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ORIGINAL ARTICLE

Potentializing the effects of GM1 by hyperbaric oxygen therapy in acute experimental spinal cord lesion in rats

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Study design: Experimental, controlled, animal study.
Objectives: To evaluate the effect of GM1 ganglioside, hyperbaric oxygen and both in combination, in the treatment of experimental spinal cord lesions in rats.
Setting: Brazil.
Methods: Thirty-two Wistar rats with spinal cord lesions were divided into four groups: one group received GM1 ganglioside, one was submitted to hyperbaric oxygen therapy (HBOT), the third received both treatments and the fourth received no treatment (control).
Results: There were no significant differences between the groups in the histological analysis, for any of the variables (necrosis, hemorrhage, hyperemia, cystic degeneration, $P>0.06$). Neither were there any significant differences in the comparison of left and right sides in the functional tests ($P>0.06$ for all). No significant differences were found in the locomotor ratings, in the comparison of groups at 2, 7, 21 and 28 days after the surgical procedure. However, in the evaluation on day 14, group 3, which received the combined therapy, showed a significantly higher Basso Beattie and Bresnahan score than the other groups ($P=0.015$).
Conclusion: The therapeutic effect of GM1 in locomotor evaluation of rats submitted to spinal cord lesion is anticipated by HBOT.
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Keywords: hyperbaric oxygenation; G(M1) ganglioside; spinal cord injuries

Introduction

Totipotent, pluripotent or multipotent, autologous or homologous tissue and stem cell growth factors have been tested in neuronal regeneration after spinal cord lesion. However, because of the short follow-up and evaluation period, there are still no concrete results, and they remain as a future possibility for cure.[1]

The pharmacological therapies currently under investigation are calcium channel blockers, naloxones, gangliosides lazaroids, dimethyl-sulfoxide and α-methylparatyrosine. Corticoids (particularly dexamethasone and methylprednisolone)[2] and gangliosides, have already been protocoled for use in humans. Methylprednisolone acts by decreasing cerebral edema and increasing blood flow, stabilizing the cell membrane and inhibiting lipid peroxidation, with a consequent decrease in the formation of free radicals. A systematic review, published in 2000, showed that high doses of methylprednisolone are effective in the treatment of acute spinal cord lesion.[3]

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GM1 (monosialoganglioside) is a therapeutic option for the treatment of spinal cord injuries and injuries of the central nervous system.[4,5] Various properties are attributed to GM1, including a reduction in neuronal edema by the increased activity of the sodium, potassium and magnesium pumps, homeostasis of the nerve cells by the reestablishment of membrane equilibrium, and in particular, the increase of endogenous neurotrophic factors. This decreases neurone destruction after trauma, increasing the plasticity mechanisms of the injured spinal cord circuits and promoting the recovery of functional connections.[6] The analysis of the results of works involving GM1 in humans reveals an improvement in locomotor function,[7] but the interpretation of these results is complex, because of the fact that methylprednisolone was used before the administration of GM1.[8]

Hypothermia has been studied as a physical means of minimizing secondary spinal cord damage, with beneficial effects when initiated in the first 8 h after trauma.[9] However, its mortality rate is high.[10] Another physical procedure that can be used is hyperbaric oxygen therapy (HBOT),[11,12] in which high partial pressures of tissue oxygen are obtained, which are higher than the atmospheric pressure. HBOT is based on the premise that a decrease in perfusion can be compensated for by an increase in partial oxygen pressure.

Experimental studies have been carried out that seek to elucidate the possible synergism between pharmacological factors of intervention in spinal cord lesions.[13] Similarly, it is possible that HBOT and GM1 have additive or synergic effects on spinal cord contusion injury. However, the effects of this treatment have still not been clearly studied in spinal cord injury models.

This study therefore seeks to evaluate, in a standardized experimental model, the influence of the treatment in rats submitted to acute spinal cord injury with HBOT, monosialoganglioside (GM1) and a combination of both, on the functional and anatomopathological results.

Materials and methods

This is an experimental, controlled trial on the use of GM1 ganglioside combined with HBOT in rats with induced spinal cord lesions. All applicable institutional and governmental regulations on the ethical use of animals were followed during the course of this research. The research protocol was approved by the institution's ethical committee.

Animals

Young, adult, male Wistar rats were used, weighing between 300 and 340 g, at 20–21 weeks of life, healthy and with normal motricity. All the animals were submitted to moderate spinal cord lesion, as described above.[14] The 32 rats were randomly separated into four groups, with eight animals in each:

- Group 1: rats submitted to monosialoganglioside (GM1) treatment at a dose of 30 mg kg^{-1} for 7 days, starting 24 h after the trauma;
- Group 2: rats submitted to HBOT sessions with two ATM lasting 1 h, for 7 days, starting 24 h after the trauma;
- Group 3: rats submitted to the combined therapy with GM1 and HBOT, according to the protocol applied in groups 1 and 2;
- Group 4: rats not submitted to any treatment, serving as control.

Rats that died after spinal cord lesion and those that showed autophagic or mutilating behavior were excluded. Animals were also excluded that presented macroscopic spinal cord anomalies on surgery, or those that still presented normal movement after the lesion (21 points on the Basso Beattie and Bresnahan (BBB) scale).[15]

At the end of the experimental period, all the rats were killed with a lethal dose of pentobarbital (140 mg kg^{-1}) per day, administered intraperitoneally.

Laminectomy. All the rats received sodium pentobarbital anesthesia intraperitoneally (55–75 mg kg^{-1} body weight). The anesthetic took effect after 5 min and lasted for at least 2 h. After anesthesia, the animals were trichotomized in the dorsal region and positioned on the surgical table. With the aid of an optical microscope, a long, dorsal, medial incision was made in the skin and aponeurotic and muscular planes, to expose the posterior vertebral arches, from T8 to T12. The muscles inserted in the spinous process and laminae of vertebrae T9 to T12 were undermined, and the joint processes of these vertebrae exposed. Hemostasia, wherein necessary, was performed using a bipolar coagulator. Using a bone rongeur, the spinous process and laminae of vertebra T10 were removed, along with the distal half of the spinous process of T9, to expose the spinal cord and enable positioning (puncture) of the tip of the NYU Impactor device, to perform the spinal cord lesion.[13,16]

Spinal cord lesion

The protocol for spinal cord lesion has been described elsewhere[14] and consists of producing a moderate spinal cord lesion with the NYU Impactor (New York University Spinal Cord Contusion System), a computerized device developed by New York University, which is designed to produce an impact using a falling weight. The impact was produced by the falling of an impact rod weighing 10 g, from a standardized height of 12.5 mm, compressing the spinal cord for 15 s. The equipment allows the speed of the rod to be monitored by a computer, registering the exact moment the rod makes contact with the spinal cord and the length of time of the contact, using spine motion sensors (Figure 1). The animals were positioned so that NYU Impactor rod touched the exposed spinal cord at vertebra T10. This was carried out by fixing the T8 and T11 spinous processes using two clamps, fastened to the base of the NYU Impactor. This ensured that the lesions were homogenous and reproducible.

The site of the contusion injury was inspected. Where hemorrhaging was present, hemostasia was performed. The contusion site was then washed with physiological sodium chloride solution at room temperature. The muscle, fascia and skin tissue planes were closed by simple suture stitches, using 2.0 monofilament nylon.

Postoperative procedures. After producing the lesions, the animals were placed under a heat source at a temperature of 25–28 °C. The rats' bladders were pressed, to empty them. Once they had recovered from the anesthesia, the animals were allowed food and water.

The animals then received cephalothin (Keflin Neutro, Ely Lilly, Brazil) subcutaneously (25 mg kg^{-1} body weight, once a day, for 7 days) immediately after the lesion, and once a day for the next 7 days, to prevent urinary tract or wound infection. Those that presented infection received the antibiotic for 10 days, and were excluded from the statistical analysis.

Hyperbaric oxygen therapy

HBOT was applied at two ATM for 1 h day^{-1} (after equalizing the pressure) for seven consecutive days.[11,12] A tubular hyperbaric chamber was used, measuring 770 mm in length and 180 mm in internal diameter, with a useful height of 150 mm (between the platform and the upper wall), transparent, acrylic walls of 10 mm in thickness and capacity to hold 10 rats simultaneously. The hyperbaric chamber was regulated to provide 14 l of oxygen per minute.

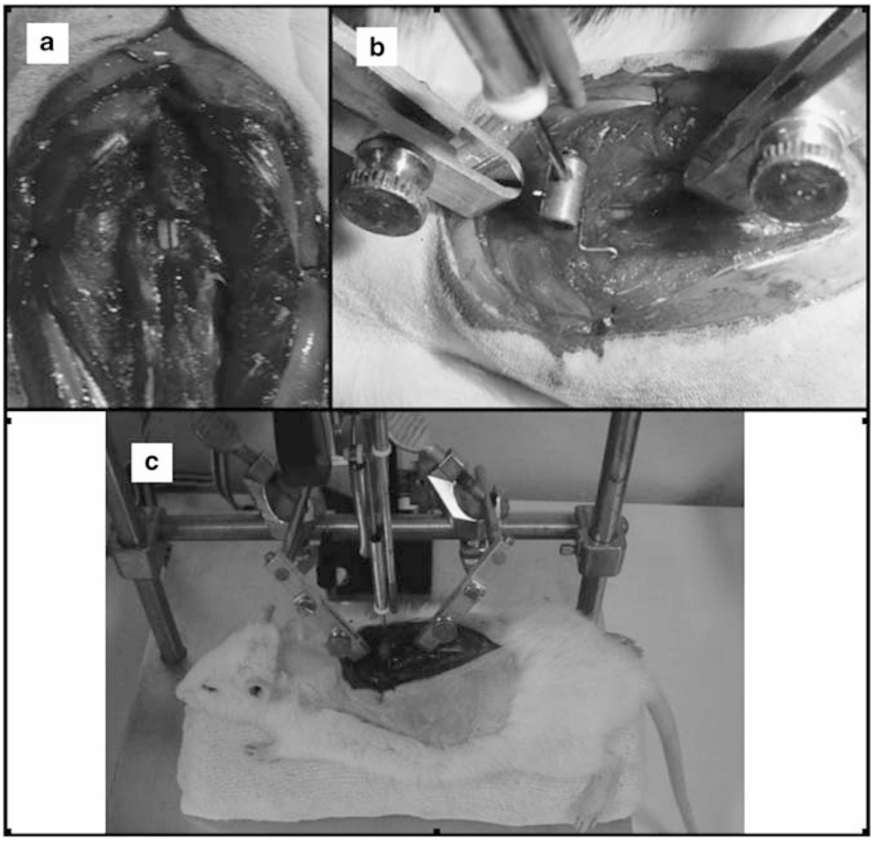

Figure 1 Procedures for spinal cord lesion production with the NYU Impactor equipment. (**a**) laminectomy, exposure of the spinal cord; (**b**) fixation; (**c**) positioning of the animal on the device.

Protocol for evaluating locomotor capacity

The recovery of locomotor capacity after spinal cord lesion was measured by the BBB scale of functional evaluation,[15] applied with simultaneous visual evaluation by two trained observers, who had no knowledge of the intervention to which each rat had been submitted (that is, they were blind to the procedure). The evaluation, carried out on days 2, 7, 13, 21 and 28 after surgery, was made by consensus or, in the case of a disagreement, the lowest value was recorded.

The experimental murine spinal cord lesion evaluation model of the MASCIS (Multicenter Animal Spinal Cord Injury Study) was adopted, standardized for Wistar rats.[17] The following indicators were observed and recorded: joint movements of the hind paw, trunk and abdomen position, dislocation of the paw and type of contact of the paw with the ground, toe coordination, contact and lifting the paw from the ground, trunk instability, and tail position in relation to the right and left sides. If the rat remained immobile for 15–20 s, movement was stimulated by touching the animal. The evaluation took 4–5 min.

Necroscopic and anatomopathological exam

In the initial necroscopic inspection, the presence of possible lesions associated with autophagia or mutilation was observed. The internal inspection was begun by removing the vertebral column, making a new extensive dorsal incision, exposing the spine and cutting a segment from T8 to T12 using scissors. Using a micro bone rongeur, all the bone structures were carefully removed, as well as the soft parts adjacent to the spinal cord, to expose it completely. Visual macroscopic evaluation of the spinal cord at the contusion site was carried out, to check for any anomaly (exclusion criteria).

Ventral incision (thoracotomy and laparotomy) was performed, to inspect the lungs, abdomen and bladder, looking for signs of empyema, condensation, flaccid neurogenic bladder (with increase in volume) or hyperemia and hematuria.

Next, the spinal cord, preserved in 10% formaldehyde solution, was sent for microscopic (optical) analysis. Histological cross-sections were made on the axial plane in the spinal cord segment, at 2 mm intervals, representing the entire lesioned area, to a length of 1 cm from the center of the lesion. This material was processed and dehydrated in a series of alcohols, then diaphanized in xylol and embedded in paraffin. Five-micra thick histological sections were produced in a microtome, half a centimeter above and half a centimeter below the central area of the lesion. The material was then fixed on glass slides stained with hematoxylin-eosin.

Three slides were prepared for each spinal cord, denominated A, B and C. Slides A and C corresponded to the proximal and distal regions of the site of the lesion, respectively, and were collected as a form of individual control in each rat. Fragment B corresponded to the site of the lesion itself.

All the fragments were graded for necrosis, hemorrhage, hypermia, degeneration of nervous substances (cystic degeneration) and cell infiltration, as absent (0), slight (1), moderate (2) and accentuated (3). The pathologists were not informed of the group of origin to which the rat spinal cord belonged (blind evaluation).

Statistical analysis

Owing to the nature of the variables, non-parametric tests were used. The Kruskal–Wallis test was used to compare the study groups, in terms of BBB score for each evaluation time, as well as for the distribution of anatomical–pathological analysis scores. The differences between the groups, wherein significant, were determined by Dunn's multiple comparison test. The comparison of mortality between the groups was performed by the χ^2 and verisimilitude ratio tests. *P*-values of <0.05 were considered significant.

Results

Table 1 shows the deaths and causes of death among the animals studied, none of which was related to the procedure itself. There was no difference between the groups, in relation to mortality ($P=0.908$). Table 1 shows that only one animal was excluded because of autophagia. There were no cases of mutilation, spinal cord macroscopic anomalies or normal movements after the spinal cord lesion (reasons that would justify further exclusions).

There were no significant differences between the groups in the histological analysis, regardless of the variable (necrosis, hemorrhage, hyperemia, cystic degeneration, $P>0.06$) or region, A, B or C, being considered. Neither were there any significant differences in the comparison of left and right sides in the functional tests ($P>0.06$ for all).

No significant difference was found in locomotor ratings in the comparison of groups at 2, 7, 21 or 28 days after the surgical procedure. However, in the evaluation on day 14, group 3, which received the combined therapy, showed a significantly higher BBB score than the other groups ($P=0.015$, Table 2).

Discussion

Combining a physical medium (HBOT) with a pharmacological method (GM1) for the treatment of spinal cord lesions, this is the first time an attempt has been made to potentialize the effects of both. The treatment can be evaluated both in terms of functional aspects (recovery of movements) and histological aspects.

As expected, in this study, there was no statistically significant difference in the results of the anatomopathological analysis with the application of each treatment in isolation, or both in combination. The low sensitivity of the hematoxylin-eosin staining method could explain the difficulty in detecting possible differences between the groups. The use of a lesion grading of 12.5 mm in height, which is smaller than that used in the majority of the other studies, may also have influenced the result.

The locomotor capacity of group 3 (which received HBOT and GM1) was significantly higher than that of the other groups in the second week of the analysis. However, in the subsequent weeks, the treatment groups and the control did not show any statistical differences between them.

These results show that the use of GM1 caused a beneficial effect in the functional recovery of the rats, which appears to have been potentialized by the use of hyperbaric oxygen in the second week after lesion. The small sample or insufficient follow-up time may explain the lack of statistical support. Even so, hyperbaric oxygenation appears to have momentarily accelerated recovery (group 3 in the second week) caused by the pharmacological therapy, while hyperbaric oxygen used in isolation (group 2) did not cause any benefit. It is worth discussing whether the time of use of the HBOT should not have been greater than that used.

In experiments with animal models, various application times of the HBOT were used, from 15 min after the lesion,[12] to 2[18] to 4 h.[19] In humans, it is recommended[20] that hyperbaric therapy be initiated within 4 h of the spinal cord lesion. In this study, the start of therapy in the hyperbaric chamber occurred 24 h after the spinal cord model lesion was produced in the animals, and it is possible that the effects of HBOT would be more intense if administrated earlier, a possibility that should be investigated in future studies.

Other studies[12,18,19,21] use oxygen under pressure at two–five ATA in animals submitted to induced spinal cord lesion. Above these pressures, the side effects, such as necrosis of the cells of the anterior horn of the spinal cord in rats, or enzyme degradation by oxidation in the brain, may occur.[22] Although there is no precise and definitive data on the ideal

Table 1 Deaths and causes among the Wistar rats studied

	Deaths	*Time of deaths*	*Causes*	*Remaining rats*
Group 1	2	First and second weeks	Urinary tract infection in one and not identified in the other	6
Group 2	1	Second week	Urinary tract infection	7
Group 3	2	First week	Urinary tract infection in one and not identified in the other	6
Group 4	2	First and second weeks	Urinary tract infection in one and autophagia in the other	6
Total	7			25

Table 2 Locomotor scores[15] according to the group of Wistar rats submitted to medullar lesion

Variables	*Group 1* (n = *16*)	*Group 2* (n = *16*)		*Group 3* (n = *16*)	*Group 4* (n = *16*)
Score at 2 days					
Mean (s.d.)	0.06 (0.25)	0.56 (1.03)		0.13 (0.34)	0.19 (0.40)
Median	0	0		0	0
Minimum–maximum	0–1	0–3		0–1	0–1
Comparison			$P = 0.239$		
	n = *14*			n = *12*	n = *14*
Score at 7 days					
Mean (s.d.)	2.57 (1.87)	2.50 (2.22)		5.25 (3.31)	3.14 (1.96)
Median	2.5	2		5	3
Minimum-maximum	0–5	0–7		0–11	0–7
Comparison			$P = 0.074$		
	n = *12*	n = *14*		n = *12*	n = *12*
Score at 14 days					
Mean (s.d.)	4.83 (3.35)	5.79 (3.75)		10.00 (3.74)	5.83 (4.15)
Median	4.5	5.5		10.5	5
Minimum–maximum	1–12	1–13		3–14	2–14
Comparison			$P = 0.015$		
	n = *12*	n = *14*		n = *12*	n = *12*
Score at 21 days					
Mean (s.d.)	9.67 (3.60)	10.71 (3.99)		13.17 (3.61)	10.25 (4.92)
Median	10.5	12		14	12
Minimum–maximum	4–14	3–17		5–16	2–16
Comparison			$P = 0.077$		
	n = *12*	n = *14*		n = *12*	n = *12*
Score at 28 days					
Mean (s.d.)	15.08 (2.54)	13.00 (4.17)		15.42 (2.58)	12.25 (3.86)
Median	16	14.5		16	13
Minimum–maximum	10–18	4–17		11–19	4–16
Comparison			$P = 0.057$		

pressure for experimentation in rats, this study opted for a pressure of two ATA.[11,19]

The National Acute Spinal Cord Injury Studies (NASCIS-2 and NASCIS-3) protocol, which is widely used, results in an improvement in neurological function in acute spinal cord lesion. Gangliosides (GM1) have shown a neurotrophic effect, but appear to inhibit the neuroprotective effects of the methylprednisolone and its mechanism of action, although this is not well elucidated, and this drug is still in the experimental stage.[5,13] It is observed that in clinical practice, the use of drugs is restricted to a coadjuvant action in acute spinal cord injury, and more evidence is required of its mechanism of action and effectiveness.

This experimental research offers evidence that there appears to be a beneficial effect in the use of GM1 in the functional recovery in spinal cord lesion, an effect that is potentialized through the combination of GM1 and HBOT.

Conclusions

The therapeutic effect of GM1 on the motricity of Wistar rats submitted to spinal cord lesion is anticipated, through the use of HBOT.

Conflict of interest

The authors declare no conflict of interest.

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