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Doctoral Thesis

Exercise for studying Type 1 Diabetes in a Non-Obese Diabetic (NOD) mouse model

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ABBREVIATIONS

Name	Full Name
T1DM	Type 1 Diabetes
T2DM	Type 2 Diabetes
BB rat	BioBreeding rat
IFN-ϒ	interferon gamma
IL-1β	interleukin-1 beta
MHC	major histocompatibility complex
NO	nitric oxide
NOD	Non-Obese Diabetic (mouse strain)
PCR	polymerase chain reaction
STZ	streptozotocin
TNF-α	Tumour necrosis factor alpha

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Abstract

Exercise for studying Type1 Diabetes in a Non-Obese Diabetic (NOD) mouse model

The incidence of Type 1 Diabetes Mellitus (T1DM) is markedly growing in the past two decades. For the management of this disease, physical exercise has been recommended as a cost-effective treatment throughout the global health system. The Non-Obese Diabetic (NOD) mouse represents a well-established experimental model analogous to human T1DM as it is characterized by a progressive autoimmune destruction of pancreatic β-cells. This thesis explored the uses of a mouse motorized treadmill to study the effects of exercise in NOD mice.

Body mass, blood glucose level, immunological soluble factors, muscular performance and islets of Langerhans architecture were monitored during 12-week moderate-intensity endurance training in female NOD mice. After 12 weeks of training, no differences were registered as to diabetes incidence (50 vs 45%) and mean glycemia between sedentary controls and mice on exercise (190±34 vs 163±38 mg/dl, mean and SD). Exercise capacity dimished in the exercising-mice with respect to controls (work, distance, VO2max, $p<.05$). Preliminary data from a morphometric analysis of pancreata indicated the presence of larger infiltrates along with increased endocrine cell areas in the NOD exercising-mice. A higher infiltrate-to-islet ratio was observed in exercising-mice with respect to the controls. An exercise-induced weight loss was also detected.

Among key anti- and pro-inflammatory cytokines: TNF-α, MIP-1β and IL-10 resulted to be lower at end of the training in the exercising animals with respect to pre-training values (1353±2 vs 1355±2.3; 984.6±12 vs 1001±37; 396±8.1 vs 407±27 MFI, respectively, $p<.05$) whereas IL-2P40 was higher in exercising-mice compared baseline (543±12 vs 539±15 MFI, $p<.05$).

Further studies are needed to clarify the utility of the NOD mouse model to mimic and investigate the exercise effects in T1DM, immunomodulation and inflammation. Specifically, dose-response studies in which exercise will be administered to NOD mice at various levels of intensity will be necessary to determine the optimal regimen of physical exercise having clear-cut preventive effects on the development of T1DM.

INTRODUCTION

Habitual physical activity and exercise training are emerging as a therapeutic component for the enhancement of health and wellness to prevent chronic diseases and age-related loss of functional capacity [1,2]. Active life style effectively lowers weight gain, an important risk factor for heart disease, diabetes and several chronic diseases, for which exercise could be preventing, protecting and reverting [3]. Diabetes is one of the costly and burdensome chronic diseases for the society, widely spreading throughout the world [4]. In this thesis I emphasized the management of non-obese diabetic (NOD) mouse model susceptible to spontaneously develop T-cell mediated autoimmune diabetes in order to mimic the characters of human type 1 diabetes mellitus (T1DM). T1DM results from an autoimmune assault of insulin-producing pancreatic β-cells, leading to an absolute deficiency in insulin synthesis and secretion [5]. Physical exercise is frequently recommended in the management of T1DM and type 2 diabetes mellitus (T2DM) and can ameliorate glucose uptake by increasing insulin sensitivity and reducing body adiposity [6].

Rodent model has a long history in the field of diabetic research; it has potentially exhibited analogous features of multiple human diseases, and for this reason, it is being applied to know the pathogenesis and management the autoimmune diseases [7]. NOD (non-obese diabetic) mouse is currently the best available animal model of T1DM, since it develops disease spontaneously and shares many genetic and immunopathogenic features with human T1DM. Consequently, the NOD mouse has been extensively studied and has made a tremendous contribution to our understanding of human T1DM [8].

NOD strain has a great tendency to exhibit a wealth of insights into the inherently complicated processes involved in autoimmune disease. There are very few study conducted over the in vivo response of immune system in relation to exercise and the occurrence of health impairment associated with cell-mediated immunity evaluation of specific antibody production [9]. The existing evidence favored the exercise modulatory action on immunocyte dynamic and, possibly, on immune function. Several cytokines are expressed in animal models of type 1 diabetes [10,11,12] and the pattern of the network in which these cytokines co-operate is very complex. A

specific cytokine might either amplify or counteract the effects of other cytokines. Moreover, the action of a cytokine can be concentration- and time-dependent.

Diabetes mellitus is a group of metabolic disease characterized by a hyperglycemic state, resulting from defective insulin secretion [13]. The increasing incidence of diabetes requires a better understanding of the pathogenesis and management of the disease. A common pathology in both type 1 and type 2 diabetes is the loss of beta-cell mass and function to meet the metabolic demands [14]. Islet of Langerhans is a crucial organ located inside the pancreas, playing significant role in glucose homoeostasis. Islets are typically composed by four different type of cells: α, β, δ and pancreatic polypeptide (PP), respectively for secretion of glucagon, insulin, somatostatin and polypeptide. Many studies reported the modulating action of exercise in which definitely contributes to maintain of homeostasis.

The global prevalence of diabetes is 230 million, among the 4·9 million patients suffered from T1DM, and the incidence of T1DM is rising by 3–5% each year causing serious socio-economic problems in worldwide [15]. It develops because of a complex interaction between environmental factors and a genetic background. Observational data for the monozygotic twins suggest that the genetic component share around 30-40% of the total risk [16,17,18] and environmental factors have also been considered as candidates contributing for this change in disease incidence in the recent decades [19,20,21]. Physical exercise is extremely important to decrease the burden of morbidity and mortality of the disease.

In spite of intensive observational studies that have carried out during the past decades, the use of physical exercise as preventive strategy against T1D is yet unclear. Particularly, several benefits have been enlightened as meaningful indications for the management of disease. Therefore, it seems necessary to set up a novel attempt to translate innovative result from NOD mouse model that can help the understanding and the cure of this disease in future.

1.1 Animal models for human Type 1 Diabetes

It is a long history of using animal models in the diabetes research. There is extensive literature available on animal models such as mouse, rat, transgenic, knockout for studying diabetes and its pathogenesis. There are potential options to set a specific timeframe to know the several specific characteristic of the disease. The subject of small rodent such as mice or rat, usually gains great attention for laboratory research due to economic and it is easy to handle. Hence, pancretomised rat and mice play a major role in finding a desirable objective crucial for the pathogenesis and management of T1DM. Thus animal model may lead to a preferable spectrum of disease-studies in thefuture.

Rodents models such as rat and mice are easily accessible, and because of their short gestational period and life span, can be monitored for many generations under a limited timeframe as compared to other animals (dog, cat and primates).

In vivo models of T1DM have been categorized into two classes:

a) Induced model of diabetes Introduction of external chemical agents such as alloxan, streptozotocin (STZ), viral infection or pancreasectomy represent a general cause for onset of disease.

b) Spontaneous model of diabetesTwo animal model BB (Bio Breading) rat and NOD (Non Obese Diabetic) mice spontaneously develop T1D. They are developed from many generations in bread laboratory by selecting hyperglycemic environment. There is a more pronounced gender bias in disease manifestation, compared to both humans and BB rats. Approximately 80% of female and 10% of male NOD mice become diabetic under pathogen-free conditions, but castration of males at an early age has been reported to increase the incidence of diabetes in male NOD mice [22].

a) Induced Model of Diabetes

The cytotoxic glucose model analogous to alloxan and STZ is the most common agent-responsible model for the study of diabetes. The substantial action of both chemicals is toxic for pancreatic β cells. Action of chemical is rapidly generated for diabetogenesis, particularly STZ

administration destroyes β cells within 15 minute [23]. The action onset of diabetes with STZ treated mice is exclusively rapid: mice rapidly show spontaneous disease progression and pathological symptoms of pre diabetes such as autoantibody formation and gradual insulitis [24,25].

b) Spontaneous model of diabetes

There are two important animals widely considered for the spontaneous development of T1D:

- BB rat
- NOD mouse.

The BB rat was developed for the first time at Bio-Breading Laboratories of Canada Ltd in 1974 and referred many characteristics of T1D found in humans. However, this animal model has shown lymphopenia, which includes severe complications of autoimmune disease. [26]. BB rat is also not easy to manage, requiring special housing and handling procedure, as they show high susceptibility of infection, specifically for respiratory tract infections, which are usually fetal.

The second rodent model, the so-called NOD mouse was developed in 1974, but its perfect evaluation was carried out later on [27]. This model is presently regarded as the most important spontaneous model for studying the pathogenesis of human T1D; these strain show a high proportion of similarity as found in humans.

Bio- Breading rat

The Diabetes-prone BB rat is the most widely used rat model for studying autoimmune diabetes; it was developed in the 1970s from a colony of outbred Wistar rats in Canada (in the Bio-Breeding Laboratories) [28]. Like NOD mouse, the BB rat develops T-cell dependent autoimmune diabetes, which is also characterized by islet auto-antibodies, as well as GAD antibodies. However, in contrast with the NOD mouse, the phenomenon of insulitis has many similarities with humans, begins 2-3 weeks before the clinical initiation of the disease, and it does not start with peri-insulitis and Th1-lymphocytes predominate in the procedure [25,26,29,30]. At about the age of 8-16 weeks, the BB rat becomes hyperglycaemic and

insulinopaenic, with polyuria and polydipsia already evolved. Though, unlike NOD mouse, ketoacidosis is very severe in the BB rat and as in humans, lethal if not treated with insulin [27,28, 31, 32].

1.2 NOD Mouse characteristics

NOD mouse has been considered the best model for spontaneous development of human T1DM, since it progresses as autoimmune type of disease paralleled to that one occurring in humans [33]. The condition prior to diabetes has shown infiltration of macrophages and lymphocytes into the pancreatic islets (Insulitis). This state progresses the complete destruction of pancreatic β cells mass. Insulitis starts around 4-6 weeks of age and diabetes occurs between 12-18 weeks predominantly in females. NOD mouse produces glutamic acid decarboxylase (GAD) [34] islets cell antibodies (ICA) and insulin autoantibodies (IAA) [35], which have also been responsible for the development of T1DM in human.

The strain of NOD mouse initially established at the Shionogi Laboratory in Japan from an inbred cataract shionogi (Cts) strain. The research was performed with objective to develop a sub-strain with raised blood glucose score in order to monitor diabetes like condition on cataract development. Primarily in 1974 one of the strains of Ct female mice was identified, as not only higher blood glucose level but showing the clinical picture of T1D. Unfortunately, these mice could not survive and died before producing offspring. However sub-line was preserved for further six years before, another mouse with similar characteristic was found. This bread was successively bread and resulted in the development of NOD mouse in 1980 [36]. The NOD mouse was developed by selectively breeding offsprings from a laboratory strain that in fact was first used in the study of cataract development [37,38]. Insulitis appeared during the period of 4-5 weeks, followed by subclinical β cell destruction and reduction of circulating insulin concentrations. Frank diabetes typically occurs between 12 and 30 week of age. An autoimmune lesion involving lymphocytic infiltration and destruction of the pancreatic β-cells leads to hypoinsulinemia, hyperglycemia, ketoacidosis, and death. There is a larger gender difference with 90% of females and 20% of males to expanding diabetes in NOD mice. [39].

Type 1 diabetes is a polygenic disease. Both in human T1DM and in NOD mouse, the primary susceptibility gene is located within the MHC. NOD mouse provided not only essential information on type 1 diabetes pathogenesis, but also valuable insights into mechanisms of immunoregulation andt olerance. Importantly, it allows testing of immuno-intervention strategies potentially applicable to man.

My thesis focused on NOD mouse as a therapeutic tools to test the efficacy of exercise training to modulate the immunological and physiological changes.

1.3 Anti- and pro-inflammatory cytokines in NOD mice

TNF-α is a proinflammatory cytokine that has been shown to be a critical mediator of the inflammatory responses characterizing autoimmune diseases in general [40]. Indeed, its role in the destruction of beta islet cells, although not being the focus of this particular thesis, has emerged as an active area of research [41,42]. The production of TNF-α has been detected by both thymocytes and stromal elements within the thymus [43] and has been shown to regulate both the proliferation, apoptosis and the maturational transition of theDN (CD4_/CD8_) subset [44].

The first study suggesting that exercise induced a cytokine response reported that plasma obtained from human subjects after exercise, and injected intra peritoneally into rats, elevated rectal temperature. In 1986, two studies were published that indicated that the level of IL-1 increased in response to exercise.

Thus far, TNF-α has been shown to have a dual role in the progression of T1DM. Neonatal exposure to TNF-α can exacerbate T1DM onset, while adult exposure to TNF-α can avert the disease entirely. Antibodies to TNF-α, administered over the same time periods have the ability to reverse these effects in both cases. The mitigating effects of adult administration have been attributed to TNF-α ability to attenuate TCR signaling, thereby suppressing the autoimmune inflammatory response in animals destined to become diabetic. Recent evidence suggests that the presence of TNF-α early in the life of NOD mice, destined to become diabetic, can reduce the frequency of regulatory CD4þCD25þ T cells to a degree significant enough to increase the

incidence and severity of T1DM. Conversely, anti-TNF-α antibodies can boost this cell population enough to avert disease entirely.

Proinflammatory cytokines are obvious candidates for precipitating early b-cell dysfunction. From studies of cell lines and isolated islets in vitro, cytokine signaling leads to the production of inducible nitric oxide synthase (iNOS) in the short term (hours) and to activation of the unfolded protein response and endoplasmic reticulum (ER) stress in the longer term (days) [45-46]. Whether the development of ER stress is directly attributable to nitric oxide accumulation itself or secondarily to other cytokine-derived signals remains arguable. Nonetheless, it has been proposed that ER stress may contribute to the susceptibility of b-cells to dysfunction in NOD mice [47-48].

1.4 Physical Exercise and Immune System

Over the past 2 decades, a variety of studies have demonstrated that exercise induces considerable physiological changes in the immune system. The interactions between exercise stress and the immune system provide a unique opportunity to link basic and clinical physiology and to evaluate the role of underlying stress and immunophysiological mechanisms. It has been suggested that exercise represents a quantifiable model of physical stress [49]. Several clinical and physical stressors such as surgery, trauma, burn, and sepsis directly stimulate a pattern of hormonal and immunological responses that have similarities to that of exercise. While other combinations of neural-endocrine-immune interactions have been examined by using a variety of psychological models, furthermore the exercise model provides a further option to establish these links using a physical stress paradigm. This thesis also reviews favorable effects of exercise immunology [50,51,52,53,54,55,56,57,58] and focuses on underlying endocrine and cytokine mechanisms.

Hypothesis

Physical exercise has a well-established role to accelerate the immune response against chronic diseases such as type 2 diabetes, heart disease and cancer. I hypothesized that physical training might delay or prevent onset of type 1 diabetes, modulate various immunological and metabolic factors including body mass, blood glucose level, immunological soluble factors, muscular performance and beta cell mass, contributing positively to glycemic homeostasis.

AIM of Thesis

The purpose of this project is to examine how moderate-intensity exercise training may exert a protective effect on glycemic proflile, exercise capacity and inflammatory markers in a non-obese diabetic (NOD) mouse model.

Objectives

1. To evaluate body mass variations, blood glucose level in exercised NOD mice with respect to age matched control mice.

2. To quantitative estimate immunological soluble factors and muscular performance in exerciser NOD mice compared with age matched control mice.

3. To compare β cell mass and islets architecture of exercising- and sedentary NOD mice.

METHODS

The Institutional Animal Care Committee of University of Miami has given its approval to run these animal studies. All NOD mice were purchased from the Charles Rivers ® and single-housed under pathogen free conditions, providing regular chow food and water ad libitum inside the animal facilities of University of Miami, Miller School of Medicine.

NOD female mice, 6-week old were randomly classified into two groups:

1. Treadmill Exercise group (20 units)

2. Sedentary age matched control (20 units)

Exercise-group was trained on the mouse treadmill at a moderate intensity (12m/min), 5 days/week over the period of 12 weeks.

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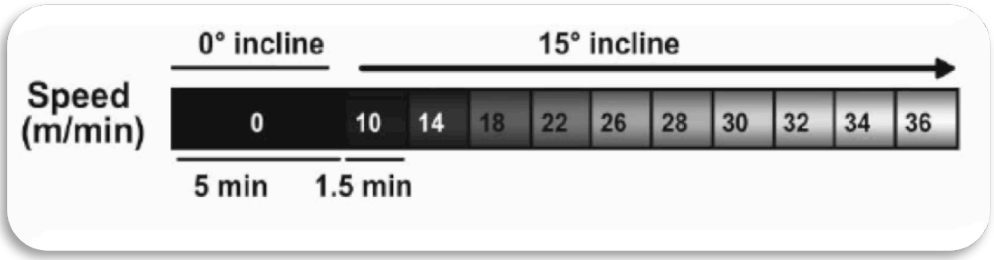
3.1 Treadmill Exercise

The treadmill, model Eco 3/6 from Columbus Instruments (Columbus Instruments, Columbus, OH) has the capability of exercising up to six mice in individual lanes. A stimulus can be created using the electrical shock grids, and grids can be enabled or disabled individually for each lane. The intensity and repetition rate of the stimulus is user controlled. All data collection and analysis was performed manually.

1. Acclimation: Prior to training, mice firstly placed for acclimation over a period of 5 min undisturbed in the treadmill respective lanes.
2. Under a chronic exercise regime, mice were trained at a speed of 12 m/min for 30 min on 5 consecutive days/wk for 12 weeks (0° slope).
3. Muscular performance test exercise capacity during a higher-speed was determined during the peak oxygen uptake (VO_2) challenge. To determine VO_2 peak, mice were placed on the treadmill for 5 min at a 0° incline and 0 m/min. The mice were then challenged with 1.5-min intervals of increasing speed at a 15° incline. The protocol is shown schematically in here:



The increasing speeds used in the protocol are 10, 14, 18, 22, 26, 28, 30, 32, 34, 36, 38, and 40 m/min. The protocol was performed until exhaustion which is defined as remaining on the shocker plate for more than 8-10 seconds.

4. Cleanup: As possible to clean almost every time of the treadmill with a mild solution of detergent and 70% ethanol. In particularly use a plastic tray underneath the treadmill that routinely collects feces and urine. It used to clean after finishing of experiment. In addition, carefully prevent urine from the control surfaces of the treadmill.

3.2 Mouse Body weight measurements

NOD mice body weight was accessed by Mettler Toledo Electronic balance.

3.3 Blood Collection procedure

- Firstly Aseptic condition applied around the work place.

- Blood sample collected gently due to it may cause stress in mice (stressful conditions can increase blood hormone- and glucose levels.

- Maintained precaution during the separation of blood without contacting skin and fur of mice.

- Tail yields a mixture of arterial and venous blood. Collection must be performed carefully since it affect the glycemia. Blood was collected from small incision of tail tip.

- Blood glucose concentration was measured using the Accu-Chek Advantage meter (Roche,Indianapolis, IN).

3.4 Cytokine measurements

Cytokines in the serum were tested using a mouse cytokine array kit (Quansys Biosciences, West Logan, UT, USA), including TNF-α, MIP-1β , IL-2P40 pro inflammatory cytokine and IL-10 anti-inflammatory cytokine.

Table 1. Reagents used for the processing of immunohistochemical analysis

S.No.	Source	Supplier	Cat
1	Wash Buffer	Biogenex	HK583-5K)
2.	Buffered Formalin 10%"	Sigma-Alderich	HT5014
3	PBS Buffer	Life Technology	AM9624
4.	Antigen Decloaker	Biocare medical	CB910M
5.	Guinea Pig anti Insulin	Biogenex	AR029-5R Polyclonal
6.	Mouse anti Glucagon	Sigma-Alderich	G2654 Monoclonal clone K79bB10
7	Alexa Fluor 488 goat anti-guinea pig	Invitrogen	A11073
8.	Alexa Fluor 568 $F(ab')_2$ fragment goat anti-mouse	Invitrogen	A11019

Immunofluorescence analysis of insulin, glucagon and pancreatic cell with exercised and control mice.

NOD pancreata were obtained from 18 animals (~20 week-old) which were sacrificed by exposure to CO_2 followed by cervical dislocation. Pancreatic tissue sample obtained from dissection process.

3.5 Immunohistochemistry.

Blocks of mouse pancreas (0.5 cm^3) were fixed in Buffered Formalin 10%" (=Formaldehyde 4%) paraformaldehyde for 4 hr, section (14 cryo protected in sucrose, and cut on cryostat (40 µm) after a rinse with PBS triton X 100 (0.3%) section were incubated in blocking solution (PBS-Triton X-100 and universal blocker reagent, Biogenex San Raman CA). Section were

incubated in universal blocker reagent (Biogenex) for 5-10 min, rinsed again in Optimax wash buffer. Thereafter, sections were incubated 4 hours (25° C) with primary antibodies, Guinea Pig anti Insulin (Biogenex AR029-5R Polyclonal), Mouse anti Glucagon (Sigma G2654 Monoclonal clone K79bB10), diluted the primaries antibody with supersensitive Wash Buffer 1x draw the circles with Pap-Pen, putting power block 30% FBS only inside the circle, usually 100-200 uL, 30min-1h at room temperature, and transfer all the slide section in the cold room at 4C for "Overnight" (O/N) 16 hours incubation. Immunostaining was visualized by using Alexa Fluor conjugated secondary antibodies, include Alexa Fluor 488 goat anti-guinea pig , Alexa Fluor 568 $F(ab')_2$ fragment goat anti-mouse, added wash buffer to cover the whole slide and wash buffer 10 to 12 times to remove unconjugated peptide and residue. For staining of all infiltarate nuclei, DAPI was used. Slides were mounted with ProLong Anti Fade (Invitrogen). In experiments process to distinguish primary antibodies, they were incubated with corresponding control peptides. Mounted with Glycerol (one drop over each section), centralized the cover glass on top of the slide and around the cover glass seal with nail polisher. At the end, all the slides were maintained at 4 ˚C in the dark cold room and visualized by the microscope in 24-48 hours.

3.6 Confocal Imaging

Confocal image of randomly selected slide islets, with histological evaluation were acquired confocal laser scanning microscope and comparative studies have carried out with pancreatic sections containing islets were examined for expression of different endocrine cells. Glucagon and insulin proportion immune staining were acquired with a Hamamatsu camera attached to Zeiss Axiovert 200 M at DRI, University of Miami, USA with virtual slice image captured with 10X objective. The proportion of contacted cells was expressed as a percentage of total number of cells in the type. I used automated method to quantify all content.

3.7 Automated Quantification

The quantification of cellular composition (i.e. β, α and nuclei) has accessed by using macro written for Image J (http//rsbweb.nih.gov/ij/), a macro custom-written script license-free for quantification of interest in each application.

RESULTS

Body weight and exercise performance

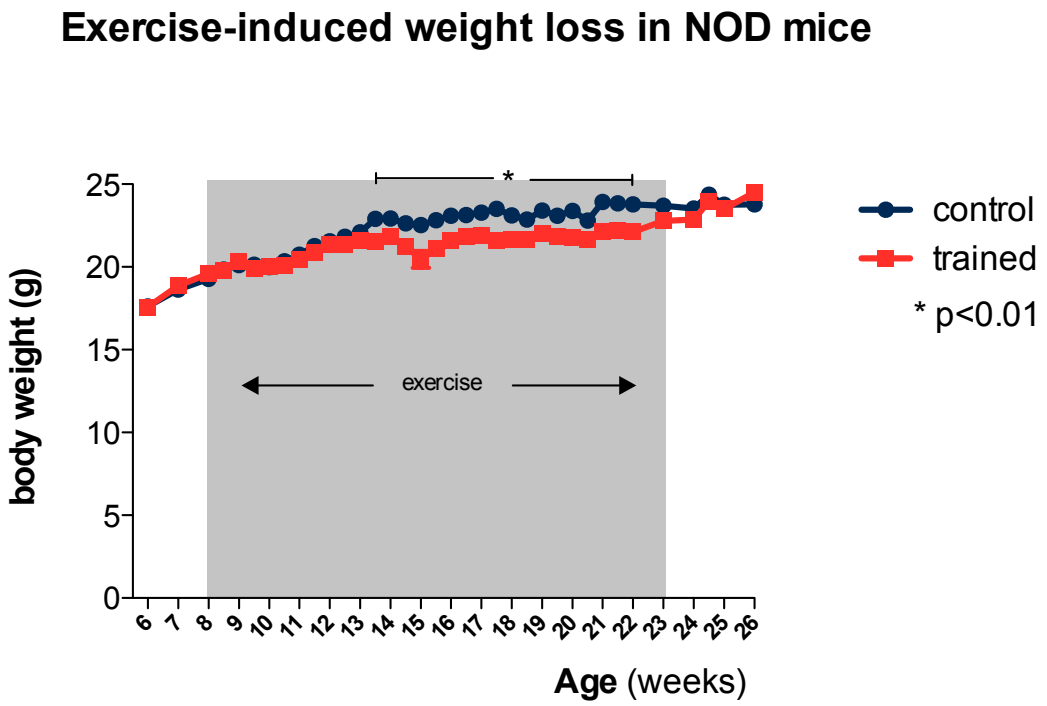


Figure 1

An exercise-induced weight loss was registered in the trained mice after 6 weeks of training as compared to the sedentary mice (-7% p<.01). NOD exercising mice were leaner than their age-matched controls for 10 weeks, from age of 13 weeeks to age of 22 weeks (Figure 1).

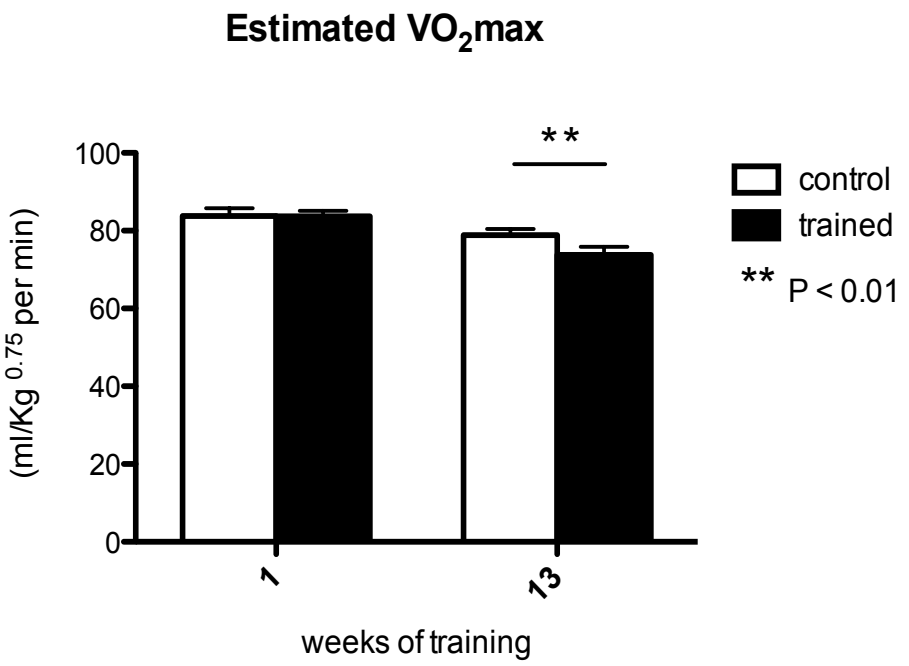


Figure 2

As for exercise capacity in general, there was a decrease of estimated VO_2max at the end of the exercise period in the NOD trained mice with respect to the sedentary controls (73.8±7.3 vs 79±6.3 ml/Kg0.75/min, $p<.05$) (Figure 2).

Figure 3

Similarly, maximal running speed assesed during submaximal exercise test was found to be slightly decreased, even though not significantly, in the NOD execising mice with respect to the controls at the end of the 12-week training period (Figure 3).

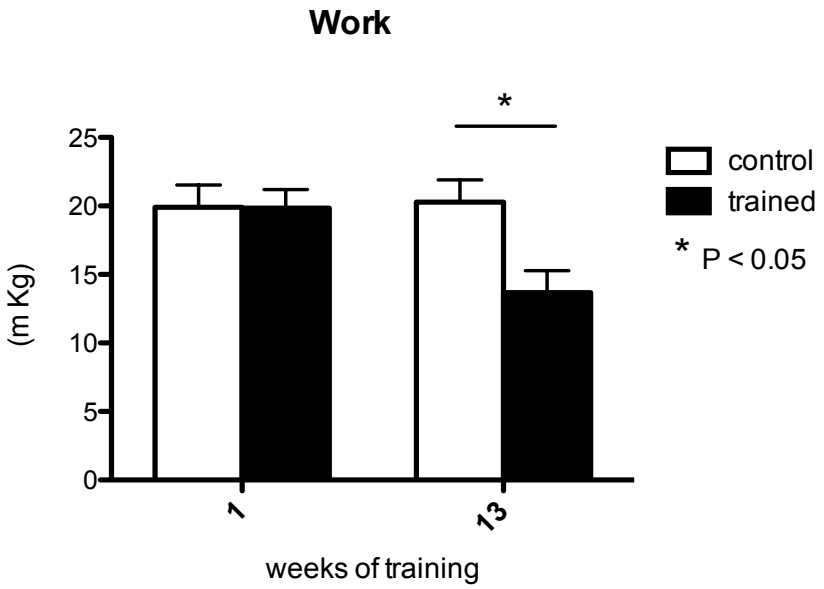


Figure 4

While there was not difference in work performance (as indicator of exercise capacity) at baseline in both exercise and control group, it reduced significantly in the NOD execising-mice with respect to the controls at the end of the 12-week training period (13.7±5.8 vs 20.3±6.7mKg, P<.05) (Figure 4).

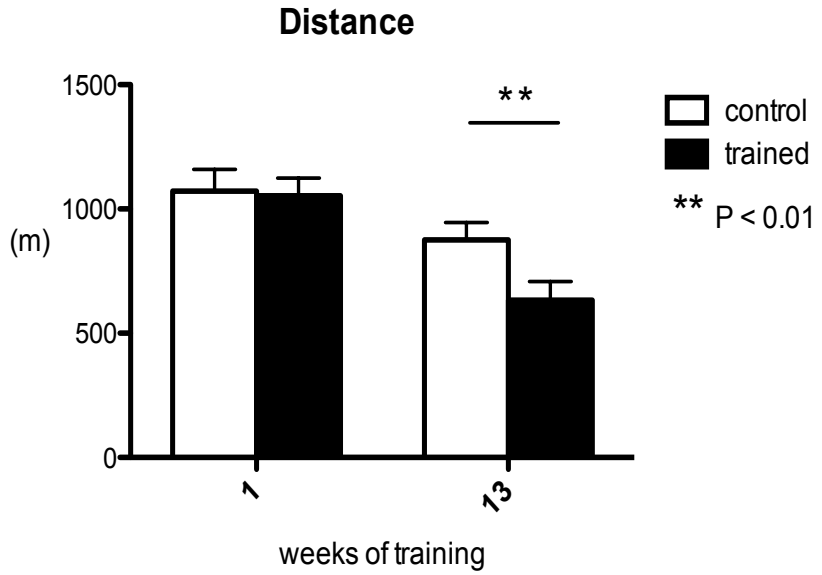


Figure 5

A significant reduction in the distance covered during the submaximal perfomance test was also found in the NOD-exercising mice with respect to the age-matched controls (634±271 vs 876±291m, P<.05) (Figure 5).

Blood glucose level

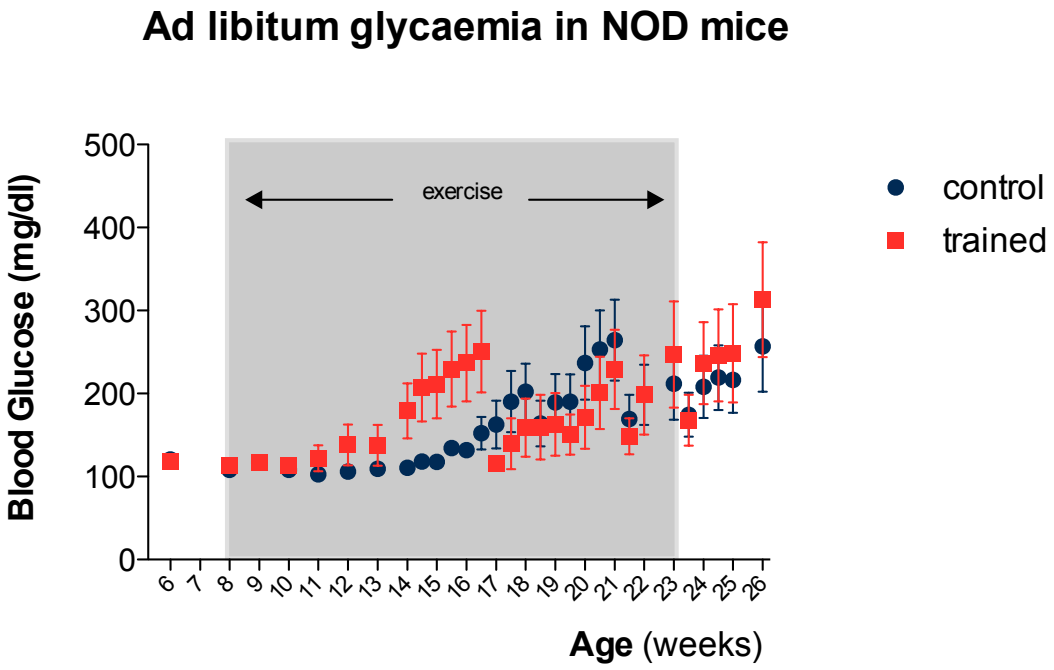


Figure 6

After 12 weeks of training, no differences were registered as to diabetes incidence (50 vs 45%) and mean glycemia between controls and mice on exercise (190±34 vs 163±38mg/dl, mean and SD) (Figure 6, 7).

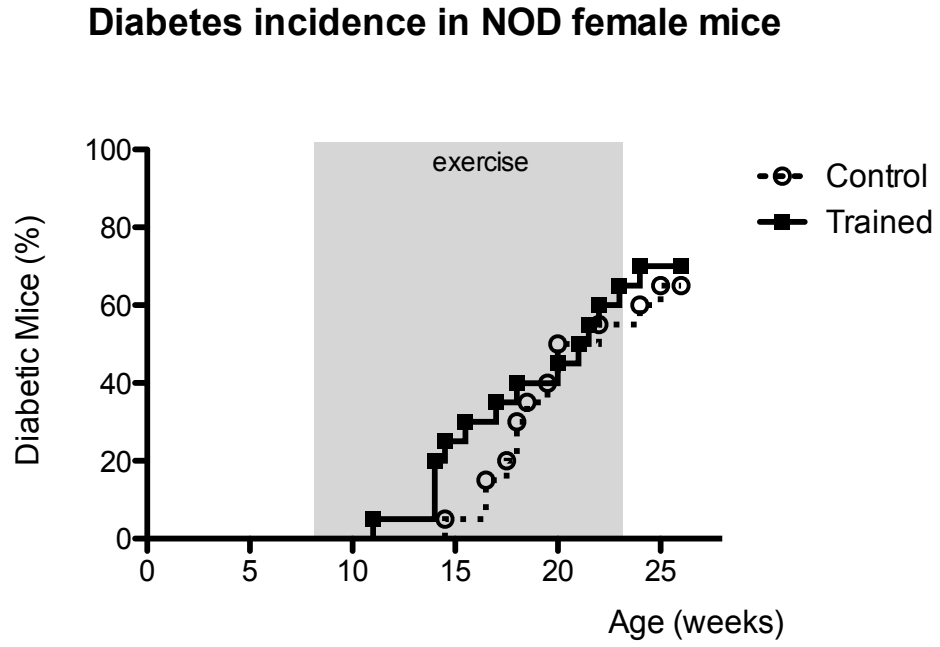


Figure 7

The evaluation of the incidence of diabetes in NOD mice undergone to exercise with respect to sedentary control, registered insignificant data (50 vs 45%, P<.05), that means that exercise performance was unable tocounteract the development of the T1DM disease (Figure 7).

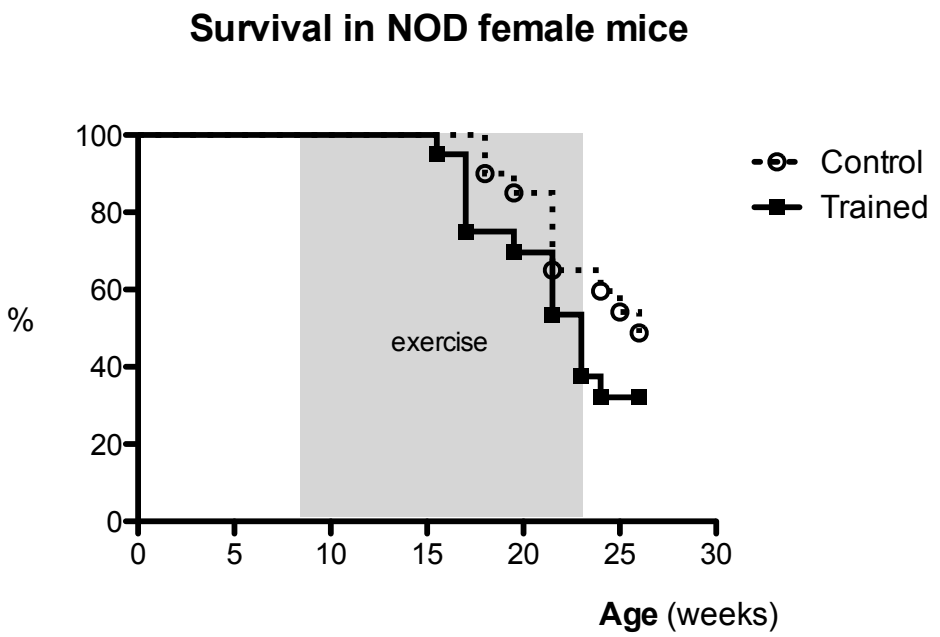


Figure 8

Survival rate in both groups of mice declined progressively, with no statistical difference (Figure 8) .

Cytokines

Table 2. Quantitative estimation of the list of pro-inflammatory and anti-inflammatory markers.

	BASELINE		8 Weeks		12,5 Weeks	
Cytokines *(MFI)*	**Control**	**Trained**	**Control**	**Trained**	**Control**	**Trained**
G-CSF	557,22976	627,0998824	572,21356	742,7788421	566,63136	639,242
GM-CSF	339,1155556	335,2545143	315,3367389	316,2091857	316,2463111	333,4168071
IFN-gamma	1180,28222	1032,617294	906,960955	878,2623053	878,755295	883,76195
IL-1 beta	312,49472	312,9124294	312,93075	313,2094158	312,93075	313,55365
IL-2	2031,339175	1939,213635	1832,872675	1814,162753	1810,691125	1825,617807
IL-4	4253,94175	4270,965	4272,1075	4252,805263	4279,07675	4254,317143
IL-6	972,71193	999,9113412	978,33306	996,0182526	997,382445	992,5197214
IL-10	396,22503	406,3973059	393,78545	399,9831684	402,98079	396,3992857
IL-2 P40	543,52146	545,0943824	540,862685	539,4593316	539,495315	542,7835143
IL-13	62,78049546	63,22044161	53,95591698	54,09984156	53,46363207	53,24785714
MCP-1	48,650625	48,18663529	48,937455	47,60646316	48,746235	48,34330714
MIP-1 beta	1002,7734	1001,444353	1004,6671	991,6998947	994,6109	984,62
MIP-2	76,4131	72,51440714	74,0655	74,40087143	76,4131	74,6524
VEGF	2196,94206	2063,674632	1929,19218	1928,216141	1926,92486	1931,091429
TNF-alfa	1353,475065	1355,225388	1354,01522	1353,881726	1354,508405	1353,90115

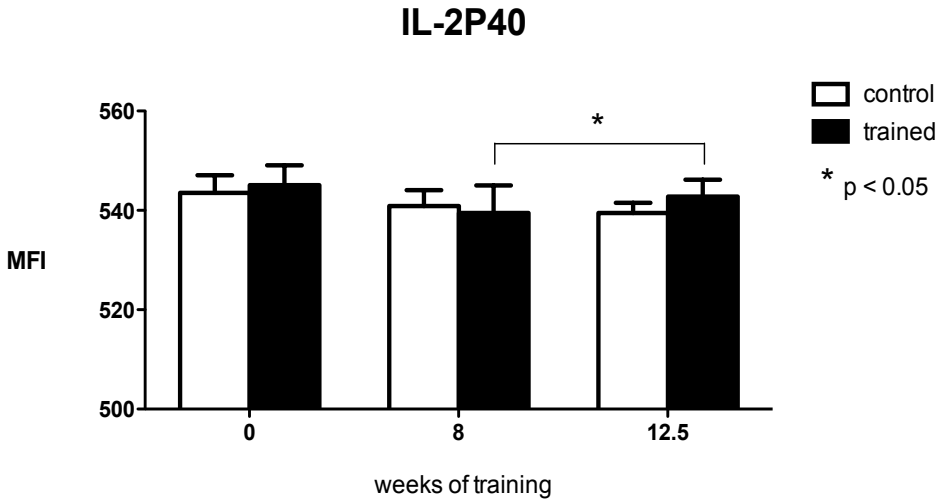


Figure 9

IL2p40 is recognized the play an specific role in T cell development, particularly in immune mediated T cells. IL2p40 The protein is a pleiotropic cytokine produced primarily by antigen presenting cells and has multiple effects on T lymphocytes and natural killer cells in terms of stimulating cytotoxicity, proliferation, production of other cytokines and Th1 subset differentiation. In these studies, IL-2P40 was higher in exercising-mice at the end of the training period as compared to 8 weeks of training (543±12 vs 539±15 MFI, p<.05) (Figure 9).

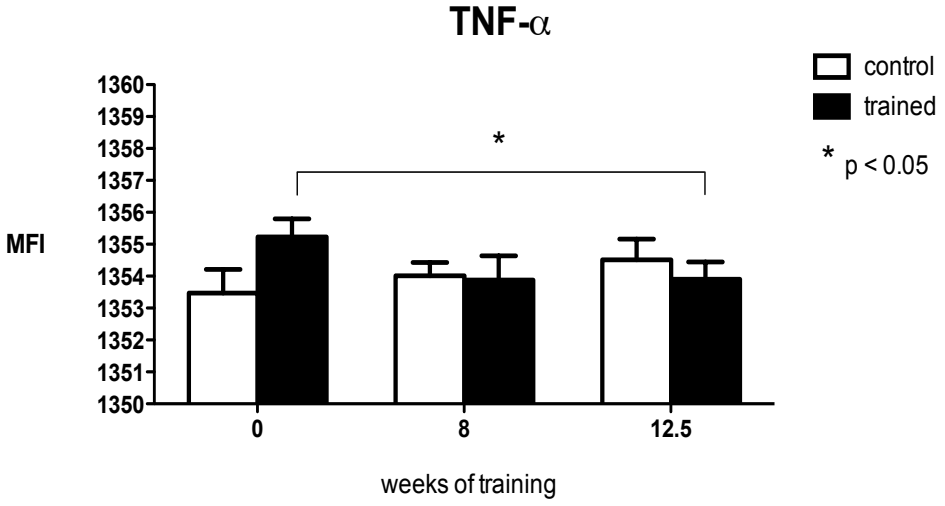


Figure 10

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine with potent immunomodulatory activity. The association of TNF-α with T1DM is frequently demonstrated in several studies. In these studies, there a was a significant decrease at the end of the 12-week training program in the NOD-trained mice with respect to the pre-training values (1353±2 vs 1355±2.3 MFI; $p<0.05$) (Figure 10).

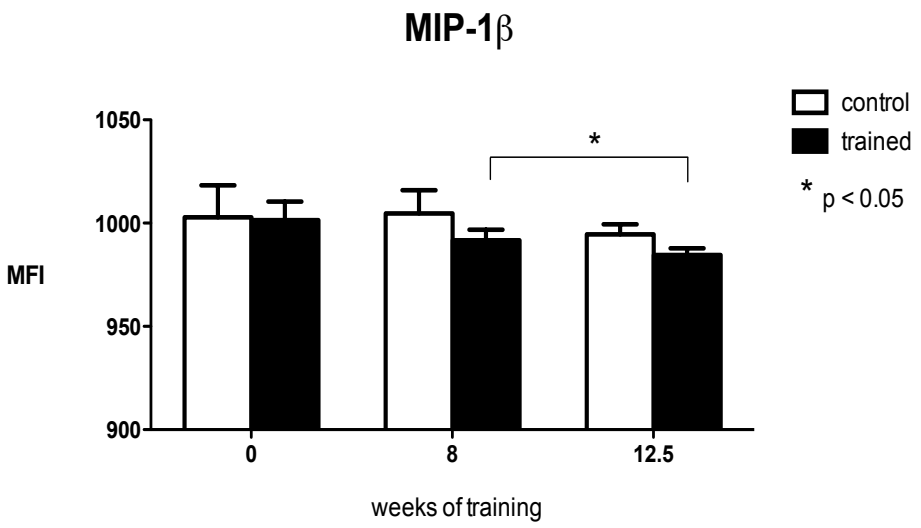


Figure 11

MIP-1β level was measured at 3 distinct timepoints over the duration of 12 week of exercise. At the end of 1st week, the MIP-1β value was unchanged in both group of animals while at the end of 12 weeks of training, the exercising animals showed a lower level of MIP-1β with respect to their training levels registered at 8 weeks (984.6±12 vs 1001±37 MFI; p<.05) (Figure 11).

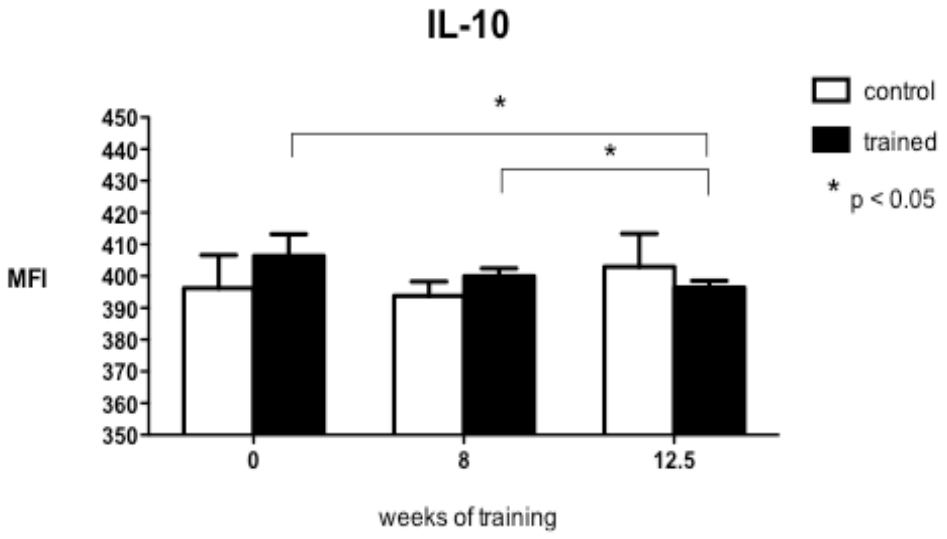


Figure 12

IL-10, is an anti-inflammatory cytokine, also known as human cytokine synthesis inhibitory factor (CSIF). In these experiments, NOD exercising animals showed lower levels of IL-10 with respect to pre-training values (396±8.1 vs 407±27 MFI, $p<.05$) and as compared to 8 weeks of training (396±8.1 vs 399 MFI, $p<.05$), respectively (Figure 12).

Islets architecture: morphometric analysis of pancreata

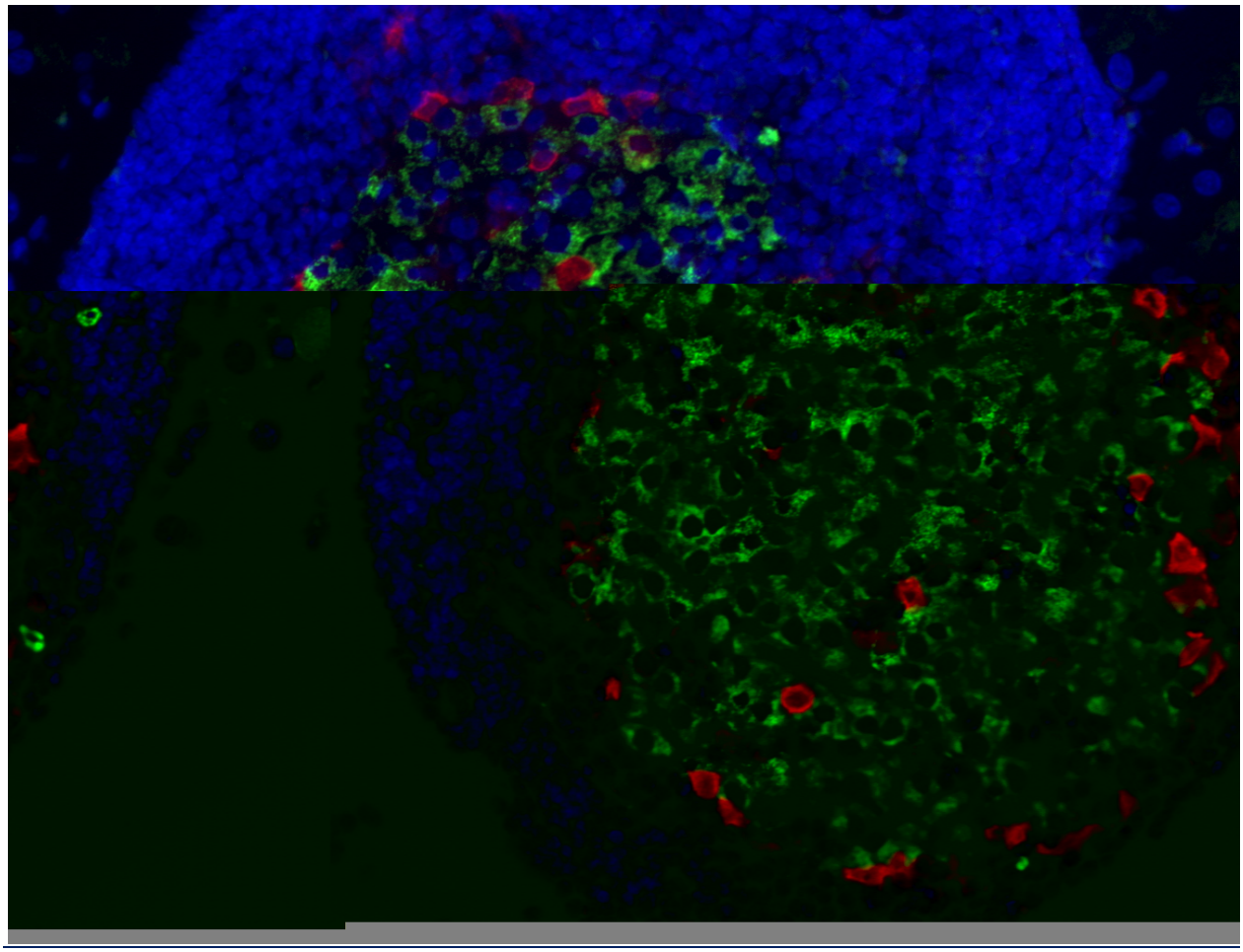


Figure 13

Islet of Langerhans visualized by immunofluorescence microscopy (Figure 13). This figure is a representative immunestaining of pancreatic sections, from a NOD mouse, on exercise. The entire tissue section is captured by a modified method of "virtual slice image capture" using a microscope with a 10x objective. Each virtual slice taken at multiple fluorescent channels is merged into one composite (shown as insulin in green, glucagon in red, and nuclei in blue, (DAPI)).

Quantitatively, the cell composition of this mouse islets was >70% made by β cells and <20% by α-cells. However, cell numbers may inevitably be dissimilar between studies and observation,

because every islets shows unique characteristics. Differences are also related to animals and species, as we can see in the averages below.

Table 3. Quantitative estimation of alpha cells and infiltrate in NOD mice on exercise and controls.

	Control_infiltrate	Control_alpha_cells	Exercise_infiltrate	Exercise_alpha_cells
Mean:	455.85	260.133	234.925	153.656
Median:	407.45	242.65	184.5	128.125
Variance:	42153.3	32260.6	33737.4	13806.9
Std. Dev.:	205.313	179.612	183.677	117.503
Std. Err.:	83.8186	73.3265	64.9398	41.5436
Skewness:	0.698317	0.293363	0.790993	0.4397
Minimum:	224	51.5	55.5	35
Maximum:	773.3	508.3	564.4	327
Sum:	2735.1	1560.8	1879.4	1229.25
N:	6	6	8	8

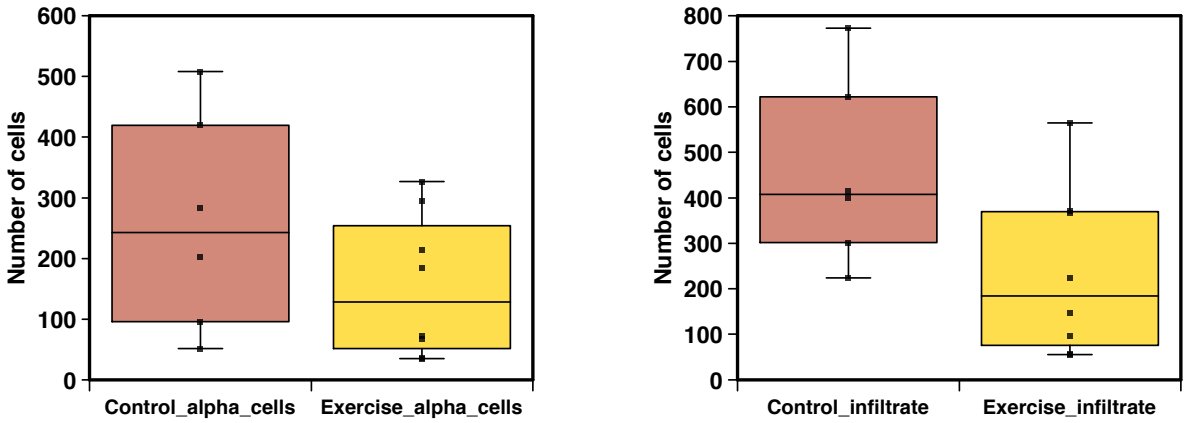


Figure 14

Scatterboxes of the averaged number of alpha-cells (left) and infiltrate (right) in NOD trained-mice and controls. No significant differences were found between the two groups (Figure 14).

DISCUSSION

As exercise training is always used in addition to insulin treatment in the management of T2DM (less in T1DM), I hypothesized that moderate-intensity, regularly-practised, exercise also exerts a potent immunomodulatory effect, countering the activation of innate immune responses and inflammation, i.e. the environmental factors that contribute to the diabetes epidemic. Therefore, the purpose of this study was to examine how moderate-intensity exercise training might control T1DM progression, exerting a protective effect on glycemic proflile, exercise capacity and inflammatory markers in NOD mice.

The non-obese diabetic (NOD) mouse is a model for autoimmune diabetes in humans. The NOD mouse exhibits spontaneous autoimmunity that causes diabetes through demise of insulin-secreting pancreatic islets. There are several studies investigating the effects of exercise in inbred rodents with naturally occurring type 2 diabetes or streptotozocin-induced diabetes, however the paucity of studies in murine models of autoimmune diabetes underpins the usefulness of the NOD mice for investigating theì impact of exercise training on T1DM progression.

Female NOD mice have an important tendency for spontaneous development of T1DM, especially after 10 weeks of age. However 78% of these animals spent at least 20 weeks of time to develop autoimmune T1DM. In this thesis, NOD mice after 12 weeks of physical training did not show major differences as to the incidence of diabetes (50 vs 45%).

Metabolic outcomes

Aaron et al [59] raveled both sedentary T1DM and T2DM mice exhibit exercise intolerance, which is substantially improved by chronic exercise training, although T1DM mice recorded moderate weight loss after the 8 weeks of training. Another work on exercise training was carried out in voluntarily using running wheels and forcefully on a treadmill for a period of 12 weeks. At the end of the voluntary training protocol, mice were 5% lighter than their sedentary counterparts [60]. In my thesis, after 6 weeks of training on female NOD mice I found a significant weight reduction in the exercising mice as compared to the controls. However I could not ascertain whether this difference was due to diabetic cachexia, exercise or simply different food habits.

Yet, the mechanisms by which exercise regulates blood glucose level remain unclear in T1DM. In one of the first investigations by Becker-Zimmermann et al, proposed beneficial effects of exercise training that used animal model of obesity-associated insulin resistance [61] demonstrated that mild exercise training (treadmill running) by older (25-wk-old) obese Zucker rats could significantly improve glucose disposal during an oral glucose tolerance test and reduced the exaggerated insulin response to a glucose challenge. Moreover, these investigators showed that exercise training by younger (7-wk-old) obese Zucker rats could prevent the deterioration of glucose tolerance experienced by these animals as they develop into adulthood. This exercise training-induced improvement in the whole body insulin sensitivity of obese Zucker rats was soon confirmed with swim training [62].

Exercise training has been known to be effective in T2DM by increasing insulin sensitivity, however there is incomplete knowledge how exercise acts in T1DM. Coskun et al. [63] with a STZ-induced experimental model of T1DM, they showed that exercise training is effective not only in increasing insulin sensitivity but for β cell protection also. They examined possible usefulness of the light-, moderate- and heavy-exercise training which it had therapeutic, preventive, and protective effect in diabetes by decreasing oxidative stress and preservation of β cell integrity. Such β cell damage often displays extensive degranulation when examined histologically, and it is clinically associated with development of diabetes in some model animal for T2DM [64].

Exercise performance

Multiple studies have assessed total spontaneous running distance of diabetic rodent models. Woodiwiss et al. [65] reported that diabetic rats with blood glucose values of 20.4 mM (368 mg/dL) ran the same distance, albeit at a modestly slower average speed, than normal rats with glucose levels of 5.6 mM (101 mg/dL). On the other hand, Keller et al. [66] found that NOD mice with blood glucose values of 25 mM (283 mg/dL) ran less than half that of normal mice with glucose values of 6 mM (108 mg/dL). In addition, Rowland and Caputo [67] found that streptozotocin-diabetic hamsters with blood glucose values of 398 mg/dL exercised half to two-thirds that of normal animals with glucose values of 116 mg/dL when maintained in a light-dark cycle (14:10), and that the extent of exercise in diabetic animals was reduced even further to 25% of control when animals were housed in continuous light. The results of the previous studies

along with the present study suggest that mild to moderate degrees of diabetes may or may not diminish exercise activity, whereas animals with more severe hyperglycemia, as in the present study, exercise less than normal.

At the start of this study, there were obviously no differences trained and control NOD mice as to muscular performance characteristics. However, the total physical capacity and estimated caloric cost of the running activity gradually fell down when T1DM disease was expanding inside the animal body. That indicated that the complication of diabetes predominated over the normal health, gradually making immune system and physical strength poor along with the attempt of physical exercise to combat the disease. Nevertheless, no signs of improvement were detected. Thus NOD strain was therefore not perfectly defending the severity of disease.

Morphometric analysis of pancreata

I hypothesized that physical training could be exerting a protective function for the islets at the onset of T1DM, because observational study with type 2 diabetes in animal model, long-duration of aerobic exercise showed to enhance islet β-cell proliferation, elavated β-cell mass, and a partial sparing of the abnormal islet morphology recognized in the sedentary diabetic rats [68].

Morphological evaluation of the islets cell from my exercised-trained mice failed to demonstrate differences compared to the sedentary diabetic group. My findings are analogous to a previous report investigating the effect of the physical training on the distribution of α-, β-, and δ-cells and pancreatic polypeptide cells in the islets of streptozotocine induced diabetic rats [69]. Additionally, another study explored the influence of exercise related to β cell function in T1DM: exercise was partially able to harness β cell damage, decrease lipid peroxidation and increase antioxidative enzymes [70].

The difference in exercise protocols may explain the conflicting outcome. In my protocol, similar to the previously cited by Howarth et al., which also showed no change in β-cell numbers, the exercise protocol was initiated after the induction of diabetes [69]. In the Coskun et al. study, the aerobic exercise protocol was initiated four weeks prior to the induction of diabetes and the exercise continued for another eight weeks to the termination of the experiment [70]. Thus, exercise may be able to protect β-cells if initiated prior to the onset of the disease but has limited or no ability to rescue the β-cells once lost.

One important difference must be highlighted between the previous studies and the current work. In the Coskun et al. paper, the serum glucose measurements were statistically lower in the exercising animals than the sedentary diabetic rats. Thus, one cannot rule out the possibility that any improvements in *β*-cell numbers or function were due to the lowered blood glucose values. In the present study, blood glucose levels were not significantly different between the exercised and sedentary diabetic mice at the termination of the study. This was accomplished by inducing severe diabetes without insulin treatment, and it provided an advantage when interpreting the data, because any changes were directly related to the effects of exercise without a reduction in blood glucose, since it is known that high glucose is toxic to islet cells [71]. Also, maintaining statistically similar blood glucose readings at the termination of the study ensured that glucotoxicity was not a factor.

This study's initial objective was to assess pancreatic β-cell numbers and physiology during immune-mediated islet destruction by following the insulin-positive–to–glucagon-positive cell ratios and other parameters. I was surprised to observe that the relative frequency of the two endocrine subsets consistently pointed to an unexpected depletion of α-cells, along with the expected β-cell loss at diabetes onset. It is important to point out that I cannot accurately determine the absolute number of α- or β-cells in the pancreas, only their relative proportion [72].

Cytokines fluctuations

Aerobic exercise reduced the appearance of proinflammatory cytokines in islet cells [73]. It is generally accepted that auto-reactive T-cells mediate the destruction of the pancreatic β-cells in T1DM. For this issue, possibilities that β-cell destruction is a cell-mediated disease and both CD8+ cytotoxic and CD4+ helper cells might be responsible in the diabetogenic process. Th1 and Th2 cells negatively cross-regulate each other's function through their respective cytokines. Th1 cytokines (e.g. IFN-γ) induce Th1 activity and suppress Th2 activity, whereas Th2 cytokines (IL-4 and IL-10) promote Th2 cells while inhibiting Th1 activity and cytokine release.

According to a recent hypothesis, a shift in the physiological Th1/Th2 immune balance can lead to pathologically increased immune response and consequently, a T-cell mediated autoimmune destruction of β-cells. There is evidence for the implication of T-cells in the development and progression of T1D in humans and NOD mice. Both CD4+ and CD8+ T-lymphocytes are crucial

during the early and late stages of disease in mice [74]. The therapeutic effect might be the suppression of Th1 determined pathogenic/destructive autoimmune process by induction of a defensive, hsp60 specific Th2 response. The immunization can presumably activate further regulatory T-cells to prevent β-beta cell destruction caused by Th1 cells.

It is believed from previous studies that the infiltration of immune cells, such as Th 1 cells and macrophages, into the islets and subsequent insulitis are hallmarks of the pathogenesis of T1DM. Activated T cells and macrophages release several pro-inflammatory cytokines, such as IL-1b, IFN-g and TNF-a, which are believed to be important mediators leading to b-cell destruction in T1DM [76,77,78,79,80].

Moreover, it should be acknowledged that even if TNF-α is crucial pro inflammatory marker in the progression of autoimmune diabetes in mice, relevant cytokines and environmental factors might efficiently substitute for the lack of TNF-α [79,80]. In summary, there is evidence that physical training effectively modulates the value of TNF-α can delay the development of T1DM in NOD mice. Plasma TNF-α as a proinflammatory marker has been investigated under physical exercise interventions in different other studies [81,82,83] .

Overall comments

NOD mice undergoing physical training and metabolic alterations are linked with glycemic homeostasis. These methods are likely to open a new therapeutic avenue for the treatment of T1DM. In this context it is important to establish training protocol techniques that allow screening of methodology that effects on islets cell differentiation, islets cell signaling and islets cell pathology.

CONCLUSIONS

In this thesis I implemented a set-up to show the advantages of animal models under exercise regime for studying immunomodulation, inflammation, and disease-progression in type 1 diabetes mellitus. Particularly, moderate-intensity exercise induced a mild anti-infllammatory effect in NOD female mice as reflected by fluctuations in some cytokines such as TNF-α and MIP-1β.

Exercise did not worsen glycemic conditions of NOD mice nor it was able to favorably control glycemia in mice on training.

Interstingly, no clear mechanisms linked immune cell infiltration and islet dysfunction, opening new avenues of investigations as to the islets architecture and NOD pancreata under exercise- and T1DM-stresses.

Further studies are needed to clarify the utility of the NOD mouse model to mimic and investigate the exercise effects in T1DM, immunomodulation and inflammation. Specifically, dose-response studies in which exercise will be administered to NOD mice at various levels of intensity will be necessary to determine the optimal regimen of physical exercise having clear-cut preventive effects on the development of T1DM.

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