

**PERIPHERAL TRANSCUTANEOUS ELECTRICAL NERVE
STIMULATION AND GAIT ANALYSIS IN RATS WITH
SPINAL CORD INJURY**

by

Berfuğ Yaren Karaharman

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**PERIPHERAL TRANSCUTANEOUS ELECTRICAL NERVE
STIMULATION AND GAIT ANALYSIS IN RATS WITH
SPINAL CORD INJURY**

APPROVED BY:

Prof. Dr. Burak Güçlü
(Thesis Advisor)

Assist. Prof. Pınar S. Özbay

Prof. Dr. Berrak Yeğen

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ACADEMIC ETHICS AND INTEGRITY STATEMENT

I, Berfug Yaren Karaharman, hereby certify that I am aware of the Academic Ethics and Integrity Policy issued by the Council of Higher Education (YÖK) and I fully acknowledge all the consequences due to its violation by plagiarism or any other way.

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ABSTRACT

PERIPHERAL TRANSCUTANEOUS ELECTRICAL NERVE STIMULATION AND GAIT ANALYSIS IN RATS WITH SPINAL CORD INJURY

Electrical stimulation strategies are investigated in the literature to recover sensorimotor impairments in spinal cord injury. In this study, transcutaneous electrical nerve stimulation (TENS) (pulse width: 0.3 ms, pulse frequency: 2 Hz, amplitude: $2 \times$ motor threshold, duration: 30 min. at 3 days/week) was applied bilaterally to the tibial nerve rats with spinal cord injury (SCI). The study included sham (n=10), SCI (n=15), NS-sham (n=6), and NS-SCI (n=18) with endpoints at day 1 (D1), day 7 (D7), month 1 (M1), month 2 (M2). After T8-T9 laminectomy, contusion-type SCI was induced by using a computer-controlled custom-made impactor device (contactor diameter: 2.3 mm, peak force: 0.9-2 N, displacement: 1.75 mm, duration: 0.5 s). The severity (moderate-to-severe) of the injury and recovery was measured Basso, Beattie ve Bresnahan (BBB) locomotor rating scale. The mechanical withdrawal threshold was measured by von Frey filaments. DeepLabCut system was used to track each paw from high-speed (120 fps) video recordings and gait parameters (stance/swing/stride durations, limb duty factor, stride length, footprint area) were extracted in Matlab before detrending for speed. Average BBB scores were significantly decreased after the injury (D1: 0). The recovery was higher at NS-SCI (BBB: 17.5) than SCI (BBB: 8) ($p = 0.003$) at M2. vF scores also improved but were not statistically different between SCI and NS-SCI ($p = 0.495$). In gait parameters, the facilitative effect of TENS was observed up until D14; however, there was a reversal trend in limb duty factor and footprint area after D14. Overall, TENS had positive effects on locomotor function and stepping after SCI. However, synchronized application of TENS with stepping may have better results in terms of gait parameters in future studies.

Keywords: Contusion, Spinal Cord Injury (SCI), Transcutaneous Electrical Nerve Stimulation (TENS), Gait, Locomotor function

ÖZET

OMURİLİK YARALANMASI OLAN SIÇANLARDA PERİFERİK TRANSKÜTANÖZ ELEKTRİKSEL SİNİR STİMÜLASYONU VE YÜRÜME ANALİZİ

Literatürde omurilik yaralanması sonrasında sensorimotor bozuklukları iyileştirmek için çeşitli elektriksel stimülasyon stratejileri araştırılmıştır. Bu çalışmada, transkütanöz elektriksel sinir stimülasyonu (TENS)(darbe genişliği: 0.3 ms, darbe frekansı: 2 Hz, genlik: $2 \times$ motor eşik, süre: 30 dk. haftada 3 gün) omurilik yaralanmalı (OY) sıçanlarda tibial sinire bilateral olarak uygulandı. Çalışmaya sham (n=10), SCI (n=15), NS-sham (n=6) ve NS-SCI (n=18) grupları ve son noktalar 1. gün (D1), 7. gün (D7), 1. ay (M1) ve 2. ay (M2) olarak belirlendi. T8-T9 laminektomiden sonra, bilgisayar kontrollü özel yapım impaktör cihazıyla (kontaktör çapı: 2,3 mm, tepe kuvveti: 0,9-2 N, yer değiştirme: 1,75 mm, süre: 0.5 s) kontüzyon tipi omurilik yaralanması induklendi. Yaralanmanın ve iyileşmenin şiddeti (orta-şiddetli) Basso, Beattie ve Bresnahan (BBB) lokomotor derecelendirme ölçeği ile ölçüldü. Mekanik geri çekilme eşiği von Frey filamentleri ile ölçüldü. Yüksek hızlı (120 fps) video kayıtlarından her bir patiyi izlemek için DeepLabCut sistemi kullanıldı ve yürüyüş parametreleri (basma/salınım/adım fazları, basma fazı oranı, adım uzunluğu, pati alanı) hız için detrending yapılmadan önce Matlab’da çıkarıldı. Ortalama BBB skorları yaralanma sonrasında önemli ölçüde azaldı (D1: 0). M2’de iyileşme NS-SCI’da (BBB: 17.5) SCI’dan (BBB: 8) daha yüksekti ($p = 0.0031$). vF skorları da iyileşti ancak SCI ve NS-SCI arasında istatistiksel fark bulunamadı ($p = 0.495$). Yürüme parametrelerinde, TENS’in olumlu etkisi D14’e kadar gözlemlendi; ancak, D14’ten sonra basma fazı oranı ve pati alanında tersine bir eğilim oldu. Genel olarak, TENS’in OY sonrası lokomotor fonksiyon ve adımlama üzerinde olumlu etkileri olmuştur. Bununla birlikte, TENS’in adım atma ile senkronize uygulanması, gelecekteki çalışmalarda yürüme parametreleri açısından daha iyi sonuçlar doğurabilir.

Anahtar Sözcükler: Kontüzyon, Omurilik Yaralanması (OY), Transkütanöz Elektriksel Sinir Stimülasyonu (TENS), Yürüme, Locomotor fonksiyon

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LIST OF ABBREVIATIONS

SCI	Spinal Cord Injury
TENS	Transcutaneous Electrical Nerve Stimulation
BBB	Basso, Beattie, and Bresnahan
WHO	World Health Organization
QOL	Quality Of Life
ISNCSCI	International Standards for Neurological Classification of Spinal Cord Injury
ASIA	American Spinal Injury Association
CCS	Central Cord Syndrome
BSS	Brown-Sequard Syndrome
ACS	Anterior Cord Syndrome
PCS	Posterior Core Syndrome
ATP	Adenosine TriPhosphate
DNA	Deoxyribo Nucleic Acid
ECM	Extracellular Matrix
MP	Methyl-Prednisolone
FDA	Food Drug Agency
BDNF	Brain-Derived Neurotrophic Factor
CSPG	Chondroitin Sulfate Proteoglycans
TLR	Toll-Like Receptor
PLGA	Poly (Lactic-co-Glycolic Acid)
PLL	Poly-L-Lysine
BM-MSCs	Bone Marrow Mesenchymal Stem Cells
iPSC-NS/PCs	Induced Pluripotent Stem Cell-Derived Neural Stem/Progenitor Cells
NPCs	Neural Progenitor Cells
EES	Epidural Electrical Stimulation
FES	Functional Electrical Stimulation Technology
AGATHA	Automated Gait Analysis Through Hues and Areas

GAITOR	Gait Analysis Instrumentation and Technology Opti-
	mized for Rodents
PrAnCER	Paw-Print Analysis of Contrasts Enhanced Recording
MIA	Monoiodoacetate
HADYЕК	Institutional Animal Experiments Local Ethics Committee
NS	Neurostimulation



1. INTRODUCTION

1.1 Motivation and Aim

Several alternative strategies of neuromodulation/stimulation therapy have been tested in the literature to help with sensorimotor losses in spinal cord injury. Most of them fail in terms of applicability to the clinic because they are invasive or require complicated and dangerous operations. Therefore, effective non-invasive strategies to improve sensorimotor impairments in spinal cord injury are increasingly needed. In this study, the main objective is to observe and understand the effect of peripheral transcutaneous electrical nerve stimulation (TENS) on locomotor recovery in rats with acute and chronic spinal cord injury with the help of gait analysis.

1.2 Outline

The purpose of this study is to investigate the recovery effect of transcutaneous electrical nerve stimulation (TENS) on locomotor functions of rats after spinal cord injury (SCI). Custom-made impactor devices and developed hardware systems to recognize rat paws from video recordings and analyze gait parameters are unique parts of this study. The background part explains SCI with its causes, effects, and pathophysiology, various treatment strategies including electrical stimulation methods, and rat models for SCI including contusion type. Also, Basso, Beattie, and Bresnahan (BBB) locomotor rating scale, mechanical withdrawal threshold test in addition to gait parameters and their analysis methods are explained based on the literature at the background section. The materials and method part includes how this study was conducted, beginning from the contusion-type SCI model, TENS application, and measurement techniques which are BBB locomotor rating scale, the mechanical withdrawal threshold experiment, and gait analysis. Results are presented with proper graphs for BBB, mechanical withdrawal, and each gait parameter (stance/swing/stride durations,

limb duty factor, stride length, and footprint area) after detrending for speed effect. Statistical analysis results are explained in the result section in addition to figures. The application success of SCI model and results of this study are discussed in the discussion part via comparison with literature studies. Also, subject number, gait parameter analysis system, and using anesthesia are specified as limitations of this work, and proposals for future studies such as increasing subject number, investigating underlying mechanism of neuroprotection after TENS application and high-frequency treatment application are mentioned as possible future works in the discussion part. Finally, the improvement effect of TENS treatment after SCI until the late acute phase is summarized in the conclusion part.

1.3 Novelty

The foremost novelty of this study is the peripheral bilateral application of non-invasive transcutaneous electrical nerve stimulation (TENS) to stimulate the tibial nerve in the medial malleolus of rats. Also, DeepLabCut software is trained to track the paw movements of rats from the video recordings of walking rats according to study and hardware system needs. Matlab is used to extract gait parameters instead of open-source or commercial systems.

2. BACKGROUND

2.1 Spinal Cord Injury

Spinal Cord Injury (SCI) is one of the multidimensional disorders that leads to physical dependence, psychological distress, morbidity, and economic burden on society [1]. Over the decades, the global incidence of SCI increased. Only in the US, 255.000 to 383.000 people are affected by SCI, as reported by the National Spinal Cord Injury Statistical Center [2] and more than 15 million people live with SCI globally according to the World Health Organization (WHO) database [3]. Patients with SCI not only struggle with physical inadequacy but also face psychological effects such as depression and anxiety (depression prevalence after SCI is around 22.2% in population) [4]. The quality of life (QOL) is defined as how a person sees themselves concerning the culture and beliefs that they live in, as well as with their goals, expectations, standards, and concerns by WHO [5]. Several studies have been conducted in the literature to investigate the quality of life (QOL) of patients with SCI. In one study, 55.3%, the majority, of the participants (SCI patients) complain about their QOL [6]. In another study, SCI patients are mostly compliant about their physical quality compared to psychological or social relations [7]. In total, it can be said that SCI affects the QOL of the patients with respect to physiological, psychological, and social relations.

2.1.1 Spinal Cord Injury Causes, Effects, and Pathophysiology

Spinal Cord Injury is a complex neurological condition that results from trauma such as car accidents or falls, and non-traumatic reasons as malignancy and degeneration. This damage prevents a bundle of nerves or nerve fibers in the spinal cord from sending signals to and receiving signals from the brain [8]. Disruption in the nerve function causes sensory, motor, and autonomic function loss [9].

SCI patients have experienced a wide range of symptoms from psychological to physical impairments. It is found that psychiatric symptoms (eg. fatigue, and suicidal ideation) and cognitive impairments (eg. tasks based on processing speed and executive function) that SCI patients go through influence their functioning. Moreover, mood disorders are more vulnerable for these patients [10]. Paralysis, pain or pressure in the neck, or back, numbness or loss of sensation in hands and feet, and bladder and bowel control loss can be listed as general physical conclusions of SCI [11]. However; types, periods, and degrees of physical impairments are contingent on the damage severity and location of the spinal cord. If the spinal cord injury occurs at the lower part of the spinal cord, only lower body parts and legs (paraplegia) are affected whereas if the upper part of the spinal cord is damaged, all limbs overcome (tetraplegia or quadriplegia) [1]. The International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) was developed for the classification of SCI and it consists of three central neurological summary scores which are motor scores, sensory scores, and impairment scale grade of American Spinal Injury Association (ASIA) [12]. According to The ASIA, based on severity SCI is investigated as complete or incomplete spinal cord injury. Grouping is done according to a range of motion which describes the movement potential of a joint or series of joints in the body and is an important indicator of joint flexibility. Loss of total motor and sensory function, or any nerve communication below the damage site, is called complete spinal cord injury. On the other hand, injuries with some residual motor or sensory function below the part of the injury are called incomplete injuries. As seen in Table 2.1 [13], complete injury is indicated as Grade A, while incomplete ones are graded as B to E on the AIS scale. AIS Grade B patients have no motor but have some sensory functions. Injuries are designated as Grade C if the motor grade is less than 3 below the neurological level of injury, while those classified as Grade D have a motor grade of at least 3 below the neurological level of injury. On the other hand, Grade E patients have abnormal reflexes, however; they have normal motor and sensory functions [13].

Incomplete spinal cord injuries are further investigated based on their mechanical damage and recovery prognosis as clinical syndromes: Central Cord Syndrome (CCS), Brown-Séquard Syndrome (BSS), Anterior Cord Syndrome (ACS), and Poste-

rior Core Syndrome (PCS). Based on a database of Spinal Cord Injury Model Systems, CCS (30%) is the most common syndrome compared to ACS (10%) and BSS (3%) among incomplete tetraplegia cohort (N=1649) between January 2011 and May 2020 [14].

Table 2.1

American Spinal Injury Association Impairment Scale. According to ASI muscle strength and sensory grading, grade 3 means active movement, full ROM against gravity [13].

A	Complete	No motor or sensory function is preserved in the sacral segments S4 - S5.
B	Incomplete	Sensory function preserved but not motor function is preserved below the neurological level and includes the sacral segments S4 - S5.
C	Incomplete	Motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade less than 3.
D	Incomplete	Motor function is preserved below the neurological level, and at least half of key muscles below the neurological level have a muscle grade of 3 or more.
E	Normal	Motor and sensory function are normal.

Central Cord Syndrome is a damage that results from hyperextension injury located in the central site of the spinal cord, predominantly in the cervical region. Hence, by positioning the motor tracts of the lower extremities along the lateral side of the spinal cord, the motor functions of the upper extremities are more damaged than those of the lower extremities [15]. Also, pain and temperature sensing loss are examined due to traversing in front of the central canal of second-order sensory fibers earlier joining the opposing side of the spinothalamic tract. Brown-Séquard Syndrome or unilateral cord syndrome results of damage to the dorsal columns, lateral corticospinal, and spinothalamic tract [16]. In this syndrome patients lose their pain and temperature sensation as CCS, however; this loss is contralateral to the injury as difference from the CCS. Also, light touch, motor function, proprioception, and vibration sense are lost ipsilaterally in BSS [17]. Anterior Cord Syndrome is caused by dominant damage on the ventral part of the spinal cord because of flexion injury or blood supply disruption from the anterior spinal artery [18]. In other words, ACS results in ischemia within the

anterior spinal artery. Unlike Central Cord and Brown-Séquard Syndrome, vibration proprioceptive sensation, and fine touch carried by the dorsal column of the spinal cord are not impaired [19]. The Posterior Cord Syndrome results in spinal cord impingement at the posterior location such as posterior epidural hematoma or depressed laminar fractures. Patients with PCS face proprioception loss (position, deep touch, and vibratory sense) whereas no motor dysfunction is experienced [16]. Lateral spinal cord sections are given according to cord syndromes are given in Figure 2.1 with crucial parts of the spinal cord [17].

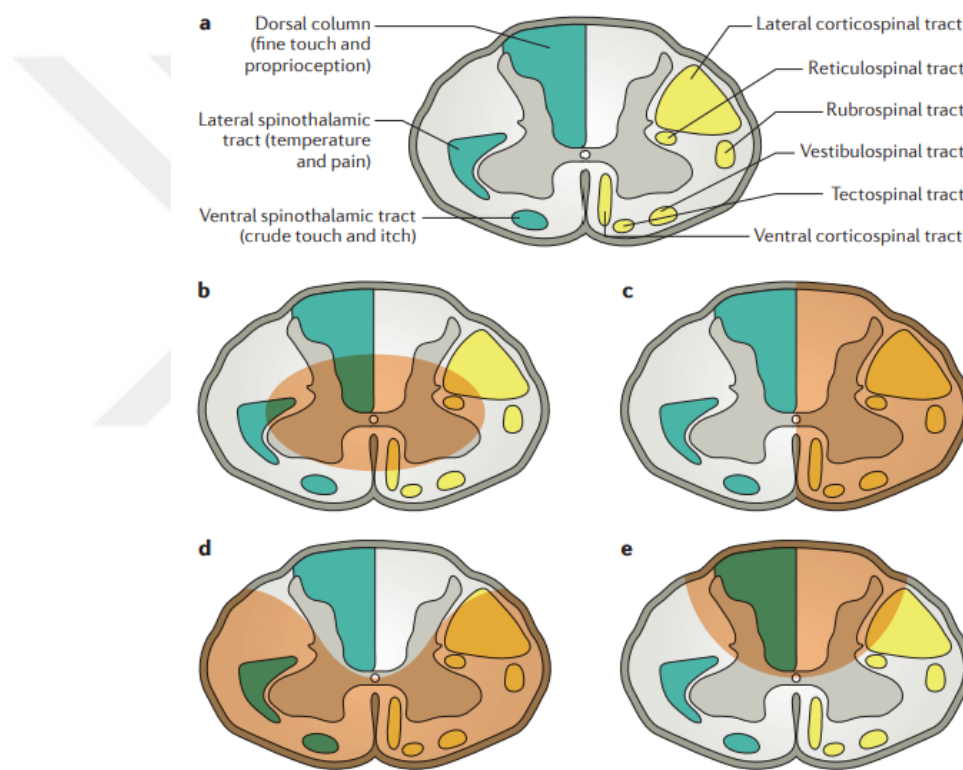


Figure 2.1 Incomplete Spinal Cord Injury Syndromes. A. Major ascending (blue) and descending (yellow) sensory tracts are shown. Orange schematic abbreviations show the spinal cord's affected part based on spinal cord injury syndromes. B. Central cord syndrome, C. Brown-Séquard Syndrome, D. Anterior cord syndrome, E. Posterior cord syndrome [17].

Primary and secondary injuries are seen in spinal cord injuries over time. Immediately after the damage, paralysis can occur as a primary injury or it can develop over time as a secondary injury because of bleeding, swelling in the spinal cord, and cell death [20]. There are three phases in the traumatic SCI which are acute (hours to days), intermediate (days to months), and chronic (> 6 months) phases. In the acute phase, instant mechanical interruption and dislocation of the vertebral column

as a result of the primary injury promote compression of the spinal cord. Neurons and oligodendrocytes are damaged, vasculature is impaired and the blood-spinal cord barrier is put at risk because of traumatic damage. The cascade of events is initiated which leads to more crucial dysfunctions [17]. Ruining the microvascular supply of the spinal cord ends with cell dysfunction and death [21]. Spinal cord swelling caused by an inflammatory response and blood-spinal cord barrier damage induces subsequent mechanical pressure of the cord [17]. Microglial cells are activated by releasing potassium, ATP, and DNA after continuous cell death. Activated microglia with other inflammatory cells penetrate the site where cell death occurs. Free radicals byproducts of phagocytic inflammatory cells, enhance necrotic and apoptotic cell death [22]. Ischaemia, negatively affected by impaired autoregulatory cord vasculature, continues to increase cell death. In the intermediate and chronic phase, functional remodeling such as remyelination attempts, extracellular matrix (ECM) adjustments, and vascular reorganization are seen [23]. An astroglial scar is becoming an inhibitor of regeneration by growing. Axonal regrowth and cell migration are limited via cystic cavities fusion [17]. In both rostral, and caudal parts of the spinal cord, atrophic changes have occurred, and according to the site, areas of white and gray matter are decreased [24]. During the chronic phase, degenerative changes are not only occurring in the spinal cord but also observed in the brain [25]. According to literature, for instance, M1-type microglia initiation can enhance the chronic inflammation of the brain [26]. Pathophysiology of phases impacts on the treatment strategies of spinal cord injury.

2.1.2 Spinal Cord Injury Treatment Strategies

Significant knowledge has been gained from basic scientific and clinical research on the molecular, cellular, and system-level processes of damage resulting from spinal cord injury. However, studies are still ongoing to find effective treatment methods. In the acute phase of the SCI, surgery is the first clinical treatment strategy for most of the SCI patient. The timing of the surgery after SCI is crucial for its success. Early decompression after SCI is safer and advantages, however; there is not any evidence showing that early surgeries provide better results [27]. Nevertheless, acute spinal cord

injury patients are more prone to be operated on. But still, there are some criteria for patients to get surgical interventions such as dislocation type of injury should be seen, and the presence of cord compression is indicative of progressive neurological deficit [28]. In addition to the advantages of surgery, there are some challenges such as therapeutic hypothermia optimal temperature decision, and risk of morbidity and mortality [29]. As clinical interventions drugs and surgeries are the most common treatment options for SCI.

Neuroprotective drugs are also preferred in the acute phase of the SCI to enhance neuroprotection and neuroinflammation via infiltration of inflammatory cells, and reduction of oxidative stress. Cell transplantation and small molecular compounds are used for scar formation regulation and nerve regeneration in the intermediate phase while the combination of biomaterials and/or stem cells and physical regulation promotes countervailing neural function recovery and neural circuit reconstruction [23]. Advanced therapeutic strategies in SCI Bioactive substances regulating, Biomaterials transplantation, Cell therapy, and Physical controlling can be investigated.

Various pharmacological molecules are used in the treatment of SCI to decrease the effects of injury. Among these drugs, only Methylprednisolone (MP) is approved by the FDA. This drug improves blood supply, reduces secondary inflammatory responses, promotes neurotrophic factor secretion, and recovers the blood - spinal cord barrier [30]. However, the usage of this drug is still controversial because of its off-label use and side effects [31]. In one systematic review, potential disease-modifying agents are identified and it is found that atorvastatin, estradiol, melatonin, and methylprednisolone have positive effects on SCI progression while ethanol and morphine have negative effects [32].

Bioactive substances such as small-molecule compounds, neuropathic factors, and exosomes have an impact on axonal regeneration and microenvironment improvement that enhance the functional recovery of SCI [33]. After the intervention of brain-derived neurotrophic factor (BDNF), for instance, axonal regeneration, remyelination, synaptic reformation, and transmission are examined [34]. In another study, Schwann

cell-derived exosomes decreased chondroitin sulfate proteoglycans (CSPG) deposition via enhancing toll-like receptor (TLR2) expression on astrocytes which resulted in further functional recovery of SCI mice [35]. The main advantage of biomaterial transplantation is the targeted drug delivery via using nanomaterials as nanocarriers. This approach increases the bioavailability of drugs and mobilization [23]. Nanoparticles, collagen, and PLGA-PLL can be used as nanomaterials in the treatment of SCI via the reduction of scar tissue, and improvement of the regenerative capacity of neurons and axons [36].

Since the regeneration abilities of mature neurons are reduced or disappear after cell damage or death at the injury site, it is crucial to revitalize cellular life in the SCI. Therefore, cell therapy is a favorable strategy for inflammatory response regulation via the secretion of cytokines and growth factors, advancing axonal regeneration and nutritional support. Also, the microenvironment of the damaged site is affected via cell transplantation which has positive effects on functional outcomes [23]. For this purpose, stem cells have been studied over the years and it is shown that different types of stem cells have an impact on the improvement of SCI outcomes in various ways. Transplantation of bone marrow mesenchymal stem cells (BM-MSCs) to the damaged site enhances the survival cell number in addition to motor function via forming bundles that bridge the epicenter of the injury [37]. Another study showed that transplantation of human induced pluripotent stem cell-derived neural stem/progenitor cells (iPSC-NS/PCs) aids functional recovery via axonal remyelination [38]. Neural progenitor cells (NPCs) are adapted as oligodendrocytes and increase myelination. NPCs decrease the secretion of pro-inflammatory cytokines/chemokines via downregulation of the cytokine production genes. In addition, these stem cells promote hindlimb locomotor function recovery [39].

Rehabilitation programs include physical therapy, occupational therapy, and other intervention methods to improve the function, mobility, and quality of life of SCI patients. It is based on improving the functional and mechanical ability of the patients in addition to preventing secondary complications. Rehabilitation also impacts on patient's place in society to help cope with independence loss of patients [20]. With

the help of key elements of physical rehabilitation such as stretching and strengthening activities, rehabilitation helps patients regain function or improve remaining ones. Certain regions of the spinal cord are stimulated via rehabilitation which provides motor and sensory function regain and local inflammation reduction in the spinal cord [23]. Rehabilitation approaches are changing and becoming varied to provide more accurate and advanced therapy for patients by developing technologies and strategies [40].

More advanced and appropriate treatment strategies are developing and becoming varied over the years by developed technologies in the field. Electrical stimulation-mediated neuroplasticity, for instance, has been widely investigated in recent years to induce neural recovery and repair. There are two ways to apply electrical stimulation to the patient whether invasive or non-invasive. Epidural electrical stimulation (EES) as invasive, functional electrical stimulation technology (FES), and transcutaneous electrical nerve stimulation (TENS) as non-invasive are mostly preferred techniques in the clinic. The application of EES involves the surgical placement of an electrical stimulation device on the epidural area according to the SCI site. In the literature it is shown that functional recovery including motor functions and hemodynamics of SCI patients is provided via EES [23]. Despite the numerous studies that demonstrate the efficacy of EES as a treatment strategy for SCI, the fact that EES is an invasive method has a deleterious effect on the applicability of this treatment strategy. Unlike EES, FES as a non-invasive method is used to stimulate the undamaged peripheral nervous system for sensory and motor benefits. Small current pulses are applied to stimulate the muscles in FES. For recovery of upper extremities functions FES is well used whereas it is combined with physical rehabilitation strategies such as wheeled walkers or stationary bicycles [17]. This non-invasive neuromodulation strategy induces the motor and sensory circuits recovery [41] and reduces systemic complications whereas strengthens neuroplasticity [42]. Transcutaneous electrical nerve stimulation (TENS) is another non-invasive technique that involves delivering electrical impulses via electrodes placed on the skin over the target nerve groups to stimulate the targeted nerves. TENS is a safe, cheap, and easy-to-use approach that relieves muscle spasticity after SCI [43]. It helps relieve pain even neuropathic pain in SCI patients [44],[45]. It may affect the same spinal networks as epidural electrical stimulation and results in improved motor

function and gait parameters [46], especially when combined with locomotor training [47].

2.1.3 Spinal Cord Injury Models in Rats

The utilization of rat models is of paramount importance in the evaluation of treatments, the comprehension of injury pathology, and the assessment of the recovery of locomotor and sensory functions. These models possess a sophisticated comprehension of neuronal circuitry changes that occur after injury, thus highlighting methodologies that can be employed to enhance regeneration, counteract inhibitory factors, and rescue cells in the acute injury phase [48].

As is well documented, SCI can be induced in rats through a variety of methods, including contusion, compression, distraction, dislocation, and transection. In addition to these physical methods, chemical agents have also been used to induce SCI in rats [49].

Compression models are based on prolonged cord compression which could be part of the spinal cord after acute shock. Dislocation of and burst fractures are the main reasons that the compression mechanism is used as the SCI model. The distraction model aims to mimic the tension forces in SCI via stretching the cord in a controlled manner. This model is still being investigated and developed to replicate the SCI more accurately [49]. Vertebral column displacement is applied in the next model by using an advanced device [50]. Transaction (complete and partial) models are widely used for investigating neural regeneration and following functional improvement. Unfortunately, this model is not appropriate to examine the pathophysiology of SCI. Chemical agents are used for imitating secondary injury cascade after traumatic SCI. For investigating the molecular mechanism of the SCI, this model is advantageous. However, characterization of the trauma, delivery location, and reproducibility are limitations of this model [49].

Contusion-type models are made by using developed devices. Contusion devices were initially designed as weight drop tubes, which subsequently became more complex by including sensors and/or modifications for controlling the movement of the weight (e.g. new generation MASCIS devices) [51]. The most advanced contemporary devices utilize impactors that are computer-controlled and incorporate sensors for the monitoring of mechanical parameters (e.g. OSU impactor) and feedback control (e.g. Infinite Horizon impactor) [52]. To generate repeatable and controllable injury models, an ideal device should satisfy certain criteria. Displacement (or velocity) and force should be measured at the probe tip, and either one should be controlled during the time course of the impact. It is also imperative that the impactor probe does not rebound, and that the initial contact parameters are specified. The advantage of the contusion-type models is that they have equivalent results with human spinal cord injuries within the scope of transient and acute injuries [49].

2.2 Measurement Technics

To investigate the effect of SCI and recovery after damage based on treatment strategies, laboratory rats are generally preferred since behavioral, physiological, and anatomical experiments can be examined easily. With the help of behavioral methods, adjustments on the spinal cord injury and mechanical/neurological function can be tested. These tests are used not only to understand the functional impacts of the injury but also to observe the effect of applied treatment strategies on prognosis. Various behavioral experiments are performed at pre- and post-treatment stages to examine sensorimotor system differentiations [53]. For this purpose locomotor tests (eg. BBB test, BBB sub-scoring test, automated walkway test, Footprint analysis test, kinematic analysis), motor tests (eg. inclined plane, limb hanging and grip strength test), sensory tests (eg. hot plate - based tests, cold sensitivity - based tests, von - Frey filaments), sensory-motor tests (eg. rope walk testing, narrow beam test), autonomic tests (eg. urinary bladder function), and electrophysiological tests (eg. evoked potentials - transcranial, and motor evoked potentials - intracranial - intraspinal tests) can be applied in the laboratory [54].

Since proper locomotion needs accurate coordination between limbs and strengths of the muscle group, locomotor tests that are mostly based on open-field tests, test striated muscles of the proximal forelimb, hindlimb, and tail. The weight support is the crucial point for the locomotion which also affects spatiotemporal parameters [55]. Moreover, as discussed in 2.1.1 Causes, Effects, and Pathophysiology of Spinal Cord Injury part, sensory responses are also decreased or lost after injury. Therefore, evaluation and measurements of sensory system outcomes after SCI is another important step to understanding the recovery. Sensory system tests are used for identifying hypoactivity (decrease or loss of sensory functions) or hyperactivity (hypersensitivity, allodynia) of the touch based on temperature changes or pain [54].

2.2.1 Basso, Beatie, and Bresnahan (BBB) Test

Basso, Beatie, and Bresnahan (BBB) locomotor rating scale is the most common locomotor test that is widely used for SCI rat studies. This test is based on a rating scale according to rat walking. This rating scale is formed based on a sequence of locomotor recovery patterns observations [56]. This rating scale consists of grades from 0 to 21 where grade 21 means normal/coordinated limb movements while grade 0 represents no locomotor activity at all. Scores 0 to 7 represent the early phase of recovery which indicates isolated joint movements (hip, knee, and ankle). Scores 8 to 13 mean intermediate recovery phase which indicates paw placement, stepping, and coordinated locomotor movements between fore and hind limbs. Scores 14 to 21 mean late phase of recovery which represents predominant paw position, trunk stability, toe Clearance, and tail position. This scoring system is given in Table 2.2 more deeply [56]. Video camera usage is advised during the training of examiners and to compare different experiment groups. BBB rating scale is based on 10 categories to score the recovery which are limb movements based on joints (hip, knee, and ankle), trunk position, plantar paw placement, weight-supported stepping, forelimb-hindlimb coordination, toe clearance (pathological sound of hindlimb during stepping), predominant paw position, trunk instability, and tail position [56]. Pre-operational training of rats is recommended but not mandatory which is a big advantage of the BBB rating scale.

Table 2.2
Basso, Beattie, Bresnahan Locomotor Rating Scale [55].

Score	Description
0	"No observable hindlimb (HL) movement"
1	"Slight movement of one or two joints, usually the hip and/or knee"
2	"Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint"
3	"Extensive movement of two joints"
4	"Slight movement of all three joints of the HL"
5	"Slight movement of two joints and extensive movement of the third"
6	"Extensive movement of two joints and slight movement of the third"
7	"Extensive movement of all three joints of the HL"
8	"Sweeping with no weight support or plantar placement of the paw with no weight support"
9	"Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight supported dorsal stepping and no plantar stepping"
10	"Occasional weight supported plantar steps, no forelimb (FL)-HL coordination"
11	"Frequent to consistent weight supported plantar steps and no FL-HL coordination"
12	"Frequent to consistent weight supported plantar steps and occasional FL-HL coordination"
13	"Frequent to consistent weight supported plantar steps and frequent FL-HL coordination"
14	"Consistent weight supported plantar steps, consistent FL-HL coordination; and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance or frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping"
15	"Consistent plantar stepping and consistent FL-HL coordination; and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact"
16	"Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off"

17	"Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off"
18	"Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off"
19	"Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and lift off; and tail is down part or all of the time"
20	"Consistent plantar stepping and consistent coordinated gait; consistent toe clearance; predominant paw position is parallel at initial contact and lift off; tail consistently up; and trunk instability"
21	"Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability, tail consistently up"

2.2.2 Mechanical Withdrawal Threshold

Mechanical allodynia which results in enhanced sensitivity to innocuous stimuli as sensory responses and mechanical withdrawal thresholds are tested in rodents via von Frey filaments. The gold standard for measuring mechanical thresholds in rodents is still manual von Frey. For the application of the test, usually, Plexiglas boxes with mesh or fine-grid bottom are used where animals are placed, as seen in Figure 2.2 [57]. The test can be applied while the animal is in the researcher's hand. In this case, the food reward system is recommended to avoid animal recognition of experimental filaments [54]. A von Frey filament that transfers force (mostly 0.008 - 300 N or 2.5 - 125g) is applied until the filament bends to the plantar side of the hind paw as Figure 2.2. Each filament is applied for 2 to 5 s with 3 to 5 minutes rest between each case [54, 57]. When the animal shows any nocifensive behaviors (licking or shaking the paw, brisk paw withdrawal), the response is observed as positive and the filament force is noted as mechanical threshold. The dorsal side of the hind paw of the abdomen can also be used as a testing surface, even though the plantar surface of the hind paw is

widely preferred [57].

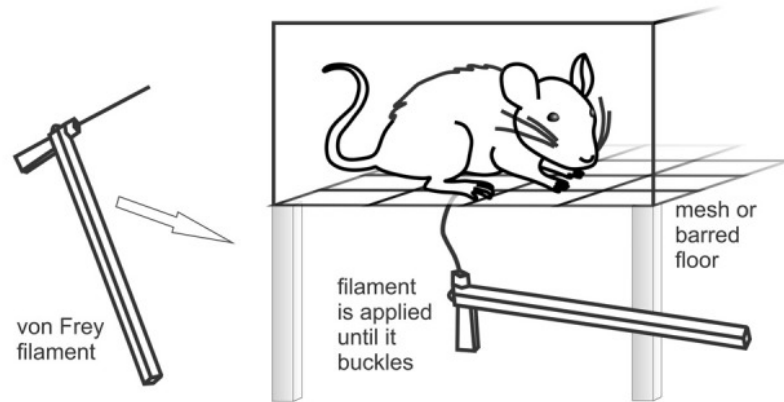


Figure 2.2 Manuel von Frey Application to Rodent [56].

2.3 Gait Analysis in Rats

In addition to the BBB rating scale, gait analysis has an important role in investigating locomotor function changes in rats. Gait analysis of rodents involves the quantitative assessment of their walking patterns and movements. This analysis is used in scientific research to study the effects of various treatments, diseases such as spinal cord injury, and genetic mutations on the movement patterns of rats [57]. Various gait parameters can be classified into four categories dynamic paw parameters (eg. stance, stand, and swing durations, duty factor), static paw parameters (eg. Base-of-Support, stride length, maximum print area), coordination parameters (eg. regular step patterns, regulatory index, locomotor speed), and others (eg. swing speed, stand index) [58]. These gait parameters are investigated more deeply in the following section (2.3.1 Gait Parameters).

2.3.1 Gait Parameters

The most commonly used gait parameters for locomotor recovery investigation are explained below:

1. Average speed (cm/s): This parameter is calculated by dividing the walked distance (cm) by the walking time (s) [58]. Evaluation of this parameter is critical because walking speed affects the gait parameters as well as the analysis [59],
2. Print area(mm²): Total area of the paw that contacts directly to the floor mean-time the stance phase. Also, the maximum print area indicates the maximum paw area that contacts the floor through whole stance phases [60],
3. Stance duration (s): This parameter indicates the duration between the moment when a paw starts to touch the floor (foot - strike) and the moment when it stops contact (toe - off) [61],
4. Swing duration (s): This parameter indicates the difference between stride and stance duration [61],
5. Stride duration (s): This parameter represents the time difference between foot-strikes of the same paw [61],
6. Stride distance (cm): The distance between the consecutive steps of the same paw [60].
7. Limb duty factor (%): This parameter is the percentage of stance duration in step cycle (stance duration + swing duration) [60].

2.3.2 Devices and Methods for Gait Analysis

Over the years many technologies have developed for gait analysis. Since methods performed with paw painting are likely to contain individual errors, video-based autonomous systems developed for gait parameters have come to the fore in recent years. With the help of these advanced technologies, individual errors and required time are reduced in addition to the ability of advanced measurements which can not be done by the naked eye [58]. Gait analysis via video of rats involves recording the rats' movements on a transparent treadmill or another flat surface and then analyzing the video footage to quantify their gait parameters. Over the years CatWalk [62], DigiGait

(treadmill) [61], MouseWalker [63], AGATHA [64], GAITOR [65], Paw-Print Analysis of Contrasts Enhanced Recording (PrAnCER) [66], systems have been developed on this basis. CatWalk and AGATHA which are the two most prominent are investigated.

The CatWalk system was first studied in 2001 on spinal cord injury rats. This video-based system can extract some of the parameters which are interlimb coordination, swing duration, paw print areas, stride length, and base of support, while rats are walking through the walkway. This system is based on a rat walkway, a light source, a mirror, a video camera, and a designated algorithm. The walkway is made of 8 cm apart plexiglass walls and $109 \times 15 \times 0.6$ cm glass floor and a mirror under the walkway with an angle of 45° which provides a view of stepping during the walking of animals. Also, the light source is through the walkway as a white florescent tube. The whole experiment is applied in the dark room. Light coming from the florescent tube directly enters the distal edge of the walkway and is only seen when the paw contact with the glass floor (Figure 2.3) [62]. This system was able to detect each stepping and extract each paw image throughout the walkway. However, the limitation of this system was that data acquisition was only applied if the animal had weight support stepping frequently [62]. Therefore, it was not possible to evaluate the injury severity and recovery process in the acute phase.

In the following years, the CatWalk system was developed (CatWalk XT by Noldus) and commercialized. Now, the system can be used for multiple central and peripheral nervous system diseases. The basics of the system are not changed which means CatWalk XT consists of a glass walkway that is lighted with a green source this time, a high-speed digital camera, and software for detecting and analyzing paw and gait parameters which is more sophisticated than this time (the CatWalk XT system is given in Figure 2.4) [58],[67]. With the help of the CatWalk XT system, 58 gait parameters including basic ones such as stand and swing duration, duty factor, and print area can be identified even with impairments. The main difference between the CatWalk XT system with the treadmill based gait analysis system is the light source and its usage. The green light is reflected in the glass walkway and only escapes from the walkway when the paw of the animal contacts to the glass and this reflection is

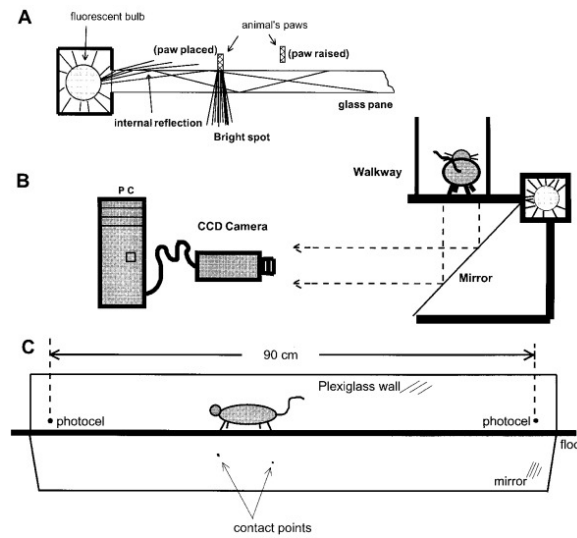


Figure 2.3 CatWalk Setup and Principles. A. The CatWalk setup uses light from a fluorescent tube to illuminate the edge of a glass sheet. When an object touches the surface, light escapes and is scattered by the object, making the point of contact visible. B. The setup consists of a corridor for animals to cross, with a mirror placed at a 45° to monitor the floor. C. Data is collected as the animal breaks infrared beams at the start and end of the corridor [61].

detected by the help of a resolution camera below the walkway. This principle ensures that only paw prints that are actually in contact with the glass floor are recognized [68]. Also, each paw print can be analyzed via this system, and a gait map is obtained as an example in Figure 2.5 [58]. However, this system has also some limitations such as selecting the appropriate gait parameters to understand or examine the disease model optimally [68].

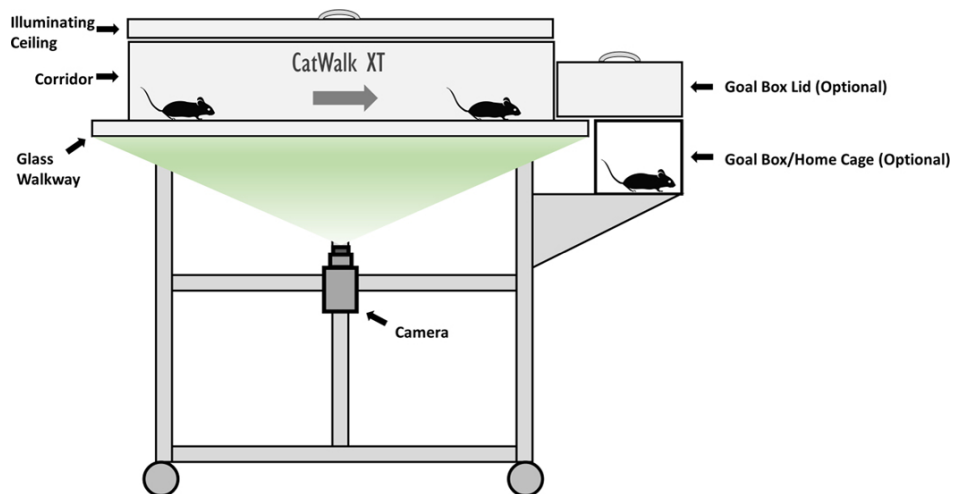


Figure 2.4 CatWalk XT system [66].

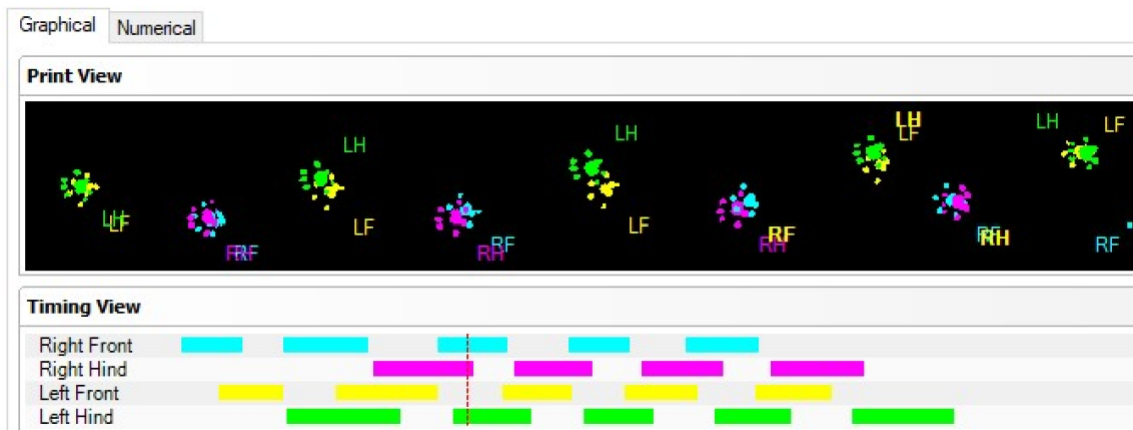


Figure 2.5 Example for paw print and gait map [57].

Digigait system (treadmill) is used to quantify different gait parameters, by quantitative coordination analysis via Virtual Dub free software [61]. In the following years, gait parameters are quantified by free walking of rats without a treadmill system. MouseWalker is developed which is made up of four main parts: the fTIR floor and walkway wall, which provide the surface for the mouse to move on, the supporting posts that hold the apparatus in place, the 45° mirror which is used to reflect the image of the mouse for analysis and the background light which provides illumination for the apparatus. This apparatus uses an LED light strip, either a cool white LED for black and white cameras or a colored LED for color cameras, which is attached to a 3/8-inch U-channel aluminum base LED mount. The LED strip is mounted on a 9.4-mm thick acrylic glass walkway measuring 8 by 80 cm, with a black cardboard strip covering the contact areas between the LED and acrylic glass. The acrylic glass walkway is constructed by gluing all four sides together using epoxy glue and cable ties. Gait parameters are determined via MouseWalker software which is written in MATLAB, and the MouseWalker platform and the software scene are given in Figure 2.6 [63].

The automated Gait Analysis Through Hues and Areas (AGATHA) system is another system that is open source for all researchers as difference from the CatWalk XT. This system is basically similar to the first CatWalk system [62], both the lateral

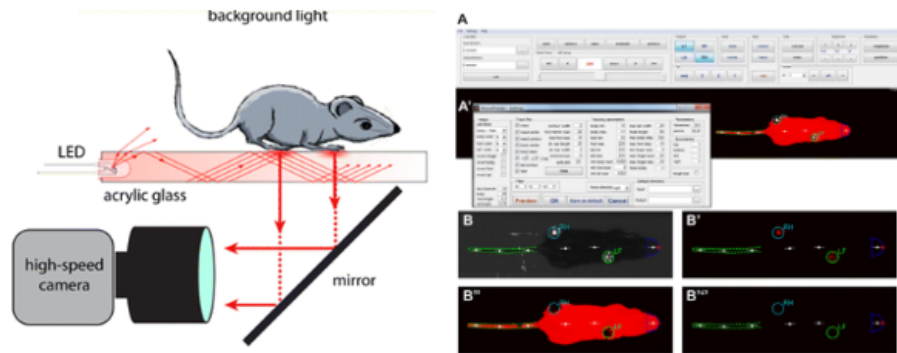


Figure 2.6 Digait (treadmill) system. The left schematic image shows the MouseWalker platform, the right visual shows the MouseWalker software interface consists of a (A) single window for loading, tracking, and editing videos, with a (A') settings window that allows for customization of detection and display options. Users can choose to (B) visualize tracking in various ways, such as simultaneously with the original video, only with the footprints (B'), only with the body contour (B''), or none at all (B''') [62].

and lower paw images of the rats while walking are recorded with a high-speed camera, the view is shown in Figure 2.7 [64]. Foot-strike locations are approximated with the help of a lateral view of the animal (Figure 2.7a) and the spatial location of the raw paw paw prints is extracted from the ventral view (Figure 2.7b). Then, isolated paw prints are analyzed via color thresholds between two frames (foot-strike and toe-off) (Figure 2.7c). Finally, each paw prints are analyzed from the whole video according to their spatial locations, and the hind paw sequence is examined (Figure 2.7d). The speed of the walking animal is calculated from the centroid of the animal from the ventral view. AGATHA can detect the same frames when the same video is analyzed over time which shows the repeatability of the system. However; manual digitization gives more accurate results than the AGATHA according to the study [64].

In 2018, new open-source code was introduced to the literature for gait parameters. The GAITOR Suite is designed to make preclinical gait analysis more accessible to researchers by providing hardware and software customization options for a broad range of applications. The GAITOR Suite has been tested in 4 models of disease and injury, such as a monoiodoacetate (MIA) injection model of joint pain, a sciatic nerve injury model, an elbow joint contracture model, and a spinal cord injury model. The GAITOR Suite was able to identify unique compensatory gait patterns in each model, indicating that it is a useful tool for detecting changes in gait in rodent models of

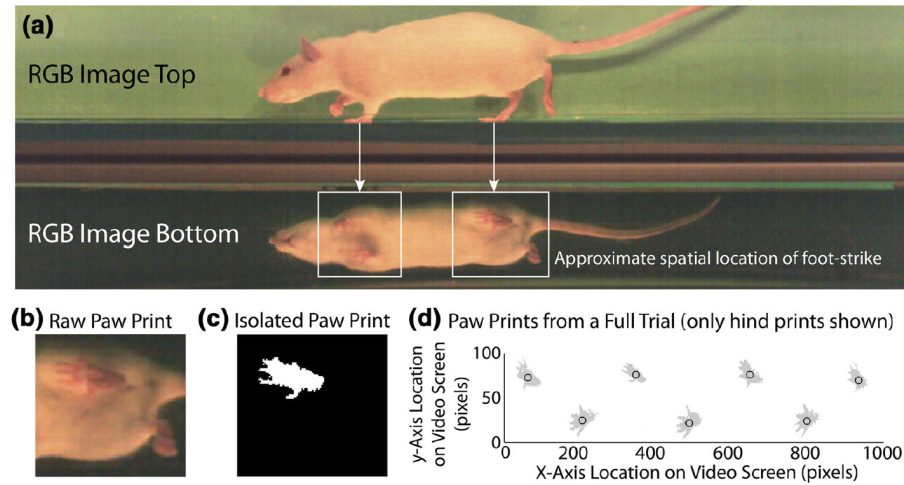


Figure 2.7 View of walking rats with on AGATHA system [64].

diverse injuries and diseases. This can improve the selection of preclinical models, the assessment of the effects of diseases, and the evaluation of potential therapies. The GAITOR Suite is being made available to the research community as an open resource at www.GAITOR.org, to promote and improve the use of gait analysis in preclinical rodent models [65].

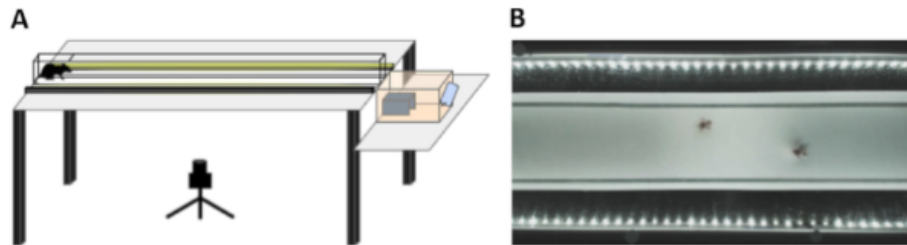


Figure 2.8 PrAnCER system. A. The rat is made to walk on a clear walkway with a floor that has been made translucent using vellum. The floor is illuminated by LED strips placed along the walkway's length and positioned in such a way that the level of light is between the animal's feet and body. This allows for a clear recording of the animal from below, while the rat moves toward the goal box which is the home cage, B. A still image taken from a video recording illustrates how the translucent floor allows for clear visibility of the rat's paws while the body of the rat remains mostly undetectable [65].

PrAnCER was developed as a software program for automated quantification of gait, which uses contrast-enhanced recordings produced by a translucent floor to isolate the rat's paw prints as it walks. The program can measure a variety of spatiotemporal gait parameters. It is confirmed that PrAnCER is consistent and accurate when compared with manual scoring of gait parameters. In this system, videos are

recorded only from the below part of the walkway as shown in Figure 2.8, where the walkway is lightened via LED lights. To determination of gait parameters, the total contact area of paws is observed and according to the time difference between steps, temporal parameters are examined [66].



3. MATERIALS AND METHOD

Figure 3.1 shows the general study flow and applications in this study with different end points (D1, D7, D14, M1, M2). Contusion-type SCI was induced on rats in two groups (SCI and NS-SCI). Throughout the study, TENS application was applied to NS-sham and NS-SCI groups. BBB scoring, mechanical withdrawal threshold, and gait analysis of all rats were observed at pre-op, D1, D7, D14, M1, M1.5, and M2 and results were analyzed.

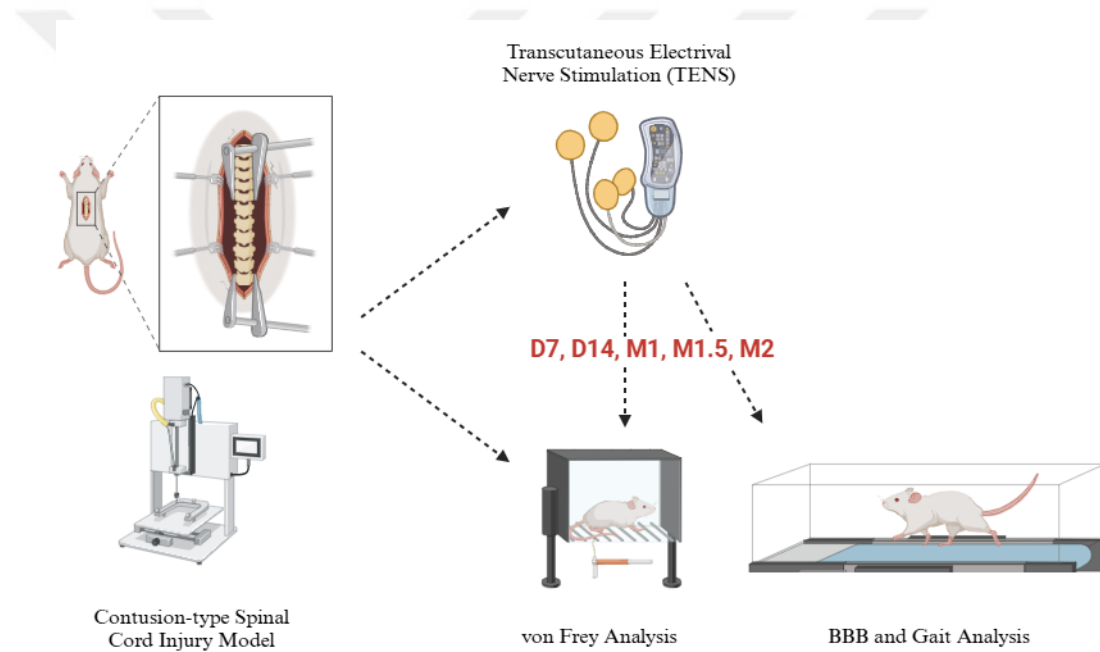


Figure 3.1 Method illustration of the study.

3.1 Subjects

A total of 64 adult Wistar albino rats (aged between two to four months) of both sexes were subjected to a contusion-type spinal cord injury in this study. The rats were divided into groups of 4 which are sham, SCI, NS-sham, and NS-SCI to be terminated at different times during the acute and chronic stages. Standard BBB

locomotor rating scale was used initially to assess the operation's severity and results of 49 adult animals (24 males, 25 females) with moderate-to-severe SCI were investigated. The study consisted of sham (n=10), SCI (n=15), NS-sham (n=6), and NS-SCI (n=18) groups with rats terminated at D7, M1, and M2 post-op. The study was approved by the Bogazici University Institutional Animal Experiments Local Ethics Committee (HADYEK) and conducted at the Bogazici University Vivarium.

3.2 Contusion - Type SCI Model

The surgery involved performing laminectomy on the rats under isoflurane anesthesia at the T8-T9 vertebrae level, followed by creating a unilateral contusion using the weight reduction method. Computer-controlled custom-made impactor device (contactor diameter: 2.3 mm, peak force: 0.9-2 N, displacement: 1.75 mm, duration: 0.5 s) was used to produce moderate-to-severe injury as observed with the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale. Computer-controlled custom-made impactor device, surgery system, and view of the vertebra before, during, and after the surgery are given in Figure 3.2. Sham laminectomy surgery was also performed on two groups (sham and NS-sham) without causing spinal cord injury. All surgical procedures were carried out by Pınar Kuru Bektaşoğlu (MD-PhD) and contusion was performed by Prof. Dr. Burak Güçlü in the Experimental Animal Production and Care Unit (Vivarium) of Bogazici University, with the assistance of the responsible veterinarian and other team members. Postoperative animal care, controls, and necessary interventions such as urine and fecal output control, weight monitoring, neurogenic bladder emptying, and administration of painkillers or antibiotics were conducted by the Bogazici University Vivarium team.

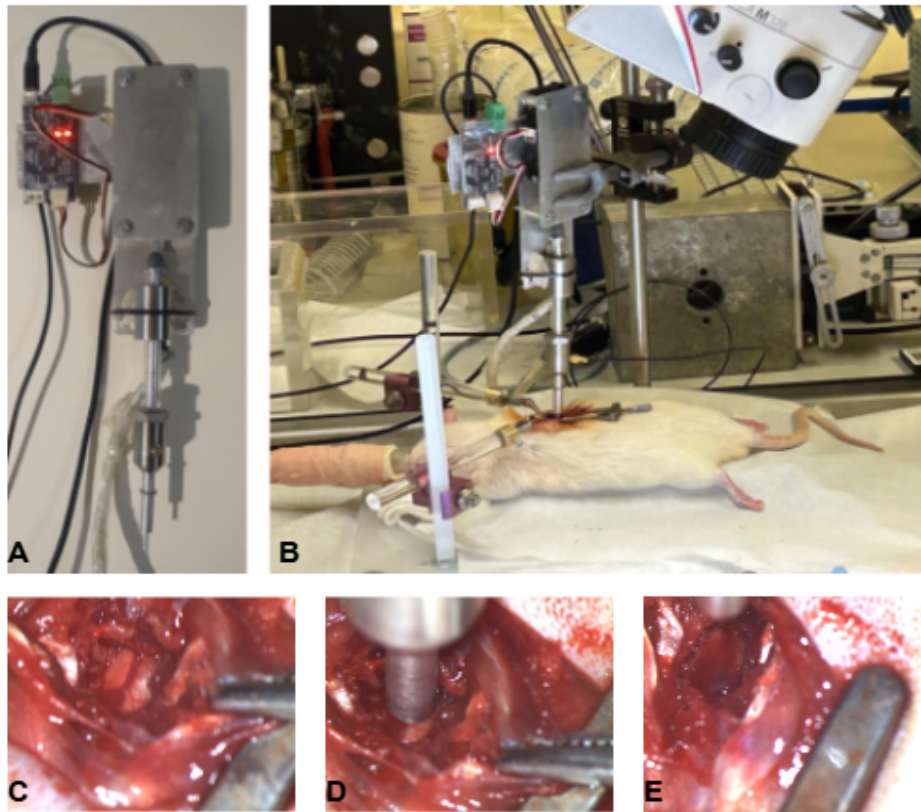


Figure 3.2 Surgery images. A. Computer-controlled custom-made impactor device, B. Rat placed in the impactor device for contusion-type SCI model under isoflurane anaesthesia, C. View of the vertebra before impact, D. View of the vertebra during impact, E. View of the vertebra after impact.

3.3 Application of Transcutaneous Electrical Nerve Stimulation (TENS)

A standardized transcutaneous electrical nerve stimulation (TENS) was applied in the Bogazici University Vivarium to activate the propriospinal system. The TENS (NeuroTrac TENS, VM Verity Medical Ltd., lot: ECS300A-OM-EN25-17-06-19) was applied (pulse width: 0.3 ms, pulse frequency: 2 Hz, amplitude: $2 \times$ motor threshold) to the peripheral tibial nerve bilaterally while rats were in ketamin-xylazine anaesthesia by the help of Bogazici University Vivarium team. The application was made to stimulate the tibial nerve. Two sets of standard ECG electrodes (each pair contains both - and + wires) were put on each hind limb of rats. Positive wires from each electrode set were placed on the medial malleolus whereas negative wires were placed to the dorsal part of the hind paws (Figure 3.3). The rats in the neurostimulation groups (NS-sham

and NS-SCI) were taken for neuromodulation three times a week for 30 minutes each time.

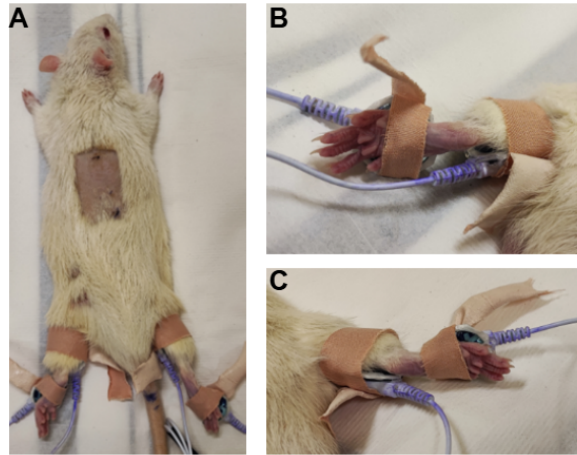


Figure 3.3 Transcutaneous Electrical Nerve Stimulation (TENS) application to rats. A. View of rat in anesthetized at bilateral TENS application, B. Left hindlimb view with electrodes placed medial malleolus and dorsal part of the hind paw, C. Right hindlimb view with electrodes placed medial malleolus and dorsal part of the hind paw.

Animals receiving neuromodulation (TENS) were subjected to treatment for two distinct durations, namely acute and chronic. The acute group ($n=11$) received a total of 80-90 minutes of treatment over a period of one week (D7). Furthermore, a subset of the chronic group ($n = 7$) received TENS for a total of 350-380 minutes over a period of one month (M1), while another subset ($n = 7$) was exposed to TENS for a total of 740-750 minutes over a period of two months (M2).

3.4 BBB and von Frey Analysis

Basso, Beattie, and Bresnahan (BBB) test was conducted via evaluating each recorded walking videos which are used for gait parameters based on numbering scale at Table 2.2. BBB scores were examined for each hind limb (both for right and left) of rats. The Paw sounds captured in the videos were meticulously analysed to ensure adequate toe clearance.

In the von Frey test, a series of monofilaments of increasing stiffness were applied

to the plantar surface (bottom) of an animal's hind paw in 10 trials with 120-s intervals. The application was done while the animals were in a cage with a fine-grid bottom. The rat's response to the stimulus, such as withdrawing the hindlimb or flinching, was recorded. The percentage of positive responses is calculated for each filament, and the filament that elicits a 60% response rate was considered the mechanical threshold. The 100-g filament was used as a cut-off in this test, as it was typically the stiffest filament that could be applied without causing tissue damage.

3.5 Gait Analysis from Videos Recordings

Custom-made walking platform was used (Width: 14 cm, Mirror width: 25.5 cm, Parkour length: 150 cm, Height: 50 cm, Height from glass panel to top: 30 cm, Height from glass panel to bottom: 20 cm, Camera to parkour distance: 87 cm, Thickness/Frame: 3.5 cm, Mirror placement angle: 45°), platform visual is given in Figure 3.4, for rats to walk and to record their videos. The platform was illuminated by a light source positioned to illuminate the area between the mirror and the glass on which the animals walked, and video recordings were conducted in a dark room. Videos of walking rats were recorded by a high-speed camera (120 fps). For each subject group, videos were recorded before and after (pre-op, D1, D7, D14, M1, M1.5, and M2) the operation. Fore and hind paws of the rats were dyed with surgical skin marker pen (BayCare) to enhance the contrast between paws and the animal body.

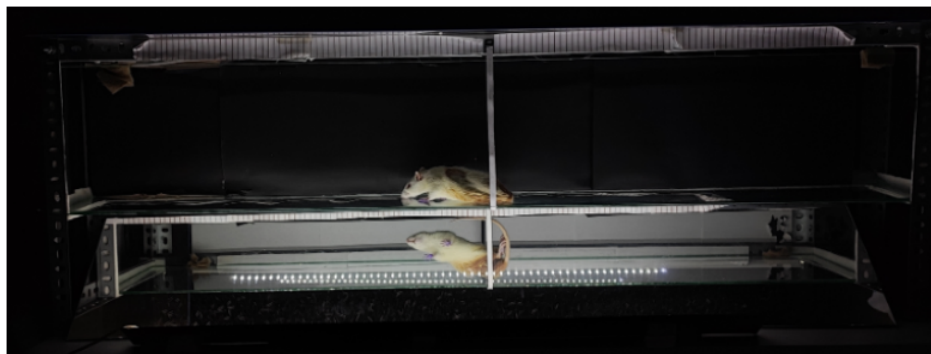


Figure 3.4 Custom-made rat walking platform.

Raw recorded videos were edited via Python. In the editing process, periods of

inactivity during which the animals were not walking were removed from the videos, while the video quality (e.g. speed, resolution, sound) was left unaltered. Videos of the animals actually taking at least 10 consecutive steps were utilized for analysis. Two videos for each subject were analyzed for each week. Gait parameters were obtained with the help of Perver Atilla İnce who is a member of the Tactile Research Laboratory. During this process, the hind and fore paws were tracked using DeepLabCut software, gait maps were obtained via tracked paws (Figure 3.5), and gait parameters (stance/swing/stride durations, limb duty factor, stride length, and maximum footprint area) were extracted in Matlab. Gait parameters were obtained, and a curve was fitted to the gait parameters based on speed in other words gait parameters detrended for speed. Statistical analysis was applied to residual gait parameters.

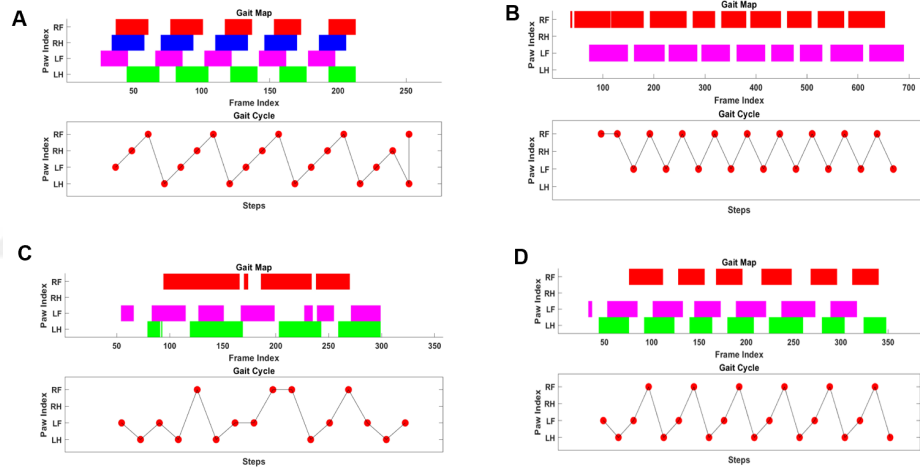


Figure 3.5 Example of Gait Map from NS-SCI group. A. The extracted gait map of one rat before the SCI operation, B. The extracted gait map of the same animal from the following day of the operation, C. The extracted gait map of the same rat from D7, and D. The extracted gait map from the M1.

Gait maps were used to check the tracked paws from the video and they are compared with the extracted gait parameters to detect errors or outliers.

3.6 Statistical Analysis

The data sets were analyzed using various statistical methods. A two-way ANOVA after align rank transform (ART) [69] were applied to BBB and von Frey re-

sults to observe time and group effects. For the gait parameters (stance/swing/stride durations, limb duty factor, stride length, and maximum footprint area), Wilcoxon rank-sum test with Bonferroni corrections were used to compare experimental groups and time effect.



4. RESULTS

Results were collected before the operation and after the operation for each rat at several endpoints. Data from 49 adult Wistar albino rats were evaluated and statically analyzed (the selection criteria are explained in the 4.1. BBB and von Frey Results section). Qualitative observations such as hindlimb usage, urine output condition, or hematuria were made after the operation and after days if needed. Just after the contusion-type SCI, almost all animals were unable to use their two hind paws and stand on their two hindlimbs. Paraplegia or hemiplegia was observed in the moderate-to-severe animals on the next day of the operation and in the first week. 12 animals experienced urine output and hematuria (except one of them) problems. During the first few post-operative days (mostly the first three days), each animal showed signs of soreness/pain. The weight of the animals was also monitored throughout the procedure. A limit of 10% weight loss on the day of surgery was set. As no animal fell below this limit, no termination was made.

4.1 BBB and von Frey Results

Moderate-to-severe injured rats were selected based on their BBB scores on D1 and D7. Rats with BBB scores less than 1 at D1 and less than 9 at D7 were designated as mild-to-severe for the injury criterion. 49 adult Wistar albino rats as a sham (n=10), SCI (n=15), NS-sham (n=6), and NS-SCI (n=18) fit this criterion and the results of these 49 animals were evaluated in this study.

BBB scores range from 21 to 0, as indicated by the BBB scale (Table 2.1). As illustrated in Figure 4.1, the BBB scores of rats were observed to change according to their respective groups over the course of the study. Scores were assigned for each hindlimb of each animal. Subsequently, BBB scores from both the right and left hind paws were observed, yielding an average single BBB score for each animal. As demonstrated in Figure 4.1, the BBB scores for the sham and NS-sham groups remained at

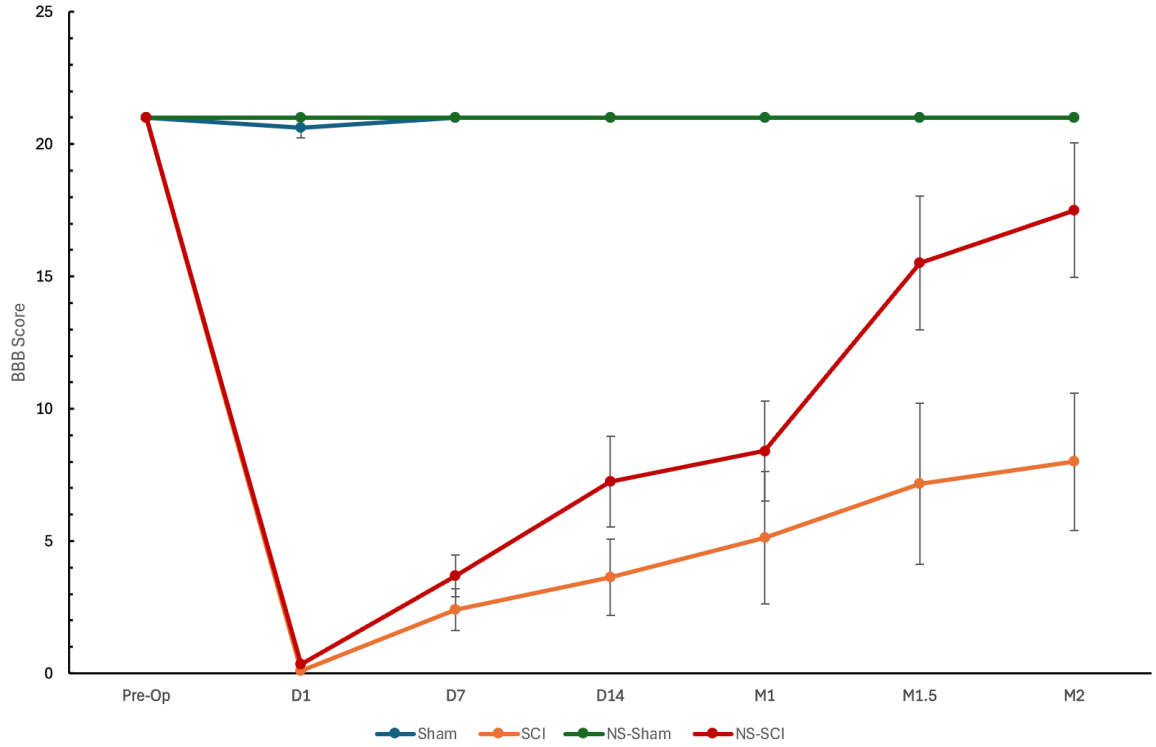


Figure 4.1 BBB Scores of groups through time changes. Blue: Sham group, Orange: SCI group, Green: NS-Sham group, and Red: NS-SCI group.

21 for all time points (pre-op, D2, D7, D14, M1, M1.5, and M2). In contrast, the BBB score decreased to 0 immediately after the operation (D1) in the SCI and NS-SCI groups, afterwards increasing over time. However, as shown in Figure 4.1, the BBB score of the SCI group that did not receive TENS treatment was lower than the NS-SCI group that received treatment at all times after D1.

Aligned rank transform ANOVA was applied to investigate the effects of group, time, and group-time interactions on BBB scores. Both group ($p = <0.001$) and time ($p = <0.001$) have significant main effects on BBB scores. To understand the group and time difference Bonferonni correction is applied. There are no significant differences detected between sham and NS-sham groups ($p = 0.051$) as expected. However, the SCI group has significantly different BBB score changes compared to the sham ($p = <0.001$), NS-sham ($p = <0.001$), and NS-SCI ($p = 0.003$) groups. Moreover, the NS-SCI group is significantly different from the sham ($p = <0.001$) and NS-sham ($p =$

<0.001) groups based on BBB scores. Therefore, it can be said that TENS treatment has a significant effect on BBB scores. Moreover, significant differences are detected between time intervals including data from all groups. Significant changes are seen between D1 and D14 ($p = <0.001$) and between D7 and D14 ($p = <0.001$). However, after two weeks to two months, no significant improvement is detected, D14 vs. M1 ($p = 1.00$) and D14 vs. M2 ($p = 0.115$). By the end of the second month (M2), the BBB score of the SCI group was 8, while the BBB of the treated NS-SCI group was 17.5. Even though there is no significant improvement after D14 to M2, BBB scores significantly recover from D1 to M2 ($p = <0.001$) including data from all groups. Moreover, the group and time interaction effect ($p = <0.001$) was significant which means that the alterations in BBB scores across the groups over time are not uniform. Time group interaction effect was significantly seen between SCI and NS-SCI groups ($p = 0.003$). Consequently, it can be concluded that by the end of the second month, the NS-SCI group had nearly reached the level of the sham/NS-sham group with respect to BBB scores, indicating their ability to perform regular stepping movements. In contrast, the SCI group was still unable to perform regular weight-bearing stepping even at the end of the second month in terms of BBB development.

In the present study, mechanical withdrawal threshold analysis was established to ascertain the mechanical threshold shift in the context of somatosensation. Throughout the experimental phase, data were meticulously collected from both hind paws of each animal. Each von Frey filament was applied ten times to the hind paw of the animal until it exhibited signs of curling. In instances where the rats exhibited behaviors indicative of discomfort (e.g., pulling, itching, licking, etc.) on at least six of these ten applications, the force of the filament was recorded as the mechanical threshold. As demonstrated in Figure 4.2, the von Frey data recorded at Pre-Op resulted in analogous outcomes for all groups (mechanical threshold ~ 60 g). Immediately after the operation (D1), no significant change was observed in the data of the sham groups (sham and NS-sham, mechanical threshold ~ 55 g), while the mechanical threshold of the injury groups (SCI and NS-SCI) increased to ~ 150 g. This outcome indicates that the mechanical withdrawal of the animals decreases after the injury ($p = <0.001$). By the end of two months, the sham and NS-sham groups have similar von Frey thresholds

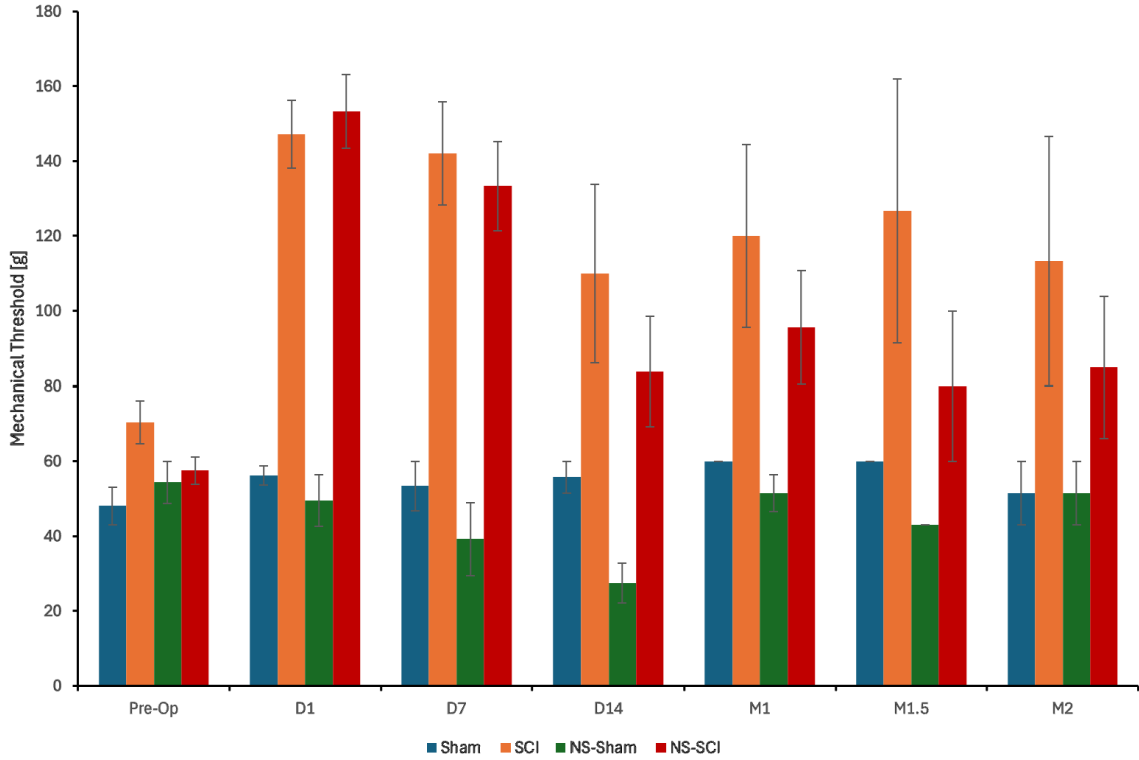


Figure 4.2 von Frey Mechanical Thresholds of groups through time changes. Blue: Sham group, Orange: SCI group, Green: NS-Sham group, and Red: NS-SCI group.

($p = 0.957$) which is not different from the pre-operation ($p = 0.691$). An enhancement was observed up to D14 in both injury groups. The mechanical withdrawal threshold of the NS-SCI group (mechanical threshold ~ 85 g) is lower than the SCI group (mechanical threshold ~ 110 g); however, no substantial discrepancy was identified between the treated (NS-SCI) and untreated (SCI) groups ($p = 0.495$). This finding suggests that both SCI and NS-SCI groups underwent recovery in terms of mechanical withdrawal threshold over time following the surgical intervention, especially until D14 ($p = <0.001$). However, this recovery can be attributed to the effects of time rather than the specific treatment administered. Overall, there was a trend for both SCI and NS-SCI groups over time-based on mechanical withdrawal thresholds.

4.2 Gait Analysis

Since the treadmill was not used in this study, the animals freely walked on the platform. Walking rats at different speeds would affect the gait parameters and thus the overall analyses. Therefore, to eliminate the speed variable and to evaluate the changes in the gait parameters independently from speed, each gait parameter was detrended according to the speed, and residual gait parameters were obtained (Figure 4.3). All statistical analyses and examinations on the gait parameters were done by using residual gait parameters after detrending for speed.

After the operation, injured rats were not able to use their hind paws (as explained in the section 4.1 BBB and von Frey Results) so for all gait parameters no data can be collected for SCI groups except Pre-Op and M1-2. Also, the graphs of gait parameters in the following sections highlight the statistically significant differences between the NS-SCI and NS-Sham groups.

4.2.1 Stance Duration

Figure 4.4 gives the residual stance duration from pre-op to end of two months for NS-SCI and sham groups. At the beginning of the experiment (Pre-Op) there is no difference between sham vs. NS-sham ($p = 0.09$), NS-sham vs. NS-SCI ($p = 0.09$), and SCI vs NS-SCI ($p = 0.09$). After one week (D7), again sham and NS-sham groups have no difference in terms of stance duration ($p = 0.93$). Also, the treated group (NS-SCI) has no significant difference from both sham ($p = 0.083$) and NS-sham ($p = 0.053$) groups. At the D14, the NS-SCI group shows similar stance duration results with both sham ($p = 0.228$) and NS-sham ($p = 0.498$) while there is not any difference was detected between sham and NS-sham ($p = 0.574$). After the first month (M1-2), even though NS-SCI gave similar results with sham groups (sham $p = 0.666$, and NS-sham $p = 0.076$), there is no evidence for the effect of TENS on the improvement ($p = 0.081$).

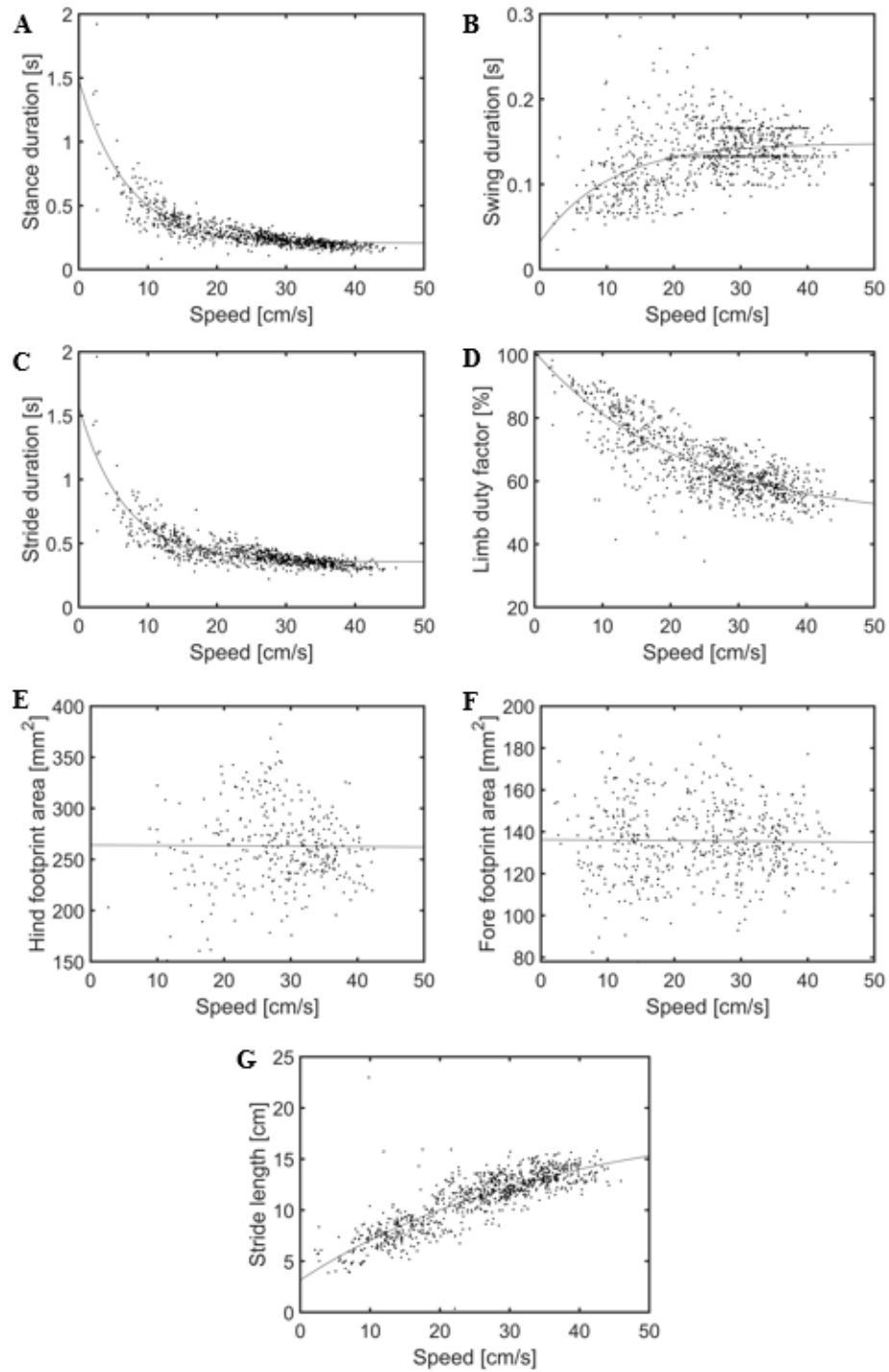


Figure 4.3 Detrending Gait Parameters for Speed. A. Detrending map of stance duration (s) to speed (cm/s), B. Detrending map of swing duration (s) to speed (cm/s), C. Detrending map of stride duration (s) to speed (cm/s), D. Detrending map of limb duty factor (%) to speed (cm/s), E. Detrending map of hind footprint area (mm²) to speed (cm/s), F. Detrending map of fore footprint area (mm²) to speed (cm/s), G. Detrending map of stride length (cm) to speed (cm/s).

In addition to residual stance duration results, raw stance duration recordings were examined to understand the speed effect on gait parameters and raw stance du-

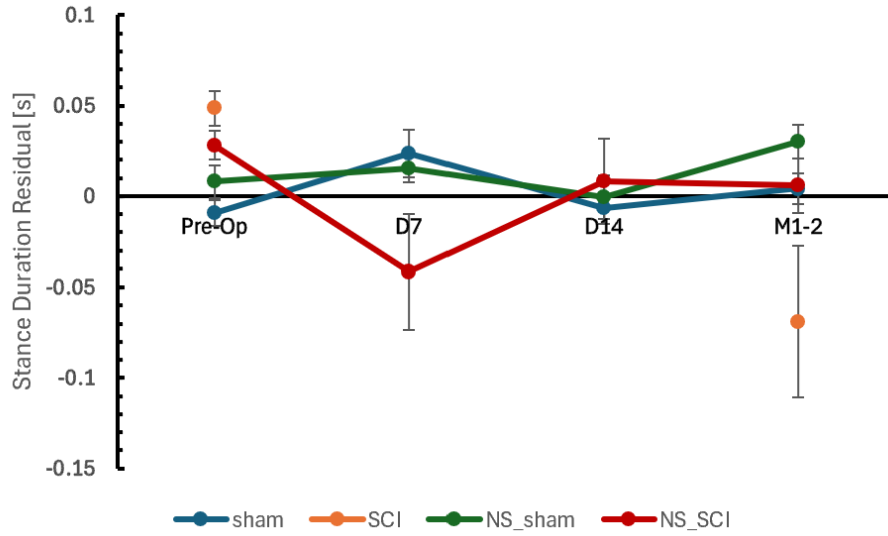


Figure 4.4 Residual Stance Duration Graph through time changes. Blue: Sham group, Green: NS-Sham group, and Red: NS-SCI group.

ration results are given in Figure 4.5. Since the effect of speed is very high on gait parameters, it cannot be compared with raw data.

4.2.2 Swing Duration

Residual swing duration change over the time for NS-SCI and sham groups is given in Figure 4.6. Before the operations, both sham groups ($p = 0.599$) and both SCI groups ($p = 0.822$) gave similar swing durations which indicated there were no significant differences between groups based on swing duration. Over time, no significant differences were detected between sham and NS-sham groups for each time point (D7: $p = 0.085$, D14: $p = 0.798$, M1-2: $p = 0.749$). Also, the treatment effect on the improvement of the swing duration was observed until D14 (NS-sham vs. NS-SCI: $p = 0.852$). However; after D14, the reversal effect was examined at M1-2 and no TENS effect on the recovery was observed (SCI vs. NS-SCI: $p = 0.371$, NS-sham vs. NS-SCI: $p = 0.04$). Moreover, the SCI group had better improvement at the end of two months compared to the NS-SCI group (sham vs. SCI: $p = 0.003$).

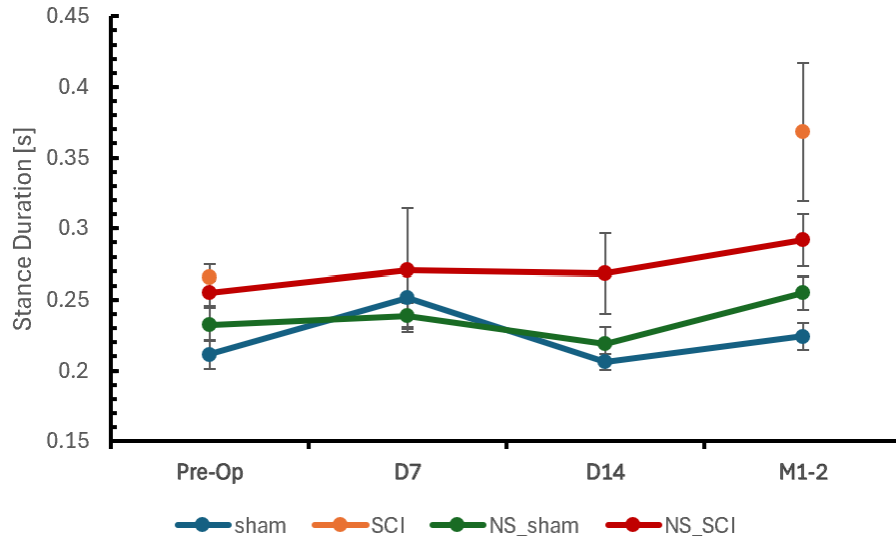


Figure 4.5 Raw Stance Duration Graph through time changes. Blue: Sham group, Green: NS-Sham group, and Red: NS-SCI group.

4.2.3 Stride Duration

The preoperative stride duration data for the sham (sham vs. NS-sham: $p = 0.22$) and SCI groups (SCI vs. NS-SCI: $p = 0.118$) were similar in the stance and swing duration parameters. Until one month, sham and NS-sham groups gave identical results for stride duration (D7: $p = 0.544$, D14: $p = 0.382$) whereas in the chronic phase (M1-2) difference between sham groups was significantly meaningful ($p = 0.036$). Until D14, the treated SCI group significantly improved the stride duration parameter (NS-sham vs. NS-SCI: $p = 0.414$), as seen in Figure 4.7. In the late chronic phase (M1-2) NS-SCI group also showed significant recovery and gave close results with NS-sham group ($p = 0.692$). However, the non-treated SCI group also showed considerable recovery ($p = 0.369$) in the late chronic phase and no differences were observed between the treated and non-treated groups ($p = 0.966$) in terms of stride duration.

4.2.4 Limb Duty Factor

Residual limb duty factors for sham, NS-sham, and NS-SCI groups are given in Figure 4.8 at pre-op, D7, D14, and M1-2 time points. Before the spinal cord injury

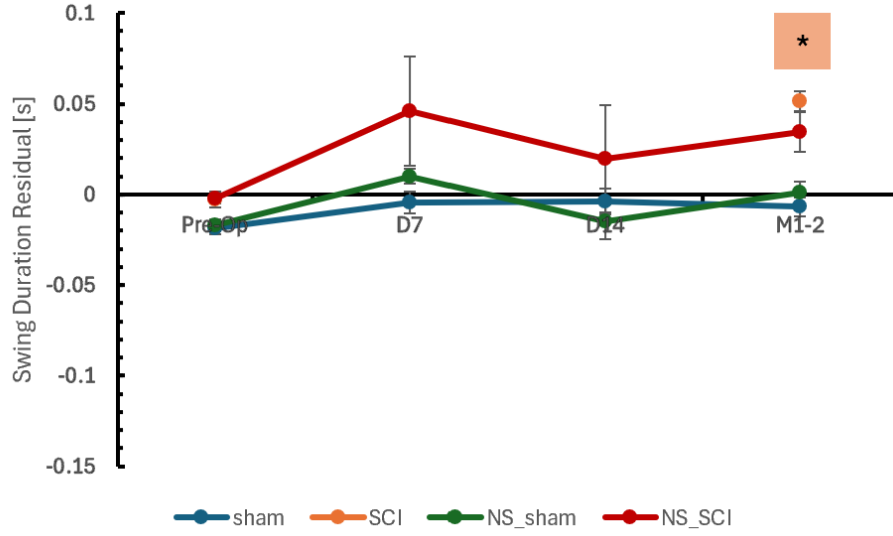


Figure 4.6 Residual Swing Duration Graph through time changes. Blue: Sham group, Green: NS-Sham group, and Red: NS-SCI group.

and sham surgeries, the limb duty factor of all groups was analyzed and there were no differences between any groups (sham vs. SCI: $p = 0.744$, sham vs. NS-sham: $p = 0.267$, sham vs. NS-SCI: $p = 0.626$, NS-sham vs. NS-SCI: $p = 0.103$, SCI vs. NS-SCI: $p = 0.127$, SCI vs. NS-sham: $p = 0.381$) in terms of limb duty factor parameter at the Pre-Op time point. Over time, no differences were observed between sham and NS-sham (D7: $p = 0.175$, D14: $p = 0.645$, M1-2: $p = 0.777$). TENS received SCI group has significant improvement until D14 (NS-sham vs. NS-SCI D7: $p = 0.212$, D14: $p = 0.662$). However, after two weeks to two months (M1-2), treatment had the opposite effect and there was a significant difference between NS-SCI vs. NS-sham groups ($p = 0.004$, Bonferroni corrected) and sham vs. NS-SCI groups ($p = <0.001$, Bonferroni corrected) in the limb duty factor parameter. Also, the SCI group has similar outcomes with the NS-SCI group at M1-2 ($p = 0.126$) which means treatment has a reverse effect after D14.

4.2.5 Stride Length

Stride length is a gait parameter related to length and not to time, in contrast to most gait parameters (stance duration, swing duration, stride duration) for which

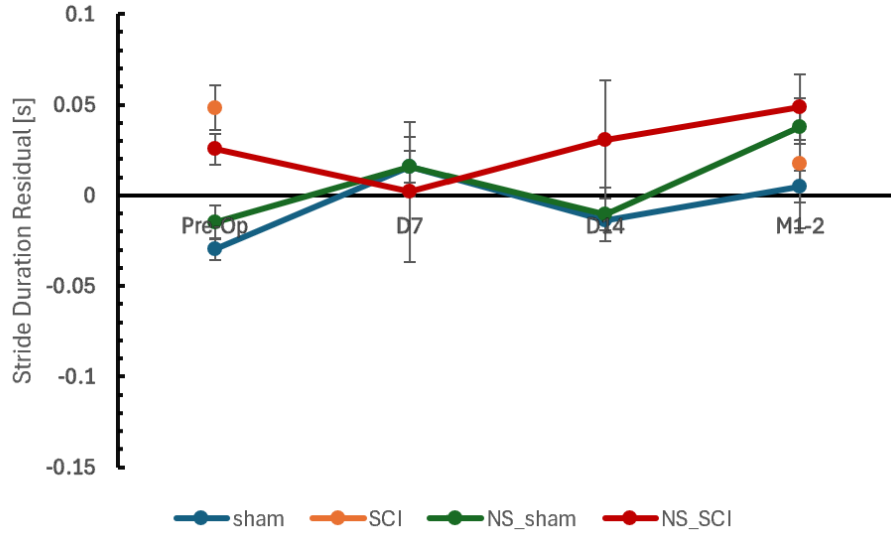


Figure 4.7 Residual Stride Duration Graph through time changes. Blue: Sham group, Green: NS-Sham group, and Red: NS-SCI group.

results have been presented in previous sections. For this parameter, data were collected from each group before the operation and these data were compared. According to the results obtained, no significant difference was found between sham groups (sham vs. NS-sham: $p = 0.425$) and SCI groups (SCI vs. NS-SCI: $p = 0.063$) before the operation. After one week, no difference between sham and NS-sham groups was observed ($p = 0.215$) and the treated group showed recovery in terms of stepping length (sham $p = 0.75$, NS-sham $p = 0.617$). At the end of the second week, sham groups gave similar results again ($p = 0.574$); however, both sham ($p = 0.043$) and NS-sham ($p = 0.043$) groups have different stride length results compared to the NS-SCI group which means treatment has no significant effect on the improvement of the treated group. This result persists at the end of the chronic phase. As given in Figure 4.7, even though there is no significant difference between the sham and NS-SCI group ($p = 0.343$), the non-treated SCI group has the same result ($p = 0.887$). Also, the SCI and NS-SCI group had the same improvement at the end of the second month ($p = 0.371$) which indicates treatment has no significant effect on the stride length parameter.

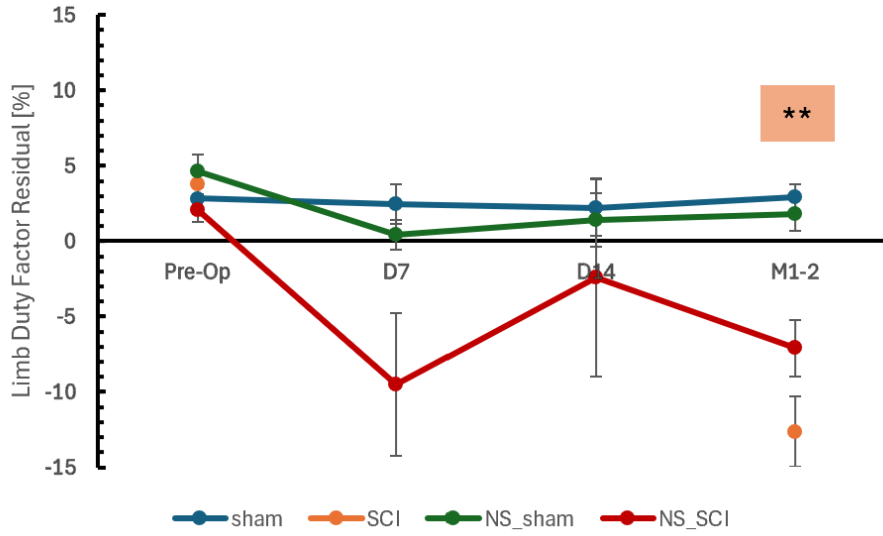


Figure 4.8 Residual Limb Duty Factor through time changes. Blue: Sham group, Green: NS-Sham group, and Red: NS-SCI group.

4.2.6 Hind Paw Footprint Area

As in stride length, hind limb area is a time-independent gait parameter. Before the operations, the maximum areas of the hind paws of the rats in each group during gait were found and compared statistically. No significant difference was found between any groups (sham vs. SCI: $p = 0.104$, sham vs. NS-Sham: $p = 0.267$, sham vs. NS-SCI: $p = 0.1$, NS-sham vs. NS-SCI: $P = 0.653$, SCI vs. NS-SCI: $p = 0.814$, SCI vs. NS-sham: $p = 0.873$). Sham groups have identical throughout the experiment (D7: $p = 0.708$, D14: $p = 0.721$, M1-2: $p = 0.25$). However, the NS-SCI group is significantly different from both sham ($p = <0.001$, Bonferroni corrected) and NS-sham ($p = 0.001$, Bonferroni corrected) groups at the end of the first week. Yet, the NS-SCI group showed improvement at D14 and gave similar results with sham groups (sham: $p = 0.755$, NS-sham: $p = 0.755$) given in Figure 4.10. Unfortunately, the treatment effect can not be observed at the M1-2 time point since the SCI group is not significantly different from the sham ($p = 0.813$) and NS-SCI ($p = 0.371$) groups. Also, observing the significant difference between NS-sham and NS-SCI groups ($p = 0.001$, Bonferroni corrected) which confirms that there is no positive effect to be gained from TENS treatment after D14.

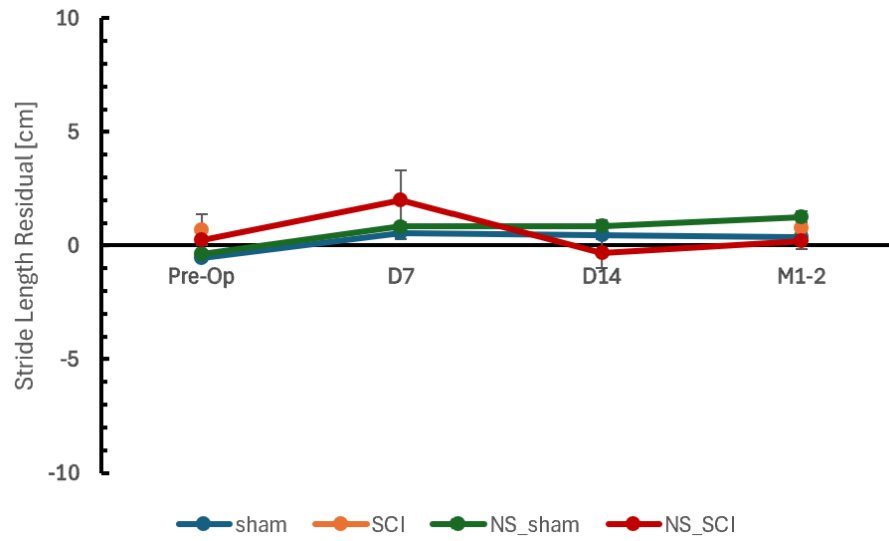


Figure 4.9 Residual Stride Length through time changes. Blue: Sham group, Green: NS-Sham group, and Red: NS-SCI group.

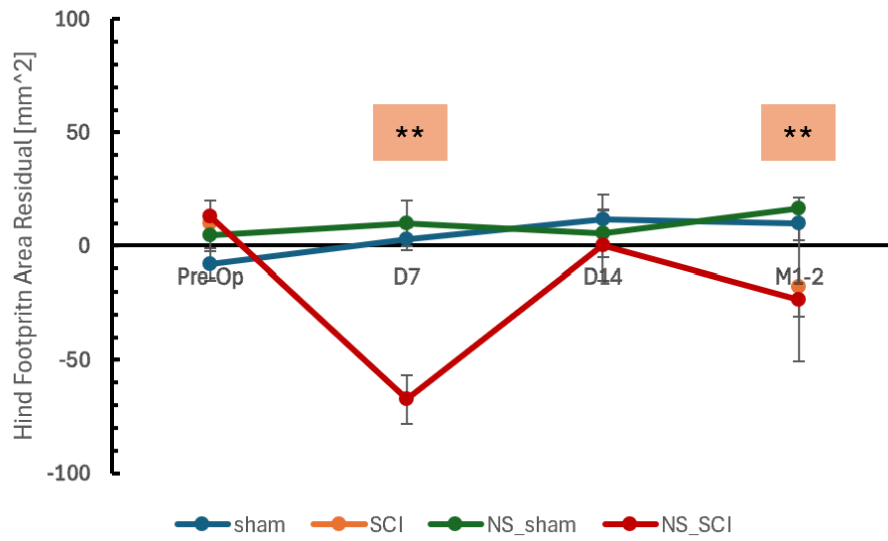


Figure 4.10 Residual Hind Footprint Area through time changes. Blue: Sham group, Green: NS-Sham group, and Red: NS-SCI group.

Overall for all gait parameter results (4.2.1. Stance Duration, 4.2.2. Swing Duration, 4.2.3. Stride Duration, 4.2.4. Limb Duty Factor, 4.2.5. Stride Length, 4.2.6. Hind Paw Footprint Area) it can be said that NS-SCI group showed some trends and significant improvement based on gait parameters particularly up until D14; however, reverse effect was observed in terms of swing duration, limb duty factor, and hind footprint area at M1-2 time point.

5. DISCUSSION

Firstly, a contusion-type spinal cord injury model was chosen to constitute a spinal cord injury model in rats. This type of SCI model provides advantages for various reasons such as understanding the treatment effects and it allows for more reliable translational research. Functional recovery after contusion-type SCI was investigated widely. By the help of the contusion-type SCI model, therapeutic interventions can be evaluated via behavioral tests to understand locomotor function recovery after the injury [70]. Moreover, the neuroprotective effects of the treatment strategies or underlying recovery mechanisms based on anatomical and histological changes can be easily investigated vis contusion-type SCI [71]. Therefore, to understand the underlying neuroprotective effect of transcutaneous electrical nerve stimulation as a therapeutic intervention following this study, the contusion-type SCI model was the perfect fit.

BBB locomotor rating scale is the most reliable and validated system to evaluate the SCI model severity and recovery over time based on locomotor functions. With the help of this scoring system, each stage of SCI can be distinguished and detected which allows for a detailed understanding of the locomotor recovery [56]. BBB scoring system was used to evaluate the severity of the SCI model in rats in this study. BBB scores can also be used to question whether or not the model created is reliable. In our study, BBB scores of SCI and NS-SCI groups decrease to BBB: 0-1 just after the injury and increase over time (Figure 4.1). In other articles in which the contusion type of spinal cord injury has been investigated, studies have been carried out on rats in which the BBB has dropped to very low levels (BBB: around 1) after the injury operation [61],[72],[73],[74]. Also, in our study, SCI and NS-SCI groups were paralyzed and were not able to use their right and left hind limbs. In the literature, several studies are showing that severely damaged animals are paralyzed after the injury [75],[76]. Moreover, several animals have neurogenic bladder problems with unable to urinate in injured groups in our study. Bladder impairments and urinating problems are seen after SCI [77] and they were studies to overcome these problems in the literature [78],[79].

Therefore, it can be said that our contusion-type SCI model is well established and worked properly to investigate the recovery effect of TENS.

von Frey experiment is mostly used for observing mechanical allodynia and hyperalgesia after spinal cord injury in rats. This test is mostly used for evaluating the sensitivity to mechanical stimuli where the sensitivity increases after SCI. This increased sensitivity represents the central sensitization and neuropathic pain. Literature showed that thermal hyperalgesia and tactile allodynia were detected in the fore- and hind paws of rats as early as 7 days after injury and persisted for at least 42 days. The contralesional hind paws of the rats showed a slight increase in paw withdrawal threshold to tactile stimuli after SCI, which corresponded to ipsilesional forelimb motor deficits that resolved over time [80]. Mechanical withdrawal thresholds are also examined via von Frey filaments on rats [81]. The other study shows that rats with severe injuries consistently had decreased withdrawal thresholds to touch and cold on their hind paws. These changes developed over several weeks after the injury. They also found that rats with severe injuries showed increased sensitivity to mechanical stimulation on their torso for the entire 10 weeks of the study [82]. In this study, the mechanical withdrawal threshold was observed after the injury in both SCI and NS-SCI groups in the early stages, D1 and D7 (Figure 4.2), and these results improve over time (at D14) although they are not completely healed.

Transcutaneous electrical nerve stimulation has a recovery effect on sensory responses after SCI in the clinic. Repeated TENS application decreases H-reflex excitability [83]. TENS advances the upper extremities function [84] in addition to the reduction in pain in SCI patients [44]. However, there are also studies in the literature that demonstrate the opposite of these clinical studies, i.e. that TENS has no significant effect on tactile withdrawal [85]. In this perspective, the von Frey results of this study are consistent with the literature in showing a reduction in allodynia over time, but no significant effect of TENS on sensory recovery. One reason for concluding that TENS has no significant effect on sensory recovery may be the frequency used in this study. High-frequency applications are much more effective in sensory recovery and improving allodynia [86]. Therefore, a similar study can be performed at high frequency

and the effect of the frequency difference on sensory recovery can be investigated.

Gait parameters are the computable indicators for observing the recovery of locomotor functions after SCI in rats. The speed of the rat is a crucial restriction on gait parameters since it has a significant effect, particularly on some of the gait parameters. Firstly, speed directly affects interlimb coordination and effective coordinated stepping. The study showed that rats have better interlimb coordination at higher speeds which decreases the coordination mistakes on the stepping pattern of rats instead of lower velocity after SCI [61],[87]. Not only coordination but also stride length is affected by the speed variation after SCI. Lower speed causes lower stride length after SCI [87]. Even though stride length was decreased after the injury, it was shown that the reason for it was not the functional impairment but instead the reduced speed [88]. Moreover, time-dependent gait parameters such as stance and stride durations are also altered based on speed. Both stance and stride durations change inversely proportional to speed in spinal cord-injured rats [61]. To observe the speed effect on gait parameters, the results of stance duration were compared by using residual and non-residual data. As explained in section 4.2.1 Stance Duration, although no significant difference was found between the sham groups, the fact that the NS-SCI group gave different meanings compared to the two different sham groups (sham and NS-sham) makes it difficult to evaluate the results appropriately. Despite the absence of any discrepancy between the sham groups in the late chronic phase, the fact that the treated SCI group exhibited contrasting results in comparison with two different sham groups engenders a dichotomy with regard to the interpretation of the effect of treatment on recovery. The reason for this discrepancy would be the speed effect on gait parameters. Since the speed decreases following injury, the velocity effect on the gait parameters should be eliminated to evaluate the gait parameters correctly. Therefore, gait parameters are detrending for speed (Figure 4.3), and residual gait parameters were examined.

In this study, transcutaneous electrical nerve stimulation was applied as a treatment strategy for improving locomotor function in SCI rats. BBB scores decreased significantly following the injury and increased over time (Figure 4.2). SCI group only

reached BBB: 8 at the end of two months which is expected compared to literature (SCI groups reach 6.5-7 BBB score at the end of 6 weeks in the study) [89]. Also, TENS applied from the tibial nerve significantly affects injured rats and has an improved effect on locomotor recovery until the early chronic phase (D14). However, in the literature, the improvement of rats treated with TENS from their dorsal part only reaches around BBB: 7 after a month [90]. This comparison shows that our application is more successful in terms of recovery time. One of the reasons why we get an earlier response to treatment with TENS application may be that we stimulate the tibial nerve because the tibial nerve provides sensory and motor communication between the lower extremities of the body and the brain. Also, stimulation of the tibial nerve modulates reflex pathways that have a similar effect with sacral neuromodulation so improves locomotor recovery after SCI [91].

In terms of gait parameters, studies show that the gait parameters of SCI rats improved until the late chronic phase of the (M1-2) injury; however, they cannot reach the sham group [74],[92]. In our results, we also observe that SCI groups only catch sham group criteria for some gait parameters except swing duration (section 4.2.2) and limb duty factor (section 4.2.4). On the other hand, the NS-SCI group shows significant recovery up until D14; however, we saw a reverse effect of the TENS for parameters of swing duration, limb duty factor, and hind footprint area after D14. One reason for this result could be the maladaptive spinal plasticity effect of TENS after D14 [93]. Hence, the electrical nerve stimulation therapeutic approach gives better results with simultaneous application with stepping, it is thought that the reason for the reversal effect due to TENS being not synchronized with stepping [47],[94].

Even though epidural electrical nerve stimulation has significant improvements on gait and locomotor function [95], application of EES is very challenging and it causes adverse effects during the application in clinic [96]. Also, the placement location of the EES is critical where the misplacement causes a reduction in the efficacy of the application [97] and EES could have variable outcomes in patients [98]. Even though advanced technology EES studies continue and their efficiency is proven [99], they remain limited because they require advanced technology and professional application.

On the other hand, TENS offers a more advantageous option compared to EES thanks to its ease of application management, not requiring advanced technology or not posing critical risks during application. Furthermore, the fact that a study has shown that TENS stimulates the same neural network as EES and leads to recovery [46] shows how important it is to investigate the mechanism and better understand the effects of this method on recovery.

5.1 Limitations

The first limitation of this study is the speed variety in the study. As explained at the beginning of this section, speed directly affects gait parameters. The fact that residual gait parameters were used after the detrending method to eliminate the speed effect was a limitation in comparing our gait parameter results with the literature directly.

A further limitation is the inability to apply TENS concurrently with stepping. Although the synchronization of nerve stimulation with stepping has demonstrated enhanced efficacy in promoting spinal plasticity and facilitating locomotor recovery, the practical challenges associated with the simultaneous application of TENS and stepping have hindered the widespread adoption of this approach. This situation could affect the efficacy of TENS.

Finally, the evaluation of the gait analysis from the walking videos is another limitation. Video analysis could lead to misreading of paw interaction with the ground which alters the gait parameters directly. Moreover, using a custom-made walking platform in addition to a lab-developed DeepLabCut system specifically for this study further diversifies the limitations of gait analysis. It also limits the direct comparison of gait parameters with literature since commercial platforms were used mostly in literature.

5.2 Future Work

In this study, the number of subjects is limited so the study should be extended with the higher number of subjects. Even though the positive effect of TENS application on locomotor function recovery is shown in this study, the molecular mechanism and neural circuitry that underlies this neuroprotective and neuroinflammatory effect should be investigated. Moreover, no significant sensory recovery was observed in this study. Therefore, TENS application could be applied in high frequencies to understand the sensory and allodynia recovery. After a wide investigation of rats, TENS application could be applied to people with SCI in the clinic for the following translational applications.

5.3 Conclusion

This study shows TENS has a positive effect on the locomotor recovery of rats with SCI until the early chronic phase of the injury. However, the results show that the treatment did not improve gait parameters between the early and late chronic phase. Therefore, applying transcutaneous nerve stimulation simultaneously with stepping would have better recovery results on the locomotor ability of rats after the injury.

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