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Postprandial Glucose, Insulin and Incretin Responses Differ by Test Meal Macronutrient Ingestion Sequence (PATTERN Study)

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Running Head: Food intake sequence and glycemcic, insulinemic and incretin responses

The trial was registered at clinicaltrials.gov as NCT03533738

1 Abstract

2 **Background:** Previous studies have shown that the sequential order of consuming different food
3 components significantly impacts postprandial glucose and insulin excursions in prediabetes and
4 type 2 diabetes, but the causative mechanisms in healthy humans remain ill-defined.

5 **Objective:** Using a typical Asian meal comprising vegetables, protein (chicken breast), and
6 carbohydrate (white rice), the aim of this study was to examine the effect of food intake
7 sequence on postprandial glucose, insulin and incretin secretions in healthy adults.

8 **Design:** Sixteen healthy Chinese adults participated in a randomized, controlled, crossover meal
9 trial. Subjects consumed in random order 5 experimental isocaloric meals that differed in the
10 food intake sequence of vegetables, protein and carbohydrate. Glucose, insulin, incretins and
11 satiety markers were measured over 3 h.

12 **Results:** There were significant food intake sequence x time interaction effects on plasma
13 glucose, insulin, glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-
14 1 (GLP-1) concentrations ($P<0.001$). In comparison with rice consumed first followed by
15 vegetable and meat (R-VM), the overall postprandial glucose response was significantly
16 attenuated after the food intake sequence of vegetable first, followed by meat and rice (V-MR) or
17 meat first, followed by vegetable and rice (M-VR) or vegetable first followed by meat and rice
18 (V-M-R) or vegetable, meat and rice consumed together (VMR). The insulin iAUC (0-60) was
19 significant lower after V-M-R than M-VR, VMR and R-VM. V-M-R food intake sequence
20 stimulated higher GLP-1 release than other meal sequences. However, GIP response was lower
21 after V-MR and V-M-R than M-VR and R-MR food intake sequences.

Conclusions: Food macronutrient intake sequence can considerably influence its glycemic, insulinemic and incretin responses. V-M-R food intake sequence attenuates the glycemic response to a greater degree with accentuated GLP-1 stimulation without any increased demand for insulin. The sequence of food intake has great potential as a novel and simple behavioral strategy to modulate glycemic response in healthy adults.

Keywords: food intake sequence, glycemic response, insulinemic response, incretin, healthy subjects

41 INTRODUCTION

42 In recent years, the prevalence of diabetes in the Asian population has increased and evidence
43 has indicated that the risk of diabetes in Asians may be higher than that of other racial groups
44 such as Caucasians of the same body mass index [1]. One of the main reasons purported for the
45 increasing diabetes prevalence is due to the use of white rice as a form of staple food. White rice
46 has been demonstrated and classified as a high glycemic index (GI) food, especially in Asian
47 (Chinese and Japanese) populations [2]. It is now widely recognized that diet plays a critical role
48 in the etiology and management of the chronic diseases, especially diabetes and obesity. The
49 Asian diets are characterized by rice being the main staple and are consumed as part of a mixed
50 meal that consists of a mixture of protein, fat and dietary fiber. Examining the impact of mixed
51 meals on glycemic responses of rice is important, as any effective method that blunts glycemic
52 excursions deserves to be advocated to the public. Previous studies have demonstrated that the
53 glycemic responses can be manipulated when eaten as mixed meals due to the differential impact
54 of the macronutrients and fiber content of the meal as a whole [3-6].

55
56 Recent research has focused on the preload presented before a main meal is consumed to reduce
57 the glycemic response induced by carbohydrates. An earlier study has shown that ingestion of fat
58 (olive oil) 30 min before a carbohydrate meal markedly slows gastric emptying and attenuates
59 the postprandial rises in glucose, insulin, and GIP, but stimulates GLP-1, in type 2 diabetes [7].
60 A recent study reported that preloads with milk proteins 30 minutes prior to bread significantly
61 lowered postprandial glycemia and insulinemia when compared to co-ingestion [8]. However, as
62 such preloads may increase overall energy intake, it would be a strategic and cost-effective
63 advantage if we can formulate simple dietary interventions capable of attenuating glycemic

responses. One promising approach is to optimize the sequence of ingestion of macronutrients during meals. However, few studies have attempted to examine the possible effects of meal sequence on glycemic and insulinemetic responses. A study done on Japanese patients with type 2 diabetes over a 24-month period has reported that a simple meal plan of ‘eating vegetables before carbohydrate’ achieved better glycemic control than an exchange-based meal plan [9]. One recent study investigated the effects of meat proteins before rice in type 2 diabetes patients and healthy controls. The findings lend credence that meal sequence can be an important regulator of gastric emptying and postprandial glucose elevation mediated through GLP-1 and glucagon secretions [10].

The comparison among the food intake sequence including vegetable, protein source and carbohydrate is limited especially using typical Asian diets. In our present study, we aimed to compare the effects of vegetables (a type colloquially termed xiao bai chye) or/and protein (chicken) before and after rice intake, in a typical Asian mixed meal, on postprandial glucose excursion, insulin and incretin secretion in healthy Chinese adults.

SUBJECTS AND METHODS

Subjects

Sixteen healthy participants [13 male and 3 female; mean ($\pm$ SD) age: 25.8 ± 4.8 y (range: 21-38); body mass index (in kg/m^2): 22.0 ± 2.0 (range: 18.7-24.8)] of Chinese ethnic background were recruited for the study by means of advertisements and personal communications. At screening, a health assessment was performed and a health questionnaire requiring potential participants to declare food allergies/intolerance/restriction, chronic diseases and smoking habits

was administered. Those who fulfilled all the inclusion criteria [body mass index (BMI) between 18 to 25 kg/m²; blood pressure (BP) – systolic BP less than 140 mmHg and diastolic BP less than 90 mmHg; 21 to 40 years; fasting blood glucose less than 6.0 mmol/L; not on prescription medication, non-smoking, no genetic or metabolic diseases] were enrolled into the study. The females enrolled attended all the test sessions during the luteal phase of the menstrual cycle.

The study was conducted at the Clinical Nutrition Research Centre (CNRC), Singapore Institute for Clinical Sciences (SICS), Singapore from November 2017 to March 2018, according to the guidelines laid down in the Declaration of Helsinki. All procedures involving human participants were approved by the Domain Specific Review Board of National Healthcare Group (2017/00742). Written informed consent was obtained from all eligible participants before participation.

Study design

This study was a randomized, crossover, non-blinded design consisting of five sessions separated by a 1-week washout period to minimize any carryover effects. Participants were also asked to refrain from any unusual exercise and activity the night before the study sessions. All test sessions lasted approximately 4 hours and were identical in all respects except for the sequence in which the test meal was consumed. At each session, participants arrived at the CNRC laboratory at 8:30 am after an overnight fast of 10 to 12 hours. Upon arrival at the laboratory, participants were first allowed to rest for 10 min. Baseline capillary blood samples were taken by finger prick using the Accu-Chek, sterile, single-use, lancing device (Abbott) to measure glucose. Following the finger-prick blood glucose measurement, an indwelling intravenous cannula was inserted into a forearm vein by a phlebotomy-trained state registered nurse and a

baseline blood sample was obtained. Subsequently, participants consumed the test meal in different order at a comfortable pace and finished it within 15 min. Finger-prick capillary and venous blood samples were collected at 15, 30, 45, 60, 90, 120, 150 and 180 min intervals following the start of the meal. Appetite ratings were also measured using validated visual analog scales (VAS) to record their subjective feelings of hunger, fullness, desire to eat and prospective food consumption at similar time points. Blood glucose concentration was measured using the HemoCue Glucose 201 analyzer (HemoCue AB, Angelholm Sweden). 250 ml water was supplied during the test meal and the study session. An ad libitum lunch was served in a climate-controlled dining room (ambient temperature: $22 \pm 1^{\circ}\text{C}$). To minimize the effects of social interactions on the quantity of food consumed, subjects ate alone until comfortably full. Quantity of food consumed (evaluated after each subject finished and left) was only revealed after the study to avoid biasing the quantity eaten if this was made known at the outset. **Supplemental Figure 1** shows subject enrollment and the final sample size after excluding the screen failures and withdrawn subjects.

Test meal sequence

Participants were served the same test meal to be consumed in different sequences in the morning on five separate days in a randomized order. The 5 test meal food intake sequences were as followed: (1) vegetables first before meat and rice (V-MR), (2) meat first before vegetables and rice (M-VR), (3) vegetables first, meat second before rice (V-M-R), (4) vegetables, meat and rice together (VMR), (5) rice before vegetables and meat (R-VM). The rice used was Thai Hom Mali fragrant rice (Double FP, Singapore) and was cooked using an electric rice cooker (SONA, Model SRC 2067). Each rice portion (63.2 g of raw rice / 50 g of available

carbohydrate) was cooked individually using 180 mL of water. The chicken used was breast meat without skin (Pasar brand, NTUC Fairprice, Singapore) and served in a portion size of 100 g. The chicken was cooked by steaming in an aluminum foil wrapper after it was cut into 0.5 cm cubes. A saucepan of water was brought to the boil on an induction cooker, and the steamer with the chicken portions was placed on top to cook for exactly 10 min. The leafy vegetables (Xiao Bai Chye, Pasar NTUC Fairprice, Singapore) was served in a portion of 180 g and boiled in the water for 3 min. Compositional information was obtained from the food suppliers. The nutrient composition of the test meal is shown in **Supplemental Table 1**. The test meals were freshly prepared in the morning of the test days and served to the participants within 30 min of preparation.

Blood glucose and insulin analysis

Capillary blood was obtained by finger prick using the Accu-Chek sterile, single-use lancing device (Roche, Germany). Before a finger prick, subjects were encouraged to warm their hand to increase blood flow. To minimize plasma dilution, fingertips were not squeezed to extract blood but were instead gently massaged starting from the base of the hand moving towards the tips. The first two drops of expressed blood were discarded, and the next drop was used for testing. Blood glucose was measured using the HemoCue Glucose 201 analyzer (HemoCue AB, Angelholm Sweden). Venous blood samples collected on the test days were centrifuged at 1500 g for 10 minutes at 4 °C, and serum was aliquoted and stored at –80 °C until being analyzed for insulin concentrations later. Serum insulin concentrations were determined using a Cobas e411 (Roche, Hitachi, USA), where the intra- and inter-assay CVs were <5% and <6% respectively.

Incretin hormones analysis

Blood samples for plasma total GLP-1, GIP and active ghrelin were collected in 3 mL of ice-chilled EDTA-treated tubes containing ¼ tablet of protease inhibitor and 30 µL Dipeptidyl Peptidase IV (DPP-4) inhibitor (Merck, Millipore). Total plasma GLP-1 was measured by ELISA (Merck, Millipore) and both of the intra and inter-assay CVs were below 15%. Total plasma GIP and active ghrelin was measured by Luminex (Merck, Millipore)

Data processing and statistical analysis

The iAUC was calculated by using the trapezoidal rule [11] for glucose, insulin, total GLP1, GIP and active ghrelin concentrations. All areas below baseline were excluded from the calculations. All analysis were performed with SPSS software (version 23.0; SPSS Inc). Two factor repeated-measures analysis of variance (ANOVA) were performed to analyze the effects of treatment, time and their interaction on outcome variables measured over the study period, including blood glucose, insulin, total GLP-1, GIP and active ghrelin responses and satiety markers. When a treatment and time interaction was statistically significant, one-factor ANOVA with general linear model, followed by Bonferroni's post-hoc tests for multiple comparison to investigate the effect of treatment and each time point measurement. Previously published studies of the analysis of glycemic response in humans have been based on only 12 subjects, as reviewed by the FAO/WHO [12] to take into account the inter-individual variations, and this number is comparable to that used in similar study[13]. Hence, a sample size of 16 ought to be adequately powered to detect the outcome differences in the present study. P values <0.05 was considered statistically significant. Values are presented as mean ± SEMs unless otherwise indicated.

RESULTS

Sixteen healthy Chinese adults (13 male, 3 female) completed the study. The participants consumed the test meals without any reluctance; overall liking did not differ among test meals sequence.

Postprandial plasma glucose response

There were no significant differences in the fasting concentrations of glucose among the five different experimental days. The postprandial glucose responses to the 5 food intake sequences are shown in **Figure.1**. There were significant time effects ($P<0.001$), treatment effects ($P<0.001$) and interaction effects ($P<0.001$). There was a significant increase in blood glucose after the M-VR, VMR and R-VM food intake sequences, but not after V-MR and V-M-R at 15 min compared with baseline. Postprandial glucose levels were significantly lower at 15, 30, and 45 min following the V-M-R food intake sequence, compared with VMR and R-MR. The V-MR and M-VR food intake sequences also showed reduced postprandial glucose levels at 15, 30 and 45 min compared with R-VM. The mean amplitude of glycemic excursions, incremental glucose peak, iAUC 0-1 h and iAUC 0-2 h were significant lower after V-MR, M-VR, V-M-R and VMR food intake sequences compared with R-VM. The M-VR food intake sequence also showed reduced overall glucose characteristic compared with VMR (Table 3). There was no significant difference among the V-MR, M-VR and V-M-R food intake sequences. The absolute glucose concentration to five different food intake sequences over 180 min was shown in **Supplemental Figure 2**.

Postprandial serum insulin response

There were no significant differences in the fasting concentrations of insulin among the five different experimental days. The postprandial insulin responses to the 5 food intake sequences are shown in **Figure.2**. There were significant time effects ($P<0.001$), treatment effects ($P=0.006$) and interaction effects ($P<0.001$). There was an increase in serum insulin immediately after the M-VR, VMR and R-VM food intake sequences but not after V-MR and V-M-R. There were significant treatment effects at time 15, 30 and 45 min. Insulin concentration was significantly higher after R-VM, VMR and M-VR food intake sequences compared with V-MR and V-M-R at the 15 min time-point. Serum insulin concentration was significant lower after V-MR and V-M-R food intake sequences compared with R-VM at the 30 min time-point. The M-VR food intake sequence induced higher serum insulin response compared with V-MR and V-M-R at the 45 min time-point. There were significant treatment effects on the mean amplitude of insulineric excursions, incremental insulin peak, iAUC 0-1 h and iAUC 0-2 h. However, after the 5 treatments were adjusted by Bonferroni's corrections, R-MR, VMR and M-VR food intake sequences induced significant higher insulin iAUC 1h compared with V-M-R. M-VR iAUC 1h was higher than V-MR (Table 4). The absolute insulin concentration to five different food intake sequences over 180 min was shown in **Supplemental Figure 3**.

Postprandial plasma GLP-1 response

There were no significant differences in the fasting concentrations of GLP-1 among the five different experimental days. The postprandial GLP-1 responses to the 5 food intake sequences are shown in **Figure 3**. There were significant time effects ($P<0.001$), treatment effects ($P<0.001$) and interaction effects ($P<0.001$). There was an increase plasma GLP-1 immediately after all the 5 food intake sequences. When compared with V-M-R, GLP-1 concentrations were significantly lower after M-VR, VMR and R-VM at 60 min, whereas GLP-1 concentration was

higher at the end of the study after the R-VM food intake sequence than M-VR. There were significant treatment effects on the overall iAUCs for plasma GLP-1, such that plasma GLP-1 was greater after V-M-R food intake sequence than VMR (Table 3). The absolute GLP-1 concentration to five different food intake sequences over 180 min was shown in **Supplemental Figure 4**.

Postprandial plasma GIP response

There were no significant differences in the fasting concentrations of GIP among the five different experimental days. The postprandial GIP responses to the 5 food intake sequences are shown in **Figure 4**. There were significant effects for time ($P<0.001$) and treatment $\times$ time interaction effects ($P<0.001$), but no treatment effects ($P=0.461$). There was a prompt increase in plasma GIP after M-VR, VMR and R-VM food intake sequences, whereas GIP concentration after V-MR and V-M-R food intake sequence were unchanged in the first 30 min compared with fasting concentration. There were significant treatment effects on the overall iAUC 1 h ($P<0.001$) and iAUC 2 h ($P=0.013$) for plasma GIP, whereas no significant difference was found for the overall iAUC 3 h ($P=0.287$). Plasma GIP iAUC 1h was greater after R-VM and M-VR food intake sequences compared with V-MR, whereas R-VM stimulated higher GIP than V-M-R and VMR food intake sequences. M-VR food intake sequence stimulated higher GIP overall iAUC 2 h than V-MR ($P=0.039$). The absolute GIP concentration to five different food intake sequences over 180 min was shown in **Supplemental Figure 5**.

Postprandial active ghrelin response and appetite sensations

There were no significant differences in the fasting concentrations of active ghrelin among the five different experimental days. For active ghrelin, there were no significant effects for time

($P=0.105$), treatment ($P=0.172$) and treatment x time interaction effects ($P=0.068$) (**Figure 5**).

There was slightly reduction in ghrelin concentration following all the food intake sequences except R-VM in overall 3 h compared to the baseline as shown in Figure 5. However, there was slightly increase in ghrelin concentration after R-VM food intake sequence. Over the 3 h periods, the iAUC for ghrelin did not differ significantly among the five food intake sequences. The absolute active ghrelin concentration to five different food intake sequences over 180 min was shown in **Supplemental Figure 6**.

There were no differences in hunger, fullness, desire to eat, or prospective consumption among all the five food intake sequences and also no significant difference was found for the buffet lunch consumption (**Supplementary Table 2**).

DISCUSSION

In this study, we demonstrate that, in healthy adults, the order of food consumption with rice based meal significantly impacts postprandial glucose, insulin and incretin response. Compared with rice first, followed by vegetable and meat (R-VM) food intake sequence, all the other food intake sequences (V-MR, M-VR, V-M-R and VMR) attenuated postprandial glucose. Vegetable consumed first followed by meat and rice (V-MR) and vegetable first, followed by meat and followed by rice (V-M-R) food intake sequences induced a similar slow initial elevation in glucose concentrations, followed by a slow secretion of insulin and GIP and a higher GLP-1 secretion. However, meat first, followed by vegetable and rice (M-VR) induced a rapid secretion of insulin, GLP-1 and GIP and had an attenuated glucose response. The findings in the present study indicate that eating vegetables, protein before carbohydrate present a simple and novel

eating strategy to attenuate postprandial glycemic response without any compensation of insulin secretion in healthy subjects.

Postprandial glycemia is a significant risk factor for diabetes and cardiovascular disease [14, 15]. Postprandial glucose spikes are more strongly associated with atherosclerosis than fasting plasma glucose or HbA1c level [16]. Many factors may influence the postprandial glucose excursion and digestion/absorption of carbohydrate in the small intestine. These include the rate of digestion, type of carbohydrate, meal composition, meal size et al. In our previous study, we reported that co-ingesting chicken, oil or vegetable with white rice considerably influences its glycemic and insulinemic responses. Co-ingesting white rice with all three components attenuates the glycemic response to a greater degree than when it is eaten with any single one of them, and that this is not at the cost of an increased demand for insulin[17]. In the present study, we replicated the typical mixed chicken rice meal incorporating vegetables, protein (chicken breast) and white rice to compare the effects of food intake sequence on postprandial glucose response. The current results show that co-consumption of vegetable, meat and rice (VMR) induced a relative higher glycemic, insulinemic and GIP response, but lower GLP-1 response compared with V-MR and V-M-R. Combined with previous results, we found that although consumption of vegetable and protein with carbohydrate together attenuated glycemic and insulinemic responses, manipulating food intake sequence (eating vegetable before protein and carbohydrate or eating vegetable, followed by protein and then carbohydrate) exert even better effects on postprandial glycemic control in healthy adults.

In our present study, we found that V-MR and V-M-R food intake sequences induced a similar attenuated glucose and insulin response compared with the R-VM and VMR sequences. There are large spikes in glucose and insulin responses in less than an hour following R-VM and VMR food intake sequences, whereas no such changes were seen with V-MR and V-M-R. Previous studies demonstrated that eating “vegetables before carbohydrate” attenuate the postprandial glucose and insulin levels in type 2 diabetes participants and normal subjects [18] and the long term glycemic improvement in patients with Type 2 diabetes [19]. The vegetables used in the previous study [20] was 500 g and it contained 21 g of dietary fiber which exceeded the amount proposed for our present study (180 g, around 2 g dietary fiber). This suggested that even a small preload of vegetables when consumed prior to carbohydrate attenuated postprandial glycemic and insulineremic responses in healthy participants. Dietary fibers have the effects of hampering the diffusion of glucose and postponing the absorption and digestion of carbohydrates, thus resulting in lowered postprandial blood glucose[21]. Previous studies proved that the consumption of protein and vegetables first, followed by carbohydrate, reduced both postprandial glucose and insulin responses in type 2 diabetes [22]. These results indicated that dietary carbohydrate consumed after vegetables were digested slowly and required less insulin for subsequent metabolic disposal.

It is well established that co-ingestion of protein (concentrate/hydrolysate) or amino acids with carbohydrate lowers the postprandial glycemic response compared to carbohydrate alone in both healthy and type 2 diabetes subjects [23-26]. Protein and amino acid ingestion can stimulate postprandial insulin secretion and, as such, can manipulate the postprandial glycemia [27]. Recently, there has been an increasing interest in the use of “preload” to reduce postprandial

glycemia. The concept of preload generally describes the ingestion of a small amount of macronutrient administered a short interval before an actual full meal, generally within an hour. This allows the preload to increase insulin secretion and reduce the rate of gastric emptying in advance of the main nutrient load, by stimulating the release of incretins (GIP and GLP-1) and other gut hormones (e.g. CCK) [28]. Recent publications have demonstrated that a small amount of protein was able to blunt the glycemic excursion to a greater extent when given before rather than consumed together with a carbohydrate meal [29, 30]. Consistent with previous studies, we also demonstrated that preloading either soy milk or dairy milk results in greater reduction in glycemic and insulinemic responses compared to co-ingestion alone [31]. However, in our current study, we found that the M-VR food intake sequence induced a lower glycemic response associated with greater insulin secretion compared with V-M-R and V-MR. Modifying the rate of nutrient absorption is a therapeutic approach for diabetes. A possible explanation for the attenuated glycemic and insulinemic responses observed with the V-M-R and V-MR food intake sequences is delayed gastric emptying and consequently slower rates of carbohydrate digestion and glucose absorption, which suggested the critical role of vegetable fibers in moderating lower glycemic and insulinemic responses. Unfortunately, gastric emptying rate was not measured in the current study.

It was recently shown that vegetables first over 10 min, and 10 min later, followed by protein and carbohydrate together over 10 min which is similar as V-MR food intake sequence in our current study but with different amount of macronutrients composition attenuated postprandial glucose response and requiring less insulin compared with the carbohydrate first, followed protein and vegetables together in prediabetes[32]. The food order results was consistent with

our current study. Unfortunately neither gastric empty nor incretin hormones were measured in the previous study.

The hormones GLP-1 and GIP were shown to be potent determinants of the postprandial insulin secretion and thus play an essential role in the regulation of postprandial glycemia [33]. Infusion of GLP-1 delays the gastric emptying rate and attenuates blood glucose [34]. The significantly higher GLP-1 response was clearly attained after the V-M-R food intake sequence. Surprisingly, the increase in GLP-1 concentrations after V-M-R seen in this study did not stimulate insulin release. We did not observe stimulatory effects of GLP-1 on postprandial insulin release after V-M-R food intake sequence which was consistent with other studies [35, 36]. GLP-1 infusion in a lower rate, decreased postprandial glycemia and insulinemic responses explained by a slowing of gastric emptying in healthy subjects [37]. Hitoshi et al [38] reported preload fish or meat 15 min before rice enhanced GLP-1 secretion and delayed gastric emptying, but was not correlated with changes of insulin secretion. However, M-VR food intake sequence induced a higher insulin response and enhanced GLP-1 secretion in our current study which suggested vegetable consumed first or not may play an important role in the incretin secretion. Controlled studies in humans on fiber consumption on the responses of these incretin hormones are scanty and discrepant. A dose of 1.7 g psyllium did not evoke measurable effects on gastric emptying and postprandial GLP-1[39]. On the other hand, M-VR, VMR or R-VM promoted GIP secretion but V-MR and V-M-R did not. GIP is known to be an incretin which stimulates glucose-dependent insulin secretion [40]. GIP response after different food intake sequence was similar with insulin response in our current study. Higher GIP promotes high fat diet-induced fat accumulation[41], suggesting that M-VR, VMR especially R-VM chronically could result in increased fat

accumulation and increased insulin resistance. Hence the chronic effects of protein preload prior to carbohydrate on the therapeutic of diabetes needs be extensively examined in future. Ghrelin is a hunger hormone which potently stimulates appetite and quantity of food intake in humans[42]. We did not observe any significant difference in ghrelin level and quantity of food consumed among the different food intake sequences. It seems ghrelin level did not decrease immediate after R-VM food intake sequence and its level is much reduced in M-VR. Food intake sequence effects on satiety and quantity of food consumed needs further investigation.

Potential limitations in our present study are the uneven distribution of male and female subjects and the lack of measurement of gastric emptying rate. Due to the difficulty in venous cannulation for female subjects, some female subjects dropped out during the study. A fairly small change in the gastric emptying rate is shown to affect the magnitude and timing of postprandial blood glucose. The underlying mechanism of how the sequence of food intake affects postprandial glycemic response remains largely unknown, however the detection of an increased GLP-1 level can be an important contributing factor for the blood glucose lowering effect of V-M-R food intake sequence. Remaining questions include how V-M-R food intake sequence impacts the gastric emptying rate. The stringent study design and the type, amount of meals and absence of intervals among the individual macronutrients simulated the real typical Asian eating behavior and represented the strengths of our study.

In conclusion, this study shows that ingestion of vegetables, followed by protein and then carbohydrate attenuated postprandial glycemic excursion, insulin secretion and GIP

concentration and increased GLP-1 concentrations but had no effect on satiety and ghrelin concentration in healthy subjects. The sequence of food intake presents a novel, simple gustatory strategy to attenuate postprandial glycemic response in healthy subjects. Our findings provide a simple but effective way to reduce postprandial glucose and prevent type 2 diabetes. Such studies are important in translating science into practice necessary for consumers to include food intake sequence as a strategy to reduce the risk of developing diabetes.

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Authors' contributions

The authors' responsibilities were as follows: SLJ, ML and JH contributed to the design of the study; SLJ, GHJ and PG recruited the subjects and carried out the study; SLJ and GHJ wrote the first draft of the manuscript; ML and JH critically revised the manuscript. All authors read and approved the final manuscript. None of the authors had any conflicts of interest.

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Table 1. Characteristics of glycemic excursion in healthy subjects ¹

	V-MR	M-VR	V-M-R	VMR	R-VM	P value
Mean amplitude of glycemic excursions (mmol/L)	6.98 ± 0.26 *	6.81 ± 0.19 * #	6.80 ± 0.28 *	7.49 ± 0.24 *	8.31 ± 0.27	<0.001
Incremental glucose peak (mmol/L)	2.33 ± 0.27 *	2.19 ± 0.21 *#	2.16 ± 0.32 *	2.74 ± 0.23 *	3.69 ± 0.25	<0.001
iAUC 0-1 h (mmol/L x min)	68.65 ± 8.85 *	69.69 ± 8.44 * #	46.27 ± 8.87 * #	98.63 ± 9.49 *	135.14 ± 7.29	<0.001
iAUC 0-2 h (mmol/L x min)	150.74 ± 18.96 *	127.08 ± 17.63 * #	127.38 ± 23.65 *	168.46 ± 19.93 *	238.37 ± 19.32	<0.001
iAUC 0-3 h (mmol/L x min)	190.58 ± 24.61	178.11 ± 25.10 *	178.42 ± 32.93	198.83 ± 24.00 *	260.76 ± 22.99	0.021

¹ All values are means ± SEMs; n=16. iAUCs were calculated by using the trapezoidal rule.

* P<0.05 compared with R-VM sequence; # P<0.05 compared with VMR sequence; 1-factor ANOVA, adjusted by Bonferroni's correction for multiple comparisons; iAUC: incremental area under the curve.

Table 2. Characteristics of insulinemic excursion in healthy subjects ¹

	V-MR	M-VR	V-M-R	VMR	R-VM	P value
Mean amplitude of insulinemic excursions (μU/ml)	66.72 ± 10.87	80.35 ± 9.23	67.50 ± 10.15	71.18 ± 7.07	93.91 ± 13.78	0.032
incremental insulin peak (μU/ml)	58.96 ± 10.06	72.22 ± 8.35	55.41 ± 9.99	62.65 ± 6.43	85.56 ± 12.90	0.02
iAUC 0-1 h (μU/ml x min)	1341.08 ± 259.31 &	2365.65 ± 277.63	1102.71 ± 281.25*#&	2136.82 ± 197.47	2909.55 ± 427.63	<0.001
iAUC 0-2 h (μU/ml x min)	3658.67 ± 567.36	4609.68 ± 555.89	3205.59 ± 450.47	4047.70 ± 470.47	5573.41 ± 902.77	<0.001
iAUC 0-3 h (μU/ml x min)	5253.42 ± 884.10	6009.50 ± 725.99	4956.25 ± 709.16	5093.17 ± 651.71	6564.13 ± 1113.87	0.091

¹ All values are means ± SEMs; n=16. iAUCs were calculated by using the trapezoidal rule.

* P<0.05 compared with R-VM sequence; # P<0.05 compared with VMR sequence; & P<0.05 compared with M-VR sequence. 1-factor ANOVA, adjusted by Bonferroni's correction for multiple comparisons; iAUC: incremental area under the curve.

Table 3. iAUCs for blood plasma GLP1 and GIP concentrations in the first 60, 120 and overall 180 min in response to 5 different food intake sequences in healthy subjects ¹

	V-MR	M-VR	V-M-R	VMR	R-VM	P value
GLP-1 iAUC 0-1 h (pmol/L x min)	310.4 ± 37.4	321.9 ± 36.7	359.3 ± 39.8	221.3 ± 28.9	291.6 ± 29.2	0.026
GLP-1 iAUC 0-2 h (pmol/L x min)	611.2 ± 54.1 *	739.4 ± 72.4 *	815.9 ± 114.7*	448.1 ± 42.3	506.2 ± 60.2	<0.001
GLP-1 iAUC 0-3 h (pmol/L x min)	947.5 ± 86.5	1074.0 ± 101.3	1206.0 ± 188.7 *	773.4 ± 72.0	932.0 ± 97.8	0.016
GIP iAUC 0-1 h (pg/mL x min)	3908.3 ± 695.7 #&	6845.0 ± 628.6	4013.6 ± 1113.2 #	6887.7 ± 1258.1 #	10445.2 ± 1485.9	<0.001
GIP iAUC 0-2 h (pg/mL x min)	14878.4 ± 1669.0 &	19098.9 ± 1096.2	15716.7 ± 2117.9	16827.4 ± 2201.1	21276.7 ± 2714.4	0.013
GIP iAUC 0-3 h (pg/mL x min)	26380.5 ± 2778.9	30133.1 ± 2285.9	28619.0 ± 3316.6	26016.5 ± 3304.1	29403.8 ± 3402.5	0.287

¹ All values are means ± SEMs; n=16. iAUCs were calculated by using the trapezoidal rule.

* P<0.05 compared with VMR; # P<0.05 compared with R-VM; & P<0.05 compared with M-VR sequence. 1-factor ANOVA, adjusted by Bonferroni's correction for multiple comparisons; iAUC: incremental area under the curve; GLP-1: glucagon-like peptide-1; GIP: glucose-dependent insulintropic polypeptide.

Figure legends:

Figure 1. Mean fasting and postprandial glucose response to five different food intake sequences over 180 min (A). iAUC for blood glucose concentration in the overall 180 min after the meal (B) (n=16). Data were analyzed by using 2-factor repeated-measures ANOVA for comparison among the five food intake sequence treatments over 180 min. There were significant effects for treatment ($P<0.001$), time ($P<0.001$) and treatment $\times$ time interaction ($P<0.001$); post hoc comparisons were adjusted by Bonferroni's correction. * $P<0.05$ compared with R-VM sequence; # $P<0.05$ compared with VMR; & $P<0.05$ compared with V-M-R sequence. iAUCs were calculated by using the trapezoidal rule. Values are means $\pm$ SEMs.

Figure 2. Mean fasting and postprandial insulin response to five different food intake sequences over 180 min (A). iAUC for blood insulin concentration in the overall 180 min after the meal (B) (n=16). Data were analyzed by using 2-factor repeated-measures ANOVA for comparison among the five food intake sequence treatments over 180 min. There were significant effects for treatment ($P=0.029$), time ($P<0.001$) and treatment $\times$ time interaction ($P<0.001$); post hoc comparisons were adjusted by Bonferroni's correction. * $P<0.05$ compared with R-VM sequence; # $P<0.05$ compared with VMR; & $P<0.05$ compared with M-VR. iAUCs were calculated by using the trapezoidal rule. Values are means $\pm$ SEMs.

Figure 3. Mean change from baseline postprandial GLP-1 response to five different food intake sequences over 180 min (A). iAUC for blood GLP-1 concentration in the overall 180 min after the meal (B) (n=16). Data were analyzed by using 2-factor repeated-measures ANOVA for comparison among the five food intake sequence treatments over 180 min. There were

significant effects for treatment ($P<0.001$), time ($P<0.001$) and treatment $\times$ time interaction ($P<0.001$); post hoc comparisons were adjusted by Bonferroni's correction. * $P<0.05$ compared with V-M-R sequence; # $P<0.05$ compared with R-VM. iAUCs were calculated by using the trapezoidal rule. Values are means $\pm$ SEMs. GLP-1, glucagon-like peptide 1

Figure 4. Mean change from baseline postprandial GIP response to five different food intake sequences over 180 min. iAUC for blood GIP concentration in the overall 180 min after the meal (B) (n=16). Data were analyzed by using 2-factor repeated-measures ANOVA for comparison among the five food intake sequence treatments over 180 min. There were significant effects for time ($P<0.001$) and treatment $\times$ time interaction ($P<0.001$), but no significant treatment effect ($P=0.234$); post hoc comparisons were adjusted by Bonferroni's correction. * $P<0.05$ compared with R-VM sequence; # $P<0.05$ compared with M-VR; & $P<0.05$ compared with VMR. iAUCs were calculated by using the trapezoidal rule. Values are means $\pm$ SEMs. GIP: glucose-dependent insulintropic polypeptide.

Figure 5. Mean change from baseline postprandial active ghrelin response to five different food intake sequence over 180 min. iAUC for blood ghrelin concentration in the overall 180 min after the meal (B) (n=16). Data were analyzed by using 2-factor repeated-measures ANOVA for comparison among the five food intake sequence treatments over 180 min. There were no significant effects for time ($P=0.105$), treatment ($P=0.172$) and treatment $\times$ time interaction ($P=0.068$). iAUCs were calculated by using the trapezoidal rule. Values are means $\pm$ SEMs.

Figure

Figure 1

Figure 2.

Figure 3.

Figure 4.

Figure 5.

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