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Tea polysaccharide ameliorates glucolipotoxicity-induced oxidative damage and apoptosis of RIN-m5F cells through Nrf2/HO-1 pathway

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Abstract: Glucolipotoxicity (GLTy) could cause the islet β cell dysfunction in metabolic diseases such as diabetes. Tea polysaccharide (TPS) has the potential to ameliorate islet damage, but its protective effects on GLTY-induced islet β cell damages and its mechanism remain unclear. The RIN-m5F cell damage model was constructed by GLTy induction. The effects of TPS on cell damage were investigated from apoptosis, oxidative stress, inflammatory response, cell senescence and cell function. MTT, ELISA, immunofluorescence, flow cytometry, WB and kits were used to detect cell viability, insulin secretion level, inflammatory factors, antioxidant enzyme activity, cell apoptosis rate and related protein expression. Si-Nrf2 was used to verify whether TPS can activate Nrf2/HO-1 signaling to play a protective role. TPS can increase the cell viability, the positive rate of EdU, insulin secretion, anti-inflammatory factor level, and the activities of SOD and CAT, reduce apoptosis, proinflammatory factor levels and the expression of p21, p53 and p16 proteins. By activating Nrf2/HO-1 pathway, TPS alleviated oxidative stress damage, thus playing a protective role against GLTY-induced cell damage. TPS effectively reduced GLTY-induced RIN-m5F cells damage through activating Nrf2/HO-1 pathway, providing a new idea and potential drug selection for the treatment of diabetes.

Keywords: Tea polysaccharide, glucolipotoxicity, RIN-m5F cells, Nrf2/HO-1, oxidative stress, inflammation, cell senescence

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INTRODUCTION

Diabetes is a chronic metabolic disorder characterized by high blood sugar levels and insulin resistance, affecting 529 million people globally in 2021, with projections indicating that over 131 billion individuals will have diabetes by 2050 (GBD 2021 Diabetes Collaborators, 2023). Type 2 diabetes, the predominant form, is marked by insulin resistance and insufficient insulin secretion, causing hyperglycemia and posing a significant threat to human health (Tinajero and Malik, 2021). Dysfunction of islet β cells is a crucial factor in the development of diabetes, and glucolipotoxicity (GLTy). is a key contributor to this dysfunction (Poitout *et al.*, 2010). GLTy results from prolonged exposure to high-sugar and high-fat conditions, damaging islet β cells through complex mechanisms involving oxidative stress, apoptosis, inflammation, and cellular aging (Lytrivi *et al.*, 2020). Oxidative stress is central to GLTy-induced damage, as excessive reactive oxygen species (ROS). disrupt cellular antioxidant balance, leading to damage of proteins, lipids, DNA, and other biomolecules, ultimately causing cell injury (Vela-Guajardo *et a l.*, 2021). Moreover GLTy triggers the release of inflammatory cytokines, which exacerbate islet β cell damage (Akash *et al.*, 2018, Hong *et al.*, 2018). Apoptosis is another critical pathway induced by GLTy, with upregulation of ap optosis-related proteins resulting in programmed cell death (Lin *et al.*, 2023). Additionally, GLTy accelerates cellular senescence, diminishing the cells' ability to proliferate and maintain function (Yan *et al.*, 2025). Thus, identifying interventions that can effectively mitigate GLTy-induced islet β cell damage is vital for diabetes prevention and treatment

Tea polysaccharide (TPS) as a natural polysaccharide, is mainly found in tea, with a complex structure, consisting of a variety of monosaccharides and having anti-diabetic properties by the abilities of anti-inflammatory and antioxidant, which acts an important role in managing the condition and its complications (Fan *et al.*, 2022, Zhao *et al.*, 2022). TPS can eliminate free radicals and decrease the inflammatory factors in cells (Yan *et al.*, 2018). In addition, TPS also can improve insulin secretion function and promote the expansion of islet β cells. However, the protective effect of TPS on GLTy-induced islet β cell damage and its specific mechanism are not completely clear, and further studies are needed.

Nuclear factor erythroid-2 related factor 2 (Nrf2). is a principal transcription factor. that can respond to oxidative stress injury in cells. When cells are stimulated by oxidative stress, Nrf2 will be separated from Keap1 and transferred to the nucleus. The combination of antioxidant stress elements (ARE). promotes HO-1 and other antioxidant oxidase genes expression, and enhances the antioxidant capacity of cells (Ma, 2013, Loboda *et al.*, 2016, Ndisang, 2017). HO-1 is a key effector molecule in the pathway (Facchinetti, 2020). The activation of Nrf2/HO-1 can effectively reduce a variety of cell injuries,

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including myocardial ischemia-reperfusion injury and neurodegenerative diseases (Wang *et al.*, 2021, Hu *et al.*, 2022). However, the role of this pathway in GLTy-induced islet β cell injury and its relationship with TPS remain unclear and need to be further explored.

In the early stage, this project team used the subcritical water extraction method to extract TPS using the discarded tea branches after Shaoxing spring tea harvesting as raw materials. The yield of TPS is 1.72 times that of traditional water extraction method. Temperature is a key factor in the subcritical water extraction process, and the inhibition rate of TPS obtained at different temperatures on α-amylase activity is obvious. This study will explore the differences in activity of TPS obtained at different temperatures. By constructing a GLTy-induced islet β-cell RIN-m5F damage model, the protective effect of TPS on cell damage was systematically evaluated from the aspects of cell apoptosis, oxidative stress, inflammatory response, cell aging and cell function, and si-Nrf2 was used. Verify the key role of Nrf2 in it. It is hoped that this study will provide some reference materials for TPS as a potential drug selection and mechanism for the treatment of metabolic diseases such as diabetes.

MATERIALS AND METHODS

Cell culture and GLTy-induced damage model establishment

Rat islet β-cell cells RIN-m5F (STM-CL-7005, Shanghai Sitaimer Biotechnology Co., Ltd) were inoculated into RPMI-1640 complete medium and cultured at 37°C and in 5% CO_2 situation. Follow-up experiments were performed after the cells grew to 70%-80% confluent. According to the experimental needs, the required cells were cultured for 24 h and then treated with drug as follows: Control group (NC): PBS, high glucose group (HG): 45 mmol/L glucose, high fat group (HF): 0.2 mmol/L sodium palmitate, high glucose and high fat group (HG+HF, MC): 22.5 mmol/L glucose and 0.1 mmol/L sodium palmitate, TPS intervention group: MC+TPS (0.0625 mg/mL). Subsequent experiments were performed 24 hours after the drug action.

MTT assay

The drug was added according to the grouping for experiments. After the drug effect was completed, 20 μL of MTT (BS186-1g, Biosharp, Anhui, China) solution was supplied and cultivated in darkness for 4 hours. Microplate reader (BIOBASE-EL10B, BIOBASE, China) detected the absorbance OD490 value at 490 nm and calculated the cell viability.

LDH assay

The culture medium was harvested and subjected to centrifugation. As the instructions of LDH assay kit (40209ES76, Yesen, Shanghai, China) shows, 60 μL LDH dyeing solution was given after 120 μL sample supplied, mixed and incubated for 30 minutes without light. The absorbance value at 490 nm was determined by micro plate reader and the relative release of LDH was calculated

Flow cytometry assay

Cells were collected and apoptosis was detected by Annexin V-FITC/PI apoptosis detection kit (BB4101, BestBio, Shanghai, China). In short, the cells were re-suspended with 400 μL Annexin V binding solution, followed by 5 μL Annexin V-FITC dye and PI dye for 15 min and 5 min, respectively and transferred to the flow tubules immediately by flow cytometry (CytoFLEX S, Beckman Coulter, USA). detected and analyzed the apoptosis rate.

EdU staining

Cell proliferation was evaluated using the EdU kit (E-CK-A377, Elabscience, Wuhan, China). Replace fresh culture medium, add EdU reagent and make its final concentration of 10 μM. After 2 h of culture, remove the medium. 1 mL 4% paraformaldehyde was aded for 15 min, then 1 mL 0.3% Triton X-100 for 20 min and 500 μL Click solution for 30 min in dark. Finally, 500 μL of DAPI solution per well was added and incubated for 5 min in dark. Under a fluorescence microscope (LWD300-38LFT, CEWEI, China), the cell proliferation level was evaluated.

RT-qPCR assay

RNA was extracted with Trizol (10606ES60, Yeasen, Shanghai, China) reverse transcribed (K1691, Thermo, Massachusetts, USA) to cDNA and amplified by qPCR using primers and SYBR mix. GAPDH was the reference gene. Calculation was by 2^{-Ct}. Table 1 shows the Primer sequence (5'-3').

TUNEL staining

Cells were fixed and translatable, stained with Tunel kit (E-CK-A321, Elabscience) and incubated at 37°C with protease K for 20 min for permeability. Then the cells or tissues were added to the buffer for 10 min. TUNEL and DAPI solution were added in order and incubated in dark for 1 h and 5 min respectively. Apoptotic cells with green fluorescence were observed and photographed. TUNEL-positive cell proportion was analyzed with ImageJ.

DHE staining

DHE kit (S0064S, Beyotime Biotech Inc, Shanghai). was used for staining. The DHE dye solution was diluted at 1:1000 to prepare the dyeing working solution, then kept at 37°C for 20 min. After sealing the plate, the fields were photographed and fluorescence intensity was analyzed by Image J.

DCFH-DA flow analysis

ROS assay kit (BC005, Kelu Biotechnology Co., LTD., Wuhan). was used to detect ROS levels. Cells were incubated with 10μM DCFH-DA and detected by flow cytometry.

SOD, MDA, CAT and GSH-PX level detection

Sample protein was extracted and determined the concentration by BCA kit (P0009, Beyotime Biotech Inc) and then the content of MDA and GSH was determined. MDA Assay Kit (abs580011, absin, Shanghai) was used to detect MDA content. The standard product was diluted and related operations were carried out in strict accordance with the instructions. In strict accordance with rat superoxide dismutase (SOD). ELISA kit (SP12914, Saipei Biotechnology, Wuhan), rat Catalase (CAT) enzyme linked immunosorbent assay kit (JL21028, JONLNBIO, Shanghai), Rat glutathione peroxidase (GSH-PX). enzyme-linked immunosorbent assay kit (JL21016, JONLNBIO, Shanghai) was operated according to the instructions.

SA-β-gal staining

SA β-Galactosidase Staining Kit (C0602, Beyotime Biotech Inc, Shanghai) was used to stain. Follow the instructions of each step carefully and take clear photos after staining.

Determination of insulin secretion level

Cells were treated under different culture conditions and then stimulated by fresh medium with 20 mM glucose for 1 hour. The supernatant was collected by centrifugation at low temperature and detected strictly according to the instructions of the Rat Insulin ELISA Kit (KE20008, Proteintech). The absorption value at 450 nm was detectermined.

Determination of inflammatory factors of culture supernatant

The culture medium supernatant was collected by centrifugation, strictly according to instructions of TNF-α, IL-1β, IL-6 and IL-10 ELISA Kit (PT516, PI303, PI328, PI525, all are from Beyotime Biotech Inc, Shanghai) for follow-up experiments, the absorbance value at 450 nm was determined and the levels of these factors were calculated.

Immunofluorescence

After the cells were fixed and permeated, they were incubated with 5%BSA for 1 h and then incubated with Anti-Nrf2 (1:500, 80593-1-RR, Proteintech) at 4ºC overnight. After washing, Fluorescent Sec. Abs. (1:500, A0423, Beyotime) was given and cultured without light for 1 h, After the nucleus is stained, cells were observed and photographed. The fluorescence intensity was analyzed quantitatively by Image J.

Si-Nrf2 transfection

Small interfering RNA targeting Nrf2 (si-Nrf2) and negative control (si-NC) from Gemma Gene (China). Cell transfection was performed as recommended in the instructions. After 24 h, the inhibition efficiency of these sirna was verified by WB assay.

Western blot (WB)

Total protein was extracted, quantified and separated, then transferred to a PVDF membrane. After blocking, primary antibodies were added and incubated at 4℃ overnight, followed by secondary antibody incubation for 1.5 h.. The developing reagent is added to the system (JP-K900, Shanghai Jinpeng Analytical Instrument Co., LTD, China). for exposure and photographing. Antibody information is as follows: PARP (13371-1-AP), Cleaved-PARP (60555-1-IG), RIP3 (17563-1-AP), caspase3 (82202-1-RR)., Cleaved-caspase3 (25128-1-AP), Bcl2 (26593-1-AP), Bax (50599-2-IG), GLUT2 (20436-1-AP), GLP-1R (26196-1-AP)., Pdx1 (20989-1-AP)., iNOS (18985-1-AP), COX2 (27308-1-AP), MCP1 (26161-1-AP), p53 (10442-1-AP), p21 (28248-1-AP), Nrf2 (80593-1-RR), HO-1 (10701-1-AP), NQO1 (67240-1-IG), β-actin (20536-1-AP), HRP-Goat Anti-Rabbit (RGAR001), HRP-Goat Anti-Mouse (RGAM001), they were from Proteintech, USA. p-RIP3 (91702), MafA (79737) and p16 (29271). were from Cell Signaling Technology, USA. Refer to the instruction manual for antibody dilution ratio.

STATISTICAL ANALYSIS

All assays were repeated at least 3 times with a sample size of 3 per group. Data were analyzed using SPSS 26.0 software and expressed as mean ± standard deviation. Group comparisions were analysed by unpaired *t* test or one-way ANOVA, with Tukey's HSD for post hoc analysis. Graphs were created using GraphPad Prism 9.0. $*P < 0.05$ denotes statistical significance.

RESULTS

High glucose and lipid situation inhibits RIN-m5F cells activity

High glucose (HG) or high lipid (HF) alone treated cells for 24 h decreased the RIN-m5F cells viability, with the largest decrease in RIN-M5F cells viability when treated in combination (fig. 1A). In addition, the RIN-m5F cells viability was decreased in a time-dependent manner in the HG+HF group (fig. 1B). Considering the feasibility of cell damage repair, the subsequent use of HG and HF-24 h was selected to induce RIN-m5F cells damage. HG+HF can significantly increase LDH release after 24 h (fig. 1C). TUNEL test results appeared the apoptosis rate was highest in HG+HF group (fig. 1D-1E). Cleaved caspase 3 is the activated form of caspase-3, which is a key executive protein in the process of apoptosis. When cells receive the apoptotic signal, caspase-3 is Cleaved and activated, forming cleaved caspase3. Cleaved-caspase3 transmits apoptosis signals by cutting a series of downstream targets, resulting in cytoskeleton destruction and DNA repair mechanism failure, and Cleaved-PARP is the product of PARP cutted (Eskandari and Eaves, 2022). p-RIP3, a phosphorylated form of RIPK3, is associated with cell membrane rupture and cell necrosis (Zhou *et al.*, 2024).

Table 1: Primer sequence

Gene	Forward primer	Reverse primer
GLUT2	AGTCACACCAGCACATACGA	AGAGGGCTCCAGTCAACGA
GLP-1R	CATCGCTTCAGCCATCCTTG	CAGCCGTGCTATACATCCACTTG
Pdx1	TGGAGCTGGCAGTGATGTTGA	TCAGAGGCAGATCTGGCCAT
MafA	AGCGGCACATTCTGGAGAG	TTGTACAGGTCCCGCTCCTT
GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT

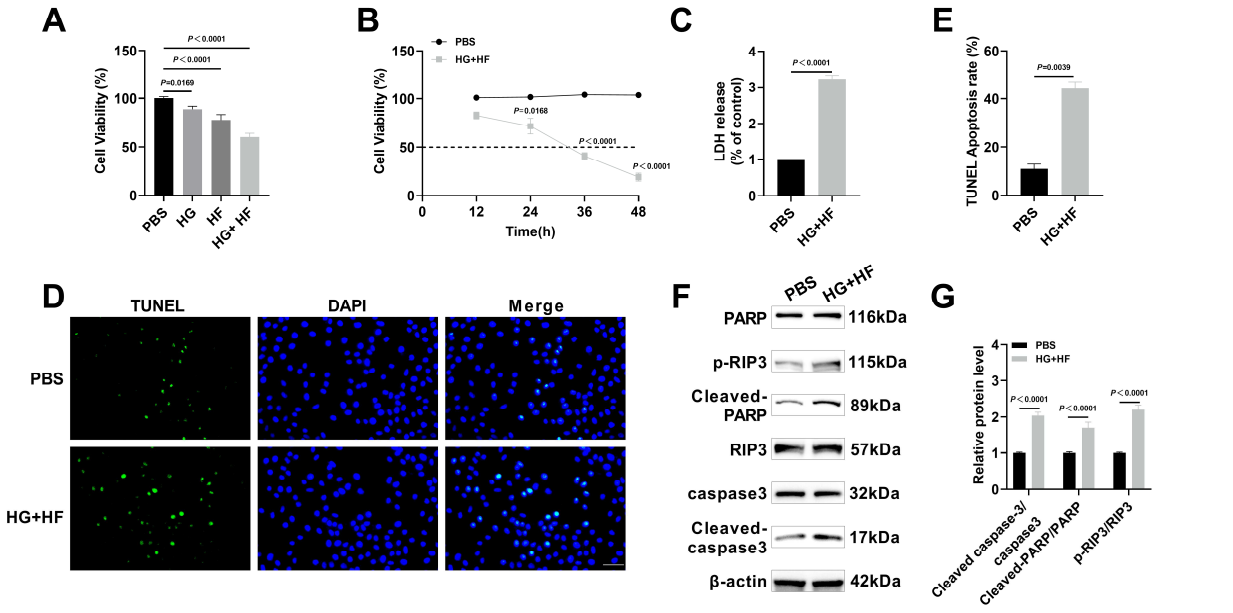


Fig. 1: Establishment of RIN-m5F cell damage model.
(A) MTT assay showed that HG and HF could reduce RIN-m5F cell viability to some extent, but HG+HF could further significantly reduce RIN-m5F cell viability. (B) MTT assay determined that HG+HF could reduce RIN-m5F cell viability in a time-dependent manner. (C) HG+HF can significantly increase LDH release. (D-E) TUNEL staining showed that HG+HF could significantly increase the apoptosis rate of RIN-m5F cells. (F-G) WB assay was used to detect the effects of HG+HF on apoptosis-related proteins. (*n*=3)

Cleaved-caspase3, Cleaved-PARP and p-RIP3 levels were increased in the HG+HF group, indicating the occurrence of RIN-m5F cells apoptosis (fig. 1F-1G). Therefore, in this study, 22.5 mmol/L glucose and 0.1 mmol/L sodium palmitate were added for 24 h to induce RIN-m5F cells damage using GLTy.

TPS attenuates the GLTy-induced proliferation inhibition and apoptosis promotion effect in RIN-m5F cells

TPS at 0.0125-0.0625 mg/mL had no adverse effect on RIN-m5F cells in NC and MC groups, while TPS at 0.125 and 0.25 mg/mL significantly reduced cell viability (S fig. 1A-1B). Since 0.0625 mg/mL of TPS treatment for 24 h was already able to increase cell viability, we chose this concentration to treat RIN-m5F cells for 24 h for subsequent experiments. RIN-m5F were treated with TPS extracted at different temperatures for 24 h and TPS extracted at 130°C significantly improved cell viability (fig. 2A). Therefore, TPS extracted at 130°C was selected for follow-up experiments. Compared with MC group, EdU positive rate of RIN-m5F cells was significantly higher after TPS co-treatment (fig. 2B-2C). Meanwhile, TUNEL experiment showed that TPS extracted at 130°C could significantly reduce the apoptosis rate of the damaged RIN-m5F cells (fig. 2D-2E). In addition, TPS could promote the Bcl2 expression, down-regulate the Bax and Cleaved caspase3 proteins levels (fig. 2F-2G), indicating the protection of TPS in the apoptosis.

TPS can improve the function damage of the damaged cells

The detection showed that after TPS treated, the insulin secretion was effectively improved (fig. 3A). Pancreatic and Duodenal Homeobox 1 (Pdx1) could regulate insulin synthesis and secretion (Zhang *et al.*, 2022a). TPS can significantly offset the decrease of Pdx1 expression caused by GLTy (fig. 3B-3C). Glucose transporter 2 (GLUT2). senses plasma glucose levels and controls glucose uptake by β cells, thereby regulating insulin secretion (Thorens, 2015). Glucagon-like peptide-1 receptor (GLP-1R) binds to glucagon-like peptide-1 (GLP-1).

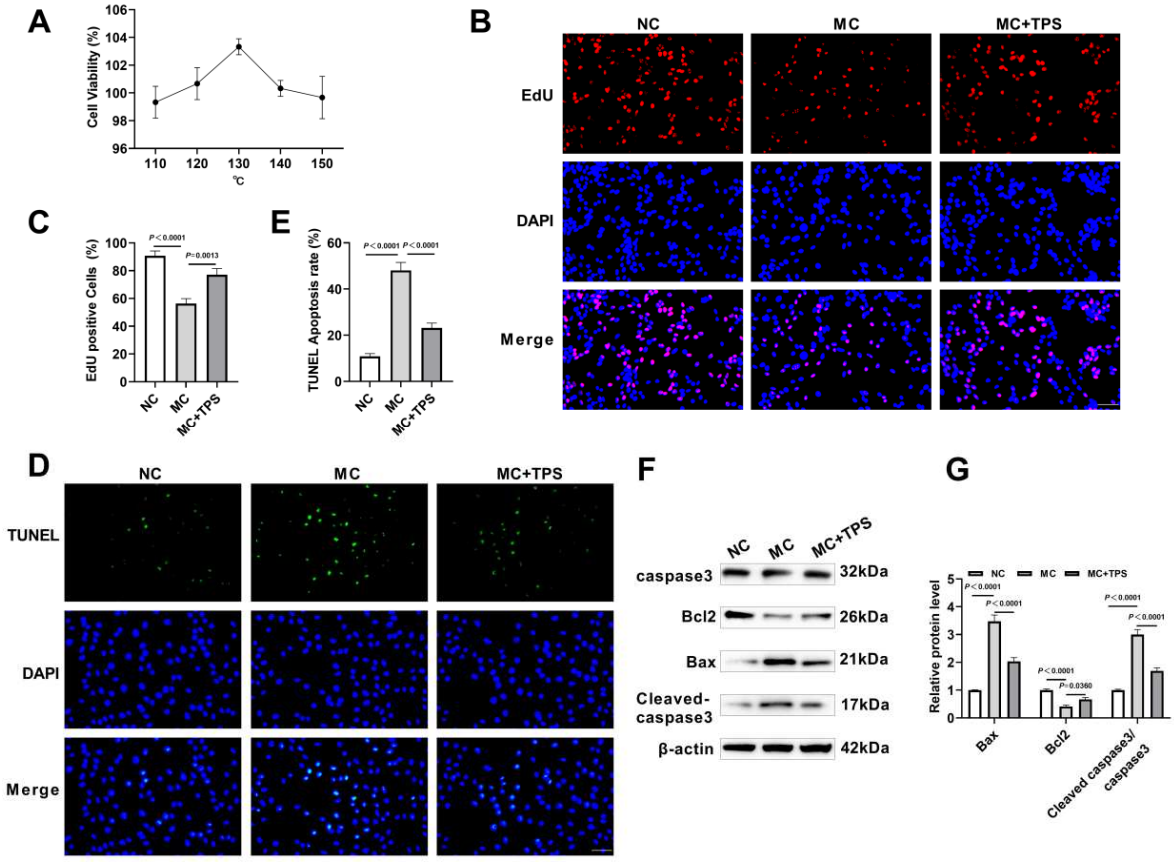


Fig. 2: Effects of TPS on damaged RIN-m5F cells in proliferation and apoptosis.
(A) MTT showed that TPS extracted at different temperatures could enhance the cell viability to a certain extent, and TPS extracted at 130℃ had the best effect. (B-C) EdU staining showed that TPS could accelerate proliferation (40×, 50 μm). (D-E) TUNEL staining showed that TPS could restrain the apoptosis (40×, 50 μm). (F-G) WB assay was used to detect the effect of TPS on apoptosis-related proteins. (*n*=3)

To regulate blood glucose (Wan *et al.*, 2023) MafA, as a transcription factor, is key to the maturation and functional maintenance of islet β cells by regulating the insulin genes levels. It works in coordination with other transcription factors such as Pdx1 to jointly regulate the function of islet β cells and maintain the normal secretion of insulin (Nishimura *et al.*, 2022). The effect of TPS on functional proteins of RIN-m5F cells was further detected, and the levels of GLUT2, GLP-1R, Pdx1 and MafA expression were up-regulated after TPS use compared with MC group (fig. 3D-3I). These results indicated that TPS could ameliorate GLTy-induced functional impairment by restoring the expression of functional related proteins.

TPS alleviates RIN-m5F cells inflammation induced by GLTy

Diabetes is a chronic inflammatory disease. Inflammatory factors can cause insulin resistance (Li *et al.*, 2023a). From the results, GLTy could lead to the exacerbation of cellular inflammatory response. The use of TPS could significantly improve the levels of these cytokines, indicating that TPS could effectively alleviate the GLTy-induced inflammatory response (fig. 4A-4D).

iNOS is an enzyme that is induced to express mainly in response to inflammatory stimuli and catalyzes L-arginine to produce nitric oxide (NO). In diabetic patients, NO produced by iNOS may participate in diabetes-related inflammatory response. The up-regulated expression of MCP-1 and COX-2 induces inflammatory cell infiltration and aggravates tissue damage. By inhibiting the expression or activity of iNOS, MCP-1 and COX-2, diabetes-related inflammatory response can be alleviated and improve diabetic complications (Amirshahrokhi and Zohouri, 2021, Bahadoran *et al.*, 2024). In the study, TPS down-regulated the iNOS, MCP1 and COX2 proteins levels (fig. 4E-4F), indicating that TPS's anti-inflammatory capacities.

TPS alleviates GLTy-induced oxidative stress and cell senescence of RIN-m5F cells

Hyperglycemia can induce a variety of oxidative stress reactions, damage islet β cells, inhibit their insulin secretion function and exacerbate insulin resistance, thus promoting the occurrence and development of diabetes (Darenskaya *et al.*, 2021). DHE staining showed that TPS could significantly reduce the superoxide level induced by GLTy and the production of intracellular superoxide (fig. 5A-5B).

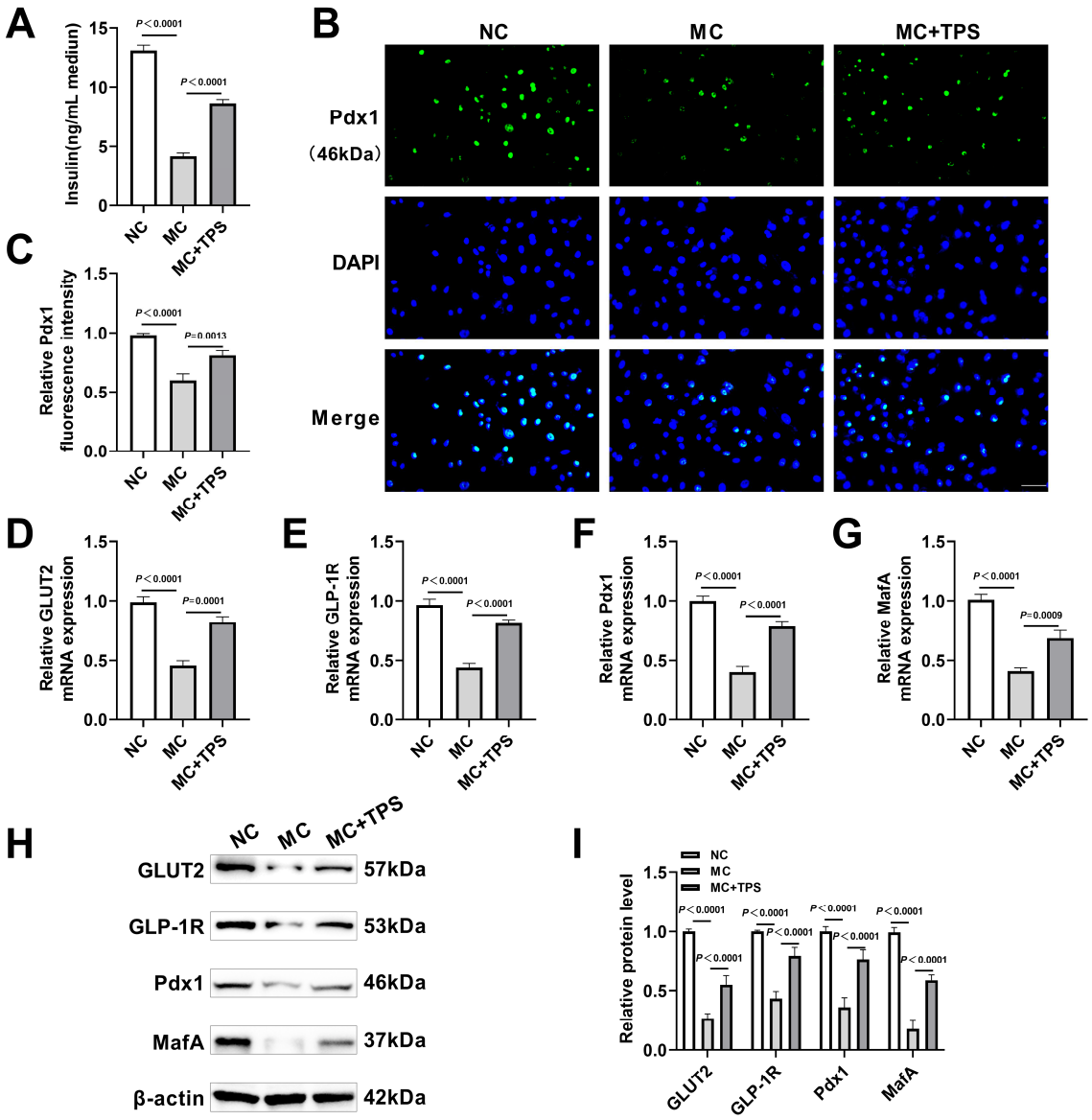


Fig. 3: Effect of TPS on RIN-m5F cells function.

(A) ELISA test showed that TPS significantly increased the ability of RIN-m5F cells to secrete insulin. (B-C) Immunofluorescence assay showed that TPS could increase the level of Pdx1 (40×, 50 μm). (D-G) TPS significantly increased the mRNA levels of GLUT2, GLP-1R, Pdx1 and MafA. (H-I) TPS significantly increased GLUT2, GLP-1R, Pdx1 and MafA protein levels. (*n*=3)

In addition, TPS can effectively reduce intracellular ROS levels (fig. 5C-5D). The MC group exhibited reduced antioxidant capacity, but TPS effectively restored antioxidant enzyme activity and lowered oxidative stress, indicating a protective effect on damaged cells (fig. 5E-5H). Additionally, aging, which is linked to chronic inflammation and cellular dysfunction, contributes to the decline in islet β cell function (Akinola, 2016). The results of SA-β-gal staining showed that senescent cells in MC+TPS group were reduced, indicating that TPS could alleviate senescence of RIN-m5F cells (fig. 5I-5J). The expression levels of p21, p53 and p16 proteins in the MC+TPS group were lower than the MC group (fig. 5K-5L) indicating that TPS could inhibit the cell aging signaling pathway, leading to the senescence can be alleviated.

TPS activates Nrf2/HO-1 pathway to reduce GLTy-induced RIN-m5F cells damage

Nrf2 protein level was seen decreased in the si-Nrf2 group and HO-1, NQO1 and other proteins levels were also downregulated accordingly, indicating that si-Nrf2 was successfully constructed and Nrf2 gene was effectively silenced (fig. 6A-6B). Immunofluorescence detection revealed GLTy significantly reduced the fluorescence intensity of Nrf2 in RIN-m5F cells, while TPS could increase it effectively, indicating that TPS could effectively activate Nrf2 (fig. 6C-6D) and Nrf2, HO-1 and NQO1 proteins levels were also up-regulated (fig. 6E-6F).

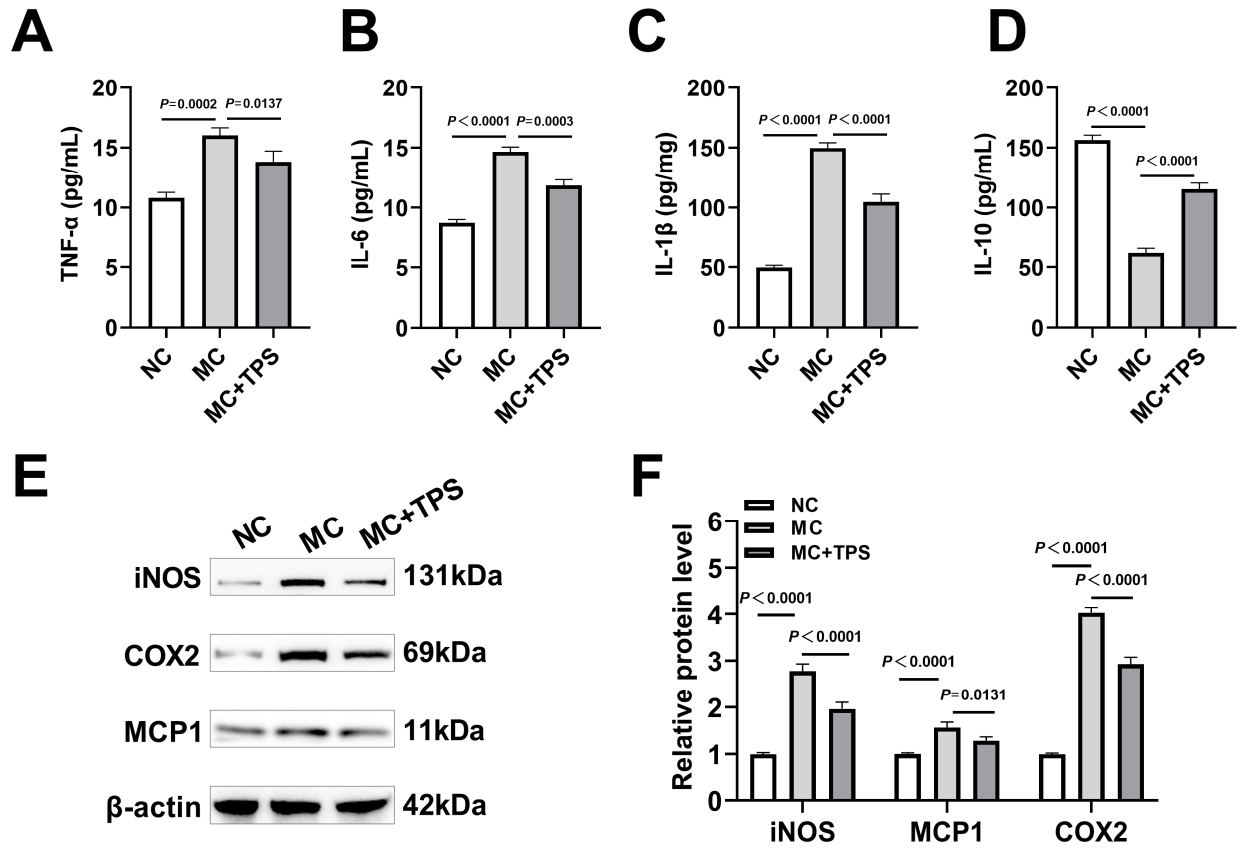


Fig. 4: TPS could inhibit the inflammation of RIN-m5F cell induced by GLTy.
(A-D) TPS significantly decreased the levels of IL-6, IL-1β and TNF-α and increased the levels of IL-10. (E-F) WB results indicated TPS could interfere significantly the high levels of iNOS, MCP1 and COX2 proteins associated with inflammation. (*n*=3)

When Nrf2 is knocked down, the effect of TPS is significantly reduced and likewise, Nrf2, HO-1 and NQO1 levels are decreased. The apoptosis rate of cells after knocking down Nrf2 was higher than that in MC+TPS group (fig. 6G-6H).

WB results showed that the Bax and Cleaved caspase3 protein levels in the MC+TPS+si-Nrf2 group were higher, while Bcl2 protein was lower than those in the MC+TPS group (fig. 6I-6J) indicating that si-Nrf2 partially counteracts the inhibitory effect of TPS on RIN-m5F cell apoptosis. Meanwhile, si-Nrf2 inhibited the antioxidant enzyme activity of RIN-m5F cells and increased lipid peroxidation products (fig. 6K-6M). In MC+TPS+si-Nrf2 group, the levels of IL-6, IL-1β and TNF-α were higher and the levels of IL-10 were lower than those in MC+TPS group (fig. 6N-6Q) indicating that the effect of TPS on reducing the inflammatory response of RIN-m5F cells was inhibited after Nrf2 knockdown. In addition, TPS up-regulated insulin secretion while si-Nrf2 down-regulated insulin secretion (fig. 6R). Protein level detection results si-Nrf2 down-regulated the levels of GLUT2, GLP-1R and Pdx1 proteins related to RIN-m5F cells (fig. 6S-6T). Thus, this study proved that TPS can activate Nrf2/HO-1 signaling pathway and after knocking down Nrf2, the protective effect of TPS on RIN-m5F cells was partially cancelled.

DISCUSSION

As a drink with a long history, tea is considered to be associated with a lower risk of type 2 diabetes (Li *et al.*, 2023b). The active ingredients in tea include tea polyphenols, theanine, caffeine and TPS (Khan and Mukhtar, 2018, Chen *et al.*, 2023). TPS is a kind of polysaccharide complex with biological activity in tea.

Most of them are acidic glycoproteins that bind to proteins and are mainly composed of sugars, proteins, pectin and other components (Wang *et al.*, 2015, Yao *et al.*, 2022). Studies show that TPS can decrease blood sugar and lipids, and boost antioxidant capacity and immunity (Du *et al.*, 2016). TPS content in tea is low, extraction methods affect yield, composition, structure and activity (Xiang *et al.*, 2023). Therefore, it is of great significance to improve the extraction process and increase the yield. Current TPS extraction methods mainly include hot water, enzyme, ultrasonic, and microwave-assisted extraction (Chen *et al.*, 2016). Subcritical water is in a certain pressure range, the water is heated to a high temperature of 100 ~ 374°C, the water still remains liquid.

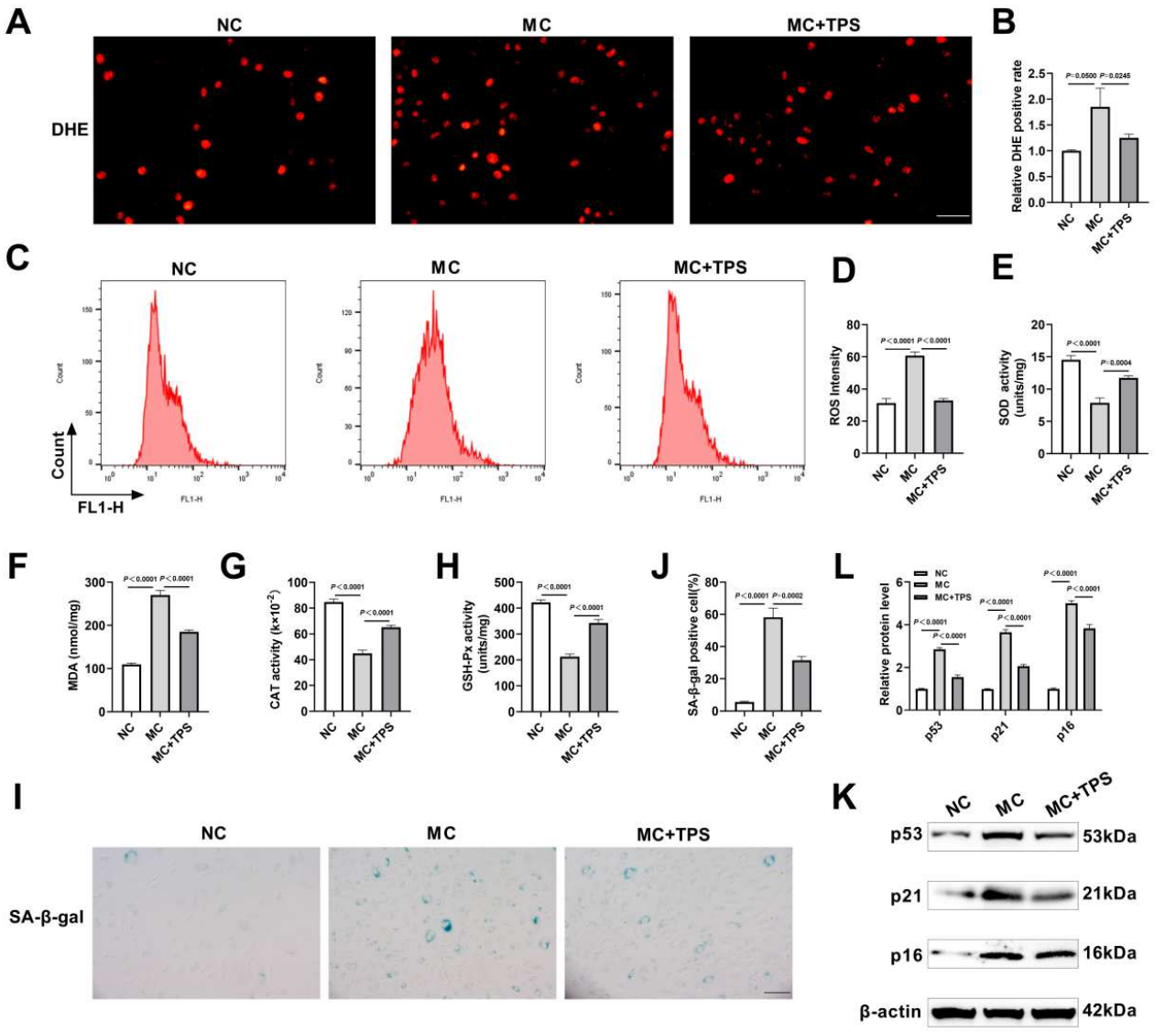
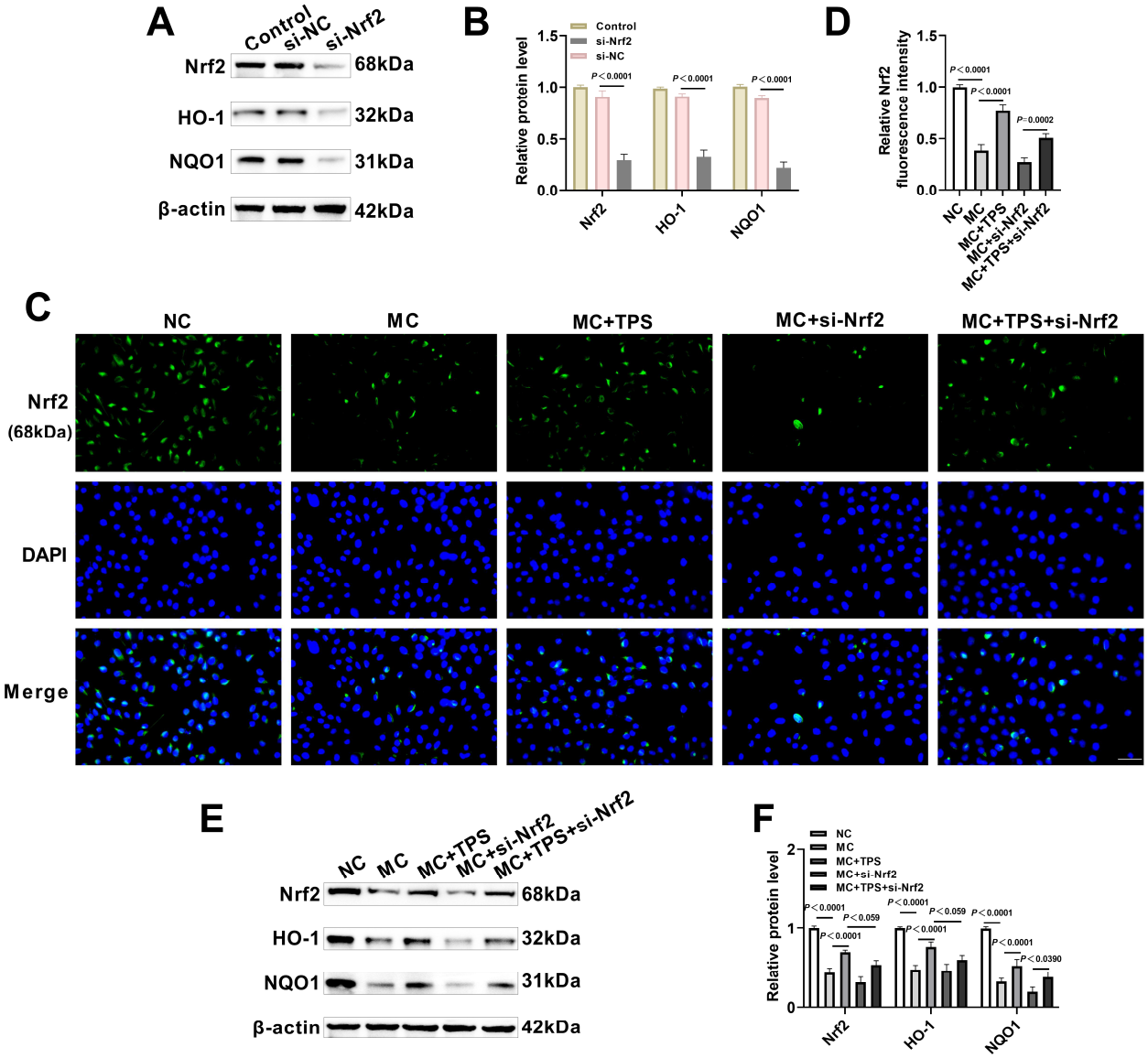


Fig. 5: TPS could improve oxidative stress and senescence of damaged RIN-m5F cells.
(A-D) DHE staining and DCFH-DA detected the ROS level (40×, 50 μm). (E-H) The kits detected levels of oxidative stress factors. (I-J) SA-β-gal staining indicated that TPS could reduce the number of senescent cells (20×, 100 μm). (K-L) WB assay analysed the reduction of the levels of aging related proteins p21, p53 and p16 after TPS treated. (*n*=3)

With the increase of temperature, the polarity, dielectric constant, surface tension and viscosity of water decrease, so as to achieve the purpose of selective extraction of target components. Subcritical water extraction technology has the advantages of environmental friendliness, high extraction rate, simple operation and low production cost, and is a new plant polysaccharide extraction technology that is easy to realize industrial production (Cheng *et al.*, 2021, Ti *et al.*, 2022). In the early stage, the project team obtained a series of TPS extracts by controlling different temperatures, and its yield was greatly improved. The role of TPS in metabolic diseases has been widely reported (Chen *et al.*, 2019) therefore, this group focused on the activity of TPS in improving diabetes.

Firstly, GLTy-induced damage model of islet β cells RIN-m5F was successfully constructed, and by MTT experiment, TPS extracted at 130ºC could significantly increase the viability of RIN-m5F cells, and reduce the apoptosis rate.

Apoptosis is one of the important mechanisms of islet β cell failure, and many drugs have received attention because of their inhibition of RIN-m5F cell apoptosis (Ding *et al.*, 2012, Zhang *et al.*, 2022b). Bax and Cleaved caspase3 are pro-apoptotic proteins, while Bcl2 is an anti-apoptotic protein(Ashrafizadeh, 2024). TPS down-regulated Bax and Cleaved caspase3 proteins levels, and up-regulated Bcl2 protein level to protect islet β cells from GLTy damage and maintain their normal physiological function.



Continue Fig 6…

In addition, it has been shown that LBP (40-160 mg/kg body weight) can effectively reduce blood glucose levels and improve insulin resistance in diabetic nephropathic mice, and attenuate glomerular and tubular damage (Wan *et al.*, 2022). However, due to the inconsistency of the experimental models, it is difficult to directly compare the effect of its action. Subsequently, we will construct a diabetic mouse model to further explore the therapeutic effect of TPS on diabetes mellitus.

The antioxidant defense mechanism of islet β cells is weak, and they are easily affected by REDOX imbalance caused by excessive reactive oxygen species and active nitrogen substances (Lenzen, 2008, Novoselova *et al.*, 2021). Inhibiting oxidative stress can protect islet cells from GLTy damage by enhancing antioxidant defenses (Li *et al.*, 2019, Xia *et al.*, 2020). Polydatin reduces lipid peroxidation, improves antioxidant status, inhibits pancreatic inflammation and thus alleviates pancreatic β cell damage (Yousef *et al.*, 2021). Camellia polysaccharide can significantly increase the activities of antioxidant enzymes SOD and CAT, reduce the content of MDA, and reduce oxidative stress, thus protecting microglia from oxidative damage (Cai *et al.*, 2024). In this study, through DHE staining, DCFH-DA detection and kit detection, it was found that TPS can effectively reduce the levels of intracellular superoxide and ROS, improve the activity of antioxidant enzymes such as SOD, CAT and GSH-PX, and reduce the content of MDA. This indicates that TPS has a strong antioxidant capacity, which can remove free radicals and reactive oxygen species in cells and reduce the damage of cells caused by oxidative stress. The antioxidant effect of TPS may be related to the abundant hydroxyl and carboxyl groups in its structure, which can react with free radicals and neutralize their activity (Zhou *et al.*, 2004).

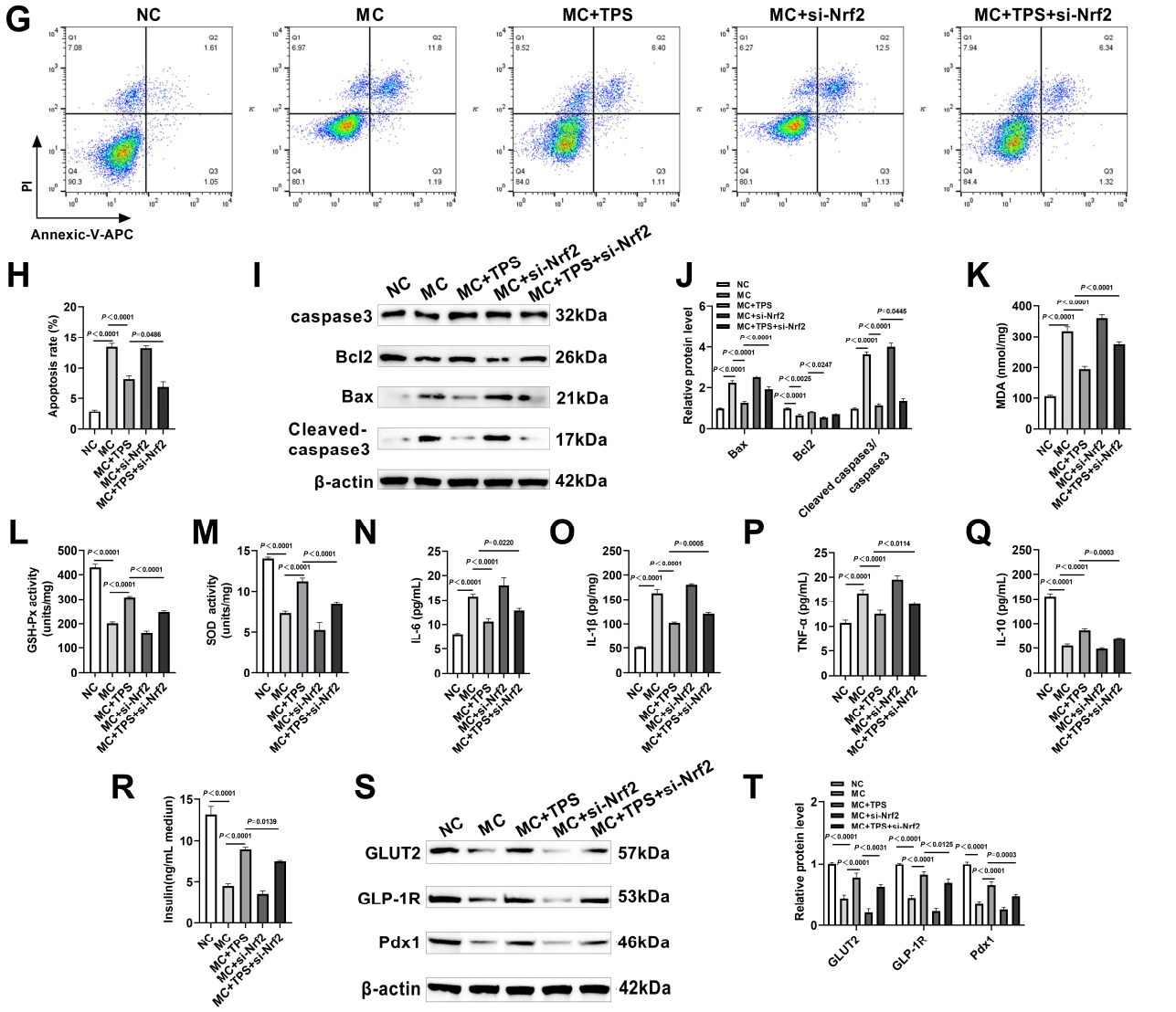


Fig. 6: TPS could activate the Nrf2/HO-1 pathway to ameliorate cell damage.
(A-B) WB assay showed that Nrf2, HO-1 and NQO1 expression were reduced after Nrf2 successfully knocked down. (C-D) Immunofluorescence assay showed that si-Nrf2 could significantly weaken the Nrf2 expression level increased in the TPS group (40×, 50 μm). (E-F) WB showed that TPS could significantly increase the expression levels of Nrf2, HO-1 and NQO1, while si-Nrf2 had the opposite effect. (G-H) Si-Nrf2 could partially counteract the effect of TPS on the apoptosis rate. (I-J) WB assay detected the changes of apoptosis-related proteins induced by different treatments. (K-M) Si-Nrf2 inhibited the increase of SOD and GSH-PX activities induced by TPS, and increased the level of MDA. (N-Q) Si-Nrf2 can increase the levels of IL-6, IL-1β and TNF-α, and decrease the levels of IL-10. (R) TPS up-regulates insulin secretion while si-Nrf2 down-regulates insulin secretion. (S-T) Si-Nrf2 down-regulates the levels of GLUT2, GLP-1R, Pdx1 proteins related to RIN-m5F cell function. (n=3)

Notably, TPS has good bioactivity and biocompatibility, and feeding 800 mg/kg BW of TPS for two weeks effectively increased serum SOD activity in carp and reduced the level of inflammatory factors, improving immunity (Yang *et al.*, 2022). Hsu *et al.* reported that daily gavage of 2500 mg/kg BW of green tea extract for 28 d did not adversely affect body weight, serum biochemistry, and urinalysis in mice (Hsu *et al.*, 2011).

Inflammation plays a pivotal role in the pathogenesis of metabolic diseases such as diabetes, contributin to insulin resistance and β-cell dysfunction through inflammatory process (Lee and Olefsky, 2021, Guo *et al.*, 2022). Targeted inflammation has been used in the treatment of diabetes for many years (Rayego-Mateos *et al.*, 2023). for the reason of the correlation with type 2 diabetes (Martínez-Ramírez *et al.*, 2021, Khdair *et al.*, 2023). In this study, by regulating the inflammatory signals, TPS inhibits inflammatory response and reduces the damage of inflammation to islet β cells. In addition, cell senescence is another important factor leading to the decline of islet β cell function (Narasimhan *et al.*, 2021). In addition, TPS exerted a

protective effect on islet function by inhibiting the cellular senescence pathway and delaying the cellular senescence process while increasing the insulin secretion level of damaged RIN-m5F cells. Liraglutide prevents damage to RIN-m5F cells induced by glycolipid toxicity by restoring Pdx1 expression (Kornelius *et al.*, 2019). Studies have pointed it out that increasing GLP-1R level can activate the Pdx1 signal and increase insulin secretion to combat glucose toxicity (Hu *et al.*, 2014). In this study, TPS can up-regulate the GLUT2, GLP-1R, Pdx1 and other proteins expression related to cell function, providing a potential drug option for the treatment of diabetes.

Activating the Nrf2/HO-1 pathway has something to prevent the podocyte injury in diabetic nephropathy (Lv *et al.*, 2023). and alleviates diabetic cardiomyopathy by inhibiting inflammation (Wu *et al.*, 2022). In this study, we detected Nrf2 translocation by immunofluorescence and found that TPS can significantly promote Nrf2 expression. Nrf2 promotes HO-1, NQO1 and other antioxidant enzyme genes level, and enhances the antioxidant capacity of cells (Duan *et al.*, 2022).. TPS can effectively activate Nrf2 and up-regulate the expression of Nrf2, HO-1, NQO1 and other proteins. By constructing si-Nrf2, this study further verified the key role of Nrf2 in the protection of TPS. The results of si-Nrf2 construction showed that after Nrf2 silencing, the protective effects of TPS on apoptosis, oxidative stress, inflammation and cell function were inhibited. This indicates that Nrf2 is an important target for TPS to play a protective role, and its activation state directly affects the protective effect of TPS. So far, this study successfully screened the TPS extract with good activity, and systematically studied the mechanism of its protection of islet β cells.

CONCLUSION

This study systematically investigated the effects of TPS on GLTy-induced islet β cell RIN-m5F damage and its mechanism, providing an important experimental basis for TPS clinical application in diabetes mellitus. As a kind of natural polysaccharide, TPS has good biocompatibility and safety and its multi-target and multi-pathway protective effect makes it have broad application prospects in the treatment of metabolic diseases such as diabetes. In addition, this study also explored the key role of Nrf2/HO-1 pathway, providing an important theoretical basis for subsequent drug development and clinical application. This study was mainly conducted in *vitro* cell models, lacking validation in *vivo* animal models and could not fully simulate the complex physiological environment in human body and the role and mechanism of TPS in other metabolic diseases need to be further explored. Future studies could further explore whether TPS exerts a protective effect through other signaling pathways and its role in other metabolic diseases and evaluate its clinical potential, including safety, efficacy and dose-dependent.

Supplementary Data

https://www.pjps.pk/uploads/2025/09/SUP1756896660.pdf

Ethical approval

This study is an *in-vitro* experiment with cells as the research object and does not involve ethical approval.

Data availability statement

The data supporting the findings of this study can be obtained from the corresponding author, upon request.

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Author's contribution

Haiyan Liu: Developed and planned the study, performed experiments and interpreted results. Edited and refined the manuscript with a focus on critical intellectual contributions.

Jianwei Zhang: Participated in collecting, assessing and interpreting the data. Made significant contributions to data interpretation and manuscript preparation.

Liangrong Zhu: Provided substantial intellectual input during the drafting and revision of the manuscript.

The final version of the manuscript has been reviewed and approved by all authors.

Conflict of interest

The authors affirm that they do not have any financial conflicts of interest.

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