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Acute Citrulline-Malate Oral Supplementation does not Improve Post-Aerobic-Exercise Autonomic Response in Normotensive and Hypertensive Subjects: a Pilot Randomized Controlled Study

A Suplementação Oral Aguda de Citrulina-Malato não Melhora a Resposta Autonômica Pós-Exercício Aeróbico em Indivíduos Normotensos e Hipertensos: um Estudo Piloto Randomizado Controlado

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Abstract

The present study was designed to investigate whether citrulline malate (CM) supplementation might influence post-aerobic-exercise autonomic response in normotensive and hypertensive subjects. Forty individuals (20 normotensives and 20 hypertensives) were randomly assigned to one of the four experimental groups (normotensive-placebo [NP], normotensive-CM [NC], hypertensive-placebo [HP], and hypertensive-CM [HC]). The participants ingested CM (6 g) or placebo dissolved in water (100 ml) 120 min before the exercise session. The exercise session was conducted on a treadmill and consisted of 40 min of running/walking at 60-70% HRreserve. The heart rate variability (HRV) was recorded continuously for 60 min post-exercise. In normotensive subjects at "post-30", LF increased and HF decreased after the CM supplementation (16% [*P*=0.041] and -32% [*P*=0.037], respectively). No significant differences were found in "pre", "post-30" and "post-60" considering the pooled (NP, NC, HP, and HC) z-scores for time and frequency HRV domains. These results suggest that a single dose of CM supplementation does not promote significant effects on post-exercise autonomic modulation in normotensive and hypertensive subjects.

Keywords: Autonomic Nervous System. Citrulline Malate. Hypertension. Exercise.

Resumo

O presente estudo foi desenvolvido para investigar se a suplementação de citrulina malato (CM) pode influenciar a resposta autonômica após exercício aeróbico em indivíduos normotensos e hipertensos. Quarenta indivíduos (20 normotensos e 20 hipertensos) foram aleatoriamente designados para um dos quatro grupos experimentais (normotenso-placebo [NP], normotenso-CM [NC], hipertenso-placebo [HP] e hipertenso-placebo [HP] e hipertenso-CM [HC]). Os participantes ingeriram CM (6 g) ou placebo dissolvido em água (100 ml) 120 minutos antes da sessão de exercícios. A sessão de exercícios foi realizada em esteira e consistiu em 40 minutos de corrida/caminhada a 60-70% da FCres. A variabilidade da frequência cardíaca (VFC) foi registrada continuamente por 60 minutos após o exercício. Nos normotensos "pós-30", houve aumento para LF e redução para HF após a suplementação de CM (16% [P=0,041] e -32% [P=0,037], respectivamente). Não foram encontradas diferenças significativas nos momentos "pré", "pós-30" e "pós-60", considerando os escores-z combinados (NP, NC, HP e HC), tanto para os indicadores de domínio do tempo, quando para os de domínio da frequência. Estes resultados sugerem que uma dose única de suplementação de CM não promove efeitos significativos na modulação autonômica após exercício em indivíduos normotensos e hipertensos.

Palavras-chave: *Sistema Nervoso Autônomo. Citrulina Malato. Hipertensão, Exercício.*

1 Introduction

nlike heart rate (the number of heartbeats per minute), the heart rate variability (HRV) is the fluctuation in the time intervals between adjacent heartbeats[1]. HRV is generated by heart-brain interactions and dynamic non-linear autonomic nervous system process, therefore, it reflects regulation of parasympathetic-sympathetic balance, blood pressure, gas exchange, heart and vascular tone[2].

HRV monitorization following an "exercise session" might provide useful insight into autonomic stress reactivity. This is consonant with the "reactivity hypothesis"[3], which suggests that cardiovascular responses to a stressor may be predictive of certain diseases[4]. It is well documented that, both aerobic and resistance exercise elicit post-exercise modulations in HRV[5,6] and that these modulations are related to the vascular blood flow and blood pressure response[7]. Therefore, vasodilatation physiological response can be related to the HRV modulation.

Some substances, like "L-citrulline" help to trigger vasodilatory responses. The citrulline-malate (CM) is composed by a combination of L-citrulline (a non-essential amino acid that has a key role in the arginine-nitric oxide system, increasing nitric oxide [NO] biodisponibility[8]) and malate (or acid malic) - a salt predominantly found in apples. Despite low CM concentrations can be provided by nutritional

sources in regular food, citrulline availability is mainly produced endogenously through two different pathways: 1) NO co-product (secondary amount) and 2) ornithine carbamylation (principal amount) by metabolites (glutamine, proline, and arginine) in only two cell types (enterocytes and hepatocytes)[9]. The citrulline produced in the liver is all channeled to the urea cycle, thus, small or negligible amounts of citrulline are directed to the circulation[10]. On the other hand, the citrulline produced by enterocytes enters the circulation system, bypasses the liver, and enters the kidneys (and other tissues) for arginine synthesis[11,12]. For this reason, it is suggested that CM supplementation could be an efficient strategy to increase extracellular arginine levels, which is recognized as the NO synthesis precursor[13]. In this line, some studies have indicated that CM supplementation increases plasmatic NO metabolite concentration[14,15], an important peripheral dilate mediator.

Therefore, it is possible that CM supplementation improves post-aerobic-exercise autonomic response in normotensive and hypertensive subjects. To our knowledge, there are no other studies investigating the acute CM supplementation effects on HRV after aerobic exercise. This comprehension could be of clinical relevance since it is known that especially hypertensive subjects exhibit impairments in autonomic modulation. Furthermore, it is important to establish whether high blood pressure can influence the post-exercise autonomic nervous system response to acute CM supplementation, as understanding aspects of these interrelationships are crucial to perform safe non-pharmacological treatment for hypertension. Thus, the present study was designed to investigate whether CM supplementation might influence post-aerobic-exercise autonomic response in normotensive and hypertensive subjects.

2 Material and Methods

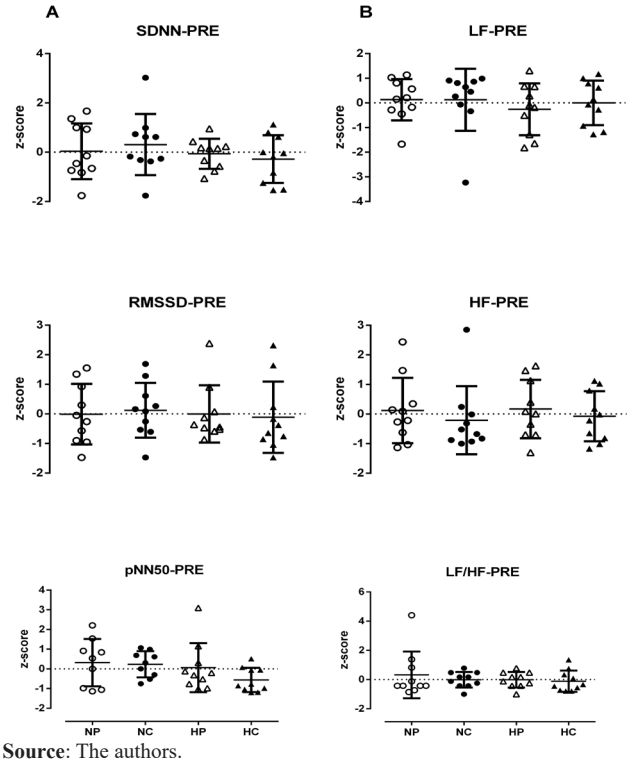
2.1 Participants

After sample size calculation (see statistical analysis session), 40 individuals (20 normotensives and 20 hypertensives), sedentary (less than 150 minutes per week of moderate physical activities) participated in the study. Volunteers were women or men, adults, without osteoarticular disabilities, and with medical authorization to exercise. Participants were recruited from the university community. The study followed the Declaration of Helsinki and was approved by the Institution Ethics Committee (78697617.4.0000.0108). All participants were informed about the methods before providing written informed consent.

2.2 Study design

This was an acute, randomized, parallel-groups clinical trial (Figure 1) to evaluate the effects of a single CM supplementation dose on the post-aerobic-exercise autonomic response in normotensive and hypertensive individuals. The participants were randomly allocated (using a random number table - https://www.random.org/) into four different experimental groups (Normotensive-Placebo [NP]; Normotensive-CM [NC]; Hypertensive-Placebo [HP]; Hypertensive-CM [HC]). Participants were not taking beta-blockers and were asked to refrain from caffeine and alcohol for 24 h before the experimental session and advised not to make changes to their regular lifestyles other than the assigned interventions. Anthropometric measures were taken before the rest period.

Figure 1 - Study design



Source: The authors.

The participants ingested a sachet, which contained 6 grams of CM or placebo (corn starch) dissolved in water (100 ml). The selected CM dose was based on previous studies[16,17]. The substances were ingested 120 min before the exercise session. The exercise was conducted on a treadmill and consisted of: a 5-min warm-up (50-65% HRreserve); 40-min running/walking at 60-70% HRreserve; and a 5-min progressive cooldown. After the exercise session, the HRV was recorded continuously for 60 min. Testing was conducted in the morning at the same time of day 9:00 am (±1 h) in a quiet, temperature-controlled room (23 °C ±1 °C).

2.3 Anthropometry

Weight was measured using a digital anthropometric scale (Urano, OS 180A, Canoas, Brazil), with an accuracy of 0.1kg and height was measured by a stadiometer with an accuracy of 0.1cm, in accordance with the procedures described by Gordon et al.[18]. The body mass index (BMI) was defined as the body mass (kg) divided by the square of the body height.

2.4 Heart rate variability measures

Heart rate variability was monitored during the rest periods (Figure 1) using a cardiac monitor (Polar RS800CX, Kempele, Finland), previously validated[19]. The participants remained seated in a calm, quiet, and thermoneutral (22 °C to 24 °C) environment during monitoring. The recorded R-R intervals were transferred to a computer using specific software (Polar Pro-Trainer software, Kempele, Finland). Fast Fourier Transformation was applied to quantify the low (LF) and high (HF) frequencies into normalized units, in accordance with the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology[20].

The time-domain analysis was obtained by SDNN (standard deviation of the NN interval), RMSSD (the square root of the mean of the squares summing of differences among the adjacent NN intervals), and pNN50 (NN50 count divided by the total number of all NN intervals) indices. The range interval analysis (Figure 1) was 10 min (rest, prior to exercise), and 30 min (post-exercise) using Kubios HRV, version 2.2 (Kuopio, Finland).

2.5 Blood pressure

The BP measurements were taken with an oscillometric device (Omron MX3 Plus, Bannockburn, USA) previously validated for clinical measures in adults[21]. The participants remained seated (rest period - Figure 1) in a calm, quiet, and thermoneutral (22 °C to 24 °C) environment for 20 min. BP was measured three times during the rest period (at 10 min, 15 min, and 20 min). The resting BP value was considered as the average of these three measurements. The BP measurements were taken according to the American Heart Association recommendations[22].

2.6 Statistical analysis

Assuming a standard deviation of 6 normalized units for the LF_{nu}, an alpha of 5%, and a desired statistical power of 95%, detecting a minimum difference of 20 normalized units[23], 6 subjects were required in each group.

Box plots, which provide a spatial representation of the concentration distributions spread, were used to identify anomalous values among the global population of measurements for each HRV indices. In each plot, the box represented the interquartile range and whiskers delineated the region occupied by ±1.5 times the interquartile range beyond the box boundaries. For this study, points plotting above or below the whiskers were identified as potential outliers. Histograms were also examined to confirm that these points were located at distribution extremes for each dataset.

The data are reported as mean and standard deviation. An independent sample t-test was used to compare mean characteristics between normotensive and hypertensive participants. One-way analysis of variance (ANOVA) was used to compare the participants' characteristics among groups (NP, NC, HP, and HC). Fisher multiple comparisons were employed to examine differences between pairs of trials.

To compare the absolute values among the experimental groups, firstly, the sphericity Mauchly's test was applied and the Greenhouse-Geisser correction if necessary. Next, these data were compared with a one-factor repeated measures general linear model. Fisher multiple comparisons were employed to examine the differences between pairs of trials.

Effect size from the paired two-sample t-test was calculated (d=mean/SD) between "pre" vs "post-30" and "post-60" for all HRV indices (d-effects: small ≥ 0.2, medium ≥ 0.50, large ≥ 0.80).

The pooled Z-score for each period ("pre", "post-30" and "post-60") was calculated. One-way analysis of variance (ANOVA) was used to compare the mean z-score among the groups (NP, NC, HP, and HC). Fisher multiple comparisons were employed to examine the differences between pairs of trials.

Statistical significance was defined as P<0.05. The statistical analysis was generated using SPSS (New York, USA), version 20, for windows.

3 Results and Discussion

The participants' characteristics are shown in Table 1. The hypertensive subjects presented higher values for age (28.5±6.6 vs 61.4±17.3 [years] - P<0.001), weight (69.0±10.3 vs 76.9±12.9 [kg] - P=0.040), height (1.68±0.07 vs 1.62±0.11 [m] - P=0.048), body mass index (24.3±2.56 vs 29.3±4.5 [kg/m^2] - P=0.001), waist circumference (79.7±7.4 vs 99.8±9.4 [cm] - P<0.001), systolic (115±12 vs 135±17 [mmHg] - P<0.001), and diastolic (73±7 vs 81±7 [mmHg] – P=0.003) resting blood pressure.

Table 1 – Participants' characteristics

	NP (n=10)		NC (n=10)		HP (n=10)		HC (n=10)		F	*P*
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Age (years)	27.7	7.4	29.9	7.8	52.0	15.3*†	58.6	8.7*†	22.836	<0.001
Weight (kg)	70.9	19.5	76.4	14.4	79.7	17.2	72.5	13.1	0.595	0.622
Height (m)	1.69	0.10	1.71	0.09	1.61	0.09†	1.58	0.09*†	4.560	0.008
BMI (kg/m^2)	24.4	4.6	25.9	3.3	30.8	6.5*†	29.2	5.8	3.124	0.038
WC (cm)	79.3	13.6	84.4	11.2	98.5	14.9*†	99.1	11.2*†	6.081	0.002
SBP (mmHg)	116	17	120	12	137	12*†	142	20*†	6.362	0.001
DBP (mmHg)	72	9	74	8	86	11*†	86	10*†	6.057	0.002

NP= normotensive-placebo; NC= normotensive-citrulline malate; HP= hypertensive-placebo; HC= hypertensive-citrulline malate; SD= standard deviation; BMI= body mass index; WC= waist circumference; SBP= systolic blood pressure; DBP= diastolic blood pressure; *P<0.05 vs NP; †P<0.05 vs NC.
Source: Research data.

Table 2 presents the absolute HRV changes in the different experimental groups. Considering NP, a significant increase in SDNN (25% [post-30]) and decrease in RMSSD (-56% [post-30]) and pNN50 (-85% [post-30] and -55% [post-60]) was identified when compared with the "pre". Additionally, a significant reduction in SDNN (-42% [post-60]) and a significant increase in RMSSD (75% [post-60]) and pNN50 (200% [post-60]) was identified when compared with "post-30". In the NC, a significant reduction in RMSSD (-59% [post-30]) and pNN50 (-84% [post-30] and -53% [post-60]) was identified (versus "pre"). Also, a significant increase in RMSSD (85% [post-60]) and pNN50 (200% [post-60]) was identified when compared with "post-30". Considering HP, a significant increase in LF (27% [post-30] and 23% [post-60]) and LF/HF (96% [post-30]) and decrease in RMSSD (-60% [post-30]) and HF (-45% [post-30] and -37% [post-60]) was identified when compared with the "pre". Additionally, a significant increase in RMSSD (67% [post-60]) and reduction in LF/HF (-29% [post-60]) was identified when compared with "post-30". In HC, a significant increase in SDNN (67% [post-30]), LF (22% [post-60]) and LF/HF (67% [post-30] and 77% [post-60]) and reduction in pNN50 (-89% [post-30) and HF (-45% [post-60]) was identified (versus "pre"). Also, a significant reduction in SDNN (-48% [post-60]) was identified when compared with "post-30". In normotensive subjects at "post-30", LF increased and HF decreased after the CM supplementation (16% [*P*=0.041] and -32% [*P*=0.037], respectively).

Table 2 - Heart rate variability component variations

	Placebo						Citrulline Malate					
	Pre		Post-30		Post-60		Pre		Post-30		Post-60	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Normotensives												
SDNN (ms)	72	40	90	42*	52	9*†	82	44	97	35	63	20
RMSSD (ms)	45	29	20	12*	35	23†	49	27	20	10*	37	11†
pNN50 (%)	20	16	3	4*	9	0*†	19	9	3	3*	9	8*†
LFnu	70	15	70	27	72	26	70	23	81	7#	76	8
HFnu	37	22	28	24	27	23	30	23	19	7#	24	8
LF/HF	4.5	5.3	5.5	3.4	5.3	3.2	3.4	1.8	4.8	2.1	3.8	1.9
Hypertensives												
SDNN (ms)	69	22	117	65	83	62	61	34	102	42*	53	19†
RMSSD (ms)	45	28	18	7*	30	7†	42	35	17	12	21	12
pNN50 (%)	17	16	4	5	14	15	9	8	1	1*	7	12
LFnu	62	19	79	12*	76	9*	67	17	77	17	82	15*
HFnu	38	19	21	12*	24	9*	33	17	23	17	18	15*
LF/HF	2.8	2.9	5.5	.8*	3.9	.5†	3.0	2.4	5.0	.1*	5.3	3.5*

*Significantly different from the pre-intervention (*P*<0.05); †Significantly different from the post-30 (*P*<0.05); #Significantly different from the placebo (*P*<0.05)
Source: Research data.

Table 3 presents the effect sizes from the paired t-test (rest vs post-30 and post-60) for each group. NP showed a large increase (post-30) and decrease effect (post-60) for SDNN. NP also showed a large decrease effect for RMSSD (post-30), pNN50 (post-30 and post-60) and a moderate decrease effect for HF (post-60). Considering NC, a large decrease effect was identified for RMSSD (post-30) and pNN50 (post-30 and post-60). HP showed a large decrease effect for RMSSD (post-30) and HF (post-30 and post-60). HP also showed a large increase effect for LF (post-30 and post-60) and LF/HF (post-30). Considering HC, a large decrease effect was identified for pNN50 (post-30) and HF (post-60). Additionally, a large increase effect was identified for SDNN (post-30), LF (post-60), and LF/HF (post-60).

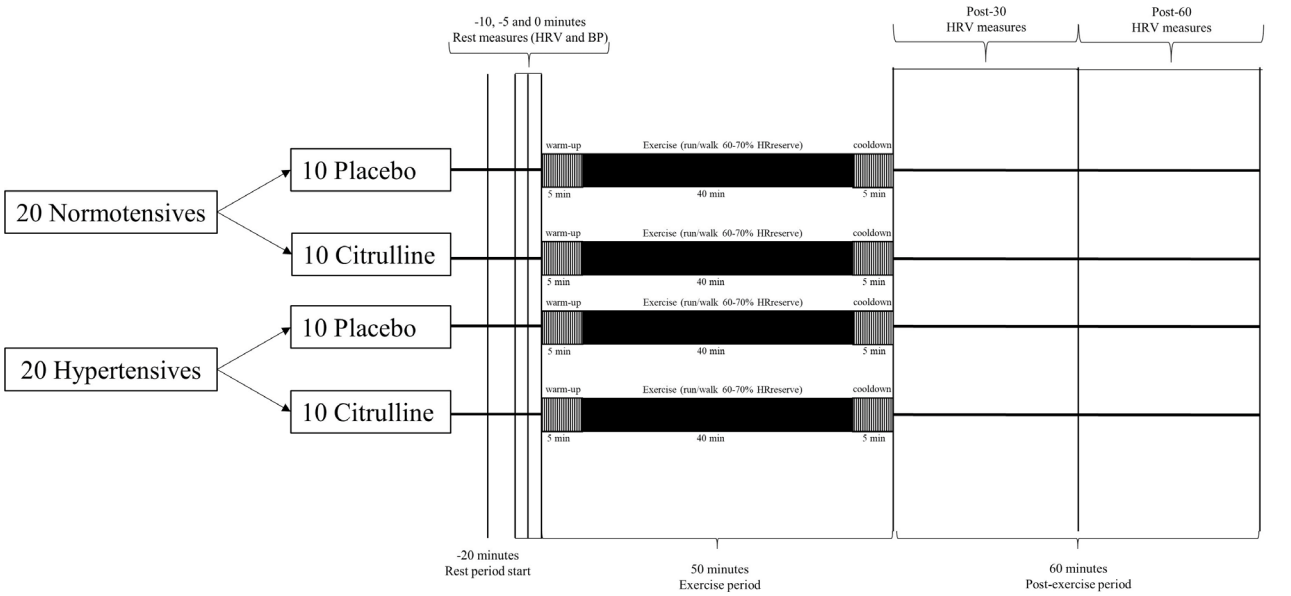
Table 3 - Effect size for Paired t-test (versus pre [d=mean/SD])

	Placebo				Citrulline Malate			
	Post-30		Post-60		Post-30		Post-60	
	ES	*P*	ES	*P*	ES	*P*	ES	*P*
Normotensives								
SDNN (ms)	0.8	0.039	-0.9	0.015	0.3	0.457	-0.5	0.146
RMSSD (ms)	-0.8	0.031	-0.3	0.311	-1.1	0.012	-0.5	0.145
pNN50 (%)	-1.3	0.005	-1.1	0.010	-2.0	0.001	-1.1	0.020
LFnu	0	0.956	0.1	0.804	0.5	0.178	0.3	0.395
HFnu	-0.5	0.164	-0.7	0.047	-0.5	0.178	-0.3	0.405
LF/HF	0.3	0.414	0.3	0.433	0.6	0.118	0.2	0.552
Hypertensives								
SDNN (ms)	0.8	0.052	0.2	0.592	1.1	0.032	-0.8	0.089
RMSSD (ms)	-1.3	0.028	-0.6	0.185	-0.7	0.074	-0.5	0.223
pNN50 (%)	-0.7	0.106	-0.2	0.519	-1.0	0.030	-0.1	0.799
LFnu	1.0	0.014	1.0	0.013	0.6	0.099	1.2	0.004
HFnu	-1.0	0.014	-1.0	0.013	-0.6	0.099	-1.2	0.004
LF/HF	0.8	0.028	0.6	0.072	0.6	0.087	1.0	0.030

ES= Effect size.
Source: Research data.

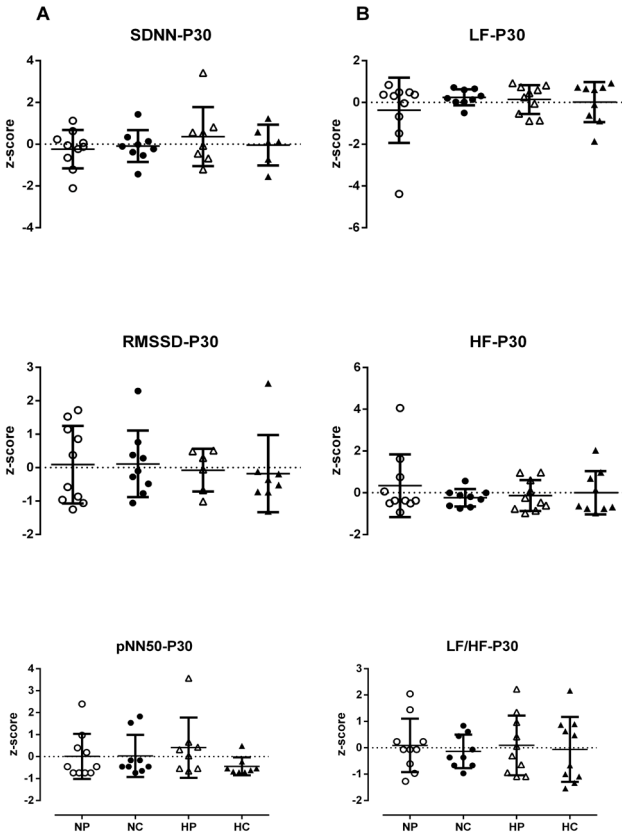
The pooled (NP, NC, HP, and HC) z-scores for time (panel A) and frequency (panel B) HRV domains are presented in Figure 2 (pre), Figure 3 (post-30) and Figure 4 (post-60). No significant differences were found in "pre", "post-30" and "post-60" for all time and frequency components.

Figure 2 - Pooled z-score comparisons at Pre



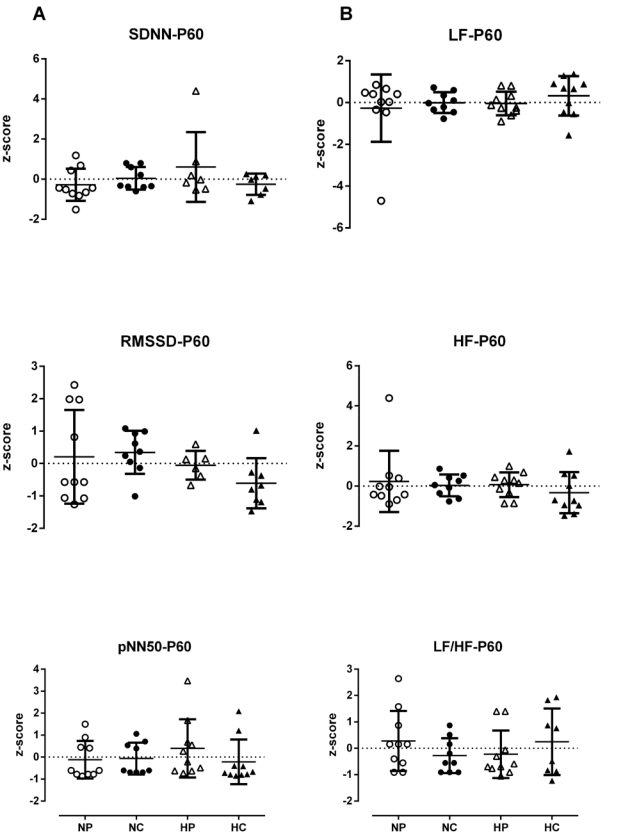
NP= normotensive-placebo (open circles); NC= normotensive-citrulline malato (closed circles); HP= hypertensive-placebo (open triangles); HC= hypertensive-citrulline malate (closed triangles).
Source: Research data.

Figure 3 - Pooled z-score comparisons at P30



NP= normotensive-placebo (open circles); NC= normotensive-citrulline malate (closed circles); HP= hypertensive-placebo (open triangles); HC= hypertensive-citrulline malate (closed triangles).
Source: Research data.

Figure 4 - Pooled z-score comparisons at P60



NP= normotensive-placebo (open circles); NC= normotensive-citrulline malate (closed circles); HP= hypertensive-placebo (open triangles); HC= hypertensive-citrulline malate (closed triangles).
Source: Research data.

The purpose of this study was to analyze the CM acute supplementation responses between normotensive and hypertensive subjects. Our results showed that there were not a considerable CM supplementation acute effects on autonomic activity for both, normotensives and hypertensives. The main finding of this study was that a single CM dose does not seem to be sufficient to induce improvements in sympathovagal balance in normotensive and hypertensive subjects. To the best of our knowledge, this is the first report on acute CM autonomic responses between normotensives and hypertensives considering standardized scores.

In view of the absolute changes, our findings indicate that normotensives are more susceptible to acute sympathovagal modulations after a single CM supplementation dose since only normotensive individuals presented LF increase and HF decrease during the first 30 minutes after half-life period. It is noteworthy that this modulation does not persist in the second period (post-60), revealing a transitory acute sympathovagal modulation. A recent preliminary report[24] demonstrated that eight weeks of L-citrulline supplementation (6 g/day) improved cardiac autonomic function in sedentary postmenopausal women. In this scenario, it is possible to hypothesize that the most striking autonomic modulations can only be revealed with chronic CM supplementation.

There is currently near-universal agreement in the literature that reports of statistical procedures such as null hypothesis significance tests should be accompanied by an appropriate measure of the effect magnitude. Thus, our standardized results (by effect size) showed a similarity with our unstandardized results. Curiously, hypertensive subjects presented HRV modulations in the frequency domain. Perhaps this is related to age since HRV time-domain measurements declined with age[25]. It is important to highlight that hypertensive subjects (regardless of CM supplementation) showed a large increase effect for LF. The LF band (0.04-0.15 Hz) was previously called the baroreceptor range because it mainly reflects baroreceptor activity during resting conditions[1]. LF power may be produced by the parasympathetic and sympathetic nervous system, and blood pressure regulation *via* baroreceptors[20], primarily by the parasympathetic nervous system[26], or by baroreflex activity alone[27]. In resting conditions, the LF band reflects baroreflex activity and not cardiac sympathetic innervation[28]. It is well documented that the cessation of exercise causes a transient reduction in arterial pressure that is referred to as post-exercise hypotension (PEH)[29]. This reduction effect has been observed following aerobic[30] and resistance[31] exercises. Hypertensive subjects present more pronounced PEH than normotensive subjects[31], this fact can explain our results, once baroreflex activity is critically involved in the blood pressure response modulation to exercise[32].

As the heart rate variability indicators are related to several factors such as age[33] and blood pressure status[34], in this study measures were standadized (z-scores) to enable the identification of relative changes in the analyzed HRV components. No significant differences were found for all time and frequency components. It is important to emphasize that mechanisms by which CM could increase the sympathovagal balance are hypothetical. Baroreflex sensitivity is a potential mediator. Chowdary et al.[35] demonstrated an improvement in baroreflex sensitivity after a single dose of L-citrulline supplementation. Considering that the adrenergic stress on the cardiovascular system is influenced by baroreceptor function to sustain appropriate blood pressure[36], it is possible that L-citrulline supplementation can improve baroreflex sensibility and that this effect may be time-dependent.

Despite all methodological care, some aspects should be considered. This was an acute experiment conducted to compare the possible effects of a single CM supplementation dose on post-aerobic exercise autonomic responses in normotensive and hypertensive subjects. However, it should not be assumed that underlying mechanisms for chronic changes in HRV are identical to those of acute responses[37]. Furthermore, interpretation of HRV as reflecting certain aspects of cardiac autonomic activity is complicated due to the fact that rather than being a direct measure of autonomic nerve activity, HRV quantifies the end-organ response modulation, i.e., the heart[38].

4 Conclusion

These results suggest that a single CM supplementation dose does not promote significant effects on post-exercise autonomic modulation in normotensive and hypertensive subjects.

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