Clinical and Fundamental Aspects of Complex Regional Pain Syndrome Type I

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Clinical and Fundamental Aspects of Complex Regional Pain Syndrome Type I

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Clinical and Fundamental Aspects of Complex Regional Pain Syndrome Type I

An academic essay in Medical Sciences

Doctoral Thesis

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from Radboud University Nijmegen
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according to the decision of the council of deans
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For my parents

To Victorine & Wouter

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Chapter 1

Introduction

INTRODUCTION

Complex Regional Pain Syndrome (CRPS) is a potentially incapacitating syndrome, that may occur after a minor injury or an operation applied to a limb. CRPS has impact on all tissues and may impair all functions of that extremity, leading to severe disability and almost intractable pain. The spectrum of symptoms and signs is wide. The early phase is characterized by signs and symptoms of inflammation including excessive pain and impaired motor and sensory function. The late phase is characterized by trophic changes of all tissues. In approximately 10 percent of the cases CRPS occurs without a preceding trauma. Formerly CRPS was denominated as causalgia, algodystrophy, Sudecks atrophy or reflex sympathetic dystrophy. In 1994 the International Association for the Study of Pain (IASP)[1,2] coined the term Complex Regional Pain Syndrome type I (CRPS I). In CRPS type II (causalgia) nerve damage must be present.
This thesis focuses only on CRPS I, as this syndrome is more prevalent than CRPS II. The pathophysiological mechanism of CRPS I is still controversial. Currently, there are several theories that explain the signs and symptoms of CRPS I[3]. In the psychosocial theory it is assumed that patients with CRPS I have a psychological predisposition. Inactivity has been suggested as a causal factor. The reflex sympathethic dystrophy theory states that an abnormally hyperactive sympathetic reflex is responsible for the signs and symptoms[4]. Paul Sudeck was the first to propose that CRPS I could be the result of an exaggerated inflammatory response after injury or operation of an extremity[5].
The reported incidence of CRPS I varies between 5.5 - 26.2 per 100,000 persons[6,7]. The incidence of CRPS I in adults is estimated 1 - 2% after fractures, 2 - 5% after a peripheral nerve damage and in 8 - 24% after a wrist fracture[8,9].

CRPS I IN CHILDREN

Complex Regional Pain Syndrome type I in children was regarded rarely occurring until the 1970s, when several case series were reported[10]. Currently there is increased awareness and the number of small case series and clinical outcome studies is rapidly growing[11-13]. Failure to diagnose CRPS I leads to delayed management, unnecessary investigations and improper treatment, which may worsen the situation and aggravate suffering. In children the clinical presentation of CRPS I is, to a certain extent, different from adults and adolescents, reason why the syndrome sometimes is not recognised in an early stage[14].
We have retrospectively studied all patients under 16 years of age with CRPS I, seen at the Radboud University Nijmegen Medical Centre in the time period 1980 - 2003 and compared these data with 951 adult patients with CRPS I (Chapter 2). This study is the first that compares children and adults with CRPS I, and contains also the largest number of paediatric CRPS I patients evaluated. Reported results of treatment of CRPS I in children are usually more favourable and

seem better than in adults[13,14]. Therefore we also investigated the quality of life (QoL) in adults who have been treated for childhood-onset CRPS I (Chapter 3).

INFLAMMATION

Although the clinical presentation of CRPS I is well known[8,15,16], the mechanism of the (chronic) pain and the underlying pathophysiology still remain subject of debate. In the acute phase CRPS I is characterized by signs and symptoms of inflammation within the periphery of the affected extremity. Observations supporting the inflammatory theory include:

(a) The dominance of clinical signs and symptoms of inflammation at onset - such as unexplained, severe pain, oedema, difference in skin temperature, difference in skin colour, and impaired function - in the affected extremity[8],
(b) Increased extravasation of indium-labeled immunoglobulin as a sign of increased capillary permeability for macromolecules[17],
(c) The involvement of oxygen free radicals[18-20],
(d) Successful treatment with radical scavengers such as dimethyl sulfoxide (DMSO), mannitol[18,19], N-acetyl-l-cysteine and vitamin C[21-23],
(e) Higher levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in blister fluid in the involved extremity in comparison to the unaffected extremity[24] and elevated levels in venous blood in CRPS I patients of IL-8 and soluble TNF receptor I/II[25] and
(f) Successful intervention in a pilot study with anti-TNF[26].

RADICALS

One of the most important features of inflammation is an excessive production of Reactive Oxygen and Nitrogen Species (RONS). Both clinical and experimental evidence support the theory that an excessive regional inflammatory response, including the production of RONS, contributes to the development of CRPS I and causes tissue damage[8,17,27,28]. We hypothesized that the increased capillary permeability, as part of an exaggerated inflammatory response, may be due to endothelial damage caused by oxygen radical species directly or mediated by polymorphonuclear leukocytes. To verify this hypothesis we performed a clinical study in which we analyzed radiolabeled autologous leukocyte imaging of both hands in patients with CRPS I of one upper extremity after a fracture or operation of the hand, comparing the affected with the unaffected hand (Chapter 4).

Several animal models are currently used to study the underlying mechanism(s) resulting in CRPS. In the animal model described by Bennet and Xie[29], a peripheral mononeuropathy is induced in the rat by placing loosely constrictive ligatures around the common sciatic nerve

at mid-thigh level, leading to nerve damage by chronic compression. Following this type of partial peripheral nerve injury, also known as the chronic constriction injury (CCI) model, the rat develops sensory, autonomic and trophic abnormalities and alterations in pain sensation, resembling the signs and symptoms as seen in CRPS patients. In this CCI model, formation of oxygen free radicals and nitric oxide have been shown to contribute to the hyperalgesia[30-32]. In order to analyse oxidative stress in the CCI model, we used recently developed spin-trap techniques and determined endogenous antioxidants such as glutathione, ceruloplasmin and transferrin (Chapter 5).

As RONS are closely involved in the development of CRPS I, our department developed an animal model of free radical induced soft tissue damage by continuous intra-arterial infusion of the free radical donor tert-butylhydroperoxide (Tert-BuOOH) in one hind limb of non-anaesthetized rats, without inducing ischemia[33]. In this model injection with the antioxidant N-acetyl-l-cysteine, which can replenish the intracellular antioxidant glutathione, reduced pain sensations, reduced vascular permeability, reduced the observed soft tissue damage and shortened the repair period[34]. To further investigate oxidative stress in this animal model of free radical induced soft tissue injury, we determined RONS and measured antioxidant levels (Chapter 6).

VENOUS OXYGEN SATURATION

Stolte et al. found in 1970 elevated venous oxygen saturation (S_vO_2) levels in CRPS I patients[35]. High S_vO_2 levels at rest are characteristic of a condition in which oxygen extraction is impaired, as it reflects the inability of tissues to utilize oxygen despite sufficient supply[36]. Furthermore, phosphorus nuclear magnetic resonance spectroscopy (PNMRs) in skeletal muscle of limbs with CRPS I, points to cellular hypoxia by abnormally high inorganic phosphate to phosphocreatine ratios at rest[27] and by rapid depletion of phosphocreatine upon skeletal muscle exercise followed by a slow recovery of phosphocreatine levels at subsequent rest[36]. In patients with acute CRPS I, our department also found significantly elevated S_vO_2 levels, obtained from the vena cubiti in the affected upper extremity, as compared to samples obtained from the unaffected contralateral limb[36,37]. However, in CRPS I patients any invasive procedure, such as performing a venous puncture, may increase the severity or recurrence of CRPS I in an affected limb, or induce CRPS I in a healthy limb[8]. In view of this, we measured peripheral oxygen saturation levels with a non invasive pulse oximeter, but could not detect any differences (data not published). We also assessed capillary oxygen saturation levels and lactate levels in the affected limb of acute CRPS I patients (Chapter 7). We assessed oxygen utilization in our animal model of free radical induced soft tissue injury, mimicking clinical signs and symptoms of CRPS I, by investigating S_vO_2 and lactate levels (Chapter 6). In Chapter 8 we examined patients with late dystrophic or atrophic end stage CRPS I requiring amputation, in order to look for evidence of diminished

oxygen consumption, by analyzing venous blood samples, and performing microscopic analysis and mitochondrial function studies on tissue obtained from the amputated extremity.
Based on the high S_vO_2 levels found, we hypothesised four possibilities: first, a deficient autoregulation of microcirculation, resulting in a widely open capillary bed, while only a small fraction of the oxygen supplied is consumed due to hyper-perfusion of some capillaries and inadequate perfusion of others; second, the presence of increased flow through non-nutritive pathways, such as through arterio-venous anastomoses[38]; third, a diffusion problem for oxygen between the erythrocytes and the mitochondria, especially due to changes in the endothelial cell and the capillary basement membrane; and finally, minimal oxygen extraction due to mitochondrial dysfunction. Mitochondrial dysfunction seemed a plausible cause for the high S_vO_2 levels found, as dysfunctional mitochondria might explain the PNMRs findings in CRPS I patients, with increased anorganic phosphate to creatine phosphate ratios at rest, pointing to cellular hypoxia[27]. However, mitochondrial function has never been investigated in CRPS I patients before. We assessed in vitro mitochondrial function by using a model of isolated skeletal muscle mitochondria subjected to the radical donor Tert-BuOOH[39], which we also have used in our animal model (Chapter 6). We also found a manner to investigate mitochondrial function in CRPS I patients, without the risk of inducing or exacerbating CRPS I in an affected limb, by analyzing amputated extremities of chronic end stage CRPS I patients on mitochondrial dysfunction (Chapter 9).

Chapter 10 summarizes our main conclusions and formulates recommendations for future research in the field of CRPS I.

AIMS OF THIS THESIS:

- To study the clinical presentation and prognosis of CRPS I in children and adults.

- To study some fundamental aspects of inflammation in CRPS I; (1) by investigating the role of oxygen free radicals in patients and in animal experiments and (2) by investigating S_vO_2 levels and factors possibly involved in impaired oxygen extraction, such as capillary density, capillary membrane thickness, and mitochondrial function.

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Chapter 2

Complex Regional Pain Syndrome type I in children

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ABSTRACT

Background:

Complex Regional Pain Syndrome type I (CRPS I) is a potentially incapacitating syndrome which can occur after a minor injury or operation to a limb. It is a disorder characterized by pain, sensory and motor disturbances. CRPS I is well known in adults but a relatively new diagnostic entity in children. The clinical presentation of CRPS I in children is, to some extent, different from adults and therefore sometimes not recognized early. The aim of this study was to search for differences in patient characteristics between children and adults with CRPS I.

Methods:

We have performed a retrospective chart review of 78 children (age < 16 year) with CRPS type I and compared the data with those of 951 adults with CRPS type I.

Results:

The child population consisted predominantly of girls, and older children (median age 13 years). The child population differed from adults in that the skin temperature of the involved extremity at onset was more often cooler, that the lower extremity was involved more frequently, and that neurological and sympathetic symptoms were less pronounced.

Conclusion:

In several aspects, CRPS I in children has a different presentation than in adults.

INTRODUCTION

Complex Region Pain Syndrome type I (CRPS I) is a potentially incapacitating syndrome which can occur after a minor injury or operation to a limb. In approximately 10 percent of the patients, CRPS I occurs without a previous injury[1]. CRPS I has an impact on all tissues and can impair all functions of that extremity, possibly resulting in severe impairment and therapy-resistant pain. The pathophysiological mechanism of CRPS I is not yet elucidated. Several mechanisms are hypothesized in the generation and maintenance of CRPS I such as a psychological predisposition, inactivity, increased sympathetic activity and an exaggerated regional inflammatory response[2]. Paul Sudeck was the first to propose that CRPS I could be the result of an exaggerated regional inflammatory response in the affected extremity after injury[3]. Later on this hypothesis was corroborated by findings that in the acute phase extravasation of Indium-immunoglobulin[4], increased concentration of leucocytes[5] and oxygen free radicals could be demonstrated within the affected part[6,7].
Formerly CRPS was denominated causalgia, algodystrophy, Sudecks atrophy or reflex sympathetic dystrophy. In 1994 the International Association for the Study of Pain (IASP)[8,9] coined the term Complex Regional Pain Syndrome type I (CRPS I). In CRPS type II (causalgia) nerve damage must be present.
The incidence of CRPS I in adults is estimated at 1 - 2% after a fracture, 2 - 5% after a peripheral nerve lesion and even 8 - 24% after a fracture of the distal radius[1,10]. The clinical presentation of CRPS I in children is, to some extent, different from adults and therefore sometimes not recognized early. We have retrospectively studied all charts of patients under 16 years of age with CRPS I, seen at the Radboud University Nijmegen Medical Centre during 24 years.

PATIENTS AND METHODS

We performed a retrospective chart analysis of the history, signs and symptoms and treatment of all children under age of 16 years with CRPS I, seen between January 1980 and January 2004 at the Department of Paediatric Surgery and General Surgery. Diagnosis was based on the criteria formulated by Veldman et al[1]. The following diagnostic criteria were used in establishing the diagnosis of CRPS I:

1. Presence of at least 4 of the following 5 signs and symptoms: unexplained diffuse pain and tenderness in the distal part of the extremity, difference in skin colour in relation to the healthy symmetrical limb, diffuse oedema, difference in skin temperature in relation to the healthy symmetrical limb and limited range of movement.
2. The above signs and symptoms increase during exercise.

3. The above signs and symptoms are present in an area much larger than the area of primary injury or operation and including the area distal to the primary injury.

These data were compared with 951 adult CRPS I patients, which have been selected on the basis of the criteria of Veldman et al. from all consecutive patients seen during the period January 1984 till September 1997 for signs and symptoms of CRPS I at the outpatient clinic of the university hospital, the Department of General Surgery.
These adults were documented prospectively according to an extensive protocol and the data entered into a computerized patient record file. Part 1 of this file consists of data obtained by a questionnaire, including age at the time of the visit to our outpatient clinic, age at the onset of the disease, gender, the initiating cause of the CRPS I (e.g., trauma, surgery, spontaneous, others), the patient's description of the skin temperature of the affected extremity at the onset of the CRPS I, the presence of CRPS I in another extremity, and modes of treatment before the patient visited our outpatient clinic. Part 2 of the file included data obtained by physical examination of the patient as described by Veldman et al. and Van der Laan et al.[1,11]. The pain within the affected area was scored by the visual analogue scale. Oedema (present or absent) skin colour (blue, red, normal) and skin temperature (cold, warm, normal) of the affected extremity were assessed clinically and documented in comparison to the unaffected contralateral limb.
Differences between children and adults in characteristic were described by means or proportions and the corresponding confidence intervals for the difference. Continuous and categorical variables were compared using the t-test and the chi-square test, respectively. Differences between children and adults in time-to-event variables were described by median interval times as estimated by the Kaplan-Meier method and compared using the Breslow-Gehan-Wilcoxon test. A p-value below 0.05 was taken to indicate statistical significance.

RESULTS

In total we identified 78 children (< 16 years) and 951 adults (age > 16 years) with CRPS I fulfilling the criteria of Veldman et al. Sixty children (76.9%) and 840 adults (88.3%) fulfilled the IASP criteria for CRPS I[8,9], and none fulfilled the criteria for CRPS II. Clinical characteristics of both populations are shown in Table I. In the paediatric population 85.9% were female, comparing to 74.9% in the adult population ($p = 0.03$). The median age in the paediatric population was 13.0 years (range 5 - 16 years) and 43.8 years (range 17 - 96) in the adult population. In children one upper extremity was affected in 23.3%, one lower extremity in 72.6% , both upper or both lower extremities in 4.1%. The upper extremity in adults was more frequently involved (60.8%) than the lower extremity in children ($p < 0.001$). In children, CRPS I was caused by a minor injury in 62.5%, usually as a result of an ankle sprain. In 6 children (8.3%) no precipitating factor could be identified (Table 1). In children CRPS I appeared to be more common during the winter season

Table 1. Clinical characteristics of adults and children with CRPS I.

	Adults % (n/n_ev)	Children % (n/n_ev)	*p*-value
Gender			0.03
Female	74.9 (712/951)	85.9 (67/78)	
Male	25.1 (239/951)	14.1 (11/78)	
Median age in years (range)	43.8 (16 - 96)	13 (5 - 16)	
Upper/lower			< 0.001
Upper extremity involved	60.8 (578/951)	23.3 (17/73)	
Lower extremity involved	39.2 (373/951)	72.6 (53/73)	
Upper and lower extremity involved	0	4.1 (3/73)	
Left/right			< 0.001
Right	51.5 (402/780)	47.4 (37/78)	
Left	48.5 (378/780)	48.7 (38/78)	
Bilateral involvement	0	3.8 (3/78)	
History of trauma			< 0.001
None	10.6 (98/926)	8.3 (6/72)	
Mild (contusion, sprain/strain)	32.2 (298/926)	62.5 (45/72)	
Severe (fracture, post surgical)	57.2 (530/926)	29.2 (21/72)	

n_ev = number of evaluable patients

(32.4%) as compared to spring (23.5%), summer (20.6%) and fall (23.5%) ($p = 0.46$). In adults the incidence of injuries was equally distributed between seasons.

The signs and symptoms at first consultation at our outpatient clinics are summarized in Table 2. Not from all patients data were complete. The main complaints in both populations were pain, a difference in skin colour and skin temperature, a limited active range of motion, and an increase in complaints after exercising the affected extremity. In the paediatric population the affected extremity was mostly cold (71.8%) with a median temperature difference of -1.3°C and blue discoloration of the skin. In the adult population the affected extremity mostly was warm (55.1%). In the paediatric population the median visual analogue score (VAS) was 7 (range 0 - 10, SD 2.8), in the adult population 4 (range 0 - 10, SD 2.6) ($p < 0.001$).

The estimated median interval between injury and CRPS I symptoms was 0.14 weeks (range 0 - 313.1, interquartile range 0 - 2) for adults (n = 647) versus 0.57 weeks (range 0 - 26.7, interquartile range 0.14 - 2.71) for children (n = 64). This is significant longer than adults ($p = 0.0009$, Breslow-Gehan-Wilcoxon test). The estimated median interval between CRPS I symptoms and first attendance at our outpatient clinic was 19.1 weeks (range 0 - 1043.6, interquartile range 9.1 - 49.4) for adults (n = 719) versus 11.9 weeks (range 0 - 205.2, interquartile range 3.7 - 28.1) for children (n = 66). This is significantly shorter than for adults ($p = 0.0002$, Breslow-Gehan-Wilcoxon test).

During the study period several treatment strategies for children with CRPS I have been implemented; consisting of a combination of medical treatment (scavengers, vasodilators, pain medication), physical therapy and psychological counselling.

Table 2. Signs and symptoms of adults and children with CRPS I.

Signs and symptoms	Adults (%) (n /n-ev)	Children (%) (n /ev)	95%-CI for difference	*p*-value
Inflammatory				
Pain	99.9 (950/951)	97.4 (76/78)	[0.5 , 8.8]	<0.01
Difference in skin colour	93.3 (887/951)	82.1 (64/78)	[4.1 , 21.3]	<0.01
Oedema	77.5 (737/951)	39.7 (31/78)	[26.3 , 48.2]	<0.01
Difference in skin temperature	90.9 (864/951)	87.2 (68/78)	[-2.4 , 13.0]	<0.01
- Cooler	44.9 (427/951)	71.8 (56/78)	[-36.2, -15.6]	<0.01
Unexplainable limited range of motion	90.1 (857/951)	62.8 (49/78)	[17.2 , 38.5]	<0.01
Increase of complaints after exercise	82.3 (763/927)	70.5 (55/78)	[2.5 , 22.9]	0.7
Neurological				
Hypaesthesia	75.4 (674/894)	46.2 (36/78)	[17.9 , 40.2]	<0.01
Hyperpathy	80.8 (705/873)	52.6 (41/78)	[17.1 , 39.4]	<0.01
Dyscoordination	47.3 (365/771)	23.4 (18/77)	[12.8 , 32.8]	<0.01
Tremor	43.6 (371/850)	22.1 (17/77)	[10.6 , 30.1]	<0.01
Involuntary movements	28.5 (212/744)	23.1 (18/78)	[-5.5 , 14.0]	0.3
Skeletal muscle spasm	21.7 (185/851)	21.4 (6/28)	[-18.0 , 11.9]	1.0
Paresis	93.2 (670/719)	48.7 (38/78)	[33.4 , 55.4]	<0.01
Pseudo paralysis	17.5 (152/867)	18.2 (14/77)	[-11.0, 6.9]	0.9
Myoclonus	8.1 (51/630)	14.1 (11/78)	[-15.6 , 0.5]	0.07
Atrophy				
Skin	40.2 (347/864)	7.8 (6/77)	[23.6 , 37.7]	<0.01
Nails	26.1 (216/827)	11.7 (9/77)	[4.9 , 20.7]	<0.01
Subcutaneous tissue	25.9 (200/771)	6.5 (5/77)	[11.1 , 24.3]	<0.01
Skeletal muscle	45.8 (374/816)	32.5 (25/77)	[1.8 , 23.4]	0.02
Sympathetic signs and symptoms				
Abnormal sweating	42.3 (343/810)	23.4 (18/77)	[7.9 , 27.7]	<0.01
Complications				
Chronic infection	5.5 (24/440)	1.3 (1/77)	[-1.8 , 6.9]	0.1
Spontaneous haematoma	47.2 (178/377)	6.5 (5/77)	[31.4 , 47.0]	<0.01

n_ev = number of evaluable patients
Not in all the charts all symptoms were documented

Twenty-two children (28.2%) suffered a renewed episode of CRPS I. In fifteen of these cases this was the result of a new injury. Five children were seen with 2 new episodes, one child with three, and two children with four new episodes of CRPS I. In 60% of these relapses, the previously affected extremity was affected again. The signs and symptoms in the renewed episode were similar to those found at the first presentation in our hospital. At the moment of the retrospective chart review, 7 children were still being treated for CRPS I in our hospital.

DISCUSSION

Once considered uncommon, currently there is increased awareness and recognition of CRPS I in children. Complex Regional Pain Syndrome type I is mainly seen in children between the ages 12 to 14 years, with a range of 5 to 17 years[12-16]. The youngest child reported with CRPS I was 2.5 years old[17]. Why CRPS I develops predominantly in adolescent girls (in our study median age was 13 years) is not known[18]. Oestrogen dependent pain responses may explain sex differences[19], while also psychosocial problems in this age group may play an important role[20]. No accurate data are available concerning the incidence and prevalence of CRPS I in children. In the present study, a minor injury, mostly an ankle sprain, induced CRPS I in 45% of the cases. In children no initiating event was found in 46% of the cases[21], in our study this was only 8.3%. In children several signs and symptoms can be attributed to CRPS I[13,14,16,22-24]. The most common complaint is pain, excessive in relation to the severity of injury. In our study, children with CRPS I presented mainly with unexplained severe pain, with a colour and temperature difference (mostly cooler), an impaired active range of motion of the affected extremity. In one report of 70 children with CRPS I, pain was present in all, mechanical allodynia in 86%, oedema in 77%, a temperature difference in 77% (cold extremity) and a difference in skin colour in 73%[13,22,23]. These findings compare reasonably with the present findings, except for oedema, which was seen in only 39.7% of our cases. This difference may be explained by the delayed presentation at our outpatient clinic, and/or by the fact that treatment had started prior to presentation. In the present series the upper extremity was affected in 23.3% of the patients, comparable to previous reports mentioning percentages between 13 and 39%[13,22,23]. Although documented in all series, it is not known why CRPS I in children presents more frequently in the lower extremity[24].

Our results show there are significant differences between adults and children with CRPS I. Most remarkable differences are that in children with CRPS I; female predominance, lower extremity involvement, mostly colder temperature difference and less oedema compared to the opposite in adults with CRPS I. Despite these differences the present study shows that also in children CRPS I is characterized by classical signs and symptoms of inflammation - pain, oedema, discoloration, changes in temperature and impaired function. Also in children CRPS I develops more often after a minor injury. Treatment results seem comparable to these found in adults. Long term prognosis in children is similar to adults, according to a quality of life study in adults with onset of CRPS I in childhood[25]. In previous studies, CRPS I recurrence rates ranged from 28% to 33%[14,21]. We also found a 28.2% recurrence rate between enrolment and follow-up. The child and his parents have to be instructed and prepared for a possible relapse, possibly following a new injury. Indeed a relapse may lead to anxiety, fear and pain, resulting in immobility and recurrence of CRPS I-like symptoms.

There is no consensus concerning the criteria for diagnosing CRPS I in children[13]. In our opinion the criteria of Veldman et al. for CRPS I in adults can also be applied to children[1]. From our study group 60 (76.9%) children and 840 (88.3%) adults also fulfilled the IASP criteria for CRPS I[8,9], none fulfilled the criteria for CRPS II.

Several protocols have been described for the treatment of CRPS in children, with various results. Most studies involved small groups or case reports. Treatment protocols currently recommended for children with CRPS I are physical therapy; advice on posture and movement, stimulation of the normal usage of the affected extremity, and psychological intervention to reduce fear of movement[21].
We have provided clinical and experimental evidence that an excessive regional inflammatory response, including the production of reactive oxygen free radicals, may be involved in causing tissue damage in CRPS I[1,4,26,27]. Also the therapeutic effect of free radical scavengers has been well documented in adults[28,29]. We have managed children with CRPS I accordingly to a treatment protocol including scavengers. This retrospective study does not allow to make firm conclusions as to the treatment of children with CRPS I with scavengers and peripheral vasodilators, a major confounding factor being the individualized co-medication and co-therapy (i.e. doses were adapted to each individual patient). Further investigations by means of randomized controlled trials should be performed to evaluate and validate the effect of scavenger therapy and peripheral vasodilators in children with CRPS I.

CONCLUSION

In this retrospective chart review 78 children (age < 16 year) with CRPS I are described and compared to 951 CRPS I adult patients. The child population consisted predominantly of girls and older children (median age 13 years).The child population differed from adults in that the skin temperature of the involved extremity at onset was more often cooler, that the lower extremity was involved more frequently, and that neurological and sympathetic symptoms were less pronounced. Increased awareness of CRPS I in children, acquaintance with signs and symptoms, use of diagnostic criteria and if necessary referral to a clinic with experience in treating children with CRPS I may lead to decreasing pain, suffering and quicker restore of function in children and adolescents with CRPS I.

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Chapter 3

Quality of life in adults with childhood-onset of Complex Regional Pain Syndrome type I

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ABSTRACT

Background:

The clinical presentation of Complex Regional Pain Syndrome type I (CRPS I) in children differs compared to the presentation in adults. Reported results of treatment of CRPS I in children are usually more favourable and seem better than the reported treatment of adults with CRPS I. We investigated the quality of life (QoL) in adults who have been treated for childhood-onset CRPS I.

Methods:

We performed a retrospective chart review on signs, symptoms and treatment of all patients, seen and treated for CRPS I in childhood (age < 16 years). At one time point a survey was sent by mail to all adult patients with onset CRPS I in childhood with a postal reminder after one month. The first part of the survey consisted of questions focused on the experience of chronic pain and other current complaints in the affected extremity. The second part consisted of a generic health-related quality of life instrument (SF-36).

Results:

Forty-two patients (75%) responded to our survey. The median follow-up period was 12 years (SD 4.7; range 2 - 22). Fifty-two percent of all patients complained about pain at the time of follow-up. Of the 12 symptoms and signs, 4 are improved, 1 is worse and the remainder are unchanged. Fifteen patients (33%) experienced one or more documented relapses. General health and physical functioning (2 out of 8 scales on the SF-36) were lower in patients compared to those of the literature.

Conclusion:

In contrast to the literature, the prognosis of childhood-onset CRPS I seems less favourable than usually reported, and is comparable to the prognosis of the adult-onset CRPS I in view of a decreased quality of life and a large relapse percentage (33%) at long-term follow-up.

INTRODUCTION

Complex Region Pain Syndrome type I (CRPS I) is a potentially incapacitating syndrome which can occur after a minor injury or limb operation. In approximately 10 percent of patients, CRPS I occurs without previous injury. CRPS I impacts all tissues and can impair all functions of the affected extremity, possibly resulting in severe impairment and therapy-resistant pain[1]. At our department, children were managed according to a standardized treatment protocol, consisting of free radical scavenger and vasodilator treatment, attention to painful trigger points and physical therapy, similar to the treatment for adults with CRPS I[2]. Since 1995, psychological counselling was added to the standard treatment protocol. Reported results of treatment of CRPS I in children are usually favourable and seem better than treatment reports of adults with CRPS I[3-5]. We therefore hypothesized that the quality of life is better in adults with childhood-onset CRPS I as compared to adults with adult-onset CRPS I. We conducted a follow-up study of adults with childhood-onset CRPS I at the Radboud University Nijmegen Medical Centre to evaluate their physical, social and psychological effects at long-term follow-up. These effects were measured with a health-related quality of life (HRQoL) instrument. The aim of this study was to investigate the HRQoL of adults patients with a childhood-onset of CRPS I.

PATIENTS AND METHODS

At first we performed a retrospective analysis of the history, signs and symptoms and treatment of all patients who have been seen and were treated for CRPS I in childhood (age < 16 years) between January 1980 and December 2003 at the Radboud University Nijmegen Medical Centre (RUNMC). Data were collected from patient charts and office notes and collected in a database. CRPS I was diagnosed based on the criteria as formulated by Veldman et al[1]. The following diagnostic criteria were used since the start of this study:

1. Presence of at least 4 of the following 5 signs and symptoms: unexplained diffuse pain and tenderness in the distal part of the extremity, difference in skin colour in relation to the healthy symmetrical limb, diffuse oedema, difference in skin temperature in relation to the healthy symmetrical limb and limited range of movement.
2. The above signs and symptoms increase during exercise.
3. The above signs and symptoms are present in an area much larger than the area of primary injury or operation and including the area distal to the primary injury.

After identification of the patients with childhood-onset CRPS I, a survey was sent by mail with a postal reminder after one month to only the adult patients (age > 16 years) with childhood-onset CRPS I. The survey was divided into two parts. The first part of the survey consisted of

questions focused on the experience of chronic pain and other current complaints in the affected extremity. These results were compared with the signs and symptoms, documented at the first consult. The second part consisted of a HRQoL instrument, namely the Dutch version of the Medical Outcome Study Short Form-36 (SF-36)[6]. The SF-36 is made up of 36 items and standardized response choices. This instrument has been used in a variety of studies and also in several studies on musculoskeletal disorders and CRPS I. Psychometric properties of this instrument have been studied in detail and are considered adequate[7-9]. The 36 items are converted to eight scales representing generic health domains. These eight health domains are: physical functioning (PF), role function-physical aspect (PR), bodily pain (BP), general health perception (GH), vitality (VT) social functioning (SF), role function - emotional aspect (RE) and mental Health (MH). Higher scores represent a better HRQoL in the particular domain PF, RP and BP are a measure of physical dimensions, RE and MH measure mental dimensions. GH, VT and SF correlate with both physical and mental domains.

All statistical analyses were conducted using SPSS (Statistical Package for the Social Sciences version 12.01 for Windows; SPSS Inc., Chicago, IL). The paired-sample t-test was used to compare the signs and symptoms between first consult and follow-up. A p-value of < 0.05 was considered statistically significant. To identify possible prognostic factors regarding the relationship between short and long-term outcome measures (signs and symptoms as noted in the first part of the survey), location of CRPS I, and the history of trauma was estimated with linear regression analysis.

RESULTS

Sixty-two adult patients were identified with childhood-onset CRPS I (age < 16 years), according to the Veldman et al. criteria, and were approached to participate in this study[1]. One patient died from severe CRPS I (euthanasia), two patients refused for unknown reasons and three patients could not be located. Hence, 56 patients were available for the study. Forty-two (75%) responded to our survey (Table 1). At first consult, when the diagnosis of CRPS I was made, the median age of these patients was 13.2 years (SD 2.2, range 6 - 16). When the survey was conducted, the median age of the patients was 25.5 years (SD 4.8, range 16 - 34). Thirty-seven (88%) were female. The median follow-up period was 12 years (SD 4.7; range 2 - 22). All were Caucasian.

Table 2 shows the signs and symptoms both from the first consult and at follow-up. Fifty-two percent of all patients complained about pain at the time of follow-up. The initial median documented visual analogue score (VAS) at first visit at our outpatient clinic was 6.0 (SD 2.4; range 2 - 10), and at follow-up was 4.5 (SD 2.4; range 1 - 8.5). Patients reported that this pain increased with exposure to cold (36%) and/or heat (10%). Ten patients (23.8%) did not have any

Table 1. Clinical characteristics of 42 adult patients with onset CRPS I in childhood.

Characteristics	Descriptives
Female (%)	37 (88)
Median age in years (range)	25.5 (16 - 34)
Median follow-up in years (range)	12 (2 - 22)
History of trauma (%)	37 (88)
Upper extremity involved (%)	8 (19)
Lower extremity involved (%)	32 (76)
Upper and lower extremity involved (%)	2 (5)
Left (%)	23 (55)
Right (%)	17 (42)
Bilateral involvement (%)	2 (5)

Table 2. Signs and symptoms at time of first visit and at follow-up of 42 adult patients with onset CRPS I in childhood (< 16 years).

	First consult n (%)	Follow-up n (%)	*p*-value
Pain	40 (95.2)	22 (52.4)	< 0.01*
Increase of complaints after exercise	25 (59.5)	24 (57.1)	0.82
Hypersensitivity	6 (14.3)	14 (33.3)	0.03*
Decrease in tactile sense	21 (50.0)	8 (19.0)	< 0.01*
Difference in skin colour	31 (73.8)	12 (28.6)	< 0.01*
Difference in skin temperature	36 (85.7)	19 (45.2)	< 0.01*
Swelling	14 (33.3)	8 (19)	0.35
Limited range of motion	25 (59.5)	19 (45.2)	0.57
Involuntary movements	10 (23.8)	7 (16.7)	0.78
Tremor	13 (30.9)	5 (11.9)	0.08
Decrease in muscle strength	15 (35.7)	15 (35.7)	0.66
Dyscoordination	11 (26.2)	5 (11.9)	0.32

* = $p < 0.05$

complaints at follow-up. Recurrence of signs and symptoms which could be attributed to CRPS I, after treatment were found in 63% of the patients, but in the patient charts and office notes, only an objectively documented recurrence rate of 33% was found.

Results of the SF-36 are shown in Table 3. Analyzing gender differences, a significant difference was observed in 6 out of 8 domains ($p < 0.05$). On all eight domains, males scored better than females.

The linear regression analysis showed that the longer the follow-up period since the first attendance at our outpatient clinic, the better patients scored on the domain role physical ($p = 0.02$). The age of the patient at the time of first consult did not significantly predict the outcome of the HRQoL scores, except for the general health domain. Neither the history of trauma nor the location of CRPS I was found to be prognostic factor.

Table 3. Long-term follow-up SF-36 means scores (SD) of 42 Dutch adults with onset CRPS I in childhood.

	Adults with onset CRPS I in childhood n = 42
1. Physical functioning	82.9 (20.4)
2. Role functioning physical	77.4 (36.1)
3. Bodily pain	79.8 (25.01)
4. General health	58.7 (25.9)
5. Vitality	67.7 (23.2)
6. Social functioning	83.03 (22.2)
7. Role functioning emotional	92.9 (22.7)
8. Mental health	78.0 (19.1)

DISCUSSION

The clinical presentation of Complex Regional Pain Syndrome type I (CRPS I) in children differs compared to the presentation in adults[5]. CRPS I is seen specifically in children from 12 to 14 years, with a range of 5 to 17 years[5,10-14]. The youngest child reported with CRPS I was 2.5 years[15]. The reason for the predilection of adolescent girls to develop CRPS I is unknown,[16] although some suggest that psychosocial aspects may play a role[17]. No accurate data are available concerning the prevalence and incidence of CRPS I in children. After a median follow-up period of 12 years, only 23.8% of the patients was free of complaints. The reported long-term outcome of the children in our study appeared to be less favourable than previously published in other studies (Table 4)[4,10-13,18,19,24]. All of these studies, however, had a much shorter follow-up period.

To our knowledge, this is the first description of long-term outcomes expressed by health-related quality of life (HRQoL) in adults with childhood-onset CRPS I. Contrary to our hypothesis, the results of the present study showed that CRPS I outcome and prognosis in children seem comparable to adults. Most adults with childhood-onset of CRPS I have a poorer HRQoL as

Table 4. Outcome of CRPS I in childhood (< 16 years).

Study	Children n*	Favourable outcome n (%)	Mean follow-up years
Tan et al. (this study)	42	10 (24)	11.5
Wilder et al.[11]	70	32 (46)	2
Sherry et al.[4]	49	43 (88)	5.3
Murray et al.[13]	43	40 (93)	0.5
Greipp et al.[18]	27	1 (4)	4.2
Stanton et al.[12]	36	25 (69)	unspecified
Lee et al.[24]	25	25 (100)	2.5
Bernstein et al.[10]	20	12 (60)	2.4
Low et al.[19]	20	18 (90)	2

* Final number of patients analyzed

compared to a standard Dutch age-matched group (16 - 40 years) of 1585 healthy subjects of the general population[20]. Analyzing all 8 items of the SF-36, adults with childhood-onset CRPS I seem to have a lower physical health status but seem to have better emotional and mental health function as compared to normal controls. This emphasizes the major impact of this disease in patients confronted with CRPS I in childhood. Children may develop different coping strategies in these psychosocial domains, a phenomenon whose end result is also observed in adults operated in childhood for a benign disease[21]. However, the observations of these studies are in contrast to the observation, that paediatric cancer survivors usually have a good HRQoL and a higher rating of physical health and social-emotional functioning, possibly related to the change in the survivors outlook on life that resulted from the cancer experience, by which they may be much happier with the various details of life[22]. When comparing our results with 65 Dutch adult patients with CRPS I diagnosed and treated in adulthood (mean age 50.2 years, SD 14.9), with a mean follow-up of 5.5 years for 3 HRQoL domains, comparable results for role physical 77.4 (36.1) versus 71.2 (40.8) and social functioning 83 (22.2) versus 82.7 (23.3) were observed[7]. The higher social emotional functioning we found in the childhood-onset CRPS I group 92.9 (22.7) as compared to the Dutch adolescent age-matched group ((84.1 (32.3)) and as compared to the adult-onset group ((78 (36.9)), may be explained by the higher proportion of females in this specific group, who may have different coping strategies than males[23].

In line with the literature reports that describe a high recurrence rate of 27.5 - 50% in children[12,13,24], in our study a (well-documented) recurrence rate of 33% was found. Children and parents could therefore be prepared for the occurrence of episodes of recurrent complaints, which may lead to anxiety, fear and pain, and to immobility and renewed occurrence of CRPS I like symptoms. We preventively instructed them how to handle these episode of recurrent complaints by themselves, and most children were able to do so with occasionally some help in the first episode of the recurrence.

There are a number of alternative explanations why our findings, that long-term results after childhood-onset CRPS I do not differ much from adult-onset CRPS I, and are rather different from earlier literature reports. First, this study has all the drawbacks of a retrospective study. Second, the patient population studied, was comprised only of unselected patients referred to our university tertiary referral centre, which functions as the single reference centre for CRPS I in the Netherlands. It is therefore likely that the CRPS I patient populations evaluated elsewhere consisted of a selected group of patients (selection bias) and also different therapeutic approaches may have been applied[25]. Primary care physicians and other general paediatricians would often consult or refer patients with CRPS I directly to the RUNMC. It can thus be argued that the patients included in our study do not comprise of a smaller subset of more severely affected patients, but constitutes a large, and probably unbiased group of children and adolescent with CRPS I. Our multidisciplinary approach warrants equal treatments strategies for all

patients. In our study group more females than males were included compared to the standard population. All other social demographics were almost equally distributed among the CRPS I patient group and the control groups. From many HRQoL studies and also in our study it is known that males show a slightly better HRQoL,[20,26] but due to small sample size this pattern could not become visible. Third, there can be a definition bias as other studies used different inclusion criteria for the diagnosis of CRPS I. Currently there is no consensus concerning the criteria for this diagnosis in children. In our opinion the Veldman and Goris criteria,[1] which have been developed by the RUNMC are more likely to be specific for CRPS I or II than the criteria used by Sherry and Weinman[16]. Retrospectively, we found that 81% of our patients fitted the criteria for CRPS I according to the International Association for the Study of Pain (IASP), and did not fulfill the criteria for CRPS II[27,28]. Fourth, baseline data were not available per patient so we had to use the healthy standard population as a substitute baseline to compare the HRQoL measurement with the test point after the onset of CRPS I. Finally, one of the components of our treatment strategies with free radical scavengers and vasodilators has barely been used outside of Europe. One could argue that these medical interventions could have been responsible for the less favorable outcome described by other studies, but it is questionable that this is very likely. Psychological treatment was not part of the standard treatment for children with CRPS I before 1995, though on indication by the multidisciplinary team psychological consultation was asked before 1995. Further investigations by means of randomized controlled trials should be performed to evaluate and validate the effect of scavenger therapy and peripheral vasodilators in children with CRPS I.

In conclusion, while previous publications generally suggests a more favourable prognosis for childhood-onset CRPS I, our series showed that at long-term follow-up, a considerable proportion of patients continued to experience moderate pain and they have modest reductions in median HRQoL sub scores, compared to the control group. Recurrent episodes are also common in one-third of cases. Therefore children treated for childhood-onset CRPS I do not have a better quality of life as compared to adult-onset CRPS I.

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Chapter 4

Leukocytes in Complex Regional Pain Syndrome type I

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ABSTRACT

Background:

The pathophysiology of Complex Regional Pain Syndrome type I (CRPS I) is unclear. An inflammatory reaction may cause the syndrome in which leukocytes may play an important role.

Methods:

In this pilot study of six patients with acute warm CRPS I, we performed radiolabeled autologous leukocyte scans of both hands, in order to assess leukocyte accumulation. Comparison was made with the unaffected limb, and with three control patients with a Colles' fracture without CRPS I.

Results:

Images of the CRPS I patients obtained 4 hours after leukocyte injection provided the clearest results. At 4 hours post-injection, there was clear, asymmetrical leukocyte accumulation in the affected extremity with a mean ratio of 1.49 ± 0.19. In control patients, no asymmetry was observed between hands (mean ratio 1.09 ± 0.06), indicating the absence of specific leukocyte accumulation. There was a statistically significant difference between CRPS I and control subjects 4 hours post injection ($p = 0.012$).

Conclusion:

We found a significantly increased accumulation of leukocytes in patients with CRPS I. This is the first study to show a possible role for leukocytes in the pathophysiology of acute CRPS I.

INTRODUCTION

Complex Regional Pain Syndrome type I (CRPS I) is a poorly understood syndrome which may occur in an extremity after even a minor injury or operation. Although the clinical signs and symptoms of CRPS I are well known, the underlying pathophysiology remains unclear. Sudeck hypothesized that an excessive inflammatory reaction may cause the syndrome[1]. Observations supporting the inflammatory theory include:

(a) The dominance of clinical signs and symptoms of inflammation - such as unexplained severe pain, oedema, a difference in skin temperature, a difference in skin colour - in the affected extremity at the onset of CRPS I[2],
(b) An increased extravasation of indium-labeled immunoglobulin as a sign of increased capillary permeability for macromolecules[3],
(c) A therapeutic response to various oxygen radical scavengers[4],
(d) Recently higher levels of IL-6 and TNF-α in blister fluid in the involved extremity in comparison to the uninvolved extremity[5] and
(e) Successful intervention in a pilot study with anti-TNF[6].

In the acute phase, CRPS I is characterized by signs and symptoms of inflammation within the affected extremity[2]. We hypothesized that CRPS I is an exaggerated inflammatory response and that the increased capillary permeability can be due to endothelial damage caused by oxygen radical species directly or mediated by polymorphonuclear leukocytes.
In this study we performed radiolabeled autologous leukocyte imaging of both hands in patients with CRPS I of one upper extremity after a fracture or operation of the hand and compared that with the unaffected contralateral hand. A second control consisted of patients with a Colles' fracture who did not develop CRPS I. This control group was chosen considering the high incidence of CRPS I after a Colles' fracture (8 - 37%)[2,7]. Our goal was to asses, in the acute phase of CRPS I, accumulation of leukocytes in the affected extremity, which could point to a possible role for leukocytes in the pathophysiology of acute CRPS I.

METHODS

The study was performed in the out-patient clinic of the Departments of General Surgery, Radboud University Nijmegen Medical Centre and the Canisius Wilhelmina Hospital Nijmegen. All new patients presenting with signs and symptoms of acute warm CRPS I, after preferably a Colles' fracture were invited to participate in the study. Patients with abnormalities in the contralateral hand were excluded. The study protocol was approved by the Regional Ethical

Committee Arnhem-Nijmegen. Written informed consent was obtained from all patients. The following diagnostic criteria were used in making the diagnosis of CRPS I[2]:

1. Presence of at least 4 of the following 5 signs and symptoms: unexplained diffuse pain and tenderness in the distal part of the extremity, difference in skin colour in relation to the healthy symmetrical limb, diffuse oedema, difference in skin temperature in relation to the healthy symmetrical limb and limited range of movement.
2. The above signs and symptoms increase during exercise.
3. The above signs and symptoms are present in an area much larger than the area of primary injury or operation and including the area distal to the primary injury.

All patients were managed according to a standardised treatment protocol[8]. Skin temperature was measured three times at the dorsal surface of the hand, with an infrared (ear) thermometer (Generic Sherwood medical First Temp "Genius" Digital Ear Thermometer), held 1 cm above the skin surface. The pain was scored according to the visual analogue score (VAS) for pain. Approximately 50 ml of blood was drawn from the antecubital vein of the unaffected extremity. Leukocytes were isolated and labelled with 200 MBq ^{99m}Technetium- Hexamethylpropyleneamine oxime (^{99m}Tc- HMPAO), as described by Peters et al.[9] and re-injected in the cubital vein. HMPAO is an efficient leukocyte label, and labels granulocytes with more stability than mononuclear leukocytes[10,11]. After one and 4 hours gamma camera images were acquired from both hands using a gamma camera (Orbiter; Siemens Medical Systems, Inc., Hoffman Estate, IL, USA). The images were analyzed by drawing regions of interest on both hands. The leukocyte accumulation was expressed as the ratio of uptake between the affected and unaffected hand. Also the specific leukocyte accumulation at the former fracture site was measured, expressed as the ratio of uptake between the fracture site (if present) and the identical contralateral region. Statistical analysis was performed using the unpaired samples t-test and the Spearmen rank correlation test. Results were considered significant when $p < 0.05$.

RESULTS

Included were four female and two male patients with acute warm CRPS I (mean age 54 years; range 42 - 66 years). In 5 patients the left extremity was involved. As shown in Table 1, the mean interval between trauma and leukocyte imaging was 71 days (range 35 - 194 days). The mean interval between the start of symptoms and leukocyte imaging was 18 days (range 1 - 46 days). The mean score on the VAS was 4.7 (range 0 - 10), the mean difference in skin temperature was 2.6° C (range 0 - 5°C).

The three control patients were all female, with a mean age of 62 years (range 52 - 72 years) (Table 1). Leukocyte scans were performed 60 days after the initial trauma. No control patient

Table 1. CRPS I patient and control patient characteristics, WBC uptake ratio affected *versus* healthy hand, 4 hours after injection and WBC uptake ratio specific at the fracture site, 4 hours after injection.

Nr	Gender Age (yrs)	Trauma or operation	4 hrs p.i.	4 hrs p.i. fracture site	Time after trauma to CRPS I	Time after trauma to scan	Time after CRPS I to scan
1	F 66	Dupuytren operation	1.65	No fracture	42 days	47 days	5 days
2	F 53	Colles' fracture, 5 weeks plaster of Paris	1.44	2.85	39 days	44 days	5 days
3	F 66	Colles' fracture, external fixation	1.31	1.44	158 days	194 days	36 days
4	M 45	Metacarpal III and V fracture internal fixation with K-wires	1.77	1.52	34 days	35 days	1 day
5	M 42	Colles' fracture and scaphoid fracture, internal fixation with plate and 12 weeks plaster of Paris	1.27	1.6	41 days	55 days	14 days
6	F 54	Distal antebrachial fracture, external fixation	1.49	1.57	6 days	52 days	46 days
7	F 52	Control Colles' fracture, 5 weeks plaster of Paris	1.06	1.74		60 days	
8	F 72	Control Colles' fracture, 6 weeks plaster of Paris	1.06	1.56		60 days	
9	F 63	Control Colles' fracture, 6 weeks plaster of Paris	1.16	1.88		61 days	

p.i. = post injection

had any signs or symptoms indicating CRPS I. All fractures presented with signs and symptoms pointing to a normal healing process at the time of measurement and all control patients were free of complaints.

The images of CRPS I patients obtained 4 hours after leukocyte injection provided clearest results (Figure 1). At 4 hours post-injection, there was clear, asymmetrical leukocyte accumulation in the affected extremity with a mean ratio of 1.49 ± 0.19. In control patients, no asymmetry was observed between hands (mean ratio 1.09 ± 0.06), indicating the absence of specific leukocyte accumulation. There was a statistically significant difference between CRPS I and control subjects 4 hours post injection ($p = 0.01$). In the CRPS I patients, the accumulation in the affected extremity showed a negative correlation with the interval between trauma and imaging ($r = -0.77$) however this was not significant $p = 0.07$ with the Spearmen rank correlation test (Figure 2). In both CRPS I patients and controls, some activity was noted at the former fracture site. In the mean ratio of leukocyte accumulation at the former fracture site was similar for CRPS I patients and for the controls (1.80 ± 0.59 and 1.73 ± 0.16, respectively, $p = 0.74$).

Figure 1. WBC uptake of patient no. 6, 4 hours after injection. There is a clear, visually asymmetrical leukocyte accumulation in the affected right hand. Some activity was noted at the former fracture site (*arrow*), the distal antebrachial fracture.

DISCUSSION

In this study, we found evidence of increased leukocyte accumulation in acute CRPS I after a trauma or operation of the hand. This accumulation seemed to decrease with the interval between trauma and imaging; however this was not statistically significant probably due to the small numbers. In contrast, some 9 weeks after the Colles' fracture, no specific leukocyte targeting was found in control patients without CRPS I. In CRPS I patients, increased leukocyte accumulation is most probably due to prolonged extravasation and accumulation of leukocytes which suggests an ongoing inflammatory response, even after the fracture or trauma had healed.

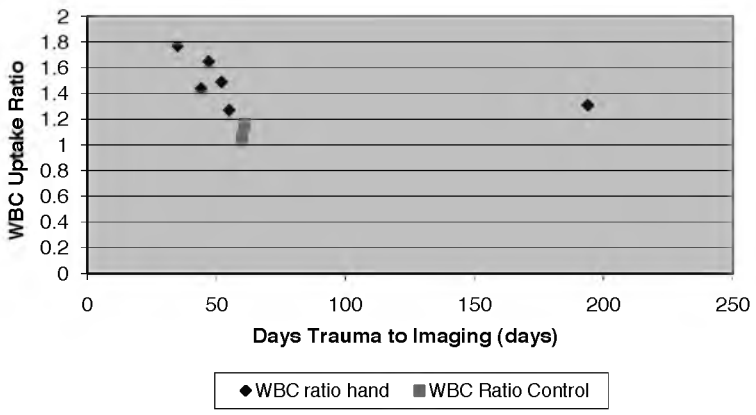

Figure 2. WBC uptake ratio affected *versus* healthy hand, 4 hours after injection *versus* time trauma to imaging (days).

Leukocytes can be categorized into three main groups: granulocytes, monocytes and lymphocytes. Lymphocytes are known to intervene in immune responses such as secreting cytokines, killing cells, or the production of antibodies. Monocytes and macrophages participate in inflammation by synthesizing numerous mediators and eliminating various pathogens. The main type of granulocytes is the neutrophil, also called the polymorphonuclear leukocyte (PMN)[12]. The PMN or segmented neutrophil is the end-stage of full maturation of the granulocyte. This cell type is involved in the pathophysiology of many processes variable from ischemia-reperfusion[13] to adult respiratory distress syndrome (ARDS), rheumatoid arthritis, inflammatory bowel diseases[12] and probably also CRPS I. ^{99m}Tc- HMPAO has been demonstrated to show a preference for granulocytes[11]. The PMN capability to inflict damage has been credited to its ability either to release toxic granule components or to generate reactive oxygen metabolites. By oxidatively inactivating a series of key proteinase inhibitors and simultaneously activating latent proteinases, PMN are able to exert more damage and with greater specificity than with oxidants alone[14]. Aggregation to the endothelium, causing the release of proteases and release of oxygen radical species[15], and plugging of the microcirculation, causing a no-reflow phenomenon can also inflict ischemia-reperfusion induced tissue injury[16]. Another mechanism which could aggravate cellular damage is induction of vascular contraction by inactivation of endothelial nitric oxic by PMN-derived oxide, and promotion of release of proinflammatory arachidonic acid metabolites[13].

The first stage of fracture repair is characterized by extravasation of blood followed by aseptic inflammation. The haematoma at the fracture site is invaded by a variety of blood elements including polymorphonuclear leukocytes. Although it has been generally accepted that this inflammatory response is beneficial, there are reports that PMN may aggravate the tissue damage if inflammatory stimuli are chronically directed against host tissue or are not properly down regulated[14]. Most of the CRPS I and control patients in this study were in the phase of fracture

repair, which explains the persisting accumulation of granulocytes at the former fracture site compared with the normal contralateral hand in both groups. This accumulation at the former fracture side diminishes in time.

To our knowledge, this is the first study which directly points to a possible role of leukocytes in the acute phase of CRPS I. Currently no histology is available in the acute phase of CRPS I, since in the acute phase any additional injury may trigger an increase of severity or recurrence of CRPS I.

In an experimental model of acute CRPS I, which uses free radical donor infusion in one extremity of the rat, vascular permeability and considerable soft tissue damage was induced[17]. In this animal model, we have shown that PMN depletion decreased the increased vascular permeability of the capillaries to some extent. This finding is in agreement with a study describing that free radicals are primarily responsible for increased vascular permeability of skeletal muscle in ischemia-reperfusion[18] and that PMN's induce an additional effect on vascular permeability in ischemia-reperfusion[19]. Both free radical generating capacity and release of proteases by PMN's could contribute to the vascular permeability[16,20,21]. Another explanation for the amplification of oxidative stress injury by PMN's may be the adherence of PMN's to endothelial cells, which triggers endothelial cells to generate additional free radicals[15,20].

One study examined nitric oxide production by peripheral monocytes stimulated with interferon-γ (IFN-γ) in CRPS I patients. Although no differences were found between the samples taken from the contralateral and ipsilateral extremity, a significant increase in absolute NO release in response to IFN-γ was present compared to controls[20,22].

Further research should be conducted, focused on the function of leukocytes at the direct site of CRPS I.

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Chapter 5

The oxidative response in the CCI model of neuropathic pain

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ABSTRACT

Background:

In the chronic constriction injury (CCI) model of rat neuropathic pain, oxidative stress as well as the antioxidants superoxide dismutase and reduced glutathione are important determinants of neuropathological and behavioural changes. Studies in the CCI model observed (indirect) signs of inflammation. We, therefore, investigated the level of oxidative stress and antioxidant enzymes in skeletal muscle tissue of the rat hind paw and (jugular vein) plasma at day 7 after nerve-injury.

Methods:

The level of reactive oxygen and nitrogen species (RONS) was determined as a measure of oxidative stress. Reduced glutathione (GSH) levels and the ceruloplasmin/transferrin ratio were determined as measures of overall antioxidant activity. RONS and overall antioxidant activity were measured in skeletal muscle tissue of the hind paw and jugular vein plasma. The level of RONS in muscle was determined using spin trapping combined with electron paramagnetic resonance spectroscopy. Using electron paramagnetic resonance spectroscopy, we also determined plasma levels of transferin and ceruloplasmin. Glutathione (GSH) levels were determined using high-performance liquid chromatography.

Results:

In skeletal muscle tissue, the level of RONS was lower in nerve-injured hind paws than in controls. The plasma level of RONS did not differ between nerve-injured and control rats. In skeletal muscle tissue, the level of GSH was higher in nerve-injured hind paws than in controls. The ceruloplasmin/transferrin ratio tended to be higher in (jugular vein) plasma of nerve-injured rats as compared to controls.

Conclusion:

This study shows that, at day 7 after nerve-injury, oxidative stress-induced changes are present in skeletal muscle tissue of the rat hind paw. Our findings of a decreased level of RONS in combination with an increased level of the antioxidant GSH suggest that an overshoot of antioxidant activity overrules initial oxidative stress.

INTRODUCTION

Oxidative stress may cause cell damage through increased oxidant generation, decreased antioxidant protection, and/or failure to repair oxidative damage. Cell damage is induced by reactive oxygen and nitrogen species (RONS), which are either free radicals, reactive anions containing oxygen atoms or molecules containing oxygen atoms that can either produce free radicals or chemically activated by them. Under normal conditions, RONS are cleared from cells by the action of superoxide dismutase, catalase or glutathione, as well as the antioxidant vitamins C and E. In pathological conditions, however, intracellular RONS level can cause severe cell damage and even cell death.

In the chronic constriction injury (CCI) model of rat neuropathic pain, it has been shown that oxidative stress as well as antioxidants superoxide dismutase and reduced glutathione are important determinants of neuropathological and behavioural consequences[1]. Heat hyperalgesia is reduced by systemically injected antioxidants[2,3]. Likewise, systemic injection of the reactive oxygen species scavenger phenyl-*N*-*tert*-butylnitrone relieves mechanical allodynia in the spinal nerve ligation (SNL) model of neuropathic pain[4]. In the CCI model, the role of oxidative stress has been investigated and confirmed only in neural structures, more specifically in the injured sciatic nerve and spinal cord. However, one should realize that other studies in the CCI model observed (indirect) signs of inflammation also in non-neural tissues. Among these are increased skin blood flow and skin temperature at an early stage after nerve injury[5] as well as oedema and increased extravasation of polymorphonuclear leukocytes in skeletal muscle tissue[6]. These inflammatory changes may also involve oxidative stress. We, therefore, investigated the level of oxidative stress and antioxidant enzymes in skeletal muscle tissue obtained from the nerve-injured hind paw and in jugular vein plasma at day 7 after nerve-injury.

Measurement of free radical concentrations is difficult, because they are extremely short lived and only produced in minute quantities. Assessing the level of radicals is generally performed by indirect methods such as the determination of the level of xanthine oxidase, which catalyzes the formation of superoxide, and the effect of antioxidant treatment on levels of lipid hydroperoxides (products of lipid peroxidation caused by free radicals)[7]. Transferrin is an iron binding protein, which prevents free iron to form toxic quantities of free radicals, thus having important antioxidant qualities. Ceruloplasmin also is an important antioxidant. Both are important extra cellular endogenous antioxidants. Transferrin and ceruloplasmin were determined as measures of antioxidant activity (AOA) in plasma. Glutathione (g-glutamyl-cysteinylglycine) is a sulfhydryl (-SH) antioxidant, antitoxin, and enzyme cofactor. The antioxidant "reduced glutathione" tripeptide is conventionally called glutathione (GSH). We assessed the level of transferrin, ceruloplasmin and glutathione as measures of oxidative stress.

MATERIALS AND METHODS

Animals

We used adult male Sprague-Dawley (Animal Research Laboratories, Himberg, Vienna, Austria) rats weighing 300 to 400 gram (mean 372 gram). The animals were granted free access to standard laboratory chow and water during a 7-day adaptation period after delivery to our experimental unit. All animals were maintained in a room at 22°C with a 12-h light-dark cycle. Prior to the experiment, the rats were fasted overnight with free access to water. The protocol of the study was approved by the Animal Protocol Review Board of Vienna and followed the requirements defined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (publication NIH 86-23, revised 1985).

Surgical procedure

The rats were anaesthetized by intra peritoneal injection of ketamine 70-mg/kg body weight and xylazine 10-mg/kg body weight subcutaneously. The animals were kept on a temperature-controlled surgical board (38 ± 1°C) and allowed to breathe spontaneously. Animals were divided into two groups; neuropathic (n = 9) and controls (n = 9). Rats of the control group were not subjected to any kind of surgery. In the neuropathic group the common left sciatic nerve was exposed at the level of the middle thigh by blunt dissection through the biceps femoris, using a microscope with 40x magnification. Proximal to the sciatic nerve trifurcation, about 7 mm of nerve was freed of adhering tissue and 4 ligatures (4/0 catgut, Ethicon, Johnson & Johnson, Brussels, Belgium) were tied loosely around it with about 1 mm spacing (Figure 1). The length of nerve thus affected was 4 - 5 mm long. The ligatures were tied in a way that the nerve was just barely constricted. The incision was closed in layers with catgut 4/0 (Ethicon), the skin with mersilene 2/0 (Ethicon).

Sampling of plasma and skeletal muscle tissue

On the day of surgery as well as before instrumentation for trap application on day seven, clinical signs and symptoms were assessed as described in detail previously[8]. To determine the oxidative status on day seven after nerve-injury, the rats were anaesthetized and maintained under anaesthesia by a mixture of 0.8% isoflurane and room air for the duration of the operation. The animals were kept on a temperature-controlled surgical board (38 ± 1°C) and allowed to breathe spontaneously. Following the induction of anaesthesia, the jugular vein was catheterised under aseptic conditions with a polyethylene catheter (PE50, Clay Adams, Parsipany NJ) filled with heparinised Ringer's solution (8 units/mL). A first heparinised blood sample (1.5 mL) was withdrawn, and blood volume replaced by 1.5 mL Ringer's solution. Plasma was separated by centrifugation during 10 minutes at 3000 *g* (12.000 rpm at 6°C). Subsequently, the spin trap 1-hydroxy-3-carboxy-pyrrolidine (CPH), developed to detect superoxide radicals and peroxinitrite radicals (RONS), was intravenously administrated in five animals in each group[9].

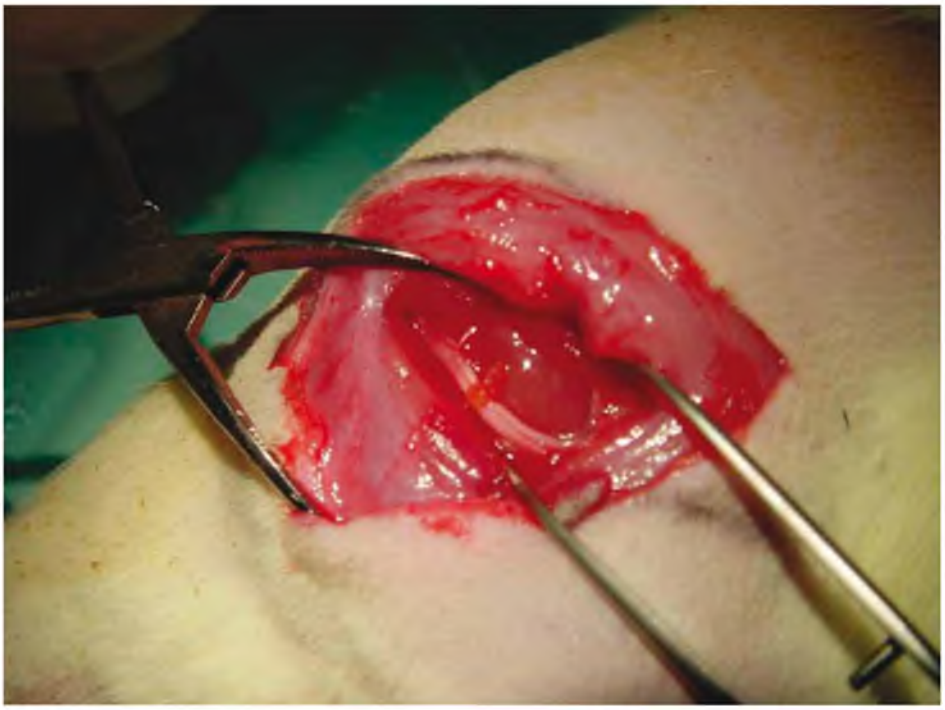

Figure 1. The chronic constriction injury (CCI) model of rat neuropathic pain; proximal to the sciatic nerve trifurcation, about 7 mm of nerve was freed of adhering tissue and 1 ligature (4/0 catgut, Ethicon, Johnson & Johnson, Brussels, Belgium) is tied loosely around (in total 4 ligatures with about 1 mm spacing will be placed).

At the end of the CPH infusion period of 30 minutes, tissue samples were obtained of the left gastrocnemius muscle. In animals without CPH infusion (n = 4), gastrocnemius muscle samples were collected by freeze clamping, and directly frozen in liquid nitrogen for GSH analysis in duplicate.

Clinical signs and symptoms

On both hind paws, a mark was placed above the ankle joint to perform identically localized measurements. Skin temperature was measured on the plantar area of both hind paws using a surface electrode (diameter = 0.6 cm; Keith, Geneva, Ohio, USA) and the temperature difference between both paws was noted. The circumference of the paw was measured using a string. The function of the left hindlimb was observed in a perspex cage 26 x 26 x 26 cm after 5 minutes habituation. During 5 minutes the time of different position of the operated paw was noted according to the spontaneous pain scale of Attal[10]. The neutral position is the position when the operated paw is posed normally on the floor of the cage. The position is not neutral if the paw rests lightly on the floor with the toes in plantar flexion, or only partially (with the heel or

the internal edge of the paw) or is completely elevated. Walking function was assessed during 5 minutes. If necessary, the rat was stimulated to walk. The walking function was noted as impaired when the rat showed a shuffling gait. Grasping function was assessed by letting the rat grasp the edge of the perspex cage 5 times. Grasping function was impaired if no grasping occurred.

Mechanically and thermally induced pain reflexes were assessed. Mechanical sensitivity was tested by foot withdrawal in response to mechanical stimulation with Von Frey filaments (North Coast Medical, San José, USA) of two bending forces (5.16 gram and 12.5 gram). The rats were placed in a perspex cage with a wire mesh floor (26 x 26 x 26 cm). After 5 minutes accommodation, a Von Frey filament was applied 10 times (once every 5 seconds) to the plantar surface of both hind paws and the frequency of foot withdrawal was noted[11]. Thermal pain was measured on a heated floor of 40 ± 1°C. After 5 minutes of accommodation the pain was scored according to the spontaneous pain score of Attal[10].

Determination of level of RONS, ceruloplasmin, and transferrin

Heparin plasma was used for electron paramagnetic resonance (EPR) analysis[12,13]. Three hundred fifty µL of plasma was placed in a 1 mL syringe and immediately frozen in liquid nitrogen. Then, the sample was pressed out of the syringe and moved to a fingertip liquid nitrogen Dewar for electron paramagnetic resonance (EPR) analysis. The EPR spectra of RONS (CPH-adducts) were recorded at liquid nitrogen temperature as described in detail previously[12,13] using a Bruker EMX EPR spectrometer at the following setting: microwave frequency 9.431 GHz, modulation frequency 100 kHz, microwave power 31 mW, modulation amplitude 15 G; gain 10^5). The double integrals of transferrin (g-factor 4.31) and ceruloplasmin (g-factor 2.05) signals were calculated and compared with those obtained from standard iron (III) and Cu (II) complexes. The standard iron (complexes were prepared by mixing 10 millimol desferrioxamine B with different amounts of iron ions ($FeSO_4 \times 7H_2O$). Desferrioxamine iron complex formed in this mixture was used as a standard. Standard Cu (II) solutions were prepared by adding different amounts of copper ions ($CuSO_4 \times 5\,H_2O$) to 10 millimol diethyldithiocarbamate (DETC) solved in 10% Bovine serum albumin. Copper DETC complex formed in this mixture was used as a standard. The final iron/copper concentrations were ranging from 0 to 25 µM. Upon reaction with RONS CP-H is transformed into a stable $CP^{\bullet}$ radical (3-carboxy-proxyl). Standard solutions of 3-carboxy-proxyl were used to quantify RONS levels in plasma and skeletal muscle tissue.

Determination of level of glutathione in skeletal muscle tissue

Tissue samples, frozen in liquid nitrogen, were homogenized in 0.4 M $HClO_4$ using a ball mill. After centrifugation acid extracts were stored at -20°C or analyzed immediately by means of ion-pair reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC-System included a Spherisorb S3ODS-2 column (3 µm, 125 mm x 4 mm ID; Phenomenex, Torrance, CA), a Rheodyne 7125 injector (Rheodyne, Cotati, CA), a PU-980

HPLC pump (Jasco, Great Dunmow, United Kingdome), a pulsation dampener (Shodex, Tokyo, Japan), a Coulochem 5100 A electrochemical detector (ESA, Bedford, MA) equipped with a 5011 analytical cell (+ 0.4 V). Data acquisition and analysis was done with an analog interface AI 406 and System Gold software (both Beckman Coulter, Fullerton, CA). The mobile phase consisted of 0.1 mol/L sodium acetate, 0.1 mol/L sodium hydrogen phosphate, 400 mg/L sodium dodecyl sulfate adjusted to pH 2.0, and 2.5% acetonitrile (vol/vol). Calibration curves for reduced glutathione (GSH) were established daily. Oxidized glutathione (GSSG) was measured after enzymatic reduction with the following assay: 100 µL acid extract were incubated with 50 µL 2.5 millimol N-Ethylmaleinimid (NEM) and 760 µL 0.1 M phosphate buffer pH 7.0 to mask endogenous GSH. Excess of NEM was exhausted with 50 µL 3mM Sodium 2-mercaptoethanesulfonate. GSSG was reduced to GSH by addition of 20 µL 2 mM NADPH and 20 µL 12 units Glutathione reductase and GSH was measured by HPLC. Glutathione levels were corrected for skeletal muscle wet weight and protein content in order to prevent a potential effect of the amount of skeletal muscle oedema.

Histology

Parts of the gastrocnemius muscles were quickly frozen in isopentane cooled to -120°C by liquid nitrogen. Sections (thickness 10 micron) were cut in a cryostat (at - 20°C) and stained with hematoxylin and phloxine.

Additional sections were stained according to standard enzyme histochemical methods for myofibrilar ATPase (for fiber typing), and for succinic dehydrogenase (SDH) and cytochrome oxidase (COX) to show mitochondrial activity. The main chemicals for the enzyme histochemical methods were purchased from Roche, Germany (ATP) and Sigma, Germany [nitro blue tetrazolium and diaminobenzidine for the SDH and COX stain respectively]; succinate used for the SDH method is a general purpose reagent. The middle remaining parts of the left and right gastrocnemius muscles were immediately fixed *in toto* by immersion in cold phosphate-buffered paraformaldehyde 4%, pH 7.3. From proximal to distal, 4 transversal slices were taken, dehydrated and embedded in Paraplast. Sections of 5 micron in thickness were stained with hematoxylin and eosin (HE). In these sections skeletal muscle fibers, blood vessels, and surrounding tissues were light microscopically examined on the presence of structural changes.

Statistical analysis

All data were analyzed using SPSS (Statistical Package for the Social Sciences version 11.0 for Windows; SPSS Inc., Chicago, IL). and are expressed as median with standard errors of the mean. Statistical analysis was done using the Mann-Whitney U test for unpaired and the Wilcoxon signed rank test for paired samples. Statistical significance was considered when $p < 0.05$.

RESULTS

Clinical signs and symptoms

After 1 - 2 days, rats developed an abnormal gait and posture, guarding behaviour and sudden licking of the neuropathic hind paw (NP). Skin temperature difference between the plantar side of the NP and the control paw (CP) was -0.06 ± 0.1°C before the operation, increasing to 1.3 ± 0.4°C after 7 days, which was significant according to the nonparametric (2 related samples) Wilcoxon signed rank test ($p = 0.008$).

The percentage increase of the NP circumference was 4.3 ± 1.7% ($p = 0.011$). Redness of the plantar side of the NP was obvious in 56% of the rats as compared to the CP. An impaired walking and grasping function was observed in 89% of the NP's. All operated rats developed a claw position of the NP. Pain observation scores were obtained pre-operatively and after 7 days. Comparison of the pain scores was performed by calculating the difference between the pre-operative and postoperative values by the nonparametric Wilcoxon (2 related samples) signed rank test. Before the surgical intervention, mechanical stimulation (n = 8) with von Frey filament showed negligible withdrawal reactions. Mechanical stimulation with a 5.16 g filament resulted in a withdrawal percentage of 0% pre-operatively, and of 2.2 ± 2.2% seven days after the operation ($p = 0.317$). Mechanical stimulation with a 12.5 g von Frey filament resulted in a significantly increased withdrawal percentage of 0% pre-operatively to 17 ± 5.5% ($p = 0.027$). Pre-operative observation of the rats on the heated plate showed normal behaviour, with a heat pain score of 0.06 ± 0%. Seven days after the operation the heat pain score difference increased to 0.97 ± 0.1%, which was significant according to the nonparametric (2 related samples) Wilcoxon signed rank test ($p = 0.011$). See Table 1 for all clinical data.

Table 1. Clinical signs and symptoms (data are presented as mean and standard error of the mean).

Parameter	Neuropathic animals pre-operative	Neuropathic animals day 7 post-operative	*p*-value
Skin temperature (°C) difference left-right foot	-0.06 ± 0.1 (°C)	1.3 ± 0.4 (°C)	0.008
Circumference	2.31 ± 0.08 (cm)	2.41 ± 0.06 (cm)	0.011
Colour difference	0%	56%	0.025
Impaired function left paw in (%) of the rats observed	0%	89%	0.005
Mechanical pain 5,16 g filament	0.224 ± 0.04	2.2 ± 2,2	0.317
Mechanical pain 12,5 g filament	0 ± 0.04	17 ± 5.5	0.027
Heated pain	0.06 ± 0	0.97 ± 01	0.011

Table 2. Plasma (jugular vein) concentrations of reactive oxygen nitrogen species (RONS), transferrin, ceruloplasmin, and antioxidant activity (AOA) in nerve-injured rats and controls (data are presented as mean and standard error of the mean).

Parameter	Neuropathic animals	Control animals	*p*-value
RONS [nmol/cm^3 plasma]	5.19 ± 0.47 (n = 5)	4.70 ± 0.18 (n = 3)	0.66
Transferrin (μM Fe (III))	25.21 ± 2.74 (n = 8)	25.82 ± 2.09 (n = 7)	0.73
Ceruloplasmin (μM Cu(II))	6.20 ± 0.64 (n = 8)	4.57 ± 0.51 (n = 7)	0.083
AOA (Cp/Tr ratio)	0.224 ± 0.04 (n = 8)	0.148 ± 0.02 (n = 7)	0.15

Table 3. Muscle tissue concentrations of reactive oxygen nitrogen species (RONS) and glutathione (GSH) in nerve-injured rats and controls (data are presented as mean and standard error of the mean).

Parameter	Neuropathic animals	Control animals	*p*-value
RONS [nmole/cm^3 tissue]	2.80 ± 0.46 (n = 5)*	5.49 ± 0.78 (n = 3)	0.025
GSH [U/mg protein]	8.81 ± 0.32 (n = 3) *	5.72 ± 0.35 (n = 4)	0.034

* = $p \leq 0.05$

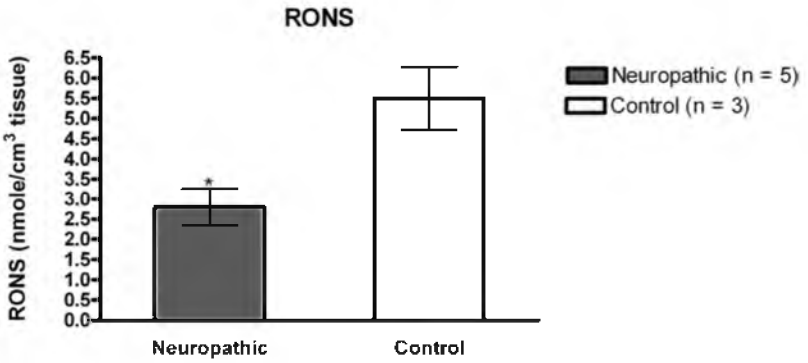

Figure 2. Muscle tissue concentrations of reactive oxygen nitrogen species (RONS) in nerve-injured rats and controls (data are presented as mean and standard error of the mean).
* = $p \leq 0.05$

Levels of RONS, ceruloplasmin, and transferrin

The levels of RONS, ceruloplasmin, and transferrin in both nerve-injured and control rats are presented in detail in Table 2. In skeletal muscle tissue, RONS levels were lower (2.80 ± 0.46 vs. 5.49 ± 0.78, $p < 0.05$) in nerve-injured paws (n = 5) than in controls (n = 3) (Table 3 and Figure 2). Jugular vein plasma levels of RONS did not differ (5.19 ± 0.47 vs. 4.70 ± 0.18, p = NS) between nerve-injured rats (n = 5) and controls (n = 3).

Jugular vein plasma levels of ceruloplasmin tended to be higher (6.20 ± 0.64 vs. 4.57 ± 0.51, p = 0.083) in nerve-injured rats (n = 8) than in controls (n = 7). Jugular vein plasma levels of transferrin did not differ (25.21 ± 2.74 vs. 25.82 ± 2.09, p = NS) between nerve-injured rats (n = 8) and controls (n = 7). Consequently, the ceruloplasmin/transferrin ratio in plasma tended to be higher (0.224 ± 0.04 vs. 0.148 ± 0.02, p = 0.083) in nerve-injured rats (n = 8) than in controls (n = 7).

Figure 3. Muscle tissue concentrations of glutathione (GSH) in nerve-injured rats and controls (data are presented as mean and standard error of the mean).

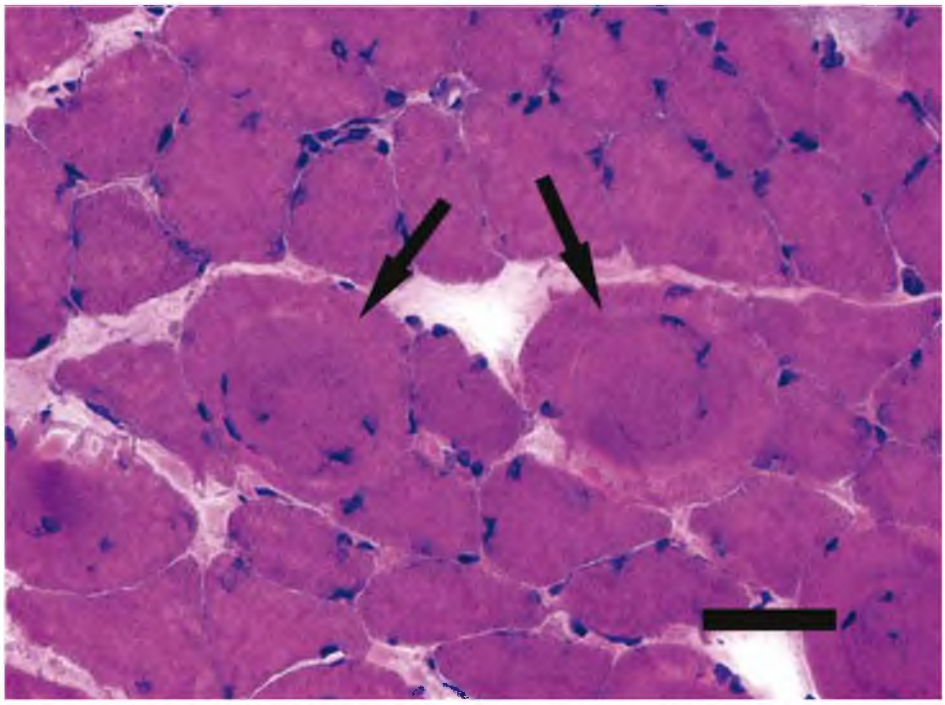

Figure 4. Rat leg muscle. The 2 arrows point to 2 abnormally large whorled muscle fibers. In these fibers a circular splitting line partly bordered by nuclei (dark) is recognizable (Hematoxylin-Phloxine stain, bar: 50 micron).

Levels of glutathione in skeletal muscle tissue

In skeletal muscle tissue, tissue reduced glutathione levels were higher (8.81 ± 0.32 vs. 5.72 ± 0.32, $p = 0.034$) in nerve-injured paws (n = 3) than in controls (n = 4) (Table 3 and Figure 3).

Histology

Histological analysis of skeletal muscle distally of the nerve constriction, showed no damage on a standard HE staining, but sections from frozen skeletal muscle locally revealed some whorled fibers suggestive (Figure 4) of regeneration of skeletal muscle[14]. Control contralateral muscles were normal.

DISCUSSION

This study in the chronic constriction injury (CCI) model of rat neuropathic pain demonstrates that the level of oxidative stress in skeletal muscle tissue of nerve injured hind paws, as indicated by the level of RONS, is decreased at day 7 after nerve injury. In contrast, (antioxidant) glutathione levels in skeletal muscle tissue of these rats are increased at this time-point. These findings indicate that in the CCI model, oxidative stress-induced changes are present not only in the injured sciatic nerve but also in skeletal muscle tissue of the ipsilateral hind paw. Our findings of a decreased level of RONS in combination with an increased level of the antioxidant glutathione suggest that a surplus of antioxidant activity prevails initial oxidative stress.
The plasma level (jugular vein) of RONS did not differ between nerve-injured and control rats indicating that the decrease of RONS in skeletal muscle tissue of CCI rats results from local changes in the nerve-injured hind paw rather than from systemic changes. The ceruloplasmin/transferrin ratio in plasma (jugular vein), reflecting systemic antioxidant activity, tended to be higher in the neuropathic group than in controls. This tendency towards an increased ratio is caused by the fact that ceruloplasmin levels were higher in nerve-injured rats whereas transferrin levels did not differ between groups. Since ceruloplasmin is an acute phase protein, its increase most likely merely results from the surgical procedure one week earlier. The oxidative stress induced changes observed in our study suggest that the inflammatory changes observed in muscle tissue by others[15] also result from oxidative stress.

As alluded to before, Tal et al.[2] observed an increased level of RONS in the nerve-injured hind paw, more specifically at the site of nerve injury. In contrast, we observed a decrease in the level of RONS in skeletal muscle tissue of nerve-injured hind paws. This dissimilarity may result from the fact that in our study, tissue specimens were obtained from non-traumatized muscle distal to the site of ligation, whereas in Tal's study RONS levels were determined at the site of nerve ligation. Alternatively, this difference may result from differences between tissues, i.e., nerve and muscle, in the level of antioxidant response to oxidative stress. Our observation that oxidative stress and antioxidant activity are involved in the pathophysiologic mechanisms underlying signs and symptoms in the CCI model is in line with studies showing that antioxidants have a beneficial effect on neuropathic pain[2,4].

Low dose ketamine has been reported to have anti-inflammatory effects, with a maximum effect at a sub-anaesthetic dose of 10 mg/kg. This anti-inflammatory effect of ketamine weans of with increasing dose; since all rats received a high dose ketamine (70 mg/kg), it is unlikely that this factor has influenced the outcome of our study[16].

In conclusion, our study in CCI rats shows that oxidative stress-induced changes are present not only in the injured sciatic nerve but also in skeletal muscle tissue of the ipsilateral hind paw. Our findings of a decreased level of RONS in combination with a marked elevation of antioxidant enzymes in skeletal muscle and plasma suggest that a surplus of antioxidant activity prevails initial oxidative stress.

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Chapter 6

Intra-arterial tertbutyl-hydroperoxide infusion induces an exacerbated sensory response in the rat hind limb, and is associated with an impaired tissue oxygen uptake

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ABSTRACT

Background:

To investigate oxidative stress and oxygen extraction mechanisms in an animal model of continuous intra-arterial infusion of a free radical donor, and in an in vitro model using isolated mitochondria.

Methods:

Tertbutyl-hydroperoxide (Tert-BuOOH, 25 mM) was infused for 24 hrs in the left hind limb of rats to induce soft tissue damage (n = 8). After seven days we assessed local sensory response, tissue oxygen consumption, oxygen radicals and antioxidant levels. In vitro mitochondrial function was measured after stimulation of isolated mitochondria of skeletal muscle cells with increasing doses of Tert-BuOOH.

Results:

Tert-BuOOH infusion resulted in an increased skin temperature (p = 0.04), impaired function and a significantly increased pain sensation (p = 0.03). Venous oxygen saturation levels (p = 0.01) and the antioxidant ceruloplasmin (p = 0.04) were increased. Tert-BuOOH inhibited mitochondrial function in vitro.

Conclusion:

Induction of free radical formation in the rat hind limb results in an exacerbated sensory response and is associated with impaired oxygen-extraction, which likely results from mitochondrial dysfunction caused by free radicals.

INTRODUCTION

Pain after limb injury gradually disappears within days to weeks. There are however some patients, in which pain remains and becomes chronic as part of the Complex Regional Pain Syndrome type I (CRPS I), formerly known as reflex sympathetic dystrophy or Sudecks dystrophy. It is a painful, potentially disabling syndrome that usually affects the distal part of the extremity. The disorder is characterized by a variety of autonomic and vasomotor disturbances, of which diffuse pain, spreading oedema, temperature disturbances, colour changes and functional impairment are most prominent[1,2]. There are various theories on the pathophysiology of this process[3]. Amongst these are immunologic alterations, changes in plasticity of the sympathetic and central nervous system, and neurogenic inflammation. Neurogenic inflammation is caused by mechanical and/or chemical activation of sensory neurons and/or small diameter sensory nerve fibers. As a result, inflammatory mediators such as substance P and calcitonin gene-related peptide are released from the distal endings of these sensory nerves causing vasodilatation, plasma extravasation, and hypersensitivity. Various studies have demonstrated that local release of these neuropeptides induces oxidative stress reflected by an excessive production of reactive oxygen and nitrogen species (RONS)[4,5]. RONS have been associated with neuropathic pain and the development of chronic pain such as in CRPS I[6], but the mechanism of action of RONS involved in the development of pain and the underlying mechanisms are not yet fully understood[7,8]. Recently, we have studied specific RONS-related pathophysiological changes in a chronic constriction injury rat model of neuropathic pain by means of recently developed spin-trap and electron paramagnetic resonance spectroscopy (EPR) techniques[9]. It was found that oxidative stress-induced changes are present not only in the injured sciatic nerve but also in skeletal muscle tissue. Drawback of the chronic constriction injury model is that it resembles CRPS type II (nerve injury present) and not CRPS type I[10].

It has been suggested that RONS play a role in the pathophysiology of CRPS I through impaired oxygen extraction similar to conditions like the adult respiratory distress syndrome (ARDS), the systemic inflammatory response syndrome (SIRS) and multi organ dysfunction syndrome (MODS)[11-14]. High mixed venous oxygen saturations and elevated lactate levels at rest are characteristic of a condition with impaired oxygen extraction as it reflects the inability of tissue or muscle to utilize oxygen despite sufficient supply[11].

Aim of this study was to investigate oxidative stress, as measured by RONS and antioxidant levels, and oxygen extraction, as measured by blood gas analysis, in a known animal model of free radical induced soft tissue injury without inducing ischemia, mimicking clinical signs of CRPS I[15,16]. Second aim was to relate oxygen extraction to mitochondrial function. This was done by using an in vitro model of isolated skeletal muscle mitochondria that were subjected to Tert-BuOOH[17].

EXPERIMENTAL procedures

Animals

Sixteen adult male Sprague-Dawley rats (Animal Research Laboratories, Himberg, Vienna, Austria) weighing 350 to 400 gram and eight adult male Sprague-Dawley rats (Animal Research Laboratories, Himberg, Vienna, Austria) weighing between 260 to 300 gram were used for in vivo and in vitro experiments, respectively. The animals were granted free access to standard laboratory chow and water during a 7-day adaptation period after delivery to our experimental unit. All animals were housed at 22°C with a 12-h light-dark cycle. Prior to the experiment, the rats were fasted overnight with free access to water. The protocol of the study was approved by the animal protocol review board of Vienna and followed the requirements defined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (publication NIH 86-23, revised 1985).

Study design

Rats underwent a 24-hour arterial infusion of Tert-BuOOH into the hind limb, inducing free radical associated soft tissue damage. At day seven anaesthetized experimental (Tert-BuOOH infused) and control rats (no infusion) received catheters in the jugular veins for blood sampling to assess oxygen radicals and the antioxidants ceruloplasmin and transferrin. Both femoral veins were punctured in the experimental animals for blood gas analysis. Thereafter all rats were killed and biopsies were taken from the gastrocnemius muscle to determine RONS and glutathione. Throughout the experimental period rats were observed daily for (pain) behaviour. Skin temperature, hind limb circumference and mechanical and heat pain responses were measured at day 1 before Tert-BuOOH infusion and at day 7 before anaesthesia.

Surgical procedures

At day one, eight randomly assigned rats were anaesthetized by intraperitoneal injection of ketamine 70-mg/kg body weight and subcutaneous injection of xylazine 10-mg/kg body weight. The animals were kept on a temperature-controlled surgical board (38 ± 1°C) and allowed to breathe spontaneously. The rats in the infusion group (n = 8) were anticoagulated intravenously with 100 units/kg heparin sodium. A polythene cannula (i.d. 0.28 mm; o.d. 0.61 mm, Laboratoire Portex, France) was placed retrogradely in the left superficial epigastric artery. The tip of the catheter was positioned at the junction of the superficial epigastric artery and common femoral artery, thus preventing impairment of arterial blood flow to the hind limb. The catheter was fixed with polyester sutures 6/0 (Dagrofil, Braun, Melsungen, Germany) and sealed with glue (Histoacryl, Braun, Melsungen, Germany). The other end of the cannula was tunnelled subcutaneously over the back of the rat to its head and connected to a flexible stainless swivel system. The swivel was connected to an infusion system. The rats were then awaked and placed in a specially equipped box being able to walk freely in the cage with the

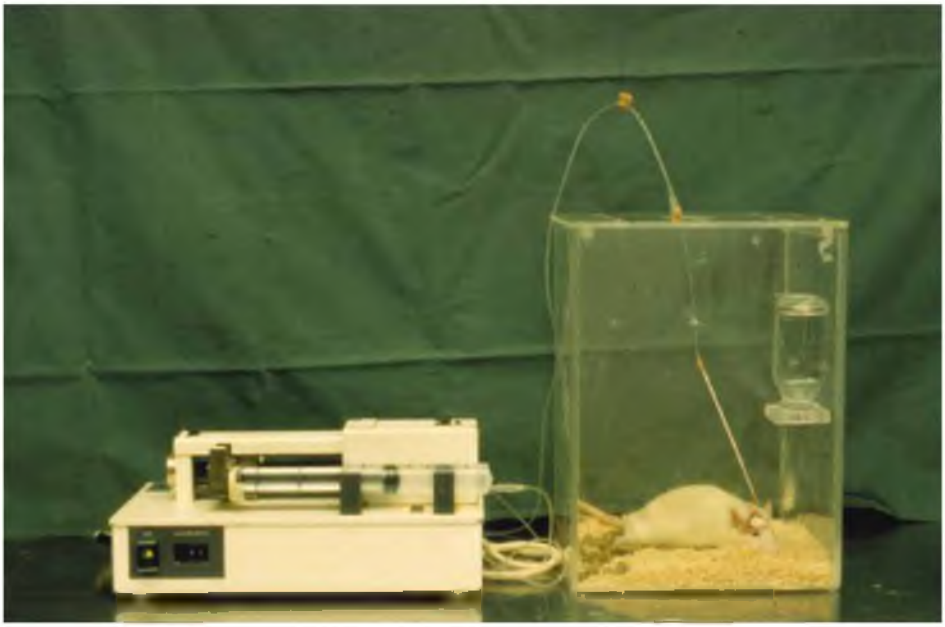

Figure 1. Infusion model in which the left superficial epigastric artery is cannulated. The other end of the cannula is tunnelled subcutaneously over the back of the rat to its head and connected to a flexible stainless swivel system. The swivel was connected to an infusion system. The rat is than placed in a specially equipped box, and able to walk freely in the cage (photo courtesy of L. van der Laan).

possibility of intra-arterial infusion in the hind limb without compromising blood flow (Figure 1). After cannulation, infusion was started immediately. During 24 hrs, the rats were infused with Tert-BuOOH (Sigma, St. Louis, USA) dissolved in saline to a final concentration of 25 mM (pH 6.8) with heparin (2,5 U/mL) , in a final dose of 0.6 mmol. Infusion of Tert-BuOOH leads to free radical formation and free radical induced soft tissue damage[15,18-20]. After 24 hrs the infusion system was disconnected. All rats were monitored daily for spontaneous pain and other abnormal behaviour.

At day 7 experimental rats (n = 8) and control rats (n = 8) were anaesthetized by a mixture of 0.8% isoflurane and room air for the duration of the procedure. The animals were kept on a temperature-controlled surgical board (38 ± 1°C) and allowed to breathe spontaneously. Following induction of anaesthesia, the jugular vein was catheterised under aseptic conditions with a polyethylene catheter (PE50, Clay Adams, Parsippany, NJ., USA) filled with heparinised Ringer's solution (8 units/mL). A first heparinised blood sample (1.5 mL) was collected for measurements of antioxidants, and blood volume was replaced by 1.5 mL Ringer's solution. Subsequently, the spin trap 1-hydroxy-3-carboxy-pyrrolidine (CPH), detecting superoxide radicals and peroxynitrite radicals (RONS), was intravenously administered in five experimental and five control animals[21]. At the end of the 30 minutes CPH infusion period, blood samples (1.5 mL) from the jugular vein were collected again for measurement of RONS. Blood samples were

also drawn by direct puncture of both femoral veins (2 x 0.5 mL) in experimental rats for blood gas analysis. Thereafter, all animals were killed and a piece of the left (infused) and left (controls) and right (infused contralateral) gastrocnemius muscle was excised and immediately frozen in liquid nitrogen for RONS and glutathione analysis.

Skin temperature, limb circumference, pain behaviour and evoked pain responses

Skin temperature was measured on the plantar side of both hind paws using a surface electrode (diameter = 0.6 cm; Keith, Geneva, Ohio, USA). The circumference of the hind paw just above the ankle was measured using a string. The relative change of the volume of the operated hind limb to the preoperative situation served as a parameter for contour alterations. Colour of the left plantar foot was observed and compared to that of the right foot. The neutral position, grasp, and walking function of both hind paws were recorded by observation of each rat for 5 minutes. The function of the left hind limb was noted as impaired when the rat had either a clench paw, did not show grasping or showed a shuffling gait, respectively. Observation of pain signs was performed preoperatively, and after 7 days after disconnection from the infusion system. Three different pain forms were assessed according to tests performed in the classical neuropathic pain[22] or peripheral inflammation[23,24] model in the rat: spontaneous pain, mechanically induced pain, and thermally induced pain. Spontaneous pain behaviour of rats was observed in a perspex cage of 26 x 26 x 26 cm after a 5-min habituation. The time of different positions of the lesioned paw was noted, according to the scale of Attal et al.[25] which varied from 0 (the operated paw is pressed normally on the floor) to 5 (the animal licks the operated paw). The spontaneous pain was calculated by the formula $t_1 + 2t_2 + 3t_3 + 4t_4 + 5t_5/300$ sec, where t_1, t_2, t_3, t_4, and t_5 are the times (in sec) spent in categories 1, 2, 3, 4, or 5, respectively. Mechanical sensitivity was tested by foot withdrawal in response to mechanical stimulation with Von Frey filaments (North Coast Medical, San José, USA) of two bending forces (5.16 g and 12.5 g). To this end, the rats were placed in a perspex cage with a wire mesh floor (26 x 26 x 26 cm). After 5 minutes accommodation, a Von Frey filament was applied 10 times (once every 5 seconds) to the plantar surface of the paw during which the frequency of foot withdrawals was noted[26]. Thermally induced pain was assessed on a heated floor of 40 ± 1°C. Following 5 minutes accommodation, the level of thermally induced pain was scored following the next 5 minutes according to the pain score of Attal et al.[25]. For example, if the operated paw was pressed normally on the floor during 5 minutes (300 seconds), this was scored as 0, the rat was not in pain. If the rat demonstrated constant licking of his operated paw, this was scored as 5x300/300 = 5.

Blood gas analysis

Blood gas analysis including oxygen tension (pO_2), pH, oxygen saturation (S_vO_2), and lactate level was done in heparinised femoral vein blood samples immediately after bilateral puncture, using a Radiometer ABL 625 Blood Gas Analyzer (Copenhagen, Denmark).

Determination of level of RONS, ceruloplasmin and transferrin in plasma

Plasma of jugular vein heparinised blood samples was separated by centrifugation during 10 minutes at 3000 g (12.000 rpm at 6°C). Heparin plasma was used for electron paramagnetic resonance (EPR) analysis[27,28]. Three hundred fifty µL of plasma was put in a 1 mL syringe and immediately frozen in liquid nitrogen. The sample was then pressed out of the syringe and put in a liquid nitrogen Dewar for electron paramagnetic resonance (EPR) analysis. The EPR spectra of RONS (CPH-adducts) were assessed at liquid nitrogen temperature as described in detail previously[27,28] using a Bruker EMX EPR spectrometer at the following setting: microwave frequency 9.431 GHz, modulation frequency 100 kHz, microwave power 31 mW, modulation amplitude 15 G; gain 10^5. The double integrals of transferrin (g-factor 4.31) and ceruloplasmin (g-factor 2.05) signals were calculated and compared with those obtained from standard iron (III) and Cu (II) complexes. The standard iron complexes were prepared by mixing 10 millimol desferrioxamine B with different amounts of iron ions ($FeSO_4$ x $7H_2O$). Desferrioxamine iron complex formed in this mixture was used as standard. Standard Cu (II) solutions were prepared by adding different amounts of copper ions ($CuSO_4$ x 5 H_2O) to 10 millimol diethyldithiocarbamate (DETC) solved in 10% bovine serum albumin. Copper DETC complex formed in this mixture was used as standard. The final iron /copper concentrations ranged from 0 to 25 µM. Upon reaction with RONS, CP-H is transformed into a stable $CP^{\bullet}$ radical (3-carboxy-proxyl). Standard solutions of 3-carboxy-proxyl were used to quantify RONS levels in plasma.

RONS and glutathione in skeletal muscle tissue

For reactive oxygen and nitrogen species (RONS) analysis, a small piece of skeletal muscle tissue (approximately three hundred fifty µL) was put in a 1 mL syringe and immediately frozen in liquid nitrogen. The sample was then pressed out of the syringe and put in a liquid nitrogen Dewar for electron paramagnetic resonance (EPR) analysis as described in the previous paragraph.

For glutathione analysis, the remaining tissue sample was frozen in liquid nitrogen and homogenized in 0.4 M $HClO_4$ using a ball mill. After centrifugation acid extracts were stored at -20°C or analyzed immediately by means of ion-pair reversed phase HPLC with electrochemical detection. The HPLC-System included a Spherisorb S3ODS-2 column (3 µm, 125 mm x 4 mm ID; Phenomenex, Torrance, CA), a Rheodyne 7125 injector (Rheodyne, Cotati,CA), a PU-980 HPLC pump (Jasco, Great Dunmow, United Kingdome), a pulsation dampener (Shodex, Tokyo, Japan), and a Coulochem 5100 A electrochemical detector (ESA, Bedford, MA) equipped with a 5011 analytical cell (+ 0.4 V). Data acquisition and analysis was done with an analog interface AI 406 and System Gold software (both Beckman Coulter, Fullerton, CA). The mobile phase consisted of 0.1 mol/L sodium acetate, 0.1 mol/L sodium hydrogen phosphate, 400 mg/L SDS adjusted to pH 2.0, and 2.5% acetonitrile (vol/vol). Calibration curves for reduced glutathione (GSH) were established daily. Oxidized glutathione (GSSG) was measured after enzymatic reduction with the following assay: 100 µL acid extract were incubated with 50 µL 2.5 millimol N-Ethylmaleinimid

(NEM) and 760 µL 0.1 M phosphate buffer pH = 7.0 to mask endogenous GSH. Excess of NEM was exhausted with 50 µL 3mM Sodium 2-mercaptoethanesulfonate. GSSG was reduced to GSH by addition of 20 µL 2 mM NADPH and 20 µL 12 units glutathione reductase and GSH was measured by HPLC. Glutathione levels were corrected for skeletal muscle wet weight and protein content in order to correct for skeletal muscle oedema.

Study design in vitro experiment

Eight adult male Sprague-Dawley rats (Animal Research Laboratories, Himberg, Vienna, Austria) weighing 280 gram ± 21 gram were killed and the hind paw skeletal muscles were quickly harvested and stored. Rat skeletal muscle mitochondria were prepared similar as described previously with heart mitochondria[17]. Isolated skeletal muscle mitochondria were incubated with Tert-BuOOH and subjected to various reagents in a closed system in vitro to assess changes in the rate of oxygen consumption.

Mitochondrial function

Immediately after harvesting mitochondria were stored at 0°C for 4 - 5 hours in a buffer containing 0.25M sucrose, 10 mM Tris _HCl, 0.5 mM EDTA (pH 7.2), and 0.5 g/L essentially fatty acid-free bovine serum albumin (BSA). Respiration rates were determined with an Oxygraph-2k Respirometer (Oroboros Ltd, Innsbruck, Austria). Skeletal muscle mitochondria were first mixed with an incubation buffer containing 80mM potassium chloride, 5mM potassium phosphate, 20mM Tris-HCl, 1mM DETAPAC and 0.1% BSA (pH 7.4). State 2 rate of respiration was defined as the respiration of mitochondria upon addition of a Krebs cycle substrate only. The transition to State 2 respiration was achieved by addition of glutamate/malate (5mM + 5mM). After respiration reached a steady state, 0.125 mM ADP was added for transition to State 3 respirations. After all ADP was transformed to ATP the State 4 respiration was achieved. The ratio State 3 rate /State 4 rate is called the respiration control index (RCI) as it reflects the coupling of oxidation and phosphorylation. The mitochondria were preincubated for 2 min with either increasing concentrations of Tert-BuOOH (final concentrations 0 - 25 mM) or with double distilled water used as a control. The kinetics of oxygen consumption rate was analyzed using a custom-made Excel program.

Statistical analysis

All data were analyzed using SPSS (Statistical Package for the Social Sciences version 16.0 for Windows; SPSS Inc., Chicago, IL). and are expressed as mean with standard error of the mean. RONS, transferrin, ceruloplasmin, and glutathione levels were compared between experimental and control rats, and in experimental rats between left (infused) and right (infused contralateral) hind limb using the Mann-Whitney U test. Skin temperature, limb circumference and pain responses of the left hind limb were compared with the right hind limb using the Wilcoxon signed rank test for paired samples. This test was also used to compare blood gas analysis

data between the left and right hind limb. For mitochondrial analysis, statistical analysis was performed by one-way ANOVA followed by a post hoc test for the least-significant difference. Statistical significance was defined as a p-value < 0.05.

RESULTS

Most rats developed an abnormal gait and posture (63%), guarding behaviour (63%), and sudden licking (75%) of the hind paw 1 - 2 days after the beginning of infusion (Figure 2). Before infusion skin temperature difference between the left and right hind paw was 0.06 ± 0.1°C. Seven days later, the skin temperature of the infused left hind paw, as compared to the right, was on average 1.7 ± 0.4°C (p = 0.043) higher. The average increase of the infused hind paw circumference was 2.3 ± 1.5% (p = 0.039). The volume increase of the left foot compared to the preoperative situation was 8.2 ± 3.4% after seven days. Colour difference was present in 63% of the rats. Spontaneous pain was almost absent after one week. Mechanical stimulation with both 5.16 g and 12.5 g von Frey filaments showed negligible withdrawal reaction before infusion. Seven days after infusion, withdrawal was significantly more frequent when stimulating with the 5.16 g filament (15 withdrawal responses out of 80 applications)[19 ± 10%; p = 0.068]

Figure 2. After 24 hrs of continuous infusion (1 mL/hr) of Tert-BuOOH (25 mM). The infused left hind paw showed increased skin temperature, increased circumference, redness of the plantar skin, impaired function and increased pain sensation, while the contralateral right hind paw these signs were absent (photo courtesy of L. van der Laan).

or the 12.5 g filament (30 withdrawal responses out of 80 applications) [38 ± 15%; $p = 0.027$] compared to before Tert-BuOOH infusion. Preoperative observation of the rats on the heated plate showed normal behaviour, with a pain score of 0.04 ± 0.1. Seven days after infusion the heat pain score of the infused hind paw was significantly increased to 0.76 ± 0.5 ($p = 0.028$), whereas the right limb showed a normal response.

Blood gas analysis

Venous oxygen saturation and venous oxygen tension of the infused left side were significantly higher than those of the infused contralateral right side, and lactate levels on the infused side tended to be higher (Table 1). These findings were compatible with an impairment of oxygen extraction in the infused hind paw.

Levels of RONS, ceruloplasmin and transferrin in plasma

The levels of reactive oxygen and nitrogen species (RONS), ceruloplasmin, and transferrin are presented in Table 2. In jugular vein plasma no difference was observed in RONS levels between infused rats and controls. Jugular vein plasma levels of the antioxidant ceruloplasmin (CP) were significantly higher in infused rats than in controls (7.04 ± 1.01 versus 4.57 ± 0.51, $p = 0.037$), whereas no differences were observed in transferrin (TR) plasma levels (23.59 ± 4.0 versus 25.82 ± 2.09, $p = 0.35$), on day seven. The antioxidant activity (AOA = CP/TR ratio) tended to be higher in infused rats than in controls (0.31 ± 0.1 versus 0.148 ± 0.02, $p = 0.063$).

Levels of RONS and glutathione in skeletal muscle tissue

In skeletal muscle tissue, no difference was observed in RONS levels between infused left side versus control animals in Table 3. RONS levels, however, were elevated compared to the

Table 1. Venous blood gas analysis, left (infused) versus right (contralateral) hind paw.

Parameter	Infused left hind limb	Contralateral right hind limb	*p*-value
$pO_{2\ (mm\ Hg)}$	38.25 ± 1.46	31.38 ± 1.55	0.012
pH	7.36 ± 0.01	7.36 ± 0.01	1.0
Lactate (mmol/L)	1.35 ± 0.13	1.21 ± 0.08	0.12
S_vO_2 (%)	53.4 ± 1.98	40.3 ± 2.51	0.012

Table 2. Plasma (jugular vein) concentrations of reactive oxygen nitrogen species (RONS), transferrin, ceruloplasmin and antioxidant activity (AOA) in Tert-BuOOH infused rats and controls (data are presented as mean and standard error of the mean).

Parameter	Infused animals	Control animals	*p*-value
RONS [nmol/cm^3 plasma]	4.36 ± 0.83	4.70 ± 0.18	0.51
Transferrin (μM Fe (III))	23.59 ± 4.0	25.82 ± 2.09	0.35
Ceruloplasmin (μM Cu(II))	7.04 ± 1.01	4.57 ± 0.51	0.037
AOA (Cp/Tr ratio)	0.31 ± 0.1	0.148 ± 0.02	0.063

Table 3. Muscle tissue concentrations of reactive oxygen nitrogen species (RONS) and glutathione (GSH) in Tert-BuOOH infused rats left infused limb versus right not infused contralateral hind limb, and left infused hind limb versus left hind limb of controls animals (*p*-value indicates left versus right hind limb and infused versus control animal respectively).

Parameter	Experimental animals		Control animals	*p*-value
	Infused left hind limb	Contralateral right hind limb	Left hind limb	
RONS [nmol/cm^3 tissue]	4.48 ± 0.73	2.53 ± 0.14	5.49 ± 0.78	0.11/0.51
GSH [U/mg protein]	7.22 ± 0.75	5.38 ± 0.35	5.72 ± 0.35	0.08/0.14

non-affected right side on day seven after Tert-BuOOH infusion (4.48 ± 0.73 versus 2.53 ± 0.14, $p = 0.11$). Glutathione levels tended to be higher in infused rats than in controls and in the contralateral right side (7.22 ± 0.75 versus 5.38 ± 0.35, $p = 0.08$).

Mitochondrial function

Tert-BuOOH had a pronounced inhibitory effect on mitochondrial function in State 3 with increasing doses of Tert-BuOOH (Figure 3). The effect was non-significant in State 2. The respiration control index (RCI), reflecting the coupling of oxidation and phosphorylation in mitochondria, was significantly decreased. This decrease was due to a reduced rate of respiration in State 3.

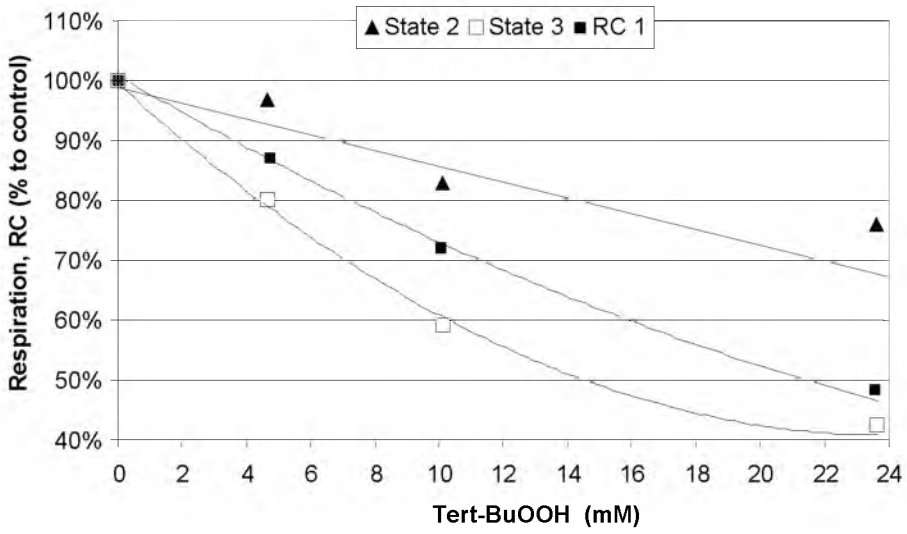

Figure 3. Dose effect of Tert-BuOOH on the respiration rate in State 2 and State 3, and the Respiratory Control Index (RCI) in rat skeletal muscle mitochondria. Respiration and RCI are both expressed in percentage to control values (in the absence of Tert-BuOOH). State 2 rate of respiration was defined as the respiration of mitochondria upon addition of a Krebs cycle substrate only.

DISCUSSION

This study demonstrates that local induction of oxygen free radical formation results in a chronic state of both local and systemic oxidative stress and impaired oxygen extraction. The same inducer of free radicals impairs mitochondrial function by interfering with ATP synthesis indicating a possible mode of action inducing oxidative stress. The data confirms that continuous intra-arterial infusion of a pro-oxidant compound causes long term behaviour in rats that is indicative of chronic pain, increased sensitivity for non-painful stimuli and elevated skin temperature. These are all elements of CRPS I. Signs of chronic pain and inflammation in the rat hind paw in this locally induced oxygen free radical model are similar to those observed in animal models of chronic neuropathic pain, such as the chronic constriction injury model (CCI model)[22].

Tert-BuOOH was chosen because it reportedly induces oxidative tissue injury in hepatocytes, renal tubules, the brain and skeletal muscle[19,20,29-31]. Tert-BuOOH is metabolized in the cell by glutathione (GSH) peroxidase to glutathione disulfide (GSSG) and tert-butanol. However GSH can be overwhelmed by using an excess Tert-BuOOH, leading to the formation of the free radicals tert-butoxyl and tert-butylperoxyl[18]. The free radical derivatives of Tert-BuOOH can induce oxidative injury as a result of lipid peroxidation and mitochondrial dysfunction[19,20]. In a previous study infusion of the vehicle of Tert-BuOOH (saline with heparin) no signs or symptoms of neuropathic pain were found, histology of skeletal muscle and arteries was unaffected, and there was no increase in vascular permeability as measured by 99mTc_IgG[15,16]. Accordingly, we have chosen to use only a non-infusion group as control.

Day seven was chosen for long term measurement of changes in both oxidant and anti-oxidant activity induced by 24 hours infusion of Tert-BuOOH because signs and symptoms of inflammation are still present on this day[32] which was confirmed by the present study. Seven days after infusion, RONS levels in femoral vein plasma of affected hind paws were not higher than those found in controls, challenging our hypothesis. However, measurement of free radical concentrations is difficult, because they are extremely short lived and only produced in minute quantities. Moreover, jugular vein plasma levels of ceruloplasmin were found to be higher in infused rats than in controls indicating an increased level of systemic antioxidant activity abolishing free radical formation. It is not known when this compensatory response commences during the seven days period of the experiment. Multiple time related observations and measurements would be needed to adequately characterize the biological response to Tert-BuOOH infusion.

We have found significantly elevated venous oxygen saturation levels and a tendency of higher venous lactate levels in the infused hind limb seven days after Tert-BuOOH infusion. High mixed venous oxygen saturations and elevated lactate levels at rest are characteristic of a condition with impaired oxygen extraction as it reflects the inability of tissue or muscle to utilize oxygen despite sufficient supply[11]. There are several causes for the high saturations levels. First, a deficient autoregulation of the microcirculation, resulting in a widely open

capillary bed, while only a small fraction of the oxygen supplied is consumed. Second, the presence of an increased flow through non-nutritive pathways, such as through arteriovenous anastomoses[33]. Thirdly, a diffusion problem for oxygen between the erythrocytes and the mitochondria, probably located in the endothelial cell and capillary basement membrane. Finally, reduced oxygen extraction may be present due to mitochondrial dysfunction. With respect to the first possibility, no published reports are available for a defective autoregulation within healthy skeletal muscle in human or rats. As to the second possibility, arteriovenous anastomoses were proven to be present in cat skeletal muscle[34], but were not found in human temporal skeletal muscle[35]. We also could not find this type of information on rats. As to the possibility of a diffusion problem, we observed in this animal model that most arteries were normal, although in previous experiments at day 7 some necrosis of endothelial cells and medial layers of the vascular wall was observed[32]. In this study, histological analysis revealed normal arteries, making a diffusion problem unlikely. Mitochondrial dysfunction remain as a plausible cause for the high S_vO_2 levels found. Tert-BuOOH induced oxidative stress is known to damage mitochondria by mitochondrial swelling resulting in mitochondrial dysfunction and reduction of their respiratory activity[36]. The observed mitochondrial swelling was attributed to an increased inner mitochondrial membrane permeabilization through protein and lipid peroxidation. In the present in vitro experiments Tert-BuOOH impaired respiratory function mitochondria due to decreased respiration in State 3, reflecting the rate of ATP synthesis and did not lower State 2 respiration, reflecting the permeability of the mitochondrial membrane. This implies that the mitochondrial energy generating system, reflected by State 3 respiration and the ATP production, is a target for RONS in this model. These data clearly show that Tert-BuOOH affects complex I of mitochondria inhibiting the electron transfer. Combining data from both experiments support the concept of Tert-BuOOH mediated mitochondrial dysfunction, causing impaired oxygen extraction. This concords with recently published studies showing that the contribution of the mitochondria in neuropathic pain was ATP dependent and not dependent on inflammatory markers[37].

It was hypothesized that signs and symptoms of chronic pain syndromes are caused by impaired oxygen extraction secondary to RONS induced mitochondrial dysfunction. An argument in favour of our hypothesis on the involvement of free radicals in the development of signs and symptoms in this rat model of chronic pain is the reduction of pain sensation, skeletal muscle and nerve damage and the shortened repair periods after administration of the free radical scavenger N-acetyl-l-cysteine, as shown in a previous study[32]. An argument challenging our hypothesis regards the possible microvascular dysfunction in skeletal muscle, which is associated with allodynia due to muscle ischemia[38]. Indeed we have observed after one week some degree of skeletal muscle necrosis, though the degree of tissue necrosis was variable in our rats and not very profound[32].

In conclusion, this study shows that Tert-BuOOH, an inducer of oxidative stress and increased RONS generation, causes behaviour indicative of chronic pain in the rat hind limb, increased responsiveness to otherwise non-painful mechanical/thermal stimuli as well as increased skin temperature. These changes are associated with an impaired oxygen-extraction, which may result from mitochondrial dysfunction caused by free radicals as shown in the in vitro experiment.

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Chapter 7

Capillary blood gas analysis in Complex Regional Pain Syndrome, a pilot study

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ABSTRACT

Background:

The pathophysiology of Complex Regional Pain Syndrome type I (CRPS I) is still a matter of debate. An inflammatory reaction may cause the syndrome. Increasing evidence points to a role for impairment of oxygen metabolism in the affected limb.

Methods:

In this pilot study (16 patients) we performed capillary blood gas analysis in extremities with acute CRPS I, in order to assess oxygen saturation and lactate levels. Comparison was made with the unaffected limb of capillary blood pH, pO_2, SaO_2, and lactate and glucose concentrations.

Results:

No statistically significant differences could be found.

Conclusion:

Capillary blood gas analysis is not useful to detect changes in oxygen saturation and lactate concentrations in CRPS I.

INTRODUCTION

Complex Regional Pain Syndrome type I (CRPS I) is a poorly understood syndrome which may occur in an extremity after even a minor injury or operation. In the acute phase CRPS I is characterized by signs and symptoms of inflammation within the affected extremity. Although the clinical signs and symptoms of CRPS I are well known, the underlying pathophysiology remains unclear. Sudeck[1] hypothesized that an excessive inflammatory reaction may cause the syndrome. Recent studies supporting the inflammatory theory include:

1) The dominance of clinical signs and symptoms of inflammation - such as unexplained severe pain, oedema, difference in skin temperature, difference in skin colour - in the affected extremity at the onset of CRPS I[2],
2) An increased extravasation of indium-labeled immunoglobulin as a sign of increased capillary permeability for macromolecules[3],
3) An increased deposition of lipofuscin as a sign of oxidative stress[3],
4) Increased systemic concentrations of bradykinin and calcitonin gene-related peptide[3],
5) A therapeutic response to various oxygen radical scavengers[4],
6) Increased lactate concentrations within the skin of the affected extremity in patients with CRPS I[5],
7) Higher concentrations of interleukin (IL)-6 and tumor necrosis factor-α (TNF-*α)* in the involved extremity in comparison with the uninvolved extremity[6].

In earlier studies, we found significantly elevated oxygen saturation levels in venous blood samples, obtained from the vena cubiti in the affected upper extremity, compared with the unaffected contralateral limb[3,7]. Therefore we were interested in assessing capillary oxygen and lactate levels in the affected limb.
In this pilot study we performed capillary blood gas measurements in the extremities of the affected limb in patients with acute CRPS I in order to asses oxygen saturation and lactate concentration compared with the unaffected contralateral limb.

METHODS

The study was performed in the out-patient clinic of the Department of Surgery, Radboud University Nijmegen Medical Centre. All new patients presenting with signs and symptoms of acute CRPS I were invited to participate in the study. Excluded were patients with abnormalities in the contralateral limb. The study protocol was approved by the Human Ethical Committee Arnhem-Nijmegen.

The following diagnostic criteria were used in making the diagnosis CRPS I[2]:

1. Presence of at least 4 of the following 5 signs and symptoms: unexplained diffuse pain and tenderness in the distal part of the extremity, difference in skin colour in relation to the healthy symmetrical limb, diffuse oedema, difference in skin temperature in relation to the healthy symmetrical limb and limited range of movement.
2. An increase in the above signs and symptoms during exercise.
3. The signs and symptoms above were present in an area much larger than the area of primary injury or operation and including the area distal to the primary injury.

All patients were non-fasting and were managed according to a standardized treatment protocol[3]. After given informed consent, they were brought to a climate-controlled room maintained at 24°C and allowed to acclimatize for approximately 15 minutes. Capillary blood was collected from a finger or toe of the CRPS I extremity, followed by sampling from the symmetrical part of the contralateral unaffected limb. The average time between blood collection and blood gas analysis was 7.5 minutes (range 3 - 18 minutes). The blood gas analyses were performed with a Chiron 865 blood gas analyser (Bayer BV, Mijdrecht, The Netherlands). Skin temperature was measured in triplo with an infrared (ear) thermometer (First Temp "Genius" Digital Ear Thermometer; Sherwood Medical, Crawley, United Kindome) held 1 cm above the skin surface. The difference between warm and cold is based on the initial skin temperature at the onset of the complaints[3]. The pain was scored according to the visual analogue score (VAS) for pain, in which the patient is asked to draw a mark on a 10 cm long line. The left side of the line is marked "No pain whatsoever" and the right side "The worst pain imaginable". Subsequently the mark is measured in centimetres, resulting in a VAS from 1 and 10.
Statistical analysis was performed using the paired t-test. Results were considered significant when $p < 0.05$.

RESULTS

Sixteen patients (4 men, 12 women) were included in this study, with mean (range) age being 48 (16 - 70) years. Twelve patients (75%) had CRPS I within an upper extremity. In 10 patients (62.5%) the right limb was involved. The clinical signs and symptoms started after a minor injury in 10 patients, after an operation in five and spontaneously in one. The mean (range) interval between the start of symptoms and capillary puncture studies was 7 (0 - 22) months. The mean (range) score on the visual analogue painscore was 4.3 (1 - 10) and the mean difference in skin temperature was -0.8 (range -5.10 to +1.80)°C.

No statistical significance could be found, comparing the laboratory data between the CRPS I limb and the normal limb (Table 1). From five blood samples taken (three in warm CRPS I and

Table 1. Results of capillary blood gas analyses from patients with Complex Regional Pain Syndrome type I (CRPS I), comparing the affected and unaffected limb.

	All patients (n = 16)		**Warm CRPS I (n = 7)**		**Cold CRPS I (n = 9)**	
	CRPS I/ Normal	***p***	**CRPS I/ Normal**	***p***	**CRPS I/ Normal**	***p***
pO_2	9.47 / 9.11 kPa	0.25	10.09 / 9.04 kPa	0.03*	9.05 / 9.14 kPa	0.81
pH	7.42 / 7.41	0.33	7.41 / 7.41	0.82	7.42 / 7.42	0.66
Glucose	6.4 / 6.3 mmol/L	0.45	6.6 / 6.4 mmol/L	0.18	6.3 / 6.3 mmol/L	0.69
Lactate	2.1 / 2.1 mmol/L	0.98	1.9 / 2.3 mmol/L	0.12	2.2 / 1.9 mmol/L	0.55
S_aO_2**	95.8 / 96.2%	0.24	97.4 / 95.9%	0.51	95.5 / 96.2%	0.11

* $p < 0.05$

** Based on n = 11 patients (4 warm CRPS I and 7 cold CRPS I patients)

two in cold CRPS I patients), no co-oximetry parameters could be calculated and measured due to clotting of the sample. As a consequence the co-oximetry parameters are based on 11 patients. When dividing the patients according to the skin temperature difference, there was a significant increase in pO_2 in the warm CRPS I limb (10.09 kPa, range 7.24 - 12.11 kPa) versus the healthy limb (9.04 kPa, range 6.8 - 10.74 kPa), while lactate concentrations were lower but not statistically significant. In the cold group, oxygen saturation tended to be lower.

DISCUSSION

These results suggest that the differences found in this pilot study merely reflect the differences in arterial blood flow through the skin in the affected extremity. In a warm extremity with CRPS I, arterial blood flow has been shown to be significantly increased, while being significantly reduced in cold CRPS I[3]. Consequently tissue pO_2 and O_2 saturation values follow the same trend, while lactate concentrations depended on the dilution factor caused by the arterial blood flow. In addition, in the study of Birklein et al.[5], at least six out of nine patients were mentioned to have a cold skin temperature, possibly explaining the higher blood lactate concentrations found.

CONCLUSION

In this study we could not determine significant differences in pO_2, oxygen saturation and lactate concentration in capillary blood samples from the affected versus the unaffected contralateral limb in patients with CRPS I. In capillary blood, pO_2 and oxygen saturation were close to arterial values. Therefore capillary blood gas analysis may not be sufficiently sensitive to detect increases in venous oxygen saturation and lactate production in this condition.

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Chapter 8

Impaired oxygen utilization in skeletal muscle of CRPS I patients

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Submitted

ABSTRACT

Background:

The purpose of this study was to evaluate oxygen extraction and utilization in end stage chronic Complex Regional Pain Syndrome type I (CRPS I) patients undergoing amputation and to relate these to muscle histology of the amputated limb.

Methods:

In 25 patients with severe CRPS I, requiring amputation of the affected limb, venous blood samples and in 11 patients skeletal muscle specimens were analyzed.

Results:

The mean venous oxygen saturation (S_vO_2) value (94.3 ± 4.0%) of the affected limb was significantly higher than S_vO_2 values found in healthy subjects (77.5 ± 9.8%) pointing to a severely decreased oxygen diffusion or utilization within the affected limb. Histological analysis showed a significant decrease of type I fibers and a significant increase of type IIB fibers. Ultrastructural investigations of soleus skeletal muscle capillaries revealed thickened endothelial cells and thickened basement membranes. Muscle capillary densities were decreased in comparison with literature data. High venous oxygen saturation levels were partially explained by impaired diffusion of oxygen due to thickened basement membrane and decreased capillary density.

Conclusion:

Venous oxygen saturation levels are significantly increased in chronic end stage CRPS I patients, corresponding with impaired oxygen diffusion. The abnormal skeletal muscle findings points to severe disuse but only partially explain the impaired diffusion of oxygen; mitochondrial dysfunction seems a likely explanation in addition.

INTRODUCTION

Complex Regional Pain Syndrome type I (CRPS I) is a poorly understood disease which may occur in an extremity after even a minor injury or operation. The reported incidence of CRPS I varies between 5.5 - 26.2 per 100.000[1,2]. Although the clinical presentation of CRPS I is well known,[3-5] the mechanism of the (chronic) pain and the underlying pathophysiology remains subject of debate. An excessive regional inflammatory response, including the production of reactive oxygen free radicals, may be involved in both acute and chronic CRPS I patients[6]. In acute CRPS I patients elevated venous oxygen saturation levels have been found[7] and cellular hypoxia is present as measured by phosphorus nuclear magnetic resonance spectroscopy[8]. In chronic CRPS I patients there are only scarce data on oxygen extraction, utilization and cellular hypoxia pointing at similar pathophysiology compared with acute CRPS I. Histological analysis in CRPS I patients by means of a muscle biopsy would be advantageous to evaluate cellular changes explaining the before mentioned findings, tough is not advisable because any additional injury or invasive procedure may increase the severity or recurrence of CRPS I in an affected limb or induce CRPS I in a healthy limb[3].

We had the opportunity to measure venous oxygen saturation levels and perform histological and electron microscopic analysis of muscle specimens in patients undergoing amputation of an extremity, because of therapy-resistant, severe chronic CRPS I. The focus of this study was on muscle fibers and capillaries because in a small pilot study by our research group, a decrease of type I fibers, atrophic fibers and severely thickened basal membrane layers of the capillaries of skeletal muscle was demonstrated,[9] pointing at repeated episodes of cell death and cell regeneration[10].

The objective of this study was twofold; To measure venous oxygen saturation levels in a large group of chronic end stage CRPS I patients, and to analyze cellular changes in their skeletal muscle possibly explaining venous oxygen saturation.

PATIENTS AND METHODS

Patients

Patients who were advised to undergo amputation of an extremity, because of therapy-resistant, severe chronic CRPS I were selected. Before the decision to amputate, all patients had been extensively, but unsuccessfully treated with sympathetic blockades, free radical scavengers, occupational therapy and pain-relieving drugs. Amputation of the affected extremity was necessary, because CRPS I had resulted in a non-functional limb with severe hyperalgesia. The indication for amputation was previously documented in a large study of patients with CRPS I[11]. All patients gave informed consent for blood sampling prior to amputation and patients with lower leg amputation for histological and electron microscopic analyses of the amputated

limb. The study was approved by the university hospital's ethical committee and was in adherence with the principles of the Declaration of Helsinki. Patients were operated according to a standard operative technique under general anaesthesia.

Blood samples

After visualizing the main vessels and before clamping, blood gas samples (2 mL) were obtained of the superficial femoral (above knee amputation), popliteal (through knee amputation), or brachial vein (through or above elbow amputation), and promptly analyzed with the Rapidlab 865 (Bayer) for pO_2, pH, lactate, glucose and venous oxygen saturation (S_vO_2). In the first ten patient in addition to venous blood samples, arterial blood gas analysis was performed.

Skeletal muscle specimens

Fresh skeletal muscle specimens of approximately 20 x 20 x 20 mm were harvested from the middle of the tibialis anterior, gastrocnemius and soleus muscles immediately after amputation. The skeletal muscle specimens were divided into two pieces: one specimen for light microscopy (LM) for measurement of fiber type percentage and capillary density (number of capillaries/mm^2), and one for electron microscopy (EM) for measurement of capillary diameter (in micron), and capillary basement membrane thickness (in micron).

For light microscopy a standard batch of staining techniques was carried out on freshly frozen transversely cut (10 micron) sections; a hematoxylin and phloxine stain and various enzyme histochemical reaction techniques including myofibrillar ATPase, to identify muscle fiber type, and succinic dehydrogenase (SDH) and cytochrome oxidase (COX) to detect mitochondrial activity and the presence of sharply demarcated regions without mitochondrial activity (also known as cores).

Muscle fiber type was determined by using ATPase stained sections (the darkest brown fibers represent type I fibers in the ATPase pH 4.2 or 4.6 stain; in the ATPase pH 4.6 stain the unstained fibers represent type IIA fibers, the intermediately stained fibers represent type IIB); 100 - 200 muscle fibers in a representative microscopic field were classified in this way by means of a drawing microscope. To estimate muscle fiber atrophy or fiber hypertrophy (four muscle fibers per muscle were measured) both the smallest and largest type I and type II fibers were drawn using the drawing microscope. Each drawn fiber area was transformed into a circle by hand using a template with several circular diameters. The matching diameter served as the measure for fiber size.

Endothelial cells were visualized to measure capillary density, with the indirect immunohistochemical peroxidase method, using monoclonal antibodies against HLA-DR (Class II). Capillary density was determined by taking the mean after analyzing 2 small regions (0.136 mm^2) in a muscle section representing an estimated minimum and maximum density per muscle per

patient. The analyses were done by counting the number of capillaries from HLA-DR stained sections (Figure 1D) by using a drawing microscope.
Electron microscopy (EM) was performed using a transmission electron microscope (JOEL type 1200EX/II) to measure the capillary basement membrane thickness (CBMT) and other capillary characteristics of soleus muscle. We choose to analyze soleus muscle, because this muscle was most affected on light microscopy. For EM fresh skeletal muscle specimens were fixed in glutaraldehyde, postfixed in osmium tetroxide, embedded in Epon, cut, and stained with uranylacetate and leadcitrate. The first ten observed capillaries per specimen during systematic screening were photographed at 6K (6000 times) magnification. CBMT was determined by averaging the two interceptions of the short elliptical axis with the basement membrane (BM) of the often elliptically cut capillary. Pericyte interceptions were not included in the measurements. Endothelial cell thickness and lumen diameter were calculated in the same way.

Controls

Blood gas control samples were not taken from the contralateral extremity, considering the risk of inducing CRPS I in that extremity. Patients matched for age and sex, undergoing elective surgery with general anaesthesia (removal of osteosynthesis material, laparoscopic cholecystectomy, excision of breastlump and treatment of anal fistula) were used as controls for blood gas analysis. Immediately after induction of anaesthesia and before incision, blood samples (2 mL) were taken from the femoral vein and promptly analyzed with the Rapidlab 865 (Bayer) for pO_2, pH, lactate, glucose and venous oxygen saturation (S_vO_2).
For fiber type I and II and capillary density data available in literature were used as reference. Sample size, mean and standard error of the mean were recorded from these studies[12-21].
No control specimens of soleus muscle were available to obtain control data on capillary basement membrane thickness. Therefore we used data of quadriceps muscle biopsies from an age matched healthy control group, included in a study on hyperthermia[22]. A correction factor of 1.5 was applied because the mean BM of lower leg (soleus) muscle is on average 1.5 times thicker than that of quadriceps capillaries attributed to increased venous hydrostatic pressure[23].

Statistical analysis

Mean venous oxygen saturation levels (S_vO_2) of the CRPS I patients were compared with the mean S_vO_2 of control patients by their 95% confidence intervals (CI). The unpaired sampled t-test was used to compare the morphological data (mean and standard error of the mean) compared with literature values. Regression analysis (curve estimation, quadratic model) was used to correlate age, CRPS I duration and morphological data (muscle fiber type parameters (type I and type IIB), capillary densities of all three different muscle specimens, and EM capillary parameters) with S_vO_2. A p-value < 0.05 was considered significant.

RESULTS

Patients

Twenty-five patients who underwent an amputation were included in this study (Table 1). All patients fulfilled the diagnostic criteria of the International Association for the Study of Pain (IASP)[4] and Veldman et al.[3]; discoloration of the skin, severe limitation of the active range of motion, extreme paresis, severe spontaneous pain, hyperalgesia and allodynia, and atrophy of skin, subcutaneous tissue and skeletal muscles were present in the affected extremity. Twenty-one were lower extremity amputations (7 through knee, 14 above knee), and 4 upper extremity amputations (three above elbow and one through elbow). The mean age at operation was 38 years (range: 17 to 61 years). The mean duration of CRPS I symptoms was 7.1 years (range 6 months to 24 years). In 81% of the cases CRPS I was induced by a minor injury, an operation, a contusion or sprain. Seven patients were smokers and 8 patients still used vasodilator drugs as treatment for CRPS I. None of the patients had migraine, were diabetic, had gastro-intestinal dysmotility problems, anxiety or were known with central or peripheral nervous system disease or peripheral vascular disease.

Blood samples

Arterial values (n = 10) were normal (data not shown). Venous oxygen saturation levels (n = 25) were significantly higher compared to the healthy matched control population (n = 16) undergoing elective surgery under general anaesthesia (removal of osteosynthesis material in 6, laparoscopic cholecystectomy in 2, excision of breastlump in 4, and treatment of anal fistula in 4). The pooled mean of the 25 CRPS I patients S_vO_2 was 94.3% (95%-CI: 92.6 - 95.9%). In the healthy matched control population (n = 16) the pooled mean was 77.5% (95%-CI: 72.3 - 82.7%) ($p < 0.001$) (Table 2). No differences were found in glucose or lactate levels between CRPS I patients and the healthy matched control population.

Skeletal muscle specimens

On light microscopic examination the skeletal muscle specimens of lower leg amputations (n = 11) showed no signs of cellular inflammation, necrosis or oedema. In Table 3 the most frequently found morphologic abnormalities are summarized, including a significantly decreased percentage of type I fibers and a significantly increased percentage of type IIB fibers. In CRPS I patients, atrophic type I and type II fibers were frequently found, often with an angular appearance, and in most specimens (Figure 1C) some centrally located unstained regions (cores) were present (especially in type I fibers) as identified by SDH, COX, and ATPase stains. Type IIB fiber percentages were twice normal in tibialis anterior and gastrocnemius muscle and were even increased with a factor 3 in the soleus muscle. Capillary density was decreased in soleus and tibialis anterior muscle as compared to literature data (Table 4).

Table 1. Twenty-five patients with therapy-resistant severe chronic CRPS I.

Nr	Sex	Age (y)	Etiology	Duration CRPS I (y)	Indication	Amputation level
1	Female[#]	35	Sprain	4	Persistent infection, severe oedema, no function	Through knee
2	Female[#]	61	Spontaneous	21	Persistent infection, no function	Above knee
3	Male[#]	35	Sprain	4	No function, severe oedema	Above knee
4	Female	58	Spontaneous	1	No function	Through elbow
5	Female[#]	34	Minor injury	3	No function, extreme equinovarus	Above knee
6	Female[#]	37	Minor injury	16	Persistent infection, severe oedema, no function	Above knee
7	Female[#]	53	Minor injury	5	No function	Above knee
8	Female[#]	21	Minor injury	5	Oedema, chronic ulceration, no function	Above knee
9	Female[#]	36	Postoperative	5	No function, persistent infection	Above knee
10	Female[#]	28	Injury	8	No function, chronic ulceration	Above knee
11	Female[#]	33	Sprain	7	Severe oedema, chronic ulceration, no function	Through knee
12	Female[#]	24	Minor injury	10	No function, chronic ulceration	Through knee
13	Female	47	Contusion	4	No function	Above knee
14	Female	40	Spontaneous	5	Severe oedema chronic ulceration	Above knee
15	Female	39	Postoperative	10	No function, hygienic problem	Above elbow
16	Female	26	Sprain	3	No function, extreme equinovarus	Through knee
17	Female	41	Spontaneous	2	No function, dystonia	Through knee
18	Female	45	Minor injury	12	No function	Through knee
19	Female	22	Sprain	9	No function	Above knee amputation
20	Female	58	Phlebotrombosis	1	Oedema, chronic infection	Above knee
21	Female	32	Minor injury	10	No function, hygienic problem	Above knee
22	Male	31	Minor injury	1	Severe oedema, skin problems, clenched fist syndrome, no function	Above elbow
23	Male	55	Postoperative	4	No function	Above elbow
24	Female	17	Injury	5	No function, chronic ulceration	Above knee
25	Female	41	Injury	24	No function	Through knee

[#] Patients with histology samples

Table 2. Blood samples from CRPS I patients and control patient (data are presented as mean and standard error of the mean).

	Venous (n = 25)	Control (n = 16)
pO_2	11.0 ± 3.7 kPa	6.1 ± 1.2 kPa**
pH	7.36 ± 0.06	7.38 ± 0.01
Lactate	1.7 ± 1.0 mmol/L	1.7 ± 0.7 mmol/L
Glucose	6.6 ± 1.7 mmol/L	5.3 ± 0.2 mmol/L
S_vO_2	94.3 ± 4.0%	77.5 ± 2.5%**

**$p < 0.01$

Table 3. Characteristics of skeletal muscle fiber type of CRPS I patients and literature (data are presented as mean and standard error of the mean).

Skeletal muscle type	CRPS I patients	Literature normal healthy patients	Reference nr
	Type I fibers (percentage)		
Soleus	26.4 ± 6.4 (n = 11)	86.4 ± 4.6** (n = 6)	12
Tibialis anterior	55.4 ± 5.5 (n = 11)	73.4 ± 4.2** (n = 6)	12
Gastrocnemius	22.5 ± 3.6 (n = 11)	50.8 ± 1.9** (n = 6)	12
	Type IIB fibers (percentage)		
Soleus	51.3 ± 7.2 (n = 10)	13.0 ± 1.6** (n = 8)	13
Tibialis anterior	19.8 ± 5.1 (n = 11)	8.8 ± 2.4* (n = 8)	13
Gastrocnemius	57.2 ± 5.5 (n = 11)	24.2 ± 2.3** (n = 8)	13

* $p < 0.05$
**$p < 0.01$

Electron microscopy showed a significantly thickened (multiplicated) basement membrane (BM) (Figure 1A), swollen endothelial cells (increased endothelial cell thickness) and a smaller capillary lumen (Table 5). In some patients necrotic capillaries (without endothelial cells but identifiable by their BM (Figure 1B)) were seen. The mean capillary BM thickness of the soleus muscle was 0.67 micron (SD 0.17), compared to 0.34 micron (SD 0.07) in the quadriceps muscle of the control group (Table 5). Comparing the corrected control values (factor 1.5)[23] with soleus muscle specimens from CRPS I patients, the difference was statistically significant.

Using regression analysis correlation coefficients were calculated for S_vO_2 versus age, CRPS I duration, the muscle fiber type parameters (type I and type IIB), capillary densities of soleus, gastrocnemius, and tibialis anterior muscle, and EM capillary parameters. There was a weak significant correlation between the S_vO_2 and BM thickness (R = 0.64, p = 0 .048). Other correlation coefficients were not significant (Figure 2).

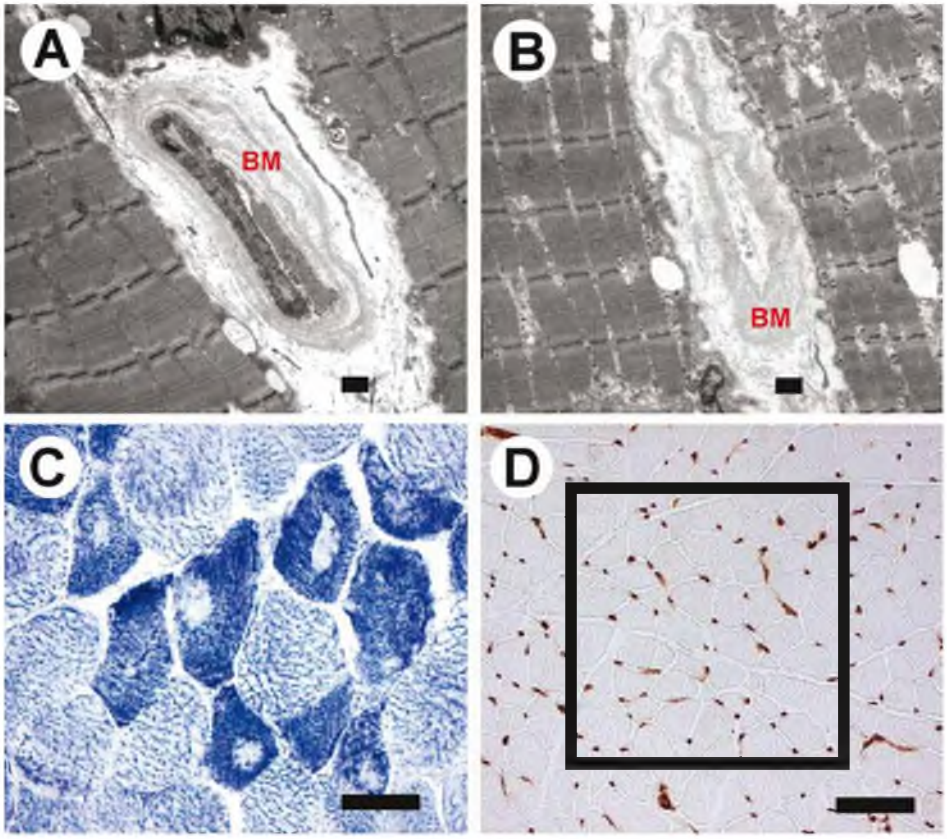

Figure 1. Light (C and D) and electron microscopy (A and B) of soleus muscle from patient 7 (A) and 10 (B, C, and D) (black bars represent 1 micron in A and B, 50 micron in C, and 100 micron in D).

DISCUSSION

The main outcome of this study was threefold; venous oxygen saturation was elevated in this large group of chronic end stage CRPS I patients, histological findings pointed to disuse atrophy in chronic CRPS I patients, and basement membrane thickness correlated with oxygen saturation levels. Based on the 95% -CI, the mean S_vO_2 of CRPS I patients was 94.3%, while the calculated mean from controls was 77.5%. Control values are not biased, because healthy subjects of 18 - 50 years,[24] healthy subjects exercising[25] and patients undergoing femoro-popliteal bypass grafting[26] showed a calculated venous oxygen saturation of 83%, which is also significantly lower in comparison with the 94.3% in CRPS I patients. High mixed venous oxygen saturations and high lactate levels at rest are characteristics of sepsis, of acute respiratory distress syndrome, systemic inflammatory response syndrome, and multiple organ dysfunction syndrome[27]. This condition has been called "impaired oxygen extraction" as it points to the inability of tissue or muscle to utilize oxygen despite an adequate oxygen supply[7,28]. In

Table 4. Capillary density (number/mm^2) in CRPS I patients and literature (data are presented as mean and standard deviation).

Skeletal muscle	CRPS I patients	Literature normal healthy patients	Reference nr
Soleus	299.8 ± 91.1 (n = 11)	288 ± 58 (n = 4)	14
		417 (range 333 - 592) (n = 12)**	15
		503.2 ± 76.3 (n = 6)**	16
Tibialis anterior	302.5 ± 66.4 (n = 11)	387± 53 (n = 28)**	17
		394 ± 44 (n = 30)**	18
		403 ± 49 (n = 6)**	19
Gastrocnemius	301.8 ± 81.5 (n = 11)	365 ± 56 (n = 4)*	14
		257 ± 44 (n = 23)	20
		271 ± 80 (n = 20)	21

* $p < 0.05$
** $p < 0.01$

Table 5. Characteristics of capillaries of soleus muscle of CRPS I patients compared with healthy control quadriceps muscle (data are presented as mean and standard deviation).

Capillaries:	CRPS I patients (n = 11) Soleus (micron)	Controls (n = 10) Quadriceps (micron)
Total diameter	5.46 ± 0.47	4.94 ± 0.62*
Diameter without BM	4.11 ± 0.32	4.27 ± 0.56
BM thickness	0.67 ± 0.17	0.34 ± 0.07**
Endothelial cell thickness	0.93 ± 0.41	0.59 ± 0.23*
Lumen diameter	2.25 ± 0.91	3.10 ± 0.59*

* $p < 0.05$
** $p < 0.01$

our patient group, arterial oxygen delivery seems sufficient, given a normal cardiac output (as reflected by a normal heart rate and the absence of cardiovascular disease) and normal arterial oxygen saturation values. Besides high venous oxygen saturation levels, increased lactate production points to impairment of oxygen metabolism. Birklein et al. found in CRPS I patients elevated skin lactate levels using microdialysis, though normal venous blood lactate taken from the antecubital veins[29]. This is consistent with our findings of normal lactate and may be attributed to adaptation of the cells to cellular hypoxia. Venous lactate also hardly reflects tissue lactate, as the skin contributes only 5% to the whole body lactate turnover. Moreover, blood circulation or blood glucose utilization influence the venous lactate level[29].

The previous pilot study of skeletal muscle tissue biopsies in chronic CRPS I patients showed a decrease in type I fibers, many atrophic fibers and severely thickened basal membrane layers of the capillaries[9], the latter finding being similar to histologic abnormalities observed in muscles of diabetes patients[10]. In this study we confirmed these findings but in addition detected fibers (especially type I) with centrally located unstained regions (cores) with SDH, COX, and ATPase stains. The outspoken atrophy and the central core lesions are more in accordance with disuse atrophy[30-33]. In a study of 14 patients with amputated extremities following therapy resistant

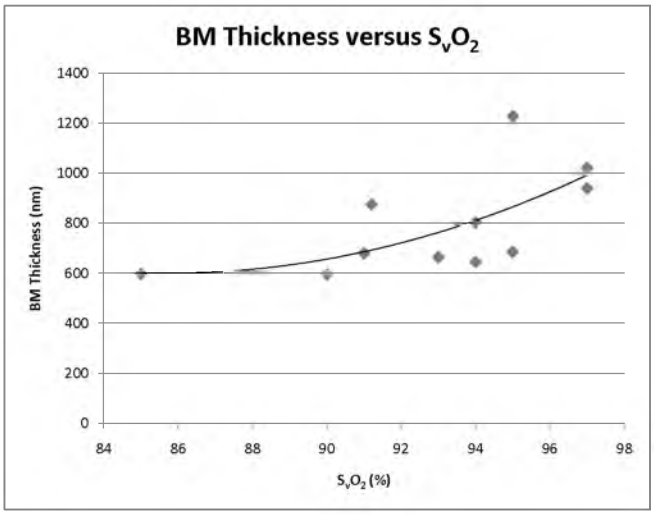

Figure 2. Scatter diagram of: BM thickness and S_vO_2 values. S_vO_2 and BM thickness show a significantly positive linear correlation. Correlation-coefficient R is 0.64 and R^2 is 0.41. BM = Basement membrane; S_vO_2= Venous saturation of oxygen; nm = nanometer (1000 nm = 1 micron).

longstanding CRPS I, both type I and type II fiber atrophy was found[34]. In 5 out of 14 patients signs of morphological denervation or reinnervation (as seen by type grouping) were observed and the authors rather concluded to a neuropathic or vascular cause than to disuse as the most important factor[34]. These clear signs of type grouping were observed in some intrinsic foot muscles. Generally, type grouping of type I or type II fibers points to muscle fiber reinnervation[35]. However, type grouping can also occur normally in some muscles[36]. In contrast to our study, we did not find type grouping in the samples of the investigated large muscle.

Low capillary density and thickened capillary wall might explain impaired oxygen extraction due to impaired diffusion. To detect and measure capillary density HLA-DR immune staining was preferred above the usual amylase-PAS method, because it gives better contrast against background and is therefore more accurate for detecting capillaries[37-39]. Capillary density was moderately decreased in soleus and tibialis anterior muscle, probably caused by disuse-adapted regression of the capillary network[40], while capillaries revealed normal total diameters. The BM thickness in skeletal muscle in our patients was increased by about 50% and venous oxygen saturation levels correlated with BM thickness; the thickest BMs were measured in patients with the highest S_vO_2, reflecting low oxygen extraction levels. At rest, however, a 50% increase in BM thickness will not lead to hypoxia since in sedentary people oxygen demand is low[41]. It seems therefore that the found BM thickness (50% increase, maximal contribution a factor 2 thicker) is related to oxygen consumption levels, implying that other mechanisms than BM thickness may be involved.

Type I fibers normally contain the highest and type IIB fibers the lowest number of mitochondria. We have found a significant decrease in type I fibers, pointing to a considerable loss of mitochondria in the lower leg muscles in our CRPS I patients. Notably, lower leg muscles predominantly contain type I fiber. By using the ultrastructural data regarding mitochondrial content in various fiber types[42], it was calculated that the structural mitochondrial loss in CRPS I patients amounts to respectively 49, 16, and 45% for soleus, tibialis anterior and gastrocnemius muscle, assuming a twofold decreased diameter of type IIB fibers. The above percentages of loss in the number of mitochondria found in soleus and gastrocnemius muscles are significantly higher compared to the 17% relative loss found in volunteers after a 42-day period of bed rest in the vastus lateralis muscle[43]. Most likely this significant loss of mitochondria is due to the fact that CRPS I patients do not exercise, because moving muscles of the affected extremity induce or increase pain. Another explanation is the increased fatigability, which is present in more than half of CRPS I patients from the very beginning[3]. Our histological findings of muscles of CRPS I patients best correspond with disuse atrophy coupled with decreased mitochondrial activity and increased fatigability[44].

There is clinical and experimental evidence that an excessive regional inflammatory response, including the production of reactive oxygen free radicals, is involved in CRPS I and causes tissue damage[3,8,45,46]. Serum lipid peroxidation products and salivary antioxidative parameters are increased in CRPS I patients[6]. Increased production of oxygen radicals can be a consequence of mitochondrial respiratory chain dysfunction. Defective mitochondrial function might also explain the nuclear magnetic resonance spectroscopy findings in CRPS I patients, with increased anorganic phosphate to creatine phosphate ratios at rest, pointing to cellular hypoxia[8]. The in sedentary people observed morphological abnormalities cannot entirely explain the high venous oxygen saturation values. A likely contributing factor is the presence of defective mitochondria explaining the impaired oxygen extraction found because muscle mitochondria are the decisive structures for oxygen metabolism. The decrease in type I fibers and thus the loss of mitochondria support the hypothesis of mitochondrial involvement. We are currently investigating the role of mitochondrial dysfunction in CRPS I, in order to further elucidate the pathophysiology of CRPS I.

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Chapter 9

Mitochondrial dysfunction is involved in the pathophysiology of Complex Regional Pain Syndrome type I

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ABSTRACT

Background:

Reactive oxygen species (ROS) are known to be involved in the pathophysiology of Complex Regional Pain Syndrome type I (CRPS I). Since the mitochondrial respiratory chain is a major source of ROS, we hypothesized that mitochondria play a role in the pathophysiology of CRPS I.

Methods:

The hypothesis was tested by studying mitochondrial energy metabolism in muscle tissue from amputated limbs of CRPS I patients.

Results:

We observed that mitochondria obtained from CRPS I muscle tissue displayed reduced mitochondrial ATP production and substrate oxidation rates in comparison to control muscle tissue and in comparison with muscle tissue from patients with arterial occlusive disease (AOD). Moreover, we observed reactive oxygen species ROS evoked damage to mitochondrial proteins and reduced MnSOD levels in CRPS I patients. No ROS evoked damage to mitochondrial proteins was observed in AOD patients while MnSOD levels were increased in these patients.

Conclusion:

The observation of a reduced mitochondrial energy production combined with reactive oxygen species induced damage in muscle tissue from CRPS I patients provides evidence for the involvement of mitochondrial dysfunctioning in the pathophysiology of CRPS I.

INTRODUCTION

Complex Regional Pain Syndrome type I (CRPS I) is a severe, disabling, and painful disease which may occur in an extremity after a trauma or injury. In the Dutch population the estimated incidence is 4000 per year, which includes approximately three times more females than males[1]. Clinical features include spontaneous and stimulus-evoked pain, oedema, vasomotor and sudomotor disturbances, motor dysfunction, and trophic changes, without evidence for peripheral nerve injury according to IASP criteria[2,3]. Despite growing knowledge of the pathophysiology of CRPS I, the primary cause of the syndrome is still unknown. In a recent study of 41 randomized controlled trials about treatment of CRPS I only limited evidence to formulate recommendations for treatment of CRPS I was found[4]. Possibilities for therapeutic intervention are therefore limited and include pharmacological pain relief, administration of radical scavengers[5,6], anti-inflammatory treatment, treatment with biphosphonates[7] and physical therapy. There are indications for an impaired oxygen metabolism in skeletal muscle of CRPS I patients[8]. In physiological processes like development, and pathological circumstances like tumorigenesis, cells are exposed to hypoxia or reduced oxygen levels. Under these circumstances elevated levels of reactive oxygen species (ROS) are produced, acting as second messengers by activating hypoxia-inducible factors (HIFs) that stimulate the cells to maintain normal ATP synthesis[9]. Under hypoxic conditions, muscle tissue fails to maintain a normal redox state, resulting in an increased production of ROS, and subsequent cell injury or dysfunction[10]. Serum lipid peroxidation products and salivary antioxidative parameters, known to be induced by ROS, are increased in CRPS I patients[11]. Increased ROS production can be a consequence of mitochondrial respiratory chain dysfunction, part of the mitochondrial energy generating system (MEGS). We hypothesized that mitochondrial dysfunctioning due to lack of oxygen and subsequent elevated ROS production is involved in the pathogenesis of CRPS I. To test this hypothesis, we examined the mitochondrial energy generating system in affected muscle tissue of CRPS I patients and in arterial occlusive disease (AOD) patients with hypoxia in an affected limb but without CRPS I characteristics.

METHODS

Patients

Eight patients (seven females, one male) with severe, chronic CRPS I of an extremity, fulfilling the diagnostic criteria of the IASP[3] and Veldman et al.[12] were included in this study. All patients had been extensively, though unsuccessfully, treated with sympathetic blockades, free radical scavengers, occupational therapy and pain-relieving drugs. Amputation of the affected extremity was advised because CRPS I had resulted in a non-functional limb with severe hyperalgesia, impairing other functions. The indication for amputation was previously documented

in a large study of patients with CRPS I[13]. Six male patients suffering from AOD were included in this study. The study was approved by the University Hospital Ethical Committee and was performed in adherence with the principles of the Declaration of Helsinki. Informed consent was obtained to acquire blood samples and muscle tissue of the amputated extremities to perform the biochemical studies. All measured parameters were compared with the control muscle samples published before[14]. In these control muscle samples we found no statistically significant differences between males and females. Blood gas samples were obtained at the beginning of the operation, after visualizing the superficial femoral vein and artery, and before clamping, and promptly analyzed with the Rapidlab 865 (Bayer).

Biochemical and molecular genetic studies

The MEGS encompasses the whole chain of mitochondrial enzymatic reactions involved in the generation of ATP, including: (I) transport of pyruvate and other substrates into the mitochondrion, (II) oxidation of pyruvate by the pyruvate dehydrogenase complex to acetyl-CoA, NADH and CO_2 , (III) oxidation of acetyl-CoA to CO_2 in the tricarboxylic acid cycle to yield NADH and $FADH_2$, (IV) oxidation of NADH and $FADH_2$ in the respiratory chain with subsequent coupled ATP production by complex V, and (V) transport of ATP out of the mitochondrion by the adenine nucleotide translocator. Immediately after amputation of the affected limb, fresh muscle specimens (M. gastrocnemius of seven CRPS I patients, and six AOD patients, and the M. extensor digitorum of one CRPS I patient) were obtained and put in ice-cold sample buffer as described before[14]. Within one hour, mitochondrial enriched fractions were prepared and the MEGS capacity was measured as described[14]. In two CRPS I patients, a fresh sample could not be obtained and instead the muscle sample was snap frozen in liquid nitrogen immediately after collection. Mitochondria-enriched fractions were prepared essentially following the same procedure as for fresh muscle samples. As frozen samples are not suitable for analysis of substrate oxidations or ATP production, only respiratory chain enzyme activities were measured in these samples. In both fresh and frozen samples, respiratory chain complexes II, III, IV and citrate synthase were measured as described before[11,15-18]. Respiratory chain complex I in the control samples and the CRPS I samples was measured as described by Fischer et al.[11], complex I in the AOD samples was measured as described by Janssen et al.[15]. To compare the three groups mutually, a relative complex I activity was calculated by expressing complex I activity for each patient of a distinct group as a percentage of the mean control value for the group in question. Citrate synthase activity was measured as a mitochondrial marker enzyme. Protein was measured by the method of Lowry et al.[19]. The complete mitochondrial genome was studied in muscle tissue of the CRPS I patients with the Affymetrix Gene Chip® Human Mitochondrial Resequencing Array 2.0, according to the manufacturer's instructions with minor modifications (www.affymetrix.com).

Immunochemical studies on muscle mitochondria

Mitochondrial fractions were isolated as described[11]. Isolated mitochondrial fractions were subjected to immunoblotting for carbonylated proteins, manganese superoxide dismutase (MnSOD) and porine. Protein carbonylation, as a measure of oxidative damage, was studied using the Oxyblot™ Oxidized Protein Detection Kit (Chemicon) in isolated muscle mitochondrial fractions according to the manufacturer's instructions with minor modifications (www.millipore.com/catalogue/item/s7150#). IRDye™ 680 conjugated goat-anti-rabbit IgG (Licor) was used as second antibody. MnSOD was studied with rabbit-anti-MnSOD (Stressgen) as first antibody and IRDye™ 800 conjugated goat-anti-rabbit IgG (Licor) as second antibody. Porin was used to normalize for the amount of mitochondrial protein in each lane. For detection of this protein Mouse Anti-porin 31-HL (Ab-3) (Calbiochem) was used as primary antibody. IRDye™ 800 or IRDye™ 680 conjugated goat-anti-mouse IgG (Licor) were used as second antibodies for the Oxyblot and the MnSOD blot, respectively. The blots were scanned on a Licor Odyssey scanner. Missing data due to limited availability of muscle sample are MnSOD in patients 4 and 5 and oxyblot in patients 4 and 6 of the CRPS I group.

Statistics

Biochemical data of the MEGS and the respiratory chain enzymes of the CRPS I patients, the AOD patients and 42 control individuals[14] were compared using the non-parametric Wilcoxon test. Differences were considered to be statistically significant if $p \leq 0.01$.

Table 1. S_vO_2 observed in controls, CRPS I and AOD patients included in this study.

	Gender	Age (years)	Duration of CRPS I (years)	S_vO_2 (%)
CRPS I patient				
1	Female	38	4	85
2	Female	34	3	91
3	Female	60	21	92
4	Female	57	1	93
5	Male	34	4	97
6	Female	41	24	99
7	Female	24	10	94
8	Female	20	5	95
AOD patient				
1	Male	62		59
2	Male	78		nd
3	Male	80		70
4	Male	46		52
5	Male	71		88
6	Male	76		91
Control patients				77.5
n = 16	Controls mean (range)			(72.3 - 82.7)

nd = not determined

Table 2. Biochemical analysis of the mitochondrial energy generating system (MEGS) in muscle tissue of controls, CRPS I and AOD patients.

	Muscle investigated	pyr+mal[a]	mal+pyr[a]	suc+acc[a]	ATP[a]
CRPS I patient					
1	M. gastrocnemius	5.13	6.61	4.08	52.7
2	M. gastrocnemius	0.87	1.20	nd	10.6
3	M. gastrocnemius	5.17	5.58	3.46	44.7
4	M. extensor digitorum	3.91	4.77	2.88	46.6
5	M. gastrocnemius	1.64	1.46	1.37	1.9
6	M. gastrocnemius	2.51	2.95	1.89	23.6
7	M. gastrocnemius	nd[b]	nd[b]	nd[b]	nd[b]
8	M. gastrocnemius	nd[b]	nd[b]	nd[b]	nd[b]
AOD patient					
1	M. gastrocnemius	7.81	6.57	4.78	53.2
2	M. gastrocnemius	4.62	4.07	3.61	48.5
3	M. gastrocnemius	3.67	3.90	3.56	37.3
4	M. gastrocnemius	6.46	6.82	4.26	62.8
5	M. gastrocnemius	7.78	6.19	3.54	39.5
6	M. gastrocnemius	4.99	4.53	2.25	41.8
Control patient	Controls (observed range)	3.45-7.99	3.28-8.80	2.03-4.18	36.0-81.7
	Controls range (mean±2sd)	3.51-8.01	3.78-8.96	2.04-4.26	36.1-76.8
	n	42	41	33	40
	p Wilcoxon test: Controls versus CRPS I	0.002	0.005	0.517	0.002
	p Wilcoxon test: Controls versus AOD	0.963	0.104	0.08	0.047
	p Wilcoxon test: AOD versus CRPS I	0.078	0.262	0.1	0.2

[a] Substrate oxidation rates and ATP production rates are given in nmol $^{14}CO_2$ or ATP/hour. mU citrate synthase (CS), respectively.

[b] As in CRPS I patients 7 and 8 a frozen muscle sample was examinated, ATP production and substrate oxidation rates could not be measured.

nd = not determined

RESULTS

Table 1 gives the venous oxygen saturation values measured in venous blood of the affected limb of CRPS I patients 93.3 ± 1.5%, AOD patients 72.0 ± 7.7% and 16 healthy control subjects (77.5 ± 2.5%, Chapter 8) just prior to the amputation, showing that the S_vO_2 values were increased in the CRPS I patients and in two AOD patients compared to healthy control patients undergoing elective surgery. We examined the mitochondrial energy metabolism in muscle prepared from the amputated limbs. The MEGS parameters (oxidation rates of [1-^{14}C]pyruvate + malate + ADP, [U-^{14}C]malate + pyruvate + ADP, and the ATP production rate from oxidation of pyruvate) were significantly lower in the CRPS I group compared to the control group, and

Table 3. Biochemical analysis of the respiratory chain enzymes in muscle tissue of controls, CRPS I and AOD patients.

	Muscle investigated	Complex I[d]	Complex II[b]	Complex III[b]	Complex IV[b]	CS[c]
CRPS I patient						
1	M. gastrocnemius	107	566	3322	1674	171
2	M. gastrocnemius	85	545	2242	1597	26
3	M. gastrocnemius	64	457	3047	1078	105
4	M. extensor digitorum	97	627	2738	1471	43
5	M. gastrocnemius	57	357	1942	1339	46
6	M. gastrocnemius	73	441	2050	1449	28
AOD patient						
1	M. gastrocnemius	83	577	3331	1517	38
2	M. gastrocnemius	88	844	3610	1252	17
3	M. gastrocnemius	98	741	3154	949	86
4	M. gastrocnemius	63	737	3376	1081	70
5	M. gastrocnemius	77	680	2867	1839	72
6	M. gastrocnemius	67	840	3518	1341	55
Control patient	Controls (observed range)[a]	62-192	333-853	2057-4964	1201-2938	23-178
	Controls range (mean±2sd)[a]	53-147	359-884	1899-4569	1099-2563	22-168
	n	41	35	35	42	42
	p Wilcoxon test: Controls versus CRPS I	0.045	0.024	0.034	0.076	0.115
	p Wilcoxon test: Controls versus AOD	0.031	0.036	0.555	0.021	0.016
	p Wilcoxon test: AOD versus CRPS I	1.0	0.006	0.016	0.522	1.0
CRPS I patient	**Muscle investigated**	**Complex I[b]**	**Complex II[b]**	**Complex III[b]**	**Complex IV[b]**	**CS[c]**
7	M. gastrocnemius	65	nd	1192	878	nd
8	M. gastrocnemius	108	nd	1289	1088	nd
	Controls (observed range)[a]	101-389	nd	1020-2530	520-2080	nd

[a] Samples from CRPS I patients 1 - 6 and the AOD patients were "fresh" muscle biopsies, whereas for CRPS I patients 7 and 8 the biopsies were frozen after collection. Therefore samples were processed in a different manner and different control values apply.

[b] Complex II, III and IV activity in fresh muscle tissue are given in mU/U CS. Complex I and III activity in frozen muscle.

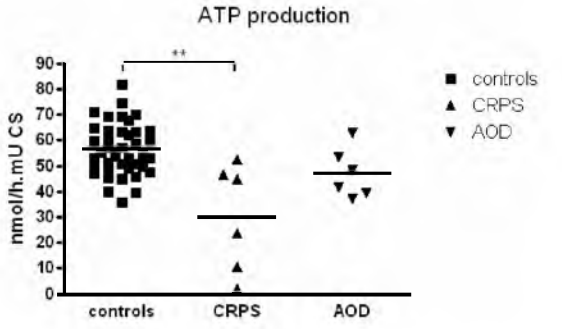
[c] CS activity is given in mU/mg protein

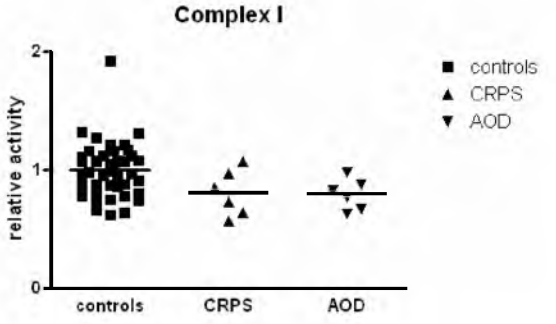
[d] Complex I activity is given as a relative activity as described in the methods section.

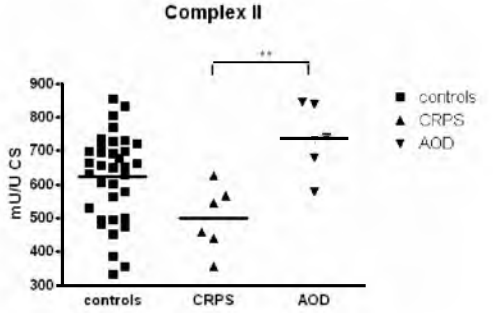
nd = not determined

did not significantly differ between the AOD patients and controls (Table 2). Although all values of the MEGS parameters measured in the AOD patients were within the control range, we found no statistically significant difference between the CRPS I and the AOD group. All MEGS parameters were significantly decreased in three out of six CRPS I patients. The oxidation

Figure 1. ATP production rates, complex I and complex II activities in muscle of controls, CRPS I patients and AOD patients.





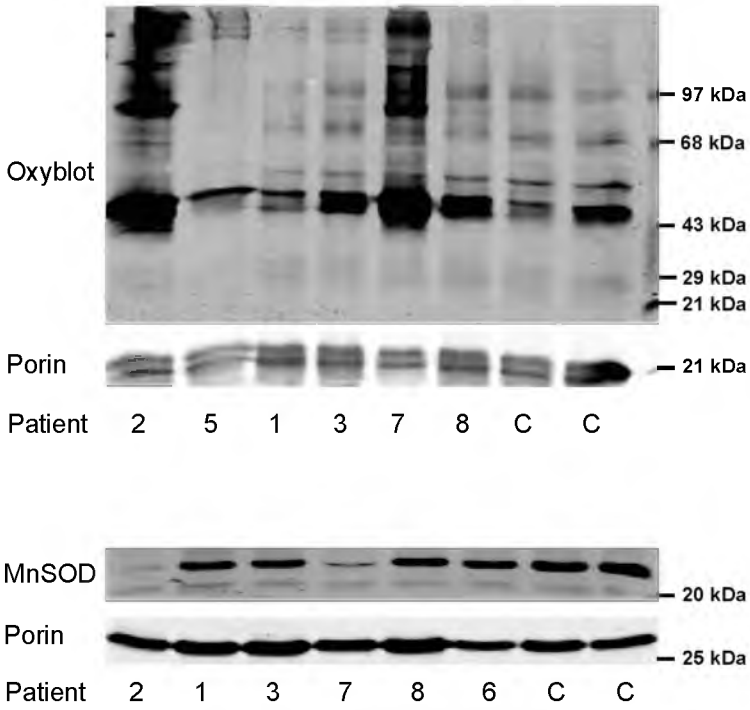


Dot plots for the ATP production rates, complex I activity and complex II activity. ATP production is expressed in nmoles ATP/hour.mU citrate synthase, complex I as a relative activity (see methods section), and complex II as mU/U citrate synthase.

■ Controls, ▲ CRPS I patients, ▼ AOD patients.

**: $p \leq 0.01$ in the Wilcoxon test.

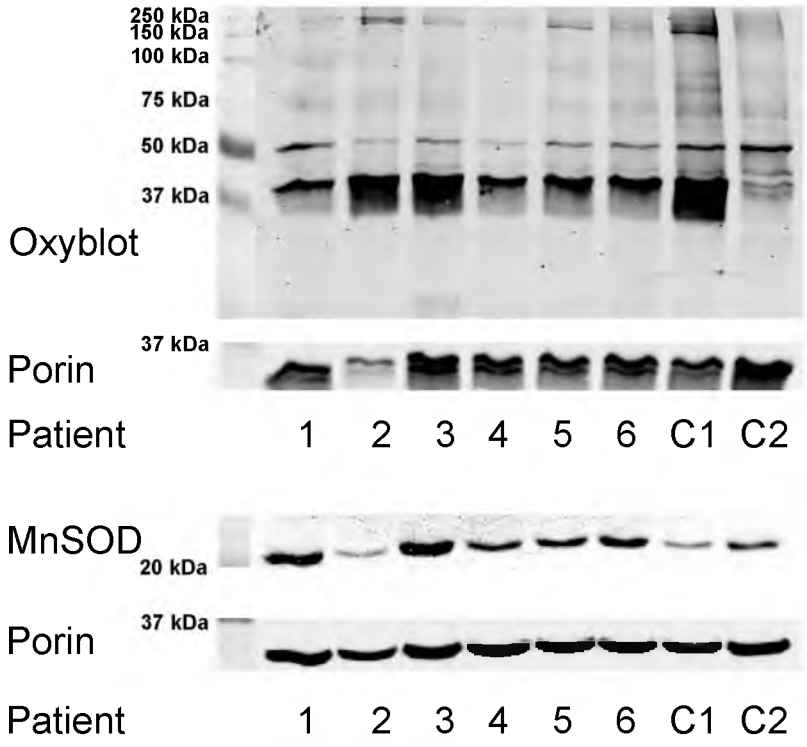
Figure 2. Immunoblots for oxidatively damaged mitochondrial proteins (Oxyblot) and MnSOD in muscle mitochondria from CRPS I patients.



Patients 2 and 7 show an increased amount of oxidatively damaged proteins (top panel) and reduced MnSOD levels (bottom panel). The immunoblot for porin from the same gel is presented below each single blot. Molecular weights indicated on the right are based on a molecular weight marker run on the same gels. Patient numbering is the same as in Table 1.

rate of [1,4-^{14}C]succinate + acetylcarnitine was significantly decreased in two of five patients. We found no significant differences for the respiratory chain enzymes complex I, III and IV between the three groups (Table 3). Complex II activity was significantly increased in the AOD group compared to the CRPS I group (Table 3). All respiratory chain enzyme activities were normal in the CRPS I and the AOD patients, except complex IV activity that was slightly, but significantly, decreased in one CRPS I and two AOD patients. In one of two CRPS I patients we found a decreased activity of complex I in frozen muscle tissue. Typical dot plots for the MEGS parameters (ATP production rate) and respiratory chain enzymes (complex I and II activity) are presented in Figure 1. In search for evidence of elevated ROS, we tested for the presence of oxidatively damaged proteins in CRPS I and AOD muscle. In all the CRPS I patients we found a varying degree of increase of oxidatively damaged mitochondrial proteins (Figure 2). In patients 2 and 7 a strong increase in oxidatively damaged proteins was observed. In patients

Figure 3. Immunoblots for oxidatively damaged mitochondrial proteins (Oxyblot) and MnSOD in muscle mitochondria from AOD patients.



Patient 2 shows an increased amount of oxidatively damaged proteins in relation to the amount of the mitochondrial marker protein porin (top panel) and increased amounts of MnSOD in patients 1, 3, 4, 5 and 6 (bottom panel). The immunoblot for porin from the same gel is presented below each single blot. Molecular weights indicated on the left are based on a molecular weight marker run on the same gels. Patient numbering is the same as in Table 1.

1, 3, 5, and 8, there was an clear increased amount of oxidatively damaged proteins with a molecular weight of approximately 250 KDa, while in proteins of lower molecular weight the level of oxidative protein damage appeared to be similar to controls. Although the nature of these damaged proteins is as yet unknown, these results clearly show an increase in oxidatively damaged proteins in muscle tissue of CRPS I patients. In patients 2 and 7 showing the highest amounts of oxidatively damaged proteins, we found a decreased level of MnSOD (Figure 2). Patient 2 also showed the strongest decrease in the MEGS parameters (Table 2). In one AOD patient (patient 2) an increased amount of oxidatively damaged proteins was found and the amount of MnSOD was more or less increased in five patients and normal in patient 2 (Figure 3).

DISCUSSION

MEGS activity in muscle tissue and also in other tissues is dependent on normal oxygen availability. Under hypoxic conditions, both the oxidation rate of pyruvate and the ATP production rate will be decreased due to a decreased oxidation of NADH and $FADH_2$ by the respiratory chain. Under these conditions, pyruvate will be converted to lactic acid, resulting in lactic acidosis. During chronic and extreme hypoxic conditions, muscle tissue fails to maintain a normal redox state. This results in an increased production of reactive oxygen species (ROS), followed by cell injury or dysfunction[10]. CRPS I patients suffer from severe dysfunction of an affected extremity, sometimes requiring amputation of the affected limb. In this study, we found that venous oxygen saturation values, obtained at the level of amputation of the affected limb just prior to the surgical procedure, were increased. This is indicative for a decreased oxygen diffusion or utilization within the affected limb[8]. Previously, P-NMR spectroscopy studies have revealed a disturbed phosphate/phosphocreatine metabolism in skeletal muscle of the affected limb, an indication for mitochondrial dysfunctioning[20]. In the present study we observed a reduced mitochondrial energy generating capacity in muscle tissue of CRPS I patients. In a second group of patients, suffering from arterial occlusive disease (AOD), we found that the venous oxygen saturation values were either increased or decreased, concluding that also in these patients oxygen metabolism is disturbed in the affected limb. However, in this group of patients all the MEGS parameters showed normal activities. CRPS I patients react different from AOD patients on hypoxic conditions. Moreover, in the CRPS I patients complex II activity was significantly decreased compared to the AOD patients. From recent studies it is known that impaired tricarboxylicacid cycle flux, particularly if it is caused by decreased activity of complex II, might result in decreased mitochondrial energy production and in overproduction of free radicals. Accumulation of succinate subsequently leads to inhibition of prolyl hydroxylases (PHDs) thereby stabilizing hypoxia-inducible factors (HIFs). These HIFs bind to hypoxia-responsive elements so activating the transcription of more than two hundred genes that allow cells to adapt to the hypoxic condition[21]. As mentioned before, mitochondrial dysfunction caused by a decreased oxygen availability will lead to an increase of ROS, resulting in oxidatively damaged (carbonylated) mitochondrial proteins[22]. In all CRPS I patients, but only in one of the AOD patients, we found a varying degree of increase of oxidatively damaged mitochondrial proteins while the amount of MnSOD was decreased in two CRPS I patients and more or less increased in five AOD patients.

Our observations are compatible with the involvement of free radicals in the pathophysiology of CRPS I that has been reported previously[5,6]. An increase in lipid peroxidation products in serum and increased antioxidative products in serum and saliva of CRPS I patients has been observed[23]. Their patients showed increased SOD activity in saliva, so in all probability Cu/Zn-SOD instead of the mitochondrial MnSOD. Little is known about the possible genetic background of CRPS I. Recently, Higashimoto et al. described eight children with mitochondrial disease and

clinical features meeting the IASP diagnostic criteria for CRPS I[24]. In two of them decreased respiratory chain complex activities were measured and in six of them the clinical features and family history pointed towards a possible mtDNA mutation. In four of their patients mtDNA transitions were detected, all known as polymorphisms in the mtDB database[25]. Whether these polymorphisms are risk factors to develop CRPS I is unknown. In our patients we performed sequence analysis of the entire mtDNA but found no pathogenic mtDNA mutations (data not shown).

In summary, our study is the first to reveal mitochondrial dysfunction and ROS pathology in muscle tissue from CRPS I patients. At this moment, it is unclear whether the mitochondrial dysfunction that is apparent at the end-stage of CRPS I could also be present in earlier stages of the disease, and further studies are needed to obtain an answer to this question. The observations in muscle from AOD patients indicate that the mitochondrial dysfunction seen in CRPS I is not a general phenomenon of severe, hypoxic muscle disease. On the basis of our findings we postulate that an impaired mitochondrial energy metabolism is involved in the pathogenesis of CRPS I.

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Chapter 10

Summary and General Discussion

CRPS I IN CHILDREN

Complex Regional Pain Syndrome type I (CRPS I) is a potentially incapacitating syndrome that may occur after a minor injury or operation to a limb. It is a disorder characterized by pain as well as sensory and motor disturbances. CRPS I is well-known in adults[1] but a relatively new diagnostic entity in children. The clinical presentation of CRPS I in children is to some extent different from that in adults and may therefore not be recognized in an early stage of the disease. In Chapter 2, we provided a retrospective chart review of 78 children (age < 16 years) with CRPS type I and compared the data with those of 951 adults with the same syndrome. Our study, the largest group of paediatric CRPS I patients evaluated, shows that there are significant differences between the two groups. We found that the child population consisted predominantly of older girls with a median age of 13 years. Children differed from adults in that a lower extremity was involved more frequently, the skin temperature at onset was more often lower than the contralateral extremity, and neurological and sympathetic symptoms were less pronounced. Treatment protocols currently recommended for children with CRPS I are physical therapy including advice on posture and movement, stimulation of normal usage of the affected extremity, and psychological intervention to reduce fear of movement[2]. We used a protocol with free radical scavengers, vasodilators (if indicated by a lower skin temperature in the affected extremity) and physical therapy in these groups. Our study did not lead to firm conclusions regarding the optimal treatment strategy for children with CRPS I. Increased awareness of CRPS I in children, familiarity with symptoms, use of diagnostic criteria and, if necessary, referral to a clinic with experience in treating children with CRPS I may help alleviate pain and suffering and more quickly restore function.

Reported results of treatment of CRPS I in children are usually more favourable than in adults[2,3]. Therefore we investigated quality of life (QoL) in adults who have been treated for childhood-onset CRPS I (Chapter 3). Patients with onset of CRPS I in childhood (age < 16 years) completed a generic health-related quality of life instrument (SF-36). Results were compared with scores in the standard population. We obtained complete SF-36 scores in 54 of 78 patients. Of these, 12 were still younger than 16 years and were excluded from the study. The median follow-up from onset of CRPS I was 12 years (range: 2 - 22). Fifteen patients (33%) experienced one or more documented relapses. General health and physical functioning (2 out of 8 scales on the SF-36) were significantly lower in patients compared to the standard population. Contrary to earlier reports[2,3], patients diagnosed with and treated for CRPS I in childhood did not score significantly better than adults[4]. In conclusion, the prognosis of patients with CRPS I onset in childhood seems comparable with the prognosis of those diagnosed at adult age.

INFLAMMATION

The clinical presentation of CRPS I is well-known[1,5,6], but the mechanism of the (chronic) pain and its underlying pathophysiology still remain unsolved. In the acute phase in adults, CRPS I is characterized by symptoms of inflammation in the periphery of the affected extremity. Both clinical and experimental evidence support the theory that an excessive regional inflammatory response, including production of reactive oxygen and nitrogen species (RONS), may contribute to the development of CRPS I and cause tissue damage[1,7-9]. Leukocytes are thought to play a significant role in inflammatory processes. To assess leukocyte accumulation, we performed radiolabeled autologous leukocyte scans of both hands in patients with CRPS I of one upper extremity after a fracture or an operation of the hand. Results from the affected hand were compared with the unaffected contralateral hand (Chapter 4). We found a significantly increased accumulation of leukocytes in patients with CRPS I. Free radical-generating capacity and release of proteases by leukocytes can contribute to the increased vascular permeability observed in CRPS I patients[7,10,11]. Adherence of leukocytes to endothelial cells may trigger the generation of additional free radicals, resulting in an exaggerated inflammatory reaction[11,12].

RADICALS

One of the most important features of inflammation is excessive production of reactive oxygen and nitrogen species (RONS). We studied the effect of RONS in two known animal models that mimic symptoms of CRPS.

In the chronic constriction injury (CCI) rat model[13], we investigated levels of oxidative stress and antioxidant enzymes in skeletal muscle tissue of the hind paw and (jugular vein) plasma. We demonstrated (Chapter 5) that, in skeletal muscle tissue, the level of RONS was lower in nerve-injured hind paws than in controls. Plasma level of RONS did not differ between nerve-injured and control rats. In skeletal muscle tissue, the level of reduced glutathione (GSH) was higher in nerve-injured hind paws than in controls. The ceruloplasmin/transferrin ratio tended to be higher in plasma of nerve-injured rats compared to controls. We found that, at day 7 after nerve injury, oxidative stress-induced changes are present in skeletal muscle tissue of the rat hind paw. Our findings of decreased levels of RONS in combination with marked elevation of antioxidant enzymes in skeletal muscle and plasma suggest that a surplus of antioxidant activity precedes initial oxidative stress. Oxidative stress and antioxidant activity are involved in the pathophysiologic mechanisms underlying symptoms in the CCI model, in line with studies showing that antioxidants have a beneficial effect on neuropathic pain[14,15].

We studied (Chapter 6) the level of RONS in our animal model of continuous intra-arterial infusion of a free radical donor in one hind limb of non-anaesthetized rats[16]. Infusion of Tertbutylhydroperoxide (Tert-BuOOH, 25 mM) for 24 hrs in the left hind limb of rats resulted in regional

symptoms of inflammation and exacerbated sensory responses. We did not find a difference in RONS levels between experimental and control animals, or between infused and non-infused contralateral hind limbs. Jugular vein plasma levels of ceruloplasmin and tissue levels of GSH were found to be higher in infused rats than in controls; this suggests an increased level of systemic antioxidant activity that prevents and/or neutralizes free radical formation.

VENOUS OXYGEN SATURATION

Increasing evidence points to a role for impaired oxygen metabolism in the affected limb. In earlier studies, we found significantly elevated S_vO_2 levels in blood samples obtained from the antecubital vein in the affected upper extremity as compared to S_vO_2 in the unaffected contralateral limb[17-19]. Therefore we were interested in assessing capillary oxygen and lactate levels in the affected limb. In our infusion animal model, we investigated oxygen extraction as measured by S_vO_2 and lactate levels. We found significantly elevated S_vO_2 levels and a tendency toward higher venous lactate levels in the infused hind limb seven days after Tert-BuOOH infusion (Chapter 6). In Chapter 7, we described a pilot study of 16 patients in whom we performed capillary blood gas analysis in extremities with acute CRPS I in order to assess oxygen saturation and lactate levels. Comparing the affected and unaffected limb as to capillary blood pH, pO_2, S_aO_2, and lactate and glucose levels we did not find statistically significant differences. We concluded that capillary blood gas analysis is not useful to detect changes in oxygen saturation and lactate and glucose levels in CRPS I patients.

In patients with severe CRPS I requiring amputation of the affected limb, we analyzed venous blood samples and skeletal muscle specimens (Chapter 8). S_vO_2 values (mean S_vO_2 94.5%) were significantly higher than in control patients (mean S_vO_2 83%), and normal values reported in the literature (mean S_vO_2 67.2 to 71.2%[19,20]), pointing to a severely decreased oxygen diffusion or utilization within the affected limb. Skeletal muscle histologic specimens showed changes compatible with disuse atrophy. Ultra-structural investigations of skeletal muscle capillaries revealed a decrease in capillary density, while endothelial basement membranes were moderately thickened. We conclude from these data that the high S_vO_2, which suggests defective oxygen metabolism, cannot fully be explained by an oxygen diffusion problem resulting from decreased capillary densities and/or basement membrane thickening. We therefore hypothesized that the high S_vO_2 found may also be due to defective oxygen utilization at the mitochondrial level. Tert-BuOOH-induced oxidative stress is known to damage mitochondria by mitochondrial swelling, resulting in reduction of their respiratory activity[21]. We have assessed mitochondrial function in vitro by stimulating isolated mitochondria in rat skeletal muscle cells with increasing doses of Tert-BuOOH. We found that the mitochondrial energy generating system, as reflected by State 3 respiration and ATP production, is a target for RONS in this model.

These data support the concept that Tert-BuOOH induces mitochondrial dysfunction, causing impaired oxygen extraction (Chapter 6).
To study our hypothesis in CRPS I patients, we studied mitochondria in affected muscle tissue from amputated limbs of CRPS I patients (Chapter 9). We observed that mitochondria obtained from CRPS I muscle tissue display reduced ATP production and substrate oxidation rates in comparison to control muscle tissue. There was a relevant correlation between S_vO_2 levels and several parameters of the mitochondrial energy generating system. Moreover, we found evidence that reactive oxygen species caused damage to mitochondrial proteins and reduced manganese superoxide dismutase (MnSOD) levels. These observations indicate that mitochondrial dysfunction is involved in the pathophysiology of CRPS I.
On the basis of our findings and those of others, we postulate the following model for the pathogenesis of CRPS I: after the triggering event has occurred, the pathological process starts with a regional excessive inflammatory response in the area of injury, including the generation of reactive oxygen species, leading to excessive pain and impaired function, reduced oxygen utilization, mitochondrial dysfunction, and finally to late severe pain, tissue dystrophy and atrophy. This model is in agreement with the previously observed positive effects of neutralization of reactive oxygen species by radical scavengers,[22,23] inhibition of the inflammatory response[24] and physical therapy[25,26]. Although mechanisms to stimulate the mitochondrial energy generating system in patients with primary mitochondrial disorders are still limited, new therapies are emerging[27] and these may be of benefit to CRPS I patients in the future.

GENERAL DISCUSSION

Our research has focused on the epidemiology, clinical aspects and quality of life in children with CRPS I, and on the underlying pathophysiological mechanism of CRPS I in general. In particular, we have focused on inflammation, oxygen free radicals and venous oxygen saturation.
In our clinic, children with CRPS I have been treated similarly to adults, according to a treatment protocol that includes scavengers, peripheral vasodilators (if indicated by a lower skin temperature in the affected extremity) and pain medication. Over time, psychological consultation has been added, as part of a multidisciplinary approach. Although results have been encouraging, we encountered some patients who did not respond well to this treatment protocol.
No studies of scavenger therapy and vasodilators have been published in children with CRPS I. We have noted several side effects of vasodilator therapy such as headache and mild hypotension, both of which also have been observed in adults with CRPS I. Scavenger therapy side effects include skin rash and gastrointestinal complaints. Based on the results of our studies and the observed side effects of vasodilator and scavenger therapy we currently cannot recommend the use of these therapies in children with CRPS I. As suggested in other studies[2,3], we modified our treatment protocol to put more emphasis on physical therapy, advice on posture

and movement, stimulation of normal usage of the affected extremity, and psychological intervention to reduce fear of movement.

While previous publications generally show a very favourable prognosis for paediatric-onset CRPS I, the results of our case series differ. At long term follow-up, some patients continue to experience moderate pain and have modest reductions in quality of life, as found in adults. Our treatment strategies using free radical scavengers and vasodilators if indicated have seldom been used outside of Europe. It may be argued that these medical interventions have been responsible for the less favourable outcome compared to other studies, but we believe this is unlikely. In children, a high relapse percentage of 27.5 - 50% was observed[28-30]. In our study, there was a documented relapse percentage of 33%. A prospective study with free radical scavengers (and vasodilators if indicated), combined with a quality of life study should be conducted in patients with CRPS I in childhood to determine effectiveness in this group. With the promising results of the intensive function-oriented physical therapy or Pain Exposure in Physical Therapy (PEPT or Macedonian therapy)[31], this method is currently being investigated in adults with CRPS I, but may also be applicable in children with CRPS I.

The experimental studies in this thesis were performed to obtain insight into the pathophysiology of CRPS I, with a focus on inflammation, oxygen free radicals and S_vO_2. We have found increased accumulation of polymorphonuclear leukocytes, the main type of granulocytes seen in the acute phase of CRPS I. These are a source of free radicals and release proteases, which contribute to increased vascular permeability. Various anti-inflammatory treatments have been tested. Blocking TNF seems to have a positive effect[32], as does the use of corticosteroids[24]. The use of the antioxidant N-acetyl-l-cysteine was successful in the infusion animal model of free radical induced soft tissue injury[33] and has also been shown effective in patients[23,34]. If these results are confirmed, these substances may provide alternative treatment options for CRPS I patients.

In the chronic constriction injury model of neuropathic pain and the animal model of free radical related soft tissue damage, a sensory response with symptoms mimicking CRPS was found. We also noted diminished RONS levels in skeletal muscle, and both models showed an increased antioxidant status as reflected by elevated systemic levels of ceruloplasmin and local GSH levels in skeletal muscle tissue. This effect may be attributed to operative trauma and sham surgery in the control group. A wider sampling frame and including more control groups would probably have benefited our results. In several studies, RONS are proposed to play an important role in chronic pain, and there may be a small and localized increase in RONS levels at the injured peripheral nerve and/or spinal cord in chronic conditions[14,15]. Determining RONS and glutathione levels of peripheral nerves in dorsal root ganglia or in the spinal cord in both models may be more relevant.

In the animal model of free radical related soft tissue injury, we hypothesized that symptoms of chronic pain syndromes are caused by impaired oxygen extraction secondary to RONS-induced

mitochondrial dysfunction. An argument in favour of our hypothesis on the involvement of free radicals in the development of symptoms in this rat model of chronic pain is that, as we have shown in a previous study, administration of the free radical scavenger N-acetyl-l-cysteine results in reduced pain sensations, reduced skeletal muscle and nerve damage, and shortened repair periods[33]. However, a limitation of our research with regard to our hypothesis concerning impaired oxygen extraction is that we did not measure muscle oxygen consumption, muscle perfusion or intracellular pO_2. We have performed flow measurements with a Transonic (T206) 0.5 V flow probe (0.25 - 0.5 mm vessels), and with the laser Doppler perfusion (Moor High Resolution Laser), with a wavelength 633 nm. We could not establish reliable baseline measurements due to technical and logistical difficulties with these apparatus, possibly related to the form of anaesthesia used. Validating the model with another form of anaesthesia or other forms of oxygen flux measurements (e.g. near infrared spectroscopy) will be important next steps.

Elevated S_vO_2 are found also in chronic end-stage CRPS I patients. Comparison of our measurement of S_vO_2 levels with results in the literature are complicated by several factors, such as multiple venous sampling sites from different muscle groups that may vary randomly or systematically in their oxygenation state, as well as effects of anaesthesia and surgical exposure on local blood flow. Furthermore, investigator bias may be presented in the literature selected for comparison. Nevertheless, S_vO_2 values were significantly increased in all our CRPS I patients, compared to an healthy matched control population. Ideally, control skeletal muscle biopsies should have been obtained from the unaffected extremity, but such control samples were not taken in view of the risk of inducing CRPS I in that extremity. Therefore we have chosen to compare our data with data from the literature.

In patients with CRPS I, muscle weakness and exercise-induced pain are present in the affected extremity from the beginning[1]. As the syndrome progresses, CRPS I patients are less able to perform skeletal muscle work because less (functional) muscle tissue is present. The resulting increased load on the remaining deteriorated muscle fibers gives rise to increased pain upon exercise. In this way, in a vicious circle of pain and disuse atrophy results in pseudo-paralysis in 15% of patients[1].

In the present study, we found that patients with severe CRPS I, requiring amputation of the affected limb, have a significantly increased S_vO_2 level in this limb. We also found several abnormalities in skeletal muscle tissue compatible with disuse atrophy, including decreased numbers of type I fibers and increased numbers of type IIB fibers. Capillary basement membrane thickness was also increased and capillary densities decreased. Based on high saturation levels we hypothesized four possibilities: Firstly, a deficient auto-regulation of microcirculation, resulting in a widely open capillary bed, while only a small fraction of the oxygen supplied is consumed due to hyper-perfusion of some capillaries and inadequate perfusion of others. Secondly, the presence of increased flow through non-nutritive pathways, such as through arteriovenous anastomoses[35]. Thirdly, a diffusion problem for oxygen between the erythrocytes and the mitochondria, especially the endothelial cell and capillary basement membrane. Finally,

minimal oxygen extraction due to mitochondrial dysfunction. With respect to the first possibility, no published reports are available indicating a defective auto-regulation within healthy skeletal muscle, poor in mitochondria, resulting in high S_vO_2 levels. As to the second possibility, arteriovenous anastomoses were proven to be present in cat skeletal muscle[36], but were not found in human temporal skeletal muscle[37]. Regarding the role of endothelial cells, we found significant changes in quadriceps capillaries in relation to the control specimens, such as an increase in endothelial cell thickness and in basement membrane thickness. Endothelial cell thickness cannot be a factor of importance for oxygen diffusion because capillary diameters, measured without the basement membrane, were not significantly different from controls. The capillary basement membrane thickness (CBMT) in skeletal muscle in our patients was increased by about 33%. This increase in thickness will certainly limit oxygen diffusion during maximum exercise. However, this 33% increase in thickness at rest cannot be a factor for tissue hypoxia, as oxygen demand at rest is normally 10 times lower than during maximum exercise for sedentary people[38]. Mitochondrial dysfunction is a plausible cause for the high S_vO_2 levels found. Indeed, in a rat model of disuse, an important decrease (3 to 4 times) in mitochondrial function was found in the gastrocnemius muscle[39]. In a comparable experiment with rabbits, heavily damaged mitochondria were observed via electron microscopy[40]. Among our patients, a large fraction of the remaining mitochondria may have been damaged or functionally inactive, while their numbers were decreased. We also found loss of mitochondrial activity with SDH and ATPase stains. High S_vO_2 combined with low oxygen extraction is well known in sepsis[41]. Apparently, functionally defective mitochondria cannot optimally metabolize the high oxygen supply in this condition, resulting in decreased ATP production even in the presence of increased tissue oxygen partial pressures.

Mitochondrial dysfunction may explain the deep skeletal muscle pain during exercise seen in CRPS I patients and explains the high S_vO_2 levels. Our data did not show increased lactate levels at rest, but increased levels probably would have been found during exercise. Artificial acidosis induced pain in the CRPS I affected extremity only and not in the healthy contralateral extremity[42]. This discrepancy can be explained by the additive effect of an already increased level of lactate in the affected extremity.

We have observed a reduced mitochondrial energy-generating capacity in muscle tissue of CRPS I patients that correlated with the S_vO_2 values in the affected extremity. Mitochondrial dysfunction will lead to an increased production of oxygen radicals, resulting in oxidatively damaged (carbonylated) mitochondrial proteins[43]. In the affected muscle of all patients, we found an increase in oxidatively damaged mitochondrial proteins. Our observations are compatible with the involvement of free radicals in the pathophysiology of CRPS I, as reported previously[44,45]. Future studies may focus on further determination of radical involvement in CRPS I by using spin trapping techniques in patients and analyzing and quantifying the mitochondrial dysfunction.

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Chapter 11

Summary in Dutch

SAMENVATTING

KLINISCHE EN FUNDAMENTELE ASPECTEN VAN HET COMPLEX REGIONAAL PIJNSYNDROOM TYPE I

INLEIDING

Het Complex Regionaal Pijnsyndroom (CRPS), een tot op heden slecht begrepen ziektebeeld, is een abnormale reactie in een extremiteit of deel daarvan na een - vaak gering - ongeval of na een operatie. Bij ongeveer 10% van de patiënten ontstaat de aandoening spontaan. CRPS kan alle weefsels en alle functies van een extremiteit aantasten en kan ernstige invaliditeit en moeilijk te behandelen pijn veroorzaken.
Voor dit ziektebeeld worden verschillende benamingen gebruikt, zoals causalgie, algodystrofie, Sudeck-dystrofie, sympathische reflexdystrofie en posttraumatische dystrofie. Omdat tot op heden de pathofysiologie niet eenduidig is vastgesteld, wordt het ziektebeeld sinds enige tijd benoemd als Complex Regionaal Pijnsyndroom type I (CRPS I) door de International Association for the Study of Pain (IASP)[1]. Men spreekt van CRPS type II (CRPS II) indien er een aantoonbaar zenuwletsel aanwezig is.
Dit proefschrift handelt met name over CRPS I, omdat het meer voorkomt dan CRPS II. Het klachtenpatroon van een patiënt met CRPS I bestaat voornamelijk uit pijn, die spontaan optreedt of ontstaat na een extremiteitletsel dat niet in verhouding staat tot de ernst van het letsel. Vroege tekenen zijn: temperatuurverschil, kleurverschil, zwelling, een beperking van de functie of beweegbaarheid en krachtverlies. Later optredende tekenen kunnen symptomen zijn van zenuwpijn; zoals een brandend gevoel, dysesthesie (overgevoeligheid voor bepaalde huidprikkels), paresthesie (onjuiste gevoelsgewaarwording), mechanische allodynie (stoornis in de pijngewaarwording) en hyperalgesie (verhoogde gevoeligheid voor pijn), of symptomen van autonome dysfunctie; zoals cyanose (blauwe verkleuring van de huid), mottling (vlekkerig worden van de huid), hyperhidrosis (transpireren), oedeem (zwelling), koude van de extremiteit en verandering in haargroei.

Dit proefschrift concentreert zich op:

- De klinische presentatie en prognose van CRPS I bij kinderen en volwassenen;

- Enkele fundamentele aspecten van ontsteking in CRPS I; (1) de rol van zuurstofradicalen in humane en proefdierstudies, (2) de rol van veneuze zuurstofsaturatie en factoren verantwoordelijk voor zuurstofdiffusie.

HET COMPLEX REGIONAAL PIJNSYNDROOM TYPE I BIJ KINDEREN

CRPS I verloopt bij kinderen ten dele anders dan bij volwassenen en wordt hierdoor vaak niet herkend. In Hoofdstuk 2 van dit proefschrift beschrijven we een onderzoek bij 78 kinderen met CRPS I (leeftijd < 16 jaren) en vergelijken we het ziektebeeld met 951 volwassen CRPS I patiënten. De gemiddelde leeftijd bij presentatie ligt tussen de 11 en 14 jaar, gemiddeld 13 jaar. Net zoals bij volwassenen komt CRPS I bij kinderen vaker voor bij het vrouwelijke geslacht. CRPS I bij kinderen wijkt in enkele opzichten af van het ziektebeeld bij volwassenen. Bij kinderen is bij aanvang van het ziektebeeld het aangedane ledemaat meestal kouder dan het andere niet aangedane ledemaat, en is de onderste extremiteit vaker aangedaan (60 - 87%) dan de bovenste.

De medische behandeling bij volwassenen is gericht op remming van de ontsteking, pijnstilling en, bij koude CRPS I, verbeteren van de perifere doorbloeding en het geven van houdings- en bewegingsadviezen. Tegenwoordig wordt meer aandacht besteed aan fysiotherapie en verbetering van de functie van de aangedane extremiteit. Ook bij kinderen wordt naast de ontstekingsremmende behandeling en de psychologische begeleiding, de fysiotherapie meer en meer benadrukt. Verscheidene behandelmodaliteiten worden beschreven met wisselende resultaten. De meeste onderzoeken betreffen echter kleine patiëntengroepen dan wel casuïstische mededelingen. Consensus over behandeling van CRPS I bij kinderen bestaat niet. Ook onze resultaten laten niet zien welke behandeling voor CRPS I bij kinderen het beste is. Toekomstig onderzoek zal dit moeten uitwijzen.

Bij kinderen met pijnklachten van het houdings- en bewegingsapparaat dient CRPS I in de differentiaaldiagnose te worden opgenomen. Alertheid met betrekking tot de diagnose en het volgen van een protocol zijn de sleutel tot de behandeling van kinderen met CRPS I. Bij ernstige klachten is snelle verwijzing gewenst naar een specialist of een specialistische groep die ervaring heeft met deze aandoening.

Volgens de literatuur is de prognose van CRPS I bij kinderen vaak beter dan bij volwassen[2-4]. In Hoofdstuk 3 hebben wij de kwaliteit van leven onderzocht bij volwassenen die op kinderleeftijd behandeld zijn voor CRPS I. Deze groep patiënten heeft een standaard vragenlijst ingevuld, waarvan de resultaten werden vergeleken met die van de algemene bevolking. Wij ontvingen een compleet ingevulde enquête van 54 van de 78 patiënten. Van deze groep waren 12 patiënten jonger dan 16 jaar. Zij zijn niet meegenomen in de studie. De gemiddelde follow-up sinds het ontstaan van CRPS I op kinderleeftijd bedroeg 12 jaren (2 - 22 jaren). Vijftien patiënten (33%) beschreven een of meer recidieven. Twee items van de kwaliteit-van-leven-studie, algemene gezondheid en fysiek functioneren, scoorden slechter dan bij de gemiddelde bevolking. Wij kwamen tot de conclusie dat de prognose van patiënten met CRPS I op kinderleeftijd vergelijkbaar is met volwassenen die op latere leeftijd CRPS I hebben gekregen. Dit in tegenstelling tot wat algemeen wordt aangenomen.

ONTSTEKING

De klachten waarmee patiënten met CRPS I zich presenteren zijn algemeen bekend[5-7], maar het onderliggende ziektemechanisme van met name de chronische pijn blijft onopgehelderd. In de acute fase wordt CRPS I bij volwassenen gekarakteriseerd door tekenen van ontsteking in het aangedane ledemaat. Zowel klinisch als experimenteel onderzoek ondersteunen de theorie dat een overmatige locale ontstekingsreactie, inclusief de productie van actieve zuurstof en stikstofradicalen, betrokken zijn bij het ontstaan en ontwikkelen van CRPS I en verantwoordelijk zijn voor de geobserveerde weefselschade[5,8-10]. Een radicaal is een vrij atoom of molecuul dat veel schade kan aanrichten. Witte bloedcellen spelen een belangrijke rol in allerlei ontstekingsreacties. Om een eventuele stapeling van witte bloedcellen te beoordelen in een aangedaan ledemaat, hebben wij witte bloedcellen radioactief gelabeld en scans gemaakt van beide handen en polsen van patiënten die aan een van deze bovenste extremiteiten CRPS I hebben ontwikkeld na een botbreuk of een operatie. Op basis van deze scans werd een vergelijking gemaakt tussen de aangedane en niet aangedane zijde (in Hoofdstuk 4).
Wij vonden een significant verhoogde stapeling van witte bloedcellen bij patiënten met CRPS I. De vrije zuurstofradicalen genererende capaciteit en het vrij maken van diverse schadelijke enzymen door witte bloedcellen zijn verantwoordelijk voor de verhoogde doorlaatbaarheid van de vaatwand, zoals men heeft kunnen waarnemen bij patiënten met CRPS I[8,11,12]. Vastkleven van witte bloedcellen aan cellen van de vaatwand kan leiden tot additionele vrije radicalen en resulteren in een overmatige onstekingsreactie[12,13].

RADICALEN

Een belangrijk onderdeel van ontsteking is het vrijkomen van een overmatige hoeveelheid reactieve zuurstof en stikstof radicalen. Wij hebben het effect van deze reactieve zuurstof en stikstofradicalen bestudeerd in twee diermodellen, die symptomen laten zien die overeenkomen met het klinisch beeld van CRPS. In het chronische constrictie ratmodel[14] wordt een ligatuur rondom de zenuw (nervus ischiadicus) naar de achterpoot van de rat gelegd. Wij onderzochten in dit model de mate van oxidatieve stress en de aanwezigheid van antioxidanten (stoffen die ongewenste oxidatie en vrije radicaalvorming kunnen voorkomen, zogenaamde vrije radicaalvangers) in spierweefsel van de aangedane achterpoot van de rat en in plasma afkomstig van de vena jugularis (halsader). Wij hebben in Hoofdstuk 5 aangetoond dat bij ratten, in spierweefsel het niveau van de zuurstof- en stikstofradicalen lager was in de aangedane poot ten opzichte van normale ratten. Er was geen verschil in reactieve zuurstof- en stikstofradicalen in het plasma. In spierweefsel vonden wij dat het niveau van het lichaamseigen antioxidant glutathion hoger was bij aangedane ratten dan bij normale ratten. In het plasma bleek het antioxidantniveau ook veel hoger te zijn bij aangedane ratten ten opzichte

van normale ratten. Ook zagen wij na 7 dagen zichtbare veranderingen die passen bij door zuurstofradicalen geïnduceerde weke delenschade in de achterpoot van de rat. Deze bevindingen van lagere waarden van zuurstofradicalen in combinatie met een duidelijk verhoging van de antioxidanten in zowel spierweefsel als plasma suggereren dat deze verhoging wellicht voorafgegaan is door oxidatieve stress (aanwezigheid van een overmatige hoeveelheid reactieve zuurstofverbindingen). Oxidatieve stress en een verhoogde antioxidantactiviteit zijn betrokken in het ziektemechanisme in dit chronische constrictie ratmodel, wat in overeenstemming is met andere studies waar antioxidanten een gunstig effect hebben op zenuwpijn[15,16].
In Hoofdstuk 6 onderzochten wij een ander diermodel, waarbij continu de vrije radicaal donor tertbutyl-hydroperoxide (Tert-BuOOH) werd geïnfundeerd in een achterpoot van wakkere ratten[17]. Infusie van Tert-BuOOH (in een dosis van 25 mM) gedurende 24 uur in de linkerachterpoot van een rat, resulteerde in symptomen en verschijnselen van een overmatige sensorische reactie, passend bij CRPS I. Wij vonden geen verschil tussen experimentele en niet behandelde ratten voor wat betreft reactieve zuurstof- en stikstofdeeltjes, noch tussen de geïnfundeerde en niet-geïnfundeerde achterpoot. Het endogene antioxidant ceruloplasmine in plasma uit de vena jugularis en glutathion uit spierweefsel waren hoger in de aangedane poot in vergelijking met normale ratten. Dit suggereert een verhoogde systemische antioxidantactiviteit, die wellicht vrije radicaalformatie verhindert.

VENEUZE ZUURSTOFSATURATIE

Er bestaan steeds meer aanwijzingen dat bij CRPS I sprake is van een verstoring in het zuurstofmetabolisme in het aangedane ledemaat. In enkele studies uit het verleden vonden wij verhoogde veneuze zuurstofsaturatie (S_vO_2) waarden in bloed dat werd afgenomen uit de vena cubiti (elleboogsvene)[18-20]. Daarom waren wij geïnteresseerd in het bepalen van de capillaire zuurstof en lactaatspiegels in het aangedane ledemaat. Wij onderzochten in ons infusiediermodel de zuurstofextractie, die werd bepaald door S_vO_2- en lactaatspiegels. Wij vonden een significant verhoogde S_vO_2-spiegel en een tendens naar hogere veneuze lactaatwaarden in de geïnfundeerde ledemaat, 7 dagen na Tert-BuOOH-infusie (Hoofdstuk 6). In Hoofdstuk 7, beschrijven we een pilotstudy van 16 patiënten bij wie wij een capillaire bloedgasanalyse hebben verricht in extremiteiten met acute CRPS I. Vergelijking van capillaire bloeduitslagen pH, pO_2, S_aO_2, lactaat en glucose tussen de aangedane en niet aangedane ledemaat, toonde geen statistische significante verschillen. Wij concluderen hieruit dat de capillaire bloedgasanalyse niet nuttig is om verandering in zuurstofsaturatie en lactaatspiegels te detecteren in patiënten met CRPS I.
Bij patiënten die vanwege een zeer ernstige CRPS I een amputatie van het aangedane extremiteit moesten ondergaan, hebben wij veneuze bloedgassen en spierbiopten onderzocht (Hoofdstuk 8). De S_vO_2 waarden (gemiddeld S_vO_2 van 94,5%) waren significant hoger dan bij

controlepatiënten (gemiddeld S_vO_2 van 83%), en ook hoger dan normaalwaarden die beschreven zijn in de literatuur (gemiddelde S_vO_2 waarde tussen 67,2 tot 71,2%)[20,21]. Dit wijst in de richting van een ernstig verminderde zuurstofdiffusie en verbruik in de aangedane extremiteit. Skeletspierbiopten tonen verandering die passen bij inactiviteitsatrofie. Onderzoek van de capillairen in de skeletspier tonen een verminderde capillaire dichtheid, terwijl de endotheliale basaalmembraan verdikt is. Beiden kunnen de gevonden hoge S_vO_2, niet helemaal verklaren. Wij hypothetiseren daarom dat de hoge S_vO_2 tevens wordt veroorzaakt door een probleem in het zuurstof verbruik op mitochondriaal niveau.
Bekend is dat Tert-BuOOH geïnduceerde oxidatieve stress in staat is mitochondria te beschadigen door zwelling, waardoor er een vermindering optreedt van de respiratoire capaciteit[22]. Wij hebben de mitochondriële functie in vitro onderzocht door geïsoleerde mitochondria van rattenspieren te stimuleren met oplopende doses Tert-BuOOH. Wij vonden dat het mitochondriële energie genererende systeem het doelwit is van zuurstof- en stikstofradicalen in dit model. Deze data ondersteunen de gedachte dat Tert-BuOOH in staat is tot mitochondriële dysfunctie, leidend tot een verminderde zuurstofextractie (Hoofdstuk 6). Om onze hypothese bij patiënten met CRPS I te onderzoeken, analyseerden wij mitochondria van aangedane spieren van patiënten die een amputatie moesten ondergaan in verband met ernstige CRPS I (Hoofdstuk 9).
De mitochondria van deze patiënten tonen een verminderde ATP-productie en substraat-oxidatie-ratio in vergelijking met controlespieren. Er is een duidelijke correlatie tussen de S_vO_2 en diverse parameters van het mitochondriële energie genererende systeem. Ook vonden wij bewijs dat reactieve zuurstofdelen belangrijke mitochondriële eiwitten beschadigen en resulteren in een verminderde hoeveelheid van het antioxidant enzym manganese superoxide dismutase (MnSOD). Deze observaties wijzen erop dat mitochondriële dysfunctie een belangrijke rol speelt in het ziektemechanisme van CRPS I.

Op basis van onze bevindingen en van andere studies postuleren wij het volgende model, dat verantwoordelijk is voor de pathogenese van CRPS I:
Nadat er een triggering event (uitlokkend moment) heeft plaatsgevonden, start het proces met een overmatige regionale ontstekingsreactie in het gebied rondom het oorspronkelijke letsel. Hierdoor worden reactieve zuurstofdeeltjes gegenereerd die verantwoordelijk zijn voor pijn, verminderde functie, verminderde zuurstofverbruik, mitochondriële dysfunctie en uiteindelijke tot late chronische pijn, weefseldystrofie en atrofie. Dit model is in overeenstemming met eerdere beschreven positieve effecten ter neutralisatie van reactieve zuurstofdeeltjes door middel van radicaalscavengers (zuurstofradicaalwegvangers)[23,24], inhibitie van het ontstekingsproces[25] en fysiotherapie[26,27]. Hoewel de mogelijkheden beperkt zijn om het mitochondriële energie genererende systeem in patiënten met primaire mitochondriële ziekten te stimuleren, zijn er nieuwe therapieën op komst[28], welke wellicht in de toekomst van nut kunnen zijn voor patiënten met CRPS I.

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Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

Edward Tan

Curriculum Vitae

CURRICULUM VITAE

Edward Tan was born on the 21th October 1971 in Nijmegen, the Netherlands. He attended his pre-university education, including Greek and Latin language and culture at the Stedelijk Gymnasium in Nijmegen. In 1990 he started medical school at the Catholic University of Nijmegen (i.e. Radboud University Nijmegen). During his study he was an active board member of the Nijmegen Medical Faculty Union, member of the Medical Fraternity SO.DA. NO.GO., student member of the Medical Educational Committee on clinical rotations, and chair of the Netherlands' Medical Student International Committee (NeMSIC) (now the International Federation of Medical Students' Associations (IFMSA Netherlands). He was also an active First Aid/Basic Life Support instructor, who started lobbying on getting the First Aid / Basic Life Support course back into the training program of medical students in Nijmegen, which was achieved in 2005. He attended clinical clerkships in Jakarta and Semarang, Indonesia and at the Mayo Clinics, Rochester (MN), in the United States of America.

In 1997 he graduated in Medicine with honours (cum laude). He started his residency in General Surgery in 1999 at the Radboud University Nijmegen Medical Centre (head: professor R.J.A. Goris, followed by professor R.P. Bleichrodt). In 2002 and 2003 he continued his surgical training at the Canisius-Wilhelmina Hospital, also in Nijmegen (head: E.D.M. Bruggink). During this period of time he was the founder and first chair of the cooperation of residents unions of the CARS hospitals (Canisius-Wilhelmina Hospital, Alysis care group, Radboud University Nijmegen Medical Centre and the St. Maartenskliniek), and organized several successful congresses. In 2005 he started his fellowship in Trauma surgery at the Radboud University Nijmegen Medical Centre (head: professor A.B. Van Vugt) and worked as a HEMS-physician (Helicopter Emergency Medical System) at the Lifeliner 3 (Nijmegen).

Since 2007 he works as a military trauma surgeon at the Radboud University Nijmegen Medical Centre. He is involved in the training of medical students, nurses and surgical residents and is an instructor at various advanced life support courses (ATLS®, APLS®, ALS) and is course director of the Definitive Surgical Trauma Care® course. He is an experienced scuba-diver and diving-physician. As active reservist of the Royal Dutch Army, he has been deployed several times to Afghanistan. The work of this thesis was started during his residency. Part of the experimental work has been performed at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria.

Edward has a ten-year-old son, Wouter, and is happily married to Victorine Janssen. They live in Nijmegen, the Netherlands.