Spinal Cord Contusion in Rats Treated with Systemic Hypothermia. Experimental Cold-inducible Protein Expression

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ABSTRACT

Introduction: Traumatic spinal cord injury is the leading cause of motor disability worldwide, and the WHO considers it a priority. This study sought to investigate the effects of therapeutic hypothermia following spinal cord contusion. **Materials and Methods:** Male rats that underwent experimental spinal cord contusion were used. For this purpose, four experimental groups were created (n=6 per group): a) control, b) lesion in normothermia (24°C, sacrificed 12h after the injury), c) lesion in normothermia (24°C, sacrificed 24h after the injury), and d) hypothermic injury (8°C for 180 min, sacrificed 24h after the injury). The expression of cold-inducible RNA-binding protein (CIRBP), Caspase-3, and NeuN was studied. **Results:** At 24 hours, spinal cord damage raised CIRBP expression slightly while also increasing Caspase-3 significantly. All of this was accompanied by images of damaged motor neurons in the anterior horn. In animals treated with hypothermia, high expression of CIRBP and very low levels of Caspase-3 were observed, which were indistinguishable from controls. Furthermore, the number of viable motor neurons was partially restored. **Conclusions:** The experimental model developed in this study was effective at inducing spinal cord injury, demonstrating neuronal protection through hypothermia. The increased expression of CIRBP in the spinal cord of rats with injury and hypothermic treatment when compared to the normothermic group suggests the possibility of using substances that increase CIRBP as therapies for the treatment of contusive spinal cord injuries.
Keywords: Contusion; hypothermia; spinal cord; CIRBP; rat; injury.
Level of Evidence: I

Contusión medular en ratas tratadas con hipotermia sistémica. Expresión de proteínas inducibles por frío experimental

RESUMEN

Introducción: La lesión traumática de la médula espinal es la principal causa mundial de discapacidad motora y una prioridad para la OMS. El objetivo de esta investigación fue estudiar el efecto de la hipotermia terapéutica tras una contusión medular. **Materiales y Métodos:** Se utilizaron ratas macho a las que se les generó una contusión medular. Se formaron cuatro grupos (6 animales por grupo): a) de control, b) con lesión en normotermia (24 °C, sacrificados 12 h después de la lesión, c) con lesión en normotermia (24 °C, sacrificados 24 h después de la lesión) y d) lesión en hipotermia (8 °C, durante 180 min, sacrificados 24 h después de la lesión). Se estudió la expresión de la CIRBP, la caspasa-3 y la Neu-N. **Resultados:** La lesión medular aumentó ligeramente la expresión de CIRBP a las 24 h y, de manera importante, la de caspasa-3, todo acompañado por imágenes de motoneuronas dañadas en el asta anterior. En los animales tratados con hipotermia, se observó una alta expresión de CIRBP y niveles muy bajos de caspasa-3, que no se distinguen de los controles. El número de motoneuronas viables se restauró parcial-

mente. **Conclusiones:** Este modelo experimental resultó eficaz para inducir una lesión medular, demostró la protección neuronal mediada por hipotermia. El aumento de la expresión de CIRBP en la médula espinal de ratas con lesión e hipotermia comparado con el del grupo normotérmico abre el camino para un posible uso de sustancias que incrementen la CIRBP como terapéutica para las lesiones medulares contusivas.

Palabras clave: Contusión; hipotermia; médula espinal; CIRBP; rata; lesión.

Nivel de Evidencia: I

INTRODUCTION

Every year, around 500,000 people worldwide suffer spinal cord injuries, increasing their likelihood of dying prematurely.[1] According to the National Rehabilitation Service of Argentina, in 2013, 2,176,123 patients with disabilities were identified (54% males and 46% females), of whom 30% suffered from a pure motor disability.[2] This creates the need to seek new therapies to improve the quality of life of patients. Our proposal is to investigate the therapeutic use of hypothermia. In our laboratory, we have shown that it can minimize central nervous system damage in mouse models of perinatal asphyxia and optic nerve trauma, with encouraging outcomes.[3-13] A slight reduction in body temperature protects the central nervous system against various types of damage.[3,5,8,12-14]

Numerous publications show that mild levels of hypothermia after injury improve neurological function and reduce histopathological damage in the bone marrow.[14-18] The neuroprotective role of hypothermia is well established in experimental animals and in patients with cardiac arrest (Hakim et al., 2018), hypoxic-ischemic encephalopathy (Yum et al., 2018), traumatic brain injury (Leng, 2017), and other diseases (Zhu et al., 2015). Although the neuroprotective mechanisms of hypothermia in different diseases vary and have not yet been fully determined, neuroprotection has been commonly attributed to its effect on decreasing metabolic rate, reducing radical generation, improving inflammation, inhibiting excitotoxicity and apoptosis. Increased expression of different cold-inducible proteins such as CIRBP (cold-inducible RNA binding protein) and RBM3 (RNA binding motif protein 3) has been described. CIRBP, also known as CIRP or heterogeneous ribonucleoprotein A18 (hnRNP A18), is an 18 kDa protein composed of 172 amino acids whose gene in humans is located on chromosome region 19p13.3. Like other members of the hnRNP family, CIRBP binds to messenger and ribosomal RNA present in the cell and regulates its half-life, the potential expression of multiple genes and thus its function. As with other RNA-binding proteins, CIRBP has been shown to be able to modulate apoptosis by playing an anti-apoptotic role in hypothermia situations. In rat neuronal cells, this effect appears to occur through the mitochondrial apoptosis pathway, as they show decreased expression of proapoptotic molecules (Bax, Bad, Bak, Cycs, Apaf1, caspase-9 and caspase-3). Recently, we have shown increased expression of CIRBP in the anterior horn the spinal cord of rats subjected to systemic hypothermia.[7,9,11,12,19-24] Taking this background into account, the objective of this study was to evaluate the effects of cold on spinal cord protection by expressing CIRBP in motor neurons of the anterior horn of the spinal cord of rats exposed to spinal cord injury and treated with systemic hypothermia. To do this, we applied our hypothermic model as a treatment for spinal contusion (MASCIS®),[25] and studied the expression of CIRBP as a protein induced by hypothermic treatment, and that of caspase-3 and NeuN to study neuron viability.

MATERIALS AND METHODS

Model and experimental design

The procedures were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (CCAC 2002; CCAC 2003). The protocol was approved by CICUAL, resolution No. 970/2015 (Universidad de Buenos Aires, Argentina). The Sprague Dawley rat (developed from Winstar) is an exogamous, multipurpose breed of albino rat widely used in medical research. Its advantages include its docility, ease of use, and our laboratory experience with this breed, which we have been working with in hypothermia since the 1980s.

Twenty-four 60-day-old adult male Sprague-Dawley rats were used and distributed into four groups (6 per group): a) control, b) with 12 h NT injury: injury in normothermia at 24 °C and euthanized 12 h after injury, c) with 24 h NT injury: injury in normothermia at 24 °C and euthanized 24 h after injury, and d) with 24 h HT injury: injury in hypothermia at 8 °C, in a cold room, for 180 min and euthanized 24 h after injury.[24] Animals were administered intraperitoneal anesthesia with a mixture of xylazine (Rompun®, Bayer, Kiel, Germany) 10 mg/kg body weight and ketamine (Ketolar®, Pfizer, Alcobendas, Madrid, Spain) 60 mg/kg body weight. The animal was placed on the surgical platform with a bolster under the thorax to help separate the spinous processes and a warm IV bag at 38 °C was used as a bed to prevent hypothermia throughout the procedure. The thoracic spinal cord was surgically exposed through a dorsal incision and laminectomy from T9 to T11. The spinal contusion was caused with the MASCIS® impactor, according to published protocols. Briefly, it entails dropping a 10 g bar that is elevated to 25 mm high, resulting in a 24 g damage load when it directly impacts the dorsal area of the rat spinal cord in T9-T10.[25] After the contusion, the wound was surgically closed and the animals were placed in individual boxes for recovery under standard vivarium conditions at 24 °C (groups with 12 h NT injury and 24 h NT injury), or exposed to hypothermia at 8 °C, in a cold room, for 180 min, after the injury and then transferred for recovery under standard vivarium conditions at 24 °C (group with 24 h HT injury). Hypothermia control was performed as detailed in previous studies of the research group.[7-13] The vivarium has light/dark cycles of 12/12 hours. Tylenol® was used for postoperative analgesia (65 mg/kg). Euthanasia was by decapitation 12 or 24 h after the injury as indicated above. Tissue processing was performed following the procedure described in previous publications.[12,26]

Hematoxylin & eosin

Spinal cords from the injured thoracic region were immersed in paraffin, cut into 5 μm thick coronal sections with a microtome (Leitz, Lauda MGW, Germany), and placed on gelatin-coated slides. Spinal cord slices were hydrated with xylene, followed by decreasing alcohol concentrations, and finally stained with hematoxylin-eosin. The stained sections were observed with an optical microscope (Carl Zeiss Axiophot, Germany) connected to a digital camera (Olympus, Q-Color 5, USA) and photographed for analysis using the NIH Image program (Wayne Rasband, 1995, NIH, Research Services Branch, NIMH, Bethesda, MD, USA).

Immunohistochemistry

Spinal cord sections were rehydrated, endogenous peroxidase was inhibited with H_2O_2 (3% in methanol) and nonspecific binding was blocked with incubation in normal goat serum. Sections were then incubated in a wet room overnight at 4°C with rabbit polyclonal anti-CIRBP primary antibodies (Proteintech®, UK, Cod. 00055668) in a 1:1000 dilution or mouse monoclonal anti-NeuN antobdies (Abcam, Cod. ab177487) in a 1:1000 dilution. The anti-NeuN antibody recognizes a molecule with a molecular mass of 46/48 kDa known as neuron specific nuclear protein (NeuN or "NEUronal Nuclei"), which is located in the DNA-binding domain of most viable neurons in the central nervous system, and thus was used in this study to quantify neuronal viability. The next day, immunoreactivity was developed with species-specific biotinylated secondary antibodies: anti-rabbit (Vector Labs, USA, catalog number PK-6101) or anti-mouse (Abcam Labs, USA ab6788) as appropriate, both at 1:200 dilution. Labeling was detected by diaminobenzidine, using the commercial kit SK-4100 (Vector Laboratories, USA). The analysis of the degree of response and the number of immunoreactive cells was performed with an optical microscope (BX40, Olympus Optical Corporation, Tokyo, Japan), coupled to a digital camera (390CU 3.2 Megapixel CCD Camera, Micrometrics, Spain), using the program Micrometrics SE P4 (Standard Edition Premium 4, Micrometrics, Spain). Three animals per experimental group were analyzed.[27]

Tissue processing for Western blot

The membrane was incubated with rabbit anti-CIRBP antibody (Proteintech®, UK) at a 1:1000 dilution and mouse monoclonal anti-CIRBP-3 antibody at a 1:500 dilution (Santa Cruz Biotech. A2921, USA). The membrane was then incubated with rabbit anti-IgG antibody (Amersham Pharmacia Biotech, USA) at a 1:5000 dilu-

tion or mouse anti-IgG at a 1:3000 dilution (Amersham Pharmacia Biotech, USA) as appropriate. The ECLTM detection kit (Amersham™) was used for development. The bands were visualized with the UVP Biospectrum/ Biolite imaging system (Analytik Jena). Three animals per experimental group were analyzed. As a loading control, the membrane was incubated with mouse anti-ß-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) in a 1:5000 dilution. The molecular weight of the protein bands was estimated using a commercial protein ladder (PageRuler®, Fermentas UAB, Vilnius, Lithuania).

Image analysis

Images of sections with hematoxylin-eosin staining or immunostaining were obtained by microscopy and digitized. The quantification of immunopositive neurons was performed as previously published by our research group. [3,7-13] Spinal cord sections were selected to be comparable between animals. The relative optical density of immunopositive neurons and the number of immunoreactive neurons were determined with ScionImage image analysis software. In anti-NeuN staining, viable motor neurons were those with a homogeneous label in the nucleus and cytoplasm. Six sections per antibody were analyzed and 10 fields per section were counted using 40X magnification. A label was considered immunopositive when its optical density exceeded 4 times or more the optical density of the background. For the quantification of the optical density of the bands obtained by Western blot, ImageJ software (NIH, USA) was used. The CIRBP and caspase-3 optical density values obtained in the Western blots were normalized according to the corresponding ß-actin optical density value and the results were expressed as mean value of the group ± standard deviation and as a percentage in relation to the control group. All experiments were performed in duplicate.

Statistical Analysis

The data obtained was loaded into a database that was analyzed with the statistical package InfoStat version 2020 and graphed using the GraphPad Prism program (version 5.0 for Windows, GraphPad Software). To determine if the differences observed between the groups were statistically significant, a one-way analysis of variance (ANOVA) was performed followed by Tukey's test. Before testing the hypothesis, the assumptions of normality were verified using the modified Shapiro-Wilk test and the assumptions of homogeneity of variances were verified using the Lavene test. A difference $p < 0.05$ was considered statistically significant.

RESULTS

Western blot

A band of ≈20.5 kDa was obtained indicating CIRBP expression, 37 kDa for caspase-3 and 42 kDa for actin (Figure 1A). For CIRBP, a significant increase in expression can be observed in the 24 h HT injury group compared to the control and 12 h NT injury group (Figure 1B). In terms of caspase-3 expression, a significant increase is observed in the 12 h and 24 h NT injury groups compared to the control group. It should be noted that rats treated with hypothermia (24 h HT injury) had a significant decrease in caspase-3 levels compared to both groups injured in normothermia, becoming indistinguishable from the control group (Figure 1C).

Morphology

We obtained images of the anterior horn of the spinal cord corresponding to Rexed laminae VII, VIII, and IX, and we analyzed the alpha motor neurons using hematoxylin-eosin histology and immunohistochemistry for CIRBP and NeuN.
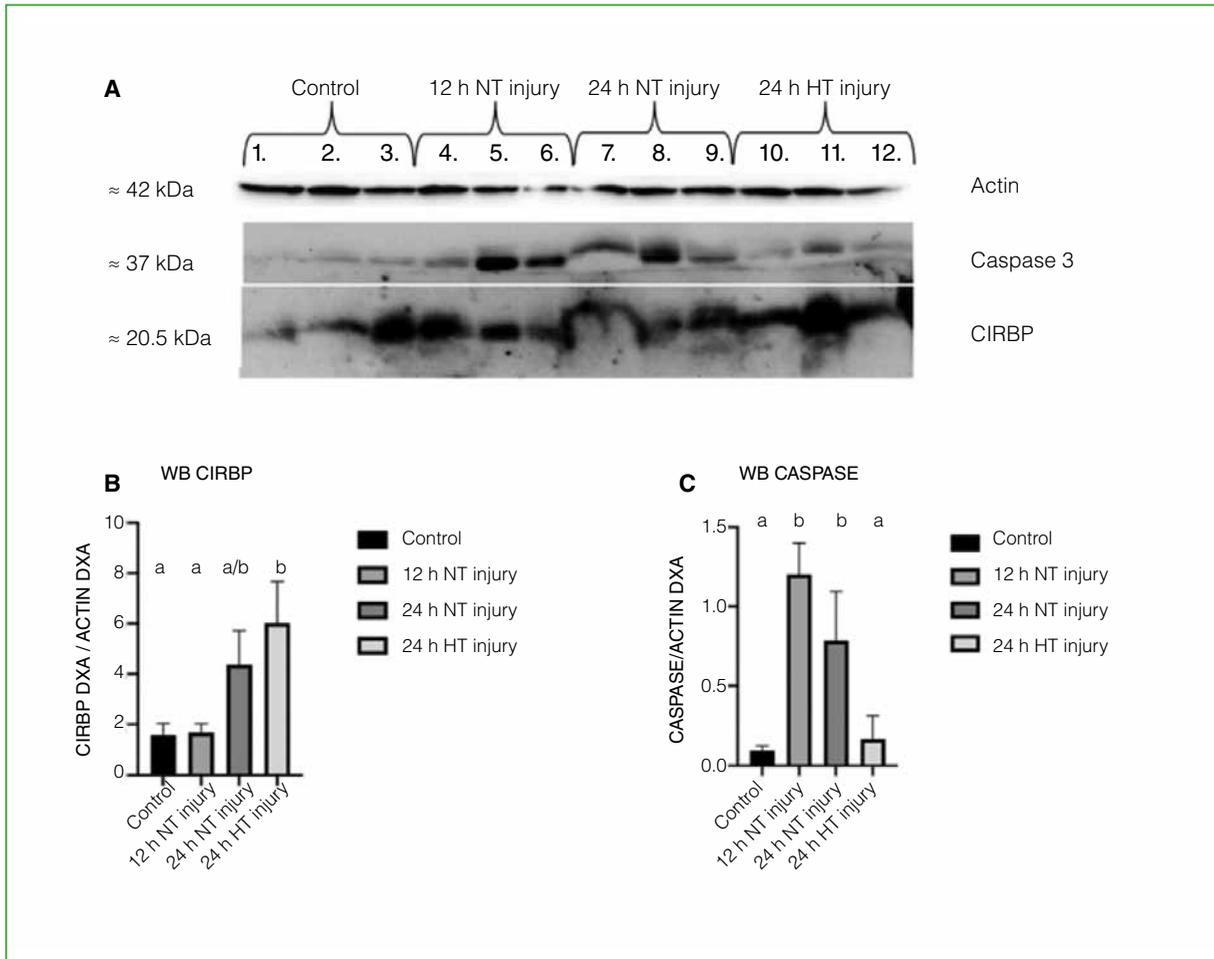
Figure 1. A. Western blot of spinal cord homogenates from all 4 groups, immunostaining for CIRBP, caspase-3 and actin loading control. **B.** Semi-quantification plot of CIRBP bands by densitometry and analysis, ANOVA test and Tukey's test. A significant difference is observed between the 24 h HT lesion group and the control group (a/b $p < 0.05$). **C.** Semi-quantification plot of caspase-3 bands by densitometry and analysis, ANOVA test and Tukey's test. A significant difference is observed between the 12 h NT and the 24 h NT injury groups, and the control and 24 h HT injury groups ($p < 0.05$). NT = normothermia; HT = hypothermia.

Hematoxylin & eosin

In the controls, the structure remains unaltered, with an H-shaped distribution of gray matter and visible large motor neurons. In the slices of the other experimental groups, there is a loss of cohesion, as evidenced by an area of early degeneration and the disappearance of the neurological structure, giving place to a lesional cavitation caused by spinal cord injury with some blood elements. (Figure 2).
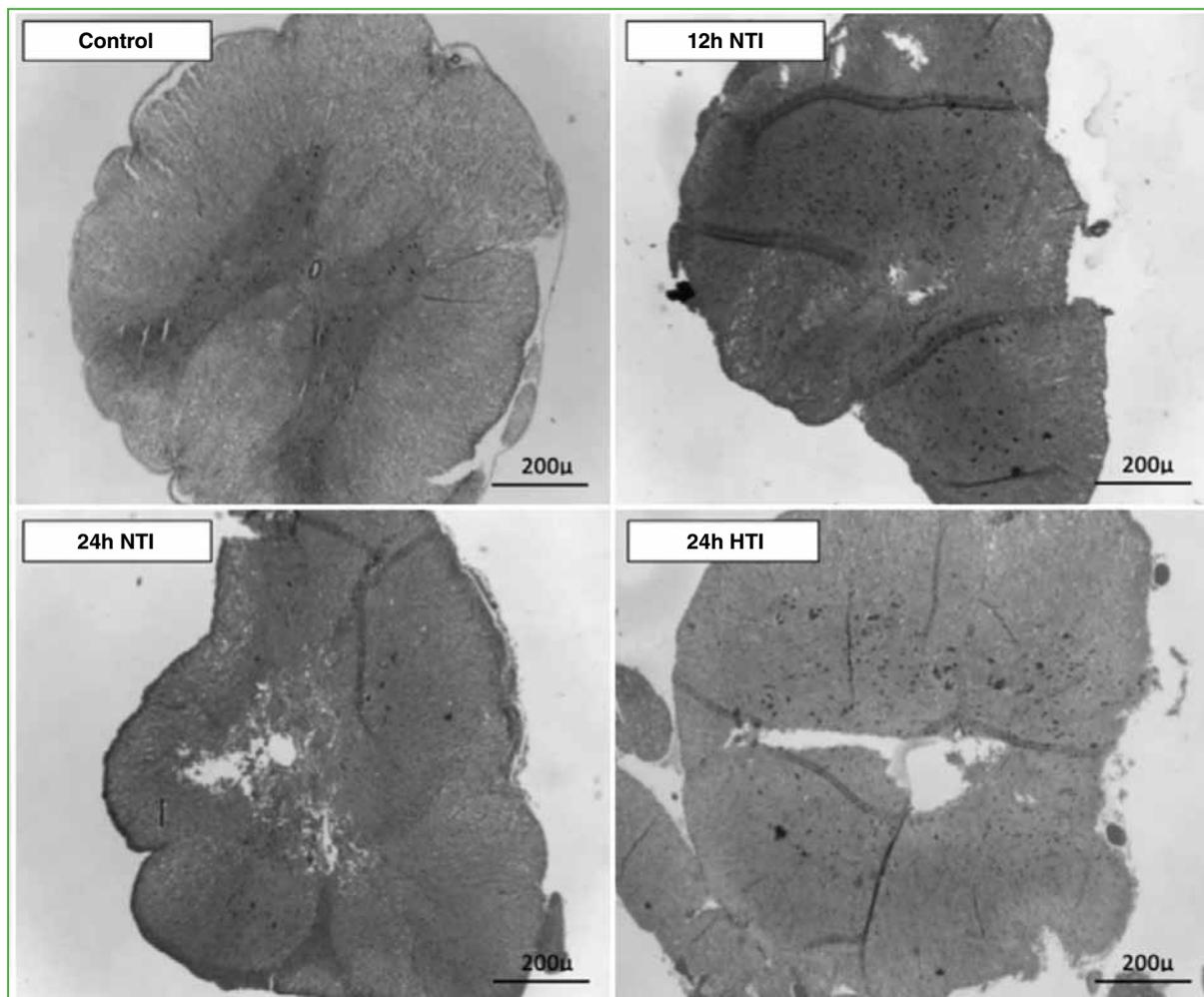
Figure 2. Spinal cord sections stained with hematoxylin & eosin. x10 magnification. Cohesion loss is marked by an area where early degeneration occurs and the usual neurological structure disappears, giving way to post-injury cavitation with some blood elements.

NT = normothermia; HT = hypothermia.
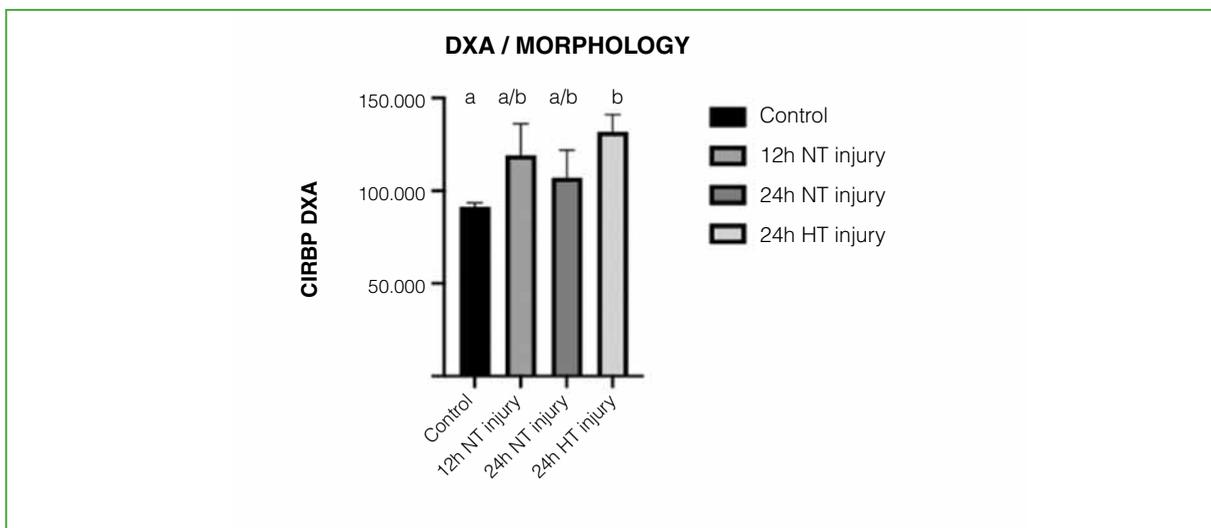
Immunohistochemistry
CIRBP Expression

In the control group, CIRBP shows a weak immunoreactive label with cytoplasmic distribution and its expression is slightly higher in the nucleus and neuropil. In the images of 12 and 24 h NT injured rats, the label was somewhat higher than that of the control group. The group with 24 h HT injury exhibited intense labeling in the nucleus and in the form of cytoplasmic "granules", in the alpha motor neurons and the surrounding neuropil (Figure 3).

When quantifying the optical density for CIRBP, a significant increase in the optical density of CIRBP was observed in the 24 h HT injury group compared to the control group ($p = 0.01$), without differences when compared to the rest of the experimental groups (Figure 4, Table 1).
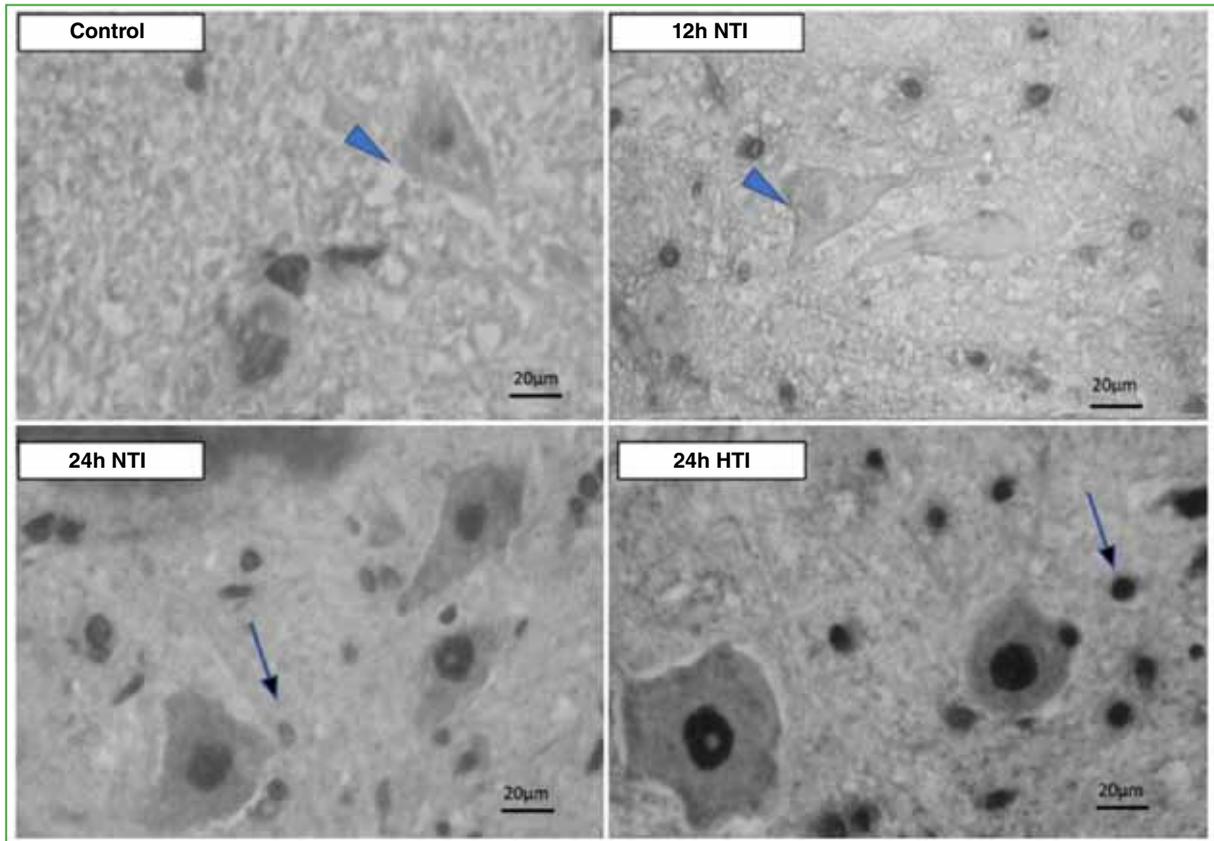
Figure 3. CIRBP. Comparative slices. x40 magnification. Magnification of Rexed laminae VII and VIII. Motor neuron with weak labeling in the controls and within 12 h of the injury (arrowhead). The label increases after 24 h in normothermia with an expression greater than 24 h with hypothermia. Glial cells with higher concentration in injured groups (arrows). NT = normothermia; HT = hypothermia.

Figure 4. Densitometric quantification plot of immunohistochemistry with anti-CIRBP antibodies in spinal cord sections of three rats per group: control, with 12 h and 24 h NT injury, and with 24 h HT injury. As observed in previous micrographs, there is a progressive increase in CIRBP expression, which is lower in control animals, moderate between 12 h NT and 24 h NT, and significant with 24 h HT. The analysis with the ANOVA test and the Tukey test revealed a significant difference between the control group and the group with 24 h HT injury (a/b $p<0.001$). NT = normothermia; HT = hypothermia.

Table 1. Relative optical density (ROD) of CIRBP in immunohistochemistry.

Groups	Average difference	95% confidence interval	Significant	p
Control vs. Normothermia 12 h	-27785	From -59942 to 4372	No	0.0924
Control vs. Normothermia 24 h	-15734	From -47892 to 16423	No	0.4462
Control vs. Hypothermia 24 h	-40419	From -72577 to 8262	Yes	0.0161
Normothermia 12 h vs. Normothermia 24 h	12051	From -20107 to 44208	No	0.6436
Normothermia 12 h vs. Hypothermia 24 h	-12634	From -44792 to 19523	No	0.6110
Normothermia 24 h vs. Hypothermia 24 h	-24685	From -56842 to 7472	No	0.1424

In the Tukey test, the significant difference between the control group and the 24 h HT group is observed; although the increase in CIRBP is observed in the NT injury groups, the difference is not significant between them as well as with the controls.

Cell viability by NeuN expression

Qualitatively, when assessing the immunohistochemical distribution of NeuN in the groups with NT injury, there is a decrease in the intensity of nuclear staining, with a perinuclear halo and eccentric nucleus, cytoplasmic retraction with irregular membrane. Some cells look like "ghost neurons", and the increase in glial cells is seen (Figure 5).
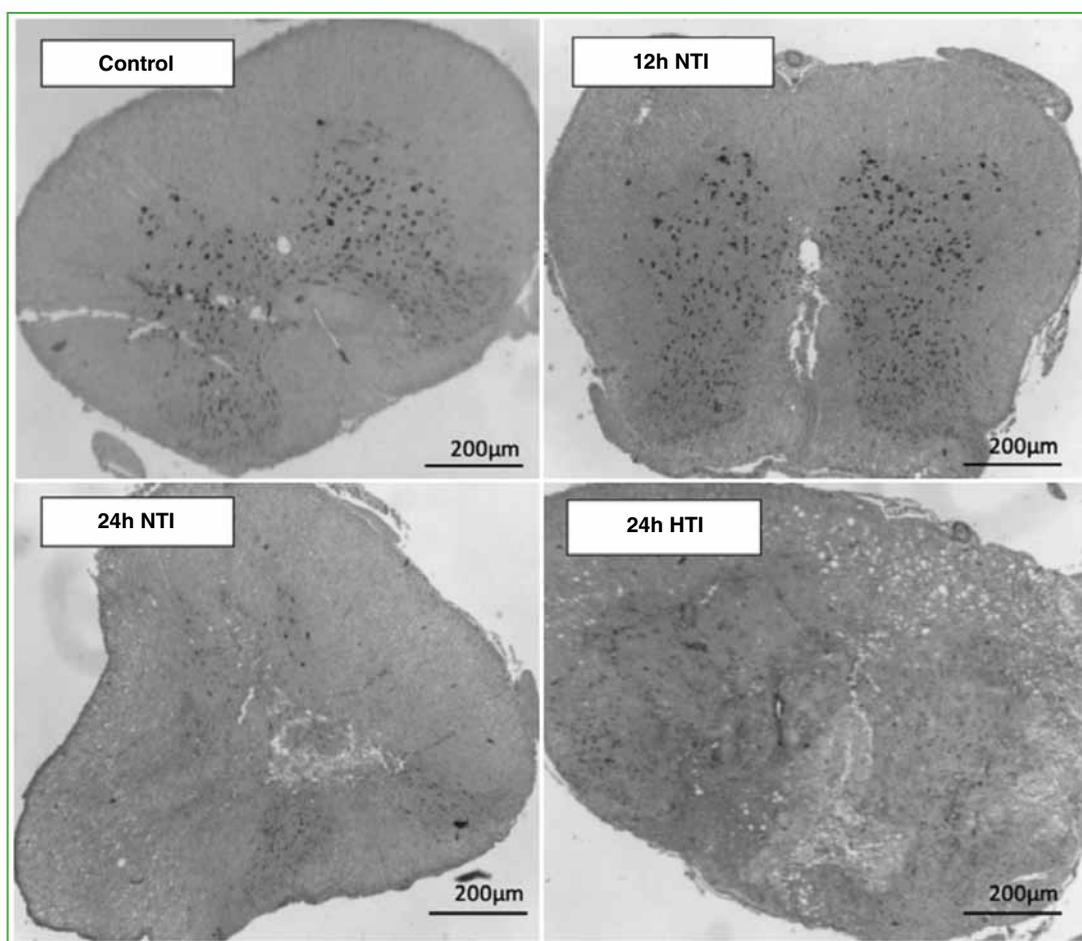

Figure 5. NeuN. Comparative slices. Magnification x4. Marked staining can be observed in both the control and the 12 h NT injury groups, as well as the 24 h HT injury group.

However, in the group with 24 h HT injury, neurons in the process of death were observed, but in fewer numbers (Figure 6).
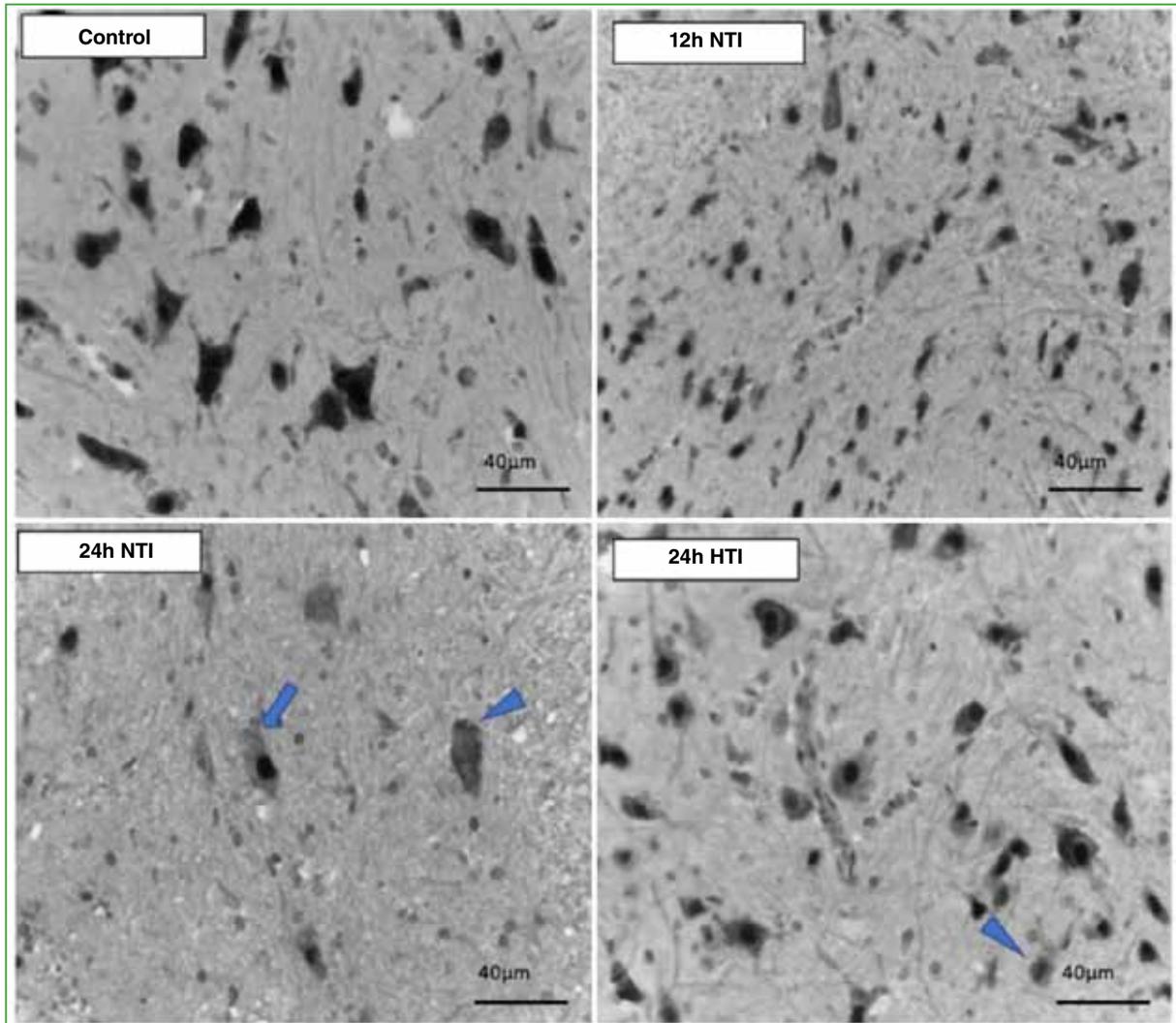
Figure 6. NeuN. Comparative slices. Magnification x2. Controls with strong label and surrounding glia without label for NeuN. The 12 h NT slices show a slight increase in glia and loss of staining in some neurons, something much more marked in the 24 h NT group, with ghost neurons (arrowhead) or neurons with eccentric nuclei (arrow). The 24 h HT injury looks similar to that of the 12 h NT group, with some motor neurons dying and mild staining loss (arrowhead).

When counting the number of viable neurons per field in spinal cord sections stained with NeuN immunolabeling, which marks the nerve fiber of vital cytoplasms and nuclei and is lost when the cell is dead or dying, cells with a central nucleus and homogeneous cytoplasmic staining were counted. A significant reduction was observed in the 12 h and 24 h NT injury groups compared to the control group (p <0.0001) (Figure 7, Table 2).

**MOTOR NEURONS EVERY 10 40X FIELDS**
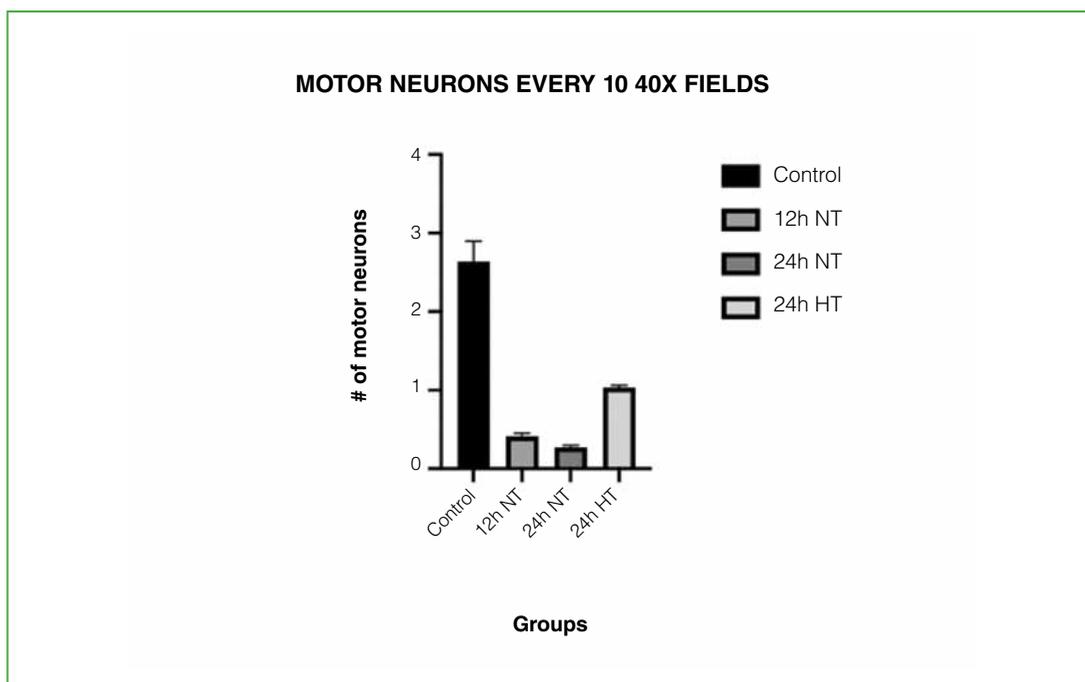
Figure 7. Densitometric quantification plot of immunohistochemistry with anti-NeuN antibodies in spinal cord sections of three rats per group: control, 12 h and 24 h NT, and 24 h HT. As observed in the previous micrographs, there is a loss of expression with progressive neuronal destruction between 12 h and 24 h of the NT injury group. In the group with 24 h HT injury, a persistence of viable motor neuron structure is observed. The analysis using the ANOVA test with the Tukey test revealed a significant difference between the control group and the group with 24 h HT injury (a/b p<0.001).

Table 2. Tukey multiple comparison test. NeuN immunohistochemistry

Groups	Average difference	95% confidence interval	Significant	p
Control vs. Normothermia 12 h	9.947	From 6.355 to 13.54	Yes	<0.0001
Control vs. Normothermia 24 h	10.54	From 6.946 to 14.13	Yes	<0.0001
Control vs. Hypothermia 24 h	6.227	From 2.635 to 9.819	Yes	0.0024
Normothermia 12 h vs. Normothermia 24 h	0.5917	From -3.000 to 4.184	No	0.95
Normothermia 12 h vs. Hypothermia 24 h	-3.72	From -7.312 to -0.1279	Yes	0.0426
Normothermia 24 h vs. Hypothermia 24 h	-4.312	From -7.904 to -0.7195	Yes	0.0206

Tukey multiple comparison test. Cell count under x40 magnification every 10 viable cell fields with anti-NeuN antibodies in the anterior horn of the spinal cord. There is a significant difference between groups, except between groups with 12 h and 24 h NT injury.

We also found a significant increase in the number of viable neurons in the 24 h HT injury group compared to both NT injury groups (p <0.002). However, neuron levels in hypothermia did not reach control group levels (Figures 6 and 8).
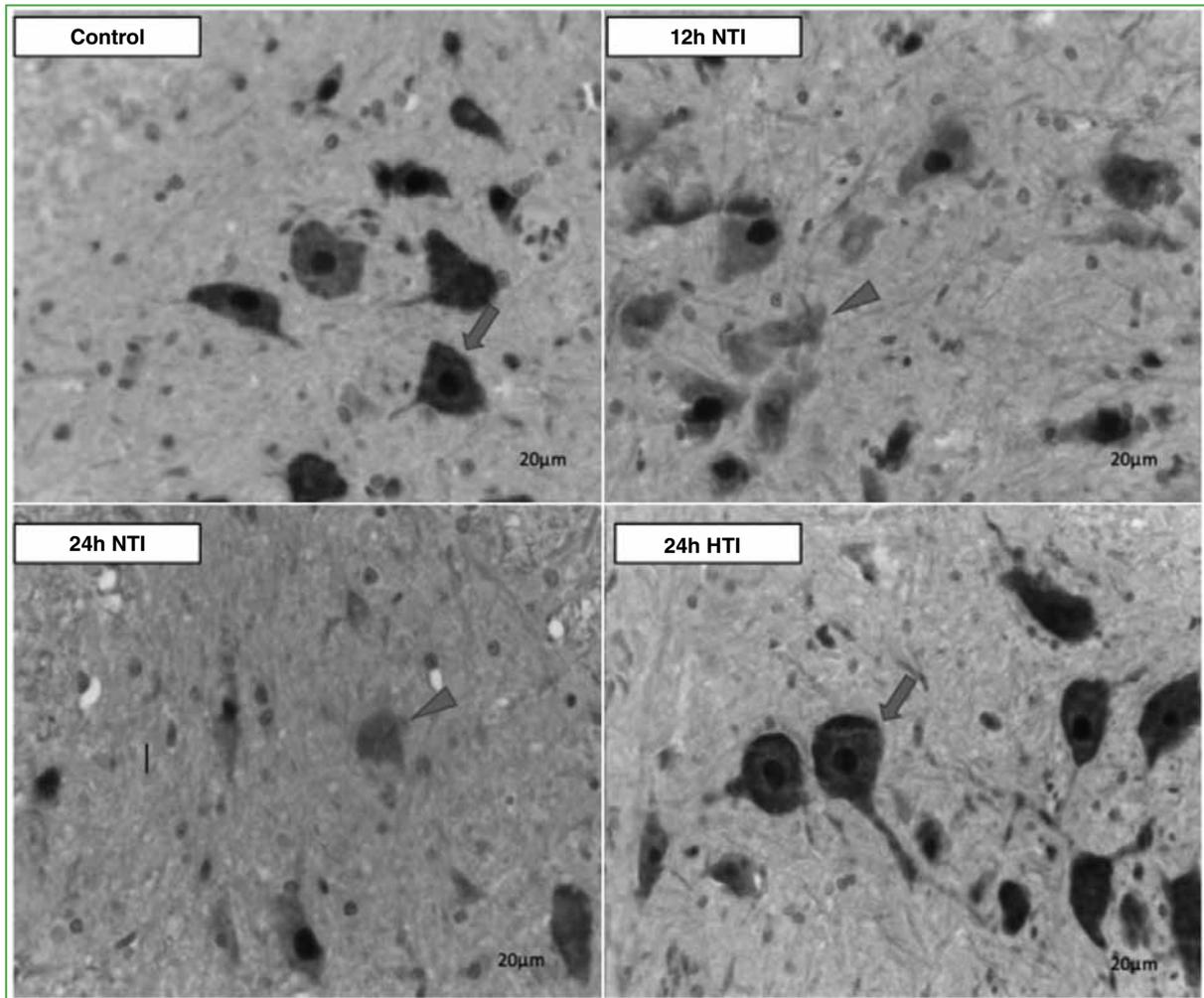
Figure 8. NeuN. Comparative slices. x40 magnification. NeuN staining with marked nuclear intensity can be observed in the control and 24 h HT injury groups, as well as moderate staining in the cytoplasm of motor neurons (arrows). Whereas, in the groups with 12 h and 24 h NT injury, neurons in the process of death are visualized (arrowhead).

DISCUSSION

Animal models of spinal cord injury are currently being developed to ensure that they are reliable, consistent, and that they reproduce the injuries seen in humans.[28,29] Questions remain as to their validity and whether they are comparable with clinical conditions in humans, which highlights the limitations of the article, particularly as only anterior horn motor neurons were evaluated. Some reviews have examined spinal cord injury models and their potential uses in mimicking this picture.[30,31]

Possible cold therapies to prevent neuronal damage after spinal cord injury are of two types: local and systemic. Local therapy has been used in the prophylaxis of spinal cord ischemia during aortic surgery. In this case, hypothermia by epidural cooling provides cytoprotection;[32] however, due to the complexity of the procedure, systemic

hypothermia is chosen.[33] Its risks include increased infection rates, renal insufficiency, necrosis of exposed areas, and scabies, so its management should be cautious. Evidence suggests using the latter when there is an acute spinal cord injury.[34]

CIRBP is considered a cytoprotective protein that accelerates cell recovery from stress.[35,36] Zhang et al. stated that it is a telomerase modulator at both 32 °C and 37 °C. CIRBP associates with the active telomerase complex through direct binding of the telomerase RNA component (TERC) and regulates the localization of telomerase in Cajal bodies.[37,38] The possible interaction of these bodies with small nuclear ribonucleoproteins could have implications for protection phenomena generated at the nuclear level. In 2019, Mingyue Liu (https://doi.org/10.1016/j.jtcvs.2018.08.100) described neuroprotective effects by reducing blood-brain barrier degradation in rats. In addition, Li-hui Chen published a study on hypothermia in 2013 (https://doi.org/10.21203/rs.3.rs-2628773/v1) and observed that overexpression of CIRBP in neurons could reduce OGD/R-induced (oxygen-glucose deprivation and reoxygenation) release of oxygen-reactive substances by reducing malondialdehyde levels and increasing superoxide dismutase and glutathione levels, reducing OGD/R-induced neuronal apoptosis by downregulating caspase-3 expression and upregulating Bcl-2 expression (known effects of CIRBP).

At the experimental level, systemic hypothermia has neuroprotective properties in cerebral and spinal cord ischemia.[39-41] Trials to perform it in experimental models generate great difficulties due to the costs and complexity of the systems.[42,43] Most have two drawbacks: invasiveness and costs.

In previous research, we have determined that brief exposure to cold induces the expression of CIRBP in the spinal cord of rats at the thoracic level (T8-T9-T10), its expression is significantly higher in animals exposed to hypothermia with different expression kinetics and time location post-intervention.[12] Based on this model, we move to a new stage where we evaluate the spinal cord injury and its cold treatment, measuring the expression of CIRBP and the number of viable neurons after injury.

The use of the MASCIS® impactor is proven to generate spinal cord contusion injury and is useful for various types of study, for example, that published by Colón et al., in 2017,[44] with the use of tamoxifen as post-injury treatment. In this study, rats were given a thoracic contusion (T10) using the impactor, and given placebo or tamoxifen granules (15 mg, for 21 days) at intervals of 0, 6, 12 and 24 hours. Euthanasia was performed 2, 7, 14, 28 or 35 days after injury to study molecular and cellular changes in acute and chronic stages. Immediate or deferred therapy (6 hours after injury) improved locomotor function and increased white matter tissue integrity and neuronal survival.[44] Although our study was not focused on chronic stages, the immediate outcomes (24 hours) of administering cold after moderate contusion were encouraging.

Research on the pathophysiological mechanisms of spinal cord injury has led to a classification scheme of primary and secondary injury.[45,46] The secondary type is divided into acute phase (0 to 48 h), subacute phase (2 weeks) and chronic phase (following the subacute phase and it can last years).[47] It is important to highlight and remember the authors that have made this difference, since the post-traumatic therapeutic action must be based on the treatment of secondary injury mediated by the inflammatory cascade, cytokines, proapoptotic proteins, as well as the causes associated with the relative hypoxia generated by multiple factors, such as the reduction of systemic blood pressure due to shock (may be due to pain, or neurogenic, or hypovolemic, or a combination of all), hypoxemia due to reduction of ventilation generated by states of coma or due to the reduction in excursion of the thoracic cage in inspiration and expiration due to pain.[48]

The caspase family regulates the execution of mammalian apoptosis. Caspase-3 cleaves several essential downstream substrates involved in the expression of the apoptotic phenotype *in vitro* (gelsolin, PAK2, fodrin, nuclear lamins and the inhibitory subunit of DNA fragmentation factor). Caspase-3 activation *in vitro* can be triggered by upstream events, leading to cytochrome c release from the mitochondria and subsequent activation of procaspase-9 by Apaf-1. Studies in rats showed that the upstream and downstream components of the caspase-3 apoptotic pathway are activated after contusive spinal cord injury and occur early in neurons in the injury site and hours or days later in oligodendroglia adjacent to and distant from the site of injury.[49] In our study, we saw the presence of necrosis and apoptosis mediators, such as caspase-3, whose manifestation is greater in groups with 12 h and 24 h NT injury, their reduced presence in the group with 24 h HT injury is statistically significant, which, in part, would be explained by the fact that cold and, in particular, CIRBP intervene in both apoptosis and cell death processes by blocking them.

CIRBP works by blocking several of these inflammatory cascades, cell death and apoptotic pathways. Although we did not attempt to mark all of these pathways in this study, we did observe that there are more viable neurons (immunopositive NeuN) in the HT group than in the NT groups, which is similar to those injured at 12 h versus 24 h in normothermia, indicating that this protein has most likely inhibited these cascades.

As limitations of the study, we must clarify that it was conducted with a small number of animals in order to adapt the research to the standards of good use and care of laboratory animals. Therefore, this research should be continued, increasing the sample size, as well as testing in other rat breeds and animal models, in order to translate this research into medical practice in the future.

GENERAL CONCLUSION

The use of cold therapy was associated with increased CIRBP expression and decreased death of motor neurons in the anterior horn of the spinal cord of injured rats. This encourages further research into cold therapy in spinal cord contusion injuries.

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