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Collagen and reticular fibers in left ventricular muscle in diabetic rats: Physical exercise prevents its changes?

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ABSTRACT

Diabetic cardiomyopathy contributes to the high incidence of mortality in both types of diabetes. We aimed to investigate the histochemical aspects of collagen and reticular fibers in the cardiac muscle and evaluate the influence of physical exercise on these aspects. Wistar rats were divided in 4 groups: sedentary control (SC), trained control (TC), sedentary diabetic (SD) and trained diabetic (TD). Diabetes was induced with alloxan (35 mg/kg). Training program consisted of swimming 1 h/day with a load of 4.8% of body weight for TD and 5.2% for TC, during 8 weeks. At the end of the experiment, samples of the left ventricle were collected, fixed in Bouin and processed on historesin. Sections were stained with periodic acid of Schiff, picrosirius-hematoxylin and ammoniacal silver. The PAS technique shows that individuals of group SD presented more intense reaction that the other groups. Picrosirius-hematoxylin technique showed a possible deposition of collagen fibers in SD. The TD group presented a reaction a lot similar to the controls' for both techniques', showing a possible prevention of this deposition. These results indicate that physical exercises might have an important role on the prevention of some negative alterations caused by experimental diabetes.

1. Introduction

Type 1 Diabetes mellitus is characterized by an absolute insulin deficiency caused by destruction of β-pancreatic cells. The main characteristic of the individual with diabetes is the occurrence of hyperglycemia, but other symptoms can be observed as polyuria, polydipsia, glycosuria, polyphagia and the increase in the presence of ketone bodies in urine and blood (Leon, 1987).

Experimentally, type 1 diabetes can be induced by the application of specific drugs like alloxan and streptozotocin, which selectively destroys the pancreatic beta cells causing a permanent hypoinsulinemia (Lerco et al., 2003; Wei et al., 2003).

Diabetic patients are considered in high risk for vascular disorders affecting the heart, brain, kidneys and peripheral vessels. These diabetic patients have presented a significant increase in mortality, mostly in recent decades (American Diabetes Association, 2003; Alexander et al., 2000), and cardiovascular disease is the leading cause of morbidity and mortality for both types of diabetes (Smanio, 2007).

Studies have suggested that diabetes may cause left ventricular dysfunction (Cosyns et al., 2007), one of the most common cardiovascular complications of diabetic patients (Cosson and Kevorkian, 2003), directly resulting in increased susceptibility to heart failure. The high left ventricular mass and wall thickness, as well the reduced left ventricular ejection fraction have also been reported for diabetic patients (Stratmann et al., 2010).

The left ventricular dysfunction also may be an early sign of diabetic cardiomyopathy (Cosson and Kevorkian, 2003), a disease that is believed to contribute to the high incidence of cardiac dysfunction and mortality from both types of diabetes (Fein, 1990; Trost and LeWinter, 2001), independent of other factors such as hypertension. Although the processes related to diabetic cardiomyopathy are not yet well known, it is speculated that they are linked to the reduced energy production due to decreased mitochondrial respiration and activity of dehydrogenases, the dysfunction of regulatory proteins and contractile impairment in the homeostasis of intracellular calcium (Li et al., 2003) and the deposition of interstitial collagen, both type I and type III.

Currently, regular exercise, along with insulin therapy and meal planning, have been regarded as one of the main approaches in the treatment of type 1 diabetes, aiming to approximate the metabolic conditions of the patient to a normal physiological state, preventing or delaying the chronic complications of diabetes (De Angelis et al., 2006).

Therefore, aim of this study is to evaluate, through qualitative analysis, the histological and histochemical damage caused by diabetes in experimental left ventricular muscle of diabetic rats and

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to evaluate the effects of exercise on the possible recovery or prevention of these changes.

2. Methods

2.1. Animals

This study was approved by the Ethical Committe in Research and Scientific Merit of the Universitary Center Hermínio Ometto - UNIARARAS (protocol number 634/2008). Adults male Wistar rats (333.6 ± 31.9 g; 70 days old) from the Central Animal Breeding Center, São Paulo State University, Botucatu campus, were used in the experiments. They were kept at 25 °C with a light/dark cycle of 12 h/12 h, and received Purina® rat chow and water ad libitum.

2.2. Diabetes induction and experimental design

Diabetes was induced by an intravenous injection (35 mg/kg b.w.) of Alloxan (Sigma). After 5 days, blood samples were obtained with animals in the fed state to determine the plasma glucose concentration. Rats that were not diabetic (glucose < 11.2 mmol/L) were eliminated from the study. For the study, the rats were randomly allocated to one of four groups ($n = 5$ per group): sedentary control (SC), trained control (TC), sedentary diabetic (SD), and trained diabetic (TD).

Training included daily swimming 1 h/day, 5 days/week, for 8 weeks, with a load of 4.8% (for diabetic animals) and 5.2% (for healthy animals), corresponding to the maximal lactate steady state (Gobatto et al., 2001).

2.3. Animal evaluation and sacrifice

At the end of the experiment, rats from each group were kept at rest for 48 h after the last exercise session, without fasting. The blood was collected and centrifuged at 3000 rpm for 10 min and from the serum glucose analysis was performed. Based on mean blood glucose, three animals most representative of each group were chosen to perform the histochemical analysis. The animals were sacrificed with prior anesthesia in CO_2 chamber. After sacrifice, portions of the left ventricle of the selected animals were collected and fixed in Bouin.

2.4. Histochemical analysis

Tissues were embedded in historesin and microtome sectioned. The sections were then stained with PAS (Mcmanus, 1946), for detection of polysaccharides; Picrosirius-hematoxylin (for determination of total collagen) and ammoniacal silver (for reticular fibers), adapted from Junqueira and Junqueira (1983). Slides with the stained sections were mounted with Canada balsam and photographed with light microscope Leica DM2000, Leica camera DFC280, with the IM50 software. A qualitative analysis of the slides was performed, based on the intensity of the reaction with the ventricular muscle.

2.5. Statistical analysis

All results were expressed as mean ± standard deviation. Statistical comparisons were made by analysis of one-way (ANOVA) with post hoc Bonferroni or Kruskal–Wallis with post hoc Dunn, with significance level $p < 0.05$.

3. Results

Table 1 summarizes the serum glucose value previously to sacrifice and Table 2 presents the qualitative analysis from histochemical techniques for every individual of each group.

The technique to evidence polysaccharides (PAS) shows that the individuals of group SD (Fig. 1C) presented a more intense reaction when compared to the control groups (Fig. 1A and B), as much in the sarcoplasm as in the endomysium. The animals of TD group (Fig. 1D) presented a reaction in the sarcoplasm very similar to the one observed on the control groups; on the other hand, the recovery was not that evident on the endomysium.

The technique of picrosirius-hematoxylin (Fig. 2) showed a little increase on the concentration of collagen fibers on the endomysium of the animals from SD group (Fig. 2C) when compared to the control groups (Fig. 2A and B), showing a possible deposition of this kind of fiber. The TD group (Fig. 2D) presented a reaction a lot similar to the ones seen on the SC and TC groups.

The ammoniacal silver technique did not show too much of a difference among the individuals of the four groups, except that the animals of group SD (Fig. 3C) had a little higher reaction in comparison with the animals of the groups SC, TC and TD (Fig. 3A, B and D, respectively).

4. Discussion

Diabetic animals showed a characteristic hyperglycemia, which is considered the main factor, at cellular level, responsible for the morphological damage caused by diabetes. The hyperglycemia seems to be the central mechanism triggering the processes that lead to the ultimate pathologic changes of myocardial hypertrophy, fibrosis, and collagen deposition (Aneja et al., 2008). This condi-

Table 1
Serum glucose (mmol/L).

	Treatment groups ($n = 8$)			
	Sedentary control (SC)	Trained control (TC)	Sedentary diabetic (SD)	Trained diabetic (TD)
Glucose	2.89 ± 0.38	2.69 ± 0.18	$14.36 \pm 2.36^{a,b}$	$12.54 \pm 2.26^{a,b}$

The values are the mean ± SD. a ≠ of SC; b ≠ of TC; c ≠ of SD; d ≠ of TD (one-way ANOVA, post hoc Bonferroni; Kruskal–Wallis, post hoc Dunn; $p < 0.05$).

Table 2
Qualitative characterization of the results from histochemistry techniques.

Histochemistry technique/rat	SC1	SC2	SC3	TC1	TC2	TC3	SD1	SD2	SD3	TD1	TD2	TD3
PAS-endomysium	++	++	++	++	++	++	+++	+++	+++	+++	++	+++
PAS-fiber	++	+	+	++	+	+	++	++	++	+	+	+
Picrosírius-Hematoxylin	++	+	++	++	++	++	+++	++++	++++	+++	++	++
Ammoniacal silver	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

(+) weak reaction with the staining technique; (++) moderate reaction with the staining technique; (+++) strong reaction with the staining technique; (++++) intense reaction with the staining technique.

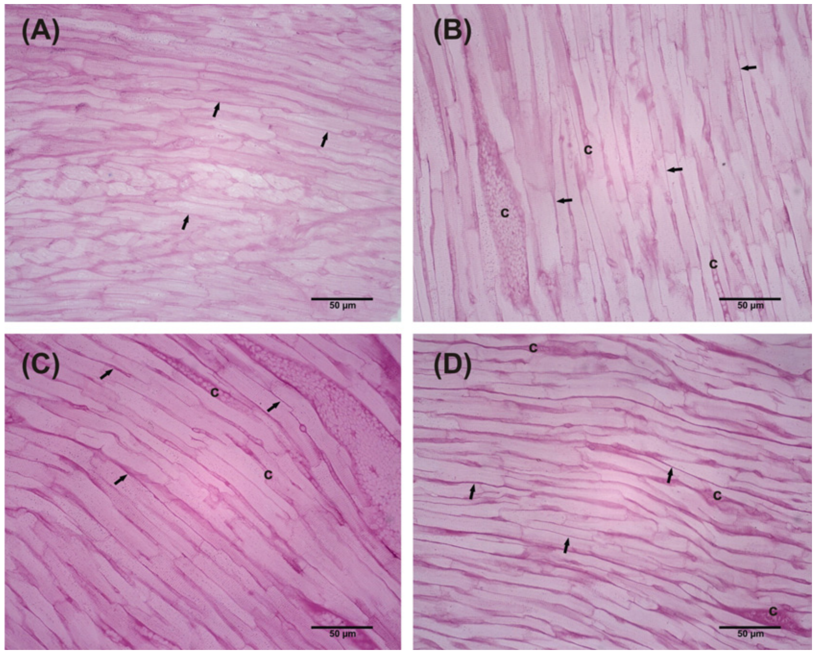

Fig. 1. Myocardium subjected to the technique PAS (400×). (A) SC Group, (B) TC Group, (C) SD Group, (D) TD Group. Arrows = endomysium, c = capillary vessels. Observe the increase on the intensity of reaction in the sarcoplasm and in the endomysium of the SD group (C) and the reduction of this intensity to levels close to the normal for the sarcoplasm of TD group (D).

tion causes an oxidative stress and activates messenger pathways that lead to cardiac fibrosis and cell death. The link between hyperglycemia and the development of diabetic cardiomyopathy seems to involve the accumulation of advanced glycated end products (Aragno et al., 2008).

Practicing physical exercises regularly is well known as an effective way to prevent numerous chronic diseases, such as diabetes. This regular practice improves the metabolic control on diabetic individuals, an important component on the treatment of diabetes mellitus (American Diabetes Association, 1994).

Several studies have shown the benefit effects of exercise on the control of glucose levels, both on animal experimentation and as on humans (Hardin et al., 1995; Aronson et al., 1997; Gobatto et al., 2001; Gomes et al., 2005, 2009). In the present work, although not statistically significant, exercise promotes a slight decrease in blood glucose levels. This reduction may have been sufficient to prevent some morphological changes caused by hyperglycemia. The muscle contractile stimulus lead to the translocation of the GLUT4 to the plasmatic membrane by the AMPK's signalers pathway (Machado et al., 2006), improving the glucose caption.

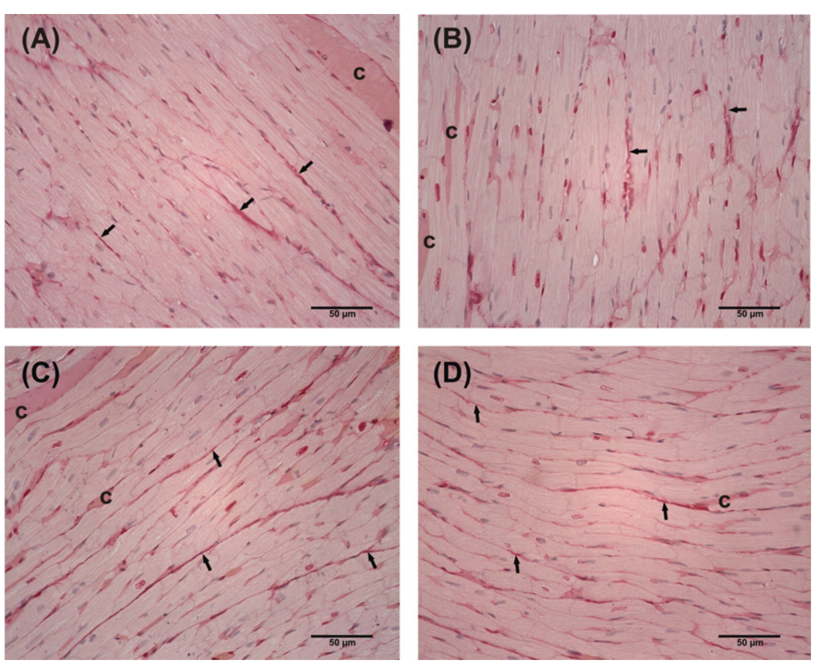

Fig. 2. Myocardium subjected to the technique picrosirius-hematoxylin (400×). (A) SC Group, (B) TC Group, (C) SD Group, (D) TD Group. Arrows = collagen fibers, c = capillary vessels. Observe the increase of the presence of collagen fibers in (B) and the reduction of the levels close to normal (D).

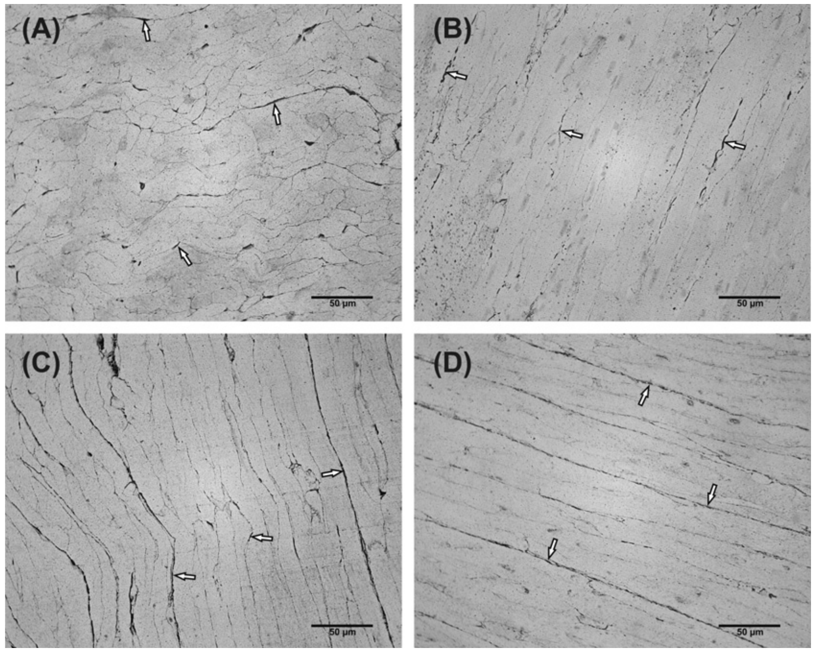

Fig. 3. Myocardium subjected to the technique ammoniacal silver (400×). (A) SC Group, (B) TC Group, (C) SD Group, (D) TD Group. Arrows = reticular fibers, c = capillary vessels. Observe the presence of reticular fibers shown by the arrows in all groups. The SD group (C) presented a little increase on the reaction for this technique, while the TD (D) group was very similar to the controls.

The increase on the intensity of the PAS technique reaction on the SD group animals' sarcoplasm evidences an increase on the polysaccharides concentration possibly due the increase of free fatty acids on these diabetic animals' myocardium that leads to the production of a glycogen spare (positive PAS) on the myocardium (Luciano and Lima, 1997), generating a higher reaction with the PAS technique on the SD group. The TD group showed a lower intensity of reaction in the sarcoplasm, that evidences a recovery on the polysaccharides concentration to levels close to control groups (SC and TC). This recovery is possibly due to the improvement of the metabolic conditions of these animals caused by the physical exercise, reducing the necessity of the production of glycogen spare.

On the endomysium, the more intense reaction is probably a result of the collagen deposition, being this possibly of type III (Cosson and Kevorkian, 2003) and type IV collagen, both positive to PAS (Junqueira and Carneiro, 2004; Feener and King, 1997).

Myocardial fibrosis and collagen deposition are the primary structural changes observed in diabetic cardiomyopathy (Aneja et al., 2008). The collagen fibers I and III are considered the main structural components of the myocardial interstice, and the increase of these fibrous components might influence the systolic and diastolic contractions negatively (Jalil et al., 1989).

Collagen interacts with glucose resulting in glycated collagen that undergoes further chemical modification to form advance glycation end products. The advance glycation end products are a stable form of crosslinked collagen and are thought to contribute to arterial and myocardial stiffness, endothelial dysfunction, and atherosclerotic plaque formation (Aneja et al., 2008).

Despite the great amount of studies related to the alterations on the balance of collagen types present on the cardiac musculature caused by diabetes, this balance still needs better elucidation.

The raise on the quantity of collagen fibers on the SD group, seen on the present paper through the picrosirius-hematoxylin technique, could be a sign of an initial process of fibrosis, a histopathological alteration commonly found on diabetic patients' myocardium. Shimizu et al. (1993) showed that there is a substantial accumulation of types I, III and VI collagen on diabetic individuals' myocardial interstice and Aragno et al. (2008) observed a deposition of types I and IV collagen on the left ventricle of diabetic rats. Moreover, Shimizu et al. (1993) also pointed out that the interstitial fibrosis on the myocardium is significantly larger on diabetic individuals and that much of this fibrosis is made of collagen fibers. However, studies performed on animal models on diabetes induction by streptozotocin have not found alterations on the amount of type III collagen after 18 weeks of diabetes (Nemoto et al., 2006).

The TD group presented a reaction very close to the ones observed in both controls groups (SC and TC), showing that the physical exercise helped to prevent the prejudicial alterations caused by diabetes perhaps due to the improvement of the metabolic state of these animals. The slight reduction of hyperglicemia may have reduced the negative effects of the oxidative stress and the others metabolic pathways that trigger the collagen deposition.

When animals of TC group were analyzed, we observed a slight increase of the intensity of reaction for Picrossirius-Hematoxilin technique that indicates a possible deposition of collagen in the cardiac muscle. This is likely to occur because the exercise may increase myocardial collagen content (Bartosov et al., 1969; Kiiskinen and Heikkinen, 1976), possibly due an increase in myocardial prolyl 4-hydroxylase, an enzyme with an activity level positively correlated with collagen biosynthesis (Takala et al., 1991; Thomas et al., 2000).

The ammoniacal silver technique evidences reticular fibers, which are rich on collagen type III. Through this technique it was possible to observe a light increase on the intensity of the SD group's reaction when compared to the control groups (SC and TC). This result shows that there might have occurred a deposition of collagen type III on the animals from SD group, represented by the fibers evidenced on the reaction, possibly due to an initial state of fibrosis that could have been developed and reached an advanced level, as Shimizu et al. (1993) observed on humans. However, the low specificity of this technique does not allow us to go on a deeper analysis about the type III collagen, one of the components most affected by diabetes.

5. Conclusion

Based on the results observed, it is possible to conclude that the regular practice of physical exercises might have an important role on the prevention, or even the re-establishment, of some of the negative alterations caused by diabetes on animal models. However, studies that involve morphological, biochemical and molecular alterations still are necessary for the complete understanding of changes caused by these complex metabolic disorders that characterize diabetes.

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References

Alexander, C.M., Landsman, P.B., Teutsch, S.M., 2000. Diabetes mellitus, impaired fasting glucose, atherosclerotic risk factors, and prevalence of coronary heart disease. Am. J. Cardiol. 86, 897–902.
American Diabetes Association, 1994. Position Statement, Diabetes mellitus and exercise. Diabetes Care 17, 924–937.
American Diabetes Association, 2003. Clinical practice recommendations. Diabetes Care 26 (1), 1–156.
Aneja, A., Tang, W.H.W., Bansilal, S., Garcia, M.J., Farkouh, M.E., 2008. Diabetic cardiomyopathy: insights into pathogenesis, diagnostic challenges, and therapeutic options. Am. J. Med. 121 (9), 457–478.
Aragno, M., Mastrocola, R., Alloatti, G., Vercellinatto, I., Bardini, P., Geuna, E., Catalano, M.G., Danni, O., Boccuzzi, G., 2008. Oxidative stress triggers cardiac fibrosis in the heart of diabetic rats. Endocrinology 149, 380–388.
Aronson, D., Violan, M.A., Dufresne, S.D., Zangen, D., Fielding, R.A., Goodyear, L.J., 1997. Exercise stimulates the mitogen-activated protein kinase pathway in human skeletal muscle. J. Clin. Invest. 99, 1251–1257.
Bartosov, D., Chvapil, M., Korecky, B., Poupa, O., Rakusan, K., Turek, Z., Vizek, M., 1969. Growth of muscular and collagenous parts of rat heart in various forms of cardiomegaly. J. Physiol. 200 (2), 285–295.
Cosson, S., Kevorkian, J.P., 2003. Left ventricular diastolic dysfunction: an early sign of diabetic cardiomyopathy? Diabetes Metab. 29, 455–466.
Cosyns, B., Droogmans, S., Weytjens, C., Lahoutte, T., Van Camp, G., Schoors, D., Franken, P.R., 2007. Effect of streptozotocin-induced diabetes on left ventricular function in adult rats: an in vivo Pinhole Gated SPECT study. Cardiovasc. Diabetol. 6, 30.
De Angelis, K., Pureza, D.Y. Da, Flores, L.J.F., Rodrigues, B., Melo, K.F.S., Schaan, B.D., Irigoyen, M.C., 2006. Efeitos Fisiológicos do Treinamento Físico em Pacientes Portadores de Diabetes Tipo 1. Arq. Bras. Endocrinol. Metab. 50 (6), 1005–10013.
Feener, E.P., King, G.L., 1997. Vascular dysfunction in diabetes mellitus. Lancet 350, 9–13.
Fein, F.S., 1990. Diabetic cardiomyopathy. Diabetes Care 13, 1169–1179.
Gobatto, C.A., Mello, M.A.R., Sibuya, C.Y., Azevedo, J.R.M., Santos, L.A., Kokubun, E., 2001. Maximal lactate steady state in rats submitted to swimming exercise. Comp. Biochem. Physiol. 130, 21–27.
Gomes, R.J., Caetano, F.C., Mello, M.A.R., Luciano, E., 2005. Effect of chronic exercise on growth factors in diabetic rats. J. Exerc. Physiol. 8, 16–23.
Gomes, R.J., Leme, J.A.C.A., de Moura, L.P., de Araujo, M.B., Rogatto, G.P., de Moura, R.F., Luciano, E., Mello, M.A.R., 2009. Growth factors and glucose homeostasis in diabetic rats: effects of exercise training. Cell Biochem. Funct. 27 (4), 199–204.
Hardin, D.S., Azzarelli, B., Edwards, J., Wigglesworth, J., Maianu, L., Brechtel, G., et al., 1995. Mechanisms of enhanced insulin sensitivity in endurance-trained athletes: effects on blood flow and differential expression of GLUT 4 in skeletal muscles. J. Clin. Endocrinol. Metab. 80, 2437–2446.
Jalil, J.E., Doering, C.W., Janicki, J.S., Pick, R., Shroff, S.G., Weber, K.T., 1989. Fibrillar collagen and myocardial stiffness in the intact hypertrophied rat left ventricle. Circ. Res. 64, 1041–1050.
Junqueira, Carneiro, 2004. Histologia Básica. Editora Guanabara Koogan, Rio de Janeiro.
Junqueira, L.C.U., Junqueira, L.M.M.S., 1983. Técnicas básicas de citologia e histologia. Editora Santos, São Paulo.
Kiiskinen, A., Heikkinen, E., 1976. Physical-training and connective tissues in young mice heart. Eur. J. Appl. Physiol. Occup. Physiol. 35 (3), 167–171.
Leon, A.S., 1987. Exercise testing and exercise prescription for special cases. Diabetes, 115–133.
Lerco, M.M., Spadella, C.T., Machado, J.L.M., Schellini, S.A., Padovani, C.R., 2003. Caracterização de um modelo experimental de Diabetes Mellitus induzido pela aloxana em ratos. Estudo Clínico e laboratorial. Acta Cir. Bras. 18 (2), 132–142.
Li, S., Culver, B., Ren, J., 2003. Benefit and risk of exercise on myocardial function in diabetes. Pharmacol. Res. 48, 127–132.
Luciano, E., Lima, F.B., 1997. Metabolismo de ratos diabéticos treinados submetidos ao jejum e ao exercício agudo. Rev. Ciênc. Bioméd. 18, 47–60.
Machado, U.F., Schaan, B.D., Seraphin, P.M., 2006. Transportadores de Glicose na Síndrome Metabólica. Arq. Bras. Endocrinol. Metab. 50 (2), 177–189.
Mcmanus, J.F.A., 1946. Histological demonstration of Mucin after periodic acid. Nature 158, p.202.
Nemoto, O., Kawaguchi, M., Yaoita, H., Miyake, K., Maehara, K., Maruyama, Y., 2006. Left ventricular dysfunction and remodeling in streptozotocin-induced diabetic rats. Circ. J. 70, 327–334.
Shimizu, M., Umeda, K., Sugihara, N., Yoshio, H., Ino, H., Takeda, R., Okada, Y., Nakanishi, I., 1993. Collagen remodelling in myocardia of patients with diabetes. J. Clin. Pathol. 46, 32–36.
Smanio, P., 2007. Doença cardiovascular em Mulheres diabéticas assintomáticas. Arq. Bras. Endocrinol. Metab. 51 (2), 305–311.
Stratmann, B., Gawlowski, T., Tschoepe, D., 2010. Diabetic cardiomyopathy—to take a long story serious. Herz 35 (3), 161–168.
Takala, T.E.S., Ramo, P., Kiviluoma, K., Vihko, V., Kainulainen, H., Kettunen, R., 1991. Effects of training and anabolic-steroids on collagen-synthesis in dog heart. Eur. J. Appl. Physiol. Occup. Physiol. 62 (1), 1–6.
Thomas, D.P., Zimmerman, S.D., Hansen, T.R., Martin, D.T., McCormick, R.J., 2000. Collagen gene expression in rat left ventricle: interactive effect of age and exercise training. J. Appl. Physiol. 89 (4), 1462–1468.
Trost, S., LeWinter, M., 2001. Diabetic cardiomyopathy. Curr. Treat. Options Cardiovasc. Med. 3, 481–492.
Wei, M., Ong, L., Smith, M.T., Ross, F.B., Schmid, K., Hoey, A.J., Burstow, D., Brown, L., 2003. The streptozotocin-diabetic rat as a model of the chronic complications of human diabetes. Heart Lung Circ. 12, 44–50.