

EVALUATION OF THE SENSORIMOTOR CONTROL OF RATS WITH CHITOSAN/FIBROIN 3D-SCAFFOLDS IMPLANTS

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Summary: *Thousands of orthopedic surgical procedures are performed to restore or replace tissues that have been injured by disease or trauma. Normally, the treatment is done by autograft or allograft. They allow for improvement of the patient's life quality although there are some drawbacks including the risk of infection and transplant rejection. Biomaterials have been widely studied and shown to be a potential substitute for conventional treatment. Several materials are used for the preparation of scaffolds, such as chitosan (CHI) and fibroin (SF). This research aimed to evaluate the sensorimotor control of rats with CHI/SF 3D-scaffolds implants. Twelve male Wistar rats (300-400g) were divided into two groups (control and CHI/SF) and submitted to calvarium surgery. These were anesthetized by thiopental sodium (50 mg/kg body wt.), the frontoparietal area was trichotomized and then, the subcutaneous tissue was dissected, in order to expose the calvarium. A fragment from the middle portion of the parietal bone was removed with the aid of a surgical motor. In the control group, the critical defect was filled only with blood clot, while in the CHI/SF group it was filled with the CHI/SF 3D-scaffold. After that, the skin was repositioned and closed using a chirurgic suture. Each animal received a post-surgery intraperitoneal injection with anti-inflammatory and analgesic (1.1 mg/kg body wt.). Functional tests were conducted for evaluating thermal and secondary-mechanical (cutaneous) sensitivities thresholds, and the four-legged grip strength. These tests were conducted immediately before and 7, 14 and 21 days after the surgical procedure. Differences among groups were addressed by means of one-way ANOVA with Tukey's post-hoc test and they were considered significant for $p \leq 0.05$. The secondary-mechanical (cutaneous) results can be put similarly but with a decaying response after 14 days only for the CHI/SF group. Nevertheless, the data were statistically similar among both groups regardless of the time frame. The results showed that the thermal sensitivity test, for control and CHI/SF groups, showed an increase in the reaction time at 50 °C in the postoperative*

period, that was maintained in subsequent analysis periods, when compared to the preoperative period. Concerning the four-legged grip strength test, a statistically similar behavior was observed in the groups regardless of the evaluation time. These results suggest that the clinical differences in all the tests were due to surgical procedure, not by applying CHI/SF 3D-scaffolds. Therefore, according to the employed methodology, it can be concluded that CHI/SF 3D-scaffolds do not change the mechanical and thermal sensitivity, neither the motor performance of rats. Other 3D-scaffolds are being tested as part of this research project and histological tests are been performed.

1 INTRODUCTION

Approximately 10% of the fractures occurred in humans have delayed bone healing. Therefore, some biomaterials are produced in order to fill empty spaces at this repair area and offer support for cell growth and neovascularization [1,2]. All the materials used as synthetic scaffolds offer positive and negative aspects for its application in bone tissue engineering. However, is essential that they have good mechanical, chemical, physical and biological properties, making them potentially suitable for use *in vivo*, reducing the rehabilitation time of patients [3-5].

A three-dimensional porous scaffold (3D-scaffolds) ensures a suitable environment for the regeneration of the injured area, allowing the cells to organize and develop in a similar environment to the original tissue. A scaffold-cell combination is crucial for the success of the surgical procedure [5,6]. Currently, there is a wide variety of biomaterials and production techniques for the manufacture of such implants. Ideally, they all must have some basic requirements, such as biocompatibility, biodegradability, good mechanical properties, similar architecture to the treated area, be reproducible, commercially viable and have low cost [2,7-10].

3D-scaffolds based on chitosan (CHI) are mechanically flexible and easily molded into different shapes. The silk fibroin (SF), in turn, presents a component of stability and mechanical properties due to their β -sheet structure. It provides module balance, tensile strength and elongation, which contributes to the good toughness and ductility properties that can be controlled by the way of preparing the 3D-scaffolds. The union of these biomaterials in a 3D-scaffold should ally biocompatibility with good mechanical strength, modeling and flexibility. Moreover, their production is relatively inexpensive, since these materials are readily available [11-14].

Generally, to evaluate the behavior of small animals in front of a new experimental intervention, the use of tests to evaluate possible changes in mechanical and thermal sensitivity becomes necessary, as well as probable differences in grip strength performance after surgery [15-18]. To date, no systematic studies were reported on the development of 3D-scaffolds using the two materials of interest here applied to neuroscience at *in vivo* tests. Therefore, the aim of this work was to evaluate the sensorimotor control of rats with chitosan/fibroin 3D-scaffolds implants.

2 METHODS AND MATERIAL

2.1 Production of 3D-scaffolds

Silk fibroin (SF) extracted from *Bombyx mori* cocoon was purchased from Huzhou Xintiansi Bio-tech Co., Ltd. (China) while the chitosan (CHI), derived from crab shell, deacetylation degree 85%, was purchased from Sigma-Aldrich (USA). Initially CHI 2% (w/v) was dissolved in 1% aqueous acetic acid. The 3D-scaffolds were prepared using a dispersion of SF (1:1 wt.%) in the CHI solution. To obtain a good homogenization the suspensions remained under magnetic stirring for one day and afterwards sonicated (Ultrasonic Cleaner Thomton 750USC, Unique, Brazil) for 10 min. Subsequently, this suspension was poured into polytetrafluoroethylene mold (height, 40 mm; diameter, 90 mm), frozen for 24h at -20°C and lyophilized (Liotop L108, Liobras, Brazil) for 24 h. The dry samples were removed from the molds and neutralized in a 0.1% (wt.%) NaOH aqueous-ethanolic (8:2 vol.%) solution for 3h. Afterwards samples were washed for 30 min with ultrapure water (UHQ, Purelab, USA) and cross-linked with 2.5% (w/v) sodium tripolyphosphate solution for 3h, followed by washing for 30 min with ultrapure water. The crosslinking step was necessary because the degradation rate of chitosan was too fast, as observed in previous tests (data not show). Finally, the 3D-scaffolds were obtained after another cycle of freezing and lyophilization.

2.2 Physicochemical characterization and *in vitro* evaluation

The physicochemical evaluation and study of the biological behavior *in vitro* of the CHI/SF 3D-scaffolds in contact with STRO+1A, MC3T3-E1 and SaOs-2 cells was held at the Institute of Materials Science of Mulhouse (IS2M, France). According to the standard methodology used, the 3D-scaffolds were considered biocompatible, presenting an interconnected porous structure capable of promoting cell adhesion and proliferation, and osteogenic differentiation of the different cell types tested [19].

2.3 *In vivo* evaluation

2.3.1 General Considerations

Twelve male Wistar rats were used (300-400 g) from the vivarium of Tiradentes University, after approval by the Ethics Committee for Animal Research of the Federal University of Sergipe (CEPA/UFS-1813). The animals were kept in environment temperature (23-25°C) and with fume hood in light-dark cycle of 12 hours, were placed in polypropylene cages with maximum capacity of 05 rats per cage, and, after surgery, they have been allocated individually. The tests were performed during the light cycle, with food and water available *ad libitum*. The use of laboratory animals in the proposed activity was justified by the fact that these 3D-scaffolds have great potential for use in humans. Thus, it would be impractical to suggest alternative models over the use of animals.

2.3.2 Surgical aspects

The animals received general anesthesia under intraperitoneal injection of 50 mg/kg thiopental sodium (Cristália, Brazil). After that, was performed trichotomy of frontoparietal

region of the animal's head, with the help of scissors and razor, with subsequent sterilization using iodized alcohol. One mucoperiosteal incision was made with a scalpel blade No. 10, half-moon shaped in the skull and with the aid of a Molt periosteal and chisel of Ohsenbein No. 01. The total thickness flaps were elevated, widely exposing the cortical bone of the region.

A fragment from the middle portion of the parietal bones, with the help of a motor and a surgical handpiece 16:1, was removed by means of a surgical trephine drill with 5 mm inner diameter and 8.5 mm of outside diameter under abundant and continuous irrigation of saline solution. The dura mater was not disturbed. After removal of internal and external cortical boards, critical defects transfixated with 5 mm in diameter were filled only with blood clot in the control group, while the CHI/SF group, the defect was filled with the CHI/SF 3D-scaffold. Then, the skin was repositioned and sutured with black silk line No. 03 (Johnson & Johnson, Brazil). After the surgery, was administered intraperitoneally 1 mg/kg of injectable Banamine pet (Schering-Plough Veterinary, Brazil) for postoperative antiinflammatory and analgesia induction. The animals were monitored until the end of the anesthesia and accommodated in individual polypropylene boxes, being in recovery for a period of 3 to 5 days from the post-operative period to the beginning of testing.

The methodology of such surgical procedure proposed for this project has already been employed in the study of Valiense et al. [20], where the authors used spherical implants of carbonated apatite in critical defect of rat calvaria in order to observe the effects of this material in bone repair.

2.3.3 Groups

The animals were randomized into Group I (Control) and Group II (CHI/SF). In the first group, the surgical procedure was performed without the placement of a 3D-scaffold, being the defect filled with blood. The second group has the surgical defect performed with the placement of the 3D-scaffold based on chitosan and fibroin. Figure 01 shows the 3D-scaffold of Group II (CHI/SF) after the implantation.



Figure 1: Implanted CHI/SF 3D-scaffold.

2.3.4 Experimental development

Two days before the surgery the animals were acclimated and after that, tests of the secondary-mechanical (cutaneous) sensitivity threshold, thermal sensitivity threshold and four-legged grip strength were performed on the 7th, 14th and 21th day. The animals were discarded only after completion of the 21 day of testing, by an intraperitoneal injection of 150 mg/kg of sodium thiopental (Cristália, Brazil). Figure 02 illustrates the experimental development through the timeline.

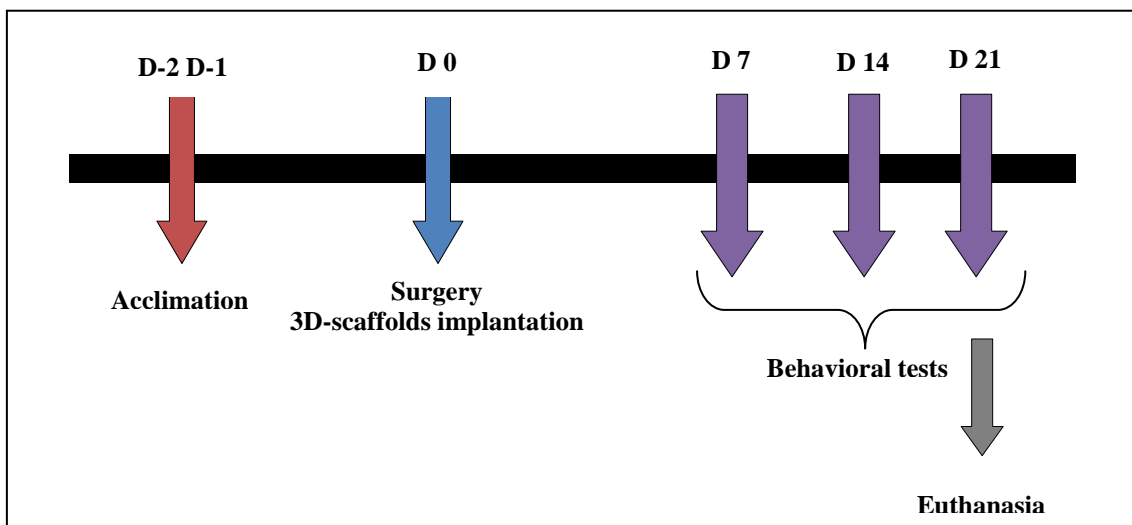


Figure 2: Experimental development timeline.

2.3.5 Mechanical and thermal sensitivity

The secondary-mechanical (cutaneous) threshold was measured by the electronic von Frey Digital Analgesymeter (Model EFF-302, Insight, São Paulo, Brazil). The tip coupled to the equipment's head was applied five times on the central area of the hind paws. The stimulus was discontinued when the paw was withdrawn. The arithmetic average of five applications was recorded as mechanical nociceptive threshold and the difference in values at different times of the experiment was considered to evaluate the threshold oscillations [21].

To evaluate the thermal threshold it was used the Hot Plate (Model EFF-361, Insight, São Paulo, Brazil). In this test, response latency was considered the period that the animals remained on a heated metal plate ($50\pm 5^{\circ}\text{C}$), until reaction to the thermal stimulus characterized by the behavior of shaking or licking its paws [22].

2.3.6 Grip strength performance

Four-legged grip strength was measured by the tension force imposed on the grid platform from the Grip Strength Meter (Model EFF-305, Insight, São Paulo, Brazil). The animal was placed on the grid for three consecutive times, so that it simply remained supported on all

four paws and then removed by the tail. Thus, the animal applied a grip strength on the grid that was recorded by the device, using the value of the arithmetic average of the results recorded [18].

3 RESULTS AND DISCUSSION

According to the results of the secondary-mechanical threshold in the control group, it was observed that after the 7th day after surgery there was a slight increase in mechanical threshold in relation to the preoperative period. In addition, this increase was progressive in the 14th and 21th evaluation days (Figure 03). According to Pogatzki; Raja [23] the following test applications could limit the reduction of the withdrawal threshold due to learning by repetition of the stimulus. This explains the gradual increase of the threshold according to the time measured, in 7th, 14th and 21th days. Nevertheless, there were no statistical differences between the evaluation periods ($p>0.05$).

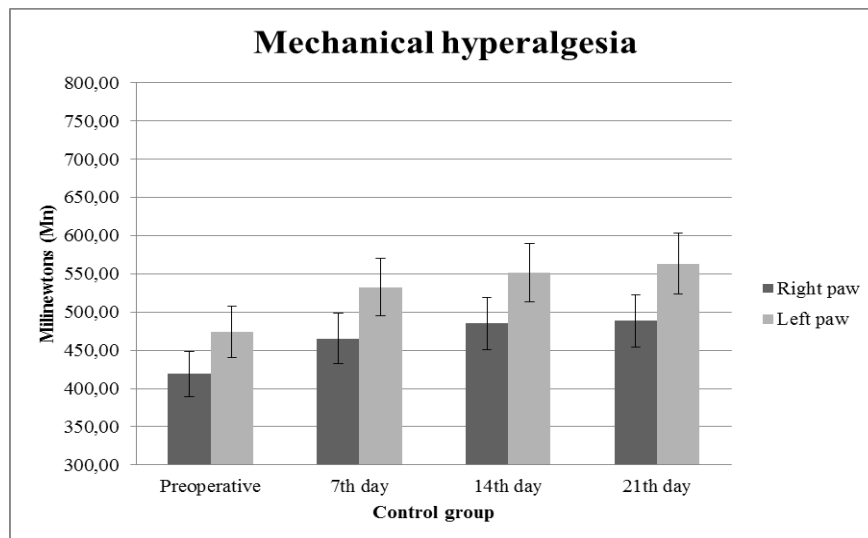


Figure 3: Mechanical hyperalgesia from control group in the preoperative period, and on the 7th, 14th and 21th days postoperatively ($p>0.05$, between the periods analyzed).

In CHI/SF group, there was a decrease of the mechanical hyperalgesia values on the 14th and 21th days postoperatively (Figure 04). However, the results were considered statistically similar ($p>0.05$). According to Bertolini et al. [24] the increase of the secondary-mechanical threshold may demonstrate decrease on the hyperalgesia state. This would indicate an increase in pain response in the 14th and 21th day of the CHI/SF group, although results were not statistically significant ($p>0.05$). It was not possible to compare the results of this research with similar studies because it was the first one to developed the proposed methodology.

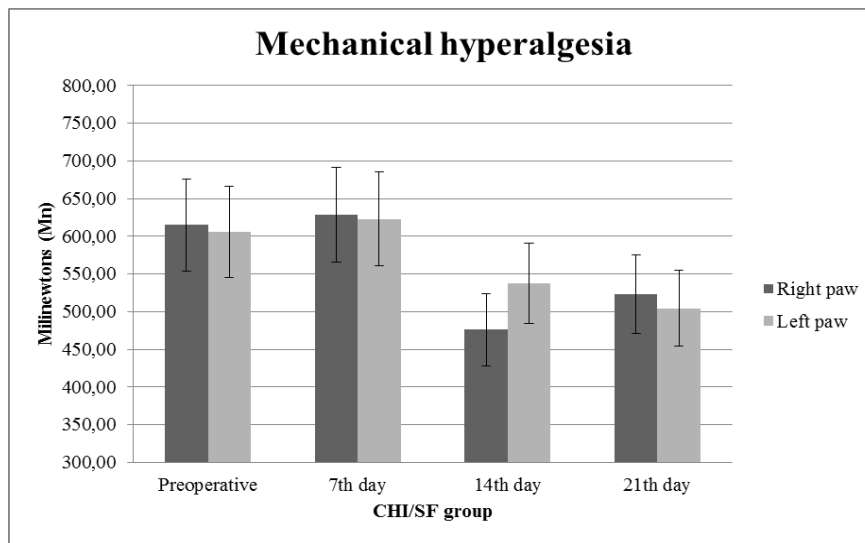


Figure 4: Mechanical hyperalgesia of CHI/SF group in preoperative period, and on the 7th, 14th and 21th days postoperatively ($p>0.05$, between the periods analyzed).

When comparing the 02 groups in relation to the studied time periods, it was observed that the behavior of animals against mechanical threshold was similar ($p>0.05$), which may be inferred that the use of the produced 3D-scaffold did not affect this analysis.

During the analysis of thermal hyperalgesia, it was observed that the behavior of the animals was demonstrated by licking of the front or hind paws in all groups, regardless of the period of analysis. The time required for this response preoperatively was $6,73\pm 2,82s$ in the control group and $6,10\pm 1,23s$ in the CHI/SF group ($p>0.05$). On the 7th day after surgery there was an increase in response time in the groups analyzed in comparison to the preoperative period ($p<0.05$), and this response was maintained in subsequent analysis periods. When the control group was compared to the CHI/SF group, it was observed that the behavior of animals facing this analyzed parameter remained statistically similar ($p>0.05$).

According to Tembhrne; Sakarkar [25], increased thermal sensitivity threshold using the Hot Plate may indicate loss of the perception of pain attributed to possible nerve damage. The absence of statistical differences between the control group and the CHI/SF group suggests that the increase in thermal sensitivity threshold may have occurred due to the surgical procedure and not by the application of the tested 3D-scaffold.

Finally, the last parameter analyzed was the function of motor performance through Grip Strength Meter. The groups were seen as homogeneous in the preoperative period ($p>0.05$), but on the 7th day analysis there was an increase in grip strength in both, with a significant difference from the first analysis ($p<0.05$). This increased strength remained on the 14th and the 21th day of analysis in both groups (Figure 05).

Tembhrne; Sakarkar [25] compared the grip strength between normal and diabetic rats and found that the grip strength reduction indicated muscular weakness and induced neuropathy. Then, the increase in grip strength on both groups may indicate neuromuscular integrity maintenance even after surgical procedure. Figure 05 shows data obtained through

Grip Strength Meter. Moreover, when compared control and CHI/SF groups, there is no statistic difference, showing that the 3D-scaffold implantation did not influence the results on this parameter ($p>0.05$).

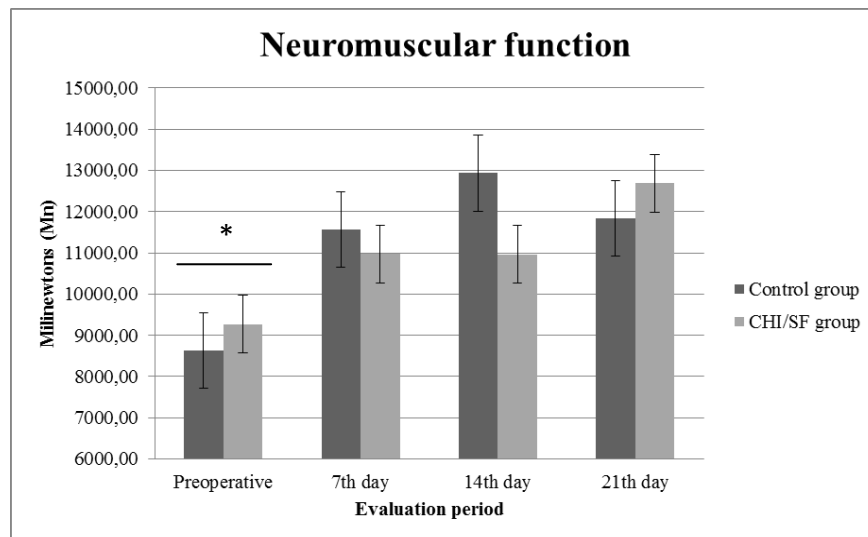


Figure 5: Mechanical hyperalgesia of CHI/SF group in preoperative period, and on the 7th, 14th and 21th days postoperatively (* $p<0.05$), between the periods analyzed).

4 CONCLUSION

As it can be concluded by the applied methodology, the use of 3D-scaffolds based on chitosan and silk fibroin do not interfere significantly in sensorimotor control of rats after the surgical procedure. Probably, the slight changes occurred were attributed solely to the surgery. It is suggested that other types of scaffolds and other evaluation techniques can be used to reaffirm the findings of this work.

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