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Effect of phenylbutazone administration on the enteroinsular axis in horses with insulin dysregulation

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Abstract

Background: Phenylbutazone is prescribed for laminitis-associated pain and decreases glucose and insulin responses to an oral glucose test (OGT) in horses with insulin dysregulation (ID).

Hypothesis/Objectives: Investigate the effect of phenylbutazone administration on the enteroinsular axis in horses.

Animals: Sixteen horses, including 7 with ID.

Methods: Randomized cross-over study design, with horses assigned to treatment with phenylbutazone (4.4 mg/kg IV q24h) or placebo (5 mL 0.9% saline). On Day 9 of treatment, an OGT was conducted, followed by a 10-day washout period, administration of the alternative treatment, and repetition of the OGT. Glucose-dependent insulinotropic polypeptide (GIP), and active glucagon-like peptide 1 and 2 (aGLP-1 and GLP-2) concentrations were determined by ELISA. The effects of ID status and treatment on peptide concentrations were assessed using *t* tests and analyses of variance.

Results: Horses with ID had significantly higher maximum GIP concentrations (Cmax) than controls (median, 279.1; interquartile range [IQR], 117.5-319.4 pg/mL vs median, 90.12; IQR, 74.62-116.5 pg/mL; $P = .01$), but no significant effect of ID was detected on aGLP-1 and GLP-2 concentrations. In horses with ID, phenylbutazone treatment significantly decreased GIP Cmax compared with placebo (168.1 ± 59.26 pg/mL vs 242.8 ± 121.8 pg/mL; $P = .04$), but no significant effect of phenylbutazone was detected on aGLP-1 and GLP-2 concentrations.

Conclusion and Clinical Importance: Glucose-dependent insulinotropic polypeptide, aGLP-1 and GLP-2 do not mediate the decrease in glucose and insulin concentrations observed after phenylbutazone administration. Only GIP was repeatedly associated with ID status, calling into question the role of the enteroinsular axis in ID.

Abbreviations: aGLP-1, active glucagon-like peptide 1; ANOVA, analyses of variance; AUC, area under the curve; BCS, body condition score; Cmax, maximum concentration; CNS, cresty neck score; DDP-4, dipeptidyl peptidase IV; EDTA, ethylenediaminetetraacetic acid; EP3, guanine receptor E-class prostanoid 3; GIP, glucose-dependent insulinotropic polypeptide; GLP-2, glucagon-like peptide 2; HAL, hyperinsulinemia-associated laminitis; HPLC, high powered liquid chromatography; ID, insulin dysregulation; IR, insulin resistance; LC-MS, liquid chromatography mass spectrometry; LOQ, limit of quantification; MFA, meclofenamic acid; mFSIGTT, modified frequently sampled intravenous glucose tolerance test; NSAID, non-steroidal anti-inflammatory drug; OGT, oral glucose test; PGE_2, prostaglandin E_2.

KEYWORDS
endocrinology, equine metabolic syndrome, hyperinsulinemia, laminitis, non-steroidal anti-inflammatory drugs, obesity, oral glucose test

1 | INTRODUCTION

Hyperinsulinemia refers to an increased amount of circulating insulin occurring basally or postprandially.[1] An oral glucose test (OGT) assesses insulin secreted in response to gastrointestinal glucose and from stimulation of the enteroinsular axis.[2,3] The enteroinsular axis involves hormones, known as incretins, that are secreted from the gastrointestinal tract in response to carbohydrate, amino acid and fat intake, promoting insulin secretion.[2,4] Incretins glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) have been investigated in ponies and are reported to be responsible for 2% and 23%, respectively of insulin secretion, with blood glucose concentration being the main driver responsible for approximately 75%.[2,5] Another peptide hormone, not a part of the enteroinsular axis, is glucagon-like peptide (GLP-2). Unlike the incretins mentioned above, it is not directly insulinogenic, but intestinotrophic meaning that it promotes growth of the intestinal epithelium, which in turn increases the capacity for nutrient absorption.[6] Increased baseline GLP-2 concentration has been reported in hyperinsulinemic ponies, and it is thought that this increased concentration might contribute to the increased glucose absorption observed in ponies with hyperinsulinemia in response to an OGT.[6] Therefore, it was hypothesized that increases in both GIP and GLP-1, along with GLP-2 contributed to the development of hyperinsulinemia in ponies.[2,6]

Hyperinsulinemia has been demonstrated to cause laminitis in a number of induction studies.[7,8] This type of laminitis is known as hyperinsulinemia-associated laminitis (HAL) and has been reported to make up to 94% of all laminitis cases.[9] Because of the highly painful nature of laminitis, pain relief is required, which in the equine veterinary industry will most commonly be phenylbutazone.[10] Phenylbutazone is a non-steroidal anti-inflammatory drug (NSAID) that is a nonselective cyclooxygenase inhibitor, inhibiting the downstream production of thromboxane and prostaglandins.[11,12] Although these products are involved in the inflammatory processes, they also have important roles in homeostasis, with prostaglandin E_2 (PGE_2) having inhibitory effects on insulin secretion by the pancreas.[13] It has also been demonstrated that PGE_2 has inhibitory effects on GLP-1 signaling and GIP action on the promotion of insulin secretion in the pancreas.[13,14] These findings suggest that treatment with NSAIDs could minimize these inhibitory effects on the pancreas and lead to increased insulin secretion. However, we previously have described a decrease in insulin and glucose concentrations during the OGT in horses with ID receiving phenylbutazone.[15] It is therefore possible that GIP, GLP-1, and GLP-2 could play a role in this alteration, and that the effect of NSAIDs may be different among species with respect to the insulin pathway.

Although most studies pertaining to the enteroinsular axis have been performed in ponies, our objective was to investigate the effect of phenylbutazone administration on GIP, GLP-1, and GLP-2 secretion in horses with ID as a mechanism to explain the decreases in insulin and glucose concentrations previously reported. We hypothesized that if GIP, GLP-1, and GLP-2 contribute to insulin and glucose concentrations in horses, phenylbutazone administration will decrease these peptide concentrations in horses with ID.

2 | METHODS

2.1 | Study design

All procedures were approved by the Institutional Animal Ethics Committee. Samples for this investigation were collected as part of an associated study previously described.[15] Briefly, a randomized crossover study was conducted over 2 consecutive years in November and December (summer in the Southern Hemisphere) with half of the horses completing the trial 1 year and the other half the next year. Horses underwent a week of acclimatization, with body weight, body condition score (BCS)[16] and cresty neck score (CNS)[17] recorded. The initial randomly assigned treatment consisted of either 4.4 mg/kg phenylbutazone (phenylbutazone sodium 200 mg/mL and sodium salicylate 50 mg/mL) administered IV once daily or a placebo treatment of 5 mL of 0.9% saline IV once daily. On the 9th day of treatment, horses underwent an OGT as previously reported.[15] Briefly, horses received 0.75 g/kg of dextrose dissolved in 2 L of warm water via a nasogastric tube. Blood samples were collected at 0, 15, 30, 60, 90, and 120 minutes, for the measurement of GIP, GLP-1, and GLP-2 concentrations. After a 10-day washout period, horses received the alternative treatment and then underwent another OGT and sampling protocol. Horses were kept in individual dirt yards, with unrestricted access to lucerne hay (analysis provided previously[15]) and water, and were exercised on an automated horse walker for 30 minutes, 3 times a week, at a walk.

2.2 | Animals

Horses were identified as having ID or as controls based on their insulin concentration during the OGT and insulin sensitivity index from a modified frequently sampled IV glucose tolerance test (mFSIGTT),[15,18] when receiving the placebo treatment. Horses were classified as having ID if they had an insulin concentration >80 μIU/mL at 120 minutes during the OGT and an insulin sensitivity index $<1.0 \times 10^{-4}$ L/μIU/min based on a mFSIGTTT (carried out as part of a concurrent study), with control horses conversely having an insulin concentration <80 μIU/mL and a sensitivity index $>1.0 \times 10^{-4}$ L/μIU/min.[3,19,20] Sixteen horses were recruited from the previous study; 7 horses were identified as having ID and 9 were identified as controls (Table 1).[15]

TABLE 1 Details of control horses and horses with ID.

	Control horses (n = 9)	Horses with ID (n = 7)	*P* value
Age (year)	13 [10.5-15.0]	15 [11.0-19.0]	.28
Weight (kg)	538.8 [497.0-564.6]	596.4 [524.8-676.8]	.17
BCS	5 [5-5]	8 [7-8]	<.0001
CNS	2 [2-2]	3 [3-4]	<.0001
OGT 120-min Insulin (μIU/mL)	16.40 [7.58-22.35]	291.0 [216.0-369.0]	.0002
Insulin sensitivity index ($\times 10^{-4}$ L/mIU/min)	5.04 [2.63-6.67]	0.39 [0.14-0.74]	.0002
Phenylbutazone concentration (μg/mL)	0.99 ± 0.51	0.71 ± 0.19	.19

Note: Data presented as median and interquartile range. Presented as median [interquartile range] or mean ± SD; Mann-Whitney test or *t* test carried out between groups, $P < .05$ considered significant.
Abbreviations: AUC, area under the curve; BCS, body condition score; CNS, cresty neck score; ID, insulin dysregulation; OGT, oral glucose test.

2.3 | Assays

Blood samples were collected into pre-chilled EDTA tubes (BD Vacutainer), then placed into ice, centrifuged at 1370*g* for 10 minutes and plasma was collected into microtubes and stored at −80°C until analysis. The ELISAs previously validated for horses were used to measure aGLP-1 and GIP concentrations following the manufacturer's instructions (EZGLPHS-35K and EZHGIP-54K, Millipore Corporation).[2,21] The range of quantification for aGLP-1 was 0.14-100 pm with an interassay coefficient of variation of 9.7%. The range of quantification for GIP was 8.2-2000 pg/mL with an interassay coefficient of variation of 9.1%. Glucagon-like peptide 2 was measured using an ELISA previously validated for use in horses (EZGLP2-37K, Millipore Corporation), but the wash step after sample incubation was removed from the manufacturer's instructions because of expected low concentrations.[6] For this assay, the range of quantification was 1-64 ng/mL with an interassay coefficient of variation of 8.9%.

Phenylbutazone concentration was measured on Day 7 of treatment (24 hours after the last dose on Day 6) using liquid chromatography mass spectrometry (LC-MS) at Racing Analytical Services Limited Laboratory, as previously reported.[15]

2.4 | Data analysis

Data analysis was performed using GraphPad Prism (Version 9.5; GraphPad Software, LLC). A Shapiro-Wilk test was used to check data for normality of continuous variables. Normally distributed data are presented as mean ± SD and non-normally distributed data presented as median (interquartile range [IQR]). Significance was set at $P < .05$. Results below the limit of quantification (LOQ) for the aGLP-1 and GLP-2 assay was given the value of LOQ/$\sqrt{2}$.[22,23]

To determine the effect of dextrose administration (time) and ID status on the concentrations of GIP, aGLP-1, and GLP-2 during the OGT, 2-way repeated measures analyses of variance (ANOVA) with Šídák's multiple comparisons post hoc test were carried out on the horses with ID and control horses when receiving the placebo treatment. Areas under the curve (AUC) were calculated using the trapezoidal method. Unpaired *t* tests or Mann-Whitney *U* test were used to compare AUC, maximum concentrations (Cmax) and baseline concentrations between horses with ID and control horses, depending on the distribution.

Two-way repeated measures ANOVAs, with Tukey's Honest Significant Difference post hoc test, also were used to investigate the effect of dextrose administration (time) and phenylbutazone treatment within each group (horses with ID or control horses). Paired *t* tests or Wilcoxon signed rank tests were used to make comparisons between treatments for AUC, Cmax and baseline concentrations. One-tailed tests were used to investigate the hypothesis of decreased incretin concentrations in horses with ID receiving phenylbutazone.

3 | RESULTS

3.1 | GIP

A significant effect of ID status and dextrose administration on GIP concentrations was observed during the OGT ($P = .02$ and $P < .0001$, respectively; Figure 1A), but no timepoint reached significance with post hoc tests. Horses with ID had a larger GIP AUC compared with the control horses (horses with ID, median, 30 095; IQR, 10 588-30 991 pg/mL × minute vs control horses, median, 8929; IQR, 7112-12 291 pg/mL × minute; $P = .02$; Figure 1B). No significant effect of ID status was found on GIP baseline concentrations (horses with ID, 117.6 ± 78.09 pg/mL vs control horses, 55.71 ± 41.94 pg/mL, $P = .06$). Horses with ID had a higher GIP Cmax compared with control horses (horses with ID, median, 279.1; IQR, 117.5-319.4 pg/mL vs control horses, median, 90.12; IQR, 74.62-116.5 pg/mL; $P = .01$; Figure 1C).

In horses with ID, dextrose administration resulted in a significant increase in GIP concentration ($P = .003$; Figure 1A), but no significant effect of phenylbutazone administration was detected ($P = .14$) and no timepoint reached significance with post hoc tests. Phenylbutazone administration did not significantly decrease GIP AUC (phenylbutazone: 16 045 ± 4759 pg/mL × minute vs placebo: 23 155 ± 12 075 pg/mL × minute; $P = .07$; Figure 1B). No significant effect of phenylbutazone treatment on baseline GIP concentrations was identified in horses with

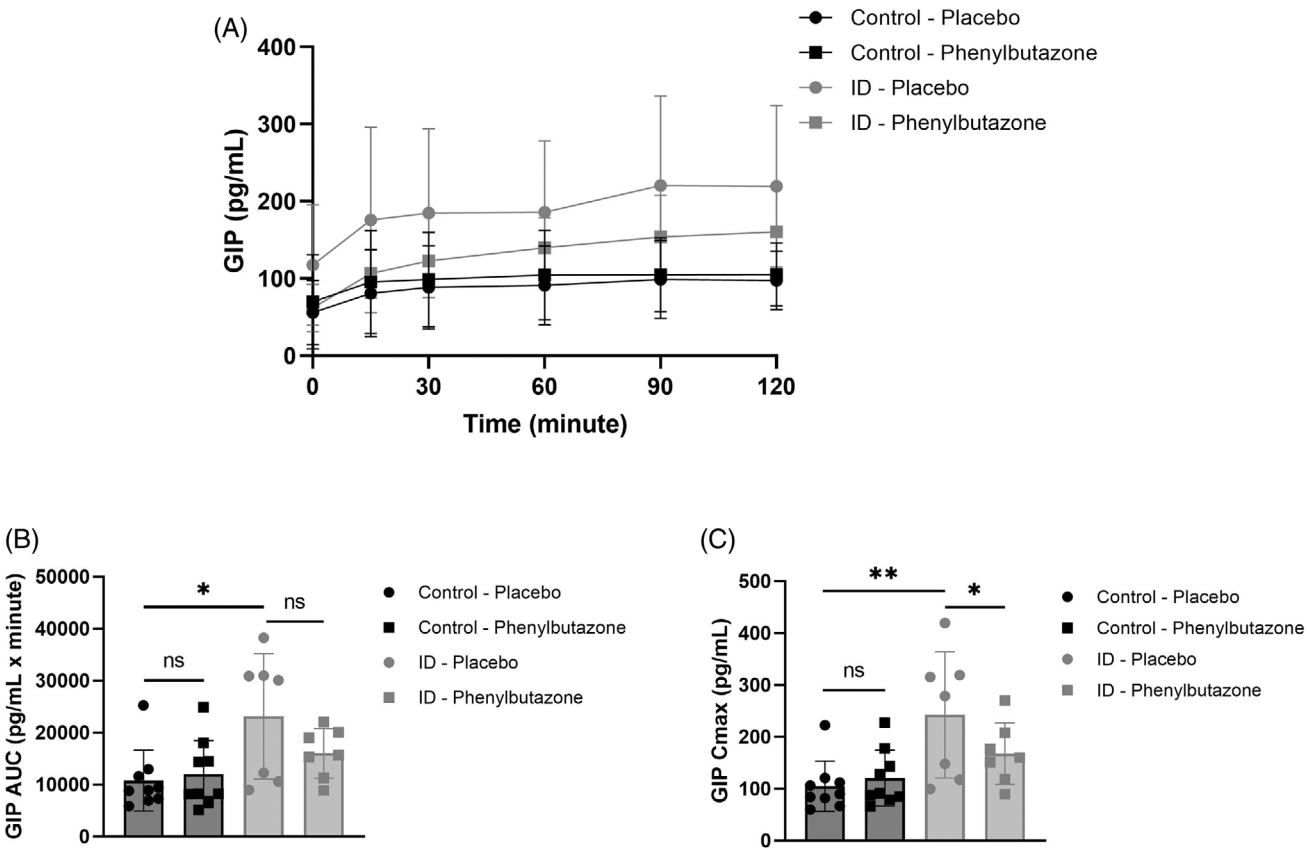

FIGURE 1 Plasma GIP concentrations during the oral glucose test (OGT; panel A), area under the curve (AUC; panel B) and maximum concentration (Cmax; panel C) for horses with insulin dysregulation (ID; n = 7; gray circle for placebo and gray square for phenylbutazone) and control horses (n = 9; black circle for placebo and black square for phenylbutazone). *P < .05; **P < .01; ns, not significant.

ID (phenylbutazone: 61.98 ± 30.83 pg/mL vs placebo: 117.6 ± 78.09 pg/mL; P = .08). However, a significant decrease in GIP Cmax was observed when the horses with ID received phenylbutazone (phenylbutazone: 168.1 ± 59.26 pg/mL vs placebo: 242.8 ± 121.8 pg/mL; P = .04; Figure 1C).

In control horses, dextrose administration during the OGT resulted in a significant increase in GIP concentration (P = .003; Figure 1A), but no significant effect of phenylbutazone administration was detected (P = .14) and no timepoint reached significance with post hoc tests. No significant effect of phenylbutazone treatment on GIP AUC was identified (phenylbutazone, median, 8230; IQR, 7470-16 257 pg/mL × minute vs placebo, median, 8929; IQR, 7112-12 291 pg/mL × minute; P = .37; Figure 1B), baseline GIP concentrations (phenylbutazone, median, 26.45; IQR, 18.06-135.7 pg/mL vs placebo, median, 39.38; IQR, 19.86-91.69 pg/mL; P = .28) or GIP Cmax (phenylbutazone, median, 91.65; IQR, 81.98-160.7 pg/mL vs placebo, median, 90.12; IQR, 74.62-116.5 pg/mL; P = .21; Figure 1C) in control horses.

3.2 | aGLP-1

A significant effect of dextrose administration (P < .0001) on aGLP-1 concentrations was noted, but no significant effect of ID status was detected (P = .05; Figure 2A) and no timepoint reached significance with post hoc tests. The aGLP-1 AUC was significantly higher in horses with ID compared with the control horses (horses with ID, median, 1661; IQR, 1573-1812 pM × minute vs control horses, median, 1279; IQR, 971.3-1399 pM × minute; P = .04; Figure 2B). Baseline aGLP-1 concentrations were significantly higher in horses with ID compared with the controls (horses with ID: 2.75 ± 1.48 pM vs control horses: 1.31 ± 0.96 pM; P = .03). No significant effect of ID status was found on aGLP-1 Cmax (horses with ID: 18.10 ± 4.73 pM vs control horses: 14.19 ± 3.40 pM; P = .07; Figure 2C).

In horses with ID, a significant effect of dextrose administration on aGLP-1 concentrations was observed (P < .0001), but no significant effect of phenylbutazone treatment was detected (P = .55; Figure 2A) and no timepoint reached significance with post hoc tests. Phenylbutazone administration in horses with ID did not significantly change the aGLP-1 AUC (phenylbutazone, median, 1141; IQR, 946.2-2007 pM × minute vs placebo, median, 1661; IQR, 1573-1812 pM × minute; P = .34; Figure 2B), baseline aGLP-1 concentrations (phenylbutazone: 1.64 ± 1.41 pM vs placebo: 2.75 ± 1.48 pM; P = .11) or Cmax compared to the placebo (phenylbutazone: 16.81 ± 5.13 pM, vs placebo: 18.10 ± 4.73 pM; P = .22; Figure 2C).

In control horses, a significant effect of dextrose administration on aGLP-1 concentrations was observed (P < .0001), but no

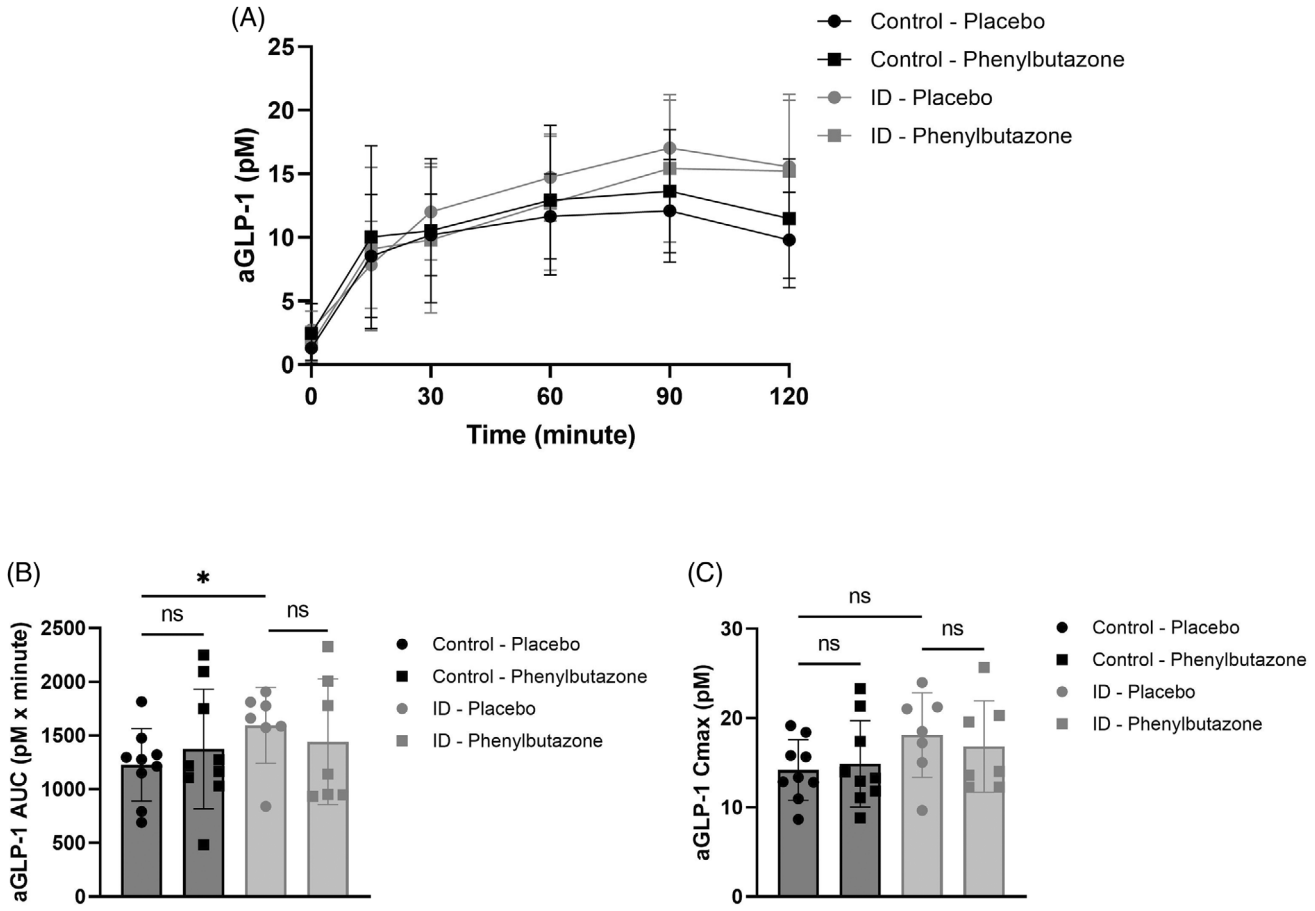

FIGURE 2 Plasma aGLP-1 concentrations during the oral glucose test (OGT; panel A), area under the curve (AUC; panel B) and maximum concentration (Cmax; panel C) for horses with insulin dysregulation (ID, n = 7; gray circle for placebo and gray square for phenylbutazone) and control horses (n = 9; black circle for placebo and black square for phenylbutazone). *$P < .05$; ns, not significant.

significant effect of phenylbutazone treatment was detected ($P = .39$; Figure 2A) and no timepoint reached significance with post hoc tests. Phenylbutazone administration did not significantly change aGLP-1 AUC (phenylbutazone: 1375 ± 558.0 pM × minute vs placebo: 1226 ± 336.7 pM × minute; $P = .22$; Figure 2B), baseline aGLP-1 (phenylbutazone: 2.46 ± 2.36 pM vs placebo: 1.31 ± 0.96 pM; $P = .09$) or Cmax in control horses (phenylbutazone: 14.88 ± 4.84 pM vs placebo: 14.19 ± 3.40 pM; $P = .35$; Figure 2C).

3.3 | GLP-2

During the OGT, a significant effect of dextrose administration on GLP-2 concentrations was identified ($P = .002$) but no effect of ID status was found ($P = .76$; Figure 3A) and no time point reached significance with post hoc tests. No significant effect of ID status on GLP-2 AUC was observed (horses with ID, median, 74.70; IQR, 46.8-206.1 ng/mL × minute vs control horses, median, 46.80; IQR, 46.80-239.8 ng/mL × minute; $P = .83$; Figure 3B), baseline GLP-2 concentrations (horses with ID, median, 1.23; IQR, 0.39-2.63 ng/mL vs control horses, median, 0.39; IQR, 0.39-2.95 ng/mL; $P > .99$) and Cmax (horses with ID, median, 1.23; IQR, 0.39-2.63 ng/mL vs control horses, median, 0.39; IQR, 0.39-3.05 ng/mL; $P = .83$; Figure 3C).

In horses with ID, a significant effect of dextrose administration × phenylbutazone treatment was detected ($P = .01$) but no significant effect of dextrose administration or phenylbutazone treatment was identified individually ($P = .06$ and $P = .14$, respectively; Figure 3A) and no timepoint reached significance with post hoc tests. No significant effect of phenylbutazone treatment was identified on GLP-2 AUC (phenylbutazone, median, 46.8; 46.8-85.11 ng/mL × minute vs placebo, median, 74.70; IQR, 46.8-206.1 ng/mL × minute; $P = .11$; Figure 3B) and phenylbutazone treatment did not significantly alter GLP-2 baseline concentrations (phenylbutazone, median, 0.39; IQR, 0.39-0.43 ng/mL vs placebo, median, 1.23; IQR, 0.39-2.63 ng/mL; $P = .06$) or Cmax in horses with ID (phenylbutazone, median, 0.39; IQR, 0.39-1.26 ng/mL vs placebo, median, 1.23; IQR, 0.39-2.63 ng/mL; $P = .11$; Figure 3C).

In control horses during the OGT, a significant effect of dextrose administration was noted ($P = .03$) but no effect of phenylbutazone treatment ($P = .68$) or dextrose administration × phenylbutazone treatment ($P = .80$; Figure 3A) was detected and no timepoint reached significance with post hoc tests. No significant effect of phenylbutazone treatment on

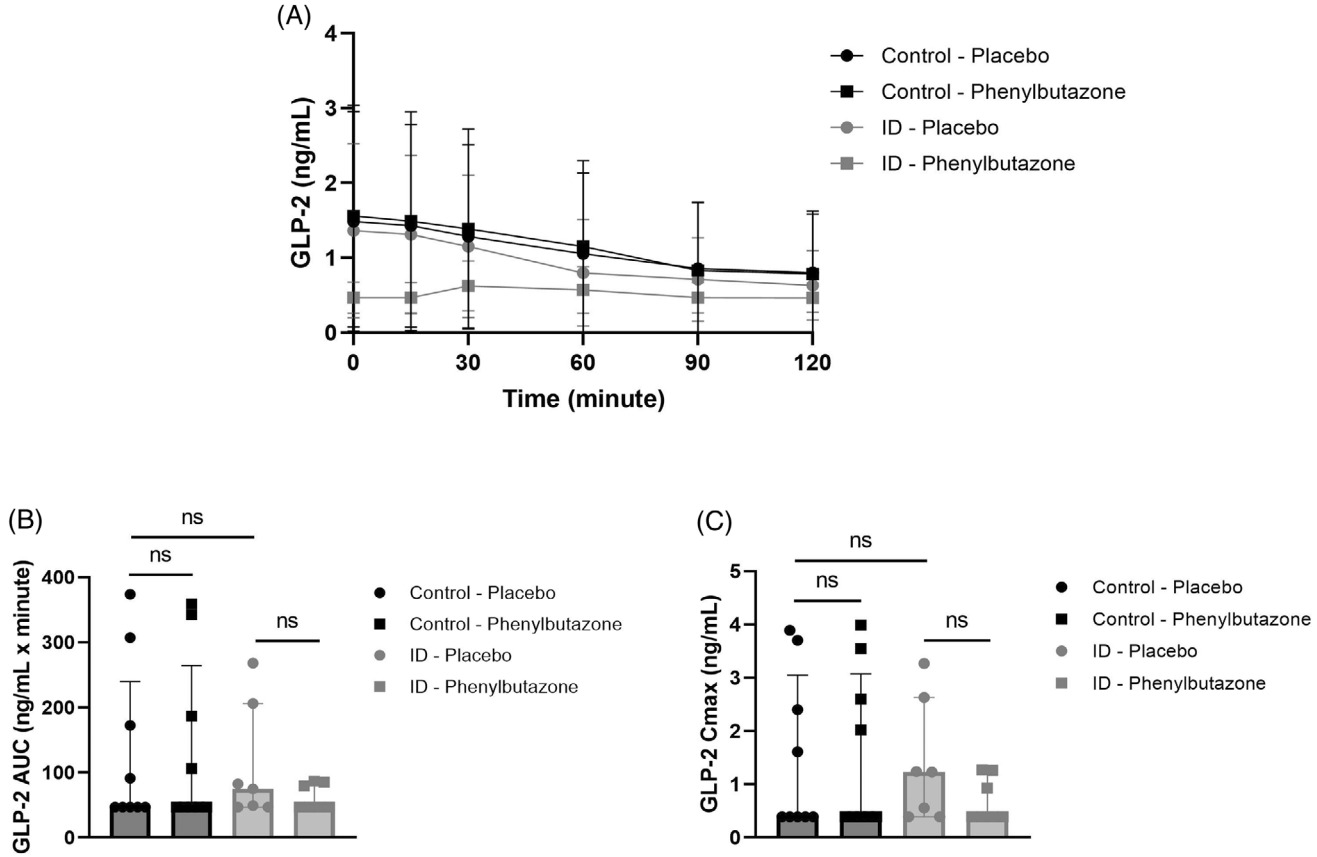

FIGURE 3 Plasma GLP-2 concentrations during the oral glucose test (OGT; panel A), area under the curve (AUC; panel B), and maximum concentration (Cmax; panel C) for horses with insulin dysregulation (ID, n = 7; gray circle for placebo and gray square for phenylbutazone) and control horses (n = 9; black circle for placebo and black square for phenylbutazone). ns, not significant.

GLP-2 AUC was observed in control horses (phenylbutazone, median, 46.8; IQR, 46.8-264.7 ng/mL × minute vs placebo, median, 46.8; IQR, 46.8-239.8 ng/mL × minute; $P = .41$; Figure 3B), baseline GLP-2 concentration (phenylbutazone, median, 0.39; IQR, 0.39-3.04 ng/mL vs placebo, median, 0.39; IQR, 0.39-2.95 ng/mL; $P = .50$) or Cmax (phenylbutazone, median, 0.39; IQR, 0.39-3.07 ng/mL vs placebo, median, 0.39; IQR, 0.39-3.05 ng/mL; $P = .50$; Figure 3C).

4 | DISCUSSION

We investigated the role of GIP, GLP-1, and GLP-2 as a potential pathways for the decreased insulin and glucose concentrations observed after phenylbutazone administration in horses with ID.[15] The results show that (a) ID status had a significant effect on GIP and on aGLP-1, but not on GLP-2 and (b) phenylbutazone administration to horses with ID only led to a significant decrease in GIP Cmax with no significant effect detected on aGLP-1 or GLP-2.

Previous studies have documented a significant difference in aGLP-1 and GIP concentrations between ponies with ID and their control counterparts, hypothesizing that, in ponies, aGLP-1 and GIP might contribute to the development of post-prandial hyperinsulinemia in an additive manner.[2] Our results also demonstrated significantly higher concentrations of GIP in horses with ID corroborating a potential role of GIP in the development of ID in horses. It is well established that GIP stimulates insulin secretion by binding to its receptor in the beta cells of the pancreas, and it also promotes proliferation and survival of beta cells and stabilizes blood glucose concentrations.[24] Nonetheless, although statistically significant, the overall contribution of GIP might be clinically limited, because previous research has indicated that GIP accounts for only 2% of total insulin secretion in ponies.[2] On the other hand, aGLP-1 has been reported to contribute 23% of insulin secretion in ponies suggesting that it could have a larger role in the development of ID in horses.[2] By binding to its receptor in the pancreatic islets, aGLP-1 initiates insulin secretion along with promoting beta cell proliferation and survival.[25] We identified a significant increase in aGLP-1 AUC in horses with ID, but no other test reached significance, suggesting potentially limited effects of this incretin in the development of ID. Several studies have failed to describe a significant difference in aGLP-1 between controls and horses with ID.[5,21,24,26,27] Possible explanations for these discrepancies include the inclusion of horses in our study rather than ponies. The initial study describing the role of GIP and aGLP-1 only included ponies, and it is becoming clear that some key metabolic differences exist between horses and ponies, and therefore results obtained in ponies might not be valid in horses.[26,28] Taken together, our data

suggest that, although there is a statistically significant effect of PO dextrose administration on both GIP and aGLP-1, their clinical relevance and actual impact on the development of hyperinsulinemia in horses might be more limited than what has been described in ponies and requires further investigation.

As an intestinotrophic incretin, GLP-2 has been considered as a possible contributor to increase glucose absorption in ponies with ID,[6] which in turn might contribute to hyperinsulinemia. Baseline GLP-2 previously has been reported to be increased in ponies with ID,[6] but a significant difference was not observed in our study for baseline GLP-2 concentrations or at any other time points during the OGT between horses with ID and controls. In addition, we observed a significant decrease in GLP-2 concentrations in response to PO dextrose, whereas previous reports in ponies described an increase in GLP-2 concentrations.[6] Although only a few studies have evaluated the role of GLP-2 in equids, the discrepancies between previous studies and ours again could be explained by the use of horses compared to ponies that might have metabolic differences.[26,28] Also, limited studies have been completed by laboratories other than the laboratory that validated the assay. Therefore, differences in repeatability might occur under different laboratory conditions. Additionally, previous reports have assigned the value of 0.275 ng/mL to values below the limit of detection previously reported as 0.55 ng/mL for equine samples,[6,29] which might influence results. Finally, studies have demonstrated that glucose data in the investigation of ID had limited repeatability and limited diagnostic value.[30] This observation suggests that mechanisms facilitating glucose absorption might not play a crucial role in the development of ID in horses and indicates that the role of GLP-2 requires further investigation.

Although phenylbutazone administration is associated with a significant decrease in glucose and insulin concentrations, a significant effect only was observed on GIP Cmax in horses with ID, and no other significant differences were detected. This lack of effect could indicate the limited role played by incretins in the secretion of insulin in horses with ID and suggest that another mechanism may play a more important role in insulin and glucose dynamics. Among those possible mechanisms, a change in peripheral tissue insulin sensitivity could be investigated. Because such an increase in insulin sensitivity would decrease blood glucose concentration (which accounts for 75% of insulin secretion) and therefore result in lower pancreatic stimulation as previously reported where dietary restriction and exercise improved insulin sensitivity and decreased insulin concentrations.[31] Another explanation could be the sensitivity of the assays. The ELISA used, although previously validated for use in equine samples, is manufactured for use in humans and rodents, and this factor potentially could decrease the specificity of the equine incretins because of differences in their sequence.[32] Also, ELISAs have decreased sensitivity compared with other methods of detection such as high performance liquid chromatography (HPLC), especially for low concentrations.[33] Most of the samples analyzed, in our study and in others, are close to the lower limit of detection of the assays where assay accuracy decreases, suggesting a possible systematic bias. Similarly, many of the previous studies have reported wide variations in incretin concentrations,[21,24,27] which also was observed in our study and might have contributed to the decreased ability to identify statistically significant or clinically relevant differences. Given our current results, from power calculations with a desired power of 0.8, at least 117 horses for aGLP-1 and 17 horses for GLP-2 would have been required to detect statistically significant differences between phenylbutazone and placebo suggesting that if an effect were present, it would likely be minimal. Another factor that might have prevented our ability to detect a significant effect is the absence of use of dipeptidyl peptidase IV (DPP-4) inhibitor. Previously, the difference between GIP and aGLP-1 concentrations collected in tubes with DPP-4 inhibitor or EDTA was investigated.[2] A small but not significant difference between the tubes was reported and results from EDTA tubes were considered valid,[2] and therefore they were chosen for use in our study. Another limitation is that our study was carried out over 2 years, introducing possible year-to-year differences. However, both trials were carried out during the same time period each year (November and December, summer in the southern hemisphere) and no horse was sampled over 2 years. Horses underwent a week of acclimatization and were kept in dirt yards with a controlled diet of lucerne hay to minimize potential effects of diet, including pasture composition. The order of phenylbutazone or placebo administration also was randomized with the horses acting as their own controls to account for the variability of OGT.[15] The dose of phenylbutazone was selected because it is the labeled dose for the management of laminitis, but other doses and other schedules are commonly used in equine practice, and it is possible that more frequent administration of a lower dose (2.2 mg/kg q12h) could have altered the enteroinsular axis in a different way. Finally, our study also was conducted in non-laminitic horses, and it is likely that because of the presence of inflammation, phenylbutazone could have altered the enteroinsular axis differently in horses with acute HAL. Inclusion of actively laminitic horses and administration of saline to those horses was not judged ethical.

Our aim was to investigate the roles of GIP, GLP-1, and GLP-2 as a potential mechanism to explain decreased glucose and insulin concentrations in horses with ID receiving phenylbutazone. No clinically relevant changes induced by phenylbutazone were detected in GIP, aGLP-1 and GLP-2 concentrations suggesting that the changes observed are likely mediated by another mechanism. In addition, only concentrations of GIP, accounting for 2% of insulin secretion in horses, were repeatedly significantly different between horses with ID and control horses, suggesting that incretins might have a lesser role in the pathogenesis of ID than anticipated.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the IACUC (SVS/153/19) of The University of Queensland.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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