The effect of oral and intravenous dextrose on C-peptide secretion in ponies[1]

M. A. de Laat[2], J. J. van Haeften and M. N. Sillence

Earth, Environmental and Biological Sciences, Queensland University of Technology (QUT), Brisbane, Queensland, 4001, Australia.

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[2]Corresponding Author: melody.delaat@qut.edu.au

ABSTRACT: Managing equine hyperinsulinemia is crucial for preventing laminitis, but our understanding of the mechanisms involved in insulin dysregulation in this species is incomplete. C-peptide is co-secreted with insulin, but is resistant to hepatic metabolism and can be used to study insulin dysregulation. This study examined C-peptide secretion in serial blood samples collected after oral and i.v. dextrose (0.75 g/kg) administration to 9 ponies (body condition score 7.1 ± 0.5). The ponies were designated as hyperinsulinemic (HI) or normoinsulinemic (NI) responders before the study, using oral glucose tests and fasted glucose-to-insulin ratios, and responses were compared between the 2 groups. C-peptide concentrations increased ($P < 0.01$) rapidly from fasted levels after both oral and i.v. dextrose, with similar area under the concentration-time curve (AUC) for both tests and a significant correlation with $AUC_{insulin}$. The $AUC_{C\text{-}peptide}$ was similar in HI and NI ponies after i.v. dextrose, indicating similar pancreatic capacity for both groups. However, for oral dextrose the $AUC_{C\text{-}peptide}$ and the $AUC_{insulin}$ were markedly higher ($P < 0.05$) in the HI ponies, indicating a greater secretion rate of these peptides. Slower insulin clearance might have also contributed to the larger $AUC_{insulin}$ in HI ponies, but this hypothesis requires further investigation with specific measures of hepatic insulin clearance.

Key words: C-peptide, equine, horse, hyperinsulinemia, insulin, laminitis

INTRODUCTION

Insulin dysregulation is increasingly recognized in horses and ponies and is usually coincident with increased fat mass, insulin resistance (**IR**) and/or amplified insulin responses to ingested carbohydrate (Frank and Tadros, 2014). Laminitis, the painful disengagement of vital soft-tissue structures located between the pedal bone and hoof wall, can be initiated by hyperinsulinemia (Asplin et al., 2007; de Laat et al., 2010). Often diagnosed in association with laminitis, insulin dysregulation is now also frequently detected before the onset of lamellar dysfunction and this represents an opportunity to prevent laminitis. Development of new treatment options for insulin dysregulation is limited by incomplete understanding of the mechanisms involved in equine hyperinsulinemia.

Proinsulin, comprising conjoined insulin and C-peptide, undergoes sequential, enzymatic cleavage to produce insulin and C-peptide in proportionate amounts (Steiner, 2011). Studies in other species have shown that after secretion, insulin is substantially extracted during its first-passage through the liver, before reaching the systemic circulation. In contrast, the hepatic clearance of C-peptide is negligible and its half-life is ~6 times longer than that of insulin, so circulating concentrations are usually several-fold higher (Marques et al., 2004). Thus, in humans, C-peptide concentrations are used to approximate insulin secretion and pancreatic β-cell activity (Fujita et al., 2015). Further, examination of C-peptide and insulin ratios can provide an indication of hepatic insulin clearance (Meier et al., 2005). C-peptide has also recently been reported to have receptor binding capability and independent biological functions pertinent to laminitis pathophysiology, such as stimulation of blood flow and insulinomimetic actions (Marques et al., 2004; Wang et al., 2012; McKillop et al., 2014; Yosten et al., 2014).

Given current research and clinical interest in equine insulin dysregulation, it is surprising that C-peptide has only been examined in a small number of studies (Geor et al., 2010; Toth et al., 2010). Clearly, further investigation of C-peptide may be rewarding for equine metabolic disease. Thus, this study aimed to compare C-peptide and insulin responses to oral and i.v. glucose in ponies with and without insulin dysregulation, to test 2 hypotheses: firstly, that hyperinsulinemia results from increased insulin production rather than decreased insulin clearance; secondly, that hyperinsulinemia associated with the oral consumption of carbohydrates is not necessarily associated with peripheral IR (i.e. that it may occur in ponies that show no impairment in their response to i.v. dextrose).

MATERIALS AND METHODS

Sample population

Nine, university-owned, mixed-breed ponies (Table 1) were stratified for insulin responsiveness using both basal and dynamic tests one week before the study. This was done using fasted glucose-to-insulin ratios (**FGIR**) and a standard oral glucose test (**OGT**, 1 g/kg dextrose in a low glycemic ration; serum collected at 0 h and 2 h); (Frank and Tadros, 2014). Ponies with an FGIR below 4.5, and a serum insulin concentration of > 520 pM 2 h after oral dextrose, were classified as hyperinsulinemic (**HI**). An FGIR above 10, and a serum insulin concentration < 222 pM 2 h post dextrose, was designated as normoinsulinemic (**NI**). The ponies were all overweight (body condition score 7.1 ± 0.5), with the HI group having evidence of regional adiposity (Henneke et al., 1983; Carter et al., 2009).

Hepatic and renal function were assessed in all ponies with routine biochemistry testing for glutamate dehydrogenase, gamma glutamyl transferase, albumin:globulin (hepatic function)

and urea, creatinine (renal function). The ponies remained healthy: hepatic and renal function parameters within the accepted reference ranges for the assays used, and free from laminitis. Water intake was unrestricted. Ethical approval for the study was granted by the University Animal Ethics Care and Use Committee (SVS/QUT/109/13).

Study design

Initially, dextrose (0.75 g/kg; lower dose than OGT selected for palatability reasons) was administered orally to ponies as a component of an otherwise low glycemic ration. Seven days later, dextrose (0.75 g/kg) was administered i.v. via an indwelling catheter (Mila, Erlanger, KY) placed aseptically in the left jugular vein. The rate of glucose infusion was individualized for each pony, to match their rate of feed intake which was measured during the oral test (the average infusion time was 52.2 ± 3.1 min). The ponies were fed the same low glycemic base ration for both tests to ensure that the total energy intake was equivalent in the two parts of the study; 200 g bran (12.6 MJ/kg DM ME, 16.6% CP, 16% starch), 500 mL tap water, 0.3% BW lucerne chaff (10.9 MJ/kg DM ME, 24.4% CP, <2% starch, WSC 8%). No feed refusals, or adverse reactions to the oral or i.v. dextrose doses, were observed. Tests were performed at 0800 h after an overnight fast. Blood samples (6 mL) were collected before (0 h) and after dosing at 30, 60, 90, 120, 180 and 360 min, with additional sampling at 15 and 45 min for the i.v. test. Blood glucose was analysed immediately using whole blood with a handheld glucometer validated for use in horses by the investigators (de Laat et al., 2012). The remaining blood was placed into plain tubes, allowed to clot and then centrifuged (10 min, 1500 x *g*) to facilitate serum separation. The serum was dispensed into multiple aliquots before freezing and storage at -80˚C to avoid repeated freeze/thaw cycles.

Assays

Serum insulin concentrations were measured with an equine-specific ELISA (Mercodia, Uppsala, Sweden) validated in-house (intra-assay CV 5.9%; inter-assay CV 7% (low-range) and 6.6% (high-range)). In the absence of a suitable assay for C-peptide, 7 commercially available ELISAs were evaluated to determine their suitability for use with equine samples. Multi-donation pooled samples expected to contain 'low', 'mid' and 'high' C-peptide concentrations were prepared using archived equine serum samples with known insulin concentrations. The expected C-peptide concentration was predicted based on the premise that the secretion of C-peptide occurs in a 1:1 ratio with insulin. In addition to the pooled samples, OGT samples (0 and 2 h) from the 9 ponies above were included with each assay to provide further information.

The assays were assessed for cross-reactivity with equine serum, precision and accuracy. The specificity of these assays for measuring equine C-peptide, and its cross-reactivity with equine insulin cannot be determined precisely, until these peptides are available in pure form for use as assay standards. The same samples, assayed in duplicate, were used to validate all ELISAs, and the standards were assayed in triplicate. Cross-reactivity between equine C-peptide and the antibody supplied with each kit was determined by the level of antibody binding that occurred in the presence of equine serum. Precision was assessed by the intra-assay CV for 6 replicates of the high, mid and low-range pooled samples. Where an assay was deemed acceptable (CV < 10%), inter-assay CV was also calculated. Recovery after the addition of an assay standard was assessed for each pooled sample, and was used as an indicator of potential assay interference, for example from binding proteins. Pooled samples were incubated overnight with a mid-range standard (1:1) to allow equilibration before analysis. Recovery on dilution of

pooled samples with assay buffer (1:1, 3:4, 1:2 and 1:4) was used to assess assay accuracy, with results assessed for linearity (r^2).

Statistical analyses

Serial time-points were compared with repeated measures ANOVA using a general linear model with post-hoc analysis (Holm-Sidak method). Comparison of **AUC** (area under the curve determined by the trapezoidal method) results for the i.v. and oral tests was made with a paired t-test. Comparisons between HI and NI groups used an unpaired t-test. Correlation co-efficients were estimated using Pearson's test, and linearity was determined by simple linear regression. The fraction of the insulin cleared from the systemic circulation was estimated using the equation $\frac{[C-peptide]-[Insulin]}{[C-peptide]}$ and calculated as the percent difference. This estimate of clearance relies on the assumptions that C-peptide and insulin are co-secreted in equimolar amounts and that the clearance of C-peptide is negligible (Rubenstein et al., 1972). Data were analysed with SigmaPlot v. 12.5 (Systat software Inc, San Jose, CA) and are reported as mean ± SEM. Significance was set at $P < 0.05$.

RESULTS

C-peptide assay evaluation

Results of the ELISA kits that demonstrated sufficient cross-reactivity are reported in Table 2. Four of the 7 assays evaluated failed to demonstrate sufficient cross-reactivity with equine C-peptide (Alpha diagnostic #0040, Alpco 80-CPTRT-E01, Millipore EZHCP-20K, Mercodia 10-1136-01). Of the remaining 3 assays 1 returned unacceptable results for linearity, recovery and precision. The only "equine-specific" kit tested (immunogen of the detection

antibody is a synthetic peptide corresponding to equine C-peptide aa57-87 conjugated to bovine thyroglobulin) did show cross-reactivity, but also performed at a sub-optimal level with poor precision and variable recovery values. The remaining ELISA (Abcam, Cambridge, UK) demonstrated cross-reactivity and good linearity, but needed to be improved, particularly for the samples likely to contain low concentrations of C-peptide. This assay was selected for further optimization and was re-evaluated using pre-processed samples.

To remove large proteins and contaminants, serum samples (200 μL) were passed through a low-protein-binding, cellulose acetate filter (0.22 μm, Co-star Spin-X, Corning Inc, NY, USA) by centrifugation for 45 s (12,700 x g). This simple and rapid processing step improved the assay precision and accuracy for high and mid-range samples, but not for low-range pooled samples that fell below the lowest standard. The inter-assay CV was 10.1%. The linearity of diluted mid and high-range samples was excellent. However, the linearity of the low-range samples after processing could not be determined given that these samples were below the lowest standard. As such, we suggest that the safe limit of detection for C-peptide in horse serum when using this test is ~57 pM. Expected results (based on serum insulin concentration) were consistent with observed results for the 18 filtered OGT screening samples during validation of this assay (data not shown) and results were consistent with the anticipated behaviour of C-peptide in a biological system. Given that the kit standards were of human origin for C-peptide and porcine origin for insulin, it is important to stress that the reported concentrations of insulin and C-peptide actually represent human and porcine equivalents of the immunoreactive peptides.

Intravenous vs. oral glucose

C-peptide concentrations increased ($P < 0.01$) rapidly from basal, fasted levels to peak 2-3 h after oral and i.v. dextrose administration (Fig. 1). Overall, C-peptide concentrations during the 6 h sampling period were similar with both tests, with no difference in AUC (oral: 4745 ± 1250, i.v.: 5210 ± 1089 pM.h/L). C-peptide concentrations were not significantly different from baseline levels 6 h after either the i.v. (296 pM), or oral (652 pM; $P = 0.067$), test (Fig. 1). As expected, the pattern of insulin secretion in response to oral and i.v. dextrose mirrored that of C-peptide (Fig. 1), with C-peptide concentrations remaining higher than insulin throughout both tests (mean insulin-to-C-peptide ratio < 1 at all time-points).

The insulin AUC was lower ($P < 0.01$) for the oral test (1999 ± 449 pM.h/L), compared to i.v. (3072 ± 278 pM.h/L), which may suggest differences in insulin clearance during tests, given that $AUC_{C\text{-}peptide}$ did not differ between tests. The fractional difference in insulin and C-peptide concentrations indicated that the clearance of insulin was greater ($P < 0.05$) 90 min after dosing for the oral test, compared to the i.v. test (62% vs. 28%, respectively), which likely reflects the time taken for absorption (Fig. 1). Lastly, the $AUC_{C\text{-}peptide}$ showed a positive correlation with $AUC_{insulin}$, for both the oral and i.v. test (Fig. 2).

Differences due to insulin responsiveness

The secretion of C-peptide after oral dextrose was significantly lower in NI ponies, than HI ponies (Fig. 3A). However, the C-peptide response to i.v. dextrose administration was similar in both HI and NI ponies (Fig. 3B). In addition, the C-peptide concentration remained elevated ($P < 0.05$) in the HI group 6 h after oral dextrose consumption. The AUC was larger ($P < 0.05$) for C-peptide, insulin and glucose in HI ponies, compared with NI ponies, for the oral test, but not different between groups for the i.v test (Fig. 4). Within groups, the AUC for C-peptide,

183 insulin and glucose was lower ($P < 0.05$) after oral dextrose consumption than after i.v. dextrose
184 in NI ponies, while the opposite was observed ($P = 0.05$) for the HI group for $AUC_{C\text{-peptide}}$ (Fig.
185 4).

186 Using C-peptide to calculate the fractional clearance of insulin can be instructive in
187 determining the extent of insulin metabolism. However, statistical analyses of clearance data to
188 compare groups were not undertaken, due to the variability in these data (related to small sample
189 size). Visual inspection of these data may indicate that decreased clearance of insulin occurs in
190 HI ponies e.g. the fraction of insulin cleared 60 min after dextrose administration was ~60% in
191 NI ponies for both tests, and only 30% and 10% in HI ponies for the for the oral and i.v. tests,
192 respectively. The mean insulin-to-C-peptide ratio for basal, fasted samples was consistent across
193 tests and did not differ between HI and NI groups (HI; 0.72 ± 0.13, NI; 0.71 ± 0.16).

194 **DISCUSSION**

195 This study has demonstrated that C-peptide concentrations increase markedly after oral
196 carbohydrate ingestion in some ponies, consistent with the hypothesis that hyperinsulinemia
197 occurs in these ponies as a result of increased insulin secretion, and not solely a decrease in
198 insulin clearance. We have also shown that insulin secretory responses to oral carbohydrates can
199 differ in ponies that have similar responses to i.v. dextrose, consistent with the hypothesis that
200 peripheral IR is not required for hyperinsulinemia.

201 To our knowledge, this is the first report of the C-peptide response to oral dextrose in
202 horses or ponies. Our data clearly indicate that C-peptide secretion increases at a similar rate
203 after both i.v. and oral dextrose in ponies, and this is consistent with findings in other species
204 (Hampton et al., 1986; Montgomery et al., 1996; Frangioudakis et al., 2008). An increase in C-

peptide release after an i.v. glucose bolus (at a lower dose rate of 300 mg/kg) has also been reported previously in horses (Toth et al., 2010), with C-peptide concentrations remaining elevated for only 100 min post-bolus, compared to 180 min in the current study. Notably, the secretion of C-peptide was 7-fold higher in HI ponies after oral dextrose consumption, compared to NI ponies. This finding is consistent with the greater insulin release that has been reported after carbohydrate-rich meal consumption in horses previously classified as insulin dysregulated/resistant, compared to healthy animals (Bamford et al., 2014). Given that there was no apparent difference in the response to i.v. dextrose between groups in the current study, use of the terms insulin-resistant and insulin-sensitive would have been misleading in the present context.

Despite a similar C-peptide and insulin response to i.v. dextrose in both groups of ponies, the NI ponies responded to the slower absorption of oral dextrose as expected, with less marked insulin and C-peptide release. However, the HI ponies responded with greater magnitude to oral dextrose, than to i.v. delivery, suggesting that orally absorbed glucose may have initiated an additional pancreatic stimulus, such as the release of incretin hormones, in this group. Incretin hormones, such as glucagon-like peptide-1 and glucose-dependent insulinotropic peptide, are secreted in response to an oral carbohydrate load, and in addition to glucose, stimulate insulin secretion in several species (Dupre, 2005; Baggio and Drucker, 2007; Yoder et al., 2010). Evidence of a functional enteroinsular axis has been shown in horses/ponies previously (Dühlmeier et al., 2001; Bamford et al., 2015), and we have recently shown that post-prandial incretin release varies with metabolic function in ponies (de Laat et al., 2015). The marked insulin secretion by the pancreas after oral carbohydrate in HI ponies places them at significant

risk of laminitis, and studies to further elucidate the casual mechanism behind this disproportionate response are urgently needed.

The finding that C-peptide and insulin secretion was enhanced in HI ponies also highlights the important question of the mechanism of hyperinsulinemia in the horse. In theory, increases in circulating insulin could result from either increased production or reduced clearance. Although we cannot eliminate an alteration in clearance as a contributing factor, our results point to increased production as a major factor. This increased insulin production could result from either the actions of insulin secretagogues, as discussed above, or differences in glucose dynamics. These include less glucose uptake by peripheral tissues leading to a prolonged glucose stimulus, more glucose absorption from the gut, or less hepatic clearance of glucose in HI ponies. While further investigations are required to examine the relative contribution of independent pancreatic stimuli, such as glucose and/or incretin hormones, or feedback mechanisms on insulin secretion, we have recently demonstrated that ponies can be hyper-responsive to oral carbohydrate without changes in peripheral glucose uptake or clearance (de Laat et al., 2015). Nevertheless, our results are consistent with the theory that hyper-responsiveness to oral carbohydrates is a driver of hyperinsulinemia in the horse, which may or may not result in tissue IR (de Laat et al., 2015). Furthermore, our present results do not indicate any difference in peripheral insulin responsiveness between the small groups of HI and NI ponies examined here, despite marked differences in their oral response.

The finding that C-peptide concentrations were consistently higher than insulin after i.v. and oral dextrose was expected, and is most likely a reflection of the fact that C-Peptide undergoes minimal hepatic metabolism, which is not the case for insulin (Polonsky et al., 1986). First-pass hepatic metabolism and excretion rates for insulin have been reported as 50% in the

dog and up to 80% in humans (Meier et al., 2005). Data from the current study suggests that insulin clearance rates are around 30% in the basal, fasted state, increasing to a peak of ~60% after dextrose administration. However, these estimates are based on low sample numbers, as well as the assumption that the heterologous assay systems used accurately reflect the relative amounts of C-peptide and insulin in the circulation. Studies that sample directly from the portal vein and assays using equine standards are required to measure hepatic clearance accurately. Thus, the subject of hepatic insulin clearance and its contribution to equine hyperinsulinemia is less well defined at this point.

In summary, our results support the hypothesis that the hyperinsulinemia observed when certain ponies ingest large quantities of non-structural carbohydrates is the result of increased insulin secretion, with possible changes in insulin metabolism playing a secondary role. Further, this increase in secretion is not necessarily associated with peripheral tissue IR, but is likely the result of either incretin action and/or alterations in glucose dynamics. The implication of our findings is that future research and diagnostic methods should focus on hepatic and gastrointestinal function as primary factors in insulin dysregulation to unlock the cause of this condition and provide new therapeutic approaches to the prevention of laminitis.

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344

345 **Table 1:** Sample population characteristics

Group	n	Sex	Age (years)	BCS (/9)	CNS (/5)	FGIR	2 h Insulin (pM)
NI	4	1 F; 3 M	11.8 ± 2	6.5 ± 1	1.3 ± 0.8[a]	15 ± 2.1[b]	136 ± 16[c]
HI	5	3 F; 2 M	13 ± 3.3	7.4 ± 0.5	2.8 ± 0.4	2.6 ± 0.4	764 ± 111

346 [a]$P = 0.04$, [b]$P = 0.0002$, [c]$P = 0.01$

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Table 2: Evaluation of ELISAs assessed for measurement of equine C-peptide.
Repeat evaluation of the Abcam assay with filtered samples is indicated in brackets.

Assay	Capture Antibody	Standards	Intra-assay CV (%)	Recovery on addition (%)	Linearity (r^2)	LOD[1] (pM)
[2]**Abcam**	Human	Recombinant				3.31
Low	monoclonal	human	23.8 (-)	49.0 (79.0)	-	(57.0)
Mid			10.4 (10.5)	68.6 (98.9)	0.90 (0.98)	
High			10.6 (7.7)	91.0 (92.1)	0.99 (0.99)	
[3]**Biosource**	Mouse	Recombinant				33.1
Low	monoclonal	(unknown)	10.3	128		
Mid			20.1	82.3	0.86	
High			37.3	71.3	0.63	
[4]**USCN**	Human	Recombinant				4.3
Low	monoclonal	human	67.9	320		
Mid			55.3	295	-0.46	
High			33.1	284	0.91	

[1]Limit of detection for each kit as indicated by the manufacturer, not the actual limit of detection for equine C-peptide;
[2]Abcam, Cambridge, UK, ab178641; 3Biosource, San Diego, CA, MBS019653; [4]USCN, Houston, TX, CEA447Hu

FIGURES

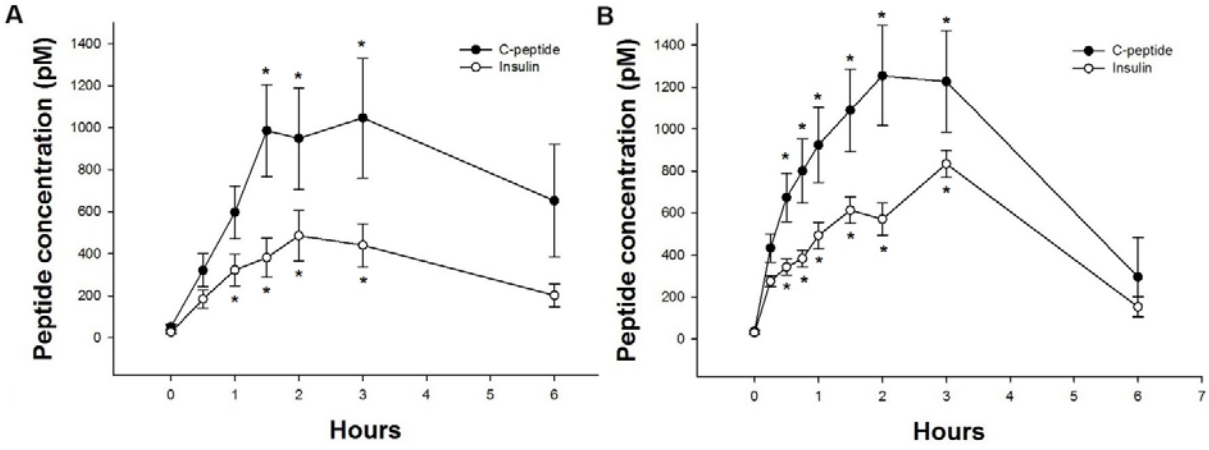

Fig. 1. Serum C-peptide concentrations (●) were similar in response to oral (**A**) and i.v. (**B**) dextrose administration (0.75 g/kg) to 9 ponies. Insulin (○) concentrations in response to both oral, and i.v., dextrose showed a similar pattern, but at reduced concentrations compared with C-peptide (●). *$P < 0.05$ compared with 0 h time-point.

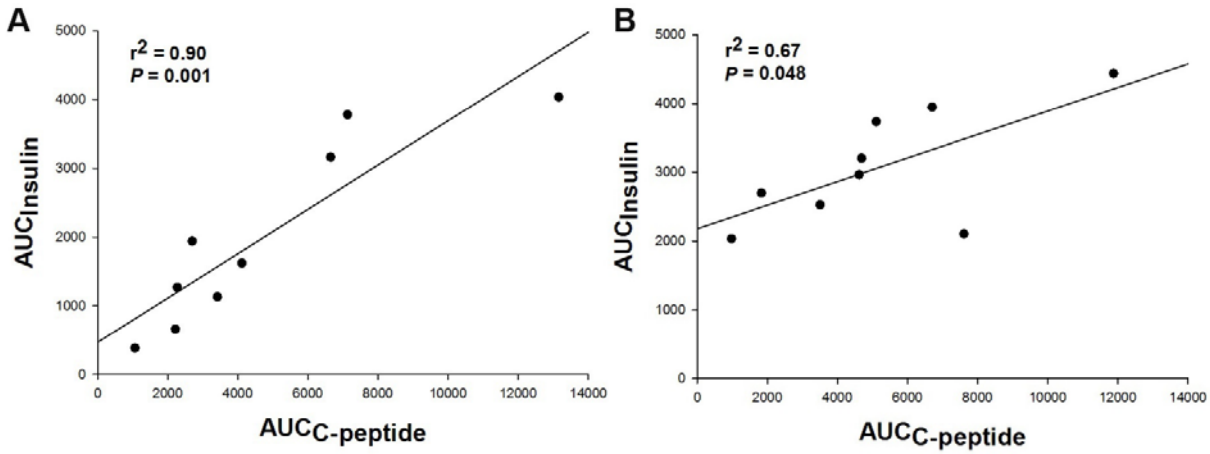

Fig. 2. C-peptide and insulin responses to oral and intravenous dextrose are positively correlated. **A:** The AUC for C-peptide over a 6 h sampling period after oral administration of dextrose (0.75 g/kg) in 9 ponies was strongly correlated with AUC for insulin (slope = 0.322, y intercept = 470). **B:** The AUC for C-Peptide over a 6 h sampling period after i.v. dextrose administration (0.75 g/kg) was also correlated with the AUC for insulin (slope = 0.171, y intercept = 2181).

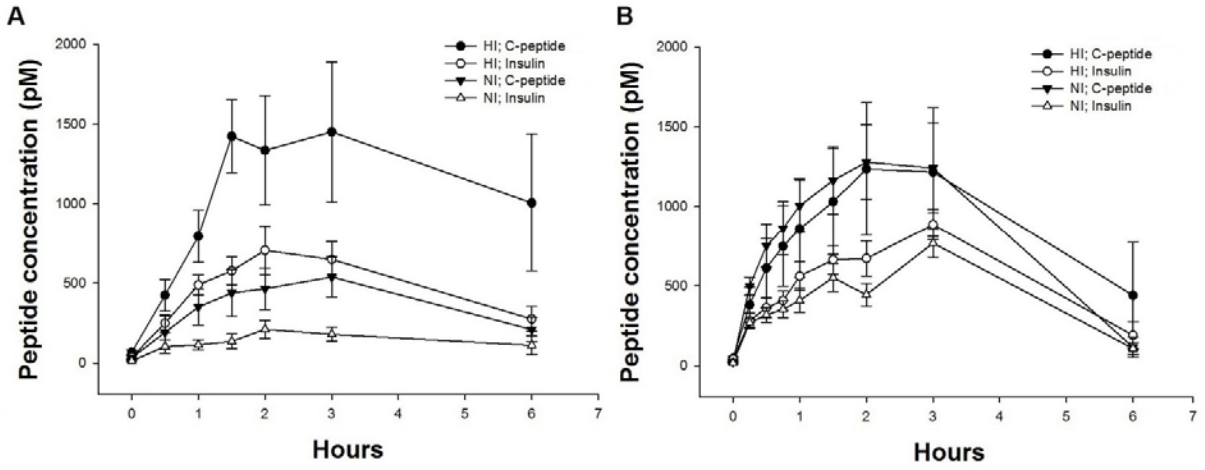

Fig. 3. A: Despite similar basal, fasted C-peptide concentrations, oral dextrose results in a larger C-peptide response in HI ponies (●) than NI ponies (▼), with C-peptide concentration remaining elevated at 6 h post-dosing. **B:** The secretion of C-peptide (closed symbols) and insulin (open symbols) after i.v. dextrose is similar between hyperinsulinemic (HI; circles) and normoinsulinemic (NI; triangles) ponies. A similar pattern, but of lower magnitude, is seen for serum insulin secretion in the two groups (open symbols).

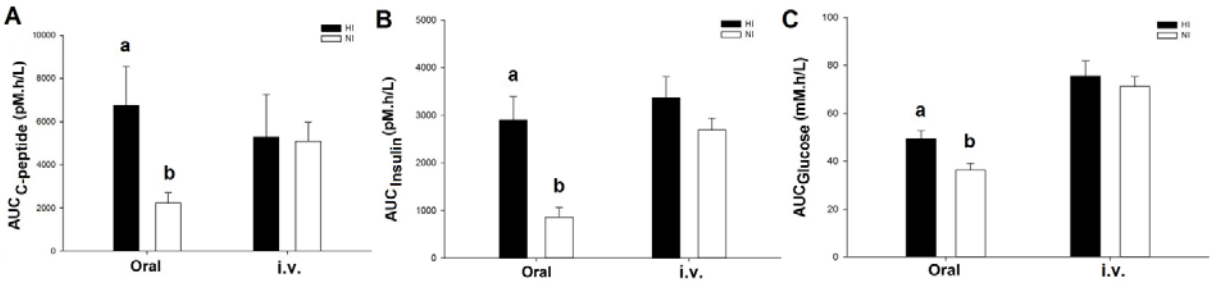

Fig. 4. The area under the concentration-time curve (AUC) is greater (a) after oral dextrose (0.75 g/kg BW) for C-peptide (**A**; $P = 0.01$), insulin (**B**; $P = 0.01$) and glucose (**C**; $P = 0.02$) in hyperinsulinemic (HI) ponies, compared to normoinsulinemic (NI) ponies. There was no difference between groups after i.v. dextrose administration for any metabolite. Within groups, NI ponies secrete less (b) C-peptide (**A**; $P = 0.03$), insulin (**B**; $P = 0.02$) and glucose (**C**; $P = 0.003$) after oral dextrose, compared with i.v. administration.