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INVESTIGATION OF THE EFFECTS OF HUYET RONG GERMINATED RED RICE ON GENE EXPRESSION IN DIABETIC MOUSE

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ABSTRACT

Diabetes mellitus is an autoimmune disease that causes hyperglycemia due to the destruction of pancreatic beta cells and deficiency in insulin synthesis. Rates of diabetes increased from 2.7% in 2002 to 5.4% in 2012 and 7.3% in 2020, making it the 7th most leading cause of death in Vietnam. Many studies have shown that dietary change can help alleviate the disease symptoms via improving metabolic control, dyslipidemia, and oxidative stress levels. Huyet Rong red rice, a domesticated rice cultivar rich in vitamins, essential trace elements, antioxidant polyphenols, fiber and a low glycemic index in nature, has become an attractive diet for patients with diabetes. For these reasons, this study aims to investigate the effects of Huyet Rong germinated red rice on the expression of diabetic associated genes, *GLUT-2*, *GLUT-4*, *IR*, *IRS1*, *NFKB1*, and *GSK-3*, in diabetic mouse models using the RT-qPCR method. Our findings reveal that the germination process has significantly increased the level of antioxidant agents, γ-oryzanol and γ-aminobutyric acid, in Huyet Rong red rice grains, rising by 1.2 and 15.1 folds, respectively. RT-qPCR analysis also reveals that the diet supplemented with germinated Huyet Rong red rice flour possesses some positive effects on the STZ-induced mice via increasing the expression of *GLUT2*, *NFKB1*, *IRS1*, and *GSK-3* that are involved in glucose transportation, insulin signaling, and inflammatory and oxidative responses.

Keywords: Diabetes mellitus, Huyet Rong, *Mus musculus*, germinated red rice, RT-qPCR analysis.

INTRODUCTION

Diabetes mellitus is a metabolic disorder that causes increased blood glucose (Briscoe, 2006). Based on the characteristics of the disease, diabetes is divided into two main types. Type 1 diabetes (T1DM) is an autoimmune disease caused by the destruction of beta cells in the pancreas (Klak *et al.*, 2020). Type 2 diabetes (T2DM) is caused by insufficient production of insulin from the pancreas and insulin resistance (Eizirik *et al.*, 2020). In the world, the rate of diabetes is predicted to increase rapidly from 463 million (9.3%) people in 2019 to 578 million and 700 million (increased by 0.9% and 1.6%) by 2030 and 2045, respectively (Saeedi *et al.*, 2019). In which, the majority of cases (>90%) are diagnosed with T2DM. In Vietnam, diabetes is the 7th leading cause of death; statistic studies reveal that the rate of diabetes increased twice in a decade (2.7% in 2002 and 5.4% in 2012) and reached 7.3% in 2020 (Ngoc *et al.*, 2020). The hyperglycemic condition of diabetic disease can lead to a wide range of severe complications, including heart disease, nerve damage, eye and kidney problems, and death in people without proper treatment.

Previous studies showed the interaction between genetic factors and dietary intake in the regulation of diabetes development. Zhang and colleagues have found that a variant of the adiponectin-encoding gene, *ADIPOQ*, which is involved in regulating glucose levels and fatty acid breakdown, interacted positively with diets supplemented with legumes and edible fungi, resulting in a reduction in the risks of T2D complications (Zhang *et al.*, 2023). Dietary changes are one of the most effective ways to reduce the symptoms of the disease due to their effects on metabolic improvements, including better glycemic control, correction of dyslipidemia and reduction of oxidative stress (Imam *et al.*, 2012; Weng *et al.*, 2019). Specifically, Nguyen et al. (2021) reported that feeding induced diabetic mice with a germinated brown rice (GRB) diet, produced by replacing carbohydrate sources with GBR flour, greatly improved the stability of glycemic index and reduced levels of total cholesterol (3.89 mmol/L) and triglycerides (2.06 mmol/L) after 4 weeks (Nguyen *et al.*, 2021). Feeding of GBR diet for 8 weeks also significantly increased insulin levels and decreased blood glucose levels in a C57BLKS/J-db/db diabetic mouse model through its abilities of protecting liver, kidney and pancreatic tissues from oxidative damage (Lee *et al.*, 2019).

Huyet Rong red rice is a domesticated rice cultivar that has been widely cultivating in Africa, India, and Southeast Asia (Boue *et al.*, 2016). Huyet Rong red rice is unpolished rice that is famous for its richness in vitamins (E, B1, B3, B6), essential trace elements (Mn, Mg, Cu, Fe, Zn) and several antioxidant polyphenols (anthocyanin, γ-oryzanol) as well as its abundance in fiber and low glycemic index in nature (Boue *et al.*, 2016; Prasad *et al.*, 2019). These properties have made it a preferable diet for patients with metabolic disorders, including diabetes, than white rice (Prasad *et al.*, 2019).

For those reasons, in this study, we aimed to investigate the effect of germinated Huyet Rong red rice on the expression of genes that are involved in the glucose transport, insulin signaling, and inflammatory and oxidative response pathways in diabetic mice. Firstly, we determined the optimal concentrations of streptozotocin for the induction of diabetes in a high-fat diet (HFD)-fed Swiss albino mouse model, and then the effects of the GRR diet on the expression of diabetic

associated genes (*Glucose Transporter-2* (*GLUT-2*), *GLUT-4*, *Insulin Receptor* (*IR*), *Insulin receptor substrate 1* (*IRS1*), *Nuclear Factor Kappa B1* (*NFKB1*), and *Glycogen Synthase Kinase 3* (*GSK-3*)) were evaluated using the RT-qPCR method. Findings in this study will help to identify the correlation between nutrition and the expression of disease-related genes, thereby providing valuable information for future nutrigenomic research in combating diabetes in humans.

MATERIAL AND METHODS

Animals

Male Swiss albino mice (*Mus musculus* var. albino) were obtained from the National Veterinary Joint Stock Company (VETVACO) and housed in standard plastic cages having sterile rice husk as bedding and maintained in the conditions of controlled luminosity (12-hours light/12-hours dark), 24 ± 2°C and 60 - 70% humidity. Fifty 6-8-week-old mice (20 ± 2g) were used for all experiments. All the experimental procedures were conducted in accordance with the guidelines of the Ethics Committee of the Institute of Genome Research.

Preparation of germinated red rice

Huyet Rong rice grains (*Oryza punctata*) were provided by Hong Van Dien Bien Trading Company Limited, Dien Bien, Vietnam. De-husked rice grains were soaked in warm water overnight and allowed to germinate for 3 days until the shoot reached 0.5-1.0 cm in length. The germinated red rice (GRR) was dried at 50°C for 24 hours and milled into flour. Compositions of GRR were analyzed by the ACQUITY UPLC system (Waters, USA) according to the manufacturer's guidance. The GRR flour was stored in 4^{o}C for subsequent experiments.

The germination rate was calculated by dividing the number of seeds sprouted by the number of total seeds, then multiplied by 100. At least 300 seeds were counted.

Experimental design

Mice were divided into 4 groups. Group 1 (n = 10; control) was fed with a standard regular diet (SRD), Groups 2-4 (n = 16 each) were fed with a high fat diet (HFD; 40% fat, 30% carbohydrate, 30% protein) containing a soybean and coconut oil mixture (1:2; v/v) as the source of fat for 8 weeks. All mice were allowed to drink water *ad libitum* during all experiments.

To induce diabetes, mice in Groups 2-4 were injected intraperitoneally with different concentrations (30, 35 and 40 mg/kg, respectively) of streptozotocin (STZ; Sigma, USA) dissolved in 0.1M citrate buffer (pH: 4.5), while mice in Group 1 were injected with 0.1 M citrate buffer. Post injection, Groups 1-4 were given access to their corresponding diets and maintained under the same housing conditions. Post-10-day period, an intraperitoneal glucose tolerance test (IPGTT) was conducted using a glucometer (Accu-Chek Instant, Roche, Switzerland). Mice were fasted overnight, and their blood glucose levels were recorded before (baseline) and after a glucose injection (50%, 2 g of glucose/kg body mass) at 30 and 60-minute intervals. Mice with a blood glucose level of higher than 11.1 mmol/L were considered to be diabetic.

Diabetic mice from a group were divided into 2 sub-groups and then fed with either a GRR diet (made by replacing the source of carbohydrate in the HFD diet with GRR

flour) or a HFD diet, and maintained at the same housing conditions for 6 weeks (Shen *et al.*, 2015). Subsequently, IPGTT was conducted again to measure the blood glucose level. For all groups, body weight was measured at 2-week intervals. Mice of all groups were sacrificed at the end of the experiment, and liver tissues were harvested for subsequent experiments.

Complementary DNA synthesis and RT-qPCR analysis

Total RNA was extracted from homogenized liver tissues using the RNeasy kit (Qiagen, Germany) following the manufacturer's instructions. The concentration and quality of RNA samples were measured using a Nanodrop™ spectrophotometer (ThermoFisher, USA). CDNA was synthesized from 1.0 µg of total RNA using the ProtoScript® II First Strand cDNA Synthesis Kit (NEB, Vietnam) following the manufacturer's instructions. The synthesized cDNA samples were then stored at -20°C for subsequent experiments.

RT-qPCR analysis was performed on a LightCycler® 96 instrument (Roche, Switzerland) using the Luna® Universal One-Step RT-qPCR Kit (NEB, Vietnam). RT-qPCR reactions (10 µL) consisted of 1X Master mix, 1.0 mM of each primer and 50 ng of cDNA sample. The amplification cycle was carried out at 95°C for 10 minutes (1 cycle) and 95°C for 15 seconds and 60°C for 40 seconds (40 cycles). The used primers were listed in Table 1. The reference genes, *PPIA* and *HPRT1*, were used to normalize the RT-qPCR data (Matoušková *et al.*, 2014; Secio-Silva *et al.*, 2023). RT-qPCR analysis was performed with 3 replicates. The RT-qPCR data were analyzed using Livak's $\Delta\Delta^{Ct}$ method (Livak and Schmittgen, 2001).

Table 1: List of primers used for RT-qPCR analysis

Primer	Sequence (5'-3')	Amplicon (bp)	Gene name	Accession number
GLUT2	F: ATCACCGGAACCTTGGCTTT R: CAGCTTTCCGGTCATCCAGT	151	*Glucose transporter 2*	NM_031197.2
GLUT4	F: CGGCTCTGACGATGGGGAA R: TTGTGGGATGGAATCCGGTC	199	*Glucose transporter 4*	NM_009204.3
IRS1	F: TGGGAGATTCCAACACCAGC R: ATCTTCGGCAGTTGCGGTAT	206	*Insulin receptor substrate 1*	NM_010570.4
NFKB1	F: GAAATTCCTGATCCAGACAAA AAC R: ATCACTTCAATGGCCTCTGTGT AG	194	*Nuclear factor kappa B subunit 1*	NM_008689.3
IR	F: TCCTGAAGGAGCTGGAGGAGT R: CTTTCGGGATGGCCTGG	89	*Insulin receptor*	NM_010568.3

GSK-3	F: TTCTGTTCGGCTACCTTCGG R: AGCCGGAACCAATCAGAGAC	136	*Glycogen synthase kinase 3*	NM_19827.7
PPIA	F: TGGCAAGCATGTGGTCTTTGG R: GATCTTCTTGCTGGTCTTGCC	100	*Peptidylprolyl isomerase A*	NM_008907.2
HPRT1	F: GGTTAAGCAGTACAGCCCCA R: TCCAACACTTCGAGAGGTCC	73	*Hypoxanthine phosphoribosyl transferase 1*	NM_013556.2

Statistical analysis

All data were analyzed using a T-test method on the Microsoft Excel program. The *p*-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Compositions of germinated Huyet Rong red rice grains

Modification of rice flour through the process of germination of rice grains has been found to be one of the most effective methods for the production of a suitable rice-based product to reduce the symptom of diabetes (Weng *et al.*, 2019). It is due to the fact that the nutritional quality and chemical compositions of rice grains have altered via a range of biochemical activities during the process of germination (Moongngarm *et al.*, 2014). In this study, Huyet Rong red rice grains showed a great rate of germination post 3-day-germination period, accounting for 95.3 ± 1.5%. The considerably high rate of germination indicates that the processed rice grains possess a high level of overall quality. The flours of non-germinated (NGRR) and GRR grains were then used as samples for the nutritional composition analysis (Figure 1). The results revealed significant differences in compositions between the NGRR and GRR flours. Particularly, the carbohydrate content of GRR was 2.5% lower than that of the NGRR. In contrast, GRR has a higher content of protein and lipid than its counterpart, increased by 2.5 and 1.1%, respectively (Table 2). More importantly, GRR have been found to possess a higher amount of antioxidant agents, γ-oryzanol (1.2 folds) and γ-aminobutyric acid (GABA; 15.1 folds) post germination (Table 2). These data are comparable with germinated brown rice from previous studies (Sitanggang *et al.*, 2021; Tsou *et al.*, 2024). Furthermore, both γ-oryzanol and GABA have also been found to ameliorate diabetes by protecting pancreatic *β*-cells against apoptosis, and regulating hepatic cholesterol metabolism as well as the expression levels of genes involved in lipogenesis, cholesterol metabolism and fatty acid oxidation in HFD-fed and obese mice (Masuzaki *et al.*, 2019). These data strongly suggest that Huyet Rong rice grains are a healthy substitute for white rice and a potential alternative diet for the treatment of diabetes.

Figure 1. Preparation of germinated Huyet Rong red rice flour. (A) De-husked and polished rice grains. (B) Three-day-old GRR grains, the shoot reached 0.5-0.7 cm in length. (C) Dried GRR grains after 24 hours at 50°C. (D) GRR flour. Bars = 1 cm.

Table 2: Compositions of non-germinated and germinated Huyet Rong red rice grains.

Compositions	Red rice	Germinated red rice
Carbohydrate	78.0%	75.5%
Protein	8.2%	10.7%
Lipid	2.1%	3.2%
Humidity	10.8%	9.2%
Ash	0.9%	1.4%
γ-Oryzanol	17.3 mg/100 g	20.5 mg/100 g
γ-Aminobutyric acid	6.9 mg/100 g	104 mg/100 g

Development of diabetic mouse model

Measurement of weight of HFD-fed mice before diabetic induction

Post 8 weeks of feeding of the SRD and HFD diets, it is clearly visible that the body weight of mice fed with the HFD diet was increasing to a higher level than that of SRD-fed mice (Figure 2). Specifically, SRD-fed mice had a body weight gain from 20.7 g to 33.9 g, which was equivalent to an increase of 1.64 folds after 8 weeks. In contrast, HFD-fed mice have developed obesity conditions, which were evidenced by a significantly increased body weight of 50.9, 51.9 g and 53.2 g in Groups 2-4, respectively; their weight gain reached a higher level of 2.0, 2.2, and 2.4 folds than their controls after 8 weeks, respectively. Diets high in saturated fatty acids (e.g., lards, butter, ghee and coconut oil) have been reported to be more obesogenic than mono- and poly-unsaturated fats (e.g., olive, canola and sesame oils), and they have been demonstrated to be highly associated with hyperglycemia (He *et al.*, 2020; Heydemann, 2016). Given that our results were consistent with previous studies, therefore, the obtained HFD-fed obese mice were ready for induction of diabetes by STZ injection.

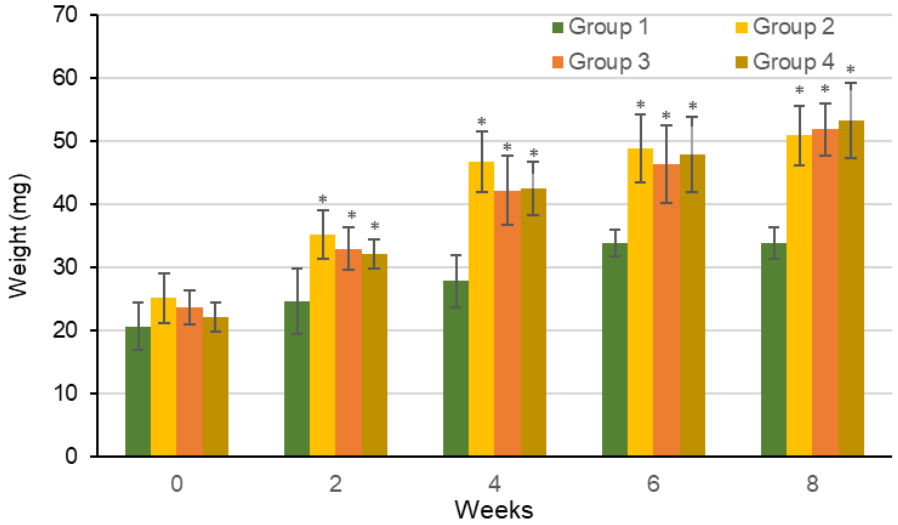

Figure 2. Measurement of weight of HFD-fed mice before diabetic induction. After 8 weeks, the weight of HDF-fed mice (Groups 2, 3 and 4) was significantly higher than that of standard diet-fed mice (Group 1). An asterisk represents a significant difference in comparison with the corresponding control group in each interval (*p*-value < 0.05).

Identification of optimal concentration of STZ for diabetic induction

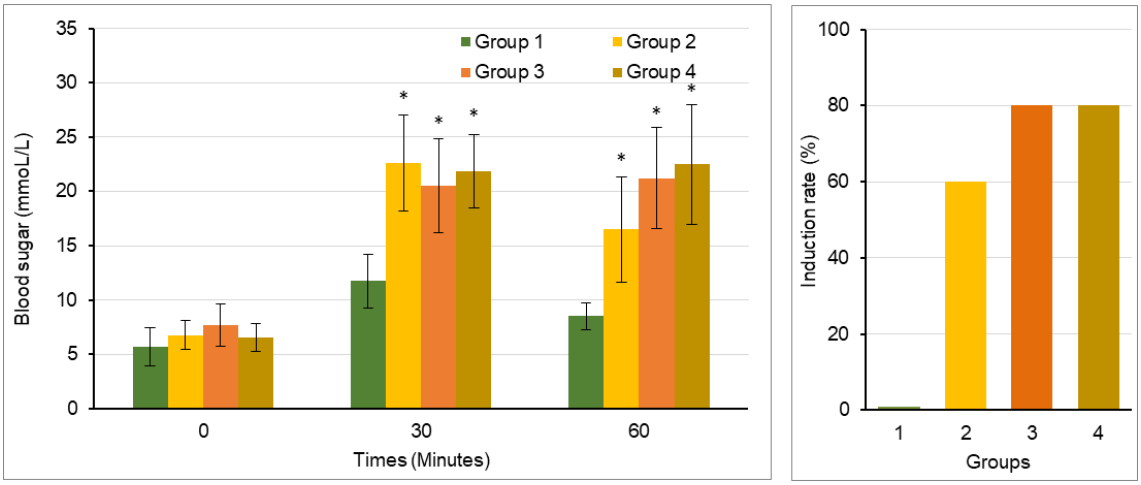

Figure 3. Measurement of blood glucose levels by IPGTT test (A) and rate of diabetic induction (B) in control (Group 1), and STZ-induced mice (Groups 2, 3, and 4). An asterisk represents a significant difference in comparison with the corresponding control group in each interval (*p*-value < 0.05).

To identify the optimal dose of STZ for diabetic induction, blood glucose levels of tested mice were measured by the IPGTT method. Figure 3A reveals that the blood glucose of mice in all groups (1-4) sharply increased to 14.6, 22.6, 20.6, and 21.8 mmol/L, respectively. Group 1 mice, receiving only a citrate buffer injection,

successfully decreased their blood glucose levels to 8.5 mmol/L at the 60-minute mark. However, the blood glucose levels of mice in Groups 2-4, which were injected with a dose of 30, 35, and 40 mg STZ/kg, remained above 11.1 mmol/L 60 minutes post-injection, a threshold at which that indicates a diabetic condition (Akinlade *et al.*, 2021; Skovsø, 2014). Among the 3 evaluated concentrations of STZ, injections of 35 and 40 mg STZ per kg body weight returned the highest rate of diabetic induction, accounting for 80% (Figure 3B). Our findings demonstrated that administering a dose of 35 mg/kg of STZ serves as an effective concentration for the induction of diabetes in mice subjected to a high-fat diet. These diabetic mice were then used for subsequent experiments before their livers were harvested for RT-qPCR analysis.

Expression of diabetic associated genes by RT-qPCR

Evaluation of RT-qPCR primers

Prior to RT-qPCR analysis, primers used in this experiment were evaluated by gel electrophoresis and melting curve methods. Figure 4 reveals that all 8 pairs of primers assessed can effectively amplify the cDNA template. They all yielded a single amplicon of expected size, i.e., *HPRT1* (73 bp), *PPIA* (100 bp), *GLUT2* (151 bp), *GLUT4* (199 bp), *GSK-3* (136 bp), *IR* (89 bp), *IRS1* (206 bp), and *NFKB1* (194 bp). These data have been confirmed by melt curve analysis, where a single peak indicates that all assessed primer pairs were highly specific and only amplified the intended product. Collectively, our data indicated that the selected primers were suitable primers for RT-qPCR analysis.

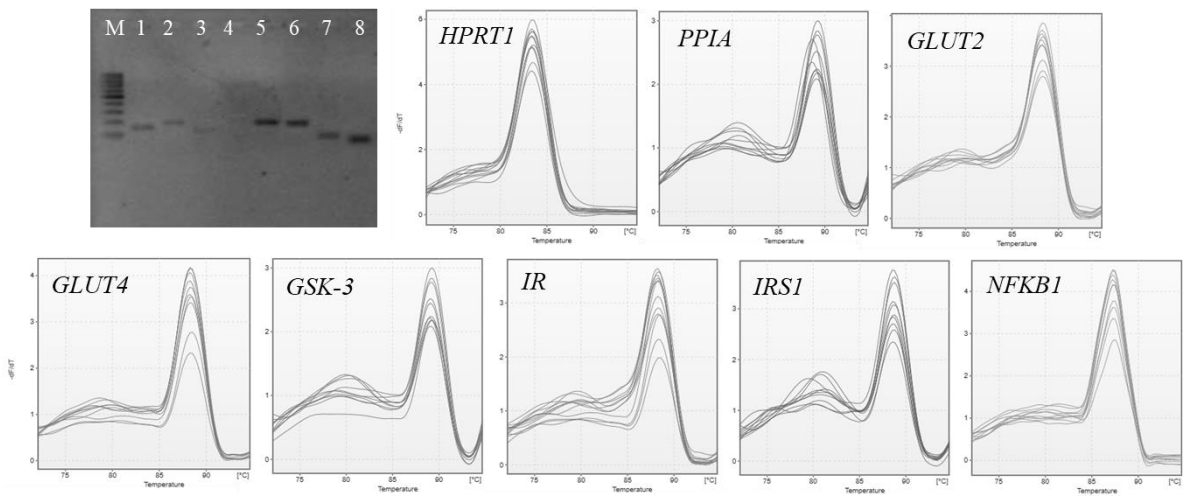

Figure 4. Evaluation of RT-qPCR primers by gel electrophoresis and RT-qPCR melt curves. All primers amplified a single product (single band at expected size and single melt curve peak). M: GeneRuler 100 bp DNA ladder; 1-8: *GLUT2*, *GLUT4*, *GSK-3*, *IR*, *IRS1, NFKB1, PPIA* and *HPRT1*.

Comparison of gene expression in control and diabetic mice

Complementary DNA from control mice (Group 1) and STZ (35 mg/kg)-induced diabetic mice subjected to the HFD (Group 3a) and GRR diets (Group 3b) was used to evaluate the expression levels of *GLUT2*, *GLUT4*, *GSK-3*, *IR*, *IRS1*, and *NFKB1* using RT-qPCR analysis. Our study revealed that expression of *GLUT2* significantly increased

in diabetic mice in Group 3a (subjected to the HFD diet), exhibiting a 4.6-fold change. On the other hand, the increased expression of *GLUT2* rose by just 2.1-fold in mice that were subjected to the GRR diet for 6 weeks. GLUT2 is an insulin independent glucose uniporter located in pancreatic β cells, liver, kidney, and intestine, which plays a crucial role in glucose homeostasis via processes of detecting glucose signals (high blood glucose levels), inducing insulin secretion and regulating the transmembrane transport of glucose into the bloodstream (Shen *et al.*, 2024). Our data are comparable with previous reports, which proved that diabetes causes an increase of up to 67.5% in the expression of *GLUT2* in liver and pancreas (David-Silva *et al.*, 2013), and an accumulation of GLUT2 transporter in diabetic mice (Marks *et al.*, 2003; Shen *et al.*, 2024). Besides GLUT2, GLUT4 is an insulin dependent glucose transporter that facilitates glucose uptake by the cells in the presence of an insulin signal. Figure 5 shows a reduction in *GLUT4* expression in diabetic mice in Groups 3a and 3b compared to their control group counterparts. However, the reduction in expression of *GLUT4* has been found to be statistically insignificant. This outcome is anticipated since *GLUT4* primarily participates in glucose transportation in adipose and muscular tissues, with a minor expression level in liver (Chadt and Al-Hasani, 2020).

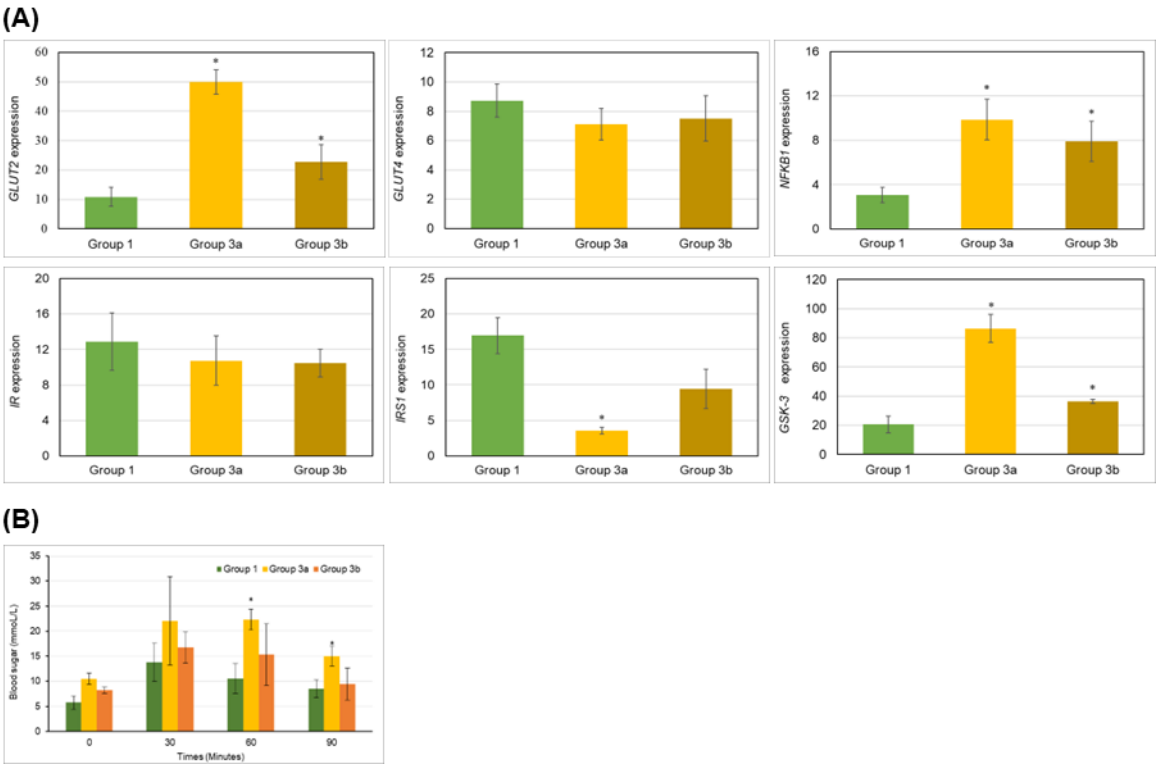

Figure 5. Gene expression of diabetic associated genes in control and diabetic mice by RT-qPCR analysis (A) and measurement of blood glucose levels by IPGTT test (B). Group 1: Control mice that were fed with SRD; Group 3a: STZ (35 mg/kg body weight)-induced mice that were fed with HFD; Group 3b: STZ (35 mg/kg body weight)-induced mice that were fed with GRR for 6 weeks. An asterisk represents a significant difference in comparison with the corresponding control group in each interval (*p*-value < 0.05).

GSK-3 is a serine/threonine protein kinase that mediates the phosphorylation of serine and threonine amino acid residues. In cells, GSK-3 serves as a negative regulator of glycogenesis in response to surplus glucose molecules, with its activity being inhibited by insulin (Eldar-Finkelman and Krebs, 1997; Wang *et al.*, 2022). In the absence of an insulin signal, the activity of GSK-3 increases, resulting in the suppression of the hepatic glycogen storage for future needs and the development of hyperglycemia. Increased expression of *GSK-3* isoforms has also been reported in studies using various mouse models exhibiting insulin resistance and type 2 diabetes (Teli and Gajjar, 2023). This expression trend is evident in our study; specifically, *GSK-3* was strongly expressed in mice in Groups 3a, increasing by 4.2 folds, but its expression was significantly reduced in mice subjected to the GRR diet for 6 weeks, accounting for 2.4 folds. In addition, GSK isoforms have also been identified as negative regulator of IR and IRS1, which are proteins that mediate insulin signaling pathway by recruiting a wide range of regulator subunits (Marushchak and Krynytska, 2021). Figure 5 reveals that the expression of *IRS1* in mice from Groups 3a and 3b was negatively correlated with *GSK-3*, which significantly decreased by 4.8 folds and 1.8 folds, respectively. Interestingly, there was a decrease in the expression of *IR*; however, the changes were negligible regardless of the mice's health conditions and dietary intake.

In this study, the expression of *NFKB1* has been found to significantly increase in mice from Groups 3a and 3b by 3.2 and 2.6 folds, respectively. NFKB1 is a ubiquitous family of transcription factors that regulates the anti-inflammatory and oxidative responses (Meyerovich *et al.*, 2018). Our results are consistent with those of Tantiwong *et al.* (2010), who found that the activity of the *NFKB* increased by 2.7 folds in obese and T2DM subjects, and Lu *et al.* (2015), who found that the NFKB increased the expression cytoprotective genes and activated the anti-inflammatory pathway in the liver of adult mice. Together, these data suggest that the anti-inflammatory and ROS scavenging pathways can be triggered in the tested mice.

Collectively, the RT-qPCR-derived data indicate that there are clear improvements in mice in Group 3, which were administered with the GRR diet for 6 weeks, in terms of glucose transportation, glucogenesis, and anti-inflammatory and oxidative response pathways. The diabetic symptoms in those mice comparatively less severe than those of their counterparts in Group 3a, which were fed with the HFD diet till the end of the experiment. It can be due to the high amount of antioxidant agents (γ-oryzanol and GABA; Table 2) in the GRR, which may protect pancreatic *β*-cells and stimulate insulin secretion (Masuzaki *et al.*, 2019). These data were backed up with the results of the IPGTT test. Particularly, the blood glucose increased rapidly in all 3 groups after 30 minutes of injection and gradually dropped over the following 60 minutes. However, mice in the control Group 1 exhibited the most rapid restoration of blood glucose to normal level (below 11.1 mmol/L), followed by Group 3b which reach 9.4 mmol/L after 90 minutes. In contrast, the blood glucose level of mice in Group 3a persisted at a high level of 15.0 mmol/L at the end of the experiment. Our findings have established a relationship between nutrition, specifically germinated red rice, and the expression of genes associated with the glucose transport, insulin signaling, and

inflammatory and oxidative response pathways in diabetic mice, thereby providing valuable insights for future nutrigenomic research aimed at combating diabetes in humans. Nevertheless, additional experiments are necessary to validate the beneficial effects of GRR on reducing diabetic symptoms.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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