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ORIGINAL STUDY

Qualitative analysis of Substance P, NK1-receptor and nerve ingrowth in Substance P-treated ruptured rat Achilles tendon

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Substance P has a stimulating effect on fibroblast proliferation, collagen organization, and angiogenesis in ruptured and subsequently sutured rat Achilles tendon. This effect is also reflected in the biomechanical properties of the tendon. The aim of this study was to substantiate the effect of exogenous substance P on endogenous substance P, NK-1 receptor, and nerve ingrowth in an *in vivo* tendon-healing setting.

Ninety-six male Sprague-Dawley rats were randomly assigned to one of four groups and injected with saline, substance P (10^{-6} µmol/kg BW and 10^{-8} µmol/kg BW) associated with neutral endopeptidase inhibitors, or neutral endopeptidase inhibitors alone into the paratendinous region of the ruptured and subsequently sutured Achilles tendons from the second until the sixth day postoperatively. Substance P, NK-1 receptor, and nerve ingrowth (PGP 9.5) were analysed using immunofluorescence at four different time points : one, two, four and six weeks postoperatively.

In all groups substance P was predominantly expressed in the extracellular matrix during the first two weeks, corresponding to fibroblast proliferation, and first disappeared from the saline group in the proliferative phase. In contrast, substance P was not expressed in the blood vessel wall during the first two weeks, when angiogenesis was most pronounced. NK-1 receptor was almost always expressed in the blood vessel wall and in the extracellular matrix during this period and disappeared progressively afterwards. No **nerve ingrowth was identified.**

Exogenously administered substance P in sutured rat Achilles tendon rupture does not stimulate sensory nerve ingrowth, but seems to have a booster effect on endogenous substance P for fibroblast proliferation via autocrine/paracrine stimulation.

Keywords : Achilles tendon rupture ; immunofluorescence ; SP ; NK-1 receptor ; PGP 9.5.

INTRODUCTION

The temporal expression of neuropeptides in the natural healing of Achilles tendon rupture has been documented in rats, suggesting an essential role in tendon repair. During the early inflammatory phase, one to two weeks post rupture, the local concentration of substance P (SP) increases. SP is a

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neuropeptide and one of the main mammalian tachykinins, which is widely distributed in the central and peripheral nervous systems (*27*). In addition to its nociceptive and proinflammatory actions, SP has been found to exert trophic effects in different tissues by preferentially activating a specific receptor, the NK-1 receptor (*19,28,32*).

This observation has led us to hypothesize that in the days immediately following Achilles tendon rupture, local SP secretion plays a crucial role in fibroblast proliferation and increase in capillaries. Previous studies reported the effect of paratendinously injected SP on ruptured and subsequently sutured rat Achilles tendons. Histologically, a dose-dependent effect on fibroblast proliferation was demonstrated and was correlated with a faster collagen organization in the ensuing weeks. Biomechanically, SP improved tendon healing by enhancing stress at maximal load and work to maximal load (*10,31*).

Scarce published data exist on the effect of exogenous SP on endogenous SP, its receptor, and sensory nerve ingrowth in tendon. Therefore, we performed a qualitative analysis to localize SP, the NK-1 receptor and PGP 9.5, a sensitive neural/nerve sheath marker in non-neoplastic tissues (*11*).

Four predefined regions in the tendon were studied : pre-existing tendon tissue, immature wound healing histologically characterized by myxoid tissue and the presence of young collagen fibres, scar tissue characterized by extensive mature collagen production, and paratenon.

MATERIAL AND METHODS

Intervention

The right Achilles tendon of 96 male Sprague-Dawley rats (2 months old) was ruptured in the midpart, using blunt scissors. The suture was made with a modified Kessler stitch. After surgery, the rats were randomly assigned to one of eight cages, with a maximum of three per cage with animals randomly divided into four groups. Each group consisted of 24 rats, and each animal was injected paratendinously from the second until the sixth postoperative day with SP 10^{-6} µmol/kg BW combined with thiorphan (T) and captopril (C), with SP 10^{-8} µmol/kg BW combined with T and C, or with T and C alone. To inhibit local degradation of SP, the injection was preceded by an infiltration of thiorphan 1 µmol/kg BW and captopril 30 µmol/kg BW. Thiorphan and captopril are both inhibitors of neutral endopeptidase, a membrane-bound surface peptidase by which SP is degraded and inactivated (*28*). The control group was injected with saline. A 23-gauge needle was inserted from proximal to distal and from posterior to anterior at a 30° angle to the skin. The insertion site was approximately 1 cm above the proximal end of the skin incision. After bone contact was established, the needle was withdrawn slightly, and the solutions were slowly injected. Afterwards, the rats were allowed to move freely in their cage with unrestricted access to water and food. In each group, six rats were killed at one, two, four, and six weeks postoperatively. The experiments were performed with the approval of the Animal Ethics Committee of the Ghent University Faculty of Medicine and Health Sciences (Ref. ECP 11 02/34). For detailed practical information about material and methods we refer to our previous papers (*10,31*).

Immunofluorescence

Four predefined regions in the tendon were studied : pre-existing tendon tissue (region 1), immature wound healing histologically characterized by mixoid tissue and immature collagen fibres (region 2), scar tissue characterized by extensive mature collagen production (region 3), and paratenon (region 4).

From each biopsy specimen H&E slides were made and the different regions were localized on each slide (fig 1). All staining was performed simultaneously.

Using a tissue microarray processor (MTA-1, manual tissue arrayer, Beecher Instruments) three tissue punches of each region were obtained and transferred into a new paraffin block. From this block, paraffin sections 3 to 4-µm thick were cut and used for indirect immunofluorescence with monoclonal antibodies raised against SP (Abcam, ab 14184-50), NK-1 receptor (Chemicon, AB 58000) and PGP 9.5 (Abcam, ab 8189-250) for 72 h at 4°C. As the secondary antibody, Alexa Fluor (495,488) (Invitrogen NV, Belgium) was incubated for 1 h at room temperature. To exclude non-specific binding of the primary antibodies, negative controls of only reaction buffer instead of diluted primary antibodies were used. To avoid non-specific binding of the secondary antibody, goat serum (Dako) was applied for 30 min to each slide. A series of different antibody dilutions was applied on rat tissue to obtain the optimal concentration for each antibody. All antibodies were diluted in Tris-HCl buffer

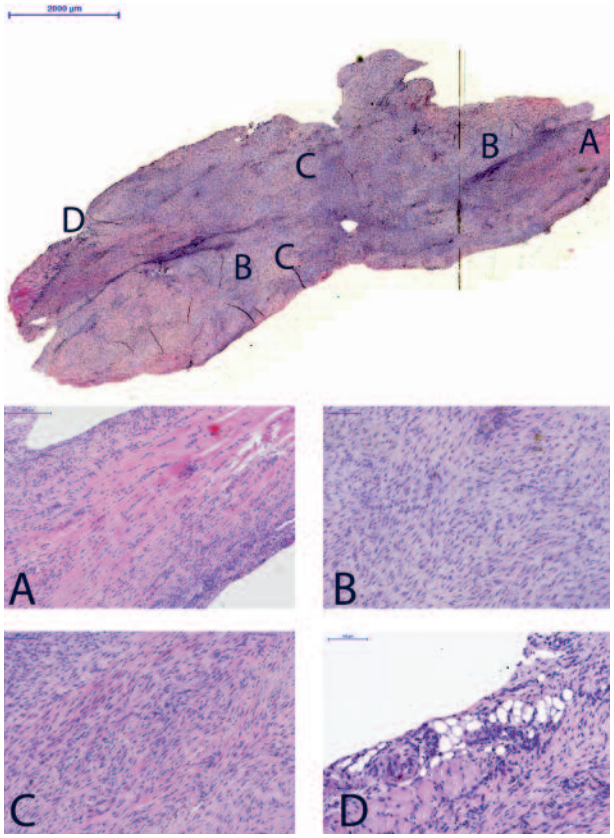

Fig. 1. — General histological overview of a whole mounted sutured rat Achilles tendon (H&E stained). Four predefined regions were selected. A : pre-existing tendon tissue (region 1) ; B : immature wound healing histologically characterized by myxoid tissue and immature collagen fibres (region 2) ; C : scar tissue characterized by extensive mature collagen production (region 3) ; D : paratenon (region 4).

(Dako) ; the concentrations of the primary antibodies were 1/500 for SP, 1/175 for NK-1 receptor, 1/20 for PGP 9.5, and 1/5000 for all secondary antibodies. As the nuclear counter stain, DAPI (4',6-diamidino-2-phenylindole) was used at a concentration of 1/5000. The immunofluorescent-stained slides were analysed using a fluorescence microscope (BX-40 Olympus, Japan).

Because of the negative results of PGP 9.5 (see results section), the mounted longitudinal section of the rat Achilles tendon was entirely stained with antibodies directed against PGP 9.5, S 100 (general nerve marker), NSE, CD 56 (N-CAM) and BCL 2, to rule out missing of nerve ingrowth by punching parts of the regions.

Statistical analysis

An independent lab technician assigned a code number to each sample. SP, NK-1 receptor and PGP 9.5 expression were scored as positive (+) or negative (0) in the blood vessel wall and in the extracellular matrix (ECM) for each region. A negative score indicated that no immunofluorescence was detected in any of the three punches per region per rat. A positive score per rat indicated that at least one punch per region showed immunofluorescence expression for the parameter studied.

Finally, six rats were killed in each group at the different time points and were scored as negative if they had a negative score for the same parameter. Positivity was scored as +, ++ or +++ if one or two rats, three or four rats, and five or six rats, respectively, scored positive for the parameter studied.

Interobserver reliability between the two blinded investigators was examined using the intraclass correlation coefficient (ICC).

At the end of the study, one of the examiners broke the code of each sample.

RESULTS

Tables I, II, III and IV summarize the results of the qualitative analyses.

Table I shows the expression of SP and NK-1 in the blood vessel wall and the ECM in the four different groups and regions in the first week (see table I). In contrast to SP results, the NK-1 receptor could be demonstrated in the blood vessel wall in all groups and all regions. In the ECM, SP and the NK-1 receptor were expressed in all regions and all groups (fig 2).

Table II demonstrates the expression of SP and NK-1 in the blood vessel wall and the ECM in the four different groups and regions in the second week. SP was not expressed in the blood vessel wall in any group, whereas the NK-1 receptor was always expressed in all regions and all groups. In the ECM, SP and NK-1 receptor expression was present in all regions in the saline group and in the group injected with SP 10^{-6} µmol/kg BW + T and C. In the group injected with SP 10^{-8} µmol/kg BW + T and C, SP and the NK-1 receptor were not expressed paratendinously in the ECM, contrary to the other regions. In the group injected with T and

Table I. — Qualitative analysis of SP and the NK-1 receptor in week 1. Immunofluorescence is markedly positive + (1-2 rats per group) or negative (0) in the blood vessel wall (BVW) and extra cellullar matrix (ECM) in the different regions

	saline				SP 10^{-6}				SP 10^{-8}				T and C			
	BVW[a]		ECM[b]		BVW		ECM		BVW		ECM		BVW		ECM	
	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1
R[c]1	0	+++	+++	+++	0	+++	+++	+++	0	+++	++	++	0	+	++	++
R 2	0	+++	+++	+++	0	+++	++	++	0	+++	+++	+++	0	+++	+++	+++
R 3	0	+++	+++	+++	0	++	+++	+++	0	+++	+++	+++	0	+++	+++	+++
R 4	0	+++	+++	+++	0	+++	+++	+++	0	+++	+++	+++	0	+++	+++	+++

[a] blood vessel wall, [b] extracellular matrix, [c] region.

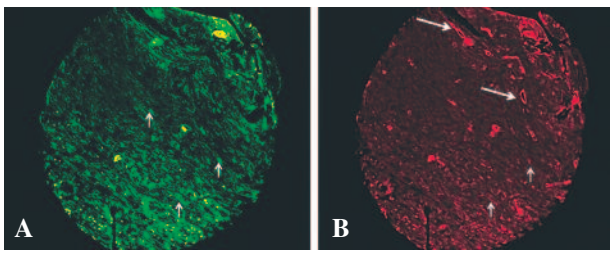

Fig. 2. — A. SP expression (green fluorescence) showing positivity in the ECM (small arrow) in myxoid tissue of rat injected with SP 10^{-6} µmol/kg BW + T and C and sacrificed one week postoperatively. Magnification 400×. B. NK-1 receptor expression (red fluorescence) showing positivity in the ECM (small arrow) and in the blood vessel wall (long arrow) in mixoid tissue of rat injected with SP 10^{-6} µmol/kg BW + T and C and sacrificed one week postoperatively. Magnification 400×.

C, SP expression was absent from regions 2 and 3, and NK-1 receptor expression was absent from region 2.

Table III illustrates the expression of SP and NK-1 in the blood vessel wall and the ECM in the four different groups and regions in the fourth week. In the saline group SP was not expressed, either in the blood vessel wall, or in the ECM. The NK-1 receptor was expressed in the blood vessel wall in all regions, whereas in the ECM, it was not expressed paratendinously. In the SP-treated groups and the group injected with T and C, the expression of SP and NK-1 varied in the different regions as shown in table III.

Table IV reflects the SP and NK-1 expression in the blood vessel wall and the ECM in the four different groups and regions in the sixth week. In the saline group SP was never expressed either in the blood vessel wall or in the ECM. The NK-1 receptor was still expressed in the blood vessel wall in all regions, whereas it disappeared from the ECM, except in one rat paratendinously. In the SP-treated groups the expression still varied in the different regions as shown in table IV. In the group injected with T and C, SP and the NK 1-receptor were expressed in the blood vessel wall in region 2 in only two rats. In the ECM, the NK1-receptor was expressed only in region 2 in a single rat.

None of the four regions stained for PGP 9.5. The ICC was 0.945 (CI 95%).

DISCUSSION

SP is a neuropeptide that is widely distributed in the central and peripheral nervous system (*27*). In the periphery, temporal expression of SP has been demonstrated in the healing of ruptured rat Achilles tendon, which is consistent with specific actions in injury, pain and regeneration (*1*). In a previous study focusing on regeneration, SP was injected paratendinously into sutured rat Achilles tendon ruptures in an attempt to promote tendon healing. From the second until the sixth day postoperatively, SP was injected at two concentrations (SP 10^{-6} µmol/kg BW and SP 10^{-8} µmol/kg BW), followed by T and C injection. Two control groups were included : saline alone and C and T alone. The endopeptidase inhibitors were administered in association with SP to slow down its degradation (*21*).

The selected concentrations of SP were derived from other studies (*21,22,23*). Although Ackermann

Table II. — Qualitative analysis of SP and the NK-1 receptor in week 2. Immunofluorescence is markedly positive + (1-2 rats per group) or negative (0) in the blood vessel wall (BVW) and extra cellullar matrix (ECM) in the different regions

	saline				SP 10^{-6}				SP 10^{-8}				T and C			
	BVW[a]		ECM[b]		BVW		ECM		BVW		ECM		BVW		ECM	
	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1
R[c]1	0	+++	+++	+++	0	++	++	++	0	++	++	++	0	+++	++	++
R 2	0	+++	++	++	0	+	++	++	0	++	++	++	0	+++	0	0
R 3	0	+++	++	++	0	++	+	++	0	++	+	+	0	+++	0	+
R 4	0	++	+	+	0	+++	++	++	0	++	0	0	0	+++	++	++

[a] blood vessel wall, [b] extracellular matrix, [c] region.

Table III. — Qualitative analysis of SP and the NK-1 receptor in week 4. Immunofluorescence is markedly positive + (1-2 rats per group) or negative (0) in the blood vessel wall (BVW) and extra cellullar matrix (ECM) in the different regions

	saline				SP 10^{-6}				SP 10^{-8}				T and C			
	BVW[a]		ECM[b]		BVW		ECM		BVW		ECM		BVW		ECM	
	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1
R[c]1	0	++	0	+	0	+++	++	++	0	++	0	0	0	+++	0	0
R 2	0	++	0	+	0	0	0	+	+	++	+	+	0	+++	0	0
R 3	0	++	0	+	0	+	0	+	0	++	0	+	+	++	+	+
R 4	0	+++	0	0	+	++	++	++	0	+	+	+	+	++	+	+

[a] blood vessel wall, [b] extracellular matrix, [c] region.

Table IV. — Qualitative analysis of SP and the NK-1 receptor in week 6. Immunofluorescence is marked positive + (1-2 rats per group) or negative (0) in the blood vessel wall (BVW) and extra cellullar matrix (ECM) in the different regions

	saline				SP 10^{-6}				SP 10^{-8}				T and C			
	BVW[a]		ECM[b]		BVW		ECM		BVW		ECM		BVW		ECM	
	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1	SP	NK1
R[c]1	0	++	0	0	0	+	0	0	0	+	++	++	0	0	0	0
R 2	0	++	0	0	+	++	0	++	+	+	0	0	+	+	0	+
R 3	0	++	0	0	0	0	0	0	0	+	0	0	0	0	0	0
R 4	0	++	0	+	+	++	+	+	0	0	0	0	0	0	0	0

[a] blood vessel wall, [b] extracellular matrix, [c] region.

et al found that both the absolute concentration of neuropeptides and the rate of change are relevant, we used the same concentration from the second until the sixth day (*1*).

In the natural healing of rat Achilles tendon rupture, Ackermann *et al* identified a shift from SP-positive nerve fibres in the loose connective tissue in the inflammatory phase to the tendon tissue proper in the regenerative phase. In our previous study, a dose-dependent proliferative effect of SP on fibroblast proliferation and angiogenesis was clearly demonstrated in ruptured and subsequently sutured rat Achilles tendon. This effect was most pronounced in the first week, compared to the control group. In the second week, the number of fibroblasts declined in all groups but, contrary to the number of capillaries, the difference between the SP-treated groups and the control group remained significantly higher (*10*) (fig 3 & 4).

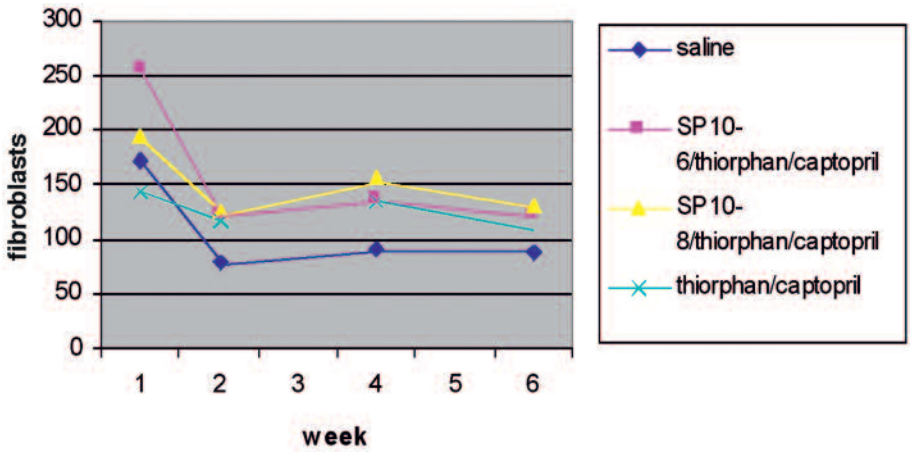

Fig. 3. — Mean number of fibroblasts per field at 1, 2, 4, and 6 weeks in the different groups

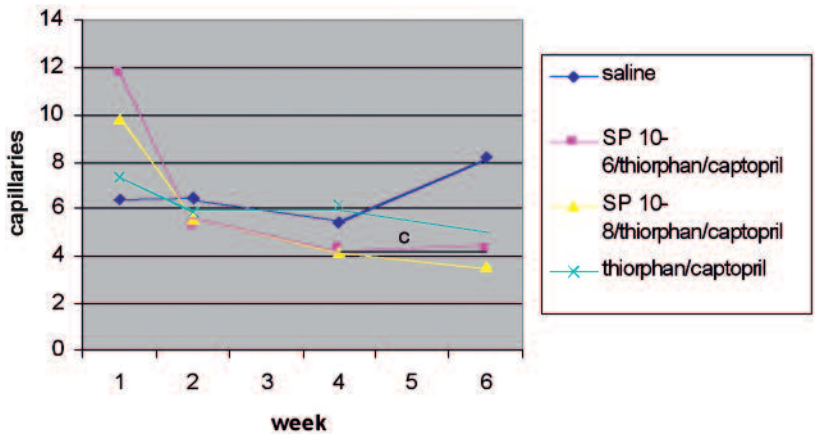

Fig. 4. — Mean number of capillaries per field at 1, 2, 4, and 6 weeks in the different groups

The present study was designed to observe and localize the effect of exogenous SP on its receptor and sensory nerve ingrowth. A difference between the blood vessel wall and the ECM in terms of localization emerged in relation to the stimulatory role of SP in angiogenesis and fibroblast proliferation, respectively.

In the first week, SP expression was expected to be present in the ECM and blood vessel wall in all groups because fibroblast proliferation and angiogenesis were most pronounced at the end of the inflammatory phase in all groups. This prediction corresponded with our findings for SP in the ECM. However, in the blood vessel wall, SP expression could not be detected in any of the groups. SP is known to induce autocrine and paracrine stimulation of fibroblasts, which can explain the constant presence of SP in the ECM (*5*). The progressive disappearance of SP from the ECM in the proliferative phase was faster in the saline group than in the SP-treated groups and was parallel to the decline of fibroblast proliferation in the different groups.

In contrast, no autocrine stimulation by endothelial cells has been reported in literature. We hypothesize that the stimulation of angiogenesis, which was most pronounced in the SP-treated

groups, is the result of the administered SP, which was degraded on day 7 and resulted in a negative immunofluorescence staining.

A quantitative analysis of SP would be interesting for additional information on the concentration of endogenous SP in the different groups, but this was beyond the scope of this study.

We expected a predominant expression of SP in region 2 and region 3, where fibroblast proliferation was most pronounced. However, SP was also expressed in region 1 and 4 in the majority of rats. In the paratenon three main types of synovial cells are present in the ECM, which are suggested to play an important role in inflammatory conditions (*17*). SP has been shown to participate in the regulation of synoviocyte proliferation (*25*). Consequently, the expression of SP at the paratenon could contribute to its role in regeneration as well as in inflammation.

NK-1 receptor expression was studied because SP has the highest affinity for NK-1, which is also the receptor in pain and neurogenic inflammation (*2,18*). NK-1 receptor expression was always present when SP was expressed in the ECM. When SP disappeared from the ECM, the NK-1 receptor could still be expressed. This result fits with the concept that neuropeptides such as SP regulate the number and sensitivity of their own receptors by several feedback mechanisms (*33*). Contrary to SP, the NK-1 receptor was always expressed in the blood vessel wall, in the first week in all groups and slowly disappeared afterwards. The permanent presence of the NK-1 receptor may not only be related to angiogenesis, but also to its role in plasma extravasation, which in turn makes it possible for other (pro)inflammatory and growth factors carried via the circulation to pass the blood vessel wall and to participate in the healing process (*6,12,33*).

PGP 9.5 immunofluorescence was performed to evaluate nerve ingrowth, which could be stimulated by SP. Strikingly, no PGP 9.5 immunoreactivity was present in any of the groups. This finding contrasts with those of Ackermann *et al*, who demonstrated nerve ingrowth in the natural healing of rat Achilles tendon rupture (*1*).

In the inflammatory phase, SP-positive nerve fibres were predominantly expressed in the loose connective tissue surrounding the paratenon. We did not explore this region. In the regenerative phase, Ackermann *et al* demonstrated active nerve ingrowth where SP was expressed mainly in the proper tendinous tissue. In our study, SP was predominantly expressed in the ECM in the first week (end of the inflammatory phase) and presented as punctate spots, not as linear. Consequently, SP expression in our study is likely to be released by the fibroblasts via autocrine and paracrine secretion after stimulation with the administered SP. From this perspective, RT-PCR could have confirmed our findings. In a recent study , the presence of SP and the NK-1 receptor was demonstrated in tenocytes of the human Achilles tendon by immunohistochemistry and in situ hybridization (*3*).

The reason why we found no nerve ingrowth in the tendon tissue proper is unclear. In the study of Ackermann *et al* the ruptured rat Achilles tendons were not sutured, and the rats were allowed to move freely in their cage without immobilization. This design left open the possibility for retraction of both ends, which could result in a gap making it physically easier for new nerve ingrowth to enter the repair site. This approach is in contrast to our study in which the two ends of the tendon were brought together by a modified Kessler stitch. Sutures were utilized to achieve tendon apposition to minimize scar tissue formation, promote collagenization, and avoid the presence of any non-tendinous tissue between the two ends (*26*). Therefore, it would be of interest to know whether the new nerve ingrowth documented by Ackermann *et al* was the result of sprouting, e.g., from the nerves in the loose connective tissue around the paratenon, or occurred via an extrinsic pathway.

Several substances have been studied in an attempt to shorten healing time (*4,13,14,15,16,30,35,36,37*). A strong point of the present study is the inclusion of an immunofluorescence evaluation, with results that complemented the previous histopathologic findings.

Despite the stimulating effect of SP, some care should be taken in extrapolating these results to humans because we used blunt scissors to transect the Achilles tendon, which does not represent a true rupture. The majority of acute Achilles tendon

ruptures result in a typical horse-tail or mop-end appearance, which researchers have not been able to reproduce experimentally (*20*). Moreover, in humans Achilles tendon rupture often occurs in subclinical pre-existing disorders while our experiment was on healthy tendons (*23*).

Another factor worth considering may be that human ruptured Achilles tendons are immobilized for at least six weeks, while the rats in our study were allowed to move freely (*34*). In this context, mechanical stimulation might have contributed to fibroblast proliferation and angiogenesis. The detrimental effects of immobilization on tendons appear to be related, at least in part, to a significant reduction in sensory neuropeptide receptor expression (*7,8*). However, to neutralize the side effects of mobilization, we did include a control group of saline-treated rats in our study.

Another interesting aspect that remains to be addressed is examination of the contralateral tendons of all rats and comparison of these with a group of normative tendons. In case of a difference, the results would allow study of any centrally mediated effect in addition to the peripheral mechanisms investigated in the present study.

Finally, because SP stimulates proliferation of skin fibroblasts, it would be of interest to determine whether it also positively influences the notoriously problematic healing of the overlying skin following open Achilles tendon repair (*9,38*).

We hypothesize that SP not only affects fibroblasts and endothelial cells but also has an effect on immune cells, which could be induced by influencing other growth factors and cytokines. Further research is necessary to explore this pathway.

CONCLUSIONS

Our findings demonstrate that the administration of SP stimulates angiogenesis and fibroblast proliferation in ruptured rat Achilles tendons, and has a booster effect on endogenous substance P for fibroblast proliferation via autocrine/paracrine stimulation. Sensory nerve ingrowth was not stimulated. Although care should be taken in extrapolating these results to humans, SP administration after tendon rupture might be of interest as a potential new approach to the treatment of Achilles tendon rupture.

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