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ARTICLE

Industry Research

Effect of mulberry fruit extract on glucose fluxes after a wheat porridge meal: a dual isotope study in healthy human subjects

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BACKGROUND: Previous research has shown the efficacy of mulberry extracts for lowering post-prandial glucose (PPG) responses. The postulated mechanism is slowing of glucose absorption, but effects on glucose disposal or endogenous production are also possible. This research assessed the effect of a specified mulberry fruit extract (MFE) on these three glucose flux parameters.
METHODS: The study used a double-blind, randomized, controlled, full cross-over design. In 3 counter-balanced treatments, 12 healthy adult male subjects, mean (SD) age 24.9 (2.50) years and body mass index 22.5 (1.57) kg/m^2, consumed porridge prepared from ^{13}C-labelled wheat, with or without addition of 0.75 g MFE, or a solution of ^{13}C-glucose in water. A co-administered ^{2}H-glucose venous infusion allowed for assessment of glucose disposal. Glucose flux parameters, cumulative absorption (time to 50% absorption, $T_{50\%abs}$), and PPG positive incremental area under the curve from 0 to 120 min ($+iAUC_{0-120}$) were determined from total and isotopically labelled glucose in plasma. As this exploratory study was not powered for formal inferential statistical tests, results are reported as the mean percent difference (or minutes for $T_{50\%abs}$) between treatments with 95% CI.
RESULTS: MFE increased mean $T_{50\%abs}$ by 10.2 min, (95% CI 3.9–16.5 min), and reduced mean 2 h post-meal rate of glucose appearance by 8.4% (95% CI −14.9 to −1.4%) and PPG + $iAUC_{0-120}$ by 11% (95% CI −26.3 to −7.3%), with no significant changes in glucose disposal or endogenous production.
CONCLUSIONS: The PPG-lowering effect of MFE is primarily mediated by a reduced rate of glucose uptake.

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INTRODUCTION

High post-prandial glucose (PPG) responses are a risk marker for (pre-) diabetes, and sustained reductions in PPG exposures may reduce the risk of diabetes and its complications [1–4]. Multiple human trials with mulberry (*Morus alba* L) fruit or leaf extracts (MFE, MLE) have shown these can reduce PPG responses to a carbohydrate load [5–8]. These extracts contain 1-deoxynorjirimycin (DNJ), which may slow carbohydrate digestion by inhibiting alpha-glucosidase (AG) [7, 9]. AG inhibition is an established mechanism to reduce the rate of glucose release and PPG response from foods rich in glycemic carbohydrates [10–12]. Our previous research showed that an MFE dose as low as 0.37 g (containing ~2 mg DNJ) consistently reduced PPG responses to 0rice meals, with no apparent evidence of malabsorption or intolerance [8, 13].

While reduced rates of glucose absorption due to AG inhibition is the likely mechanism for the PPG-lowering effect of mulberry extracts, effects on glucose uptake have not been directly confirmed in vivo. Józefczuk et al. [14] reported that addition of MLE to cornflakes reduced post-meal breath $^{13}CO_2$ by ~20% over 2 h. That suggests effects on post-prandial glucose uptake, but does not exclude possible effects on post-absorptive glucose metabolism or storage. PPG response profiles are an integrated result of changes in rates of three glucose flux parameters: rate of appearance from exogenous glucose (RaE), endogenous glucose production (EGP, mainly from hepatic glycogen), and glucose clearance rate (GCR, disposal into tissues). Variation in RaE can largely explain variation in food effects on PPG [15], but there are examples where changes in RaE do not parallel PPG responses [16–20]. This can occur where an intervention has larger impacts on systemic glucose disposal or production.

To determine RaE, changes in blood glucose originating from the food product must be distinguished from the other flux parameters. Using the dual tracer method [16], subjects consume a ^{13}C-isotope labelled glucose source (e.g. starch) while intravenously infused with a second glucose tracer (D-[6,6-2H$_2$] glucose) that allows determination of glucose disposal (disappearance rate of total glucose, RdT). EGP is the difference between the rate of total glucose appearance (RaT) and RaE. An issue that may affect interpretation of RaE using this method is a potential gap in the ^{13}C-glucose mass-balance, where not all of the tracer appears in blood [21]. This may result from "loss" of tracer during its first pass through the gut wall and liver, an effect assumed to be larger if

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RaE is faster. However, where losses of exogenous glucose are small, variation in the quantitative rate and cumulative appearance of the labelled exogenous glucose in circulation represents variation in rates of absorption.

The present study tested the effect of adding MFE to a wheat porridge (WP) made from ^{13}C-labelled wheat. The primary purpose was to quantify the effect of MFE on the time required to absorb 50% of the dose ($T_{50\%abs}$), RaE and other glucose kinetic parameters. Breath $^{13}CO_2$ was collected to assess changes in the oxidative metabolism of exogenous glucose. Lastly, post-prandial insulin, gastric inhibitory polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and glucagon were measured, to assess possible changes in hormones influencing glucose fluxes. An oral ^{13}C-labelled glucose-in-water solution was included as an additional treatment, to assess the potential first-pass loss of absorbed tracer from a simple food format with high RaE.

STUDY DESIGN, SUBJECTS, MATERIALS AND METHODS

Subjects

Subjects were healthy, normal-weight, normoglycemic males (see Supplementary Table 1 for in- and exclusion criteria). This was an exploratory trial and a formal power calculation was not possible, as there were no directly relevant prior data with which to estimate outcome variability under these test conditions. Based on feasibility and the diminishing gains in precision with greater sample sizes, 12 subjects were planned to be allocated to treatments [22].

Twenty-two individuals were recruited from a database at the clinical study site (Quality Performance Service [QPS] Netherlands B.V.; Groningen, NL), and 12 subjects randomized to the experimental interventions (Supplemental Fig. 1). A screening visit was used to inform and instruct potential participants, obtain signed informed consent and assess eligibility. Four of the 22 individuals were ineligible to participate, three were eligible but not needed, and 3 were retained as reserves. Ethical approval was obtained from the Medical Ethics Review Committee Brabant (Tilburg, NL) on 1 December 2015, and interventions took place from 26 January through 10 February 2016. The protocol was registered at www.clinicaltrials.gov (number NCT02662738) before undertaking any procedures.

Study design

The study used a double-blind, randomized, controlled, full cross-over (within-subject) balanced treatment order design [23]. The 3 treatments were: ^{13}C-labelled WP + MFE, ^{13}C-labelled WP + placebo, ^{13}C-labelled glucose in water. Using computer-generated randomization, individuals not involved with the study assigned each subject an identification number, assigned each treatment product a code, and allocated subjects to a treatment sequence. Personnel and subjects involved with the study were blinded as to the nature of the test products until after the blind data review. A password-protected file with the subject and treatment codes was provided to the study coordinator, to be accessed only if early de-blinding of the study was necessary.

Following the single-day screening visit, subjects attended the study site for three intervention visits separated by at least one week. Subjects were instructed to minimize changes in their habitual diet and activity during the study period. They were also asked to refrain from consuming ^{13}C-rich foods (Supplementary Table 2) for 3 days preceding the experiments, and from exercise and alcohol consumption the day prior to each test day. On the evening before intervention days, subjects were admitted to the clinic to consume a standardized evening meal and follow a minimal physical activity pattern. All participants fasted overnight (from 19.30 until consumption of the test product), with water allowed up to 1 h prior to test product administration.

Study test products and product administration

Details of the sourcing and preparation of the MFE and study test products are in the Supplementary Material. In brief, WP meals containing ~50 g available carbohydrates were prepared from 72.5 g of a standard commercial wheat mixed with 2% universally ^{13}C-labelled wheat. Sealed sachets containing 0.75 g MFE (0.5% DNJ) or placebo, identical in weight and appearance, were supplied to the test site. The contents of the sachets were stirred into the individual WP servings according to the treatment allocation schedule. At time (t) = 0 min, subjects consumed the WP and 100 mL tap water within 15 min.

The additional treatment was an oral solution of 50 g glucose containing by weight 2% of ^{13}C universally-enriched glucose (>99 atom% ^{13}C, food grade, Cambridge Isotope Laboratories, Tewksbury MA, USA) and 98% unlabelled glucose in 250 ml total water volume. The glucose solution was also consumed within 15 min.

Consumption of WP or solutions was monitored by a physician for completeness. Beginning from 1 h after starting consumption of test products, subjects were allowed to drink 150 ml water every subsequent hour. The volume of water consumed on the first test day was recorded and the same volume consumed on subsequent intervention days. Six hours after start of consumption of the meal the subjects were offered lunch and departed the test site.

$^{2}H_2$-labelled glucose infusions

On intervention days subjects had a venous catheter inserted in both forearms, one for the infusion of $^{2}H_2$-labelled glucose and the other for blood collections. At $t = -122$ min, one 27 ml bolus of sterile D-[6,6-$^{2}H_2$]-glucose (5.6 mg/kg body weight) solution was infused within 2 min, and thereafter a continuous infusion of 0.07 mg/kg body weight per minute (20 ml/h) was started and maintained for 8 h. A hospital pharmacy (University Medical Centre Groningen; Groningen, NL) prepared the infusion solutions and bolus syringes.

Sampling and measurement of glucose, insulin, GLP-1, GIP and glucagon

Venous blood samples were drawn before (at −30 and −5 min, averaged as baseline), and at 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min after start of test product (WP or water) consumption for plasma glucose measurements. The blood samples taken up to 240 min were also used to determine concentrations of insulin in serum, and glucagon, and (for WP treatments only) GLP-1 and GIP in plasma. Details of the analytical methods are in the Supplementary Materials.

Tracer analysis and calculation of glucose kinetic parameters

All samples were analyzed by gas chromatography (GC) quadrupole mass spectrometry (MS) (SSQ7000; Thermo-Finnigan, San Jose CA, USA). Derivatives were separated on a AT-5MS 30 m × 0,25 mm ID (0.25 µm film thickness) capillary column (Alltech, Breda, The Netherlands) [21, 24]. The initial step in data analysis was to adjust the fractional distribution of glucose isotopologues determined by GC/MS for the natural abundance of ^{13}C atoms [25]. Calculations of glucose kinetic parameters (RaT, RdT and RaE) were based on the non-steady-state equations of Steele et al. [26] as modified by De Bodo et al. [27]. We used an approach suggested by Radziuk et al. [28], including the assumption that the clearance rates of all glucose isotopologues, i.e., tracers and tracee, are identical. Furthermore, the volume of distribution for glucose was considered to be 200 ml/kg and the pool fraction 0.75 [29]. EGP was determined as the difference between RaT and RaE. GCR was derived from RdT and the prevalent plasma glucose concentrations [20]. The cumulative appearance of glucose was calculated from the RaE and time (RaE × time) to construct cumulative absorption curves, corrected for the amount already

disposed. The time to reach 50% absorption ($T_{50\%abs}$) was calculated from the cumulative absorption curves.

Breath sampling for $^{13}CO_2$

Breath samples were taken for $^{13}CO_2$ analysis at 2, 4 and 6 h after consumption of the test meals, as an indicator of changes in the contribution of meal-derived glucose to post-prandial fuel oxidation. Details on the collection and analysis of breath samples are in the Supplementary Material. Data for breath $^{13}CO_2$ are expressed relative to total CO_2 production, estimated as 300 mmol/m^2 body surface area per hour at rest [30, 31].

Safety analysis

Adverse events (AE) were recorded during study days by the study physician, using predefined criteria for severity and relatedness. All AEs were followed up to resolution or until there was no further change. The final outcome of any AEs that continued beyond the end of the study would be determined by further contact with the subject or their personal physician.

Statistical analysis

Glucose, glucose flux parameters, hormones (insulin, glucagon, GLP-1 and GIP) and breath $^{13}CO_2$ data were all summarized as area under the curve over a period of 120 min after the test product was consumed ($AUC_{0\text{-}120}$). The AUC was calculated using the trapezoidal rule [32, 33]. For glucose, the positive incremental AUC ($+iAUC_{0-120}$) was calculated by subtracting (120*baseline values) from the AUC_{0-120}, while the total AUC ($tAUC_{0-120}$) was calculated for insulin, RaE, GCR, glucagon, GLP-1, GIP and breath $^{13}CO2$. For EGP a decremental AUC ($dAUC_{0-120}$) was calculated by subtracting the AUC_{0-120} from (120*baseline EGP). Similar calculations were also used to derive the data values reported over a period of 240 min.

Statistical analyses were carried out on log-transformed tAUC, +iAUC or dAUC using linear mixed models with subjects as a random effect. The model included treatment, baseline characteristics and visit number as fixed effects. All statistical analyses on efficacy endpoints were based on within-subject differences. The results were expressed as a percentage change using the control meal (WP without MFE) as a reference and its 95% confidence interval (CI). Following common convention and for ease of interpretation, where the 95% CI for the % change does not include a null effect (zero), results are described as "statistically significant". Treatment means were obtained from the Least squares (LS) mean via back transformation. No formal inferential statistical tests were planned or performed, as the study was not explicitly powered to do so given the small number of subjects.

Deviations from registered protocol

The registered protocol was ambiguous regarding the primary outcome (^{13}C- and $^{2}H_2$-glucose) but clear that the study was intended to quantify "the time needed to absorb 50% of the apparent total of available exogenous carbohydrate (RaE * Time)." $T_{50\%abs}$ is therefore reported here as the primary outcome, in the context of other glucose flux parameters. Exploratory analyses on metabolomics, anti-oxidants and DNJ concentrations will be reported elsewhere.

RESULTS

Study population, compliance and adverse events

The 12 subjects allocated to treatments were males with a mean (SD; range) age 24.9 (2.50, 21–30) years and body mass index 22.5 (1.57; 19.2–24.7) kg/m^2 at enrolment. All subjects completed all treatments and follow-up, with no drop-outs or missed sessions (Supplemental Fig. 1). There were no major deviations from protocol and no missing data points. Subjects completed all meals on all occasions within the allowed time. Intention-to-treat and per-protocol data sets were therefore identical, and the complete data set used in analyses. Two subjects reported in total 4 mild AEs, one unrelated to treatment and others having no clear relation with any specific study treatment product. All AEs were resolved before the end of the trial. The blinded randomization remained intact until after final data-lock.

Glucose kinetic parameters (fluxes)

The mean cumulative glucose appearance in plasma from exogenous sources for all three treatments is shown in Fig. 1.

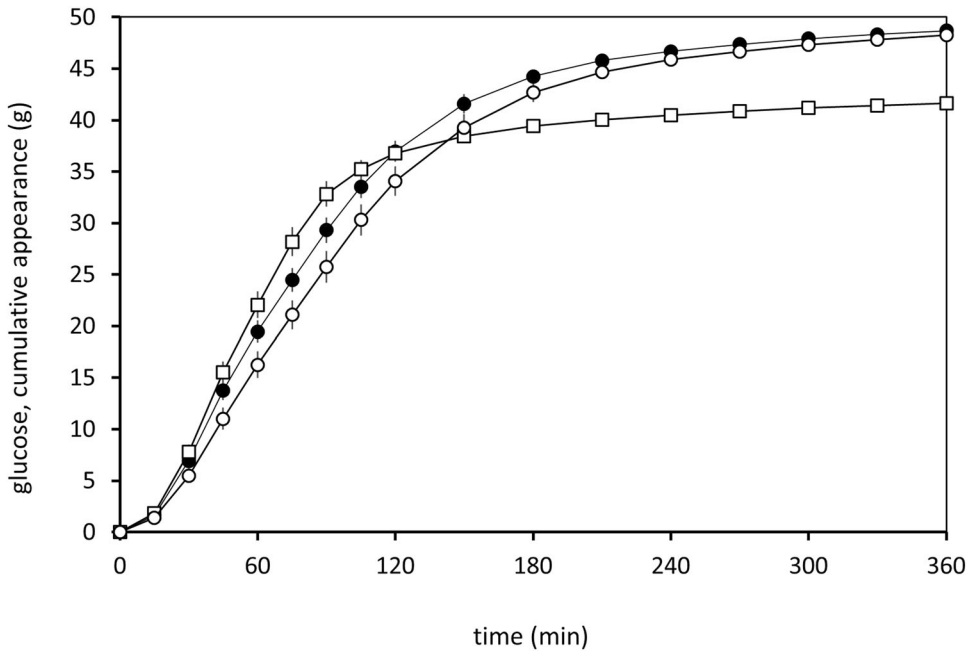

Fig. 1 Cumulative glucose appearance. Mean (±SEM; $n = 12$) cumulative glucose appearance in plasma at timepoints following consumption of ~50 g available carbohydrate from a wheat porridge meal + placebo (closed circle) or with the addition of 0.75 g MFE (open circle), or a glucose drink (open square).

Table 1. Glucose flux parameters (mean and change vs. control).

Flux parameter	WP + placebo (control)			WP + 0.75 g MFE			Change vs control[a]			Glucose drink			Change vs control[a]		
	Mean	95% CI		Mean	95% CI		Mean	95% CI		Mean	95% CI		Mean	95% CI	
$T_{50\%abs}$, min	75	70	81	86	80	91	10.2	3.9	16.5	58	53	64	−17.4	−23.7	−11.1
RaE $AUC_{0–120}$, (μmol/kg) min	2620	2466	2782	2399	2258	2548	−8.4	−14.9	−1.4	2613	2460	2776	−0.2	−7.3	7.4
RaE $AUC_{0–240}$, (μmol/kg) min	3320	3253	3389	3264	3197	3332	−1.7	−4.3	0.9	2880	2821	2940	−13.3	−15.5	−10.9
GCR $AUC_{0–120}$, (ml/kg) min	454	405	509	436	389	489	−3.9	−13.2	6.4	494	440	554	8.8	−1.8	20.7
GCR $AUC_{0–240}$, (ml/kg) min	820	778	865	816	774	860	−0.5	−5.7	4.9	833	789	878	1.5	−3.9	7.1
EGP $AUC_{0–120}$, (μmol/kg) min	627	578	680	595	549	646	−5.1	−11.7	2.1	659	607	715	5	−2.7	13.4
EGP $AUC_{0–240}$, (μmol/kg) min	1142	1052	1240	1212	1116	1316	6.1	−4.7	18.3	880	808	957	−23	−31.3	−13.7

$N = 12$ subjects, within-subjects analyses.
MFE mulberry fruit extract, *tAUC* total area under the curve, *CI* confidence interval, $T_{50\%abs}$ time required to absorb 50% of the dose, *RaE* rate of appearance of exogeneous glucose, *GCR* glucose clearance rate, *EGP* endogenous glucose production rate, *AUC* area under the curve (120 or 240 min), *WP* wheat porridge.
[a]Percent change from WP control except $T_{50\%abs}$ (expressed as mins).

After 360 min, a mean (95% CI) of 48.7 (47.9–49.5), 48.3 (47.2–49.3), and 41.6 (40.8–42.4) g of exogenous glucose had appeared in plasma, following consumption of the WP with placebo (control), WP with MFE, and the glucose drink, respectively. The glucose kinetic parameters (Table 1) show that the addition of MFE to WP significantly extended $T_{50\%abs}$ and reduced RaE over 120 min, with no significant effects on GCR or EGP.

Post-prandial glucose response

The mean plasma glucose response to wheat WP was significantly reduced by the addition of MFE (Fig. 2 and Table 2).

Insulin, GIP, GLP-1 and glucagon

Serum insulin concentrations did not significantly differ between treatments (Supplemental Table 3). Plasma GIP, GLP-1 and glucagon responses over 120 or 240 min were all similar for WP with and without the addition of MFE (Supplemental Table 4).

Breath $^{13}CO_2$

The addition of MFE to WP resulted in a mean 11.8% (95% CI −22.5 to 0.3) lower percent of ingested $^{13}CO_2$ dose expired per hour over 120 min (Supplemental Table 5).

DISCUSSION

This study adds to other research on the effect of mulberry extracts on PPG [5–8, 13], and confirms that MFE reduces the rate of systemic appearance of glucose from dietary carbohydrates. The results are consistent with the generally-proposed mode of action of DNJ-containing mulberry extracts [7, 14, 34]. The study benefited from a realistic carbohydrate load and well-specified source of MFE, at a dose and DNJ content previously shown to reduce PPG responses [8].

In a recent systematic review and meta-analysis of published glucose flux studies, each 10% reduction in RaE was associated with ~7% reduction in PPG, roughly consistent with observed relationships in the present study. The mean reduction in PPG here (11%) was however somewhat less than in our previous trials with MFE added to rice [8, 13]. MFE also significantly reduced PPI in those studies, whereas there was a negligible effect here. Differences may reflect differing test products and subject populations. There may also be less confidence in the estimated effect sizes here, as previous trials were substantially larger and explicitly powered for PPG as a primary outcome. Comparisons with mulberry extracts tested by other research groups have limited value because of the differing source material (invariably MLE, not MFE), content of DNJ or other bioactive components [34], and other aspects of test materials and conditions.

As noted in the introduction, RaE may not directly reflect glucose absorption, if there are losses of labelled glucose before it appears in the systemic circulation. In this study it seems reasonable to interpret RaE after WP consumption as a characteristic of exogenous glucose absorption, because cumulative absorption data for these treatments show that >95% of the exogenous ^{13}C-label appeared in plasma by 6 h. This indicates minimal losses of exogenous glucose due to malabsorption or pre-systemic removal in the gut wall or liver. In contrast, <85% of the ^{13}C-labelled glucose from the glucose drink appeared in plasma. Thus, from that rapidly absorbed oral solution, more of the label appears to have been lost before entering the systemic circulation. It is possible that this value for glucose overestimates 'first-pass' losses, though it is within the range reported in other studies (Table 1 from [35]). We are not aware of any similar analyses directly comparing a glucose drink to a starchy food, but other studies have reported a lower cumulative appearance of glucose from meals with a higher (vs lower) glycemic index or faster (vs slower) apparent starch digestibility [36, 37].

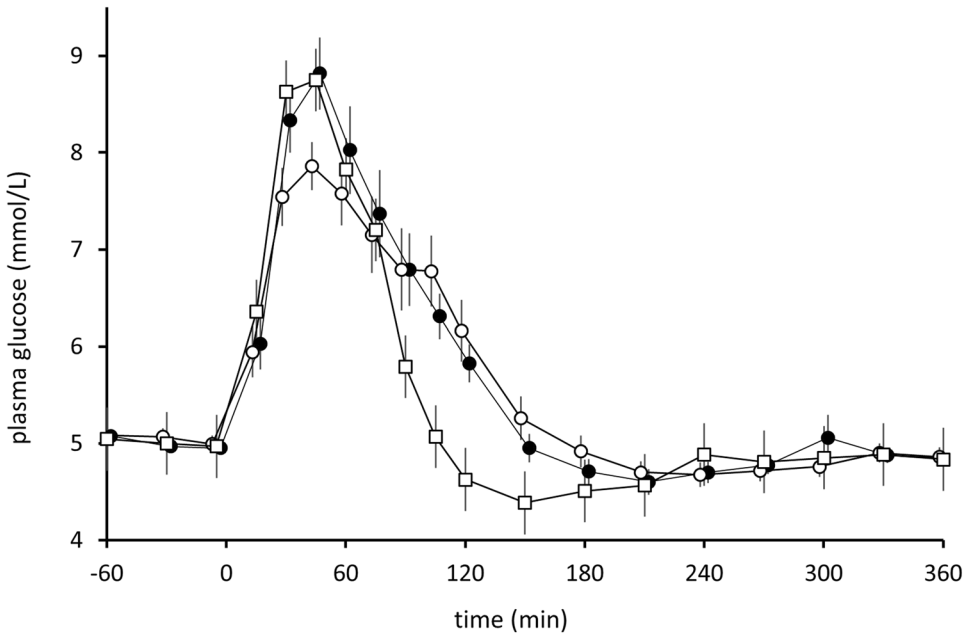

Fig. 2 Plasma glucose response. Mean (±SEM; $n = 12$) plasma glucose per timepoint after consumption of ~50 g available carbohydrate as a wheat porridge meal + placebo (control, closed circle), the same meal with addition of 0.75 g MFE (open circle) and a 50 g glucose drink (open square). Data points jittered for clarity.

Table 2. Positive incremental AUC values for glucose from 0–120 min ($+iAUC_{0\text{-}120}$) after ~50 available carbohydrate as wheat porridge meal without MFE (control), with the addition of 0.75 g MFE, and after a 50 g glucose in water.

Treatment	$+iAUC_{0\text{-}120}$ mmol/L min			% change vs control		
	Mean	95% CI		Mean	95% CI	
WP + placebo (control)	236	180	310	(reference)	(reference)	
WP + 0.75 MFE	210	160	276	−11.4	−26.3	−7.3
Glucose drink	218	166	287	−7.5	−23.1	11.2

$N = 12$ subjects, within-subjects analyses.
MFE mulberry fruit extract, *+iAUC* positive incremental area under the curve, *CI* confidence interval, *WP* wheat porridge.

MFE therefore slowed the rate but not total cumulative absorption of glucose from WP. Minimal effects on gastrointestinal hormones GIP or GLP-1 suggest that absorption was not displaced toward more proximal regions of the intestine, and there was also no effect of MFE on glucagon levels. The absence of effects on these hormones is also consistent with the absence of significant changes in the GCR and EGP flux measures.

Although these results seem clear and consistent, there are potential methodological weaknesses that should be considered. Most significantly, use of a third isotopic label may have benefits over the dual isotope method modelled in a non-steady (e.g. post-prandial) state [38]. However, it is uncertain if there would be large differences between the dual- and triple-isotope methods in practice, or under what conditions this might occur [39]. We also used a pool fraction of 0.75, consistent with our previous research and other studies of the effects of dietary interventions on glucose fluxes [16, 17, 21, 40, 41]. However, a value of 0.65 is commonly applied, and there is longstanding uncertainty over the most appropriate value, though probably limited impact within this range [28, 38, 42]. These methodological considerations may influence quantitative parameter estimations, but we do not believe they alter the overall conclusions for our primary research question on the mechanism of action of MFE. Given the within-subject study design and closely similar test foods, it seems unlikely that the specific methods would introduce significant, treatment-specific directional bias in $T_{50\%abs}$, RaE or other flux parameters. Moreover, observed effects on measures of PPG and breath $^{13}CO_2$, both physiologically related to but derived independently from the flux parameters, closely corresponded to the results for RaE in direction and magnitude.

While this trial successfully achieved its primary objective, the scope of the research was limited. There are many possible additional postprandial measures such as C-peptide that could be used to fully characterize the profile of acute physiological responses to MFE. Furthermore, although there is evidence suggesting benefits of sustained consumption of mulberry products for cardiometabolic risk factors [43, 44], measures of responses following repeated or chronic consumption of MFE would also be helpful to establish its full range of health effects.

Overall, together with other research on mulberry extracts, we conclude that this source and dose of MFE reduces PPG mainly by slowing but not reducing glucose uptake from carbohydrate-rich foods. Along with other assurances of safety and efficacy, this mechanistic understanding may help in considering potential applications of MFE as an ingredient in commercial foods or supplements intended to help control blood glucose.

DATA AVAILABILITY

The datasets generated during the current study are not publicly available as the participants did not give express consent for this, but are available on reasonable request, noting that some caveats may apply.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study design and protocol development, data interpretation, and writing and reviewing of the manuscript. A-RH managed the interface between the sponsor and study site, and monitoring of data collection. HH was primarily responsible for the statistical design and analyses, which were agreed with all authors in advance of study recruitment. All authors have seen and approved the manuscript, and agreed that any questions related to the accuracy or integrity of any part of the work are appropriately investigated, resolved and documented.

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COMPETING INTERESTS

HMB and A-RH are employees of Unilever, the sponsor of the study and a manufacturer of carbohydrate-containing foods. GSD, HH and DJM were employees of Unilever at the time the research was designed and conducted, but have no current affiliation with the company, and no other competing interests in the topic of this research.

ETHICAL APPROVAL

The study was conducted according to the guidelines of the Declaration of Helsinki, and the protocol approved by the Medical Ethics Review Committee Brabant (Medische Ethische ToetsingsCommissie Brabant, Tilburg, The Netherlands) on 1 December 2015. All participants provided written informed consent. The protocol was registered at www.clinicaltrials.gov (number NCT02662738) prior to undertaking any procedures.

ADDITIONAL INFORMATION

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