Expression of Cold-Inducible Proteins in Rat Spinal Cord Subjected to Systemic Hypothermia

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ABSTRACT

Introduction: Traumatic spinal cord injury is the main cause of motor disability in developed and underdeveloped countries, being a priority interest to the WHO. The effect of hypothermia on the expression of CIRBP (cold-activated protein) in the anterior grey column of 60-day-old male albino Sprague-Dawley rats was studied at the structural and biochemical levels and proposed as a possible therapeutic approach. **Materials and Methods:** 24 rats were randomly divided into two groups; normothermia (n = 6), at 24º C, and hypothermia, (n = 18) at 8º C for 180 minutes and euthanized at 12, 24, and 48 h post-treatment. Western blot and immunohistochemistry for CIRBP were used. **Results:** A progressive increase in the expression of CIRBP was observed from 12 to 48 hours, with statistically significant values after 24 and 48 hours compared to controls. **Conclusion:** This experimental model demonstrated efficacy, accessibility, and economy to generate systemic hypothermia, which provides a novel range of therapeutic strategies. The increase in the expression of cold-inducible proteins in the rats' spinal cords allows us to study the benefit of hypothermia at the molecular level for the first time, being of utmost importance for therapeutic studies in spinal cord injuries.

Keywords: Hypothermia; spinal cord; CIRBP; rat.

Level of Evidence: I

Expresión de proteínas inducibles por frío en la médula espinal de rata sometida a hipotermia sistémica

RESUMEN

Introducción: La lesión traumática de la médula espinal es la principal causa de discapacidad motora en el mundo, y representa una prioridad para la Organización Mundial de la Salud. Se estudió, a nivel estructural y bioquímico, el efecto de la hipotermia sobre la expresión de la CIRBP (proteína activada por frío) en el asta anterior de la médula de ratas Sprague-Dawley albinas macho de 60 días, planteándola como terapéutica posible. **Materiales y Métodos:** Se dividió a 24 ratas en dos grupos: normotermia a 24 ºC (n = 6) e hipotermia a 8 ºC (n = 18), durante 180 min, sacrificadas a las 12, 24 y 48 h después del tratamiento. Se utilizó Western blot e inmunohistoquímica para la CIRBP. **Resultados:** Se observó un aumento progresivo de la expresión de la CIRBP de 12 a 48 h en las motoneuronas del asta anterior. Los valores fueron estadísticamente significativos entre los grupos de 24 h y 48 h comparados con los de los controles. **Conclusiones:** Este modelo experimental resultó eficaz, accesible y económico para generar hipotermia sistémica y abre un abanico de estrategias terapéuticas. El aumento en la expresión de las proteínas inducibles por frío en la médula espinal de ratas permite, por primera vez, estudiar el beneficio que aporta la hipotermia a nivel molecular, lo que resulta de suma importancia para estudios de terapéuticas en las lesiones medulares.

Palabras clave: Hipotermia; médula espinal; CIRBP; rata.

Nivel de Evidencia: I

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INTRODUCTION

In the world, about 500,000 people suffer spinal cord injuries each year; this increases between two and five times the probability of dying prematurely. Low- and middle-income countries have the worst survival rates. The direct lifetime cost ranges from $1.1 million to $4.6 million per patient. In North America, there are 1.3 million patients who generate a global expense of between 1.43 and 5.98 billion dollars (*National Spinal Cord Injury Statistical Center* 2014/2021).[1] The National Rehabilitation Service of Argentina published that there were 2,176,123 patients with some type of disability, of which 54% were men, and 30% had a pure motor disability (*Anuario Estadístico sobre Discapacidad, Ministerio de Salud de la Nación Argentina*, 2013).[2] This problem generates the need for new therapeutic strategies to reduce the sequelae of patients with spinal cord injuries. Within them are laboratory studies on hypothermia.

In our laboratory, we have applied therapeutic hypothermia to reduce central nervous system damage produced in an experimental model of severe perinatal asphyxia, with encouraging results in spinal and ocular disease,[3-10] and in models of optic nerve trauma.[11] The reduction of body temperature protects the nervous system and the retina against damage of various kinds by decreasing metabolism by reducing reactive oxygen species and inhibiting the toxic release of nitric oxide.[3,5,8,12-14] Hypothermia during perinatal asphyxia has preventive effects against damage to the central nervous system[3,5,13,15] and has been proposed as a neuroprotective treatment in humans.[13,15-17]

In terms of spinal cord injury, the literature shows that relatively mild levels of hypothermia after injury improve function and reduce histopathologic damage.18-22 The intrinsic processes by which cold produces tissue protection remain unknown. However, the expression of different cold-inducible proteins has been described, such as cold-inducible RNA binding protein (CIRBP) and RNA binding motif protein 3 (RBM3)[7,9,11,23-28] that could be of great relevance.

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.[29] As with other RNA-binding proteins, CIRBP is capable of modulating apoptosis, playing an antiapoptotic role in hypothermic situations.[30,31] In rat neuronal cells, this effect seems to occur through the mitochondrial apoptosis pathway, since they show a decrease in the expression of proapoptotic molecules (Bax, Bad, Bak, Cycs, Apaf1, caspase-9, and caspase-3).[30] The exhaustive study of cold-inducible proteins (CIRBP and RBM3) could open a new horizon regarding their relationship with tissue protection mechanisms.

Despite the great potential applications of hypothermia, there is an inherent problem: the difficulty of applying cold to specific organs or regions of the body due to their ability to self-regulate temperature.[32] To apply hypothermia to internal organs, such as the brain, it is necessary to cool the blood with an external circulation system,[33] without forgetting that hypothermia can cause adverse side effects, such as a decreased immune response or renal failure.[34]

Based on previously published experience,[10,28,35] we decided to apply our simple and inexpensive experimental model, useful in in vivo assays, for spinal cord cooling and to study the expression of CIRBP at the structural level by means of immunohistochemistry and immunofluorescence, and at the molecular level using Western blot in the spinal cord of rats exposed to cold.

Considering everything previously described, the objective of this study was to evaluate the expression of CIRBP in motor neurons of the anterior horn of the spinal cord of rats exposed to this model of systemic hypothermia.

MATERIALS AND METHODS

Model and experimental design

All procedures related to the handling and treatment of animals were carried out under the *National Institutes of Health Guidelines for the Care and Use of Laboratory Animals*-CCAC 2002; CCAC 2003 and the *Society for Neuroscience* guidelines, 1992. The experimental protocol was reviewed and approved by the CICUAL Committee *(Comité Institucional para el Uso y Cuidado de Animales de Laboratorio*), Provision No. 970/2015 (*Universidad de Buenos Aires, Argentina*).

Twenty-four 60-day-old adult male Spargue-Dawley rats were used, divided into two groups: group 1, normothermia (control, n = 6) at a controlled temperature of 24 ºC and group 2 (hypothermia, n = 18) in a cold room at 8°C for 180 min. After hypothermia, they were placed in individual boxes and kept under standard animal facility conditions at 24 ºC, with light/dark cycles of 12/12 h, until euthanasia by guillotine decapitation, as described by Contartese et al.[35] and Larrayoz et al.[28]

Obtaining and processing the tissue

The animals were euthanized at 12, 24 and 48 h after treatment. The marrows were extracted and immediately submerged in a 4% paraformaldehyde fixation solution in 0.1 M phosphate buffer, pH 7.4, at 4 ° C for 2 h and then transferred to a sucrose solution in increasing concentrations of 10%, 20%, and 30% in 0.1 M phosphate buffer at 4 °C, for three successive days.

Hematoxylin & eosin

To study the structural alterations of the paraformaldehyde-fixed marrows, they were dehydrated with increasing concentrations of ethanol followed by xylene and embedded in paraffin. 5-10 µm thick sections were obtained using a microtome (Leitz, Lauda MGW, Germany), placed on gelatinized slides, and stained with hematoxylin-eosin. Finally, the slides were mounted with DPX synthetic resins (Fluka®, Switzerland). The 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 (developed by Wayne Rasband, 1995, NIH, Research Services Branch, NIMH, Bethesda, MD, USA). Three animals per experimental group were analyzed.

Immunohistochemistry

The fixed and cryoprotected marrows were embedded in Tissue-tek TEC®, frozen at –70 °C, and cut into 20 µm thick sections with a Leitz Lauda cryostat. The sections were mounted on slides previously coated with 1.5% gelatin. The medullary sections were incubated overnight at 4 ºC with rabbit polyclonal anti-CIRBP antibody (Proteintech, UK, Cod.00055668) at a 1:1000 dilution, followed by a biotinylated anti-rabbit secondary antibody (Vector Labs, USA). The third layer was incubated for one hour with the streptavidin peroxidase complex (ABC Complex, Vector Labs, USA). 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 with 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]

Immunofluorescence

Rabbit polyclonal anti-CIRBP primary antibody (Proteintech®, UK, Cod.00055668) was used at a 1:1000 dilution, which was detected with Alexa Fluor® 488 donkey anti-mouse IgG secondary antibody (Life Technologies, USA, Lot: 1423052) in a 1:300 dilution. DAPI (4',6-diamino-2-phenylindole, Sigma-Aldrich, USA) was used as a specific nuclear marker at a dilution of 1:1000. Finally, the sections were evaluated and photographed with a Nikon C1 Plus confocal laser microscope (Nikon Inverted Research Microscope Eclipse Ti, Nikon Corp., Tokyo, Japan) and the images were analyzed with the EZC1 program (EZ-C1 v3.9 Software, Nikon Ltd., Tokyo, Japan). Three animals per experimental group were analyzed.

Tissue processing for Western blot

The procedure described by Rodrigo et al.[36,37] was followed. Homogenate supernatants of protein concentration determined by the Bradford method were used. Electrophoresis was carried out under denaturing conditions on SDS polyacrylamide gels. The bands were transferred at 350 mA for 90 min onto PVDF membranes. After the transfer, the membrane was blocked with a solution of TBS with Tween 20 at 0.05% v/v (TBST) containing skimmed milk powder at a concentration of 3% w/v (TBST-milk) for 1 h under gentle agitation. The blocking solution was discarded and the membrane was incubated with a rabbit anti-CIRBP antibody (Proteintech®, United Kingdom) diluted 1:3000 in TBST-milk for 16 h, under gentle agitation at 4 °C. After three washes with TBST under gentle agitation for 10 min each, it was incubated with an anti-rabbit IgG antibody (Amersham

Pharmacia Biotech, USA) conjugated with horseradish peroxidase diluted 1:5000 with TBST-milk for 1 h, under gentle agitation. Washes were carried out with TBST and then TBS under gentle agitation for 10 min each; then, the images were developed with the ECL™ detection kit (Amersham™) and the bands were visualized with a 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-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) in a 1:5000 dilution, for 16 h under gentle agitation at 4 °C. After three washes with TBST under gentle agitation for 10 min each, it was incubated with a goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Jackson Immuno Research Laboratories, Pennsylvania, USA) diluted 1:5000 with TBST-milk for 1 h under gentle agitation. As mentioned before, washings and development were performed.

Image analysis

The images obtained in microscopy and Western blot were digitized. For both immunohistochemistry and immunofluorescence, the spinal cord sections were selected so that they were comparable between the different animals. For the same technique, all images were taken on the same day, under standardized capture conditions, to avoid the effect of external variability. Relative optical density, cell area, reactive area, and thickness were determined with an image analysis program (ScionImage). A label was considered immunopositive when its optical density exceeded 4 times or more the optical density of the background. For the digital adjustment of the brightness and contrast of the images, the image editing program Adobe Photoshop (Adobe Photoshop CS5, Adobe Systems Inc., Ontario, Canada) was used. The CIRBP values obtained in the Western blots were normalized according to the corresponding ß-actin value and the results were expressed as the mean value of the group ± the 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 of $p < 0.05$ was considered statistically significant.

RESULTS

Western blot

As a result, a single band with a molecular weight of 19 kDa was obtained, indicating the specific expression of CIRBP in the spinal cords of the rats studied (Figure 1). CIRBP expression was significantly increased in hypothermic rats euthanized at 24 h compared to control rats ($p < 0.05$, one-way ANOVA with Tukey's test); however, no significant differences were observed in hypothermic animals at 48 h compared to the other groups studied.

Morphology

We obtained images of the anterior grey column corresponding to Rexed laminae VII, VIII, and IX, and we analyzed the alpha motor neurons both with classical hematoxylin-eosin histology and by immunohistochemistry and immunofluorescence.

Hematoxylin & eosin

In the marrow section of the control rats (Figure 2), the anterior grey column and the surrounding white matter were observed with the motoneurons corresponding to the gray matter, which measure between 30 and 70 µm in diameter, among them, there are small interneurons, such as Renshaw cells. The same area was evaluated both by immunohistochemistry and immunofluorescence (Figures 3 and 4).

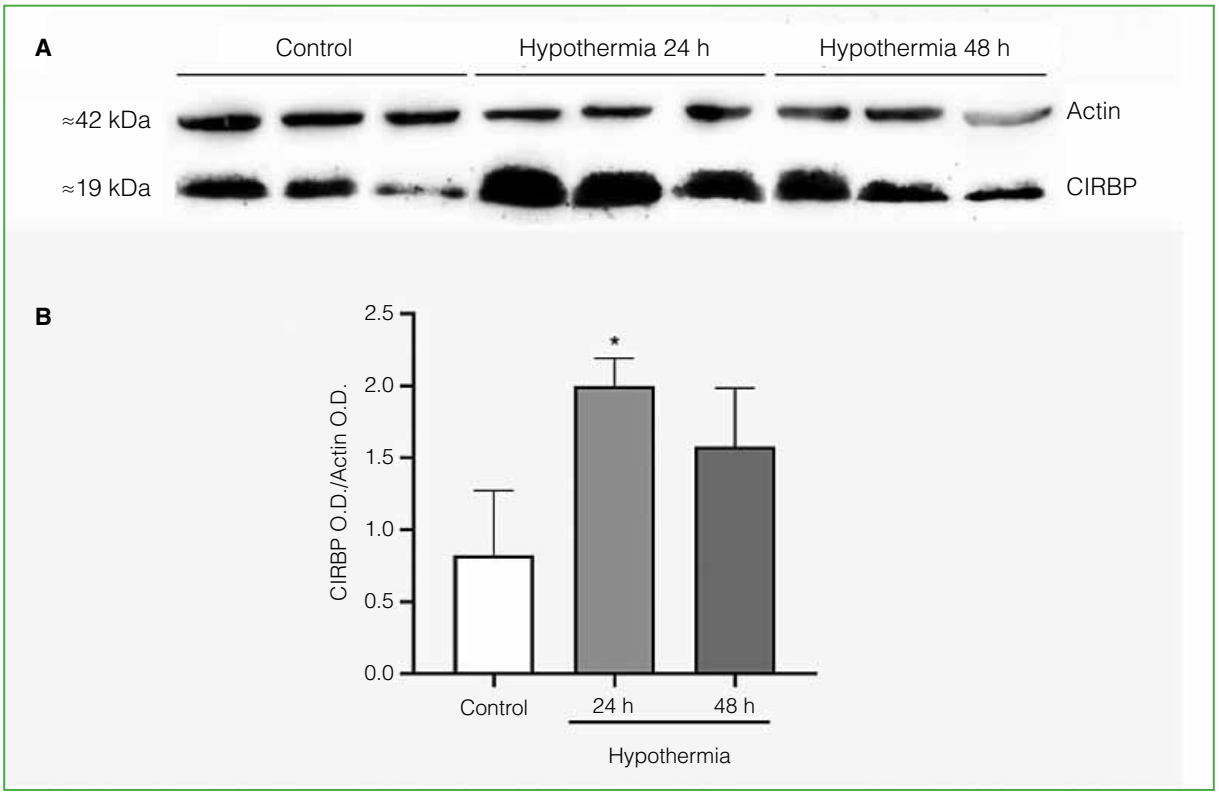

Figure 1. Anti-CIRBP and anti-actin Western Blot of spinal cord homogenates (**A**) from three rats from the control group and from the hypothermia group 24 h and 48 h after hypothermia. By semi-quantification of the bands (**B**) with densitometry and subsequent analysis with the one-way ANOVA test with Tukey's test, a significant difference was observed between the hypothermic group at 24 h and the control group (*p <0.05).

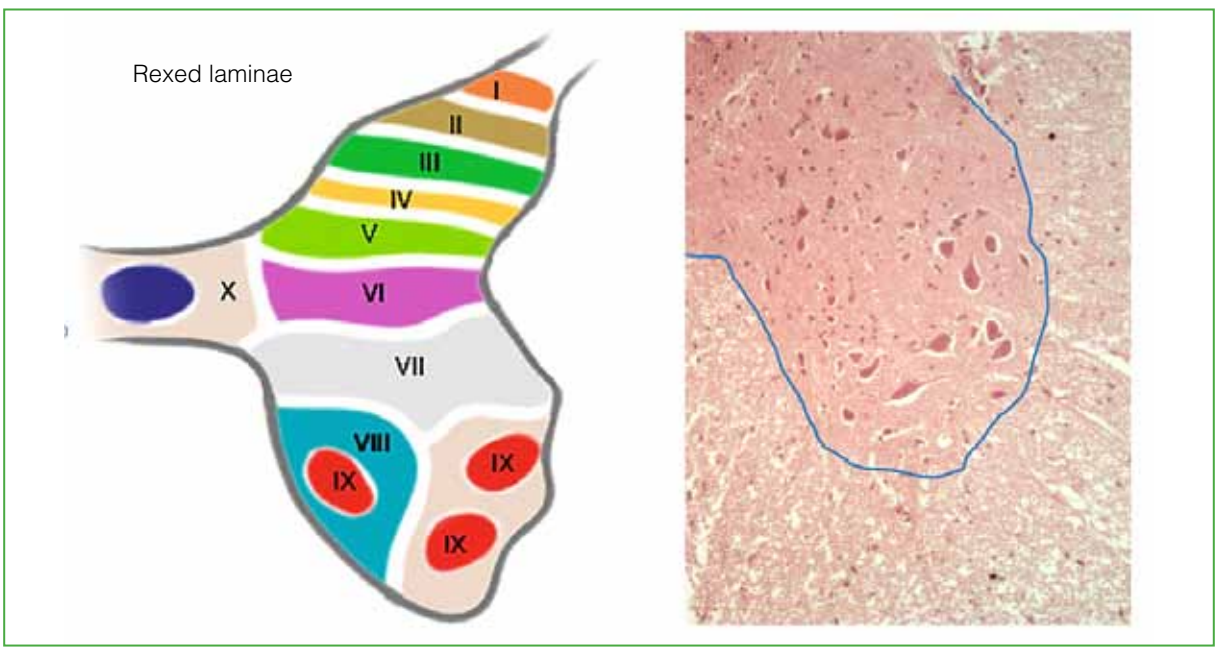

Figure 2. Spinal cord sections stained with hematoxylin & eosin technique. Magnification of the anterior grey column corresponding to Rexed laminae VII, VIII and IX, to observe the alpha motor neurons in detail.

Immunohistochemistry

In some sections of the control group, the antibody to CIRBP showed no label and, in others, there was a weak label in the neuropil (Figure 3). In the spinal cords of hypothermic rats, at 12 h post-treatment, mild CIRBP-specific immunoreactivity was observed in the nucleus of ventral horn alpha motor neurons, whereas the 24-h and 48-h post-hypothermic groups showed a large increase in the expression of CIRBP both in the nucleus and nucleolus, and in the cytoplasm (in the form of "lumps"), of these cells (Figure 3).

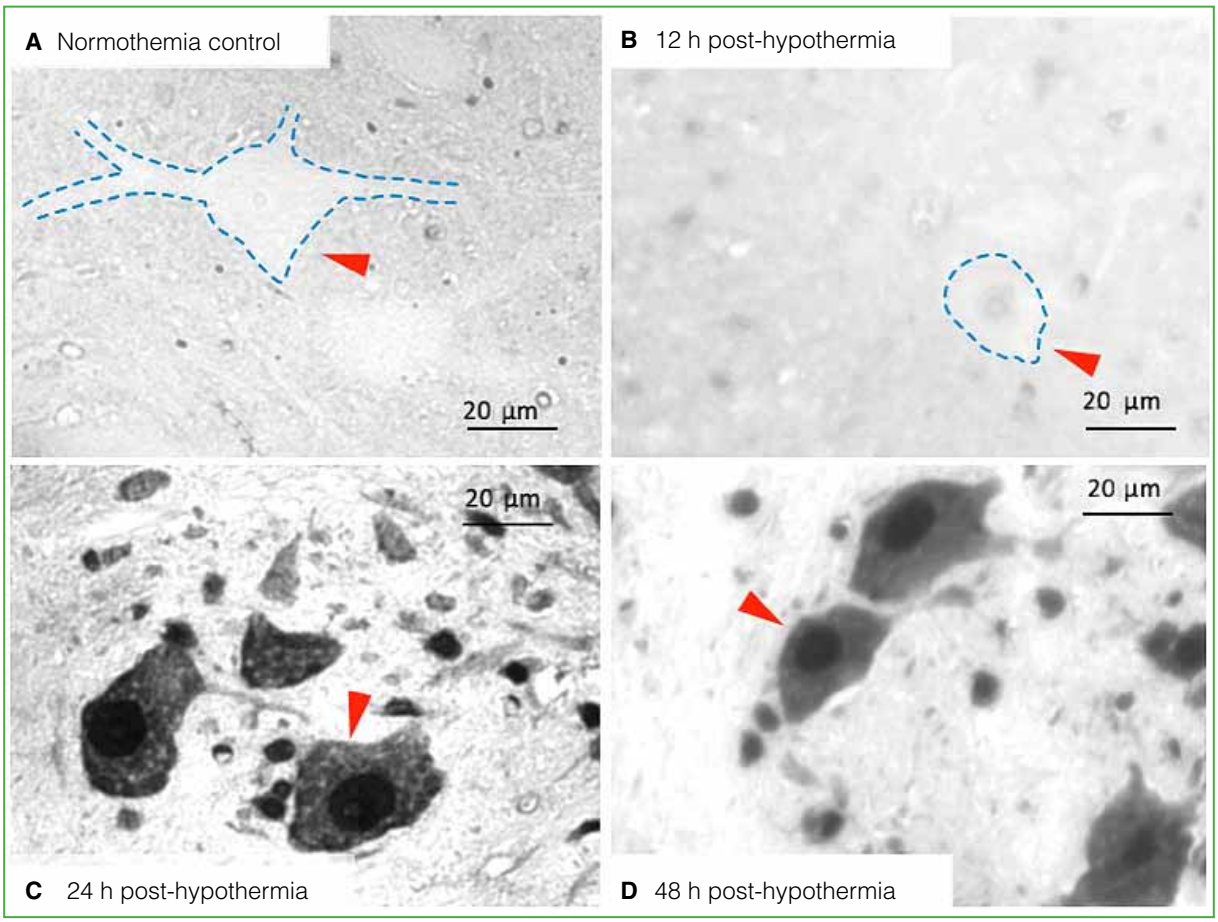

Figure 3. CIRBP immunohistochemistry. Comparative sections with x40 increase of the times between the control and hypothermic groups, of the anterior grey column. Magnification of Rexed lamina IX, motor neuron detail (red arrows). The absence of labeling or weak labeling is observed in the neuropil of the normothermic group, for which the contour of the motor neuron is indicated with a dotted line (**A**). It was slightly higher in the nucleus and nucleolus at 12 h of hypothermia (**B**) and very intense in the nucleus and cytoplasm of the motoneuron at 24 h (**C**) and 48 h (**D**) post-hypothermia.

Immunofluorescence

The results of this technique coincided with those observed in immunohistochemistry (Figure 4).

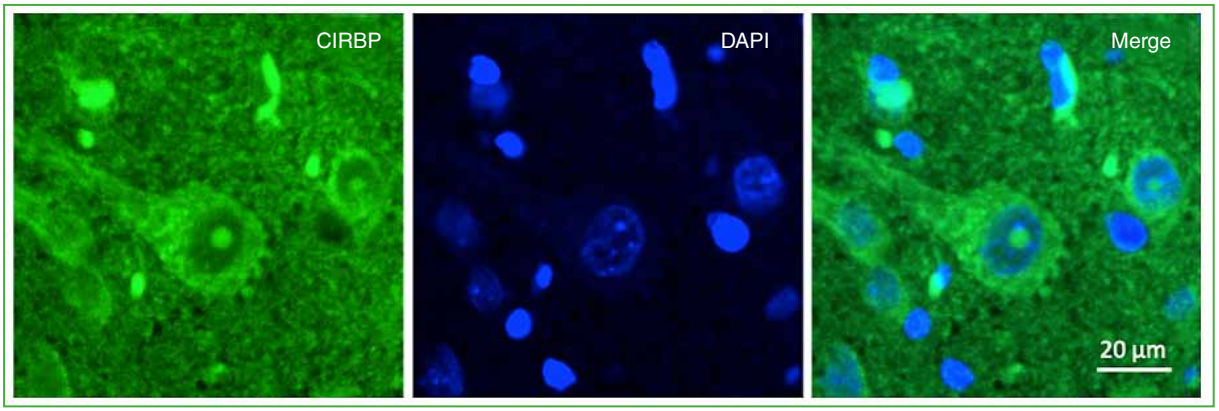

Figure 4. Immunofluorescence. Photograph of the alpha motor neuron of the anterior grey column after hypothermia, corresponding to Rexed lamina IX. CIRBP (green) and DAPI (blue). An increase in cytoplasmic fluorescence is observed in the form of a 'lump' with nucleolar labeling.

Image analysis

Immunohistochemistry with light microscopy revealed significant differences in CIRBP expression with hypothermic treatment. A progressive increase in optical density was determined for CIRBP, which was scarce in normothermia, slight at 12 h post-hypothermia in the nucleus, and increased in the nucleus and at the cytoplasmic level at 24 h and 48 h (Figure 5).

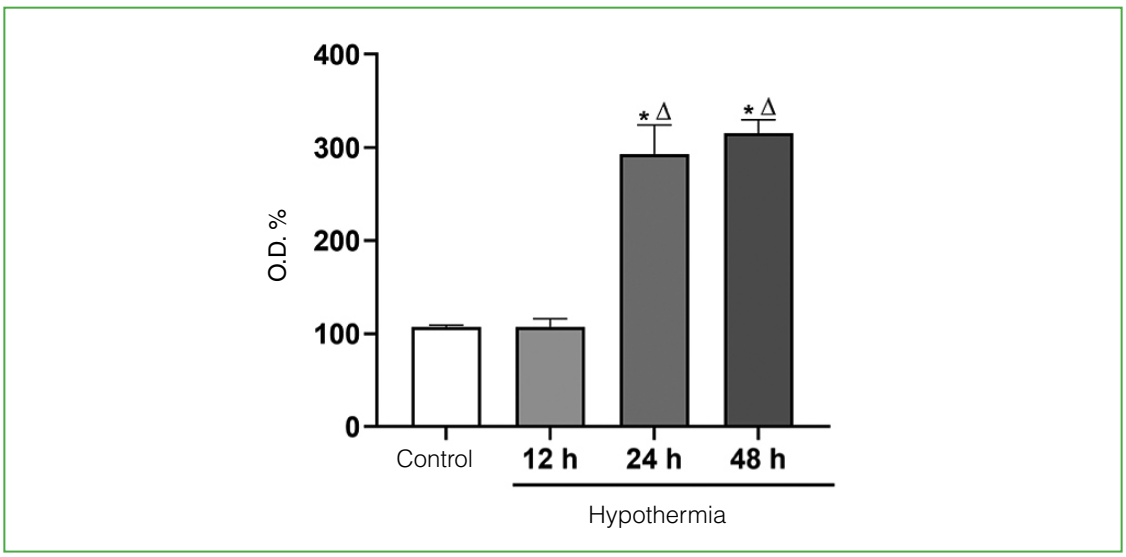

Figure 5. Immunohistochemistry with anti-CIRBP antibodies of spinal cord tissue sections of three rats from the control group and the hypothermic group at 24 h and 48 h. As observed in the microphotographs, there is a progressive increase in CIRBP immunolabeling. It is minimal in control animals, while in the group of animals treated with hypothermia, it ranges from mild at 12 h to moderate/intense at 24 h and 48 h post-hypothermia. The quantification of the bands with densitometry and the subsequent analysis with one-way ANOVA with Bonferroni test evidenced a significant difference between the control group and the groups of rats treated with hypothermia at 24 h and 48 h (*p <0.001), and between the group of hypothermic rats at 12 h and the groups of rats treated with hypothermia at 24 h and 48 h (*p < 0.001).

The statistical evaluation of the relative optical densities showed that the groups have differences, particularly after 24 h post-hypothermia, where the concentration increases, especially at the nuclear level, and then at 48 h, it is intensely observed in the cytoplasm. in the form of lumps ($p < 0.001$, one-way ANOVA with Bonferroni test) (Figure 5).

DISCUSSION

Currently, there is no cure for spinal cord injury. For this reason, models of spinal cord injury in animals are continually being developed that are reliable, consistent and that reproduce the injuries observed in humans.[38-40] Questions remain about their validity and whether they are comparable with clinical conditions in humans. Some reviews have examined spinal cord injury models and their potential uses in mimicking this condition.41-43 Possible cold therapies, proposed to prevent neuronal damage from 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 through epidural cooling provides cytoprotection,44 but due to the complexity of the procedure, systemic hypothermia is chosen.[45] Evidence suggests using the latter when there is an acute spinal cord injury.4CIRBP is considered a cytoprotective protein that accelerates cell recovery from stress.47,48 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 the Cajal bodies.30,49 The possible interaction of these bodies with small nuclear ribonucleoproteins could have implications for protection phenomena generated at the nuclear level, a topic that is currently being studied in detail.

Systemic hypothermia trials in experimental models generate great difficulties due to costs, the complexity of the systems and the management of circulating water coolers,[50] induced coma in a cold room,[51] or even transrectal systems that require several hours. Various models have been developed, but most have two drawbacks: they are invasive and expensive.

In our laboratory, we have determined that brief exposure to cold ("hypothermic shock") induces the expression of CIRBP and RBM3 in the retina. We have observed a significant increase in CIRBP at the nucleotide level 6 h after hypothermia. For this reason, we used groups subjected to hypothermia starting at 12 h.[7,9,11] We were able to observe the presence of CIRBP in the controls, which was considered as the basal expression of the nervous tissue of the anterior horn of the spinal cord. . We obtained a slight increase in CIRBP expression at 12 h post-hypothermia. The phenomenon is observable in the comparative evaluation of densities that was carried out both in Western blot and in immunohistochemistry preparations. We also observed a different location of the immunofluorescent label in the animals at 12 h post-hypothermia (nucleolus and cytoplasm), where an apparent nucleolar label and the appearance of cytoplasmic "lumps" positive for CIRBP could be seen. In the initial times, the label increased in the nucleolus and the perinuclear cytoplasm, then decreased at the nucleolar level, but increased in the cytoplasm in the form of lumps, and so did the expression in the neuropil. The results presented are somewhat similar to those previously published by our laboratory in retinal studies, where the increase in CIRBP and RBM3 in their various layers is observed at 12, 24 and 48 h after the application of hypothermia.[10,27]

In summary, the presence of CIRBP in the spinal cord at the thoracic level (T8-T9-T10) is found both in animals at room temperature and in animals exposed to hypothermia. Its expression is significantly higher in animals exposed to hypothermia with different expression kinetics and localization between 12, 24, and 48 h after the intervention.

To date, no description of the protein expression or the localization of this protein in the spinal cord of hypothermia models has been published, which confirms the originality of the task that we are carrying out. In this model, the effects of cold and the expression of cold-inducible proteins in the short and medium term were observed for the first time, and this could be useful for the study of bone marrow pathologies, providing valuable information on the pathophysiological mechanisms that are triggered in the normal patient and the one subjected to hypothermia.

Based on what was observed and developed in our laboratory with this model, we can affirm its usefulness to produce systemic hypothermia from the exposure of animals to moderate cold (8 ºC of room temperature) that decreases body temperature by around 2 ºC, generating biochemical changes with a sufficient stimulus to increase the expression of CIRBP.

The advantages of the model are that it is non-invasive and produces a state of global, transient, and reversible hypothermia, reproducing what happens in situations of exposure to cold. Finally, the cost of this system and its complexity are extremely low, only a cold room (refrigerator) at 8 °C, a cage, and a timer are required.

GENERAL CONCLUSION

The presented systemic hypothermia model stimulates the increase of CIRBP in the spinal cord of adult male rats, expressing itself histologically and molecularly.

In memory of my father, Dr. Luis Aníbal Sarotto, 1935-2022.

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