# NEUROMODULATORY EFFECTS OF ULTRASOUND STIMULATION ON CERVICAL CORD INJURED RATS

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**Rakib Uddin AHMED**

**A thesis submitted in partial fulfillment of the requirements for the degree of**

**Doctor of Philosophy**

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## Certificate of originality

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Rakib Uddin Ahmed (Name of student)

# Abstract

Spinal cord injury is a devastating neurological condition that affects millions of people worldwide each year. Several neuromodulatory approaches have been applied to restore the function of injured individual, among which neuromodulation therapy has shown most promising results, so far. There is, however, some limitations persist with electrical neuromodulation therapies. Invasive method of spinal cord neuromodulation requires surgeries while non-invasive method shows limited improvements to restore hand function. However, the mechanism is yet unknown. In addition to the electrical and magnetic stimulation, therapeutic effect of ultrasound stimulation on neurons has recently been explored. The non-invasive therapeutic effect of low-intensity pulsed ultrasound is a novel approach that can modulate the neuronal function following a traumatic injury in brain and peripheral nerve. Because of the safety and efficacy low-intensity pulsed ultrasound stimulation has shown promising results in neurorehabilitation. The objective of this thesis was to investigate potential functional effects with ultrasound stimulation in chronic cervical spinal cord injured animal models via behavioural and electrophysiological studies. It was found that, the forelimb reaching and grasping success rate was higher during low-intensity pulsed ultrasound stimulation than without stimulation in rats with cervical cord injury. Furthermore, the rats showed higher grip forces in ultrasound stimulation group compared to the control group. However, the effect is not enough to restore the full forelimb function. Hence, a combinatory neuromodulatory approach was conducted to restore the hand function in cervical cord injured rats. First, a serotonergic agonist drug (Buspirone) with different doses was administered intraperitoneally to find out an optimum dose. The result showed that low doses of serotonergic agonist significant

improved forelimb function in rats over high to medium dose group rats. After finding the optimum dose, a combinatory neuromodulatory approach- ultrasound and drug-based treatment was provided to the injured rats and compared to the only stimulation and control groups. The combination group of rats has shown the consistent recovery in the forelimb reaching and grasping task over the control group of rats indicated the potential of the approach. This thesis summarizes the therapeutic effects of low-intensity pulsed ultrasound and serotonergic against neuromodulation on forelimb functional recovery including reaching and grasping after cervical spinal cord injury, and offers a clinical translation of a novel non-invasive neuromodulation therapy for the paralyzed.

# PUBLICATIONS ARISING FROM THE THESIS

## Peer-reviewed journal articles

- **Ahmed, R. U**., Alam, M., & Zheng, Y. P. (2019). Experimental spinal cord injury and behavioral tests in laboratory rats. *Heliyon*, 5(3), e01324. DOI: 10.1016/j.heliyon. 2019.e01324.
- **Ahmed, R. U**., Edgerton, V.R., Li, S., Zheng, Y. P., Alam, M. Buspirone dose-response on forelimb functional recovery in cervical spinal cord injured rats. ***(Under review).***
- **Ahmed, R. U.,** Alam, M., Li, S., & Zheng, Y. P. A novel combinatory approach ultrasound neuromodulation along with drug to restore the functions in cervical cord injured rats. (***In preparation).***

## Conference proceedings

- **Ahmed, R. U**., Alam, M., Li, S., & Zheng, Y. P. Ultrasound neuromodulation to restore the functions in cervical cord injured rats. International Neuromodulation Society's 14$^{th}$ World Congress, Sydney, Australia, 2019. DOI: 10.1111/ner.12958.
- **Ahmed, R. U.,** Edgerton, V.R., Li, S., Zhong, H., & Zheng, Y. P., Alam, M. Buspirone dose-response on forelimb functional recovery in cervical spinal cord injured rats. American Society for Experimental Neurotherapeutics 22$^{nd}$ Annual Meeting, Bethesda, MD, USA, 2020. DOI: 10.1007/s13311-020-00896-5.

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# TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

5-hydroxytryptamine (5-HT)

Analysis of variance (ANOVA)

Brain-derived neurotrophic factor (BDNF)

Corticospinal tract (CST)

Electromagnetic Spinal Cord Injury Device (ESCID)

High intensity focused ultrasound (HIFU)

Infinite Horizon (IH)

Irvine, Beatties and Bresnahan (IBB)

Low-intensity pulsed ultrasound (LIPUS)

Mesenchymal stem cells (MSCs)

Multicenter Animal Spinal Injury Study (MASCIS)

Pulse Repetition Frequency (PRF)

Spinal cord injury (SCI)

Spatial-peak temporal-average intensity ($I_{SPTA}$)

Ultrasound (US)

# Chapter 1: Background

*The work described in this chapter has been published (Ahmed et al. Heliyon. 2019).*

## 1.1 Introduction

Traumatic spinal cord injury (SCI) is a devastating neuronal dysfunction that affects a large number of people worldwide. Though the exact prevalence of SCI is unknown, it is estimated that every year a significant number of people get involved with this injury. In the United States approximately 17,000 new cases are recorded each year that is around 54 cases per million of their population (NSCISC 2016) while in China around 60,000 new cases of SCI are reported each year caused by vehicle crash, construction works, mining and other accidents (Qiu 2009).

In addition, bullet wounds or other form of violence (26%), sports accidents (7%) and falls from high land (22%) are major causes of SCI in USA.(DeVivo et al 2002). Incidence and prevalence of SCI varies worldwide and no exact information can be found (Singh et al 2014). It has been reported that in Australia, the incidence is 16 cases, and in western Europe 15 cases per million individuals (Cripps et al 2011). Traumatic SCI most commonly occurs in male (79.8%) compare to female (20.2%) (Chen et al 2016). While the average age range for SCI lies between 15 to 30 years. However, for older population (but less than 50 to 60 years) the incidence rate also increased from 4.6% in 1970 to 13.2% in 2008 indicates that incidence of SCI is rising (Devivo 2012, DeVivo & Chen 2011, van den Berg et al 2010). Following SCI, the patients and their families face considerable physical, social and financial problems. It has been estimated that approximately US$1.1-4.6 million per patient are spent over their lifetime (Rankin et al 2017). The injury affects spinal cord

functions completely or incompletely by impairing motor, sensory and autonomic reflexes. There is no cure for SCI at the moment (Heselmans 2020). However, there are several approaches including cell treatment, neurostimulation, pharmacological modulation and rehabilitative strategies have been carried out to find a possible solution for SCI patients (Alam et al 2017, Assinck et al 2017, Edgerton & Roy 2012). Although epidural stimulation method showed significant improvement in SCI patients (Angeli et al 2018, Angeli et al 2014, Harkema et al 2011) this invasive method requires major surgeries.

### *1.1.1 Anatomy and physiology of spinal cord*

Spinal cord is a cylindrical structure that begins from the foramen magnum of the skull and run through the vertebral canal. In human, it terminates at the lower border of the lumbar vertebra **(Fig. 1a).** Three membranes cover the spinal cord; the dura mater, arachnoid mater and pia mater. Within the subarachnoid space, cerebrospinal fluid surrounds the spinal cord.

Spinal cord is composed of an inner core of gray matter which is surrounded by white matter. Gray matter contains mainly cell bodies, axon and dendrite terminals, interneurons and glial cells. In sagittal section, gray matter is an “H” shaped structure and divided into dorsal horn (left and right), intermediate grey, ventral horn (left and right) **(Fig. 1b).**

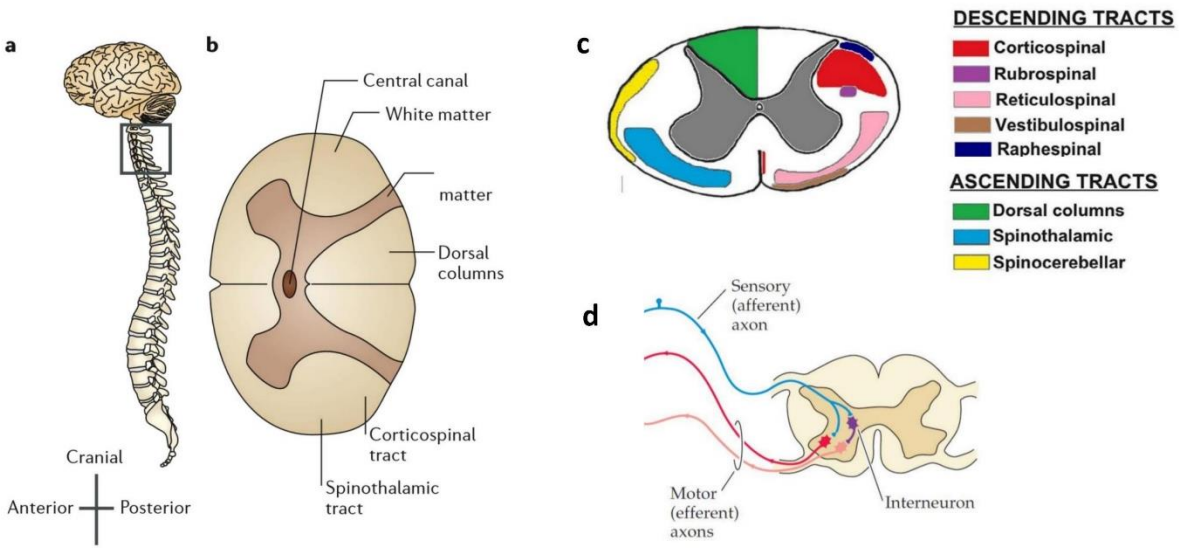



**Fig. 1**: **Anatomy and physiology of spinal cord. a)** Schematic view of the human spinal cord; **b)** cross-section view; and **c)** location of human ascending and descending tracts (S= sensory and M=motor tracts); **d)** transmissions of neural impulse through the sensory and motor neuronal fiber. Reprinted from (Halterman 2005, Thuret et al 2006, Watson & Kayalioglu 2009).

White matter is composed of mainly myelinated axons which contain two different tracts- ascending and descending tracts. Sensory information transmits to the central nervous system through the ascending tracts. It mainly carries information related to the pressure, vibration, movement and proprioception. Descending tracts originate from the cortical areas of brain **(Fig. 1c)**. Different motor activities such as locomotion, posture, balance, muscle tenacity are carried out through these tracts. Spinal sensory neuron from dorsal horn leaves through the intervertebral foramen and enter into the dorsal root ganglion which is comprised of cell bodies of pseudounipolar neurons. Similarly, ventral root leaves from ventral horn of the spinal cord and carry neuronal motor fibers **(Fig. 1d)**. Distal to the ganglion dorsal and ventral roots join to form a spinal nerve that later forms peripheral nerves (Arthur J. Vander 2001).

### *1.1.2 Types of spinal cord injury*

The levels and types of injury depend on the location and severity of the neural circuits being damaged due to the injury. It can be broadly categorized as incomplete or complete lower-limb paralysis (paraplegia), or both limb paralysis (quadriplegia). Incomplete injury involves partial severed spinal cord with the injured persons retaining some functions. While in complete injury, the spinal cord is completely severed (in clinical measures) and no neurological function present below the level of injury (**Fig. 2a)**. However, it was found that most clinically complete SCI patients residue some or few anatomical connections across the lesion (Awad et al 2015), and thus possess the potentials for future recovery via neuroplasticity (Alam et al 2016).

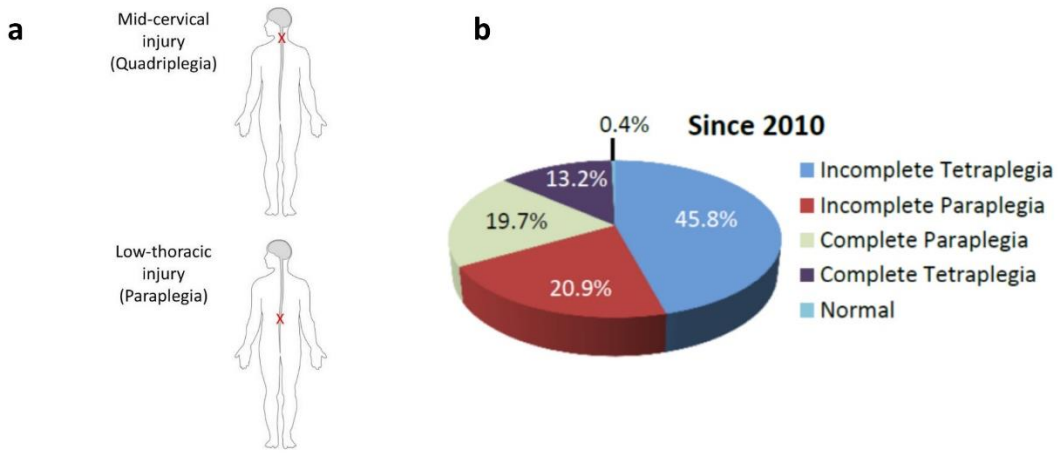



**Fig. 2: Spinal cord injury level a)** Different level of injury in patient. Injury at higher cervical level can lead to difficulty including breathing, coughing, speaking and all limb paralysis. The lower level of injuries results lower limb paralysis, bladder and bowel dysfunctions and other complicated issues. Adapted from (Alam et al 2016). **b)** SCI fact sheet; Adapted from National SCI statistical center, 2017.

According to the National Spinal Cord Injury and Statistical center (Patel et al 2017), incomplete injuries are more frequent condition than the complete injuries **(Fig. 2b)**. At the cervical level incidence is higher around 60%, while at thoracic and lumbosacral level the injury is respectively 32% and 9% (Chen et al 2016).

### *1.1.3 Pathophysiological events after SCI*

Traumatic injury events are categorized into two major groups- primary and secondary injury. Initiation of primary injury event occurs following a mechanical trauma, dislocation and disruption of the vertebral column that causes compression and/or transection of the spinal cord. Consequently, the blood-brain barrier disrupts and results in immediate neuronal death.

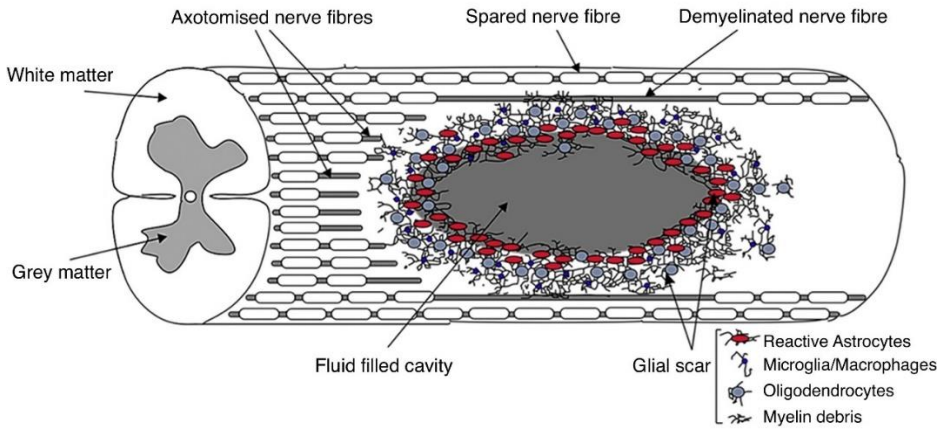



**Fig 3**: **Pathological events after complete and incomplete spinal cord injury**. The injured spinal cord lesion includes three distinct areas- **i**) a central fluid-filled cavity (gray color), **ii**) an outer glial scar lesion that is composed of astrocytes, macrophages and oligodendrocytes (red border) and **iii**) outer spared neuron, some of them are functional and have the capacity to regenerate. Adapted from (McCanney et al 2017).

Following mechanical injury leads to several biological and biochemical events that elucidate the damage of the spinal cord and termed as a secondary event. It includes several

events such as- hemorrhage, reactive cell infiltration, alteration of cell membrane permeability, glutamate excitotoxicity, free radical production, lipid peroxidation, cytokine production, ion-channel modulation, apoptosis and later glial scar formation **(Fig. 3)** (Liu et al 2011, Mautes et al 2000, Obrenovitch & Urenjak 1997). The events occur over time from days to months, and further neurological damages took place and carry into the chronic phase (>1 year) (Maynard et al 1979).

### *1.1.4 Complications after SCI*

SCI patients suffer acute and chronic medical complications. Common complications related to their daily activities such as – bladder and bowel dysfunction, muscle spasticity, pain, pressure sores, temperature regulation and other psychological problems. Compared to other complications, bladder and bowel dysfunction are the most common sequelae after a SCI. Two patterns of neural bowel dysfunction are normally reported after SCI- upper and lower motor neuron bowel dysfunction. Upper motor neuron bowel dysfunction results from the injury at the sacral level and lower motor neuron bowel dysfunction from the sacral spinal cord roots or peripheral nerve innervation (Stiens et al 1997). Lower motor neuron dysfunction is associated with constipation, hemorrhoids and abdominal distension (Coggrave et al 2009). Complete SCI patients develop muscle spasticity because of disconnection from the upper motor neuron which results in the flaccidity of muscle. Patients often develop infections because of not fully completion of bladder expression. Furthermore, atrophy of muscles is also a common sequela of complete SCI. For atrophy, lower limb muscles are most affected due to the loss of contractile protein early after SCI (Castro et al 1999).

## 1.2 Rat model for SCI

Spinal cord injury (SCI) is responsible for damaging the sensory, motor and autonomic functions of an individual (Kjell & Olson 2016). To address this for developing and testing therapies, over the past few decades animals have been used (Zhang et al 2014). Both large and small animal models are commonly used to study the injury mechanism and to assess the therapeutic outcomes after a controlled SCI in laboratory settings (Cheriyan et al 2014). Large animals, especially dogs and cats were used in the early 1950s to1970s (Kwon et al 2015). Afterward, rodent models became popular and were then standardized as a suitable animal model for SCI experiments. In rodent models, rats and mice are mostly used to evaluate the functional and morphological changes after SCI (Metz et al 2000, Sharif-Alhoseini et al 2017, van den Brand et al 2012). However, in the mouse model, following a SCI, evidence suggests that a modest degree of regeneration occurs in the injured area and no typical cyst-like structure is formed, while such type of axonal regeneration is not found in rat and non-human primates (Kjell & Olson 2016). In contrast, after an injury in rats, a large fluid-filled cystic cavity is formed which is similar to the human pathology after SCI and thus useful for preclinical studies (Lee & Lee 2013). Rats are quickly adaptive to the experimental environments and the given tasks for sensorimotor evaluations. Furthermore, high availability, low infection rate after surgery, and ease of maintenance make the rat a prime candidate for translational research. Based on the above, laboratory rats are now widely used as experimental SCI models.

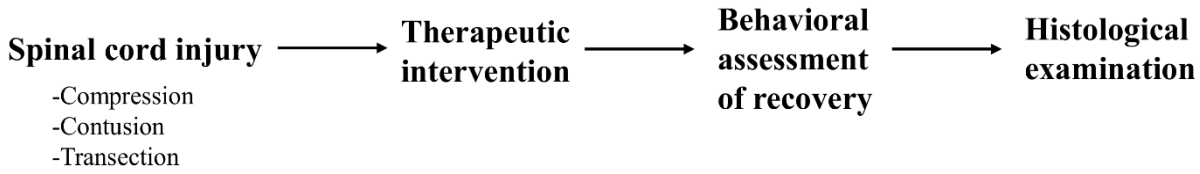



**Fig 4: Overall experimental approach of developing a translational therapy for SCI**. SCI is induced in a rat via one of the three commonly applied techniques: compression, contusion and transection. After experimental injury, several therapeutic interventions are used to regenerate the damaged neuronal pathways. To assess the functional recovery, several behavioral tests are performed based on the injury level and finally, histological examination is performed to find out the anatomical changes.

In this translational research, several rat injury models were developed to examine the neurophysiological responses after a SCI. Numerous approaches are being applied to regenerate the damaged axon across the lesioned area and confirmed immunohistochemically and by protein analysis (Olson 2013). Furthermore, apart from histological analysis, behavioral responses are also studied as a primary measure to assess the recovery of spinal pathways **(Fig 4)**.

### 1.2.1 Injury models

Experimental injury models are designed to explore the physiological changes after SCI to evaluate the treatments to repair the broken spinal cord. Laboratory rats are commonly used to evaluate neural repair after an experimental SCI. Although some SCI experiments have been conducted in non-human primates, more demanding protocols, critical manpower, health safety and a large amount of investment in the experimental setup and management make the primate model much more difficult and less used than rat injury models (Sharif-Alhoseini & Rahimi-Movaghar 2014). In rat SCI experiments, three different injury models

are widely utilized: compression, contusion and transection models. Among these, contusion and compression are the most common types of injury found in humans. Hence, to evaluate the neuronal changes and behavioral outcomes, these models are essential (Sharif-Alhoseini et al 2017).

### 1.2.2 Compression model

In humans, flexion and axial compression (burst fracture) are responsible for displacing bone fragments into the vertebral canal and consequently SCI occurs. However, the timing of compression can affect the outcome of pathology (Abdullahi et al 2017). To address this problem, in rats the same model of injury is carried out to create persistent spinal canal occlusion that is similar to human acute SCI. This injury is varied at different levels of the spinal cord. For example, at lumbar level, compression injury impairs the ejaculation reflex in male rats (Kozyrev et al 2016). There are several ways to create a compression model for rats such as calibrated forcep compression, clip compression and baloon compression (Abdullahi et al 2017). However, the clip compression model at the thoracic level is much more correlated with the functional and histological outcome (Poon et al 2007).

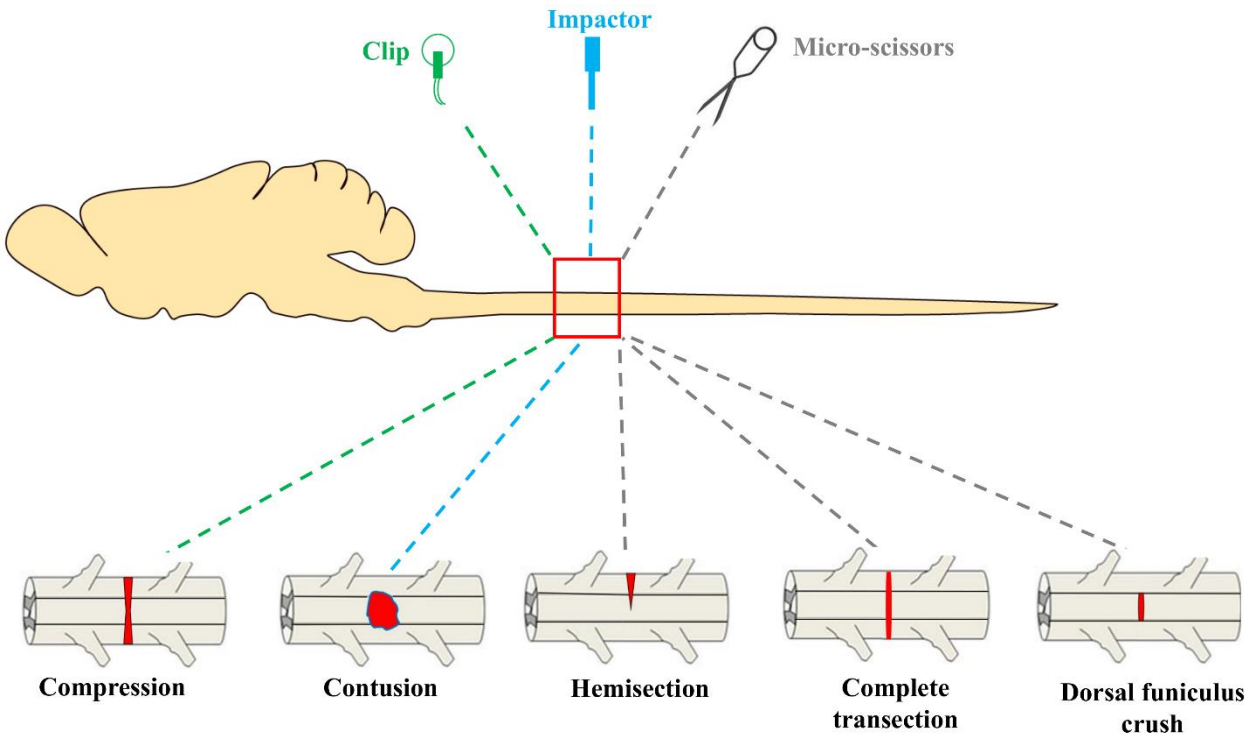



**Fig 5: Different injury models in rats' spinal cord.** In the compression model a clip is used to initiate the injury. For the contusion model of injury an impactor is dropped from a predefined distance. In the transection model of injury, a surgical blade is used to carry out different types of transection injury. Modified from (Walker & Xu 2012).

The acute clip compression model is the first non-transection model that was developed in 1978 (Rivlin & Tator 1978) and is widely used for experimental injury in rats. It is an inexpensive method where a special clip is used to compress the spinal cord (Tator & Poon 2009). Under inhalation anesthesia, a laminectomy is performed at a desired level of the spine by retracting muscles and removing the spinous process and body of vertebrae. A modified aneurysm clip **(Fig 5)** is then inserted extradurally and maintained for 60 sec and then the clip is removed to produce an acute injury (Moonen et al 2016). Muscles and connective tissues are then sutured, and the skin is closed. The injury model is useful to

study the minimal loss of neurons after a SCI. Moreover, for secondary injury mechanism the model is also useful (Sun et al 2017).

### 1.2.3 Contusion model

In humans, compression injury is caused by mainly falls from a height or by a physical impactor that crushes the spinal vertebral column which further leads the bony parts to compress the spinal cord (Kjell & Olson 2016). In rats, to get the same type of injury, the contusion model is probably the most suitable method (Young 2002). Among the several anatomical locations, in thoracic position contusion injury is commonly used to evaluate the forward and backward locomotion as well as recovery of the spinal tract (van Gorp et al 2014). Usually, the injury is induced by an impactor which is used to drop a 10-gm rod onto the spinal cord (Young 2009). Several impactors are available worldwide to serve this purpose. In Multicenter Animal Spinal Injury Study (MASCIS), the New York university impactor, Infinite Horizon (IH) impactor and Ohio State University Electromagnetic Spinal Cord Injury Device (ESCID) are most commonly used. In MASCIS, a 10-gm computer-controlled load is used to induce a SCI in rats (Gruner 1992). After laminectomy, at a desired spinal level, muscles and spinous process are removed according to the diameter (usually 2.5 mm) of the impactor **(Fig 5)**. Height of the impactor, drop weight and time are previously set up on a computer before initiation of the injury. After the injury, subdural hemorrhage is commonly found which is later washed with saline (Young 2009). Finally, muscles and skin are sutured. Another reliable commercially available impactor is Infinite Horizon (IH) which is also used to produce SCI rat models. System software is implemented to apply impactor force to the spinal cord. This injury renders an ideal model of SCI by reducing the variability among other existing devices (Scheff et al 2003).

### 1.2.4 Transection model

The transection model of injury is the most widely utilized method that can give the information of desired neural tracts at different spinal level after injury. To investigate the neuronal and functional improvements after different transplantation therapies, transection models of SCI are commonly used (Lukovic et al 2015). This injury model is quite stable and assessment can be performed within four weeks of the injury (Wang et al 2014). The spinal cord transection model is also useful to evaluate axonal regeneration and behavioral responses after SCI (Flores-Leal et al 2017). Transection injury cannot produce any condition similar to contusion injury. However, this injury is useful to investigate the damaged tract recovery after experimental therapies. Two main transection models (complete and incomplete) are used in rats. In the incomplete transection model (often called hemisection model), different portions of spinal cord are cut such as lateral hemisection, dorsal hemisection and dorsal funiculus crush to carry out different experiments. However, incomplete surgical transections are not considered relevant to human SCI (Steward & Willenberg 2017). A complete injury requires a complete transection of the spinal cord including both ascending and descending tracts. To carry out a complete transection injury after laminectomy, microdissection scissors are inserted to cut the spinal cord at a desired level and depth. After an injury gelfoam is inserted into the transection site to minimize the bleeding and to confirm the separation of the spinal cord. After some complete transections ventral parts of the axons may be spared, which is responsible for some residual motor function of the hindlimb (Shah et al 2012). For an incomplete transection injury iridectomy scissors are used to cut both the dorsal and ventral columns of the spinal cord from lateral to the midline by closing the tip of the scissors

(Arvanian et al 2009). Muscles and skin incision are then sutured, and the rat is examined for therapeutic approaches and behavioral tests after recovery. Complete and incomplete transection injuries are not very similar to the clinical SCI of humans; they are rather utilized for neuroscience and neurology research to study the neuronal circuits and pathways (Steward & Willenberg 2017).

Transection injury can give information on both ascending and descending tract functions which is useful in neuroscience research (Sharif-Alhoseini et al 2017). Compared to other injury models there is no need for a special device to transect the spinal cord. Before transecting the spinal cord, it is essential to understand the neurophysiology of the different anatomical structures of the nervous system. A complete transection injury event involves the dissection of both ascending and descending tracts (Cregg et al. 2014). Special care should be taken for the pain management and urinary bladder management after a complete transection. To study specific spinal tract activity such as dorsal and dorsolateral corticospinal tract (CST), an incomplete transection (or hemisection) injury can be applied. After therapeutic interventions, this injury can also provide information regarding axonal tract regeneration. The hemisection model of injury is suitable at the level of cervical region CST which is responsible for forelimb reaching tasks. To regenerate axonal tracts for translational research after any therapeutic approach or cell transplantation, the transection model of injury can be a choice for experiments (Bregman et al 2002). However, the transection injury model is unable to study the secondary injury mechanism.

### 1.2.5 Selection of an optimum injury model

Pathological and behavioral functions that alter after a SCI depend on the site and type of the injury. A correct injury model can give a proper understanding of the system and the

cellular-level changes after a SCI. Furthermore, an experimental SCI model is necessary to evaluate the safety and reliability of a new therapeutic intervention before translating it to humans (Schwab & Bartholdi 1996). Every injury model has advantages and disadvantages over other models. Some selection criteria (listed in **Table 1**) are necessary to select the most optimum one for a specific study.

**Table 1: Selection of an optimum injury model**

| Contusion | Compression | Transection |
|---|---|---|
| • The model is useful for neuroprotective mechanism study (Pinzon et al 2008).<br>• Ideal injury model for studying pathology and secondary injury mechanism (Sharif-Alhoseini et al 2017).<br>• Suggested for translational research. | • Simple and reliable method (Marques et al 2009).<br>• Less invasive method (Abdullahi et al 2017).<br>• Useful for cell transplantation therapy<br>• Suitable model for translational research (Tator & Poon 2009). | • To study specific pathway function and regeneration (Lukovic et al 2015).<br>• Easy to trace the axonal tract (Lukovic et al 2015).<br>• Precise control over the injury.<br>• Suitable model for neuroscience research. |

Based on the different aspects of lesion and behavioral tests, experimental rat models have advantages and disadvantages over other animal models. For example, for therapeutic

research of forelimb muscles and movements, the transection injury model is useful (Steward & Willenberg 2017). To evaluate motor recovery, skilled reaching tasks in rats would be useful. Similarly, drug and cell treatments are useful to study the regeneration of the CST in the spinal cord that assists the forelimb movements (Dai et al 2009, Girgis et al 2007). Hence, it is essential to choose the right injury model based on the specific research question one might have in SCI research.

### 1.2.6 Behavioral examinations

The fundamental organization of a rat's spinal cord is not much different from that of the human spinal cord. Before and after an experimental SCI in rats, behavioral tests are conducted to understand the alteration of neurophysiology of the spinal cord neural network. Furthermore, a behavioral test helps to trace any improvement of a specific function at different stages of therapeutic intervention. As an example, after a cervical cord injury, the forelimb muscles lose the ability to contract volitionally to grasp anything. Furthermore, the co-ordinations between the muscles are also lost (Alam et al. 2017). Different muscles are involved in different skilled movements such as reaching, grasping, and withdrawal of the limb which can be topographically mapped in the spinal cord region as shown in **Fig 6** (McKenna et al 2000). After an experimental SCI in the desired location, alteration in the spinal cord and neurological function can be confirmed after behavioral assessment. Hence, behavioral recovery is one of the fundamental tests after any therapeutic intervention of SCI. Laboratory rats are widely used to evaluate different types of behavioral tests that involve neurological functions.

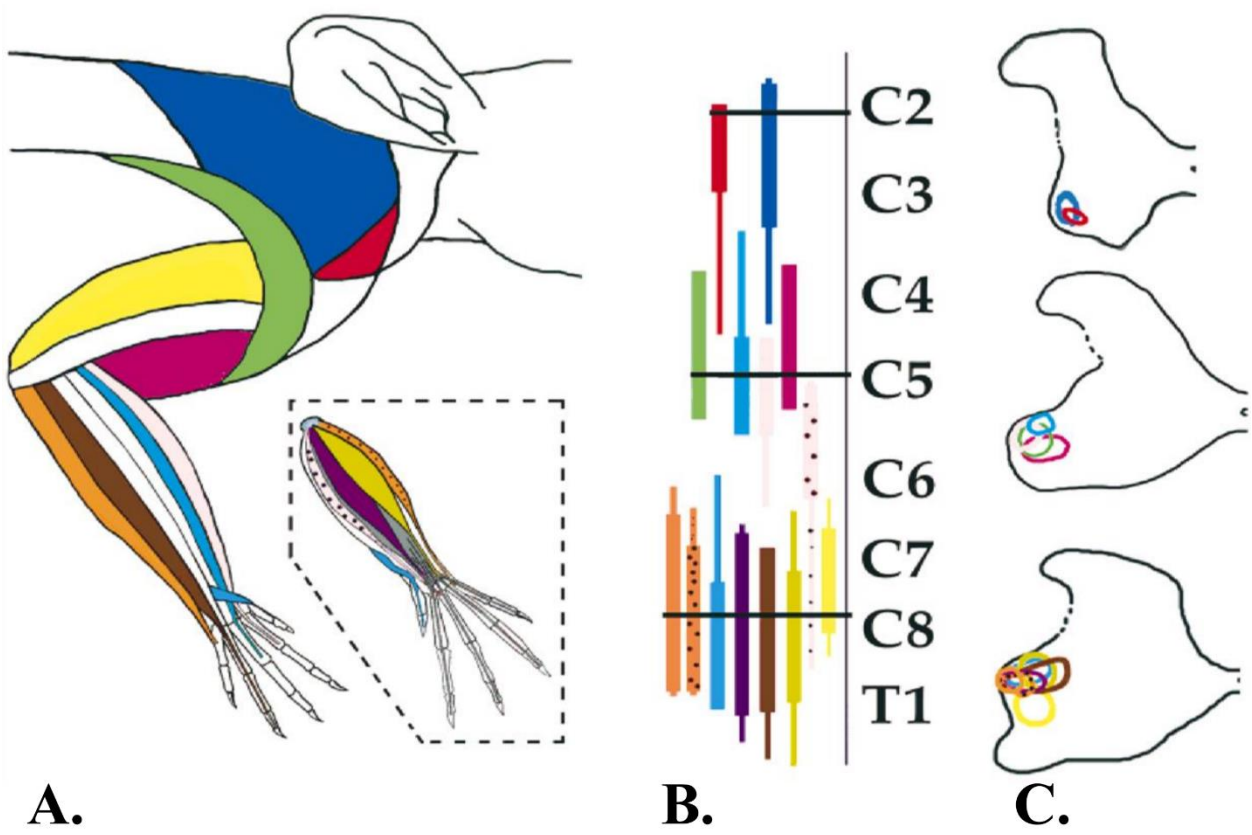



**Fig 6**: **Schematic representation of forelimb muscles in relation to cervical spinal cord** **A)** shoulder and arm muscles; **B)** their rostrocaudal topography of motoneuron columns; and **C)** the relative position in the transverse plane at three levels of the spinal cord of a laboratory rat. Adapted from (McKenna et al 2000).

Different behavioral tests are carried out to estimate the pre- and post-injury functions of various neurological tracts of the sensorimotor system (Muir & Webb 2000). These tests are mainly categorized based on data collection procedures and functional organization of the nervous system (Kunkel-Bagden et al 1993, Muir & Webb 2000). According to the data collection procedure, these tests are categorized as: 1) endpoint measures, in which a certain behavior receives a score according to some predefined scale, e.g. number of pellets eaten in a given time; 2) kinematic measurements, which range from a qualitative description of movements to continuous quantitative measurements, e.g. Basso-Beattie-

Bresnahan (BBB) scale; 3) kinetic measurements, which quantify the force produced by the limbs, e.g. forelimb grip strength tests; and 4) electrophysiological measurements, e.g. motor and somatosensory evoked potentials (Muir & Webb 2000). Based on the neurological tracts used (Sedy et al 2008), these behavioral examinations can be divided into: 1) Motor test, e.g., limb grip strength test, forelimb reaching task; 2) Locomotor test, e.g. BBB scale, swim test; 3) Sensory test, e.g. Von Frey filament test, test for hot and cold sensation; and 4) Sensorimotor test, e.g ladder walking test, grid walking test, rope climbing and walking test.

### 1.2.7 Motor test

Descending tracts of motor neurons convey information to the lower motor centers in the spinal cord that are responsible for transferring information to the muscle fiber to initiate movements of the limbs (Schomburg 1990). The CST is one of the foremost descending tracts of the motor system that plays a vital role in skilled motor tasks by conveying volitional information to the limbs. The degree of CST injury is strongly co-related to the impairment of skilled motor function after a SCI (Raineteau & Schwab 2001). In rats, the CST arises from the sensorimotor cortex and projects to laminae III-VI in the ventral part of the spinal dorsal column. Less than 5% of the CST from the cerebral cortex terminates in the ipsilateral side of the spinal cord (Brosamle & Schwab 1997). Moreover as a descending motor tract, the CST highly dominates forelimb and hind limb control (Iwaniuk et al 1999). However, CST projections are less developed in the lumbosacral enlargement of the spinal cord and thus it has less effect on hindlimb stepping (Muir & Whishaw 1999). After a cervical injury, motor control of the forelimb is profoundly affected due to the impairment of the CST. Motor functions may be restored after skilled motor learning

training that assists in the reorganization of the CST (Starkey et al 2011). To assess the motor recovery of descending tracts, several functional tests have been developed. These tests include evaluation of the motor functions of different limbs after complete and incomplete SCI. Among these tests the Irvine, Beatties and Bresnahan (IBB) scale, skilled forelimb reaching task and limb grip strength test are the most common to evaluate the motor functions after an experimental SCI in laboratory rats.

### 1.2.8 Skilled forelimb reaching task

To evaluate motor function after a cervical SCI and treatments, a skilled forelimb reaching task is commonly evaluated for rats (Whishaw et al 1993). The task is time-consuming and needs several weeks of training to induce motor learning for forelimb food pellet retrieval **(Fig 7 )**. First, rats are need to habituated with the apparatus and special food pellet (usually sugar pellet) for one to two weeks. A special Plexiglas chamber (40 cm × 25 cm × 30 cm) with a 1-2 cm wide opening for grasping the food pellet from a pit is normally used **(Fig 9)**.

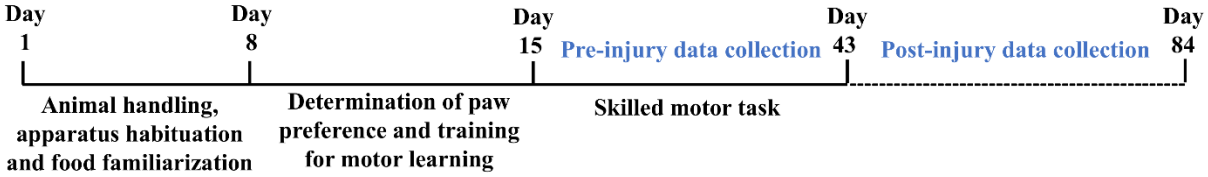



**Fig 7**: **A typical schedule for skilled forelimb reaching tasks**. The first week is essential for animal handling, apparatus habituation and food familiarization for the rats. The following week is for determining the rats' preferred paw for forelimb reaching. During the next 4 weeks, rats are trained in the forelimb reaching task. At the end of 4 weeks of training, a baseline video of rats' reaching and grasping behavior is recorded followed by an experimental SCI. To evaluate the injury and the recovery after different treatments, typically 6 weeks of reaching tests of food pellets are required. The reaching scores are

then compared with the baseline for the statistical significance of difference to evaluate different treatment effects on forelimb functional recovery.

Food restriction is needed from 3 days prior to starting the training. Training is required for several weeks to master the reaching and grasping of the food pellets. A pellet should be placed on a pit platform in front of the box slit and to ensure that the rat approaches the opening in a continuous manner. Ten pellets for warm up and 20 pellets per task are usually used to evaluate the reaching behavior. Qualitative and quantitative assessments of a rat's skilled reaching task are performed after 4 weeks of training (5 sessions/week) (Whishaw et al 2003a); (Alam et al 2017). For qualitative assessment, 10 components of movements (digits to the midline, digits flexed, elbow in, advance, digits extended, arpeggio, grasp, supination I and II) are rated. Animals' reaching is rated on a 3-point scale. For normal reaching a score of "0" and for abnormal movement "1" is used. If there is no movement then "2" is given (Whishaw 2000, Whishaw et al 1993) **(Fig 8)**. For quantitative assessment, the success rate of a rat's reaching is usually calculated. It includes the total number of pellets retrieved and eaten. The following equation is used for reaching success rate estimation:

$$\text{Success rate} = \text{Number of pellets} / \text{Number of trials} \times 100$$

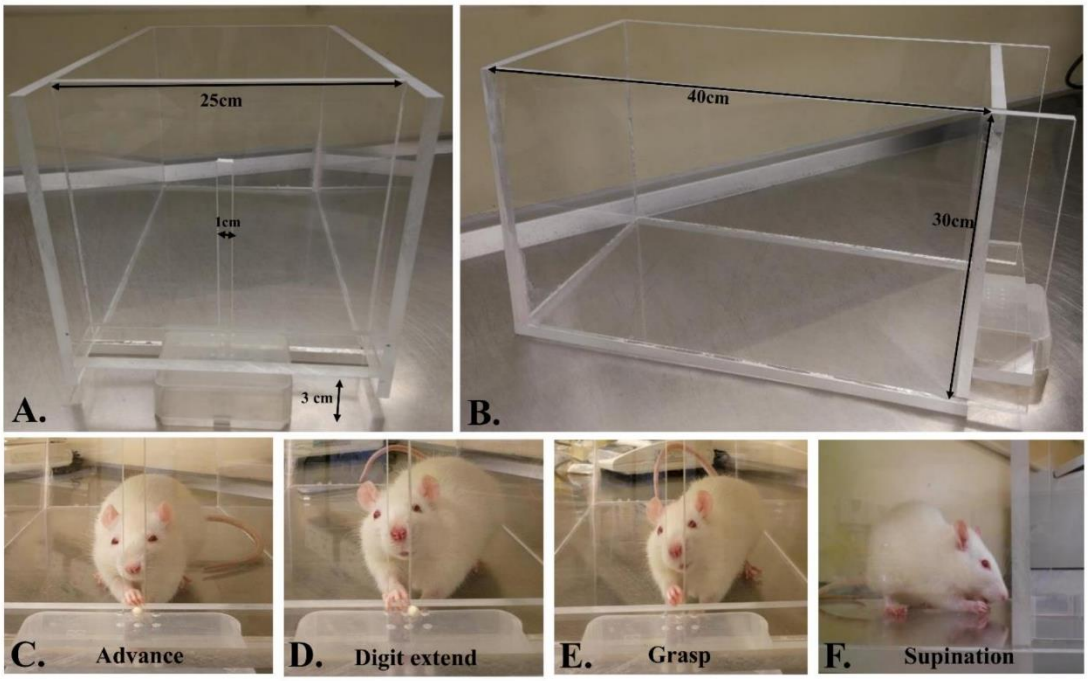



**Fig 8**: **Skilled forelimb reaching task for a laboratory rat**. **A)** A special Plexiglas chamber is designed to evaluate the forelimb reaching task. A 1 cm wide opening in the front of the box is used to extend the forelimb to grasp a food pellet from a pit platform which is 3 cm above the base. **B-E)** Different stages (advance- forelimb is advanced through a slot to the platform, digit extension- forepaw is extended toward the pellet, grasp- paw grasps the pellet and supination- paw is withdrawn from the slot and a pellet is successfully taken into the mouth) of reaching and grasping of a sugar pellet by a trained Sprague Dawley rat.

Furthermore, the amount of time required for grasping the pellets can also be measured to evaluate forelimb functional recovery (Chan et al 2005). A major limitation of the skilled forelimb reaching task is that partial lesion may not impair the reaching success and qualitative scores including forepaw aiming, supination and food pellet release. However, it is also expected that complete

restoration of the damaged corticospinal pyramidal tract will not restore the original behavioral condition (Piecharka et al 2005).

### 1.2.9 Irvine, Beatties and Bresnahan (IBB) scale for forelimb function

The IBB scale is a 10-point scale to identify recovery of both proximal and distal forelimb functions after unilateral cervical SCI. Unlike the forelimb reaching task, acclimatization of rats and food deprivation is not necessary for this test (Irvine et al 2010). First, a rat is placed in a cylindrical Plexiglas chamber for acclimation to the food. When the rat is well adapted to the apparatus and food, grasping and eating behavior are recorded by a video camera. Various sizes (spherical and doughnut) of cereals are generally used to assess forelimb function. From the recorded video, elbow position (extended or flexed), supporting of the paw (contact or noncontact), position of forepaw (clubbed, extended or flexed), digit movements and grasping method are evaluated (Inoue et al 2013). However, visual inspections by the experimenter may affect the experimental assessment and outcomes.

### 1.2.10 Limb grip strength test

A grip strength test is used to evaluate both forelimb and hindlimb functions (Meyer et al 1979). Furthermore, the quantitative strength of flexor muscles can also be measured by this test. Grip strength measurement is important because it can predict any functional deficits in limbs after a SCI. It is also useful to evaluate the motor function (Anderson et al 2005). Anderson and colleagues also showed that after hemisection of the cervical spinal cord rats completely lose their ability to grip anything for two weeks, whereas after complete hemisection, rats lose the gripping ability permanently (Anderson et al 2005). The grasping ability of a rat's forelimbs is evaluated as described previously (Alam et al

2015). Acclimatization of rats to the testing apparatus is necessary before starting the test. At the beginning, a rat forepaw is placed to grasp a bar (1 mm diameter) that is attached to a force transducer. The rat is then gently pulled away from the bar. The sensor reads the pull strength until the rat releases the bar. Maximum grip strength is then measured from the recorded force. For each forelimb, several testing sessions are usually conducted to evaluate the maximum grip strengths of forepaws. This method is a simple procedure to evaluate the recovery over time. The response is immediate and the rats are always motivated to grasp. However, after daily repeated testing, rats may hesitate to grasp if they feel an unpleasant sensation when lifted by the tail (Bertelli & Mira 1995).

In patients, one of the priorities is to restore arm and hand function after a cervical injury. To study this in laboratory rats, skilled motor tasks are essential. One of the skilled motor tasks includes forelimb function for food-pellet reaching which helps to evaluate the motor tract before and after an experimental cervical SCI. However, in the food-pellet reaching task, a food restriction is necessary that may affect the sensitivity of the result (Alexander & B. 2012). To study forelimb proximal and distal digit movements a food pellet reaching task could be a better choice of test.

An appropriate behavioral test should be chosen to understand the effects of new therapeutic interventions on experimental animals. Limitations of the behavior tests should be considered before proceeding with the test. For specific tract recovery, a behavior test should be chosen very wisely. For example, to evaluate the motor recovery, especially after CST injury, skilled reaching task in rats is useful. Similarly, for drug and cell transplantation treatments for sensorimotor recovery, different grid walking tests can be considered. Behavioral assessment should also be chosen on the basis of reliability,

validation and sensitivity (Basso 2004, Basso et al 1995, Fouad et al 2013). The overall criteria for selecting a particular behavioral test are summarized in **Table 2.**

**Table 2.** ***Selection of an appropriate behavioral test***

| **Motor test** | **Locomotor test** | **Sensory test** | **Sensorimotor test** |
|---|---|---|---|
| • To study volitional movements including arm and digit movements (Irvine et al 2010).<br>• Qualitative and quantitative assessment of forelimb function (Whishaw et al 2003a).<br>• To test different neuronal tract functions (Whishaw et al 1993). | • To study the central pattern generator (CPG) mechanism (Guertin 2012).<br>• To study flexor and extensor neural network organization (Kiehn 2006). | • To examine sensory integrity and improvement after any neurological injury (Detloff et al 2012).<br>• To evaluate the afferent tract function (Lavrov et al 2008). | • To examine somatosensory and sensorimotor integration (Moreno-López et al 2016).<br>• To assess gait dynamics (Rigosa et al 2015).<br>• To evaluate the coordination between left and right limb movements (Metz & Whishaw 2002). |

### 1.3 Strategies for SCI repair

Following a SCI, several pathological events are developed that affect neuronal functions by forming glial scars and hindrance to axonal regeneration and recovery (Nas et al 2015, Rolls et al 2009). Therapeutic approaches in present days focus on preventing the severity of primary and secondary injuries related events following SCI. Until now, several approaches including cell treatment (Assinck et al 2017) neurostimulation (Harkema et al 2011), pharmacological modulation (Pantovic et al 2005) and physical rehabilitation have been applied to improve the conditions of SCI subjects **(Fig 9).** Epidural stimulation showed significant improvement; however, the approach cannot singularly improve the function without extensive physical therapy (David Darrow et al 2019).

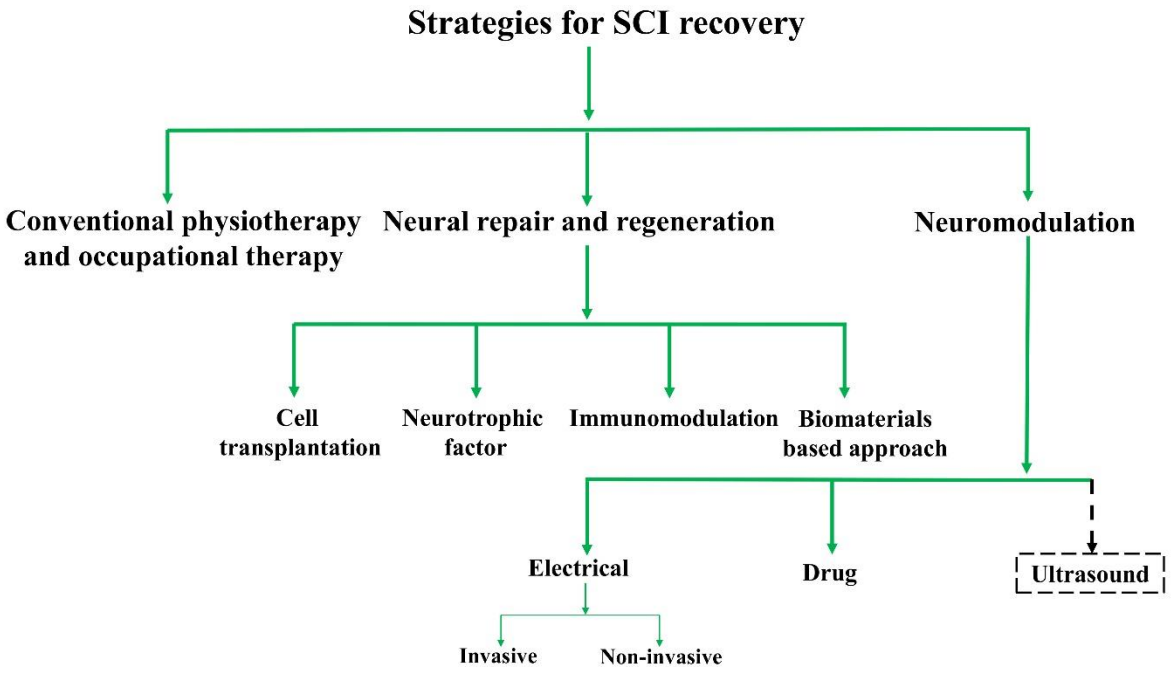



**Fig 9**: **Strategical approaches following SCI**. The recovery approaches from SCI include both neural regeneration following the neural repair and neuromodulation. Neural regeneration can be achieved by cellular, immunomodulatory and biomaterial-based

techniques. Alongside the neuromodulation is a popular approach to modulate the damaged spinal cord to restore the function.

Electrical neuromodulation in spinal cord has the potentials to optimize the recovery. In contrast, combinatorial approaches such as low doses of pharmacological agents, electrical stimulation and motor training have shown significant functional improvements in SCI animal models and human subjects (Angeli et al 2018, Duru et al 2015, Formento et al 2018, Gill et al 2018, Wagner et al 2018, Zhao et al 2013).

### *1.3.1 Cell transplantation and neurotrophic factor*

Stem cells have the potential to improve sensorimotor function through regeneration and remyelination of axonal fibers (Gu et al 2010, Khankan et al 2016). Clinical uses of some stem cells have shown promising improvement in the motor recovery after spinal cord injury (El-Kheir et al 2014, Mendonca et al 2014, Satti et al 2016). In the United States till now almost 1200 clinical trials have been carried out by using the stem cell therapies (Sabbah & Behfar 2020). Recently a phase I clinical trial of autologous adipose tissue-derived mesenchymal stem cells has shown a favorable improvement following a cervical injury in one patient with no side effects (Bydon et al 2020). The study suggested meaningful improvement compared to the other clinical trials. However, the study is still ongoing.

Besides this in recent years stem cells have shown also some promising results in an animal model of SCI and human clinical trial as neuroprotector and immunomodulator. Among these neural stem cells, mesenchymal stem cells, olfactory ensheathing cells, schwann cells, activated macrophages, embryonic stem cells and induced pluripotent stem cells are remarkable (Curtis et al 2018, Gensel & Zhang 2015, Kanno et al 2015, Khankan et al

2016, Nagoshi & Okano 2017). Neural stem cells act as a neuroprotective agent by promoting functional serotoninergic neurons through the modulation of Bcl-2 expression that mainly prevents apoptosis (Chen et al 2007). Moreover, as an immunomodulator, mesenchymal stem cells (MSCs) and neural stem cell can easily migrate to the injury site to modulate the injured area (Nakajima et al 2012). Among stem cells, MSCs can easily migrate to the injured area and trigger out anti-inflammatory effect (Pineau & Lacroix 2007). Neural stem cell has a robust immunomodulatory activity after transplantation of SCI to reduce neutrophil infiltration, regulate macrophage activation and attenuate inflammatory cytokines (Cheng et al 2016). Besides as an immunomodulator, stem cells can secrete neurotrophic factors that also facilitate neuronal regeneration and currently under investigation (Gransee et al 2015, Ide et al 2016).

Neurotrophic factors are small molecules of polypeptides that is crucial for axonal regeneration, neuroprotection and ion channel regulation (Huang & Reichardt 2001). Neurotrophins work predominantly by guiding neural cell survival, increase synaptic activity by releasing neurotransmitters, control synaptic plasticity and facilitate axonal regeneration and dendritic branch (Chao 2003). NT-3 trigger out the action by mainly axonal regeneration in the CST region. Brain-derived neurotrophic factor (BDNF) protects both rubrospinal and corticospinal neurons. In chronic spinal cord injuries, combination therapy of BDNF with cell transplantation promoted axonal regeneration into peripheral nerve grafts (Plunet et al 2002). Likewise, neurotrophin nerve growth factor (NGF). also acts as a neuroprotector in SCI by inhibiting the oxidative base apoptosis (Salinas et al 2003).

### *1.3.2 Immunomodulation*

Following injury, the inflammatory cell infiltration in the injured region can carry out an avoidable detrimental effect of an individual. Infiltration of M1 macrophage in the injured region associated with tissue damage, peroxidation and fibrosis. However, M2 macrophages disappear (Monteiro et al 2018) after the injury. The immunomodulatory approaches have been developed to protect the neural tissue and combat the inflammation immediately after spinal cord injury. In animal model recent years systematic administration of IL-4 for 7 weeks promotes motor neuron and oligodendrocyte numbers in the injured area. Moreover, the animals regained weight support and the inducible nitric oxide (iNO) concentration reduced indicates the role in neuroprotection (Lima et al 2017). In recent years stem cells have also shown some promising results in animal model of SCI as a immunomodulator. Among stem cells, MSCSs can easily migrate to the injured area and trigger out ant-inflammatory effect (Liu et al 2016). Comprising the above findings, the initial result of immune modulation is not fully satisfactory for SCI recovery.

### *1.3.3 Neuromodulatory approaches*

Neuromodulation is used to alter the neuronal activity using drug or electrical or magnetic interferences. In injured patients spinal cord neuromodulation is used to restore voluntary and/or autonomic functions. In recent years, several approaches of neuromodulation using electrical, magnetic or pharmacological agents (drugs) have been used to modulate the neural activity (James et al 2018).

**i. Electrical neuromodulation**

There are several methods of spinal cord electrical stimulation **(Fig 10)**. Electrical stimulation can be delivered to the spinal cord via invasive and non-invasive methods. Invasive methods include epidural and intraspinal stimulation that requires low stimulation current. While non-invasive stimulation includes transcutaneous painless electrical stimulation to reactivate the spinal cord through the sensory interneuronal pathway (Sayenko et al 2015).

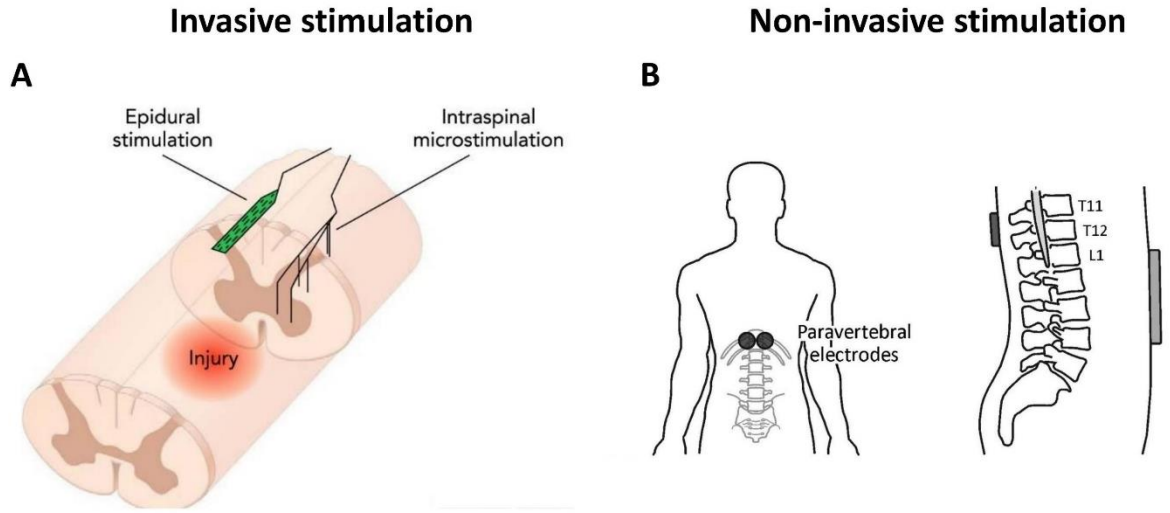



**Fig 10: Electrical neuromodulation**. A) Invasive stimulation including epidural and intraspinal stimulation; B) Non-invasive transcutaneous stimulation. Adapted from (Ievins & Moritz 2017, Minassian et al 2012)

In 1967, Shealy and colleagues at first stimulated the spinal cord via a single electrode at 10-50 Hz frequency for relief of chronic intractable pain (Shealy et al 1970). Later, bipolar electrodes at high frequency (100-200 Hz) stimulation were used to relieve the pain while the current remaining well below the level for tissue damage (Shealy et al 1967). In recent years, several research groups have been working to restore the functions after SCI in

animal models (Bamford & Mushahwar 2011, Edgerton et al 2008). Epidural electrical stimulation has shown significant improvement in cervical SCI rats and quadriplegic patients (Alam et al 2017, Lu et al 2016). More recently, Coutrine and his colleagues targeted neurotechnologies to restore the neuronal activity of spinal cord injured individual those who suffered more than four years (Wagner et al 2018).

Intraspinal electrical stimulation is rarely done in humans. In animal study, intraspinal stimulation can elicit a wide variety of functional capabilities (Giszter et al 2000, Holinski et al 2016). In cervical injured rats, intraspinal electrical stimulation facilitates motor function and lasts for weeks even after stimulation ceased (Sunshine et al 2013). Furthermore, in monkeys intraspinal stimulation can improve the reaching and grasping function (Zimmermann et al 2011). Intraspinal electrical stimulation to the ventral spinal cord directly activates motorneuron to facilitate the single joint movement. Moreover, when stimulation applied to the lamina of spinal cord it activates axons and interneurons directly (Ranck 1975).

Non-invasive electrodes on the surface of the skin over the vertebral column are used to stimulate the spinal cord, and termed as transcutaneous spinal cord stimulation. Because of non-invasive nature and no need for surgery, the method is more attractive compared to the epidural and intraspinal electrical stimulations. Epidural and transcutaneous stimulation can target the same neural structures. A 10-kHz frequency with 100mA of current is passed through the skin to activate the spinal cord without causing any pain (Gerasimenko et al 2015). The stimulation can modulate the excitability of spinal and supraspinal circuits and thus can improve the upper extremity strength (Inanici et al 2018). Clinical studies have shown that when stimulation applied different spinal levels along with rehabilitation

training, the treatment can improve locomotion in chronic spinal cord injured patients (Yury P. Gerasimenko 2015). Recently, a case study has shown a patient after 21 years of paralysis regained volitional control of lower limb and standing ability after transcutaneous stimulation (Alam et al 2020). However, the mechanism yet not understood. It is assumed that transcutaneous stimulation is likely less accurate in targeting the specific spinal cord (Hofstoetter et al 2018).

**ii. Cortical neuromodulation**

Transcranial magnetic stimulation is another neuromodulatory approaches applied on the skull to elicit the motor activity from the cortical neuron. When applied with motor activity, stimulation can increase the excitability of cortical neuron (Sriraman et al 2014). In chronic tetraplegic condition, a 10Hz stimulation combined with repetitive practice can improve the hand function (Gomes-Osman & Field-Fote 2015). Moreover, in motor incomplete SCI patients high-frequency magnetic stimulation can lead to improve the gait (Kumru et al 2016). This model of stimulation could be useful if the anatomical and neuromodulatory effects are known after stimulating throughout the injury period.

**1.4 Future perspectives**

Recently, another non-invasive neuromodulatory approach, called trans-spinal magnetic stimulation has shown some hopeful results in translational research. In mouse model following a thoracic contusion of injury, exposure of trans-spinal magnetic stimulation at a rate of 15Hz remarkably improves the functional recovery (Leydeker et al 2013). Furthermore, in the injured tissue elevation of the excitatory neurotransmitter, glutamate

was found higher. However, in cervical injured patient trans-spinal magnetic stimulation can reduce the excitability of the CST (Nakagawa & Nakazawa 2018).

# Chapter 2: Aims and objectives

## 2.1 Rationale of the current study

Although epidural electrical neuromodulation for spinal cord injured patient has shown promising results, some limitations still persist. This invasive method requires a surgical implantation of stimulating electrodes and optimization of the locations of electrodes (Alam et al 2016). Although the parameters used in the epidural stimulation is difficult to translate in non-invasive stimulation because of the application of current through the skin (Ievins & Moritz 2017) some promising result already demonstrated in the upper extremity recovery (Inanici et al 2018, Parag Gad 2018, Rodionov et al 2019) In addition to the electrical and magnetic stimulations, the therapeutic effects of ultrasound stimulation on neurons has recently been explored (Blackmore et al 2019, Tyler et al 2018, Tyler et al 2008). Low-intensity pulsed ultrasound (LIPUS) is a novel non-invasive neuromodulation approach that could be used for rehabilitation after a neurological disorder such as spinal cord injury. Besides this, the safety and efficacy of LIPUS have shown promising results on brain (Jiang et al 2016, Legon et al 2014, Mueller et al 2014, Tufail et al 2010, Yuan et al 2016) and peripheral nerve injuries (Matthew et al 2018, Ni et al 2017). To find a successful non-invasive neuromodulation strategy, firstly, therapeutic ultrasounds on the spinal cord were investigated in SCI animal model in the current study. Moreover, it has been demonstrated that epidural electrical stimulation along with serotonergic agonists can transform nonfunctional spinal circuits into functional after a complete transection in rats with weight-bearing bipedal locomotion (Courtine et al 2009, Dominici et al 2012, van den Brand et al 2012). To carry out a similar effect in cervical cord injured rats, in the present

study ultrasound stimulation was delivered along with a serotonergic agonists drug-Buspirone to facilitate the forelimb functional recovery in rats.

## 2.2 Research hypothesis

From the above rationale, it was hypothesized that

***1) Ultrasound stimulation to the injured cervical spinal cord neuromodulates motor functions in rats.***

***2) Ultrasound neuromodulation along with serotonin agonists improve forelimb function in cervical cord injured rats.***

# Chapter 3: Ultrasound neuromodulation of the injured spinal cord

## 3.1 Therapeutic ultrasound

Ultrasound is a mechanical sound wave and it is inaudible due to its frequency of 20 kHz or above. Medical ultrasound, also known as ultrasonography is a diagnostic imaging technique based on the application of ultrasound. It is used to see the structures of internal body by recording the echoes from different tissues. Besides the imaging purpose, therapeutic value of ultrasound stimulation is also significant because of its biophysical effects on the tissues. Ultrasound stimulation carries out the therapeutic action by penetrating the deep tissues. Sonic energy can be transformed into heat through solid concentrations of tissue material (Schwan 1956). The ultrasonic reaction on biological tissue was extensively studied by Lehman (Lehmann 1953). It has been shown that heating caused by ultrasound plays a significant role in therapeutic conditions, although the study was done by using high-intensity ultrasound. Currently, high intensity focused ultrasound (HIFU) is used as a thermal source for tissue ablation, tissue hyperthermia and microbubble-mediated blood-brain barrier opening to deliver the drug in the brain (Hynynen et al 2001, Kim et al 2008). The physical and biological effects of high-frequency sound waves of higher intensity at first were studied by Wood and Lomis (G.F.S 1928). Then it was studied by another group of researchers on the nervous tissue (Fry et al 1950). High intensity (~35 W/cm$^2$) ultrasound was used to irradiate the dorsal surface over the frog lumbar enlargement of the spinal cord for 4 sec. After irradiation, the frog exhibited hind limb paralysis along with elevation of the temperature (1 to 2ºC). Other studies also showed that an increase of 2 to 3ºC could cause various changes in biological tissue during musculoskeletal and neurological disorders with HIFU (Zhang et al 2017a).

Comparing with high-intensity stimulation, LIPUS has shown promising results without any side effects on the musculoskeletal system (Cook et al 2001, Enwemeka et al 1990, Zhang et al 2017b). It does not cause any tissue damage because of its low thermal effect and intensity.

### 3.2 Effects of ultrasound on the central nervous system

Thermal effects of high-intensity ultrasound has an high clinical significance for non-invasive surgeries (Naor et al 2016, Zhang et al 2017a). However, heating tissue with high intensity can cause irreversible damage in visual cortex activity (Fry et al 1958). Meanwhile, in therapeutic ultrasound low-intensity is used to modulate neuronal membrane potentials and synaptic transmissions through the non-thermal activation of ion-channels (Tyler et al 2008) **(Fig 11).** Some other studies have shown that low-intensity ultrasound can modulate the cortical, thalamic and hippocampal regions in animal model (Lee et al 2016, Li et al 2016, Robert et al 2018, Yoo et al 2011). However, the exact mechanism of this neuromodulation is still unclear. Acute modulation of brain in animal model has shown safe and paved the way for human studies.

The effects of low-intensity ultrasound on spinal cord is not well-studied. Takagi and colleagues at first explored the role of ultrasound in frog spinal cord (Takagi et al 1960). The action of ultrasound on spinal reflex discharge was studied in that experiment. After 20 sec of stimulation small spontaneous discharge was recorded on the ventral root. When higher intensity was used to stimulate the spinal cord, spontaneous discharge again appeared. However, beyond a certain level, the discharge started to decrease and disappear.

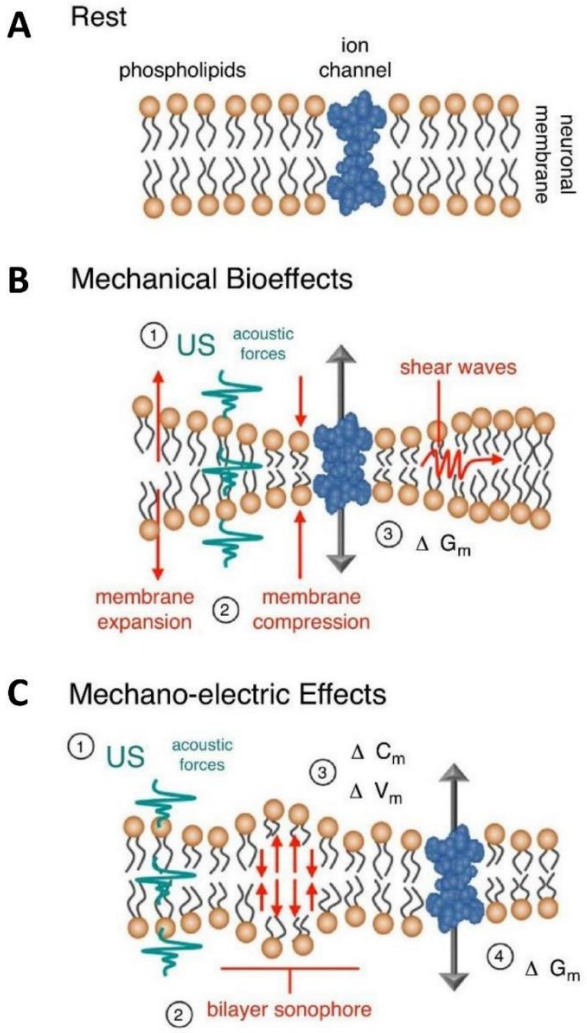



**Fig 11: Proposed mechanism of the neuronal membrane and ion channel alteration during a low-intensity ultrasound stimulation**. **A)** Resting condition of the neuronal membrane and ion channel; **B)** (1) Acoustic pressure causes a mechanical effect on membrane and ion channels (2) membrane compression (3) changes of membrane conductance. **C)** (1) and (2) Acoustic forces causes the formation of bilayer sonophore (3) and (4) changes in the membrane capacitance (Cm), voltage (Vm) and membrane conductance (Gm). Adapted from (Tyler et al 2018).

### 3.3 Effect of ultrasound on peripheral nerve

In addition to brain modulation, ultrasound can also modulate the peripheral nerve both *in vitro* and *in vivo* (Jiang et al 2016, Saffari et al 2017). Electrophysiological recordings from frog sciatic nerves have shown that ultrasound has a reversible effect (Takagi et al 1960). In this study, researchers found that high-intensity ultrasound has an inhibitory effect compared to the low intensity. However, the reason behind this is still unrevealed. A recent study has demonstrated that cavitation produced by the ultrasound can modulate crab peripheral nerve (Saffari et al 2017). A study in rat model has shown that focused ultrasound can stimulate the sciatic nerve similar to electrical stimulation (Matthew et al 2018). However, a previous study has shown that focused ultrasound decrease the nerve action potential indicated that ultrasound could be used to block nerve conduction either temporarily and permanently (Colucci et al 2009). In peripheral nerve injury, autograft with LIPUS significantly induced faster axonal regeneration (Jiang et al 2016).

In recent years significant research has been conducted to elucidate the therapeutic effect of ultrasound stimulation in several neurological disorders such as stroke, Parkinson disease and pain management (Leinenga et al 2016). However, no study of therapeutic ultrasound on the SCI recovery has yet been conducted. Furthermore, the motor behavior and the effects of ultrasound on the SCI neuromodulation are unknown. To address this, in the present thesis study, therapeutic ultrasound on spinal cord was investigated in SCI animal model. In this study, for the first time, I have investigated the effects of LIPUS on injured spinal cord and the motor behavior study following cervical cord injury.

All the following experimental procedures were approved and performed under the strict guidelines of Animal Subjects Ethics Sub-committee of The Hong Kong Polytechnic

University and the Department of Health – Animals (control of experiments) of the Hong Kong S. A. R. government.

### 3.4 Experimental subjects

Twenty-five healthy adult female Sprague Dawley rats (240-280 gm body weight) were used in this study. Because of the low feed conversion ratio and easy to handle the female rats were used in this study. The rats were housed at a constant temperature (25 ºC) in the Centralized Animal Facilities at The Hong Kong Polytechnic University. The daylight cycle was maintained on a regular 12 hr. light/dark. Fresh food and ad-libitum water were provided.

### 3.5 Experimental design

To accustom with the forelimb reaching and grasping task, first 1-2 weeks the rats were handled to familiarize with a special Plexiglas chamber (40 cm × 25 cm × 30 cm; with a 1-2 cm wide opening) for grasping the food pellet from a pit (Ahmed et al 2019) **(Fig 13)**. At the same time, the rats were also familiarized with the special 45 gm dustless food pellets (Bio-serv®, Flemington, NJ, USA) to grasp and eat by the preferred forepaw. After two weeks of handling, apparatus habituation, food familiarization and preferred paw reaching for forelimb grasping task the rats were trained to grasp the pellet from the pit in a consecutive manner. Food restrictions were provided before starting the training. To master on reaching and grasping task, 45 mg dustless pellets (Bio-serv®, Flemington, NJ, USA) were given in the food pit. The pellets were placed on a pit platform in front of the box slit and ensured that the rat approaches the opening in a consistent manner. Ten pellets for warm-up and 20 pellets per task were usually used to evaluate the reaching behavior.

Quantitative assessments of rat's skilled reaching task were performed for six weeks as described previously (Whishaw & Tomie 1989).

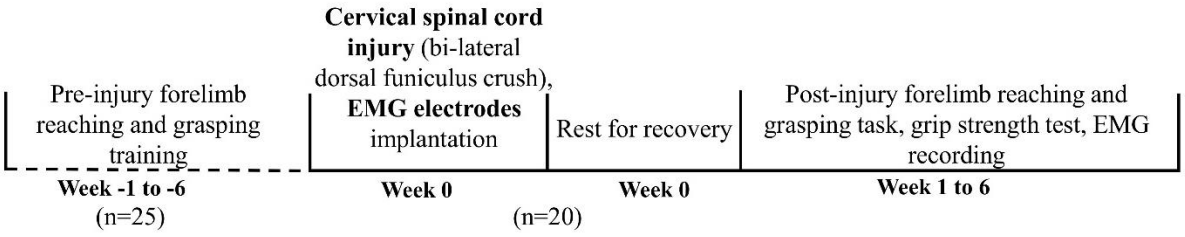



**Fig 12: Experimental setup**. 25 rats were trained for six weeks for forelimb reaching and grasping task. After familiarization, skilled learning and accustom to the task, 20 rats achieved 75% success rate after 6 weeks of training and included for the SCI surgery and EMG electrodes implantation. After one week of recovery from injury the rats were categorized into two groups – ultrasound stimulation group (US, n=11) and control group (Control, n=9) for evaluation of therapeutic ultrasound stimulation in the injured cervical cord.

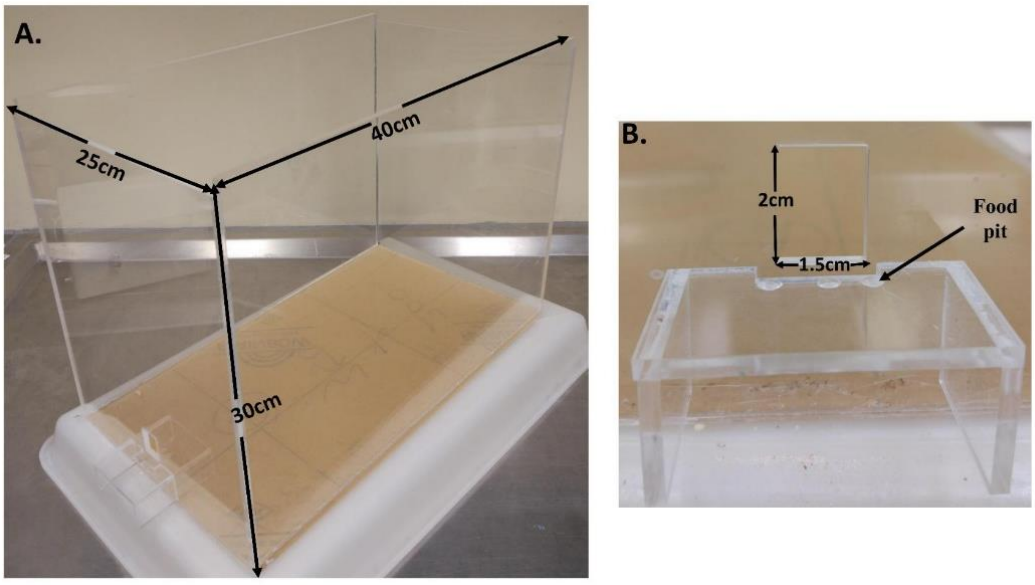



**Fig 13: Food pellet chamber;** A) Special Plexiglas chamber for reaching and grasping task; B) Small pit for food pellet. A small platform was attached outside the pit to place the

food pellets. Additional small dips were made on the platform for additional stabilities of the pellets.

Following six weeks of successful training, nineteen rats showed high success rates and were included for the surgery. The rats were prepared for the experimental SCI surgery to deficit forelimb motor function (**Fig 12**). The dorsal funiculus was crushed at C4 level to induce significant motor deficits. At the same time, EMG electrodes were implanted in the forelimb extensor digitorum and flexor digitorum muscles. After recovering from the injury, the rat's forelimb reaching task success rates were collected. To get unbiased results the rats were divided into two balanced groups- US stimulation (n=11) and Control group (n=9) (**Fig 12**). Post-injury data were collected once a week for 8 weeks and compared with the control group rats.

### 3.6 Pre-injury success rate

Twenty-five rats were trained for 6 weeks after one week of handling, habituation of the food pellets and behavior chamber to master the reaching and grasping task. At day-10 the success rate was found 18.75 ± 4.22 which gradually increased to 57.75 ± 3.58 until day 20 **(Fig 14)**. And at week -6 the average value of success rates was recorded~75%. Following 6 weeks of training, spinal cord injury was carried out in twenty rats.

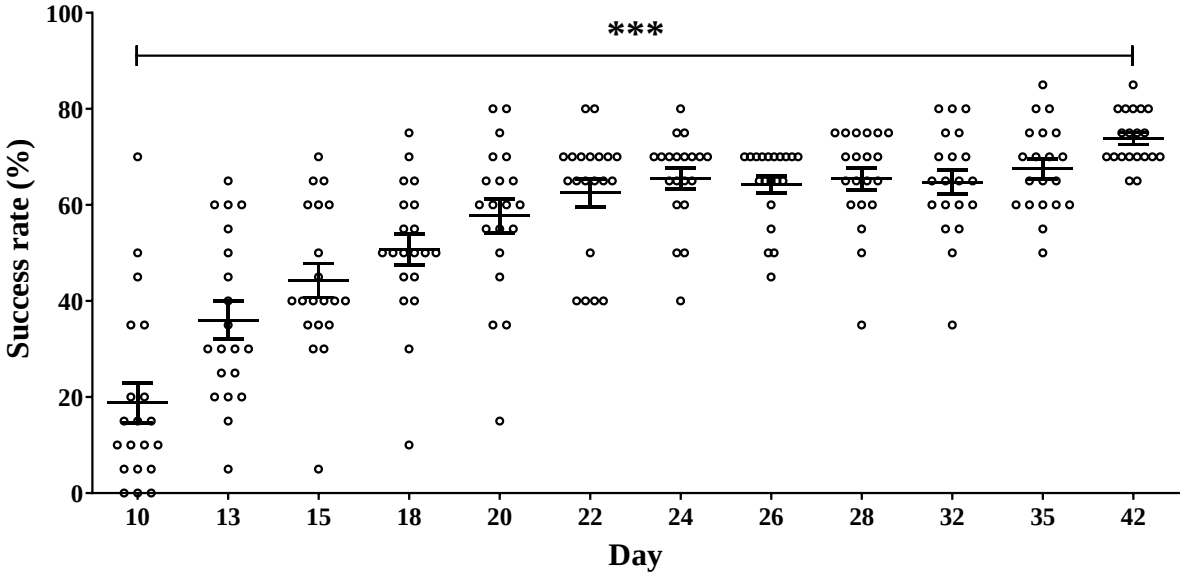



**Fig 14**: **Reaching and grasping learning curve of intact rats** (n=20). Significant difference (****P<0.001,* one-way ANOVA) was found at day-42 compared to the day-10 of reaching and grasping success rates (Mean ± SEM).

### 3.7 SCI surgery

To carry out a significant forelimb motor deficit incomplete SCI was carried out in twenty rats. The rats were first anesthetized with isoflurane gas (5%) and maintained (1.5-2%) via a facemask throughout the surgery. The rat's body temperature was maintained at 37 ºC using a heating pad (Thermostar Homeothermic Blanket, RWD®, China). Before the surgery, an analgesic Buprenorphine HCL (Buprenex®, 0.5mg/kg, S.C.) was administered. The surgical sites (head and cervical region) were carefully shaved and disinfected by povidone-iodine (Betadine®, Mundipharma, Switzerland) followed by 70% ethanol. To carry out an incomplete SCI a longitudinal midline incision was made dorsal to the cervical spinal column (Alam et al 2017). Laminectomy was performed by removing fascia and reflecting underlying spinal muscles over the C2-C6 vertebra to isolate the spinous process

with the rongeurs. At C4 level by using a bone nibbler, the spinal cord was exposed. To produce an incomplete injury at C4 level dorsal funiculus was crushed by inserting tips of a fine sharp forceps (2 mm wide and 2 mm deep) as described by García-Alías and colleagues (García-Alías et al 2009) (**Fig 15**). A 2-mm object was used after each surgery to measure the length of injury at C4 level. Hemostat was carried out by placing small cotton.

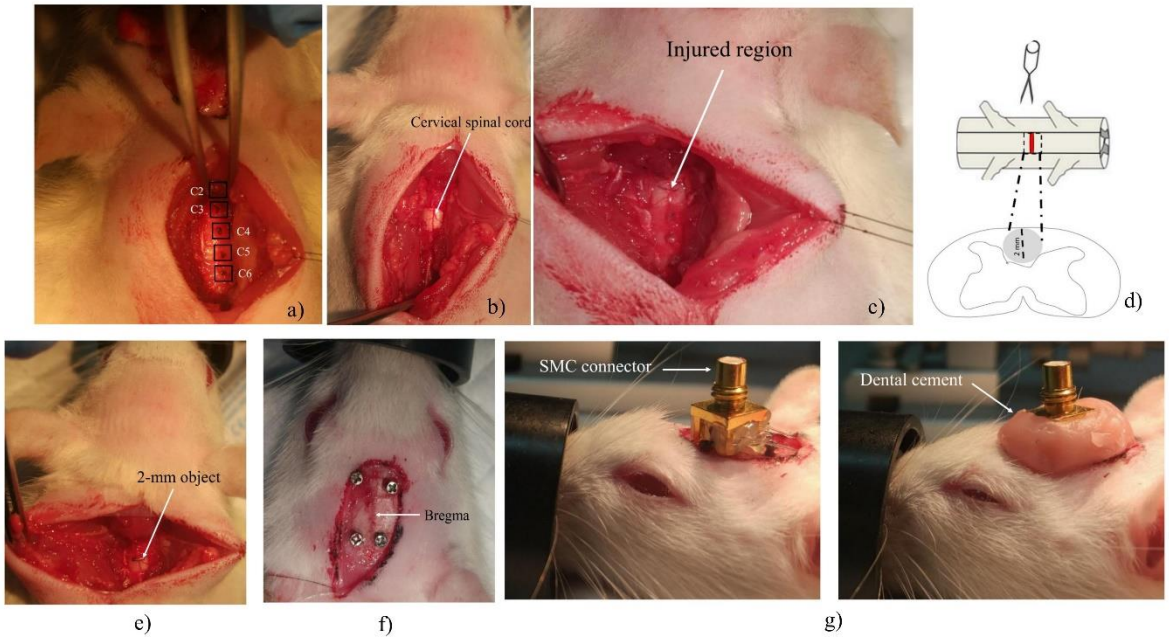



**Fig 15: Experimental spinal cord injury in trained rats**. **a)** After incising the skin and retracting the muscles layer the cervical spinous processes (C2-C6) are carried out for laminectomy; **b)** Laminectomy carried out at C4 level; **c & d)** Sharp fine forceps used to crush the dorsal funiculus (2 mm deep and 2 mm wide) at C4 level; **e)** A 2-mm object was used to measure the length of the injury; **f)** Skin incision was made along the midline of the skull to place the stainless steel screws; **g)** After placing the screws a short miniature connector (SMC) connector was placed on the midline of the skull and dental cement was used to fix it.

### 3.8 Preparation of ultrasound discs for implant

To deliver therapeutic ultrasound in behaving rats, an implanted ultrasound probe is used in this study. As ultrasound probe piezoelectric discs (PZT-8, Beijing Quanxin Ultrasonic Co. Ltd, China) were utilized. To transmit electric power to the piezoelectric disc, two (5-cm each) multi-stranded Teflon-coated stainless-steel wires (Conner wire, Chatsworth, CA, USA) were connected **(Fig 16a)**. The other ends of the wires were connected to the pins of a head connector (SMC) to mount on the head of the rat. The piezoelectric element was then coated with a biocompatible material as described previously (Alam et al., 2019). In brief, by the dip-coating method, the piezo element was coated at first with polyurethane. After 12 hr of drying at 75°C in a hot air oven (OF-02G, JEIO TECH Co. Ltd., South Korea), another FDA-approved biocompatible compound silicone (Shantou Chaonan Xiancheng Hengchang Silicone Material Factory, China) was applied over the first layer. After encapsulation, the entire probe was sterilized by UV-ray.

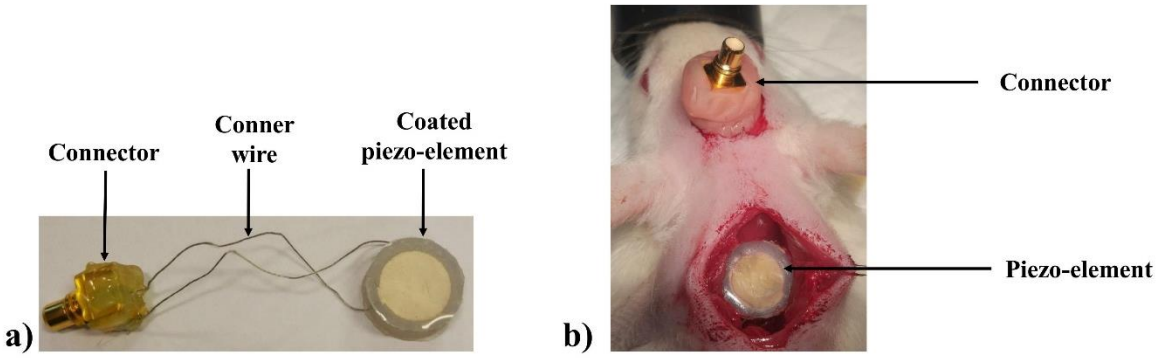



**Fig 16: Ultrasound stimulation probe; a)** A piezoelectric element was coated with silicone and connected to a connector via Teflon coated wires; **b)** The probe was placed on the cervical region after dorsal column crush and the connector was mounted on the head using dental cement.

### 3.9 Mounting the connector and placing the ultrasound probe

To anchor the connector on the skull, the rats were placed on a stereotaxic frame. A skin incision was made along the midline of the skull. Fascia and muscles over the skull were reflected laterally. The skull was dried and drilled to put the stainless steel screws into the bone **(Fig 15f)**. The SMC connector (RS components®, Taiwan) was then placed between the screws. Dental cement was used to fix the connector **(Fig 15g)**. The ultrasound probe was then placed at the C4 level of the spinal cord where dorsal funiculus was crushed and fixed by suturing the wires with the adjunct muscles **(Fig 16b)**. The muscles and connective tissue over the cervical region were sutured by using 4.0 Vicryl (ETHICON®, NJ, USA) and skin was closed with continuous 4.0 Ethilon suture.

### 3.10 EMG electrodes implantation

To record the muscle activities during reaching and grasping tasks EMG electrodes were implanted in rats. The objectives of implanting the electrodes were to record the extensor and flexor activity before and after the ultrasound stimulation. From each group, 2 rats were selected to implant the EMG electrodes at preferred forelimb extensor and flexor digitorum muscles.

The head plug of the EMG connector was placed on the skull after retracting the skin and connective tissue. To place the electrodes at paw muscles, the Teflon-coated stainless-steel wires (AS631, Cooner Wire, USA) were carried out to the incised forelimb distal flexor and extensor digitorum muscles area. A blunt forceps was used to pass the electrodes subcutaneously at the muscle belly. After placing the electrodes, a sharp forceps was used to retract the fascia and find out the muscle belly. To fix the electrodes a 27-gauze needle

was inserted in the muscle belly and the Teflon coated wires were passed in the muscles. A part (~0.5 mm) of the Teflon from the wires were removed to make EMG electrodes. The electrodes were then anchored tightly at both ends by using 4.0 Ethilon sutures. To confirm the position of the electrodes, electrical stimulation was delivered through the connector to observe muscle contraction. After confirmation, the wires were then coiled subcutaneously to relieve the stress. Ultrasound probe connector was then fixed on the skull after drilling screws onto the skull. Finally, dental cement was applied to affix the connector.

### 3.11 Post-surgery care

After the surgery, the rats were transferred to a temperature and humidity controlled incubator (AEOLUS Incubator, ICU-1801, USA) to recover from the anesthesia. After recovering from the injury the rats were transferred to the individual home cage. Post-surgery oral antibiotic Enrofloxacin (Baytril®, 0.5mg/kg, s.c) was administered for six days to prevent infection and analgesic Buprenorphine HCL (Buprenex®, 0.5mg/kg, S.C.) was administered twice daily for three days to suppress pain. Fresh fruits were provided in the cage for faster recovery. After 10 days of recovering from the surgery, reaching and grasping scores were collected.

### 3.12 Ultrasound stimulation parameters

For LIPUS stimulation, acoustic frequency 1 MHz, 20% duty cycle, Pulse Repetition Frequency (PRF) 1 kHz was used to generate pulsed ultrasound as described before (Wei et al 2014a). Previously, it was found that low intensity (~30 $mW/cm^2$) can help to recruitment stem cell requirements in facilitating fracture healing (Wei et al 2014a) and modulate the ion channel in neuron (Tyler et al 2008). The same parameter was used for

this study.  A coaxial cable was used to deliver the current from a 50W power amplifier to the ultrasound probe (**Fig. 17**). Ultrasound stimulation was provided for about 10 minutes during the reaching and grasping behavior task. Because it is already known that for any spinal cord stimulation first few minutes is needed to modulate the neuron (Alam et al 2017). Hence, the first 5 minutes of stimulation was provided to modulate the neuron and the next 5 minutes were used to calculate the forelimb reaching and grasping task success rate. The time slot was enough to grasp the pellets.

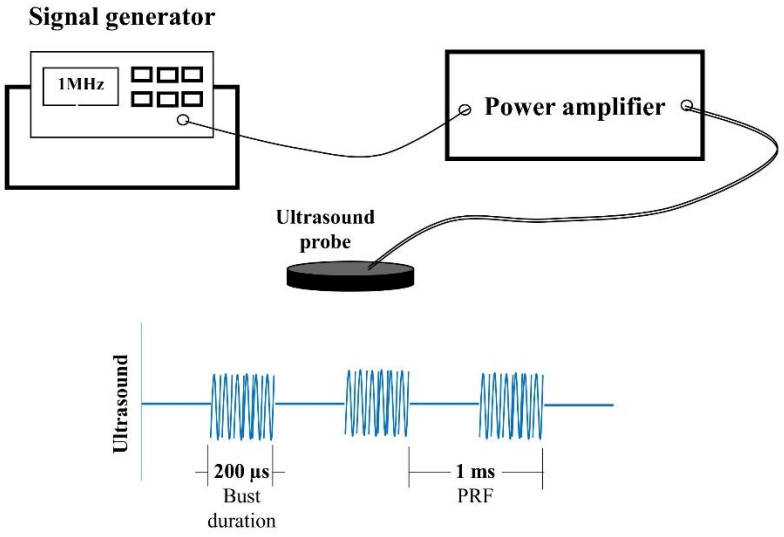



**Fig 17: Ultrasound stimulation parameter**. A custom-made ultrasound probe with an acoustic frequency of 1 MHz, 20% duty cycle and 1 ms (PRF) pulse repetition frequency were used to generate the LIPUS.

### 3.13 Ultrasound intensity measurement

Before placing the ultrasound probe on the cervical region the intensity was measured by using a needle hydrophone (HNP-1000, Onda Corporation, USA). The probe was placed inside a water tank and the hydrophone was placed rostrally to the probe at a 4-mm distance (**Fig. 18**). To supply the voltage to the ultrasound probe, a coaxial cable was

connected to the connector and the intensity was recorded by the hydrophone. The average ultrasound intensity was found around 67.35 mW/cm$^2$ ($I_{SATA}$).

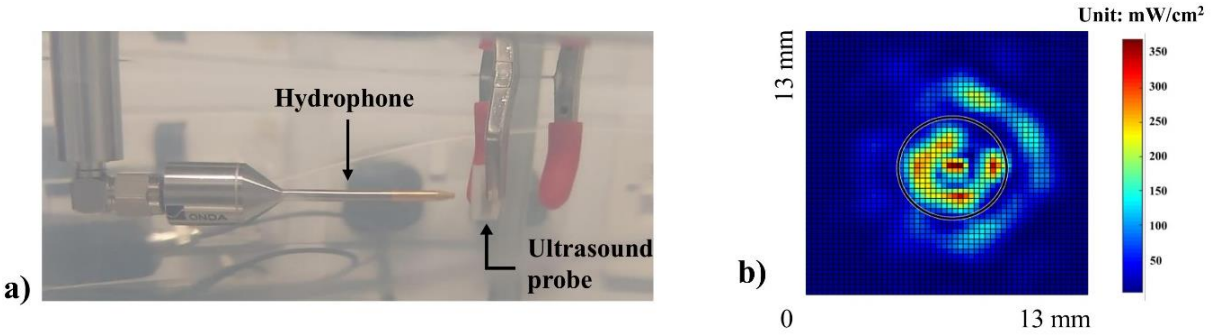



**Fig. 18: Ultrasound intensity measurement in a water tank. a)** In a water tank, a hydrophone was placed front side of the tank. **b)** Scanning result from one ultrasound probe ($I_{SATA}$ value = 67.35 mW/cm$^2$).

To measure the intensity of pulsed ultrasound inside the vertebral canal, an *ex-vivo* experiment was conducted.

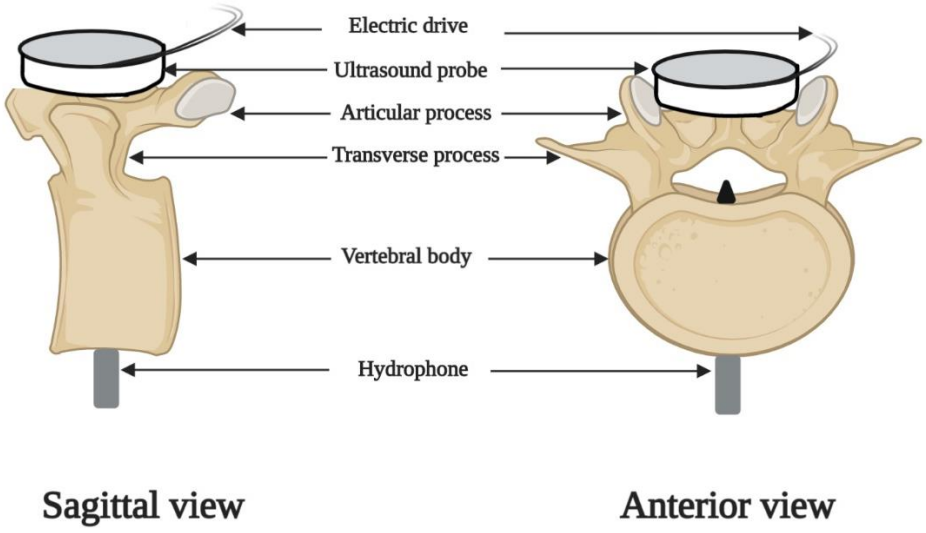



**Fig. 19: *Ex-vivo* setup to measure the ultrasound intensity inside the vertebral canal** (10 mm diameter probe used to generate ultrasound; acoustic frequency 1 MHz, 20% duty cycle, PRF 1 kHz).

Previously, a vertebra was collected from one rat to measure the intensity inside the vertebral canal (**Fig. 19**). The vertebral body was drilled by an electric micro driller and the needle of the hydrophone was inserted within the body of the vertebra to measure the intensity of the ultrasound. From *ex-vivo* experiments, the average intensity of the ultrasound signal reaching the spinal cord area was found ~32 $mW/cm^2$ that was calculated by a point measurement at 4 mm distance from the ultrasound probe. This intensity was used during the behavior test.

### 3.14 Data analysis and statistics

Reaching and grasping success rates were calculated as described before (Alam et al 2017, Whishaw et al 2003b). Quantitative assessment was calculated from each rat. To calculate the success rate pre-injury 20 pellets and post-injury 30 pellets (during-stimulation 20 and post-stimulation 10) were given. A two-point scoring system was used where 0= failed to grasp the pellet at the first attempt and 1= first attempt to grasp the pellet. Pre- and post-injury success rate was calculated to find out the difference.

Two-tailed pair test was used to determine the success rate of reaching and grasping rate between pre- vs. post-injury. Two-way analysis of variance (ANOVA) with Tukey post-hoc test was used to determine the difference among the success rates for during- and post-ultrasound therapeutic stimulation. The maximum grip force from two groups was also analyzed by using two-way ANOVA with Tukey post-hoc test from week -1 to week -6.

For EMG analysis, video footage of reaching and grasping (successful and unsuccessful) attempts were examined frame by frame in a media player. The EMG signals were

bandpass filtered at 10-1000 Hz and amplified 1000 times using an analog amplifier (Model 1700 Differential AC Amplifier, AM Systems, United States). After filtering the amplified EMG signal was rectified and area under the curve of the rectified signal was then calculated by “trapz function”: A data acquisition system (Power1401-3A, Cambridge Electronics Design Ltd., United Kingdom) was used to digitize the EMG signals. A software (Signal, Cambridge Electronics Design Ltd., United Kingdom) was used to visualized the signals and synchronized with the video during the reaching and grasping task of extensor and flexor muscle EMGs. The difference between groups was considered as significant if $p<0.05$. Statistical analysis was performed using Prism (GraphPad Prism Software, version 8.4.2, USA) and MATLAB (Math Works Inc., Natick, USA).

## 3.15 Results

### *3.15.1 Pre-injury vs Post-injury success rate*

Behavior data was collected once a week from one-week post-injury. The rats were placed in a Plexiglas chamber with a 1 cm wide opening. Dustless sugar pellets were placed on a pit platform in front of the box slit to ensure that the rat approaches the opening in a consistent manner. After one week of recovery from the injury, the success rates were measured and compared to the pre-injury baseline. The success rate dropped significantly ($p=0.0381$) compared to the pre-injury score.

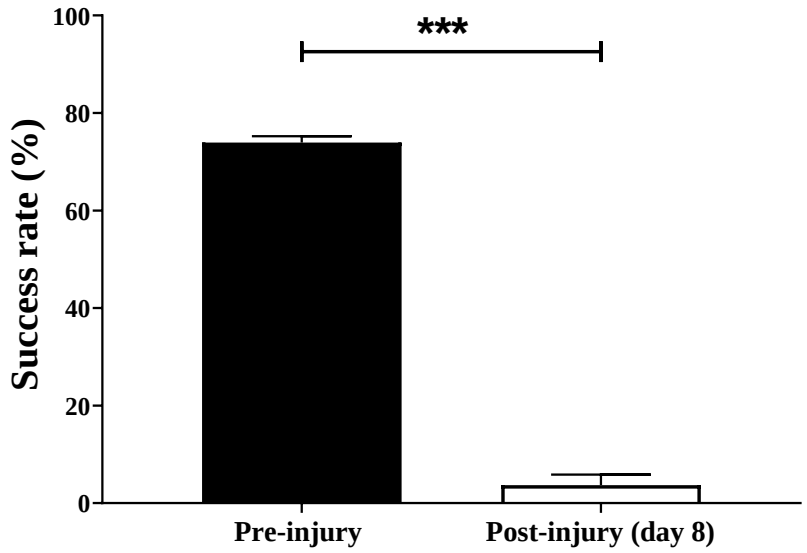



**Fig. 20**: **Pre-injury and post-injury success rates of forelimb reaching and grasping tasks**. The success rate dropped after one week of recovery from the injury (****p < 0.001*, paired t-test, n= 19).

The finding indicates that there was a significant motor deficit induced due to the injury **(Fig. 20)**. Most of the rats lost their grasping function. However, some of them have little grasping ability. At day-8 the rats were tested to grasp the pellet. Following the tests, the rats were ranked according to the success rates. The rats were than divided into two balanced score groups (US and Control group) by bin selection method. Success rates were collected for all US group rats during- and post-ultrasound stimulation.

### *3.15.2 LIPUS facilitates forelimb reaching and grasping function in rats*

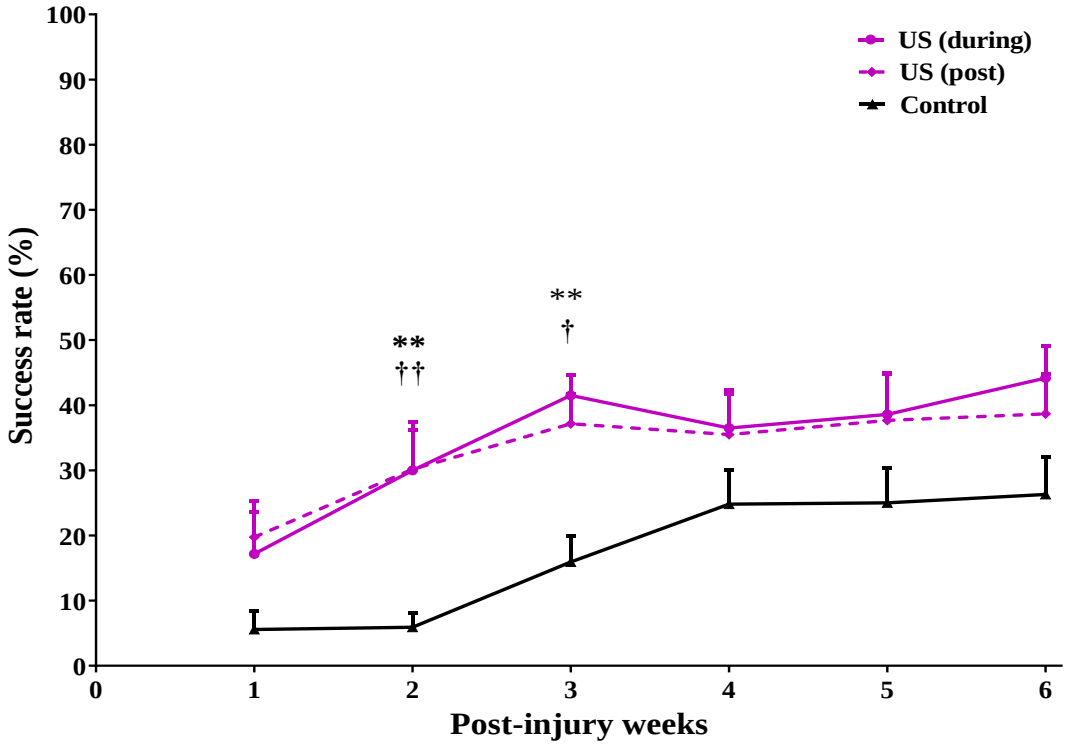



**Fig. 21: Average success rates (mean ±SEM; US, n=10 and control, n=9) of reaching and grasping tasks in US and control group rats.** Solid pink line indicates the scores when the US rats receiving the ultrasound stimulation and dotted pink line is the scores immediately after the stimulation. The black line indicates the success rates of the control group rats that did not receive ultrasound stimulation. At week-2 and 3 during ultrasound stimulation, significant (***p < 0.01*, two-way ANOVA, Tukey post-hoc test) improvement of reaching scores was found compared to the control group. Post-stimulation the success rate was also found significantly higher than control group scores at week -2 (***p < 0.01*, two-way ANOVA, Tukey post-hoc test) and week-3 (*† P<0.05*, two-way ANOVA). One of the rat did not attempt the task and was excluded from the results.

The success rate in US stimulation group was found higher compared to the control group from week 1 to 6. The US group rats had significant improvement at week-2 (30.00 ± 6.28 vs 5.92 ± 2.20; p =0.0079), (30.16 ± 7.31 vs 5.92 ± 2.20; p =0.0074) and week-3 (41.50 ± 3.11 vs 15.92 ± 3.92; p =0.0044), (37.167 ± 4.54; p =0.0223) compared to the control

group rats **(Fig. 21)**. However, from week-4 onward no significant improvements were found. At week-6 little more improvements were observed during (44.16 ± 4.98) and post (38.66 ± 6.15) stimulation, compared to the control group (26.30 ± 5.76). However, the score at week-6 was not significant like week-2 and 3 scores.

### *3.15.3 EMG activity*

During reaching and grasping EMG were recorded from extensor digitorum and flexor digitorum muscles at 1 to 3-week post-injury. The raw EMG signals are presented in **Fig. 22.**

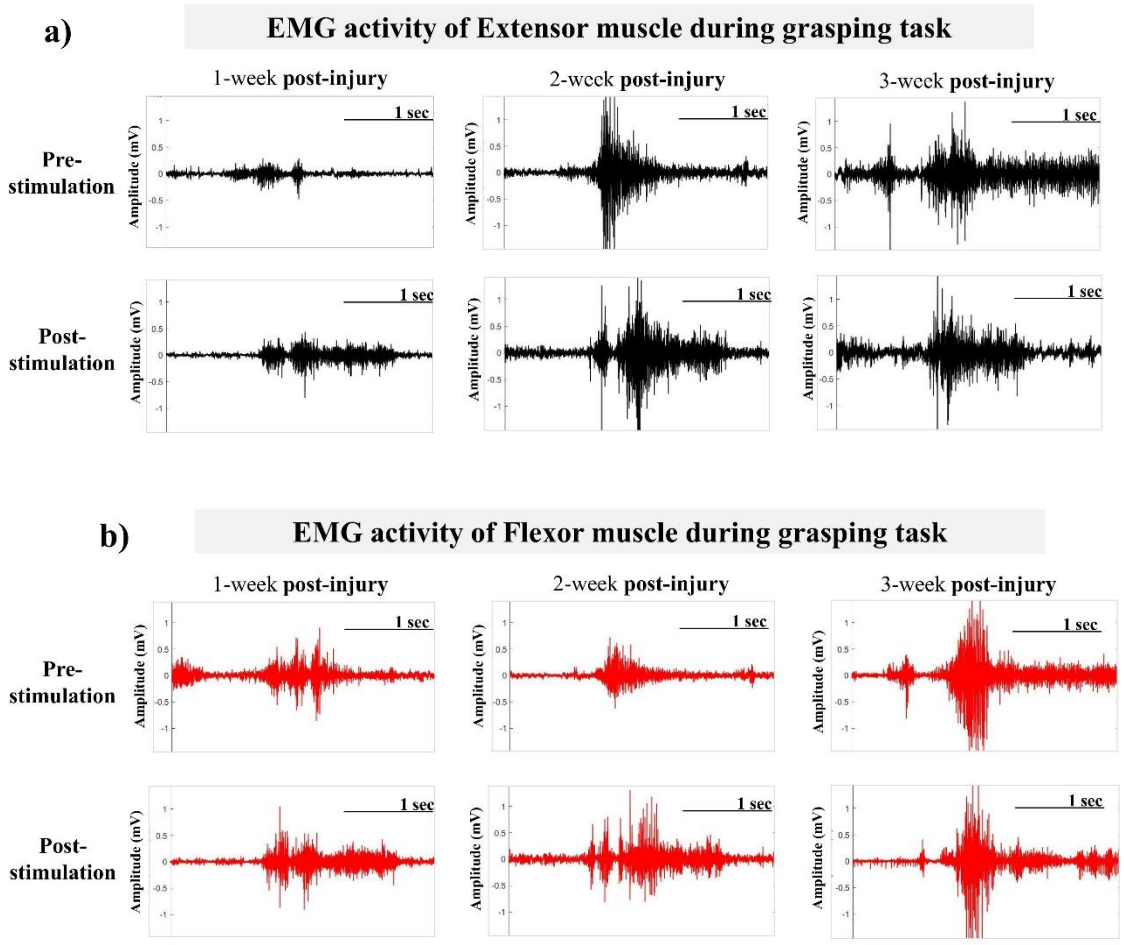


**Fig. 22: Raw EMG signals from a)** extensor digitorum and **b)** flexor digitorum muscles during the forelimb reaching and grasping task at 3 different post-injury weeks. One representative rat EMG is presented in the figure.

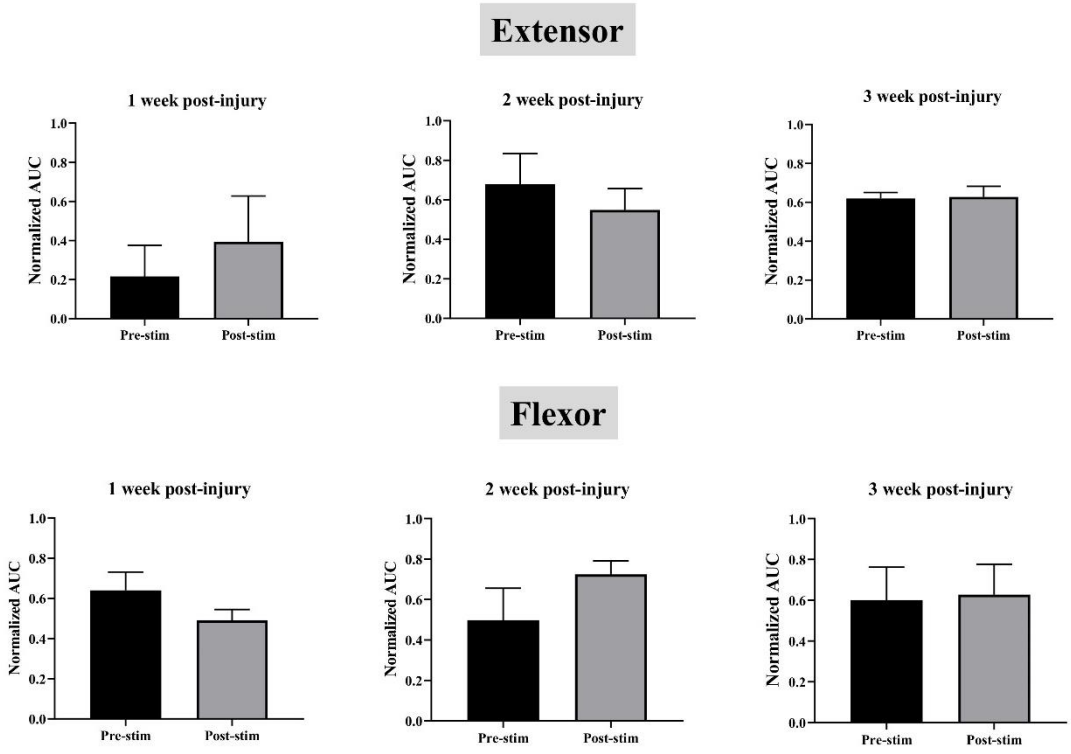
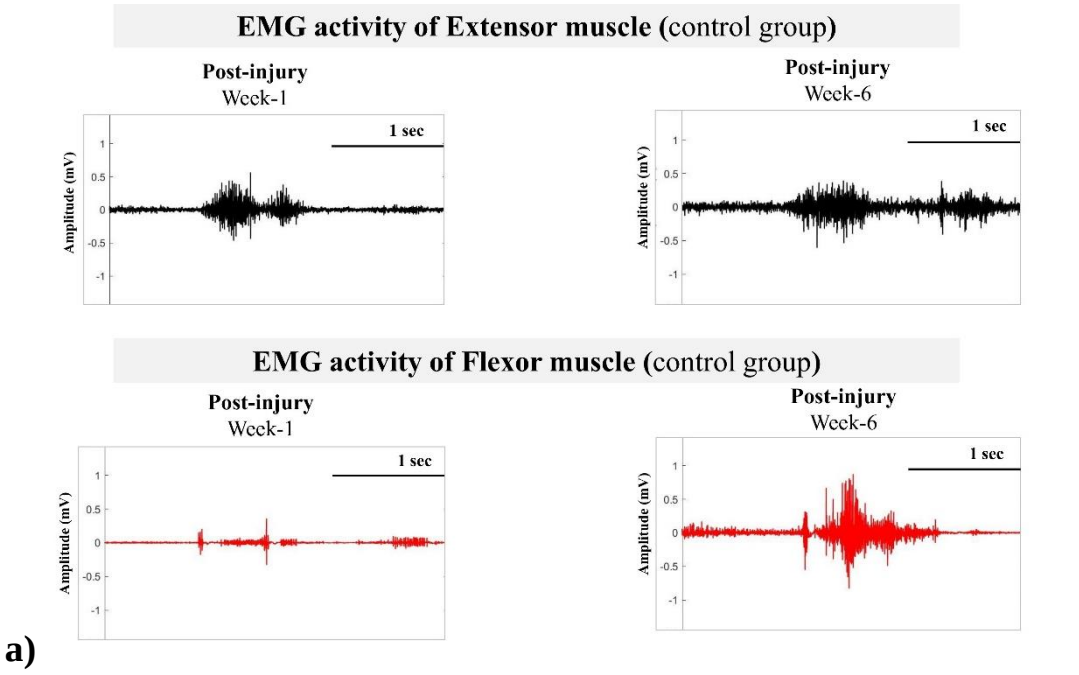



**Fig: 23. Normalized area-under-the-curve (AUC) of EMG signals from extensor digitorum and flexor digitorum muscles of US group rats during single pellet reaching task** (n=10 trials/rat). Data are presented as mean and SEM.

EMG signals of extensor digitorum and flexor digitorum muscle values were normalized to calculate the AUC. Values were compared to the pre-stimulation and post-stimulation at week-1, 2 and 3 (**Fig. 23**). At week-1 post-injury, the normalized value of extensor muscle increased after the simulation. However, at week-2 the value decreased and no difference was found at week-3 pre- and post-stimulation. In contrast, the flexor muscle at week-1 the values decreased after stimulation and increased at week-2. Likewise, the extensor muscle, in flexor muscle no differences were found at week-3. This indicates after a 3-week of

post-injury the stimulation cannot facilitate reaching and grasping tasks like week-1 and 2. However, no statistically significant differences were found at 3 different weeks.



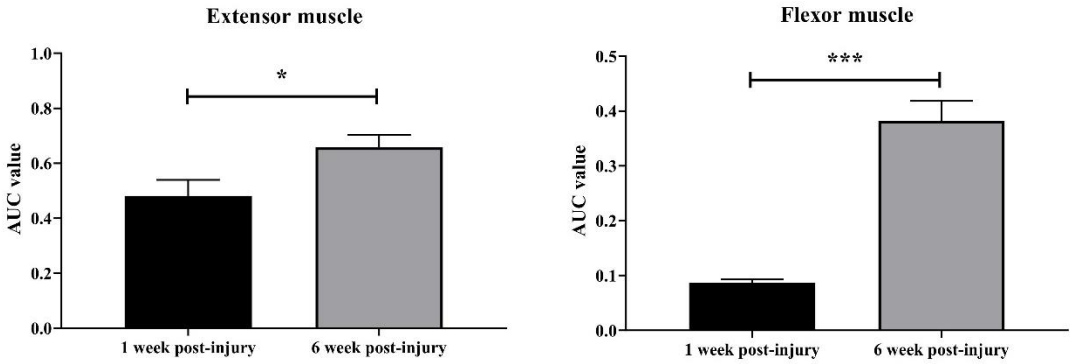




**Fig. 24: Comparison of (mean ± SEM) AUC values of extensor and flexor muscles EMG at week-1 and 6 during reaching and grasping task; a)** Raw EMG signal and **b)** AUC value of extensor and flexor digitorum muscles at week -1 and week-6. Data are presented as mean ± SEM. Significant differences were observed in extensor muscle (*$p < 0.05$, unpaired two-tailed t-test) and flexor muscle (***$p < 0.001$, unpaired two-tailed t-test).

From **Fig. 24a** it is observed that the control group rat muscles amplitude increase without any stimulation after injury similar as found before (Alam et al 2017). In control group at week-6 post-injury the AUC value increased from week-1 in both extensor (0.48 ± 0.05 to 0.65 ± 0.04, $*p < 0.05$, unpaired two-tailed t-test) and flexor (0.09 ± 0.006 to 0.38 ± 0.03, $***p < 0.001$, unpaired two-tailed t-test) muscles (**Fig. 24b**).

### *3.15.3 Role of LIPUS in forelimb grip force*

Following skilled reaching and grasping test, grip strength test of 11 rats (US group, n=6 rats, and Control group, n=5 rats) were determined by using a custom made grip strength meter as described before (Alam et al 2015). Each week after the forelimb reaching and grasping task the grip force was recorded. Rats were held for grasping the grid that connected to a force sensor. To record the grip force, the rats were gently pulled from the grid.

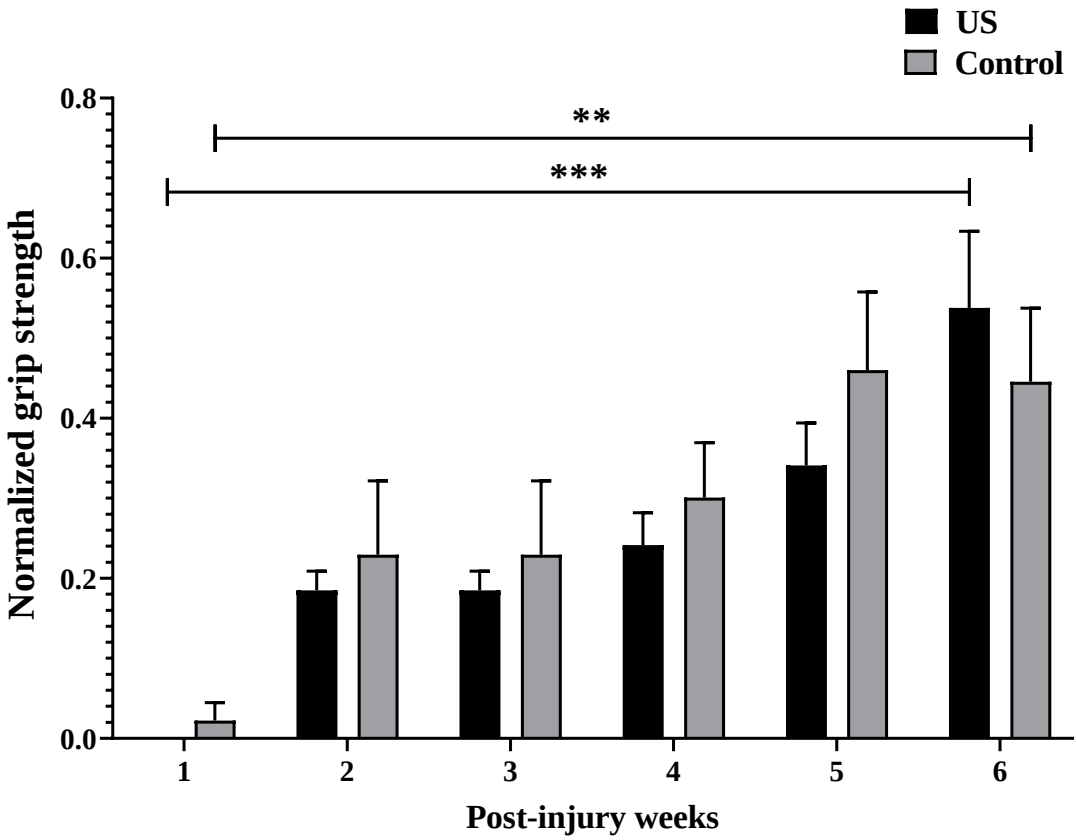



**Fig. 25: Normalized grip force.** Data are presented as mean and SEM (US group, n=6; and Control group, (n=5). Grip force values were normalized to the maximum values. Significant improvements were found in US group rats (****p < 0.001*, two-way ANOVA, Tukey post-hoc test) and in control group at week 6 (***p < 0.01*, two-way ANOVA, Tukey post-hoc test) compare to the post-injury week-1

The grip strength force of each week's values was averaged and the maximum value from pre-injury to week-6 was calculated. The values of each week were normalized from each rat according to the rule ($X = \frac{X-Xmin}{Xmax-Xmin}$) (Alam et al 2015). Significant muscle strength improvements were found at week- 6 compare to the week-1 of post-injury in US group **(Fig. 25)**. However, in the control group, little significant improvements were found at week- 6. In addition, no significant differences were observed between the two groups at any week.

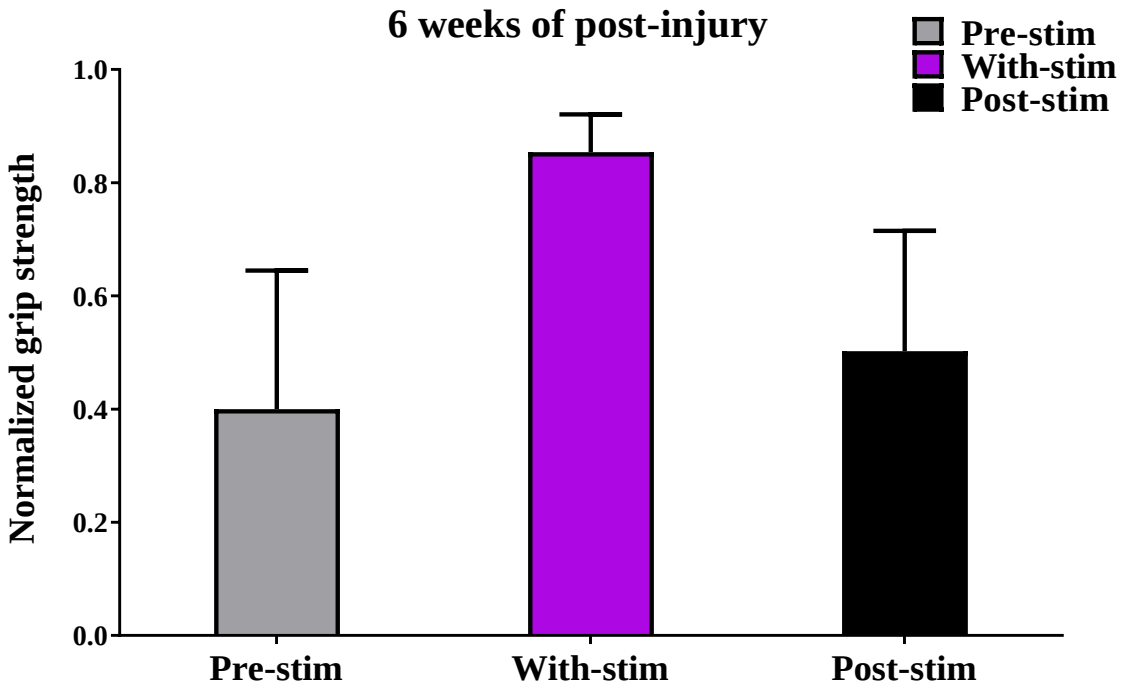



**Fig. 26: Average (Mean ± SEM, n=5) maximum grip strength pre-, with- and post-ultrasound stimulation**. The grip force value was calculated from the peak output value of the sensor in the grip strength meter after 6 weeks of post-injury.

The grip strength test from the other five rats was tested pre-, during- and post-ultrasound stimulation at six-week post-injury (**Fig. 26**). Although the result was not statistically significant, the rats had shown higher grip forces during ultrasound stimulation. Furthermore, the post-stimulation grip strength is little higher than the pre-stimulation suggesting a therapeutic effect of ultrasound stimulation.

### 3.16 Summary and discussion

In the present study, the effects of LIPUS was evaluated via forelimb grasping task and grip strength in chronic incomplete spinal cord injured rats. Moreover, two different antagonistic muscle responses were also evaluated. Incomplete cervical injury at C4 level

showed a significant deficit in motor function because of the disruption of the motor neuronal network.

Previous studies have demonstrated that epidural electrical stimulation in the cervical spinal cord can facilitate reaching and grasping function in the same injury type animals (Alam et al 2017). However, electrical stimulation is an invasive method that requires surgery when a targeted stimulation is needed. Besides, the contacted thin wires can be moved and damage the neuronal tissue to carry out a secondary inflammation to form a tertiary scar. In comparison to electrical stimulation, ultrasound with a low-intensity can be delivered to the nervous tissue to recover the neuronal tract (Tyler et al 2018). LIPUS is one of the choices of neuro-stimulation because of its penetration and modulation capability in the neurons (Blackmore et al 2019). In our experiment skilled motor reaching and grasping task was used to evaluate and compare at different conditions: during- and post- ultrasound stimulation. The effects of ultrasound stimulation on the success rate was found higher during-stimulation compared to post-stimulation. However, after week-3 post-injury, no significant difference was found and the success rate was higher compared to the control group of rats. The possible cause is after 3 weeks the astrocytic scar is become matured and converted to fibrotic scar (Kjell & Olson 2016) and ultrasound could not facilitate the recovery behind the chronic scar. Moreover, at the end of week-6, the success rate was increased during-stim than the post-stimulation success rate. To recover the neuronal tract from injury weekly LIPUS treatment can facilitate grasping function in the injured rats. Previously, it was reported that after crush injury the neuropathic tissue is more sensitive to US than the normal healthy tissue (Ni et al 2017) (Tych et al 2013). The results also reflected in the experiment.

Forelimb grip force measurement is a useful tool for measuring the recovery for incomplete injury (Anderson et al 2005). Likewise, forelimb reaching and grasping task, the task is not skilled (Ahmed et al 2019). The distal flexor muscle is responsible for grasping and gripping ability of rats that are controlled by CST. Moreover, the gripping ability is also determined by some part of the reticulospinal tract that descends in the medial part of the ventral column (Anderson et al 2005). From our experiment, it is also noticeable that after partial injury in control group rats recover the gripping ability. The finding is similar to the result published by Anderson and his colleagues (Anderson et al 2005). The grip force is mainly combined with all forelimb muscle functions that include flexor digitorum, extensor digitorum, brachialis, triceps brachii, etc. Following injury, the forelimb muscles lost their grasping function because of no supraspinal input to the flexor muscle. However, other forelimb muscle function doesnot affect significantly after the injury. Hence, the grip force result is not consistent with the forelimb reaching and grasping task. Moreover, in our experiment, normalized grip force data showed that the force tends to increase in the US group and a significant improvement was found at week-6 compared to the week-1. In addition, at week-6 the grip forces were tested from five rats pre-, during- and post-ultrasound stimulation (**Fig. 26**). Although the result was not statistically significant, the rats have shown higher grip forces during ultrasound stimulation. Moreover, the post-stimulation grip strength is little higher than the pre-stimulation suggesting the therapeutic effects of our pulsed ultrasound. The two different behavior tasks suggest the therapeutic effect of ultrasound during stimulation is higher than the post-stimulation.

In our study, the EMG data has shown that at week-6 in the control group following injury the muscle activation increased compared to the pre-injury that was previously described

by Alam. M., et al., 2017. The hyperexcitation condition of the neural network may cause more energy facilitation in the distal forelimb muscles compared to the immediate after injury. Moreover, in US group antagonistic activity of the two different muscle extensor and flexor was found at week-1 and 2 post-injury. The flexor muscle activity increased after stimulation at week-2 indicating the increase of grasping rate that is similar to the forelimb reaching and grasping success rate (**Fig. 23**). However, after 2 weeks of post-injury, no differences were observed in extensor and flexor muscles before and after stimulation.

The current chapter examined the effect of LIPUS on cervical injured rats. In summary, the LIPUS effect on SCI rats was found higher during and post-stimulation. Moreover, the flexor muscle responses were found higher after the stimulation indicating the effect of LIPUS. However, only the therapeutic ultrasound approach is not enough for functional recovery after cervical cord injury in rats. A combination of repair strategy is much needed for maximum recovery (Olson 2013). Combinatorial approaches such as low doses of pharmacological agents and electrical stimulation have shown functional improvements in SCI animal models and human subjects (Courtine et al 2009, Duru et al 2015, Freyvert et al 2018). A serotonergic against drug-based neuromodulation with electrical stimulation is one of the popular approaches for locomotion recovery in spinal cord injured rats (Courtine et al 2009). As a serotonin agonist, Buspirone previously has shown forelimb functional recovery (Jin et al 2015). However, the dose responses were not studied. To find out the effective dose on cervical cord injured rats next experiment was designed and conducted that described in the following chapter.

# Chapter 4: Serotonergic neuromodulation in the injured spinal cord

## 4.1 Introduction

Spinal cord injury in the cervical cord region damages both sensorimotor and motor function (Freund et al 2012). Moreover, the serotonergic system is disrupted and dysregulated after the injury. Serotonin (5-hydroxytryptamine or 5-HT) is a monoaminergic neurotransmitter that plays an important role in the modulation of the neural network and locomotion activity. In addition to the locomotion activity serotonin plays a major role in the gain control of volitional limb movements (Wei et al 2014b). Alteration of this serotonergic system following SCI accountable for various degrees of paralysis (Nardone et al 2015).

Previously, it has shown that serotonin modulates the neuronal pathway by modulating the interneuron and motor neuronal pathway (Schmidt & Jordan 2000). The results suggest that there is a connectivity of supraspinal and interneuronal pathways in spinal circuits. In rat cervical spinal cord, the distribution of serotonin localized in the gray matter VII and X region indicating the forelimb motor control (Newton & Hamill 1988). Moreover, in spinal cord effect of serotonin vary because of the receptor and mode of action. Functionally, 7 receptors of serotonin present that modulate the neuronal activity (Kalinina et al 2019).

Following SCI, systemic application of the targeted serotonin-receptor agonists with other neuromodulatory approaches can facilitate modulation of the neural network (Courtine et al 2009, Musienko et al 2011, van den Brand et al 2012). Among the several receptors $5HT_{2A}$, $5HT_{2C}$ and $5HT_{7}$ agonists have modulation capability in hind limb stepping (Bos et al 2013, Fouad et al 2010, Liu & Jordan 2005). As a $5HT_{2}$ receptor agonist quipazine has a

high affinity in the $5HT_{2A}$ and $5HT_{2C}$ receptors, Moreover, 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OHDPAT) has also the affinity for $5HT_7$ and $5HT_{1A}$ receptors to modulate the CPG activity (Antri et al 2003, Courtine et al 2009, Sławińska et al 2012). In addition to the systemic administration of 5HT agonist, the grafting brainstem serotonin receptor neurons into the spinal cord can control the locomotion after spinal cord injury. Both $5HT_{2A}$ and $5HT_7$ receptors have a robust influence in controlling the CPG activity in grafted rats (Sławińska et al 2013). Moreover, $5HT_{2A}$ receptors can control motor neuronal activity in addition to CPG control pattern of locomotion. As a receptor activation, $5HT_2$ receptor agonist alone has the potential to facilitate the persistent sodium current inward and firing the motorneuron with and without chronic spinal cord injury (Harvey et al 2006). Although, pharmacological therapy by serotonin agonists has shown improved functional recovery in standing and stepping in SCI paraplegics and limited progress has been made on improving arm and hand functions using such neurochemical modulation (Freyvert et al 2018).

Buspirone, a partial agonists act as a receptor for 5-$HT_{1A}$ and dopamine $D_2$ autoreceptors. As a weak affinity to 5-$HT_2$ receptors, Buspirone also used as an anxiolytic drug to improve brain functions through enhancing neural plasticity after injuries or diseases (Loane & Politis 2012). In recent studies on SCI patients, it has been shown that electrical stimulation to the spinal cord, when combined with regular Buspirone administration can restore voluntary control of hand function (Freyvert et al 2018) and locomotor function (Gerasimenko et al 2015). Previous reports have shown that low doses of Buspirone can stimulate the somatodendritic 5-$HT_{1A}$ receptors in an *ex-vivo* experiment from the bovine brain (Peroutka 1985) and can trigger motor activity in rat model (Shireen & Haleem

2005). However, high doses of Buspirone works on dopaminergic neurons in the presynaptic terminal and trigger out its activity (Dhavalshankh et al 2007). In Parkinson's diseases, a dose of 2mg/kg b.w. Buspirone can reduce locomotor activity and has also shown this effect in other neurological disorders (Lundblad et al 2005).

Likewise, $5HT_2$ receptor agonist, it has been assumed that Buspirone as a $5HT_{1A}$ receptor agonist can modulate the interneuronal activity by repetitive firing in chronic conditions (Harvey et al 2006). But the exact dose-response of such monoaminergic drugs on improving arm and hand functions in cervical SCI is unknown. To evaluate this, in the current chapter, I investigated the dose-responses of Buspirone treatment on reaching and grasping function after a cervical spinal cord injury in rats.

**4.2 Experimental design**

Twenty-four adults healthy female Sprague-Dawley rats (230±20 grams b.w.) were used in this experiment. Adlibitum food and water were provided before starting the pre-injury forelimb reaching and grasping task training. Bodyweight was constantly monitored. The room temperature (24ºC) and humidity (40%) maintained according to the guidelines. Animal handling was done for one week- before the forelimb reaching and grasping training as described in **Chapter 3**. The rats were trained to reach and grasp the pellet from the pit platform (**Fig. 14**). Thirteen rats demonstrated a success rate around 70% in the task and the rest showed a minimum 60% success rate (**Fig. 27**).

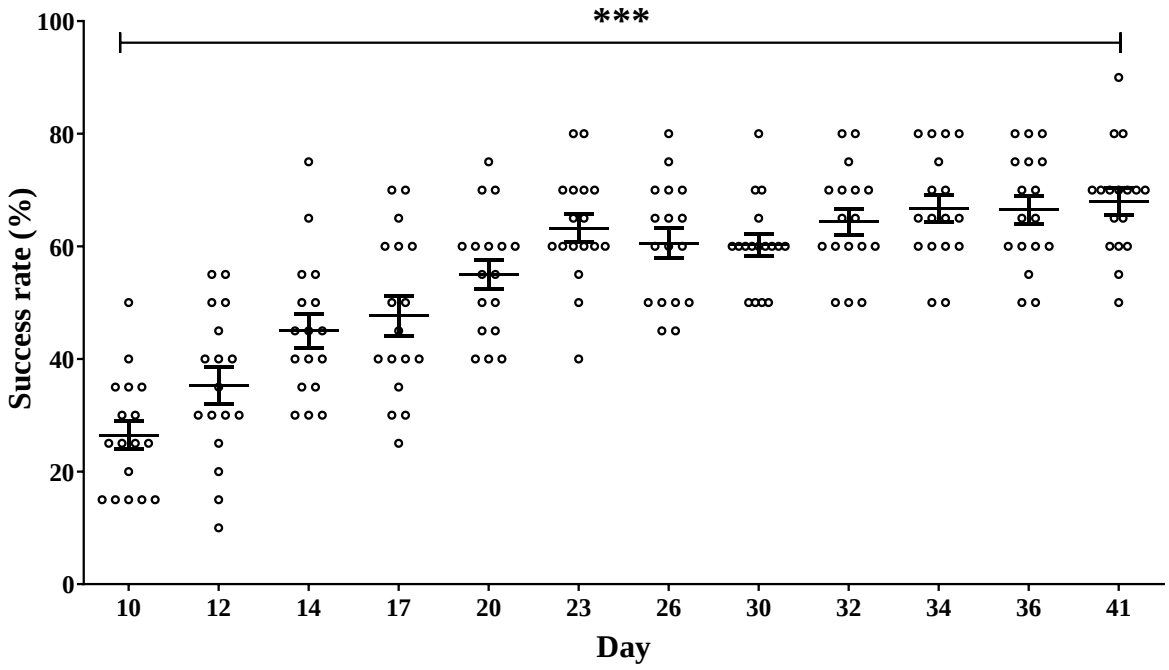



**Fig. 27: Reaching and grasping the learning curve of intact rats** (n=17). Significant differences (****P<0.001*, one-way ANOVA) were found at day-42 compared to the day-10 of reaching and grasping the success rate (Mean ± SEM).

During the training 30 pellets were provided to grasp pellets from the pit. Successful 6 weeks of training, the best seventeen rats were selected for the SCI experiment (**Fig. 28**). During the surgery, the EMG electrodes were implanted in the preferred paw distal muscles-extensor and flexor digitorum.

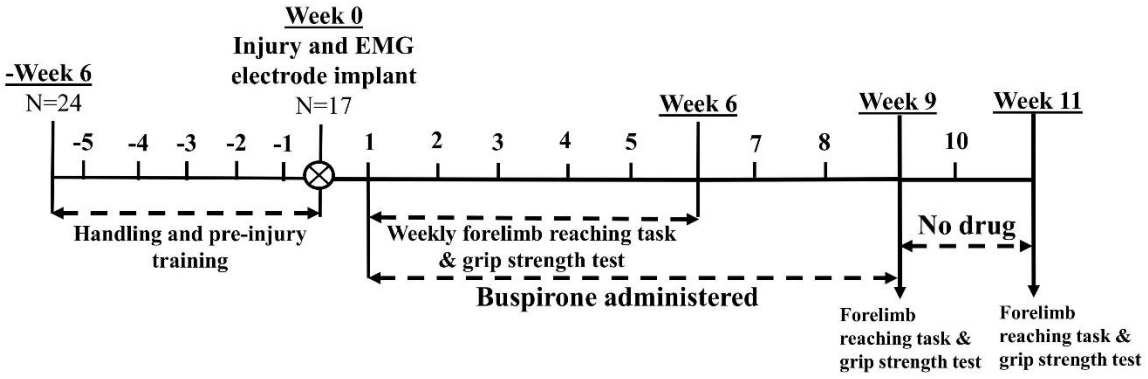


**Fig. 28**: **Experimental design**. Seventeen rats were trained to reach and grasp the pellet for 6 weeks. After skilled reaching and grasping training task, the rats received dorsal funiculus crush at C4 level and subsequent implantation of EMG electrodes in the preferred paw. After one week of recovering from injury, the rats were ranked and divided into three balanced dosages groups of Buspirone; **Group A** with low dose (1.5mg/kg b.w.; n=5), **Group B** with medium dose (2.5mg/kg b.w.; n=6) and **Group C** with high dose (3.5mg/kg b.w.; n=6). Weekly forelimb reaching task and grip strength tests were done for week-6 continuously. The animals were then tested at week-9 and finally, the drug was withdrawn and retested at week-11.

The surgical procedure and EMG electrodes implantation procedure is the same protocol as mentioned in chapter-3. After surgery, the rats were placed in an incubator by administering the analgesic and antibiotic. The rats were then moved to a single individual home cage. Fresh fruits and juice were provided from the cage early recovery.

### 4.3 Drug treatments

At day-7, the rats were tested to reach and grasp the sugar pellet. The rats were then ranked and divided into three balanced dosages groups- **Group A** with low dose (1.5mg/kg b.w.; n=5), **Group B** with medium dose (2.5mg/kg b.w.; n=6) and **Group C** with high dose (3.5mg/kg b.w.; n=6). Buspirone (Tocris®, UK) was previously prepared by dissolving 1mg/1ml ultrapure distilled water (Invitrogen®, USA) and administered intraperitoneally into different doses groups of SCI rats up to 9 weeks post-injury.

Within 1 hour of Buspirone administration, the behavior tests and EMG recording were done. Each animal was videotaped from the front while retrieving food pellets with a video camera. After each forelimb reaching and grasping task and EMG recordings, the grip strength test of the rats was recorded once per week for 9 weeks, as described in **Fig. 28**. To see the long term effect of the drug, after 9 weeks, the drug administration was ceased,

and the behavior tests and electromyography recordings were recorded only at week-11 post-injury.

## 4.4 Results

### *4.4.1 Pre-injury vs Post-injury success rate*

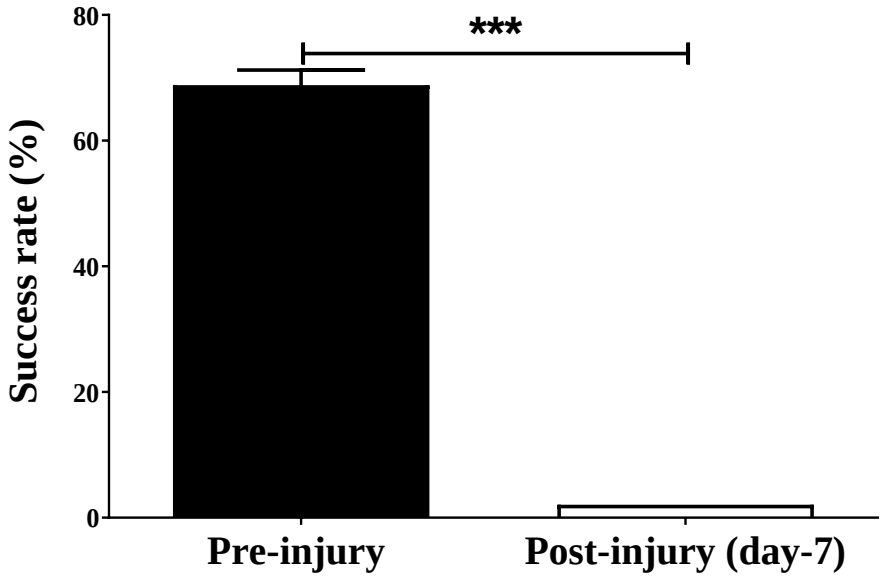



**Fig. 29**: **Pre-injury vs post-injury success rate**. After the injury, the success rate dropped significantly (****p < 0.001*, paired t-test).

Following injury, the animals lost the grasping function. However, the animals can place their forelimb in the food pit and can use it as locomotion. The post-injury forelimb post-injury forelimb grasping success rates were calculated and compared with the pre-injured animals. Forelimb reaching scores dropped significantly 1 week- after the cervical cord injury (68.82 ± 2.33 vs 2.05 ± 1.25, *p <0.05*; paired t-test) (**Fig. 29**).

### *4.4.2 Low dosage of Buspirone facilitate forelimb grasping function*

All rats, according to their different doses groups, the drug Buspirone was administered for 9 weeks. The grasping success rate was measured after the drug administration. The animals in Group-A and B started to increase their grasping function at week-4 and 5. The success rate of each group of animals was compared with week-1. In **Group-A**, differences were found at week-4 and-5, respectively (40.33 ± 6.33 and 41.99 ± 4.89, $**p < 0.05$, one way ANOVA). From week-6 to -11 a significant difference was found compared to the post-injury week-1 (47.33 ± 6.53, 46.33 ± 5.22 and 45.33 ± 4.54,$**p < 0.05$, ANOVA) (**Fig. 30a**).

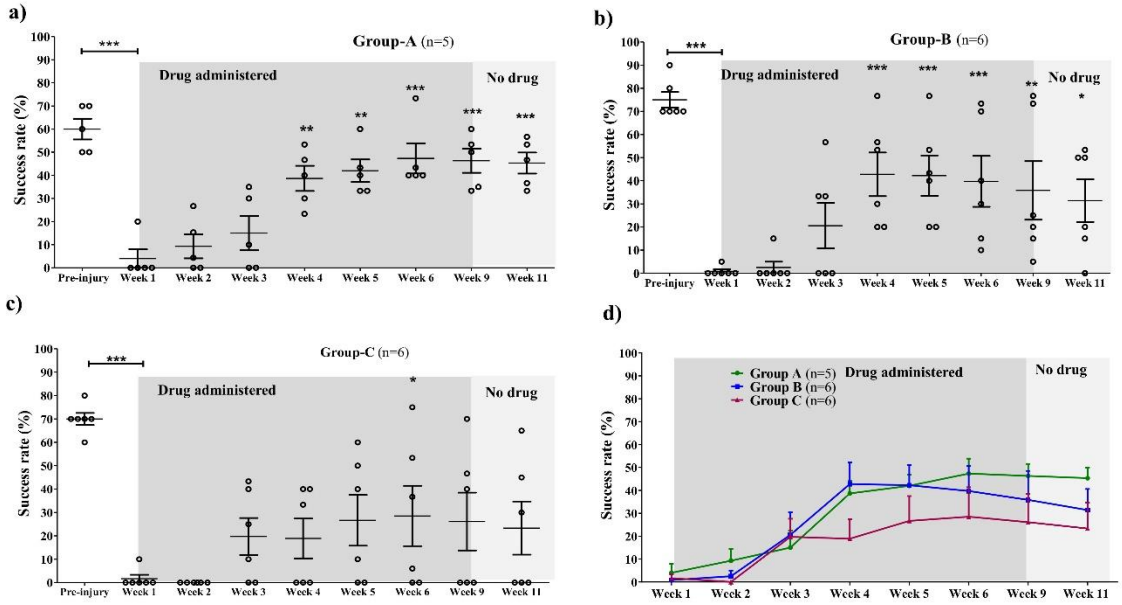



**Fig. 30**: **Reaching and grasping scores of three different groups after Buspirone administration. a)** Mean (±SEM) success rates of **Group-A** animals. Week-1 vs week-4 and 5 ($**p < 0.05$, one-way ANOVA, Friedman test). Week-1 vs week-6,9 and 11 ($***p < 0.05$, one-way ANOVA, Friedman test). **b)** Mean (±SEM) success rates of **Group-B** animals. Week-1 vs week-4 and 5 ($***p < 0.05$, one-way ANOVA, Friedman test). Week-1 vs week-6 and 9 ($**p < 0.05$, one-way ANOVA, Friedman test). Week-1 vs week-11 ($*p$

$< 0.05$, one-way ANOVA, Friedman test). **c)** Mean (±SEM) success rates of **Group-C** animals. No significant difference was found. **d)** Overall success rates of all three groups.

Moreover, **Group-B** rats significantly improved the forelimb grasping function after week-3 (42.77 ± 9.40, 42.21± 8.57 and 39.72 ±11.01; ***$p < 0.05$, ANOVA) compared to week-1 (**Fig. 30b**). At week-9 (35.83 ± 12.68) and 11 (31.38 ± 9.23) behavior test was done to see the long-term dose effect of the 2mg/kg b.w. of Buspirone. The success rate dropped at week-11 after the cessation of Buspirone administration. The animals in **Group-C** did not show any significant improvement after the administration of Buspirone at 3.5 mg/kg b.w (**Fig. 30c**). The success rates of all three groups were compared with each other (Fig. **30d**). No significant differences were found. However, in Group-A rat's higher success rates were found at week-11 compared to the other two groups. In **Group-B** and **C** the scores increased after 2 weeks. However, in **Group-B** rats, the reaching scores increased up to 5 weeks (58.68 ± 12.21). At week-6 the success rate dropped (39.65 ± 11.82) while the **Group-C** rats were found a little improvement (44.16 ± 19.46). Moreover, the **Group-A** rats have shown improvement of grasping function after week-3 to 9 (24.22 ± 11.61, 63.53 ± 5.90, 69.97 ± 5.62, 80.25 ± 11.47 and 78.44 ± 9.07) and it also persists after cessation of Buspirone administration (77.73 ± 10.40). In addition, **Group-B** and **C** rats the reaching scores dropped after Buspirone withdrawal (40.11 ± 11.87 and 34.22 ± 16.95). At the end of week-11, low dose rats **Group-A** showed an average of 60% improvement over medium dose **Group-B** to high dose **Group-C** rats.

### *4.4.3 Low dosage of Buspirone treatment improves distal muscle co-ordination*

Raw EMG signals from the distal forelimb muscles- extensor and flexor digitorum muscles were recorded during grasping task (**Fig. 31**). The EMG signals were recorded during

week-1 of post-injury after the Buspirone administration. Until week-6, grasping task was carried out weekly. After week-6 the grasping task was done only at week-9 and withdrawal of the drug at week -11 and subsequently, EMGs were recorded. Videotape footage of the forelimb reaching task of each rat was examined frame by frame to identify the components of the grasping.

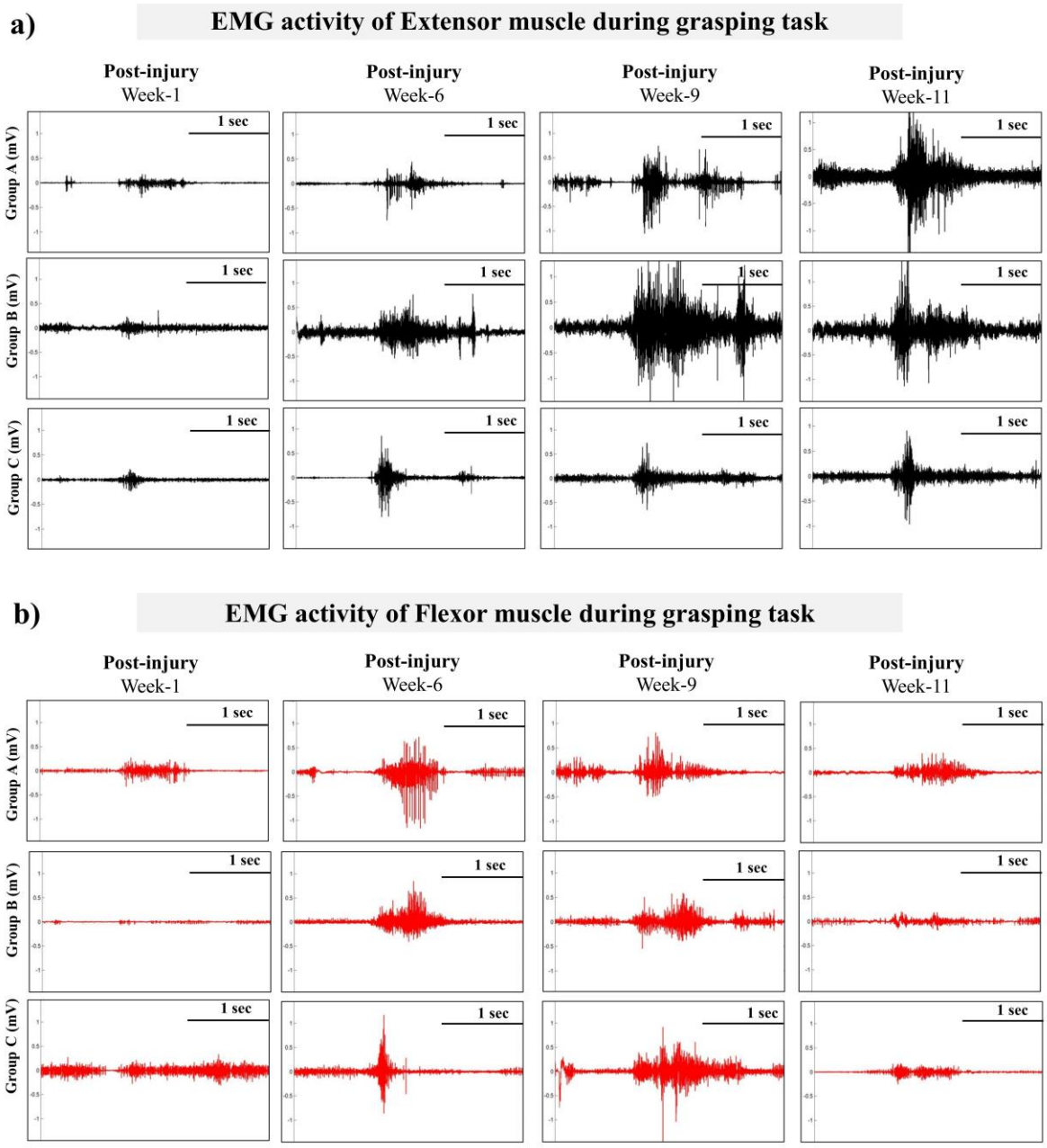


**Fig. 31**: **Effects of different dosages of Buspirone on forelimb a) Extensor digitorum and b) Flexor digitorum muscles during reaching and grasping task.**

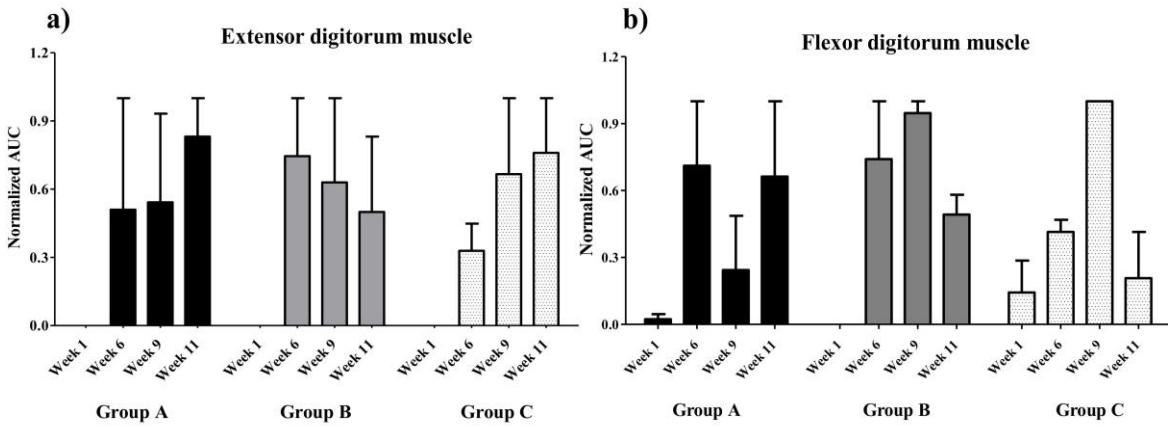



**Fig. 32: Normalized AUC value of distal muscles.** Normalized area-under-the-curve (AUC) of a) extensor and b) flexor muscles were recorded during the single pellet grasping task (n=30 trials/animal) after Buspirone administration in three different groups. Two animals were selected from each group for the analysis. EMG patterns over time were evaluated by ANOVA. Data are represented as mean and SEM value.

In **Group-A** and -**C** the normalized AUC value of extensor muscle increase gradually after week-6 (**Fig. 32a**). The increasing pattern is persistent even after the withdrawal of the drug. In contrast, **Group-B** rats because of the spasticity in extensor muscle the value decreased. The dose was not enough to reduce the extensor muscle spasticity. Distinct responses of flexor muscle were found in each group compared to the extensor muscle. In **Group-A** rats after week-6, the flexor muscle response dropped at week-9 and dramatically increased at week-11 after the withdrawal of the drug. The finding indicates a low dose (1.5mg/kg b.w) of Buspirone has a long term effect of flexor muscle recovery. In contrast, **Group-B** and **C** rats the responses of flexor muscle increased gradually until week-9 and

dropped after the cessation of the drug administration. However, no significant difference was found in each group (**Fig. 32b**).

### *4.4.4 Low-dosage of Buspironetreatment improves forelimb grip strength*

Post-injury average maximum grip force of **Group A** rats were recorded 6.83 N (week-1) to 17.80 N (week-5) (**Fig. 33a**). For **Group B** rats, the values were recorded from 4.95 N (week-1) to 13.42 N (week-5) (**Fig. 33b**). In **Group C** the values increased from 3.34 N (week-1) to 12.15 N (week- 4) (**Fig. 33C**).

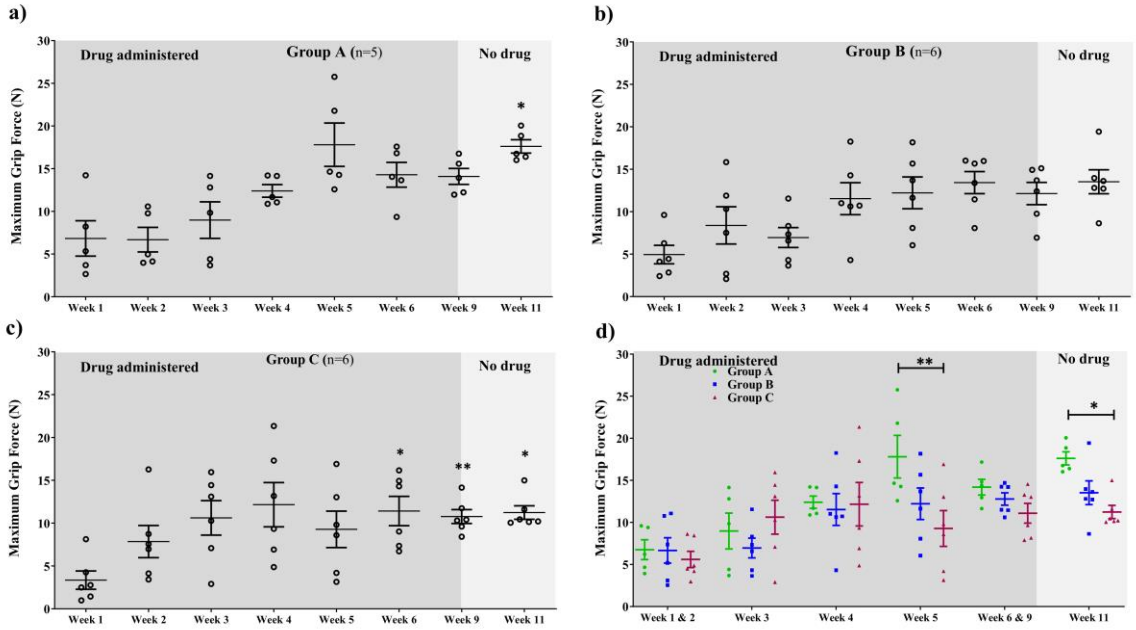



**Fig. 33**: **Maximum grip force of three different groups after Buspironeadministration**. **a)** Mean (±SEM) maximum grip force of **Group A** rats. A significant improvement was found at week-11 (*$p<0.05$, one way ANOVA, Tukeys post-hoc test) compared to week-2 and week-9 vs week-11 (*$p<0.05$, one way ANOVA, Tukeys post-hoc test) **b)** Mean (±SEM) maximum grip force value of **Group B** animals. **c)** Mean (±SEM) maximum grip force of **Group C** animals. The high doses group rats showed a significant improvement at week-6 and week-11(*$p<0.05$, one way ANOVA, Tukeys post-hoc test) compared to the week-1; week-1 vs week-9 (**$p<0.01$, one way ANOVA, Tukeys post-hoc test). **d)** Mean (±SEM) maximum grip force of three groups. **Group-A** showed significant grip strength at

week-5 ( ***P<0.01* one way ANOVA, Tukeys post-hoc test) and week-11 (**P<0.05*, one way ANOVA, Tukeys post-hoc test) compared to the **Group-C**.

However, at week-6 and 9, the value dropped to 14.28N and 14.09N, respectively in **Group-A** rats (**Fig. 33a**). A similar effect of Buspironewas also found in **Group-B** and **C** rats. In **Group-B** rats, the value was boosted from 4.95N (week-1) to 13.42N (week-6) and dropped at week-9 to 12.13N (**Fig. 33b**). Moreover, the **Group-C** animals maximum value lifted from 3.34N (week-1) to 12.15N (week-4) and dropped to 10.77N (**Fig. 33c**). While at week-11, after the withdrawal of the drug the values were lifted to higher in all groups of animals respectively (17.61N, 13.52N, and 11.24N) (**Fig. 33d**).

### *4.4.4 Normalized success rate vs grip force*

No significant relation was found between the forelimb reaching score and grip force in the early post-injury weeks. In **Group-A** rats a consistent pattern of recovery was found in week-6, 9 and 11 (**Fig. 34a**). However, in

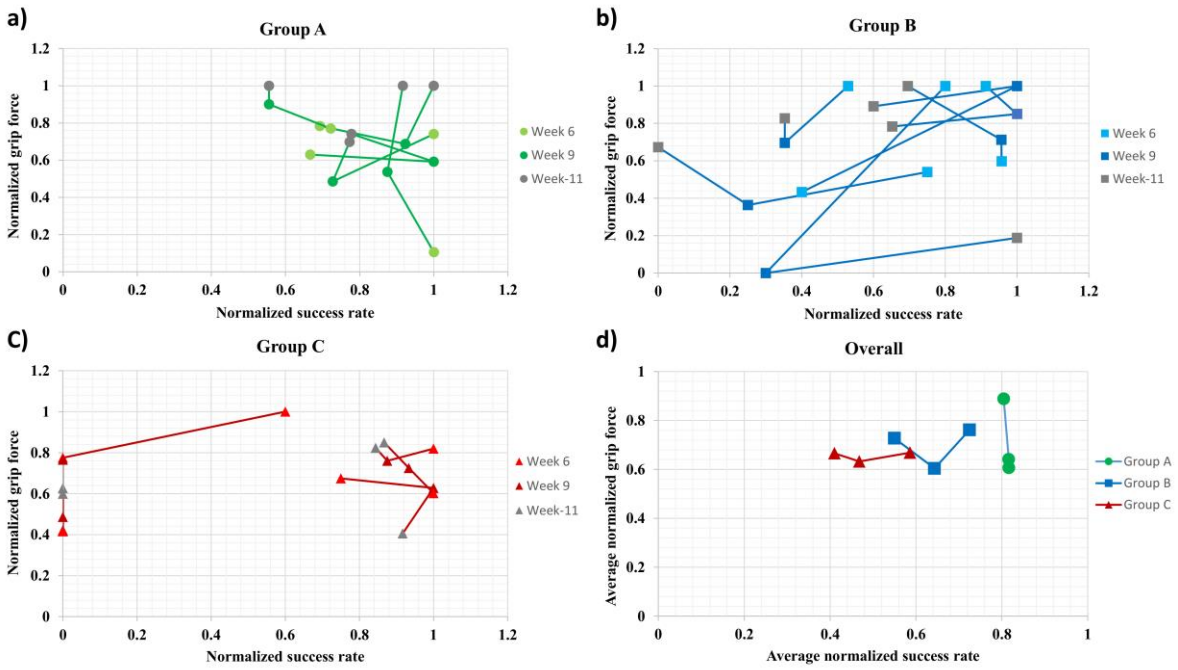



**Fig. 34: Normalized success rate vs. normalized grip force at different post-injury weeks. a-c)** The maximum grip force and the success rate was normalized and plotted for each rat at week 6,9 and 11. **d)** Overall relationship of three groups at week-6,9 and 11.

**Group-B** rats recovery of the motor task increased at week-6 at the optimum level and after two weeks of the interval the improvement decreased (**Fig. 34b**). The finding indicates that each week motor task has an influence on the functional recovery. After 9 weeks of withdrawal of Buspironethe recovery of **Group-B** rats decreased a significant way. Moreover, at week-11 in **Group-B** one of the rat did not improve. Likewise, the **Group-A** rats, **Group-C** rats has a consistent pattern of recovery (**Fig. 34c**). However, two animal didn't improve their success rate, although a little improvement was found in grip strength.

### 4.5 Summary and discussion

As a serotonin agonist, Buspirone is an anxiolytic drug that has a high affinity for $5HT_{1A}$ receptors. Moreover, some antagonistic property of the drug was found in the dopamine

receptor (Loane & Politis 2012). In tetraplegic patients, Buspirone has shown improvement of forelimb grip strength along with electric stimulation (Freyvert et al 2018). However, the effect of the drug was not strong enough to carry out the recovery. A preliminary study has shown that as a serotonin agonist Buspirone can significantly modulate the serotonergic neurotransmission system after spinal injury in rat model to improve the forelimb reaching task (Jin et al 2015). However, the muscle coordination of different doses during the grasping task has not been discovered. In this chapter, I have demonstrated that different dosages of Buspirone, a partial serotonin agonist have distinct effects on the forelimb functional recovery after a dorsal funiculus injury in rat model.

A recent study of Buspirone has shown an acute effect on hindlimb stepping, paw placement, facilitate locomotion (Renaud Jeffrey-Gauthier et al 2018) and elevated frequency-dependent depression of H-reflex after a complete spinal cord lesion in mouse model (Develle & Leblond 2020). Comprising this study suggests that the role of serotonergic agonist drug Buspirone on improving the injured circuitry of locomotion.

A limited study has been conducted on the dose-dependent effects of Buspirone in animal model. Earlier results have shown the effects of the doses of Buspirone on behavior and cerebral glucose metabolism in rat model. A low dose (0.4 mg/kg) reduced cerebral metabolic rates for glucose in rats compared to the high doses (4 and 40 mg/kg). It was shown that the low dose of Buspirone can preferentially activate the 5-HT1A receptor (Freo et al 1995). For pain, the dose of 0.1 mg/kg of Buspirone reduced pain threshold in the hot plate test while higher-doses (1.0 and 2.0 mg/kg) increased it (Haleem et al 2018). As a nootropic drug, Buspirone can also improve the acquisition and retention of memory in the water maze test in rat model. However, higher doses (1.0 and 2.0 mg/kg) showed a

distinct effect (Haleem et al 2018). The dose-dependent event of Buspirone has an influence in the brain striatum. A low dose (1 mg/kg) Buspirone stimulates 5-HT1A receptors and decreases striatal metabolism compared to the higher dose (5 mg/kg). The finding also suggests that a low dose could help to release dopaminergic neurons that could be useful in reducing parkinsonian-like effects (Shireen & Haleem 2005).

In Parkinson's disease, a 1mg/kg b.w of dose did not impair the rotarod performance, while higher doses (2 and 4 mg/kg b.w.) significantly reduced the rotarod performance 25% and 20%, respectively (Dekundy et al 2007). Buspirone has an influential role in dopamine-dependent behavior in rats. A low dose of Buspirone can significantly increase the synthesis and availability of dopamine in the pre-synaptic terminal, whereas the higher dose can only block the postsynaptic dopaminergic receptors (Dhavalshankh et al 2007). These findings emphasize the importance of Buspirone doses in neurologic disorders.

The effect of different Buspirone doses has a robust effect on modulating the neural circuits following a cervical cord injury in rats. A dose of 1.5mg/kg b.w. for 9 weeks showed significant improvement compare to the week-1 post-injury. From week-7 to 8, the animals reaching and grip strength were not tested to ensure the spontaneous neuronal recovery might not have any influence. Moreover, **Group-A** rats at week-9 and the success rate was also found the same as week-6; even after withdrawal of the Buspirone, success rate and grip strength test were almost consistent with a slight decrease compared to the week-9, while in other groups dropped in a continuous manner. The distal forelimb muscles during reaching and grasping were increased in Group-A rats at week-11 while in **Group-C** animal the muscle response decreased after the withdrawal of the drug. The results suggest

that a low dosage of Buspirone can modulate the supraspinal network and has a robust effect in grasping function.

The main finding of the study described in this chapter is that the low dose of Buspirone can markedly improve the success rate of grasping food pellets and forelimb muscle synergies in rats. Furthermore, the improvement is consistent even after 2 weeks of withdrawal of the drug. However, only the Buspirone administration cannot facilitate recovery. To find out the maximum synergistic effect of US and drug-based combinatory neuromodulatory approach, the next experiment was conducted that described in the following chapter.

# Chapter 5: Ultrasound and drug-based combinatory neuromodulation for SCI recovery

## 5.1 Introduction

Non-invasive mechanical neuromodulation via LIPUS has shown positive effects on axonal regeneration and improvement of the rate of nerve autograft after peripheral nerve injury (Chang et al 2005, Crisci & Ferreira 2002). LIPUS treatment has also shown enhanced viability and proliferation of induced pluripotent stem cell-derived – neural crest stem cells in cultured condition (Lv et al 2013, Zhang et al 2009).

From recent studies, it is found that single modal therapy for SCI hardly results in any significant functional improvements (Olson 2013). Thus, to obtain better functional recovery, a combination of different treatment modalities are being increasingly applied in experimental studies with promising results (Alto et al 2009, Courtine & Sofroniew 2019, Hutson et al 2019). In orthopedic disorders, synergistic effects of LIPUS and stem cells can enhance bone growth in fracture condition by stimulating some growth factors (El-Mowafi & Mohsen 2005, Hiyama et al 2007, Nolte et al 2001). As described in chapter 3, LIPUS following cervical cord injury has shown promising results in cervical cord injured rats. The neuromodulatory effect of therapeutic ultrasound thus has a significant role in the spinal cord repair following an injury. However, the effect appeared not strong enough (see chapter 3) for the injured rat to recover the function significantly. Hence, the current study examines if a combined effect a serotonin agonist drug Buspirone along with LIPUS can significantly improve forelimb motor function in SCI rats. Buspirone has already shown significant improvement in the spinal cord recovery in SCI rats (Jin et al 2015). However,

the optimum dosage for forelimb functional recovery was unknown. In this thesis, I have determined an optimum dose of Buspirone for forelimb fine motor control recovery (see **Chapter 4**). It was found that a low dose of 1.5 mg/kg b.w. is more beneficial for the recovery of forelimb functions that the higher doses (2.5 and 3.5 mg/kg b.w). So, in the present study, I hypothesized that the LIPUS and Buspirone (1.5 mg/kg b.w.; i.p.) may provide a better recovery from the cervical cord injury in rats. This will help to explore efficacy of a novel combinatorial approach of ultrasound and drug treatment for SCI.

In this chapter, the effects of ultrasound stimulation and drug-based novel combined approach will be investigated to find the recovery after an incomplete cervical injury in rats.

### 5.2 Experimental design

Twenty-four adults healthy female Sprague-Dawley rats (230±40 grams b.w.) were used in this experiment. Adlibitum food and water were provided before starting the pre-injury forelimb reaching and grasping task training. Bodyweight was constantly monitored. The room temperature (24°C) and humidity (40%) maintained according to the guidelines. Animal handling was done for one week- before the forelimb reaching and grasping training as described in **Chapter 3** (**Fig. 35**).

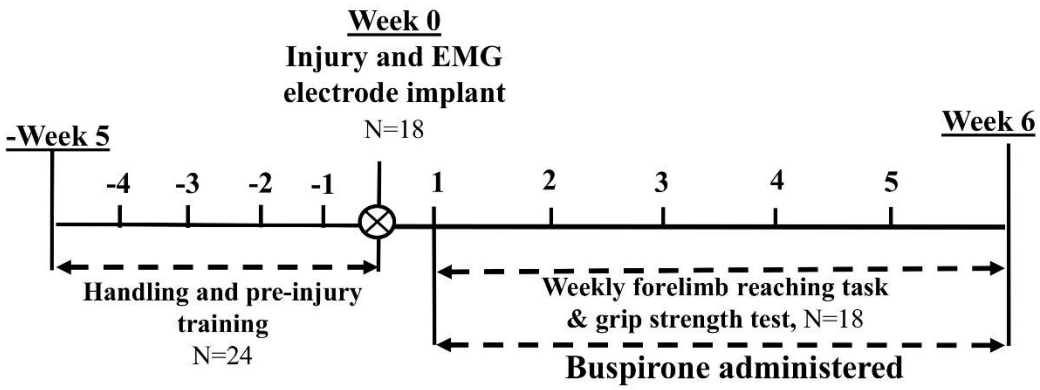



**Fig. 35**: **Experimental setup**. 24 rats were trained for six weeks for forelimb reaching and grasping. After familiarization, skilled learning and accustomed to the task 18 rats achieved average 65% success rate at week-5 of training and included for the SCI surgery and EMG electrodes implantation. After one week of recovering from the injury 18 rats have shown interest in the forelimb reaching and according to the success rate ranking, the rats were categorized into three balanced groups– ultrasound stimulation group (US, n=6), Combination treatment group (US + drug treatment, n=7) and Control group (n=5) for the evaluation of the combination effect of therapeutic ultrasound and the drug in injured cervical cord.

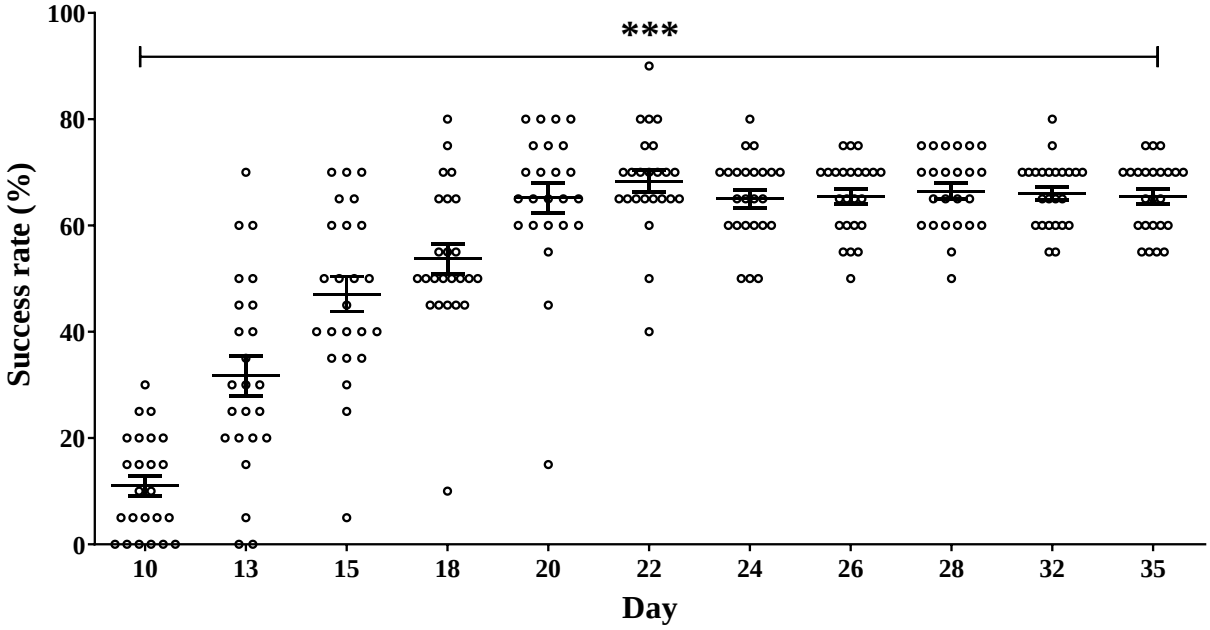


**Fig. 36**: **Reaching and grasping the learning curve of intact** rats (n=18). Significant differences (****P<0.001*, one-way ANOVA) were found at day-35 compared to the day-10 of reaching and grasping the success rate (Mean ± SEM).

During the training 20 pellets were provided to grasp pellets from the pit. Successful 5 weeks of training, the best eighteen rats were selected for the SCI experiment (**Fig. 36**). However, after the injury, three rats did not attempt the forelimb reaching and grasping tasks and excluded from the test. However, the grip force data were recorded from all the 18 rats; US group (n=6), Combined group (n=7) and Control group (n=5). The surgical procedure, preparation of the ultrasound probe and EMG electrodes implantation procedure are the same as described in **Chapter 3**.

### 5.3 US stimulation and drug treatment

After 5 minutes of therapeutic stimulation, the behavior task was conducted by using the parameters and intensities mentioned in chapter 3. Ultrasound stimulation was delivered for 10 minutes during the reaching and grasping behavior task. A dose of 1.5 mg/kg b.w of Buspirone was administered (i.p.) in the combination group rats daily for 6 weeks.

### 5.4 Results

#### *5.4.1 Improvement of forelimb fine motor function*

After recovering from the injury, food restrictions were provided to the rats to perform the reaching and grasping task. Buspirone was administered at a dose of 1.5mg/kg b.w. every day for 6 weeks.

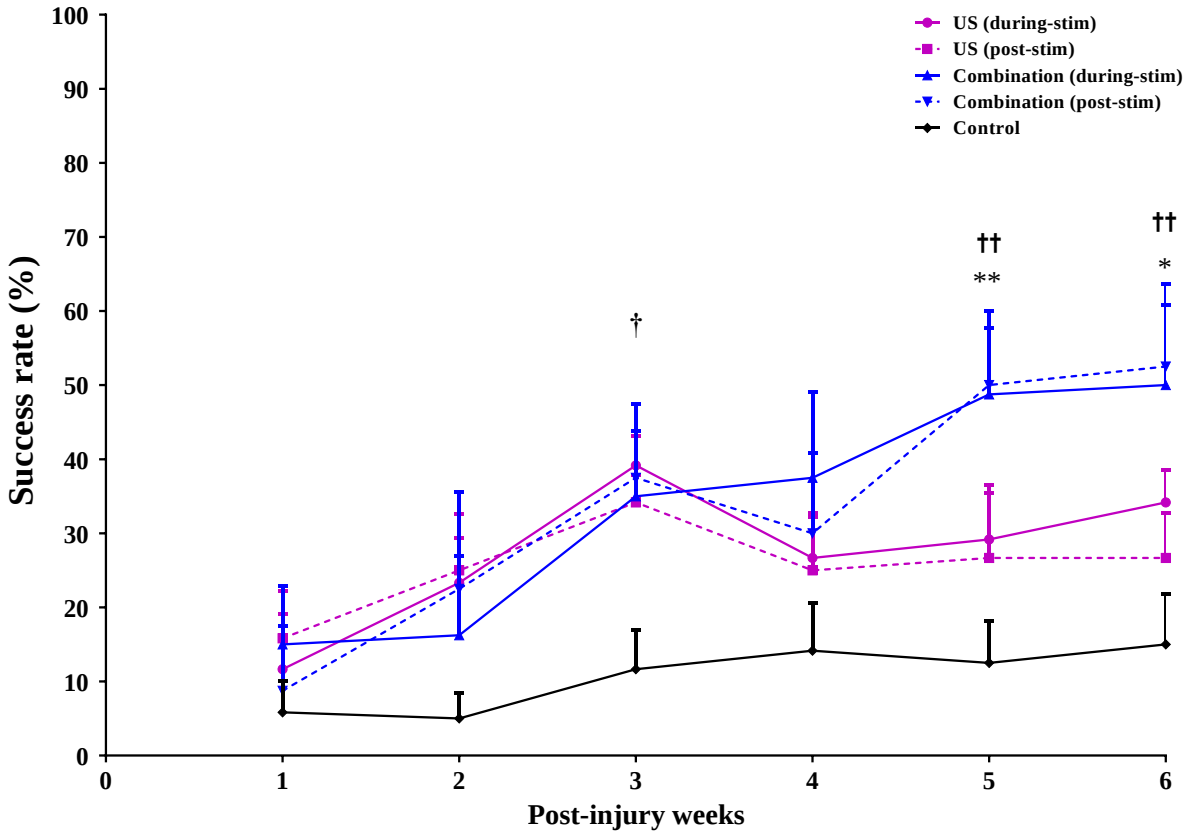



**Fig. 37**: **Average success rates (mean ±SEM; US, n=6, combined, n=4 and control, n=5) of reaching and grasping tasks of three groups of rat.** The solid pink line indicates the success rates of the ultrasound group rats when receiving the ultrasound stimulation and dot line is the immediately after stimulation success rates. Solid blue line indicates the success rates of the combination group rats when receiving the ultrasound stimulation and dot line is the immediately after stimulation success rate. The black line indicating the success rate of the control group of rats that did not receive any stimulation and drug treatment. At week- 3 during ultrasound stimulation, significant († $p < 0.05$, two-way ANOVA, Tukey post-hoc test) improvement of reaching score was found in US group compared to the control group. During stimulation the success rate of combined group was found significantly higher compared to the control group at week-5 and 6 (**$p < 0.01$ and *$p < 0.05$ two-way ANOVA, Tukey post-hoc test). However, at week-5 and 6, the post-stimulation success rate was found significant (††$P<0.01$, two-way ANOVA, Tukey post-hoc test) compare to the control group rats.

The success rate in US stimulation group and combined group were found higher compared to the control group reaching and grasping success from week 1 to 6 post-injury treatment. A significant improvement was found at week-3 during ultrasound stimulation (39.167 ± 3.96 vs 11.667 ± 5.27; p =0.0079) (**Fig. 37**). However, after week-3 the success rate dropped in US stimulation group and did not show any significant improvement until week-6. Conversely, the combined group rats improved significant forelimb functional recovery in a consistent manner compared to the US stimulation groups, at week-5 and 6 (48.75 ± 8.98 vs 12.50 ± 5.73; p =0.0097 and 50.00 ± 10.80 vs 15.00 ± 6.83; p =0.0139) during stimulation compared to the control group. Post–stimulation, the improvement was also found significantly higher at week -5 and 6 compared to the control group (50.00 ± 10.00 vs 12.50 ± 5.73; p =0.0067 and 52.50 ± 11.08 vs 15.00 ± 6.83; p =0.0067).

### *5.4.2 EMG activity*

At week-1 and 5 of post-injury EMG signals were recorded from three representative rats. The raw figures are presented in **Fig. 38 and 39**.

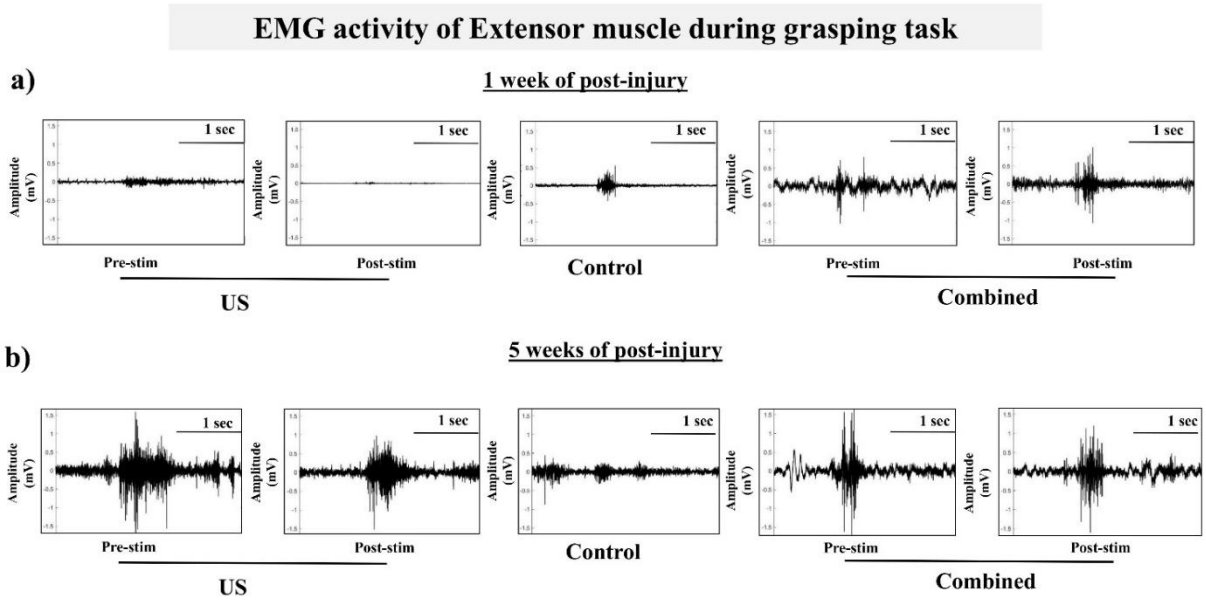


**Fig. 38**: **Raw EMG signals from extensor muscle at a) 1-week of post-injury and b) 5-weeks of post-injury from three rats presented in the figure.**

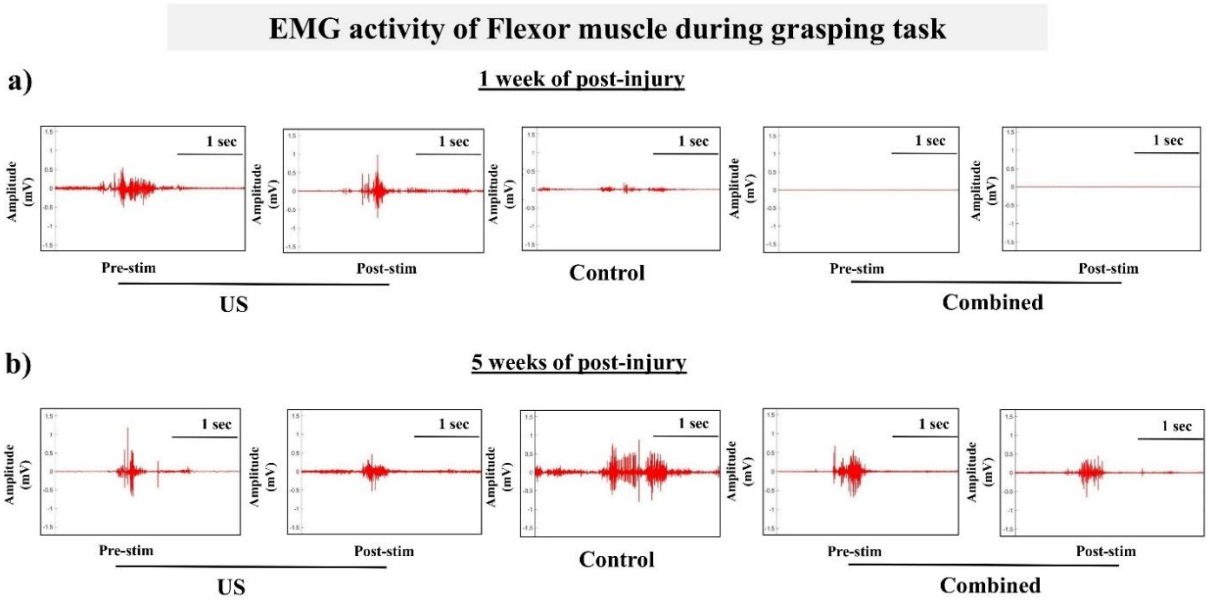
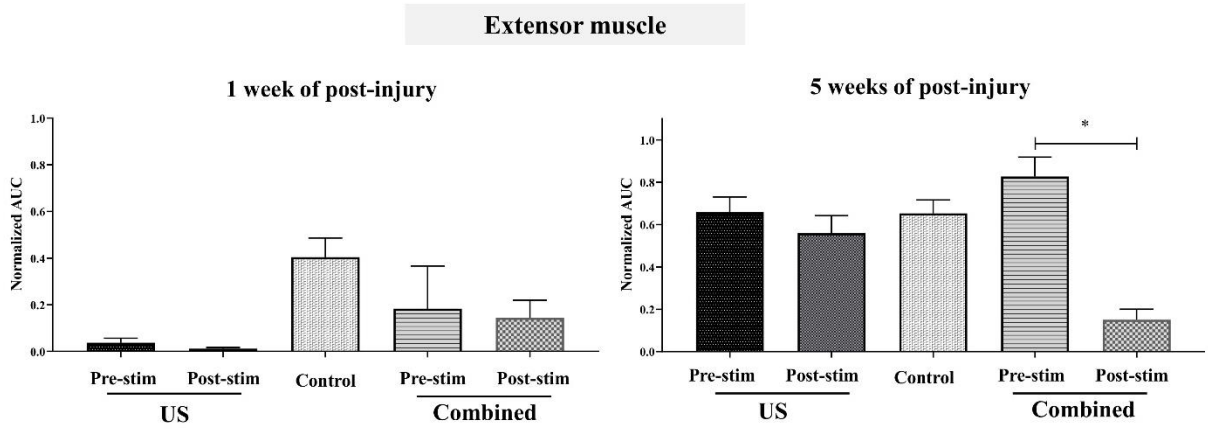



**Fig. 39**: **Raw EMG signals from flexor muscle at a) 1-week of post-injury and b) 5-weeks of post-injury from three rats are presented.**

**a**)



**b)**

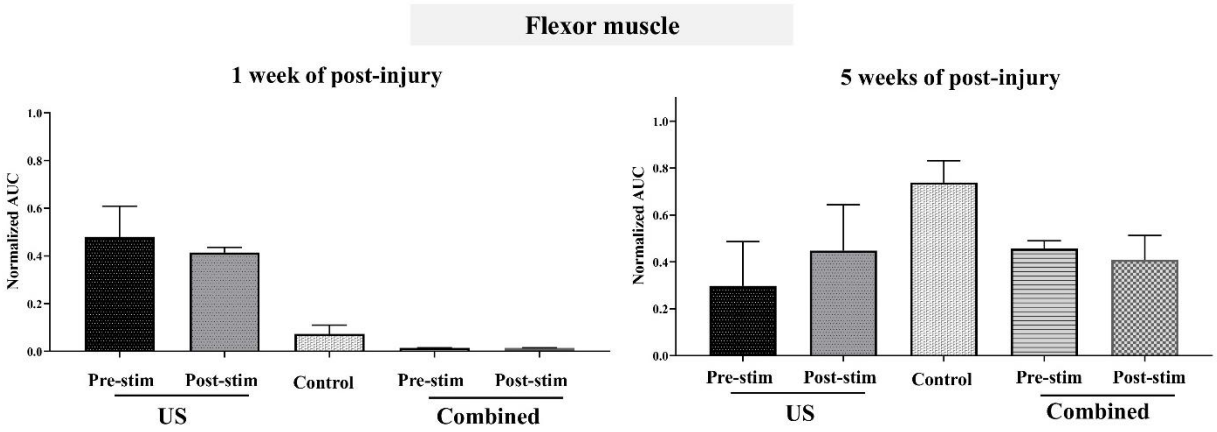



**Fig. 40**: **Normalized area-under-the-curve (AUC) of EMG signals** from **a)** extensor digitorum and **b)** flexor digitorum muscles of three group of rats during the single pellet reaching task (n=10 trials/rat). A significant difference was found at 5 weeks of post-injury in combined group of extensor muscle (*$p < 0.05$ two-way ANOVA, Tukey post-hoc test). Data are presented as mean and SEM.

To calculate the normalized AUC of EMG signals of extensor and flexor digitorum muscle the values were normalized as described in **Chapter 3**. From US and combined group, the normalized values were compared to the pre- and post-stim with the control group at 1 and 5 weeks of post-injury (**Fig. 40**). At 1 week of post-injury, the normalized value of extensor muscle decreased after the simulation in combined and US groups. Moreover, a significant decrease of muscle activity was found at 5-weeks of post-injury in the extensor muscle compared to the pre-stimulation (0.82 ± 0.09 vs 0.15 ± 0.05; p= 0.0025) (**Fig. 40a**). A similar response was found in the flexor muscle at week-1 in the extensor muscle. However, 5 weeks of post-injury the flexor muscle values were increased in the US group and decreased in combined group rats after stimulation.

### *5.4.3 Maximum grip force*

After each week of reaching and grasping task maximum grip force was determined as described in **Chapter-3**. The maximum grip force of US group rats was found 8.75 N (week-1) to 14.56 N (week-6). For combined group rats, the value was from 8.72 N (week-1) to 13.58 N (week-6). In the control group rats a similar increasing trend, from 7.63 N (week-1) to 13.79 N (week-6) (**Fig. 41**). This reconfirms what was found in **Chapter 3**, that the forelimb grip strength may not be dependent on the treatment modalities.

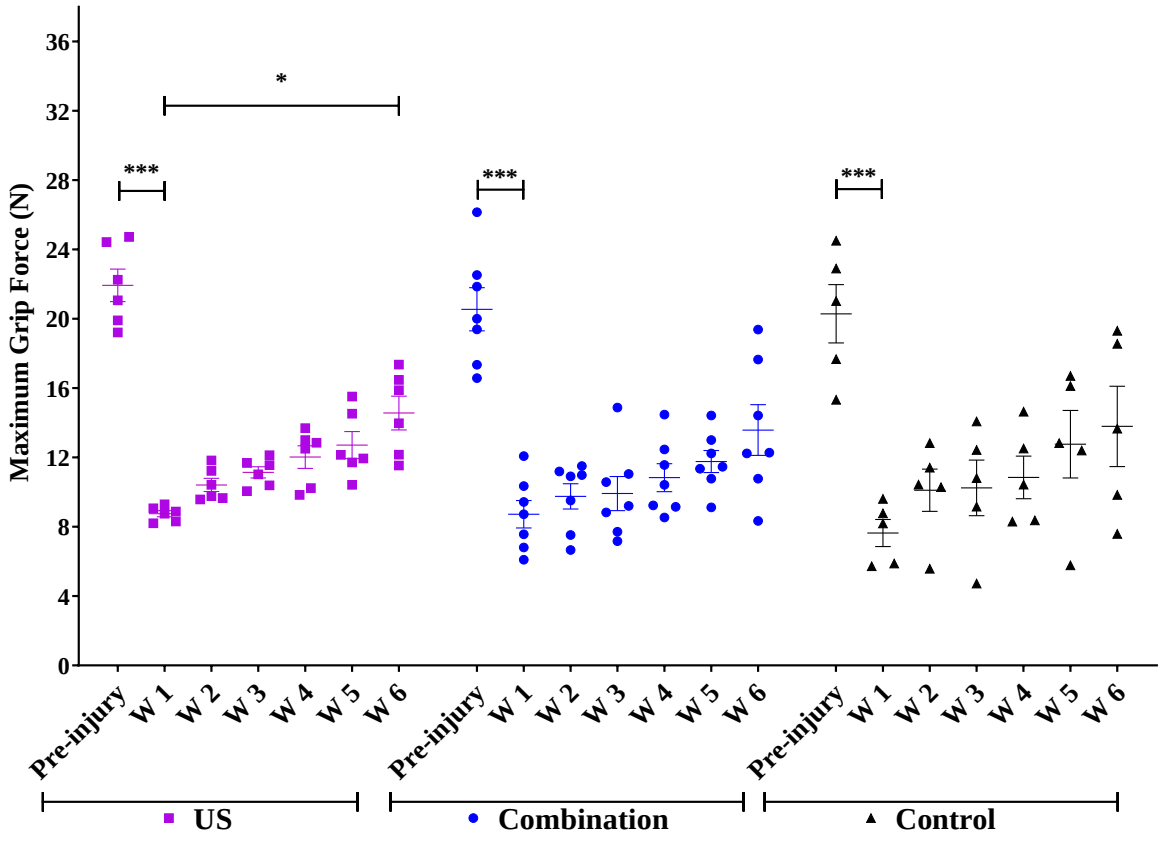



**Fig. 41**: **Maximum grip force of three different groups (mean ±SEM; US, n=6, combined, n=7 and control, n=5).** At week-1 of injury, the grip force dropped significantly compared to the pre-injured condition in three group of rats (US group, 21.93 ± 0.93 vs 8.75 ± 0.17; Combined group, 20.55 ± 1.24 vs 8.22 ± 0.79 and control group

20.28 ± 1.68 vs 7.63 ± 0.78; ***$p < 0.001$, two-way ANOVA, Tukey post-hoc test). At week-6 US group rats improved the grip force (*$p < 0.05$ two-way ANOVA, Tukey post-hoc test) compared to their week-1 of injury.

### 5.5 Discussion

In this chapter, a synergistic effect of LIPUS and serotonergic drug has shown a significant role in modulating neural networks after cervical cord injury and improving functions. The maximum success rate of US group rats was found at week-3, however, afterward, the success rates decreased. The findings of forelimb reaching and grasping task of US group stimulation support the result of **Chapter 3** of the US stimulation rats. Similar to other single treatment approaches, LIPUS stimulation as a single approach could not facilitate significant functional recovery in chronic condition of SCI, in this case after 3 weeks post-injury. Several reasons can be hypothesized. One reason could be the formation of scar in the injured region which may prevent the LIPUS effect. After an incomplete spinal cord injury in rats the astrocytic scar has formed and become mature after 2-3 weeks of injury (Kjell & Olson 2016). In chronic condition, the mature scar later developed to fibrotic scar after infiltration of fibroblasts. It can be assumed from the experiment that after 3 weeks of injury the intensity could not facilitate the recovery because of the chronic scar. Moreover, in a previous study it has already shown that after three weeks, ultrasound driven piezoelectric voltage dropped because of the acoustic impedance of the growing scar around the ultrasound probe (Li et al 2020). The same events may have happened in the US stimulation rats. In another aspect, during and post-ultrasound stimulation no big differences were found throughout the period. To get the most from the neuromodulation, even for the ultrasound stimulation was given for 5 min before the reaching task. It is

expected that the time is enough to neuromodulate the cervical cord and improve forelimb function. Similar to other neuromodulation strategies, the stimulation effect may last for several minutes post stimulation. To answer this critical question, after turning off the ultrasound stimulation reaching score was measured immediately. This scoring lasted upto about 5 min post-stimulation. The non-significant difference between the scores during and post-stimulation suggest the stimulation was neuromodulatory. The combination group rats shown a consistent recovery in the forelimb reaching and grasping task similar to the Buspirone treated rats mentioned in the **Chapter 4**. So, the improvement following 4 weeks post-injury maybe mainly due to the drug.

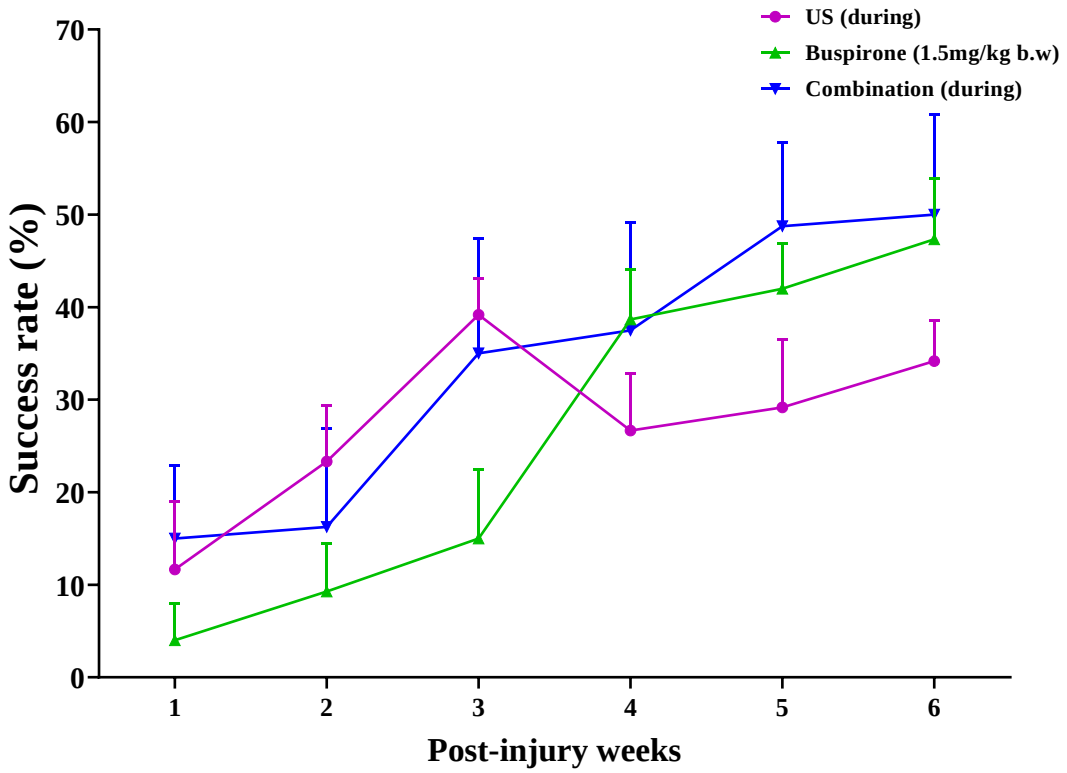



**Fig. 42**: **Comparison of ultrasound group (n=6, during stimulation), Buspirone group (n=5, 1.5 mg/kg b.w.) and Combination group (n=4, during stimulation) rats forelimb reaching and grasping success rates (data presented as mean ± SEM value**).

The comparison of three different groups of rats forelimb reaching and grasping tasks success rates are presented in **Fig. 42**. In combination group rats the forelimb reaching and grasping task success rates at week-6 is similar to the finding of low dose of Buspirone treated rats. However, in combined group of rats week-1 to 3 the success rates is much higher compare to the low dose of Buspirone treated rats of **Chapter 4**. But, from week-4 the success rate is almost similar to the low doses of Buspirone administered rats. A little improvement of success rate was found at week-6 in combined group of rats compared to the low dose of Buspirone treated rats**.** This comparison suggests that the LIPUS and Buspirone has a synergistic effect on the forelimb reaching and grasping task recovery in injured rats.

## Chapter 6: Conclusions and future directions

### *Conclusions*

A majority of human SCI involved dysfunction of the upper extremity. To restore upper extremity functions in SCI tetraplegics, several strategies have been investigated over the years with little or no improvements for severely injured patients. In this aspect, experimental animal model of rat plays a major role to translate the basic research to human. In the recent year's ultrasound neuromodulation has emerged as new prospect in neural rehabilitation research. Spinal cord neuromodulation via LIPUS is still unexplored avenue in SCI recovery research. Furthermore, combination therapy of non-invasive neurostimulation by LIPUS and serotonergic against neuromodulation is entirely a unique approach. The findings of this thesis includes possible functional recovery by ultrasound spinal cord stimulation and a definite recovery of forelimb functions when the ultrasound stimulation is combined with the serotonin drug-based neuromodulation in incomplete cervical cord injured rats. Based on these results, it can be stated that this new and innovative approach will open a new era in spinal cord injury repair research and translation.

### *Limitations and future directions*

Finding recoveries after the loss of upper extremity functions due to a cervical injury in the recent years drew more attention because of high clinical significance. Following skill training, rats used their forelimbs for feeding after a successful grasping of food palate. The reaching and grasping behavior in rats is quite similar to humans. This allows the behavioral task to be a powerful tool to translate in clinical conditions (Klein et al 2012). Although, the finesse digit control is less developed in rodents than primates (Courtine et al

2007). The use of non-human primates could be more useful to investigate the behavioral and electrophysiological changes. Histological evaluation was not included in the present study. However, histological data cannot give the functional information of neural activity and muscle synergies (Lavrov et al 2006). To address this, longitudinal electrophysiological measurements were conducted in the present thesis study to find out the extensor and flexor muscle activities during the behavioral task.

Therapeutic ultrasound treatment as a neuromodulation is a promising tool in the several neurological disorders (Leinenga et al 2016). Very few studies had been conducted of ultrasound stimulation in rat injury model (Blackmore et al 2019). Most previous studies of ultrasound stimulation were related to the brain and peripheral nerve stimulation (Kim et al 2015, Mehić et al 2014, Ni et al 2017, Tych et al 2013, Younan et al 2013). However, the responses of ultrasound stimulation are not known in the rat spinal cord. For the first time, in this thesis, I have explored the effects of therapeutic ultrasound stimulation in rat spinal cord after an experimental injury. In combined group, only four rats showed interest in reaching and grasping task. Thus the samples were not large enough to show the significance for all the post-injury weeks. With a larger sample size, the result will be more conclusive. Another limitation of this study is the potential variability of the custom made ultrasound probe. Although the intensity of the implanted ultrasound probe of all the rats were in the range of 60-70mW/cm$^2$, it is difficult to ensure if the intensity values remained the same for all implanted probes throughout the implant period. With a more reliable rigorously tested ultrasound probe can be used, the results will be more accurate. In previous study in cat model it was shown that ultrasound has a stimulation effect on spinal reflex (Shealy & Henneman 1962). In the present study, we did not investigate the

modulation effect of therapeutic ultrasound in the spinal reflex. Hence, more studies are needed to investigate the ultrasound effect on spinal circuits.

It was also discovered that ultrasound with serotonergic agonist drug has more therapeutic effects than sole approach of ultrasound stimulation. However, the mechanism of this recovery is still known and beyond the scope of this thesis. In the present thesis, the effect of therapeutic ultrasound stimulation was explored on cervical injured rats reflected in behavioral and electrophysiological studies. However, a histological examination whether the therapy induces a permanent change to the injured nervous system or not was not evaluated. Alteration of descending tract information could give the information of the recovery mechanism.

The thermal effect of ultrasound was not evaluated in this study. Previously, in brain stimulation, it was shown that a short period of LIPUS stimulation can produce mechanical bio-effects through the skull by producing the thermal effect (Legon et al 2014). It has been assumed that thermal effect of ultrasound has a significant role could modulate the neural network. More studies are needed to understand the thermal mechanisms of ultrasound neuromodulation in the cellular and molecular changes of neurons and neural circuits.

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