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Nature and mechanism of peripheral nerve damage in an experimental model of non-freezing cold injury

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Non-freezing cold injury (NFCI), so called trench foot, is a condition characterised by a peripheral neuropathy, developing when the extremities are exposed for prolonged periods to wet conditions at temperatures just above freezing. Classically, military personnel are affected, with 14% of casualties in the Falklands conflict afflicted. Clinically, NFCI is characterised by a well-defined acute clinical picture and chronic sequelae. Little is known regarding the pathophysiology and treatment of this condition. Opinions vary as to the type of nerve fibres most susceptible to damage and proposed mechanisms of injury include direct axonal damage, ischaemia and ischaemia/reperfusion. A series of investigations has been performed to clarify which populations of nerve fibres are more susceptible to damage, and to elucidate the exact mechanism of nerve injury. An *in vivo* rabbit hind limb model, subjected to 16 h of cold immersion (1–2°C), provided the basis of this study.

Nerve specimens were examined by semi-thin sectioning for myelin fibre counts, by electron microscopy to assess the unmyelinated fibre population, and fine nerve terminals in plantar skin were assessed immunohistochemically. The results showed that large myelinated fibres were preferentially damaged, while small myelinated and unmyelinated fibres were relatively spared. Nerve damage was found to start proximally and extend distally with time.

Serial temperature measurements identified a warm–cold interface in the upper tibial region of immersed limbs. As this was the initial site of injury, this suggested that a dynamic balance exists in the cold immersed limb between the protective effects of cooling and the damaging effects of ischaemia. The non-invasive technique of near infrared spectroscopy was used to measure changes in tissue oxygen supply and utilisation and blood volume. The findings supported the hypothesis that an interface is created at the site of initial nerve damage in the upper tibia, where cyclical ischaemia–reperfusion injury occurs.

Non-freezing cold injury (NFCI) is the term applied to the condition which develops in humans when tissues are cooled for prolonged periods, in a wet environment, at temperatures just above their freezing point (−0.55 to 15°C). As a consequence, tissue damage occurs which can involve both the hands and feet, although the lower extremity is the more often affected. The greatest number of cases have occurred during times of war, in military personnel forced to remain outdoors in harsh environmental conditions *(1)*. During the early years of World War I (1914–1918), infantry bogged down in the static trench warfare characteristic of the Western Front (Fig. 1), developed the classical 'trench foot', which is one clinical manifestation of NFCI *(2)*. A conservative estimate suggests that 115361 men were incapacitated. Until this time NFCI was frequently confused with frostbite, a freezing cold injury, and it was only then that a positive distinction was made between the two. More recently, 70 out of 666 British casualties evacuated to hospital ships during the Falklands Conflict were affected *(3)*. Isolated civilian cases have been reported intermittently in peacetime after accidental cold exposure in outdoor sportsmen, the homeless, and those who are unable to protect themselves because of impaired consciousness or mental disability *(4,5)*. The modern

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Figure 1. Officers of the 12th Royal Irish Rifles wading through mud of a fallen-in communication trench, the result of a thaw after weeks of snow and frost. Western Front, Essigny, France, 7 February 1918. This illustrates the typical conditions giving rise to trench foot. (Reproduced with kind permission of the Imperial War Museum, Q10681.)

day peacetime equivalent of trench foot is probably unrecognised and underdiagnosed.

The clinical features of NFCI, many of which are secondary to peripheral nerve damage, have been comprehensively described *(6,7)*. The severity of injury and symptoms have been shown to be proportional to the duration of cold exposure *(8–10)*. In the acute stage, sensory modalities of pain and temperature are most severely affected, though all modalities of nerve function are involved to some extent *(7,11)*. Subsequently, motor weakness and muscle wasting can develop while, in severe cases, tissue necrosis and gangrene can occur. Features of autonomic disturbance, characterised by hyperhidrosis, cold sensitivity and inappropriate vascular temperature responses, are the typical chronic sequelae *(12)*.

The high morbidity and the military and economic consequences of this condition have periodically provoked clinical and experimental research. Investigators have endeavoured to increase their understanding of NFCI and its pathophysiology, so that possible treatment or prophylaxis options could be identified. Early clinical investigations were flawed as they failed to distinguish between freezing and non-freezing injury, while experimental studies were limited by relatively poor investigative techniques *(8,13)*. However, substantial nerve damage was produced when unanaesthetised animals were exposed to cold and wet for long periods *(13,14)*. More recently, modern techniques have been available, but anaesthetic restraints have meant that only minor nerve injuries have been produced *(15,16)*. Isolated nerve cooling models have been used in several animal species *(16–18)* in an attempt to produce more severe injury in anaesthetised animals. However, these experimental procedures did not re-create the true physiological conditions in which NFCI develops. A rabbit whole hind limb model has been used by previous researchers *(8,15,16)* and has the advantage of being a more exact representation of the conditions producing nerve damage in man.

In spite of the research interest, opinions remain divided regarding the type of nerve fibres most susceptible to damage and no consensus exists regarding the exact mechanism of injury. To clarify which populations of nerve fibres are most affected, we have performed a series of studies into the morphological changes occurring in nerve trunks and terminals and assessed their progression with time. By increasing the duration and frequency we have produced a more severe nerve injury than has previously been achieved experimentally in anaesthetised subjects, which allowed some clarification on the mechanism of nerve injury.

Experimental model

The rabbit hind limb model of NFCI formed the basis of our investigations and was a modification of the technique used by Kennett and Gilliatt *(16)*. In a typical study both denuded hind limbs of an anaesthetised and ventilated New Zealand White rabbit (3.5-4.0 kg) were suspended through an aperture in the platform on which the rabbit rested. The right hind limb (test limb) was immersed in a water bath at 1–2°C, to 2 cm above the knee; while the left hind limb, acting as an unimmersed control, was suspended outside the bath and carefully insulated from it. Systemic cooling was minimised using a body blanket, heated pad and radiant lamp. Core temperature, heart rate, FiO_2 (35–40%) and $EtCO_2$ were monitored continuously; arterial oxygen saturation was maintained at 100% and adequate hydration ensured. A continuous intravenous infusion of ketamine (60 mg/ml) and xylazine (8 mg/ml) allowed light surgical anaesthesia to be maintained for up to 22 h. All experimental procedures were conducted in compliance with Home Office regulations and requirements.

Susceptibility of different subpopulations of nerve fibres to damage

No consensus agreement exists from previous investigations as to the vulnerability of nerve fibres to damage in NFCI, with different authors reaching different conclusions. There is evidence against a relationship between nerve fibre size or type and susceptibility to cold *(10,13)*. However, clinical as well as experimental evidence *(14)* would suggest that small myelinated and unmyelinated Aδ and C fibres are most susceptible to damage; indeed the nerve modalities conveyed via these fibres appear most severely affected, as they are in other conditions with similar clinical features *(19)*. Other authors have suggested that the converse is true and cold affects predominantly large myelinated fibres *(17,18,20)* and the most recent experimental evidence has provided some evidence for this *(16)*.

Myelinated fibres

To investigate damage to myelinated fibres, samples of nerve were harvested from immersed and non-immersed limbs at 3 days ($n = 10$) and 7 days ($n = 12$) after a 16 h cold immersion study. Further samples were obtained 7 days ($n = 10$) after the second of two 16 h periods of cold immersion, separated by 1 week, for a total cold exposure of 32 h. Computerised image analysis was performed on transverse semi-thin sections (0.7 μm) cut from resin-embedded samples of upper tibial, mid-tibial and medial plantar nerves and stained for myelin with thionine and acridine orange. Undamaged fibres were counted and their diameters calculated. In order to assess whether damage varied with fibre size, the axons were divided into four groups (quartiles) according to diameter (<5 μm, 5.0–6.99 μm, 7.0–8.99 μm, >9 μm) and statistical comparisons were made using analysis of variance (ANOVA).

At 3 days after 16 h immersion, only a few sporadic larger diameter fibres showed evident damage, with axoplasmic shrinkage and myelin disruption. The immersed limb had significantly fewer undamaged fibres ($P < 0.001$) when all sizes were considered. At 7 days after 16 h immersion, widespread pan-fascicular demyelination, axonal degeneration and endoneural oedema were evident, with the greatest reduction in the largest fibres (>9 μm, $P < 0.001$). There was some non-significant reduction in numbers for smallest fibres (<5 m diameter) in immersed nerves (Fig. 2).

Damage varied with the position of the nerve in the limb (Table I). At 3 days after single immersion, significant damage was seen proximally at the knee (just below the surface of the water) and mid-tibial positions, while at 7 days the changes became significantly worse on descending the limb (for fibres >9 μm, $P = 0.01$ and of the fibres irrespective of size, $P = 0.03$). The differences between test and control nerves were significant for all positions ($P = 0.01$)

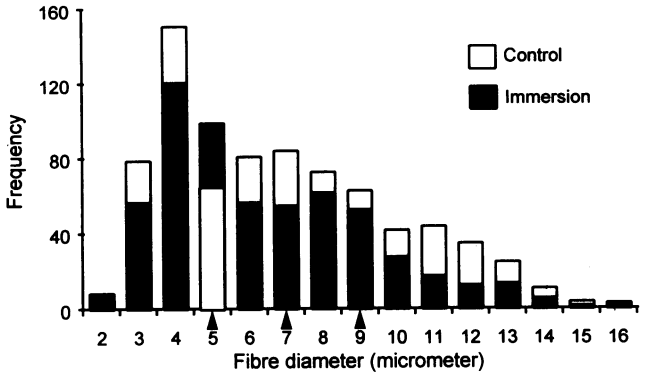

Figure 2. The typical bimodal distribution of undamaged myelinated fibres arranged according to diameter at the mid-tibial position in immersed (test) and unimmersed (control) limbs 7 days after a single 16 h study. This illustrates a reduction in fibres of all diameters in immersed limbs compared with controls and that the proportion of damaged fibres appears greater for larger than for smaller diameter fibres.

Table I. Mean number of undamaged myelinated fibres per image for immersed and non-immersed limbs according to position and time

Position	*Mean number of fibres*		*Difference in means*
	Non-immersed limb	*Immersed limb*	
16 h day 3			
Knee	271.8 ± 6.59	238.1 ± 6.59	33.7
Mid-tibia	259.5 ± 6.59	219.5 ± 6.59	40
Foot	230.6 ± 6.59	217.9 ± 6.59	12.7
16 h day 7			
Knee	259.4 ± 8.97	236.9 ± 8.97	22.5
Mid-tibia	243.4 ± 8.97	184.1 ± 8.97	59.3
Foot	239.8 ± 8.97	166.5 ± 8.97	73.3

All values expressed as mean ± sem

At 7 days after a double immersion, the qualitative morphological features of damage were essentially the same as those after a single immersion (Fig. 3). Quantitatively, there were significantly fewer undamaged fibres of all sizes in immersed limbs ($P < 0.001$), with the greatest reduction in the largest fibres (>9 μm, $P = 0.01$).

The changes in nerve damage identified with time (greater at 7 days than at 3 days) support the concept that nerve damage commences proximally, possibly at a warm–cold interface, at a site in the upper tibial region of the immersed limb, in agreement with the work of Kennett and Gilliatt *(16)*. However, the damage produced after double immersion (16 h + 16 h) was not significantly greater than that produced by single immersion (16 h).

Unmyelinated fibres

The assessment of damage to unmyelinated nerves is notoriously difficult owing to their small size (0.4–2.4 μm diameter) and rapid rates of recovery after injury. Relying solely on fibre counts to indicate damage can be misleading and accessory criteria such as increases in the number of the smallest fibres and the presence of Schwann cell bands, small Schwann cell projections or empty Schwann cell units may be useful indicators of injury *(21)*. In our studies, ultra-thin sections were prepared for electron microscopy from mid-tibial nerve samples harvested at 7 days after single and double cold immersion. Total and damaged unmyelinated fibre counts were made for each section along six random non-adjacent EM grid edges (×15 000) magnification). To allow an assessment of unmyelinated axon diameters by direct measurement, photographs were taken of three randomly selected low-power fields (×4000).

No qualitative morphological damage to unmyelinated fibres was obvious for either immersion duration. There were no significant reductions in the mean total number of undamaged fibres and no significant increases in the number of unmyelinated fibres containing swollen or disrupted mitochondria in immersed limbs relative to controls. The only significant feature suggestive of

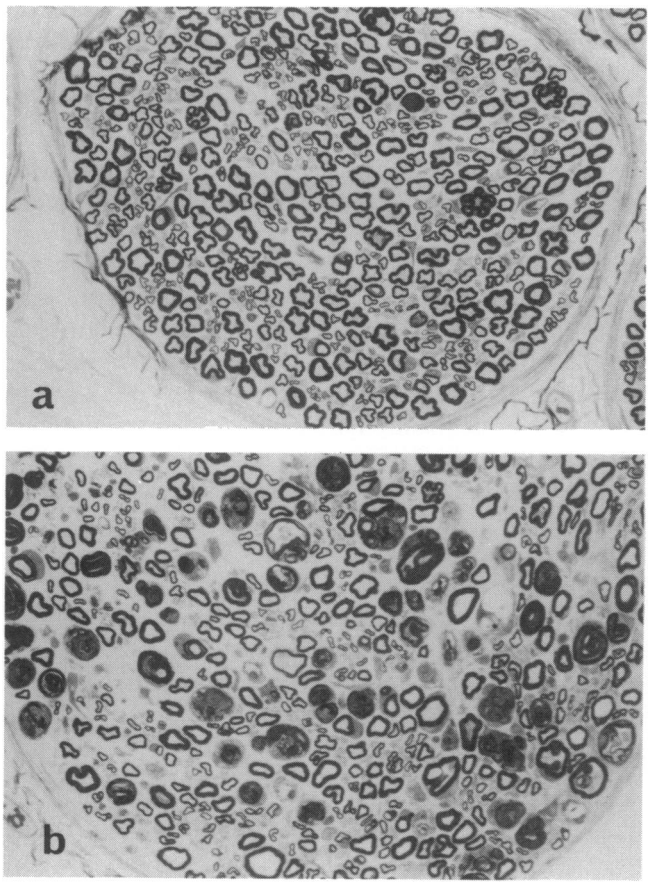

Figure 3. Transverse sections of (*a*) unimmersed and (*b*) immersed nerve at the mid-tibial position of a single subject (16 h study, day 7). Notice the darkly stained myelin sheaths of undamaged small, medium and large diameter fibres in the control nerve. Note the widespread damage to large myelinated fibres with disruption of the myelin sheath and loss of axoplasm and the relatively normal appearance of the smaller fibres in the injured nerve. (Thionine and acradine orange, mag×233.)

unmyelinated fibre damage was a reduction in the mean axon diameter in immersed limbs compared with controls after double immersion only (0.89 ± 0.02 and 0.99 ± 0.02 μm respectively, $P = 0.02$). This would suggest that unmyelinated nerves become damaged only in more extreme injuries.

Terminal nerve fibres

Fine terminal nerve fibres in the dermis of plantar skin were also studied in full-thickness plantar skin biopsies harvested at 7 days after 16 h and 32 h cold immersion. In these the nerves were immunostained with antisera to protein gene product 9.5 (PGP), a pan-neuronal structural marker; calcitonin gene related peptide (CGRP), a functional marker for sensory nerves; S100 a Schwann cell marker; and CD31 a vascular endothelial marker. Quantitative assessment of subepidermis nerve fibres was carried out using computerised image analysis and expressed as percentage staining per field.

In the single immersion group, significant decreases were observed in immersed limbs compared with controls, for fibres immunostained for PGP 9.5 ($0.98 \pm 0.33\%$ and $2.0 \pm 0.33\%$, respectively, $P = 0.01$), CGRP (0.94 ± 0.16 and 1.41 ± 0.12, $P = 0.008$) and S100 (1.09 ± 0.18 and 2.04 ± 0.12, $P < 0.001$). Similar significant changes were induced by double immersion, with no statistical difference from results of single immersion. Staining to CD31 did not identify significant changes to endothelial cells in either group.

Mechanism of nerve fibre injury

It is likely that the aetiology is NFCI is complex and multifactorial, while the exact mechanism of nerve fibre injury remains obscure. Early clinical studies implicated factors such as immobility, malnutrition, fatigue, stress and constricting footwear to be partially responsible for producing the syndrome *(6)*. Large and Heinbecker *(13)*, suggested that ischaemia was the mechanism contributing to structural nerve damage. Later studies measuring tissue Po_2 in NFCI models *(8,22)* have demonstrated that hypoxia and local tissue anoxia are present subcutaneously and intramuscularly during cooling. Recent evidence using a whole rabbit hind limb model *(16)*, suggests that axonal injury is the direct result of cold injury, consistent with previous studies *(4,17)*. However, there has been no demonstration of histological muscle damage *(16)*, which is against an ischaemic aetiology, as in these conditions muscle is more susceptible to damage than nerve *(23)*.

Ultrastructural changes in the subcutaneous microcirculation of hamsters after repeated NFCI, closely resembled the changes expected after an ischaemia–reperfusion injury *(24)*. The formation of oxygen derived free radicals, which is implicated in ischaemia–reperfusion insults, have been demonstrated during re-warming of a rabbit hind limb, cooled to 0°C for 20 min *(16)*. Clinical evidence adds further support to an ischaemia–reperfusion mechanism, as recurrent exposure to repeated cooling and re-warming cycles, produce progressively more severe NFCI symptoms *(8,9)*.

In a rabbit model, it has been demonstrated that the initial site of injury is confined to a small region of the upper tibial nerve in the cold immersed limb, at a level below the water line *(16)*. This proposed the existence of a balance between the injurious effects of ischaemia and the protective effects of cooling. At the site of injury, our hypothesis is that this balance is disrupted and the protective effects lost, with local fluctuations giving rise to a repetitive ischaemia–reperfusion injury. We also hypothesise that a warm–cold interface exists at the site of initial nerve injury. To test these hypotheses, serially placed thermocouples were employed to measure temperatures within the experimental limbs and the non-invasive technique of near infrared spectroscopy (NIRS) was used to identify evidence of a possible ischaemia–reperfusion injury *(25)*.

Serial temperature measurements

Thermocouples contained within 21G hypodermic needles were inserted percutaneously so as to lie adjacent to the tibial nerve and secured at five sites along both immersed ($n=6$) and control ($n=6$) limbs, between the foot and the knee, to allow continuous temperature recordings for the full duration of the experiment.

During immersion, the rabbits' core temperature decreased with the duration of cold exposure (38.1 ± 0.2°C pre-immersion; 31.9 ± 0.6°C after 16 h (mean ± sem). On removal of the limbs from the cold wet environment, body temperatures rose to 34.1 ± 0.5°C after 3 h of re-warming. A natural temperature gradient was found to exist in all limbs before any experimental procedure. Baseline temperatures at the knee were consistently higher than those in the foot by an average 8.6 ± 0.8°C (mean ± sem). In unimmersed control limbs, temperatures fell by 6–7°C over the whole 16 h period, which could be related to systemic hypothermia associated with the cooling of the immersed limb. The largest temperature difference between any two adjacent probes was found between the two most distal measurement points and was of the order of 3°C. At no time did tissue temperatures fall below 16°C, which is consistent with the absence of morphological nerve damage *(1)*. In the most distal three positions of the immersed limb, temperatures decreased to that of the water bath (1–2°C) within 3–4 h and remained constant until the end of immersion. Warmer temperature in the upper tibia implies that a positive temperature gradient ascended the immersed limb, as well as in the unimmersed limbs (Fig. 4). However, the largest gradient was found to exist between the two most proximal probes with a large temperature drop occurring in the upper tibia (mean decrease 7.0 ± 1.6°C), identifying this as the site of a warm–cold interface in the immersed limb. Large temperature increases were seen during re-warming at all probe positions. In one study an increase in temperature was associated with limb movement during immersion. The maximum increase (from 7.7°C to 17.4°C) was recorded at the knee position.

Serial temperature measurements in tissue adjacent to the tibial nerve have shown marked cooling at all sites in the immersed limb down to temperatures which have previously been associated with the development of NFCI *(1)*. The positive temperature gradients found ascending the limb, particularly evident in the immersed limbs, in the upper tibial region, supports the hypothesis of a warm–cold interface at the site of initial nerve damage

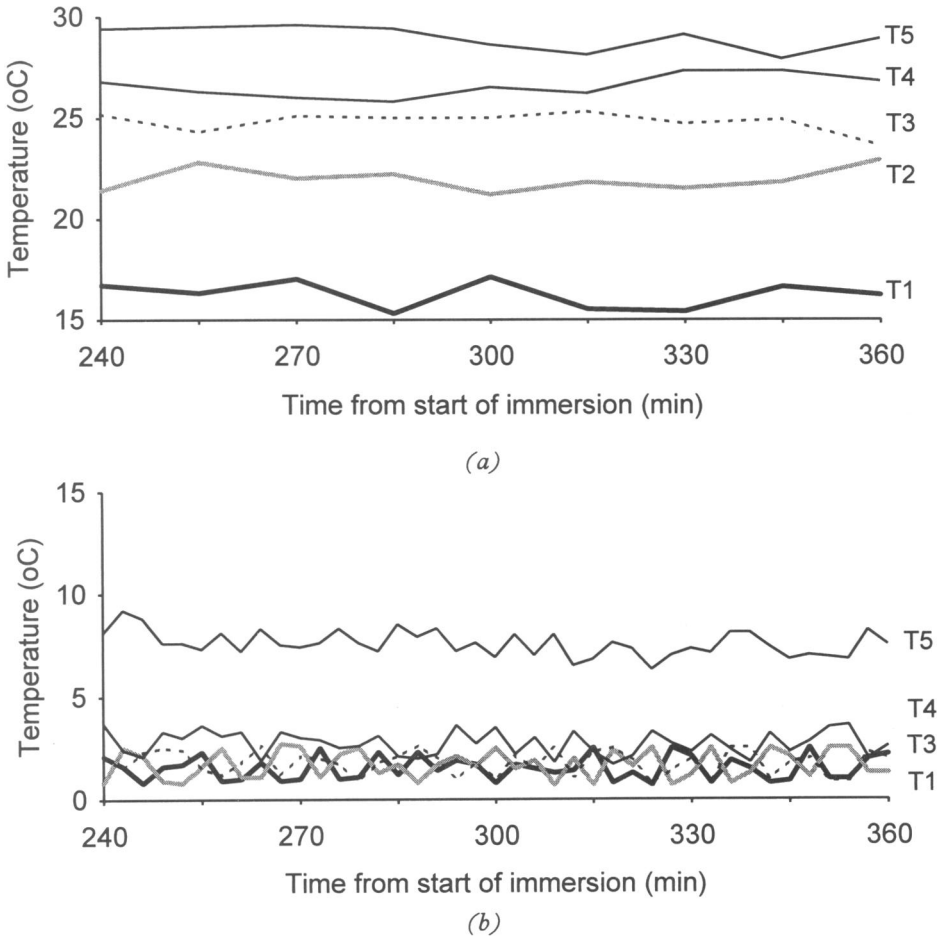

Figure 4. Typical temperature changes occurring in *(a)* an unimmersed and *(b)* a cold immersed rabbit hind limb during a representative 2 h period of cold immersion, from individual experiments, illustrating the temperature gradients in each. (T1 = foot position, T3 = Mid-tibial position, T5 = knee position.)

(16). During re-warming the immediate and rapid increase in tissue temperature corresponds to the reperfusion effect, as the return of warm oxygenated blood to a previously shut down vascular bed is the main heat source, with radiant heat from the surrounding environment responsible for only minor re-warming. In previous experimental NFCI, transient rises in muscle temperature have been observed in association with motion of the chilled limb *(8,9)*. The relatively large increase in tissue temperature associated with movement of the limb during cooling is again indicative of a reperfusion insult. In man in conditions producing NFCI, it is likely that this type of reperfusion associated with movement is occurring continuously.

Evidence to support the role of ischaemia–reperfusion injury

NIRS allows us to monitor non-invasively the continuous real-time changes of concentration in oxy- and deoxy-haemoglobin (HbO_2 and Hb) and oxidised cytochrome aa3; as demonstrated in the rabbit hind limb in different experimental conditions including arterial, venous and total vascular occlusion *(26)*. Hence, information is provided on the changes in tissue oxygen supply and intracellular oxygen availability and utilisation. The summation of Hb and HbO_2, gives changes in total haemoglobin (Hb_T), reflecting changes in blood volume and providing an indication of blood flow and perfusion, while the oxygenation index ($Hb_D = HbO_2 - Hb$) reflects net changes in haemoglobin oxygenation independent of changes in blood volume.

In this study the NIRS probes were fixed in three positions (knee, mid-tibial or mid-metatarsal level) and changes in NIRS parameters were measured continuously in test and control limbs during 16 h of immersion and 3 h of re-warming. Changes in suspended limbs were also monitored in normothermic conditions at the mid-tibial position for 16 h. Although various changes were recorded at all positions in response to cold immersion, blood pressure remained constant for the duration of the experiments, indicating that this parameter had no confounding effect on the monitored NIRS changes.

Knee position

The main features were an increase in total haemoglobin in both immersed and control limbs (9.57 ± 7.40 and 18.32 ± 3.48 μM respectively, $n = 10$, mean ± sem) and a minor decrease in the oxygenation index, progressing with the duration of immersion. The Hb_D change in the immersed limbs (-9.90 ± 5.69 μM) appeared greater than in controls (-0.92 ± 3.50 μM). However, these differences were not significant for either Hb_D or Hb_T. Deoxygenated Hb levels remained relatively stable, while the main changes were a consequence of HbO_2 increases, suggesting that the main features are due to an arterial effect. Immobility and dependency in both immersed and unimmersed limbs lead to reduced oxygen demands, vascular stasis and pooling which is the most likely explanation of these changes.

Mid-metatarsal (foot) position

In immersed limbs ($n = 5$), large decreases were seen in both blood volume and oxygenation index compared with unimmersed limbs over the 16 h period with a stable Hb (Hb_T, -32.23 ± 4.66 and $+18.0 \pm 3.19$ μM, $n = 5$; and Hb_D, -32.33 ± 2.12 μM and $+17.81 \pm 3.0$ μM, respectively, $n = 5$). These changes are consistent with complete vascular shutdown owing to vasoconstriction and a cut-off of oxygenated blood. When compared with controls, the large decreases in both parameters were highly significant ($P < 0.001$). Although these are not absolute measures of tissue oxygenation, these changes are highly indicative of an ischaemic environment in which nerve damage can be produced.

Mid-tibial position

In immersed limbs ($n = 12$) three patterns of change were identified, all significantly different from control values ($P \leqslant 0.008$). Oscillations in Hb_T and Hb_D ($n = 3$) were measured with a periodicity of approximately 4 h and amplitudes of Hb_T 20.7 ± 2.76 μM and Hb_D 14.79 ± 1.95 μM. These changes are representative of cyclical oxygenation and deoxygenation (Fig. 5), direct evidence of cyclical ischaemia reperfusion occurring at this site of the warm–cold interface. Concentrations of Hb were stable. Other patterns of change were as for the immersed foot ($n = 2$) and as for the immersed knee ($n = 7$). This variable pattern of changes seen between animals might indicate that the exact site of initial injury varied slightly between individuals.

For all unimmersed limbs, re-warming produced little change in any of the NIRS parameters, while limbs which had been cold immersed were associated with a reactive increase in oxygenated and total haemoglobin (blood volume) maximal after 1 h. The NIRS changes recorded during re-warming at all positions within immersed limbs were consistent with reactive hyperaemia and a reperfusion insult.

The overall effects of prolonged anaesthesia at room temperature were similar to those occurring in the control limb of animals undergoing cold immersion, in that the main features were an increase in total haemoglobin ($+17.81 \pm 2.84$ μM) and a minor decrease in oxygenation index (-7.55 ± 4.29 μM) with time. However, in these cases the changes were a consequence of increases in Hb, with stable HbO_2, consistent with the suggestions that in the unimmersed limb the changes are a result of immobility and venous stasis, while the arterial delivery of oxygenated haemoglobin remains unimpaired.

Nerve damage was more severe distally than proximally, 7 days after injury. Thus the morphological changes may be the consequence of a sequence of events initiated by ischaemia–reperfusion at the upper tibial level. Distal to this point, the nerve then undergoes Wallerian degeneration in the ensuing 7–10 days. In

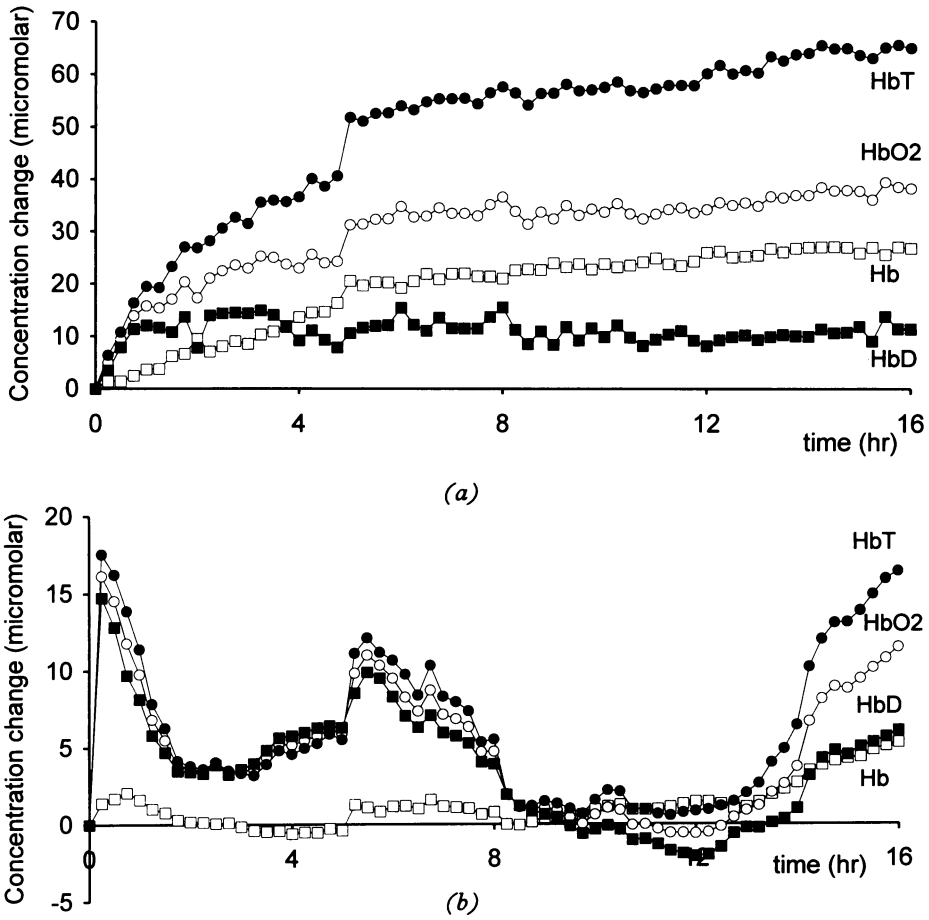

Figure 5. Typical concentration changes occurring in NIRS parameters monitored at the mid-tibial level in *(a)* an unimmersed and *(b)* a cold immersed limb during 16 h of cooling, from a single experiment in which oscillations were a feature.

addition, at re-warming, a further reperfusion insult will probably lead to added nerve damage throughout the immersed limb. The damage will be more extreme in the foot as prolonged ischaemia is more likely to be associated with a significant insult on reperfusion *(27)*.

Conclusions

In summary, this combined approach has clearly demonstrated that there is nerve damage as a consequence of NFCI in the rabbit model. A reduction in the number of myelinated fibres of all sizes was seen, with the greatest reduction in large diameter fibres, a feature consistent with ischaemic neuropathy. Initially, nerve damage appeared proximally, though the severity of injury spread and increased distally with time. Unmyelinated fibres showed only minor damage. Consistent with the injury seen in the nerve trunk, significant changes were found in the fine nerve terminals in the skin. The possible role of an ischaemic mechanism and ischaemia–reperfusion in the aetiology of NFCI has been investigated in a rabbit hind limb model using *in vivo* temperature monitoring and the non-invasive technique of NIRS. The resulting evidence has suggested that both of these mechanisms may contribute to the nerve injury.

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