 24, 32, and 48 h after surgery. If symptoms were apparent, mice received another sc injection of 0.1 mg/kg bw buprenorphine.

2.4 Behavioural Tests

Seven days after surgery, the animals were tested using various behavioral tests to evaluate their locomotor activity, anxiety, motor coordination, and learning. None of the tests was based on negative reinforcement. The experimental schedule was as follows:

D1 Surgery
D7 Locomotor activity
D8 Elevated plus maze
D9 Marble burying
D10 Social recognition memory
D11 Rota rod
D14 D17 Hole Board
D19 The animals were sacrificed, and the brains were collected to determine the infarct volume.

2.5 Locomotor Activity

A three-dimensional computerized system (Acti-Mot, TSE, Bad Homburg, Germany) was used to measure locomotor activity. The system consisted of four identical boxes (46 × 46 × 50 cm). The horizontal and vertical movement of the animals was measured through their interruption of infrared light beams from cells (sixteen per frame) located at the edges of the apparatus. Beam interruptions caused by the horizontal movements of the animals were detected and registered at a high spatial and temporal resolution (x-y axis). Rearing was measured via an identical frame in the z-axis, which was mounted at a height of 6 cm. The illumination level in the sound-reduced testing room was 30 lx. The boxes were cleaned and wiped before the first test and after each test. The animals were placed in the test box for 20 min. Activity was measured in terms of total activity time, representing time spent in horizontal movement + time spent in vertical training.

2.6 Elevated Plus Maze

The elevated plus maze is an accepted test for measuring situational anxiety [15]. The apparatus was made of black polyvinyl chloride and had two open and two closed arms (50 × 10 × 40 cm) raised 50 cm above the floor. The floor of the arms was smooth, with a light intensity of 30 lux. A mouse was placed on the central platform of the apparatus facing a closed component. A camera on the ceiling of the test room was used to score and tape the animal's behavior from an adjacent room for 7 min. The number of entries into open and closed arms, time spent in open arms, and time spent in closed arms were measured, and the %time spent in open arms (related to a total time of 420 seconds) was calculated. Entry was defined as placing all paws into the respective compartment of the maze. The maze was cleaned and dried after each trial.

2.7 Marble Burying

The marble burying test is used to quantify object-related anxiety, obsessive-compulsive, and repetitive behavior in rodents [16-19].

Mice were placed individually in housing cages containing 12 marbles arranged in a grid pattern on top of 5 cm Cobb bedding for 30 min [20]. After the mice were returned to the home cages, the number of buried marbles (more than 75%) was counted.

2.8 Social Recognition Memory

Social recognition memory reflects the ability of mice to recognize and remember familiar individuals. The brain structures involved in social recognition memory have been described elsewhere [21, 22].

To investigate the social recognition memory of the sham and MCAO mice, we utilized a social discrimination paradigm consisting of a training session in which the subject mouse was presented with a novel mouse from another strain and a recall session in which the subject animal was allowed to investigate the familiar animal from the training session and an additional mouse from a third strain.

The tests were conducted in a 46 × 46 × 50 cm plastic box. The illumination level was 30 lux, and the animals' behavior was scored via a video camera in an adjacent room. The mice were habituated to the test box the day before testing for 15 min. Before the first test and following each test, the box was cleaned and wiped. In trial 1, the counterpart mouse was presented inside a wire containment cup, and social interaction lasted 7 minutes. The time spent in direct contact between the animals was scored as sociability. Afterward, the animals were returned to their home cage. Thirty min later, a second trial was conducted. Here, the test mouse was exposed to the mouse from trial one (familiar) and an additional mouse from a different strain (unfamiliar). The time spent in direct contact was measured. The Retention Index RI was calculated according to the formula:

$$\mathrm{RI} = \frac{(\mathrm{t_{unknown}} - \mathrm{t_{known}})}{(\mathrm{t_{unknown}} + \mathrm{t_{known}})}$$

2.9 Rota-Rod Test

The rota-rod test is used to test motor coordination. The test was performed with the rota-rod apparatus (TSE, Bad Homburg, Germany). The mice were forced to run on a rotating drum with speeds starting at 3 rpm, accelerating to 20 rpm within 60 s. The delay time before they fell from the rotating rod was recorded.

2.10 Hole Board

The hole-board test is recognized and accepted for anxiety and spatial memory [23-25]. The Acti-Mot apparatus (TSE, Bad Homburg, Germany) was utilized for this test. The boxes had a hole board (46 cm × 46 cm) containing 16 equally spaced holes (Ø 1 cm). Horizontal activity and head dipping were measured by the interruption of infrared light beams from cells located in the frames of the apparatus 1 cm below (head dipping) or 4 cm above (locomotor activity) the hole board. The illumination level in the sound-reduced testing room was 30 lx. The animals were placed on the hole board for 10 min at about the same time on three consecutive days. The boxes were cleaned and wiped before the first test and after each test.

2.11 Measurement of Infarct Volume

Twenty-four h after the final hole-board tests, the animals were deeply anesthetised with pentobarbital (60 mg/kg ip) and decapitated. The brains were removed quickly from the skull and frozen in isopentane (-40°C). Coronal sections (50 µm) were cut and stained with cresyl violet. JPG

images from all departments were analyzed using Adobe Photoshop CC2015 to measure the infarct area. To evaluate brain edema, the infarct volume was calculated according to the formula used by Kim et al. [26]. The total infarct volume was calculated as the sum of the infarct volumes from all slices, giving a three-dimensional approximation of the total infarct volume.

2.12 Statistics

Statistical analyses were performed using a two-way ANOVA followed by ANOVA to detect group differences. The independent factors were surgery (sham vs. MCAO) and treatment (saline vs. FP given before or immediately after MCAO). Data were further analyzed using one-way ANOVA followed by post hoc Bonferroni testing. Statistical analyses of hole-board data were performed using a repeated measure ANOVA. The significance threshold was fixed at 0.05.

3. Results

3.1 Locomotor Activity

Locomotor activity was quantified 7 days after surgery. Two-way ANOVA revealed no effects of surgery $F_{1,41}$ = 0.035, p = 0.85 and treatment $F_{2,41}$ = 0.99, p = 0.38 and no surgery x treatment interaction $F_{2,41}$ = 0.07, p = 0.93, Figure 1.

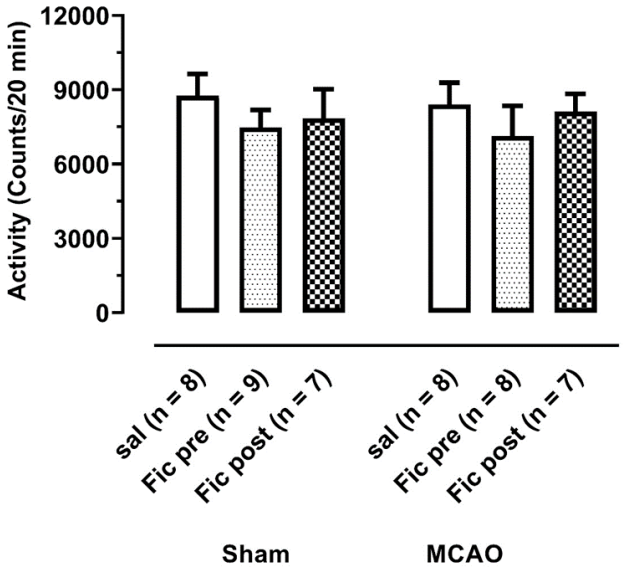

Figure 1 Effect of 50 mg/kg Ficus platyphylla extract given 1 h before or immediately after occlusion of the middle cerebral artery (MCAO) on locomotor activity measured on day 7 after surgery. Sal = saline. Mean ± SEM. There were no significant differences between the control and treated groups.

3.2 Elevated Plus Maze

Situational anxiety was quantified 8 days after surgery. As shown in Figure 2, there were no significant differences between the groups of mice in terms of any parameter. Number of arm changes: surgery $F_{1,41}$ = 3.6, p = 0.065, treatment $F_{2,41}$ = 0.53, p = 0.9, and no surgery x treatment

interaction $F_{2,41}$ = 0.29, p = 0.7. Percentage time spent on open arms: surgery $F_{1,41}$ = 0.3, p = 0.04, treatment $F_{2,41}$ = 0.73, p = 0.48, surgery x treatment interaction $F_{2,41}$ = 1.53, p = 0.229.

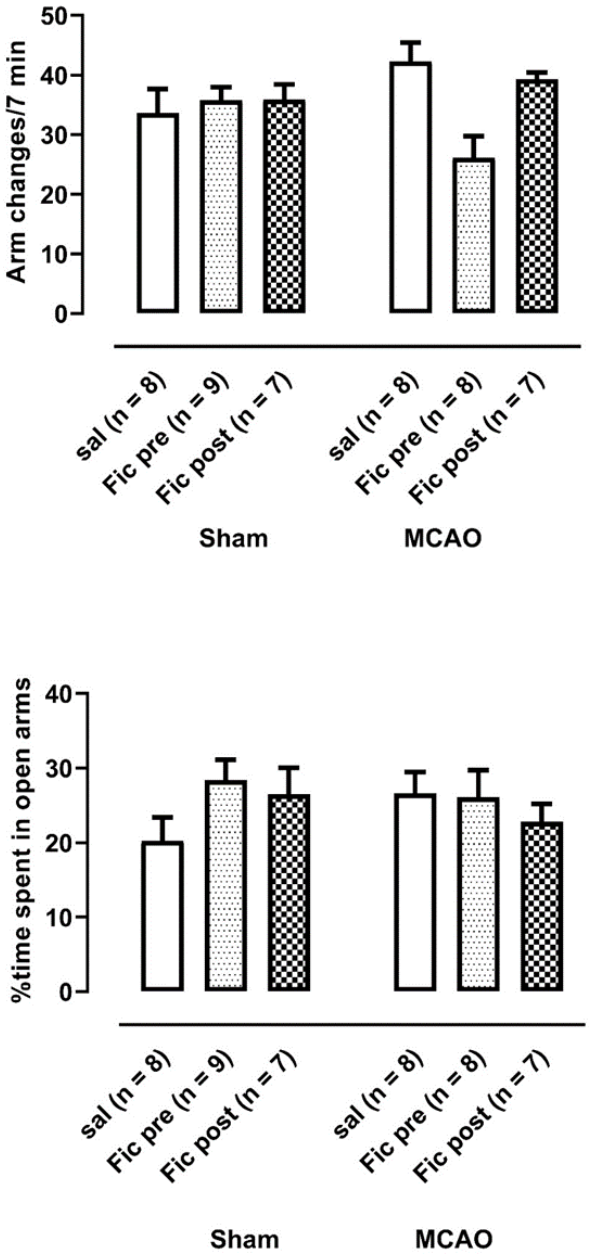

Figure 2 Effect of 50 mg/kg Ficus platyphylla extract given 1 h before or immediately after occlusion of the middle cerebral artery (MCAO) on elevated plus-maze behavior measured on day 8 after surgery. Sal = saline. Mean ± SEM. There were no significant differences between the control and treated groups.

3.3 Marble Burying

As shown in Figure 3, the groups had no significant differences regarding the number of buried marbles. Surgery $F_{1,41}$ = 0.5, p = 0.49, treatment $F_{2,41}$ = 0.41, p = 0.66, and no surgery x treatment interaction $F_{2,41}$ = 1.36, p = 0.27.

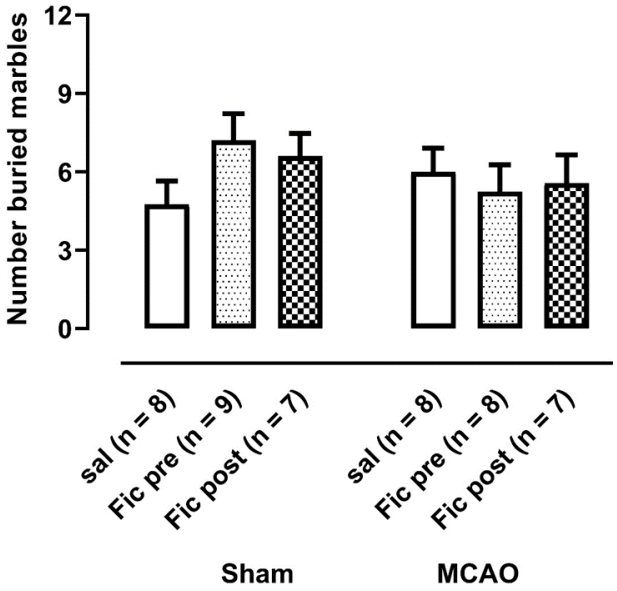

Figure 3 Effect of 50 mg/kg Ficus platyphylla extract given 1 h before or immediately after occlusion of the middle cerebral artery (MCAO) on marble-burying behavior measured on day 9 after surgery. Sal = saline. Mean ± SEM. There were no significant differences between the control and treated groups.

3.4 Social Recognition Memory

As shown in Figure 4, there were no differences between the animals in terms of sociability: surgery $F_{1,41} = 0.13$, $p = 0.72$, treatment $F_{2,41} = 0.06$, $p = 0.99$, and no surgery x treatment interaction $F_{2,41} = 0.36$, $p = 0.7$. In terms of social recognition, there was no effect of surgery $F_{1,41} = 0.2.43$ $p = 0.13$, and no effect of treatment $F_{2,41} = 0.14$, $p = 0.87$, but a significant surgery x treatment interaction $F_{2,41} = 5.6$, $p = 0.007$). Mice from the sham group differed significantly $F_{2,21} = 3.63$, $p = 0.044$. Post hoc Bonferroni testing revealed a considerably lower RI in the group treated with FP before surgery $p < 0.05$. The difference between sal-treated controls and FP immediately after surgery was insignificant ($p = 0.53$). In MCAO animals, there was a significant difference between the treatment groups $F_{2,20} = 4.07$, $p = 0.033$. The animals treated with FP had higher RIs (Bonferroni test, $p < 0.05$).

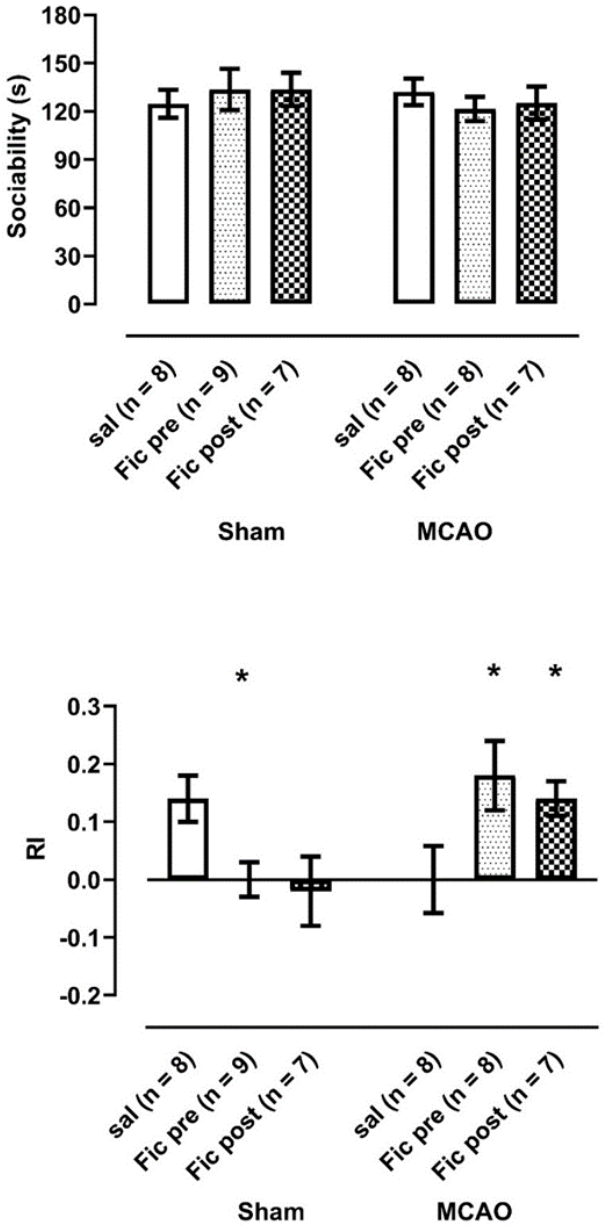

Figure 4 Effect of 50 mg/kg Ficus platyphylla extract given 1 h prior to or immediately after occlusion of the middle cerebral artery (MCAO) on social behaviour measured on day 10 after surgery. Above is sociability; below is the social recognition index RI. Sal = saline. Mean ± SEM. * $p < 0.05$. There were no significant differences between control and treated groups in Sociability.

3.5 Rota Rod

Performance on the rota rod is shown in Figure 5. The length of time mice were able to remain on the rota rod was relatively similar for all groups tested. There were no significant differences related to surgery $F_{1,41} = 0.11$, $p = 0.91$ or treatment $F_{2,41} = 2.3$, $p = 0.61$, and no surgery x treatment interaction $F_{2,41} = 1.04$, $p = 0.36$.

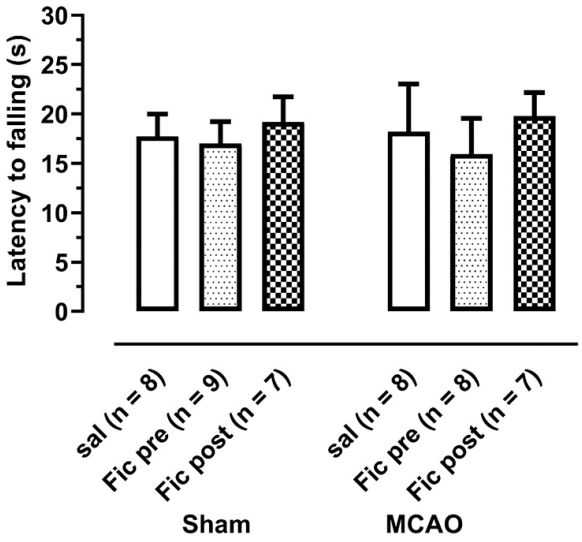

Figure 5 Effect of 50 mg/kg Ficus platyphylla extract given 1 h before or immediately after occlusion of the middle cerebral artery (MCAO) on rota-rod performance measured on day 11 after surgery. Sal = saline. Mean ± SEM. There were no significant differences between the control and treated groups.

3.6 Hole Board

As shown in Figure 6, a learning effect was represented by decreasing exploratory activity over the test period $F_{2,82} = 4.43$, $p = 0.033$, Greenhouse-Geisser adjustment. There was no effect related to surgery $F_{2,82} = 1.66$, $p = 0.19$ or treatment $F_{4,82} = 0.51$, $p = 0.7$, and no surgery x treatment interaction $F_{4,82} = 0.96$, $p = 0.43$.

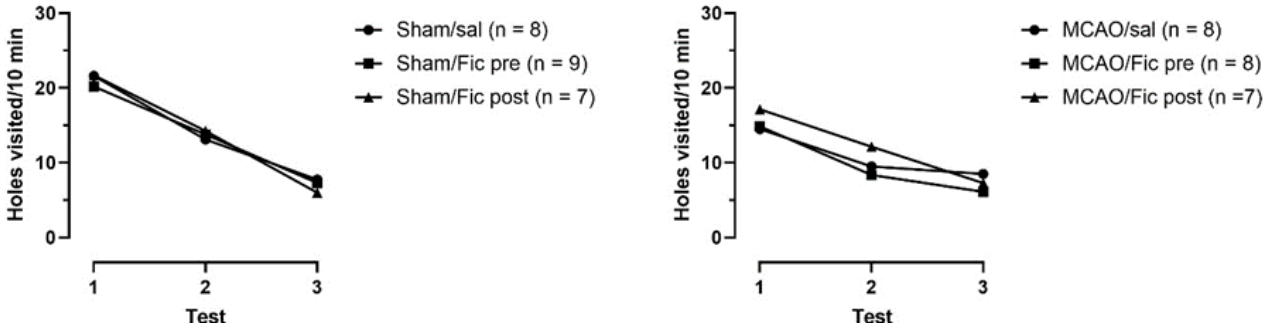

Figure 6 Effect of 50 mg/kg Ficus platyphylla extract (Fic) given 1 h before or immediately after occlusion of the middle cerebral artery (MCAO) on hole-board exploration measured on days 14-17 after surgery. Sal = saline. Mean. For clarity, the SEM has not been given. There were no significant differences between the control and treated groups.

3.7 Lesion Size

Histomorphological analysis of the brains demonstrated that the ipsilateral hemisphere of all animals exhibited pathological alterations. As shown in Figure 7, the animals treated with 50 mg/kg FP 1 h before or immediately after MCAO had smaller infarct sizes than those given saline $F_{2,14} =$

5.44, p = 0.021. The difference between MCAO/sal vs. MCAO/FP before MCAO and MCAO/sal vs. MCAO/FP immediately after MCAO is significant (p < 0.05).

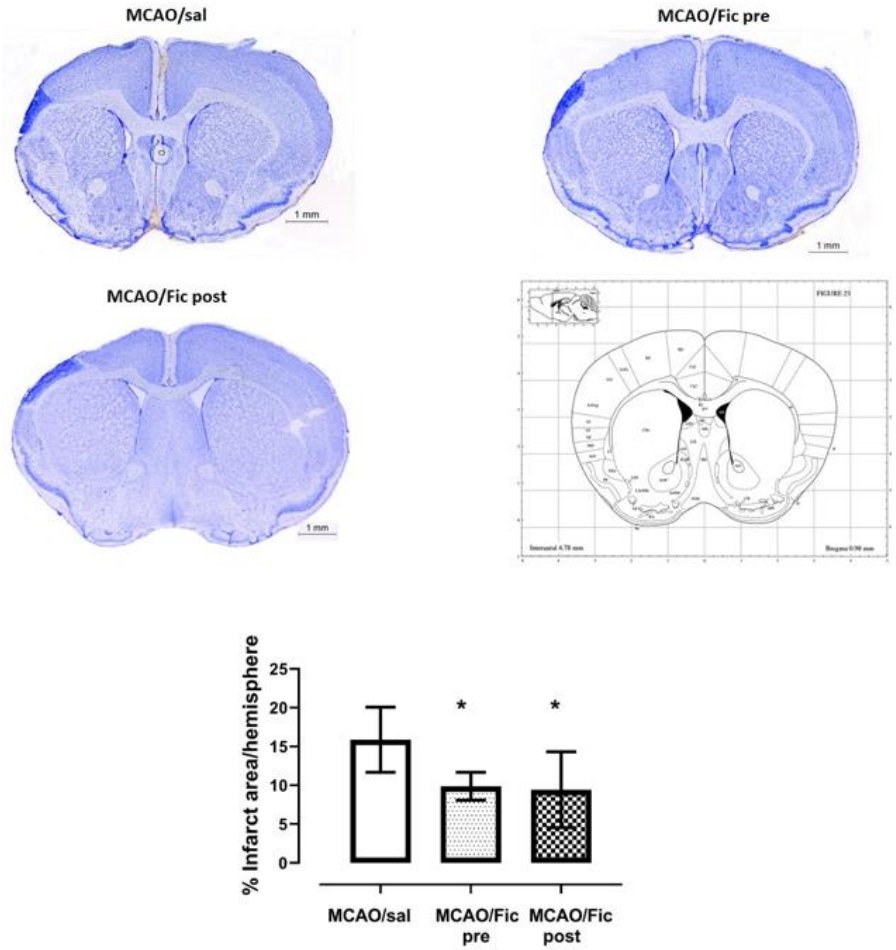

Figure 7 Effect of 50 mg/kg Ficus platyphylla extract (Fic) administered 1 h before or immediately after the middle cerebral artery (MCAO) occlusion on %age infarct volume/hemisphere in mice. Five animals were used per group. * p < 0.05. Reference from [27].

4. Discussion

In the present study, we investigated the long-term effect of Fic-induced hypothermia on functional recovery after ischemia-induced brain damage caused by MCAO. Ten days after MCAO, we found an impairment in social recognition alleviated by hypothermia (Figure 4). Interestingly, there was no impairment in hole-board habituation (Figure 6). Moreover, we did not detect a delayed effect of MCAO on locomotor and emotional behavior or motor coordination (Figures 1-3, 5).

Using the MCAO model of ischaemic stroke, we recently investigated the effect of Fic-induced hypothermia on stroke volume. The brain collected 24 h after the insult exhibited typical tissue loss, reduced by hypothermia. GFAP was found to be upregulated [11] in the brains. In other experiments, a high incidence of GFAP positivity was found on day 14 and day 21 after MCAO [28]. Moreover, these authors found a positive reaction for S100 proteins, which are regarded as damage-associated molecular pattern molecules. Interestingly, these proteins were also detectable in the subthalamic

area [28]. It is well known that reactive astrocytes exert a neuroprotective function in terms of glutamate re-uptake, forming antioxidants, and stimulating neurogenesis [29]. In the present study, brains were collected 19 days after MCAO. The contour of the coronal sections appeared regular, which might be due to astrocyte activation and gliotic scar formation (Figure 7).

Astrogliosis, which occurs after an ischaemic insult, is a complex phenomenon. It might accelerate or retard functional recovery [30, 31]. To investigate active recovery, the animals were tested using a battery of ethologically derived tests for locomotor and anxiety-related behavior and learning performance. There were no significant differences between the experimental groups in terms of locomotor activity (Figure 1), anxiety-related behavior (Figure 2 and Figure 3), and motor coordination (Figure 5). This provides us with a reliable basis on which to compare the learning performance of the animals. As shown in Figure 4, MCAO did not affect sociability but significantly impaired social memory. This impairment was counteracted by Fic-induced hypothermia. Interestingly, MCAO and hypothermia did not interfere with hole-board exploration.

Interruption of the cerebral blood flow results in neuronal necrosis, surrounded by the penumbra region [32]. In mice, the infarct area includes the cortex, striatum, thalamus, hypothalamus, and hippocampus [32-35]. The hippocampal formation is involved in both types of learning [24, 36]. Detailed analysis revealed that the ventral CA1 and the dorsal CA2 played a unique role in social memory [37, 38]. Moreover, this type of learning requires a subpopulation of neurons in the prefrontal cortex [22]. Previous experiments performed 96 h after permanent MCAO showed that treatment with rosmarinic acid improved the animals' performance in the Y-maze, the object recognition tests, and the Morris water maze [29]. These tests are related to frontal-subcortical circuits [39], the hippocampal CA1 region [40], as well as the striatum and cortex [41]. It would appear that MCAO caused impairment in more complex learning tasks, including social recognition (Figure 4). It has previously been discussed that restorative effects on memory are due to synaptogenic activity and inflammatory action [29]. Similar beneficial mechanisms initiated due to Fic-induced hypothermia have been described in previous experiments [11].

Locomotor and emotional behavior tests appear to provide less robust data when assessing long-term functional impairments after permanent MCAO in mice. We propose that behavioral tests based on more complex neuronal circuits might be one tool that might be used to detect long-term neurological damage.

Author Contributions

Martin Helmuth and Axel Becker performed the experiments. Ben A. Chindo participated in the investigation and data analysis, and Axel Becker and Ben A. Chindo wrote and edited the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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