Acute effects of blood flow restriction resistance exercise on endothelial function and platelet aggregation

Efeitos agudos do exercício contrarresistência com restrição de fluxo sanguíneo na função endotelial e agregação plaquetária

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ABSTRACT

Objective: To compare endothelial function and platelet aggregation after resistance exercise performed with low-intensity blood flow restriction (LI-BFR) or free blood flow (LI-FreeBF) and high intensity with no blood flow restriction (HI-FreeBF) in healthy adults. Methods: Ten healthy men (23 ± 3 years) performed three experimental trials involving bilateral leg press and knee extension in a randomized crossover design: LI-BFR (3 x 15 reps at 30% 1 RM), LI-FreeBF (3 x 15 reps at 30% 1 RM), and HI-FreeBF (3 x 8 reps at 80% 1RM). BFR was maintained at 50% of the individual total occlusion pressure during the three sets, and it was released after the end of set 3. Brachial artery flow-mediated dilation (FMD) was measured with ultrasound with Doppler before and after exercise. Blood was collected to determine nitrite levels and platelet aggregation. Results: None of the volunteers reported any adverse reactions during the exercise protocols. A 3 x 2 ANOVA with repeated measures in both factors (condition vs. time) indicated no significant main effects or interactions for FMD, basal and peak brachial artery diameter, and shear rate. Plasma nitrite levels and platelet aggregation did not differ among the three exercise conditions nor pre-post resistance exercise. Conclusion: Our results indicate that lower limbs resistance exercise performed at low or high intensities and with or without BFR does not affect endothelial function, nitrite levels, and platelet aggregation. These findings indicate that such exercise conditions do not seem to represent cardiovascular risk from a hemostatic point of view in healthy adult men.

Keywords: oxyhemoglobin; deoxyhemoglobin; muscle strength; resistance exercise; hemodynamics.

RESUMO

Objetivo: Comparar a função endotelial e a agregação plaquetária após exercícios contrarresistência de baixa intensidade com restrição do fluxo sanguíneo (BI-RFS) ou fluxo sanguíneo livre (BI-FSLivre) e alta intensidade com fluxo sanguíneo livre (AI-FSLivre) em adultos saudáveis. Métodos: Dez homens saudáveis (23 ± 3 anos) realizaram três ensaios experimentais envolvendo leg press bilateral e extensão de joelho em um delineamento cruzado randomizado: BI-RFS (3 x 15 repetições a 30% 1 RM), BI-FreeBF (3 x 15 repetições a 30% 1 RM), e HI-FreeBF (3 x 8 repetições a 80% 1RM). A RFS foi mantida em 50% da pressão de oclusão total individual durante as três séries, e foi liberada após o final da série 3. A dilatação fluxo-mediada da artéria braquial (DILA) foi medida com ultrassom com Doppler antes e após o exercício. O sangue foi coletado para determinar as concentrações de nitrito e a agregação plaquetária. Resultados: Nenhum dos voluntários relatou qualquer reação adversa. A ANOVA 3 x 2 com medidas repetidas em ambos os fatores (condição vs. tempo) não indicou efeitos principais ou interações significativas para DILA, diâmetro basal e de diâmetro máximo da artéria braquial, nem força de cisalhamento. Nitrito plasmático e agregação plaquetária não diferiram entre as três condições de exercício nem pré-pós exercício. Conclusão: O exercício contrarresistência de membros inferiores realizado em baixa ou alta intensidade, com ou sem RFS não afeta a DILA, os níveis de nitrito, nem a agregação plaquetária. Os achados indicam que tais condições de exercício não parecem representar risco cardiovascular do ponto de vista hemostático em adultos saudáveis.

Palavras-chave: oxiemoglobina; desoxiemoglobina; força muscular; treinamento de força; hemodinâmica.
Introduction

Endothelial dysfunction is an important variable involved in cardiovascular morbidities such as atherosclerosis, hypertension, and coronary artery disease [1]. It has been shown that resistance exercise (RE) can improve endothelial function in adults [2]. Nevertheless, despite favorable long-term adaptations to training, a single RE bout can transiently worsen endothelial function and increases platelet aggregation during and soon after an individual takes part in an exercise session [3].

When associated with blood flow restriction, resistance exercise (BFR-RE) has been recognized for its favorable effects on strength and hypertrophy [4]. However, there is scarce evidence available on their effects on vascular health.

Brachial artery flow-mediated dilatation (FMD) serves as an index of nitric oxide (NO)-mediated endothelium-dependent vasodilator function in humans and it is regarded as a surrogate marker of vascular/endothelial function and cardiovascular disease [5].

Few studies have investigated the effects of low-intensity BFR-RE on FMD. Evidence from chronic studies demonstrated improvements [6], impairments [7], and no significant differences in FMD following RE performed under free blood flow (FreeBF) or BFR [8,9]. The only study investigating the acute effects of BFR-RE on endothelial function [10] pointed to a decrease in FMD after a single bout of handgrip exercise under BFR.

An acute increase in blood shear stress and the inherent decrease in oxygen muscle saturation caused by BFR-RE may positively affect endothelial health. Increased production of NO and activation of vascular endothelial growth factor transcription [11,12] stimulates angiogenesis and improves endothelial function. However, exercise can also trigger factors with a negative impact, such as increased platelet aggregation [13].

Studying the balance between the vasoactive substances that favor vascular function and those that can impair it is of paramount importance since an exercise session seems to be able to activate both [10]. Given the scanty body of evidence on BFR-RE protocols involving major muscle groups and real-world protocols, this study’s objective was to compare endothelial function and platelet aggregation after low-intensity BFR-RE, low-intensity FreeBF, and high-intensity resistance exercise in healthy adults.

Methods

Participants

Ten healthy male (23 ± 3 years) undergraduate physical education students participated in this study. Subjects signed a written informed consent form before the experimental procedures. The study was conducted based on the ethical standards in Resolution 510/16 of the Brazilian National Health Council, according to the
recommendations defined in the Declaration of Helsinki for research with human beings, signed at the 59th Assembly of the World Medical Association in 2008. The institutional review board at the State University of Rio de Janeiro approved the study protocol (#3.125.780).

The morphological and health variables of the participants are presented in Table I.

**Study protocol**

The study was conducted in a randomized and crossover-controlled trial model. After the initial two visits, each subject was randomly assigned to all three treatment conditions by drawing with no case reposition.

Subjects reported to the laboratory on five occasions. The first visit was used to explain the experimental procedures, take anthropometric measures and collect data on pre-participation screening (Physical Activity Readiness Questionnaire - PAR-Q, Sheppard, 1988). On the second visit, blood samples were drawn for characterizing the participants’ lipemic profile, and maximal dynamic strength tests (one-repetition maximum - 1RM) in the bilateral leg press and knee extension were recorded.

In the third, fourth, and fifth sessions, participants underwent knee extension and leg press exercise routine under three experimental conditions, in random order separated by three days of wash-out period: 1) low intensity with restricted blood flow (LI-BFR); 2) low intensity with free blood flow (LI-FreeBF), and 3) high intensity with free blood flow (HI-FreeBF). Brachial artery flow-mediated dilation (FMD) was measured by ultrasound before and after exercise in each experimental condition. Blood samples were collected to determine nitrite and platelet aggregation values.

The steps of the experimental procedures are shown in Figure 1.

**Figure 1 - Experimental design**

**Determination of arterial occlusion pressure**

A 17 cm x 68 cm wide nylon cuff (Tyco Welch Allyn DS44-11) was used to identify the artery occlusion pressure (AOP). The participants were asked to lie down in the prone position, with the cuff applied to the proximal-most portion of the left thigh. Then, the 40 mm linear array ultrasound probe (LOGIQe, GE Health Systems,
Minas Gerais, Brazil) was positioned over the popliteal artery in Doppler mode. The cuff was continuously and slowly inflated until the pulse was silent. After silence, the flow was slowly released to detect the pulse onset and inflated again to silence to confirm AOP detection. The average AOP found was 152.0 ± 8.0 mmHg, and the average pressure applied throughout the exercise conditions with BFR was 76.0 ± 4.1 mmHg.

**Maximal dynamic strength tests**

One-repetition maximum tests (1RM) was performed in leg press and knee extension isotonic machines. Participants warmed up with an estimated 50% of 1RM, using the following equation: 1RM = 100 x load / (102.78 – 2.78 x rep) (Nascimento et al., 2007). From the predicted value of 1RM, the participants performed three to five attempts in an incremental fashion, with intervals of 5 min, until the heaviest load that could be successfully lifted once was determined.

The estimated 1RM observed was 167.0 ± 17.9 kg for leg press, and 89.0 ± 18.8 kg for knee extension.

**Resistance exercise**

All subjects were subjected to three experimental conditions at random. The low-intensity condition with blood flow restriction (LI-BFR) and free blood flow (LI-FreeBF) was performed at 30% 1RM. The high-intensity condition with free blood flow (HI-FreeBF) was performed at 80% 1RM in leg press and knee extension exercises.

For the leg press, the applied load was 50.1 ± 5.4 kg for LI-BFR and LI-FreeBF and 133.6 ± 14.3 kg for HI-FreeBF. For knee extension, the correspondent load was 26.7 ± 5.7 kg and 71.2 ± 15.1 kg, respectively.

In the conditions LI-BFR and LI-FreeBF, subjects performed three sets of 15 repetitions. In the HI-FreeBF condition, the individuals performed three sets of eight repetitions, always with a 1-min interval between sets in both exercises.

During BFR conditions, a 17 cm x 68 cm wide nylon cuff (Tycos Welch Allyn DS44-11) was inflated and maintained at 50% of the individual occlusion pressure. The cuff pressure was released only after the completion of the third set.

**Brachial artery hemodynamics**

Brachial artery flow-mediated dilation (FMD) was performed using a two-dimensional color spectral Doppler ultrasound equipped with a 14-MHz linear transducer (Logic e, GE Health Systems, Brazil). Measures were obtained before and immediately after each exercise condition, with the subject lying in the supine position. The probe was placed on the right arm’s anteromedial face perpendicular to the forearm’s centerline, 5-10 cm below the antecubital fossa and over the artery. Basal and post-occlusion diameters were continuously measured between the intima-lumen-intima interfaces. The occlusion was maintained for 5 minutes using a 17 cm x
68 cm nylon cuff (Tycos Welch Allyn DS44-11) on the arm to apply pressure slightly 50 mmHg above the systolic arterial pressure, which was confirmed by the lack of a pulse on the Doppler screen. The procedure was recorded for a total duration of 8 min: 1-min basal, 5-min of blood occlusion, and 2-min after cuff deflation.

The same investigator performed all tests. FMD was calculated as the percentage change in artery diameter after flow release (peak diameter) about the basal diameter.

Brachial artery diameter and shear rate (4 times blood velocity divided by diameter) were calculated: basal diameter and basal shear rate corresponded to the average of the records obtained every second during the first minute preceding the cuff inflation. Peak diameter and peak shear rate were automatically detected as the highest values obtained during the final two minutes of recording after cuff release. Off-line analyses of diameters and shear rate were performed using automated edge-detection software (Cardiovascular Suite, Quipu, Pisa, Italy).

Previous unpublished data from our laboratory showed a high test-test reliability of FMD measurements, with intraclass correlation coefficients of $R = 0.83$ and $R = 0.78$ for intraday and inter-day measurements, respectively. The absolute typical error of measure was 0.8 % and 1.38 %.

**Blood samples**

A trained nurse performed all blood collections in this study. Biochemical analyzes were performed by a blinded investigator, not familiar with the testing procedures.

At baseline, blood samples (5 mL) were obtained by venipuncture after a 12-hour fast. Levels of fasting total cholesterol, high-density lipoprotein cholesterol, low-density-lipoprotein cholesterol, and triglycerides were determined enzymatically with a Roche/Hitachi 917 system (A. F. Hoffmann-La Roche AG, Basel, Switzerland) and standard kits.

Nitric oxide production and platelet aggregation were assayed before the basal FMD procedure and immediately after FMD was performed post-exercise. A 5 mL sample of blood was drawn from the antecubital vein using a sterile needle. For nitrite, blood samples were centrifuged at 4,000 rpm for 5 min to separate the plasma that was stored at -80 °C. The protein levels were quantified by bicinchoninic acid assay (BCA kit, BioAgency, Brazil), and the absorbance was read at 562 nm (TP-Reader, Thermoplate, Brazil). The Griess assay assessed nitrite levels. The samples were mixed with an equal volume (1:1) of Griess reagent (0.1% n-(1-naphthyl) ethylene-diamine dihydrochloride, 1% sulfanilamide, and 2.5% H3PO4). The absorbance was measured at 540 nm using a 96-well microplate reader (TP-Reader, Thermoplate, Thermo Fisher, Waltham, MA, USA).

The platelet aggregation analysis was performed within 2 hours after blood collection. Firstly, platelet-rich plasma was obtained by centrifugation at 200 g for 15 min. Subsequently, the platelet-poor plasma was obtained by centrifugation at 900
for 10 minutes, according to the method of Yun-Choi et al. (2000). Platelet count
was adjusted to 150.000 cells/µL with platelet poor plasma. Platelet aggregation was
quantified according to Born’s technique (1962) in an optical aggregometer (Aggro/
Link Model 810-CA. Chrono-Log, EUA) at a temperature of 37o C, using ADP as ago-
nist (ADP at concentrations of 5.0 uM).

**Statistical analysis**

All data are expressed as mean and standard deviation. Shapiro-Wilk normali-
ty test for small samples was used to verify data departure form normality. A two-way
ANOVA with repeated measures on two factors (3 x 2, condition vs. time) was used
to identify differences in FMD and blood variables between LI-FreeBF, LI-BFR, and
HI-FreeBF conditions. The Bonferroni post-hoc test was used to detect specific diffe-
rences when a significant main effect was found. All analyses were performed using
a commercial software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY:
IBM Corp). The level of statistical significance was set at a P-value < 0.05.

The power of the test was determined based on the F-test statistics family,
using an ANOVA with repeated measures and within-between factors interaction.
Post-hoc analysis identified the study’s power at 0.74. For this result, an effect size of
0.40 was considered, an error α: 0.05, for a sample size of 9 participants (there was one
sample loss for platelet aggregation and other sample loss to nitrite), in three con-
ditions (LI-FreeBF, LI-BFR and HI-FreeBF), two measures repeated over time (pré vs
post treatment), a correlation between repeated measures of 0.8 and non-sphericity
correction of 1.

**Results**

**Participants characteristics**

None of the volunteers reported any adverse reactions during the exercise
protocols. All participants negatively answered the PAR-Q questionnaire.

All variables tested did not show any statistical significance departure from
normality.

**Hemodinâmica da artéria braquial**

Dados ultrassonográficos de qualidade suficiente não foram coletados para
três participantes, e seus dados foram excluídos da análise hemodinâmica final.

Os resultados da ANOVA 3 x 2 para diâmetro basal e de diâmetro de pico da ar-
téria braquial, shear rate basal e shear rate de pico (Tabela II) medidos antes e após os
três protocolos de exercício indicaram que não houve efeitos principais significativos
ou interações condição versus tempo. Não foram encontradas diferenças significati-
vias para efeitos principais ou interação condição versus tempo para DIILA (Figura 2).
Table I - Participants characteristics

| Variables                         | Mean (± standard deviation) |
|-----------------------------------|-----------------------------|
| Age (years)                       | 23 (± 3)                    |
| Body mass (kg)                    | 77.4 (± 13.1)               |
| Stature (cm)                      | 176.4 (± 5.2)               |
| Body fat (%)                      | 9.2 (± 4.4)                 |
| Systolic blood pressure (mmHg)    | 118.1 (± 4.6)               |
| Diastolic blood pressure (mmHg)   | 82.2 (± 6.0)                |
| Heart rate (bpm)                  | 82.7 (± 4.3)                |
| Total cholesterol (mg/dL)         | 140.7 (± 13.6)              |
| High density lipoprotein (mg/dL)  | 50.5 (± 9.9)                |
| Low density lipoprotein (mg/dL)   | 56.8 (± 15.8)               |
| Triglycerides (mg/dL)             | 140.7 (± 13.6)              |

Table II - Brachial artery hemodynamics responses to the three experimental resistance exercise conditions

| Variable                        | LI-BFR                  | LI-FreeBF               | HI-FreeBF               |
|---------------------------------|-------------------------|-------------------------|-------------------------|
| **Basal diameter (mm)**         |                          |                         |                         |
| Pre                             | 4.1 (± 0.5)             | 4.2 (± 0.5)             | 4.0 (± 0.5)             |
| Post                            | 4.1 (± 0.5)             | 4.2 (± 0.5)             | 4.2 (± 0.6)             |
| **Peak diameter (mm)**          |                          |                         |                         |
| Pre                             | 4.5 (± 0.5)             | 4.5 (± 0.5)             | 4.4 (± 0.6)             |
| Post                            | 4.6 (± 0.4)             | 4.5 (± 0.5)             | 4.6 (± 0.7)             |
| **Basal shear rate (s⁻¹)**      |                          |                         |                         |
| Pre                             | 97.1 (± 59.2)           | 134.4 (± 100.2)         | 103.4 (± 32.4)          |
| Post                            | 127.0 (± 79.1)          | 118.7 (± 58.7)          | 121.6 (± 61.5)          |
| **Peak shear rate (s⁻¹)**       |                          |                         |                         |
| Pre                             | 495.4 (± 164.6)         | 564.7 (± 67.5)          | 474.9 (± 104.5)         |
| Post                            | 589.4 (± 148.4)         | 597.6 (± 91.4)          | 492.2 (± 137.5)         |

Data are reported as means ± SD for seven participants; LI-BFR = low-intensity blood flow resistance exercise; LI-FreeBF = low-intensity resistance exercise with free blood flow; HI-FreeBF = high-intensity resistance exercise with free blood flow.

**Platelet aggregation**

Results from 3 x 2 ANOVA showed no statistical differences in platelet aggregation in any of the three exercise conditions at pre-post resistance exercise neither condition x time interactions (Figure 3).
LI-BFR = low intensity with restricted blood flow; LI-FreeBF = low intensity with free blood flow; HI-FreeBF = high intensity with free blood flow; mean and standard deviation. No differences were statistically significant for \( P < 0.05 \)

**Figure 2** - Brachial artery flow-mediated dilation measured at pre- and post-exercise under different experimental conditions

LI-BFR = low intensity with restricted blood flow; LI-FreeBF = low intensity with free blood flow; HI-FreeBF = high intensity with free blood flow; mean and standard deviation. No differences were statistically significant for \( P < 0.05 \)

**Figure 3** - Platelet aggregation measured at pre- and post-exercise under different experimental conditions

Nitrite

No significant main effects or condition x time interactions were observed for nitrite levels. Figure 4 shows the nitrite levels before and after the exercise bouts. The average increment between pre and post-exercise was \( 4.0 \pm 1.9 \) \( 4.0 \pm 1.9 \) \( \mu M \) for LI-BFR, \( 1.1 \pm 0.6 \) \( \mu M \) for an LI-FreeBF, and \( 4.6 \pm 5.1 \) \( \mu M \) for HI-FreeBF.
LI-BFR = low intensity with restricted blood flow; LI-FreeBF = low intensity with free blood flow; HI-FreeBF = high intensity with free blood flow; mean and standard deviation). No differences were statistically significant for P < 0.05.

Figure 4 - Blood nitrite levels measured at pre- and post-exercise under different experimental conditions.

Discussion

The present study was conducted to identify the effects of different acute resistance exercise conditions with and without blood flow restriction on endothelial function, blood nitrite concentrations, and platelet aggregation. To the best of our knowledge, it is the first study to investigate the effects of different acute BFR-RE involving major muscle groups on endothelial function, blood nitrite concentrations, and platelet aggregation. The main finding was that bilateral lower limbs exercises performed under blood flow restriction did not threaten vascular function in healthy males.

Participants of this study had homogeneous physical and metabolic characteristics since anthropometric and lipemic profiles were within the recommended levels for adults [15,16]. Furthermore, none of them reported any discomfort during or after exercising on the three experimental conditions.

Analyzing the peak shear rate (SR) values at a visual inspection, it was possible to observe a higher increase between before and after exercise bouts in the conditions LI-BFR (75.22 ± 203.33 s⁻¹) and HI-FreeBF (60.63 ± 62.24 s⁻¹), compared to LI-FreeBF values (19.78 ± 54.92 s⁻¹). Although no statistically significant differences were observed, numerical values of higher increments sought in the LI-BFR and HI-FreeBF conditions denote the high physical effort, defined by the participants’ difficulty in completing the sets and repetitions proposed under these conditions, which may have influenced the more significant increases in the peak SR in these conditions [17]. As well as the peak SR, the basal SR values underwent higher nume-
ric modifications under LI-BFR and HI-FreeBF conditions than in LI-FreeBF. However, these changes were not statistically significant either.

Tinken et al. [18] demonstrated that when the exercise increases SR, an augment in FMD is expected in response, since the higher the SR, the greater the mechanical action of blood flow on endothelial cells, promoting the release of vasoactive substances that promote vasodilation. Nevertheless, no significant acute increase in FMD was observed in the present study after the three exercise conditions. Probably the fail in identifying significant changes was influenced by the great variability of the data.

Controversially, Paiva et al. [10] studied the effects of FMD values 15 min after a single session of bilateral dynamic handgrip exercise (20 min with 60% of the maximum voluntary contraction, 15 contractions per minute) and reported that the addition of BFR to the protocol blunted the increase in FMD observed after the same exercise without BFR. The authors attributed the findings to the higher oscillatory SR and the production of reactive oxygen species caused by the BFR in the arm exercised under restriction.

However, the present study did not assess shear rate patterns or reactive oxygen species, impairing the interpretation of data. Therefore, our findings are limited to the observation that different intensities of resistance exercise with or without BFR produce similar immediate effects on endothelial function.

Acute periods of augmented retrograde SR have been observed to impair endothelial function [19]. Shear patterns in non-exercising limbs may vary according to the different modalities of lower limb exercise [20]. Concerning resistance exercise effects, Thomas et al. reported that three sets of 10 RM of knee extension caused only a trivial and short-lived increase in antegrade shear rate and no significant change in retrograde flow immediately following exercise [21]. Less is known about RE-BFR, but it is possible to speculate that the exercises protocols tested in the present study should not have notably increased the retrograde SR, and therefore, should not have had harmful effects on FMD.

Concerning platelet aggregation, no deleterious effect was provoked by the application of BFR to resistance exercise. It is known that part of the endothelial dysfunction is related to platelet adhesion, as platelets are activated when there is an injury in the endothelium [22]. Although chronic physical training plays an important role in preventing manifestations of cardiovascular diseases, including atherosclerosis [23], an acute strenuous exercise bout increases platelets activation and aggregation, promoting inflammatory processes [24]. An explication to the attenuation in the inherent increase in platelet aggregation observed in the present study might be attributed to the training status of the participants, all resistance trained. According to Creighton et al. [25], after an acute bout of heavy resistance exercise, platelet activation markers appear to be lower in individuals who are resistance trained.

As stated earlier, plasma levels of nitrite, a suitable and reliable indicator of systemic NO production [26], did not present statistically significant changes in res-
response to the exercise protocols applied. These findings are according to Boeno et al. [27], who developed a study very similar to the present one. They compared the effect of either LI-BFR, LI-FreeBF, and HI-FreeBF resistance exercise (upper and lower limbs) on nitric oxide byproduct levels and antioxidant enzyme activity in healthy young men. They demonstrated that one session of LI-BFR resistance exercise was not capable of modulating plasma NOx levels. However, when compared with the levels in condition HI-FreeBF, these levels were significantly higher after exercise.

It is important to emphasize that NO has antiatherogenic properties and exerts inhibitory actions for platelet adhesion, activation, and aggregation [28]. From a hemostatic point of view, the non-elevation of platelet aggregation associated with maintaining nitrite levels may demonstrate that this method can be safe to be practiced, as it does not increase the risk of vascular thrombus formation.

It is important to note that we used an exercise protocol involving only lower limbs in the present study. However, earlier studies have indicated that vascular responses to exercise may also be evident in arteries not directly feeding the active skeletal muscle, with evident changes in brachial artery FMD following lower limb exercises [29].

It is recognized some limitations in the interpretation of the results of the present study. The hemodynamic responses were examined only immediately after the exercise bout, and observation in the time course of responses would be more appropriate. We did not assess shear rate pattern and reactive oxygen species production, which are essential factors influencing vascular responses. Further studies are needed to elucidate the role of these variables in endothelial function and platelet aggregation.

**Conclusion**

In summary, our results indicate that lower limbs resistance exercise in low or high intensities and with or without BFR as used in the present study could not significantly affect the responses induced in endothelial function, nitrite levels, and platelet aggregation. These findings indicate that such exercise conditions do not seem to represent cardiovascular risk from a hemostatic point of view, at least to healthy adult men.

**Conflict of interest**
All authors declare that there is no conflict of interest regarding this study and manuscript.

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**Author’s contributions**
**Conception of the study:** Gomes PSC, Meirelles CM, Fernandes Junior ML; **Study design:** Meirelles CM, Gomes PSC, Fernandes Junior ML; **Data collection:** Fernandes Junior ML; **Biochemical analysis:**
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