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Research Article

Potential Effects of Ethanol Extracts of Propolis on Insulin Secretion and Metabolomics in MIN6 Cells

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Abstract

To investigate the potential effects of ethanol extract of propolis (EEP) on glucose metabolism in MIN6 cells. EEP was obtained by treating propolis with anhydrous ethanol. The optimal safe concentration of EEP against MIN6 cells was determined to be 50 μg/mL by the MTT method. The main measurement indexes included insulin secretion, glucose uptake and apoptosis rate under low and high glucose conditions. In addition, the levels of the differential metabolites in MIN6 cells were identified by metabolomics. EEP can effectively promote the proliferation of MIN6 cells. Under low glucose conditions, glucose uptake increased; under high glucose conditions, insulin secretion of MIN6 cells was enhanced, while the apoptosis rate was reduced. Moreover, after EEP treatment, the differential metabolites of low glucose group and high glucose group were mainly amino acids, carbohydrate and organic acids. EEP protected and promoted insulin secretion in MIN6 cells under high glucose conditions, which is associated with amino acid metabolism, mitochondrial metabolism, nicotinate and nicotinamide metabolism, and bile acid metabolism. EEP was significantly improves the glucose uptake and insulin secretion in MIN6 cells. It mainly regulated metabolites of MIN6 cells such as amino acids, carbohydrate, organic acids etc.

Keywords: Ethanol extract of propolis; MIN 6 cell; Insulin secretion; Metabolomics

Introduction

Recently, the prevalence of diabetes and obesity has increased rapidly, especially in developing countries such as China, India, etc. Indeed, China has become the largest diabetes country in the world [1]. For example, by 2017, a large population study based on a nationally representative sample of the mainland Chinese population showed that 11.2% of adults (according to WHO criteria) or 12.8% of adults (ADA criteria including the addition of HbA1c) aged 18 and older living in China had diabetes [2]. Type 2 diabetes mellitus (T2DM) is mainly caused by insulin resistance (IR) and hypoinsulinism. T2DM can promote multiple organ damage and various complications, including diabetic nephropathy, cardiomyopathy and myocardial infarction [3]. In addition, increasing health expenditures have led to increased financial pressure on diabetic patients and it is necessary to find novel as well as cheap anti-diabetic agents with minimal side effects for the treatment of this chronic disorder [1]. Weight loss and exercise training can serve as an effective intervention [4], while some anti-diabetic drugs are still needed to control the glucose level in the blood. Nevertheless, there are some adverse side effects associated with use of oral antidiabetic drugs, including hypoglycemia, osteoporosis and heart failure, which greatly limits their clinical application. Evidences have demonstrated that herbal medicines and their active ingredients can display significant anti-diabetic properties, with less toxicity and side effects [3].

As a traditional natural medicine, propolis exists as a viscous solid jelly formed by mixing plant resins collected by worker bees from the honeybee insects such as *Apis mellifera* L. and their maxillary

glands, wax glands and other secretions. It was officially included in the Pharmacopoeia of the People's Republic of China in 2005. A number of previous studies have shown that propolis contains a variety of physiologically active components and can be widely used in medicine, daily chemicals, agriculture as well as food [5-7]. Hence, it has been employed as a valuable bee product [8].

In addition, prior clinical studies have also indicated that propolis supplementation can effectively control the glucose level in blood and antioxidant status of T2DM patients and its mechanism of action may be related to the improvement of antioxidant indexes of serum. For example, the glutathione level in serum in patients taking propolis was observed to increase significantly, lactate dehydrogenase activity was markedly reduced [9,10], carbonyls level of serum decreased and the levels of IL-1β and IL-6 in serum was significantly increased [10], whereas TNF-α level in serum significantly decreased [10,11]. Moreover, different propolis can display varying trends in modulating the levels of the various inflammatory factors in patients, and thus can display differential effects on diabetes biomarkers such as fasting blood glucose (FBG) and insulin [12], and as such, there is a lack of research on the potential impact of the different doses of propolis.

It has been demonstrated that Propolis and Ganoderma compound tablet, Taiwan green propolis extracts (TGPE) can significantly improve the weight loss of rats. It was found that compared with the model group, FBG and glucose tolerance was observed to decrease significantly [13,14]. Oral propolis capsules can significantly inhibit the increase of FBG and triglycerides (TG), control the blood glucose, regulate lipid metabolism and improve insulin sensitivity in T2DM rats [15]. Taiwan green propolis extracts can effectively delay the progression of T2DM, reduce the degree of pancreatic β cell depletion, attenuate inflammation in rats, and suppress the generation of reactive oxygen species (ROS). Additionally, the serum of the TGPE treatment group displayed higher levels of leptin and adiponectin, and promoted the expression of liver genes perixisome proliferation-activated receptor alpha (PPAR-α) and cholesterol-7a-hydroxylase (CYP7A1), which was related to catabolism and removal of lipids14. Propolis has also been found to be effective in improving the intestinal microflora of mice with diabetes, repairing the intestinal mucosal damage of mice with diabetes, it can also facilitate the recovery of intestinal microflora, and increasing the short-chain fatty acids as well as other metabolites of intestinal microbes, which may be one of the mechanisms by which propolis can functionally ameliorate hyperglycemia [16,17]. Additionally, propolis can alleviate diabetes complications, such as kidney disease, retinopathy, foot ulcers and non-alcoholic fatty liver [18].

Recently, studies on the changes of metabolites in T2DM patients upon using metabolomics technologies such as ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) [19] and ultra high-performance liquid chromatography coupled with quadrupole-time-of-flight tandem mass spectrometry (UHPLC-QTOF-MS) [20] have been reported. In addition, possible biomarkers and corresponding metabolic pathways have been determined according to the difference in metabolites produced, thus providing an important theoretical basis for effectively preventing and treating T2DM [21]. For instance, Ma et al. quantitatively analyzed 87 different kinds of metabolites in the blood and urine of T2DM patients, including biomarkers related to metabolism of amino acids, lipids and carbohydrates [22]. Moreover, targeted metabolomics was used to identify pre-diagnostic biomarkers for T2DM in obese people in the United Arab Emirates. It has been established that early detection of changes in biomarker and metabolites patterns can be used for treat and prevent the early stage of diabetes and before the full outbreak of T2DM [23]. In animal model experiments, ^{1}H-NMR-based metabolomics were used to potentially determine 61 kinds of T2DM-related metabolites in the db/db mouse model, but due to some limitations associated with the study, including limited coverage of some potentially important metabolites (e.g., bile acid and lipids), further studies are needed to reveal these important regulatory effects [24].

It has been demonstrated that ethanol extraction of propolis is the most effective way to obtain high content of antioxidants [25]. Moreover, ethanol extracts of propolis (EEP) contain several key natural compounds such as flavonoids and polyphenols, which can effectively regulate T2DM and could be developed into potent anti-diabetic drug candidates with minimal side effects, but the relevant mechanisms underlying their actions have not been fully elucidated. MIN6 cells are an ideal model for studying the function of islet cells. This study primarily aimed to investigate the possible effects of EEP on glucose metabolism in MIN6 cells, using metabolomics to find differential metabolites and corresponding metabolic pathways that might be modulated and to explore the mechanisms of action of EEP on T2DM, which can provide useful lead for identification of novel natural products for the management of diabetes.

Materials and Methods

Reagents and instruments

Main instruments. Stat Fax-2000 Microplate Reader (Awareness Technology, USA), Thermo Scientific Dionex Ultimate 3000-Q Exactive Mass Spectrometer (Thermo Fisher, USA), ACQUITY UPLC BEH C18 (2.1×50mm, 1.7μm, Waters, USA), Accuri C6 Plus Cytometer (BD, USA), VC 130PB ultrasonic processor (Sonic Materials, USA), ALC-210.3 Electronic balance (Startorius, Germany), AVANCE III 400 MHz NMR spectrometer (Bruker Biospin, Switzerland), 5 mm nmr tubes (NORELL, USA).

Main Reagents. MIN6 cell strain (Shanghai EK-Bioscience Biotechnology Co., Ltd, China); penicillin- streptomycin, RPMI 1640 Medium (HyClone, USA); dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT), Hepes, albumin bovineV(BSA) (Solarbio, Beijing, China); fetal bovine serum (FBS) (Zhejiang Tianhang Biotechnology Co., Ltd., China); acetonitrile (HPLC) (Merck KGaA, Germany); ethanol (HPLC), K_2HPO_4, NaH_2PO_4, NaCl, KCl, NaOH (Sinopharm Chemical Reagent Co., Ltd, China); $MgCl_2$ (Tianjin Guangcheng Chemical Reagent Co.,Ltd, China); $CaCl_2 \cdot 6H_20$ (Tianjin Damao chemical reagent factory, China); glucose kit (glucose oxidase method), insulin assay kit (Nanjing Jiancheng Bioengineering Institute, China); Annexin V-FITC/PI apoptosis assay kit (Biosharp, China), D_2O (99.9%, 0.05% TSP) (Tenglong weibo technology Co., LTD, China).

Extracttion of propolis and compositions analysis

Anhydrous ethanol was added to propolis at the ratio of 1:10, ultrasonic treatment at 40℃ for 3 hrs, left overnight, and the supernatant was filtered and collected. The above steps repeated three times. The ethanol extract of propolis (EEP) was obtained by combining the supernatant, rotating evaporation and freeze-drying, and stored at -20℃. Before the experiment, EEP was redissolved in anhydrous ethanol and filtered by 0.22 μm filter membrane. The chemical compositions of EEP were analyzed by ultrahigh-performance liquid chromatograph Q extractive mass spectrometry (UHPLC- QE-MS). Thermo Science™ XCalibur™ was used to export the analyzable total ion current graph, and combine with Compound Dicsoverer 3.1 software to process the raw data for spectral analysis. Identify and characterize spectral peaks through metabolomics databases (e.g. HMDB, KEGG, mzCloud).

MIN6 cell culture

MIN6 cells were cultured in RPMI-1640 complete medium (containing 10% fetal bovine serum and 1% penicillin-streptomycin) at 37℃ and 5% CO_2, the cells were adhered to 80%-90% and then subcultured.

Determination of safe administration concentration of ethanol extract of propolis to MIN6

The well-growing MIN6 cells were seeded into a 96-well plate with 4×10^4/well, and 200 μL complete culture medium was added to each well. After the cells were attached to the wall for 24 hrs, the original medium was discarded and the blank group (0μg/mL) and the control group (0.1% ethanol), 10μg/mL EEP 20μg/mL EEP 50μg/mL EEP 100μg/mL EEP 250μg/mL EEP and 500μg/mL EEP groups were cultured for 24 hrs. The cell activity was measured by MTT assay, each group was provided with three wells.

Cell supernatant acquisition and insulin stimulation test

The well-growing MIN6 cells were seeded into a 24-well plate with 2×10^5/well, and 1 mL complete culture medium was added to each well. After the cells were attached to the wall for 24 hrs, the original medium was discarded, washed gently with KRBH buffer, and then pre-incubated with KRBH for 2 hrs to make the cells sensitized to glucose. Using fresh KRBH buffer, the experiment were divided into four groups: low glucose control group (5.5mmol/L Glu, LG), low glucose plus EEP group (5.5mmol/L Glu + 50μg/mL EEP, LG + EEP), high glucose control group (25mmol/L Glu, HG), high glucose plus EEP group (25mmol/L Glu + 50μg/mL EEP, HG + EEP), incubated at 37℃ 5% CO_2 for 1 h. The supernatant of the cells was collected by 1.5mL EP tube and centrifuged at 3000rpm for 15min, and stored at -20℃.

Determination of glucose uptake in MIN6 cells

The well-growing MIN6 cells were seeded into 24-well plate with 2×10^5/well, and 1 mL complete culture medium was added to each well. After the cells were attached to the wall for 24 hours, the original medium was discarded, gently washed twice with KRBH buffer, and then incubated for 2 hours with KRBH buffer containing 50μg/mL EEP. Then, add 5.5mmol/L and 25.0mmol/L glucose respectively, incubation at 37℃ 5% CO_2 for 20min, and collect the supernatant for detection.

Apoptosis assay by flow cytometry.

The cells treated with glucose and EEP for 24 hours, wash them twice with cold PBS, digest the cells with trypsin, add fresh culture solution to stop digestion, then gently blow to uniform single cell suspension, centrifuge to discard the culture medium, add PBS and centrifuge again for 2 times, and count the cells under microscope (the number of cells is controlled at 2×10^5/well). The apoptosis kit was used for determination.

Nuclear magnetic resonance spectroscopy

Cell extraction. MIN6 cells were divided into blank control group (0mmol/L Glu, C), blank control plus EEP group (0mmol/L Glu + 50μg/mL EEP, C + EEP), LG group, LG + EEP group, HG group and HG + EEP group. After the cells were treated for 24 h at 37℃, the original culture solution was discarded, the cells were rinsed three times with PBS and 4 mL ice-cold methanol was added to quench the cells. Scraping the cell and a mixture of methanol, chloroform, and ultrapure water (4:4:2.85 v/v/v) was added to the cell suspension. The cell suspension was vortexed in an iced water bath. The cells were sonicated 9 times for 1 min with 1 min intervals on ice and then kept on ice for 15 min, followed by centrifugation at 12000rpm for 4min at 4℃. Repeated three times and the aqueous phase was collected. The supernatant was rotating

evaporated and then dissolved in D_2O. After centrifugation, the supernatant was lyophilized in a freeze dryer and the residue was dissolved in 650μL phosphate buffer solution with D_2O (0.185M KH_2PO_4:0.225M K_2HPO_4:D_2O = 9:81:100 v/v/v). Finally, after centrifugation at 12,000 rpm for 4 min at 4°C, 550 μL of the supernatant was transferred into a 5 mm NMR tube and stored at −80°C.

Nuclear Magnetic Resonance Spectroscopy. All ^1H NMR spectra were obtained using an AVANCE 400 III superconducting Fourier transform NMR spectrometer equipped with ^{13}C, ^1H double resonance optimization of 5 mm CPTCI triple resonant trans-cryogenic probe. The proton resonance frequency was 400.104 MHz using the zg30 pulse sequence. The spectral width (SWH) was 12019.230 Hz. The number of cumulative scans (NS) was 256 and the number of empty times (DS) was 2. The experimental temperature (TE) was 298 K, and the spectrum was processed using Topspin3.2.

Chemometrics Analysis. The ^1H NMR spectra were analyzed by MestreNova 6.1.0 (Mestrelab Research, Santiago de Compostela, Spain) versus the exponential window function (Exponential: 0.5) to improve the signal-to-noise ratio. Manual correction of phase with baseline was carried out and TSP was used for calibration. The peak of 4.575-5.20 ppm was the cut off in MIN6 cells. The integral width (bin) was 0.002 and the integration interval was -0.5-9.5; they were normalized to the total area of the spectrum. The integral data was saved in.txt format. After opening the file with Excel, the water peak was removed and the data was introduced into SIMCA software for principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA).

Metabolite Identification. The data were imported into the Human Metabolomics Database (HMDB) (http://www.hmdb.ca/metabolites), Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/metabolomics/db_find/index.php), and other public databases to identify qualitative research. Differential metabolites were identified.

Signal Pathway Enrichment Analysis. The KEGG (https://www.kegg.jp/) was used for the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analysis, and the MetaboAnalyst 5.0 (http://www.metaboanalyst.ca/faces/ModuleView.xhtml) was used for signal pathway enrichment analysis.

Data analysis

Statistical analysis used GraphPad Prism 8 and SPSS version 24.0. The results are expressed in mean±SD. Differences were analyzed by Student's t test. $p < 0.05$ was considered statistically significant.

Results and analysis

Chemical composition of EEP

Using thermo Science ™ XCalibur™, the analyzable total ion flow diagram is derived, as shown in Fig.S1. CD software was used to identify 926 different substances in EEP, among which the substance with a peak area greater than 109 was identified as shown in Table S1. According to the area normalization method that was used to calculate the content of qualitative compounds, it was found that flavonoids accounted for 47.4%, mainly including genistein, daidzein, naringenin, isoliquiritigenin, sakuranetin, olmelin, hispidulin, isorhamnetin and tricin, etc., and polyphenols accounted for 7.5%, mainly including coniferyl ferulate and caffeic acid phenethyl ester, etc. In addition, carbohydrate accounted for 0.9%, mainly including sucrose and hexose, coumarins accounted for 11.9%, mainly containing imperatorin, coumarin, etc., and organic acids accounted for 3.8%, mainly including phenprocoumon, (E)-p-coumaric acid, etc. The results suggested that the main components of EEP were flavonoids.

Optimized concentration of EEP and its effects on glucose uptake as well as insulin secretion in MIN6 cells under different conditions.

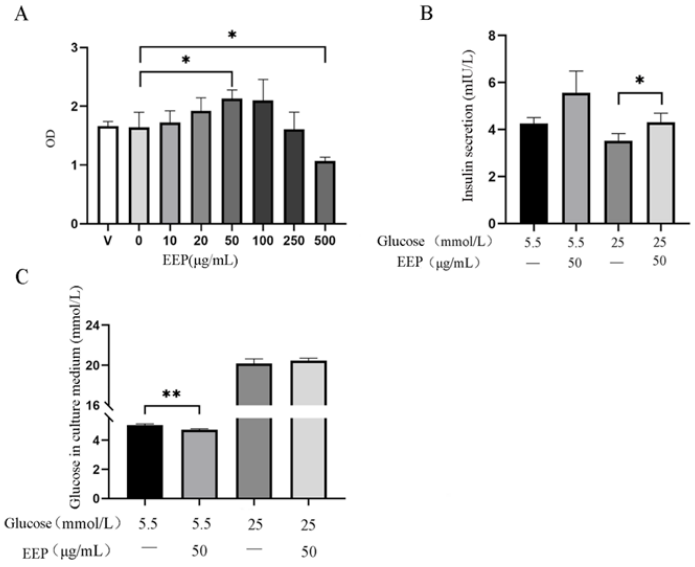

Figure 1 A: MTT assay; B: Insulin secretion detection test; C: Glucose intake test.

Note: *$p < 0.05$, **$p < 0.01$ vs Control group.

As shown in Fig.1A, MIN6 cell activity upon treatment with 10, 20, 50 and 100 μg/mL EEP increased, while MIN6 cell proliferation activity upon exposure to 250 and 500 μg/mL of EEP decreased significantly after 24 h. Specifically, the OD value was the highest

when the EEP concentration was 50 μg/mL, and it was found that 50 and 500 μg/mL EEP groups were significantly different from the control group ($p < 0.05$). Hence, EEP concentration was set as 50 μg/mL for the following experiments.

As shown in Fig. 1B, after MIN6 cells were treated with 50 μg/mL EEP, insulin secretion increased markedly. When glucose concentration was 5 mmol/L, EEP promoted insulin secretion significantly ($P < 0.05$), and when glucose concentration was 5 mmol/L, EEP promoted insulin secretion to not a significant extent, thereby suggesting that 50 μg/mL EEP can effectively increase insulin secretion during high glucose.

As shown in Fig.1C, compared with the control group, when glucose concentration was 5.5 mmol/L, after addition of 50 μg/mL EEP, the glucose concentration of the culture medium decreased remarkably with a significant difference ($p < 0.01$). However, when glucose concentration was 25 mmol/L, the glucose concentration of the culture medium did not decrease significantly, thus suggesting that EEP can functionally enhance glucose uptake by MIN6 cells under low glucose conditions.

Effects of EEP on apoptosis rate

As shown in Fig. 2A, the cell survival rate of the control group with high glucose was significantly lower than that of the normal cell control and EEP treatment substantially inhibited apoptosis of MIN6 cells induced by high glucose. The cell necrosis rate was slightly increased, and the overall cell survival rate was significantly increased ($p < 0.01$). However, compared with the low glucose group, no significant difference was observed despite the increased cell survival rate, thus indicating that EEP displayed an inhibitory effect on high glucose induced apoptosis of MIN6 cells, and the overall cell survival rate was increased significantly.

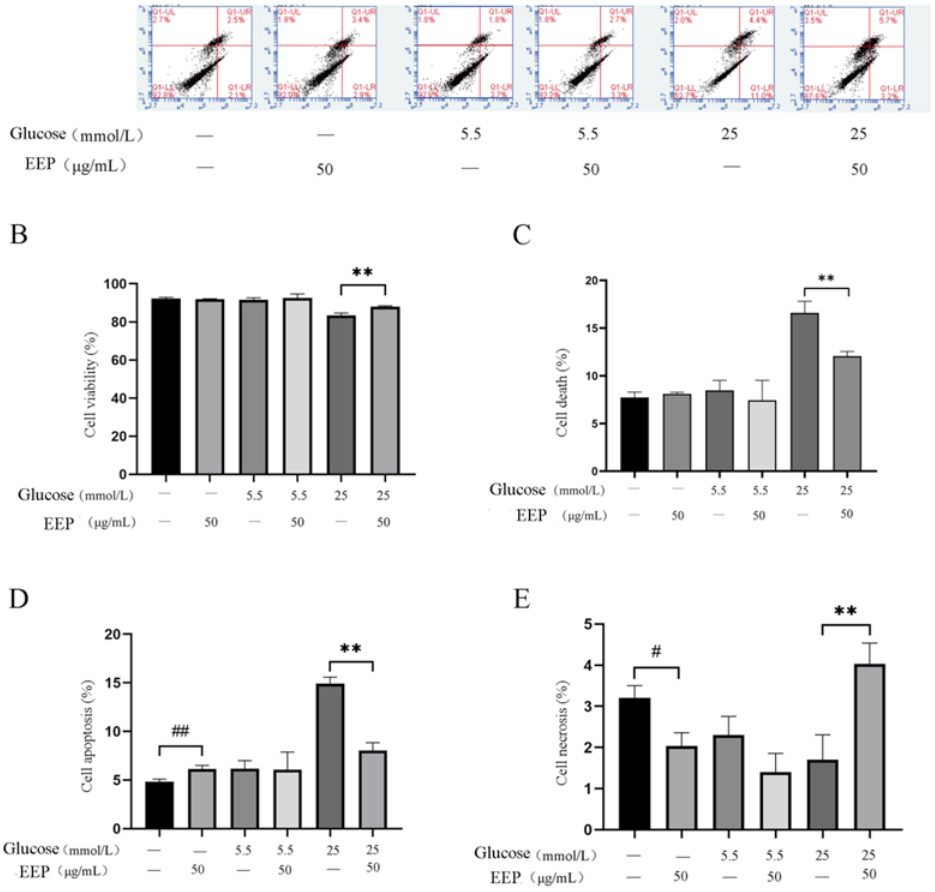

Figure 2: Effect of EEP on apoptosis of MIN6 cells. **(A)** Flow cytometry apoptosis diagram; **(B)** Cell viability rate; **(C)** Cell death rate; **(D)** Cell apoptosis rate; **(E)** Cell necrosis rate.

Note: #$p < 0.05$, ##$p < 0.01$ vs Blank group, **$p < 0.01$ vs HG (25 mmol/L Glu).

Multivariate statistical analysis

We imported the integrated area under the chemical shift of the NMR spectrum into the SIMCA-P14.1 software in excel format for multivariate statistical analysis. Principal component analysis (PCA) revealed differences between the LG and LG+EEP (Fig.3A), LG and HG (Fig.3B), HG and HG+EEP (Fig.3C). but the separation effect is not significant due to the large difference between sample groups and high dispersion. In addition, we also compared C and C+EEP, C and LG, C and HG, LG and HG, as shown in Fig.S2.

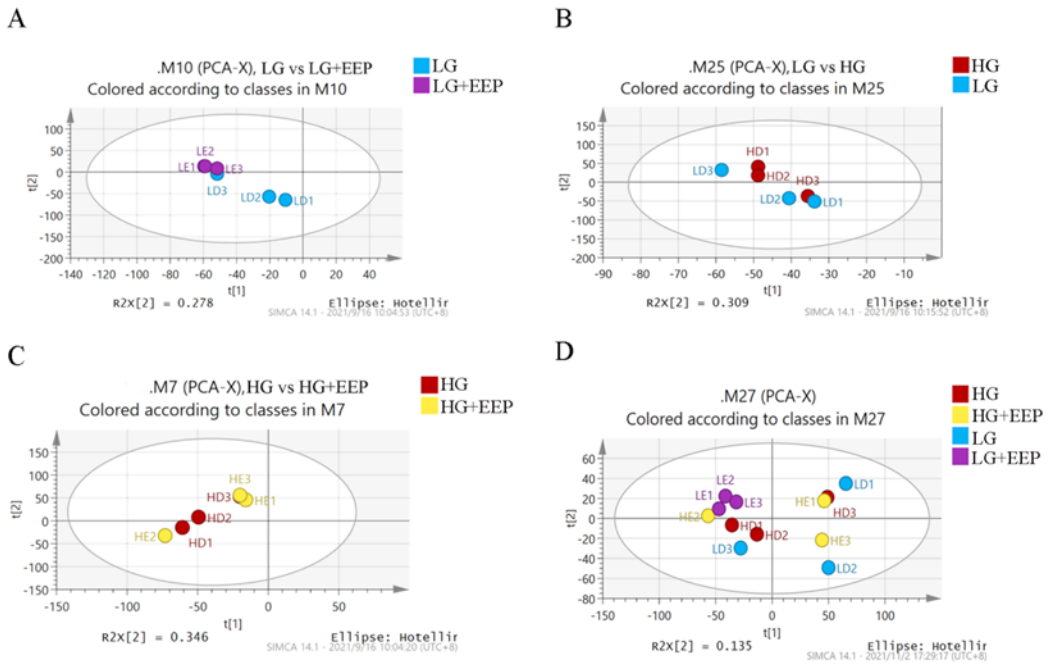

Figure 3: Multivariate statistical analysis. **(A)** LG vs LG+EEP; **(B)** LG vs HG; **(C)** HG vs HG+EEP; **(D)** HG, HG+EEP, LG, LG+EEP.

Identification of differential metabolites by metabolomics

$p < 0.05$ was used to filter the differential metabolites, there were 61 differential metabolites in LG and LG +EEP, 75 differential metabolites in LG and HG, and 43 differential metabolites in HG and HG + EEP (Table.S2). Due to the large number of differential metabolites, according to KEGG related metabolic pathways and pathway enrichment analysis, differential metabolites closely related to metabolic pathways were selected, as shown in Table 1. Additionally, combined with changes of the differential metabolites between groups, the alterations of metabolites was mainly concentrated in amino acids, carbohydrate, and organic acids. Under low glucose conditions, after EEP treatment, levels of amino acids, carbohydrate and organic acids was increased; after induction of high glucose, carbohydrate and homoserine, levels of amino acids such as methionine was increased, but the levels of histidine decreased compared with the low glucose group. Moreover, compared with the HG group, after EEP treatment, the levels of cellobiose and tryptophan was decreased, but the levels of tyrosine metabolites such as norepinephrine and adrenaline was decreased, while the level of L-thyroxine increased. Additionally, NADH appeared to increase. The differential metabolite information of C and C+EEP, C and LG, C and HG, LG and HG is shown in Table.S3-S5.

Table 1 Differential metabolites information table

		LG vs LG+EEP		LG vs HG		HG vs HG+EEP	
Title	**Matching shifts**	**P value**	**Trend**	**P value**	**Trend**	**P value**	**Trend**
			Amino acids				
Guanidineacetic acid	3.781	0.000	↑				
1-Methyl-L-histidine	3.058, 3.676	0.001	↑	0.002	↑		
L-Cysteine	3.044	0.001	↑				
L-Ornithine	3.043	0.001	↑				
L-Tyrosine	3.055	0.001	↑				
Argininosuccinic acid	3.269	0.003	↑				
L-Phenylalanine	3.271	0.003	↑				
L-Homoserine	3.774, 3.843	0.003	↑	0.014	↑		
L-Cystine	3.564	0.004	↑				
L-Threonine	1.318	0.004	↑				
gly-pro	3.574	0.004	↑				
N-Acetyl-L-alanine	1.317	0.014	↑				
S-Adenosyl-L-homocysteine	3.081, 3.801	0.010	↑	0.031	↑		
L-Alanine	1.471	0.014	↑				
L-Serine	3.952	0.014	↑				
L-Norleucine	1.339	0.018	↑				
3,5-Diiodo-L-tyrosine	3.842			0.014	↑		
L-Methionine	3.85			0.014	↑		
3,4-Dihydroxy-L-henylalanine	3.912			0.020	↑		
L-Histidine	7.826			0.023	↓		
N-acetyl-L-aspartic acid	4.379			0.024	↓		
trans 4 Hydroxy-L-proline	4.336			0.038	↓		
L-Tryptophan	3.3054, 3.292					0.026	↓
			Carbohydrate				
Lactulose	3.575	0.002	↑				
D-(+)-Maltose	3.266	0.003	↑				
D-Allose	3.774, 3.688	0.003	↑	0.002	↑		
L-(+)-Arabinose	3.775	0.003	↑				
D-Fructose-6-phosphate	3.555	0.004	↑				
Lactose	3.564, 3.808	0.004	↑	0.031	↑		
D-(-)-Fructose	3.806, 3.683	0.013	↑	0.002	↑		
D-Galacturonic acid	3.809, 4.396	0.042	↑	0.016	↓		
D-(+)-Mannose	3.851			0.014	↑		
D-(-)-Tagatose	3.849			0.014	↑		
L-(-)-Fucose	3.848			0.014	↑		

D-Ribose 5-phosphate	3.842			0.014	↑		
D-Trehalose	3.44			0.018	↑		
D-(+)-Glucose	3.401			0.029	↑		
1,6-anhydro-β-d-glucose	3.668			0.034	↑		
D-Cellobiose	3.611, 3.294			0.043	↑	0.026	↓
		Organic acids					
Chenodeoxycholic acid	1.325, 1.3205, 1.7924	0.004	↑	0.039	↑	0.027	↓
L-(+) Lactic acid	1.318	0.004	↑				
L-Malic acid	2.355	0.035	↑				
Pyruvic acid	2.364	0.035	↑				
Quinaldic acid	7.873			0.015	↑	0.018	↓
Shikimic acid	4.385			0.016	↓		
Quinolinic acid	7.439					0.044	↓
Nicotinic acid	7.504					0.045	↓
		Others					
Spermidine	1.777, 3.044	0.001	↑	0.039	↑	0.027	↓
Epinephrine	3.271, 6.953	0.003	↑			0.039	↓
NADH	7.517			0.010	↓	0.002	↑
Betaine	3.895			0.020	↑		
Tryptamine	7.706			0.024	↓		
Spermine	1.786, 1.777			0.039	↑	0.027	↓
myo-Inositol	3.609			0.043	↑		
Serotonin	3.305					0.026	↓
(+)α-tocopherol	1.781					0.027	↓
3-(2-hydroxyethyl)indole	7.127					0.028	↑
Indole	7.126					0.028	↑
L-Thyroxine	7.131					0.028	↑
(-)-Norepinephrine	6.953					0.039	↓

Analysis of metabolic pathways

In order to study the metabolic pathways of biomarkers related to glucose-free, low-glucose, high-glucose conditions and those after EEP treatment, the KEGG database was used to explore their potential interactions and MetPA was used to further confirm the influence of these distinct metabolic pathways (Fig. 4). The metabolic pathways and effects of C and C+EEP, C and LG, C and HG, LG and HG are shown in Fig.S3.

Moreover, pathway enrichment showed that after the low glucose addition of EEP, it mainly changed the pathways such as those associated with glycine and serine metabolism, urea cycle, homocysteine degradation, methionine metabolism, catecholamine biosynthesis, etc. High glucose mainly changes the methionine metabolism, cardiolipin biosynthesis, betaine metabolism, glycerol Phosphate Shuttle, histidine metabolism of MIN6 cells. After high glucose was added to EEP, it mainly changed nicotinate and nicotinamide metabolism, tryptophan metabolism, caffeine metabolism and vitamin B6 metabolism (Fig. 5).

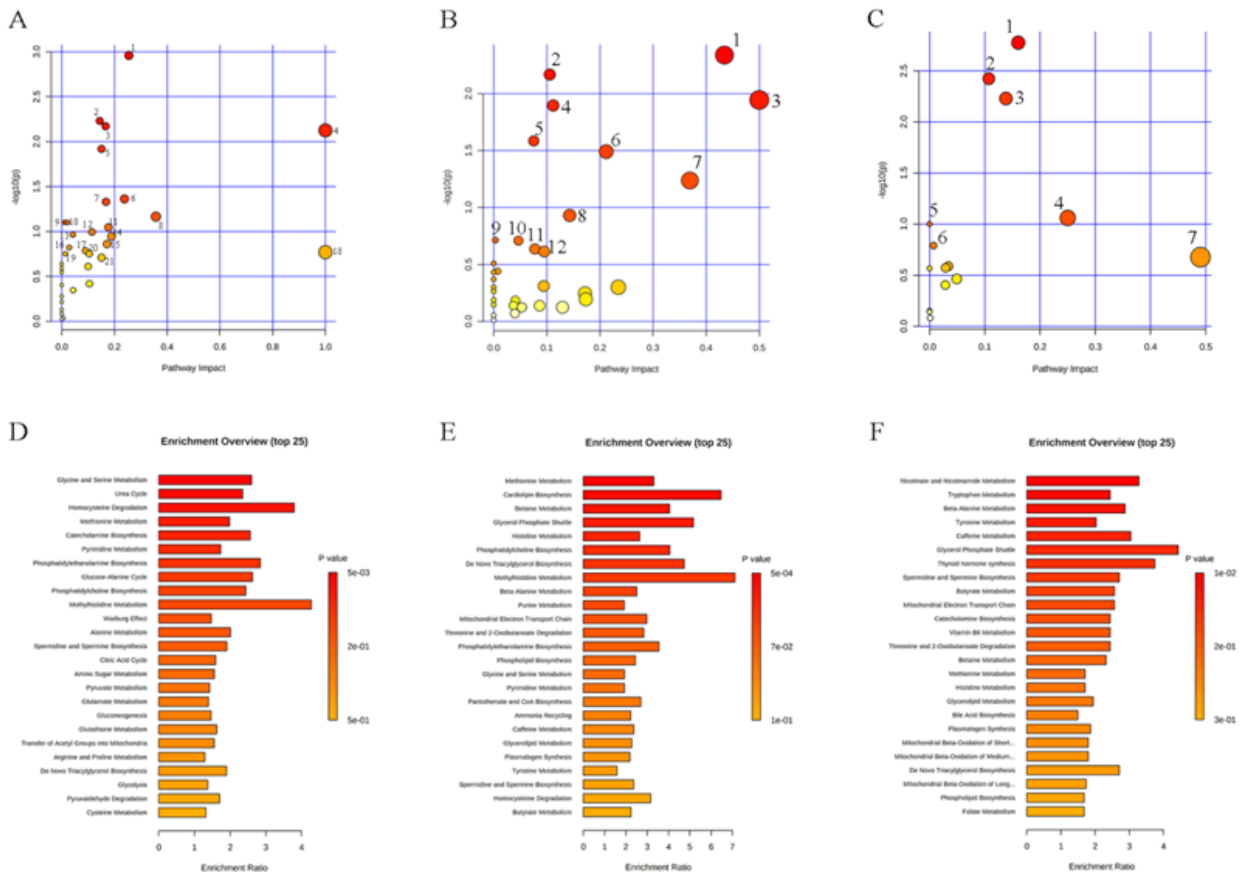

Figure 4: Overview of metabolic pathways **(A)** LG vs. LG+EEP (1) Glycine, serine and threonine metabolism; (2) Cysteine and methionine metabolism; (3) Aminoacyl-tRNA biosynthesis; (4) Phenylalanine, tyrosine and tryptophan biosynthesis; (5) Pyrimidine metabolism; **(B)** LG vs. HG (1) Histidine metabolism; (2) Pyrimidine metabolism; (3) Riboflavin metabolism; (4) beta-Alanine metabolism; (5) Purine metabolism; **(C) HG vs. HG+EEP** (1) beta-Alanine metabolism (2) Tyrosine metabolism (3) Nicotinate and nicotinamide metabolism (4) Tryptophan metabolism (5) Riboflavin metabolism) and metabolite enrichment of differential metabolites; **(D)** LG vs LG+EEP; **(E)** LG vs HG; **(F)** HG vs HG+EEP.

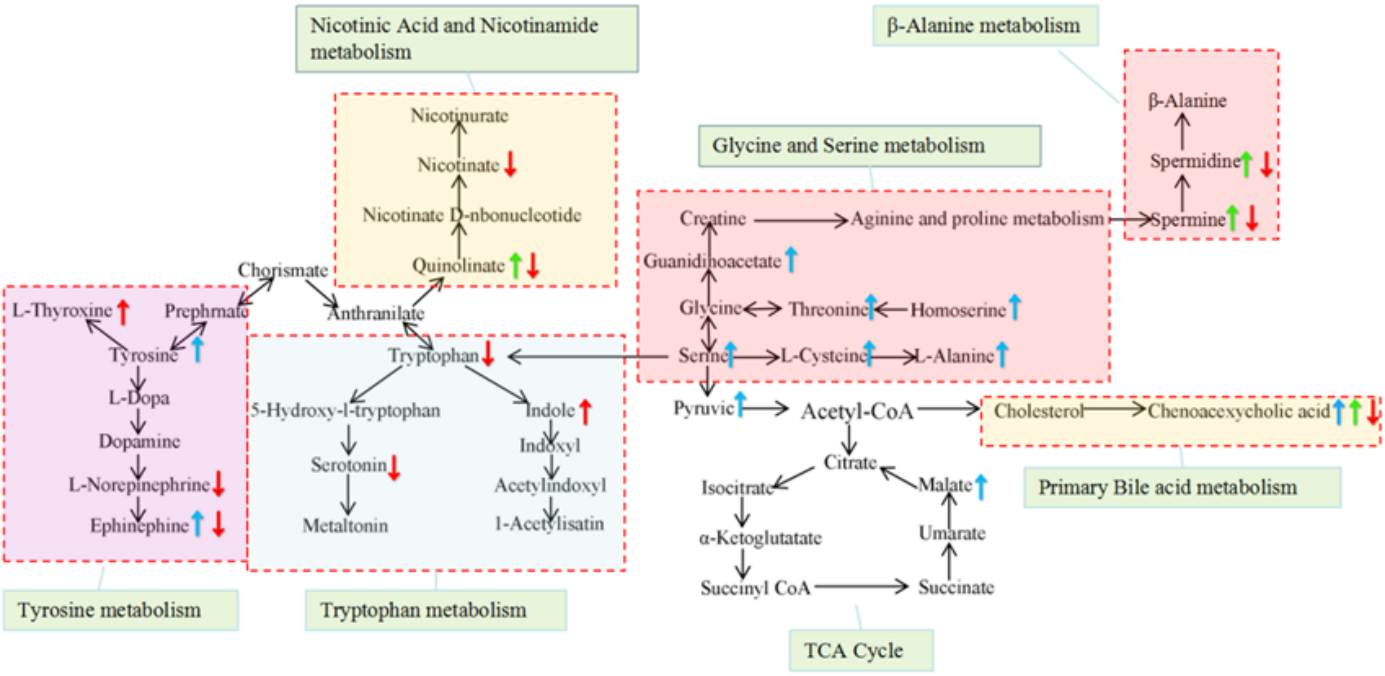

Figure 5: Comparation of the general biosynthetic pathways of LG vs LG+EEP, LG vs HG, HG vs HG+EEP related metabolites. ↑↓indicate LG vs LG+EEP, ↑↓**indicate** LG vs. HG, and ↑↓indicate significant changes in metabolites between HG vs. HG+EEP.

Discussion

The main components of EEP used in the study included polyphenols carbohydrate, coumarins, and organic acids, which was similar to the previous analysis of Luxi EEP using GC-MS. However, the contents of these components was significantly different, for example, the flavonoids in the EEP used this time accounted for 47.4%, which was higher than the 29.3% in the Luxi EEP, which might be due to the differences in instrument used and propolis origin [8]. However, the results of the both the studies proved that the main component in EEP is flavonoids. Flavonoids have been reported to exert substantial anti-diabetic effects, such as reducing oxidative stress in T2DM and enhancing the function of glucose transporters [26]. Propolis has been widely used for assist diabetes treatment because of its important functions in regulating blood glucose, preventing and treating diabetes. The therapeutic effects of EEP on MIN6 cell may be related to the promotion of glucose uptake, enhancement of insulin secretion, inhibition of apoptosis, regulation of amino acids metabolism mitochondria metabolism, nicotinic acid and nicotinamide metabolisms and bile acid metabolism in these cells.

Metabolism of amino acids

Under low glucose conditions, after EEP treatment, the levels of glycocyamine, cysteine, serine, threonine, alanine and homoserine increased significantly, and they actively participated in the metabolism of both glycine and serine. Glycine, a multifunctional amino acid is involved in the regulation of a variety of metabolic pathways, which are essential for many human physiological processes [27], and may interfere with the regulation of insulin level [28]. Additionally, the norleucine level was observed to be increased. In freshly isolated rat adipocytes, leucine and its analogue norleucine can activate the mammalian target of rapamycin (mTOR)-signaling pathway [29]. In addition, previous studies have shown that L-leucine can serve as an anabolic compound for promoting growth and tissue repair. Its anti-diabetic effects have also been reported, which were attributed to its ability to mTOR signaling pathway to stimulate the muscle glucose uptake [30]. This might be the reason that EEP can effectively promotes glucose uptake by MIN6 cells under low glucose conditions. Additionally, EEP treatment led to enhanced ornithine level in the urea cycle under low glucose conditions, which can be attributed

to enhanced energy metabolism in MIN6 cells.

At glucose concentration of 25 mmol/L, EEP treatment significantly promoted the insulin secretion in MIN6 cells, decreased the levels of tryptophan, serotonin, adrenaline as well as norepinephrine, and increased the level of thyroid hormone. Increased level of thyroid hormone can also effectively reduce the glucose level in blood in several ways, such as by increasing the insulin secretion of pancreatic β cells, and affecting the uptake of glucose by adipose muscle tissues [31]. Peterhoff et al. reported that adrenaline can functionally inhibit insulin secretion by activating α2-adrenaline receptors (ARs) inhibition [32]. EEP can lead to a marked decrease in the levels of adrenaline and norepinephrine, which can potentially stimulate insulin secretion in MIN6 cells. Additionally, adrenaline and norepinephrine are involved in tyrosine metabolism, and serotonin is involved in tryptophan metabolism. A number of studies have indicated that tryptophan could impair insulin secretion, whereas tryptophan metabolites inhibited the synthesis of pro-insulin, and glucose as well as leucine-induced insulin release in rats [33]. Tyrosine and tryptophan belong to the class of aromatic amino acids, and have been shown to be closely related to T2DM, as increased levels of aromatic amino acids in serum will be accompanied by a higher risk of T2DM [34,35]. However, EEP treatment led to a significant reduction in the metabolism of both tryptophan and tyrosine in MIN6 cells, which might be one of the mechanisms for alleviating T2DM.

Mitochondria metabolism

Glucose is the main stimulus of insulin secretion, and its metabolism in pancreatic β-cells can be achieved by tightly combining glycolysis with mitochondria metabolism [36]. Nevertheless, high glucose level is harmful to natural pancreatic β-cells and can cause apoptosis when exposed for a long time [37]. This study also proved the hypothesis that high sugar can lead to a decrease in MIN6 cells insulin secretion capacity, thereby causing apoptosis. Intact cells are essential for maintaining the cell structure and function, however, osmotic regulation plays a vital role in maintaining the structure as well as function [38]. Betaine and inositol are important osmotic pressure regulators found in astrocytes [39]. Additionally, compared with the control group, the level of inositol in the hippocampus of diabetes obese rats was found to be increased to avoid ion-induced protein function disturbance [40]. In this study, after high-glucose treatment, the levels of betaine and inositol in MIN6 cells increased significantly compared to low-glucose-treated cells. It can be thus speculated that an increase in levels of betaine and inositol might be due to the increased demand for regulation of osmotic pressure by MIN6 cells after high-glucose exposure, which indicated that MIN6 cells might exhibit self-protection behavior under high glucose stress.

Glycolysis-derived NADH is also important for ATP synthesis, because it can provide the reducing equivalent of the electron transfer chain (ETC) through modulating glycerol-3-phosphate and malate--aspartate shuttles, and inhibiting these two shuttles at the same time can substantially reduce the release of insulin. Hyperglycemia damages mitochondria metabolism and reduces the ATP required by glucose stimulated insulin secretion (GSIS). Lack of ATP can damage the closure of K_{ATP} channels, which can leads to the membrane depolarization, calcium influx and insulin granule exocytosis, which can further increase blood sugar and thus effectively trigger a vicious circle [41]. Meanwhile, the coupling of NADH and mitochondria ETC is crucial for pancreatic β-cell function as well as energy metabolism, and restoration of mitochondria bioenergy is also very important for the treatment of T2DM. Moreover, experimental results have shown that the NADH level decreased and the glycerol phosphate shuttle pathway was inhibited under high glucose conditions. After EEP treatment, it was observed that the NADH level rose remarkably, and the glycerol phosphate shuttle and the mitochondria ETC pathway were activated, thus indicating that the function of mitochondria has been restored at least to a certain extent, and thereafter the ability of insulin secretion was also restored.

Metabolism of nicotinic acid and nicotinamide

Nicotinic acid is an important nutritional supplement. Although it is beneficial to human health, studies have shown that nicotinic acid can increase FBG, reduce glucose stimulated insulin secretion (GSIS) as well as insulin secretion and impair glucose tolerance. It can also be combined with nicotinic acid receptor GPR109a to stimulate sodium-glucose cotransporter 1 (SGLT1) and glucose-transporters 2 (GLUT2) jejunal glucose uptake in diabetes mediated by hyperglycemia, thereby impairing fasting blood glucose [42]. Sun et al. reported that the accumulation of nicotinic acid (a form of Vitamin B3) significantly increased the production of insulin level in plasma and H_2O_2, impaired glucose metabolism, and caused oxidative stress and IR [43]. Oxidative stress could impair the function and completeness of mitochondria [44]. The results of this study revealed that EEP treatment led to substantially reduced level of nicotinic acid under high glucose conditions. In summary, propolis can reduce blood glucose by attenuating the intake of carbohydrates in the gastrointestinal tract and intestinal cells [45]. The decrease in nicotinic acid level caused by EEP may be one of the possible mechanisms for restoring T2DM.

Metabolism of bile acids

Under high glucose conditions, chenodeoxycholic acid related to metabolism of the bile acids changed significantly after EEP intervention. Chenodeoxycholic acid, one of the most abundant primary bile acids in the human body, belongs to hydrophobic bile acid, which can effectively destroy cell membranes, promote the production of ROS, and ultimately lead to cell apoptosis as well as necrosis. It can also activate the death receptors through mitochondria pathway and induce ER stress to trigger apoptosis of liver cells [46]. Additionally, findings of Chinese T2DM hamster liver proteomics proved that bile acid metabolism was abnormal in Chinese hamsters under T2DM state [47], and bile acid can promote T2DM progression through stimulating liver gluconeogenesis, glycogen synthesis, energy consumption, inflammation and other mechanisms [48]. Under high glucose conditions, chenodeoxycholic acid level has been reported to be increased. Chenodeoxycholic acid level decreased significantly after EEP treatment, thus indicating that EEP might protect MIN6 cells by down-regulating chenodeoxycholic acid level.

Conclusions

EEP is rich in flavonoids, which can significantly improve the glucose uptake by MIN6 cells under low glucose conditions and insulin secretion in MIN6 cells under high glucose conditions. Meanwhile, EEP treatment can also significantly reduce cell damage and decrease cell mortality. The metabolomics results showed that EEP can mainly regulate metabolites of MIN6 cells (e.g., amino acids, carbohydrate, organic acids), and the protection mechanism was related to metabolism of amino acids, restoration of mitochondria metabolism, and down-regulation of metabolism of nicotinic acid and nicotinamide as well as bile acid. However, the experimental results also indicated that the changes in some metabolites after EEP treatment under low glucose conditions were consistent with those under high glucose conditions, such as S-adenosyl-L-homocysteine, carbohydrates, etc., and the each group glucose level of differential metabolites changed significantly, which still needs further research.

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