Acute Effects of Functional Electrical Stimulation and Inspiratory Muscle Training in Patients With Heart Failure: A Randomized Crossover Clinical Trial

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Abstract

Background: Heart Failure is a multisystem disorder, which includes autonomic dysfunction.

Objective: To evaluate the acute effects of Functional Electrical Stimulation (FES) and Inspiratory Muscle Training (IMT) on autonomic control, endothelial function and inflammatory cytokine levels in patients with HF.

Methods: Randomized crossover trial including 12 patients undergoing three randomized interventions: FES, IMT, and FES+IMT, with a 1-week interval between sessions. IMT was performed for 15 minutes with 30% of the maximal inspiratory pressure. FES was performed in the vastus lateralis and vastus medialis muscles, at 20Hz for 30 minutes. The autonomic control was measured using beat-to-beat blood pressure monitoring (Finapres); the endothelial function, using the flow-mediated dilation technique (FMD); and inflammatory cytokine levels were assessed before and after the sessions.

Results: Autonomic control after FES decreased regarding LF/HF (p=0.01) and LFn.u (p=0.03), and increased regarding mean RR (p=0.005). Increased mean RR was observed after IMT (p=0.005) and after FES+IMT (p=0.02). No differences were found in FMD and blood lactate concentration. As regards the cytokines, FES led to a decrease in TNF-α levels (pre vs. 24 hours post, p = 0.05). IMT resulted in increased IL-10 levels (pre vs. 24 hours post, p=0.05) and decreased TNF-α levels (1 hour post vs. 24 hours post, p = 0.03). No difference was observed when the two interventions were associated.

Conclusion: FES, IMT, and FES+IMT changed the autonomic control without changing the endothelial function. FES and IMT separately changed inflammatory cytokine levels. Clinical Trials: NCT01325597. (Int J Cardiovasc Sci. 2016;29(3):158-167)

Keywords: Breathing Exercises; Heart Failure; Electric Stimulation; Randomized Controlled Trial.

Introduction

Heart Failure (HF) is the main health problem in the United States,¹ and the estimated costs for 2015 amount to 44.6 billion dollars.² In Brazil, in 2012, there were 248,271 hospital admissions for HF; 23,119 of them resulted in death, with costs amounting to R$ 266,253,192.00.³ HF is a multisystem disorder including autonomic dysfunction, which is characterized by abnormalities in reflex mechanisms, such as cardiopulmonary reflexes, baroreflex and chemoreflex.⁵ Treatment is currently based on pharmacological and non-pharmacological therapies for the autonomic changes, such as exercise training,⁶ functional electrical stimulation (FES)⁷-⁸ and inspiratory muscle training (IMT).⁹ A meta-analysis analyzed the effects of FES in patients with HF, showing increased peak oxygen uptake (VO₂ peak)¹⁰ and 6-min walking distance in comparison with the control group.¹¹ Similarly, the effect of IMT on these patients shows improvements in functional capacity.⁹,¹¹ respiratory
and peripheral muscle strength, as well as relieve of symptoms of dyspnea and depression.\textsuperscript{12}

However, few studies have evaluated the acute effect of these therapies on this population. Labrunée \textit{et al}\textsuperscript{13} verified that FES promoted a significant decrease in sympathetic nervous activity, with no changes in hemodynamic or ventilatory parameters. Regarding IMT, no studies evaluated the acute effect of this intervention on the autonomic control of these patients, although studies have been conducted in healthy individuals.\textsuperscript{14}

Nonetheless, no studies have assessed the effects of the association of these two therapies on this population. Therefore, this study aimed to evaluate the acute effect of FES and IMT on the autonomic nervous system, endothelial function, cytokines and lactate levels in patients with heart failure.

\section*{Methods}

\subsection*{2.1. Experimental design}

This study is a randomized crossover trial that was approved by the Ethics Committee of the Federal University of Health Sciences of Porto Alegre (UFCSPA) under number 075/05, and the Institute of Cardiology under number 4546/10. Informed written consent was obtained from all participants in accordance with standards established by the latest revision of the Declaration of Helsinki, from June 2014 to May 2015. This study is registered in Clinical Trials under the number NCT01325597.

\subsection*{2.2. Participants}

Patients were consecutively selected at the pre-transplant outpatient clinic and in the echocardiography sector of the Institute of Cardiology of Rio Grande do Sul / University Cardiology Foundation. The inclusion criteria were patients of both genders with heart failure (NYHA functional class II, III or IV); ejection fraction <40\%, as determined by echocardiography; and pharmacological therapy maintained for at least a month prior to inclusion in the study. Patients were excluded if they had unstable ventricular arrhythmia up to three months prior to the beginning of the study; peripheral vascular disease; acute respiratory disease; unstable angina; aortic valve stenosis; current cigarette smoking; chronic kidney disease or were on hemodialysis; fever and/or infectious disease; and malignancies.

\subsection*{2.3. Interventions}

\subsection*{2.3.1 Functional Electrical Stimulation}

The FES protocol was performed with a functional electrical stimulation device (Electrical Physiological Stimulator – LYNX – FMUSP, São Paulo, Brazil) with the following parameters: 20 Hz frequency; pulse width: 0.5 ms; stimulation time: 5s; resting time: 10 s, for 30 min or until muscle fatigue. The intensity was individually adjusted, considering the patients’ ability to promote full knee extension and comfort during contractions. Adhesive surface electrodes (Spes, Italy, 50 x 90 cm) were positioned in the motor areas of the vastus lateralis and vastus medialis muscles as determined by muscle mapping with knees flexed at 60°.

\subsection*{2.3.2 Inspiratory Muscle Training}

The IMT protocol was performed using a linear resistive breathing device (Threshold®, New Jersey, USA) for 15 minutes according to each patient’s respiratory rate, maintaining diaphragmatic breathing. Training was performed using a load of 30\% maximal inspiratory pressure, which was assessed by a digital vacuometer model MVD 300 (Globalmed, Porto Alegre, Brazil), according to the methodology proposed by the American Thoracic Society\textsuperscript{15}. The values were calculated based on the equations proposed by Neder \textit{et al}.\textsuperscript{16}

\subsection*{2.3.3. Functional Electrical Stimulation + Inspiratory Muscle Training}

Training with the association of these two interventions (FES+IMT) was performed first with IMT, immediately followed by FES, as described above.

\section*{Outcomes}

In this study, autonomic control was considered the primary outcome; secondary outcomes were endothelial function and blood lactate measured pre- and post-intervention, and inflammatory cytokines measured pre-, 1 hour post- and 24 hours post-intervention.

\section*{Autonomic control assessment}

The autonomic nervous system control was non-invasively assessed using a pressure monitor Finapres (Ohmeda 2300, Monitoring Systems, Englewood, USA) by recording beat-to-beat blood pressure.
A pressure cuff was placed around the intermediate phalanx of the right middle finger; the upper limb and the cuff were maintained at the midaxillary line level throughout the procedure. Digital signal conversion was obtained using the POWER LAB 4/35 software with LabChart. The systolic peak was automatically detected from the pulse waves, and the interval between each wave (heart pulse period) was used to construct the tachogram and analyze the heart rate variability (HRV) in the time and frequency domains. Analyses were carried out using the Kubios HRV software (version 2.1 Department of Applied Physics, University of Eastern Finland, Kuopio).

The following time-domain HRV analysis parameters were determined: mean of RR intervals (normal consecutive beats, ms, Mean RR); mean heart rate (Mean HR); square root of the mean of the sum of the squares of differences between adjacent normal RR intervals (RMSSD, ms); and the number of successive RR pairs that differed by more than 50 ms (NN50, count). The following frequency-domain HRV parameters were determined: low-frequency component (LF: 0.04 - 0.15 Hz); high-frequency component (HF: 0.15 - 0.4 Hz); and LF/HF.

Pressure was recorded before and after the intervention, with the patient in the supine position for 10 minutes, in an air-conditioned room (approximately at 23°C), and always in the morning.

Assessment of endothelial function

A high-resolution ultrasonography equipment (EnVisor CHD, Philips, Bothell, WA, USA) was employed to assess the endothelial function non-invasively. A high-frequency vascular transducer (3-12 MHz; L12-3, Philips, Bothell, WA, USA) was used to obtain longitudinal images of the brachial artery walls.

Assessment of blood lactate levels

Blood lactate levels were measured by capillary puncture with the lactate analyzer Accutrend Plus - Roche. Measurements were taken pre, immediate-post- and 1 hour post- intervention.

Assessment of inflammatory cytokines

The inflammatory cytokines Interleukin-10 (IL-10), Interleukin-6 (IL-6) and Tumor Necrosis Factor Alpha (TNF-α) were measured by enzymatic assays (Elisa), using commercial kits and following the manufacturer’s instructions. Colorimetric reactions were read at 450 nm in a microplate reader. Plasma cytokine levels were determined from values obtained from a standard curve with different dilutions of the recombinant protein.

Randomization and blinding

The order of intervention was randomized with data generated by a software available at www.random.org; afterwards, data were maintained in allocation concealment, in opaque envelopes kept in a remote place. A blinded researcher generated the numeric sequence of patients selected based on inclusion and exclusion criteria. The numeric sequence was kept secret until the exact time when the intervention began. Researchers were blinded to the outcomes of groups.

Statistical analysis

Data are presented as mean ± standard deviation and median and interquartile range. The Shapiro-Wilk test was performed to assess data normality. The primary outcome of this study was autonomic control, assessed by HRV. The sample size calculation was based on an alpha level of 5% and beta level of 90%. For mean difference and standard deviation calculations, the results of sympathetic nervous activity found by Labrunée et al. were used as reference. The sample size calculation resulted in 12 patients in each protocol (FES, IMT, and FES+IMT).

For comparisons between groups and timepoints, the Friedman test was used and, when necessary, the Wilcoxon test (pre- and post- interventions), with a statistical significance of 5%. Analyses were performed using the SPSS (Statistical Package for the Social Sciences) software version 22.0 for Windows.

Results

Fifty patients were selected, 12 of whom fulfilled the eligibility criteria and completed the study, as shown in the flowchart in Figure 1.

Of the 12 patients included in the study, 54.6% were women, with dilated cardiomyopathy as the main etiology of HF (63.6%) and a mean ejection fraction of 36.8%; the majority (63.6%) were in NYHA...
class III. All the patients were taking beta-blockers and statins. Additional sample characteristics are presented in Table 1.

In the autonomic control analysis, the FES protocol showed a significant increase in mean RR intervals (mean RR, \( p=0.04 \)) and a significant decrease in mean heart rate (mean HR, \( p=0.05 \)), in addition to decreased ratio of sympatovagal balance (LF/HF, \( p=0.01 \)) and the low-frequency component corresponding to sympathetic nervous system activity (LF\textsubscript{n.u}, \( p=0.03 \)).

The IMT protocol showed increased mean RR (\( p=0.005 \)) and decreased mean HR (\( p=0.005 \)). In the FES+IMT group, there was an increase of mean RR (\( p=0.02 \)) and a decrease of mean HR (\( p=0.02 \)). No significant difference was observed in other autonomic control variables. These data are shown in Table 2.

Regarding the endothelial function, values for the pre- and post-intervention periods were as follows: FES protocol = [8.9(5.9-18.3) vs. 12.2(5.0-16.9) \( p=0.7 \)]; IMT protocol = [8.5(4.6-11.2) vs. 10.6(3.9-16.9) \( p=0.6 \)]; FES+IMT protocol = [8.0(5.2-16.0) vs. 12.3(6.4-17.5) \( p=0.3 \)]. However, these values did not show significant differences.

In the assessment of blood lactate levels, none of the interventions showed significant differences at any of the three timepoints when they were measured (pre, post, 1 hour post). When FES was used, the values were pre= 3.2 [2.7-3.8] mmol, post= 3.4 [2.2-4.1] mmol and 1 hour post= 3.4 [2.2-4.7] mmol, \( p=0.8 \). In the IMT protocol, the lactate values were pre= 3.6 [2.4-5.0] mmol; post= 3.4 [1.9-4.9] mmol and 1 hour post= 3.6 [2.7-4.6] mmol, \( p=0.5 \). In protocol FES+IMT, the lactate values...
Table 1
Baseline clinical characteristics of the study population

<table>
<thead>
<tr>
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<th>n=12</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>66.1 ± 7.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.9 ± 5.3</td>
</tr>
<tr>
<td>Gender (%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>45.4</td>
</tr>
<tr>
<td>Female</td>
<td>54.6</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td></td>
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<tr>
<td>DBP (mmHg)</td>
<td>113.2 ± 16.6</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>68 ± 12.2</td>
</tr>
<tr>
<td>MIP (% predicted)</td>
<td>68.2 ± 7.7</td>
</tr>
<tr>
<td>MEP (% predicted)</td>
<td>70 ± 15</td>
</tr>
<tr>
<td>Etiology of Heart Failure (%)</td>
<td></td>
</tr>
<tr>
<td>Dilated</td>
<td>105 ± 21</td>
</tr>
<tr>
<td>Ischemic</td>
<td>63.6</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>36.4</td>
</tr>
<tr>
<td>NYHA functional classification, (%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>36.4</td>
</tr>
<tr>
<td>III</td>
<td>63.6</td>
</tr>
<tr>
<td>Medications, (%)</td>
<td></td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>100</td>
</tr>
<tr>
<td>Statin</td>
<td>100</td>
</tr>
<tr>
<td>Diuretic</td>
<td>90.9</td>
</tr>
<tr>
<td>Antiplatelet</td>
<td>90.9</td>
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<tr>
<td>ACE-Inhibitor</td>
<td>72.7</td>
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<tr>
<td>Digitalis</td>
<td>54.4</td>
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<tr>
<td>Hypoglycemic</td>
<td>45.5</td>
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<tr>
<td>Nitrates</td>
<td>18.2</td>
</tr>
<tr>
<td>Angiotensin Receptor Blocker</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Data as mean ± SD. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate, MIP: maximal inspiratory pressure, MEP: maximal expiratory pressure; LVEF: left ventricular ejection fraction; ACE: angiotensin converting enzyme.

were pre= 3.4 [2.7-4.2] mmol, post= 3.3 [2.1-4.4] mmol and 1 hour post= 3.4 [2.4-4.1] mmol, p=0.5.

Concerning inflammatory cytokine levels, the FES intervention significantly reduced TNF-α levels (pre vs. 24 hours post, p=0.05). In IMT, there was a significant increase in IL-10 levels (pre vs. 24 hours post, p=0.05) and a significant decrease in TNF-α levels (1 hour post vs. 24 hours post, p=0.03). Yet, when FES and IMT were associated, the inflammatory cytokine levels did not show significant differences. The values are shown in Table 3.

**Discussion**

The results of this study show that after a session of FES, IMT, or association of FES+IMT, there were changes in the autonomic cardiovascular control and inflammatory cytokine levels in patients with HF,
with no changes in the endothelial function or blood lactate levels. After FES, the sympathetic compound (LF), sympatovagal balance (LF/HF) and heart rate (HR) were decreased, whereas the HRV was increased. Furthermore, after IMT, HR was reduced and HRV increased; the association of these two techniques resulted in increased HRV and decreased HR.

To the best of our knowledge, this is the first study to demonstrate the acute effect of the association of the two interventions on these outcomes, and there

<table>
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<th>Table 2</th>
<th>Results of the analysis of heart rate variability</th>
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<tbody>
<tr>
<td></td>
<td>FES</td>
</tr>
<tr>
<td></td>
<td>PRE</td>
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<tr>
<td>Frequency-Domain</td>
<td></td>
</tr>
<tr>
<td>LF n.u</td>
<td>60.7 [30.8-69.4]</td>
</tr>
<tr>
<td>HF n.u</td>
<td>39.3 [30.6-69.2]</td>
</tr>
<tr>
<td>LF/HF</td>
<td>1.5 [0.44-2.26]</td>
</tr>
<tr>
<td>Time-Domain</td>
<td></td>
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<tr>
<td>Mean RR (ms)</td>
<td>889.3 [808.2-1043.7]</td>
</tr>
<tr>
<td>Mean HR (1/min)</td>
<td>67.5 [58.1-74.2]</td>
</tr>
<tr>
<td>NN50 (count)</td>
<td>4.0 [3.0-42.0]</td>
</tr>
</tbody>
</table>

Values expressed as median and interquartile range; * significant difference between pre and post intervention (Wilcoxon test p < 0.05); FES: Functional Electrical Stimulation, IMT: Inspiratory Muscle Training, LF nu: low frequency component normalized; HF nu: high frequency component normalized; LF / HF ratio between the LF and HF components; Mean RR- Mean intervals RR; Mean HR: Mean heart rate, RMSSD: square of the sum of differences between adjacent normal RR intervals to the average square root; NN50: Difference between RR normal intervals greater than 50 ms.

<table>
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<th>Table 3</th>
<th>Results of inflammatory cytokines</th>
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<tbody>
<tr>
<td></td>
<td>FES</td>
</tr>
<tr>
<td></td>
<td>PRE</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>[0.15-1.42]</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>[0.43-0.86]</td>
</tr>
<tr>
<td>TNFa</td>
<td>11.52</td>
</tr>
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<td></td>
<td>[10.08-15.38]</td>
</tr>
</tbody>
</table>

p < 0.05 Friedman test. Equal letters differ. Data as median and interquartile range; FES: Functional Electrical Stimulation; IMT: Inspiratory Muscle Training; IL-10: Interleukin 10; IL-6: Interleukin 6; TNFa: Tumor necrosis factor alpha.
is no information to compare the results found. However, few studies evaluated the effect of FES and IMT separately. In our study, we found that FES promotes changes in the autonomic control, with a reduction in LF and LF/HF. Labrunée et al. found a significant reduction in the sympathetic nervous activity after FES employing different low-frequency modalities in the same population. However, Kang et al. did not observe any differences in healthy individuals.

It is possible that central mechanisms are involved in the reduction of sympathetic activation, as occurs with the use of Transcutaneous Electrical Nerve Stimulation (TENS), in which cutaneous stimulation is able to modify the baroreflex sensitivity. Another possibility relates to the metaboreflex, which can be increased or attenuated depending on the type of muscle contraction and exercise intensity.

Furthermore, mechanisms such as the strength of muscle contraction, the duration of the treatment and the electrical stimulation frequency used in this study (20Hz) may have determined the changes observed in the autonomic control and cytokine production. Moreover, lactate levels did not change in our analysis, showing that FES (according to our protocol) promoted low-intensity exercise.

Regarding the endothelial function, our study did not find significant differences in flow-mediated vasodilation of the brachial artery after FES. In healthy individuals, it is expected that conventional physical exercise will acutely reduce the flow-mediated dilation of the brachial artery, followed by normalization of the values. These results depend on factors such as intensity, type and duration of exercise. Therefore, the stimuli caused by the interventions in our study were not sufficient to produce acute changes in the endothelial function. Moreover, the long-term assessment of the endothelial function after FES training has shown a significant increase of flow-mediated vasodilation of the brachial artery in patients with HF. This effect probably occurs because of the improvement of mechanisms that change the endothelial function, such as nitric oxide production and inflammatory response.

Regarding the inflammatory cytokines, our results show significantly reduced levels of TNF-α comparing pre- vs. 24 hours post-FES. During sessions of acute physical exercise, it is expected that pro-inflammatory cytokine levels will increase, as opposed to what is found in chronic exercise. After 6 weeks of FES training, patients with HF achieved a significant reduction of TNF-α levels and higher IL-10/TNF-α rates. Hence, the acute modifications of the interleukins assessed in our study could be, in part, one of the mechanisms that lead to the chronic adaptation observed in studies with chronic FES interventions.

Regarding IMT, our results show that, despite a significant increase in HRV caused by increased RR intervals, there were no significant alterations in the sympathetic and parasympathetic components. No studies have evaluated the acute effects of training on this population. Our results can be explained by the low overload applied (30% MIP) and corroborate the findings of Plentz et al. ‘s study which demonstrated that, in healthy subjects, the load did not affect the autonomic control. However, when performed with a higher load (60% MIP) an increase in the LF component was observed. Nonetheless, Mello et al. reported that after 12 weeks of IMT with 30% MIP a decrease in the sympathetic modulation and increase in the parasympathetic modulation were observed. These findings show that adaptation and improvement of the systems result from treatment intensity and duration.

The patients in our study presented values under 70% (MIP), indicating inspiratory muscle weakness; moreover, there was a significant decrease in HR after IMT and there was no significant difference in blood lactate levels. IMT, however, can attenuate the metaboreflex exacerbation and possibly improve exercise tolerance in patients with HF.

Taken together, these responses indicate that 30% MIP can be considered a load that does not promote metabolic stress.-

Concerning endothelial function, there were no significant changes in the values of flow-mediated dilation after IMT, indicating that this intervention does not acutely produce sufficient stimuli to modulate or modify this response. Laoutaris et al. demonstrated that even chronically, low and high intensity IMT (15% and 60%, respectively) did not promote modifications in the endothelium-dependent vasodilation in patients with HF.
Regarding the inflammatory cytokines, in our study, the results demonstrated a significant increase of IL-10 (pre vs. 24 hours post) and decrease of TNF-α levels at timepoints 1 hour post vs. 24 hours post IMT. This result was also observed when IMT was assessed chronically by Laoutaris et al., who found a significant reduction of TNF-α receptor I in its soluble form after 10 weeks of IMT with 60% MIP. This finding can be explained by the overload imposed on the diaphragm by the IMT, resulting in improved aerobic capacity and consequent reduction of blood cytokine levels. The same effect occurs when physical exercise is performed.

As for the association of FES+IMT, there are no studies evaluating the association of the two techniques with these outcomes.

In relation to the autonomic control, a significant difference in HRV due to increased mean RR and decreased heart rate, we did not find significant differences between sympathetic and parasympathetic components, endothelial function, blood lactate and inflammatory cytokines. Although there are no studies evaluating the association of these two techniques, it was expected that it would promote an increased mean RR and decreased HR, since separately both techniques produced the same response. Regarding the sympathetic and parasympathetic components, it is possible that these results have occurred because of the way the techniques are applied. Although the application of FES had promoted a reduction in LF and LF/HF, TMI alone did not change these components, and this may have neutralized the effect of FES.

The endothelial function and blood lactate did not change when the two techniques were applied in a combined form. This result was also expected because the techniques alone did not promote changes in these variables and we believe that the low intensity of the two techniques contributed to this result.

In our study, the combination of two techniques did not result in any significant difference in the levels of inflammatory cytokines, unlike shown in studies that evaluated the effect of these two techniques used alone and in the long term.

**Study limitations**

As limitations of this study, we did not assess the metaboreflex and chemoreflex, which could have shown more specifically the involvement of each of these mechanisms in the autonomic control related to the interventions. Another noteworthy factor is a possible residual effect, also called carry-over effect, present due to the study design, which can influence its internal validity. Despite the randomized order of interventions for each patient, and the one-week washout period between sessions, it is possible that effects of learning and familiarization with the techniques by the patients are present.

**Conclusion**

This study found that one session of functional electrical stimulation, inspiratory muscle training, or both interventions combined resulted in autonomic changes with improved HRV, without alterations in endothelial function and blood lactate levels. FES and IMT alone led to a decrease in pro-inflammatory cytokine levels in cardiac patients. The effects observed in this study may partly explain the therapeutic and physiological adaptations that occur in patients with heart failure undergoing these interventions for long periods. However, further studies with greater sample sizes are necessary to obtain a more powerful analysis and to confirm the changes observed here.

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**Author contributions**

Conception and design of the research: Nicolodi GV, Sbruzzi G, Macagnan FE, Dipp T, Macedo ACP, Casali KR, Plentz RDM. Acquisition of data: Nicolodi GV, Dipp T, Macedo ACP. Analysis and interpretation of the data: Macagnan FE, Plentz RDM. Writing of the manuscript: Nicolodi GV, Sbruzzi G, Dipp T, Plentz RDM. Critical revision of the manuscript for intellectual content: Sbruzzi G, Macagnan FE, Casali KR, Plentz RDM.

**Potential Conflict of Interest**

No potential conflict of interest relevant to this article was reported.
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Study Association
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