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# Modulations of behavioral consequences of minor cortical ischemic lesion by application of free radicals scavengers

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**Abstract.** Functional and morphological consequences of ischemic lesions are partially related to the production of reactive oxygen species (ROS). The aim of the study was to create a unilateral photothrombic lesion with minimal morphological changes and minor sensorimotor and cognitive deficits and also to test whether the application of ROS scavengers after the end of induction of ischemia had improved the functional outcome.

Adult Wistar male rats were randomly divided into five groups: naive control, sham operated animals, animals with induced ischemia, and two groups of animals with induced ischemia and subsequent ROS scavenger application –melatonin or tempol. The group subjected to ischemia showed a significant decline in performance in sensorimotor tests and the Morris water maze (MWM) test, compared to control animals. Tempol (50 mg/kg, i.p.) did not improve sensorimotor function and did not change spatial learning. Melatonin (100 mg/kg, i.p.), on the contrary, resulted in a significant improvement in animals' performances. All the ischemia subjected animals had increased speed of swimming in the MWM test, compared to the control group.

Our findings showed that subsequent application of ROS scavengers improve ischemia outcomes, with melatonin being more potent. Conversely, neither melatonin, nor tempol decreased swimming speed cased by ischemia.

**Key words**: Cortical ischemia — Cognitive impairment — Behavior — ROS scavenger

**Abbreviations:** BB test, Beam Balance test; MWM, Morris water maze; ROS, reactive oxygen species; RT, Rotarod test; rRT, reverse Rotarod test.

## Introduction

The severity of disabling consequences associated with cortical ischemia is related to the size of the lesion, its location and how quickly reperfusion of the affected zone occurs, assuming that it does occur (Zhao et al. 2002). Diminished recovery is due to production of reactive oxygen species (ROS). ROS are able to increase the lesion size and alter functional consequences by damaging the hypoperfused zone of ischemic, but still vital, tissue. In addition to ischemic tissue damage, ROS formation during reperfusion further worsens the clinical outcomes.

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Photothrombic ischemic lesions, introduced by Dietrich and colleagues (Dietrich et al. 1984), mimic the naturally occurring thromboembolisms in strokes. Green-light activation of circulating photosensitive dye triggers endothelial damage *via* ROS, leading to platelet aggregation and microvascular stasis (Dietrich et al. 1987). This model of ischemia offers the advantage of precisely determining the location of the lesion, its size and severity.

Gross ischemic lesions have typical functional consequences. While distinguishing between functional changes becomes more difficult with smaller and more superficial lesions, they offer an opportunity to study the potential for improving the outcomes of such lesions.

Decline in cognition is a common symptom in stroke patients. The measure of the cognitive ability of these patients allows for predicting the outcome of the ischemic lesions (Pohjasvaara et al. 2002). In animal models, it is difficult to

specify separately cognitive changes linked to motivation, planning, and execution of movements. Therefore a battery of sensorimotor tests, in addition to the Morris water maze (MWM) test (Baldi et al. 2003), are useful tools for estimating these cognitive changes.

The aim of this study was to investigate whether minor unilateral photothrombotic ischemic lesion in the cortical sensorimotor area will produce distinct behavioral traits. It was expected that even a small superficial lesion would produce a deterioration of motor skills and cause certain changes in cognition.

The second part of the work was targeted at testing the effect of ROS scavengers on improving the motor skills and conditions in our model after an induction of ischemia. The scavenger application during the crucial period of increased ROS generation in relatively high dose was expected to abrupt the cascade and improve outcome of ischemia by prevention of oxidative ROS damage.

## Materials and Methods

### *Animals and reagents*

Sixty male Wistar rats (ANLAB, Czech Republic), 200–250 g, were randomly divided into five groups of 12 animals. One group was subjected to photothrombic cortical ischemia (Ischemia); two other groups, in addition to induction of ischemia, received the ROS scavengers –tempol (IschTemp) or melatonin (IschMel). The fourth group was sham operated (Sham) and the fifth was a naïve intact control group (Control). Rats were housed in groups of four under 12-hour light/dark cycles and received food and water ad libitum.

All the chemicals used were supplied by Sigma-Aldrich®. Ischemia was induced under the general anesthesia: ketamine 100 mg/kg i.p. and xylazine 16 mg/kg i.m. The photosensitive die Rose Bengal – 4,5,6,7-Tetrachloro-2′,4′,5′,7′-tetraiodofluorescein disodium salt, 20 mg/ml/kg, 0.9% NaCl solution, was used. The following ROS scavengers and antioxidants were used: tempol – 4-hydroxy-TEMPO, 50 mg/kg, in 2 ml $H_2O$ solution; and melatonin –N-Acetyl-5-methoxytryptamine, 100 mg/kg in 2 ml of 2% Tween 80 solution.

All experiments were performed according to the guidelines of the Ministry of Health of the Czech Republic. The animal protocols were approved by the Ethics committee of the Third Faculty of Medicine, Charles University in Prague.

### *Induction of ischemia*

The general anesthesia was induced by application of 100 mg/kg ketamine i.p. and 16 mg/kg xylazine i.m. Exposed animals' skulls were irradiated following i.v. application of Rose Bengal. A beam from a high-powered green-light laser (power density = 50 mW/mm$^2$, illuminated area <1 mm$^2$) was subsequently centered on 3 points (6 min each) of the right side of the skull. Anteroposterior and lateral coordinates of the points were: 0, 5; 0.5, 4.1 and –0.5, 4.1. According to Palomero-Gallagher and Zilles (2004) the following cortical areas were involved: primary motor, premotor, and primary somatosensory (Fig. 1, right). After laser irradiation, the scalps were sutured and the animals were allowed to recover in their home cages until the beginning of the sensorimotor tests. Animals were placed into a dark environment during their recovery, in order to prevent photochemical injury to the retina as well as supporting the potentiation of the effect of melatonin (Talaei et al. 2010). Ten minutes after irradiation, the animals from the IschTemp and IschMel groups, still under anesthesia to exclude injection stress, received an i.p. injection of tempol or melatonin respectively. Sham operated animals received saline solution i.v. (0.9% NaCl, 1 ml/kg) and were also subjected to the laser irradiation; otherwise, a similar experimental protocol to the induction of ischemia was used. Intact control animals were kept in a dark environment for the same amount of time as experimental groups, i.e. until the testing phase started 24 hours after induction of ischemia. Thereafter animals were returned to the normal light/dark cycled regime –12 h light/12 h dark.

### *Sensorimotor tests*

Twenty four hours after induction of ischemia animals were tested only with the Beam Balance test (BB1) to avoid melatonin-related circadian rhythm influence on their performance. This test was aimed to asses gross vestibulomotor function (Murphy et al. 1995). Rats were positioned on a wooden bar (400 mm length, 10 mm diameter, 800 mm vertical height); the objective was to remain balancing on the wooden bar for 60 s. A maximum of 10 trials were allowed to meet the objective.

Forty-eight hours after induction of ischemia, the rats were further tested by the Beam Balance test 2 (BB2), Rotarod test (RT), reverse Rotarod test (rRT) and MWM Visible platform acquisition.

In the BB2 test, the objective was changed from 60 to 120 s of balancing. In the BB2, as well as BB1, the number of trials needed to meet the objective was recorded. The RT was performed in order to assess motor deficits and vestibulomotor and tactile function. The animals were placed on a cylinder (115 mm diameter, 6 rpm speed of rotation) in an opposing manner to that of the cylinder rotation. The animals' objective was to remain on the cylinder for 120 s in a maximum of 10 trials. In the rRT, rats were placed on a cylinder oriented in the same direction as that of the rotation. The objective was for the rats to reorient themselves on the cylinder and remain there for 120 s. In both tests, the number of trials needed to meet the objective was recorded.

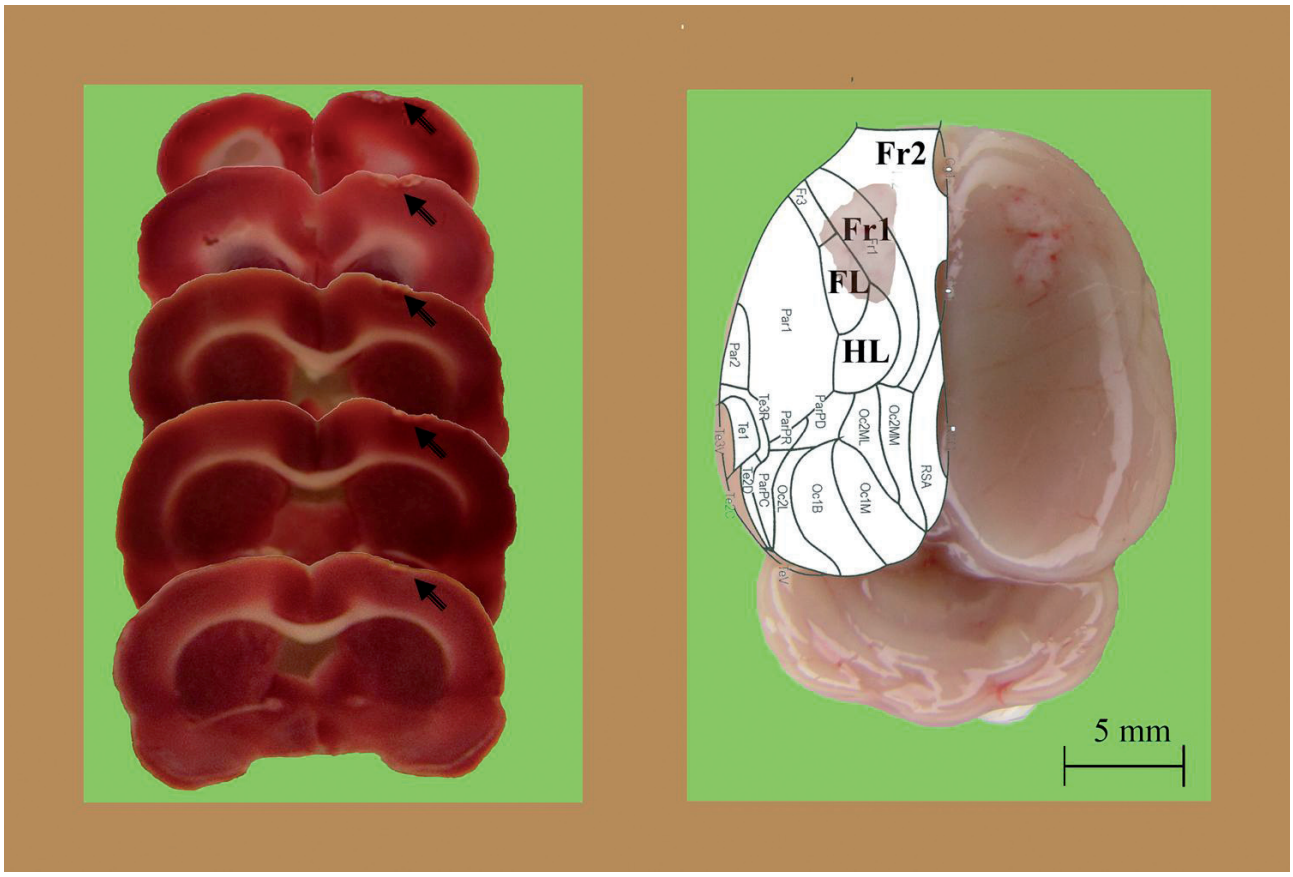


**Figure 1.** Morphological depiction of the ischemic lesion. Left: the ischemic lesions on the brain slices are pointed out by arrows. Right: native photograph of the animal's brain with the ischemic lesion at the frontal part of the right hemisphere. On the left hemisphere were superimposed cortical areas. The shadow area corresponds to the location and extension of the lesion on the opposite hemisphere. The following ares were involved: Fr1, frontal cortex, area 1 (primary motor); Fr2, frontal cortex, cortex area 2 (premotor, supplementary motor); FL, parietal cortex, forelimb area (primary somatosensory); HL, parietal cortex, hindlimb area (primary somatosensory); Oc, occipital cortex; Par, parietal cortex; RSA, agranular retrosplenial cortex; Te, temporal cortex.

The MWM Visible platform acquisition test was used to test the rats' motoric functions. The rats were placed in a water tank (1.98 m diameter, 19–20°C water temperature) with a black plastic platform, 10 mm above the waterline. The animals were allowed to swim for 60 s; if the rat reached the platform, it was allowed to remain there for 30 s before starting the next trial. If the animal failed to locate the platform within 60 s, it was manually guided there by the experimenter and allowed to rest for 30 s. Eight trials were performed. The latency in platform acquisition, the total distance moved and the mean velocity were calculated in software EthoVision (Noldus, The Netherlands).

*Spatial learning and memory MWM tests*

Seventy-two hours after ischemic-induction, place navigation testing was started. The test is aimed to assess animals' spatial orientation, in terms of learning the position of a hidden under the water platform with use of extra-maze clues. This test was conducted over six consecutive days with eight trials *per* day. The trial protocol was similar to the Visible platform acquisition test, however, the platform was transparent and hidden 20 mm below the water level. The latency in platform acquisition, the total distance moved, the mean velocity, and the search error (Gallagher et al. 1993) were calculated using the EthoVision video-tracking system.

Twenty-four hours after the last navigation trial, memory retention was evaluated using the Probe test. Rats were allowed to swim for 60 s in the absence of an escape platform. The percentage of time spent in the goal quadrant, the quadrant in which the escape platform was positioned during learning, the number of goal quadrant entries and the number of crossings over the area where the platform was previously located were recorded and quantified.

*Morphology*

Under the general anesthesia, the animals were perfused transcardially with a 0.9% NaCl solution (8–10°C), 24 hours after the Probe test. The animals' brains were removed immediately after the perfusion and cut into coronal slices (thickness = 500 μm) at the level of the laser irradiation. The 2,3,5-triphenyltetrazolium chloride reduction test (Khan et al. 2000) was used to detect mitochondrial survival. Digital photographs of the slices were taken and evaluated for signs of ischemia.

*Statistical analysis*

Data were tested for normality of distribution using the Kolmogorov-Smirnov test. The Kruskal-Wallis test followed by Dunn's Multiple Comparison post-test was used for comparison of number of trials in Beam Balance and Rotarod tests. One way ANOVA tests followed by Tukey's multiple comparison test single were used for evaluation of Visible platform acquisition and Probe tests results. Two-way ANOVA with repeated measures followed by Bonferroni post-tests were used for comparison of parameters in the test of spatial learning. Differences were considered significant if $p < 0.05$.

## Results

All of the animals which were irradiated by the laser beam survived and recovered. The observations of spontaneous motor activity of the animals in their home cages did not confirm any gross functional disturbances. Ischemic lesions were minor but distinguishable in each animal and were maximally transcortical in the Ischemia group (Fig. 1). Scavenger-treated animals had lesions reaching maximally into the IV–V cortical layers; there were light changes around areas of superficial necrosis. In such diminished lesions, many times ($n = 4$), it was not possible to distinguish its borders.

### *Sensorimotor changes*

*Beam balance 1 and Beam balance 2 tests*

There were no significant differences in the number of trials between either of group in the BB1 test performed 24 hours after induction of ischemia.

In the BB2 test performed 48 hours after the induction of ischemia (Fig. 2A), the Ischemia group showed a significant increase in the number of necessary trials compared to the Control ($p \leq 0.001$) and Sham animals ($p < 0.05$). IschMel and IschTemp groups did not differ significantly in the number of trials needed to meet the objective either from Ischemia or Control and Sham. Also, there was no statistically confirmed difference between Control and Sham animals in their performance.

*Rotarod and reverse Rotarod tests*

The RT performed 48 hours after the induction of ischemia did not reveal any significant disturbances in vestibulomotor and tactile functions in experimental animals. There were no any significant differences in performance of Control, Sham and experimental animals (Ischemia, IschMel, IschTemp). However, in the rRT (Fig. 2B) followed RT, the Ischemia group performed more poorly than the Control ($p \leq 0.001$) and Sham ($p \leq 0.01$). There was no difference in perform-

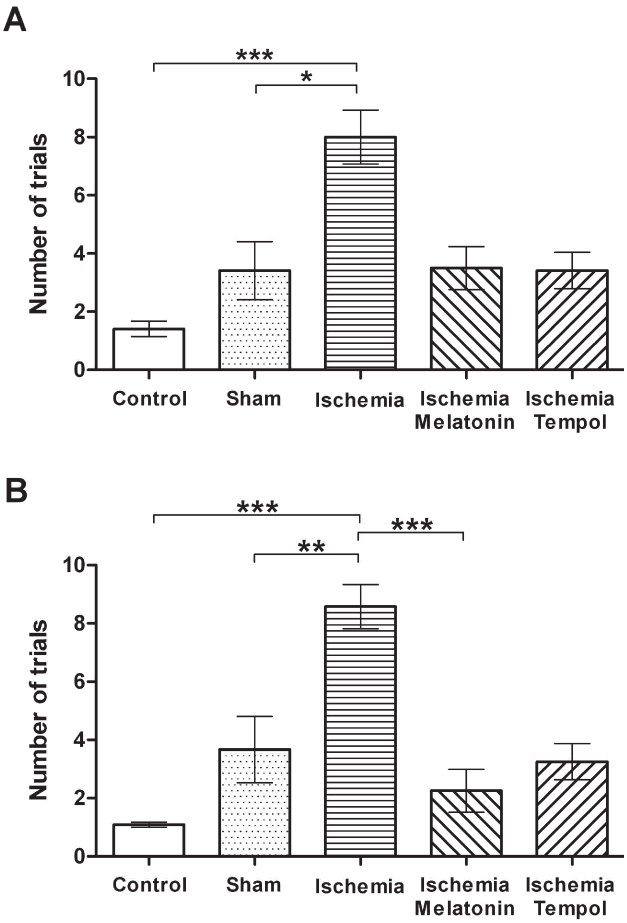


**Figure 2.** Sensorimotor tests results. **A.** Performance of each group individually in the BB2 test (mean ± SEM), which was conducted 48 hours after laser irradiation. Animals in Ischemia group needed more trials to meet the objective in comparison to the Control ($p \leq 0.001$) and Sham animals ($p < 0.05$). There were no other statistically confirmed differences in the performance between the groups. **B.** Performance of each group in rRT (mean ± SEM). The performance of the Ischemia group was worse in respect to Control ($p \leq 0.001$) and Sham animals ($p \leq 0.01$). The group with melatonin application following the induction of ischemia had lower number of trials needed to meet the objective ($p \leq 0.001$). There were no statistically confirmed differences in the performance between the rests of groups. * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

ance of Control ans Sham animals. Also, the application of ROS scavenger melatonin decreased the number of trials needed for reaching the objective (IschMel $p \leq 0.001$). The group of animals with tempol application did not differ in performance from either group.

*Visible platform acquisition test*

In the MWM Visible platform acquisition test, we did not find any significant difference in the latencies of platform aquisition.

One-way ANOVA of distances traveled to platform exhibited $p \leq 0.001$ and $F_{(4, 55)} = 6.458$. According to the Tukey's multiple comparison test, the IschTemp group showed a significant increase in the distance moved during trial compared to Controls ($p \leq 0.001$), Sham ($p \leq 0.01$) and IschMel ($p \leq 0.01$). No other significant differences in distances moved were determined.

ANOVA of the mean velocity showed $p \leq 0.001$, $F_{(4, 55)} = 6.48$. There was a significant increase of swimming velocity in the Ischemia ($p \leq 0.001$) and IschTemp ($p \leq 0.01$) groups compared to the Control. Ischemia animals also swam faster than their Sham ($p < 0.05$) counterparts. The IschMel group did not display any significant changes compared to the Ischemia or Control groups.

***Spatial learning and memory changes***

All the animals, regardless of treatment, demonstrated learning abilities during the 6-day trial period, as dem-

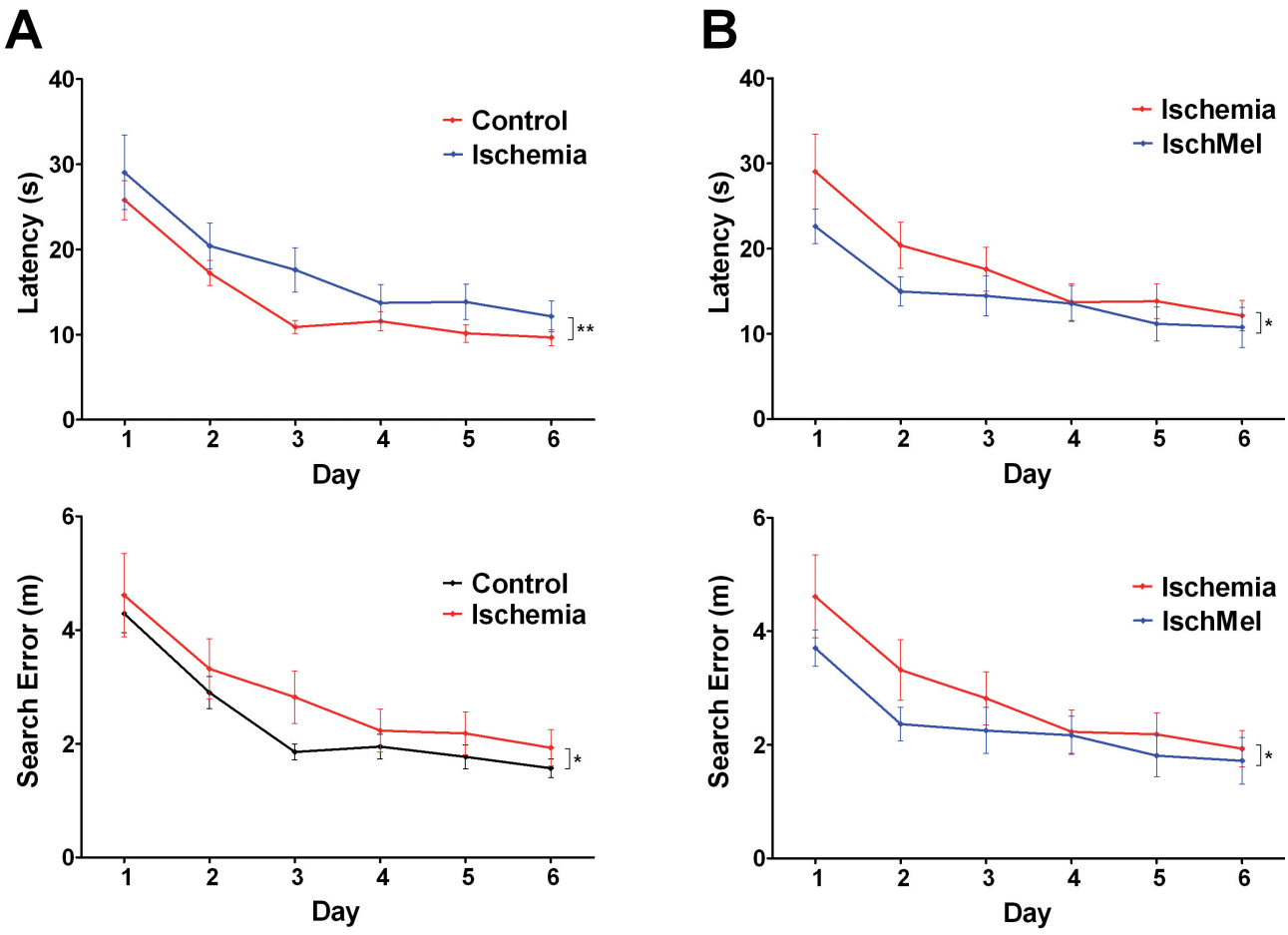


**Figure 3.** Spatial learning in place navigation tests. **A.** The swimming latencies and cumulative distances (search errors) in the place navigation test (mean ± SEM) of Ischemia and Control groups. The animals with induced ischemia needed a significantly longer time to find the hidden platform (ANOVA: $p \leq 0.01$, $F_{(4, 55)} = 7.41$). Search errors in ischemic animals were also significantly higher in respect to the Control group (ANOVA: $p < 0.05$, $F_{(4, 55)} = 3.95$). **B.** Comparison of the swimming latencies and search errors of ischemia-induced and melatonin treated (IschMel) animals (mean ± SEM). Melatonin treated group had shorter time needed to find the platform (latency) than the Ischemic animals (ANOVA: $p < 0.05$, $F_{(4, 55)} = 4.67$). The search error was also lower in melatonin treated group (ANOVA: $p < 0.05$, $F_{(4, 55)} = 3.93$). * $p < 0.05$; ** $p \leq 0.01$.

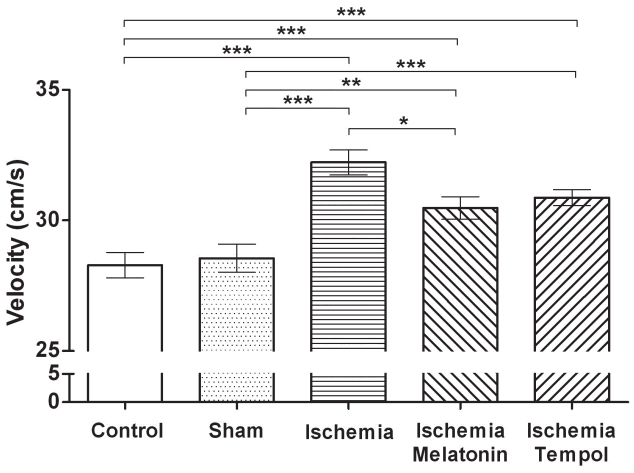


**Figure 4.** Mean swimming velocities. The graph depicts the mean swimming velocities during the place navigation test (mean ± SEM; note that the y axis is interrupted). The significant differences are indicated by asterisks (*). Regardless of the treatment type, animals with ischemic lesion showed higher velocity compared to the Control and Sham animals. Melatonin treatment after the induction of ischemia caused the significant decrease in swimming velocity in comparison to untreated animals with ischemic lesion. Significant ANOVA results: control *vs.* ischemia: $p \leq 0.001$, $F_{(4, 55)} = 37.74$; *vs.* melatonin: $p \leq 0.001$, $F_{(4, 55)} = 12.39$; *vs.* tempol: $p \leq 0.001$, $F_{(4, 55)} = 19.85$). The significance in the Sham treatment vs. ischemia: $p \leq 0.001$, $F_{(4, 55)} = 27.97$; *vs.* melatonin: $p \leq 0.01$, $F_{(4, 55)} = 8.09$; *vs.* tempol: $p \leq 0.001$, $F_{(4, 55)} = 13.19$. The significance of the melatonin treatment *vs.* ischemia $p < 0.05$, $F_{(4, 55)} = 5.53$. * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

onstrated by the decreased latencies ($p \leq 0.001$, $F_{(4, 55)} = 43.41$), the moved distance ($p \leq 0.001$, $F_{(4, 55)} = 44.57$) and the search error ($p \leq 0.001$, $F_{(4, 55)} = 38.34$). Overall the Ischemia group performed poorly compared to the Control (Fig. 3A) and the IschMel (Fig. 3B) groups. It showed an increase in the latencies, search error (for significance see Fig. 3) and the moved distance (Control: $p \leq 0.001$, $F_{(4, 55)} = 15.5$; IschMel: $p \leq 0.01$, $F_{(4, 55)} = 8.45$). Ischemia group also differed from the Sham group in the distance moved ($p \leq 0.01$, $F_{(4, 55)} = 8.61$). Tempol-treated animals were significantly worse than the Control in latency to find the platform ($p \leq 0.01$, $F_{(4, 55)} = 6.74$) and distance moved ($p \leq 0.01$, $F_{(4, 55)} = 8.45$). They differed from the Sham ($p < 0.05$, $F_{(4, 55)} = 4.84$) and IschMel ($p < 0.05$, $F_{(4, 55)} = 4.78$) groups only in longer distances moved. Unexpectedly for such a minor cortical lesion, all ischemia induced animals, regardless of treatment, showed an increased mean swimming velocity compared to the Control and Sham groups (Fig. 4). Ischemia induced animals also had higher velocity than the IschMel group.

The Memory retention conducted by the Probe test was not affected.

## Discussion

The superficial ischemic lesion of the cortex caused certain sensorimotor, spatial learning changes, and hyperactivity in presented model. The disturbance of the sensorimotor function was observed in the tests when animals had to learn a new motor skill. The animals also showed modification of the learning strategy that caused an increase in search error during spatial learning. The sign of hyperactivity was an increase in swimming velocity observed in Morris water maze tests.

The present study showed that the ischemic damage of sensorimotor cortex develops over 48 hours to cause detectable changes in function. The performance of experimental animals was significantly reduced 48 hours after induction of ischemia in the test of static postural reactions (gross vestibular function) – BB test (Alexis et al. 1995). The results showed that the Control and Sham animals had been able to learn a new motor skill, while the ischemic group lost this ability. There was evidence of slight disturbances in motor coordination as well. It was also observed that ischemia caused the worsening of the animals' performance in the reverse Rotarod test (Žáčková 1984). The animals had to learn to reorient themselves to the direction opposite to the rotation of cylinder, in addition to being able to remain on cylinder for 120 s. Deterioration of performance in animals subjected to ischemia in this test clearly demonstrated the role of the sensorimotor cortex in the learning of new motor skills. On the other hand, no disturbances in the classic Rotarod test assessing dynamic postural reactions of the animals (Hamm et al. 1994; Donát 1999) were observed in this study. Hence the conclusion that the presented superficial cortical lesions did not lead to a significant reduction in gross motor functions followed by this test. The results of sensorimotor tests demonstrated higher sensibility of the motor learning to a minor ischemic insult of the cortex than motor function itself.

The spatial learning ability in the Ischemic animals was also affected. Even though all the animals were able to learn the position of the platform hidden under water, there was a significant increase in the time (latency parameter) needed by the ischemic animals to find the platform. The role of motor learning must be also taken in account for understanding the results from MWM tests.

The hyperactivity was present in animals with the cortical lesion. It was observed 48 hours after ischemia induction in Visible platform acquisition test and it lasted through whole spatial learning period. Similar increase in locomotor activity was already described in gerbils with global cerebral ischemia (Gerhardt and Boast 1988; Cuzzocrea et al. 2000) and mouse models of focal cerebral ischemia (Winter et al. 2005; Kilic et al. 2008). In the model of minor and superficial lesion such an increase was unexpected. The possible explanation of the mechanisms of increased locomotion in the present model

is the involvement of the cortico-striatal pathways, which could cause higher levels of noradrenaline in the striatum (Winter et al. 2005). The abnormally high firing rates from the damaged neurons would increase swimming velocity of the animals from the experimental groups. Therefore, further biochemical tests for measurement of catecholamine levels would beneficial.

In the two groups that received ROS scavenger treatment ten minutes after induction of ischemia, milder disturbances of function, in comparison to the pure ischemic animals, was observed. Melatonin is a potent antioxidant and ROS scavenger, it has both intracellular and extracellular effects (Reiter 1998; Karbownik et al. 2001; Hung et al. 2008). In the present experiment, the effect of melatonin developed over 48 hours (BB1 test showed no significant changes in comparison to Control and Sham operated) and it lasted over the entire experimental period (9 days in total). However, significant neuroprotection required very high doses of melatonin (100 mg/kg).

Tempol is a stable, low-molecular weight piperidine nitroxide, widely used in animal studies for prevention of oxidative tissue damage. It is a potent membrane-permeable ROS scavenger with superoxide dismutase-like activity. Doses used in experiments varied widely from 5 to 200 mg/kg as well as the routes of administration (i.p., i.v, single or continuous injection).

In present study it was observed the great difference in parameters in respect to ischemic animals the after single dose of tempol (50 mg/kg, i.p.). Despite it, the difference was not statistically significant. Thus, we can not confirm the positive effect of this scavenger 48 hours after the induction of ischemia in sensorimotor tests. There was almost no effect of tempol observed in test of spatial learning and memory. It has been stated by Thiemermann (2003) in his review work on tempol, that the dose needed to have protective effects had to be relatively high (up to 100 mg/kg). On the other hand, Thiemermann describes the exposure of cells to higher concentrations of tempol as potential risk of tissue damage *via* tempol-induced oxidative stress and apoptosis. According to this author the lower dose of tempol in this experiment could explain the reason why the effects were not impressive in this study. Scavenger activity of tempol was also described with use of lower doses than in this experiment (Rak et al. 2000). Thus, more investigations would be needed to determine the appropriate dosage of tempol required in inducing protective effects in ischemia/reperfusion models.

Unpredicted findings were observed in the performance of Sham animals. Minor cortical defects were observed on morphology slices in some animals. In addition, the spatial learning curves analysis showed that the findings in most of the test parameters did not differ from the Control, naive animals but also from the ischemic group. It was observed that, even though presumed thermal injuries affected spatial navigation tasks, they did not cause hyperactivity, similar to that seen in the ischemic animals. Then the hyperactivity could be related to the ischemic component of the photothrombotic lesion but not only to the tissue defect itself.

In conclusion, we must recall that even a minor cortical lesion is capable of causing mild sensorimotor learning deficits and a decline in MWM performance. Our findings confirmed the expected effects of ROS scavengers on ischemia induced animals. We suppose that the development of post-ischemic hyperactivity is not directly related to oxidative stress itself but more to the ischemia of the sensorimotor cortex.

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