Physical Exercise Improves Insulin Sensitivity of Rats Exposed to Cigarette Smoke

EXERCISE AND
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ORIGINAL ARTICLE

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ABSTRACT

Goal: Smoking can cause cardiovascular diseases and reduction on insulin sensitivity. This study evaluated the effect of smoking and associated moderate physical activity on the insulin sensitivity in the heart by GLUT4 gene expression. Methods: Male Wistar rats were divided into 4 groups: (C) control, (EX) exercised, (SS) sedentary smoker and (ES) exercised smoker. SS and ES groups were submitted to cigarette smoke exposition, 30 min/2x a day/60 days. Ex and EF groups performed running on a treadmill, during 60min/60 days. GLUT4 protein and mRNA contents analysis was performed by Western Blotting and RT-PCR, respectively. Results: The results showed that neither smoking nor physical activity changed body weight (C: 364.7 ± 9.7, EX: 372.4 ± 7.2, SS: 368.9 ± 6.7, ES: 376.4 ± 7.8 g) and heart weight (C: 1.12 ± 0.05; EX: 1.16 ± 0.04; SS: 1.14 ± 0.05; ES: 1.19 ± 0,05g). Insulin sensitivity was reduced in sedentary smoker group, and exercise improved this condition (C: 3.7 ± 0.3; EX: 5.28 ± 0.5 *; SS: 2.1 ± 0.7 *; ES: 4.8 ± 0.09 **; *P <0.05 vs C, ** P <0.05 vs. SS). mRNA and protein contents did not change among the groups. On the other hand, smoking caused reduction, and exercise provoked increase in GLUT4 total content per gram of heart (C: 119.72±9.98; EX: 143.09±9.09; SS: 84.36±10.99*; ES: 132.18±11.40# AU/ g tissue, *P<0.05 vs C, #P<0.01 vs SS). Conclusion: We concluded that smoking reduces insulin sensitivity and the cardiac ability in uptaking glucose, which can be reversed by moderate physical exercise.

Keywords: glucose transporter, smoking, insulin resistance, type 2 diabetes.

INTRODUCTION

Smoking is currently one of the main public health problems. It is clear the tobacco participation in the increase and/or aggravation of cardiovascular, pulmonary, circulatory diseases as well as countless types of cancer, contributing to increase of population morbidity and mortality[1].

It is known that smoking alters the cellular metabolism in many aspects, among which, we can name alteration in the insulin sensitivity with development of the resistance to the hormone, which if not treated in time could lead to the development of a type 2 diabetes scenario [2-4]. Smoking is clearly associated with increase of abdominal fat (higher waist-hip ratio)[5,6], increase of free fatty acids as well as glycerol mobilization, dyslipidemias episodes (increase of LDL and decrease of HDL), endothelial dysfunction, increase in blood viscosity and hypercoagulability status[7,8]. Smoking is also associated with a low level systemic inflammation status[9] and oxidative stress[10], characterized by the increase pro-inflammatory cytokines[11]. The literature mentions that the increase of pro-inflammatory cytokines participates in the genesis of insulin resistance, since it can interfere in the insulin signaling[12-14].

It is known that at the cellular level, insulin resistance is associated with alteration in the insulin signaling way, reduction of the GLUT4 glucose transporter expression or alteration in the GLUT4 translocation to the plasmatic membrane of adipose and muscular cells, which finally causes lower biological activity of the hormone, disturbing the glucose homeostasis[15,16].

The contractile activity also stimulates the glucose uptake, promoting higher amount of GLUT4 glucose transporters in the sarcolema by the increase of translocation, regardless of the insulin presence[17]. Research suggests that there are different GLUT4 intracellular compartments; one stimulated by insulin and the other stimulated by exercise, and the combination of the two results in additional effects concerning the glucose transportation[18,19]. There is evidence that due to the increase in the [AMP]:[ATP] and [creatine]:[phosphocreatine] ratios by the tissue needs during the muscle contraction, there is activation of a kinase termed 5'-AMP activated protein kinase (AMPK) which would participate in the GLUT4 translocation stimulated by the activity[18,20].

The heart can greatly uptake glucose for being a very active muscle tissue. It is known that smoking is one of the main causes of cardiovascular and circulatory diseases. Increase in the expression of GLUT4 glucose transporter in cardiac muscle increases glucose oxidation in the myocardium, favoring cardiac performance[21]. Thus, physical activity practice appears as an important method to fight the deleterious consequences caused by smoking, altering the risk of smokers to develop insulin resistance and probable diabetes, as well as improving cardiac capacity.

Within this context, this investigation had the aim to quantify the GLUT4 protein and RNAm in the cardiac muscle of smoking rats and submitted to moderate physical activity on treadmill for characterization of sensitivity to peripheral insulin and possible role of the cardiac tissue.

METHODS

Animals and training program: Wistar male rats were kept in an animal facility at mean temperature of 22 ± 2ºC, with 12hour luminosity cycles, from 7:00 to 19:00 (light period) and from 19:00 to 7:00 (dark period), and fed with standard food and water ad libitum. The animals were sorted in four groups: (C) control sedentary; (EX) control exercised; (SS) sedentary smoker and (ES) exercised smoker. Groups SS and ES were submitted to 4 cigarettes smoke/time, for 30 minutes, two times/day, during 60 days. A closed box divided in two compartments was used as inhaling chamber for passive smoking. One of the box compartments was used for placement of four lit cigarettes on a support, and the other compartment was reserved for the animals' placement. A source of compressed air with flow of 10L/min was connected to the side where the cigarettes were in order to allow the cigarettes combustion and provide the smoke conduction to the other side of the box where the rats were placed. On the animals' compartment there was also a hole for air drainage, through which the mixture exhaustion occurred.

Groups EX and ES performed running protocol on treadmill for small animals. The treadmill was triggered by a 12V motor which provided a 10-meter per minute velocity for sliding on the belt and consequently animal's induced movement under study. The training session had duration of 60 minutes, performed during 60 days, five days per week, being characterized as moderate physical exercise, since there was not increase of intensity.

All experimental procedures were approved by the Ethics in Research Committee of FCT-UNESP, Presidente Prudente Campus, file 262/2008.

Insulin sensitivity evaluation: in order to evaluate the insulin tolerance in vivo, an insulin tolerance test (ITT) was performed. Ione week prior to sacrifice, six animals from each group were intraperitoneally anesthetized (i.p.) with ketamine chlorhydrate and xylazine chlorhydrate (60mg/kg PC). A small incision on the distal section tip of the animals' tails was done for blood samples collection. The first collection occurred before the insulin administration (basal). Subsequently, 0.5UI/kg PC of regular insulin (Novolin 100U/ml, Novo Nordisk, Denmark) was administrated. Glycemia dosing was performed at every five minutes, at times 0 (basal) at 20 min after insulin administration. Glycemia was verified through reagent bands and glucose meter (Biocheck TD-4225 / Bioeasy Diagnóstica Ltda. / MG – Brazil). Later on, the fall Constant was calculated (kITT, %/min.) from the linear regression of the glycemia concentrations obtained during the test [22].

Samples acquisition: 24 hours after the last training session, the animals were anesthetized with ketamine chlorhydrate and xylazine chlorhydrate (60mg/kg PC) i.p. for acquisition of the blood and tissue samples. After anesthesia, the cardiac muscle was removed and 0.1g for RT-PCR separated and the remaining for Western Blotting.

GLUT4 protein quantification – Western Blotting: The sample was weighed, homogenized and centrifuged in TRIS HCl 10mM; EDTA 1mM, sucrose 250mM, pH 7.4 buffer, obtaining the total fraction of cellular membranes[22]. The total protein concentration was detected by the Lowry method[23]. Equal amounts of proteins were made soluble in Laemmli buffer, subject to SDS-PAGE (10%) and later transferred to nitrocellulose Hybond-C Super membrane (GE healthcare, AMERSHAM Biosciences, UK). After 1-hour block with 3% of skim Milk, the membranes were incubated with anti-GLUT4 antibody (Chemicon International, Temecula California, USA), dilution 1:3000 in PBS (NaCl 0.8%, $Na_2HPO_4[12H_2O]$ 0.115%, KCl 0.02%, KH_2PO_4 0.02%), added from bovine serum albumin (BSA 8%), during three hours at 37°C. Incubation with secondary antibody was performed (rabbit anti-IgG), marked with peroxidase enzyme (HRP) (GE Helthcare, Amersham, UK), diluted 1:6000 in blocking solution during one hour. After wash in buffer, the sample was exposed to the ECL reaction (Luminol 1.1%, P-Coumaric Acid 0.48%, Tris 1M pH 8.5 11.1%, H_2O distilled) for two minutes and subsequently exposed to the Hyperfilm (IGF – Corporation, New Jersey, USA) for detection of the resulting bands. The film was photographed by a camera (Gel Logic 100, Kodak Molecular Imaging, USA) and the images were analysed by densitometry in the Scion Image software for Windows (Scion Corporation, USA).

RT-PCR: RNAm semi-quantification of the GLUT4: The muscle tissue samples were processed in homogenizer model OMNI TH – USA (Lodan) with TRIZOL Reagent (Invitrogen, USA) for extraction of total RNA. The RT-PCR assay was then performed. Reverse transcription was performed according to the manufacturer's instructions (Invitrogen, USA), using five milligrams of total RNA extracted from the heart, RT M-MLV Reverse Transcriptase enzyme (200 U/mL; Invitrogen®, USA), and dNTPs mixture (10mM each). One-microliter aliquots of the RT final product (cDNA) were added to the 10pmol/μL mixture of GLUT4 specific primers (Sense: 5'- CCCCTCCAGGGCAAAGGAT - 3'; Antisense: 5'-TCCTGGAGGGGAACAAGAA - 3' – 203pb fragment), specific buffer, dNTPs mixture (10 mM), GoTaq DNA Polymerase 5U/μL enzyme (Promega, USA), and distilled water, in final volume of 50μL. The PCR reactions were performed in a Thermocycler trade mark Techne, model Endurance TC-312 (Techne Inc. New Jersey, USA) at different temperatures, with 28 cycles, and 54°C of annealing temperature. The amplified products were submitted to the 2.0% agarose gel electrophoresis and 0.02% ethidium bromide. The samples in the gel were visualized with UV light and the images acquired in photovideodocumentation equipment (Mod. Gel Logic 100 with epiluminescence, Kodak Molecular Imaging, USA). The GLUT4 gene RNA expression was normalized by the expression of the constitutive protein gene β-actin (Sense: 5' – ATGAAGATCCTGACCGAGCGTG – 3'; Antisense: 5' – CTTGCTGATCCACATCTGCTGG – 3'; fragment of 512pb; annealing temperature: 54°C and 24 cycles), calculated by the ratio between the values of the gene under interest densitometry and of the constitutive gene.

Statistical analysis: Data were analysed by descriptive statistics, with the results presented as mean ± MSE. Statistical evaluation of the results was done through means comparison, using parametric ANOVA test with post-test whenever necessary (Bonferroni). The differences between groups were considered significant when P value was lower than 0.05.

RESULTS

It can be seen in table 1 that the mean body weight and the cardiac tissue weight on the sacrifice day were not different between the studied groups, evidencing thus that neither the smoking imposed, nor the physical exercise had effect on the morphometric parameters of the studied animals. Fasting glycemia was also similar for all groups.

Table 1. Fasting glycemia, body weight and cardiac tissue weight.

Group	Fasting glycemia (mg/dL)	Body weight (g)	Heart weight (g)
C	148.0 ± 10.0 (n = 15)	364.77 ± 9.7 (n = 18)	1.12 ± 0.05 (n = 17)
EX	131.5 ± 7.0 (n = 19)	372.42 ± 7.2 (n = 21)	1.16 ± 0.04 (n = 19)
SS	139.5 ± 9.6 (n = 18)	368.95 ± 6.7 (n = 22)	1.14 ± 0.05 (n = 22)
ES	130.6 ± 10.2 (n = 18)	376.42 ± 7.8 (n = 21)	1.19 ± 0.05 (n = 18)

The glycemia decrease constant (kITT) (figure 1) evidenced lower peripheral sensitivity to insulin in the SS group compared to the remaining groups (C: 3.7 ± 0.3; EX: 5.3 ± 0.5*; SS: 2.1 ± 0.7* #; ES: 4.8 ± 0.098* &; n = 6, *P < 0.05 vs. C, #P < 0.03 vs. EX; &P < 0.05 vs. SS). On the other hand, physical exercise reverted this scenario.

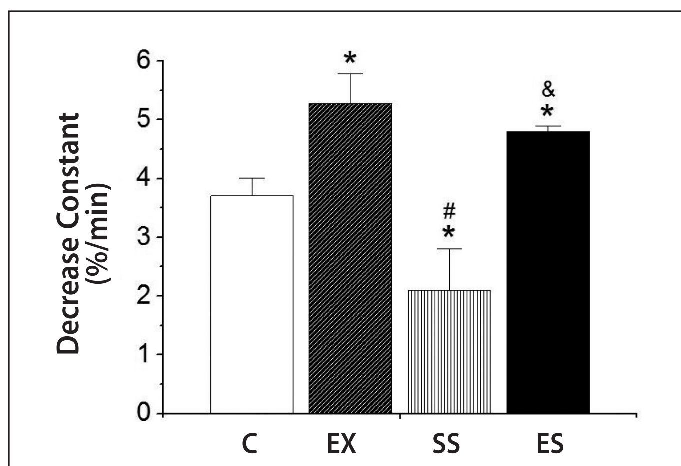

Figure 1. Insulin tolerance test in vivo (ITT). Mean +/- MSE of the glycemia decrease constant (kITT, expressed in %/min.), calculated from the linear regression of the glycemia concentrations obtained in the times 0-20 minutes of the test. Three to six animals per group were used. Control group (C), Exercised control (EX), sedentary smoker (SS) and exercised smoker (ES). Significant values when *P < 0.05 vs. C, #P < 0.03 vs. EX; &P < 0.05 vs. SS. (ANOVA and Bonferroni as post-test).

The imposed smoking was not sufficient to cause alteration in the RNAm content of the GLUT4 glucose transporter when compared to the non-smoking group (C). Alteration in the RNAm content of GLUT4 in the exercised groups EX and ES was not observed either when they were compared to their respective pairs C or SS (C: 75.7 ± 9.7; EX: 82.12 ± 8.5; SS: 75.9 ± 7.08; ES: 76.73 ± 7.8 AU, n = 10) (figure 2).

Assessment of the GLUT4 protein amount expressed in arbitrary units by µg of protein (AU/µg of protein), did not expose significant differences among groups (C: 100.9 ± 6.2; EX: 109.2 ± 4.7; SS: 95.9 ± 7.3; ES: 107.9 ± 6.4 AU, n = 10), evidencing a clear correlation between protein content and RNAm content for the GLUT4 gene in the cardiac tissue (Figure 3).

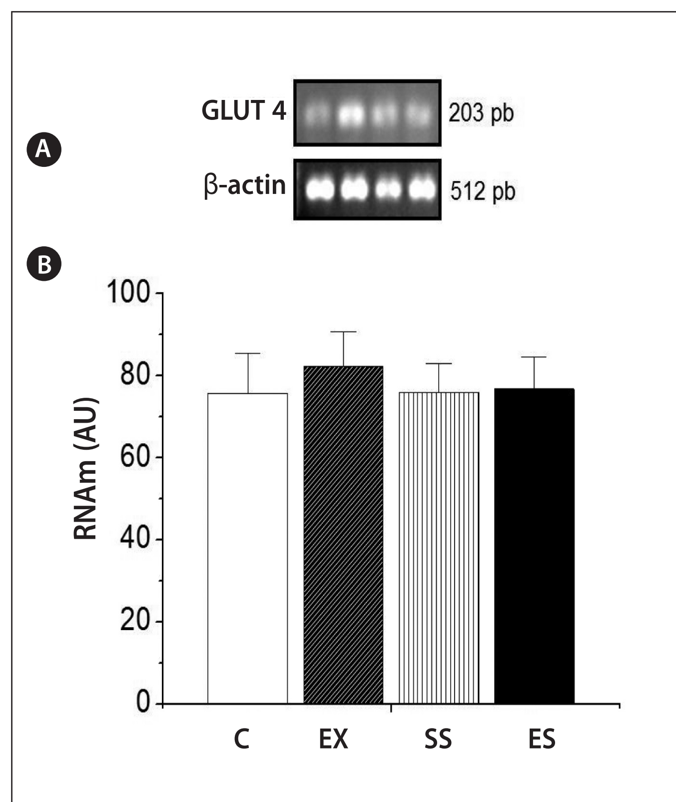

Figure 2. RNAm content of GLUT4 corrected by the RNAm content of β-actin in the cardiac tissue of the control (C), control exercised (EX), sedentary smoker (SS) and exercised smoker (ES) groups. In A) typical images obtained in RT-PCR assay evidencing RNAm of GLUT4 and of β-actin in the same group presentation sequence; in B) representative chart of the values obtained in 10 different experiments, results expressed in AU (arbitrary units),as mean ± MSE.

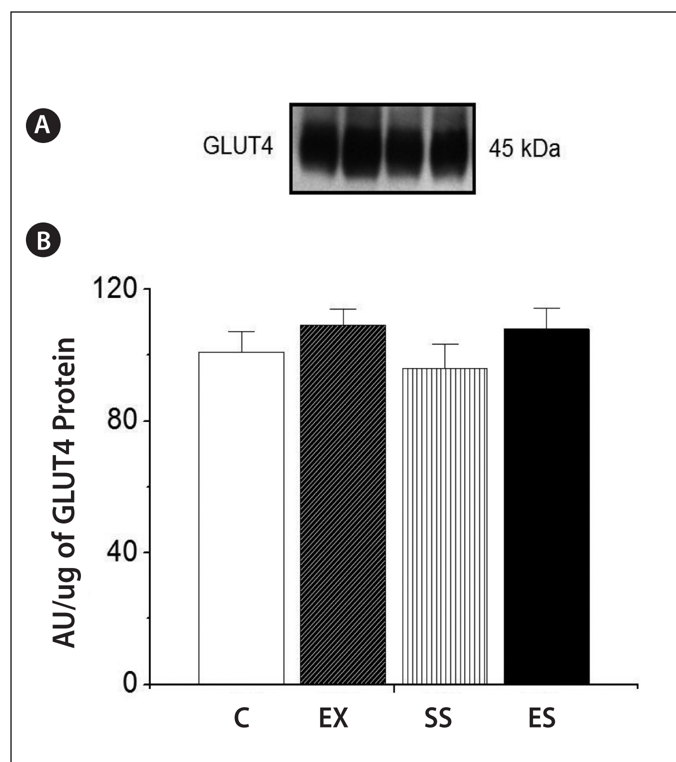

Figure 3. GLUT4 protein content in fraction of total membranes of cardiac tissue of the control group (C), control exercised group (EX), sedentary smoker group (SS) and exercised smoker group (ES). 30 ʃg of protein were submitted to the electrophoresis and immunodetection, as described in the materials and methods section. In A) typical self-radiography obtained by the Western Blotting assay in the same group presentation sequence. In B) representative chart with values obtained in 10 different experiments, expressed in arbitrary units per microgram of protein (AU/ug protein) as mean and mean standard error.

The total GLUT4 content per gram of cardiac tissue was calculated from these results expressed in AU/µg of protein and of protein recovery values [22]. It was observed that smoking caused reduction and that exercise caused significant increase of the total amount of GLUT4 per gram of cardiac tissue (C: 119.72 ± 9.98; EX: 143.09 ± 9.09; SS: 84.36 ± 10.99*; ES: 132.18 ± 11.40# AU-g tissue, *P < 0.05 vs. C, #P < 0.01 vs. SS, n = 10 animals) (Figure 4).

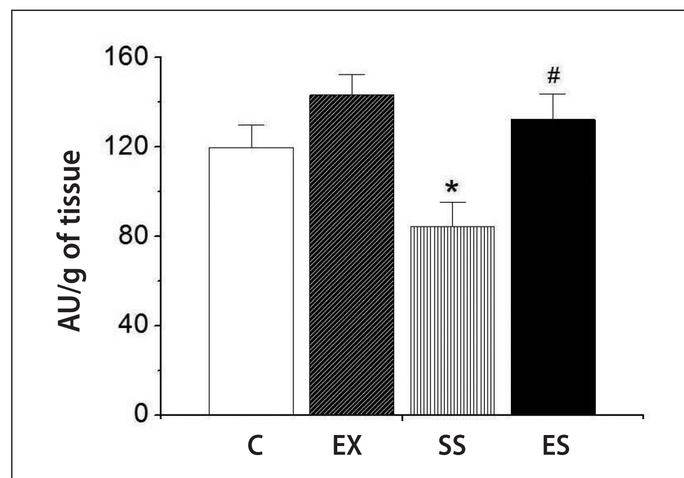

Figure 4. Total content of GLUT4 protein per gram of tissue, fraction of total membrane of cardiac tissue of the control group (C), control exercised group (EX), sedentary smoker group (SS) and exercised smoker group (ES). 30 ʃg of protein were submitted to the electrophoresis and immunodetection, as described in the materials and methods section. Values expressed in arbitrary units per gram of tissue (AU/g tissue) as mean and mean standard error. Significant values when *P < 0.05 vs. C and #P < 0.01 vs. SS (ANOVA and Bonferroni as post-test).

DISCUSSION

Increasing evidence indicates that smoking, both active and passive, is associated with insulin resistance scenario as well as decrease of glucose tolerance[3,4,8]. Insulin resistance (IR) is a characteristic of the metabolic syndrome (MS) and type 2 diabetes (DM2) and involves target-tissues, such as adipose tissue, the liver, skeletal and cardiac muscles[24].

Smoking causes inflammation of the airways and low-level systemic inflammation[9] by the activation of macrophages, neutrophils and T lymphocytes, which release proteases and reactive oxygen species (ROS). Increase of oxidative stress leads to activation of redox-sensitive transcription factors, which are critical to the transcription of pro-inflammatory cytokines[11]. There are reports that the secretion of these pro-inflammatory citokines participates in the IR genesis [14] and studies showed that the subclinical inflammatory reaction with presence of IL-1, IL-6 and TNF-α play an important role of the DM2 pathogenesis[25].

The current literature presents that physical exercise has been recommended to prevent and treat IR and DM2, since it can increase the capacity of glucose transportation by the muscle regardless of the insulin activity[26], through the increase of expression and translocation of GLUT4 glucose transporters to the plasmatic membrane of the cardiomyocytes[16].

In the present study, the weight of the studied animals did not vary between groups. A probable hypothesis for this fact would be the smoking time (60 days) which the animals were exposed to in the study. Perhaps this exposure time has not been sufficient to affect the food intake and thus, cause reduction in the mass incorporation of the studied animals, consequently not promoting body weight alteration. Nevertheless, in the literature[27], it was verified that weight and food intake of male rats exposed to smoke for only 30 days were lower than the rats not exposed to it.

Although the fasting glycemia is not altered with smoking, important reduction in the insulin sensitivity was verified in the smoker animals. On its turn, moderate physical exercise improved this situation in the presence of and absence of smoking, demonstrating hence that physical exercise practice is crucial to improve the glycemic homeostasis as well as increase the peripheral insulin sensitivity. Some research points out that physical exercise determines improvement in insulin activity[28] and that, in the heart, the contractile and hypoxic stimuli caused by exercise lead to GLUT4 translocation to the plasmatic membrane by the signaling way of AMPK (5' AMP-activated protein kinase)[16]. The triggering factor of the decrease in the insulin sensitivity when exposed to cigarette smoke is yet to be determined.

The analysis of the GLUT4 protein content results in AU/µg of protein showed that smoking did not alter the transporter expression in the cardiac musculature. These results are correlated with the amount of RNAm found in the smokier group. Investigations in the literature state that nicotine may interfere in the GLUT4 translocation, but not directly in its expression [29]. Thus, a priori, the results found here are in agreement with the reports from the literature.

However, when the total GLUT4 amount was analysed per gram of cardiac tissue, which represents the capacity of the tissue to uptake glucose, it was verified that smoking caused reduction of 24% compared to the control, and that physical exercise practice associated with smoking caused increase of 56%. Correlation of these values with insulin sensitivity found in the studied animals may suggest that the heart is interfering in the body's glycidic homeostasis, despite representing small tissue mass when compared to the skeletal musculature for example, a tissue which also suffers insulin influence.

Additionally, it was verified that exercise per se increased the heart capacity to uptake glucose, since increase in the amount of GLUT4 protein was detected per gram of tissue in the exercised control animals. Such increase may contribute to better glucose oxidation n the myocardium and improve cardiac performance in the exercise. This mechanism of glucose uptake increase by the GLUT4 increase is important to the myocardic protection during an ischemia[30].

Therefore, the results here presented let us conclude that smoking, besides all the alterations previously mentioned in the literature involving cardiopulmonary and vascular diseases, can also be deleterious to the glycidic homeostasis, causing harm to the capacity of the cardiac tissue to uptake glucose. Recurrent moderate physical exercise seems to be an important method to fight the deleterious effects caused by smoking, especially improving the glucose uptake by the myocardium and the peripheral insulin sensitivity.

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All authors have declared there is not any potential conflict of interests concerning this article.

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