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RESEARCH ARTICLE

No additive effect of acetaminophen when co-ingested with caffeine on cycling performance in well-trained young men

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Abstract

We investigated the effect of caffeine and acetaminophen on power output during a 6-min performance test, peripheral fatigue, and muscle protein kinase A (PKA) substrate phosphorylation. Fourteen men [age (means ± SD): 26 ± 6 yr; $\dot{V}O_{2max}$: 63.9 ± 5.0 mL·min^{-1}·kg^{-1}] completed four randomized trials with acetaminophen (1,500 mg), caffeine (5 mg·kg body wt^{-1}), combined caffeine and acetaminophen (caffeine + acetaminophen), or placebo. Mean power output during the 6-min performance test (placebo mean: 312 ± 41 W) was higher with caffeine (+5 W; 95% CI: 1 to 9; P = 0.017) and caffeine + acetaminophen (+6 W; 95% CI: 0 to 12; P = 0.049) than placebo, but not with acetaminophen (+1 W; 95% CI: −4 to 7; P = 0.529). Decline in quadriceps maximal isometric voluntary torque immediately after the performance test was lower (treatment × time; P = 0.035) with acetaminophen (−40 N·m; 95% CI: −53 to −30; $P < 0.001$) and caffeine + acetaminophen (−44 N·m; 95% CI: −58 to −30; $P < 0.001$) than placebo (−53 N·m; 95% CI: −71 to −39; $P < 0.001$) but was similar with caffeine (−54 N·m; 95% CI: −69 to −38; $P < 0.001$). Muscle phosphocreatine content decreased more during the performance test (treatment × time; P = 0.036) with caffeine + acetaminophen (−55 mmol·kg dry wt^{-1}; 95% CI: −65 to −46; $P < 0.001$) than placebo (−40 mmol·kg dry wt^{-1}; 95% CI: −52 to −24; $P < 0.001$). Muscle net lactate accumulation was not different from placebo (+85 mmol·kg dry wt^{-1}; 95% CI: 60 to 110; $P < 0.001$) for any treatment (treatment × time; P = 0.066), being +75 mmol·kg dry wt^{-1} (95% CI: 51 to 99; $P < 0.001$) with caffeine, +76 mmol·kg dry wt^{-1} (95% CI: 58 to 96; $P < 0.001$) with acetaminophen, and +103 mmol·kg dry wt^{-1} (95% CI: 89 to 115; $P < 0.001$) with caffeine + acetaminophen. Decline in muscle ATP and glycogen content and increase in PKA substrate phosphorylation was not different between treatments (treatment × time; $P > 0.1$). Thus, acetaminophen provides no additive performance enhancing effect to caffeine during 6-min maximal cycling. In addition, change in PKA activity is likely not a major mechanism of performance improvement with caffeine.

NEW & NOTEWORTHY Here, we show that acetaminophen does not provide additive performance improvement to caffeine during a 6-min cycling ergometer performance test, and that acetaminophen does not improve performance on its own. Neither substance affects peripheral fatigue, muscle glycolytic energy production, or phosphorylation of muscle proteins of importance for ion handling. In contrast to previous suggestions, increased epinephrine action on muscle cells does not appear to be a major contributor to the performance enhancement with caffeine.

athletes; doping; muscle metabolism; paracetamol; supplement

INTRODUCTION

Ingestion of substances to improve exercise performance has been used since antiquity (1, 2). Today, the World Anti-Doping Agency (WADA) restricts the use of most substances with the potential to increase performance in competitive sport (3), and therefore an area of increasing interest is the use of permitted supplements to provide performance enhancement (4–6). For this purpose, caffeine and acetaminophen are among the most common supplements used by athletes (7, 8). However, although both substances on their own may enhance performance during short-term high-intensity exercise (5, 9–11), only a few studies have investigated their potential additive effects. Considering the marginal differences between gold and silver medal performances at events such as the Olympic Games (9), the concept of combining permitted supplements to provide even modest performance enhancements is intriguing for athletes, but nonetheless relatively unexplored in controlled studies.

The mechanisms behind the performance enhancing effect of caffeine are presumably due to several factors (9, 10, 12). One contributing mechanism is thought to be an increased release of epinephrine and norepinephrine, which is generally observed during intense exercise with prior caffeine ingestion (13–15). Epinephrine and norepinephrine stimulate surface beta-adrenoceptors and may affect skeletal muscle substrate utilization through the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling

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pathway. For example, venous epinephrine infusion during moderate intensity exercise has been shown to increase glycolytic energy production and glycogenesis (16, 17), and during maximal intensity exercise, stimulation of the cAMP/PKA pathway with beta$_2$-adrenoceptor agonists also increases glycolytic energy production (18). Further affected by PKA signaling are proteins regulating muscle K^+ and Ca^{2+} homeostasis, as subunits of the Na^+/K^+-ATPase and sarcoplasmic reticulum calcium ATPase (SERCA) are activated by PKA phosphorylation (19–21). Indeed, caffeine has also been shown to decrease muscle interstitial K^+ accumulation in vivo (22). These observations underlie the hypothesis that caffeine improves exercise performance by stimulating myocellular processes that regulate energy turnover, contractile strength, and ion handling. However, no studies have explored the effect of caffeine on muscle PKA activation. Instead, early studies provided insight into the role of beta-adrenergic action in caffeine-induced performance improvement by indirect measures such as plasma lactate and muscle glycogen utilization (13, 14, 23–25). An alternative approach is measurement of global phosphorylation state of substrates of the canonical beta-adrenergic signaling pathway (PKA), thus providing a direct measure of epinephrine and norepinephrine stimulation on skeletal muscle. Based on the effects of epinephrine and norepinephrine on PKA-signaling and glycolytic energy production, along with the increased epinephrine and norepinephrine release induced by caffeine, it could be speculated that increased PKA signaling explains some of the performance enhancement previously observed with caffeine ingestion.

The putative mechanisms underlying performance improvement with acetaminophen are less studied, but generally thought to be through decreased perception of exertion (26, 27) [as has also been reported for caffeine (12, 28)]. For example, Mauger et al. (27) observed that ingestion of 1,500 mg acetaminophen before a 16-km cycling time trial increased mean power output on average 2% compared with the placebo trial, whereas ratings of perceived exertion were unchanged. In another study, acetaminophen was shown to preserve power output during eight repeated wingate sprints without increased ratings of perceived exertion (11). Thus, evidence indicates that a higher power output can be produced at a given perceived exertion with acetaminophen. In addition, acetaminophen has been reported to preserve muscle contractile force after fatiguing isolated quadriceps contractions (29), thus raising the possibility that acetaminophen plays a role in counteracting peripheral fatigue development.

Despite evidence of performance enhancement with caffeine and acetaminophen ingestion, only one study has explored their potential additive effect. Tomazini et al. (6) found that caffeine improved performance during a 4-km time trial whereas combined caffeine and acetaminophen ingestion did not improve performance compared with placebo. Although those results indicate that acetaminophen blunted the effect of caffeine, a caveat of the study by Tomazini et al. is that the participants were not well-trained ($\dot{V}O_{2max} \approx 39\ mL \cdot kg^{-1} \cdot min^{-1}$). Considering that one of the putative mechanisms of acetaminophen and caffeine ingestion is reduced sensation of exertion, it is conceivable that the magnitude of the response in elite athletes, who are more accustomed to overcoming exercise-induced exertion, will not be as pronounced as observed in untrained individuals. In addition, there are indications of differences in the response to exogenous substances between well-trained and untrained individuals. For example, in a recent study, Porcelli et al. (30) observed that the ergogenic effects of dietary nitrate supplementation on 3-km running performance were only evident in low- and moderately trained individuals ($\dot{V}O_{2max}$ of ≈38 and ≈52 $mL \cdot min^{-1} \cdot kg^{-1}$, respectively), but not in well-trained athletes ($\dot{V}O_{2max}$ of ≈72 $mL \cdot min^{-1} \cdot kg^{-1}$). With caffeine, differences between untrained and well-trained individuals have also been observed in peripheral fatigue development (31) and during a 3-km time trial (32). Thus, extrapolation of performance data from untrained to well-trained individuals is not always possible, and data are needed to confirm previous findings of the ergogenic potential of co-ingestion of caffeine and acetaminophen in well-trained populations. In addition, previous studies have not investigated peripheral fatigue development or other putative mechanisms, such as skeletal muscle metabolism, that may underlie potential performance changes with caffeine and acetaminophen. Specifically, the effects on skeletal muscle lactate production, PKA signaling, and markers of ion handling warrant further investigation.

Thus, in the present study we investigated the effect of single and combined ingestion of caffeine and acetaminophen on performance in a 6-min cycling performance test and on peripheral fatigue in well-trained young men. In addition, we examined the effect on skeletal muscle PKA signaling response and markers of K^+ and Ca^{2+} handling through phosphorylation of known regulatory subunits of SERCA and the Na^+/K^+-ATPase as well as rating of perceived exertion during the exercise.

METHODS

Participants and Ethics Approval

Healthy young men were recruited through online posters as well as flyers at University of Copenhagen. Inclusion criteria were age between 18 and 45 yr, $\dot{V}O_{2max} > 60\ mL \cdot kg^{-1} \cdot min^{-1}$, and body mass index $< 26\ kg \cdot m^{-2}$. Exclusion criteria were allergy toward the study medication, smoking, and chronic illness. Based on a recent study showing that habitual caffeine consumption does not influence the effect of caffeine on performance, we did not exclude participants based on their habitual caffeine consumption (33). Withdrawal criteria were unacceptable side effects, complications related to the study, or noncompliance with the study protocol. The study was approved by the committee on Health Research Ethics of the Capital Region of Denmark (H-17002476) and was performed in accordance with the Declaration of Helsinki. Caffeine, acetaminophen, and placebo (lactose monohydrate) were delivered by the Hospital Pharmacy of Region H (Copenhagen, Denmark) in accordance with Good Manufacturing Practice and were administered in gelatin capsules that looked identical and were administered in the same number at all trials.

All participants received written and verbal information about the aims and contents of the study as well as possible risks involved, including associated side effects of the study drugs. Each participant gave his written and oral informed consent before inclusion in the study. All tests were carried

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out at Department of Nutrition, Exercise and Sports (NEXS), University of Copenhagen, Denmark.

Eighteen participants were initially included in the study. Of these, 14 completed the study, and 4 dropped out (1 due to injury unrelated to the study and 3 due to personal reasons). Participants were engaged in sports such as cycling, triathlon, running, and swimming. Characteristics of the participants who completed the study are presented in Table 1.

Assessment of Eligibility Criteria

Eligibility criteria were assessed during a screening where participants were examined by a medical professional to exclude unknown cardiovascular or respiratory disease. Hereafter, whole body composition was determined by dual-energy X-ray absorptiometry (DXA, Lunar DPX-IQ, Version 4.7 E, Lunar Corporation, Madison, WI). Then, $\dot{V}O_{2max}$ was determined by indirect calorimetry (Oxycon Pro, Viasys Healthcare, Hoechberg, Germany), during an incremental test on a bicycle ergometer (Monark LC7, Monark Exercise AB, Vansbro, Sweden) starting at 150 W and increasing 25 W·min^{-1} until exhaustion. Participants who fulfilled all inclusion criteria and none of the exclusion criteria completed a 6-min familiarization performance test on a bicycle ergometer, and one further familiarization performance test conducted on a separate day.

Study Design

This study was a double-blinded, randomized, placebo-controlled trial. After the screening and familiarization visits, participants' trial order was randomized in a counter-balanced manner. The trials were: caffeine (5 mg·kg^{-1} body wt) + placebo, acetaminophen (1,500 mg) + placebo, caffeine + acetaminophen, and placebo (lactose monohydrate). Allocation of participants and administration of study medication was conducted by personnel with no further affiliation to the experimental trials or data analysis. Sample size was determined for the primary response measure for a linear mixed model repeated-measures design. Effect size and standard deviation were estimated from a previous study (4). An overview of the experimental trials is presented in Fig. 1.

Experimental Trials

On experimental days, participants arrived at the laboratory and were administered the study drugs with water. Participants then rested for 60 min to allow time to obtain a systemic effect of the ingested caffeine and acetaminophen (34, 35). Then, participants performed a standardized warm-up consisting of 3 × 4 min at 40%, 50%, and 65% incremental peak power output (iPPO; 149 ± 15, 186 ± 19, and 241 ± 24 W for 40%, 50%, and 65% of iPPO, respectively) as determined from the incremental test at the screening visit. At 3 min during each workload, participants were asked about their rating of perceived exertion (RPE) on a modified Borg-scale ranging from 1 to 10. After the warm-up, participants' maximal voluntary contraction torque (MVC) and contractile properties of the right quadriceps muscle were measured. Then, a biopsy sample was obtained from the vastus lateralis muscle of the left thigh with a 4-mm Bergström needle with suction (36). Before muscle sampling, an incision was made through the skin and fascia at the belly of the vastus lateralis muscle during local anesthesia (2 mL lidocaine without epinephrine, Xylocaine 20 mg·mL^{-1}, AstraZeneca, Cambridge, UK). Muscle biopsy samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis. A catheter was placed in an antecubital vein of the right arm for blood sampling. Then, a 6-min performance test was started, in which participants were instructed to exert themselves maximally, producing as high a power output as possible during the 6 min. The performance test was initiated 10 min after the MVC and 15 min after the warm-up. Blood samples were drawn before, and in 2-min intervals during the performance test, as well as 2 and 4 min after completion of the performance test and immediately analyzed for lactate, glucose, and K^+ in a blood gas analyzer (ABL 800 FLEX, Radiometer, Copenhagen, Denmark). Pulmonary gas exchange was measured continuously starting 4 min before and finishing at the end of the performance test. Immediately upon completion of the performance test, a biopsy from m. vastus lateralis of the right thigh was obtained and immediately frozen in liquid nitrogen, followed by measurements of MVC and contractile properties.

All experimental trials were identical apart from the drug administration and were conducted at the same time of day for each participant to avoid influence of circadian rhythm. Participants were asked to record and replicate their dietary intake on experimental days and to abstain from caffeine and analgesics for 24 h, as well as strenuous physical activity for 48 h before each experimental visit. Experimental trials were separated by at least 1 wk to ensure complete washout of the study drugs and to allow participants to be fully recovered before the next experimental trial.

Table 1. *Participant characteristics (n = 14)*

Age, yr	26 (±6)
Height, cm	182 (±6)
Weight, kg	74.0 (±7.7)
Lean mass, kg	61.7 (±6.1)
Fat mass, kg	9.9 (±2.6)
Fat%	13.8 (±3.3)
$\dot{V}O_{2max}$, mL·min^{-1}	4,719 (±538)
$\dot{V}O_{2max}$, mL·min^{-1}·kg^{-1})	63.9 (±5.0)

Values are mean (±SD). $\dot{V}O_{2max}$, maximal oxygen uptake.

Experimental Procedures

Performance test.

The 6-min performance test was performed on a bicycle ergometer (Monark LC7, Vansbro, Sweden) connected to a computer where cadence and power output were recorded with Monark software (Monark Test Software, v. 3.3). Participants rested on the bicycle ergometer for 4 min for determination of resting oxygen uptake. After the 4-min rest, participants pedaled against a brake load of 10 N at a cadence of 70–80 rpm (≈75 W) for 30 s, after which the 6-min performance test started. Each participant's brake load was calculated individually with a formula employing iPPO and average rpm from the incremental test and based on experience from previous studies in our laboratory (37, 38): Load (N) = $\left(\frac{\text{iPPO}}{11}\right) + \frac{\text{average rpm incremental test}}{(-10)} + 10$. The brake load (gearing) during the performance test was fixed and could

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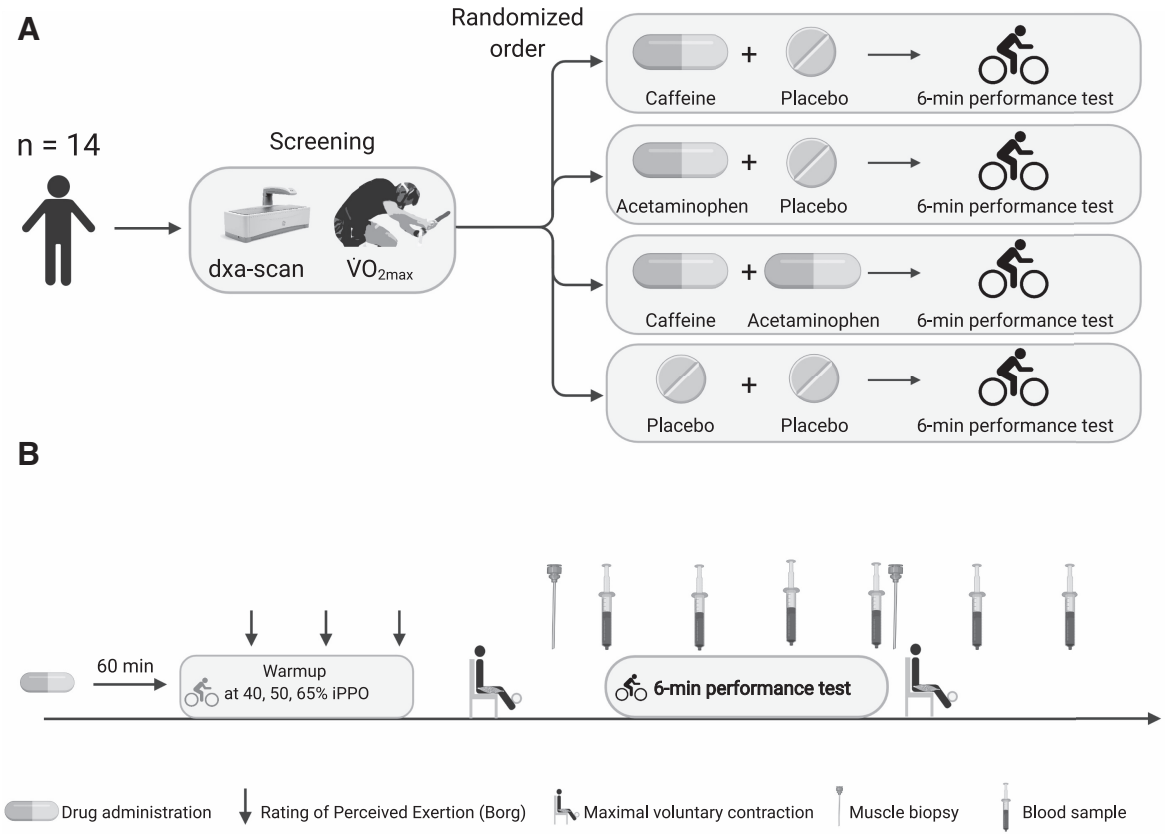

Figure 1. Graphical presentation of the study (*A*) and trial days (*B*). DXA, dual-energy X-ray absorptiometry; iPPO, incremental peak power output, $\dot{V}O_{2max}$, maximal oxygen uptake.

not be adjusted, hence change in power output was solely through changes in rpm. The same individual brake load was used for each participant's experimental trial. During the performance test, participants were informed about elapsed time every 30 s, except for the final minute where the time was given every 15 s. Participants were blinded for rpm, and minimal verbal encouragement was provided to standardize testing conditions. Bike ergometer settings were recorded and repeated at subsequent trials. Participants were allowed to bring their own cycling shoes.

Measurements of MVC and contractile properties.

Measurements of contractile properties of the quadriceps muscle were performed with participants seated on a customized chair, with their right leg secured to a strain gauge (Vishay Precision Group, Tedea Huntleigh, Model No. 615) by velcro tape placed just above the malleoli. The right leg was visually adjusted to 90° flexion, and velcro tape was secured around the hip to limit force production to the quadriceps muscle. Participants were instructed to hold on to metal bars secured to the table during contraction. The distance from the patella to the malleoli was measured to calculate torque, and to be reproduced. Before initiation of the test, self-adhesive electrodes (PALS Platinum, Model 895240, Axelgaard Manufacturing CO., LTD. DK, 8520 Lystrup, Denmark) were placed on the right thigh above rectus femoris and vastus lateralis at 25% and 75% of the distance measured between spina iliaca superior anterior and the base of the patella.

To familiarize participants, three submaximal contractions at self-estimated 30%, 50%, and 70% of maximal voluntary contraction were performed. Then, three electro-stimulations were delivered with constant-current electro-stimulation (Digitimer, model DS7AH, Welwyn Garden City, England) at 300, 600, and 999 mA with 400 V for 200 μs, separated by ≈30 s, to gradually familiarize to the subsequent stimulation intensity employed during the MVC protocol. Each MVC session consisted of a 3–4 s maximal voluntary contraction under strong verbal encouragement with no visual feedback. When a plateau in force production was reached, an electro-stimulation was delivered to create a superimposed twitch. A potentiated twitch was delivered ~1 s following relaxation.

The strain gauge signal was received by an amplifier (AD instruments, Octal bridge Amp, model ML228, Australia) connected to a computer. Data were recorded at 1 kHz and analyzed in LabChart 7 (AD instruments, v. 7.3.8). The following parameters were determined: MVC (N·m): highest torque during a maximal voluntary contraction; peak twitch (PT) torque (N·m): highest torque developed during potentiated stimulation 1 s following relaxation from MVC; time-to-peak twitch torque: time from potentiated stimulation to peak twitch torque; half-relaxation time (ms): time from peak twitch torque to half of peak twitch torque. The highest MVC and peak twitch (PT) value, and the lowest half relaxation time (HRT) and time to peak twitch (TPT) value from

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each experimental trial were used. PT was employed as a measure of peripheral fatigue, in accordance with other studies of peripheral fatigue (31, 39). Voluntary activation (VA) was calculated as described by Bachasson et al. (40), using the following equation: $VA = \left[1 - \left(\frac{T_{SI}}{T_{Pot}}\right)\right] \times 100$, where T_{SI} is the superimposed twitch on top of MVC and T_{Pot} is the potentiated twitch after relaxation. In cases where T_{SI} was delivered slightly after MVC, a correctional equation was used as described by Strojnik and Komi (41): $VA = [100 - (T_{SI} - T_b) \times \frac{\left(\frac{T_b}{MVC_{peak}}\right)}{T_{Pot}}] \times 100$, where T_b is the torque immediately before T_{SI} and MVC_{peak} is the highest MVC value obtained without any stimulation.

Muscle Metabolites and Glycogen Determination

Muscle samples were freeze-dried at −20°C for 48 h and at room temperature for 2 h. Freeze-dried muscle tissue was dissected free of fat, blood, and connective tissue under a microscope. For determination of muscle lactate, ≈1 mg dry wt of muscle tissue was homogenized in a buffer containing 0.6 M perchloric acid and 1 mM EDTA, neutralized to pH 7.0 with 2.2 M $KHCO_3$, and stored at −80°C until analyzed for muscle lactate fluorometrically (42). Muscle glycogen content was determined by extraction of ≈2 mg dry wt muscle tissue in 1 N HCl and hydrolyzation at 100°C for 3 h, followed by the hexokinase method, as previously described (42). ATP and PCr were determined with muscle tissue extracted in a solution of 1.5 M perchloric acid and 1 mM ethylenediaminetetraacetic acid, neutralized to pH 7.0 with 2.2 M $KHCO_3$, and stored at −80°C until analyzed enzymatically, as previously described (18).

Immunoblotting and SDS-PAGE

Protein contents were determined by Western blotting as described previously (43). Briefly, ≈2 mg dry wt muscle tissue was homogenized for 1 min at 30 Hz on a shaking bead-mill (TissueLyser II, Qiagen, Valencia, CA) in ice-cold lysis buffer containing: 10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 2 mM Na_3VO_4, 10 mM NaF, 2 mM PMSF, 1 mM EDTA (pH 8), 1 mM EGTA (pH 8) 10 μg·mL^{-1} aprotinin, 10 μg·mL^{-1} leupeptin, and 3 mM benzamidine. Samples were rotated end-over-end for 30 min at 4°C and centrifuged (18,320 *g*) for 20 min at 4°C after which the supernatant was collected. The protein concentration of each sample was determined in triplicate with a BSA kit (Thermo Fisher Scientific, MA) and samples were created in duplicate with 6× Laemmli buffer (7 mL 0.5 M Tris-base, 3 mL glycerol, 0.93 g DTT, 1 g SDS, and 1.2 mg bromophenol blue) and ddH_2O to achieve equal protein concentration. Equal amounts of protein were loaded in wells of precasted 4%–15% gels (Bio-Rad Laboratories, CA) with all samples for each participant loaded on the same gel. Proteins were then separated according to their molecular weight by SDS-PAGE and semi-dry transferred to a PVDF membrane (Millipore A/S, Copenhagen, Denmark). Membranes were blocked for 15 min in either 2% skim milk or 3% BSA in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) before an overnight incubation in primary antibody at 4°C and a subsequent incubation in horseradish peroxidase conjugated secondary antibody at room temperature for 1 h. Bands were visualized with ECL (Millipore) and recorded with a digital camera (ChemiDoc MP Imaging System, Bio-Rad Laboratories). Bands were quantified using Image Lab version 6.0 (Bio-Rad Laboratories) and determined as the total band intensity adjusted for background intensity.

Primary antibodies used were: 5′AMP-activated protein kinase α (AMPKα, No. 2531, Cell Signaling Technology, MA), p-AMPKα^{Thr172} (No. 2531, Cell Signaling Technology), acetyl-CoA carboxylase (ACC, No. 3676, Cell Signaling Technology), p-ACC^{Ser79} (No. 07-303, Sigma-Aldrich), PKA substrate (No. 9621, Cell Signaling), phospholamban (PLN, No. PA5-19351, Pierce—Thermo Fischer Scientific), p-phospholamban^{Thr17} (No. SC-17024, Santa Cruz Biotechnology, Santa Cruz, CA), phospholemman/FXYD1 (No. 13721-1-AP, Proteintech Group, Chicago, IL), unphosphorylated FXYD1 (AB_FXYD1). Secondary antibodies used were Alexa-350 goat anti-rabbit (1:5,000; Invitrogen P10994, Life Technologies Denmark) and Alexa-555 donkey anti-mouse (1:1,000; Invitrogen A31570).

Statistical Analysis

Statistical analysis was performed in SPSS version 25.0 (IBM, Armonk) and tested for normality with Shapiro–Wilk test and QQ-plots. Data were normally distributed and are presented as means [±standard deviation (SD)]. Outcome statistics are presented as mean effect size (±95% confidence interval [CI]) and *P* values to represent probability. *P* values below 0.05 were considered statistically significant. Between-treatment differences were estimated by repeated measures two-tailed linear mixed modeling with treatment and time as fixed factors, and participant as random factor. Between-group differences were estimated with pairwise comparisons. Power output data were analyzed with a repeated measures one-factor linear mixed model ANOVA with power output as fixed factor and participant as random factor. Due to failure of power output recording, power output data from one trial (caffeine + acetaminophen) was excluded from analysis for one participant.

RESULTS

Performance Test

During the 6-min performance test, mean power output was 312 ± 41 W for placebo, 317 ± 43 W for caffeine, 313 ± 45 W for acetaminophen, and 317 ± 44 W for caffeine + acetaminophen. There was an effect of treatment ($P = 0.009$), with greater mean power output with caffeine (+5 W; 95% CI: 1 to 9; $P = 0.017$) and caffeine + acetaminophen (+6 W; 95% CI: 0 to 12; $P = 0.049$) than placebo, whereas acetaminophen alone did not affect mean power output significantly (+1 W; 95% CI: −4 to 7; $P = 0.605$). There were no apparent differences in mean power output between caffeine and caffeine + acetaminophen ($P = 0.689$; Fig. 2*A*). Pacing during the performance test expressed in 1-min intervals (Fig. 2*B*) revealed no apparent differences between individual time points (treatment × time $P = 0.998$). No order effect was evident, as mean power output was not significantly different ($P = 0.995$) between trial visits (first visit: 314 ± 42 W; second visit: 315 ± 42 W; third visit: 314 ± 43 W; fourth visit: 315 ± 38 W).

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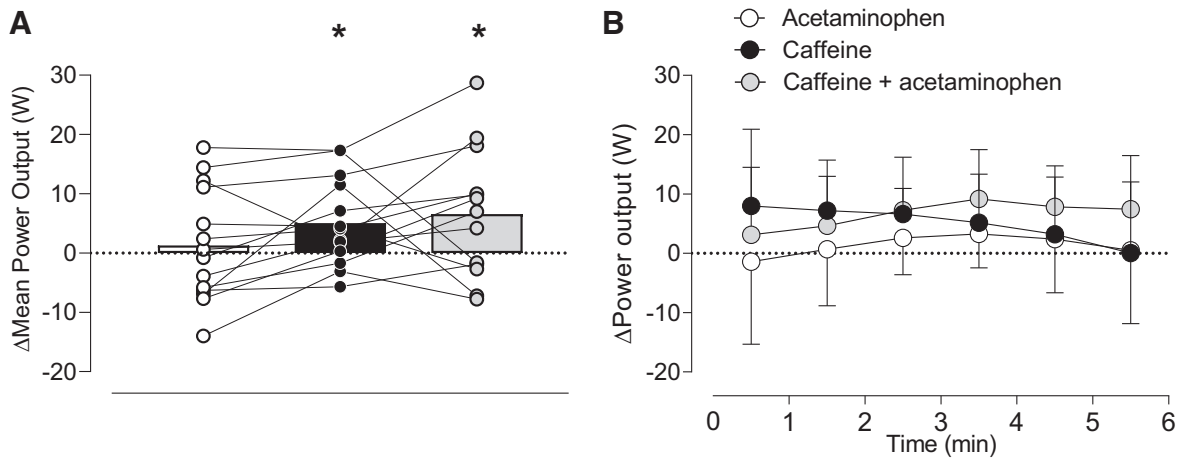

Figure 2. Individual and mean (bars) changes in mean power output compared with placebo (*A*) and mean power output in 1-min intervals compared with placebo (*B*) after treatment with caffeine (5 mg·kg body wt^{-1}; black circles), acetaminophen (1,500 mg; white circles), or combined caffeine and acetaminophen (gray circles) during a 6-min performance test in highly trained men. $n = 14$ participants at all trials except caffeine + acetaminophen which was $n = 13$. *Different from placebo ($P \leq 0.05$). Data in (B) are means ± 95% CI.

Quadriceps Contractile Function

Before the 6-min performance test, there were no significant treatment differences in MVC ($P = 0.682$), voluntary activation ($P = 0.889$), peak twitch torque ($P = 0.713$), and time to peak twitch torque ($P = 0.820$), whereas half relaxation time was lower with caffeine than placebo ($P = 0.024$). Treatment affected the decline in MVC with the performance test, as both acetaminophen and caffeine + acetaminophen displayed a lower ($P = 0.024$ and $P = 0.049$, respectively) decline than placebo (Table 2), whereas caffeine did not affect the decline from before to after the performance test ($P = 0.849$). Peak twitch torque, time to peak twitch torque, half relaxation time, and voluntary activation were not significantly different between treatments, before or after the performance test, but a main effect of time was evident, where voluntary activation ($P = 0.001$) and peak twitch torque decreased ($P < 0.001$) with the performance test, whereas half relaxation time lengthened ($P < 0.001$; Table 2).

Muscle Metabolites and Glycogen

Before the performance test, muscle ATP ($P = 0.283$), phosphocreatine (PCr; $P = 0.452$), and glycogen ($P = 0.257$) content were not significantly different between treatments, but muscle lactate content was 5.1 mmol·kg dry wt^{-1} higher with caffeine ($P = 0.007$), but not with caffeine + acetaminophen (+3.1 mmol·kg dry wt^{-1}; $P = 0.059$), than placebo (Table 3). During the performance test, the net decline in muscle PCr content was higher with caffeine + acetaminophen than placebo ($P = 0.017$) and caffeine ($P = 0.005$), but not different from acetaminophen ($P = 0.269$). Muscle lactate accumulation was not higher than placebo with any of the treatments, but caffeine + acetaminophen resulted in higher muscle lactate accumulation than with caffeine ($P = 0.025$) and acetaminophen ($P = 0.035$). There were no apparent differences with treatment in net decline in muscle ATP and glycogen content during the performance test (Table 3).

Muscle Protein Phosphorylation

Before the start of the performance test, there were no apparent differences in phosphorylation ratios of 5′AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), phospholamban (PLN), phospholemman (FXYD1), and protein kinase A (PKA) substrates (all $P > 0.05$; Fig. 3). During the performance test, phosphorylation increased for ACC (main effect of time $P < 0.001$), PLN ($P < 0.001$), FXYD1 ($P = 0.002$), and PKA substrates ($P < 0.001$), with no difference between treatments (treatment × time, $P > 0.1$ for all), whereas phosphorylation of AMPK did not change significantly ($P = 0.111$; Fig. 3). Overall, greater (main effect of treatment $P = 0.007$) AMPK phosphorylation was evident only with caffeine + acetaminophen ($P = 0.003$), whereas it was unchanged with caffeine ($P = 0.055$) and acetaminophen ($P = 0.059$) compared with placebo.

Plasma Lactate, Glucose, and K^+

There were no apparent differences in plasma concentrations of lactate, glucose, and K^+ during the performance test (treatment × time point: $P = 0.622$, $P = 0.737$, and $P = 0.378$, respectively). Main effects of treatment during the performance test were evident for a higher plasma glucose with caffeine ($P < 0.001$) and caffeine + acetaminophen ($P < 0.001$; Fig. 4*B*) than placebo, and lower plasma K^+ with caffeine ($P = 0.001$) and caffeine + acetaminophen ($P = 0.026$) than placebo (Fig. 4*C*). In recovery, there were no apparent differences in plasma concentrations of lactate, glucose, and K^+ (treatment × time point: $P = 0.791$, $P = 789$, $P = 0.968$), but there was a main effect of treatment for higher plasma lactate with caffeine + acetaminophen ($P = 0.040$) than placebo, and higher plasma glucose with caffeine ($P < 0.001$) and caffeine + acetaminophen ($P < 0.001$) than placebo.

Oxygen Uptake

Oxygen uptake was not different between treatments during the performance test (treatment × time point: $P = 0.997$). Participants reached 97.0 ± 6.4%, 96.5 ± 5.3%, 96.1 ± 8.0%, and

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Table 2. *Contractile function of the quadriceps muscle before (Pre), after (Post), and expressed as delta-change (Δ) after a 6-min performance test with prior ingestion of placebo, caffeine (5 mg·kg body wt^{-1}), acetaminophen (1,500 mg), or combined caffeine and acetaminophen in highly trained men*

	Placebo (n = 14)			Caffeine (n = 14)			Acetaminophen (n = 14)			Caffeine + Acetaminophen (n = 14)			Interaction (treatment × time)
	Pre	Post	Δ (95% CI)	Pre	Post	Δ (95% CI)	Pre	Post	Δ (95% CI)	Pre	Post	Δ (95% CI)	P Value
MVC, N·m	232 (±54)	179 (±56)	−53 (−71 to −39)	224 (±59)	170 (±74)	−54 (−69 to −38)	224 (±49)	184 (±52)	−40[a,b] (−53 to −30)	229 (±61)	185 (±74)	−44[a,b] (−58 to −30)	0.035
VA, %	91.8 (±4.7)	80.1 (±8)	−11.7 (−15 to −8)	91.1 (±5.5)	78.4 (±11)	−12.7 (−17 to −8)	91.2 (±5.6)	79.1 (±12)	−12.1 (−18 to −7)	90.6 (±4.9)	79.8 (±7)	−10.9 (−14 to −8)	0.651
PT, N·m	81 (±21)	45 (±11)	−35 (−50 to −23)	80 (±21)	45 (±20)	−35 (−48 to −24)	77 (±20)	47 (±14)	−30 (−40 to −19)	77 (±17)	46 (±12)	−31 (−41 to −21)	0.243
TPT, ms	76 (±6)	79 (±14)	3 (−6 to 11)	77 (±9)	75 (±9)	−1 (−10 to 6)	75 (±8)	76 (±12)	1 (−7 to 7)	76 (±10)	75 (±12)	−1 (−8 to 7)	0.119
HRT, ms	42 (±15)	57(±19)	15 (8 to 21)	35 (±11)*	46 (±18)	12 (3 to 25)	41 (±16)	53 (±22)	12 (3 to 20)	43 (±14)	61 (±20)	18 (8 to 28)	0.450

Data are means (±SD) for Pre and Post values and mean (±95% CI) for Δ values. HRT, half relaxation time; MVC, maximal voluntary contraction torque; PT, peak twitch torque; TPT, time to peak twitch torque; VA, voluntary activation. [a]Different from placebo ($P \leq 0.05$); [b]Different from caffeine ($P \leq 0.05$); *Different from placebo at Pre ($P \leq 0.05$).

Table 3. *Skeletal muscle metabolites and glycogen before (Pre), after (Post), and expressed as delta-change (Δ) after a 6-min performance test with prior ingestion of placebo, caffeine (5 mg·kg body wt^{-1}), acetaminophen (1,500 mg), or combined caffeine and acetaminophen in highly trained men*

	Placebo (n = 14)			Caffeine (n = 14)			Acetaminophen (n = 14)			Caffeine + Acetaminophen (n = 14)			Interaction (treatment × time)
	Pre	Post	Δ (95% CI)	Pre	Post	Δ (95% CI)	Pre	Post	Δ (95% CI)	Pre	Post	Δ (95% CI)	P Value
ATP	25.9 (±4.2)	23.1 (±4.0)	−2.8 (−6.0 to −0.4)	29.7 (±4.1)	23.6 (±5.4)	−6.1 (−8.9 to −3.7)	27.2 (±3.7)	23.9 (±3.9)	−3.3 (−5.1 to 0.0)	26.7 (±5.2)	24.4 (±5.2)	−2.3 (−4.7 to 0.0)	0.061
PCr	74.0 (±14.7)	34.4 (±17.0)	−39.6 (−51.8 to −23.6)	74.2 (±15.6)	39.4 (±20.5)	−34.7 (−47.1 to −25.4)	81.1 (±17.2)	29.9 (±14.2)	−51.2[b] (−60.1 to −38.0)	77.7 (±19.5)	23.0 (±7.9)	−54.7[a,b] (−64.9 to −45.6)	0.036
Glycogen	489 (±113)	392 (±104)	−98 (−137 to −59)	482 (±135)	397 (±141)	−85 (−121 to −49)	474 (±118)	375 (±112)	−98 (−161 to −36)	449 (±89)	332 (±81)	−117 (−143 to −91)	0.285
Lactate	6.8 (±3.4)	92.2 (±42.0)	85.3 (60.0 to 110.0)	11.9 (±6.2) [a]	86.9 (±44.3)	74.9 (50.8 to 99.1)	8.1 (±7.0)	84.2 (±32.7)	76.2 (57.7 to 95.6)	9.9 (±9.3)	112.6 (±26.4)	102.7[b,c] (88.5 to 114.9)	0.066

Data are means (±SD) for Pre and Post values and mean (±95% CI) for Δ values. All values are mmol·kg dry wt^{-1}. ATP, adenosine triphosphate; Cr, creatine; PCr, phosphocreatine. [a]Different from placebo ($P \leq 0.05$). [b]Different from caffeine ($P \leq 0.05$). [c]Different from acetaminophen ($P \leq 0.05$).

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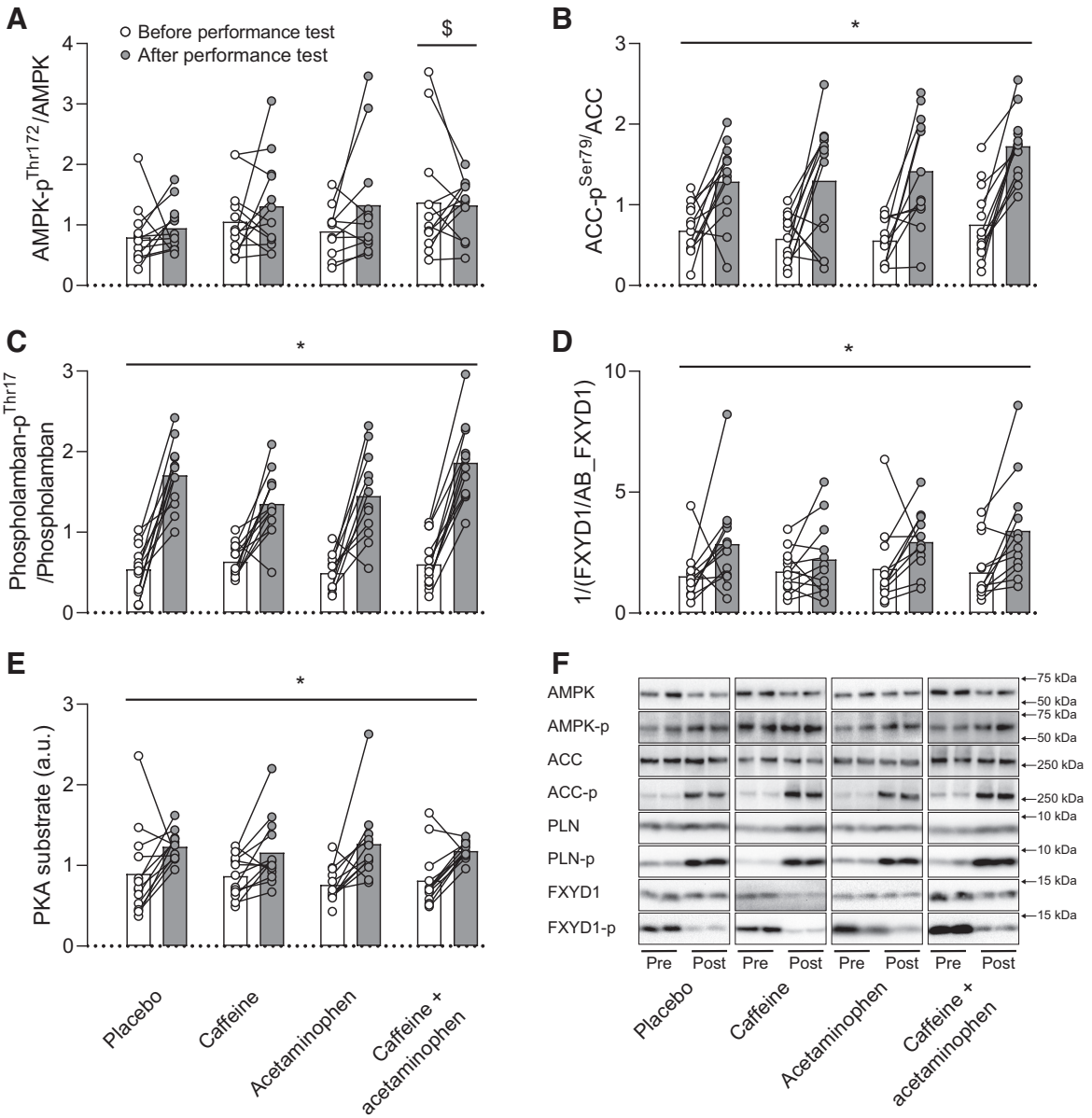

Figure 3. Mean (bars) and individual (circles) values for muscle phosphorylation ratios of 5′AMP-activated protein kinase (AMPK; *A*), acetyl-CoA carboxylase (ACC; *b*), phospholamban (PLN; *C*), phospholemman (FXYD1; *D*), and total phosphorylation of protein kinase A (PKA) substrates (*E*) before (white circles) and after (gray circles) a 6-min performance test after treatment with placebo, caffeine (5 mg·kg body wt^{-1}), acetaminophen (1,500 mg), or combined caffeine and acetaminophen in highly trained men. Representative blots from before (Pre) and after (Post) the performance test (*F*). Note that the AB_FXYD1 antibody recognizes primarily unphosphorylated FXYD1, hence the increased signal intensity at Pre in (*F*). $n = 14$ at all trials, except in acetaminophen at the Post time point where one sample is missing due to inadequate tissue yield. *Main effect of time ($P \leq 0.05$). $Main effect of treatment ($P \leq 0.05$).

98.3 ± 5.7% of individual $\dot{V}O_{2max}$ obtained during the incremental test for placebo, caffeine, acetaminophen, and caffeine + acetaminophen, respectively, during the performance test.

Rating of Perceived Exertion

Rating of perceived exertion during the warm-up at 40%, 50%, and 65% iPPO was not different between treatments (treatment × workload interaction: $P = 0.562$; Table 4).

DISCUSSION

The primary finding of the present study was that co-ingestion of acetaminophen did not increase mean power output during a 6-min performance test more than caffeine alone in highly trained young men. In addition, neither caffeine nor acetaminophen decreased peripheral fatigue, and the performance improvement with caffeine could not be explained by increased skeletal muscle PKA-signaling affecting glycolytic energy production, or phosphorylation of ion handling proteins.

Caffeine and combined caffeine and acetaminophen treatment increased mean power output during the 6-min performance test with 1.6% and 2.1%, respectively, which is comparable to other studies examining caffeine ingestion before high-intensity exercise (9). This increase was attributed to caffeine ingestion, as acetaminophen alone did not

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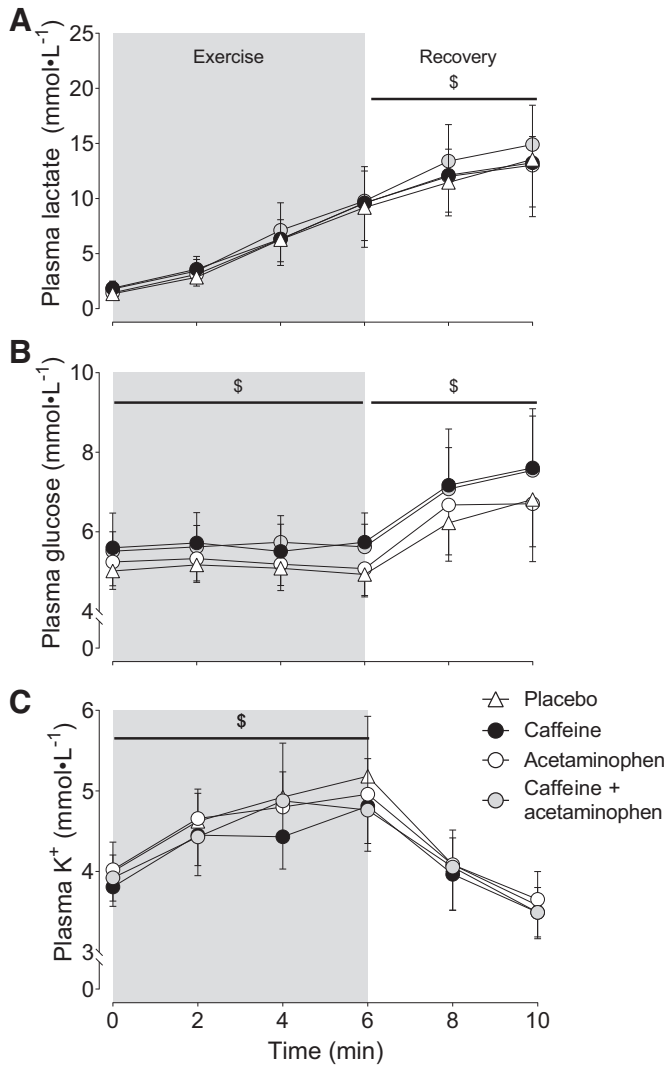

Figure 4. Plasma lactate (*A*), glucose (*B*), and K^+ (*C*) before, during, and in recovery from a 6-min performance test after treatment with placebo (white triangles), caffeine (5 mg·kg body wt^{-1}; black circles), acetaminophen (1,500 mg; white circles), or combined caffeine and acetaminophen (gray circles) in highly trained men. n = 14 in all trials. Data are means (±SD). $Main effect of treatment.

improve mean power output, and acetaminophen did not additively increase performance when co-ingested with caffeine (Fig. 2*A*). In agreement, Tomazini et al. (6) compared the effect of caffeine and acetaminophen on performance during a 4-km cycling time trial (duration ≈7 min) and observed a ≈2% increase in performance with caffeine (5 mg·kg body wt^{-1}), although this was not evident with co-ingestion of acetaminophen. Some methodological differences exist between the current study and that by Tomazini et al., one being the difference in training status of the participants, who in the study by Tomazini et al. were untrained and with a lower $\dot{V}O_{2max}$ than the participants in the present study (39 vs. 64 $mL \cdot kg^{-1} \cdot min^{-1}$). Exogenous substances do not necessarily elicit comparable responses in trained and untrained individuals, as has been reported in several studies (30–32). For example, when participants were divided into low and high performers based on initial time trial performance in a study by Santos et al. (31), caffeine only increased peripheral fatigue in the low performers. Similarly, in a study by Boyett et al. (32), untrained responded more than trained individuals in 3-km time trial performance with caffeine. It can be assumed that some of the discrepancies between untrained and well-trained individuals can be explained by well-trained individuals being accustomed to exercise-induced pain, and thereby deriving less benefit from any potential dampening of pain sensation from both caffeine and acetaminophen. Nonetheless, in the present study, the performance enhancing effects of caffeine were evident among well-trained athletes confirming the relevance for elite athletes, where finishing time differences of less than 1% may decide the outcome in competitive events lasting less than 8 min (9). Conversely, the discrepancy between the present study and that by Tomazini et al. regarding combined ingestion of caffeine and acetaminophen cannot be readily explained but may possibly be attributed to differences in training status or the employed testing protocol.

The lack of additional performance enhancement with co-ingestion of acetaminophen in the present study aligns with the observation that acetaminophen on its own had no effect on performance. This is in contrast to studies reporting improved power output after acetaminophen intake during cycling exercise of shorter [3 min (44)] and longer [≈26 min (27)] durations than employed in the present study. As the dose of acetaminophen administered in those studies (1,000–1,500 mg) and training status of participants ($\dot{V}O_{2max}$ ≈60 $mL \cdot min^{-1} \cdot kg^{-1}$) were comparable with the present study, the discrepancies between the present and previous studies are not easily explained but may be related to exercise duration. Nonetheless, the current findings do not support a performance enhancing effect of acetaminophen during short-term high-intensity exercise.

Although the effects of combined caffeine and acetaminophen ingestion on cycling performance have been investigated previously, the effects on skeletal muscle fatigue and metabolism have not been investigated. In the present study, peripheral fatigue levels after the 6-min performance test were not different between treatments (Table 2), with peak twitch torque being ≈40% lower after the performance test in all treatments. In addition, the decline in voluntary activation was similar between treatments. However, acetaminophen attenuated the decrease in post-exercise voluntary contraction torque. One other study reported a similar finding where acetaminophen lowered the decline in maximal voluntary torque during 60 × 3 s repeated quadriceps maximal

Table 4. *Rating of perceived exertion (0–10 Borg scale) with prior ingestion of placebo, caffeine (5 mg·kg body wt^{-1}), acetaminophen (1,500 mg), or combined caffeine and acetaminophen in highly trained men*

	Placebo	Caffeine	Acetaminophen	Caffeine + Acetaminophen
40% iPPO (RPE)	2.1 (±0.5)	1.8 (±0.8)	2.1 (±0.7)	1.8 (±0.7)
50% iPPO (RPE)	3.6 (±0.7)	3.1 (±0.8)	3.6 (±1.0)	3.4 (±0.8)
65% iPPO (RPE)	5.6 (±1.2)	5.2 (±1.4)	5.4 (±1.1)	5.1 (±1.2)

iPPO, incremental peak power output; RPE, rating of perceived exertion. n = 14 for all trials.

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contractions, which was related to a greater post-exercise electromyography (EMG) amplitude than placebo (29). Another study showed an attenuated decline in EMG-amplitude during the last part of a 3-min ergometer performance test with acetaminophen (44). Although those studies indicate that motor neuron drive may explain the attenuated decrease in maximal voluntary contraction torque with acetaminophen, this is inconsistent with the unaltered level of voluntary activation observed in the present study. In addition, the difference in voluntary contraction torque between treatments in the present study is minor (≈3%) and possibly related to the fact that pre-exercise voluntary contraction torque was slightly lower with acetaminophen than placebo. Nevertheless, based on the observations of the decrease in peak twitch torque being unaffected by treatment, caffeine and acetaminophen do not seem to affect peripheral fatigue after high-intensity exercise.

A putative mechanism for the performance enhancement with caffeine is through stimulation of muscle β-adrenoceptors. Indeed, in the present study, increases in plasma glucose indicated that epinephrine and norepinephrine concentrations were increased by caffeine. However, this did not appear to contribute to the performance enhancement as even though, and perhaps because, PKA substrates were markedly phosphorylated by exercise, there were no detectable differences between treatments. Likewise, muscle lactate and AMPK-phosphorylation at rest indicated that caffeine (and to some extent acetaminophen) induced a signaling response in muscle, but this too was overshadowed by the phosphorylation induced by exercise. Increases in PKA activity induce a shift in skeletal muscle substrate utilization (45), but this could not explain the performance enhancement with caffeine. Although combined ingestion of caffeine and acetaminophen indeed resulted in greater muscle lactate content associated with the higher mean power output (Table 3), this was not the case with caffeine alone despite the similarly increased mean power output (Fig. 2*A*). Likely, this is explained by the differences in pacing strategy with treatments (Fig. 2*B*). Thus, the difference in muscle lactate accumulation may primarily reflect the difference in power output during the last part of the performance test rather than the entire test. Indeed, combined ingestion of caffeine and acetaminophen displayed on average ≈5 W higher mean power output than the other treatments during the latter half of the performance test (Fig. 2*B*), thus possibly explaining the discrepancy in muscle lactate. This is further supported by the higher muscle PCr depletion with combined ingestion (Table 3). Further in support of unchanged muscle metabolism with caffeine was the observation that glycogen content decreased to the same extent between treatments, which is in agreement with other studies (14, 24). Lastly, despite an overall lower plasma K^+ with caffeine, muscle protein phosphorylation indicated no effect on skeletal muscle ion handling, as markers of Na^+/K^+-ATPase and SERCA activity were phosphorylated by exercise regardless of treatment. However, Mohr et al. (22) observed lowered interstitial K^+ concentrations during knee-extensor exercise with caffeine despite unaltered plasma K^+ levels, suggesting that plasma K^+ may not be a sensitive marker of interstitial K^+ concentrations. Nevertheless, increased beta-adrenergic action on skeletal muscle PKA-signaling, glycolytic energy production, or phosphorylation of ion handling proteins does not seem to explain the increased performance with caffeine.

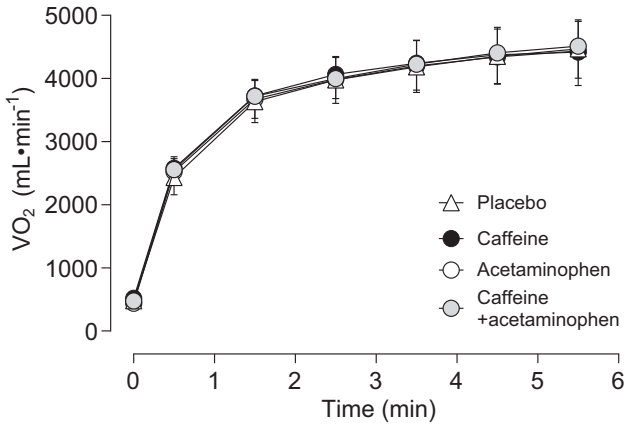

Figure 5. Oxygen uptake ($\dot{V}O_2$) during a 6-min performance test after treatment with placebo (white triangles), caffeine (5 mg·kg body wt^{-1}; black circles), acetaminophen (1,500 mg; white circles), or combined caffeine and acetaminophen (gray circles) in highly trained men. Data are means ± SD.

Likewise, aerobic energy production did not seem to contribute to the observed performance enhancements (Fig. 5), and rating of perceived exertion during submaximal exercise was unaffected, in contrast to previous studies showing that both caffeine and acetaminophen may result in lower ratings of perceived exertion during fixed workloads (46, 47) or similar ratings of perceived exertion despite increased workloads (27, 48). One possible explanation for this discrepancy may be that the participants in the present study were asked to rate exertion at relatively low workloads, and it cannot be excluded that exertion would have been rated differently during the performance test where intensity was much higher or if higher power outputs had been used during the standardized warmup.

In summary, co-ingestion of acetaminophen and caffeine did not increase mean power output during a 6-min performance test more than caffeine alone. Neither caffeine nor acetaminophen decreased peripheral fatigue and the performance improvement with caffeine could not be explained by increased β-adrenergic action on skeletal muscle, glycolytic energy production, or phosphorylation of ion handling proteins. Thus, there is no additive effect of acetaminophen on performance enhancement with caffeine, and acetaminophen does not improve performance on its own during a 6-min performance test. In addition, change in skeletal muscle PKA activity by increased beta-receptor stimulation does not appear to be a major mechanism of performance improvement with caffeine.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.M.C., M.H., and J.B. conceived and designed research; S.J. and K.E. performed experiments; S.J. and K.E. analyzed data; S.J., K.E., P.M.C., M.H., and J.B. interpreted results of experiments; S.J. prepared figures; S.J. drafted manuscript; S.J., K.E., P.M.C., M.H., and J.B. edited and revised manuscript; S.J., K.E., P.M.C., M.H., and J.B. approved final version of manuscript.

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