J. vet. Pharmacol. Therap. **31**, 156–166, doi: 10.1111/j.1365-2885.2008.00942.x.

Caffeine-induced hyperactivity in the horse: comparisons of drug and metabolite concentrations in blood and cerebrospinal fluid

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Vickroy, T. W., Chang, S.-K., Chou, C.-C. Caffeine-induced hyperactivity in the horse: comparisons of drug and metabolite concentrations in blood and cerebrospinal fluid. *J. vet. Pharmacol. Therap.* **31**, 156–166.

The goals of this study were to elucidate the temporal and quantitative relationships between caffeine and its major bioactive metabolites in blood and cerebrospinal fluid (CSF) and to characterize the pharmacokinetic–pharmacodynamic relationship for caffeine-induced changes in spontaneous locomotor activity in the horse. We hypothesized that caffeine and its metabolites distribute efficiently into the CSF to antagonize adenosine A1 and A2a receptors and that spontaneous locomotor activity correlates well with caffeine and/or metabolite concentrations in CSF and blood. A microdialysis system was developed to allow simultaneous monitoring of locomotor activity and collection of CSF and blood samples for pharmacokinetic analysis. CSF concentrations of caffeine and its metabolites were evaluated to determine the percentage of central adenosine receptor blockade by the established standard inhibition curves. Caffeine increased the spontaneous locomotor activity for up to 4 h in a dose-dependent manner. After 3 mg/kg caffeine administration, blood caffeine concentration as well as locomotor activity increased sharply to near peak level while CSF caffeine concentrations exhibited a slow rise to a steady-state 75 min later. High correlation coefficient was found between locomotor activity and caffeine concentrations in blood ($R^2 = 0.95$) and in CSF ($R^2 = 0.93$). At 3 mg/kg dosage, theophylline was the only detectable caffeine metabolite in the CSF. The concentrations reached in the CSF were sufficient to partially block central adenosine A1 (14% blockade) and A2a (11% blockade) receptors. There were no statistically significant differences between the pharmacokinetics of caffeine in the blood and CSF. This study provides novel evidence that locomotor stimulation in horses is closely correlated with caffeine concentrations in the blood and CSF and, furthermore, is consistent with blockade of central adenosine receptors.

(Paper received 13 August 2007; accepted for publication 24 December 2007)

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INTRODUCTION

Caffeine is one of numerous substances that have been used illegally to improve the athletic performances of human and nonhuman competitors. While studies have demonstrated that caffeine is most beneficial for competitions that entail prolonged or rigorous physical activity, the cellular changes that underlie caffeine-enhanced performance remain incompletely understood. Previous studies have revealed that caffeine elicits numerous cellular and physiologic changes that could increase athletic endurance, including improvements in cardiac function (Evoniuk *et al.*, 1987), increased skeletal muscle contractility (Sinclair & Geiger, 2000) as well as the well-recognized psychomotor stimulant effects in the central nervous system (CNS). While most evidence indicates that psychomotor stimulation derives from caffeine-induced antagonism of forebrain A1 and A2a adenosine receptors in humans and laboratory animal species (see review by Fredholm *et al.*, 1999), it is possible that other actions may contribute to the behavioral effects of caffeine, including influences on central noradrenergic (Ferre *et al.*,

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1996), dopaminergic (Daly, 1993; Ferre *et al.*, 1996), cholinergic (Forloni *et al.*, 1986), and GABAergic pathways (Mukhopadhyay & Poddar, 1995; Ferre *et al.*, 1996).

In several mammalian species, one of the best-characterized and readily quantifiable behavioral effects of caffeine is a pronounced and dose-dependent stimulation of spontaneous locomotor activity. Hyperactivity has been used previously as an index of CNS stimulation by caffeine and other xanthine compounds in a number of laboratory animal species, including mice, rats, and squirrel monkeys (Glowa & Spealman, 1984; Kaplan *et al.*, 1991). Caffeine-induced hyperactivity has long been recognized to be highly dose-dependent in rodents (Thithapandha *et al.*, 1972; Kaplan *et al.*, 1991; Uzbay *et al.*, 2007), and in humans (Kaplan *et al.*, 1997) and correlates closely with whole brain drug concentrations (Fredholm *et al.*, 1983; Kaplan *et al.*, 1989). As caffeine is a relatively small lipophilic compound, it distributes effectively into cerebrospinal fluid (CSF) and brain tissue (Nakazono *et al.*, 1992). There appears to be a strong correlation between caffeine occupancy of CNS adenosine receptors and the intensity of motor stimulation in several species (Spealman, 1988; Kaplan *et al.*, 1993), but similar correlation has not been well established for horses.

Although the CNS actions of caffeine have been studied extensively in laboratory animals, it is unclear what parallels can be determined to the drug's actions in other species. In horses, for example, caffeine exhibits significant pharmacokinetic differences, including a much longer biological half-life (13 h compared with 1–3 h in rodents and humans; Fredholm, 1995) and a distinct pattern of metabolite formation (Aramaki *et al.*, 1991). Two published reports have confirmed that caffeine produces robust and dose-dependent locomotor stimulation in horses (Greene *et al.*, 1983; Queiroz-Neto *et al.*, 2001) at doses ≥2 mg/kg. However, correlations between caffeine/metabolite concentrations and the increased locomotor activity have not been established. In addition, although we have reported previously that caffeine is a potent competitive antagonist at A1 and A2a receptors in the equine forebrain *in vitro* (Chou & Vickroy, 2003); CSF concentrations of caffeine and metabolites in the horse have not been studied such that CNS adenosine receptor blockade could be evaluated in relation to the motor stimulative effects. Therefore, knowledge of CNS concentrations of caffeine and metabolites are essential to elucidate the relationship between adenosine receptor antagonism and motor stimulation in the horses.

In an attempt to better understand the motor stimulant actions of caffeine in horses, we developed a double-probe *in vivo* microdialysis sampling technique that enables simultaneous measurement of drug in blood and CSF with concurrent monitoring of spontaneous locomotor activity. The overall goals of this study were to elucidate the temporal and quantitative relationships between caffeine and its major bioactive metabolites in blood and CSF and to characterize the pharmacokinetic–pharmacodynamic (PK-PD) relationship for caffeine-induced changes in spontaneous motor activity.

MATERIALS AND METHODS

Experimental subjects

Six clinically healthy horses including five Thoroughbreds and one quarter horse (two males and four females; ages 3–20 years old) were used for this study. The horses were housed in a fenced paddock with free access to hay and water prior to use in this investigation. On the morning of each experiment, the subject was transferred to a lighted temperature-controlled interior stall (3.6 by 3.6 meters) and given free access to hay and water. Except for a short period during which microdialysis probes were implanted, horses were kept in this stall for the duration of the experiment. All procedures and experimental protocols used in this investigation were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee prior to the performance of this study.

Venous and CSF microdialysis

The semi-automated microdialysis model for free-moving horses was carried out as described previously (Chou *et al.*, 2001). As illustrated in Fig. 1, two battery-powered micro-infusion pumps and a microfraction collector were secured in a clear plastic box that was bolted to a wooden base and affixed to an exercise saddle on the horse's back. Each horse was implanted with two linear-type microdialysis probes (LM-10; Bioanalytical Systems, Inc., Lafayette, IN, USA) that had a 10 mm membrane length and 6 kDa molecular weight cutoff dialysis section. After surgical preparation by antiseptic solution (Exidine-4; Baxter, Deerfield, IL, USA) of the designated implantation area (see below), one probe was folded gently in half and passed through a sterile 14-gauge Teflon catheter (5½ inch length; Abbocath-T, Abbott Ireland, Ireland) after the catheter had been inserted into the left jugular vein. The procedure for insertion of a CSF probe was modified from that described above. After the horse was sedated with 0.5 mg/kg xylazine (xyla-ject Phoenix Pharmaceutical Inc., St Joseph, MO, USA) injected into the contralateral jugular vein to that catheterized for drug measurements, an 8-inch long, 16-gauge stainless spinal needle (Mila International, Inc., Florence, KY, USA) was inserted in the subarachnoid space caudal to the sixth lumbar spinous process. Correct placement was confirmed following the removal of the stylet and 3 mL of CSF was withdrawn by a syringe. Presence and clarity of the CSF sample confirmed positioning in the subarachnoid space. A single LM-10 microdialysis probe, that previously had been folded in half and glued two 25-cm small-bore polyethylene tubing that functioned as a handle, was passed through the spinal needle until the probe membrane tip was fully extended through the needle. The probe was then maintained in place as the needle was withdrawn slowly. By using this approach, the probe membrane remained well within the subarachnoid space. All procedures were conducted under aseptic conditions. After both probes were implanted, they were perfused independently

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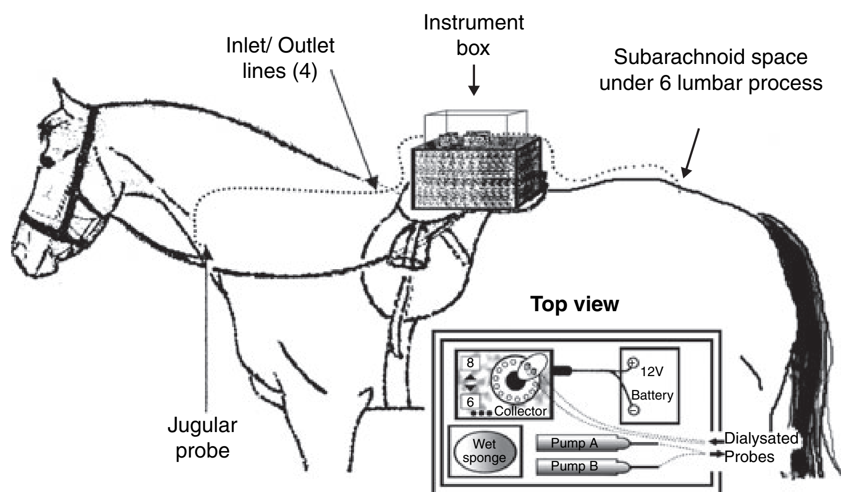

Fig. 1. Schematic representation of instrument box and implantation sites (jugular vein and the sixth lumbar process) for microdialysis probes in horses.

with sterile 0.9% (w/v) sodium chloride solution at 5 μL/min for 30 min. Following the initial washout period, probes were perfused at a flow rate of 2 μL/min. This flow rate was maintained throughout the experiment and the perfusates were collected through presterilized polyethylene tubing by a programmable dual-channel microfraction collector (CMA-142, CMA Microdialysis, Inc., Stockholm, Sweden) at preset intervals of 10 or 30 min. The delay time associated with dead volume of the tubing was determined in each experiment and incorporated into all data calculations.

Gentamicin study

To demonstrate that the insertion of the spinal probe did not compromise the integrity of the blood–brain barrier (BBB) and thereby promote mixing of drug between blood and CSF, gentamicin sulfate (6.6 mg/kg, i.v.) was administered to the horse and evaluated for its relative penetration across the BBB. Gentamicin was selected for this purpose owing to its well-recognized inability to penetrate the mammalian BBB (Jawetz, 1995). Time-matched dialysate samples from CSF and jugular vein at various time points (0.5, 1, 2, 3, 4, 5, 6, and 7 h after gentamicin injection) were assayed for gentamicin sulfate content with an enzyme-linked immunosorbent assay (International Diagnostic System Corp., St Joseph, MI, USA). The gentamicin concentration was estimated from a standard curve constructed with seven standard drug concentrations ranging from 25 pg/mL to 2 ng/mL. The limit of detection is <10 pg/mL by the producer's instruction.

Drug administration and sample collection

Immediately before each experiment, anhydrous caffeine (Sigma Chemical Co., St Louis, MO, USA) was dissolved at a final concentration of 60 mg/mL in sterile saline that contained 48 mg/mL sodium benzoate (Sigma Chemical Co.). The resulting caffeine solution was administered by i.v. bolus injection (approximately 30 sec) into the jugular vein at a dose rate of 3 mg/kg. Dialysate samples were collected from CSF and jugular vein probe outputs 30 min prior to caffeine injection; this was followed by sample collections at 10-min intervals during the initial 100-min period after caffeine administration followed by 30-min collection intervals during the next 5-h period. The dialysate samples were collected in preweighed vials. This allowed individual sample sizes to be measured and corrections for variations in the volume of individual fractions. The microvials containing dialysate samples were covered tightly with Parafilm (American National Can, Menasha, WI, USA) and stored at below 4 °C until analysis on the following day.

Drug analysis

Concentrations of caffeine, theophylline, and theobromine in jugular vein and cerebral spinal fluid (CSF) dialysates were determined by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) as described earlier (Chou *et al.*, 2001). Caffeine, theophylline, and theobromine were separated on a Lichrosphere RP C-18 column (150 × 4.6 mm; 5 μm particle size; Supelco, Inc., St Louis, MO, USA) with mobile phase containing 10 mM NaH_2PO_4 (pH 4.8), acetonitrile, and tetrahydrofuran (90:7:3 v/v) at ambient temperature and 0.8 mL/min flow rate. Samples were injected using a fixed-volume (19.6 μL) loop and methylxanthines were detected at a UV wavelength of 273 nm. Values for drug concentrations in samples were extrapolated from linear standard curves for each compound (0.005–5 μg/mL). The limits of detection for theobromine, theophylline, and caffeine were 2, 4 and 10 ng/mL, while limits of quantitation were 5, 10 and 20 ng/mL, respectively. Intra-assay and interassay variations were all <9%.

Evaluation of spontaneous motor activity

Horses for the behavioral study were kept at pasture when not being used for an experiment. On the morning of each experiment, the horse was transferred to the stall and given free access to hay and water. The entrance to the stall was shielded by a dark-green curtain to reduce distractions. Except for the short period before caffeine was injected and the behavioral

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observations were begun, horses were kept in this stall for the duration of the experiment. Immediately before drug injection, the hay bag and feed bucket were removed to reduce purposeful movements caused by food or water consumption.

Prior to the actual behavioral experiments, preliminary pilot studies were carried out in three horses at three different caffeine dose rates (see below) to validate the use of limb movements as an indicator of motor activity. Horses were brought to the stall at least 2 h before the experiment to acclimate to the environment and to the microdialysis equipment box and saddle. Caffeine–benzoate solution at doses of 0.5, 1.5, and 3 mg/kg were given i.v. to the horses and motor activity was monitored for 90 min. The motor activity was measured by either counting the steps of a white-tape marked left thoracic limb or the total number of grids (90 × 90 cm grid) crossed by the same limb during successive 5-min intervals. Control tests were conducted in which horses were given either normal saline or vehicle solution (2.4 mg/kg benzoate dissolved in saline). In some cases, the evaluation of caffeine-treated horse was performed on the same subject following the evaluation of no-drug treatment. Observation was made by two independent observers situated about 5 ft above the stall.

The actual behavioral evaluation was conducted in conjunction with CSF and jugular vein microdialysis in six horses. Direct blood sampling was not performed to avoid behavioral effects associated with disturbance to the horse. The motor activity was videotaped from 1 h prior to 7 h after caffeine administration and was later evaluated from tape recordings by counting steps of the tape-marked front thoracic limbs within each 5-min period. A step was defined as a movement of the leg that resulted in a change of location of the foot. Therefore, scratching, pawing, and stamping were not counted (Greene *et al.*, 1983).

Pharmacokinetic analyses

Pharmacokinetic parameters were determined for caffeine in blood and the CSF for each horse using noncompartmental analysis, which only assumes first-order kinetics and requires determination of the area under the curve (*AUC*) from dose time to infinity, area under the moment curve (*AUMC*) and the excretion rate. The following first-order equation:

$$C_t = C_1 \bullet e^{-\lambda 1 \bullet t} + C_2 \bullet e^{-\lambda 2 \bullet t}$$

was fit to the data from jugular vein dialysates to determine these values whereas another first-order equation:

$$C_t = C_1 \bullet e^{-\lambda 1 \bullet t} + C_2 \bullet e^{-\lambda 2 \bullet t} - (C_1 + C_2) \bullet e^{-\lambda 3 \bullet t}$$

which allows for accumulation as well as disappearance of drug, was fit to the data from CSF. C_t is the drug concentration at time t; C_1, C_2, λ_1, λ_2, and λ_3 were the model values that were fitted to the data. The weighted nonlinear best fit for each mathematical model was performed with a computerized algorithm based upon minimization of the sum of the squared deviations (Caceci & Cacheris, 1984). Pharmacokinetic parameters were calculated based on noncompartmental kinetics (Gibaldi & Perrier, 1982) according to equations described previously (Chou *et al.*, 2001).

Data analyses and statistics

Data from motor stimulation (step counts per 5 min) were recalculated to comply with the intervals incurred in time-matched CSF and jugular vein concentrations of caffeine by microdialysis. The analysis of correlation was performed by nonlinear regression procedure (NLIN) using a least-square method (Ratkowsky, 1990). A NLIN segmented model (Jennrich & Sampson, 1968) was applied to evaluate correlations between caffeine concentrations in CSF or jugular vein dialysates and motor activity using SAS PROC NLIN and PROC GLM (SAS procedures guide, SAS Institute, Cary, NC, USA). A Student's *t*-test was used to test for differences between pharmacokinetic parameters derived from caffeine concentrations in CSF and jugular vein. A *P*-value of <0.05 was accepted as being statistically significant.

RESULTS

Assessment of blood–brain barrier integrity following subarachnoid probe implantation

As illustrated in Fig. 2a, i.v. injection of gentamicin (6.6 mg/kg) produced a peak drug concentration of 66 μg/mL in blood. Throughout the 6-h sampling period, the plasma concentrations of gentamicin decreased in a linear fashion with an apparent half-life of 2.6 h, as revealed by noncompartmental pharmacokinetic analysis. By comparison, the concentration of gentamicin in CSF was greater than three orders of magnitude lower than blood and never exceeded 0.003 μg/mL (3 ng/mL) throughout the entire sampling period (Fig. 2b). Direct comparison of time-matched samples from blood and CSF revealed that gentamicin concentrations ranged from 4700- to 234 000-fold lower (0.02–0.0004%) in CSF compared to blood (Fig. 2c). In addition to the extremely low drug concentrations in CSF, there was no consistent temporal pattern (increase or decrease) for gentamicin in CSF during the 7-h sampling period.

Kinetic profiles of caffeine and theophylline in venous blood and CSF

Following i.v. caffeine administration (3 mg/kg), time-matched dialysate samples were collected from separate microdialysis probes in venous blood and CSF and assayed off-line for caffeine and its two major metabolites (theophylline and theobromine). As illustrated in Fig. 3a, caffeine attained a blood concentration of 5000 ± 500 ng/mL immediately following injection and declined by nearly 50% to a concentration of 2600 ± 150 ng/mL by the end of the 7-h sampling period. Noncompartmental pharmacokinetic analysis revealed an apparent $t_{1/2}$ of 11.20 h and an apparent volume of distribution [$V_{d(ss)}$] of 0.76 ± 0.07 L/kg in blood (Table 1). In comparison, the concentration–time profile for caffeine exhibited a different profile in CSF (Fig. 3b) insofar as the time to achieve peak drug concentration was delayed by approximately 90 min and the maximum caffeine concentration was significantly lower

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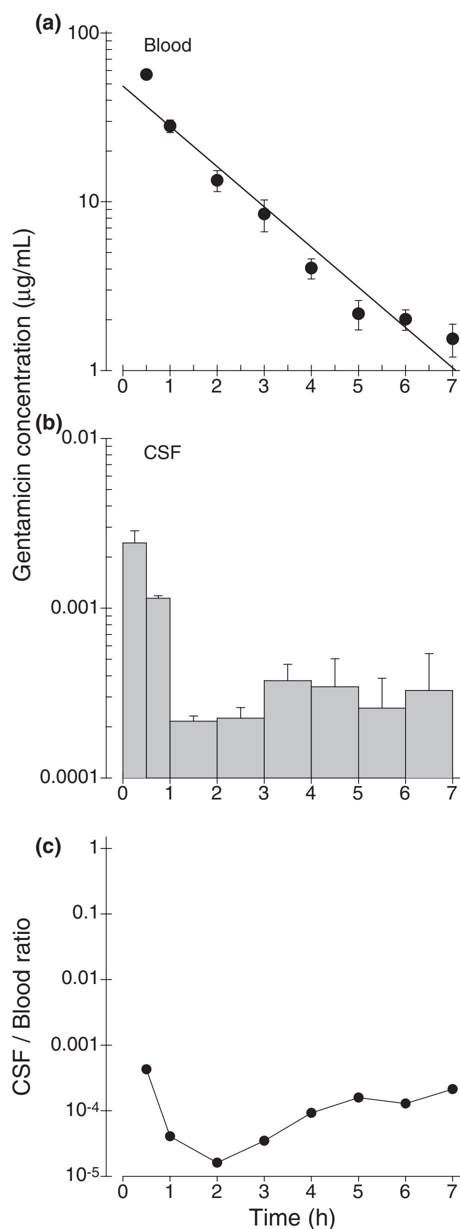

Fig. 2. Concentration–time profiles for gentamicin in blood (a) and cerebrospinal fluid (CSF; b) following gentamicin administration (6.6 mg/kg, i.v.). Time-matched CSF to blood ratio of averaged gentamicin concentration is shown in panel (c) ($n = 6$).

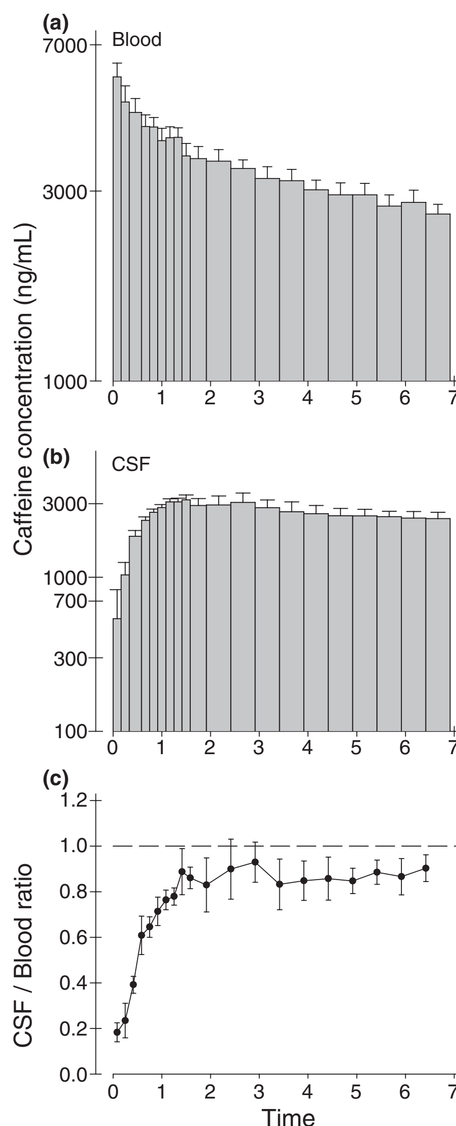

Fig. 3. Concentration–time profiles for caffeine dialysate (mean ± SEM, $n = 6$) in blood (a) and cerebrospinal fluid (CSF; b) following caffeine administration (3 mg/kg, i.v.). Ratios of CSF to blood caffeine dialysate concentrations at each sampling time are shown in panel (c).

(3200 ± 250 ng/mL; $P < 0.05$) than that present in blood. As illustrated in Fig. 3c, caffeine concentrations in CSF remained at 87 ± 4% of drug concentrations in blood following the initial equilibration period. Results from non-compartment analysis revealed kinetic parameters for caffeine in CSF [apparent $t_{½}$ = 10.56 h; apparent $V_{d(ss)}$ = 0.93 ± 0.14 L/kg] that did not differ significantly from parameters derived for caffeine in blood (Table 1).

Table 1. Derived pharmacokinetic parameters for caffeine in venous blood and CSFs

	Jugular vein	CSF
$t_{½}$ (h)	11.20	10.56
$V_{d(ss)}$ (L/kg)	0.76 ± 0.07	0.93 ± 0.14
Cl (mL/min/kg)	0.40 ± 0.06	0.62 ± 0.12
AUC (μg·h/mL)	69.9 ± 8.8	48.0 ± 7.4
MRT (h)	18.1 ± 3.3	15.0 ± 2.9

Values are presented as mean (±SEM) for six horses administered caffeine (3 mg/kg, i.v.). Harmonic mean is used to present half-life.
AUC, area under the curve; CSF, cerebral spinal fluid.

In addition to caffeine concentrations, dialysates from venous blood and CSF were analyzed for the two major *des*-methyl bioactive caffeine metabolites (theophylline and theobromine) that are known to be formed in horses and several other species. As illustrated in Fig. 4, theophylline was undetectable initially for periods of 60 min in blood and 75 min in CSF following

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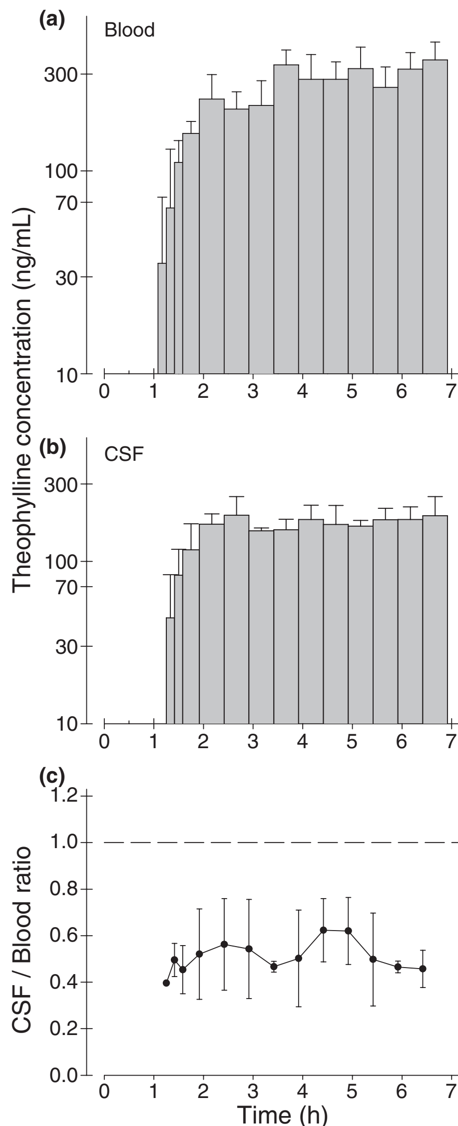

Fig. 4. Concentration–time profiles for theophylline dialysate (mean ± -SEM, $n = 4$) in blood (a) and cerebrospinal fluid (CSF; b) following caffeine administration (3 mg/kg, i.v.). Ratios of CSF to blood theophylline dialysate concentrations at each sampling time are shown in panel (c).

i.v. caffeine administration. Subsequent to this early lag phase, theophylline concentrations rose gradually to achieve steady-state concentrations of approximately 300 ng/mL and 160 ng/mL in blood and CSF, respectively. Despite the difference between absolute concentrations of theophylline in blood and CSF, it appeared that drug concentrations in these compartments exhibited similar temporal patterns. Direct comparison of time-matched samples from blood and CSF (Fig. 4c) revealed that theophylline concentrations in CSF remained within the range of 40–60% of blood theophylline concentrations throughout the 7-h sampling period with a time-averaged value of 52 ± 2% for all dialysate samples.

Additional comparisons were carried out within individual subjects to further delineate the relationships between caffeine

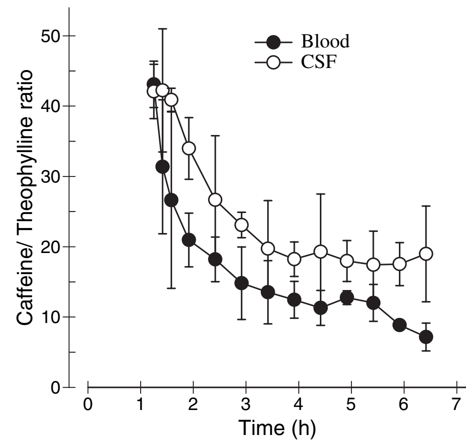

Fig. 5. Ratios of caffeine to theophylline dialysate concentrations in blood and cerebrospinal fluid after 3 mg/kg caffeine administration i.v. Data shown are mean ± SEM for six horses.

and its principal metabolite in blood and CSF. As illustrated in Fig. 5, blood dialysates from all subjects contained substantially higher concentrations of the parent drug caffeine compared to theophylline, with initial caffeine/theophylline ratios of 43 ± 3.3 and 42 ± 3.9 in blood and CSF, respectively. In both compartments, the caffeine/theophylline ratios appeared to decrease in an exponential manner with a drop of approximately 50% during the first 1-h sampling period. At later time points, however, differences began to emerge between venous blood and CSF. Comparison of the caffeine/theophylline vs. time plots for both compartments revealed a significantly greater decline in venous blood vs. CSF at time points >4 h after caffeine administration ($P < 0.025$; Mann–Whitney U-test). All analyses for theobromine failed to detect any of this caffeine metabolite in CSF throughout the entire sampling period.

Motor stimulant effects of caffeine

As illustrated in Fig. 6a, thoracic limb movements increased significantly within 5 min following caffeine administration at all three dosages. The average thoracic step counts per 5-min period exhibited a dose-dependent response to caffeine with a 20-fold increase observed at 3 mg/kg caffeine (Fig. 6b) compared to the control. Following the rapid initial rise in activity, step counts remained near peak activity throughout the 90-min monitoring period in horses given 3 mg/kg caffeine whereas animals given lower doses exhibited a moderate decline in activity levels.

Based on the results of these initial dose–response studies, it appeared that doses of 1 and 3 mg/kg caffeine yielded similar peak of motor activity, although responses appeared to be more reliable at the higher caffeine dose. Therefore, subsequent studies of motor activity in conjunction with microdialysis-based measurements of caffeine concentrations in venous blood and CSF were carried out using the 3 mg/kg dose in six horses. Results from this study revealed that thoracic limb step counts were very low prior to drug administration but increased

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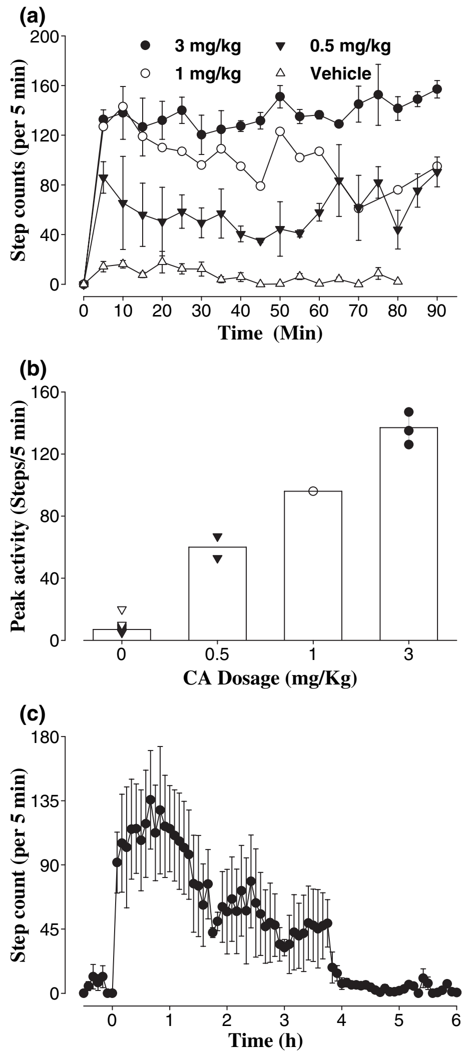

Fig. 6. Stimulant effects of caffeine on locomotor activity. Horses were injected i.v. with either vehicle (2.4 mg/kg benzoate in saline) or increasing doses of caffeine (0–3 mg/kg). Locomotor activity was measured by step counts (mean ± SEM, n = 1–3) of left thoracic limb per 5-min period (a). A dose–response plot for caffeine dosage and peak locomotor activity in the first 90 min is shown in panel (b). Average step counts from 30 min before to 6 h after caffeine (3 mg/kg, i.v.) administration is shown in panel (c) (n = 6).

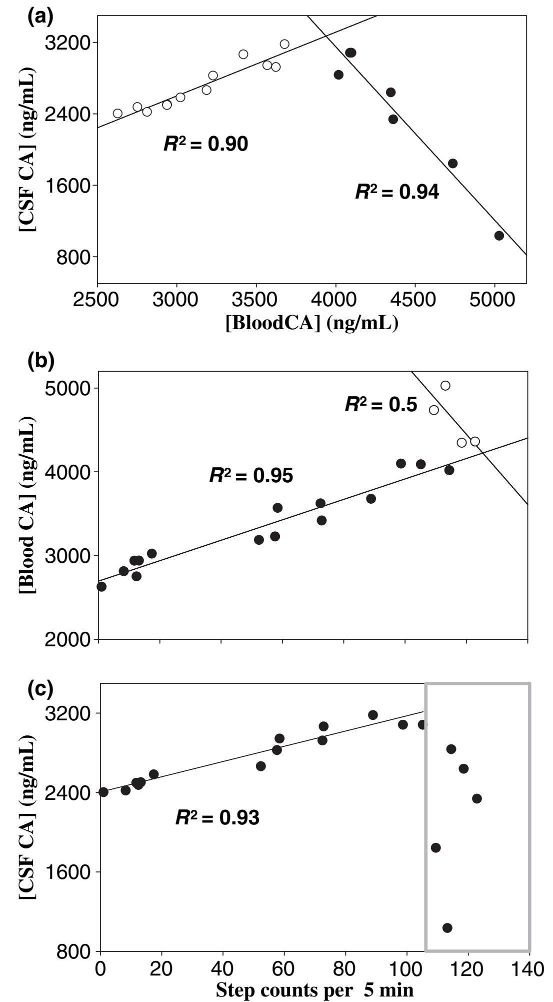

Fig. 7. (a) Correlation of blood and cerebrospinal fluid (CSF) caffeine concentrations. Nonlinear regression analysis using segmented model revealed two linear fits intercept at caffeine concentration of 3928 ng/mL in the blood. The correlation coefficient (R^2) for blood caffeine concentrations below and above 3928 ng/mL is 0.9 (slope = 0.7) and 0.94 (slope = −1.9), respectively. (b) Correlation of step counts per 5-min period to blood dialysate concentrations of caffeine. Nonlinear regression plots revealed two regression lines intercept at blood caffeine concentration of 4191 ng/mL. The R^2 for blood caffeine concentrations below 4191 ng/mL was 0.94 and above 4191 ng/mL was 0.5. (c) Correlation of step counts per 5-min period to CSF dialysate concentrations of caffeine. CSF concentrations of caffeine correlated well with locomotor activity when step counts per 5 min were below 105 (R^2 = 0.93).

by six- to sevenfold immediately following caffeine treatments. The motor activity then remained at near peak level for approximately 80 min before it was reduced by half and was maintained at half maximal activity for another 2 h. The step counts returned to baseline level 4 h after drug injection (Fig. 6c). A moderate increase in motor activity was found between 105 and 125 min in this study.

Correlation between caffeine levels and motor activity

Caffeine concentrations in CSF correlated well with caffeine concentrations in venous blood (Fig. 7a). During the distribution phase of caffeine into CSF, a correlation coefficient (R^2) of 0.94 was determined with a slope of −1.9 indicating the almost twofold slower increase of caffeine concentrations in CSF relative to the decrease of caffeine concentrations in venous blood (Fig. 7a). Subsequently, caffeine exhibited parallel elimination from CSF and venous blood with a good correlation (R^2 = 0.9; Fig. 7a). On the other hand, motor activity was well correlated (R^2 = 0.95) with caffeine concentrations in serum (Fig. 7b), and in the CSF (R^2 = 0.93) when the step counts per 5 min was below 105 (Fig. 7c).

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DISCUSSION

The current study highlighted the capacity of microdialysis to ascertain CSF drug concentrations continuously and in concert with behavioral evaluation. This approach has enabled the investigation of motor stimulation effects by caffeine with respect to drug concentrations in the CSF, which is presumed the biophase for stimulant drug actions. Brain concentrations of caffeine, in turn, when compared to the affinity of caffeine to adenosine receptors, allow the examination of the underlying mechanism of caffeine in relation to adenosine receptor antagonism.

In this study, motor stimulation by caffeine was a consistent effect in all horses although the intensity of the stimulation varied between individual horses. The increase in motor activity by caffeine was dose-dependent over the range of 0.5–3 mg/kg (Fig. 6b) and the effect persisted up to 4 h at the highest dose (Fig. 6c). Our results agree well with previous study (Queiroz-Neto *et al.*, 2001) in the intensity and duration of motor stimulation at the same dosage (3 mg/kg); at slightly higher caffeine dosage (5 mg/kg), the intensity and duration both doubled. A similar duration of motor activation has been reported in mice following 20 mg/kg of caffeine (Kaplan *et al.*, 1991). This time period, although similar in length, marked a significant difference between these two species insofar as the 4-h motor stimulation represented less then one-half of the half-life of caffeine in equine CSF ($t_{½}$ =10.56 h). In comparison, motor stimulation persisted for a period equivalent to three half-lives in mouse brain. In other words, while the motor stimulant effect of caffeine in the horse diminished at caffeine concentrations within 80% of C_{max} in CSF, motor stimulation in mice is detectable until the concentration of caffeine in the brain decreases to <15% of the C_{max} concentration. Assuming adenosine receptor antagonism is the major mechanism responsible for caffeine motor stimulation in both species; such disparities seem to be very significant considering that the K_d values for adenosine A1 and A2a binding sites in horse brain are very comparable to those in mice (Maemoto *et al.*, 1997; Alexander & Millns, 2001). The whole brain caffeine concentrations in the mice when motor stimulation became insignificant was about 2 μg/g (brain tissue) while at the disappearance of motor stimulation, free CSF caffeine concentrations in the horse was around 2.5 μg/mL. Although direct comparison of these two concentrations (μg/g vs. μg/mL) is apparently inappropriate, the presence of significant concentrations of caffeine metabolites in mouse brain (theophylline, theobromine, and paraxanthine at 0.5–1 μg/g brain tissue vs. 0.2 μg/mL theophylline and no detectable paraxanthine in horse CSF) may be a reasonable explanation for the prolonged motor stimulation period in mice compared to horses. Caffeine has been known to produce a pattern of biphasic motor stimulation in mice and rats where at higher doses (>20 mg/kg) a decline in maximum motor activity occurs (Daly, 1993). It was also suggested that an optimal window (10–20 μg caffeine/g brain tissue) is required in order for a maximal stimulant effect to occur (Thithapandha *et al.*, 1972). Although the present study provides no evidence for a biphasic dose–response relationship for caffeine in horses, it is possible that higher drug doses could reveal such relationship.

To exhibit motor stimulant effects through CNS, it is important for the drug to effectively cross the BBB. The integrity of the BBB following probe implantation was assessed through studies with the antibiotic drug gentamicin. Gentamicin is a widely used aminoglycoside antibiotic that normally does not penetrate the BBB and has a molecular weight that is comparable to caffeine. Results from those experiments indicated that an extremely small fraction (<0.02%) of the drug in systemic circulation was found in CSF. If one assumes that a similar extent of leakage occurs with caffeine, the overall contribution to CSF drug concentrations from blood stream would be insignificant insofar as caffeine would be increased by <1 ng/mL in CSF. In addition to the preservation of the integrity of BBB function, the current study reveals that caffeine diffuses into CSF more efficiently than theophylline. The average ratio for caffeine concentration in CSF relative to blood was 0.87 for caffeine and 0.53 for theophylline for the period of 2–6 h following caffeine injection, indicating caffeine and theophylline concentration in CSF was 87% and 53%, respectively, of their concentrations in the blood. The finding of a difference in distribution efficiency across BBB between caffeine and theophylline has been reported previously in rats (Stahle *et al.*, 1991). However, the time required for caffeine to reach equilibrium in horse CSF was longer and theophylline distributed into horse CSF almost twice as efficiently as in rat brain (CSF/blood ratio 55% vs. 25%). In rats, brain concentrations of caffeine equilibrate quickly and maintain a constant ratio relative to drug concentrations in blood. However, in the CSF of horses, caffeine concentrations do not equilibrate until 90 min following drug administration thereby causing a lengthy delay in the establishment of a steady CSF/blood ratio. The slower equilibration in equine CSF may be explained by differences between CNS compartments (striatum vs. CSF), larger brain volume and the use of a relatively low caffeine dose (3 vs. 20 mg/kg) in horses. The uneven distribution is supported further by the finding that the caffeine:theophylline ratio is higher in the CSF than in venous blood. The difference in blood–brain distribution has been attributed to differences in the extent of protein binding for these two structurally similar compounds. While the protein binding of caffeine to plasma macromolecules is approximately 10% in most mammalian species, the binding of theophylline to plasma protein is 30% in the horse (Chou *et al.*, 2001). Therefore, in addition to anatomical and physiologic factors among animal species, the higher protein binding of theophylline than caffeine may explain in part the lower CSF/blood ratios of theophylline in equine reported here.

The motor-stimulant effects of caffeine have been associated most closely with competitive antagonism of adenosine A2a receptors that are co-localized and functionally interact with brain dopamine receptors (Ferre *et al.*, 1996; Fredholm *et al.*, 1999). The role of A1 adenosine receptor in the stimulation of motor activity is less conclusive (Kaplan *et al.*, 1993; Marston *et al.*, 1998; Fredholm *et al.*, 1999). Animal studies have shown that the minimal dose range of caffeine to produce significant locomotor

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effects ranges from 1.5 to 10 mg/kg and were similar in many species including horses (Greene *et al.*, 1983; Queiroz-Neto *et al.*, 2001). In humans, nonhuman primates and rodents, this dose range can be converted to brain concentrations of caffeine (1–10 μM) within which the adenosine receptor blockade may occur to induce locomotor stimulation (Stahle *et al.*, 1990). The present study provides the first direct evidence that, in the horse, CSF concentrations of caffeine, are sufficient to at least partially block adenosine A1 and A2a receptors. With the application of microdialysis, free CSF concentrations of methylxanthines are determined so that brain tissue binding and serum protein binding of methylxanthines are eliminated. In this way, there should be no overestimation of brain methylxanthine concentrations unlike previous studies that relied upon measurements in whole brain samples. Therefore, a more realistic estimation of brain methylxanthine concentrations that may account for adenosine receptor binding is expected. After 3 mg/kg caffeine injection, CSF caffeine concentrations were estimated at 15–20 μM (2.5–3.5 μg/mL; Fig. 2) in the first 2 h during which time motor activity was significantly elevated. This concentration, although lower than the affinities (K_i) of binding of caffeine to equine adenosine A1 (77 μM) and A2a (38 μM) receptors we have demonstrated *in vitro* in an earlier study (Chou & Vickroy, 2003), the inhibition curve of caffeine to these two receptor subtypes indicated that caffeine concentrations at 10–20 μM is estimated to block approximately 6% of A1 and 9% of A2a receptors in equine forebrain tissue. Theophylline was also detected in CSF from as early as 75 min after caffeine injection; although concentrations were significantly lower (0.2 μg/mL≅1 μM) than caffeine. Considering that the binding affinity of theophylline at adenosine receptor was higher (7 μM for A1 and 16 μM for A2a receptor) than caffeine (Chou & Vickroy, 2003), we believe that theophylline may also contribute to motor stimulation effect in view of its ability to block about 7.6% of adenosine A1 and 1.5% of A2a receptors at current CSF concentrations. When combined, approximately 14% of A1 and 11% of A2a receptors are blocked by the caffeine and theophylline concentration reached in equine CSF. Although whether or not this seemingly low percentage of blockade provided sufficient antagonism to elicit hyperactivity observed in the horses remained to be studied, based on the theory of spare receptor, it is not impossible for a drug to exert high pharmacologic effects at low (<10%) receptor occupancy. Synergistic effects between adenosine A1 and A2a receptors might also be possible (Nikodijević *et al.*, 1991). Finally, we have noticed that motor activity exhibits a small but reproducible latent increase at a time that coincides with the appearance of theophylline in CSF (Figs 4 & 6c). Therefore, the possibility remains that caffeine metabolites may contribute to motor stimulation following caffeine administration in horses.

Perhaps the most interesting finding in this study is the marked disparity between the time courses for motor activation and caffeine concentration in CSF. These results are not surprising in view of the delayed time course for caffeine appearance in CSF and remains at or near its peak level when the motor response develops fully and declines. Considering that the maximal stimulant effects of caffeine in rats and mice occur within a finite range of drug concentrations in the brain and it was clear from Figs 3 & 6 that motor activity started to decline before caffeine reached C_{max} in the CSF; it is possible that the existence of optimal concentration window found in rodents also exists in horses and contributes at least in part to the inability of maintaining motor activity at high caffeine concentrations. However, whether or not this effect alone can cause a significant decrease in motor activity is questionable.

Alternatively, although CSF is in close anatomical contact with brain tissues and has been one of the better resources for accessing drug concentrations in the brain, CSF concentrations of drug from subarachnoid space may not be temporally correlated with drug concentrations at presumed sites of action within the forebrain in view of the distance CSF has to travel in a large animal like horse to the lumbosacral space where it was sampled. Before the volume and circulation rate of CSF in the horse could be well characterized, concerns remain that caffeine and theophylline concentrations recovered from the lumbosacral CSF probe may have been diluted or delayed compared to the concentrations of these compounds in the bio-phase of adenosine receptor sites. In practice, to analyze the distribution characteristics of a drug in CNS, the transfer of drug between blood and brain, blood and CSF, and also between brain and CSF must all be considered. Although it is generally accepted that there is no tight barrier for drug permeability in ependyma, the thin epithelial membrane lining the ventricular system of the brain and the spinal cord canal between the CSF and brain tissue, it is also known that the diffusion rate of certain drugs in the brain ECF is quite slow (Nakazono *et al.*, 1992). Previous studies in rats have suggested that while caffeine distribution from blood to CSF and brain tissue was almost equally efficient and no appreciable accumulation of caffeine was observed in the brain, there was undetectable diffusion of caffeine between CSF and brain (Nakazono *et al.*, 1992). If this is also true in horse, the caffeine concentrations in CSF may not reflect changes of caffeine concentrations in brain tissue accurately. Whether brain concentrations of caffeine and theophylline are temporally and quantitatively reflected by their CSF concentrations at lumbosacral subarachnoid space warrants further investigation. Nevertheless, we did find that at step counts below 105 per 5 min there was good correlation between motor activity and CSF caffeine level (R^2 = 0.93). Based on the dose–responsive motor activation observed in this and previous study (Queiroz-Neto *et al.*, 2001), in which higher motor activity was associated with higher caffeine dose, the improved correlation at step counts below 105 per 5 min supported earlier discussions (Daly, 1993) that a biphasic dose–response also exists for horses and the degree of motor activation is associated with a proper concentration window of caffeine and metabolites.

In conclusion, one must keep in mind that, even though the primary action of caffeine may be to block adenosine receptors, the motor stimulant effects of caffeine may arise from interactions with multiple sites (Mukhopadhyay & Poddar, 1995) that lead to secondary effects on many classes of neurotransmitters (Fredholm *et al.*, 1999; Ribeiro, 1999) and maybe other biological effectors. Recently, the involvement of nitrergic system

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(nitric oxide synthase) in the psychostimulant effect of caffeine in mice has been proposed (Uzbay *et al.*, 2007), highlighting new discoveries in this area. It appears that prediction of motor stimulation based solely on drug concentration would be less accurate without consideration of other receptor systems such as dopaminergic and benzodiazepine receptors. Use of microdialysis in combination with the assessment of motor stimulant effects presents a great potential for elucidating the questions above. For example, microdialysis could be used to determine drug concentrations directly from the targeted brain region in concert with measurement of neurotransmitter concentrations. Microdialysis is also capable of delivering selective compounds (e.g. adenosine antagonists) to brain areas rich in specific receptor subtypes and allow for monitoring of pharmacologic and behavioral responses simultaneously. The current study provides the first demonstration of CSF microdialysis in free-moving horses and should prove to be useful in the study of the mechanism by which adenosine receptor selective compound(s) may contribute to the motor stimulation by caffeine in horses.

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