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The role of minimal nerve injury in a new animal model for CRPS

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ABBREVIATION

ANOVA	analysis of variance
aU	arbitrary unit
AWMF	Association of the Scientific Medical Societies in Germany
CCI	chronic nerve constriction injury
CGRP	calcitonin gene related peptide
CPIP	chronic post-ischemia pain
CRPS	complex regional pain syndrome
DNI	distal nerve injury
DRG	dorsal root ganglion
ETR	exaggerated tissue trauma
FCD	functional capillary density
Fig	figure
FITC	fluoresceinisothiocyanate
GABA	gamma-aminobutyric acid
GM-GSF	granulocyte macrophage-colony stimulating factor
IASP	International Association for the Study of Pain
IL	interleukine
i.p	intraperitoneal
I-R	ischemia-reperfusion
mA	milliampere
min	minute
ms	millisecond
N	Newton
NADH	nicotinamide adenine dinucleotide hydride
NGF	nerve growth factor
NK	neurokinin
RSD	reflex sympathetic dystrophy
SEM	standard error mean
SIP	sympathetic independent pain
SMP	sympathetic maintained pain
SNS	sympathetic nervous system
SP	substance P
Tab	table
TNFR	tumor necrosis factor receptor
TNF-α	tumor necrosis factor-alpha
UV	ultraviolet

1 Abstract

Complex regional pain syndrome (CRPS) is a chronic pain disease characterized by autonomic changes and usually develops in an extremity after tissue trauma. CRPS 1 is traditionally defined by the absence of a specific nerve injury. However, recently, a minimal nerve injury has been proposed to play a pathophysiologic important role in CRPS 1. We combined a closed soft tissue trauma and a minimal nerve injury to establish a new animal model in rats, in order to study the effects of tissue trauma and minimal nerve injury on the emergence of CRPS-like symptoms.

The closed soft tissue trauma was induced in the hindlimbs of Sprague Dawley rats with a pneumatic impactor. Additionally, the ipsilateral sciatic nerves were crushed with an instrumented clamp to cause a minimal nerve injury (30 Newton, 20s). The experiment was performed on a total of 42 rats. The rats which received closed soft tissue trauma and minimal nerve injury served as the clamp group (N=21). The other set of animals with closed soft tissue trauma and sham operation of the nerve served as the sham group (N=21). Pain behavior observations and assessment of autonomic changes (paw temperature and paw volume) were performed on day 0, 2, 4, 7, 14, 21 and 28. In vivo fluorescence microscopy was performed in the traumatized muscle on day 2, 7 and 28 in order to assess the regional inflammation and tissue perfusion. Nerve conduction was applied in the operated sciatic nerve to assess its conductive function. Additionally, muscle cell apoptosis in the traumatized muscle was assessed by both in vivo fluorescence microscopy and caspase-3 immunohistochemistry.

As a result, shortly after the surgery the clamp group with closed soft tissue trauma and minimal nerve injury developed pronounced signs of neuropathic pain (spontaneous pain, hot and cold allodynia and mechanical hyperalgesia), while the sham group remained pain free. Besides the neuropathic pain, increased temperature, edema formation, regional inflammation, tissue perfusion failure, tissue hypoxia and enhanced muscle cell apoptosis appeared in both the clamp and the sham group. However, most symptoms of neuropathic pain in the clamp group and muscle cell apoptosis in both groups vanished within the 28 days of observation. The autonomic changes, regional inflammation and microcirculatory deterioration disappeared in both groups at 7 day.

CRPS-like symptoms were detected in our animal model. The minimal nerve injury led to a pronounced neuropathic pain. The closed soft tissue trauma induced posttraumatic sequelae, such as regional inflammation, circulatory deterioration and muscle cell apoptosis. However, most of the symptoms were transient and completely disappeared within 28 days. Thus, the closed soft tissue trauma and minimal nerve injury are not sufficient to induce an ongoing CRPS.

2 Zusammenfassung

Das Komplexe Regionale Schmerzsyndrom (CRPS) ist eine Erkrankung, die durch Schmerz, sensible, motorische und autonome Veränderungen charakterisiert ist und typischerweise nach einem Extremitätentrauma auftritt. Hierbei wird in CRPS 1 ohne nachweisbaren und CRPS 2 mit definiertem - Nervenschaden unterschieden. In letzter Zeit wurde jedoch auch für das CRPS 1 eine pathophysiologisch bedeutsame minimale Nervenverletzung postuliert. Wir kombinierten ein geschlossenes Weichteiltrauma mit einer minimalen Nervenverletzung bei der Ratte um an diesem neuen Tiermodell das Auftreten CRPS-typischer Symptome zu untersuchen.

Das geschlossene Weichteiltrauma wurde standardisiert mittels Druckbolzen auf den Hinterlauf von Sprague Dawley Ratten appliziert. Zusätzlich erfolgte eine Nervenquetschung am ipsilateralen N. ischiadicus (30 Newton für 20s). Tiere, die eine Weichteil- und Nervenverletzung erhielten, wurden als “Clamp Gruppe” bezeichnet (n=21). Als Kontrolle dienten 21 weitere Tiere, die das Weichteiltrauma erhielten, bei denen der N. ischiadicus lediglich dargestellt und nicht weiter traumatisert wurde. Das Schmerzverhalten, sowie autonome Veränderungen (Temperaturunterschiede und Ödem der Pfoten) wurden an Tag 0, 2, 4, 7, 14, 21 und 28 gemessen. Um die lokale Entzündungsreaktion und Gewebeperfusion zu beurteilen wurde an Tag 2, 7 und 28 eine *in vivo* Fluoreszenzmikroskopie des ipsilateralen M. soleus durchgeführt. Die Muskelzellapoptose wurde sowohl *in vivo* fluoreszenzmikroskopisch , als auch immunhistochemisch bestimmt. Zur Darstellung der neuronalen Konduktivität führten wir eine Messung der Nervenleitlatenz des N. ischiadicus durch.

Bereits kurz nach der Traumatisierung zeigten alle Tiere der “Clamp Gruppe” ein erhebliches neuropathisches Schmerzverhalten (Spontanschmerz, Kälte- und Wärmeallodynie und mechanische Hyperalgesie), während die Tiere der Kontrollgruppe keinerlei Schmerzverhalten demonstrierten. Neben dem neuropathischen Schmerz stellten sich Veränderungen in Pfotentemperatur, Muskelödem, regionaler Entzündung im verletzten Muskel, Muskelzellapoptose und Gewebeperfusion und -oxygenierung in beiden Versuchsgruppen dar. Die Symptome des neuropathischen Schmerzes waren über den Beobachtungszeitraum rückläufig und erreichten zum Ende der Untersuchung das Ausgangsniveau. Die autonomen

Veränderungen und Parameter der lokalen Entzündung und Mikrozirkulation lagen bereits ab dem 7. Tag wieder im Normbereich.

In diesem neuen Tiermodell konnten wir Symptome des CRPS induzieren. Die minimale Nervenverletzung führte zu einer deutlichen neuropathischen Schmerzsymptomatik. Die zusätzlich durchgeführte Weichteiltraumatisierung erzeugte typische posttraumatische Folgeerscheinungen wie regionale Entzündung, Störungen der Mikrozirkulation, und Muskelzellapoptose. Allerdings waren die meisten Symptome nur vorübergehend und zum Ende der Untersuchung nicht mehr nachweisbar. Die Kombination von Weichteiltrauma und minimaler Nervenverletzung waren In diesem Tiermodell nicht geeignet, ein anhaltendes CRPS zu erzeugen.

3 Introduction

3.1 Background

In the American civil war, an uncommon pain disease was reported by Dr. Silas Weir Mitchell. He found that some soldiers with gunshot wounds developed a severe painful condition which could not be explained by the initial trauma. The initial gunshot wound in the limb led to an intense and persisting pain in the remote extremity. The pain was described as burning and the affected patients often refused to be examined. Besides the pain, glossy red skin was also reported in some of the cases. This pain condition was first considered to be due to nerve trunk irritation. Mitchell tried several treatments which included leeches, electrostimulation, massage and blistering. However, none of these methods led to satisfying results. Mitchell used the term "causalgia" (combination of Greek word "Kausis" and "Algos" which mean "hot" and "pain") to describe the commonly observed burning pain [1, 2].

In 1900, the posttraumatic pain associated with edema and bone atrophy was independently described by Sudeck [3]. He proposed an exaggerated inflammatory response to trauma or operation being the major cause and coined the term "inflammatory bone atrophy" (Sudeck atrophy) [4].

In the early 20th century, the French surgeon Leriche made detailed reports about causalgia in traumatized limbs. He suggested sympathectomy as a therapy, but sympathectomy was still not able to resolve the pain condition [5]. In 1946, based on the observations of Leriche, Evans proposed a theory of sympathetic dysfunction. He believed that the development of exaggerated pain was due to the hyperactivity of the sympathetic nervous system. In his theory, continuous nociceptive input generated by a trauma affects the spinal cord and enhances the output signal of the sympathetic nervous system and further leads to symptoms in the affected extremity. The term "Reflex Sympathetic Dystrophy (RSD)" was put into use in order to reflect the assumed sympathetic pathophysiology [6].

Later on, many terms based on the clinical features, evidence of nerve lesion, trophic changes, responses to treatment and different theories emerged due to poor understanding of pathogenic reasons. This diversity of terms also led to confusion, misdiagnosis and mistreatment of this pain disease [3]. In this situation, a more accurate and widely accepted nomenclature was in sore need. In 1993, the International Association for the Study of Pain (IASP) reviewed the nomenclature and

developed a set of diagnostic criteria. In 1994, by the common consent of participating scholars, the term Complex Regional Pain Syndrome (CRPS) was announced [7].

3.2 Definition

Complex Regional Pain Syndrome (CRPS) is a chronic pain disease distinguished by neuropathic pain, sensory disturbance, temperature alteration, edema, sudomotor and trophic changes.

Based on the presence of nerve injury, CRPS can be subdivided into two types, CRPS 1 and CRPS 2.

CRPS 1 develops after trauma without specific nerve injury and was formerly known as reflex sympathetic dystrophy (RSD) or Sudeck atrophy.

CRPS 2 develops after trauma combined with verified nerve injury and was formerly termed causalgia.

3.3 Epidemiology and prognosis

CRPS usually develops after acute tissue trauma or operation. A study in the United States showed an incidence of 5.5/100,000 person per year [8]. Another higher incidence (26.2/100,000) was reported in the Netherlands. CRPS develops more often in the upper limbs. More females than males are affected, with a ratio of approximal 3:1. Advanced age is another risk for CRPS [9]. Fracture is the most common initial event, followed by surgery, crush injury, sprain and even minimal injury [10]. Distal radius fracture is the most common cause of CRPS 1. The incidence has been described to be between 8% to 39% [11-13].

In a prognostic study of CRPS, 6 years after the onset, 30% of the patients had recovered and 54% were considered as stable. However, 16% of the patients reported severe progressive symptoms. Of the 54% patients with a stable condition, 41% could resume their former job completely, 28% could resume work with an adjusted occupation or reduced working hours. The other 31% were not able to work anymore [14]. The outcome of CRPS seems to be grim, less than 1/3 of the patients recovered, a large proportion of the rest lost their work ability, some patients still need long-term treatment (pharmacologic, invasive treatment, or physiotherapy) [14]. In 1998, the average cost for the health-care (excluding physiotherapy) of a CRPS patient was € 5700/year in the Netherlands [15]. The resulting high unemployment and expensive treatment put a heavy socioeconomic burden to both patients and the state.

3.4 Diagnosis and clinical presentation of CRPS

Early recognition and prompt treatment can improve the outcome of CRPS [16]. However, the early signs of CRPS are often unspecific. Pain, numbness, swelling, and stiffness are also normal symptoms seen after trauma or operation, which leads to misinterpretation and misdiagnosis [12]. The lack of distinct early signs and effective diagnostic tools usually contributes to a delayed diagnosis. The diagnosis of CRPS is based primarily upon the anamnesis and symptoms elicited during physical examination. The IASP diagnostic criteria were published in 1994. These diagnostic criteria have a high sensitivity (0.99) but unsatisfying specificity (0.41), leading to over-diagnosis [3]. The Budapest diagnostic criteria (2003) (**Tab. 1**) were published with an improved specificity (0.68). A subset of Budapest criteria were developed with a higher specificity (0.79) for research purpose [17].

Tab. 1. Budapest clinical diagnostic criteria for CRPS [17]

1. Continuing pain, which is disproportionate to any inciting event.
2. Must report at least one symptom in three of the four following categories: • Sensory: hyperalgesia and/or allodynia • Vasomotor: temperature asymmetry and/or skin color changes and/or skin color asymmetry • Sudomotor/edema: edema and/or sweating changes and/or sweating asymmetry • Motor/trophic: decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nail, skin)
3. Must display at least one sign at time of evaluation in two or more of the following categories: • Sensory: hyperalgesia (to pinprick) and/or allodynia (to light touch and/or deep somatic pressure and/or joint movement) • Vasomotor: temperature asymmetry and/or skin color changes and/or asymmetry • Sudomotor/edema: edema and/or sweating changes and/or sweating asymmetry • Motor/trophic: decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nail, skin)
4. There is no other diagnosis that better explains the signs and symptoms.

3.4.1 Pain

Continuing pain, which is out of proportion to the initial trauma, is the main feature of CRPS. The pain usually manifests near the site of injury, it can spread to the whole limb and even to the opposite side [18]. The spontaneous pain is usually described as burning or shooting pain and can be heightened by emotional or physical stress. The intensity and duration of pain can vary from case to case and the symptoms can get worse over time [14].

3.4.2 Sensory disturbance

Sensory disturbance, especially the exaggerated evoked pain is another key feature of CRPS. Exaggerated evoked pain includes allodynia and hyperalgesia [19]. The term allodynia refers to pain experienced upon innocuous stimulation, like a slight touch or even the air flow in the affected region. Hyperalgesia describes a hypersensitivity to noxious stimuli that are slightly painful under normal conditions.

3.4.3 Vasomotor dysfuntion

Vasomotor dysfunction includes altered skin temperature and/or color. In most cases of CRPS, the affected extremities are abnormally warm, usually 1˚C warmer [20]. The warm skin can become unusually cold over time [21]. However, about 30% patients experience cold CRPS from the beginning with decreased temperature in their affected limbs [22].

3.4.4 Sudomotor dysfuntion and edema

Patients with CRPS often show altered sweating, especially in CRPS 1. Hyperhidrosis is more common than hypohidrosis [23]. Edema is quite common in CRPS affected limb, it is usually observed in the early stage and diminishes over time [24].

3.4.5 Motor and trophic changes

Motor and trophic changes are frequently observed in CRPS. Most of the patients report motor function disturbances, which include motor weakness, tremor and dystonia [20, 25]. Abnormal hair and nail growth can happen in the affected area. In the chronic stage, atrophy of muscle and skin as well as contractures can appear [24]. Pain, edema formation and consequent contractures can severely limit the range of motion of CRPS affected limbs.

3.5. Treatment and therapy

Due to the puzzling nature of the disease and lack of pathophysiologic understanding, the therapy for CRPS is an unresolved issue. The current treatment for CRPS is mainly aiming on symptom control and rehabilitation therapy.

3.5.1 Physical therapy

Physical therapy is the most frequently applied method. Common methods include transcutaneous electrical nerve stimulation, progressive weight bearing, tactile desensitization, massage and contrast bath therapy [26]. Physical therapy is proven to reduce pain, improve the motor function and abate trophic changes such as contracture and vascular compromise in the late stage [26]. Physical therapy should be applied below the pain threshold, since the symptoms can be exaggerated by a painful stimulation [27, 28].

3.5.2 Pharmacotherapy

Current pharmacotherapy for CRPS is mostly symptomatic treatment. According to the therapeutic guideline of Association of the Scientific Medical Societies in Germany (AWMF), analgesics, bisphosphonates, calcitonin, free radical scavengers and corticosteroids can be considered for treating CRPS. Opioids are usually applied in the acute stage of intense pain, but they are thought to be less effective for treating chronic pain [29]. Sodium channel blockers were found to reduce spontaneous pain and specific characteristics of evoked pain [30]. The administration of antidepressants can alleviate neuropathic pain and manage the comorbidities of CRPS, such as depression and sleep disorder [31]. Gabapentin, which is an anticonvulsant, is also proven to have analgesic effects in neuropathic pain and can lead to pain relief in some CRPS cases [32, 33]. However, in a double blind study, the pain score of the gabapentin treated group showed no difference compared to the control group [34]. Besides the pain management, the inflammation-like symptoms such as redness, swelling and heat are treated with NSAIDs and corticosteroids. Since neurogenic inflammation is a possible mechanism of CRPS, the administration of anti-inflammatory drugs can be promising, though more consistent studies are needed to support its effectiveness [35].

3.5.3 Sympathetic blocks

Sympathetic blocks can be applied secondarily in some patients with CRPS. The patients with sympathetic maintained pain (SMP) are likely to have relief after the procedure. However, this method can not help the patients with sympathetic

independent pain (SIP). Sympathetic blocks should be applied together with physical therapy [36].

3.5.4 Spinal cord stimulation

Spinal cord stimulation is an invasive therapeutic method for CRPS. A stimulator can be implanted in the epidural space or directly over the nerves located outside the central nervous system. The signal generated by the device can stimulate the spinal cord directly to control the pain. Besides the stimulator implantation, a drug pump can also be implanted to continuously deliver analgesics centrally instead of oral administration. Though the spinal cord stimulation is effective, noninvasive methods are preferred [37].

3.6 Pathophysiology

The pathophysiology of CRPS is still largely unknown. Numerous studies addressing the pathophysiology of CRPS have been carried out during the past decades. It has become commonly accepted that multiple mechanisms are involved.

3.6.1 Peripheral sensitization

A peripheral sensitization can be triggered by the initial trauma of CRPS. After being traumatized, the primary afferent nociceptors and Schwann cells of injured nerves secrete neuropeptides such as substance P (SP), calcitonin gene related peptide (CGRP) and several inflammatory factors (TNF-α, IL-1β and IL-6) [38]. Those factors activate their receptors expressed on the primary nociceptors and lead to increased excitability and decreased firing threshold, which can make an innocuous stimulation painful and exaggerate a noxious stimulation [39]. At the same time, the continuous nociceptive input generated by the peripheral nociceptors may affect the sensory neurons in the spinal cord and contribute to a central sensitization.

3.6.2 Central sensitization

Central sensitization is an increased excitability of nociceptive neurons in the spinal cord or higher nervous centre. Central sensitization is thought to be triggered by repeated nociceptive afference [40]. The sensory inflow generated by the initial trauma is carried into the spinal cord by primary afferent fibers. This nociceptive input leads to a central expression of neuropeptides (SP, bradykinin and CGRP) and excitatory amino acid glutamate in the spinal cord [41]. Those factors act on the relative receptors in the nociceptive neurons of the dorsal horn and increase their excitability [42]. When neurons in the dorsal horn are sensitized, they increase spontaneous activity and

decrease the threshold to peripheral stimulation [42]. These altered activities turn the neurons in the dorsal horn into an amplifier for peripheral nociceptive input and finally lead to allodynia and hyperalgesia [41, 43].

The initial trauma can induce peripheral sensitization. Peripheral sensitization may generate continuous nociceptive input and cause a central sensitization. It is likely that the peripheral sensitization and central sensitization coincide in CRPS rather than happen separately.

3.6.3 Neurogenic inflammation

Patients with CRPS frequently develop inflammation-like symptoms such as redness, swelling, altered temperature and pain. Some researchers proposed that CRPS is an inflammatory disease [44]. As we mentioned in the section of peripheral sensitization, the primary afferent fibers, especially the C fiber has an efferent neurosecretory function. It can release neuropeptides (SP and CGRP) and TNF-α following a nerve lesion [45]. Several studies found an up-regulation of neuropeptides and TNF-α in CRPS affected limbs [46-48]. SP induces extravasation of plasma protein and subsequent edema formation [45]. CGRP is a potent vasodilator which can induce temperature and color change [49]. TNF-α is a proinflammatory factor which can initiate the secretion of inflammatory factors. It can also act on the TNF receptor-1 which is expressed on the primary nociceptors to enhance this excitability and lead to pain sensitization [50]. At the same time, some negative results of neurogenic inflammation were also reported. In some studies, the proliferation of immunocytes and regional levels of inflammatory factors were assessed in CRPS patients. However, no difference was found between CRPS cases and control [51-53]. Those negative findings make the proposal of a neurogenic inflammation somewhat controversial. Anyhow, a neurogenic inflammation may explain the early features of CRPS, but it is also interesting to note that the inflammation-like symptoms such as redness, edema and heat are usually absent in the chronic stage of CRPS, but the pain can still persist [8]. This may imply that in the chronic stage, the pain can be independent from the inflammatory processes.

3.6.4 Sympathetic dysfunction

Some of the symptoms found in CRPS can be explained by a dysfuntion of the sympathetic nervous system (SNS). In the early stage, the sympathetic outflow is found to be inhibited in CRPS cases [54]. The inhibited sympathetic outflow leads to cutaneous vascular vasodilation. As a result, the affected limb can be red and warm,

which is a common feature of CRPS. As a compensation of weakened sympathetic function, both the density and sensitivity of the adrenoceptors are elevated [35, 55]. Wasner reported exaggerated vasoconstriction to cold challenge in CRPS affected limbs, but the level of circulating norepinephrine was surprisingly low [56]. This may indicate an excessive sympathetic reaction caused by the compensatory up-regulation of the adrenoceptors, even if the outflow of SNS is low. This consequent heightened sympathetic vasoconstriction may explain why a warm CRPS can turn into a cold type in the chronic stage. Excessive vasoconstriction can impair the local circulation and oxygen exchange, and thus leads to tissue hypoxia and acidosis, which can be a reason for pain [57]. On the other hand, the alpha-adrenoceptors expressed on the nociceptive afferents are also up-regulated. Circulating adrenaline can directly activate these receptors and contribute to sympatho-afferent coupling, which means a persistent pain can be maintained by sympathetic outflow [58, 59]. Sympathetic dysfunction may be responsible for some of the features of CRPS. However, sympathetic blockade can not help most of the patients with CRPS, which suggests that other mechanisms are also involved [60].

3.6.5 Genetic factors

Genetic factors are assumed to be involved in the development of CRPS. Herlyn et al found a significant association between the α_{1a}-adrenoceptor gene and CRPS 1 which developed after a radius fracture [11]. In another study, Vaneker et al reported the association between a promotor polymorphism in the gene of TNF-α and CRPS 1 [61]. However, no single genetic cause can be linked to the occurrence of CRPS so far.

3.6.6 Minimal nerve injury in CRPS 1

CRPS 1 is traditionally defined by the absence of a nerve lesion. Recently, the current definition of CRPS 1 has been challenged. Oaklander et al related an evidence of epidermal neurite loss in the CRPS 1 affected limbs of humans [62, 63]. They proposed that a minimal nerve injury is involved and CRPS 1 is rather a small fiber neuropathy [64, 65]. The “small fibers” are refer to small-diameter unmyelinated fibers (C fiber) and thin myelinated fibers (Aδ fiber), both of which are main primary nociceptive afferent fibers [64]. Those fibers are easily damaged during a trauma or an operation [65]. Compared with a nerve trunk lesion, a minimal nerve injury is usually undetectable by physical examination or surface nerve conduction [64]. This may explain why a minimal nerve injury has not been described in CRPS so far. Though the evidence of

neuropathy in CRPS 1 was reported, the distinct mechanism how a minimal nerve injury could lead to CRPS 1 is still not clear.

Other factors such as cortical reorganization and psychological factors have also been implicated in the pathophysiology of CRPS [66, 67]. However, those theories remain in the stage of assumption. The mechanism how CRPS occurs and develops is still to be further clarified.

3.7 Animal models

Patients with CRPS typically develop intense neuropathic pain. The pain can be exaggerated by touch or physical examination and lead to a progress of the symptoms or even trigger a relapse [27, 28]. Because of this, many studies of CRPS are based on animal models. In the past decades, several animal models have been established in order to study the pathophysiology and symptomatology of CRPS. The common animal models are as follows.

3.7.1 Sciatic nerve chronic constriction injury model (CCI model)

In 1988, Bennett and Xie developed an animal model of neuropathic pain. In this model, the sciatic nerve of a rat was isolated from surrounding tissue, 4 ligatures were tied loosely around it in order to produce continuous stimulation [68]. CCI led to persistent neuropathic pain and trophic changes such as muscle atrophy and abnormal claw growth in the animals. This method is easy to perform and reproducible, which makes CCI become a classical model to study the neuropathic pain and its long-term effect. However, it is limited to modeling CRPS 2 which is defined by a specific nerve lesion.

3.7.2 Tibial fracture model

Fracture is the most common cause for CRPS 1. A fracture model in rodents was established by Guo et al in order to study posttraumatic CRPS 1 [69]. In this model, a tibial fracture was induced with adjusted pliers. Subsequently, the affected hindlimb was wrapped in a casting tape for 4 weeks. Another group without fracture was also immobilized for control. Tibial fracture and immobilization induced CRPS-like symptoms such as allodynia, edema, plasma protein extravasation and periarticular osteoporosis. Interestingly, the control group also developed similar symptoms after an immobilization for 4 weeks. This finding indicates that a postoperative immobilization may prompt the development of CRPS, but at the same time, the effect of a tibial fracture in this model becomes unclear.

3.7.3 Chronic post-ischemia pain model (CPIP model)

A novel hypothesis was proposed by Coderre et al in 2004. They speculated that CRPS 1 is due to deep-tissue ischemia and inflammation. Based on this idea, an ischemia-reperfusion (I-R) model was established. A tourniquet was placed around the ankle of anesthetized rats for 3 hours. Hyperemia, edema and neuropathic pain were observed following reperfusion. Evaluation of inflammatory parameters showed elevated proinflammatory factors early after the reperfusion [70]. Additionally, the I-R injury led to signs of sympathetic dysfunction, including up-regulation of α-adrenoceptors and enhanced sensitivity to catecholamines. Coderre and colleagues believe that both CRPS 1 and CRPS 2 are initiated by the same event, a microvascular I-R injury that follows a trauma. However, different results appeared in the study of Ludwig et al, after a ligation to the femoral artery of rats for 3 hours and subsequent reperfusion, no trophic change or pain was observed [71]. The finding of Ludwig et al does not support the proposal of I-R injury being the major cause of CRPS. Compared with the method of femoral artery ligation, a whole limb constriction in the I-R model is likely to cause a nerve compression and thus rather CRPS 2.

3.7.4 Needlestick distal nerve injury model (DNI model)

Oaklander et al reported a loss of epidermal neurites (29%) in the CRPS 1 affected limbs of humans [62]. This evidence showed a relationship between neuropathy and CRPS 1 which is traditionally defined to be without any nerve lesion. Oaklander et al proposed that CRPS 1 is induced by an injury of the small nerve fibers [64]. Based on this idea, a needlestick distal nerve injury model (DNI) was developed in rats [72]. Here a penetrating injury of the tibial nerve with needles of 3 different diameters (18G, 22G and 30G) was induced. As a result, mechanical hyperalgesia and loss of epidermal neurites were detected. Interestingly, only a portion of the animals from the DNI group developed these symptoms which are independent on the needle diameter. These findings match what we see in the clinic that most of patients with a trauma do not develop CRPS. Additionally, this study also reported pain sensitivity and neurites loss in the innervating area of uninjured nerves and even in the contralateral limb, which can reflect the spread of symptoms in CRPS. However, some of the animals with neuropathic pain have already recovered in 14 days, but the pain in patients with CRPS is usually more long-lasting. This model has successfully reproduced several

features of human CRPS 1, but it is still not able to fully model the progressive symptoms of CRPS.

3.7.5 Exaggerated tissue trauma (ETR model)

In order to study the pathophysiology of CRPS 1, an animal model of exaggerated tissue trauma in rats was developed by Gradl et al [73]. In this model, a standardized soft tissue trauma was induced in the hindlimbs of rats by a pneumatically driven impact device. A supernatant of the traumatized muscular tissue was prepared by homogenization and centrifugation. This mediator-enriched supernatant was infused to the limbs of rats with a standardized soft tissue trauma in order to induce an exaggerated tissue trauma (ETR). Animals which received standardized soft tissue trauma (TR), infusion of supernatant of non-traumatized muscle (STR) and untreated animals (CO) were included for control. As a result, the ETR group developed a temporal mechanical hyperalgesia for 14 days, but no hot or cold allodynia was detected, while the other groups were pain-free. The animals with soft tissue trauma showed enhanced inflammatory responses in the traumatized muscle, the posttraumatic inflammation was found most pronounced in the ETR group. However, the method of ETR only induced some temporal CRPS-like symptoms instead of an ongoing CRPS.

4 Goal in research

The current animal models are of great help in understanding the pathophysiology and symptomatology of CRPS. However, none of them can fully reproduce the development of CRPS. The animal model of exaggerated tissue trauma by Gradl et al has produced pronounced regional inflammation and CRPS-like symptoms. However, it failed to induce ongoing signs of CRPS. At the same time, the current concept of CRPS 1 and CRPS 2 being two different entities has been challenged by Oaklander et al. They reported the evidence of neuropathy in CRPS 1 which is traditionally defined by the absence of a nerve lesion and proposed an injury to small nerve fibers being the cause of CRPS 1. Tissue trauma is the most common initializing event of CRPS 1. However, it is not clear that whether a minor nerve injury plays an essential role besides the trauma. To answer this question, we combined a closed soft tissue trauma and an extra minimal nerve injury in order to establish a new model of CRPS. Further assessments of pain behavior, autonomic changes, regional inflammatory response, microcirculation and cell apoptosis were performed with various techniques, in order to clarify the following questions:

- Can a single soft tissue trauma lead to neuropathic pain and other symptoms of CRPS?
- Can a combined injury of closed soft tissue trauma and a minimal nerve injury lead to CRPS-like symptoms?
- Are the combination of closed soft tissue trauma and a minimal nerve injury sufficient to induce ongoing symptoms of CRPS?

5 Material and Methods

5.1 Animals

The experiments were performed in male Sprague Dawley rats (body weight 250-300g; Charles River Labortaries, Sulzfeld, Germany) which were kept on water and standard laboratory chow ad libitum. The experimental protocol was approved by the local animal rights protection authorities (LALLF 7221.3-1.2-025/11 (1.1-090/10)) and followed the National Institutes of Health guidelines for the care and use of laboratory animals.

5.2. Experimental groups

42 animals were divided into two groups, the sham group and clamp group (n=21 for each group). Based on the duration of experiment, both groups were divided into 3 subgroups (2 days, 7 days and 28 days group, n=7 for each subgroup). The grouping method, experimental duration and interventions are given in **Tab. 2**.

Tab. 2. Group information

	2 days	7 days	28 days	interventions
sham	7	7	7	soft tissue trauma + sham operation
clamp	7	7	7	soft tissue trauma + minimal nerve injury

5.3 Surgical procedure

5.3.1 Closed soft tissue trauma

Animals were anesthetized with 6% pentobarbital-sodium (55mg/kg body weight i.p, Narcoren, Merial, Hallbergmoos, Germany). The left hindlimb was shaved and fixed into a plastic mold which fits the shape of the rats' limb in order to avoid kinetic energy loss due to movement (**Fig .1**). By means of a pneumatically driven impact device (custom made by the workshop of the Medical Faculty, University of Rostock, Germany), a standardized soft tissue trauma was induced on the lateral tibial compartment of the hindlimb, resulting in a high energy and velocity impact trauma in the lower extremity [74].

The pneumatic impact device consists of a compressed nitrogen gas source and an adjustable impactor. The parameters of impact were selected to a velocity of 7m/s, a deformation depth of 11mm and a contact duration of 100ms. The diameter of the impactor is 10mm[75, 76]. After the tissue trauma, the traumatized limb was carefully examined to exclude fracture and skin laceration. The soft tissue trauma was induced

in a total of 42 rats.

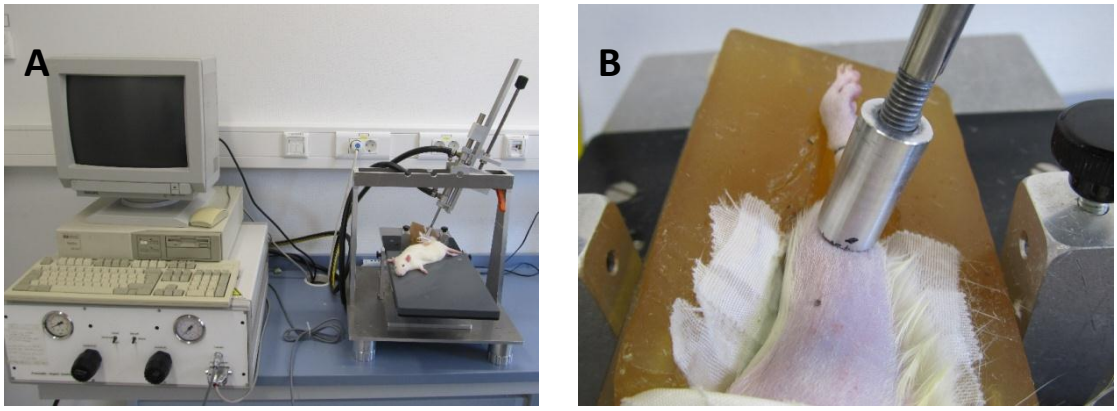

Fig. 1. Induction of the closed soft tissue trauma. A: The pneumatically driven impact device; B: The left hindlimb of a rat was fixed in a plastic mold to be traumatized by the impactor.

5.3.2 Minimal Nerve Injury

The animals were placed on a heating plate immediately after the soft tissue trauma to keep the body temperature at 37˚C. After preparing and disinfecting the skin, an incision of 1cm length was made on the left thigh. The surgical approach was made bluntly through the biceps femoris. The left sciatic nerve was exposed at the level of the middle of the thigh and carefully separated from adhering tissue by blunt dissection [68]. The minimal nerve injury was induced with an instrumented clamp which was connected to a computer. The force and duration of clamping were monitored online. The clamping force of 30 Newton (2xlock of the clamp) and duration of 20s were selected since this procedure induces a moderate nerve injury without complete nerve damage (several different settings of crush injury were tested in the previous trails (data not shown)). The sciatic nerve was placed between the two pincers of the clamp, when the monitored clamping force showed a steady waved course around the baseline, the pincers were closed with 2 locks and the nerve was clamped for 20s (**Fig. 2**).

On the other hand, the sciatic nerve of the sham group was only exposed without being clamped. After the surgical procedure, animals were placed back to their cages, allowed to recover from anesthesia and had free access to food and drinking water.

5.4 Pain assessment

To assess the neuropathic pain, spontaneous behavior, pain behaviors to hot, cold and mechanical stimuli were observed on different days. The day of surgery was defined as day 0, behavior was observed on day 0 prior surgery and served as

baseline. After surgery, observation was carried out on day 2, 4, 7, 14, 21 and 28. For the observation, the experimental animals were placed in a transparent plexiglass box (22cm x 16cm x 15cm) (custom made by the workshop of the Medical Faculty, University of Rostock, Rostock, Germany) which provides good ventilation and perfect vision. Different stimulations were applied by changing the bottom plate of the box (**Fig. 3**).

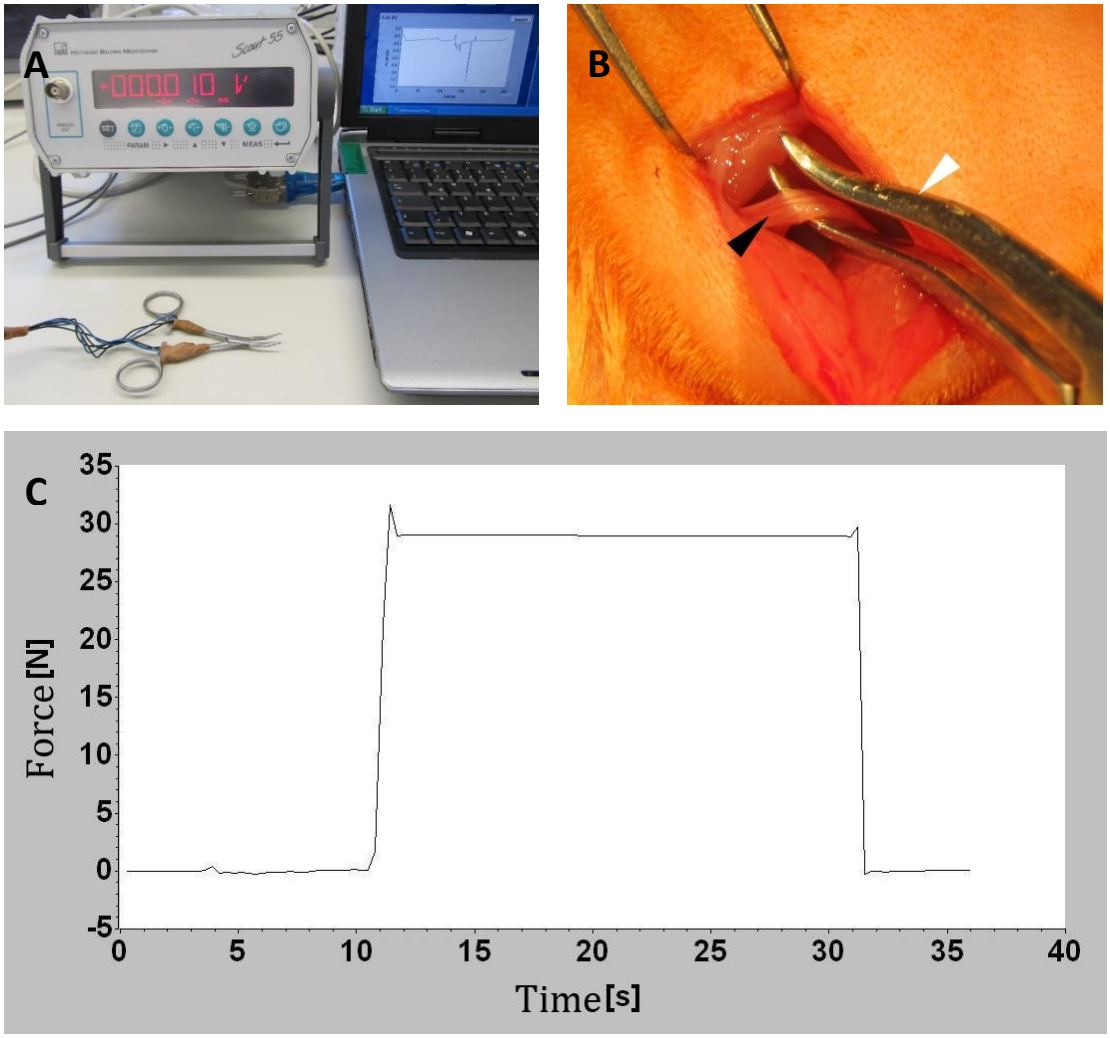

Fig. 2. Induction of the minimal nerve injury. A: The instrumented clamp which is connected to a computer for monitoring; B: The exposed sciatic nerve (black arrow) and the instrumented clamp (white arrow); C: Clamping force and duration were recorded online.

5.4.1 Pain behavior observation

Spontaneous pain was observed when the animals were placed on an ordinary plastic plate under room temperature (21°C). The hot and cold stimulation was applied by using a heating (40°C) or a cooling plate (4°C). Before each observation, the animals

were allowed to habituate to the environment for 10min without any intervention from the observer.

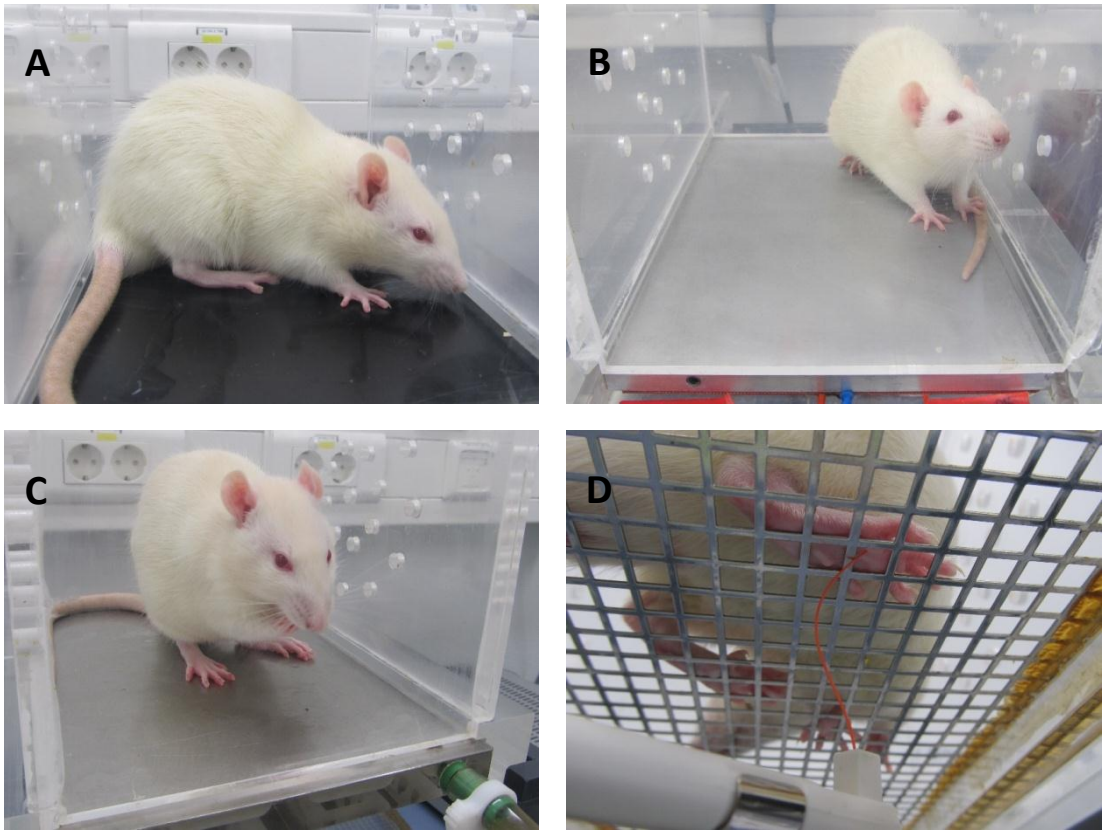

Fig. 3. Pain behavior observation. A: The ordinary plate (21°C); B: The heating plate (40°C); C: The cooling plate (4°C); D: The metal mesh plate for mechanical stimulation.

The observation of each pain type includes 3 trials. Each trial lasted for 300s, a total period of 900s for each observation was recorded. The criteria of pain behavior used in the experiment are as following: 0=the paw is pressed normally on the floor; 1=the paw rests slightly on the floor and the toes are in a ventroflexed position; 2=the paw rests slightly on the floor and the external edge is suspended; 3=only the heel is pressed on the floor, the paw is in an inverted position; 4=the whole paw is elevated; 5=the animal licks the operated paw (**Fig. 4**) .The duration of different positions was recorded during the observation. Based on the scale of different positions, a quantification of pain behavior was performed and the numerical pain index was calculated with the given formula [77]:

0*T0+1*T1+2*T2+3*T3+4*T4+5*T5/900

T0 to T5 are the durations (s) spent in the corresponding positions (0 to 5). Each observation lasted for 900s (3 x 300s). An average pain index was calculated to reflect the intensity of pain.

Fig. 4. The criteria used for quantification. Six possible positions of the operated hind paw are rated from 0 to 5 in order to quantify the pain behavior. Adapted from Attal et al [77].

5.4.2 Mechanical stimulation

The animals were placed on a metal mesh plate with 0.6x0.6cm cells. After the period of habituation, mechanical stimulation was applied to the plantar region of the operated paw with 6 calibrated von Frey filaments ranging from 0.6g to 15.0g (North coast medical, USA). The mechanical stimulation was performed when the paw was pressed on the floor. Stimulation was applied until the filament bent. At this moment, the fibers' force was fully exerted. Every single trial contains 6 stimulations of filaments at a frequency of 1/s. The same procedure was repeated using each filament in an ascending order from 0.6g to 15.0g. Five trials were performed each time. An interval of 3min was allowed between each trial. The result was expressed as a withdrawal frequency towards each filament (paw withdrawals/5 * 100%=% withdrawal frequency) [78].

5.5 Measurement of paw temperature

Skin temperature of bilateral paws was measured on different days (day 0, 2, 4, 7, 14, 21 and 28). Animals were anesthetized with isoflurane and a surface electrode (skin temperature probe 400^{TM}, Covidien, USA) was placed on the plantar region of the paw (**Fig. 5A**). The temperature of each paw was noted and the difference between

the operated side and the control side was calculated. The temperature difference was finally expressed as an absolute value, since the operated paw could become either abnormally warm or cold.

5.6 Measurement of paw volume and edema

The volumes of both paws were measured following the temperature measurement. The paws of both sides were immersed in a calibrated cylinder filled with water until the fluid level just reached the proximal tip of the glabrous surface of the heel [74] (**Fig. 5B**). The volume difference between both sides was calculated (volume difference= operated side - control side) and served as a parameter of edema. In addition, edema in the traumatized muscle was assessed ex vivo. On the relevant final days of the experiment (day 2, 7 and 28), the animals were put to death and the tibialis anterior muscles of both sides were excised. The wet weight of the muscles was measured immediately. After that, the muscles were kept and dried in a laboratory oven (60°C) for 72 hours, the dry weight was then measured. A dry/wet weight ratio was calculated. A fraction of operated side to control side was later calculated with the formula below and served as an indice of muscular edema:

Edema fraction = (dry/wet ratio of operated side) / (dry/wet ratio of control side).

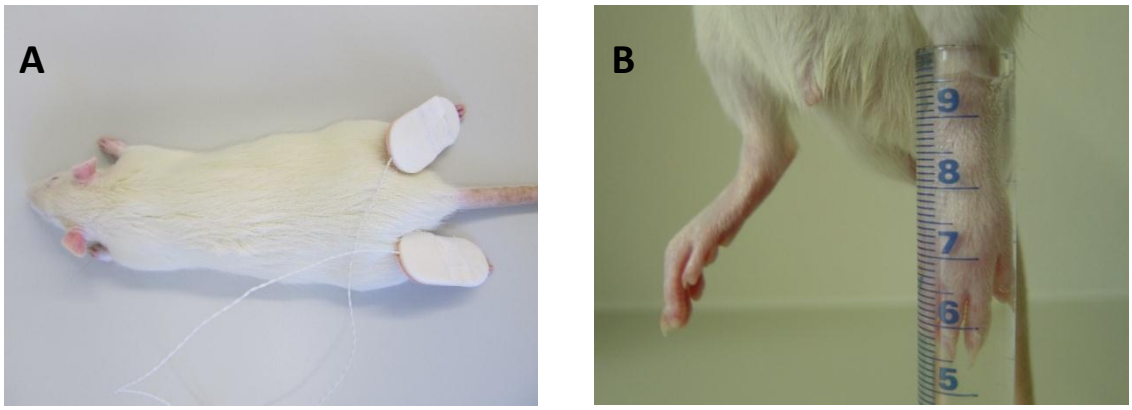

Fig. 5. Measurement of temperature (A) and volume (B). A: Two surface electrodes were placed onto the plantar region of the paws in order to measure the temperature; B: The paw of an anesthetized rat was immersed in a calibrated cylinder filled with water to measure the volume.

5.7 Nerve conduction study

Nerve conduction was performed on the final experimental day (day 2, 7 and 28) to assess the conductive function of the sciatic nerve. Pentobarbital-anesthetized animals were placed on a warm plate to maintain the body temperature (37°C). The sciatic nerve of the operated side was carefully exposed and freed from the

surrounding tissue. For the nerve conduction, a ground electrode was placed in the subcutaneous tissue between stimulating and recording site [79]. Two recording needle electrodes were positioned in the tibialis anterior muscle and the flexor muscle in the paw. Electrical stimulation was performed in the operated sciatic nerve by a stimulator (A320, World Precision Instruments, Berlin, Germany) (**Fig. 6**). The stimulations (0.2V, 300ms) were applied to both the proximal and the distal trunk of the injured site. The conduction curves were recorded and analyzed off line with the program LabVIEW (National Instruments, USA). To analyze the conductive curve, the starting point of descending branch of the curve was marked. The conductive time (ms) from zero (the starting point of the motor evoked potential) to the beginning of descending branch was calculated by program. After that, the conductive latency between the proximal and distal site was calculated with the following formula:

Conductive latency (ms) =conductive time (proximal) - conductive time (distal).

The conductive latency of sham group without nerve injury served as baseline.

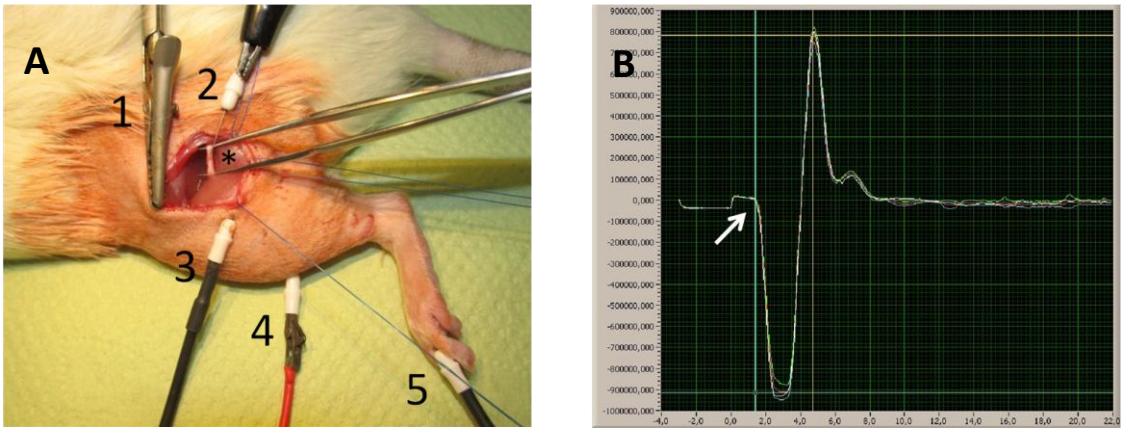

Fig. 6. Nerve conduction study. A: The left sciatic nerve (*) was exposed, the electrodes were placed in respective positions. 1: the positive pole of the stimulator; 2: the negative pole of the stimulator; 3: the ground electrode; 4: the recording electrode in the tibialis anterior muscle; 5: the recording electrode in the muscle of the paw; B: The starting point of the descending branch (white arrow) was marked on the curve in order to calculate the conductive time.

5.8 In vivo high resolution fluorescence microscopy

In vivo high resolution fluorescence microscopy was performed in the traumatized soleus muscle on the final days of the experiment (day 2, 7 and 28). After the assessment of nerve conduction, subsequent surgery was performed. A cervical median approach was made through skin and the platysma, the skin and the platysma were pulled to both sides in order to expose the trachea and the carotid arteries

which ascend along the side of the trachea. The trachea was intubated and connected to a mini ventilator (TSE-994500, Technical & Scientific Equipment, Germany). Mechanical ventilation was applied (room air, 0.8ml/100g body weight; 17 breaths/min). The right carotid artery was carefully freed from the carotid sheath and further separated from the vagus and the sympathetic nerve. The distal side of the carotid artery was ligated. A tiny transverse incision was made proximally to the ligature. A catheter (PE-50; Portex, Hythe, Kent, UK) was placed into the carotid artery. The catheter was fixed for later administration of fluorescent dyes and continuous monitoring of central hemodynamics (Sirecust; Siemens, Germany).

The traumatized soleus muscle was surgically prepared for the in vivo fluorescence microscopy. The affected hindlimb was fixed and a longitudinal incision was made in the skin and subcutaneous fascia in the tibial anterolateral region. The fascia was retracted posteriorly in order to expose the soleus muscle. The soleus muscle was stretched moderately to allow sufficient surface for scanning. After a plane surface was prepared, a piece of cover glass was placed on the muscle and the space between the cover glass and the muscle was filled with 37°C warm physiological saline to prevent the muscle from drying. The plane surface of the muscle had to be as horizontal as possible to give convenient access for the following microscopic examination (**Fig. 7A**). The animals were allowed to recover from the surgery for 30 min. During this period, the animals were adjusted to physiological hemodynamic and ventilation parameters (mean arterial blood pressure 100–120mmHg; pO_2 70-90mmHg; pCO_2 30–40mmHg; pH 7.35–7.45).

For in vivo microscopic examination, a high resolution fluorescence microscope (B46-0013d, Zeiss, Germany) equipped with a blue filter (excitation/emission 465-495nm/>505nm), a green filter (510-560nm/>575nm) and an ultraviolet filter (UV filter) (340-380nm/>400nm) was used in the experiment. During the fluorescence microscopy, the whole surface of the soleus muscle was scanned and microscopic images of 5 different sites were chosen for assessment. Before administration of fluorescent dyes, NADH autofluorescence was measured by exposition to ultraviolet epi-illumination for 10s and observation with the UV filter (40x). Fluoresceinisothiocyanate (FITC)-labeled dextran (15mg/kg body weight, Sigma, Germany) and Rhodamine 6G (0.15mg/kg body weight, Sigma, Germany) were injected intra-arterially to enhance the contrast of the microvascular network and stain the leukocytes in vivo [38]. The capillaries were observed under the blue filter

(40x). Subsequently, 5 flowing venules were identified under the blue filter (20x). Leukocyte movement was observed in the same venules with the green filter (20x). The optical source was shaded with a shutter during the intervals of observation in order to avoid phototoxic effects. Microscopic images were captured with a charge-coupled device video camera (FK-CM-2412-2-01-IQ-R4, Pieper, Germany) and recorded onto an S-VHS video system for subsequent offline analysis.

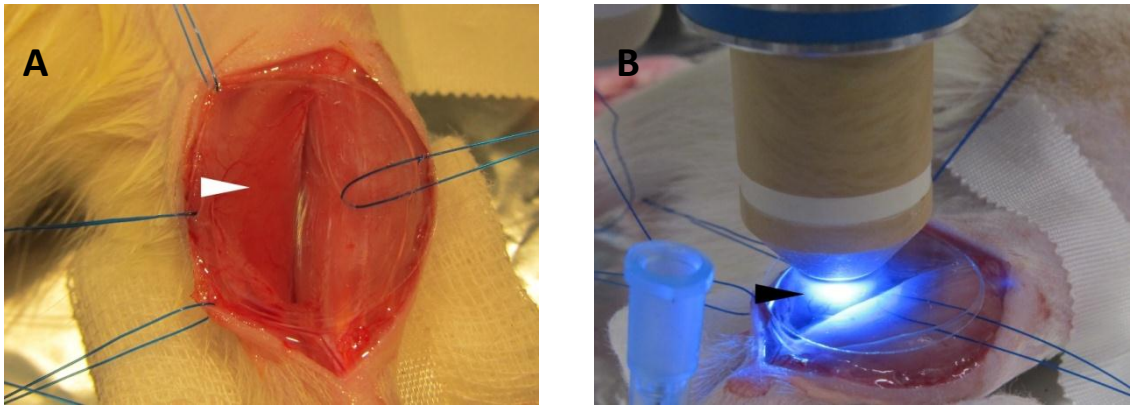

Fig. 7. In vivo fluorescence microscopy. A: The traumatized Soleus muscle (white arrow) was prepared for microscopic examination; B: The soleus muscle (black arrow) was scanned with a fluorescence microscope.

5.9 Microcirculatory analysis

The microscopic images recorded on video tapes were analyzed offline using a micro-circulation image analysis program (CapImage, Zeintl, Heidelberg, Germany). To assess the NADH autofluorescence, the image frame on the 10th second was taken for analysis. The program-assisted gray level determination was performed on the inter-capillary tissue in order to avoid the interference of the microvascular structures. The functional capillary density (FCD) was defined as the overall length of perfused capillaries in each observation field. The functional capillaries in the dynamic images were marked manually, the whole length of the capillaries and the total area of observation field were calculated by the program Capimage. The value of FCD was given as "cm/cm^{2}" (overall length/total area).

The leukocyte-endothelial interaction was assessed by recording the dynamic images of post-capillary venules for 30s. Based on the behavior, leukocytes inside post-capillary venules were divided into 3 types which include floating leukocyte (passes quickly with the blood flow), rolling leukocyte (rolls along the vessel wall and the velocity is below 40% of normal) and adhering leukocyte (adheres firmly to the vessel wall without moving). Leukocytes were counted according to the different behavior.

Rolling leukocytes was expressed as a percentage ((rolling cell) / (rolling cell + floating cell) %). Diameter and length of the analyzed venule section were also measured to calculate the area of the endothelial surface (mm^2), assuming cylindrical micro-vessel geometry. The leukocyte adherence was expressed as nonmoving leukocytes per endothelial surface (sticking cell / area of endothelial surface) (n/mm^2) [74]. For the analysis of vascular permeability, a program-assisted grey level determination was performed on both intra-vascular area and the tissue surrounding area. The parameter of vascular permeability was expressed as a "leakage fraction" (extra-vascular grey level / intra-vascular grey level) which reflects the extent of vascular permeability.

For analysis of NADH autofluorescence, the functional capillary density (FCD), leukocyte rolling, leukocyte adherence and the vascular permeability, 5 observation fields were analyzed for each parameter and the results were finally given as a mean value.

5.10 Assessment of muscle cell apoptosis

In vivo apoptotic cell death was observed under the fluorescence microscope. Staining of myocyte nuclei was performed by intra-arterial injection of bisbenzimide (Hoechst 33342; 10mmol/kg) [74]. The bisbenzimide marked cells were identified under the UV filter (40x). The apoptotic cells i.e. those showing typical signs of apoptosis such as nuclear condensation and fragmentation, were counted and expressed as "n/observation field". 10 observation fields were taken for assessment, the result is given as a mean value.

The muscle cell apoptosis was further assessed ex vivo. After fluorescence microscopy, animals were put to death and the traumatized soleus muscle was excised and fixed with 4% formalin. Paraffin sections of muscular tissue were prepared for immunostaining. To assess the cell apoptosis, cleaved caspase-3 staining was performed with a rabbit anti-cleaved caspase-3 antibody (1:400, No.9661, cell Signaling Technology, Frankfurt, Germany). The sections were analyzed under a light microscope (BX 51, Olympus, Japan) (400x). The caspase-3 positive cells were counted. The area of each observation field is 1 mm^2 and the result was expressed as n/mm^2. 25 different observation fields were analyzed, the result was finally given as a mean value.

5.11 Baseline setting

For the assessment of pain behaviors, temperature and volume asymmetry, the intragroup values of day 0 were taken as baseline. In vivo fluorescence microscopy and immunohistochemistry were performed in another 6 healthy animals. The results of the healthy animals served as baseline for ex vivo assessment of muscular edema, regional inflammation, microcirculation and muscle cell apoptosis.

5.12 Statistical analysis

Statistical evaluation was performed with SigmaStat (Sigmastat; Jandel Scientific, San Rafael, CA, USA). Based on the normality and equal variance across groups, data was evaluated with analysis of variance (ANOVA), followed by Holm-Sidak test. Results are given as mean ± Standard Error of the Mean (SEM) and statistical difference was set at $P<0.05$.

6 Results

In general, the experimental animals were well kept and had gradually gained weight during the study (**Tab. 3**). No behavior of autotomy was observed. No animal was ruled out due to infection or other diseases.

Tab. 3. The weight gain of animals within 28 days

	day 0	day 2	day 4	day 7	day 14	day 21	day 28
sham	273±6 g	282±7 g	293±7 g	312±9 g	369±5 g	409±5 g	435±5 g
clamp	281±4 g	291±6 g	302±6 g	316±6 g	361±9 g	395±10 g	421±11 g

* n=7 for each group, data are given as mean ± SEM.

6.1 Neuropathic pain

In order to assess the pain, the method of behavior observation was carried out. Based on the different positions which were presented in the affected paws, the pain was rated and quantified.

On day 0 prior surgery, spontaneous pain or nociceptive responses to hot and cold was absent in all the animals. During the test of mechanical stimulation, animals showed slight withdrawal reactions to von Frey filaments ranging from 6.0g to 15.0g, but rarely responded to forces from 0.6g to 2.0g.

Shortly after the surgery up to day 7, pronounced signs of neuropathic pain were observed in the clamp group. These signs include spontaneous pain, hot and cold allodynia and mechanical hyperalgesia. Spontaneous pain and allodynia decreased over day 14 and 21 and completely vanished within 4 weeks. Mechanical hyperalgesia was also found to be alleviated, but it did not disappear within 28 days. In the sham group with sole soft tissue trauma pain did not develop.

6.1.1 Spontaneous pain

Spontaneous pain is the pain behavior expressed under room temperature (25˚C) without further stimulation. On day 2, the affected paws of the clamp group commonly showed position 1 or position 2, which reflect a spontaneous pain. As shown in **Fig. 8B**, the index of spontaneous pain was found to be elevated on day 2, peaked between day 4 and day 7. The pain index went back to baseline on day 21 representing the paw placed normally on the floor.

6.1.2 Hot and cold allodynia

On day 2, the animals of the clamp group demonstrated pronounced responses to hot and cold simulations. They behaved restlessly during the observation, which produced a stark contrast with the calm behavior of the sham group. Besides position 1 and 2, positions indicating high pain intensities such as position 4 (elevating the paw) and position 5 (licking the paw) were also observed. As shown in the graphs (**Fig. 8C and D**), the pain indices of the hot and cold allodynia were elevated on day 2 and reached the peak between day 4 and day 7 being parallel with the spontaneous pain. However, hot and cold allodynia lasted until day 21 and went back to baseline on day 28.

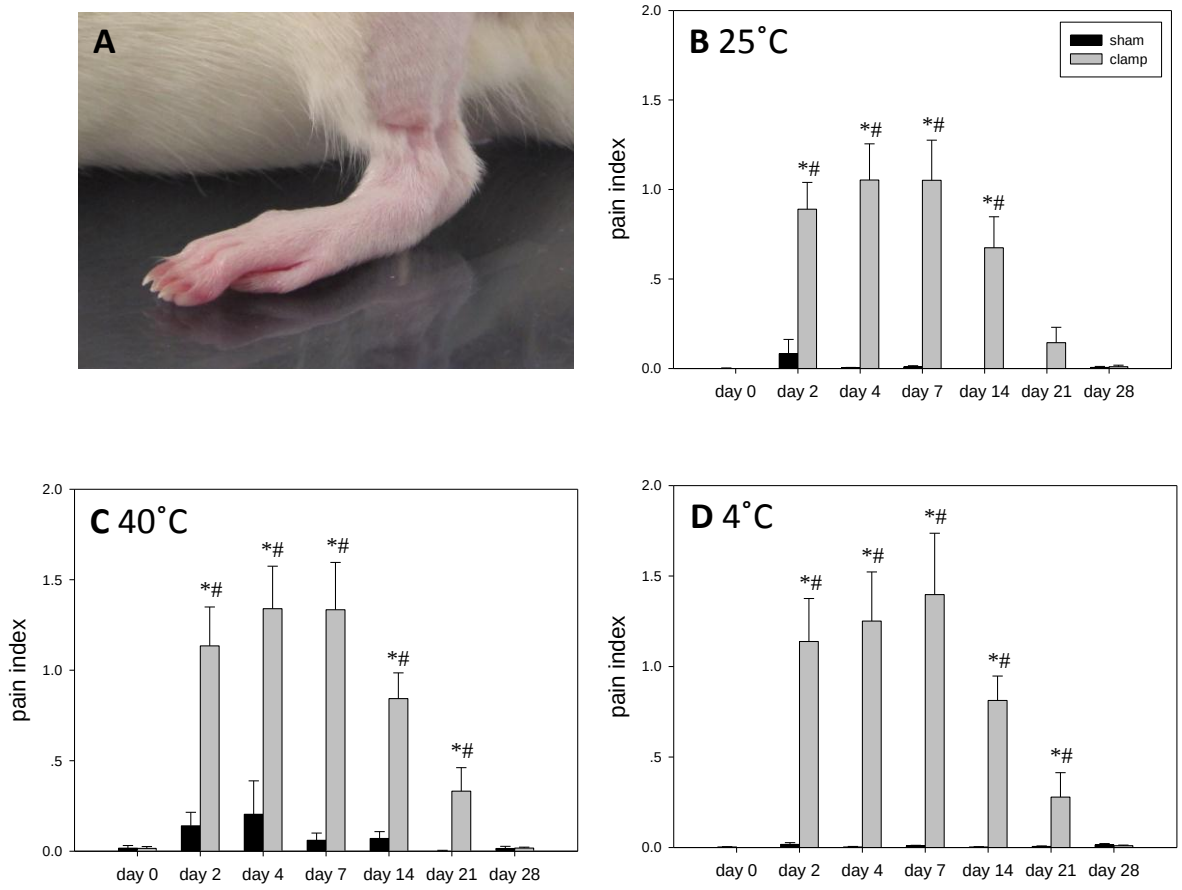

Fig. 8. Assessment of neuropathic pain. A: An animal from the clamp group showed position 1 in the operated limb; B: Index of spontaneous pain; C: Index of hot allodynia; D: Index of cold allodynia. Values are given as mean ± SEM; Two Way ANOVA followed by Holm-Sidak Test, * P<0.05 vs sham group, # P<0.05 vs day 0.

6.1.3 Mechanical hyperalgesia

Mechanical stimulation was applied with 6 calibrated von Frey filaments ranging from 0.6g to 15.0g. The animals of the clamp group showed a heightened response to the mechanical stimulation, especially to higher masses (6.0g, 10.0g and 15.0g). Since the

stimulations rated from 6.0g to 15.0g were also able to evoke withdrawal responses in healthy animals, the relatively enhanced reaction after surgery was interpreted as mechanical hyperalgesia. The mechanical hyperalgesia appeared along with other signs of pain. As shown in **Fig. 9**, the withdrawal frequency of the clamp group was found to be elevated on day 2 and most pronounced between day 4 and day 7. After day 7, it began to decline. This trend of decline continued until the final day of experiment. On day 28, compared with the readings in the first week, the mechanical hyperalgesia of the clamp group was found to be still alleviated and showed significant difference to that of the sham group. Compared with other signs of neuropathic pain, the mechanical hyperalgesia seems to be more long-lasting. In addition, the withdrawal responses to lower forces (0.6g to 2.0g) were also enhanced in the clamp group. A significantly heightened reading (2.0g) was detected on day 7, which can be interpreted as a sign of mechanical allodynia. This significant reading disappeared at day 14.

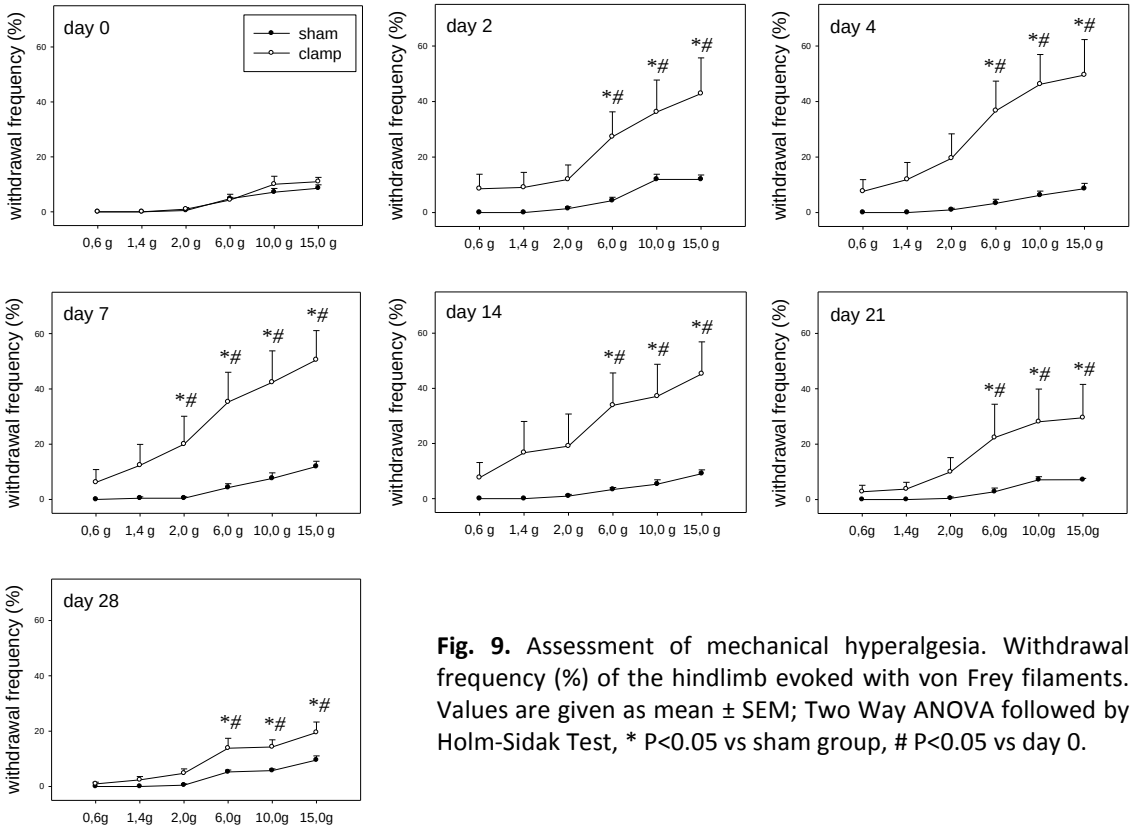

Fig. 9. Assessment of mechanical hyperalgesia. Withdrawal frequency (%) of the hindlimb evoked with von Frey filaments. Values are given as mean ± SEM; Two Way ANOVA followed by Holm-Sidak Test, * P<0.05 vs sham group, # P<0.05 vs day 0.

6.2 Nerve conduction study

The conduction curve and latency of the sham group was altered over 28 days and served as baseline (mean ± SEM=0.09 ± 0.03ms). After the surgery in animals of the

clamp group, the electromyography showed abnormal curves on day 2 and day 7 and the assessment of latency measurement was not possible. This condition was found improved on day 28, approximately normal curves were commonly detected in the clamp group. Compared with the baseline, a significantly prolonged latency could be assessed in the clamp group (mean ± SEM=0.25 ± 0.08ms) (**Fig. 10**).

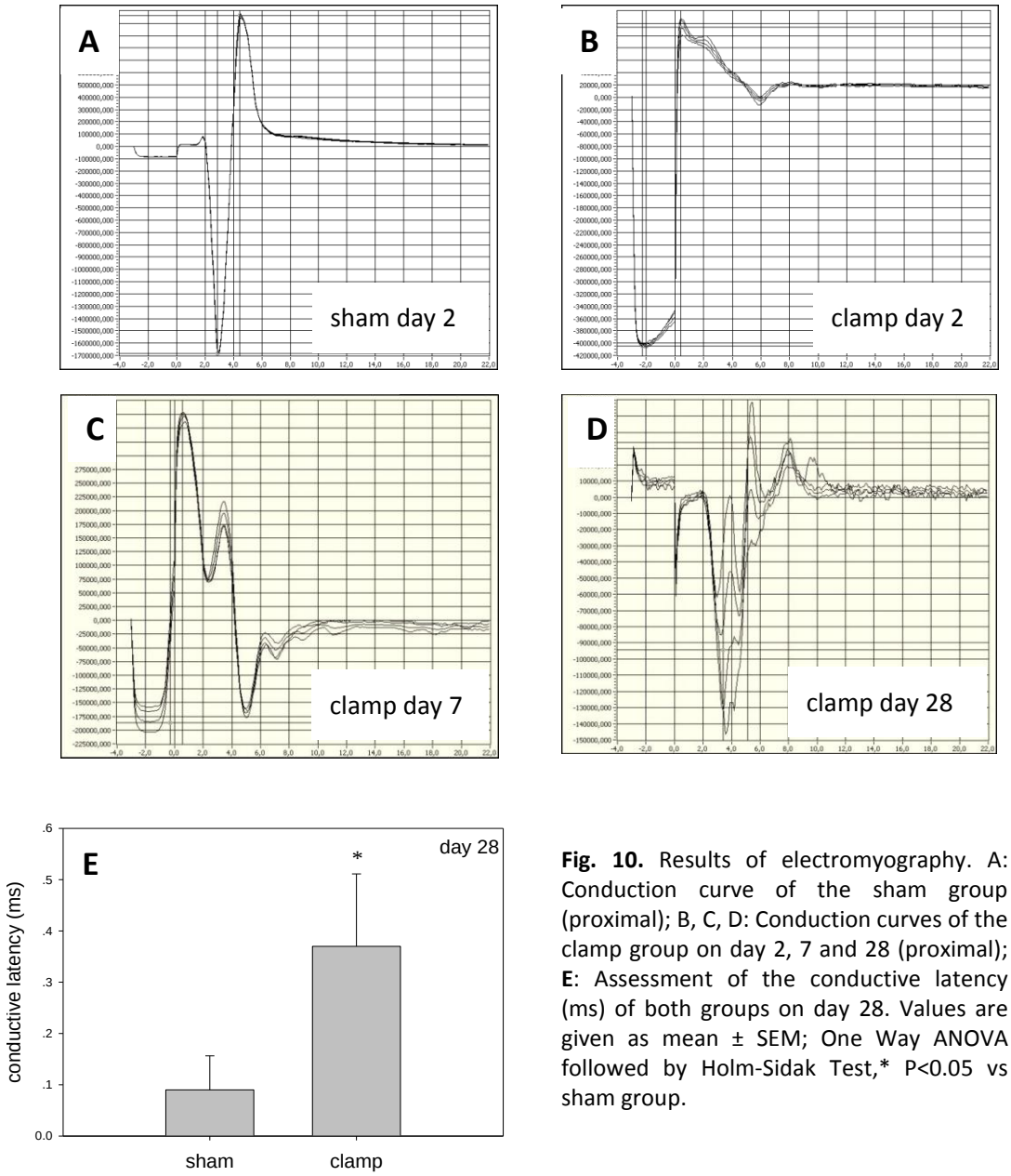

Fig. 10. Results of electromyography. A: Conduction curve of the sham group (proximal); B, C, D: Conduction curves of the clamp group on day 2, 7 and 28 (proximal); **E**: Assessment of the conductive latency (ms) of both groups on day 28. Values are given as mean ± SEM; One Way ANOVA followed by Holm-Sidak Test,* P<0.05 vs sham group.

6.3 Paw temperature asymmetry

The temperature differences between the operated paw and the control side were calculated on different days. According to the value at day 0, there is a natural temperature asymmetry between the paws of both sides. The natural difference is less than 0.4˚C. After surgery, both groups showed temporally enhanced temperature

asymmetries. The temperature difference was finally expressed as absolute value, because the operated paw may become either warmer or colder after surgery, but in our study, the operated paw was always warmer than the control side (0.83˚C-0.90˚C). As shown in **Fig. 11A**, the enhanced temperature asymmetries in both groups appeared on day 2 and day 4 and went back to baseline on day 7. The clamp group showed no difference compared with the sham group.

6.4 Assessment of edema

The volumes of both paws were measured and differences between both sides were calculated subsequently. There was a volume increase in both groups on day 2, which indicates edema formations in the traumatized muscle. The muscular edema of both groups was transient since the readings went back to baseline on day 7. No intergroup difference appeared (**Fig. 11B**).

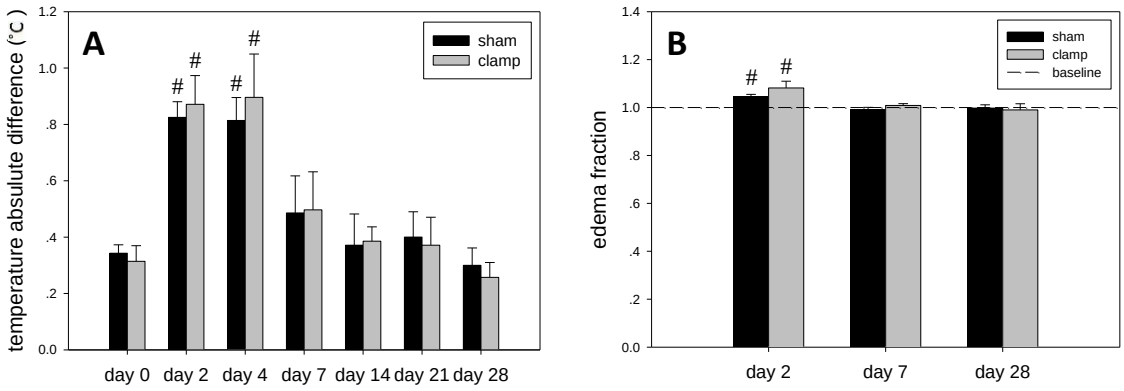

Fig. 11. A: Assessment of temperature asymmetry (˚C). Values are given as mean ± SEM; Two Way ANOVA followed by Holm-Sidak Test. # P<0.05 vs day 0; B: Assessment of intramuscular edema fraction. Values are given as mean ± SEM; One Way ANOVA followed by Holm-Sidak Test, # P<0.05 vs baseline.

6.5 Assessment of microcirculation

Signs of microcirculatory deterioration and regional inflammation in the traumatized soleus muscle were observed by in vivo fluorescence microscopy and analyzed offline. After the surgery, both the sham and clamp group developed regional inflammatory responses, tissue perfusion failure and sign of hypoxia on day 2. However, the inflammation and signs of microcirculatory deterioration were almost absent in both groups on day 7. No evidence of persistent inflammation was found.

6.5.1 Tissue perfusion failure and signs of tissue hypoxia

NADH is an entry enzyme of oxidative phosphorylation in the mitochondria. Elevated levels of NADH can be interpreted as signs of tissue hypoxia [80]. As shown in **Fig. 12**, a heightened autofluorescence of NADH was observed in both groups under epi-illumination on day 2, which reflected tissue hypoxia after surgery. The NADH autofluorescence of both groups went back to baseline at day 7 and day 28.

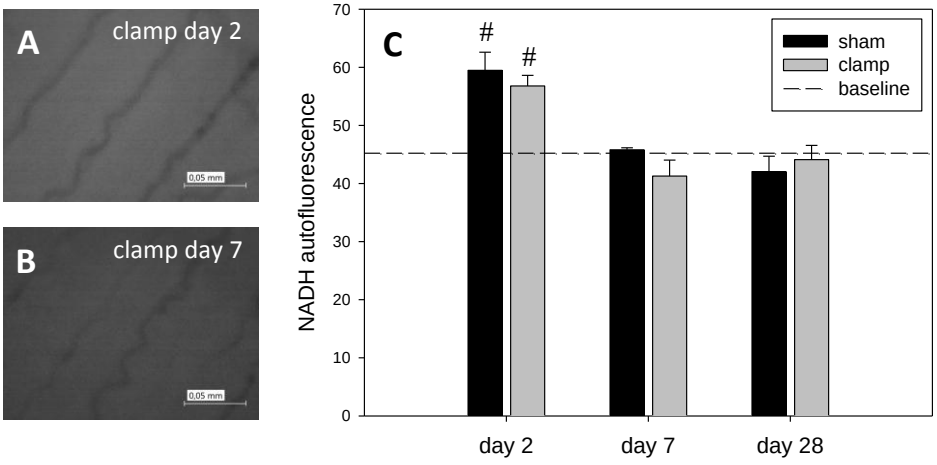

Fig. 12. Assessment of NADH autofluorescence. A: A heightened autofluorescence of NADH was observed on day 2; B: The NADH autofluorescence returned to baseline on day 7; C: Index of NADH autofluorescence (arbitrary unit (aU)). Values are given as mean ± SEM; One Way ANOVA followed by Holm-Sidak Test, # P<0.05 vs baseline.

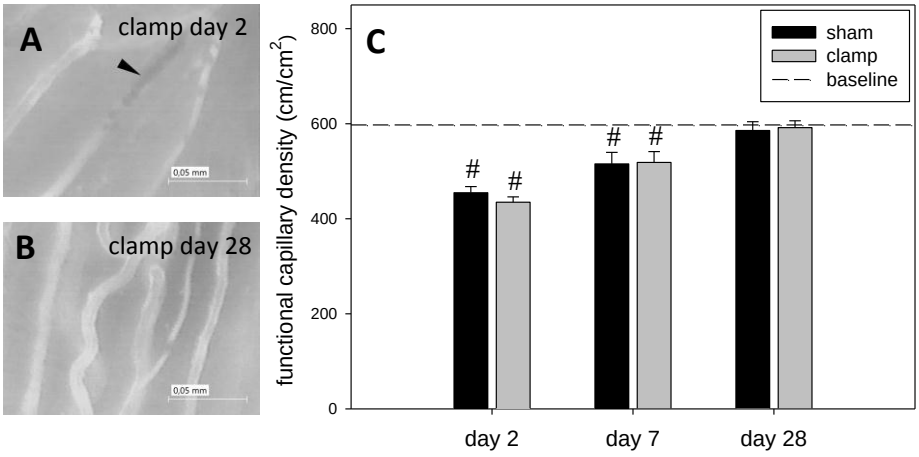

Fig. 13. Assessment of functional capillary density (FCD). A: Thrombosis in a capillary on day 2 (black arrow); B: Elevated FCD on day 28; C: Quantitative anlysis of the FCD (cm/cm^2). Values are given as mean ± SEM; One Way ANOVA followed by Holm-Sidak Test, # P<0.05 vs baseline.

The functional capillary density (FCD) represents the number of regional nutritive capillaries and reflects the condition of tissue perfusion. FCD was found lowered in both groups on day 2 and day 7 when compare to the baseline values. Besides the

decreased number in capillaries, thrombosis was also observed in some microvessels (**Fig. 13**). FCD was finally detected to reach baseline values of approximately 600 cm/cm^2 on day 28. There were no statistical significant differences of the NADH autofluorescence and the FCD between the sham and clamp group.

Regression analysis showed an inverse correlation between FCD and the NADH autofluorescence in both groups i.e. the higher the FCD is, the lower the NADH autofluorescence is (**Fig. 14**). This result indicates that the extent of tissue hypoxia is closely related to FCD which reflects the tissue perfusion.

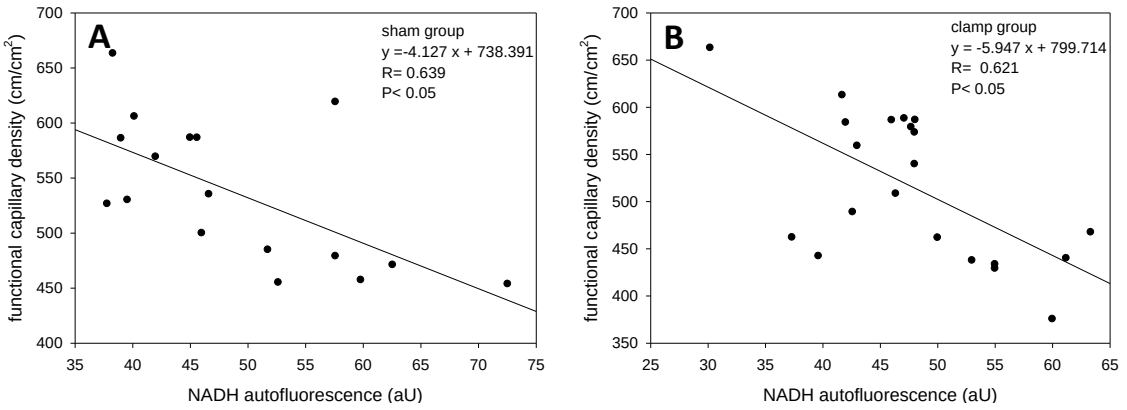

Fig. 14. Linear regression analysis between FCD and NADH autofluorescence over 28 days. A: Regression analysis in sham group; B: Regression analysis in clamp group. R= regression coefficient.

6.5.2 Regional inflammation

Pronounced signs of regional inflammation in the skeletal muscle were observed in both groups on day 2. These signs include enhanced interaction between leukocytes and vascular endothelium as well as increased vascular permeability.

The enhanced interaction between leukocytes and vascular endothelium was reflected by an increased fraction of rolling leukocytes (leukocytes which moved along the vessel wall at a velocity less than 40% of normal) and number of adhering leukocytes (leukocytes which adhered firmly to the endothelium without moving). This enhanced interaction was commonly observed even in fast flowing vessels (**Fig. 15**). Increased vascular permeability developed along with the enhanced leukocyte-endothelial interaction. Under normal conditions, the fluorescent dye is restricted inside the vessels due to its high molecular size. When the vascular permeability increases, the macromolecular dye can leak into the surrounding tissue. As we can see from **Fig. 16**, the enhanced leakage of fluorescent dye was observed in both

groups on day 2 which indicated an increased vascular permeability. The vascular permeability of both groups recovered on day 7.

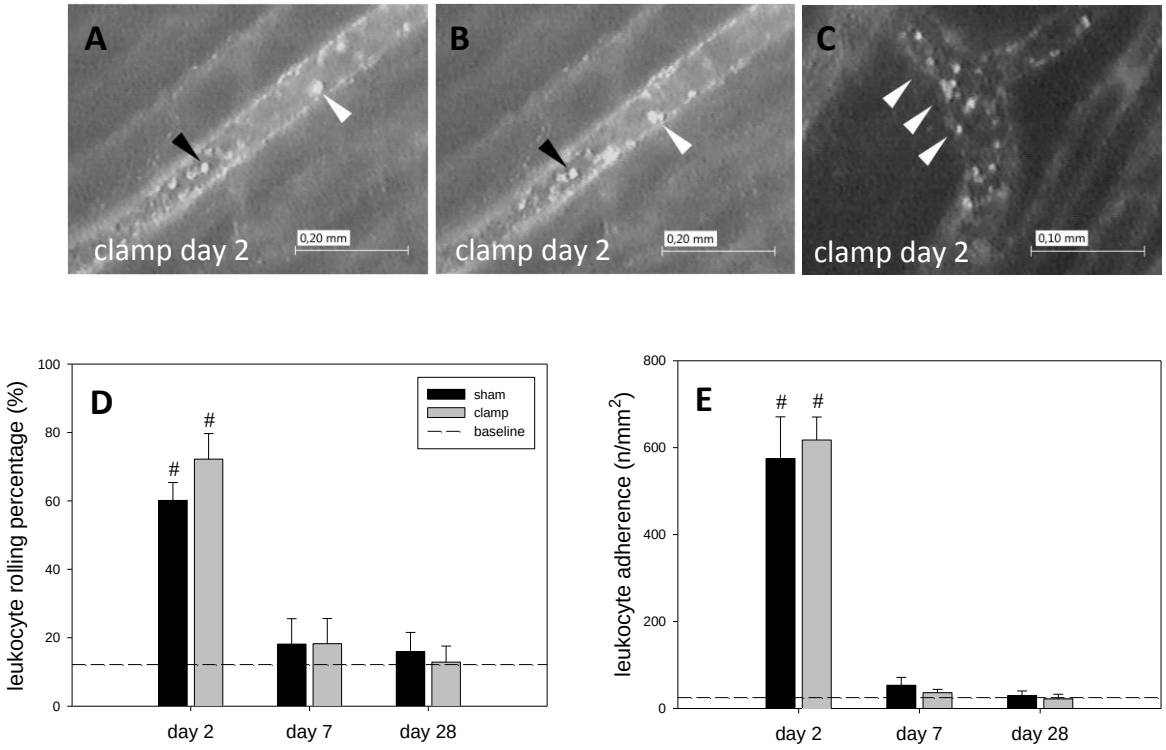

Fig. 15. Assessment of leukocyte-endothelial interaction. A, B, C: Representative images of enhanced leukocyte-endothelial interaction. Beside adhering leukocytes (A, B: black arrow), a rolling leukocyte was moving in a low velocity (A, B: white arrow) (a time gap of 3s between A and B); Increased adhering leukocytes were observed on day 2 (C: white arrows); D: Leukocyte rolling fraction (%); E: Leukocyte adherence (n/mm^2). Values are given as mean ± SEM; One Way ANOVA followed by Holm-Sidak Test, # P<0.05 vs baseline.

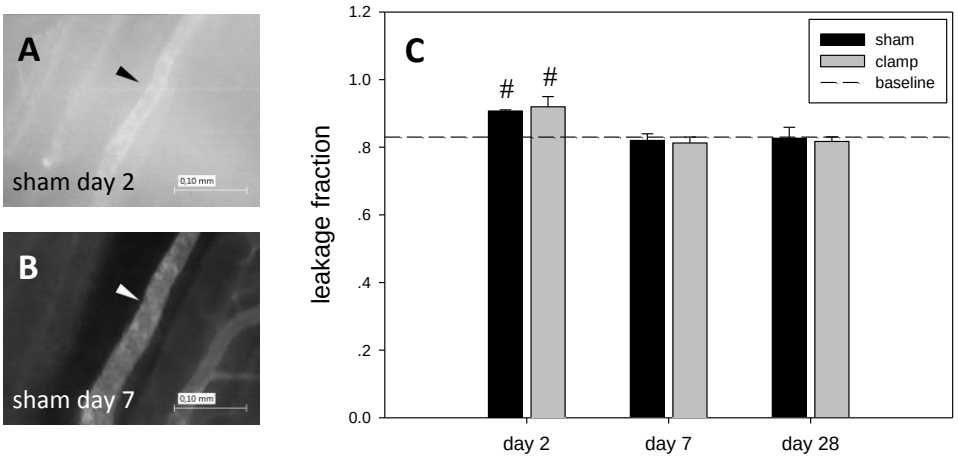

Fig. 16. Assessment of vascular leakage. A: The fluorescent dye leaked into the surrounding tissue due to increased vascular permeability (black arrow); B: The vascular permeability was restored on day 7 (white arrow); C: Vascular leakage fraction. Values are given as mean ± SEM; One Way ANOVA followed by Holm-Sidak Test, # P<0.05 vs baseline.

As shown in **Fig. 15** and **Fig. 16**, the signs of regional inflammation were observed in both groups on day 2 and disappeared on day 7. The clamp group showed no statistical significant difference to the sham group. No evidence of persisting inflammation was detected since all the inflammatory parameters went back to physiological values within 7 days.

6.6 Muscle cell apoptosis

Muscle cell apoptosis in the traumatized soleus muscle was assessed by both in vivo fluorescence microscopy and caspase-3 immunohistochemistry. Enhanced cell apoptosis in the traumatized muscle was detected in both groups. Under the fluorescence microscope apoptotic cells with nuclear condensation were observed. Quantification analysis showed a heightened number of cells with signs of apoptosis on day 2 and day 7 in both groups (**Fig.17**). This finding matched with the results of the caspase-3 cell staining (**Fig.18**). Caspase-3 positive cells were also observed under the light microscope in both groups on day 2 and day 7. This enhanced cell apoptosis was resolved within 28 days and almost absent in both groups. The apoptosis in the clamp group was slightly more pronounced than that of the sham group, however, no statistical significant difference was detected.

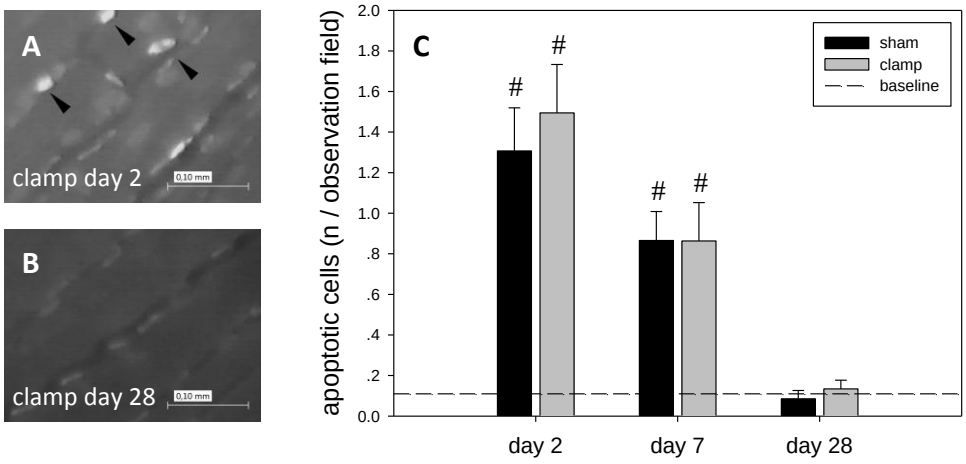

Fig.17. A: Assessment of cell apoptosis by in vivo fluorescence microscopy. A: Bisbenzimide marked myocytes with nuclear condensation were observed on day 2 (black arrow); B: Apoptotic cells were rarely found on day 28; C: Quantitative analysis of cell apoptosis. Values are given as mean ± SEM; One Way ANOVA followed by Holm-Sidak Test, # $P<0.05$ vs baseline.

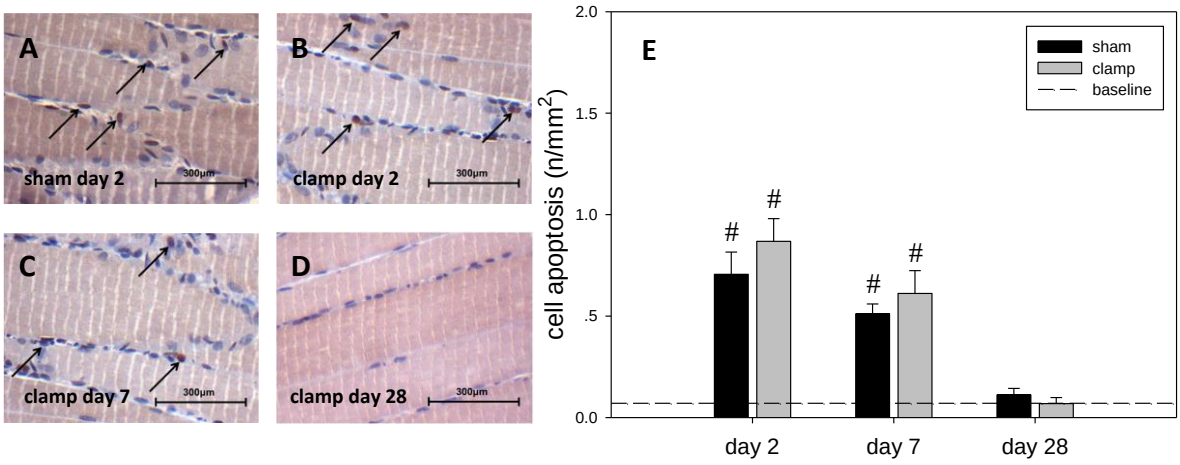

Fig.18. Assessment of cell apoptosis by caspase-3 immunohistochemistry. A, B, C, D: Representative images of caspase-3 staining under light microscope. Increased caspase-3 positive cells (black arrows) were commonly observed within 7 days in both groups (A, B, C). Apoptotic cell death was absent on day 28 (D); E: Quantitative analysis of caspase-3 staining. Values are given as mean ± SEM; One Way ANOVA followed by Holm-Sidak Test, # $P<0.05$ vs baseline.

7 Discussion

CRPS 1 is traditionally thought to be distinguishable from CRPS 2 by the absence of a definite nerve lesion. However, this current dogma of CRPS 1 has been challenged by a series of reports describing minimal nerve injury as a pathophysiologic important feature of CRPS 1 [64.65]. In our study, we combined a closed soft tissue trauma and a minimal nerve injury in order to establish a novel animal model for CRPS 1. A controlled closed soft tissue trauma was applied in the hindlimbs of rats, leading to an acute high-energy impact injury. In addition, the ipsilateral sciatic nerve which is the dominant nerve trunk of the hindlimb was slightly crushed with an instrumented clamp to cause a minimal nerve injury. In the pilot experiment, several settings (force, duration) of the crush injury were tested. The optimized parameters (30 Newton, 20s) were chosen in order to induce a recognizable nerve lesion.

Sensory disturbance, especially pain, is usually the chief complaint of human CRPS. The patients can experience spontaneous pain, allodynia (to a light touch or temperature sensation) and hyperalgesia (to painful stimulation such as pinprick) [15, 81]. In order to assess the pain, spontaneous behavior and nociceptive responses to different stimuli (hot, cold and mechanical stimulations) were observed and quantified. After the surgery, the minimal nerve injury initiated a quick onset of neuropathic pain in the clamp group. The animals developed pronounced signs of neuropathic pain, which included spontaneous pain, hot and cold allodynia and mechanical hyperalgesia. Abnormal positions of the operated paw, which are interpreted as guarding behaviors, were commonly observed in the clamp group. Besides the aberrant postures, the animals with nerve lesion also behaved anxiously and restlessly, especially under the condition of hot (40˚C) and cold (4˚C), which were tolerable to the animals prior surgery. These findings mirror hot and cold allodynia in human, which stands for abnormal pain incited by innocuous stimuli. When applying the mechanical stimuli, the animals with the minimal nerve injury often ran around or elevated their paws to escape from the stimulation. Elevated withdrawal frequencies to mechanical stimuli ranging from 6.0g to 15.0g have been observed from day 2, which indicated mechanical hyperalgesia. The withdrawal responses to lower masses (0.6g, 1.4g and 2.0g) were also found to be enhanced after surgery, but only a

response to 2.0g reached statistical significance on day 7. However, this can be a transient sign of mechanical allodynia.

In contrast, the sham animals showed no sign of pain and behaved calmly during the observation. The single soft tissue trauma did not produce any symptoms of pain. In our animal model, the quick onset of neuropathic pain was dependent on the minimal nerve injury.

Neuropathic pain appeared rapidly after the minimal nerve injury. One possible reason may be hyperexcitability of the peripheral nociceptors. Crush injury to the nerve usually leads to damage of axons and the severity of axonal damage is related to the intensity of the injury [82]. We speculate that the minimal nerve injury led to a partial damage to the affected nerve, meaning that some of the axons were damaged during the surgery and others survived. It was found that partial nerve damage is more likely to induce neuropathic pain than whole nerve transection [40]. The damaged fibers do not directly participate in pain generation. However, they may sensitize adjacent surviving fibers to generate more pain [83, 84]. Wu and his colleagues performed ligation and transection of the L 5 spinal nerve in rats and caused neuropathic pain [83]. Interestingly, spontaneous activity was also recorded in the uninjured L 4 spinal nerve. This spontaneous activity was proven to originate from peripheral nociceptors [83]. In another study of Ali et al, a lesion was induced in the L 6 spinal nerve, but spontaneous activity and adrenergic sensitivity were found in cutaneous C fibers which arose from the adjacent uninjured spinal nerves [84]. These studies indicate that a nerve lesion can lead to changes in uninjured nerves and produce pain. After a nerve lesion, proinflammatory factor (TNF-α), neuropeptides (SP and CGRP) and nerve growth factor (NGF) are secreted by the peripheral nociceptors and Schwann cells of the injured nerve fibers [39, 58, 83]. Since the injured and uninjured nerve fibers coexist in the clamped sciatic nerve, the adjacent surviving neighbors are exposed to the factors released from injured axons. This may be a reason why the sensory disturbance can spread in CRPS related pain symptoms.

TNF-α is released by Schwann cells after a nerve lesion [85, 86] and is proven to play an important role in neuropathic pain [87, 88]. Local application of TNF-α can induce hot hyperalgesia and mechanical allodynia [89-91]. Sommer et al found that the hot hyperalgesia following a chronic nerve injury can be attenuated by a TNF-α inhibitor [92]. TNF-α induced pain can be a direct effect on the primary afferent neurons. TNF receptor-1 and 2 (TNFR-1 and TNFR-2) are expressed in both the dorsal root ganglion

(DRG) and peripheral nociceptors. They are found to be up-regulated following a nerve injury [93-95]. TNF-α activates its receptors in the peripheral nociceptors and leads to a decreased threshold to innocuous stimuli, enhanced response to noxious input and spontaneous activity [93,96]. Besides the direct effect to the primary afferent neurons, TNF-α also has suppressive effects on myelin formation and may impair the nervous conduction [97]. As a proinflammatory factor, TNF-α initiates a cascade of inflammatory factors (IL-1, IL-6 and IL-8). Those factors can induce warmth, redness and edema which are commonly observed in both inflammation and CRPS [89, 98].

SP and CGRP may sensitize the peripheral nociceptor and produce mechanical allodynia via IL-1 β signaling [99]. Besides the neuropeptides, NGF may also play a role in the development of pain since anti-NGF treatment was found to attenuate the pain behavior in the tibial fracture model [100]. After the Schwann cells, another source of NGF in the affected region is the keratinocyte. It was found that SP stimulates the neurokinin-1 (NK-1) receptor expressed on keratinocyes and mediates an up-regulation of NGF and TNF-α [101]. A peripheral axon injury can also lead to changes in the affiliated cell body in the DRG and the neurons in the dorsal horn [58, 83, 102]. Neuropeptides (SP, CGRP) and their receptor proteins were also up-regulated centrally in the dorsal horn following a peripheral nerve injury [42, 83]. The peripherally released neuropeptides (SP, CGRP) and NGF can be taken up and transported to the central neurons by afferent nerve fibers (mainly C fibers) [83, 103]. SP can activate the neurokinin-1 G-protein–coupled receptor in the dorsal horn and lead to a long lasting membrane depolarization [104, 105]. CGRP participates in the development of central sensitization by activating the postsynaptic CGRP-1 receptor [106]. The expression of the CGRP-1 receptor proteins can be enhanced by NGF [13]. This complicated network suggests a synergy effect of different factors.

Except the different factors released after nerve lesion, myelin damage and remodeling can be another reason of neuropathic pain. Clamping injury can damage the myelin sheath of small myelinated fibers (Aδ-fibers) [82]. The myelin sheath is essential for maintaining a stable velocity of conduction and preventing the interferences between different axons. Damage to the myelin is often the cause of sensory disorders. Demyelination is the main pathological change of multiple sclerosis, Guillain-Barre syndrome and diabetic neuropathy which are also characterized by neuropathic pain and sensory disturbance [107]. Zhu et al reported that neuropathic pain developed immediately after cobra venom induced demyelination in a sciatic

nerve if rat [108]. In this study, besides the conductive impairment of A-fibers, the unmyelinated C fibers were also affected. Spontaneous antidromic activity and hyper-excitability of C-fiber polymodal nociceptors were detected following demyelination. The antidromic activity of C fibers was reduced by stimulating the A-fibers in the ipsilateral sciatic nerve and appeared again when stopping the stimulation [108]. This may suggest that the normal activity of myelinated A-fibers can inhibit the ectopic activity of C-fibers. It was proposed that the GABAergic and glycinergic inhibitory neurons in the dorsal horn, which were activated by the input of A-fibers, are responsible for maintaining normal sensory signaling [109, 110]. Activation of inhibitory interneurons can depress the central terminals of C-fibers and prevent the antidromic activity and consequent hyperexcitability of peripheral nociceptors [108].

As in our animal model, the impaired nerve conduction of the sciatic nerve was proven by electromyography, which can be an evidence of myelin damage. An impaired input of A-fibers might lead to dysfunction of the central inhibitory interneuron and hyper-excitability of peripheral nociceptors of C-fibers. The surviving nociceptors might also be sensitized by the factors which were released after partial nerve damage. Those changes would lead to pronounced symptoms of neuropathic pain which appeared rapidly after nerve clamping. As we can see from the present results, the ratings of spontaneous pain and allodynia were found elevated on day 2, peaked between day 4 and day 7. Then the ratings of pain began to decline towards zero. As a result, the spontaneous pain and allodynia vanished on day 28. The upward curve represented the progressive onset of neuropathic pain while the downward curve could be interpreted as a process of recovery.

Haftek and colleagues examined the microstructure of clamped nerves by electron microscopy in 1968. He found that, besides the damage of axons and myelin, the integrity of the endoneurial tubes and Schwann cell basal lamina altered [82]. In our study, we clamped the sciatic nerve slightly in order to induce a minor injury. So we postulate that the integrity of the endoneurial tubes and Schwann cell basal lamina of the clamped nerve were still intact. Intact membrane tubes offer a good environment for regeneration, since the broken axons were still in their parent tubes. The endoneurial tubes can guide the regenerating axons towards their appropriate targets. Remyelination can also be well supported by the intact Schwann cell basal lamina [111]. As a result, the process of nerve regeneration can be faster and more accurate after a minimal nerve injury. The nerve begins to regenerate in a few hours after the lesion.

The regenerating axons sprout from the proximal side of the injured nerve and grow into the endoneurial tubes [111]. Those newborn sprouts fire easily and can develop spontaneous activity, which may lead to pain [83], as seen in the present study.

At the same time, the distal segment of the injured nerve began the regeneration by Wallerian degeneration. Wallerian degeneration is an essential part of the nerve regeneration [111]. It happens after a nerve injury and begins in the distal stump of the injured axons, usually 24-36 hours after injury [112]. The Wallerian degeneration includes three stages, which are axon and myelin degeneration, debris clearance and regeneration [113]. The axons and myelin in the distal stump degenerate soon after the nerve injury. Schwann cells and macrophages are activated and begin to phagocytize the myelin fragments and axonal debris. Of note, several inflammatory factors (IL-1, IL-6, TNF-α), cytokine (GM-CSF) and nerve growth factor (NGF) are released by the activated Schwann cell [114]. TNF-α can sensitize the peripheral nociceptors, as mentioned above. The neuropeptides may affect the nociceptive afference via IL-1 β signaling [99]. Those factors might play an important role in the development of neuropathic pain, since they can affect the surviving fibers which are mixed with the degenerating nerve fibers. In a study of La Fleur M et al, the level of TNF-α was found to increase progressively and peak on day 7 distal to a crushed nerve [115]. An earlier peak was showed in the study of Rotshenker et al, they reported a peak of TNF-α and IL-1β on day 2 after nerve lesion [114]. As in our model, the pain ratings of spontaneous pain and allodynia were found elevated on day 2 and peaked between day 4 and day 7. The burst of neuropathic pain in the first week after nerve lesion can be closely related to the production of several factors during Wallerian degeneration. The neuropathic pain was found to be alleviated on day 14 and vanished after 28 days, which can be a consequence of a down-regulation of pain-inducing factors.

The quick production of pain-inducing factors is followed by a secretion of anti-inflammatory factors such as IL-10. IL-10 is mainly produced by the subsequently recruited macrophages. IL-10 gradually down-regulates the inflammatory factors, finally bring the cytokine secretion in Wallerian degeneration to a conclusion in 2 or 3 weeks [114]. This down-regulation of pain-inducing factors may be reflected by the downward curves of neuropathic pain in the present study.

After the stage of debris clearance, the regeneration begins. Schwann cells line up in the endoneurial tubes and synthesize NGF, which attracts axonal sprouts from the proximal segment of the injured nerve [114, 116]. Remyelination happens at the same

time. Schwann cells form a line termed Bands of Bungner within the endoneurial tube, guiding the production of new formed myelin [117]. The remyelinated fibers regain their function gradually along with the remyelination. As described above, the activity of myelinated A-fibers may activate the interneurons in the dorsal horn and regulate the nociceptive afference. The regained activity of A-fibers can depress the hyper-excited polymodal nociceptors. As a result, the remyelination is parallel with the relief of pain. As we can see from our study, the gradually recovered nerve conduction is reflected by the electromyography. Following the anomalous readings on day 2 and day 7, the clamp group showed approximately normal curves on day 28, but with a prolonged latency. The regenerated myelin is thinner than normal and the internodes between the newly formed Ranvier nodes are also shorter, which can explain the slowed velocity and prolonged latency [118].

Besides the spontaneous pain and allodynia, a mechanical hyperalgesia was also detected in the clamp group. Several studies suggest that, mechanical hyperalgesia is due to changes of the central nervous system [42, 119, 120]. One possible mechanism of hyperalgesia is a activity dependent central sensitization, which stands for central sensitivity induced by repeated noxious stimulations [42]. In a study, Wall and colleagues applied a brief low frequency electrical stimulation to the afferent C-fiber for 20s and induced pain hypersensitivity for up to 90min [42]. In another study of Hathway et al, the electrical stimulation for 5min led to a mechanical hypersensitivity for 48 hours [121]. This hypersensitivity may be induced through activation of TRPA1 and TRPV1 channels in the dorsal horn neurons [122, 123]. When applying mechanical stimulation in our study, an interesting phenomenon was observed. The first trial of stimulation usually did not induce many withdrawal responses. The withdrawal frequency began to increase with the second or the third trial. The first trial of repeated mechanical stimulation might serve as an induction of mechanical hypersensitivity under the condition of nerve lesion.

Of note, compared with other signs of neuropathic pain, the mechanical hyperalgesia was more durable. After day 7, a trend of alleviation was detected in the mechanical hyperalgeisa as we could find that the response frequency of clamp group had greatly decreased on day 28. However, the response frequency of the clamp group was still significantly higher than that of the sham group on the final day of experiment, which reflects a prolonged mechanical hyperalgesia. This result is in accordance with the findings of Rotter et al. In this study, spontaneous pain, hot and cold allodynia and

mechanical hyperalgesia also developed after a sciatic nerve crush injury (25 Newton, 30s). Rotter and colleagues performed observations for a longer period of 42 days. The animals with nerve injury were still showing a mechanical hyperalgesia on day 42 when other signs of pain had already disappeared [127]. These findings suggest that hyperalgesia may be the most persistent type of neuropathic pain, which develops after a nerve lesion.

Besides the neuropathic pain, human CRPS also presents autonomic changes, such as red and glossy skin, altered temperature and edema [15]. Some of these symptoms are similar to inflammatory responses. As it was already mentioned above, neuropeptides (SP, CGRP) are released by peripheral nociceptors following a nerve lesion [58]. The neuropeptides may lead to changes as plasma extravasation and vasodilation as well as to warmth, redness and swelling [103]. Based on this, some researchers proposed a neurogenic inflammation in CRPS [44].

In the present study, the alterations of temperature and volume in the paws were assessed. Subsequently, we performed in vivo high resolution fluorescence microscopy in order to check the inflammatory response and perfusion failure in the traumatized muscle. As a result, we found an inflammatory response, local perfusion failure and temperature asymmetries in the clamp group. However, these symptoms comparably appeared in the sham group. This may suggest that these changes are results of a posttraumatic inflammation following the blunt muscle trauma rather than a neurogenic inflammation.

On day 2, signs of muscular inflammation and local perfusion failure were observed in both groups. The high-energy soft tissue trauma led to an acute damage to the small vessels and capillaries in the traumatized area and contributed to a regional inflammation with increased vascular permeability and plasma leakage as well as enhanced leukocyte-endothelial interaction) [124]. Capillary damage and posttraumatic muscular edema contributed to decreased FCD and further impairment of tissue oxygen exchange, which was reflected by an elevated level of NADH autofluorescence. The rapidly developed regional inflammation and microvascular deterioration were almost absent on day 7. The temperature asymmetries were heightened in both groups on day 2 and day 4. The operated side was warmer than the contralateral side. This can be explained by an inflammatory vasodilation. The temperature asymmetries also resolved on day 7 along with the local inflammation. Besides the temperature asymmetry, no volume change was detected in the remote paws and the edema was

restricted to the traumatized muscle. All the signs of inflammation and autonomic changes were alleviated within 7 days, but the neuropathic pain in the clamp group was still ongoing. In summary, this finding does not support a neurogenic inflammation being the reason of neuropathic pain in the present study.

The enhanced apoptosis in both groups was confirmed by both fluorescence microscopy and caspase-3 immunohistochemistry. This enhanced apoptosis can be a result of direct cell injury by the closed tissue trauma [74]. The subsequently released TNF-α also has a weak apoptosis inducing effect and it might particularly promote the apoptosis in the acute phase of regional inflammation [71]. Interestingly, there was no difference detected between the clamp and the sham group, though a nerve injury is usually a reason for apoptosis in the innervated muscle [125, 126]. In another study of Rotter et al, crush injuries were applied to both the soleus muscle and the ipsilateral sciatic nerve of rats. They found that compared with the groups with single muscle injury, muscle cell apoptosis was enhanced in the animals with both muscle and nerve injuries on day 1 and day 7 [127]. As in our study, the muscle cells apoptosis in the clamp group was also found to be more pronounced on day 2 and day 7, but no statistical significant difference was detected. The reduction of apoptosis in both groups within the consequent 28 days may reflect the process of recovery from tissue trauma in 28 days.

Our animal model successfully induced neuropathic pain which is the most important symptom of clinical CRPS. It is reasonable to state that a nerve lesion, even a minimal nerve injury, is sufficient to induce neuropathic pain for several weeks. This finding confirms the close relationship between nerve lesion and neuropathic pain. Compared with the needlestick distal nerve injury model (DNI), the current method mimics the trauma situation in humans better. However, as in our study, animals with minimal nerve injury presented all the phenotypes of pain. Additionally, the course of pain showed a high consistency. All the animals of the clamp group developed neuropathic pain on day 2 and recovered from most of the pain symptoms on day 28. This high reproducibility may let the minimal nerve injury become a standard method for producing temporal neuropathic pain. However, it is not able to perfectly model the natural incidence and pain course of human CRPS, since only a fraction of people with trauma or nerve lesion would develop CRPS and the courses of pain are very different from case to case. This is also the problem which most of the animal models suffer from [69, 70, 73]. Interestingly, this point was emphasized in the DNI model by

Oaklander et al. They used the term “prevalence” to describe the data since only a portion of the experimental animals with DNI developed neuropathic pain and other symptoms, which may better model a prevalence of human CRPS [72].

On the other hand, the spontaneous pain, hot and cold allodynia in the present study disappeared in 28 days. The mechanical hyperalgesia lasted longer, but it also showed a trend of alleviation, which might be attribute to the process of nerve regeneration, as the mechanical hyperalgesia in the clamp group disappeared later. Actually, most of the human CRPS cases can also remit or recover spontaneously, but the pain in human CRPS usually persists longer, sometimes from months to years. In the DNI model, some animals with mechanical hyperalgesia have already recovered on day 14 and only one rat developed cold sensitization [72]. The mechanical hyperalgesia induced in the model of exaggerated soft tissue trauma (ETR) also lasted for only 14 days while hot and cold allodynia were absent [73]. In our study, the minimal nerve injury was accompanied with several phenotypes of pain (spontaneous pain, hot and cold allodynia and mechanical hyperalgesia) for longer period, but it is still not able to induce ongoing pain. The results of electromyography reflected the conductive function of the injured nerve and also presented a process of recovery in 28 days. This trend of recovery proceeded along with the alleviation of neuropathic pain. This finding in the present study may provide an evidence to support the idea that myelin damage is of pathophysiological importance in the development of neuropathic pain.

Besides the neuropathic pain, inflammatory responses, muscular edema and temperature changes were detected in the operated limbs. However, those changes were transient in nature, similarly to what Gradl et al found in the model of exaggerated soft tissue trauma (ETR) [73]. These symptoms are more likely to represent posttraumatic sequelae induced by the closed soft tissue trauma instead of being neurogenic, since both groups with tissue trauma developed regional inflammation, muscular edema and altered temperature regardless the presence of nerve lesion. This is in agreement with the results of the ETR model [73, 74]. Accordingly, the neurogenic edema and temperature alteration were also absent in the DNI model [72].

Loss of epidermal neurites and small fiber degeneration were reported in the human CRPS 1 affected limbs [64], which indicated a connection between nerve lesion and CRPS 1, but the distinct mechanism how a minor nerve lesion leads to ongoing changes in CRPS 1 can still not be explained. The method of chronic nerve constriction injury (CCI) is capable to induce long-lasting neuropathic pain and trophic changes,

but it is more likely to cause a remarkable nerve lesion which is typical for CRPS 2. Questions of how a minor nerve lesion and which method of minor nerve injury can induce ongoing autonomic and trophic changes are still not clear. Of note, in the present study, the minimal nerve injury produced pain, while the closed soft tissue trauma was responsible for the regional inflammation, perfusion failure, autonomic changes and muscle cell apoptosis. In the clinic, most of the nerve lesions are accompanied by tissue trauma, so the features of human CRPS 1 are possibly due to a combined effect of minor nerve lesion and tissue trauma.

In summary, this novel animal model of closed soft tissue trauma combined with a minimal nerve injury has successfully produced CRPS-like symptoms, such as neuropathic pain and trophic changes. However, the high prevalence and short term of the symptoms are still not able to fully model the development of human CRPS. Anyhow, we have established a framework of new animal model for CRPS, different settings and this method of nerve lesion can be applied for further studies.

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11 DECLARATION OF ORIGINALITY

I hereby declare on oath,

- that I have produced this doctoral thesis by myself and did not use any sources and resources other than the ones stated,

- that I did not have any other illegal help, and

- that the present doctoral thesis has not been presented in any shape or form for examination at any other national or international institution than at the Medical Faculty, University of Rostock.

Rostock, October 16, 2012

Zi Wang