METHODS

Comparative Evaluation of Two Methods for Studies of Experimental Focal Ischemia: Magnetic Resonance Tomography and Triphenyltetrazoleum Detection of Brain Injuries

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The volumes of foci of injuries, evaluated by T2-suspended MRT images and analysis of histological sections stained by triphenyltetrazoleum chloride, were compared on a model of unilateral intravascular blocking of the middle cerebral artery branch. The two methods for evaluation of foci of lesions gave close results, correlating with the severity of neurological deficiency in animals subjected to ischemia, manifesting in behavioral tests.

Key Words: *cerebral ischemia; magnetic resonance tomography; triphenyltetrazoleum chloride*

Cerebral ischemia is caused by temporary or permanent disorders in blood supply to the brain or its part, which can be a result of embolism, thrombosis, or hemodynamic disorders. A local reduction of blood delivery to the brain causes exhaustion of energy reserves in this zone, which often leads to development of a cascade of neuronal injuries, involving hyperactivation of glutamate receptors, production of free radicals, infiltration of macrophages, and other pathological processes [5,10]. The problem of cerebral ischemia and pharmacological correction of this condition is one of the priority problems of modern experimental and clinical neurology and neurosurgery, requiring creation of adequate models and methods for evaluation of ischemic effects.

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Today many experiments are carried out on a model of ischemia initiated by unilateral intravascular blocking of the middle cerebral artery by means of a capron thread. The thread is inserted through *a. carotis communis* to the site of *a. cerebri media* ramification from *a. carotis interna* [6,11]. This method for induction of focal ischemia leads to development of a standard (by size and location) focus of ischemic necrosis. The size of the focus is evaluated in sections prepared by the standard histological methods. Magnetic resonance tomography (MRT) has been used for this purpose in recent years [2,4,9]. This method is characterized by some advantages over histological methods: it is noninvasive, the process can be studied over time, and the lesions can be rapidly evaluated. However, it involves a detailed comparison of the morphometrical results obtained by MRT with the histological findings and comparison of both with the results of behavioral tests, evaluating the neurological defi-

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ciency in animals. By the present time just few reports on the problem are available and the reported data are miscellaneous.

Using a model of unilateral intravascular blocking of the middle cerebral artery branch, we compared the volume of the focus of lesions evaluated using T2-suspended MRT images and the sections prepared by the standard histological methods. The severity of neurological deficiency was evaluated by behavioral tests.

MATERIALS AND METHODS

The study was carried out on 15 male outbred rats (220-250 g). The animals were handled in accordance with the requirements formulated in Directions of the European Community Council 86/609/EEC on the use of animals for experimental studies.

The animals were divided into 2 groups: 1) sham-operated (n=7) and 2) rats with ischemic cerebral infarction (n=8). The rats were daily weighed. The animals received chloralhydrate anesthesia (300 mg/kg intraperitoneally).

Brain ischemia was induced by insertion of a silicone-coated Nylon thread [13] till blocking of the middle cerebral artery, as described previously [8,14]. The bloodflow was blocked during 60 min, after which the thread was removed from the vessel and blood supply to the middle cerebral artery basin was restored. Body temperature was maintained at the level of 37.0±0.5°C during and after the operation. Group 1 animals were subjected to the same procedures without crossing the vessels and thread insertion.

All behavioral tests were carried out 24 h before the operation, thus determining the basal values. Neurological deficiency of animals was evaluated on days 4 and 7 after the intervention by the limb stimulation test and on day 7 postoperation by the cylinder test. Animal behavior was evaluated using double blind control.

The degree of asymmetry in the use of the fore limbs during spontaneous exploration of the cylinder walls was evaluated in the cylinder test [12].

The sensorimotor recovery of the rat limbs was evaluated by the modified limb stimulation test [7]. This test consiss in reaction of the hind and fore limbs to tactile, proprioceptive, and visual stimulation. The test included 7 trials for the left and right sides of the body. Disorders in the limb work were evaluated by scoring: 2 points: the rat completely fulfilled the test; 1 point: the test was fulfilled with a more than 2 sec delay and/or incompletely; 0 points: the rat did not react to limb stimulation.

The brain of all experimental animals was studied by MRT on days 1 and 7 after the operation. All MRT experiments were carried out on a BioSpec 70/30 device (Bruker) with 7 T magnetic field induction and 105 mT/m gradient system.

The radiofrequency signal was transmitted using a linear transmitter with an inner diameter of 72 mm. A surface receiver coil for the rat brain was used for detecting the radiofrequency signal. The T2-suspended images were obtained using the RARE (Rapid Acquisition with Relaxation Enhancement) spin echo-based pulsed sequence with the following parameters: TR=6000 msec, TE 63.9 msec, section thickness 0.5 mm, visual field 4.2×3.1 cm, matrix 256×384, resolution 0.164×0.164 mm/pixel. Total scanning time was 4 min 48 sec. The animals were anesthetized by chloralhydrate and placed into the positioning device with a stereotaxis and thermoregulation systems. The following parameters were monitored during MRT imaging: ECG, respiration rate, and rectal temperature using Small Animal Monitoring and Gating System (SA Instruments Inc.). The MRT images were analyzed using ImageJ 1.38x software (National Institutes of Health).

On day 7 after MRT study the animals were injected with chloralhydrate overdose and decapitated. The brain was rapidly removed and washed in saline. Coronal sections (2 mm) were sliced using a special device with grooves for precise slicing. The slices were stained by 1% triphenyltetrazoleum chloride during 10 min at 37°C and fixed in 10% formalin solution in 0.1 M phosphate buffer [3]. The slices were scanned from both sides at a resolution of 2400 dpi in order to obtain images. Sites not stained by tetrazoleum were considered as necrotic zones. The infarction area, including the cortex and subcortical structures for each section, was measured using software for image analysis. The infarction volume (V) was evaluated by the formula:

$$V=d\times\Sigma Ai,$$

where ΣAi is the sum of damaged areas in all sections and d is the thickness of slices.

The infarction volume, percentage of brain edema, and percentage of injury were evaluated for the entire hemispheric volume using T2-suspended MRT images, obtained on days 1 and 7 after ischemia [2].

The data were statistically processed using Statistica 6.0 software (StatSoft). The normality of the sign distribution in the sample was evaluated by Shapiro—Wilk's W test. Statistical significance of differences in behavioral tests was evaluated using Mann—Whitney's U test. The data are presented as the mean±error in the mean.

RESULTS

The cortex with the sensorimotor zones and striatum are the main targets of injury in the model of ischemia used in our study. In the sections they look clear (unstained) (Fig. 1). Magnetic imaging of the rat brain 24 h after ischemia showed the zone of injury as hyperintense signal in T2-suspended images in comparison with intact white matter at the expense of development of the cytotoxic edema. This method, similarly as staining by triphenyltetrazoleum chloride (TTC), showed that the damaged structures were predominantly the brain cortex and the sublying striatal area (Fig. 2, *a*). According to morphometry on day 1, the volume of damaged hemisphere was 14±3% larger in comparison with the intact hemisphere because of edema caused by ischemia. On day 7 after ischemia initiation the volume of the damaged hemisphere reduced significantly and was just 3±1.2% larger than that of the contralateral hemisphere. Magnetic imaging showed a trend to a reduction of the impaired zone hyperintensity in comparison with day 1 after ischemia (Fig. 2, *a*, *b*). Morphometric values obtained by TTC staining of the brain on day 7 after ischemia (Fig. 2, *c*) showed the size of damaged zone very close to the MRT result (Table 1). The true size of the involved zone (with consideration for the severity of edema of the damaged zone) on days 1 and 7 did not precisely coincide according to MRT and TTC method, but the absolute values were rather close (Table 1). No injuries were detected by MRT and histological studies in group 1 animals. According to MRT, on day 1 the volume of injury was 55.0±3.3%, on day 7 it was 59.3± 3.3% of the entire hemisphere. These data indicate that in our model the main process of formation of the ischemic focus took place during the first 24 h after ischemia.

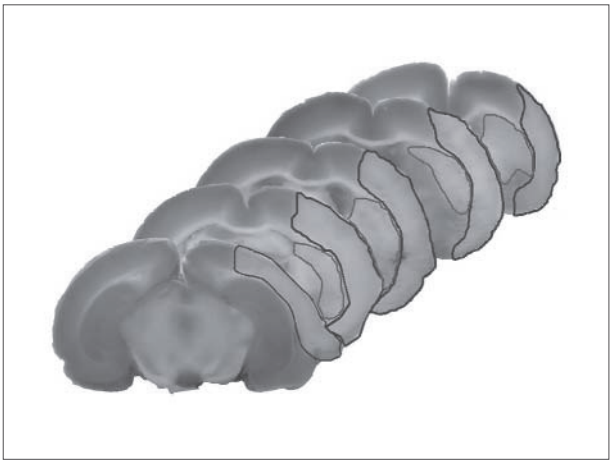

Fig. 1. Standard picture of lesions in the cerebral cortex (outlined with a black contour) and the sublying striatum after ischemia initiation by blocking of the middle cerebral artery with a capron thread. TTC method for detection of ischemic focus in sections, day 7 after ischemia.

These morphological values confirm that 1-hour blocking of the middle cerebral artery caused significant damage to the cerebral cortex and striatum of rats, which were well visualized at the histological level and by MRT. Quantitative data on the volume of the foci and their location, obtained by the TTC method, were close to the values calculated from analysis of MRT images (Table 1), which completely correlated with the sensorimotor disorders in the limbs of these animals, shown by behavioral tests (Fig. 3). Independent use of the contra- and ipsilateral fore paws in the cylinder test before induction of ischemia was 19 and 13% of all tactile contacts with the cylinder wall, respectively (Fig. 3, *b*). On day 7 after the operation the use of the damaged paw (contralateral to the damaged cerebral hemisphere) reduced statistically significantly (2-fold), reaching 10%. This decrease was compensated for by more active use of the ipsilateral paw (4-fold, or 53% in comparison with group 1; $p<0.05$). It is noteworthy that 4 and 7 days after ischemia the neurological deficiency was incompletely, but statistically significantly ($p<0.05$) compensated (Fig. 3, *a*). In parallel with development of changes in neurological deficiency, group 2 rats exhibited a stubborn body weight loss, which

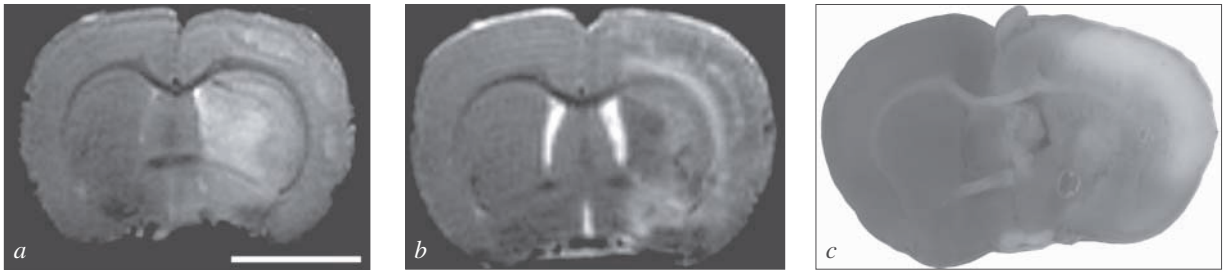

Fig. 2. Comparison of ischemic injury in the same site of the brain on days 1 (*a*) and 7 (*b*) after ischemia (MRT method) and on day 7 (*c*) (TTC method). Scale: 5 mm.

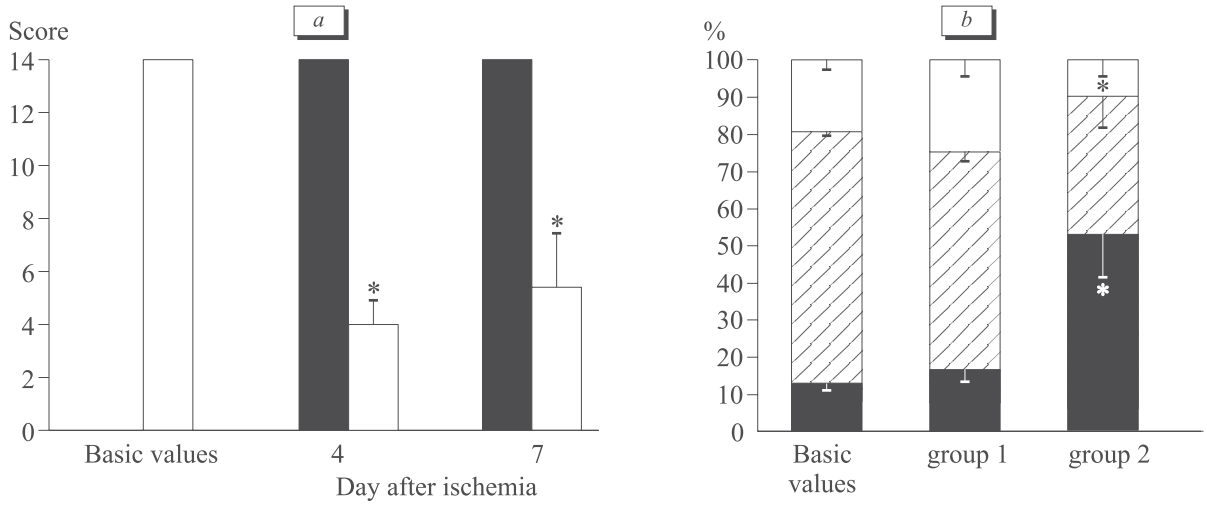

Fig. 3. Pronounced neurological deficiency in animals exposed to ischemia: limb stimulation test on days 4 and 7 (*a*) and cylinder test on day 7 (*b*) after ischemia. *a*) dark bars: group 1; light bars: group 2; *b*: dark segment: ipsilateral paw; cross-hatched segment: both paws; light segment: contralateral paw. $p<0.05$ *vs.* group 1.

TABLE 1. Volumes of Cortical and Striatal Injuries in Focal Cerebral Ischemia in Rats with Consideration for Edema of the Damaged Zone on Days 1 and 7 after Ischemia

Day after ischemia	Size of injury in cerebral zones, mm^3			
	MRT		TTC method	
	cortex	striatum	cortex	striatum
1	192±28	57±3.7	—	—
7	199±29	47±5.3	217±25	57±6.9

was the maximum (18.0±3.6% of body weight before the intervention) on day 4 and regressed by day 7 (9.3±3.3%). In group 1 body weight loss was observed only during the first 2 days after the operation and was negligible. Such body weight changes were observed previously in animals subjected to ischemia [1].

Hence, the results of behavioral tests, indicating neurological deficiency in animals subjected to ischemia and the data of standard histological TTC test were in good correlation with morphological data on the severity of brain injury, obtained by MRT. The advantages of MRT (the method is noninvasive, the process can be studied over time, the injuries can be rapidly evaluated) indicate the efficiency and good prospects of MRT as a method for studies of the mechanisms of brain injury development in experimental focal ischemia and for evaluating the efficiency of new neuroprotectors.

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