



## Identifying glial scar tissue using infrared thermography: a spinal cord injury pilot study

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<https://doi.org/10.36105/psrua.2021v1n1.03>

### ABSTRACT

**Introduction:** Glial scarring after a spinal cord injury (SCI) can represent both a physical and a molecular barrier for axonal regeneration and thus its removal has been found to be helpful in the recovery process. For this removal to be feasible in humans, an efficient method is needed to clearly identify glial tissue without inflicting more damage. **Objective:** To evaluate infrared thermography as a tool for identifying glial scar tissue in chronic SCI. **Material and methods:** An exploratory experimental pilot study was performed on Sprague-Dawley rats divided into sham and SCI (T9). All animals were subjected to a baseline thermography performed after a laminectomy that was either followed by closure of the surgical planes (sham group) or injury infliction (SCI group). Five weeks later, a second thermography was performed. Afterward, the spinal cord (T8-T10) was removed and processed for glial fibrillary acidic protein (GFAP) immunohistochemistry, which was used as a gold standard for identifying reactive astrocytes and glial scar. All animals received the same care throughout the study. **Results:** The thermography did not reveal a statistical difference for the baseline values ( $p = 0.24$ ); however, a significant difference in thermography values was found 5 weeks later ( $p = 0.01$ ). This difference significantly correlated with astrocyte counts at the site of injury ( $r = -0.57$ ;  $p = 0.03$ , Spearman's correlation). **Conclusions:** Infrared thermography could be useful to evaluate the extent of glial scar after SCI.

**Key words:** spinal cord injury; infrared thermography; glial scar.

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## RESUMEN

**Introducción:** La cicatriz glial de una lesión de médula espinal (LME) es una barrera física y molecular para la regeneración axonal, por lo que su resección es útil en la recuperación en modelos animales. Para que esta resección sea factible en humanos, es necesario un método claro de identificación de tejido cicatricial sin provocar mayor daño. **Objetivo:** Este estudio busca evaluar la utilidad de la termografía infrarroja en la identificación de la cicatriz glial en LME crónica. **Materiales y métodos:** Se realizó un estudio piloto exploratorio experimental con ratas Sprague-Dawley divididas en un grupo sham y un grupo de LME (T9). Se realizó una termografía basal poslaminectomía en todos los animales, seguida de cierre de la herida o LME y se obtuvo una segunda termografía 5 semanas después. Se retiró la médula (T8-T10) y se realizó inmunohistoquímica anti-GFAP (proteína ácida fibrilar glial) como estándar para el marcaje de células de la glia. Todos los animales recibieron los mismos cuidados. **Resultados:** No hubo diferencia estadística entre los valores basales de la termografía ( $p = 0.24$ ); sin embargo, 5 semanas después se observó una diferencia significativa en los valores presentados en la zona de lesión de los animales con LME ( $p = 0.01$ ). Esta diferencia presentó una correlación significativa con la cantidad de astrocitos ( $r = -0.57$ ;  $p = 0.03$ , prueba de correlación de Spearman). **Conclusiones:** La termografía puede ser de gran utilidad para conocer la extensión de la cicatriz glial observada después de una LME.

**Palabras clave:** lesión de médula espinal; termografía infrarroja; cicatriz glial.

## 1. INTRODUCTION

Depending on the level and severity of the injury, spinal cord injury (SCI) patients are subject to dealing with life-long medical and economic consequences that have a detrimental effect on their quality of life.<sup>1,2</sup> With this in mind, researchers have studied possible treatment options that can either improve or even permanently re-establish the functions of the spinal cord. These strategies approach the problem through the pathophysiology of the injury, and this has led to therapies such as cyclooxygenase inhibition, antioxidants, apoptosis inhibitors, and immunophilin ligands, among others.<sup>3</sup> Nevertheless, despite the success of some of these therapies, a review of several studies suggested that removing the glial scar before administering any of these therapies is the best strategy to promote neurological recovery.<sup>4</sup>

After SCI, a glial scar begins to form in the acute stage and remains active throughout the chronic phase. The scar is mainly constituted by astrocytes that undergo reactive astrogliosis (hypertrophy, increased proliferation, and secretion of GFAP, vimentin, nestin, cytokines, and proteoglycans), constituting a physical barrier to prevent damage expansion.<sup>5</sup> Nevertheless, this glial scar also acts as a physical and chemical barrier for neural growth and axonal regeneration.<sup>4,6</sup> Different studies have shown that the glial scar has a very dynamic function and is equally important during the early stages of injury as it is restrictive in the chronic one. Initially, these cells release endothelial growth factors and vasoconstrictors to prevent more bleeding, antioxidants to counteract the glutamate-induced excitotoxicity, and regenerating factors such as FGF-2 and S100 $\beta$  for tissue restoration.<sup>7</sup> In the long run, however, these effects diminish or disappear and what remains is a physical and molecular barrier for restoration, largely due to the increase in chondroitin sulfate proteoglycans (CSPGs)

secreted from the scar, which in turn inhibit axonal growth.<sup>8</sup> In line with this, several studies have proven in experimental models that the surgical removal of this scar tissue, alone or in combination with other therapeutic strategies, can be successful in improving motor capacity below the injury site.<sup>9</sup> That is why the design of a method to precisely identify and successfully remove the glial scar without creating more damage remains an important area of research. This problem can be addressed by using an infrared thermography camera. This device processes thermal data through electromagnetic radiation and converts it into an electronic image.<sup>10,11</sup> The camera detects heat from the studied tissue. Since the scar has a low metabolic activity, and thus lowers the temperature, infrared thermography might help recognize the extent of the glial scar. This would provide an opportunity to carefully remove the scar without inflicting further damage to the neural tissue.<sup>12</sup> The aim of this study was to determine whether infrared thermography could effectively identify glial scar in an injured spinal cord.

## 2. MATERIALS AND METHODS

An experimental pilot study with laboratory animals was carried out. The research protocol was approved by the IRB and the Internal Committee for Laboratory Animal Use and Care (CICUAL) at Universidad Anáhuac México.

### 2.1 Experimental design

Since this was an exploratory study, the research team used a criteria sample size of 11 Sprague-Dawley female rats ranging in weight from 210 to 250 grams, with no prior diseases or

interventions. All animals were subject to a laminectomy (T9), followed by a baseline thermography of about 1.5 cm proximal and caudal to T9. Then, randomization determined which animals were immediately closed (sham,  $n = 4$ ) and which were injured (experimental,  $n = 7$ ). Both groups received the same post-surgical care for 5 weeks, after which the same area was exposed again for a second thermography. This timeline allowed for the glial scar to gradually evolve from the subacute stage into a more mature and less active tissue within 2 weeks post-injury.<sup>13</sup>

## 2.2 Tissue management

The animals were euthanized by perfusion with prior heparin and anesthesia (sodium pentobarbital). Then, the spinal cord was removed and fixed with paraformaldehyde for 24 h and 30% sucrose for 72 h; it was then frozen with Tissue-Tek. Longitudinal sections of the spinal cord were used in GFAP immunohistochemistry, with GFAP as the gold standard in reactive astrocyte identification to have a comparative parameter for the thermography. In every case, 40- $\mu\text{m}$  sections were made until visualizing the central canal, and three consecutive sections were used in immunohistochemistry. Finally, sections with the most gliosis were chosen and the area with the most GFAP marking was photographed (20x).

## 2.3 Laminectomy and experimental SCI

After applying anesthesia using intramuscular xylazine/ketamine (10 mg/kg/50 mg/kg), careful skin and muscle tissue incisions allowed for visualization of the 9<sup>th</sup> thoracic vertebra and its removal to expose the spinal cord. The animals in the experimental group were then subjected to a moderate contusion injury using an IH Spinal Cord impactor (Precision System & Instrumentations) with a force of 200 kdyn at a 2-mm distance. Polyglycolic acid and nylon sutures were used to close muscle tissue and skin, respectively. After surgery, 1 mL saline solution and 0.1 mL ketoprofen (intraperitoneal) were administered.

## 2.4 Postoperative care

The animals were housed in pairs with food and water *ad libitum*. They received manual bladder voiding for two weeks and a prophylactic dose of 0.1 mL enrofloxacin (subcutaneous) every other day during the first week post-op. Oral acetaminophen (0.2 mL) was administered every 12 h for the first two weeks. The animals received veterinary care for the whole duration of the experiment as established in the National Institutes of Health guidelines<sup>14</sup> and the Official Mexican Standard NOM-062-ZOO-1999.<sup>15</sup>

## 2.5 Infrared thermography

A FLIR E6-XT infrared camera was used and always maneuvered by the same person at a 10-cm distance (verified with a measuring ruler) straight above the exposed tissue to get a clear image for analysis. The data from these images was analyzed using the accompanying software (FLIR Thermal Analysis and Reporting) by pinpointing the exact T8-T10 zone in each thermogram and obtaining the temperature.

## 2.6 Immunohistochemistry

The removed spinal cords (T8-T10) were embedded with Tissue-Tek and placed in a cryostat at  $-22\text{ }^{\circ}\text{C}$  to make 40- $\mu\text{m}$  longitudinal sections until visualizing the central canal; 3 consecutive sections were taken for immunohistochemistry. The sections were incubated in PBS with 3% hydrogen peroxide, rinsed with PBS, and then incubated in PBS and 0.3% Triton. Subsequently, the primary antibodies were added (rabbit polyclonal GFAP antibody, 1:1000; Merck) at  $4\text{ }^{\circ}\text{C}$  for 2 nights. Once rinsed with PBS, samples were incubated with donkey anti-rabbit IgG antibody (1:500, Millipore) at room temperature for 1.5 h. Samples were rinsed with PBS and incubated in A+B conjugate (Vector PK 6100) for 1.5 h. They were rinsed again with PBS, and finally, the DAB kit (Vector SK-4100) was applied. The sections were placed on gelatinized slides and covered with Cytoseal 60 and a coverslip.

## 2.7 Astrocyte counts

In every case, the section with the most gliosis was analyzed with 20x magnification, always focusing on the most marked region within the analyzed section. This selected region was photographed and analyzed using ImageJ to count the marked astrocytes for further analysis. This software recognized GFAP-marked astrocytes and allowed the researcher to manually count them.

## 3. STATISTICAL ANALYSIS

The Shapiro-Wilk test showed that the thermography data did not have a normal distribution ( $p = <0.001$ ), but the astrocyte count data did show a normal distribution ( $p = 0.15$ ). Therefore, a Mann-Whitney U test was used to compare intergroup thermography, a Student's t-test compared astrocyte counts, and a Spearman's correlation determined the correlation between thermography and gliosis (assessed by Image J program for astrocyte count). SPSS v.22 and GraphPad Prism 5 were used for analysis.



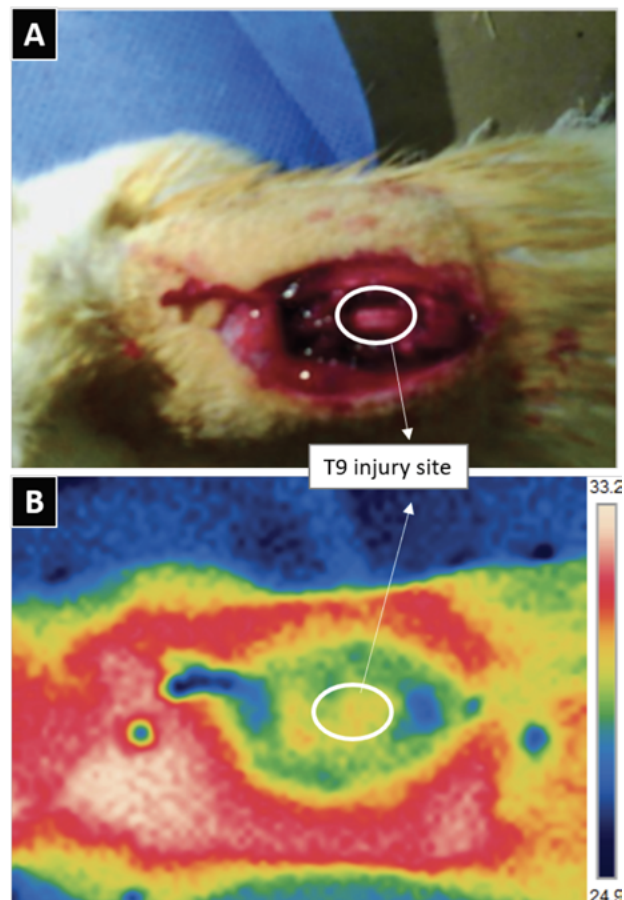
#### 4. RESULTS

Table 1 shows the general results obtained from this study: baseline and 5-week thermography as well as astrocyte count for the same spinal cord segment. A preliminary difference can be observed in GFAP-marked astrocytes in the sham vs. the experimental group. In addition, lower temperatures are shown by the injured animals (in the second thermography) compared to the temperatures in the *sham* group; further analyses detailed in the figures below were performed to draw valid conclusions.

Figure 1 shows a randomly selected infrared thermogram taken from one of the animals in the study (immediately after the laminectomy). The images were used for further analysis given that they also save the temperature range of the exact moment they were taken and allow for the exact determination of the temperature of any area within the image.

Figure 2A shows the thermography analysis that proved no difference existed before the intervention. Both groups demonstrated very similar baseline temperatures (*sham* 30.45 mean  $\pm$  0.50 SD, experimental 29.91  $\pm$  0.46,  $p = 0.15$ ). Figure 2B, however, illustrates how the temperature in the injury zone varies significantly between the groups 5 weeks later (*sham* 32.68  $\pm$  0.32, experimental 30.39  $\pm$  1.35,  $p=0.01$ ).

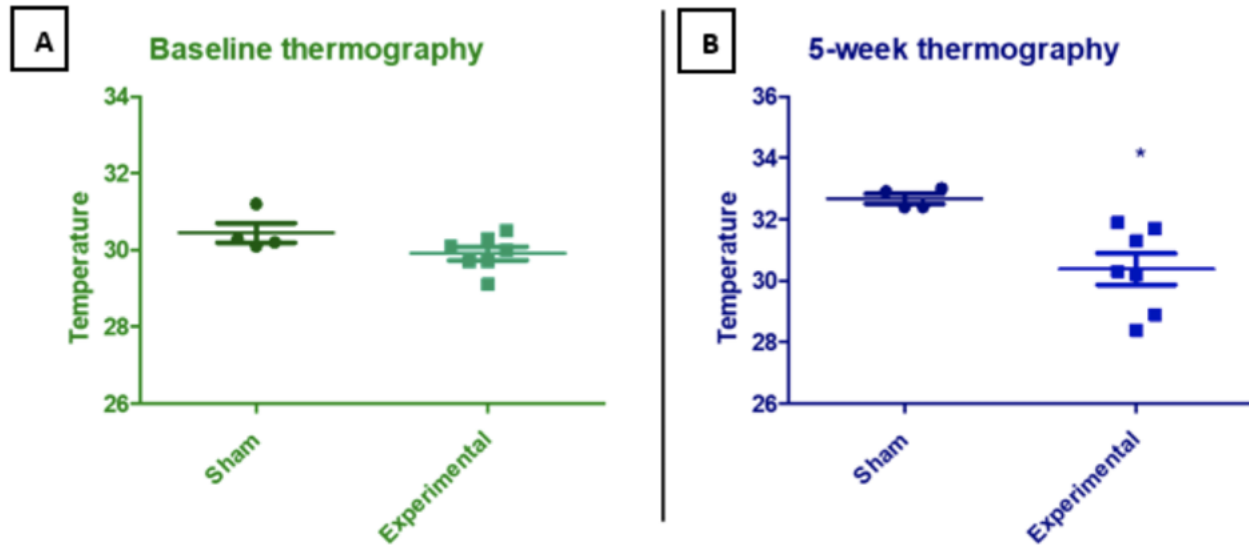
Figure 3 is also a randomly selected microscopic image to portray how astrocytes were marked with anti-GFAP immunohistochemistry for the astrocyte count. In Figure 4, the difference in astrocyte counts between groups was evaluated, finding a significant difference between the *sham* (13.75  $\pm$  2.98 mean  $\pm$  SD) and the experimental group (54.71  $\pm$  7.63) ( $p = 0.0001$ ). This difference reveals



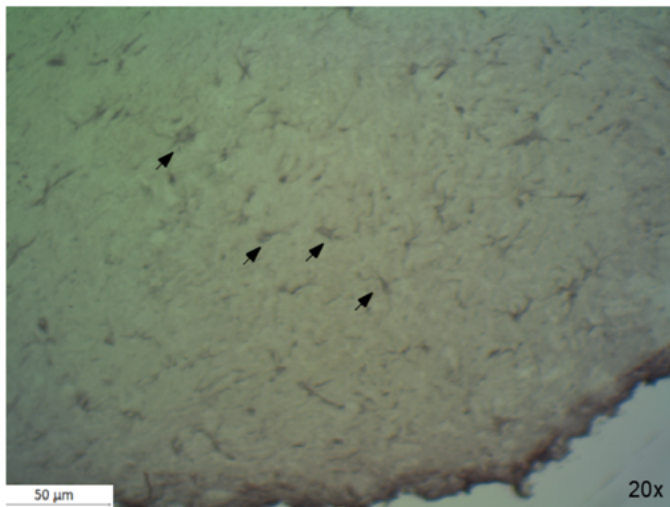
**FIGURE 1.** Infrared thermogram taken immediately after the laminectomy, before group assignment. A. Simultaneous picture capture with thermogram; the circled area was later analyzed. B. Thermogram of the picture shown above (A) allows for precise temperature evaluation. Temperature range (24.5–33.2°C) automatically calibrated is shown to the right. Images were randomly selected for graphic representation of a thermography.

**TABLE 1.** Thermography and gliosis results per animal.

Animals	Baseline thermography	5-week thermography	Number of GFAP-marked astrocytes
<i>Sham</i> (1)	31.2 °C	32.4 °C	10
<i>Sham</i> (2)	30.2 °C	32.9 °C	15
<i>Sham</i> (3)	30.1 °C	32.4 °C	13
<i>Sham</i> (4)	30.3 °C	33 °C	17
<i>Experimental</i> (1)	30.1 °C	30.2 °C	70
<i>Experimental</i> (2)	30.3 °C	28.9 °C	52
<i>Experimental</i> (3)	29.7 °C	31.7 °C	54
<i>Experimental</i> (4)	30 °C	30.3 °C	51
<i>Experimental</i> (5)	30.5 °C	31.3 °C	58
<i>Experimental</i> (6)	29.7 °C	28.4 °C	46
<i>Experimental</i> (7)	29.1 °C	31.9 °C	52



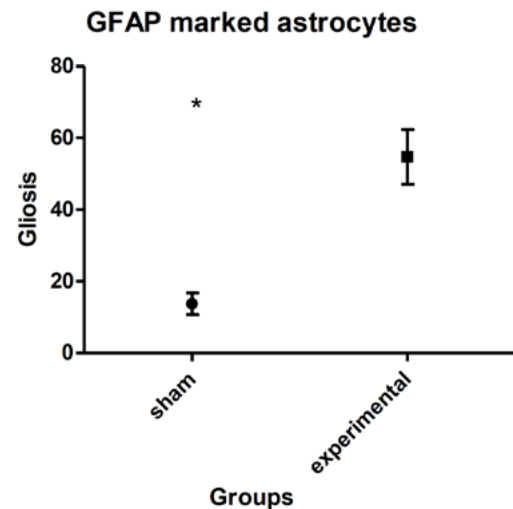
**FIGURE 2.** N = 4, 7 (*sham*, experimental). **A.** Mann-Whitney U test found no statistical difference between temperatures of the groups;  $p = 0.15$ . **B.** Mann-Whitney U test found a statistical difference in temperatures between groups;  $p = 0.01$ . Data shown as mean  $\pm$  SEM.



**FIGURE 3.** Anti-GFAP immunohistochemistry. Microscopic 20x image of a histologic section of injured spinal cord (after 5 weeks) with anti-GFAP immunohistochemistry. The arrows point at some of the marked astrocytes.

the number of activated astrocytes at the site of SCI vs. an unharmed spinal cord. In addition, it provides an important evidence of GFAP as a precise identifier of such cells in the glial scar.

Finally, Figure 5 shows the correlation between the two variables, thermography, and glial scar (represented by GFAP-marked astrocytes). A negative  $r$  of 0.57 with a statistical significance ( $p = 0.03$ ) establishes that the lower the temperature is, the higher the gliosis in that same area is (a pattern seen in the injured animals). Moreover, the graph



**FIGURE 4.** GFAP-marked astrocytes. N = 4, 7 (*sham*, experimental). Student's t-test found a statistical difference between the number of marked astrocytes by immunohistochemistry in the two groups;  $p = 0.0001$ .

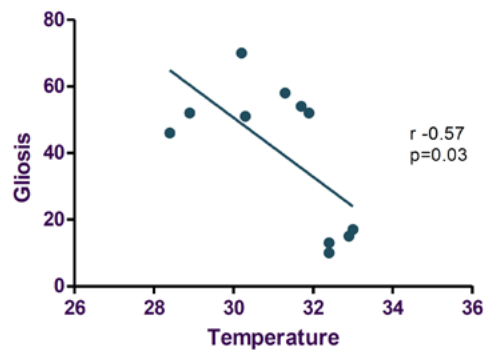
showed two different distributions among the animals in the *sham* vs. the experimental group since the animals without SCI consistently had higher temperatures and lower gliosis, reinforcing our hypothesis.

## 5. DISCUSSION

The results show that there are temperature changes in injured vs. healthy tissue in the spinal cord and, in the case of SCI by contusion, these changes correlate with the amount



### Thermography and anti-GFAP immunohistochemistry correlation



**FIGURE 5.** Thermography and anti-GFAP immunohistochemistry correlation.  $N = 4, 7$  (*sham*, experimental). Spearman's correlation found a negative correlation ( $r = -0.57$ ) between temperature and gliosis ( $p = 0.03$ ). Each dot represents an animal; the animals in the *sham* group show higher temperatures and less gliosis than those in the experimental group.

of gliosis. This leads us to understand that temperature is a useful criterion for identifying healthy vs. scar tissue in the spinal cord. Considering the current research efforts surrounding glial scars and their role in SCI recovery and axonal regeneration, these findings are significant in putting forth novel strategies. Exploring noninvasive ways to identify this tissue is pivotal in terms of developing viable treatment options.<sup>16</sup>

This scar tissue is currently being thoroughly investigated since, even though the scarring process is very important for recovery in the early stages after a SCI, this same scar becomes a recovery barrier during the chronic stages of SCI.<sup>17,18</sup> Accordingly, a study by Rodríguez-Barrera R, *et al.* proved that chronic scar removal combined with immunization with neural-derived peptides resulted in changes that led to improved motor function.<sup>9</sup> Tom EJ, *et al.* showed the success in using the bacterial enzyme chondroitinase ABC for CSPG digestion in combination with a graft, resulting in functional axonal regeneration in an SCI model.<sup>19</sup> Zhang S, *et al.* used rose Bengal scar ablation with promising results, including a significant increase in myelination.<sup>20</sup> Other research groups have tried to remove this scar without favorable results, either because the removal was performed in acute stages after SCI or because their method for identifying the scar tissue was invasive and had effects of its own.<sup>21-23</sup>

Some reports, however, do not recommend glial scar removal based on findings regarding a continuous role the glial scar plays in guiding axonal regeneration throughout chronic stages of SCI. This should be considered and studied further, even though most of the evidence supports scar removal at these chronic stages of injury.<sup>24</sup>

The present study proved equal temperature in the studied spinal cord area before the intervention, and lower temperature in that same area of the injured animals vs. those in the *sham* group after 5 weeks. Since all the animals underwent laminectomy, this elicited an inflammatory response expected after any intervention, which leads to a rise in local temperature. Still, the animals subjected to a SCI after the laminectomy exhibited lower local temperatures at the site of injury 5 weeks later compared to those in the control group. This was to be expected since the *sham* animals were also subjected to a laminectomy and, therefore, there was an inflammatory response. However, the animals with SCI had tissue necrosis and vascular changes besides inflammation.<sup>25</sup> The variations in temperature were subtle but statistically significant when compared between groups.

It is important to consider that this technique is innovative and there is no universal standardized use. The research team took the necessary precautions, including always taking the same distance, always with the same operator, and in random order between all animals, as to decrease possible bias. Nevertheless, even taking these precautions, there is some unavoidable human error as with every operator-dependent machine, as well as temperature variations during the interventions that could be further monitored and controlled in later studies. Future projects could blind the analysis from the operation for better results.

The immunohistochemistry results were also fairly expected since there was a significant rise in astrocyte count in the spinal cords of those animals in the experimental group,<sup>26</sup> and the correlation results further strengthen the hypothesis of the study.

This is a pilot study with a small animal sample and thus additional data are needed to proceed towards clinical research. Still, the concept is solid and should encourage this line of research that ultimately seeks to improve the lives of SCI patients.

## 6. CONCLUSIONS

Our results show that infrared thermography is effective in detecting minimal temperature changes in live tissue. The lower temperatures detected in the spinal cord tissue were associated with higher gliosis among animals in the experimental group (SCI). These findings can be explored in new studies on chronic scar removal in SCI patients without inflicting more damage to the surrounding healthy tissue.





## 7. ACKNOWLEDGMENTS

This research would not have been possible without the contribution of the Physiology Department at Universidad Nacional Autónoma de México.

## 8. CONFLICT OF INTERESTS

The study was funded by the Research Department at Universidad Anáhuac México.

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