

CLINICAL SIGNS AND SYMPTOMS OF ACUTE REFLEX SYMPATHETIC DYSTROPHY IN ONE HINDLIMB OF THE RAT, INDUCED BY INFUSION OF A FREE-RADICAL DONOR

L. VAN DER LAAN ¹, P. KAPITEIN ¹, A. VERHOFSTAD ², T. HENDRIKS ¹, R. J. A. GORIS ¹

The acute phase of reflex sympathetic dystrophy (RSD) is characterized by the classical signs and symptoms of inflammation (rubor, calor, dolor, tumor and impaired function). As free radicals are involved in acute inflammation, we studied the effects of free radicals in an animal model, especially as to signs and symptoms found in acute RSD. Awake rats were given continuous intra-arterial infusion (1 ml/h) in the left hindlimb, with saline (n = 6) or the free-radical donor tert-butylhydroperoxide (tert-BuOOH, 25 mM, n = 6). During a 24-h infusion period the skin temperature, volume, skin color, function and pain reactions of the paws were observed. After 24 h the rats were killed and both gastrocnemius muscles were histologically analyzed. Infusion with tert-BuOOH induced in the left paw an increased skin temperature, increased volume, redness of the plantar skin, impaired function and increased pain sensation, while these acute RSD signs and symptoms were absent in the saline infused animals. The alterations in pain sensation (spontaneous, mechanical and thermal pain) were similar to findings in the neuropathic animal model. The gastrocnemius muscles of the saline infused rats and the contralateral gastrocnemius muscle of the tert-BuOOH infused rats showed no histological tissue damage. In the left gastrocnemius muscle free-radical-related damage was visible. Induction of free-radical formation in one hindlimb of awake rats mimics the acute signs and symptoms of acute RSD, with alterations in pain sensation as found in the classical neuropathic animal model of RSD, as well as in acute RSD patients.

Keywords : reflex sympathetic dystrophy ; inflammation ; free radicals.

Mots-clés : dystrophie réflexe sympathique ; inflammation ; radicaux libres.

INTRODUCTION

Reflex sympathetic dystrophy (RSD) is a syndrome occurring mostly after minor trauma or operation on an extremity (22). The various denominations used for RSD such as Sudeck's atrophy, causalgia, algodystrophy, peripheral trophoneurosis, or Babinsky-Froment sympathetic paralysis, vary according to the country concerned, the precipitating factor, or the medical specialty treating the patient. At the moment, there is no consensus about the pathophysiology of RSD. Until recently it was generally accepted that RSD is the result of an abnormal orthosympathetic reflex. However, blockade of the orthosympathetic system is not always an effective treatment (7, 11, 14). As a consequence of these negative therapeutic results RSD was recently renamed complex regional pain syndrome (CRPS) at a

¹ Department of Surgery, University Hospital Nijmegen, Nijmegen, The Netherlands.

² Department of Pathology, University Hospital Nijmegen, Nijmegen, The Netherlands.

Correspondence and reprints : L. van der Laan, Department of Surgery, University Hospital Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands.

special consensus workshop in Orlando, Florida (19). Another opinion, which does RSD patients much harm, is that this group of patients has a psychological or social predisposition for developing RSD. There is no prospective study that proves this theory (3). In addition, a psychosocial abnormality was not reported for RSD patients in a large study of chronic pain patients (6). Another mechanism for the pathophysiology of RSD, introduced by Sudeck in 1942, involves an exaggerated inflammatory response to injury or operation (20). In a prospective study of Veldman *et al.* (22), it was confirmed that the acute phase of RSD is characterized by classical signs and symptoms of inflammation (edema, increased skin temperature, redness of the skin, limited range of motion and pain). Scintigraphic analysis of acute RSD patients supported this exaggerated inflammatory theory (15). Goris suggested that free radicals may play a role in the pathogenesis of RSD, based on the findings that acute RSD responds well to treatment with oxygen radical scavengers (8-10), and the finding of exaggerated amounts of lipofuscin in skeletal muscle biopsies of patients with RSD (21). The classical neuropathic pain model of Bennet in the rat is the currently used animal model of RSD (2, 12). In this model similar alterations in pain sensations (spontaneous pain, mechanical pain and thermal pain) are observed as present in RSD patients (16). Mechanically-induced pain sensations of RSD patients are allodynia, defined as pain from normally innocuous stimuli, and hyperpathia, defined as increased sensitivity to painful stimuli.

The aim of this study was to observe the effects of free radicals in an animal model, especially with respect to signs and symptoms found in acute RSD.

MATERIALS AND METHODS

Animal model

Adult male Wistar rats (weight 320-400 g) were anesthetized with atropine S.C. (0.25 mg/kg), fluanison (10 mg/ml)/fentanyl (0.2 mg/ml) mixture I.M. (0.445 mg/kg) and I.P. midazolam hydrochloride (4.5 mg/kg). During the surgical procedure, body temperature was maintained between 37°C and 38°C

using a heating pad. Microsurgically, the left superficial epigastric artery was cannulated with a polythene cannula (ID 0.28 mm ; OD 0.61 mm ; laboratoire Portex, France), retrograde with the tip at the orifice of the femoral artery. The other end of the cannula was extended subcutaneously over the back to the head of the rat and connected to a flexible infusion system. After the operation, anesthesia was terminated with I.M. naloxone (2 ug/kg). The result of the surgical procedure was a nonanesthetized rat connected to a flexible infusion system, with continuous intra-arterial infusion in the femoral artery of one hindlimb without concurrent ischemia. Infusion (1 ml/h) was started immediately after cannulation of the superficial epigastric artery and continued for a period of 24 h. Six rats were infused with the free-radical donor tert-butylhydroperoxide (tert-BuOOH ; Sigma, St. Louis, USA), dissolved in saline to a final concentration of 25 mM with heparin (2.5 U/ml), and six rats were infused with saline plus heparin (control group). The experimental protocol was approved by the Animal Ethics Review Board of the Faculty of Medicine, University of Nijmegen.

Analysis of signs and symptoms

The same criteria as used to diagnose RSD in patients at our outpatient clinic of the department of surgery (22), were examined in all rats, namely difference in skin temperature compared with the other hindlimb, diffuse edema of the foot, difference in skin color compared with the other hindlimb, limited active range of motion of the hindlimb, and unexplained diffuse pain of the hindlimb.

Skin temperature and volume of both feet were measured just before the operation and 1, 4, and 24 h after starting the infusion. The skin color of the feet was observed after 1, 4, and 24 h after starting the infusion, while the function of both hindlimbs was observed after 24 h. Skin temperature was measured on the plantar region of both hind paws using a surface electrode (d = 0.6 cm ; Keithly, Geneva, Ohio, USA), and the temperature difference between the two feet was calculated. Volume of the foot was quantitated by submersion of the paw in a calibrated cylinder filled with water. The percentage change of the volume of the operated hindlimb to the preoperative situation served as a parameter for contour alterations. Color of the left plantar foot was observed and compared to the right untreated foot. Impairment of the function of the left hindlimb was noted if the rat showed a

shuffling gait. Observation of pain signs was performed preoperatively and after 24 h of infusion. Assessment of pain was performed according to various methods utilized in the classic neuropathic pain model in the rat as described by Bennet *et al.* (2), including: spontaneous pain, mechanically-induced pain and thermally-induced pain. Spontaneous behavior of rats was observed in a perspex cage of 25 × 25 × 40 cm after 5 min habituation. For 5 min, the length of time the paw was held in various positions was noted, according to the scale of Attal *et al.* (1), which varies from 0 representing the operated paw pressed normally on the floor to 5 representing the animal licking the operated paw. The score over the 5-min period provides an index of spontaneous pain intensity for each rat. The spontaneous pain was calculated by the formula:

$$t1 + 2t2 + 3t3 + 4t4 + 5t5 / 300 \text{ s}$$

where t1, t2, t3, t4 and t5 are the lengths of time (in s) spent in categories 1, 2, 3, 4 or 5, respectively. In order to quantify mechanical sensitivity of the plantar hind paw, we measured foot withdrawal in response to mechanical stimuli with Von Frey filaments (North Coast Medical, San Jose, CA, U.S.A.) of two different bending forces (5.16 g and 46.5 g), according to the method described by Chaplan *et al.* (5). After 5-min accommodation in the cage, a Von Frey filament was applied 10 times (once every 5 s) to the plantar surface of the left foot and the frequency of foot withdrawal was noted. Thermal pain was observed on the hot and cold plate. After a behavioral accommodation period of 5 min on a floor heated to 40°C (19), the heat-related pain was scored according to the spontaneous pain method for 5 min (see above). Following the heat experiment, the floor was chilled to 4°C, and the rat was allowed to accommodate for 5 min. Behavior of the left hind paw on the chilled plate was observed and scored by the same procedure.

Histology

Rats were reanesthetized after 24 h of infusion. Left and right gastrocnemius muscles were dissected and immediately fixed in toto by immersion in cold phosphate buffered paraformaldehyde 4%, pH 7.3. Subsequently, the animals were killed. From proximal to distal, 4 transverse slices were taken, dehydrated and embedded in Paraplast. Sections of 5 µm in thickness were stained with hematoxylin and eosin (HE). In these sections structural changes in skeletal muscle fibers were examined by light microscopy.

Statistical Analysis

Quantitative data were expressed as mean plus standard error of the mean. The measurements at the various time points of the tert-BuOOH infused rats and the saline infused rats were compared by the Mann-Whitney test. Pain scores of the two groups were compared by relating the difference between the post — and preoperative values of the various groups. The level of significance was set at $p < 0.05$.

RESULTS

Signs and symptoms

During the 24-h tert-BuOOH infusion, skin temperature of the left foot increased to levels higher than found preoperatively, and higher than both legs of the saline infused animals (table I - $p = 0.002$). Cannulation followed by saline infusion had no effect on skin temperature of the operated hind paw. The volume of the left foot increased gradually during continuous tert-BuOOH infusion and was significantly higher than before the operation ($p < 0.01$ - fig. 1). Saline infusion did not change the left foot volume.



Fig. 1. — A typical example of the feet after 24 h tert-BuOOH infusion. The left foot (right on the figure) is swollen and has a red skin colour, while the contralateral foot is normal.

Redness of the plantar side of the left foot was visible after 4 h of tert-BuOOH infusion in 83% of the rats and was still visible in 5 tert-BuOOH infused rat after 24 h of infusion. This phenomenon was not found in the saline infused rats. After 24 h

Table I. — The inflammatory signs and symptoms (with spontaneous and thermal pain) of TBOOH (tert-butylhydroperoxide) infused rats (n = 6) or saline infused rats (n = 6) during a period of 24 h (* = p < 0.05)

Signs and symptoms	Pre-operative		1 h		4 h		24 h	
	Saline	TBOOH	Saline	TBOOH	Saline	TBOOH	Saline	TBOOH
Skin temperature (°C) difference left-right foot	-0.03 ± 0.03	-0.02 ± 0.04	-0.11 ± 0.06	1.02 ± 0.29 *	-0.02 ± 0.07	1.10 ± 0.08 *	-0.03 ± 0.05	0.73 ± 0.10 *
Volume increase (%) left foot to pre-operative situation			0 ± 0	5.0 ± 2.2 *	0 ± 0	10.7 ± 2.7 *	0 ± 0	14.2 ± 4.5 *
Red skin colour of the left foot present in the rats (%)	0	0	0	83	0	100	0	83
Impaired function of the left paw present in the rats (%)	0	0					0	83
<i>Pain sensation</i>								
— spontaneous pain	0 ± 0	0 ± 0					0.02 ± 0.02	0.87 ± 0.15 *
— heated plate	0.03 ± 0.06	0.15 ± 0.07					0.18 ± 0.07	1.36 ± 0.23 *
— chilled plate	0.05 ± 0.12	0.11 ± 0.09					0.02 ± 0.02	0.87 ± 0.25 *

of infusion, impaired function was observed in 83% of tert-BuOOH treated animals, while saline infused rats and the contralateral leg of the experimental rats demonstrated normal function.

Pain observation scores were obtained preoperatively and after 24 h of infusion. Statistical analysis of the pain score, found in tert-BuOOH and saline infused rats was performed by calculating the difference between the preoperative and postoperative (after 24 h of infusion) value of both groups. Before the surgical intervention, the spontaneous pain score was 0 in all rats. Infusion with tert-BuOOH induced a significant increase in the spontaneous pain score as compared to saline ($p = 0.004$). Before the surgical intervention, mechanical stimuli with the 5.16-g Von Frey filament showed negligible withdrawal reactions in contrast to the 46.54-g Von Frey filament (fig. 2). The 5.16- and 46.54-g Von Frey filaments induced an increased withdrawal percentage in the tert-BuOOH group compared to the saline control group. This difference was only significant with the 5.16-g filament ($p = 0.004$). Preoperative observation of the rats on the heated or cold plate showed normal behavior. Infusion with tert-BuOOH induced a significant increase in the heat and cold pain score compared to saline infusion ($p < 0.004$).

Histology

Light microscopic examination of the left and right gastrocnemius muscles of saline infused animals and the contralateral gastrocnemius muscles of tert-BuOOH infused rats showed intact polygonal skeletal muscle fibers, no edema or leukocytes. After 24 h of tert-BuOOH various stages of cellular degeneration were present with severe edema and leukocyte infiltration.

DISCUSSION

In this novel animal model we evaluated the effects of intra-arterial infusion of tert-BuOOH, inducing the formation of free radicals tert-butoxyl and tert-butylperoxyl (18). These free-radical products of tert-butylhydroperoxide induce oxid-

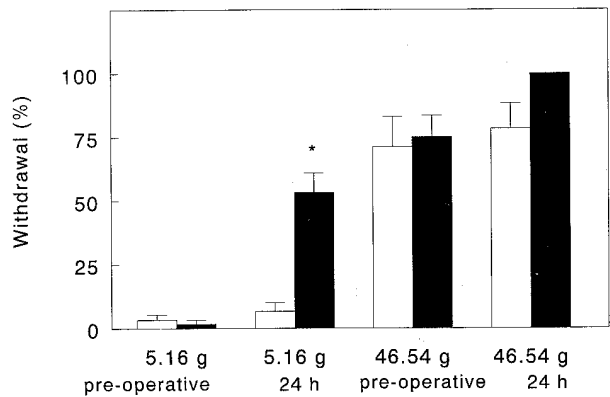


Fig. 2. — Mechanically-induced pain of the infused hindlimb by stimuli with a Von Frey filament with a force of 5.16 g. or 46.54 g. Tert-BuOOH infused ($n = 6$): (■) and saline infused animals ($n = 6$): (□) (* = $p < 0.01$).

ative injury based on lipid peroxidation and mitochondrial dysfunction (4, 13, 17).

Within 1 h after starting the tert-BuOOH infusion, signs also found in acute RSD (increased skin temperature, edema and redness of the skin) were visible. This fast reaction on the exposure to free radicals is in agreement with the *in vitro* kinetic effect of tert-BuOOH (17).

Observation of changes in function and response to painful stimuli were performed after 24 h of infusion to avoid interference from the anesthesia. Impaired function induced by 24 h of tert-BuOOH infusion was indicated by a shuffling gait.

Assessment of pain was performed according to methods used in the classical mononeuropathic pain model in the rat, a frequently used animal model of RSD (2). After 24 h of tert-BuOOH infusion the spontaneous pain-related behavior was significantly altered with regard to the preoperative situation. However, in the mononeuropathic animal model significant changes in spontaneous pain behavior of the affected hind limb are found only 5 days postoperatively (1). This difference in delay between free-radical initiated pain and neuropathic-induced pain is interesting because clinically the pain of RSD is already present immediately after the initiating trauma and not after a longer period as in a peripheral nerve lesion. Mechanical pain sensations in the free-radical animal model were measured by Von Frey

filaments. By this technique, the two mechanical pain sensations present in RSD patients (allodynia and hyperpathia) were found in this animal model (16). For interpretation of the nociceptive effect of thermal plate compared to the spontaneous pain behavior of the rat, we used the spontaneous pain formula of Attal also for the behavior of the rat on the thermal plate. In the free-radical infused animal, the heated plate induced significantly increased pain behavior, as compared to spontaneous pain behavior. In contrast, on the chilled plate tert-BuOOH infused rats exhibited pain behavior comparable to their spontaneous pain behavior. This nonnoxious method for indicating allodynia for cold may not be sensitive enough in contrast to the thermal struggle test. The other possibility is that the free-radical animal model and RSD free-radical-induced soft tissue damage does not induce cold allodynia in the present experimental setting.

Histological analysis of the left gastrocnemius muscles after 24 h tert-BuOOH infusion showed various stages of cellular degeneration in combination with accumulation of interstitial fluid and leukocytes. The infused free radicals induce damage only within the infused paw, because in the gastrocnemius of the noninfused paw no cellular damage was observed. To our knowledge histological analysis of skeletal muscle of acute RSD patients has never been performed. Therefore it was not possible to compare histologically free-radical-damaged skeletal muscles to acute RSD-affected skeletal muscle.

It is concluded that in our animal model it is possible to induce free-radical damage in one hindlimb of nonanesthetized animals. Continuous intra-arterial infusion of tert-BuOOH in this animal model causes acute clinical inflammatory signs and symptoms comparable to those found in acute RSD, with a pain syndrome as found in the classical neuropathic animal model of RSD and in acute RSD patients.

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SAMENVATTING

L. VAN DER LAAN, P. KAPITEIN, A. VERHOFSTAD, T. HENDRIKS, R. J. A. GORIS.

De acute fase van Reflex Sympathische Dystrofie (RSD) wordt gekenmerkt door de klassieke ontstekings symptomen (rubor, calor, dolor, tumor en functio laesie). Daar zuurstofradicalen een belangrijke rol hebben in acute ontsteking, werd in een diermodel het effect van zuurstofradicalen bestudeerd, met speciale aandacht voor de klinische tekenen en symptomen van RSD. Middels cannulatie werd de linker achterpoot van wakkere ratten continu intra-arterieel geïnfundeerd (1 ml/uur), met fysiologisch zout (n = 6) of de vrije zuurstofradicaaldonor tert-butylhydroperoxide (tert-BuOOH) (25 mM) (n = 6). Gedurende de infusie periode van 24 uur werd de huidtemperatuur, volume, huidskleur, functie en pijnreacties van de poten gemeten en gescoord. Na 24 uur werden de ratten afgemaakt en de gastrocnemius spieren histologisch geanalyseerd. Tert-BuOOH infusie induceert in de linker achterpoot

een verhoogde huidtemperatuur, zwelling, rode verkleuring van de voet zool, verminderde functie en pijnsensaties. Deze bevindingen werden niet waargenomen in de controle groep. De veranderingen in pijnsensaties (spontane, mechanische en thermische pijn) vertoont overeenkomst met de bevindingen in het neuropathische diermodel. De gastrocnemius spieren van fysiologisch zout groep en de contralaterale gastrocnemius spieren van de tert-BuOOH geïnfundeerde ratten vertoonden geen histologisch waarneembare schade. In de linker gastrocnemius spier was vrije zuurstofradicaal gerelateerde schade zichtbaar. Geïnduceerde vrije zuurstofradicaal vorming in een achterpoot van wakkere ratten geeft overeenkomstige tekenen en symptomen van acute klinische RSD, met veranderingen in pijnsensaties zoals die in het neuropathische diermodel van RSD optreden.

RÉSUMÉ

L. VAN DER LAAN, P. KAPITEIN, A. VERHOFSTAD, T. HENDRIKS, R. J. A. GORIS. La perfusion de donneurs de radicaux libres au niveau de la patte du rat induit une symptomatologie caractéristique de dystrophie réflexe sympathique aiguë.

La phase aiguë de la dystrophie réflexe sympathique (DRS) est caractérisée par une symptomatologie inflammatoire (rougeur, chaleur, douleur, gonflement et perte fonctionnelle). Les radicaux libres étant impliqués dans les phénomènes inflammatoires aigus, les auteurs ont étudié les effets des radicaux libres dans un modèle animal, en particulier en ce qui concerne la symptomatologie caractéristique de DRS aiguë. Une perfusion intra-arterielle continue (1 ml/h) a été réalisée au niveau de la patte arrière gauche de rats éveillés, soit avec du sérum physiologique (6 rats), soit avec un donneur de radicaux libres tert-butylhydroperoxide (tert-BuOOH, 25 mM — 6 rats). Pendant la perfusion de 24 heures, la température cutanée, le volume, la coloration cutanée, la fonction et les réactions aux douleurs des pattes ont été observées. Après 24 heures, les rats ont été euthanasiés et les muscles jumeaux des 2 pattes arrière ont été étudiés sur le plan histologique. La perfusion de tert-BuOOH a induit au niveau de la patte gauche une augmentation de température cutanée, une augmentation de volume, une rougeur de la peau plantaire, une perte fonctionnelle et une augmentation des sensations douloureuses. Cette symptomatologie de DRS aiguë ne fut pas retrouvée chez les animaux perfusés à l'aide

de sérum physiologique. Les altérations de sensation douloureuse (douleur spontanée, mécanique et thermique) furent similaires aux résultats obtenus dans le modèle d'animal neuropathique. Les muscles jumeaux des rats perfusés à l'aide de sérum physiologique, et du muscle controlatéral des animaux perfusés de tert-butylhydroperoxyde (tert-buOOH) n'ont pas révélé d'altération histologique. Par contre, au niveau du muscle jumeau du côté perfusé

de tert-butylhydroperoxyde (tert-buOOH), des lésions induites par les radicaux libres furent observées. En conclusion, l'induction de la formation de radicaux libres au niveau d'une patte de rat éveillé reproduit la symptomatologie caractéristique de la DRS aiguë, avec altération des sensations douloureuses comme observé dans le modèle classique neuropathique ou chez les patients présentant une DRS.