RESEARCH ARTICLE

Red Rice Bran Ethanol Extract Reduces IL-1β as the Risk of Pancreas Fibrogenesis in Type 2 Diabetic Rat Model

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Abstract

BACKGROUND: Oxidative stress and inflammation contribute to pancreatic cell dysfunction that promote insulin resistance in type 2 diabetes (T2D). Red rice bran contains bioactive substances with anti-inflammatory and antioxidant properties which improved insulin resistance in obese mice. However, no studies have explored the potential of ethanol extract of red rice bran (EERRB) to prevent T2D progression, particularly pancreatic fibrosis complications. This study was conducted to investigate the effect of EERRB on inflammation measured with interleukin (IL)-1β and fibrosis of pancreatic tissue in a rat model of T2D.

METHODS: Rats were induced with streptozotocin and nicotinamide to induce diabetes, and then separated into five groups. One group received no treatment, while the other four received 9 mg/kg/day acarbose, 165, 330, or 660 mg/kg/day EERRB orally for 21 days. Immunohistochemistry was conducted on pancreas tissues to measure the expression of IL-1β, while pancreatic fibrosis was assessed with Masson's Trichrome staining.

RESULTS: EERRB reduced the expression of pro-inflammatory cytokine, IL-1β, in pancreas tissue in a dose dependent manner. Significantly lower IL-1β expression were found in group receiving 660 mg/kg/day EERRB (10%) compared to diabetic with no treatment group (50%) (p<0.0001). Additionally, the IL-1β expression in the highest dose of EERRB group was comparable to the group receiving acarbose (10%).

CONCLUSION: This finding suggests the beneficial effect of EERRB in the hyperglycemic condition that causes oxidative stress through blocking the IL-1β expression, hence alleviating the inflammation in pancreas tissue, and have a tendency in preventing pancreatic fibrosis progression, a process implicated in T2D pathogenesis.

KEYWORDS: diabetes, inflammation, pancreatic fibrosis, red rice bran

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Introduction

Diabetes is a global health problem with 1.4 millions deaths based on global burden of disease study.(1,2) The burden of diabetes is rising, particularly in low- and middle-income countries, which account for over 80% of global diabetes-related mortality.(3) It is also projected that Southeast Asia will have one of the highest rates of diabetes prevalence in the next two decades.(4) The increasing prevalence of diabetes is a global health concern and type 2 diabetes (T2D) accounts for over 90% of all diabetes cases is associated with abnormal insulin secretion, insulin resistance, and a decrease in insulin's compensatory response.(5)

T2D is defined as persistent high blood glucose levels that is caused by an insufficient amount of insulin due to oxidative stress and inflammation. These factors lead to pancreatic cell dysfunction and insulin resistance, as well as altered insulin secretion.(6-8) Evidences indicate that interleukin (IL)-1β, either independently or in conjunction with tumor necrosis factor (TNF)-α and interferon (IFN)-γ, facilitates β-cell failure and apoptosis.(7) An excess of nutrients and metabolic activity cause a chronic inflammatory condition in diabetes mellitus leading to pancreatic fibrosis. This fibrosis was mediated by activation of substances typically found in inflammatory signaling cascade.(9) In particular, hyperglycemia was reported to induce the production of IL-1β, which contribute to the damage of pancreatic tissue.(10)

Prior studies utilizing ultrasonography and computed tomography (CT) have demonstrated a reduction of 7–22% pancreatic volume in individuals with T2D compared to those without glucose intolerance. The reduction of cell numbers, increasing fat accumulation, and pancreatic fibrosis were hypothesized underlying those conditions.(11) It was known that pancreatic tissue damage could promote fibrosis. This damage can result in cell necrosis and/or apoptosis, leading to the generation of cytokines and growth factors.(7,12) Afterwards, macrophages engulf the injured cells, and the produced cytokines stimulate the growth and conversion of local fibroblasts into myofibroblasts.(13)

Effective diabetic patient management focuses primarily on preventing chronic hyperglycemia-related injuries. Moreover, randomized controlled clinical trials have consistently demonstrated that maintaining a strict glycemic level is effective in preventing diabetic complications.(14) The therapeutic approaches to controlling glycemic levels have advanced in recent decades. Nevertheless, anti-diabetic drugs can lead to severe hypoglycemia and impairments in liver and kidney function.(15) Despite the various constraints linked to the use of current synthetic antidiabetic drugs, the attempt to develop novel antidiabetic therapies derived from natural sources persists.(16)

Rice is a widely consumed cereal grain that serves as a staple diet for half of the world's population. The rice milling process produces a variety of byproducts, including 10.5% of whole-grain rice and 20% of rice husk, germ, and seed coat, collectively known as rice bran.(17) Red rice bran contains various bioactive phytochemical including flavonoids, anthocyanins, vitamin E (tocotrienols and tocopherols), and γ-oryzanol. Anthocyanins, a subtype of flavonoids, are responsible for the red-purple pigment in the ethanol extract of red rice bran (EERRB).(18) The crude extract of red rice possesses anti-cancer, anti-inflammatory, antioxidant, anti-obesity, and ameliorated insulin resistance.(17) EERRB at dose 330 and 660 mg/kgBW/day effectively reduced fasting blood glucose level in T2D rat model induced by streptozotocin (STZ) and nicotinamide (NA).(18)

The pancreatic fibrosis was mediated by activation of substances typically found in inflammatory signaling cascade.(9) One of the identified inflammation markers that has been linked to the damage in pancreatic β-cells is IL-1β, a cytokine that promotes inflammation and affects key metabolic processes such as insulin production and β-cell failure as well as apoptosis.(7,19) Macrophages in pancreatic β-cells are the primary sources of IL-1β production. The IL-1 receptor antagonist (IL-1Ra) tightly controls the levels of IL-1β. The pancreatic β cells exhibit a high level of IL-1 receptor (IL-1R) compared to other cells. Maintaining the right balance between IL-1β and IL-1Ra levels is important for controlling the response of β-cells and the progression of T2D.(20)

The above evidence strongly supports EERRB's potential for ameliorating T2D. However, previous studies have not yet explored the potential of EERRB to hinder the progression of T2D, particularly pancreatic fibrosis complications. Therefore, this study was conducted to determine whether EERRB prevents the expression of IL-1β as a pro-inflammatory cytokine and inhibits pancreatic fibrosis formation.

Methods

Preparation of Diabetes Rats Model

Rats were intraperitoneally injected with 65 mg/kg STZ and 230 mg/kg NA (STZ-NA) to induce diabetes. NA was dissolved in normal saline and injected 15 minutes before STZ administration. STZ was mixed with citrate buffer (PH 4.5) immediately before use. STZ-NA injections were given once daily for five days. Following this 5-days STZ-NA administration, fasting blood glucose (FBG) was measured using a glucometer. Rats classified as diabetic (FBG>150 mg/dL 3-days post STZ-NA induction) were included in the study.(21)

EERRB Preparation and Phytochemical Analysis

The red rice bran (*Oryza sativa* L.) (PT. Bakti Indonesia, Magelang, Indonesia) used in this study was originally locally cultivated in Magelang. The extraction was started

by soaking the red rice in 96% ethanol for 7 days (1 gram bran in 6 mL ethanol). During the extraction process, it was stirred regularly on a shaker every 6 hour for 5 minutes (speed 150 rpm) at room temperature. After that, we squeezed the extract and filtered it with Whatman filter paper followed by evaporation using rotatory evaporator at 30°C. The red rice bran ethanol extract was prepared as previously described.(18)

In this study, the phytochemical analysis to measure the concentration of total flavonoid and anthocyanin was conducted using UV-Vis Spectrophotometry at wavelength between 535 nm and 700 nm. This was based on previous study measuring total anthocyanin levels of ethanol extract of Red Andong Leaf Extract.(22) Total flavonoid and anthocyanin content were 456.40 mg/100 gram and 340.24 mg/100 gram, respectively.

Animal Grouping and Treatments

A total of 35 rats (male, strain Wistar, ±8 weeks old) were obtained from Centre of Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. Rats were housed individually in a room with controlled temperature (21-22°C), lighting (lights on at 7 AM off at 7 PM) and humidity (45-60%). A 7-days habituation period was given before experimentation. Standard chow, which was Comfeed AD II (Japfa Comfeed Indonesia, Jakarta, Indonesia) and water were available *ad libitum* throughout the experimental period. Rats were assigned into 5 groups randomly each comprising of 7 rats: diabetes only group (STZ-NA), diabetes group receiving acarbose treatment at a dose of 9 mg/kg/day (STZ-NA + ACA), diabetes group receiving EERRB at the dose of 165 mg/kg/day (STZ-NA + 165EERRB), 330 mg/kg/day (STZ-NA + 330EERRB), and 660 mg/kg/day (STZ-NA + 660EERRB), respectively. EERRB was orally administered daily using a gastric probe for 21 days. The experimental procedure was depicted in Figure 1. All experiments in the study protocol were approved by The Research Ethics Committee Faculty of Medicine Universitas Sebelas Maret Indonesia (No. 58/UN27.06.6.1/KEP/EC/2021).

Immunohistochemistry (IHC) Analysis for IL-1β Expression and Pancreatic Fibrosis Assessment

Pancreas tissue was harvested, and fixed in 4% paraformaldehyde, and then embedded in paraffin wax. For IHC processing, the tissue was firstly dewaxed, followed by antigen retrieval process. Tissue was put in a microwave oven at 90°C for 3 minutes with Tris-EDTA antigen retrieval buffer (pH=9). The tissue was then let to cool down for 15 minutes and washed with phosphate-buffered saline (PBS) twice, each lasting for 5 minutes. After that, 3% H_2O_2 was applied to block endogenous peroxidase and then washed with running water for 5 minutes. The tissue was then blocked with 10% normal donkey serum (NDS) for 10 minutes followed by primary antibody incubation at 4°C for 18 hours. Rabbit primary antibody against IL-1β (Cat. No. A20527; Abclonal, Woburn, MA, USA) was used. Staining for IL-1β was performed using TrekAvidin-HRP Label as per the manufacturer's instructions (Cat. No. STHRP700 L10; Biocare Medical, Pacheco, CA, USA). Tissues were incubated with 3,3-diaminoben-zidine tetrahydrochloride (DAB) as chromogen and hematoxylin eosin for counterstaining. Meanwhile, to assess pancreatic fibrosis, Masson's Trichrome staining procedure was used. All sections were analyzed by light microscopy BX53 (Olympus, Tokyo, Japan).

Manual examinations of IL-1β expression and fibrosis were conducted by two pathologists who assessed the entire field of view of pancreatic tissue. The results obtained were subsequently compared between the two pathologists. IL-1β expression was evaluated by assessing the percentage of pancreatic β cells that exhibited positive staining within the cell cytoplasm. Data were expressed as percentages. While, the results of pancreatic fibrosis assessment were subsequently evaluated by assessing the percentage of fibrosis (indicated by blue staining) within the islets of Langerhans pancreatic tissue. The data were subsequently categorized into scores: 0 that representing negative, 1 that indicating 1-25%, 2 that representing 26-50%, 3 that representing 51-75%, and 4 that representing 76-100%.

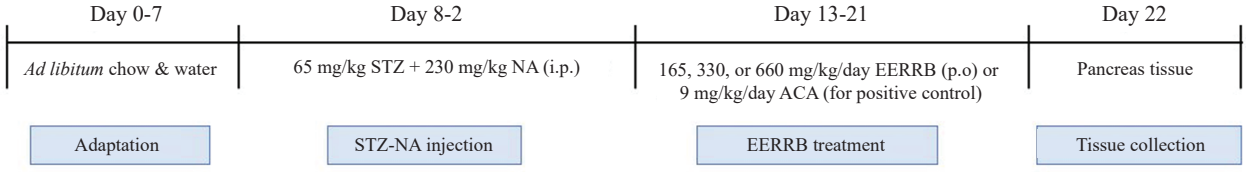

Figure 1. Timeline of the study. i.p: intraperitoneal, p.o: per oral.

Statistical Analysis

All data analysis were performed using GraphPad Prism Version 10 (GraphPad software, Sunnyvale, CA, USA). To assess the effect of EERRB on IL-1β expression and the degree of pancreas tissue fibrosis, one-way ANOVA followed by Tukey's multiple comparisons test was used. Data are expressed as mean±SEM. Significance levels were set at $p<0.05$.

Results

EERRB Reduced The Expression of IL-1β

Figure 2 demonstrated that the group of rats with STZ-NA induction exhibited significantly elevated levels of IL-1β compared to the other groups, suggesting that administrating STZ and NA could trigger the production of pro-inflammatory cytokines, which promotes the T2D development. IL-1β had been associated with the damage in pancreatic β-cells and T2D.

When comparing the expression of IL-1β in the pancreas tissues, analysis by one-way ANOVA showed that there was a significant difference between groups ($p<0.0001$). Post hoc analysis by Tukey's multiple comparison test revealed that rats treated with acarbose and EERRB at a dose of 165, 330, and 660 mg/kg/day had significantly lower IL-1β expression than diabetic rats with no treatment (STZ-NA group) ($p=0.0000$, $p=0.0194$, $p=0.0008$, $p=0.0000$, respectively) (Figure 2 and Figure 3). The IL-1β expression in STA-NA group was 50%, whilst STZ-NA + 660EERRB was 10%. Additionally, the IL-1β expression in the highest dose of EERRB group was comparable to the STZ-NA + ACA (10%). It can be seen that there was no significant mean difference (95% CI: -21.95 - 21.95) between the STZ-NA + 660EERRB and STZ-NA + ACA, thereby suggesting that EERRB successfully suppressed IL-1β in this T2D rats model. Detail comparison value of IL-1β expression between groups could be found in Table 1.

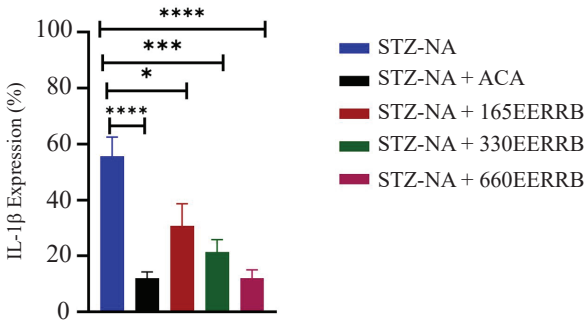

Figure 2. Expression of IL-1β in pancreas tissue following interventions.

EERRB Did Not Affect The Fibrosis of Pancreatic Tissues

In T2D, an excess of nutrients and metabolic activity might led to a chronic inflammatory condition could result in pancreatic fibrosis. When comparing the degree of tissue fibrosis of the pancreas tissue, analysis by one-way ANOVA showed that there was no significant difference between groups ($p=0.1407$) (Figure 4 and Figure 5). Although, no significant difference was found between those group, the degree pancreatic fibrosis in diabetes group receiving

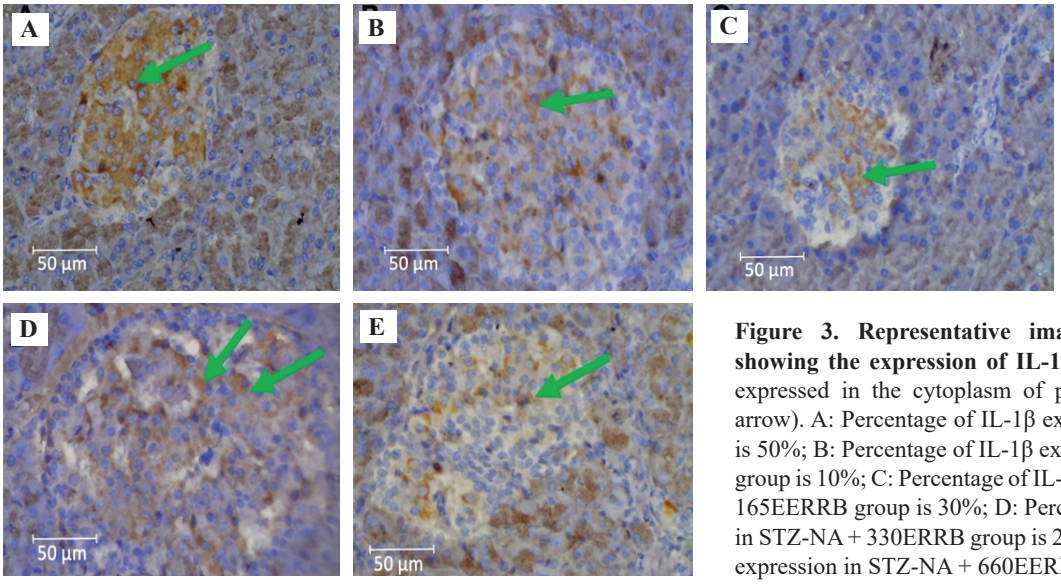

Figure 3. Representative images of pancreas tissue showing the expression of IL-1β in each group. IL-1β is expressed in the cytoplasm of pancreatic beta cell (green arrow). A: Percentage of IL-1β expression in STZ-NA group is 50%; B: Percentage of IL-1β expression in STZ-NA +ACA group is 10%; C: Percentage of IL-1β expression in STZ-NA + 165EERRB group is 30%; D: Percentage of IL-1β expression in STZ-NA + 330ERRB group is 20%; E: Percentage of IL-1β expression in STZ-NA + 660EERRB group is 10%.

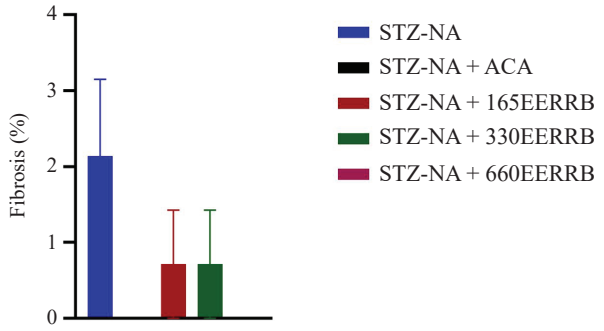

Figure 4. The degree of fibrosis in pancreas tissue following interventions.

treatments were lower (below than 1%) compared to STZ-NA group (2.14%). Additionally, both STZ-NA + 660EERRB and STZ-NA + ACA also had similar degree of pancreatic fibrosis, which was 0%.

Discussion

T2D is a complex disease defined by insulin resistance, low-grade chronic inflammation, and is known as a persistent inflammatory condition characterized by elevated levels of TNF, ILs, and adipokines.(19) Cellular stress leads to the development of insulin resistance, β-cell dysfunction, and eventually T2D. Insulin resistance might be induced by many cells signaling proteins involved in systemic inflammation, such as TNF-α, IL-1β, and interferon-γ, resulting in hyperglycemia.(23)

Results of this study showed that rats induced with STZ-NA exhibited significantly elevated levels of IL-1β compared to the other groups. This present finding is in line with previous study (18), revealing that the STZ-NA induction causes elevation of FBG levels above the normal threshold of 126 mg/dL. Rats that were administered STZ-NA had mimicking T2D condition and serves as an established model for T2D, since NA could minimize the harmful effect of STZ on the destructions of β-cell pancreas and allow the remaining β cells to still be responsive to glucose stimulation.(24)

In this study, administrating three different doses of EERRB effectively suppressed IL-1β in a dose-dependent manner. Supporting the anti-inflammatory effect of EERRB, a previous study demonstrated that the red rice polar extract fraction, which contains high levels of phenolic compounds and proanthocyanidins, effectively reduced the production of TNF-α, IL-6, and nitric oxide (NO) in lipopolysaccharide-induced Raw 264.7 macrophages. This anti-inflammatory effect might be achieved by suppressing the nuclear factor kappa-B (NF-κB) pathway and other inflammatory signaling pathways.(25) Feruloylated oligosaccharides, which are a form of ferulic acid bound to carbohydrates, extracted from rice bran, have been shown to inhibit TNF-α, IL-1β, IL-6, and NO.(26)

The bran of red rice is rich in bioactive substances, including flavonoids, anthocyanins, proanthocyanidins, protocatechuic acid, ferulic acid, γ-oryzanol, and vitamin E. The presence and interaction of phenolic chemicals, specifically anthocyanins and proanthocyanidins, are likely responsible for giving the antioxidant, metabolic, and anti-inflammatory effects.(27) Additionally, the rich abundance of acylated steryl glucosides, flavonoids, ferulic acid, policosanol, resveratrol, and plasma metabolites in rice bran

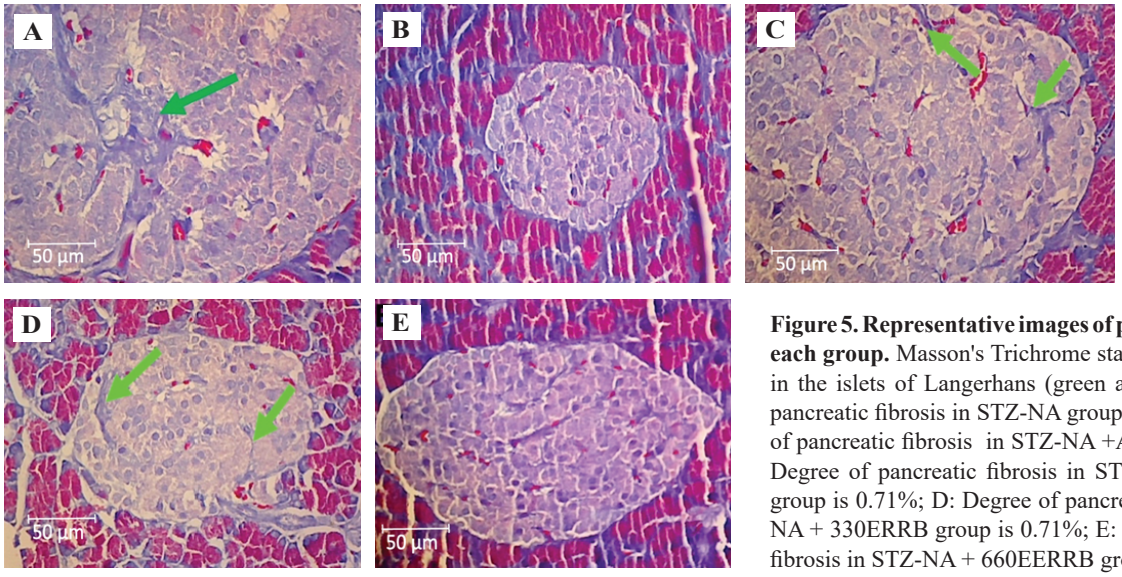

Figure 5. Representative images of pancreatic fibrosis in each group. Masson's Trichrome staining reveals fibrosis in the islets of Langerhans (green arrow). A: Degree of pancreatic fibrosis in STZ-NA group is 2.14%; B: Degree of pancreatic fibrosis in STZ-NA +ACA group is 0%; C: Degree of pancreatic fibrosis in STZ-NA + 165EERRB group is 0.71%; D: Degree of pancreatic fibrosis in STZ-NA + 330ERRB group is 0.71%; E: Degree of pancreatic fibrosis in STZ-NA + 660EERRB group is 0%.

mediated its antioxidant, anti-inflammatory, and free radical scavenging properties.(23) A higher overall flavonoid content was found in red rice compared to black rice varieties, with the majority found in the bran layer instead of the endosperm. Anthocyanin and proanthocyanidin phytochemicals are predominantly located in the rice bran layer.(28) It serves as the basis of the flavonoids and anthocyanins analysis, which in this study which were found 456.40 mg/100 gram and 340.24 mg/100 gram, respectively for total flavonoid and anthocyanin content. While, at a dose of 50 and 200 mg/kg/day administered for 4 weeks, anthocyanin from *Padus racemose* (956 mg/100 mg) was able to reduce FBG in STZ-induce diabetic mice, an effect likely mediated by its antioxidant and anti-inflammatory.(29) In addition, flavonoid obtained from *Scutellaria baicalensis* Georgi (*S. baicalensis*) could alleviate inflammation in diabetic mice at a dose of 400 mg/kg when administered for 3 weeks. (30) The dose administered in this study was comparable to those used in the two previous studies.

A high glucose concentration can stimulate proliferation and generation of extracellular matrix proteins in pancreatic interstitial cells. Furthermore, the diabetes condition led to a slight increase in the proliferation of islet cells.(31) It was shown that growing pancreatic stellate cells (PSCs) under high glucose conditions boosted their proliferation rate, upregulated the expression of α-smooth muscle actin (α-SMA), and enhanced the production of collagen.(32) Hence, this study has explored the potency of EERRB in inhibiting pancreatic fibrosis in a T2D rats model. This study results indicated that EERRB might help to suppress pancreatic fibrosis, as evidenced by a lower pancreatic fibrosis score in EERRB treatment groups compared to the STZ-NA group, even though it did not demonstrate statistical significance. A previous study found that the pancreatic tissue of T2D Wistar rats received 1000 mg/kg BW of red rice bran aqueous extract for 12 weeks have pancreatic morphologies reminiscent of normal rats. (17) In addition, a dose of 2205 or 4410 mg/kg/day of rice bran water extract for 4 weeks diminished pancreatic abnormalities in high-fat diet-induced obese rats.(33) Therefore, EERRB still may become a promising therapy to attenuate pancreatic fibrosis. The insignificant result in this present study might happen due to a low dose, or a short time of intervention compared to previous studies. A higher dose or a longer duration of treatment is suggested for further studies to explore the mechanism of EERRB in pancreatic fibrosis.

The pathophysiology of T2D related to pancreas fibrosis and the role of EERRB to alleviate T2D is illustrated in Figure 6. Rice bran has been found to contain a variety of beneficial compounds through phytochemical analysis, including phytosterols, flavonoids, and phenolic compounds. (34) Interestingly, EERRB compared to the brown rice bran extract exhibited a greater concentration of total flavonoids (962.38 and 788.21 mg QE/100 g dry matter, respectively), as well as a greater capacity to scavenge free radicals when prepared in optimal conditions which were EC_{50} values of 41.3 and 33.6 µg/mL, respectively.(35)

Phenolics, the most prominent phytochemicals found in whole grains, are recognized as natural antioxidants. They function by scavenging free radicals, thereby reducing the occurrence of oxidative stress-related harm to important biological components like lipids, proteins, and DNA. (36) Flavonoids enhance the ability of β-cells to secrete

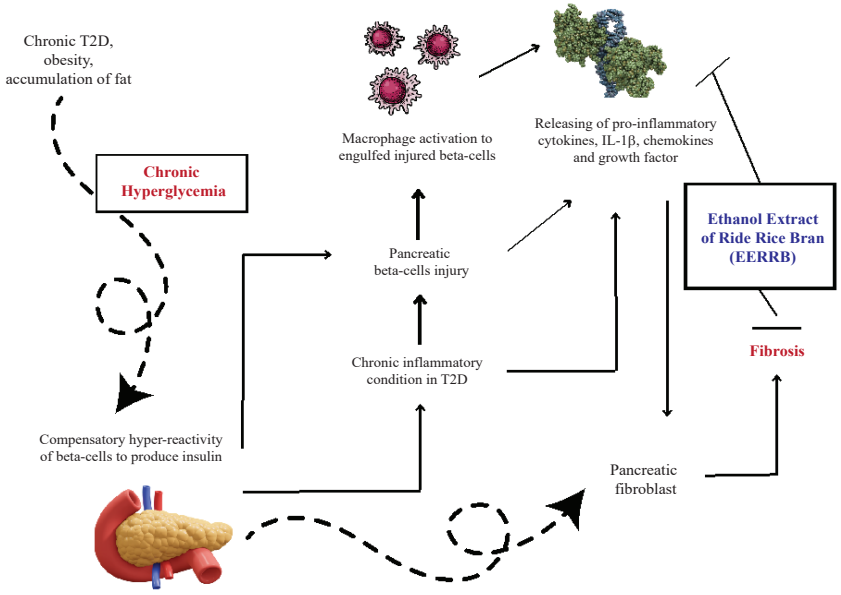

Figure 6. The role of EERRB in the pathophysiology of T2D related to pancreas fibrotic. EERRB effectively inhibited IL-1β in the T2D rat model and suppress pancreatic fibrosis.

insulin and improve their survival mechanisms. Moreover, flavonoids are believed to protect β-cells viability by reducing levels of reactive oxygen species (ROS), activating anti-apoptosis pathways, and inhibiting the formation of nitric oxide. This process helps counteract the harmful effects of glucotoxicity, lipotoxicity, and cytokines. (37) These flavonoids and phenolic components probably underlie the tendency of red rice bran ethanol extract to inhibit the progression of pancreatic fibrosis in T2D model mice by suppressing the inflammatory process.

This study used acarbose as a positive control and demonstrated its ability to inhibit IL-1β in a mouse model of T2D. Acarbose has been documented in diminishing chronic inflammation in T2D from its tendency to suppress IL-6, IL-1β, and TNF-α after 12 months of therapy.(39) Additionally, acarbose was also shown to significantly reduce the levels of IL-6 and CRP in individuals with T2DM after 7 months of treatment compared to a placebo.(40) The proven capacity of acarbose to attenuate inflammation, as mentioned before, may underlie its propensity to impede the advancement of pancreatic fibrosis, as demonstrated in this study. This study observed at inflammatory process and pancreatic fibrosis by only by examining IL-1β expression and tissue structure using IHC. While IL-1β can represent the level of inflammation, examining other inflammation parameters such as TNF-α, IL-6, or nitrit oxide and adding other fibrosis parameters, such as TGF-β, or matrix metalloproteinase (MMP)-1 and type 1 collagen would provide more comprehensive understanding of the molecular mechanism underlying EERRB effect in preventing TD2 complication, *i.e*., pancreatic fibrosis. Further studies should point off those other parameters to increase the impact of this study.

Conclusion

This present study demonstrated EERRB significantly reduces the expression of pro-inflammatory cytokine, IL-1β, in pancreas tissue of T2D rats in a dose dependent manner. The degree of IL-1β reduction at the dose of 660 mg/kg/day was comparable to that of acarbose effect. Therefore, this study has found that EERRB using all of these doses successfully suppressed IL-1β in this T2D rats model, although these doses were not significantly adequate to attenuate pancreatic fibrosis. However, at all doses, EERRB still had a lower pancreatic fibrosis score, indicating a tendency to inhibit pancreatic fibrosis. This finding further provides a rationale for the development of EERRB as an alternative treatment for T2D.

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Authors Contribution

RDY and BW were involved in concepting and planning the research, BW performed the data acquisition/collection, MM performed the experimental data analysis and designed the figures, RDY drafted the manuscript, RDY and MM aided in interpreting the result, NW and DN reviewed the manuscript, DNP finalized the manuscript. All authors took parts in giving critical revision of the manuscript and approved the final version of the manuscript.

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