Effect of a divided caffeine dose on endurance cycling performance, postexercise urinary caffeine concentration, and plasma paraxanthine

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This study compared the effects of a single and divided dose of caffeine on endurance performance and on postexercise urinary caffeine and plasma paraxanthine concentrations. Nine male cyclists and triathletes cycled for 90 min at 68% of maximal oxygen uptake, followed by a self-paced time trial (work equivalent to 80% of maximal oxygen uptake workload over 30 min) with three randomized, balanced, and double-blind interventions: 1) placebo 60 min before and 45 min into exercise (PP); 2) single caffeine dose (6 mg/kg) 60 min before exercise and placebo 45 min into exercise (CP); and 3) divided caffeine dose (3 mg/kg) 60 min before and 45 min into exercise (CC). Time trial performance was unchanged with caffeine ingestion ($P = 0.08$), but it tended to be faster in the caffeine trials (CP: 24.2 min and CC: 23.4 min) compared with placebo (PP: 28.3 min). Postexercise urinary caffeine concentration was significantly lower in CC (3.8 μg/ml) compared with CP (6.8 μg/ml). Plasma paraxanthine increased in a dose-dependent fashion and did not peak during exercise. In conclusion, dividing a caffeine dose provides no ergogenic effect over a bolus dose but reduces post-exercise urinary concentration.

The majority of laboratory studies demonstrating the ergogenic effect of caffeine ingestion on performance administer caffeine as a single dose 1 h before exercise (3, 6, 12–15, 23, 25). This is largely to ensure a peak plasma concentration during exercise. However, it is unknown whether this is the optimal timing of caffeine administration to maximize its ergogenic effect. Few studies have measured the plasma concentration of caffeine during exercise or examined its variability in exercising subjects.

To date, five studies have investigated the effects of repeated caffeine administration during exercise (7, 19, 21, 26, 29), and not all observed a performance-enhancing effect. Of these, only two have employed a self-paced time trial, a protocol more reliable (20) and representative of competition, as an endurance performance measure (7, 21). Thus there is limited available information on the effects of the timing of caffeine ingestion during simulated competition.

As a central nervous system stimulant, caffeine is classed as a prohibited substance by the International Olympic Committee (IOC) and other sporting bodies. However, because caffeine is present in many commonly ingested foodstuffs, 12 μg/ml is the permissible postexercise urinary threshold under which no doping offense is recorded. Performance-enhancing effects have been observed with postexercise urinary levels well below this threshold (14, 25), so it is uncommon for a positive result to be registered (9). There is substantial inter-subject variability in the metabolism and elimination of caffeine (4, 21, 25), particularly during exercise (11). Thus urinary caffeine concentration as a marker of caffeine consumption may not accurately reflect dose or plasma levels.

Both Cox et al. (7) and Kovacs et al. (21) measured postexercise urinary caffeine concentrations after caffeine ingestion during exercise. The former study showed no effect of splitting the dose on urine concentrations, but the latter observed lower concentrations (2.5 μg/ml) after a split dose than previous studies [5.8 μg/ml, 6.1 μg/ml (28), and 4.8 μg/ml (25)] with a bolus dose. It is possible that splitting the caffeine dose

CAFFEINE IS ONE OF THE MOST widely consumed drugs in the world and is known to be ingested by sportspeople to augment performance. Ample laboratory-based (6, 8, 13–16, 19, 21, 25) and some field-based evidence (3, 23) demonstrate the beneficial effects of caffeine on endurance exercise performance. However, few reports document the incidence of its use as an ergogenic aid in the athletic population and its ideal pattern of delivery.

There is some evidence that caffeine-containing cola drinks are commonly consumed by endurance cyclists during their event, particularly toward the latter stages (24). Sport or “energy” drinks and carbohydrate gels that contain caffeine (or caffeine analogs such as guarana) have recently become widely available. Such products are designed for consumption both before and during endurance exercise.

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delays the appearance in the urine but still provides a similar ergogenic effect to a bolus dose.

Paraxanthine, the primary metabolite of caffeine, accounts for >80% of caffeine degradation (22). It is pharmacologically active as an adenosine-receptor antagonist and thus potentially ergogenic (2, 17, 18, 27), presenting difficulties in determining whether caffeine alone is responsible for the effects on exercise performance. Little is known about paraxanthine kinetics during exercise.

The purpose of this study was threefold: 1) to compare the ergogenic effects of caffeine administration before exercise with administration before and during simulated endurance cycling performance, 2) to test the hypothesis that dividing the dose would reduce the postexercise urinary concentration, and 3) to observe the plasma kinetics of paraxanthine during exercise after the single and divided dose.

METHODS

Subjects. Nine healthy and well-trained cyclists and triathletes agreed to participate in the study. The subjects [age 25.5 ± 5 (SE) yr, weight 76.4 ± 6.9 kg, maximal oxygen uptake (Vo2 max) 5.5 ± 0.3 l/min] were nonsmokers. Each subject was fully informed about the experimental procedures and possible risks before giving informed, written consent. The protocol was approved by the Human Ethics Committee of The University of Sydney.

Preexperimental protocol. Subjects reported to the laboratory before the start of the experiment for an incremental test to measure their submaximal oxygen uptake and Vo2 max on an electronically braked cycling ergometer (Lode Excalibur Sport, Groningen, The Netherlands). All subsequent exercise tests were done on the same ergometer. The submaximal test required each subject to cycle in a stepwise fashion for 8 min at four submaximal workloads (100, 175, 250, and 325 W) at a constant, self-selected cadence. The Vo2 max test followed, beginning 5 min after the last submaximal workload, and required a linear increase in power from 0 W at 1 W/s until volitional fatigue. A relationship between oxygen consumption in the last minute of each submaximal workload and power was developed and used to calculate a workload equivalent to both 70 and 80% of Vo2 max.

Experimental protocol. The study was a randomized, double-blind, placebo-controlled, balanced factorial trial. Subjects were tested on three occasions where gelatine capsules were given to ensure compliance. The protocol was approved by the Human Ethics Committee of The University of Sydney.

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Experimental protocol. The study was a randomized, double-blind, placebo-controlled, balanced factorial trial. Subjects were tested on three occasions where gelatine capsules containing either lactose (placebo) or caffeine were administered 1 h before exercise and 45 min into exercise. The treatments administered in a double-blind manner were placebo-placebo (PP), single caffeine dose (6 mg/kg)-placebo (CP), and caffeine (3 mg/kg)-caffeine (3 mg/kg) (CC). The subjects were assigned randomly to one of three test sequences (PP-CP-CC, CP-CC-PP, or CC-PP-CP), each containing three subjects. After the test, subjects were asked whether they could determine which treatment they had received. The tests were administered on the same day of the week, with an interval of 7 days between tests.

Subjects arrived at the laboratory after an overnight (12 h) fast. They were encouraged to drink adequate water on the previous evening and on arising to ensure full hydration. Subjects were asked to refrain from ingesting products containing caffeine for 48 h before presentation and to refrain from heavy exercise 24 h before testing. To minimize variation in preexercise muscle glycogen status, subjects were required to repeat the same training and food selection for subsequent tests. Food and training diaries were collected for the 48 h before presentation. Written and verbal reminders were given to ensure compliance.

At the start of each test, a catheter was inserted in the dorsal aspect of the hand. The catheter was kept patent by flushing with saline every 15 min. A resting blood sample (~60 min) was obtained. The test dose (caffeine or placebo) was administered, and the subject remained seated for 1 h. Further samples were collected after 30 min of rest (~30 min) and just before the beginning of exercise (0 min).

Exercise consisted of a 5 min warm-up at a workload of half that calculated to elicit an oxygen uptake of 70% Vo2 max. Subjects then cycled for 90 min at the 70% Vo2 max workload. During this first 90 min, the ergometer was set in hyperbolic mode so that the work rates were independent of cadence. At 90 min, the ergometer automatically changed to linear mode (workload was proportional to cadence in a relationship defined by the linear factor) and 30-min self-paced time trial was performed. The linear factor was calculated according to the formula

$$W = L^2(rpm)$$

where L is the linear factor and was determined in a way where W represented the work rate eliciting 80% Vo2 max and rpm was the average pedaling rate in the final stages of the Vo2 max test.

The time trial required the subject to complete a target amount of work (i.e., that eliciting 80% of Vo2 max for 30 min) as quickly as possible. Subjects were aware of the end point and were verbally encouraged to complete the time trial as quickly as possible.

During all tests, the ambient temperature was 22°C and the relative humidity varied between 50 and 60%. A constant fan speed was provided at all times during exercise, and the same self-selected hydration protocol was used during each test.

Blood sampling. Further samples were obtained at 30, 45, 60, and 90 min of exercise and at the completion of performance. Blood was drawn into a 5-ml syringe and divided between a lithium-heparinized tube (2 ml) and a fluoridated tube (2 ml). The tubes were immediately inverted, placed on ice, and later centrifuged (model T J-6 centrifuge, Beckman), and the serum was aliquoted into plastic tubes and frozen at −20°C.

Analyses. Serum caffeine and paraxanthine levels were determined by using fully automated HPLC (model 510, Waters, Milford, MA). Samples were deproteinated by mixing 100 μl of 0.8 M perchloric acid with 100 μl of serum. After vortex mixing, the proteins were removed by centrifugation at 14,000 g (room temperature) for 3–4 min. A 125-μl aliquot of the supernatant was neutralized with 10.7 μl of 4 M sodium hydroxide. Samples were centrifuged for 5 min at 2,000 g and then transferred to plastic 200-μg tubes, respun for 5 min at 2,000 g to remove air bubbles, and placed in the autosampler. The method involved a dilution factor of 2.17. The deproteinized sample (100 μl) was injected by autoinjector and eluted isocratically with the elution buffer [potassium phosphate (3.47 g)–methanol (300 ml)] for 20 min. The mobile phase was filtered through a 0.45-μm filter and degassed under vacuum before use. Eluted peaks were detected by ultraviolet absorbance at 274 nm, and peak areas were used for quantitation by using a four-point standard curve. The elution time for caffeine was 7.7 min and for paraxanthine was 3.3 min. The temperature was ambient (range 21–24°C), and the flow rate was constant at 1.2 ml/min. Standards were run in duplicate at the beginning of and halfway through each assay.
Urine samples were collected at the beginning and end of the test. Immediately after collection, urine samples were stored at −20°C for later analysis of caffeine by using the HPLC system. Urine volume throughout the duration of the test was also recorded.

Statistical analyses. Dependant variables were analyzed by a repeated-measures ANOVA. Polynomial functions of time were chosen in the contrasts to characterize outcome variables measured at several distinct time points. Different treatment groups were then compared by using the appropriate interaction terms in an ANOVA table. All statistical analyses were performed using specialized statistical software (SPSS version 8.2). Statistical significance was accepted at $P \leq 0.05$. All data are expressed as means ± SE unless otherwise stated.

RESULTS

Eight of nine subjects completed all aspects of the trial. One subject was excluded from analysis because he was unable to complete the time trial when given the single dose (CP) of caffeine. The subject experienced nausea and nervousness, which have both been commonly associated with caffeine ingestion. None of the subjects reported habitual consumption of large amounts of caffeine (<250 mg caffeine/day). During the three trials, mean body weight loss during the entire experiment, corrected for urine output, was 1.26 ± 0.6 kg, indicating no significant differences in hydration status between trials. The mean fluid ingested during the exercise protocol was 1,444 ± 309 ml.

Time trial performance. Repeated-measures ANOVA revealed no main effect of sequence of ingestion on performance time ($P = 0.080$). However, there was a clear trend for the time trial to be completed significantly faster after the ingestion of caffeine (average with CP and CC of 23.8 ± 2.8 min) compared with placebo (PP: 28.3 ± 3.1 min) (Fig. 1).

Plasma caffeine levels. Before each trial, subjects showed zero levels of caffeine, confirming their compliance to abstaining from caffeine-containing products before testing. During the experiment, the plasma caffeine levels increased in a dose-related manner after the ingestion of the caffeine capsules, and no caffeine was present when placebo was ingested (Fig. 2A). Plasma caffeine levels were significantly elevated at −30 min ($P = 0.000$) and reached a peak within 90 min after CP administration. With CC there was an initial peak between 0 and 30 min followed by a slow decline until the second caffeine dose when plasma caffeine levels increased again and continued to rise until the end of the time trial. Plasma caffeine concentration was significantly greater in the CP compared with the CC trial until −60 min into exercise ($P = 0.001$), after which the caffeine concentrations were similar ($P = 0.483$). Maximum caffeine concentrations were 7.3 and 6.7 µg/ml for CP and CC, respectively.

Paraxanthine. The presence of paraxanthine in the plasma was significantly higher in the CP compared with the CC trial ($P < 0.05$). Paraxanthine concentration continued to increase after caffeine ingestion but showed a different relationship to the previously described plasma caffeine levels. The increase in paraxanthine levels occurred at a slower rate to caffeine, and no peak concentration was obtained (Fig. 2B).

Urinary caffeine content. There were no significant differences among trials in the volume of urine produced, but it was less after exercise than before. Urine production during the 1-h preexercise period was 228 ± 21, 247 ± 27, and 233 ± 31 ml for PP, CP, and CC, respectively. No caffeine was detected in urine after PP. Mean postexercise caffeine levels for CP and CC were 6.89 ± 0.73 µg/ml (3.70–9.06 µg/ml) and 3.82 ± 0.34 µg/ml (2.35–6.16 µg/ml), respectively ($P = 0.002$; Fig. 3). Between-subject variations in postexercise urinary caffeine levels were large.

When the initial dose of caffeine (3 vs. 6 mg/kg) was considered, there was a significant correlation between

![Fig. 1. Performance times to complete target amount of work for the 3 trials. PP, placebo 60 min before and 45 min into exercise; CP, single caffeine dose (6 mg/kg) 60 min before exercise and placebo 45 min into exercise; CC, divided caffeine dose (3 mg/kg) 60 min before and 45 min into exercise. Values are means ± SE for 8 subjects.

![Fig. 2. Plasma caffeine (A) and paraxanthine (B) concentrations (conce) during rest and exercise. Values are means ± SE for 8 subjects. VO$_{2max}$, maximal oxygen uptake; TT, time trial. *Significantly lower compared with CP, $P < 0.05$. **Significantly different from previous value, $P < 0.05$.](http://jap.physiology.org/DownloadedFrom10/2020-254.4)
The present study does not mirror these previous findings because single and split dosing. Type II error is the unlikely cause because of the small difference between the two caffeine trials, and others (7) have had similar findings.

Paraxanthine exhibits similar pharmacological and ergogenic properties to caffeine (2, 18). Its effects on skeletal muscle have been demonstrated in vivo to reduce plasma potassium concentrations (27) and in vitro to increase calcium concentration transiently to subcontraction levels in resting skeletal muscle (17). Paraxanthine concentrations were significantly higher throughout the test after the single dose compared with the divided caffeine dose; maximum levels were 1.4 and 1.1 μg/ml, respectively. However, with both treatments, paraxanthine continued to increase throughout exercise such that no peak was obtained. Therefore, when plasma caffeine levels were decreasing, paraxanthine was still rising. These results are consistent with the findings of earlier studies. Caffeine doses of 2 and 4 mg/kg gave peak paraxanthine concentrations of 1.4 μg/ml at 300 min and 1.6 μg/ml at 540 min, respectively (2). In six spinal cord-injured subjects, paraxanthine concentration increased gradually throughout 180 min without plateauing, after administration of 6 mg/kg caffeine (27). Similarly, a constant elevation of paraxanthine after caffeine ingestion (4 mg/kg) to a peak concentration of 1.15 μg/ml was measured at 180 min with no indication of a plateau (18).

The relative contributions of caffeine and paraxanthine to performance enhancement remain unclear. It has been suggested that paraxanthine formation may be quantitatively more important in having pharmacological effects than previously believed (22). Nonetheless, no conclusions can be made to determine effects of caffeine on performance and therefore did not include a single dose for comparison. These studies also used concomitant carbohydrate supplementation, making comparison between the findings of the present study with these investigations difficult. The results of the present study, like those of Cox et al. (7), indicate that dividing a caffeine dose for ingestion during exercise provides no ergogenic effect on endurance cycling performance over a bolus dose given before exercise. It is possible that the maximal ergogenic effect of caffeine occurs with doses ≤3 mg/kg (7, 15). Therefore, ingestion of more caffeine before or during exercise will have no further ergogenic effect.

Fig. 3. Postexercise urinary caffeine concentration in CP and CC trials. Values are means ± SE for 8 subjects. *Significantly lower compared with CP, P < 0.05.

Fig. 4. Relationship between initial caffeine dose and final urinary caffeine concentration (r = 0.735, P = 0.001; n = 8 subjects).
the impact of paraxanthine during exercise in this study.

The amount of caffeine excreted is consistent with values reported in other studies that used caffeine doses up to 9 mg/kg, which observed significant improvements in endurance performance with caffeine doses that produce urinary levels well below the IOC threshold of 12 µg/ml (14, 15, 21, 28). We believe this is the first study to report a lower postexercise urinary caffeine concentration of divided-dose caffeine administration as opposed to single-dose administration. The mean caffeine level present in the urine was almost twofold greater in the single-dose trial compared with divided-dose trial. These results support those of Kovacs et al. (21) that lower urinary caffeine levels were due to spreading the dose throughout the duration of exercise.

Because total caffeine intake was identical in both trials, the timing of administration must have influenced urine concentration. This is reflected by the significant positive correlation between initial caffeine dose and the final urine concentration (Fig. 4), suggesting that the second dose of caffeine did not influence the urinary caffeine levels at the time of collection. A comparison of previous observations support these findings. Split administration of a 4.5 mg/kg dose (21) achieved markedly lower urinary caffeine concentrations (2.5 µg/ml) than a similar (5 mg/kg) single dose [4.8 µg/ml (25) and 5.8 and 6.1 µg/ml (28)].

Two important implications can be drawn from the urine results. First, larger ergogenic doses of caffeine may not exceed the IOC limit if the dose were divided throughout exercise. Second, the timing of caffeine intake and urinary sampling has a large influence on the subsequent caffeine concentration in the urine. Therefore, the caffeine levels present in the urine are not always an accurate indication of total caffeine intake. It is possible that an individual could manipulate the urinary caffeine concentration at the completion of competition, by altering the timing of caffeine ingestion.

Our results suggest that urine is not the best method for doping analysis of caffeine. Current urine analysis is based solely on the 1–3% excreted as unmetabolized caffeine and does not account for the metabolites cleared by the kidney. Paraxanthine, like caffeine, is a potent competitive adenosine antagonist that can contribute to the ergogenic effects of caffeine (2, 17, 27). Furthermore, paraxanthine concentrations continue to rise after completion of exercise (2). Although the paraxanthine concentration can be measured in the urine, its excretion is highly variable (1) and, like caffeine, could be influenced by event duration, individual metabolism, and environmental conditions. Other more accurate methods, such as analysis of caffeine metabolite ratios in the urine, have been suggested (10).

In conclusion, caffeine ingestion did not significantly alter endurance cycling performance compared with a placebo, although it tended to have an ergogenic effect. Furthermore, there was no significant effect of timing of caffeine delivery on performance; however, dividing the caffeine dose resulted in substantially lower postexercise urinary caffeine levels. This latter effect may present a potential loophole to accurately determining an athlete’s caffeine intake via urine analysis.

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