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Original Article *Özgün Araştırma*

A MODIFICATION OF THE CHRONIC CONSTRICTION INJURY MODEL OF NEUROPATHIC PAIN: SILK USAGE INSTEAD OF CHROMIC CATGUT

Nöropatik Ağrı Kronik Sıkıştırma Hasarı Modelinde Bir Değişiklik: Krome Katgüt Yerine İpek Kullanımı

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ABSTRACT

Objective: In the chronic constriction injury (CCI) neuropathic pain rat model, usage of chromic catgut causes intense neuroinflammation and also undesirable conditions such as autotomy. This causes difficulties in the application and interpretation of pain behaviour tests. In this study, it was aimed to investigate the suitability of the use of surgical silk as a suture material in the CCI model, based on behavioural tests and certain biomarkers.

Material and Methods: CCI model was created by loosely ligating the right sciatic nerves of rats with surgical silk. Tactile allodynia, thermal and mechanical hyperalgesia were evaluated on the 2nd, 7th, 14th, 21st and 29th postoperative days. Expressions of glial fibrillary acidic protein (GFAP), signal transducer and transcription activator 3 (STAT3) and its phosphorylated form (p-STAT3) were evaluated from the spinal cord tissue by western blotting technique.

Results: Sciatic nerve-ligated rats did not lose weight but gained weight on days 14, 21, and 29 like control rats. Statistically significant decreases were found in pain thresholds in three different pain tests after the operation. The decrease in the pain threshold was accompanied by a statistically significant increase in GFAP protein expression in the spinal cord on days 2 and 14 simultaneously. No statistically significant difference was found in the expression of STAT3 or p-STAT3 protein in the spinal cord at any time.

Conclusion: The absence of weight loss and autotomy expected from the classical CCI model, the reversal of the decrease in the pain threshold, the concomittant increase in GFAP protein expression in the spinal cord, and the absence of change in STAT3 and p-STAT3 expression in our study suggest that the model we created by tying 3 loose silk knots on the sciatic nerve has a milder course of neuroinflammation.

Keywords: *Allodynia, hyperalgesia, catgut, silk*

ÖZ

Amaç: Kronik konstriksiyon yaralanması (KKY) nöropatik ağrı sıçan modelinde krome katgüt kullanımı yoğun nöroinflamasyona ve ototomi gibi istenmeyen durumlara neden olmaktadır. Bu durum ağrı davranış testlerinin uygulanmasında ve yorumlanmasında zorluklara neden olmaktadır. Bu çalışmada, KKY modelinde cerrahi ipeğin dikiş materyali olarak kullanımının uygunluğunun davranış testleri ve bazı biyobelirteçlere dayalı olarak araştırılması amaçlandı.

Gereç ve Yöntemler: KKY modeli, sıçanların sağ siyatik sinirlerinin cerrahi ipek ile gevşek bir şekilde bağlanmasıyla oluşturuldu. Taktil allodini, termal ve mekanik hiperaljezi ameliyat sonrası 2., 7., 14., 21. ve 29. günlerde değerlendirildi. Glial fibriller asidik protein (GFAP), sinyal dönüştürücü ve transkripsiyon aktivatörü 3 (STAT3) ve bunun fosforile edilmiş formunun (p-STAT3) ekspresyonları, omurilik dokusundan *western blot* tekniği ile değerlendirildi.

Bulgular: Siyatik siniri bağlanan sıçanlar kilo kaybetmedi, ancak kontrol sıçanları gibi 14, 21 ve 29. günlerde kilo aldı. Operasyon sonrası üç farklı ağrı testinde ağrı eşiklerinde istatistiksel olarak anlamlı düşüşler saptandı. Ağrı eşiğindeki azalmaya, aynı anda 2. ve 14. günlerde omurilikteki GFAP protein ekspresyonunda istatistiksel olarak anlamlı bir artış eşlik etti. Omurilikte STAT3 veya p-STAT3 proteininin ekspresyonunda herhangi bir zamanda istatistiksel olarak anlamlı bir fark bulunmadı.

Sonuç: Klasik KKY modelinden beklenen kilo kaybı ve ototomi olmaması, ağrı eşiğindeki düşüşün tersine dönmesi, buna eşlik eden omurilikte GFAP protein ekspresyonunda artış, STAT3 ve p-STAT3 ekspresyonunda değişiklik olmaması çalışmamızda siyatik sinir üzerine 3 adet gevşek ipek düğüm atarak oluşturduğumuz modelde nöroinflamasyonun daha hafif seyrettiğini göstermektedir.

Anahtar Kelimeler: *Allodini, hiperaljezi, katgüt, ipek*

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INTRODUCTION

Neuropathic pain (NP) is an important health problem that is difficult to diagnose and treat. NP manifests itself in various clinical signs because of nerve damage or dysfunction in the central or peripheral nervous system. Major clinical manifestations of NP are allodynia (pain to non-painful stimuli) and hyperalgesia (exaggerated pain response to a painful stimulus) (1).

There are historically many experimental animal models of NP and other chronic pain syndromes. One of them is the chronic constriction injury (CCI) which is the first model of partial peripheral mononeuropathy described by Bennett and Xie in 1988. The CCI model allows us to observe the increase in chemical and thermal sensitivity, cold and mechanical allodynia, and motor disturbances observed in patients with NP, in addition to findings such as intraneural oedema, focal ischemia, and Wallerian degeneration. Four knots are loosely tied with chromic catgut to the surgically dissected sciatic nerve (2). The tightness of the knots must be adjusted so that the epineural circulation is not disturbed. One of the disadvantages of the model is that the tightness of the knots around the sciatic nerve varies according to the person performing the procedure (3,4). It causes avoidance behaviours such as not putting weight on to the operated side of the animal. Ipsilateral autotomy (70%) may be seen (2). Autotomy and avoidance behaviour can lead to difficulties in performing pain tests. While chromic catgut is preferred in rats in general, findings have been published showing that the use of silk in mice can create a better neuropathic pain model than the use of catgut (5).

Activation of astrocytes plays a significant role in the pathogenesis of neuropathic pain. Increased glial fibrillary acidic protein (GFAP levels and astrogliosis (astrocyte hypertrophy, morphological cell body enlargement and thickening of cell extensions) are often used in pain studies as indicators of astrocyte activation (6). Astrocytes have a significant role in both the initiation and chronicity of neuropathic pain and inflammatory pain (7). The larger the increase in GFAP staining, the greater the degree of hyperalgesia (8).

The main intermediate filaments of astrocytes are vimentin and GFAP. During astrocyte development, early vimentin dominance shifts to GFAP as the astrocyte matures (9). GFAP is thought to have the function of increasing astrocyte endurance and mechanical strength. It is generally accepted that an increase in GFAP is a reliable indicator of astrocyte activation (10).

Peripheral nerve damage causes IL-6 production; IL-6 activates the JAK2-STAT3 system and increases the amount of active form, phosphorylated signal transducer and transcription activator 3 (p-STAT-3) on spinal microglia. Blocking the STAT-3 pathway by the JAK2 inhibitor AG490 attenuated both mechanical allodynia and thermal hyperalgesia in sciatic nerve-ligated (SNL) rats (11).

In this study, we aimed to evaluate the use of silk in the CCI model by performing pain tests in rats and determining the expression of two reliable biomarkers, GFAP and STAT-3. The main reason for our choice of silk is that we think it will cause less tissue reaction compared to chromic catgut.

MATERIALS AND METHODS

Animals

Adult, male and female Wistar rats weighing between 200-300 g were obtained from Ege University Laboratory Animal Application Research Center (EGEHAYMER) before starting the study**.** The rats were kept in plastic cages with sawdust on the floor for one week for adaptation to the environment, with one rat in each cage. They were fed with standard rat chow and water *ad libitum* in a 12-hour night-12-hour day cycle at 24±2°C. The study protocol was approved by the Local Animal Ethics Committee Ege University (Approval no: 2017-028). All experiments were conducted in accordance with the "Guidelines for Animal Experimentation of the International Association for the Study of Pain (IASP)".

Experimental Groups

The animals were divided into 7 experimental groups in the study. Control (C) and sham operated (SO) groups (n=9 and n=8 respectively) were allowed to live for 29 days. In SNL rats (n=30), the SNL2 group was allowed to live for 2 days, the SNL7 group for 7 days, the SNL14 group for 14 days, the SNL21 group for 21 days, and the SNL29 group for 29 days.

Induction Of Neuropathic Pain Model

The CCI model was applied using silk instead of chromic catgut. Experimental animals were anaesthetized by intraperitoneal administration of ketamine-xylazine (50-20 mg/kg). The right main Sciatic nerve was exposed by blunt dissection of the biceps femoris muscle at the level of the right thigh. Three knots were placed around the nerve with 4.0 silk, tight enough, not to disrupt the epineural circulation, and with 1 mm spacing. After suturing the muscle and fascia, the skin was sutured with 3.0 silk. Wound cleaning was done with surgical antiseptic solution and the animals were placed in their cages to keep for maximum 29 days until sacrifice. Pain thresholds were followed for 29 days in control animals without any surgical procedure. In the sham-operated group, the same procedures were performed without tying the sciatic nerve.

Behavioral Tests

Dynamic plantar esthesiometer (Ugo Basile, Italy) for allodynia, plantar test (Ugo Basile, Italy) for thermal hyperalgesia and analgesia meter (Ugo Basile, Italy) for

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mechanical hyperalgesia were used in experimental animals before and after the operation, and the tests were applied to both paws. Before the test, the animals were kept in wire mesh compartments for about 30 minutes to allow them to adapt to the environment.

Paw withdrawal times were recorded in seconds for the plantar test and esthesiometer, and in grams, for the analgesia meter, when the animal pulled its paw. Tests were applied at a maximum of 50 g and not exceeding 20 seconds to prevent skin sensitivity and not to cause thermal damage. Three measurements were made at five-minute intervals in each animal and the average was taken. Data obtained were compared with the measurements performed before the operation and the pain threshold was expressed as a percentage change. The percentage change was calculated as:

Percent (%) change in pain thresholds=(measured value/baseline value)*100

Protein Expression With Western Blot Technique

L3-L4 spinal cord segments of sacrificed animals were taken. Tissues (100mg/ml) were transferred in homogenization buffer (20 mM Tris hydrochloric acid [HCL], 2 mM ethylene glycol tetraacetic acid [EGTA], 5 mM ethylene diamine tetraacetic acid [EDTA], 10 mM dithiotreitol [DTT], 0.5 mg/ml aprotinin, 0.001 mg/ml pepstatin, 0.001 mg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1% phosphatase inhibitor cocktail, in pH:7.7) were transferred on the ice. After centrifugation at 1400 RPM +4°C for 30 minutes, the protein samples remaining in the supernatant were taken in Eppendorf tubes. Protein concentrations were measured by the classical Lowry method according to the absorbance values read at 750 nm wavelength the spectrophotometer and recorded as µg/µl. 50 µg total protein samples were mixed with 1/4 of 4X Laemmeli buffer and boiled at 100°C for 3 minutes and then loaded onto 4% stacking and 10% separating sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (12). The proteins were transferred onto the Nitrocellulose membrane. The membrane was blocked by incubation in a blocking buffer for 1 hour at room temperature. Membranes cut from the appropriate places according to the molecular weight of the protein to be studied were incubated overnight at +4°C with primary antibodies (Anti-GFAP Millipore MAB360 1:2000, Anti-STAT3 Abcamab119352 1:1000, Anti-pSTAT3 Abcamab76315 1:1000, Anti-beta Actin SIGMA A5441 1:20000). Afterwards, the membranes were washed with washing buffer 3 times at 5 minutes intervals and incubated with secondary antibodies (Anti Mouse IgG SIGMA GENA9311:10000, Anti-rabbit IgG SIGMA GENA9341:10000) in blocking buffer for 2 hours at room temperature. The membranes were covered with freshly prepared electrochemiluminescence (ECL) solution (RPN2236 ECL AMERSHAM) in the dark room. After 5 minutes of ECL incubation, band images were obtained by scanning with a chemiluminescence device (LI-COR C-DIGIT Blot Scanner). Densitometric analyses of the bands were performed using the image Studio Digits v4.0 program. Obtained values were normalized with beta-actin values.

Statistical Analysis

No formal a priori sample size calculation was made before the experiments. The sample size was determined to be at least 6 in each group, considering similar studies and our previous experience. Considering possible losses, 6-9 animals were used in each group.

All numerical data were presented as mean±standard error, and categorical data were summarized using frequency and ratio values. In behavioural tests, the percent changes in pain threshold were obtained for each measurement point by comparing the pre-operative (day 0) pain thresholds of each animal to the pain thresholds measured on the postoperative days (2., 7., 14., 21. and 29. days). In Western Blotting analysis, the densitometric values of STAT3, p-STAT3 and GFAP bands were normalized by the densitometric values of the β-actin bands incubated simultaneously.

All data from behavioral tests and body weight measurements were analyzed with parametric tests as they were normally distributed as confirmed by the Shapiro-Wilk test. However, protein expression data obtained from western blot analyzes did not follow a normal distribution; therefore, nonparametric tests were used for these data.

Paired t-test was used to test the significance of the difference between body weights before and after the experiment in each group. The assumption of normality in numerical variables was checked with the Shapiro-Wilk test. In parallel with the results, repeated measurement ANOVA was used to evaluate the effects of time (day 2, day 7, day 14, day 21 and day 29), group (control-operated) and interaction in pain tests. In the variables that were found to have a significant interaction, the time effect was evaluated separately in the control and operated groups with repeated measurement ANOVA, and when the result was found to be statistically significant, binary time comparisons were made with Bonferroni correction. Comparisons between groups were made using a separate t-test using all data on each day. The Mann-Whitney U test was used to test for differences between groups, as the data on protein expression did not fit the normal distribution.

Statistical analyses were performed using the IBM SPSS Statistics 25.0 (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) package program. The level of significance was determined as 0.05 in all analyses.

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RESULTS

The body weights of C and SNL rats before and after the experiment are shown in Table 1. Weight loss did not occur in any of the experimental groups; the rats continued their normal development. Rats in C and SNL14, SNL21 and SNL29, but not SNL2 and SNL7 groups, gained weight significantly.

Table 1: Body weight of rats (grams) in control and sciatic nerve ligated (SNL) groups

GROUPS	BEFORE	AFTER
Control	240.2±5.2	271.1±10.0*
SNL2	280.3±9.8	291.7±16.2
SNL7	264.8±6.4	273.2±12.6
SNL14	250.0±11.2	296.3±15.6*
SNL21	235.8±10.2	301.7±21.3**
SNL29	214.8±5.5	313.3±24.7**

SNL: Sciatic nerve-ligated
*Data are presented as mean ± standard error of mean, n= 6-9. *p<0.05; **p<0.01 after vs before*

In addition, when the clinical characteristics of rats with NP was evaluated, there was no deterioration in general condition or any death in rats. However, especially in the first week after the operation, rats refrained of giving the body weight to the operated side and excessive licking behaviour was observed in rats.

Behavioral Pain Tests

Evaluation of allodynia in dynamic plantar esthesiometer

Pain thresholds in the right paw (operated side) of the SNL group on days 2 and 7 were statistically significantly reduced when compared to the right paws of the C group (p<0.001; p<0.001; Figure 1A) and SO group (p<0.01; p<0.001; Figure 1A). In the left paw, there was no statistically significant change in pain thresholds over time (Figure 1B).

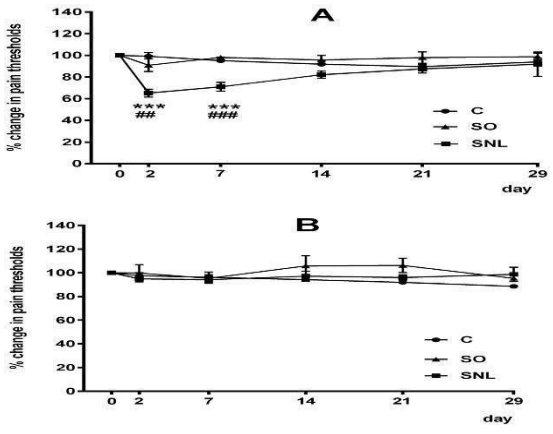

Figure 1: Percent change of pain thresholds of the right paw (A) and contralateral paw (B) in the experimental groups as measured by dynamic plantar esthesiometer. Data are presented as mean ± standard error of mean, n=8-30. *p<0.05; **p<0.01 and ***p<0.001 SNL vs C; # p<0.05; ## p<0.01 and ### p<0.001 SNL vs SO. C: Control, SO: Sham-operated, SNL: Sciatic nerve ligated

Evaluation of thermal hyperalgesia in the plantar test

Pain thresholds in the right paws of the SNL group on the 2nd, 7th, 14th and 21st days after the operation were significantly reduced compared to the right paws of the C group (p<0.001; p<0.001; p<0.05 and p<0.01, respectively; Figure 2A). Differently, pain thresholds decreased significantly on the 2nd and 7th days when SNL group compared to the SO group (p<0.001; p<0.001, respectively; Figure 2A). The right paw pain thresholds of the SO group were significantly decreased on the 2nd, 14th and 21st days compared to the C group (p<0.001; p<0.01; p<0.01; respectively; Figure 2A). In the left paw, there was no statistically significant change in pain thresholds over time compared to the other two groups (Figure 2B).

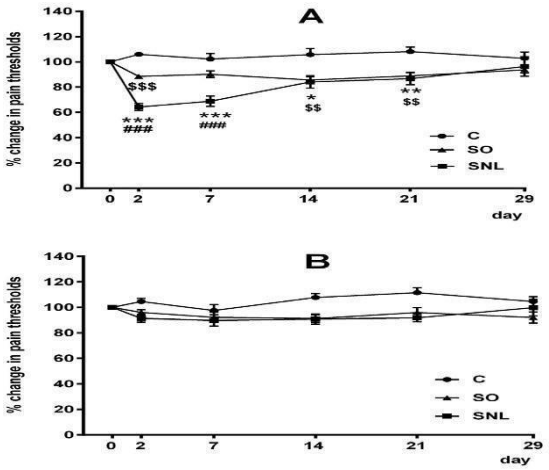

Figure 2: Percent change of the pain thresholds of the right paw (A) and contralateral paw (B) in the experimental groups as measured by plantar test. Data are presented as mean ± standard error of mean, n=8-30. *p<0.05; **p<0.01 and ***p<0.001 SNL vs C. # p<0.05; ## p<0.01 and ### p<0.001 SNL vs SO. $ p<0.05; $$ p<0.01 and $$$ p<0.001 SO vs C. C: Control, SO: Sham-operated, SNL: Sciatic nerve ligated.

Evaluation of mechanical hyperalgesia in analgesia meter

Pain thresholds were significantly reduced in the right paws of the SNL group on the 2nd, 7th, 14th and 21st days after the operation, when compared to the right paws of C group rats (respectively p<0.001; p<0.001; p<0.01); p<0.05; Figure 3A). Likewise, the pain thresholds were reduced in the right paws of the SNL group on 2, 7, 14, and 21 days after the operation, when compared to the SO group rats (p<0.001; p<0.001; p<0.001; p<0.05, respectively; Figure 3A). In the left paw, there was no statistically significant change in pain thresholds over time compared to the other two groups (Figure 3B).

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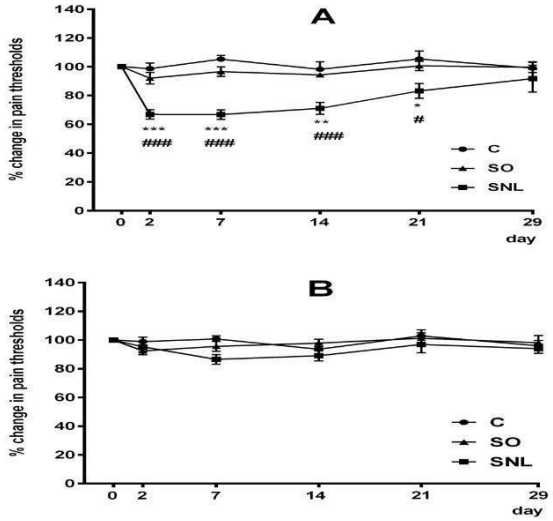

Figure 3: Percent change of the pain thresholds of the right paw (A) and contralateral paw (B) in the experimental groups as measured by analgesia meter. Data are presented as mean ± standard error, n=8-30. *p<0.05; **p<0.01 and ***p<0.001; SNL vs C; # p<0.05; ## p<0.01 and ### p<0.001 SNL vs SO. C: Control, SO: Sham-operated, SNL: Sciatic nerve ligated.

Western Blotting Results

Evaluation of GFAP expression

GFAP expression in the spinal cord tissue of rats in the SNL group was found to be statistically significantly higher on the 2nd and 14th days than in the C group (p<0.05; Figure 4). As can be seen in the Figure 4, although GFAP expression increased on the 7th day, statistical significance could not be demonstrated due to individual differences between rats.

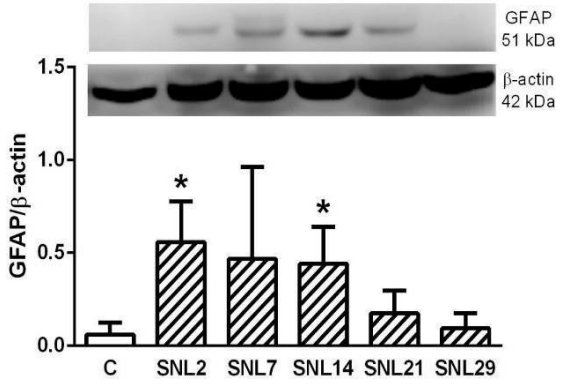

Figure 4: GFAP protein expression in the spinal cord tissue of control (C) and sciatic nerve ligated (SNL) rats. Data are presented as mean ± standard error, n=4-5. *p<0.05; SNL vs C. C: Control, SNL: Sciatic nerve ligated

Evaluation of STAT3 and p-STAT3 expression

The expression of STAT3 (Figure 5A) and p-STAT3 (Figure 5B) in the spinal cord tissue of rats in the SNL group did not differ statistically significant from the C group.

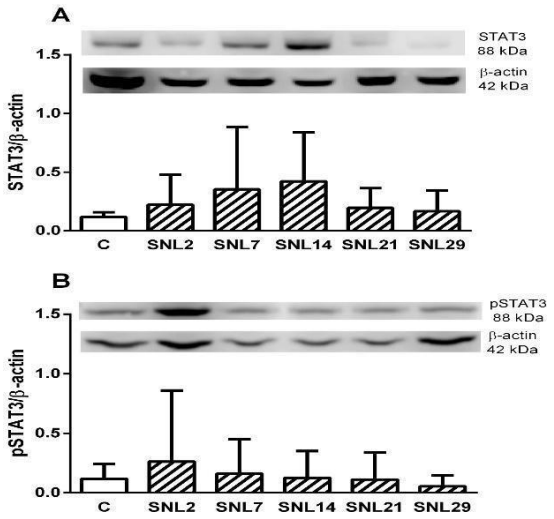

Figure 5: STAT3 (A) and p-STAT3 (B) protein expression in spinal cord tissue of control (C) and sciatic nerve ligated (SNL) rats. Data are presented as mean ± standard error, n=6. C: Control, SNL: Sciatic nerve ligated

DISCUSSION

With our modification, we prevented postoperative weight loss and general condition deterioration in rats. A clinical condition called chromodacryorrhea (red tear), which is rarely seen in the early postoperative period in the classical CCI model, was not seen in our study (2). The rate of "autotomy" differs between publications with CCI model, nevertheless, "mild to moderate autotomy" has been reported at a rate of up to 70% (2,13). Autotomy is the mutilation of the affected paw by the animal itself. Autotomy may cause hypoesthesia or normal responses to mechanical or thermal stimuli and may result in no nociceptive reactions occurring in the respective rats (14). In our study autotomy was not observed in any of the animals and the rats were more stable clinically; therefore, it was thought that the silk used as suture material did not cause such intense inflammation as chromic catgut.

Looking at the behavioural tests, thermal and mechanical hyperalgesia were especially prominent on the 2nd, 7th, 14th and 21st days of the disease, and all pain thresholds returned to their preoperative values on the 29th day. Differently, the decrease in pain threshold returns after the 14th day of dynamic plantar esthesiometer. These findings indicate that tactile allodynia disappeared earlier than thermal and mechanical hyperalgesia in our model. In a comparative study using silk and catgut in rats, it was reported that both methods induced mechanical allodynia and mechanical hyperalgesia, but neuropathic pain symptoms remained stable for up to 56 days in catgut use and weakened in 21 and 28 days when silk was used (15). During our study, the sham operation caused thermal hyperalgesia compared to the control group. It

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has been shown that sham operation leads to long-lasting pain avoidance behaviour (16). There are also studies in the literature showing that sham operation causes both thermal hyperalgesia and mechanical allodynia (17). In our study, the sham operation caused only thermal hyperalgesia. A decrease in the pain threshold was accompanied by a concomitant increase in GFAP protein expression in the spinal cord. Although clinically mild neuropathy in our study, pathology at the level of GFAP protein expression in the spinal cord indicates that central sensitization and therefore inflammation at the spinal cord level can occur even in this mild neuropathic pain animal model.

We found that GFAP expression increased in the spinal cord, especially on the 2nd and 14th days of neuropathic pain. Our findings support the argument that astrocytes play a role in both the initiation and maintenance of chronic pain (18). The absence of statistically significant increase in GFAP expression on the 21st and 29th days is parallel to the attenuation of the clinical characteristics of hyperalgesia and allodynia in rats. This finding supports the idea that spinal astrocytes play an important role in the persistence of allodynia and hyperalgesia in chronic pain conditions (19).

The second parameter evaluated at the spinal cord level in our study is the expression of both total and phosphorylated form of STAT3. Likewise, it has been shown that activation of the STAT3 pathway is required for damage-related astrocyte proliferation in the neuropathic pain model created by L5 or L6 SNL, and the STAT3 pathway itself plays a role in the continuity of tactile allodynia (20). In a study by Dominguez et al. in the L5-L6 SNL model, total STAT3 protein expression in the spinal cord tissue of neuropathic rats did not change, while phosphorylated STAT3 protein increased significantly on days 1 and 2 after nerve injury with increase lasting up to 15 days and expression decreased to control levels on day 21 (11). Contrarily, both total STAT3 and p-STAT3 protein expression was not different from the control in our study. Considering the protein expressions, it is noteworthy that total STAT3 tended to increase in the first 14 days (see Figure 5A) and p-STAT3 in the second day (see Figure 5B). However, this increase could not be statistically proven because there was too much variation between individual animal responses. On the other hand, when the original Western Blotting bands and the data in the graph presented in the study of Dominguez et al. are examined, it is noteworthy that this increase was not observed on the 7th day (11). In another study, STAT3 mRNA expression was evaluated by RT-PCR technique in the spinal cord dorsal horn in the L5-L6 SNL model, and mRNA expression was found to be high on the attached side on the 5th day after ligation (20). Therefore, there are expression differences at the translational and transcriptional levels for total STAT3 and p-STAT3 protein in publications. In addition, although it has been stated that there is STAT3 activation in the spinal cord as a common response in different nerve injury models, information about the timing of activation is also contradictory (21).

This study has several limitations. Due to using only young adult rats, our findings may not be generalizable to different age groups. The high individual variations observed in western blot analyzes may have affected the statistical significance of changes in protein expressions. However, it should not be overlooked that statistically significant results were obtained. The sample size in our study is consistent with similar studies using the CCI model (3). Obtaining significant differences in statistical analysis suggests that the sample size in the groups is sufficient. However, using larger sample groups in future studies may increase the generalizability of the results. Although our study design did not include a direct comparison with chromic catgut, future studies comparing both techniques in the same experimental setup may provide clearer information on the differences between these two materials.

The absence of weight loss and autotomy, the earlier reversal of the decrease in pain thresholds, the increase in GFAP, and the lack of change in STAT3 and p-STAT3 expression collectively indicate that the use of silk in the CCI model resulted in a milder neuroinflammation. Further studies are required to evaluate other mediators of neuroinflammation at different steps of glial activation (IBA-1, p-38 and phosphorylated p-38 as glial biomarkers, cytokines such as TNF-α and IL-1β etc.) at the spinal cord level in this model.

Conflict Of Interest: There is no conflict of interest between the authors.

*Researchers' Contribution Rate Statement***:** Concept/Design: RSŞ, AÖ, SÜG; Analysis/ Interpretation: RSŞ, AÖ, SÜG; Data Collection: RSŞ, AÖ, SÜG; Writer: RSŞ, AÖ, SÜG; Critical Review: RSŞ, AÖ, SÜG; Approver: RSŞ AÖ, SÜG.

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Ethics Committe Aproval: The study protocol was approved by the Ege University Faculty of Medicine Local Animal Ethics Committee (2017-028).

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