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ORIGINAL ARTICLE

Investigating circadian clock gene expression in human tendon biopsies from acute exercise and immobilization studies

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Abstract

Purpose The discovery of musculoskeletal tissues, including muscle, tendons, and cartilage, as peripheral circadian clocks strongly implicates their role in tissue-specific homeostasis. Age-related dampening and misalignment of the tendon circadian rhythm and its outputs may be responsible for the decline in tendon homeostasis. It is unknown which entrainment signals are responsible for the synchronization of the tendon clock to the light–dark cycle.

Methods We sought to examine any changes in the expression levels of core clock genes (*BMAL1, CLOCK, PER2, CRY1,* and *NR1D1*) in healthy human patellar tendon biopsies obtained from three different intervention studies: increased physical activity (leg kicks for 1 h) in young, reduced activity (2 weeks immobilization of one leg) in young, and in old tendons.

Results The expression level of clock genes in human tendon in vivo was very low and a high variation between individuals was found. We were thus unable to detect any differences in core clock gene expression neither after acute exercise nor immobilization.

Conclusions We are unable to find evidence for an effect of exercise or immobilization on circadian clock gene expression in human tendon samples.

Keywords Tendon · Circadian clock · Gene expression · Exercise · Immobilization · Aging

Abbreviations

BMAL1	Brain and muscle Arnt-like protein-1
BMI	Body mass index
CLOCK	Circadian locomotor output cycles kaput
CRY1	Cryptochrome 1
NR1D1	Nuclear receptor subfamily 1 group D member 1
PER2	Period 2
RPLP0	60S acidic ribosomal protein P0
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SCN	Suprachiasmatic nuclei
SD	Standard deviation

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Introduction

The circadian clock is an evolutionarily conserved system that allows life on earth to coordinate their physiology and behavior to the 24-h day. The core components of the molecular pacemaker responsible for driving the circadian rhythm in the mammalian central clock, the suprachiasmatic nuclei (SCN), is identical in all peripheral clock tissues, including tissues of the musculoskeletal system (Dibner et al. 2010). There is a large breadth of data that strongly suggest that disrupted circadian rhythms in peripheral tissues, as a result of sleep disorders, evening screen time, shift work and aging, can contribute to the development of diseases, including cancer and metabolic diseases (Roenneberg and Merrow 2016). Understanding how peripheral clocks are synchronized and aligned to the

light–dark cycle is crucial for understanding the link between the circadian clock and health.

The endogenous circadian rhythms of skeletal muscle (McCarthy et al. 2007), cartilage (Gossan et al. 2013), tendon (Yeung et al. 2014), and intervertebral disc (Dudek et al. 2017) drive tissue-specific rhythmic gene expression that regulates tissue homeostasis (Dudek and Meng 2014). The tendon clock regulates the homeostasis of the collagen-rich extracellular matrix (Yeung et al. 2018 preprint). The collagen fibrils in the matrix enable tendon to undergo repeated cycles of mechanical loading, and therefore, its maintenance has important consequences for biomechanics. Arrhythmic mouse tendons with a mutation in *Clock* or deletion of *Bmal1* have premature aging phenotypes, and aged wild-type tendons have a dampened circadian rhythm that is misaligned with the SCN (Yeung et al. 2018 preprint; Yeung et al. 2014). Scheduled exercise can entrain the circadian rhythms of skeletal muscle and lung tissue clocks (Sasaki et al. 2016; Wolff and Esser 2012). Whether acute changes in the level of physical activity itself can explain clock gene changes, in humans, is not known.

The findings of a recent systematic review concluded that exercise is a zeitgeber for the human circadian system, where multiple studies demonstrated phase shifts in hormone secretion and body temperature cycles (see Lewis et al. 2018 for a systematic review and references therein), but the effect of exercise on individual peripheral clock rhythms has not been fully investigated. Exercise is known to induce glucocorticoid release and generate heat in musculoskeletal tissues, including tendon (Ker 1981; Riemersma and Schamhardt 1985), and both these effects are able to entrain peripheral clocks (Gossan et al. 2013; Sasaki et al. 2016; Tahara et al. 2015). Glucocorticoids can activate glucocorticoid response elements upstream of circadian gene promoters, including *Per2* (Balsalobre et al. 2000; Reddy et al. 2009, 2012). The addition of dexamethasone can synchronize ex vivo tissues and cells in culture, including mouse tendons and primary mouse and human tendon cells, by driving *Period* expression (Balsalobre et al. 2000; Yeung et al. 2014). Thus, reduced exercise-induced glucocorticoid release could potentially modulate the tendon circadian clock circuitry.

The aim of this study was to examine the relationship between acute physical activity, as well as prolonged immobilization, and the expression of core clock genes in human tendons in vivo. We hypothesized that physical activity may be a mechanism of entrainment for the tendon clock and that inactivity may contribute to the decline in circadian performance.

Materials and methods

Study design

Human patellar tendon biopsies were obtained from three different intervention studies. Participant information for the first study (acute exercise) was as previously described (Heinemeier et al. 2013) and the study is summarized in Fig. 1a. Briefly, 31 healthy, moderately physically active young men were randomized into three groups: group A [age 23.5 ± 3.0 (mean ± SD), body mass index (BMI) 23.2 ± 2.0; $n = 10$), group B (age 23.5 ± 3.0; BMI 23.4 ± 2.0; $n = 11$), and group C (age 23.9 ± 2.5; BMI 23.4 ± 2.0; $n = 10$)]. 10–14 days prior to the experiment, the subjects performed a warm-up followed by a max test at 35 one-legged kicks per minute with a starting resistance of 0.5 kg in a modified Krogh ergometer as previously described (Miller et al. 2005). Every 3 min, 0.5 kg resistance was added and this continued until the subjects could no longer maintain the correct kicking frequency. The highest attainable workload was defined as W_{max}. On the day of the experiment, all subjects performed 1 h of leg kicks at 67% of W_{max} and 35 kicks per minute (2100 total kicks).

Participant information for the second study, the immobilization of young men, was as previously described (only the placebo group) (Boesen et al. 2013) and the study is summarized in Fig. 2a. Briefly, ten young, healthy and physically untrained men (age 22.1 ± 2.2; BMI 22.6 ± 2.2) were subjected to 2 weeks of unilateral immobilization of a randomly selected limb.

Participant information for the third study, the immobilization of old men, was as previously described (only the placebo group) (Dideriksen et al. 2017) and the study is summarized in Fig. S1A. Briefly, six healthy, moderately physically active men (age 71.8 ± 7.7; BMI 24.1 ± 1.3) were provided with 20 g of whey protein supplement (Lacprodan, Arla Foods Ingredients Group, Viby J, Denmark) twice daily throughout the study period to ensure that they had sufficient amounts of protein and essential amino acids to optimize their nutritional conditions (Farup et al. 2014). The first protein supplement was taken 2 days before the immobilization period. For both young and old subjects, a lightweight fiber cast was applied from just above the malleoli to just below the groin, positioned at 50° of the knee joint flexion to minimize walking ability. This method of immobilization has been demonstrated to induce substantial muscle atrophy in short-term immobilization studies (Suetta et al. 2009). They were also instructed to perform all activities on crutches and to perform venous pump exercises several times a day to prevent potential formation of deep venous thrombosis and isometric contraction of the quadriceps of the immobilized leg.

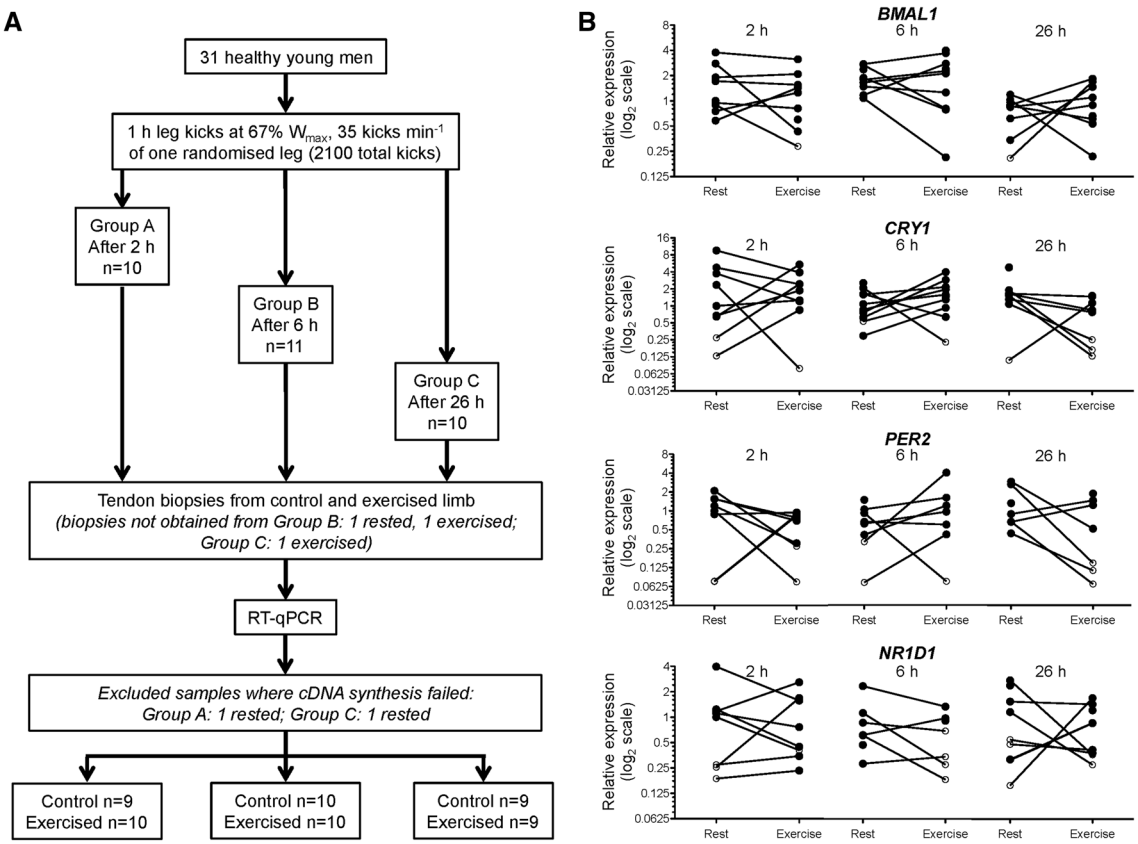

Fig. 1 Clock gene expression in young patellar tendon after physical activity. **a** Schematic of the workflow of the acute exercise study. **b** Patellar tendon biopsies were taken at 2, 6, and 26 h after 1 h of leg kicks or rest. The expression of *BMAL1*, *CRY1*, *PER2,* and *NR1D1* was analyzed by RT-qPCR. Expression was normalized to *RPLP0*. No significant differences were found ($p > 0.05$). Open circles represent maximum possible values in the samples, where the level was below our detection limit of one molecule. Connecting lines indicate paired samples

All subjects gave written informed consent and were informed of the risks associated with the study. All studies were approved by the local Ethical Committee of the Capital Region Copenhagen (H-KF 01-213/02, H-4-2010-010, and H-1-2010-007) in accordance with the Declaration of Helsinki II. All participants were non-smokers, did not take any medication, and did not have prior injuries to their lower extremities, including the knee joints.

Tendon biopsy

The skin over the biopsy site was sterilized and treated with local anesthetics (1% lidocaine). The biopsies were taken from the patellar tendon with a Bard Magnum Biopsy Instrument (C.R. Bard Inc., Covington, GA, USA) and a 14 G needle. In the acute exercise study, exercise was performed and completed in the morning, before 12:00 pm, biopsies were taken from both rested and exercised legs, in a randomized order, at 2 h (group A), 6 h (group B), or 26 h (group C) after the exercise. In the immobilization studies, biopsies were taken from the control limb 2 days prior to the immobilization period (baseline control) and from the immobilized limb after the immobilization period. Biopsies were obtained from the young individuals between 9:00 am and 12:00 pm and from the old individuals at 11:00 am ± 30 min. The weight of the biopsies was ~ 5 to 10 mg, cleared of adipose tissue and blood and snap frozen in liquid nitrogen and store at − 80 °C.

RNA isolation and measurements

RNA was isolated from tendon biopsies as described in detail previously (Heinemeier et al. 2013). Tendon biopsies (5–10 mg) were homogenized in 1 ml TRI Reagent (Sigma) in a 2 ml tube containing five stainless steel balls (2.3 mm ∅) and one sharp silicon carbide particle (1 mm ∅) (both from BioSpec Products) by shaking at speed level 4 for 15 s in a FastPrep-24 Classic Instrument (MP Biomedicals) at 4 °C, six times, with cooling on ice between each shaking step. After, bromo-chloropropane was added to separate the sample into aqueous and organic phases.

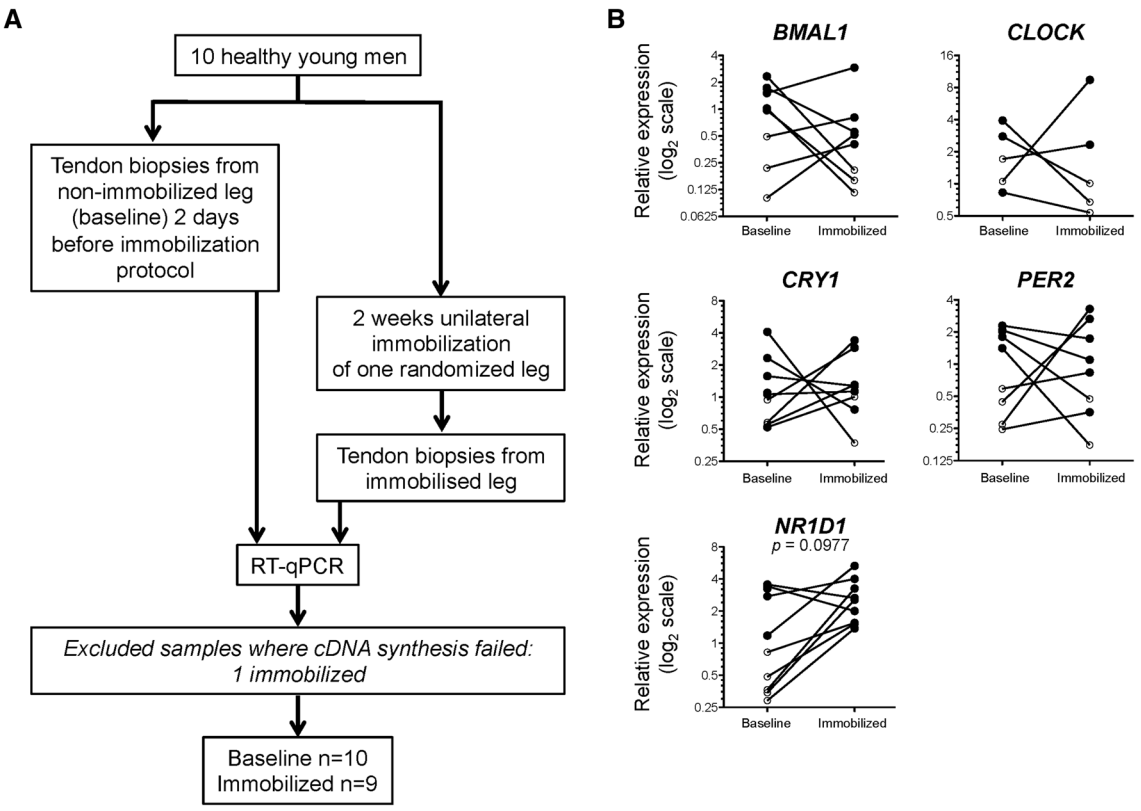

Fig. 2 Clock gene expression in young patellar tendon before and after 2 weeks of immobilization. **a** Schematic of the workflow of the immobilization study. **b** Patellar tendon biopsies were taken after 2 weeks of lower leg immobilization. Controls were tendon biopsies taken from the contralateral leg at baseline before immobilization. The expression of *BMAL1*, *CLOCK*, *CRY1*, *PER2,* and *NR1D1* was analyzed by RT-qPCR. Expression was normalized to *RPLP0*. No significant differences were found ($p > 0.05$). Open circles represent maximum possible values in the samples, where the level was below our detection limit of one molecule. Connecting lines indicate paired samples

RNA was precipitated from the aqueous phase using isopropanol, in the presence of 120 µg glycogen. The RNA pellet was washed with ethanol and subsequently resuspended in RNase-free water. Total tendon RNA was measured using Quant-iT RiboGreen assay (Thermo Fisher Scientific). The RNA yield in ng total RNA per mg tendon tissue was: study one (2 h rest 53 ± 17, 2 h exercised 44 ± 10, 6 h rest 50 ± 21, 6 h exercise 48 ± 16, 26 h rest 40 ± 16, 26 h exercise 40 ± 9), study two (baseline 92 ± 12, immobilized 55 ± 23), and study three (baseline 15 ± 15, immobilized 15 ± 11). There were no significant differences between the two legs in any group. As the RNA yield was too low for gel electrophoresis, RNA integrity was evaluated by absolute amounts of reference gene targets (*RPLP0* and *GAPDH*), which did not vary to a degree indicating mRNA breakdown. The standard curves in PCR were performed in duplicates and the samples as single measurements on 150, 250 pg and 200 pg cDNA for the three studies, respectively, due to limited material remaining.

Real-time RT-qPCR

The amount of mRNA for *BMAL1*, *CLOCK*, *PER2*, *CRY1,* and *NR1D1* was measured with reverse-transcription real-time PCR and normalized to the amount of 60S acidic ribosomal protein P0 (*RPLP0*) mRNA, as previously described in detail (Doessing et al. 2010). *RPLP0* was used as the reference gene in this study because it is one of the most stable for circadian studies (Kosir et al. 2010). Accession number of target transcript was searched using Basic Local Alignment Search Tool (BLAST) to identify the region of the sequence that could be amplified for all variants of the target. Intron-spanning primers, which would not cover or amplify common single-nucleotide polymorphisms were designed using Primer-BLAST. Potential primer dimer formation was visualized using Beacon Designer Free Edition of the qPCR Design Software (Premier Biosoft). Primer sets where there was no potential of 3′ overhangs in priming were selected. The predicted PCR product was checked using BLAST to ensure that only the target transcript will be amplified. Validation of primers was performed using a

diluted series of known number of molecules of oligonucleotides (Ultramere, Integrated DNA Technologies) corresponding to the PCR product. Primer sequences used: *BMAL1* (NM_001030272.2; GGGCTGGGGCAGGAAAAATAG, GAGCCACAGCTAGAAGGCGATG), *CLOCK* (NM_001267843.1; GCCCAACCCCTTCTGCCTCTTC, CGTCGGGATCTTGGTTGGTGT), *PER2* (NM_022817.2; AACCAGCCCACCTGCTCCTACC, GCTGGGAACTCG CATTTCCTCTT), *CRY1* (NM_004075.4; GCAGATGTG TTTCCCAGGCTTTTC, TAGCTGCGTCTCGTTCCT TTCC), *NR1D1* (NM_021724.4; GCAAGAGCACCAGCA ACATCAC, GCAACGTCCCCACACACTTTACAC) and *RPLP0* (NM_053275.3; GGAAACTCTGCATTCTCGCTT CCT, CCAGGACTCGTTTGTACCCGTTG).

Due to the limited amount of material and the low expression of the circadian genes, in a substantial number of cases the PCR reactions were negative due to the detection limit of one molecule. As we use a standard curve with known number of molecules this detection limit could be assessed, hence undetected samples were assigned a level of one molecule (indicated with open circles in figures) to enable comparison with the other samples. Note that this preference of assigning an artificial molecule to undetectable samples in the RT-qPCR analysis may result in the mRNA level calculated using the artificial molecule being greater than the mRNA level calculated from the detected value. For example, in Fig. 1b for *NR1D1* 26 h after rest or exercise, we have two paired sample sets, where the *NR1D1* mRNA values calculated from applying the artificial molecule after rest were greater than the *NR1D1* mRNA values calculated from detected values after exercise. The reason for this is due to variation in the reference gene RPLP0 that we used to perform the normalization. For example, the detected *RPLP0* values from the two aforementioned paired sample sets were between 1500 and 1700 after rest and ~ 3500 after exercise and the detected *NR1D1* values were ~ 1.7 for the latter and an artificial *NR1D1* molecule was assigned to the after rest samples. The result is that one artificial *NR1D1* molecule normalized to 1500–1700 *RPLP0* molecules is greater than ~ 1.7 detected *NR1D1* molecule normalized to ~ 3500 *RPLP0* molecules. From a statistical point of view, this analysis approach creates the least bias. Furthermore, in the three independent studies analyzed, there was only one other instance, where the mRNA level calculated using the artificial molecule is greater than the mRNA level calculated from the detected value, in Fig. 1b for *NR1D1* 6 h after rest or exercise.

Two exercised samples and one young immobilization sample failed the cDNA synthesis. In the instances where both samples of a pair were artificially assigned one molecule, these samples were excluded from further analyses. Furthermore, a few measurements failed the melting curve test and were also excluded from further analyses.

Statistical analysis

Statistical analyses were performed using Prism v5.0 (GraphPad Software). Non-parametric Wilcoxon matched-pairs signed rank tests were performed on each paired data set. Differences were considered significant when $p < 0.05$. Data are shown as individual data or mean ± SD.

Results

To determine whether a single bout of exercise could have acute effects on the tendon clock, we analyzed tendons biopsied from young individuals at 2 or 6 h after 1 h of leg kicks by RT-qPCR and compared them to biopsies from the contralateral rested leg. We also analyzed tendons biopsied 26 h after exercise to determine any longer term effects. Messenger RNAs for the core clock genes examined were detectable in most of the samples, except *CLOCK* that was only detected in nine randomly distributed samples (data not shown). No obvious trend could be observed in the expression of any of the circadian clock genes, *BMAL1*, *CRY1*, *PER2,* or *NR1D1*, in tendons at 2, 6, or 26 h after exercise or rest (Fig. 1b). Variation in response to acute exercise could be observed between individuals at all three timepoints and no significant differences were found.

Next, we examined tendon biopsies taken before and after 2 weeks of immobilization of young individuals. All core clock genes examined were detected but not in all the samples. No obvious patterns were observed in the expression of clock genes, *BMAL1*, *CLOCK*, *CRY1,* or *PER2* between baseline and immobilization except a trend of increased *NR1D1* expression after immobilization (Fig. 2b). However, no significant differences were found between baseline and post-immobilization *NR1D1* expression levels.

We also analyzed tendon biopsies from old individuals before and after 2 weeks of immobilization. Many samples from old tendon biopsies were excluded from the analyses due to incorrect melting curves, likely due to very low abundance of the target mRNA. No significant differences were found in the expression level of *BMAL1*, *CLOCK*, *CRY1*, *PER2*, or *NR1D1* before or after immobilization (Fig. S1B).

Discussion

Elucidating how peripheral clocks are synchronized is key to understanding the mechanisms linking the circadian clock and health. Dampening and misalignment of the tendon circadian clock correspond to increase in risk of developing tendinopathies in mice (Yeung et al. 2014). The aim of this study was to investigate whether physical activity is an entrainment signal for the human tendon clock. We

were unable to detect any significant differences in samples obtained from the previous acute exercise and immobilization intervention studies, and here, we discuss why circadian conclusions cannot be drawn.

Non-light entrainment signals integrate complex periodic changes in the environment into the circadian network, which makes the system slow to adapt to circadian challenges, e.g., jet lag, but allows it to be more robust and protected from unwanted phase shifts, and allows synchronization of peripheral clocks to be adapted to sustained zeitgebers, e.g., food availability (reviewed by Husse et al. 2015). The integrated circadian network would explain why a single bout of exercise during the early morning might not affect the expression of core clock genes in young tendon. It is possible though that regular timed physical exercise could entrain the tendon clock, especially in older individuals. Peripheral clocks of aged mice are more susceptible to non-light zeitgebers, such as timed feeding, and are entrained faster than peripheral tissues in young mice (Tahara et al. 2017). This phenomenon in aged peripheral tissues is thought to be due to decline of input from the SCN, as faster entrainment of peripheral tissues by non-light zeitgebers also occurs in SCN-lesioned mice (Husse et al. 2014; Saini et al. 2013).

It was surprising that 2 weeks of immobilization also did not have any significant effects in young biopsies and low expression in older tendons meant we were unable to determine any immobilization effects in aging either. Furthermore, due to batch variations in timing, subject selection, sampling, RNA purification, and cDNA synthesis between the three studies, we could not compare the baseline data between young and old tendons to examine any age effects on core clock gene expression.

Several limitations of both the acute exercise and immobilization intervention studies, from which the tendon biopsies were analyzed, could explain why we were unable to find any significant differences: (i) low expression of clock genes in human tendon; (ii) systemic effects of acute exercise on the non-immobilized legs; and (iii) large variation in humans.

(i) The presence of a circadian rhythm in primary human tendon cells has been demonstrated previously (Yeung et al. 2014), but it is unknown whether the in vivo microenvironment has an effect on it. In the present study, we observed that some measurements fell below the detection limit of the assay, especially in the older tendon samples, which makes assessing the tendon clock at a single timepoint more challenging. In this present study, we artificially assigned one molecule to those samples, in order not to introduce bias by excluding these non-detected values or to assign a C_t value of 40, which would set all undetected samples with extremely low values (McCall et al. 2014). In our setup, we can estimate our real detection limit (from the standard curve with known molecules). Therefore, by setting undetected samples to one molecule, we at least can estimate the maximum possible level in that sample.

(ii) The contralateral control 'rested' leg was used in the acute exercise study and we cannot rule out any systemic effects of the 1 h of leg kicks may have on the 'rested' tendon. Therefore, having the mobility of the one leg in the immobilization studies might have also mitigated the effect of immobilization on the tendon of the immobilized leg. Possible solutions would be to include a cohort of non-exercised individuals for the acute exercise study and a cohort of bed rest patients for the immobilization study.

(iii) There are diurnal differences in the mechanical properties of human tendons (Pearson and Onambele 2005, 2006), which may be indicative of diurnal gene expression differences. Care was taken to exercise and sample tendons at the same time of day (within a 3-h period) within each of the studies. However, as these studies were not optimized for circadian investigation, information on individual chronotypes (e.g., Munich ChronoType Questionnaire, Circadian Energy Scale) was not acquired, and the difference in chronotypes in humans makes it difficult to ensure that we are sampling all tendons at the same point in the circadian cycle. Large variation in human samples makes circadian studies based on small sample sizes and a single timepoint difficult. Unfortunately, multiple biopsies from tendon are not recommended due to the fact that just a single needle biopsy activates growth factor signaling for several months in the human tendon (Heinemeier et al. 2016). Recent computational approaches have been successful in determining the circadian phase of an individual's internal biological time with single timepoint samples from skin and blood (Wittenbrink et al. 2018; Wu et al. 2018). Alternatively, identification of tendon-specific, clock-controlled secreted factors would enable non-invasive tracking of the circadian clock at multiple timepoints in human tendons.

In conclusion, a combination of low expression levels of clock genes and a large variation between human chronotypes, no significant effects of acute physical activity or prolonged immobilization on core clock gene expression in tendon could be determined. Therefore, further investigations with careful considerations into circadian study design are required to determine whether physical activity is a synchronizer for the human tendon clock.

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Author contributions C-YCY and MK conceived the study; KMH performed the exercise study; APB and KD performed the immobilization study; PS designed the analyses; C-YCY and PS performed data

collection and statistics; C-YCY drafted the manuscript; C-YCY, PS, KMH, and MK edited the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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