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Original Article

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# Calcitonin effect on Achilles tendon healing. An experimental study on rabbits

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**Abstract**

**Objective:** A positive potential effect of Calcitonin (CT) on Achilles tendon healing was investigated as well as the ability of MRI to follow the tendon healing process. **Materials and Methods:** A standardized tenotomy of the Achilles tendon was performed on forty-two rabbits. Twenty-one animals received daily 21 IU /kg Calcitonin intramuscularly (treatment group CT) during the experiment and the remaining received saline solution (control group P). Seven animals from each group were killed at one, two and three weeks postoperatively. All animals had serial MRI scans and tendon samples underwent biomechanical and histological testing. **Results:** For both groups, animals of the same subgroup showed statistically significant difference in signal intensity values of MRI between the 1st and 3rd week ($p<0.001$) and between the 2nd and 3rd week ($p<0.001$). Signal intensity values of MRI didn't show any differences between animals under treatment and controls measured at 1st ($p=0.23$), 2nd ($p=0.23$) and 3rd ($p=0.53$) postoperative week. Tendon samples from group CT showed statistically significant difference in ultimate tensile strength compared to controls at 2 ($p<0,0005$) and 3 ($p<0,0005$) weeks post-surgery. Histology showed a positive Calcitonin effect at all tendon healing stages. **Conclusion:** It is suggested that Calcitonin enhances Achilles tendon healing process.

**Keywords:** Calcitonin, Tendon Healing, MR Imaging, Biomechanical Testing, Achilles Tendon/Histology

## Introduction

Achilles tendon is a common site of acute and overuse injuries and its healing is a time consuming process[1]. The best treatment for Achilles tendon ruptures is still unclear[2-6]. Several studies have been conducted in an attempt to reduce tendon healing time by using ultrasound[7-10], by applying early controlled motion and tensile stress across the tendon[11-14], by galvanic stimulation[15], by administering growth factors[16], bone morphogenetic proteins[17], chondromorphogenetic proteins[18], and non-steroid inflammatory drugs[19,20].

Calcitonin has an extensively studied biological inhibitory effect on osteoclastic bone resorption[21], a stimulus effect on osteocyte function[22-24], and a less studied stimulus effect on osteoblast function[25-27]. It has also been shown that calcitonin stimulates chondrocytes[28-30], acts as an anti-inflammatory agent, promotes bone density and toughness[31], and enhances fracture healing[32-35]. We therefore hypothesized that calcitonin could possibly enhance tendon healing too, based on the fact that fibroblasts, chondroblasts and osteoblasts originate from the same precursor cells and on the fact that there are calcitonin receptors in tendons[36-41].

We present the results of an experimental study in which the calcitonin effect was evaluated on Achilles tendon healing and was proved by histological, biomechanical and magnetic resonance imaging (MRI) tests.

## Materials and methods

Forty-two adult male New Zealand white rabbits, aged three months and weighing 2.5 Kg on average (2.4 to 2.6 Kg)

The authors have no conflict of interest.

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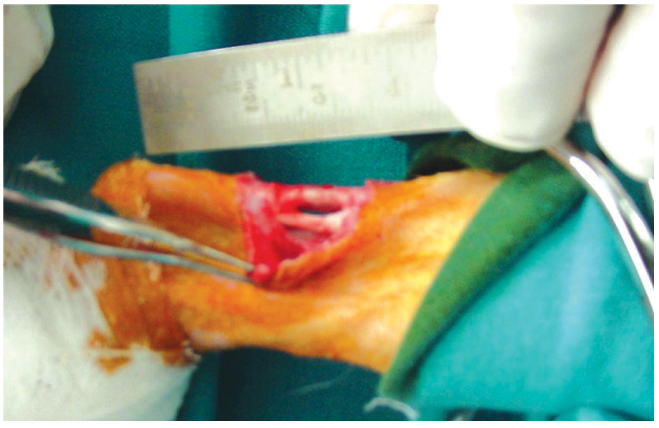

**Figure 1.** The standardized experimental partial Achilles tendon tenotomy is shown.

at the start of the study, were used in two groups CT and P (CT=treatment group, P=control group). The animals were randomly divided into six equal subgroups (CT1, CT2, CT3, P1, P2, and P3) of seven rabbits and were kept under standard experimental conditions (Numbers 1, 2, 3=subgroups according to the week of postoperative animal sacrifice). Under general anaesthesia, induced by cetamine, mitazolam and atropine, a longitudinal skin incision of two centimetres was performed over the postero-medial aspect of the right Achilles tendon of all animals. The Achilles tendon was dissected free from surrounding tissues and the epitendon was split longitudinally. Then, a standardized full-thickness hole was performed, using a belt hole opener causing partial tenotomy of 50% of the tendon width, 3cm proximal to its calcaneal insertion. The epitendon was sutured with an absorbable suture and the skin was closed using a Nylon suture 3-0 (Figure 1). Postoperatively, the animals were left free to walk bearing their full weight. The animals of group CT received salmon calcitonin intramuscularly (21 IU/kg) daily and those of group P (control group) received a saline solution injection daily intramuscularly, all starting from the operative day till the end of the 3rd postoperative week. Two prophylactic veterinary antibiotic doses of 5 mg/Kg/24h of enrofloxacilin (Bayer/Veterin) were given to all the animals.

Animals of subgroups CT1 and P1 were sacrificed at one week postoperatively, those of subgroups CT2 and P2 at two weeks, and those of subgroups CT3 and P3 at three weeks (this timing of intervals corresponded to the advancing stages of tendon healing accordingly)[18]. The animals were sacrificed using a high i.v dose of 0.5g Trapanal (Altana Pharma). All the right Achilles tendons of all these animals were dissected proximally 5cm above the calcaneal insertion, using a standard ruler, and distally the tendons were harvested including a piece of the calcaneus on the tendon. Then the tendons were biomechanically tested and histologically examined.

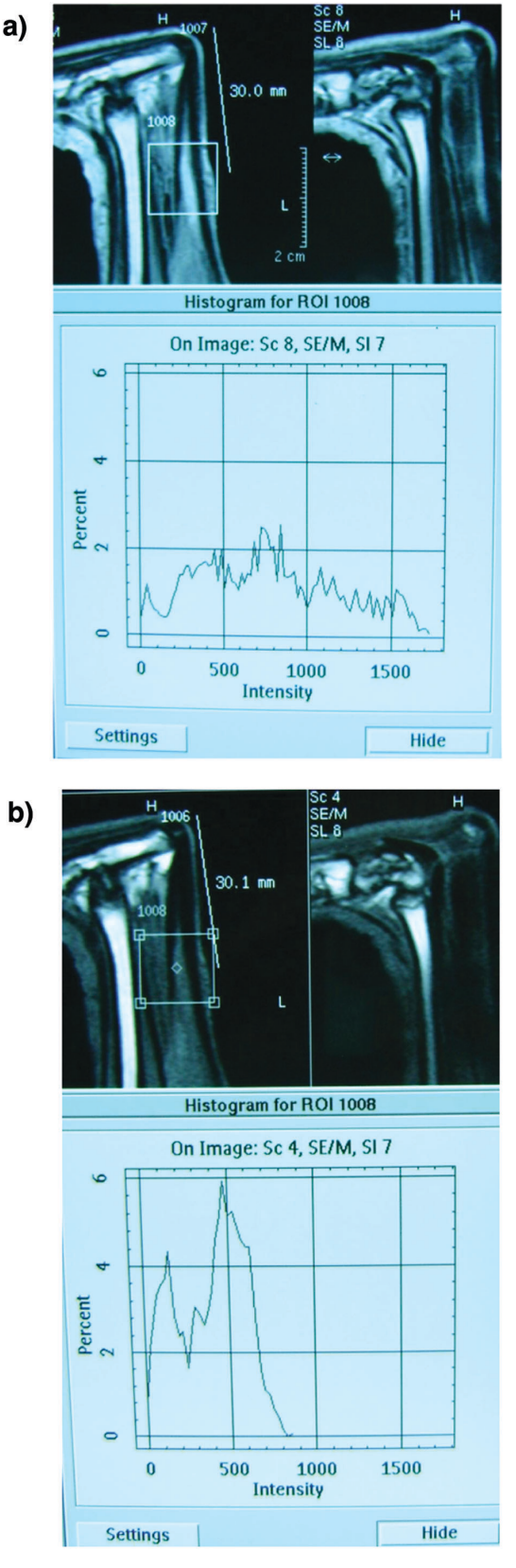


**Figure 2.** The sagittal fat suppressed contrast enhanced T1-w SE MR images, show the histograms of a region of interest with peak signal intensity values a) at 7 days in a treated animal and b) at 21 days in the same treated animal.

MR imaging examinations were performed at 1st, 2nd and 3rd week postoperatively in all animals and before the animals were sacrificed, using a 1.0T scanner (Philips Intera, Best, The Netherlands) with a dedicated surface coil (Figure 2). Signal intensity was measured as the peak value in the area of tenotomy (located 3 cm proximal to the tendon calcaneal insertion) in the contrast enhanced images. A region of interest with an associated histogram rather than a single point measurement was selected for accurate depiction of the peak value (Figure 2). The peak value was selected instead of the mean one, as the latter might induce errors due to the small size of the tissue under study. Animals underwent scanning under general anaesthesia with the leg being well fixed in the isocenter of the magnet. Two pulse sequences in a sagittal and coronal plane were applied to all animals: a) T2-w TSE (TR/TEms: 2000/120, 2 NSA, 3 mm slice thickness, 14 cm FOV) and b) contrast-enhanced (after i.v. injection of 0.2 mmol per kilogram of body weight of gadodiamide, Omniscan; Nycomed, Oslo, Norway) T1-w Spin Echo (CE/FS/T1-w SE) with fat suppression (TR/TEms: 400/16, 3 NSA, 3 mm slice thickness, 14 cm FOV). The T2-w images were used for assessing the evolution of signal changes in the tenotomy area but were not used in the statistical analysis. The highest value of the signal intensity measured in the area of the tenotomy, was recorded on the contrast-enhanced images[42].

For biomechanical testing, tendons were fixed between two metal clamps and pulled at a constant standard velocity of 1 mm/sec until failure using a Frank-Universal Testing Machine 81803II/PC (Frank Co, Bern, Switzerland). Specimens were kept wet in normal saline with the tests being performed within twenty minutes after harvesting. Load-Deflexion curves were taken and from these the structural tendon mechanical parameters of ultimate tensile strength (N), stiffness (N/mm), and toughness (N·mm) were recorded and studied[43].

Following biomechanical testing, tendon specimens were fixed in 10% phosphate buffered formalin, embedded longitudinally in paraffin and stained with heamatoxylin and eosin (H/E) for histological evaluation. Sections were examined under light microscopy and polarized light. All specimens were tested by (LK-pathologist) in a blind fashion to avoid biased estimation for the degree of healing process. Cellular and microscopical changes of the tendon were estimated according to the classification system of Thomas et al.[19]. The specimens were graded as either "relatively mature" were tendon morphology was restored or "relatively immature" were cellular fibrous tissue, inflammatory cells, neovascularity, disturbance of collagen orientation were prevailed.

Experimental procedures were reviewed and approved by an Institutional Animal Care Committee in accordance with the current National legislation for experimentation in animals (Animal ethic approval reference No=K/1948/13-5-99).

Statistical analysis was performed using a two-way ANOVA model with interaction, including treatment factor and the time factor as main effects. When significant interactions did emerge, within group comparisons were also made, using one-way ANOVA and Tukey's HSD test .The Chi Square test with Yates'correction was also used in order to compare tendon healing maturity between specimens of different groups. Statistical significance was set at $P<0.05$. All analyses were carried out with SPSS 10.0.5 for Windows (SPSS, Chicago, Ill., USA). Results are reported as means ± standard deviations (SDs).

| | 1st week | 2nd week | 3rd week |
|---|---|---|---|
| | Peak signal intensity values (SI) | | |
| Treated group | 1087.5±89.9+ | 1342.8± 100.8+ | 266.4±32.5 |
| Control group | 1212.5±92.8+ | 1671.4±110+ | 306.4±40.2 |
| +p<0,05 vs 3rd week | | | |

**Table 1.** Magnetic resonance imaging. Average (± standard deviation) peak MRI signal values in the different groups of animals and time intervals are presented.

## Results

**MR imaging:** Comparing treated group of animals (CT1, CT2, CT3) with those of control groups (P1, P2, P3) a non statistically significant (ss) difference on peak signal intensity values could be detected at the 1st week ($p=0.23$), 2nd week ($p=0.23$) and 3rd week ($p=0.53$) time intervals (Table 1). In contrast, in the treated animals of group CT, a decrease of peak signal intensity values was observed which was of ss difference between the 1st and 3rd week ($p<0.001$), and between the 2nd and 3rd week ($p<0.001$) time intervals. Similarly, comparing control animals of group P, a decrease of peak signal intensity values was observed which was also of ss difference between the 1st and 3rd week ($p<0.001$), and between the 2nd and 3rd week ($p<0.001$) time intervals.

**Biomechanical testing:** Comparing treated group of animals (CT1, CT2, CT3) with those of control groups (P1, P2, P3), regarding the parameter of ultimate tensile strength, ss difference was found at the 1st week ($p<0.005$), the 2nd week ($p<0.0005$) and the 3rd week ($p<0.0005$) time intervals (Table 2). A ss difference was also found when the parameter of stiffness was evaluated at the 1st week ($p=0.005$), the 2nd week ($p<0.0005$) and the 3rd week ($p<0.0005$) time intervals. A similar ss difference was also found when the parameter of toughness was also evaluated at the 1st week ($p=0.005$), the 2nd week ($p<0.0005$) and the 3rd week ($p<0.0005$) time intervals. Comparing treated animals of group CT, a progressive increase of ultimate tensile strength, stiffness and toughness values was observed which was of ss difference between the 1st and 3rd week ($p=0.001$) and between the 2nd and 3rd week ($p=0.001$) time intervals. Comparing control animals of group P, a progressive increase of ultimate tensile strength, stiffness and toughness values was observed which was of ss difference between the

| | 1st week | 2nd week | 3rd week |
|---|---|---|---|
| **Ultimate tensile strength (N)** | | | |
| Treated group | 48.1±3.8+ | 71.21± 5.6+ | 101.03 ±10.1 |
| Control group | 34.7±2.9*+ | 46.58± 7.9*+ | 69.6 ±5.8* |
| **Stiffness (N/m)** | | | |
| Treated group | 14.6±1.4+ | 23.8±1.9+ | 33.33±3.8 |
| Control group | 8.3±2.1*+ | 15.44±3.6*+ | 23.39±2.4* |
| **Toughness (Nm)** | | | |
| Treated group | 0.05±0.01+ | 0.10±0.02+ | 0.32±0.05 |
| Control group | 0.03±0.01*+ | 0.06±0.01*+ | 0.11±0.03* |
| *p<0,0005 vs treated group , +p<0,05 vs 3rd week | | | |

**Table 2.** Biomechanical testing. Average (± standard deviation) values of biomechanical testing parameters in the different groups of animals and time intervals are presented.

| | 1st week | 2nd week | 3rd week |
|---|---|---|---|
| **Relative immaturity** | | | |
| Treated group | N=4 | N=1 | N=1 |
| Control group | N=7* | N=7* | N=6* |
| **Relative maturity** | | | |
| Treated group | N=3 | N=6 | N=6 |
| Control group | N=0* | N=0* | N=1* |
| *p<0,05 vs treated group | | | |

**Table 3.** Histological tendon healing. Qualitative grouping of tendon samples (n: number of samples) in the different groups of animals and time intervals.

1st and 3rd week (p=0.01) and between the 2nd and 3rd week (p=0.01) time intervals.

**Histology:** At the 1st postoperative week, microscopical images prepared from longitudinal sections of specimens, from both CT1 and P1 subgroups, revealed an inflammatory response at the borders of the hole site, consistng mainly of lymphocytes and fewer eosinophils, neutrofils and giant cells. There were new-formed blood vessels and exuded plasma cells, erythrocytes and macrophages and patches of fibrin. Specimens from the treated CT1 subgroup showed a great number of newly formed blood vessels and an increased cellularity especially at the edge of the tenotomy, consisting mainly of precursors of fibroblasts compared to those specimens of the control subgroup P1 where the inflammatory cells had infiltrated the collagen fibers of the tendon (Figure 3A H/E). Polarized images showed the arrangement of the collagen fibers that were congregated in the group CT1 and dispersed in group P1 (Figure 3A POL). At the 2nd postoperative week, specimens of both subgroups CT2 and P2 showed a degree of collagen maturation. Most fibers in group CT2 were numerous, short, and arranged perpendicularly to the long axis of the tendon. Inflammatory cells had increased in group P2 in comparison to group CT2 (Figure 3B H/E). Polarized images showed newly formed collagen fibers haphazardly arranged in group P2 (upper right corner of Figure 3B Plc POL) whereas in group CT2 only old intact fibers had polarized (lower left corner of Figure 3B CT POL). Finally, at the 3rd postoperative week, in specimens from both CT3 and P3 subgroups more mature collagen fibers had formed in a linear fashion intermingled with inflammatory cells. Specimens of subgroup P3 showed less mature collagen fibers (upper left corner of Figure 3 Plc POL) in comparison to the intact tendon (lower right corner of Figure 3 Plc POL). Polarized images of subgroup CT3 (Figure 3C CT POL) showed advanced maturity of collagen fibers oriented almost parallel to the long axis of the tendon which were almost undistinguishable from the intact tendon observed in control subgroup P3 (lower right corner Figure Plc 3C).

According to the classification system of Thomas et al.[19] our specimens from treated (CT) and control (P) groups were divided into two categories a) relatively immature and b) relatively mature, taking into consideration their morphological characteristics and cellularity. The score was statistically analyzed. Specimens from group CT appeared to be ss more mature at the 1st week (p=0.05), at the 2nd week (p=0.05) and at the 3rd week (p=0.05) postoperatively when compared to those of group P (Table 3).

## Discussion

Calcitonin has been extensively used, in the past, as an antiosteoclastic agent for the treatment of metabolic bone diseases[21,44]. However, there is evidence of its effects on other musculoskeletal system cells, as well as of its direct or indirect effect on fracture healing[22-35]. The working hypothesis of this experimental study, that calcitonin might enhance tendon healing as well, was based on the above studies and on the fact that all musculoskeletal cells originate from the same precursor cells[36,37]. Also, there is evidence from recent studies of the stimulating effect of calcitonin gene related peptides[38]. Our results suggest positive findings, mechanically and structurally, in the use of calcitonin for tendon repair. This study suggests a potential new indication using calcitonin for tendon repair. A possible pathway of how calcitonin results in an accelerated tendon healing process is based on published information[39-41] regarding gene-related peptide receptors of calcitonin in the Achilles tendons of rats and rabbits. The authors believe that calcitonin influences tendon healing by acting directly on these receptors and that this interesting possibility should be further investigated in the future.

In this study, calcitonin was administered in doses similar to those used in studies evaluating the effect of calcitonin on fracture healing and no adverse effects were observed[31,33,35]. As a tendon model, the Achilles tendon was used because it is easily accessible surgically and because its anatomical and

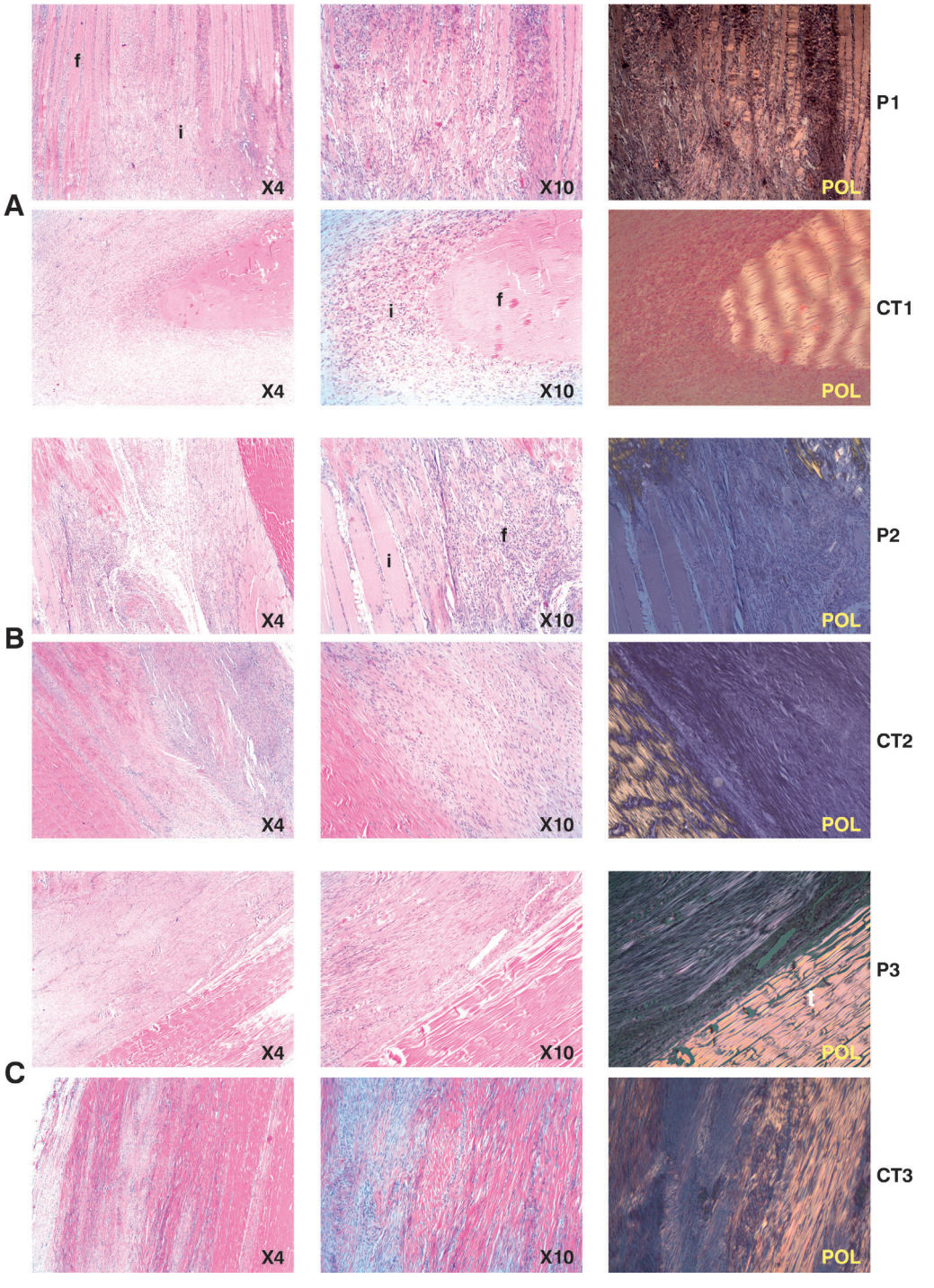


Figure 3. **A)** Images of longitudinal sections from P1specimens (Plc), revealed at the tenotomy edge inflammatory infiltration (i) in-between collagen fibers (f) whereas the CT1 specimens showed an increased cellularity (H/E staining, X4 and X10 magnification). Polarized images indicated the arrangement of the collagen fibers which were dispersed in P1 group and were congregated in CT1 group (X10 magnification, POL). **B)** Images from P2 specimens showed a degree of collagen synthesis and the presence of inflammatory cells (H/E staining, X4 and X10 magnification). Polarized images showed newly formed collagen fibers haphazardly arranged (X10 magnification, POL upper right corner). In CT2 group most fibers were numerous, short, and arranged perpendicularly to the long axis of the tendon (H/E staining, X4 and X10 magnification). Polarization appeared only in old intact fibers (X10 magnification, POL, lower left corner). **C)** In P3 images more mature collagen fibers than P2 were formed in a linear fashion intermingled with inflammatory cells (H/E staining, X4 and X10 magnification). Polarization showed less mature collagen fibers (X10 magnification, POL, upper left corner) in comparison to intact tendon (t) (X10 magnification, POL, lower right corner). CT3 images showed advanced maturity of collagen fibers oriented parallel to the long axis of the tendon (H/E staining, X4 and X10 magnification). Polarized CT3 image showed collagen fibers almost undistinguished from the intact tendon observed in P3.

functional characteristics in rabbits are similar to those in humans. A standardised full-thickness hole in its mass, covering almost 50 percent of the tendon width was made in order to avoid structural and mechanical failure of the tendon, to allow non-operative tendon healing treatment and to permit full weight bearing for the rabbit after the operation. Other models of tendon tears needing surgical repair and temporary protection or leg unloading were not chosen due to the fact that they involve serious confounding factors. Moreover, the avoidance of plaster cast protection facilitated MRI imaging performance and early mobilization of the tendon avoided potential deposits of local chondral or osteoid formation as has been described when administering morphogenetic proteins[18]. Early mobilization also remains a dominant element of non-operative treatment of tendons[4, 45-47].

The results of the present study show that calcitonin in doses as administered improves the tendon healing process in rabbits although this study was designed to evaluate the effect of different dosages. Histological sections show enhanced maturation of healing tissue in treated animals compared to those untreated. A calcitonin effect on fibroblast proliferation was observed, possibly similar to that reported on osteoblasts and chondrocytes[25-30], an effect which can be explained by the fact that all these musculoskeletal cells originate from the same primitive cells[36,37] and by the fact that there are calcitonin receptors in tendons[36-41]. It remains to be shown whether calcitonin affects fibroblast proliferation directly or if it has an indirect action on mesenchymal cells. This question cannot be answered by this study due to its design. All parameters of biomechanical testing show a progressive improvement with calcitonin administration and treated animals present superior changes compared to those untreated, at all time intervals. Biomechanical changes were more pronounced in the parameters of ultimate tensile strength and toughness and were chronologically matched with histological changes. It remains to be shown whether or not calcitonin enhances tendon healing in humans in whom the tendon tear area is often fissured and degenerated[48]. There are also questions related to the human therapeutic dose and the timing of calcitonin administration that remain to be answered. Perhaps the availability of oral calcitonin administration in the future will facilitate further research in this field taking into serious consideration the fact that calcitonin has no serious side effects[44].

The diagnosis of Achilles tendon rupture is based on history and clinical examination. Evaluation of the tendon healing process is difficult and subsequent management decisions are often based on the patient's age, local vascular condition and the time elapsed since the start of treatment. Imaging findings are useful in optimising the treatment planning[49-52]. MR imaging is known to provide important information regarding Achilles tendon pathology. Colour and power Doppler ultrasound are equally accurate. Also, MR imaging is reproducible, is not operator-dependent and can assess other structures such as cartilage and bone marrow[49, 53-55].

In this study MR imaging scans were performed in order to evaluate progress during the tendon healing process. The T2-w TSE sequence was used to evaluate morphological changes in the healing area after the tenotomy. Contrast-enhanced MR imaging is considered as the method of choice for the evaluation of tendon repair and therefore it was used for the quantitative evaluation of signal alterations[50-53]. In our study, the 2nd week time interval was the earliest time in which the progress of tendon healing process could be detected. MR imaging, although efficacious in detecting quantitative progressive tendon healing changes in each group separately, failed to discriminate between treated animals and controls. This discrepancy could be explained by the intense enhancement of immediate postoperative changes, which occur in any tissue, and may mask the discrete effects of calcitonin.

Newer developments with high field scanners (3-7 T), multichannel phased array coils and fast perfusion imaging sequences might contribute to an increase in the accuracy of MR imaging in assessing the difference between treated and untreated animals. Such a capability may allow for a better assessment of human Achilles tendon healing in a non- invasive way, facilitating decisions about treatment management.

In conclusion, calcitonin administration in doses as mentioned enhances healing behaviour of standardised Achilles tendon tears in rabbits and this was shown by histological and biomechanical means. MR imaging, though able to detect progressive tendon healing changes in each group separately, failed to differentiate treated from untreated groups of rabbits. It is of clinical importance nevertheless that MR imaging was able to monitor the progression of the healing process. Further research is needed in order to evaluate the possible direct effect of calcitonin on fibroblasts and on human degenerative tendon tears.

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