Brain (1997), **120,** 631–646

The pathogenesis of non-freezing cold nerve injury
Observations in the rat

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Summary
Non-freezing cold nerve injury is uncommon in civilian practice, but may reach epidemic proportions in war zones. Studied since the time of Hippocrates, its aetiology has remained elusive. We sought to replicate experimentally, a peripheral nerve cold temperature gradient, since this has been emphasized in clinical descriptions. Our observations, in the rat, of the vasa nervorum show that cold-induced intravascular aggregation is followed by a 'no-reflow' phenomenon which culminates in endothelial damage and delayed thrombotic occlusion.

Key words: sciatic nerve; cold injury; animal model; nerve blood flow; ischaemia

Abbreviations: MAP = mean arterial blood pressure; NBF = nerve blood flow

Introduction
The effects of cold on peripheral nerves have been studied since the time of Hippocrates. However, it has been during military operations that cold-induced neuropathies have commanded most attention. The devastating events of cold nerve injury were well documented in Napoleon's Russian Campaign, the Crimean War, the British Mission to Tibet, the First and Second World Wars, the Korean conflict and the Falklands War. In some campaigns, instances of cold neuropathy were so numerous that military operations were seriously jeopardized (Smith *et al*., 1915*b*; Greene, 1941; Ungley and Blackwood, 1942; Blackwood and Russell, 1943; Blackwood, 1944*a*, *b*; Lewis and Moen, 1952; Dunning, 1964; Buckels *et al*., 1967; Payne, 1984).

Although less frequent in peacetime, cold-induced neuropathy is a well-known risk in mountaineering (Carter *et al*., 1988) and an occupational hazard for fishermen (Semsarian, 1994). Iatrogenically, such lesions may occur as a complication of cryotherapy (Drez *et al*., 1981; Bassett *et al*., 1992) and following open heart surgery (Chandler *et al*., 1984; Efthimiou *et al*., 1991).

Early investigators suggested that cold-induced nerve lesions might be primarily ischaemic in origin (Large and Heinbecker, 1944; Denny-Brown *et al*., 1945). It was reasoned that stagnation of blood might lead to thrombus formation (Friedman, 1945). However, this concept was not accepted by the majority of subsequent investigators because of a lack of convincing experimental support (Lewis and Moen, 1952; Sayen *et al*., 1960; Nukada *et al*., 1981; Kennett and Gilliatt, 1991*a*, *b*). We, in agreement with other workers, found in pilot studies that cooling sciatic nerve for 3 h, to constant temperatures as low as 2°C, did not result in vascular occlusions or conspicuous morphological change in cooled vasa nervorum. Controversy has therefore existed regarding the pathogenesis of peripheral nerve cold injury.

A dominant theme in many early clinical descriptions of cold-induced neuropathy was that it often developed during periods in which temperature varied (Smith *et al*., 1915*a*). In this series of experiments of cold-induced neuropathy, we have therefore sought to replicate such temperature variation. Our principal aim was to re-explore the role of ischaemia in non-freezing cold nerve injury.

Material and methods
Animals
This study was approved by the Committee on Ethics in the Care and Use of Laboratory Animals, University of Otago, Dunedin, New Zealand. Eighty-eight adult, male Wistar rats, weighing 300–350 g, were housed in plastic cages and water and purina chow made available, *ad libitum*. Rats were randomly divided into six experimental groups (10 animals

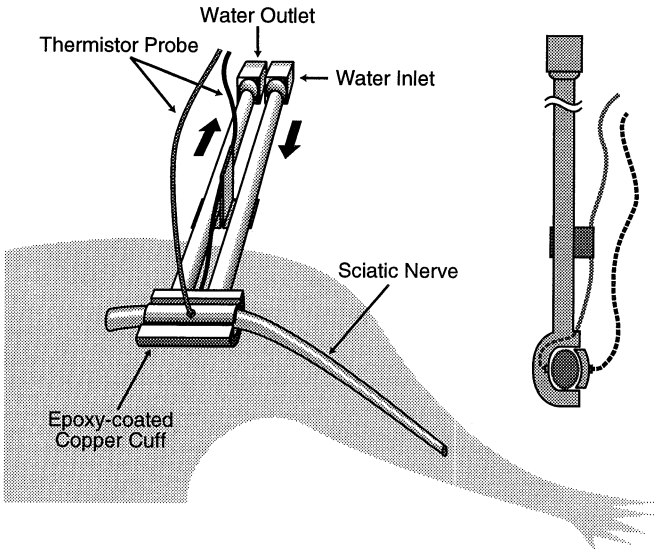


Fig. 1 Schematic drawing of the experimental design. A 10 mm copper cuff, through which either cool or warm water could be circulated, was placed under the sciatic nerve. The nerve was first cooled through gradients of 1–5°C for 3 h and the nerve temperature then rapidly restored to 37°C.

per group), and control groups, for nerve blood flow (NBF) measurements (18 animals) and for mean systemic arterial blood pressure (MAP) observations (10 animals).

Anaesthesia was induced by intraperitoneal xylazine (10 mg/kg) and ketamine (90 mg/kg). These drugs were continued at half this dosage every 20–30 min, as necessary.

Once the animal was anaesthetized, the right sciatic nerve was exposed by a lateral incision over the proximal femur at midthigh level. About 15 mm of nerve was mobilized proximal to its division into posterior tibial and common peroneal branches. Care was taken to avoid injuring epineurial vessels.

Apparatus

For local cooling of the sciatic nerve, we used a small cuff made from copper tubing, shaped into a hollow semicircle and coated with epoxy resin (Fig. 1). A distance of 10 mm separated water inlet and outlet tubes. Nerve temperature was monitored by a thermistor incorporated into the base of the cuff. A second thermistor rested lightly on the surface of the nerve, opposite the cuff. Once the cuff was correctly positioned, immediately distal to the origin of the inferior gluteal nerve, the temperature difference between the two thermistors did not exceed 0.5°C.

Nerve cooling and rewarming

Paraffin (liquid paraffin BP) was applied to the nerve, and infiltrated between the cuff and the underlying muscle tissue. Focal cooling of the sciatic nerve at a mid-thigh level was achieved by a water/ethylene glycol mixture, circulated through the cuff with an electric circulator (Cole-Parmer Instrument Co., Chicago, IL, USA). The cooling sequence was 1°C 20 min, 2°C 10 min, 3°C 10 min, 4°C 10 min and 5°C 10 min. This cycle was repeated twice to achieve 3 h of cooling. An electrical blanket, placed beneath the rat, maintained a rectal temperature of 37–37.5°C. Immediately following nerve cooling the sciatic nerve was rewarmed to 37.5°C.

In control animals, the sciatic nerve was positioned on the cuff but water temperature maintained at 37.5°C for 3 h.

Nerve blood flow

Sciatic NBF was measured before, during and following cold induction, using a 0.5 mm diameter fiberoptic probe, connected to a laser Doppler flowmeter (Model ALF-21, Advance Co., Japan). The probe, placed vertically on the epineurium, was held in position by a magnetic micromanipulator, seated on an iron plate to minimize vibration. The probe both delivered incident laser illumination and collected reflected light from the same site. Care was taken in placing the probe to avoid epineurial microvessels, to ensure that flow measurements represented integrated capillary flow within the fascicle rather than surface epineurial flow. A clear ultrasonic transmission gel (Eko Gel, 39044 Egna Italy) was used as an interface between the nerve and probe to eliminate light scatter.

Prior to nerve cooling, a 10 min stable NBF baseline was first established, using a chart recorder (Rikadenki Kogyo Co., Tokyo, Japan). NBF measurements were then continuously monitored during the cooling period and for the ensuing hour of nerve rewarming. NBF data was collected at 10 min intervals for a duration of 1 min and readings for each group averaged at the same time points. Changes in NBF were expressed in flow units, which were then calculated to obtain a percentage of pre-cooling baseline values. At the completion of NBF measurements, silk sutures (6.0) were used to define the proximal and distal extent of the hypothermic nerve injury.

When re-examining NBF prior to sacrifice, the previously cooled segment of sciatic nerve, identified by suture markers, was carefully re-positioned on the cuff. No water was circulated through the cuff. The laser Doppler probe was placed over the centre of the cooled nerve, and NBF recorded for 1 h.

Control NBF measurements were obtained using the same cuff, with and without water circulating at 37°C.

Group 1 animals were sacrificed immediately after the completion of 1 h of nerve rewarming following the 3 h period of nerve cooling. The remaining experimental animals were sacrificed following a second NBF assessment, at 12 h (Group 2), 24 h (Group 3), 2 days (Group 4), 3 days (Group 5) or 5 days (Group 6).

Arterial pressure

Since NBF may be altered by variation in systemic blood pressure, MAP was monitored in 10 experiments. In these

assessments, the left common carotid artery was exposed and ligated rostrally. A polyethylene catheter (PE50), containing saline-sodium heparin (50 IU/ml) was then implanted into the proximal carotid artery. It was connected to a pressure transducer (Statham P23 AC, Hato Rey, Puerto Rico) at heart level, which led to a chart recorder (Ink Writing Oscillograph, Model 79 Wu 8P40, Grass Instrument Co., Quincy, Mass., USA). MAP and NBF measurements were continuously recorded for 3 h without nerve cooling (five rats), or with nerve cooling (five rats). These rats were then euthanized with an overdose of pentobarbital sodium.

Microcirculation

Epineurial sciatic nerve arterioles, venules and capillaries were observed and photographically recorded, with a dissecting microscope and camera (SZ-PT, Olympus, Japan). Six animals were examined in each group, before, during and after nerve cooling.

Morphology

At a predetermined time, most control and cooled sciatic nerves were fixed *in situ* for 30 min with 4% glutaraldehyde in 0.025 M cacodylate buffer (pH 7.4). In a minority of experiments, sciatic nerves were fixed by perfusing 150 ml of Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.025 M cacodylate buffer at pH 7.40) through the abdominal aorta.

Cooled sciatic nerve segments, together with proximal and distal nerve segments, were then fixed in 2% glutaraldehyde overnight, at room temperature. Nerve segments were then washed in 0.025 M cacodylate buffer for 30 min, post-fixed in 1% osmium tetroxide in 0.025 M cacodylate buffer for 2 h, dehydrated in ethanol for 50 min, immersed in propylene oxide for 20 min and embedded in Epon 812. Transverse and longitudinal semithin sections were cut, using an ultracut microtome (Reichert-Jung Ultracut, Austria) and stained with methylene blue or phenylenediamine for light microscopy. Thin sections, cut with a diamond knife, were stained with uranyl acetate followed by lead citrate and examined in a Philips 410 electron microscope.

Statistical analysis

All statistical calculations were carried out on an Apple Macintosh Computer using the Statview program. NBF statistics were performed using a paired Student's t test for comparison. Values were expressed as mean $\pm$ SD. Significance was assumed for $P < 0.05$.

Results

Nerve blood flow

A 3 h cold injury to sciatic nerve, with a sequential gradient between 1 and 5°C, resulted in a significant and irreversible

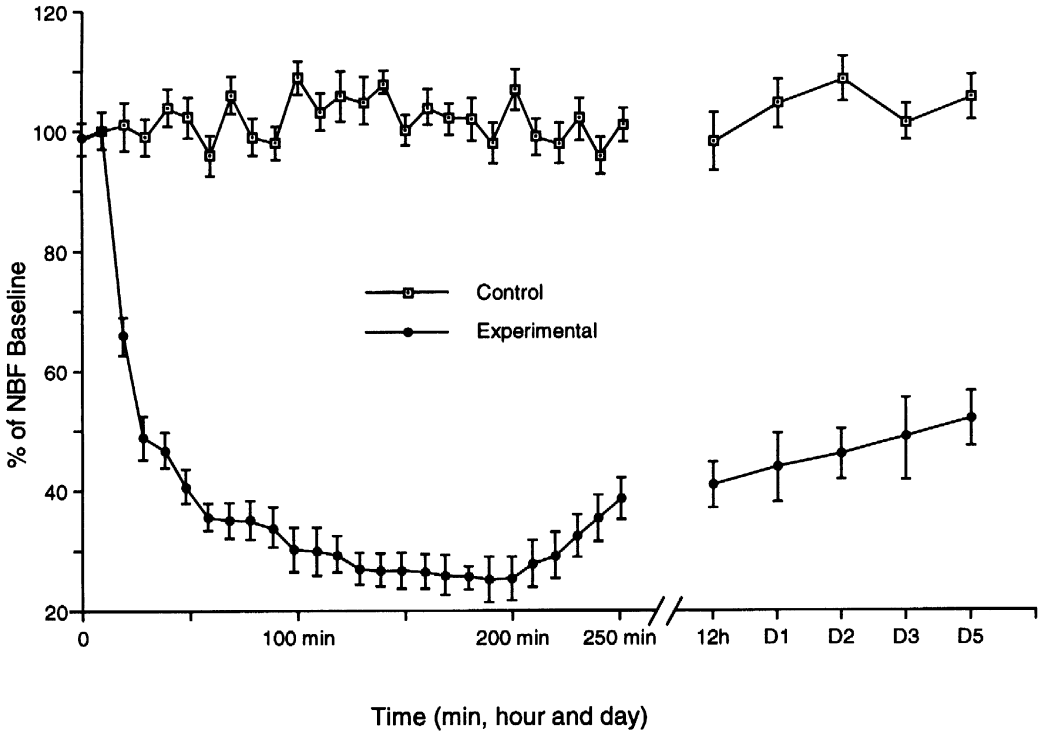


Fig. 2 Laser Doppler mean NBF measurements in control and experimental animals at 10 min intervals during nerve cooling and rewarming (up to 250 min) and at follow-up examination immediately prior to sacrifice (at various times up to 5 days). Note that the NBF falls steeply over 20 min and reaches its nadir (25% of baseline) 180 min after the onset of cooling. NBF remains significantly reduced up to 5 days after cold injury.

Table 1 *Representative sciatic NBF measurements before, during and after nerve cooling*

Sampling time point	NBF values*	
	Perfusion unit	% of baseline
10 min†	31.8 ± 1.4	
30 min	15.5 ± 1.2	48.7 ± 3.7
70 min	11.2 ± 1.0	35.2 ± 3.1
130 min	8.6 ± 0.8	27.0 ± 2.5
190 min‡	8.0 ± 1.6	25.1 ± 3.7
250 min	12.4 ± 1.1	39.0 ± 3.5
12 h	12.9 ± 1.2	40.6 ± 3.8
Day 1	13.8 ± 1.8	43.4 ± 5.7
Day 2	14.4 ± 1.4	45.3 ± 4.4
Day 3	15.3 ± 2.1	48.1 ± 6.6
Day 5	16.2 ± 1.5	50.9 ± 4.7

NBF = nerve blood flow (means ± SD) obtained by averaging all data at the same time points in 60 rats. *All NBF values were significantly different from the baseline at 10 min ($P < 0.05$). †Baseline value. ‡Commencement of nerve rewarming.

reduction in NBF (Table 1). Mean NBF in cooled sciatic nerves fell progressively to reach a nadir of 25% of baseline (Table 1). One hour after rewarming, sciatic NBF was still significantly reduced, at 39% of baseline. When cooled sciatic nerves were re-examined at intervals of 12 h to 5 days, mean NBF values remained significantly depressed (Table 1, Fig. 2). By contrast, NBF in control animals showed no significant alteration during the 3 h period of observation, or at re-examination prior to sacrifice. Likewise there was <10% variation in MAP during experiments and these slight variations in MAP had no influence on NBF.

Microcirculation

Normothermic observations

Under a dissecting microscope, sciatic nerve epineurial arterioles, metarterioles, capillaries and venules were observed prior to cooling (Figs 3A and 4A). Spontaneous irregular contractions in metarterioles, 'vasomotion', were noted. Blood flow through capillaries was seen to be rapid and non-laminar. In larger vessels, erythrocytes were often confined to an axial stream, leaving a peripheral plasma layer. Reversed flow was not seen except through venule–venule bridges. There was no stasis and no aggregation of erythrocytes.

Hypothermic observations

After 0–30 min. As the nerve temperature fell toward 1–5°C, flow rates in arterioles slowed considerably, but were largely unaffected in metarterioles. Flow remained rapid in capillaries although plasma skimming, where a thin layer of plasma separated erythrocytes from the intima, was prominent. Flow in venules varied between slow and normal with an infrequent laminar line.

After 30–60 min. Flow through arterioles, metarterioles, capillaries and venules became progressively slower, to reach a sluggish or static state with erythrocytes no longer discrete, but having a granular appearance (Fig. 4B). Many vessel walls now became irregular in outline and the diameter of capillaries was reduced.

After 60–120 min. Intravascular aggregation of red cells was now very prominent. Flow in most capillaries ceased entirely by the end of this period. Arterioles and metarterioles became constricted, and their vessel walls irregular. Flow in these vessels was often no longer laminar and vasomotion became inconspicuous. It was during this phase that venules became slightly dilated prior to becoming constricted. Their blood flow was no longer laminar and transient rouleaux formations were frequently noted (Fig. 4C). Indeed flow in some venules was restricted to the passage of an occasional isolated erythrocyte.

After 120–180 min. Constriction and irregularity of arterioles, metarterioles and venules became more marked (Fig. 3B). Flow, in these vessels, as judged by the movement of erythrocytes, was sluggish or static. Blood flow in venules became progressively slower, with prominent rouleaux formations and occlusive aggregates (Fig. 4D).

Observations during nerve rewarming

After 1 h of rewarming, a minority of arterioles and venules showed a slight increase in diameter towards normal (Fig. 3C). The majority of arterioles and venules remained irregularly constricted and where occluded arterioles or venules reopened they were poorly perfused (Figs 3C and 4E). Capillaries remained for the most part markedly constricted, and reflow was seen only in an occasional capillary. Occlusive aggregates persisted in 70% of cooled nerves.

Vascular pathology

Light microscopic examination of cold lesioned nerve after 1 h of rewarming revealed many empty epineurial and endoneurial vessels suggesting a 'no-reflow phenomenon' (Figs 5A and 6A). Twelve hours to 1 day after nerve cooling, red cells and platelets had begun to aggregate within the vasa nervorum (Figs 5C and 6B). Endothelial cells in cooled nerve were markedly swollen at 12 h (Fig. 7A). By 2–5 days after nerve cooling, many epineurial and endoneurial vessels were thrombosed (Figs 5D and E, 6C and D, and 7B).

Within 12 h of cooling, nerves showed severe oedema (Fig. 5B). It was first evident in the subperineurium and around endoneurial capillaries. Later it appeared more diffusely within the endoneurium and split myelin lamellae. It began to decrease with the onset of blood vessel thrombosis (Fig. 5E).

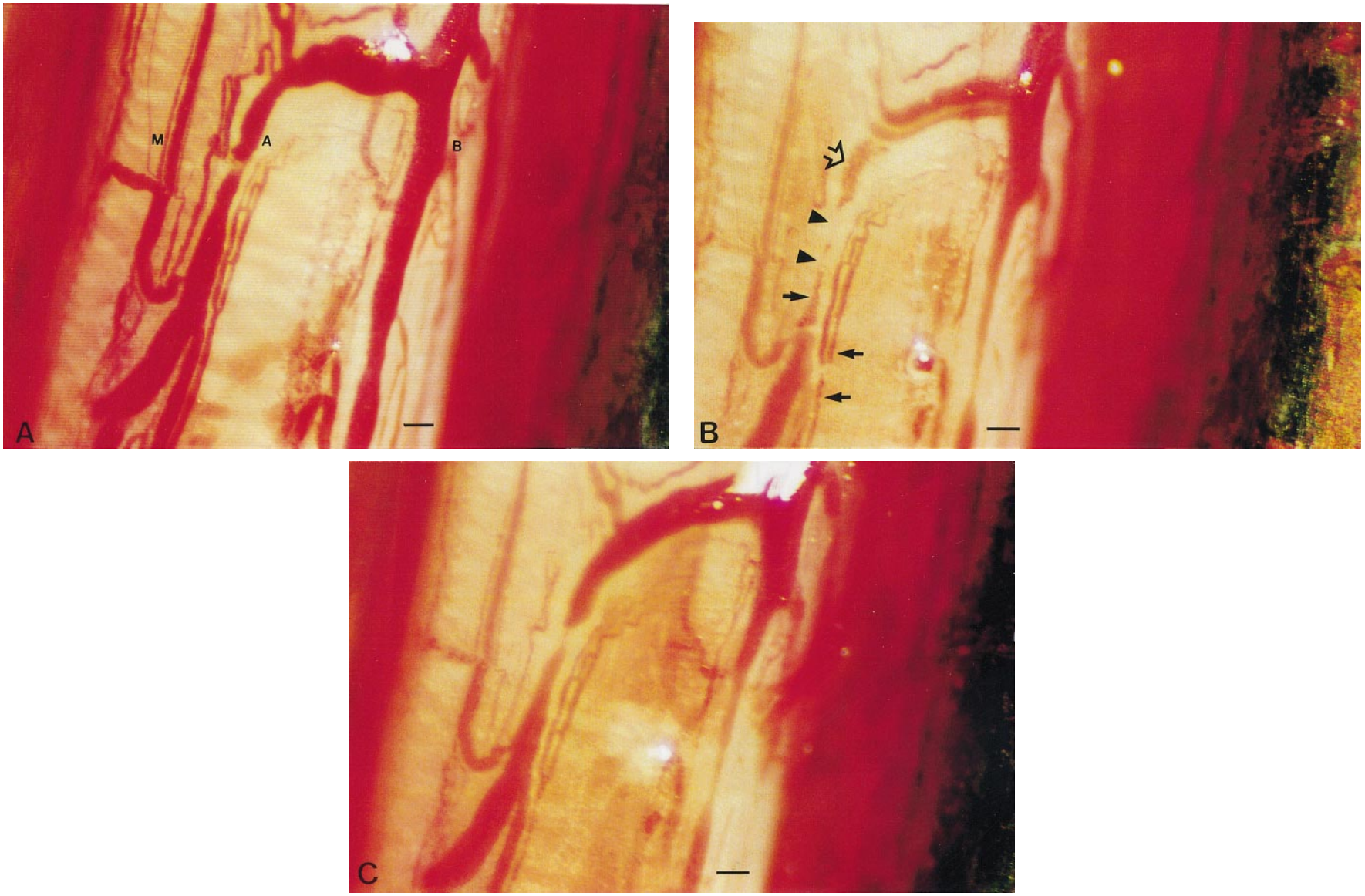
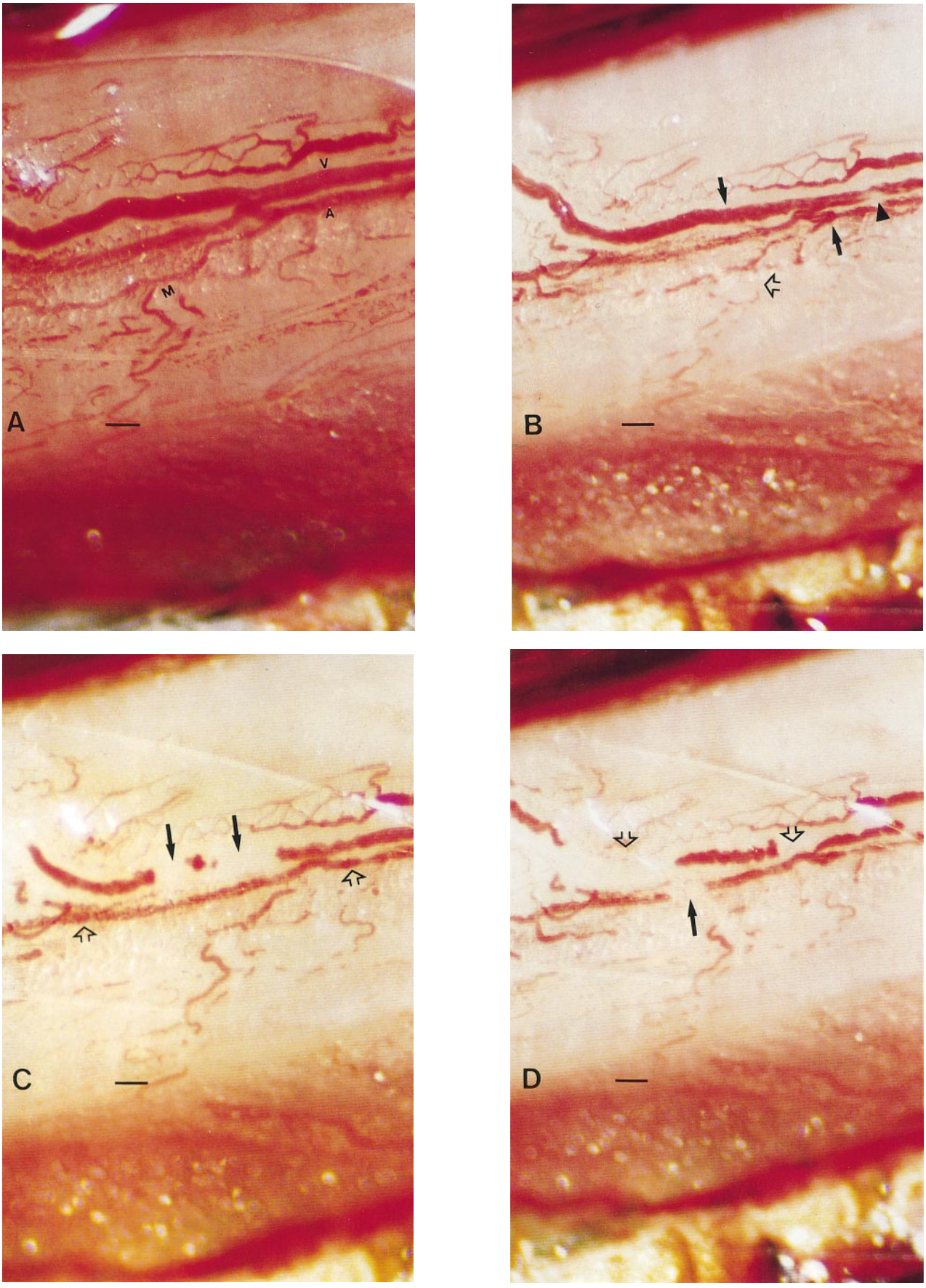


Fig. 3 Sciatic nerve epineurial microcirculation before, during and following nerve cooling (1–5°C). (**A**) During normothermia (37.5°C) an epineurial arteriole with its major branches (A and B) and metarterioles (M) were identified by vasomotion. (**B**) Marked constriction of the arteriolar branches and metarterioles after 3 h of cooling at 1–5°C. Note granular aggregation (arrows), rouleau formation (open arrow) and occlusive aggregates (arrow heads). (**C**) Arteriolar circulation after 1 h of rewarming (37.5°C). Note improved but still impaired blood flow in branch B and focally restricted reflow in branch A. Bars represent 100 μm.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Nerve fibre morphology

Early axonal degeneration, in the form of 'empty' or 'dark' axons, was seen 24 h after nerve cooling (Fig. 5C). By 2–5 days after cooling, when many endoneurial blood vessels were thrombosed, injured nerves showed severe pathological changes. Myelinated fibre pathology included dark swollen axons, inappropriately thin myelin and attenuated axons (Fig. 5D and F). On electron microscopic examination after nerve cooling, intra-myelinic oedema was noted at 12 h, and shrunken axons at 2 days (Fig. 8A and B). In contrast, unmyelinated fibres were largely spared following cold injury, and no pathological changes were seen in control sciatic nerve.

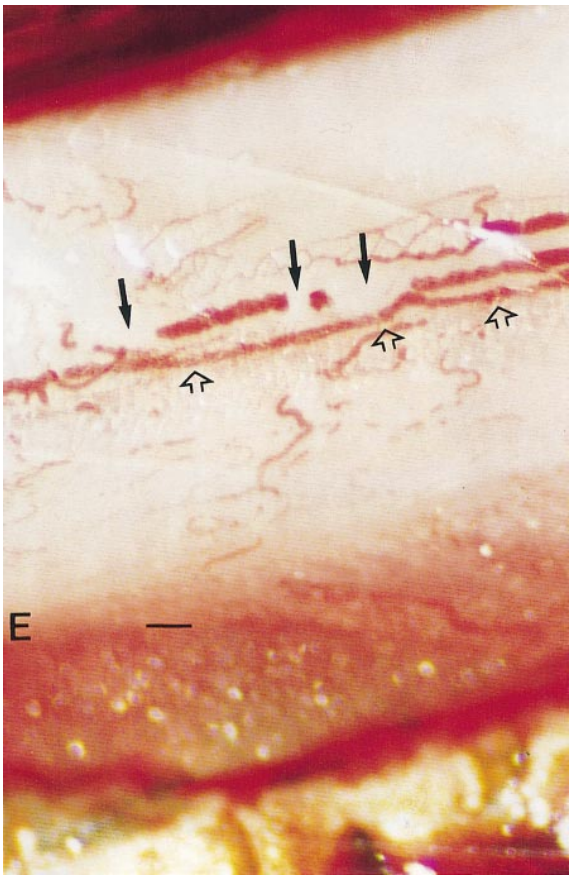

Fig. 4 Sciatic nerve epineurial microvessels before, during and following nerve cooling (1–5°C). (**A**) Normothermia. Arteriole (A), venule (V) and metarterioles (M) have a normal appearance. (**B**) One hour after the commencement of nerve cooling the diameter of both the arteriole and venule are reduced by ~40%. Under a dissecting microscope, erythrocytes present a granular appearance in both vessels (arrows). Note occlusive aggregations (open arrow) in metarteriole and the suggestion of leucocyte clumping in the venule (arrow head). (**C**) Two hours after nerve cooling segmental occlusive aggregates are seen in the venule (arrows). The arterioles contain prominent rouleaux (open arrows). (**D**) Three hours after nerve cooling there is stasis of flow in both vessels. An occlusive aggregate (arrow) is now seen in the arteriole and those in the venule have extended (open arrows). (**E**) After 1 h of nerve rewarming (37.5°C) the venule still exhibits multiple segmental occlusions (arrows). Erythrocyte granulations (open arrows) in the arteriole indicate poor reperfusion. Bars represent 100 μm.

Discussion

There has been a long-standing controversy regarding the pathogenesis of peripheral nerve cold injury. Although early investigators raised the possibility of ischaemia (Smith *et al*., 1915*b*; Lake, 1917; Ungley and Blackwood, 1942; Blackwood and Russell, 1943; Large and Heinbecker, 1944; Denny-Brown *et al*., 1945), most subsequent researchers argued against the concept of ischaemia causing the selective and widespread loss of myelinated fibres that is the hallmark of cold-injured nerve (Lewis and Moen, 1952; Sayen *et al*., 1960; Nukada *et al*., 1981). Alternative suggestions were a direct effect of cold on nerve fibres, vasogenic or cytotoxic oedema, and a disruption of axoplasmic transport (Fernandez *et al*., 1970; Grafstein *et al*., 1972; Basbaum, 1973; Kennett and Gilliatt, 1991*a*, *b*).

Temperature variation has often been stressed in cold injury. Common precipitants have been exposure of a frost-bitten limb to the warmth of a fire prior to re-exposing the limb to cold, and variations between the intense cold of night and the less severe cold of day (Smith *et al*., 1915*a*; Richards, 1944; Friedman, 1945). The injurious effects of temperature variation might be due to (i) a mismatch between different tissue temperatures and that of the perfusing blood; (ii) reperfusion injury; (iii) alternating constriction and vasodilatation with subsequent excessive exudation and the production of harmful metabolites; or (iv) changes in membrane permeability and a rise in intracellular calcium (Richards, 1944; Kennett and Gilliatt, 1991*b*). In this study, an animal model of cold neuropathy was therefore developed to produce focal cyclical cooling of nerve.

The longer cooling time in this model, compared with most previous investigations (3 h), was again suggested by clinical reports. Many human cases of severe cold injury have suffered from cold exposure for days (Lewis and Moen, 1952). Similarly, the time-course of non-freezing cold injury can be spread over weeks (Friedman, 1945). It would seem under many circumstances that time is as important as temperature, as to whether or not a subject will suffer a cold-induced neuropathy.

Acute experimental cold lesions are ideally suited for laser Doppler quantification since this technique allows for rapid and continuous recordings (Nesbitt and Acland, 1980; Rundquist *et al*., 1985; Takeuchi and Low, 1987; Low *et al*., 1989; Myers and Heckman, 1989). Moreover, a laser Doppler flowmeter measures the blood-cell flux in a conical volume beneath the probe, related to the number and velocity of blood cells, rather than the absolute volume of blood cells in the field. Finally, alterations in the NBF can be related to pathological changes at the same level, since the method is non-invasive (Nukada *et al*., 1992). Using this technique, it was possible to monitor the dynamic changes in NBF closely, before, during and after nerve cooling.

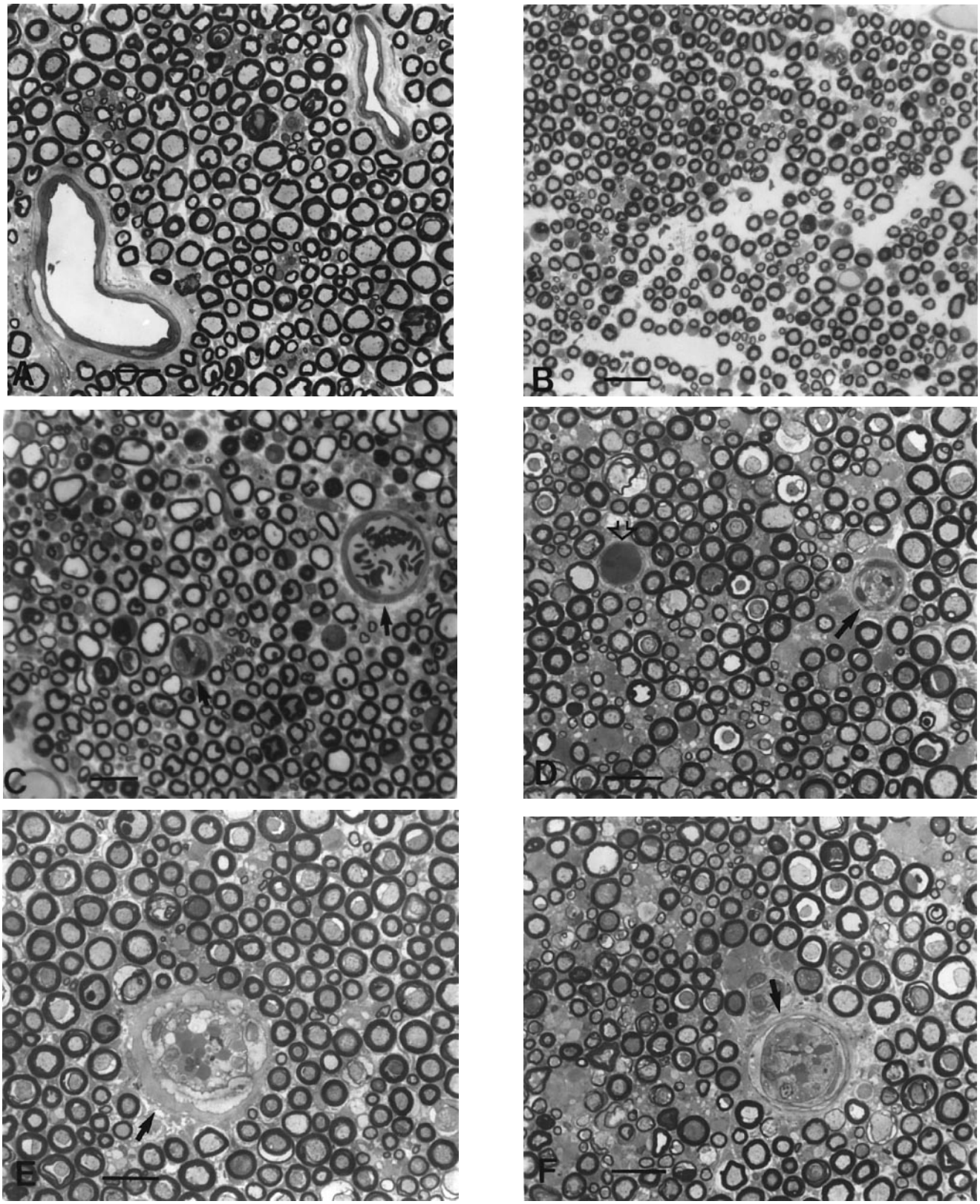

Fig. 5 Transverse sections of rat cooled sciatic nerve. (**A**) 'Empty' endoneurial vessels 1 h after rewarming. (**B**) Endoneurial oedema seen 12 h after nerve cooling. (**C**) Axonal degeneration prominent 1 day after nerve cooling. Note red cell aggregations in endoneurial vessels (arrows). (**D**) Severe axonal degeneration including dark, 'empty' and shrunken axons 2 days after nerve cooling. An endoneurial vessel with swollen endothelium is thrombosed (arrow) and another packed with red cell aggregates (open arrow). (**E**) Severe axonal degeneration with prominent intramyelinic oedema, 3 days after cooling. Note the thrombosed endoneurial vessel with markedly swollen, degenerating endothelial cells (arrow). (**F**) A thrombosed endoneurial vessel with more advanced nerve fibre degeneration 5 days after cooling (arrow). Bars represent 25 μm.

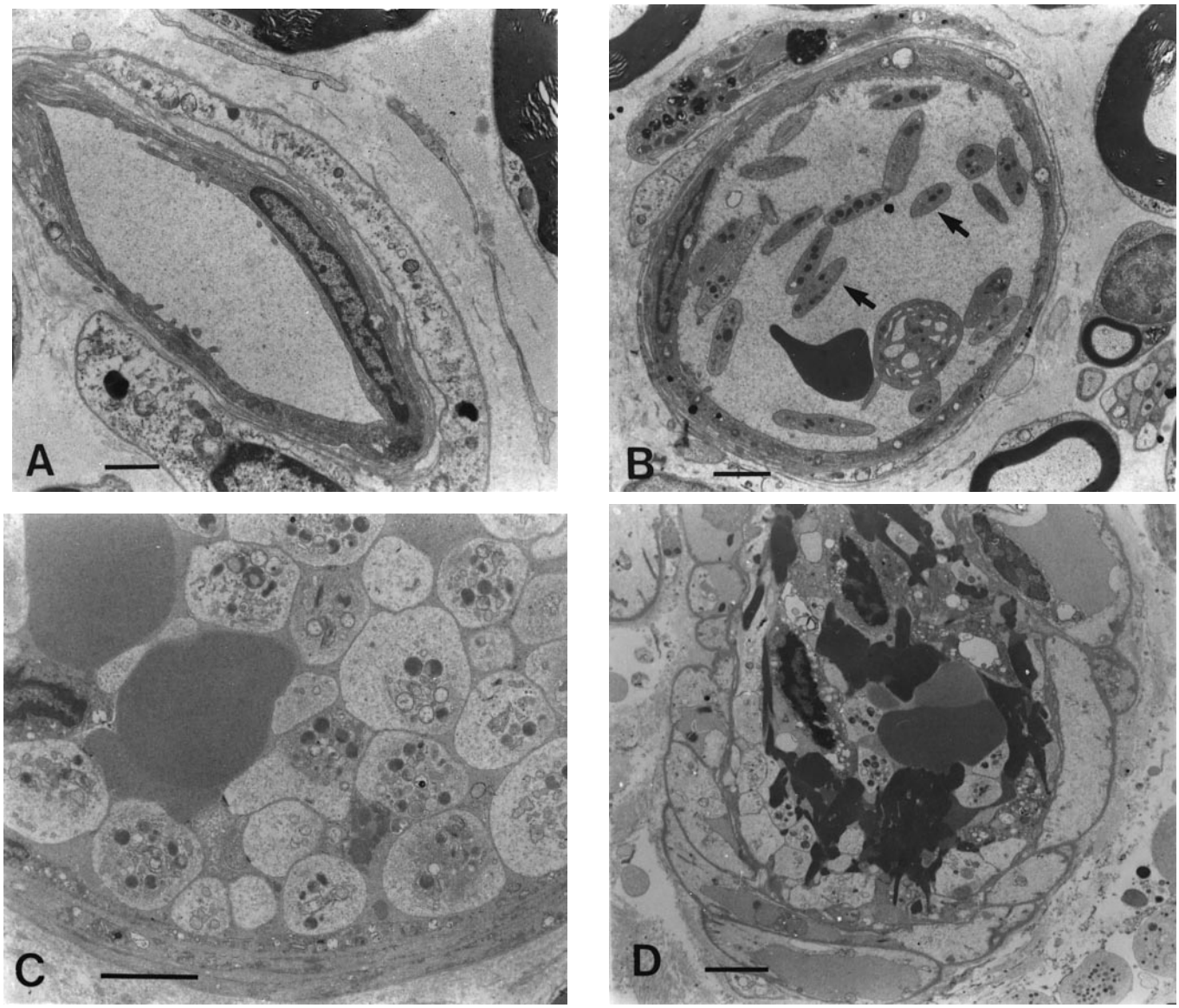

Fig. 6 Electron micrographs of endoneurial vessels in cooled sciatic nerve. (**A**) An empty capillary with a degenerating pericyte 1 h after nerve rewarming. Bar represents 2 μm. (**B**) Aggregating platelets (arrows) 24 h after cooling. Bar represents 2 μm. (**C**) Platelets, adherent to the endothelium of a venule, show variable degrees of degranulation without pseudopod formation, 48 h after nerve cooling. Two red blood cells are trapped within this platelet thrombus. Bar represents 1 μm. (**D**) A thrombus formed of platelets, red blood cells and fibrin 5 days after nerve cooling. The blood vessel wall is necrotic. Bar represents 2 μm.

The results of this study show a significant and sustained reduction of nerve blood flow following a non-freezing cold injury. Even after 60 min of nerve rewarming, the NBF was still only 39% of that seen pre-injury. Similarly, NBF recordings 5 days after cold injury, were still only 50% of baseline levels.

There is thus compelling evidence that a principal result of nerve hypothermia is a failure of the nerve microcirculation. There are several possible explanations for a cold-induced impairment of flow within the vasa nervorum.

(i) Increased blood viscosity has been reported during hypothermia (Eckstein *et al*., 1942; Lynch and Adolph, 1957; Eiseman and Spencer, 1962; Grossman and Lewis, 1964; Virgilio *et al*., 1964; Fukusumi and Adolph, 1970). This has been attributed to an absolute and relative increase in the concentration of globulin and fibrinogen. The increased viscosity is therefore considered to be caused by cooling *per se*. According to molecular theory, cooling results in a decreased distance between molecules, with a resultant increase in number of molecular collisions (Suzuki and Penn, 1965). Increased viscosity of blood could thus account for slowing of blood flow. This effect will be exaggerated in capillaries and small venules where flow rates (shear rates) are low, thus causing a further increase in viscosity and setting up a vicious circle (Wells, 1964).

(ii) Our observations indicate that early in nerve hypothermia there is marked aggregation of blood cells within the epineurial capillary circulation, compounded by a

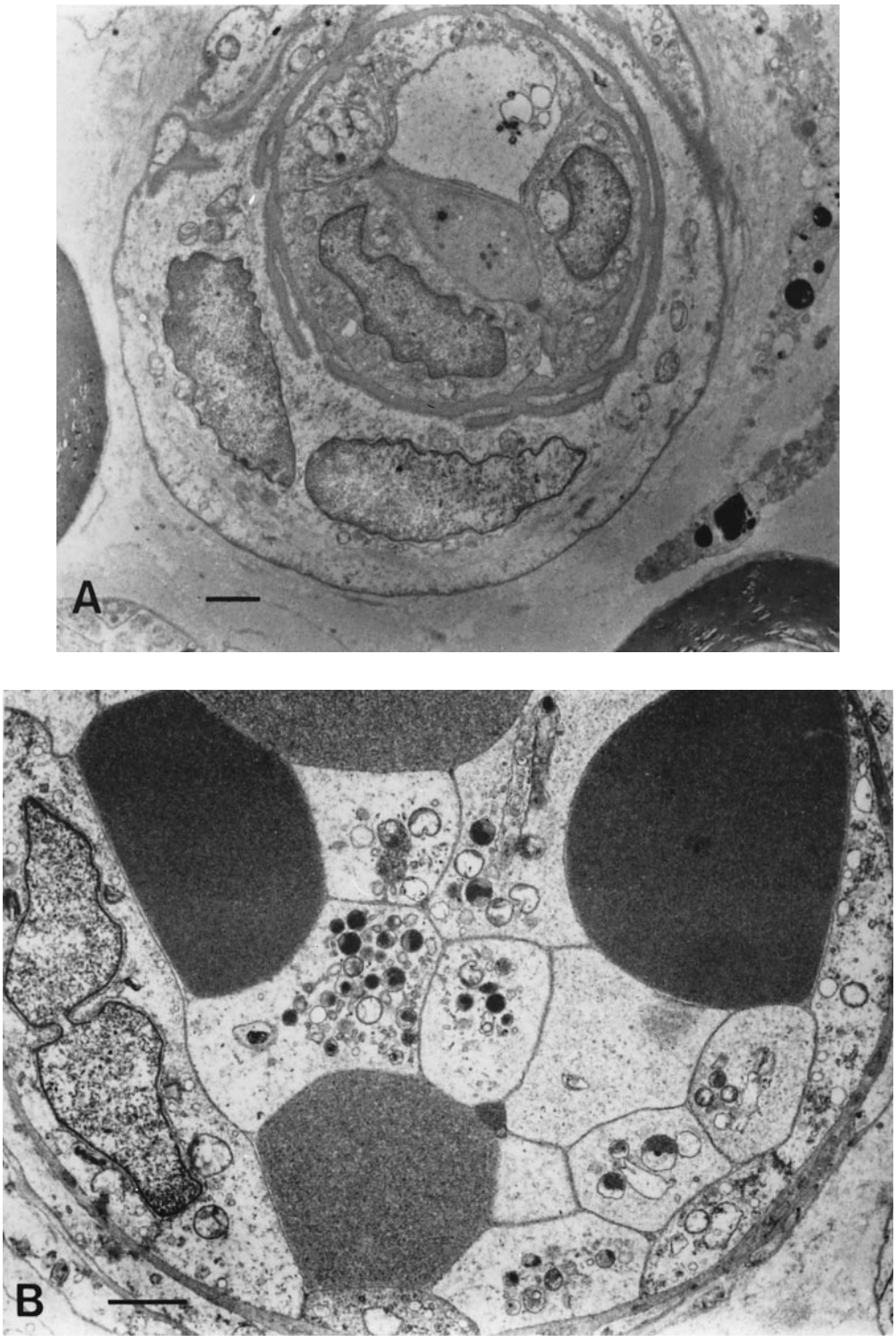

Fig. 7 Electron micrographs of cooled endoneurial vessels. (**A**) An endoneurial capillary lumen narrowed by swollen endothelial cells 12 h after nerve cooling. (**B**) A thrombosed endoneurial capillary seen in rat sciatic nerve, 2 days after cooling. Bars represent 1 μm.

constriction of arterioles and metarterioles. Intravascular erythrocyte aggregates have also been a conspicuous finding in the microcirculation during generalized hypothermia (Bigelow *et al*., 1950; Gelin and Löfström, 1955; Blatters and Horvath, 1958; Bigelow, 1959; Löfström, 1959; Löfström and Zederfeldt, 1959; Long *et al*., 1963; Bigelow, 1964; Bond *et al*., 1964; Grossman and Lewis, 1964; Schs *et al*., 1964). The development of cold-induced aggregates has been attributed to a number of causes (Fåhraeus, 1921; Gelin and Löfström, 1955; Löfström and Zederfeldt, 1957; Ditzel, 1959;

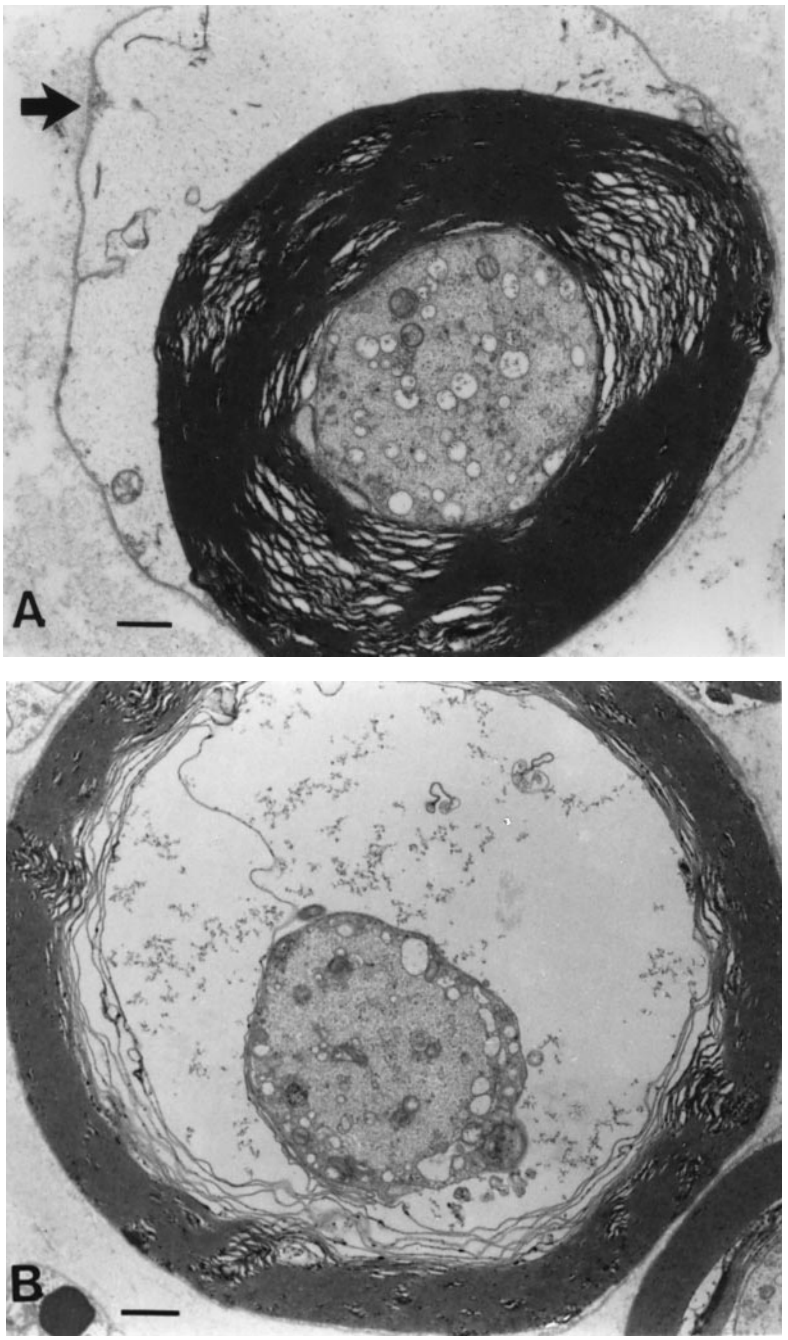

Fig. 8 Electron micrographs of cooled sciatic nerve fibres. (**A**) A rat sciatic nerve fibre, 12 h after nerve cooling, illustrating myelin unravelling and intramyelinic oedema (arrow). (**B**) A rat sciatic nerve fibre 2 days after cooling, exhibiting a shrunken axon and marked periaxonal oedema. Bars represent 1 μm.

Löfström, 1959; Löfström and Zederfeldt, 1959; Gelin and Zederfeldt, 1961; Wells, 1964). (a) An absolute increase in the concentration of total plasma proteins during generalized hypothermia results from a rapid leakage of fluid from the vascular compartment (Löfström, 1959). The decrease in circulating plasma volume without evidence of a loss of proteins in induced hypothermia intensifies erythrocyte aggregation (Sturkie, 1947; Rodbard *et al*., 1951; Gelin and Löfström, 1955; Löfström, 1959). (b) An increase in the relative proportion of high to low molecular weight substances in the plasma. (c) A reduction in blood flow (Ditzel, 1959; Pories *et al*., 1962; Wells, 1964). However, this latter viewpoint has been disputed by others (Löfström, 1959), and it is pertinent that early in the present experiments there was marked slowing of flow or stasis, without aggregate formation. We have no satisfactory explanation for the development of aggregates noted in our experiments. While segmental arterial hypotension at the cooling site and reduced rate of blood flow may have contributed to their development, we do not believe that they are the sole factors responsible.

Such an aggregation of blood cells will diminish the surface available for gas exchange, predominantly at capillary level. The equatorial diameter of red cells is greater than the diameter of most capillaries and it is only by a change of shape that red cells are able to transverse capillaries. Aggregates of red cells have never been observed to pass through capillaries and it is likely during hypothermia that they block the entrance to capillaries, with resultant tissue hypoxia (Suzuki and Penn, 1965).

(iii) Peripheral resistance has been reported to be increased during hypothermia (Bigelow *et al*., 1950; Jude *et al*., 1957; Kuhn and Turner, 1959; Delin *et al*., 1964; Goodyer, 1965; Rittenhouse *et al*., 1971; Zarins and Skinner, 1973). Increased flow resistance will result in a reduced pressure head and so contribute to sluggish flow in capillaries and venules. Factors possibly responsible for resistance changes are increased sympathetic stimulation (Kuhn and Turner, 1959; Kondo *et al*., 1974; Kopf *et al*., 1975) and blood viscosity (Lynch and Adolph, 1957).

(iv) A 'no-reflow phenomenon' after cold injury was suggested in this study by the physiological finding of persistently reduced NBF during the recovery phase and the morphological observation of many collapsed and empty endoneurial capillaries. The 'no-reflow phenomenon', characterized by a lack of post-ischaemic reperfusion, was first described with reference to rabbits, rendered globally ischaemic (Ames *et al*., 1968). These authors found areas of impaired vascular filling which increased with prolongation of the ischaemic time. Lundborg (1970, 1975) showed that a 'no-reflow phenomenon' could also occur in the peripheral nervous system if the duration of ischaemia was sufficiently long. The occurrence of 'no-reflow' raises the possibility that ischaemia may modify blood vessels and impair the return of blood flow, with irreversible parenchymal damage as a secondary event. Thus endothelial cells may play a crucial role in bringing about a 'point of no return.' The phenomenon of 'no reflow' after cooling is probably due to a combination of factors, including a reduction in calibre of small vessels (arteriolar and met-arteriolar spasm), endothelial oedema and postischaemic hypotension (Kowada *et al*., 1968).

(v) Widespread but delayed thrombotic occlusion of the vasa nervorum was undoubtedly a potent factor in inducing poor nerve perfusion in the latter part of this hypothermic study. A possible explanation for this delayed thrombosis is platelet activation, occurring as a consequence of cold-induced endothelial damage. Swollen endothelial cells and adjacent platelet aggregations both favour this concept. Previous work in our laboratory has shown that non-freezing nerve injury distorts endothelial cells and disrupts their 'tight' junctions (Nukada *et al*., 1981). Such endothelial surface changes are likely to change the luminal surface from an anticoagulant surface to a procoagulant one, and thus promote platelet adherence. It is well known that the circulating cellular element most intimately involved with injury to blood vessels is the platelet (Warren and Bono, 1970). Once platelets are stimulated to adhere to a vessel wall, their granular contents are released. In turn, these contents promote aggregation of additional platelets. Aggregation is also enhanced by the release of Von Willebrand factor, which is adhesive to GP1b membrane protein and fibrinogen. Activated platelets also release adenosine diphosphate and thromboxane A2, which further recruit platelets. Thrombin itself may stimulate a further release of platelet granules and the subsequent recruitment of new platelets. Ultimately platelet aggregates will occlude injured blood vessels and arrest flow.

An alternative explanation is that cold haemagglutination plays a role in thrombus formation, as described in cold neuropathies (Stats and Bullowa, 1943). Greene (1943) found that haemolysis was a frequent, if not constant feature of cold injury. Release of haemoglobin might affect blood vessels directly and masses of residual stroma from destroyed erythrocytes could block the circulation.

Further points for consideration are the release of thrombokinase from warmed red cells following cooling and subsequent thrombosis (Lake, 1917) and the release of H-substance from tissue, damaged by cold (Lewis, 1941).

This experimental evidence of ischaemia in non-freezing cold injury is supported by clinical investigations. Friedman (1945), in a study of 14 cases of trench foot (a non-freezing cold injury), found numerous vessels containing erythrocyte thrombi of the type formed in stagnant blood. In addition, some vessels contained mixed thrombi composed of red cells, platelets, hyaline material and enmeshed leucocytes. Typically in these patients, thrombus formation was delayed.

As in other 'cold' studies (Basbaum, 1973; Nukada *et al*., 1981), we observed in this investigation a degeneration of myelinated fibres with unmyelinated fibres largely preserved. This pathology is attributable to myelinated fibres being more vulnerable to both ischaemia and anoxia (Dahlin *et al*., 1989; Fujimura *et al*., 1991; Xu and Pollock, 1994). That cold-injured myelinated fibres were damaged by ischaemia is supported by the morphological findings of dark, often swollen axons, inappropriately thin myelin and attenuated axons. These findings are typical of morphological changes seen in other models of nerve ischaemia (Korthals and Wisniewski, 1975; Korthals *et al*., 1978). Swollen dark axons are a manifestation of accumulated axonal organelles. This pathology most likely results from an interruption of fast axoplasmic transport, an early pathological reaction to ischaemia/hypoxia (Ochs, 1971; Korthals and Wisniewski, 1975; Griffin *et al*., 1977; Tsukita and Ishikawa, 1980). Attenuated axons may be seen where ischaemia impairs slow neurofilamentous transport (Friede and Samorajski, 1970; Hoffman *et al*., 1983; Hoffman *et al*., 1984; Nukada and Dyck, 1984; Nukada *et al*., 1986). Occasional demyelination is a secondary event in ischaemia, a result of acute and severe alteration of axons (Chopra and Hurwitz, 1967; Eames and Lange, 1967; Asbury *et al*., 1970; Weller *et al*., 1970; Beckett and Dinn, 1972; Griffiths and Duncan, 1979; Vital and Vital, 1985).

In the present experiments, oedema following cold injury was shown mainly to have a diffuse distribution in the

interstitium, and only occasionally to be compartmentalized within axon, myelin sheath or Schwann cell soma. It is well known that cold injury of peripheral nerve is followed by endoneurial oedema (Basbaum, 1973). The progressive increase in endoneurial oedema after cold injury may be related to the absence of endoneurial lymphatics (Sunderland, 1965), or to the abnormal vascular permeability of endoneurial vessels caused by the release of biogenic amines (histamine or serotonin) from mast cells, an early pathological event in hypothermic nerve injury (Olsson, 1968). Although the origin of intramyelinic fluid is not known it has been suggested that since nodes of Ranvier are in direct contact with the extracellular space, a major pathway for oedema probably exists via the nodal region (Graham *et al*., 1976). A more likely possibility for endoneurial oedema is that following cold-induced ischaemia, endothelial tight junctions are disrupted (Nukada *et al*., 1981). It is possible that oedema increases the endoneurial fluid pressure and influences NBF through constriction of the transperineurial microcirculation (Beggs *et al*., 1991; Kalichman and Myers, 1991; Myers *et al*., 1991). It is of importance that oedematous nerves have been noted to be susceptible to ischaemic injury (Myers *et al*., 1986), perhaps in part as a result of increased intercapillary distances in the endoneurium (Low *et al*., 1985; McManis *et al*., 1986).

It has been suggested that suspension of axoplasmic transport plays an important role in the pathogenesis of nerve cold injury (Lewis and Moen, 1952; Sayen *et al*., 1960; Basbaum, 1973; Nukada *et al*., 1981; Kennett and Gilliatt, 1991*a*, *b*). There are now many studies which show that low temperature blocks or slows axoplasmic transport (Fernandez *et al*., 1970; Grafstein *et al*., 1972). Axoplasmic arrest is seen in mammalian peripheral nerve between 0°C and 10°C but transport resumes with nerve rewarming after cold-blocks lasting up to 22 h (Ochs and Smith, 1975). Arrested axoplasmic transport has been shown to fully recover after 7 h of anoxia without evidence of axonal degeneration (Leone and Ochs, 1978). On this evidence, a 3 h blockade of axonal flow would not account for the severe axonal degeneration seen in this hypothermic study.

We conclude from the results of this peripheral nerve investigation that ischaemia plays a principal role in the pathology of non-freezing cold injury.

Acknowledgements

We wish to thank Mr Ken Miller for helpful advice regarding the photography of vasa nervorum and Dr Alex Dempster for stimulating discussions.

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Received August 13, 1996. Revised October 29, 1996.
Accepted November 22, 1996