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Cutaneous innervation before and after one treatment period of acupuncture

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Short title:

Acupuncture and cutaneous innervation

Key words

Skin, acupuncture, peripheral nerve, CGRP, PGP 9.5, VR1

Conflicts of interest

None declared.

Summary

Background The effect of acupuncture on nociceptive pain is well documented, but effects on nociceptive itch have been contradictory.

Objectives To evaluate possible effects of acupuncture on the occurrence, distribution and function of sensory nerve fibres in human skin.

Methods Ten subjects were treated by inserting ten acupuncture needles subcutaneously in the upper-lateral aspect of one buttock. The subjects were recruited from an acupuncture clinic and were undergoing specific acupuncture treatment for their disorders. The needles were stimulated (rotated to and fro) twice during the twice-weekly 25-minute sessions over five weeks. Skin biopsies, diameter 3mm, were taken before and 3 to 6 days after local acupuncture. Antibodies against pan-neuronal marker, PGP 9.5, calcitonin gene-related peptide, CGRP, vanilloid receptor VR1 and μ-and δ-opioid receptors were employed to study sensory unmyelinated nerve fibres that transmit nociceptive pain and itch. A histamine prick test using planimetry was used to record experimental itch after acupuncture on the treated area and on the corresponding control skin respectively, and a visual analogue scale was used to evaluate itch.

Results The number of CGRP nerve fibres/ biopsy sectionwas reduced from 36.0$\pm$3.3 to 21.3$\pm$4.0 ($p=0.05$) after the treatment. The PGP 9.5-immunoreactive nerve fibres/ biopsy were found both in the epidermis and in subpapillary dermis. The total number of PGP 9.5 immunoreactive nerve fibres 249.8$\pm$16.7 to 211.8$\pm$12.0 ($p=0.03$). The PGP 9.5 immunoreactive nerve fibres occurring in the dermis appeared more fragmented after the acupuncture compared to pre-treatment. The VR 1 immunoreactivity was found both in the free nerve fibres and in kite-like formations, possibly mast cells, throughout the

dermis, sometimes occurring around hair follicles. The number of VR1 immunoreactive elements was not significantly influenced by the acupuncture, 33.5±4.6 vs 43.0±4.4 (p=0.09). No immunoreactivity was found in the skin against μ-and δ-opioid receptors with the antibodies used in this study. Neither histamine-induced itch nor cutaneous responses were influenced by acupuncture.

Conclusions The present data indicates an effect of acupuncture on neuropathic itch but not histamine-mediated itch. Our findings support the opinion that the pain-relieving effects of acupuncture partly depend on its effect on the peripheral innervation.

Introduction

Needle acupuncture is traditional Chinese art of healing, about 3000 years old, for the treatment of certain diseases and symptoms. Thin needles are inserted through the skin to varying depths, often into the underlying muscle, here called "muscle acupuncture." Many modifications of the method have been described and the concept of acupuncture is not well defined.[1]

There is now reasonable evidence that acupuncture has a clinically relevant pain-relieving effect on certain forms of chronic nociceptive pain.[2, 3, 4, 5]

Acupuncture also seems to affect the function of the autonomic nervous system in various ways. For example, peripheral circulation increases after acupuncture,[6]nausea and vomiting can be relieved, and salivary flow rates can be improved in patients with different forms of xerostomia.[7, 8]

Several physiological mechanisms have been suggested for the pain-relieving effect of acupuncture. Spinal and supraspinal endorphin release has been proposed, as has the

involvement of other transmitters like serotonin and norepinephrine. [9, 10] In addition to the central neurochemical changes there are also peripheral changes after acupuncture. The insertion of a needle into the tissues induces direct changes close to the needle (in all different tissues penetrated) and through axon reflexes. The flare reaction (reddening, vasodilatation) is often seen locally around the acupuncture needles. This vasodilatation in the skin, due to axon reflexes, has been recognised for a very long time and the mechanisms have been clarified in detail.[11] The stimulation of Aδ or C fibres releases vasoactive and pro-inflammatory neuropeptides (e.g. CGRP, SP, NKA, opioids, galanin, somatostatin and VIP). It has been shown that the concentration of the neuropeptides VIP and CGRP in saliva from xerostomic patients increases after acupuncture.[12]

Opiate receptors have been found on nociceptive afferents in inflammatory conditions.[13] The different endorphins are secreted from inflammatory cells in the tissue after an injury. [13] This accumulation may lead to a peripheral opioid analgesia some days after an injury and might be initiated by acupuncture needles in the tissue. [14]

In a recent retrospective study, symptomatic relief of neuropathic pruritus was reported in 12 of 16 patients treated with acupuncture.[15] Twelve patients had symptoms of brachioradial pruritus, seven of notalgia paresthetica and four of meralgia paresthetica. Relapse occurred in 37% of patients within 1-12 months following treatment. Localized neuropathic itch such as notalgia paresthetica, brachioradial pruritus and other peripheral neuropathies display aberrant sensory innervation.[16, 17] The question is whether this sensory innervation may be a target for acupuncture. In other words, does acupuncture exert any effects on sensory nerve fibres in the skin?

We addressed this question by studying skin biopsies from gluteal uninvolved skin before

and a few days after acupuncture treatments. Antibodies against pan-neuronal marker, PGP 9.5 and calcitonin gene-related peptide, CGRP, served to evaluate sensory innervation in the skin. Immunohistochemical markers for a thermoreceptor, vanilloid-receptor, VR1 (capsaicin- receptor), and μ-and δ-opioidreceptors responsible for perception of pain respectively were employed to study sensory unmyelinated nerve fibres that transmit nociceptive itch and pain. Histamine induced flare, wheal and itch were recorded in order to evaluate the function of the sensory nerve fibres after acupuncture.

Materials and methods

Patients and procedures

Ten patients were treated with acupuncture because of pain or other dysfunctional disorder. All patients were women and their mean age was 55, with a range from 31 to 75 years. Table 1 shows a summary of information concerning the age, and treatment diagnosis**.** Muscle acupuncture was used and all patients responded well to the treatment given. Therapeutical acupuncture needles were not inserted in the area used experimentally in this study.

In each session, the patients were given their normal acupuncture treatment first and then the experimental procedure. This consisted of ten acupuncture needles subcutaneously (to a depth of about about 10 mm) in a circle with a diameter of about 20 mm. The needles were 0.30 mm thick and 30 mm in length. All needles were inserted in the same upper-lateral aspect of one buttock. No needle sensation, de-qi feeling (numbness, slight pain), was searched for. The needles were stimulated (rotated to and fro) twice during the

25-minute sessions. Punch biopsies (3 mm in diameter) were taken from the upper, lateral aspect of one buttock one week before the local acupuncture. The needles were inserted 1 cm around the first punch biopsy. Acupuncture was then applied twice weekly for 5 weeks. The second biopsy was taken from the skin of the buttocks, some distance from the scar after the first biopsy, 3-6 days after the tenth local acupuncture treatment. Informed consent was obtained in all cases. The study was approved by the Ethics Committee of the Lund University Medical Faculty and has been conducted according to Declaration of Helsinki principles.

Processing of biopsies

The biopsies were fixed by immersion overnight in a mixture of 2% formaldehyde and 0.2% picric acid solution in a 0.1mol/l phosphate buffer (pH 7.2) and then thoroughly rinsed in a Tyrode solution containing 10% sucrose. They were then frozen on dry ice, after which 10μm thick sections were cut serially in a cryostat. The sections were then processed for immunocytochemistry, using antibodies against the neuropeptide calcitonin gene-related peptide (CGRP) and the pan-neuronal marker PGP 9.5. The antibodies against CGRP, raised in guinea pig (working dilution 1: 1200, Euro Diagnostica, Malmö, Sweden), were used to demonstrate the thin sensory C-fibres.[18] The PGP 9.5 antibodies (working dilution 1:200, Ultraclone, Cambridge, UK) were used to visualize the various cutaneous nerve fibres. PGP 9.5 is a cytoplasmic constituent present in all parts of the neuron, and in both the cell body and all the processes. Data on the specificity of the antibodies employed has been presented elsewhere.[18] Antibodies against the vanilloid receptor VR 1, which were raised in rabbit (working dilution 1: 600, Euro Diagnostica,

Malmö, Sweden), were used to identify capsaicin-sensitive structures.[18] Vanilloid capsaicin, the major pungent agent contained in hot pepper, is known to release neuropeptides from sensory nerve fibres.[19] No fluorescence was detected in preabsorption tests with excess amount of homologous antigen (100 microgram of synthetic peptide per ml antiserum at working dilution).

Antibodies against μ- and δ-opioid receptors were raised in rabbit (working dilution for μ- opioid-receptors 1:200 and 1: 400, for δ-opioid-receptors 1: 400 and 1. 800, ImmunoStar Inc, Hudson, WI, USA).[20, 21]

Three consecutive sections of each of the biopsies were studied. The microscope used was a Leica Aristoplan epifluorescence. The number of immunoreactive nerve fibres in the epidermis and the dermis was assessed visually at magnification of x 250. All the immunopositive nerve fibre fragments in the whole biopsy section were counted. Blind evaluation of the biopsies was performed by a single observer. Micrographs were taken using Kodak Tri-X 400 film.

Histamine responses

In five individuals (No 5, 7, 8, 9, 10 in Table I), histamine (Soluprick^{R}, ALK, Denmark) was ´pricked` into the area treated by acupuncture on the upper lateral aspect of the buttock and on the other corresponding, untreated side. Evaluation regarding erythema and infiltration was made after 5 and 20 minutes resp using planimetry. In addition, itch evoked by histamine was evaluated after 5 minutes using VAS.[22]

Statistical analysis

Statistical evaluation was based on the mean of the counts in the three sections taken

from each of the ten patients before and after the treatment respectively. Results are expressed as mean ± SEM. Wilcoxon signed ranks test was used for comparing the skin biopsies before and after all treatments.

Results

Skin biopsies

CGRP-immunoreactive nerve fibres were mostly localized in subpapillary dermis and only occasionally found in the epidermis (Figure 1 a, 1 b). The number of CGRP nerve fibres was reduced from 36.0±3.3 to 21.3±4 (p=0.005) after the treatment (Figure 4a). The PGP 9.5-immunoreactive nerve fibres were found both in the epidermis and in subpapillary dermis (Figure 2 a, 2 b). The number of PGP 9.5-immunoreactive nerve fibres in the biopsies was significantly reduced from 249.8±16.7 to 211.8±12.0 (p=0.03) (Figure 4b).

The VR 1 immunoreactivity was found both in free nerve fibres and in kite-like formations throughout the dermis, sometimes occurring around hair follicles (Figure 3a, b). The number of VR1 immunoreactive nerve fibres seemed to be amplified by the acupuncture from 33.5±4.6 to 43.0±4.4 (p=0.09), after the treatment (Figure 4a).

There was no immunoreactivity in the skin seen with antibodies against μ-and δ-opioid receptors used in this study although they were capable of demonstrating neuronal elements in dorsal horn of the rat spinal cord; δ-opioid receptors present in nerve fibers and μ-opioid receptors in nerve cell bodies (unpublished observations).

The immunohistochemical data are summarised in Table 2.

Histamine reaction

Repeated acupuncture did not influence prick test reactions to histamine. The mean flare reaction on the treated skin was 8.6±4.6cm^2 compared to 8.2±4.4cm^2 on the control side. The mean wheal response was 0.8±0.1 cm^2 vs 0.9±0.5 cm^2 on the control side. The histamine-evoked itch (VAS) was 24% vs 18% on the control side.

Discussion

We have shown that the innervation of the skin using a pan-neuronal marker PGP 9.5 was reduced after a treatment period of ten acupuncture sessions. The density of CGRP-immunoreactive nerve fibres, representing sensory innervation in the skin, was also reduced after the treatments. Such a reduction of sensory nerve fibres conducting the itch sensation may help to reduce itch. A proliferation and sprouting of cutaneous nerve fibres has been found in some conditions of itch such as notalgia paresthetica and in pruritus of uremia respectively.[23, 24] Our findings would explain the symptomatic relief of local pruritus reported in 12 of 16 patients treated with acupuncture.[15]

A reduction of cutaneous nerve fibres was found after other treatments of itch such as topical capsaicin or phototherapy. [25, 26] Another treatment of itch that has been developed recently is cutaneous field stimulation (CFS), which activates C-fibres electrically and induces a reduction of intraepidermal PGP 9.5 nerve fibres only, leaving the intradermal fibres unaffected.[27] The difference between acupuncture and CFS in their effects on cutaneous innervation may be explained by the respective positions of the needles and electrodes. While acupuncture needles in this study were positioned subcutaneously, 15-20 mm deep, the electrodes of CFS are 2 mm long and protrude mainly into the epidermis

when the flexible rubber plate is applied to the skin.

VR1 immunoreactivity was found both at free nerve fibres and in kite-like structures in dermis. Although not confirmed by a routine histology or specific markers, the form and distribution of the VR1 immunoreactive cells around the hair follicles suggest that they are mast cells.[28] The number of VR1-IR structures, including nerve fibres and mast cells, in the skin was not influenced significantly by acupuncture. This finding is in accordance with a previous study on the effects of phototherapy on cutaneous innervation.[26] A reduction of VR1 immunoreactve nerve fibres might be expected, as the number of sensory CGRP-IR nerve fibres was reduced, and VR1 co-localized with the neurotransmitters such as CGRP and substance P.[29, 30] that only a subpopulation of VR1-IR nerve fibres are identical with CGRP-IR nerve fibres.[26]

As the respective numbers of epidermal and dermal nerve fibres were reduced, a reduction in the axon reflexes, such as the flare reaction to histamine prick test, might be expected. In this study histamine-induced responses, including itch, did not seem to be influenced by acupuncture. This finding should, however, be interpreted with some caution because we only tested five patients. This contrasts with a recently published study where a single acupuncture inhibited the wheal and itch but not flare induced by histamine.[31] An effect on itch would imply an inhibition of the nerve conduction and the axon reflex resulting in flare, while wheal, on the other hand, is an effect of vascular permeability. [32] Nerve blockade, using anaesthetics like Xylocain, almost totally inhibits the flare reaction to histamine while its effect on wheal is only partial.[32] In the present study we were interested in the effects of serial acupuncture on local cutaneous innervation, which may have implications for treatment of itch. However, all the patients

had undergone acupuncture at other sites (Table 2), possibly adding general effects on itch and explaining why there was no difference between the two sides. Such effects may be explained by generation of endogenous opioid peptides in several organ systems. However, we failed to find any immunoreactivity in the skin toward μ-and δ-opioid receptors used, although the antibodies used gave positive immunostaining in the rat spinal cord. An occurrence of an isomer of a μ-receptor, 1A, in human skin and of a δ-opioid receptor in rat skin has been reported previously.[33, 34] In both these studies, the immunoreactive opioid receptors were confined to sensory nerve fibres, co-existing with CGRP.[33, 34] Further studies are needed to localize opioid receptors in human skin.

Our finding of a local effect of acupuncture on peripheral innervation casts new light on the clinically relevant pain-relieving effect of acupuncture on chronic nociceptive pain.[2,3,4,5] In some painful nociceptive conditions, such as osteoarthritis (of the hip joint), endometriosis, rotator cuff syndrome (of coracoacromial ligament) or Achilles tendinosis, density of peripheral nerve fibres has been shown to be increased compared to normal, non-inflamed tissue. [34, 35, 36, 37, 38] A reduction of peripheral nerve fibre density where the needles are inserted locally at the pain-generating process might be one explanation for the pain-relieving effects in nociceptive pain conditions. On the other hand acupuncture has no better effect than placebo on different forms of neuropathic pain.[2] A possible explanation would be that peripheral nerve density is reduced in some forms of neuropathic pain, such as diabetes neuropathy, burning mouth syndrome, and HIV-associated sensory neuropathy [40, 41,42]

In conclusion, we found a reduction of the cutaneous CGRP and PGP 9.5 immunoreactive nerve fibre density following serial acupuncture in ten treatments. This

finding would explain the alleviation of nociceptive pain and indicate an effect of acupuncture on itch conditions associated with proliferation of sensory nerve fibres. The VR1-immunoreactivity, mainly confined to nerve fibres and mast cells, was not significantly changed by acupuncture. It may explain why repeated acupuncture influenced neither the itch nor the size of prick test reactions to histamine in this study. It seems, however, that acupuncture may be used to treat neuropathic itch. Our findings might also give rise to more research concerning peripheral mechanisms of acupuncture.

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Legends

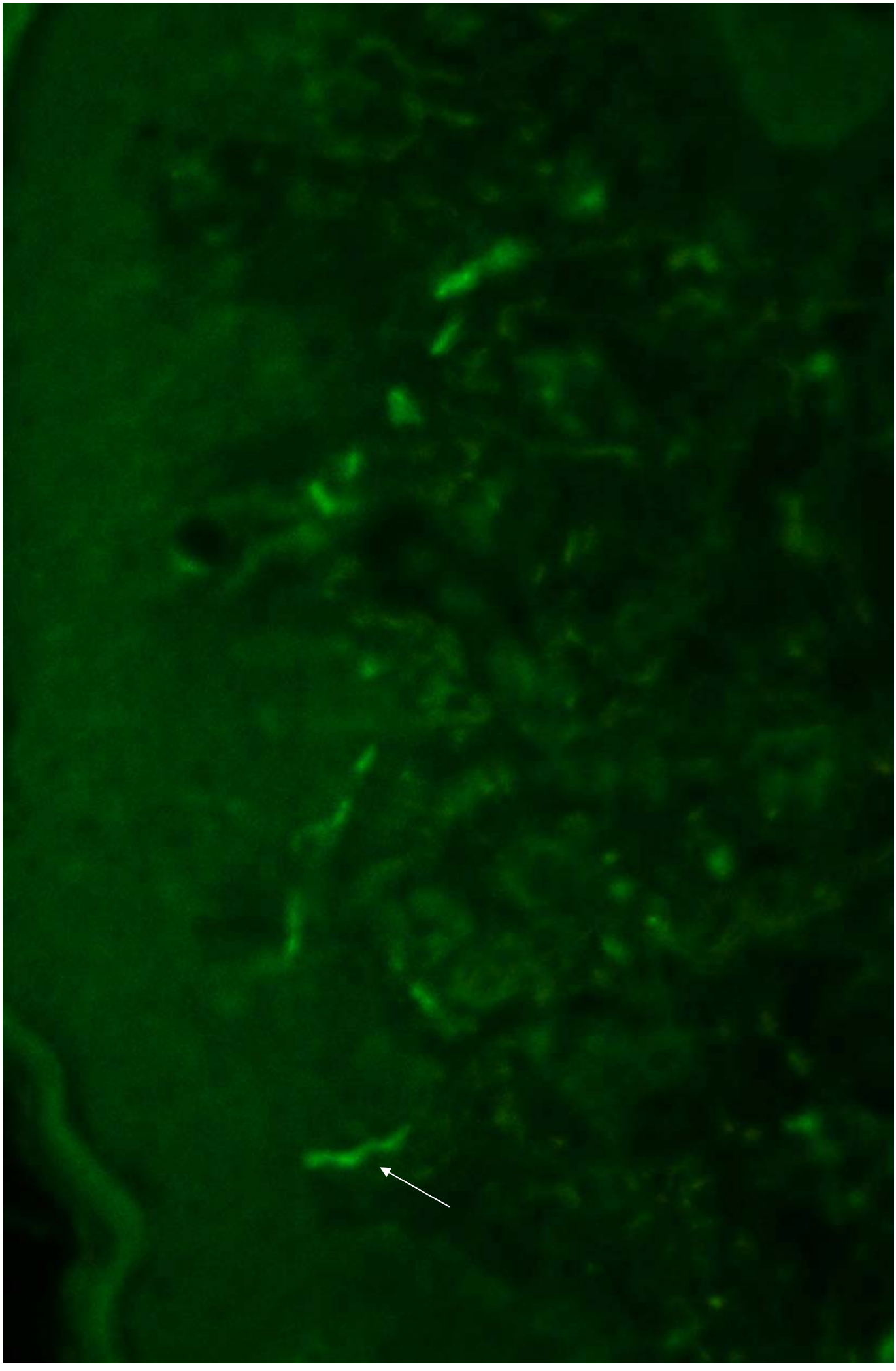
1. Skin biopsies from patient 4 taken prior to (a) and after (b) serial acupuncture resp immunostained for PGP-9.5. The total cutaneous innervation shown. The PGP-immunoreactive nerve fibres are distributed both in the epidermis and dermis (a), and the number is reduced after the treatments (b).Magnification x 175 (a) and x 250 (b) respectively. The arrows show examples of nerve fibers.

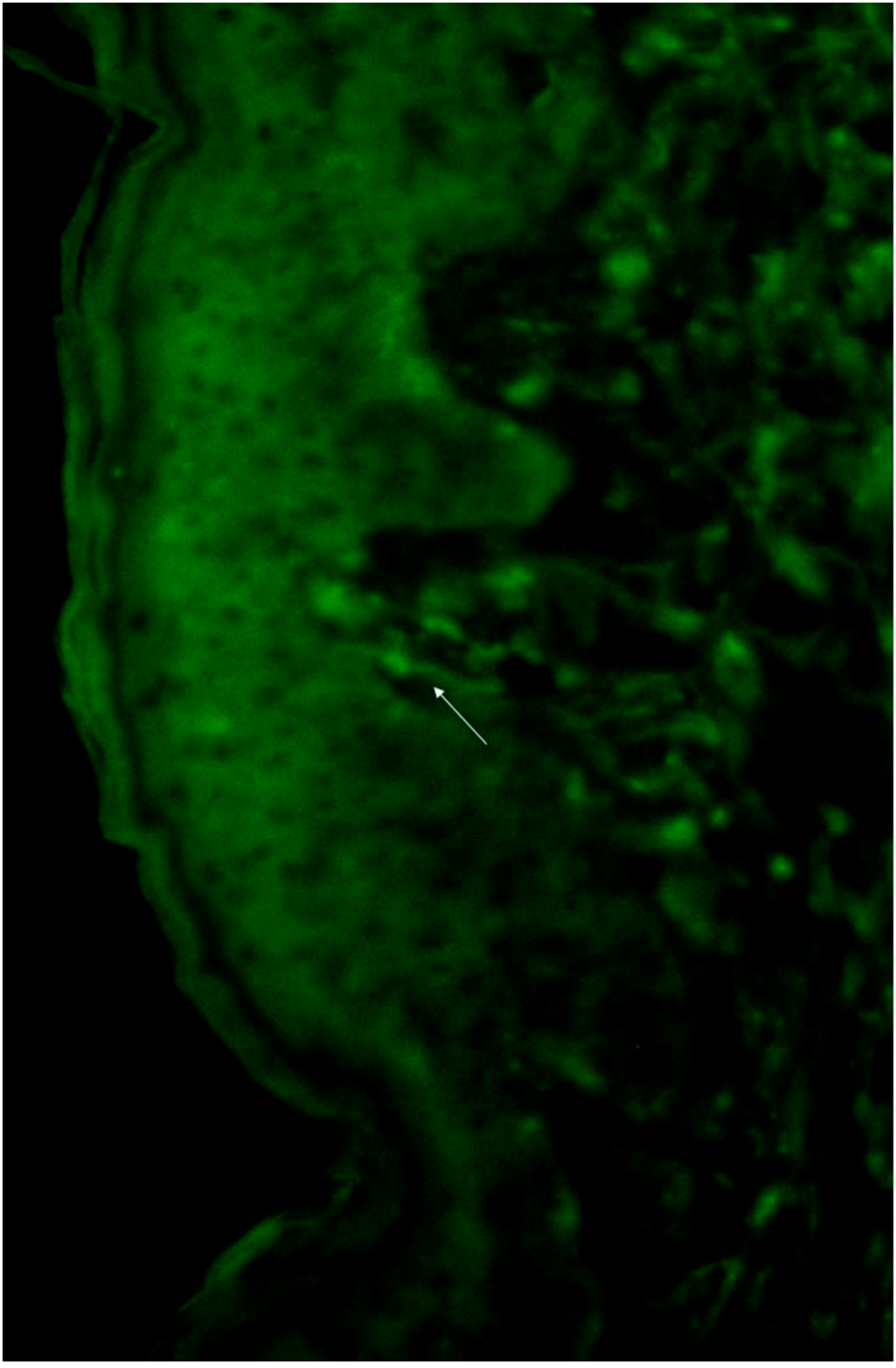
2. Skin biopsies from patient 6 taken prior to (a) and after (b) serial acupuncture respectively, immunostained for CGRP, and showing sensory innervation. The number of both intraepidermal and dermal nerve fibres is reduced after acupuncture. Magnification x 175 (a) and x 250 (b) respectively. The arrows show examples of nerve fibers.

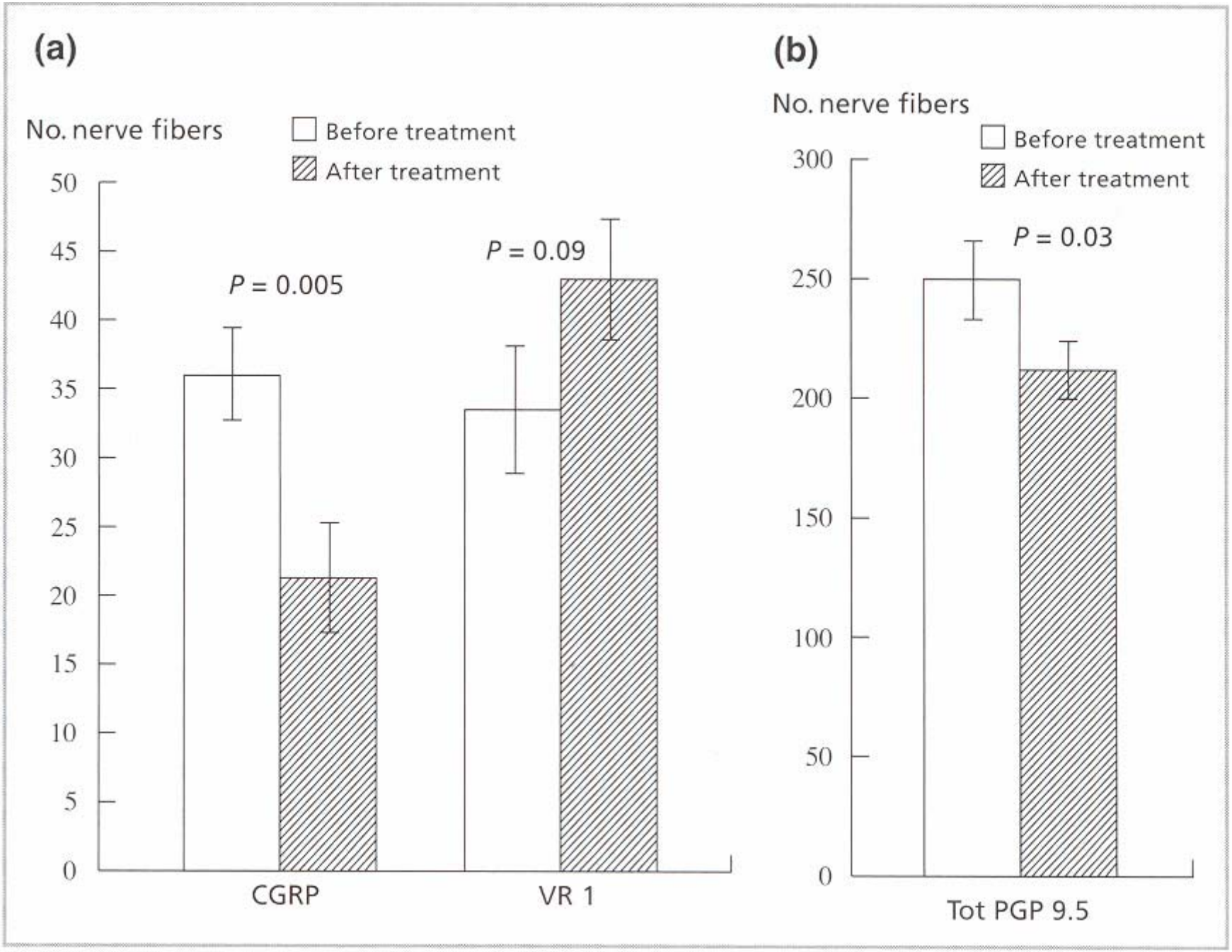
3. Skin biopsies from patient 8 taken prior to (a) and after (b) serial acupuncture ectively, and immunostained for capsaicin receptor, VR1. The arrows show distribution on mast cells (a, b) and free nerve fibres (b). Magnification x 175 (a, b).

4. Nerve fibre density in 3mm punch biopsies from skin before and after 10 treatments with acupuncture. The mean number of CGRP-IR nerve fibres ($\pm$SEM) found after

acupuncture is reduced compared to the number before the treatment, $p = 0.005$ (a). The density of VR1-IR structures ($\pm$SEM) including both mast cells and nerve fibres found prior to and after acupuncture is not significantly altered ($p = 0.09$) (a). The number of PGP-IR nerve fibres ($\pm$SEM) found after acupuncture is reduced compared to the number prior to the treatment, $p= 0.03$ (b).






(a)
No. nerve fibers
Before treatment
After treatment
50
45
40
35
30
25
20
15
10
5
0
P = 0.005
P = 0.09
CGRP
VR 1
(b)
No. nerve fibers
Before treatment
After treatment
300
250
200
150
100
50
0
P = 0.03
Tot PGP 9.5