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Effect of Exercise Training on Heart Sympathetic Activity and Lung Function in Mexican obese Adolescents

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Abstract

Introduction: The aim of this study was to evaluate the short term effect of a moderate-intensity physical activity program on heart sympathetic activity and lung function in Mexican obese adolescents.

Material and methods: we performed a longitudinal study in 38healthy obese adolescents (24 female and 14 male). Subjects were submitted to aerobic exercise training during 90 minutes for 16 weeks, 5 days per week. Before and after the intervention program, blood pressure, anthropometric measurements, glucose, insulin, and leptin levels were evaluated, spirometry and 60 minutes electrocardiograph monitoring were performed.

Results: After the exercise program, VO_{2max}s increased in the entire group with the highest increase in men. The BMI reduced by 4%, body fat by 2.4% (95% CI, 1.6-3.2) in women and 2.2% (95% CI, 0.6-3.8) in men. Also heart rate and systolic blood pressure (SBP) diminished. Change in SBP was associated with change in adiponectin levels (β = -0.47, p = 0.0001). LF/HF index was associated with changes in body fat (β = -0.39, p = 0.002) and SDDN was associated with changes in body mass index (β = 0.04, p = 0.04) and body fat (β = -1.04, p = 0.02).

Conclusions: A moderate-intensive exercise for 90 minutes a day at short term significantly diminished adiposity, has a positive effect on heart sympathetic activity and improves lung function in obese adolescents.

Keywords

Heart sympathetic activity, Lung function, Obese adolescents, Exercise program

Introduction

Obesity is a prevalent metabolic disorder in large parts of the developing world. In 2004, the age adjusted rates of obesity and overweight reported in the third National Health and Examination Survey (NHANES III) was 16.0% for children [1]. In Mexico, the National Health and Nutrition Survey 2006 showed a 23.3% and 9.2% for overweight and obesity prevalence respectively in 12-19 years old [2], this represents a relative increase of 7.8% and 33.3% in overweight and obesity respectively when compared with the National Nutrition Survey 1999. In 2012 Mexico ranked first in the world in childhood obesity and second in adult obesity, according to the National Nutrition Survey 2012 (ENSANUT). National prevalence of overweight and obesity in school was 19.8% and 14.6% respectively. In Guanajuato, the prevalence of overweight and obesity was 19.4% and 17.5% respectively [3].

Obesity is associated with a chronic inflammatory response [4], which has been proposed to have an important role in cardiovascular disease. Furthermore, Hispanics are one of the populations especially prone to develop obesity and its comorbidities [5].

Literature examining the association between obesity and lung function is conflicting. It has been established that standing height is an important anthropometric variable in predicting spirometric data in children. However, the power of height in predicting lung volumes was significantly reduced in overweight males. Obese children may show low Forced Expiratory Volume in one second (FEV_1) /Forced Vital Capacity (FVC) index related to an increase in FVC, rather than a decrease in FEV_1 [6].

A recent study found that controlled aerobic exercise program, without weight loss, reduced hepatic and visceral fat accumulation, and decreased insulin resistance in obese adolescents [7]. However, there is scarce information evaluating the short term effects of a moderate intensity physical activity program on hemodynamic, heart sympathetic activity and lung function in obese Mexican adolescents.

Materials and Methods

We performed a longitudinal study in 38 asymptomatic obese

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adolescents (BMI ≥ 95th percentile, according to the growth charts from the Centers for Disease Control and Prevention) [8]. A sample size of 38 subjects was calculated using that statistic [9], according to detect a difference of at least 20% in FEV_1 values before and after the exercise program, considering a mean and standard deviation of 3.0 ± 0.6 L with an alpha of 0.05, and a power of 0.90.

Adolescents from 12 to15 years were recruited from a public high-school. All of them were healthy, non-smokers, without chronic illnesses, and none had participated in athletics or regular exercise before this study. Participants read and signed an informed consent document prior to the study. All experimental procedures were approved by the local ethical committee and were carried out in accordance with the ethical guidelines laid down in the Helsinki Declaration.

Training programs

All subjects were submitted to aerobic exercise training during 90 minutes for 16 weeks, 5 days per week according to the European Youth Heart Study recommendations [10]. The exercise program used in this study was specifically designed by investigators (L.B. and G.J.), and it consisted of the following: 1) Warm up (10 minutes): Sports-specific callisthenics such as push-ups and sit-ups, 2) Moderate effort (30 minutes): The participants performed competition skills, including take-downs, escapes, running etc., 3) Recreational activities (30 minutes): swimming, basket-ball, volley-ball, and foot-ball, 4) Flexibility (15 minutes) and 5) Stretching (5 minutes).

Physical activity was assessed by the International Physical Activity Questionnaire [11]. It is expressed as metabolic equivalents (METs) which represent the ratio of the energy expended during a specific activity to the resting metabolic rate. METs were assigned to each physical activity according to the Compendium of Physical Activities [12], as values of 2.5, 4.0, and 6.0 for low, moderate, and intense activity respectively. Overall fitness was assessed according to the 12-min walk-run test (12 mWRt) [13], to estimate the maximal oxygen intake (VO_{2max}).

After 10 days of exercise according to the initial physical fitness assessment, all obese children were capable to perform a 90 min training session at 60-70% of maximal heart rate. Participation in the exercise training program averaged 88.4% of scheduled visits.

Recommended feeding

All adolescents were instructed to go on a weight-reduction diet by a standard appointment at the nutrition department. Dietary assessment was carried out by one experienced qualified dietitian to minimize inter-individual bias among different professionals. Food quantities were estimated using Nutrikcal a nutritional software package developed by Ogali (Mexico, D.F.).

Anthropometric measurements

All measurements were performed by the same technician, using the methodology of the International Society for the Advancement of Kineanthropometry (ISAK). Weight was measured with a portable scale with a 125 kg maximum capacity and a +/- 100 g error. Height was measured with astadiometer with a sensitivity of 0.1 cm. Body-mass index (BMI) was estimated by dividing weight (kg) by height2 (m2). The percentage of body fat was estimated by the BC-420 impedance meter MA (TanitaCorp., Tokyo, Japan).

Biochemical measurements

Venous blood was obtained after 12 hours overnight fasting to measure leptin, adiponectin, glucose, lipids and insulin levels. Insulin resistance was estimated by the homeostatic model assessment-insulin resistance index (HOMA-IR) with the following formula: fasting serum insulin (mU/mL) fasting plasma glucose (mmol/L)/22.5 [14].

Blood glucose concentration was measured using the glucose oxidase method (Ortho Clinical Diagnostics, Johnson_Johnson). The intra-assay coefficient of variation (CV) and inter-assay CV were 1.9% and 7.5% respectively. Serum insulin was measured with a solid phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). The intra and inter assay coefficients of variation were 5.2 and 7.3%, respectively. Serum leptin was measured with an immunoradiometric assay (IRMA) (Diagnostics Systems Laboratories, Inc., Webster, Texas). The intra- and inter assay coefficients of variation were 3.7 and 5.2%, respectively.

Measurement of heart sympathetic activity

Blood pressure was obtained by a mercury sphygmomanometer with a cuff covering two-thirds of the right arm. After 15 minutes in rest, two readings were made within a five-minute interval, and the average was registered. The sympathetic activity was evaluated with 60 minutes electrocardiograph monitoring at rest in sitting position using a three-channel Holter recorder (model GBI-3S, Galix Biomedical Instrumentation Inc.). The tapes were analyzed in Holter Galix software, to obtain the heart rate variability as the standard deviation (SD) of all the normal R-R intervals (SDNN). Spectral analysis was carried out with a direct fast Fourier transform providing the total (0.01-1.00 Hz), low (0.04-0.15 Hz) and high (from 0.15 to 0.40 Hz) frequency power. We obtained the low frequency (LF)/high frequency (HF) ratio as a measure of sympathetic activity [15]. Ectopic beats were identified and excluded from analysis.

Measurement of lung function

Lung function was measured by forced spirometry. The spirometry was performed with a spirometer EasyOne® (NDD, Technopark darned Switerland), which meets the diagnostic criteria for precision, accuracy and linearity, established by the American Thoracic Society [16]. To carry out follow the recommendations of the ATS and the following parameters were obtained: FVC, FEV_1 and FVC/FEV_1 ratio. The quality of spirometric tests was assessed by several criteria in addition to the automatic evaluation done by the software device, According to ATS, three acceptable tests were obtained, recorded the best value by the spirometric software. Another indicator of quality was reproducibility. FEV_1 and FVC were considered reproducible according to ATS criteria when the best two trials differed by not more than 200 mL. A total of 97.5% of the tests achieved reproducibility within 150 mL fulfilling the 2005 ATS-ERS criteria [17,18]. Reference values of Hankinson et al. [19] for Mexican-Americans were used. The presence of spirometric values below the 5th percentile of reference values were considered abnormal. The obstructive pattern was defined by the diminution of the FEV_1 and FEV_1/FVC index, the restrictive pattern by diminution of the FVC, with normal FEV_1/FVC index and mixed pattern by diminution of FVC and FEV_1.

Statistical considerations

Results are expressed as mean ± SD or as median (95% CI) according to variables´ distribution. Differences between genders were assessed by χ^2 for proportions. The Mann-Whitney U test or Student´s t-test were used for continue variables displaying non-normal or normal distribution respectively. For comparison of continue variables before and after the exercise program we performed ANOVA test and Tukey honest significant difference as post-hoc test. In addition, we performed ANCOVA test considering gender as co-variable. Considering that systolic blood pressure has been a better predictor risk of cardiovascular events than diastolic blood pressure levels [20], forward stepwise multiple regression analysis was used to study the clinic and biochemical predictors of change in these variables. We considered F value to enter and F value to remove set to $p < 0.05$ and $p < 0.10$, respectively. All data were analyzed using the STATISTICS software version 6.0 (Stat soft Inc. Tulsa OK).

Results

We included 38 adolescents, 24 female and 14 male. Adolescents showed glucose levels near 100 mg/dL although none was diagnosed as diabetic. Physical activity in all participants was classified as poor, and was significantly lower in men than in woman (4.6 ± 1.0vs. 5.5

Table 1: Baseline clinical and biochemical characteristics of obese adolescents according to gender.

Variable	Female n = 24	Male n = 14	*p*
Age (years)	13.2 ± 0.7	12.8 ± 0.7	0.16
BMI (kg/m$^{2)}$	32.0 ± 3.7	29.6 ± 3.3	0.04
% Bodyfat	40.9 ± 2.7	32.7 ± 6.7	0.006
Glucose* (mg/dL)	**90.6 ± 8.8**	**97.7 ± 9.0**	**0.001**
Insulin$ (mU/mL)	6.2 (3.1-9.4)	4.9 (1.7-8.2)	0.04
HOMA-IR	1.3 (0.7-2.0)	1.1 (0.4-1.9)	0.11
Leptin (ng/mL)	24.3 (17.4-32.3)	19.9 (15.2-22.1)	0.08
Adiponectin (ng/mL)	12.7 (4.9-22.7)	6.3 (2.5-12.9)	0.16
FVC (liters)	3.576 ± 0.6	3.650 ± 0.8	0.08
FVC (% predicted)	114.8 ± 19.5	111.0 ± 12.5	0.4
FEV_1 (liters)	3.029 ± 0.5	2.955 ± 0.6	0.17
FEV_1 (% predicted)	153.4 ± 22.3	105.6 ± 14.6	0.17
FEV_1/FVC%	84.7 ± 5.2	82.1 ± 6.1	0.48
PEF (liters)	6.587 ± 1.37	6.605 ± 1.5	0.43
PEF (% predicted)	107.0 ± 22.0	104.0 ± 19.6	0.11
SBP (mmHg)	127.5 ± 8.5	125.3 ± 7.7	0.44
DBP (mmHg)	86.6 ± 7.3	82.1 ± 5.0	0.04
VO_{2max} (mL/kg/min)	16.3 ± 3.7	19.3 ± 3.5	0.01
HR (bpm)	**91.4 ± 16.0**	**97.8 ± 9.6**	**0.26**
SDNN (ms)	83.5 (67.9 - 99.1)	65.7 (51.9 - 79.6)	0.07
LF/HF	3.6 (3.0 - 4.3)	5.0 (3.8 - 6.3)	0.15

Variables are shown as mean± SD or mean (95%CI).

p: difference between genders.

*Glucose conversion factor to SI 0.05551; $Conversion factor to SI 7.175.

HOMA-IR: homeostatic model assessment-insulin resistance index.

FVC: forced vital capacity

FEV_1: forced expiratory volume at 1 second

FEV_1/FVC: forced expiratory volume at 1 second/forced vital capacity

PEF: peak expiratory flow

SBP: systolic blood pressure

DBP: diastolic blood pressure

VO_{2max}: maximal oxygen intake

SDNN: standard deviation of all the normal R_R intervals

LF/HF: low-frequency/high-frequency index.

± 1.0METs), respectively (p = 0.01). Obese adolescents showed low values in FEV_1/FVC index with average value of 84 (95% CI: 81-85). At baseline, female adolescents showed higher body mass index, body fat, glucose, insulin and diastolic blood pressure than male adolescents, but lower VO_{2max} levels (Table 1).

Physical activity and anthropometric changes

After the exercise program, 90% of participants were classified as performing moderate physical activity, the distance traveled in 12 min WRt were significantly increased, 1.3vs.1.6 km, (p = 0.0001) for women and 1.4 vs.1.8 km in men (p = 0.0001). The VO_{2max} also improved, with an average increase of 6.3 ± 1.8 mL/kg/min in women and 8.8 ± 1.7 mL/kg/min (5.3 - 13.6) in men (p < 0.001 in both).

Total caloric intake decreased after the intervention program in the entire group (2077 ± 562 vs. 1639 ± 535 Kcal, p = 0.03), the average intake of macro nutrients was 51.8 ± 18.7% of carbohydrates, 14.0 ± 6.5% of proteins, and 31.0 ± 13.1% of fat, without difference by gender at the end of the study.

Eight (21.0%) adolescents did not show weight reduction during the program. However, the average weight reduction was 4% (1.6 kg/m^2) in the entire group. Body fat decreased 2.4% (1.6-3.2) in women (p = 0.0001) and 2.2% (0.6 - 3.8) in men (p = 0.01). As shown in table 2, there was a reduction in serum glucose, insulin, HOMAIR, and leptin, whereas adiponectin increased 71%, and the statistical significance persisted independently of gender.

Sympathetic activity

After the exercise program, heart rate significantly decreased, and this reduction was higher in men than in women (3 and 9 beats per minute on average, respectively). Systolic blood pressure decreased by 8mmHg (95% CI, 2.8-13.7) in women and 11 mmHg (95% CI, 4.8-17.2) in men. Diastolic blood pressure decreased by 17.5mmHg (95% CI, 13.2-21.0) in the entire group without difference by gender, whereas SDNN show significant increase by 20. 1ms (95% CI, 6.8-33.4) in women and 16.8ms (95% CI, 8.0-25.6) in men. No significant changes were observed in the low-frequency/high-frequency index in both genders (Table 2).

In multiple regression analysis, change in systolic blood pressure was associated with change in adiponectin levels (β = -0.47, p = 0.0001). LF/HF index was associated with changes in body fat (β = -0.39, p = 0.002) and SDDN was associated with changes in body mass index (β = 0.04, p = 0.04) and body fat (β = -1.04, p = 0.02).

Lung function

We observed improvement in lung function parameters, FVC (% predicted) increased by 5.2% (95% CI, 1.0-9.3) in women (p = 0.016) and 8.0% (95% CI, 2.9-16.0) in men (p = 0.042). FEV_1 (% predicted) showed no significant changes in women, but it increased by 6.2% (95% CI, 1.9-13.3) in males (p = 0.05) and PEF (% predicted) showed a lot of variability between genders, but an overall non-significant increase by 4.7% (p = 0.08) after the exercise program. In multiple regression analysis the change in FEV_1 was associated with change in VO_{2max} (β = 0.904, p = 0.02), and body fat (β = -0.56, p = 0.001).

Discussion

Obesity prevalence has increased in Mexican adolescents. This disease is associated with a chronic inflammatory response, which has been proposed to have an important role in cardiovascular disease especially in adults. Hispanic adolescents seem to have a high risk of being obese during adulthood. We report the effect of physical training program on heart sympathetic activity and lung function in Mexican obese adolescents.

Obesity and sedentary physical activity are important cardiovascular risk factors [21,22]. The American Heart Association concluded that primary prevention is the key to decreasing obesity and limiting its societal impact, particularly since the prevalence of obesity is increasing in children and adolescents. In our study, all obese adolescents showed low physical activity and VO_{2max} levels according to charts for Spanish children [23]. However, they improved their exercise capacity and aerobic energy metabolism, which have been associated with reduction in local inflammatory activation.

In our study, body fat decreased in both genders and change in BMI was significantly related to diminish in systolic blood pressure. This can be explained by changes in hormonal production according to adipocytes size. Growth of adipose tissue mass involves both hypertrophia and hyperplasia of adipocytes. Fat cell size has been related to different adipokine production of adipose tissue. Long-term-steady-state weight reduction in obese subjects resulted in marked decreases in fat cell volume, leptin secretion, and serum leptin concentrations compared with control subjects, but an increase in adiponectin levels, despite similar percent body fat [20]. Furthermore, exercise reduces adipocyte hypertrophy and up-regulation of peroxisome proliferator-activated receptor (PPAR) gammain rats with metabolic syndrome [24], which could explain at least in part the reduction in glucose, leptin, and the increase in adiponectin levels in our study.

Evidence exists that leptin levels are reduced in physical active individuals independent of BMI [24,25], and that leptin is associated with C reactive protein(CRP) levels [26]. Consequently, physical activity could decrease interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) levels and, ultimately, CRP production, by reducing obesity and leptin and increasing adiponectin and insulin sensitivity [27,28]. Even more, some of these effects may be mediated by modification of cytokine production from other sites, besides adipose tissue, such as skeletal muscles.

Adiponectin levels were negatively associated with SBP levels

Table 2: Anthropometric, biochemical and hemodynamic variables before and after exercise program in obese adolescents.

Variable	Baseline	After exercise	Delta (CI 95%)	*p*	*p**
Weight (kg)	78.4 ± 12.5	75.2 ± 13.6	-3.2 (1.9 - 4.4)	0.0001	0.004
BMI (kg/m^2)	31.1 ± 3.6	29.5 ± 3.9	-1.6 (1.0 - 2.1)	0.0001	0.0001
Bodyfat (%)	37.8 ± 6.0	35.5 ± 6.2	-2.3 (1.6 - 3.1)	0.0001	0.0001
Glucose* (mg/dL)	93.2 ± 9.4	89.1 ± 8.1	-4.1 (1.7 - 7.6)	0.02	0.08
Insulin$ (mU/mL)	5.7 (3.5 - 8.0)	3.3 (1.9 - 4.6)	-2.4 (1.0 - 3.8)	0.001	0.001
HOMA-IR	1.2 (0.7 - 1.8)	0.7 (0.4 - 1.0)	-0.5 (0.3 - 0.8)	0.0001	< 0.0001
Leptin (ng/mL)	23.2 (19.5 - 26.9)	17.8 (14.79 -20.9)	-5.4 (3.2 - 7.4)	0.0001	0.003
Adiponectin (ng/mL)	15.1 (10.2 - 20.2)	26.1 (19.2 - 33.0)	10.8 (6.5 - 15.1)	0.0001	0.0006
HR (bpm)	93.7 ± 14.2	88.4 ± 12.0	-5.3 (1.9 - 9.7)	0.02	0.02
SBP (mmHg)	126.7 ± 8.2	117.3 ± 10.3	-9.3 (5.3 - 13.3)	0.0001	0.0001
DBP (mmHg)	85.0 ± 6.8	67.6 ± 6.4	-17.3 (14.4 - 20.2)	0.0001	0.0001
VO_{2max} (mL/kg/min)	18.8 ± 3.3	27.3 ± 6.5	8.5 (6.5 - 10.4)	0.0001	0.0001
SDNN (ms)	58.0 (50.2 - 65.8)	70.8 (65.9 - 88.0)	18.9 (10.2 - 27.6)	0.0001	0.0002
LF/HF	4.4 (3.5 - 5.2)	3.8 (3.5 - 4.8)	-0.5 (0.2 - 1.0)	0.52	0.42
FVC (% predicted)	113.4 ± 17.2	119.6 ± 17.2	6.2 (2.4 - 10.0)	0.002	0.003
FEV_1 (% predicted)	112.1 ± 17.6	135.8 ± 17.8	23.7 (8.1 - 34.2)	0.0001	0.0001
FEV_1/FVC%	83.4 ± 5.7	83.0 ± 6.5	-0.5 (0.1 - 0.7)	0.53	0.42
PEF (% predicted)	105.9 ± 20.9	110.6 ± 19.2	4.7 (1.7 - 10.1)	0.05	0.003

Variables are shown as mean ± SD or mean (95% CI).

p: difference between before and after exercise program

*p value adjusted for gender.

*Glucose conversion factor to SI 0.05551; $Conversion factor to SI 7.175.

HOMA-IR: homeostatic model assessment-insulin resistance index

HR: Heart rate (beats per minute)

SBP: systolic blood pressure

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VO_{2max}: maximal oxygen intake

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FVC: forced vital capacity

FEV_1: forced expiratory volume at 1 second

FEV_1/FVC: forced expiratory volume at 1 second/forced vital capacity

PEF: peak expiratory flow.

in multiple regression analysis, this supports the hypothesis that adiponectin reduces the inflammation status of vasculature, and in turn blood pressure levels [28]. It has been reported that adiponectin dose dependently produces inhibition of the expression of adhesion molecules vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) andendothelial-leukocyte adhesion molecule-1 (E-selectin), reducing monocyte's phagocytic activity and decreasing the accumulation of modified lipoproteins in the vascular wall [29]. Weight loss is a potent inducer of adiponectin levels, however they are affected by many other factors including gender, age, and lifestyle [30]. So, the increase in physical activity even in those children without weight loss during follow up could explain the increase in adiponectin levels.

In this study the rate of change in BMI appears to be more significant than the absolute level of BMI in influencing adolescents SBP, this supports the hypothesis that the possible mechanisms for the development of hypertension in obesity may include insulin resistance, sodium retention, increased sympathetic nervous system activity, activation of the renin-angiotensin-aldosterone system, and altered vascular function [31].

Sympathetic nervous system activity is increased in obesity, particularly sympathetic activity to the kidney and skeletal muscle [32]. The probable reasons for over activation of the sympathetic nervous system in obesity include hyperinsulinemia and/or insulin resistance, increase in leptin, adiponectin, or other adipokines, rennin-angiotensin system over activity, and lifestyle factors [30], which could explain the findings of our study in which the changes in heart rate were associated with the change in leptin levels. The change in SBP was associated with change in adiponectin levels. Other measurements of adiposity such as reduction in body fat was associated with final SDNN and LF/HF index showing the interaction of obesity on heart sympathetic activity.

The effect of obesity on lung function is still not entirely clear, the published results are controversial. Some authors report changes in lung function related to the degree of obesity [33,34], while others report no association between lung function and anthropometric measurements [35,36]. In the present study, we evaluated the lung function of adolescents without respiratory symptoms or a history of respiratory disease. At baseline, 33% of adolescents had a higher percentage of predicted FVC and FEV_1, this suggests that obese adolescents may present hyperinflation. 36.8% were classified with obstructive lung disease (FEV_1%/FVC ≤ 80), and this condition reduced to 26.3% after the training program. Height has been identified as an important predictor factor of spirometric variables resulting collinearity with BMI. Because of our small sample size, our observation that the effects of obesity on FEV_1/FVC are related to an increase in FVC, has to be interpreted with caution. After an exercise program of 16 weeks is expected that adolescents increase their muscle mass, BMI may reflect increased muscular mass and be correlated with higher lung volumes and therefore influence the observed relationship with FVC and FEV_1, respectively. However, we did not evaluate change on diet, so it is difficult to consider change in BMI and body fat only related to the exercise program.

The main strengths of this study were the high performance of a training program, according to the proposed by the European Youth Heart Study [10], considering that clustering of cardiovascular disease risk factors has recently proved a better measure of cardiovascular health in children than single risk factors [23]. However, a limitation of this study is the small sample size and that we did not have control group.

Conclusions

In conclusion, a 16 weeks programme involving 90 minutes of aerobic exercise 5 days per week significantly diminished adiposity, has a positive effect on heart sympathetic activity and improves lung function in Mexican obese adolescents who are especially prone to develop metabolic and hemodynamic complications. However, other studies are necessary to evaluate whether the decrease in BMI was only secondary to the exercise program or also related to change on diet.

Declaration of Conflicts of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Authors' Contributions

BLS carried out the design of the study and the acquisition of data, performed the laboratory analysis, the statistical analysis and interpretation of the data and drafted and revised the manuscript. JMGM and NAL performed the laboratory analysis, the statistical analysis and interpretation of the data and drafted and revised the manuscript. ENL participated in the acquisition and analysis of data and the laboratory analysis. MLM and RTG helped to interpret the data and to draft the manuscript. All authors read and approved the final manuscript.

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