

**Baicalein, a novel anti-diabetic compound**

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### **ABSTRACT**

Both in type 1 (T1D) and type 2 diabetes (T2D), the deterioration of glycemic control over time is primarily caused by an inadequate mass and progressive dysfunction of  $\beta$ -cells, leading to the impaired insulin secretion. Thus, the search for agents to protect  $\beta$ -cell and enhance its function is important for diabetes treatment. Studies have reported that baicalein, a flavone originally isolated from the roots of Chinese herb *Scutellaria baicalensis*, has various claimed beneficial effects on health, such as anti-oxidant, anti-viral, anti-thrombotic, and anti-inflammatory effects. However, it is unclear whether it exerts an anti-diabetic action. Here, we present evidence that baicalein may be a novel anti-diabetic agent. Specifically, dietary intake of baicalein significantly improved hyperglycemia, glucose tolerance, and blood insulin levels in high-fat diet (HFD)-fed middle-aged diabetic mice, which was associated with the improved islet  $\beta$ -cell survival and mass. Baicalein treatment had no effect on food intake, body weight gain, circulating lipid profile, and insulin sensitivity in HFD-fed mice. In in-vitro studies, baicalein significantly augmented glucose-stimulated insulin secretion in insulin-secreting cells (INS1) and promotes viability of INS1 cells and human islets. These results demonstrate that baicalein may be a naturally occurring anti-diabetic agent by directly modulating pancreatic  $\beta$ -cell function.

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## GLOSSARY OF TERMS

### A

AMPK: 5' AMP-activated protein kinase

ATP: Adenosine triphosphate

ADP: Adenosine diphosphate

### B

BrdU: Bromodeoxyuridine

Bcl-2: B-cell lymphoma 2

Bcl-xl: B-cell lymphoma-extra large

BW: Body weight

### C

cAMP: Cyclic adenosine monophosphate

CDKs: Cyclin-dependent protein kinases

CREB: CRE-binding protein

COX-2: Cyclooxygenase-2

CB: Citrate buffer

## **D**

DMSO: Dimethyl sulfoxide

## **E**

ELISA: Enzyme-Linked ImmunoSorbent Assay

## **F**

FBS: Fetal bovine serum

## **G**

GSIS: Glucose stimulated insulin secretion

GTT: Glucose tolerance test

GLP-1: Glucagon like peptide-1

GI: Gastrointestinal

GSH-Px: Glutathione peroxidase

## **H**

HI: Heat inactivated

HF: High fat

HFD: High fat diet

## **I**

INS1: Clonal rat pancreatic  $\beta$ -cells

IP: Intraperitoneal injection

iNOS: Inducible NO synthase

## **K**

K<sub>ATP</sub>: ATP-dependent potassium channels

KRBB: Krebs-Ringer bicarbonate buffer

## **L**

LPH: Lactase phlorizin hydrolase

## **M**

MTS: 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

Mcl-1: Myeloid cell leukemia 1

## **N**

NO: Nitric oxide

NF- $\kappa$ B: Nuclear factor kappa-light-chain-enhancer of activated B cells

NMDA: *N*-methyl-D-aspartate

NADH: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

## **P**

PKA: Protein kinase A

PKC: Protein kinase C

PGE<sub>2</sub>: Prostaglandin E2

## **R**

ROS: Reactive oxygen species

## **S**

STZ: Streptozotocin

SOD: Oxidoreductase dismutase

SE: Standard error

## **T**

T1D: Type 1 Diabetes

T2D: Type 2 Diabetes

TNF- $\alpha$ : Tumor necrosis factor-alpha

## Chapter 1: INTRODUCTION

Diabetes mellitus is a complex metabolic disorder characterized by abnormalities in insulin secretion and action, which leads to hyperglycemia (1). Type 1 diabetes (T1D) is a T-cell-mediated autoimmune disease leading to the destruction of pancreatic  $\beta$ -cells, whereas type 2 diabetes (T2D) is due to a combination of peripheral insulin resistance and loss of functional  $\beta$ -cell mass (2-5). Diabetes is a growing public health problem in the United States, presently affecting 25.8 million or 8.3% of the American population (6). This number is still increasing in Americans, which is predicted to double by 2025 (7). The medical costs associated with this disease in 2007 reached \$218 billion in the United States, which included drug therapy and surgical treatment (6). In both T1D and T2D, inadequate  $\beta$ -cell mass and  $\beta$ -cell dysfunction leading to impaired insulin secretion are central to the deterioration of glycemic control (8). Therefore, the search for novel and cost-effective agents that can enhance  $\beta$ -cell function and increase  $\beta$ -cell mass are important to provide an effective treatment for diabetes.

*Scutellariae baicalensis Georgi*, a medicinal plant, grows in some Asian countries including Siberia, Far East of Russian, Mongolia, and China and other Eastern Asian countries (9; 10). The root of *Scutellariae baicalensis Georgi*, also called *Huangqin* in Chinese, has been used as an ingredient in traditional Chinese medicine formulations for thousands of years (11). According to Chinese medicine, *Huangqin* has various beneficial effects on health. For example,

it can lower blood pressure and decrease lipid content in the blood. There have been 295 compounds isolated from *Huangqin*, more than 40 of which are flavonoids (12; 13). Baicalein, baicalin, wogonin, and wogonoside are presumably the main bioactive components in *Huangqin*. Recently, baicalein and its glucuronide baicalin have drawn wide attention due to their possible health effects. Baicalein may have potential beneficial effects in diabetes-related complications (14-17), cancers (18; 19), cardiovascular disease (20), inflammation (21), bacterial infections (22; 23), and oxidative stress (24-26), although some of these reports remain controversial and the mechanisms of these effects are unclear.

Studies on whether baicalein has an anti-diabetic effect are scarce. It was reported that baicalin is effective in the treatment of streptozotocin-induced diabetes in rats (15). The mitochondrial damage caused by diabetes was well controlled by baicalin by protecting the integrity of the inner membrane of the mitochondria (15). Another study showed that long-term administration of baicalin has potential therapeutic benefits in rats fed high-fat diet, including a reduction in the body weight gain and the levels of circulating serum cholesterol and free fatty acid, as well as amelioration of lipid accumulation in the liver and systemic inflammation markers (14). Although emerging evidence suggests that baicalein may have effects on diabetes related complications, it is unknown whether baicalein has an anti-diabetic effect. Moreover, whether this compound exerts a beneficial effect on pancreatic  $\beta$ -cells is lacking.

As aforementioned, loss of functional  $\beta$ -cell mass and  $\beta$ -cell dysfunction are central to the development of diabetes (8). Thus, search for novel and cost-effective agents that can increase  $\beta$ -cell mass and/or enhance  $\beta$ -cell function is extremely important to provide effective

treatment for diabetes and thereby decrease the burden of morbidity from diabetes and related complications. In the present study, I evaluated the anti-diabetic potential of baicalein and further determined its effects on insulin secretion and  $\beta$ -cell viability.

## Chapter 2: REVIEW OF LITERATURE

### Flavones

Flavones are a group of flavonoids with a 2-phenylchromen-4-ketone (2-phenyl-1-benzopyran-4-ketone) backbone (Fig.A). Flavones are mostly found in cereals and herbs and the individual's intake of flavones from the diet is between 1 to 3 mg per day in U.S. adults (27). Recently, flavones have increasingly received attention in research community and public due to their putative beneficial effects against some degenerative diseases such as atherosclerosis, osteoporosis, diabetes mellitus, and certain cancers (28). Natural flavones include baicalein, apigenin, luteolin, wogonin, tangeritin, scutellarein, and many others.

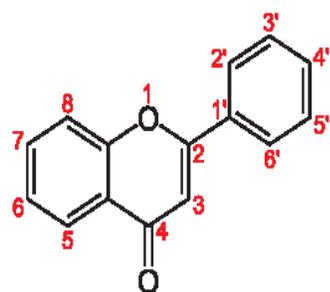
### Baicalein

#### Sources, structure, and chemistry

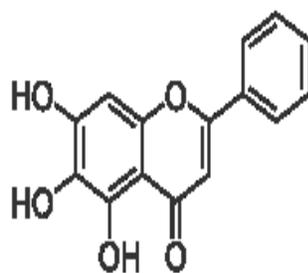
As an important member of flavones, baicalein is originally isolated from *Huangqin* (Fig.B). *Huangqin* has been widely used in Chinese traditional medicine for various claimed health benefits. There are 295 compounds isolated from *Huangqin*, including flavonoids, phenylethanoid glycosides, iridoid glycosides, diterpenes, triterpenoids, alkaloids, phytosterols and polysaccharides. There are over 40 flavonoids identified from *Huangqin*, which are primarily conjugated with a glucose molecule to form glycosides. Baicalein, baicalin, wogonin,

and wogonoside are thought to be the main bioactive components in *Huangqin*, and their ratios to the dry material were about 5.41%, 10.11%, 1.3%, 3.55%, respectively (29). The flavones extracted from *Huangqin* have been shown to exert anti-oxidant (30), anti-viral (31; 32), anti-thrombotic (20; 33), anti-inflammatory (34), and anti-cardiovascular effects (35). As shown in Fig.C, *Huangqin* contains four major flavones: baicalein (5,6,7-trihydroxyflavone), wogonoside (wogonin-7-glucuronic acid), baicalin (7-glucuronic acid, 5,6-dihydroxy-flavone), and wogonin (5,7-dihydroxy-8-methoxyflavone) (29).

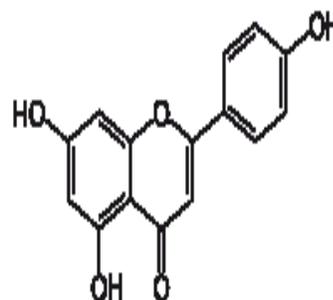
Baicalein is a yellow crystalline solid, and its chemical formula is  $C_{15}H_{10}O_5$ , with a molecular weight of 270.25 g/mol. As shown in Fig.A, the chemical structure of baicalein consists of three phenolic rings; with three hydroxyl groups at C-5, C-6, and C-7, respectively.



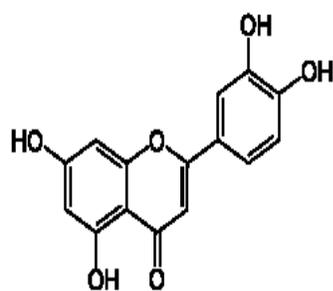
Common structure



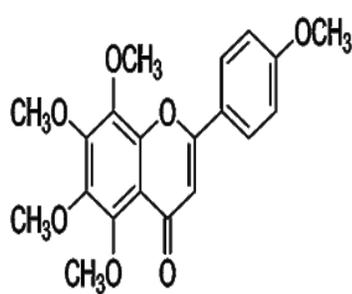
Baicalein



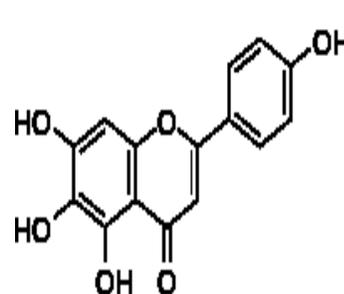
Apigenin



Luteolin



Tangeritin



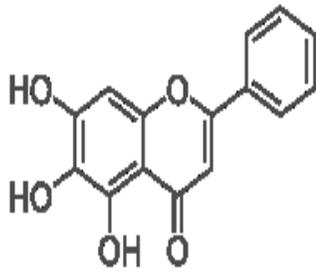
Scutellarein

**Fig.A. Structures of flavones (36)**

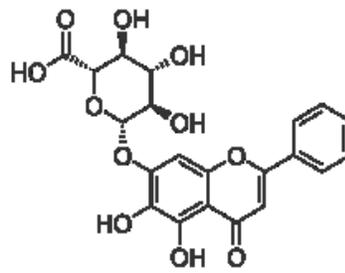


**Fig.B. *Scutellariae baicalensis Georgi* (upper) and its dried root**

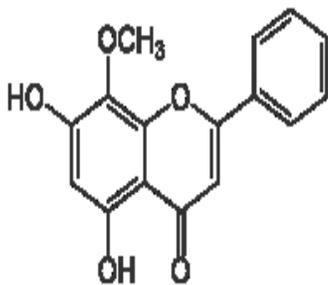
***Huangqin* (lower) (37). Used under fair use, 2012**



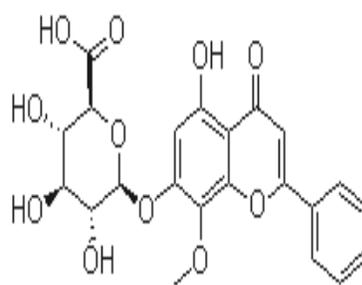
**Baicalein**  
(5,6,7-trihydroxyflavone)



**Baicalin**  
(7-glucuronic acid, 5,6-dihydroxy-flavone)



**Wogonin**  
(5,7-dihydroxy-8-methoxyflavone)



**Wogonoside**  
(wogonin-7-gluronic acid)

**Fig.C. Structures of baicalein, wogonin, wogonoside and baicalin**

derived from *Huangqin* (29). Used under fair use, 2012

## **Absorption and metabolism**

Flavones occur in plants primarily in the form of glycosides, which means that the flavones are conjugated to a glucose molecule. When ingested, glucose-conjugated baicalein undergoes hydrolysis by the intestinal bacteria enzymes such as  $\beta$ -glucosidases or lactase phlorizin hydrolase (LPH), releasing the corresponding bioactive aglycones (baicalein) (38). Baicalin is the glucuronide of baicalein (37). It was reported that baicalein can more efficiently pass through the intestinal epithelium than baicalin (39; 40). The aglycone baicalein can permeate easily through the monolayer from the lumen to the blood stream due to its high lipophilicity and low molecular weight. Baicalin however exhibits limited permeability due to its relatively high hydrophilicity and large molecular weight (41; 42). Interestingly, it was found that baicalin is only moderately absorbed in the stomach, whereas baicalein can be well absorbed from the stomach and small intestine, but the absorption from the colon is limited (43). However, after ingestion, baicalein undergo extensive metabolism in the intestine and liver in the form of glucuronidation (38). A major portion of baicalein therefore is retained within the intestinal mucosal cells and transformed into baicalin and then transported to the circulation system (39).

It was reported that after oral administration of baicalein, the plasma concentration of total baicalein including its conjugated metabolites like baicalin and baicalein sulfate (44), can reach up to 10  $\mu$ M and can maintain at 1  $\mu$ M for 36 h (45; 46). The glucuronide conjugates of baicalein from the circulation can enter the small intestine through biliary excretion, which will undergo hydrolytic cleavage through intestinal  $\beta$ -glucosidases (47). A large part of the enteric excretion

of baicalein will pass through the intestinal epithelium and regenerate baicalin again, which will then be transported to the bloodstream (45). After ingestion and transformation, baicalin is distributed to many tissues and organs in the body. It was reported that in vitro human serum albumin binding of baicalin in human plasma is in the range of 86% to 92% (48). Zhiyan et al reported that baicalin can penetrate blood-eye barriers and enter the lens (49). Huang et al reported that baicalin also can penetrate the blood-brain barriers to get into the brain tissue (50). So the baicalein conjugates circulation may play an important role for its distribution through the bloodstream. While widely distributed in the body after ingestion, the potential beneficial or harmful roles of baicalein in the tissues are largely unclear.

## **Pancreatic $\beta$ -cells and diabetes**

### **Diabetes mellitus**

Diabetes mellitus is a complex metabolic disorder characterized by abnormalities in insulin secretion and action, which leads to hyperglycemia. There are two main types of diabetes, T1D and T2D. T1D is a chronic autoimmune-mediated disease characterized by destruction of pancreatic  $\beta$ -cells resulting in absolute insulin deficiency (51-53). T1D, primarily caused by genetic and environmental factors, mostly occurs in children (54; 55). While it is well established that T1D is due to the destruction of pancreatic  $\beta$ -cell (56), the exact pathogenic process is still not completely known. Some studies suggest that T1D development is associated with the infection of some virus, such as coxsackievirus B, mumps virus, enteroviruses, and rubella (57; 58). However, it is generally accepted that  $\beta$ -cell destruction is mediated by T-lymphocytes that infiltrate into the islets, causing immune response and ultimate destruction  $\beta$ -cell apoptosis (2). It

was found that islet cell auto-antibodies are present in the serum of 90% newly diagnosed diabetic patients and multiple auto-antibodies are reported to cause progressive  $\beta$ -cell autoimmunity (59-61). Successful islet transplantation can normalize hyperglycemia in T1D (62-64). However, the supply shortage of qualified islets, side effect of utilization of immune suppression drugs, and progressive apoptosis of transplanted islets limit the wide application of this approach. Therefore, developing efficient and low-cost prevention strategy is necessary.

T2D is a metabolic disorder, which causes high blood glucose along with insulin resistance and insulin deficiency (3). T2D is a major risk factor for cardiovascular disease, strokes and kidney failure (65). Several reports indicate that genetic factors are very important in the pathogenesis of T2D, with a family history of diabetes present in about 50% of first degree relatives (66; 67). Insulin resistance is important to the etiology of T2D, which could be caused by defective insulin receptors on the target cells because of inflammation (68-71). At the onset of T2D, The body can still manage blood glucose homeostasis by increasing  $\beta$ -cell mass and function to secrete more insulin to compensate for increased insulin demand due to insulin resistance (5; 72; 73). The burden of increased insulin demand can cause progressive loss of  $\beta$ -cell mass and  $\beta$ -cell dysfunction, which will then lead to defective ability of pancreatic  $\beta$ -cell to secrete insulin. Therefore insulin resistance can progress to overt T2D when  $\beta$ -cells are unable to compensate for increase insulin demand (74; 75). Indeed, those with T2D always manifest increased  $\beta$ -cell apoptosis and reduced  $\beta$ -cell mass (76-79). As such, a strategy that promotes  $\beta$ -cell survival and mass can potentially provide a therapeutic means to prevent the onset of

diabetes (80). Therefore, the search for novel agents that can promote islet survival is extremely important to provide effective treatment for this disease.

### **Pancreatic $\beta$ -cell and insulin secretion**

The mammalian pancreas is functional as both an endocrine and exocrine gland. It consists of two different types of parenchyma tissues. Exocrine gland cells are called acini, which produce digestive enzymes, while endocrine gland consists of islet of Langerhans. Islet of Langerhans only accounts for 1-2% of total adult pancreatic tissue, but it plays a critical role in regulating physiological homeostasis in the mammalian body (81). There are four main groups of cells in islet:  $\beta$ -cells consisting of 65-80% of islet that makes and produces insulin,  $\alpha$ -cells consisting of 15-20% of islet cell which secrete glucagon,  $\delta$ -cells consisting of 3-10% of islet that makes somatostatin, and PP cells consisting of 3-5% of islet which generate pancreatic polypeptide (3).

Insulin is central to regulating carbohydrate and fat metabolism in the body. The insulin secretion elicited by insulinotropic agents such as glucose, is biphasic, and a transient response of 4-10 min duration is considered the first phase, followed by a gradual increase in secretion rate referred as the second phase (82)(Fig.D). Following cellular glucose uptake, glycolysis and subsequent tricarboxylic acid cycle increase cellular adenosine triphosphate/adenosine diphosphate ratio (ATP/ADP), which leads to the closure of  $K_{ATP}$  channels and then the depolarization of the cell membranes. This event activates voltage-dependent  $Ca^{2+}$  channels and

consequently triggers  $\text{Ca}^{2+}$  influx, which ultimately leads to insulin release into the blood stream (83). Activation of the  $\text{K}_{\text{ATP}}$  channel-dependent pathway plays the key role in the first phase of glucose-stimulated insulin release. The first phase insulin release can be amplified by second messengers such as cyclic adenosine monophosphate (cAMP) (84). Following the nadir in the first phase, the second phase starts which is characterized by a gradually increasing rate of release to a plateau after a further 25 to 30 min. The second phase is mainly due to the  $\text{K}_{\text{ATP}}$  channel-independent pathways, although the mechanisms involved are not fully understood. However, several hypotheses have been proposed, which involve protein kinase C (PKC), protein kinase A (PKA), cAMP, protein acyl transferases and phospholipase A2 (84).

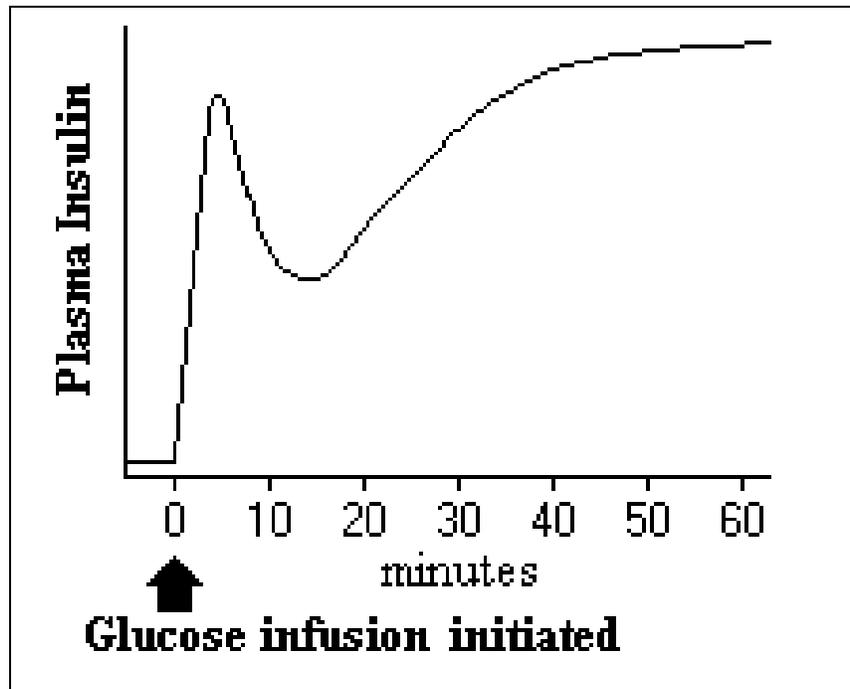


Fig.D. Glucose stimulated biphasic insulin secretion (85). Used under fair use, 2012

## **Pancreatic $\beta$ -cell mass**

In both T1D and T2D, inadequate  $\beta$ -cell mass and  $\beta$ -cell dysfunction leading to impaired insulin secretion are central to the deterioration of glycemic control (8). Pancreatic  $\beta$ -cell mass is regulated by the relative balance of neogenesis, proliferation, and cell death (86-88). Neogenesis is defined as the formation of new  $\beta$ -cells by the differentiation of precursor cells (89). This process, which can determine  $\beta$ -cell population, normally stops after birth. However, under certain condition, neogenesis can be activated. It was reported that neogenesis can be activated after severe injury to endocrine or exocrine pancreatic tissue (89). In addition, some hormones such as incretins also can activate neogenesis from precursor cells (90). Pancreatic  $\beta$ -cell proliferation is primarily responsible for postnatal growth and mass expansion of  $\beta$ -cells (90). At the beginning of T2D,  $\beta$ -cell proliferation will increase  $\beta$ -cell mass causing more insulin secretion to compensate for insulin resistance. Pancreatic  $\beta$ -cell proliferation is regulated by certain stimuli such as glucose, insulin, and growth factors, like glucagon-like peptide-1 (GLP-1), and some  $\beta$ -cell cycle regulators, such as cyclins and cyclin-dependent kinases (cdks) (90; 91). Pancreatic  $\beta$ -cell apoptosis occurs in T1D and also cause loss of  $\beta$ -cell mass in T2D (92; 93). Therefore, search for novel agents that can preserve or regenerate functional  $\beta$ -cell mass by stimulating  $\beta$ -cell proliferation, inhibiting  $\beta$ -cell apoptosis, and/or inducing  $\beta$ -cell neogenesis, is an important strategy to treat diabetes.

## **Baicalein and Diabetes**

### **Health effects of baicalein**

It has been reported that baicalein possesses some important biological activities. A number of studies reported that baicalein can prevent several chronic diseases such as cardiovascular and certain cancers. In addition, it may have anti-oxidant (25; 26) and anti-inflammatory (21) effects. However, the exact mechanisms for these actions are not clear.

During aerobic metabolism, reactive oxygen species (ROS), including superoxide ( $\cdot O_2^-$ ), hydroxyl radicals ( $\cdot OH$ ) and non-radical  $H_2O_2$ , are constantly generated by cells as normal by-products (94). Although moderate amount of ROS plays an important role in defense against pathogens and in regulation of cellular functions and intracellular signaling, high levels of ROS induce oxidative stress that can lead to different diseases (95). Superoxide dismutase (SOD), a cytoplasmic enzyme, can defend against oxidative stress by scavenging  $\cdot O_2^-$  (94). Baicalein is capable of transferring free radicals and to acting as a chelator of redox-active metal ions (96). In addition, it was reported that baicalein can improve SOD and glutathione peroxidase (GSH-Px) activities in the cortex and hippocampus in rats (97).

Baicalein has been shown to protect from inflammation *in vitro* and in various animal models (98). The anti-inflammatory effect of the baicalein may be at least partly due to its inhibition of nitric oxide (NO) production via down-regulation of several inflammation-

associated genes such as inducible NO synthase (iNOS), cyclooxygenases, and lipoxygenases in RAW 264.7 macrophages (99). Recent findings suggest that prostaglandin E2 (PGE<sub>2</sub>), the pro-inflammatory product of elevated cyclooxygenase-2 (COX-2) activity, plays a direct role in malignant progression of most solid tumors (100). Baicalein were shown to suppress PGE<sub>2</sub> synthesis and inhibit COX-2 expression (101). Chemokines such as Interleukin-4 and tumor necrosis factor-alpha (TNF- $\alpha$ ) and chemokine receptors are critical mediators of inflammation. It was shown that baicalein can inhibit the binding of chemokines like interleukin-8 to human leukocytes (102). However, the physiological relevance of these *in vitro* findings is unclear.

It was reported that oral administering of 20 mg/kg baicalein inhibits the growth of established prostate tumors by about 55% (103). The anti-cancer effects of baicalein are probably due to its ability to scavenge ROS or directly inhibit cell cycle genes and induce cell apoptosis (29). Nuclear factor- kappa B (NF- $\kappa$ B) is an inducible transcription factor that controls the expression of over one hundred genes involved in immunity, inflammation, proliferation, and apoptosis. And NF- $\kappa$ B is considered to be a survival factor that activates expression of various anti-apoptotic genes such as anti-apoptotic proteins Bcl-2, Bcl-xL, and Myeloid cell leukemia 1 (Mcl-1) that promotes survival of many types of tumors (104). Baicalein treatment suppressed NF- $\kappa$ B activity and the expression of Bcl-2, Bcl-xL and Mcl-1 in myeloma cells (105). Baicalein may also inhibit tumor cell growth by inducing cell cycle arrest at several checkpoints. For example, exposure of breast and lung cancer cells to baicalein decreased Cyclin D1 protein levels (106). Consistently, baicalein treatment also reduced the expression of Cyclin D1 and D3 in prostate cancer cells (107).

### **Whether baicalein has an anti-diabetic action is unknown**

As aforementioned, baicalein has been previously investigated for its potential beneficial effects on various human degenerative diseases. However studies on whether baicalein has an effect on diabetes are very limited. Evidence shows that oxidative stress plays a potential role in the initiation of diabetes (108) and baicalein has been reported to exert an anti-oxidant activity (109). Baicalin, the glucuronide of baicalein, is the main metabolite in the blood stream after baicalein injection (46). Waisundara et al gave baicalin to streptozotocin-induced diabetic rats and found that baicalin can protect the integrity of the inner membrane of the mitochondria that was damaged by diabetes (15). Guo et al found that baicalin treatment reduced elevated concentrations of serum cholesterol, free fatty acid, and insulin in rats fed a high-fat diet. Further, it was found that baicalin reduced the systemic inflammation markers and diminish lipid accumulation in the liver (14). Baicalein was found to improve metabolic syndrome through an AMPK-dependent mechanism in high-fat diet-fed mice (16). In the present study, I found that baicalein decreases blood glucose levels, improves circulating insulin concentration and preserves islet  $\beta$ -cell mass in obese diabetes mice, suggesting that this compound may have an anti-diabetic effect.

## **Significance**

As aforementioned, diabetes is growing public health problem in the United States, presently affecting 25.8 million or 8.3% of the American population. This number is still increasing in Americans, and is predicted to double by 2025. It is recognized that loss of  $\beta$ -cell mass and function is central to the development of diabetes. Therefore, the search for novel and cost-effective compounds that can promote  $\beta$ -cell survival and enhance  $\beta$ -cell function is important to provide effective treatment for diabetes. In the present study, I investigated whether baicalein can promote  $\beta$ -cell mass and protect  $\beta$ -cell function, thereby preventing T2D using a relevant mouse model. We found for the first time that baicalein improves hyperglycemia, circulating insulin levels, and preserves functional islet mass in middle-aged obese diabetic mice. These results will form the basis for developing novel and low-cost botanical agents for diabetes prevention and treatment.

## Chapter 3: MATERIALS AND METHODS

### Reagent and materials

RPMI-1640 media (RPMI) was purchased from Sigma-Aldrich (St. Louis, MO); CMRL-1066 media (CMRL) was from Mediatech, Inc. (Holly Hill, FL); heat-inactivated fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). Baicalein (98% pure) for *in vitro* studies was purchased from Sigma-Aldrich (St Louis, MO). Stock solutions of baicalein at 20 mM were dissolved in sterilized Dimethyl sulfoxide (DMSO) and stored at -80 °C before use. Baicalein (98% pure by HPLC) for *in vivo* studies was purchased from Xi'An Yile Bio-Tech Company, China; ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA) kits were obtained from Merckodia (Winston-Salem, NC); aqueous one solution cell proliferation assay kits were from Promega (Madison, WI); all other chemicals were from Sigma-Aldrich. Glucose was dissolved in sterile water and stored at -80 °C.

### Cell and human islet culture

INS1 cells were cultured as previously described (110) in RPMI medium containing 11.1 mM glucose and supplemented with 10% FBS, 1 mM sodium pyruvate, 10 mM HEPES, 2 g/L sodium bicarbonate, 50  $\mu$ M  $\beta$ -mercaptoethanol, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Medium was changed every 2-3 days until cells were approximately 70%

confluence. Human islets were isolated from cadaver organ donors in the Islet Cell Resource Centers administered by Southern California Resources Center & Southern California Islet Consortium at National Medical Center (Duarte, CA). The islet purity was 90-99% and viability was 80-99%. Before the experiment, the islets were maintained in CMRL medium containing 10% FBS.

### **Insulin secretion assay**

For determining the effect of baicalein on GSIS, INS1 cells were cultured in a 96-well plate in RPMI medium containing 11.1 mM glucose and 10% HI FBS at 37°C for 48 h until the cells became 50%-60% confluence. The cultures were then switched to RPMI medium containing 3 mM glucose and 2% FBS. After 12 h, cells were washed with Krebs-Ringer bicarbonate buffer (KRBB; 129 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 5 mM NaHCO<sub>3</sub>, 0.1% BSA, and 10 mM HEPES, pH 7.4) one time, followed by stimulation with 0.1, 1, 5, 10, or 20 μM baicalein in either 3 or 20 mM glucose at 37°C for 30 min. Insulin in the supernatants was measured by ELISA.

### **Cell proliferation assay**

INS1 cells were incubated with various concentrations of baicalein or vehicle in RPMI medium containing 5.5mM glucose and 2% FBS at 37°C. 24 h later, the cultures were continued for an additional 4 h in the presence of bromodeoxyuridine (BrdU), an analog of thymidine which incorporates into newly synthesized DNA, therefore labeling replicating cells. Cell

proliferation was assessed by BrdU incorporation measurements with an ELISA cell proliferation assay kit as previously described (111).

### **Cell viability assay**

INS1 cells or human islets were incubated with various concentrations of baicalein or vehicle in RPMI medium containing 5.5mM glucose and 2% FBS at 37°C. 48h later, the cultures were continued for an additional 4h in the presence of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), a tetrazolium compound, which was reduced by cells into a colored formazan product that was soluble in culture medium. Cell viability was assessed by the absorbance measurements at 490nm with a plate reader.

### **Animals and treatment with baicalein**

Retired breeder male C57BL/6NCr mice (8 months old) were purchased from NCI-frederick (Frederick, MA). Animals were housed in a room maintained on a 12h light/dark cycle under constant temperature (22–25°C) with ad libitum access to food and water. The protocol of this study was reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Tech. After an initial adaption period for one wk and the mice were divided into 6 groups (n=10 mice/group) with initial fasting blood glucose and body weight balanced among groups. Mice were fed a standard rodent chow diet, a high-fat diet (HFD) (60 kcal% fat, corn oil substitutes for soybean oil) with or without baicalein throughout the experiment. After 4 wk of treatment, mice received an intraperitoneal injection (i.p.) of streptozotocin (STZ) dissolved in

0.1 M cold sterile sodium citrate buffer (pH 4.5) at 40 mg/kg daily for 3 consecutive days. Control mice received i.p. of saline. Body weight and food intake were measured weekly throughout the study. Body composition was measured before injection STZ and before sacrifice.

### **Plasma glucose and insulin measurement**

Fasting blood glucose were recorded every two wk before STZ injection and weekly after STZ in tail vein blood sample using a glucometer (Roche, IN). At the end of feeding experiment, mice were fasted for 12h before blood was drawn. Fasting plasma insulin levels were measured using ultrasensitive rat ELISA kits.

### **Glucose tolerance and insulin tolerance tests**

For glucose tolerance test, mice were fasted overnight and injected i.p. with a single bolus of glucose (1 g/kg BW). Glucose levels were measured at time points of 0, 15, 30, 60 and 120 min after glucose administration. For insulin tolerance test, mice were injected i.p. with insulin (0.5 units/kg BW), and blood glucose levels were measured at 0, 15, 30, 60, and 120 min after insulin administration.

### **Immunohistochemistry and $\beta$ -cell mass**

After mice were euthanized, the pancreata were dissected and fixed in 4% (vol/vol) formaldehyde buffer (pH7.2). A series of tissue sections (6  $\mu$ m thickness) were prepared by

AML Laboratories, Inc (Baltimore, MD). Tissue sections, mounted on glass slides, were stained with insulin antibody firstly, and then an immunofluorescence stain (FITC-conjugated secondary antibody) would be followed for insulin to detect  $\beta$ -cells. The  $\beta$ -cell area was measured using images acquired from insulin-stained (green fluorescence) pancreatic sections. The  $\beta$ -cell mass were calculated by dividing the area of insulin-positive cells by the total area of pancreatic tissue. The images of islet were taken by Nikon camera along with microscope.

### **Statistical analysis**

All data were analyzed using student's t-test and are expressed as mean  $\pm$  standard error (SE). Difference between control and treatment was considered significant at  $p < 0.05$ .

## Chapter 4: RESULTS

### **Dietary intake of baicalein improves glucose tolerance in obese mice.**

To determine if the better glycemia control by baicalein is through protecting  $\beta$ -cell function, we performed a glucose tolerance test prior to injection of STZ. There was a significant increase in plasma glucose levels (Fig. 1A) and  $AUC_{\text{glucose}}$  (Fig. 1B) in the HFD fed mice compared with 0.5g/kg baicalein-treated mice. Our results showed that dietary intake of baicalein significantly improved glucose tolerance in obese mice. These results suggest that baicalein may promote pancreatic  $\beta$ -cell insulin secretory function in response to glucose challenge.

### **Dietary intake of baicalein does not improve insulin sensitivity in obese mice.**

Insulin resistance is important to the etiology of T2D, and usually occurs in obesity. To determine if dietary intake of baicalein could increase insulin sensitivity in obese mice, we performed insulin tolerance test prior to injection of STZ. There was little difference in plasma glucose levels (Fig. 2A), and the  $AUC_{\text{glucose}}$  (Fig. 2B) was similar between HFD fed and baicalein-treated mice. Our results showed that dietary intake of baicalein does not improve insulin sensitivity in obese mice. These results suggest that baicalein may regulate blood glucose level but not due to improve insulin sensitivity.

### **Baicalein has no effect on food intake in obese diabetic mice.**

To exclude the possibility that baicalein might change mouse appetite, thereby modulating food intake, which could contribute to the effect of baicalein in diabetic mice, we monitored food intake throughout the experiment. Before STZ injection, body weight has no difference in the control and baicalein-treated group (data not shown). Our results show baicalein did not alter food intake (Fig.3), suggesting that the protective effect of baicalein is not due to alternation of food intake.

### **Dietary intake of baicalein ameliorates hyperglycemia in obese diabetic mice.**

To assess whether baicalein can prevent diabetes, we fed C57BL/6NCr mice (male, 8 months old, retired breeder) with a normal diet, a high fat, or high fat diet containing 0.25 g, 0.5 g, or 1.0g/kg baicalein. After 4 wk of dietary treatment, streptozotocin (STZ, 40 mg/kg BW) was administered intraperitoneally for 3 consecutive days to induce mild- to moderate-level of diabetes mediated by a destruction of islet  $\beta$ -cell in mice (112). Our data showed that baicalein significantly ameliorated STZ-induced hyperglycemia in diabetic mice fed 0.25 and 0.5 g/kg baicalein, with 0.5 g/kg baicalein producing the maximal protective effect (Fig.4).

### **Dietary baicalein intake prevents body weight and body fat loss in obese diabetic mice.**

With severe insulin deficiency, the body will show symptoms such as frequent urination and unusual weight loss (113). To investigate if baicalein can prevent body weight loss, we measured body weight and composition before and after injection of STZ. The results show that body weight and fat and muscle mass have no difference in the control and baicalein-treated group (data not shown). Our results showed that dietary ingestion of 0.25 and 0.5 g/kg baicalein prevents the loss of body weight secondary to the development of diabetes (Fig. 5A) and dietary ingestion of 0.5 g/kg baicalein also prevents fat loss (Fig. 5B) in obese diabetic mice. However, dietary ingestion of baicalein has no influence on muscle mass (Fig. 5C).

#### **Dietary baicalein intake improves blood insulin levels in obese diabetic mice.**

To determine if the better glycemia control by baicalein is the result of the improved islet function, we measured insulin levels in plasma of control and baicalein-treated mice. As shown in Fig. 6, plasma insulin levels in mice fed with 0.25 and 0.5 g/kg baicalein were significantly higher as compared to those in non-treated diabetic mice, suggesting that baicalein may ameliorate hyperglycemia primarily through preserving islet  $\beta$ -cell function.

#### **Dietary intake of baicalein improves islet $\beta$ -cell mass in obese diabetic mice.**

Since STZ causes diabetes by destroying islet  $\beta$ -cells (114), and our previous studies showed that baicalein might protect  $\beta$ -cell function, we then examined whether baicalein treatment preserved  $\beta$ -cell mass in diabetic mice. We found that STZ administration severely

decreased  $\beta$ -cell mass and disrupted the islet architecture (Fig. 7). However, dietary intake of baicalein significantly improved islet  $\beta$ -cell mass and preserved its structure in diabetic mice.

### **Baicalein does not enhance $\beta$ -cell proliferation *in vitro*.**

It was reported that several flavonoids such as genistein can stimulate  $\beta$ -cell proliferation (115). We considered the possibility that baicalein could increase  $\beta$ -cell mass by stimulating  $\beta$ -cell proliferation. However, our results showed that baicalein at 0.1 $\mu$ M to 20 $\mu$ M concentrations had no effect on INS1 cell proliferation as determined by BrdU incorporation assay (Fig.8).

### **Baicalein promotes GSIS in INS1 cells.**

In animal study, we found that baicalein may promote  $\beta$ -cell function and insulin secretion in response to glucose. To examine whether baicalein directly enhances GSIS in  $\beta$ -cells, we cultured INS1 cells in the presence or absence of baicalein for 30 min. Baicalein augments GSIS in INS1 cells exposed to 3 mM or 20 mM glucose (Fig.9).

### **Baicalein promotes viability of INS1 cells and human islets.**

Our animal study shows that baicalein preserves functional  $\beta$ -cell mass. To investigate the mechanism underlying this protective action by baicalein, we assessed if baicalein affects the viability of human islets and INS1 cells. Our results showed that baicalein dose-dependently

increases both human islet (Fig. 10A) and INS1 cell (Fig. 10B) viability, with 5-20  $\mu\text{M}$  exerting a significant protective action.

## Chapter 5: DISCUSSION

Baicalein is a major active constituent in the Chinese medicinal herb *Huangqin*. Previous studies showed that it has potential beneficial effects on several chronic diseases. Recent studies showed that baicalein and its glucuronide baicalin may exert beneficial effects on diabetes mellitus or diabetes-related complications (14-16). However, whether and how baicalein exerts anti-diabetic effects is unclear. Loss of functional  $\beta$ -cell mass and  $\beta$ -cell dysfunction are central to the development of diabetes. It is increasingly suggested that chronic hyperglycemia is the leading cause of pancreatic  $\beta$ -cell dysfunction and loss of  $\beta$ -cell mass in vivo, thereby making the major contribution to the deterioration of glycemic control and the overt development of type 2 diabetes (116). Thus, the search for novel agents that promote  $\beta$ -cell survival and thereby preserve functional  $\beta$ -cell mass may provide an effective strategy to prevent the onset of diabetes. In the present study, we found that dietary intake of baicalein can ameliorate hyperglycemia in obese diabetic mice by preserving  $\beta$ -cell mass and protecting  $\beta$ -cell function (Fig 4, 6, 7). In this context, it is tempting to speculate that baicalein may be a plant-derived novel anti-diabetic compound by protecting pancreatic  $\beta$ -cell mass and function.

The reported plasma concentration of baicalein in rats through oral administration could reach to more than 5 $\mu$ M and its glucuronide baicalin could reach 10 $\mu$ M (38; 45). To consider the potential biological relevance of baicalein on  $\beta$ -cell function, the doses of baicalein used in the

present study overlap with the physiologically achievable levels in the plasma following dietary intake. Therefore, our results showing that 5 $\mu$ M baicalein can improve the viability of INS1 and human islet cells and stimulate insulin secretion may be biologically relevant. In our animal study, we fed the mice with a diet containing 0.25g/kg, 0.5g/kg, or 1g/kg baicalein, which are equivalent to the amount of 25mg/kg BW/d to 100mg/kg BW/d, given the food intake of 5g/day/mouse with 50g of individual BW. We chose this dose range because it is similar to the recommended dose taken by humans (117).

Loss of  $\beta$ -cell mass and function play a critical role to the development of both T1D and T2D. Therefore, regeneration or preservation of functional  $\beta$ -cell mass could provide an effective strategy to prevent and treat diabetes. The results in the present study showed baicalein could increase  $\beta$ -cell mass in obese diabetic mice (Fig.7). However, baicalein could not enhance  $\beta$ -cell proliferation by BrdU incorporation assay *in vitro* (Fig.8). One possible reason is that baicalein increases  $\beta$ -cell mass *in vivo* due to protection of  $\beta$ -cells from apoptosis. Moreover, after ingestion, baicalein undergo extensive metabolism in the intestine and liver in the form of glucuronidation (38). Baicalin was more stable in the circulation system than baicalein. Several studies showed the concentration of baicalin was several fold larger than baicalein in the blood stream after oral administration of baicalein (45). Therefore, baicalein metabolites, but not baicalein per se, may exert beneficial effects *in vivo* after ingestion baicalein, which remains to be determined.

It is well characterized that glucose induces insulin secretion through glycolysis and mitochondrial oxidation in  $\beta$ -cells. These events cause the increase in intracellular ATP/ADP ratio, which sequentially leads to closure of  $K_{ATP}$  channels, depolarization of the cell membranes, then activate voltage-dependent  $Ca^{2+}$  channels and consequently trigger  $Ca^{2+}$  influx, and exocytosis of insulin-containing granules (83). Therefore, the effect of baicalein on insulin secretion is at least partially mediated through improving mitochondria metabolism, thereby ATP generation in  $\beta$ -cells (116). Our *in vitro* study showed dose-dependent concentration of baicalein could increase the cell viability in both INS1 cell and human islets (Fig.10). Increased cell viability would produce more nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), leading to enhance mitochondrial function to increase ATP production, which may in turn enhance insulin secretion in  $\beta$ -cells. While the result from the present study show that baicalein treatment improves clonal  $\beta$ -cell and human islets viability, it was still unclear if baicalein could protect  $\beta$ -cell from apoptosis, then preserve  $\beta$ -cell function during the development of diabetes, which need further investigation.

Our results showed that baicalein could significantly stimulate insulin secretion in INS1 cells (Fig.9). However, the mechanism is unclear. It is well established that cAMP signaling plays an important role in normal pancreatic  $\beta$ -cell function including insulin secretion (119). Liu et. al. reported that genistein, a flavonoid from soybean, acutely stimulated insulin secretion in pancreatic  $\beta$ -cell through a cAMP-dependent protein kinase pathway (120). It was shown that baicalein exerts some beneficial effects in intestinal epithelial cells through the cAMP/PKA cascade (121). In addition, baicalein reportedly promotes memory function through NMDA receptor-dependent activation of cAMP response element-binding protein (CREB)

phosphorylation (122). CREB is a transcriptional factor downstream of the cAMP/PKA pathway (123). However, it is unknown whether baicalein can activate the cAMP/PKA pathway in  $\beta$ -cells, leading to insulin secretion.

In summary, we found for the first time as to our knowledge that dietary supplementation of baicalein could significantly ameliorate hyperglycemia and increase blood insulin levels, concomitant with the improved functional islet mass, in obese diabetic mice. In addition, baicalein directly improves  $\beta$ -cell and human islet viability and insulin secretion, suggesting that this natural compound may have a novel anti-diabetic action. Future study should be directed at elucidating the molecular mechanism by which baicalein stimulates insulin secretion and promotes  $\beta$ -cell survival.

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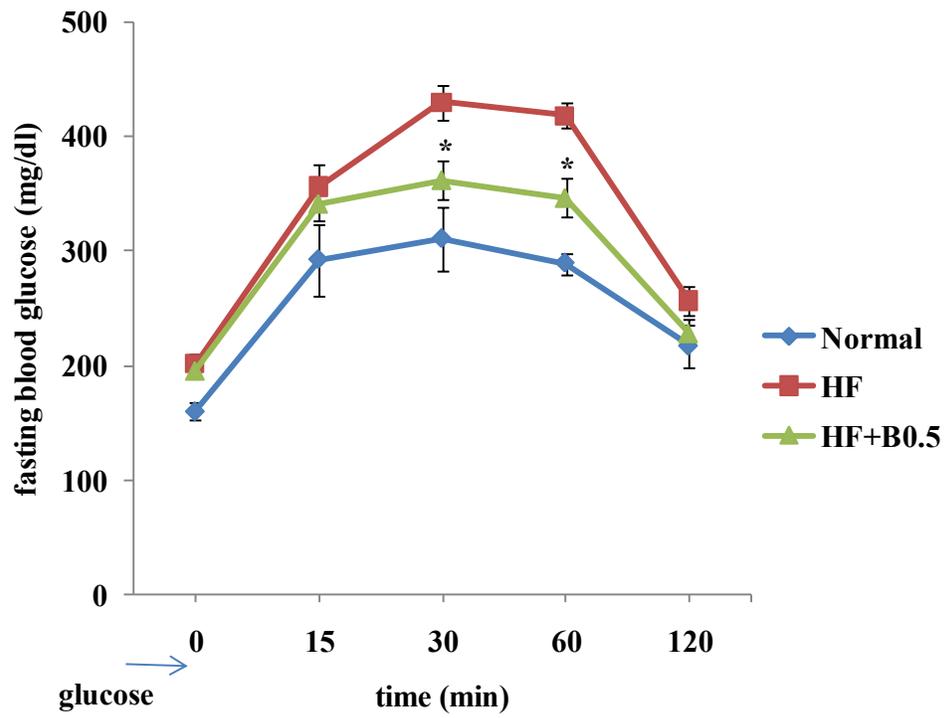
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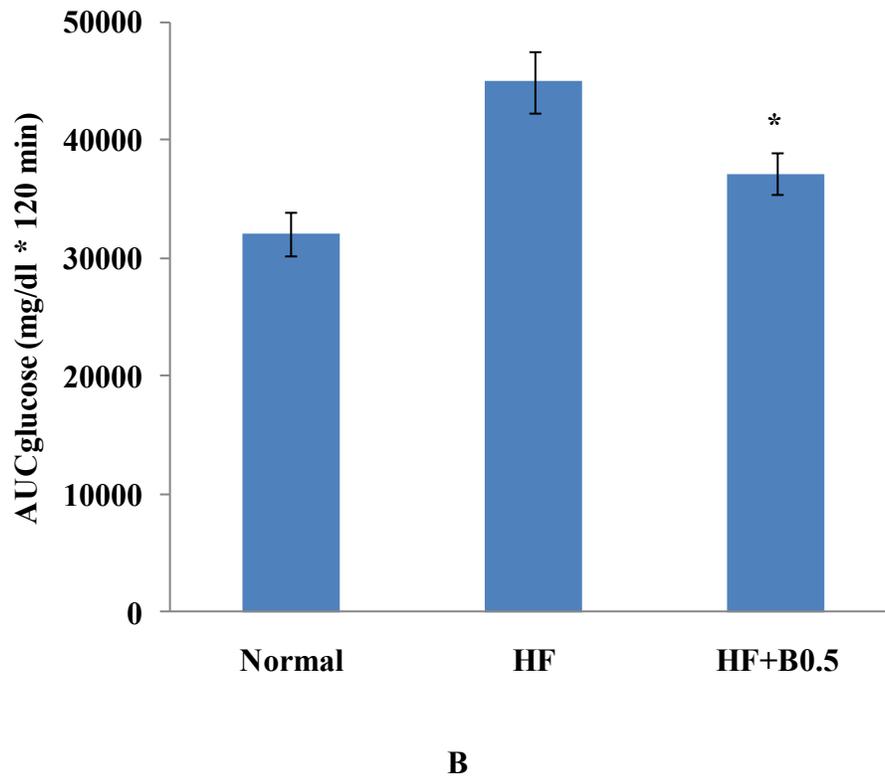
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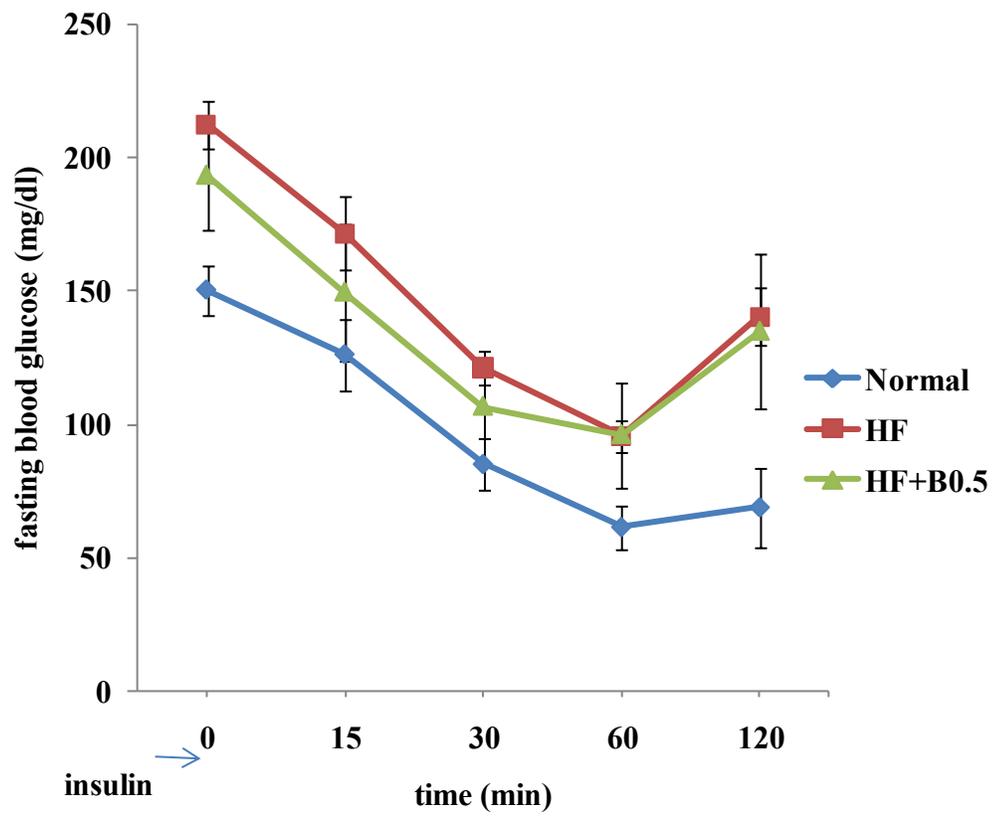
# FIGURES



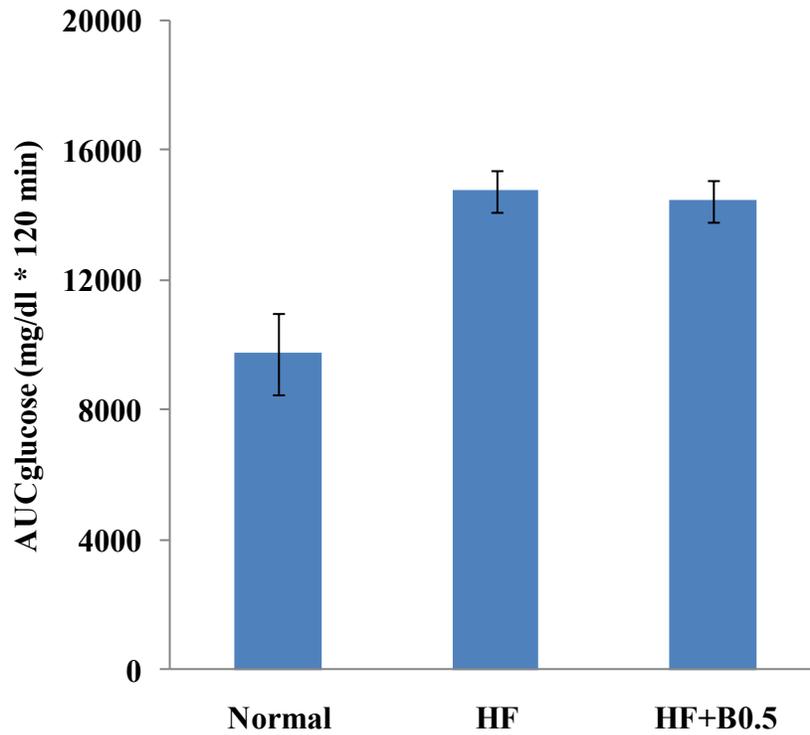
A



**Fig.1. Dietary intake of baicalein improves glucose tolerance in obese mice**      Glucose tolerance test was performed in HF alone treated mice and baicalein-treated mice. The experiment was administered when mice fed high-fat diet for 4 wk. Mice were fasted for 12 h; 1g glucose/kg BW was delivered for each mouse via i.p. injection. Fasting blood glucose (A) was measured at 0, 15, 30, 60 and 120 min after glucose injection.  $AUC_{\text{glucose}}$  (B) was calculated using the trapezoidal rule. Results are means  $\pm$  SE. (n= 10) \*,  $p < 0.05$  vs HF.



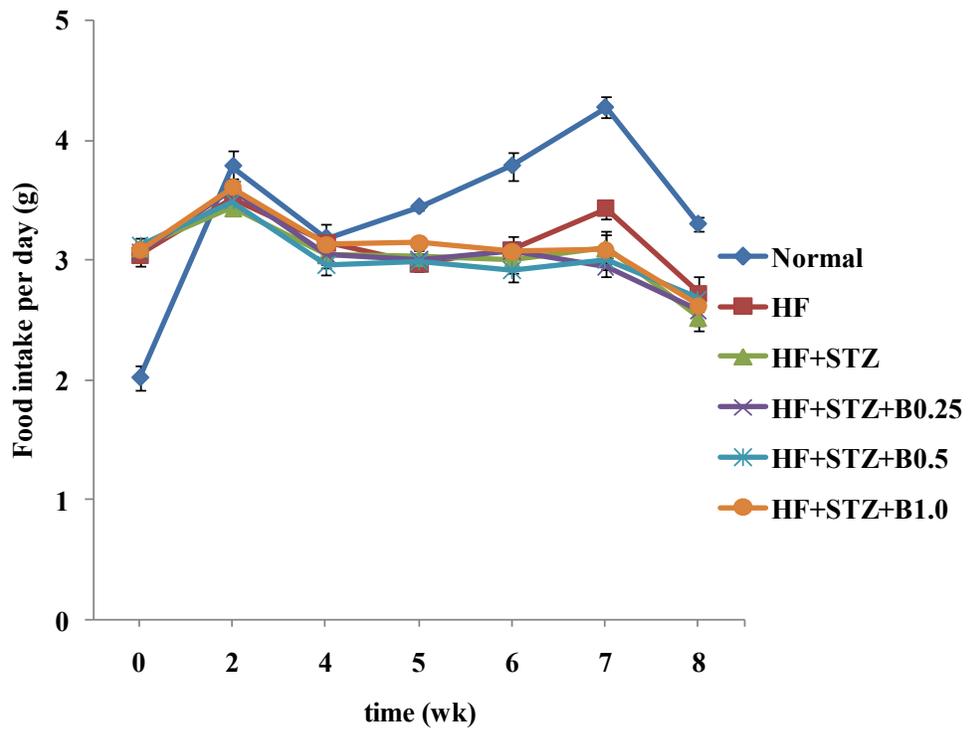
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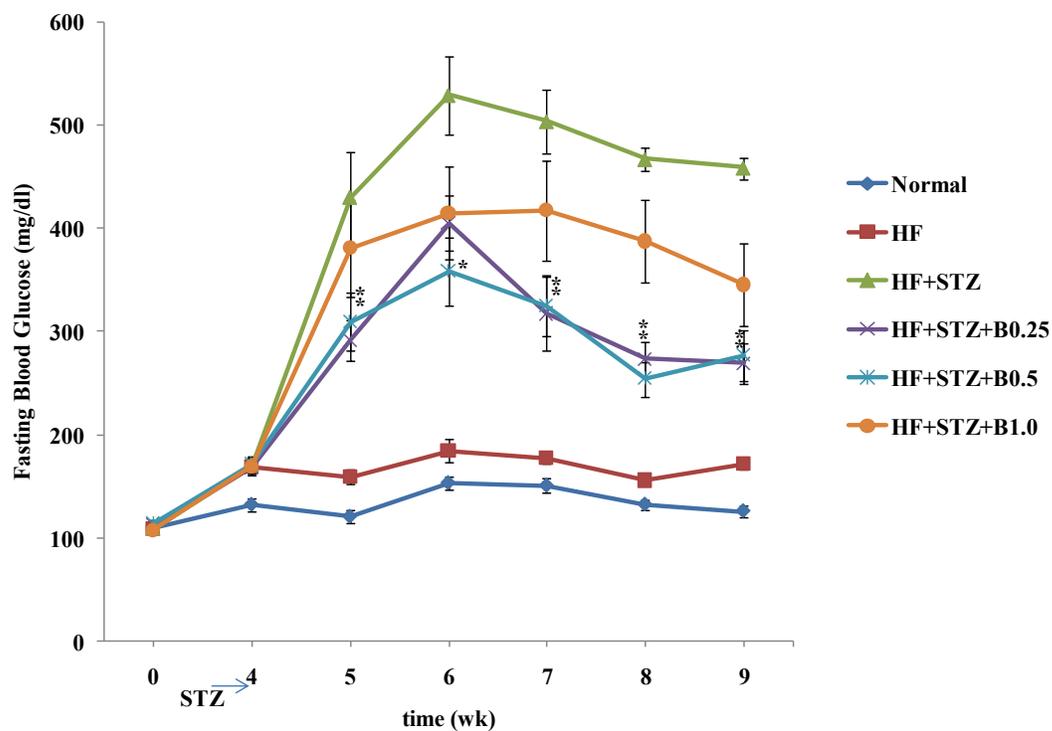
**B**

**Fig.2. Dietary intake of baicalein does not improve insulin sensitivity in obese mice**

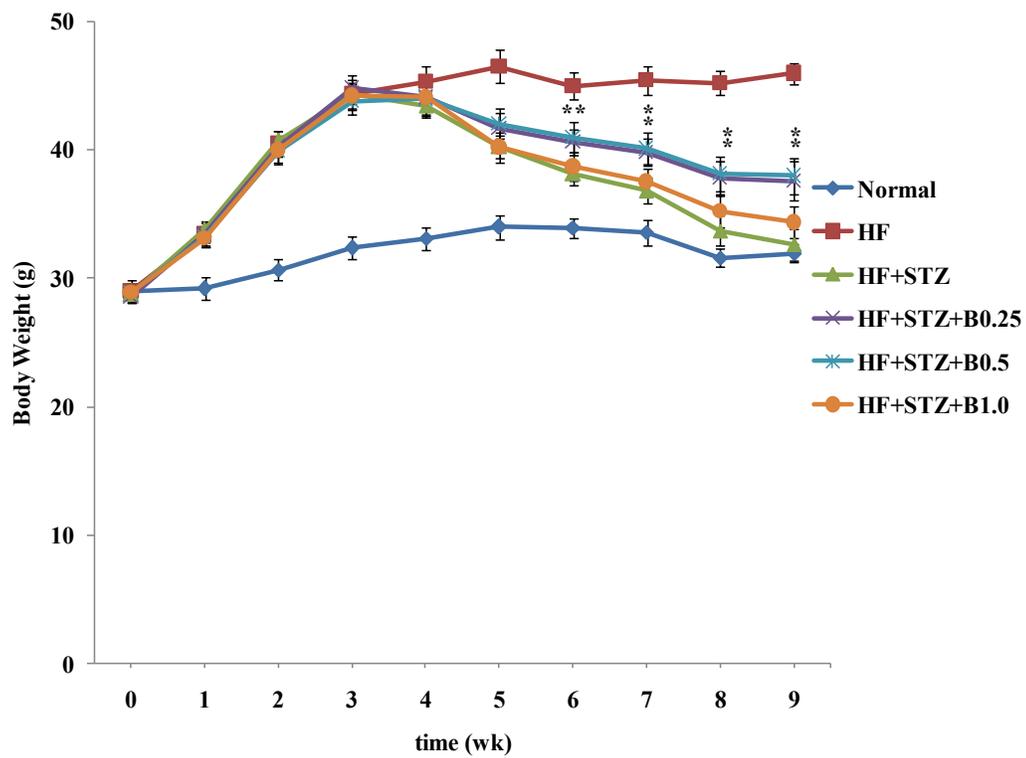
Insulin tolerance test was performed in HF alone treated mice and baicalein-treated mice. The experiment was administered when mice fed high-fat diet for 4 wk. Mice were fasted for 12 h; 0.5 units insulin/kg BW was delivered for each mouse via i.p. injection. Fasting blood glucose (A) was measured at 0, 15, 30, 60 and 120 min after insulin injection.  $AUC_{\text{glucose}}$  (B) was calculated using the trapezoidal rule. Results are means  $\pm$  SE. (n=10)



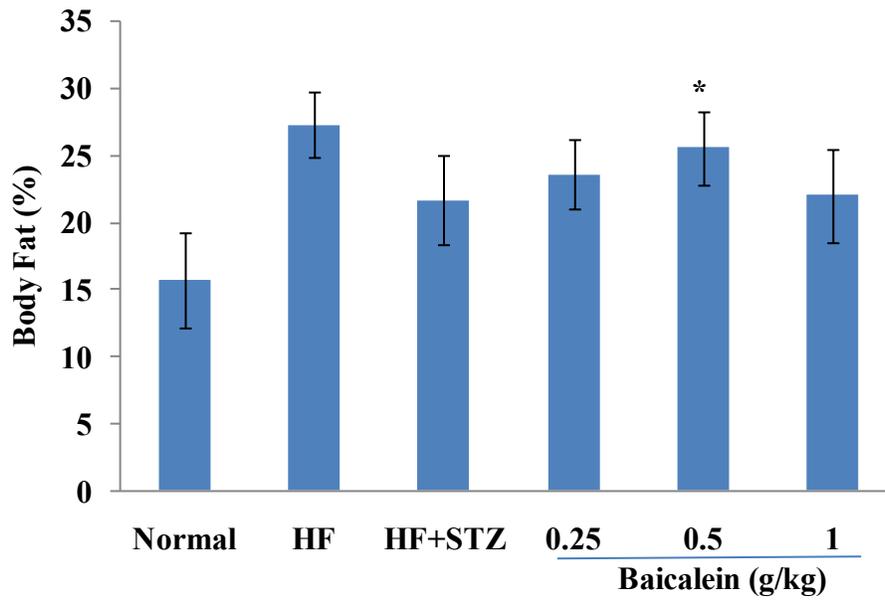
**Fig.3. Baicalein has no effect on food intake in obese diabetic mice** Food intake was monitored in control or treatment group every week. STZ was injected in wk 4. Data were obtained from 10 mice each group.



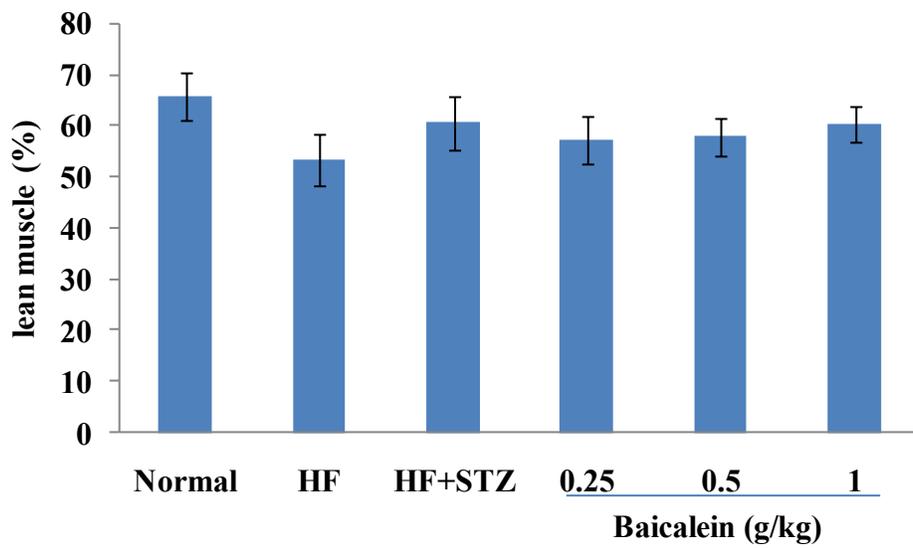
**Fig.4. Dietary intake of baicalein ameliorates hyperglycemia in obese diabetic mice** C57BL/6NCr male mice (8 months old) were fed with baicalein diet (0.25, 0.5, or 1 g/kg diet). Aged-matched normal mice were fed with normal diet. Aged-matched high fat (HF) mice were fed with HF diet without injection STZ. Fasting blood glucose was obtained every week after injection STZ. \*,  $p < 0.05$  vs HF+ STZ.



A

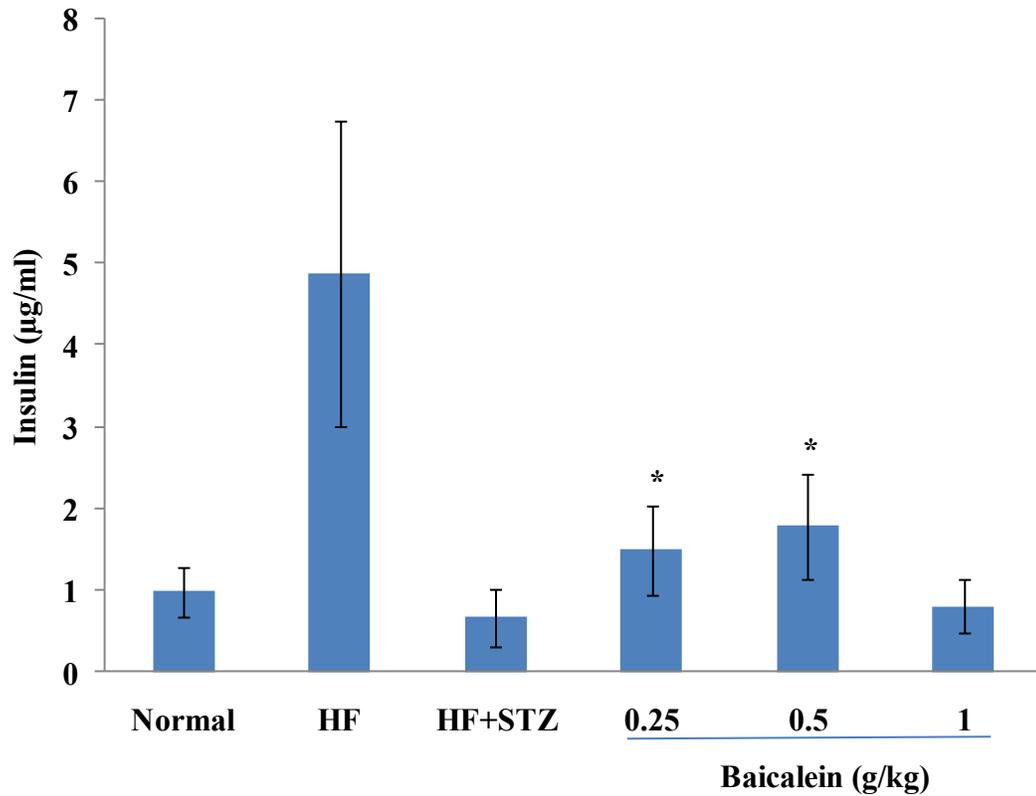


**B**

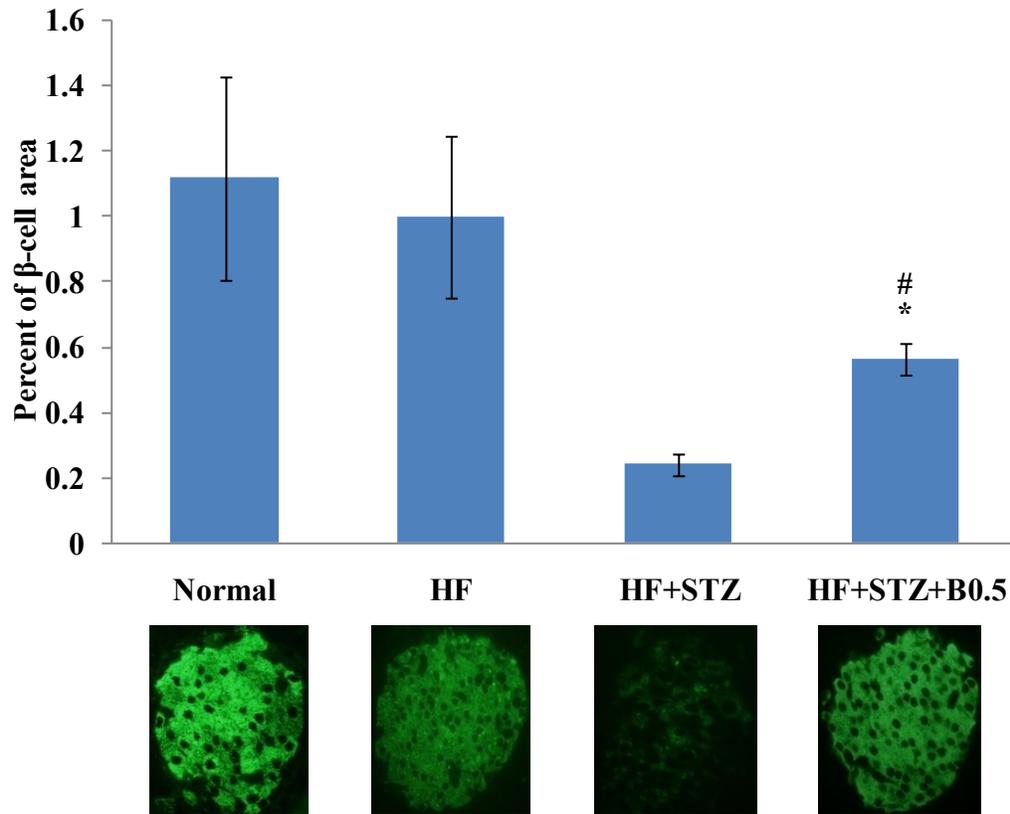


**C**

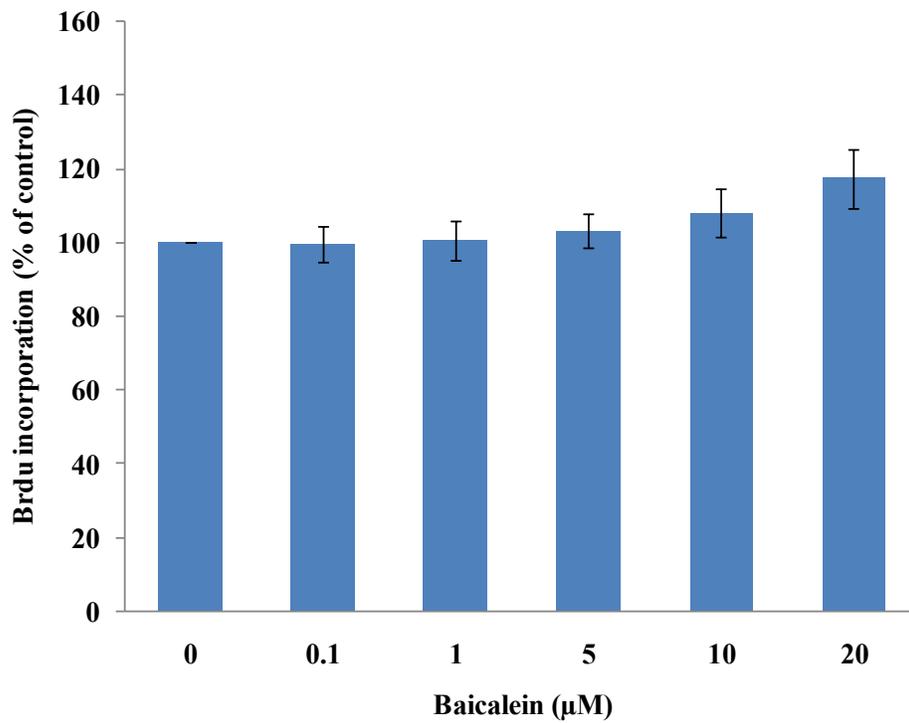
**Fig.5. Dietary baicalein intake prevents body weight and body fat loss in obese diabetic mice** STZ was injected in wk 4. Body weight (A) was monitored in control or treatment group every week. Body fat (B) and muscle mass (C) was monitored two wk after injection STZ. Dietary baicalein ingestion significantly prevented body weight and fat loss. Data were obtained from 10 mice each group. \*,  $p < 0.05$  vs HF+ STZ.



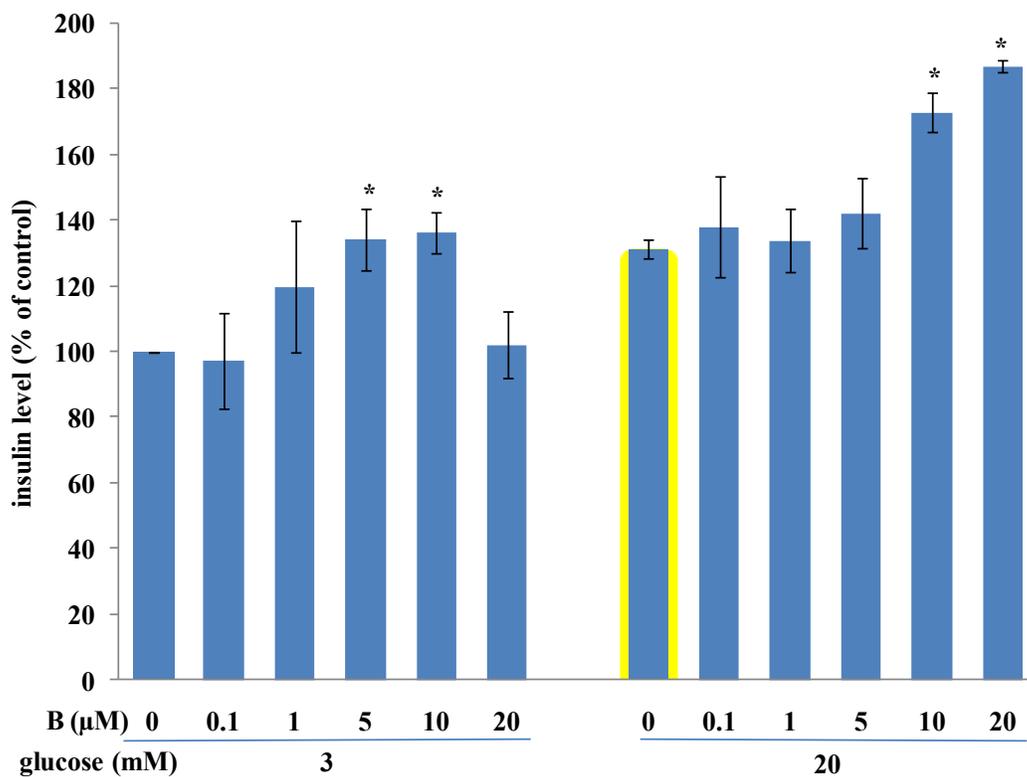
**Fig.6. Dietary baicalein intake improves blood insulin levels in obese diabetic mice**  
 Plasma insulin concentration was determined by ELISA after mice were sacrificed. Insulin level is significantly higher in baicalein-treated group than that of HF+ STZ alone treated mice. Data were obtained from 10 mice each group. \*,  $p < 0.05$  vs HF+ STZ.



**Fig.7. Dietary intake of baicalein improves islet  $\beta$ -cell mass in obese diabetic mice**  
 Pancreatic area sections were stained with insulin antibody after mice were sacrificed. Data were obtained from 10 mice each group. \*,  $p < 0.05$  vs HF+ STZ alone treated mice. #,  $p < 0.05$  vs normal group.



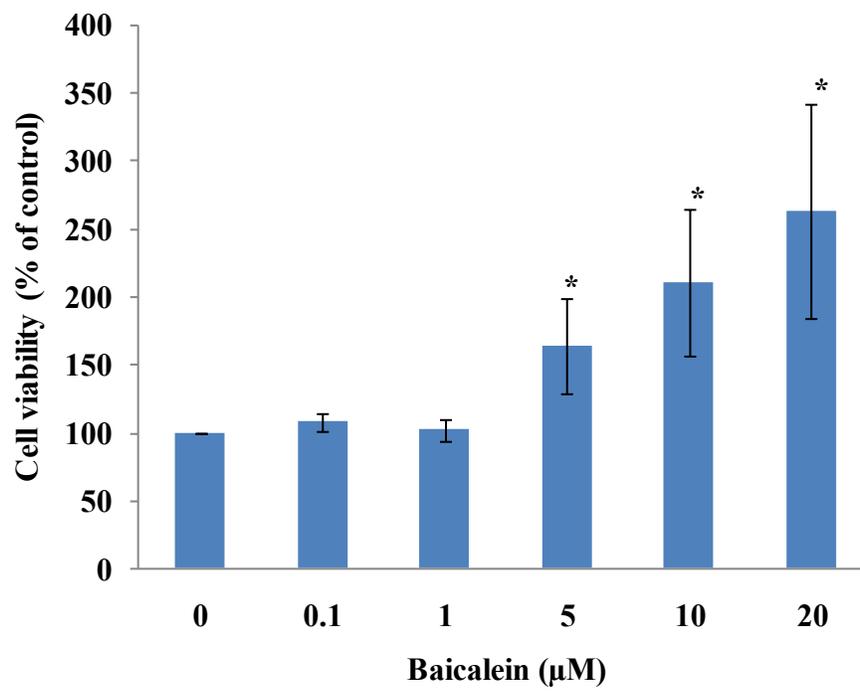
**Fig.8. Baicalein does not enhance  $\beta$ -cell proliferation *in vitro*** INS1 cells were incubated with various concentration of baicalein (0.1, 1, 5, 10, or 20 $\mu$ M) or vehicle in RPMI medium containing 5.5mM glucose and 2% FBS. Cell proliferation was determined by measuring DNA synthesis using BrdU assay. Data were expressed as mean  $\pm$ SE from at least 4 repeats.



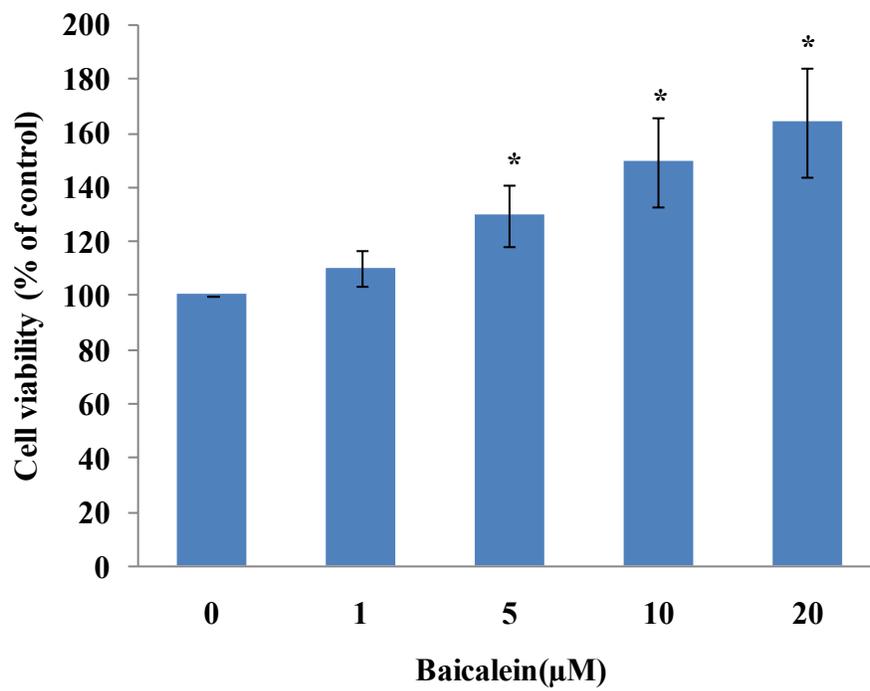
**Fig.9. Baicalein promotes GSIS in INS1 cells**

INS1 cells were incubated in KRBB

buffer with various concentrations of baicalein in the presence of 3mM or 20mM glucose at 37°C for 30min. Each treatment would have 5 repeats. Insulin secreted into supernatants was measured by Elisa. Data were expressed as mean ±SE. Each data was at least 4 repeats. \* P<0.05 vs control.



A



## B

**Fig.10. Baicalein promotes viability of INS1 cells and human islets** Human islets (around 100 islets/well) (A) and INS1 cells (cell density  $10^5$ /ml) (B) were cultured in reduced RPMI (5.5mM glucose, 2% FBS) in the presence of different concentration of baicalein (0.1, 1, 5, 10, or 20  $\mu$ M) or vehicle for 24 h. The results were tested by promega cellTiter 96® aqueous one solution assay. Data were expressed as mean  $\pm$ SE and each data was at least 4 repeats. \*  $p < 0.05$  vs control.