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Resistant starch (RS), a novel endogenous inert marker for detecting glucose absorption of small intestine with sweeteners administration in mice

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Abstract

Resistant starch could be degraded by the fermentation of colonic microorganisms in the large intestine of mammals, but not in the small intestine. In this study, we established a novel strategy by using resistant starch as an endogenous marker to determine the glucose absorption of the small intestine of laboratory animals. By optimization of the classical enzymatic method of starch measurement, the demand for the sample weight was reduced by 90%. Moreover, the amount of resistant starch in normal feed was detectable without any extra addition. The value of small intestine glucose absorption of mice was similar when using resistant starch and titanium dioxide as inert markers. The fermentation of resistant starch by intestinal microorganisms in the small intestine was demonstrated not disturbing the detection of glucose absorption significantly. Artificial sweeteners exposed ICR mice showed different glucose absorption which indicated, first, resistant starch can be used as a novel endogenous marker in the small intestine of small animals; second, although glucose tolerance did not change in mice after short-term exposure to artificial sweeteners, there were significant changes in glucose absorption associated with it; third, the short-term exposure resulted in no significant change in glucose tolerance.

Keywords: Resistant starch, Titanium dioxide, Small intestine, Glucose absorption, Artificial sweeteners

Introduction

To date, several well-established animal and cell-based models are used for studying intestinal absorption, including in vitro (Caco-2 cells model), ex vivo (Ussing chamber) or in situ (small intestinal perfusion models) and in vivo animal models [1]. For the evaluation of intestinal glucose absorption rate, the in vitro cell models have several limitations such as wide variation with passage number, variability between different laboratories [2–4]. The ex vivo or in situ methods of intestinal perfusion technique are simple for estimating intestinal absorption rate. However, as in all ex vivo/in situ models, the tissue viability of the intestinal respiratory system has a significant effect on the results [1]. In addition, the barrier imposed by the freshly isolated animal intestine may result in slower absorption rates than those obtained in intact animals [5]. Starch digestion and glucose absorption in the small intestine are very complicated, including the nervous reflex from the taste receptors, induction of hormones, and secretion of enzymes into the gastrointestinal tract and increase of the absorption rate. As

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reviewed above, the in vitro cell models and ex vivo or in situ methods may not be suitable for the evaluation of small intestinal glucose absorption rate. Herein, the in vivo animal models were used in this study. The main advantage of in vivo animal models is the presence of an intact system.

As a classical method, the glucose absorption rate of lots of animals had been investigated in intact individuals before. However, the drawbacks of the chemical analysis of intestinal samples are obvious [6]: first, always need an extra addition of inert marker, which is inconvenient and hard to detect. Moreover, as a normal use inert marker, chromic oxide (Cr_2O_3) is toxic for the animals; second, need a large amount of content for each test. So far as we know, the smallest animal that had enough content samples from the small intestine and could be investigated by the in vivo method (intact individuals) is in chick [7]. To study the glucose absorption rate in the laboratory model animals (such as murine) in large scales conveniently, the discovery of novel inert marker is necessary, and the determination method of the starch and novel inert marker is needed to be optimized for much fewer content samples.

After analyzing the composition of normal feed of the laboratory model animals, we found resistant starch has the potential to work as a novel inert marker without extra addition. According to the bioavailability of starch in the human small intestine, starch is divided into three categories. Ready digestible starch refers to starch molecules that are quickly digested and absorbed in the small intestine. Slowly digestible starch is starch that can be fully digested and absorbed in the small intestine but at a slower rate. Resistant starch (RS) refers to starch and its degradants that are not digestible in the small intestine of healthy humans and are fermented in the large intestine [8, 9]. 99.99% of human intestinal microorganisms are located in the colon [10]. RS could be degraded in the large intestine by the fermentation of colonic microorganisms, but not in the small intestine [11–13].

At present, more than 20 kinds of artificial sweeteners with the characteristics of metabolic inertia have been widely used in the food and pharmaceutical industries in various countries and regions. Epidemiological studies have found that the use of artificial sweeteners may increase the risk of obesity and metabolic disorders [14–17]. According to some studies, when refined or rapidly digested carbohydrates are included regularly in our diets, glucose absorption rates are increased, and blood glucose and insulin responses are usually enhanced, resulting in dietary related chronic diseases such as diabetes and obesity [4, 18, 19]. Thus, the glucose absorption rate of the gastrointestinal tract is considered to be an important parameter to evaluate the risk of the highly glycaemic index of daily food.

In this study, the glucose absorption of the small intestine was investigated by analyzing the intestinal contents in vivo. Resistant starch was considered as a novel and endogenous non-absorbable inert marker in normal feed to correct for the intestinal fluid volume. The detection of resistant starch in the normal feed or synthetic feed (standard ingredients add the RS), and the dispersion and propulsion features of the small intestine of Sprague Dawley (SD) rats that feeding with synthetic feed (standard ingredients add the RS and TiO_2) was calculated to compare with the classical method. With the modification of the standard method for the determination of resistant starch, the glucose absorption of different sweetness (the intestine of sweet was converted relative to 2% sucrose as sweetness 1 [20]) of Acesulfame K and Saccharin (Sweetness is 250 and 300 times relative to the same concentration of sucrose [21]) stimulus ICR mice for 1 week and feeding with synthetic feed (standard ingredients add the RS and TiO_2) were also detectable by using few small intestinal contents. The resistant starch, a novel and endogenous inert marker, was also useful in the determination of other nutrients absorption rate in the small intestine, especially the duodenum and jejunum.

Materials and methods

Animals, housing and feeding managements

The principles for the care and use of laboratory animals, approved by the Animal Use and Care Committee of the Chinese Academy of Sciences, were observed in the conduct of this study. All experiments were approved by the Animal Ethics Board, University of Zhejiang Gongshang University.

Thirty male 300 g Sprague Dawley (SD) rats and 40 male 6 weeks ICR mice were obtained from the SLAC Laboratory Animal Co. LTD (Shanghai, China). Five of each species were housed in one cage and allowed to acclimatize for 1 week to adjust to a temperature of 24 °C, with 12 h of light and 12 h of darkness. Water and food were supplied ad libitum. And then, SD rats were randomly distributed to the 6 groups (n = 5) and were fed on different diets (from No.1 to No.6, Table 1) for 2 weeks. The experimental diets differed only in their proportions of high-amylose corn starch [Novelose330, National Starch & Chemical Company, USA, 41% resistant starch type 3 (RS3)]. At the same time, ICR mice were randomly distributed to the 8 groups (n = 5) and were fed on the No.1 diet and were exposed to different sweetener drinks for 2 weeks. Drinks differed in their kinds and sweetness (Table 2).

Table 1 Composition of experimental diets

Diet number	1	2	3	4	5	6
Feed ingredient	**Content**					
Wheat	34%					
Maize	20–25%					
Soybean meal	21%					
Fish meal	4.0%					
Premixtures	4.2%					
Bran	3.8%					
Soybean oil	2.0%					
Soybean	2.0%					
Yeast	2.0%					
Grass powder	0.5%					
Maltodextrin	0.5%					
Titanium dioxide	1.0%					
High-amylose corn starch	–	1%	2%	3%	4%	5%

High-amylose corn starch containing ~ 41% resistant starch

Table 2 The sweetness of different sweeteners and their concentration

	Concentration of acesulfame K, g/L	Concentration of saccharin, g/L
Sweetness of 8	0.8	0.53
Sweetness of 12	1.2	0.8
Sweetness of 16	1.6	1.07

The oral glucose tolerance test (OGTT) was performed on experimental animals during the last day of the study. Glucose was administered orally (2 g/kg body weight) to overnight fasted-animals. Blood was drawn from the tail vein and serum samples collected immediately before the start of the OGTT. Glucose levels were measured by a glucometer (Roche) immediately before and 15, 30, 60, 90, and 120 min after oral administration of glucose.

Sample preparation

At the end of the feeding period, all the animals were killed by an intracardiac overdose of sodium-pentobarbital (Macklin, Shanghai, China) and their small intestines were immediately removed and divided into 10 segments (SD rat) or 5 segments (ICR mice): duodenum, upper and lower jejunum, and upper and lower ileum [22], all the contents from the small intestine of SD rats were used for comparison of two markers, resistant starch (RS) and TiO_2 (Aladdin, Shanghai, China), while the contents in the duodenum and upper jejunum of ICR mice were used for testing total sugar absorption. The contents of each segment were then removed into an empty tube and placed on ice. These samples were kept frozen until further processing. Before using, samples were homogenized through shaking with two 5 mm stainless steel beads (Jingxin Technology, Shanghai, China) in a tissuelyser (Tissuelyser-24, Jingxin Technology, Shanghai, China) at 65 Hz for 2 × 2 min.

Chemical analyses

Starch and RS contents of intestinal samples were measured mainly according to the analysis procedure provided by the Resistant Starch Assay Kit (catalog number K-RSTAR, AOAC Official Method 2002.02 and AACC Method 32–40 [23] Megazyme International Ireland Ltd. Co., Wicklow, Ireland) with the following modification for few contents from the small intestine of laboratory animals. In brief, 400 µL of the mixture (pancreatic a-amylase, 10 mg/mL, and amyloglucosidase, 3 U/mL,) was added to each 100 mg of sample (in a 1.5 mL tube, T1) and incubated in a shaker with 200 strokes/min for 16 h at 37 °C. After incubation, 400 µL of ethanol (99%) was added and stirred vigorously (stop reaction), and then centrifuged at 12,000*g* for 2 min. The supernatant was moved to a new 1.5 mL tube (T2) carefully and 800 µL of 50% (v/v) ethanol was added to the residue and stirred in; this was followed again by centrifugation and removal of the supernatant. 50% ethanol-washing step was repeated forth. All the supernatants from the washing step were joined into the tube T2 (glucose from all the digestible starch in one sample). 250 µL 2 M KOH was added to the residue (T1), to dissolve the residue for 20 min with gentle stirring in an ice water bath; at which point 1.2 M sodium acetate buffer (1 mL, pH 3.8) and amyloglucosidase (15 µL, 3300 U/mL) were added to stop the reaction. Samples were incubated in the water bath

at 50 °C for 30 min and centrifuged at 12,000*g* for 3 min. To 10 µL of the supernatant from T1 (RS) or T2 (digestible starch), 300 µL of glucose oxidase–peroxidase-aminoantipyrine (GOPOD, > 12,000 U/L glucose oxidase; > 650 U/L peroxidase; 0.4 mM 4-aminoantipyrin) were added respectively and the mixture was incubated in the water bath at 50 °C for 20 min. Absorbance was measured using a 96-well microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA) at 510 nm. Sodium acetate buffer (0.1 M, pH 4.5) was used as a blank, glucose with different concentrations (start from 500 mg/mL in 0.2% benzoic acid, end at 0.8 mg/mL) was tested as a standard curve. The glucose concentration of liquid in T1 or T2, named C1 or C2, was calculated depending on the standard curve.

The amount of titanium dioxide in intestinal samples was measured [24] with the following modification in the reaction system for fewer contents from the small intestine of laboratory animals. In brief, 200 mg of each digesta sample were weighed to porcelain crucibles for 1.5 h at 300 °C for the sake of carbide and followed with 13 h at 580 °C in order to get dry-ash. 4 mL H_2SO_4 (7.4 M) was added to each crucible upon cooling. All the samples were then gently boiled for approximately 50 min until completely dissolved. After cooling the solutions and wash water were poured quantitatively into a plastic tube. 8 mL H_2O_2 (30%) was added to each plastic tube and the contents were diluted to 12 mL with distilled water. Absorbance was measured using a 96-well microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA) at 410 nm. The mixture of 7.4 M sulphuric acid solution and hydrogen peroxide (30%) was used as a blank. Titanium dioxide with different concentrations (start from 50 µg/mL, end at 3.125 µg/mL) was tested as a standard curve. The analyses were performed in triplicate.

Calculations

The mass of digestible starch or resistant starch was determined as $V1 \times C1/0.9$ or $V2 \times C2/0.9$ (V1 or V2 represent the total volume of the liquid in T1 or T2) and titanium dioxide was determined as $12 \times C3$ [C3 represent titanium concentration in the sample solution (µg/mL)]. The mass of total starch was calculated as the mass of digestible starch plus that of resistant starch.

The quality rate of titanium dioxide and resistant starch was determined as Eq. 1:

$$R = \frac{M_1(\text{Titanium dioxide})/100\,\text{mg}}{M_2(\text{Resistant starch})/100\,\text{mg}} \quad (1)$$

The relative total starch was determined as Eq. 2:

$$\text{Relative total starch} = \frac{\text{Total starch}}{\text{Resistant starch}} \quad (2)$$

Percentage of total carbohydrates about the experimental group compared with the control group was determined as Eq. 3:

$$\text{Percentage of total carbohydrate \%} = 100 \times \frac{\text{TC/RS (Duodenum, Experimental group)}}{\text{TC/RS (Duodenum, Blank group)}} \quad (3)$$

Percentage of absorption of glucose was determined from the glucose/reference substance ratios at any intestinal segment as Eq. 4:

$$\text{Glucose absorption \%} = 100 \times \left[1 - \frac{\text{TC/RS (segment)}}{\text{TC/RS (Duodenum)}}\right] \quad (4)$$

Measurement of blood glucose levels

The animals were monitored for blood glucose changes during the last week of experiments. Blood glucose was

Table 3 Effect of sweeteners on body weight, food intake and drink consumption

Sweetness	Control	Acesulfame K			Saccharin		
		8	12	16	8	12	16
Body weight gain (g)	3.48 ± 0.17	3.59 ± 0.12	3.49 ± 0.06	3.72 ± 0.09	3.36 ± 0.11	3.52 ± 0.10	3.47 ± 0.10
Food intake (g)	4.56 ± 0.14	4.67 ± 0.12	4.57 ± 0.20	4.39 ± 0.07	4.30 ± 0.09	4.33 ± 0.14	4.15 ± 0.06
Drink consumption (mL)	4.72 ± 0.09	6.58 ± 0.10***	7.96 ± 0.11***	7.79 ± 0.18***	6.59 ± 0.13***	7.58 ± 0.03***	7.59 ± 0.10***

Data are expressed as Mean ± SD (n = 5 per group)

***$p < 0.001$ represents a significant difference when compared with the control group

measured directly in small samples of venous blood (tail nick) using a glucometer (Bayer) [25].

Results and discussions

Bodyweight, food intake and drink consumption

As showed in Table 3, there were no significant effects of sweeteners on body weight and food intake. Nevertheless, a significant increase in drink consumption ($p < 0.001$) was found in an animal group fed with acesulfame K or saccharin solution when compared to the control group which drinks water only.

Optimization of RS measurement

The determination of RS was based on a Megazyme assay kit (find more details in the methods mentioned above). For each test, one kilogram sample is needed as the manufactory's recommendation. In this study, we try to investigate the contents of the small intestine of murine (small animals) in the laboratory on large scales. Thus, some amendments for the method were made: first, the sample weight was reduced to 100 mg (10% of the recommendation) for each test while the reaction buffer system was also decreased to 10% to maintain the detestability of the trace RS in the small intestine of small animals; second, all the reactions were done in a 1.5 mL tube. To improve the reaction efficiency, a stainless steel bead was added into the tube, shaking with the content sample and buffers; third, to maintain the accuracy of measurement, the 50% ethanol-washing step was repeated forth to remove trace of glucose contamination from the hydrolysis of digestible starch. These amendments ensured that in the majority of cases, the determination of RS content of samples from the intestine of small laboratory animals was accurate and high-throughput. Compared with titanium dioxide, the analysis method of resistant starch can be easily detected, and more samples can be operated at one time.

Table 4 The measurement value of different content of resistant starch in normal feed or synthetic feeds

Diet number	Theoretical value of RS added/100 mg	Measure value/100 mg
No.1 feed	–	0.69 ± 0.074 mg
No.2 feed	0.2 mg	0.93 ± 0.068 mg
No.3 feed	0.4 mg	1.12 ± 0.057 mg
No.4 feed	0.6 mg	1.37 ± 0.045 mg
No.5 feed	0.8 mg	1.45 ± 0.072 mg
No.6 feed	1.0 mg	1.58 ± 0.059 mg

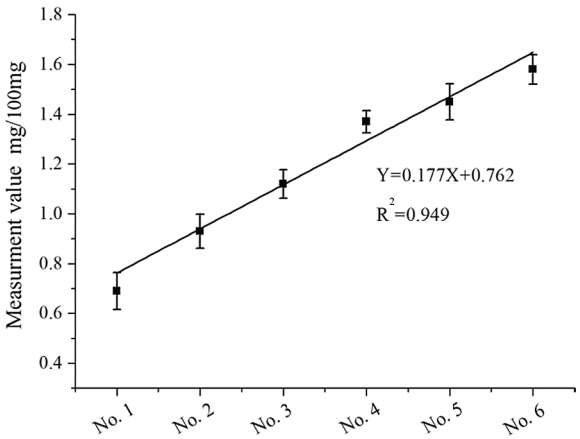

Fig. 1 The fitting curve of different contents of resistant starch in normal and synthetic feeds

Optimization of the addition of RS as an inert marker in feed

To evaluate the proper additive value of RS, six synthesis diets with different amounts of high-amylose corn starch added (No.1–No.6 as shown in Table 1) were used. It was shown that the content of resistant starch was about 0.69% in No.1 feed without any addition of high-amylose corn starch. The measurement values of resistant starch in other synthetic feeds were shown in (Table 4). The fitting curve of measurement values of resistant starch in No.1–No.6 feeds were shown in (Fig. 1) and the judgment coefficient of the goodness of fit (R^2) is 0.949. The purpose of this experiment proved that resistant starch in ordinary feed (0.69% RS) can be measured with the optimized method which from supporting the resistant starch has the potential of becoming a marker that no exogenous addition is required. This result implied that the amount of endogenous RS in the No.1 feed was enough for precise detecting when using RS as a potential inert marker.

Comparison of RS and TiO_2 as an inert marker in rats

SD rats feeding with No.1 feed were used in this assay, the same segment of small intestine content sample was analysed to evaluate the amount of resistant starch and titanium dioxide. It showed that the trend of resistant starch change is similar to that of titanium dioxide in ten different positions distributing along the small intestine in orders (Fig. 2). The ratio (TiO_2/RS) was approximately 0.8 (Fig. 2).

It was shown that there was a minor difference between the curves of measurement at the position of jejunum; however, a repeatable result was achieved when using resistant starch or titanium dioxide as an inert marker

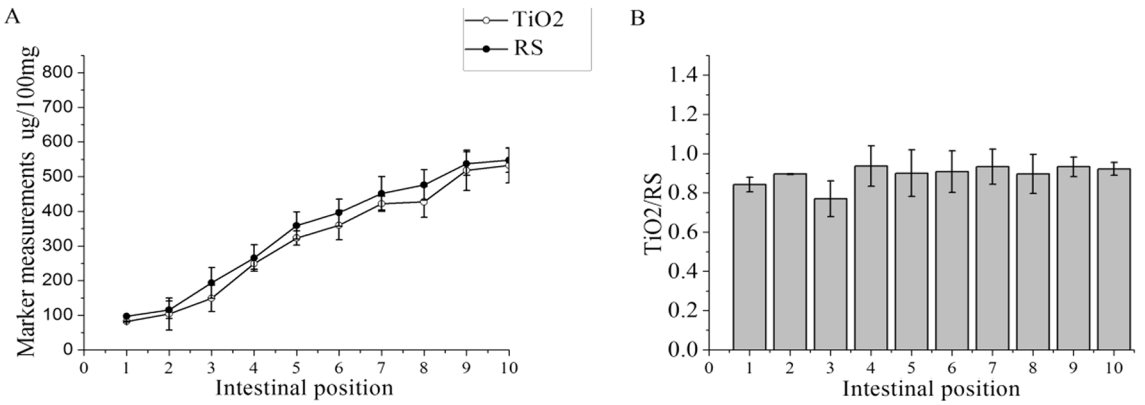

Fig. 2 Comparison of two inert markers. The amount of two markers of SD rat small intestine contents in different positions was detected (**A**) and the ratio between RS and TiO_2 calculated by the formula mention above was shown (**B**). *$p < 0.05$*

to correct the relative mass of carbohydrate in intestinal samples. In our study, the dispersion and propulsion features of resistant starch was shown to be similar to titanium dioxide in small intestine of murine, which means RS could be used as a novel inert marker as well as titanium dioxide.

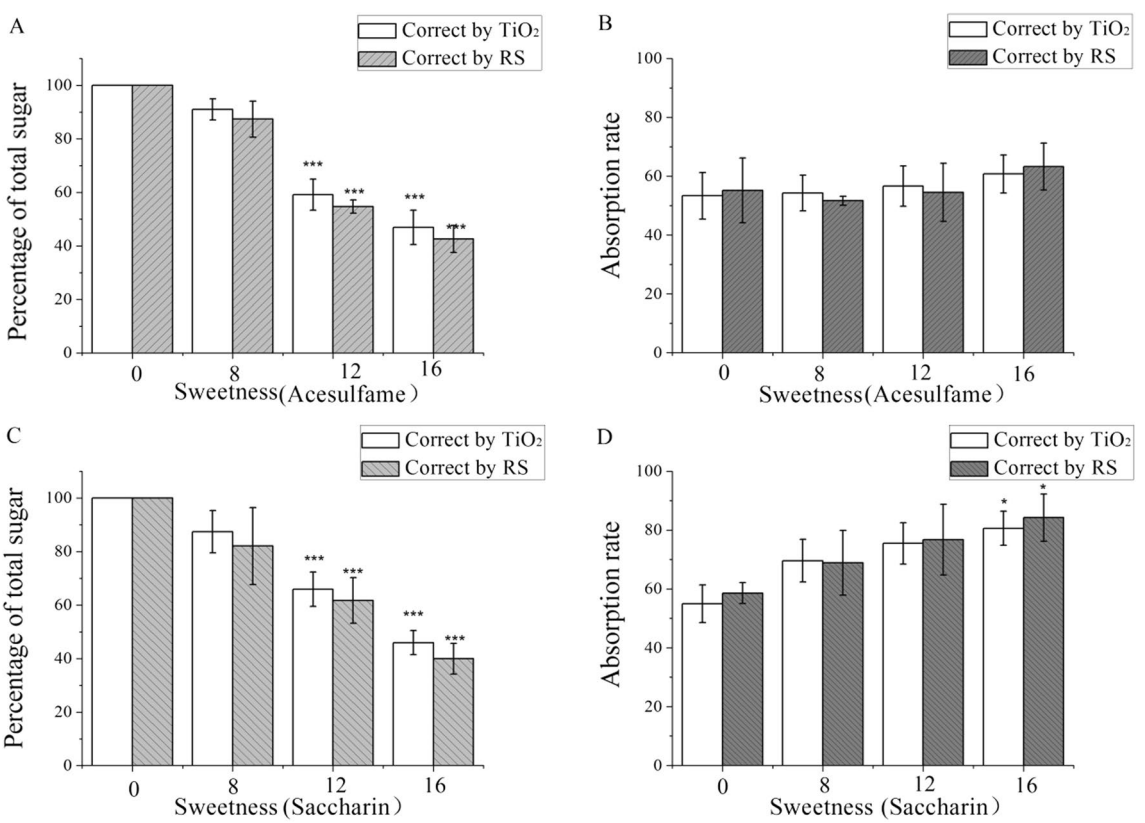

Fig. 3 Using TiO_2 or RS as the inert marker respectively, the percentage of total carbohydrates in the duodenum (**A**) and absorption rate in upper jejunum (**B**) were calculated as the formula mention above, based on the data achieved from the intestinal samples of acesulfame K exposed mice. The same experiment was carried out using saccharin as the stimuli and was indicated (**C** and **D**). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$*

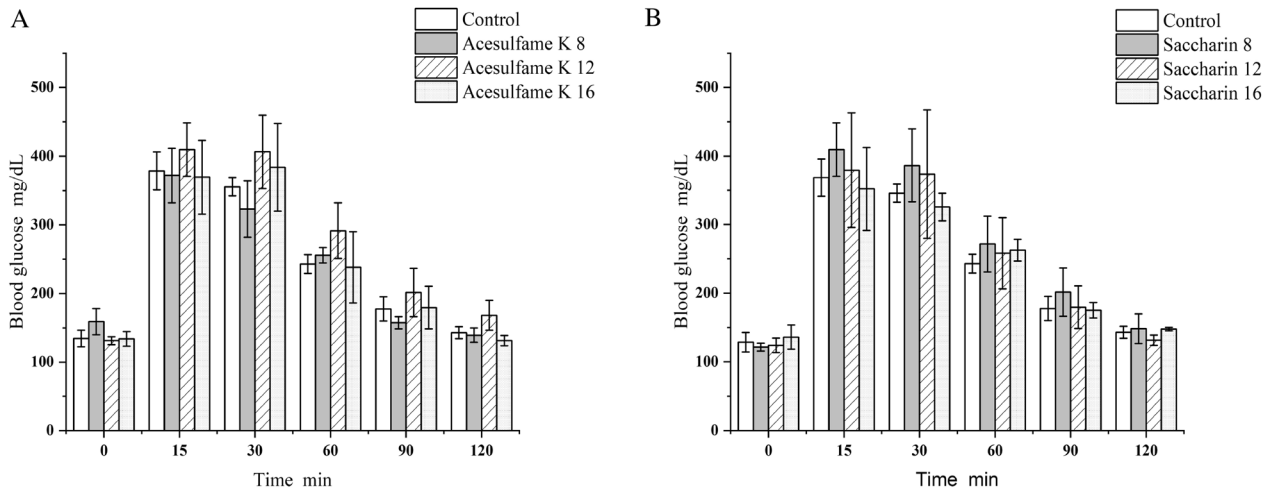

Fig. 4 The plasma glucose level of acesulfame K-drunk mice (**A**) and saccharin-drunk mice (**B**) at different time intervals during OGTT

Glucose absorption rate of small intestine in murine

The purpose of this trial was to determine whether the novel inert marker RS and optimized method work well in ICR mice. The intestinal contents of sweetener (acesulfame K and saccharin) exposed mice were prepared. The relative percentage of total carbohydrates in the duodenum and the absorption rate of the segments between the duodenum and upper jejunum were calculated to evaluate the change induced by sweetener exposure.

The amount of titanium dioxide in the contents samples was detected as well as that of RS (Fig. 3). The results showed that no matter which inert marker was used to correct the value achieved, the calculated relative percentage and the absorption rate is similar. In particular, the significant change between the sweetness 12 and 16 groups compared to the sweetness 0 group was repeated when using different inert markers. It was worth noting that the standard deviation of the data normalized by the inert marker RS was slightly larger than that of titanium dioxide, which indicated that the measurement of RS is more variable. These results showed that titanium dioxide is more suitable for experiments that demanding quantitation with high resolution. However, in some semi-quantitative experiments, the resistant starch is an inert marker that was easy to be detected and high-throughput.

Results of sweeteners exposed ICR mice indicated that the relative percentage of total carbohydrates in the duodenum of high sweetness exposed mice significantly declined compared to that of the water-exposed group. The relative percentage of total carbohydrates in the duodenum of sweetness 12 and 16 of acesulfame K exposed mice were approximately ~ 50% and ~ 35% of the water-exposed group, The relative percentage of total carbohydrates in the duodenum of sweetness 12 an 16 of saccharin exposed mice were approximate ~ 55% and ~ 40% of the water-exposed group. In a word, artificial sweeteners significantly increased total sugar absorption in the duodenum of 1 week exposed mice. It was worth noting that in the segment of the duodenum the absorption of carbohydrates increased with the sweeteners of increasing sweetness. But in the upper jejunum, the absorption of glucose had no significant change no matter in acesulfame K exposed mice or saccharin exposed mice. The use of artificial sweeteners can lead to metabolic syndrome such as obesity and type 2 diabetes, but its mechanism has not been consistently approval. Collectively, our results implied that the increasing glucose absorption of the small intestine was enhanced by the artificial sweeteners exposed, which maybe correlates to some metabolic syndrome.

Plasma glucose levels

The plasma glucose levels of experimental animals were observed by OGTT. There was no significant change in glucose tolerance after short-term exposure to artificial sweeteners (Fig. 4). This is different from previous studies [25, 26]. The possible reason is that the blood glucose shown in this experiment is a short-term experimental result, while the change of glucose tolerance caused by sweetener is a phenomenon caused by long-term accumulation. It's worth noting that the determination method of glucose absorption described in this study was designed just for the small intestine, but not applicable for the large intestine where more microorganisms lived, further more, amylose could affect insulin and glucose metabolism in human and mice in a long or short term administration [27, 28].

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Authors' contributions
Conceptualization, YW, QS, HJ and SC; data curation, YW, QS, HJ, SY, XX, XG and JD; funding acquisition, YW, QS, LC and SC; investigation, LC, MS and SC; methodology, QC, LC, SC, SC and HJ; project administration, SC; supervision, LC and SC; writing—original draft, YW, QS, HJ and SC. All authors read and approved the final manuscript.

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Availability of data and materials
The data presented in this study are available in article. The Sprague Dawley (SD) rats and ICR mice were obtained from the SLAC Laboratory Animal Co. LTD (Shanghai, China). The high-amylose corn starch (Novelose330) was purchased from National Starch & Chemical Company, USA.

Declarations

Competing interests
There is no competing interest within this article.

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