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Article

8 Weeks of 2s-Hesperidin Supplementation Improves Power Output at Estimated Functional Threshold Power and Maximum Power in Amateur Cyclist

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ABSTRACT

2S-hesperidin is a flavanone (flavonoid) found in high concentrations in citrus fruits. It has an antioxidant and anti-inflammatory effect, improving performance in animals. This study investigated the effects of chronic intake of an orange extract (2S-hesperidin) or placebo on aerobic-anaerobic and metabolic performance markers in amateur cyclists. A double-blind, randomized, placebo-controlled trial was carried out between late September and December 2018. Forty amateur cyclists were randomized into two groups: one taking 500mg/day 2S-hesperidin and other taking 500 mg/day placebo (microcellulose) for 8 weeks. All participants completed the study. Performance and metabolic aerobic-anaerobic markers were measured using incremental and rectangular tests by indirect calorimetry. The anaerobic power was determined using Wingate tests. After 8 weeks supplementation, there was a significant increase in the incremental test in estimated functional threshold power (FTP) (3.23%; $p \leq 0.05$) and maximum power (2.68%; $p \leq 0.05$) with 2S-Hesperdin compared to placebo. In the rectangular test, there was a significant decrease in VO_2 (-8.26%; $p \leq 0.01$) and VO_2R (-8.88%; $p \leq 0.01$) at VT2 in placebo; however, there were no significant differences between groups. In the Wingate test, there was a significant increase ($p \leq 0.05$) in peak and relative power in both groups, but without significant differences between groups. Supplementation with an orange extract (2S-hesperdin) 500mg/day improves estimated FTP and maximum power performance in amateur cyclists.

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Keywords: flavonoid; polyphenols; orange extract; performance; endurance; aerobic; anaerobic; nutrigenomic; sport nutrition

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1. INTRODUCTION

Hesperidin is a flavonoid found mainly in citrus fruits [1], reaching high concentration in sweet orange (*Citrus sinensis*) [2]. Due to its chemical structure, including a chiral carbon (C-2), Hesperidin can be present as S or R isomer (**Figure 1**). 2S-hesperidin is the predominant natural form in citrus fruits [3], but industrial processing leads to the transformation of the natural S isomer into the R isomer (**Figure 1**) [4]. The bioavailability of the two isomers is different, for instance a 5.2-fold higher efficiency in the glucuronidation has been observed for S-hesperetin compared to R-hesperetin in vitro, without any significant change in the sulfonation kinetics [5]. Clinical trials have demonstrated the therapeutic effects of hesperidin and its metabolites in various diseases (e.g., neurological and psychiatric disorders, cardiovascular diseases, etc.) due to its anti-inflammatory properties, antioxidants, lipid reducers and insulin sensitizers [6-9]. In view of its effects, the pharmaceutical and nutritional industries have extensively marketed hesperidin. However, little attention has been paid to the effects of hesperidin on physical performance.

Insert figure 1

Regarding performance, only one study has investigated the acute effect of 2S-hesperidin in humans. Martínez *et al.* [10] showed that after ingesting one single 500 mg dose of either 2S-hesperidin or placebo (cross-over study) 5 hours before

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the test, trained cyclists significantly improved average power (2.3%), maximum speed (3.2%) and total energy (∑ 4 sprint test) (2.6%) with Cardiose® supplementation in the best sprint of the four repeated sprint test (30 s duration). No significant changes were observed in any of these variables with placebo.

In humans, chronic supplementation of hesperidin has also been studied. Pittaluga *et al.* [11] investigated the effect of 250 ml of red-orange juice (ROJ), which has a high content of hesperidin, on exercise performance (incremental test) in healthy, trained older women. Following 4 weeks of consumption of ROJ (3 per day), these older women significantly increased their work capacity by 9.0% compared to placebo (-1.5%). Another chronic study evaluated the effect of a 4-week supplementation of 2S-hesperidin (500 mg/day) in trained cyclists and observed significant increases in average power output (14.9 W = 5.0%) in a 10 min time-trial test on a cycle ergometer, whereas those that consumed placebo had a non-significant increase in average power output (3.8 W = 1.3%) [12].

The effect of long-term intake of hesperidin has also been investigated in animal studies. Biesemann et al. [13] observed that 6-weeks of hesperetin supplementation (main metabolite of hesperidin) (50 $mg \cdot kg^{-1} \cdot d^{-1}$) improved running performance (exercise time) in aged mice. De Oliveira et al. [14] found that four weeks of hesperidin consumption (100 mg/kg body mass) enhanced the antioxidant capacity in the continuous swimming group (183%) and decreased the lipid

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peroxidation (TBARS) in the interval swimming group (-45%) in rats. This study also found an improvement in endogenous antioxidant enzymes, such as reduced glutathione (GSH), oxidized glutathione (GSSG) and GSH:GSSG ratio. In the same line, a recent study in trained animals reported that intake of hesperidin for 4 weeks improved performance and prevented immune alterations induced by exhausting exercise [15]. Recently, one parallel-group study has shown improvements in the time until exhaustion (58%) on maximal exercise test at 3 weeks of a 5-week chronic supplementation of 2S-hesperidin (200 mg/kg), but not in placebo group [16]. In the same study, it was observed an enhancement of the antioxidant state (superoxide dismutase (SOD), glutathione peroxidase (GPx)) in the lymphoid and hepatic tissue after the test until exhaustion in the rats that consumed 2S-hesperidin.

Another flavonoid, quercetin, has also demonstrated to improve the 5 km running performance time (-11.3% quercetin group; -3.9% control group) after its 14 day supplementation (250 mg/d) by trained triathletes [17]. A systematic review that included 13 randomized controlled trials found that cocoa-derived flavonoid (epicatechinand catechin, and oligomeric procyanidin) supplementation did not affect performance [18]. Thus, there may be some specificity regarding the type of flavonoid that affects physical performance.

The mechanisms by which chronic intake of hesperidin may improve performance

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are associated with increased activation of AMP-activated protein kinase (AMPK) [19,20] and nuclear respiratory factor 2 (NRF2) [6], leading to improved mitochondrial biogenesis and antioxidant status, respectively [21,22]. In addition, hesperidin has the ability to improve nitric oxide synthesis (NO) [23], which may improve glucose utilization in exercise and increase blood flow to the muscles, promoting an increase in nutrient and oxygen delivery to the muscle [24]. More detailed human studies are needed to determine precisely what molecular mechanisms explain the effects of hesperidin.

It has been hypothesized that some molecules with anti-inflammatory and antioxidant activity may interfere with exercise-generated adaptations causing a decline in performance when ingested chronically [25]. However, there is controversy on this issue, since supplementation of polyphenols, such as quercetin, have been shown to improve performance [26]. To solve this question, future studies on polyphenols (specifically flavonoids) are needed to clarify which pathways or receptors are activated to help explain the possible or lack of improvements in performance.

Based on the understanding behind the mechanism of hesperidin in vitro, as well as the scientific evidence presented above, hesperidin is a good candidate for improving performance. Hesperidin strongly increases intracellular ATP compared to the AMPK activator 5-Aminoimidazole-4-carboxamide

 doi:10.20944/preprints202009.0341.v1

ribonucleotide (AICAR), even when AICAR concentration has been increased by 10-fold (100 μM) [13]. In addition, hesperetin (10 μM) has been shown to increase intracellular ATP by 33% and mitochondrial spare capacity by 25%, as well as establish an antioxidant state.

Much of the aforementioned investigations have used maximal exercise intensities and acute intake protocols, and little is known about how supplementation of 2S-hesperidin affects submaximal and maximal exercise intensities with long-term consumption. We hypothesised that chronic intake of 2S-hesperidin would improve performance at submaximal and maximal exercise intensities. Therefore, the aim of this study was to examine the chronic effects of 2S-hesperidin (500 mg, Cardiose®) supplementation on performance (generated power) in an incremental test (high aerobic component) at FatMax, ventilatory threshold 1 and 2 (VT1 and VT2) and at power maximum, and in a Wingate test (high anaerobic component). The secondary objective was to evaluate whether hesperidin supplementation modified metabolic (O_2 and CO_2) and energy substrate (carbohydrates and fats) markers during a rectangular test that could explain a possible enhancement in performance.

2. METHODOLOGY

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2.1 Participants

Forty healthy, male amateur cyclists participated and completed the study (**Table 1**). All the participants had to meet the following inclusion criteria: 18-55 years, BMI of 19-25.5 $kg \cdot m^{-2}$, at least 3 years of cycling experience and training for 6-12 $h \cdot wk^{-1}$. Volunteers were excluded if they: a) were smokers or regular alcohol drinkers, b) had a metabolic, cardiorespiratory or digestive pathology or anomaly, c) had an injury in the prior 6 months, d) were supplementing or medicating in the prior 2 weeks and/or e) had non-normal values in the blood analysis parameters. First, participants were informed about the procedures, and a signed informed consent was obtained. The study was conducted according to the guidelines of the Helsinki Declaration for Human Research [27] and was approved by the University's Ethics Committee.

Table 1. Baseline general characteristics and training variables of participants.

	2S-Hesperidin	Placebo	p-value
Age (years)	35.0 (9.20)	32.6 (8.90)	0.407
Body mass (kg)	71.0 (6.98)	70.4 (6.06)	0.773
Height (cm)	175.3 (6.20)	176.5 (6.10)	0.541
BMI ($kg \cdot m^{-2}$)	23.1 (1.53)	22.6 (1.43)	0.292
BF (%)	8.9 (1.63)	9.0 (1.64)	0.803
VO_{2MAX} ($L \cdot min^{-1}$)	3.99 (0.36)	3.98 (0.63)	0.971
VO_{2MAX} ($mL \cdot kg^{-1} \cdot min^{-1}$)	57.5 (6.97)	57.9 (9.53)	0.880
HR_{MAX} (bpm)	184.9 (11.11)	183.2 (8.68)	0.593
VT1 (%)	50.9 (5.63)	50.0 (4.78)	0.610
VT2 (%)	84.9 (5.85)	84.1 (5.70)	0.644
Training variables	**2S-Hesperidin**	**Placebo**	**p-value**
Total distance (km)	1121.12 (534.99)	1082.43 (810.46)	0.868

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HR_{AVG} (bpm)	144.76 (8.88)	137.48 (13.11)	0.067
W_{AVG} (W)	174.86 (15.79)	163.47 (32.49)	0.435
RPE	6.34 (0.82)	6.33 (1.16)	0.975

Values are expressed as mean (SD). BMI = body mass index; BF = body fat; $VO_{2máx}$ = maximum oxygen volume; VT1 = ventilatory threshold 1 (aerobic); VT2 = ventilatory threshold 2 (anaerobic); Total distance = of all the training sessions carried out during the study period; HRavg = average heart rate of all the training sessions carried out during the study period; Wavg = average power output of all training sessions during the study period.

175

176

177 **2.2 Study design**

178 A double-blind, parallel and randomized experimental design was performed.
179 Participants were divided into two groups: experimental (2S-hesperidin; n=20)
180 and control (Placebo; n=20). Total distance of usual training was balanced to make
181 it similar between groups (**Table 1**). Participants consumed two capsules at the
182 same time of either 2S-hesperidin (500 mg) (Cardiose®, produced by HTBA
183 (HealthTech BioActives – Murcia, Spain)) or placebo (microcellulose) for 8 weeks.
184 Specifically, Cardiose® is a natural orange extract that, due to its unique
185 manufacturing process, maintains most of the natural hesperidin isomeric form
186 (NLT 85% 2S-Hesperidin). Cyclists were instructed to take the supplement along
187 with breakfast and to continue their usual diet and training schedule. Subjects in
188 both groups were instructed not to consume foods high in citrus flavonoids
189 (grapefruit, lemons or oranges) for 5 days prior to and during the study, this was
190 verified by diet recalls records.

191 **2.3 Procedures**

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192 Participants visited the laboratory on seven occasions. Visit 1 consisted of a
193 medical examination and blood extraction to determine health status. When urine
194 samples were collected on visit 2 in the fasted state, both groups consumed the
195 supplements under the supervision of an investigator, which was followed by a
196 standardized breakfast. On visits 2 and 5, a 24-hr diet recall and a Wingate test
197 were performed. On visits 3 and 6, another 24-hour diet recall was conducted,
198 followed by an incremental test until exhaustion on a cycle ergometer. On visits 4
199 and 7, the 24-hour diet recall was repeated, and participants performed a
200 rectangular test on the cycle ergometer (**Figure 2** and **Tables 2**). Prior to each
201 testing session (visits 2, 3, 4, 5, 6 and 7), a standardized breakfast composed of 95.16
202 g of carbohydrates (68%), 18.86 g of protein (14%) and 11.30 g of lipids (18%) was
203 prescribed by the sport nutritionist.

204 ***Insert figure 2***

Table 2. Between-group comparisons in dietary intake of cyclists.

	Pre-intervention			Post-intervention		
	2S-Hesperidin	**Placebo**	**p-value**	**2S-Hesperidin**	**Placebo**	**p-value**
Kilocalories	2163.60 (519.02)	2100.18 (515.77)	0.708	1974.09 (377.97)	2133.51 (437.98)	0.237
Carbohydrates (g)	245.72 (73.46)	221.93 (69.68)	0.312	216.58 (63.47)	248.26 (58.15)	0.117
Protein (g)	113.50 (25.21)	115.20 (25.37)	0.837	108.97 (23.05)	101.52 (23.67)	0.332
Lipids (g)	80.75 (27.24)	83.52 (23.65)	0.739	71.48 (17.61)	71.59 (18.89)	0.985

Values are expressed as mean (SD). The mean values correspond to the average of all 24-hour diet recall data collected at pre-intervention (visits 2, 3 and 4) and post-intervention (visits 5, 6 and 7). * indicates significant differences ($p \leq 0.05$).

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206 **2.3 Testing**

207 ***2.3.1 Medical exam***

208 A medical examination, performed by the research centre's medical doctor and
209 including health history, resting electrocardiogram and examination
210 (auscultation, blood pressure, etc.), was used to confirm that the volunteer was
211 healthy enough to be enrolled in the study.

212 ***2.3.2 Maximal test***

213 Incremental step with final ramp test was performed on a cycle ergometer using a
214 metabolic cart (Metalyzer 3B. Leipzig, Germany) to determine maximal fat
215 oxidation zone (FatMax), VT1 and VT2 and maximal oxygen consumption
216 (VO_{2max}). Participants began cycling at 35W for 2 min, increasing then by 35W
217 every 2 min until RER>1.05, initialling then the final ramp ($+35W \cdot min^{-1}$) until
218 exhaustion. To ensure VO_{2max}, at least 2 of the following criteria had to be achieved:
219 plateau in the final VO_2 values (increase ≤ 2.0 $ml \cdot kg^{-1} \cdot min^{-1}$ in the 2 last loads),
220 reaching maximal theoretical HR (220 – age)·0.95), RER ≥ 1.15 and lactate ≥ 8.0
221 $mmol \cdot l^{-1}$. VT1 was determined using the criteria of an increase in $VE \cdot VO_2^{-1}$ (VE =
222 pulmonary ventilation) without further increase in $VE \cdot VCO_2^{-1}$ and departure from
223 the linearity of VE, whereas VT2 corresponded to an increase in both $VE \cdot VO_2^{-1}$ and
224 VE/VCO_2^{-1} [28,29]. All VT1 and VT2 assessments were made by visual inspection of
225 graphs in which were time-plotted against each relevant respiratory variable
226 measured during testing. Ventilatory thresholds were obtained using the

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ventilatory equivalents method described by Wasserman [30]. FTP was defined as the highest average power output (PO) that can be maintained for 1 hour [31]. The estimated functional threshold power (FTP) was calculated using the following equation [32]:

$$FTP\ (W) = Pmax\ (W) \times 0.865 - 56.484$$

2.3.3 Rectangular test

Rectangular test was performed on a cycle ergometer using the power output values resulting from the maximal test (FatMax, VT1 and VT2). Participants exercised continuously from FatMax to VT1 and to VT2 for 10 min without rest. Cardiorespiratory variables (VO_2, VO_2R, carbohydrate oxidation (CHO), fat oxidation (FAT) and cycling economy) were determined for each metabolic zones.

2.3.4 Wingate test

Wingate test (WAnT) consisted of an all-out, 30-s sprint on a cycloergometer (Monark Ergomedic 894E Peak Bike, Vansbro, Sweden). Breaking resistance was held constant at 7.5% of each individual's body mass [33]. All participants were verbally encouraged to pedal as fast as possible during the entire sprint. Absolute and relative (i.e., to body mass) peak power and anaerobic capacity were calculated.

2.3.5 Blood samples

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For blood analytics, two samples were taken; one in 3-mL tube with ethylenediaminetetraacetic acid (EDTA) and another in 3.5-mL tube with polyethene terephthalate (PET). Red blood cell count was carried out in an automated Cell-Dyn 3700 analyser (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, haemoglobin, haematocrit and haematimetry indexes were determined. These data were used to verify the health status of the subjects and were not included in the study.

2.3.6 Urine samples

Main hesperidin metabolites were analysed in participants' urine. Urine samples, corresponding to the collection of urine 24 h before (V2) and after (V7) the supplementation in both groups for each participant, were frozen in liquid nitrogen after collection and thawed for its analysis. For analysis, 50 µL of urine were mixed with 100 µL of water with 1% formic acid containing the internal standard. Then, the mixture was injected into LC-MS/MS (UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 Series Agilent Technologies, Sta. Clara, CA, USA). Metabolites were quantified by external standard calibration, using rac-Hesperetin-d3 as the internal standard (Supplementary material).

2.4 Statistical analysis

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Statistical analysis was carried out using IBM Social Sciences software (SPSS, v.21.0, Chicago, IL, USA). Data are presented as mean ± SD. Levene and Shapiro-Wilks tests were performed in order to check for homogeneity and normality of the data, respectively. Depending on the normality and homogeneity outcomes obtained, paired T-test or Wilcoxon signed-rank test were carried out to examine within-group pre-post differences. Likewise, between-group comparison was calculated using ANCOVA test or Mann–Whitney U test, using pre-test values as covariates (to eliminate any possible bias possibility caused by the initial level of each group in the different dependent variables). Furthermore, the rectangular test data analysis was done using repeated measures T-test to obtain within-group differences when comparing the different time points. Relationships between levels of excreted hesperidin metabolites in urine and other evaluated parameters were analysed using Pearson correlation analysis (r). Significance level was set at $p \leq 0.05$.

3. RESULTS

3.1 Hesperidin metabolites urine

Different hesperidin metabolites, mainly hesperetin glucuronides and sulfates, were analysed in the urine of the participants after Cardiose® intake. The main metabolite detected was hesperetin-3-glucuronide, representing 78.9±5.0% (n=20) of the total, while hesperetin-7-glucuronide and hesperetin-7-sulfate made up

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6.9±2.9% (n=20) and 14.7±4.1% (n=20) of the excreted metabolites. Despite the similarities in the excreted metabolites profile, a large interindividual variability was observed in the excreted amount, with hesperidin metabolites ranging from 2.3 to 37.5 μmol. These differences between subjects indicate differences in the absorption and excretion of hesperidin, which have been previously reported [34].

3.2 Maximal test on a cycle ergometer

Figure 3 shows the pre- and post-intervention values and changes in VT1 and VT2 power, estimated FTP and maximum power achieved during the maximal test.

At VT1 there was no significant differences in pre-post power neither in 2S-hesperidin group (-3.72% = -6.00 W; p=0.437) nor in Placebo group (3.42% = 5.25 W; p=0.453), without significant differences in VT1 power changes between groups (p=0.423). At VT2, there was a non-significant pre-post decrease in power output in Placebo (-3.11% = -8.90 W; p=0.264), and no significant changes were observed in 2S-hesperidin group (1.04% = 2.90 W; p=0.642). Comparison between groups showed no significant changes (p=0.299).

Interestingly, 2S-hesperidin group significantly increased pre-post maximum power (1.93% = 7.40 W; p=0.049) and estimated FTP (2.33% = 6.40 W; p=0.049). In contrast, Placebo group showed no significant changes in estimated FTP (-0.90 % = -2.51 W; p=0.387) and maximum power (-0.75% = -2.90 W; p=0.388) during the intervention. When comparing changes between groups, there was a significant

 doi:10.20944/preprints202009.0341.v1

increase in estimated FTP (3.23% = 8.91 W; p=0.042) and maximum power (2.68% = 10.32 W; p=0.042) in 2S-hesperidin group versus placebo.

Additionally, there was a positive significant correlation between the levels of excreted hesperidin metabolites in urine and the difference in maximum power (r=0.701; p<0.001) and estimated FTP (r=0.725; p<0.001) in the supplemented group.

Insert figure 3

3.3 Rectangular test on a cycle ergometer

At FatMax, there was a significant pre-post decrease in fat oxidation (FAT) (p=0.007) and efficiency (p=0.010) in Placebo group, whereas the 2S-hesperidin supplemented group showed no changes in evaluated parameters (**Table 3**). No significant differences were found for between-group comparisons.

At VT1, there was a significant increase pre-post in carbohydrate oxidation (CHO) (p=0.020) and a significant decrease pre-post in fat oxidation (p=0.003) in Placebo group, but no changes were observed in 2S-hesperidin (**Table 3**). No significant changes were found betweengroups.

After the supplementation period, there was a significant decrease in VO_2 (-8.26%; p=0.002) and VO_2R (-8.88%; p=0.002) at VT2 in Placebo group, in contrast to 2S-

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328 hesperidin, which showed no significant changes (**Table 3**). Between-group
329 comparison showed a trend to a decrease (p=0.074) in VO_2R for placebo versus 2S-
330 hesperidin group.

Table 3. Changes in metabolism, energy substrate, energy and energy efficiency in FatMax, ventilatory threshold 1 (VT1) and ventilatory threshold 2 (VT2) during the rectangular test.

	2S-Hesperidin			Placebo		
	Pre-intervention	Post-intervention	p-value	Pre-intervention	Post-intervention	p-value
			FatMax			
VO_2 ($L \cdot min^{-1}$)	2.23 (0.50)	2.02 (0.37)	0.063	2.27 (0.48)	2.10 (0.57)	0.151
VO_2R ($ml \cdot kg^{-1} \cdot min^{-1}$)	31.45 (6.17)	28.54 (5.43)	0.060	32.40 (6.82)	29.51 (6.99)	0.100
CHO ($g \cdot min^{-1}$)	2.20 (0.58)	2.01 (0.37)	0.169	2.20 (0.50)	2.27 (0.56)	0.521
FAT ($g \cdot min^{-1}$)	0.29 (0.90)	0.26 (0.14)	0.247	0.32 (0.14)	0.21 (0.14)	0.007
Efficiency (%)	26.68 (2.95)	26.05 (3.90)	0.411	26.94 (2.79)	24.62 (2.27)	0.010
			VT1			
VO_2 ($L \cdot min^{-1}$)	2.19 (0.39)	2.10 (0.35)	0.396	2.10 (0.41)	2.09 (0.47)	0.961
VO_2R ($ml \cdot kg^{-1} \cdot min^{-1}$)	31.05 (5.34)	29.62 (5.20)	0.357	29.96 (5.84)	29.64 (6.37)	0.824
CHO ($g \cdot min^{-1}$)	2.08 (0.47)	2.07 (0.30)	0.974	1.86 (0.47)	2.19 (0.49)	0.020
FAT ($g \cdot min^{-1}$)	0.31 (0.10)	0.27 (0.15)	0.184	0.35 (0.12)	0.23 (0.14)	0.003
Efficiency (%)	26.55 (2.62)	25.25 (5.38)	0.250	27.49 (3.25)	25.86 (5.85)	0.282
			VT2			
VO_2 ($L \cdot min^{-1}$)	3.49 (0.43)	3.36 (0.41)	0.135	3.63 (0.52)	3.33 (0.54)	0.002
VO_2R ($ml \cdot kg^{-1} \cdot min^{-1}$)	49.48 (6.83)	48.25 (6.84)	0.211	51.90 (8.17)	47.29 (7.76)	0.002
CHO ($g \cdot min^{-1}$)	5.11 (1.18)	5.42 (1.37)	0.349	5.53 (1.45)	5.25 (1.13)	0.369
FAT ($g \cdot min^{-1}$)	0.04 (0.08)	0.04 (0.09)	1.000	0.02 (0.06)	0.01 (0.03)	0.334
Efficiency (%)	20.58 (3.09)	19.65 (3.37)	0.272	20.15 (2.25)	20.20 (4.30)	0.965

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Values are mean (SE). VO2 = volume of oxygen uptake; VO2R = body mass oxygen consumption; FatMax = intensity at which maximum fat oxidation is given; VT1 = ventilatory threshold 1 (aerobic); VT2 = ventilatory threshold 2 (anaerobic); CHO = carbohydrate oxidation; FAT = fat oxidation; efficiency = percentage.

3.4 Wingate test

Table 4 shows the results of the parameters evaluated during the Wingate test prior and after supplementation, which are also summarized in **Figure 4**.

In the 2S-hesperidin group, there were significant increases in absolute (4.9% = 35.48 W; p=0.001) and relative (4.3% = 0.44 W·kg-1; p=0.004) initial power (first five seconds of the test), as well as in absolute (6.1% = 49.78 W; p<0.001) and relative (5.6% = 0.64 W·kg-1; p=0.001) peak power. Also, there was a trend to an increased power at maximum speed (4.4% = 33.99 W; p=0.051) and a descending trend in time at peak power (-18.1% = -641.2 ms; p=0.052) after the supplementation with 2S-hesperidin. Non-significant changes were observed in time at maximum speed.

Placebo group showed a significant increase in absolute (6.1% = 47.18 W; p=0.016) and relative peak power (5.6% = 0.64 $W \cdot kg^{-1}$; p=0.014), and a significant decrease in time at maximum speed (-13.2% = -929.2 ms; p=0.001). Non-significant changes were observed in absolute and relative initial power, power at maximum speed and time at peak power for placebo.

Between-group comparison only reported a trend to decrease in time at maximum speed (-12.5% = -878.35 ms; p=0.059) in Placebo compared with 2S-hesperidin.

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Table 4. Changes in performance parameters in the Wingate test.

	2S-Hesperidin			Placebo		
	Pre-intervention	**Post-intervention**	**p-value**	**Pre-intervention**	**Post-intervention**	**p-value**
Initial power absolute (W)	718.78 (143.05)	754.26 (143.09)	0.001*	712.50 (103.46)	742.96 (101.78)	0.084
Initial power relative (W)	10.16 (1.82)	10.59 (1.78)	0.004*	10.13 (1.38)	10.56 (1.29)	0.078
Absolute peak power (W)	810.83 (160.26)	860.61 (170.37)	>0.001*	792.04 (100.96)	840.23 (118.93)	0.016*
Relative peak power (W)	11.46 (2.04)	12.10 (2.27)	0.001*	11.29 (1.37)	11.93 (1.49)	0.014*
Power at maximum speed (W)	759.95 (156.45)	793.53 (132.23)	0.051¤	746.29 (110.30)	754.34 (96.14)	0.709
Time at peak power (ms)	3541.40 (1722.52)	2900.20 (923.99)	0.052 ¤	3193.40 (1218.48)	2816.90 (1013.54)	0.138
Time at maximum speed (ms)	7208.65 (1098.24)	7157.85 (2005.11)	0.888	7024.35 (1347.65)	6095.20 (957.33)	0.001*

Values are mean (SE). *Within-group significant changes (p≤0.05) ¤Within-group trend to significant changes (p=0.05-0.010)

****Insert Figure 4****

4. DISCUSSION

The main objective of this study was to evaluate the effects of chronic intake of 2S-hesperidin on aerobic and anaerobic performance in amateur cyclists. For this purpose, participants were supplemented for 8-weeks with 500 mg Cardiose®, a natural extract of sweet orange (*Citrus sinensis*) which contains hesperidin in its natural 2S form (NLT 85% 2S-Hesperidin). Following the 8-week intervention, 2S-

 doi:10.20944/preprints202009.0341.v1

hesperidin supplementation led to significant improvements in submaximal and maximal intensity exercise performance in the incremental tests versus placebo. There was a significant decrease in VO_2R at VT2 in placebo, but not in 2S-hesperidin, in the rectangular test. In addition, a decrease in time to peak power and an increase in power at maximum speed in the Wingate test were observed in 2S-hesperidin. Thus, Cardiose® does have a positive impact in the performance of amateur cyclists.

The bioavailability of hesperidin is a factor that must be taken into account when examining its effectiveness, since the average maximum peak blood plasma concentration occurs after 5-7 hours of its ingestion and is almost eliminated post-24h [35]. However, the excreted metabolites in urine has been shown to reach at maximum levels at post-24 h with continued remnants after 48 h [35]. It is interesting to mention that the area under the curve was more than doubled (0.5L orange juice; 4.19μmol h/l vs 1l orange juice; 9.28 μmol h/l) at 24 h when high doses of hesperidin were consumed (1l orange juice = 444 mg hesperidin) [35]. This indicates that high doses increase exposure to the body of 2S-hesperidin metabolites than low doses (222 mg/l). The dose that the cyclists in our study consumed was equivalent to more than one liter of orange juice, with the high carbohydrate load that it entails. The metabolites of hesperidin that appear mainly in the blood are glucuronides (87%) and sulfoglucuronides (13%) [35]. These results are very similar to those found in this study.

 doi:10.20944/preprints202009.0341.v1

Another key factor in the metabolism and absorption of 2S-hesperidin is the intestinal microbiota. In particular, Amaretti et al. [36] established that the species *Bifidobacterium catenulatum* and *Bifidobacterium pseudocatenultum* had the ability to hydrolyze hesperidin, because in their genome they have the gene encoding for the enzyme α-L-rhamnose (limiting enzyme), which contributes to the release of aglycone from certain routine-conjugated polyphenols, such as hesperidin. A recent study suggests that the contradictory finding regarding the intake of hesperidin in humans may be due, in part, to the interindividual variability in its bioavailability, which highly depends on the α-rhamnosidase activity and the composition of the gut microbiota [37]. On the other hand, hesperidin has shown to have a probiotic effect by promoting the growth of some beneficial bacterial species in the colon, the key role being the production of short-chain fatty acids (SCFA) (*Bifidobacterium spp.*, *Lactobacillus spp.*, or *Akkermansia muciniphila*) [37]. SCFA are absorbed with healthy effects on the permeability of the intestinal barrier and the distal organs and tissues. In addition, hesperidin has the ability to inhibit the growth of harmful bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Prevotella spp.*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, among others [37]. SCFA are key mediators of mitochondria energy metabolism and act as ligands for free fatty acid receptors 2 and 3 (FFAR2, FFAR3) that regulate glucose and fatty acid metabolism, sirtuin 1(SIRT1), which plays a role in mitochondrial biogenesis via PGC-1α deacetylation [38]. Therefore, the intake of 2S-hesperidin could improve performance through a prebiotic effect modulating intestinal

 doi:10.20944/preprints202009.0341.v1

microbiota by modulating the production of SCFA that interacts with transcription factors and genes.

It is well known that having high VO_{2max} (74 ml·kg^{-1}·min^{-1}) is a key factor for high-level mountain and road cyclists [39]. Another important factor in cyclists' performance is their ability to produce high levels of power. Hawley *et al.* [40] reported high correlations between maximum power output (PPO)-VO_{2max} ($r=0.97$, $p<0.0001$) and PPO and 20-km (TT) cycle time ($r=-0.91$, $p<0.001$) in trained cyclists. Similar correlations were found between VO_{2max} and FTP when testing untrained recreational cyclists and moderately trained cyclists [32]. Therefore, the improvement of VO_{2max} and FTP would indicate an increase in performance. Regarding flavonoid supplementation, a previous study reported a 5% increase in absolute power output in a 10-min time trial (TT) after 4 weeks of 2S-hesperidin intake (500 mg) in cyclists [12]. These findings are in line with our results where we found performance improvements in eFTP and maximum power after 2S-hesperidin intake, with positive correlations with the excretion of metabolites in urine. Therefore, an increase in power production at high intensity is a key factor in cycling performance. In fact, some authors have indicated that the main factor that differentiates high-level cyclists from the rest of the cyclists is their power production capacity versus their VO_{2max} [41]. From our findings, an improvement in FTP and peak power output after chronic intake of 2S-hesperidin would improve the performance of endurance athletes for competition. Our hypothesis

 doi:10.20944/preprints202009.0341.v1

is that chronic intake of 2S-hesperidin could help generate or maintain adaptations at the mitochondrial level and of the endogenous antioxidant system in a period where the volume and intensity of training is decreasing, as in the study we conducted (late September-mid December). Therefore, the placebo group would have decreased their performance in maximum power and FTP due to the loss of adaptations achieved during the cycling post-season.

In addition, performance improvements have also been seen in animals following chronic intake of hesperidin and hesperetin (hesperidin metabolite) [13,15,16]. Biesemann et al. [13] found that old mice taking hesperetin for 8 weeks (50 mg/kg/d) maintained performance is a test until exhaustion, but mice taking placebo declined by about 100 s from baseline. This indicates an anti-aging effect, supported by improved muscle fiber. Biesemann et al. [13] prior to the study presented above, performed a screening of possible molecules that significantly increased oxygen consumption and ATP levels in myotubes, finding that one of the most potent compounds (screening of 7949 molecules) was the flavanone hesperetin, increased intracellular ATP by 33% and mitochondrial spare capacity by 25%. This increase in ATP at the cellular level was justified by an increase in gene expression of the peroxisome proliferator-activated receptor-gamma coactivator 1- α (PGC-1α) and NRF2, also, it increased the level of proteins of PGC-1α and of complexes I, III and IV of the electron transport chain in the mitochondria, in muscle cells (in vitro) [13]. In this experiment, hesperetin

 doi:10.20944/preprints202009.0341.v1

444 specifically increased spare capacity which is considered an indicator of
445 mitochondrial fitness/flexibility [42,43]. In addition, hesperetin has shown
446 increased activation of AMPK in liver cells [20] and fibroblasts [19]. AMPK is a
447 sensor of cellular energy status that plays a central role in skeletal muscle
448 metabolism, regulating muscle exercise capacity, mitochondrial function and
449 contraction-stimulated glucose uptake [44].

450 Considering that PGC-1α and AMPK are an important transcriptional masters
451 regulators of mitochondrial biogénesis (↑ biogenesis mitochondrial and oxidative
452 capacity) [44,45] y NRF2 which is an essential regulator in the control of cellular
453 redox homeostasis y controls glutathione synthesis (reactive oxygen species (ROS)
454 scavenging) [46]. This indicates that the prevention of performance loss in old rats
455 after intake of hesperetin is due to improved mitochondrial biogenesis and
456 endogenous antioxidant status [13]. These findings are similar to those found in
457 our study in the rectangular test, whereas there was a significant decrease in VO_2R
458 in placebo, however, was maintained in the 2S-hesperidin group, with decreased
459 in the oxidation of fats at FatMax and VT1 and in the CHO oxidation at VT1was
460 observed in placebo. This effect could be due to the loss of oxidative capacity
461 mediated by reduced activation de PGC-1α (↓ mitochondrial content) [13]. A
462 decrease in oxygen consumption values in the ventilatory thresholds and in
463 maximum exercise has been associated with a decrease in power outputs in
464 professional cyclists after 3-weeks of cycling competition [47]. These results suggest

 doi:10.20944/preprints202009.0341.v1

that the chronic intake of 2S-hesperidin can prevent the decline in VO_2R, which is related with a decrease in the ability to produce power in cyclists.

It should be noted that the hypothesis of improved performance after ingesting 2S-hesperidin in our study is based on findings discovered by in vitro and animal studies. Therefore, it is necessary to carry out more mechanistic studies to determine of the action of hesperidin or hesperetin in human muscle.

In addition, in vitro experiments with hesperetin have shown an increase in GSH/GSSG due to increased GSH and decreased GSSG, since hesperetin upregulates glutamate-cysteine ligase modifier subunit (Gclm9), which is the rate-limiting enzyme of glutathione synthesis [13]. In this study, SOD and catalase expression was not changed. However, in a rat model with pleurisy, the antioxidant activity of hesperidin reduced the production of ROS in the liver and increased the liver activities of CAT and SOD [48]. It should be noted that scavenging activity hesperidin neutralizes reactive oxygen species, such as superoxide anion, generated during conditions of oxidative stress, like intense physical exercise [49].

Estruel-Amades et al. [16] observed that five weeks of supplementation with 2S-hesperidin (200 mg/kg three days per week) prevented an increase in ROS and decline in SOD and CAT activity after a test until exhaustion in the thymus and spleen of mice with an intensive training plan. This study also showed an improvement in performance (distance covered) of 58% after 3 weeks of

supplementation in a test intervention until exhaustion. Sin embargo, Recently, Ruiz-Iglesias et al. [15] found that intake of 2S-hesperidin (200 mg/kg three days per week) for 5 weeks improved performance and prevented exercise-induced immune system alterations after testing to exhaustion in a trained rat model. Citrus flavanone (hesperidin and hesperetin) has the ability to modulate cellular antioxidant defenses through the Nrf2-ARE pathway, which regulates gene expression of antioxidant enzymes, such as SOD, CAT, HO-1 and GPx, decreasing intracellular pro-oxidants [50].

It is well known that ROS production during exercise may be related to decreased performance, since it may cause oxidative damage to the mitochondria and muscle contractile proteins and may interfere with the excitation–contraction coupling process [51]. The balance between oxidant production and antioxidant removal is vital to the regulation of cellular functions [52]. But if the antioxidant response is insufficient or the production of ROS is chronically increased, the body will not be able to restore the level of redox homeostasis by increasing ROS concentration, which would lead to altered gene patterns and an inability to adapt to increased oxidative stress [25]. Therefore, antioxidant substances (flavonoids → 2S-hesperidin) may help neutralize free radicals and thereby prolong skeletal muscle integrity and prevent a decline in performance [53]. Based on the scientific evidence found between the relationship of 2S-hesperidin supplementation and the improvement of endogenous antioxidant status and sports performance, there

 doi:10.20944/preprints202009.0341.v1

507 does not appear to be a clear pattern of antioxidant enzyme enhancement, since
508 different effects have been found and most studies were conducted in animals or
509 *in vitro* and few in humans. However, there are indications that hesperidin intake
510 improves endogenous antioxidant status. This may be due to the type of sample
511 (animal or human, sedentary or athletic, male or female, etc.), the type and amount
512 of molecule used, differences in intestinal microbiota, pharmacodynamics and
513 pharmacokinetics, the duration of the study and the type of test used. Future
514 studies are needed to decipher what mechanisms regulate 2S-hesperidin in the
515 complicated and interconnected endogenous antioxidant system in humans. Our
516 hypothesis is that 2S-hesperidin could improve performance in amateur cyclists
517 by modulating gene components, such as AMPK and PGC-1α, that enhance
518 energy production combined with a 2-way antioxidant effect: a direct pathway
519 where 2S-hesperdin removes ROS directly and by enhancing the expression of
520 NRF2 that controls endogenous antioxidant capacity.

521 Other factors that are important for success for endurance athletes are high power
522 levels and anaerobic capacity that are essential physiological requirements for
523 mountain cyclists [54,55]. Besides, one study has identified that anaerobic power is
524 a key performance factor for mountain cyclists [56]. Martínez *et al.* [10] observed
525 improvements in average power (2.3%) and maximum speed (3.2%) during a
526 repeated 30-s sprint test in amateur cyclists following an acute intake of 2S-
527 hesperidin. Although there are no previous studies that have evaluated the effect

 doi:10.20944/preprints202009.0341.v1

of chronic hesperidin intake on maximum anaerobic capacity, Gelabert-Rebato *et al.* [57] found improvements in average power (5.0%) during a Wingate test after intake of polyphenols (mangiferin and luteolin). The results of these two studies are in line with the results obtained in our research after performing the Wingate test (high anaerobic component) with 2S-hesperidin in post-intenvention, since several performance markers (initial power, absolute and relative peak power, power at maximum speed and time at peak power) were improved in this test. Therefore, taking into account described Wingate test results, as well as previous findings reported by other studies about the importance of anaerobic capacity in cyclists' performance, it is evident that the chronic intake of 2S-hesperidin could contribute to improving the competitions results of these athletes.

At the molecular level, an in vitro study has demonstrated a great inhibitory effect of the enzyme xanthine oxidase (XO) (81.3%) with the exposure of 200 μM of hesperitin, showing a dose-dependent inhibition of xanthine oxidase with an IC50 value of 16.48 μM comparable to that 2.07 μM of the positive control allopurinol (a drug clinically prescribed for gout treatment). Xanthine oxidoreductase has the ability to reduce molecular oxygen to superoxide, but at low oxygen and pH stresses, as seen during prolonged sprints [58,59], repeated sprints [60], and post-exercise ischemia [61]. Therefore, a possible decrease in ROS production under high anaerobic conditions (Wingate test = sprint 30s) by XO inhibition through the action of hesperetin could decrease muscle damage and function, avoiding a loss

 doi:10.20944/preprints202009.0341.v1

549 in performance. This mechanism would work in parallel with the direct
550 neutralizing action of ROS by hesperidin and the improvement of the endogenous
551 antioxidant system by the activation of NRF2 [13,49,50].

552 On the other hand, it is known that performance is not limited by the delivery of
553 oxygen to the muscles during a single sprint exercise under normal conditions at
554 sea level [62]. The most probable explanation for why 2S-hesperidin
555 supplementation may have improved performance is the enhancement of
556 mitochondrial bioenergy, which could be negatively affected by high levels of ROS
557 produced [13] during repeated sprint exercise [63].

558 In addition, several authors have described a stimulating effect of nitric oxide
559 production after hesperidin supplementation. Rizza *et al.* [23] observed an increase
560 in endothelial activity NO synthase to produce NO after exposure of bovine aortic
561 endothelial cells to hesperetin, which promoted an increase in flow-mediated
562 dilation in individuals with metabolic syndrome. In addition, Liu *et al.* [64] showed
563 an increase in gene expression of endothelium nitric oxide synthase improving NO
564 synthesis by exposure to hesperetin in endothelial cells. NO can relax human
565 vascular cells (vasodilatación) [65], which leads to improved blood flow during rest
566 and exercise [66]. Vasodilation is a physiological mechanism used not only for the
567 supply of oxygenated blood, but also for the delivery of glucose, lipids and other
568 nutrients to a variety of tissues [67]. Theoretically, increased blood flow would
569 increase the delivery of O_2 and nutrients (e.g. amino acids and glucose) to

 doi:10.20944/preprints202009.0341.v1

exercising skeletal muscle, thus aiding exercise performance during high intensity (conditions of hypoxia) [68].

At the metabolic-molecular level, we hypothesize that improvements in performance at the submaximal and maximal levels after 2S-hesperidin supplementation may be related with 2S-hesperidin ability to activate key metabolic factors, such as AMPK [23] and NRF2 [50]. In general, an increase in PGC-1α activity, via an increase in activation of the intracellular signaling pathways AMPK [69], promotes the activation of NRF2 [70], modifying the transcription of key genes involved in mitochondrial biogenesis, antioxidant status and metabolism. Modifications in these transcription factors have shown performance improvements in endurance athletes [21]. Therefore, 2S-hesperidin has the ability to promote muscle-level adaptations of endurance athletes, which could improve their performance in competitions. In contrast, the improvement in the Wingate test could be due to an improvement in the synthesis of NO and the endogenous antioxidant state and the direct action of 2S-hesperidin by neutralizing ROS.One limitation of our study is the lack of having muscle biopsies to examine the possible mechanisms that could explain these improvements due to financial restrictions. They could have provided valuable.

PRACTICAL APPLICATIONS

 doi:10.20944/preprints202009.0341.v1

590 The data found in this research shows how chronic intake of 2S-hesperidin
591 enhances performance in FTP and maximum power. Advances in these areas of
592 intensity are crucial for improving results in cycling competitions. Furthermore,
593 as observed in the rectangular test, 2S-hesperidin has the ability to maintain
594 oxygen consumption and fatty acid oxidation levels in VT2, in periods with a
595 decrease in training exercise volume and intensity (i.e., this study was conducted
596 in the off-season). It also showed a positive effect on high-intensity 5s exercise,
597 which could help improve performance in short duration sports where strength-
598 power involvement is high. Given the effects reported by 2S-hesperidin, sports
599 nutritionists would have other ergogenic aids available to improve the
600 performance of their athletes. In this period, cyclists had decreased the volume
601 and intensity of training with respect to other periods of the year. This is an
602 important aspect to consider when comparing our results with other studies, as
603 the outcomes could be different due to the volume and intensity of usual training
604 during the testing time period.

605

606

607 **5. CONCLUSIONS**

608 Supplementation with 2S-hesperidin (Cardiose®) during eight weeks promotes
609 improvement in estimated FTP and maximum power in amateur cyclists during
610 an incremental test. Furthermore, the supplementation with 2S-hesperidin may

611 prevent a possible power loss in VT2 (rectangular test) in training periods with
612 less volume and load. These findings support the use of 2S-hesperidin as a natural
613 new ergogenic aid, which can help cyclists improve both their anaerobic and
614 aerobic performance.

615

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625

626 **Authors' contributions**

627 Conceptualization, F.J.M.N., C.M.-P. and P.E.A.; methodology, F.J.M.N., C.M.P.
628 and

P.E.A.; formal analysis, F.J.M.N., C.M.P. and J.C.V.; investigation, F.J.M.N., C.M.P. and J.C.V.; resources, F.J.M.N., C.M.P. and J.C.V.; data curation, F.J.M.N., C.M.P. and J.C.V.; writing—original draft preparation, F.J.M.N.; writing—review and editing, F.J.M.N., C.M.P. and J.C.V.; visualization, C.M.P.; supervision, C.M.P. and P.E.A.; project administration, C.M.P. and P.E.A.; funding acquisition, P.E.A. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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894

895 **FIGURES**

896 **Figure 1.** Structure of hesperidin enantiomers S and R and their metabolites

897 hesperetin, produced by the intestinal microbiota. Modified from Li et al [71].

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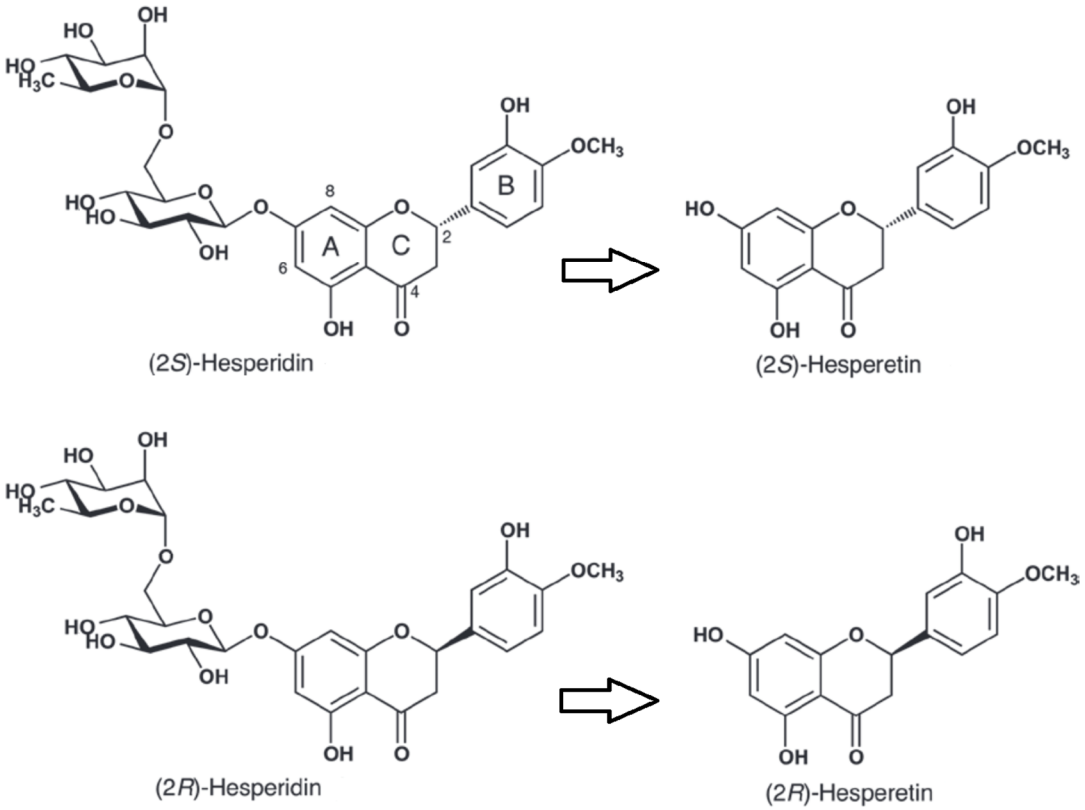

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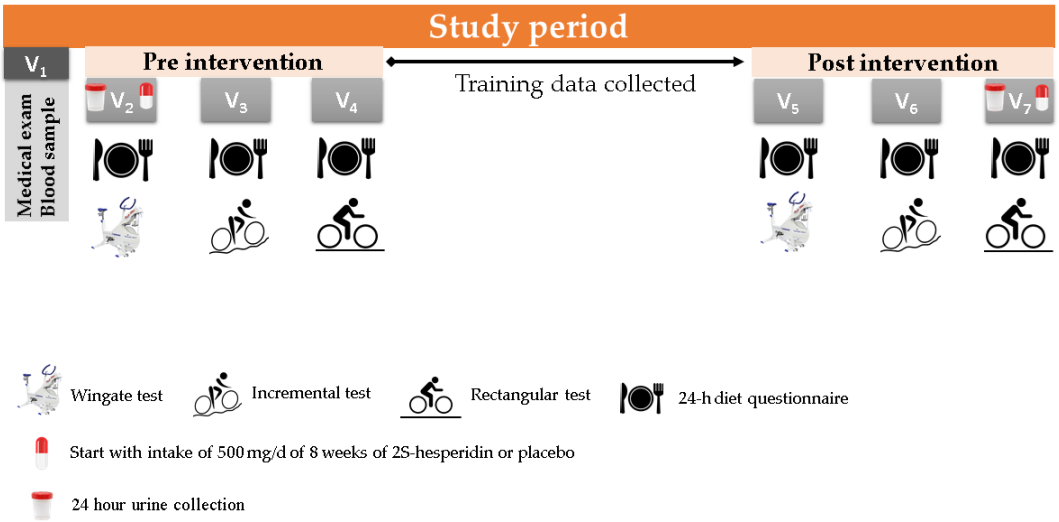
899 **Figure 2.** Study planning with explanation of the different visits (V 1-7).



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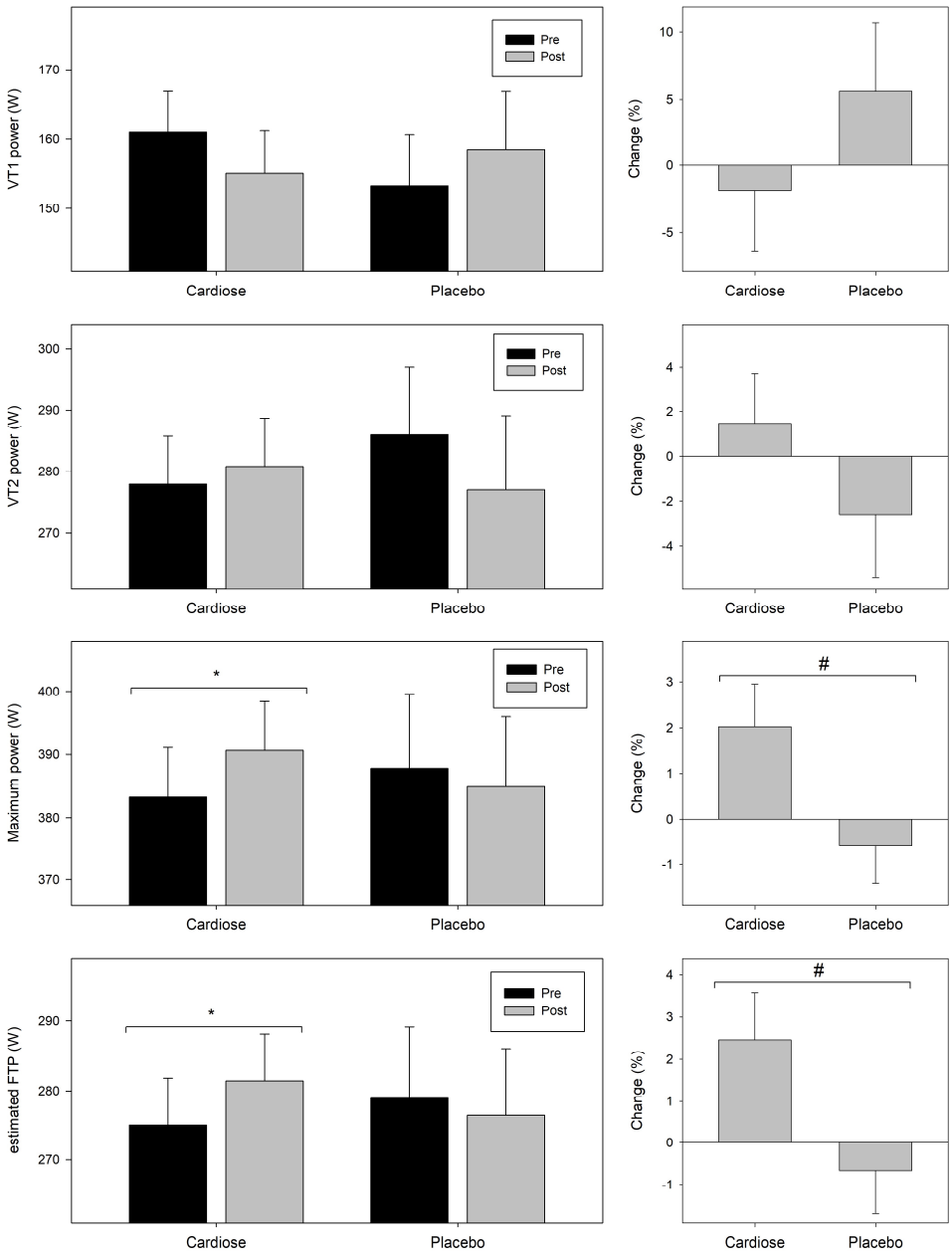
901 **Figure 3.** Changes in ventilatory 1 (VT1) power, ventilatory threshold 2 (VT2)
902 power, estimated functional threshold power (FTP) and maximum power during
903 the maximal test. Values are mean±SE *Within-group significant changes (p≤0.05).
904 #Between group significant changes (p≤0.05).



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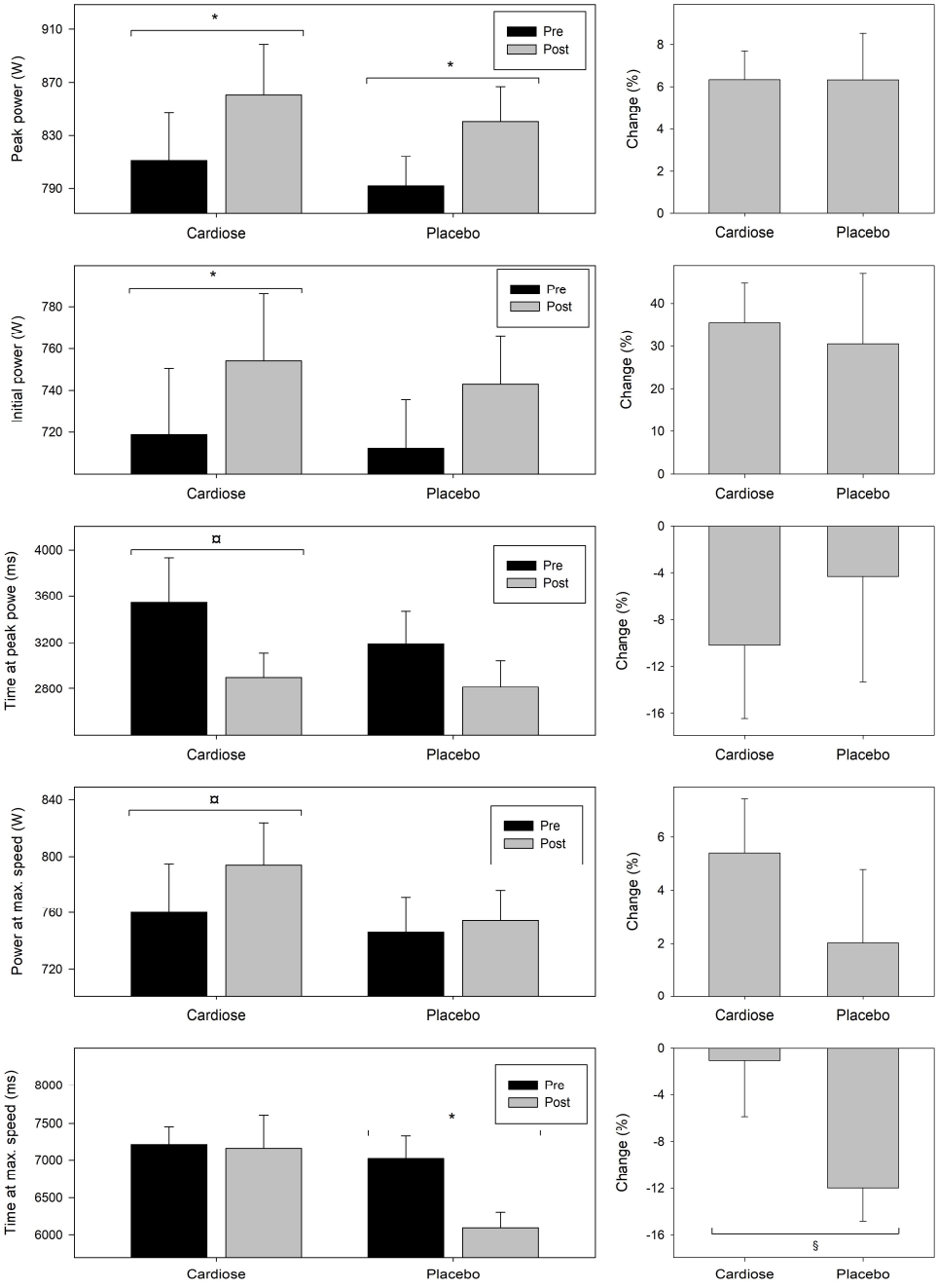
906 **Figure 4.** Changes in parameters evaluated during the Wingate test prior and after
907 supplementation. Values are mean±SE *Within-group significant changes (p≤0.05).
908 ¤Within-group trend to significant changes (p=0.05-0.010). § Between group trend
909 to significant changes (p=0.05-0.010).



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