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Long-term behavioral assessment of function in an experimental model for ischemic stroke

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Abstract

Middle cerebral artery occlusion (MCAO) in rats is a well-studied experimental model for ischemic stroke leading to brain infarction and functional deficits. Many preclinical studies have focused on a small time window after the ischemic episode to evaluate functional outcome for screening therapeutic candidates. Short evaluation periods following injury have led to significant setbacks due to lack of information on the delayed effects of treatments, as well as short-lived and reversible neuroprotection, so called false-positive results. In this report, we evaluated long-term functional deficit for 90 days after MCAO in two rat strains with two durations of ischemic insult, in order to identify the best experimental paradigm to assess injury and subsequent recovery. Behavioral outcomes were measured pre-MCAO followed by weekly assessment post-stroke. Behavioral tests included the 18-point composite neurological score, 28-point neuroscore, rearing test, vibrissae-evoked forelimb placing test, foot fault test and the CatWalk. Brain lesions were assessed to correlate injury to behavior outcomes at the end of study. Our results indicate that infarction volume in Sprague-Dawley rats was dependent on occlusion duration. In contrast, the infarction volume in Wistar rats did not correlate with the duration of ischemic episode. Functional outcomes were not dependent on occlusion time in either strain; however, measureable deficits were detectable long-term in limb asymmetry, 18- and 28-point neuroscores, forelimb placing, paw swing speed, and gait coordination. In conclusion, these behavioral assays, in combination with an extended long-term assessment period, can be used for evaluating therapeutic candidates in preclinical models of ischemic stroke.

Keywords

Ischemic stroke; CatWalk; long-term functional recovery; middle cerebral artery occlusion; rat strain

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Disclosure/Conflict of Interest
No conflict of interest to declare.

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1. Introduction

Stroke is the leading cause of adult disability in the industrialized world (Lloyd-Jones et al., 2010). Many studies have identified neuroprotective compounds in experimental cerebral ischemia models (Demers et al., 2005; Fox et al., 1993; Green et al., 1995; Roussel et al., 1992); however, none so far have been translated to a clinically effective therapy (Lakhan et al., 2009). This dearth of clinically effective treatments may have been due to transient protective activity and the timing of administration prior to and following ischemic onset. Additionally, there is high variability among different studies in the severity of ischemic brain injury based on model, strain, vendor, age, and cerebral artery occlusion time (Aspey et al., 1998; Oliff et al., 1995; Rewell et al., 2010; Walberer et al., 2006). It has also been reported that changes in duration of ischemia could alter the therapeutic effect of the same drug (Bardutzky et al., 2005). Therefore, there is a need to develop a consistent, non subjective outcome measures that can detect long-term sensorimotor deficit following middle cerebral artery occlusion (MCAO). Such a model could be advantageous in our efforts for discovery of novel therapeutic strategies for ischemic stroke.

It has been shown that different rat strains display different behavioral and histological outcomes after experimental periods of ischemia (Aspey et al., 2000; Bardutzky et al., 2005; Oliff et al., 1995; Walberer et al., 2006). Sprague-Dawley (SD) rats are reported to develop ischemic damage faster than Wistar-Kyoto rats and develop larger ischemic lesions compared to Wistar rats in a permanent MCAO model (Bardutzky et al., 2005; Walberer et al., 2006). However, transient MCAO induces larger ischemic lesions and formation of edema in Wistar rats (Walberer et al., 2006). Similarly, SD rats are reported to have the smallest infarct lesion compared to both Wistar and Fischer-344 rats after transient MCAO (Aspey et al., 2000). Due to these apparent differences during infarction and injury progression, there is a need for more longitudinal studies in this area (Back et al., 2004). To our knowledge, there has not been a long-term comparison of cerebral damage and functional recovery in these strains of rats, which are interchangeably used in stroke preclinical research studies.

Several studies have investigated the sensorimotor sequela following rat models of cerebral ischemia (Bederson et al., 1986; Borlongan and Sanberg, 1995; Wakayama et al., 2007; Wang et al., 2008; Woodlee et al., 2005; Zhang et al., 2000). Long-lasting behavioral deficits following transient MCAO have been reported in a cross-midline vibrissae-evoked forelimb placement test up to one month, same side placement up to 120 days, and the rearing test up to five weeks in Long-Evans rats (Woodlee et al., 2005). In addition, it has been reported that the rotarod and adhesive removal test can detect behavioral deficits in Wistar rats up to three weeks after two-hour MCAO (Zhang et al., 2000). It has also been shown that the 18-point composite neurological score (composite neuroscore) can be used to detect deficits after up to one week of recovery in Wistar rats subjected to one hour of MCAO (Garcia et al., 1995). The cylinder test has been used in SD rats in a three month study; however, this was done in an MCAO model induced by vasoconstriction, which shows functional recovery to baseline within one month (Hicks et al., 2008).

In this report, we aimed to compare two common rat strains, Sprague-Dawley and Wistar, with two occlusion times, 60 and 75 minutes, and measure long-term histological and behavioral outcome. We evaluated behavioral tests capable of detecting long-term deficits up to twelve weeks after ischemic injury in order to identify ischemic models and behavioral tests which could be reliably used for screening in pharmacological studies.

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2. Materials and Methods

2.1. Subjects and experimental groups

A total of 18 Wistar and 49 SD male rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). In the first study, 18 rats from each strain were sub-divided into two experimental groups subjected to MCAO for 60 minutes; Wistar (n=9) and SD (n=9); and MCAO for 75 minutes; Wistar (n=9) and SD (n=9). In a follow-up experiment, 31 additional SD male rats were used. Twenty-one underwent MCAO for 65 minutes and 10 had sham surgery. Occlusion time was altered from the first experiment in attempt to optimize the model for increased behavioral deficit while minimizing increased infarct volume. Rats were exposed to 12-h light/dark cycles and had access to food and water ad libitum. All experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee of Stanford University and were performed based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Sufficient actions were considered for reducing pain or discomfort of subjects during the experiments.

2.2. Surgical procedure

Rats weighing 300-350 g were subjected to transient MCAO as previously described (Memezawa et al., 1992) with minor modifications. Briefly, a 0.37 mm diameter monofilament with silicon coating (Doccol Corporation, Redlands, CA) was used to occlude the origin of middle cerebral artery (MCA). In the first study, the filament was removed 60 or 75 minutes after MCAO. For the second study, rats were subjected to 65 minutes of MCAO. The body temperature was maintained at 37°C using a homeothermic blanket (Harvard Apparatus, Holliston, MA) during the surgical procedures. For sham surgery, rats were anesthetized and a neck incision was made. The incision site was sutured closed at the end of the procedure. Neurological impairment was assessed using a scoring method described previously (Bederson et al., 1986) in order to confirm successful occlusion.

2.3. Behavioral tests

All rats were handled and trained at least three days prior to the start of the experiment. Behavioral tests were conducted one day prior to surgery and continued 1, 2, 3, 4, 6, 8, 10, and 12 weeks after surgery by a blind and experienced observer. In order to stratify the groups and minimize variability, animals with composite neuroscore greater than 16 on day two after MCAO were excluded. The rearing test, composite neuroscore, 28-point neuroscore, foot fault, and vibrissae-evoked forelimb placing test were run in parallel with the CatWalk to verify long-term behavioral deficits. Animals recovered to baseline performance in the foot fault test after four weeks, and the test was discontinued.

2.3.1. Rearing test—The rearing test was designed to evaluate locomotor asymmetry by observing forelimb activity while rearing against the wall of a clear plastic cylinder (Schallert et al., 2000). It has been cautioned that rats easily habituate to a cylinder and become inactive (Schallert and Woodlee, 2005). To prevent this habituation, an empty-lidded cage with dimensions 18.5 inch length by 10.25 inch width and 8 inch height was used in this study rather than a cylinder. The large arena allowed for increased horizontal ambulation which stimulated rearing behavior throughout the duration of the study. Each animal was allowed to freely explore the arena for up to five minutes. A total of 20 rears were scored by the use of the right paw, left paw, or both paws during rearing. The following equation was used to calculate percentage of impaired paw usage: [(Impaired paw + ½ both paws) / Total rears] * 100.

2.3.2. 18-point composite neurological score—The composite neuroscore comprises 6 different neurological tests: (1) spontaneous activity, (2) symmetry in limb movement, (3)

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forepaw outstretching, (4) climbing, (5) body proprioception, and (6) response to vibrissae touch. Each test is scored with a maximum of 3 points based on a set of pre-determined criteria described elsewhere (Garcia et al., 1995). The scores for each test were summed for a highest possible score of 18, indicating no neurological deficit, and the lowest score of 3, for animals with the most severe impairment.

2.3.3. 28-point neuroscore—The 28-point neuroscore was used to evaluate sensorimotor function and was modified from methods previously described (Lenzlinger et al., 2004). This neuroscore uses eleven tests with a cumulative maximum score of 28: (1) circling (maximum 4 points), (2) motility (maximum 3 points), (3) general condition (maximum 3 points), (4) righting reflex when placed on back (maximum 1 point), (5) paw placement of each paw onto a table top (maximum 4 points), (6) ability to pull self up on a horizontal bar (maximum 3 points), (7) climbing on an inclined platform (maximum 3 points), (8) grip strength (maximum 2 points), (9) contralateral reflex (maximum 1 point) and (10) contralateral rotation when held by the base of tail (maximum 2 points), and (11) visual forepaw reaching (maximum 2 points). Scoring was determined on a scale starting from 0 for severe impairment to the maximum score for healthy function.

2.3.4. CatWalk test—CatWalk (Noldus Information Technology, Wageningen, The Netherlands), a quantitative gait analysis system, was used for assessment of gait following experimental stroke (Lubjuhn et al., 2009; Wang et al., 2008). Prior to the first testing day, the animals were trained to traverse a glass walkway towards their home cage. On subsequent training days, three complete runs across the walkway were recorded by a camera positioned below. If an animal failed to complete a run within 8 seconds, walked backwards, or reared during the run, the animal was given an additional re-run. The average of three runs was reported. Additionally, if an animal was unable to complete runs during any experimental day due to weakness from surgery, the lowest score for the particular group was assigned to the impaired animal. The experiment took place in the dark, and the glass walkway was illuminated with beams of light allowing the paws to reflect light as they came in contact with the glass floor. An experienced observer who was blind to the experimental group labeled each paw on the recorded video and paw-related parameters were reported. A full description of gait-related parameters measured by the CatWalk is defined (Table 1). Pixels below the light intensity of 20 units on a 0-255 arbitrary scale were filtered out. Seven animals from the MCAO group and two from the sham group were unable to complete the CatWalk task or be detected by the CatWalk apparatus on day two and/or one week post-op due to severe impairment. In this case, they were assigned the lowest or highest value for the group on that particular day to indicate the worst score.

2.3.5. Vibrissae-evoked forelimb placing—Forelimb placing assesses the ability of rats to sense tactile stimulus from the vibrissae and subsequently elicit a motor response of forelimb placing (Schallert et al., 2002). In this test, the animal's body is held gently such that the paws are suspended off the experimenter's hand. The animal is then brought laterally to a table top while the vibrissae brush against the edge of the table, allowing the animal to reach the edge with its forepaw. This is repeated ten times on each side. The number of successful paw placements on to the edge of the table is recorded. A cross-midline variation to the test is also performed. Here, the animal is held on its side and successful placements of the opposite paw to the stimulated whisker are recorded.

2.3.6. Foot fault test—The foot fault (Columbus Instruments, Columbus, OH) is used to measure the coordination of an animal walking across a horizontal ladder (Colle et al., 1986). The ladder width is 6.5 inches, with 0.3125 inch beams 1 inch apart. The animal is trained to walk across a ladder 0.25 inch above a steel plate. The plate is then charged once

during training so that if the animal's paw comes in contact with the plate, the animal is lightly shocked. The animal learns consequently to avoid the steel plate by keeping its paws on the ladder beams during each run. Foot misplacements during the run and time to complete each run are counted. The average performance of three consecutive runs is reported.

2.4. Infarct volume

After completion of the last behavioral test time-point, rats were anesthetized with isoflurane and transcardially perfused with 0.9% saline and 3% paraformaldehyde. The brains were removed, post-fixated for 24 hours, and stored in a saline solution with 20% sucrose and 3% PFA before sectioning. The collected brain tissues were sectioned 40 um thick by cryostat in coronal sections and stained with hematoxylin and eosin (H&E) (Wu, 1940). Five sections were selected at approximately 3, 2, 1, 0 and −3 mm from Bregma and scanned into the computer. In each section, an outline was traced around the contralateral hemisphere and the ipsilateral hemisphere using ImageJ (NIH, USA). Infarct area (mm^2) was calculated by subtracting the area of the ipsilateral hemisphere from the contralateral hemisphere. Infarct volume was calculated by the mean cavity area of two adjacent sections multiplied by the distance between the two sections. The total volume was the sum of the volumes for each section.

2.5. Statistical analysis

Data are presented as mean ± SEM. $P<0.05$ is considered statistically significant. Repeated measure two-way ANOVA was used for statistical evaluation of recovery time-points post-surgery for all the groups. The Bonferroni *post hoc* test was used when appropriate. The Student's *t*-test and Mann-Whitney U test were used to compare infarction volume when appropriate based on normality. A Pearson correlation coefficient evaluated the relationship between infarction volume and behavioral outcomes. Statistical analyses were conducted using GraphPad Prism (La Jolla, Ca).

3. Results

3.1. Wistar and SD rats display functional deficit in the rearing test following 60 and 75 minutes of MCAO

The rearing test was used to measure forepaw asymmetry in this model involving unilateral injury. Prior to ischemic injury, both SD and Wistar rats showed no bias of left and right forepaw usage while rearing. Both strains displayed statistically significant differences from the baseline two days after surgery which was dependent on duration of MCAO; Wistar (2-way ANOVA, effect of occlusion time $F_{1, 24} = 5.041$, $P < 0.05$) and SD rats (2-way ANOVA, effect of occlusion time $F_{1, 26} = 5.353$, $P < 0.05$). In Wistar rats, the 75 minute group displayed consistently lower scores than the 60 minute group during the recovery period. However there was no statistically significant difference between these two groups (Fig. 1A). SD rats also showed similar results during recovery between 60 and 75 minutes of occlusion (Fig. 1B). Inter-strain differences between Wistar and SD rats were not statistically significant at 60 or 75 minutes.

3.2. Neurological deficit following 60 and 75 minutes of MCAO

The composite neuroscore assessed neurological deficit following the ischemic injury. All groups showed significant impairment after surgery compared to baseline (Fig1C and D). While Wistar rats showed initial deficit dependant on occlusion time (2-way ANOVA, effect of occlusion time $F_{1, 24} = 4.519$, $P < 0.05$), SD animals did not show any significant differences based on occlusion time. No other significant differences in the 18-point

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neurological score was detected during the recovery period between 60 and 75 minutes of MCAO in Wistar and SD rats.

3.3. Long-term histological outcome following 60 and 75 minutes of MCAO

Histology three months after MCAO was used to measure infarct size. No significant differences in brain infarction were detected at three months of recovery in Wistar rats subjected to 60 (8.63 ± 2.75) or 75 minutes (9.01 ± 2.93) of MCAO. However, SD rats subjected to 75 minutes of MCAO had a significantly larger infarction volume (16.24 ± 1.93) than SD rats subjected to 60 minutes (7.00 ± 2.24) of MCAO (Fig. 1E: $P < 0.05$). Representative microphotographs of the coronal brain section of the Wistar and SD rats subjected to 60 and 75 minutes of MCAO stained with H&E are shown (Fig. 1F). There was no significant correlation between infarction volume and behavioral deficit after twelve weeks of recovery in the rearing test and composite neuroscore for either Wistar or SD rats (Table 2). However, there were statistically significant correlations between the infarction volume at twelve weeks and composite neuroscore at weeks one and two for SD rats subjected to 75 minutes MCAO, week one for Wistar rats subjected to 60 minute MCAO, and week four for Wistar rats subjected to 75 minutes MCAO. A significant correlation between infarction volume and the behavioral outcome in the rearing test was observed at two weeks of recovery following 75 minutes MCAO in Wistar rats.

3.4. Mortality

No mortality was observed in SD rats subjected to 60 minutes of MCAO, while one death occurred in the Wistar group during surgery. Three Wistar rats subjected to 75 minutes of occlusion died within the first week of recovery, indicating 33% mortality. There were three deaths in the 75 minute MCAO SD group within the first week as well, including one additional death after three weeks of recovery, yielding 44% mortality. None of the surviving animals were excluded from analysis. These preliminary behavioral and histological studies resulted in selection of 65 minutes of MCAO in SD rats as a model of focal ischemia for further assessment of behavioral assays suitable for the evaluation of long-lasting functional deficits after experimental stroke.

3.5. Functional deficits of SD rats subjected to 65 minutes MCAO

SD rats subjected to 65 minutes of MCAO showed significantly less body weight gain compared to the sham group during the twelve week recovery period (Fig. 2A: 2-way ANOVA, effect of surgery $F_{1, 232} = 5.940$, $P < 0.05$). This group also displayed significant impairment in the usage of the affected forepaw in the rearing test (Fig. 2B: 2-way ANOVA, effect of surgery $F_{1, 232} = 30.71$, $P < 0.0001$). A significant impairment on day two ($P < 0.001$), week one ($P < 0.01$), week two ($P < 0.05$), week four ($P < 0.001$), week eight ($P < 0.05$), and week twelve ($P < 0.01$) was detected post-MCAO. The 28-point neuroscore also showed a statistically significant behavioral deficit post-MCAO with recovery over time (Fig. 2C: 2-way ANOVA, effect of surgery $F1, 232 = 39.78$, $P < 0.0001$). A significant impairment on day two through week two ($P < 0.001$), weeks four and six ($P < 0.01$), and week twelve ($P < 0.05$) was also detected. The ischemic group showed general neurological deficit in the composite neuroscore over the twelve-week recovery period (Fig. 2D: 2-way ANOVA, effect of surgery $F_{1, 232} = 51.30$, $P < 0.0001$). Further analysis showed MCAO caused significant impairment in this task on day two through week two ($P < 0.001$) and week four ($P < 0.05$).

In the vibrissae-evoked forelimb placing tests, significant impairment was observed contralateral to the brain infarction in right whisker stimulation-right paw placement (Fig. 3A: 2-way ANOVA, effect of surgery $F_{1, 232} = 5.668$, $P = 0.024$) but not in the control side (Fig. 3B). Post hoc analysis showed significant deficit two days post-surgery on the right

side (P < 0.001). Deficit in the cross-midline variation was also observed in right whisker stimulation-left paw placement (Fig. 3C: 2-way ANOVA, effect of surgery $F_{1, 232} = 5.957$, $P = 0.021$) and in the left whisker-right paw (Fig. 3D: 2-way ANOVA, effect of surgery $F_{1, 232} = 5.106$, $P = 0.032$). In the foot fault test, SD rats subjected to MCAO surgery showed significant impairment and rapid recovery in both foot fault count (Fig. 3E: 2-way ANOVA, effect of surgery $F_{1, 232} = 15.98$, $P = 0.0004$) and time to complete run (Fig. 3F: 2-way ANOVA, effect of surgery $F1, 232 = 13.16$, $P = 0.001$). Day two showed significant impairment in both parameters by post hoc analysis (P < 0.001).

3.6. Gait impairment in CatWalk following MCAO

The CatWalk assessment of sensorimotor function post MCAO in rat demonstrate long-lasting deficit for several weeks in behavioral parameters related to the hindpaw. A summary of gait parameters detected by CatWalk in SD rats subjected to 65 minutes of MCAO are provided in Table 3. Additional post hoc analysis showed SD animals have significant impairment on day two in regularity index (P < 0.001), stand index of the right forepaw (P < 0.001), swing duration of the right hindpaw (P < 0.001), stride length of the left and right hindpaws (P < 0.001), base of support for the forepaws and hindpaws (P < 0.001), paw area of the right forepaw (P < 0.001) and left forepaw (P < 0.05), coordination of left forepaw stance during right hindpaw swing (P < 0.001), coordination of the left forepaw stance during left hindpaw swing (P < 0.01), and coordination of the left hindpaw stance during left forepaw swing (P < 0.05). Deficits during the first week include the paw areas of the right forepaw and hindpaw (P < 0.01) and left forepaw (Table 3: P < 0.05). Base of support was impaired in the forepaws during week two (P < 0.01). The most significant parameters analyzed by CatWalk are described in further detail below, including the swing speed, intensity, duty cycle, and interlimb coordination of girdle pairs (Figs. 4-7).

Swing speed—MCAO induced a significant decrease in swing speed in the right forepaw (Fig. 4A: 2-way ANOVA, effect of surgery $F_{1, 232} = 6.726$, $P = 0.015$), left forepaw (Fig. 4B: 2-way ANOVA, effect of surgery $F_{1, 232} = 7.708$, $P = 0.010$), right hindpaw (Fig. 4C: 2-way ANOVA, effect of surgery $F_{1, 232} = 23.55$, $P < 0.0001$) and left hindpaw (Fig. 4D: 2-way ANOVA, effect of surgery $F_{1, 232} = 13.63$, $P < 0.001$). All paws showed significant recovery over time post-surgery. Post hoc analysis of the data showed that swing speed for all paws was significantly reduced on the second day after MCAO ($P < 0.001$). However, there was no significant difference in this parameter one week after surgery in the forepaws and ipsilateral hindpaw. In contrast, a sustained impairment was detected in the contralateral hindpaw for the duration of the study which was statistically significant up to 4 weeks ($P < 0.05$).

Paw intensity—Animals subjected to MCAO applied significantly less paw pressure during locomotion on all four paws; right forepaw (Fig. 5A: 2-way ANOVA, effect of surgery $F_{1, 232} = 16.44$, $P = 0.0003$), left forepaw (Fig. 5B: 2-way ANOVA, effect of surgery $F_{1, 232} = 9.644$, $P = 0.004$), right hindpaw (Fig. 5C: 2-way ANOVA, effect of surgery $F_{1, 232} = 13.62$, $P < 0.001$), and left hindpaw (Fig. 5D: 2-way ANOVA, effect of surgery $F_{1, 232} = 6.803$, $P = 0.014$). A gradual recovery of the paw pressure deficit during the recovery time was detected in all four paws. Post hoc analysis revealed significant differences in right ($P<0.001$) and left ($P<0.01$) forepaws at two days and one week post-surgery.

Duty cycle—Animals subjected to MCAO displayed a decrease in duty cycle in the affected right hindpaw only which varied over time (Fig. 6C: 2-way ANOVA, effect of surgery $F_{1, 232} = 10.95$, $P = 0.003$). The right forepaw showed animals subjected to MCAO have a significantly delayed recovery over time compared to sham operated animals (Fig.

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6A: 2-way ANOVA, effect of surgery $F_{1, 232} = 2.373$, $P = 0.134$). The left hindpaw and forepaw showed no significant differences compared to the sham animals (Fig. 6B and D) Post hoc analysis of the data showed significant differences in the right hindpaw duty cycle at two days and one week post-MCAO ($P<0.05$).

Interlimb coordination—No significant differences between the MCAO and sham group were detected when placing the left forepaw during right forepaw stance (Fig. 7A). The right forepaw showed delayed placement during left forepaw stance (Fig. 7B: 2-way ANOVA, effect of surgery $F_{1, 232} = 9.218$, $P = 0.005$). MCAO led to faster placement of the left hindpaw during right hindpaw stance (Fig. 7C: 2-way ANOVA, effect of surgery $F_{1, 232} = 20.16$, $P = 0.0001$). When the left hindpaw is in stance, there is a significant difference between sham and MCAO groups during right hindpaw placement (Fig. 7D: 2-way ANOVA, effect of surgery $F_{1, 232} = 10.25$, $P = 0.003$). Post hoc analysis showed that the left forepaw anchor with right forepaw target had a deficit at two days ($P<0.001$) and two weeks ($P<0.05$) post-surgery. In addition, the right hindpaw anchor with left hindpaw target showed a deficit at day two ($P<0.05$) and week two ($P<0.01$).

4. Discussion

Many experimental studies have identified potential neuroprotective treatments against ischemic stroke; however none so far have been translated to a clinically effective treatment (Lakhan et al., 2009). The lack of translation of these discoveries to clinically effective treatments may have been due to a lack of routine assessment of long-term behavioral outcomes alongside the pathological outcome. In this study we aimed to identify behavioral assays which could be conducted over the long term for assessment of the functional deficits following experimental stroke. We have clearly shown that SD rats display larger brain infarction when subjected to a longer ischemic episode in comparison to Wistar rats, which did not display such a time-response. Furthermore, it seems that there is no direct correlation between long-term functional outcomes and the duration of MCAO in these two strains. A correlation between behavior and brain injury was detected early on but not beyond one month of recovery. Our results indicate that long-term neurological deficits from weeks to months are detectable using the composite neuroscore, rearing test, and 28-point neuroscore. Gait analysis demonstrated significant long-lasting deficits for several weeks in areas including swing speed, paw pressure, duty cycle, and coordination of girdle paws. A more pronounced behavioral deficit was detectable in the affected hind paw during recovery. This will provide a sensitive assessment tool for the evaluation of performance of the hind paw after stroke.

4.1. Histological outcome after experimental stroke

Although several studies demonstrate SD rats are less susceptible to ischemic injury than Wistar rats in reperfusion models (Aspey et al., 1998; Oliff et al., 1995; Walberer et al., 2006), our results indicate that after three months of recovery, SD rats were more sensitive than Wistar rats. SD rats subjected to 75 minute MCAO had a larger infarct volume than SD rats subjected to 60 minute MCAO, whereas increasing the occlusion time had no effect on the infarction volume of Wistar rats. This discrepancy between strains may be due to larger collateral circulation in Wistar rats in comparison to SD rats, leading to more blood flow in the brain during MCAO. It has been shown that infarction volume does not necessarily differ between MCAO and MCAO with concurrent occlusion of the contralateral CCA, a result dependant on strain and vendor (Oliff et al., 1995). This result suggests longer occlusion time causing more cerebral damage in SD rats, compared to Wistar rats. Delayed mortality 3 weeks after MCAO in SD rats but not Wistar rats further supports this.

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Additionally, it has been demonstrated that interstrain differences in cerebral blood flow can vary the progression of ischemic damage over time (Bardutzky et al., 2005). The severity of ischemic damage, as our results verify, is dependent on the timing and duration of assessment, with ischemic damage progressing differently in different strains of rats during the reperfusion period. It is notable that the recovery period used in our study is much longer than the follow-up period for histological outcome in previous studies (Aspey et al., 1998; Oliff et al., 1995; Walberer et al., 2006). This emphasizes the need to measure the extent of cerebral damage in experimental models of the stroke over a longer period of time.

4.2. Behavioral outcome after experimental stroke

We have shown that MCAO in rats leads to short-term as well as long-lasting behavioral deficits up to three months after the injury. Our results also show no direct correlation between the extent of infarct and behavioral outcomes after three months of recovery. However, significant correlations between the final infarction volume and earlier behavior outcomes were apparent in the composite neuroscore and rearing tests. Fewer occurrences of statistically significant correlations in the rearing test may indicate that asymmetric limb motor function is not affected by cerebral infarction as much as the composite neuroscore, which involves more sensory and general activity tasks. Lack of correlation between infarction size and behavioral outcomes may furthermore highlight that ischemic damage in one cerebral hemisphere can cause an adaptive response by the intact hemisphere to restore cross-midline sensorimotor functions over time (Jones et al., 2009; Nudo, 2007; Woodlee et al., 2005). The variability in the results from different tests further indicates the need to run a multitude of behavioral assays when evaluating the functional deficit and recovery in these experimental models of ischemic stroke. In addition, we generally observed that functional recovery was not dependant on occlusion time; however, the initial deficit post-surgery was. This finding again stresses the importance of long-term assessment when evaluating the injury itself and potential therapeutic strategies.

Automated gait analysis was used in this rat model as a novel method of measuring motor deficit following MCAO, in addition to the rearing test, composite neuroscore, paw placement, foot fault, and 28-point neuroscore. While these tests can evaluate gross asymmetry and neurological function, the CatWalk can show detailed impairment of each individual paw and overall gait pattern. A previous study reported gait impairment in a distal model of MCAO combined with bilateral occlusion of the CCA (Wang et al., 2008). Investigators in this report demonstrated a gait impairment five weeks post-MCAO in paw intensity, area, placement, lateral paw support time, and phase dispersion of ipsilateral paws when the impaired forepaw is in stance and hindpaw is in swing. Our findings show short-term deficits recovering within two weeks post-MCAO including intensity, stride length, stand index, duty cycle, and the timing of the placement of the impaired forepaw in relation to the contralateral forepaw, along with longer-lasting deficits in paw swing speed and the timing of placement of the contralateral hindpaw in relation to the impaired hindpaw. A decrease in intensity is in accordance with previous studies suggesting damage to the motor cortex causes reduced weight bearing (Starkey et al., 2005; Wang et al., 2008). In the distal MCAO model, chronic effects are observed in relative paw placement and step phase of ipsilateral paws (Wang et al., 2008). However, in the suture model used in this study, we did not observe these impairments either transiently or over time. A slower swing speed, particularly in the affected hindlimb, was observed. In addition, the step phase of hind limbs was altered transiently, indicating an asymmetrical limp in the distal model (Wang et al., 2008), but our model shows long term-impairment in both hind and forelimbs, where the affected forepaw is quicker to place than the unaffected forepaw, and the unaffected hindpaw is slower to place than the affected hindpaw. These apparent differences could be

explained by the injuries developed in the model used here in the subcortical and striatum areas.

5. Conclusion

Our results indicate that SD rats are more responsive to MCAO time than Wistar rat. Furthermore, we have shown that infarction volume does not correlate to behavioral differences *per se* in either of these two strains. In addition, long-term assessment through parallel behavioral tests is needed when testing novel therapeutic candidates due to the interstrain variability of the progression of ischemic damage and functional recovery. We have demonstrated that long-term deficits following cerebral ischemia are measurable through multiple tests. To this end, the Catwalk is a useful tool that can be used in conjunction with traditional sensorimotor tests, including the rearing test, 18-point composite neurological score, vibrissae-evoked forelimb placing, foot fault, and 28-point neuroscore. Finally, abnormalities detected in hind paw movement by catwalk post MCAO are long-lasting and can be used for long term assessment of function post injury.

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Highlight

- Duration of cerebral ischemic episode affects rat strains differently
- Infarct volume, but not necessarily behavior, differs between strain after ischemia
- Several sensorimotor deficits are still measurable 3 months after cerebral ischemia
- CatWalk can evaluate gait deficit in individual paws, especially in the hindpaw
- Functional recovery needs to be assessed long-term with parallel behavior tests

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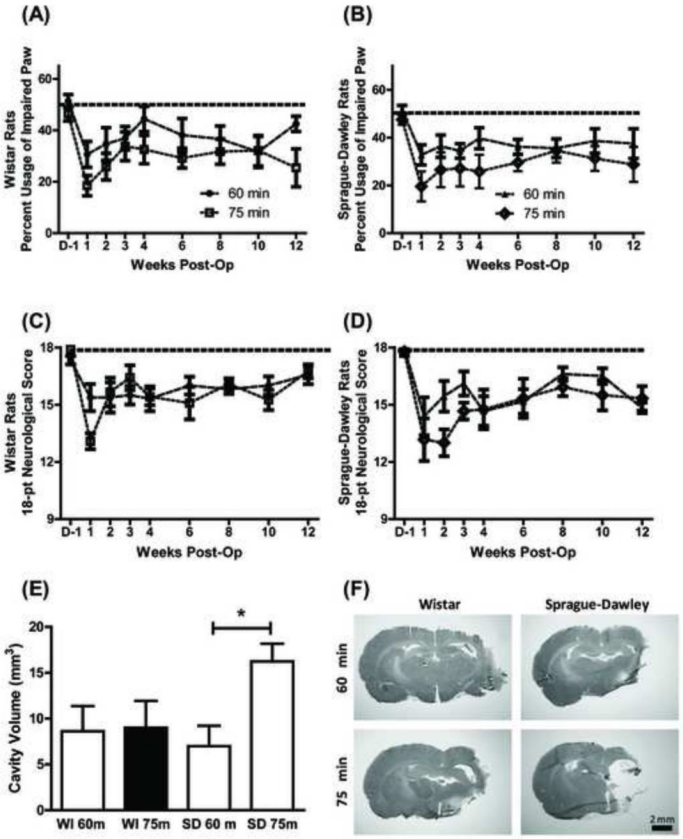

Fig. 1.

Functional deficits and histological outcomes after MCAO. (A) Percentage of impaired forelimb usage during the rearing test for Wistar rats subjected to MCAO (60 mins, n = 8; 75 mins, n = 6). (B) Percentage of impaired forelimb usage for SD rats subjected to MCAO (60 mins, n = 9; 75 mins, n = 6). (C) The composite neuroscore for Wistar rats subjected to MCAO (60 mins, n = 8; 75 mins, n = 6). (D) The composite neuroscore for SD rats subjected to MCAO (60 mins, n = 9; 75 mins, n = 6). (E) Cavity volume three months post-MCAO (60m, 60 minutes MCAO; 75m, 75 minutes MCAO, * $p<0.05$ Mann-Whitney U-test). (F) A representative image from each group with a cross section at −1.80 mm from Bregma. (Error bar: S.E.M. The X-axis, D indicates days).

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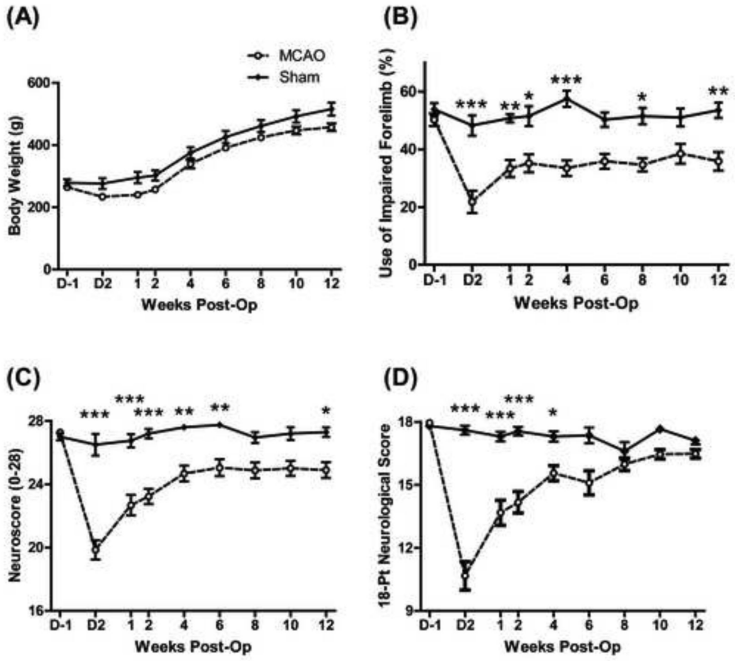

Fig. 2.

Effect of 65 minute MCAO on SD rats. (A) Body weight decreases after MCAO. (B) Asymmetry of forelimb use during the rearing test is observed up to twelve weeks post-MCAO. (C) Functional deficits persist for twelve weeks in the 28-point neuroscore. (D) The composite neuroscore shows MCAO causes significant impairment for up to four weeks. (Error bar: S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ Bonferroni post hoc. The X-axis, D indicates days) MCAO n = 21, sham n = 10.

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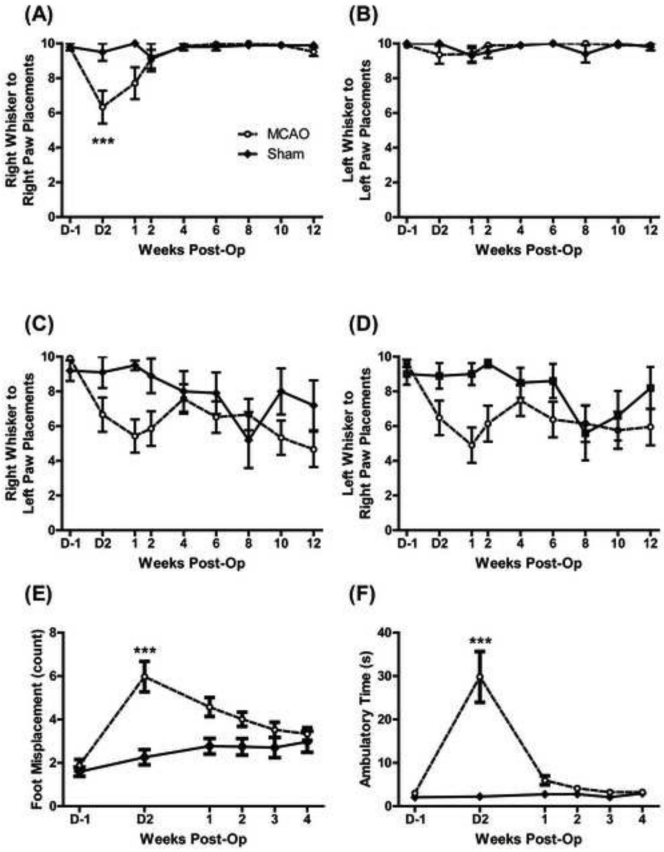

Fig. 3.

Short-term deficits observed in SD animals subjected to 65 minute MCAO. (A) Right whisker stimulation of the right forepaw is impaired post-op. (B) Left whisker stimulation of the left forepaw remains intact. (C) Right whisker stimulation of left paw placement shows deficit. (D) Deficit also observed in left whisker stimulation of right paw placement. (E) Increase in foot misplacements in the foot fault test. (F) Increase in trial duration during foot fault test. (Error bar: S.E.M. *** $p<0.001$ Bonferroni post hoc. The X-axis, D indicates days) MCAO n = 21, sham n = 10.

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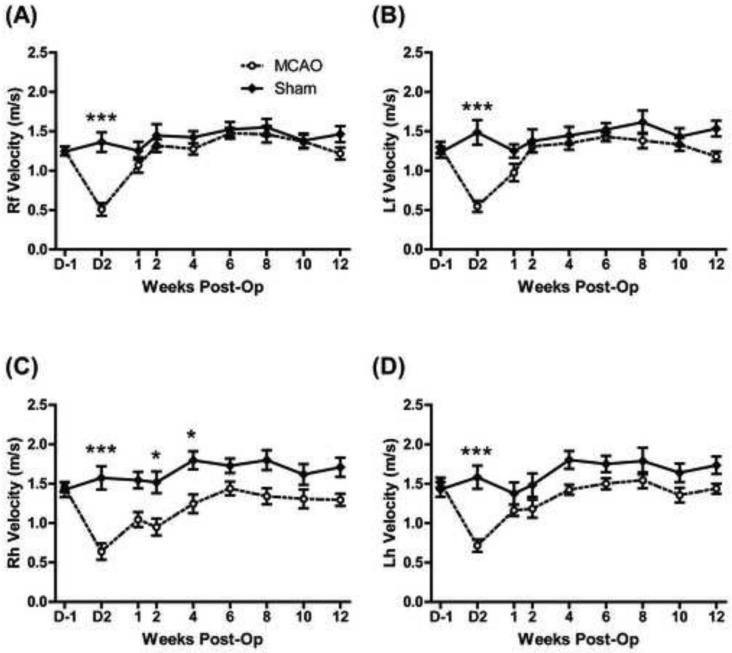

Fig. 4.

Sixty-five minute MCAO causes a decrease in paw swing speed in SD rats. (A) Right forepaw, Rf. (B) Left forepaw, Lf. (C) Right hindpaw, Rh. (D) Left hindpaw, Lh. (Error bar: S.E.M. * $p<0.05$, *** $p<0.001$ Bonferroni post hoc. The X-axis, D indicates days.) MCAO n = 21, sham n = 10.

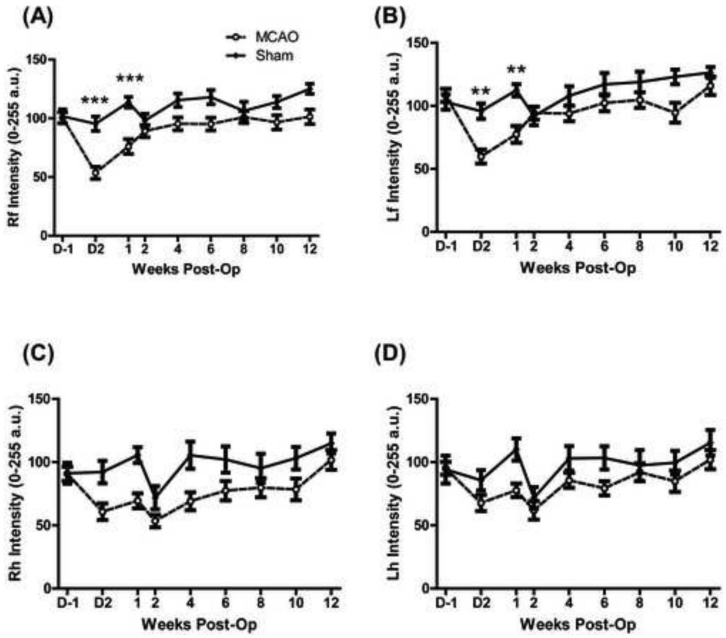

Fig. 5.

Effect of 65 minute MCAO on paw pressure exerted on the walkway in terms of pixel brightness or intensity. (A) MCAO reduced the paw pressure applied during locomotion in the right forepaw, Rf. (B) Decreased paw pressure during locomotion in the left forepaw, Lf. (C) Decreased paw pressure in the right hindpaw, Rh. (D) Decreased paw pressure in the left hindpaw, Lh. (Error bar: S.E.M. ** $p<0.01$, *** $p<0.001$ Bonferroni post hoc. The X-axis, D indicates days.) MCAO n = 21, sham n = 10.

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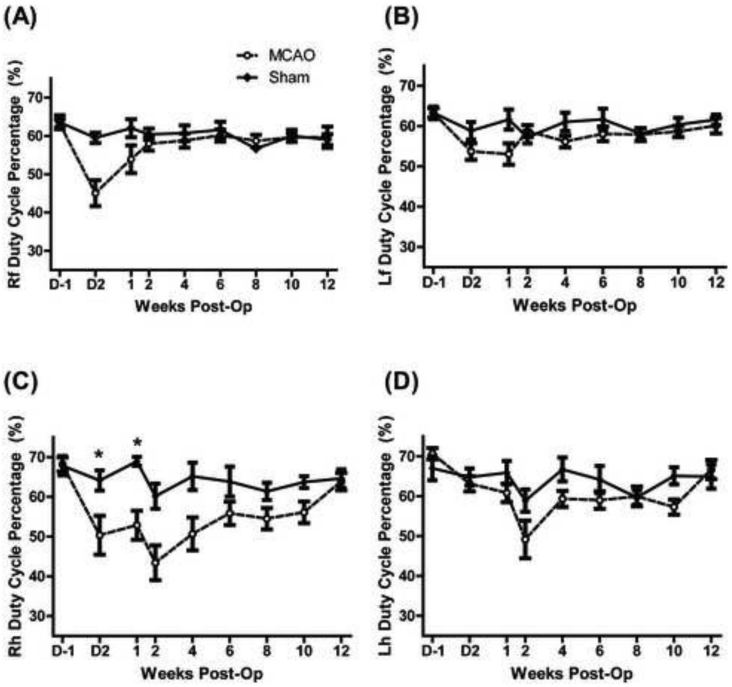

Fig. 6.

Effect of 65 minute MCAO on paw duty cycle, or the percentage of time a paw is in stance in relation to the whole stride cycle. (A) Right forepaw, Rf, remains intact post-MCAO. (B) No deficit in left forepaw, Lf. (C) The fraction of time the right hindpaw, Rh, is in stance accounts for less of the stride cycle in SD animals subjected to MCAO compared to sham-operated animals (* $p<0.05$ Bonferroni post hoc). (D) No deficit in left hindpaw, Lh. (Error bar: S.E.M. The X-axis, D indicates days.) MCAO n = 21, sham n = 10.

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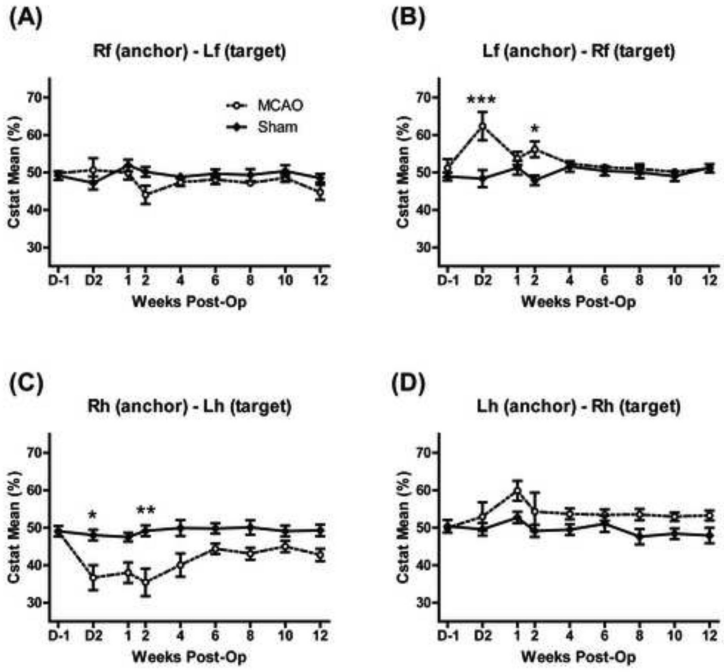

Fig. 7.

Interlimb coordination of girdle paws after 65 minute MCAO in SD rats. (A) Left forepaw, Lf, remains intact during swing. (B) Delayed placement of the right forepaw, Rf, during swing. (C) Left hindpaw, Lh, positions faster when the right hindpaw, Rh, is in stance. (D) The right hindpaw is delayed during swing while the left forepaw is in stance. (Error bar: S.E.M. Anchor is the paw in stance. Target is the paw in swing. The X-axis, D, indicates days. Cstat mean refers to the temporal phase relationship between two paws during stride cycles. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ Bonferroni post hoc.) MCAO n = 21, sham n = 10.

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Table 1

CatWalk gait parameter definitions

Parameter (unit)	Definition
Run duration (s)	Time duration of entire run
Regularity (% index)	Four times the number of step sequences over the total number of paw steps
Ab stride pattern (% usage)	Step sequence from Lf to Rh to RF to LH
Print position (mm)	Distance from former forepaw position to consecutive hindpaw position
Base of support (mm)	Distance between girdle paw pairs
Support (% time standing)	Percentage of time two paws are in simultaneous contact with the floor
Phase lag (%)	Percentage of time the target paw takes to step in relation to the step cycle of the anchor paw
Stand duration (s)	Time duration of the paw in contact with the floor during a step cycle
Swing duration (s)	Time duration between two consecutive steps of the paw during a step cycle
Stride length (mm)	Distance the paw travels from one step to the next
Stand index (index ratio)	Index is an indication of the speed the paw is lifted from the floor
Paw area (mm^3)	Size of the paw in contact with the floor
Max contact at (% time)	Percentage of time the paw takes to get to maximum contact with floor over the total stand duration
Swing speed (m/s)	Stride length over the swing duration
Intensity (0-255 a.u)	Pressure of paw in contact with the floor
Duty cycle (% time)	Percentage of time the paw accounts for the total step cycle of that paw
Interlimb Coordination (%)	Similar to Phase lag however assumes that the target paw placements can not preceed anchor paw

Rf - Right forepaw; Lf - Left forepaw; Rh - Right hindpaw; Lh - Left hindpaw

Anchor paw - paw in stance; Target paw - paw in swing

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Table 2

The relationship between cavity volume three months after MCAO and behavioral outcome in the rearing test and 18-point composite neurological score

	Rearing Test								18-point Composite Neurological Score							
	Wistar 60m		Wistar 75m		SD 60m		SD 75m		Wistar 60m		Wistar 75m		SD 60m		SD 75m	
	Pcc	*P* value	Pcc	*P* value	Pcc	*P* value	Pcc	*P* value	Pcc	*P* value	Pcc	*P* value	Pcc	*P* value	Pcc	*P* value
Day-1	0.045	0.916	0.146	0.782	−0.536	0.215	−0.197	0.709	0.530	0.177	−0.049	0.926	0.037	0.937	−0.019	0.972
Week 1	−0.456	0.257	−0.765	0.076	−0.660	0.107	−0.792	0.060	−0.759	0.029*	−0.721	0.106	−0.750	0.052	−0.930	0.007**
Week 2	−0.351	0.393	−0.878	0.021*	−0.457	0.303	−0.503	0.309	−0.627	0.096	−0.632	0.178	−0.627	0.132	−0.822	0.045*
Week 3	−0.128	0.762	−0.627	0.183	0.066	0.888	−0.773	0.071	−0.349	0.397	−0.480	0.335	−0.590	0.163	−0.449	0.372
Week 4	−0.143	0.735	−0.383	0.453	−0.307	0.503	−0.425	0.401	−0.608	0.110	−0.940	0.005**	−0.622	0.136	−0.068	0.899
Week 6	−0.140	0.741	−0.516	0.295	−0.287	0.532	−0.564	0.244	−0.500	0.208	−0.156	0.768	−0.701	0.079	−0.218	0.679
Week 8	−0.234	0.577	−0.019	0.972	0.094	0.841	−0.382	0.455	−0.271	0.516	−0.510	0.301	−0.317	0.488	−0.467	0.351
Week 10	−0.514	0.192	−0.664	0.150	0.435	0.329	−0.497	0.316	−0.240	0.568	0.723	0.105	0.099	0.832	0.761	0.079
Week 12	−0.332	0.422	−0.585	0.223	0.419	0.350	−0.237	0.651	−0.523	0.183	−0.071	0.894	0.203	0.662	0.366	0.476

60m, 60 min MCAO; 75m, 75 min MCAO; SD Sprague Dawley

Pcc- Pearson Correlation Coefficient

* $P < 0.05$,

** $P < 0.01$

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Table 3

CatWalk parameter statistics for measuring functional recovery in gait

	Effect					
	MCAO surgery		Recovery Time		Interaction	
	F Value	P value	F Value	P value	F Value	P value
Run duration	1.941	0.174	6.202	< 0.0001 ***	3.893	0.000
Regularity	5.639	0.024 *	2.641	0.009	2.055	0.041
Ab stride pattern	2.012	0.167	1.966	0.052	2.847	0.005
Print position						
right	0.673	0.419	1.026	0.417	2.799	0.006
left	0.004	0.953	0.946	0.479	1.183	0.310
Base of support						
forepaw	27.340	< 0.0001 ***	5.203	< 0.0001 ***	0.911	0.508
hindpaw	16.510	< 0.001 ***	3.285	0.001	0.751	0.646
Support						
ipsilateral	1.635	0.211	4.641	< 0.0001 ***	1.704	0.098
diagonal	0.203	0.656	2.447	0.015	1.070	0.385
girdle	0.583	0.451	2.010	0.046	0.778	0.623
Phase lag						
Diagonal						
Rf -> Lh	2.089	0.159	1.063	0.390	1.757	0.087
Lh -> Rf	3.572	0.069	1.573	0.134	1.910	0.059
Lf -> Rh	5.695	0.024 *	1.526	0.149	2.158	0.032
Rh -> Lf	9.158	0.005 **	2.282	0.023	1.348	0.221
Ipsilateral						
Rf -> Rh	0.318	0.577	0.986	0.448	2.126	0.034
Rh -> Rf	4.559	0.041 *	1.811	0.076	0.621	0.760
Lf -> Lh	2.082	0.160	1.197	0.301	2.498	0.013
Lh -> Lf	1.231	0.276	1.820	0.074	2.578	0.010
Stand Duration						

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	Effect					
	MCAO surgery		Recovery Time		Interaction	
	F Value	P value	F Value	P value	F Value	P value
Rf	0.996	0.327	2.765	0.006	3.389	0.001
Rh	0.004	0.949	3.195	0.002	1.658	0.110
Lf	1.859	0.183	4.175	0.000	2.138	0.033
Lh	0.916	0.347	7.474	< 0.0001 ***	1.571	0.134
Swing duration						
Rf	2.096	0.158	4.661	<0.0001 ***	1.889	0.063
Rh	14.850	0.001 ***	0.933	0.490	1.293	0.248
Lf	2.216	0.147	4.574	<0.0001 ***	2.645	0.009 **
Lh	5.698	0.024 *	1.013	0.427	1.544	0.143
Stride length						
Rf	1.194	0.284	17.150	<0.0001 ***	3.023	0.003 **
Rh	2.051	0.026 *	14.500	<0.0001 ***	2.273	0.023 *
Lf	1.668	0.207	24.970	<0.0001 ***	3.634	<0.001 ***
Lh	4.259	0.048 *	20.690	<0.0001 ***	3.764	<0.001 ***
Stand index						
Rf	7.532	0.010 *	2.125	0.034 *	1.895	0.062
Rh	0.051	0.824	1.601	0.125	1.787	0.081
Lf	1.174	0.288	2.591	<0.010 **	2.296	0.022 *
Lh	0.726	0.401	2.173	0.030 *	2.380	0.018 *
Paw area						
Rf	22.360	<0.0001	8.818	<0.0001 ***	1.421	0.188
Rh	14.370	<0.001 *	4.372	<0.0001 ***	1.116	0.353
Lf	8.304	0.007 **	8.297	<0.0001 ***	2.008	0.046 *
Lh	11.330	0.002 **	3.817	<0.001 ***	0.728	0.667
Max contact at						
Rf	1.721	0.200	2.178	0.030 *	0.445	0.893

$watermark-text $watermark-text $watermark-text

	Effect					
	MCAO surgery		Recovery Time		Interaction	
	F Value	P value	F Value	P value	F Value	P value
Rh	0.036	0.851	1.457	0.174	1.844	0.070
Lf	1.018	0.321	3.249	0.001**	0.409	0.915
Lh	0.000	0.990	2.805	0.006**	3.048	0.003**

Rf - Right forepaw; Lf - Left forepaw; Rh - Right hindpaw; Lh - Left hindpaw

Anchor paw -> Target paw

*$P < 0.05$,

**$P < 0.01$,

***$P < 0.001$