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Research Report

Cerebral embolic ischemia in rats: Correlation of stroke severity and functional deficit as important outcome parameter

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ABSTRACT

The embolic MCA occlusion model in rats is used for recanalisation studies in acute stroke. In addition to the determination of lesion size, the assessment of functional outcome may improve the value of this model. Male Wistar rats were submitted to MCA clot embolism or sham surgery. In order to achieve a larger variety of lesion volume, 2 subgroups (each 7 animals) were subjected to differently sized emboli (30 and 40 mm). Follow-up period was 6 days. Outcome assessment consisted of a test battery including parallel bar crossing, observation of behaviour in an open field and an 8-arm maze and a neurological score with ten different sensorimotor and coordinative items. Animals were perfusion-fixed on day 7 (blinded examination). For both subgroups, there were significant impairments with regard to performance on the Neuro score, parallel bar crossing and maze exploration. Improvement was only partial during the follow-up period. On follow-up day 6, there was still a significant correlation between total infarct volume and functional outcome on the Neuro score ($R=0.80$, $p=0.0006$) and the exploration behaviour in the maze ($R=0.66$, $p=0.01$). Application of emboli with a length of 40 mm caused more functional impairment and a more extended lesion volume compared with 30 mm. We present outcome tests that provide quantitative and objective tools to test functional impairment in rats following embolic stroke.

1. Introduction

Efficacy of intravenous thrombolysis for acute stroke treatment is limited due to a relatively poor recanalisation rate (Mori et al., 1992) and due to incomplete functional recovery in the majority of treated patients (The National Institute, 1995; The ATLANTIS, 2004). Animal experiments can help to improve the existing therapeutic strategies. A model of thromboembolic occlusion is required to investigate the safety and efficacy of new antithrombotic therapies and to evaluate combination therapies with neuroprotective agents and new recanalisation strategies such as ultrasound mediated throm-

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bolysis (Daffertshofer et al., 2004; Nedelmann et al., 2005; Schneider et al., 2006; Zhang et al., 2005). Different animal models of embolic cerebral vessel occlusion have been developed (Busch et al., 1997; Krueger and Busch, 2002; Zhang et al., 1997a,b). The evaluation of these models is mainly based on conventional endpoints such as ischemic lesion volume. In order to resemble human studies of neuroprotective and recanalisation therapies as closely as possible and to improve the value of experimental evaluation in animal models, the assessment of the functional outcome is increasingly recommended.

Functional impairment of animals subjected to brain ischemia has been studied after induction of ischemia with different models of transient or permanent middle cerebral artery (MCA) occlusion (Zausinger et al., 2000; Ding et al., 2001a,b, 2002). Different motor and behavioural tests have been validated for their appropriateness to assess various degrees of brain damage (Reglodi et al., 2003; Rogers et al., 1997; Roof et al., 2001). However, there has not yet been a study that investigates the value of different functional outcome parameters in an embolic occlusion model. Zhang and co-workers introduced a composite neurological severity score for evaluation after embolic MCA occlusion (Chen et al., 2001; Zhang et al., 2005). However, no correlation studies between infarct volume and functional outcome were performed. The purpose of this study was therefore to evaluate different functional outcome parameters for their potential to detect various degrees of functional impairment and to discriminate between different infarct sizes after embolic MCA occlusion in rats. Functional evaluation included an extended neurological score with 10 different motor, coordinative and sensory items, a parallel bar crossing test, different tasks in an 8-arm maze and observation of spontaneous behaviour.

2. Results

2.1. *Physiological variables and mortality*

A total of 23 rats was included in the study, with 7 surviving animals per experimental group. The physiological parameters of the 23 animals remained within the normal physiological range throughout the experimental procedure.

After termination of anesthesia, all animals recovered to normal vigilance. Two animals in the group that was subjected to a clot length of 40 mm deteriorated clinically and died within 2 days after ischemia. Postmortem histological evaluation revealed extensive infarction of the right hemisphere with signs of cerebral edema and displaced ventricles. These two animals were excluded from the analysis.

2.2. *Local cerebral blood flow and infarct volume*

The mean baseline lCBF did not differ in the three groups. In all animals subjected to clot embolism, the MCA occlusion was verified by a sustained decrease in local perfusion monitored at the right cortical surface. CBF measurements at the different time points of the experiment are shown in Fig. 1. Fifteen minutes after application of the blood clot, lCBF had dropped significantly in both groups subjected to embolism, compared

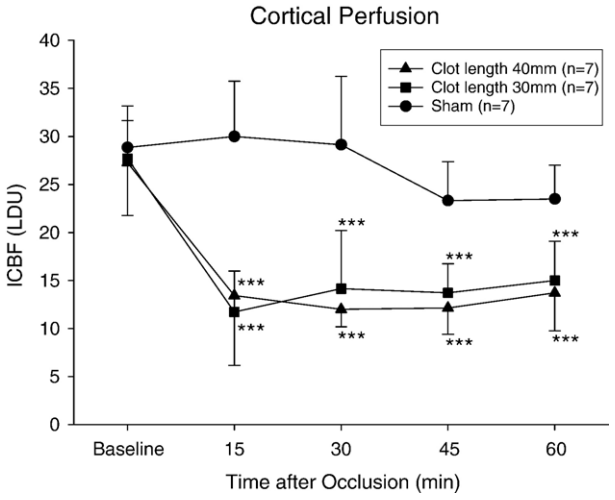

Fig. 1 – Local cerebral blood flow was measured using LDF over the right parietal cortex before and at different time points after occlusion. Values are mean ± SD. ***$p<0.001$ as compared to sham (difference between 30 and 40 mm was not significant).

to baseline and compared to the sham group ($p<0.001$ for both groups, as compared to sham). lCBF remained significantly reduced throughout the observation period. There was no difference in the extent of decrease of lCBF between the two groups with embolism.

The total infarct volume was 53.22 ± 43.15 mm^3 in animals subjected to 40 mm clots and 19.86 ± 18.77 mm^3 with 30 mm clots. The difference in lesion volume between these two groups was not significant ($p=0.18$). Regression analysis revealed no correlation between the infarct volume and the reduction of lCBF at the different time points ($R=-0.14$; $p=0.63$ at 30 min). The sham-operated rats had no infarction in any part of the brain.

2.3. *Neurological evaluation*

Neurological impairment during the follow-up period, as assessed with the Neuro score, is shown in Fig. 2. Animals in both groups with embolism recovered partially from injury, but impairment, compared to sham, remained significant throughout the observation period.

Additionally, there was a significant difference in the performance between the two groups on days 1, 2, 5 and 6. Taking all animals subjected to MCA occlusion, there was a strong correlation between lesion size and the extent of impairment on the Neuro score (day 1: $R=0.78$; day 2: $R=0.70$; day 3: $R=0.71$; day 4: $R=0.67$; day 5: $R=0.78$; day 6: $R=0.80$; Fig. 5A). Correlation on each observation day was significant with $p<0.01$.

On the basis of these results, the animals were additionally evaluated using two different scoring systems that are described in the literature (see Neurological score section). This was done to compare our Neuro score with other existing methods of outcome evaluation. Use of the scoring system described by Zea Longa et al. (1989) did not result in significant correlations for any of the observation days (e.g. day 6: $R=0.30$,

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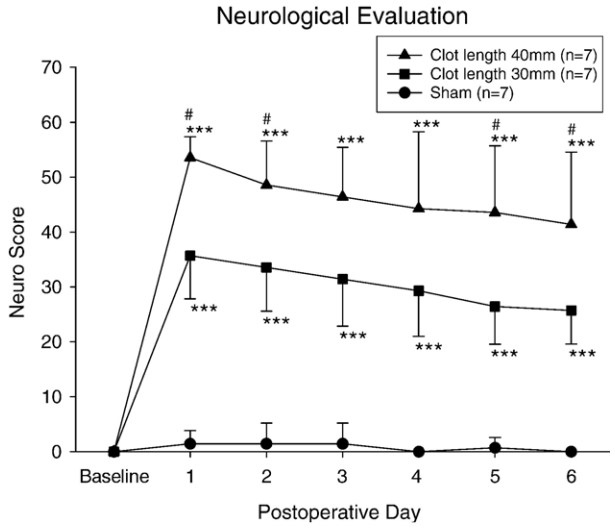

Fig. 2 – Graph of neurological scores before MCA occlusion and during a 6 day examination period. Although there was slight improvement in the performance of the animals, neurological impairment remained statistically significant for both groups with embolism, as compared to sham-operated animals (*$p<0.001$). The difference between the groups with embolism was significant on days 1, 2, 5 and 6 ($^{\#}p<0.05$). Values are mean ± SD.**

$p=0.28$). The neurological score described by Zausinger et al. (2000) revealed significant results throughout the follow-up period, however, compared to our score, the strength of the correlation was consistently lower (e.g. day 6: $R=0.51$, $p<0.05$).

2.4. *Parallel bar crossing*

Performance on the parallel bars was strongly impaired in both groups of animals subjected to embolism (Fig. 3). Both groups partially recovered during the follow-up period. Compared to baseline and to sham, only the performance of the animals subjected to a clot length of 40 mm remained significantly impaired with regard to the traversing time and the number of foot faults. There were statistically significant differences between the two groups with embolism on days 2 to 4 with regard to traversing time and throughout the observation period with regard to the number of foot faults. The extent of correlation of the infarct volume with the number of foot faults decreased during follow-up (day 1: $R=0.76$; day 2: $R=0.76$; day 3: $R=0.65$; day 4: $R=0.53$; day 5: $R=0.41$; day 6: $R=0.33$). These results were significant up to day 4 ($p<0.05$).

Correlation of the infarct volume with the traversing time dropped to a non-significant level after day 2 of the observation period (day 1: $R=0.83$, $p<0.001$; day 2: $R=0.66$, $p<0.05$).

2.5. *Maze and observation of spontaneous behaviour*

The results of the animals' performance in the 8-arm maze and of the observation of spontaneous behaviour in the open field are shown in Table 1. Except for the results of the turning angle during exploration, there were no significant differences between the two groups with embolism and the sham group throughout the observation period. Furthermore, regression analysis of these parameters did not reveal any significant correlations between the total infarct volume and these parameters (data not shown).

However, analysis of the turning angle of the animals during exploration of the maze revealed a strong tendency of the animals with embolism to explore the maze towards their right. Results were significant for both groups as compared to sham on day 2 and additionally for the 40 mm group at the end of the observation period (Fig. 4). Furthermore, the correlation

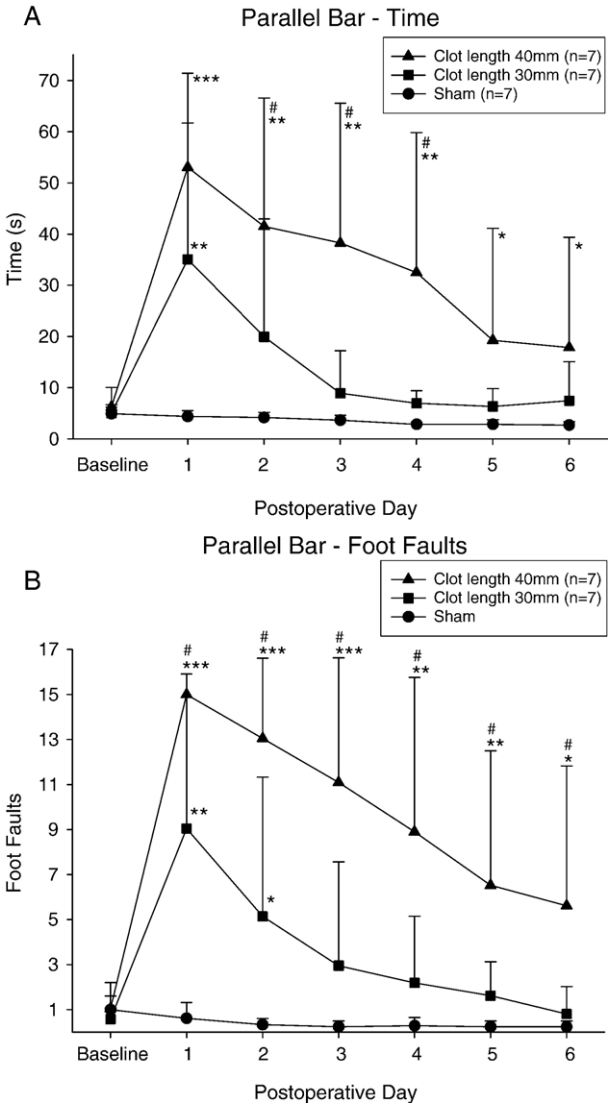

Fig. 3 – Graphs showing the performance on the parallel bar crossing test with regard to traversing time (A) and the number of foot faults (B). Compared to baseline and to sham, the performance of the group subjected to 40 mm clots remained significantly impaired. Comparison between groups revealed significant differences on days 2 to 4 with regard to traversing time and throughout the observation period with regard to the number of foot faults. Values are mean ± SD. *$p<0.05$ compared to sham. **$p<0.01$ compared to sham. ***$p<0.001$ compared to sham. $^{\#}p<0.05$ between groups with embolism.

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Table 1 – Results of functional testing on day 6 in 8-arm maze and open field

	Sham	Clot length 30 mm	Clot length 40 mm
Maze			
Velocity (cm/s)	15.02±0.79	13.31±1.64	12.05±2.02
Time in maze (s)	70.29±11.23	132.80±45.38	124.26±46.00
Exit found	7	5	6
Number of visited arms	5.14	4.71	4.14
Turning angle (degree)	−24.47±29.65	47.68±34.18	122.06±5.03*
Open field			
Velocity (cm/s)	11.01±0.88	10.15±0.91	9.94±0.68
Movement (%)	74.16±3.82	76.61±5.94	81.33±2.79
Distance moved (m)	24.86±3.01	23.88±3.25	24.45±2.20

Values are given as mean±SD, except for "exit found" and "number of visited arms", which are total numbers.
Movement (%): portion of time the animal was in movement.
Number of animals is 7 per group.
* $p<0.01$ compared to sham; a negative angle signifies a turn to the left (see Experimental procedures for details).

between total infarct volume and the turning angle was statistically significant throughout the observation period (day 1: $R=0.68$; day 2: $R=0.87$; day 3: $R=0.74$; day 4: $R=0.84$; day 5: $R=0.83$; day 6: $R=0.66$; Fig. 5B). Correlation was significant on each observation day with $p<0.05$.

3. Discussion

The present study was designed to evaluate different tests of functional outcome after embolic MCA occlusion in rats. Our principle finding was that embolic intracranial vessel occlusion leads to lasting functional impairment that can be detected and quantified by different outcome tests. This is of particular importance for further studies on therapeutic effects, in which a solid quantification is the basis for the differentiation of multiple treatment paradigms.

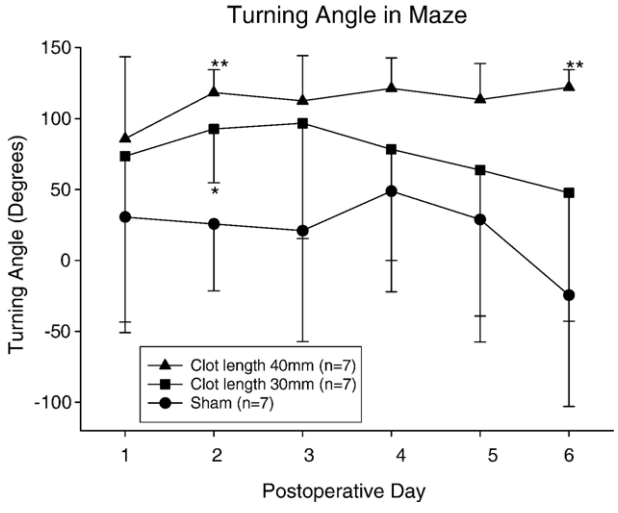

Fig. 4 – Turning angle during exploration in 8-arm maze (see Experimental procedures section for details). Values are mean±SD. *$p<0.05$ as compared to sham. **$p<0.01$ as compared to sham.

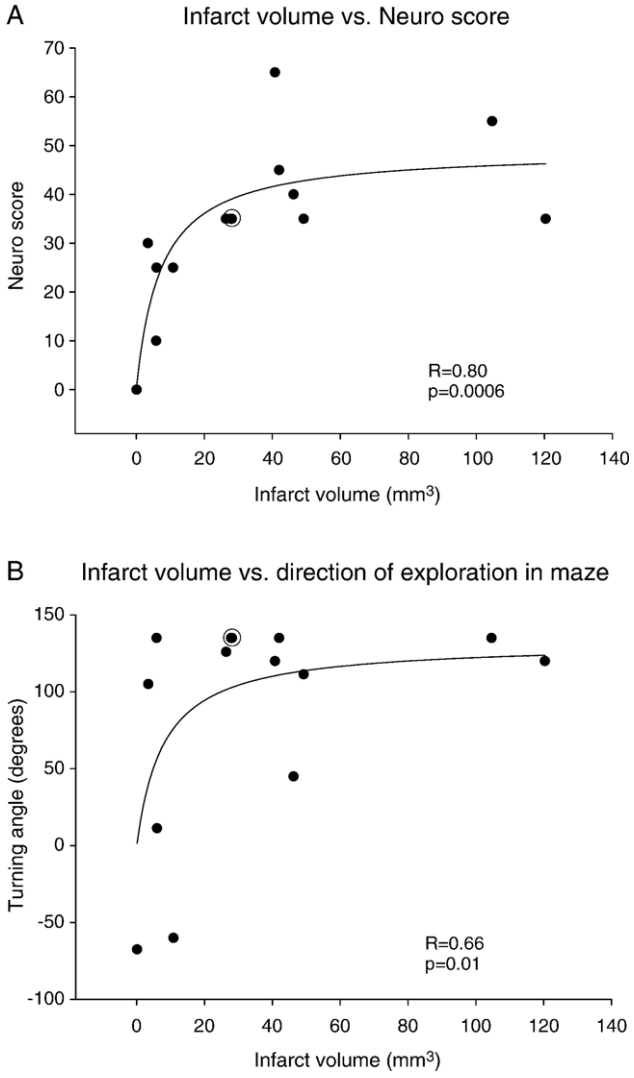

Fig. 5 – Scatter plots showing the correlation between the infarct volume and the functional outcome as determined by the Neuro score (A) and the direction of exploration in the 8-arm maze, expressed as the relative turning angle per passage through the central chamber (B). Correlation was significant throughout the observation period. Results on day 6 of follow-up are shown. The encircled points represent 2 overlapping points.

Behavioural testing in addition to histology will improve the effective evaluation of potential therapeutic interventions after embolic MCA occlusion. Assessment of functional outcome more closely reflects the approach in human clinical studies on recanalisation. Furthermore, purely relying on infarct volume does not allow the detection of abnormal function after ischemia in cases of normal or near-normal histology (Jaspers et al., 1990; Yamaguchi et al., 1995). This is demonstrated by studies that found significant effects in functional outcome assessment, although therapeutic interventions appeared to be ineffective based on histological evaluation

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(Hattori et al., 2000; Kawamata et al., 1996; Colbourne and Corbett, 1994).

The most consistent and lasting results were obtained by use of our Neuro score, which displayed significant differences throughout the observation period (compared to sham and between groups with embolism) and showed a strong correlation with the ischemic lesion volume. Furthermore, we are the first to describe an outcome assessment based on exploration behaviour of the animals in an 8-arm maze. This assessment is based on the observation that animals with right hemispheric lesions tend to explore the maze towards their right. An evaluation score of the animals' turning angle gave significant results at the end of the follow-up (day 6) and a significant correlation with the infarct volume throughout the observation period. Compared to these outcome tests, assessment on parallel bars seems of less value. Observation of spontaneous behaviour in an open field does not help to detect impairment in animals subjected to embolic intracranial vessel occlusion.

To obtain different sizes of cerebral infarction, we injected two different volumes of blood clot into the internal carotid artery. This approach has been used before in a mouse model of embolic stroke (Zhang et al., 2002). The initial reduction of cerebral perfusion did not show any correlation with infarct volume after 7 days and did not differ between the two groups. The reason for this observation is not well understood, but may possibly reflect a difference in the stability of the differently sized clots. The results emphasize the importance of a well defined clot size for this embolic stroke model. These mechanisms should be further investigated in future studies.

3.1. *Neurological score*

The Neuro score includes testing for different motor and non-motor deficits that are not evaluated by more conventional outcome scores, which are mainly based on assessment of basic motor functions (Gerriets et al., 2004; Li and Stephenson, 2002; Zausinger et al., 2000; Zea Longa et al., 1989). Compared to other elements of the test battery used in this study, it detected impairment of the animals more reliably throughout the observation period. Scoring of functional deficits remained statistically significant even in animals with smaller infarct sizes, as seen in animals subjected to a clot length of 30 mm. The correlation of lesion volume with impairment on the Neuro score remained strong until the end of the follow-up period. Detection of impairment in animals with smaller lesions is of particular importance in animal studies concerned with therapeutic effects, such as recanalisation studies, as it will facilitate differentiation between various treatment paradigms.

To further evaluate the value of our Neuro score, the results were compared with two existing scoring systems. Both of these scores are based on observation of motor deficits. To our knowledge, the 5-point score, originally described by Zea Longa et al. (1989), has never been validated for its ability to differentiate between various infarction volumes. Application of this score in our sample of ischemic animals did not reveal any correlation between the infarct volume and the values obtained with the score. Most of our animals displayed only mild deficits on this score (failure of left forepaw extension), which did not allow the differentiation of impairment severity. A modified 6-point motor scale was introduced by Zausinger et al. (2000), which displayed a significant correlation with lesion volume in a group of animals subjected to ischemia by use of the intraluminal thread model. This score additionally tests for instability to lateral push. Inclusion of this item improved differentiation between various extents of functional impairment in our sample of ischemic animals. However, compared to our score, the strength of correlation was consistently lower throughout the follow-up. Thus, an evaluation score purely based on motor functions only partially reflects the variety of functional deficits that occur in cerebral ischemia. A comprehensive neurological score, comprising different motor, coordinative and sensory items, more closely reflects the overall functional deficit of the animals (Chen et al., 2001; Reglodi et al., 2003).

The inclusion into the score of a semiquantitative evaluation of motor functions on the parallel bars reflects motor testing under more difficult conditions and allows a more precise differentiation of motor impairment. The torso twisting test (Petullo et al., 1999) proved to be a sensitive measure and gave pathological results in animals that otherwise displayed only slight disability. Hemianopia was found to be very helpful for assessment of non-motor impairment. A considerable number of animals showed signs of hemianopia. The animals scored when there was repeatedly no reaction to visual stimuli approaching from the left. In addition, it was found helpful to include observations of spontaneous behaviour in our evaluation, as in animals with hemianopia, exploration behaviour is strongly directed towards the right. To get more information on the extent of hemianopia, this novel item was separately evaluated in the 8-arm maze (see below). Sensory impairment was detected in five animals, absence of whisker movements on the left side in one animal. There was no loss of hearing and no loss of consciousness in any of the animals. Omission of these two items from the evaluation did not change the level of significance and the strength of correlation.

3.2. *Maze*

The most important finding of the different tests performed in the 8-arm maze is the result of the relative turning angle during the exploration. There were differences between the groups with embolism and the sham group that were statistically significant at day 2 and at the end of the observation period. The correlation between infarct volume and the relative turning angle showed a significant positive relationship throughout the follow-up.

This test was designed following the observation that rats prefer to turn to the side ipsilateral to the infarction. Healthy animals display a more random exploration behaviour, which is reflected by the group of sham-operated animals, that show no clear directional preference during the follow-up period (Fig. 4). This preference towards the right cannot be explained by a contralateral muscular weakness (which, if at all, results in a drifting or circling towards the left (Reglodi et al., 2003)), but is evidently due to the inadequate perception of the sensory (visual, vestibular, and possibly somesthetic) stimuli received by the contralateral side of the body. This is supported by the observation that animals that scored for hemianopia in the

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Neuro score showed this exploration behaviour most strongly. The significant correlation between the relative turning angle and the infarct volume shows that this score, to some extent, enables quantification of hemianopia. Some impaired animals were reluctant to explore the maze, which reduced the number of animals available for analysis. However, exploration activity improved during the follow-up, with all animals exploring the maze at day 6. This led to a more reliable data and statistical significance at the end of the observation period and shows that testing throughout the whole period is required.

The determination of the turning angle is a novel approach to quantification of functional impairment. This test may be valuable in future studies since it tests for deficits that are not detected by more conventional outcome scores based only on motor functions. An asymmetry test of movement has also been described in the corner test (Zhang et al., 2002). This test, however, was evaluated in embolic ischemia in mice and in a model of intracerebral hemorrhage (Hua et al., 2002) and has, to our knowledge, not been performed in an ischemic model in rats.

The other parameters tested in the maze failed to reveal any differences between the groups with embolism. With regard to the time within the maze and the number of animals that found the exit, all animals in the different groups displayed a slope of learning, however, again without any difference between the groups. The velocity of movement and the number of visited arms did not change throughout the course of the experiment.

3.3. *Parallel bar crossing*

At the beginning of the follow-up period, the parallel bar crossing test revealed strong impairment in most animals subjected to ischemia. However, in contrast to our findings with the Neuro score and the turning angle in the maze, impairment was not consistent over the follow-up period. Most animals displayed a marked recovery of motor functions within the first post-ischemic days, a phenomenon that has been previously observed in balance tests by other investigators (Ding et al., 2001a,b; Reglodi et al., 2003). Parallel to functional recovery, the strength of correlation between infarct volume and impairment dropped to non-significant levels from day 5 (number of foot faults) and day 3 (traversing time). This slope of recovery is a limitation of this outcome test. Significant results at the beginning of the observation period suggest a restriction of outcome evaluation to the first 2 days after induction of ischemia. Ding and co-workers (Ding et al., 2001a,b, 2002) used a scoring system rather than the total number of foot faults or duration of performance (0: no foot faults; 1: mean <1 fault; 2: >/=1 fault; 3: >2 faults; 4: >3 faults; 5: cannot traverse). Use of this scoring system in our sample did not change the significance levels of our data (data not shown).

In conclusion, our study demonstrates the relationship between the functional outcome and the extent of ischemia in embolic MCA occlusion in rats. We are the first to describe evaluation tools in embolic MCA occlusion that reliably allow us to differentiate between various degrees of impairment. Furthermore, the tests that showed most consistent results are easy to perform. According to our data, the Neuro score and the exploration behaviour in the 8-arm maze are the most suitable as evaluation tools in outcome studies. Assessment of performance on the parallel bars as an integrative item in the Neuro score is helpful as it allows a more differentiated evaluation of motor impairment. However, quantitative assessment on the parallel bars seems of less value as the slope of functional recovery was steepest in these tests. Assessment of spontaneous behaviour in an open field may well be omitted from further analyses.

Thus, our data provide valuable information for future studies on therapeutic recanalisation strategies in the embolic MCA occlusion model in rats.

4. Experimental procedures

4.1. *Animal preparation*

23 male Wistar rats weighing 383±49 g (mean±SD) were used in the present study. Animals were purchased from Charles River Laboratory (Sulzfeld, Germany). All experiments were performed in accordance with the German animal protection legislation and were approved by the regional ethics committee (Az 1.5 177-07/051-43).

Rats were anesthetized by intraperitoneal injection of chloral hydrate (initially, 36 mg/100 g body weight; maintenance, 36 mg/h), orally intubated and mechanically ventilated throughout the entire operation procedure. A thermostatically regulated heating pad was used to maintain rectal temperature at 37.0 °C. The tail artery was cannulated with a PE tube for continuous monitoring of arterial blood pressure and for blood sampling to measure pH, blood gases, hemoglobin, electrolytes, glucose and lactate. Animals were weighed at baseline and before perfusion fixation.

4.2. *Induction of brain ischemia and measurement of CBF*

Thromboembolic cerebral ischemia was produced as previously described by Zhang et al. (1997b). In short, the right and left common carotid arteries (CCA), the right external carotid artery (ECA) and the right internal carotid artery (ICA) were exposed. ECA branches were cauterised, the distal ECA ligated and the stump mobilized. Both CCAs and the ICA were temporarily clamped and a modified PE 50 catheter (0.28–0.30 mm outer tip diameter) was inserted into the ECA and gently advanced about 15 mm into the ICA, being then about 2 mm from the MCA origin. A previously prepared blood clot was injected into the catheter and placed in the distal ICA. The catheter was withdrawn and the puncture site cauterised. The clip on the left CCA was removed at 5 min, the clips on the right ICA and CCA at 10 min after injection.

For the preparation of the blood clot, 200 μl of blood was drawn from the tail artery and mixed with 4 NIH units of thrombin (human alpha thrombin, Enzyme Research Laboratories, South Bend, USA). The blood was filled into a 20 cm piece of a PE 50 catheter (inner diameter 0.86 mm) and left at room temperature for 2 h. The resulting blood clot string was removed from the catheter and gently washed in saline. The required piece of blood clot was cut from the string and drawn into the injection catheter. In order to achieve a larger variety of lesion size for functional evaluation and to test for an

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optimum clot size, 2 subgroups each with 7 surviving animals were subjected to differently sized emboli (lengths 30 and 40 mm). Seven sham-operated animals served as controls.

Local cerebral blood flow (lCBF) was monitored in the cerebral cortex of the supply territory of the right middle cerebral artery (MCA) by laser Doppler flowmetry (LDF; Laserflo™ BPM^2, Vasamedics, St. Paul, MN, USA). The skull was exposed by a sagittal midline skin incision. A right sided burr hole was drilled about 1 mm posterior to the bregma and 4 mm lateral to the midline, covering an area of 3×4 mm. The drill tip was continuously flushed with saline to avoid thermal injury to the cortex. Care was taken to keep a thin layer of the skull and the dura intact. lCBF was sequentially measured at 15 different sites, which is the number we previously recommended to keep the deviation from the true CBF low (Soehle et al., 2001). Care was taken to obtain flow readings only from areas free of large pial vessels. The mean lCBF of each group was averaged from the median of these 15 measurements. The mean value was used for further analysis and is expressed in laser Doppler Units (LDU). Scans were performed at baseline conditions, and 15, 30, 45 and 60 min after induction of cerebral ischemia.

Seven days after ischemia, the rats were re-anesthetized with chloral hydrate and perfused transcardially with 4% paraformaldehyde. The brains were removed, embedded in paraffin, and cut into 3 μm thick coronal sections at 400 μm intervals. The brain slices were stained with hematoxylin and eosin. The infarct areas were assessed planimetrically (OPTIMAS 6.51, BioScan Inc) by a blinded examiner. For each brain, slices were measured encompassing the entire infarct. Only areas of pannecrosis consisting of the loss of affinity for hematoxylin that affects all cell types (neuronal, glial and vascular) were measured. Infarct volume was calculated by multiplying the infarct area of each slice by the distance (400 μm) between successive slices.

4.3. *Functional evaluation*

All behavioural tests were performed between 1:00 pm and 4 pm. A 5 min resting period was allowed between each type of test. Functional evaluation was performed on preoperative day 1 (baseline) and on a daily basis from postoperative days 1 to 6. Parallel bar crossing included a 2 day training period prior to baseline.

4.3.1. *Neurological score*

Neurological deficits at baseline and after induction of ischemia were studied by use of a score with ten different items. The score was designed to test for different motor, coordinative and sensory functions. Each item scored 0 (no impairment) or 10 (impairment), except for spontaneous walking (0: normal gait; 5: drifting/circling; 10: unable to walk on ground) and parallel bar traversing (to test for motor functions under more difficult circumstances; 0: no impairment; 5: impairment (slowing, foot faults); 10: unable to cross). Thus, overall functional impairment was scored from 0 (no impairment) to 100 (no reaction to any stimuli).

The items included were as follows: inability to fully extend left forelimb; instability to lateral push from right; torso twisting (in this test, animals were gently lifted by the tail and impairment was assumed when animals flexed their body to the left and remained in that position in three consecutive attempts); walking on ground (see above); semiquantitative assessment of walking on parallel bars (see above); whisker movements on left side (present or absent); consciousness (normal or no reactions to stimuli); hearing (normal or no reaction to acoustic stimuli); sensory (normal or no reaction to left sided touch or prick); left-sided hemianopia (normal or repeatedly absent reaction to visual stimuli approaching from left).

For further validation, our Neuro score was compared with two motor scores, which are both described in the literature. One score, originally described by Zea Longa et al. (1989), is based on assessment of spontaneous motor functions on a 5-point scale (0: no deficit; 1: failure to extend left forepaw fully; 2: circling to the left; 3: falling to the left; 4: no spontaneous walking). The other score additionally includes testing of lateral instability (Zausinger et al., 2000) (5: no deficit; 4: consistent flexion of the left forelimb; 3: reduced resistance to lateral push toward the paretic side; 2: circling to the left when pulled by the tail; 1: spontaneous circling to the left; 0: no spontaneous motion).

4.3.2. *Parallel bar crossing test*

Two parallel wooden bars (diameter 1.8 cm; length 1 m; distance between bars: 2.5 cm) were positioned horizontally, 50 cm above a soft pad in case the rat falls. The room was quiet and darkened. The rat was placed at one side of the bars and a light and noise source behind the rat was switched on, serving as an adverse stimulus. After crossing the bars, the rat entered a dark chamber and the adverse stimulus was switched off. The time required to cross the bar and the number of foot faults were counted, with a maximum count of 60 s and 15 foot faults. Inability to cross the bars and falling were counted as 60 s and 15 foot faults. With each rat, three trials were performed in sequence and the scores of each trial were averaged.

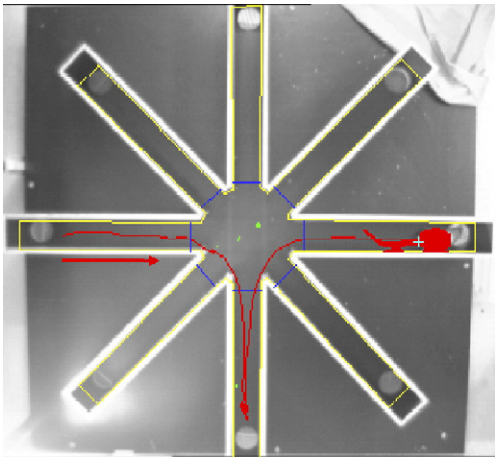

Fig. 6 – Movement of a test animal through the 8-arm maze as viewed by the tracking software. The yellow and blue lines represent predefined areas of interest within the maze (central chamber and arms). The red line shows tracking of the animal's movement (arrow: direction of movement). As the animal had two 90° turns to the right, this would result in a relative turning angle of +90° (see Experimental procedures section for further definition).

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4.3.3. *Maze*

The animals were tested in an 8-arm wooden maze installed in an illuminated, quiet room. Each roofless arm (64×10×20 cm) projected from a central chamber, resulting in an angle of 45° between neighbouring arms. At its far end, each arm contained a hole in the floor leading to a cage. All but one hole were closed by a grid. The rat was placed in the arm opposite the one with the opening, and a noise source was switched on for the duration of the trial. The trial ended after 300 s of exploration or as soon as the rat exited the maze into the accessible cage. The path of each rat was recorded with a camera installed above the maze and by use of a tracking software (EthoVision™, Noldus Information Technology, Utrecht, Netherlands; Fig. 6). Principle evaluation parameter was the direction of movement during exploration. This item was included following the observation that animals with right-hemispheric ischemia display a tendency to turn more to their right when exploring the maze. The turning angle was determined as a measure of this tendency and defined as the deviation from straight movement. Because the maze has 8 arms, a turn to the neighbouring arm results in a deviation of 135°, omitting one arm a turn of 90°, and omitting two arms a turn of 45°. Turns to the right were counted as a positive angle, turns to the left as negative. The angles of every turn during one session were added and divided by the total number of passages through the central chamber to obtain a relative angle that describes the mean angle per turn.

Further evaluation parameters were: time in maze (exploration time), velocity of movement, exit found, and the number of visited arms without an opening (maximum count of 7; animals that did not find the exit within 300 s were automatically scored 7).

4.3.4. *Observation of spontaneous behaviour*

Observation of spontaneous behaviour was carried out in an open field, consisting of a roofless black box (75×75×39 cm) in a dark and quiet room. Each session consisted of observation of a single rat for a period of 300 s. The movement of the animal was registered and analysed automatically (EthoVision™, see above). Evaluation parameters were velocity of movement, the total distance moved and the amount of time the animal was in movement (expressed as percent of total time).

4.3.5. *Statistical analysis*

Statistical analysis was performed using SPSS 12.0 for Windows. Comparison of infarct volume between the groups was performed by the Mann–Whitney *U*-test. Differences in functional outcome between all groups were assessed by an analysis of variance (ANOVA) for repeated measures followed by an LSD post hoc test. For regression analysis of the infarct volume and the different functional outcome parameters, a Spearman rank order correlation was performed. Values are expressed as means ± SD. A p-value < 0.05 was considered to be statistically significant.

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