Article

Discovery Medicine 2023; 35(178): 861–867
https://doi.org/10.24976/Discov.Med.202335178.81

Marein Alleviates High Glucose-Induced Human Retinal Microvascular Endothelial Cell Injury by Up-Regulating *SNHG7* Expression

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Published: 1 October 2023

Background: Marein has been shown to possess therapeutic effects against diabetic retinopathy, but whether it can protect against high glucose (HG)-induced human retinal microvascular endothelial cell (HRMEC) injury remains unclear. Our study aimed to explore the effect of marein on HG-induced HRMEC injury and the mechanism underlying this purported therapeutic effect.

Methods: HRMEC was divided into normal glucose group, high glucose (HG) group, HG+marein low, medium and high (L, M, H) concentrations group, HG+pcDNA group, HG+pcDNA-small nucleolar RNA host gene 7 (*SNHG7*) group, HG+marein+si-negative control (NC) group, and HG+marein+si-*SNHG7* group. Flow cytometry and Western blotting were performed to determine apoptosis rate and apoptosis-related protein levels. Superoxide dismutase (SOD) activity, lactate dehydrogenase (LDH) level and malondialdehyde (MDA) content were detected to assess cellular oxidative stress. *SNHG7* expression was examined using real-time quantitative PCR.

Results: After treatment with low, medium and high concentrations of marein, apoptosis rate, Bax level, LDH level and MDA content were decreased, while B-cell lymphoma-2 (Bcl-2) level, SOD activity, and *SNHG7* expression were promoted in HG-induced HRMEC injury in a concentration-dependent manner ($p < 0.05$). After overexpression of *SNHG7*, apoptosis rate, Bax level, LDH level and MDA content were decreased, while Bcl-2 level and SOD activity were enhanced in HG-induced HRMEC injury ($p < 0.05$). In contrast, *SNHG7* knockdown reversed the effect of marein on HG-induced HRMEC injury.

Conclusions: Marein could alleviate HG-induced HRMEC injury by up-regulating *SNHG7* expression.

Keywords: marein; *SNHG7*; high glucose; HRMEC; injury; oxidative stress

Introduction

Diabetic retinopathy (DR) is one of the major microvascular complications of diabetes, which is characterized by sustained hyperglycemia-induced damage to the retina [1,2]. Fortunately, traditional Chinese medicine offers medications that can prevent and treat DR by suppressing the oxidative stress pathway [3,4]. Therefore, uncovering the molecular mechanisms of these medications in protecting against DR is crucial for the development of potential molecular drugs for DR. Studies have reported that *Coreopsis tinctoria* Nutt (CTN) possesses hypoglycemic, lipid-lowering, and antioxidant effects. Furthermore, it has been shown to play a role in early DR treatment by down-regulating the expression of vascular endothelial growth factor (*VEGF*) and intercellular adhesion molecule 1 (*ICAM1*) in the retina [5]. Marein is one of the main chemical components in the ethyl acetate extract of CTN, which has been found to improve angiotensin II/hypoxia-induced abnormal glycolipid metabolism in cardiomyocytes [6]. Besides, it has been suggested that marein has a protective effect on islet cell injury induced by high glucose (HG) and high fat [7]. In addition, marein improves DR by reducing sodium-glucose cotransporter-2 (SGLT2) protein expression and activating AMP-activated protein kinase (AMPK) pathway [8]. However, the effect of marein on high glucose (HG)-induced human retinal microvascular endothelial cell (HRMEC) injury and the mechanism underlying its beneficial effect remain obscure.

Studies have suggested that small nucleolar RNA host gene 7 (*SNHG7*) may be a potential therapeutic target in DR, which can repress the endothelial-mesenchymal transition of HG-induced HRMEC [9]. Moreover, it has been reported that *SNHG7* inhibits angiogenesis in HG-induced HRMEC during DR progression [10]. In our previous studies, we found that marein could regulate the mRNA expression of *SNHG7*. However, whether marein mediates HG-induced HRMEC injury by regulating *SNHG7* remains unclear. The aim of this study was to reveal the underlying molecular mechanism by which marein improves the progression of DR. On the basis of the above, we proposed and verified the hypothesis that marein regulates *SNHG7* expression to alleviate HG-induced HRMEC injury.

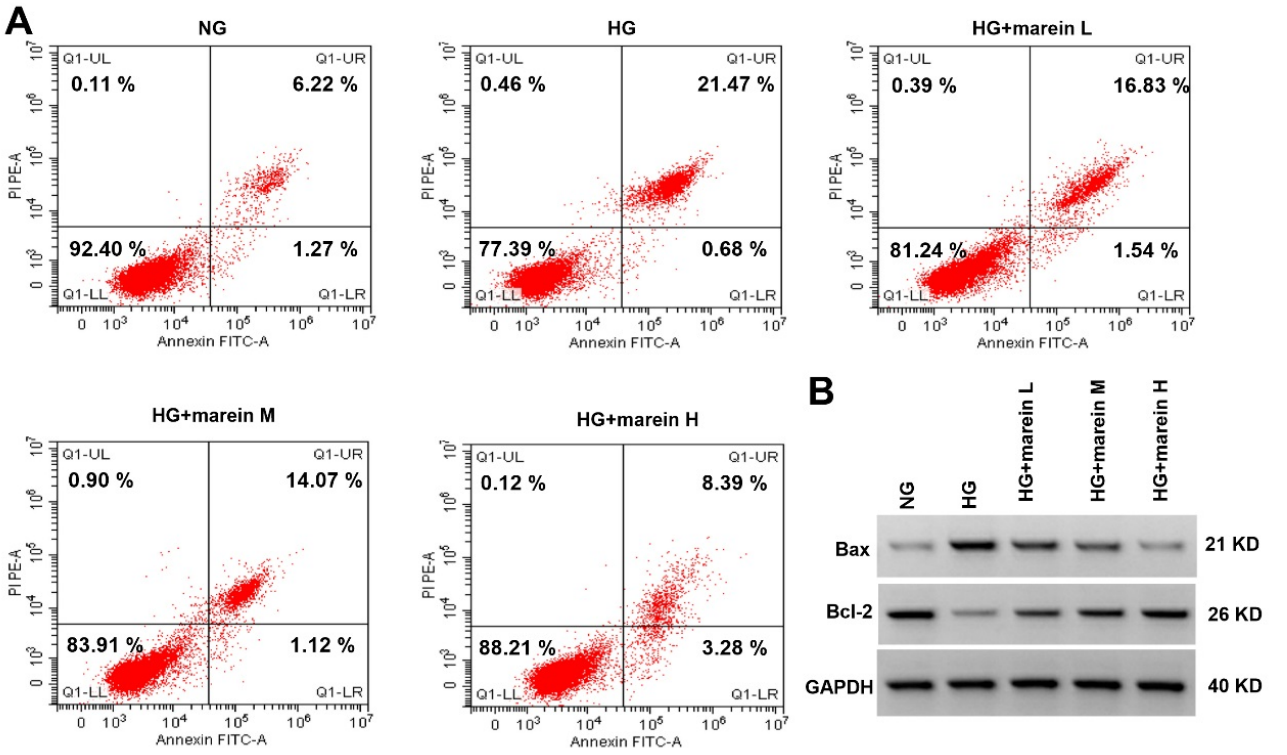

Fig. 1. Effect of marein on high glucose (HG)-induced human retinal microvascular endothelial cell (HRMEC) apoptosis. (A) Cell apoptosis rate was analyzed by flow cytometry. (B) Apoptosis-related protein levels were examined by means of Western blotting (n = 3). NG, normal glucose; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Bcl-2, B-cell lymphoma-2; FITC, fluorescein isothiocyanate.

Materials and Methods

Cell Treatment and Grouping

HRMEC (CP-H130) bought from Procell (Wuhan, China) was tested for mycoplasma and certified by short tandem repeat (STR) analysis. Cells were cultured with Dulbecco's Modified Eagle Medium (DMEM; D5921, Sigma-Aldrich, St. Louis, MO, USA) containing 5.5 mmol/L and 30 mmol/L glucose (D9434, Sigma-Aldrich, St. Louis, MO, USA), and were then categorized as normal glucose (NG) group and HG group, respectively. After the cells reached 60% confluence, Lipofectamine 3000 (L3000075, Invitrogen, Carlsbad, CA, USA) was used for transfecting pcDNA *SNHG7* overexpression vector (pcDNA-*SNHG7*), small interfering RNA against *SNHG7* (si-*SNHG7*: F 5′-CGAUUCUUAAGUUCUGCUAUU-3′, R 5′-UAGCAGAACUUAAGAAUCGGG-3′) and their controls (pcDNA and si-NC: F 5′-GGAGUAGGGAGCAAACCUAUAGGAA-3′, R 5′-UUCCUAUAGGUUUGCUCCCUACUCC-3′) into HRMEC. After transfection for 48 h, cells were harvested for functional experiments.

HRMEC was treated with 50 μmol/L (low), 100 μmol/L (medium), 200 μmol/L (high) marein (535-96-6, derived from cancer nutrition therapy (CNT), HPLC 90%–99%, Naturewill, Chengdu, China), in combination with 30 mmol/L glucose, and were labeled as HG+marein L, M and H groups, respectively. HRMEC was transfected with pcDNA and pcDNA-*SNHG7*, and treated with 30 mmol/L glucose, which were denoted as HG+pcDNA group and HG+pcDNA-*SNHG7* group, respectively. HRMEC was transfected with si-NC/si-*SNHG7* and treated with 200 μmol/L marein and 30 mmol/L glucose, which were labeled as HG+marein+si-NC group and HG+marein+si-*SNHG7* group, respectively.

Flow Cytometry

HRMEC was suspended with binding buffer, followed by staining with Annexin V-fluorescein isothiocyanate (V-FITC) and propidium iodide (PI, BB-4101, BestBio, Shanghai, China). Cell apoptosis rate was assessed using a flow cytometer (LSRFortessa TM X-20, BD Biosciences, San Diego, CA, USA).

Western Blotting

Total proteins were extracted from HRMEC by using Protein Extraction Kit (BB-3101, BestBio, Shanghai, China), and protein concentration was determined using BCA Kit (BB-3401, BestBio, Shanghai, China) and the absorbance was read by SpectraMax i3x microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The 4% stacking gel and 10% separating gel were prepared and installed in an electrophoresis tank. Protein samples (20

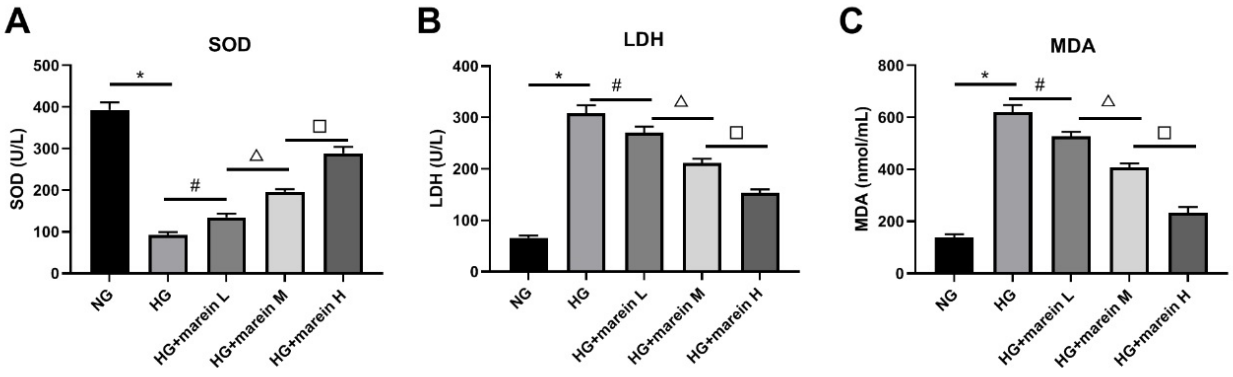

Fig. 2. Effect of marein on HG-induced oxidative stress in HRMEC. (A) Superoxide dismutase (SOD) activity. (B) Lactate dehydrogenase (LDH) level. (C) Malondialdehyde (MDA) level (n = 3). Notes: $^{*}p < 0.05$ compared to NG group; $^{\#}p < 0.05$ compared to HG group; $^{\triangle}p < 0.05$ compared to HG+marein L group; $^{\square}p < 0.05$ compared to HG+marein M group.

Table 1. Effect of marein on HG-induced HRMEC apoptosis.

Groups	Apoptosis rate (%)	Bax level (fold)	Bcl-2 level (fold)
NG	7.69 ± 0.43	0.14 ± 0.01	0.78 ± 0.06
HG	22.89 ± 1.34*	0.68 ± 0.05*	0.20 ± 0.02*
HG+marein L	19.37 ± 0.85$^{\#}$	0.50 ± 0.04$^{\#}$	0.37 ± 0.03$^{\#}$
HG+marein M	16.64 ± 0.80$^{\#\triangle}$	0.38 ± 0.03$^{\#\triangle}$	0.52 ± 0.05$^{\#\triangle}$
HG+marein H	12.38 ± 0.61$^{\#\triangle\square}$	0.23 ± 0.02$^{\#\triangle\square}$	0.65 ± 0.05$^{\#\triangle\square}$
F	144.984	125.673	78.833
p	<0.05	<0.05	<0.05

Data are expressed as mean ± standard deviation; n = 3.

Notes: $^{*}p < 0.05$ compared to NG group; $^{\#}p < 0.05$ compared to HG group; $^{\triangle}p < 0.05$ compared to HG+marein L group; $^{\square}p < 0.05$ compared to HG+marein M group.

μg) were separated by means of sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then, the separated proteins were transferred onto PVDF membranes. After blocking with non-fat milk, the membrane was incubated with anti-Bax (ab263897, 1:1000, Abcam, Cambridge, MA, USA), anti-B-cell lymphoma-2 (Bcl-2) (ab59348, 1:500, Abcam, Cambridge, MA, USA), or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2500, ab9485, Abcam, Cambridge, MA, USA) antibody overnight at 4 °C, followed by the incubation with goat anti-rabbit immunoglobulin G (IgG) (ab205718, 1:20,000, Abcam, Cambridge, MA, USA) antibody at room temperature for 1 h. Protein blots were developed using enhanced chemiluminescence (ECL) Reagent (BB-3501, BestBio, Shanghai, China) in Tanon 2500 chemiluminescence imaging system (V2.0, Tanon, Shanghai, China), and the gray value was evaluated by ImageJ software (V10.0, National Institutes of Health, Bethesda, MA, USA).

Detection of Cell Oxidative Stress

Superoxide dismutase (SOD) activity, lactate dehydrogenase (LDH) level and malondialdehyde (MDA) content in HRMEC were measured using corresponding detection kits (KFS388, KFS353 and KFS380, respectively, Baiaolaibo, Beijing, China) according to manufacturers' instructions.

Real-Time Quantitative Reverse-Transcription PCR

Total RNAs were extracted from the treated cells using TRIzol reagent (15596018, Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using Prime-Script RT Reagent Kit (RR716, Takara, Tokyo, Japan). Then, qRT-PCR was performed using SYBR Premix Ex Taq™ II (RR820A, Takara, Tokyo, Japan) in PCR system (ABI7500, Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the thermocycling conditions as follows: 95 °C for 1 min, 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. The sequences of primers used in this experiment are listed below: *SNHG7*: F 5′-GTGGGCACTGAGAATGGTTT-3′, R 5′-AAGTGCCCG AGCTTCAGATA-3′; *GAPDH*: F 5′-CTCTGCTCCTCC TGTTCGAC-3′, R 5′-CGACCAAATCCGTTGACTCC-3′. Relative expression of tested genes was normalized by *GAPDH* expression and calculated by $2^{-\Delta\Delta Ct}$ method.

Table 2. Effects of *SNHG7* overexpression on apoptosis and oxidative stress of HRMEC.

Groups	*SNHG7* (fold)	SOD (U/L)	LDH (U/L)	MDA (nmol/mL)	Apoptosis rate (%)	Bax (fold)	Bcl-2 (fold)
HG+pcDNA	1.00 ± 0.00	94.21 ± 10.05	311.34 ± 13.55	624.37 ± 35.06	22.93 ± 1.13	0.70 ± 0.07	0.21 ± 0.01
HG+pcDNA-*SNHG7*	5.22 ± 0.15*	321.29 ± 14.59*	93.49 ± 6.86*	171.13 ± 12.48*	9.49 ± 0.56*	0.19 ± 0.01*	0.70 ± 0.06*
t	48.728	22.201	24.844	21.095	18.458	12.492	13.953
p	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

Data are expressed as mean ± standard deviation; n = 3.

Note: *$p < 0.05$ compared to HG+pcDNA group.

Table 3. Effect of *SNHG7* knockdown on HG-induced HRMEC apoptosis.

Groups	*SNHG7* (fold)	Apoptosis rate (%)	Bax (fold)	Bcl-2 (fold)
HG+marein+si-NC	1.00 ± 0.00	11.48 ± 0.67	0.23 ± 0.02	0.65 ± 0.07
HG+marein+si-*SNHG7*	0.25 ± 0.03*	20.56 ± 1.04*	0.79 ± 0.05*	0.27 ± 0.03*
t	43.301	12.712	16.813	8.642
p	<0.05	<0.05	<0.05	<0.05

Data are expressed as mean ± standard deviation; n = 3.

Note: *$p < 0.05$ compared to HG+marein+si-NC group.

Statistical Analysis

Quantitative data are expressed as mean ± standard deviation, and analyzed by SPSS 20.0 software (IBM, Armonk, NY, USA). Comparison was assessed by Student's *t*-test (2 groups) or one-way analysis of variance (ANOVA) (multiple groups), and LSD *t*-test was used to compare between 2 groups. Differences were considered statistically significant when $p < 0.05$.

Results

Marein Inhibited HG-Induced HRMEC Apoptosis

Cell apoptosis rate and Bax level were higher ($p < 0.05$), while Bcl-2 level was lower ($p < 0.05$) in HG group than in NG group. Besides, cell apoptosis rate and Bax level were decreased ($p < 0.05$), while Bcl-2 level was increased ($p < 0.05$) in HG+marein L, M and H groups in a marein concentration-dependent manner (Fig. 1, Table 1). These findings confirmed that HG induced HRMEC apoptosis, which could be reversed by marein.

Marein Suppressed HG-Induced Oxidative Stress in HRMEC

Compared to NG group, SOD activity was decreased ($p < 0.05$), while LDH level and MDA content were increased ($p < 0.05$) in HG group. Besides, SOD activity was enhanced ($p < 0.05$), while LDH level and MDA content were reduced ($p < 0.05$) in HG+marein L, M and H groups in a marein concentration-dependent manner (Fig. 2). These data supported the role of marein in suppressing HG-induced oxidative stress in HRMEC.

Marein Promoted SNHG7 Expression in HG-Induced HRMEC

Compared to NG group, *SNHG7* expression was lower in HG-induced HRMEC ($p < 0.05$). Also, the *SNHG7* expression in HRMEC in HG+marein L, M and H groups was increased ($p < 0.05$) in a marein concentration-dependent manner (Fig. 3). These findings suggested that marein enhanced *SNHG7* expression in HG-induced HRMEC.

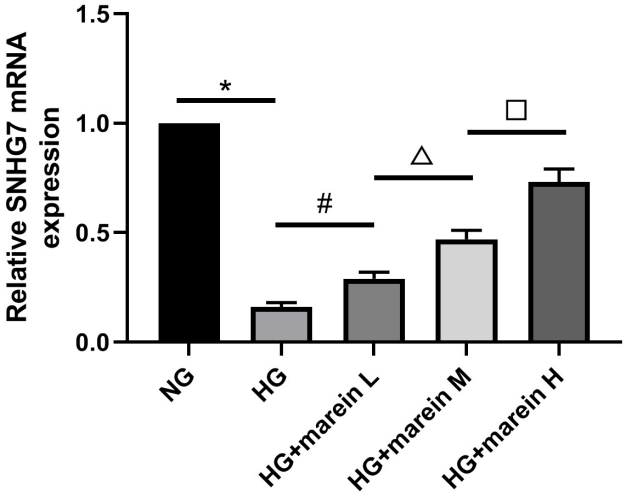

Fig. 3. Effect of marein on small nucleolar RNA host gene 7 (*SNHG7*) expression in HG-induced HRMEC. qRT-PCR was used for the measurement of *SNHG7* mRNA expression in each group (n = 3). Notes: *$p < 0.05$ compared to NG group; $^{\#}p < 0.05$ compared to HG group; $^{\triangle}p < 0.05$ compared to HG+marein L group; $^{\square}p < 0.05$ compared to HG+marein M group.

Table 4. Effect of *SNHG7* knockdown on HG-induced oxidative stress in HRMEC.

Groups	SOD (U/L)	LDH (U/L)	MDA (nmol/mL)
HG+marein+si-NC	290.84 ± 15.16	152.36 ± 10.43	238.82 ± 26.06
HG+marein+si-*SNHG7*	114.75 ± 9.35*	283.33 ± 13.48*	598.02 ± 28.19*
t	17.124	13.310	16.203
p	<0.05	<0.05	<0.05

Data are expressed as mean ± standard deviation; n = 3.

Note: *$p < 0.05$ compared to HG+marein+si-NC group.

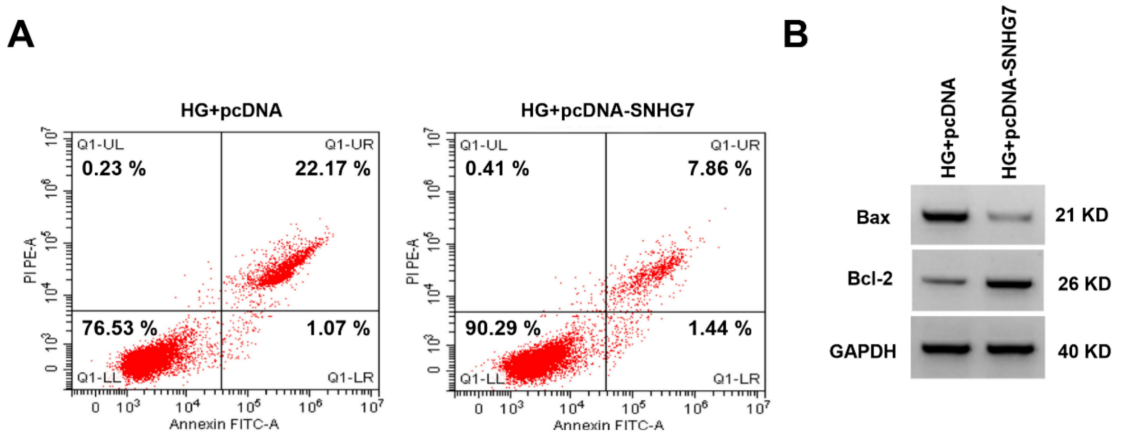

Fig. 4. Effect of *SNHG7* overexpression on HG-induced HRMEC injury. (A) Cell apoptosis rate was examined using flow cytometry. (B) Western blotting was used to detect apoptosis-related protein levels (n = 3).

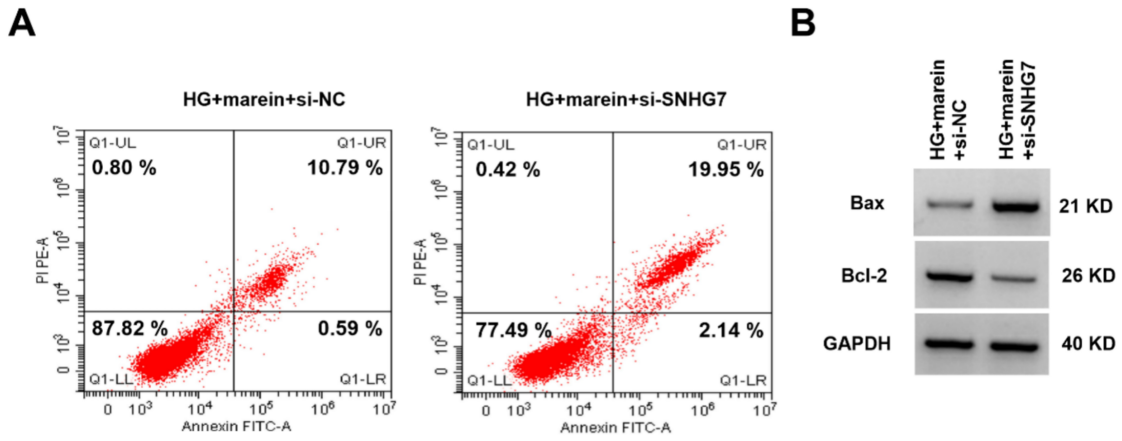

Fig. 5. Effect of *SNHG7* knockdown on marein-mediated protection of HRMEC from HG-induced apoptosis. (A) Cell apoptosis rate was examined using flow cytometry. (B) Apoptosis-related protein levels were analyzed by means of Western blotting (n = 3). NC, negative control.

Overexpression of SNHG7 Alleviated HG-Induced HRMEC Injury

Compared with HG+pcDNA group, *SNHG7* expression, Bcl-2 level, and SOD activity were higher ($p < 0.05$), while LDH level, MDA content, apoptosis rate and Bax level were lower ($p < 0.05$) in HG+pcDNA-*SNHG7* group (Fig. 4, Table 2). These results corroborated the ability of *SNHG7* in alleviating HG-induced HRMEC injury by suppressing apoptosis and oxidative stress of the cells.

SNHG7 Knockdown Reversed the Protective Effect of Marein on HG-Induced HRMEC Apoptosis

Compared with HG+marein+si-NC (negative control) group, *SNHG7* expression was decreased in HG+marein+si-*SNHG7* group ($p < 0.05$). *SNHG7* knockdown reduced Bcl-2 level but raised the apoptosis rate and Bax level in HG-induced HRMEC treated with marein ($p < 0.05$) (Fig. 5, Table 3). These results confirmed that silencing of *SNHG7* negated marein's protection of HRMEC from the HG-induced apoptosis.

SNHG7 Knockdown Reversed Marein-Mediated Suppression of HG-Induced Oxidative Stress in HRMEC

Compared with HG+marein+si-NC group, SOD activity was lower ($p < 0.05$), while LDH level and MDA content were higher ($p < 0.05$) in HG+marein+si-*SNHG7* group (Table 4). These data suggested that *SNHG7* knockdown abolished the marein-mediated suppression of HG-induced oxidative stress in HRMEC.

Discussion

Oxidative stress plays a critical role in DR development [11,12]. Traditional Chinese medicine and its extracts contain active compounds that can target multiple mechanisms, including oxidative stress-related pathways, so as to prevent or delay the progression of DR [13,14]. Studies had reported that marein, a flavonoid compound of CTN, can inhibit HG-induced fibrosis and inflammation in rat mesangial cells [15]. Importantly, marein could alleviate HG-induced human nucleus pulposus cell injury [16] and prevent PC12 cell apoptosis induced by methylglyoxal [17]. Collectively, these studies indicated that marein has a protective effect on cells. In this study, we treated HG-induced HRMEC with different concentrations of marein. Our findings revealed that cell apoptosis rate, Bax level, LDH level and MDA content were decreased, while Bcl-2 level, SOD activity and *SNHG7* expression were increased in a marein concentration-dependent manner. These results indicated that marein could suppress HG-induced HRMEC apoptosis and oxidative stress.

SNHG7 has been shown to participate in regulating the pathogenic processes of many diseases, such as osteoarthritis [18] and pituitary adenomas [19]. It has been demonstrated that *SNHG7* knockdown aggravated hemorrhagic shock, reperfusion-induced apoptosis and oxidative stress in pregnant rats [20], while overexpression of *SNHG7* could relieve neuronal apoptosis and oxidative stress induced by oxygen glucose deprivation/re-oxygenation (OGD/R) [21]. This study showed that *SNHG7* was underexpressed in HG-induced HRMEC. After *SNHG7* was overexpressed, the apoptosis rate and oxidative stress of HG-induced HRMEC were suppressed, indicating a protective role of *SNHG7* in repressing HG-induced HRMEC injury. We also found that marein could upregulate *SNHG7* expression. Additionally, rescue tests revealed that *SNHG7* silencing negated marein-mediated suppression of HG-induced HRMEC injury, confirming that marein suppressed HG-induced HRMEC injury by up-regulating *SNHG7* expression.

There are some limitations to this study. Although our results showed that the beneficial effects of marein are correlated with *SNHG7*, these findings did not contribute to a deeper understanding of the underlying mechanisms, which warrants comprehensive and in-depth investigation in future. Besides, this study investigated the impact of marein on *SNHG7* only, but its impact on other genes and associated pathways were not explored. Our future research will focus on conducting genome or transcriptome sequencing on different groups to observe the gene expression changes between HG group and HG+marein H group, and performing gene enrichment analysis to elucidate the relevant pathways.

Conclusions

In conclusion, marein could inhibit HG-induced HRMEC injury by up-regulating the expression of *SNHG7*. Therefore, these new findings support the use of marein in DR treatment.

Availability of Data and Materials

The data and materials of this experiment are available.

Author Contributions

SMJ, YHF and MMC designed the research study. JX performed the research. RFD provided help and advice on the experiments. GYL analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This work was supported by the Science and Technology Development Special Fund of Shenzhen Longgang District (No. LGKCYLWS2020087 and LGWJ2021-145), Shenzhen Innovation of Science and Technology Commission (No. SZXK039).

Conflict of Interest

The authors declare no conflict of interest.

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