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***Morphological, molecular and hormonal adaptations to early morning vs.
afternoon resistance training***

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Abstract

It has been clearly established that maximal force and power is lower in the morning compared to noon or afternoon hours. This morning neuromuscular deficit can be diminished by regularly training in the morning hours. However, there is limited and contradictory information upon hypertrophic adaptations to time-of-day specific resistance training. Moreover, no cellular or molecular mechanisms related to muscle hypertrophy adaptation have been studied with this respect. Therefore, the present study examined effects of the time-of-day specific resistance training on muscle hypertrophy, phosphorylation of selected proteins, hormonal concentrations and neuromuscular performance. Twenty five previously untrained males were randomly divided into a morning group ($n=11$, age 23 ± 2 yrs), afternoon group ($n=7$, 24 ± 4 yrs) and control group ($n=7$, 24 ± 3 yrs). Both the morning and afternoon group underwent hypertrophy-type of resistance training with 22 training sessions over an 11-week period performed between 07:30-08:30 h and 16:00-17:00 h, respectively. Isometric MVC was tested before and immediately after an acute loading exclusively during their training times before and after the training period. Before acute loadings, resting blood samples were drawn and analysed for plasma testosterone and cortisol. At each testing occasion, muscle biopsies from m. vastus lateralis were obtained before and 60 minutes after the acute loading. Muscle specimens were analysed for muscle fibre cross-sectional areas (CSA) and for phosphorylated p70S6K, rpS6, p38MAPK, Erk1/2, and eEF2. In addition, the right quadriceps femoris was scanned with MRI before and after the training period. The control group underwent the same testing, except for MRI, between 11:00 h and 13:00 h but did not train. Voluntary muscle strength increased significantly in both the morning and afternoon training group by 16.9 % and 15.2 %, respectively. Also muscle hypertrophy occurred by 8.8 % and 11.9 % (MRI, $p<0.001$) and at muscle fibre CSA level by 21 % and 18 % ($p<0.01$) in the morning and afternoon group, respectively. No significant changes were found in controls in muscle strength and CSA. Both pre- and post-training acute loadings induced a significant ($p<0.001$) reduction in muscle strength in all groups, not affected by time of day or training. The post-loading phosphorylation of p70S6^{Thr421/Ser424} increased independent of the time of day in the pre-training condition, whereas it was significantly increased in the morning group only after the training period ($p<0.05$). Phosphorylation of rpS6 and p38MAPK increased acutely both before and after training in a time-of-day independent manner ($p<0.05$ at all occasions). Phosphorylation of p70S6^{Thr389}, eEF2 and Erk1/2 did not change at any time point. No statistically significant correlations were found between changes in muscle fibre CSA, MRI and cell signalling data. Resting testosterone was not statistically different among groups at any time point. Resting cortisol declined significantly from Pre to Post in all three groups ($p<0.05$). In conclusion, similar levels of muscle strength and hypertrophy could be achieved regardless of time of the day in previously untrained men. However, at the level of skeletal muscle signalling, the extent of adaptation in some parameters may be time-of-day dependent.

Introduction

In a person unaccustomed to morning exercise, voluntary and artificially evoked muscle strength (maximum and explosive), has been repeatedly reported to be on average 5-10% lower in the morning hours compared to the rest of the day (e.g., Araujo et al., 2011, Callard et al., 2000, Castaingts et al., 2004, Coldwells et al., 1994, Deschenes et al., 1998, Gauthier et al., 1996, Giacomoni et al., 2005, Guette et al., 2005a, Mora-Rodriguez et al., 2012, Nicolas et al. 2005., Sedliak et al., 2008). This phenomenon has been termed as “morning neuromuscular deficit” due to the fact that that inputs from the central nervous system are, at least partly, an important source (for a review see Sedliak 2013).

The first direct evidence that morning neuromuscular deficit can be diminished by regularly training in the morning hours over period of several weeks was reported by Souissi et al. (2002). Since then, several papers have been published confirming that morning resistance training performed regularly for at least 5 weeks can reduce morning – afternoon difference in short-term maximal performance, whereas regular afternoon training preserves or even amplifies the morning – afternoon difference in performance in adult and also children population (Sedliak et al. 2008, Souissi et al. 2012, Chtourou 2012 a, b, Chaouachi, et al., 2012, Zbidi et al., 2016). Importantly, the magnitude of strength and power gains during a training period are similar regardless of time of day of training. A recent study of Chtourou et al. (2015) showed that also a detraining phenomenon in time-of-day specific adaptations exists. In their study, the time-of-day specific improvements in strength during a 14-week period, comparable to those described in the above studies, returned to baseline values after 5 weeks of detraining. However, the mechanisms responsible for time-of-day specific adaptation in neuromuscular performance and also for detraining can be only speculated. For instance, diurnal temperature variation, normally coinciding with diurnal variation in muscle

strength and power in untrained subjects, was not modified by the training regimens or during the detraining period (Chtourou et al. 2015). Furthermore, the myoelectrical activity (EMG signal) did not show any significant diurnal variation before nor after the time-of-day specific training (Sedliak et al. 2008).

Besides neural mechanisms, local, intracellular mechanisms may also contribute to the diurnal variation in muscle strength and power. For example, early morning resistance loading may induce significantly higher between-subject variation in some muscle growth- or metabolism-related signalling pathways compared to the same acute loading later in the day (Sedliak et al. 2012). Phosphorylation of specific proteins in protein kinase B/muscle target of rapamycin/p70 ribosomal S6 kinase signalling pathway (Akt/mTOR/p70S6K) and to some extent also in mitogen-activated protein kinases (MAPK) signalling pathway has been shown to positively regulate muscle growth (Terzis et al. 2008; Shi et al. 2009; Bodine et al. 2001). Further, resistance exercise primarily aimed at increasing muscle hypertrophy is a potent stimulus to the increase of mTOR and MAPK signalling (Hulmi et al. 2009; 2010; Drummond et al. 2009, Terzis et al. 2008). At least signalling through rapamycin sensitive mTOR complex 1 (mTORC1) is needed to induce protein synthesis after resistance exercise (Drummond et al. 2009).

There is limited information upon hypertrophic adaptations to time-of-day specific resistance training. In the first study, Sedliak et al. (2009) concluded that 12-week resistance training in the morning and afternoon hours was similarly effective when aiming for muscle hypertrophy of the lower limbs. However, the average gains in muscle mass were half of the size typically seen in a similar population of subjects and training duration (Wernbom et al. 2007), probably because a combination of high resistance/high speed and hypertrophy training loadings was

applied. For instance, Hulmi et al. (2012) showed that a high-load neural protocol of relatively short time under contraction does not fully activate signalling pathways leading to hypertrophy.

On the contrary, the second study on this topic showed that a 24-week combined strength- and endurance-training program induced larger gains in muscle mass in the evening training groups compared to the morning groups (Kuusmaa et al. 2016, in press). In both the above mentioned studies the time-of-day specific resistance/combined training did not affect diurnal variation in resting testosterone and cortisol, and induced similar magnitude of adaptations in strength performance regardless of the time of day.

Performing mixed training programs as in the works of Sedliak et al. (2009) and Kuusmaa et al. (2016) may lead to reduced/altered muscle gains compared to typical hypertrophic type of training as defined by Kraemer and Hakkinen (2008). In addition, so far no cellular or molecular mechanisms related to muscle hypertrophy adaptation have been studied with respect to the time of day. Therefore, the present study, a part of the same project with previously published data from acute responses (Sedliak et al. 2012) focuses now on the hypertrophic training response. More precisely, we aimed to examine effects of the time-of-day specific, purely hypertrophic type of resistance training on skeletal muscle hypertrophy measured both at a macro-and microscopic level, further on phosphorylation of selected proteins, systemic hormonal concentrations and neuromuscular performance.

Materials and Methods

Subjects

Forty-eight subjects replied to recruiting flyers placed on several free advertising boards. Out of them, thirty-two men met the following inclusion criteria, collected by questionnaires and during the recruiting interview: a clinically healthy male between 20 to 30 years of age with no medication within the last 14 days, a non-smoker, regular sleep pattern with sleep duration ranging from 6 to 9 hours per night, no history of strength training, removing its chronic effects on muscle signalling (e.g. Galpin et al. 2016), other regular physical activity not more than once a week during the past 3 years, no history of shift work and classification as a “neither type” in the self-assessment questionnaire to determine morningness-eveningness (Horne & Ostberg 1976).

Subjects were randomly distributed to either training groups (24 subjects) or a control group (8 subjects). Subjects belonging to the training groups were further pair-matched based on their pre-training maximum isometric leg extension strength and body mass index and the pairs were randomly divided into two time-of-day-specific training groups: morning (n=12) and afternoon (n=12).

Out of these 32 subjects, six subjects in the morning and afternoon group were excluded due to five or more missed training sessions; one subject in control group terminated the study without stating the reason.

Twenty five subjects (n=11, n=7 and n=7 in the morning, afternoon and control group, respectively) successfully accomplished the entire experiment and only their data are presented in the “Results” section. The groups’ age and anthropometrical characteristics at the pre-training state were as follows (mean \pm SD):

morning group: age 23 ± 2 yrs, body mass 77.8 ± 5.7 kg, height 182 ± 8.1 cm

afternoon group: age 24 ± 4 yrs, body mass 77.0 ± 5.2 kg, height 180 ± 8.1 cm

control group, age 24 ± 3 yrs, body mass 79.1 ± 6.2 kg, height 179 ± 8.8 cm

This study was conducted in accordance with the ethical standards of the journal (Portaluppi et al., 2010), complied with the Declaration of Helsinki and was approved by the Ethics Committee of the Faculty of Physical Education and Sports, Comenius University, Bratislava, Slovakia. An informed consent form was read and signed by each individual subject prior to the investigation.

Experimental design

In general, the experiment consisted of an 11-week training period and two testing sessions placed 7 to 10 days before and 5 to 7 days after the first and the last training session, respectively. All physical testing and resistance training sessions were conducted between February and July of the same year in the facilities of the Faculty of Physical Education and Sports, Comenius University, Bratislava, Slovakia. Blood and biopsy sampling and magnetic resonance imaging were performed by medical professionals in a nearby hospital. Subsequent laboratory analyses of muscle tissue specimens were carried out in the laboratories of the Norwegian School of Sport Sciences (Oslo, Norway - immunohistochemistry) and University of Jyväskylä (Jyväskylä, Finland – Western blotting). Blood samples were analysed at the Faculty of Natural Sciences, Comenius University in Bratislava, Slovakia.

Both the morning and afternoon group underwent identical strength testing and training protocols differing only with regards to the time of day of testing and training. The morning and afternoon group were tested and exercised exclusively between 07:30 - 08:30 h and 16:00

- 17:00 h, respectively. The control group was tested between 11:00 h and 13:00 h equally to the training groups but did not perform any resistance training during the experimental period.

Familiarization and pre-testing

Subjects underwent one pre-testing session held between 11:00 h and 14:00 h five to seven days prior to the actual experiment. Firstly, body height and body mass were measured. Subsequently, subjects were familiarized with the testing protocol and apparatus and then performed three repetitions of isometric maximum voluntary bilateral leg extension at the knee angle 107° (MVC) on a custom built, computer controlled linear motor-powered leg press dynamometer, described in details elsewhere (Sedliak et al., 2012). During MVC, subjects were seated on the seat with arms placed on the chest. Two adjustable padded bars, connected with the backrest, were placed on the shoulders to prevent any upwards movement during exercise. Knee angle was set to 107°, hip angle to 110 ° and ankle angle to 90°. Participants were instructed to push with maximal effort for 3 – 4 seconds against the footrest platform using both anterior and posterior thigh and hip muscles while keeping firm contact with the seat and backrest. Loud verbal encouragement was given. An analogue signal, converted to digital via a 12-bit AD converter was sampled by the computer with the sampling frequency of 1000 Hz and analyzed with custom-made software. Out of three MVC pre-testing trials, the trial with the highest peak force was taken for further analyses.

Time-of-day-specific test protocol

The time-of-day-specific testing sessions were applied twice to each individual subject - during March (Pre-training testing) and during June and July (Post-training testing).

Subjects were instructed to refrain from alcohol, caffeine, sexual and strenuous physical activity 48 hours prior and during the test day. Light- and medium-speed walking shorter than 20 min per a bout was allowed. They were also asked to go to bed between 22:00 and 23:00 h and get up between 05:45 and 06:15 h in the morning of the testing day. No naps were allowed on the testing day. Compliance with the requirements for the activity and sleep patterns were visually verified from wrist movement analyzer data (Actiwatch, Cambridge, United Kingdom) worn by subjects during the last 16 – 24 h before the test.

The subjects were scheduled to report at the laboratory between 06:45-07:15 h, 10:15-10:45 h and 15:15-16:45 h for the morning, control and afternoon group, respectively. Immediately after arrival, subjects were given carbohydrate rich, low-protein low-fat meal. The meal consisted of a bun with margarine and strawberry jam (total average values per 1 portion: energy 251 kcal, carbohydrates: 46 g, protein: 4.4 g, fat: 5.5 g) and was scheduled approximately 20 minutes before the first pre-loading muscle biopsy. The rationale for this standardized meal was to diminish possible differences in nutritional status. For the morning group, this was a first meal after an overnight fast. For the control group, the standard meal was the second meal during the day after breakfast (07:00 – 07:30, cereals and milk). For the afternoon group, the standard meal was the third meal during the day after breakfast (07:00 – 07:30, cereals and milk) and lunch (12:00 – 12:30 h, meat with rice or potatoes). Clear written instructions were given to subjects with respect to the amount and contents of the breakfast and lunch.

Subjects then underwent testing procedures in the following order: pre-loading muscle biopsy, pre-loading blood sampling, pre-loading MVC (Pre MVC), acute loading protocol, post-loading MVC (Post MVC) and post-loading muscle biopsy (Figure 1).

Figure 1

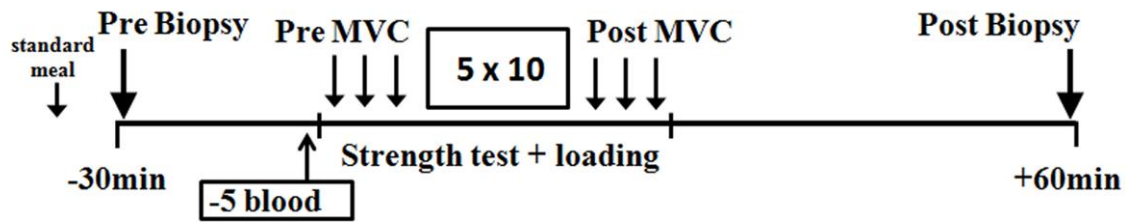


Figure 1. Chronology of the time-of-day-specific test protocol. Pre and Post – before and after acute heavy resistance loading, respectively, MVC - maximum isometric voluntary contraction, 5x10 – five sets of ten repetitions of maximum isokinetic voluntary contractions;

“-30min”, “-5 blood” and “+60min” stands for biopsy sampling 30 minutes and blood sampling 5 minutes before the first bout of Pre MVC and 60 minutes after the last bout of Post MVC, respectively. The morning and afternoon group were tested with this protocol between 07:30 - 08:30 h and 16:00 - 17:00 h, respectively. The control group was tested between 11:00 h and 13:00 h but did not perform any resistance training during the experimental period.

Muscle biopsies

At each testing occasion, two muscle biopsy samples were obtained; 30 minutes before the first bout of pre-loading MVC (pre-loading biopsy) and 60 minutes after the last bout of post-loading MVC (post-loading biopsy, Figure 1). Biopsy samples were taken from the m. vastus lateralis muscle with a 5-mm Bergström biopsy needle and with manual suction, midway between the upper region of patella and greater trochanter. The pre-loading and post-loading biopsy sample was always taken from the right and left vastus lateralis, respectively. Muscle biopsy specimens were cleaned of any visible connective and adipose tissue as well as blood. One part of the biopsy sample was frozen immediately in liquid nitrogen and the second part selected for immunohistochemistry was mounted in a Tissue-Tek compound (Cat#4583;

Sakura Finetek, Torrance, CA, USA) and quickly frozen in isopentane cooled on liquid nitrogen. All samples were stored at -80°C until assay.

Strength test, blood sampling and acute loading protocol

After arriving from the hospital (cca 20 min post biopsy), subjects rested 5 minutes in a supine position and then blood samples were drawn from the antecubital vein using a needle and syringe. A standardized warm-up protocol consisting of brief dynamic stretching movements, fifteen unloaded parallel squats and one trial of approximately 50% MVC in testing conditions was performed after blood sampling. Subsequently, three trials of Pre MVC were measured in the same manner as described above. After a 2 minute-rest period, an acute loading protocol consisting of five sets of ten repetitions of isokinetic bilateral leg extension was performed on the dynamometer with rest intervals of 1.5 minutes between the sets. Movements of the pedals started from 90° in knee joint and ended approximately 0.08 m before legs were fully extended. Speed of the pedals was $0.2\text{ m}\cdot\text{s}^{-1}$. Subjects were required to push with a maximal voluntary effort both in the eccentric and concentric phase of all movement cycles. Loud verbal encouragement was given during each repetition. Two minutes after the acute loading exercise protocol 3 trials of MVC were carried out again (Post MVC).

In addition to Pre- and Post-training testing, MVC only was tested in both training groups prior the first training session at week 3, 6 and 9 (Mid 1, Mid 2 and Mid 3) using an identical protocol described above.

Tissue and blood processing

Muscle specimens were later homogenized in ice-cold buffer (20mM HEPES, pH 7.4, 1mM EDTA, 5mM EGTA, 10mM MgCl₂, 100mM β-glycerophosphate, 1mM Na₃VO₄, 2mM DTT, 1% Triton X-100, 0.2% sodium deoxycholate, and 1% phosphatase inhibitor cocktail (P 78443; Pierce, Rockford, USA)) at a dilution of 15 ml/mg of wet weight muscle. Homogenates were rotated for 30 min at 4°C, centrifuged at 10 000 g for 10 min at 4°C to remove cell debris, and stored at -80°C. Total protein content was determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, USA) in triplicates with an automated KoneLab device (Thermo Scientific, Vantaa, Finland).

Western immunoblot

Aliquots of muscle lysate were solubilized in Laemmli sample buffer and heated at 95°C to denaturise proteins. Samples containing 30 µg of total protein were separated by SDS-PAGE for 60 min at 200 V using 4–20 % gradient gel on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Both samples from each subject were run on the same gel. Proteins were transferred to PVDF membranes at 300 mA constant current for 2 h on ice at 4°C. The uniformity of protein loading was checked by staining the membrane with Ponceau S and by re-probing the membrane with an antibody against α-actin (Sigma, Saint Louis). Membranes were blocked in TBS with 0.1 % Tween 20 (TBS-T) containing 5 % non-fat dry milk for 1 h and then incubated overnight at 4°C with commercially available rabbit polyclonal primary phosphospecific antibodies. Antibodies recognized phosphorylated (p-) p70 ribosomal S6 kinase (p70S6K) at Thr³⁸⁹ and Thr⁴²¹/Ser⁴²⁴, ribosomal protein S6 (rpS6) at Ser^{240/244}, p38 mitogen-activated kinase (p38 MAPK) at Thr¹⁸⁰/Tyr¹⁸², extracellular signal-

related kinase 1/2 (p44/p42) (Erk1/2) at Thr²⁰²/Tyr²⁰⁴, and eukaryotic elongation factor 2 (eEF2) at Thr⁵⁶ (Cell Signaling Technology, USA).

The primary antibodies were diluted in TBS-T containing 2.5% non-fat dry milk 1:1000-1:2000 except for p-eEF2, which was diluted 1:5000. Membranes were then washed in TBS-T, incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG; Cell Signaling Technology, USA) diluted 1:25 000 in TBS-T with 2.5 % milk for 1h followed by washing in TBS-T.

Phosphorylated proteins were visualized by an enhanced chemiluminescence method according to the manufacturer's protocol (SuperSignal West femto maximum sensitivity substrate, Pierce Biotechnology, Rockford, USA) and quantified (band intensity x volume) using a Chemi Doc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories). The results were normalized to Ponceau S. Our earlier experiments demonstrated a proportional linear relationship between protein loaded and Ponceau S in quantification between 5 and 60 µg of total protein loaded (Hulmi et al. 2012).

The membranes described above were incubated in Restore Western blot stripping buffer (Pierce Biotechnology) for 30 min and reprobbed with appropriate antibodies for detection of the total expression levels of Akt, rpS6, p38 and Erk 1/2 (Cell Signaling Technology) and p70S6K (Santa Cruz Biotechnology) by immunoblot analysis as described above. The rationale was to verify whether the total protein content of the signalling proteins analysed significantly changes from pre- to post-loading condition.

Immunohistochemistry

Muscle specimens were also analysed for muscle fibre cross-sectional areas (CSA). Serial cross sections (8 μm) were cut using a microtome (CM3050; Leica Biosystems GmbH, Wetzlar, Germany) at -20°C and mounted on Superfrost Plus microscope slides (Thermo Scientific), air-dried and stored at -80°C until further analysis.

The muscle cross sections were blocked for 30 min with 1% bovine serum albumin (BSA; A4503, Sigma Life Science, St. Louis, MO, USA) in a phosphate buffer saline and 0.01% Tween20 solution (PBS; 524650, Calbiochem, EMD Biosciences CA, USA; Tween20; Sigma-Aldrich, MO, USA), before incubated with antibodies against myosin heavy chain type 2 (1: 500; SC71; gift from Prof. S. Schiaffino) and dystrophin (1:1000; ab15277, Abcam) diluted in the blocking solution overnight at 4°C . The muscle sections were then incubated with appropriate secondary antibodies (Alexa Fluor, A11005 and A11001, Invitrogen, Carlsbad, CA, USA). The muscle sections were washed 2-3 x 10 minutes in PBS-t between each step and finally mounted with cover glass and visualized with 20x magnification objectives, using a high resolution camera (DP72, Olympus Corp., Tokyo, Japan) attached to an Olympus microscope (BX61) with a fluorescence light source (X-Cite 120PCQ, EXFO Photonic Solutions Inc., Mississauga, ON, Canada). The individual fibre cross sectional areas and fibre type were analysed with TEMA software (CheckVision, Hadsund, Denmark). Based on the fibre type staining, values were calculated for type I and type II fibres, separately. A mean of 534 fibres (range 113-1192) were analysed on each cross section.

Muscle fibre CSA are presented as means of both the type I and type II fibres as there was significant correlation between training-induced relative changes in type I and type II CSA ($r^2=0.892$, $p<0.001$).

Blood analyses

Blood samples were centrifuged at 2500 x g for 15 min at 4°C. Plasma was aspirated and kept at -80 °C until measurement. Plasma testosterone and cortisol concentrations were determined with radioimmunoassay (RIA) using commercial kits according to the manufacturer instructions (DRG International, Inc., USA). All samples for hormone analyses were run within a single assay with intra-assay variation coefficients of 2.5 % and 2.6 % for testosterone and cortisol, respectively.

Magnetic resonance imaging (MRI)

It should be noted that only subjects belonging to the training groups but not the controls underwent MRI. The entire length of right quadriceps femoris was scanned with magnetic resonance imaging (Magnetom Avanto 1.5T, Siemens AG, Munich, Germany) at rest 8 – 10 days before the first training session and again 5 – 7 days after the final training session on different occasions compared to strength tests. Typical morphological images were acquired in the axial planes by using proton density fast spin echo sequence. The axial image slice spacing was 8.5 mm. The CSA of rectus femoris and vasti muscle group were analyzed from the images with freely available Osiris 4.0 software. The reproducibility of drawing the areas was high with a correlation coefficient of 0.98 and mean error of 0.37 ± 0.24 % when analyzed by the same person. The statistical analyses yielded no differences in training-induced changes between the rectus femoris and vasti muscle group. Therefore, in order to simplify the MRI data presentation, the cross-sectional areas of both rectus femoris and vasti muscles were merged together and presented as quadriceps femoris (QF) CSA. A mean of 5 QF CSA from the middle part of the thigh (from the 6th to 11th slice counted from the distal part) was further used for statistical analyses.

Resistance training

Training sessions were performed twice a week, separated from each other 48 h to 72 h, differing only with regards to time of day between the morning and afternoon group. At the 2nd and 10th training session, a 1 repetition maximum (RM) for each exercise was calculated from an individual's 3 to 6 RM performance. Resistance training was progressive with slightly decreased training load and volume at week 4, 7 and 11. All training sessions included leg presses, knee flexions and knee extensions as the main exercises performed always at the beginning of training session followed by 5 upper body and core exercises. For the lower body exercises, duration of 3 and 2 seconds for the concentric and eccentric part of each repetition, respectively, were required in order to increase time under the tension and hence maximise muscle hypertrophy (Wernbom et al., 2007). During the first five weeks, the number of repetitions per leg exercises ranged from 10 to 15 with relative intensity of 40% up to 60% of 1 RM performed in 3 sets. In the second half of the training period, the number of repetitions per leg exercises ranged from 8 to 12 with the relative intensity of 50% up to 80% of 1 RM performed in 4 sets, rest intervals 2 - 2.5 minutes between the sets. During each leg exercise the load was individually adjusted in order to achieve technical failure at least within the last set from the 2nd training week onwards. All training sessions were supervised.

In order to maximize the anabolic effect of training, a 25 g-dose of whey protein (60-30-10 % of concentrate-isolate-hydrolysate mixture, respectively, BIO5 Whey Better, Bratislava, Slovakia) mixed with 0.4 l of water was given to each subject immediately after the training session. Consumption of ~20–25 g of whey or other rapidly absorbed protein has been recommended to maximally stimulate muscle protein synthesis after resistance exercise in young healthy individuals (Churchward-Venne et al., 2012). The control group was also given the same protein supplement for a home use. They were instructed to consume it twice a week throughout the experimental period as a separate meal between breakfast and lunch.

Statistical analyses

Standard descriptive statistics (mean \pm SD) were calculated. Data at each time-point were analyzed for normality using the Shapiro-Wilk test. When normally distributed, a Levene's test verified the equality of variances in the samples.

GLM for repeated measures was used to analyse force, MRI and hormonal data with a main effect of training (pre to post) and training*group interaction. When main effect and/or interactions were significant, paired-samples T-test was performed to analyse within-group differences.

Due to violation of normality, non-parametric statistical methods were used to compare changes in phosphorylation of selected proteins. Related-samples Wilcoxon signed ranks test was used to examine pre-to-post changes with groups merged together (acute loading effect only) and within the groups separately. To compare percentage changes between the groups in the phosphoprotein data, Mann-Whitney U test for independent samples was used.

Pearson's correlation coefficients were calculated for relative training-induced changes in MVC, QF CSA, muscle fibre CSA and also between relative acute changes in phosphorylation of selected proteins both before and after the training period and hypertrophy measures with groups merged together.

The level of significance was set at $p < 0.05$.

Results

Muscle strength and hypertrophy

Voluntary muscle strength increased significantly in both the morning and afternoon training group by 16.9 % and 15.2 %, respectively, during the 11-week training period (training main effect $p < 0.01$, all absolute values in Table 1). There was no statistically significant difference in maximum force production between the training groups at any time points (Figure 2). Muscle strength did not significantly change in the control group (Table 1). Both Pre- and Post-training acute loading induced a significant ($p < 0.001$) reduction in voluntary muscle strength, not affected by time of the day or training. At Pre, the average decrease was 34.5 ± 18.1 %, 34.3 ± 7.9 % and 30.8 ± 11.8 % in the morning, afternoon and control group, respectively. At Post, the respective values were 29.5 ± 13.2 %, 30.0 ± 12.4 % and 31.4 ± 13.9 %.

Table 1

	Morning group		Afternoon group		Control group	
	Pre	Post	Pre	Post	Pre	Post
body mass (kg)	77.8 ± 5.7	78.2 ± 6.1	77.0 ± 5.2	77.6 ± 6.9	79.1 ± 6.2 kg	79.8 ± 7.2 kg
isometric force (N)	3456.89 ± 1171	$4040.97 \pm 1354^{**}$	3656 ± 1588	$4212 \pm 1469^*$	3433 ± 1389	3342 ± 1142
mean QF CSA (mm^2)	7721 ± 795	$8385 \pm 828^{***}$	8689 ± 1519	$9674 \pm 1495^*$	not measured	not measured
mean fibre CSA (μm^2)	5249 ± 809	$6356 \pm 1127^*$	6069 ± 1180	$7002 \pm 1090^*$	6138 ± 1016	5755 ± 1107
cortisol ($\mu\text{g/dL}$)	35.6 ± 8.8	$30.5 \pm 6.1^*$	31.3 ± 7.9	$23.6 \pm 8.4^*$	30.6 ± 8.1	$24.4 \pm 8.5^*$
testosterone (ng/mL)	5.85 ± 2.76	5.19 ± 1.91	5.08 ± 0.91	5.36 ± 0.73	5.52 ± 2.01	6.49 ± 1.93

Table 1. Absolute values (mean \pm SD) of selected anthropometrical, performance, morphological and hormonal parameters measured before (Pre) and after (Post) 11-week time-of-day specific resistance training in the morning, afternoon and control group. *, **, *** - statistically significant difference compared to Pre values at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. (QF CSA represents quadriceps femoris cross-sectional area obtained by magnetic resonance imaging and fibre CSA represents muscle fibre cross-sectional area from histological data).

Figure 2

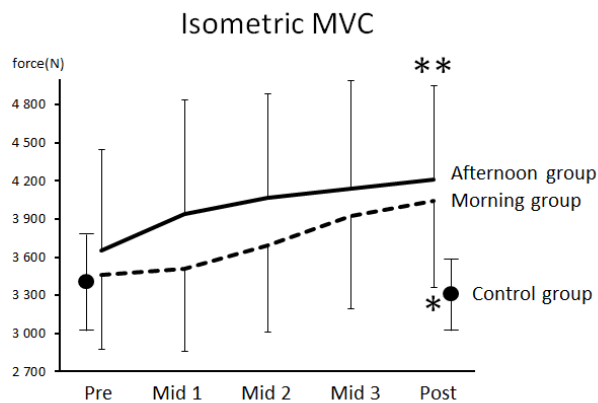


Figure 2 Maximum isometric voluntary force (mean \pm SD) measured on a leg-press dynamometer before (Pre), during (Mid 1 – week 3, Mid 2 – week 6, Mid 3 – week 9) and after (Post) 11-week time-of-day specific resistance training in the morning (Morning - dashed line) and afternoon (Afternoon – continuous line) training group. The control group was measured at Pre and Post occasion only (black dots).

**, ** - statistically significant difference compared to Pre values at $p < 0.05$ and $p < 0.01$ respectively.*

At macroscopic level, mid-part of QF CSA increased significantly by 8.8 % and 11.9 % in the morning and afternoon group, respectively, when measured by MRI (training main effect $p < 0.001$), with no significant difference between the two training groups (Figure 3, panel A, and Table 1).

At microscopic level, quadriceps femoris fibre size CSA increased significantly in the morning and afternoon by 21 % and 18 %, respectively, but did not change in the control group (-6 %, training main effect $p < 0.01$, Figure 3, panel B, and Table 1). The increase in

fibre CSA was similar for type I and type II fibres in both the morning group (20 vs. 22 % respectively) and the afternoon group (17 vs. 18 % respectively).

Figure 3

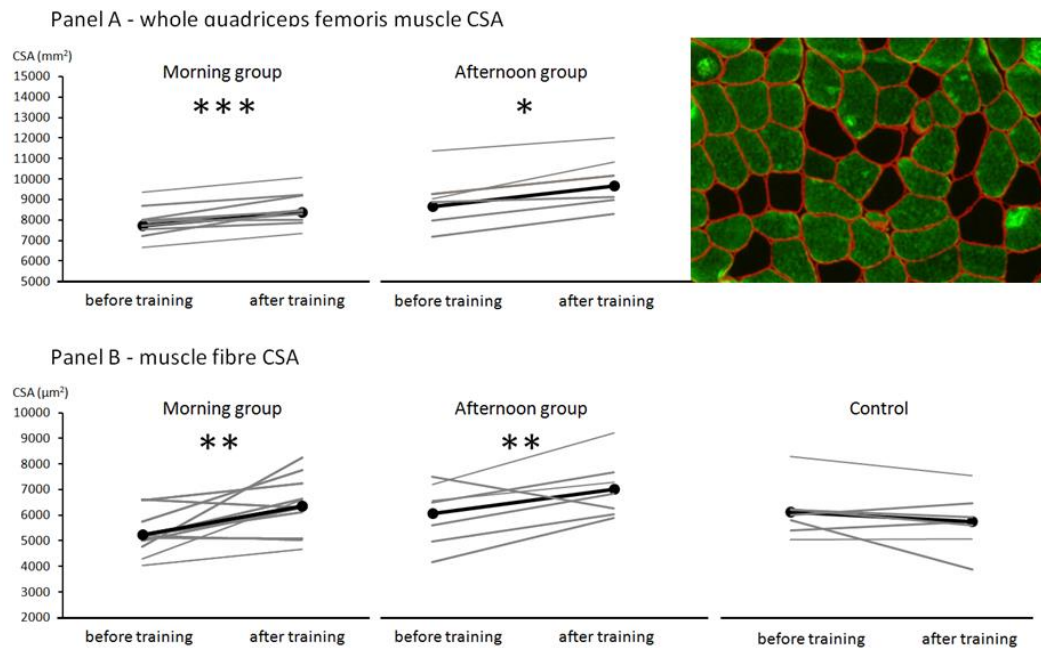


Figure 3 Cross-sectional areas of the mid-part of quadriceps femoris muscle before and after 11-week time-of-day specific resistance training in the morning (Morning) and afternoon (Afternoon) training groups and the control group (Control) obtained from magnetic resonance imaging (Panel A) and from histological data (panel B). Thick lines represent group mean values, thin lines represent individual data.

Right upper corner: a representative image from immunohistochemistry. Muscle fibres with myosin heavy chain type 2 (green colour). Magnification – 20x.

*, **, *** - statistically significant difference between Pre- and Post training within a given group at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

There were no significant correlations between morphological changes (measured by MRI and immunohistochemistry) and MVC.

Cell signalling and hormonal concentrations

The post-loading phosphorylation of p70S6^{Thr421/Ser424} increased independent of the time of day in the pre-training condition, whereas it was significantly increased in the morning but not in the afternoon group after the training period (between-training group difference $p < 0.05$, Figure 4). The phosphorylation of rpS6 and p38MAPK increased after acute resistance loading both before and after training in a time-of-day independent manner ($p < 0.05$ at all occasions, not shown). On the contrary, the phosphorylation of p70S6^{Thr389}, eEF2 and Erk1/2 did not change significantly due to acute resistance loading at any time point (not shown).

No statistically significant correlations were found between changes in muscle fibre CSA, MRI and cell signalling data.

Figure 4

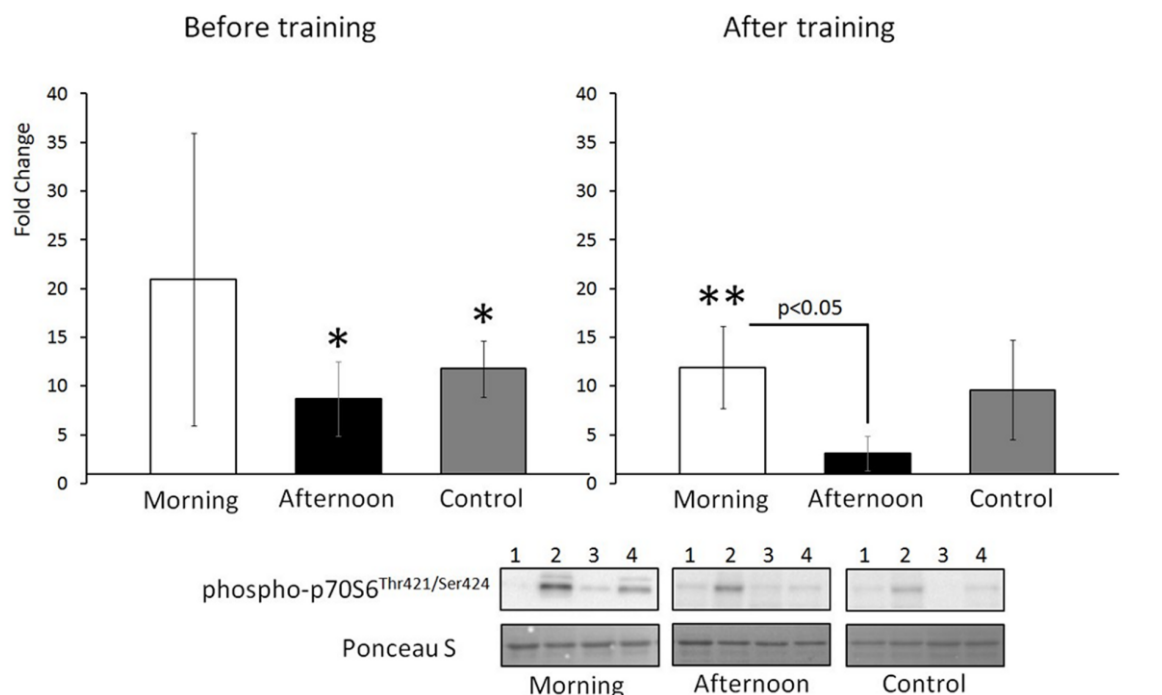


Figure 4 Fold changes (mean \pm SD) in phosphorylation of p70S6 Thr421/Ser424 induced by an acute bout of resistance exercise before and after 11-week time-of-day specific resistance training in the

morning (Morning) and afternoon (Afternoon) training group and non-training controls. Below are shown representative Western blot images in the three groups at various time points as follows:

1 and 2– Pre-loading and Post loading, respectively, before training

3 and 4– Pre-loading and Post loading, respectively, after training

**, ** - statistically significant increase in phosphorylation compared to pre-loading state in a given group at $p<0.05$ and $p<0.01$ respectively.*

$p<0.05$ – statistically significant difference between the morning and afternoon group at a given p level value.

Resting total testosterone concentrations were not statistically different among groups at any time point (Table 1). Resting total cortisol concentrations declined significantly from Pre to Post in all three groups (training main effect $p<0.05$, Table 1). The morning training group showed a tendency for higher cortisol concentrations at both Pre and Post compared to the afternoon and control group, however, the difference was not statistically significant ($p=0.081$ and $p=0.066$, respectively).

Discussion

The main results of the present study were that similar levels of muscle strength and hypertrophy could be achieved regardless of time of the day during 11 weeks of strength training in previously untrained men. However, we observed that, at the level of skeletal muscle signalling, the extent of adaptation in some parameters may be time-of-day dependent. On the contrary, time-of-day specific training did not affected resting levels of testosterone and cortisol.

Skeletal muscle strength and hypertrophy

The 11-week time-of-day specific training resulted in significant improvements in maximum isometric strength of 16.9 % and 15.2 % in the morning and afternoon group, respectively. It should be mentioned that that our results on isometric strength gains might be underestimated to some extent. The actual training modality was of dynamic nature and therefore the isometric testing was not training-specific. Importantly, the magnitude of strength gains was similar regardless of time of day of training and comparable to previous studies dealing with the time of day effects (Sedliak et al. 2008, 2009 Souissi et al. 2002, Souissi et al. 2012, Chtourou 2012 a, b, Chaouachi, et al., 2012, Zbidi et al., 2016, Kuusmaa et al. 2016). Furthermore, time of day did not affect the fatigue levels induced by the experimental acute resistance loading and there was only a slight insignificant decrease in the fatigue index in both training groups after the training – from approx. 34 % before the training to 30 % after the training. Based on these relative values, a strong physiological stimulus was delivered to the muscles examined at all acute loading occasions irrespective of the time of day. This may suggest that also the actual training-induced fatigue was similar in the morning and afternoon training groups throughout the training period.

The present findings on maximum muscle strength were also reflected in the hypertrophic adaptations of the participants, although with no significant correlations between morphological changes and MVC. Clearly, training responses may vary between loading protocols and the present results can be generalized only to the type of loading protocol used. No significant effect of time of day was found in muscle hypertrophy either at microscopic levels of muscle fibre CSA or at macroscopic level of the mid-part of quadriceps femoris CSA. Average muscle fibre CSA were rather large in the present study, especially in the control group with average CSA of both type I and II fibres over $6000 \mu\text{m}^2$. However, also the study of Staron et al (1994) on untrained men reported fibre CSA larger than $6000 \mu\text{m}^2$ for IIA type well over $5000 \mu\text{m}^2$ for type I and type IIB. Data from MRI showed similar gains in the mid-part of quadriceps femoris CSA of 8.8 % and 11.9 % in the morning and afternoon group, respectively. Our results are well in line with the data of Kuusmaa et al. (2016) showing mean increases of vastus lateralis CSA of 9.5 % and 12 % in the morning and afternoon group, respectively, measured by ultrasound, after the first 12 weeks of combined resistance and endurance training.

Interestingly, it seems that moderate endurance cycling training preceding or following the actual resistance training did not interfere with muscle mass gains in their group of previously untrained men (Kuusmaa et al. 2016, Mikkola et al. 2012). On the contrary, Sedliak et al. (2009) showed during 12-week resistance training only 2.7% and 3.5% increases in quadriceps femoris CSA in the morning and afternoon groups, respectively. The main reasons for the blunted hypertrophic adaptation could be that half of the training sessions were focused on maximum strength and power with rather low training volume and time under contraction, which are among the key hypertrophic factors. In addition, the subjects were also pre-conditioned by 12-week resistance training before starting with the actual time-of-day-

specific resistance training.

Overall, it seems that training duration and training status (Galpin et al. 2016) could play some role in the time-of-day-specific adaptation. As mentioned above, Kuusmaa et al. (2016) did not find significant differences in hypertrophy after 12 weeks. However, after 24 weeks men training in the evening hours had increased CSA significantly more compared to men training in the morning. At present, it is difficult to explain these findings. The main question is whether these various responses to time-of-day-specific resistance training were real effects of time of day or caused by various confounding factors. It is of course impossible to make a concluding statement based on the above findings although many possible confounding factors were controlled in the present study. For instance, the training volume of both training groups was identical in relative measures. Beside thorough sleep and dietary habits instructions, a post-training recovery drink containing whey protein and maltodextrin was provided after every session to all participants for a better control of nutrient intake and timing, except for the acute resistance loading protocol with muscle biopsy. It has been shown that supplementation of whey protein before and/or immediately after heavy resistance exercise increases anabolic signalling in human skeletal muscle (Hulmi et al. 2009, Lane et al. 2017). However, data on signalling pathways involved in skeletal muscle hypertrophy support the possibility of hypertrophic adaptations being time-of-day-dependent. Sedliak et al. (2012) have suggested that strength training in the morning may not provide optimal stimulus for some individuals because of large inter-individual variability in protein signalling after morning compared to the evening strength training.

Cell signalling

It is of course questionable to what extent there is causality between high morning between-

subject variability in the acute responses (cell signalling) showed by Sedliak et al (2012) and long-term adaptations (cell hypertrophy). However, data from the present study suggest that time-of-day-specific long-term adaptations may exist also in signalling pathways. More specifically, the p70 ribosomal S6 kinase (p70S6KThr421/Ser424) exhibited reduction in its phosphorylation response to resistance exercise bout after 11 weeks of afternoon resistance training. Similarly decreased p70S6K phosphorylation, and thus activity within the Akt/mTOR/p70S6K signalling pathway has been reported in resistance-trained, but not endurance-trained men, after resistance exercise (Coffey et al. 2006). Interestingly, Galpin et al. (2016) showed that also Erk1/2 expression (not measured in the present study) is down-regulated after chronic resistance training in well-trained weightlifters and powerlifters. In contrast, in men training between 07:30 – 08:30 h for 11 weeks, elevated levels of phosphorylated p70S6K persisted in a similar way as in untrained controls. It could be speculated that unchanged phosphorylation of p70S6^{Thr421/Ser424} after the period of morning resistance training may compensate for some other contributing factors (e.g., hormonal, metabolic, myogenic) that are less/more activated in the morning compared to the afternoon hours, resulting in similar outcome in cell hypertrophy measures. Indeed, animal models have indicated a circadian variation in the mRNA expression of the myogenic regulatory factors MyoD, myogenin, MuRF1, Akt1, and ribosomal protein S6, and also in phosphorylated levels of Akt and ribosomal protein S6 in rodent skeletal muscle (Andrews et al. 2010, Shavlakadze et al. 2013). Alternatively, it may be that the phosphorylated p70S6K at Thr421/Ser424 may not have an effect on the activity of the enzyme as no between-group changes were observed in the phosphorylation of rpS6 downstream to p70S6K.

In the present study, the rest of signalling kinases did not exhibit time-of-day-specific adaptations but some of them were affected by the acute loading. rpS6 phosphorylation was

increased in response to acute resistance loading in line with studies using untrained subjects (e.g., Dreyer et al. 2010, Hulmi et al. 2009, Terzis et al. 2010). Phosphorylated p38 MAPK, responding to both anabolic (e.g., muscle cell stretch, functional overload) and catabolic stimuli (e.g., tumor necrosis factor α) (Jin and Li 2007, Norrby and Tagerud 2010, Wretman et al. 2001) was also elevated after the resistance loading in line with previous reports in humans (Cochran et al. 2010, Hulmi et al. 2010, Terzis et al. 2010). Terzis et al. (2008) reported that acute post-loading increase in p70S6^{Thr389} correlated with muscle mass gained during a training period. This was not the case in the present study. Besides no significant correlation between p70S6^{Thr389} and muscle mass, p70S6^{Thr389}, eEF2 and Erk1/2 did not change significantly due to acute resistance loading at any time point. Reasons may be various. Regarding phospho-Erk1/2, Tannerstedt et al. (2009) and Hulmi et al. (2010) found significant exercise-induced elevations but their timing of post-loading biopsy was closer to cessation of exercise compared to the present experiment. For eEF2, resistance exercise has been shown to decrease eEF2 phosphorylation (increases eEF2 activity) in some (Dreyer et al. 2010, Mascher et al. 2008), but not all studies (Hulmi et al. 2009). Although several signalling pathways have been consistently shown to be activated at the 60 min post-exercise, it is possible that we may have missed phosphorylation of some of the variables in the present study. Further studies with more biopsy time-points are needed in the future to get a more complete picture of the signalling responses after time-of-day-specific training as well as actual measurements of muscle protein synthesis.

Hormonal concentrations

Resting total testosterone concentrations were not affected by time-of-day specific training in the present study. This is a typical finding with these types of resistance training studies as the training frequency of 2-3 sessions per week allows for at least 48 hours of recovery.

Consequently, sufficient time for photic and social contact factors to reset any possible phase-shifting effect caused by the exercise training on the circadian rhythm including hormonal concentrations is probably achieved (Duffy et al. 1996, Teo et al. 2011). Interestingly, the afternoon group showed similar hormone concentration compared to the morning and control group, although taken in the afternoon between 15:15-16:45 h. Typically, diurnal variation with lower afternoon and evening values compared to the morning hours is present in young men (Van Cauter *et al.* 1996, Veldhuis *et al.* 1987). It is difficult to explain slightly elevated afternoon testosterone levels in the afternoon group, moreover being within the physiological range for the local male population.

Cortisol results were somewhat clearer with this respect. The morning training group exhibited strong tendency for higher cortisol concentrations both before and after the training period when compared to the afternoon and control group as known from the literature (Van Cauter *et al.* 1996). Similarly to our previous study (Sedliak et al. 2007), resting cortisol concentrations were significantly higher before the training period compared to the post-training values. Since the same phenomenon was present also in the control group, it seems likely that anticipation of stressful events, e.g., previously inexperienced muscle biopsy, caused an increase in resting cortisol during the first testing occasion (Mason et al. 1973, Michaud et al., 2009) rather than the effect of time-of-day-specific resistance training.

Conclusions

In previously untrained young men, comparable gains in lower extremity muscle mass and strength can be achieved regardless of whether training is performed in the morning or in the afternoon hours. This seems to hold true when the training period last from 10 to 12 weeks. As to the mechanisms for the similar adaptations to training, resting testosterone and cortisol

values and majority of protein signalling pathways were unaffected by the time-of-day-specific training. However, p70S6^{Thr421/Ser424} kinase exhibited reduction in its phosphorylated portion after the afternoon but not morning resistance training. It could be speculated that unchanged phosphorylation of p70S6^{Thr421/Ser424} after the period of morning resistance training may compensate for some other contributing factors (e.g., hormonal, metabolic, myogenic) that are less/more activated in the morning compared to the afternoon hours, resulting in similar outcome in cell hypertrophy measures.

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Declaration of Interest statement

Conflict of interest: The authors state that there is no conflict of interest.

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