Gamma-Oryzanol Attenuates Myocardial Fibrosis in Type 2 Diabetic Rats Through Modulation of Glycaemic Control and Lipid Metabolism

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ABSTRACT

INTRODUCTION: Diabetes mellitus (DM) is a major public health concern, with a prevalence of over 11% worldwide and expected to rise to 13% by 2050. Cardiovascular disease (CVD) is the leading cause of death among people with diabetes, responsible for roughly one-third of all deaths. In preclinical research, γ-oryzanol (γ-ORZ), a bioactive substance derived from rice bran oil, has demonstrated promising antidiabetic effects. This study used a rat model of type 2 diabetes mellitus (T2DM) to evaluate the cardioprotective effects of γ-ORZ. **MATERIALS AND METHODS:** Fifty-four male Sprague-Dawley rats (200–250 g) were randomly allocated into six groups (n=9): non-diabetic control, non-diabetic + γ-ORZ (100 mg/kg), diabetic control, diabetic + metformin (300 mg/kg), diabetic + γ-ORZ (100 mg/kg), and diabetic + metformin + γ-ORZ (same doses as above). A single injection of streptozotocin (40 mg/kg) was administered after six weeks on a high-fat diet to develop type 2 diabetes. Treatments were given orally for a period of four weeks. **RESULTS:** γ-ORZ significantly lowered fasting blood glucose, glycated haemoglobin (HbA1c), insulin resistance (HOMA-IR), triglycerides (TG), and total cholesterol (TC), while increasing high-density lipoprotein (HDL) levels ($p<0.05$). No significant differences were observed in body weight, food intake, or serum insulin levels ($p>0.05$). Histopathological examination revealed improved cardiac tissue structure in γ-ORZ-treated diabetic rats compared to diabetic controls. **CONCLUSION:** γ-ORZ exhibited notable cardiometabolic benefits in diabetic rats through modulation of glucose and lipid metabolism. These findings support its potential as a natural adjunct therapy for managing diabetes and associated cardiovascular complications.

KEYWORDS: Type 2 diabetes; diabetic heart disease; hyperglycaemia; insulin resistance; diabetic dyslipidaemia

INTRODUCTION

The prevalence of cardiovascular disease (CVD) among individuals with diabetes continues to rise, with reported rates of 46.0% in North America and the Caribbean and 42.5% in Southeast Asia. Overall, diabetic patients exhibit approximately twice the risk of developing CVD compared to non-diabetic individuals,[1] and a two-to fourfold higher risk of developing coronary artery disease (CAD).[2]

Diabetic heart disease (DHD) serves as an umbrella term encompassing three major pathologies associated with diabetes-related complications: coronary artery disease (CAD), cardiac autonomic neuropathy (CAN), and diabetic cardiomyopathy (DCM).[2] Among these, CAD is the most prevalent manifestation, accounting for approximately 29.4% of all CVD cases.[3] The pathophysiological mechanisms underlying DHD are multifactorial, driven primarily by poor glycaemic control and dyslipidaemia. These metabolic disturbances

initiate a cascade of interrelated molecular and cellular processes, including the formation of advanced glycation end products (AGEs), oxidative stress, chronic inflammation, endothelial dysfunction, and impaired insulin signalling, ultimately culminating in adverse cardiac outcomes.[4-8]

Chronic hyperglycaemia alone is sufficient to induce oxidative stress and inflammatory responses that compromise endothelial function. The resultant endothelial injury increases arterial stiffness and accelerates atherogenesis by promoting atherosclerotic plaque formation.[9] Dyslipidaemia, another hallmark of insulin resistance, is typically characterised by elevated triglyceride concentrations and reduced high-density lipoprotein (HDL) cholesterol levels. Insulin resistance alters the activities of lipoprotein lipase and hepatic lipase, leading to disrupted lipoprotein metabolism and the accumulation of atherogenic lipoproteins. This dyslipidaemic profile constitutes a critical risk factor for arterial plaque deposition and the subsequent development of CVD.[10,11]

Although pharmacological agents such as metformin remain central to diabetes management, limitations related to cost, tolerability, and patient adherence have stimulated interest in alternative therapeutic strategies. γ-oryzanol, a bioactive phytochemical derived from brown rice and rice bran oil, has demonstrated antihyperglycaemic, antioxidant, and lipid-lowering properties in both preclinical and clinical models, suggesting its potential utility in the management of type 2 diabetes mellitus (T2DM) and its associated complications.[12] A systematic review[13] further highlighted the capacity of γ-oryzanol to ameliorate diabetes-related complications, particularly diabetic neuropathy and nephropathy, through its antihyperglycaemic, anti-inflammatory, antioxidative, and antidyslipidaemic mechanisms. However, evidence regarding its efficacy in diabetic cardiovascular complications, specifically DHD, remains scarce. The present study, therefore, aims to address this gap by evaluating the cardioprotective potential of γ-oryzanol in comparison with metformin, a standard antidiabetic agent.

MATERIALS AND METHODS

Preparation of γ-Oryzanol and Metformin Solutions

The γ-oryzanol powder used was purchased from SolarBio Life Sciences, Beijing, China (CAT No. YS157671), while the metformin brand used was Diabetmin, manufactured by Hovid Berhad, Malaysia. γ-Oryzanol and metformin solutions were prepared separately. γ-Oryzanol (5 g) was dissolved in 50 mL of 4% Tween-80 to obtain a final concentration of 100 mg/mL, while metformin (15 g) was dissolved in 50 mL of distilled water to achieve a final concentration of 300 mg/mL. Both solutions were freshly prepared weekly and stored at 4 °C.

Animals

Based on the hormonal analysis (glucose, insulin, and HOMA-IR) from an earlier study, the sample size was calculated using PS Power Software (version 3.1.6).[14] The ultimate sample size determined using these parameters was 54 rats (n=9 per group). All animals were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM) Health Campus, following approval by the Institutional Animal Care and Use Committee of USM (USM/IACUC/2022/(137)(1229). Animals were housed three per opaque polypropylene Type I cage, with sufficient bedding, under controlled conditions (temperature:

23 ± 1°C; relative humidity: 50 ± 5%); a 12-hour light/dark cycle was maintained. Standard commercial food pellet diet ([SPD], Gold CoinFeedmills, Port Klang, Malaysia) and water were provided ad libitum.

Induction and Assessment of Obesity and T2DM

Fifty-four adult rats were split into two groups at random: an obese group and a control group. For six weeks, the obese rats (n=36) were fed a high-fat diet (HFD) developed based on the previously described method[15] to promote obesity, whereas the control group (n=18) was provided an SPD. The HFD was composed of 50% SPD, 38% pure ghee, 8% full-cream milk powder, and 4% white sugar. The diet provided an energy density of approximately 5.61 kcal/g, whereas the SPD produced an energy density of 3.27 kcal/g.

Rats were deemed obese if their Lee Obesity Index score was greater than 315. This threshold was applied based on recent literature, which defines this value as indicative of obesity in rat models.[16,17] Moreover, this higher threshold was selected to provide a stringent definition of obesity in the diabetic model and to reduce potential confounding effects of diabetes-associated changes in lean body mass.

After an overnight fast, 40 mg/kg of streptozotocin (STZ) was dissolved in 0.1 M sodium citrate buffer, pH 4.5, and injected intraperitoneally to induce diabetes. On the other hand, a volume of citrate buffer solution equivalent to the body weight of the animals was administered to the normal diet group.[18] To reduce mortality from hypoglycaemia, rats were given drinking water enriched with 15 g/L of sugar for a full day.[19] After seven days of STZ injection, rats were diagnosed with diabetes if their fasting blood glucose levels were more than 11.1 mmol/L, as determined by a glucometer taken from the tail vein.[20]

Animal Groupings and Treatment

Six groups (n=9) of diabetic and non-diabetic rats were randomly assigned and given the following treatments:

Group 1: Non-diabetic control rats (NC) administered with 4% Tween 80 solution equivalent to their body weight.
Group 2: Non-diabetic rats administered with γ-oryzanol (N+γ-ORZ) at a dose of 100 mg/kg.
Group 3: Diabetic control rats (DC) administered with 4% Tween 80 solution equivalent to their body weight.
Group 4: Diabetic rats administered with Metformin (D+MTF) at a dose of 300 mg/kg
Group 5: Diabetic rats administered with γ-oryzanol (D+γ-ORZ) at a dose of 100 mg/kg
Group 6: Diabetic rats administered the combination of metformin and γ-oryzanol (D+MTF+γ-ORZ) at the same previous dose.

For four weeks, oral gavage was used to deliver all treatments every day. The dose of γ-oryzanol was chosen based on a previous study by Ghatak [21]

Assessment of Physical Parameters

The body weights of the rats were recorded at baseline (Week 0) and at the end of the treatment period (Week 4), and intergroup differences were subsequently analysed. Food and water intake were measured daily

at the cage level by weighing the amounts provided and the remaining quantities after 24 hours. Any spillage was collected and weighed separately, then subtracted to determine the net food consumption. Daily intake was expressed as the mean intake per rat by dividing the net cage consumption by the number of rats housed per cage.

Assessment of Biochemical Parameters

All animals were treated for four weeks according to their assigned experimental groups. At the end of the treatment period, animals were euthanised, and blood samples were collected for the assessment of glycaemic and lipid parameters. Serum was separated and stored at -20°C until analysis.

Glycaemic Parameters

Final fasting blood glucose (FBG) was measured from the tail vein following an overnight fast using a glucometer (ACCU-CHEK Guide Me; Roche Diagnostics, Germany). Serum glycated haemoglobin (HbA1c) and insulin concentrations were determined using commercially available ELISA kits (Cat. Nos. ELK5370 and ELK2370, respectively), in accordance with the manufacturers' instructions. Insulin concentrations were converted to standard units (pmol/L), while HbA1c was reported as absolute concentrations from serum, since total haemoglobin was not measured, consistent with the ELISA kit specifications. Insulin resistance was evaluated using the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) according to the following formula:

HOMA-IR = [fasting glucose (mmol/L) x fasting insulin (μIU/ml)]/22.5.[22]

Lipid Parameters

Serum lipid profile parameters, including total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C), were measured using Siemens Healthineers assay kits: Atellica CH Cholesterol_2 (REF 11097609), Atellica CH Triglycerides_2 (REF 11537222), and Atellica CH HDL Cholesterol (REF 11537213). All analyses were performed according to the manufacturer's instructions using an automated analyser (Atellica CH Analyser, Siemens Healthineers, Germany). Results were expressed in mmol/L.

Histological Examination of Heart Tissue

The left ventricle was excised and immediately fixed in 10% formaldehyde. Fixed tissues were dehydrated in a graded series of alcohols, cleared in xylene, and embedded in paraffin. Serial sections (5 μm thick) were prepared using a microtome. Sections were stained with haematoxylin and eosin (H&E; [Sakura manual staining, Finetek Japan]) for general histological examination and with Masson's trichrome (MT stain kit, Cat. No. K035, Diagnostic BioSystems, USA) to assess fibrosis. Histological images were captured using the Motic digital slide assistant (version 1.0.7.61b, Motic Asia, Hong Kong) at 10× magnification and saved as TIFF files. Fibrotic areas were quantified using ImageJ software (National Institute of Health, Bethesda, Maryland), as previously described.[23]

Data Analysis

All data were analysed using GraphPad Prism version 10.4.2 (633) (GraphPad Software, San Diego, CA, USA). Comparisons between all groups were performed using one-way analysis of variance (ANOVA)

followed by Tukey's post hoc test. Data are presented as mean ± standard deviation (SD), and a *p*-value of <0.05 was considered statistically significant.

RESULTS

Effects of γ-Oryzanol on Physical Parameters in Rats

The effects of γ-oryzanol on daily food intake, daily water intake, and body weight are displayed in Table I. The results show that γ-oryzanol treatment significantly reduced water intake in diabetic rats compared with the diabetic control group (p=0.0001), although intake remained higher than in the normal control group.

However, it showed no significant effects on daily food intake (p=0.0734) and weekly body weight (p=0.4933) compared with the diabetic control group.

Table I: Effects of γ-oryzanol on daily food, daily water intake, and body weight in diabetic and non-diabetic rat groups.

Groups	Daily water intake (ml/day)	Daily Food intake (g/day)	Body Weight (g)		
			Week 0	Week 4	Gain/Loss
NC	38.85±5.46^{a}	25.21±1.56^{a}	349.80±24.80^{a}	385.90±23.00^{a}	36.10±1.80
N+γ-ORZ	35.30±5.53^{a}	24.24±1.80^{a}	345.70±9.58^{a}	367.60±19.62^{a}	21.90±10.04
DC	65.44±4.28^{b}	21.62±2.14^{b}	419.20±27.19^{b}	357.40±23.04^{a}	-61.80±4.15
D+MTF	43.42±4.19^{c}	18.87±2.00^{c}	421.00±17.00^{b}	397.50±18.88^{b}	-23.50±1.88
D+γ-ORZ	56.89±4.11^{d}	20.62±1.36^{b}	423.20±31.71^{b}	376.80±23.02^{a}	-46.40±8.69
D+MTF+γ-ORZ	48.52±5.21^{e}	18.96±2.11^{c}	422.30±20.37^{b}	391.60±23.10^{b}	-30.70±2.73

All values are expressed as mean ± SD. Group NC: non-diabetic control; Group N+γ-ORZ: non-diabetic rats treated with γ-oryzanol (100 mg/kg); Group DC: diabetic control; Group D+M: diabetic rats treated with metformin (300 mg/kg); Group D+γ-ORZ: diabetic rats treated with γ-oryzanol (100 mg/kg); Group D+MTF+γ-ORZ: diabetic rats treated with metformin (300 mg/kg) and γ-oryzanol (100 mg/kg). Groups sharing at least one common superscript letter ($^{a–e}$) are not significantly different, whereas groups with no common letters differ significantly (p<0.05).

Effects of γ-Oryzanol on Biochemical Parameters in Rats

The effects of γ-oryzanol on FBG, HbA1c, insulin, HOMA-IR, TC, TG, and HDL are presented in Table II. The results illustrated that γ-oryzanol substantially decreased FBG (p=0.0223), HbA1c (p=0.0005), HOMA-IR (p=0.0296), TC (p=0.0001), TG (p=0.0001) and raised HDL (p=0.0090) levels but revealed no significant impact on insulin levels (p=0.9672) in the γ-oryzanol-treated diabetic group compared with the diabetic control group.

Table II: Effects of γ-oryzanol on FBG, HbA1c, insulin, HOMA-IR, TC, TG, and HDL in diabetic and non-diabetic rat groups.

Groups	FBG (mmol/L)	HbA1c (µg/mL)	Insulin (pmol/L)	HOMA-IR	TC (mmol/L)	TG (mmol/L)	HDL (mmol/L)
NC	5.44±0.46^{a}	197.3±62.47^{a}	0.17±0.09^{a}	0.25±0.08^{a}	2.00±0.25^{a}	0.60±0.10^{a}	0.66±0.05^{a}
N+γ-ORZ	4.63±0.33^{a}	193.0±45.47^{a}	0.16±0.07^{a}	0.24±0.10^{a}	1.90±0.28^{a}	0.58±0.16^{a}	0.75±0.03^{a}
DC	28.31±3.45^{b}	443.6±46.22^{b}	0.22±0.06^{a}	1.48±0.40^{b}	4.40±1.65^{b}	10.21±6.38^{b}	0.83±0.10^{b}
D+MTF	11.94±3.80^{c}	278.0±81.72^{c}	0.18±0.08^{a}	0.69±0.31^{c}	2.51±0.53^{c}	3.27±1.76^{c}	0.95±0.06^{c}
D+ORZ	23.75±3.80^{d}	312.4±53.83^{c}	0.19±0.04^{a}	1.00±0.46^{c}	2.33±0.26^{c}	2.60±0.78^{c}	0.96±0.09^{c}
D+MTF+γ-ORZ	12.43±2.15^{c}	257.5±38.41^{c}	0.18±0.04^{a}	0.69±0.21^{c}	2.36±0.58^{c}	3.50±1.93^{c}	0.96±0.12^{c}

All values are expressed as mean ±SD. Different superscripts ($^{a–d}$) indicate significant differences between groups ($p<0.05$). Note that group definitions are the same as in Table I.

Effects of γ-Oryzanol on Myocardial Histological Changes

Figure 1 presents the effects of γ-oryzanol on heart tissue in rats assessed using H&E and Masson's trichrome stains. In the diabetic control group (DC), proinflammatory cell infiltration (arrowhead), disarrayed myocardial striations (dotted arrow), and increased interstitial fibrosis (asterisk) were markedly observed.

Conversely, these pathological changes were reduced in the treatment groups (metformin, γ-oryzanol, and combination). Figure 2 illustrates the graphical representation of interstitial and perivascular fibrosis findings from the Masson's trichrome stain assessed using ImageJ software.

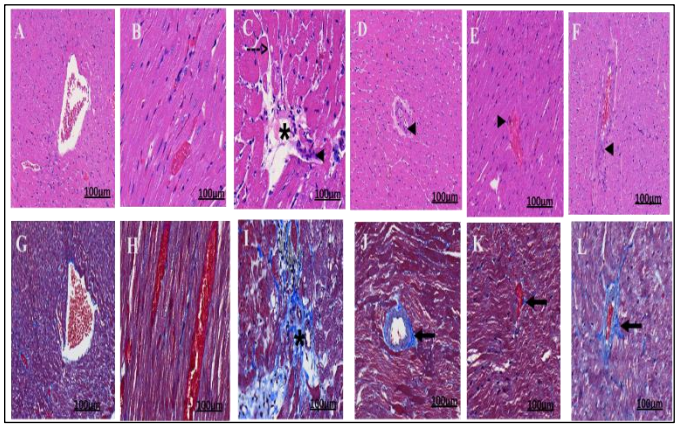

Figure 1: Representative photomicrographs of myocardial histology across experimental groups.

Panels A-F show haematoxylin and eosin (H&E) stained sections, while panels G-L show Masson's trichrome stained sections: A, G: Non-diabetic control group showing normal myocardial architecture with intact cardiomyocytes and minimal collagen deposition; B, H: Non-diabetic rats treated with γ-oryzanol showing preserved myocardial structure comparable to controls; C, I: Untreated diabetic group showing marked myocardial disorganisation, inflammatory cell infiltration, and extensive interstitial collagen deposition.; D, J: Diabetic rats treated with metformin showing partial improvement in myocardial architecture with reduced fibrosis; E, K: Diabetic rats treated with γ-oryzanol showing improved cardiomyocyte alignment and attenuated collagen accumulation; F, L: Diabetic rats treated with combined γ-oryzanol and metformin showing moderate structural restoration and reduced fibrotic changes. Black arrows (→) indicate perivascular fibrosis, arrowheads (→) indicate inflammatory cell infiltration, asterisks (*) indicate collagen deposition, and a dotted arrow (→) indicates disarrayed myocardium. Images are representative of nine animals per group. Original magnification ×10; scale bar = 100 µm.

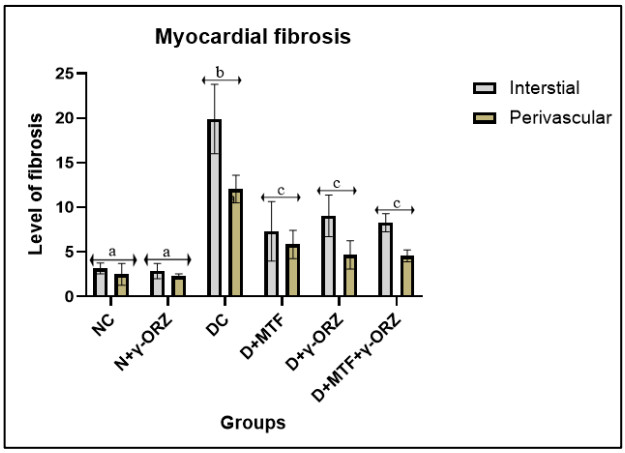

Figure 2: Quantitative analysis of myocardial fibrosis in experimental rat groups assessed by Masson's trichrome staining and analysed using ImageJ software. Fibrosis is expressed as the percentage collagen-positive area of myocardial tissue. Data are presented as mean ± SEM. Group NC: non-diabetic control; Group N+γ-ORZ: non-diabetic rats treated with γ-oryzanol (100 mg/kg); Group DC: diabetic control; Group D+M: diabetic rats treated with metformin (300 mg/kg); Group D+γ-ORZ: diabetic rats treated with γ-oryzanol (100 mg/kg); Group D+MTF+γ-ORZ: diabetic rats treated with metformin (300 mg/kg) and γ-oryzanol (100 mg/kg). Columns sharing different superscript letters($^{a-c}$) indicate significant differences between groups ($p<0.05$).

DISCUSSION

To the best of our knowledge, this is the first study to clarify the cardioprotective potential of γ-oryzanol in diabetic conditions, compare its effectiveness with that of a common antidiabetic medication, and investigate potential synergistic interactions between the two interventions. According to the findings of this study, γ-oryzanol significantly reduced excessive water intake, indicating partial amelioration of polydipsia but had no significant impact on food intake or body weight. In contrast, a previous study[24] reported differing results for both diabetic and non-diabetic rats. This discrepancy may be attributed to variations in experimental duration, as the present study employed a four-week intervention period, whereas the comparative study used an eight-week protocol. Therefore, duration-dependent effects cannot be excluded.

The observed reduction in water intake is likely due to the antihyperglycaemic effects of γ-oryzanol, which enhance metabolic homeostasis, alleviate osmotic diuresis, and consequently reduce thirst. However, water intake remained higher than that of the normal control group, likely due to persistent hyperglycaemia leading to continued osmotic diuresis. This observation aligns with the modest reductions in fasting blood glucose and HbA1c levels observed in the γ-oryzanol-treated group, suggesting that while γ-oryzanol improves polydipsia and glycaemic control, it does not completely restore glycaemic homeostasis.

All diabetic groups experienced some degree of weight loss during the four-week treatment period. γ-oryzanol did not significantly attenuate weight loss compared with the untreated diabetic control group, in contrast to the metformin-treated and combination-treated groups, which exhibited more pronounced improvements. Although all diabetic groups showed lower food intake compared to the non-diabetic groups, the untreated diabetic group still demonstrated relatively higher consumption than the diabetic-treated groups. However, γ-oryzanol treatment did not significantly reduce food intake relative to the untreated diabetic control, whereas the metformin-treated and combination-treated groups demonstrated more notable improvements.

Gamma-oryzanol significantly improved FBG, HbA1c, and HOMA-IR without affecting serum insulin levels. A similar pattern of effects, with significant reductions in FBG and HbA1c, and no notable changes in serum insulin or HOMA-IR, was reported in a previous study involving diabetic patients.[25] In contrast, an earlier study using a rodent model of metabolic syndrome demonstrated a significant improvement in HOMA-IR, suggesting possible model-dependent variability.[26]

The observed antihyperglycemic effects of γ-oryzanol may be attributed to its ability to reduce glucose absorption in the gastrointestinal tract by inhibiting α-amylase and α-glucosidase enzymes.[27] Additionally, γ-oryzanol may alleviate insulin resistance by upregulating glucose transporter-4 (GLUT-4) expression in skeletal muscle.[28] Given the strong correlation between inflammation and insulin resistance, the improvement in HOMA-IR may also result from enhanced adiponectin secretion. This anti-inflammatory adipokine promotes insulin sensitivity and reduces resistance.[29]

The antidyslipidaemic effects of γ-oryzanol are among its most consistently reported pharmacological actions.[13] In the present study, γ-oryzanol significantly influenced total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL) levels. The effects on TC and TG were consistent with previous findings in rodent models of diabetes mellitus,[30–32,] while the observed increase in HDL levels aligns with earlier reports.[21,33]

These lipid-modulating effects may result from γ-oryzanol's ability to inhibit the incorporation of cholesterol into micelles, thereby reducing intestinal cholesterol absorption and enhancing faecal excretion.[34] Additionally, γ-oryzanol may exert inhibitory effects on 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. This rate-limiting enzyme catalyses the conversion of HMG-CoA to mevalonate in the cholesterol biosynthetic pathway, as previously documented.[35] Collectively, these mechanisms likely contribute to the observed reduction in blood cholesterol levels in the present study.

The histological findings supporting the biochemical results obtained in this study align with recent findings from a study.[36] In diabetes, chronic hyperglycaemia and dyslipidaemia promote myocardial fibrosis through the excessive generation of advanced glycation end products (AGEs) and reactive oxygen species (ROS), activation of NF-κB and TGF-β/Smad signalling, reduced nitric oxide (NO) bioavailability, and lipid peroxidation, as reflected by increased malondialdehyde (MDA) levels. These metabolic disturbances drive cardiomyocyte injury, fibroblast activation, and the progression of interstitial and perivascular fibrosis, ultimately leading to myocardial stiffness and the disruption of normal cardiac architecture.[37-40] By improving glycaemic and lipid homeostasis, γ-oryzanol may mitigate these pathological changes, thereby reducing oxidative stress, inflammatory signalling, and profibrotic remodelling, which leads to the preservation of myocardial architecture in the treated animals. However, the absence of direct measurements of cardiac analysis markers, oxidative stress, and inflammatory markers remains an essential limitation of the study.

Furthermore, the combination of γ-oryzanol with metformin provided only modest and statistically insignificant improvements in HbA1c and HOMA-IR, suggesting a possible overlap in their mechanisms of regulating glycemic and lipid metabolism, consistent with mechanisms reported in previous studies.[21,41,42] However, the precise pathways underlying this interaction remain unclear and warrant further investigation.

Findings from the non-diabetic group indicate that the beneficial effects of γ-oryzanol are more pronounced under pathological metabolic conditions. Overall, these results highlight the potential of γ-oryzanol as a natural adjunctive therapy for diabetic heart diseases.

CONCLUSION

In conclusion, γ-oryzanol attenuated myocardial fibrosis in type 2 diabetic rats, potentially through modest improvements in glycaemic control and lipid metabolism. While the combination with metformin did not produce significant additive effects, these findings highlight the potential of γ-oryzanol as an adjunctive therapy for diabetic heart disease. Further studies are warranted to clarify the underlying molecular mechanisms.

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CONFLICT OF INTEREST

The publishing of this report has no conflicts of interest, as declared by all authors.

INSTITUTIONAL REVIEW BOARD (ETHICS COMMITTEE)

The Universiti Sains Malaysia Institutional Animal Care and Use Committee's rules (USM IACUC) were strictly adhered to in executing all experimental procedures, as contained in the approval USM/IACUC/2022/(137)(1229).

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