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Editorial office

LITHUANIAN SPORTS UNIVERSITY
Sporto str. 6, LT-44221 Kaunas, Lithuania
Tel. +370 37 302636
Fax +370 37 204515
E-mail zurnalas@lsu.lt
URL www.lsu.lt/en/scientific-journals_BJSHS

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Spausdino UAB *Vitae Litera*, Savanorių pr. 137, Kaunas, LT-44146

CHANGES IN RESTING SALIVARY TESTOSTERONE, CORTISOL AND INTERLEUKIN-6 AS BIOMARKERS OF OVERTRAINING

Travis Anderson, Simon Haake, Amy R. Lane, Anthony C. Hackney
University of North Carolina, Chapel Hill, NC, USA

ABSTRACT

Background. Overtraining (OVT) is a concern for many athletes. Immunological (increased interleukin-6 [IL-6]) and hormonal (increased cortisol [C], decreased free testosterone [fT]) biomarkers have been analyzed during training to detect OVT development.

Methods. This study determined if resting levels of salivary IL-6, T, and C change during a pre-season resistance training (RT) program in 20 Division I American football players (mean ± SD: age = 19.1 ± 1.1 years; height = 185.4 ± 6.7 cm; mass = 102.0 ± 22.2 kg; body fat = 14.7 ± 7.6%). 1RM squat, bench press and Olympic-style clean, IL-6, C and T were assessed at baseline (WK1), week 4 (WK4), week 6 (WK6) along with psychological status (PS) to determine affective state.

Results. 1RM (bench press: 121.6 ± 36.3 kg vs. 127.4 ± 35.9 kg, squat: 187.2 ± 30.2 kg, 190.9 ± 28.1 kg, clean: 116.8 ± 14.6 kg, vs. 119.2 ± 14.5 kg), IL-6 (1.42 ± 1.77 pg/mL vs. 5.60 ± 12.57 pg/mL) and C (2.57 ± 2.46 nmol/L vs. 5.33 ± 4.94) increased significantly from WK1 to WK6 ($p < .05$), fT decreased significantly (417.44 ± 83.63 pmol/L vs. 341.10 ± 87.79 pmol/L) from WK1 to WK6 ($p < .05$). PS was minimally affected during the study. Significant biomarker changes were detected, but no OVT was induced (i.e. performance improved).

Conclusion. Therefore, directional changes in these biomarkers may not be sufficiently reflective of OVT in RT programs.

Keywords: stress, hormones, biomarkers, anabolic-catabolic.

INTRODUCTION

Elite level strength and conditioning coaches are tasked with optimizing player performance. To achieve these goals, strength coaches often train athletes throughout the year at, or near, their maximal physical capacity using a process called overreaching. In the overreaching process, one or more training factors (e.g. modality, duration, intensity, volume) are increased beyond what the athlete is typically accustomed to in order to elicit a super-compensatory response (i.e. overload principle) (Meeusen et al., 2006). When overreaching training is excessive however, it can lead to a state known as "overtraining," characterized by a rapid deterioration in performance that does not respond to a rest or regeneration period (Lehmann, Foster, & Keul, 1993). An athlete who is overtrained may display a myriad of physiological and psychological symptoms including chronic fatigue, decreased performance, depression, apathy towards training, and sleeplessness. Research findings suggest these symptoms may be largely attributed to a compromise in the immunological and neuro-endocrine systems (Robson, 2003).

Intense or prolonged bouts of exercise can lead to the production and subsequent elevation of pro-inflammatory cytokines. These cytokines, such as interleukin-1α, interleukin-1β, and interleukin-6 (IL-6) are part of an acute pro-inflammatory immune response to physiological

stressors. During periods of overreaching pro-inflammatory cytokines may reach chronically elevated levels. Dr. Lucille Smith developed the 'Cytokine Hypothesis' to explain overtraining in athletes, specifically proposing that excessive production and/or heightened sensitivity in tissues to the specific cytokine IL-6 is the principle factor leading to the physiological changes in the overtrained state (Robson, 2003; Smith, 2000).

Research suggests that the neuro-endocrine system is also a valuable parameter to analyze when determining whether an athlete is overtrained (Hackney, 2006). During intense-prolonged exercise, the neuro-endocrine system releases hormones that moderate the metabolic response to exercise. These hormones also play an important role in the functioning of the immune system during exercise. Cortisol, the major human glucocorticoid hormone released in response to physical and psychological stress, is a strong effector factor of the immune response (Hackney & Waltz, 2013). Additionally, it is well documented that in response to exercise training stress (i.e. if excessive) basal testosterone levels will become suppressed in men, perhaps due to the inhibitory actions of cortisol (Urhausen, Gabriel, & Kindermann, 1995). With the above in mind, the purpose of this study was to evaluate select immunological and hormonal biomarker (salivary cortisol, testosterone, IL-6) responses of men in a six-week intensive pre-season training program to examine for signs of overtraining.

METHODS

Subjects were twenty moderate to highly strength-trained healthy male subjects (mean ± SD: age = 19.1 ± 1.1 years; height = 185.4 ± 6.7 cm; mass = 102.0 ± 22.2 kg; body fat = 14.7 ± 7.6%) recruited from a Division I collegiate American Football team. All subjects were upper-classmen and constituted a variety of non-lineman positions. All signed a written informed consent in compliance with the Declaration of Helsinki. For eligibility subjects must have had full participation in team training activities for a minimum of 3 days a week for 3 months prior to the study and have had a two year history of resistance training consisting of at least one training session per week.

Each subject was assessed at six separate sessions. Anthropometric data (age, height, mass, body fat percentage) were collected at the first session. Body fat percentage was determined through triplicate skinfold measurements at select sites (abdomen, chest, and thigh) using skinfold calipers (Skyndex, Fayetteville, AR) and body fat percentage was calculated using the Jackson-Pollock equation (Golding, 2000). Salivary samples were collected at Week 1 (baseline), Week 4 and Week 6 while psychological status responses and body mass were collected weekly. The modified version of the Recovery-Stress Questionnaire for Athletes (REST-Q) was used to monitor psychological status over the course of the study, administered to the subjects on the morning of saliva sample collection.

Prior to collection of saliva samples, subjects were first asked to rinse their mouths with water, spit to eliminate particles, and then allow saliva to accumulate; samples were then collected via passive drool sampling technique. The time of day (afternoon; 15:00–17:00 H) for all salivary collections remained relatively consistent over the course of the study for each subject (± 15 minutes). Samples were stored on ice until transported to a storage freezer (–80° C) until later analysis.

Saliva samples were assessed for IL-6, free testosterone (fT) and cortisol (C) concentrations. Stored saliva samples were allowed to thaw and were then centrifuged at 3000 x *g* at 4 degrees C to remove any particulate matter. The resulting supernated saliva specimens were assayed for cortisol, IL-6, and fT using high sensitivity enzyme immunoassays (Salimetrics, State College, PA, USA).

Training load, intensity, and volume were controlled by strength and conditioning staff and monitored by the principle investigator to ensure progressions remained within $\pm$ 10% of baseline load. Muscle strength was assessed pre- and post-training using 1 repetition maximum (1RM) for bench press, back squat and Olympic-style clean. Each training session consisted of a 10–15 minute warm-up comprised of three separate stations (core/abdominal exercise, dynamic/static stretching and shoulder exercises), each lasting 3–5 minutes. Post warm-up, training consisted of whole body and body part isolated resistance training exercises including back squat, bench press, power clean, hang clean, incline bench press, lunges, and several assistance exercises (Table 1, all exercises). Each training session began with one of the primary exercises, progressing to near-maximum intensity. Supplementary exercises were completed after completion of the main lifts. Total session length was between 45–60 minutes.

Table 1. **Exercises used throughout the 6-week training period**

Primary	Lower Body	Upper Body		Compound
Bench Press	Front Squat	DB Overhead Press	DB Curl	Hang Clean
Back Squat	BB Lunges	Push Ups	BB Curl	
Power Clean	RDL	Incline Bench Press	BB Row	
	Calf Raises	Incline DB Press	Pull Ups	
	Deadlift	Front Press	BB Shrugs	
		Close-Grip Bench Press		

Training volume increased gradually over the course of the study through manipulation of total sets completed per exercise and an increase in the number of exercises completed. Relative intensity (training stimulus as percentage of maximum capability), total training load, and training load for the primary exercises were catalogued to ensure an appropriate training stimulus was being applied.

Beginning on the second week of training conditioning runs were completed twice a week on Tuesdays and Thursdays. These conditioning sessions were executed immediately after the completion of the resistance training session and consisted of 100 or 300-meter runs at a brisk predetermined pace. Each week, running volume was increased to create a higher physiological demand on the athlete. On the fifth and sixth weeks of training, the 300-meter runs were replaced with a speed/agility circuit lasting 45 minutes. The circuit consisted of 4 separate drills completed for a total of 8 minutes, with 2 minutes rest between drills.

Statistical Analysis. This quasi-experimental study was powered based upon anticipated biomarker responses, for a two-sided test with an effect size of ≥ 0.50 and a power (β) of 0.80. The sample size required to show statistical significance with these parameters was calculated to range from 12 to18 subjects (Cohen, 1992).

Separate one way within subjects repeated measures ANOVA were used to analyze IL-6, fT, C, body mass, and the REST-Q categories over measurement time. Tukey post-hoc tests were used to determine significant mean differences between levels within each ANOVA. Separate paired samples t-tests were used to determine changes in muscular strength assessments. Significance for all statistical tests was set a priori at $\alpha \leq 0.05$.

RESULTS

Weekly body mass (kg) of subjects is displayed in Table 2 and increased significantly by the conclusion of the study.

Each of the subjects had completed 1RM on all major lifts used in the study (bench press, back squat, and power clean) prior to involvement and hence were familiar with the procedures performed per National Strength and Conditioning Association guidelines (Fry & Kraemer, 1991). 1RM results during the six-week study period were catalogued. Pre- and post-training 1RM results are displayed in Table 3.

Table 2. **Weekly body mass (mean ± *SD*)**

	Pre$^{\gamma}$	Week 2	Week 3	Week 4	Week 5	Week 6
Body Weight (kg)	102.0 ± 22.2	102.5 ± 22.3*	102.8 ± 22.1*	103.0 ± 22.4*	103.3 ± 22.4*	102.8 ± 22.2

Notes. *Indicates significant change from baseline. γ Beginning of study (week 1) ($p < .05$).

Table 3. **Performance responses of main resistance exercise lifts (mean ± SD)**

Lift	Pre-Study Max (kg)	Post-Study Max (kg)	Change (%)
Bench Press	121.6 ± 36.3	127.4 ± 35.9*	+4.8 ± 4.2
Back Squat	187.2 ± 30.2	190.9 ± 28.1*	+2.0 ± 3.1
Power Clean	116.8 ± 14.6	119.2 ± 14.5*	+2.1 ± 3.3

Note. *Significant differences from respective pre-study value ($p < .05$).

Hormone (units)	Pre$^{\gamma}$	Week 4	Week 6
Cortisol (nmol/L)	2.57 ± 2.46	3.67 ± 3.42	5.33 ± 4.94*
IL-6 (pg/mL)	1.42 ± 1.77	4.19 ± 8.27	5.60 ± 12.57*
Free Testosterone (pmol/L)	417.44 ± 83.63	456.00 ± 100.98	341.10 ± 87.79*

Table 4. **Salivary biomarker concentrations (SI units) mean ± *SD*)**

Notes. *Significant difference from Week 1 trial (p < .05). γ Beginning of week 1.

Affective Category	Pre-Study	Week 2	Week 3	Week 4	Week 5	Week 6
Anger	1.56 ± 1.2	1.38 ± 1.5	1.05 ± 1.2*	1.36 ± 1.7	0.83 ± 1.3*	1.15 ± 1.4*
Depression	1.67 ± 1.6	1.81 ± 1.4	1.32 ± 1.3*	1.46 ± 1.6	1.62 ± 1.8	1.83 ± 1.9
Fatigue	1.85 ± 1.3	1.91 ± 1.6	1.46 ± 1.4*	1.54 ± 1.5	1.44 ± 1.4*	1.56 ± 1.4
Vigor (Motivation)	1.43 ± 1.4	1.10 ± 1.2*	1.32 ± 1.5	1.23 ± 1.5	1.06 ± 1.3	0.92 ± 1.1*

Table 5. **REST-Q score by affective category (mean ± *SD*)**

Notes. *Significant difference from respective pre-study (Week 1) baseline measure (p < .05). Scale range: 1 (Low) – 5 (High).

The mean (± *SD*) resting salivary C, IL-6 and fT responses over the six-week study period are displayed in Table 4. C trended upward throughout the study. The increase from Week 1 to Week 4 was not significant (p = .236). However, the difference between Week 1 and Week 6 was significant, showing an increase of approximately 110% (p = .004).

There were no significant differences between means for resting IL-6 over the course of the study (p = .170). However, due to the large variability in the responses, the data was transformed (log base 10) and re-analyzed according to literature recommendations (Hackney & Viru, 2008). This analysis showed a significant increase in IL-6 values from Week 1 to Week 6, an increase of approximately 300% (p = .001).

fT did not change significantly from week 1 to week 4 (p = .411). There was, however, a significant reduction from week 1 to week 6, a decrease of approximately 20% (p = .007).

Scores for each affective category on the REST-Q are displayed in Table 5. All affective categories scores (anger, depression, fatigue and vigor) were significantly reduced from baseline at various points during the study (see Table 5 for specific points of significance; *N.B.*, increased scores meant greater amounts of affective status, except for vigor where increased scores meant decreased status).

DISCUSSION

Our aim was to investigate the combined responses of C, IL-6 and fT of American football athletes during an intensive pre-season training regimen. It was hypothesized that significant resting, basal increases in C and IL-6, and a decrease in fT would occur; and, potentially impair weight lifting performance due to increased sensations of fatigue, illness, and stress developing in accordance with the "Cytokine Hypothesis" of overtraining. Athletes who are overtrained have a variety of specific physiological and psychological symptoms – weight loss, performance decline, increased fatigue-depression, and reduced vigor-motivation (Hackney, 2006; Smith, 2000; Urhausen et al., 1995). By the end of the 6-week training period in this study there were significant increases in each of the three 1RM exercises (performance improved), body weight increased and no adverse psychological changes occurred; in fact, slight positive affective changes developed. Based upon accepted criteria, the subjects in this study were not symptomatic of overtraining. Yet, the biomarker responses (decreased fT, increased C and IL-6) observed are indicative of the theoretical changes supposedly reflective of an overtrained state as has been proposed in the literature by several research groups (Hackney, 2006; Kraemer & Ratamess, 2005; Robson, 2003; Smith, 2000; Urhausen et al., 1995).

Notably all the subjects were experienced weight lifters who had strength performances commensurate with elite American football athletes (Fry & Kraemer, 1991; Hoffman & Kang, 2003; Ware, Clemens, Mayhew, & Johnston, 1995). Also, they were supervised in their training by both a qualified strength coach and research team

member who designed and executed their strength-conditioning program utilizing a scientific, progressive overload methodology (Burke et al., 2001; Hoffman & Kang, 2003). This program was intended to improve the athlete's performance, as well as subject them to a rigorous regimen associated with development of overtraining (Fry & Kraemer, 1991). It is also critical to note the resting biomarker levels observed prior to the exercise intervention reflect valid resting levels, based on reference ranges provided by the assay manufacturer (Salimetrics, USA) as well as reported in other studies (Cox, Pyne, Gleson, & Callister, 2008; Cullen, Thomas, Webb, & Hughes, 2015; Minetto et al., 2005; Rossi, 2006). Therefore, it is reasonable to state that our training protocol was designed and executed correctly and our initial biomarker levels were appropriately normal, and hence are not confounding factors in this study's outcomes. This leaves the question why then did we see significant biomarker changes, which according to theory should reflect overtraining, but the overtrained state did not develop?

Evidence suggests the feasibility of the "Cytokine Hypothesis" as a causative means for developing overtraining is sound and logical (Robson, 2003; Robson-Ansley, Blannin, & Gleeson, 2007; Robson-Ansley, de Milander, Collins, & Noakes, 2004; Smith, 2000). We feel, however, that what is lacking is development of an understanding towards the magnitude of biomarker changes necessary to reflect the overtrained state. In other words, not just a direction of change, but how much of a change from normal healthy values is necessary to signal adaptive disruption. This type of recommendation is lacking in the literature and is vitally needed to allow for biomarker monitoring to become more viable as a means for use in the monitoring of athletes.

CONCLUSIONS

Current findings point to the need for investigators to recognize that the directional changes of endocrine-immune biomarkers may not reflect a pathology state until the magnitude of that change reaches a critical level. Therefore, criteria "cut-points" for the magnitude of such changes are needed and future research should address this point to improve the diagnostic capability of biomarkers in sports physiology.

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Conflict of interest. The authors declare that they have no conflict of interest.

Ethical standards. The experiments conducted in this study comply with all guidelines and regulations within the USA.

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Corresponding author **Travis Anderson**
Endocrine Section – Applied Physiology Laboratory
Department of Exercise & Sport Science
University of North Carolina
Chapel Hill, NC, 27599-8700 USA
Tel/Fax 541-760-8547 / 919-962-0489
Email tanders2@live.unc.edu

EFFECT OF KINETIC RETURN ANKLE FOOT ORTHOSIS IN PATIENT WITH INCOMPLETE SPINAL CORD INJURY: CHANGES OF THE GAIT PATTERN. A CASE REPORT

Margot Bergmann[1], Mari Alvela[1], Priit Eelmäe[1,2], Doris Vahtrik[3], Helena Gapeyeva[3]
Haapsalu Neurological Rehabilitation Centre[1], Haapsalu, Estonia
Centre of Excellence in Health Promotion and Rehabilitation[2], Haapsalu, Estonia
Institute of Sport Sciences and Physiotherapy, University of Tartu[3], Tartu, Estonia

ABSTRACT

Background. The variety of orthotics available induces a purpose for estimation of their influence of functional mobility for individual needs in people with incomplete spinal cord injuries (ISCI). The aim of the study was to investigate the effect of the use of kinetic return ankle foot orthosis (KRAFO) on gait pattern in case of ISCI.

Methods. Ankle and knee joint kinematic and kinetic characteristics during gait with and without KRAFO were studied in a 34-year-old man with ISCI (fracture v.C5) using 3D motion analysis system (Vicon Motion Systems Ltd., UK) including two dynamographic platforms (AMTI, USA). Ankle and knee joint angles at initial contact and mid-stance, ankle dorsiflexion and foot progression angle in swing phase and ankle joint push-off values in stance phase were analysed.

Results. An excessive dorsiflexion in right ankle joint at initial contact, in mid-stance and in swing-phase occurred when walking without the orthosis, which decreased (105, 57 and 73%, respectively, $p < .01$) with the use of KRAFO. Orthoses use evoked the decrease (77%, $p < .01$) in peak foot progression angle. Ankle joint peak push-off power was low without the use of KRAFO and decreased even more (28%, $p < .05$) with the use of orthosis. Decreases of knee joint flexion angle at initial contact and in mid-stance (29 and 23%, respectively) with the use of KRAFO were not significant as compared to gait without orthosis.

Conclusions. Walking with KRAFO improved ankle and knee joint stability, providing a decrease in ankle kinematic characteristics but ankle joint push-off power did not change. Further studies are needed to compare the effect of KRAFO in comparison with other orthoses on gait pattern in case of ISCI in accordance with the patient-centric approach for rehabilitation process management.

Keywords: spinal cord injury, clinical gait analysis, ankle foot orthosis, kinematic and kinetic characteristics of gait.

INTRODUCTION

Spinal cord injury (SCI) is a traumatic event that affects conduction of both sensory and motor signals across the site of lesion. Autonomic nervous system could be affected as well; therefore patients' physical, psychological and social well-being is interrupted (Kirshblum et al., 2011; Singh, Tetreault, Kalsi-Ryan, Nouri & Fehlings, 2014). One of the complications after the injury is skeletal muscle atrophy. Smaller cross-sectional area is related to the loss of central activation and insufficient loading. The chronic stages of injury also include connective tissue infiltration in skeletal muscles (Gorgey & Dudley, 2007).

The ability to restore gait function is the "ultimate goal" for incomplete spinal cord injured patients (ISCI) and the ability to just stand is not enough. However, if patients are able to walk independently, they usually want to improve the quality of their gait (van der Salm et al., 2005).

A large amount of impairments that affect the gait pattern of ISCI patients are related to foot clearance problems in swing phase, i.e. excessive plantarflexion, limited knee or hip flexion, eversion of ankle joint. Due to the clearance problems, initial contact is also impaired. In the case of typical gait pattern, ankle joint is dorsiflexed to a neutral position in terminal swing, so that heel strike can occur at initial contact. If plantarflexion in swing phase is present, compensatory movements (i.e. unstable pelvic movement, compensatory functions of contralateral leg) are performed to rise foot from the floor (van der Salm et al., 2005). Patients with central neurological disorders have also reduced ability to push off from the floor with ankle joint. Low push-off values are caused by weakness of plantarflexors. An excessive work by hip joint could be used to compensate this reduced ability to push off (Bregman, Harlaar, Meskers & de Groot, 2012). During initial and mid-swing phases, changes are caused by stiffness of plantarflexors and also by weakness of ankle dorsiflexors. During terminal swing, in addition to ankle joint position, the position of whole lower limb could be affected by a decrease in angular velocity that is generated by hip flexors (Barbeau, Ladouceur, Mirbagheri & Kearney, 2002).

A conventional approach to correct drop-foot is custom-made ankle-foot orthotics (AFO), that keeps ankle joint in neutral position and does not allow the foot to drop in swing phase of gait (Kottink et al., 2004; Zou et al., 2014). One of the most used AFOs is posterior leaf spring (PLS) AFO, which is made of thermoplastics. PLS AFOs have very low energy storage and energy return capabilities; therefore they do not help patients during propulsion and do not generate enough energy to push off from the ground (Zou et al., 2014). Use of PLS AFO may contribute to disuse atrophy of calf muscles (Meier, Ruthsatz & Cipriani, 2014). AFO immobilizes inversion, eversion, abduction, adduction and plantarflexion, allowing only dorsiflexion of ankle joint, so AFOs with support around foot and/or ankle can improve medio-lateral stability of ankle during stance phase (Meier et al., 2014; Slijper, Danielsson & Willen, 2014). As the use of AFO prevents drop-foot, significant reduction in muscle activity of tibialis anterior is also noticed while wearing the orthosis; therefore prolonged usage could lead to atrophy (Meier et al., 2014).

Nowadays modern orthotics are available that provide more dynamic design - a kinetic return AFO (KRAFO) (ToeOFF®, Allard, USA) (Figure 1). Using KRAFO allows normal biomechanical function in talocalcaneal and talotarsal joints compared to PLS AFO, which causes additional calf muscle activity during gait (Meier et al., 2014). Prefabricated KRAFOs are used to stabilize ankle joint in anterio-posterior direction (Slijper et al., 2014). Also KRAFOs return energy during third rocker of gait to assist with propulsion, which also causes more muscle activity in plantarflexors (Meier et al., 2014).

Figure 1. **Kinetic return ankle foot orthosis**

KRAFOs are also known as carbon-composite AFOs. Among other things, KRAFOs can be used to improve ankle joint power and plantarflexor moment (Bregman et al., 2012; Zou et al., 2014). KRAFO has properties that enable storage of energy at the begging of stance phase and a return of this energy at the end of stance phase. The return of energy in late stance phase should increase push-off values and therefore reduce the need for different compensation strategies (Bregman et al., 2012).

Instrumented clinical gait analysis (CGA) that is conducted in laboratory is more precise than observational gait analysis as objective information could be collected, such as electromyography, kinetic and kinematic data. Joint movement could be observed in all three planes and forces that act on the body are recorded by dynamographic platforms. All the collected data is compared at the exactly same percentage of gait cycle (Chambers & Sutherland, 2002).

This case study was conducted to evaluate whether using KRAFO improves gait pattern of ISCI patient. As carbon-composite orthosis are relatively new, only few studies have been conducted. KRAFO is probably a proper orthosis also for patients with ISCI, as it is for other central neurological disorders that have been researched already (Bregman et al., 2012). As the main deviations of gait in ISCI patients are impaired initial contact, drop-foot in swing and low push-off values in the end stance phase, KRAFO could be a proper solution for this patient group as it is expected to solve all above mentioned deviations.

The aim of the current study was to evaluate ankle and knee joint angles at initial contact and mid-stance, ankle dorsiflexion and foot progression angle in swing phase and ankle joint push-off power in the end of stance phase during gait with and without kinetic return ankle foot orthosis in patients with ISCI. We hypothesized that while using KRAFO (1) it would be possible to keep ankle joint in neutral position during initial contact and mid-stance and therefore to reduce semiflexion in knee joint; (2) an excessive ankle joint dorsiflexion and foot progression angle in swing phase would decrease; (3) ankle joint push-off values would increase.

METHODS

Subject. A man aged 34 years with traumatic ISCI (fracture of 5th cervical vertebrae), American Spinal Injury Association Impairment Scale (AIS) grade D (motor incomplete) volunteered to participate in the present study. Patient's body mass was 90 kg, height 200 cm and body mass index 22.5 kg/m^2. Time since injury was 4.5 years at the moment of current analysis. The patient was able to walk independently and did not need any walking aids. KRAFO was used to correct his gait pattern for about a week. The patient wore KRAFO for approximately 12 hours per day. Prior to injury, the patient was an amateur athlete (extreme sports), with training load of 6–8 hours per week. The patient read and signed a written informed consent form in regard to the Declaration of Helsinki principles. The study had an approval from the ethics committee.

Measurement procedures. Prior to gait analysis, a thorough physical therapy assessment was carried out by two physiotherapists (PT) – all of the measurements were carried out by the same therapist and assisted by other. Muscle strength of lower extremities was assessed using Modified Oxford Scale (min grade 0/5 – no contraction; max grade 5/5 – movement against gravity with full resistance). Length of hamstring muscles was assessed by popliteal angle and length of plantarflexors by Silverskjöld test. Modified Tardieu scale was used to measure muscle tone of knee extensors and flexors and of plantarflexors (min T0 – no resistance throughout passive movement; max T4 – unfatigable clonus (> 10 s) occurring at a precise angle; R1/R2 – spasticity angle; R1 – angle of catch seen at fast velocity; R2 – full range of motion achieved when muscle is at rest and tested at slow velocity) (Li, Wu & Li, 2014). Goniometer was used to measure joint range of motion (ROM). Leg length was measured with non-elastic tape from anterior superior iliac spine to medial malleolus. Body balance was assessed by three tests: Tinetti Balance Assessment Tool (≤ 18 high; 19–23 moderate; ≥ 24 low risk of falls) (Vaught, 2001), Romberg's test and standing on one leg. Selective motor control (SMC) of tibialis anterior muscle was assessed with modified Trost scale (min grade 0 – only patterned movement observed, total synergy; max grade 2 – completely isolated movement observed, no synergy) (Zwaan, Becher & Harlaar, 2012). After PT assessment, instrumented CGA was conducted firstly with regular shoes and afterwards using KRAFO on right foot. All the measurements and data capturing were conducted within one day.

Kinematics and kinetics of gait. Three-dimensional gait analysis system with eight infrared cameras (MX-T20 cameras, 100 Hz; Vicon Motion Systems Ltd., UK) was used to capture kinematic data and two dynamographic platforms (AMTI, USA) were used to capture kinetic data. In addition two video cameras (Basler, USA) were used to capture video data from sagittal and coronal planes. Data was captured with Nexus 1.4.1. software and presented for interpretation with Polygon 3.1. software (Vicon Motion Systems Ltd., UK). Reflective markers were placed according to Davis model (Baker, 2006). The patient walked over-ground on an eight meter walkway. Fifteen trials were performed for each condition – gait with and without KRAFO and mean data was used for analysis. Duration of resting pause between trials was 3 to 5 minutes, when the patient sat on a spine-supporting chair.

Statistical analysis. MS Excel software was used to analyse the gait characteristics. Data are arithmetic means and standard deviations (*SD*).

Outcome parameters during walking with regular shoes and with KRAFO were compared using Student's paired samples *t*-test. Change (in percent) for outcome parameters as compared to initial level was calculated applying the formula (*b*-*a*)/*a*, where *a* presents data for walking without orthosis and *b* – data for walking with use of the KRAFO. The lowest level of significance was set to $p < .05$

RESULTS

Pre-testing patient evaluation. Data of the patient's pre-testing investigation is presented in Table 1.

The subject had 2 cm leg length discrepancy (right > left). The obtained data demonstrated an increase in Popliteal angle and therefore a decrease in length of hamstring muscles bilaterally. Length of gastrocnemius muscles was also decreased bilaterally, which was measured using Silverskjöld test. Spasticity was greater distally than proximally; clonus occurred in soleus and gastrocnemius muscles. Muscle strength was in moderate level ranging from grade four to five. Right tibialis anterior muscle had impaired selective motor control. Body balance impairment was found while eyes were closed or standing on right leg, but overall falling risk was estimated as low.

Ankle joint characteristics during gait. Characteristics of ankle and knee joint during walking with and without the use of KRAFO are presented in Table 2 and the example of change of ankle joint kinematics in sagittal and transversal planes is demonstrated in Figure 2.

At initial contact, an excessive dorsiflexion of right ankle joint was noted while walking with regular shoes, which decreased with using KRAFO. Dorsiflexion angle was reduced by 10.4˚, which is 105% less ($p < .01$) than angle with regular shoes while walking with KRAFO.

When walking without KRAFO, an apparent dorsiflexion in mid-stance in right ankle joint was found. Use of KRAFO reduced dorsiflexion angle by 10.4˚ in ankle joint in the middle of stance phase, which is 57% less ($p < .01$) than angle without orthosis.

The patient demonstrated an excessive dorsiflexion in swing phase in right ankle joint; peak dorsiflexion in swing phase exceeded reference values while walking without KRAFO.

Table 1. **Data of patient's pre-testing investigation**

Measure	Sin	Dex
Leg length discrepancy (cm)	2 (dex > sin)	
Popliteal angle (˚)	40	50
Silverskjöld test (knee 90°/0°)	(25/0)	(20/–5)
Spasticity (R1˚/R2˚)		
Rectus femoris muscle	T0	T2 (30/150)
Hamstrings	T2 (90/100)	T2 (55/90)
Soleus muscle	T3	T4
Gastrocnemius muscle	T3	T3
Muscle strength (points)		
Knee Extensors	5	5
Hamstrings	5	5
Dorsiflexors	5	4+
Plantarflexors	5	4
Selective motor control of tibialis anterior muscle (points)	2	1
Ankle instability	+	+
Balance		
Tinetti test (points)	Score 25	
Romberg test	Eyes closed unsecure	
Standing on one leg (s)	OK	5 (compensating)

Table 2. **Gait characteristics of ankle and knee joints with and without the orthosis in incomplete spinal cord injured patient**

Gait characteristic	Without KRAFO (mean ± *SD*)	With KRAFO (mean ± *SD*)	Percentages (%)	Change	*p*-value
Ankle joint angles (°)					
Dorsiflexion at initial contact	9.9 ± 2.7	–0.5 ± 1.1	105	↓	.003
Dorsiflexion at mid-stance	18.3 ± 2.3	7.9 ± 1.6	57	↓	.003
Dorsiflexion max in swing	19.8 ± 0.3	5.4 ± 0.5	73	↓	.001
Foot progression in swing	44.8 ± 3.7	10.3 ± 2.0	77	↓	.0001
Knee flexion angles (°)					
Initial contact	18.2 ± 1.9	12.9 ± 5.4	29	↓	.19
Mid-stance	15.7 ± 3.7	12.0 ± 0.6	23	↓	.16
Ankle power (W/kg)					
Max power generation	1.5 ± 0.2	1.1 ± 0.1	28	↓	.03

Note. KRAFO – Kinetic return ankle foot orthosis. Change (%) for outcome parameters as compared to initial level was calculated by formula (*b-a)/a*, where *a* presents data for walking without orthosis and *b* – data for walking with use of the KRAFO. The arrow shows the decrease of gait characteristics with orthosis as compared to without orthosis demonstrating the improvement, except for ankle push-off values.

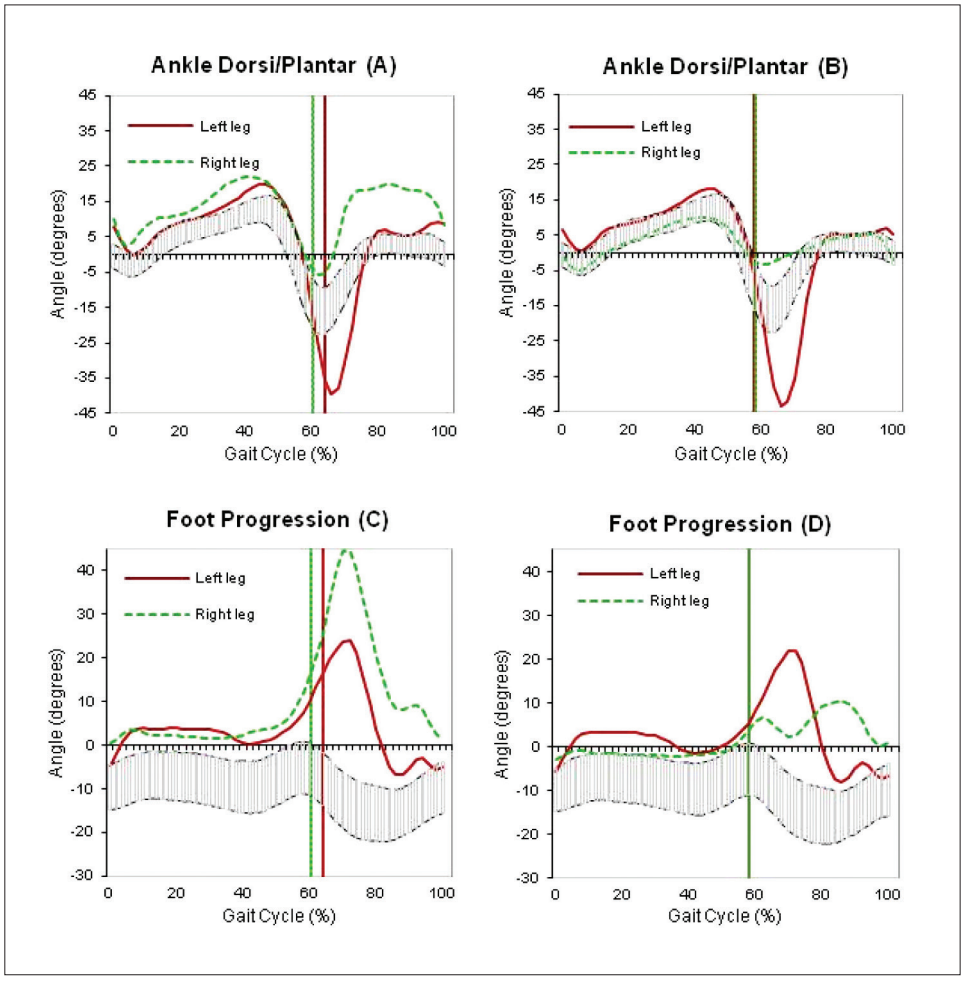

Figure 2. **Ankle joint kinematic characteristics in patient with ISCI: In sagittal plane for dorsal/plantar flexion angle during walking without (A) and with kinetic return ankle foot orthosis (KRAFO) (B); in transversal plane for foot progression angle during walking without (C) and with KRAFO (D)**

Note. KRAFO for the right leg was used.

Corresponding dorsiflexion value decreased 14.4° while walking with orthosis, which is 73% less ($p < .01$) than angle without KRAFO.

Instability of ankle joint caused extensive movement towards internal rotation on foot progression characteristics in the beginning of

swing phase while walking with regular shoes. Using KRAFO improved ankle stability and foot progression angle approached normal values. Peak internal rotation reduced 34.5˚ while walking with KRAFO which is 77% less ($p < .01$) than peak angle with regular shoes.

The example of change of ankle power characteristics is presented in Figure 3. Push-off values in ankle joint were low while walking with regular shoes and decreased even more (0.4 W/kg) while walking with orthosis. Peak push-off value decreased by 28% ($p < .05$) with the use of KRAFO as compared to walking with regular shoes.

Knee joint characteristics during gait. The example of change of knee joint kinematics in sagittal plane is demonstrated in Figure 4. Initially contact semiflexion occurred in knee joint, which was reduced to some extent (5.3˚) while walking with KRAFO, which is 29% less ($p > .05$) than walking with regular shoes.

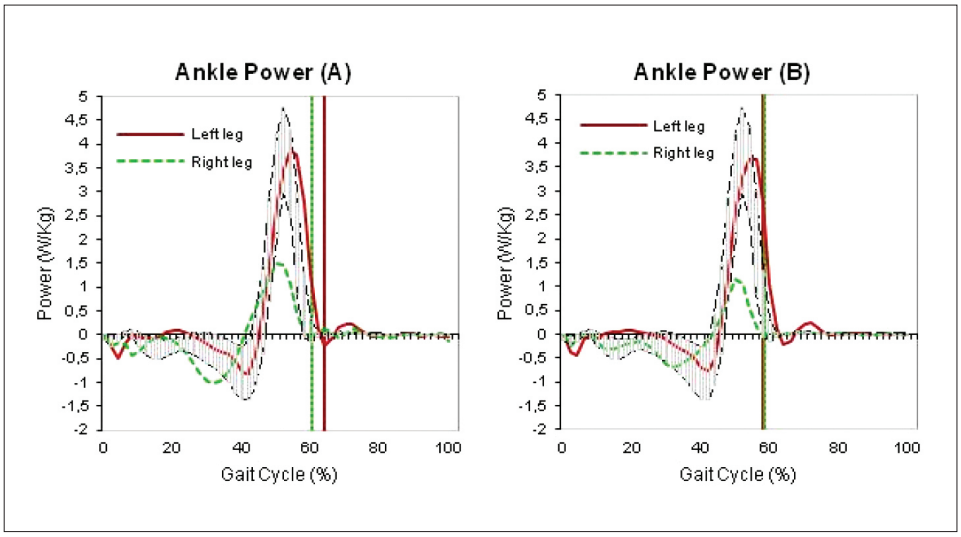

Figure 3. **Ankle joint power characteristics in patient with ISCI: Push-off values during walking without (A) and with kinetic return ankle foot orthosis (KRAFO) (B)**

Note. KRAFO for the right leg was used.

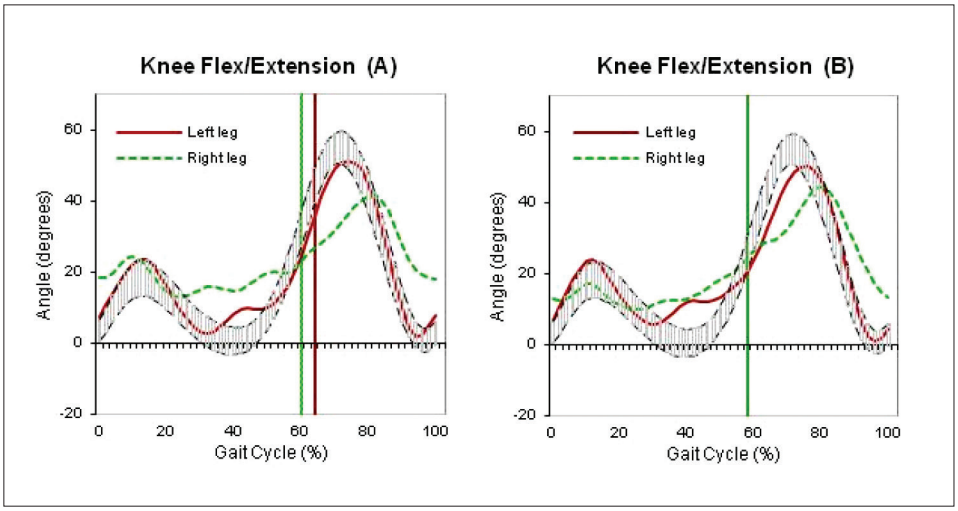

Figure 4. **Knee joint kinematic characteristics in sagittal plane in patient with ISCI: Flexion/extension angle during walking without (A) and with kinetic return ankle foot orthosis (KRAFO) (B)**

Note. KRAFO for the right leg was used.

Knee joint was in semiflexion also in mid-stance while walking without orthosis. Knee flexion values in mid-stance while walking with KRAFO decreased by 3.7˚ which is 23% less ($p > .05$) as compared to gait without orthosis, but it did not completely reach reference values. The changes of knee joint range of movement characteristics were not statistically significant.

DISCUSSION

The current study was conducted to evaluate the acute effects of carbon-composite KRAFO in ISCI patient. The main focus was on ankle joint range of motion in sagittal and transverse planes; knee joint ROM in sagittal plain and also on ankle power changes. The main findings of the study

were that using KRAFO in a patient with ISCI (1) reduced ankle joint dorsiflexion and knee joint flexion at initial contact and mid-stance; (2) reduced ankle joint dorsiflexion and foot progression angle in swing phase; (3) push-off values of ankle joint did not change significantly.

An excessive dorsiflexion in ankle joint at initial contact was caused by semiflexion in knee joint and also because of the patient's initiative to improve clearance in swing. Semiflexion in knee joint causes an apparent dorsiflexion because tibia is moved anteriorly in relation to foot, causing bigger dorsiflexion ROM. The use of KRAFO helped to reduce dorsiflexion and knee joint flexion at initial contact.

Dorsiflexion in stance phase was also caused by excessive knee flexion. During typical gait pattern, almost full extension should occur in knee joint in mid-stance, but in this case semiflexion occurs instead in both walking types – with and without orthosis. Plantarflexion-knee extension couple (PF-KE) plays an important role in controlling knee joint. In case of full knee extension, knee extensors become relatively insufficient and control only the first phase of knee extension. The second phase, where maximal knee extension is achieved, is controlled by plantarflexors. If the second phase is missing, it leads to a mild crouch gait, even though quadriceps muscle strength may be normal (Brunner & Rutz, 2013). In this case the second phase is impaired and PF-KE was insufficient; therefore ground reaction force (GRF) aligned behind the knee joint centre and mild crouch occurred. Plantarflexor muscle activation was insufficient and knee extensor muscle activation was excessive. At loading response and mid-stance, external knee flexion moment is increased, which causes knee extensors to work excessively to avoid collapse. The use of KRAFO in case of patient with ISCI improved PF-KE; GRF aligned more towards the knee joint centre, therefore knee semiflexion in stance phase was reduced causing less dorsiflexion. Also muscle work in stance phase was improved due to better biomechanical alignment.

At terminal stance and pre-swing ankle joint moves towards plantarflexion and eccentric contraction in plantarflexors occur. As the length of right plantarflexor muscles is decreased, not enough energy is stored to the muscles; therefore energy that is generated at push-off is also decreased (Bregman et al., 2012). While walking with KRAFO, plantarflexion ROM is even more limited due to the design of orthosis and plantarflexors do not elongate enough, therefore push-off values of right ankle joint are decreased while walking with orthosis. In this case the patient has moderate strength during plantarflexion and muscles are able to generate some energy, therefore KRAFO may even interrupt muscles to work independently and increase in push-off values is not noticeable. Meier et al. (2014) found that the use of KRAFO improved calf muscle circumference compared to plastic AFO in patients with neurological disorders. For example, plastic AFO immobilises calcaneus in a subtalar neutral position and therefore diminishes functional capacity of the calf muscle group. In our study, the patient had moderate plantarflexion strength and use of KRAFO immobilizes muscles and probably calf muscle group atrophy should be seen if AFO is used for a long time in contrast to Meier et al. (2014) study. Results of our study are in line with study conducted by Bregman et al. (2012) where using spring-like AFO increase in maximum ankle power generation was not found. In their study, data of gait analysis using AFO in patients with neurological deficit who had some ankle joint function with not complete drop-foot was studied. They also state that this type or orthosis may contribute to reduction of calf muscle activity.

Drop-foot that is caused by weakness and/or impairment of selective motor control of muscles is a very common problem in ISCI patients (van der Salm et al., 2005). To avoid tripping and falling, dorsiflexors are activated as much as possible to rise the foot from the ground. In our study an over-activity of dorsiflexors was a cause for excessive dorsiflexion in swing phase – muscles pulled the foot of the ground too much as they had moderate strength and SMC grade 1, so the patient was able to dorsiflex ankle joint and did that excessively to ensure that he did not fall. The use of orthosis does not allow excessive dorsiflexion due to its mechanical properties. Therefore dorsiflexion is reduced in the swing phase and muscles do not have to work excessively to rise the foot from the ground. Ankle joint is kept in a neutral position. Meier et al. (2014) stated that wearing orthosis could cause tibialis anterior muscle atrophy which could be a problem in this case also. The patient is used to activate dorsiflexor muscles excessively to rise the foot, but if he wears orthosis there is no need to activate them anymore. Therefore longer wearing period could cause muscle disuse atrophy.

In transverse plane, foot progression angle in stance phase is turned towards internal rotation to some extent. During toe-off and at the begging

of swing phase, rapid movement towards internal rotation occurs that reduces in mid-swing. The main causes for the rapid movement are ankle joint instability and modest weakness of dorsiflexor muscles. While walking with KRAFO, foot progression angle moves more towards reference values and rapid movement right after the toe-off and in swing phase is also reduced. Meier et al. (2014) claim that the use of KRAFO allows to improve biomechanical function in talocalcaneal and talotarsal joints, but does not allow an excessive movement; therefore it stabilizes the ankle joint.

The patient has leg length discrepancy (right leg, which is more affected, is 2 cm longer than left leg), which could also be a reason for deviated gait characteristics. The patient may unconsciously try to compensate the leg length discrepancy by increasing flexion in right leg. This could also be the reason for semiflexion in knee joint and therefore excessive dorsiflexion in ankle joint. As functional leg length was not measured (from navel to medial malleolus), no strong conclusions or recommendations could be made.

Practical impact. The **s**tudy of acute effects of using a KRAFO demonstrated improved ankle and knee joint flexion in stance phase and improved foot progression and dorsiflexion in swing phase. Using a kinetic return AFO has positive effect on ISCI patient's gait pattern on both stance and swing phases concerning ankle joint kinematics. The study with more subjects could be performed for the evaluation of the effect of KRAFO on kinetic characteristics of ankle joint during gait.

Strengths and limitations of the study. The present study strength feature is the fact that KRAFOs are relatively new and have not been tested on ISCI patients. Our study provides detailed information about the acute effect of KRAFO on ankle and knee joint characteristics. Our pilot study results demonstrate that the use of KRAFO does improve gait characteristics of ISCI patient and provide a good insight for future research with greater patient population.

Limitation of the study is that despite that case gives personal information we cannot generalize the results of the present case study to a wider population. Electromyography should also be conducted to declare that use of KRAFO reduces the activity of calf muscle group.

As the patient did not receive any gait training with orthosis and he had worn it for a short period of time, gait pattern could be affected. Only acute effects of the KRAFO were evaluated. The influence of different orthosis on gait characteristics should have been compared for ISCI patient.

CONCLUSIONS

It can be concluded that in case of a patient with ISCI, immediate effect of the use of KRAFO demonstrated improvement in ankle joint dorsiflexion and knee joint flexion at initial contact and in mid-stance. The use of KRAFO reduced ankle joint dorsiflexion and foot progression angle at swing phase. However, ankle power at push-off did not change significantly due to moderate plantarflexor strength and limited function of ankle joint. Further studies are needed to compare the effect of KRAFO in comparison with other orthosis (for example, carbon-composite AFO) on gait pattern in case of ISCI in accordance with the patient-centric approach for management of the rehabilitation process.

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Corresponding author **Margot Bergmann**
Haapsalu Neurological Rehabilitation Centre
Sadama 16, Haapsalu, Estonia, 90502
Tel. +37253414574
Email bergmannmargot@gmail.com

SEX DIFFERENCES IN RELIABILITY OF TESTS TO ASSESS COGNITIVE FUNCTION

Vaida Berneckė[1, 2], Kazimieras Pukėnas[1], Marius Brazaitis[1]
Lithuanian Sports University[1], Kaunas, Lithuania
Šiauliai State College[2], Šiauliai, Lithuania

ABSTRACT

Background. The purpose of this study was to identify whether the learning effect, fatigue, motivation, effort and/or sex-specific neural, physiological and morphological factors influenced the results of the test–retest reliability of tests to assess cognitive function.

Methods. The sample included ten men (age 21.2 ± 0.4 years; body mass 79.5 ± 8.3 kg) and ten women (age 22.0 ± 1 years; body mass 60.0 ± 10.0 kg). Participants accomplished six tests (three for memory and three for attention) four times, i.e. two times (with 24 hours' break) on successive days (teaching) and two times (with 48 hours' break) on the third and fifth days (re-testing to assess the reliability). The reliability was assessed by calculating the average of the population, standard deviation, and intraclass correlation coefficient (*ICC*).

Results. In males and females, measurements of attention function were highly reliable over time (*ICC* > .84). The ICCs for volume of spatial memory were above .79, for memory of even number recognition above .57 for both genders and for memory of figure recognition .00 for males and .79 for females.

Conclusion. In young healthy males and females, measurements of attention function were highly reliable over time. Meanwhile, reliability for volume of spatial memory was good/high for both sexes, but reliability of memory for even number recognition was insufficient for both sexes and results from memory of figure recognition showed good reliability for women and insufficient reliability for men.

Keywords: memory, attention, test-retest, reproducibility, gender.

INTRODUCTION

Neuropsychological assessments are designed to measure cognitive functions in both healthy and clinical populations and remain important tools for research studies, clinical diagnoses, patient outcomes, and intervention monitoring (Kueider, Parisi, Gross, & Rebok, 2012; Zygouris & Tsolaki, 2014). Why are working memory and attention so important in cognitive control and why are reliability studies of tests assessing cognitive function essential? One reason for this is because the healthy human brain comprises remarkable complexity in both its structural architecture and functional communication networks (Bullmore & Sporns, 2009). Working memory is critically important in cognition and seems necessary for many cognitive abilities, such as reasoning, language comprehension, planning and spatial processing (D'Esposito, 2007). This system is critical for virtually all forms of "online" cognitive processing, as evidenced by robust correlations with measures of fluid intelligence, scholastic aptitude (Cowan et al., 2005) and is central to much of human behaviour (LaRocque, Lewis-Peacock, Drysdale, Oberauer, & Postle, 2013). Attention facilitates target processing during both perceptual and post perceptual stages of processing, and functionally dissociated processes have been implicated in the maintenance of different kinds of information in working memory (Awh, Vogel, & Oh, 2006). Posner and Petersen (1990) have proposed that the sources of attention form a specific system of anatomical areas, which

can be further broken down into three networks. These networks carry out the functions of alerting, orienting, and executive control (Fan, McCandliss, Sommer, Raz, & Posner, 2002). Thus, although it is clear that these processes are closely intertwined, the nature of these interactions depends upon the specific variety of attention or working memory that is considered (Awh, Vogel, & Oh, 2006). In psychological research, cognitive complexity often is used to refer to high-level cognitive processes – mainly problem solving, reasoning, and decision making – and their interaction with more basic processes such as perception, learning, motivation, and emotion (Knauff & Wolf, 2010; Osman, 2010). As Knauff and Wolf (2010) pointed out, there is a second important aspect to complexity – the complexity given by the environment with which the agent has to interact (Osman, 2010). The flexibility and adaptiveness of a cognitive system depends highly on its ability to learn from previous experience (Schmid, Ragni, Gonzalez, & Funke, 2011). Elements relevant to the solution process are large (complexity), highly interconnected (connectivity), and dynamically changing over time (dynamics). Extensive empirical research has demonstrated that performance varies in systematic ways over time as a result of biological variability, time awake, time on task, circadian rhythms, learning effect and a variety of other factors that impact the effectiveness and efficiency of cognitive processing and are all factors that can affect data between and within trials (Weir, 2005).

There are other ways in which test-retest reliability can be defeated: examiners can fall short through lack of competence (Bauer et al., 2012). Moreover, subjects may show up for an assessment session without adequate reading glasses, or having taken cold medication that affects their alertness (Kipps & Hodges, 2005), be suffering from a severe headache or illness, the effects of motivation also might play a role in the performance (Barr, 2003). It appears that even in healthy volunteers the learning trajectories may differ by neuropsychological domain, age and education of participants (Beglinger et al., 2005). For the all aforementioned reasons, in the present research we chose a short, simple test that does not necessitate the use of expert examiners, participants were young, healthy, and had the same educational level and accomplished two training sessions before reliability assessment with 48 h between test-retest sessions. Also, tests were conducted in a low traffic, quiet environment to enable the participant to concentrate solely on the assessment and not be distracted by the surroundings. For reliability analysis, we selected from "Effecton" studio (2006) three attention (the test for the assessment of complex reaction, test for the search of visual objects, the test for attention transfer) and three memory tests (the test for the volume of spatial memory, test for even number recognition, test for figure recognition) that comprehensively assess main characteristics of attention and WM described above.

Another reason justifying the need for repeatability experiments are gender-related brain anatomical (Allen, Damasio, Grabowski, Bruss & Zhang, 2003; Gur, Gunning-Dixon, Bilker, & Gur, 2002; Shikhman, 2007), functional differences (Speck et al., 2000), as well as among other differences circulating gonadal hormones have impact on cognition (Gur et al., 2000; Mathew, Wilson, & Tant, 1986). The literature has addressed numerous findings that support several brain anatomical, gender-related brain differences, such as males tend to have larger brain volume, while the gray-to-white ratio tends to be grater in females (Allen et al., 2003; Gur et al., 2002; Shikhman, 2007), also males having a significantly larger left versus right planum temporale area, a difference that is not significant in females (Kulynych, Vladar, Jones, & Weinberger, 1994). A larger splenium in females versus a larger genu in males is one aspect of the gender-related differences in the dimensions of the corpus callosum (Dubb, Gur, Avants, & Gee, 2003) and hippocampal volumetric sex-differences (Maller, Réglade-Meslin, Anstey, & Sachdev, 2006). Both post-mortem and imaging studies have found that relative to brain size, women have larger volumes in the hippocampus (Filipek, Richelme, Kennedy, & Caviness, 1994), caudate nucleus (Filipek et al., 1994; Murphy et al., 1996), anterior cingulate gyrus (Paus et al., 1996) dorsolateral prefrontal cortex and planum temporale (Schlaepfer et al., 1995). In contrast, the relative volumes of the amygdala (Giedd et al., 1996) and paracingulate gyrus (Paus et al., 1996) are consistently larger in men (Andreano & Cahill, 2009). From a neuroimaging perspective, Filippi et al. (2013) research has shown that there are gender differences in functional connectivity during resting state; i.e. the authors found that women had greater intrinsic functional connectivity inclusive of the cingulate, dorsolateral prefrontal

cortex, and the inferior frontal gyrus, while men demonstrated increased functional connectivity in parietal regions, characteristics that the authors attribute to potential strategy differentiation. These observed differences could help explain the disparity in performance between the genders on various cognitive tasks, as well as bringing into question the possibility of inherent neural network differences (Hill, Laird, & Robinson, 2014).

The above-described facts suggest that the disparity in performance between the genders in various cognitive tasks is evident, as well as bringing into question whether the possibility of distinct consistency in test-retest reliability between the genders exists. According to the evidence, women are more likely than men to show significantly greater activations in the prefrontal regions (Goldstein et al., 2005), which have been implicated in encoding and retrieval of visuospatial information (Carter et al., 1998; Mayberg, 1997; Poldrack et al., 1999; Smith & Jonides, 1999; Wagner, 1999). Moreover, the availability of dopamine transporters (Lavalaye, Booij, Reneman, Habraken, & van Royen, 2000; L. H. Mozley, R. C. Gur, P. D. Mozley, & R. E. Gur, 2001; Staley et al., 2001) and plasma serotonin levels are also higher in women than in men (Ortiz, Artigas, & Gelpi, 1988) when estrogens and dopamine enhance working memory (Berman et al., 1997; Jacobs & D'Esposito, 2011; Shaywitz et al., 1999) and estradiol increases emotional arousal (O'Neal, Means, Poole, & Hamm, 1996). Besides, Barral and Debu (2004) suggest that while males are faster than female at aiming at a target, the females are more accurate. Regarding to reviewed literature about neurobiological sex differences relevant to attention and memory, we hypothesize that higher reliability will be expected for women than for men.

METHODS

Experimental approach to the problem. The experiment was designed aiming at assessing the reliability of tests of cognitive functions (memory and attention) depending on gender. The functions of memory and attention were measured by the same examiner over four days, the research participants accomplished two familiarization sessions with 24 hours break and two sessions for test-retest reliability with 48 hours break. Our aim was to identify whether the learning effect, fatigue, motivation, effort and/or sex-specific neural, physiological and morphological factors influenced the results of the test–retest sessions. The dependent variables included measures of short term memory: memorization, storage and recall; for attention: stability, concentration, distribution and transfer. The independent variables were the two identical trials (three standard memory and three attention tests) over two days and sex group (women vs men).

Organization and procedure of the research. The research was conducted in the Sports Science and Innovation Institute at Lithuanian Sports University. The research participants were introduced to the aims, procedure and possible inconveniences of the research. Young and healthy students of Lithuanian Sports University: ten men (age 21.2 ± 0.4 years; body mass 79.5 ± 8.3 kg; stature 184 ± 4.3 cm; BMI 23.5 ± 2.4 kg/m^2; fat free mass 65.8 ± 5.4 kg; mean ± SD) and ten women (age 22.0 ± 1 years; body mass 60.0 ± 10.0 kg; stature 168.0 ± 6.0 cm; BMI 21.0 ± 2 kg/m^2; fat free mass 44.0 ± 5.0 kg; mean ± SD) participated in the research. They accomplished the tests four times, i.e. two times (with 24 hours' break) on successive days (teaching) and two times (with 48 hours' break) on the third and fifth days (re-testing to assess the reliability). The research participants had to complete six tests (three tests for memory and three for attention); the tests were presented in random order. The accomplishment of all tests lasted approximately for 20 minutes. The participants completed the tests in a quiet environment: they were not disturbed by other people, noise, music or other distracters. The tests of the memory and attention were described elsewhere (Bernecke et al., 2012).

Statistical analysis. The reliability of research results was assessed by calculating the average of the population, standard deviation, and intraclass correlation coefficient (*ICC*). The *ICC*s were used to analyse the correlations between the values obtained on different days (Singh et al., 2011). The *ICC* was computed as a single-measure *ICC* using a two-way random-effect model (absolute agreement). The level of significance was set at $p < .05$. Statistical analyses were performed using IBM SPSS Statistics software (v. 22; IBM Corporation, Armonk, NY).

RESULTS

Reliability assessment of memory tests related to gender. The data from two test-retest sessions for all three memory tests in male and female subjects are presented in Table 1. The results of the average length of number sequence in *testing the volume*

of spatial memory showed good reiteration of the results for both genders. The results of the intraclass correlation coefficient of the average number of guessed symbols of testing *the amount of numbers memorization* showed high reliability for males and good reliability for females. The results of testing *even number recognition* revealed poor reliability for both genders. The results of testing *memory for figure recognition* demonstrated good reliability for the females, whereas insufficient reliability has been found for males, in addition, females recognized significantly greater number of figures than males (Table 1).

Reliability assessment of attention tests related to gender. The data from two test-retest sessions for all three attention tests in males and females are presented in Table 2. The results of the *test of complex reaction* assessment showed high reliability for both genders. The results of testing *the search for image samples* revealed high reliability for males and females; besides, males were significantly faster in task accomplishment compared with the females. The results of assessing the reliability of testing *attention transfer* revealed high reliability for females and good reliability for males (Table 2).

Table 1. **Results of the reliability assessment of memory tests for both genders**

Gender/ ICC	Volume of spatial memory				Memory for even number recognition		Memory for figure recognition	
	Average length of numeric sequence		Average number of guessed symbols		Number of correct answers		Number of correctly recognized figures	
	Test	*Re-test*	*Test*	*Re-test*	*Test*	*Re-test*	*Test*	*Re-test*
Male	7.00 ± 0.72	6.79 ± 0.66	6.77 ± 0.73	6.51 ± 0.69	10.5 ± 2.22	10.6 ± 2.95	7.5 ± 0.53*	7.2 ± 1.23*
ICC‘	.87#		.91#		.57		.00	
Female	6.52 ± 0.75	6.68 ± 0.56	6.3 ± 0.68	6.46 ± 0.6	11.14 ± 2.32	12.07 ± 2.7	8.4 ± 0.70	8.2 ± 0.42
ICC“	.79#		.82#		.68#		.79#	

Notes. Values are shown as mean and standard deviation. *ICC*’ – intraclass correlation coefficient for males. *ICC*” – intraclass correlation coefficient for females. *$p < .05$, compared with female. # significant, $p < .05$.

Table 2. **Results of the reliability assessment of attention tests for both genders**

Gender/ ICC	Test of complex reaction		Test of search for image samples		Test for attention transfer	
	Time of reaction (ms)		Average time of the accomplishment of five tasks (s)		Speed of accomplishing the task (s)	
	Test	*Re-test*	*Test*	*Re-test*	*Test*	*Re-test*
Male	598.56 ± 44.66	585.6 ± 51.98	36.6 ± 6.93*	36.4 ± 6.11*	179.0 ± 40.73	165.2 ± 33.64
ICC‘	.93#		.97#		.84#	
Female	600.21 ± 57.09	609.29 ± 64.0	29.20 ± 5.53	30.00 ± 5.62	167.14 ± 45.32	168.71 ± 41.67
ICC“	.93#		.96#		.94#	

Notes. Values are shown as mean and standard deviation. *ICC*’ – intraclass correlation coefficient for male. *ICC*” – intraclass correlation coefficient for female. *$p < .05$, compared with females. # significant, $p < .01$.

DISCUSSION

Our study focused on sex-specific test-retest reliability of responses to tests assessing memory and attention. The tests of memory and attention were found to be stable over a 48-hour period for both genders with one exception for figure recognition memory for the males. In the present study the two familiarization sessions were accomplished with 24 hour brake. Two training sessions were chosen because many authors affirmed that the most prominent learning effect occurs between the first and second test-retest sessions. As Falleti, Maruff, Collie, and Darby’s (2006) study illustrated that

performance generally improved from the first to the second assessment on the CogState battery. Moreover, after the second assessment, the performance of the group stabilized and improved no further on any of the cognitive measures (Falleti et al., 2006). Meanwhile, Claus, Mohr, and Chase (1991) demonstrated improvement across three weekly test sessions in which alternate forms were used. Also, Benedetto et al. (1995) demonstrated minimal learning after three trials across six tests from the ANAM library. According to Beglinger et al. (2005), the dual baseline may be beneficial with two to three practice sessions before a treatment is measured. The results from our study have confirmed that two training sessions are sufficient to achieve good test repeatability for both genders.

The length of test–retest interval is another important factor to consider (Barr, 2003). One must contemplate the additional range of factors influencing the comparability of test settings at baseline and retesting (Barr, 2003). There are several benefits in investigating changes in performance over very short test-retest intervals (Falleti et al., 2006). First, estimates of the magnitude of practice can be derived under conditions most optimal for improvement to occur (Falleti et al., 2006). Second, the use of very short intervals minimizes the extent to which the individuals will undergo any physical or psychological changes that could give rise to true cognitive change (i.e., changes in sleep patterns, stage in menstrual cycle) (Falleti et al., 2006). Therefore, estimates of improvement in these conditions would be more likely to reflect only measurement-related factors and not include any effects of normal biological variability that are known to cause subtle changes in cognition and which operate over weeks or months (Bland & Altman, 1996). For the above discussed reasons in this study we have chosen the 24-hour break between test-retest sessions for teaching and 48-hour break for reliability experiment. Results of this inter-day reliability study showed a high stability between sessions for the attention in both genders (*ICC* > .93) with one exception for test of attention transfer for male (*ICC* = .84). Similar results were found in our previous intra-day experiment (Bernecke et al., 2012) where *ICC* values ranged from good to high (*ICC* > .86). The results from our inter-day and intra-day experiments showed good/high stability for volume of spatial memory test. Meanwhile, insufficient reliability was obtained for memory of even number recognition (inter-day experiment) and for figure recognition during both inter-day and intra-day experiments.

Observed differences between males' and females' brain anatomical and functional characteristics could help explain the disparity in performance between the genders on various cognitive tasks (Hill et al., 2014). In general, the gender-related differences include a wide range of processing skills (González-Garrido, Gómez-Velázquez, Sequeira, Ramos-Loyo, & López-Franco, 2013). It has been shown that females recall better the appearance of others better than males (Mast & Hall, 2006) and score higher on tasks involving manipulation of phonological and semantic information, episodic and semantic memory, verbal learning, verbal analytical working memory (WM), object location memory, fine motor skills, perceptual speed and writing skills (Hedges & Nowell, 1995). Our results support the proposition that women better recall than men, we established that women significantly better performed figure recognition task. Moreover, good stability for the test-retest results were established for the women (*ICC* = .79) and unstable for men (*ICC* = .00). In general, women were more likely than men to show significantly greater activations in the hypothesized prefrontal regions, despite the same performance as the men (Goldstein et al., 2005). These regions included middle, inferior, and orbital prefrontal regions, which have been implicated in encoding and retrieval of visuospatial, semantic, and phonological information and inhibitory functions associated with orbitofrontal cortex (Botvinick, Nystrom, Fissell, Carter, & Cohen, 1999; Carter et al., 1998; Mayberg, 1997; Poldrack et al., 1999; Smith & Jonides, 1999; Wagner, 1999). While males tend to score higher on tasks involving mathematical (Lynn & Irwing, 2008), spatial (Kaufman, 2007; Lejbak, Crossley, & Vrbancic, 2011), object (Lejbak et al., 2011), visuospatial working memory (VSWM), fluid reasoning, and positional reconstruction, or when spatiotemporal analyses are required (Lejbak, Vrbancic, & Crossley 2009; Ramos-Loyo & Sánchez-Loyo, 2011). In rodents, exogenous estradiol can enhance the consolidation of object recognition (Luine, Jacome, & Maclusky, 2003), water maze navigation (Packard & Teather, 1997), and inhibitory avoidance (Rhodes & Frye, 2004). However, we have not found any significant differences between genders for volume of spatial memory or for even number recognition. Repeatability for the volume

of spatial memory was good/high for the men and good for the women, repeatability for memory of even number recognition was insufficient for both genders. Many authors have established that males have faster reaction times than females and female disadvantage is not reduced by practice (Adam et al., 1999; Dane & Erzurumlugoglu, 2003). In the present study, test of search for image samples best reflects the reaction because there is minimum probability for the mistake and can be performed easily and fast. However, the results of the aforementioned test showed faster reaction time for the female subjects compared to the male ones ($p < .05$); the reliability results were high for both genders ($ICC > .96$). Obviously, females were faster because during this test they had to find the numbers quickly and we believe that this was the case because it has been established that females are more accurate (Barral & Debu, 2004) and have a better object location memory than males (Hedges & Nowell, 1995). In our case women showed better test-retest reliability ($ICC = .94$) then men ($ICC = .84$) for test of attention transfer, the test timing does not differ significantly between genders. Equal high reliability have been found between genders ($ICC = .93$) for the test of complex reaction and also the test timing does not differ significantly.

CONCLUSION

In conclusion, in young healthy males and females, measurements of attention function, such as tests of complex reaction, search for image samples and attention transfer are highly reliable over time. Whereas reliability for volume of spatial memory are good/high for both genders, reliability of memory for even number recognition is insufficient for both genders, and results from memory of figure recognition showed good reliability for females and insufficient reliability for men.

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Corresponding author **Vaida Bernecke**
Lithuanian Sports University
Ryto str. 11, Sutkūnai, Šiauliai area
Lithuania
Tel. +37068871470
Email vaidabernecke@gmail.com

REDUCED CITRATE SYNTHASE ACTIVITY EFFECT ON OXYGEN CONSUMPTION RATES IN ISOLATED MITOCHONDRIA FROM MICE LIVER AND MUSCLES

Andrej Fokin[1], Rasa Žūkienė[2], Aivaras Ratkevičius[1]
Lithuanian Sports University[1], Kaunas, Lithuania
Vytautas Magnus University[2], Kaunas, Lithuania

ABSTRACT

Background. Liver and skeletal muscles play the major role in metabolism. Mitochondria are of particular importance in functioning of these organs. We tested the hypothesis that reduced citrate synthase (CS) activity could induce improved fatty substrate and carbohydrate oxidation in mitochondria extracted from liver and hind limb muscles of mice.

Methods. Eight mice each of 12-week-old control C57B6/J (B6) and congenic B6.A-(rs3676616-D10Utsw1)/Kjn (B6.A) mice were studied. The mitochondria were isolated by differential centrifugation method followed by assessment of mitochondrial respiration and citrate synthase (CS) activity. Mitochondrial respiration was measured as oxygen consumption with Clark-type oxygen electrode by using polarography system. CS enzyme activity was measured spectrophotometrically.

Results. The activity of CS was by ~32% lower for mitochondria for B6.A compared to B6 mice (603.9 ± 135.6 U/g and 894.2 ± 193.2 U/g, respectively). Mitochondrial respiration did not differ significantly between the strains.

Conclusions. 30% reduction in citrate synthase activity does not impair mitochondrial respiration.

Keywords: mitochondrial respiration, insulin resistance, β-oxidation.

INTRODUCTION

Mitochondrial dysfunction might contribute to such conditions as obesity, insulin resistance and type II diabetes (Christe et al., 2013; Yang et al., 2012). Both obesity and type 2 diabetes are associated with poor performance of mitochondria (Houmard, 2008). The misbalance between β-oxidation of fatty acids and substrate flux through Krebs cycle seems to be of special importance for functioning of mitochondria and, therefore, the development of obesity and diabetes (Koves et al., 2008). It means that the balance between oxidation of fatty acids and oxidation of carbohydrates in citric acid cycle is one of the common mechanisms to regulate performance of mitochondria. There is evidence that citrate or citric acid, an intermediate of the mitochondrial Krebs cycle, plays a role in controlling this balance (Ruderman, Saha, Vavvas, & Witters, 1999). Cytosolic citrate is converted by ATP citrate lyase (ACL) to acetyl CoA, the substrate for acetyl CoA carboxylase (ACC) in the synthesis of malonyl CoA. Malonyl CoA can inhibit carnitine palmitoyl transferase 1 (CPT) and thus interfere in with fatty acid oxidation. Low rates of fatty acid oxidation contribute to metabolic disorders. It can be speculated that reduced levels of CS activity might be beneficial in promoting fatty acid oxidation under conditions of excessive substrate supply.

Citrate synthase (CS) has often been used as a mitochondrial marker in both animal and human studies (Hamilton & Booth, 2000; Rabol, Boushel, & Dela, 2006). Mammalian CS is encoded by a single nuclear gene. After translation in the cytosol, CS is

transported into the mitochondrial matrix, where it functions as the first and rate-limiting enzyme of the citric acid cycle and thus plays a decisive role in regulating energy generation and ROS production of mitochondrial respiration. A missense mutation of Cs might therefore alter mitochondrial function (Johnson, Gagnon, Longo-Guess, & Kane, 2012). Liver, skeletal muscles, adipose tissue and pancreas are believed to play the major role in insulin resistance (Bouderba et al., 2012). In current study we have tested mitochondrial oxidation of different substrates in liver and skeletal muscles. We investigated mitochondrial respiration in mice with H55N polymorphism which is associated with reduced CS activity (Ratkevicius et al., 2010).

The aim of the study was to test the hypothesis that low citrate synthase (CS) activity could improve fatty substrate and carbohydrate oxidation in mitochondria. Thus we investigated whether CS activity and respiration differ between mitochondria derived from C57BL/6J (B6) strain and congenic B6.A-(rs3676616-D10Utsw1)/KjnB6 (B6.A) strains of mice. B6.A strain carries the A/J allele in the genomic region containing the *Cs* gene on otherwise B6 strain background.

METHODS

Animals. All the procedures were approved by the Lithuanian State Food and Veterinary Service (No. 0223). Mice were kept in standard cages (cage dimensions: 267 x 207 x 140 mm) at 20–22º C temperature and 55 ± 10% humidity with 12/12- h light/dark cycle. Mice fed for standard rodent diet (58.0% kcal from carbohydrate, 28.5% kcal from protein, 13.5% kcal from fat; LabDiet 5001, LabDiet, St. Louis, USA) and received tap water *ad libitum*. For mitochondria experiment 12 week-old B6 and B6.A mice were used. B6 and eight B6.A mice of male and female sex (n = 8 in each group) were studied.

Mitochondria isolation and respiration. Following euthanasia by the cervical dislocation, liver and hind limb muscles were quickly excised and placed into separate 80 ml ice cold 0.9% KCl solution. After 3 min, liver and muscles were briefly minced with surgical scissors. Additionally, muscles were incubated for 5 min in ice cold isolation medium A (150 mM sucrose, 75 mM KCl, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, pH = 7.4) supplemented with 2 mg/ml proteinase (type XXIV, Sigma P8038). Liver was filled homogenized in medium H (250 mM sucrose, 10 mM TRIS, 3 mM EGTA, pH = 7.7) by electric Potter-Elvehjem homogenizer with 10 strokes at 750 rpm. After the incubation, 20 ml of ice cold isolation medium B (250 mM sucrose, 20 mM MOPS, 0.1 mM EGTA, pH = 7.4) supplemented with 1 mg/ml of BSA was added into minced muscles and the final mix was homogenized with 10 strokes at 750 rpm. Homogenates were transferred to the centrifuge tubes. Mitochondria were isolated by differential centrifugation (Zukiene, Nauciene, Ciapaite, & Mildaziene, 2010). For liver mitochondria there were 3 steps of centrifugation: 800 x g for 5 min, 6800 x g for 10 min and 6800 x g for 10 min, for muscle mitochondria: 800 x g for 10 min, 10 000 x g for 10 min and 10000 x g for 10 min. After the 2nd centrifugation the supernatant was removed and 10 ml of ice cold isolation medium B was added to re-suspend the muscle mitochondrial pellet. In case of liver mitochondria, medium M (250 mM sucrose, 5 mM TRIS, pH = 7.3) was added to re-suspend mitochondrial pellet. Mitochondrial pellets were kept on ice throughout the experiment. The protein concentration was determined by a modified burette method using bovine serum albumin (BSA) as a standard. Final mitochondrial suspensions were used immediately for respiration measurement or stored at –80ºC until enzyme activity analysis.

Mitochondrial respiration was measured as oxygen consumption (O_2 nmol min^{-1} mg^{-1} protein, VO2) at 37°C in 1.5 ml glass vessel equipped with Clark-type oxygen electrode by using polarography system. The following respiratory substrates were used: 1) 5 mM glutamate plus 5 mM malate (G + M); 2) 5 mM pyruvate plus 5 mM malate (P + M); 3) 0.25 mM malate plus 0.005 mM palmitoyl-carnitine (M + PC); 4) 5 mM glutamate plus 5 mM malate plus 0.005 mM palmitoyl-carnitine (GM + PC); 5) 5 mM pyruvate plus 5 mM malate + 0.005 mM palmitoyl-carnitine (PM + PC); 6) 5 mM succinate plus 0.001 mM rotenone (Suc + Ro). Rotenone inhibits role respiration complex I and was used in to measure VO_2 which is associated with respiration complex II (Bouderba et al., 2012). 1 ml of incubation solution 6 (IT6) (KCl 110 mM, creatine monohydrate 50 mM, TRIS 20 mM, KH_2PO_4 5 mM, Mg ($MgCl_2 \cdot 6H_2O$) 2.5 mM, pH = 7.2) was used in the experiments. Mitochondrial non-phosphorylating state 2 respiration (V2) was initiated by adding mitochondria into IT6 containing substrates. State 3 respiration (V3) was initiated by adding 1 mM of ATP which is constantly converted to ADP by creatine- creatine phosphokinase system in the medium.

Citrate synthase activity assay. CS activity was measured as in our previous studies (Ratkevicius et al., 2010). Briefly, 10 ml of mitochondria lysate was added to start the reaction in 990 ml of reaction reagent which then consisted of 100 mM triethanolamine-HCl, DTNB (100 µM), 0.25% Triton-X (vol/vol), 0.5 mM oxaloacetate, 0.31 mM acetyl CoA with pH adjusted to 8.0. The wave length of the spectrophotometer was set 412 nm and the molar extinction coefficient of 13,600 $M^{-1} \cdot cm^{-1}$ was used in calculations of the maximum CS activity (Vmax) during the first 2 min of the reaction at room temperature (~21°C). CS from porcine heart was used as a standard (C3260-200UN, Sigma-Aldrich, UK) for assay calibration. Protein concentration of the mitochondrial lysates was determined using the Bradford Assay (Bio-rad, Hertfordshire, UK). CS activity was expressed in units or U (mmol min^{-1}) per gram (g) protein.

Statistical analysis. 2-way ANOVA test and Bonferroni post-test were used to evaluate differences between the strains in ATP stimulated mitochondrial respiration. Unpaired *T*-test was performed to observe enzyme activity differences. Values of $p < .05$ were considered as statistically significant. Results are presented as mean ± *SD*.

RESULTS

CS activity was reduced by 32% in mitochondrial samples (cor. spec. 894.2 ± 193.2 in B6 mitochondria compared to 603.9 ± 135.6 U/g in B6.A mitochondria) in mice with CS polymorphism (B6.A, $n = 8$) compared to control (B6, $n = 8$), $p < .05$ (Figure 1). There was no difference between male and female mice.

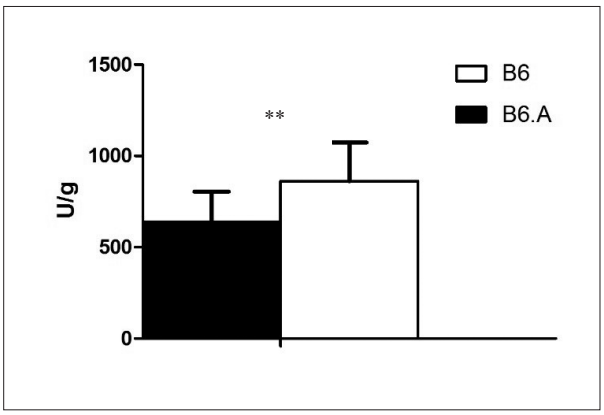

Figure 1. **Citrate synthase' activity (U/g) in B6 and B6.A mice mitochondria**

Note. $^{**}p < .05$.

No significant differences in V3 were observed between mitochondria from B6 and B6.A liver or muscles (Figures 2 and 3, respectively). There was a tendency of increased respiration with PM and PM + PC substrates in B6.A mice compared to B6 both in liver and muscles mitochondria.

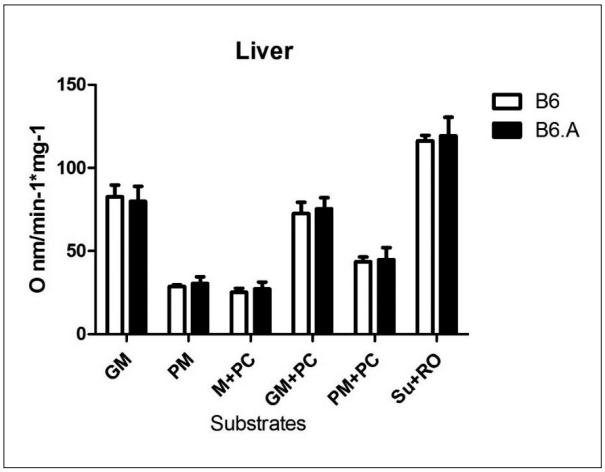

Figure 2. Comparison of three liver mitochondria respiration states between B6 and B6.A mice

Note. The following substrates were used: GM – glutamate plus malate, PM – pyruvate plus malate, M + PC – malate plus palmitoylcarnitine, Su + RO – succinate plus rotenone.

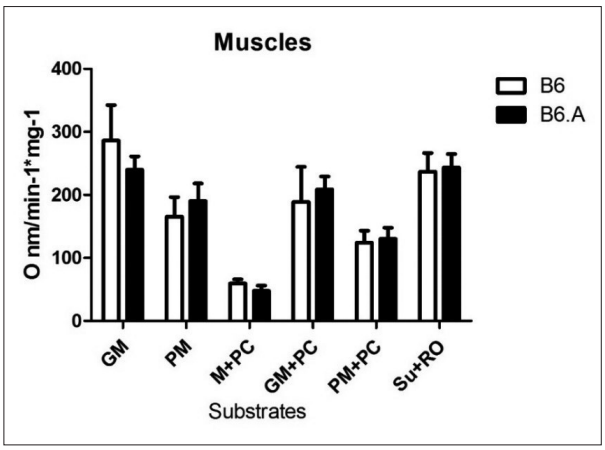

Figure 3. **Comparison of three muscle mitochondria respiration states between B6 and B6.A mice**

Note. The following substrates were used: GM – glutamate plus malate, PM – pyruvate plus malate, M + PC – malate plus palmitoylcarnitine, Su + RO – succinate plus rotenone.

In liver mitochondria, V3 was higher with PM + PC substrates compared to PM substrate in both strains ($p < .05$) (Figure 2). V3 with GM + PC compared to GM without an addition of PC fatty substrate remained constant in both strain and did not differ (B6: GM 82.53 ± 17.62 and GM + PC 72.58 ± 13.38 min^{-1} mg^{-1} protein, respectively; B6.A: GM 79.83 ± 24.14 and GM + PC 75.48 ± 17.38 min^{-1} mg^{-1} protein, respectively). VO2 at

V3 under SU + RO conditions was higher than measured with other substrates in both B6 and B6.A mice liver mitochondria ($p < .05$).

In muscle mitochondria, unlike in liver mitochondria, V3 with PM + PC was lower compared to PM in both mice strain ($p < .05$) (Figure 3). Respiration V3 with GM + PC compared to GM was also lower in both strains, but not statistically significantly. The respiration of SU + RO was high as in liver mitochondria in B6 and in B6.A mice. With mitochondria isolated from muscle, we also noticed a net decline in respiratory chain activity under M + PC condition (low VO_2 rates compared to respiration with other substrates, Figure 3).

DISCUSSION

The main aim of the study was to show if low citrate synthase (CS) activity could improve fatty substrate and carbohydrate oxidation in mitochondria. We studied CS activity and respiration in mitochondria derived from B6 and B6.A mice. B6.A strain carries the A/J allele in the genomic region containing the Cs gene on otherwise B6 strain background. Previous studies showed that A/J mice show 50–65% reduction in muscle tissue derived CS activity compared to other mouse strains despite similar levels of *Cs* mRNA and lack of differences in CS and cytochrome c protein content (Ratkevicius et al., 2010). In agreement with these results, we found ~32% lower mitochondrial CS activity in B6.A mice compared to B6 mice. However, we did not observe any significant difference in mitochondrial respiration between these strains. We measured CS activity without any prior interventions. There is some evidence to suggest that CS activity increases after cycling to exhaustion at 75% of peak O_2 uptake, whereas activities of marker enzymes for fatty acid oxidation (β-hydroxyacyl-CoA dehydrogenase) and glycolysis (phosphofructokinase) were unaffected (Tonkonogi, Harris, & Sahlin, 1997). This suggests that CS activity might vary before and after various interventions.

We did not observe any significant differences in mitochondrial respiration between B6 and B6.A mice with reduced CS activity. Mitochondrial respiration differed significantly in the presence and in the absence of palmitoylcarnitine (PC) which is a fatty substrate. Indeed, carbohydrate oxidation in liver mitochondria was faster with addition of PC (PM + PC) compared to PM alone. Those differences might exist because of a strong link between β-oxidation of fatty substrates and oxidation of carbohydrates in citric acid cycle (Rogge, 2009). In muscle, mitochondrial respiration inhibition with PC can be explained by ANT inhibition (Ciapaite et al., 2006).

In isolated mitochondria oxygen consumption rates and mechanics differ from intact cells. There are several studies which show significant difference in VO_2 of mammals before and after high fat diet (Bourbera et al., 2012). In summary, our study did not reveal any association between reduced CS activity and mitochondria respiration rates in mice under normal conditions without any external interventions, e.g. hyperthermia (Zukiene et al., 2010).

CONCLUSIONS

1. B6.A-(rs3676616-D10Utsw1)/KjnB6 mice have reduced citrate szynthase activity in comparison with C57BL/6J mice.

2. Oxygen consumption rates in liver and muscles mitochondria were similar in B6.A-(rs3676616-D10Utsw1)/KjnB6 and in C57BL/6J mice.

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Corresponding author **Andrej Fokin**
Lithuanian Sports University
Sporto str. 6, LT-44221 Kaunas
Lithuania
Tel. +37060914960
E-mail andfok@stud.lsu.lt

ACTIVITY OF UPPER BODY MUSCLES IN DOUBLE POLING AND SKIERG WORKOUT

Roman Horyna[1], Radka Bačáková[1], Martina Chrástková[1], Jan Sedlák[2], Roman Čmejla[2] , Bronislav Kračmar[1]
Charles University in Prague[1], Prague, Czech Republic
Czech Technical University in Prague[2], Prague, Czech Republic

ABSTRACT

Background. The aim of the study was to compare the involvement of upper body muscles during double poling and SkiErg Concept 2 workout and verify its specificity for cross-country skiing.

Methods. Ten elite Czech cross-country skiers performed double poling and SkiErg workout. Electromyography of selected upper body muscles and cycle characteristics were analysed. To monitor the electrical activity of muscles, we used the device ME6000. Data were analysed using Mega Win and MATLAB software version R2012b.

Results. Relative poling phase during double poling was 30.30 ± 2.02% and during SkiErg workout 54 ± 3.36%. Pre-activation of trunk flexors was significantly higher during double poling due to high and forward body position before pole plant. Pre-activation of trunk flexors was not significantly different as pre-activation of shoulder and elbow extensors during SkiErg workout. Deactivation of these muscles came significantly later during SkiErg workout.

Conclusion. SkiErg cannot be considered a specific training method for cross-country skiing. It can be recommended to obtain specific power, but long-term application may cause disruption of double poling technique, especially timing of trunk flexors, shoulder and elbow extensors.

Keywords: cross country skiing, double poling, SkiErg, upper body, EMG.

INTRODUCTION

Cross-country (XC) skiing is one of the most demanding endurance sports and displays a great variety and multiplicity of performance determinants. There are two basic skiing techniques, the classic style and skating style. Important technique of the classic style is double poling (DP). DP is mainly used on flat and slightly downhill and steep sections of the track, which is achieved at high speed locomotion (Fabre, Balestreri, Leonardi, & Schena, 2010). The importance of DP has increased during the last two decades. It is not only used for mass starts and sprints, but also for long distance (Saltin, 1997; Stöggl, Lindinger, & Müller, 2006). This technique put more emphasis on upper body muscles (Holmberg, Lindinger, Stöggl, Eitzlmair, & Müller, 2005), which requires the necessary level of their specific strength. Cross-country skiers use a wide range of imitation drills due to the absence of snow in summer and sometimes also in winter. SkiErg Concept 2 (SkiErg) is often used for imitation drills in training (Figure 1). SkiErg workout is part of the test battery of the Czech national team in XC skiing. Imitation drills on SkiErg are ranked among semi-specific training methods, as well as roller skiing and other imitation drills (Hottenrot & Urban, 2004).

Several studies have been performed to investigate physiological aspects (Hoffman et al., 1998; Saltin, 1997; Staib, Im, Caldwell, & Rundell, 2000), fewer studies – kinematic and kinetic aspects (Hoffman et al., 1995; Holmberg

Figure 1. **SkiErg "Concept 2"**

et al., 2005; Horyna, Finková, & Kračmar, 2012; Millet, Hoffman, Candau, & Clifford, 1998; Smith, Fewster, & Braudt, 1996) and very few studies-kinesiological aspects (Chrástková, Bačáková, Špulák, Kračmar, & Čmejla, 2012; Chrastkova, Bacakova, Spulak, Cmejla, & Kracmar, 2013; Holmberg et al., 2005; Horyna, Bačáková, Špulák, Kračmar, & Čmejla, 2014; Suchý & Kračmar, 2008; Zoppirolli et al., 2013) of the DP technique. Holmberg et al. (2005) showed that muscles were engaged in sequential order starting with trunk and hip flexors, followed by shoulder extensors and the elbow extensor triceps brachii during DP. They found two DP strategies with different kinematic, kinetic and kinesiological characteristics. Nilsson, Tinmark, Halvorsen, and Arndt (2013) found a similar change in electromyography (EMG) activity while increasing the speed of locomotion or increasing the horizontal resistance during DP. Zoppirolli et al. (2013) suggest that the stretch-shortening cycling effectiveness of the triceps brachii and latissimus dorsi muscles is a major determinant of DP performance.

So far, no study compared the kinesiology aspects and timing activation of relevant muscles during SkiErg workout with DP. Hottenrot and Urban (2004) considered SkiErg as a semi-specific training method, but it was not supported by any biomechanical or kinesiological studies. The aim of the study was to compare the involvement of the upper body muscles during DP and SkiErg workout. Comparing both types of locomotion can clarify the intra-individual level of coordination similarity or difference and specificity of the SkiErg for XC skiing training.

METHODS

Subjects. Ten elite male Czech XC skiers, 18 ± 1.1 years old, 179 ± 6.1 cm, and 68.5 ± 5.8 kg volunteered to participate. All subjects were familiar with SkiErg workout, both as part of their training and in testing. They had a classical pole length 85 ± 2% of body height. All the skiers were fully acquainted with the nature of the study before they gave their written informed consent to participate. The research techniques and protocol were approved by the Ethics Committee of Charles University, Prague, Czech Republic. Parental permission of young athletes under 18 years old is available at the authors upon authorization of the Ethics Committee.

Overall design. Intra-individual comparative analytical study investigated two types of locomotion: DP and SkiErg workout. DP was performed once on a 150 m section of the track with a slight incline of 1°. We analysed 20 cycles for each subject. Probands used his own XC skis adequately prepared for the snow conditions. DP and SkiErg workout were performed with the same intensity at the anaerobic threshold (80–85% of maximum heart rate). Probands have experience with this pace during training. SkiErg drag factor was chosen the same as in testing. For the evaluation of the measured data, the period was selected where the motion stereotype was stabilized. We observed two variables: cycle duration and muscle activity (timing).

EMG measurements. For kinesiological analysis we used EMG method which is non-invasive (De Luca, 1997). EMG measurement was completed with synchronized video recording using two cameras: SONY HDR-SR12 with a maximum video resolution of 1920 x 1080 pixels and frame rate 25 frames per second. For EMG recording we used a mobile device – ME 6000 Biomonitor (Mega Electronics, Kuopio, Finland) with sampling frequency 1000 Hz. This device was carried on athlete's body. Ag/AgCl electrodes

(Medico Lead) were positioned on the belly of each muscle, longitudinally with respect to the underlying muscle fibres, in accordance with standard recommendations.

Electrodes were placed on the following muscles: *m. obliquus externus abdominis dx (OBLe); m. rectus abdominis dx, pars superior (RA); m. pectoralis major dx, pars sternocostalis (PMa); m. triceps brachii dx, caput longum (TRI);* and *m. latissimus dorsi dx (LD).* Laterality was not an observed variable.

The obtained data were analysed using software Mega Win and Matlab. In cooperation with the Faculty of Electrical Engineering of the Czech Technical University in Prague we created an original algorithm for the evaluation of the EMG data.

Each channel of raw EMG signal was individually high-pass filtered (20 Hz, Butterworth, 6-th order) for the elimination of artefacts. Further the EMG signals were rectified and filtered by down-pass filter (20 Hz, Butterworth, 6-th order) for calculation of linear EMG envelope. The cut-off frequency 20 Hz of down-pass filter was used due to the dynamic of movement and accuracy of detection muscle activity in linear envelope. Lower value of cut-off frequency reduced time accuracy of muscle activity detection. The cut-off frequency increase does not improve the detection accuracy and reduces the robustness of detection algorithms.

The boundaries of movement cycles used for the calculation of average EMG envelopes are defined as time positions of consecutive local maxims in manually selected EMG channel. For this purpose, channels with specific EMG activity character are preferred. In each multi-channel EMG record, a channel with one significant peak in movement cycle was selected for the identification of movement cycle boundaries. The identified boundaries of individual movement cycles are used for linear envelope signal segmentation. Signal segments are linearly interpolated to uniform length 1000 points which correspond to 1 second with used sample frequency and are sufficient to the characterization of EMG average envelopes.

The EMG activity detection, respectively the timing of onsets and cessations of muscle contractions identification, is based on thresholding of EMG envelope signal, analogically applied in other research (De Luca, 1997; Hug & Dorel, 2009; Konrad, 2005). The improved threshold algorithm is used for EMG activity detection and is described in detail in the research of Špulák, Čmejla, Bačáková, Kračmar, and Satrapová (2014). The EMG average envelope is utilized in order to improve detection results in combination with threshold detector used for each EMG channel separately. The threshold is set to 25% of difference between peak and minimum envelope value in relevant movement cycle.

The detected EMG activity with synchronously recorded video is obtained. The ground contact and highest position of the hand timing is identified in the video. One DP cycle was defined as the period from the start of the pole ground contact to the start of the subsequent pole ground contact, during SkiErg workout from the highest position of the hand to the subsequent highest position of the hand. This process was used for time normalization to 0 – 100% scale of DP cycle. The EMG activity is converted to relative scale and timing in several DP cycles is analysed.

The study is focused on the involvement of upper body muscles during two types of locomotion. We observed:

a) pre-activation as the average time between the moment of activation of selected muscles and the moment of pole ground contact (respectively the highest position of the hand during SkiErg workout).

b) post-activation as the average time between the moment of pole ground contact (respectively the highest position of the hand during SkiErg workout) and the moment of deactivation of selected muscles.

Cycle time, absolute and relative poling times were determined for each DP and SkiErg workout cycle.

Statistics. A Shapiro-Wilk's test ($p > .05$) and visual inspection of their histograms showed that all of the biomechanical values were approximately normally distributed for all types of locomotion. The data are presented as means ± standard deviations. A one-way analysis of variance ANOVA was conducted to evaluate the influence of locomotion type on the parameters of interest. The independent variable, type of locomotion, involved two groups: double poling and SkiErg workout. Post hoc comparisons to evaluate pairwise differences among locomotion means were conducted with the use of Tukey HSD test.

All statistical analyses were performed using the SPSS 11.0 Software for Windows (SPSS Inc., Chicago, IL, USA) and statistical significance defined as an α value of .05 or less.

RESULTS

Kinematic variables are shown in Table 1. The cycle time was 0.99 ± 0.13 s during DP and 1.19 ± 0.15 s during SkiErg workout. The poling time was 0.30 ± 0.02 s during DP and 0.64 ± 0.04 s during SkiErg workout, corresponding to 30.30 ± 2.02% and 53.8 ± 3.36% of the cycle time. All variables showed a significant difference.

Table 2 shows the values of muscles activation, which are further illustrated graphically (Figure 2). The timeline is transferred from the absolute (s) to relative time (%) due to comparison.

Table 1. **Cycle and poling time characteristics of DP and SkiErg workout**

Parameter	DP	SkiErg	*p*-value
Cycle time (s)	0.99 ± 0.13	1.19 ± 0.15	.004
Poling time (s)	0.30 ± 0.02	0.64 ± 0.04	.000
Relative poling time (%)	30.30 ± 2.02	53.8 ± 3.36	.000

Note. DP – double poling.

Table 2. **Average duration of muscle activity during DP and SkiErg workout (% of average cycle duration)**

Parameter	DP	SkiErg	*p*-value
Pre-activation OBLe	17.17 ± 5.67	7.27 ± 4.90	.001
Post-activation OBLe	18.75 ± 5.68	25.32 ± 4.56	.032
Pre-activation RA	17.3 ± 5.09	5.79 ± 4.01	.000
Post-activation RA	16.39 ± 5.99	25.21 ± 4.22	.001
Pre-activation PMa	5.15 ± 4.51	3.64 ± 4.01	1.000
Post-activation PMa	20.61 ± 7.64	28.74 ± 5.53	.028
Pre-activation TRI	4.84 ± 4.36	2.01 ± 2.36	.194
Post-activation TRI	26.97 ± 5.15	33.08 ± 4.53	.021
Pre-activation LD	1.59 ± 1.00	1.77 ± 1.22	1.000
Post-activation LD	26.53 ± 5.85	33.27 ± 3.87	.009

Note. OBLe – obliquus externus abdominis; RA – rectus abdominis; PMa – pectoralis major; TRI – triceps brachii; LD – latissimus dorsi; DP – double poling.

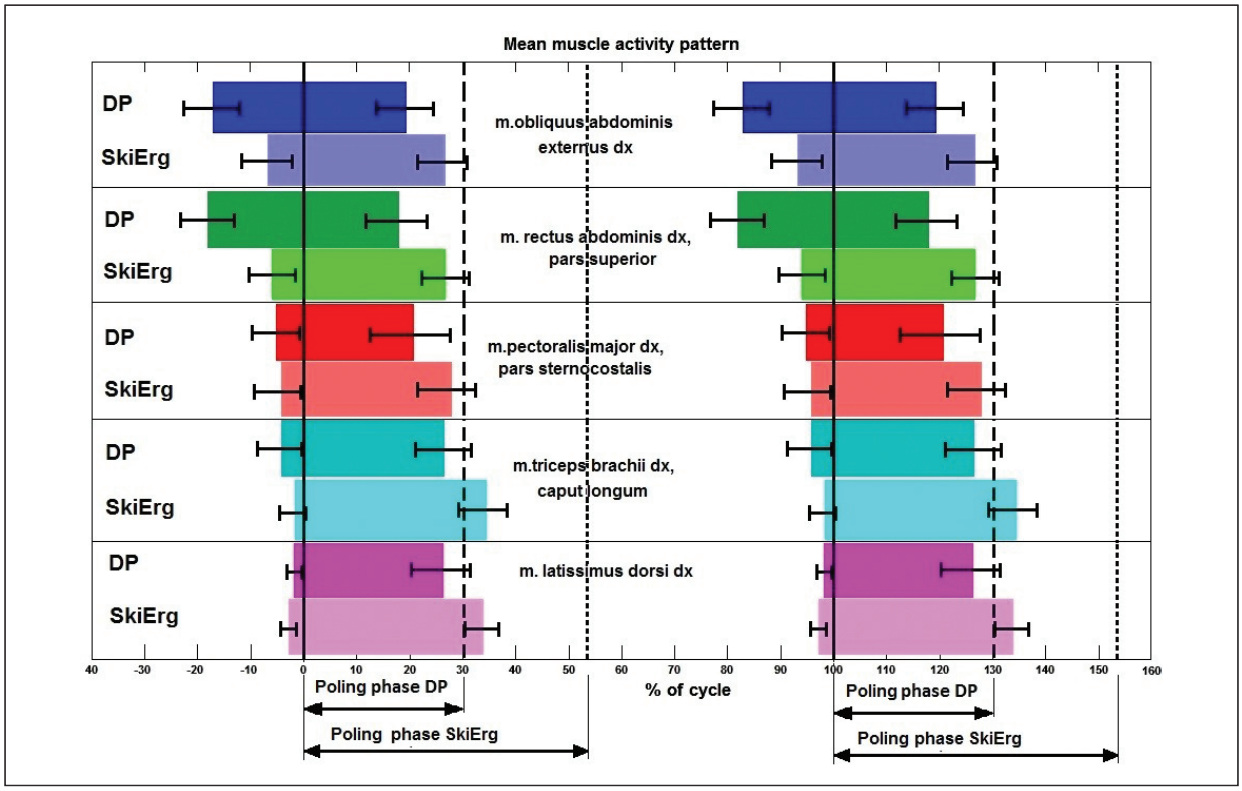

Figure 2. **Relative activation time of selected muscles during DP and SkiErg workout in two average cycles**

Note. The data are mean values. DP – double poling, m. – musculus, dx – dexter.

The abdominal muscles reached considerable pre-activation during DP. OBLe (respectively RA) were activated 17.17 ± 5.67% (respectively 17.3 ± 5.09%) before pole plant. Pre-activation of these muscles during SkiErg workout reached significantly lower values of 7.27 ± 4.90% (5.79 ± 4.01%). They switched off according to a "first in, first out" pattern, 18.75 ± 5.68% (respectively 16.39 ± 5.99%) after pole plant during DP and 25.32 ± 4.56% (respectively 25.21 ± 4.22%) during SkiErg workout.

Pre-activation of PMa as an antagonist with stabilizing function to LD was 5.15 ± 4.51% during DP and 3.64 ± 4.01% during SkiErg workout. The difference was not significant. PMa was switched off by 8.13% later during SkiErg workout than during DP.

Differences between activation of both propulsion muscles TRI and LD during both types of locomotion were not significant. Pre-activation reached values ranging from 1.59 to 4.84%. TRI (respectively LD) was deactivated by 6.11% (6.74%) later during SkiErg workout in comparison to DP.

DISCUSSION

Average cycle time during DP was 0.99 s, which corresponds to the study of Stöggl et al. (2006) with 1.01 s. Holmberg et al. (2005) found 1.13 s, Nilsson et al. (2013) 1.075 s and Zoppirolli et al. (2013) 1.09 s. The above mentioned authors conducted their measurements on the same level of intensity around the anaerobic threshold and the differences may be due to the nature of locomotion. They were measured under laboratory conditions on roller skis "in vitro". Average cycle time during SkiErg workout was 1.19 s, which is 20% longer compared with DP. It is due to longer poling time during SkiErg workout. Average poling time was 0.30 s during DP, which corresponds to the study Holmberg et al. (2005), which also indicate 0.30 s. Nilsson et al. (2013) found 0.32 s, Zoppirolli et al. (2013) found 0.31 s. Stöggl et al. (2006) found the average poling time of 0.51 s with inclination of 3°. We measured the poling time of 0.64 s during SkiErg workout, which is 113% longer compared with DP. There is a critical point during DP, where the hand with the pole reached the highest position with no angular velocity. The body is in a high starting position with distinctly extended hip, knee, and ankle joints and a clear forward shift of the body weight (forward lean). Then begins trunk flexion and angular velocity starts to increase followed by pole plant, in which the skier does not expend force from the zero level as it is during SkiErg workout, where forward lean position does not occur and propulsive phase begins at the moment of the highest position of the hand.

Coordination patterns (muscle sequencing) shown in Figure 2 point to the existence of muscle chains. The first one switched on trunk flexors (RA and OBLe). PMa starts to activate as the second and TRI with LD as the third. Pre-activation difference between the locomotion of these muscles is not significant and reaches a range from 0.18 to 2.83%. They switched off according to a "first in-first out" pattern during DP, which corresponds to the measurements of Holmberg et al. (2005) and Horyna et al. (2014). The first was deactivated trunk flexors (RA and OBLe) followed by PMa, TRI and LD. Holmberg et al. (2005) found later deactivation of TRI compared to LD, which may be caused by longer cycle, when it is time to complete extension of the elbow joint. Zoppirolli et al. (2013) found the pre-activation of the TRI 4.3% during DP, which corresponds to our measurements (4.8%). Pre-activation of the LD was found 5.5%, which does nott correspond to our data (1.59%). Lower pre-activation of the LD compared to TRI was found also by Nilsson et al. (2013). Holmberg et al. (2005) drew similar conclusions as Zoppirolli et al. (2013). They found longer pre-activation of LD compared to TRI. In the same order LD and TRI were deactivated. High activation of shoulder extensors PMa and LD was found by Holmberg et al. (2005). PMa has a double function with extension of the shoulder joint in the first part of poling phase, and a stabilizing function as antagonist to LD.

Significantly higher pre-activation of the trunk flexors RA and OBLe compared to other muscles was during DP. Earlier studies have focused on shoulder and elbow extensors without exploring possible important role of the abdominal muscles (Hoffman et al., 1995; Millet et al., 1998; Smith et al., 1996). Holmberg et al. (2005) and Nilsson et al. (2013) found a high level of EMG activity in RA and OBLe, indicating their important role in DP. They assume that this sequential pattern is involved in creating a low angle in the hip joint during poling, which leads to additional propulsive force.

Pre-activation of trunk flexors RA and OBLe is significantly lower during SkiErg workout due to the missing forward lean position. Trunk flexion occurs simultaneously at the moment of pulling handle of the SkiErg. Therefore the difference

between pre-activation of trunk flexors and other muscles is not significant.

Deactivation of all selected muscles occurs 7–8% later during SkiErg workout compared to DP. Prolonged post-activation of LD, TRI and PMa during SkiErg workout shows the consequences of an artificial instrument for simulating locomotion. The construction of the simulator does not allow creating the timing of poling as on the snow. Poling is longer, looser and its character is directed more towards the isokinetic contraction, while the propulsion effect during DP is based on the explosive muscular work. SkiErg workout has strength-endurance character, whereas DP has explosive-strength-endurance character.

Simultaneous activation timing of the abdominal muscles with other muscles shows again the artificial motion during SkiErg workout. The muscles in the muscle chains on the ventral side of the body do not create an optimal starting position (attitude) in which "punctum fixum" is formed for the work of the main propulsion muscle LD and the main antigravity muscle PMa (corresponding to the character of the movement during DP). Their coordination has the character of generalized movement without differentiation of muscle functions compared to DP with using trunk flexors for creating optimal situation for propulsion and antigravity muscles function of the shoulder girdle.

CONCLUSION

Muscle involvement during DP and SkiErg workout is different. Relative poling phase during DP is 30.3% with explosive-strength-endurance character of performance, while relative poling phase of SkiErg workout is 53.8% with isokinetic muscle contraction and strength-endurance character of performance.

Pre-activation of trunk flexors is significantly higher during DP due to forward lean position of the body, which is missing during SkiErg workout. The trunk flexors are involved in locomotion with minimal advance of the other propulsion muscles, which shows the artificial character of the movement with a lack of muscle function differentiation.

SkiErg cannot be considered as a specific training method for cross-country skiing. It can be recommended to obtain specific power, but long-term application may cause disruption of double poling technique, especially the timing of trunk flexors, shoulder and elbow extensors. Further investigation in this area should focus on kinetic and kinematic aspects when using DP and SkiErg workout.

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Corresponding author **Roman Horyna**
Charles University, Faculty of Physical Education and Sport
José Martího 269/31
162 52 Prague 6 – Veleslavín
Czech Republic
Tel. +420777615904
E-mail romhor@centrum.cz

CHANGES IN THE MINERAL STATUS IN THE ORGANISMS OF YOUNG ATHLETES WITHIN A ONE-YEAR TRAINING CYCLE

Tatiana Krupskaja[1], Ludmila Loseva[1], Marija Pushkareva[1], Olga Tsivunchyk[1], Slavamir Anufrik[1], Kazys Milasius[2], Marija Pečiukonienė[2]
Yanka Kupala State University of Grodno[1], Grodno, Belarus
Lithuanian University of Educational Sciences[2], Vilnius, Lithuania

ABSTRACT

Background. Concentration of trace elements in the hair allows to get an idea how they are taken by the organism over a long period of time and to study relative correlations with different genetic, dietary and environmental factors. Research aim was to identify changes in the macro- and microelement status for young athletes involved in different sports activities depending on the preparation period within one-year training cycle.

Methods. A total of 78 young athletes aged 12–17 years, 32 of which were swimmers (group I), 17 – tennis players (group II) and 29 – Taekwondo athletes (group III) participated in the study. Biological material (hair samples), the volume of 0.1–0.15 g were taken for experimental studies in three periods of time: preparatory, competitive and transition period within a one-year training cycle. Hair samples were analyzed using the method of X-ray fluorescence (XRF) in order to detect multiple elements. For quantitative analysis, 8 chemical elements (sulfur, potassium, calcium, iron, copper, zinc, strontium and selenium) were determined in a single hair sample.

Results and conclusion. A non-invasive method to determine mass fraction of the chemical elements (sulfur, potassium, calcium, iron, copper, zinc, lead, chlorine, bromine, strontium, selenium) in a hair sample is an informative method for assessing the physiological response of the body (young athletes) with physical activity at different stages of training. The statistical analysis of the obtained results revealed the dependence of the concentration of trace elements from a kind of sports activity and stage of preparation for young athletes.

Keywords: chemical elements, X-ray fluorescence analysis, young athletes, preparation stage.

INTRODUCTION

In today's training and competition requirements to the major functional systems of the body of an athlete are extremely high, which can lead to a deep depletion of its functional reserves. Thus, the role of the various tools helping high performance and ensuring efficiency in adaptive processes sharply increased (Jeukendrup & Gleeson, 2010). Sufficient concentration of minerals and trace elements is an important factor for increasing the athlete's performance, efficiency of training and recovery (Benardot, 2000; Wolf & Manore, 2007). Obviously, non-balanced nutrition leads to the certain insufficiency of substances and trace elements along with extremely high physical activities. A matter of fact is that the trace elements are an essential part of many enzyme complexes, hormones and vitamins. Thus, the functioning of almost all regulatory systems of the body depends on the balance of trace elements (Hunt & Groff, 1990; Maughan, 1999). Low concentrations of minerals and electrolytes are involved in the formation of the cytoskeleton of cells; they circulate in enzyme complexes and substances which are responsible for the supply of oxygen. They have a strong influence on the ion balance adjusting the sensitivity of nerve and muscle cells to maintain

the acid-base balance of the organism (Clarkson, 1991, 2000). This all is an essential addition to the solid system of training contributing to more rapid and effective solutions, which, undoubtedly, stimulate and increase sportsmanship (Manore & Thomson, 2000).

Thus, the most important point for a young athlete is to make a right choice of sports activity. There are various methods to determine the most appropriate sports activities, such as rapid tests and techniques, monitoring of functional state of the body, methodology of pedagogical testing, assessment of general physical development, screening of psychosomatic parameters during physical education, etc. It is necessary to maintain a daily balance of vitamins, macro- and microelements in the human body. These substances are involved in the processes of regulation of metabolism and play a significant role in the processes of adaptation to physical stress (Close et al., 2006; Олейник, Гунина, & Сейфулла, 2010). In the recent years, hair sample analysis became wide popular along with the study of blood, plasma and urine in order to determine concentrations of macro- and microelements (Contiero & Folin, 1994; Gordon, 1985; Kuangfei et al., 1999; Paschal, Di Pietro, Philips, & Gunter, 1989). Concentration of the trace elements in the hair shows how they accumulated them in an organism within a long period of time and describes correlations between various factors, such as genetic, nutritional and environmental (Noguchi, Itai, Kawaguchi, Takahashi, & Shinsuke, 2012; Pavlov, Agadzhanian, Alisievich, & Chekhovskikh, 1989; Radomska, Graczyk, Konarski, & Adamowicz, 1991). Nevertheless, concentrations of macro- and microelements in a hair sample depend on the age, sex and place of residence (Afridi, Kazi, Jamali, Kazi, & Shar, 2006; Grabeklis, Lakarova, Eisazadeh, & Skalny, 2011; Sturaro, Parvoli, Doretti, Allegri, & Costa, 1994). Thus, the experimental data definitely show that the concentration of elements in the hair describes the status of all systems of the organism and a single hair sample is first of all, an integral indicator of the mineral status. Furthermore, there is not enough information about the mineral status for young athletes of different sex and age, involved in different sports at various stages of training within a one-year cycle. This study aimed to find solutions to the above mentioned challenges. Purpose of the study was to identify changes in macro- and microelement status for young athletes involved in different sports, depending on the period of training within a one-year training cycle.

METHODS

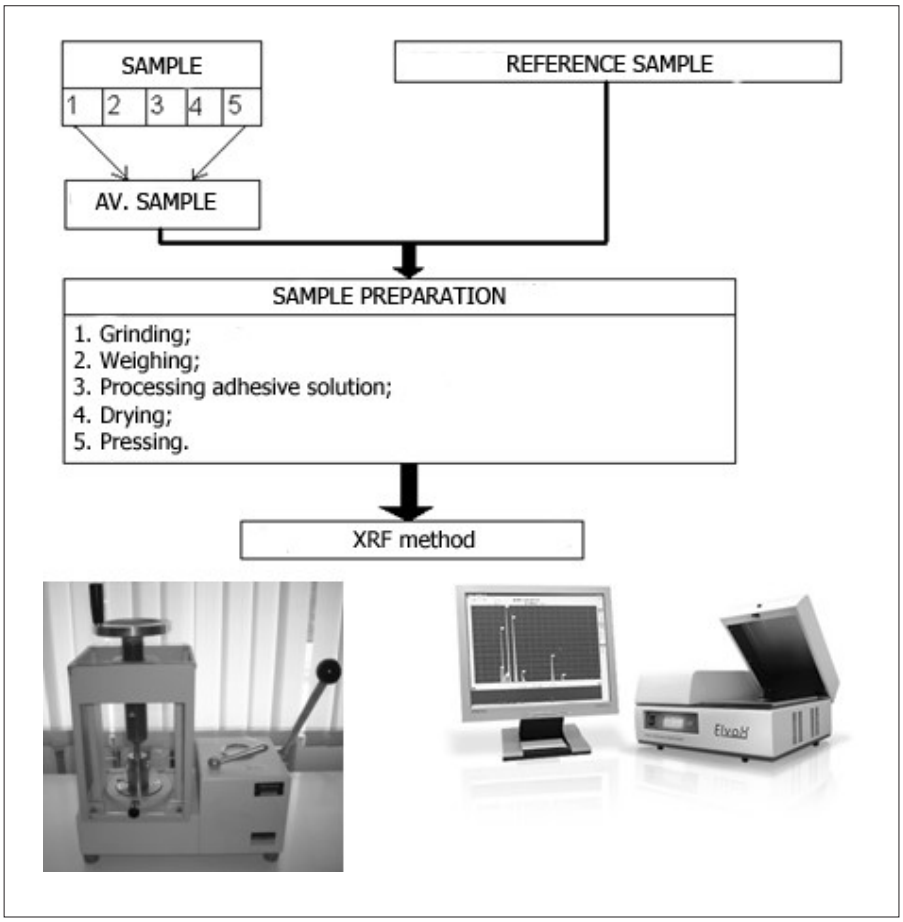
Research participants were 3 groups of young athletes (swimming, tennis and taekwondo) aged 12–17 years. A total of 78 young athletes aged 12–17 years, 32 of which were swimmers (group I), 17 – tennis players (group II) and 29 – Taekwondo athletes (group III). The athletes who participated in the study had chosen sport as a main subject in the educational and sports facilities in Minsk with specific training programs for each sports activity. The selection of the biological material - hair samples, the volume of 0.1–0.15 g were taken in three periods of a one-year training process - in the preparatory, competitive and transition period. Hair multi-element analysis was done by using the method of X-ray fluorescence (XRF), shown in Figure. A single hair sample was used for the quantitative analysis of the concentration of 8 chemical elements (sulfur, potassium, calcium, iron, copper, zinc, strontium and selenium). Obtained results were compared with the references of chemical and biological samples and the average (interquartile range), described by various scientists in population-based studies (Hops, 1977; Qian, Chiao, Wu, & Tian, 1990).

Advantages of the XRF method:

- possible to obtain spectrum review of all elements in one dimension;
- quick information;
- minimum sample preparation without destroying a sample;
- possible to study a samples on different matrices;
- low energy consumption and chemical reagents;
- affordable with low cost of sample analysis.

The obtained data was processed by methods of mathematical statistics. The results of the average (X) described for all parameters in the different groups along with the standard deviation (S). Reliability in differences of element balance in the body of young athletes in different training periods was determined by the *t*-test (Student's *t*-test). Pearsons' correlation analysis of macro- and microelements interrelations was carried out. "Statistica 7.0" program was used for statistical data processing.

Figure. **XRF method. Stages of sample preparation**



RESULTS

Studies of macro- and microelements in the hair of young athletes from different sports showed the changes of elemental balance depending on the stage of preparation (Table). The accuracy of the identified differences in the average of trace elements on the 1st and 2nd stages of preparation in a group of young swimmers was increased for Ca, Cu, Se (significance level of $p < .001$), while K and Fe dropped at the same level of reliability. Also, Zn, S and Sr remained unchanged. The third phase of the study showed an increase of Ca, Cu and Se compared to the first stage of the study as well as Fe compared to the second stage. In the transition period K, Zn, Fe and Sr was lower than in the preparation period. Ca did not increase significantly in the hair of young tennis players during a yearly training ($p > .05$) while, S and Sr showed significant changes ($p < .001$). Concentration of K, Cu and Fe was down to the lower level ($p < .05$). Concentration of Ca was detected as significant increasing the dynamics for taekwondo athletes in the competition; however, in the transitional period the level of this element was the lowest ($p < .001$). The lowest concentration of K, Cu, Fe and S was detected in the competition period. All changes were considered statistically significant. Concentration of Zn was the highest in the competitive period. Concentration of S and Sr was the lowest in the transition period.

Correlations analysis between micro elements for children in different kinds of sports showed certain regularity. Only one correlation (straight average) between the iron and potassium was found for swimmers. For children who play tennis, strontium and selenium correlated with sulfur and zinc correlated with potassium and calcium accordingly. Correlation with potassium was showed as the opposite while with calcium-directly. Such with increasing zinc, concentration of potassium decreased but concentration of calcium increased. Even more correlation highlights found for taekwondo athletes. Sulfur negatively correlated with potassium, calcium, selenium, iron and strontium as: $r = .63$, $r = .68$ and $r = .74$, accordingly.

Table. **Changes in concentration of macro – and microelements in hair samples of young athletes within one-year training cycle (X ± S)**

Sports	Periods of training	Ca	K	Zn	Cu	Fe	S	Se	Sr
Reference µg/g		**550–1700**	**70–170**	**120–200**	**9–30**	**10–30**	**21000–49000**	**0.3–1.2**	**0-3**
Swimmin (n = 32)	Preparatory	1222.5 ± 107.84	184.00 ± 17.46	147.9 ± 4.23	7.6 ± 0.55	20.2 ± 1.93	35514.4 ± 1639.06	0.5 ± 0.05	4.6 ± 0.47
	Competitive	1552.4 ± 103.9	77.4 ± 9.34	146.7 ± 3.81	9.7 ± 0.55	5.2 ± 0.59	35629.7 ± 974.77	1.2 ± 0.45	4.7 ± 0.48
	Transitory	1329.9 ± 73.44	103.8 ± 8.3	139.00 ± 3.55	8.3 ± 0.47	10.6 ± 2.15	33271.1 ± 1537.66	0.6 ± 0.07	3.8 ± 0.21
Significance of changes	t I–II	t = 16.7 $p < .001$	t = 11.8 $p < .001$	t = 1.6 $p > .05$	t = 4.3 $p < .001$	t = 38.4 $p < .001$	t = 0.5. $p > .05$	t = 4.2 $p < .001$	t = 1.8 $p > .05$
	t I–III	t = 5.4 $p < .001$	t = 8 $p < .001$	t = 3.4 $p < .01$	t = 3.7 $p < .01$	t = 25.5 $p < .001$	t = 1.6. $p > .05$	t = 1.4 $p > .05$	t = 2.3 $p < .05$
Tennis (n = 17)	Preparatory	542.6 ± 85.22	293.5 ± 58.27	108.3 ± 6.18	7.9 ± 0.42	21.3 ± 2.28	34328.4 ± 3179.11	0.7 ± 0.06	3.9 ± 0.41
	Competitive	536.7 ± 68.04	271.9 ± 64.74	104.3 ± 6.24	8.0 ± 0.53	9.07 ± 1.25	39558.9 ± 3206.40	0.7 ± 0.08	6.0 ± 0.55
	Transitory	549.1 ± 72.96	258.3 ± 62.86	104.4 ± 7.05	7.3 ± 0.44	11.4 ± 2.05	36518.0 ± 1583.04	0.6 ± 0.06	4.5 ± 0.49
Significance of changes	t I–II	t = 1.3. $p > .05$	t = 0.2. $p > .05$	t = 1.6. $p > .05$	t = 1.5 $p > .05$	t = 27.5 $p < .001$	t = 14.1 $p < .001$	t = 1.0 $p > .05$	t = 35.5 $p < .001$
	t I–III	t = 0.2. $p > .05$	t = 2.6 $p < .05$	t = 1.7 $p > .05$	t = 2.5 $p < .05$	t = 14.9 $p < .001$	t = 3.1 $p < .01$	t = 2.7 $p < .05$	t = 13.2 $p < .001$
Taekwondo (n=29)	Preparatory	585.0 ± 112.02	274.00 ± 39.33	138.2 ± 9.95	8.2 ± 0.40	24.6 ± 2.28	37233.4 ± 2757.97	0.6 ± 0.06	3.7 ± 0.42
	Competitive	616.4 ± 146.24	126.5 ± 23.06	155.3 ± 22.68	7.1 ± 0.47	10.1 ± 0.50	29148.6 ± 1121.24	0.5 ± 0.03	2.7 ± 0.32
	Transitory	394.9 ± 42.6	251.8 ± 36.65	114.5 ± 6.41	7.3 ± 0.44	13.3 ± 1.86	31462.4 ± 1486.36	0.4 ± 0.04	2.6 ± 0.24
Significance of changes	t I–II	t = 2.1 $p < .05$	t = 26.4 $p < .001$	t = 3.1 $p < .01$	t = 13.5 $p < .001$	t = 20.9 $p < .001$	t = 12.1 $p < .001$	t = 4.6 $p < .001$	t = 14.9 $p < .001$
	t I–III	t = 6.5 $p < .001$	t = 0.7 $p > .05$	t = 4 $p < .001$	t = 11.0 $p < .001$	t = 13.7 $p < .001$	t = 11.4 $p < .001$	t = 5.2 $p < .001$	t = 18.8 $p < .001$

DISCUSSION

Calcium is one of the major macro elements. It plays an essential role in bone formation, muscle contraction and mediated neuronal processes; Ca is important for blood clotting and ATP degradation, it regulates the activity of different enzymes. Ca deficiency leads to osteoporosis and weak muscle contraction. Thus, the lack of Ca must be constantly replenished. Paschal et al. (1989) found out that children and teenagers aged 12–14 years had the highest need of calcium. Similar data were obtained by Kozielec, Drybańska-Kalita, Hornowska, and Sałacka (1996) who studied the needs of trace elements for inhabitants of the north of Poland. Our study showed that swimmers had significantly greater concentration of this element in the hair. Potassium is involved in nerve impulse transmission; it supports homeostasis and regulates muscle tonus. Lubkowska (2009) discovered a significant difference of this element in the hair of the Polish students different for women and men. In our study, the highest concentration of potassium (above the reference level) was detected in the hair of tennis and taekwondo athletes. Zinc is a part of enzymes, also involved in the metabolic processes in the synthesis of proteins and nucleic acids. It is a hematopoietic element. Długaszek, Skrzeczanowski, and Kaszczuk (2014) noted that concentration of this element should be higher in the hair of women. Contiero and Folin (1994) concluded that a higher concentration of zinc in the body helps to increase body weight and muscles for athletes. Our research has shown that concentration of Zinc was higher in the hair of young swimmers compared to other athletes. Copper determines growth and hemopoietic processes along with degradation of glycogen and glucose; it enhances lipolysis activity and accelerates the absorption of iron and hemoglobin synthesis. González-Reimers

et al. (2014) showed that the concentration of Cu in the body depended on the degree of fatigue. With more significant fatigue, concentration of Cu strongly decreases. Our research has shown that concentration of Cu was on the lower level of the reference standards for young athletes. Iron is an essential element in the body for oxygen transport and as an integral part of hemoglobin and myoglobin. While it is involved in immune responses, it plays an important role for growth, hematopoiesis and influences enzymatic activity. There are many factors that determine iron deficiency in the body of athletes. Our research has shown that in all our participants there was a lack of this element during the competition period. During the recovery period concentration of Fe increased but has not reached the level of the preparation period. Selenium is an opposite to the degeneration of the fibres, which causes a lack of vitamin E. According to Momcilovic, Moroviic, Prejac, Skalnaya, & Ivicic (2006), deficiency of selenium has a negative influence on muscle activity. The results of our study showed that concentration of Se in the hair of swimmers was the most. Sulfur is part of the protein, amino acids and certain hormones involved in digestion processes. It is as integral part of insulin that helps to regulate blood sugar. Concentration of this element did not exceed the reference limits in all our studies. Strontium is very important for the bone formation, so its concentration is crucial in childhood and adolescence. In our studies the highest level (6.0 ± 0.55 μg / g) of strontium was detected in the hair of tennis players in the competitive period which significantly exceeded the reference limits.

CONCLUSIONS

Based on the results of different groups of young athletes in swimming, tennis and taekwondo in the preparatory, competitive and transition periods for a one-year training cycle, we can draw the following conclusions:

1. Roentgen-fluorescent (XRF) method is a high-specific and an informative tool to determine concentration of macro- and microelements in the hair of young athletes at the different stages of the preparation for the annual cycle. The statistical analysis of the obtained results for chemical elements in the hair of young athletes revealed the related correlation between concentration of the elements, sport activities, the preparation phase and the age. Results of our studies might be useful for optimizing the training process and the nutrition of young athletes.

2. It is shown that concentration of calcium and zinc was significantly higher in the hair of swimmers compared to other athletes. There was a significant decrease of iron in the competitive period for that group of athletes.

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Corresponding author **Kazys Milašius**
Lithuanian University of Educational Sciences
Studentu str. 39, LT-08106 Vilnius
Lithuania
E-mail kazys.milasius@leu.lt

HIGH GROWTH DUMMERSTORF MICE HAVE REDUCED SPECIFIC FORCE OF SLOW AND FAST TWITCH SKELETAL MUSCLE

Petras Minderis[1], Andrej Fokin[1], Aivaras Ratkevičius[1, 2]
Lithuanian Sports University[1], Kaunas, Lithuania
University of Aberdeen[2], Foresterhill, Scotland, UK

ABSTRACT

Background. Mouse strains differ in body and skeletal muscle mass. It is commonly believed that specific force is a constant value irrespective of muscle mass. We hypothesised that excessive muscle hypertrophy might compromise force output.

Methods. We studied force generating capacity and muscle mass of isolated soleus (SOL) and extensor digitorum longus (EDL) muscles in 14–15-week-old males of C57BL/6J, BEH+/+ and DUH mice (n = 7 per strain). In addition, muscles of young (4–5 weeks old, n = 7 per strain) BEH+/+ and DUH mice were also studied. Specific forces were calculated as isometric tetanic force divided by the estimated physiological cross-section area (PCSA) of the muscles.

Results. DUH strain generated lower specific force (p < .01– .001) than both C57BL/6J and BEH+/+ strains in SOL (110 ± 20 vs. 146 ± 28 and 164 ± 8 mN/mm^2, respectively) and EDL muscles (74 ± 18 vs. 101 ± 19 and 95 ± 11 mN/mm^2, respectively). There were no differences between muscles of young and adult mice (p > .05). C57BL/6J and BEH+/+ generated similar specific force.

Conclusions. Our results show that body mass is not associated with reduction in specific force of skeletal muscles in mice. It seems that age did not affect specific force either. However, the heaviest DUH mice had lower specific force in both slow twitch SOL and fast twitch EDL compared to BEH+/+ and C57BL/6J mice. It appears that DUH strain could be a useful model in studying factors limiting specific force of skeletal muscle.

Keywords: muscle hypertrophy, muscles mass, specific force, mice.

INTRODUCTION

Skeletal muscle is the most abundant tissue constituting 40–50% of body mass in vertebrates. Muscle function is positively associated with physical fitness and plays a critical role in health and well-being (Cohen, Nathan, & Goldberg, 2015). Loss of muscle mass and function is observed in ageing, after musculoskeletal trauma as well as in various chronic diseases such as neuromuscular disorders, cancer, diabetes, sepsis and HIV (Cohen et al., 2015).

Muscle mass is to a large extent determined by genetic factors (Pescatello, Devaney, Hubal, Thompson, & Hoffman, 2013). However, only few genes affecting muscle function are known. For instance, dysfunction of *Mstn* gene results in substantial hypertrophy of skeletal muscles in mice and humans (McPherron, Lawler, & Lee, 1997; Schuelke et al., 2004). Identification of genes and physiological mechanisms that mediate their effects on skeletal muscles might help to develop new therapeutic strategies against various conditions associated with muscle dysfunction.

It is often assumed that muscle mass reflects muscle force generating capacity (Jones, Bishop, Woods, & Green, 2008), but neurological factors may interfere with force production in humans as

well (Degens, Erskine, & Morse, 2009). Moreover, specific force or muscle force per physiological cross sectional area (PCSA) tends to be higher in type 2 than type 1 muscle fibres (Bottinelli, Schiaffino, & Reggiani, 1991; Krivickas, Dorer, Ochala, & Frontera, 2011; Stienen, Kiers, Bottinelli, & Reggiani, 1996; Young, 1984). Little effort has been spent in search for other factors modulating specific force. Identification of inbred mouse strains that differ in specific force of isolated muscles excluding neurological influence could be an important initial step, which would facilitate search for the relevant genetic factors and physiological mechanisms.

It has been established that C57BL/6J, Berlin high (BEH+/+) and Dummerstorf high (DUH) strains of mice differ significantly in body and muscle mass (Amthor et al., 2007; Lionikas et al., 2013a). It is often speculated that an increase in pennation angles of muscle fibres can reduce specific force in skeletal muscles showing excessive hypertrophy (Amthor et al., 2007). The main aim of this study was to test the hypothesis that specific muscle strength decreases with increase in muscle mass. We compared specific force in extensor digitorum longus (EDL) and soleus (SOL) muscles in C57BL/6J, BEH+/+ and DUH strains. Skeletal muscles undergo significant growth from young age to adulthood (Agbulut, Noirez, Beaumont, & Butler-Browne, 2003; Gokhin, Ward, Bremner, & Lieber, 2008). Thus we studied skeletal muscles of adult and young mice before they reach adult body and muscle size. This allowed discriminating between effects of mouse strain and mass size of specific muscle force.

METHODS

Animals. All procedures involving mice were approved by the Lithuanian State Food and Veterinary Service (No. 0223 in 2012). All mice were housed in standard cages under the same environmental conditions (12:12 h light-dark cycle at 21–23^{0}C) with ad libitum access to food and water. 14–15 week-old males of C57BL/6J, BEH+/+ and DUH strains were studied. In addition, muscles of young mice (4–5 weeks old) of both large growth strains (BEH+/+ and DUH) strains were also studied in order to examine strain effects on muscle force before the onset of muscle growth. BEH+/+ mice was generated by crossing BEH mice which have dysfunctional *Mstn* gene with the Berlin Low (BEL) strain and then repeatedly backcrossing the offspring to BEH using marker assisted selection for the wild type allele (+) myostatin (Amthor et al., 2007; Lionikas et al., 2013b). Therefore, BEH+/+ mice have normally functioning myostatin as both C57BL/6J and DUH. Animal number in each group was the same (n = 7).

Muscle properties. Mice were euthanized by cervical dislocation and SOL or EDL was excised with 5-0 silk suture tied securely to the proximal and distal tendons. The muscle was then fixed between two platinum plate electrodes in 100 ml Radnoti tissue bath filled with the Tyrode solution (in mM: 121 NaCl, 5 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 0.4 NaH_2PO_4, 0.1 NaEDTA, 24 $NaHCO_3$, 5.5 glucose) that was bubbled with a gas mixture of 95% O_2 and 5% CO_2 at pH 7.4. The bath was maintained at room temperature of ~22–25^{0}C during all experiments. The muscle was suspended vertically in the bath with the proximal tendon attached securely to the lever arm of muscle test system (1200A-LR Muscle Test System, Aurora Scientific Inc., Canada) and distal tendon to an iron hook. Muscle length was increased in steps every 30 s just after delivery of electrical pulse to evoke a twitch contraction. This procedure was continued until twitch force did not increase with the increase in muscle length. The muscle was then photographed with the length scale in the background to assess muscle length with a precision of 0.1 mm. The muscle was kept at this optimal length (L_0) during the assessment of contractile properties. Firstly, single twitch was generated and peak twitch force was measured. Twitch contraction time was assessed as the time from the beginning of the contraction to the peak of twitch force. Twitch half relaxation time was measured as the time taken for force to decline from peak to 50% of peak value. Afterwards, the muscle was subjected to 300 ms (EDL) and 900 ms (SOL) trains of stimuli at 25, 50, 75, 100, 150 and 200 Hz for assessment of peak tetanic force and force-frequency relationship. Specific tetanic force was calculated as peak tetanic force divided by muscle physiological cross-sectional area (PCSA). PCSA was estimated by dividing muscle wet weight by the product of fibre length (L_f), and the density of mammalian skeletal muscle (1.06 g/cm^3) as described previously (Brooks & Faulkner, 1988). L_f/L_0 ratios of 0.45 and 0.70 were used in these calculations for adult mice EDL and SOL respectively, and L_f/L_0 ratios of 0.45 and 0.71 were used for young mice EDL and SOL respectively

(Brooks & Faulkner, 1988). The muscle was freed from tendons, blotted and weighed (Kern, ABS 80-4, Germany) following all the measurements.

Statistical analysis. All analyses were performed using SPSS 20.0 package for Windows. The Shapiro-Wilk test was used to determine the normality of the variables in the strains. Differences between strains were assessed by one-way ANOVA test. LSD test was applied for *post hoc* comparisons. For all statistical tests, the level of significance was set a priori at $p < .05$. All data are presented as means ± *SD*.

RESULTS

Mice body and skeletal muscle morphometric properties are presented in Table. Adult animals of all three strains were significantly different in all these parameters ($p < .05– .001$). DUH strain was heaviest and had largest muscles compared to other two strains ($p < .001$). BEH+/+ mice were intermediate by these parameters between C57BL/6J and DUH. Muscle contribution to the overall body mass differed between these strains. C57BL/6J mice had larger ($p < .05–.001$) ratios of muscle to body weight than BEH+/+ and DUH, especially for SOL. According to this parameter, muscle contribution to body weight was smallest in BEH+/+ mice while DUH strain showed intermediate values between BEH+/+ and C57BL/6J strains. Other morphometric parameters such as muscle and fibre length as well as PCSA were associated with the body size as mice differed significantly in body mass. Young mice of both larger strains (BEH+/+ and DUH) were selected by body weight to match the C57BL/6J strain. They did not differ in this parameter, but the rest morphometric parameters like weight and PCSA of muscles were lower ($p < .05–.001$) compared to C57BL/6J strain.

Table. **Morphometric properties of soleus (SOL) and extensor digitorum longus (EDL) muscle in C57BL/6J, BEH+/+ and DUH strains of mice**

	Young		Adult		
	BEH+/+	**DUH**	**C57BL/6J**	**BEH+/+**	**DUH**
SOL properties					
BW (g)	25.0 ± 1.9***	27.1 ± 2.3***	26.4 ± 0.7	51.8 ± 4.0†††	77.6 ± 9.2†††‡‡‡
MW (mg)	6.1 ± 0.6***†††	7.5 ± 0.9***††	10.5 ± 0.9	13.5 ± 1.1††	25.0 ± 3.6†††‡‡‡
MW/BW (%)	0.024 ± 0.002†††	0.027 ± 0.003**†††	0.040 ± 0.004	0.026 ± 0.003†††	0.032 ± 0.003†††‡‡‡
L_0 (mm)	11.3 ± 0.7***†††	10.9± 0.6***†††	13.0 ± 0.7	14.3 ± 0.4†††	15.1 ± 0.4†††‡
L_f (mm)	8.0 ± 0.5***†††	7.8 ± 0.5***†††	9.1 ± 0.5	10.0 ± 0.3†††	10.6 ± 0.3†††‡
PCSA (mm^2)	0.71 ± 0.06***†††	0.90 ± 0.08$^{***\dagger\#}$	1.10 ± 0.11	1.31 ± 0.12†	2.23 ± 0.29†††‡‡‡
EDL properties					
BW (g)	25.2 ± 1.8***	25.7 ± 0.9***	25.8 ± 1.5	49.1 ± 3.7†††	80.5 ± 6.2†††‡‡‡
MW (mg)	9.0 ± 0.4***†	9.4 ± 0.6***†	10.7 ± 0.5	14.7 ± 1.0†††	30.7 ± 2.4†††‡‡‡
MW/BW (%)	0.037 ± 0.00***†††	0.036 ± 0.003††	0.042 ± 0.003	0.030 ± 0.002†††	0.038 ± 0.003 †‡‡
L_0 (mm)	13.6 ± 0.8	13.4 ± 0.7***	13.3 ± 0.5	14.2 ± 0.8†	16.9 ± 0.7†††‡‡‡
L_f (mm)	6.1 ± 0.4	6.0 ± 0.3***	6.0 ± 0.2	6.4 ± 0.3†	7.6 ± 0.3†††‡‡‡
PCSA (mm^2)	1.39 ± 0.12***†	1.48 ± 0.13***†	1.69 ± 0.13	2.17 ± 0.17†††	3.81 ± 0.38†††‡‡‡

Note. Values are means ± *SD*; BW, body weight; MW, muscle weight; MW/BW, muscle weight to body weight ratio; L_0, optimal muscle length; L_f, optimal fibre length; PCSA, physiological muscle cross-sectional area. $^{**}p < .01$, $^{***}p < .001$ vs. adult mice of the same strain; $^{\dagger}p < .05$, $^{\dagger\dagger}p < .01$, $^{\dagger\dagger\dagger}p < .001$ vs. C57BL/6J, $^{\ddagger}p < .05$, $^{\ddagger\ddagger\ddagger}p < .001$ vs. BEH+/+; $^{\#}p < .05$ vs. young BEH+/+.

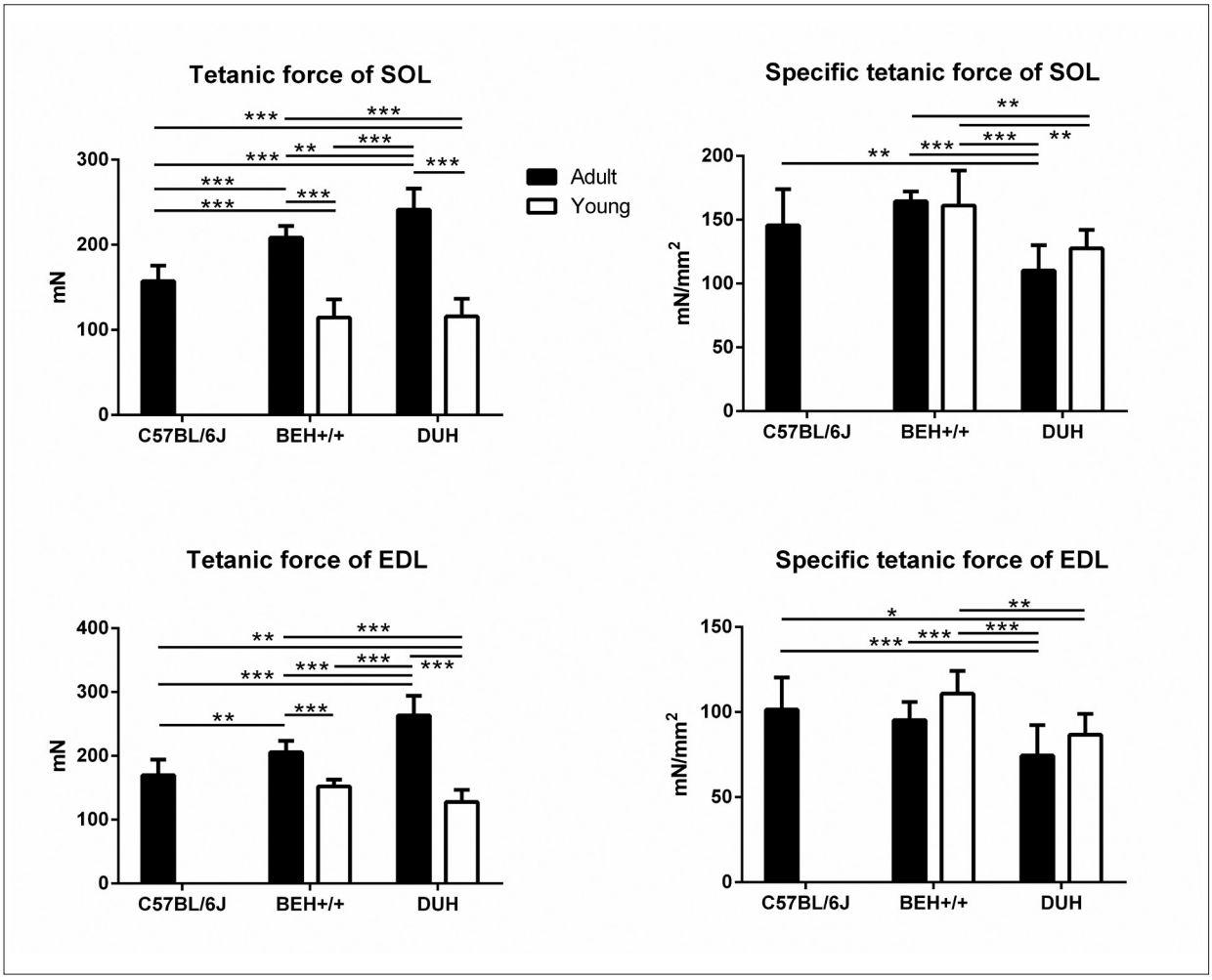

Figure 1. **Tetanic force generation capacity of soleus (SOL) and extensor digitorum longus (EDL) muscles for C57BL/6J, BEH+/+ and DUH strains**

Note. Values are means ± *SD*. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, respectively.

Data on contractile properties of the skeletal muscle are presented in Figure 1. The positive relationship between skeletal muscle size and their peak tetanic force was observed so that muscles of larger strains were stronger. Thus, SOL and EDL muscles of DUH mice were strongest ($p < .01– .001$) than muscles of both BEH+/+ and C57BL/6J mice, and SOL and EDL muscles of BEH+/+ mice were stronger ($p < .01– .001$) than muscles of C57BL/6J mice. However, there were differences between the strains when peak tetanic force was normalized to PCSA in order to calculate specific tetanic force. DUH strain had lowest ($p < .01– .001$) specific tetanic force for both SOL and EDL compared to other two strains. Young DUH mice also showed lower specific force ($p < .05– .01$) than the other strains for the both muscles. There were differences neither between C57BL/6J and BEH+/+ strains nor between young and adult mice of the same strain. The tendency toward increments of this parameter in young vs. adult mice was observed.

We have also assessed twitch speed as twitch contraction time and half relaxation time (Figure 2). Contraction time and half relaxation time of SOL and EDL muscles did not differ significantly between adult strains. Twitch speed, especially when assessed by half relaxation time, tended to be increased in young mice compared to adult mice. This was the most apparent on in young DUH mice which differed ($p < .05– .001$) from the other strains in these properties.

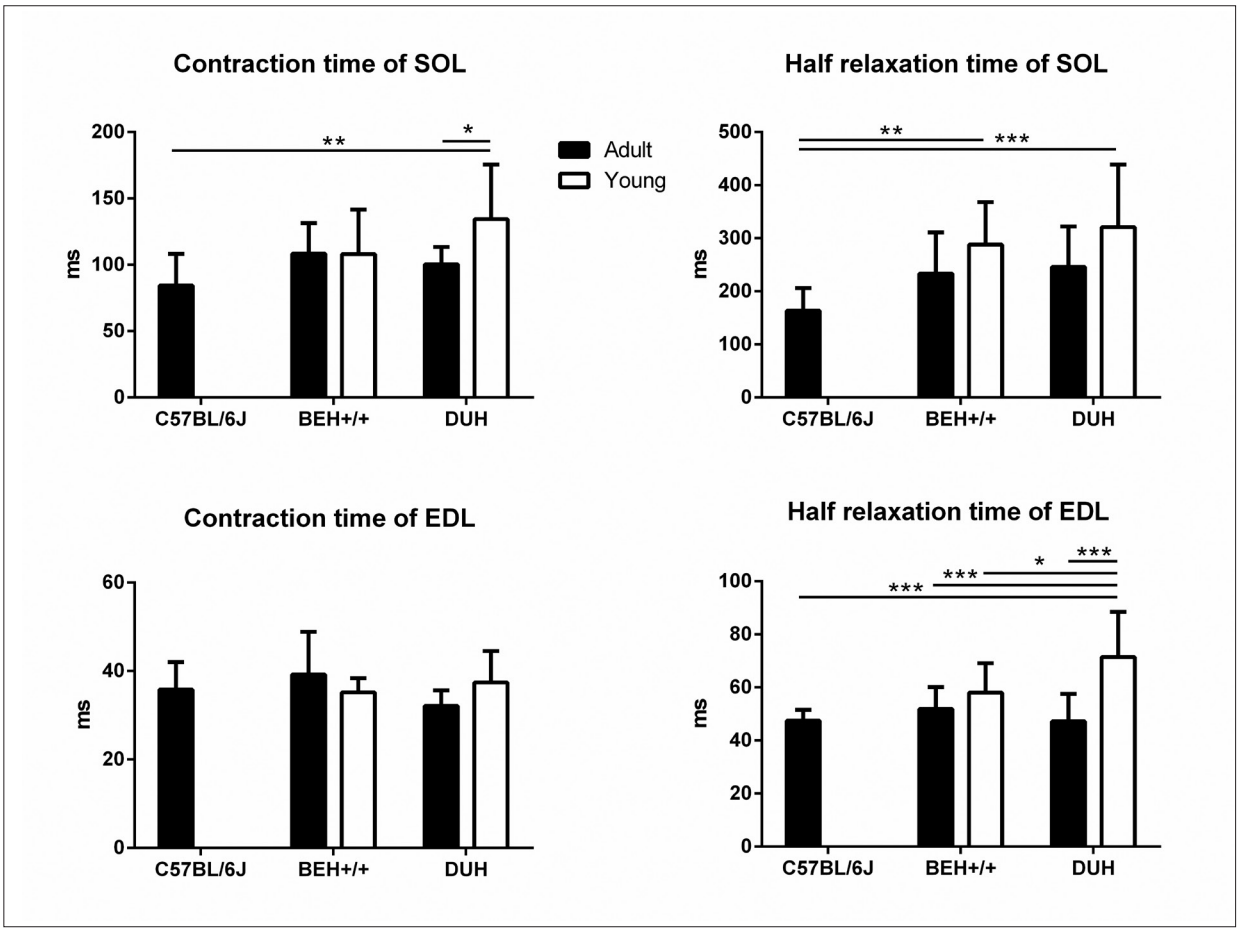

Figure 2. **Twitch velocities of soleus (SOL) and extensor digitorum longus (EDL) muscles for C57BL/6J, BEH+/+ and DUH strains**

Note. Values are means ± *SD*. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, respectively.

DISCUSSION

The main aim of the study was to test the hypothesis that increase in body and muscle mass is associated with reduction in specific force of skeletal muscles in mice. The results of the study do not agree with this hypothesis. C57BL/6J and BEH+/+ mice did not differ in specific force of SOL or EDL despite two-fold differences in body mass. Specific muscle force was also similar in young and adult mice which differed several fold in body mass. Interestingly, however, the heaviest DUH mice had reduced specific force in both SOL an EDL.

We studied C57BL/6J mice which had similar body mass as many other inbred strains (Reed, Bachmanov, & Tordoff, 2007) and compared this strain with two high growth strains, i.e. BEH+/+ and DUH strains. The BEH+/+ and DUH strains were generated by selection for muscularity and/or body weight score over more than 30 generations (Bünger et al., 2004). These strains were referred to as strains with large protein accretion and thus with large muscle mass (Varga et al., 1997; Bünger et al., 2004). Muscle of BEH strain is associated with the Compact mutation in the *Mstn* gene which contributes to doubling mass of most muscles (Varga et al., 1997; Amthor et al., 2007; Minderis et al., 2015). Myostatin factor was eliminated in this study as BEH+/+ mice with the wild type allele were examined. This was done to eliminate specific effects of myostatin gene which has been associated with low specific force in mice (Minderis et al., 2015). Interestingly, however, muscle mass to body mass ratio of both BEH+/+ and DUH was lower than in C57BL/6J mice. Thus, enlargement of tissues other than skeletal muscles is probably of major importance in determining body mass of BEH+/+ and DUH mice.

We did not find any differences in specific force between young and adult mice. Our hypothesis

was that adult mice would show lower specific force than young mice. Muscle hypertrophy leads to a decrease in specific force if it associated with dramatic increase in muscle fibres diameter without increase in attachment area for muscle fibres at the myotendinous junction (Degens, 2012). Mice undergo significant increase in muscle mass in the process of maturation. It could be that this is matched by an increase in attachment area for muscle fibres so that pennation angles of muscle fibres do not change significantly in spite of dramatic changes in muscle mass. These assumptions are supported by in vivo human studies which show that maturation has no effect on architecture of muscle fibres (Morse et al., 2008; O'Brien, Reeves, Baltzopoulos, Jones, & Maganaris, 2010). In addition, prepubertal children and adults have the same specific force of quadriceps (O'Brien et al., 2010). Interestingly, however, specific force of gastrocnemius was higher by 21% in pubescent boys compared to adult men (Morse et al., 2008). This was not due to changes in moment arm length, muscle architecture or antagonist coactivation and the authors attributed these findings to possible discrepancies in measuring PCSA between boys and men. In our case, however, there might be genetic differences in muscle architecture between the mouse stains. These genetic effects could explain low specific force in DUH mice as muscle fibre pennation angle is an important determinant of muscle force output (Ikegawa et al., 2008; Kawakami, Abe, Kuno, & Fukunaga, 1995).

In addition to body and skeletal muscle mass, muscle fibre-type composition is also determined by genetic factors. Lionikas et al. (2013 a) showed that distinct strains differing in muscle mass also differ in muscle fibre-type composition. DUH mice have twice as many type 1 fibres than C57BL/6J (64 ± 11 vs. 31 ± 2%) in SOL while BEH mice with the homozygous Compact allele in *Mstn* gene had 35 ± 2% type 1 fibres (Lionikas et al., 2013a). A constitutive myostatin knock out results in an approximately 20% increase in the relative content of type 2 fibres at the expense of type 1 fibres in SOL (Girgenrath, Song, & Whittemore, 2005). Thus it is likely that BEH+/+ mice with wild type myostatin might be intermediate by proportion of type 1 fibres between C57BL/6J and DUH mice. Twitch speed measurements, especially half relaxation time, suggest that SOL of C57BL/6J is faster than in the other two strains. This might suggest that lower specific force in DUH is somehow associated with greater amount of slow type 1 fibres (Bottinelli et al., 1991; Krivickas et al., 2011; Stienen et al., 1996; Young, 1984). However, this explanation can hardly be valid for EDL which contains only negligible quantities of type 1 fibres.

It is also possible that contractile protein levels are lower in DUH mice compared to BEH+/+ and C57BL/6J mice. The average myonuclear domain (MND) is larger in myostatin-deficient mice which show excessive muscle hypertrophy compared to wild type mice (Qaisar et al., 2012). Muscle fibres with large MNDs show lower levels of contractile proteins (Qaisar et al., 2012). MND is not fixed and can increase in response to growth stimulus (Van der Meer, Jaspers, & Degens, 2011). Qaisar et al. (2012) have also proposed a hypothesis about a size threshold for MND beyond which muscle fibres are not able to maintain adequate myofibrillar protein levels and number of functioning cross-bridges. According to Lionikas et al. (2013a) DUH mice has a larger CSA of both type 1 and 2A fibres than C57BL/6J strain in SOL and larger CSA of type 2A but not type 1 fibres in SOL than myostatin-deficient BEH which demonstrates lower specific force. As isoforms of type 2 fibres are in both SOL (2A) and EDL (2B, 2X and very few 2A) muscles in considerable amounts they could be as a potential candidate for lower specific force due to enlarged MNDs in type 2 fibres. The drawback of this theory is that it cannot explain why young DUH mice with 3-fold smaller muscle mass (i.e. significantly smaller muscle fibres) and therefore supposedly smaller MNDs compared to adult counterparts have lower specific force as well.

One might argue that it is not reliable to compare skeletal muscle function between muscles of young mice with ongoing developmental hypertrophy and mature muscles of adult mice. We did not find any studies where force generation capacity was compared between young and adult mice of DUH and BEH+/+ strains. However, 2–3 month old C57BL/6J mice did not differ from 9–10 month mice in specific force of SOL and EDL (Brooks & Faulkner, 1988). Approximately 2-fold and 3-fold increase in skeletal muscle mass was observed in the BEH+/+ and DUH strains from the age of 4–5 weeks to 14–15 weeks in our study. Several studies show that skeletal muscles of ~1 month old mice are already displaying all characteristics typical for mature muscle (Agbulut et al., 2003, Gokhin et al., 2008). During the first days after birth skeletal muscles of mice have lower

density of contractile material and show different fibre type composition, but then catch up with adult muscle within several weeks. Following a period of 21 days after birth SOL and EDL muscles display a sequential transition from embryonic to neonatal and eventually to adult myosin heavy chain (MyHC) isoforms though few differences in a proportion of adult MyHC isoforms in muscles was still remaining after 21 days (Agbulut et al., 2003). Gokhin et al. (2008) demonstrated a robust increase in a myofibrillar packing from 48 to 93% in mice tibialis anterior muscle fibres with an accompanying increase in maximum isometric tension to a 6-fold following a period of 28 days postnatal. Collectively, this evidence suggests that young mice of 28–35 days as in our study should demonstrate a force generation capacity comparable to adult mice. Small differences in contraction time and half relaxation time between young and adult mice herein might be associated with still unfinished transition processes in composition of MyHC isoforms.

There are also methodological issues to consider when assessing specific force. Some investigators calculate muscle force relative to a muscle mass while others normalize force to muscle CSA. The calculations of specific force by dividing the absolute force with muscle mass are common in studies where muscle lengths are similar between the animal groups. In our case, however, there were significant differences in muscle length between strains and age groups. It appears that differences in muscle length have to be accounted when comparing muscles mass of mice in our study. This involved calculation of muscle CSA. There are two different methods for assessment of muscle CSA. Some investigators calculate an anatomical CSA (ACSA) while others estimate the physiological CSA (PCSA). It appears that PCSA provides a better reflection of muscle force generating capacity since skeletal muscles differ in the length of muscle fibres as the ratio of fibre to muscle length (L_f/L_0) is 0.70 and 0.45 for SOL and EDL of C57BL/6J mice, respectively (Brooks & Faulkner, 1988). We used these ratios to evaluate a fibre length indirectly for a PCSA calculation in all three strains as we were not able to find any information about fibre to muscle length ratios for BEH+/+ and DUH mice. Thus, an applied ratio if it is inaccurate might result in an underestimation or overestimation of a real PCSA of these strains. However, we suppose that if there are any differences in a fibre to muscle ratio between C57BL/6J and larger strains it should not be dramatic. Indeed, in SOL muscle of the ICR mice strain weighing 40–50 g (very similar to the BEH+/+ mice) showed L_f/L_0 ratio of 0.71 (Choi & Widrick, 2009; Widrick & Barker, 2006). EDL muscles of WI/HicksCar rats, which are significantly larger animals than mice, showed L_f/L_0 ratio of 0.4 which is also similar to C57BL/6J mice (Carlson & Faulkner, 1998).

Another methodological issue may concern viability of muscles differing in size during an ex vivo procedure. As muscles of the DUH strain are much thicker it might be argued that deeper fibres of these muscles are affected by hypoxia. Segal and Faulkner (1985) demonstrated that ex vivo SOL and EDL (~70–90 mg) of rats show good contractile performance over significant periods of time when incubated as in our experiment using temperatures not exceeding 25 ^{0}C. Moreover, such temperature ensures an adequate O_2 diffusion and similar to fresh muscles glycogen content.

CONCLUSIONS

Results of the study do not agree with the hypothesis that increase in body mass is associated with reduction in specific force of skeletal muscles in mice. Interestingly, however, the heaviest DUH mice had reduced specific force in both SOL an EDL. It appears that this mouse strain could be an interesting model in studying factors limiting specific muscle force.

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Corresponding author **Petras Minderis**
Lithuanian Sports University
Sporto str. 6, LT-44221 Kaunas
Lithuania
Tel. +37060467644
E-mail petras.minderis@lsu.lt

MUSCLE WASTING AFTER 48 HOURS OF FOOD DEPRIVATION DIFFERS BETWEEN MOUSE STRAINS AND IS PROMOTED BY MYOSTATIN DYSFUNCTION

Petras Minderis[1], Indrė Libnickienė[1], Aivaras Ratkevičius[1, 2]
Lithuanian Sports University[1], Kaunas, Lithuania
University of Aberdeen[2], Aberdeen, Scotland, UK

ABSTRACT

Background. Genetic factors play an important role in determining muscle mass. Indeed, myostatin dysfunction is associated with a pronounced muscle hypertrophy. The aim of our study was to test the hypothesis that starvation induced muscle wasting differs between BEH+/+ and C57BL/6J strains of mice and myostatin dysfunction prevents muscle wasting in BEH strain.

Methods. 18-week-old males of C57BL/6J, BEH+/+ and BEH were subjected to 48 h food deprivation (FD). C57BL/6J mice were representatives of classic mouse strain. BEH mice which differ from BEH+/+ mice by Compact mutation in the *Mstn* gene represented a model for myostatin dysfunction. All mice were divided into experimental and control groups. The control groups consisted of mice fed ad libitum. Seven mice were studied in each group. Mice were weighed before as well as 24 h and 48 h after FD which was followed by dissection and weighing of the hindlimb skeletal muscle.

Results. BEH and BEH+/+ mice showed a similar (16.9 ± 1.4% vs. 19.3 ± 2.4%, $p > .05$) loss of body mass while loss of body mass in C57BL/6J mice was the greatest (24.8 ± 1.9%, $p < .001$) after FD. The loss of muscle mass was significant in both BEH ($p < .001$) and C57BL/6J ($p < .01$) mice, but it was below the level of significance ($p > .05$) in BEH+/+ mice.

Conclusions. Myostatin dysfunction promotes muscle atrophy after FD. During short periods of FD, BEH+/+ mice are more resistant to body and muscle loss compared to C57BL/6J mice.

Keywords: myostatin deficiency, food withdrawal, starvation, muscle atrophy.

INTRODUCTION

Skeletal muscle mass comprises 40-50% of body mass and is associated with health and well-being of humans (Wolfe, 2006). On the other hand, muscle wasting is often a consequence of many chronic diseases which have a deteriorating effect on the quality of life (Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013). Dietary manipulations which are often associated with a caloric restriction can also cause a decrease in both body and muscle mass (Matsakas et al., 2013). Extent of muscle wasting depends on the magnitude of caloric restriction and food deprivation (FD) or fasting causes the most significant muscle wasting (Collins-Hooper et al., 2015). There is a significant amount of evidence suggesting that caloric restriction has a beneficial effect on health as judged by changes in body composition and lipoprotein profile (Anderson & Weindruch, 2012). However, the major concern is muscle wasting during those dietary manipulations. It would be beneficial to design strategies to prevent or reduce loss of muscle mass during fasting or caloric restriction. This is especially important in view of the fact that malnutrition and starvation are common amongst the ill, venerable and ageing (Elia & Stratton, 2000). Indeed, age related loss of muscle mass might exaggerate the negative effects of caloric restriction on skeletal muscles (Yanai, 2015).

Myostatin, which belongs to TGF-β superfamily of secreted growth factors, is a negative regulator of skeletal muscle mass (McPherron, Lawler, & Lee, 1997). Mammalian species with a constitutive myostatin knockout (KO) show pronounced muscle hypertrophy (McPherron et al., 1997, Schuelke et al., 2004). Inhibition of myostatin in adult mice can also increase muscle mass (Whittemore et al., 2003; Personius et al. 2010), since myostatin inhibition increases protein synthesis and decreases protein breakdown in skeletal muscles (Lipina, Kendall, McPherron, C., Taylor, & Hundal, 2010; Schiaffino et al., 2013). Myostatin expression is upregulated in many pathological conditions leading to muscle wasting (Costelli et al., 2008; Gonzalez-Cadavid et al., 1998; Gruson, Ahn, Ketelslegers, & Rousseau, 2011; Plant et al., 2010). Thus myostatin inhibition might be a useful intervention protecting skeletal muscle from atrophy under various pathological conditions and ageing. In fact, myostatin inhibition leads to partial improvements in muscle mass and function of *mdx* mice, a model for Duchenne muscle dystrophy (Bogdanovich et al., 2002). Evidence about effects of myostatin inhibition on skeletal muscles during various catabolic states is sparse and ambiguous. It appears that myostatin KO prevents from glucocorticoid-induced muscle atrophy (Gilson et al., 2007) but not from muscle wasting after hindlimb suspension (McMahon et al., 2003).

It is unclear if myostatin inhibition could prevent from muscle wasting during FD. The results of a few studies in this area are contradictory (Allen, Cleary, Lindsay, Loh, & Reed, 2010; Collins-Hooper et al., 2015). Both of these studies used C57BL6/J mice. However, effects of myostatin inhibition are likely to be strain dependent as mouse strains vary significantly in body mass, muscle mass as well as muscle fibre composition (Lionikas et al., 2013a). Berlin high (BEH) strain was generated after breeding mice for protein accretion over more than 30 generation (Bünger et al., 2004). It is likely that this strain carries several gene variants which favour accretion of muscle mass. Physiological mechanisms, which are triggered by these gene variants, might interact with myostatin in a different way than in C57BL/6J strain. Thus, it is important to study effects of myostatin inhibition using various mouse strains.

Our primary aim was to test a hypothesis that myostatin dysfunction prevents loss of muscle mass during FD. We compared muscle wasting in BEH strain with dysfunctional myostatin and BEH+/+ strain which carries the functional myostatin. The secondary aim of our study was to examine effects of genetic background on muscle wasting during FD. Thus we compared C57BL/6J and BEH+/+ mice with preserved myostatin function as is the case for many other strains of mice.

METHODS

Experimental animals. All experimental procedures involving mice were approved by the Lithuanian Republic Alimentary and Veterinary Public Office (no. 0223 in 2012 and no. 10 in 2014). 18 week-old males of BEH+/+, BEH and C57BL/6J were used. BEH mice carry MstnCmpt-dl1Abc (Compact; Cmpt) mutation in both *Mstn* alleles (Varga et al., 1997). This mutation causes 12-bp deletion in the *Mstn* gene sequence. As a result, the BEH mice are lacking the functional myostatin (Amthor et al., 2007; Lionikas et al., 2013a, b). BEH+/+ mice with normal myostatin function were generated by crossing BEH mice with the Berlin Low (BEL) strain and then repeatedly backcrossing the offspring to BEH using marker assisted selection for the functional myostatin allele (+) (Amthor et al., 2007; Lionikas et al., 2013b). The breeding pairs of C57BL/6J mice were obtained from the Jackson laboratory (USA) whereas the breeding pairs of the BEH and BEH+/+ were a generous gift of prof. Lutz Bünger.

Before experiments mice were bred and housed in the animal facilities of Lithuanian Sports University. They were kept in standard cages, one to five individuals per cage at a temperature of 20–21ºC and 40–60% humidity with the normal 12/12-h light/dark cycle reversed. Animals were fed standard chow diet (58% kcal from carbohydrate, 28.5% kcal from protein, 13.5% kcal from fat; LabDiet 5001, Saint Louis, USA) and received tap water ad libitum.

Experimental protocol. Two mice of the same strain and similar body weight were assigned either to the control or FD intervention, respectively. The control mouse was provided with ad libitum access to food and water. The FD mouse had ad libitum access to water, but did not receive any food for 48 h. Mice were weighed at 0 h, 24 h and 48 h of the intervention (Kern 440–45N, Germany). Seven pairs of mice from each of three strains (BEH+/+, BEH and C57BL/6J) were studied.

At the end of the experiment the mice were euthanized by the exposure to CO_2. Immediately afterwards, the heart and the skeletal muscles including gastrocnemius, plantaris, soleus, tibialis anterior and extensor digitorum longus were dissected and weighed (Kern, ABS 80-4, Germany). Before weighing the muscles were freed from all visible tendons and blotted dry rapidly on filter paper.

Muscle weights were assessed with a precision of 0.1 mg. The skeletal muscle mass was calculated as a sum of masses of all five dissected muscles.

Statistical analysis. All analyses were performed using Prism 6.0 and SPSS 20 software for Windows. All data were tested for normality using the Shapiro-Wilk test. Control and fasted mice were compared with unpaired Student's t-test. One-way ANOVA with Bonferroni's post hoc test was used for comparing strains. For all statistical tests, the level of significance was set a priori at $p < .05$. All data are presented as means ± *SD*.

RESULTS

Data on body mass of mice are presented in Figure 1. Before experiments, BEH mice were heavier by 16% ($p < .001$) than BEH+/+ mice while C57BL/6J mice were approximately two fold ($p < .001$) lighter than both BEH+/+ and BEH mice. FD induced a decrease ($p < .001$) in body mass of all three strains. The loss of body mass was greater during the initial 24 h period of FD compared to the subsequent 24–48 h period ($p < .001$).

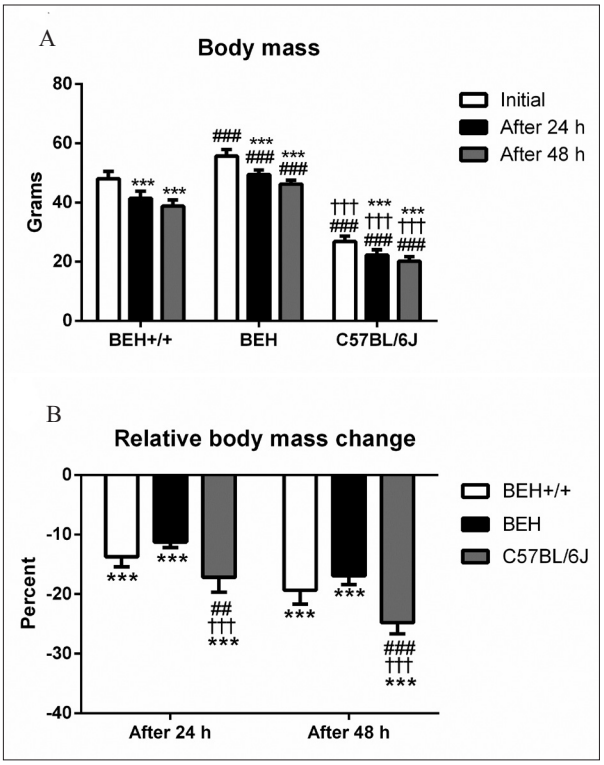

Figure 1. **(A) Body mass as well as (B) percentage change in body mass of BEH+/+, BEH and C57BL/6J mice after 24 and 48 h of food deprivation (FD)**

Notes. The relative change in body mass for FD mouse was calculated using the data of the control mouse that initially was matched by weight and belonged to the control group. Values are means ± *SD*; ***$p < .001$ vs. initial or previous time point, respectively; $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ vs. BEH+/+; $^{\dagger\dagger\dagger}p < .001$ vs. BEH.

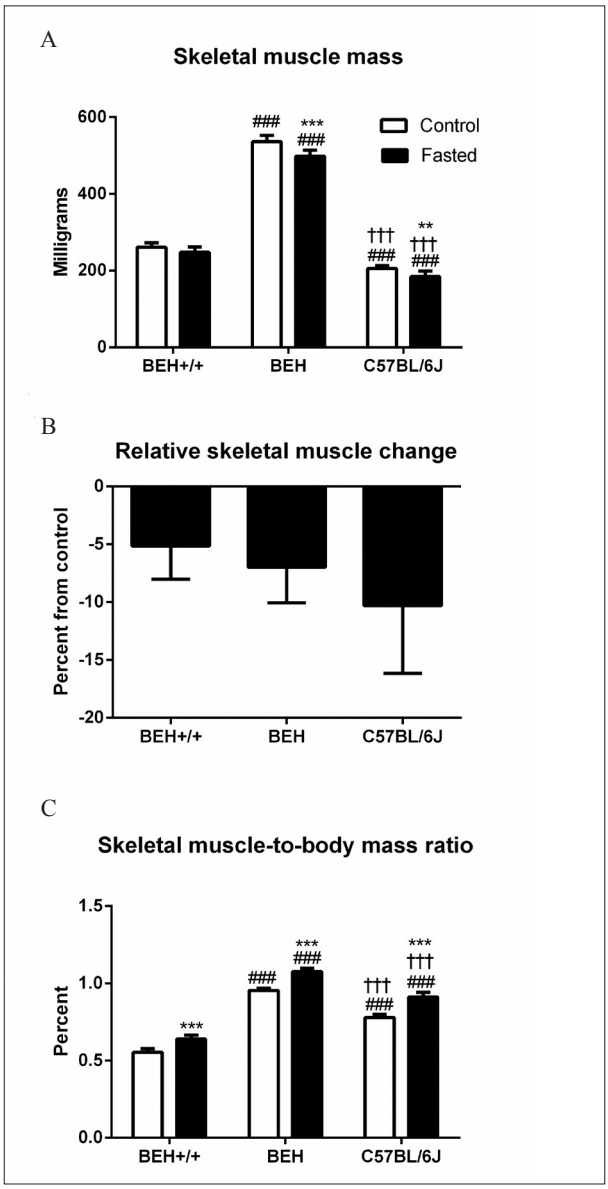

Figure 2. **(A) Muscle mass, (B) percentage change in the muscle mass and (C) muscle to body mass ratio in control and fasted BEH+/+, BEH and C57BL/6J mice**

Notes. The muscle mass was calculated as the summed weight of gastrocnemius, plantaris, soleus, tibialis anterior and extensor digitorum longus muscles. The relative change in muscle mass for fasted mouse was calculated using the data of the control mouse as in Figure 1. Values are means ± *SD*; **$p < .01$, ***$p < .001$ vs. control; $^{\#\#\#}p < .001$ vs. BEH+/+; $^{\dagger\dagger\dagger}p < .001$ vs. BEH.

C57BL/6J mice showed greater relative decrease in body mass than both BEH+/+ ($p < .01$) and BEH mice ($p < .001$). There were no differences between BEH+/+ and BEH mice which showed smaller ($p < .001$) changes in body mass than C57BL/6J mice over the entire 48 h period of FD.

Data on skeletal muscle mass are presented in Figure 2. The skeletal muscle mass depended on mouse strain ($p < .001$). Muscle mass of BEH mice was ~105 and ~160% greater than in BEH+/+ and C57BL/6J mice, respectively. We also evaluated

the contribution of skeletal muscle to overall body mass by calculating a ratio of muscle to body mass. Skeletal muscle to body mass ratio of BEH mice was by ~72% and ~23% greater compared to BEH+/+ and C57BL/6J mice, respectively. This ratio for C57BL/6J strain was by ~41% greater than for BEH+/+ mice. FD caused a decrease in muscle mass of BEH ($p < .001$) and C57BL/6J ($p < .01$) mice, but had little effect on muscle mass of BEH+/+ mice (Figure 2A). A relative decrease in muscle mass tended to be greater for both BEH and C57BL/6J mice compared to BEH+/+ mice though the differences were not significant (Figure 2B). Muscle to body mass ratio increased ($p < .001$) after FD in all three strains.

Data on changes in specific skeletal muscles are presented in Table. BEH mice had greater mass of all skeletal muscles than BEH+/+ mice with differences ranging from 1.7 to 2.6 times for soleus and plantaris, respectively. C57BL/6J mice had the smallest muscles which were two-three fold lighter than in BEH mice and 5–27% lighter than in BEH+/+ mice. There was a significant variation in extent of atrophy between the skeletal muscles after FD. 48 h FD induced a significant wasting of the gastrocnemius mass in BEH+/+ ($p < .05$), BEH ($p < .001$) and C57BL/6J ($p < .01$) mice. Plantaris lost weight in C57BL/6J ($p < .01$) and BEH mice ($p < .01$), but remain unchanged in BEH+/+ mice. Soleus became lighter in C57BL/6J ($p < .05$) and BEH mice ($p < .05$), but did not change in BEH+/+ mice. The weight of the tibialis anterior muscle mass decreased only in C57BL/6J mice ($p < .05$). Extensor digitorum longus showed loss of mass in all three strains, i.e. BEH+/+ ($p < .01$), BEH ($p < .01$) and C57BL/6J ($p < .05$). In summary, BEH+/+ mice experienced loss of mass in two muscles (gastrocnemius and extensor digitorum longus), BEH in four muscles (gastrocnemius, plantaris, soleus and extensor digitorum longus) and C57BL/6J in all five muscles (gastrocnemius, plantaris, soleus, tibialis anterior and extensor digitorum longus).

Heart mass is presented in Figure 3. BEH mice had similar heart mass to BEH+/+ mice. C57BL/6J mice had the lightest hearts ($p < .001$). Heart to body

Table. **Muscle mass for BEH+/+, BEH and C57BL/6J mice from the control (CON) and food deprivation (FD) groups**

Strain	Group	GAS (mg)	PL (mg)	SOL (mg)	TA (mg)	EDL (mg)
BEH+/+	CON	154.1 ± 9.8	17.2 ± 1.2	10.0 ± 0.6	66.6 ± 2.4	12.9 ± 0.6
	FD	142.6 ± 7.7*	16.6 ± 1.6	9.9 ± 0.7	66.9 ± 5.7	11.6 ± 0.8**
BEH	CON	323.6 ± 12.1$^{\#\#\#}$	44.0 ± 1.9$^{\#\#\#}$	17.3 ± 1.0$^{\#\#\#}$	123.4 ± 6.1$^{\#\#\#}$	27.3 ± 1.2$^{\#\#\#}$
	FD	299.8 ± 8.7 $^{\#\#\#}$***	39.5 ± 2.6 $^{\#\#\#}$**	16.2 ± 0.5$^{\#\#\#}$*	117.6 ± 7.1$^{\#\#\#}$	24.9 ± 1.2$^{\#\#\#}$**
C57BL/6J	CON	121.8 ± 5.3$^{\#\#\#\dagger\dagger\dagger}$	16.3 ± 0.4†††	8.2 ± 0.5$^{\#\#\#\dagger\dagger\dagger}$	48.9 ± 2.2$^{\#\#\#\dagger\dagger\dagger}$	10.0 ± 0.6$^{\#\#\#\dagger\dagger\dagger}$
	FD	109.2 ± 9.1$^{\#\#\#\dagger\dagger\dagger}$**	14.6 ± 1.2†††**	7.4 ± 0.7$^{\#\#\#\dagger\dagger\dagger}$*	43.8 ± 4.0$^{\#\#\#\dagger\dagger\dagger}$*	9.1 ± 0.6$^{\#\#\#\dagger\dagger\dagger}$*

Note. Values are means ± *SD*; GAS, gastrocnemius; PL, plantaris; SOL, soleus; TA, tibialis anterior; EDL, extensor digitorum longus. *$p < .05$, **$p < .01$ and ***$p < .001$ vs. control, $^{\#\#\#}p < .001$ vs. BEH+/+, $^{\dagger\dagger\dagger}p < .001$ vs. BEH+.

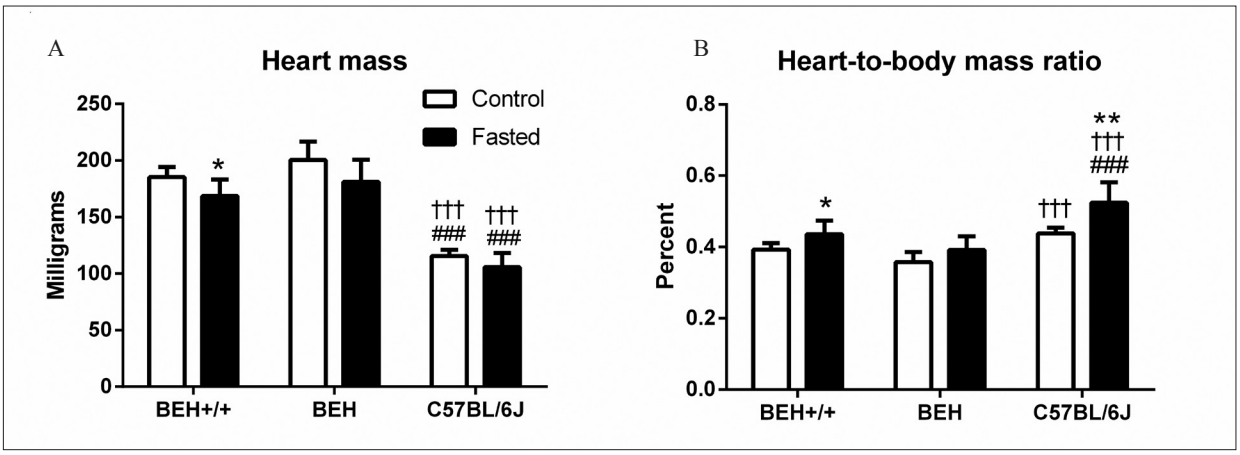

Figure 3. **(A) Heart mass and (B) heart mass to body mass ratio in control and fasted mice of BEH+/+, BEH and C57BL/6J strains**

Note. Values are means ± *SD*; *$p < .05$, **$p < 0.01$ vs. control; $^{\#\#\#}p < .001$ vs. BEH+/+; $^{\dagger\dagger\dagger}p < .001$ vs. BEH.

mass ratio was the greatest ($p < .001$) in C57BL/6J mice and this was especially clear after 48 h FD. A trend towards reduced heart mass was observed in all mice after FD, but it was significant ($p < .05$) only for BEH+/+ mice. Heart to body mass ratio increased in BEH+/+ ($p < .05$) and C57BL/6J mice ($p < .01$) after FD.

DISCUSSION

The main aim of this study was to examine effect of myostatin dysfunction and genetic background on body and skeletal muscle atrophy after 48 h food deprivation (FD). Firstly, we hypothesized that myostatin dysfunction might prevent muscle atrophy. However, our results do not confirm this hypothesis. On the opposite, muscle mass of BEH mice showed greater atrophy compared to BEH+/+ mice. Secondly, we have hypothesized that FD induced muscle atrophy will differ between the strains of mice due to genetic factors. Indeed, C57BL/6J mice showed greater loss of body and muscle mass than BEH+/+ mice. This might be due to higher content of body fat in BEH+/+ mice than C57BL/6J though potential differences in metabolic rate between the mice could also play the role.

Myostatin dysfunction or myostatin KO is associated with profound muscle hypertrophy in various mammalian species (McPherron et al., 1997; Schuelke et al., 2004). Indeed, BEH mice with myostatin dysfunction showed two fold greater muscle mass compared to the BEH+/+ mice of the same genetic background, but with the preserved myostatin function. Increased skeletal muscle mass is a consequence of both hypertrophy and hyperplasia of muscle fibres (McPherron et al., 1997). The regulation of fibre number by myostatin most likely results from direct effects of myostatin on proliferation and/or differentiation of myoblasts during the development (Lee, 2004), whereas the regulation of fibre size is a result of increased protein synthesis (Lipina et al., 2010; Welle, Bhatt, & Pinkert, 2006) and/or activation of satellite cells (McCroskery, Thomas, Maxwell, Sharma, & Kambadur, 2003). Interestingly, myostatin deficiency seems to have little effect or even causes a slight reduction in the size of other organs (Bünger et al., 2004; Lin et al., 2002). It is probably associated with limited expression and activity of myostatin in tissues other than skeletal muscle (McPherron et al., 1997; Ji et al., 1998). Furthermore, studies with myostatin KO species clearly demonstrate that myostatin dysfunction leads not only to increased skeletal muscle mass but also to decreased fat content (Lin et al., 2002; McPherron et al., 1997; McPherron & Lee, 2002). Mean total body fat mass was reduced by 70% in myostatin KO mice compared to wild type (WT) mice (McPherron & Lee, 2002). In confirmation of these findings by other investigated, BEH mice differed little from BEH+/+ in body mass though had marked greater muscle mass in our study as well. In general, the physiological role of myostatin remains to be investigated in greater detail. There is a clear evidence for skeletal muscle accretion in myostatin deficiency and an increase of myostatin expression during various catabolic diseases (Costelli et al., 2008; Gonzalez-Cadavid et al., 1998; Gruson et al., 2011; Plant et al., 2010). These findings suggest that inactivation of myostatin might be a useful strategy in preserving muscle mass during various conditions and physiological challenges. Indeed, myostatin targeting by antibodies ameliorated muscle wasting in *mdx* mice which serve as a model for Duchenne muscle dystrophy (Bogdanovich et al., 2002; Wagner, McPherron, Winik, & Lee, 2002). However, effects of myostatin targeting are less clear under conditions of caloric restriction or fasting which also lead to loss of muscle mass.

Myostatin expression is increased in skeletal muscles of mice after 48 h FD, but myostatin is not essential for muscle atrophy since myostatin KO mice undergo a substantial muscle atrophy as well (Allen et al., 2010). Interestingly, Allen et al. (2010) found no differences in muscles atrophy between myostatin KO and WT mice after 24 h FD, but after 48 h FD fast twitch muscles of myostatin KO mice lost less weight compared to muscles of WT mice. Our results seem to contradict these findings. BEH mice with dysfunctional myostatin showed greater muscle atrophy compared to BEH+/+ mice irrespective of the muscles examined. It might be argued that these discrepancies between studies could be due to differences in mouse strains used in these studies. Indeed, we used BEH mice while Allen et al. (2010) studied C57BL/6J mice. However, recent results of Collins-Hooper et al. (2015) on C57BL/6 also contradict those results of Allen et al. (2010). Collins-Hooper et al. (2015) showed that during 24 h of FD C57BL/6 mice with myostatin KO mice lost more muscle mass than WT mice and this difference did not depend on the type of muscles studied. Interestingly, caloric restriction of 40% applied over 5 weeks also resulted in greater muscle atrophy in myostatin

KO compared to WT mice (Matsakas et al., 2013). Thus, a significant amount of evidence suggests that mice with myostatin deficiency show greater muscle wasting compared to the WT mice during food restriction.

Lean body mass or fat-free mass is the major factor determining basal metabolic rate (BMR), which is substantially variable indicator among individuals (Johnstone, Murison, Duncan, Rance, & Speakman, 2005). Higher BMR proposes a higher energy expenditure which would be translated into an increased loss of body and skeletal muscle mass during FD. However, myostatin KO mice are not showing higher BMR than WT counterparts when BMR is normalized to lean mass or total body mass (Guo et al., 2009; McPherron & Lee, 2002). This might be associated with unchanged or even decreased size of internal organs in myostatin KO mice, which also play a significant role in overall BMR (Konarzewski & Diamond, 1995). Irrespective of BMR, myostatin KO mice seem to have metabolic changes in skeletal muscle. Although there were no differences in the rate of whole body lipid oxidation, but reduced lipid oxidation in skeletal muscle and increased glucose utilization was observed in myostatin KO mice (Guo et al., 2009). This is in agreement with findings that skeletal muscle of myostatin deficient mice is more glycolytic (i.e. having more fast twitch fibres) than muscle of WT mice (Girgenrath et al., 2005). Thus, the evidence suggests that alteration in the metabolism rather than increased metabolic rate might be of major importance in greater FD-induced muscle wasting in myostatin-deficient mice compared to WT controls.

We have also studied the effect of mouse strain on FD induced muscle atrophy. Our results show that BEH+/+ mice experience greater FD-induced muscle atrophy than C57BL/6J mice. It is well known that muscle proteins are broken down to amino acids which are released into circulation and used for gluconeogenesis and greater levels of fat can reduce mobilization of muscle protein for energy during starvation (Cuendet et al., 1975; Runcie & Thomson, 1970). C57BL/6J mice are approximately two fold lighter than BEH+/+, but have greater muscle to body mass ratio than BEH+/+. Thus C57BL/6J mice have greater relative muscle mass and might have less fat than BEH+/+ mice. Lower fat mass might promote greater utilization of skeletal muscle protein for energy in C57BL/6J mice compared to BEH+/+ mice. Differences in between the strains might also be of importance for muscle wasting during FD. Smaller animals have higher mass specific BMR which is related to larger surface area to volume ratio compared to bigger animals (Hochachka, Darveau, Andrews, & Suarez, 2003). It is, however, unclear if this prediction derived from studies of different mammalian species of markedly different body masses can be transferred into mouse strains.

Specific skeletal muscles showed differences in muscle atrophy after FD. Soleus muscle tended to show smaller atrophy compared to other skeletal muscles in all three mouse strains. This might be at least partially related to fibre type composition. When atrophy is induced by inactivity, unloading or denervation slow twitch muscles are affected more than fast twitch muscles (Roy, Baldwin, & Edgerton, 1991; Schiaffino et al., 2013). When atrophy is loading independently and associated with increased hormone or cytokine signalling, then it seems that fast twitch muscles and/or fast twitch fibres show greater susceptibility to muscle atrophy (Roy et al., 1991). In agreement with our findings, Li and Goldberg (1976) also found that soleus muscle is less sensitive to starvation compared to the faster contracting muscles and proposed that these differences might be due to lower sensitivity of slow twitch muscles to corticosteroids compared to fast twitch muscles (Goldberg & Goodman, 1969; Livingstone, Johnson, & Mastaglia, 1981). Myostatin KO mice have a higher content of fast twitch fibres (Girgenrath et al., 2005). Higher content of fast twitch fibres in skeletal muscles might be a key factor in causing greater atrophy in BEH than BEH+/+ mice after FD. Indeed, soleus showed a significant loss of muscle mass only in BEH mice.

In summary, our results show that BEH mice with myostatin dysfunction have greater muscle mass, but experience more severe muscle atrophy when subjected to FD compared to BEH+/+ mice with functioning myostatin. These findings do not support the hypothesis that myostatin targeting could preserve skeletal muscle mass during food restriction. However, BEH mice show constitutive loss of myostatin function and might not be the most appropriate model to evaluate effects of myostatin inhibition on muscle atrophy. An overt phenotype differences between BEH and BEH+/+ were established before the experiment with FD. Studies with conditional myostatin KO or inhibition during the period of FD are needed to test this hypothesis appropriately. We have also found that C57BL/6J mice show greater FD induced muscle atrophy than BEH+/+ mice. We propose that lower levels of body

fat and higher metabolic rate could potentially be responsible for this increased rate of muscle atrophy in C57BL/6J mice compared to BEH+/+ mice.

CONCLUSIONS

1. Mice with myostatin dysfunction are more sensitive to muscle atrophy after FD supposedly due to reduced fat mass and increased fast twitch fibre content in skeletal muscle.

2. C57BL/6J mice show greater loss of muscle mass during FD compared to BEH+/+ mice. This could also be due to lower fat mass in C57BL/6J mice compared to BEH+/+ mice. However, differences in metabolic rate and muscle fibre type composition might also play a role.

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Corresponding author **Petras Minderis**
Lithuanian Sports University
Sporto str. 6, LT-44221 Kaunas
Lithuania
Tel. +37060467644
E-mail petras.minderis@lsu.lt

COLLEGE ATHLETES' PERCEPTIONS OF COACHING BEHAVIOURS: DIFFERENCES BETWEEN INDIVIDUAL AND TEAM SPORTS

Aleksandra Aleksic-Veljkovic, Dusanka Djurovic, Ivana Dimic, Rifat Mujanovic, Kamenka Zivcic-Markovic
University of Niš, Niš, Serbia and Montenegro

ABSTRACT

Background. The aim of this research was to examine differences between athletes' perception of coaching behaviors in individual and team sports.

Methods. College athletes (N = 100) participated in the study. Three questionnaires were administered to the athletes: Demographic questionnaire, Leadership Scale for Sports and Negative Coaching Behavior Questionnaire.

Results. The results of this study revealed the significant differences among athletes' perception of coaching behaviors in individual and team sports. Individual athletes in this study gave higher ratings to training and instruction, social support and positive feedback leader behavior from their coaches. Also, athletes from individual sports had smaller scores on two dimensions and total score of negative coaching behavior questionnaire.

Conclusion. Those findings suggest that the behavior of the coach directed towards improving the performance of athletes` was higher evaluated from athletes in individual sports. Further studies should provide more information about coaches' behavior during the competitive.

Keywords: Demographic questionnaire, Leadership Scale for Sports, Negative Coaching Behavior Questionnaire.

INTRODUCTION

A good coach-athlete interaction tends to enhance motivation, induce pleasant emotions, and create a satisfactory and positive climate under the training and competition conditions (Bortoli, Robazza, & Giabardo, 1995), weather we take individual or team sports in consideration. Recently many researchers have examined this problem (Bortoli et al., 1995; Fallis, 2013; Jurko, Tomljanović, & Čular, 2013; Kenow & Williams, 1999; Siekanska, Blecharz, & Wojtowicz, 2013; Williams, Jerome, & Sartain, 2003) Poland, participated in the study. They represented both individual (n = 50, considering athletes' perceptions about their coaches at the training) and competition or game. They examined athletes of different ages and different levels of competition.

The relationship between coach and athlete is a very complex phenomenon which is affected by many variables. Also, this relationship influences the development of athletes and their sports career. The attitudes of athlete and coach are like a two-way street and it is important to examine how athlete experiences or evaluates their coach and their behavior. Individual and team sports reflect different expectations of the coaches and athletes and their relationship. The way athletes notice their coaches behavior's affects all included, as well as the sports achievement, and it is influenced by many psychological variables (attitudes, emotions, goals).

The evaluation of athletes can be influenced by three groups of variables: situational, such as the nature of the sport, the level and the nature of competition, the atmosphere in the team, and then the variables mostly related to individual differences between coaches and athletes: gender, age, attitudes, motives, goals. The third variable is the coach's

perceptions of the behaviors of their athletes (Kenow & Williams, 1999; Smoll & Smith, 1989).

Siekanska et al. (2013)Poland, participated in the study. They represented both individual (n = 50 examined how actual and former athletes in different sports levels perceived coaching behavior. Eighty competitive college athletes (44 males and 36 females; 21.89 ± 1.48 years of age; 8.35 ± 3.65 years of competitive experience) participated in the study. They represented both individual ($n = 50$) and team sports ($n = 30$). The participants responded to a demographic survey and the Coaches' Behaviors Survey and it was confirmed that coaches who perceived their athletes as more skilled, also treated them differently. Female athletes as compared with male athletes, more frequently pointed at the leniency in coach's behavior towards highly skilled athletes, and perceived it as a factor inhibiting athletic development. Additionally, women often found individualization of the training process as a behavior reinforcing development. Less achieving athletes more often pointed out to "a post-training session interest in the athlete" as directed only towards more achieving counterparts; however, they indicated "leniency and favoring" less often than the athletes with international achievements. They also listed "excessive criticism" as a type of behavior hindering development, but they indicated coaches' "authoritarianism and distance" less frequently than the more accomplished counterparts.

On the sample of 240 young athletes, both boys and girls, practicing in sport individually or in a team, of three age classes, 10–11, 13–14, 16–17 (20 subjects in each cell), Bortoli et al. (1995) obtained information on their perceptions on their actual coach and their ideal coach. The results clearly showed that athletes wished to have a better coach. A coach was evaluated by up to five athletes. Analysis of variance with repeated measures on the last factor was performed. Age, sport, and questionnaire forms main effects and their interaction were significant ($p < .05$). A follow-up analysis of variance on each of the two questionnaire forms was then applied. The analysis gave the following results: (I) athletes, in general, would have liked to have, better behavior from their coaches than the ones they actually had ($F = 153.44$, $p < .0001$), younger athletes ($F = 3.59$, $p < .05$) and athletes of team sports ($F = 4.36$, $p < .051$ gave better evaluations of their coaches; the interaction of gender, age, and sport was also significant ($F = 15.40$, $p < .05$). The results confirmed the general wish of youngsters to have a better coach and emphasize the need to improve a positive coach-athlete relationship (Barnett, Smoll, & Smith, 1992).

The atmosphere and the general relationship between athletes in the team are associated with leadership of the coaches. They depend on whether the coach is focused on improving the performance of athletes in a variety of physical training segments, or focused solely on the result, that is, to win the contest. If the coach is focused on performance, he or she gives positive feedback to athletes thereby rewarding their efforts, progress and good teamwork. On the other hand, coaches focused on the result predominantly use penalties when players do something wrong in training and competition, and thus encourage competitiveness among teammates, not cooperation (Jurko et al., 2013). The aim of this research was to examine if there were any differences between athletes' perception of coaching behaviors in college athletes in individual and team sports in Serbia.

METHOD

Procedure. Procedure of testing followed the earlier investigations (Kenow & Williams, 1999). Testing took place prior to a practice session. No games or competitions occurred within two days of the testing session in order to avoid potential response distortion. Three forms of a questionnaire were administered to the athletes: Demographic questionnaire, Leadership Scale for Sports (LSS) and Negative Coaching Behavior Questionnaire (NCBQ).

Demographic questionnaire contained questions about sports experience, the start of the sport career, years of training with current coach and time spent with coach per week.

Leadership Scale for Sports (LSS) had five dimensions: Training and Instruction (13 questions), Democratic Behavior (9 questions), Autocratic Behavior (5 questions), Social Support (8 questions), Positive Feedback (5 questions). The LSS contained 40 items that ask athletes to indicate the frequency with which their coach engages in specific types of coaching behavior. Item responses are based on a 5-point Likert scale, ranging from "*never"* to "*always",* and scores for each scale were produced by summing the item responses and dividing by the number of items in that category. The LSS has so far been used to measure the preferences of athletes for specific leader behavior for the coach, and the

perception of athletes regarding the actual leader behavior of their coach (Chelladurai & Saleh, 1980; Dallas, Kirialanis, & Mellos, 2014).

Negative Coaching Behavior Questionnaire (NCBQ) was used to examine frequency of the undesirable forms of coaching behaviors. The NCBQ had three dimensions: Insensitivity to Athletes' Wellbeing, Negative Feedback and Result Orientation. The reliability of subscales was satisfactory: Insensitivity to Athletes' Wellbeing (.89), Negative Feedback (.85) and Result Orientation (.78) (Greblo, 2011; Jurko et al., 2013).

Subjects. College athletes from individual sports ($n = 50$) and team sports ($n = 50$) participated in the study (see Table 1). All subjects had at least one full season of playing experience under their current coach. Subjects participated voluntarily and with the guarantee of anonymity. We contacted the athletes only after obtaining the coaches' permission. The Ethics Committee of the Faculty of Sport and Physical Education, University of Niš verified that this investigation complied with all ethical standards for scientific investigations involving human participants.

Statistical analysis. The statistical analysis was conducted employing the SPSS 20.0 software. Basic descriptive statistical data were calculated for the analyzed quantitative variables. For the comparisons the analysis of variance for interactions was used. The results where p was lower than the accepted level of significance ($p < .05$) were considered statistically significant.

Table 1. **Information about the athletes**

Athletes	Individual (N = 50)				Team (N = 50)			
Variable	Min	Max	Mean	SD	Min	Max	Mean	SD
Sports experience	2	24	8.60	5.31	3	17	9.46	4.09
Start of the sport career (years)	3	19	8.52	3.73	4	15	8.66	2.78
Training with current coach (years)	1	24	6.14	4.71	1	10	3.00	2.05
Spent time with coach per week (hours)	1	70	12.88	12.32	2	45	12.28	8.80

RESULTS

The descriptive statistics of preferences on five dimensions of leader behavior of individual and team sports athletes are shown in Table 1. The mean score of the five dimensions of Leader Behavior (LSS) and three dimensions and total score of Ne-gative Coaching Behavior Questionnaire (NCBQ) and results of ANOVA are shown in Table 2.

From Table 2 it is evident that the statistically significant differences existed between athletes from individual and team sports in five dimensions and total score of NCBQ.

Table 2. **Descriptive Statistics and ANOVA results**

Athletes	Individual (n = 50)		Team (n = 50)					
Variable	Mean	SD	Mean	SD	Sum of Squares	Mean Square	F	p
Training & Instruction	4.24	.68	3.91	.71	2.69	2.685	5.552	**.020**
Democratic Behavior	3.59	.72	3.32	.79	1.84	1.838	3.230	.075
Autocratic Behavior	2.70	.69	2.87	.87	.74	.740	1.203	.275
Social Support	3.64	.76	3.29	.86	3.06	3.062	4.669	**.033**
Positive Feedback	4.36	.76	3.90	.81	5.20	5.198	8.462	**.004**
Insensitivity to athletes' wellbeing	1.73	.79	2.35	.99	9.77	9.766	12.129	**.001**
Negative feedback	1.28	.47	1.81	.96	6.97	6.970	12.145	**.001**
Result orientation	2.92	1.04	3.12	.99	1.05	1.051	1.018	.315
NCBQ	1.97	.50	2.43	.77	5.12	5.123	12.103	**.001**

DISCUSSION

The results of this study revealed the significant differences between different athletes' perceptions of coaches' behaviors in individual and team sports, as the *F*-ratio was found higher than the required value to be significant. Individual athletes in this study gave higher ratings to training and instruction, social support and positive feedback leader behavior dimension from their coaches. Also, athletes from individual sports had smaller scores on two dimensions and total score of negative coaching behavior questionnaire. Rhind, Jowett, & Yang (2012) concluded that athletes who performed in individual sports also perceived that their coach felt closer, more committed, and complementary than athletes who performed in team sports, similar to our results.

This finding suggests that the behavior of the coach directed towards improving the performance of athletes was higher evaluated by athletes in individual sports. The coaches of individual sports gave more instruction to athletes about performance of the skills, techniques and tactics of their sports and organization activities. Also, athletes in individual sport appreciated the coaches' concern for the welfare of athletes, creating a positive environment and interpersonal relationships. Results show that the behavior of a coach related to reinforcing athletes and recognizing and rewarding good performances was more rated in individual sports.

Those results can be explained by the dynamics between the athlete and the coach, which are different in individual and team sports. Researchers believe that in individual sports the coach and athlete operate on a "one-to-one" with the focus on individual development and progression. Coaches and athletes have more opportunity to develop dependent relationships because they rely on each other, while in team sports this relationship is more formal, hierarchical and flexible because it is training a group of athletes. In the team sport we have synergy between players and performance of the team (Olympiou, Jowett, & Duda, 2008)Duda, & Yin, 2000.

The behavior of the coach which is oriented to the training of sports skills, support and positive feedback leads to an increase in faith of athletes and their possibilities. Coach who shows positive emotions and manners in his behavior, use constructive criticism, takes care of the needs of athletes. On the other hand, this kind of behavior leads to the creation of a positive working atmosphere, encourages the confidence of athletes (Williams et al., 2003).

Baker, Yardley, & Cote (2003) examined the moderating effect that athlete's sports type (i.e. individual or team) may have on the relationships among seven coaching behaviors (mental preparation, technical skills, goal setting, physical training, competition strategies, personal rapport, and negative personal rapport) for predicting coaching satisfaction. Moderated multiple regression analyses indicated that each of the seven coaching behaviors was a significant main effect predictor of coaching satisfaction. However, sports type (i.e. team or individual sports) was found to moderate six of the seven relationships: mental preparation, technical skills, goal setting, competition strategies, personal relationship, and negative personal relation in predicting satisfaction with the coach. These findings indicate that high coaching satisfaction for athletes in team sports is influenced to a greater extent by the demonstration of these behaviors than it is for individual sport athletes.

Our research results show that there is no difference between athletes of individual and team sports in terms of the perception of a democratic and authoritarian style of the coach. These results are somewhat inconsistent with previous research which shows that coaches in individual sports prefer more democratic behaviors. They leave athletes to participate in the decision-making process on the objectives, tactics and strategy of performance, as opposed to autocratic behavior coach that decisions are made by himself without consulting with athletes (Loughead & Hardy, 2005; Terry, 1984). These results of our study can be linked to the situational factor because at the moment our researched athletes were out of the competition. During the competition season, the styles of the coaches' behaviour may be more pronounced.

CONCLUSION

The results of our study indicate that the perception of the quality of the relationship between coach and athlete is not necessarily caused by autocratic or democratic style of coaching. Both, in individual and team sports the quality of the relationship between coach and athlete is important to coaching behavior characterized by providing social support to athletes, supporting positive emotional attitude, constructive criticism and authentic concern. In this context, the obtained

results showed that the athletes in individual sports preferred democratic behavior of the coach, which means that the coach leaves athletes to participate in the decision-making process on the objectives, tactics and strategy of the competition or game as opposed to coaches' autocratic behavior where decisions are made by them without consulting the athletes. Recommendation for further research is to provide longitudinal studies about coaching behavior during the competitive season considering the differences between types of sports, for example aesthetic, combat or power sports.

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Corresponding author **Aleksandra Aleksic-Veljkovic**
University of Niš
Carnojevica str. 10a, 18000 Niš
Serbia and Montenegro
E-mail aleksic.veljkovic@gmail.com

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