Effect of Microencapsulated Cocoa Polyphenols on Macro- and Microvascular Function after Eccentric Exercise

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Abstract: Background: Evidence has demonstrated that non-habitual exercise, such as eccentric exercise, can increase reactive oxygen species and induce endothelial dysfunction, which plays a central role in the development of cardiovascular disease. Polyphenol-rich foods, such as cocoa, have been widely investigated in vascular function due to their antioxidant effect. Aims: The goal of this study was to evaluate the impact of microencapsulated cocoa (MC) polyphenols in the flow-mediated dilation (FMD) response and forearm muscle oxygenation (StO2) parameters after an eccentric exercise. Methods: Thirteen physically active adults were enrolled in a randomized, double-blind, and crossover study. FMD and StO2 were evaluated before and after 24 h, 48 h, and 72 h of eccentric exercise and MC or placebo supplementation. Results: No significant difference in FMD response and StO2 parameters was observed after MC and placebo (p > 0.05). Conclusions: A single dose of MC did not change FMD and muscle StO2 parameters after eccentric exercise in healthy individuals.

Keywords: flow-mediated dilation; endothelial function; muscle oxygenation; polyphenols; bioactive compounds

1. Introduction

Endothelial dysfunction is characterized by an imbalance in vasodilatory (nitric oxide, prostacyclin, adenosine triphosphate, etc.) and vasoconstrictor factors (angiotensin, thromboxane, endothelin-1, etc.) that can increase the risk of cardiovascular events, such as acute myocardial infarction and cerebrovascular diseases [1–3]. The reduced nitric oxide (NO) bioavailability, a critical molecule that regulates vascular tone, can occur by increased reactive oxygen species (ROS), which has been demonstrated to be greater after strenuous physical exercise [4,5]. For example, previous studies have shown that non-habitual exercise, such as eccentric exercise, could increase ROS production and induce vascular dysfunction [6,7]. Stacy et al. (2013) [7] demonstrated that eccentric unilateral contraction of the biceps brachii muscles (2 sets of 25 repetitions) leads to impairment of local endothelial function (FMD response) in healthy male adults. The authors have reported that eccentric exercise-induced muscle damage results in increased muscle blood flow and muscle oxygen saturation to the muscle to repair the muscle damage caused by exercise. This scenario, however, can contribute to a transient reduction in FMD response that can persist up to 96 h after exercise [7]. Moreover, Oliveira et al. (2020) [8] demonstrated that a single resistance exercise session decreases endothelial function (measured by flow-mediated...
dilation (FMD)), suggesting that intense exercise can acutely induce impaired endothelial dysfunction. Notably, a 1% increase in FMD value was associated with a 9% decrease in future risk of cardiovascular events [9]. Stacy et al. (2013) demonstrated a reduction of FMD from approximately 9% to 4% 48 h after eccentric exercise. Therefore, strategies to minimize exercise-induced endothelial dysfunction are interesting to investigate [10,11].

Nutritional interventions are a practical approach to reverse chronic and transient endothelial damage [10–12]. Among them, polyphenols-rich foods have gained popularity in the scientific community because of their positive effects on endothelial function [13,14]. It appears that the antioxidant property of polyphenols is one of the main reasons for the cardiovascular benefits due to the ability of polyphenols to neutralize ROS, preventing their interaction with NO and the subsequent formation of oxidizing agents [15].

Cocoa, produced from the cocoa bean (Theobroma cacao), is a good source of polyphenol compounds with potent antioxidant properties [16]. Furthermore, the polyphenols present in cocoa have been demonstrated to have vascular benefits [17]. Besides that, polyphenols from cocoa are sensitive compounds that can suffer impacts on absorption, solubility, digestibility, and metabolism, which may compromise their biological effect [18]. Studies demonstrated that only 5–10% of the total polyphenols in foods are absorbed by the intestine [19]. Therefore, it becomes essential to develop effective delivery systems to increase the bioavailability of food compounds. Food microencapsulation is an emerging and alternative technology [20] that may contribute to improving nutrient stability and bioavailability. Enhancing the polyphenol bioavailability with microencapsulation technology could reduce the need to ingest a higher dose of polyphenols, potentiating vascular effects [20]. It has been shown that the area under the blood concentration-time curve after the oral administration of nano-particle curcumin was 27-fold higher than that of curcumin powder in healthy participants [21]. This evidence suggests that small particles (as generated by the spray drier) can induce a positive effect due to increased polyphenol bioavailability.

Recently, a single dose of microencapsulated cocoa has been shown to mitigate eccentric exercise-induced vascular dysfunction after 30 min exercise [11]. However, it has not been demonstrated whether cocoa supplementation can affect muscle recovery 72 h post-eccentric exercise. Considering the worldwide concern about the risks of advancing cardiovascular diseases, the present study investigated whether microencapsulated cocoa polyphenols could attenuate macro- and microvascular dysfunction for up to 72 h after eccentric exercise. It is important to assess macro- and microvascular function whenever possible, given that it is a different entity and presents a distinct impact in a cardiovascular event [22]. Microencapsulated cocoa polyphenols were hypothesized to attenuate macro and microvascular dysfunction induced by eccentric exercise for up to 72 h in healthy individuals.

2. Methods

2.1. Participants

Thirteen participants, six men and seven women, who were physically active (at least 12 months engaged in resistance training performed ≥ 3 times a week) adults were included in this study. Female individuals were assessed during the menstrual period to avoid potential variations in vascular response related to estrogen concentration during the menstrual cycle [23]. Exclusion criteria included participants taking antioxidant supplements and anti-inflammatory, antibiotics, or anti-hypertensive medication. Hypertensive and overweight individuals were also excluded from the study. The study was approved by the Institutional Ethics Committee (n° 4.859,763). Clinical Trials Registry (ReBEC) (RBR-3bc3rnb).

2.2. Study Design

All participants came to the laboratory for nine days to perform eccentric exercise and intake the microencapsulated cocoa or placebo (PLA) intervention in a double-blind,
Experimental design of the study.

2.3. Microencapsulated Cocoa/Placebo Preparation

Maltodextrin was mixed with Cocoa powder (100% Cocoa Nestlé®, São Paulo, Brazil) for supplementation. The solution containing cocoa powder and maltodextrin was dried using a mini spray dryer to prepare a microencapsulated product. The process was performed using an inlet temperature of 160 °C, feed rate of 30%, and airflow of 70%. Volunteers consumed 25 g of microencapsulated cocoa (80 mg of total flavonoids) or PLA (low-cocoa chocolate milk powder containing approximately 9 mg of total flavonoids) dissolved in 250 mL of water. The dosage of 80 mg of cocoa flavonoids was chosen based on a previous study [26] demonstrating improved endothelium-dependent vasodilation as evaluated by FMD after cocoa supplementation. Total flavonoids were evaluated by diluting microencapsulated cocoa in methanol (1:5). The cocoa solution was mixed with 2% Aluminum chloride in methanol (v/v). The absorbance of the mixed solution was measured at 415 nm in a spectrophotometer (Model Lambda 25 UV/VIS, PerkinElmer, Waltham, MA, USA) after 10 min. Total flavonoid content was expressed as quercetin equivalent [11]. The principal investigator generated the balanced permutation randomized sequences using an online platform and allocated individuals to groups as they were consecutively recruited.Both participants and investigators were blinded to the groups.

2.4. Eccentric Exercise Protocol

The participants performed an elbow flexion and extension exercise with an isokinetic dynamometer (Humac Norm, CSMi Medical Solutions, Stoughton, MA, USA) in the eccentric (flexion)—concentric (flexion) mode. The exercise was performed with the participants in an adjustable bed with Human Norm equipment. The exercise movement was performed with a joint range of motion from 0° to 90°, beginning with concentric
elbow flexion, followed by eccentric elbow extension. The participants performed six sets of 10 maximal voluntary contractions using the non-dominant limb at an angular velocity of 30°·s⁻¹ in both the extension (active movement) and flexion (passive movement) phases, with a recovery period of 1 min between exercise sets. Verbal encouragement was given during exercise protocol to ensure maximal efforts during exercise. Previous evidence has shown that this exercise protocol was sufficient to induce muscle [27] and vascular damage [11].

2.5. Flow-Mediated Dilatation

Analysis of macrovascular endothelial function by FMD of the brachial artery was performed concurrently with analysis of muscle oxygen saturation by near-infrared spectroscopy (NIRS) as previously described and according to guidelines presented elsewhere [23,28]. The participants laid down in a bed, and the ultrasound’s transducer was placed on the medial aspect of the dominant arm (not exercised limb), approximately 2 cm above the participants’ medial epicondyle of the humerus. Thus, the longitudinal image of the brachial artery was obtained and recorded during 1 min of baseline, 5 min of vascular occlusion (utilizing Hokanson E20 AG101, Bellevue, WA, USA—250 mm Hg cuff pressure), and 3 min of post occlusion reperfusion. Changes in the brachial vessel (artery) diameter data were detected and continuously recorded throughout the FMD procedure using edge-detection and wall-tracking software (Cardiovascular Suite version 4.2.1, Quipu srl, Pisa, Italy). Percent FMD (%) was determined as the percent change from baseline to peak arterial diameter.

2.6. Muscle Oxygen Saturation Measurement

Muscle oxygen saturation (StO₂) was evaluated using a commercially available portable near-infrared spectroscopy (NIRS) device (PortaMon, Artinis, Medical Systems), which was placed on the participant’s flexor-dominant forearm muscles. The NIRS system was connected to a personal computer via Bluetooth for data acquisition (10 Hz), analog-to-digital conversion, and further analysis of the raw data (i.e., no filters were used) using native software (Oxysoft version 2.1.6; Artinis Medical Systems). After a 30-s baseline StO₂ analysis, a pneumatic cuff placed in the cubital fossa of the participant’s arm was inflated to supra systolic levels (i.e., 250 mmHg) for 5 min. After releasing the cuff (reperfusion phase), StO₂ was recorded for 3 min. The following NIRS parameters were considered for statistical analysis: (a) desaturation rate (StO₂downslope), which corresponds to the StO₂ downslope (the rate of change of StO₂ per second) during vascular occlusion; (b) resaturation rate (StO₂upslope), which corresponds to the StO₂ upslope (10-sec window) during the reperfusion phase; (c) magnitude of O₂ resaturation (ΔStO₂) was calculated as the difference between the maximum and minimum value achieved during the reperfusion phase. The StO₂upslope and the magnitude of O₂ resaturation have been used as a parameter of microvascular reactivity [24]. The desaturation rate was informative data since it represents the magnitude of tissue ischemia during the vascular occlusion test [29].

2.7. Statistical Analysis

The data’s normality, homogeneity of variances, and sphericity were examined with the Shapiro-Wilk, Levene, and Mauchly tests, respectively. An a priori power analysis (G*Power version 3.0.1) was performed using a two-way ANOVA test with repeated measures within factors [30,31].

To detect a difference between PLA and microencapsulated cocoa in flow-mediated dilation and StO₂ parameters, an analysis of variance (repeated measures two-way ANOVA) was performed. The post-hoc test was used when the F value was significant. The α level of significance was set at 0.05. The data were shown as mean ± standard deviation.
3. Results

Table 1 shows the thirteen participants were randomized and completed the study. The participants were 25 ± 4 years old, 66 ± 12 kg body weight, and body mass index (BMI) of 23.5 ± 3 kg/m².

Table 1. Baseline characteristics of the participants.

<table>
<thead>
<tr>
<th>Variables</th>
<th>MC</th>
<th>PLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>25 ± 4</td>
<td></td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>66.8 ± 12</td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.5 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

Clinical

<table>
<thead>
<tr>
<th>Variables</th>
<th>MC</th>
<th>PLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mm Hg)</td>
<td>112 ± 8</td>
<td></td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>73 ± 7</td>
<td></td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>64 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

DBP = diastolic blood pressure, HR = heart rate, SBP = systolic blood pressure. Values are reported as mean ± standard deviation (SD).

3.1. Flow-Mediated Dilation

There was no significant main effect (for time) and interaction (treatment*time) for baseline artery diameter (p = 0.359 and p = 0.473, respectively), peak artery diameter (p = 0.248 and p = 0.614, respectively), and FMD (p = 0.408 and p = 0.615, respectively) (Table 2 and Figure 2). Supplementary Material shows a figure with FMD individual data of the participants (Figure S1).

Table 2. Flow-mediated dilation (FMD) and tissue oxygen saturation (StO₂) before (T0) and after (T24, T48, and T72) ingestion of a single dose of microencapsulated cocoa (MC) and (PLA).

<table>
<thead>
<tr>
<th>Variables</th>
<th>T0</th>
<th>T24</th>
<th>T48</th>
<th>T72</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD (%)</td>
<td>MC</td>
<td>9.9 ± 3.9</td>
<td>11.1 ± 5.6</td>
<td>14.02 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>11.7 ± 4.2</td>
<td>11.6 ± 4.1</td>
<td>12.1 ± 6.1</td>
</tr>
<tr>
<td>BAD (mm)</td>
<td>MC</td>
<td>0.32 ± 0.09</td>
<td>0.32 ± 0.08</td>
<td>0.33 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>0.30 ± 0.09</td>
<td>0.33 ± 0.08</td>
<td>0.32 ± 0.07</td>
</tr>
<tr>
<td>PAD (mm)</td>
<td>MC</td>
<td>0.35 ± 0.1</td>
<td>0.35 ± 0.1</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>PLA</td>
<td>0.35 ± 0.1</td>
<td>0.37 ± 0.08</td>
<td>0.36 ± 0.09</td>
</tr>
</tbody>
</table>

Figure 2. Brachial artery flow-mediated dilation (FMD%) parameters at baseline and after 24 h, 48 h, and 72 h eccentric exercise and microencapsulated cocoa or placebo supplementation.
Table 2. Cont.

<table>
<thead>
<tr>
<th>Variables</th>
<th>T0</th>
<th>T24</th>
<th>T48</th>
<th>T72</th>
</tr>
</thead>
<tbody>
<tr>
<td>StO$_2$upslope (% s$^{-1}$)</td>
<td>MC 2.1 ± 0.5</td>
<td>2.01 ± 0.6</td>
<td>1.9 ± 0.6</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>PL 2.04 ± 0.3</td>
<td>1.8 ± 0.6</td>
<td>1.9 ± 0.7</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>StO$_2$downslope (% s$^{-1}$)</td>
<td>MC -0.08 ± 0.02</td>
<td>-0.09 ± 0.02</td>
<td>-0.08 ± 0.03</td>
<td>-0.08 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>PL -0.08 ± 0.01</td>
<td>-0.08 ± 0.02</td>
<td>-0.07 ± 0.02</td>
<td>-0.09 ± 0.01</td>
</tr>
<tr>
<td>△StO$_2$ (%)</td>
<td>MC 32.5 ± 7.7</td>
<td>32.4 ± 6.9</td>
<td>36.01 ± 7.5</td>
<td>33.7 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>PL 32.3 ± 6.7</td>
<td>31.8 ± 6.1</td>
<td>35.8 ± 6.2</td>
<td>31.2 ± 6.1</td>
</tr>
</tbody>
</table>

Values are reported as mean ± standard deviation (SD). △StO$_2$ = magnitude of O$_2$ resaturation.

3.2. Muscle Oxygen Saturation Parameters

There was no significant main effect regarding time and interaction (treatment x time) for StO$_2$downslope ($p = 0.277$ and $p = 0.354$, respectively), StO$_2$upslope ($p = 0.366$ and $p = 0.609$, respectively), and magnitude of muscle StO$_2$ ($p = 0.317$ and $p = 0.176$, respectively) (Table 2). Supplementary Material shows a figure with StO$_2$upslope and magnitude of muscle StO$_2$ individual data of the participants (Figure S1).

4. Discussion

The present study investigated the effect of a single dose of microencapsulated cocoa on macro- (FMD response) and microvascular (oxygen resaturation rate) function over 72 h after a single round of eccentric exercise. Our finding revealed that microencapsulated cocoa polyphenols did not affect both FMD response and muscle StO$_2$ parameters in response to eccentric exercise.

Eccentric exercise was expected to reduce FMD response and remain lower up to 72 h after exercise. Eccentric exercise has been reported to induce muscle damage due to muscle ruptures caused by intensity exercise, generating low-grade inflammation [27]. As a result, macro- (FMD response) and microvascular (StO$_2$ parameters) functions could be impaired since inflammation and excessive ROS production (induced by exercise) can decrease NO bioavailability [11,32]. FMD response is clinically relevant since FMD is a predictor of incident cardiovascular events in population-based adults and is considered a biomarker of NO production [9]. In addition, microvascular function assessed by NIRS has been reported to present a strong association with cardiovascular risk factors [33]. With antioxidant properties, cocoa polyphenols could counteract excessive ROS production, which may increase the NO bioavailability. In this scenario, vascular damage would be mitigated after cocoa supplementation [32].

A previous study from our laboratory has shown reduced FMD response in 12 young participants 30 min after performing six sets of 10 repetitions of eccentric exercise [11]. Choi et al. (2019) [32] also found lower FMD response after eccentric elbow flexion exercise in healthy individuals. Stacy et al. (2013) [7] showed that two sets of 25 repetitions of unilateral maximal eccentric biceps brachii muscle contractions impaired FMD response <5% to <9% 48 h after the exercise in healthy young individuals. In line, Caldwell et al. (2016) [34] observed a <4% to <6% impaired femoral artery FMD response after eccentric knee extensor exercise.

The increased oxidative stress could explain the acute reduction of FMD response observed after eccentric exercise, as shown in the abovementioned studies [7,32]. For instance, Goldfarb et al. (2005) [35] demonstrated that plasma malondialdehyde, an oxidative stress biomarker, increased 48 h after four series of twelve repetitions of elbow flexions. These results suggest that the increase in ROS production induced by eccentric exercise may affect endothelial function since ROS interact with vasodilator molecules, such as NO, potentially generating oxidizing substances, such as the peroxynitrite. These substances can act by decreasing NO bioavailability and increasing oxidative stress in the vascular environment, resulting in impaired endothelial function (FMD response) [36].

In contrast to the abovementioned evidence, the present study did not observe impairment in FMD response and muscle oxygenation parameters (microvascular parameters)
after a single eccentric exercise session. This fact may be related to the protocol utilized in this study, as the macro- and microvascular function was assessed between 24 and 72 h after eccentric exercise instead of assessing minutes after exercise (acute effect). Furthermore, the variety of exercise protocols used in previous studies may be involved in a discrepancy in results [7]. For example, Stacy et al. (2013) [7] used two series of 25 repetitions of elbow flexion, observing an attenuation in the endothelial function. Our protocol consisted of six sets of ten repetitions of elbow flexion, which may explain the lack of a negative effect on macro- and microvascular function in the present study. However, vascular dysfunction has been shown after similar eccentric exercises (six sets of ten repetitions) [11]. In addition, it has been demonstrated that muscle damage may be attenuated after a second of the same exercise protocol [37]. Since this study utilized a crossover design, the participants underwent the same exercise protocol on two occasions, separated by a washout period. However, we have recently demonstrated that the eccentric exercise protocol adopted in this study induced vascular damage in a crossover design [11], suggesting that performing the same exercise protocol does not appear to influence the magnitude of vascular damage.

No significant changes in FMD and muscle StO$_2$ parameters after microencapsulated cocoa supplementation were observed. In contrast, previous studies have demonstrated a positive impact of cocoa ingestion on vascular function [38]. It appears that a similar dose of cocoa flavonoids can induce an increase in vascular function in different populations [38]. The lack of effect of microencapsulated cocoa ingestion in vascular function could be due to the absence of vascular dysfunction induced by eccentric exercise. Previous studies have shown a positive effect of cocoa supplementation in healthy individuals [26]. Thus, independent of the impact of eccentric exercise in generating vascular dysfunction, cocoa ingestion could increase FMD response. In addition, the absence of cocoa supplementation in increasing FMD and muscle StO$_2$ parameters can be associated with the supplementation protocol utilized in this study (vascular function assessment by 24 h after exercise). Thus, multi-day supplementation (>15 days) could induce different findings. However, it has been shown microencapsulated cocoa can attenuate the reduction in FMD caused by eccentric exercise in healthy individuals, suggesting that a single dose can also positively affect FMD response [11].

**Experimental Consideration**

A limitation of this study was that the participants underwent the same exercise protocol on two occasions (crossover design), which could create a repeated bout effect. However, a previous study has reported similar strength losses immediately post-exercise in the initial and repeated bouts [20]. In addition, a previous study used a similar exercise protocol to the one utilized in the present study. This study showed significant biomarkers of muscle damage (reduced muscle force, muscle soreness, and plasma myoglobin) between the bouts of exercise, suggesting that the exercise protocol was sufficient to induce muscle damage [27]. Moreover, it is important to mention that the participants enrolled in the present study were trained in resistance exercise, which may have contributed to the absence of the FMD effect in this study. For example, Stacy et al. (2013) [7] reported that resistance training reduced FMD 1 h after exercise and remained lower for 96 h in nonresistance-trained male subjects.

Additionally, Buchanan et al. (2017) [39] reported that FMD in sedentary individuals was reduced after resistance training, while FMD was unaffected in exercise-trained individuals. It suggests that sedentary individuals may have a more robust impairment in FMD after resistance training. Moreover, the present study did not evaluate the bioavailability of total flavonoids in an in vitro study to determine the quality of total flavonoids and polyphenols. However, we have previously reported that a single dose of 80 mg of cocoa flavonoids in microencapsulated cocoa supplements attenuated vascular dysfunction induced by exercise, suggesting that supplementation with microencapsulated cocoa utilizing a low dosage (80 mg of total polyphenols) positively affects humans [11]. A limitation of our study protocol was that we offered a single dose of microencapsulated cocoa (only on
the first day of the experiment—before exercise protocol) and assessed vascular function over 72 h after exercise protocol. Likely, multi-day supplementation of microencapsulated cocoa over 72 h after exercise could positively impact vascular function since offering cocoa flavonoids more often might increase the pool of flavonoids in plasma, inducing different findings observed in the present study. In addition, administering a higher dose of cocoa flavonoids could also have a different impact on vascular function over 72 h after exercise. Therefore, future studies are warranted to investigate the effect of higher and/or prolonged doses of cocoa flavonoids to better understand if the lack of effect seen in this study was due to the chosen dosage.

5. Conclusions
This study reported that eccentric exercise did not reduce FMD muscle StO$_2$ parameters in trained individuals over 72 h after exercise. In addition, a single dose of microencapsulated cocoa polyphenols did not also affect FMD muscle StO$_2$ parameters. Future studies investigating the impact of eccentric exercise in other populations and the effect of multi-day microencapsulated cocoa polyphenol supplementation are warranted to delineate better the effect of eccentric exercise and microcapsulated cocoa supplementation in trained individuals.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jvd3030019/s1, Figure S1: Data showing flow-mediated dilation (FMD), upslope of oxygen saturation signal during first seconds after cuff deflation (StO$_2$pslope), and the magnitude of StO$_2$ signal after cuff deflation (∆StO$_2$) before (baseline), 24, 48, and 72 h after microencapsulated cocoa (MC) or placebo (PLA).

Author Contributions: G.V.d.O. contributed substantially to FMD and NIRS analysis, data acquisition, statistical analysis, and interpretation. O.J.F.R.J. contributed to the recruitment of the participants. L.V.M.d.S., M.V.-S. and G.V.d.O. wrote the manuscript. T.S.A. contributed to reviewing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Federal University of Rio de Janeiro, Campus Macaé (protocol code 36846720.7.0000.5699 and date of approval in 18 November 2020).

Informed Consent Statement: All participants signed the consent form after being fully informed about the study’s nature and purpose, and all experimental procedures followed the ethical standards of the Declaration of Helsinki. Brazilian Registry of Clinical Trials (ReBEC): RBR-3bc3rn.

Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: The authors have no conflicts of interest that are directly relevant to the content of this manuscript.

References


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