Identification of genipin as a potential treatment for type 2 diabetes

By

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ABSTRACT

Type 2 diabetes (T2D) is a chronic metabolic disease characterized by hyperglycemia, insulin resistance, and the dysfunction of β-cells. While there are several therapies for T2D, there is no effective treatment that can reverse the functional decline of pancreatic β cells in T2D patients. Glucagon-like peptide-1 (GLP-1) is a peptide hormone secreted by human intestinal L cells, which can stimulate the proliferation and differentiation of β cells and promote glucose-stimulated insulin secretion (GSIS), thereby playing a critical role in maintaining glycemic homeostasis. Recently, GLP-1-based medications have been developed for treating T2D. However, most of the GLP-1-based drugs are expensive and have significant adverse effects. Therefore, development of safer and more convenient agents that can mimic the physiologically fed state to promote endogenous GLP-1 secretory function of intestinal L-cells to improve glucose homeostasis holds great potential for the prevention and treatment of T2D. This project aimed to examine whether natural compound genipin promotes intestinal GLP-1 secretion and exerts anti-diabetic effects. I found that genipin rapidly increased GLP-1 secretion from intestinal L-cells, with 10 and 100 μM concentration inducing significant incretin hormone release. L-cells exposed to genipin displayed a rapid increase in intracellular $[Ca^{2+}]_i$ and the activity of phospholipase C (PLC). Inhibition of PLC ablated genipin-stimulated Ca^{2+} increase and GLP-1 secretion, suggesting that genipin-induced GLP-1 release from the cells depends on the PLC/Ca^{2+} pathway. In vivo, genipin reduced the non-fasting and fasting blood glucose levels, improved insulin resistance, and protected again high fat diet-induced liver damage. All together, these data indicate that genipin is a naturally occurring anti-diabetic agent, which could be a pharmaceutical lead for developing anti-diabetic drugs.

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General Audience Abstract

More than 34 million Americans are suffering from diabetes, with over 90% of these cases being type 2 diabetes (T2D). Loss of β-cell mass and function is central to the deterioration of glycemic control over time in T2D. Therefore, preservation or improvement of β-cell mass and its insulin secretory function could prevent and treat T2D. While there are several pharmaceutical therapies for T2D, no effective treatment is available for reversing functional decline of pancreatic β-cells in T2D patients. It has been well recognized that glucagon-like peptide-1 (GLP-1), which is an incretin hormone secreted from intestinal L-cells, plays a critical role in maintaining glycemic homeostasis via potentiating glucose-stimulated insulin secretion and promoting β-cell proliferation. This present work is to determine whether natural compound genipin promotes intestinal GLP-1 secretion and thus exerts anti-diabetic effect.

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DEDICATION

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LIST OF ABBREVIATIONS

A

ALT: Alanine aminotransferase

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

AUC: Area under the curve

B

BW: Body weight

BSA: Bovine serum albumin

C

cAMP: Cyclic adenosine monophosphate

C_{max}: Maximum concentration

CNS: Central nervous system

D

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

DN: Diabetic nephropathy

DPP-4: Dipeptidyl peptidase 4

E

ELISA: Enzyme-linked immunosorbent assay

F

FFA: Free fatty acid

FBS: Fetal bovine serum

FoxO1: Forkhead box class O1

G

GBM: Glomerular basement membrane

G6Pase: Glucose-6-phosphatase

GIP: Glucose-dependent insulinotropic peptide

GLP-1: Glucagon-like peptide 1

GLUT2: Glucose transporter 2

GLUT4: Glucose transporter 4

GSIS: Glucose stimulated insulin secretion

GTT: Glucose tolerance test

GPCR: G-protein coupled receptor

H

HFD: High fat diet

HP-β-CD: Hydroxypropyl-β-cyclodextrin

HPLC: High-performance liquid chromatography

I

IBMX: 3-isobutyl-1-methylxanthine

IP_3: Inositol trisphosphate

IR: Insulin resistance

ITT: Insulin tolerance test

J

JNK: c-Jun N-terminal kinase

K

KRB: Krebs Ringer Buffer

M

MRT: Mean residence time

P

PDX-1: Duodenal homeobox 1

P-gp: P-glycoprotein

POMC: Pro-opiomelanocortin

PPARa: Peroxisome proliferator-activated receptor

PEPCK: Phosphoenolpyruvate carboxykinase

PLC: Phospholipase C

PI3-K: Phosphatidyl inositol 3-kinase

POMC: Proopiomelanocortin

PTT: Pyruvate tolerance test

R

ROS: Reactive oxygen species

S

SGLT1: Sodium-glucose co-transporter 1

T

TCF7L2: T-cell factor 7-like 2

$T_{1/2:}$ Half-life

T2D: Type 2 diabetes

TG: Triglycerides

TRPM5: Transient receptor potential channel M5

U

UCP2: Uncoupling protein 2

UPLC-Q/TOF MS: Ultraperformance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry

W

WT1: Wilms' tumor 1 gene

CHAPTER ONE

Introduction

Background

Diabetes is a group of metabolic diseases, which is one of the major health challenges around the world [1-3]. In the United States, 10.5% of the population has diabetes, with approximately 90-95% of all diagnosed diabetes cases belonging to type 2 diabetes (T2D) [3]. T2D is characterized by insulin resistance and varying degrees of insulin synthesis and secretion defects caused by pancreatic β-cell dysfunction [4-6]. Metformin has been widely used for the treatment of T2D. While it is effective in ameliorating hyperglycemia primarily via decreasing hepatic glucose production and enhancing insulin sensitivity, it is unable to stop the progressive decline in β-cell function and mass [7-10]. Therefore, search for agents that promote β-cell function and mass could be an effective strategy in the prevention and treatment of T2D [11].

It is well recognized that a group of intestinal hormones can increase insulin secretion in response to ingested nutrients[12]. There are two major incretin hormones, glucagon-like peptide-1 (GLP-1) and sugar-dependent insulinotropic peptide (GIP), of which GLP-1 plays a more critical role in maintaining glycemic homeostasis [13-15]. GLP-1 is primarily secreted by L-cells, which are enteroendocrine cells primarily located in the ileum and colon. In addition to augmenting glucose-stimulated insulin secretion (GSIS) while suppressing glucagon secretion [16, 17], GLP-1 was also shown to promote the survival and regeneration of pancreatic β-cells in rodent models [18]. Further, GLP-1 delays stomach emptying, induces satiety, and reduces body weight gain in animal models of obesity [19]. However, it was found that postprandial GLP-1 response is

significantly impaired in patients with T2D [20]. Therefore, inducing intestinal GLP-1 secretion or activating the GLP-1 signaling system could be an effective strategy for the treatment of T2D.

Lately, GLP-1 based drugs, including GLP-1 analogs and dipeptidyl peptidase-4 (DPP-4) inhibitors that inhibit the breakdown of GLP-1, have been developed to treat T2D [21-23]. However, patients receiving GLP-1 based drugs may require injections and are expensive, and some patients experience side effects such as nausea and vomiting [24]. More importantly, the use of incretin-based drugs raised substantial concerns of a possible increased risk of pancreatitis and C-cell hyperplasia [24-26]. Furthermore, DPP-4 is a ubiquitous enzyme involved not only in the enzymatic cleavage of numerous other peptide substances other than GLP-1, but also is in non-enzymatic interactions with some proteins [27], highlighting a wide range of biological functions of DPP-4. Therefore, indiscriminate inhibition of DDP-4 may affect a spectrum of important physiological functions of DDP-4 other than the degradation of GLP-1.

Given these above-mentioned reasons, there is still an immense prospect for developing safer and more convenient agents that can promote endogenous GLP-1 secretory function of intestinal L-cells to improve glucose homeostasis, thereby preventing and treating of T2D. Many natural compounds including monoterpenes have been recently investigated for the treatment of T2D [28]. Genipin, a small molecular agent isolated from fruit of gardenia jasminoides Elli, is a monoterpene [29, 30]. It has been reported that genipin is a potent inhibitor of uncoupling protein 2 (UCP2) which is a negative regulator of glucose-stimulated insulin secretion (GSIS), thereby regulating the level of blood glucose [31]. It is unclear whether this compound has antidiabetic activity and whether it can promote GLP-1 secretion and β-cell function. Therefore, my thesis research aimed at investigating whether genipin has antidiabetic effects by stimulating the secretion of GLP-1.

Hypothesis

From my preliminary studies searching for potential plant-derived antidiabetic compound, I found that genipin can directly target intestinal L-cells, leading to the secretion of GLP-1. Based on this exciting result, I hypothesize that **genipin may have an anti-diabetic effect via promoting GLP-1 secretion**. To test this hypothesis, I propose the following specific aims:

Specific Aim 1. Determine the cellular mechanism by which genipin induces GLP-1 secretion from L-cells

I hypothesize that genipin stimulates phospholipase C (PLC), leading to intracellular Ca^{2+} release, thereby GLP-1 secretion from L-cells.

Specific Aim 2. Explore the anti-diabetic effect of genipin in vivo.

I hypothesized that genipin plays a role in anti-diabetic impact by increasing GLP-1 level in diabetic mice.

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CHAPTER TWO

Literature Review

Abstract

More than 34 million Americans are suffering from diabetes, with over 90% of these cases being type 2 diabetes (T2D). Loss of β-cell mass and function and insulin resistance are central to the deterioration of glycemic control over time in T2D. Therefore, preservation or improvement of β-cell mass and its insulin secretory function could prevent and treat T2D. Genipin, a metabolite from geniposide, is mainly generated from gardenia jasminoides Elli, and has been reported to exhibit antidiabetic effects. However, the main targets and mechanisms for genipin to exert antidiabetic roles are unclear. This chapter reviews the chemistry, pharmacokinetic, and its potential antidiabetic effects of genipin. Attention is also given to recent studies exploring the cellular mechanisms of genipin actions that may relate to its antidiabetic effect. Further, a brief overview of possible toxicity is provided in the context of developing therapeutic approaches using this compound.

Key words: genipin; geniposide; type 2 diabetes; insulin resistance; β-cell dysfunction; UPC2

1. Introduction

Diabetes is a rapidly growing chronic disease in the US and around the world, with 463 million adults being affected globally in 2019, which is projected to reach 700 million by 2045 [1]. Type 2 diabetes (T2D) is the most common type of diabetes, accounting for approximately 90% to 95% of all diabetes cases [2]. Clinical studies have shown that insufficient physical activity, overnutrition, cigarette smoking, alcohol, and obesity are the major risk lifestyle factors of T2D [3]. Specifically, obesity is estimated to account for about 55% of T2D cases [4]. T2D and its complications have caused millions of deaths worldwide [5]. In the US, the average life expectancy of T2D patients is shortened by 2 years [6]. Approximately 12% of global health expenditure in 2015 was used to treat T2D and its related complications [7].T2D is a result of chronic insulin resistance and loss of β-cell function and mass [8-10]. Insulin resistance in T2D results in reducing glucose uptake by muscles and adipocytes, and weakening insulin-mediated intracellular activities [11, 12]. Constant insulin resistance will progress to T2D when β-cells become unable to secrete adequate amounts of insulin to compensate for decreased insulin sensitivity, which is largely due to pancreatic β-cell dysfunction and apoptosis [8-10]. Classic anti-diabetic drugs include α-glycosidase inhibitors that can delay the absorption of intestinal glucose into blood by competitively inhibiting various glucosidase enzymes located in the small intestine; sulfonylureas and meglitinide that could promote insulin secretion; and thiazolidinediones which increase the insulin sensitivity of the liver, muscle, and adipose tissue [13]. Recently, GLP-1-based drugs, including GLP-1 analogues and dipeptidyl peptidase-4 (DPP-4) inhibitors that inhibit the breakdown of GLP-1, have been developed for treating T2D [14-16]. However, some patients with these treatments suffer from plenty of side effects such as gastrointestinal complications and gradual loss of drug sensitivity after long-term usage [17-19].

Prlonged medications are required since T2D is a chronic disease, which leads to financial burdens for some lower-income households [20]. Hence, development of safer and cheaper antidiabetic agents holds great potential for the prevention and treatment of T2D. In this review, attention will be given to highlight the related research performed in terms of chemistry, pharmacokinetic properties as well as antidiabetic effects of genipin in T2D.

2. Sources and Chemistry of Genipin

Genipin (Figure. 1), an aglycone derived from geniposide, is a major constituent in the fruits of gardenia jasminoides Elli (gardenia, Rubiaceae family). It can also be generated from an iridoid glycoside geniposide by the intestinal enzyme β-glucosidase [14, 15]. Genipin was identified from Rubiaceae in the 1960s [16], and genipin and geniposide were then extracted from more than 30 different plants [17]. Some of these plants, including G. jasminoides (Zhizi in Chinese), Eucommia ulmoides Oliv. (Duzhong in Chinese), and Rehmannia glutinosa Libosch. (Dihuang in Chinese), in traditional Chinese medicine have been used to treat cancer, inflammation, and diabetes for hundreds of years [18-20].

Genipin can be generated by hydrolysis of geniposide [23, 24], which primarily occurs via microbial transformation and enzymatic hydrolysis in the intestine [22, 24, 25]. It was demonstrated that *Penicillium nigricans* could be used to ferment gardenia to produce genipin, with a conversion rate of 95% [25]. The yield of genipin via immobilized β-glycosidase hydrolysis was 47.81% with purity over 98% (HPLC) as reported [24]. With a molecular formula of $C_{11}H_{14}O_5$ (Figure. 1), genipin mainly consists of two units of 5-carbon building blocks called isoprene. Thus, it belongs to the large group of monoterpenes [26, 27]. As monoterpenes have been shown to exert

anti-diabetic activities of [28], it is speculated that genipin may also have anti-diabetic properties [29-32].

3. Pharmacokinetic Properties

The pharmacokinetics of genipin have been previously investigated using both in vivo and in vitro models, which will be summarized below.

3.1. Absorption

Pharmacokinetic studies have shown that geniposide is hydrolyzed to genipin by bacterial β-glucosidase enzymes in the intestine before uptake into enterocytes [33]. However, genipin is a highly non-polar hydrophobic molecule, indicating poor bioavailability, as the solubility of compounds plays a key role in their absorption. In addition, the transportation of genipin is dependent on P-glycoprotein (P-gp), which is a transporter extensively located on the intestinal epithelium that pumps drugs from the enterocytes back into the intestinal lumen [36]. Thus, a specific strategy to improve the solubility can enhance its absorption [34]. Hydroxypropyl-β-cyclodextrin (HP-β-CD) is a oligosaccharide containing 7 D-(+)-glucopyranose units with the property of improving the water solubility of various compounds [35]. By using HP-β-CD, the absorption rate and permeability of genipin were elevated by 1.60- and 2.46-fold, respectively, due to improved water solubility of genipin and inhibition of genipin returning to lumen by suppression of the P-gp ATPase [37].

3.2. Metabolism

Genipin can be transformed from its glycosides geniposide by intestinal bacteria enzymes (β-glucosidase) in animals [23, 38]. It was reported that there are 24 bacterial species capable of hydrolyzing geniposide into aglycone genipin [39]. However, genipin is not the only metabolite of geniposide. In the presence of bacterial and animal esterase, genipin can be hydrolyzed to genoposidic acid [40]. Genipin can be further metabolized or transformed upon absorption. Using ultraperformance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (UPLC-Q/TOF MS), it was reported that there were a total of 10 major metabolites (G1-G10) identified in rat plasma, urea, feces, and bile, with 10 metabolites detected in the bile, 6 in the urine, 2 in the plasma, and 1 in the feces [40]. The metabolism of genipin includes demethylation, ring-opening, cysteine-conjugation, hydroformylation, glucuronidation, and sulfate conjugation, in which the sulfated and glucuronidated conjugates of genipin were the main metabolites [41]. A recent study investigated the metabolism and pharmacokinetics of genipin and geniposide in rats. It was shown that after oral administration of 100 and 200 mg/kg of genipin, they were primarily metabolized to genipin sulfate, whereas the parent forms of genipin and geniposide were not detectable in the blood [42]. In another study, bioavailability of geniposide was examined. The results showed that after oral administration of 1200 mg/kg hot-water extract of Garden fruits (equivalent to 216.4 mg geniposide with only a trace amount genipin) to the rats, the plasma concentrations of geniposide reached peak levels (approximately 103.1 μg/ml) at 30 min post oral administration, but genipin was hardly detectable, which peaked at about 60 minutes (approximately 0.07 μg/ml) as analyzed by using high-performance liquid chromatography (HPLC) with a column switching system [43]. It was

estimated that the half-life ($T_{1/2}$) of genipin after oral administration to rats was 4.86±2.55 h, the mean residence time (MRT) was 7.86±3.61 h, the mean area under the plasma drug concentration-time (AUC) was 90.60±13.44 ng/mL/h, and the maximum plasma concentration (C_{max}) was 17.41±5.27 ng/mL [44]. These results indicate that the bioavailability of genipin is very low, or it can be rapidly metabolized upon absorption. Therefore, it is necessary to be cautious when interpreting in vitro data for physiological relevance.

3.3. Excretion

In the presence of oxygen, genipin can spontaneously react with amino acids to yield water-soluble blue pigments [45, 46]. While the specific mechanism of the blue pigment formation is unclear, it was speculated that the formation of genipin-based blue pigments may be due to the oxygen radical-induced polymerization and dehydrogenation of several intermediate pigments [47, 48]. However, the substances of blue pigments may be the main form of excretory genipin [49, 50]. The excretion rate of genipin through urine and feces was estimated to be 0.82% and 24.34%, respectively [50].

4. Antidiabetic potential of genipin

Diabetes is a metabolic disease characterized by hyperglycemia, which is usually caused by genetic factors, immune dysfunction, inflammation, free radicals, mental factors, and other pathogenic factors acting on the body, leading to dysfunction of pancreatic islets, insulin resistance, etc [51-53]. Long-term high blood glucose causes chronic impairment and dysfunction of various

tissues, particularly the eyes, kidneys, heart, blood vessels, and nerves [54]. T2D, which is also called adult-onset diabetes and usually develops after middle age, accounts for more than 90% of diabetic patients [55]. The principal characteristics of T2D are chronic insulin resistance and β-cell dysfunction and apoptosis [8-10]. In China, G. jasminoides fruits were recorded to treat “Xiaoke" (T2D) as early as thousands of years ago. However, the underlying mechanism was poorly understood. As genipin is a major component in Jasminoide fruits, it is intriguing to speculate that this compound may exert an antidiabetic action. In the following sections, the review will discuss recent advances in understanding the cellular action of genipin and geniposide with focuses on their effects in regulating glucose homeostasis.

4.1. Genipin is a UCP2 inhibitor

Uncoupling protein 2 (UCP2) is a mitochondrial inner membrane carrier protein widely expressed in many tissues, including pancreatic islets, gastrointestinal tract, liver, adipose tissue, skeletal muscle, immune system, and brain [56, 57]. UCP2 was demonstrated to mediate the leakage of protons across the inner mitochondrial membrane and inhibit ATP production from glycolysis and consequently reduces glucose-stimulated insulin secretion (GSIS) from beta-cells [58]. Therefore, UCP2 is considered to be a negative regulator of GSIS [59], which adversely affects glucose homeostasis. Indeed, knockout of UCP2 in mice enhanced islet ATP production and insulin secretion [59, 60]. Conversely, diabetic rats showed higher UCP2 mRNA and protein levels in pancreatic islets [61, 62]. Consistently, UCP2 was also found to play a role in obesity and T2D pathogenesis in humans [29, 63]. Thus, UCP2 may be a potential therapeutic target for developing treatment for T2D.

Recent studies demonstrated that genipin may be a promising agent in maintaining insulin–glucose homeostasis by inhibition of UCP2 [58, 64-67]. It was found that inhibition of UCP2 by genipin in pancreatic islets increases ATP production, leading to the closure of K(ATP) channels and therefore increases in insulin secretion [58, 64]. It is now recognized that the central nervous system (CNS) also plays an important role in maintaining glucose homoeostasis. Specifically, hypothalamic POMC neurons are glucose sensing neurons, and systemic glucose rises cause the closure of the ATP-induced K(ATP) channels, leading to the depolarization of the neurons and increase in firing rate, which subsequently inhibits food intake and stimulate energy expenditure [68-70]. It was shown that disruption of glucose sensing in POMC neurons impaired systemic glycemic control in mice, confirming that they are involved in the regulation of blood glucose control. Indeed, glucose sensing by POMC neurons is impaired in obese and diabetic mice, and emerging evidence showed that obesity-induced UCP2 expression inhibits glucose sensing in glucose-excited POMC neurons [71]. Therefore, genipin could potentially enhance the firing rate of POMC neurons by inhibiting UCP2, thus improving the ability of POMC neurons to regulate glucose homeostasis [71].

Diabetic nephropathy (DN) is one of the most important complications of diabetes. The main structural changes of DN include mesangial expansion, glomerular basement membrane （GBM）thickening, podocyte damage, and eventually glomerular sclerosis [72-74]. Specially, the expression of Wilms' tumor 1 gene (WT1), a specific marker of podocytes, and podocins, are reduced in DN patients with podocyte damage [75]. It was shown that treatment with genipin suppressed hyperglycemia-induced DN progression in a murine model of DN induced by intraperitoneal injection of streptozocin (STZ) [65]. The protective effect of genipin on DN may be achieved by down-regulation of UCP2 in the kidney, thereby enhancing the expression of WT1

and podocin in podocytes. In addition, genipin attenuated glucose-induced albumin leakage caused by the podocyte monolayer, thereby ameliorating podocyte injury and protecting the kidney from damage caused by hyperglycemia [65].

4.2. Genipin promotes insulin sensitivity

Insulin secreted by pancreatic β-cells plays a pivotal role in energy metabolism and blood glycemic control [76, 77]. It is well recognized that insulin resistance (IR), defects in insulin action, and impaired β-cell function are key features in T2D [78]. Subjects with IR will progress to overt diabetes if β-cells fail to secrete adequate amount of insulin to compensate for the defects in its action [79]. Therefore, preventing IR in peripheral tissues should be effective in preventing T2D [77].

Genipin may have the potential to alleviate IR. One study found that genipin dose-dependently stimulates glucose uptake in C_2C_{12} myotubes [80]. Mechanistically, genipin is able to activate insulin receptor substrated-1(IRS-1) and subsequently provoke the phosphatidyl inositol 3-kinase (PI3-K)/Akt signaling pathways and calcium concentrations, thereby leading to glucose transporter 4 (GLUT4) membrane translocation and glucose uptake [80]. Paradoxically, it was shown that genipin impaired insulin signaling and consequently suppressed insulin-stimulated glucose uptake in 3T3-L1 adipocytes via UCP2 inhibition-mediated activation of the ROS/c-Jun N-terminal kinase (JNK) 1/2 pathway [66].

The liver plays a vital role in maintaining glucose homeostasis [81], and obesity and T2D are always intricately associated with chronic liver disorders such as nonalcoholic fatty liver disease [82, 83]. Hepatic IR and the increased hepatic glucose production are considered some of the early pathological changes in T2D subjects [84-86]. In HepG2 cells exposed to chronic

hyperlipidemia, genipin treatment inhibited the accumulation of intracellular lipids while greatly increasing the abundance of peroxisome proliferator-activated receptor alpha (PPARa) mRNA [30]. It was also reported that geniposide suppressed hepatic glucose production through activating AMPK, which subsequently induced the phosphorylation and therefore inactivation of acetyl-CoA synthase (ACC) and forkhead box class O1 (FoxO1) activity. In addition, the expression level of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), two rate-limiting enzymes in the gluconeogenic pathway, were reduced [87]. It is suggested that geniposide and genipin may inhibit hepatic gluconeogenesis by regulating the AMPK-FoxO1 signaling pathway. IR development is consistently associated with age [88]. In aged rats, genipin could alleviate hepatic IR and hyperinsulinemia via relieving hepatic oxidative stress and mitochondrial dysfunction [89]. Collectively, these *in vitro* and *in vivo* studies demonstrated that genipin could increase insulin sensitivity, but the exact mechanism underlying this action is still unclear.

4.3. Effect of genipin on β-cell function

Significant loss of functional β-cell mass over time in patients with T2D plays a central role in the deterioration of blood glucose control [90-92]. Therefore, preservation of pancreatic β-cell survival and function is critical in the prevention and treatment of T2D [93, 94]. While there is no evidence that genipin can regulate β-cell function, it was demonstrated that geniposide can protect against β-cell apoptosis and improve its survival [95-97].

It has been established that both chronic hyperglycemia and high levels of plasma free fatty acids (FFA) can lead to mitochondrial oxidative stress, which subsequently causes pancreatic β-cell apoptosis [95, 98]. It was shown that geniposide treatment attenuated apoptosis of INS-1 cells exposed to chronic palmitate. This protective effect was associated with improved Akt and GLP-

1R signal that led to enhanced pancreatic and duodenal homeobox 1 (PDX-1) expression in palmitate-treated INS-1 cells, indicating that geniposide may prevent lipotoxicity-induced β-cell apoptosis through the GLP-1R signaling pathway [95, 97]. Geniposide also has been shown to protect against glucotoxicity-induced apoptosis of INS-1 cells via AMPK-mediated increase in Bcl-2/BAX protein ratio [96, 97].

In addition to preventing β-cell apoptosis, another potential therapeutic strategy is to regenerate functional β-cells. It has been established that the Wnt/β-catenin pathway is a key regulator of insulin secretion, and β-cell survival and regeneration [99-101]. Intriguingly, geniposide could significantly increase the activity of T-cell factor 7-like 2 (TCF7L2), which is a key transcriptional factor for the Wnt/β-catenin signaling pathway, an important developmental pathway. Accordingly, geniposide induced ductal cell differentiation via activating TCF7L2 and stimulating the JAK2/STAT3 pathway in exocrine cells isolated from mouse pancreas. *In vivo*, geniposide improved β-cell survival and glucose homeostasis in C57BL/6J mice fed a high-fat diet and db/db mice [102]. Therefore, geniposide might be a promising natural compound to promote β-cell function.

5. Toxicology

Some studies have shown that long-term and high-dose administration of gardenia induced hepatotoxicity in mice and rats [103-105], which has always been a major problem hindering use as a clinical medication. As a product of gardenia, the toxicity of genipin is unclear, although some research has reported that genipin may protect liver function [106, 107]. In an *in vitro* study, a comparative analysis of the differences in hepatotoxicity of geniposide and genipin showed that incubating HepG2 cells with different concentrations of geniposide (20~1 000 μmol) did not

produce significant cytotoxic effects. On the contrary, exposure of HepG2 cells to high dose of genipin (50 μmol to 1000 μmol) induced oxidative stress and hepatotoxicity in a concentration-dependent manner [108], suggesting that genipin at relatively high doses may exert hepatotoxicity, but its physiological relevance is unclear. *In vivo,* genipin can convert to genipin dialdehyde mediated by intestinal microbiota, which could modify lysine residues of hepatic proteins. Hence, the underlying mechanism for hepatotoxicity may be ascribed to genipin dialdehyde [109].

In vivo, it was reported that one-time oral administration of a high dose of genipin (200 mg/kg) to male Sprague–Dawl rats caused the mortality rate to be as high as 78% (7/9) during 48h. However, rats given 100 mg/kg genipin had a survival rate of 100% [110]. The LD_{50} value of genipin was identified as LD_{50} (oral, rat): >50 mg/kg, LD_{50} (oral, mouse): 237 mg/kg, and LD_{50} (i.p. mouse): 190 mg/kg [111]. *In vitro*, genipin at moderate concentrations (generally 0.5–5 mM) has been shown to be cytotoxic, which may be cell line-dependent [112]. Overall, further research is needed to determine the potential beneficial and side effects of these compounds.

6. Conclusion

In this review, the pharmacokinetic and potential antidiabetic effects of genipin were discussed. Genipin can be generated from geniposide by intestinal enzyme β-glucosidase and decomposed into a variety of metabolites in vivo. Recent studies show that genipin as an inhibitor of UCP2 holds great potential in alleviating insulin resistance and improving β-cell function (Figure 2), and therefore may be a promising natural compound with anti-diabetic actions. However, there remain many unanswered questions. First, the pharmacokinetics of genipin such as its bioavailability and metabolism are still largely unknown. Second, although the herbs containing geniposide and genipin have been medicinally used, the optimal doses in terms of their

safety are unclear; and third, the studies exploring its antidiabetic efficacy and underlying mechanism are still scarce.

7. Figures

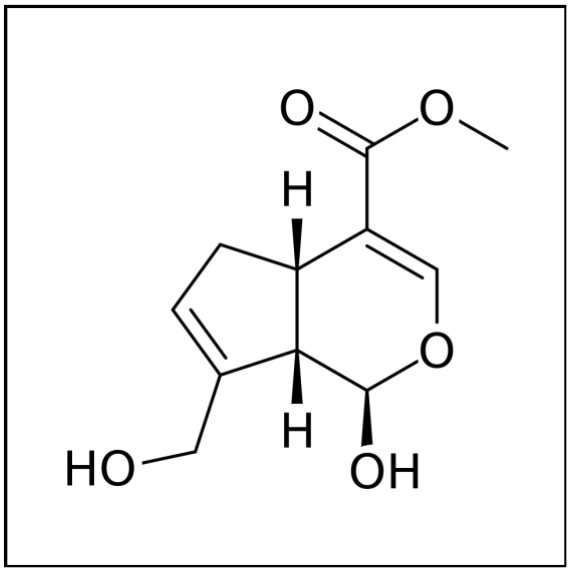

Figure 1. Structure of genipin

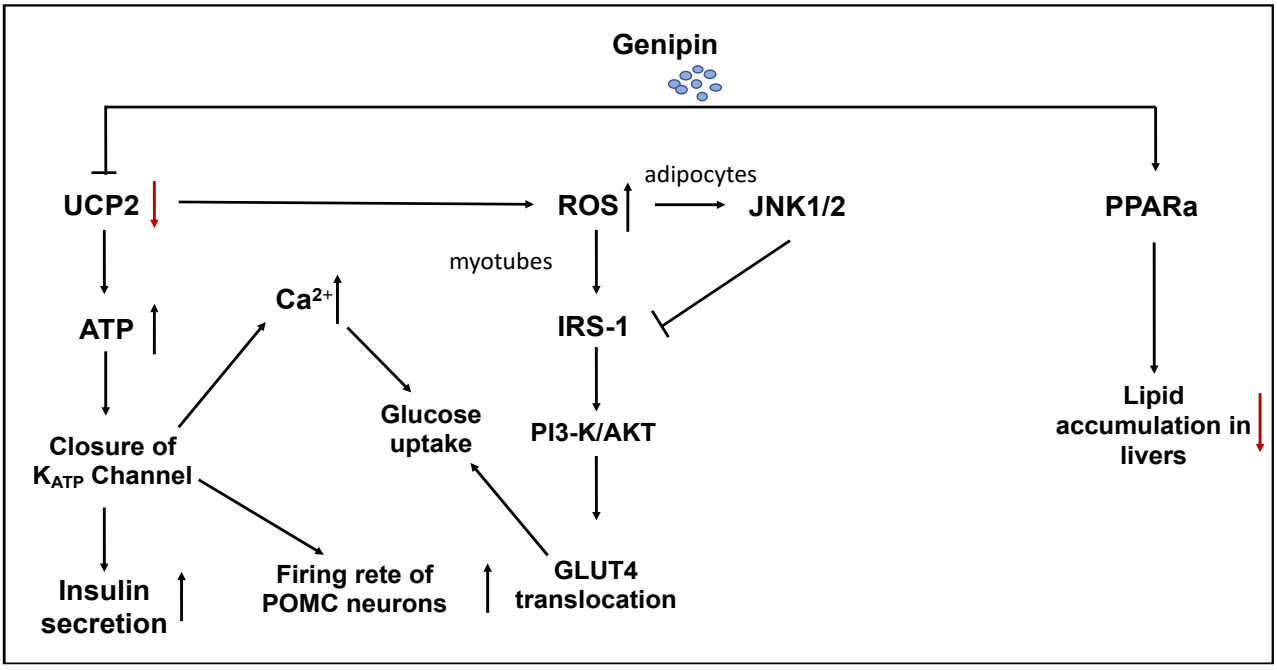

Figure 2. Schematic diagram of mechanisms of genipin in T2D

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CHAPTER THREE

Identification of genipin as a potential treatment for type 2 diabetes

Abstract

The prevalence of Type 2 diabetes (T2D) has been rising dramatically in many countries around the world. The main characteristics of T2D are insulin resistance and dysfunction of β-cells. While there are several pharmaceutical therapies for T2D, no effective treatment is available for reversing functional decline of pancreatic β-cells in T2D patients. It has been well recognized that glucagon-like peptide-1 (GLP-1), which is an incretin hormone secreted from intestinal L-cells, plays a vital role in regulating glycemic homeostasis via potentiating glucose-stimulated insulin secretion (GSIS) and promoting β-cell function. I found that genipin, a natural compound from gardenia jasminoides Elli, can directly target intestinal L-cells, leading to the secretion of GLP-1. Incubation of the cells with genipin elicited a rapid increase in intracellular Ca^{2+}. Inhibition of PLC ablated genipin-stimulated Ca^{2+} increase and GLP-1 secretion, suggesting that genipin-induced GLP-1 release from the cells is dependent on the PLC/Ca^{2+} pathway. In vivo, acute administration of genipin stimulated GLP-1 secretion in mice. Chronically, treatment with genipin via oral gavage at 50 mg/kg/day for 6 weeks reversed hyperglycemia and insulin resistance in HFD-fed diabetic mice. Moreover, genipin alleviated the impaired lipid metabolism and decreased lipid accumulation in the liver of HFD-fed diabetic mice. These results suggest that naturally occurring genipin might be a novel agent for the treatment of T2D and diet-induced fatty liver disease.

Key words: Genipin, GLP-1, L-cells, insulin resistance, lipid accumulation, mice.

Introduction

Diabetes is still a global health issue worldwide, and the population of diabetic patients has been steadily increasing in recent years. More than 90% of diabetic cases are type 2 diabetes (T2D) [1]. It is well recognized that a group of intestinal hormones can increase insulin secretion in response to ingested nutrients, thus exerting incretin effects [2]. There are two major incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), of which GLP-1 plays a more critical role in maintaining glycemic homeostasis [3-5]. GLP-1 is primarily secreted by L-cells, which are enteroendocrine cells primarily located in ileum and colon [6]. In addition to augmenting glucose-stimulated insulin secretion (GSIS) while suppressing the secretion of glucagon from pancreatic α-cells [7, 8], GLP-1 was also shown to promote the survival and regeneration of pancreatic β-cells in rodent models [9]. Further, GLP-1 analogs delay stomach emptying, induce satiety, and reduce body weight gain in animal models of obesity [10]. While in patients with T2D, postprandial GLP-1 response is significantly impaired [11]. Therefore, inducing intestinal GLP-1 secretion or activating the GLP-1 signaling system could be an effective strategy for the treatment of T2D.

Genipin is a small molecule in fruits of gardenia jasminoides Elli and genipa americana, and it can also be generated from an iridoid glycoside geniposide by the intestinal enzyme β-glucosidase [12, 13], Geniposides have been used to treat cancer, inflammation, metabolic diseases and diabetes in traditional Chinese medicine for hundreds of years, while the underlying mechanism is poorly understood [14-16]. Interestingly, recent studies demonstrated that genipin is an inhibitor of uncoupling protein 2 (UCP2), a mitochondrial inner membrane carrier protein expressed in various tissues [16]. It was found that inhibition of UCP2 by genipin in pancreatic islets increases ATP production, leading to closure of K(ATP) channels and subsequently increases

in insulin secretion [17, 18]. Given that UCP-2 may also participate in regulating GLP-1 secretion [19], I investigated whether genipin induces GLP-1 secretion both in L-cells and in mice, and further explored the antidiabetic potential of this compound using HFD-fed diabetic mice.

Materials and Methods

Chemicals

Genipin was purchased from BOC Sciences (Shirley, NY) with purity > 98%; U73122 was from Tocris Bioscience (Pittsburgh, PA); DMEM media were from Hyclone (GE Healthcare Bio-Sciences, Pittsburgh, PA); fetal bovine serum (FBS) was obtained from GenClone (Genesee Scientific, EL Cajon, CA); penicillin-Streptomycin was from Sigma (St. Louis, MO); poly-D-lysine was from MP Biomedicals (Solon, OH); trypsin-EDTA and GLP-1 ELISA kits for in vitro experiments were from MilliporeSigma (Burlington, MA); fluro-4AM was from ThermoFisher Scientific (Waltham, MA); bovine serum albumin (BSA); diprotin A, methylcellulose, IBMX, cyclic AMP ELISA kit, alanine aminotransferase (ALT) kit, and triglycerides colorimetric assay kit were from Cayman (Ann Arbor, MI); leptin was from Bertin Pharma (Montigny-le-Bretonneux, France); GLP-1 ELISA kits for plasma assay were from Crystal Chem (Elk Grove Village, IL); CellTiter-Blue® cell viability assay was from Promega (Madison, WI); glucose meter was from AgaMatrix (Salem, NH); and all the other chemicals were from Sigma (St. Louis, MO).

Cell culture

Mouse GluTag L-cells were maintained in the Dulbecco's Modified Eagle Medium/low glucose (DMEM, 5.5 mM glucose) supplemented with 10% fetal bovine serum (FBS), and 1%

penicillin streptomycin [20]. Cells were kept at 37 ºC with 5% CO_2 until reaching 80%-85% confluence, and were then trypsinized with 0.05% trypsin-EDTA, and seeded into poly-D-lysine pre-coated plates. The passage number of GluTag L-cells in all the experiments was between 25 and 35.

Measurement of GLP-1 secretion

To perform the GLP-1 secretion assay, GluTag L-cells were seeded into 24-well plates coated with poly-D-lysine 24 h before the study. On the following day, cells (~80% confluence) were incubated with Krebs Ringer Buffer (KRB, 129 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 10 mM HEPES, 5 mM $NaHCO_3$, pH 7.4) supplemented with 0.2% BSA for 30 min and then treated with genipin (0.1 µM, 1 µM, 10 µM) dissolved in DMSO, 2 mM butyrate (positive control), or vehicle with comparable volume of DMSO for 1 h. To rest the role of PLC, cells were preincubated with 10 µM U73122 for 30 min and then treated with 10 µM or 100 µM genipin for 1 h. Supernatants were collected for measuring GLP-1 levels using an GLP-1 assay kit. Cells were then lysed with 0.1 M HCl to measure the protein contents and GLP-1 levels were normalized to the protein concentrations from the same samples.

Cell viability assay

GluTag cells were seeded into 96-well tissue culture microplates with the density of 1×10^4 cells/well. After 24 h, cells were treated with different concentrations of genipin (0, 0.01 µM, 0.1 µM, 1 µM, 10 µM, 50 µM and 100 µM) for 1 h. Cell viability were then examined using a CellTiter-Blue® cell viability assay kit. Briefly, 20 µl of CellTiter-Blue® reagent was added to

each well and cells were incubated at 37 ºC for another 1 h. Cell viability were measured by recording fluorescence at 540nm/590nm.

Measurement of intracellular calcium concentrations ($[Ca^{2+}]_i$)

GluTag cells were seeded into black 96-well microplate at a density of 3.0 x 10^4 cells/well and cultured in low glucose DMEM supplemented with 1% FBS and 1% penicillin streptomycin for 16 h as previously described [21]. Cells were then washed with KRB and loaded with 2 µM Fluo-4AM in Ca^{2+}-free KRB buffer at 37ºC in the dark for 1 h. The cells were washed again and incubated in Ca^{2+}-free KRB buffer for 30 min at room temperature to allow for fura-4AM de-esterification. For intracellular Ca^{2+} measurement, basal signals of Fluro-4AM- loaded cells were recorded for 10 sec and Ca^{2+}-free KRB buffer containing 10 µM genipin or 2 mM butyrate (positive control) was then injected. The fluorescence intensities were recorded every second for 240 acquisition cycles at 495 nm excitation and 518 nm emission using a Spectro fluorophotometer (FLUOstar OPATIMA, Cary, NC).

Measurement of intracellular cyclic adenosine monophosphate (cAMP)

GluTag cells were grown to 80% confluence in 24-well plates and washed with pre-warmed KRB before treatment. Cells were then exposed to various concentrations of genipin, 0.2 mM IBMX, or vehicle. After 20 min, culture medium was removed, and cells were washed twice with ice-cold KRB. Monolayers were immediately lysed using 0.1 M HCl on ice for 20 min, and the lysates were used for measuring intracellular cAMP contents according to the manufacturer's manual and cAMP levels were normalized to the protein concentrations from the same lysed samples.

Animal studies

C57/BL6J male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All procedures performed in animal studies were approved by Institutional Animal Care and Use Committee at Virginia Tech. All mice were housed under 23ºC and 12 h light-dark cycle with ad libitum access to food and water.

The effect of acute administration of genipin on GLP-1 secretion

To determine whether genipin stimulates GLP-1 secretion in vivo, 10-week-old C57/BL6 male mice fed a standard chow diet (SD) were randomly divided into 2 groups (n=6/group). After being fasted for 5 h, blood samples were collected into EDTA tubes (Microvette) pre-coated with 50 μM diprotin A. Mice were then given genipin (50 mg/kg) or vehicle (2% methylcellulose) via oral gavage. Blood samples were drawn from tail after 15 min and total plasma GLP-1 concentrations were measured using a mouse GLP-1 ELISA kit.

Chronic effects of genipin in obese diabetic mice

Male C57/BL6J mice of 7 weeks of age were either fed a SD (n=11) or a HFD (n=22 mice) with 60% calorie from fat (Research Diet Inc., New Brunswick, NJ). After 10 weeks when mice fed a HFD became obese, insulin resistant, and glucose intolerant, as shown in our recent study [22], diet-induced obese mice were divided into two groups (n=11/group) with comparable body weight (BW) and blood glucose, and then administered genipin (50 mg/kg, once a day) or vehicle (2% methylcellulose) via oral gavage for 6 weeks. Age-matched healthy control mice fed a SD were given vehicle for 6 weeks. BW, food intake, fasting, and non-fasting blood glucose were

measured weekly, and body composition was determined by using NMR Lean/Fat Analyzer for small animals (Bruker, Billerica, MA) at the beginning and the end of the study.

Pyruvate tolerance test (PTT), insulin tolerance test (ITT), and glucose tolerance test (GTT)

PTT, ITT, and GTT were measured at 4^{th}, 5^{th}, and 6^{th}, respectively. For PTT, mice were fasted for 16 h and then the basal blood glucose levels were measured. Afterwards, mice were given pyruvate (1g/kg BW) or vehicle via intraperitoneal (i.p.) injection. Blood samples were drawn from tail vein at 0, 15, 30, 60, and 120 min for measuring glucose using a glucose meter. For ITT, mice were fasted for 5 h followed by i.p. injection of insulin (1.5U/kg BW). Blood was drawn at 0, 15, 30, 60, and 120 min and blood glucose levels were measured. For GTT, mice were fasted for 14 h and then a bolus of glucose (1g/kg BW) was administered via i.p. injection. Blood glucose levels were measured at 0, 15, 30, 60, and 120 min post-injection of glucose.

Histological analysis

At the end of the study, liver samples were collected after euthanasia of the mice and fixed in 4% paraformaldehyde. Embedding and sectioning of liver samples were performed by AML Laboratories Inc. (Jacksonville, FL, USA). Tissue sections were de-paraffinized with xylene, rehydrated in graded ethanol solutions, and stained with hematoxylin and eosin (H&E) as previously described [23].

Fecal lipid extraction and measurement

Mice feces were collected for 3 consecutive days at the beginning and the last week of the treatment. Feces were weighed and dried and lipids were then extracted in chloroform-methanol

(2:1) mix solution. Suspensions were centrifuged at 1,000 x g for 10 min at room temperature. The chloroform: methanol phases were collected for lipid measurement as described [24].

Biochemical analyses

Blood samples were collected by cardiac puncture immediately after mice were euthanized, and blood samples were transferred into EDTA pre-coated tubes then centrifuged at 1000 g for 15 min at 4 °C. Plasma was collected from the supernatant and stored at -80 °C. Plasma triglycerides (TG) and leptin were measured using assay kits. Livers were harvest from control and genipin treated mice after the blood sample collection and stored at -80 °C. Liver triglyceride was extracted and tested according to the method provided by the same triglyceride colorimetric kit. Plasma alanine aminotransferase (ALT) activity was measured using a colorimetric kit.

Statistical analysis

The *t*-tests were used to determine the statistical difference in first and last week' data from body composition and fecal lipid, respectively; other data were analyzed by ANOVA and significance were determined by student *t* test by using JMP 15 pro (SAS Institute Inc, Cary, NC). The $P < 0.05$ was considered a significant difference.

Results

Genipin may have no toxicity

To examine whether genipin has toxic effect on cells, GluTag cells were treated with various of concentrations of geipin for 1 h and cell viability were examined by using a CellTiter-Blue® cell viability assay kit. As shown in Fig. 1, genipin (0.01-100 μM did not affect cell viability up to 1 h of exposure, suggesting that this dose range of genipin may not exert cytotoxicity to the cells.

Genipin increased GLP-1 secretion in L-cells

To explore whether genipin is capable of targeting L-cells to stimulate GLP-1 secretion, GLUTag cells were treated with various concentrations of genipin (0, 0.1, 1, 10, 100 μM) or 2 mM butyrate as control for 1 h. The result showed that genipin was potent in stimulating GLP-1 secretion from L-cells, with 10 μM ($P < 0.05$) and 100 μM ($P < 0.01$) of genipin significantly inducing GLP-1 release, although the magnitude of this effect was lower than that of butyrate ($P < 0.001$) (Fig. 2).

Genipin-induced GLP-1 release did not depend on cAMP

To explore how GLP-1 secretion from L cells is induced, intracellular cAMP levels were measured. To this end, GluTag cells were treated with 10 or 100 μM concentrations of genipin or 0.2 mM IBMX, and then the levels of intracellular cAMP were measured in the cell lysates. As shown in Fig. 3, genipin did not enhance the accumulation of intracellular cAMP, while the cAMP concentrations were significantly increased in IBMX treated-cells (1.43-fold compared with vehicle-treated cells), indicating that genipin-induced GLP-1 secretion is independent of cAMP.

Genipin induced-GLP-1 secretion was mediated via PLC/Ca2+signaling

Rapid increases in intracellular Ca^{2+} is critical for triggering GLP-1 secretion [25]. To further explore whether genipin induced GLP-1 secretion via increasing intracellular Ca^{2+} concentration, I preincubated GLUTag cells with Fluo-4am followed by adding 10 μM or 100 μM genipin or 2mM butyrate. The result showed that the cells exposed to genipin induced a rapid increase in $[Ca^{2+}]_i$ (Fig. 4A), although the magnitude was lower than that elicited by 2 mM butyrate.

To determine whether genipin induced-GLP-1 secretion were PLC-dependent, GluTag cells were pre-treated with U73122, a specific antagonist of phospholipase C (PLC) [26]. After 30 min, genipin-induced GLP-1 secretion was measured. As shown in Fig. 4B, blockage of PLC abolished genipin-induced GLP-1 secretion from L-cells, indicating that genipin induced-GLP-1 release was mediated via the PLC/Ca^{2+}signaling pathway.

Acute administration of genipin increased circulating GLP-1 levels in mice

Since genipin induced GLP-1 secretion in L-cells, I next tested whether it could stimulate GLP-1 secretion in vivo. Mice were fasted for 5 h, then blood was drawn before and 15 min after giving genipin (50 mg/kg) or vehicle via oral gavage. There were no differences in basal GLP-1 concentrations between the genipin and control groups (13.0 ± 1.6 pM vs. 12.3 ± 1.2 pM), but acute administration of genipin significantly increased plasma GLP-1 levels as compared to the control (14.4 ± 3.9 pM vs. 10.9 ± 1.7 pM) (Fig. 5).

Genipin decreased blood glucose in HFD-fed diabetic mice

When fed HFD for 10 weeks, C57/BL6J mice became obese, insulin resistant, and glucose intolerant as compared with SD fed mice (data not shown). The diet-induced obese mice were then orally administration with 50 mg/kg genipin or vehicle once daily for 6 weeks. As shown in Fig 6, genipin treatment significantly lowered fasting blood glucose after 3 wks of treatment as compared with the obese control group (Fig. 6A). Non-fasting blood glucose levels in mice treated with genipin were also significantly lower at the end of 2-wk of treatment, but this difference disappeared at the end of 3 weeks of treatment (Fig. 6B).

Genipin improved insulin resistance in HFD-fed diabetic mice

Since genipin has shown the potential to alleviate hyperglycemia, I next assessed whether it could promote insulin sensitivity and reverse glucose dysregulation in diabetic mice caused by HFD. In this regard, I performed PTT, ITT, and GTT at the 4^{th}, 5^{th}, and 6^{th} week, respectively. As shown in Fig. 7, obese mice displayed the impairment of pyruvate, insulin, and glucose tolerance. Genipin treatment significantly improved insulin resistance (Fig. 7A, B) and glucose intolerance (Fig. 7C, D), whereas it had no significant effect on pyruvate tolerance (Fig. 7E, F), suggesting that genipin may not modulate liver gluconeogenesis.

Genipin did not alter BW and food intake in HFD-fed diabetic mice

BW and food intake were measured every week during treatment with genipin. Genipin treatment moderately reduced BW as compared with the baseline BW (43.64 ± 5.0 g vs. 45.23 ± 5.11 g), but this change did not reach statistical significance (Fig. 8A). In addition, daily food intake between HFD group and genipin treated mice did not differ (Fig. 8B). Further body composition of genipin-treated and untreated obese mice was similar Fig. 8C-F). Consistently,

genipin treatment had no effect on plasma leptin (Fig. 8G), a hormone mainly acting on regulation of appetite and fat storage [27]. Hence, genipin regulation of glucose homeostasis was not due to reducing appetite and BW.

Genipin improved lipid profile in HFD-fed diabetic mice

As expected, HFD feeding significantly increased plasma TG levels in obese mice as compared with SD mice (Fig. 9A). However, plasma triglyceride concentrations in genipin-treated mice were almost restored to the levels comparable to those in the lean mice (Fig. 9A). To determine whether genipin affects lipid absorption, I collected the feces at the first and the last week of the treatment and measured lipid contents. The results showed that oral administration of genipin increased triglyceride contents in feces (Fig. 9B), suggesting that genipin may inhibit lipid digestion and/or absorption in the intestine.

As the liver plays a critical role in lipid metabolism [28], the morphology of liver was examined. Consistent with the increases of plasma TG in HFD-fed mice, the livers of these mice were oversized, and color pale yellow compared to the livers from SD mice (Fig 9E). However, the livers from genipin-treated mice were similar to those from SD mice. In addition, the ratio of liver weight to BW and hepatic TG in HFD mice were significantly higher than those of SD mice ($P < 0.01$), in which these patterns were reversed by genipin treatment (Fig. 9C, D). Consistently, the liver histology examination showed that obese mice displayed significantly higher accumulation of liver lipid droplets relative to those from SD mice, which however were largely reversed by genipin treatment (Fig. 9F), suggesting that genipin restored the impaired hepatic lipid metabolism caused by chronic HFD feeding. Next, I measured ALT activity, which is a sensitive marker for liver function [29]. Serum ALT activity in obese mice was drastically higher than that

of lean mice, whereas it was significantly reduced in genipin-treated mice (Fig. 9G), suggesting that genipin can protect against liver damage caused by chronic HFD feeding.

Discussion

It is well recognized that GLP-1 plays a critical role in maintaining glycemic homeostasis by augmenting glucose-stimulated insulin secretion (GSIS) and promoting pancreatic β-cell mass and survival [3-5, 30]. It was found that GLP-1 secretion in response to ingested nutrients is significantly impaired in patients with T2D [31], which may contribute to the dysfunction of pancreatic β-cells and development of T2D. Therefore, inducing intestinal GLP-1 secretion could be a strategy for the prevention and treatment of T2D. In this study, I explored the effect of genipin, a natural agent generated from the fruits of gardenia jasminoides, on GLP-1 secretion and glycemic control in a diet-induced insulin resistant and glucose intolerance mouse model. The results indicated that genipin was able to increase GLP-1 secretion both in vitro and in vivo. At the molecular level, genipin may induce GLP-1 secretion via activating through the PLC / Ca^{2+} pathway. In vivo, genipin reduced non-fasting and fasting blood glucose levels, improved insulin resistance, and protected again high fat diet-induced heptosteatosis. Therefore, genipin may exert GLP-1-mediated anti-diabetic effect and protect against obese-related liver damage.

Secretion of GLP-1 from intestinal L cells is mainly regulated by the intake of macronutrients, primarily fatty acids [32-34], although glucose [35, 36], amino acids, and dietary fibers [37] may also induce GLP-1 release. In addition, a variety of neuroendocrine factors such as neurotransmitters and neuropeptides released by the enteric nervous system as well as the secreted hormones from other enteroendocrine cell types, such as acetylcholine [38] and gastrin-releasing peptide [39], have been implicated in the regulation of GLP-1 secretion. However, treatment strategies based on these mechanisms have not been successfully developed to treat T2D. To investigate the signaling pathway that mediates genipin-stimulated GLP-1 secretion, I first

tested whether genipin increases intracellular [Ca^{2+}], which is critical for triggering GLP-1 secretion [40]. I found that incubation of the cells with genipin induced a rapid increase in intracellular [Ca^{2+}] $_{i..}$ I then examined the effect of genipin on the activity of PLC, which hydrolyzes PIP2 to the Ca^{2+}-mobilizing second messenger IP3, thereby elevating intracellular [Ca^{2+}], [41]. In the present study, I performed a series of experiments in vitro with the results indicating that genipin activation of GLP-1 secretion is mediated via the PLC/Ca^{2+} signaling pathway. The mechanisms of GLP-1 secretion in L-cells are various. Glucose is able to elevate intracellular [Ca^{2+}]i and subsequently triggers GLP-1 secretion through the phospholipase C (PLC)/ inositol trisphosphate (IP3)/Ca^{2+}-sensitive transient receptor potential channel M5 (TRPM5) pathway [41]. Our results suggested that genipin is capable of increasing GLP-1 release by inducing a rapid increase in intracellular [Ca^{2+}]$_i$ in L-cells, which is consistent with the previous studies.

Genipin was recently identified as an inhibitor of UCP-2, which is expressed in various tissues and was reported to participate in the regulation of insulin–glucose homeostasis [42-46]. Inhibition of UCP-2 by genipin was found to reverse pancreatic islet dysfunction and increase insulin secretion [42, 45]. More recently, it was demonstrated that UCP-2 is also involved in regulating GLP-1 secretion, and that inhibition of UCP-2 increases GLP-1 release [19]. While not directly examined, I speculate that genipin may promote GLP-1 secretion at least partially via a UCP-2-depenent mechanism. As shown in our study, genipin-improved glucose tolerance may be due to the fact that genipin induced GLP-1 secretion to enhance insulin-independent glucose disposal [47]. In addition, the data from the present study show that genipin was able to ameliorate insulin resistance in diabetic mice. Interestingly, blood glucose levels at 60 – 120 min post insulin injection, but not at the early phase (0 - 30 min) during ITT, significantly differ between HFD

control and genipin groups, indicating that genipin regulating insulin sensitivity may be complex. A study found that inhibition of UCP-2 by genipin suppressed hypoglycemia-induced glucagon secretion from pancreatic α-cells and subsequently impaired blood glucose recovery from hypoglycemia [48]. Hence, the observed effect of genipin on insulin sensitivity could be partially due to its effect on glucagon secretion from the islets, which needs further investigation.

While GLP-1 can reduce gastric emptying, food intake, and BW [49], and it was shown that genipin decreased BW gain in both rats and mice [50, 51], the present study did not demonstrate these effects. The effect of genipin on obesity might depend on the treatment period, dosage, animal models, or treatment approaches. Liver is the major organ to synthesize triglycerides. Overloaded triglycerides can accumulate in the liver leading to hepatic insulin resistance and non-alcoholic fatty liver disease (NAFLD) [52]. Our results indicated that genipin reduced hepatic lipid accumulation and elevated ALT activity by HFD feeding, which are more pronounced effects than its effect on glucose homeostasis, suggesting that this compound exerts protective effects on liver function in obese mice. This beneficial effect of genipin may not relate to UCP-2 or GLP-1[53]. Previous studies showed that genipin inhibited intracellular lipid accumulation in free fatty acid treated HepG2 cells by improving the levels of PPARα, which plays an important role in regulating nutrient metabolism and energy homeostasis [54]. However, another study reported that inhibition of UCP2 by genipin with the concentration of 5 μM significantly aggravated palmitate-induced lipid accumulation in HepG2 cells via upregulating oxidative stress [56]. Data from in vivo studies are more consistent. One study showed that genipin improved liver dysfunction via inhibiting the production of TNF-α [55]. Another study demonstrated that genipin was also shown to improve lipid metabolism in the liver via the miR-

142a-5p/SREBP-1c axis [51]. Our data thus are consistent with the results from these studies. However, how genipin protects against NAFLD is worth further investigation.

In conclusion, I found that genipin significantly induced GLP-1 secretion in GluTag L-cells and in mice. Treatment with genipin restored glucose homeostasis and insulin sensitivity in HFD-induced diabetic mice, which may be due to increasing GLP-1 release in vivo. In addition, genipin effectively improved lipid metabolism and reduced liver accumulation in mice. Given these results, genipin may be a natural agent that can promote endogenous GLP-1 secretory function of intestinal L-cells and protects against diet-induced liver damage to maintain glucose homeostasis. Therefore, genipin could be a promising lead compound for developing treatments for NAFLD and T2D.

Figures

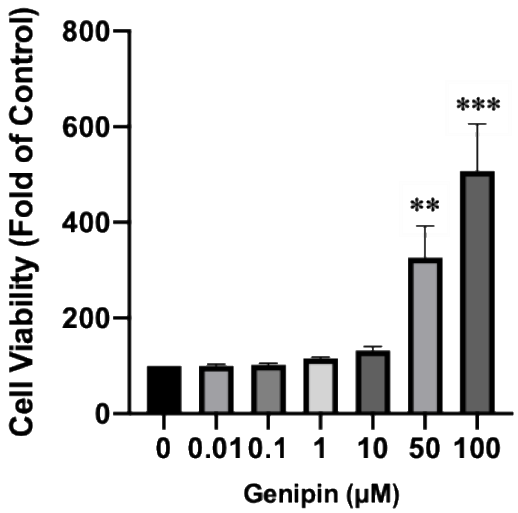


Figure 1. The cell toxicity of genipin. GluTag cells were treated with different concentrations of genipin for 1 h and cell viability was assayed by using a cell viability assay kit. Experiments were repeated three times (n=4) and data are presented as means ± SEM. *** $p < 0.001$ vs. control.

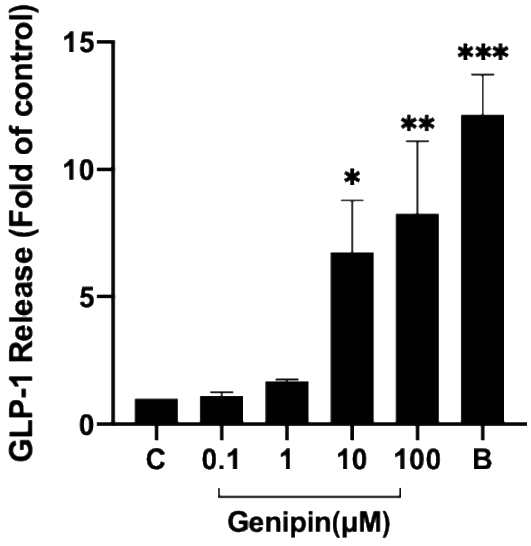


Figure 2. Genipin induced the secretion of GLP-1 in GluTag L cells. GluTag cells were treated with 0.1, 1, 10, 100 μM genipin and control (C) and 2 mM butyrate dissolved in the same solution that was used as the control treatment (B) for 1 h. Supernatants were collected for GLP-1 measurement. Experiments were repeated three times in duplicate each (n=4) and data are presented as means ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. control.

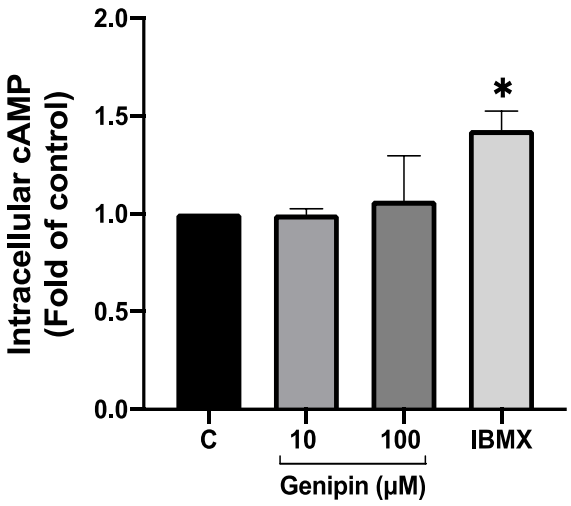


Figure 3. Genipin has no effect on cAMP production. GluTag cells were treated with different concentrations of genipin or 0.2 mM IBMX for 20 min. Cell lysates were used for intracellular cAMP measurement using an EIA kit. Data are means ± SEM, n=3. * $p < 0.05$ vs. control.

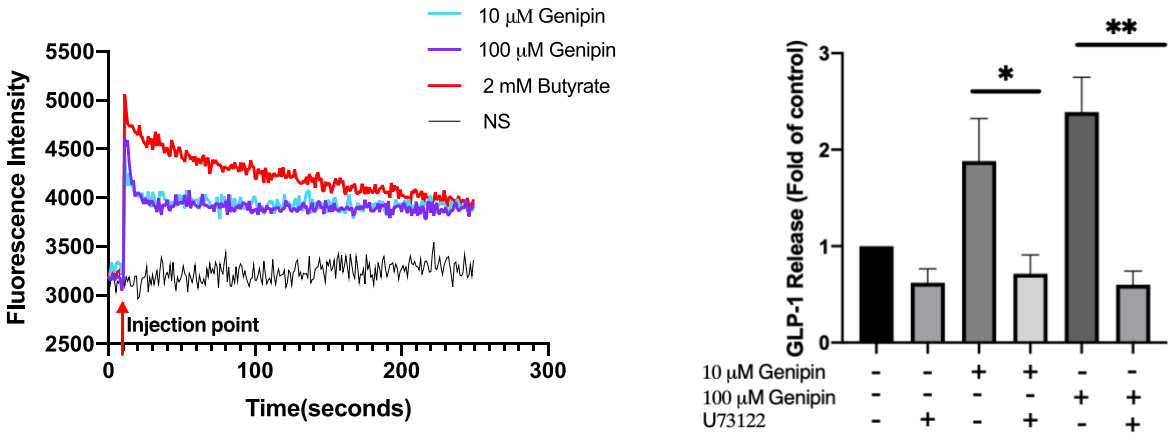


Figure 4. Genipin induced-GLP-1 release was mediated via the PLC/Ca2+signaling pathway. **(A)** Suspended GLUTag cells were pretreated with Fluo-4am and treated with genipin (10 μM, 100 μM), butyrate (2 mM), or vehicle (NS). The $[Ca^{2+}]_i$ response was measured using a fluorescence plate reader. A representative image from 3 independent experiments is shown. **(B)** GluTag cells were pretreated with 10 μM U73122 for 30 min, followed by addition of genipin (10 μM, 100 μM) or vehicle for another 1 h. Supernatants were collected for GLP-1 measurement. Data are presented as means ± SEM, n=3. * $p < 0.05$, ** $p < 0.01$ vs. U73122 pretreated group.

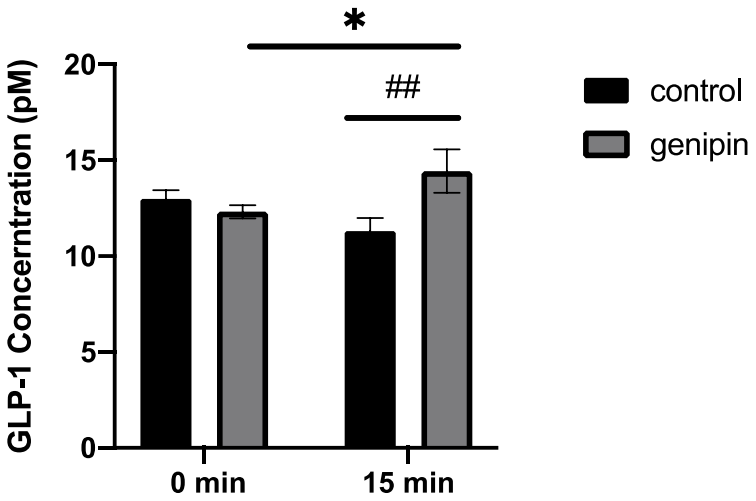


Figure 5. Acute administration of genipin increased plasma GLP-1 in vivo. Mice were orally administrated with 50 mg/kg genipin or vehicle. Blood samples were drawn before treatment (0 min) or 15 min after gavage. Treatment was repeated for one time at 1-week interval. Data are shown as means ± SEM; n =12 for each group, * $p< 0.05$ vs. 0 min; $^{\#\#}$ $p< 0.01$ vs. control.

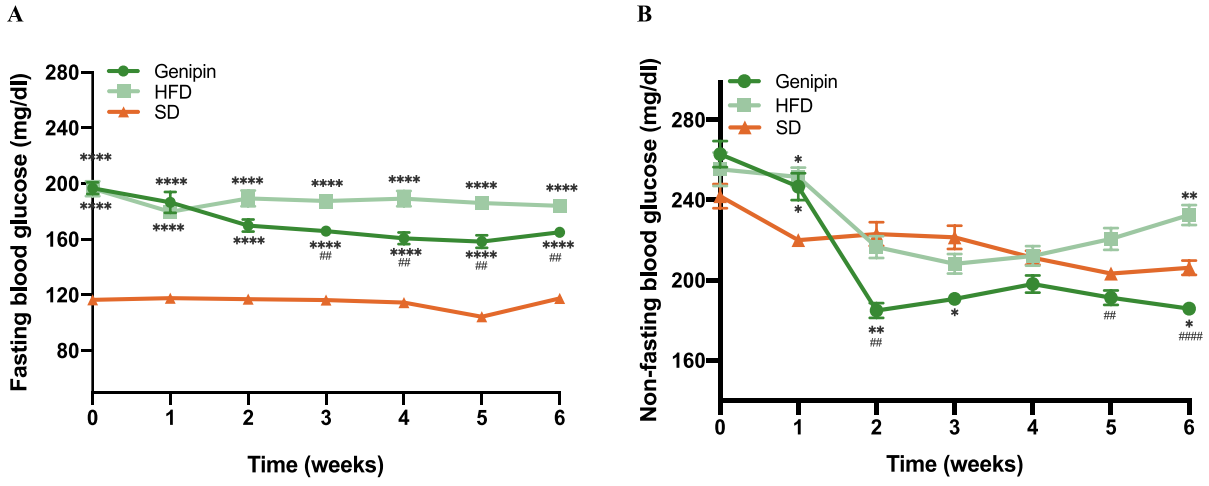


Figure 6. Genipin decreased blood glucose in HFD-induced oebese mice. Mice were fed a HFD or SD for 10 weeks, and then were given 50 mg/kg genipin or vehicle via oral gavage. Fasting blood glucose **(A)** and non-fasting blood glucose **(B)** were measured weekly during the treatment. Data are presented as mean ± SE n= 12 mice of SD, 11 mice of HFD and genipin-treated groups. * $p< 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. SD mice; $^{\#\#}$ $p < 0.01$, $^{\#\#\#\#}$ $p < 0.0001$ vs HFD mice.

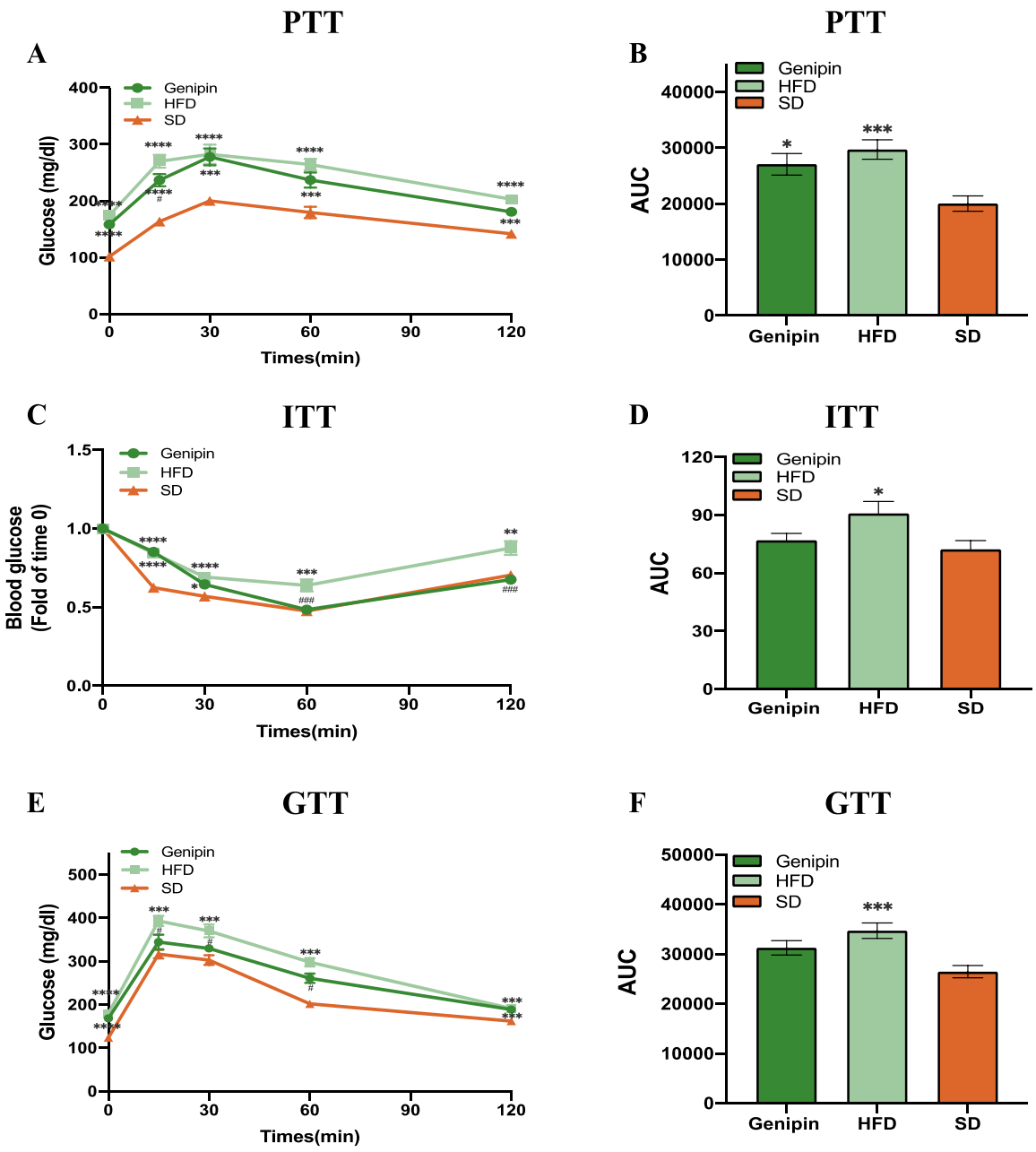
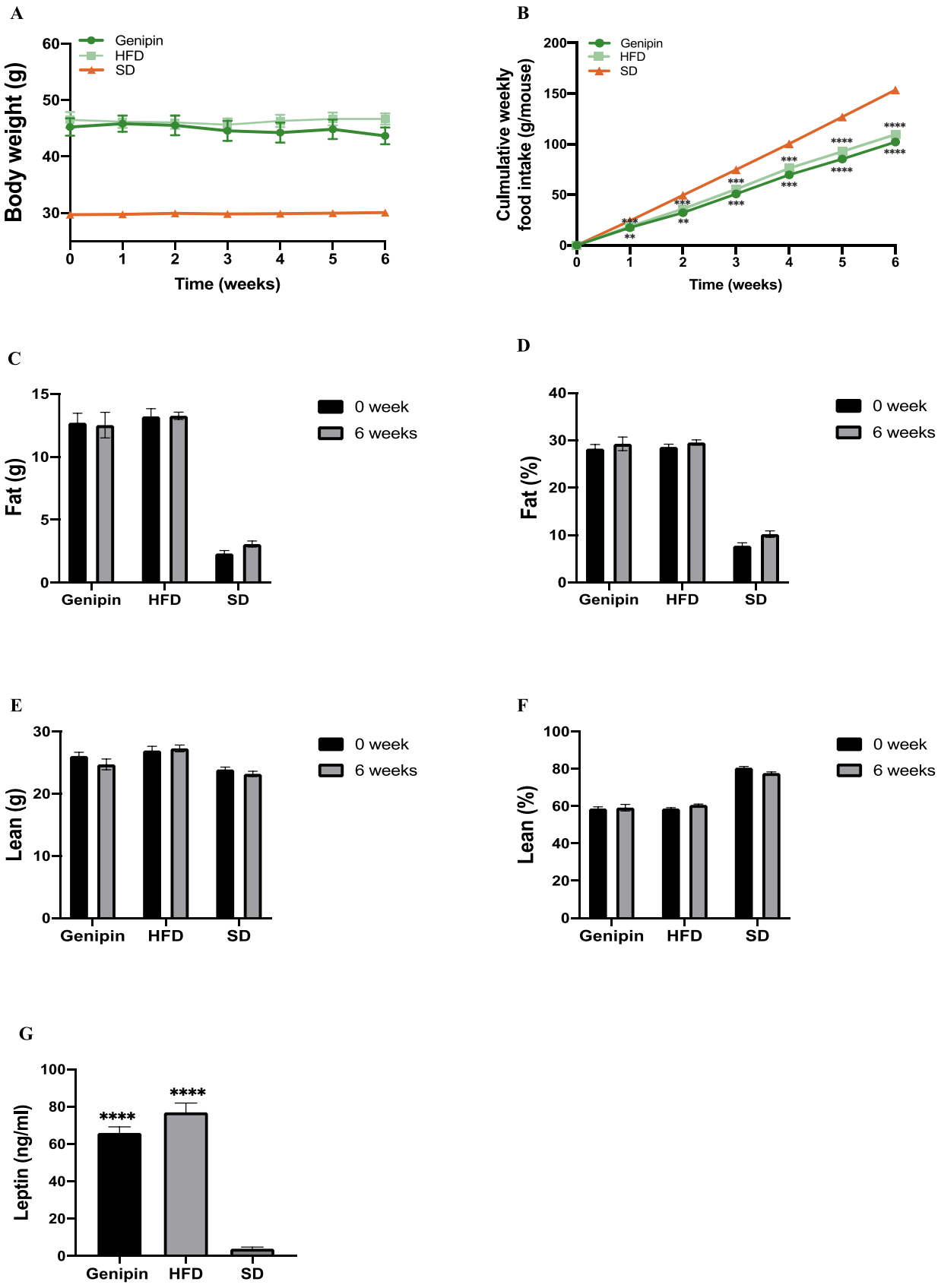


Figure 7. Genipin improved insulin resistance in HFD-fed obese mice. PTT **(A)** and GTT **(E)** were measured after 4-wk and 5-wk of the treatment, respectively. The corresponding area under the curve (AUC) are presented in **(B)** and (**F**). ITT **(C)** was performed after 5-wk of the treatment. Blood glucose was normalized to the 0 min of each group. AUC of ITT was calculated and shown in **(D)**. Data are presented as mean ± SE n= 12 mice of SD, 11 mice of HFD and genipin-treated groups. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. SD mice; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. HFD mice.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SD
Body weight (g)
Time (weeks)
B
Culmulative weekly food intake (g/mouse)
Time (weeks)
C
0 week
6 weeks
Fat (g)
Genipin
HFD
SD
D
Fat (%)
E
Lean (g)
F
Lean (%)
G
Leptin (ng/ml)

Figure 8. Genipin did not alter BW gain and food intake in obese mice. BW **(A)** and food intake were recorded weekly. Body composition **(C-F) was** measured before and after the treatment. Blood samples were collected after treatment and plasma leptin **(G)** was determined by commercial kit. Data are presented as mean ± SE or SEM; **** $p < 0.0001$ vs. SD.

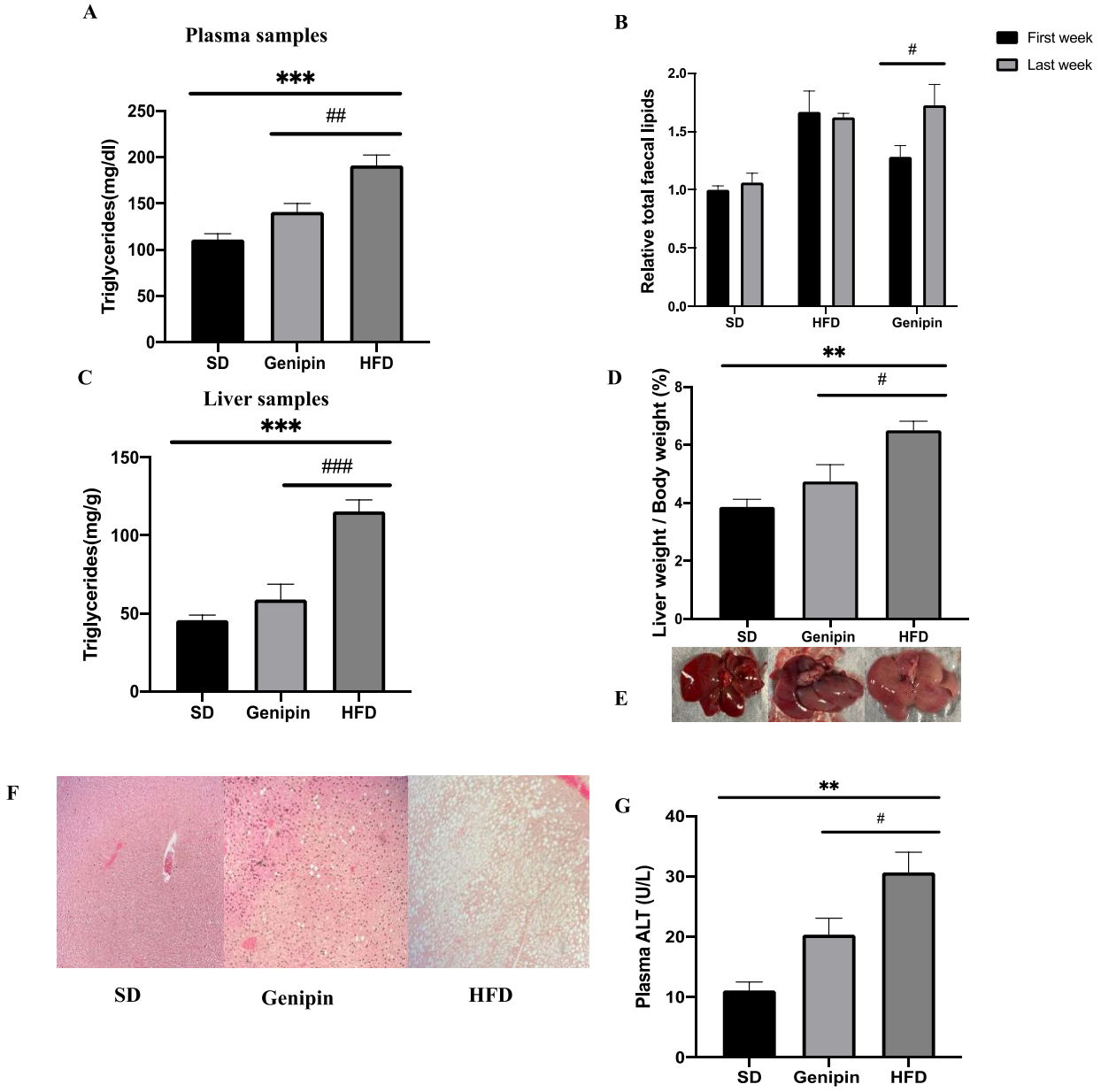

Figure 9. Genipin alleviated HFD-induced hepatic lipid accumulation and dysfunction of the livers in obese mice. At the end of 6-wk treatment, mice were euthanized and **(A)** blood samples and liver samples **(C)** were collected for measuring TG in plasma. **(B)** Lipid contents in feces collected at the 1-wk and 6-wk treatment were measured. **(D)** TG in livers were extracted and measured using a

kti. **(E)** A set of representative photos of the liver is shown **(F)** Representative Liver sections stained with H&E are provided. **(G)** ALT levels were measured in plasma using an assay kit. ** $p < 0.01$, *** $p < 0.001$ vs. SD mice; # $p < 0.05$ vs. HFD mice.

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