

Immobilization in diabetic rats results in altered glucose tolerance

A model of reduced locomotion/activity in diabetes

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Abstract

Aims Type 2 Diabetes Mellitus affects more than 350 million people worldwide. This metabolic disorder is characterized by insulin resistance, β -cell dysfunction and elevated hepatic glucose output. Patients with diabetes are hospitalized frequently (3-fold greater) and with longer admissions (30% longer) than the non-diabetic subjects. The aim of the present study was to investigate the impact of bed rest on the metabolic changes in type 2 diabetes mellitus, with particular interest in skeletal muscle mass and function and metabolism.

Methods and results 13wk old male Zucker diabetic fatty (ZDF) rats were randomly divided into two groups: control (ZDF-Con) and cage-immobilized animals (ZDF-Cage) for 28 consecutive days in a space-restricted cage. The Area Under the Curve (AUC) values for plasma glucose concentration in ZDF-Cage rats were significantly increased (approximately 4-fold as compared with ZDF-Con rats). GLUT4 gene expression in red soleus muscle of ZDF-Cage animals was reduced 2.5-fold in comparison with ZDF-Con rats. Although no apparent changes were observed either in fasting plasma glucose or insulin levels, a trend towards an increase in the HOMA-IR index and decreased levels of plasma adiponectin (-30%) were observed in ZDF-Cage animals. Moreover, ZDF-Cage rats did not lose muscle mass and force but performed a reduced total physical activity level (-22%).

Conclusions The present study results suggests that 28 days of immobilization (in a space-restriction model) significantly impaired glucose tolerance with concomitant reduced plasmatic adiponectin levels and GLUT4 expression in soleus muscle of type 2 diabetic rats.

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Introduction

Although bed rest was a common therapeutic intervention in chronically ill patients providing beneficial effects by procuring patient comfort and contributing to recovery, in the past 50 years it has become apparent that the effects of bed rest may be harmful [1,2]. Even though the practice is still commonly used for an array of conditions, randomized clinical trials failed to show bed rest as an effective treatment for any illness [3]. During prolonged bed rest (due to aging, recovery from injuries, sepsis or other pathological conditions) skeletal muscle undergoes severe loss which results in decreased physical performance [4,5]. From a clinical point of view, complications arising from immobilization or physical inactivity might worsen primary disease or trauma and might become the most relevant problem to treat rather

than the primary disorder [6]. Side effects of reduced physical activity can lead to paralysis, joint stiffness and pain, with protective limitations of motion and mental disorders and main metabolic alterations such as insulin resistance, thromboembolic disease, disuse osteoporosis, respiratory and musculoskeletal complications [7]. Excessive loss of muscle mass is a poor prognostic indicator, resulting in longer hospitalization and recovery time, impairing the efficacy of many different therapeutic treatments, as well as health increasing care cost and decreasing patient's quality of life.

Type 2 Diabetes Mellitus (T2DM) is a metabolic disorder characterized by insulin resistance, β -cell dysfunction, and elevated hepatic glucose output that affect more than 350 million people worldwide [8,9]. Decreased muscle strength, lower muscle quality, and

accelerated loss of muscle mass, especially in the lower extremities, have been documented in up to 70% adult diabetic patients [6]. Particularly, older patients with diabetes are a high risk of future mobility disability and loss of independence [10,11]. Although comorbidities (such as cardiovascular disease, obesity, vision loss, obesity, arthritis) are important factors in the development of physical disability in diabetic patients (partially explaining the risk of severe walking limitation [12]), evidence is emerging that part of the mobility reduction process is mainly due to a direct effect of diabetes on skeletal muscle [13,14]. This period of prolonged disuse or bed represents an additional problem in diabetic patients, affecting their quality of life.

Immobilization methods in rodents are mainly confined to casting [15,16] or suspension of extremities [17]. Each procedure has specific advantages and strengths which encourage its use, as well as disadvantages which limit data interpretation and differ each other in terms of the degree of reproduced inactivity [18] and distinct protein degradation profiles induced [19]. In this regard, in a previous study we have described a new disuse-induced muscle wasting animal model based on cage volume reduction, able to induce loss of muscle mass and strength in healthy animals [20]. This model reflects, in a much better way, what is encountered in human subjects in bed rest associated, for instance, with hospitalization.

Taking all this into consideration, our interest was addressed to analyse the influence of muscle disuse on the cellular and metabolic processes involved in the aetiology and evolution of T2DM. In this regard, in order to determine whether prolonged inactivity is a high-risk behaviour for diabetic patients [6,9], we aimed to reproduce, in an experimental animal model, the pathological condition of a patient affected by T2DM subjected to bed rest or reduced daily ambulatory activity for long-term period, which we induced by the cage immobilization model. The experimental model of "bed rest" used in this study (which has been previously published [20]) represents muscle unloading, gravitational load differences due to postural change (i.e.

standing versus lying down). Specifically, this study was designed to assess the contribution of reduced ambulatory activity to: I) skeletal muscle integrity and functionality, and II) whole body glucose metabolism and insulin sensitivity in skeletal muscle in an experimental rodent model of T2DM.

Experimental

Animals

The study was performed on 13 weeks-old male Zucker Diabetic Fatty (*fa/fa*) (ZDF) rats from Charles River Laboratory (*Germany*). Animals were housed in individual cages on a regular dark-light cycle (light from 8:00 am to 8:00 pm) at a constant temperature of 22°C to 24°C and humidity (40%), with free access to food and water throughout the experimental period of 28 days. After an acclimation period of one week prior to the beginning of the experiment, ZDF rats were allocated into two experimental groups (n=6 each): control (ZDF-Con) and immobilized (ZDF-Cage) [20] (Figure 1). The immobilized animals were kept for 28 consecutive days in a reduced cage (*Tecniplast 2150*), the space is restricted to 12 cm x 12 cm x 8 cm (approximately an 80% reduction of the total standard cage volume) [20]. ZDF-Con rats were used as diabetic untreated control (non-immobilized) not submitted to any immobilization procedure for the entire period of the experiment. Body weight, food and water intake were recorded daily (Figure 2). All the animals were fed the AINM93M maintenance diet (*Abbott Nutrition Laboratories, Granada*), whose formulation consists of 71.15 % carbohydrate, 12.79 % protein and 3.50 % fat [21]. All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals. They were cared for in compliance with the *Policy on Humane Care and Use of Laboratory Animals* (ILAR 2011) and in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The experimental protocol was approved by the Ethical Committee of the University of Barcelona (CEEA 313/14).

FIGURE 1. Timeline of the experimental design.

After an acclimation period of 1 week prior to the beginning of the experiment, ZDF rats were allocated into 2 experimental groups (n=6 each) according to their initial body weight. All animals were fed ad libitum with AINM93M diet. The cage immobilized (ZDF-Cage) animals were kept for 28 consecutive days in a reduced volume cage (80% reduction versus the total volume of a standard cage). The non-immobilized control animals (ZDF-Con) were kept for the entire period of the experiment in a standard cage and they were not subjected to any treatment. Grip force test was performed once a week. In order to minimize the influence of the fasting on the final body weight, body composition and physical activity data collected during the last experimental day, the OGTT was performed 4 days prior to euthanasia. Rats were daily monitored and cleaned. All the experimental groups were euthanized on day 28.

Figure 1

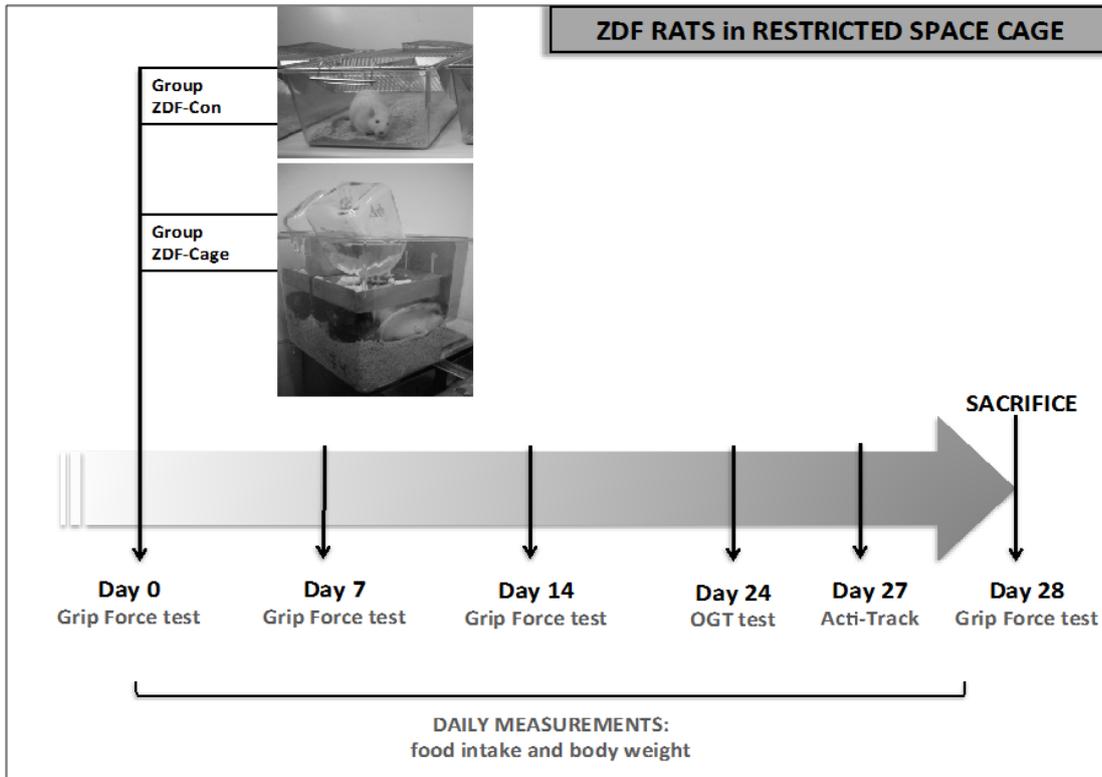
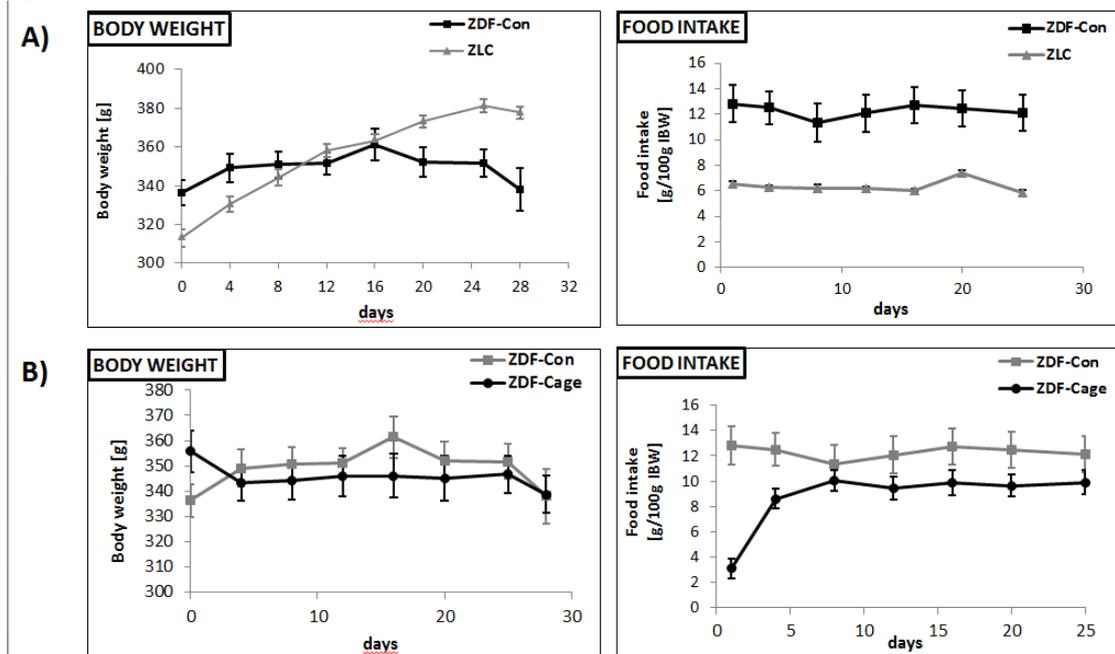


FIGURE 2. Time course of body weight growth and daily food intake in immobilized ZDF rats.

The graphs show the average growth rate and the daily food ingested of control and immobilized ZDF rats during the experiment. Lean ZDF littermate controls: ZLC; non-immobilized control animals: ZDF-Con; and cage immobilized animals: ZDF-Cage. Body weight and food intake were expressed as mean value of six animals, bars: SEM. The formula used to calculate the food intake was (total food ingested each day / 100g body weight (BW)).

A) Data were analysed by a Linear Mixed Model, being Diabetic phenotype (D) a crossed between-subjects factor, and time (T) the within-subjects factor (repeated measures). Restricted Maximum Likelihood (REML) method was used to fit the model. According to Akaike Information Criterion (AIC) and Schwarz Bayesian Information Criterion (BIC), Huynh-Feldt structure was finally chosen for the variable Body weight (T $p=0.000$; TxD $p=0.000$); Factorial Analytical of First Degree structure was finally chosen for the variable Food Intake (D $p=0.001$; T $p=0.081$; TxD $p=0.000$).

B) Data were analysed by a Linear Mixed Model, being Immobilization (I) a crossed between-subjects factor, and time (T) the within-subjects factor (repeated measures). Restricted Maximum Likelihood (REML) method was used to fit the model. According to the values of Akaike Information Criterion (AIC) and Schwarz Bayesian Information Criterion (BIC), First Order Autoregressive was finally chosen as the covariance matrix structure for the variable Body weight (I $p=0.758$; T $p=0.002$; IxT $p=0.000$); First Order Factor Analytic for the variable Food Intake (I $p=0.235$; T $p=0.000$; IxT $p=0.000$).

Figure 2

Euthanasia

Rats were euthanased at day 28. Prior to euthanasia rats were weighed and anesthetized (3:1 mixture of ketamine (Imalgene®) and xylazine (Rompun®)). Blood was collected from the aorta and post-prandial plasma separated by centrifugation at 3,500g for 10 min at 4°C and stored at -80°C. Muscles and other tissues were rapidly excised, weighed and frozen in liquid nitrogen. All tissues were stored at -80°C until analysis.

Oral glucose tolerance test (OGTT)

An OGTT was performed 4 days prior to euthanasia in order to minimize the influence of the fasting on the final body weight, body composition and physical activity data, collected during the last experimental day. Animals were fasted overnight (12 hours) with access to water *ad libitum*. Blood from non-anesthetized rats was collected in heparinised wells from a cut in the distal extreme of the tail in order to assess plasmatic fasting levels of glucose and insulin. To reduce the chance of infection, a topical germicide (*BETADINE solution*) was applied to the tail following blood collection. Blood collection (0.2 mL) was obtained

15, 30, 60 and 120 min after the glucose solution administration (1g/kg rat) by gavage. Glucose levels were measured by the Glucometer (*Accutrend, GCT, Roche Mannheim, Germany*). The animal was placed in its cage in fasting condition during the time within each evaluation. Blood was centrifuged at 13,000g for 30 sec and plasma stored at -80°C. The results of the test were analysed by calculating the Area Under the Curve (AUC) for plasma glucose concentration (expressed as mg/dL) by the “incremental area” method, in which the baseline measures are subtracted from all subsequent readings before calculating the AUC [22].

Biochemical parameters

Blood samples were collected from the heart of anaesthetized animals by cardiac puncture. Fasting plasma insulin levels were determined using the ultrasensitive rat insulin Elisa Assay Kit (*Biorbyt orb 54819, Bionova, Spain*). In order to estimate the insulin sensitivity in ZDF rats, the widely used HOMA-IR index was calculated according to the formula: [insulin (μ units/mL) \times glucose (mmol/L)]/22.5 [23,24]. Plasma triglycerides levels were established by the system *Metrolab 2300* using a spectrophotometer method. Plasma levels of interleukin-6 (IL-6) (*Diaclone, 670010192, Bionova, Spain*), serum amyloid A (SAA) (*Cusabio, csb-E08590u, Bionova, Spain*) and adiponectin (*Cusabio csb-E0727ir, Bionova, Spain*) were quantified by ELISA test according to the manufacturer’s protocol.

Physical activity

Physical activity was assessed during the last 24h before the euthanasia of the animals using the *IR ACTIMETER System* and *ACTITRAK software* (*Panlab-Harvard Apparatus, Spain*). This system uses activity sensors that translate individual changes in the infrared pattern caused by movements of the rats into arbitrary activity counts (automated system). In order to carry out the measurements, animals remained in their home cage with free access to food and water, and a frame containing an infrared beam system was placed on the outside of the cage. Data were collected for a total period of 24h separated into 12h periods. This software enables the user to analyze the general activity of each animal individually through some parameters like: locomotors activity (i.e. movements with displacement; number of movements/second), fast/slow stereotypes (i.e. movements without displacement: eat/clean; number of movements/second), distance travelled into the zone during the interval (cm), time involved in fast and slow movements and resting (without displacement, i.e. eating, sleeping, cleaning; expressed as a percentage of the total), maximum, minimum and mean speed and total activity (number of movements/second) [25].

Grip strength

Skeletal muscle force in rats was quantified by the grip-strength test once a week. The grip strength device (*Panlab-Harvard Apparatus, Spain*) comprised a pull bar connected to an isometric force transducer (dynamometer). Basically, the apparatus was positioned horizontally and the rats were held by the tail and lowered towards the device. The animals were allowed to grasp the pull bar by their forelimbs and were then pulled backward in the horizontal plane. The force applied to the bar just before the animals lost grip was recorded as the peak tension. At least three measurements were taken per rat on both baseline and test days, and the results were averaged for analysis. This force was measured in grams/grams initial body weight [26].

Fiber cross sectional area (CSA)

After the euthanasia, the soleus muscles were rapidly excised and quickly frozen in liquid-nitrogen cooled isopentane, maintaining the correct orientation to allow cross section. 10 μ m of transverse sections from the mid-belly of the muscles were cut on a cryostat at -20°C. The slides obtained were stained by haematoxylin-eosin staining protocol, mounted with permount mounting media (*Fisher, USA*) and photographed at 10x magnification. Fiber CSA was determined on randomly chosen 100 individual fibers per animal by the *ImageJ software* [27] and expressed in pixels. Differences in absolute values are due to changes in photo magnification and/or resolution. Both variables, however, were maintained fixed within each experiment.

RNA isolation and RT-PCR

Total RNA from soleus muscle was extracted by TriPure™ kit (*Roche, Barcelona, Spain*). Reverse transcription (RT) reactions were prepared using First Strand cDNA Synthesis Kit for RT-PCR (*Roche, Barcelona, Spain*) following the manufacturer’s instructions. Analysis of mRNA levels of the genes from the different proteolytic systems was performed with primers designed to detect GLUT4 gene products (Gene ID: 25139) (5'-GTTTCCAGCAGATCGGCTCTGA-3'; 5'-GCAAGGACCAGTGTCCAGTCA-3'). To avoid the detection of possible contamination by genomic DNA, primers were designed in different exons. The real-time PCR was performed using a commercial kit (*LightCycler™ 480 SYBR Green I Master, Roche, Barcelona, Spain*). The relative amount of all mRNA was calculated using comparative CT method. 18S (Ribosomal RNA) (5'-CGCAGAATCCCCTCCGACCC-3'; 5'-CCCAAGCTCCAACACTACGAGC-3') and HMbs (hydroxymethylbilane synthase) (5'-TGCCAGAGAAAAGTGCCGTGGG-3'; 5'-

TGCAAGCTCATCCAGCTTCCGT-3') mRNA was used as the invariant control for all studies. The efficiency of the PCR was evaluated using a dilution bank of RNA dilutions (ranging from 100 ng/µL to 0.08 ng/µL), and determining the slope of the regression between the resulting Cts and the logarithm of the concentration: Efficiency (%) = 100 (10^{-1/m} - 1) "m" would be the slope. A slope around -3.3 indicates an efficient amplification.

Statistical analysis

To summarize and describe the results, average (arithmetic mean) and standard error of the mean (SEM) were calculated for each studied variable. Intergroup differences were evaluated using Student's *t*-test, and linear mixed models. All the statistical analysis was performed using SPSS (version 21).

Results and Discussion

Zucker diabetic rats were immobilized (ZDF-Cage) for 28 consecutive days in a reduced cage characterized by a reduction in the volume of 80% in relation with standard cage (Figure 1).

As it can be seen in Table 1, both control and immobilized ZDF animals showed similar initial body weights. However, when compared with the non-diabetic controls (ZLC), they are clearly hyperphagic (Table 1, Figure 2) and show a significantly decreased energetic

efficiency. After 21 days of immobilization in the space-restriction model, ZDF-Cage rats ate slightly less (-24%) in comparison to the non-immobilized age-matched littermates (Table 1 and Figure 2). It is known that a period of bed rest leads to physical inactivity status with an associated lower energy requirements [28]. Interestingly, immobilized animals were not losing weight or showing any kind of wasting at the time of the study (Table 1), and presumably were able to maintain energy balance by eating slightly less. A reduction of food intake was observed during the first three days of restriction (Figure 2). This feeding behaviour was previously observed in healthy rats immobilized in the same model [20] representing a physiological response to the new environmental stress at which apparently the animals were submitted. The attenuated food intake observed was not translated into a reduced energetic efficiency (Table 1), being the blunted appetite a compensatory response to the reduced total energy intake associated with lower energy requirement due to physical inactivity.

Insulin resistance is a characteristic feature of T2DM patients and plays an essential role in the pathogenesis of the disease [29]. It is well established that decreased peripheral glucose transport and disposal in muscles and fat, increased endogenous hepatic glucose production together with inadequate inhibition of lipolysis in fat depots are keys hallmarks of insulin resistance [30].

TABLE 1 Body weight and food intake in immobilized ZDF rats.

| <i>Parameter</i> | <i>ZLC</i> | <i>ZDF-Con</i> | <i>ZDF-Cage</i> |
|--|----------------|-------------------|------------------|
| IBW (g) | 313 ± 4.6 (6) | 336 ± 6.6 (6)* | 356 ± 8.4 (6) |
| FBW 28 days (g) | 378 ± 3.1 (6) | 338 ± 10.8 (6)** | 339 ± 7.4 (6) |
| FOOD ingested 21 days (g) | 417 ± 6.7 (6) | 847 ± 90.9 (6)*** | 675 ± 48.6 (6) |
| FOOD INTAKE 21 days (g/IBW) | 133 ± 2.2 (6) | 253 ± 30 (6)*** | 192 ± 17.2 (6) # |
| ENERGETIC EFFICIENCY = [(FBW-IBW)/g food ingested] x100 | 14.6 ± 0.7 (6) | 2.0 ± 0.7 (6)*** | 0.3 ± 2.2 (6) |

In order to test the effects of reduced muscle activity on glucose metabolism in diabetic animals, fasting blood glucose and insulin levels along with blood glucose Area Under the Curve (AUC) responses were measured four days prior the euthanasia. As expected, ZDF rats showed a clear glucose intolerance (as compared with the non-diabetic controls (ZLC)) as shown when examining OGTTs (Figure 3A). Concerning the diabetic animals, a significant increase in blood glucose concentration was found within 15 minutes after glucose administration (1 g/kg, orally) in both experimental groups. While the increased glycemia was gradually diminished within 2 hours in ZDF-Con rats, the regulatory capacity of blood glucose was significantly attenuated in ZDF-Cage ones (Figure 3B). In the latter, glycemia values did not return to baseline levels after 120 min from the glucose load (Figure 3B). In parallel, the AUC values in ZDF-Cage rats were significantly increased to approximately 4-fold ($p < 0.001$) whether compared with ZDF-Con rat data (Figure 3D). ZDF diabetic animals, as compared with the non-diabetic controls (ZLC), were clearly hyperglycaemic, hypoinsulinemic and showed an increased HOMA-IR together with elevated concentrations of circulating triglycerides (Table 2). Concerning the diabetic groups, although no apparent changes were observed either in fasting plasma glucose and insulin levels (Table 2), there was a trend towards an increase in the HOMA-IR index in immobilized animals, suggesting their decreased insulin sensitivity (Table 2).

Since the major manifestation of insulin resistance is a reduced stimulated glucose disposal by skeletal muscle, we focused our attention on the effects of the immobilization on glucose transport in unloaded soleus muscle of diabetic rats. It has been previously demonstrated that insulin- and contraction-stimulated glucose uptake decreases in atrophic soleus muscle of tail-suspended rats [31]. Insulin resistance in skeletal muscle was due to the attenuated expression level of the glucose transport GLUT4 with or without insulin-stimulation, decreased GLUT4 activity or impaired GLUT4 translocation to sarcolemma [31]. Since the reduced GLUT4 content in immobilized muscle likely contributes to the deleterious impact on the altered glucose metabolism of diabetic animals [32], the gene expression of GLUT4 in the red soleus muscle was assessed by RT-PCR analysis. As expected, diabetic animals showed decreased GLUT4 expression compared with the non-diabetic controls (ZLC) (Figure 4A). Cage-immobilized animals resulted in 2.5-fold ($p = 0.05$) decrease in GLUT4 gene expression in red soleus muscle compared with the ZDF sedentary controls (Figure 4B). These reduced GLUT4 mRNA levels might result from both rapid repression of the transcription of GLUT4 gene and an increased rate of turnover of the GLUT4 mRNA by hormonal and nuclear factors [33–35]. The above results suggest that the action of insulin may be blunted in the cage-immobilized animals (as shown by the glucose tolerance curves (Figure 3)), this accounting for glucose intolerance.

FIGURE 3. Oral Glucose Tolerance Test and area under the curve in immobilized ZDF rats.

Effect of stepwise increments in blood glucose concentrations on plasma glucose measurements in ZDF immobilized animals during an oral glucose tolerance test (OGTT) performed 4 days prior the euthanasia. Lean ZDF littermate controls: ZLC; non-immobilized control animals: ZDF-Con; and cage immobilized animals: ZDF-Cage. Graphic representation of OGTT values as incremental glycaemia values (mg/dl) obtained at each time point of the curve. Glucose levels in blood were measured at different times: 0', 15', 30', 60' and 120' after oral glucose solution (2g/kg rat) administration. Each point represents a mean of observations in 6 rats. Each interval covers mean \pm SEM.

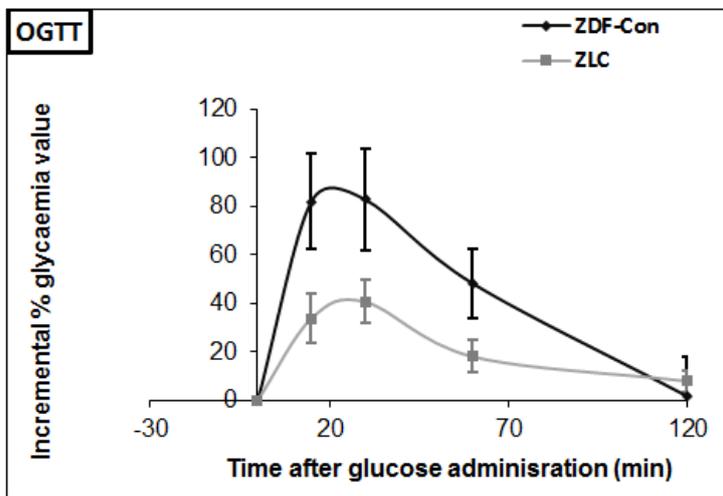
A) Data were analysed by a Linear Mixed Model, being (D) Diabetic Phenotype a crossed between-subjects factor, and time (T) the within-subjects factor (repeated measures). Restricted Maximum Likelihood (REML) method was used to fit the model. According to Akaike Information Criterion (AIC) and Schwarz Bayesian Information Criterion (BIC), Identity Scale was finally chosen for the variable glycaemia (D $p=0.006$; T $p=0.001$; DXT $p=0.000$).

B) Data were analysed by a Linear Mixed Model, being Immobilization (I) a crossed between-subjects factor, and time (T) the within-subjects factor (repeated measures). Restricted Maximum Likelihood (REML) method was used to fit the model. According to the values of Akaike Information Criterion (AIC) and Schwarz Bayesian Information Criterion (BIC), Scaled Identity was finally chosen as the covariance matrix structure for the variable glycaemia (I $p=0.000$; T $p=0.000$; IxT $p=0.000$).

C) and D) Incremental area under the curve of plasma incremental glycaemia values. Each bar represents a mean of observations in 6 rats. The line above each bar is the SEM. Values that were found to be significantly different by Student's t-test between the lean ZDF littermate controls (ZLC) and ZDF-Con (A) or ZDF-Con and ZDF-Cage group (C) are indicated by, ** $p<0.01$, *** $p<0.001$.

Figure 3

A)



B)

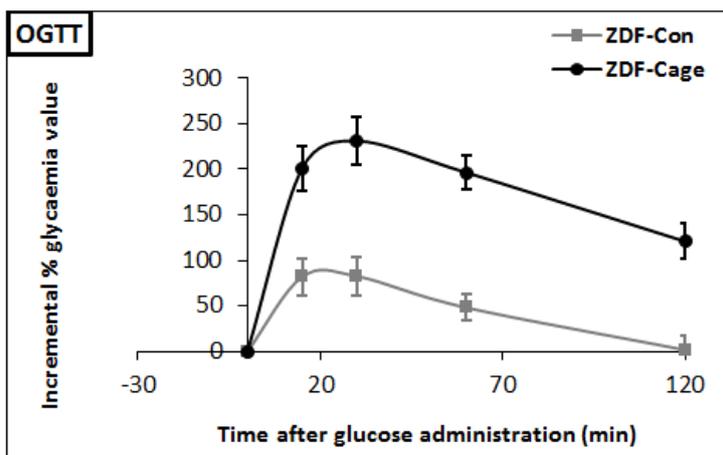


Figure 3

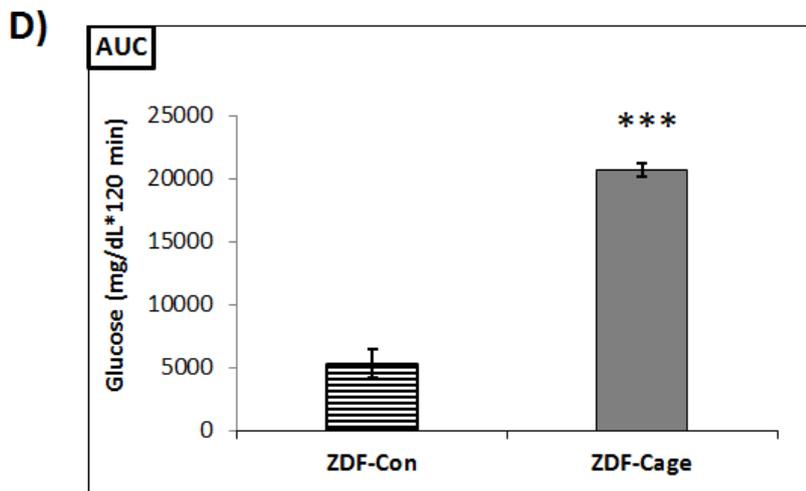
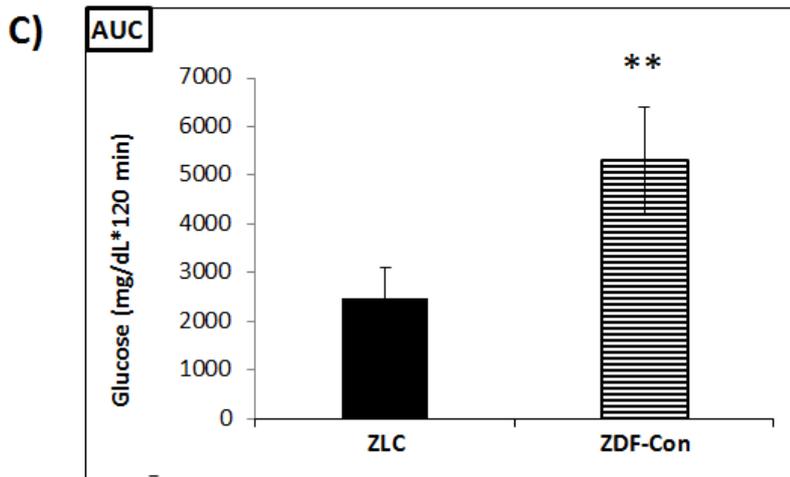


TABLE 2. Circulating parameters in immobilized ZDF rats.

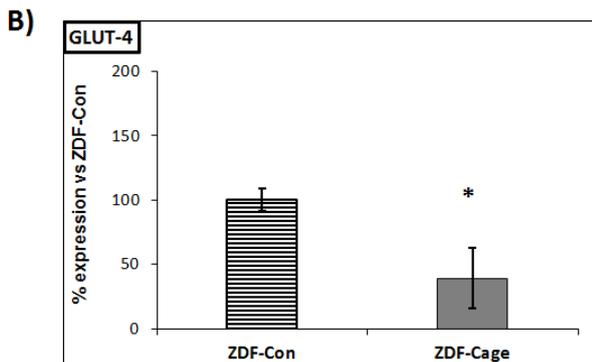
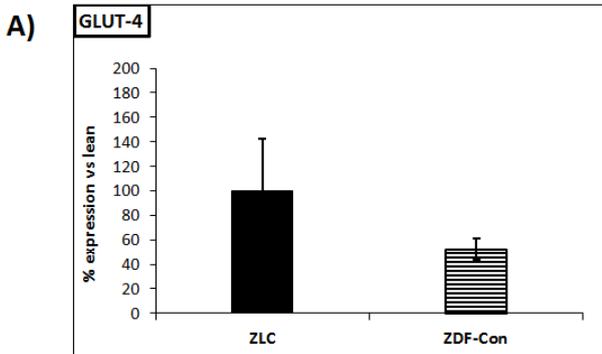
| | ZLC | ZDF-Con | ZDF-Cage |
|------------------------------|----------------|-------------------|-------------------|
| Biochemical Parameter | | | |
| Glucose (mg/dL) | 135 ± 3.3 (6) | 221 ± 8.7 (6)*** | 234 ± 15.5 (6) |
| Insulin (ng/mL) | 3.5 ± 0.7 (6) | 1.4 ± 0.4 (5) ** | 1.8 ± 0.2 (6) |
| HOMA-IR | 1.1 ± 0.2 (6) | 18.4 ± 4.2 (5) ** | 26.7 ± 4.8 (6) |
| Triglycerides (mg/dL) | 129 ± 19.1 (6) | 500 ± 53.6 (5)** | 258 ± 36.2 (6) ** |
| Adiponectin (pg/mL) | 1349 ± 118 (6) | 1359 ± 175 (5) | 947 ± 108 (6) # |
| Inflammatory markers | | | |
| SAA (ng/mL) | 928 ± 297 (6) | 277 ± 124 (6) # | 383 ± 135 (5) |
| IL-6 (pg/mL) | 91.2 ± 5.4 (6) | 92.2 ± 5.9 (5) | 94.2 ± 2.3 (5) |

Data are expressed as mean ± SEM for the number of animals indicated in parentheses. Non-immobilized control animals: ZDF-Con; and cage immobilized animals: ZDF-Cage. Fasting plasma insulin and glucose levels were measured 4 days before euthanasia and after a 12h fasting period. Insulin sensitivity of individual animals was evaluated using the validated homeostasis model assessment (HOMA) index. The formula used was as follows: [HOMA-IR] = fasting serum glucose (mg/dL)x fasting serum insulin (mM/mL)/405. Plasma insulin, adiponectin, IL-6 (Interleukin-6) and SAA (Serum Amyloid A) were assessed by ELISA. Plasma triglycerides were measured by spectrophotometric systems. Values that were found to be significantly different by Student’s t-test between the lean ZDF littermate controls (ZLC) and ZDF-Con (ZDF-Con column) or ZDF-Con and ZDF-Cage groups (ZDF-Cage column) are indicated by *p<0.05, **p<0.01, #p<0.1.

FIGURE 4 GLUT4 in immobilized ZDF rats.

The average value of HMBs and 18S were used as invariant control for all studies. Columns: mean values of five animals; bars: SEM. Values that were found to be significantly different by Student’s t-test between the lean ZDF littermate controls (ZLC) and ZDF-Con (A) or ZDF-Con and ZDF-Cage group (C) are indicated by, *p<0.05.

Figure 4



The obese phenotype of the ZDF model is due to their defect in producing leptin. If leptin action is lacking, hyperphagia causes disease of non-adipose tissues with generalized steatosis, lipotoxicity, and lipoapoptosis [36]. Enlarged adipose tissue suffers from infiltration of macrophages and therefore imbalance of pro-inflammatory and anti-inflammatory factors (i.e. tumour necrosis factor- α and IL-6) leading to inflammation, impairment of insulin sensitivity and deregulation of lipid metabolism [37,38]. Most of these adipokines (i.e. leptin, cytokines, resistin and adiponectin and plasminogen activator inhibitor-1) have been shown to inhibit adipogenesis and thereby further promote adipose tissue hypertrophy [39,40]. Increased reactive oxygen species production from accumulated fats also leads to increased oxidative stress in blood, dangerously affecting other organs including the liver and skeletal muscle [41]. Adiponectin acts as an insulin sensitizer in liver and muscle and as an anti-atherogenic signal. A deficiency of this adipokine induces obesity and decreases insulin-regulated carbohydrate metabolism, thus leading to insulin resistance. In fact, the blood level of adiponectin falls in T2DM [42]. For this reason, we assessed the inflammatory state of ZDF rats quantifying the plasmatic concentration of some adipokines, namely IL-6 and SAA. Interestingly, the decreased glucose uptake in the immobilized group was associated with decreased levels of plasma adiponectin (-30%, $p < 0.1$) (Table 2). The presence of lower levels of this cytokine confirmed the worse insulin sensitivity observed in restrained ZDF rats. However, no changes in IL-6 and SAA levels were detected between ZDF-Cage and ZDF-Con rats (Table 2). No changes were observed in either circulating adiponectin, IL-6 or SAA in the diabetic animals as compared with the non-diabetic controls (ZLC) (Table 2)

Perturbations of lipid metabolism in diabetic ZDF rats is characterized by increased circulating triglycerides (TG), higher levels of NEFA, and total cholesterol increases compared with controls [43]. Interestingly, immobilization significantly decreased the concentration of circulating TG. Indeed, the high triglyceridemia, characteristic of the animal model used [44], was reduced by 48% (Table 2). This could be a consequence of the inhibition of lipolysis promoted by physical inactivity, as previously observed in human bed rest studies [6]. The decrease of lipolysis results in less uptake of fatty acids by the liver with a subsequent reduction in the hepatic rate of triglyceride synthesis.

Diabetic animals showed reduced individual muscle masses (including heart) as compared with the non-diabetic controls (ZLC) (Table 3). Immobilization did not result in any changes in tissue or organ weights when comparing the two diabetic groups, with the exception of an increase in the weight of brown adipose tissue (BAT), as shown in Table 3. BAT is a key tissue for energy expenditure *via* fat and glucose oxidation for thermogenesis. The increased BAT mass of the immobilized ZDF rats represents an index of an enhanced body's heat production under disuse condition. In fact,

during unloading it was found an increased sympathetic nervous system tonus that supports the notion that non-shivering thermogenesis is chronically stimulated under this catabolic state [45].

Our next objective was to analyse whether disuse-induced muscle atrophy aggravated the catabolic state induced by the diabetic phenotype. Unexpectedly, 28 days of immobilization in the space-restricted cage did not alter the muscle mass in diabetic animals. Indeed, no changes in muscle weights were recorded in ZDF-Cage rats versus the ZDF-Con group (Table 3). This is in contrast to the muscle atrophy observed in soleus muscle (-13%) of healthy rats immobilized in the space-restriction model after the same period of disuse [20].

Muscle mass is a function of the size and number of muscle fibers. A marked increase in the intramuscular connective tissue, as a result of the myofibrils loss, could compensate a loss of muscle fibers resulting in no changes in muscle weight, that contributes to deterioration of the functionality and of the biomechanical properties was reported in immobilized skeletal muscle [46,47]. Therefore, we decided to examine whether some differences were present in fiber size between immobilized and free-moving animals. Interestingly, mean muscle fiber size from soleus muscle cross-sections of ZDF-Cage animals was significantly lower (20%; $p < 0.05$) than the non-immobilized diabetic ones (Figure 5B).

Muscle fiber atrophy typically lead to a decreased strength, functional capacity and ultimately increased mortality in patients with T2DM [48–50]. For this reason, we measured muscle strength by means of grip force evaluation. The diabetic groups showed decreased grip force, as compared with the non-diabetic controls (ZLC) (Table 4). ZDF-Cage rats did not show any decrease in muscle force, as compared with the non-immobilized controls (Table 4).

Indeed, an additional experiment designed to measure the physical performance was performed. Using the *Actimeter* device we were able to translate individual changes into an infrared pattern caused by movements of the animals into arbitrary activity counts (see the Experimental section for more details). As previously reported by our group, the outputs generated by the system are directly correlated with changes in body weight and muscle mass loss of the animal [51]. Control diabetic rats showed the same activity as the non-diabetic controls (ZLC) (Table 5). Interestingly, in spite of the lack of muscle weight loss, total physical activity was significantly reduced (22%, $p < 0.05$) in cage-restricted animals in the 24 hour-period before their sacrifice, as compared with the control diabetic ones (Table 5), as confirmed by lower number of stereotyped movements (-54%, $p < 0.01$) and total travelled distance performed (-35%, $p < 0.05$). Resting time of ZDF-Cage animals was also increased (+4%, $p < 0.05$), while the time involved in different type of movements was decreased.

In summary, diabetic rats immobilized for 28 days in the space restriction model do not develop

muscle mass loss, in spite of impaired physical activity. The cage-immobilized animals show glucose intolerance and increased insulin resistance as suggested by reduced plasmatic adiponectin levels and a decrease in GLUT4

mRNA muscle expression. In conclusion, altogether the results presented here clearly emphasize the importance of studying bed rest in different pathological conditions and, therefore, future studies should be encouraged.

TABLE 3 Tissue and muscle weights in immobilized ZDF rats.

| | ZLC | ZDF-Con | ZDF-Cage |
|------------------------|------------------|-------------------|------------------|
| Organs | | | |
| KIDNEY | 839 ± 20.6 (6) | 1049 ± 63.7 (6) | 951 ± 44.3 (6) |
| SPLEEN | 198 ± 2.5 (6) | 172 ± 3.6 (6) | 167 ± 3.1 (6) |
| LIVER | 4418 ± 342 (6) | 5932 ± 388 (6) | 5953 ± 249 (6) |
| CARCASS | 97942 ± 1215 (6) | 72081 ± 1181 (6) | 72430 ± 659 (6) |
| HEART | 363.2 ± 13.3 (6) | 300 ± 5.8 (6)** | 301 ± 5.2 (6) |
| Skeletal muscle | | | |
| GSN | 611 ± 12.3 (6) | 335 ± 10.5 (6)*** | 347 ± 5.4 (6) |
| EDL | 54.9 ± 1.6 (6) | 30.2 ± 1.0 (6)*** | 32.2 ± 0.7 (6) |
| TIB | 213 ± 5.2 (6) | 115 ± 2.5 (6)*** | 120 ± 2.7 (6) |
| SOL | 52.6 ± 1.5 (6) | 32.9 ± 0.9 (6)*** | 34.3 ± 0.8 (6) |
| Adipose Tissue | | | |
| WATd | 1364 ± 70.5 (6) | 3286 ± 113.2 (6)* | 3308 ± 98.0 (6) |
| WATe | 1225 ± 86.3 (6) | 2044 ± 103 (6)*** | 1865 ± 63.1 (6) |
| BAT | 141 ± 4.1(5) | 173 ± 8.9 (6) * | 231 ± 15.5 (6)** |

Data are expressed as mean ± SEM for the number of animals indicated in parentheses. Non-immobilized control animals: ZDF-Con; and cage immobilized animals: ZDF-Cage. Tissue and muscle weights are expressed as mg/100 g of initial body weight (IBW). GSN: gastrocnemius, EDL: extensor digitorum longus; TIB: tibialis; SOL: soleus. WATd: dorsal white adipose tissue, WATe: epididymal white adipose tissue; BAT: brown adipose tissue. Carcass weight (body without organs) is also expressed as mg/100 g of initial body weight (IBW). Values of bilateral tissues were obtained from the average of the two components. Values that were found to be significantly different by Student's t-test between the lean ZDF littermate controls (ZLC) and ZDF-Con (ZDF-Con column) or ZDF-Con and ZDF-Cage groups (ZDF-Cage column) are indicated by *p<0.05, **p<0.01, #p=0.1.

TABLE 4. Effects of the immobilization on muscle strength in ZDF rats.

| | ZLC | ZDF-Con | ZDF-Cage |
|-------------------|---------------|------------------|---------------|
| GRIP FORCE | | | |
| day 0 | 4.0 ± 0.1 (6) | 2.5 ± 0.1 (6)*** | 2.4 ± 0.1 (6) |
| day 7 | 3.8 ± 0.1 (6) | 2.5 ± 0.1 (6)*** | 2.3 ± 0.1 (6) |
| day 14 | 4.2 ± 0.1 (6) | 2.8 ± 0.1 (6)*** | 2.6 ± 0.1 (6) |
| day 28 | 5.0 ± 0.1 (5) | 3.0 ± 0.1 (6)*** | 2.8 ± 0.2 (6) |

Data are expressed as mean ± SEM for the number of animals indicated in parentheses. Non-immobilized control animals: ZDF-Con; and cage immobilized animals: ZDF-Cage. Grip force of the forelimbs was measured once a week and expressed as g/g initial body weight. Values that were found to be significantly different by Student’s t-test between the lean ZDF littermate controls (ZLC) and ZDF-Con (ZDF-Con column) or ZDF-Con and ZDF-Cage groups (ZDF-Cage column) are indicated by ***p<0.001.

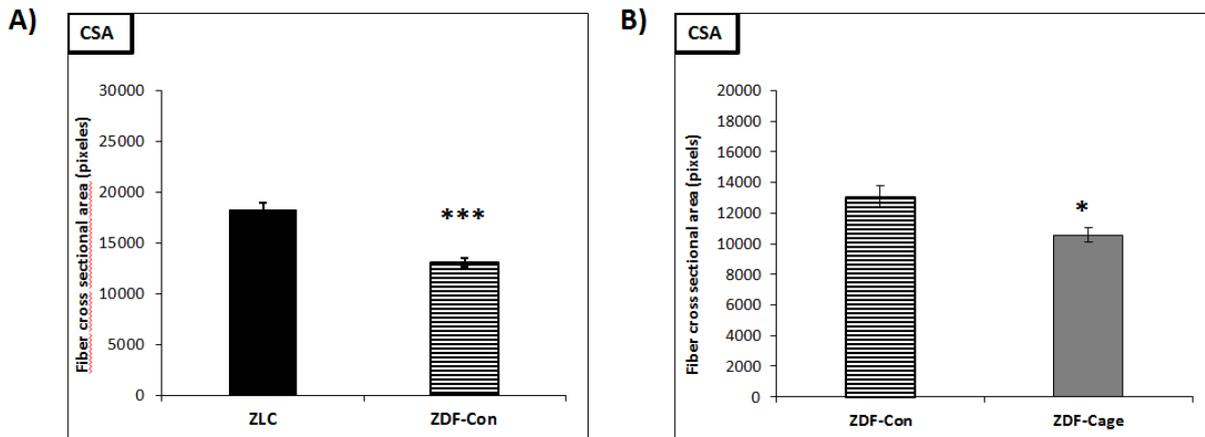
TABLE 5. Physical activity in immobilized ZDF rats

| | ZLC | ZDF-Con | ZDF-Cage |
|--|------------------|------------------|--------------------|
| Physical Activity (24h) | | | |
| TOTAL PHYSICAL ACTIVITY (activity units) | 35212 ± 3044 (6) | 32122 ± 2675 (6) | 24949 ± 958 (6)* |
| STEREOTYPED MOVEMENTS (number/second) | 4645 ± 383 (6) | 6611 ± 821 (6) # | 3018 ± 306 (6) ** |
| LOCOMOTOR MOVEMENTS (number/second) | 30567 ± 2742 (6) | 25512 ± 1979 (6) | 21931 ± 706 (6) |
| MEAN VELOCITY (cm/sec) | 0.7 ± 0.0 (6) | 0.7 ± 0.1 (6) | 0.5 ± 0.04 (6)* |
| DISTANCE TRAVELED (cm) | 56943 ± 3527 (6) | 60470 ± 8148 (6) | 39170 ± 3047 (6) * |
| Time (% of total 24h) | | | |
| Resting time | 79.9 ± 1.3 (6) | 82.1 ± 1.3 (6) | 85.3 ± 0.5 (6) * |
| Fast-movements time | 3.6 ± 0.3 (6) | 4.1 ± 0.4 (6) | 2.9 ± 0.1 (6) * |
| Slow-movements time | 16.5 ± 1.0 (6) | 13.8 ± 0.9 (6) # | 11.8 ± 0.4 (6) # |

Data are expressed as mean ± SEM for the number of animals indicated in parentheses. Non-immobilized control animals: ZDF-Con; and cage immobilized animals: ZDF-Cage. Physical activity is expressed in activity units. Stereotyped movements include movements without displacement (eating and cleaning movements); conversely, locomotor movements include movements with displacement. Mean velocity is expressed in cm/s. Total distance traveled is expressed in cm. Time is expressed as the percentage of total time (24 h). The thresholds of time are the following: time involving resting (sleeping, cleaning and eating time): [0–2] cm/s, time involving slow movements: cm/s and time involving fast movements: [>5] cm/s. Values that were found to be significantly different by Student’s t-test between the lean ZDF littermate controls (ZLC) and ZDF-Con (ZDF-Con column) or ZDF-Con and ZDF-Cage groups (ZDF-Cage column) are indicated by *p<0.05, **p<0.01, # p<0.1.

FIGURE 5 Muscle fiber size in immobilized ZDF rats.

Muscle fiber cross-sectional area (pixels) of soleus muscle was determined on randomly chosen 100 individual fibers per animal by the Matic Image Plus 2. Bars and segments represent the mean and SEM for each group (n=6). Non-immobilized control animals: ZDF-Con) and cage immobilized animals: ZDF-Cage. Values that were found to be significantly different by Student's t-test between the lean ZDF littermate controls (ZLC) and ZDF-Con (A) or ZDF-Con and ZDF-Cage group (B) are indicated by by * $p < 0.05$, *** $p < 0.001$,

Figure 5

Conflict of interests & statement of authorship

Each author has participated sufficiently, intellectually or practically, in the work to take public responsibility for the content of the article, including the conception, design, and for data interpretation. All authors have read and approved the final manuscript. All authors of this research have not conflict of interest related with employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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