Research Article

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In vitro assessment of food-derived-glucose bioaccessibility and bioavailability in bicameral cell culture system

Formüle Edilmiş Gıda Özelliklerinin Hücre Kültürü Sisteminde *İn vitro* Olarak Değerlendirilmesi

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Abstract

Background: Functional foods can help prevent metabolic diseases, and it is essential to evaluate functional characteristics of foods through *in vitro* and *in vivo* experimental approaches.
Objective: We aimed to use the bicameral cell culture system combined with the *in vitro* digestion to evaluate glucose bioavailability.
Materials and methods: Cake, almond paste, and pudding were modified by adding fiber and replacing sugar with sweeteners and polyols. Digestion process was modeled in test tubes. Rat enterocyte cells (IEC-6) were grown in a bicameral cell culture system to mimic the physiological characteristics of the human intestine. The glucose bioaccessibility and cellular glucose efflux were measured by glucose oxidase assay.
Results and discussion: The glucose bioaccessibilities of modified foods were significantly lower (cake: 2.6 fold, almond paste: 9.2 fold, pudding 2.8 fold) than the controls. Cellular glucose effluxes also decreased in the modified cake, almond paste, and pudding by 2.2, 4, and 2 fold respectively compared to their controls.
Conclusion: Our results suggest that combining *in vitro* enzymatic digestion with cell culture studies can be a practical way to test *in vitro* glucose bioaccessibility and bioavailability in functional food development.

Keywords: bioavailability; functional food; glucose bioaccessibility; glucose efflux; IEC-6 cells; metabolic syndrome.

ÖZ

Giriş: Fonksiyonel gıdalar, metabolik hastalıkların önlenmesinde önemli yer tutmaktadır. Bu nedenle, fonksiyonel gıdaların özelliklerinin deneysel yaklaşımlarla incelenmesi önemlidir.
Amaç: Bu çalışmada, *in vitro* sindirim uygulanan gıda örneklerindeki glikoz molekülünün biyoyararlanımının insan bağırsak sisteminin oluşturulduğu hücre kültürü modelinde incelenmesidir.
Materyal ve Yöntem: Kek, badem ezmesi ve puding örneklerinin formülasyonlarında, lif eklenerek ve tatlandırıcı olarak şeker alkollerinin kullanımıyla değişiklikler yapılmıştır. Örneklere test tüplerinde sindirim gerçekleştirilerek glikoz biyoerişilebilirliği saptanmıştır. Ardından, insan bağırsağının fizyolojik özelliklerini taklit edebilen sıçan enterosit hücre hattı olan (IEC-6) kullanılarak hazırlanmış ikili hücre kültürü sisteminde glikoz biyoyararlanım testi uygulanmıştır. Tüm glikoz ölçümlerinde, glikoz oksidaz enzim testi kullanılmıştır.
Bulgular ve Tartışma: Modifiye edilmiş gıdalarda glikoz biyoerişilebilirlikleri, ilgili kontrol örneklerden önemli derecede farklı bulunmuştur (kek: 2.6 kat, badem ezmesi:

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9.2 kat ve puding: 2.8 kat). Hücrelerden salınan glikoz benzer şekilde, kontrol örneklerine göre kek için 2.2 kat, badem ezmesi için four kat ve puding için two kat düşük olarak saptanmıştır.
Sonuç: Elde edilen bulgulara göre, test tüpü sindirim yönteminin hücre kültürü çalışmaları ile birleştirilmesi yaklaşımının, fonksiyonel gıda tasarımı için *in vitro* glikoz biyoerişilebilirliğinin ve biyoyararlanımının saptanmasında kullanılabilecek bir yöntem olarak düşünülebilir.

Anahtar Kelimeler: fonksiyonel gıda; metabolik sendrom; glikoz biyoerişilebilirliği; Hücresel glikoz salınımı; IEC-6 hücre hattı; biyoyararlanım.

Introduction

Type 2 diabetes mellitus (T2DM) is recognized as a severe and worldwide health concern. The number of people with diabetes in 220 countries was 415 million in 2015 and is expected to be 642 million in 2040 [1]. Thus, the issue of T2DM has received considerable critical attention. Obesity and T2DM together constitute the biggest epidemic in human history [2]. The urbanization, industrialization, nutritional transition, obesity, low physical activity, and genetic factors lead to T2DM [3]. Weight gain caused by eating sweets and desserts was equal to that of the weight gain caused by refined grain consumption [4]. Thus, the food industry was compelled to design healthier foods to prevent metabolic diseases. The term functional food is defined as "foods that have a potentially positive effect on health beyond basic nutrition, helping the promotion of optimal health conditions and reducing the risk of noncommunicable diseases" [5]. Designing low-calorie and high-fiber foods with no sugar is important for T2DM patients. In recent years, research on the design of no added sugar foods for T2DM has become very popular.

The formulation of functional foods involves various approaches, including adding dietary fiber to reduce the glucose intake in the intestinal lumen and replacing sugar with its substitutes. The rate of absorption and the effects of blood glucose and insulin concentrations vary significantly among different carbohydrate sources [6]. The system ranks the carbohydrate sources based on their effect on increasing the blood glucose levels in terms of glycemic index (GI). Furthermore, the combination of GI and all other available carbohydrate contents constitute their glycemic load (GL). Various methods have been developed to measure the GI and GL either *in vitro*, such as the digestibility of starches [7, 8] or as *in vivo* human studies [9, 10]. Although test tube studies give insight into how much of the related nutrient is released in the intestinal lumen, the cellular uptake of these nutrients by epithelial cells might change the expected results. In order to overcome this, cellular glucose efflux studies can be combined with test tube digestion methods.

The current study aimed to use a combined method of test tube digestion and in the *in vitro* cellular nutrient transport system to design functional foods for obesity and T2DM. For this purpose, almond paste, cake, and vanilla pudding were developed as sample functional foods by replacing sugar and adding fiber. We evaluated the relative food glucose accessibility and cellular glucose efflux after applying a test tube digestion and then examined the glucose transporting IEC (*Rattus norvegicus* epithelium) cell line.

Materials and methods

Chemicals and reagents

We purchased pepsin (P7000), guar (G4129), pancreatin (P7545), amyloglucosidase (A7095), invertase (I4504), D-glucose (G7021), glucose oxidase assay kit (GAGO-20), penicillin–streptomycin (P4333), and insulin (I9278) from Sigma-Aldrich (Mannheim, Germany) and Dulbecco's modified eagle media (DMEM) without glucose (A14430-01), DMEM with 4.5 g/L glucose (41965-039), fetal bovine serum (FBS, 10500-064), and L-glutamine (25030-24) from GibcoTM (MA, USA). All other chemicals were analytical grade. Unless stated otherwise, all solutions were prepared with Milli-Q water. We purchased the IEC-6 cell line (*R. norvegicus* small intestine, CRL-1592TM) from ATCC® (VA, USA).

In vitro digestion

We obtained modified samples (MSs) of almond paste, plain cake, and pudding with steviol glycoside and polyols for sweetness and inulin for fiber (Supplementary Table S1) from Takita Brand (Egepak Gıda ve Ambalaj San. A.Ş., İzmir, Turkey). We purchased the same type of control samples (CSs) without any modifications from local supermarkets in İzmir. Cake and pudding samples were prepared according to package instructions, and almond paste was ready to eat. These products did not have any nutrition or health claims and contained sucrose (Supplementary table). The *in vitro* digestion method was applied with some modifications from the protocol of Englyst, Hudson, and Englyst [11]. All experimental conditions were determined according to preliminary experiments and performed at the same time for individual samples, including sample amount, glucose output from only water or gastric digestion, and duration of sample incubations during different digestion phases. Furthermore, all the digestion experiments were performed at the same time. The amounts of samples for digestion protocol were determined regarding their initial carbohydrate contents. We included commercial white bread from a local market as an experimental conditional control. Briefly, the samples were minced to mimic the chewing action in the mouth. For

the gastric phase, 1% g (w/v) pepsin, 1% g (w/v) guar gum, and 0.05 M HCl (v/v) were prepared in 50 mL water to make a gastric fluid solution. Then, minced samples of almond paste (0.2 g), cake (0.25 g), and pudding (0.75 g) were added individually to 5 mL of gastric fluid solution and incubated for 30 min at 37 °C. At the end of the incubation period, 5 mL of 0.25 M sodium acetate solution was added to neutralize the pH. For the intestinal fluid, 3 g of pancreatin in 20 mL of water was added and vortexed for 10 min. Next, this pancreatic enzyme solution was centrifuged at 6,800 *g* for 10 min, and 15 mL of the supernatant was taken and were combined with 0.666 mL amyloglucosidase and 1 mL (10 mg/mL) invertase enzymes to finalize the intestinal fluid mixture. During the intestinal digestion phase, 2.5 mL of the intestinal fluid mixture was added in 10 mL samples from the gastric digestion phase. The cake and almond paste samples were incubated for 2 h, and the pudding sample was incubated for 4 h horizontally in an orbital shaker (Thermo Forma, 481, MA, USA) at 100 rpm and 37 °C. Furthermore, five glass balls were included in each tube to mimic the peristaltic movement during intestinal digestion phase. The samples were not suitable for cell culture experiments due to high water content and glucose concentration for cell culture glucose transport. Thus, the samples were lyophilized to concentrate the glucose in liquid digested samples, and this allowed us to have enough concentration of glucose in the smallest volume for cellular glucose efflux study.

Glucose measurement

At the end of the digestion procedure, we determined released glucose contents with a glucose oxidase assay kit (Sigma, GAGO20) spectrophotometrically with a microplate reader (MultiskanTM GO, Thermo Fisher, MA, USA), following the instructions. The glucose levels were normalized to the dry weight of food samples.

Cell culture

R. norvegicus small intestine enterocyte cells (IEC-6) were purchased from American Cell Culture Collection (ATCC, CRL-1592). The IEC-6 cells were maintained in DMEM containing 4.5 g/L glucose, 10% heat-inactivated FBS, 1% L-glutamine, 1% penicillin–streptomycin, and 0.1 unit/mL insulin. The passage numbers of the cells were kept between 15 and 25. The IEC-6 cells were grown on collagen-coated inserts (CLS3495, Corning®Transwell®) to mimic the *in vivo* conditions of the intestine system. Briefly, 1×10^6 cells/well were seeded on 12-well inserts and grown for 10 days. Transepithelial electrical resistance (TEER) of monolayer cells was measured with an EVOM instrument (World Precision Instruments, USA). It has been indicated that TEER levels of IEC-6 cells have been determined as 30–45 Ω/cm^2 [12, 13]. Thus, this value was used as a set point of TEER for our experiment.

Glucose efflux in IEC-6 cell line

Each lyophilized tube was dissolved in 2 mL glucose-free DMEM cell culture medium. The same volume of both control and test samples were used to model the same portion size consumption of foods. Around 0.5 mL of dissolved samples was added on apical sides of the inserts. The amount of the glucose-free medium that was introduced on the basolateral side was 1.5 mL for each well. The cell culture medium samples were taken from the basolateral side after 30, 60, 90, and 120 min incubation periods to monitor the glucose efflux. In this experiment, an additional 180th min time point was also added only for pudding samples. In this experiment, D-glucose was used as an experimental control whether glucose affected the cellular TEER or monolayer structure. We also monitored time dependent glucose efflux from digested samples by looking at free glucose efflux. The glucose levels were measured with the glucose oxidase assay kit and normalized total cellular protein amount.

Statistical analysis

The results were expressed as mean ± SE with at least four replicates. All analyses were performed, and figures were made in GraphPad Prism (version 6.0 for Windows, GraphPad). Initial sample weight and enzyme digestion time points were analyzed by two-way ANOVA. Digestion measurements and basolateral glucose results were analyzed by one-way ANOVA followed by Tukey's post hoc test.

Results

Effect of sample amount and enzyme incubation time on digestion process

We used CSs to determine the amounts of samples that should be used in grams and the incubation time for the individual sample types. The glucose levels from cake and almond paste did not significantly change regarding different incubation time (h) and amount (g) of samples (Figure 1A, B). However, we observed that 0.75 g and 4-h duration digestion time points showed significant glucose output from the pudding sample compared to the 2 h duration of digestion for both 0.75 and 1.5 g of sample weights (Figure 1C). When we utilized sample digestion, we also performed parallel experiments that involved the following groups: Group 1 was the reference samples in only water to evaluate the level of rapidly soluble glucose, Group 2 was the reference samples in an only gastric enzyme solution to assess the effect of gastric enzyme and low pH on glucose release from samples, and finally, Group 3 included both gastric and intestinal enzymes present in a test tube digestion procedure. We found that carbohydrate digestion enzymes significantly increased glucose output from the cake (31-fold), almond paste (16-fold), and pudding (27-fold) relative to their Group 2 samples (Figures 2A, B, and C, 3C).

Glucose bioaccessibility

After the digestion protocol was established with CSs, both CSs and MSs were utilized to digestion in the test tube.

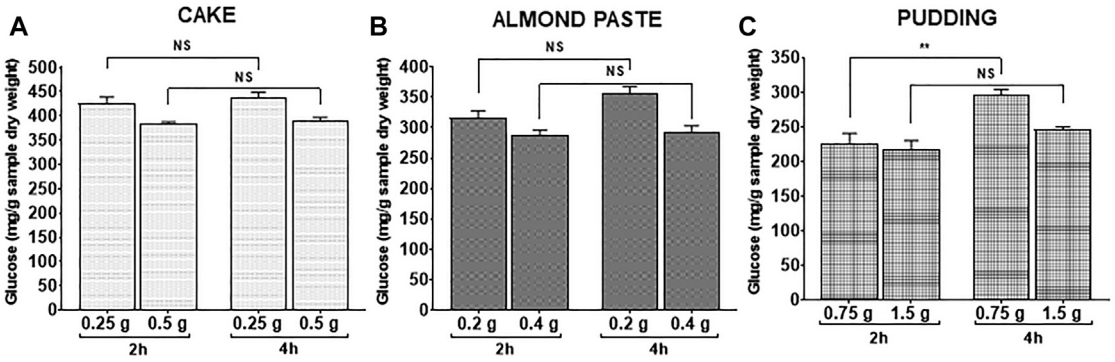

Figure 1: Determination of weight and duration for digestion of control cake, almond paste, and pudding samples. We mimicked the *in vivo* carbohydrate digestion method in a test tube. The effects of weights and time points of samples were tested to find acceptable digestion conditions for reference cake, almond paste, and pudding samples (A, B, C). Data (n=3) are presented as mg glucose of g dried weight. Significance was calculated by using one-way ANOVA followed by Tukey's post hoc analysis. NS, non-significant.

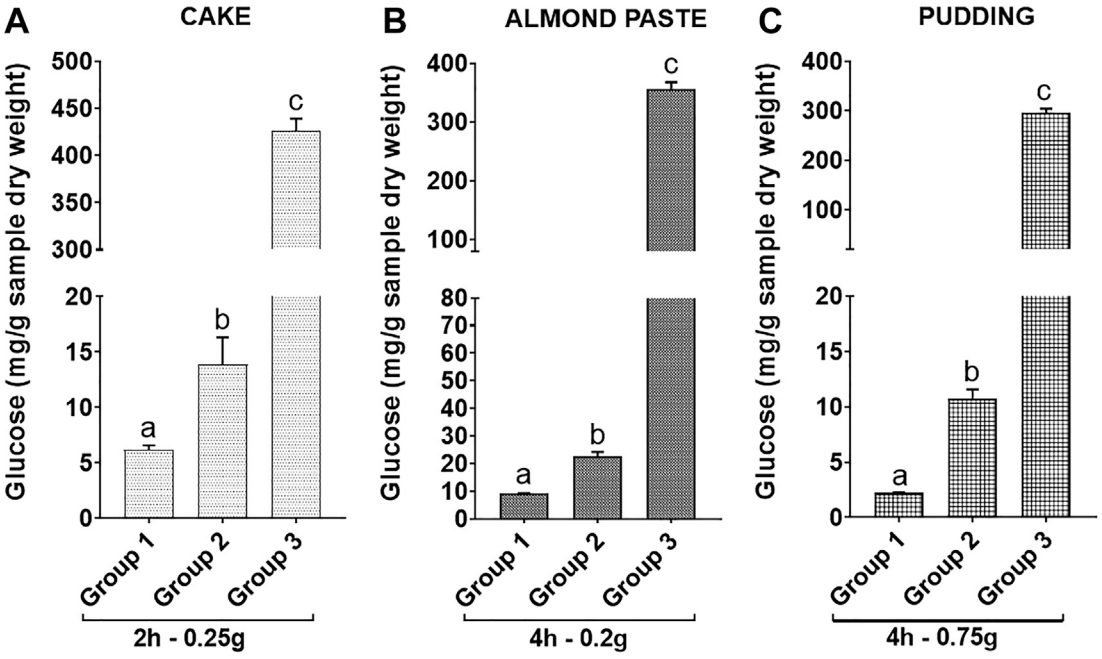

Figure 2: Determination of experimental digestion conditions. All control samples were incubated in water, only gastric enzyme solution, and both gastric and digestion enzyme solution under determined weights and digestion times. When the samples were exposed to gastric and carbohydrate digestive enzymes, glucose levels significantly increased in the cake, pudding, and almond paste sample groups (A, B, C). Data (n=3) are presented as mg glucose of g dried weight. Significance was calculated by using one-way ANOVA followed by Tukey's post hoc analysis.

[a,b,c]Statistically different from one another within each panel (at least $p<0.05$). Group 1: Control samples (gram) in water. Group 2: Control samples (gram) in the pepsin solution. Group 3: Control samples (gram) in the pepsin enzyme and carbohydrate digestion enzyme solutions.

Moreover, white bread was also included in the digestion protocol as an experimental control. White bread showed the highest glucose release among all samples (Figure 3). Besides, glucose output from carbohydrate digestion of modified cake (2.6-fold), almond paste (9.2-fold), and pudding (2.8-fold) samples were significantly ($p\leq0.05$) lower than their CSs.

Cellular glucose efflux in IEC-6 cell-line

We selected the IEC-6 cell line as an *in vitro* model. TEER values of 10 days post-confluent IEC-6 cells were ~45–50 Ω/cm^2, indicating the monolayer formation of cells. We used digested samples with the same volume to mimic the same portion sizes for both control and modified foods.

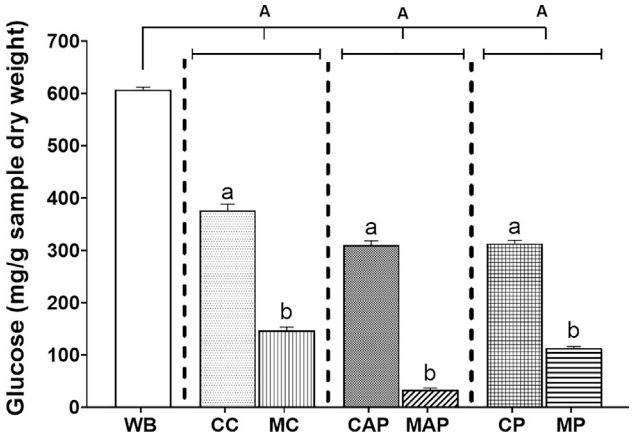

Figure 3: Glucose bioaccessibility. Modified food samples and their controls were digested in a test tube. White bread was used as an experimental control for digestion. We compared all modified samples with their control samples. Bioaccessibility of glucose in the formulated cake, pudding, and almond paste samples was lower than their controls. Data (n=3) are presented as mg glucose of g lyophilized samples. Significance was calculated by using one-way ANOVA followed by Tukey's post hoc analysis, and different small letters above the data bars indicate statistical significance (at least $p<0.05$) in a group of samples. Letter (A) indicates the significance between D-glucose treatment group and other samples of groups (at least $p<0.05$). WB, white bread; CC, control cake; MC, modified cake; CAP, control almond paste; MAP, modified almond paste; CP, control pudding; MP, modified pudding.

We found that the efflux of glucose from the modified cake (1.9, 1.6, 2.2, and 2.2 folds for 30th, 60th, 90th, 120th min time points, respectively), almond paste (4.3, 5.3, 3, and 3.7 folds 30th, 60th, 90th, 120th min time points, respectively) and pudding (3.3,1.6, 1.6, 1.5, and 2.0 folds 30th, 60th, 90th, 120th, 180th min time points, respectively) samples were significantly lower than their CSs (Figure 4). We observed that the bioaccessibility of glucose in CSs was higher than their bioavailability in the cell culture system. The glucose bioaccessibilities of cake, almond paste, and pudding were 2.6, 9.2, and 2.8 folds lower than their CSs, respectively ($p\leq0.05$). On the contrary, the glucose effluxes of these samples were 2.2, 4, and two folds lower than the CSs for cake, almond paste, and pudding, respectively ($p\leq0.05$).

Discussion

We mimicked gastric and intestinal carbohydrate digestion processes in the test tube to evaluate glucose accessibilities of the food samples. Next, rat enterocyte cells (IEC-6) were used to mimic the human intestine system and to utilize the intestinal glucose efflux from food samples. The food derived glucose and fiber levels are the two factors involved in calorie and energy intake into the human body [14, 15]. Thus, new formulations were developed and different amounts of fiber, polyols and high-intensity sweetener were used in each modified sample depending on their texture and taste. Type of the foods was selected considering the market value and consumption rates in Turkey. Therefore cake, almond paste, and pudding were involved in the study as MSs. Moreover, we selected similar types of food as CSs from local supermarkets in İzmir. Since CSs had more glucose and less fiber than MSs group, CSs samples were used to determine experimental digestion conditions (Figure 2). We hypothesized that if the enzyme

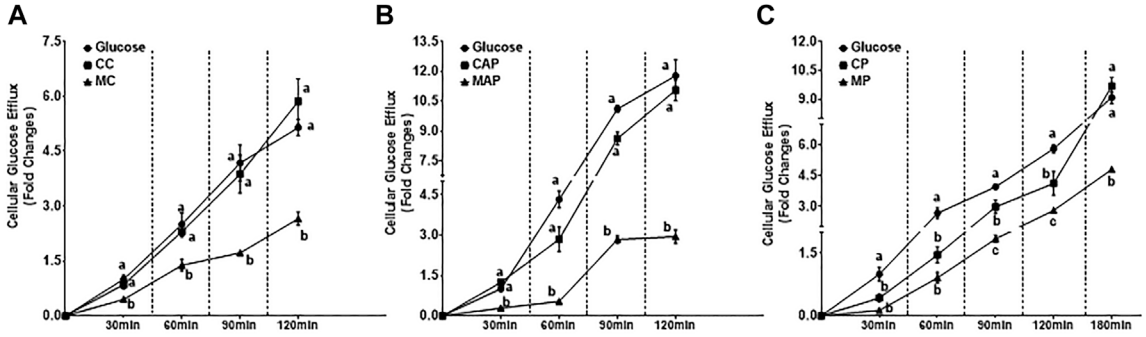

Figure 4: Cellular glucose efflux. Rat intestine enterocyte cells (IEC-6) were used as a model of the human intestine system. We grew IEC-6 cells on a bicameral cell culture insert for 10 days. TEER was measured before and after every transport study to evaluate the monolayer structure of cells. The same volume samples were introduced into the apical side of cells, and samples were collected from the basolateral side of cells at different time points. Glucose level was measured with the glucose oxidase enzyme assay and results were normalized to total protein concentration. Glucose efflux was significantly lower in formulated foods than their reference samples (A; cake, B; pudding, C; almond paste). CC, control cake; MC, modified cake; CAP, control almond paste; MAP, modified almond paste; CP, control pudding; MP, modified pudding. Data (n=4) are presented to a fold change of cellular glucose efflux. Significance was calculated by using one-way ANOVA followed by Tukey's post hoc analysis. Different small letters above the data bars indicate statistical significance (at least $p<0.05$) in a group of samples.

amount and incubation time were determined regarding CSs protocols, those parameters might also be suitable for carbohydrate digestion protocols of MSs due to their low carbohydrate levels. It might be evidence that the level of total carbohydrate was higher in CSs resulting in more glucose output relative to MSs. However, their food matrix and profiles were different so it should be tested by the digestion of the samples to determine glucose bioaccessibility from all experimental samples because the food matrix is one of the critical factors that affect food digestion [16]. Our results indicate that glucose bioaccessibilities in MSs were lower than CSs (Figure 3). White bread was included in the study as the control of digestion, and it showed higher glucose output after digestion, indicating that the digestion of carbohydrate was successfully performed in our experimental conditions.

The bioaccessibility of a nutrient refers to the amount of nutrient releases from digested foods and it does not indicate the absorbable portion of nutrients from enterocyte cells of the small intestine [17]. Intestinal nutrient absorption might depend on ingredients and matrix of foods [16], nutrient-dependent transporter protein levels on enterocyte cells [18], transport rate of nutrients [19], and the physiological condition of the person [20]. Human and animal studies are well-accepted models to investigate the functional properties of foods or evaluate the health impact of foods. Human and animal-derived cells can be used instead of *in vivo* models [21]. However, all experimental models have weaknesses and strengths. In this study, we used rat noncancerous enterocyte cells (IEC-6) to mimic the human intestine. This cell line has been widely accepted for nutrient transport studies. We observed higher cellular glucose efflux in CSs than MSs, indicating that consuming MSs might provide less glucose after consumption. The same volume of digested samples was introduced in the apical side of the bicameral cell culture system to compare cellular glucose efflux between samples. D-glucose was also used to control cellular glucose efflux in the cell culture study instead of digested white bread because the glucose solution allowed us to see if there were any effects of external factors including digested sample solution and ingredients in digested samples on cells that were grown on the bicameral system. We used the same volume of digested samples to mimic the same amount of eaten food samples. When the amount of glucose in the food samples was compared, MSs samples contained less glucose than CSs. Thus, it was not surprising to find low glucose bioaccessibility and cellular glucose efflux in the MSc. However, we intended to show the *in vitro* digestion process that can be combined with the cell culture study to estimate glucose absorption or relative glycemic features of food samples. Human colon carcinoma epithelial cell line (Caco-2) is often preferred to the model human intestine system to study bioavailabilities of nutrients and development of functional molecules [21]. We utilized noncancerous rat enterocyte cells to create the human intestinal epithelial barrier system. IEC-6 cell line can be used as an alternative to Caco-2 cells. Moreover, IEC-6 cells mimic the human intestine system when they grow on the bicameral insert system since no alternative noncancerous human intestinal cell line can be used to model the human intestine system [22, 23]. It has been indicated that *in vitro* test tube digestion approaches are performed to estimate the carbohydrate digestibility in terms of the GI. However, the correlation between the test tube and *in vivo* studies might be different due to the complex system of *in vivo* conditions [24]. Thus, test tube digestion and cell culture studies would be acceptable experimental approaches before *in vivo* studies.

To our knowledge, this is the first study that measures the glucose efflux from a food matrix after applying test tube digestion *in vitro*. *In vitro* carbohydrate digestion has been used to determine features of functional foods, including glucose bioaccessibility and glucose bioavailability. The *in vitro* carbohydrate digestion process is well established in laboratory conditions, and it can be able to mimic human carbohydrate digestion. Besides, the glucose efflux procedure was also combined with the test tube digestion procedure to gain an insight into the absorbed glucose in a formulated food product. However, lack of hormone stimulation, microbiota, and systemic responses are some of the concerns of *in vitro* bioaccessibility and bioavailability studies when compared to *in vivo* studies. Moreover, rat noncancerous enterocyte IEC-6 cells can be used in nutrient bioavailability studies.

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