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Palatability, glycemic, and insulinemic responses to various carbohydrate formulations: Alternatives for the diagnosis of insulin dysregulation in horses?

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Abstract

Background: Oral glycemic challenge (GC) tests are recommended for diagnosis of insulin dysregulation (ID). Various protocols are used, but all have limitations in terms of palatability, ease of use, variable composition, geographic availability, or some combination of these.

Hypothesis/Objective: To evaluate newly developed formulations with defined carbohydrate composition for use as oral GCs.

Animals: Thirty-four horses and ponies in various metabolic states.

Methods: Our objectives were carried out in 2 separate cross-over experiments. First, the palatability and acceptance of various GCs (2 syrups, 1 granulate) offered for free intake were compared to glucose mixed in a chaff-based diet. Subsequently, syrups were administered by syringe and compared to an oral glucose test using naso-gastric tubing (tube OGT) to investigate the glycemic and insulinemic responses. Second, these variables were compared in the best performing GC-formulations (granulate further optimized to pelleted formulation and 1 syrup) and a tube OGT. All GCs were administered with equivalent amounts of 0.5 g glycemic carbohydrates per kg body weight.

Results: Only the GC pellets were consumed completely by all horses (consumption time 5 ± 2 min). When administered by syringe, the GC syrup also was well accepted. The insulin concentrations at 120 min correlated significantly between tube OGT and GC pellets ($r = .717$; $P < .001$) or GC syrup ($r = .913$; $P < .001$). The new GC syrup and GC pellets discriminate between healthy and ID horses.

Conclusions and Clinical Significance: The GC pellets (DysChEq)™ and GC syrup can be used as palatable and well-accepted oral GC tests for assessment of ID in horses.

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; BCS, body condition score; BW, body weight; CNS, cresty neck score; EMS, equine metabolic syndrome; GC, glycemic challenge; ID, insulin dysregulation; NSC, non-structural carbohydrate; OGT, oral glucose test; OST, oral sugar test; PPID, pituitary pars intermedia dysfunction.

KEYWORDS
DysChEq, EMS, equine metabolic syndrome, glycemic carbohydrates, horse, laminitis, oral glucose challenge, oral glucose test, pituitary pars intermedia dysfunction, pony, PPID

1 | INTRODUCTION

Insulin dysregulation (ID) is recognized as the key factor involved in the development of endocrinopathic or hyperinsulinemia-associated laminitis in equids suffering from equine metabolic syndrome (EMS) and pituitary pars intermedia dysfunction.[1-5] Oral glycemic challenges (GCs) currently are recommended to assess ID in equids[6-8] and are considered superior to basal measures of insulin or glucose or both for reliable identification of ID.[6,9] However, all established test protocols based on oral application or ingestion of glycemic stimuli have limitations in terms of palatability, ease of use in the field, disclosure of the glycemic substance content or geographic availability.

The oral sugar test (OST)[10] represents a simple and straightforward testing procedure based on stimulation using commercial corn syrup (Karo-light, ACH Food Companies, Memphis, Tennessee, USA). Dosages of 0.15 mL to 0.45 mL/kg body weight (BW) syrup have been described.[11-13] The observations that higher dosages improve diagnostic accuracy so far are controversial.[13,14] A disadvantage of the higher dosage is that it might require impractically high volumes in typical horse populations, complicating the otherwise simple test procedure.

Karo light corn syrup is not readily available all around the world. Thus, modified OSTs based on Dan Sukker Glykossirap (Nordic Sugar A/S, Copenhagen, Denmark) available in Sweden or Crown Lily White corn syrup (ACH Food Companies, Ontario, Canada) available in Canada have been established to overcome this limitation.[15,16] The main drawback of OSTs based on corn syrup (or likely other sugar syrups available for human food consumption) is that the exact sugar composition is not disclosed by the manufacturer and may vary with different batches.[13]

Also frequently published is the assessment of ID with "in feed glucose challenge," based on feeding a chaff-based diet artificially enriched with variable amounts of glucose.[1,7,17,18] Seemingly, being a quite simple and time-saving procedure, this test requires the voluntary intake of the full ration within a limited period of time, which often is not achieved because of the low palatability of high amounts of glucose.[17] Training the horse to eat the feed is routinely done in research settings and may result in better acceptance but is not an option for a single test under field conditions. Thus, glucose administration via a nasogastric tube[19,20] remains the current last possible oral test option in some cases. It offers the advantage of administering an exact amount of glucose at a specific time point under standardized conditions and often is used in research settings. However, passing a nasogastric tube for a diagnostic purpose is relatively invasive and often evokes debates with owners and care takers because of the use of nose twitches and risk of epistaxis.

We evaluated liquid and solid GCs with defined composition of carbohydrates (i.e., glucose, maltose and oligosaccharides for assessment of ID in horses). Our objective was to investigate palatability as compared to an in-feed glucose test. In addition, glucose and insulin responses of the most palatable formulations in comparison to a standard oral glucose test via nasogastric tubing (tube OGT) were evaluated.

2 | MATERIALS AND METHODS

2.1 | Composition of GCs

Liquid and solid GC formulations with a defined composition of glucose, maltose and oligosaccharides (composed of glucose units) were manufactured (Table 1).

Two different liquid GC (Boehringer Ingelheim Danmark A/S, Kalundborg, Denmark) were used: Syrup A (salt flavored)—30% glucose and maltose, 37% oligosaccharides (composed of glucose units, with a degree of polymerization between 3 and 10), 2% salt and water and Syrup B (apple flavored)—28% glucose and maltose, 37% oligosaccharides (composed of glucose units, with a degree of polymerization between 3 and 10), 15% apple juice, 5% apple sugar extract, 0.05% apple aroma and water.

Solid GC (Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany): First a granulate GC based on a carrier of flour of fermented cornflakes with addition of glucose, maltose, and oligosaccharides based on glucose units was produced and employed in Study 1. A routine feedstuff analysis (AGROLAB LUFA GmbH, Kiel, Germany) resulted in 7% moisture, 2% crude protein, 1% crude fat, 0.5% ash, 0.5% crude fiber and 89% non-structured carbohydrates (NSC). Subsequently and based on results from Study 1, a pelleted GC formulation (DysChEq™, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany) was produced using an augmented carrier component based on flour of linseed and flour of maize germ and highly refined plant oils and extracts and employed in Study 2. A routine feed stuff analysis was carried out in 2 different laboratories (Eurofins Steins Laboratorium A/S, Vejen, Denmark and AGROLAB LUFA GmbH, Kiel, Germany) for the pelleted GC with comparable results of 5.7% moisture, 9.8% crude protein, 11.0% crude fat, 3.0% ash, 3.8% crude fiber and 66.7% NSC. The malto-oligosaccarides fingerprint of the pelleted GC formulation was further determined by high performance anion exchange chromatography coupled to pulsed amperometric detection (Eurofins Food Testing Netherlands B.V., Heerenveen, The Netherlands), indicating 32% glucose and maltose as well as oligosaccharides based on glucose units summing to 35%. In addition, the pelleted formulation was analyzed for the presence of sugar not solely containing glucose. Galactose and lactose were undetectable; fructose and sucrose had concentrations of <1% (weight/weight).

TABLE 1 Overview of glycemic challenges and formulations

Name	Appearance	Intake/Application route	Study	Cohort	Blood analysis
In-feed OGT	Solid	Trough fed	Study 1, phase A	Metabolically healthy	No
Tube OGT	Liquid	Tubed	Study 1, phase B	Metabolically healthy	Yes
			Study 2	Metabolically healthy and ID	
GC syrup A (salty flavor)	Liquid	Trough fed	Study 1, phase A	Metabolically healthy	No
		Syringed	Study 1, phase B		Yes
			Study 2	Metabolically healthy and ID	
GC syrup B (apple flavor)	Liquid	Trough fed	Study 1, phase A	Metabolically healthy	No
		Syringed	Study 1, phase B		Yes
GC granulate	Solid	Trough fed	Study 1, phase A	Metabolically healthy	No
GC pellets (DysChEq)™	Solid	Trough fed	Study 2	Metabolically healthy and ID	Yes

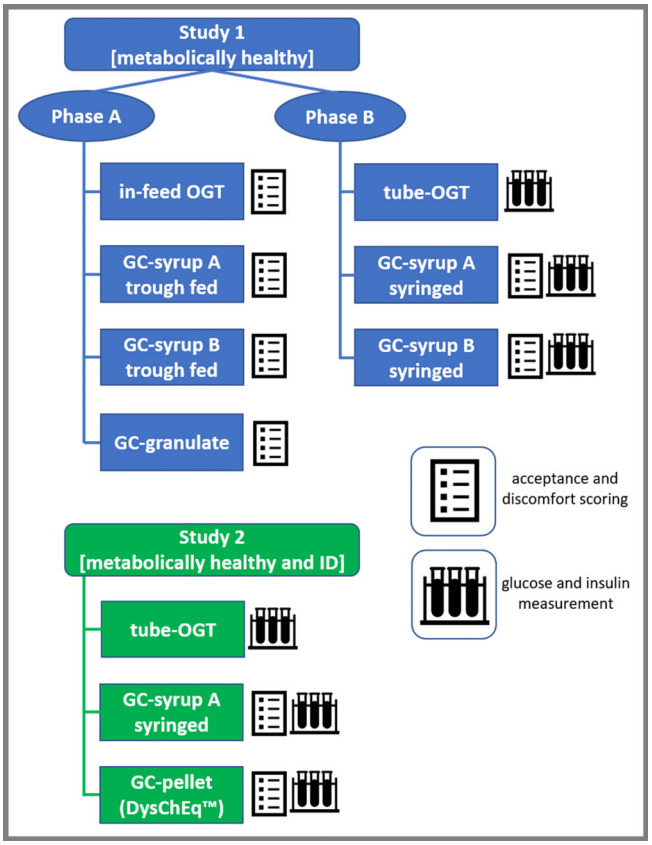

FIGURE 1 Schematic overview of the study design

TABLE 2 Definition of acceptance score and discomfort score

Acceptance score	Description—test item was…
1—very good	…accepted without problems.
2—good	…accepted with minor hesitation but eaten within 10 min, in case of liquid via syringe—administered with minor hesitation
3—acceptable	…accepted with major hesitation but eaten within 10 min, in case of liquid via syringe—administered with some reinforcement
4—unacceptable	…hardly taken up freely, in case of liquid via syringe—administered with major reinforcement or impossible
Discomfort score	**Description**
1—none	No signs of discomfort after administration
2—slight to moderate	Slight to moderate sings of discomfort noted (eg, coughing)
3—high	Pronounced signs of discomfort noted (eg, head shaking)

2.2 | Study design

The objectives of the study were carried out in 2 separate experiments (Figure 1).

2.2.1 | Study 1—Assessment of feasibility

In phase A, 3 new GC (granulate, salty flavored syrup and apple flavored syrup) as well as glucose (crystalline a-D-glucose anhydrous, C☆Dex Dextrose 02402, Cargill) mixed with chaff (in-feed OGT) were trough-fed to horses that had not been fasted in order to assess palatability (acceptance and discomfort) using a clinical score (Table 2). When trough-fed, complete and rapid voluntary uptake is mandatory for the use as an accurate challenge, thus 10 min was used as a maximal time frame. All GC were offered with equivalent amounts of 0.5 g glycemic carbohydrates per kg BW with 2 days washout between. The different GC were tested in a partial cross-over setting employing 8 healthy young horses and ponies per GC. The mean age of the French Trotter (n = 7; 2 geldings, 5 mares) and Welsh Ponies (n = 9; 6 geldings, 3 mares) was 3 ± 1 year. The mean BW was 289 ± 115 kg.

In phase B, the glycemic and insulinemic response to salty-flavored GC (Syrup A) and apple-flavored GC (Syrup B), both orally administered by syringe, was compared to glucose dissolved in 2 L water administered via nasogastric tubing (tube OGT). The GC all were performed by administration of equivalent amounts of 0.5 g

glycemic carbohydrates per kg BW, in a randomized cross-over design with 1 week washout between test days in the same cohort of horses and ponies as in phase A. Horses and ponies were fasted for approximately 12 hours overnight before the GC. Blood samples in EDTA were collected by venipuncture before testing and at 60 and 120 min after the GC. Plasma was prepared and stored frozen until analysis of insulin concentrations using an equine-optimized ELISA (Equine Insulin ELISA, Mercodia, Uppsala, Sweden) previously validated for analysis of equine insulin in serum samples[21] and internally validated for use in plasma samples (unpublished data). Plasma glucose concentrations were analyzed directly in the blood sample using a handheld point-of-care glucometer (Alphatrak 2, Abbott, Germany) previously validated for use in equids.[22]

Study 1 was carried out in winter; the horses were kept on dry lots with hay and straw as supplemental roughage.

2.2.2 | Study 2—Proof of diagnostic principle

Eighteen Icelandic Horses with variable insulinemic status were available for study 2. These research horses have been continuously endocrinologically characterized and monitored as part of several research projects.[23-26] The median age of the Icelandic Horses was 20 (10-26) years. There were 10 mares, 7 geldings, and 1 stallion. The median BW was 370 (287-415) kg. Phenotypic characteristics varied with a median body condition score (BCS)[27] of 6.5 (4-8) and median cresty neck score (CNS)[28] of 3.4 (2-4). Horses were kept under similar feeding and management conditions on a free-range barn and paddock with ad libitum feeding of hay. The study was performed in spring before transition to pasture. Horses underwent 3 different testing procedures in a sequential cross-over design with a wash out period of 1 week in between. Before testing, horses were fasted for approximately 12 hours overnight. Horses were either fed 0.75 g/kg BW of a pelleted GC formulation or syringed orally with 0.565 mL/kg BW salty-flavored GC (Syrup A), both corresponding to 0.5 g/kg BW glycemic carbohydrates or underwent standard OGT procedure with administration of glucose 0.5 g/kg BW dissolved in 2 L water via nasogastric tube. Acceptance and discomfort were assessed for feeding the GC pellets and syringing the GC syrup and scored as in study 1 (Table 2). Feeding the GC pellets by trough was restricted to 10 min. Leftovers of the GC pellets and GC syrup lost because of adverse reaction of the horses during syringing were recorded. Blood samples were collected from a jugular vein catheter before testing and after the GC for 4 hours. Blood samples were placed into fluoride oxalate tubes for the determination of glucose concentrations and into EDTA tubes for plasma preparation. Plasma was stored frozen until further analysis of insulin concentrations analyzed as described for study 1. Plasma glucose concentrations were analyzed using a colorimetric assay (GLUC3, Cobas, Roche Diagnostics GmbH, Mannheim, Germany) on an automated discrete analyzer (Cobas Mira, Roche Diagnostics GmbH, Mannheim, Germany) using fluoride oxalate samples.

2.3 | Data analysis

Data analysis was performed using GraphPad Prism software (version 9.0.0; GraphPad Inc. La Jolla, California, USA). Scoring results for acceptance, palatability and discomfort were described by median and range and compared using Wilcoxon matched pairs signed rank test. Continuous data were tested for normality using the Shapiro-Wilk normality test. Insulin concentrations were normally distributed after log transformation. Glucose and log-transformed insulin concentrations over time were analyzed using a repeated measure (RM) 1-way analysis of variance (ANOVA). Baseline corrected areas under the curve (AUC; net incremental area above baseline value) were calculated for glucose and log-transformed insulin with the trapezoidal method using the statistics software and analyzed by RM-1-way ANOVA. Effects of insulin status on GC performance were analyzed with RM-2-way ANOVA. All ANOVAs were performed with Geisser-Greenhouse correction followed by Dunnett's or Tukey's multiple comparison tests against the basal time point or standard tube OGT when appropriate. Spearman's correlation coefficient and linear regression analysis were used to examine relationships between insulin concentrations at 120 min. Statistical significance was accepted at $P < .05$. All values were expressed as median and range or median and 95% confidence intervals (CI) unless indicated otherwise.

3 | RESULTS

All horse tolerated the experiments well and no evidence of adverse effects was observed.

3.1 | Study 1—Assessment of feasibility

3.1.1 | Phase A

None of the challenges tested (in-feed OGT, new granulate and both new liquid GC formulations) achieved an overall acceptable voluntary uptake when trough-fed. Animals showed initial interest but stopped eating. The acceptance score did not differ between the tested GCs (median 4; range, 3-4). The discomfort score was not analyzed, because with hardly any uptake these data were irrelevant.

3.1.2 | Phase B

When administered via syringe, both liquid GCs were well accepted. The median (range) acceptance score for the salty-flavored GC (Syrup A) was 2 (1-3) and not different from the apple-flavored GC (Syrup B) with 2 (2-3). Similarly, the median (range) discomfort score was 1.5 (1-2) for Syrup A and 2 (1-3) for Syrup B.

For insulin, the pre-challenge concentrations were significantly lower in the tube OGT group than in both liquid GC groups (Syrup A—

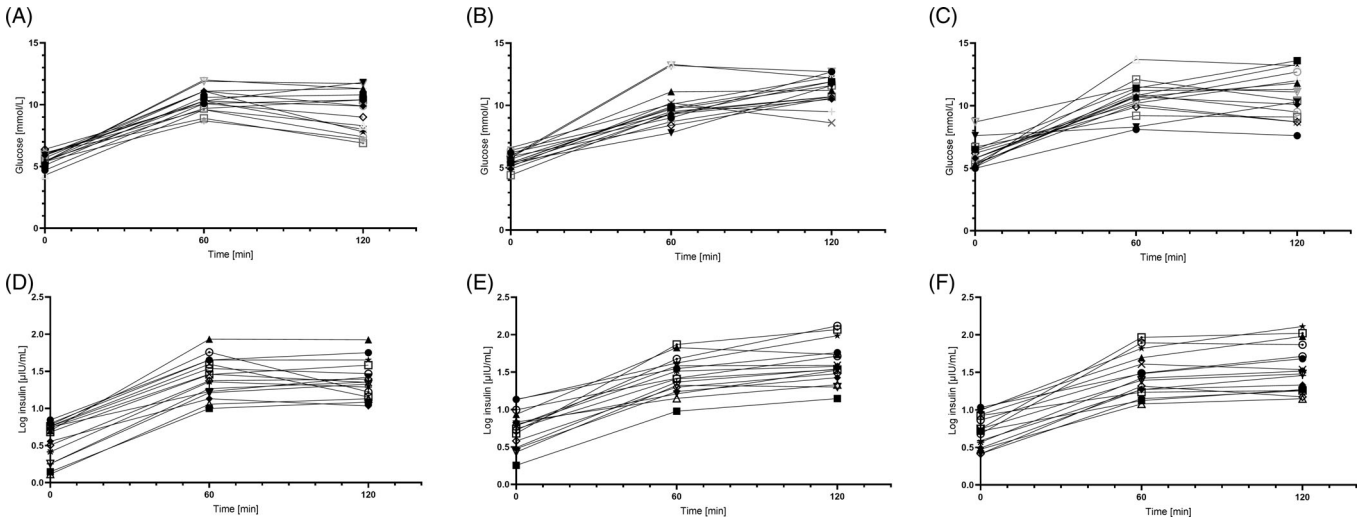

FIGURE 2 Glucose and insulin dynamics study 1. Individual glucose (A-C) and log-transformed insulin (D and E) concentrations over time in study 1 (n = 16) for tube oral glucose test (A + D), liquid glycemic challenger A (Syrup A) (B + E), and liquid glycemic challenger B (Syrup B) (C + F)

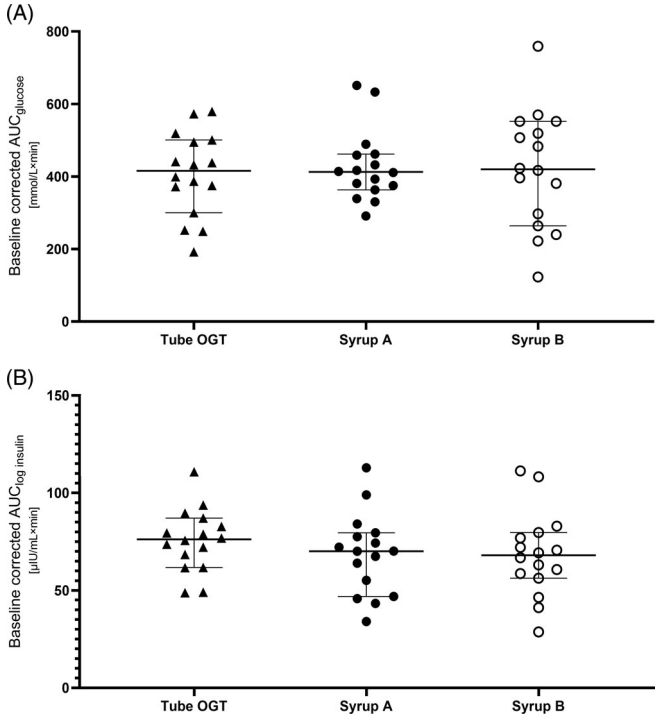

FIGURE 3 Baseline corrected area under the curve (AUC) for glucose and insulin in study 1. Scatter dot plot with median and 95% confidence intervals of baseline corrected $AUC_{glucose}$ (A; mmol/L × min) and $AUC_{log\ insulin}$ (B; µIU/mL × min) for tube oral glucose test (tube OGT) liquid glycemic challenger (GC) (Syrup A) and pelleted GC (Pellets) (n = 16). Tube OGT (black triangle), liquid GC (Syrup A) (black circle), and pelleted GC (Pellets) (black squares). For insulin, RM ANOVA were performed on log transformed data.

P = .01; Syrup B—*P* = .04). Glucose and insulin concentrations significantly increased during all 3 GC over time (both, glucose, and insulin: *P* < .001; Figure 2). The 3 GC did not result in significantly different glucose and insulin dynamics. The baseline corrected $AUC_{glucose}$ and $AUC_{log\ insulin}$ did not differ between the tube OGT and the 2 liquid GCs (Figure 3).

3.2 | Study 2—Proof of diagnostic principle

The median acceptance for the salty-flavored GC syrup A administered via syringe was 2 (1-4) and the discomfort score was 1.5 (1-3). Defense reactions during administration of the syrup varied widely among individual horses and resulted in a mean syrup loss of 4.3 ± 4.1%. For the GC pellet formulation, palatability was excellent with an acceptance score of 1 for all horses. Thus, the GC pellets were more favorable (*P* < .001) compared to the GC syrup A. Most horses showed no signs of discomfort during and after ingestion of the GC pellets, resulting in a median discomfort score of 1 (1-2). From the observed signs of discomfort, transient pronounced salivation was the most common finding. Seventeen of 18 horses consumed the GC pellets within the maximal set time frame of 10 min. The 1 horse not finishing the GC pellets in time had a negligible loss of 5 g pellets corresponding to 1.8% of the total volume and showed moderate salivation. The mean time to full consumption for all 18 horses was 5 ± 2 min.

The pre-test glucose concentrations were significantly higher before feeding the GC pellets compared to tube OGT (*P* = .01). Time significantly affected glucose concentrations after all 3 GC (*P* < .001; Figure 4A-C). The glucose concentrations quickly increased until 120 to 180 min and thereafter decreased in all 3 procedures in all horses with high individual variability. The baseline corrected $AUC_{glucose}$ was higher for the tube OGT compared to the GC syrup A (*P* = .01) and did not differ between tube OGT and GC pellets (Figure 5A).

The pre-test insulin concentrations were significantly higher before GC syrup A compared to tube OGT (*P* = .01). Insulin

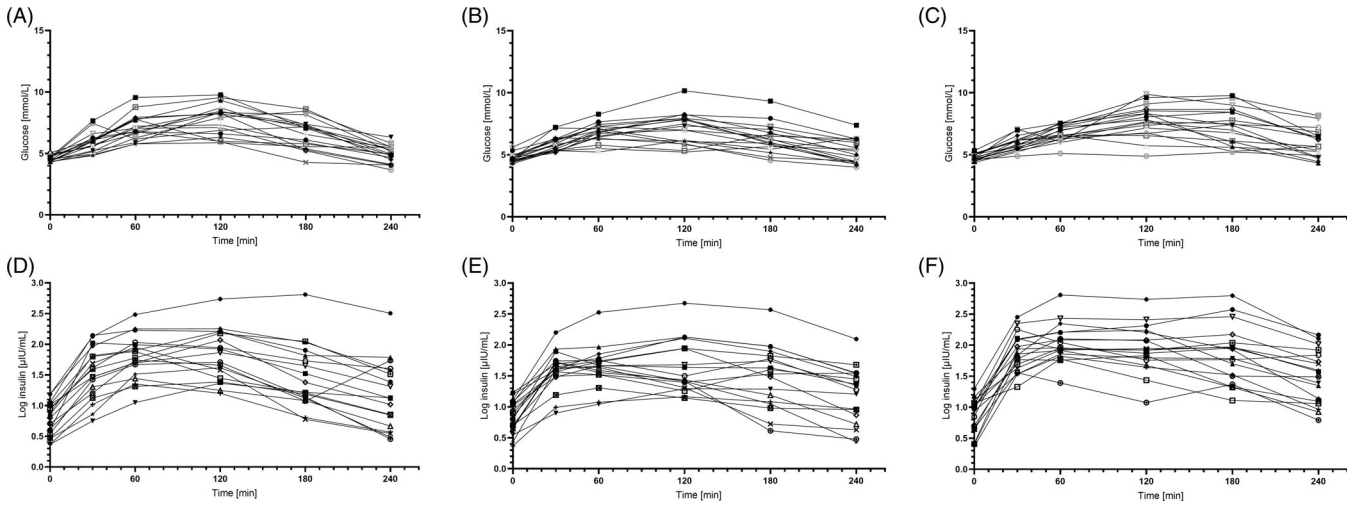

FIGURE 4 Glucose and insulin dynamics study 2. Individual glucose (A-C) and log-transformed insulin (D-F) concentrations over time in study 2 (n = 18) for tube oral glucose test (A + D), liquid glycemic challenger A (Syrup A) (B + E), and pelleted glycemic challenger (Pellets) (C + F)

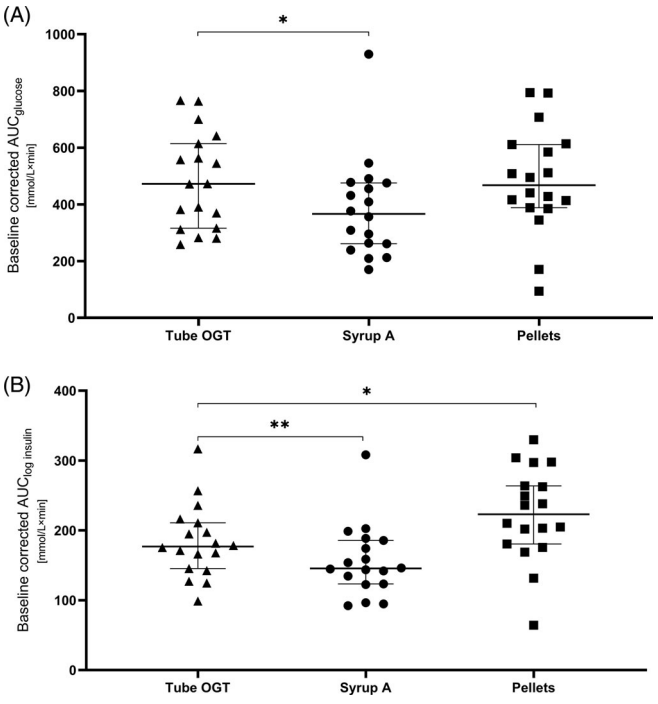

FIGURE 5 Baseline corrected area under the curve (AUC) for glucose and insulin in study 2. Scatter dot plot with median and 95% confidence intervals of baseline corrected $AUC_{glucose}$ (A; mmol/L × min) and $AUC_{log\ insulin}$ (B; µIU/mL × min) for tube oral glucose test (tube OGT) liquid glycemic challenger (GC) (Syrup A) and pelleted GC (Pellets) (n = 18). Tube OGT (black triangle), liquid GC (Syrup A) (black circle), and pelleted GC (Pellets) (black squares). For insulin, RM ANOVA were performed on log transformed data.
*P < .05; **P < .01

concentrations significantly increased in a time-dependent fashion for all 3 GC (P < .001) with high individual variability (Figure 4D,E). The overall endogenous insulin response calculated as the baseline corrected $AUC_{log\ insulin}$ was higher for the tube OGT compared to the response provoked by the GC syrup A (P = .01) and was higher for the GC pellets compared to tube OGT (P = .05; Figure 5B). Nevertheless, insulin concentrations at 120 min were strongly and positively correlated between tube OGT and GC syrup A formulation (r = .9133 and P < .001; r^2 = .9629 and P < .001) and between tube OGT and the GC pellets (r = .7172 and P < .001; r^2 = .8199 and P < .001; Figure 6).

To assess diagnostic feasibility, the results of earlier studies performed in this herd were evaluated. The enrolled horses had been continuously monitored for their insulinemic responses in oral GCs (tube-GTs) performed longitudinally over 1.5 years.[23-26] Based on these results, the horses were retrospectively classified and grouped as either non-ID (n = 5), representing animals with constant low insulin responses, intermediate insulin status (n = 7), representing those with constant, intermediate or alternating insulin responses and ID horses (n = 6), representing animals with constant pathological high insulin responses to repeated oral GCs. Insulin dynamics are shown in Figure 7. In all groups, significant effects were evident for time (non-ID: P = .004; intermediate and ID: P < .001) and the GC (non-ID: P = .04; intermediate: P = .01; ID: P = .02). In non-ID individuals, the GC pellets provoked significantly higher insulin concentrations at 120 and 240 min compared to the tube OGT (P = .03 and P = .002, respectively). In intermediate cases a significant interaction for time and GC was found (P < .05); insulin concentrations were higher for the GC pellets compared to tube OGT at 30 and 180 min (P < .05 and P = .02, respectively) and lower for the GC syrup A compared to the tube OGT at 120 min (P = .03). In consistently ID horses, the insulin concentrations were higher for the GC pellets compared to tube OGT at 60 min (P = .04).

Although some statistically significant differences were detectable in the RM ANOVAs, the new GC syrup as well as the GC pellets were able to clinically discriminate the healthy and the ID horses with the same accuracy as the tube OGT (Table 3).

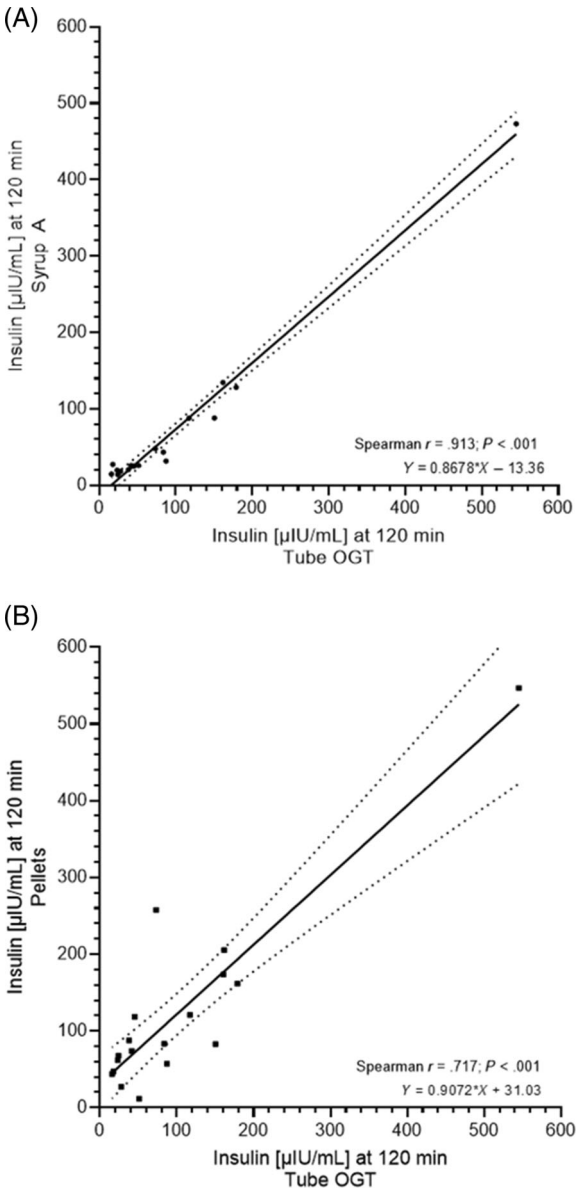

FIGURE 6 Correlation of insulin concentrations at 120 min in study 2. Spearman correlation and linear regression of insulin concentrations 120 min after the glycemic challenge with (A) tube oral glucose test (tube OGT) and liquid glycemic challenger (Syrup A) or (B) Tube OGT and GC pellets (n = 18). The dotted lines represent the 95% confidence bands of the best-fit line.

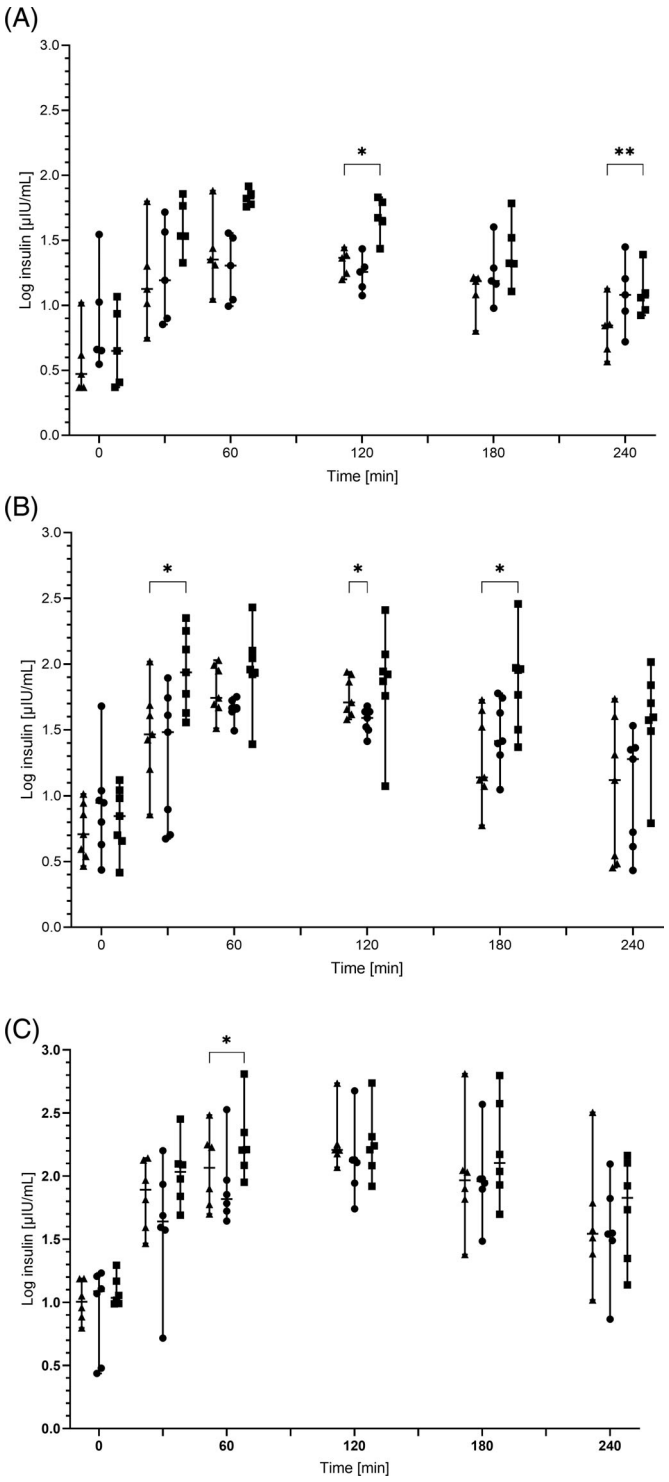

FIGURE 7 Impact of ID-status on insulin dynamics during three glycemic challenges in study 2. Scatter plot with median and range of log-transformed insulin concentrations over time in horses retrospectively grouped—healthy non-ID horses (n = 5) (A), horses with intermediate insulin status (n = 7) (B), and consistently insulin dysregulated horses (n = 6) (C). Tube oral glucose test (OGT) (black triangle), liquid glycemic challenger (Syrup A) (black circle), and pelleted glycemic challenger (Pellets) (back squares). RM ANOVA were performed on log transformed insulin data. *P < .05; **P < .01

4 | DISCUSSION

Testing for ID in horses is essential to guide dietary management and select appropriate treatment options if required. Dynamic testing is recommended to diagnose ID in horses and ponies.[6] We tested different solid and liquid GC formulations with defined carbohydrate composition employed at a dosage of 0.5 g glycemic carbohydrates per kg BW.

No evidence of laminitis or colic was observed throughout the study period in any horse, contradicting the perceived risk of eventually provoking laminitis episodes or colic because of dynamic testing

TABLE 3 Median (95% CI) insulin concentrations at 120 min after glycemic challenge in non-ID and ID-horses

	Tube OGT	Syrup A	Pellets
Non-ID horses	23.2 (15.8-27.9) μIU/mL	18.1 (11.9-27.2) μIU/mL	47.2 (27.2-67.7) μIU/mL
ID horses	161.1 (117.2-545.0) μIU/mL	131.2 (55.0-473.2) μIU/mL	167.8 (83.0-546.7) μIU/mL

for ID. The studies indicated that the GC pellets (DysChEq) and the GC syrups have potential for simple clinical usage as a safe diagnostic test for assessment of ID in equids.

Testing for ID by trough feeding can be challenging presuming complete and rapid voluntary uptake of the diagnostic feed for use as an accurate challenge.[6,17] Thus, palatability is pivotal and critical. Of the tested GC, only the GC pellets met the criterion of rapid and voluntary uptake. The GC granulate initially tested in study 1 did not perform satisfactorily and was not completely consumed by the horses and ponies, probably because of its sticky oral haptic. Moreover, glucose mixed in chaff (in-feed OGT) did result in very low palatability in our study. A limitation, potentially impacting the low overall acceptance for trough feeding of the GCs observed in phase A of study 1 assessing the feasibility of various GC, might be that the horses had not been fasted before assessment of acceptance. Despite controversial results regarding the impact of fasting or feeding state before testing for ID on insulin and glucose dynamics,[29,30] withholding feed for a certain time might have resulted in better palatability and acceptance for all GC.

In-feed OGT have been used in the past as a diagnostic test for ID.[2,6,7] However, the observed low acceptance of in-feed OGT is in accordance with our experience in daily practice and a previous study reporting the necessity of training for the in-feed OGT, and even then a variable slow (up to 45 min) uptake was observed.[17] We did not acclimatize the animals to the new GC formulations and furthermore limited the time for ingestion to 10 min allowing for an accurate challenge. After changing the formulation of the solid GC from the granulate structure used in study 1 to a pelleted structure employed in study 2, the revised pelleted GC formulation was voluntarily taken up by all 18 horses with prior fasting, but without any training after a mean of 5 min.

Moreover, the GC syrup was not voluntarily ingested when trough fed. However, when administered orally via syringe, the GC syrup was well accepted and, in most cases, easy to administer with only minor and acceptable losses, which are comparable to what is published for OST using commercially available syrups.[15] The 2 GC syrups (salty-flavored and apple-flavored) performed similarly in terms of acceptance, but also with respect to the glycemic and insulinemic responses evaluated in study 1. The Karo light corn syrup contains salt, and as such the new salty-flavored GC syrup A represents a reproducible substitute for Karo light and thus the experiments in study 2 were carried out with the salty-flavored GC syrup. The volume of the GC syrup formulations to be administered to the horses and ponies in our study was 0.565 mL/kg to achieve a dosage of 0.5 g glycemic carbohydrates per kg BW. This corresponds to approximately 280 mL for a 500 kg horse and is comparable to volume used for the currently recommended high dose Karo light corn syrup for OST.[13] Unfortunately, further concentration of the syrup to allow a reduction in the volume with similar high concentration of glycemic carbohydrates and therefore potential increase in compliance as well as ease of application was not possible.

The GC syrup and CG pellets were designed to be manufacturable with a reproducible and constant composition to be used for assessment of ID in horses. This design is a main advantage compared to syrups commercially available for human food consumption, with sometimes unknown composition or which might be subject to changes without notification. Similarly, characterization of postprandial glucose and insulin responses with standardized meal or diet challenges[1,20] or commercial pelleted feeds for horses[17] provided satisfying acceptance and resulted in reliable assessment of the postprandial insulin response. However, standardization is clearly restricted to the particular study. Thus, a variety of GCs are useful for defined experiments but are difficult to reproducibly use as a standard diagnostic test across studies, especially when a diagnostic cut-off is employed to assess the diagnosis of ID. Nevertheless, for clinical case-based decisions, using the identical diagnostic approach repeatedly (carbohydrate challenge and insulin assay) is an acceptable procedure for monitoring purposes once an initial valid diagnosis of ID has been made.[6]

The investigated GCs seem to provide substantial stimulation of the enteroinsular axis and pancreatic insulin release.[31-33] The overall glucose and insulin responses calculated as baseline corrected AUC did not differ between the tube OGT and the GC syrup or the GC pellets in metabolically healthy horses enrolled in study 1. In contrast, the $AUC_{glucose}$ was slightly lower after the GC syrup compared to tube OGT in the mixed cohort of healthy and ID horses and resulted in significantly lower overall insulinemic responses calculated as $AUC_{loginsulin}$. Whether this finding is clinically relevant, a coincidental observation, or indicates expected variability of an oral test remains unknown. Baseline corrected AUC was used to appropriately calculate the glycemic and insulinemic responses to the various GC tests and to avoid the bias generated in AUC calculation by different baseline results.

Interestingly, the $AUC_{glucose}$ for GC pellets was similar to the tube OGT in the mixed cohort of horses despite a significantly higher $AUC_{log\ insulin}$ provoked by the GC pellets. This observation might be explained by the impact of chewing and oral processing for the GC pellets. Chewing modifies starch digestion and the metabolic response to carbohydrates in humans[34] and it has been shown that the number of chews per bit increases relevant gut hormones, potentially altering glycemic and insulinemic responses. Furthermore, mastication stimulates salivation and thereby alters gastric and intestinal processing of foods by enzymatic degradation, and increased salivation was observed in 8/18 horses during trough feeding of GC pellets.

Also, modulation of intestinal glucose absorption and regulation of glucose homeostasis has been studied intensively in humans and rodents in context of obesity and metabolic diseases and highlights the impact of oral and intestinal sweet tasting by variable receptors.[35-39] Oral sensory stimulation promotes the cephalic phase of insulin secretion in humans and rodents and modulates glucose homeostasis and insulin dynamics.[40] In experiments studying the effect of intra-gastric glucose administration with and without simultaneous oral food tasting, the total glucose responses were significantly lower and the initial insulin and C-peptide responses significantly higher under simultaneous orally stimulated conditions, suggesting substantial activation of the cephalic phase insulin release.[38] Thus, our results provide evidence of similar conditions in horses and may explain the higher insulin response after the GC pellets.

The combination of healthy and ID horses in the second part of our study represents the clinically relevant population of patients. The observation that the GC pellets evoked the highest insulin response held true for both the healthy as well as the ID horses. Despite differences in the absolute level of response, the insulin responses of the GC syrup as well as GC pellets at the diagnostic relevant time point (120 min after stimulation) were strongly and positively correlated to the measurements in the reference standard tube OGT. Most importantly, the GC syrup as well as the GC pellets clearly discriminated the metabolically healthy and the ID horses.

The most important goal of our study was to evaluate if simplifying the diagnostic procedure can be achieved with the GC pellets or the GC syrup. For dynamic testing a feasible procedure for the field has been described (i.e., to instruct the owner to fast the horse and employ the challenge) and the veterinarian takes blood samples between 60 and 180 min afterward. Given the excellent palatability, this approach might indeed be feasible for the GC pellets. Especially and likely of additional advantage, the GC pellets seemed to evoke a rapid onset and prolonged plateau of the insulinemic response. This provides potential for definition of a broader time frame for the collection of a diagnostic sample and additional diagnostic certainty. The optimal time point for collection of a diagnostic blood sample already has been discussed in previous studies with disparate conclusions[15,16,18,40] and should be addressed for these new GC formulations in a larger study population.

A limitation to the transfer of the presented diagnostic procedure to a routinely used application is the fact that the determination of insulin concentrations was performed using the equine insulin ELISA, an assay that showed convincing performance in research settings but is not readily available in commercial laboratories because of the increased hands-on time for 96-well assays compared to automated, random-access immunoassay platforms. Thus, additional studies are needed to derive diagnostic thresholds for the newly developed GCs in a larger cohort of individuals with assays available in routine diagnostic laboratories. Furthermore, the repeatability of the GC performed using the solid and liquid formulations and the effect of pre-test feeding or fasting remain to be addressed.

5 | CONCLUSION

The presented pellet and syrup formulations with a standardized composition showed glycemic and insulinemic responses comparable to the reference standard (tube OGT) to assess ID in horses or ponies. Especially, the pelleted composition (DysChEq)™ had high palatability and thus provides the potential to function as a simple and standardized dynamic test to assess ID in horses and ponies.

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CONFLICT OF INTEREST DECLARATION

The study was funded by Boehringer Ingelheim Vetmedica GmbH. Tobias Warnken has consulted for and received research funding related to the EMS from Boehringer Ingelheim Vetmedica GmbH. Dania Reiche and Johanna Sonntag are employees of Boehringer Ingelheim Vetmedica GmbH. At the time of study execution and manuscript preparation Tobias Warnken was employed at the Clinic for Horses at the University of Veterinary Medicine Hannover.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

The experiments performed in study 1 have been approved by an ethics committee of Sanofi Pasteur and the Ministère de l'Enseignement Supérieur de la Recherche et de l'Innovation, France under the reference number: APAFIS#11232-2017091209197694v1. The experiments performed in study 2 have been approved by the State Office for Consumer Protection and Food Safety in accordance with the German Animal Welfare Law (LAVES) Ref.-No: 33.9-42502-04-17/2646 and 33.19-42502-05-17A0999.

HUMAN ETHICS APPROVAL DECLARATION

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

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