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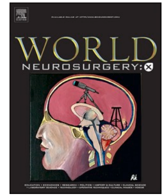

Evaluation of the Chetomin effect on histopathological features in a murine acute spinal cord injury model

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ABSTRACT

Background: Several research studies have been focused on improving the treatment and prognosis of acute spinal cord injury, as part of this initiative we investigated the use of Chetomin to reduce the inflammatory response in this pathology.
Methods: An experimental, prospective, cross-sectional study was performed using 42 Wistar rats where we analyzed the effect of Chetomin compared to methylprednisolone administered 1 and 8 h after the spinal cord injury in a murine model.
Results: Chetomin administration 8h post-injury decreased IL-6 and VEGF expression; and, and its administration 1h post-injury decreased NF-kB expression.
Conclusions: Chetomin has anti-inflammatory effects in acute spinal cord injury, whether these effects are observable with other proinflammatory markers should be investigated.

Abbreviations list

Abbreviation	Definition
3-NT	Monoclonal Nitrotyrosine
ANOVA	Analysis of variance
BSB	Blood spinal barrier
Chetomin	Dithiodiketopiperazine
CD40L	CD40 Ligand
CNS	Central nervous system
CX3CL1	Neuronal chemokine Fractalkine
CXCL12	Motif Chemokine Ligand 12
DMSO	Dimethyl sulfoxide
DUSP19	Dual Specificity Phosphatase 19
EZH2	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
FASL	Fas ligand
H&E	Hematoxylin and eosin stain
HIF-1α	Hypoxia-induced factor 1 alpha
HSP70	Heat-shock-protein 70
ICAM-1	Intracellular adhesion molecule 1
IFN-1b	Interferon beta 1 b
IFN γ	Interferon gamma
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
I.M.	Intramuscular

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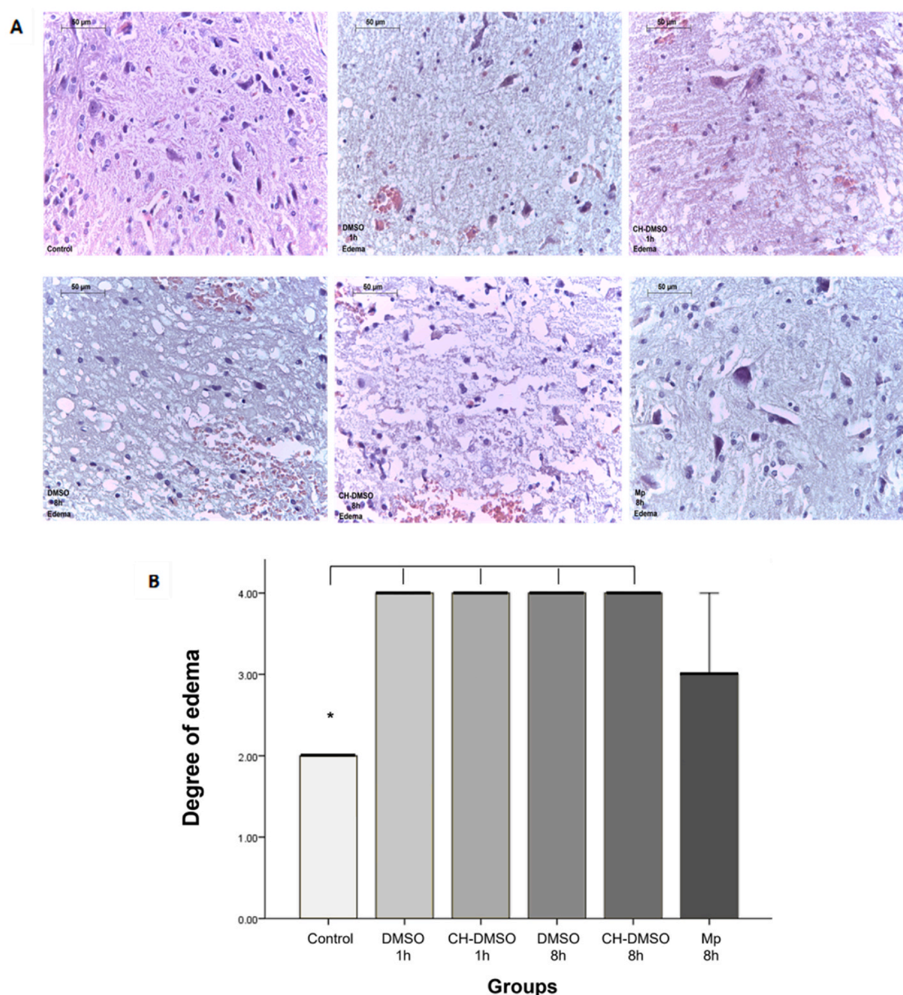

Fig. 1. A) Representative histological sections using H&E staining at 40x of the spinal cord which show the different degree of edema in all groups, it can be appreciated a section of the spinal cord from each group. B) Semi quantitative analysis, the results plotted using a Kruskal Wallis Test shows a significant difference between control group with DMSO 1h, CH-DMSO 1h, DMSO 8h and CH-DMSO 8h.

(*continued*)

Abbreviation	Definition
INCMNSZ	National Institute of Medical Sciences and Nutrition
iNOS	Nitric oxide synthase
Kg	Kilograms
L2	Lumbar vertebra 2
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
Mg	Milligrams
MP	Methylprednisolone
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa beta
NSAIDs	Non-steroidal anti-inflammatory drugs
ROS	Reactive oxygen species
RNS	Responsive neurostimulation
SC	Subcutaneous
SCI	Spinal Cord Injury
SE	Standard Error
SPSS	Statistical Package for the Social Sciences
T5	Thoracic vertebra 5
T8	Thoracic vertebra 8
T10	Thoracic vertebra 10
TGF-β	Transforming growth factor-beta
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-alpha
USA	United States
VCAM-1	Vascular adhesion molecule-1
VEGF	Vascular endothelial growth factor

1. Introduction

Acute spinal cord injury (SCI) is a traumatic condition with high morbidity and mortality. It is estimated that 12,500 new spinal cord injury cases are reported in North America every year, and men between 25 and 35 years of age are the most affected. Nearly 90 % of spinal cord injuries are caused by automobile accidents, violence, sports, or falls, affecting mainly young people.[1,2]

1.1. *Current medical management of spinal cord injury*

It is very important that these patients be carefully managed from the site of the trauma and their transport to a hospital center, as the elapsed time between injury and medical-surgical attention is fundamental.

The time that passes from trauma to medical-surgical attention is crucial in these cases, and it has been shown that performing pharmacological care within the first 8 h and spinal decompression during the first 24 h can be highly beneficial for the patient.[3]

Primary spinal cord injury directly destroys multiple tissue elements and causes neuronal death; in addition, it provokes a series of biochemical and cellular events that lead to the expansion of the lesion days or even months after the initial injury. This process is called secondary spinal cord injury and is characterized by three processes of cell

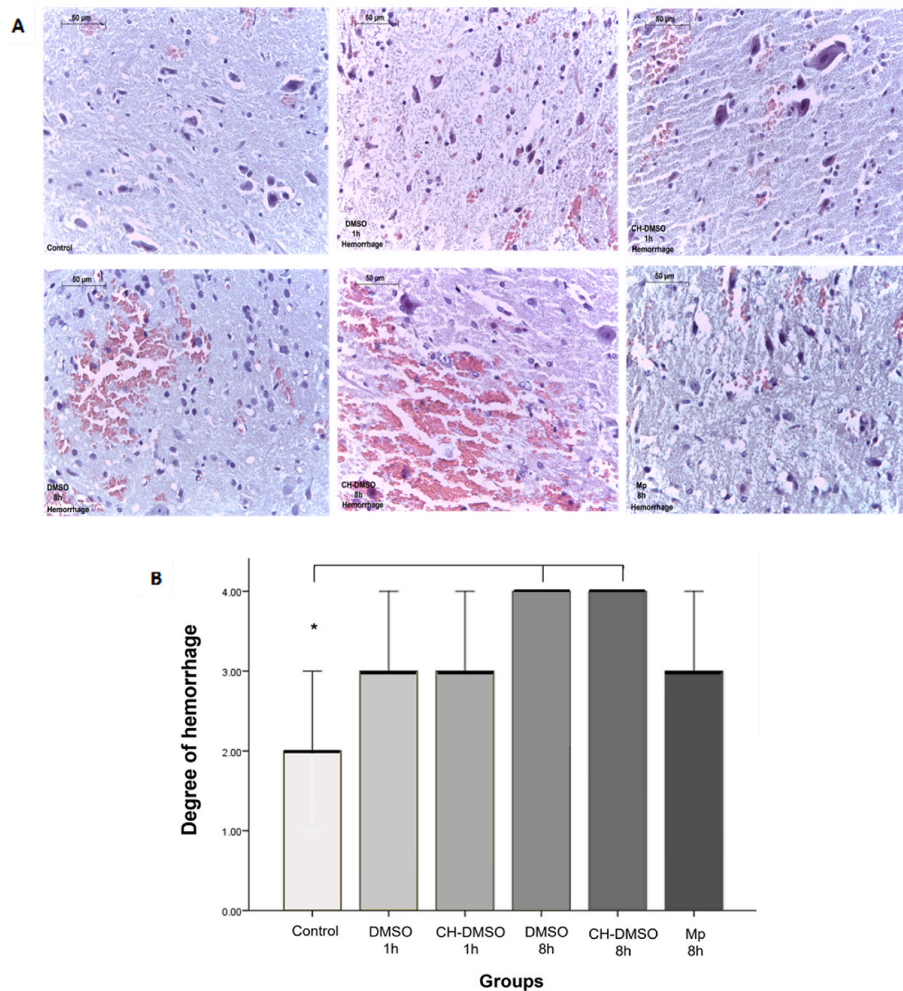

Fig. 2. A) Representative histological sections using H&E staining at 40x of the spinal cord that show the different degree of hemorrhage in all groups, it can be appreciated a section of the spinal cord from each group. B) Semi quantitative analysis, the results plotted using a Kruskal Wallis, $p < 0.05$. Test shows a significant difference between control group with DMSO 8h and CH-DMSO 8h.

death and irreversible tissue damage: necrosis, apoptosis, and atrophy.[4,5]

Administering drugs that help reduce the posttraumatic inflammatory process in the spinal cord can be very useful for reducing morbidity and mortality in these patients. The only drug routinely used in both humans and pets is methylprednisolone succinate, which can cause adverse effects.[3] Non-steroidal anti-inflammatory drugs (NSAIDs), such as naloxone, nimodipine and tirilazad mesylate have also been used and have not shown favorable results.[6–8]

1.2. Primary injury

The pathophysiology involves tumor necrosis factor alpha (TNF-α) and interleukin 1-β (IL-1β), which cause oligodendrocyte death by inhibiting glutamate transporters in astrocytes. Oligodendrocytes are thereby exposed to greater amounts of this neurotransmitter, causing excitotoxicity, apoptosis and neutrophil infiltration in the early stages of spinal cord damage.[9,10]

The response starts with the migration of microglia to the site of insult within minutes of injury, and the neuronal chemokine fractalkine (CX3CL1) acts via microglial CX3CR1 receptors to maintain microglial function in both health and disease. Molecules such as IFN-γ stimulate the release of cytotoxic mediators from microglia, including reactive oxygen species (ROS), arachidonic acid metabolites, and proinflammatory and anti-inflammatory cytokines.[11,12]

Astrocytes develop a hypertrophic phenotype, functioning to form a dense impenetrable glial scar around the lesion perimeter. Glial scar formation is thought to ward off neurotoxic events, preventing the spread of secondary degenerative processes and limiting immune cell entry into the damaged spinal cord. Although astrocytes are crucial for the activation of microglia, they can also paradoxically suppress the recruitment of microglia and T lymphocyte immune cells after injury via the production of IL-10 and IFN-1β.[13]

Neutrophils bind to blood−spinal barrier (BSB) proteins, such as vascular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), enabling infiltration into the damaged spinal cord. Once within the injury site, neutrophils phagocytose cellular debris via the production of oxidative and proteolytic enzymes, subsequently promoting the clearance of debris from the lesion epicenter and preparing the area for neuronal regeneration.[14]

The depletion of circulating neutrophils, inhibition of neutrophil-related proteolytic enzymes, and reductions in adhesion molecule levels have all been shown to confer significant neuroprotection after SCI.[15]

1.3. Secondary injury

Once secondary spinal cord injury progresses, there is an increase in local and systemic production of proinflammatory cytokines, which can be detected as elevated levels in cerebrospinal fluid minutes to hours

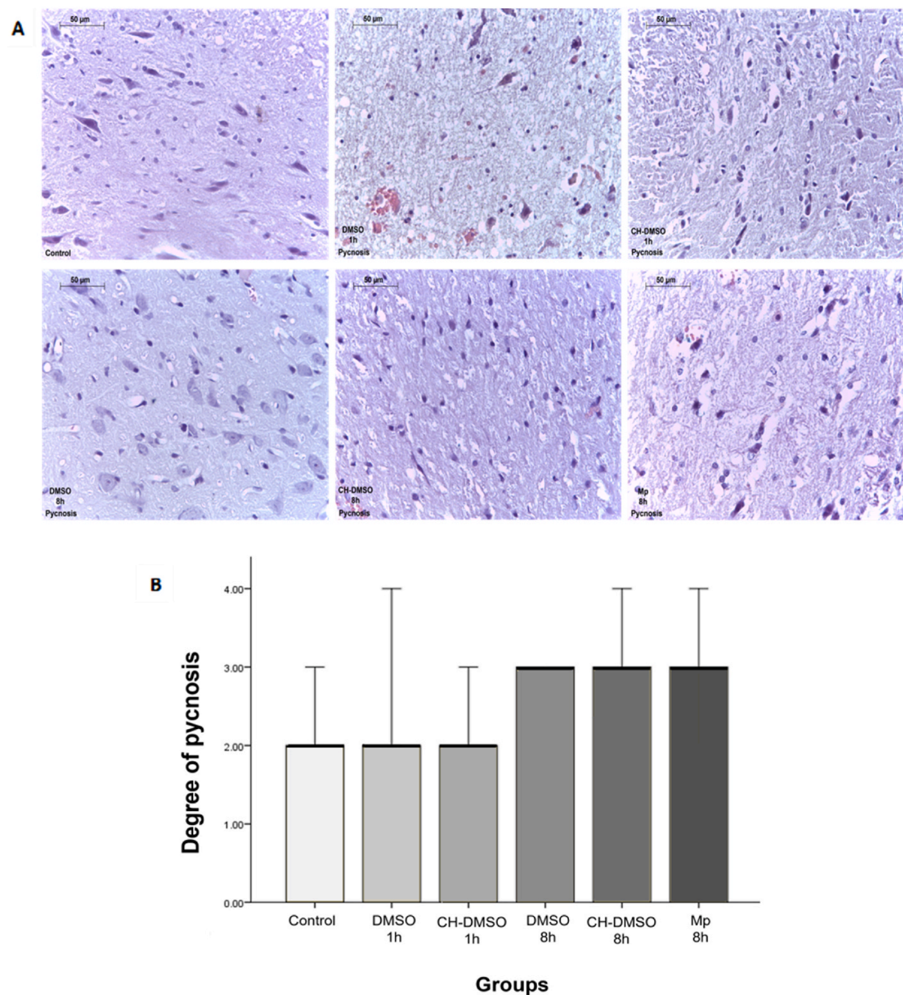

Fig. 3. A) Representative histological sections using H&E staining at 40x of the spinal cord that show the different degree of pyknosis in all groups, it can be appreciated a section of the spinal cord from each group. B) Semi quantitative analysis, the results plotted using Kruskal Wallis, $p < 0.05$. Test shows no significant difference was observed between groups.

after injury.

There are different molecular pathways of damage: TNF-α causes excitotoxicity through a series of interconnected deleterious mechanisms. First, the release of this cytokine by microglia causes glutamate release, which in turn leads to increased TNF-α production from 3 h after injury to 24 h after microglia are generated. Finally, the increase in the excitatory/inhibitory radius causes excessive calcium levels, leading to neuronal death by apoptosis.[10] In turn, chemotaxis with neutrophil infiltration occurs in the acute stages of spinal cord damage.

It has been reported that both TNF-α and IL-1β cause the death of oligodendrocytes, inhibiting glutamate transporters in astrocytes; this accumulation of glutamate is what causes the death of oligodendrocytes by excitotoxicity.[9] This cycle in which TNF-α is released causes an increase in glutamate, and there is an increase in the excitatory/inhibitory radius, increasing Ca^{++} levels and causing neuronal death by apoptosis.[10] The proinflammatory cytokine response seems to be regulated by nuclear factor kappa beta (NF-κB), the same factor that encodes different proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, which are regulated by hypoxia-induced factor 1α (HIF-1α).[16,17] HIF-1α is a transcription factor that regulates the expression of the NF-kB gene, which in turn regulates the expression of TNF-α, IL-1β, IL-6, and IL-12, among other proinflammatory cytokines.[18,19]

Microvascular disruption, ionic imbalance, increased intracellular calcium levels, glutamate excitotoxicity, mitochondrial dysfunction, arachidonic acid breakdown leading to proinflammatory cytokine expression, and inducible nitric oxide synthase (iNOS) activation contribute to ROS formation.[20]

1.4. HIF-1α and Chetomin

Dithiodiketopiperazine (Chetomin) is a metabolite obtained from the fungus Chaetomium that has antimicrobial effects. Systemic administration of Chetomin inhibited hypoxia-inducible transcription within tumors and inhibited tumor growth. Chetomin can indirectly inhibit the transcriptional activity of HIF-1α and inactivate apoptosis, activate autophagy, and promote axonal regeneration of microtubules within 28 days.[21] Chetomin is an indole alkaloid heterodimer that reflects an asymmetric dimeric structure linked at C3-N1′ and is coupled by two tryptophan fragments. This fully functional molecule contains 5 tetra substituted carbons and 6 estrogenic centers and has two dithiodiketopiperazine rings in its structure, which results in oxidation and reduction under acidic conditions.[22,23] At the molecular level, Chetomin binds and interrupts the tertiary structure of the CH1 domain of the p300 coactivator, preventing its interaction with HIF-1α,[22] thus attenuating the transcription induced by hypoxia and its relationship with HIF-1α. In addition, Chetomin can attenuate the inflammatory response (IL-6 and TNF-α release) and apoptosis via the regulation of Toll-like receptor 4 (TLR4) and HIF-1α in ischemia−reperfusion injury.[24]

Chetomin has a half-life of 4 h, coinciding with the maximum peak intratumoral concentration. There is evidence that Chetomin penetrates

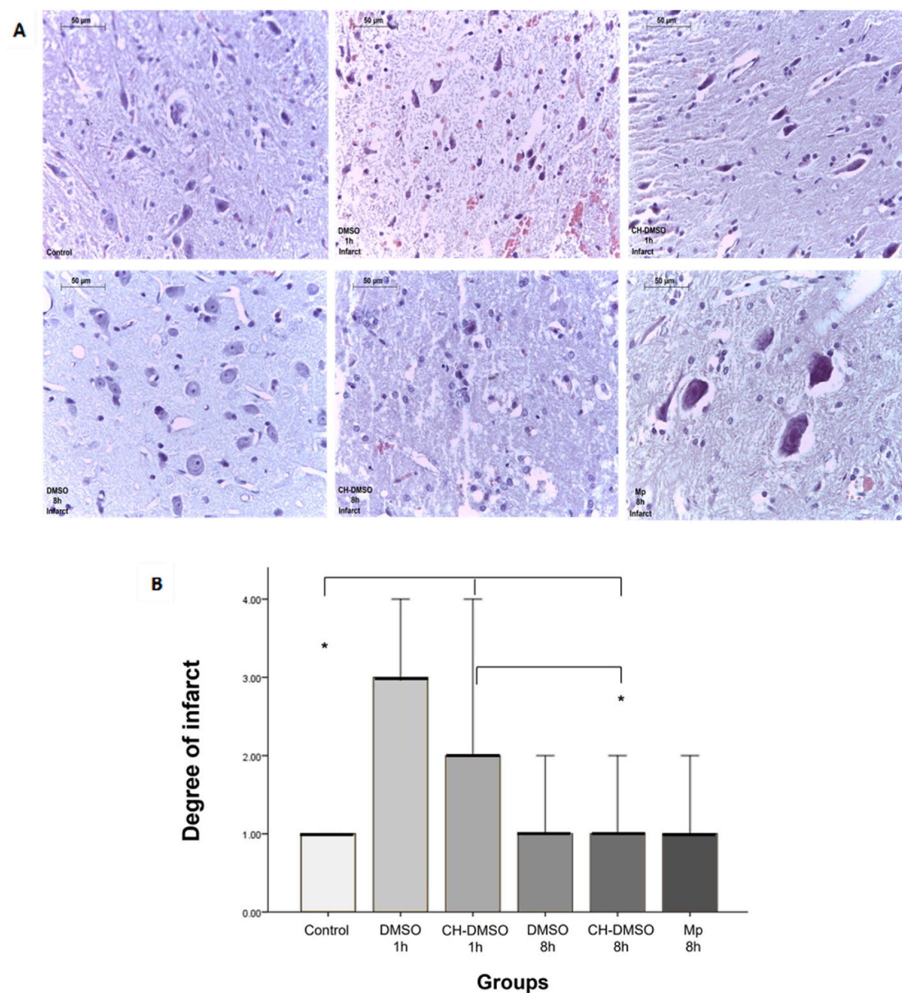

Fig. 4. A) Representative histological sections using H&E staining at 40x of the spinal cord that show the different degree of infarct in all groups, it can be appreciated a section of the spinal cord from each group. B) Semi quantitative analysis, the results plotted using a Kruskal Wallis, $p < 0.05$. Test shows a significant difference between control group with CH-DMSO 1h, CH-DMSO 8h, and group CH-DMSO 1h with CH-DMSO 8h.

the blood−brain barrier and is active in nervous tissue.[25] The effect of Chetomin on acute spinal cord injury has not been studied; if it has a positive effect, the time of its administration is unknown, and a decrease in histopathological damage can be observed. In studies on breast cancer models, Chetomin at a dose of 0.5 mg/kg inhibited HIF-1α, resulting in a reduction in tumor volume and the prevention of proliferation; thus, Chetomin is considered safe for murine models.[26]

2. Materials and methods

Forty-two clinically healthy male Wistar rats weighing 250–350 g were used. The animals were handled following NOM-062-ZOO-1999 and authorized by the Animal Research Committee of the National Institute of Medical Sciences and Nutrition (INCMNSZ) under the code number CEX-1914-18-18-1.

The animals were divided as follows. Group 1 control ($n = 7$): spinal cord extraction immediately after laminectomy without causing acute spinal cord injury. Group 2: DMSO (Zoetis, Gdl, Mexico) for 1 h ($n = 7$); treatment with vehicle (5 % DMSO; IV) 1 h after acute SCI and removal of the affected medullary segment 4 h after treatment. Group 3 CH-DMSO for 1 h ($n = 7$): Chetomin (2 mg/kg; IV) was administered 1 h after acute SCI, and the affected spinal cord segment was removed 4 h after treatment. Group 4 DMSO 8 h ($n = 7$): Treatment with vehicle (IV) 8 h after acute SCI and removal of the affected spinal segment 4 h after treatment. Group 5 CH-DMSO 8 h ($n = 7$): Treatment with Chetomin (2 mg/kg; IV) 8 h after acute SCI and removal of the affected spinal cord segment 4 h after treatment. Group 6 MP 8 h ($n = 7$): Treatment with methylprednisolone (30 mg/kg; IV) 8 h after acute SCI and removal of the affected spinal cord segment 4 h after treatment. Chetomin was dissolved in 5 % DMSO in all the experimental groups.

2.1. Surgical technique

The animals were anesthetized intraperitoneally with acepromazine-ketamine (5/10 mg/kg), meloxicam (1 mg/kg S C.), and enrofloxacin (10 mg/kg I.M.). Subsequently, orotracheal intubation was performed, and the animal model was connected to a rodent ventilator. A tidal volume of 6–8 mL/kg was used, the respiratory frequency was 60 ventilations/minute, and anesthesia was maintained with isoflurane at a 2–2.5 % minimum alveolar concentration.

The murine model was placed in the ventral decubitus position with limb fixation, and a trichotomy of the thoracolumbar vertebral region (T5-L2) was performed. Antisepsis was performed with 10 % iodopovidone, and the dorsum of the rat was incised longitudinally to expose the superficial fascia, which was dissected to reach the deep fascia, and the dorsal muscles, which were disinserted from the spine. The paravertebral space was dissected subperiosteally to reach the spinolaminar space and subsequently to the ipsilateral transverse process. Laminectomy was performed at the level of T8-T10, and extradural spinal cord clipping was performed via a vascular clip with pressure of 30 g,

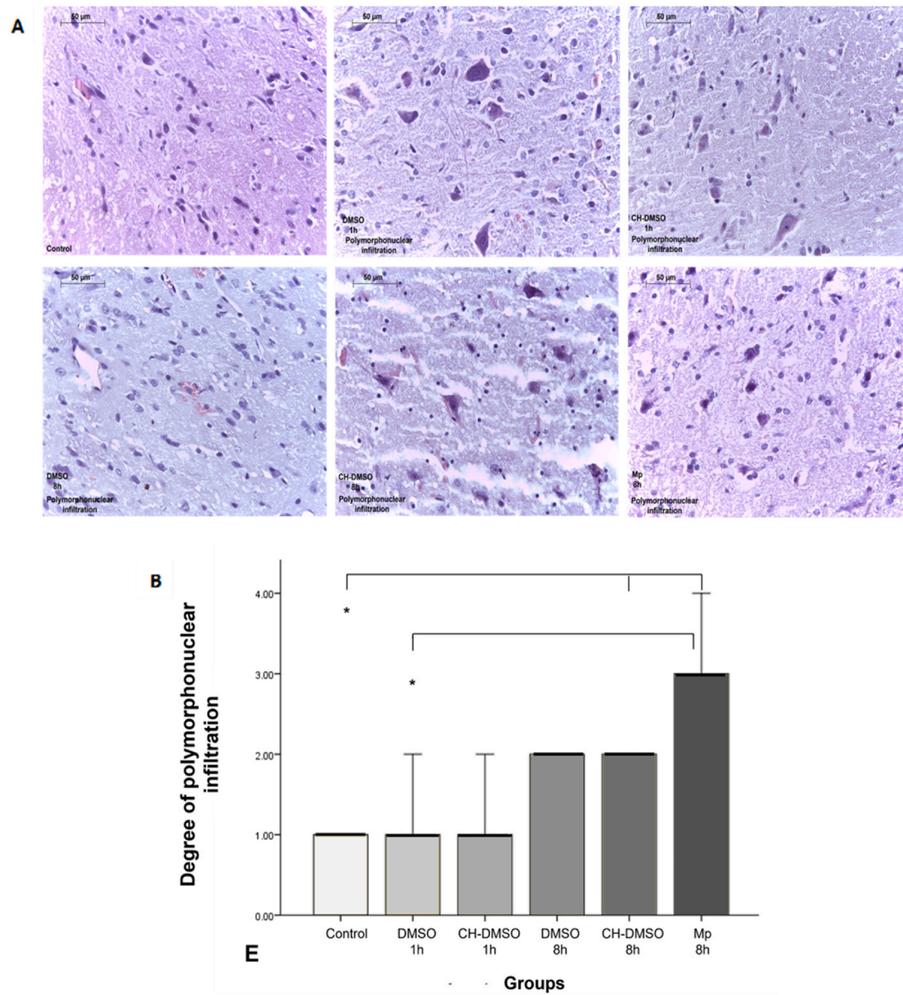

Fig. 5. A) Representative histological sections using H&E staining at 40x of the spinal cord which show the different degree of polymorphonuclear infiltration in all groups, it can be appreciated a section of the spinal cord from each group. B) Semi quantitative analysis, the results plotted using a Kruskal Wallis, $p < 0.05$. Test shows a significant difference between control group with CH-DMSO 8h, and MP 8h, also DMSO 1h with MP 8h.

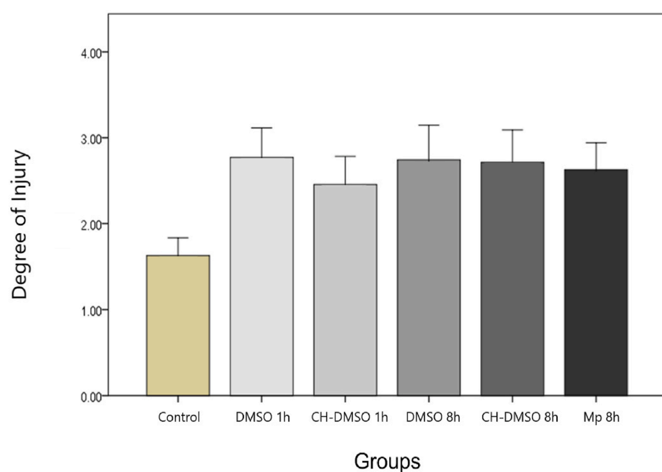

Fig. 6. Bar represents the means and their standard errors for each study group using an ANOVA test post hoc with $p < 0.05$. Considering all the variables of the histological damage of the study this this includes edema, hemorrhage, pyknosis, infarction, and polymorphonuclear infiltration, no significant difference was observed.

which was removed after 1 min of compression to achieve acute spinal cord injury in a controlled manner.[27] The nerves were sutured in planes with 5-0 absorbable monofilament sutures and the rats were moved to the recovery area where they have access to a thermal bed, water, and food ad libitum.

2.2. *Drug administration*

In the DMSO 1 h and DMSO 8 h groups, vehicle dimethyl sulfoxide (DMSO) was administered at a concentration of 5 % via IV injection. The CH-DMSO 1 h and CH-DMSO 8 h groups were administered Chetomin obtained from Sigma (MO, USA) at a dose of 0.5 mg/kg, which was subsequently diluted with DMSO at a concentration of 5 % intravenously.

2.3. *Tissue sampling*

To obtain tissue samples, 4 h after treatment, the animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal route). The animals were placed in dorsoventral decubitus for a sternotomy and intracardiac infusion of 150 mL of 4 % formaldehyde in the left ventricle to preserve the tissue; subsequently, the incision was reopened at the posterior midline level, and the spinal cord was extracted in a block from segments T8 to T10. The samples were stored in 10 cc glass containers for transport in 10 % formaldehyde at a 4:1

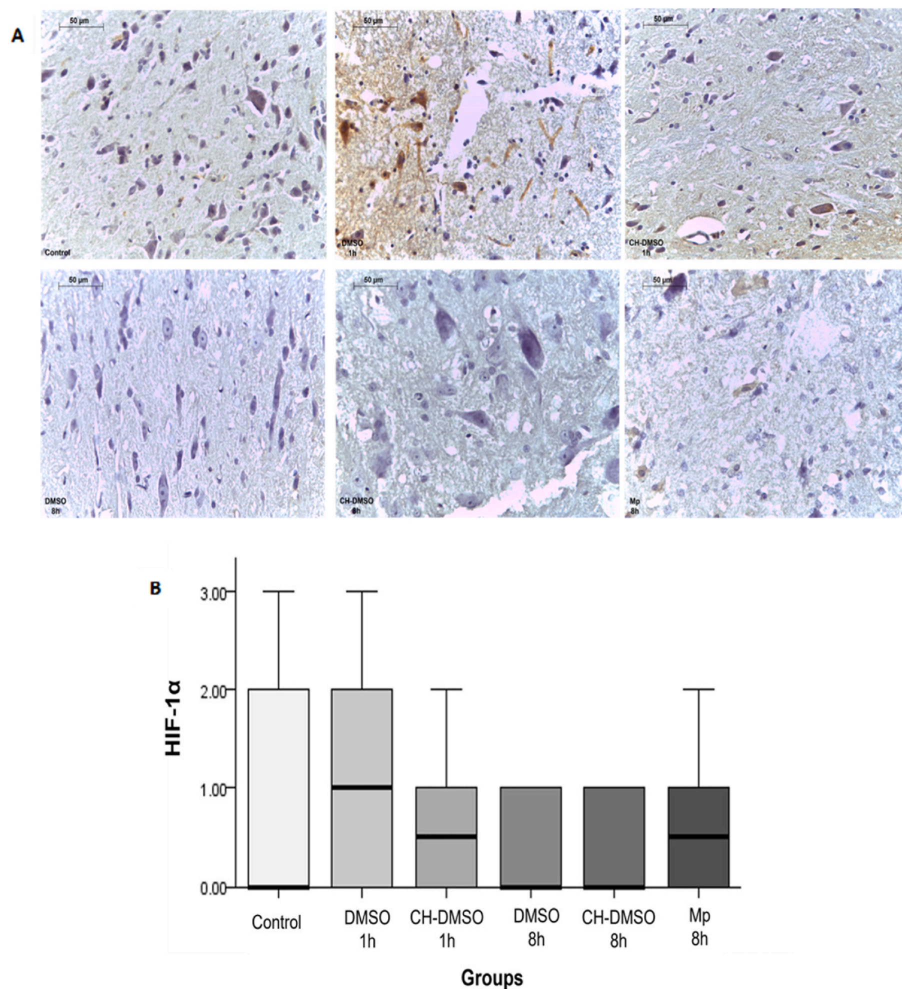

Fig. 7. A) Immunohistochemistry using an antibody of HIF-1α in the spinal cord at 40x. It can be appreciated a section of the spinal cord from each group. B) The results plotted using a Kruskal Wallis Test shows no significant difference between the groups, $p < 0.05$.

ratio to the volume of the tissue. The samples were labeled with information regarding the group to which they belonged and the experimental subject number, with the number and letter in ascending order.

2.4. Histology and immunohistochemistry

Three axial sections of the samples were fixed in 10 % formalin buffer: rostral to the lesion, at the epicenter of the lesion and caudal to the lesion. Subsequently, the samples were processed by the paraffin method. Sections were made with a 5-micron microtome taking medullary tissue from the previously indicated portions. The spinal cord tissue was stained with hematoxylin-eosin, and the slides were blindly evaluated by a neuropathologist via light microscopy with a 10x or 40x objective. Spinal cord injury was evaluated according to the following variables: edema, hemorrhage, pyknosis, infarction, and infiltration of the spinal cord parenchyma by neutrophils. The evaluation scale was as follows: 0, when this characteristic was not found in the sample. 1, found focally in a slice. 2, found focally in 2 or more slices. Finally, 3, found diffusely.

The immunohistochemical evaluation was performed by a neuropathologist with no distinction of which group each tissue belonged to. Five-micron-thick sections were tested using Bio SB reagents according to the manufacturer's instructions (BioSB, Santa Barbara Ca, USA). The antibodies used were monoclonal, NF-κB (1:1000, Santa Cruz; TX, USA); monoclonal, HIF-1α (1:500, Santa Cruz; TX, USA); monoclonal, VEGF (1:1000, Santa Cruz; TX, USA); monoclonal, IL-6 (1:1000, Santa Cruz; USA); and monoclonal, nitrotyrosine (3-NT) (1:500, Santa Cruz; TX, USA). All the samples were examined under a Leica DM 1000×microscope (Leica Microsystems), and 40× magnification images were acquired. The evaluation of each slide was carried out by a neuropathologist who was blinded to the conditions of the slides, and the staining scale scores were 0, absent. 1, light. 2, moderate. 3, severe.

2.5. Statistical analysis

Descriptive statistics are presented as the mean ± standard error (SE). Inferential statistics were performed to compare the means of the previously exposed variables between groups employing Kruskal–Wallis analysis and Tukey's post hoc variance test (ANOVA). A value less than or equal to $p = 0.05$ was considered to indicate statistical significance. The SPSS v20.0 statistical program (SPSS Inc., Chicago, Illinois, USA) was used.

3. Results

3.1. Histology

Edema formation: Compared with the control group, the groups treated with vehicle, DMSO for 1 and 8 h, CH-DMSO for 1 h or CH-DMSO for 8 h had significantly greater edema (Fig. 1A and B); however, the group treated with MP presented the same edema levels as the control group (Fig. 1B).

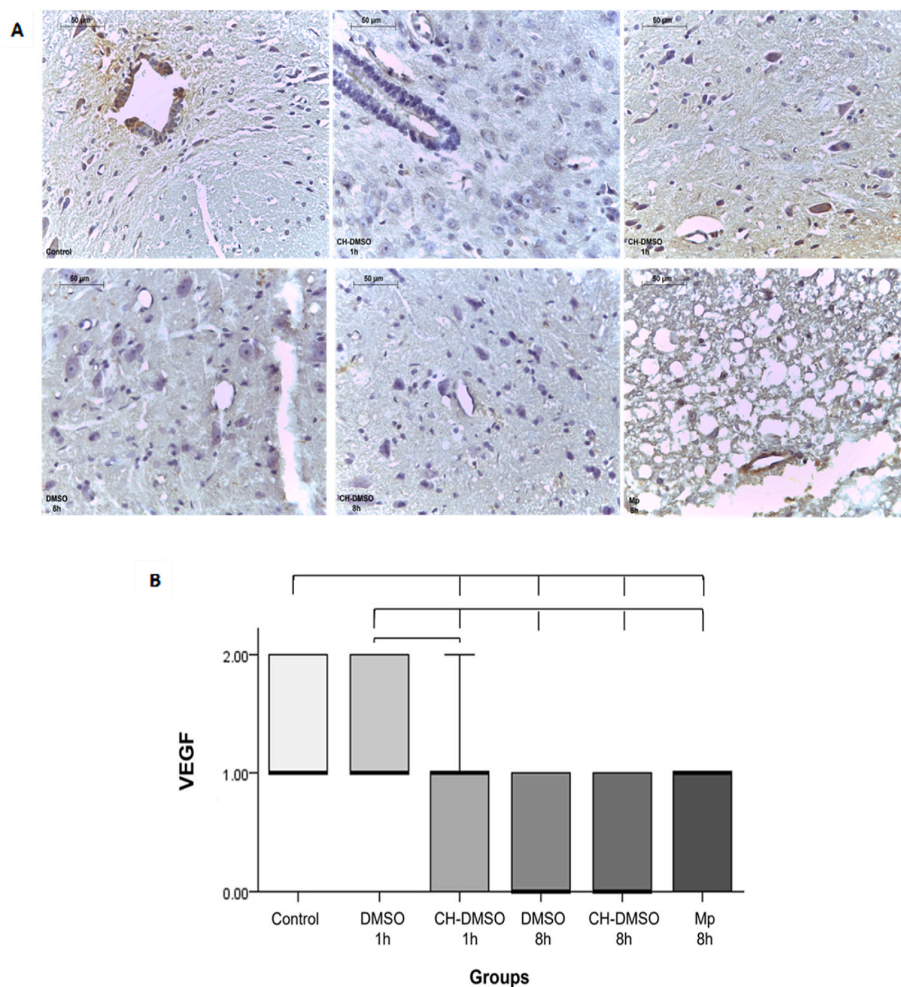

Fig. 8. A) Immunohistochemistry using an antibody of VEGF in the spinal cord at 40x, it can be appreciated a section of the spinal cord from each group. B) The results plotted using Kruskal Wallis, $p < 0.05$. Test shows a significant difference be-tween control group with CH-DMSO 1h, DMSO 8h, CH-DMSO 8h h and MP 8 h groups; between the DMSO 1 h group and the CH-DMSO 1 h, DMSO 8 h, CH-DMSO 8 h and Mp 8 h groups; and finally, between the DMSO 1 h group and the CH-DMSO 1 h group, where the administration of DMSO and Chetomin with DMSO 8 h after injury decreased the VEGF level.

Hemorrhage: Compared with those in the control group, hemorrhage was significantly greater in the 8 h DMSO group and 8 h CH-DMSO group (Fig. 2A and B); these levels were unchanged in all the remaining groups of animals.

Neuronal pyknosis: No significant difference was observed between the groups (Fig. 3A and B).

Infarct: Analysis of the infarct area revealed that the CH-DMSO 8 h group presented a significantly smaller infarct area than the control group, and the same effect was observed in the CH-DMSO 8 h group compared with the CH-DMSMO 1 h group (Fig. 4A and B), indicating that 8 h of CH-DMSO treatment can reduce the damage generated by ischemic changes.

Polymorphonuclear infiltration: There was greater infiltration in the MP 8 h group than in the CH-DMSO 8 h group (Fig. 5A and B), indicating that the remaining animals had the same levels of infiltration molecules.

Finally, an overall average of the degree of injury of all study variables was calculated, and we did not observe any changes in any of the study groups (Fig. 6).

3.2. Immunohistochemistry

HIF-1α: The analysis revealed that vehicle did not modify the presence of HIF-1α in the spinal cord after ischemic damage (Fig. 7A and B).

***VEGF*:** The presence of VEGF was significantly lower in all treated animals and in the DMSO, Chetomin and MP groups than in the control and DMSO 1 h groups (Fig. 8A and B).

In the histological section, a decrease in the IL6 concentration was evident after 8 h of CH-DMSO treatment and 8 h of MP treatment (Fig. 9A and B) compared with that in the DMSO 1 h and CH-DMSO 1 h control groups.

***NF-κB*:** The results revealed that the expression of NF-κB in spinal cord neurons was lower in the DMSO and CH-DMSO 1 h groups (Fig. 10A and B) than in the control group; however, no changes were observed in the CH-DMSO 8 h or MP groups.

3-NT: When the oxidative stress marker was analyzed, we observed that methylprednisolone administered after 8 h had a slightly lower value than that in the DMSO 1 h group (Fig. 11A and B). There was also a significant difference between the 8 h groups (CH-DMSO for 8 h and MP for 8 h) and the 1 h groups (DMSO for 1 h and CH-DMSO for 1 h) (Fig. 11A and B).

4. Discussion

Primary spinal cord injury is secondary to trauma to the spinal tissue and can only be avoided with primary prevention. The safety measures that have been implemented in cars, as well as in workplaces where workers are exposed to falls or other risk factors for spinal trauma, have contributed to the reduced prevalence of this condition; however, when there is progression to secondary spinal cord injury, there are no effective treatments.

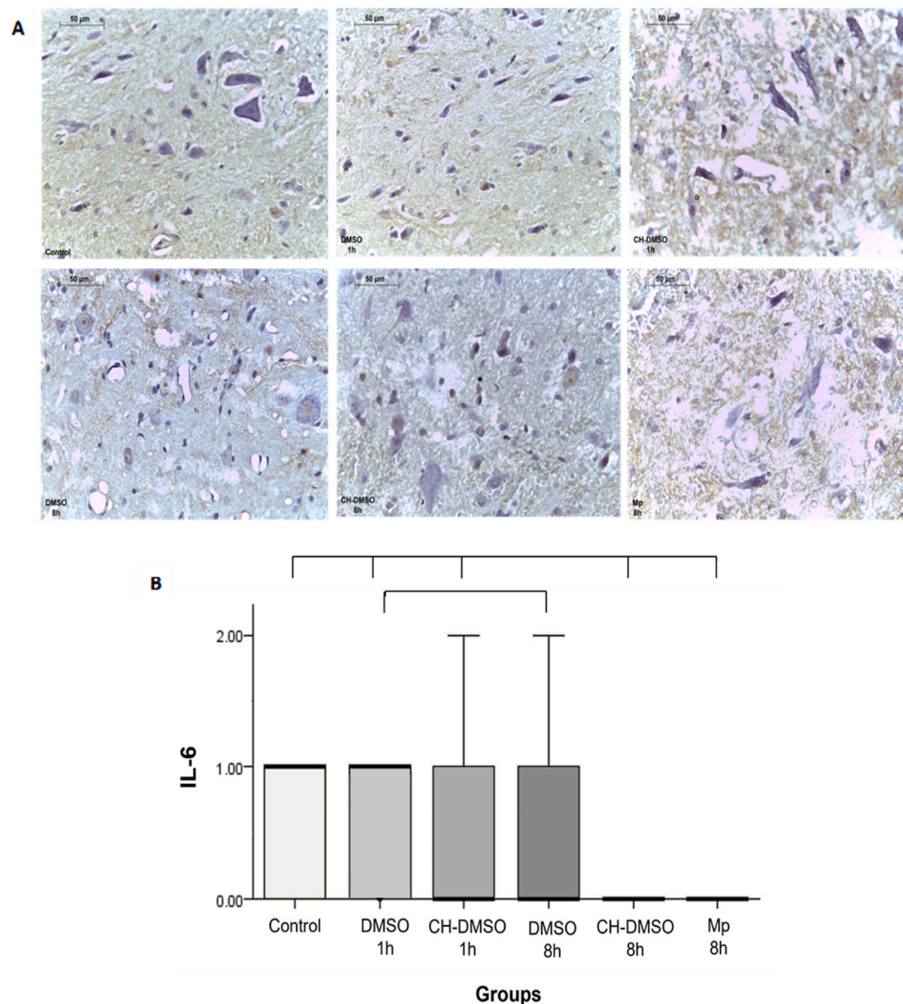

Fig. 9. A) Immunohistochemistry using an antibody against IL-6 in the spinal cord at 40 ×; a section of the spinal cord from each group is shown. B) The results were plotted using the Kruskal–Wallis test, $p < 0.05$. B) Test showing a significant difference between the control group and the DMSO 1 h, CH-DMSO 1 h, CH-DMSO 8 h and MP 8 h groups and between the DMSO 1 h group and the DMSO 8 h group, where the administration of Chetomin 8 h after injury decreased the level of IL-6.

Given that a fundamental part of the pathophysiology of acute spinal cord injury is the inflammatory response, studies have been carried out to identify therapeutic targets to reduce it. Currently, the use of methylprednisolone at high doses in the first hours of trauma is standardized and has been shown to reduce long-term sequelae[28]; however, its use is controversial due to the adverse effects that may occur, and in some studies, an increase in short-term mortality has been documented.[29]

Previous studies have demonstrated the usefulness of reducing the inflammatory process in acute spinal cord injury, as in the study performed by Bingqiang Xu and collaborators, who studied the effects of heat shock protein 70 (HSP70) reported that its expression reduces the expression of inflammatory cytokines such as IL-8 and TNF-α.[30] Moreover, other studies have demonstrated that the expression of DUSP19 in a model of acute spinal cord trauma can decrease the expression levels of NF-κB, IL8, and IL6.[31] Similarly, Min Fei Y and collaborators described that it is possible to reduce NF-κB expression through microRNA-182.[32]

Regarding the expression of HIF1-α, Shuangfei Ni and collaborators performed an experiment in which they induced inflammation in microglia through lipopolysaccharide to subsequently evaluate the effect of EZH2 on the expression of HIF1-α and found that, upon inhibition, inflammatory factors such as TNF-α, IL-6 and IL-7 decreased.[33] Guolei Zhang and collaborators also demonstrated that minocycline could have a favorable effect on the outcome of acute spinal cord injury by decreasing the expression of HIF1-α and FASL.[34]

Reports have shown that the expression of HIF1-α is not elevated in acute spinal cord injury but rather decreases 24 h after injury[35]; however, it is necessary to point out that the expression of HIF-1α is time dependent, as its expression starts approximately 3 h after neuronal hypoxia, is maintained for 18 h and decline after 24 h.[36]

There is evidence that the overexpression of HIF-1α inactivates apoptosis, activates autophagy and promotes axonal regeneration of microtubules in a period of 28 days with favorable results, but these findings warrant further investigation.

The main objective of our study was to determine whether the administration of Chetomin could decrease the inflammatory response in acute spinal cord injury. We chose Chetomin because it decreases the levels of HIF-1α, a transcription factor that regulates the NF-κB gene and can regulate the expression of TNF-α, IL-1β, IL-6, and IL-12, among other proinflammatory cytokines.

According to our results regarding NF-κB gene transcription, the lowest expression was observed at the time of Chetomin administration. NF-κB has its highest expression soon after spinal cord injury; therefore, Chetomin has a greater effect when it is administered earlier; however, unlike NF-κB, IL-6 expression is lower at 8 h after Chetomin administration. Therefore, we can infer that Chetomin, through the inhibition of HIF-1α, has a direct effect on IL-6 expression, as previously reported in a study on the activation of articular chondrocytes that initiate synovitis in ischemic osteonecrosis of the femoral head, where they demonstrated a direct relationship between HIF-1α and IL-6 expression.[37]

The inflammatory response both in spinal cord trauma and in other

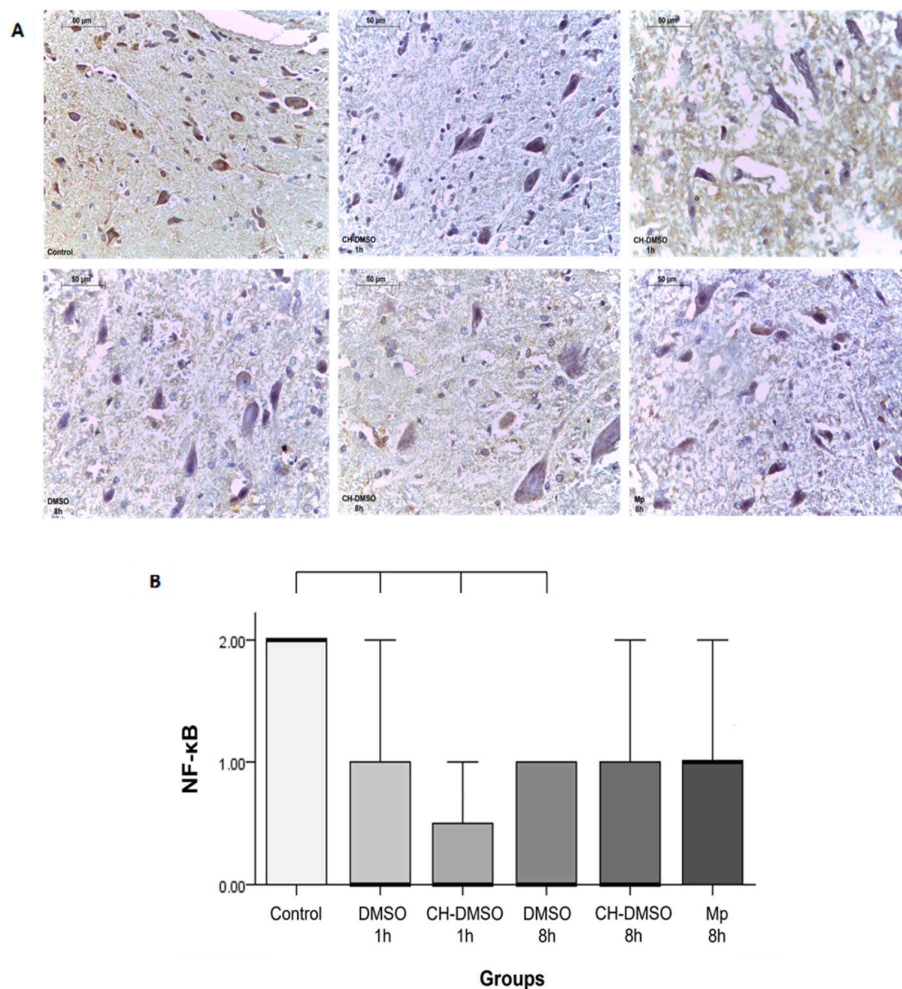

Fig. 10. A), Immunohistochemistry was performed using an antibody against NF-κB in the spinal cord at 40 × , and a section of the spinal cord from each group was obtained. B), The results were plotted using the Kruskal Wallis test, $p < 0.05$. The test revealed a significant difference between the control group and the DMSO 1 h, CH-DMSO 1 h, and DMSO 8 h groups, where the administration of Chetomin 1 h after injury decreased the level of NF-κB.

pathologies is mediated by various signaling pathways. Therefore, it is necessary to interfere with the inflammatory cascade through transcription factors such as NF-κB; however, NF-κB is not necessarily the only regulator of the immune response, as we described previously, since high levels of IL-6 are associated with lower expression of NF-κB, while lower levels of IL-6 are associated with higher expression of NF-κB, demonstrating that IL-6, despite being mediated by NF-κB, is not 100 % dependent on it.

Regarding the presence of polymorphonuclear cells in the different groups, it was shown that the group with the greatest infiltration of these cells was the methylprednisolone group. This finding is interesting because, due to the anti-inflammatory effects of this drug, we would expect a lower infiltration in this group, however, the response depends on the period of exposure to the drug and a maintenance dose; the other groups, unlike the MP group, were treated with the DMSO vehicle, which has been shown to have immunomodulatory properties that decrease IL-1β levels, inhibit NF-κB activation, and reduce the production of TNF-α, IFN-γ, and IL-2.[38] In contrast, we believe that Chetomin anti-inflammatory actions may be due to that Chetomin acts as a HIF-1α inhibitor and is consequently one of the more than 70 genes whose transcription is the master regulatory key of GLUT-1 and is related to glycolysis; therefore, this inhibition of the glycolysis pathway leads to a decrease in PMN cell activity and the production of some cytokines.[39]

When we analyzed the effects of DMSO in our model, we observed that it decreased polymorphonuclear expression in acute spinal cord injury; however, Chetomin showed better results in terms of modulating the expression of inflammatory molecules.

According to the results obtained by immunohistochemistry, the MP group presented lower levels of 3-NT than did the Chetomin group. Methylprednisolone, due to its anti-inflammatory effects, decreased the release of RNS while decreasing tyrosine nitration and was more effective than Chetomin; however, we cannot deny that Chetomin has antioxidant properties, which could be demonstrated with another methodology.

5. Conclusions

Chetomin had a better effect at the experimental level at 8 h, as evidenced by the decrease in the expression of various molecules such as IL-6 and VEGF, as well as a lower degree of ischemia and a decrease in polymorphonuclear infiltration, all these results with a significant difference. The only parameter in which the administration of Chetomin 1h had better results was in reducing NF-κB expression.

However, to appreciate a more apparent change at the histological level, a mid-term evaluation would be adequate to detect the morphological changes by treatment with Chetomin and to relate them at the molecular level, since in our study we did not find that Chetomin influences the decrease of hemorrhage, pyknosis or edema after acute spinal cord trauma. Chetomin as an anti-inflammatory agent could not be ruled out, especially because of the low presence of IL-6, the observed

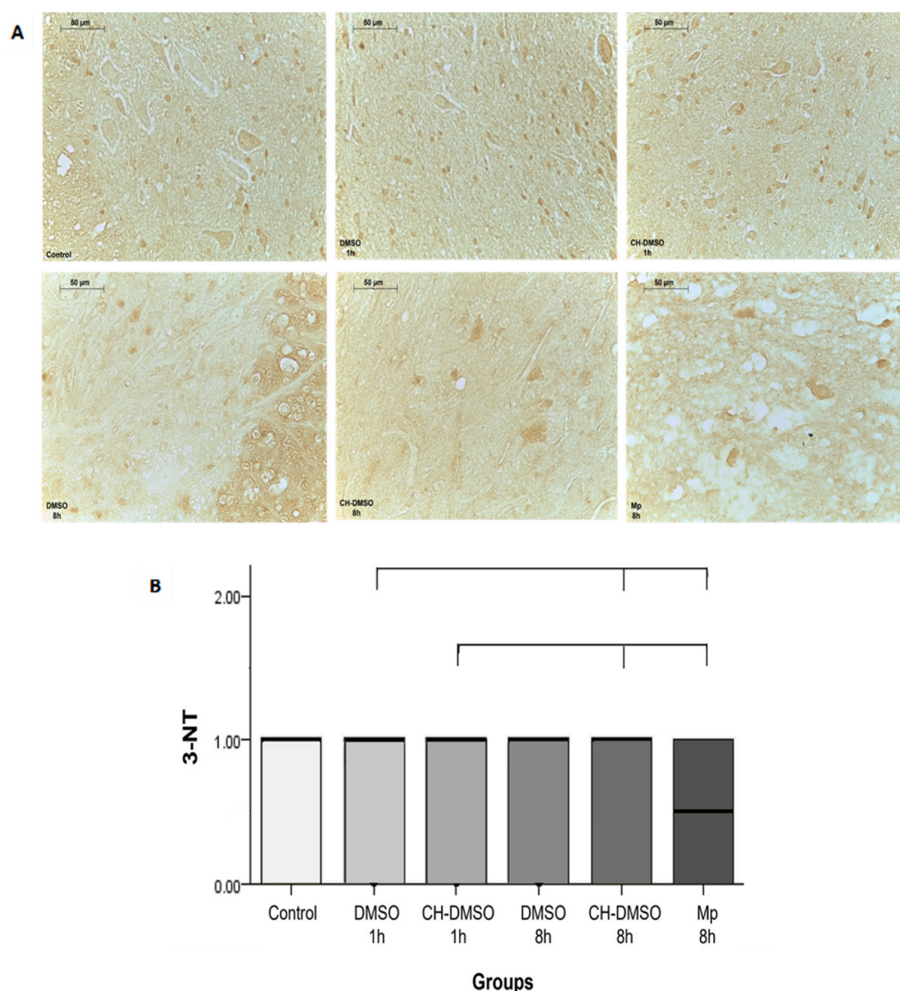

Fig. 11. Immunohistochemistry using an antibody against 3-NT in the spinal cord at 40 × revealed a section of the spinal cord from each group. The results plotted using a Kruskal–Wallis test show a significant difference between DMSO for 1 h and CH-DMSO for 8 h and between MP for 8 h and CH-DMSO for 1 h and CH-DMSO for 8 h and MP for 8 h.

low levels of NF-κB, and the lower polymorphonuclear infiltration concerning methylprednisolone treatment. Finally, it must be investigated whether these effects are observable with other proinflammatory markers and with the administration of the drug with another vehicle without associated immuno-modulatory properties. Finally, further studies of its antioxidant effect should be carried out; since in the case of 3-NT it did not have better results regarding methylprednisolone, however, its use at 8 h was better than 1 h to treatment. It's important carry out more studies extending the period of spinal cord injury by administrating the same treatment further 8 h after spinal cord injury.

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Institutional review board statement

The animals were handled following NOM-062-ZOO-1999 and authorized by the Animal Research Committee of the National Institute of Medical Sciences and Nutrition (INCMNSZ) under the code number CEX-1914-18-18-1.

Informed consent statement

"Not applicable."

CRediT authorship contribution statement

Carlos César Bravo-Reyna: Writing – review & editing, Project administration, Methodology, Conceptualization. **Vladimir Miranda-Galván:** Software, Methodology, Formal analysis, Data curation. **Gervith Reyes-Soto:** Validation, Resources, Conceptualization. **R. Vicuña:** Validation, Methodology. **Jorge Alanis-Mendizabal:** Writing – original draft, Methodology, Investigation. **Manuel Escobar-Valderrama:** Writing – original draft, Methodology, Investigation. **David Arango:** Writing – original draft, Data curation. **Claudia J. Bautista:** Methodology. **Victoria Ramírez:** Writing – review & editing, Visualization. **Gonzalo Torres-Villalobos:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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