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Research Article

Cooking Recipe as a Determinant of Glycemic Index Variation in Commercial and Natural Wheat Flour

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ABSTRACT

An *in vivo* study was conducted with various cooking recipes to select suitable wheat flour in view point of glycemic index (GI) value from the commercially available whole and plain wheat flour by comparing with natural wheat flour. We assessed the glycemic responses of 10 healthy individuals with reference food (50 g glucose) and cooked wheat roti, equivalent to 50 g carbohydrate, in hot (100°C) and normal water (25°C) recipes. To prevent residual effects, the individuals were kept overnight fasting throughout the study period. Capillary glucose levels at 0, 15, 30, 45, 60, 90 and 120 minutes after consuming the meals were assessed and used to determine the Incremental Area Under Curve (IAUC). Results showed a significant decrease ($p<0.001$ and $p<0.005$) in glucose levels of the test foods compared to reference food. The measured GI values of the samples processed with normal water were categorized as low (plain wheat flour, 52), medium (56 for whole wheat flour) and high (natural flour, 73). But all the samples were in low GI class when processed with hot water; GI was 43, 47 and 44 for whole wheat flour, plain wheat flour, and natural wheat flour, respectively. The temperature differences in the recipes are believed to be the primary cause of variation in GI. Hot water treatment provided low GI in the recipes that may be good for both healthy and diabetic people.

Introduction

Diabetes, a rapidly growing global chronic disease, is largely influenced by diet and sedentary lifestyle. In Bangladesh, 12.88 million people are affected by diabetes, and 3% of all-age deaths are caused by this condition (WHO, 2016). The prevalence of diabetes worldwide in 2021 was 537 million and will be affected 643 million by 2030 and 783 million 2045. Moreover, about 200 million more people will be affected with pre-diabetes or would be at risk of developing diabetes by 2045 (Sun *et al.,* 2022). Asians face higher risks of diabetes-related complications, including chronic kidney damage, cancer, coronary artery disease, retinopathy, hearing and mental health issues, and decreased quality of life due to socio-economic context.

Controlling carbohydrate intake for diabetes patients can be achieved by using glycemic index to select carbohydrates with less impact on blood sugar. Glycemic index (GI), in 1981 – a scale that ranks carbohydrate-containing foods based on how much they raise blood glucose levels compared to a standard reference food (Jenkins *et al.,* 1981). Foods with high glycemic index are rapidly digested leading to a quick and significant increase in blood sugar level and vice-versa. Here are three categories of foods based on GI value: High GI foods (≥70), Medium GI foods (56-70), and Low GI foods (≤55). Low GI foods include plain wheat flour, whole wheat flour, oats, barley, fruits, multi-grains, custard, skim milk, carrot, and peanuts.

GI database of Atkinson *et al.* (2008) reveals seven wheat-based foods with a mean GI of 58. Wolever *et al.,* (2008) suggested that low-GI foods' impact on short-satiety and food intake may be due to factors beyond blood glucose response, such as fiber and protein content. People are increasingly recognizing the importance of a healthy diet, leading to increased attention for wheat flour varieties like whole and refined wheat flour and their use in various foodstuffs like roti, bread, nan bread, pancakes, pizza, wafer, pasta, and biscuits due to their beneficial effects from dietary fiber (Peressini and Sensidoni, 2009) and resistant starch (a type of starch that does not digested in small intestine but pass through the large intestine and have

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no any effects on glucose concentration of blood) properties.

In developed countries, whole and refined grains are consumed, with whole grains containing bran, germ, and endosperm, and refined grains containing only endosperm (Slavin, 2000). Whole grains are more protective against chronic diseases due to their concentrated antioxidants, trace minerals, phenolic compounds, oligosaccharides, dietary fiber, and resistant starch (Slavin, 2003).

Fiber consumption increases stomach distension, increases satiety and slows nutritional absorption, therefore reduces disease risks. Moreover, resistant starch (RS) is not absorbed in the small intestine, potentially preventing colon cancer and obesity (Fässler *et al.,* 2006). Awareness of fiber and RS benefits has led to increased consumption of whole grain breads and bran breads, as whole wheat is more complex and contributes to a healthy, sustainable diet due to its indigestible carbohydrate content.

Roti, also known as chapati in Asian nations like Bangladesh, Pakistan, India, and Sri Lanka, is a staple meal that aids in diet and weight loss, particularly popular among individuals with type 2 diabetes and observed that white rice high in glycemic index value (Thondre and Henry, 2009). Roti is made with normal water; whole wheat flour and salt produce hard texture whereas the same with hot water form softer texture. The temperature increases the mechanical resistance of the gluten network. As a result, hot water denatures the proteins causing decreased gluten content, while normal water helps proteins to form gluten (Cuq *et al.,* 2000). Blending wheat flour with hot water results in starch gelatinization, which affects starch digestibility by reducing quickly digestible starch and increasing RS (Graça *et al.,* 2021).

Roti can be made in various ways, but a proper recipe ensures a more nutritious final product. The cooking method and condition determine the extent of starch gelatinization, which directly affects digestibility and GI. A low GI recipe can help maintain type-2 diabetes, obesity, and prevent other complications, making it an effective choice for a healthy diet. Having in thoughts the shortage of scientific literatures at the impact of cooking in in vivo GI of commercially available wheat and natural wheat this experiment was conducted.

Materials and Methods

Experimental location and period

An *in vivo* experiment was carried out in the Professor Muhammad Hossain postgraduate laboratory of the Department of Biochemistry and Molecular Biology, Bangladesh Agricultural University, Mymensingh Sadar, Mymensingh-2202 from June to August, 2023.

Collection and preparation of sample

Two commercial wheat flours, fresh whole wheat flour and fresh plain wheat flour, were purchased from a local market and were certified by BSTI. Natural wheat was collected from the Dairy Science field laboratory, Bangladesh Agricultural University, Mymensingh. Waste materials were removed from the natural wheat, sun dried and grinded. All the collected and prepared wheat samples were categorized according to their types as presented in the Table 1.

Table 1. Categorization of wheat flours purchased from local market

Wheat Type	Brand Name	Type of Flour
Commercial wheat flour	Fresh	Whole wheat flour
		Plain wheat flour
Natural wheat flour	–	Whole wheat flour

Selection of ten healthy individuals

For this experiment, ten healthy individuals were selected. Their biological parameters such as age, body weight, Body Mass Index (BMI), blood pressure and height were considered. Volunteers were selected based on their self-willingness, age of 25 ± 1 years, average body weight of 59.29 ± 8.18 kg and average BMI of 21.85 ± 2.55 kg/m^2. They were non-diabetic and devoid of other disorders or food allergies. Throughout the experimental period, they were asked to stick to a regular exercise routine and diet. The Diabetic Association of Bangladesh (DABHT73/2018) accepted the study protocol in accordance with the guidelines outlined in the Declaration of Helsinki. All subjects were informed prior to participation, and informed consent was obtained in writing.

Reference food

Glucose powder, GlucoMax – D (GlaxoSmithKline Bangladesh Limited) was purchased from a local pharmacy and used as a reference food for this study. Fifty (50) grams glucose was measured using an analytical balance and diluted into 250 mL water. To prevent the carry-over effect, the influence of the reference diet on blood glucose levels was evaluated every two days prior to evaluating the performance of the test foods (Brouns *et al.,* 2005). However, there was a washout period of at least seven days in between each trial of a test diet.

Test foods

Both the commercial and natural wheat flours were used as test food. The short description of test food preparation is mentioned here. In a large mixing bowl, amount of wheat flour (whole/plain flour) corresponding to 50g of carbohydrate for 10 individuals was added with a pinch of salt. Fifty (50) grams carbohydrate was determined from the described proximate analysis of test foods.

- Fresh whole wheat flour amount: 50 × 100 / 72.6 (carbohydrate content)
  = 68.87g × 10 person = 688.7 g
- Fresh plain wheat flour amount: 50 × 100 / 76 (carbohydrate content) = 65.8 g × 10 person = 658 g
- Natural whole wheat flour amount: 50 × 100 / 73.68 (carbohydrate content) = 67.86 g × 10 person = 678.6 g

Gradually water (hot/cold) added to the flour while kneading to form a dough. The dough was divided into small lemon size balls. Each ball was rolled into thin circles and dusted with flour if required while rolling. Heated tawa/non-stick pan was kept in medium heat and carefully rolled roti was placed on it. Rotis were lightly baked on both side and were kept in normal freezer for 8-10 hrs. Then the rotis were properly baked for few minutes till golden spots appear. Then foods were served for the individuals. The test meal took around 10 minutes to digest after the first bite

Proximate analysis of test foods

The nutritional qualities of the natural wheat flour were represented in the proximate analysis. The proximate analysis was carried out at the Grain Quality and Nutrition laboratory of Bangladesh Rice Research Institute, Gazipur, Bangladesh.

Moisture content

One tea spoon wheat flour was taken in crucibles for each sample and were dried in an oven at 105°C for 2 hours and cooled in desiccators. The weight of the dried crucibles was measured, and the moisture loss was expressed as a percentage. According to the established procedures of (AOAC, 2019), the moisture content (%) was determined as follows:

$$\text{Moisture content (\%)} = \frac{W_1 - W_2}{\text{Initial weight of sample(g)}} \times 100$$

where, W_1 = weight of the crucible with sample (g) and W_2 = weight of the crucible with dried sample (g)

Ash content

Ash content was determined using a standard non-enzymatical approach. To determine the ash content (%), dry samples were held in a muffle furnace for 6 hours after ignition at 600°C.

The following formula was used to compute the ash content (%):

$$\text{Ash content (\%)} = \frac{W_1 - W_2}{\text{Initial weight of sample(g)}} \times 100$$

where, W_1 = weight of the ash with crucible (g) and W_2 = weight of the empty crucible (g)

Fiber content

Dietary fiber content (%) was estimated by AOAC (2019) method and the percentage was calculated as follows:

$$\text{Dietary fiber content (\%)} = \frac{\text{Loss of weight (g)}}{\text{Weight of sample(g)}} \times 100$$

Protein content

A combination of 2 g wheat flour, $CuSO_4$, and K_2SO_4 was placed into Kjeldahl tube, followed by addition of 94% H_2SO_4, and was digested at 350°C for 1 hour. The tube was connected to the Kjeldahl apparatus for distillation, and a conical flask containing boric acid was added. The flask was then titrated with 0.2 N H_2SO4 solution. Protein content (%) was calculated as:

$$\%N = \frac{(T_s - T_b) \times \text{Normality of the } H_2SO_4 \times 0.014}{\text{Weight of sample(g)}} \times 100$$

where, %N = percentage of nitrogen present in examined sample; T_s = Titer value of sample (mL); T_b = Titer value for blank (mL); Normality of H_2SO_4= 0.2 N; Weight of sample = 0.1 g; 0.014 = mL equivalent of N_2;
% Protein = % Nitrogen × 5.5 (conversion factor for plant proteins)

Fat content

Fat content (%) was determined after continuous extraction with n-Hexane for a period of nearly 20 hours, with the help of Soxhlet apparatus. Fat content was estimated by the following formula:

$$\text{Fat(\%)} = \frac{\text{Weight of extract(g)}}{\text{Weight of sample(g)}} \times 100$$

Carbohydrate content

Total carbohydrate content (%) was measured using the formula according to FAO (2019) as follows:
Carbohydrate (%) = 100 – (% Moisture + % Ash + % Protein + % Fiber + % Fat Content)

Experimental Treatments

In the experiment two recipes were used to make dough from the commercial and natural whole wheat flour samples. They comprise –

i. Hot water (100°C) and
ii. Normal water (25°C) treatment.

Blood Glucose Measurement

Measured portions of test foods containing 50 g of available carbohydrate were served to participants after overnight (10-12hrs) fasting. Capillary finger-prick blood samples were taken at 0 (fasting), 15, 30, 45, 60, 90, and 120 minutes after starting to eat the test meal using a safety lancet and glucose stick. Blood glucose was determined using glucometers (Bioland, G-423E). The patients were instructed to stay within testing area and engage in some sedentary activities such as - reading, communicating etc.

Calculation of glycemic index

The Incremental Area Under Curves (IAUC) for the blood glucose variation during the testing of test and reference foods was calculated geometrically in the MS-Excel-2007 program by trapezoid rule ignoring the area beneath baseline value of reference food (Coulibaly *et al.*, 2021). Reference foods' IAUC was always taken to be 100. The conversion value from each test item's glycemic index was computed using the following equation and compared to the reference food. According to the procedure of Jenkins *et al.* (1981), GI categorization was performed after obtaining all the GI values. The GI for the foods was calculated using the average of all ten participants' glycemic scores. The amount of accessible carbohydrates in a typical portion of each item was multiplied, and the GI of that food was divided by 100 to get the glycemic load of each food.

Glycemic Index =

$$\frac{\text{Blood glucose IAUC value for test food}}{\text{Mean IAUC value for reference food (glucose)}} \times 100$$

Statistical analysis

For analysis of the study results such as mean, percentage (%), standard deviation (SD), standard error mean (SEM) with level of significance (p-value) were obtained. Data were statistically analyzed using SPSS for windows (version 25.0) by paired sample t test. Mean comparisons were measured for significant level at $P < 0.01$ and $P < 0.05$.

Results

Observation of the research participants

The demographic characteristics like age, body weight, height, BMI, fasting blood glucose level as well as blood pressure were obtained when they were in fasting condition. Values are shown as means with standard deviation demonstrated in Table 2. No statistical significance was observed among the anthropometric characteristics, the GI values and IAUC values.

Table 2. Anthropometric data on the research participants (ten individuals)

Parameters	Mean ± SD
Age (year)	25.3 ± 0.50
Body weight (kg)	59 ± 7.66
Height (m)	1.64 ± 0.08
Body mass index (BMI) (kg/m²)	21.85 ± 2.55
Fasting blood glucose level (mmol/L)	5.8 ± 0.2
Blood pressure	
Systolic (mm/Hg)	119.5 ± 3.69
Diastolic (mm/Hg)	78.1 ± 4.25

From the Table 2 it was observed that study individuals had an average BMI of 21.85 ± 2.55 kg/m² which was inside the acceptable BMI range (18.5 to 24.9 kg/m²) for young, healthy people (CDC, 2022). Fasting blood glucose level for non-diabetic individuals should be between 4.4 mmol/L and 6.1 mmol/L according to Alberti and Zimmet (1998). In this study selected individuals also had fasting blood glucose of 5.8 ± 0.2 mmol/L.

Nutritional properties of wheat flour brands and natural whole wheat

Nutritional value is a measure of how well-balanced a mixture of essential components, such as proteins, carbs, fats, and minerals, meet the nutritional demands of consumers in food products or diets. The estimated nutritional values of the examined natural wheat flours showed the similar results of Kulkarni *et al.* (2012). The study determined the nutritional components of natural wheat flour, including fat, protein, ash, moisture, and fiber, while brand wheat flours listed these components (Table 3). Crude fiber is crucial for preventing diabetes, colon cancer, and heart disease. Natural whole wheat flour has a fiber content of 0.46%, which was lower than obtained value (0.82%) of Mepba *et al.* (2007), but near the reported value (0.30%) of Ade-Omowaye *et al.* (2008).

Table 3. The proximate nutritional composition and cooking characteristics of test foods

Type of flour	Available carbohydrate (%)	Fat (%)	Protein (%)	Moisture (%)	Ash (%)	Dietary fiber (%)	Roti Serving (g)
Fresh-whole	72.6	1.9	11.5	-	-	-	68.87
Fresh-plain	76	1.5	9.5	-	-	-	65.79
Natural	73.3	1.3	11.3	12.7	0.94	0.46	68.21

The crude protein content of commercial and natural wheat flour ranges from 9.5% to 11.5% and similar findings were also revealed from Ahmed *et al.* (2005) where they also obtained the value for protein ranged from 10.32% to 11.58%. Carbohydrate is very important for a healthy diet and should make up 50% of daily caloric intake (Chaudhari *et al.,* 2018). The study found that the content carbohydrate percentage in wheat samples ranged from 72.6% to 76%, which was also similar to previous research by David *et al.,* (2015) and Kulkarni *et al.* (2012), where they found a range of 57.35% to 83.60% and 74.88% respectively.

Different cooking recipes on the variation of blood glucose (mmol/L) level

The study examined blood glucose levels (mmol/L) after two hours of consumption of test foods cooked in hot and normal water recipes, along with a reference food. Data were shown in Figure 1 to 4 as ± SEM. No variations were observed at 0 minutes. Each test food with two distinct recipes had its baseline value determined by the reference food's equivalent glucose level. However, both the reference food and test foods showed varying significance levels at $p< 0.01$ and $p< 0.05$.

Figure 1 indicates the blood glucose levels (mmol/L) of reference food as glucose and the same amount of carbohydrate containing roti of commercial whole and natural whole wheat flour (test foods) processed in hot water. Here hot water used as treatment and observed that at 0 minutes after consumption no significant variation were obserd for both cases but for after 20 minutes to the 2 hours the reference food and test foods showed varying significance levels at $p< 0.01$ and $p< 0.05$.

However, Figure 2 also showed the same picture of figure 1 for the reference food and plain wheat flour with hot water. It was also observed that the reference food and test foods showed significant variation at $p< 0.01$ and $p< 0.05$ levels after 20 minutes to the 2 hours.

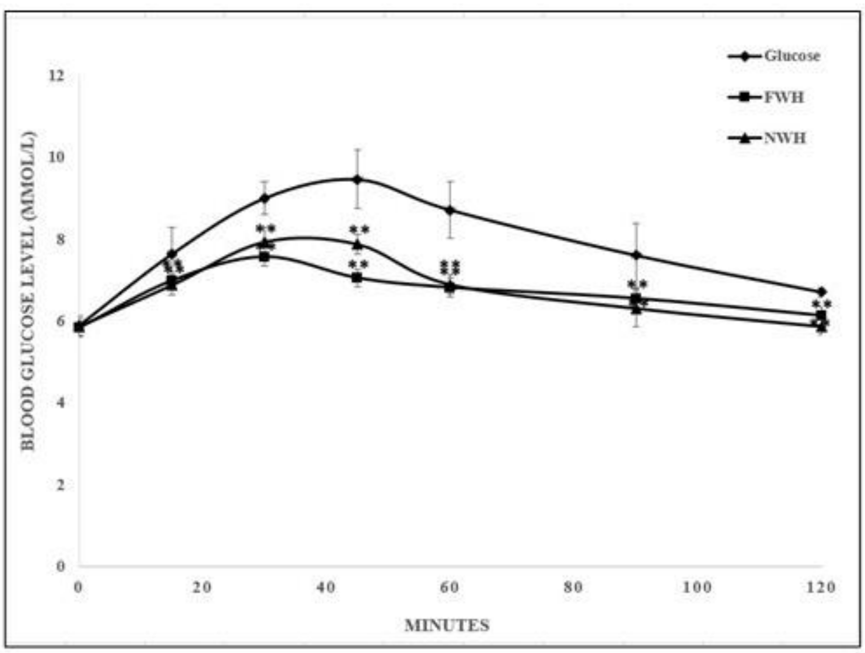

Figure 1: Comparison among the trends of blood glucose levels (mmol/L) of hot water (H) treatment for 2 hours after feeding glucose(reference food) and fresh whole (FWH) and natural whole wheat (NWH)flour (test foods) processed in hot water, with significance levels indicated by ** for $p<0.01$ and * for $p<0.05$.

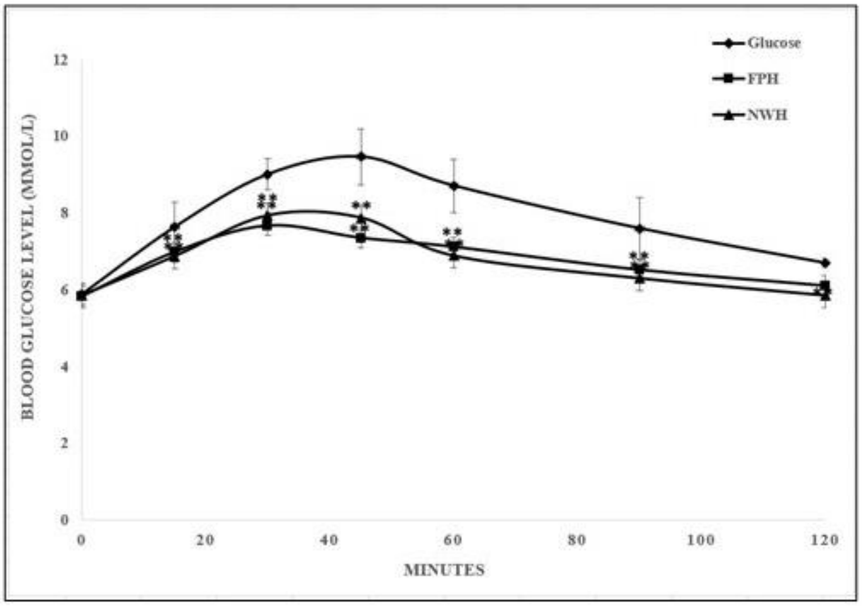

Figure 2: Trends of blood glucose levels (mmol/L) of hot water (H) treatment for two hours following the ingestion of glucose (reference food), fresh plain wheat flour (FPH) and natural wheat flour (NWH) (test foods), processed in hot water are compared; significant levels are shown by ** for p<0.01 and * for p<0.05.

On the other hand, Figure 3 showed the blood glucose levels (mmol/L) of reference food as glucose and both whole wheat flours of commercial and natural sources with normal water processing as treatment. It revealed the similar findings as others, at 0 minutes after consumption no significant variation and similar trends as others were also observed between 20 minutes to 120 minutes.

Finally at Figure 4 normal water processinf treatment was applied to the plain commercial wheat flour and natural flour. Here glucose also counted as referece food and distincted the similar result trends as above.

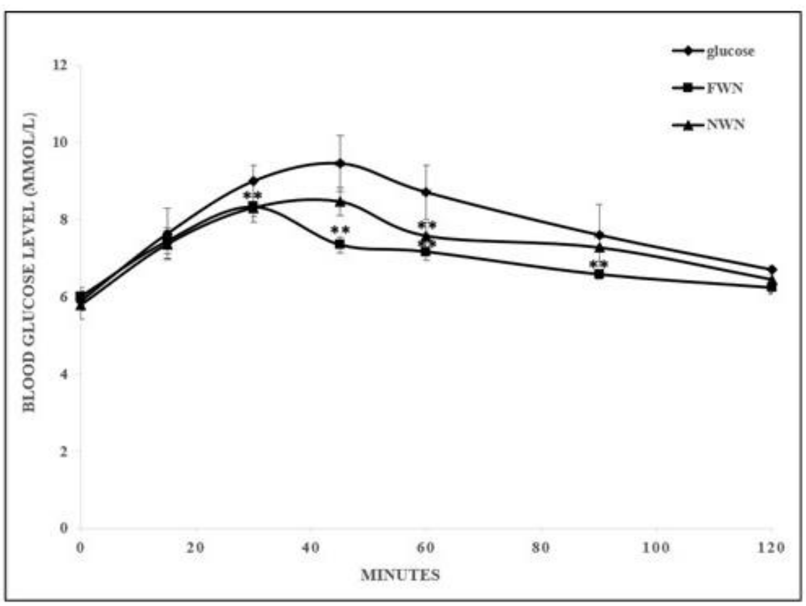

Figure 3: Comparison of the blood glucose levels (mmol/L) trends of normal water (N) treatment for two hours following the feeding of glucose (reference food), fresh whole wheat flour (FWN) and natural wheat flour (NWN) (test foods), processed in normal water. ** For p<0.01 and * for p<0.05 indicates significant levels.

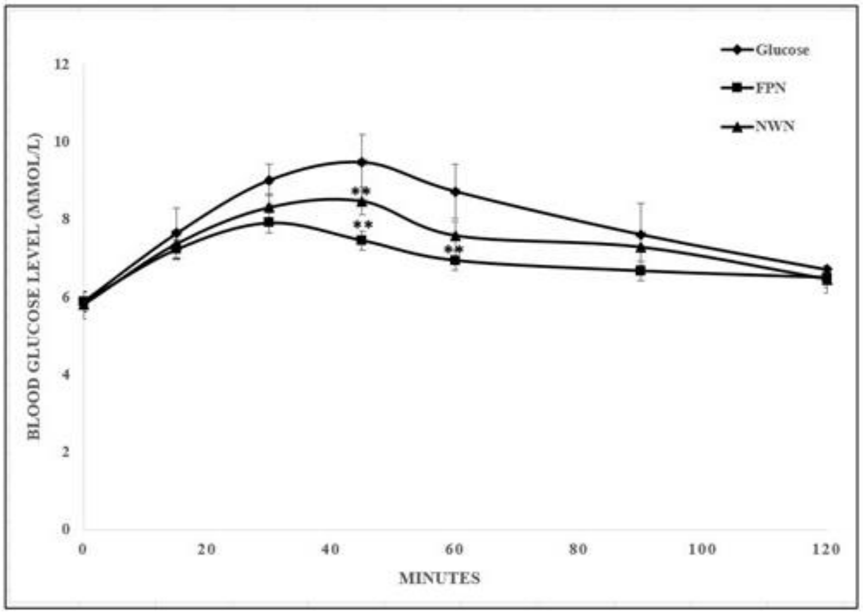

Figure 4: Comparison of blood glucose levels (mmol/L) trends of normal water (N) treatment for two hours following the ingestion of glucose (reference food), fresh plain wheat flour (FPN) and natural wheat flour (NWN) (test foods), processed in normal water. ** For $p<0.01$ and * for $p<0.05$ indicates significant levels.

Calculation of Incremental Area Under Curve (IAUC) mmol/L. min

IAUC was calculated in the Excel software (2019) based on various glucose levels (mmol/L) at glucose feed conditions, commercial wheat flour types, and natural whole wheat flour sample feed conditions at different durations or time (min). Results showed significant (1% and 5% level of significance) differences at 15, 30, 45, 60, 90 and 120 minutes time in hot water processing and at 30, 45, 60 and 90 minutes in case of normal water processing. From Table 4 it can be observed that hot water recipe exhibited lower GI value than normal water recipe.

Table 4. Incremental Area Under Curve (IAUC) variations of reference and test foods at hot water and normal water recipe with obtained GI values

Wheat flour varieties with recipes	Incremental Area Under Curve (IAUC) mmol/L.min			Obtained GI value	
	Reference food (glucose)	Hot water recipe	Normal water recipe	Hot water recipe	Normal water recipe
Fresh whole	263.75	143.625	148.12	43	56
Fresh plain	263.75	125.185	139.179	47	52
Natural whole wheat	263.75	194.25	118.286	44	73

Glycemic index values variations and classification

GI values were further obtained from the IAUC values of reference food and test foods. The overall variations of GI values of test foods cooked in two different recipes (hot and normal water) along with their categorization are shown in Figure 5. It was observed that fresh whole wheat flour hot water recipe reduced GI by 17% as compared to normal water. While fresh plain wheat and natural whole wheat flour hot water treatment also decreased GI values by 16% and 40% compared to normal water treatment, respectively (Figure 5). When fresh plain, whole and natural wheat flour were treated with normal water they were categorized as low (52), medium (56) and high GI (73) foods, respectively. But when the samples were treated with hot water all became part of the low GI (47, 43 and 44) food class according to the classification by Brand-Miller *et al.* (2003).

Discussion

Cooking or heat treatment alters starch's granular and polymeric structures, increasing its accessibility for small intestine digestion and absorption. Gelatinization, caused by heat and water, disrupts the native starch's structure, causing enzymatic destruction and enhancing its availability for absorption (Jung *et al.,* 2009). According to Allan *et al.* (2018) gelatinization temperature for wheat starch is ~60.8°C. Such finding was also observed in previous study of Ghiasi *et al.* (1982).

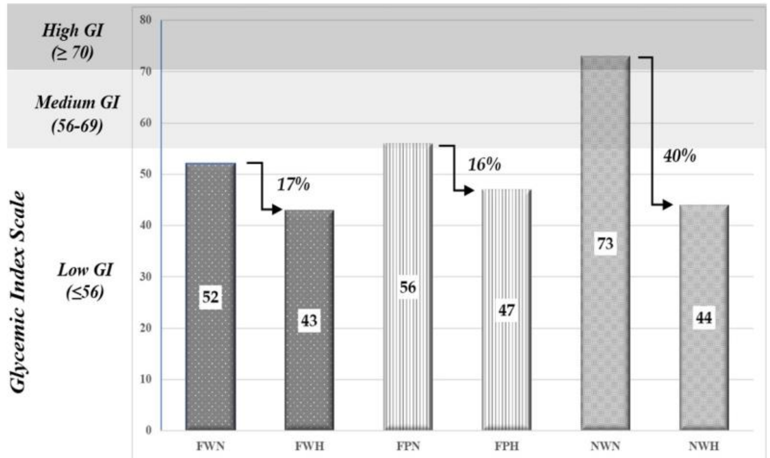

Figure 5: GI response along with categorization of different test foods at hot and normal water recipe. Where, FWN = Fresh whole wheat flour with normal water, FWH = Fresh whole wheat flour with hot water, FPN = Fresh plain wheat flour with normal water FPH = Fresh plain wheat flour with hot water NWN = Natural whole wheat flour with normal water and NWH= Natural whole wheat flour with hot water.

Furthermore, Day *et al.* (2013) indicated that salts inhibit water ingress in starch granules by electrostatic screening, reducing swelling. At 60°C, starch granules swell and absorb liquid irreversibly due to weakening hydrogen bonds in flour. Water enters the molecules, causing swelling, and the organized crystalline structure of starch is lost in gelatinized starch mixes (Ghiasi *et al.,* 1982). Flour dispersed in water at room temperature causes starch granules to slightly swell and settle to the bottom, but this process can be reversed. De Bondt *et al.* (2020) rejected the hypothesis of wheat bran affecting wheat starch gelatinization. Which means plain and whole wheat acts similarly with hot and normal water recipes. After dough making and half-baking, roti's were refrigerated. After being gelatinized, starch progressively starts to undergo rearrangement of its amylose and amylopectin macromolecules at low temperatures, which increases the crystalline structure of the starch molecules. Retrogradation is the term for this technique. Retrogradation is the process by which unstructured gelatinized or solubilized starch is changed into a more structured or crystalline state. According to the report of Chung *et al.* (2006), doing so lowers the GI value as these becomes resistant to digestive enzymes.

Fiber and heating affect starch hydrolysis rates, with fiber slowing down the process when it creates a physical barrier to hydrolytic enzymes, and stone-ground whole meal flour hydrolyzes more slowly than white flour (Snow and O'Dea, 1981). Wheat, consisting of wheat bran, endosperm, and germ, is ground into whole wheat. Plain wheat flour contains the only endosperm, is polished and bleached for whiteness. Its nutritional value is low since it contains a significant amount of gluten content and lack of major portion of proteins, fiber and vitamins. Some kinds of plain wheat in Bangladesh are processed with maize flour, which has a higher GI value (~65-70) (Mlotha *et al.,* 2016). That's why commercial plain wheat flour showed higher GI value compared with commercial whole wheat flour.

Protein-rich foods increase insulin secretion, lowering postprandial blood glucose concentrations. This is due to their starches being difficult to hydrolyze, resulting in lower glycemic index (GIs). Gluten is an example, slowing down pancreatic amylase action, thereby reducing GIs (Eleazu, 2016). This study reveals that whole wheat flour has a higher protein content of 9.5% to 11.5% compared to rice protein (7%). The study suggests that hot water denatures proteins, reducing gluten content, while normal water aids in gluten formation (Cuq *et al.,* 2000). This can be a factor that makes whole wheat flour having lower GI value. The findings suggest the best recipe based on GI variance under different conditions, but adding more brand samples to this in vivo experiment could provide a solid justification.

Conclusion

The study examined the GI values of two cooking recipes (hot and normal water) to compare the commercial wheat flour brand and natural wheat flour. A proper recipe guarantees that the dough is created properly, leading to a more delicious and wholesome end product. Hot water recipe had a lower GI value, suggesting that it would be advantageous for various therapeutic management in both normal persons and those with diabetes. Digestibility and GI are closely related to the degree of starch gelatinization, which is dictated by the cooking conditions and technique. An appropriate (low GI) supper can help preventing further problems and manage type 2 diabetes and obesity. According to the GI classification the normal-water-recipe-containing samples showed as medium and high GI class and hot-water-recipe-containing samples performed as low GI class foodstuff. Thus, it would help consumers to choose the right cooking recipe, thereby making it a more suitable option for individuals with diabetes.

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Ethics approval

The study protocol was approved by the Ethical Standard of Research Committee, BAURES (No. BAURES/ESRC/45/2024).

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