Effects of Caffeine on Locomotor Activity of Horses: Determination of the No-effect Threshold

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Caffeine is the legal stimulant consumed most extensively by the human world population and may be
found eventually in the urine and/or blood of race horses. The fact that caffeine is in foods led us to
determine the highest no-effect dose (HNED) of caffeine on the spontaneous locomotor activity of horses
and then to quantify this substance in urine until it disappeared. We built two behavioural stalls
equipped with juxtaposed photoelectric sensors that emit infrared beams that divide the stall into nine
sectors in a ‘tic-tac-toe’ fashion. Each time a beam was interrupted by a leg of the horse, a pulse was
generated; the pulses were counted at 5-min intervals and stored by a microcomputer. Environmental
effects were minimized by installing exhaust fans producing white noise that obscured outside sounds.
One-way observation windows prevented the animals from escaping outside. The sensors were turned on
45 min before drug administration (intravenous or caffeine). The animals were observed for up to
8 h after i.v. administration of 2.0, 2.5, 3.0 or 5.0 mg caffeine kg−1. The HNED of caffeine for stimulation
of the spontaneous locomotor activity of horses was 2.0 mg kg−1. The quantification of caffeine in urine
and plasma samples was done by gradient HPLC with UV detection. The no-effect threshold should
not be greater than 2.0 µg caffeine ml−1 plasma or 5.0 µg caffeine ml−1 urine. Copyright © 2001 John
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INTRODUCTION

Caffeine is a ubiquitously available and extensively used psychotropic drug that is not only consumed as a
drug but also is present in beverages such as coffee, tea, coconut, cola nut and cola drinks. More than 60
plants have been identified as containing caffeine.1

Reports from laboratories involved in the analysis of horse urine indicate that caffeine, theobromine and
theophylline are detected occasionally in the urine of sports horses. Caffeine is metabolized to many
compounds, including the dimethylxanthines, theophylline, theobromine and paraxanthine. Methylxanthines
act as central nervous system (CNS) stimulants and their administration may have an antispasmodic effect, reducing
drowsiness and fatigue and increasing the capacity for work.2 Caffeine stimulates the respiratory centres,
relaxes bronchial smooth muscle and produces complex cardiovascular effects.3 It seems to increase the
capacity for muscular work in people4 and to act on the kidney to produce diuresis.2

Caffeine is utilized in horses in disturbances of the circulation and fatigue. The drug has enhanced the
running performance of the animals5–6 and produced an increase in locomotor activity7 but this effect was small
and only occurred shortly after i.v. injection.8 The methylxanthines produce their pharmacological effects
by antagonizing the CNS depressant actions of adenosine. Specific receptors have been demonstrated for
adenosine in the CNS, and the methylxanthines bind to these receptors and antagonize the depressant actions
of adenosine analogues that penetrate the brain.7

The Association of Racing Commissioners International (ARCI) has declared that caffeine has no valid therapeutic use in racehorses: caffeine found in racehorse urine or blood samples is a class 2 violation
deserving a particularly strong penalty.

Prohibited substances of dietary or endogenous origin have been a growing concern among world
racing authorities.8 The Malayuan Racing Association (MRA)—the organization that controls horse racing in
Malaysia and Singapore—has problems concerning the interpretation of finding substances that could be of
dietary origin, like caffeine, and has set the threshold level for caffeine at 10 ng ml−1 in plasma,9 indicating
that a caffeine level of <10 ng ml−1 plasma is most likely from the horse receiving caffeine-contaminated feeds. Because caffeine has a rather long clearance time in the horse, such feeds may lead to the detection of low caffeine levels in horse blood.

Major factors in interpreting the presence of caffeine in racehorse samples are the plasma/urine caffeine

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concentration necessary to produce a pharmacological effect and the dose of caffeine that will increase locomotor activity in the horse. The objective of the present study was to determine the highest no-effect dose (HNED) of caffeine that causes increased locomotor activity in horses and the threshold of urinary concentration after administration of this dose.

**EXPERIMENTAL**

**Animals**

The study was conducted on eight English thoroughbred mares and two half-bred English mares kept on pasture and additionally offered mineral salts: *Cynodon sp. hay ad libitum* and pelleted commercial feed twice a day for a total of 6 kg day⁻¹. The ten mares were weighed and sprayed every month with a pyrethroid insecticide (Butox, Quimio SA) at the recommended dilution and wormed with 1% ivermectin (Ivomec, Merck, Sharp and Dohme). The experimental protocol was approved by the Institutional Animal Care and Use Committee at the Faculdade de Ciências Agrárias e Veterinárias (UNESP). Campus de Jaboticabal.

**Drugs**

Anhydrous caffeine was supplied by Sigma Chemical Co. (St. Louis, MO) and 0.9% sodium chloride solution was purchased from Glicolabor Ind. Farmacêutica Ltda. (Ribeirão Preto, SP, Brazil). All the caffeine doses were diluted in 250 ml of physiological saline heated to 37°C and rapidly injected i.v. immediately after preparation.

**Construction of the behavioural stalls**

We constructed two behavioral stalls equipped with juxtaposed photoelectric sensors installed at a height of 45 cm that emitted an infrared beam (Fig. 1). Each time the beam was interrupted, a pulse was generated. The number of pulses was counted at 5-min intervals and stored in a data logger (Campbell Scientific Inc, Logan, UT) connected to a microcomputer for later analysis of spontaneous locomotor activity (SLA).

The stalls prevented the animals from seeing the outer environment and were equipped with two exhaust fans that, in addition to stopping the accumulation of gases, produced a white noise that prevented the animals from hearing noise from outside. Between the stalls there was a room with the controlling equipment used. The room had two smoked-glass windows that permitted undetected observation of the animals' behaviour when the lights were turned off. The configuration of the behavioural stalls for the quantification of horse SLA was described in detail elsewhere.

**Experimental procedures**

The animals were placed in the behavioural stalls the afternoon before the experiments for habituation to the environment. At 7:00 a.m. they received feed and hay. At 9:00 a.m. the sensors were turned on and caffeine or saline (control) was administered i.v. 45 min later (time zero). The animals were observed continuously by members of the research team over a period of 8 h from the time of administration.

At the end of this period, the sensors were turned off and the data were transferred to a 1.44 M byte floppy disk for later analysis. The stalls were cleaned and new animals were placed there for the experiment of the subsequent day.

A 7 day interval elapsed between doses for each animal, except for the 5 mg kg⁻¹ dose for which a 10 day interval was allowed to elapse before the next dose.

**Data analysis**

The data logger recorded the number of interruptions of each light beam in 5-min intervals. The values were summed and the mean for each time interval was calculated. The results of SLA were calculated as the number of interruptions per minute during the time interval between the last count and the next count. For example: SLA attributed to the 5-min time refers to the number of interruptions that occurred between time zero and time 5 divided by the number of minutes (in this case, 5 min). Similarly, the SLA for the 90-min time refers to the number of interruptions that occurred between time 75 and time 90 divided by the number of minutes (in this case, 15 min).

Data were analysed statistically using the PROC GLM procedure of the SAS version 6.11 computer software, according to the following mathematical model:

\[ y_{ij} = \mu + D_i + A_{ij} + T_k + TD_{ik} + \epsilon_{ij} \]

where \( \mu \) = overall mean, \( D_i \) = effect of the \( i \)th caffeine dose (\( D_i = 0, 2.0, 2.5, 3.0 \) and 5.0), \( A_{ij} \) = nested effect of the \( i \)th dose within the \( j \)th animal, \( T_k \) = effect of the


Table 1. Summary of analysis of variance of spontaneous locomotor activity in thoroughbred mares

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine dose (D)</td>
<td>4</td>
<td>80778.0*</td>
</tr>
<tr>
<td>Within-animal dose</td>
<td>41</td>
<td>3425.52*</td>
</tr>
<tr>
<td>Time (T)</td>
<td>25</td>
<td>2400.68*</td>
</tr>
<tr>
<td>D × T</td>
<td>100</td>
<td>910.62*</td>
</tr>
<tr>
<td>Residue</td>
<td>948</td>
<td>330.15</td>
</tr>
<tr>
<td>Total</td>
<td>1118</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>71.73%</td>
<td></td>
</tr>
<tr>
<td>R²</td>
<td>87.43%</td>
<td></td>
</tr>
</tbody>
</table>

\(P < 0.0001\)

The determination of the dose–response curve

The dose–response curve was obtained by determining the area between the control curves and the curve for each dose (area under the curve). These areas were inferred in an indirect manner by cutting out and weighing their outlines on paper. For this purpose, we used graphs of the same size and scale for all determinations, (without symbols, to avoid undue variations in weight) and good quality paper. The data thus obtained were used to construct the regression curve.

Chemical determinations

After the determination of the HNED for caffeine, the animals rested for 45 days without receiving any therapeutic substance.

Plasma and urine samples were collected before the administration of the HNED (time zero) and after 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 18, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288 and 312 h of HNED injection.

The urine samples were taken with a flexible urethral catheter and stored at \(-20^\circ\text{C}\). After each collection, the urinary bladder was emptied completely. The plasma samples were collected by jugular vein puncture in vacuum tubes (Vacuum II, Labnew Ind. and With Ltda.) containing sodium heparin (14.3 U ml\(^{-1}\)). The tubes were centrifuged at 3500 rpm for 15 min and the plasma was stored frozen at \(-20^\circ\text{C}\).

The urine samples underwent a liquid–liquid extraction process at pH 9.2, according to the method described elsewhere. To extract the plasma caffeine, 1 ml of plasma was added to 1 ml of methanol–acetonitrile (3:1), which was shaken for 10 min and centrifuged at 2000 rpm.

The determination of caffeine concentrations in the samples was done by HPLC using HP 1100 equipment with a 5-μm, 125 × 4 mm 100 RP-18 column and a gradient acetonitrile–water–acetonitrile (95:5 initial proportion) solution at the mobile phase.

RESULTS

Table 1 shows a summary of the analysis of variance applied to SLA in English thoroughbred mares. It can be seen that the coefficient of variation was relatively high (71.73%), indicating wide individual variability in the measurement of spontaneous locomotor activity in horses. On the other hand, the mathematical model used explained 67.43% of the variation in the characteristic studied. Table 1 also shows that all sources of variation significantly affected (\(P < 0.0001\)) spontaneous locomotor activity. The interaction between caffeine dose and time was significant, indicating that the main effects (doses and time) should not be analysed separately. Thus, we carried out further statistical analyses to discriminate between the joint effect of dose and time.

Figure 2A showed that the administration of a dose of 2 mg caffeine kg\(^{-1}\) did not produce an SLA-stimulating effect, which was quite close to the results obtained for the control group. The observation of animal behaviour in the stalls after administration of caffeine at this dose also showed no noteworthy
change, with the animals behaving in the same manner as the saline-injected controls. After the injection of 2.5 mg kg\(^{-1}\) caffeine it can be seen that there was a marked and relatively transitory elevation in SLA between 5 and 25 min, followed by a decrease and stabilization of the values close to those observed for the control. The means for this experimental group did not differ significantly from those for the control, nor did they differ from the group injected with 5 mg kg\(^{-1}\) caffeine at 0-25 min. On the other hand there was a significant difference \(P < 0.05\) between the means obtained before and after (20 and 25 min) administration of the 2.5 mg kg\(^{-1}\) dose.

While monitoring the animals through the observation window, we noted that ca. 15 min after receiving caffeine the animals showed restlessness, a behaviour similar to that observed when they received higher doses but considerably different compared with the lower doses.

The SLA of animals tested with the dose of 3 mg caffeine kg\(^{-1}\) was markedly increased, reaching a maximum effect 20 min after injection and remaining elevated for ca. 150 min, gradually decreasing thereafter until practically reaching control values at 270 min. The mean SLA values obtained with this dose were not significantly different from those for the control group when the different doses were compared. However, within-group comparison of the different times showed a significant difference \(P < 0.005\) between time 20 and time -15 min. Five minutes after the administration of 5 mg caffeine kg\(^{-1}\) there was a marked increase in spontaneous locomotor activity in the animals tested, reaching quite elevated levels 60 min after injection. This elevation persisted at approximately these levels for at least 6 h, tending to return to basal levels thereafter. Statistical analysis indicated a significant increase \(P < 0.05\) between time 5 and time 450 min compared with the control group. Within-group analysis of the means for the different times showed a significant difference \(P < 0.05\) between the means obtained before (-30 and -15 min) injection of 5 mg kg\(^{-1}\) caffeine and those obtained at times 25, 45, 60, 75, 90, 180, 210 and 240 min after injection of the drug.

Visual observation of the animals demonstrated an increase in spontaneous locomotor activity, restlessness and stereotyped movements (head shaking, 'back and forth' movements), with one animal intensely biting the door of the stall—a behaviour similar to that observed in amphetamine-induced stereotypy.

Figure 2B presents the dose–response curve for the effect of caffeine on the SLA of horses. As can be seen, the curve that best fits the data seems to be sigmoidal (in its initial portion), demonstrating that doses of <2 mg kg\(^{-1}\) are ineffective. On the other hand, if we do not force the curve to pass through the point of zero dose versus zero response, the resulting curve is practically a growing straight line, demonstrating a good dose–response relationship.

For determination of the plasma caffeine levels, a standard curve was constructed with caffeine concentrations of 2-20 \(\mu g\) caffeine ml\(^{-1}\). The quantification limit was established in 2.0 \(\mu g\) caffeine ml\(^{-1}\). Three out of nine animals presented caffeine levels of 2.0-2.3 \(\mu g\) ml\(^{-1}\) 15 min after the injection. In the other samples, caffeine was detected but not quantified due to the low levels found, i.e. below the quantifying limit of 2 \(\mu g\) ml\(^{-1}\). One of the three animals mentioned above showed a quantifiable concentration only in the 15-min sample, another one up to 60 min and the third up to 2 h. After 8 h of administration more than half of the animals no longer showed caffeine in detectable concentrations, and at the end of 18 h no samples showed any trace of caffeine.

The urinary excretion curve for caffeine (Fig. 3) shows an increase in excretion during the first hour of administration, reaching the elimination peak at this moment (1 h).

**DISCUSSION**

The present studies demonstrated the stimulating effects of caffeine on SLA as well as other behavioural alterations in horses. The SLA increased in a dose-dependent way, with the animals showing a sustained increase with the largest doses.

It has been proposed that dopamine is the main mediator for the behavioural effects of caffeine, which is a competitive antagonist of the adenosine receptors that produces a large range of central effects. Recently, it has been proposed that caffeine increases the dopaminergic activity by antagonizing competitively the adenosine receptors. Therefore, several studies have been demonstrating that the behavioural effects observed after caffeine administration are similar to those observed after classic dopaminergic agonists such as cocaine or amphetamines, including an increase of the locomotor activity and mental excitability, in both animals and human beings.

**Figure 3.** Urinary elimination of caffeine after administration of the dose of 2.0 mg kg\(^{-1}\) iv. in English thoroughbred mares.
Besides the participation of the dopaminergic system it appears that the gabergenic and cholinergic systems are involved. In addition to calcium channels, because nifedipine has been described to block the increase of locomotor activity produced by caffeine in rats. In horses, there were significant increases (P < 0.05) of speed in gallop tests after administration of caffeine i.v. in doses of 2, 4 and 9 mg kg⁻¹. In another study with thoroughbred horses, doses of 5–10 mg caffeine kg⁻¹ improved race performance.

We did not find any reference to the determination of the HNED for caffeine in horses, but similar experiments were done with other species, mainly rats, that demonstrated that doses of 2.5–40 mg kg⁻¹ i.p. can increase the locomotor activity. These results are consistent with our findings, which indicated that for horses the smallest effective dose was 2.5 mg caffeine kg⁻¹ i.v. The higher doses used (3.0 and 5.0 mg caffeine kg⁻¹) were quite efficient at stimulating the locomotor activity of horses trotting in the stall, presenting a similar behaviour to that observed after fenatal administration.

The dose of 2.0 mg caffeine kg⁻¹ i.v. in thoroughbred mares was taken as the HNED under our experimental conditions. This result contradicts previous work with horses, which related a significant increase of speed in gallop tests with this dose, and with rats, which showed no increase of locomotor activity nor any alteration of behaviour, with larger doses (2.5 and 5 mg caffeine kg⁻¹).

The dose–response curve of caffeine on SLA showed that doses of 0.2–2 mg kg⁻¹ are ineffective, whereas larger doses cause characteristic symptoms of excitement that, in theory, could stimulate animal performance. After caffeine administration, the peak effect usually appears between 5 and 25 min and, depending on the dose, the duration of the effect can be up to 4 h.

Regarding the kinetic study, our results are consistent with the literature. The plasma analyses for caffeine showed the largest elimination rates occurring in the first 10 h after injection, when in most of the animals it was possible to detect the drug.

The half-life for caffeine in horse plasma after the administration of 4 mg kg⁻¹ i.v. was ca. 18 h. Approximately 60% of the caffeine administered orally is eliminated in the first 3 days, but its metabolites can be found in horse urine up to 10 days after administration. Using a caffeine dose twice as great as the one that we used, it was observed that the plasma concentration peaked in the first 5 min after injection, maintaining levels of 2.7 μg ml⁻¹ in the first 2 h and after that a fast concentration decrease. The results obtained in the present study are compatible with the information found in the literature.

The concentration–time curves of caffeine, theobromine, theophylline and paraxanthine in equine plasma after intravenous, intramuscular and oral administration of 2.5 mg caffeine kg⁻¹ have been determined previously. Interestingly, these authors did not observe significant differences between the different routes. On the other hand, they found a direct correlation between caffeine half-life and the weight of the animals, suggesting that this variable could be foreseen as a function of the weight of the animal. In a similar experiment, but using only the oral route and dose and evaluating the effect of exercise on the plasma caffeine and its metabolites levels, the authors observed caffeine concentrations of >2.0 μg ml⁻¹ at 10 h after administration in both experimental groups. In addition, they found no significant differences in plasma caffeine when comparing exercised animals with non-exercised animals.

Regarding the concentration of caffeine found in urine, the present work obtained similar results to those found in the literature, which demonstrated that after the administration of 4 mg caffeine kg⁻¹ i.v. in horses there was a higher elimination rate during the first day, decreasing gradually after 6 h and staying important until day 9. Greene et al. found caffeine traces up to 8 days after injection, whereas we found caffeine traces up to 10 days after administration.

In another paper the caffeine elimination curve in horses was determined after i.v. administration of a dose of 2.5 mg kg⁻¹. The authors reported concentrations close to 4 μg ml⁻¹ of urine in samples obtained up to 10 h after administration. In our studies, no sample reached that value. The highest levels that we found (4.35, 4.87, 4.11, 4.08 and 3.37 μg ml⁻¹) were obtained between 0.5 and 4 h after i.v. administration of a dose of 2.0 mg caffeine kg⁻¹.

We can conclude that the HNED of caffeine to stimulate the horse SLA measured in the behavioural stall could be set as 2 mg kg⁻¹ i.v. Therefore, the no-effect threshold should not be greater than 2 μg caffeine ml⁻¹ plasma or 5 μg caffeine ml⁻¹ urine.

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