EFFECTS OF TRAINING, DETRAINING, AND RETRAINING ON STRENGTH, HYPERTROPHY, AND MYONUCLEAR NUMBER IN HUMAN SKELETAL MUSCLE

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Running title: A search for human muscle memory

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ABSTRACT

Previously trained mouse muscles acquire strength and volume faster than naïve muscles; it has been suggested that this is related to increased myonuclear density. The present study aimed to determine whether a previously strength-trained leg (mem-leg) would respond better to a period of strength training than a previously untrained leg (con-leg). Nine men and 10 women performed unilateral strength training (T1) for 10 weeks, followed by 20 weeks of detraining (DT) and a 5-week bilateral retraining period (T2). Muscle biopsies were taken before and after each training period and analyzed for myonuclear number, fiber volume, and cross-sectional area (CSA). Ultrasound and one repetition of maximum leg extension were performed to determine muscle thickness (MT) and strength. CSA (~17%), MT (~10%), and strength (~20%) increased during T1 in the mem-leg. However, the myonuclear number and fiber volume did not change. MT and CSA returned to baseline values during DT, but strength remained elevated (~60%), supporting previous findings of a long-lasting motor learning effect. MT and strength increased similarly in the mem-leg and con-leg during T2, whereas CSA, fiber volume, and myonuclear number remained unaffected. In conclusion, training response during T2 did not differ between the mem-leg and con-leg. However, this does not discount the existence of human muscle memory since no increase in the number of myonuclei was detected during T1 and no clear detraining effect was observed for cell size during DT; thus, the present data did not allow for a rigorous test of the muscle memory hypothesis.

Key words: muscle memory, myonuclei, CSA, exercise, motor learning

NEW & NOTEWORTHY

If a long lasting intramuscular memory exists in humans, this will affect strength training advices for both athletes and the public. Based on animal experiments we hypothesized that such a memory exists and that it is related to the myonuclear number. However, a period of unilateral strength training, followed by detraining, did not increase the myonuclear number. The training response, during a subsequent bilateral retraining period, was not enhanced in the previously trained leg.

INTRODUCTION

Muscle wasting and frailty are major health problems in the aging Western population (14, 24), and strength training represents an important strategy for dealing with this problem. Considerable evidence shows that physical activity, especially strength training, can counteract loss of strength and muscle wasting in the elderly, thereby improving overall quality of life (9, 10). However, hypertrophy induced by strength training is attenuated in elderly individuals, and may be partly explained by satellite cells' reduced capacity to proliferate and generate new myonuclei (40). Therefore, an alternative or complementary strategy could be to encourage strength training at a young age, when hypertrophy and generation of new myonuclei can be achieved more easily. Mouse studies have shown that myonuclei inserted into muscle cells, in conjunction with hypertrophy, are not lost during subsequent atrophy and could potentially represent a functionally important "muscle memory" that aids hypertrophy during future overload activities (6-8, 15, 19, 26, 28). Even though the methods used for inducing hypertrophy in these studies are different from those used in physiological training in a human setting (synergist ablation, steroid administration, tail loaded ladder climbing, etc.), they provide important insight into molecular mechanisms potentially regulating the hypertrophic response.

It is generally believed that previous strength training facilitates reacquisition of muscle mass even after long intervening periods of inactivity. However, there is little direct scientific evidence for such a memory effect of strength training in humans, although some studies have shown that muscle strength, and to some degree, hypertrophy, is resilient during detraining periods, and faster gain is obtained during subsequent retraining periods (12, 17, 21, 45, 46). Muscle memory involves the central nervous system (CNS) and motor learning components since an increase in force may precede an increase in muscle mass, possibly mediated by

alterations in spinal motor neuron excitability and synaptogenesis within the spinal cord (1, 37). However, animal experiments have highlighted a memory effect residing in the muscle cells themselves (7, 8, 15, 28).

The cellular memory was originally suggested to be related to the number of myonuclei, since myonuclei recruited during hypertrophy are not lost during subsequent atrophy (7, 8), and could have a half-life in excess of 15 years in humans, as suggested by ^{14}C dating of whole muscle homogenate (44). Other possible mechanisms include long-lasting epigenetic modifications (48). In a recent study, young men who performed two identical 7-week periods of bilateral strength training, separated by 7 weeks of detraining, gained muscle mass faster during the second training period (12% vs 7%). Furthermore, the second training period was associated with a greater number of hypomethylated genes than the first (42).

In the present study, young, untrained individuals underwent a 10-week unilateral leg strength training program. Afterwards, they were detrained for 20 weeks before undergoing a 5-week, bilateral training program aimed at comparing the training response of the two legs. Muscle strength and thickness, fiber size, and number of myonuclei (derived from muscle biopsies) were measured. We hypothesized that the unilateral training period would increase muscle mass as well as the number of myonuclei, and that this increased myonuclear content would aid retraining 20 weeks later in the previously trained leg.

METHODS

Experimental Design

Fig. 1 shows a schematic illustration of the experimental design. The study was performed over 35 weeks and included two strength training periods (T1 and T2) separated by a 20-week detraining period (DT) with no training at all. During T1, unilateral strength training was performed for 10 weeks, while, during T2, bilateral strength training was performed for 5 weeks. The exercises included leg presses and leg extensions, and training usually took place three times per week. Both moderate [70% to 75% of one repetition maximum (1RM)] and heavy loading (80% to 85% of 1RM) were performed in an undulating, periodized manner. Two weeks of low-load, blood flow restricted exercise (BFRT) was incorporated into T1 (weeks 4 and 8). The purpose of this novel strength training design was to maximize hypertrophy, satellite cell activation, and fusion (33, 34, 49), with the aim of creating muscle memory in the trained leg. To ensure optimal protein intake and stimulate muscle growth, participants consumed 25 g of whey protein (One Whey, Fitnessguru Sweden AB, Stockholm, Sweden) immediately after each training session in both training periods (39). Muscle thickness (MT, m. vastus lateralis) and unilateral leg extension (1RM) of the quadriceps muscles were determined before and after each training period. Muscle biopsies (m. vastus lateralis) were obtained from the unilaterally trained leg pre- and post-T1, and from both legs pre- and post-T2. Muscle biopsies were analyzed to calculate fiber volume, fiber-specific CSA, and myonuclear number.

Participants

Study participants included 19 healthy, inactive volunteers (25 ± 1 years old, 71 ± 4 kg, 175 ± 8 cm, 10 women and nine men), who had never engaged in any regular sport or physical activity. Participants were carefully informed about the experimental design, and possible

risks related to the project, and gave written informed consent form before entering the study. Ten male subjects were originally recruited; however, one dropped out before starting the first training period. The study was approved by the Regional Ethics Committee of Stockholm, Sweden (DNR 2015/211-31/4) and was performed in accordance with the Declaration of Helsinki.

Training Protocols

All training sessions were performed at the laboratory of applied sport sciences at the Swedish School of Sport and Health Sciences. Each participant was individually supervised by a trainer for each session to ensure the training was performed correctly. The exercise equipment included a Cybex leg press (model 16110) and a Cybex leg extension (model 11051-90) (Cybex International Inc. Medway, USA). The right and left legs were randomly assigned in a counterbalanced fashion to serve either as control leg (con-leg) or memory leg (mem-leg). Only the mem-leg was trained for 10 weeks during T1, and both the mem-leg and con-leg were trained for 5 weeks during T2. The legs were trained one at a time during T2, alternating between sets. The two training periods were separated by a ~20-week DT period, during which no training was allowed.

Except for weeks 4 and 8 in T1, a conventional resistance-training program was applied three times per week (Monday, Wednesday, and Friday), with three sets of leg presses followed by three sets of leg extensions. The exercise intensity was quantified in relation to 1RM strength in the given exercise movement. The Monday session was performed with moderate intensity loads corresponding (70% to 75% of 1RM, ~10–12 reps) and the Friday session used heavy loads (80% to 85% of 1RM, ~5–7 reps) until volitional failure. The loads were increased every Monday if the subjects could perform more than their designated repetition range during the previous training session. The Wednesday session was performed using the same number of repetitions (reps) as the Monday session, but with a 10% load reduction (i.e., not

until volitional failure). There was a 1–2 min rest period between sets, and 3–4 min between exercises.

BFRT was performed five times per week at weeks 4 and 8 during T1. Participants wore a cuff (Delfi Medical low-pressure tourniquet cuff, 30–77cm) on the proximal thigh that was automatically inflated using a Zimmer A.T.S. 2000 tourniquet system (Zimmer inc. Warsaw, IN, USA). During the entire working period, the cuff was inflated and regulated to a pressure of ~100 mmHg. BFRT consisted of four sets of either leg extension or leg press on alternating days. The loads used were ~20% of 1RM for the leg extension and ~30% of 1RM for the leg press. The first two sets were submaximal; participants performed 30 reps in the first set and 10 reps in the second set. The last two sets were performed to failure and all subjects performed <10 reps during these sets. There was a rest period of 30s between sets.

1RM Strength Testing

The same Cybex leg extension machine used during training was also used for 1RM testing. Two unilateral familiarization sessions were performed one week before T1 for both the con-leg and the mem-leg. All 1RM tests were performed unilaterally at least 72 h after any exercise or biopsy and standardized to a range of motion from 90° to within less than 20° of knee flexion (where 0° represents full extension). The test was preceded by a 5-min general warm-up at 60 W using a cycle ergometer (Monark Ergomedic 893E, Monark Exercise, Varberg, Sweden), followed by an exercise-specific warm-up with 10 reps at ~50%, five reps at 70%, and a final three reps at 90% of the subjects predicted 1RM (estimated from the previous familiarization session), with 2–3 min of rest between sets. The load was then progressively increased by 2.5% to 10% for each attempt until the 1RM was reached. The rest period between each attempt was 3–5 min.

Muscle Thickness

The m. vastus lateralis MT was measured using ultrasound (Siemens Acuson S1000, Siemens Healthcare GmbH, Erlangen, Germany) with a Multi-D matrix 14L5 (5–14 MHz) 4.5-cm probe. The machine settings were standardized for all the image measurements. All measurements were performed at least 72 h after any exercise and always before the 1RM tests and biopsies, using a previously described procedure (2). Briefly, MT image measurements were recorded at 25%, 50%, and at 75% of the distance between the superior edge of the patella and the trochanter major, with the ultrasound probe placed in the transversal plane and perpendicular to the skin. An average was taken of three analyzed images taken from each location. To ensure screening reliability, all the first measurement reference points were registered in a coordinate system so that subsequent measurements could be performed at the exact same locations. All measurements were performed by an ultrasound recording specialist, with the participants lying in a supine position for ~15 min prior to the measurement.

Muscle Biopsies

Muscle biopsies were collected under local anesthesia (2% Carbocain, AstraZeneca, Södertälje, Sweden) from the mid-part of the m. vastus lateralis, proximally separated by at least 3 cm. The biopsies were obtained 5–8 days after the final training session in T1 and T2 and at least 72 h after 1RM testing. An incision was made in the skin and the fascia before obtaining two biopsies using a Weil–Blakesley chonchotome as described previously (23). The biopsy procedure typically sampled 150 mg of muscle tissue (2 × 50–100 mg). The tissue obtained was rapidly cleaned to remove blood and fat, and a muscle sample was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for later single fiber analysis. Another sample was mounted in embedding medium (OCT Cryomount, Histolab Products

AB, Gothenburg, Sweden) and frozen in isopentane, cooled to its freezing point in liquid nitrogen, for later histochemical analyses. Samples were then stored at −80°C until analyzed.

Preparation and Analysis of Frozen Sections

Sections were prepared by cutting 8 µm-thick cross sections using a microtome at −20°C (CM1860, Leica, Germany). The sections were mounted on microscope slides (Superfrost Plus, Thermo Scientific, MA, USA), air-dried, and stored at −80°C. After thawing, sections were blocked in 1% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 (PBS-t; BSA, A4503, Sigma Life Science, St. Louis, MO, USA; PBS, 524650, Calbiochem, EMD Millipore, Darmstadt, Germany; Tween 20, 437082Q, VWR International, Radnor, PA, USA) for 60 min at room temperature prior to incubating with anti-dystrophin (ab15277, AbCam, Cambridge, UK) and anti-myosin heavy chain 2 (SC-71, DSHB, Iowa City, IA, USA; deposited to the DSHB by Schiaffino, S.) antibodies overnight at 4˚C. After incubating overnight, the sections were washed three times for 10 min in PBS-t and incubated with appropriate secondary antibodies (A11005 or A11001, Molecular Probes, Life technologies, Carlsbad, CA, USA) for 60 min at room temperature. After three 10-min washes in PBS-t, the muscle sections were covered with a coverslip, mounted using ProLong Gold Antifade Reagent with DAPI (P36935, Molecular Probes), and left to dry overnight at room temperature.

Sections were imaged using a high-resolution camera (DP72, Olympus Corp., Japan) mounted on a microscope (BX61, Olympus) with a fluorescence light source (X-Cite, 120PCQ, EXFO Photonic Solutions Inc., Mississauga, ON, Canada), using a 4× 0.13 NA air objective (UPlanFL N, Olympus) for CSA analyses or a 10× 0.30 NA air objective for myonuclear analyses (UPlanFL N, Olympus).

Nuclei were defined as myonuclei if the geometrical center was located inside the inner rim of the dystrophin staining. A total of 50 type 1 and 50 type 2 fibers were included in the analysis. Individual muscle fiber CSAs were analyzed and measurements were calculated using TEMA software (Checkvision, Hadsund, Denmark). CSA analysis included 198 (range, 25–577) type 1 fibers and 374 (range, 62–988) type 2 fibers.

Preparation and Analysis of Single Muscle Fibers

Single muscle fibers were isolated by maceration (47). Fixed samples were dissected into smaller muscle tissue bundles using tweezers. As much of the non-muscular tissue as possible was removed before the muscle fiber bundle was submerged in 0.25 mL of 40 % NaOH for 3 h at room temperature. Next, 0.75 mL of PBS was added to neutralize the solution, and the dissociating biopsy was shaken vigorously (1000 rpm/Fisons Scientific Whirlimixer) for 8 min to remove all non-myofiber cells attached to single fibers. Isolated single muscle fibers were then centrifuged at 13,000 rpm for 1 min, followed by removal of as much of the liquid as possible without removing fibers. Ultrapure water was then added to a final volume of 1 mL, followed by another round of centrifugation. The procedure was repeated five times. Single fibers in solution were then poured into a petri dish, picked out one by one, placed on a glass slide (Superfrost plus, J1800AMNZ, Thermo Scientific), and mounted using Fluoromount G with DAPI (Southern Biotech cat. 0100-20). A total of 44–57 fibers were isolated from each biopsy sample. The slides were dried overnight and sealed using nail polish.

Fiber segments of equal size were analyzed by acquiring images (318.08 × 318.08 μm with an aspect ratio of 640 × 640 pixels) in different focal planes with step size of 0.70 μm on an Olympus BX61W1 upright confocal microscope, using Fluoview1000 with a 40× PlanApo (NA 0.80, Olympus) water immersion objective. A 405-nm laser was used to excite DAPI to

visualize the myonuclei (Fig. 2A), and a 633-nm laser was used to visualize the fiber autofluorescence used for volume rendering (Fig. 2B). The transmitted light detector (TD1) was used to visualize sarcomeres as well as general morphology. We ensured that each fiber segment was straight and had clearly visual nuclei. Fibers that were hypercontracted or visually damaged were not measured.

Acquired image stacks of the single fiber segments were reassembled to three-dimensional images using Imaris Bitplane 8.3.1 (Bitplane AG, Zurich, Switzerland). The volume was rendered, and myonuclei were counted automatically, then confirmed manually (Figs. 2C and D). Using a single z-plane of the TD1 image, 10 sarcomeres in the proximal and distal half of the fiber was measured manually. The number of myonuclei was counted from the whole imaged fiber segment, and to correct for differences in fiber stretch, the myonuclear number were corrected against sarcomere length and shown as the number of myonuclei per sarcomere. The myonuclear domain size (MND) was calculated from the uncorrected myonuclear number against the measured volume from the individual imaged fiber segments. Fibers with central nuclei were excluded to avoid including regenerative fibers in the analysis. All single fiber analyses were performed by the same blinded investigator.

Statistical Analysis

Data are presented as mean ± SD. Differences between groups were analyzed on data material normalized against its respective first measurement using GraphPad Prism 7 software (San Diego, CA, USA). For each comparison, repeated measures were calculated for one-way ANOVA with the Greenhouse–Geisser correction, with Sidak´s multiple comparisons test of selected pairs, and with individual variances. To correlate the two different methods used for myonuclear number count and fiber size measurements, Pearson correlation coefficients were calculated for all biopsies pooled together. A two-way repeated measures ANOVA with Sidak´s multiple comparisons test was applied to assess differences between men and women

at each time-point. Dunnett´s multiple comparisons test was applied to assess time effects within the group men and women separately. Statistical significance was determined at $p \leq 0.05$.

RESULTS

Muscle Strength

Maximal strength (measured as 1RM for unilateral leg extension) improved by 20% in the mem-leg after T1 (40 ± 12 to 48 ± 14 kg, $p < 0.01$), and 60% of this improvement was still present after the DT period. There was a trend for a 5% strength improvement in the con-leg after T1 (40 ± 12 kg to 42 ± 13 kg, $p = 0.06$). After T2, the strength in both the mem-leg (45 ± 14 to 49 ± 13 kg, $p < 0.01$) and the con-leg (41 ± 13 to 47 ± 13 kg, $p < 0.01$) improved to the same extent (Figs. 2A and B).

Muscle Thickness

After T1, an increased MT was observed in the mem-leg (20.4 ± 3.5 to 22.4 ± 3.7 mm, $p < 0.01$), whereas the con-leg was unaffected (21.0 ± 3.5 mm to 21.2 ± 3.5 mm). The increase seen in the mem-leg was lost during DT so that both legs had similar MT at the start of T2. After T2, both the mem-leg (20.5 ± 3.3 to 22.8 ± 3.6 mm, $p < 0.01$) and the con-leg (20.8 ± 3.7 to 22.7 ± 3.5 mm, $p < 0.01$) showed a similar increase in muscle thickness. The magnitude of this increase (~10%) was similar to that of T1, even though T2 was only half as long (Figs. 3C and D).

Myonuclei

The number of myonuclei was measured on cryosections as the number of nuclei with their geometrical center inside the dystrophin ring per fiber (Figs. 4B–G) and as the number of myonuclei per sarcomere length in isolated muscle fibers (Figs. 4H and I). Neither method showed any significant effect of training or detraining on the number of myonuclei.

There was a significant correlation between the results of the two different myonuclei measurement methods when all sample points were included ($r = 0.59$, $p < 0.01$).

Muscle Fiber Size

After T1, an increase of ~17% was observed in the total fiber CSA of the mem-leg (4020 ± 1086 to 4718 ± 1145 μm^2, $p < 0.01$, Figs. 5C and D). The type 1 fibers' CSA increased by ~13% (4003 ± 880 to 4533 ± 1166 μm^2, $p = 0.08$, Figs. 5E and F), and the type 2 fibers' CSA increased by ~18% (4016 ± 1357 to 4735 ± 1193 μm^2, $p < 0.01$, Figs. 5G and H). Both legs CSA remained unchanged during DT and T2. The muscle fiber volume was not significantly affected by T1, DT, or T2 in either leg (Figs. 5I and J).

As with the myonuclear number, there was also a correlation between the CSA and fiber volume ($r = 0.48$, $p < 0.01$) when all sample points were included.

Myonuclear Domain Size

As a consequence of the lack of a significant change in fiber size and myonuclear number from the single fiber analysis, MND remained unaffected by training and detraining (Figs. 6A and B). Also, there were no relationships between individual levels of MDN and changes in muscle fiber size during T1, DT, and T2 (data not shown).

Sex Differences

T1, DT, and T2 had similar effects on both sexes for all measured parameters. However, muscle strength, muscle thickness and fiber size were higher in men than in women at most time points ($p < 0.05$, Figs.7C–F). Also, the ANOVA showed a sex difference (men > women) for myonuclear number in the cryosections ($p < 0.05$, Fig. 7A), but not in the isolated muscle fibers (Fig. 7B).

DISCUSSION

The present study aimed to determine whether a previously strength-trained leg (mem-leg) would respond differently compared with a previously untrained contralateral leg (con-leg) following a 5-week bilateral strength training period. We hypothesized that the mem-leg would have an enhanced hypertrophic response compared with the con-leg, and that this would correspond to an increased number of myonuclei. However, the myonuclear number did not differ between legs, and both legs responded similar to the training.

The training program used in the present study was designed to induce both hypertrophy and satellite cell activation/fusion, thereby increasing the muscle mass as well as the mem-leg's myonuclear number in a relatively short time period (10 weeks). However, this was only partially successful. While the increases in MT (~0.14% increase per day) and fiber CSA (~0.28% increase per day) of the m. vastus lateralis muscle were similar to those observed in previous studies (5, 18, 43), the number of myonuclei remained unaffected by training. A possible explanation for this may be that the increase in fiber hypertrophy was too small (<20% in CSA and no significant increase in single fiber volume). While significant myonuclei increases have been reported at lower hypotrophy levels (<10%), more consistent increases normally occur in humans when muscle fiber hypertrophy is >20% (11, 36). In the present study, one of the inclusion criteria was lack of regular physical activity or taking part in sport on a regular basis; therefore, only very sedentary individuals were selected for the study. The purpose of doing this was to recruit individuals with similar starting points, i.e., untrained muscle fibers with no memory of training. This may have influenced the apparent lack of increase in myonuclear number as the training-induced hypertrophy may not have put the nuclei under sufficient strain, as suggested by the myonuclear domain ceiling theory (4, 27, 35). According to this theory, the existing myonuclei may be capable of supporting the

present level of muscle fiber hypertrophy (27). However, even though hypertrophy of human muscle is often associated with myonuclear accretion, it has also been suggested that accretion of myonuclei might serve other purposes than supporting de novo hypertrophy. For example, recent mouse studies show that muscle repair and remodeling are important drivers for the addition of myonuclei (31, 32).

The 20-week DT period and the following 5-week retraining period had no effect on CSA or fiber volume in either leg. However, a relatively large increase in MT was observed during retraining in both the mem-leg and con-leg (~10%). This mismatch between the muscle fiber and MT measurements could be explained partly by edema-induced muscle swelling. Damas et al. (13) showed that muscle edema may be present >72 h after the last training session of short, strength training programs in untrained individuals. Another possible explanation could be that the ultrasound scan site and the muscle biopsy sites were not the same. Ultrasound scans were performed at 25%, 50%, and 75% of the distance between the superior edge of patella and the trochanter major, and an average value was used to calculate MT. Most muscle biopsies were obtained near the mid-part of the m. vastus lateralis; however, due to the large number of biopsies, final biopsies were obtained at more proximal locations. A recent study showed that ultrasound measurement of MT is a reliable tool for monitoring hypertrophic responses, but that it is important to perform biopsies and ultrasound scans at the same location due to regional differences in hypertrophy (18). Another possible explanation is that the repeated biopsy procedure induced muscle damage, causing atrophy of the fibers (45). It is also possible that the training regime, which included both heavy strength training and BFRT, was too strenuous and exerted some detrimental effects on muscle fibers, which counteracted to what we aimed to achieve (optimization of satellite cell proliferation and myonuclear addition). However, the training was carefully periodized and the volume and effort of BFRT

(~50 repetitions per exercise and only the last two sets were performed to failure) were limited to what we have previously shown not to induce muscle fiber damage (33).

An increase of approximately 20% in strength was observed after the initial training period. Interestingly, a strength increase was also observed in the con-leg after this period. While this increase was not statistically significant ($p = 0.06$), it does suggest that motor learning may be involved in the training effects in both legs. Enhanced strength in an untrained limb after a period of unilateral training was previously described and referred to as "the cross education effect" (30). This effect is not caused by hypertrophy, but is linked to adaptations within the CNS (16). Such neurological adaptations may have played a role in the observed increased strength in the mem-leg. In fact, it is well established that neurological adaptations contribute to strength improvements in untrained individuals during the initial weeks/months of strength training (3, 22, 38). However, it is also possible that the observed strength gain was influenced by qualitative alterations within the muscle cells, such as changes in myosin concentration and cross-bridge kinetics (25, 29).

While the term "muscle memory" is also used to describe such long-lasting neurological modifications, this is misleading. This form of memory is probably largely unrelated to muscle cells' properties and should, instead, be referred to as "motor learning." Although there was a clear motor learning effect in the present study at the onset of the retraining period (the mem-leg was ~10% stronger than the con-leg), this had no impact on strength progression during this period because both legs responded to training similarly. Hence, similar to the hypertrophy findings, no memory effect was observed for strength in the present study.

Recent studies suggest that there may be an epigenetic component involved in human muscle memory (41, 42). This was not investigated in the present study, and we cannot exclude that epigenetic alterations, such as DNA-methylation, took place in the mem-leg during T1. This might explain why the participants in the present and a previous study (45) retained muscle fiber hypertrophy during several months of detraining. However, even if T1 induced epigenetic alterations, they had no detectable effect on the hypertrophic response in our study because both the con-leg and the mem-leg responded similarly to T2. Also, it is possible that the retention of fiber hypertrophy was a consequence of an increased activity level during detraining rather than epigenetic alterations. The participants were not allowed to train, but their daily activity levels were not controlled, and being part of a training intervention might have motivated them to be less sedentary during detraining. Actually, fiber CSA is not retained during detraining in participants accustomed to strength training (20), indicating that the training level is important for the detraining response.

Conclusions

Our study demonstrates that previously trained human muscles do not respond differently to a second training period compared with previously untrained muscles. However, this does not rule out the existence of human muscle memory because previous studies in mice suggest that the memory storage mechanism was associated with an increased number of myonuclei, a parameter that remained unaffected in the present experiment. Therefore, future studies are required on potential muscle memory in humans involving training both to induce hypertrophy and increase the myonuclear number.

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DISCLOSURES

The authors have no conflicts of interest, financial, or otherwise, to declare.

AUTHOR CONTRIBUTIONS

N.P., M.W., J.B., T.R. and K.G. conceived and designed research; N.P., E.E., K.T.C., I.J., M.W., M.E., K.S. and B.E. performed experiments; N.P., E.E., K.T.C., I.J., K.S. and J.B. analyzed data; N.P., E.E., K.T.C., J.B., T.R. and K.G. interpreted results of experiments; E.E. and J.B. prepared figures; N.P. and K.G. drafted manuscript; N.P., E.E., K.T.C., M.E., B.E., K.S., M.W., H.H., J.B. T.R. and K.G. edited and revised manuscript; N.P., E.E., K.T.C., M.E., B.E., K.S., M.W., H.H., J.B., T.R. and K.G. approved final version of manuscript.

ENDNOTE

At the request of the author(s), readers are herein alerted to the fact that additional materials related to this manuscript may be found at the Mendely Data Respiratory, which at the time of publication they indicate has the following DOI: 10.17632/s677jds6t2.1. These materials are not a part of this manuscript and have not undergone peer review by the American Physiological Society (APS). APS and the journal editors take no responsibility for these materials, for the website address, or for any links to or from it.

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FIGURE LEGENDS

Fig. 1. Schematic illustration of the experimental protocol. T1, first training period; DT, detraining period; T2, second training period. Red and blue rectangles denote the training periods for the memory and control leg, respectively. Only the memory leg was trained during T1, whereas both legs where trained during T2. Pink denotes BFRT performed at weeks 4 and 8. Biopsies were collected, as indicated, after at least a 48-h rest. Muscle thickness was measured by ultrasound, and the 1RM test was performed in association with all the biopsy sampling points.

Fig. 2. Effect of training (T1), detraining (DT), and retraining (T2) on leg extension strength. (A) 1RM leg extension strength (mean ± 95% CI) and (B) individual measurements and mean ± 95% CI. Measurements were normalized to pre-T1 for the respective leg. Blue represents the control leg, and red represents the memory leg (n = 19). ** $p < 0.01$, difference from previous time point for the indicated leg, underneath and above the data points, for the control and memory leg respectively. $^{\#\#}$ $p < 0.01$, $^{\#}$ $p < 0.05$, difference between the legs at the time point indicated. P = 0.06 denotes the trend for an increase from Con-pre1 to Con-post1.

Fig. 3. Effect of training (T1), detraining (DT), and retraining (T2) on muscle thickness. (A, B) Representative ultrasound images from vastus lateralis pre- and post-T1. (C, D) Muscle thickness shown normalized to pre-T1 for the respective leg, and individual measurements (mean ± 95% CI). Blue represents the control leg, red represents the memory leg (n = 19). ** $p < 0.01$, difference from previous time point for the indicated leg, underneath and above the data points, for the control and memory leg respectively. $^{\#\#}$ $p < 0.01$, difference between the

legs at the indicated time point. The outlier in (D) was a male participant with an unusually large muscle mass, despite a sedentary lifestyle with no history of training.

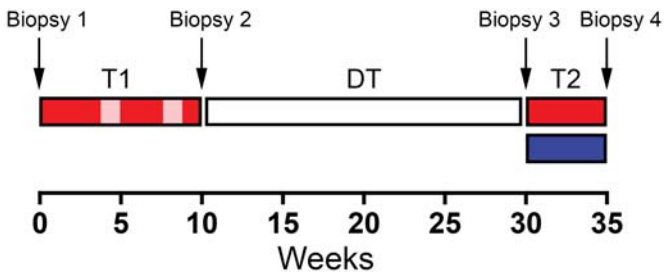
Fig. 4. Effect of training (T1), detraining (DT), and retraining (T2) on the myonuclear number. (A) Three-dimensional visualization of DAPI-stained nuclei (1), cell volume (2), and rendering of these to measure fiber volume and count nuclei, shown from outside (3) and inside (4) the fiber (see Methods for more details). (B–I) The left column shows myonuclear number (mean ± 95% CI) normalized to the first biopsy from the respective leg, and the right column shows the individual biopsy average values and group means ± 95% CI. The measurements were myonuclear number per fiber cross section for all fibers (B, C), type 1 fibers (D, E), and type 2 fibers (F, G). (H, I) Single fiber myonuclear number per sarcomere. Blue represents the control leg and red represents the memory leg (n = 19).

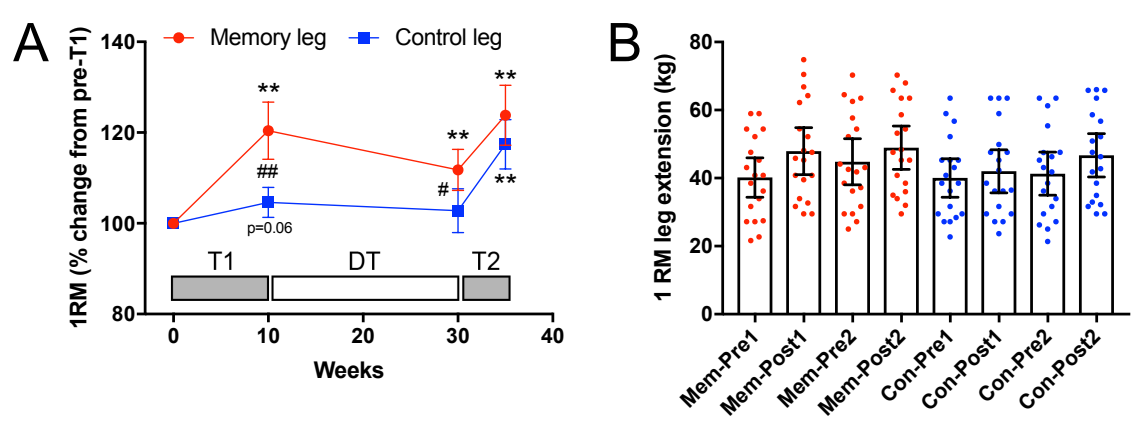
Fig. 5. Effect of training (T1), detraining (DT), and retraining (T2) on muscle fiber size. (A, B) Representative micrographs of cryosections obtained from vastus lateralis pre- and post-T1, stained for dystrophin (red) and type 2 fibers (green). (C-J) The left column shows fiber size (mean ± 95% CI) normalized to the first biopsy from the respective leg, and the right column shows individual biopsy average values and group means ± 95% CI. Measurements were CSA for all fibers (C, D), type 1 fibers (E, F), and type 2 fibers (G, H). (I, J) Single fiber volume measurements. Blue represents the control leg and red represents the memory leg (n = 17 for C-H, n = 19 for I and J). ** $p < 0.01$, difference from previous time point for that leg.

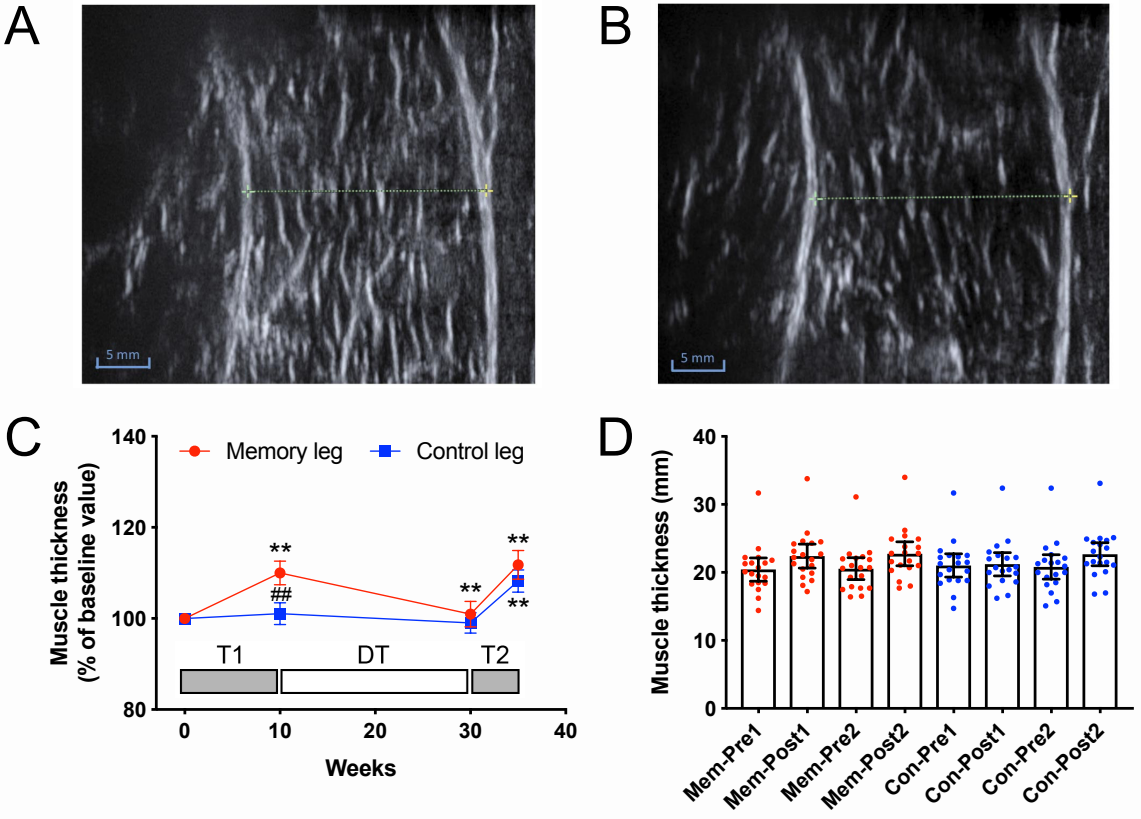
Fig.6. Effect of training (T1), detraining (DT), and retraining (T2) on myonuclear domain size. (A) Myonuclear domain size (mean ± 95% CI) normalized to the first biopsy from the

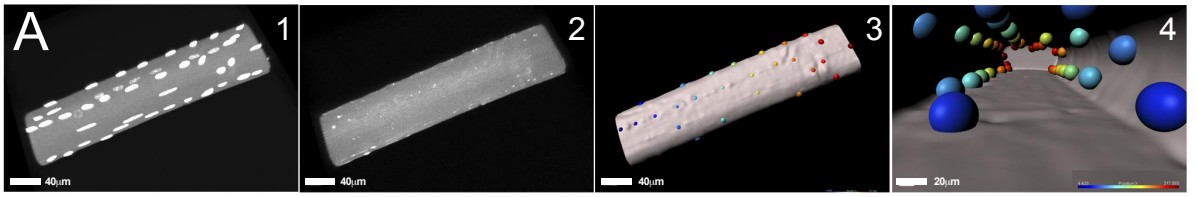
respective leg. (B) Individual biopsy average values and group means ± 95% CI. Blue represents the control leg and red represents the memory leg (n = 19).

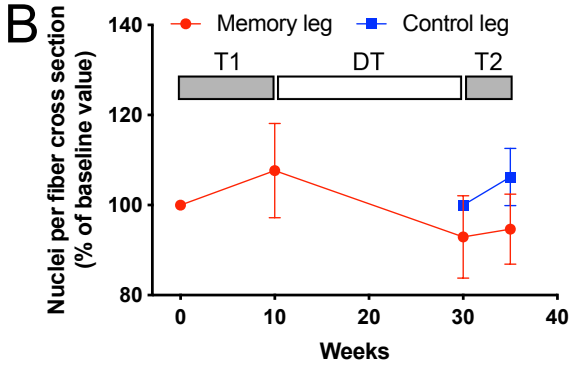
Fig.7. Sex differences during training (T1), detraining (DT), and retraining (T2). Differences between women (magenta) and men (gray) for the memory leg (group means ± 95% CI) for non-normalized values are shown for myonuclear number from cross sections (A) and single fibers (B), 1RM (C), muscle thickness (D), fiber CSA (E), and fiber volume (F). * $p < 0.05$, difference from pre-T1 at the indicated time points, underneath and above the data points, for woman and men respectively. $^{\#}$ $p < 0.05$, difference between men and women at the indicated time points.

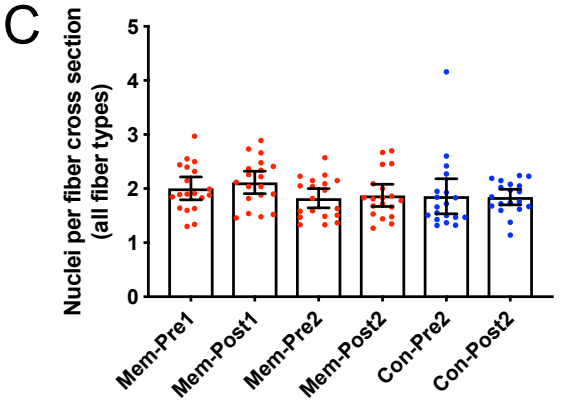

Biopsy 1
Biopsy 2
Biopsy 3
Biopsy 4
T1
DT
T2
0
5
10
15
20
25
30
35
Weeks

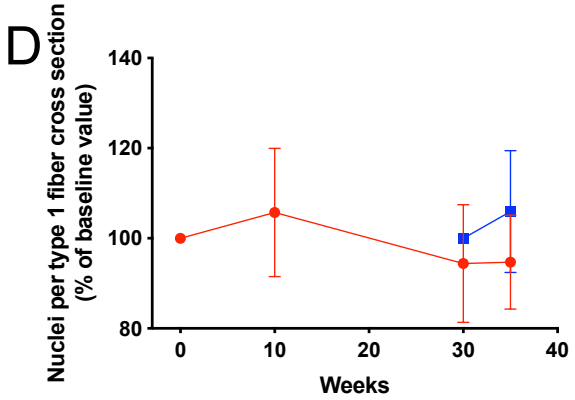

A
Memory leg
Control leg
1RM (% change from pre-T1)
140
120
100
80
**
**
**
**
##
#
p=0.06
T1
DT
T2
0
10
20
30
40
Weeks
B
1 RM leg extension (kg)
80
60
40
20
0
Mem-Pre1
Mem-Post1
Mem-Pre2
Mem-Post2
Con-Pre1
Con-Post1
Con-Pre2
Con-Post2

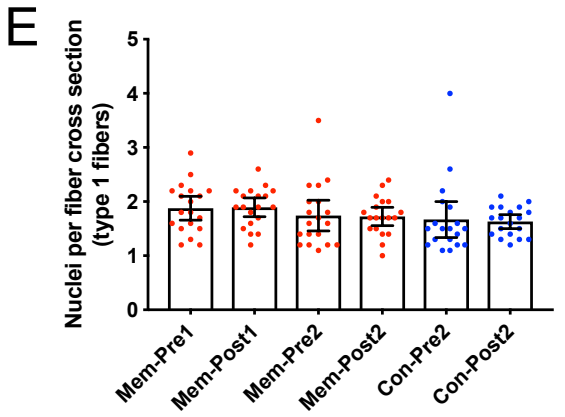

A
5 mm
B
5 mm
C
Memory leg
Control leg
Muscle thickness (% of baseline value)
140
120
100
80
**
##
**
**
**
T1
DT
T2
0
10
20
30
40
Weeks
D
Muscle thickness (mm)
40
30
20
10
0
Mem-Pre1
Mem-Post1
Mem-Pre2
Mem-Post2
Con-Pre1
Con-Post1
Con-Pre2
Con-Post2

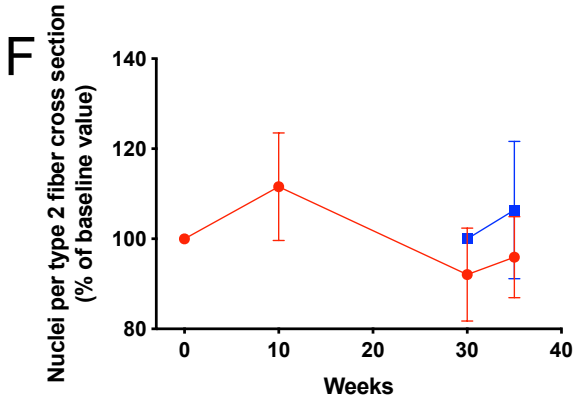

A
1
2
3
4
40µm
40µm
40µm
20µm

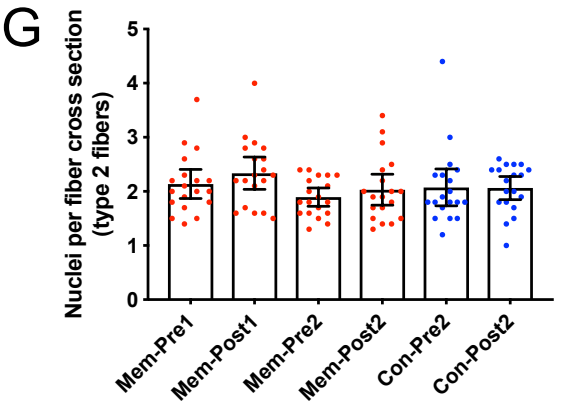

B
Memory leg
Control leg
T1
DT
T2
Nuclei per fiber cross section
(% of baseline value)
140
120
100
80
0
10
20
30
40
Weeks

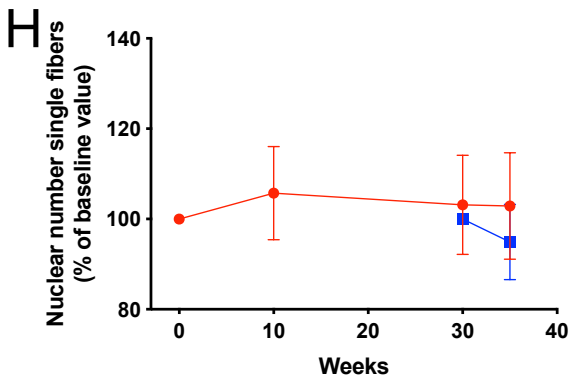

C
Nuclei per fiber cross section
(all fiber types)
5
4
3
2
1
0
Mem-Pre1
Mem-Post1
Mem-Pre2
Mem-Post2
Con-Pre2
Con-Post2

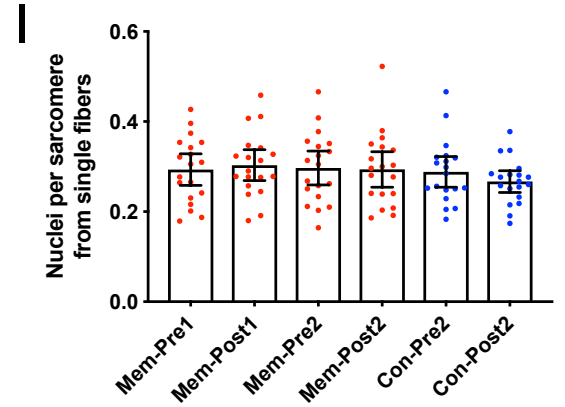

D
Nuclei per type 1 fiber cross section
(% of baseline value)
140
120
100
80
0
10
20
30
40
Weeks

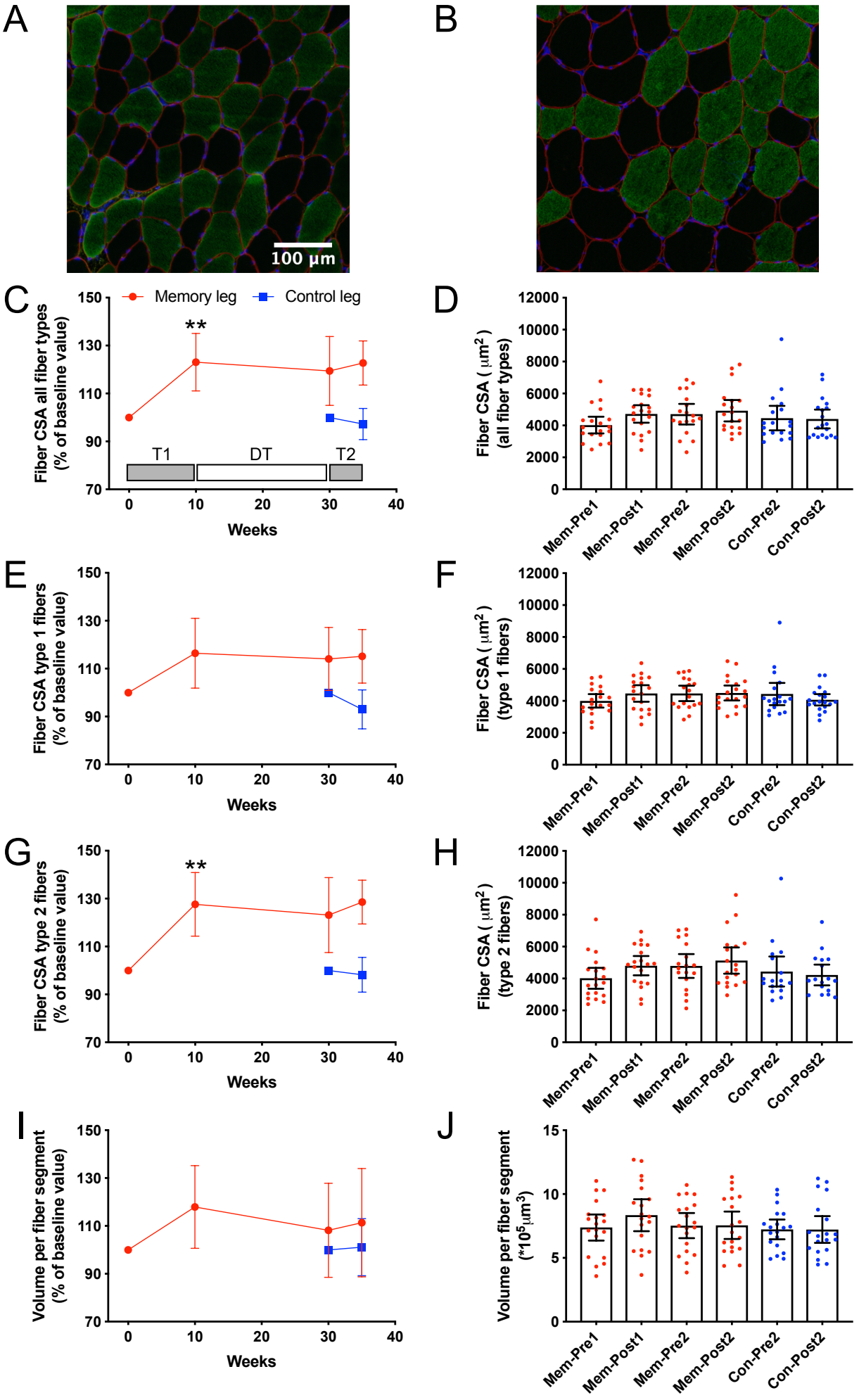

E
Nuclei per fiber cross section
(type 1 fibers)
5
4
3
2
1
0
Mem-Pre1
Mem-Post1
Mem-Pre2
Mem-Post2
Con-Pre2
Con-Post2

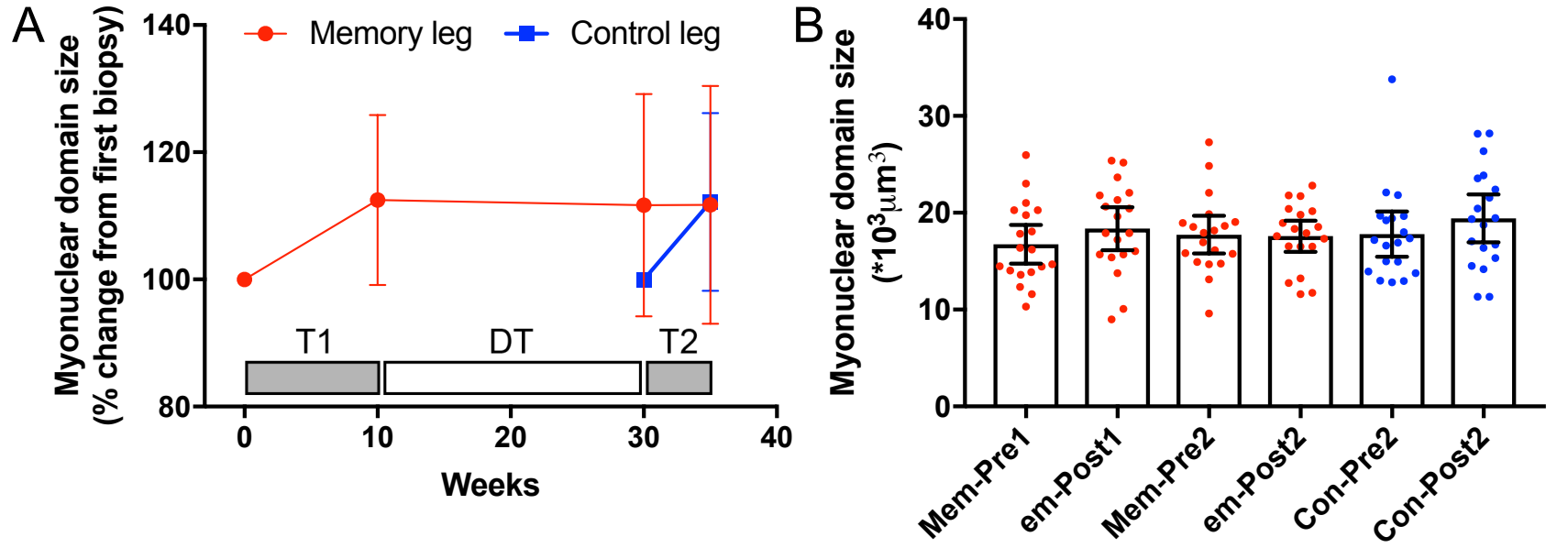

F
Nuclei per type 2 fiber cross section
(% of baseline value)
140
120
100
80
0
10
20
30
40
Weeks

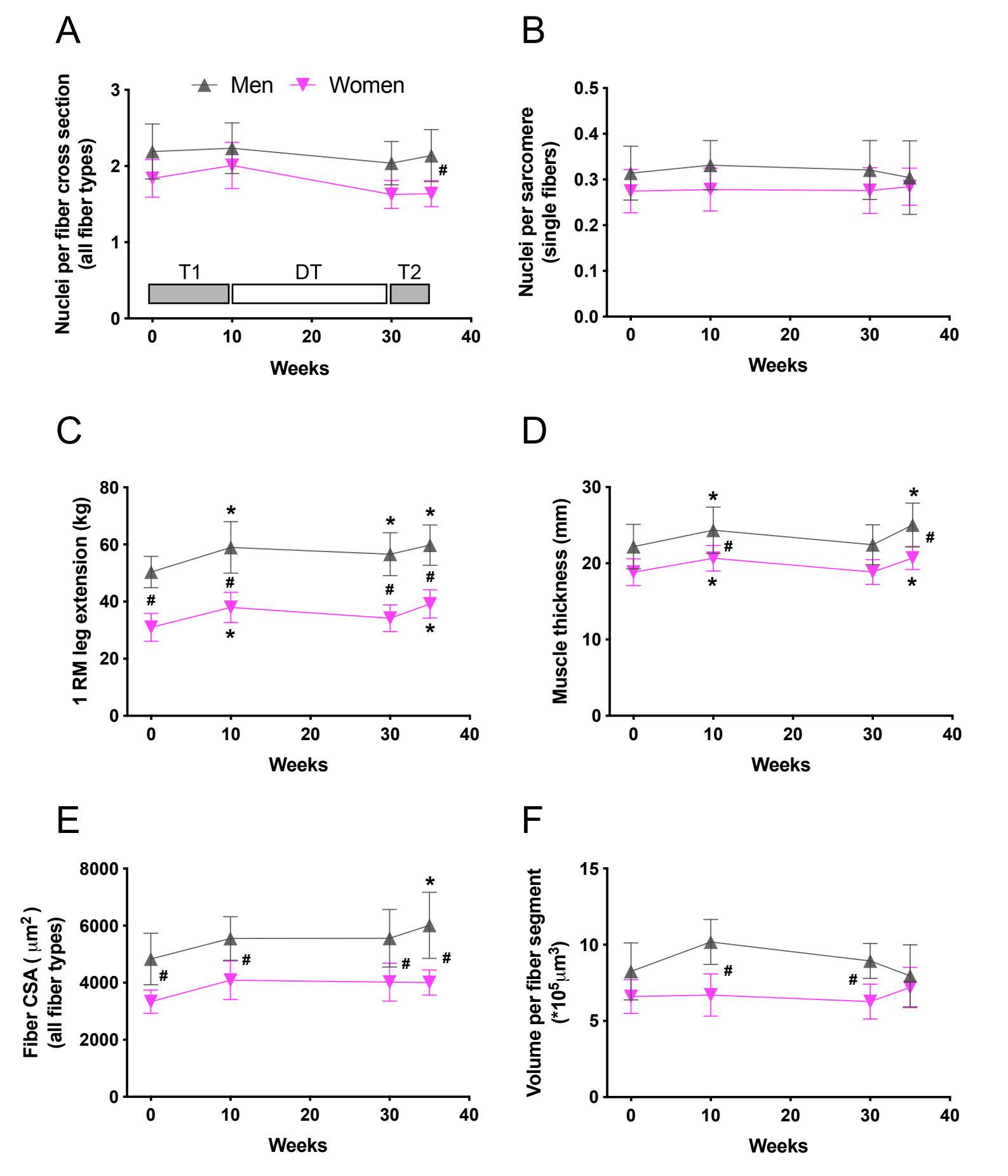

G
Nuclei per fiber cross section
(type 2 fibers)
5
4
3
2
1
0
Mem-Pre1
Mem-Post1
Mem-Pre2
Mem-Post2
Con-Pre2
Con-Post2


H
Nuclear number single fibers
(% of baseline value)
140
120
100
80
0
10
20
30
40
Weeks


I
Nuclei per sarcomere
from single fibers
0.6
0.4
0.2
0.0
Mem-Pre1
Mem-Post1
Mem-Pre2
Mem-Post2
Con-Pre2
Con-Post2


A
100 µm
B
C
Memory leg
Control leg
**
Fiber CSA all fiber types (% of baseline value)
T1
DT
T2
Weeks
D
Fiber CSA (µm2) (all fiber types)
Mem-Pre1
Mem-Post1
Mem-Pre2
Mem-Post2
Con-Pre2
Con-Post2
E
Fiber CSA type 1 fibers (% of baseline value)
Weeks
F
Fiber CSA (µm2) (type 1 fibers)
G
**
Fiber CSA type 2 fibers (% of baseline value)
Weeks
H
Fiber CSA (µm2) (type 2 fibers)
I
Volume per fiber segment (% of baseline value)
Weeks
J
Volume per fiber segment (*10^5 µm^3)


A
Myonuclear domain size
(% change from first biopsy)
140
120
100
80
Memory leg
Control leg
T1
DT
T2
0
10
20
30
40
Weeks
B
Myonuclear domain size
(*10³μm³)
40
30
20
10
0
Mem-Pre1
em-Post1
Mem-Pre2
em-Post2
Con-Pre2
Con-Post2


A
Men
Women
Nuclei per fiber cross section (all fiber types)
T1
DT
T2
Weeks
B
Nuclei per sarcomere (single fibers)
Weeks
C
1 RM leg extension (kg)
Weeks
D
Muscle thickness (mm)
Weeks
E
Fiber CSA (μm²) (all fiber types)
Weeks
F
Volume per fiber segment (*10⁵μm³)
Weeks