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Transdermal delivery of carnosine into equine skeletal muscle

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RESEARCH ARTICLE

Abstract

The dipeptide carnosine consists of β-alanine and L-histidine. It plays a major role in skeletal muscle metabolism, especially as an intracellular buffer and antioxidant. Increasing intramuscular carnosine has been shown to improve recovery from exercise and increase anaerobic threshold and time-to-exhaustion. Dietary supplementation with carnosine does not effectively increase intramuscular carnosine due to the presence of carnosinase in the blood. However, an effective transdermal delivery process could expediently increase intramuscular concentrations of carnosine. This study's objective was to examine the efficacy of a transdermal system for delivering carnosine into the skeletal muscle of horses, using a randomised, placebo controlled, crossover study. Carnosine plus a proprietary transdermal delivery agent or the agent alone (placebo) were applied to the middle gluteal muscles of 10 Thoroughbred racehorses, and muscle biopsies were taken before and 30, 60, and 120 min after application. Muscle carnosine concentration was measured using an enzyme-linked immunosorbent assay. A two-way repeated measures analysis of variance was used to test for the main effects of time and treatment (placebo or carnosine) as well as an interaction between time and treatment. Independent F-tests examined the change in intramuscular carnosine levels from baseline to each time point (30, 60, and 120 min). There was a significant main effect of treatment (P=0.004), no significant main effect for time (P=0.18), and a non-significant interaction of treatment with time (P=0.08). Mean intramuscular carnosine concentrations increased from baseline to 120 min. Compared to concentrations following placebo application, carnosine was greater by ~35% at 30 min (P=0.002) and ~46% after 60 min (P=0.044), but not at 120 min (P=0.20). The results indicated that intramuscular carnosine can be increased using a transdermal delivery system within 60 min of application which could have important implications for the health of horses, and their capacity to perform and recover from physical activity.

Keywords: carnosine, gluteal muscle, horses, transdermal, intracellular buffering, exercise

1. Introduction

Carnosine is a dipeptide that is found in the cells of almost all organisms and serves as an important molecule in mammalian metabolism (Begum *et al.,* 2005; Boldyrev *et al.,* 2013; Budzeń and Rymaszewska, 2013; Holliday and McFarland, 2000). Carnosine contains an imidazole ring with a pKA of 6.83, which is the pH range that muscle cells pass through as hydrogen ions accumulate during exercise. As such, carnosine functions as the main intracellular buffer to maintain pH during exercise and also displays robust antioxidant and antiglycation properties (Boldyrev *et al.,* 2010; Dunnett and Harris, 2010; Dunnett *et al.*, 1997; Nagai *et al.,* 2003). Due to the important nature of this molecule in metabolism, performance, and aging, carnosine has been widely studied for its ability to improve the overall health and physical work capacity of mammals.

Carnosine plays a critical role in the health and physical work capacity of horses and exists in much higher concentrations in horses than in humans. For example, intramuscular carnosine levels hover around 20-30 mmol/kg in humans, and between 30 and 170 mmol/kg in horses (Dunnett and Harris 1999; Harris *et al.*, 2006; Marlin *et al.,*

1989), which reflects an up to 8-fold difference between these mammalian species.

Currently, increasing intramuscular carnosine has been a laborious and inefficient endeavour. Oral carnosine supplementation has been largely ineffective due to one key barrier preventing the oral carnosine from accumulating in muscle tissue: the presence of the enzyme carnosinase in blood. Supplemental carnosine that is absorbed (Dunnett and Harris, 1999; Harris *et al.*, 2006; Marlin *et al.,* 1989) following ingestion is quickly degraded by carnosinase, thus preventing its accumulation in skeletal muscle (Gardner *et al.,* 1991). However, one can glean information regarding the potential of carnosine by looking at studies done with β-alanine, the availability of which is the rate-limiting precursor to endogenous carnosine synthesis. β-alanine is not broken down in the blood by carnosinase and can bioaccumulate in skeletal muscle. Indeed, several studies in human and equine athletes have shown that β-alanine supplementation results in an increase in muscle carnosine (by ~20-40%), and concurrent improvements in buffering capacity and the rate at which muscular fatigue develops during exercise (Derave *et al.,* 2007; Dunnett and Harris, 1999, Gardner *et al.,* 1991; Kendrick *et al.,* 2009).

A previous study demonstrated that carnosine passes through the skin when mixed with a pharmaceutical vehicle that facilitates the transdermal movement of carnosine (Dissette *et al.,* 2018). However, the quantitative effect of this movement on skeletal muscle carnosine concentrations has not been reported. The objective of the present study was to assess whether the application of carnosine to the skin overlying equine skeletal muscle was associated with an increase in carnosine concentrations of that muscle and, if so, to determine the time course of this increase.

2. Materials and methods

Protocol overview

The study was a randomised, placebo controlled, crossover study conducted with Thoroughbred racehorses at Washington State University. The samples were collected over a period of two-days, 14 days apart to allow for washout of the previously applied substance. Horses were randomly assigned to either the placebo arm or the transdermal carnosine arm through random number generation for the first collection period and then assigned to the other arm on the second collection period. The study was approved by the Institutional Animal Care and Use Committee at Washington State University. Muscle carnosine assays were performed at the University of Alabama Birmingham.

Horses

Ten Thoroughbred horses aged 3-8 years were used (Table 1). Horses had been in regular racetrack training until about 4 weeks before the start of the study.

Transdermal carnosine

The transdermal carnosine gel was an aqueous based formula that consists of water, glycerine, magnesium sulphate, and a proprietary carnosine-complex in which the total concentration of carnosine as a volume/volume percentage was 1.5%. The gel was applied to a 25 cm^2 area. The placebo was visually, tactilely, and olfactorily identical, but did not contain the carnosine-complex.

Table 1. Characteristics of individual horses to evaluate efficacy of transdermal delivery of carnosine to the underlying middle gluteal muscle.

Participant	Sex	Age (year)	Weight (kgs))
1	male	7	509
2	female	7	500
3	female	4	480
4	male	4	500
5	male	4	477
6	female	4	482
7	male	8	500
8	female	4	470
9	male	3	486
10	male	5	493
Mean		5.0	489.8
Standard error		0.5	4.0

Biopsy protocol

A total of 4 muscle biopsies were obtained from the middle gluteal muscle of each horse. Height, weight, age and gender were recorded. An area, ~25 cm^2 over the middle gluteal muscle was clipped and shaved, cleaned using alcohol, and locally anaesthetised by the subcutaneous injection of <5 ml 2% lidocaine. Following a sterile skin preparation, a pre-application biopsy (baseline sample) was taken (T=0) using a Bergström biopsy needle as previously described (Lindholm and Piehl, 1974). All biopsies were obtained from a single incision and were obtained at a depth of 6 cm. The biopsy site was covered and 60 ml of either the placebo or carnosine mixed with the transdermal delivery vehicle was applied to the prepared site and additional biopsies were obtained 30 (T=30), 60 (T=60), and 120 (T=120) min after the application was complete. The immediate biopsy area was cleaned prior to each biopsy to ensure aseptic conditions. The biopsy samples were snap frozen in liquid nitrogen, packaged in dry ice, and sent to the laboratory for biochemical analysis.

Quantification of intramuscular carnosine by ELISA

Briefly, muscle biopsy samples were trimmed into ~31 mg pieces and aliquoted. One aliquot of the sample was homogenised in phosphate buffered saline and 1% protease inhibitor. Samples were then spun at 10,000 rpm for 10 min and the supernatant was removed and saved for use in the commercially available ELISA that was used to determine carnosine concentrations (CAT: NBP2-75013; Novus Biological Sciences, Centennial, CO, USA); the inter- and intra-assay coefficients of variation were <4.54% and <5.31%, respectively. Cellular debris was labelled and stored for potential future use.

Statistical analysis

The data were analysed for normality using the Shapiro-Wilk test. A two-way repeated measures analysis of variance (RM-ANOVA) was used to test for the main effects of time and treatment (placebo or carnosine) as well as an interaction between time and treatment. Independent F-tests were also conducted to examine the change in intramuscular carnosine levels from baseline to each time point (30, 60, and 120 min). Alpha values were set *a priori* at <0.05.

3. Results

There was no difference between the placebo or carnosine groups at T0 for intramuscular levels of carnosine (Placebo: 2,646 [1,502-3,231] (ng/mg); Carnosine: 1,973 [1,198-2,760] (ng/mg), P=0.29, median [interquartile range]). The RM-ANOVA showed a significant main effect of the treatment (P=0.004), no significant main effect for time (P=0.18), and a trend toward an interaction of treatment by time (P=0.08) (Figure 1).

When comparing data pertaining to carnosine and placebo applications at T30, intramuscular concentrations of carnosine were increased by 35±11% (mean ± standard error), compared to -3±14% for placebo (P=0.001). At T60, intramuscular carnosine concentrations were increased by 46±17% compared to 11±16% for placebo (P=0.044). At T120, muscle levels of carnosine increased by 76±45% from the baseline value, compared to 13±16% for placebo. These changes were not significantly different (P=0.20; Figure 2).

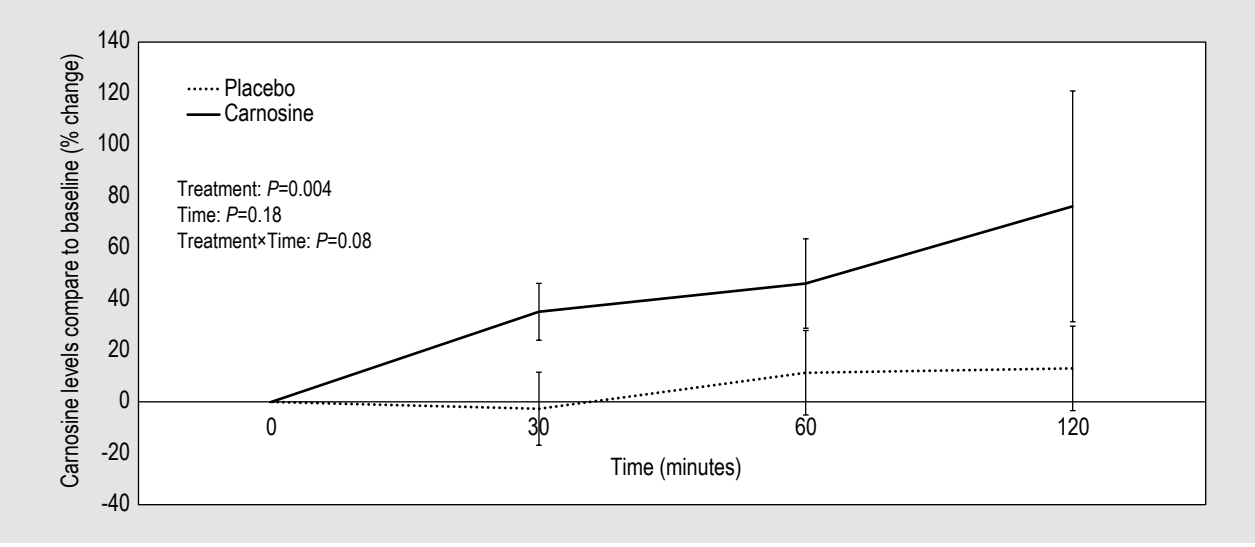

Figure 1. Effect of transdermal transfer of carnosine to the middle gluteal muscle on its concentrations (mean ± standard error) of carnosine up to 120 min after its application to the skin overlying this muscle (n=10).

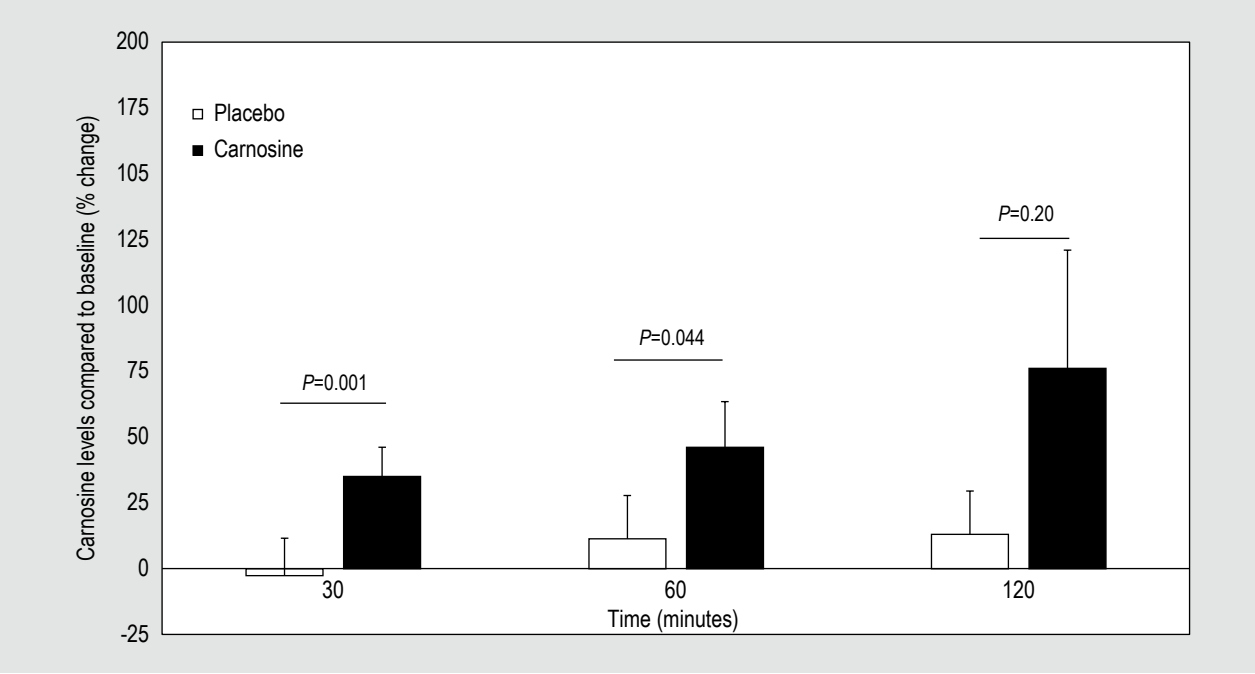

Figure 2. Comparison of changes in middle gluteal muscle carnosine concentrations 30, 60 and 120 min following the application of carnosine or placebo to the skin overlying the muscle (mean ± standard error) (n=10).

4. Discussion

Carnosine plays a critical role in maintaining intracellular pH during exercise and functions as the primary intracellular buffer in virtually all mammals. Until now, attempts to directly increase muscle concentrations of carnosine have been largely ineffective. These data present the first evidence that transdermal delivery of carnosine can increase its concentration in muscle tissue in as soon as 30 min post-application. These findings have substantial implications for the rate of, and health during, recovery, and physical work capacity of horses in all equine sports.

On average, the increases in muscle carnosine post application ranged between 30-50% from baseline concentrations within the first hour, and up to ~75% within 2 h. However, interindividual effects should also be considered, as some horses showed a minimal response (0-15%) and some horses were observed to have 200-300% increases by 2 h. These results compare favourably to previous attempts to raise intramuscular carnosine concentrations in horses by feeding β-alanine and histidine, which are the precursor amino acids to carnosine. In this previous work, 30 days of β-alanine (100 mg/kg) and histidine supplementation (12.5 mg/kg) showed a 10-40% increase in intramuscular carnosine concentrations (Dunnett and Harris, 1999). In contrast, the present study shows that delivering carnosine through a transdermal approach effectively increases intramuscular carnosine concentrations and works within 30-60 min following a single application, as opposed to 30 days of consistent feeding. This makes a transdermal approach substantially more efficacious, and possibly more cost effective.

There are also important implications surrounding the general metabolism of carnosine based on these findings. First, the present study indicates that a single, acute application increases carnosine levels substantially and that, when considering standard carnosine metabolism, the concentrations of carnosine should stay elevated above baseline values for at least an hour after a single application of transdermal carnosine (Baguet *et al.*, 2009; Spelnikov and Harris, 2019). Second, if we draw parallels from the literature regarding the feeding of β-alanine to both humans and horses, the chronic use of transdermal carnosine is likely to raise basal levels of carnosine substantially as well, with basal increases of 30-50% being reasonable targets to achieve (Derave *et al.,* 2007; Dunnett and Harris, 1999; Kendrick *et al.,* 2009). One consideration of the current study to consider is that this study did not examine the deeper muscles, and future work will be needed to determine if a transdermal approach can be utilised to increase carnosine concentrations in the deep musculature.

The present study suggests that increasing equine skeletal muscle carnosine concentrations via a transdermal delivery system is feasible. This may have several possible benefits from a health and recovery perspective, as well as from a physical work capacity viewpoint. Carnosine has been well studied as an intramuscular antioxidant. It exhibits robust free-radical scavenging ability in muscle tissue, as well as in brain tissue (Kohen *et al.,* 1988). Additionally,

it has been shown to reduce glycation in animal studies, suggesting it may help attenuate some of the protein-based mechanisms of aging (Bingül *et al.,* 2017; Kohen *et al.,* 1988). From a work capacity aspect, increasing intracellular carnosine through a transdermal approach might reduce the rate at which fatigue develops, improve recovery, and increase the work capacity of a horse. However, this requires controlled evaluation. Studies in humans have shown that higher levels of muscle carnosine lead to lower levels of fatigue (Bingül *et al.,* 2017; Kohen *et al.,* 1988; Varanoske *et al.,* 2017). This reduction in fatigue may be, at least in part, due to the fact that increasing carnosine levels directly improves the buffering capacity of muscle tissue of people in the face of hard physical work (Bingül *et al.,* 2017; Dunnett and Harris, 1999; Kohen *et al.,* 1988; Varanoske *et al.,* 2017). Improving intramuscular buffering capacity could allow a horse to maintain a high rate of glycolysis for longer due to an enhanced ability to buffer the effects of the hydrogen ion production that accompanies glycolysis. The associated reduced degrees of fatigue may substantially improve the recovery rate of the horse, as the mechanical and biochemical strain placed on the system may be attenuated at the same given workload.

In addition, improving the intracellular buffering capacity within 60 min of application of carnosine to the skin also presents an opportunity for attenuating metabolic related issues that may arise from injury or surgical interventions on the musculoskeletal system of horses (Di Paola *et al.,* 2011; Roberts *et al.,* 1998). Anaerobic metabolism that can predominate at the site of injury, trauma, or infection often leads to rapid drops in intracellular pH leading to either necrotic or apoptotic cell death. The ability to increase intracellular buffering capacity in a short time window may provide substantial benefit to the animal in these scenarios.

Another aspect of this study that deserves consideration is the fact that the present study was conducted on the musculature of the Thoroughbred racehorse which is a breed that already tend to have the highest levels of carnosine in the gluteus muscles (Mori *et al.,* 2015). These results demonstrate that even in a muscle group that has a naturally high level of carnosine, these concentrations can still be increased well above baseline by using the transdermal delivery system that was utilised here. It may be that other muscle groups that have naturally lower levels of carnosine (e.g. the masseter and triceps brachii muscles) will experience even greater increases in intracellular carnosine levels via the transdermal approach, but this requires additional research.

In summary, these results provide initial data demonstrating that a transdermal delivery system can increase carnosine concentrations in the muscle tissue of horses within 60 min of applying it to the skin. This may have important implications for the health, recovery, and performance capacities of horses competing in most equine sports disciplines.

5. Conclusions

These data are the first data to demonstrate that transdermal delivery of carnosine to the middle gluteal muscle can substantially increase intramuscular levels of carnosine and do so within 30-60 min of application. Due to the ability of transdermal carnosine to mitigate declines in performance during sustained efforts and to aid in recovery from strenuous effort, this formulation may be beneficial for horses that engage in a wide variety of athletic endeavours (e.g. racing, show jumping and polo).

Conflict of interest

BD is affiliated with and owns stock in Velocity Animal Sciences. This study was funded by Velocity Animal Sciences.

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