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Treinamento de resistência com e sem administração de insulina: uma análise do plexo cardíaco e do tecido adiposo epicárdio

Resistance training with and without insulin administration: an analysis of the cardiac plexus and epicardial adipose tissue

Entrenamiento de resistencia con y sin insulina: análisis del plexo cardíaco y del tejido adiposo epicárdico

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Resumo

O plexo cardíaco é uma vasta rede de neurônios agrupados em gânglios distribuídos por todo o miocárdio. O tecido adiposo epicárdico cobre o coração desempenhando funções importantes, como o armazenamento de lipídios. No entanto, sua expressão exagerada pode representar um fator de risco, que pode ser prevenido pela prática de atividades físicas que aumentem a capacidade propulsiva contrátil do coração. A insulina tem sido utilizada em associação com exercícios físicos para aumentar a massa muscular e melhorar o desempenho físico. Tanto a insulina quanto os exercícios foram evidenciados devido aos seus efeitos neurotróficos. O objetivo deste estudo foi verificar se a insulina associada ao treinamento de resistência poderia modificar estruturalmente o plexo cardíaco e o tecido adiposo epicárdico. Quatro grupos (n = 6) de camundongos Swiss machos foram usados: não treinado salina, não treinado insulina; treinado; treinado insulina. O treinamento foi realizado em escada vertical a 90% da carga máxima, 3 vezes / semana durante 8 semanas consecutivas. Após o período experimental, os corações dos animais foram retirados e cortes de 5 μm foram corados com Hematoxilina / Eosina, Giemsa e Picrossírius para avaliação das estruturas do plexo cardíaco. Não houve diferença significativa em relação à área e ao número total de neurônios, nem à área com colágeno. No entanto, enquanto a administração de insulina hipertrofiou os adipócitos e predispôs um ambiente inflamatório, o exercício físico exerceu papel antiinflamatório. Como conclusão, vale ressaltar que o treinamento resistido não alterou o plexo cardíaco, porém o tecido adiposo epicárdico foi reduzido, efeito antagonizado pela insulina.

Palavras-chave: Coração; Neurônios; Treinamento de resistência; Gordura epicárdica.

Abstract

The cardiac plexus is a vast network of neurons grouped into ganglia distributed throughout the myocardium. The epicardial adipose tissue covers the heart performing important functions, such as, lipid storage. However, its exaggerated expression might represent a risk factor, which can be prevented by the practice of physical activities that improves the heart contractile propulsive capacity. Insulin has been used in association with physical exercise so as to increase muscle mass and improve physical performance. Both insulin and exercise have been evidenced due to their neurotrophic effects. The purpose of this study was to ascertain whether insulin associated with resistance training could structurally modify the cardiac plexus and epicardial adipose tissue. Four groups (n = 6) of male Swiss mice were used: non-trained saline, non-trained insulin; trained saline; trained insulin. The training was performed on a vertical ladder at 90% of the maximum load, 3 times/week for 8 consecutive weeks. After the experimental period, the hearts of the animals were removed, and 5-µm sections were stained with Hematoxylin/Eosin, Giemsa and Picrossirius in order to evaluate the structures of the cardiac plexus. There was no significant difference with regard to the area and the total number of neurons, nor to the area with collagen. However, whereas insulin administration hypertrophied the adipocytes and predisposed an inflammatory environment, physical exercise played an anti-inflammatory role. As a conclusion, it is worth mentioning that resistance training did not change the cardiac plexus, however the epicardial adipose tissue was reduced, an effect antagonized by insulin.

Keywords: Heart; Neurons; Resistance Training; Epicardial fat.

Resumen

El plexo cardíaco es una vasta red de neuronas agrupadas en ganglios distribuidos por todo el miocardio. El tejido adiposo epicárdico cubre el corazón y realiza funciones importantes, como el almacenamiento de lípidos. Sin embargo, su exagerada expresión podría representar un factor de riesgo, que puede prevenirse mediante la práctica de actividades físicas que mejoren la capacidad de propulsión contráctil del corazón. La insulina se ha utilizado en asociación con el ejercicio físico para aumentar la masa muscular y mejorar el rendimiento físico. Tanto la insulina como el ejercicio se han evidenciado por sus efectos neurotróficos. El propósito de este estudio fue determinar si la insulina asociada con el entrenamiento de resistencia podría modificar estructuralmente el plexo cardíaco y el tejido adiposo epicárdico. Se utilizaron cuatro grupos (n = 6) de ratones suizos machos: solución salina no adiestrada, insulina no adiestrada; solución salina adiestrada; insulina entrenada. El entrenamiento se

realizó en escalera vertical al 90% de la carga máxima, 3 veces / semana durante 8 semanas consecutivas. Después del período experimental, se extrajeron los corazones de los animales y se tiñeron secciones de 5 μm con Hematoxilina / Eosina, Giemsa y Picrossirius para evaluar las estructuras del plexo cardíaco. No hubo diferencia significativa con respecto al área y el número total de neuronas, ni al área con colágeno. Sin embargo, mientras que la administración de insulina hipertrofió los adipocitos y predispuso un ambiente inflamatorio, el ejercicio físico jugó un papel antiinflamatorio. Como conclusión, cabe mencionar que el entrenamiento de resistencia no cambió el plexo cardíaco, sin embargo el tejido adiposo epicárdico se redujo, efecto antagonizado por la insulina.

Palabras clave: Corazón; Neuronas; Entrenamiento de resistencia; Grasa epicárdica.

1. Introduction

The best known function of insulin is to maintain glycemic control. Research has shown that its use in association with physical exercise predisposes an increase of muscle mass and improves physical performance. Part of this effect can be attributed to the fact that this hormone is highly anabolic, since it stimulates several protein synthesis pathways, including the contractile proteins, which act in synergy with the growth hormone, thus, promoting muscle hypertrophy (Zanou, et al., 2013). Moreover, insulin decreases the plasma levels of fatty acids and increases the uptake of triglycerides by the adipose tissue (Dimitriads, et al., 2011). However, its continuous use might change important cardiovascular parameters, such as blood pressure, in addition to causing dysfunctions, for example, atherosclerosis and cellular edema in the cardiac musculature (Ferrari, 2013).

On the other hand, the regular practice of physical exercise is always referred to as being beneficial for the main cardiovascular parameters. Studies have shown that exercise improves the heart contractile propulsion capacity as this practice strengthens myocardium and prevents the deposition of adipose tissue into this organ (Monti, et al., 2015). Moreover, since long-term exercises stimulate anti-inflammatory effects, they are associated with the prevention of cardiovascular diseases (Giada, et al., 2008).

Regularity in practicing exercise is also correlated with health and neuronal preservation (Ichige, et al., 2016), such as the preventing degeneration common to aging (Gama, et al., 2010).

Similarly to physical exercise, insulin has been described as a neurotrophic agent, capable of promoting proliferation, differentiation and neuronal growth, it is believed to

stimulate the expression of the TAU protein (necessary for the growth of neurons) and inhibit the expression or stabilization of tubulin mRNA (Nemoto, et al., 2011). Despite that, specific studies that correlate its use with exercise and cardiac plexus neurons were not found.

The cardiac plexus consists of a vast network of neurons grouped into ganglia which are distributed throughout the heart tissue, especially in the atrial region and the great vessels of the base of the heart . These ganglia are covered by connective tissue capsules that support and shape them. Considering their functions, these ganglia process information from the autonomic nervous system, retransmitting it to the myocardial fibers and actively participating in the regulation of the organ activity, and that is why it is called the Intrinsic Cardiac Nervous System (ICNS) (Jimenes, et al., 2017).

Part of the protection of both the ganglia and autonomic nervous system of the heart is performed by the epicardial adipose tissue, which also has important functions, such as lipid storage to supply the energy that myocardium needs and regulate the vasomotricity of the coronary arteries. Anatomical and metabolic characteristics distinguish it from other visceral fats, such as the accelerated metabolism of fatty acids and a single transcriptome enriched in genes which are associated with inflammation and endothelial function at local and systemic levels. The exaggerated expression of this tissue might represent a risk factor, triggering cardiac arrhythmia, that is, a coronary artery disease (Lacobellis, et al., 2015).

Several studies have shown that the ICNS can be structurally and functionally changed as a result of infarction, infection, aging, diabetes (Jimenes, et al., 2017). However, researches that correlate it to the use of insulin concomitant with the practice of physical exercises were not found. Therefore, considering the correlation of facts and the functional importance of the ICNS and epicardial adipose tissue, this study aimed at ascertaining whether the use of exogenous insulin and resistance training on a vertical ladder can morphologically modify the cardiac plexus and the epicardial adipose tissue of mice.

2. Materials and Methods

2.1 Animals

This study was carried out with Swiss male mice, divided into 4 groups (n=6/group): Non-trained Saline (NTS): mice not submitted to strength training on a vertical ladder that received saline; Non-trained Insulin (NTI): mice not submitted to strength training on a vertical ladder and treated with insulin; Trained Saline (TS): mice submitted to strength

training on a vertical ladder that received saline; Trained Insulin (TI): mice submitted to strength training on a vertical ladder and treated with insulin. Both insulin and saline were intraperitoneally administered (0.3 U/kg body weight) 5 days a week, at about 12 o'clock. Before and after the experimental period (which lasted 8 weeks), the animals were weighed on an appropriate scale (FILIZOLA®).

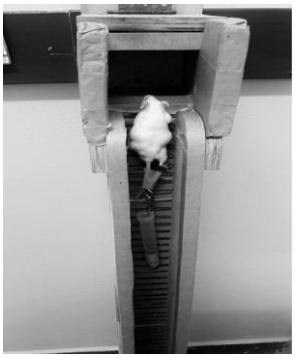
All the principles of animal experimentation established by the National Council for the Control of Animal Experimentation (CONCEA) were abided, and the experimental protocol described herein was previously approved by the Committee on Animal Research and Ethics (CARE) of the State University of Maringá (UEM), under the legal opinion number 8548200616/2016.

2.2 Resistance training model

For training the mice, a 105 cm height and 8 cm width vertical ladder was used, inclined at 80º, with steps of 2 mm width, 1 cm apart from each other (Figure 1). A 12 x 12 x 12 cm chamber was housed at the top of the ladder to serve as shelter for the resting period of the animals during the climbing series. The base of the ladder was 10 cm away from the ground, preventing the tail of the animals from having any contact with the floor. The loading system was fixed with adhesive tape on the proximal portion of the tail of the animals.

The training protocol initially considered the establishment of the maximum exercise load, since the training of the TS and TI groups was performed at 90% of this value, at a frequency of 3 times a week, during 8 weeks. The maximum load (ML) was established as the load of the last complete climbing. Each week a new ML test was performed, but starting from the ML of the previous week, to adjust the training intensity. Mice from groups SS and SI were subjected to the ML test on the first and last week of the experimental period (Pereira, et al., 2019).

Figure 1. Mouse performing the vertical ladder training.



Source: prepared by the authors based on the research data.

2.3 Heart collection and histological processing

After 8 weeks of training, the animals were intraperitoneally anesthetized with 120 mg/kg of Thiopental and 5 mg/kg of Lidocaine, and subsequently submitted to thoracotomy to remove the heart, pericardium and the vessels of the heart base.

The removed organ was weighed on an analytical scale (SHIMADZU®), then washed with PBS solution (0.1 M; pH 7.4) and stored in 4% paraformaldehyde for 48 hours. Subsequently, the heart was submitted to routine histological processing for paraffin embedding and microtome cutting.

The heart was embedded with the apex downwards and all sections were performed above the atrioventricular septum, since the number of cardiac plexus ganglia located in the atrial tissues is higher than those of the ventricular tissues.

Semi-serial cross-cuts of 5 µm were performed by using a microtome (Leica RM 2255®) from the apex-base of the heart to the base of the vessels in the atrial region, and, then, distributed in 12 slides, each one containing 5 sections (60 sections/mouse).

The slides were stained by using three histochemical techniques: 1. Hematoxylin/Eosin (H.E.) to identify and locate the cardiac plexus ganglia and visualize basic cellular aspects, such as nucleus, nucleolus and cytoplasm of the neurons and glia cells, as well as histopathological aspects; 2. Giemsa in order to complement the identification, localization and quantification of the cardiac plexus neurons, in addition to identifying the glia cells by distinguishing between higher and lower basophilic intensity neurons; 3. Picrossirus red, with polarized light to quantitatively analyze type I and type III collagen fibers associated with ganglia.

2.4 Quantitative, morphometric and morphological analysis of the cardiac plexus

The quantitative analysis of the ganglia neurons of the cardiac plexus was carried out by sampling and analysing 40 microscopic fields of each animal; all the neurons of each ganglion found were counted by using a photonic microscope (MOTIC®) 40X objective lens.

The morphometric analysis assessed the cell body area of 100 neurons of the cardiac plexus/animal distributed in different sections. Therefore, the sections were visualized under an optical microscope (MOTIC®) equipped with a camera associated with a computerized image analysis system (Image Pro-Plus 4.0).

The morphological analysis showed the structural characteristics of the cells that form the heart plexus. Thus, besides the location of the cardiac plexus, the basic cellular aspects of both neurons and glia cells were analyzed (nucleus, nucleolus, cytoplasm and shape).

2.5 Analysis of the collagen fibers of the cardiac plexus

A common light and polarized light microscope (MOTIC®) was used to differentiate the birefringence and the organization of type I and type III collagen fibers of the extracellular matrix associated with the plexus.

2.6 Histopathological and morphometric analysis of the epicardial adipose tissue

The sections of the epicardial adipose tissue stained with H.E. were observed with a 20X objective lens. Ten fields/slide/animal were analyzed. The inflammatory infiltrates were quantified according to their intensity and distribution .The intensity considered the number of cells of the immune system seen: absent (0 to 9 cells), mild (10 to 25 cells), moderate (26 to 50 cells) and intense (> 50 cells). The distribution of the inflammatory infiltrates was considered per field: focal (a single infiltrate in the visual field); multifocal (more than one infiltrate in the visual field), and diffuse (inflammatory cells diffusely distributed in the visual field) (Michailowsky, et al., 2001).

The morphometric analysis used sections stained with H.E. and measured the area of 50 adipocytes/animal in random areas. The images were captured with an optical microscope (MOTIC®) equipped with a camera, associated with a computerized image analysis system (Image Pro-Plus 4.0).

2.7 Statistical analysis

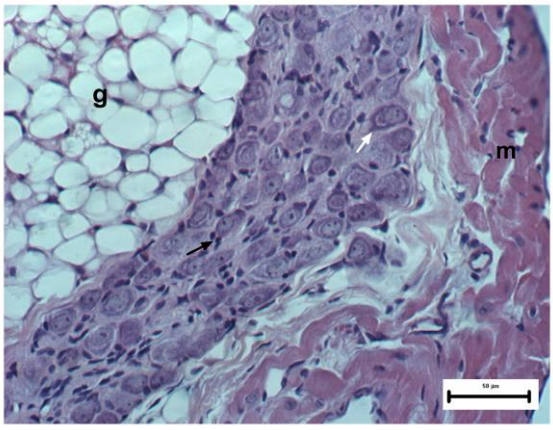
Data were compared by using BioEstat 5.4. software (Mamirauá®). Either the D'Agostino Pearson test or Shapiro-Wilk test was carried out so as to determine the data normality. Kruskal Wallis test followed by *Dunns'* post-test (*GraphPad Prism* 8®) were used. The results were expressed as mean±standard deviation, and $p < 0.05$ was considered statistically significant.

3. Results

3.1 Location and characterization of the cardiac plexus

The cardiac plexus ganglia of all the groups assessed were adjacent to the vessels of the base of the heart and immersed in their unilocular fat. The analysis of the ganglia showed that they vary in size and shape. However, whereas the neuronal bodies had a globular shape with decentralized nuclei (including some binucleated cells), the glial cells were always positioned peripherally to the neurons (Figure 2).

Figure 2. Heart ganglion of the mouse. Neurons (**white arrow**) and glial cells (black arrow); myocardium **(m)** and adipose tissue **(g).** TI Group. Hematoxylin/Eosin, 40X objective lens.

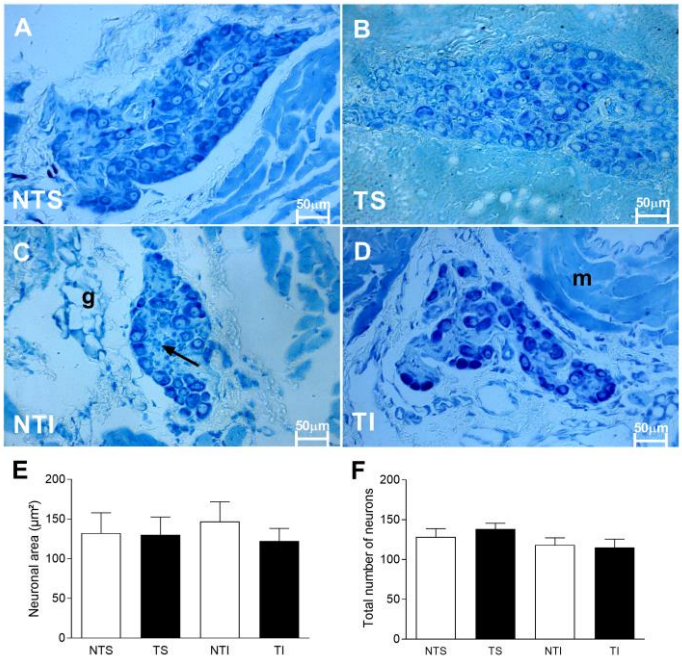


Source: prepared by the authors based on the research data.

3.2 Quantitative and morphological analysis of the cardiac plexus

Resistance training and the use of insulin did not quantitatively or morphologically change the cardiac plexus (Figure 3E,F). Therefore, there was no significant difference in relation to the neuronal area of the different experimental groups. Likewise, the total number of neurons per animal was not modified by these variables.

Figure 3. Photomicrographs obtained by using a 40X objective lens. Staining through Giemsa's technique showing the ganglia and heart plexus neurons of the NTS (A), TS (B), NTI (C) and TI (D) groups; Total neuronal area (E) and total number of neurons (F) of the cardiac plexus of the NTS, TS, NTI, TI groups**. g:** adipose tissue; **m**: myocardium; **black arrow:** glial cells. The values refer to the mean±standard deviation. *One-way* ANOVA, followed by *Tykey* post-test.



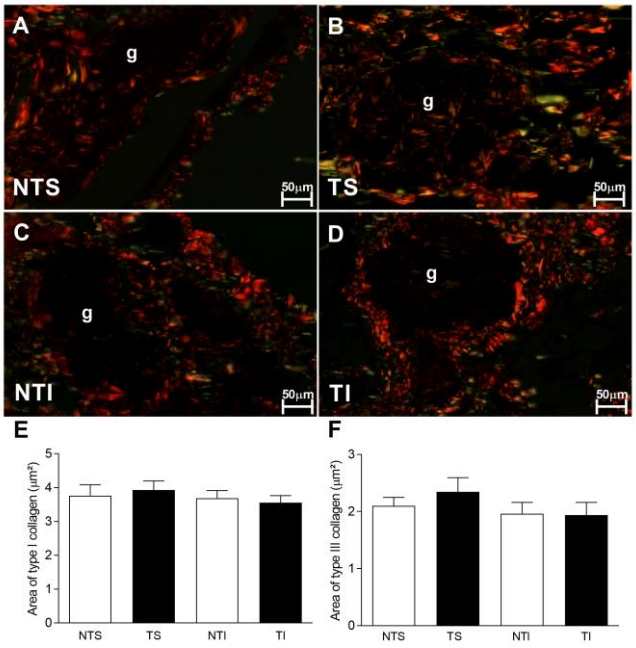
Source: prepared by the authors based on the research data.

3.3 Analysis of connective tissue associated with the cardiac plexus

The findings of the present study showed that both the neurons and the ganglia evaluated were covered by type I collagen and, to a lesser extent, by type III collagen (Figure 4A,B,C,D).

The area occupied by type I collagen fibers was not different among the experimental groups, nor it was different in relation to type III collagen (Figure E,F).

Figure 4. Photomicrographs obtained by using a 40X objective lens (**A, B, C** and **D**) showing the deposition of collagen fibers into the cardiac plexus of the NTS (**A**), TS (**B**), NTI (**C)** and TI (**D**) groups. **E**: graph showing the quantification of type I collagen fibers; **F**: graph showing the quantification of type III collagen fibers. The values refer to the mean±standard deviation. *One-way* ANOVA, followed by *Dunns'* post-test. **g**: area occupied by cardiac ganglion.

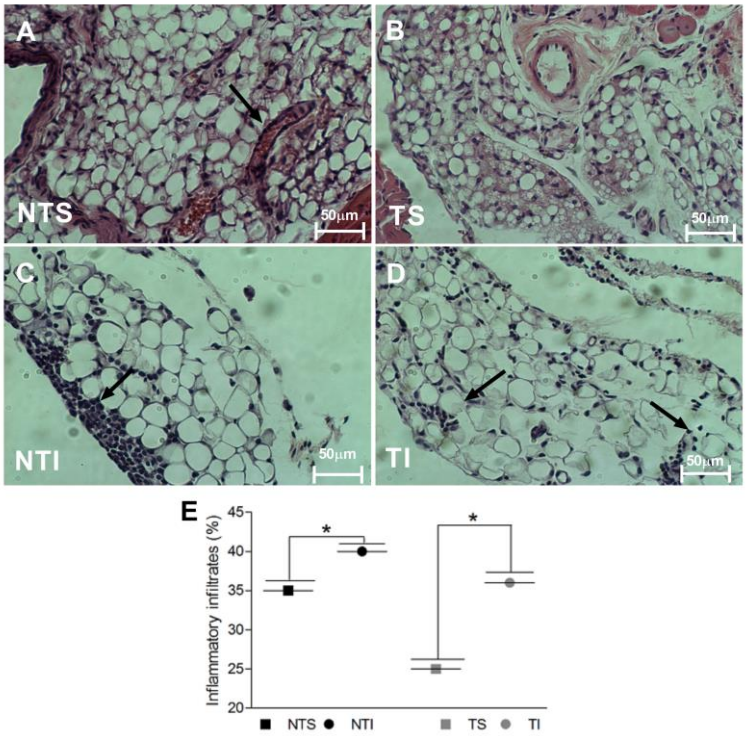


Source: prepared by the authors based on the research data.

3.4 Histopathological and morphometric analysis of the epicardial adipose tissue

The histopathological analysis of the epicardial adipose tissue evidenced a substantial increase in the percentage of inflammatory infiltrates in the NTS (35%), NTI (40%) and TI (36%) groups in relation to the TS group (25%) (Figure 5E). The NTS group showed an intense and moderate profile of inflammatory infiltrates in the epicardial fat (Figure 5C).

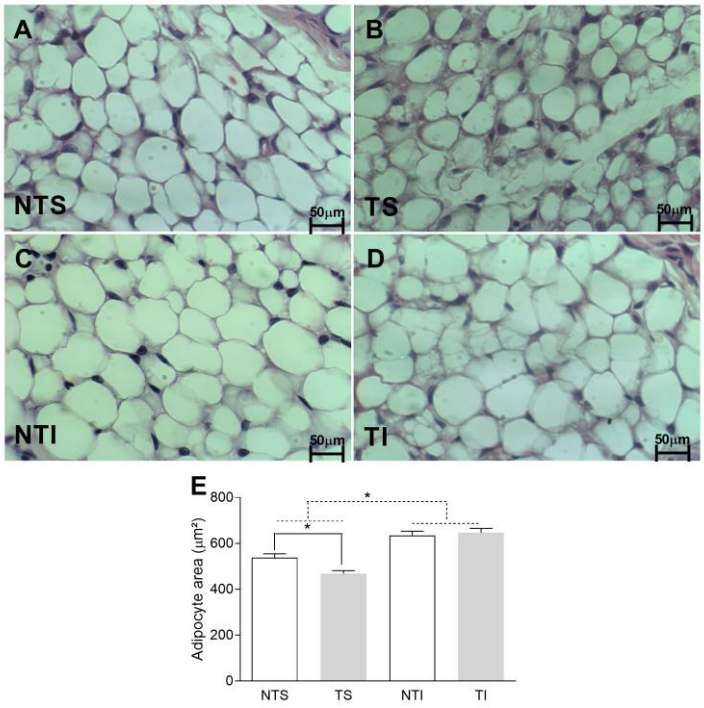
Figure 5. Photomicrographs obtained by using a 20X objective lens showing inflammatory infiltrates in the epicardial fat (black arrows) of the NTS (A), TS (B), NTI (C) and TI (D) groups**. E:** graph showing the total percentage of inflammatory infiltrates (values refer to the percentage); *One-way* ANOVA, followed by *Dunns'* post-test; *p<0.05.



Source: prepared by the authors based on the research data.

The morphometric analysis of this adipose tissue showed a significant difference in the adipocyte area, that is, lower in the TS group than in the NTS group. Furthermore, there was a significant increase in the adipocyte area in the NTI and TI groups in relation to the NTS and TS.).

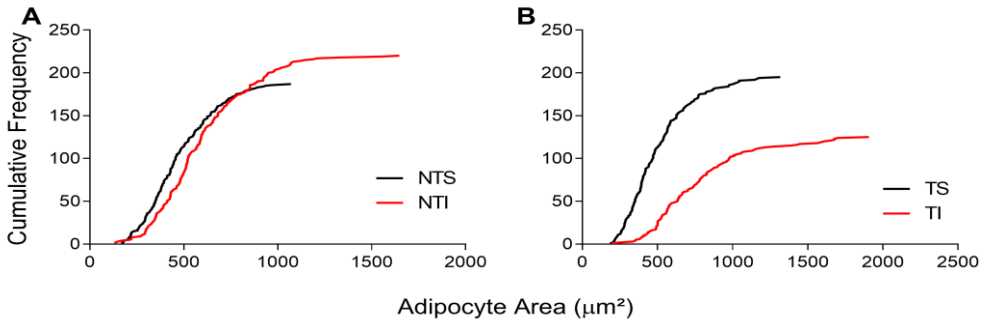
Figure 6. Photomicrographs obtained by using a 40X objective lens showing the cardiac epicardial adipose tissue of the NTS (A), TS (B), NTI (C) and TI (D) groups: graphs comparing the adipocyte area. *One-way* ANOVA, followed by *Tykey* post-test. *p<0.05.



Source: prepared by the authors based on the research data.

In addition, the cumulative distribution of adipocyte area frequencies shows that insulin adiministration played a role in adipocyte hypertrophy, while NTS and TS groups had more adipocytes between 500-1000 µm² (Figure 6.1A), the NTI and TI groups had between 1000-1500 µm² (Figure 6.1B).

Figure 6.1. Adipocyte area cumulative distributions of the epicardial adipose tissue from no-treined mice (NTS-NTI) and treined mice (TS and TI).



Source: prepared by the authors based on the research data.

4. Discussion

The present study is the first to evaluate the influence of resistance training on a vertical ladder and insulin administration on the cardiac plexus and epicardial tissue of Swiss mice. The literature review generally shows changes in the heart muscular structure. It is known that modifications in muscle mass and/or cardiac function are a reflex of morphorfunctional changes in their control systems, such as the one performed by the ICNS.

The morphological analysis of the present study on the neurons and glial cells of the cardiac plexus was in agreement with the data shown in the current literature. Therefore, the location of the ganglia was confirmed in the atrial region, near the vessels of the organ base and arranged in the middle of its unilocular fat. An elongated shape of these ganglia and the rounded shape of their neurons with their nuclei and nucleoli decentralized were also identified, which were bordered by the glial cells (Jimenes, et al., 2017).

Previous results from our research group using this same experimental model showed that there was a significant difference in relation to body weight, where trained animals (TS and TI) had less weight when compared to sedentary groups. Biochemical data showed that the use of insulin increased the dosage of hepatic glycogen and the release of basal glucose from the liver, and its use associated with training meant that the increase in exercise-induced gluconeogenesis was reduced (Pereira, et al., 2019).

No changes were identified either in the number of neurons or in the neuronal area of the different experimental groups after training and insulin administration. As previously mentioned, this fact might have resulted from the training period adopted herein, since correlated studies have recommended more than 8 weeks of training (Neto, et al., 2017) or higher weekly frequency (Rosa, et al., 2005).

A study used a moderate running protocol for 10 months to evaluate the morphological changes of the cardiac plexus due to age (Gama, et al., 2010). It is necessary that the training period is from 8 to 16 weeks so that benefits are obtained. In addition, since a training period inferior or equal to 8 weeks is equivalent to only 15 to 30% of the total life time of the animal, such a practice cannot be considered habitual (Rosa, et al., 2005).

We thought, then, that the exercise protocol used in this study was not able to change the morphological and quantitative parameters of the cardiac plexus, because, the results of the present study showed that there was no significant difference in the neuronal area of the different groups. These data differ from studies whose results showed that physical exercise reduced the neuronal area of the cardiac plexus (Gama, et al., 2010).

Finally, the results of this study showed that resistance training and insulin administration did not significantly change either the amount deposited or the proportion of types I and III collagen fibers. There is a predominance of type I collagen over type III, since this type of collagen is important for structural maintenance of expandable organs, such as the heart (Akamatsu, et al., 2007).

The histopathological analysis of epicardial fat revealed that insulin administration played an inflammatory role, whereas resistance training had a protective effect. In fact, the epicardial fat has been pointed as responsible for the production of inflammatory cytokines (TNF-α, IL-1β and IL-6) and vasoactive substances capable of locally or systemically change the endothelial function and promote cardiometabolic changes. On the other hand, this tissue is also capable of secreting adiponectins and adrenomedulins with important anti-inflammatory effects (Talman, et al., 2014).

Regular exercise minimizes the risk of chronic and metabolic cardiorespiratory diseases, partly because it exerts anti-inflammatory effects that reduce visceral adipose tissue (and consequently reduce adipocines), and partly because it predisposes to an anti-inflammatory environment (Gleeson, et al., 2011). In addition, researchers showed an increase in serum concentrations of adiponectins and IL-15 (anti-inflammatory substances), reduction in serum concentrations of resistin, leptin, and C-reactive protein (pro-inflammatory cytokines) after 24 weeks of combined training (resistance + aerobic) in obese men (Brunelli et al., 2015). Recent studies have emphazised the beneficial changes that physical training is able to induce in fat tissue, both in subcutaneous (Stanford, et al., 2015), and epicardial fat (Kim, et al., 2009).

The present results showed that in the TS group there was a decrease in the area of epicardial fat adipocytes. These findings are in agreement with current researches according to which exercise, even without changing body weight, reduces the epicardial fat and visceral fat tissue (Willund, et al., 2010). On the other hand, insulin administration increased the area of these adipocytes in the NTI and TI groups; the authors pointed out that insulin inhibits lipolysis of the unilocular fat tissue and plays an anabolic role in the fat tissue through several mechanisms, such as stimulation of the FSP27 protein, responsible for the formation of the unilocular vesicles of adipocytes (Czech, et al., 2013). Therefore, insulin administration can be harmful because, although it does not change the body weight of the animals, it increases cardiac adipocytality, which predisposes an increased risk of cardiovascular diseases.

Finally, some limitations must be taken into account. The scarcity of resources has deprived us of more specific analyzes such as biochemical measurements, pro and anti-

inflammatory cytokines and immunohistochemical techniques in cardiac tissue. However, the histological analyzes performed here can serve as a basis for more specific future studies.

5. Conclusions

The data obtained in the present study showed that the resistance training performed on a vertical ladder for 8 weeks and insulin administration did not morphologically or quantitatively change the cardiac plexus, nor the proportion of associated type I and III connective tissue. However, insulin administration predisposed to an inflammatory environment and hypertrophy of the epicardial fat, whereas exercise played an anti-inflammatory role in this fat.

Therefore, considering the functional importance of the cardiac plexus and the lack of research that correlates it with the regular practice of physical exercise, further research that investigate, for example, the influence of resistance training on the plexus in longer periods, and other training protocols, is suggeted. Such findings contribute to improve the understanding of how physical exercise is related to the ICNS neurons.

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