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Original Article

Taxifolin Attenuates Hepatic Fibrosis and Injury in High-Fat Diet and Streptozotocin-Induced Type 2 Diabetes Mice

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Abstract: This study investigated the protective effects of taxifolin (TAX), a bioflavonoid, against hepatic fibrosis and injury in the type 2 diabetes mellitus (T2DM) mouse model. T2DM model was induced in male C57BL/6 mice by high-fat diet (HFD) and intraperitoneal injection for 5 consecutive days of 40 mg/kg streptozotocin (STZ). HFD-STZ groups *were* administered TAX (80 mg/kg/day) or vehicle daily for 12 weeks. The control or wild-type (WT) group received equal amounts of the vehicle and was fed a normal chow diet. Body weight and blood glucose were checked every 4 weeks. H&E and Masson's trichrome staining were performed to assess liver fibrosis and injury. The western blot was used to determine the expression levels of key fibrotic proteins. The data showed that taxifolin treatment significantly decreased body weight, liver weight, and blood glucose in diabetic mice. In particular, liver enzymes including alanine transaminase (ALT) and aspartate transferase (AST), and triglyceride levels were significantly higher in the non-treated diabetic group than in the WT group but markedly reduced after TAX treatment. In addition, the histological analysis revealed that TAX attenuated hepatic interstitial fibrosis. Moreover, western blot data showed downregulation of fibrotic markers including TGF-β1, Col1a1, and Smad2 in TAX group treatment indicating the molecular mechanisms through which TAX exerts its hepatoprotective effects. These findings suggest that TAX holds therapeutic potential in mitigating liver fibrosis and injury associated with T2DM, providing a promising avenue for further research and potential clinical application.

Keywords: Hepatic fibrosis, type 2 diabetes, non-alcoholic fatty liver disease.

1. Introduction

According to the latest survey by International Diabetes Federation, in 2021, there are 537 million adults with diabetes globally, and it is predicted to rise to 783 million by 2045 [1]. In which, type 2 diabetes mellitus (T2DM) accounts for over 90% of all diabetes patients [2]. There is a strong link between T2DM and nonalcoholic fatty liver disease (NAFLD), which is also recognized as a

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common complication of T2DM [3]. NAFLD is found in up to 70-80% of individuals with T2DM [4]. In addition, uncontrolled T2DM, characterized by chronic hyperglycemia and insulin resistance, significantly contributes to lipid accumulation, oxidative stress, and inflammation. This, in turn, is a main risk factor for the faster NAFLD progresses from simple steatosis and hepatic fibrosis to non-alcoholic steatohepatitis, which can further advance to cirrhosis and hepatocellular carcinoma [5]. The synergistic effect of NAFLD and T2DM increases morbidity and mortality risks [3, 6]. Recently, although numerous therapies exist to manage blood glucose levels and prevent the progression of diabetes-related complications such as dipeptidyl dipeptidase-4, glucagon-like peptide-1 agents, and especially SGLT2 inhibitors, the issue of adverse effects continued to pose significant challenges [7, 8]. Thus, developing new, safe, and effective strategies to prevent diabetes-induced complications including liver injury is highly desirable.

Taxifolin (3,5,7,3,4-pentahydroxy flavanone, TAX), also known as dihydroquercetin, is a naturally occurring flavonoid found in various plants [8]. As a member of the flavonoid family, taxifolin has garnered significant attention for its potent antioxidant, anti-inflammatory, and antifibrotic properties [10]. In recent years, research has increasingly focused on the potential of taxifolin as a therapeutic agent for chronic diseases such as cardiovascular disorders, neurodegenerative diseases, and metabolic syndromes [9, 11]. Its ability to scavenge free radicals, reduce oxidative damage, and modulate key signaling pathways positions taxifolin as a potential treatment option for conditions where these processes play a critical role. Several studies have shown the benefits of TAX for NAFLD and acute liver injury [12, 13]. However, its impact and mechanism of action on hepatic fibrosis and liver injury in the condition of T2DM remaín unclear. Therefore, the present study was conducted to elucidate the effects of TAX in preventing diabetic hepatology in mice models and to advance in uncovering the mechanism of action of TAX.

2. Materials and Methods

2.1. Materials

Taxifolin (480-18-1), streptozotocin (18883-66-4), and a triglyceride quantification kit (MAK266) were purchased from Sigma Aldrich (Korea). HFD-containing 40 kcal% fat (mostly palm oil), 20 kcal% fructose, and 2% cholesterol from Research Diets INC. USA (D09100310). Blood glucose test strips and glucometer from Accu-Chek, Roche, USA. Antibodies were used in this study including transforming growth factor beta 1 (TGF-β1, MA5-44667, Invitrogen); Collagen 1 alpha 1 (Col1a1, sc-293182), and mothers against decapentaplegic homolog 2 (Smad2, sc-393312) were obtained from Santa Cruz; GAPDH (#2118, Cell Signaling Technology). In addition, other compounds or reagents and equipments used in Western blot and histological analysis were of analytical quality and obtained from reputable commercial suppliers.

2.2. Methods

2.2.1. Animal Experiments

Eight-week-old male C57BL/6 mice were purchased from Charles River (Japan). All mice were housed in controlled temperature (20-24 ℃) and humidity (40-70%) on a 12-hour light cycle and with access to standard laboratory chow and tap water *ad libitum*. After adaptive feeding for three days, mice were divided randomly into 3 groups (n=6/group): the control (WT) group was fed a normal-chow diet, while the other two groups were fed an HFD diet to induce obesity. These two groups were administered STZ at a dose of 40 mg/kg by the intraperitoneal injection for 5 consecutive days of the fourth week. TAX-treated group (WT+HFD-STZ+TAX) received once daily

oral dose of 80 mg/kg of TAX for 12 weeks, while the TAX-untreated group (WT+HFD-STZ) and WT group were administered equal amounts of vehicle via oral gavage. Body weight and blood glucose were measured every 4 weeks. At the end of the treatment, fasted mice were sacrificed by exsanguination under anesthesia by inhalation of 2-5% isoflurane in the ambient air of the room. Blood samples and liver tissue were collected for further experiments. A schematic summary of the experimental design is presented in Figure 1A.

2.2.2. Fasting Blood Glucose and Glucose Tolerance Test

Mice were fasted for 8 hours before blood glucose testing. 2 μL blood from the tail vein was collected via tail tipping and measured directly using a glucose meter with test strips. At the end of the treatment, the intraperitoneal glucose tolerance test (IPGTT) was performed, and mice were also fasted for 8 hours before intraperitoneal injection of a 20% D-glucose solution. Blood glucose levels were determined before the injection (time 0) and at different time points (30, 60, 90, 120 minutes).

2.2.3. Serum-biochemical Analysis

Fasting blood specimens were collected from the heart in commercial tubes without anticoagulants. Blood samples were left upright on the rack for 30 minutes at room temperature. After that, the serum was collected by centrifugation of blood tubes for 5 minutes at 1,000 x g. Serum samples were stored at -20 °C until analysis. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by using ALT assay kit (MET-5123) and AST assay kit (MET-5127) from Cell Biolabs, INC., USA according to the manufacturer's instructions.

2.2.4. Measurement of Triglyceride Levels in Liver Tissue

Liver samples (100 mg) were washed in phosphate-buffered saline (PBS) and homogenized in 1 mL solution of 5% Nonidet P 40 substitute (74385, Sigma-Aldrich, Korea). The samples were heated at 80 °C for 3 min and then cooled to ~ 25 °C. The samples were then centrifuged for 3 min at 5,000 x g. The supernatant was collected into a new tube and then liver triglyceride level was determined by a triglyceride quantification kit according to the manufacturer's instructions.

2.2.5. Histological Analyses

After dissecting, the liver and tibia bone were isolated, and the liver's wet weight and tibia's length were measured. A portion of the largest lobe of the liver was excised, rinsed with PBS, and fixed in paraformaldehyde for 24 hours. The tissue was then transferred to a new tube containing 50% ethanol. Fixed specimens were processed, embedded in paraffin blocks, and sectioned into 4-μm thick slices. The sections were stained using hematoxylin and eosin (H&E) and Masson's trichrome staining kits (Sigma Aldrich, Korea), and photographed with a NanoZoomer Digital Slide Scanner (Hamamatsu, Japan). Quantitative analysis of each section was performed using ImageJ 1.48 software (NIH, MD). In addition, liver damage was evaluated based on the tissue injury score classification as described in previous studies [14, 15].

2.2.6. Western Blot Analysis

Liver proteins were extracted using RIPA lysis buffer supplemented with a protease and phosphatase inhibitor cocktail (Thermo Fisher, Waltham, MA, USA). Total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk and incubated overnight at 4 °C with the primary antibodies TGF-β1, Col1a1, Smad2, and GAPDH. The following day, membranes were incubated with the HRP-conjugated secondary antibody (Jackson ImmunoResearch, USA) for 1 hour at room temperature. Protein bands were visualized using an ECL kit (WESTSAVE Up, AbFrontier, Korea), and their intensities were quantified using ImageJ software.

2.2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0.1 software (San Diego,

CA, USA). Data are presented as mean ± standard deviation (SD) or standard error (SE). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for multiple comparisons. A p-value of < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Taxifolin Reduces Body Weight and Improves Glucose Tolerance in HFD-STZ-Induced Diabetes Mice

Compared with WT mice, WT+HFD-STZ mice exhibited significantly higher body weight, elevated fasting blood glucose levels, and marked impairment in glucose tolerance impairment (Figure 1). As shown in Figure 1B, a significant reduction in body weight was observed in diabetic mice treated with TAX after 8 weeks. Additionally, TAX effectively lowered fasting blood glucose levels and improved glucose homeostasis of diabetic mice throughout 12 weeks of the treatment (Figure 1C-D). These findings confirm the successful establishment of a type 2 diabetes model in mice and align with results from previous studies [13, 16].

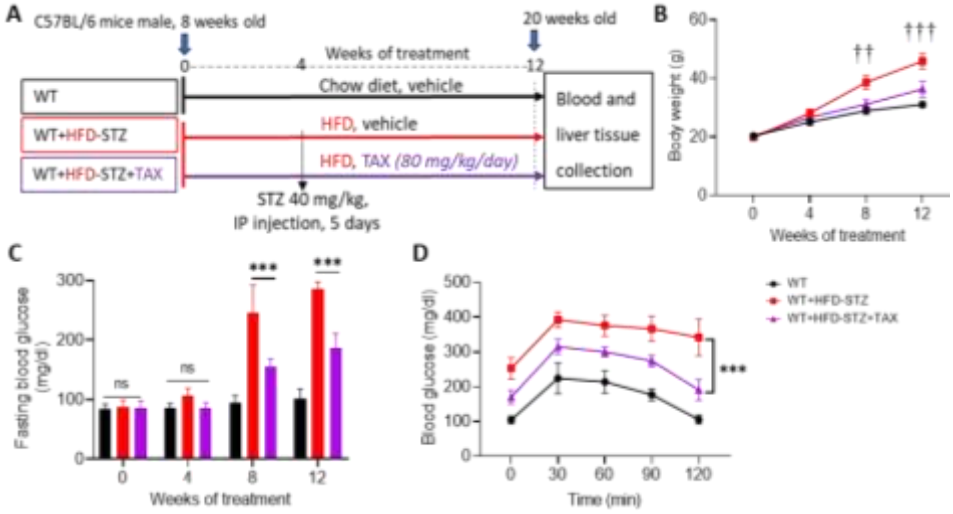


Figure 1. Effects of TAX on body weight and blood glucose of mice. A: experimental design; B: body weight (†† P<0.01, ††† P<0.001 WT+HFD-STZ vs. WT+HDF-STZ+TAX); C: fasting blood glucose; D: glucose tolerance. Data are presented as the mean±SD; ***P<0.001, ****P<0.0001, ns: not significant.

3.2. Taxifolin Attenuates Serum ALT, AST, and Liver Triglyceride Levels in HFD-STZ-Induced Diabetes Mice

The livers of diabetic mice were significantly larger, with a higher liver weight-to-tibia length ratio compared to WT mice. However, TAX-treated diabetic mice exhibited a notable reduction in liver weight (Figure 2A). T2DM in HFD-STZ-induced mice led to hepatotoxicity, evidenced by elevated serum levels of AST and ALT, as well as increased liver triglyceride levels. In addition, liver triglyceride accumulation, a hallmark of various liver diseases, including NAFLD, is characterized by excessive triglyceride build-up in hepatocytes, resulting in hepatic steatosis [3, 5, 17]. In this study, TAX treatment significantly reduced liver triglyceride levels (Figure 2B). Since liver enzymes, such as ALT and AST, are critical indicators of hepatocellular damage and liver dysfunction, their elevation correlates with the severity of liver injury [17]. To assess the potential protective effects of TAX against liver damage in diabetes mice, serum ALT and AST levels were measured. The results revealed that TAX effectively lowered both ALT and AST

levels (Figure 2C-D). These findings suggest that TAX protects hepatocytes from T2DM-induced hepatic steatosis and may mitigate associated liver dysfunction.

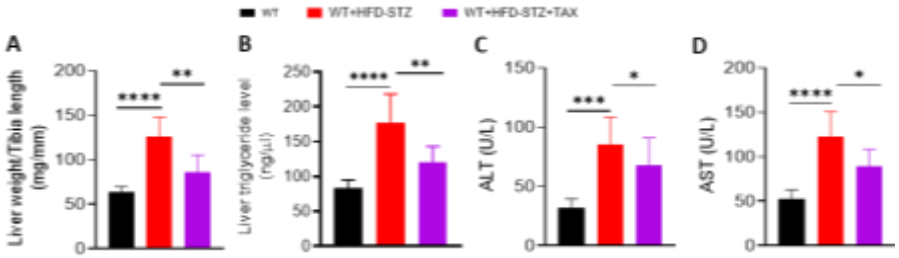


Figure 2. Effects of TAX on liver weight and serum liver function biomarkers. A: liver weight; B: liver triglyceride; C: serrum ALT level and D: serum AST level. Data are presented as the mean±SD; *P<0.05; **P<0.01, ***P<0.001, ****P<0.0001.

3.3. Taxifolin Ameliorates Liver Injury in HFD-STZ-induced Diabetes Mice

The effects of TAX on liver injury are presented in Figure 3. The WT group displayed a normal hepatocyte structure with a clear central vein, whereas the WT+HFD-STZ group exhibited severe hepatocyte degeneration, vacuolated cytoplasm, hemorrhage, and pronounced steatosis with lipid droplet accumulation (Figure 3A). In addition, the H&E-stained liver sections from the diabetic group revealed inflammatory cell infiltration and ballooned hepatocytes (black arrow), hallmark features of progression from NAFLD to non-alcoholic steatohepatitis (NASH). Histological scoring of NAFLD further confirmed a significant increase in tissue injury scores in the diabetic group compared to the WT group (Figure 3B).

In contrast, the TAX-treated diabetic group exhibited nearly normal hepatocyte morphology, reduced hemorrhage, and decreased degeneration of liver cells. These histopathological improvements were consistent with the biochemical findings, further demonstrating the protective effects of TAX against hepatotoxicity in HFD-STZ-induced diabetic mice.

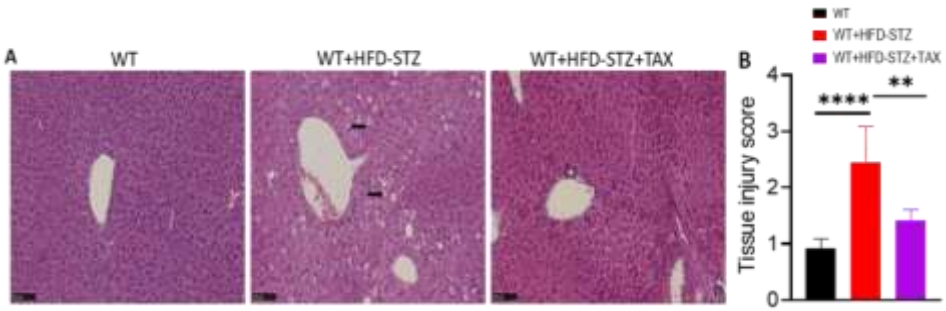


Figure 3. Effects of TAX on histopathological alteration of liver tissues in HFD-STZ-induced diabetes mice. A: representative images of H&E-stained liver sections of mice groups, magnification x400, the scale bar represents 100 μm; B: liver tissue injury score. Data are presented as the mean±SE; **P<0.01, ****P<0.0001.

3.4. Taxifolin Attenuates Hepatic Fibrosis in HFD-STZ-induced Diabetes Mice

Hepatic fibrosis, characterized by excessive accumulation of extracellular matrix components, develops as a result of chronic liver injury and prolonged activation of inflammatory responses and fibrogenesis [18]. Detecting and quantifying fibrosis are essential for evaluating liver damage and predicting disease progression. In the present study,

Masson's staining revealed increased interstitial fibrosis and collagen fiber accumulation in the livers of diabetic mice compared to WT mice (Figure 4A-B), Notably, these fibrotic changes were significantly alleviated after 12 weeks of TAX treatment, highlighting its potential to mitigate hepatic fibrosis in HFD-STZ-induced diabetic mice.

3.5. TGF-β1/Col1a1/Smad2 Pathway modulated by Taxifolin Treatment in Diabetes Mice

Next, to investigate the mechanism underlying hepatic fibrosis, we examined the TGF-β1/Col1a1/Smad2 signaling pathway. Transforming growth factor β1 (TGF-β1), a key regulator of fibrosis in various organs, plays a critical role in liver fibrosis and is considered one of the most significant fibrotic cytokines identified to date [19, 20]. Upon binding to its receptor, TGF-β1 activates Smad proteins, leading to the formation of a Smad2/Smad4 complex that translocates into the nucleus.

This complex functrions as a transcriptional regulator, binding to promoter regions of target genes, including those encoding extracellular matrix components such as Col1a1, as well as other fibrogenic factors [19, 21]. In this study, the western blot analysis revealed significantly elevated expression of TGF-β, Col1a1, and Smad2 proteins in the diabetic group compared to the WT group. However, in the TAX-treated diabetic mice, the expression levels of these fibrotic markers were markedly reduced (Figure 5A-D). These findings suggest that TAX effectively downregulates the TGF-β1/Col1a1/Smad2 signaling pathway, thereby mitigating hepatic fibrosis in diabetic mice.

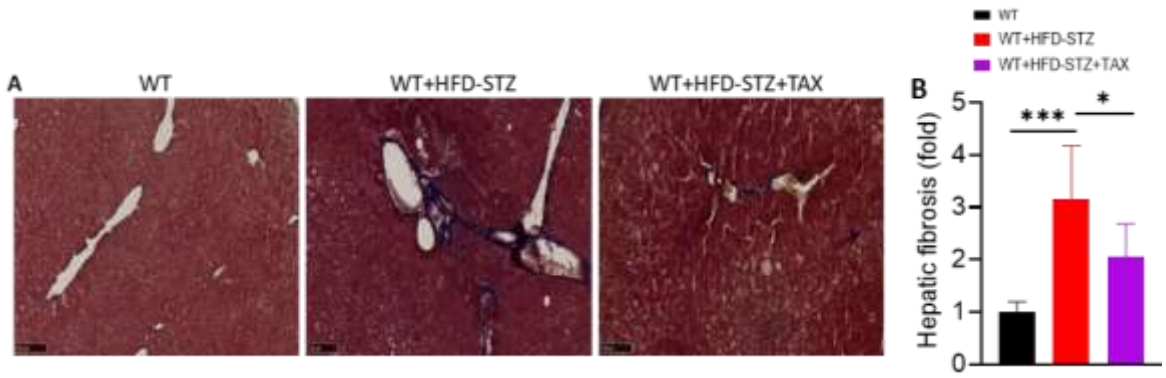
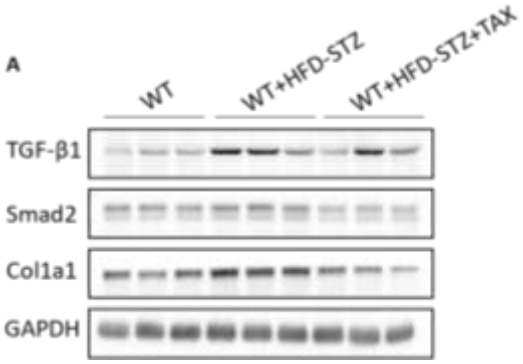


Figure 4. Effects of TAX treatment on hepatic fibrosis in diabetes mice. A: representative images of Masson-stained liver sections, magnification x400, scale bar 100 μm; B: the area of hepatic fibrosis was analyzed by ImageJ software. Data are presented as the mean±SE; *P<0.05, ***P<0.001.



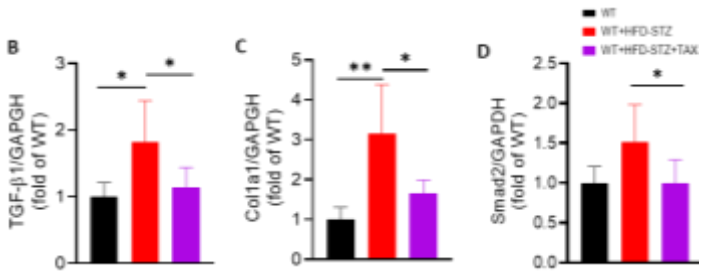


Figure 5. Effects of TAX on protein expression of hepatic fibrosis markers in diabetes mice. A: representative images of protein expression of TGF-β1, Col1a1, Smad2, and GAPDH using western blotting analysis; B-D: Quantitative analysis of these proteins, respectively. Data are presented as the mean±SD. *p<0.05, **p<0.01.

4. Conclusion

In conclusion, the key finding of the present study is that taxifolin significantly protects against hepatic fibrosis and injury induced by HFD-STZ. This protection occurs through a reduction in hepatic triglyceride accumulation, decreased levels of liver injury markers ALT and AST, and inhibition of the TGF-β1/Col1a1/Smad2 signaling pathway. These results suggest that taxifolin may have potential therapeutic applications in the management and treatment of chronic liver conditions. Further research is needed to fully explore the therapeutic potential of taxifolin and to elucidate the mechanisms underlying its anti-fibrotic effects.

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