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Original article

NEEDLE-NERVE INTERACTION IN ACUPUNCTURE: A MORPHOLOGICAL STUDY

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Summary

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Some acupuncture effects are considered to be caused by interaction with nerve structures in and around the acupoints. The aim of the present study was to investigate the nerve structures that interact with the needle in acupuncture and to present their distribution in acupoint tissues. To do this, the microscopic anatomy and its alterations in the vicinity of the needle tract formed after experimental acupuncture in ST_{36} acupoint in rats were described by histological and immunohistochemical methods. Free nerve endings were seen in the epidermis, and surrounding hair follicles and sebaceous glands in the dermis. Muscle spindles and larger nerve fibres close to blood vessels were also observed deeper, in the muscular plane. Needling of the acupoint caused destruction and displacement of hair follicles together with their free nerve endings. Deeper, some muscle spindles and smaller nerves were displaced and disrupted. Larger nerves were not destroyed, but rather pushed aside by the needle. Furthermore, needle impact also caused degranulation of mast cells near the needle tract. The findings suggest multiple ways of interaction between acupuncture needle and the nerve structures of the acupoint. Acupuncture combines destruction, disruption and displacement of nerve structures, together with additional interaction with mast cells. Those mechanisms are involved in eliciting the needling sensation and are possibly associated with the systemic effect of acupuncture.

Key words: free nerve endings, muscle spindles, needle tract, nerve fibres, Zusanli acupoint (ST_{36})

INTRODUCTION

Acupuncture points (also called acupoints) are locations on the body that are the focus of treatment in the Traditional Chinese Medicine (TCM). Acupuncture is a method of TCM, involving the insertion of thin needles into the body at acupuncture points with therapeutic intention. Some effects of acupuncture are believed to be caused by interaction of the needle with nerve structures in and around the acupoints. They are thought to be responsible not only for the sensory registration of acupuncture but also for its systemic effects. Many researchers attribute importance to the innervation of acupoints in the effects of acupuncture. Acupoints are relatively rich in nerve structures that can be activated during acupuncture, including free nerve endings, Ruffini corpuscles, Meissner corpuscles, Krause corpuscles, lamellated corpuscles, and muscle spindles (Gunn *et al.,* 1976; Dung, 1984; Zhou *et al.,* 2010). Researchers have also established the presence of neurovascular bundles passing through or close to acupuncture points (Bossy, 1984). All of them are presumed to be involved in eliciting sensation during acupuncture. A neural acupuncture unit (NAU) is a proposed term to denote the structures activated during needling of the acupoint (Zhang *et al.,* 2012).

For the subject of our research the acupuncture point Zusanli (ST_{36}) was chosen as one of the most widely used points used for treatment of gastrointestinal discomfort, nausea and vomiting, as well as of general discomfort, stress and fatigue. Evidence shows that the acupuncture at Zusanli has a central action mechanism caused by the activation of the frontal lobe, temporal lobe, anterior central gyrus, postcentral gyrus, and other brain regions associated with exercise, language, sensation and mental cognition. Stimulation of this acupoint could improve the blood flow in the brain function area, and promote the reorganisation and recovery of related neurological function (Wang *et al.*, 2004; Li *et al.*, 2015).

The anatomy of ST_{36} acupoint: epidermis, dermis, subcutis, fascia, epimysium and striated muscle, with large calibre blood vessels and nerves in the deeper striated muscle plane was already demonstrated in a previous study of ours (Dimitrov, 2012a). Following acupuncture the newly formed needle tract penetrates the epidermis, dermis, subcutis, fascia, reaches the muscle, however, deeper structures are not affected. The integrity of the tissues the needle passes through is disrupted. In another earlier study, we reported partial destruction of elastic and collagen fibres and increased degranulation of mast cells in the subcutis of the acupuncture area. The blood vessels, the glands and the hair follicles close to the needle tract were compressed, but largely undamaged (Dimitrov, 2012b). Generally, all nerve structures, associated with the skin and subcutaneous soft tissues should be inevitably involved in the morphological alterations following acupuncture. However, the data regarding the nature of those nerve structures in ST_{36} acupoint and their interaction with the needle is relatively scarce.

The aim of the present study was to examine the nerve structures that interact with the acupuncture needle and to present their distribution in tissues. This was shown in the ST_{36} acupoint in rats, by describing the newly formed needle tract following needling.

MATERIALS AND METHODS

The experiments were performed on 6 adult male Wistar rats. The experimental procedures were approved by the Research Ethics Committee at the Medical Faculty of Trakia University with all efforts made to reduce the number of animals used and their suffering. ST_{36} acupoint was located using the standard method of anatomical proportions about 5 mm below the head of the fibula under the knee joint, and 2 mm lateral to the anterior tubercle of the tibia (Lee *et al.,* 2012). The area around the acupoint was shaved and marked under the control of the skin-resistance measuring apparatus KWD-808. The rats were deeply anaesthetised with ketamine-xylazine at the appropriate dosage. Standard 0.25×13 mm steel acupuncture needles were then inserted 5 mm deep into ST_{36}, remaining in the tissues for 10 min. For histological sampling, animals were perfused first with 0.05M PBS followed by 4% PFA in 0.1M phosphate buffer, pH 7.36. Unlike ordinary needles, the acupuncture needle was thinner and did not cause major damage to adjacent tissues. After removal, the tissue defect was rapidly closed by approximation of tissues, without leaving long lasting damage. To overcome this obstacle, samples from ST_{36} acupoint with size 5×5×5 mm was excised together with the needle and postfixed overnight at 4 °C. Tissue was paraffin-embedded and microtome-sectioned in 5 µm sections; alternatively, it was cryoprotected and sectioned on a freezing microtome in 30 µm sections. In the case of paraffin embedding, the acupuncture needle remaining in the block, was used as orientation mark, and was only taken out immediately reaching its plane with the microtome blade, thereby largely preserving the needle tract. Classical histological staining techniques: H&E, Bodian, Mallory, van Gieson, and Heidenhein's Azan, and toluidine blue for demonstration of mast cells were used. Immunohistochemistry on paraffin sections was also performed. Samples were incubated with antisera against S-100 for demonstration of nerve structures and against tryptase and 5-HT for demonstration of mast cells, following a standard protocol. Sections were dewaxed and rehydrated, subsequently washed in 0.1M phosphate buffered saline (PBS), pH 7.4, incubated in 1.2% hydrogen peroxide in absolute methanol for 30 min, followed by antigen retrieval in 10 mM citrate buffer (pH 9.0) for up to 10 min in pressure cooker, rinsing with cold PBS/ Triton X-100 between steps. Sections were incubated in a humid chamber overnight at 4 ^{o}C with the primary antibody: Monoclonal Mast Cell Tryptase (clone 10D11, Leica Biosystems, Newcastle), Monoclonal Mouse Anti-Human Serotonin (clone 5HT-H209, DAKO, Denmark), Polyclonal Rabbit Anti-Crow S-10 0 (DAKO, Carpinteria, USA) in dilution 1:100. After three PBS washes, the slides were incubated with DAKO-REAL^{TM} En-Vision^{TM} detection system (DAKO) according to manufacturer's instructions and counterstained with Mayer's haematoxylin. Negative control was done by omitting the primary antibody.

RESULTS

The tissues of the acupoint and their alterations by the needle were of particular interest in our research, so in the description of our findings our focus was mainly on the effects after acupuncture. After acupuncture of ST_{36} acupoint, the integrity of the epithelium, dermis, subcutaneous connective tissue, fascia, epimysium and striated muscles was disrupted. Typical folds formation in the direction of the needle penetration was seen. Connective tissue was thickened and displaced, cells contained within it were drawn in the direction of the needle, together with larger structures. Intact nerve structures, among those displaced and destroyed by the acupuncture needle, were observed in all tissues.

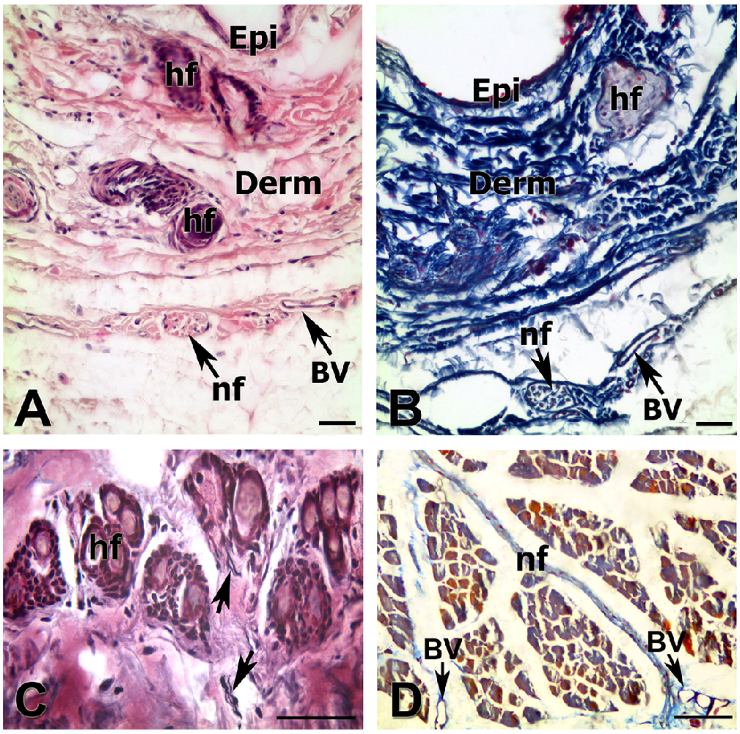

Fig. 1. Nerve structure displaced by the needle in the tissue of ST_{36} acupoint. **A**, **B.** Micrographs showing small nerve fibres: H&E-stained section (A) and Azan-stained section (B); **C**. Bodian-stained section showing free nerve endings and small nerve fibres in the vicinity of hair follicles; **D.** Large nerve fibres in striated muscle: Mallory-stained section. Epidermis (Epi), dermis (Derm), nerve fibres (nf), free nerve endings (arrow), hair follicles (hf), blood vessel (BV), striated muscle (SM). Scale bar=50 µm.

Bodian staining demonstrated free nerve endings in the epidermis. The epidermis was displaced in the vicinity of the needle tract, and the free nerve endings contained in it were largely destroyed by the acupuncture needle (Fig. 1C). Small nerve fibres in the dermis, and sebaceous glands free nerve endings in the dermis palisading around the hair follicles, displaced by the needle (Fig. 1C) were also visualised using Bodian staining. Few hair follicles with free nerve endings were destroyed by the needle. In the dermis, compressed and displaced small nerve fibres and small blood vessels were seen close to the needle tract (Fig. 3C).

Small nerve fibres were present in the subcutaneous tissue (Fig. 1A, B), as well as larger, S-100 positive nerve fibres in the deep muscular layer of the acupoint, some in immediate proximity of the needle tract (Fig. 2E, F). Nerve fibres in the dermis were commonly associated with small blood vessels.

The striated muscle plane of the acupoint contained small nerves and blood vessels as well, with some of them destroyed and displaced by the needle. The larger nerve fibres in the deep muscular plane ran parallel to larger blood vessels, as neurovascular bundles (Fig. 1D). They were not affected by acupuncture needle. Close to the small nerve fibres in the striated muscle, clusters of tryptase positive mast cells were seen (Fig. 4B).

Striated muscle contained muscle spindles as well (Fig. 2A–F). The muscle spindles were surrounded by connective tissue and were seen predominantly in the proximity of blood vessels and nerve fibres (Fig. 2D, F). Some of the muscle spindles were partially destroyed by the acupuncture needle (Fig. 2C; Fig. 3E, F), while other were unaffected. A summary of the observed nerve structures in the ST36 acupoint interacting with the acupuncture needle is given in Table 1.

Furthermore, a number of mast cells was observed in virtually all tissues of the acupoint. 5-HT positive mast cells were seen in close proximity to nerve structures, both in the intact dermis, close to hair follicles and in the vicinity of the needle tract (Fig. 3A–C). Mast cells were associated with small nerve fibres of the striated muscle (Fig. 4A-D) and muscle spindles as well (Fig. 3D). Some of them were dragged together with the displaced tissue, showing signs of degranulation

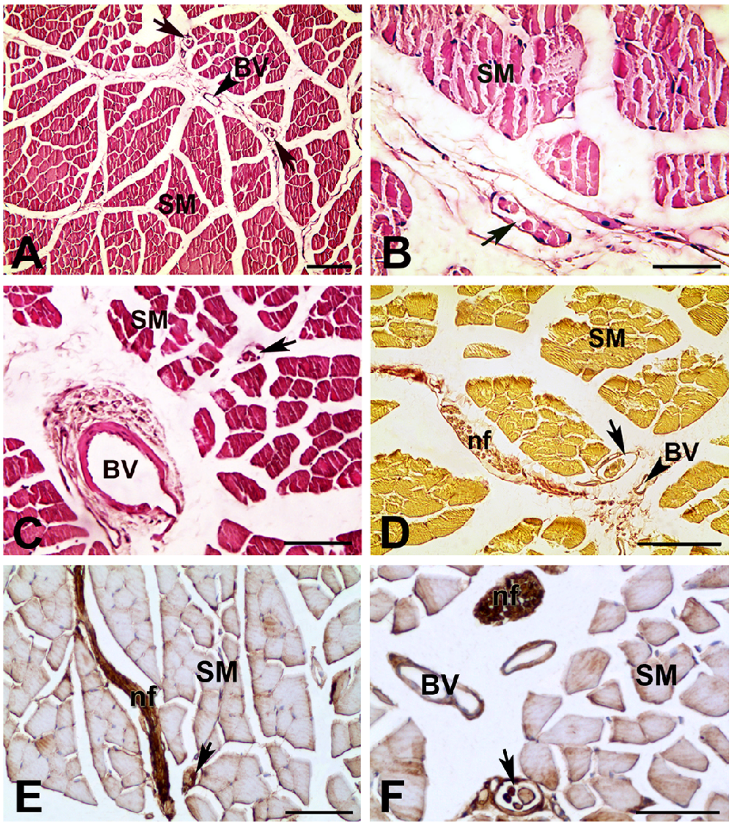

Fig. 2. Muscle spindles and nerve fibres in striated muscle in the vicinity of the needle tract. H&E-stained sections visualising muscle spindles in striated muscle **(A, B)** and partially destructed muscle spindles (**C**); **D.** Muscle spindles and nerve fibres in striated muscle: van Gieson-staining; **E, F.** Visualisation of muscle spindles and nerve fibre with S-100. Muscle spindles (arrow), nerve fibre (nf), striated muscle (SM), blood vessel (BV). Scale bar=50 µm.

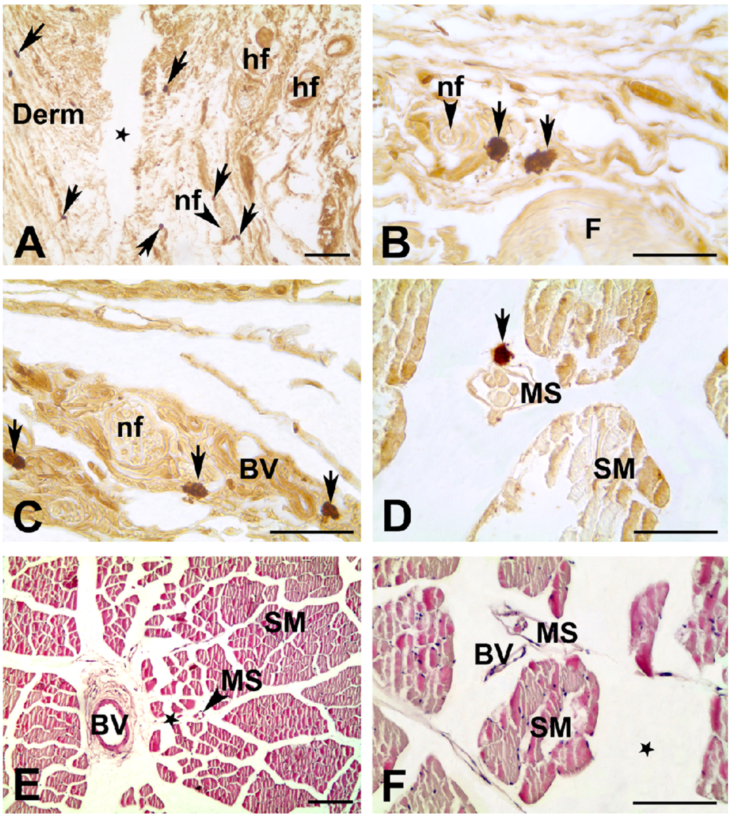

Fig. 3. A–C. Compressed and displaced nerve fibres and 5-HT-positive MCs showing signs of degranulation in the vicinity. **D.** Muscle spindles and 5-HT-positive MCs in the vicinity with signs of degranulation. **E, F.** H&E-stained sections showing compressed and destructed muscle spindles around the needle tract. Nerve fibres (nf), mast cells (arrow), hair follicles (hf), needle tract (star), dermis (Derm), fascia (F), blood vessels (BV), muscle spindles (MS). Scale bar=50 µm.

(Fig. 3B). The acupuncture needle mechanically destroyed a small number of mast cells. Their contents (as visible granules) were scattered all along the needle tract, sometimes at a significant distance from the mast cell itself.

DISCUSSION

Our results clearly demonstrate the presence of different nerve structures by S-100 immunohistochemistry and Bodian staining in the tissues surrounding the acupuncture needle tract. In the epidermis these were predominantly free nerve endings; the dermis and subcutis mostly contained free nerve endings around hair follicles and small nerve fibres parallel to blood vessels; in the striated muscle the main findings were small and large nerve fibres, as well as muscle spindles. Our findings clearly show that in tissue the acupuncture needle interacted with some nerve structures in their typical modality (free nerve endings) while displacing and more rarely destroying others (small nerve

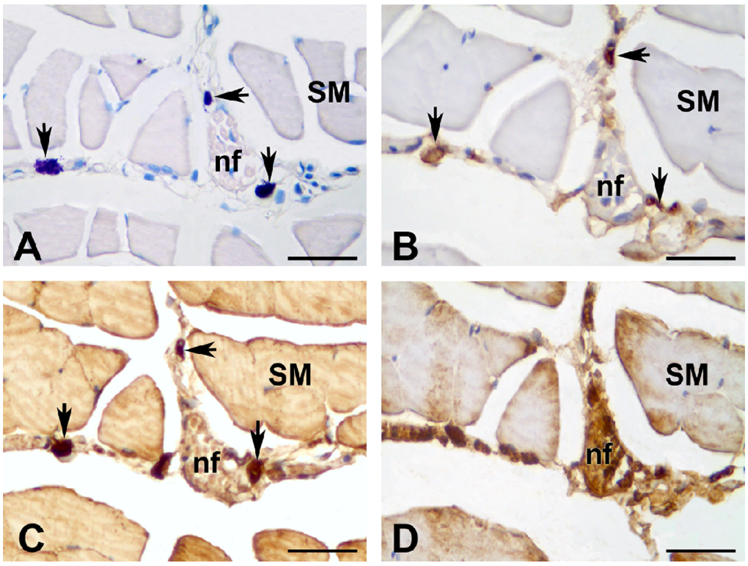

Fig. 4. Mast cells (MCs) in proximity to nerve fibres in striated muscle in the vicinity of the needle tract. **A.** Toluidine blue-stained MCs. **B.** Tryptase positive MCs**. C.** 5-HT positive MCs **D.** Visualisation of nerve fibres with S-100. MCs (arrow), nerve fibres (nf), striated muscle (SM). Scale bar=50 µm.

Table 1. Nerve structures in the tissue in the vicinity of the needle tract in Zusanli (ST_{36}) acupoint in rats

Location of nerve structures	Types of nerve structures
Epidermis	Free nerve endings
Dermis and subcutis	Free nerve endings, palisades of lanceolate nerve endings around the hair follicles, small nerve fibres
Striated muscle	Muscle spindles, small and large nerve fibres

fibres and muscle spindles in the vicinity of the needle tract).

The observed alterations following needling of the acupoint allowed concluding that they may play a major role in the response to acupuncture suggesting multiple ways of interaction between acupuncture needle and the nerves structures of the acupoint. We suggest that acupuncture combines stimulation, disruption and displacement, and destruction of nerve structures, together with additional interaction with mast cells, as seen by tryptase immunohistochemisty.

The observed nerve structures (free nerve endings, muscle spindles and nerve fibres) in the tissue adjacent to the needle tract did not largely differ from previously observed histological features of the acupoint (Tao, 1989; Dimitrov, 2012a; Dimitrov *et al.,* 2015). Those structures within and around the acupoint were suggested to be acupoint receptors (Kim *et al.,* 2006). Experimental evidence implied that the effects of acupuncture are mediated by activation of afferent nerve fibres innervating the skin and muscles (Kagitani *et al.,* 2010). For some researchers, the acupoint receptors, forming and maintaining the needling sensations in acupoints in areas of abundant musculature (such as ST36) are mostly muscle spindles (Wang & Liu, 1989). In this regard, Dung (1984) speculated that the acupoints were much more complex and their needling involved the simultaneous activation of large peripheral nerves, smaller superficial nerves, nerves passing though narrow spaces, motor points, neuromuscular junctions, blood vessels close to neuromuscular junctions, and bifurcations of peripheral nerves.

There are proofs that the stretch reflex of skeletal muscle was also involved in the acupuncture effect. It has been hypothesised that the closer to a muscle spindle a needle was inserted, the stronger the elicited stretch reflex was. Moreover, it was possible to puncture intrafusal fibres directly, inducing their direct contractions (Jin *et al.,* 2007). In our study, we demonstrated that this spindle-needle interaction was indeed possible.

Data that the acupoints were actually normal anatomical locations, and that there was nothing unusual in terms of receptors, which could contribute to their proposed special function (Yu *et al.,* 1996) were provided. The nerve density has been previously determined in skin biopsies, and it was suggested that a human acupoint does not necessary possess more, but rather less subcutaneous nerve structures compared with locations not accepted as effective for acupuncture (Wick *et al.,* 2007). Even though the density of nerve structures in acupoints is lower, they are not completely absent and are found in significant amount. In our research we did not perform a quantitative analysis of nerve structures in acupoint ST_{36}. However, the structures observed, despite their putatively lower density, cannot be ignored when describing the morphological basis for acupuncture. Their inevitable activation by needling is possibly one of the mechanisms contributing to the effect of acupuncture by neurohumoral, neuromuscular, and centrally mediated mechanisms. The lower numbers of receptors might be a factor facilitating easier manipulation and increased mechanical stimulation without causing unpleasant sensations.

Based on available data, we suggest a mechanism of mast cell-nerve interaction, involved in the acupuncture effect. Our results showed clusters of 5-HT positive mast cells in the immediate vicinity of nerve fibres, as well as close to hair follicles. In our previous studies (Dimitrov *et al.,* 2015), we have demonstrated abundant innervation around them, as well as direct serotonin release induced by acupuncture (Dimitrov *et al.,* 2017). Therefore, we should consider a possible interaction between nerves and mast cells. The idea of a neuromastocytic junction is not new; its role in acupuncture has been previously discussed (Chen & Zhang, 1987). Evidence hints towards the notion that mast cells are found intimately associated with structures of the peripheral nervous system in a variety of tissues (Johnson & Krenger, 1992). At this stage of research, such a functional association on a morphological level cannot be demonstrated but only their spatial proximity could be confirmed – nerve fibres shared perivascular localisation with mast cells. Acupuncture (mechanical stimulation) can activate mast cells, which can subsequently activate nerve cells (Yao *et al.,* 2014). It is acknowledged that manual acupuncture stimulation induced degranulation of mast cells, so mast cells were most probably involved in initiating acupuncture signals by stimulating by peripheral nerves (Sa *et al.,* 2013). The mechanism behind this could also involve direct receptor activation, as suggested by the direct neurotransmitter release caused by acupuncture (Dimitrov *et al.,* 2017). Moreover, the mediators released by the mast cells can diffuse through the loose connective tissue of the interstitum, in certain, more permeable direction (Tomov *et al.,* 2019). This "preferential permeability" of the interstitium for neurotransmitters may, at least partially, explain the connection between suggested acupuncture canals, and, for instance, pilomotor lines (Liu *et al.,* 2002).

An important finding in our study was the presence of muscle spindles in the vicinity of the needle tract, with some of them severely disrupted by the needle. Therefore, despite its small size the acupuncture needle was able to destroy some larger nerve structures. The large nerve fibres and blood vessels in the deep muscular plane of the acupoint were not damaged, but only displaced by the needle confirming the that acupuncture did not significantly disrupt the integrity of larger neurovascular structures (Dimitrov, 2012b).

CONCLUSIONS

Acupuncture combines destruction, disruption and displacement of nerve structures, which can be demonstrated on a morphological level. Additional interaction between needle and mast cells was also evident. Those mechanisms are undoubtedly involved in eliciting the needling sensation, and may play a role for the systemic effect of acupuncture.

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