TRANSDERMAL MONOSIALOGANGLIOSIDE WITH LASER IN THE TREATMENT OF SPINAL CORD LESION IN RATS

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ABSTRACT

Objectives: To evaluate the effects of monosialoganglioside (GM1) administered transdermally with laser in the recovery of spinal cord injury in rats. Methods: Forty male Wistar rats underwent spinal cord contusion using the NYU Impactor. In Group 1, the rats received 0,2 ml of saline intraperitoneally daily; in Group 2, GM1 was administered intraperitoneally at a concentration of 30 mg/kg per day; in Group 3, rats were treated daily with laser at low temperature on the skin, and in Group 4, the daily laser session also contained GM1. All the groups were treated for 42 days. The animals were evaluated by the Basso, Baettie and Bresnahan (BBB) functional scale on days 7, 14, 21, 28, 35 and 42 after the injury, and by histopathology and motor evoked potential after 42 days of injury. Results: The animals in Group 4 had higher BBB scores compared with the other groups. There were no differences between the groups, or in the comparisons over time. Histological evaluation showed no differences, and no differences were found in the motor evoked potential tests either. Conclusion: GM1 associated with the use of low-temperature laser shows no superior functional, neurological or histological results in the treatment of spinal cord lesions in rats. ***Evidence Level I, Experimental, Controlled, Animal Study.***

Keywords: Spinal cord injuries. Contusions. G(M1) ganglioside. Lasers.

Citation: Souza FI, Cristante AF, Marcon RM, Ferreira R, Santos GB, Barros Filho TE. Transdermal monosialoganglioside with laser in the treatment of spinal cord lesion in rats. Acta Ortop Bras. [online]. 2013;21(2):87-91. Available from URL: http://www.scielo.br/aob.

INTRODUCTION

There are few methods for the treatment of spinal cord lesions, and no really effective treatment is available.[1] Surgical treatment for mechanical stabilization and decompression may be performed in cases of unstable fractures with spinal cord lesion, and the use of drugs for the treatment of spinal injuries has been extensively studied. Experiments with the use of chemicals are conducted mostly with the aim of promoting nerve regeneration, and in an attempt to inactivate or reduce the secondary cascade of events that follows spinal injuries. However, only two of these drugs are already used clinically: monosialoganglioside (GM1) and methylprednisolone,[2] although there is no consensus as to the benefits of their indication.[3]

GM1 is an antineurotoxic, anti-inflammatory, neuroprotector agent, essential in neuronal excitability of myelinated and unmyelinated fibers. It also promotes neuronal development, growth, differentiation and maturation, and reduces the intensity of Walerian degeneration.[4]

Many researchers also advocate the use of physical means in the treatment of spinal injuries in an attempt to obtain better results. Hypothermia is one such alternative; it reduces post-traumatic metabolism and energy consumption, decreasing the intensity of secondary lesions, hypoxia and ischemia, as well as apoptosis of neurons and glial cells.[5-7]

A new type of laser used for drug administration was recently developed in Italy (Laser Ice Med). It allows transcutaneous penetration of particles mixed in a gel at low temperature, by means of 635nm, 50mW parallel beams. Santos et al. presented, at the European Congress of Neuroscience, their research on the effects of laser with GM1 in trauma associated with cord and peripheral nerve injury in Wistar rats. GM1, administered daily by the transdermal route for 60 days, resulted in regeneration superior to that of the control group, in the histological and functional evaluations of the sciatic nerves and spinal cords.[8]

The results of each GM1 and "laser ice" therapies are, however, still not satisfactory, which prompted us to study a possible synergy between them. The possibility that the low temperature could improve the results of the medication, as it happens in other

All the authors declare that there is no potential conflict of interest referring to this article.

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Study conducted at LIM 41 – Laboratório de Investigação Médica do Sistema Músculo Esquelético do Departamento de Ortopedia e Traumatologia da Faculdade de Medicina da Universidade de São Paulo – FMUSP, São Paulo, SP, Brasil.
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Article received on 7/23/2012, and approved on 10/9/2012.

medicine fields, should be investigated as an opportunity to treat spinal cord injuries. The objective of this study was, therefore, to evaluate the effects of monosialoganglioside (GM1) administered transdermally, and of laser at low temperatures, in the functional and histological recovery of spinal cord injury in rats.

METHODS

Study design and ethics

In this experimental, placebo-controlled trial, all institutional and governmental regulations on the ethical use of animals were followed. The research protocol was approved by the institution's Ethics Committee and the procedures are in accordance with research protocols. The animals in this study, once anesthetized, were submitted to laminectomy and to an experimental spinal cord lesion, and then to therapy with monosialotetrahexosylganglioside (GM1), laser at low temperature, or both. They were then compared with a control group receiving placebo, and evaluated functionally and by somatosensitive evoked potential, for the effects of the therapy on spinal cord lesion recovery.

Animals and anesthetic procedure

Forty Wistar male rats, aged 20 to 21 weeks, weighing 300 to 340 g, were used in this study. Rats that died at any time after the experimental spinal cord lesion were excluded. Animals were also excluded if they presented macroscopic spinal cord anomalies (malformations) detected during the surgical procedure or those that still presented normal movement after the lesion (absence of paraplegia).

The rats were anesthetized before the laminectomy and spinal cord lesion procedures with a dose of 55 to 75 mg/kg body weight of sodium pentobarbital, intraperitoneally. The anesthetic effect was obtained in five minutes, and lasted for at least 60 minutes. At the end of the experiment all the rats were weighed, and then euthanized with a lethal dose of pentobarbital (140mg/kg), intraperitoneally. All the rats were examined macroscopically, and histopathologic analysis was performed.

Laminectomy and spinal cord lesion

The laminectomy method was already described.[9,10] The spinous process and laminae of vertebrae T8 and T11 were removed to expose the spinal cord, in order to perform the spinal cord lesion.

The multicenter spinal cord lesion protocol MASCIS (Multicenter Animal Spinal Cord Injury Study), standardized for Wistar rats, was adopted.[9-11] The lesion was produced with the computerized NYU Impactor device (New York University Spinal Cord Contusion System), using a falling weight of 10 g, which compresses the spinal cord for 15 seconds. The height was standardized at 25 mm.[12,13] The device was adjusted to produce the impact between the upper margin of T9 and the lower margin of T10.

The animals received prophylactic cephalothin subcutaneously (25 mg/kg body weight) immediately, and then once a day for the next seven days, to prevent wound or urinary tract infection. Once they had recovered from the anesthesia, the animals were kept in cages, in groups of five each, with access to food and water *ad libitum*.

Groups and therapies

The animals were divided manually into four groups, with ten animals each:

Group 1 received only saline solution intraperitoneally (0.2 ml per day), and was considered the control group;

Group 2 received 30 mg/kg body weight of GM1 (TRB Pharma, Campinas, São Paulo, Brazil) diluted in 0.2 ml of saline, administered intraperitoneally, daily;

Group 3 received only laser therapy, without GM1, for three minutes daily;

Group 4 received laser for three minutes daily, with GM1 at a concentration of 30 mg/kg.

All groups were treated for 42 days. The dose of 30 mg/kg per day of GM1 was calculated according to the dissolution of the gel contained in the laser tube and the number of animals treated with each tube.[14,15]

The laser device attaches to a tube filled with frozen water-based gel, which causes local hypothermia. In Group 4, GM1 was mixed with the gel on the day prior to administration.

Functional evaluation

The recovery after spinal cord lesion was evaluated each week, for six weeks, by the Basso Beattie and Bresnahan (BBB) scale, varying from 0 to 21 points on either side.[11] The evaluation was performed simultaneously by two trained observers, who were blind to the procedure administered in each rat. The evaluation was carried out on days 7, 14, 21, 28, 35 and 42 after surgery. In the case of a disagreement, the lowest value was recorded. Each evaluation took four to five minutes per animal.

The animals were then put under anesthesia again, for the evaluation of the evoked potential, as described by Ferreira et al.[16]

Necropsy and histological analysis

The segment of spine from T8 to T12 (bone and soft parts) was removed from the sacrificed rats, and a visual macroscopic evaluation of the spinal cord at the contusion site was carried out, to check for any anomaly. The spinal cord was then removed, and sent for microscopic analysis. Histological cross-sections were made on the axial plane of the spinal cord segment, 2 mm thick, and 1cm from the center of the lesion, proximally and distally. This material was processed and embedded in paraffin. Five-micra thick histological sections were produced, 5 mm above and below the central area of the lesion. The material was then fixed on slides, stained with hematoxylin-eosin, three for each spinal cord: one for the region proximal to the lesion, one for the center and one for region distal from the lesion, therefore representing the entire injured area.

The variables evaluated were: necrosis, hemorrhage, hyperemia, degeneration of nervous substances (cystic degeneration) and cell infiltration. They were recorded as absent (0), slight (1), moderate (2) and high (3), yielding a score of between 0 and 15.[13,17] The anatomopathological examination was carried out by a single pathologist, who was blind to the group allocation.

STATISTICAL ANALYSIS

The average BBB scores were compared using a mixed effects model with two factors: group and week of assessment, considering the repeated measurements over the weeks. The effect of interaction between these factors was also evaluated.

The mixed-effects model was adapted, considering equal covariance matrices in the different groups and with an unstructured form. Initially, the distributions of the measures of amplitude and latency of the evoked potential test, in the four study groups, were compared using the nonparametric Kruskal-Wallis test. Analysis of variance (ANOVA) was then used to compare the mean amplitudes and latencies of the different groups.

The association between score level and the groups was assessed using the Fisher exact test. p-values less than 0.05 were considered statistically significant.

RESULTS

During the study period, there were nine deaths. The cause of death was identified as autophagia in six cases, and was undetermined in three. Therefore, the study was concluded with 31 animals: eight in Group 1, seven in Group 2, seven in Group 3, and nine in Group 4. There was no association between deaths and group allocation (p = 1,000, Fisher exact test). The animals' mean weight was 347 g, varying from 311 g to 402 g at the end of the experiment.

Functional evaluation

Table 1 shows descriptive measurements of the BBB score for each study group and week of assessment, with wide variation in the data over the course of several weeks. This phenomenon was observed for all the groups, and overall, the average BBB score (and standard deviation) increased from 0.9 (1.4) in the first week to 12.7 (4.4) in the sixth week of observation. Group 4 had higher average values from the second to the fifth week. In the sixth week, Group 1 stood out from the others, with the highest average observed in the study (14.8).

The adjustment of the model indicated a significant interaction effect between group and week, with $p < 0.001$. Therefore, the comparison between groups had to be made within each week, and comparison over the weeks had to be made within each group. The results of these comparisons are shown in Table 2, which shows that: (I) for each week of the evaluation, there was no evidence of significant differences between the mean values of the groups (all comparisons with $p \geq 0.215$), (II) for all the groups, the averages for the weeks are significantly different ($p < 0.001$). Thus, there was no difference in the comparison of one treatment with another, but there was a significant difference in the averages over time.

Based on these results, the analysis was continued, in order to compare the average weekly increases within each group, as shown in Table 3.

Table 1. Descriptive measurements for BBB score, according to the group and week of assessment.

Group		Week of assessment					
		1st	2nd	3rd	4th	5th	6th
Group 1 (N = 8)	**Mean**	**1.3**	**1.8**	**5.8**	**7.5**	**9.8**	**14.8**
	Standard deviation	1.6	1.7	2.3	2.4	3.2	4.2
	Median	0.5	2.0	5.0	7.0	9.5	15.0
	Minimum	0	1	4	7	8	7
	Maximum	4	4	11	13	15	20
Group 2 (N = 7)	**Mean**	**0.7**	**2.3**	**5.4**	**7.4**	**9.0**	**12.3**
	Standard deviation	1.5	2.3	1.9	3.3	4.4	4.2
	Median	0.0	3.0	6.0	7.0	9.0	13.0
	Minimum	0	0	3	3	4	5
	Maximum	4	5	8	13	15	17
Group 3 (N = 7)	**Mean**	**0.6**	**2.7**	**5.1**	**7.3**	**7.7**	**11.4**
	Standard deviation	1.5	2.0	2.6	3.3	4.5	4.9
	Median	0.0	3.0	6.0	6.0	6.0	13.0
	Minimum	0	0	0	3	2	4
	Maximum	4	5	8	11	14	18
Group 4 (N = 9)	**Mean**	**0.9**	**4.1**	**7.7**	**9.6**	**10.8**	**12.1**
	Standard deviation	1.4	3.1	3.4	3.9	4.4	4.6
	Median	0.0	3.0	7.0	9.0	11.0	12.0
	Minimum	0	0	3	5	6	7
	Maximum	3	9	13	16	19	19
Total (N = 31)	**Mean**	**0.9**	**2.8**	**6.1**	**8.0**	**9.4**	**12.7**
	Standard deviation	1.4	2.4	2.7	3.3	4.1	4.4
	Median	0.0	3.0	6.0	7.0	9.0	13.0
	Minimum	0	0	0	3	2	4
	Maximum	4	9	13	16	19	20

Table 2. p values for the comparison between weeks in each group and between groups within each week.

		p value
Comparison between groups	In the 1st week of assessment	0.829
	In the 2nd week of assessment	0.215
	In the 3rd week of assessment	0.448
	In the 4th week of assessment	0.448
	In the 5th week of assessment	0.530
	In the 6th week of assessment	0.492
Comparison between weeks	In group 1	< 0.001
	In group 2	< 0.001
	In group 3	< 0.001
	In group 4	< 0.001

p values obtained from the mixed effects model.

Table 3. p values corresponding to the comparison between two consecutive weeks within each group.

Group	Comparison of two consecutive weeks	Mean difference	p value
Group 1	2nd week - 1st week	0.5	0.492
	3rd week - 2nd week	4.0	< 0.001
	4th week - 3rd week	1.8	< 0.001
	5th week - 4th week	2.3	< 0.001
	6th week - 5th week	5.0	< 0.001
Group 2	2nd week - 1st week	1.6	0.043
	3rd week - 2nd week	3.1	< 0.001
	4th week - 3rd week	2.0	< 0.001
	5th week - 4th week	1.6	0.025
	6th week - 5th week	3.3	< 0.001
Group 3	2nd week - 1st week	2.1	0.006
	3rd week - 2nd week	2.4	< 0.001
	4th week - 3rd week	2.1	< 0.001
	5th week - 4th week	0.4	0.540
	6th week - 5th week	3.7	< 0.001
Group 4	2nd week - 1st week	3.2	< 0.001
	3rd week - 2nd week	3.6	< 0.001
	4th week - 3rd week	1.9	< 0.001
	5th week - 4th week	1.2	0.048
	6th week - 5th week	1.3	0.086

p values obtained from the mixed effects model.

Evoked potential: latency and amplitude

Table 4 presents the descriptive measures for the variables amplitude and latency in the evoked potential examination, according to the study groups. The extremely high value of latency (10.95) observed in Group 1 (case #2, whose original values for the left and right are 11.8 and 10.1, respectively), corresponds to the same case that presented an extremely low value for amplitude (81.0, original values for the left and right of 89.0 and 73.0, respectively).

The analysis indicated no significant differences between the mean latencies of the four groups (p = 0.335). However, the analysis

of residuals indicated unreasonable adjustment of the data, with unequal variances between groups. This was mainly due to the large variability in Group 1 when compared with the other groups. This finding remained the same even after attempts to transform the data. The residual analysis showed two cases requiring careful investigation, corresponding to the two extreme values of Group 1, case #1 (3.30) and #2 (10.95). The nonparametric Kruskal-Wallis test showed no significant differences between the distributions of the four groups for either the amplitude measurements ($p = 0.884$) or the latency measurements ($p = 0.118$).

The ANOVA model for the amplitude data indicated no significant differences between the averages of the four groups, with $p = 0.921$. An analysis of the residuals of the model was performed, and two points were detected for careful investigation, which correspond to extreme values of Group 3 (case #1, amplitude of 3140.5) and 4 (case #7, amplitude of 2848.0), as previously emphasized. Thus, new settings of the ANOVA model were made, which dismissed each of these points and also dismissed the two points simultaneously. Excluding case #1, the mean and standard deviation for Group 3 were reduced to 275.0 and 70.6. In Group 4, excluding case #7, these values fell to 277.7 and 158.2. In all these tests, there was no indication of significant differences between the groups (Table 5, $p > 0.5$).

The results of the analysis, excluding these points, are also presented in Table 5. Note that the removal of the extremely high value (Model B) does not alter the conclusions, since it does not indicate significant differences between the groups ($p = 0.530$). Otherwise, excluding the minimum value (Models A and C) results in models indicating significant differences between the groups. Investigating which groups were different, the two models indicated that the mean latencies of Groups 1 and 4 are different, however, they are not different from the other groups considered.

Table 4. Descriptive measures of amplitude and latency.according to groups and analysis of variance (ANOVA).

Evoked potential	Groups	N	Median	SD	Median	Minimum	Maximum
Amplitude p = 0.921	Group 1	8	544,6	596,2	243,5	81,0	1840,0
	Group 2	7	392,9	442,5	270,5	120,0	1384,5
	Group 3	7	684,4	1085,0	270,0	187,5	3140,5
	Group 4	9	563,3	869,5	262,5	150,0	2848,0
	Total	31	547,3	754,4	262,5	81,0	3140,5
Latency p = 0.335	Group 1	8	6,7	2.2	6,2	3,30	10,95
	Group 2	7	6,0	0,4	6,1	5,30	6,35
	Group 3	7	6,1	0,3	6,1	5,50	6,50
	Group 4	9	5,6	0,6	5,4	4,85	6,85
	Total	31	6,1	1,2	6,0	3,30	10,95

SD: Standard deviation.

Table 5. p values for the comparison test of the mean amplitudes and latencies in the four study groups by analysis of variance (ANOVA).

Model	Total cases considered	p value
Amplitudes		
without case #1. Group 3	30	0.791
without case #7. Group 4	30	0.647
without the above two cases	29	0.509
Latencies		
without case #1. Group 1	30	0.034
without case #2. Group 1	30	0.530
without the above two cases	29	0.044

Histological results

Histological analysis, expressed as scores for the variables absent, mild, moderate and severe, according to study groups and the site of the injury, showed no significant differences between the groups, except for the variable necrosis in the central region of the lesion ($p < 0.001$, Table 6) and hyperemia, also obtained for the central region ($p = 0.015$, see Table 6). The variable bleeding showed no difference between the groups or periods ($p > 0.20$), neither did degeneration of neural substance ($p > 0.66$) or even cellular infiltration ($p > 0.11$).

Table 6. Distribution of scores of necrosis and hyperemia. according to the group and region of the spinal cord. and differences according to Fisher's exact test.

Necrosis score		Group 1		Group 2		Group 3		Group 4	
		N	%	N	%	N	%	N	%
Proximal p = 0.186	Absent	6	75.0	4	57.1	2	28.6	6	66.7
	Discrete	2	25.0	3	42.9	2	28.6	3	33.3
	Moderate	0	0.0	0	0.0	3	42.9	0	0.0
Central p < 0.001	Absent	3	37.5	0	0.0	1	14.3	0	0.0
	Discrete	5	62.5	2	28.6	0	0.0	6	66.7
	Moderate	0	0.0	5	71.4	6	85.7	3	33.3
Distal p = 0.282	Absent	5	62.5	1	14 (3)	3	42.9	3	33.3
	Discrete	3	37.5	2	28.6	3	42.9	3	33.3
	Moderate	0	0.0	4	57.1	1	14 (3)	3	33.3
	Total	8	100%	7	100%	7	100%	9	100%
Hyperemia score		**Group 1**		**Group 2**		**Group 3**		**Group 4**	
		N	%	N	%	N	%	N	%
Proximal p = 0.132	Absent	2	25.0	0	0.0	0	0.0	2	22.2
	Discrete	4	50.0	7	100.0	5	71.4	3	33.3
	Moderate	2	25.0	0	0.0	2	28.6	4	44.4
Central p = 0.015	Absent	1	12.5	0	0.0	1	14.3	0	0.0
	Discrete	7	87.5	2	28.6	1	14.3	4	44.4
	Moderate	0	0.0	4	57.1	5	71.4	5	55.6
	Intense	0	0.0	1	14.3	0	0.0	0	0.0
Distal p = 0.390	Absent	1	12.5	0	0.0	0	0.0	1	11.1
	Discrete	6	75.0	3	42.9	6	85.7	5	55.6
	Moderate	1	12.5	4	57.1	1	14.3	3	33.3
	Total	8	100%	7	100%	7	100%	9	100%

DISCUSSION

Although far from being a subject of consensus in the scientific community, GM1 is a promising therapeutic option, especially when favorable results are obtained by enhancing its action by other means, such as physical, chemical or biological means. GM1 has been used in several clinical studies in patients with spinal cord injury, and its safety has been confirmed, with few complications, and promising results showing improvement in motor recovery.[4,18] Laser can promote transdermal absorption of proteins, peptides and other molecules, making it a good alternative route for drug administration.[19-21] Santos et al.[8] presented preliminary results in favor of the use of low temperature laser associated with GM1 in spinal cord and peripheral nerve injuries. Following this line of research, we conducted this study with rats, adding the analysis by evoked potential, an evaluation that can be considered more objective than the BBB score alone.

Evoked potential appears to be a reliable test in all cases of spinal cord injury. However, in this study, it did not show any significant differences between groups or between periods, even for the two cases that were well above average. Possibly the pathological evaluation by immunohistochemistry staining

could reveal evidences of neuronal regeneration that were not seen with the methods used in this study. However, our team is already following this research line.

In the histological analysis after six weeks of injury, there was a significantly higher concentration of moderate scores of necrosis in the rats treated with GM1 or laser, but without any correlation with other the items assessed, on with the histological or functional results. It may be that the lack of statistical significance between the groups in the histological analysis is due to the technique used.[10] Further analysis of the bone marrow histology with electron microscopy, and specific methods for staining the nerves, could produce more significant results. Observation of the descriptive results of functional assessments reveals a slight increase in the motor function of rats in all the groups over time. However, statistical analysis showed no significant differences. It would be interesting to re-evaluate them with a larger sample.

One could assume that in acute and sub-acute spinal cord injury, there is a synergy between laser, hypothermia and GM1. After a certain period of time, this combination would have a contrary effect, but this hypothesis needs to be tested in specific studies.

CONCLUSIONS

There was no statistically significant difference, in the functional, histological or motor evoked potentials assessments, between rats with moderate spinal cord injuries treated with GM1, laser, or a combination of both.

ACKNOWLEDGEMENT

The authors wish to thank TRB Pharma, Brazil, for the donation of the monosialotetrahexosylganglioside (GM1) used in this experimental study.

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