INTRODUCTION
The incidence of tendinopathy and tendon injury has risen rapidly in the last three decades. Yet the functional outcome of tendon rehabilitation is still suboptimal partly due to poor innervation and vascularisation. Recently we demonstrated that injured tendons exhibit extensive new nerve fibre ingrowth into the normally almost aneuronal tendon proper during the early regenerative phase of healing. We also noted that this new nerve fibre ingrowth is followed by a specific temporal expression of sensory neuropeptides substance P (SP) and calcitonin gene related peptide (CGRP), which have previously been reported to promote angiogenesis and tissue regeneration by stimulating proliferation of endothelial cells and fibroblasts. Intermittent pneumatic compression (IPC), commonly used in the prevention of deep vein thromboses, has recently been reported to also improve the healing of ischemic wounds and fractures. The mechanisms behind the effects of IPC, however, remain largely unclear. The aim of this study was to evaluate the neurovascular healing effects of IPC on Achilles tendon repair.

METHODS

Animal: 20 male Sprague Dawley rats (180–200 grams) were subjected to blunt rupture to their right Achilles tendons under anaesthesia and allowed free mobilization after surgery. At day 3 post rupture, under anaesthesia, the IPC mini pump (Biopress SystemTM, Flexcell International), a specially designed device for rats, treatment started. The IPC cuff inflates (3 seconds) /deflates (27seconds) in a cyclic manner to a maximum pressure of 55mmHg one hour daily over two weeks (5 rats) and four weeks (5 rats).10 rats were served as controls. Five rats of each group where eutanized and fixated with Zamboni solution at week 2 and week 4 respectively. The entire Achilles tendons were harvested and sectioned (15µm) coronally.

Histology: H&E staining was applied for analysis of the tendon structure, e.g., the amount, orientation and maturation of collagen fibers, and manual counting of blood vessels was performed under 2× magnifications as described under semi-quantitative analysis. Immunohistochemistry (IHC): Immunofluorescent staining was performed according to the avidin / biotin method with antibodies against CGRP (1:10000) and SP (1:1000) and the nerve regenerating marker GAP43 (1:2000). Double staining of GAP43 with SP and CGRP was applied. A Nikon epifluorescence microscope (Eclipse E800, Yokohama, Japan) was used to examine the sections under 20x magnifications.

Semi-quantitative analysis: For each rat, four interval sections from different longitudinal levels of the tendon were stained. Semi-quantitative image analysis was used to assess the total area occupied by nerve fibres immunoreactive to CGRP within the tendon proper. Statistical analysis was performed by the Mann-Whitney U test. P value < 0.05 was considered to denote a significant difference.

RESULTS

Histology: IPC was found to enhance tendon healing according to collagen organization and number of new blood vessels both at 2 and 4 weeks post-rupture. The increased numbers of new vessels in the IPC treated tendons were mainly seen at the rupture site and at the musculotendinous junction. IHC: Gap-43, SP and CGRP positive fibers were identified in all parts of the Achilles tendon of both groups. However, there was a clear increase in neuropeptide immunoreactivity in the rats treated with IPC. The increase in Gap-43, SP and CGRP expression was most pronounced in the tendon proper at the rupture site. CGRP-immunoreactivity at the rupture site occurred both in free nerve endings and around newly formed blood vessels (Fig1. A-B). Double staining with GAP43 verified the ingrowth of new nerve fibers (Fig1. C-D).

Semi-quantitative analysis: The number of vessels and density of CGRP were increased in both groups over time. As compared to control, the total numbers of vessels were 62% and 43% higher in the treated group at week 2 and 4 respectively (Fig2. A). The density of CGRP positive fibers in the IPC treated group was also increased 50% and 34% at week 2 and 4 respectively, compared to controls. The density of CGRP increased with almost 100% in both groups between week 2 and 4 (Fig2. B).

DISCUSSION
The present study demonstrates that intermittent pneumatic compression enhances neuro-vascular ingrowth during healing after Achilles tendon rupture. Under normal condition the tendon proper is almost devoid of nerve fibres. After rupture, however, an ingrowth of vessels and nerves occurs, suggested to be essential for tendon healing. The mechanisms of IPC leading to the simultaneous increase in new nerve fiber ingrowth and angiogenesis in healing tendons are not fully investigated. In addition to the known effects of IPC, i.e. release of nitric oxide and increase in arterial blood flow, it may prove that IPC also enhances the release of sensory neuropeptides, SP and CGRP. These neuropeptides are known to promote angiogenesis and tissue regeneration by stimulating proliferation of endothelial cells and fibroblasts. The morphologic finding of increased SP and CGRP positive nerve fibres around newly formed blood vessel would comply with a role in angiogenesis, whereas the location of non-vascular free nerve endings interspersed between collagen bundles strengthens the notion of a role in fibroblast proliferation. Altogether our findings support that IPC may play an important role for the treatment of tendon injuries. Whether the increased neuro-vascular ingrowth leads to a biomechanically improved tendon tissue remains to be studied.

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Fig1. A-D. Fluorescence photomicrographs of Achilles tendon showing single staining (green) of CGRP fibers (A-B) and double staining (yellow) of Gap43 (green) with Substance P (red) (C-D) in the connective tissue around ruptured area of control (A, C) and IPC treated (B, D) tendon at week 2 (A-B) and week 4 (C-D). Objective = 20x, Bar = 50 µm. Arrow = positive fiber. V = vessel. CT = connective tissue.

Fig2. A-B. The number of blood vessels (A) and immunoreactivities of neutral CGRP (immunofluorescent area—µm²) (B) in control and IPC treated Achilles tendon over 4 weeks. * = P value < 0.05

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