METHODS

Exercise Tests and BBB Method for Evaluation of Motor Disorders in Rats after Contusion Spinal Injury

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Contusion spinal injury of different severity was induced by the weight drop method in male rats by dropping standard weight from the heights of 6.5, 12.5, 25, and 50 mm on the spine after laminectomy at the Th9 level. The dynamics of recovery of voluntary movements was evaluated over 8 weeks after the operation by comparing the traditional semiquantitative BBB score with objective parameters of exercise tests. Exercise tests detected cessation of spontaneous recovery of impaired functions 1-2 weeks earlier than the BBB method. The maximum performance according to the rotarod and narrowing track tests was 20-40% lower than the level of voluntary movement recovery evaluated by the BBB score. No appreciable differences between the levels of maximum recovery of voluntary movements (BBB score) and swimming velocity were detected. According to morphometric analysis of the volumes of pathological cavities and intact white matter in the focus of injury, exercise tests adequately reflected the severity of spinal contusion in rats. Exercise tests used in the study are recommended for objective preclinical evaluation of the efficiency of new means for the treatment of spinal injuries.

Key Words: *spinal contusion; exercise tests; BBB method*

The pathogenesis of structural and functional disorders leading to pronounced motor deficiency was revealed in basic studies of spinal injuries (SI) on animals [1,4]. However, effective pathogenetic therapy for correction of SI consequences remains to be found [5,8].

The solution of the problem is largely impeded by imperfect modern methodologies for evaluating the efficiency of experimental therapy for SI. For example, the majority of authors evaluate functional recovery of the spinal cord (SC) by semiquantitative visual assessment of voluntary movements of rats in the "open field" by the BBB score [2]. The authors of this method distinguish three phases of recovery after SI: early (appearance of movements in the hind paw joints, which corresponds to 0-7 points), intermediate (recovery of plantar steps and normal coordination of the fore and hind paw movements; 8-13 points), and late phase (the paw is put parallel to the trunk, the thumbs are elevated above the floor during a step

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by the hind paws, *etc.*; 14-21 points). The use of the BBB score implies that two workers participate in experiment, as estimation of 1-2 point differences in the motor sphere is rather subjective. On the other hand, only so low a level of spinal function recovery can be attained in experimental therapy of SI [5]. It is obvious that preclinical trials of new means for correction of motor disorders should include methods for objective registration of motor activity parameters in laboratory animals. These methods include exercise tests, characterizing physical performance and adaptation potential of the body in general. However, there are virtually no reports on the use of these tests for evaluation of the rat functional status after SI [7].

We compared the representative value of exercise tests and the BBB method for evaluation of the dynamics of recovery of motor and coordination dysfunctions in rats after spinal cord contusion (SCC) of different severity.

MATERIALS AND METHODS

The study was carried out on outbred male albino rats (n=81) initially weighing 300-350 g. The animals were kept under standard conditions of an experimental biological vivarium with free access to water and food.

The rats were narcotized by intraperitoneal injection of a mixture of 5% ketamine (100 mg/kg) and 0.5% seduxen (3 mg/kg). After laminectomy at the Th9 level, the rat spine was rigidly fixed with metal clips by the osteal processes of Th8 and Th10 vertebrae. Spinal contusion was induced by the weight drop method: a metal rod (10 g, 2 mm in diameter) fell vertically strictly into the center (in the median line) of a visualized portion of the spine. The shock mechanism was precisely aimed into the point of injury by a stereotaxic device micromanipulator. SCC of different severity were induced by dropping the rod from different heights (6.5, 12.5, 25, and 50 mm) similarly to the conditions described by the authors of the BBB method [2].

Two series of experiments were carried out. In series I, the classical SCC model was reproduced [2,6]. SCC of different severity was reproduced in rats of 4 groups: mild (weight drop from the height of 6.5 mm; 10 rats), medium (weight drop from the height of 12.5 mm; 10 rats), severe (weight drop from the height of 25 mm; 10 rats), and extremely severe (weight drop from the height of 50 mm; 6 rats). Functional recovery was evaluated only by the BBB method. Experimental series II (study by exercise tests) was carried out on rats with mild (n=9), medium (n=18), and severe (n=18) SCC. Exercise tests were not carried out on rats with extremely severe SCC because of prolonged paraplegia of the hind limbs.

Animals used in both series of experiments were daily adapted to the open field (BBB method) for 3 days before SCC, and series II animals were additionally trained to perform exercise tests: clinging to a rotating rod in the rotarod device, moving along a narrowing track, and swimming in a narrow basin. At the end of training, the results in each test were recorded.

After SCC induction, the rats were tested weekly over 15 weeks (series I) and 8 weeks (series II) during the same hours of the day. Motor functions were tested by the following protocol: day 1, BBB and narrowing track; day 2, rotarod; day 3, swimming.

Voluntary movements of rats were evaluated by the BBB score; the animals were placed in the center of an open field (round field 110 cm in diameter with a wall) and the parameters of their movements were recorded strictly according to the recommendations of the BBB score authors [2]. An independent experimentator took part in the study for verification of values.

For training the animals to perform the rotarod test, the rats were placed on a drum 7 cm in diameter and its rotation velocity was gradually increased from 7 to 20 rpm. Before SCC the animals rapidly adapted to the procedure and could cling to the drum rotating at a velocity of 20 rpm during 180±20 sec (n=35). During testing after SCC, the time of clinging to rotating drum was recorded.

In one more test the rats were trained to swim without touching the lateral walls of the basin (120×20 cm) and get out to the platform situated on the opposite side of the basin. Before SCC the rats covered the swimming track at a velocity of 0.38±0.05 m/sec (n=35). During testing after SCC, the swimming velocity was recorded.

In the narrowing track test the rat was placed at the beginning of a 165-cm-long track, 9 cm wide at the beginning and 3-cm-wide at the end, located at the height of 80 cm above the floor and ending in the cage familiar for the animals. Intact rats actively moved towards the cage and passed the track uneventfully. After SCC, the distance from the starting point to the point of the first error (one of the hind paws missing the track) was recorded.

Material for the analysis of the relationship between functional disorders and SC injuries in experimental series II was collected 5, 30, and 60 days after SCC (exercise tests). The material was collected from rats with medium and severe injuries, 5 per group. The animals were narcotized with ketamine (150 mg/kg). Transcardial perfusion with phosphate buffer (pH 7.2) and 4% paraformaldehyde was carried out. After laminectomy and exposure of the spine, the focus of injury was visualized and a 20-mm fragment of the spine was collected 10 mm rostrally and 10 mm caudally from the epicenter. The material was fixed in 10% neutral formalin, dehydrated, and embedded in paraffin by the standard method. The areas of pathological cavities, intact white and gray matter in the focus of injury and at a distance of 1, 3, 5, 7, and 10 mm rostrally and caudally from it were measured on the transverse sections of the spine stained with methylene blue or luxol fast blue. Morphometry was carried out after digital processing of the material using the Photoshop software.

The data were statistically processed using Student's *t*, ANOVA and Mann—Whitney tests, and Pearson's correlations coefficient.

RESULTS

During 3 months of the study, the survival of rats after SCC was 100% in the group with mild injury, 97% after medium severe injury, 85% after severe, and 65% after extremely severe injuries. Mortality was observed mainly during the first 5-7 days.

Gradual recovery of voluntary movements was observed in all groups of animals; it was over 4-5 weeks after the standard SCC injury (Fig. 1). The BBB score virtually did not change after reaching the levels proportional to the severity of SCC. These results in fact completely reproduced the data obtained by the authors of the BBB method for evaluation of voluntary movements of rats after SCC [2]. Our long-term (15 weeks) monitoring of animals after SCC showed neither spontaneous improvement, nor spontaneous deterioration of voluntary movements in rats with SCC.

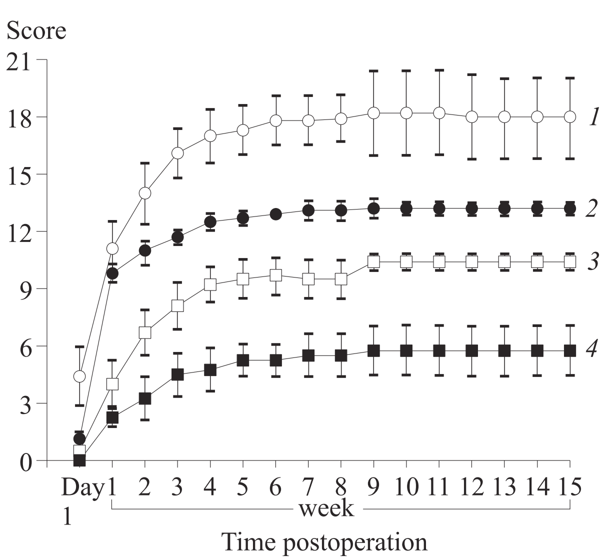

Fig. 1. Dynamics of voluntary movements recovery (BBB method) in rats with SCC of different severity. *1*) mild, *2*) medium, *3*) severe, *4*) extremely severe injury. Differences between the groups are significant ($p<0.05$) for all periods of the study.

Analysis of the results of exercise tests showed specific features in the periods and levels of spontaneous recovery of functions impaired after SCC (Table 1, Fig. 2).

Voluntary movements were restored soon in rats with mild SCC: the steps were coordinated from week 2 (more than 14 points, BBB score; Fig, 1). However, exercise tests showed no adequate improvement of performance. For example, swimming velocity, time of holding to rotating rod, and uneventful passage along the narrowing track remained low 3-4 weeks after SCC (Fig. 2). Rats with medium and severe SCC also exhibited significantly worse performance in exercise tests (rotating rod and narrowing track) and sooner (2-3 weeks after SCC) cessation of performance restoration in comparison with the level of voluntary movements recovery shown by the open field test (BBB score). The greatest motor deficiency was recorded in the rotating rod test. On the whole, the dynamics of swimming velocity recovery was virtually the same in both groups as that according to the BBB score.

Hence, the results of exercise tests and the method for evaluation of voluntary movements (BBB score) adequately reflect the severity of SCC in rats, which is confirmed by SC morphometry. For example, total area of pathological cavities measured on a transverse section of the spine 5, 30, and 60 days after injury at a fixed (1, 3, 5, 7, and 10 mm) distance from the epicenter in the rostral and caudal directions was larger in severe injury (Fig. 3). On the other hand, the volume of intact white matter of SC was lower in rats with severe SCC than in animals with medium severe injury. The differences in these zones were statistically significant ($p<0.05$) 30 days after SCC.

The severity of disorders in voluntary movements (BBB test) in rats with SCC at the Th8-Th10 level is largely determined by the volume of intact spinal white matter in the focus of injury [3]. The data indicate that this factor also determined functional recovery demonstrated in the rotating rod, swimming, and narrowing track tests. For example, 30 days after SCC (by the moment of completion of morphological organization in the focus of injury) [1], the volume of intact white matter and the results of exercise tests were in medium ($r>0.33$) and strong ($r>0.66$) correlation (Table 2). Coefficients of correlation between the volume of intact white matter and results of exercise tests were sig-

TABLE 1. Dynamics of Exercise Test Results in Rats with SCC of Different Severity (*M*±*m*)

Test	SCC severity	Week after SCC								
		before operation	1	2	3	4	5	6	7	8
Swimming, m/sec	mild	0.39±0.02	0.21±0.01	0.240±0.009	0.240±0.007	0.260±0.009	0.280±0.009	0.290±0.009	0.300±0.007	0.290±0.007
	medium	0.40±0.02	0.180±0.006	0.230±0.016	0.230±0.011	0.250±0.007	0.250±0.012	0.250±0.007	0.270±0.008	0.270±0.011
	severe	0.37±0.01	0.130±0.005**	0.140±0.001**	0.150±0.004**	0.150±0.001**	0.150±0.005**	0.160±0.005**	0.160±0.004**	0.160±0.004**
Rotating rod (20 *rpm*), sec	mild	178.6±4.3	3.7±2.18	12.60±3.03	16.20±3.57	21.00±2.59	29.10±8.03	33.70±5.21	32.70±4.69	37.40±6.57
	medium	185.1±3.1	2.10±1.78	7.90±3.29	14.10±3.57	18.10±1.58	19.00±4.13	22.40±1.58	23.6±2.5*	22.90±1.58*
	severe	174.1±4.6	0.7±0.7*	1.3±0.9**	2.40±0.96**	3.30±0.96**	4.70±1.23**	4.70±0.96**	5.90±1.11**	5.9±1.3**
Narrowing track, cm	mild	165	36.8±8.6	59.8±10.7	70.8±19.6	94.2±18.6	95.0±19.4	94.8±15.6	94.8±16.5	96.3±15.7
	medium	165	28.3±12.1	34.3±7.6	41.3±14.7	67.0±5.4*	67.0±6.7*	66.1±3.4*	68.9±5.5*	67.9±7.4*
	severe	165	—	—	—	22.0±2.1**	24.5±3.0**	26.75±2.00**	28.8±3.6**	28.75±4.50**
BBB score, points	mild	21	10.8±1.3	13.8±0.9	16.4±0.6	17.4±0.6	17.4±0.6	17.6±0.8	17.6±0.8	17.6±0.8
	medium	21	9.8±0.3*	11.2±0.6*	11.8±0.3*	12.5±0.3*	12.75±0.30*	13.0±0.5*	13.0±0.5*	13.0±0.5*
	severe	21	3.8±0.9**	6.75±1.30**	8.0±0.9**	8.75±1.00**	8.75±1.00**	8.75±1.00**	8.5±0.8**	8.5±0.8**

Note. *$p<0.05$ compared to rats with mild SCC; **$p<0.01$ compared to rats with mild and medium SCC.

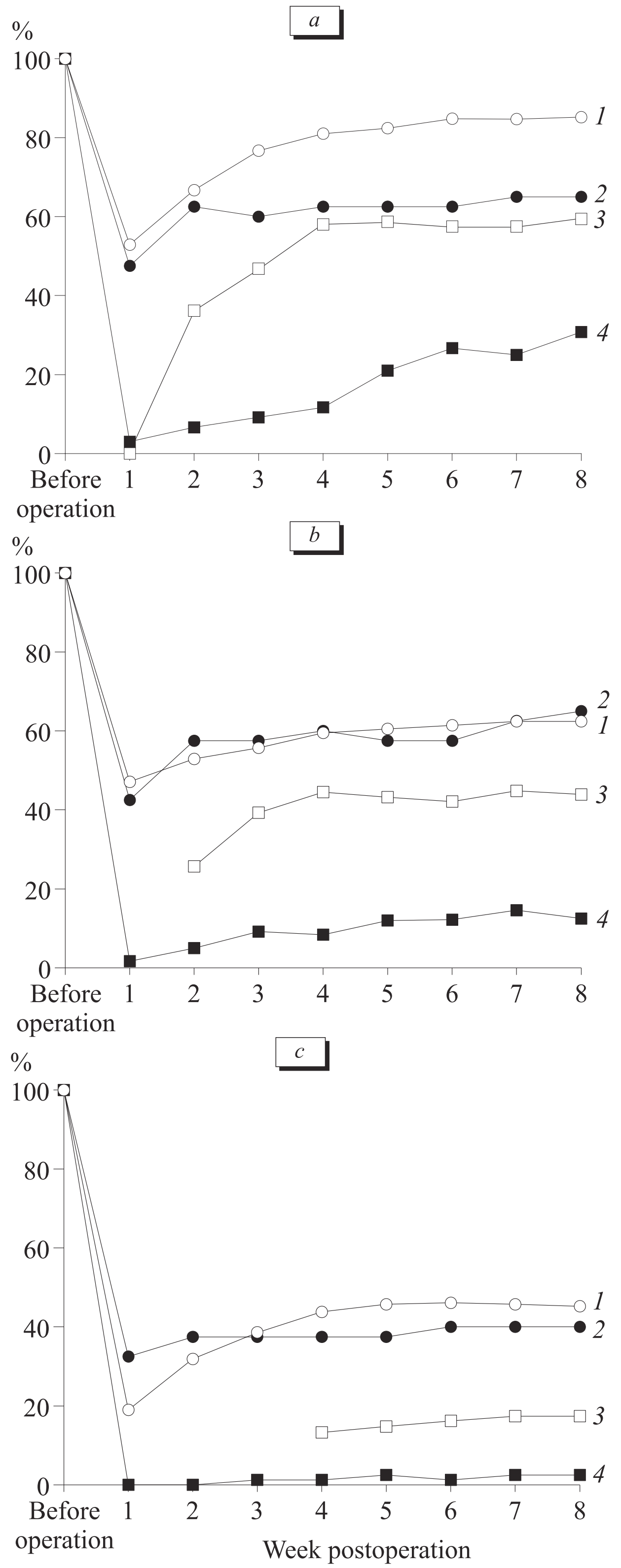

Fig. 2. Dynamics of recovery of voluntary movements (BBB test) and performance in exercise tests in rats after SCC of different severity. *a*) mild, *b*) medium, *c*) severe injury. *1*) BBB; *2*) swimming; *3*) narrowing track; *4*) rotarod. Values before SCC are taken for 100%.

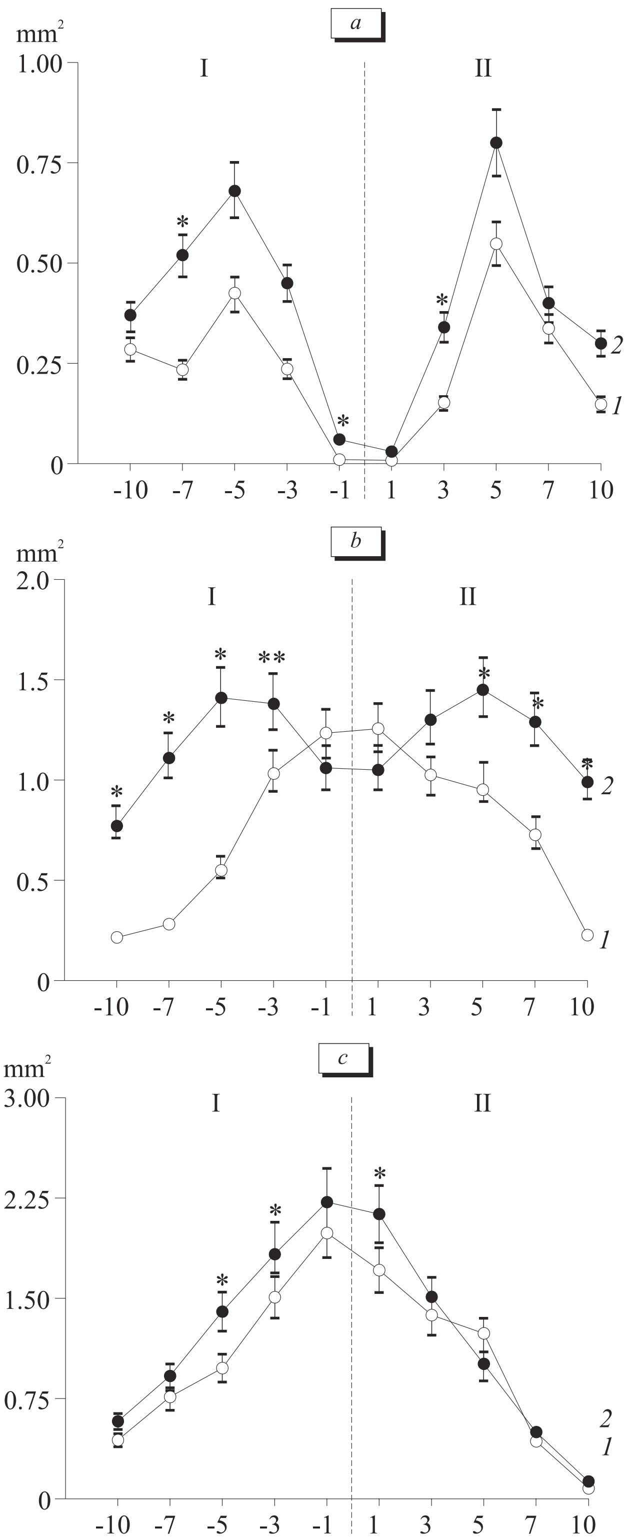

Fig. 3. Area of pathological cavities in transverse sections of the spine 5 (*a*), 30 (*b*), and 60 (*c*) days after medium (*1*) and severe (*2*) SCC. Abscissa: distance from the focus (mm). *I*: caudal direction; *II*: rostral direction. Ordinate: summary area of cavities in transverse section of the spine. *$p<0.05$, **$p<0.01$ compared to rats with medium severe SCC.

TABLE 2. Correlations between Functional Recovery Parameters and Area of Intact White Matter in the Focus of Its Injury in Rat Spinal Sections 4 Weeks after SCC of Different Severity

Severity of SCC	BBB	Rotarod	Swimming	Narrowing track
Medium	0.81	0.49	0.56	0.78
Severe	0.41	0.72	0.96	0.93

nificantly higher in animals with greater loss of white matter (severe injury) in the focus of SCC.

Comparative analysis of motor disorders normalization, evaluated by the BBB score and exercise tests, has shown that the tests more precisely indicated the type of pathological changes in the motor activity after experimental SCC of different severity. For example, exercise tests detect cessation of spontaneous recovery of impaired functions 1-2 weeks earlier than the BBB method. In addition, the maximum performance of all groups of rats with different severity of SCC in the rotarod and narrowing track tests was 20-40% lower than the levels of voluntary movements recovery, evaluated by the BBB score. On the other hand, the levels of maximum recovery of voluntary movements (BBB score) and swimming velocity virtually did not differ in rats with medium and severe SCC. This recommends the swimming test as the objective method characterizing the degree of motor disorders recovery in rats after SCC, adequate to the BBB method.

Hence, exercise tests (swimming, rotating rod, and narrowing track) objectively reflect motor recovery in rats after SCC of different severity. In combination with the BBB method, they reflect the adaptation potential and performance in general. Exercise tests used in our study are recommended for preclinical studies of the efficiency of new treatments for spinal injuries.

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