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EFFECTS OF AQUATIC EXERCISES IN A RAT MODEL OF BRAINSTEM DEMYELINATION WITH ETHIDIUM BROMIDE ON THE BEAM WALKING TEST

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Abstract – Multiple sclerosis is a demyelinating disease of the central nervous system associated with varied levels of disability. The impact of early physiotherapeutic interventions in the disease progression is unknown. We used an experimental model of demyelination with the gliotoxic agent ethidium bromide and early aquatic exercises to evaluate the motor performance of the animals. We quantified the number of footsteps and errors during the beam walking test. The demyelinated animals walked fewer steps with a greater number of errors than the control group. The demyelinated animals that performed aquatic exercises presented a better motor performance than those that did not exercise. Therefore aquatic exercising was beneficial to the motor performance of rats in this experimental model of demyelination.

KEY WORDS: multiple sclerosis, demyelination, aquatic exercises, physiotherapy.

Efeitos de exercícios aquáticos no desempenho motor de ratos submetidos a um modelo de desmielização com brometo de etídio

Resumo – A esclerose múltipla é uma doença desmielinizante do sistema nervoso central que se associa a graus variados de incapacidade. Não se sabe se a realização de intervenções fisioterapêuticas precoces têm impacto na evolução dessa doença. Utilizamos um modelo experimental de desmielinização com o gliotóxico brometo de etídio e a aplicação de exercícios aquáticos precoces para avaliar o desempenho motor dos animais. Foram quantificados o número de passos e os erros executados durante a travessia da barra elevada. Os animais desmielinizados apresentaram menor número de passos e maior quantidade de erros em comparação aos grupos-controle. Os animais desmielinizados que realizaram exercícios aquáticos apresentaram melhor desempenho motor que os animais desmielinizados que não foram submetidos à intervenção. Portanto, a realização de exercícios aquáticos mostrou-se benéfica no desempenho motor dos animais submetidos ao modelo experimental do brometo de etídio.

PALAVRAS-CHAVE: esclerose múltipla, desmielinização, exercícios aquáticos, fisioterapia.

Multiple sclerosis (MS) is a chronic neurologic disease, often disabling, whose progressive course is characterized by multiple motor deficits leading to severe disability[1]. It is characterized as an auto-immune disease that causes destruction of oligodendrocytes and, consequently, loss of the myelin sheath. This myelin loss causes a great variability of pathologic conditions[2-4]. Although its etiology is unknown[5], it is postulated that genetic and environmental factors causes self-destructive lesions in the central nervous system, specifically against the white matter[5-7]. In spite of the present emphasis on preventive health[8], neurorehabilitation, in general, and physiotherapy, specifically, are not started until the patient is severely disabled. Most patients' referrals to rehabilitation services happen when there are important functional disturbances such as limitation in gait, in transfer, diminished muscle strength or deterioration of medulla oblongata function[9,10]. These late referrals seem to be due to a lack of evidence about the effects of early physiotherapy in MS, which can be beneficial, innocuous or even harmful to these patients' rehabilitation.

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Currently, physiotherapy in the treatment of multiple sclerosis is started at latter stages of the disease, and as this happens when there are incapacitating limitations, physiotherapy remains restricted to rehabilitation. The use of experimental models of demyelination is an option to understand the biology of MS. Numerous experimental models that simulate the pathophysiology of the demyelinating process of MS have been developed. Ethidium bromide (EB) is a gliotoxic drug that induces demyelination[2,3] and it is a well described model that has improved the comprehension of the biologic process of central nervous system (CNS) demyelination[11-13].

The aim of this study was to evaluate the locomotion of EB-demyelinated rats on the beam walking test, after early physical activity with aquatic exercises and to observe its impact on the motor performance of the animals.

METHOD

The research project was approved by the Ethics and Research Committee of the Universidade Metodista de São Paulo on September 15, 2005 (project # 200/05). We used twenty-eight male Sprague-Dawley rats, which were one month old in the beginning of the experiments, kept in standard animal facility. During the experiments the animals received *ad libitum* water and pelet food (Novital).

Study protocol

The animals were anesthetized with ketamine and xylazine (5:1, 0.1 mL/100 g), shaved in the fronto-parieto-occipital region of the skull and cutaneous antisepsis was performed with 2% iodine solution. A burr-hole was made on the skull, 0.85cm postero-diagonally to the right-side of the bregma exposing the dura mater. The injections of 10 μL of 0.1% EB solution in 0.9% saline or 0.9% saline solution were carried out using a Hamilton syringe fitted with a 26 gauge removable needle, inserted in a vertical position until reaching the base of the skull, into the cisterna pontis, an enlarged subarachnoid space below the ventral surface of the pons.

The rats were randomly assigned into 4 groups of 7 animals each.

All the animals underwent an adaptation period on the elevated beam and only the first group performed adaptation to the aquatic exercises. The adaptation was done for 10 consecutive days before the surgical procedure. The initial duration of the aquatic exercises was 5 minutes with a progressive increase up to 10 minutes of exercise in a 28°C heated pool[14]. The animals were trained in the elevated beam for 5 minutes. As described by Jeffery and Blakemore[15], the elevated beam is 2 m long and 18 mm wide, with 10×10 cm^2 platforms in each end. The central 1m portion of the beam was measured and the limits marked.

Following the training period, the animals in each group underwent the surgical procedure and were then discriminated as: Group 1: Animals that were injected with 0.1% EB and performed aquatic exercises. Group 2: Animals that were injected with 0.1% EB and did not perform aquatic exercises. Group 3: Animals that were injected with 0.9% saline and did not perform aquatic exercises. Group 4: Control animals that did not perform aquatic exercises.

Right after demyelination, the first group performed aquatic exercises for 31 days, with a frequency of 3 weekly sessions of 10 minute duration.

All groups were evaluated in the beam walking test at 24 and 48 hours and at 3, 7, 11, 15, 21 and 31 days post-injection.

For the evaluation of the animal performance on the beam walking test, two video-cameras were positioned on opposite sides and all the evaluations were saved for posterior analysis. Two independent examiners participated in the recordings.

The evaluation of the groups consisted of counting the number of footsteps needed for each animal to complete the traverse of the beam and by means of a score rank that considered the analysis of the footsteps taken by the rats with the posterior limbs in relation to their positioning. Score 0 represented an adequate positioning of the posterior limb and scores 1 and 2 were considered errors and were analyzed in conjunction[15] (Table 1). In the end of each session, the score and the footsteps taken to complete the traverse were added. These data were collected when the animal walked between the 1m marks on the central part of the beam.

Statistical analysis

The results obtained from the two examiners were compared and tested for concordance with weighted kappa (κ). All variables were tested for normality with Kolmogorov-Smirnov goodness-of-fit model. All variables were considered to be normally distributed. For the comparisons among and inside groups we used Analysis of Variance Test (ANOVA). The comparison between groups, two by two, was performed with Mann-Whitney test. All p values <0.05 were considered to be significant. Statistical analyses were performed using Statistical Package for Social Science (SPSS) version 10.0 and Medical Calculator (MedCalc) version 9.0.

Table 1. Score rank for the analysis of the footsteps taken by the rats with the posterior limbs in relation to their positioning.

Score	Posterior limbs positioning
0	'Normal': foot positioned on top of the beam, no slippage
1	'Minor error': foot slip so that part of the foot was visible below the lower surface of beam, or the foot dragged along beam surface
2	'Major error': whole foot slipped below lower surface of the beam.

Table 2. Number of footsteps of four animal groups.

Groups/days	1	2	3	4	5	6	7	8
1	69.3 (2.9)	65.3 (9.2)	60.4 (1.5)	63.4 (6.4)	65.6 (7.9)	62.7 (3.9)	58.7 (4.3)	56.4 (5.7)
2	55.6 (30.2)	69.5 (8.8)	64.0 (4.3)	57.4 (6.7)	64.8 (10.0)	56.4 (10.4)	49.0 (19.1)	59.2 (5.4)
3	74.1 (4.5)	67.2 (4.1)	64.1 (3.6)	60.4 (11.2)	62.0 (4.3)	61.7 (3.9)	50.8 (12.5)	50.7 (2.8)
4	79.4 (4.2)	71.5 (3.5)	66.4 (6.5)	60.8 (12.9)	58.4 (20.0)	63.1 (3.4)	53.1 (16.7)	47.0 (14.4)

Data are presented as sum of means (standard errors) of number of footsteps observed by both observers.

Table 3. Scores performed by four animal groups.

Groups/Days	1	2	3	4	5	6	7	8
1	5.0 (3.2)	4.5 (4.7)	3.0 (1.8)	5.8 (2.4)	1.1 (1.9)	3.7 (2.8)	3.1 (1.6)	3.1 (1.9)
2	5.3 (1.5)	6.2 (3.8)	4.8 (3.6)	6.4 (4.7)	6.0 (4.2)	0.4 (0.54)	1.6 (1.6)	1.6 (0.8)
3	4.2 (2.8)	2.1 (2.3)	2.4 (2.3)	4.0 (2.8)	2.2 (2.9)	0.8 (1.5)	0.5 (1.5)	2.8 (3.0)
4	2.5 (1.9)	1.8 (1.2)	2.1 (2.7)	3.1 (2.7)	4.0 (3.6)	2.7 (2.2)	2.8 (2.9)	2.2 (2.1)

Data are presented as sum of means (standard errors) of scores observed by both observers.

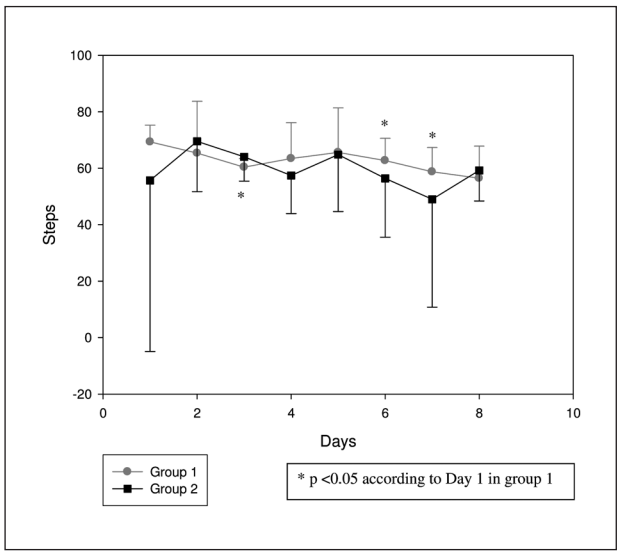

Fig 1. Number of footsteps across days of groups 1 and 2.

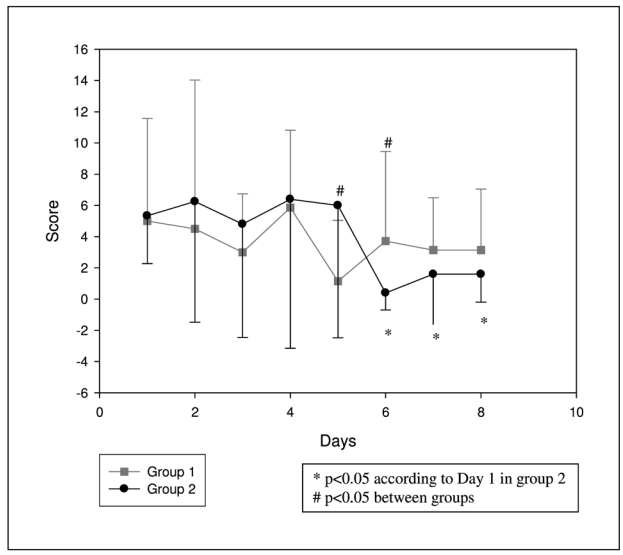

Fig 2. Scores across days in groups 1 and 2.

RESULTS

In the beginning of the experiments there were a total of 28 animals (seven in each group). However, during the surgical procedure, two animals from group 2 died after initial anesthesia and were excluded from the final results, leaving group 2 with 5 animals.

The data collected from each examiner were compared for concordance. There was an excellent concordance between examiners according to the number of footsteps required to traverse the elevated beam (κ=0.922) and also regarding scores (κ=0.921).

As there was no difference between the evaluations of the two examiners the data obtained from them were added and subsequently analyzed in conjunction.

Comparing the number of footsteps taken by the animals from the four experimental groups to traverse the elevated beam, there was a significant difference among the groups (p=0.014). By performing a second analysis, it was possible to determine that the difference found was among demyelinated (groups 1 and 2) and non-demyelinated groups (groups 3 and 4) (p=0.03). These results indicate that the demyelinated animals took lesser steps on the beam walking test at the beginning of practice, suggesting a motor performance deficit (Table 2). It was also shown that the non-demyelinated animals showed a progressive decrease in the number of steps.

When we compare motor performance among demyelinated animals that performed (group 1) or not (group 2) aquatic exercises, we notice that there was no difference among them according to the number of steps that

animals took to traverse the elevated beam across time (p=0.233). However, it is noticed a reduction in the number of steps across days in group 1, like happened with the non-demyelinated animals, suggesting a better motor performance in this group with days (Fig 1).

Focusing on scores, firstly, we noticed a difference among groups (Table 3) (p=0.021). When we look again for the performance in groups 1 and 2, it is found that the sum of scores of both examiners are different (p=0.018). By analyzing the sum of scores day by day, it is noticed that the difference between groups happened on days 5 and 6 (Fig 2). Group 1 showed fewer errors till day 5 and then, more errors on day 6.

DISCUSSION

In the present study, EB was used as a demyelinating agent in order to mimic the pathophysiologic process of demyelinating diseases such as MS. The injection of a 0.1% EB solution in the cisterna pontis determines the disappearance of local astrocytes and the formation of primary demyelinating lesions with the invasion of Schwann cells and lymphocytes in the damaged area[16]. The intracisternal injection of EB in Sprague-Dawley rats determined oligodendrocyte degeneration 72 hours after drug injection and, 6 days after the surgical procedure, the center of the lesion had most of the axons demyelinated with oligodendrocyte disappearance and the presence of abundant macrophage infiltrate containing myelin debris[17].

The results of this study show there is a difference in the motor behavior of equally demyelinated animals (groups 1 and 2) in comparison with the non-demyelinated animals during the observation period. The demyelinated animals made fewer steps than the non-demyelinated ones. Some studies report that the animals receiving EB injection develop motor deficits until day 31, but not anymore on day 35 after lesion[4,17]. The presence of these deficits may contribute to the decrease in the number of footsteps taken by the demyelinated animals, probably due to motor limitation during locomotion and uncertainty to perform the steps needed to complete the elevated beam traverse. One study correlated the motor inaptitude to oligodendrocyte loss and, consequently, to the demyelination caused by the action of EB[4]. The main point, however, was a reduction in the number of steps across days in group 1 like the non-demyelinated animals, suggesting a better motor performance in the group with aquatic exercises practice.

A possible explanation for the reduction in the number of footsteps taken by the animals in group 1 as in the non-demyelinated animals with the progression of the days may be motor learning. The performance of motor skills involves the learning concept known as "learning transfer", which comprises the ability to acquire experience and to perform skills facing a new situation[28]. Such process might have happened with our animals. The animals studied were young, which may contribute to a greater difficulty in performing the tasks, since most studies using the experimental model of EB used 3-4 month-old animals at the start of the experiments[3,4].

An additional finding of the present study is the significant difference in motor performance, as measured by number of errors, between the demyelinated animals that performed an aquatic series of exercises and those that did not. In general, group 1 animals (demyelinated + exercise) had fewer errors when compared to group 2 (demyelinated + sedentary). In clinical studies, physical exercise practice by individuals with MS has greatly benefited such patients[18-20]. One study showed that different types of exercise, including aquatic exercises, for MS individuals with mild to moderate compromise lead to improved functional activities, execution of daily life activities, as well as decreased fatigue and improved quality of life of such individuals[20]. Some aspects of the physical properties of water, such as buoyancy and viscosity, diminish the action of gravity and allow greater balance, movement amplitude, development of muscle strength and endurance, all contributing to improvement in symptomatology and functional deficits. Our study in an experimental model has similar outcomes, but adding the probable benefit of early intervention.

Although group 1 had fewer errors than group 2, in day 6 this tendency was inverted, and group 1 presented more errors than group 2. It is described the presence of a subjective and inespecific aspect able to cause disability, tiredness and even sudden decrease in muscle strength. This event is called fatigue and is one of the most frequent and generally disabling clinical findings in MS patients[10,21-23]. When comparing such difference in our results, we can also correlate it with the action of the gliotoxic drug in the EB-demyelinating model. On day 11, remyelination promoted by oligodendrocytes started and on day 15 post-injection Schwann cells began to invade the central nervous system. With this information, we conclude that the results presented during the progression by group 1 coincide with the remyelination period. One possible explanation for this finding is that aquatic exercise resembles a physical and psychological stress that would cause an immediate and progressive increase in the secretion of adrenocorticotropin by the anterior pituitary, favoring the gradual secretion of cortisol in a few minutes[24]. It is known that Wistar rats treated with dexamethasone had a deficient remyelinating process after EB injection in the brain stem[3,25,26]. Morphologic observations by Bondan et al.[27] included delayed remyelination by oligodendrocytes, persistence of demyelinated axons, presence of scarce revascularization and considerable amounts of myelin-derived

membranes in the center of the lesion. Considering that during aquatic exercise cortisol release might have occurred and correlating this physiologic event to the findings of Bondan et al.[27], we find possible that the presence of this hormone could slow the remyelinating process, imparing this way the performance of the animals in group 1. In this setting, it is relevant that new studies should be done with larger samples, following the animals for a longer period of time and, if possible, minimizing the putative action of cortisol.

Our study has some limitations that may compromise our results. First, we studied a very few number of animals. Maybe this is the reason why there were not many differences among groups. Second, although our two models (beam walking test and aquatic exercises) are valid, to the best of our knowledge, this was the first study to combine both methods. Therefore, we can not exclude possible mistakes on performing both methods.

In conclusions, early physical activity in animals subjected to brain stem demyelination with EB has a positive impact on the motor deficit caused by the lesion, as shown by the lower scores of the animals that performed aquatic exercises. These results cannot be generalized, but they corroborate the few clinical studies that showed benefit of physical activity in MS.

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