ORIGINAL ARTICLE

Histological analysis of Spinal Cord Injury treated with Amniotic Membrane

Análise histológica de lesão medular tratada com membrana amniótica

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ABSTRACT

Spinal cord injury (SCI) is one of the most harmful syndromes that affects humans due to neuronal destruction and interruption of the nerve impulse transmission between axons. The conduction of motor, sensory, and autonomic responses below the level of the injury is seriously compromised, generating high treatment costs for the health system and a reduction in quality of life, stimulating research into new treatment protocols. This study aimed to evaluate the effectiveness of a biomaterial, the amniotic membrane (AM), to treat experimentally induced SCI. 15 adult rats were divided into three groups (n = 5): S (Sham), L (SCI without treatment), and AM (SCI treated with AM). Spinal cord injury was induced in the region T9-T10 by direct trauma, free-falling a weight (10 g, 2 mm flat edge) held on a mini guillotine, 25 mm above the exposed spinal cord. A fragment of AM, obtained from the human placenta after maternal consent, was applied to the injured area only in the AM group. After 28 days, specimens from the area of spinal cord injury were excised and subjected to routine histological procedures. Data from the semi-quantitative score, obtained from a scheme that assigned different scores to regions of the spinal cord, and from the quantitative analysis were subjected to parametric statistical analysis. Results showed that Group S presented medullary tissue without changes (score 0). In contrast, Group L presented numerous areas of cavitation in the dorsal and lateral regions of the white and gray matter (9.61 ± 6.60 $p<0.001$) with an intense inflammatory infiltrate. The AM group exhibited small areas of cavitation in the dorsal and lateral regions of the white matter and part of the dorsal columns in the gray matter (0.94 ± 1,03, $p<0.001$), with few inflammatory cells. The results suggest the effectiveness of AM in the treatment of induced SCI, characterized by a reduction in the evolution of inflammatory and degenerative processes in the central nervous tissue compared to the untreated group.

Keywords: Spinal cord injury, Amniotic membrane, Biomaterial, Healing, Regenerative medicine.

RESUMO

A lesão medular (LM) é uma das síndromes mais prejudiciais que afeta os humanos como consequência da destruição neuronal e da interrupção da transmissão do impulso nervoso entre os axônios. A condução das respostas motoras, sensoriais e autonômicas abaixo do nível da lesão fica gravemente comprometida, gerando custos elevados de tratamento para o sistema de saúde, com redução da qualidade de vida, estimulando pesquisas por novos protocolos de tratamento. Este estudo teve como objetivo avaliar a eficácia de um biomaterial, a membrana amniótica (MA), no tratamento da LM induzida experimentalmente. 15 ratos adultos foram divididos em três grupos (n = 5): S (Sham), L (LM sem tratamento) e AM (LM tratado com MA). A lesão medular foi induzida na região T9-T10, por trauma direto, pela queda livre de um peso (10 g, borda plana de 2 mm) preso

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em uma mini guilhotina acima da medula espinhal exposta. Um fragmento de MA, obtido de placenta humana após consentimento materno, foi aplicado na área lesionada apenas no grupo AM. Após 28 dias, os espécimes da área da lesão medular foram excisados e submetidos a procedimentos histológicos de rotina. Os dados do escore semi quantitativo, obtidos a partir de esquema que atribuu diferentes escores às áreas da medula espinhal, e da análise quantitativa foram submetidos a testes estatísticos não paramétricos e paramétricos. Os resultados revelaram que o Grupo S apresentou tecido medular sem alterações (escore 0), enquanto o Grupo L apresentou numerosas áreas de cavitação nas regiões dorsal e lateral da substância branca e cinzenta (escore 10,0, p≤0,001) com intenso infiltrado inflamatório. O grupo AM exibiu pequenas áreas de cavitação nas regiões dorsal e lateral da substância branca e em parte das colunas dorsais, na substância cinzenta (escore 1,8, p≤0,001), com poucas células inflamatórias. Os resultados sugerem a eficácia da MA no tratamento da LM induzida, caracterizada pela redução na evolução dos processos inflamatórios e degenerativos no tecido nervoso central em comparação ao grupo não tratado.

Palavras-chave: Lesão medular, Membrana amniótica, Biomaterial, Cicatrização, Medicina regenerativa.

INTRODUCTION

Spinal cord injury (SCI) is one of the most harmful syndromes that affects humans due to neuronal destruction and interruption of the nerve impulse transmission between axons. The conduction of the motor, sensory, and autonomic responses below the level of the injury is compromised, decreasing the quality of life and generating high costs in the healthcare system[1,2].

The estimated global incidence of SCI is 40–80 people per million inhabitants. In the United States, with an incidence of 54 cases/million annually [1]. Car accidents, falls, acts of violence, and physical or sports activities are the main causes of cord injury. SCI is divided into traumatic, when consequent to an external physical impact, and non-traumatic, due to acute or chronic disease, such as a tumor or infection. The rate of traumatic SCI was higher in men (79.8%) than in women (20.2%), with prevalence at the cervical level (~ 60%), followed by the thoracic level (32) and lumbar level (32%)[2].

Chronologically, the initial phase of SCI, or primary phase, is characterized by mechanical tissue destruction, followed by vasodilation and hyperemia, mainly in the gray matter of the medullary tissue. The next phase (or acute phase, 2–48 h after injury) presents edema and hemorrhage due to rupture of the blood-spinal cord barrier (BBB). These events induce an intense inflammatory response, with the release of pro-inflammatory cytokines and interleukins (IL), and infiltration of immune cells, resulting in electrolyte imbalance, production of free radicals, increased permeability to sodium, and accumulation of glutamate and other excitatory neurotransmitters[2–6]. The intermediate phase (days to weeks after SCI) presents intense glial proliferation, elimination of necrotic debris, revascularization, and BBB restoration. The late or chronic phase (weeks to months/years after SCI) is characterized by cellular and axonal degeneration, formation of glial scare, and development of cystic cavitation filled with extracellular fluid, small bands of connective tissue, and residual macrophages covered by thin astrogliosis layer[1,3–5,7,8].

The economic, social, and clinical consequences of SCI have stimulated the search for new therapeutic approaches that currently involve early surgical procedures, vasopressor medications, and corticosteroids. Some research has evaluated the effectiveness of photobiomodulation (PBM), biomaterials, like amnion derivatives [9,10].

Studies have been carried out, in animal models and in clinical trials, to

evaluate the benefits of cell therapy. Regenerative medicine has investigated the benefits of using Amniotic Membrane (AM) patches, a low-cost and widely available biomaterial with several biological properties that can promote wound healing. AM is a biomaterial that is easy to apply in tissue repair, as a patch, without triggering an immunological response[11–15].

AM is the innermost layer of fetal membranes. It contains stem cells and several types of collagen and fibronectin, which may be involved in its ability to reduce scars and inflammatory processes, also acting as a scaffold for cell proliferation and differentiation [12,16,17]. AM has been used in ophthalmology[18] otolaryngology[14], orthopedic surgery[19], wound treatment [20], and modulation of inflammatory processes in pericarditis[21]. Shaw [17] reported that the clinical application of placental tissues holds promise for the treatment of intervertebral disc pathology and the prevention of epidural fibrosis and spinal cord injury, suggesting the potential use of amniotic membranes in spinal surgery.

The AM biocompatibility was associated with the release of immunomodulatory and anti-inflammatory mediators, present in the AM microenvironment, in addition to growth factors that are responsible for the benefits of this biomaterial in regenerative medicine[11,12,20–24]. In addition, published preclinical studies have shown that AM used as a patch has significant results in treating cardiac ischemia[21], hepatic fibrosis [13,25] and Achilles tendon injury[26].

Considering that the treatment of spinal cord injuries is a challenging problem faced by clinicians and research scientists in regenerative medicine, this study aimed to evaluate the effectiveness of the amniotic membrane in the treatment of spinal cord injury induced in rats by evaluating the inflammatory process, presence of cavitation, and necrosis areas in the spinal cord.

MATERIALS AND METHODS

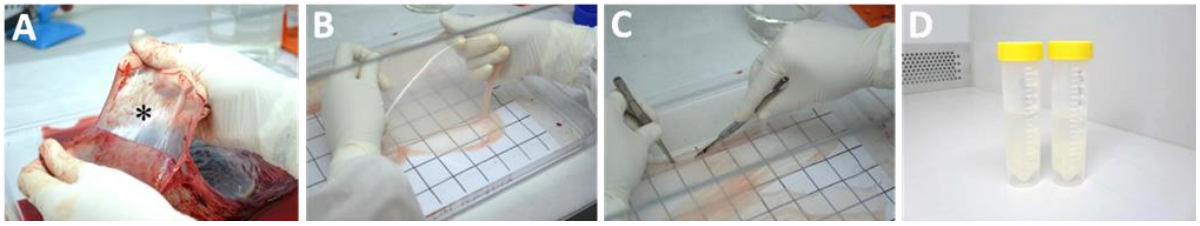
Animals and experimental groups

Fifteen male *Wistar* rats, weighing 200 ± 20 g, were housed in polypropylene cages with feed and water *ad libitum* in a controlled temperature environment (20 ± 2°C) and a light cycle of 12:12 light: dark. The rats were randomly divided into three equal experimental groups (n=5): Control group (GS): surgical procedures + simulation of SCI induction and AM application; Lesion group (GL): SCI induction without treatment; Amniotic Membrane group (GAM): SCI induction + treated with AM. All animals were euthanized after 28 days (Institutional Committee of University of Paraíba Valley, Nº. A17/CEUA/2014).

AM collection and processing

Term human placenta was obtained from cesarean section surgery after maternal consent (Human Research Ethics Committee Nº. 063/2011-PH/CEP). The donor had a normal pregnancy, a healthy medical history, and negative serological results for syphilis, human immunodeficiency virus (HIV), and hepatitis B and C[25]. The placentas were processed in a laminar flow hood under sterile conditions. The amniotic membrane was manually separated from the chorionic membrane and washed with a saline solution containing 100 U/ml of penicillin, 100 µg/ml streptomycin, and amphotericin B. The AM fragments (2 cm^2) were stored in DMEM culture medium without serum and phenol-red (50 ml), at room temperature, and were used within 24 h (Figure 1).

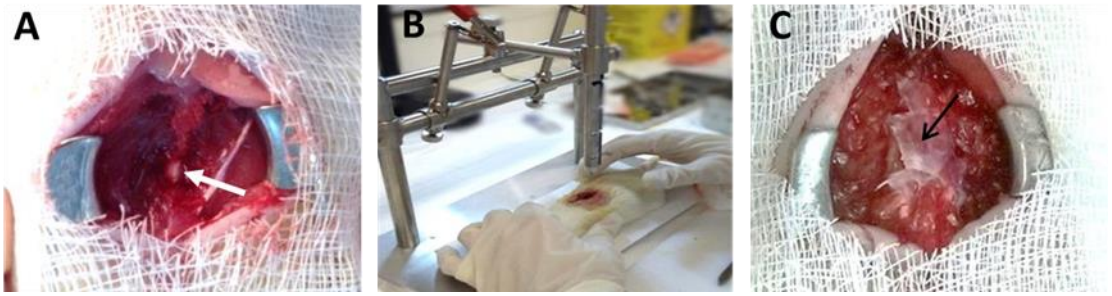
Figure 1: AM processing. (A) – AM was manually separated from the chorionic membrane; (B) – AM was washed in saline and antibiotic/antifungal solution; (C) – AM was cut into 2 cm^2 fragments; (D) – AM was stored separately at room temperature in serum-free and phenol red-free DMEN for 24h until application.



Spinal cord injury and application of AM

After the anesthetic procedures with ketamine hydrochloride (0.2 mg/kg), xylazine hydrochloride (0.1 mg/kg), and tramadol hydrochloride (0.1 mg/kg) intramuscularly, a dorsal midline longitudinal incision (2,5 cm) was made in the region T9-T10. The soft tissues were divulsed from the vertebrae, and a laminectomy was performed to expose the spinal cord segment in the L and AM groups. The spinal cord injury was induced by free-falling a weight (10 g, 2 mm flat edge) held on a mini guillotine at a height of 25 mm above the exposed spinal cord. The weight was removed (Figure 2) fifteen seconds after the free fall[27,28].

Figure 2: Spinal cord injury induction and application of AM: (A) spinal cord before SCI induction (white arrow); (B) rat position under mini guillotine to induction of SCI; (C) application of AM immediately after SCI (dark arrow).



In the group treated with amniotic membrane (AM group), a fragment of this biomaterial (a patch with 2 cm^2) was applied, keeping the mesenchymal side of the biomaterial in contact with the injured spinal cord. The edges of the AM patch were glued to the adjacent vertebrae using a drop of methacrylate glue. Subsequently, the incision was closed in two layers in all groups. Antibiotics (Enrofloxacin, 5 mg/kg, 24/24h) and analgesics (Tramadol Hydrochloride, 10 mg/kg, 12/12h) were administered to the animals intramuscularly, for five days.

Histological processing

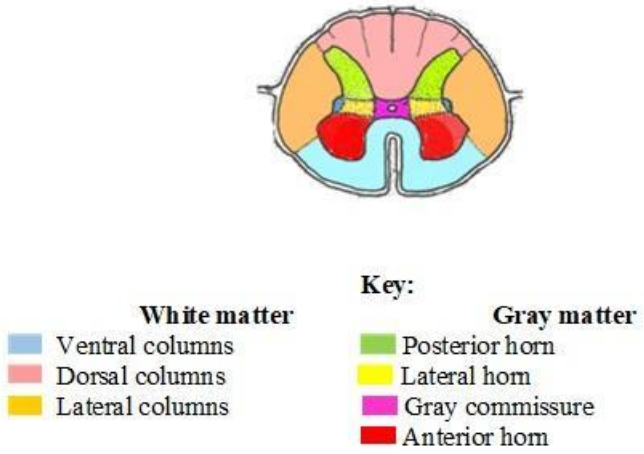
All animals were euthanized after 28 days with an overdose of anesthetic. The vertebral column and spinal cord between T8 and T12 were carefully removed and the cranial and caudal regions were identified, and fixed in 10% buffered formalin for 24 h at room temperature. The specimens were conditioned in ethylenediaminetetraacetic acid (EDTA- 20 days), dehydrated, and

embedded in Paraplast. Nine semi-serial transverse sections (4 µm thick) containing the epicenter of the injury were obtained from each sample at an interval of 50 µm along the 0.5 mm length of the spinal cord. The sections were stained with hematoxylin and eosin (H&E) and evaluated using semi-quantitative and quantitative analyses.

Semi-quantitative analysis

The total area of the gray and white matter and the cavitation area (tissue degeneration) were measured using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The results were converted to percentages. To enable the assessment of changes in the white and/or gray matter at the epicenter of the lesion (Figure 3) was created a scheme, and a score to the areas of the spinal cord.

Figure 3: Representation of anatomical regions of spinal cord.



The spinal cord cross-section was divided into seven distinct regions: ventral, dorsal, and lateral columns at white matter; anterior, lateral, and posterior horn, plus the gray commissure at gray matter.

The overall injury area of each spinal cord cross-section was divided into seven distinct regions (Figure 3): ventral, dorsal, and lateral columns of white matter; anterior, lateral, and posterior horn, plus the gray commissure of gray matter. These regions were scored as: 0 = no injury; 1 = trace injury; 2 = slight injury (injury occupying one-quarter of the region); 3 = moderate injury (injury occupying half of the region); 4 = moderately severe injury (injury occupying three-quarters of the region); and 5 = severe injury (injury occupying all of the region). A score was assigned to the injury, considering the extent of mechanical damage and the level and intensity of both the injury and inflammatory response.

H&E-stained slices were used for image analysis using NIS Elements AR 3.2 software. The regions containing the spinal cord lesions were captured using a camera (Nikon, Japan) connected to a microscope (Nikon Eclipse 80i, Japan). The lesion region was distinguished from the surrounding normal medullary tissue by the presence of inflammatory cells and areas of cavitation resulting from the central nervous system necrosis in this region.

Quantitative image analysis

Images from histological sections were captured using a Leica DM2500 Trinocular Microscope and digitalized using

Leica Application Suites LAS v3.7 program (1024×768-pixel, 24 bit/pixel resolution) with a global magnification of 50×. Digital images were processed using the ImageJ program to measure the cavitation area in all experimental groups. The lesion region was quantified using two parameters: lesion size in the rostral to the caudal direction and lesion size from lateral to lateral. The program was calibrated to image 8bit type (gray), adjusted in the 300 pixels/mm scale, and applied the contrast to delimitation and measurement of cavitation areas present in the spinal cord tissue.

Statistical analysis

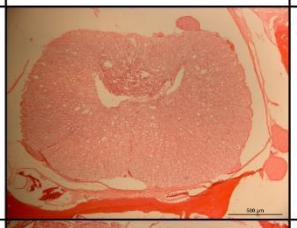
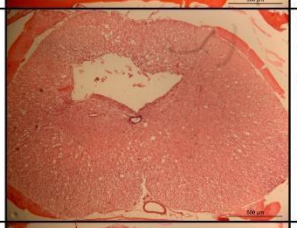
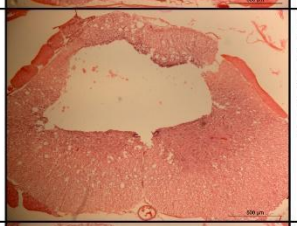
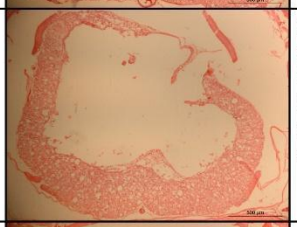
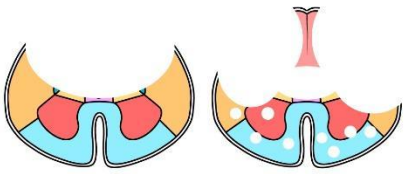
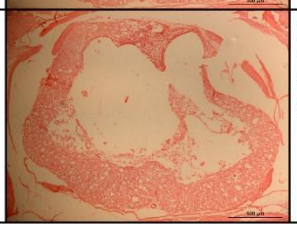
Data are reported as mean ± standard deviation (SD). Data were subjected to parametric statistical analysis using completely randomized one-way analysis of variance (ANOVA) with Tukey's post-hoc test. A value of $p<0,05$ was considered statistically significant. Data analysis was performed using Graph Pad Prism®, version 6.0, resulting in a graphical presentation of the results.

RESULTS

Semi-quantitative analysis

The histological sections of groups S, L, and AM had different characteristics regarding tissue degeneration and chronic inflammatory infiltrate. The histopathological changes in the spinal cord are shown in Figure 4.

Figure 4: The semi-quantitative spinal cord injury scoring is assigned a number from 0-5 based on the presence and size of cavities in the white and gray matter.

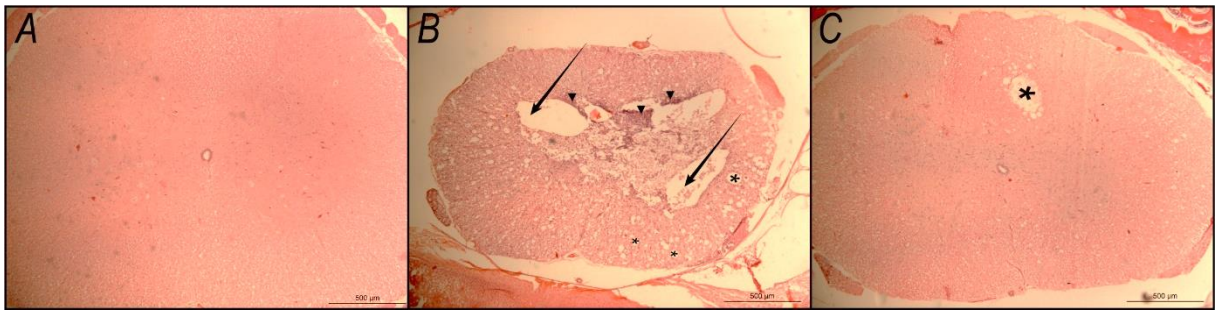
score	Illustration	Fotomicrografia	Description
1			Micro cavities in the dorsal and lateral region of white matter and in 1/3 of one of the dorsal columns of gray matter.
2			Micro cavities in the dorsal, lateral and ventral region of white matter and cavitation areas in 1/2 of both dorsal columns of gray matter.
3			Micro cavities in the lateral and ventral region of white matter and cystic appearance in the dorsal region of white matter and one or both dorsal columns of gray matter.
4			Micro cavities in the lateral and ventral region in both white and gray matter. Cavitation areas with a cystic aspect in the dorsal region affecting both the white matter and both columns and the central channel of the gray matter.
5			Micro cavities in the ventral and lateral region and cavitation area with a cystic aspect with disruption of the spinal cord limit.

Proposed score to assess the altered area after spinal cord injury: 0= no injury; 1= trace of injury; 2= mild lesion (affects a quarter of the region); 3= moderate lesion (affects half of the region); 4= moderate to severe injury (affects three-quarters of the region); and 5= severe injury (all regions affected).

In Group S, no areas of cavitation, micro cavities, or inflammatory cells were observed, either in the white or gray matter (Figure 5A). In turn, histological sections of group L showed micro cavities predominantly in the lateral and ventral regions of the white and gray matter. Furthermore, in this group, there were evident large areas of cavitation with a cystic appearance in the dorsal and lateral regions of the white and gray matter and the central canal, with intense chronic inflammatory infiltrate at its edges. In addition, microscopic visual differentiation between gray and white substances was impaired by the rupture of the structural boundary of the spinal cord and the presence of micro cavities in the lateral and ventral regions (Figure 5B).

Histological sections of the AM group revealed small areas of cavitation in the white matter and part of one or both of the dorsal horns of the gray matter. A discrete inflammatory infiltrate was observed around the micro cavities at the ventral and lateral white matter regions (Figure 5C).

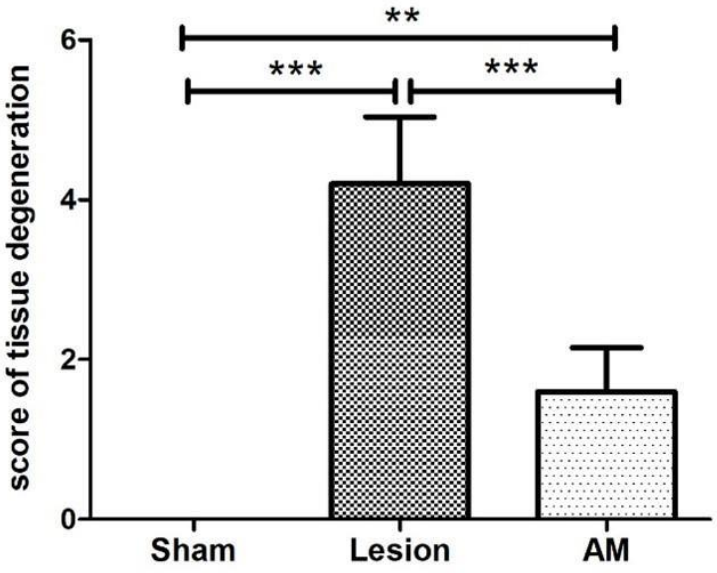
Figure 5: Histological images of Sham (A), Lesion (B), and Amniotic Membrane (C) groups showing the absence/presence of micro/cystic cavities in the white and gray matter.



microcavities (*); inflammatory cells (arrowhead ►) and the central canal (long arrow)

Semi-quantitative analysis of tissue degeneration at the epicenter of spinal cord injury in the experimental groups showed that the AM group had a significantly lower tissue degeneration score than the L group, respectively (1,6 ± 0.54 vs 4.2 ± 0.83, $p<0.01$) (Figure 6).

Figure 6: Semi-quantitative analysis of tissue degeneration at the epicenter of spinal cord injury of experimental groups.

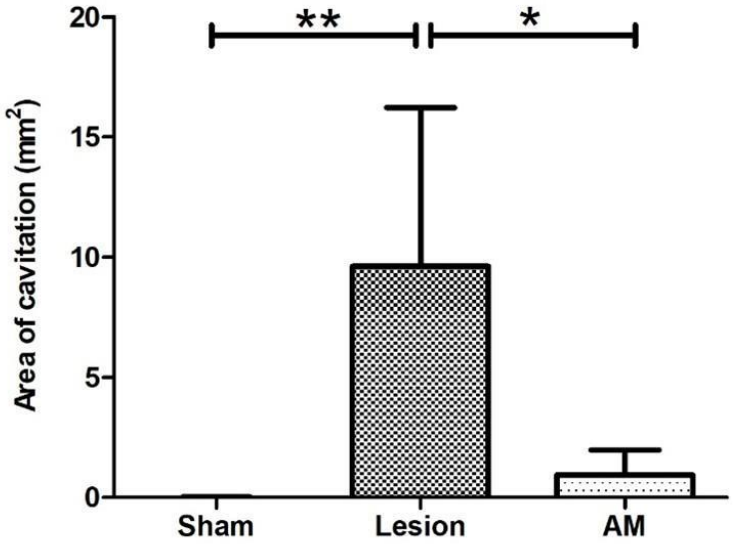


Data expressed as mean ± SD. **p<0.01; ***p<0.001.

Quantitative analysis

Cavity area size was evaluated at the rostral-caudal region, including the epicenter of the spinal cord lesion in all histological sections. The total areas without any cells or structures, considered cavitation areas, were quantified based on the total area of the spinal cord to determine the percentage of cavitation area in the epicenter of the SCI. The quantitative data obtained by the image analysis are shown in Figure 7. Following the proposed analysis, Group S presented a mean area of zero (0) regarding the cavitation area. On the other hand, the mean area of Group L was 9.61.0 compared with GS (p<0,01), while Group AM presented a mean area of 0.94, a significant reduction, compared with GL (p≤0,05). The evaluation of scores relating to tissue degeneration demonstrated similar results, with a score of zero (0) for Group S, 4.5 for GL, and 0.8 for Group AM, all with significant statistical differences (p<0,01).

Figure 7: Quantitative analysis of cavitation area at the epicenter of spinal cord injury in experimental groups.



Data expressed as mean ± SD. *p<0.05; **p<0.01.

DISCUSSION

The histological results of this study indicate the effectiveness of the amniotic membrane in the treatment of spinal cord injury induced in rats, considering the reduction of degeneration and necrosis processes in the central nervous tissue compared to the untreated group. These results encourage new research with this biomaterial for a new treatment protocol for SCI, considering the low cost, wide availability, and absence of ethical conflicts.

Although to date its clinical application is not yet indicated to treat spinal cord injuries, the high potential of this biomaterial has been demonstrated in other healing processes, as mentioned previously. In the present study, we used the release of a weight supported by a mini guillotine, inducing a compression or contusion injury in the spinal cord. This methodology simulates the type of spinal cord injury that commonly affects humans, resulting from falls or other forms of physical impact that crush the bone canal and compress the spinal cord.[1,17,27]

The requirements for improving functional results arose after the evidence that SCI in humans has different characteristics depending on the time elapsed since the injury[29]. Few studies have developed therapeutic alternatives to reverse or prevent tissue damage caused by spinal cord injury, including the use of biomaterials[30] and stem cell therapies[31]. In our study, we used a tissue transplantation strategy (patch) rather than cells, considering that AM acts mainly as a biological dressing, and a matrix and source of soluble factors that diffuse through the tissue[11,32].

The results suggested that the AM applied as a patch over the spinal cord injury area preserves the microenvironment of this biomaterial that acts to reduce the inflammatory response[11,32]. Studies have shown that the effectiveness of the amniotic membrane in tissue repair is related to the presence of growth factors, antimicrobial peptides[12], IL-1 antagonist receptors [11] and anti-inflammatory, antiangiogenic, and proangiogenic factors, among others[33]. In addition, AM is biocompatible and does not induce graft rejection by the host, as it does not contain the non-polymorphic human

leukocyte antigen (HLA-G) and little or nothing of the HLA, A, B, C, or DR antigen[11,12,20].

Placental tissues have been extensively investigated for facilitating neurological recovery, due to their neurotrophic capacity and ability to induce neural differentiation. [22]. *In vivo* animal models have shown that amnion cells can favor neural cell differentiation, reduce secondary neural damage, modulate microglial activity in the spinal cord, promote nerve fiber remyelination, and improve functional recovery[17,34].

The AM epithelial cells have properties to inhibit the proliferation, maturation, and recruitment of antigen-presenting cells (APCs). As a consequence, this biomaterial reduces the progression of the inflammatory response, attracts M2 macrophages (reparators), and prevents the proliferation of M1 macrophages[11,23,35,36]. A preclinical study by Zhou [37], using AM mesenchymal stem cells (MSCs) in rats with spinal cord injury revealed a significant increase in neurological function, suggesting that this action occurred by reducing the inflammatory response and cell apoptosis, favoring axonal regeneration. Ryu[38] compared the effects of bonemarrow–derived, adipose-derived, umbilical cord tissue, and umbilical cord blood MSCs on neural regeneration in a canine SCI model. A significant improvement in locomotion was observed in all MSC groups, with an increase in the number of surviving neurons and neurofilament-positive fibers. However, the extraction of stem cells from AM can lead to the loss of tissue factors in its rich microenvironment. The use of AM as a patch eliminates the processes of obtaining stem cells, reducing costs, and simplifying its use, preserving the components present in this biomaterial.

A study published by our research group reported positive results obtained by analyzing the movements and functional index of the sciatic nerve in rats with spinal hemisections treated with AM patches. The group treated with this biomaterial showed significant improvements in locomotion speed and sciatic functional index when compared to the untreated group [39]. The promising results point to AM as a potential alternative treatment with a positive impact on reducing the damage caused by spinal cord injuries, also observed in the present study

Secondary injuries can affect undamaged neural circuits away from the SCI site. After spinal cord injury, neurons, glial cells, and their precursors die. The descending and ascending spinal tracts are interrupted, giving rise to cavitation areas, demyelination, and astrogliosis, with relevant changes in the spinal cord cytoarchitecture [33,40]. Zhang[4] found that rupture of corticospinal axons at the SCI site resulted in the death of spinal motoneurons below the level of the lesion, with progressive atrophy of the skeletal muscle fibers innervated by these fibers. Therefore, it is extremely important to slow down or prevent the progression of necrosis. Our results indicate that the fragment of the amniotic membrane surrounding the spinal cord injury area significantly reduced the evolution of tissue necrosis, represented by the formation of cavitation areas. Thus, viable cells and fibers of the nervous tissue can maintain the functionalities that would be lost without treatment with this biomaterial.

This study is a pioneer in evaluating the application of amniotic membrane patches as an innovative protocol for treating spinal cord injury in rats. Semi-quantitative and quantitative histomorphological analyses indicated the effectiveness of this biomaterial represented by the reduction in the formation of cavitation areas in the central nervous tissue. The results indicate a delay in the evolution of the necrosis process of nerve cells and fibers, preserving the cytoarchitecture compared with the untreated group.

This is a pioneering study that aimed to evaluate an innovative protocol for treating spinal cord injury in rats with the

application of amniotic membrane patches. Semi-quantitative and quantitative histological analyses demonstrated the effectiveness of this treatment with a reduction in both, the inflammatory process and the formation of cavitation areas, in SNC. These results indicate that the AM delayed, or even prevented, the progression of the necrosis process of cells and nerve fibers, minimizing the damage associated with SCI. So, AM applied as a patch acts as a protective therapeutic intervention against nervous tissue damage after spinal cord injury.

CONCLUSION

The amniotic membrane reduces inflammatory processes and the formation of cavitation areas in the central nervous tissue after a spinal cord injury. These promising results encourage continued research into new treatment protocols for spinal cord injuries using this biomaterial, due to its wide availability and numerous properties that qualify it to reduce the damage caused by spinal cord injuries.

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Emilia Angela Lo Schiavo Arisawa, Luciana B. Sant'Anna, and Mario Oliveira Lima: Validation, Formal analysis, Funding acquisition;
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All the authors have read and agreed to the published version of the manuscript.

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Declarations

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