

FUNCTIONAL AND HISTOLOGIC EVALUATION OF HYPERBARIC OXYGEN THERAPY IN RATS WITH SPINAL CORD INJURY

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ABSTRACT

Objective: To evaluate the effectiveness of the application of hyperbaric oxygen therapy in Wistar rats with spinal cord contusion produced using computerized equipment to create impact by a falling weight, NYU Impactor. **Methods:** We evaluated 17 male rats with weights ranging from 265 to 426 g; impacts were performed with a weight of 10 g from a pre-determined height of 12.5 mm, at the tenth thoracic vertebra, after completion of prior laminectomy. The rats were randomly divided into a control group and a group treated with hyperbaric oxygen. The latter, was treated with oxygen therapy in a hyperbaric chamber for one hour daily for a period of 30 days. The assessment of locomotor recovery was conducted

on the 2nd, 9th, 16th, 23rd and 30th postoperative days, measured by the functional scale and the site of injury submitted to anatomopathological examination. **Results:** Improved locomotor recovery was demonstrated in the rats treated with hyperbaric oxygen in the initial stages of the evaluation, but at the end of the evaluation there was no statistically significant difference between the two groups. The anatomopathological examination showed structural changes of the spinal cord in both groups. **Conclusion:** Spinal cord injury in rats evolved differently in the hyperbaric oxygen therapy group compared with the control group, in the initial phase.

Keywords: Hyperbaric oxygenation. Spinal cord injury. Rats, Wistar.

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INTRODUCTION

Spinal cord injuries are incapacitating, irreversible and involve a high economic and social cost. They are characterized by partial or complete interruption of the main functions of the spinal cord, that is, of the motor, sensory and reflex functions, and by provoking neurovegetative disorders of the body segments located below the level of the injury. Their most frequent cause is traumatism, but they are also produced by tumors,¹ infection or vascular injury.²

Spinal cord injuries generally occur in young patients, are more frequent in men, and 70% are traumatic. The most common causes are car accidents, falls, firearm wounds, stab wounds, sports and recreational activities.³ Diving in shallow waters has special significance due to the high prevalence in our country.⁴

In Brazil, the prevalence of spinal cord traumatism is around 40 new cases/year/million inhabitants, about 6 to 8 thousand

new cases/year.⁵ In comparison, in São Paulo state, data from the São Paulo State Department of Health show approximately 1,750 new cases/year and a current population of nearly 250,000 patients with spinal cord injury in Brazil.

In the last two decades, several surveys have been conducted in an attempt to obtain a more effective treatment for spinal cord injury.⁶⁻⁸ All these surveys basically involve four methods of approach for acute spinal cord injury patients: surgical, pharmacological and biological approaches and that performed through physical media, consisting of hyperbaric oxygen therapy and hypothermia.

Hyperbaric oxygen therapy is a therapeutic approach based on the obtainment of high tissue oxygen partial pressures,⁹ when breathing pure oxygen inside a hyperbaric chamber, and a higher-than-atmospheric pressure.

The spinal cord injury exhibits primary or secondary mechanisms of damage to the spinal cord, the primary mechanical

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injury and the secondary injury resulting from one or more biochemical and cellular processes triggered by the primary injury.¹⁰ The concept of secondary injury was first postulated by Allen,¹¹ who proposed the existence of harmful agents present in necrotic and hemorrhagic material that cause additional damage to the spinal cord and these agents are biochemical factors.

The aim of this study is to verify the efficacy of the therapy using hyperbaric oxygen, as an isolated treatment through an experimental, controlled and randomized study on rats with a spinal cord contusion produced by computerized weight-drop impact (*NYU Impactor*). An effort is made to evaluate the effect of the therapy with hyperbaric oxygen on the spinal cord contusion, the functional evaluation of the neurological deficit through the BBB scale,¹² and the alterations found in the anatomopathological exam.

MATERIAL AND METHOD

Seventeen male *Wistar* rats with weight ranging from 265 to 426g, originating from the Centro de Bioterismo da Faculdade de Medicina da Universidade de São Paulo, were evaluated in the study. Upon arrival, all the mice were evaluated in terms of general conditions and motility, and were identified (marking of black and red stripes on the tail). Up to five rats from the same litter were allocated to each cage (40 x 60cm).

We opted to use *Wistar* rats due to their availability in our environment and of the lesser technical difficulties in the handling of these animals. The species of preference for spinal cord experiments would ideally be that of the primates, yet their use is limited due to high cost, limited availability, handling difficulties and ethical considerations.¹³ The rat may be a good alternative in these experiments since its spinal cord has cytoarchitectonic organization and vascularization similar to humans.

The following inclusion and exclusion criteria were adopted:

Inclusion criteria:

- *Wistar* rats;
- Young adult males (20 to 25 weeks of life inclusive);
- Weight between 250 and 450 grams, inclusive;
- General condition (coat and clinical state) and normal motility;

Exclusion criteria:

- Death after injury;
- Loss of tissue in the injured area;
- Spinal cord anomalies in the injured area observed macroscopically;
- Autophagy or mutilation among the animals;
- Severe infection after injury;
- Infection refractory to antibiotic therapy after injury;
- Urine infection after 10 days of treatment with antibiotic (presence of blood in the urine);
- Absence of control over urination;
- Normal movement in the first post-injury evaluation (21 points on the BBB scale [BASSO, BEATTIE and BRESNAHAN] of functional evaluation).

Formation of the experimental groups

The rats were separated randomly (by draw) and formed two groups:

- Control Group - rats submitted to slight spinal cord injury through computerized equipment for spinal cord impact by weight fall - *NYU Impactor* ("New York University Spinal Cord Contusion System - Impactor"), without subsequent treatment;
- Hyperbaric Oxygen Group - mice submitted to the same slight injury protocol with the *NYU Impactor* and, afterwards, submitted to the hyperbaric oxygen therapy protocol for 30 days (1 hour/day).

Of the 20 rats initially included, three were excluded. One rat from the Control group was excluded as it exhibited normal locomotion (21 points on the BBB scale in the first evaluation) after the injury (exclusion criterion). Two rats from the Hyperbaric Oxygen group died: one immediately after the injury and the second between the second and ninth day after the injury.

The rats were anesthetized prior to the injury with 55 to 75mg/kg of intraperitoneal Pentobarbital.

Spinal cord exposure to controlled contusion was performed using a surgical microscope. After trichotomy, an incision was made on the dorsal midline to expose the posterior arches of the vertebral column, from TVIII to TXII. The muscles inserted in the spinous processes and in the laminae from TIX to TXI were detached. The articular processes of these vertebrae were exposed. Hemostasis, when necessary, was performed with a bipolar coagulator. The spinous process, lamina of the TX vertebra and distal half of the spinous process of the TIX were removed with a micro-punch until the spinal cord was exposed, allowing the positioning of the tip of the rod (puncture) of the *NYU Impactor*.¹⁴

The participants adopted the spinal cord injury experimental model of MASCIS ("Multicenter Animal Spinal Cord Injury Study") standardized for *Wistar* rats.^{15,16}

They decided to produce slight lesions through the computerized weight-drop impact device *NYU Impactor*. The impact test consisted of the fall of an impact rod weighing 10g from a predetermined height of 12.5mm between the tip of the rod (puncture) and the spinal cord surface in free fall, through a guide tube monitored by computer (rod speed, absolute and relative deformation of the spinal cord, instant of effective contact and contact time) in order to reduce the imprecision factors.

The computerized equipment for spinal cord impact by controlled weight-drop known as the *NYU Impactor* (Figure 1) is composed of:

- Impact device (10g rod at predetermined height of 12.5mm for slight contusion, guide tube and system for monitoring position, fall speed, instant of contact, period of contact, spinal deformation and absolute and relative deformation of the spinal cord);
- Interfacing devices (instrumentation);
- IBM-PC compatible microcomputer;
- VGA video monitor;
- Interface card with parallel outlet and timer.

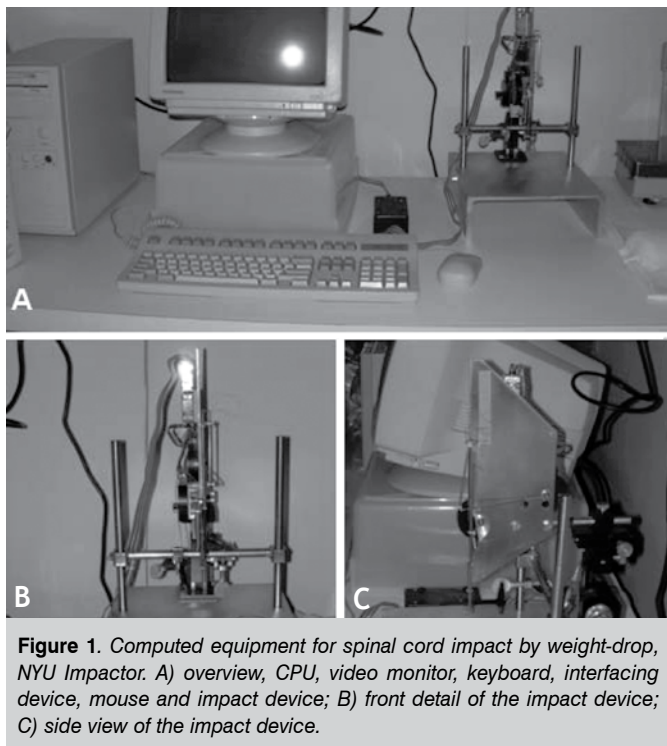


Figure 1. Computed equipment for spinal cord impact by weight-drop, NYU Impactor. A) overview, CPU, video monitor, keyboard, interfacing device, mouse and impact device; B) front detail of the impact device; C) side view of the impact device.

After the injury, the rat was placed on a heated surface, not exceeding the temperature of 38°C. The site of the contusion injury was inspected. Hemostasis was performed where there was bleeding. Then the contusion site was washed with a sterile saline solution of sodium chloride at room temperature. The muscular, fascial tissue planes and the skin were brought together with single stitch suture using 2.0 monofilament nylon thread.

The rats were submitted to antibiotic therapy to prevent and/or reduce infection in the surgical wound in the urinary tract. 25mg/kg of cefalotin (Keflin Neutro® - Ely Lilly) was administered subcutaneously immediately after the injury and once a day during the next seven days. In those that presented infection the treatment was extended up to the 10th day and if it had no effect the rat was submitted to euthanasia (exclusion criterion).

They used a tubular hyperbaric chamber that was 770mm long, with an internal diameter of 180mm and useful height of 150mm (between the platform and the upper wall) especially sized for rats, with 10mm thick transparent acrylic walls. The hyperbaric chamber featured a control valve at the entrance, a differential manometer (kgf/cm²) (aneroid barometer) for control of internal pressure, a flowmeter at the exit (l/min calibrated at 3.5kgf/cm² or 345kPa and 21°C) and a thermometer (°C). The flow of oxygen (O₂) originated from a cylinder through flexible piping (plastic). (Figure 2)

The recovery of locomotor capacity after spinal cord injury was measured using the BBB scale (BASSO; BEATTIE; BRESNAHAN) of functional evaluation.¹²

The BBB scale of functional evaluation is based on specific observational criteria, simple and non-ambiguous defini-

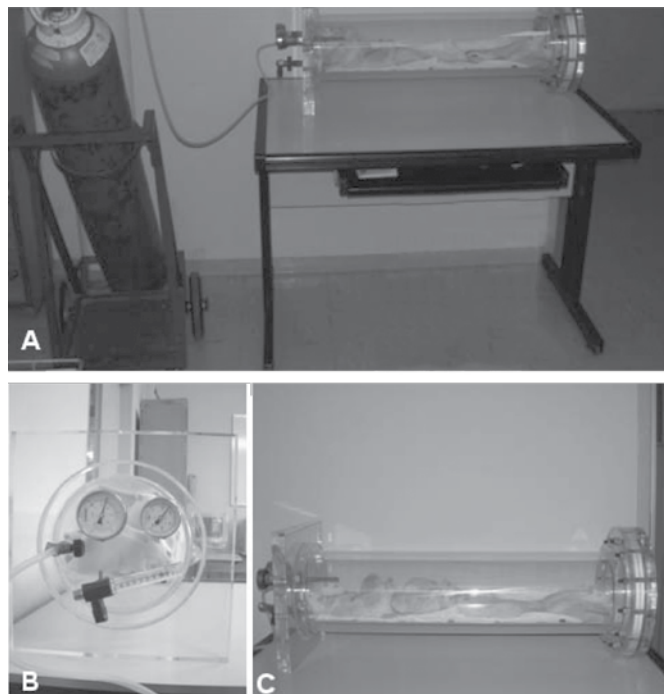


Figure 2. Hyperbaric oxygen therapy chamber: A) overview of the oxygen tube, circuit and chamber; B) right side of the chamber, inlet valve, differential manometer, thermometer and flow meter of spine; C) maintenance of up to 8 rats at the same time (1hr/day).

tions of the terms, and allows a fast and precise description of the locomotor performance.

This scale was adopted by the institutions that form MASCIS and by LETRAN. It was decided to perform a blind appraisal that consisted of visual assessment by a trained team (two observers) using the lowest evaluation.

All the rats from the Control and Hyperbaric Oxygen groups were evaluated on the 2nd, 9th, 16th, 23rd and 30th postoperative days.

The evaluation consisted of placing the rat in the center of an observation box measuring 80x80cm with 17cm high edges lined with a (dark) turquoise blue drape for greater contrast during the observation period (white rats, dark blue background). (Figure 3)

The locomotor capacity of the rats was evaluated. The observations on movement of the hind leg joints (hip, knee and ankle), trunk and abdominal position, leg displacement (swing) and form of contact of the leg with the ground, coordination, toes, contact and raising of the leg from the ground, trunk instability and relative position of the tail, in relation to the left and right sides, were noted down in a specific form. The form was used to record identity, number of postoperative days and comments, and facilitated the description of movement and score definition. The BBB scale ranges from 0 to 21 points for each side, right and left.

The evaluation of each rat was performed by two simultaneous, adequately trained and impartial observers, who were unaware of the group of origin of the rat, so as to not interfere in the results.

If the rat, positioned at the center of the box, remained im-

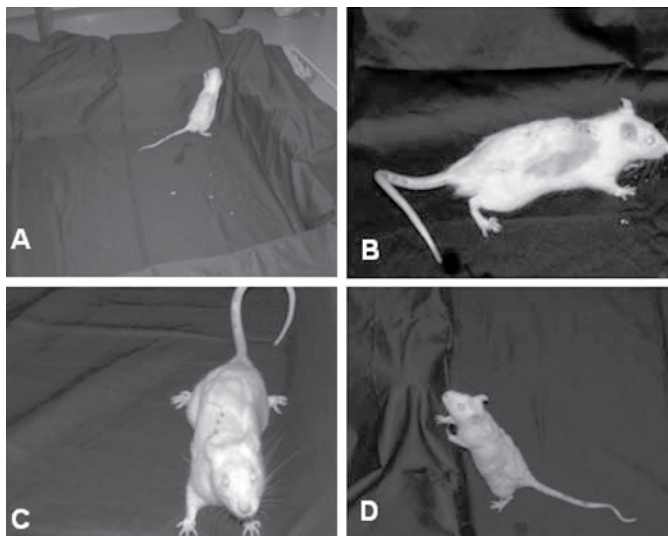


Figure 3. Determination of the locomotor capacity after injury on the BBB scale of functional evaluation. A) rat positioned in the box lined with blue drape to facilitate observation; B) rat in side view, evaluation of the movement of the hip, knee and ankle of the right hind leg and tail position; C) evaluation of the body inclination; D) rat with maximum recovery of motor capacity (21 points) after 30th postoperative day.

mobile for 15 to 20s, it was encouraged to move through pencil flicks.

The evaluation of the rat's locomotor capacity took from 4 to 5 minutes during which time the characteristics of the movement executed were noted down in the respective form.

The characteristics of consensus between the observers were noted down. In the event of a disagreement, it was decided to note down the worst characteristic (lowest score).

At the end of the experimentation period all the rats were euthanized in compliance with the legislation in force and according to the precepts of the Colégio Brasileiro de Experimentação Animal - COBEA,¹⁷ a lethal dose of intraperitoneal pentobarbital was used for this purpose.

A new extensive dorsal incision was made to remove the spinal column, exposing the spine then cutting a segment from TVIII to TXII with scissors.

All the bone structures and soft parts adjacent to the spinal cord were carefully removed with a micro-punch until it was completely exposed.

A macroscopic visual evaluation of the spinal cord was performed at the contusion site to verify any anomaly (exclusion criterion). None was found.

After this the removed spinal cord was inserted in properly identified bottles with a formaldehyde solution (10%) and sent to the Pathological Anatomy Service of the Orthopedics and Traumatology Institute of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo.

The anatomopathological study consisted of a microscopic (optical) analysis of slides stained with hematoxylin-eosin (HE). The Pathology Service evaluated and staggered (absent, modest, moderate and remarkable) the cases in terms of:

- Hyperemia;
- Degeneration of nerve substance;
- Necrosis;
- Cell infiltrate.

The pathologists were not informed of the group of origin to which the spinal cords of the rats belonged (blind evaluation).

RESULTS

On the 2nd postoperative day, the group not treated with hyperbaric oxygen therapy (control group) presented an average of 2.4 points on the BBB scale, while the treated group presented an average of 5.9 points on the scale. (Figure 4)

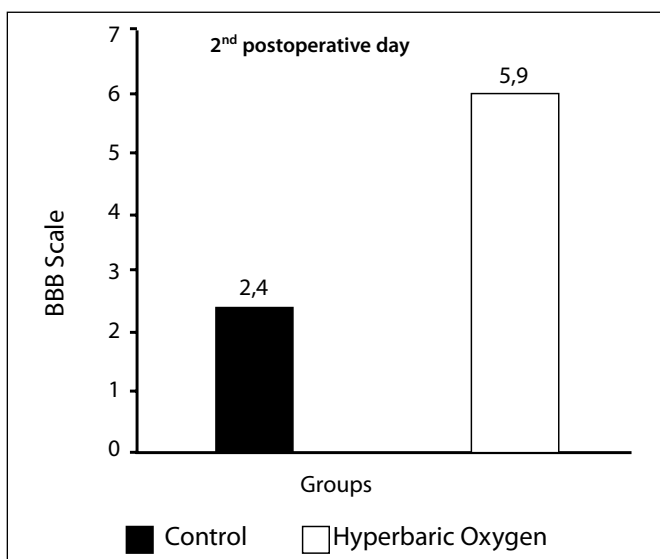


Figure 4. Evaluation of locomotor capacity on the 2nd postoperative day.

On the 9th postoperative day, the difference between the groups decreased on the motor evaluation scale, with the control group presenting 9.9 points, and the group treated with hyperbaric oxygen therapy scoring 11.9 points on the BBB scale. (Figure 5)

On the 16th postoperative day, the control group with oxygen therapy presented 12.6 points, while the group submitted to the hyperbaric chamber presented 14.4 points on the BBB scale. (Figure 6)

On the 23rd day, the control group presented 13.8 points, against 15.9 of the group that received hyperbaric oxygen therapy. (Figure 7)

Finally, on the 30th postoperative day, the evaluation verified that the control group presented 15.6 points on the BBB scale, while the treated group presented 17.4 points. (Figure 8)

In relation to the hyperemia encountered, in the control group it was evidenced that 55.6% of the rats presented a moderate degree of hyperemia, and 44.4% an accentuated degree, with no individuals having been observed with modest degrees; in the group that received hyperbaric oxygen therapy, half presented an accentuated degree of hyperemia, ¼ a moderate degree, and ¼ a discrete degree. (Figure 9)

As regards the degeneration of nerve substance, in the con-

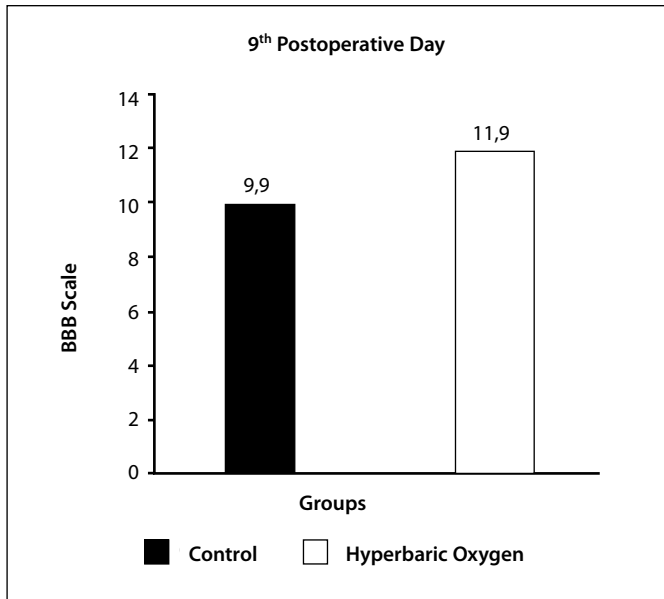


Figure 5. Evaluation of locomotor capacity on the 9th postoperative day.

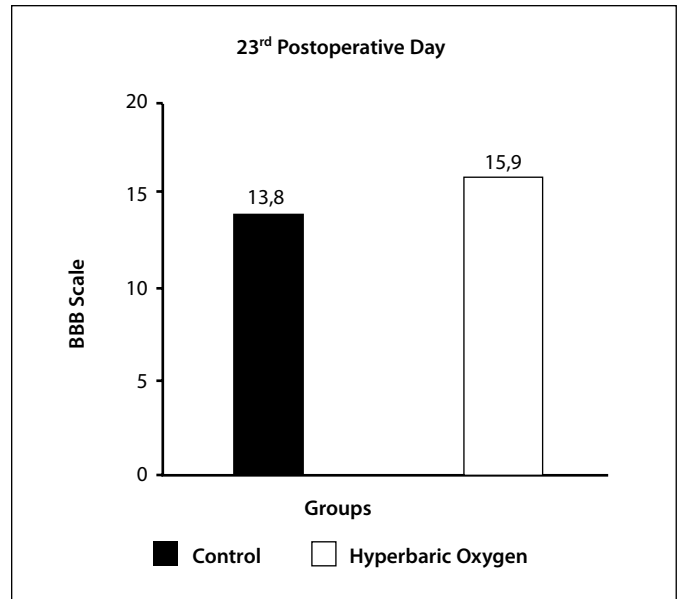


Figure 7. Evaluation of locomotor capacity on the 23rd postoperative day.

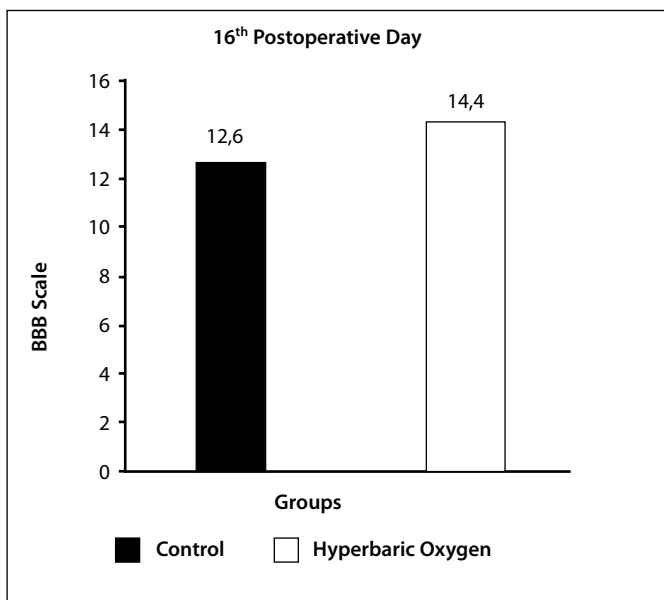


Figure 6. Evaluation of locomotor capacity on the 16th postoperative day

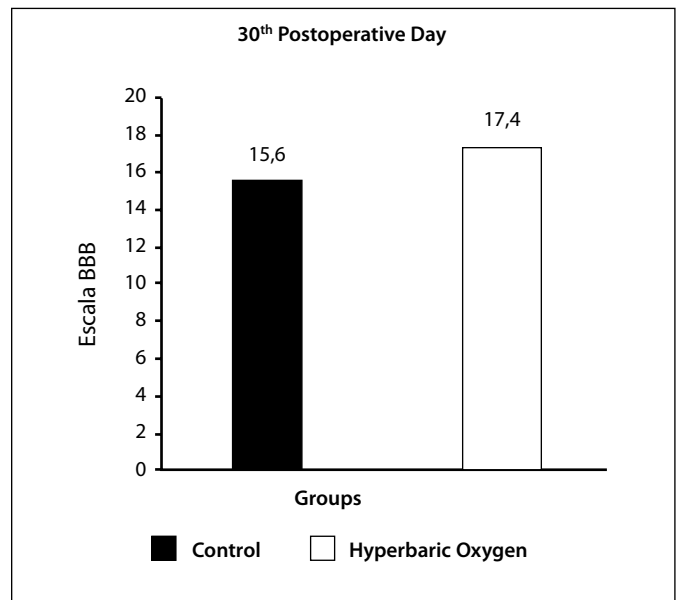


Figure 8. Evaluation of locomotor capacity on the 30th postoperative day.

control group there was remarkable degeneration in 100% of the individuals, while in the treated group, moderate degeneration was observed in 37.5% and remarkable degeneration in 62.5% of the individuals. (Figure 10)

In the tissue necrosis evaluation, the control group presented similar results to hyperemia encountered, with remarkable necrosis in 44.4%, and moderate necrosis in 55.6% of the individuals. In the treated group, 62.5% of the individuals presented accentuated necrosis, 25% moderate, and 12.5% modest signs. (Figure 11)

Finally, in the evaluation of cell infiltrate, the control group findings were 33.3% remarkable, 55.6% moderate and 11.1% absent. In the group that received therapy in a hyperbaric

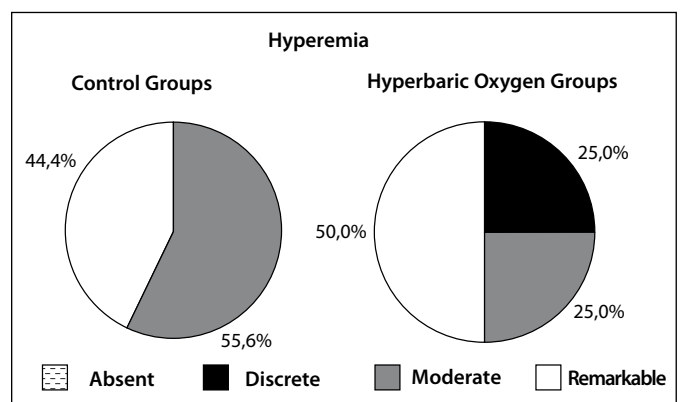


Figure 9. Evaluation of hyperemia in the two groups.

chamber, remarkable signs were observed in 37.5% moderate signs in 25% and modest signs of inflammatory infiltrate in 37.5%. (Figure 12)

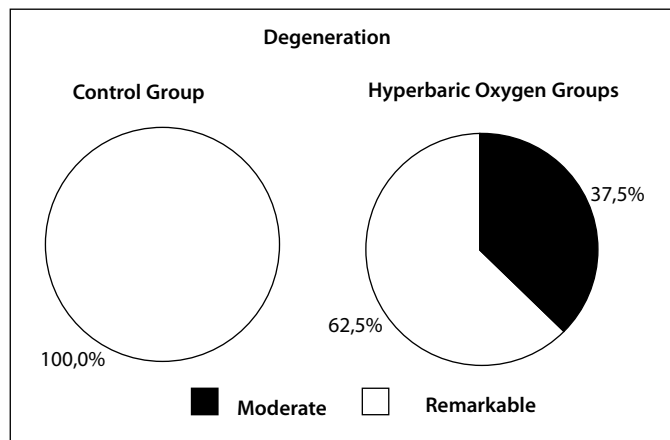


Figure 10. Evaluation of degeneration of nerve substance in the two groups.

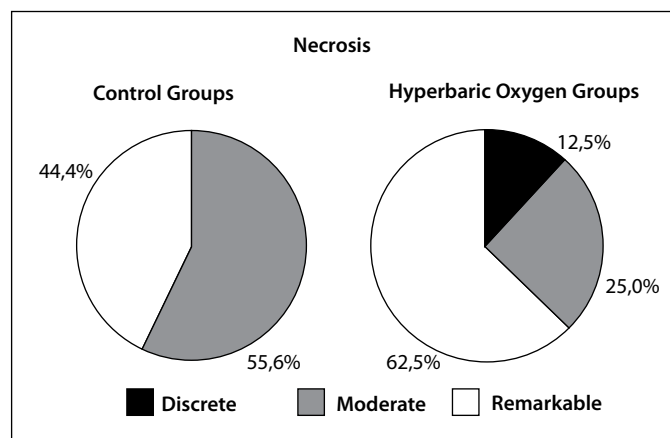


Figure 11. Evaluation of tissue necrosis in the two groups..

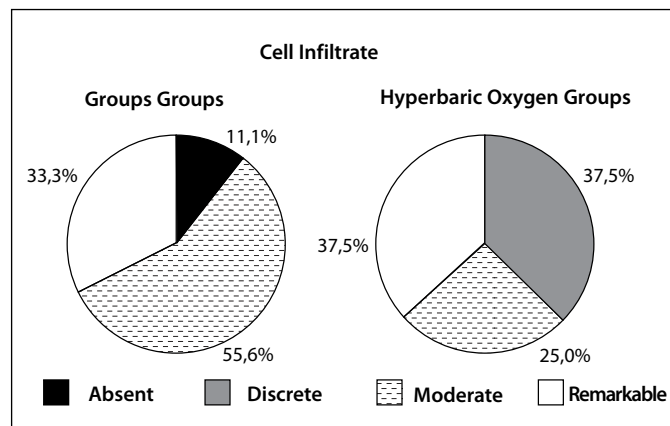


Figure 12. Evaluation of the presence of cell infiltrate in the two groups

DISCUSSION

For the evaluation of locomotor recovery of the hind limbs, the participants initially performed separate measurements of the left and right sides of the Control and Hyperbaric Oxygen

groups, on the 2nd, 9th, 16th, 23rd and 30th postoperative day. The fact that no statistically significant functional differences were proven allowed the grouping of results of the function of the left and right limbs of each group. This facilitated the absolute interpretation of results. (Figures 4 to 12) Hence a comparison was made of the differences between the general results of the Control and Hyperbaric Oxygen groups.

After euthanasia, samples of the spinal cord tissues were taken from the injury site and submitted to the anatomopathological exam, by the hematoxylin and eosin staining method, with the purpose of proving tissue lesion stigmas. This study was necessary to make sure that the traumatism had been effective in the production of the spinal cord contusion.

The locomotor capacity results, obtained by the BBB scale, of the Hyperbaric Oxygen group, were 59.3% higher than those of the Control group on the 2nd postoperative day (Figure 4) and 20.2% higher on the 9th day. (Figure 5) On the 16th day the difference was not proved. (Figure 6) Again, on the 23rd day the result of the locomotor capacity of the rats from the Hyperbaric Oxygen group was 15.2% higher than that of the Control group (Figure 7). On the 30th postoperative day the results were equal. (Figure 8)

Through these data, remarkable and accelerated locomotor capacity recovery was verified in the rats treated with hyperbaric oxygen therapy up to the 9th postoperative day. From then on, in this group, functional progression decreased gradually until the 16th day. The functional difference was once again resumed in favor of the hyperbaric oxygen group on the 23rd day and subsequent decrease to the level of equivalence that occurs on the 30th day, when the stabilization and equalization of results occurred.

It was verified with a basis on these results that from the histological point of view there was no proof of the benefit of the therapy with hyperbaric oxygen, in relation to hyperemia, degeneration of nerve substance, necrosis and cell infiltrate.

CONCLUSIONS

The anatomopathological exam proved the structural alterations of the spinal cord in a uniform manner in the two groups. The functional evaluation of the neurological deficit proved similar after the impact contusion method in the two groups. The evaluation of the recovery of locomotor capacity by the BBB scale proved effective in the interpretation of the effect of hyperbaric oxygen therapy.

The slight spinal cord injury caused to the rats evolved differently in the hyperbaric oxygen therapy group in comparison with the control group, in the initial phase (accelerator effect). The contribution of this experimental survey was the demonstration of the accelerator effect in the initial phase of recovery of the locomotor capacity of rats. With a basis on these findings, hyperbaric oxygen therapy becomes a useful tool in the therapeutic arsenal for the resolution of the intricate pathology that is spinal cord traumatism; however, supplementation with biological and cell resources should constitute the next steps to be taken. Issues such as application time, ideal pressure and mechanisms of action are put on hold.

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