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ORIGINAL STUDY

Doxycycline impairs tendon repair in rats

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Doxycycline exhibits various effects apart from its antimicrobial activity, such as inhibition of matrix metalloproteinases (MMPs). MMPs, mainly collagenases and gelatinases, are capable of degrading virtually all constituents of the extracellular matrix and are critical to connective tissue remodelling and healing. We therefore hypothesised that doxycycline would negatively influence the rat tendon healing process and impede tendon regeneration. The Achilles tendon of 60 Sprague Dawley rats was transected transversely. The animals were treated with doxycycline, 130 mg/kg body weight/day. The healing tendons were evaluated mechanically at 5, 8 and 14 days. Doxycycline significantly decreased force at failure ($p < 0.005$) and energy uptake ($p < 0.001$). Doxycycline serum concentration was 3.4 (SD 1.0) µg/ml. In conclusion, tendon healing can be affected by doxycycline at clinically relevant serum concentrations. This observation might be of relevance to further studies exploring effects of MMP-inhibitors on tendon tissue.

Keywords : tendon repair ; doxycycline ; matrix metalloproteinase (MMP).

INTRODUCTION

Doxycycline, a member of the tetracycline family, is a commonly used broad spectrum antibiotic. Alongside their antimicrobial effects, tetracyclines also inhibit matrix metalloproteinases (MMPs), and among the clinically approved tetracyclines, doxycycline is the most potent in this regard (*9*).

MMPs are a group of zinc dependent neutral proteinases that are capable of degrading almost all constituents of the extracellular matrix (*22*). MMPs are fundamental in connective tissue remodelling and healing (*6, 15*). There is also evidence that MMPs participate in tendon healing and remodelling (*12, 16*). Furthermore, *in vitro* studies have shown doxycycline inhibition of collagen synthesis (*4, 11*). Thus, inhibition of MMPs and of collagen synthesis should be detrimental to tendon healing and could negatively affect the long-term outcome of tendon rupture.

The purpose of this study was to investigate the effect of clinically relevant concentrations of doxycycline on the rat Achilles tendon healing process. Our hypothesis was that doxycycline would negatively influence the healing process and render the tendon weaker.

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MATERIALS AND METHODS

The study was approved by the local animal ethics committee for animal experiments, and followed the established guidelines.

Sixty female Sprague Dawley rats (Scanbur BK, Stockholm, Sweden) weighing 221 (SD 10)g were used for this study. The animals were allowed to acclimatize to laboratory conditions for 7 days prior to any experimental manipulation. The animals were randomised cage-wise (two rats at a time) to six groups ; 5, 8 and 14 days doxycycline treatment and 5, 8 and 14 days untreated control.

The treatment groups received doxycycline hyclate (Sigma-Aldrich, Stockholm, Sweden), administered in deionised drinking water, starting one day before the operation. Control animals received deionised drinking water. Water bottles from all cages were weighed once daily during the study period. Shortly before sacrifice, blood was collected from five randomly chosen rats of the doxycycline 14 days group for serum analysis of doxycycline concentration.

Surgical procedure

The animals were anaesthetised with isoflurane gas (Forene, Abbot Scandinavia, Solna, Sweden) and given preoperative subcutaneous injections of 9.6 mg trimetoprim-sulfoxide (Bimotrim vet., Ceva Vetpharma AB, Lund, Sweden) and 0.015 mg buprenorphine (Temgesic, Schering-Plough, Brussels, Belgium). The surgeon was blinded for treatment during the operation. All groups received the same surgical procedure of Achilles tendon transection. The skin on the left hind paw was shaved and washed with chlorhexidine. A 3 mm transverse skin incision was made over the lateral side of the Achilles tendon. The Achilles tendon complex was dissected free from other tissues and 8 mm of the plantaris tendon was removed. Thereafter, the Achilles tendon was cut transversely 3 mm proximal to the calcaneal insertion. Thus, a tendon defect was created, which was left unsutured to become filled out by a tendon regenerate. The skin was sutured. Animals were allowed free cage activity immediately after the operation. At the evaluation day, animals were killed by CO_2 asphyxiation.

Mechanical testing

Directly following sacrifice, the tendon with the attaching calcaneus was transected free from other tissues and removed. The callus diameter was measured sagittally and transversely with a digital calliper, and the cross-sectional area was calculated assuming elliptical geometry. The tendon was then fixed between two clamps, one of them a custom made calcaneal clamp holding the bone in 30° dorsiflexion relative to the direction of traction and the other sandwiching the tendon's proximal end between fine sand papers. The clamps were attached to a material testing machine (100 R, DDL Inc. Eden Praire, MN, USA) and pulled at a constant speed of 0.1 mm/s until failure. Peak force, stiffness and energy uptake at 10% droop of the curve were recorded.

Analysis of serum levels of doxycycline

Shortly before sacrifice, five randomly chosen doxycycline-treated rats in the 14 days group were anesthetised with isoflurane gas and blood was collected by cardiac puncture. The blood samples were centrifuged at 2000 $\times$ g for 10 minutes and the supernatant stored at -70° C until testing. Serum concentrations of doxycycline were determined by way of an agar well diffusion assay using *Bacillus cereus* ATCC 11778 as the test organism (Smittskyddsinstitutet, Solna, Sweden) (*13*).

Statistical analysis

Data were analyzed using a two-way ANOVA test. Because the variance in each group appeared to be proportionate to the mean value, the data was ln-transformed before analysis to produce equal variance. After ln-transformation, no significant differences between variances remained.

RESULTS

There were 4 exclusions in the 5 days group (2 controls and 2 doxycycline rats) and 2 controls in the 8 days group. This was due to post-operative complications and technical problems during mechanical testing. There were no differences in weight gain between doxycycline treated rats and controls.

All specimens ruptured in the newly formed tendon regenerate between the transection stumps.

Doxycycline significantly decreased force at failure and energy uptake over time. These results were clearly evident already on day 5. Stiffness, transverse area and stress at failure were not

Table I. — Results 5, 8 and 14 days post-transection of rat Achilles tendon. Force at failure and energy uptake were significantly decreased by doxycycline treatment

	Treatment	5 days		Decrease (%)	8 days		Decrease (%)	14 days		Decrease (%)	p*
		Mean	SD		Mean	SD		Mean	SD		
Force (N)	Control	12.8	2.7		23.6	2.6		47.3	8.1		< 0.005
	Doxycycline	9.8	3.1	23.1	20.2	5.4	14.5	41.5	9.6	12.2	
Stiffness (N/mm)	Control	5.0	1.2		7.3	1.8		14.9	2.8		> 0.05
	Doxycycline	4.2	1.3	16.3	6.2	1.5	14.4	15.4	3.0	-3.0	
Energy (Nmm)	Control	24.8	6.4		65.5	10.0		128.9	37.7		< 0.001
	Doxycycline	18.9	6.1	23.9	50.8	11.7	22.5	92.6	27.7	28.2	
Area (mm^2)	Control	7.5	1.7		4.9	1.7		9.6	1.2		> 0.05
	Doxycycline	6.3	1.4	12.5	4.7	1.4	2.5	8.4	1.6	12.0	
Stress (MPa)	Control	1.8	0.5		5.5	2.0		5.0	1.3		> 0.05
	Doxycycline	1.5	0.5	12.9	4.6	1.6	16.8	5.0	1.3	0.0	

* p-values based on ANOVA of ln-transformed values.

affected significantly (table I). Doxycycline serum concentration was 3.4 (SD 1.0) μg/ml.

DISCUSSION

Rats exposed to therapeutic concentrations of doxycycline in serum demonstrated diminished healing during the early stages of experimental tendon repair, as measured by force and energy uptake at failure. The effects were not large, and perhaps it cannot be ruled out that they are due to unspecific side-effects of doxycycline. For example, doxycycline might have caused the rats to be less active, which could render their tendons weaker due to decreased mechanical loading. However, we know of no such effects and therefore believe that the results are due to doxycycline's pharmacological effects on tendon tissue metabolism.

Impaired tissue mechanical properties were evident already on day 5, corresponding to the inflammatory and early proliferative stage of healing in this model. The measured mechanical properties describe the new tissue regenerate between the stumps. This early tendon callus consists of a loose collagenous network, rich in proteoglycans and with a large proportion of collagen type III. When tendon repair enters the remodelling phase (in our model at about 1 week) (*2*), the loose callus is gradually replaced by a more densely organized collagen, mostly type I (*19*). The impaired mechanical properties imply, not unexpectedly, that MMPs play an important role in removing the early callus tissue, when it is to be replaced. Our results suggest, that this removal is also a prerequisite for building strength. Thus, doxycycline appears to have delayed remodelling.

Doxycycline is a promiscuous molecule with several bioactive moieties. It has been shown to inhibit several MMPs at pre- and post-translational levels. Additionally, doxycycline can inhibit MMPs by indirect mechanisms, e.g. by reducing the degradation of endogenous tissue inhibitors of metalloproteinases (TIMPs) indirectly, further potentiating the MMP-inhibitory effect of doxycycline (*9*). This probably impairs essential clearing up processes necessary for regeneration and may also distort important remodelling mechanisms.

The current findings support previous communications suggesting that MMP-inhibitors impair cutaneous wound contraction via effects on myofibroblasts (*15*). The tendon callus and the dermal granulation tissue show similarities suggesting that MMP-inhibition should lead to similar effects. Other studies, however, show that both skin and intestinal wound strength are enhanced by MMP-inhibitors (*10, 20, 23*). These contrasting results imply that the effects of MMP inhibitors might be highly model-dependent. Our main finding is

therefore mostly that effects of some kind were seen also with a low dose.

MMPs might also be required for the release of growth factors, lingering in the extracellular matrix and necessary for stimulation of tenocytes and vessel ingrowth during repair. Indeed, it has been shown that doxycycline inhibits neovascularisation (*8, 14*). This could lead to impaired regeneration of wounded tissue. Tetracyclines have also been reported to have effects on levels of/expression of iNOS, IL-1b, TNF-α and PGE2, thereby inhibiting collagen synthesis, fibroblast proliferation and MMP-expression. Although results and opinions diverge (*1, 3, 7, 17, 21*), these mechanisms could help explain the findings of the present study.

The dose administered produced a serum concentration corresponding to what is normally achieved by giving 200 mg daily in humans (*18*), which confirms that we have studied the effect of a clinically relevant dose.

This study is, to our knowledge, the first to show a pharmalogical effect of MMP-inhibitors on tendon repair. The effect need not always be detrimental and, at least theoretically, there are several potentially beneficial applications of MMP-inhibitors on tendon tissue, e.g. against rheumatoid arthritis-associated tendon breakdown (*5*). We conclude that doxycycline negatively influences the early stages of experimental tendon healing but that further studies of MMP-inhibitors on tendon tissue are needed to fully explore their effects and mechanisms of action.

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