Plasma carnosine concentration: diurnal variation and effects of age, exercise and muscle damage

M. DUNNETT*, R. C. HARRIS†, C. E. DUNNETT‡ and P. A. HARRIS§

Department of Veterinary Basic Sciences, Royal Veterinary College, Hawkshead Campus, North Mymms, Hatfield, Hertfordshire AL9 7TA; †School of Sports Science, University College Chichester, Chichester, West Sussex PO19 4PE; ‡Independent Equine Nutrition, PO Box 319, Bury St. Edmunds, Suffolk IP28 6SY; and §Waltham Centre for Pet Nutrition, Melton Mowbray, Leicestershire, UK.

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Summary

This study was undertaken as part of a larger investigation into carnosine metabolism and function in the Thoroughbred horse. More specifically, we wished to evaluate plasma carnosine concentration as a potential indicator of muscle carnosine status. In contrast to man, carnosine is present in equine plasma where its presence is consistent with the absence of plasma carnosinase. A significant effect of age on plasma carnosine concentration in resting Thoroughbred horses was observed. Values in horses age 3 years and older were 11.3–14.1 µmol/l, whereas concentrations in foals and yearlings were 3.9–8.7 µmol/l (P<0.001). Lower values in young horses may reflect lower skeletal muscle carnosine concentrations. There was no significant within-day variation in plasma carnosine concentration in fed and fasted horses (P>0.05). Intense exercise resulted in a small significant increase (P<0.05) in plasma carnosine concentration (pre-exercise, 10.3 ± 1.0 µmol/l; postexercise, 12.4 ± 4.4 µmol/l). Greater increases were observed (57.6–702.3 µmol/l) following onset of exercise-induced rhadomyolysis (ERS). An apparent relationship was observed between elevated plasma carnosine and increased plasma creatine kinase (CK) and aspartate transaminase (AST) activities. Plasma carnosine concentrations did not reflect the severity of the condition as determined by clinical examination. In conclusion, elevated plasma carnosine levels are observed following exercise induced muscle damage, with the greatest elevations occurring during episodes of external rhabdomylosis syndrome. Plasma carnosine measurements could provide an alternative clinical indicator of muscle damage; and in conjunction with plasma taurine measurements may be indicative of selective *type 1* or *type 2* muscle fibre damage. However, given the complexity of the analytical technique, its applications would probably be confined to specialist referral or research centres.

Introduction

In the horse, the imidazole dipeptide carnosine (ß-alanyl-L-histidine) is present predominantly in skeletal muscle, where it is one of the most abundant small molecular weight compounds. Concentrations in equine skeletal muscle frequently exceed 100 mmol/kg dry weight (Harris *et al.* 1990). Carnosine functions as a physiological hydrogen ion (H^+) buffer (Davey 1960). Other physiological roles have also been ascribed to carnosine, including an antioxidant function (Kohen *et al.* 1988), involvement in wound healing (Nagai *et al.* 1986) and myosin ATPase activation (Avena and Bowen 1969). The presence of such high carnosine concentrations in equine skeletal muscle is central to the maintenance of intracellular pH homeostasis during intense exercise, where anaerobic glycolysis results in the accumulation of large quantities of lactate and H^+. In contrast to other small molecular weight compounds located within muscle, such as carnitine and creatine, carnosine is synthesised *in situ* from histidine and ß-alanine by the action of carnosine synthetase (EC 6.3.2.11) (Kalyanker and Meister 1959; Ng and Marshall 1976). The appearance of carnosine in plasma, therefore, may result primarily from leakage from muscle cells.

Micromolar concentrations of carnosine have been detected in plasma and serum from several species, including chicken, rabbit (Seely and Marshall 1981), rat (Kurisaki and Hiraiwa 1988), dog (Dunnett and Harris 1992) and some species of fish (Abe 1991; Abe *et al.* 1986). Carnosine is present in the plasma of human pre-term infants (mean ± s.d. 8.8 ± 7.1 µmol/l), but occurs at a lower concentration of 3.1 ± 7.5 µmol/l in older full-term infants (Valman *et al.* 1971) and is absent from normal adults (Perry *et al.* 1967; Dunnett and Harris 1992). This age-related disappearance of carnosine is correlated with an increase in serum carnosinase activity (EC 3.4.13.4). Serum carnosinase activity in children age less than 1 year is 0.14 ± 0.07 and 0.22 ± 0.15 µmol/ml/h for males and females, respectively, in contrast to adult values of 1.85 ± 0.59 to 9.66 ± 0.42 µmol/ml/h (Bando *et al.* 1984, 1986; Wassif *et al.* 1994).

Significant increases in plasma carnosine concentration as a result of increased leakage have been observed in rats following skeletal muscle damage (Kurisaki and Hiraiwa 1988). Increased leakage of carnosine from damaged muscle cells may explain some of the variability in plasma concentration seen in other studies.

Given the greater potential decline in intramuscular pH in the horse during strenuous exercise, carnosine performs a more significant role as an intracellular physiological buffer than in many other species. Despite this, no direct or indirect studies have been conducted to examine a possible relationship between plasma and muscle carnosine concentrations. Equine plasma carnosine concentrations of 1.7–5.2 µmol/l (McLean *et al.* 1987)

*Author to whom correspondence should be addressed.

and 11.3 ± 3.9 µmol/l (Dunnett and Harris 1992) have been reported. However, only small numbers of horses were studied. The aims of this study were to 1) measure the plasma carnosine concentration range in Thoroughbred horses of differing ages and genders; 2) study the 24 h variability in plasma carnosine in fed and fasted horses, and 3) investigate possible concentration changes associated with strenuous exercise and muscle damage. Plasma carnosinase activity was also measured.

Materials and methods

Blood sampling

Blood samples were obtained from the jugular vein by needle or via an indwelling catheter. Heparinised blood (5 ml) was centrifuged for 10 min at 2000 ***g*** and 4°C. Plasma was aspirated and stored at -20°C prior to analysis.

Analytical methods

Plasma carnosine (Dunnett and Harris 1992) and taurine (Jones and Gilligan 1983) concentrations were determined by high-performance liquid chromatography. Plasma carnosinase activity was determined using a minor modification (extension of incubation time to 120 min) of the method of Bando *et al.* (1984). The plasma carnosinase assay was validated by making comparative measurements on human plasma (n = 5 in duplicate). Mean ± s.d. activity in human plasma (2.08 ± 1.48 µmol/ml/h) was consistent with serum values (Bando *et al.* 1984) in the literature (1.85 ± 0.59 µmol/ml/h). Plasma aspartate transaminase[1] (AST: EC 2.6.1.1) and creatine kinase[2] (CK: EC 2.7.3.2) activities were determined by kinetic methods using commercial diagnostic reagents on a Kone Specific Autoanalyser[2].

Protocols and sampling procedures

The study was conducted within the provisions of the UK Animals (Scientific Procedures) Act 1986 and with the approval of the ethics committee of the institute.

Study A - plasma carnosine concentration and carnosinase activity in the Thoroughbred horse, and the influence of age and gender: Blood samples were collected from 112 Thoroughbred horses in training (26 males, 30 females, 25 geldings), yearlings (13 males, 13 females) and foals (2 males, 3 females) from 4 racing yards and one stud farm in the Newmarket area. All blood samples were collected between 15.00 and 17.00 h.

Study B - variation in plasma carnosine concentrations over 24 h in fed and fasted horses: Blood samples were collected at 2 h intervals from 6 resting Thoroughbred horses, age 4–7 years (5 geldings and 1 filly), over a 24 h period on 2 separate days. On the first day, each horse underwent a normal feeding regimen comprising Spillers Stud Cubes[3] (2 kg) provided at 07.00, 12.30 and 16.30 h, and hay (2–3 kg) provided with the morning and evening feeds. Water was provided *ad libitum* throughout the day. On the second day, the horses were fasted following the evening feed. Blood samples were not collected during the overnight phase of the fasting experiment. The experimental sessions were separated by a period of 2 days.

Study C - variation in plasma carnosine concentration as a result of high-intensity exercise: Six Thoroughbred horses (4 geldings, 2 fillies), age 5–13 years, were trained for 4 weeks prior to undertaking a treadmill-based standardised exercise test (SET). During the SET, horses were exercised continuously at an intensity that was increased stepwise to a point of near-maximal performance. Horses were walked at 1.6 m/s for 10 min on a level surface, followed by a succession of canters of 1 min duration on a 5° incline at speeds of 6, 8, 9, 10, 11 and 12 m/s or until the onset of fatigue, defined by the point at which they were no longer able to match the speed of the treadmill despite humane encouragement. Blood samples were collected pre-exercise and 5 min, 30 min, 2 h and 24 h postexercise.

Study D - Changes in plasma carnosine concentration following the onset of equine exertional rhabdomyolysis syndrome (ERS): Blood samples were obtained from 3 horses following the onset of episodes of ERS. The number of samples and their collection times varied in each horse. Initial samples were collected within 90 min of the onset of ERS and subsequent samples were obtained at 24, 48, 72, 60 and 120 h. The occurrence of ERS in each horse was diagnosed by the same veterinary surgeon. Established criteria were used to assess the clinical severity of the episode in each case (Harris 1989). These criteria included mobility, rigidity and swelling of the skeletal muscles, sweating, pulse and respiration rates, and urine colouration. Severity was graded on a scale from 1 to 5, with a grading of 5 signifying the greatest severity.

Statistical analyses

A 2-way analysis of variance (ANOVA) was used to identify significant effects of age and gender. Significance was declared at $P<0.05$. In the instance where significance was detected, a multiple comparison test, Fisher's Protected Least Significant Different (PLSD), was applied. Plasma carnosine concentrations at different times in fed and fasted horses and between fed and fasted horses at different times were compared using 2-way ANOVA. Pre- and postexercise plasma carnosine concentrations were compared using 1-way ANOVA.

TABLE 1: Plasma carnosine concentrations in horses of different ages and genders (n = 112)

	Plasma carnosine concentration (µmol/l)			
Age	Males (n)	Females (n)	Geldings (n)	All genders (n)
Foals	5.6 ± 1.6 (2)	5.1 ± 0.5 (3)	NA	5.8 ± 1.3 (5)
Yearlings	6.8 ± 0.8 (13)	6.5 ± 1.6 (13)	NA	6.0 ± 1.3 (26)
2	10.3 ± 3.4 (16)	11.3 ± 3.1 (12)	9.8 (1)	10.7 ± 3.2 (29)†
3	12.8 ± 3.5 (9)	12.1 ± 3.2 (6)	11.3 ± 1.4 (4)	12.2 ± 3.0 (19)†
4	14.1 (1)	12.3 ± 1.5 (4)	11.9 ± 1.1 (3)	12.4 ± 1.5 (8)†
5	NA	13.0 ± 4.4 (6)	13.4 ± 2.6 (4)	13.2 ± 3.6 (10)†
6	NA	10.4 ± 2.7 (2)	12.4 ± 3.4 (13)	12.1 ± 3.3 (15)†

†Significantly different from foals and yearlings ($P<0.001$); NA = samples not available.

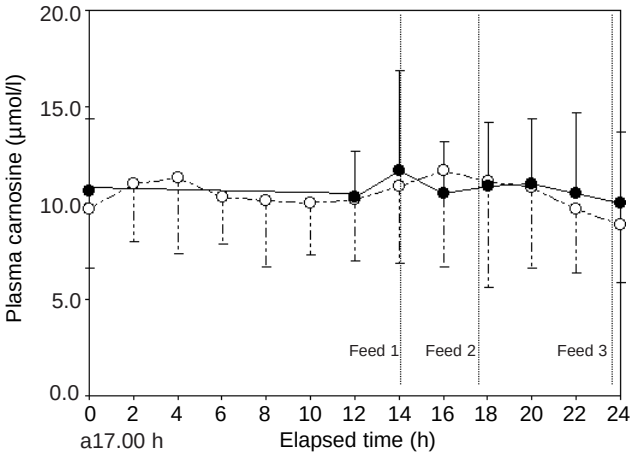

Fig 1: Changes in plasma carnosine over 24 h in fed (○) and fasted (●) horses (n = 6).

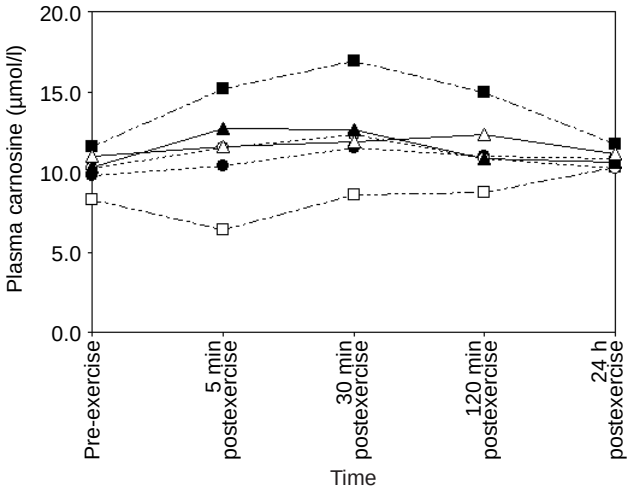

Fig 2: Comparison of plasma carnosine concentrations before and after exercise in individual horses (n = 6).

Results

Study A

Mean ± s.d. plasma carnosine concentrations in Thoroughbred horses of different ages and genders are given in Table 1. There was no significant difference in plasma carnosine concentration between males, females and geldings at any age (P>0.05). There was, however, a significant effect of age on the plasma carnosine concentration. Plasma carnosine concentrations in both foals and yearlings were approximately half those found in horses age 2–6 years (P<0.001). No significant difference in plasma carnosine concentration was evident between horses age 2–6 years. The range in plasma carnosine concentration in yearlings (3.9–8.7 µmol/l) was also much narrower than in older horses (e.g. 6.5–17.0 and 8.2–21.0 µmol/l in 2- and 3-year-olds, respectively). Plasma carnosinase activity was not detectable in samples from all horses.

Study B

Comparisons of mean ± s.d. plasma carnosine concentrations between fed and fasted horses over 24 h are shown in Figure 1. Values for all 6 horses were within the range established in the preceding study. Mean plasma carnosine concentration over 24 h in resting horses during normal feeding ranged from 8.8 to 11.7 µmol/l with a mean within-horse variance of 1.6 µmol/l. Changes in plasma carnosine concentration over 24 h during normal feeding were not significant (P>0.05). Mean plasma concentration over 24 h during fasting was 10.0–11.7 µmol/l with a mean within-horse variance of 1.5 µmol/l. Changes in mean plasma concentration over 24 h during fasting were not significant (P>0.05). Plasma concentrations were not significantly different between fed and fasted horses (P>0.05).

TABLE 2: Peak measured values in plasma carnosine and taurine concentrations and CK and AST activities in 3 horses following the onset of ERS

		Peak values measured (time after onset)			
Horse	Grade (severity)	Taurine (µmol/l)	Carnosine (µmol/l)	CK (iu/l)	AST (iu/l)
1	2–3 (Moderate)	38.4	57.6 (2 h)	1500 (1 h)	1250 (48 h)
2	2 (Mild)	63.1	143.0 (1 h)	1700 (2 h)	2300 (48 h)
3	1 (Mild)	256.0	702.3 (1 h)	37000 (24 h)	11900 (24 h)

Study C

Pre- and postexercise changes in plasma carnosine concentrations in individual animals are shown in Figure 2. Mean ± s.d. pre-exercise plasma carnosine concentration (10.3 ± 1.0 µmol/l) was within the range observed in Study A; mean peak concentration after exercise was 12.4 ± 4.4 µmol/l (P<0.05 compared to the pre-exercise concentration). Increases in plasma concentration occurred in 5 of the 6 horses. In one of these, the plasma concentration reached 17 µmol/l. The greater increase in postexercise plasma carnosine concentration in this horse was accompanied by greater increases in plasma AST and CK activities. In the remaining 4 horses, smaller increases, within the earlier observed range, were seen. For all horses, however, mean pre- and postexercise plasma AST and CK activities were within the normal clinical reference range (AST, 105–230 iu/l; CK, 16–49 iu/l), indicating minimal skeletal muscle damage. Mean pre- and postexercise AST and CK activities were not significantly different (P>0.05).

Study D

Three cases of ERS were investigated during the course of this study. The clinical details of each case are summarised below.

Horse 1: A 17-year-old Thoroughbred x Arab mare experienced an episode of ERS following approximately 10–20 min moderate exercise comprising walking, trotting and cantering over a series of 6 cross-country fences. The horse showed reluctance to move, firm and swollen gluteal muscles, and a slight discolouration of the urine. The clinical severity of the episode was graded 2–3.

Horse 2: An 11-year-old Thoroughbred mare experienced an episode of ERS following moderate exercise comprising approximately 40 min walking, 15 min trotting and 5 min half-speed uphill cantering. The horse lay down immediately on returning to the stables after exercise, but could be encouraged to stand and move. The clinical severity of the episode was graded 2.

Horse 3: A 10-year-old Thoroughbred x gelding experienced an episode of ERS; details of the exercise undertaken prior to the episode are unknown. Slight stiffness was evident, but no muscle

abnormality was apparent. All other clinical signs appeared normal. The clinical severity of the episode was graded 1.

Peak measured values in plasma carnosine and taurine concentrations and CK and AST activities in the 3 horses following the onset of ERS are shown in Table 2. ERS was associated with increases in carnosine, AST and CK in 2 horses, and carnosine, taurine, AST and CK in the third. Peak concentrations in carnosine and taurine (where relevant) occurred within 2 h; peak activities in AST and CK occurred at variable times (Table 2). The observed peak increases in carnosine, taurine (*Horse 3* only), AST and CK were above normal in each case.

The increase in plasma carnosine concentration varied from 5-fold (*Horse 1*) to 70-fold (*Horse 3*) and was related to the increases in AST and CK. An increase in plasma taurine concentration was seen only in *Horse 3*, where the greatest increases in all 3 other parameters were also observed.

Discussion

Micromolar concentrations of carnosine in plasma from Thoroughbred horses found during this study are slightly higher than the only previously reported value for equine plasma in 2 groups of Standardbred horses at rest (1.7 ± 1.7 and 5.2 ± 2.8 µmol/l) (McLean *et al.* 1987). It is possible that this discrepancy arises from methodological differences between the 2 studies, or that there exists an interbreed difference that reflects between-breed differences in muscle carnosine content. Similar values have also been reported for chick and rabbit plasma (Seely and Marshall 1981; Kurisaki and Hiraiwa 1988). The age-related increase in equine plasma carnosine concentration is similar to that for carnitine reported by Foster *et al.* (1989). The increase in plasma carnosine concentration may arise from increasing biosynthetic capacity during maturation and therefore a greater potential 'leakage' from skeletal muscle. Increasing carnosine biosynthetic capacity in muscle and thereby increasing carnosine content has been demonstrated in rats (Johnson and Hammer 1992) and man (Christman 1976). Interestingly, in man, carnosine is initially present in the blood of premature infants, at concentrations comparable with those in foals and yearlings, but subsequently declines during later infancy. This reduction coincides with an increase in plasma carnosinase activity (Bando *et al.* 1984). In contrast, plasma carnosinase activity was not detected in all horses sampled. The apparent absence of plasma carnosinase activity in the Thoroughbred horse is consistent with data from other nonprimates (Jackson *et al.* 1991).

In both man and horse, it has been established that the plasma concentrations of certain metabolites exhibit regular fluctuations during the course of the day (Orme *et al.* 1994; Youket *et al.* 1985; Zilva and Parnall 1984), the changes in some of these being related to feeding. Previous studies in horses have shown postfeeding increases in the plasma concentrations of amino acids including histidine (Johnson and Hart 1974; Russell *et al.* 1986). However, comparison of the within-horse variance in plasma carnosine concentration between fed and fasted horses (1.6 µmol/l fed, 1.5 µmol/l fasted) suggests that normal feeding has a negligible influence on the plasma concentration. Within-day fluctuations in plasma carnosine concentration were random and differences in concentration at different times were not statistically significant ($P>0.05$).

The small postexercise increases in plasma carnosine concentrations found in Study C are similar to increases observed in Standardbred horses following racing, in which the mean pre- and postexercise plasma carnosine concentrations were 1.7–5.2 and 8.3–11.0 µmol/l, respectively (McLean *et al.* 1987). It is probable that the postexercise increases are a consequence of minor sarcolemmal damage and subsequent leakage of sarcoplasmic contents into the blood. Owing to the high muscle carnosine concentration in the horse, significant increases in plasma concentration are likely to occur as a result of even minor muscle fibre damage. This possibility may explain some of the higher plasma carnosine values observed in the 112 horses in training. In these animals, blood samples would, in most instances, have been collected between 6 and 10 h postexercise. It is not impossible that some subclinical muscle damage was present in a small proportion of the horses blood-sampled.

No previous investigations of the effect of exercise-induced muscle damage on plasma carnosine concentrations have been made. A 10- to 20-fold increase in the plasma concentration, however, was observed in rats following muscle trauma (Kurisaki and Hiraiwa 1988). Evidence from Study D suggests that large increases in plasma carnosine concentration may result from muscle damage associated with ERS. The peak concentration was proportional to the magnitude of the damage, as indicated by large increases in both plasma AST and CK activities, although this did not discriminate between mild widespread damage and severe localised damage. The use of pharmacokinetic parameters determined after intravenous and intramuscular injections of carnosine may enable an estimate to be made of the mass of muscle damaged in this case.

As a result of the almost exclusive localisation of taurine in *type I* muscle fibres (Dunnett and Harris 1995) and the much greater concentration of carnosine in *type II* fibres, the appearance of increased plasma concentrations may be indicative of selective *type I* or *type II* fibre damage. Owing to the relatively low concentration of carnosine in tissues other than skeletal muscle, damage to these will result in only minimal increases in plasma carnosine concentration. It is estimated from measurements of carnosine in myocardium, the most abundant pool of carnosine after skeletal muscle (Dunnett 1995), that complete destruction of this tissue would result in a peak plasma concentration of just 30–40 µmol/l. Taurine, on the other hand, is ubiquitous in other tissues and damage to these could also increase its concentration in plasma. Skeletal muscle, however, constitutes by far the largest single pool of taurine, 75% or more in the rat (Jacobson and Smith 1968), as well as being the tissue most likely to be affected by exercise. The large increases in plasma carnosine and taurine concentrations seen in *Horse 3* are consistent with damage to both *types I* and *II* muscle fibres. Although the present data is limited, it is suggestive and may call into question the earlier proposal of Valberg *et al.* (1993) that muscle damage associated with episodes of ERS is confined to *type II* fibres. However, a much more extensive study would need to be conducted to establish this fact. Furthermore, it is also possible that, during more severe ERS episodes, *type I* fibre damage may occur consequentially to *type II* fibre damage.

Measurement of plasma carnosine concentration could offer an alternative approach to the clinical identification of skeletal muscle damage in suspected cases of ERS. Furthermore, concurrent analysis of changes in plasma carnosine and taurine concentration could provide a useful diagnostic tool to ascertain damage to *type I* and/or *type II* muscle fibres. Admittedly, given the need for specialised high-performance liquid chromatographic systems with which to perform the analysis, the application of the technique would probably be limited to specialist referral centres or research centres. Plasma carnosine measurement could provide a more selective test for muscle damage than plasma AST/CK activities. Plasma AST/CK activities are not specific indices of skeletal muscle damage.

Although CK activities are relatively high in skeletal muscle, significant activities are also present in the brain and heart; significant AST activity also occurs in the heart and liver. Therefore, increases in these parameters are also likely with various forms of soft tissue damage. As noted earlier, there are no other pools of carnosine in the body capable of causing a similar increase in the plasma concentration. Further detailed work would need to be conducted to establish whether plasma carnosine analysis could provide a more quantitative measure of the severity of muscle damage than the methods currently used.

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Manufacturers' addresses

[1]Randox Laboratories Ltd., Co. Antrim, N. Ireland.
[2]Kone Diagnostics, San Diego, California, USA.
[3]Spillers Horse Feeds, Milton Keynes, Buckinghamshire, UK.

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