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Faculteit Revalidatiewetenschappen

master in de revalidatiewetenschappen en de kinesitherapie

Masterthesis

The intrinsic muscle adaptations after disuse

Arne Budo
Gregory Roets

Scriptie ingediend tot het behalen van de graad van master in de revalidatiewetenschappen en de kinesitherapie, afstudeerrichting revalidatiewetenschappen en kinesitherapie bij inwendige aandoeningen

PROMOTOR :
dr. Pieter VAN NOTEN

2018
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Acknowledgement

This master thesis has been written to apply for a degree in physical rehabilitation at the university of Hasselt, campus Diepenbeek. The students B.A. and R.G. were very interested in the topic “The intrinsic muscle adaptations after disuse”. Due to great interest in this research domain and for the rehabilitation of multiple sclerosis, a study protocol could be made. We would like to thank our promotor Dr. P. Van Noten for his guidance in the realization of this master thesis. His experience and knowledge in this research domain have been very valuable. We also like to thank Drs. Jan Spaas for his collaboration in this research. Without their co-operation, this master thesis could not be feasible. Finally, the fellow students want to thank each other for the fluent co-operation.

Budo Arne: Heers, België, 12 augustus 2019 B.A.

Roets Gregory: Heers, België, 12 augustus 2019 R.G.

Research context

This paper describes a fundamental research experiment that is situated in between musculoskeletal and neurological rehabilitation. In more detail, this paper describes the effect of a mouse multiple sclerosis (MS) model on muscles.

Muscles react on their usage: muscle tissue increases when being trained or decreases in a state of disuse. Disuse, which results from bed rest (BR), immobilization (IMM), hindlimb suspension (HLS), disease or neurological defects, typically evokes some of the following muscle adaptations: loss in muscle mass, decreased cross-sectional area (CSA) of the whole muscle as in a single muscle fiber and less peak force. Hereby, we can assume that the effects of disuse are dependent on the cause. Another important given is the duration of disuse, which can alter these muscle characteristics to a greater or lesser extent. A decrease in muscle mass of the soleus already occur after four to seven days of HLS (Thompson, 2002). According to Kourtidou-papadeli et al. (2004), the decrease in muscle mass of the soleus reaches a plateau around 70 days of HLS.

Disuse, in this paper, results from MS. In mice, MS is mimicked by inducing an experimental autoimmune encephalomyelitis (EAE), by which they develop an increased neurological paralysis from caudal to cranial. Because disuse is commonly seen in persons who are inactive, it is interesting to discuss the effects of inactivity on muscle force compared to EAE related inactivity. Physical therapists often deal with people who are exposed to disuse-related problems. For example, there is no cure possible for people diagnosed with MS. The therapy is merely a treatment of the symptoms which include fatigue and a loss of strength. Therefore, a good understanding and knowledge of which adaptations occur and which underlying mechanism play an important role in this process, can help optimizing the rehabilitation. This explains why this topic closely matches the domain in physical rehabilitation.

The first part of our research was a literature study about the intrinsic muscle adaptations after a certain disuse protocol within a healthy population and thus MS excluded. Changes due to specific forms of disuse were described, such as IMM, BR and HLS. The second part will include MS as a cause of disuse in mice. This study consists the mice of two different studies. The first is the CAREAE-study, EAE-mice were exposed to 18 (EAE1) or 28 (EAE2) days of disuse. This study uses a healthy control (CON) group which were also evaluated after 18 days. The second study is the DOSCAR study, where mice were exposed to 56 days of disuse. The researchers performed the measurements and the analyzes of the DOSCAR study with the help of their promotor Dr. P. Van Noten. In the other studies (EAE1 and EAE2), the measurements were performed by other

researchers. Due to the same study-protocol and with the permission of the other researchers, these two studies could be analyzed together, as done in this paper.

The data collection was set up at the centre for rehabilitation research (REVAL) in Diepenbeek under the direction of Dr. P. Van Noten and Dr. Jan Spaas. This paper makes the second part of a master thesis to apply for a degree in physical rehabilitation at the University of Hasselt. It is a study promoted by Dr. P. Van Noten in collaboration with Drs. Jan Spaas. It is part of another study where the effect of carnosine on a model of EAE is studied. The central format is used as guideline and the work is done as a duo under the guidance of promotor Dr. P. Van Noten.

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1. Abstract

Background: Disuse results when muscles are exposed to inactivity. Intrinsic muscle changes like fiber-type shift, decreased cross-sectional area (CSA) or muscle mass will occur. Inactivity is commonly seen in people with a certain condition or illness. Therefore, it is important for rehabilitation purposes to know how these changes occur, to what extent and which impact disuse has on the short- and long-term.

Objectives: The main goal is to determine the effects of Multiple Sclerosis (MS)-related disuse on muscle strength, CSA, fiber-type composition and the resistance to withstand fatigue of the soleus (SOL) muscle, when exposed to short- and long-term inactivity.

Participants: Forty-four female C57BL/6 mice of ten weeks old were induced with experimental autoimmune encephalomyelitis (EAE) and developed a progressive paralysis from caudal to cranial. Different groups are: EAE1 (n=10), EAE2 (n=10) and DOSCAR (n=14). Mice were sacrificed at 18, 28 and 56 days. A healthy control group CON (n=10) was used to compare results.

Measurements: Electrical stimulation of the SOL muscle at 1Hz (twitch), 50Hz (tetanic) and 10Hz, 25Hz, 75Hz, 100Hz and 125Hz, a fatigue (FAT)-protocol (50Hz) and a five and ten-minute recovery tetanus (both 50Hz) were included for analysis. The data were analyzed using the program 'LabVIEW'.

Results: Eight-teen (EAE1) and 28 (EAE2) days of disuse resulted in significantly lower tetanic. A significant decrease in force after the FAT-protocol is seen for EAE2 and DOSCAR compared to CON. The decrease was larger and occurred faster in the DOSCAR group.

Conclusion: Fifty-six days of disuse showed a greater decrease in active force compared with EAE groups and CON after a fatigue-protocol. Therefore, the resistance to withstand fatigue is decreased (DOSCAR), which can infer that the duration of disuse plays a significant role in active force.

2. Introduction

Disuse can be described as a decrease in everyday activity compared to a normal pattern. The complete individual, or a specific body part (e.g. leg or arm), becomes physical inactive and the involved muscles are used less frequently. This results in adaptations of the muscle and neuronal tissue, but it also affects the vascular tissue and creates intracellular alterations. An accumulation of these factors could contribute to the loss of muscle mass and muscle force (Bodine, 2013; Degens & Alway, 2006; R. H. Fitts, Riley, & Widrick, 2001). Disuse is not a disease 'an sich' but results from a decrease in load and/or neuronal activation, which are predominantly associated with pathology like multiple sclerosis (MS) or an altered environment like space travel (Edgerton, Roy, Allen, & Monti, 2002; Thompson, 2002).

Muscle tissue adapts easily to different kinds of stimuli. Depending on its use, the outcome will be different. Increased mechanical load for example causes hypertrophy. When muscles are used more often, they will grow in size (Frontera & Ochala, 2015), in other words, muscle tissue is created by increasing the cross-sectional area (CSA) of individual muscle fibers (Cho, Kim, & Song, 2016) . When there is less or no mechanical load, as in disuse, a negative stimulus could cause atrophy (Brooks & Myburgh, 2014). The loss of muscle tissue can be explained as an altered balance between protein synthesis and breakdown (Cho, Kim, & Song, 2016; Rodriguez et al., 2014). A decrease in muscle CSA is hereby the result and causes this atrophy. Thompson (2002) and Fitts, Riley & Widrick (2000) discovered that antigravity muscles (consisting out of mostly type I fibers), such as the soleus (SOL), atrophy more compared with type II muscles when exposed to seven to 14 days of disuse. This was the same for seven days of spaceflight. This can be explained by the fact that antigravity muscles should be more active and stay active for a longer period of time to hold the body against gravity. Therefore, these muscles are more susceptible to a period of disuse.

Atrophy leads, as mentioned above, to the loss of muscle mass which can be up to 37% (in humans) after a model of spaceflight. Hence, the limited duration of spaceflight (Fitts et al., 2001; Fitts, Riley, Widrick, R. D., Widrick, J. J., 2000; Thompson, 2002). While performing tests on rats and mice, Stelzer and Widrick (2003) found a decrease of 25% after a seven-day model of hind limb suspension (HLS), which is equal to the changes found after immobilization (IMM) over a period of ten days (Hinkle, Donnelly, Cody, Sheldon, & Isfort, 2005), and Canon & Goubel (1995) found 59% decrease after three weeks. This loss of muscle mass is accompanied by lower peak

forces and an increase in fatigue rates, affecting the whole muscle, but also the individual muscle fibers (Cho et al., 2016; Feng, Chen, Malek, & Jin, 2016)

The decrement of peak force after HLS ranges between 21% and 25% for the SOL muscle (Hanson, Harrison, Young, Stodieck, & Ferguson, 2013; Thompson, 2002) which is equal to the changes found after IMM (Hinkle et al., 2005). This force loss is due to the number of cross-bridges in parallel, which decreases when muscle mass decreases (Thompson, 2002). Since type I muscle fibers are more sensitive to atrophy compared to type II fibers (Boonyarom, Kozuka, Matsuyama, & Murakami, 2009; Thompson, 2002), muscle atrophy changes the muscle fiber-type composition. Modeled after a HLS ranging between seven to 14 days, there is a fiber-type shift from type I to type II (47% type I and 53% type II) while under normal circumstances, the SOL muscle has approximately between 80% and 90% type I fibers and 10% to 20% type II fibers (Anderson, Almeida-Silveira, & Perot, 1999; Boonyarom et al., 2009; Canon & Goubel, 1995; Deschenes, Britt, & Chandler, 2001; Fitts, McDonald, & Schluter, 1991; Kasper, McNulty, Otto, & Thomas, 1993; Kourtidou-Papadeli et al., 2004; Ohira et al., 2006; Thompson, 2002; Yu, Gao, Feng, & Jin, 2007).

There is a difference between short-term disuse, which leads to weakness of the muscles involved, and long-term disuse, which evokes more fatigability caused by a shift from type I to type II fibers in the SOL muscle (J. A. KENT-BRAUN, 1997). This differs from the shift that occurs within a non-antigravity muscle as the extensor digitorum longus muscle (EDL), which predominantly has type II fibers. Only density of the type II fibers was decreased but there was also a slight increase of type I fibers of approximately 6% (Boonyarom et al., 2009). Depending on the duration of disuse, the changes in muscle- and muscle fiber CSA are slightly different. After 17 days of spaceflight for example, J. J. Widrick (1999) found a decrease of whole muscle CSA of 15%. After seven days of HLS, the decrease is found to range between 13% and 28.4% (Hanson et al., 2013; Stelzer & Widrick, 2003) for both type I and II fibers (Boonyarom et al., 2009; Ohira et al., 2006). These CSA changes are ranged between 35% (Hanson et al., 2013) and 50% after two weeks of HLS (Favier, Benoit, & Freyssenet, 2008). Immobilization only inflicted a 20% whole muscle CSA decrease after three weeks (Ye et al., 2013). Thus, a longer time of disuse results in a deeper decline of the CSA, as well as a decline in maximum peak force and a greater shift from type I to type II fibers (Hanson et al., 2013).

There are a lot of models to mimic disuse. Examples are HLS, Bed rest (BR), IMM and spaceflight. This are specific models to mimic disuse but are rare conditions in a human life

compared to disease associated disuse. These simulating models are used because disease cannot have any interference on disuse while in reality, disease is a much more common given. Experimental autoimmune encephalomyelitis (EAE) is another way to mimic MS disease in mammals, more specific to this study, in mice. Through EAE, an inflammatory demyelinisation can be provoked and will cause similar symptoms as in MS. It is believed that MS-related disuse is responsible for the adaptations in the muscle tissue that occur in persons with MS (J. A. KENT-BRAUN, 1997), however, this still remains unclear. Several MS studies indicate muscle atrophy, increased fatigability, less peak power and a decline in muscle CSA (A. V. NG, 2004; ARNOLD de HAAN, 2000; Carroll, Gallagher, Seidle, & Trappe, 2005; CHARLES P. LAMBERT, 2000; DENA J.P. GARNER, 2002; J. A. KENT-BRAUN, 1997). These changes are similar with the ones found after models of disuse (Kent-Braun et al, 1997). MS, however, is characterized from a central component. In other words, there is a demyelinisation of the white matter of the central nervous system (CNS) while this is not the case with HLS or IMM (Wens et al., 2014).

In conclusion, intrinsic muscle changes after disuse are not only dependent on the duration of disuse, but also on the kind of disuse model that is used.

EAE in mice models is typically used to study the CNS and rarely to study muscle adaptations, which include CSA, muscle mass and fiber-type shift. Therefore, an EAE-experiment for the short- and long term, in particular 18, 28 and 56 days has been used. When mice are induced with EAE, the first symptoms occur within nine to 14 days. Therefore, the researchers chose 18 days so that the mice will have the chance to be exposed to disuse. As mentioned above, many changes already occur after three weeks, thus 28 days was chosen. Fifty-six days was chosen to discuss the long-term intrinsic changes of disuse. Our aim will be to discuss the intrinsic muscle adaptations dependent on the duration of disuse by MS.

The first aim is to determine if there is a difference in the decrement of strength in the SOL muscle of mice at these different time periods. Moreover, we will discuss what influence the duration of disuse has on the resistance to withstand fatigue, expressed in muscle force. Our second aim is to see whether there is a correlation between disease score and its influence on muscle force of the SOL.

3. Material and methods

The results of our study are based on two studies: the first one is the CAREA-study, consisting of the groups EAE1, EAE2 and a healthy control (CON) group to compare results with. Secondly the DOSCAR study. We employed only parts of the data from both studies. Therefore, all mice except for CON were induced with experimental autoimmune encephalomyelitis (EAE). Induction of EAE was done at day 0, followed by a period without any signs or symptoms (day 0 to day 10). Following on this period, hindquarter paralysis starts to occur (day 11 to day 14). Some of the mice almost recovered completely where others keep showing signs of disease. After 18 days, the EAE1 and CON group were sacrificed. The EAE2 group was sacrificed after 28 days and the DOSCAR after 56 days (figure 1).

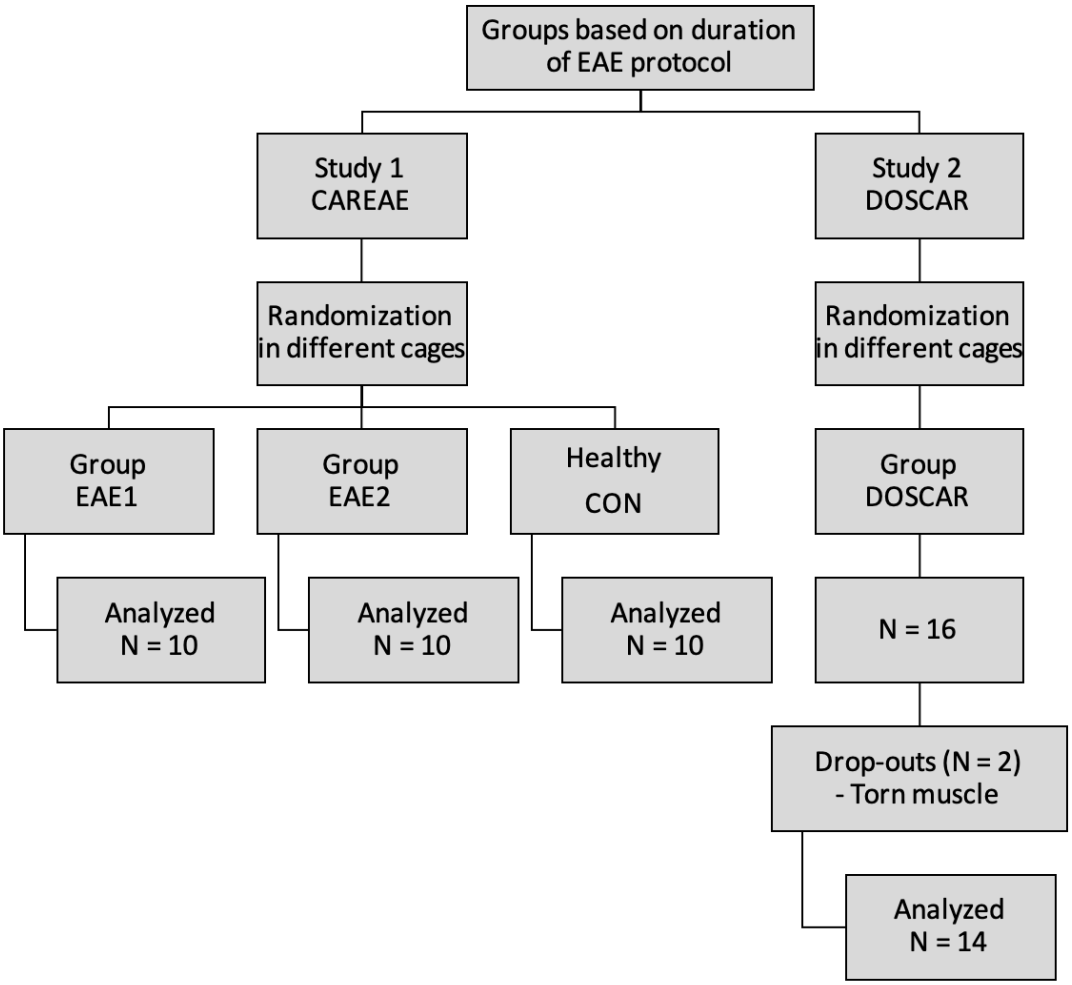

Figure 1. Flowchart group classification

3.1. Animal groups

Sixteen (DOSCAR), 20 (EAE1, EAE2) female C57BL/6 mice with an age of ten weeks old, were induced with EAE and are used for this experimental design. The healthy CON group (without EAE induction) consisted of ten mice with the same age as the others. Fourteen muscles of the DOSCAR group were used for analysis due to rupture during force measurement (N=2). The total number of analyzed mice was 44. Animals were randomly divided into cages. Mice were housed in ideal lab conditions to minimize the effect of stress, which could negatively affect EAE development. The animals were housed in a quiet environment without excessive noise or vibration and had access to food and water. The study protocol was approved by the animal Ethics committee of Hasselt University. Injection protocol can be seen in figure 2.

3.2. EAE-scoring

The paralysis starts at the tail and progresses to the trunk. The scoring is done using a scale from 0 (no symptoms) to 5 (death). By taking an EAE-score of two as start value, we can be sure that all the included mice have weakened hind legs. At this point, the mice have a limp tail and weakness or dragging of the hind legs. This is necessary because we are only interested in analyzing the muscle force when the mice are using the hind legs less than before. From this point on, we can assume that the SOL muscles are more likely to be exposed to disuse. The onset of paralysis is between nine to 14 days after immunization, with peaks of disease at three to five days after onset. On average, this means the peak of disease will maximize at approximately 18 days.

3.3. Muscle sampling

After dissection, the muscles are placed in a krebs-heinseleit buffer with a temperature of 25°C and gassed with 95% O2 and 5% CO2 gas for 15 minutes. With use of a self-written program, instant muscle force was first monitored by a software program (LabVIEW) during the experiment wherefore the muscle force was analyzed post-hoc in the same program. During dissection, a little piece of rope was attached through the tendon with a little loop at the end. Each loop is fixed so that the muscle hangs stable in between the force transducer and a micro-positioner.

3.4. Electrical stimulation

The stimulation frequency of every tetanic contraction was set at 50Hz, with a stimulation duration of 350ms and each with two minutes' rest in between. Bipolar pulses of 1ms pulse duration were used. Stimulation frequency for the fatigue (FAT) protocol was also 50Hz with a pulse duration of 350ms, but now with a rest period of 2.5 seconds between stimulations. First, rest length (L0) was determined using a few tetanic contractions (50Hz) at first passive force contribution (approximately 5mN) by small length adjustments through the micropositioner. L0 was set at highest active tension. The first contraction after L0 determination was a twitch (1Hz) followed by a tetanic contraction. After the tetanic, five other stimulations were done (10Hz, 25Hz, 75Hz, 100Hz, 125Hz) with two minutes' rest between each contraction. After this, a ten-minutes FAT protocol started. Then, tetanic recovery force after five (REC 5) and ten (REC 10) minutes was evaluated. Only the DOSCAR study received three extra pulses at 20Hz, 30Hz and 40Hz. Only the corresponding pulses were used for analyses. This could be valuable to mention since this can have some minor influences on the results in this group.

3.5. Determination of force

After the experiment, all the muscle force measurements were analyzed individually with LabVIEW. The method to analyze a single contraction was as follows; the start of the pulse was determined manually. The program automatically determined peak force and pulse duration values of this contraction. All contractions were analyzed in the same way, except for the FAT protocol, where a mean value of three pulses was calculated and used for analysis. We were interested in active muscle forces. Therefore, we used peak force and passive force to calculate the active muscle force by the following formula: maximum peak force - (minus) passive pre-contraction force. Force – Hz relationship consisted of twitch, tetanic, 10Hz, 25Hz, 75Hz, 100Hz and 125Hz and were described in absolute values as well as relatively to individual muscle CSA. Whereas the FAT protocol is described relatively to FAT 0. Muscle CSA was measured using the following formula: muscle weight (g) / (1.06 (g/cm^3) * muscle length (cm)).

3.6. Cumulative EAE-score

To determine whether an animal had been sick for a long period of time, we used the cumulative EAE-score. This is the sum of the scores given to each mouse per day. This was done to see if a

higher cumulative disease score, has an influence on the tetanic force or FAT 10 force, dependent of the animal disuse group.

3.7. Data analysis

3.7.1. Statistics

All data were analyzed using JMP pro 14. Normality of data and distribution were evaluated using the Shapiro-Wilk W test. If the data had a normal distribution, as in comparing force-Hz relationship between the groups, a one-way ANOVA was used. In case of a non-normal distribution, which was found in all of the FAT-protocols, a Wilcoxon Rank Sum each pairs test was used. The correlation between tetanic force and FAT 10 force with disease score was evaluated with spearman's rho correlation (r_s). Data were expressed as mean ± standard deviation (SD). A single factor repeated measures design was applied to determine the difference between in-muscle strength after several electrical stimulations and fatigue protocol. A 5% level of significance was used (α=0.05).

3.7.2. Source and software

The program 'LabVIEW' was used to analyze the data. Every contraction apart from the L0 determinations, was incorporated until the recovery from fatigue. Libreoffice was used as a tool to read the data from the program LabVIEW. After this, the obtained data were integrated in Excel so that graphs could be made. At last we exported these excel files into JMP pro 14.

4. Results

4.1. Muscle mass

Mean muscle mass was 0.00558g±0.00118 for EAE1, 0.00551g±0.00113 for EAE2, 0.00609g±0.00161 for DOSCAR and 0.00647g±0.00139 for CON. However, no significant differences were found between groups and compared to CON (figure 3).

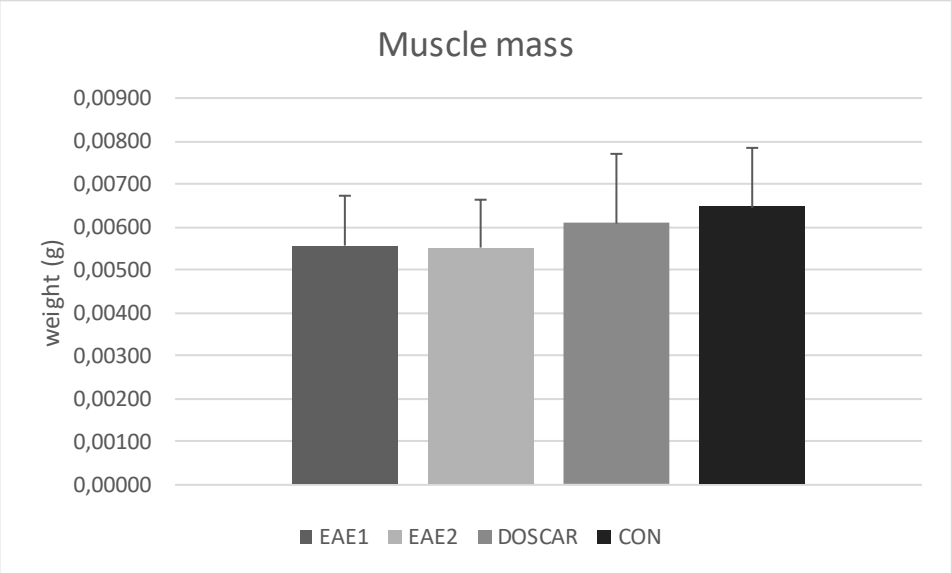

Figure 3. Means ± SD of muscle mass (g) between groups

4.2. Muscle size (CSA)

Mean CSA of the muscle was 0.00500 cm^2±0.00107 for EAE1, 0.00494 cm^2±0.00101 for EAE2, 0.00562 cm^2±0.00147 for DOSCAR and 0.00562±0.00113 for CON. However, no significant differences were found between groups and compared to CON (figure 4).

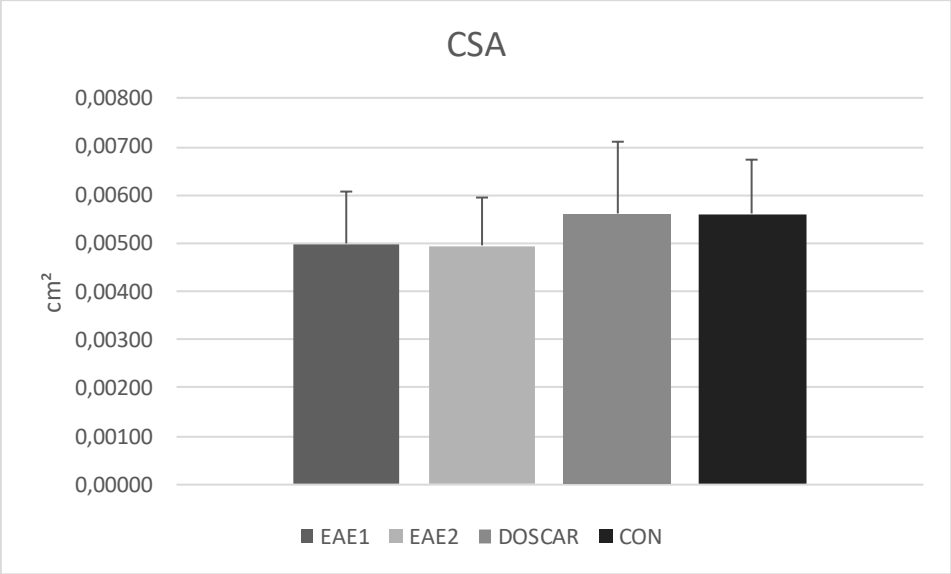

Figure 4. Means ± SD in muscle CSA (cm^2) between groups

4.3. Active Force – Hz relation

Active forces for EAE1, EAE2, DOSCAR and CON groups are listed in table 1. No differences were found in mean active twitch force between the four groups. However, analyzing tetanic forces, EAE1 and EAE2 found significant lower forces compared to CON. EAE1 decreased with 17% ($p<0.0459$) and EAE2 with 16% ($p<0.0311$). Between DOSCAR and CON group, DOSCAR decreased 14% in active force, however, this difference was not significant. At a frequency of 100Hz, there were differences in all three intervention groups compared to CON. There was 20% decrease for EAE1 ($p<0.0262$), 18% for EAE2 ($p<0.0307$) and 21% for DOSCAR ($p<0.0086$). Significantly differences were also seen when comparing the groups with CON at a frequency of 125Hz. There was 21% decrease for EAE1 ($p<0.0225$), 18% decrease in the EAE2 group ($p<0.0304$) and 23% decrease in DOSCAR ($p<0.0054$). Only the DOSCAR group reported a significant difference at 75Hz compared to CON with a decrease of 17% ($p<0.0472$).

4.4. Active Force – Hz relation relatively to muscle CSA

When comparing muscle force relatively to the individual muscle CSA. A significant decrease of 18% ($p< 0.0252$) in tetanic force (50Hz) is seen in the DOSCAR group only, compared to CON. When evaluating 75Hz, 100Hz and 125Hz, DOSCAR was also the only group compared to CON with a significant decrease of 19% ($p<0.0189$), 24% ($p<0.0025$) and 25% ($p<0.0020$), respectively. An overview can be seen in figure 5.

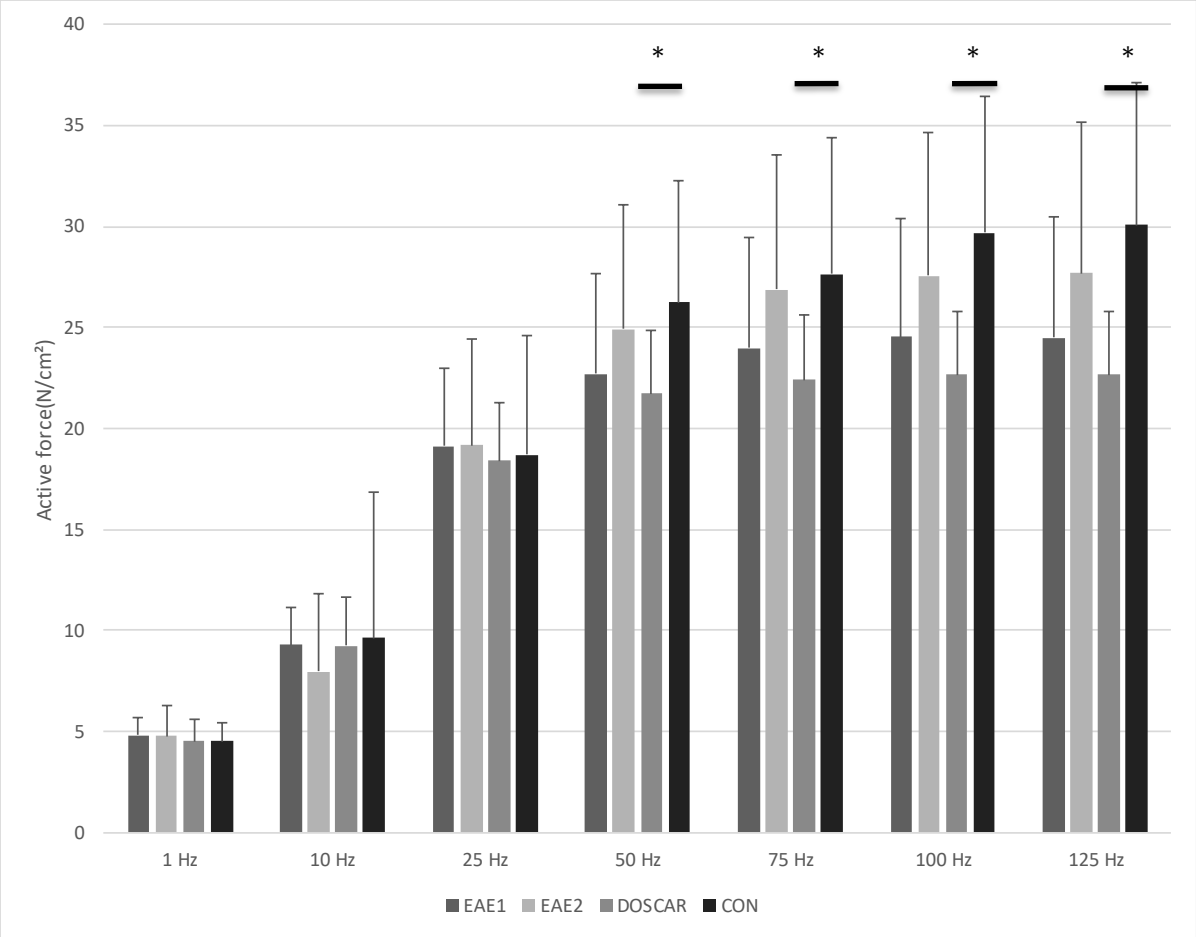

Figure 5: Mean ± SD of active forces at different frequencies for each group, expressed relatively to muscle CSA. *<0.05 indicates significant difference compared to control (CON)

4.5. Fatigue protocol

A within-group analysis over all time moments (FAT 0 until FAT 10, REC 5 and REC10) revealed a significant decrease in the first three minutes of the fatigue protocol for all groups (figure 6). Evaluating the forces in a single group, compared to the next FAT moment, significant differences are seen for the EAE1 group between FAT 0-FAT 1 ($p<0.0001$, decrease of 13%), between FAT 1-FAT 2 ($p<0.0017$, decrease of 9%) and between FAT 2-FAT 3 ($p<0.0140$, decrease of 10%). Looking at recovery, only at FAT 10-REC 5 ($p<0.0002$, increase of 41%) a significant difference is seen. For EAE2 group, significance was found between FAT 0-FAT 1 ($p<0.0001$, decrease of 14%), between FAT 1-FAT 2 ($p<0.0091$, decrease of 7%) and between FAT 2-FAT 3 ($p<0.0452$, decrease of 9%). Recovery values follow the same trend as EAE1 group, only FAT 10-REC 5 was significant ($p<0.0002$, increase of 36%). Also in the DOSCAR group significant values are found between FAT

0-FAT 1 (p<0.0001, decrease of 14%), between FAT 1-FAT 2 (p<0.0002, decrease of 10%) and between FAT 2-FAT 3 (p<0.0159, decrease of 11%). Comparing recovery values, FAT 10-REC 5 was significant (p<0.0001, increase of 42%). However, different from EAE groups, FAT 0-REC 5 was significant (p<0.0064, still 10% less). For the CON group, significant values were found for FAT 0-FAT 1 (p<0.0001, decrease of 16%), FAT 1-FAT 2 (p<0.0017, decrease of 8%) and FAT 2-FAT 3 (p<0.0058, decrease of 10%). Comparing recovery values, more differences could be seen in the CON group compared to intervention groups. FAT 10-REC 5 (p<0.0003, increase of 31%), FAT 0-REC 5 (p<0.0001, 17% less), FAT 0-REC 10 (p<0.0014, 9% less) and REC 5-REC 10 (p<0.0257, increase of 8%) were significant.

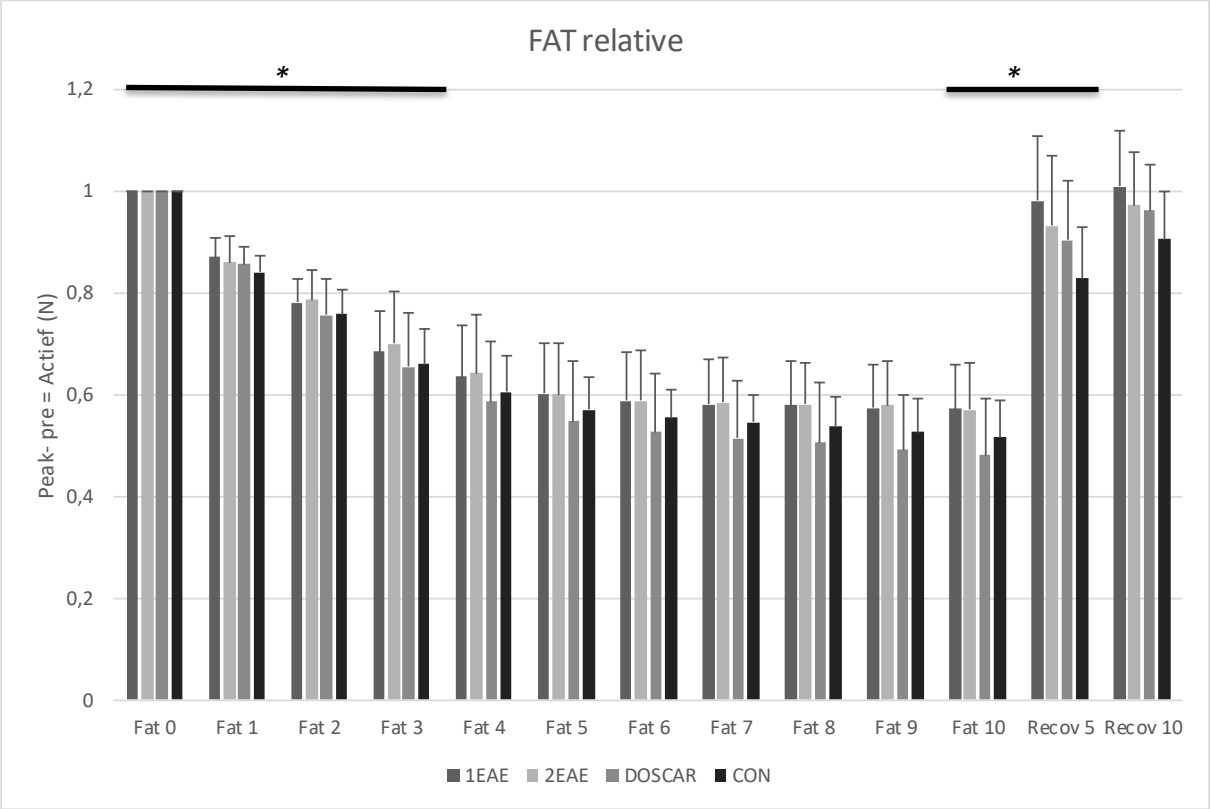

Figure 6. Mean ± SD within-group for the fatigue-protocol. Values are relative to FAT 0. *<0.05 indicates significant difference for all the groups compared to the next FAT-moment

4.6. EAE-score

Mean EAE-scores were 1.60±1.030 for EAE1 group, 1.61±0.612 for EAE2 group and 1.98±0.0.659 for DOSCAR group. There were no significant differences for EAE-scores between the three groups. Mean cumulative scores were 8.15±5.467 for EAE1 group, 27.10±9.594 for EAE2 group and 86.571±29.328 for DOSCAR group. There were differences in cumulative score for EAE1 vs EAE2 (p<0.0001), EAE1 vs DOSCAR (p<0.0001) and EAE2 vs DOSCAR (p<0.0001). Mean disease

time was 1.6 days±1.897 for EAE1 group, 6.30 days±4.923 for EAE2 group and 31.643 days±14.648 for DOSCAR group.

For cumulative score, there was no general correlation with tetanic force (r_s=-0.1333, p<0.4524). For EAE1 group, there was a strong negative correlation between tetanic force and cumulative score (r_s=-0.7212, p<0.0186). In EAE2 group, a very strong negative correlation is measured (r_s=-0.8424, p<0.0022). This correlation was not found within the DOSCAR group (r_s=-0.1980, p<0.4974) (figure 7).

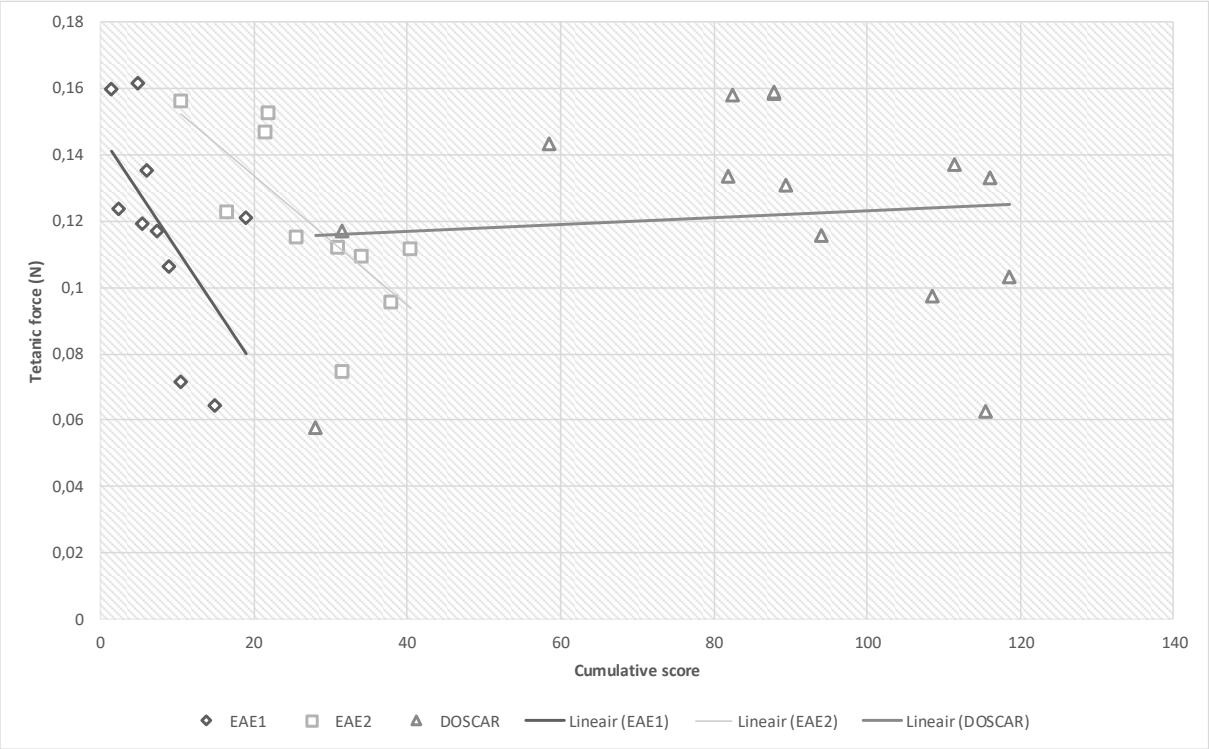

Figure 7. Correlation when comparing tetanic forces with cumulative EAE-score between EAE1, EAE2 and DOSCAR groups

When the correlation between cumulative score and FAT 10 force were analyzed, there was a general moderate negative correlation (r_s=-0.4836, p<0.0059). EAE1 group found a strong negative correlation between cumulative score and FAT 10 (r_s=-0.6727, p<0.0330) and EAE2 also found a moderate negative correlation (r_s=-0.4788, p<0.1615). Equal to tetanic force, DOSCAR also did not find any correlation between FAT 10 and cumulative score (r_s=-0.2636, p<0.4334) (figure 8).

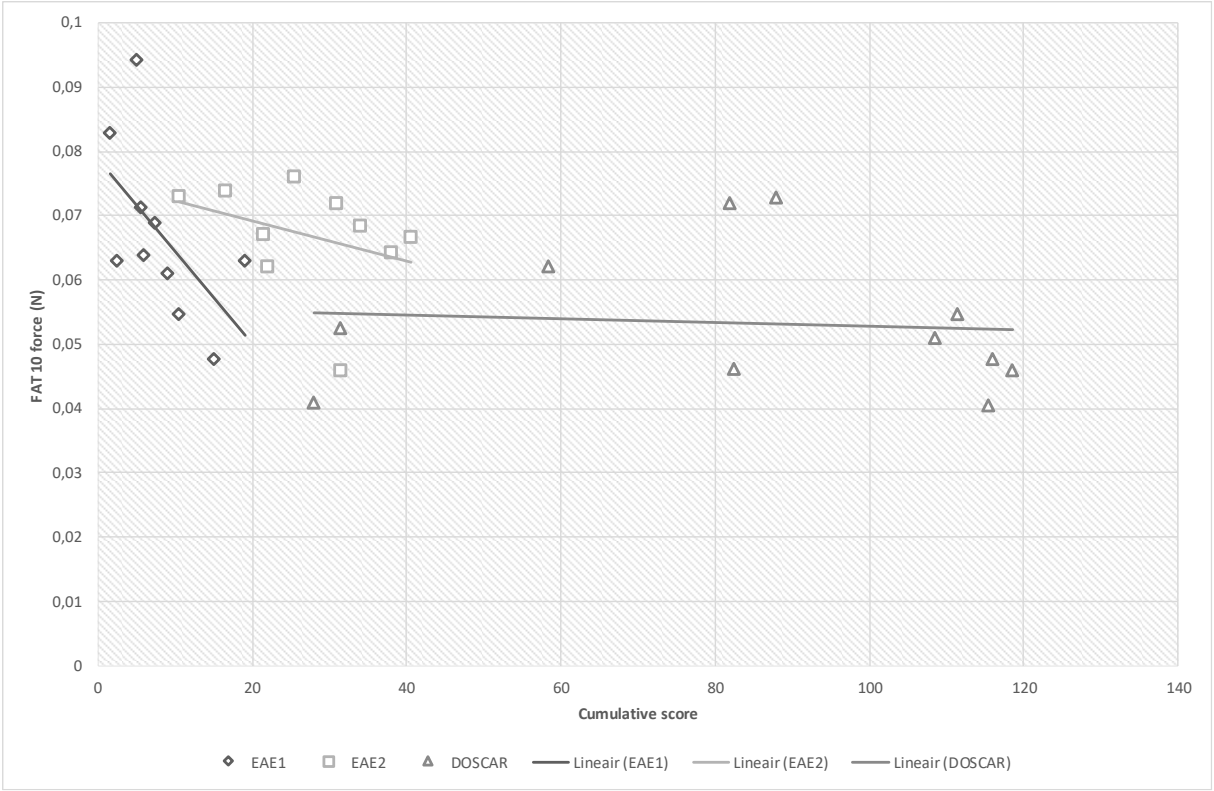

Figure 8. Correlation when comparing FAT 10 with cumulative EAE-score between EAE1, EAE2 and DOSCAR groups

5. Discussion

This study investigates a period of 18, 28 of 56 days MS-related disuse on active force production. The results of this study suggest that, after mimicking MS with an EAE experiment, the characteristics of the skeletal muscles and muscle fibers are changed.

5.1. Muscle mass and CSA

The decrease in muscle mass after 18, 28 and 56 days is not significant. This conflicts with the existing literature. Hanson et al., 2013 state that the decrease in muscle mass already appears after seven days. This is simply explained by the fact that the synthesis/breakdown rate of proteins is altered. No stimuli for synthesis will lead to breakdown. Hanson et al., 2013 also found that over time, the muscle and body mass did not further decrease. This could be due to the fact that the body, and thus the muscles involved, adapts to this new situation. This can be a reason the DOSCAR groups has a higher muscle mass compared to the other two intervention groups.

It was expected that muscle fiber CSA would decrease because it is the main component of muscle atrophy. However, no differences in muscle CSA were found between 4 groups. Boonyarom et al. (2009), Fitts et al. (1991), Hanson et al. (2013), Kasper et al. (1993) and Kim and Thompson (2013) found significant decreases of muscle CSA in the SOL between seven and 14 days. Anyhow, this study could not confirm this finding and thus no significant atrophy is seen. A possible explanation could be an increase in intramuscular fat. An intramuscular adipose tissue development was seen after three days of deconditioning, however this was only the case in human leg muscles after a model of dry immersion (Pagano et al., 2018). The research in the domain of intramuscular fat infiltration after a period of disuse is scarce. Additionally, no significant loss of muscle CSA does not mean that there are no alterations of muscle fiber CSA. Because of the possible shift to type II fibers, there are more fibers in parallel, which increases muscle CSA (Thompson, 2002).

5.2. Active Force – Hz relation

Decrease of maximal tetanic force was found after 18, 28 and 56 days of disuse. This should be due to the loss of muscle mass and contractile proteins (Fitts, McDonald, & Schluter, 1991). However, this finding was not confirmed in this study. A possible explanation remains unclear. Because of the inactivity, muscles undergo atrophy which is the loss of CSA. This atrophy is accompanied with loss of contractile proteins and a decrease of number of cross-bridges per CSA

(Fitts et al., 1991; Thompson, 2002). This loss of contractile proteins can be the reason of the altered protein synthesis which occurs in atrophy.

Fitts et al., 1991 found that the peak specific tension (when force was normalized to CSA) is dependent on the number of cross-bridges. However, the decrease of force for the DOSCAR, but not for the other groups, could imply that it is dependent on the duration of disuse. This decrease can already be seen after one week (Kasper et al., 1993) and could be due to the loss of myofilaments. Because of this, less cross-bridges are formed and forces are lower (Stelzer & Widrick, 2003). When the duration of disuse becomes longer, the number of cross bridges will decrease (Fitts et al., 1991; Thompson, 2002). This could be the reason that only the DOSCAR group has a larger decrease when force was normalized to CSA. However, the EAE2 group has higher forces compared to EAE1 group from 50 Hz to 125 Hz but these were not significant. This can be due to the fiber-type shift from type I to type II. Type II fibers are stronger and can therefore produce more force.

Looking at frequency, only significant differences were seen in the DOSCAR group, implying that duration of disuse has an influence on the active force. It could be due to the fact that the longer the disuse, the less resistant a muscle becomes to higher frequencies.

5.3. FAT-protocol and fiber-type shift

At Fat 0, all three groups are significantly different in active absolute forces compared with CON. This is due the fact that these groups were exposed to disuse where the CON group was not. When comparing the active relative forces of the DOSCAR with the other groups (EAE1, EAE2), we can see that the DOSCAR group decreases with a total of 52% until FAT 10 (significant decrease for DOSCAR compared to CON) while EAE1 and EAE2 only decreased 44% and 43% respectively. It seems that the group that have been exposed to disuse for a longer period, 56 days in particular, is less able to withstand fatigue. A possible explanation is a stronger reduction of type I fibers compared to EAE1 and EAE2 (Boonyarom et al., 2009; Thompson, 2002) (figure 9). Not using the limbs for a longer period results in less activation of the muscles. Antigravity (type I) fibers are more sensitive to disuse compared to type II fibers. This can result in a fiber-type shift from type I to type II (Thompson, 2002). However, there is no significant difference between EAE2 and DOSCAR. This can imply that in the EAE2 group, this fiber-type shift also occurs. Immunohistochemical analysis should provide insight but was not included due to time limitations. Within-group analysis, and thus the comparison between the FAT moments in a single

group (figure 6) revealed that the decrease of active force reached a plateau after FAT 4, and this for all the groups (EAE1, EAE2, DOSCAR and CON). When comparing FAT 3 with FAT 5 and FAT 6, significant differences were found, but comparing FAT 4 with FAT 5 and FAT 6 did not show any significance. Additionally, FAT 10, FAT 9, FAT 8, FAT 7 and FAT 6 did also not differ from each other. Therefore, we can state that this plateau starts from FAT 4. No significant values can be seen from this point until FAT 10. This means that there are still some type I fibers in the SOL muscle who can withstand a fatigue contraction protocol. Looking at recovery, only a difference was seen in the DOSCAR group between FAT 0 and REC 5. This means that recovery is slower compared to other groups. The same is seen in the CON group. More specific, this group also found significance between REC 5 and REC 10 which could imply more type I fibers because of the faster recuperation.

The fiber-type composition of a muscle is associated with the resistance against fatigue. Thompson (2002) stated that type I fibers are more resistant to fatigue at which the strength decreases less rapidly. This in contrast to type II fibers, which are exhausted more quickly (Boonyarom et al., 2009; Kourtidou-Papadeli et al., 2004; Thopson, 2002). After a period of disuse, a fiber-type transformation occurs and the amount of type I fibers decreases whereas type II fibers increases. However, the total amount of fibers remains the same. The decline in type I is therefore compensated by an increase in type II (Boonyarom et al., 2009). This transformation is already seen within a week of inactivity (Thompson, 2002). This can explain why in the FAT-protocol, the DOSCAR group decreases more rapidly in comparison with the CON group. However, the amount of type II fibers does not become predominant. This can explain why active forces of the three EAE groups still remains lower compared to CON during a tetanic contraction. In the healthy CON the fibers are more resistant to fatigue. When comparing the duration of disuse, there is a small difference between 18 and 28 days. In contrast, 56 days had a greater decrease than EAE1, EAE2 and CON, which results in less resistance to fatigue due to less type I fibers. This finding can also be stated by looking at figure 9. DOSCAR group decreases faster from FAT 4 (18% compared to 11 % for EAE1 and 12% for EAE2), which means the resistance to fatigue is lower. Thompson (2002) also believed that a less rapid recovery was the cause of a loss of mitochondria. The fusion rate of the mitochondria is regulated by mitofusin II. Disuse changes the expression of this protein (Feng et al., 2016) and therefore less mitochondria are expressed. This can possibly explain why the values between FAT 10 and REC 5 and REC 5 and REC 10 for CON group are significant. This group consists of more type I fibers and has therefore a shorter recuperation

time. Another possible explanation for the increased fatigue after the FAT protocol, could be due to a greater rate of glycogen depletion as well as more lactate production in type I fibers (Thomson, 2002). A possible interpretation for this phenomenon could be due to the decreased type I fibers in the SOL. The remaining fibers are more loaded to withstand fatigue and produce therefore more lactate.

However, the CON group shows lower relative forces when compared to EAE1 and EAE2. This can be explained by the fact that relative values are used. When absolute values are used, the CON group has a higher active force. Therefore, more force seems to be lost when values are made relative, compared to other groups where forces were lower.

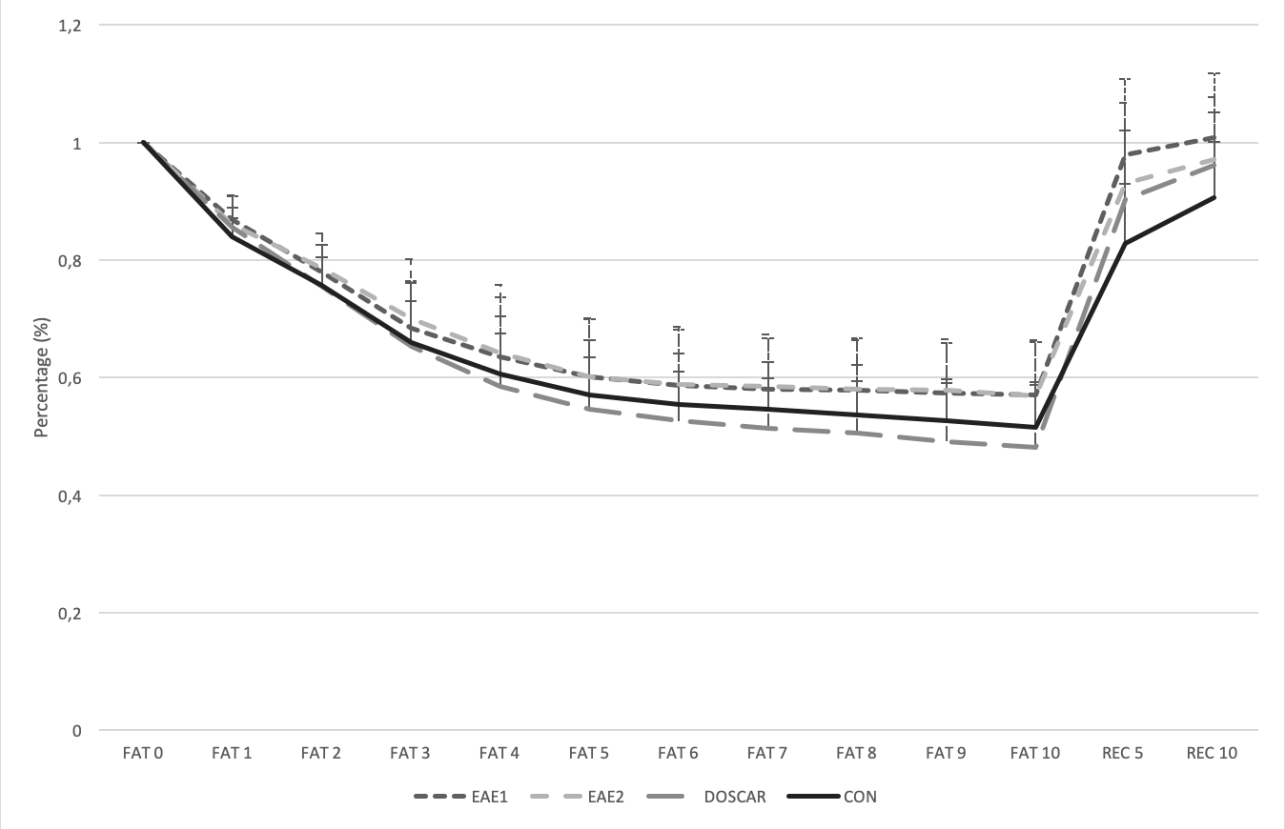

Figure 9. Mean Relative force (%) ± SD (%) for all groups during a fatigue protocol with five and ten minutes' recovery. Depletion of relative force at different FAT and recovery times between groups are shown.

5.4. EAE-score

As mentioned above, there is no general correlation between tetanic force and cumulative score. This could be due to the inclusion of the DOSCAR group, where no correlation was found. A

possible explanation for this given, could be due to outliers within this group. This means that active force values (tetanic and FAT 10) of some mice are more diffuse, which can influence the findings. Hence, no correlation can be seen (figure 7 and 8). The disease model also stabilizes after 28 days (Hookelabs.com), therefore the muscles have the time to adapt. Because of this, the correlation for the DOSCAR group can be lower compared to the EAE1 and EAE2 groups. Around 25% of the mice experience an increase in EAE-score between 20-27 days (Hookelabs.com). Therefore, it can be possible that the mice in the EAE1 and EAE2 groups did not experience this increase, while the mice in the DOSCAR group probably did. An exact reason remains unclear.

A strong negative correlation was found for the EAE1 group and a very strong for EAE2. This means that when EAE-score was higher, the tetanic force as well as FAT 10 force decreases. This was not seen in the DOSCAR group. It could be due to the fact that the environment of the muscles adapts to the disuse on the longer term. However, another explanation could not be found.

5.5. MS

It is believed that disuse related to MS is responsible for the adaptations in their muscle tissue. People with MS experience muscle atrophy, an increased fatigability, less peak power and a decline in muscle cross-sectional area (CSA). However, MS results from a central nervous defect (demyelinisation of the white matter of the central nervous system (CNS) which plays an important role in the disease progress. This central component can't be found when we solely speak about disuse. Disuse is not mediated from the central nervous system, but from an external trigger; not using the muscles anymore. (NG. et al., 2004; KENT-BRAUN et al., 1997; Wens, Eijnde, & Hansen, 2016). However, there are still a lot of similarities with other models of disuse such as HLS. There is a decrease in muscle mass and muscle fiber CSA, decline in peak force and more fatigue (NG et al., 2004; J.A.KENT-BRAUN et al., 1997, Thompson, 2002). Therefore, we could presume that the changes in MS muscle tissue are the result of disuse and less likely result from the disease itself. However, we did not look into the neural changes specifically. It can be possible that there are a lot of neural changes, such as impaired conduction velocity or altered motor units, with the same result as disuse. De HAAN et al. (2000) for example, found that the force loss was due to MS because of the reduction in maximal neural drive. The voluntary contraction of the MS subject was 11.2% lower compared to healthy subjects. NG et al. (2004) also found that changes in voluntary force development were due to central changes in MS. Therefore, the atrophy can also be due to neural changes and not only muscular. Because of this, the assumption should be

made with caution. It is however a step in the right direction in understanding the changes but there is still need for much more research on this topic. This research should also focus on neural changes and not only on muscular.

6. Limitations

6.1. Duration of disease

By giving the mice a score between zero to five, it is possible to know when they don't use the limbs anymore (EAE-score equal or above two). This EAE model is not perfect because not all mice get sick, and some mice do not stay sick once they reached a score of two. By this fact it can cause alterations in the results. For example, no correlation was found in the DOSCAR group due to outliers.

An additional analysis was done where mice were divided in groups by disease time (sick for less than one week, sick between one and two weeks and sick for more than two weeks). These results were not very clear and did not add any contribution in interpreting the results. Therefore, we excluded this part. By grouping them under being sick, it will rule out this problem. This paper contains the results of two different studies, as stated before. The two studies were executed on different time periods. This can give some issues and can explain why the DOSCAR tetanic force has no significant difference with CON group. Also Force – Hz protocol consisted of three more contractions in the DOSCAR group: A pulse of 20Hz, 30Hz and 40Hz. These extra contractions can have a possible impact on the force generated afterwards.

6.2. Execution of protocol

There were more researchers who performed the protocol. This could lead to a lower inter-rater reliability and differences in interpreting and measuring the data. Researchers were also not blinded during protocol. Therefore, an observer bias can occur. However, the protocol used was very strict and therefore the simulations of the muscles lead to reproducibility of the measurements.

7. Conclusion

Soleus muscle active force in EAE mice decreases depending on the duration of disuse compared to healthy controls. This is seen after a fatigue protocol where long term (56 days) disuse has a greater negative impact on the resistance to fatigue and recovery, than short term (18 days) disuse.

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9. Attachments

The mice were subcutaneous injected with 0.1 mL/site emulsion Antigen (MOG35-55 or MOG1-125 in complete Freund's adjuvant (CFA) in emulsion. The mice were acclimatized for seven days before immunization prior to emulsion. Immunization is performed on day zero under isoflurane anesthesia. Day one, mice were injected with Pertussis toxin (PTX).

The injection of PTX went intraperitoneally at 0.1 mL/dose which was repeated 24 hours later. The mice were controlled for signs of EAE starting on day 0 after immunization. As soon as the first signs of paralysis occurred, the mice were provided with food pellets and wet food on the floor of the cage. There was easily access to water. Hydrogel (clear H2O) can be used as a source water.

Figure 2. Injection protocol for EAE induction

Table 1

Mean ± SD force – Hz relationship (N) for different groups.

Group	EAE1	EAE2	DOSCAR	CON
	X ± SD	X ± SD	X ±SD	X ± SD
Twitch	0.0236±0.0053	0.0224±0.0044	0.0249±0.0070	0.0246±0.0038
Tetanus	0.1180±0.0318*	0.1197±0.0259*	0.1219±0.0325	0.1420±0.0155
10 Hz	0.0456±0.0087	0.0364±0.0102	0.0516±0.0170	0.0533±0.0411
25 Hz	0.0978±0.0238	0.0913±0.0196	01025±0.0256	0.1012±0.0244
75 Hz	0.1249±0.0347	0.1295±0.0294	0.1254±0.0329*	0.1494±0.0171
100 Hz	0.1289±0.0374*	0.1329±0.0328*	0.1270±0.0335*	0.1607±0.0184
125 Hz	0.1291±0.0383*	0.1336±0.0345*	0.1265±0.00330*	0.1629±0.0191

*$p < 0.05$ in comparison with CON. EAE1, 18 days; EAE2, 28 days; DOSCAR, 56 days; CON, healthy control at 18 days.

Appendix 1: Inventarisatieformulier Budo Arne

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KNOWLEDGE IN ACTION

INVENTARISATIEFORMULIER WETENSCHAPPELIJKE STAGE DEEL 2

DATUM	INHOUD OVERLEG	HANDTEKENINGEN
27/9/2018	Bespreking onderwerp thesis deel 2	Promotor: Copromotor/Begeleider: Student(e): Student(e):
18/10/2018	Bespreking thesis + data analyse uitleg	Promotor: Copromotor/Begeleider: Student(e): Student(e):
24/10/2018	Bespreking thesis + data analyse uitleg	Promotor: Copromotor/Begeleider: Student(e): Student(e):
6/02/2019	Skype sessie: bespreking vervolg data-analyse + statistiek	Promotor: Copromotor/Begeleider: Student(e): Student(e):
1/07/2019	verdere uitwerking onafgewerkte taken + bespreking	Promotor: Copromotor/Begeleider: Student(e): Student(e):
10/07/2019	Bespreking statistiek + vragen	Promotor: Copromotor/Begeleider: Student(e): Student(e):
		Promotor: Copromotor/Begeleider: Student(e): Student(e):
		Promotor: Copromotor/Begeleider: Student(e): Student(e):
		Promotor: Copromotor/Begeleider: Student(e): Student(e):
		Promotor: Copromotor/Begeleider: Student(e): Student(e):

In te vullen door de promotor(en) en eventuele copromotor aan het einde van MP2:

Naam Student(e): Arne Budo **Datum:** 9/8/2019

Titel Masterproef: De intrinsieke spieraanpassingen na disuse

1) Geef aan in hoeverre de student(e) onderstaande competenties zelfstandig uitvoerde:
   - NVT: De student(e) leverde hierin geen bijdrage, aangezien hij/zij in een reeds lopende studie meewerkte.
   - 1: De student(e) was niet zelfstandig en sterk afhankelijk van medestudent(e) of promotor en teamleden bij de uitwerking en uitvoering.
   - 2: De student(e) had veel hulp en ondersteuning nodig bij de uitwerking en uitvoering.
   - 3: De student(e) was redelijk zelfstandig bij de uitwerking en uitvoering
   - 4: De student(e) had weinig tot geringe hulp nodig bij de uitwerking en uitvoering.
   - 5: De student(e) werkte zeer zelfstandig en had slechts zeer sporadisch hulp en bijsturing nodig van de promotor of zijn team bij de uitwerking en uitvoering.

Competenties	NVT	1	2	3	4	5
Opstelling onderzoeksvraag	O	O	O	O	O	O
Methodologische uitwerking	O	O	O	O	O	O
Data acquisitie	O	O	O	O	O	O
Data management	O	O	O	O	O	O
Dataverwerking/Statistiek	O	O	O	O	O	O
Rapportage	O	O	O	O	O	O

2) Niet-bindend advies: Student(e) krijgt toelating/geen toelating (schrappen wat niet past) om bovenvermelde Wetenschappelijke stage/masterproef deel 2 te verdedigen in bovenvermelde periode. Deze eventuele toelating houdt geen garantie in dat de student geslaagd is voor dit opleidingsonderdeel.

3) Deze wetenschappelijke stage/masterproef deel 2 mag wel/niet (schrappen wat niet past) openbaar verdedigd worden.

4) Deze wetenschappelijke stage/masterproef deel 2 mag wel/niet (schrappen wat niet past) opgenomen worden in de bibliotheek en docserver van de UHasselt.

Datum en handtekening Student(e)	Datum en handtekening promotor(en)	Datum en handtekening Co-promotor(en)
9/08/2019		

Appendix 2: Inventarisatieformulier Roets Gregory

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INVENTARISATIEFORMULIER WETENSCHAPPELIJKE STAGE DEEL 2

DATUM	INHOUD OVERLEG	HANDTEKENINGEN
27/09/2018	Bespreking onderwerp thesis deel 2	Promotor: Copromotor/Begeleider: Student(e): Student(e):
18/10/2018	Bespreking thesis + data-Analyse uitleg	Promotor: Copromotor/Begeleider: Student(e): Student(e):
24/10/2018	Bespreking thesis + data-Analyse uitleg	Promotor: Copromotor/Begeleider: Student(e): Student(e):
06/02/2019	Skype sessie: Bespreking vervolg data - analyse + statistiek	Promotor: Copromotor/Begeleider: Student(e): Student(e):
01/07/2019	Verdere uitwerken van onafgewerkte taken + bespreking	Promotor: Copromotor/Begeleider: Student(e): Student(e):
10/07/2019	Bespreking statistiek + vragen	Promotor: Copromotor/Begeleider: Student(e): Student(e):
		Promotor: Copromotor/Begeleider: Student(e): Student(e):
		Promotor: Copromotor/Begeleider: Student(e): Student(e):
		Promotor: Copromotor/Begeleider: Student(e): Student(e):
		Promotor: Copromotor/Begeleider: Student(e): Student(e):

In te vullen door de promotor(en) en eventuele copromotor aan het einde van MP2:

Naam Student(e): Gregory Roets **Datum:** 09/08/2019

Titel Masterproef: De intrinsieke spieraanpassingen na disuse

1) Geef aan in hoeverre de student(e) onderstaande competenties zelfstandig uitvoerde:
   - NVT: De student(e) leverde hierin geen bijdrage, aangezien hij/zij in een reeds lopende studie meewerkte.
   - 1: De student(e) was niet zelfstandig en sterk afhankelijk van medestudent(e) of promotor en teamleden bij de uitwerking en uitvoering.
   - 2: De student(e) had veel hulp en ondersteuning nodig bij de uitwerking en uitvoering.
   - 3: De student(e) was redelijk zelfstandig bij de uitwerking en uitvoering
   - 4: De student(e) had weinig tot geringe hulp nodig bij de uitwerking en uitvoering.
   - 5: De student(e) werkte zeer zelfstandig en had slechts zeer sporadisch hulp en bijsturing nodig van de promotor of zijn team bij de uitwerking en uitvoering.

Competenties	**NVT**	**1**	**2**	**3**	**4**	**5**
Opstelling onderzoeksvraag	O	O	O	O	O	O
Methodologische uitwerking	O	O	O	O	O	O
Data acquisitie	O	O	O	O	O	O
Data management	O	O	O	O	O	O
Dataverwerking/Statistiek	O	O	O	O	O	O
Rapportage	O	O	O	O	O	O

2) Niet-bindend advies: Student(e) krijgt toelating/geen toelating (schrappen wat niet past) om bovenvermelde Wetenschappelijke stage/masterproef deel 2 te verdedigen in bovenvermelde periode. Deze eventuele toelating houdt geen garantie in dat de student geslaagd is voor dit opleidingsonderdeel.

3) Deze wetenschappelijke stage/masterproef deel 2 mag wel/niet (schrappen wat niet past) openbaar verdedigd worden.

4) Deze wetenschappelijke stage/masterproef deel 2 mag wel/niet (schrappen wat niet past) opgenomen worden in de bibliotheek en docserver van de UHasselt.

Datum en handtekening Student(e)	Datum en handtekening promotor(en)	Datum en handtekening Co-promotor(en)
09/08/2019