High intensity exercise in an animal model for Multiple Sclerosis: impact on glucose tolerance.

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Research context

The REVAL Rehabilitation Research Center of the Biomedical Research Institute of Hasselt University focuses on the rehabilitation of neurodegenerative diseases, notably Multiple Sclerosis (MS). This includes optimizing rehabilitation protocols and clinical outcome as well as investigating the underlying mechanisms of the overall therapeutic effect of MS rehabilitation. As such and in collaboration with national and international partners, REVAL researchers recently investigated the impact of different exercise intensities (light, moderate, high) and modalities (strength training, cardiorespiratory training, whole body vibration, electrostimulation, cooling) on exercise capacity, muscle contractile properties, spasticity, motor coordination. In particular, in an animal MS model (experimental autoimmune encephalomyelitis or EAE) REVAL investigators demonstrated that exercise therapy may have the potential to delay onset of hind limb paralysis[1]. The REVAL center of the Hasselt University Biomedical Research Institute has fully equipped physical training/rehabilitation facilities (Technogym). Furthermore, REVAL provides in measurement instruments to assess cardiorespiratory fitness (Jaeger, Oxycon), muscle strength (Biodex isokinetic dynamometer), body composition (DEXA scan), muscle contractile properties (histochemistry lab) and muscle energy metabolism (biochemistry lab). This master thesis is part of the doctoral research of Dr. Inez Wens, investigating the effects of different training intensities and modalities on glucose tolerance in MS and EAE. Consequently, the determination of the research design, recruitment of the participants and the specific data collection was within the context of the above described project. To become familiarized with all the experimental procedures, both students participated in similar measurements associated with parallel/similar research projects promoted by Prof. dr. Bert Op 't Eijnde, Prof. dr. Dominique Hanssen & Prof. dr. Frank Vandenabeele. Hereafter and to minimize data registration errors, both students analyzed data independently. Data interpretation was performed in close collaboration with the (co)promotor. Statistical procedures and academic writing as well as reproducing the results and discussion were accomplished by both students together.

Abstract

Background. The prevalence of impaired glucose tolerance is increased in individuals with Multiple Sclerosis (MS). Similar to other populations, it has been suggested that higher intensity rehabilitation/training programs could improve glucose tolerance in MS. However, because the use of a high intensity program was never investigated before in this population, it seems warranted to first explore this in an animal (rat) model for MS, notably experimental autoimmune encephalomyelitis (EAE).

Objective. This thesis aims to investigate the impact of high intensity exercise on glucose tolerance (GT) and insulin in EAE rats.

Methods. Control (CON) and EAE rats were divided in sedentary (CON-SED, EAE-SED) and exercise (CON-EX, EAE-EX) subgroups. Glucose tolerance was assessed by measuring the total area under the curve (tAUC) after an oral glucose tolerance test (OGTT, 2g/kg BW) immediately after a 10d training period of treadmill running (Study 1, groups indicated by '$_1$') or after a 7d recovery period following training (Study 2, groups indicated by '$_2$'). Body weight (BW), food intake (FI) and hind limb paralysis (clinical score) were monitored daily.

Results. Glucose and insulin tAUC did not differ between groups. Exercise did not affect glucose and insulin tAUC in CON and EAE. However, compared to Study 1, glucose tAUC of EAE-CON, EAE-EX and CON-EX of Study 2 increased by +24%, +41% and +25% respectively. Furthermore, in EAE exercise didn't exacerbate the clinical symptoms and improved BW recovery (+11% for EAE-EX$_2$ compared to +6% for EAE-SED$_2$ from day 1-11). Similar beneficial effects where shown for rats of Study 1 and on FI in both studies.

Conclusion. EAE nor exercise were able to affect glucose tolerance. However, glucose tAUC was increased after hindquarter paralysis.

Keywords. autoimmune encephalomyelitis, experimental; rats; exercise; glucose; insulin; rehabilitation

Introduction

Multiple Sclerosis (MS) is an autoimmune disease characterized by demyelination of the central nervous system leading to heterogeneous and complex symptoms affecting whole body functioning. As a result, individuals with MS are less physically active than their healthy controls[2].

In healthy people and other populations, physical inactivity contributes to the development of secondary health problems, such as cardiovascular diseases, elevated blood pressure, obesity and diabetes mellitus type II (T2DM), preceded by impaired glucose tolerance (IGT)[3-5]. Interestingly, Wens et al. (2013) already reported an increased risk to develop the metabolic syndrome and cardiovascular disease (CVD) in individuals with MS. In particular, this review reported that type 2 diabetes (T2DM) in MS occur in a similar, or slightly higher way comparing to the general population[6]. Furthermore, another study concluded an increased IGT prevalence in MS patients, compared to matched healthy controls, indicating metabolic defects that may increase comorbidity[7]. As described in Jeon et al. (2007), T2DM is a chronic disease, characterized initially by insulin resistance and eventually by glucose intolerance[8]. The skeletal muscle insulin resistance is defined by an elevated level of blood glucose and can be diagnosed by an oral glucose tolerance test (OGTT)[9, 10].

In general, the rehabilitation of individuals with secondary health complications and glucose intolerance consist primary on increasing physical activity[11, 12]. Some studies reported that low- to moderate exercise intensity improved glucose tolerance[13, 14], whereas other findings suggested that this level of intensity was not sufficient to improve insulin sensitivity[15-17]. Noteworthy, low- to moderate intensity exercise in individuals with MS does not result in changes in IGT[18]. Nonetheless, the effect of high intensity exercise on IGT in individuals with MS was never investigated before.

Several studies reported a beneficial impact of moderate intensity exercise on strength, exercise capacity, depression, fatigue and quality of life in individuals with MS[19-21]. Interestingly, several authors suggested that high intensity programs could result in more beneficial effects in MS patients[22-25]. However, since the use of a high intensity exercise program was never investigated in MS, and it is unclear whether this could be tolerated[22, 23], the use of an animal model seems appropriate[26].

Experimental autoimmune encephalomyelitis (EAE) in rats is one of the most widely accepted animal model of MS[27, 28]. The model is characterized by progressive hindquarter paralysis, starting at the tip of the tail at day 12-14 and increasingly to the trunk until day 17 after induction[1]. So far, the influence of EAE induction on glucose tolerance in rats was never investigated before. Nonetheless, to test the effects of insulin on the body's ability to dispose glucose in animal research, a glucose tolerance test (GTT), oral or intraperitoneal, is commonly used[29]. In the first part of this master thesis it has been concluded that insulin resistance in obese and diabetic rats improved after training[30-32]. However, physical activity in different types of rats altered the plasma glucose- and insulin concentration, as well as the insulin secretion and plasma glucose after glucose administration, in an inconsistent manner[33]. The varying type of protocol used for the exercise intervention could be a reason to clarify the different

conclusions[33]. Nevertheless, the influence of exercise on glucose tolerance in EAE rats was never investigated before.

In keeping with the above line of reasoning, this thesis aims to investigate the impact of high intensity exercise on glucose tolerance (GT) and insulin in EAE rats. It is hypothesized that EAE induction has a negative impact on GT and insulin concentration and that training improves these parameters.

Materials and methods

Animals

Female Lewis rats (n=80, age 6-7 weeks, body weight 120-170 g), obtained from the Harlan CPB, Zeist, The Netherlands, were kept in the animal facilities of Hasselt University. The animals were housed in individual cages on a light:dark cycle of 12:12h, a temperature of 22°C and a relative humidity of 22-24%. The rats were fed ad libitum with normal rat pellets (Carfil RN-01-K12, Harlan). Moreover, the National Research Council's guide for the care and use of laboratory animals was followed. The animal Ethics Committee of Hasselt University approved the study protocol in accordance with the national and European legislation.

Study design

All rats underwent an acclimatization period (-21d) which consists of handling, resting and adaptation of the new environment, to re-stabilize the behavioral and physiologic parameters. Following acclimatization and adaptation rats were registered in a treadmill running training program (SED, n=40, sedentary training and EX n=40, high intensity training). From then onwards, daily food intake and body weight were registered. During habituation (-14d) animals were familiarized to treadmill running (day -14 to -1) to reduce the treadmill-induced stress and to ascertain that every rat was able to run the imposed protocol[34]. The training durations and intensities was progressively increased until EX rats ran 1 hour at 18m/min (25° inclination). SED animals were subjected to similar daily manipulation. Consequently, the two groups were subdivided in a healthy control (CON-SED and CON-EX) and EAE group (EAE- SED and EAE-EX). The rats assigned to the EAE-SED and EAE-EX groups were induced to EAE at day 0. Thereafter, the EAE-EX and CON-EX groups were subjected to a daily training until progressive hindquarter paralysis at approximately day 11 prevented this. However, if an animal developed hindquarter paralysis before day 11, preventing daily exercise, the exercise program was immediately terminated, in accordance with the designated endpoint of exercise, where after the animal was excluded and humanely euthanized by an intracardial injection of pentobarbital sodium. Animals which completed the trainings period were divided in two groups, tested by oral glucose tolerance test (OGTT) and euthanized at two different moments (Study 1 and 2). This because suggestions have been made that one week of inactivity during the paralysis period of EAE rats, which was also applied in the control rats, may have tempered training effects [1, 35].

Study 1: Blood samples of forty rats (10 $EAE\text{-}SED_1$, 10 $CON\text{-}SED_1$, 10 $EAE\text{-}EX_1$ and 10 $CON\text{-}EX_1$) were taken immediately after the training period and before the onset of hindquarter paralysis, where after rats were euthanized. To avoid acute exercise-induced training effects, tests were performed between twenty-four and thirty-six hours after the last exercise bout.

Study 2: After completion of the training period, rats (10 $EAE\text{-}SED_2$, 10 $CON\text{-}SED_2$, 10 $EAE\text{-}EX_2$ and 10 $CON\text{-}EX_2$) were kept sedentary and allowed to a partial symptom recovery of one week. Hereafter, blood sampling, testing and euthanasia were performed (day 17).
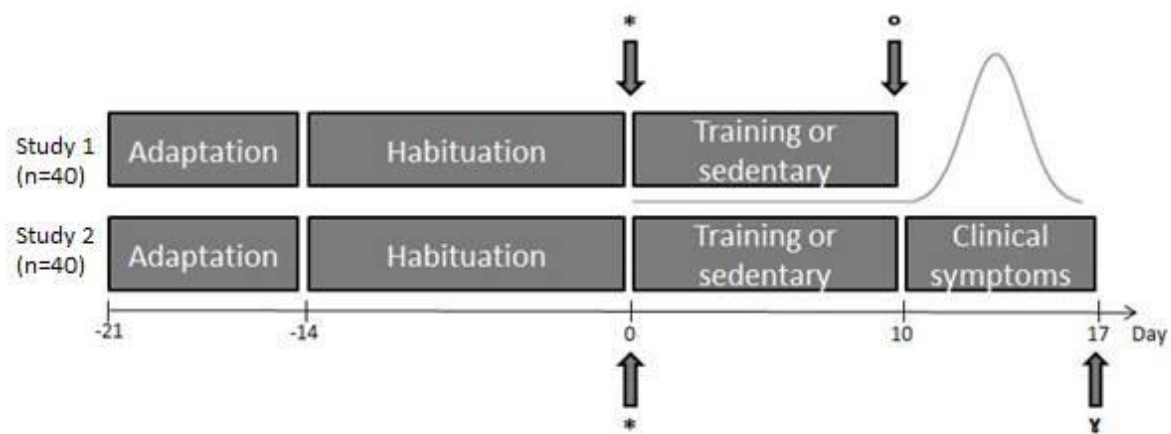
Figure 1. (Adapted with permission from Dr. Wens I.) Study design. * Start of daily training. ° Testing and euthanasia rats Study 1. [x] Testing and euthanasia rats Study 2.

EAE induction

EAE was induced in EAE subgroups by a single percutaneous injection in both footpads (100µl/foot) under isoflurane anesthesia and consisted, per animal, of 24µl purified myelin basic protein (MBP, 25mg/ml) in combination with 25µl 7RA heat killed mycobacterium tuberculosis (20mg/ml, Difco), 120µl complete Freunds adjuvant (CFA, Difco) and 31µl phosphate-buffered saline (PBS)[36].

Training

The intention of this study was to train at a high intensity just below the maximal lactate steady state (MLSS). In pathogen-free rats the MLSS is reached at a velocity of 20m/min[37-40]. Given the fact that the rats are induced with EAE, the training intensity is set at 18m/min (25° inclination). Based on Dudley et al. (1982), the duration of the exercise sessions is determined at 60 minutes, since a peak response of oxidative capacity is achieved at 60 minutes[41]. The EAE-SED and CON-SED groups were exposed to a stationary treadmill for the same amount of time as the EX groups, in order to avoid behavioral inequalities between groups[42.]

Primary outcome measures

Glucose tolerance and insulin concentration

The Oral Glucose Tolerance Test (OGTT) was used to evaluate whole body glucose tolerance. At the day of euthanasia, after a 16 hour fasting, two grams of glucose per kg body weight (50% solution; Merck KGaA, Darmstadt, Germany) was administered into the stomach of the rats through a gastric catheter. Blood was taken from a small cut in the tip of the tail immediately before (0 min) and 20, 40, 60, 80, 100 and 120 minutes after glucose administration for measurement of blood glucose levels. For measurement of serum insulin levels, venous blood was taken at 0, 60 and 120 minutes under gas anesthesia.

Blood glucose was determined by the Analox GM7 (Analis SA, Namur, Belgium) and insulin by electrochemoluminiscence ELISA (Meso Scale Discovery). Concentrations of glucose and insulin were

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measured to express glucose and insulin responses at the different time points. To have a clear view about glucose tolerance, total area under blood glucose and insulin curves (tAUC) were calculated according to the trapezoidal method. A higher tAUC value means a higher glucose/insulin value in serum[7]. To investigate the disease effect, CON-SED and EAE-SED were compared. Exercise effects of healthy control rats and EAE induced rats were calculated by comparing CON-SED vs. CON-EX and EAE-SED vs. EAE-EX respectively.

Secondary outcome measures
Body weight and food intake
Daily body weight and food intake were monitored at eight a.m. using a digital balance (Sartorius®, Germany) and expressed in grams (g).

Clinical score
From the start of the training period, symptoms were daily observed and appointed on a 0-5 scale. 0 - no signs; 0,5 - partial loss of tail tonus; 1 - complete loss of tail tonus; 2 - hind limb paresis; 3 - hind limb paralysis; 4 - moribund; 5 - death due to EAE[36]. This score was used to register a disease and training effect on clinical symptoms. Disease peak was defined as the highest clinical score of each animal.

Statistical Analysis
Statistical Package for the Social Sciences (SPSS) was used for statistical analyses. Shapiro-Wilk test was used to check normality. For comparison of data between two groups, a non-parametric independent sample t-test was performed. Significance was set at $p < 0,05$.

Results

Primary outcome measures

<u>Study 1</u>

Glucose concentration and glucose tAUC

A representative image of glucose concentrations during OGTT of all four groups is shown in Figure 2. At baseline there were no differences in glucose concentrations between groups. In addition, after glucose intake, glucose concentration changes remained comparable between groups during the complete course of the OGTT.
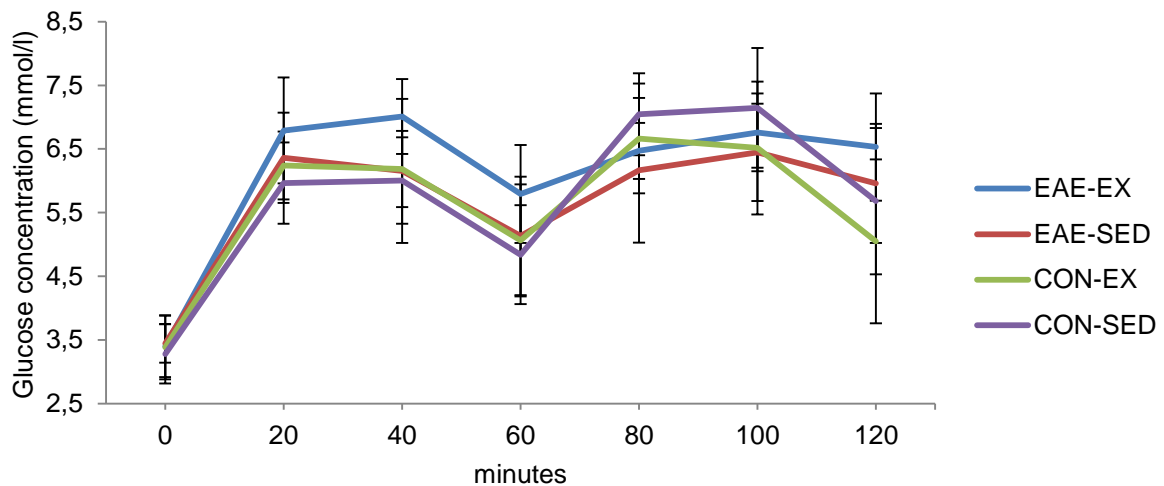

 Figure 2. Blood glucose concentration on 0, 20, 40, 60, 80, 100 and 120 minutes (Study 1, n=40). Values are means and ±SE.

Glucose tAUC from the sedentary healthy controls did not differ from the tAUC of the sedentary EAE group (Figure 3). Furthermore, there was no difference between the tAUC of the CON-SED compared to the tAUC of the CON-EX group. Likewise, the tAUC of the EAE-SED group did not differ from these of the EAE-EX.
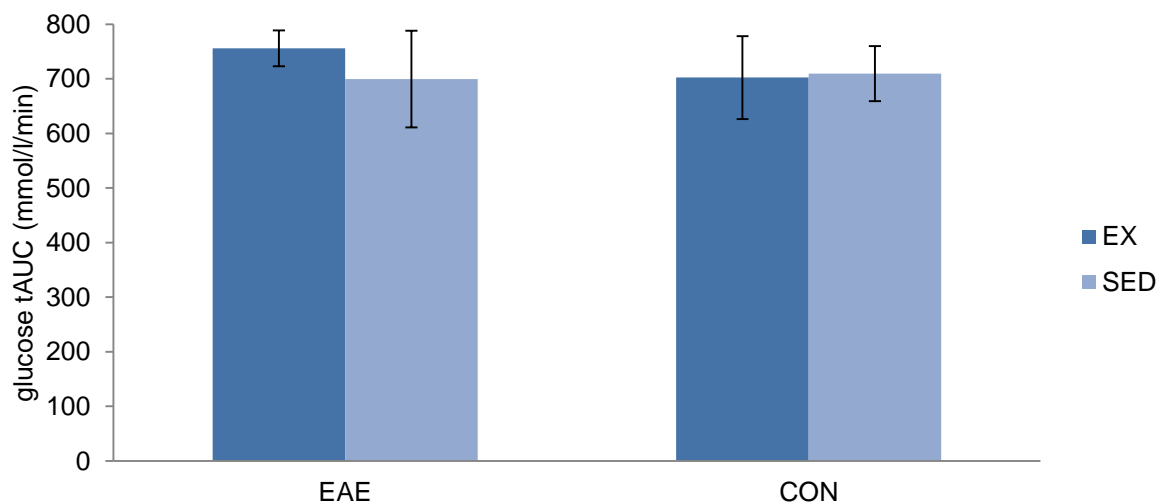

Figure 3. Glucose tAUC (Study 1, n=40) expressed in means and ± SE. No significant (p>0,05) difference compared to groups.

Insulin concentration and insulin tAUC

Serum insulin concentration of healthy control and EAE are shown in Figure 4. At baseline and after glucose intake, insulin profiles were comparable between all groups.
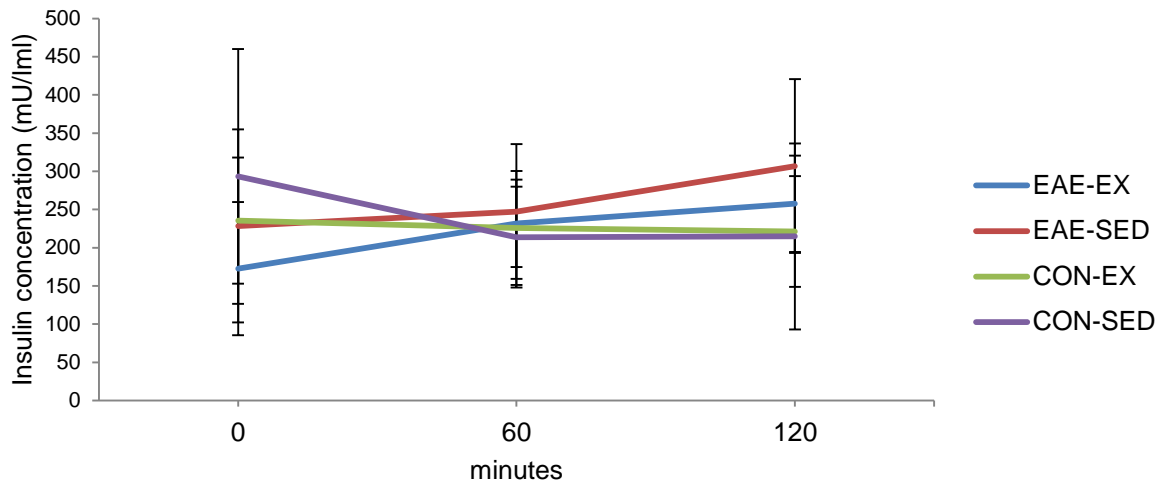

Figure 4. Insulin concentration on 0, 60 and 120 minutes (Study 1, n=40). Values are means and ±SE.

In addition, insulin tAUC did not differ between EAE-SED and CON-SED (Figure 5). Furthermore, insulin tAUC did not differ between EAE groups (EAE-EX vs. EAE-SED), nor between healthy control groups (CON-EX vs. CON-SED).
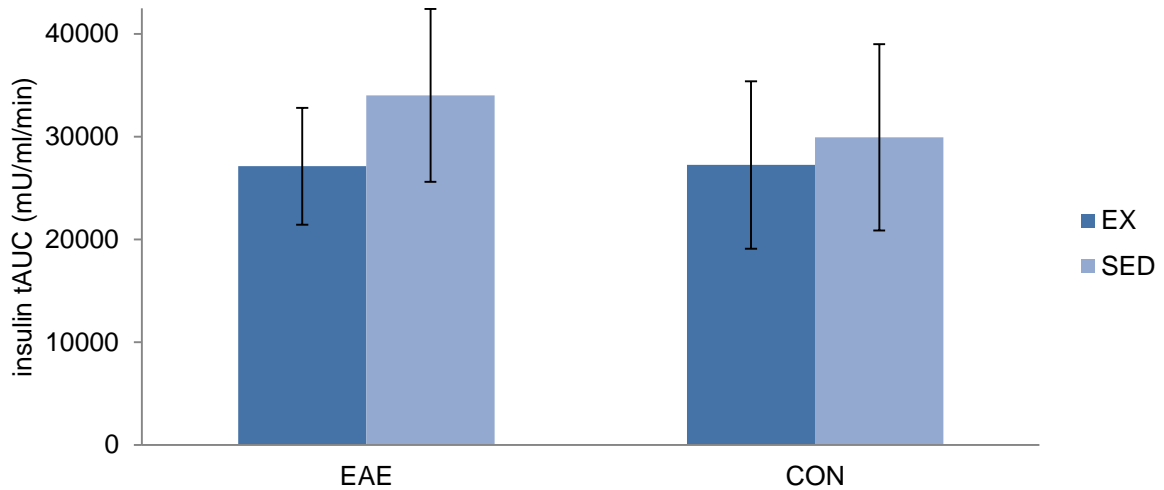

Figure 5. Insulin tAUC (Study 1, n=40) expressed in mean and ±SE. No significant (p>0,05) difference compared to groups.

Study 2

Primary outcome measures

Blood glucose and insulin concentration after OGTT of healthy control and EAE resulted in comparable graph as in Study 1 (data not shown).

Furthermore, same conclusions as Study 1 were found for glucose tolerance and insulin concentration. In particular, glucose and insulin tAUC did not differ between EAE-SED and CON-SED. Likewise, there was no significant difference for glucose and insulin tAUC between EAE groups (EAE-EX vs. EAE-SED), and between healthy control groups (CON-EX vs. CON-SED) (data not shown).

Comparison of Study 1 and Study 2

To investigate the suggested difference in glucose tolerance of Study 1 and 2, the total area under the curve of all groups from both studies were compared. As shown in Figure 6a, glucose concentration during OGTT in EAE-SED (p=0,016), EAE-EX (p=0,040) and CON-EX (p=0,049) were significantly enhanced in Study 2 compared to Study 1 with 24%, 41% and 25% respectively. Nevertheless, the insulin tAUC did not differ between the two studies in all groups (Figure 6b).
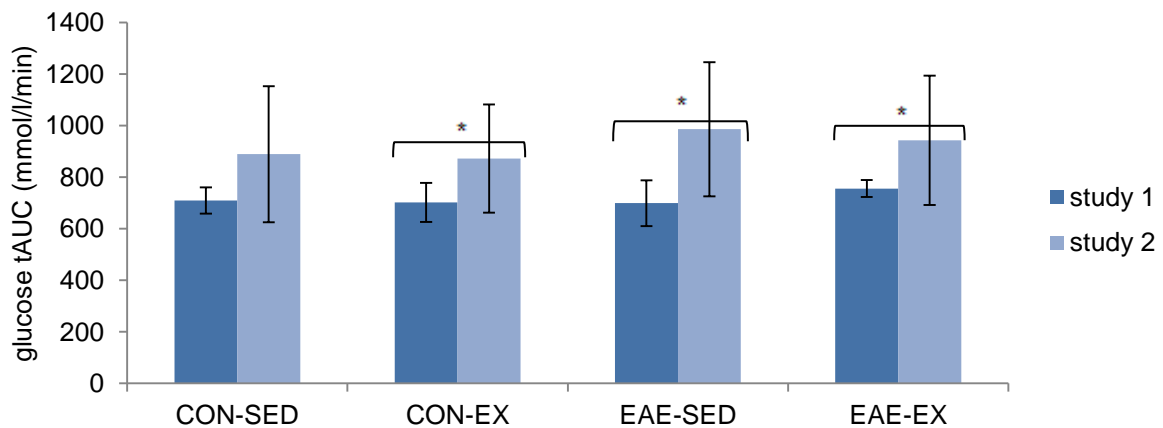

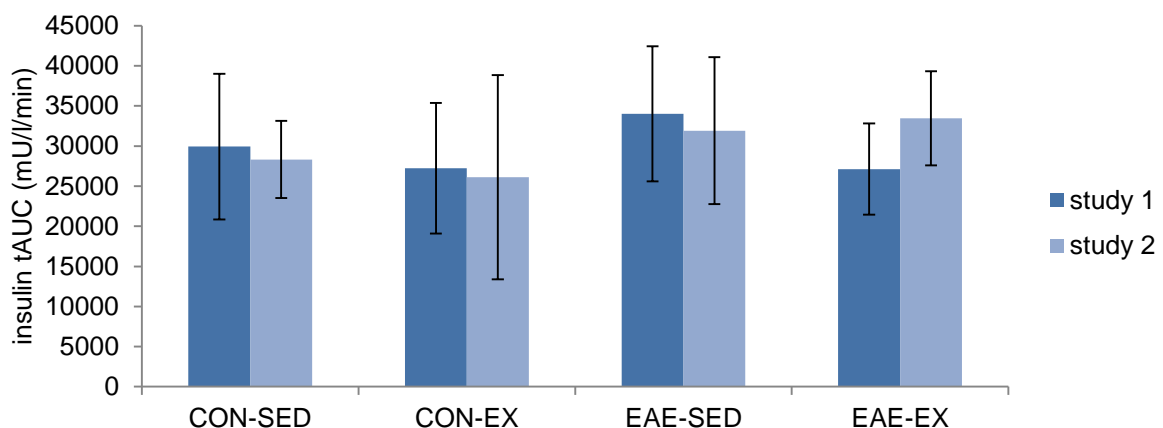**Figure 6a.** Glucose tAUC of all groups of Study 1 and 2. * significant difference between Study 1 and 2 (p>0,05)

Figure 6b. Insulin tAUC of all groups of Study 1 and 2. No significant (p>0,05) difference.

Secondary outcome measures

The body weight increased progressively during habituation period in all groups of Study 1 and 2. As shown as in Figure 7, the parameter decreased immediately after EAE induction with circa 9% (day 0-1). The body weight recuperated gradually until the onset of hindquarter paralysis (day 11, Study 1 and 2). Hereafter, the weight decreased progressively during the symptom manifestation until day 17 (only applicable for Study 2). Exercise in EAE rats resulted in a higher increase of body weight recovery (+11% for EAE-EX compared to +6% for EAE-SED at day 11). After onset of clinical symptoms, body weight reduced equally in both groups (-circa 15% at day 17). However, significance could not be evinced. Nevertheless, the process of the BW of the healthy control rats in Study 2 increased gradually after day 0, with a total difference of BW +20% at the day of euthanasia compared to the EAE rats. The course of the food intake can be illustrated in the same manner (data not shown). Also for Study 1, data had the same course as shown as in Figure 4, except the graphs stops at an earlier time period (day 11).
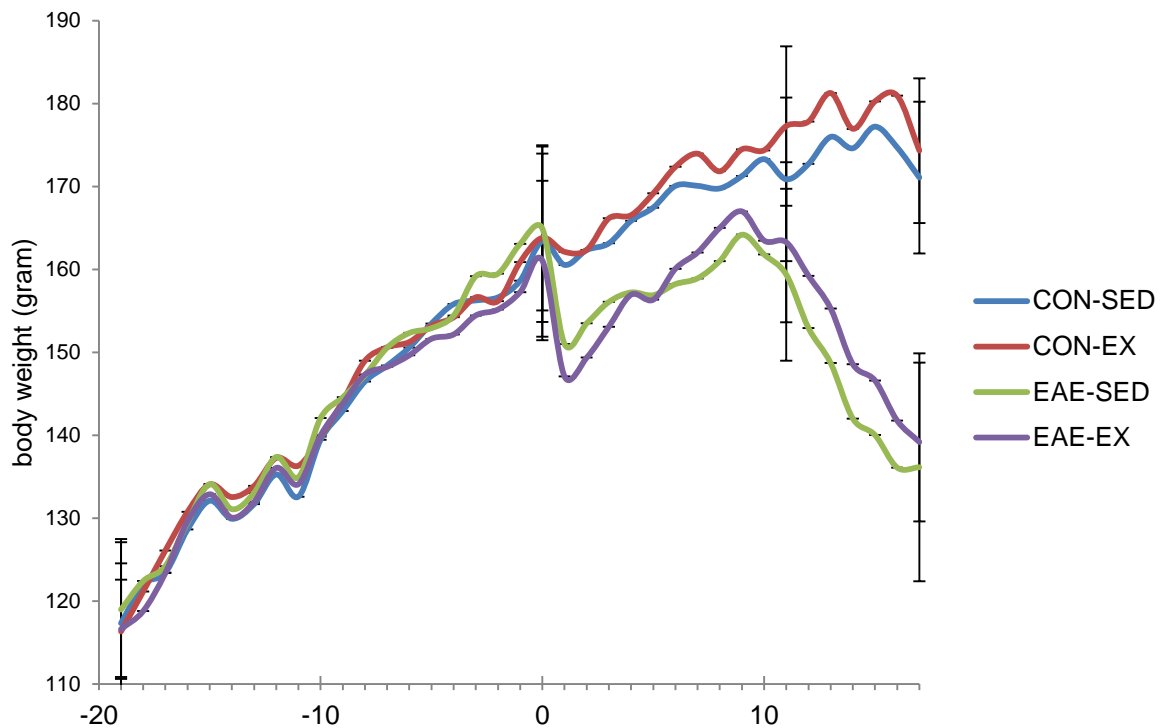

Figure 7. Body weight (gram) of rats from Study 2. Values are means ± SE at day-19, 0, 11 and 17.

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Clinical score

In Study 2, clinical scores were daily monitored from day 0 and depicted in Figure 8. There were no significant differences in clinical scores during the partial symptom recovery. Nevertheless, a delay in onset of symptoms can be noted in EAE-EX. However, significance could not be evinced.
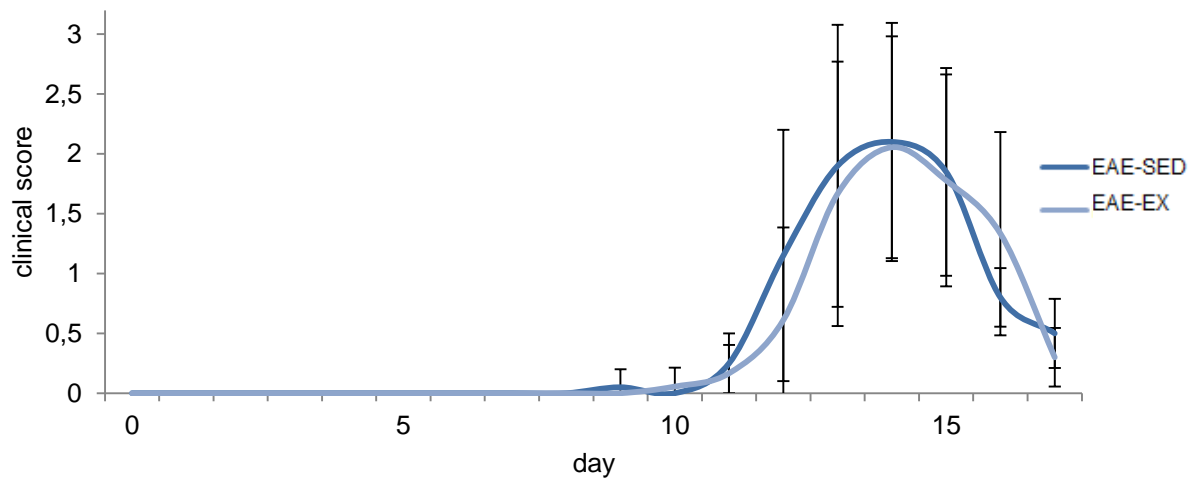

Figure 8. Clinical score of EAE rats of Study 2.

Drop out

One rat (EAE-EX) died during Study 2 by a treadmill accident and was not taken into account while analyzing the results.

Discussion

This study investigated the impact of high intensity treadmill exercise on glucose tolerance and serum insulin in EAE rats. Within the conditions of the present study, we clearly demonstrated that EAE and exercise did not alter glucose and insulin concentration during OGTT (Study 1 & 2). Furthermore, hind limb paralysis and/or inactivity (Study 2) increased glucose tAUC of EAE-SED, EAE-CON and CON-EX rats.

EAE and OGTT

Because it was assumed that rats would not withstand more than one OGTT, rats (n=80) were divided in two groups, Study 1 and 2. An OGTT was performed once in each study (day 11 and 17 respectively). Separating the rats had an negative influence on the sample size and the power. Furthermore, while observing the data during the OGTT, no peak concentration of glucose and insulin were noticed in all groups from both studies. This could be due to the long time intervals between the blood sampling. Missing the peak concentration has an adverse effect on the tAUC because more difference between tAUC could be observed when measuring the peak reaction of glucose and insulin during OGTT. Additionally, even though the OGTT is a common used tool to investigate glucose tolerance in rats, it is not assumed as the 'gold-standard'.

EAE and high intensity treadmill training

High intensity treadmill training was used to investigate the effect on GT and insulin. Suggestions were made that treadmill training is more accessible to set equal intensity parameters and assume equal workload/energy consumption compared to swimming training. Furthermore, research that applied treadmill training has shown that insulin resistance in obese and diabetic rats improved after training[31, 32], this affirms the choice of the exercise type. Wens et al 2015, investigated the effect of exercise intensities, were the high intensity exercise (HI, 18/min, 25° inclination) showed more improvements compared to low- and moderate intensity, therefore HI was selected for this study[1]. The beneficial effects of high intensity training were also suggested by previous research[22-25]. However, it is still difficult to compare studies considering the usage of varying intensity, volume and type of intervention. The intention of this study was to train just below the maximal lactate steady state (MLSS). In pathogen-free rats the MLSS is reached at a velocity of 20m/min[37-40]. Even though lower velocity was applied in this recent study, we can assume that exercise intensity was near the MLSS because inclination of 25° was added. However, lactate concentrations and spirometric values were not measured in this study to confirm this anaerobic state.

Exercise effect on primary and secondary outcome measures

The daily training didn't have an influence on the GT and IS in all groups (EAE and CON-rats Study 1 and 2). This could be due to the fact that EAE induction did not alter GT and IS in the first place. Moreover, it could be due to the period of inactivity[31], a not high enough exercise intensity[32],

frequency, volume[32], program duration[31] and/or too low sample size. Nonetheless, exercise didn't exacerbate clinical symptoms in EAE and it seems that it delayed the hindquarter paralysis onset. Furthermore, high intensity exercise diminished body weight and food intake drop after EAE-induction.

Disease effect on primary and secondary outcome measures

Despite the fact that MS patients have a higher IGT prevalence[6, 7] and EAE is the most commonly used animal model for MS[27], no studies investigated heretofore the influence of EAE induction on glucose tolerance in rats. In the recent study, no disease effect was determined in the matter of glucose and insulin values the day after the last training bout and after partial hindquarter paralysis recovery. Nevertheless, an effect of EAE induction was established in the secondary outcome measures. Furthermore, the period of clinical symptoms had an impact on the GT since glucose concentrations were higher after the symptom period (EAE_2) compared to direct after the training period (EAE_1) whereas insulin concentration didn't differ. This induces the suggestion that glucose tolerance could be influenced through decreased insulin receptor sensitivity and/or glucose transporter type 4 (GLUT4) expression during the symptom period. It can be confirmed by the fact that glucose tAUC in $EAE-SED_2$ is at a higher extent compared to $EAE-SED_1$, whereas no difference in tAUC between CON-SED is seen in both studies. However, because rats were kept sedentary during presence of symptoms, decreased GT could be due to inactivity itself. This could be explained by the fact that glucose tAUC was also decreased in $CON-EX_2$ compared to $CON-EX_1$.

EAE and MS

The findings of this study cannot be generalized to MS patients. Nevertheless, the results of this study may provide important information. The induction of EAE doesn't have an influence on glucose tolerance of these rats. To consider the higher risk of diabetes in MS, EAE could be not the right model to explore this. Also, the exercise used in this study could not be the appropriate intensity modality to alter blood glucose and insulin concentrations. Furthermore, even though the use of the high intensity exercise did not alter the clinical symptoms of EAE rats, caution should be taking when suggesting that high intensity exercise doesn't exacerbate symptoms in MS. Moreover, regular exercise at the very early phase of the disease seems useful to delay the onset of clinical symptoms and increase body weight of EAE rats. Likewise, this result should be translated with caution to MS patients.

Limitations and future directions

This research contained a couple of limitation. Based on these limitations, recommendations for future research could be made. The lack of a disease effect in glucose and insulin could be due to a small sample size. Furthermore, the sample size was divided in two. The reason of this separation was the assumption that rats would not withstand more than one OGTT. Stevens et al. (2014) shows that it is possible to do multiple OGTTs in one single rat[43]. With this recent knowledge, it would be possible to do several glucose and insulin measurements in the same rat, where there is no need for separating the study. This would mean a larger sample size, which results in a higher study power. Furthermore,

testing by the use of a hyperinsulinemic-euglycemic clamp could have been more useful than merely insulin concentration to investigate the disease effect, because this is assumed as the 'gold-standard' in assessing insulin sensitivity[44]. The absence of an exercise effect could possibly be declared by the short program duration. It would be interesting to extend the training and let it continue during the symptom period in terms of exercise effect. The latter implication is also useful to explore the underlying cause of the decreased glucose tolerance demonstrated in Study 2, namely alteration in underlying mechanism of EAE rats or due to inactivity. In addition to this, GLUT4 expression is an interesting parameter of insulin sensitivity to take into account. In combination with glucose and insulin values, a more comprehensive conclusion could be made in terms of underlying metabolic defects in EAE.

However we based our intensity level on former literature[37-40], the exact intensity of the current study is not known, while there were no lactate and/or VO2 measurements. Considering literature reports that glucose values could be influenced by resistance training in diabetic rats[45] and in humans with IGT and diabetes[46, 47], it would be interesting to apply or add this type of training in future EAE studies when investigating the influence on glucose tolerance. Additionally, the training could be more intensive by adding more frequencies and volume to the protocol.

Conclusion

In the present study, EAE and exercise did not alter blood glucose and insulin concentrations during OGTT. However, a disease effect was demonstrated when observing the body weight, food intake and clinical scores. Moreover, a period of hindquarter paralysis-induced inactivity resulted in an increased glucose tAUC in EAE rats. This may indicate metabolic defects in EAE rats due to paralysis and/or inactivity.

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