Thermal nociception using a modified Hargreaves method in primates and humans

Zhengwen Ma, PhD[a,1]
Yao Li, PhD[a,1]
Yi Ping Zhang, MD[b,1]
Lisa B.E. Shields, MD[b]
Qing Xie, MD[c]
Guofeng Yan, MS[a]
Wei Liu, PhD[a]
Guoqiang Chen, PhD[a]
Ying Zhang, PhD[d]
Benedikt Brommer, PhD[f]
Xiao-Ming Xu, MD, PhD[e]
Yi Lu, MD, PhD[g]
Xuejin Chen, PhD[a]
Christopher B. Shields, MD[b]

[a] School of Medicine, Shanghai Jiao Tong University, Shanghai, China
[b] Norton Neuroscience Institute, Norton Healthcare, Louisville, KY, USA
[c] Department of Rehabilitation Medicine, Ruijin Hospital, Shanghai Jiao Tong University, Shanghai, China
[d] Department of Rehabilitation Medicine, Xuhui District Central Hospital, Shanghai, China
[e] Spinal Cord and Brain Injury Research Group, Stark Neurosciences Research Institute, Department of Neurological Surgery, Indiana University School of Medicine, Indianapolis, IN, USA
[f] Department of Neurology, Harvard Medical School, Boston, MA, USA
[g] Department of Neurosurgery, Harvard Medical School, Boston, MA, USA

Correspondence to:
Christopher B. Shields: cbshields1@gmail.com
Xuejin Chen: chenxuejin@shsmu.edu.cn

Summary

Nociception is an important protective mechanism. The Hargreaves method, which involves measuring withdrawal latency following thermal stimulation to the paw, is commonly used to measure pain thresholds in rodents. We modified this technique to measure pain thresholds in monkeys and humans.
The modified Hargreaves method was used to quantitate pain sensitivity in eight normal rhesus monkeys, 55 human volunteers, and 12 patients with spinal cord or cauda equina lesions. Thermal stimulation was delivered at 80% of maximum output, and the duration of the stimulation was set at a maximum of 10 seconds to avoid skin injury.
The following withdrawal latencies were recorded: 2.7±0.12 seconds in volunteers and 3.4±0.35 seconds in neurologically intact monkeys ($p>0.05$). Patients with spinal cord or cauda equina lesions showed significantly increased latencies ($p<0.001$).
The modified Hargreaves technique is a safe and reliable method that can provide a validated measure of physiological pain sensation.

KEY WORDS: Hargreaves, monkeys, pain, spinal cord injury, threshold

Introduction

Sequential quantitative assessment of sensory pathways is an approach used in humans to monitor the progression of neurological diseases that affect epicritic (non-crossing) and protopathic (crossing) sensory pathways and to measure response to treatment. Objective measures, such as quantitative sensory tests, can be used to measure different sensory functions: touch, pain, pressure, vibration and temperature (Shy et al., 2003). However, these measures have limitations because they provide inconsistent results (Pavlaković and Petzke, 2010). Equipment specifically designed to measure sensation, such as Medi-Dx 7000™ (Vax-D Medical Group, Tucson, AZ) and Neural-Scan (JP Medical, Dallas, TX) are often unreliable (Tack et al., 1994). No reliable, quantitative and cross-species validated measure of physiological pain sensation is currently available, even though such measures would be valuable to assess nociception in many neurological conditions.
Transient receptor potential (TRP) channels are responsible for mediating heat and pain detection of skin in vertebrates (Glauser et al., 2011; Yao et al., 2010; Zhu, 2007). TRPV1 and TRPM8 are sensory ion channels activated by heating and cooling, respectively. These thermosensitive TRP channels are cellular

[1] Zhengwen Ma, Yao Li, and Yi Ping Zhang are co-first authors with equal contributions to this manuscript.

temperature sensors and they are crucial in the maintenance of a stable core body temperature and in responses to the ambient environment (Yang et al., 2010). Heat-evoked pain in skin is mediated by thermosensitive TRP channels via unmyelinated C fibers and transmitted to the brain via specific, segmentally crossing, spinothalamic sensory pathways in the spinal cord and brain (McGlone and Reilly, 2010; Tominaga, 2010). The receptors belonging to the heat-activated TRPV1 subfamily send a warning signal to the brain to avoid heat-induced injury (Schumacher, 2010).

The Hargreaves method was designed as a means of quantitatively measuring cutaneous pain thresholds in rodents (Hama and Sagen, 2007; Hargreaves et al., 1988; Hirose et al., 2010; Kang et al., 2010; Kocevski and Tvrdeić, 2008; Ratcliffe et al., 2011; Sandhir et al., 2011; Xu et al., 2012). The method uses a standard baseline temperature applied to the skin in unrestrained rodents. Pain thresholds are measured as hindlimb withdrawal latencies from a painful stimulus (power flux density measured in mW/cm^2) (Hargreaves et al., 1988). The painful stimulus is created using a standard thermal device, and withdrawal responses are quantitatively recorded. The time interval from thermal stimulus delivery to pain perception depends on the gradient between the heat source and the baseline temperature. The device has not previously been used in monkeys or humans. In this study, we used the Hargreaves method to measure pain thresholds in normal primates and neurologically intact volunteers.

Materials and methods

Experimental subjects

Eight adult rhesus monkeys (Macaca mulatta) were used (6 females and 2 males, age range: 43-46 months; body weight range: 4.6-5.6 kg). They were purchased in Chengdu Sichuan Province, PRC. The monkeys were housed in their own individual cages measuring 110 cm long, 82 cm wide, and 80 cm high. The cages were cleaned twice daily. Air was purified through primary and medium efficiency filters, dehumidified and maintained at a constant temperature (20-24°C). An automatic lighting system was set for 12 hours on and 12 hours off. Monkeys were allowed to drink *ad libitum* and were fed twice a day with standard puffed monkey feed (100 g each feeding) (Beijing KeAoXieLi Feed Co., Ltd, Beijing). The animals were also fed an apple (100 g) or half a banana daily, at noon, as well as nuts (50 g) and half an egg twice a week. Body temperature was controlled by placing a disposable vest on the monkey postoperatively in addition to maintaining the room temperature at 25-30°C.

Before the monkeys were sacrificed, they were deeply anesthetized with ketamine hydrochloride (50 mg/kg im) until no heartbeat could be detected using a physiological monitor (MultiParameter DigiMAP DM 8X, Digicare Biomedical Technology, Boynton Beach, FL). Immediate transcardial perfusion with 1,500 ml physiological buffered saline (PBS) was then performed, followed by irrigation with 4,000 ml of 4% paraformaldehyde in PBS.

Fifty-five neurologically healthy human volunteers were recruited for evaluation of normative pain thresholds. They comprised 29 females and 26 males, ranging in age from 22 to 55 years. Their body weight ranged from 41 to 95.5 kg. Neurologically intact monkeys and volunteers were subjected to thermal stimuli to determine their safe levels of stimulation. Twelve patients with chronic sensory deficits of the legs caused by spine trauma, metastatic neoplasm, or vascular lesions affecting the spinal cord or cauda equina (Table I) were recruited. These patients tested our hypothesis that the Hargreaves system can be used to quantitatively assess pain sensation. If a response was not elicited within 10 seconds, the thermal stimulus was discontinued. The International Standards for Neurological Classification of Spinal Cord Injuries score (Kirshblum et al., 2011) was used to record neurological deficits following non-traumatic and traumatic injuries.

No analgesic drugs were used in the monkeys or humans involved in this study.

Ethics statement

All monkey surgeries, postoperative care and behavioral studies were conducted at the Department of Laboratory Animal Sciences at Shanghai Jiao Tong University School of Medicine. All surgical interventions, treatments and postoperative animal care procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, USA, 1996). The animal use protocol for primates subjected to spinal cord injury (SCI) was approved by the Animal Experiment Ethics Committee of the Shanghai Jiao Tong University School of Medicine (Approval number: 2013074).

The human volunteers, both the neurologically intact subjects and the spinal cord-injured patients, were required to read and sign a consent form before the test was performed. The consent document included an introduction as well as information relating to the study: the aim, the risks, costs or rewards, information security, the principles of voluntary participation, and the names of the executors of the research project, contacts, authorities, and institutional supervision. These consent procedures were reviewed and approved by the Institutional Review Board (Ethics Committee, Ruijin Hospital affiliated with the Shanghai Jiao Tong University School of Medicine) (Approval number: 2013-01). All participant information was anonymous.

Hargreaves apparatus and calibration of the thermal stimulus

The Hargreaves apparatus (Harvard Apparatus, Holliston, MA) is a thermal pain stimulation and record-

ing device designed to measure hyperalgesia to thermal stimulation in unrestrained animals (Hargreaves et al., 1988). It is used extensively in pain and SCI research in animals (Horiuchi et al., 2010; Miyasaka et al., 2006; Pavić et al., 2011; Upadhya et al., 2011). The device measures the pain threshold at the plantar surface of the hindpaw by heating the footpad through a glass platform. Paw withdrawal to pain causes a sudden reduction in reflected infrared radiation, and this turns off the heat source and stops the reaction time counter. The latency of paw withdrawal is a measure of the pain threshold and can be recorded with an accuracy of ± 0.1 second. Each plantar test is calibrated via an infrared radiometer to ensure delivery of the same power flux (mW/cm^2) and, thus, a consistent nociceptive stimulus.

The heat stimulus was adjusted to 80% of maximal output. Thermal calibration curves for this system were recorded comparing temperature to duration of stimulation. A digital thermometer (TES-1311A, TES Electrical Electronic Corp., Taipei, Taiwan) was placed against the glass plate of the Hargreaves apparatus at the site of heat stimulation. The temperature of the glass plate was incrementally increased and was

Table I - Summary of 12 patients with neurological deficits.

Sex	Age	Injury level	ASIA score	Injury description	Pain Response (sec) IFIS R/L	BBT R/L
M	45	C3	B	Traumatic SCI with vertebral body fracture, treated with a C3-4 vertebral body fusion. Quadriplegia and sensory deficits below C3.	5.8/5.5	2.4/2.7
M	54	C8	B	Traumatic SCI with vertebral fracture and C7 dislocation. Anterior cervical vertebrectomy. Paraplegia and sensation deficits below C8.	10/10	10/10
M	55	T7	C	Metastatic spinal cord compression caused by T7 vertebral body tumor with epidural extension. Paraparesis of the legs with decreased sensation.	4.5/5.1	3.5/2.5
M	41	C4	D	Severe cervical myelopathy secondary to spondylosis treated by multilevel laminectomy. Minor weakness and severe right-sided numbness below C4.	10/2.3	10/4.1
F	32	L2	B	Traumatic L2 vertebral burst fracture causing a cauda equina lesion. Resultant paralysis below L2 and severe numbness of the legs.	10/10	10/10
M	66	L3	C	Traumatic cauda equina injury due to L3 burst fracture. Anterior-posterior decompression and fusion. Lower extremity weakness and moderate numbness.	5.9/7.1	7.9/5.7
F	29	T12	A	Traumatic T12 burst fracture inducing complete SCI with paraplegia, loss of sensation, and loss of sphincter control.	10/10	10/10
M	55	C5	C	Metastatic tumor of C5 with an anterior spinal cord compression. Resultant moderate weakness and moderate numbness below C5.	5.4/5.2	3.6/3.4
M	72	C4	C	Traumatic cervical SCI with OPLL treated with laminectomy and fusion from C3 to C7. Resultant moderate weakness and moderate numbness below C4.	4.9/5.4	10/10
M	62	T10	D	Dural arteriovenous fistula at T10 treated surgically with resultant damage to the thoracic spinal cord. Muscle weakness and mild numbness of the legs.	2.7/3.1	5.3/2.6
M	68	C3	D	C3 cavernous hemangioma of the spinal cord that bled. Chronic mild weakness on the right and numbness on the left below the lesion (Brown-Sequard syndrome).	2.7/5.3	3.5/3.5
M	18	L2	C	Traumatic L2-3 vertebral body burst fracture treated with decompression and fusion. Resulted in moderate weakness of the legs and numbness of legs (R>L).	5.3/4.1	8.0/6.4

Abbreviations: L=left; R=right; C=cervical; T=thoracic; L=lumbar; BBT=base of big toe; IFIS=instep front inner side; SCI=spinal cord injury; OPLL=ossification of the posterior longitudinal ligament.

recorded from the thermometer every 0.2 seconds. Thermal calibration curves were replicated three times to ensure reliability and accuracy.

Pain threshold assessment

To assess pain threshold, monkeys were restrained on a chair (Houhuang Animal Laboratory Equipment Co., Suzhou, PRC) with the height of the chair adjusted to allow their hindpaws to rest comfortably on the 35x80 cm glass platform (Fig. 1A) (Hargreaves et al., 1988). They wore goggles to avoid hindpaw withdrawal caused by visual stimulation. The lower limbs were not restrained and could be withdrawn from the glass plate when the animal experienced pain.

The plantar surface of the hindpaw was initially pre-warmed to a baseline temperature of 37°C by placing the hindpaw on the glass platform for ≥ 2 minutes before commencing thermal stimulation. The heat was applied to precise sites on the hindpaw visualized by means of a mirror located beneath the glass plate. The focused thermal stimulating beam passed through the glass plate and was applied to two sites on the hindpaw: i) between the first and second metatarsal bones and inferior to the fibular sesamoid (instep front inner side, IFIS) and ii) the base of the big toe (BBT). These sites were pre-marked to facilitate application of the thermal stimulus to the precise target (Fig. 1B). The maximal output of the infrared source was set at 80%, and the maximal duration of stimulation was 10 seconds in monkeys and humans to avoid injury to the skin. The heat stimulus was terminated as soon as the monkeys or humans (volunteers or patients) experienced pain and/or withdrew their hindpaw/foot. The latency from the onset of thermal stimulation to hindpaw/foot withdrawal was automatically recorded by an internal timer in the Hargreaves device. Pain adaptation to thermal stimulation was avoided in this study by not repeating the test in the same subject within three days (Hashmi and Davis, 2008).

The monkeys were assessed for the possible presence of skin redness and blistering at one hour and 24 hours following thermal stimulation, and pain at the stimulated site was evaluated by applying pressure to the site of stimulation. Pain in humans was assessed according to the presence of pruritis or discomfort with or without pressure. Analgesics were not administered.

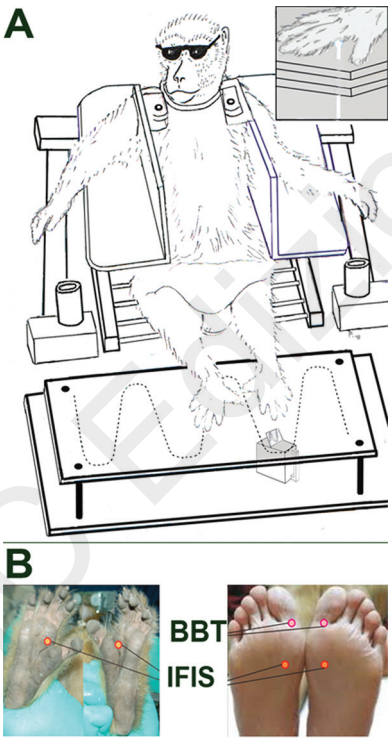

Figure 1 - Position of monkey on the Hargreaves device.
(A) Monkey in a restraining chair during measurement of withdrawal latency to thermal stimulation. The chair was adjusted so that the hindpaws rested comfortably on the glass surface of the Hargreaves device. Goggles were used to avoid any response caused by visual stimulation. The infrared beam targeted the plantar skin through the glass plate (insert). The temperature of the glass platform was set at a baseline value of 37°C before testing to reduce response variability. (B) The plantar sites of skin stimulation (orange spots) in monkeys and humans [base of big toe (BBT) and instep front inner side (IFIS)].

Histology of the thermal stimulated and non-stimulated plantar skin

At one hour and 24 hours after thermal stimulation (80% maximal stimulus for 10 seconds), two monkeys (one monkey at each time point) were anesthetized with a ketamine (200 mg)/xylazine (20 mg) cocktail and sacrificed. They were transcardially flushed with 1,500 ml PBS to remove blood, followed by irrigation with 4,000 ml of 4% paraformaldehyde in PBS (pH=7.4). Full-thickness skin specimens from the stimulated left hindpaw and from the corresponding area of the non-stimulated right hindpaw were removed and post-fixed in paraformaldehyde for 24 hours, then washed and preserved in 75% ethanol overnight. The specimens were dehydrated in ethanol at concentrations of 85%, 95% and 100%, respectively. After cleansing in xylene, the skin samples were embedded in high melting point paraffin. The skin sections were 4 µm thick. For routine H&E staining, slides were warmed to 68°C for one hour. Sections were stained in hematoxylin for 5-6 minutes at room temperature. Excess dye was removed by 10 minutes of water irrigation. The sections were rinsed in 0.5% HCl/ethanol solution, and treated with eosin for 30 seconds. After rinsing five times with 95% ethanol, stained sections were dehydrated and coverslipped for microscopic examination (Zeiss AX 10, Carl Zeiss MicroImaging, LLC, Thornwood, NY).

Immunohistochemistry staining was performed (Liu et al., 2006). Sections were warmed at 37°C overnight, rinsed with PBS (0.1 M, pH 7.4), and then treated with 3% H_2O_2 for one hour to quench endogenous peroxidase. After rinsing with PBS, sections were incubated in a blocking solution (Invitrogen, Carlsbad, CA) for one hour. Primary antibodies included mouse anti-CD68 (1:200, Abcam, Cambridge, MA), mouse anti-TNF alpha (1:100, Abcam), mouse anti-IL6 (1:200, Abcam), and

mouse anti-IL10 (1:100, Abcam). All antibodies were diluted in 1% BSA (Sigma, St. Louis, MO)/PBS (pH 7.2), and sections were incubated with the individual antibodies overnight at 4°C. On the second day, sections were incubated at 37°C with a secondary antibody (Invitrogen) for 30 minutes. After incubation with HRP-streptavidin (Invitrogen) at 37°C for 30 minutes, the reaction complex was visualized with 3,3-diaminobenzidine (Dako, Carpinteria, CA). All sections were then dehydrated with graded alcohols, cleared with xylene, and coverslipped. The specificity of labeling was confirmed by using mouse primary antibody isotype control (Invitrogen).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 6.00, GraphPad Softward, Inc., La Jolla, California, USA). All data are presented as mean ± S.D. One-way analysis of variance (ANOVA) was performed followed by LSD post-hoc or Tukey's multiple comparison tests. A p-value of <0.05 was considered statistically significant.

Results

The average withdrawal latency in the 55 volunteers was 2.7±0.12 seconds compared with 3.4±0.35 seconds in the eight neurologically intact monkeys ($p>0.05$). However, patients with spinal cord or cauda equina lesions demonstrated significantly increased latencies, and at 14/48 sites the thermal stimulation had to be discontinued at 10 seconds ($p<0.001$).

Thermal calibration of the Hargreaves device

Using 80% maximum thermal output, the temperature rose from the baseline value of 37ºC to 53.25±2.01ºC at one second, 90.53±0.36ºC at five seconds, and 111.47±2.23ºC at 10 seconds. The temperature increase was linear to the duration of exposure to the heat stimulus. Temperature curves were repeated three times, and the results were highly reproducible (±1.365, SD) (Fig. 2).

Pain thresholds of monkeys and neurologically healthy human volunteers

The latency of hindpaw/foot withdrawal in response to thermal stimulation was recorded in eight neurologically intact monkeys and 55 neurologically intact humans (Fig. 3). The mean latency to withdrawal in neurologically healthy humans was 2.7±0.12 seconds (2.6±0.17 seconds on the right and 2.8±0.17 seconds on the left). The mean withdrawal latency in the monkeys was 3.4±0.35 seconds (3.0±0.42 seconds on the right and 3.8±0.54 seconds on the left). The latency in the monkeys was slightly longer than in the neurologically healthy humans, but there were no statistically significant differences between the two species ($p>0.05$). Temperatures between 60ºC and 90ºC induced pain and resulted in hindpaw/foot withdrawal. There were no side-to-side latency differences ($p>0.05$) (Fig. 3). Ten seconds of thermal stimulation using the Hargreaves device were safe and sufficient to activate pain in neurologically healthy primates.

Pain threshold measurement using the Hargreaves method in patients with spinal cord and cauda equina lesions

The patients with sensory neurological deficits arising from damage to the spinal cord or the cauda equina exhibited prolonged withdrawal latencies (Table I). These patients, affected by numbness and hypesthesia, had decreased pain perception. These findings were quantitatively measured using the Hargreaves test. The pain latency to thermal stimulation was significantly prolonged in the patients with sensory deficits compared with the normal volunteers (Fig. 4). A delayed withdrawal or verbal response to thermal stimulation suggests that this important protective mechanism is lost. Indeed, the patients with an absent or delayed response to the

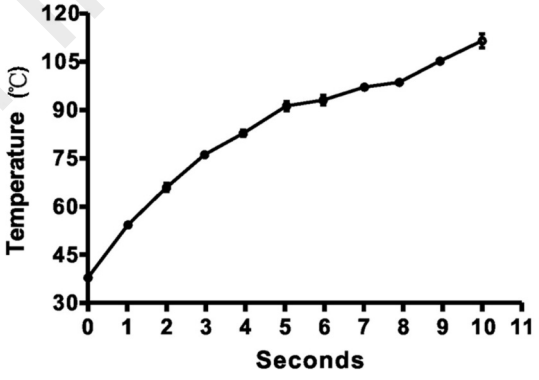

Figure 2 - Calibration curve showing temperature rise over 10 seconds.
Three trials were highly reproducible (Bar=standard deviation).

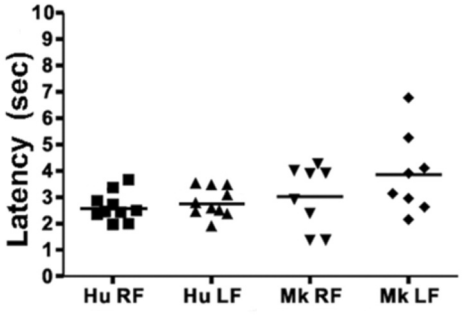

Figure 3 - Latency of foot/hindpaw withdrawal in response to laser-induced thermal stimulation causing pain in neurologically intact humans and monkeys.
Hu=human; Mk=monkey; RF=right foot/hindpaw; LF=left foot/hindpaw.

pain stimulus were more susceptible to injury. The risk of injury was greater the larger the area of skin numbness. Of the 12 patients with chronic neurological deficits, five (42%) had at least one skin site where the pain threshold latency was ≥ 10 seconds. The risk of inadvertent injury to an insensate area of skin was greatest in patients demonstrating a latency of ≥ 10 seconds in all four tested sites (two sites on each foot). Two of these five patients had a severe SCI, and one had a severe cauda equina lesion. The majority (58%) of patients with chronic neurological deficits retained their protective mechanism against thermal injury.

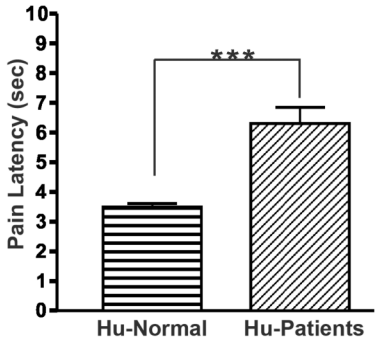

Figure 4 - Compared to neurologically intact volunteers, withdrawal latency to thermal stimulation was significantly longer ($p<0.001$) in patients with sensory deficits. The stimulation was discontinued after a latency of 10 seconds (Hu = human).

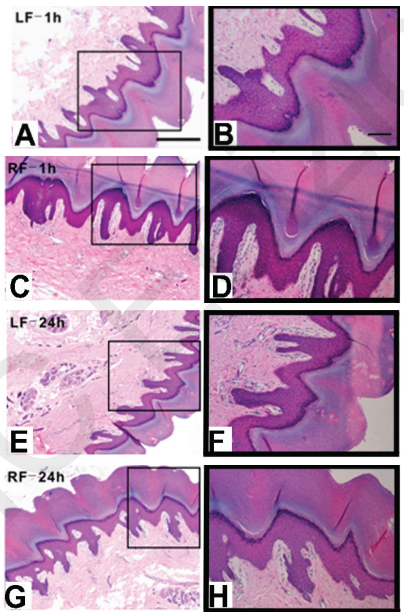

Figure 5 - Histology of the plantar skin in monkeys at 1 or 24 hours after a 10-second laser stimulation.
(A,B) One hour after thermal stimulation to the left hindpaw and (C,D) corresponding area of the non-stimulated right hindpaw. (E,F) 24 hours after stimulation to the left hindpaw and (G,H) corresponding area of the non-stimulated right hindpaw. B, D, F, and H are high power magnifications of A, C, E, and G, respectively. Epidermis, corium, and subcutaneous tissues were normal. There was no clear evidence of tissue damage or increased inflammatory cell infiltration after later stimulation at any time point. (Bar = 500 µm).

Safety of the modified Hargreaves method

The neurologically intact volunteers described a warm sensation followed by discomfort and pain after thermal stimulation. All the neurologically intact volunteers reflexively withdrew their stimulated foot from the glass plate following 8 seconds of infrared simulation and reported discomfort or a tingling sensation that lasted a few seconds to minutes. At one hour and 24 hours after thermal stimulation, neither numbness nor discomfort on palpation of the stimulated area was detected.
No hindpaw withdrawal or vocalization occurred at one or 24 hours when the area exposed to infrared thermal stimulation was palpated in the monkeys. No blisters, swelling or color changes were observed in either the monkeys or the humans. Skin samples from areas of skin subjected to 10 seconds of 80% of maximal infrared heat stimulation were harvested in anesthetized monkeys at one hour (one monkey) and 24 hours (one monkey). Histological examination of the stimulated skin showed no evidence of damage compared to normal skin harvested from the same site on the opposite hindpaw. The epidermis retained its normal cytoarchitecture, and the vessels of the dermis displayed no damage and no evidence of inflammatory cell infiltration (Fig. 5).
The expression of pro-inflammatory cytokines (TNF-α, IL-6, and IL-10) was upregulated in the deep layers of

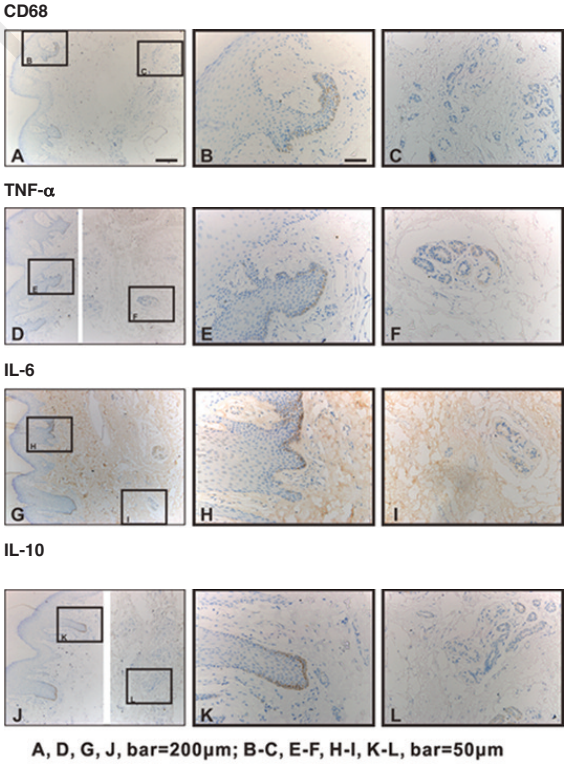

Figure 6 - Illustration of upregulation of pro-inflammatory cytokines 24 hours after heat stimulation of the skin without skin damage.
CD68 (A, B, C), TNF-α (D, E, F), IL-6 (G, H, I) and IL-10 (J, K, L) were expressed in the basal layers of the epidermis at the epidermal-dermal junction (B, E, H, K). There was no change in cytokine expression in the dermis. C, F, I, and L indicate sweat glands of the dermis.

the epidermis and dermis (Fig. 6). A painful stimulus applied to the skin could initiate inflammation, but the response was mild. There were no skin morphological changes at one or 24 hours following the thermal stimulation. No significant increase in inflammatory cell infiltration at the stimulation sites occurred. No significant changes were observed in the activity of CD-68 (Fig. 6).

Discussion

In this study we provide the first demonstration that the Hargreaves thermal test can be used as a reliable and quantitative measure to evaluate pain sensation in non-human primates and humans (cross-species validation study). The Hargreaves test was designed for use in rodents to measure paw withdrawal latency. Our experiment was designed as a preclinical study to evaluate pain threshold changes in patients with sensory deficits.

Physiological pain is a protective mechanism, and recognition of sensory loss in patients with neurological disorders may prevent injury. Noxious stimuli (electrical, thermal, chemical and mechanical) have been used clinically to test for pain (Shy et al., 2003). However, no reliable and quantitative methods for measuring pain thresholds have been developed. Infrared thermal stimulation has several advantages as a means of pain threshold testing. First, the thermal stimulus is completely absorbed by the superficial 100 µm thickness of skin, regardless of skin pigmentation. Second, it activates superficial thermosensitive nerve terminals. Third, it provides a rapid rise in temperature. The Hargreaves method uses a standardized infrared thermal stimulus to measure pain latency and threshold on pre-warmed feet (González-Ramírez et al., 2012; Fuchs et al., 2010; Hara et al., 2012; Mert et al., 2013; Montagne-Clavel and Oliveras, 1996; Nadal et al., 2006; Pavić et al., 2011; Pavlaković and Petzke, 2010). This technique has been used successfully in rodent SCI research as well as in studies of dorsal horn function and diabetic peripheral neuropathy (Hylden et al., 1989).

Our study investigated whether use of the Hargreaves test is safe in non-human primates submitted to heat stimulation of the hindpaw. The anatomy and biology of the skin of monkeys and humans share several similarities, especially regarding dermo-epithelial thickness (Taffe, 2011). Monkeys display humanoid behavioral responses to pain (Tillman et al., 1995a,b). The absence of verbal communication in non-human primate studies was a major limitation compared to studies in humans (McGlone and Reilly, 2010). The latency of hindlimb withdrawal in response to thermal stimulation can be measured using the Hargreaves apparatus. We showed that thermal stimulation at 80% maximum output for 10 seconds was harmless and elicited no behavioral or histological changes in the footpad in primates. These observations provide justification for use of the Hargreaves method in humans.

Hindpaw withdrawal to thermal stimulation in neurologically intact non-human primates and humans occurred within four seconds with a small standard deviation. Using 80% thermal stimulation set for 10 seconds, abnormal skin sensation was recognized. Loss of skin sensation was dangerous and resulted in major impairment of the protective mechanism against pain. In our study we objectively and quantitatively measured pain sensation which was found to be suppressed in patients affected by diseases involving sensory pathways of the spinal cord or cauda equina. We showed that five of the 12 (42%) patients with chronic sensory neurological deficits may, without protection, be at risk of injury (withdrawal latency ≥ 10 seconds). No patient with sensory deficits manifested shorter-than-normal latencies. This finding corresponds to the clinical setting in which no patient had an exaggerated reaction to the stimulus (hyperalgesia) (Sawatzky et al., 2008).

The presence of pain threshold alterations in humans with chronic sensory abnormalities was confirmed using this method. This test may be used to monitor progression of sensory deficits from the acute to the chronic stages of SCI, providing a quantitative profile of sensation. We performed pain threshold testing at only two sites on the plantar surface (IFIS and BBT), but this method may be expanded to study other dermatomes in the upper and lower extremities.

The thermal calibration curve was linear and highly reproducible following several trials. The calibration results in our study were different from those elicited in monkeys and humans when exposed to similar temperatures. The heat-sink phenomenon is due to rapid dissipation of heat from the plantar surface caused by subcutaneous microcirculation that transports warm blood from the plantar surface (Le Bars et al., 2001). Minor differences in hindpaw temperature can cause variations in the withdrawal latency, with an increase in the reaction time occurring as the hindpaw temperature increases (Le Bars et al., 2001). Only a small area of the hindpaw was exposed to heat in the Hargreaves test and, therefore, the heat-sink effect was minimal. Ten seconds of thermal stimulation using our protocol caused no permanent tissue damage, however, it was capable of upregulating pro-inflammatory cytokines in the skin.

The current study investigating nociceptive responses provides cross-species validation. We have expanded the use of the Hargreaves apparatus and demonstrated that our protocol was safe and sensitive in neurologically intact primates and human volunteers as well as in patients with sensory deficits following SCI or cauda equina injury. Patients with other diseases associated with sensory deficits, such as diabetic neuropathy, carpal tunnel syndrome and peripheral polyneuropathy, may also be studied quantitatively for pain threshold. This technique allows reliable screening of patients who have lost their protective mechanisms for pain perception.

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References

Fuchs D, Birklein F, Reeh PW, et al (2010). Sensitized peripheral nociception in experimental diabetes of the rat. Pain 151:496-505.

Glauser DA, Chen WC, Agin R, et al (2011). Heat avoidance is regulated by transient receptor potential (TRP) channels and a neuropeptide signaling pathway in Caenorhabditis elegans. Genetics 188:91-103.

González-Ramírez A, González-Trujano ME, Pellicer F, et al (2012). Anti-nociceptive and anti-inflammatory activities of the Agastache mexicana extracts by using several experimental models in rodents. J Ethnopharmacol 142:700-705.

Hama A, Sagen J (2007). Behavioral characterization and effect of clinical drugs in a rat model of pain following spinal cord compression. Brain Res 1185:117-128.

Hara K, Nakamura M, Haranishi Y, et al (2012). Antinociceptive effect of intrathecal administration of hypotaurine in rat models of inflammatory and neuropathic pain. Amino Acids 43:397-404.

Hargreaves K, Dubner R, Brown F, et al (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32:77-88.

Hashmi JA, Davis KD (2008). Effect of static and dynamic heat pain stimulus profiles on the temporal dynamics and interdependence of pain qualities, intensity, and affect. J Neurophysiol 100:1706-1715.

Hirose K, Iwakura N, Orita S, et al (2010). Evaluation of behavior and neuropeptide markers of pain in a simple, sciatic nerve-pinch pain model in rats. Eur Spine J 19:1746-1752.

Horiuchi H, Ogata T, Morino T, et al (2010). Adenosine A1 receptor agonists reduce hyperalgesia after spinal cord injury in rats. Spinal Cord 48:685-690.

Hylden JL, Nahin RL, Traub RJ, et al (1989). Expansion of receptive fields of spinal lamina I projection neurons in rats with unilateral adjuvant-induced inflammation: the contribution of dorsal horn mechanisms. Pain 37:229-243.

Kang M, Jung I, Hur J, et al (2010). The analgesic and anti-inflammatory effect of WIN-34B, a new herbal formula for osteoarthritis composed of Lonicera japonica Thunb and Anemarrhena asphodeloides BUNGE in vivo. J Ethnopharmacol 131:485-496.

Kirshblum SC, Burns SP, Biering-Sorensen F, et al (2011). International standards for neurological classification of spinal cord injury (revised 2011). J Spinal Cord Med 34:535-546.

Kocevski D, Tvrdeić A (2008). The effect of repeated daily measurements on paw withdrawal latencies in Hargreaves test. Coll Antropol 32 (Suppl 1):93-97.

Le Bars D, Gozariu M, Cadden SW (2001). Animal models of nociception. Pharmacol Rev 53:597-652.

Liu NK, Zhang YP, Titsworth WL, et al (2006). A novel role of phospholipase A2 in mediating spinal cord secondary injury. Ann Neurol 59:606-619.

McGlone F, Reilly D (2010). The cutaneous sensory system. Neurosci Biobehav Rev 34:148-159.

Mert T, Gunes Y, Gunay I (2013). Comparison of actions of systemically and locally administrated local anaesthetics in diabetic rats with painful neuropathy. Fundam Clin Pharmacol 27:161-168.

Miyasaka K, Nomoto S, Ohta M, et al (2006). Disturbance of response to acute thermal pain in naturally occurring cholecystokinin-a receptor gene knockout Otsuka Long-Evans Tokushima Fatty (OLETF) rats. J Pharmacol Sci 101:280-285.

Montagne-Clavel J, Oliveras JL (1996). The "plantar test" apparatus (Ugo Basile Biological Apparatus), a controlled infrared noxious radiant heat stimulus for precise withdrawal latency measurement in the rat, as a tool for humans? Somatosens Mot Res 13:215-223.

Nadal X, Baños JE, Kieffer BL, et al (2006). Neuropathic pain is enhanced in delta-opioid receptor knockout mice. Eur J Neurosci 23:830-834.

Pavić R, Pavić ML, Tvrdeić A, et al (2011). Rat sciatic nerve crush injury and recovery tracked by plantar test and immunohistochemistry analysis. Coll Antropol 35 (Suppl 1):93-100.

Pavlaković G, Petzke F (2010). The role of quantitative sensory testing in the evaluation of musculoskeletal pain conditions. Curr Rheumatol Rep 12:455-461.

Ratcliffe P, Abernethy L, Ansari N, et al (2011). Discovery of potent, soluble and orally active TRPV1 antagonists. Structure-activity relationships of a series of isoxazoles. Bioorg Med Chem Lett 21:4652-4657.

Sandhir R, Gregory E, He YY, et al (2011). Upregulation of inflammatory mediators in a model of chronic pain after spinal cord injury. Neurochem Res 36:856-862.

Sawatzky B, Bishop CM, Miller WC, et al (2008). Classification and measurement of pain in the spinal cord-injured population. Spinal Cord 46:2-10.

Schumacher MA (2010). Transient receptor potential channels in pain and inflammation: therapeutic opportunities. Pain Pract 10:185-200.

Shy ME, Frohman EM, So YT, et al (2003). Quantitative sensory testing: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology 60:898-904.

Tack CJ, Netten PM, Scheepers MH, et al (1994). Comparison of clinical examination, current and vibratory perception threshold in diabetic polyneuropathy. Neth J Med 44:41-49.

Taffe MA (2011). A comparison of intraperitoneal and subcutaneous temperature in freely moving rhesus macaques. Physiol Behav 103:440-444.

Tillman DB, Treede RD, Meyer RA, et al (1995a). Response of C fibre nociceptors in the anaesthetized monkey to heat stimuli: correlation with pain threshold in humans. J Physiol 485:767-774.

Tillman DB, Treede RD, Meyer RA, et al (1995b). Response of C fibre nociceptors in the anaesthetized monkey to heat stimuli: estimates of receptor depth and threshold. J Physiol 485:753-765.

Tominaga M (2010). [Activation and regulation of nociceptive transient receptor potential (TRP) channels, TRPV1 and TRPA1]. Yakugaku Zasshi 130:289-294.

Upadhya MA, Dandekar MP, Kokare DM, et al (2011). Evidence for the participation of cocaine- and amphetamine-regulated transcript peptide (CART) in the fluoxetine-induced antihyperalgesia in neuropathic rats. Peptides 32:317-326.

Xu C, Xu W, Xu H, et al (2012). Role of puerarin in the signalling of neuropathic pain mediated by P2X3 receptor of dorsal root ganglion neurons. Brain Res Bull 87:37-43.

Yang F, Cui Y, Wang K, et al (2010). Thermosensitive TRP channel pore turret is part of the temperature activation pathway. Proc Natl Acad Sci U S A 107:7083-7088.

Yao J, Liu B, Qin, F (2010). Pore turret of thermal TRP channels is not essential for temperature sensing. Proc Natl Acad Sci U S A 107:E125-E127.

Zhu MX (2007). Understanding the role of voltage gating of polymodal TRP channels. J Physiol 585:321-322.