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Research article

Citrus Flavonoid Supplementation Improves Exercise Performance in Trained Athletes

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Abstract

Previous studies have shown that polyphenol supplementation may be an effective strategy to improve exercise performance, due to their antioxidant character and ability to stimulate NO production. These properties may contribute to exercise performance, yet no conclusive research has been performed in exploring the direct effects of citrus flavonoids on human exercise performance. Therefore, the purpose of this study was to assess whether supplementation of a customized citrus flavonoid (CF) extract for 4 weeks improves cycling time-trial performance in trained male athletes. In a double-blind, randomized, parallel study, 39 healthy, trained males were given a daily dose of either 500 mg of a customized citrus flavonoid extract (CF) or a placebo for 4 weeks. Exercise performance was tested by means of a time-trial test on a cycle ergometer, during which participants had to generate as much power as possible for duration of 10 minutes. Absolute power output significantly increased with 14.9 ± 3.9 W after 4 weeks of CF supplementation, corresponding with a 5.0% increase, compared to 3.8 ± 3.2 W (1.3% increase) in placebo ($p < 0.05$). In addition, oxygen consumption/power ratio significantly decreased in the CF group compared to placebo ($p = 0.001$), and a trend was found in the change in peak power output in CF (18.2 ± 23.2 W) versus placebo (-28.4 ± 17.6 W; $p = 0.116$). The current study is the first convincing report that citrus flavonoid supplementation can improve exercise performance, as shown by a significant increase in power output during the exercise test.

Key words: Hesperetin, power output, antioxidant, time trial, ergometer.

Introduction

Sport nutrition and nutritional supplementation aimed at enhancing exercise performance and recovery from exercise is commonly used by athletes (Burke and Read, 1993; Froiland et al., 2004; Tscholl et al., 2010). For instance, foods high in carbohydrates and protein are consumed in order to replenish glycogen stores and promote muscle anabolism (Reid, 2013). Although exercise is known to be beneficial for improving exercise performance and preventing several pathological conditions, like cardiovascular disease, type II diabetes, metabolic syndrome, and neurodegenerative diseases, it also induces reactive oxygen species (ROS) production, which is related with several conditions leading to diseases (Davis et al, 2010; Davis et al., 2009; Halliwell, 1991; Malaguti et al., 2013; Masella et al., 2005). Exhaustive exercise increases ROS production, leading to muscle fiber damage, which eventually results in muscle fatigue (Peternelj and Coombes, 2011). However, there is growing evidence suggesting that the presence of a small stimulus, like low concentration of ROS, is able to express the transcription of major antioxidant genes. Enzymes like superoxide dismutase (SOD) and glutathione are important antioxidant defenses that protect cells from ROS-induced oxidative stress (Masella et al., 2005). Moderate exercise acts as a stimulator of the body's antioxidant defenses against oxidative damage (Gomez-Cabrera et al., 2008; Powers et al., 2011). The correlation between oxidative damage and muscle fatigue could be an important strategy for nutritional interventions to increase exercise performance. Antioxidant supplementation may be an effective strategy, considering the reactive oxygen species (ROS) scavenging effects that could lead to a reduction in muscle damage caused by prolonged exercise (Myburgh, 2014; Sachdev and Davies, 2008).

Polyphenols, including flavonoids derived primarily from fruits, have been of interest due to their antioxidant and anti-inflammatory effects (Masella et al., 2005). Previous studies showed that polyphenols derived from pomegranates, cherries and blueberries reduced muscle soreness and improved muscle strength after eccentric exercise (Bowtell et al., 2011; McLeay et al., 2012; Trombold et al., 2010; 2011). Next to potential benefits in muscle recovery, flavonoid supplementation has been shown to improve endurance exercise performance in humans (Davis et al., 2009; 2010; MacRae and Mefferd, 2006; Nieman et al., 2010).

Another point of interest is the association that has been made with stimulation of nitric oxide (NO) production and subsequent improved endothelial function with different flavonoids, including hesperidin (Rizza et al., 2011). NO acts on smooth muscle cells within the arterial wall, causing vasodilatation and thereby improving blood flow and reducing blood pressure (Cooke et al., 1997; Umans and Levi, 1995). During exercise, this allows for an increase in transport of oxygen and nutrients to the muscles and removes metabolites causing fatigue in prolonged exercise (Bescós et al., 2012; Bloomer, 2010; Wylie et al., 2013).

However, no conclusive clinical research has been performed to explore direct benefits of citrus flavonoids on exercise performance in trained athletes (Malaguti et al., 2013). Therefore, the aim of the study was to elucidate whether a customized citrus flavonoid (CF) extract sup-

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plementation for duration of 4 weeks improves cycling time-trial performance in trained male athletes. It was hypothesized that CF extract supplementation will improve time-trial performance in trained athletes.

Methods

Participants

Trained, non-smoking male participants, aged 18 – 25 y, engaging in moderate to high physical activity for a minimum of 30 minutes at least 3 times a week were included in the study. Exclusion criteria were the use of dietary antioxidant and/or vitamin supplements, and the ingestion of products containing citrus flavonoids or its metabolite 4 days prior to and during the study.

All participants gave written informed consent before participation. The study was approved by the Medical Ethics Committee of Wageningen University and conducted in full accordance with the principles of the Declaration of Helsinki of 1975 as amended in 2013 and with the Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). The study was performed at InnoSportlab Papendal, Arnhem, The Netherlands (now part of the National Sportscenter Papendal). The trial has been registered in the Clinical Trials register (NCT02787733).

Design and protocol

The study was designed as a randomized, parallel group, double-blind design to test the effects of a daily dose of 500 mg CF extract supplementation versus a placebo over a period of 4 weeks. A publicly available randomization program (http://randomizer.org) was used to randomly assign participants to one of the two interventions.

The study consisted of a familiarization test, a baseline test (Test 1) and a final test after a 4-week intervention period (Test 2). Prior to each test, participants were instructed to standardize their workouts and dietary intake and to refrain from physical exercise and alcohol for at least 24-h prior to testing. During the complete duration of the study, participants were instructed to refrain from eating foods containing citrus flavonoids, including lemons, oranges, and grapefruit.

The familiarization test consisted of a 5 min warm-up at 100 W followed by a 10 min time-trial on a cycle ergometer (SRM, Jülich, Germany), during which participants had to generate the maximal power (W) possible over the time course of 10 min. Power output (W) and heart rate (bpm) were measured continuously during the time-trial, and indirect calorimetry was used to measure oxygen consumption (VO_2; $mL \cdot min^{-1}$) during the time-trial. In addition, an estimation of maximal oxygen consumption (VO_{2max}; $mL \cdot min^{-1}$) was made. At t = 0, 9, 10 and 11 min after starting the test, participants were asked to indicate their perceived exhaustion (RPE) using a Borg scale from 6 – 20, in which 6 is no exertion at all and 20 is maximal exertion. The mean power that was measured during the familiarization test was used to determine the power for a 10 min pre-exhaustion test during Test 1 and Test 2.

The following test protocol was performed identically on both test days. First, participants had to perform a standardized pre-exhaustion test, consisting of 10 min cycling at 80% of the mean work-load as established during the familiarization test. During this pre-exhaustion phase, heart rate and exhaustion at t = 0, 9, 10 and 11 min after start of the test were measured. Subsequently, participants took a passive rest period of 25 min followed by 5 min of warm-up at 100 W, as a warm-up has been shown to improve subsequent exercise performance (Fradkin et al., 2010). Directly after that, participants had to perform a 10 min time-trial comparable to the familiarization test. The same parameters were obtained as in the familiarization test with additional exhaustion measurements at t = -2, -1 prior to start of the test. Between Test 1 and Test 2, an intervention period of 4 weeks was conducted, in which the participants administered either CF extract supplementation or placebo supplement.

Study product

BioActor BV (Maastricht, the Netherlands) supplied the CF extract, standardized for hesperetin-7-O-rutinoside 2S enantiomer, with a total rutinoside content of at least 90% (WATTS'UP®/CF extract). The study product was formulated into capsules containing 250 mg of CF extract and 10 mg Magnesium Stearate by Aminolabs (Hasselt, Belgium). The placebo capsules containing 250 mg Microcrystalline Cellulose were produced to be identical in appearance and taste. The capsules were transported and stored at room temperature. Participants were instructed to ingest two capsules with 200 mL water daily before breakfast, for 4 weeks from the first morning after Test 1 (T1) until the morning of Test 2 (T2).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows (version 22.0 Armonk, NY, USA). Only data from participants who completed the study (n = 39) were used in the final analysis. Before statistical analysis, the Shapiro-Wilk test was performed to test for normality in the test population. To determine the effects of CF supplementation on the measured performance outcomes, the mean values at baseline (T1) were compared with the mean values after 4 weeks of supplementation (T2). A paired-samples t-test was used to compare the T1 and T2 measurements within both the CF and placebo group. To compare the differences between the CF and placebo group, a two-way mixed ANOVA was performed. Data is reported as mean ± standard error of the mean (SEM) and the level of significance was set on $p < 0.05$.

Results

Study participants

Thirty-nine participants completed the study protocol, as one participant dropped out after the first test day due to an injury not related to the study. The baseline characteristics of the participants in both groups are shown in Table 1. No significant differences were found between CF extract supplementation and placebo at the start of the 4 week intervention period.

Table 1. Participant characteristics of the 39 participants that completed the study protocol. Data were obtained at baseline and values are expressed as mean (± SEM).

	Total (n=39)	Citrus Flavonoid (CF) (n=19)	Placebo (n=20)
Age (yr)	23.0 (.3)	22.7 (.4)	23.6 (.4)
Weight (kg)	74.4 (1.29)	74.1 (1.75)	74.8 (1.93)
Height (m)	1.84 (.01)	1.83 (.02)	1.84 (.02)
BMI ($kg \cdot m^{-2}$)	22.1 (.30)	22.1 (.42)	22.0 (.44)
Exercise time per week	9.6 (.6)	8.8 (.6)	10.3 (.9)
Absolute power (W)	299.3 (7.7)	298.0 (10.8)	300.5 (11.4)
Relative power ($W \cdot kg^{-1}$)	4.0 (.1)	4.0 (.2)	4.0 (.2)
Heart rate (BPM)	174 (2)	171 (4)	177 (3)
Average VO_2 ($mL \cdot kg^{-1} \cdot min$)	50.3 (1.3)	51.1 (1.6)	49.6 (2.1)
Estimated VO2max ($mL \cdot kg^{-1} \cdot min$)	57.1 (1.2)	57.9 (1.6)	56.5 (1.8)
VO_2/Power ratio	.172 (.003)	.176 (.005)	.170 (.004)
Peak power output (W)	332.5 (21.0)	359.4 (36.0)	306.8 (22.0)

No significant differences were found between CF and placebo group at baseline, $p > 0.05$.

Table 2. Within and between group comparison of the performance outcomes of the citrus flavonoid group and placebo group, with corresponding p-values. Values are expressed as mean (± SEM).

Variables	Citrus Flavonoid (CF) (n=19)				Placebo (n=20)				
	Test 1	Test 2	ΔT2-T1	< P1†	Test 1	Test 2	ΔT2-T1	<P2‡	<P3§
Weight (kg)	74.1 (1.8)	74.2 (1.74)	.1 (.1)	.406	74.8 (1.9)	74.7 (1.9)	-.1 (.4)	.718	.529
Abs P (W)	298 (11)	313 (10)	14.9 (3.9)	.001	301 (11)	304.3 (11.4)	3.8 (3.2)	.243	.032
Rel P ($W \cdot kg^{-1}$)	4.0 (.1)	4.2 (.1)	.2 (.1)	.002	4.0 (.2)	4.1 (.1)	.1 (.1)	.328	.077
HR (BPM)	170 (4)	169 (3)	-1 (2)	.578	176 (3)	177 (3)	1 (1)	.206	.276
Av VO_2	51.1 (1.6)	52.0 (1.2)	.9 (.7)	.235	49.6 (2.1)	50.2 (1.8)	.6 (.6)	.330	.736
Es VO_2max	57.9 (1.6)	57.9 (± 1.3)	.0 (.7)	.988	56.5 (1.8)	56.7 (1.6)	.2 (.9)	.784	.826
VO_2/Power ratio	.176 (.005)	.167 (.004)	-.009 (.002)	.001	.170 (.004)	.171 (.004)	.001 (.002)	.534	.001
PP output (W)	360 (36)	378 (40)	18 (23)	.444	307 (22)	279 (20)	-29 (17)	.123	.116

Abs P: Absolute power; Rel P: Relative power; HR: Heart rate; Av VO_2: Average VO_2 ($mL \cdot kg^{-1} \cdot min^{-1}$); Es VO_{2max}: Estimated VO_{2max} ($mL \cdot kg^{-1} \cdot min^{-1}$); PP output: Peak power output .†P1; P-value of within CF group differences between Test 2 and Test 1 (ΔT2-T1), compared using a paired t-test, ‡P2; P-value of within placebo group differences between Test 2 and Test 1 (ΔT2-T1), compared using a paired t-test , §P3; P-value of the difference between pre- and post-treatment between the CF and placebo group using a mixed ANOVA.

Exercise performance, power output, and VO_2

Table 2 shows the results of the performance outcomes from the participants with CF extract supplementation and placebo, before and after the 4 week intervention period. Both absolute ($p = 0.001$) and relative ($p = 0.002$) power output were significantly increased over the 4 week supplementation in the group receiving CF extract supplementation. No difference in weight was noted during the trial period in either group, making that the relative power parameter in this study did not need to be corrected for changes in weight. In the placebo group, no significant differences in absolute and relative power were found when comparing T2 with T1. The power output of each participant for the whole duration of the time-trial test was also recorded. Figure 1 shows the average power evolution profiles for both groups at baseline and after 4 weeks of CF extract supplementation.

When plotting the mean absolute power output (Figure 2A), it becomes apparent that the group receiving CF extract supplementation significantly increased ($p = 0.001$) absolute power output by 5% over the 4-week supplementation period, while the placebo group did not ($p > 0.05$). When expressed as the difference in absolute power output between test days (ΔT2 – T1), the increase in power after 4 weeks of CF extract supplementation is significant and almost 4 times higher than placebo ($p = 0.032$; Figure 2B). In addition, a trend was found for the increase in peak power output (in the first 3 seconds of workout) after 4 weeks of treatment in CF extract supplementation (18.2 ± 23.2 W) versus placebo (-29.0 ± 17.4 W; $p = 0.116$; Table 2).

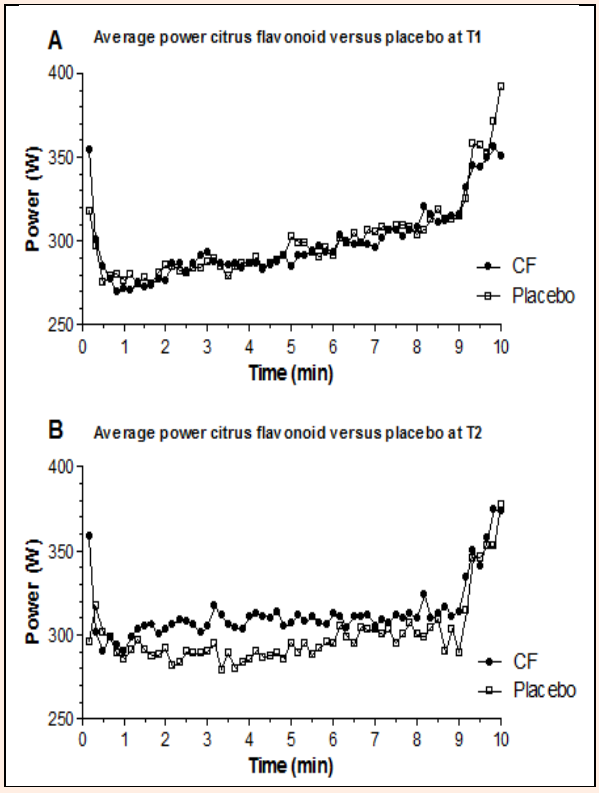

Figure 1. A) Average power evolution profiles during Test 1 for CF and placebo group. B) Average power evolution profiles during Test 2 for CF and placebo group.

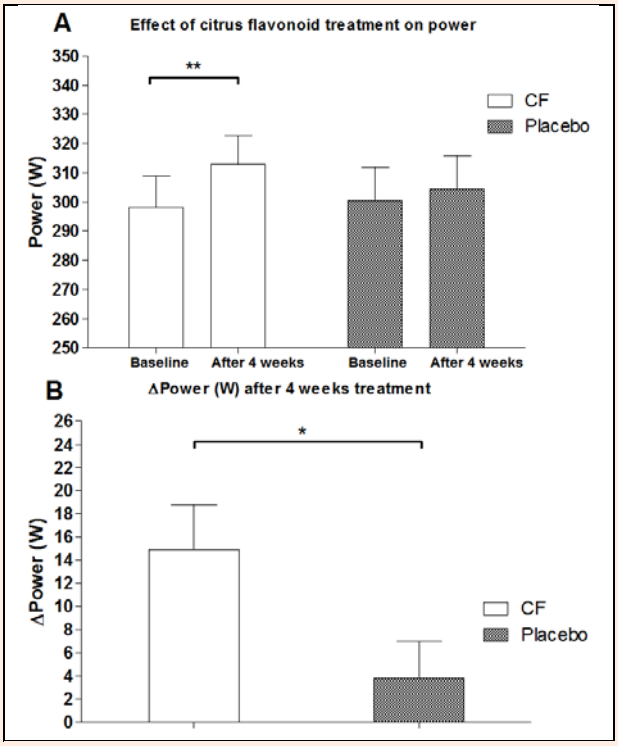

Figure 2. A) After 4 weeks of supplementation, the average power in the CF group was increased with 5% (p = 0.001), while no significant increase was noted in the placebo group (p = 0.243). Values are expressed as mean ± SEM. B) The increase in power after 4 weeks treatment compared to baseline (ΔT2-T1) is significantly higher within the CF group than within the placebo group (p = 0.032*). Values are expressed as mean ± SEM.**

In addition to power output, the VO_2 consumption was measured. No significant differences ($p > 0.05$) in VO_2 consumption were found between baseline and after four weeks of treatments in both groups (Figure 3A). When plotting the VO_2 consumption against the power output data, the results show that the VO_2/Power ratio significantly decreased ($p = 0.001$) with 5.1% in the CF extract supplementation group compared to a non-significant 0.6% increase ($p = 0.534$) in the placebo group (Figure 3B). When looking at the delta VO_2/Power ratio ($\Delta T2 - T1$), a significant between-group effect is apparent ($p = 0.001$), in which the VO_2/Power ratio decreased with 0.009 in the CF extract supplementation group and insignificantly increased with 0.001 in the placebo group.

Discussion

The present study shows that CF extract supplementation for 4 weeks significantly increased absolute power in trained athletes compared to placebo. Furthermore, body mass and VO_2 did not change in either groups, resulting in a higher amount of power produced per kilogram of bodyweight (relative power) and per unit of oxygen (VO_2/Power ratio) in the CF group compared to placebo.

Previous studies showed that the flavonoid quercetin increased exercise performance and endurance capacity (Davis et al., 2010; MacRae and Mefferd, 2006). While *in vivo* the exact mode of action of flavonoids is unclear, oxygraphic results for quercetin and hesperetin support the shared working mechanism of both compounds by which they can increase exercise performance (Dorta et al., 2005).

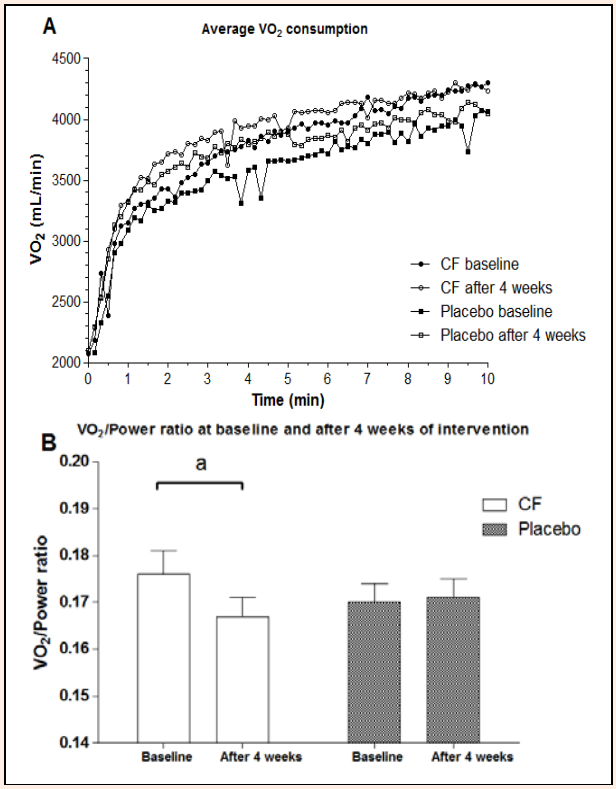

Figure 3. A) Average VO_2 consumption over time in both groups, before and after 4 weeks of intervention. B) The VO_2/power ratio was significantly decreased in the CF group compared to the ratio at baseline (p = 0.001). Values are expressed as mean ± SEM.**

In addition to their antioxidant activity, flavonoids may exert other intracellular effects and may interact at the mitochondrial level, contributing to maintain high energetic demand and redox homeostasis. It is suggested that polyphenols may have a potential effect on the functioning of the whole electron transfer chain (ETC), acting on the total mitochondrial respiration process rather than focusing on only a single specific complex (e.g. Complex I) (Santos et al., 1998). In addition, they can act, whether favorably or not, on various mitochondrial processes. Some polyphenols have even been described to act on the signaling pathways (i.e. modulating intrinsic apoptosis (Mertens-Talcott et al., 2003; Zhang et al., 2013)) and trigger mitochondrial biogenesis (i.e. inducing sirtuins) (Baur et al., 2006; Chung et al., 2010; Davis et al., 2009; Sandoval-Acuna, Ferreira and Speisky, 2014a).

The notion that hesperidin behaves in a similar manner as quercetin is supported by several studies that show that quercetin supplementation also increases exercise performance and endurance capacity (Davis et al., 2010; MacRae and Mefferd, 2006). In particular, quercetin is also known to increase mitochondrial biogenesis and

exercise tolerance (Davis et al., 2009; Sandoval-Acuna et al., 2014b). While both hesperidin and quercetin exhibit mild uncoupling activity *in vitro* (Dorta et al., 2005), it is expected that, *in vivo*, hardly any uncoupling activity of flavonoids can be anticipated (van Dijk et al., 2000). This would increase proton leak and maximal respiratory capacity, supporting the hypothesis that hesperitin induces mitochondrial biogenesis.

Another point of interest is the effect of flavonoids on mitochondrial Ca^{2+} uniporter within the cell, increasing mitochondrial Ca^{2+} concentration. Ca^{2+} regulates several pathways, including one which stimulates eNOS, contributing to an increase of NO production, and stimulating K^+ efflux (and Ca^{2+} influx), causing hyperpolarization of the cell membrane in endothelial cells (Garland et al., 2011). These results in relaxation of the smooth muscle in blood vessels and with this lowered blood pressure and increased blood flow (Reid, 2013; Umans and Levi, 1995; Wylie et al., 2013). Also, increased mitochondrial Ca^{2+} might stimulate oxidative metabolism by upregulation of pyruvate dehydrogenase and increase of mitochondrial membrane potential, allowing generation of high ATP concentrations lowering oxidative stress and muscle damage, ultimately increasing strength output (Bugger and Abel, 2010; Jouaville et al., 1999; Rutter andRizzuto, 2000). The strong increase in power output in the present study (Table 2; Figure 2) is particularly interesting as it seems in contrast with typical hypotheses that mainly an improved blood flow, and therefore better oxygen delivery to the muscles, can improve exercise performance in athletes (Andersen and Saltin, 1985; Newman et al., 2002). Indeed, the observation that the VO_{2max} remains unchanged in combination with increased power output supports the potential role of the CF extract in the oxygen maintenance process in the muscle itself, rather than just increased oxygen delivery.

Furthermore, the fact that the positive results were obtained in a study in which well-trained athletes (average 9.6 hours per week) were included, further increases the relevance of the increase in power output. Indeed, it becomes exceedingly more difficult for trained individuals to gain further increase in strength and performance compared to untrained individuals (Ahtiainen et al., 2003; Peterson et al., 2005). An increase of 5% in overall power output is comparable with observed increases after creatine use (Buford et al., 2007). Interestingly, peak power output showed a trend to increase from baseline to 4 weeks after intervention in CF compared to placebo (Table 2). Although not significant ($p = 0.112$) due to a large variation between participants, a calculated Cohen's d effect size of 2.70 shows a large magnitude of the treatment in the CF extract supplementation group on peak power output. An increase in peak power output through CF supplementation could be relevant for sports requiring immediate power generation.

To account for potential training adaptation, a familiarization test was conducted before the start of the study. But still, habituation with the test makes the athlete perform better along the study, simply because he is prepared better. Furthermore, daily exercise volume or nature (strength vs. endurance) was not recorded or standardized by participants, although the nature of training has different effect on muscle alterations and metabolism (Baars, 2006). Also, several measurements that would have had added value to the study were not performed to lower the burden for the participant. For example, VO_{2max} testing could give more insight in in aerobic fitness. Or, muscle biopsies would be of interest regarding mitochondrial number and function, for example to determine mitochondrial fractional area, an important quantitative indicator of oxidative capacity. Blood collection prior to and after exercise would have allowed for measurement of multiple blood markers for example related to oxidative stress, such as plasma malondialdehyde, reduced glutathione or cardiac troponin T, a marker for cardiac damage (Bloomer et al., 2005; Rifai et al., 1999). Both blood and muscle biopsies would have allowed a more mechanistic point of view, but were not included in this study due to their invasive character. Nevertheless, the study was placebo-controlled and the heart rate and VO_2 did not change after 4 weeks, making it unlikely that the observed effects were a result of an overall increased fitness or extra effort specifically for the CF extract study group.

Conclusions

Repeated intake of a specific CF extract may improve exercise performance in trained athletes. Therefore, this study is highly relevant for athletes to maximize their performance capacity. The improved performance might be due to improved respiratory efficiency in the mitochondria as a result of CF extract supplementation. However, further mechanistic research is needed to confirm this.

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Key points

- Oxygen-processing capacity of mitochondria in the muscles plays a key role in exercise performance and recovery.
- In a double-blind, randomized, parallel study with 39 healthy, trained males the effect of 500 mg of a customized citrus flavonoid extract (CF) on exercise performance was assessed.
- CF intake significantly increased absolute power output with 5% and significantly decreased the oxygen consumption/power ratio, as VO_{2max} remained unchanged.
- The improved performance therefore may be due to improved respiratory efficiency as a result of CF extract supplementation, however, further mechanistic research is needed to confirm this.

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