Jiedu Tongluo Tiaogan Formula Protects Pancreatic Islet Cells Against Dysfunction by Relieving Endoplasmic Reticulum Stress and Excessive Autophagy via Regulating CaMKKβ/AMPK Pathway

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Research

Keywords: JTTF, pancreatic β-cells, endoplasmic reticulum stress, excessive autophagy, CaMKKβ/AMPK

DOI: https://doi.org/10.21203/rs.3.rs-797748/v1

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Jiedu Tongluo Tiaogan Formula protects pancreatic islet cells against dysfunction by relieving endoplasmic reticulum stress and excessive autophagy via regulating CaMKKβ/AMPK pathway

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Abstract

**Background:** Endoplasmic reticulum stress (ERS) and excessive autophagy are increasingly recognized as risk factors associated with development and progression of β-cell dysfunction. Jiedu Tongluo Tiaogan Formula (JTTF) has known anti-glucotoxicity activities, but its pharmacological effects on pancreatic cell are not clearly understood. This study was designed to investigate JTTF effects/mechanisms on in vitro glucotoxicity (HG)-induced ERS and excessive autophagic damage of pancreatic cells in vitro and on in vivo pancreatic injury in db/db mice.

**Methods:** The chemical composition of a JTTF preparation were analyzed using high-performance liquid chromatographic fingerprinting. Meanwhile, cell viability, glucose-stimulated insulin secretion, insulin biosynthesis dysfunction, Ca2+ overload, ERS and excessive autophagy were assessed in JTTF-pretreated pancreatic β-cells with HG-induced injury. Hematoxylin and eosin staining and immunohistochemical analyses of pancreatic tissues revealed effects of in vivo JTTF pretreatment on development of HG-induced pancreatic injury in db/db mice.

**Results:** Five JTTF chemical components were identified. Our results revealed that JTTF
treatment protected β-cells from HG injury by increasing insulin biosynthesis and glucose-stimulated insulin secretion (GSIS), while also decreasing Ca\(^{2+}\) overload, ERS and excessive autophagy. Furthermore, protective effects of JTTF treatment against HG-induced β-cell ERS and excessive autophagy were linked to regulation of CaMKKβ/AMPK pathway functions, while JTTF administration as confirmed to reverse pancreatic injury in db/db mice.

**Conclusions:** Collectively, the results presented here indicate that JTTF may prevent islet cell dysfunction in T2DM mice by inhibiting CaMKKβ/AMPK pathway-mediated ERS and excessive autophagy. These findings enhance our understanding of mechanisms underlying beneficial JTTF-induced amelioration of T2DM.

**Keywords:** JTTF, pancreatic β-cells, endoplasmic reticulum stress, excessive autophagy, CaMKKβ/AMPK

**Background**

The prevalence of diabetes mellitus (DM) has increased dramatically in recent decades [1], with the disease becoming a serious public health problem due to its life-threatening nature and complications, such as kidney damage and slow-healing wounds [2]. DM is classified into several disease categories, with type 2 diabetes mellitus (T2DM) most prevalent [3]. Progressive deterioration in β-cells function and marked reduction of β-cells mass are classic findings in islet cells of human T2DM patients [4]. Importantly, numerous studies have demonstrated that long-term exposure of pancreatic β-cells to high glucose (HG) levels induces cell dysfunction and apoptosis, thus impairing insulin secretion function and promoting eventual development of T2DM [5]. Aside from strategies to reduce insulin resistance or promote GSIS by increasing hepatic glucose output, maintenance of healthy pancreatic β cell function is another strategy for preventing T2DM [6], since dysregulated pancreatic β-cells play critical roles in T2DM pathogenesis [7].

Recently, Traditional Chinese Medicine (TCM) has gained attention as an important source of potential bioactive compounds for use in preventing or treating T2DM. [8] TCM is an integral medicinal system based on clinical practice spanning thousands of years that has been shown to effectively treat T2DM, but with fewer side effects than associated with modern medicines. Accordingly, TCM practitioners often prescribe combinations of plant species and/or minerals, referred to as formulae, whereby each formula consist of several herbs working harmoniously together to achieve an ideal therapeutic outcome [9]. As a key TCM treatment modality, formulae have been adopted by academicians and patients for treatment of multiple organ system disorders, particularly chronic diseases and metabolic syndromes [10]. JTTF, which was formulated according to TCM theory, is composed of *Coptis chinensis* Franch (Huanglian), *Radix Rhei Et Rhizome* (Dahuang), *Astragalus propinquus* Schischkin (Huangqi), *Salvia miltiorrhiza* Bunge (Danshen) and *Bupleuri Radix* (Chaihu). In fact, we have administered JTTF formula in our clinic to thousands of patients and have observed beneficial JTTF effects for lowering blood glucose, blood lipids and body weight. Notably, one previous study demonstrated that JTTF could improve insulin resistance and reduce cell apoptosis, while stimulating glucose and lipid metabolism in rats with type 2 diabetes. Although JTTF has been reported to alleviate T2DM via various mechanisms, it is not yet certain whether JTTF can regulate autophagy, restore endoplasmic reticulum function and maintain cell homeostasis. Several mechanisms likely contribute to T2DM development, including endoplasmic reticulum...
stress (ERS), abnormal autophagy, oxidative stress, inflammation, apoptosis and mitochondrial dysfunction [11, 12]. The endoplasmic reticulum (ER) is a system of continuous flattened membranes that serve as sites for protein synthesis, folding, and maturation[13]. However, when certain external stimuli upset ER balance, protein synthesis signaling is interrupted, causing accumulations of unfolded and misfolded proteins within the ER lumen that disrupt homeostasis and ultimately lead to ERS [14, 15]. Importantly, ER homeostasis is essential for maintaining β-cell survival and normal insulin secretion, since islet cells are very sensitive to ERS damage [16]. Previous studies have revealed a strong association between ER stress and impaired glucose homeostasis, indicating a crucial role of homeostatic function in promoting T2DM onset and/or progression. Consequently, if ER stress is sustained long-term and the unfolded protein response (UPR) is not powerful enough to restore normal ER function, the ERS response will be triggered to remove stressed cells[17].

Molecular mechanisms underlying β-cell failure and dysfunction associated with T2DM reportedly result from ERS responses induced by glucolipotoxicity and autophagy dysfunction[18, 19]. Upon initiation of ER stress, cells activate a series of complementary adaptive mechanisms to cope with protein-folding challenges [20]. When these mechanisms fail to remove excess aberrantly folded proteins, ER stress may trigger autophagy dysfunction [21]. Once initiated, autophagy may progress in either direction, depending on stress severity and cell coping mechanisms for invoking survival tactics versus self-destruction via apoptosis, with the latter occurring under unbearable microenvironmental conditions [22]. Nevertheless, the relationship between autophagy and ER stress is not yet fully understood[23]. Recently reported observations suggest that β-cell exposure to high glucose conditions can easily lead to ER stress that can progress to a T2DM state [24]. Currently it is known that excessive calcium ion levels in the ER will activate CaMKKβ, causing calcium ions to be released into the cytoplasm leading to activation of AMPK by AMP. In turn, a high degree of AMPK activation can cause excessive autophagy that results in excessive consumption of integral cellular organelles and components culminating in autophagic apoptosis and cell damage [25].

In this study, we aimed to evaluate the protective effect of JTTF on HG-induced β-cell injury, while also exploring JTTF molecular mechanisms for improving insulin secretion function through inhibition of ER stress and excessive autophagy. Notably, this JTTF protective effect was shown to be mediated by the CaMKKβ-AMPK signal pathway, since addition of an AMP analog and activator of AMPK, AICAR, could abrogate beneficial JTTF effects. Taken together, the results of this study reveal a mechanism underlying JTTF protection of pancreatic β-cells that may provide helpful insights for developing a potential strategy for combatting T2DM.

Materials and Methods
Preparation of JTTF
JTTF is composed of five Chinese herbs, as shown in Table 1. The five herbs were provided by the Department of Pharmacy of the Affiliated Hospital of Changchun University of Chinese Medicine (Jilin, China). The five herbal ingredients were mixed in standard proportions then extracted according to TCM protocols. The filtered liquid was lyophilized then crushed to generate a fine powder. Powdered extracts were stored at -80 °C for future study.

We have established a method for the detection of active ingredients of JTTF via high-performance liquid chromatography (HPLC, Agilent, Santa Clara, CA, US) followed by mass spectrometric analysis performed using an AB Sciex API3200MD mass spectrometer.
connected to a Jasper HPLC System via an electrospray ionization (ESI) interface (Supplementary Fig. 1).

![Supplementary Fig. 1](image)

**Supplementary Fig. 1** JTTF levels were determined using HPLC.

**Cell Culture and Treatments**

The rat insulinoma (INS-1) cell line was purchased from the American Type Culture Collection (Manassas, VA, US). Cells were cultured in RPMI-1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS, Hyclone, USA), 100 U/ml penicillin (Biosharp, Hefei, China) and 100 mg/ml streptomycin (Biosharp) at 37 °C in a humidified atmosphere with 5% CO₂. To establish the high-glucose (HG) injury model, INS-1 cells were treated with 20 mM glucose for 48 h then cells were incubated in the absence or presence of JTTF for 48 h. Next, spent medium (with or without JTTF) was removed and replaced with fresh medium prior to incubation of cells for various periods of time with or without glucose. Next, cells were seeded into 6-, 24-, or 96-well plates depending on the particular assay. Mannitol was used as an osmolality control.

**Cell viability assay**

Cell viability, an indication of JTTF and HG cytotoxicity, was evaluated using an MTT assay. MTT solution (0.5 mg/mL in PBS) was added to each well then plates were incubated for 4 h at 37 °C. Next, 150 μL DMSO was added to each well then absorbance readings (at 570 nm) were recorded using a microplate reader (TECANA-5082, Magellan, Austria).

**Glucose-stimulated insulin secretion (GSIS)**

Cells (1 × 10⁵ cells/well) were treated with HG and/or JTTF for 48 h then were individually transferred to individual wells of V-bottomed 96-well plates. Next, plates were incubated for 60 min at 37 °C in HEPES-balanced Krebs-Ringer (KRH) bicarbonate buffer containing 114 mM
NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.16 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 25.5 mM NaHCO$_3$ and 20 mM HEPES (pH 7.2–7.4) supplemented with 0.2% (w/v) bovine serum albumin (BSA) and 2 mM glucose. The cells were then incubated for an additional 60 min in KRH-bicarbonate buffer containing 5 mM or 20 mM glucose. Subsequently, supernatants were collected to quantify secreted insulin levels using a Rat/Mouse Insulin Elisa Kit (Millipore, USA).

**Quantitative real-time PCR (qRT-PCR)**

INS cells were seeded at a density of $2 \times 10^5$ cells/well in a 6-well plate. Cells were treated with glucose and/or JTTF for 48 h followed by measurements of $INS1$, $INS2$, $PDX1$ and $MafE$ mRNA expression levels. Total RNA from cultured cells was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA). Next, 1 mg of RNA was reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Subsequently, qPCR was performed using a Bio-Rad CFX96 system with cycling parameters of 95 °C for 5 min then 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Primer sequences are listed in Table 2.

**Ca$^{2+}$ fluorescence measurement**

A novel fluorescently labeled calcium indicator, fluo-4 AM, was used to measure changes in INS cell Ca$^{2+}$ levels. Cells cultured in 6-well plates were treated with indicated drugs for 48 h then cells were immediately washed three times with ice-cold PBS and lysed in lysis buffer. Finally, the fluorescence intensity of lysates was detected with a multimode microplate reader (TriStar2, Germany) using an excitation wavelength of 494 nm and an emission wavelength of 516 nm.

**Western blot analysis**

Cell samples were lysed in RIPA buffer (Beyotime Biotechnology) then lysate proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer of proteins to membranes. After blocking with 5% non-fat milk, the membranes were incubated with specific primary antibodies (1:1000) overnight at 4 °C: GRP78 (1:1000, Abcam, ab198787), ATF6 (1:1000, Abcam, ab227830), PERK (1:1000, Abcam, ab229912), p-PERK (1:1000, Abcam, ab192591), IRE1α (1:1000, Abcam, ab37073), p-IRE1α (1:1000, Abcam, ab81936), p-mTOR (1:1000, Abcam, ab32028), mTOR (1:1000, Abcam, ab137133), ULK1 (1:1000, CST, #6439), p-ULK1 (1:1000, CST, #14202), p62 (1:1000, Abcam, ab56416), Beclin-1 (1:1000, Proteintech, No. 11306-1-AP), LC3 (1:1000, Proteintech, No. 14600-1-AP), p-AMPK (1:1000, Abcam, ab23875), AMPK (1:1000, Abcam, ab80039), CaMKKβ (1:1000, CST, #11945), p-CaMKKβ (1:1000, CST, #12716) and GAPDH (1:5,000, Bioworld, BS6007M). After incubation with appropriate secondary antibodies (1:5000) for 1 h at room temperature. Finally, specific bands were detected and quantified using a chemiluminescent imaging system (FluorChem M System, Protein Simple, San Jose, CA, USA). Proteins levels were normalized against the loading control GAPDH.

**Acridine orange (AO) staining**

INS cells were seeded into wells of 24-well plates at a density of $6 \times 10^4$ cells in 1 ml of medium per well overnight. Samples were then treated with glucose and/or JTTF for 48 h. AO (Absin Bioscience, Inc.) solution was added to each well at a concentration of 1 µg/ml, after which samples were incubated at 37°C for 15 min. After rinsing with PBS twice, slides were removed and inverted onto carriers made of sheet glass. Cell slides were then observed and imaged under a confocal microscope (Hitachi, Ltd.). The filter was excited to 488 nm and blocked at 515 nm.

**Animal studies**

To evaluate the effect of JTTF on T2DM-associated disease parameters in vivo, healthy db/db and
db/m male mice (6 weeks old) were divided into four groups with six mice in each group as follows:
Group 1: db/m mice-nondiabetic control mice (received distilled water) via oral gavage for 56 days
Group 2: db/db mice-control type 2 diabetic mice (received distilled water) via oral gavage for 56 days
Group 3: db/db mice receiving 2.4 g/kg/d JTTF via oral gavage for 56 days
Group 4: db/db mice receiving 7.2 g/kg/d JTTF via oral gavage for 56 days

All animal procedures were performed in accordance with approval of the Animal Ethics Committee of Changchun University of Chinese Medicine. During the 56-d course of treatment, body weight was measured once each week and fasting blood glucose (FBG) levels were monitored every two weeks. After 56 d (treatment completion), oral glucose tolerance testing (OGTT) was performed then mice were euthanized and serum was collected from each mouse for lipid analysis. Next, pancreatic tissues were excised, weighed, then fixed to slides for histological assessments. No animals became ill or died during the study period. All animal procedures were performed in accordance with the principles approved by the Animal Ethics Committee of Changchun University of Chinese Medicine.

**Statistical analysis**
Each experiment was repeated at least three times, with results expressed as means ± standard deviations. One-way ANOVA followed by Tukey test was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Multiple comparisons between groups were performed using the S-N-K method. A value of $p<0.05$ was considered significant.

**Results**

**JTTF protects against dysfunctional HG-induced insulin secretion by INS-1 cells.**
Before JTTF effects on insulin secretion were investigated, JTTF cytotoxicity was assessed by treating INS-1 cells with various concentrations (0-500 μg/mL) of JTTF for 24 h or 48 h followed by assessments of viable cell numbers. Treatment with a concentration of JTTF up to 500 μg/mL for 24 h or 48 h did not induce significant cytotoxicity, with cell viability remaining above 90% (Fig. 1a). Next, effects of JTTF treatment on insulin secretion were assessed for glucose-starved cells, with results shown in Fig. 1b. Cells were treated with 5.0 mM low-glucose or 20.0 mM high-glucose concentrations then the quantity of secreted insulin was measured to assess basal insulin secretion or GSIS, respectively. Following the same glucose starvation protocol, after INS-1 cells were stimulated with a high glucose concentration (20 mM), levels of INS-1 cell insulin secretion decreased as compared to the control group level. By contrast, insulin secretion function greatly improved with JTTF treatment.
Fig.1 Effects of JTTF on cell viability and insulin secretion of INS-1 cells exposed to high glucose. (a) After INS-1 cells were exposed to various JTTF concentrations (50, 100, 200 or 500 μg/mL) for 24 h or 48 h, cell viability was determined by MTT assay. (b) GSIS results. Ctrl: control group. *P < 0.05, **P < 0.01, and ***P < 0.001 versus Ctrl group (n = 3), #P < 0.05, ##P < 0.01 and ###P < 0.001 versus HG group (n = 3).

JTTF upregulates insulin biosynthesis and expression of insulin-related genes by INS-1 cells

In T2DM, dysfunctional pancreatic β-cells exhibit reduced insulin secretion, an indicator of disease progression. PDX-1 and MafA genes are involved in development of pancreatic β-cells and transcription of the insulin gene[6]. To further analyze protective effects of JTTF treatment on pancreatic β cells, INS-1 cells were exposed to high-glucose conditions. Subsequently, HG-induced impairments of INS1 and INS2 mRNA expression were reversed in the presence of JTTF, with JTTF treatment leading to increased mRNA expression observed for PDX-1 and MafA, key transcription factor genes that regulate β cell proliferation and insulin expression (Fig. 2a-d). In addition, HG exposure led to significantly reduced expression of PDX-1 and MafA proteins, which were significantly reversed by JTTF treatment (Fig.2e-f). These findings suggested that
JTTF protected INS-1 cells from HG-induced glucotoxicity.

**Fig.2** Effect of JTTF treatment on insulin-related gene expression in INS-1 cells. (a-d) mRNA levels corresponding to transcription-level expression of insulin genes (*INS1* and *INS2*) and transcription factors associated with pancreatic β cell gene expression (*PDX-1*, *MafA*), as determined via qPCR. (e) PDX-1, MafA and GAPDH protein expression levels in INS-1 cells were examined by Western blot analysis. (f) Quantification of expression levels of cleaved PDX-1 and MafA proteins in controls. Values represent mean ± SD derived from three independent experiments. Ctrl: control group. *P < 0.05, **P < 0.01, and ***P < 0.001 versus Ctrl group (n = 3), #P < 0.05, ##P < 0.01 and ###P < 0.001 versus HG group (n = 3).

**JTTF treatment alleviates calcium overload and ER stress after HG-induced injury of INS-1 cells**

Many studies have reported that ER stress-mediated injury caused by intracellular Ca^{2+} overload is involved in the pathogenesis of T2DM [26]. First, we determined whether JTTF treatment-mediated protection against HG-induced pancreatic β-cells injury was associated with
increased intracellular Ca\(^{2+}\) load. Analysis of Ca\(^{2+}\) fluorescence intensity showed that HG exposure enhanced intracellular Ca\(^{2+}\) overloading of INS-1 cells that was subsequently reduced by JTTF treatment (Fig. 3a-b). In addition, to further clarify underlying mechanisms by which JTTF alleviated HG-induced pancreatic β-cell injury, we measured expression levels of ER stress-related proteins. We found that HG exposure induced significantly increased expression of GRP78, ATF6, p-PERK and p-IRE1α, with expression levels significantly reduced by JTTF treatment (Fig. 3c-d). These results suggest that ER stress was fully activated by HG stimulation and was almost fully reversed by JTTF treatment.

**Fig. 3** Effect of JTTF on expression of ER stress indicator proteins in HG-induced INS-1 cells. (a) Intracellular Ca\(^{2+}\) levels of INS-1 cells were monitored using fluorescent Ca\(^{2+}\) indicator Fluo-4 AM. (b) Bar graph represents fluorescence intensity based on Flou-4 AM. (c) After INS-1 cells were subjected to JTTF pretreatment followed by addition of HG, intracellular proteins were assayed using Western blotting, with blots probed with antibodies against GRP78, ATF6, p-PERK/PERK, p-IRE1α/IRE1α and GAPDH. (d) Quantification of expression levels of ER stress-related proteins in controls. Values correspond to means ± SD from three independent experiments. Ctrl:
control group. *P < 0.05, **P < 0.01, and ***P < 0.001 versus Ctrl group (n = 3), †P < 0.05, ‡P < 0.01 and ‡‡P < 0.001 versus HG group (n = 3).

**JTTF decreases excessive autophagy after HG injury in INS-1 cell**

Studies have reported that increases in ER stress might stimulate accelerated autophagy through multiple mechanisms [27]. To confirm whether JTTF treatment inhibited autophagy and protected β-cells from HG-induced injury, we monitored levels of several autophagy-related proteins (mTOR, ULK1, Beclin-1, LC3, p62) in β-cells using Western blot analysis, while concurrently assessing the level of OA-stained autophagosomes in β-cells using immunofluorescence staining. As presented in **Fig. 4a and 4b**, mTOR expression decreased in untreated HG-exposed INS-1 cells (the model group) relative to untreated cells without HG exposure, but significantly increased after JTTF treatment. ULK1 levels increased in the model group, but were significantly reduced after JTTF treatment. Accumulation of Beclin-1, an essential autophagic protein involved in initial stages of autophagy, was increased in the model group, but was significantly decreased after JTTF treatment. Moreover, conversion of LC3I to LC3II, a hallmark of autophagy, was increased in the untreated model group and significantly reduced by JTTF treatment. By contrast, p62 expression was decreased in the untreated model group but was significantly increased after JTTF treatment (**Fig. 4c-d**).

Results of AO staining of autophagosomes confirmed our Western blot results. AO is a specific dye that can enter lysosomes and act as an indicator of lysosomal membrane permeability [28]. As compared with the untreated, non-HG-exposed control group, significantly greater numbers of autophagic vacuoles were observed in mice of the 48-h HG-exposed model group. After JTTF treatment, numbers of autophagic vacuoles decreased with increasing JTTF concentration in a dose-dependent manner as compared to numbers of untreated HG-exposed model group cells (**Fig. 4e-f**).
Fig. 4 JTTF reduced excessive autophagy in the HG-induced INS-1 cell injury model. (a) Western blot analyses of p-mTOR/mTOR and p-ULK1/ULK1 levels normalized to GAPDH level as loading control and (b) densitometric analyses of band intensities normalized to GAPDH level. (c) Western blot analyses of LC3II/ LC3I, p62 and Beclin-1 with GAPDH serving as loading control and (d) densitometric analyses of band intensities normalized to GAPDH. (e) Changes of
autophagosome numbers were detected by AO staining. (f) Bar graph represents fluorescence intensity based on AO staining. Ctrl: control group. *P < 0.05, **P < 0.01, and ***P < 0.001 versus Ctrl group (n = 3), †P < 0.05, ‡P < 0.01 and §§P < 0.001 versus HG group (n = 3).

Involvement of the CaMKKβ-AMPK pathway in JTTF inhibition of ER stress and excessive autophagy in INS-1 cells

Several reported studies have demonstrated that the CaMKKβ-AMPK pathway, a major pathway that regulates pancreatic ER stress and excessive autophagy, plays critical roles in maintaining the balance between ER stress and excessive autophagy associated with T2DM development[29]. To evaluate potential mechanisms whereby JTTF inhibits ER stress and excessive autophagy, the CaMKKβ-AMPK pathway was examined in pancreatic β-cells. Previous studies have shown that when homeostasis is disrupted, phosphorylated AMPK may suppress mTOR activity to release restraints on autophagic activity [30]. Here, Western blot results revealed that HG exposure led to markedly increased expression of p-AMPK, while JTTF treatment significantly decreased p-AMPK level in HG-exposed β-cells. (Fig. 5a-b)

To further confirm that AMPK regulates autophagic activation during HG-induced injury, we used AICAR, an AMPK-specific activator, to study AMPK effects on ER stress and excessive autophagy. Relative to cells exposed to HG alone, HG-exposed cells treated with AICAR exhibited significantly increased p-AMPK levels. Similarly, AICAR treatment significantly decreased intracellular levels of p-mTOR (Fig. 5c-d). These data suggest that the protective effect of JTTF on HG-induced injury was abolished by AMPK activator AICAR. Next, we performed AO staining to determine abundance levels of autophagic vacuoles and to confirm that AO-staining results aligned with our Western blotting results. As compared to cells treated with HG alone, AICAR-treated cells contained significantly more red-stained autophagic vacuoles (Fig. 5e), thus confirming that following HG-induced cell injury, ER stress and excessive autophagy are regulated by upstream CaMKKβ-AMPK signaling.
Fig. 5 JTTF attenuates HG-induced ER stress and excessive autophagy of INS-1 pancreatic cells through CaMKKβ-AMPK signaling. (a) Western blot analyses of p-AMPK/AMPK proteins, with GAPDH used as loading control and (b) densitometric analyses of band intensities normalized to GAPDH. (c) Relative changes in expression of p-AMPK/AMPK and p-mTOR/ mTOR proteins after cell exposure to HG with or without AICAR alone or AICAR combined with JTTF. (d)
Quantitative analysis of p-AMPK/AMPK protein-level expression. Bands were quantified using Image J software after normalization of intensities to GAPDH band intensities. (e) After pretreatment with JTTF and/or AICAR, levels of autophagosomes in INS-1 cells were detected by AO staining. Ctrl: control group. *P < 0.05, **P < 0.01, and ***P < 0.001 versus Ctrl group (n = 3), ^P < 0.05, ^^^P < 0.01 and ^^^^P < 0.001 versus HG group (n = 3).

Effect of JTTF on glucose and lipid metabolism in diabetic mice

No significant differences in body weights of mice across or within groups were detected during the experiment (Fig. 6a). However, we did observe significant effects of JTTF treatment (2.4 and 7.2 mg/kg), which reduced blood glucose and HbA1c levels to levels found in healthy control mice, with beneficial treatment effects accentuated by administration of high-dose JTTF as compared to low-dose JTTF (Fig. 6b-c). Intriguingly, JTTF administration also increased fasting serum insulin level as compared to that of T2DM model mice (Fig. 6d). Notably, it was observed that serum TG, TC, LDL-C and HDL-C levels were significantly higher in db/db mice as compared to levels in normal control group mice. Furthermore, JTTF treatment (2.4 and 7.2 mg/kg) of db/db mice markedly reduced TG, TC and LDL-C levels relative to corresponding levels observed in untreated db/db mice (the control group), while HDL-C levels remained unchanged (Fig. 6e-h).
Fig. 6 JTTF treatment ameliorates hyperglycemia and hyperlipidemia in db/db mice. Determination of body weight, FBG, HbA1c, Fasting serum insulin, TG, TC, LDL-C and HDL-C among control group, model group and administration groups. (M ± SD, n = 6). *P<0.05, **P<0.01, ***P<0.001, vs. normal group; #P<0.05, ##P<0.01, ###P<0.001, vs. T2DM group. HbA1c, glycated hemoglobin; TC, total cholesterol; TG, triglycerides.
JTTF protects against HG-induced β-cell injury

To evaluate effects of JTTF on T2DM indicators, *in vivo* experiments were performed using the db/db mice model, with low and high doses of JTTF administered to db/db mice (n = 6 per group). H&E-stained sections of rat pancreatic tissues from 4 groups are shown in Fig. 7a. H&E staining of pancreatic tissues of T2DM mice demonstrated that ingestion of a high-fat diet severely injured the pancreas, leading to decreased islet cell numbers, diminished pancreatic islet diameters, disordered pancreatic islet structures and appearance of vacuoles and swollen nuclei.

![H&E-stained sections of rat pancreatic tissues from 4 groups](image)

**Fig. 7** Effects of JTTF treatment on H&E staining results in mice. Analysis of H&E staining of biopsied islet cell tissue samples obtained from control, model and JTTF-treated group mice (M ± SD, n = 6, magnification × 20)

**Discussion**

Pancreatic β-cells play a central role in maintaining glucose homeostasis by secreting adequate insulin in response to hyperglycemia. However, chronic hyperglycemia in patients with T2DM causes progressive β-cell damage that leads to deterioration of β-cell insulin secretion function and eventual development of T2DM [5]. Available treatments for T2DM still rely mainly on combinations of oral antidiabetic agents with lifestyle and nutritional adjustments. Despite the continuous development of novel and improved hypoglycemic drugs, these drugs can only ameliorate hyperglycemia or temporarily improve insulin responses in target tissues; they cannot prevent worsening of β-cell dysfunction [31, 32]. By contrast, traditional Chinese formulas composed of multiple herbs act on complex diseases through multiple targets, pathways and biological processes as a suitable strategy for alleviating T2DM. Previous studies have demonstrated that suppression of ER stress and excessive autophagy may protect β-cells from damage in the HG model [33]. In this study, we used spontaneous type 2 diabetic db/db mice, a classical T2DM animal model, since this model develops diabetes spontaneously without need for
additional interventions or synthetic agents[34]. Using this model, we found that blood glucose, HbA1c, body weight and lipid levels were all significantly decreased after 8 weeks of JTTF treatment in a dose-dependent manner, thus indicating that JTTF treatment alleviated a systemic metabolic disorder in diabetic mice. We also researched the effect of JTTF on islet cell function and cell mass in HG-induced pancreatic β-cells. Our results revealed that JTTF treatment preserved β-cell function, probably via CaMKKβ/AMPK-mediated suppression of ER stress and excessive autophagy as a potential experimental mechanism responsible for beneficial JTTF therapeutic effects. (Fig.8)

**Fig.8** Schematic illustration of mechanisms underlying JTTF protection from HG-induced ER stress and excessive autophagy. Epidemiological evidence in humans and recent mechanistic studies in rodents indicate that ERS predisposes pancreatic β-cells to develop pathophysiologic disorders [35]. Due to the functional importance of the ER to the biosynthesis, folding, modification and trafficking of secretory and membrane proteins, it is critically important that β-cells maintain ER homeostasis[36]. When mechanisms that maintain ER homeostasis fail, activation of cellular dysfunction-sensing signaling pathways restore homeostasis[37]. However, excessive long-term ERS results in toxic effects that eventually lead to pancreatic β-cell dysfunction [38]. Meanwhile, the ER, one of the most important calcium storage sites in eukaryotic cells, plays an important role in regulating calcium homeostasis [39]. Another marker of ERS, GRP78, is an important chaperone protein produced during ER stress that under normal physiological conditions can bind stably to three receptor proteins, PERK, IRE1α and ATF-6, with GRP78 itself lacking biological activity[40]. When ERS occurs, PERK, IRE1α and ATF-6 dissociate from GRP78, triggering long-term robust expression of GRP78, PERK, IRE1α and ATF-6 proteins that can cause pancreatic islet cell apoptosis and β-cell dysfunction, with islet β-cell apoptosis contributing to T2DM progression. Importantly, under hyperglycemic conditions, associated, sustained ER stress triggers increases in β-cell levels of intracellular Ca²⁺, with HG-induced dysfunction of ER Ca²⁺ transport contributing to sustained β-cell ER stress [41]. These results thus emphasize the importance of Ca²⁺ levels to β-cell function, insulin action and insulin secretion that are relevant to T2DM development [42].
Furthermore, an increase in cytosolic Ca\(^{2+}\) is associated with insulin secretion disorder and is responsible for Ca\(^{2+}\)/CaMKII hyperphosphorylation, JNK activation, inflammatory signaling and increased hepatic glucose production as well [43]. Here we found that long-term exposure to HG induces ERS in INS-1 cells. Interestingly, JTTF treatment may alleviate HG-induced UPR changes and reduce release of Ca\(^{2+}\) within INS-1 cells in vitro in a dose-dependent manner.

Autophagy flux plays key roles in cellular homeostasis and is characterized by formation of double-membrane structures (autophagosomes) that engulf cytoplasmic material and subsequently fuse with lysosomes, leading to degradation of phagocytized substances. This process is readily enhanced by mild environmental stressors as a beneficial self-defense mechanism under normal conditions. However, it becomes a dysfunctional defense mechanism under conditions of severe stress, resulting in cellular injury that can progress to tissue atrophy and organ pathology [44]. Due to the fact that underexpression or overexpression of autophagy can cause disease, autophagy is rapidly upregulated in response to limited stress stimuli (e.g., hypoxia, nutrient deprivation), thus promoting cell survival. Nevertheless, when the stress is severe or persistent, excessive autophagy is induced, leading to abnormal cell function that can progress to cell senescence and death [45]. Whether autophagy produces adaptive responses to cell survival or initiates programmed cell death depends on the stage of cell growth and types of changes in the extracellular environment [46]. Several studies have shown that excessive autophagy can lead to islet apoptosis-associated β-cell autaphagic injury and death that involve several key autophagy-related genes, with LC3 widely regarded as a critical marker of autophagy initiation [47, 48]. Mechanistically, conversion of LC3-I to LC3-II triggers formation of autophagolysosomes, while p62 facilitates docking of cargo to the cell membrane [49, 50]. Another autophagy-related protein, Beclin-1, may promote autophagosome formation, since autophagy has been shown to be up-regulated by Beclin-1 overexpression. In mammalian cells, the autophagic process is initiated by inactivation of mechanistic/mammalian target of rapamycin (mTOR) [51], while autophagic activation of ULKs complexes contributes to phagophore nucleation [52]. In this study, LC3-II, ULK1 and Beclin-1 expression levels were significantly greater in HG-exposed cells than in control cells, while decreased p62 and mTOR levels were observed after HG-induced injury. Taken together, results obtained for autophagy-related proteins indicated that HG induced high autophagic flux. Moreover, JTTF dose-dependent preconditioning of cells significantly decreased autophagy accumulation relative to that in untreated HG-exposed group cells, thus confirming the protective effect of JTTF against HG induced injury via inhibition of autophagy.

Multiple lines of evidence suggest that phagophores form at ER-mitochondria contact sites during starvation [53, 54]. ERS is a major factor in autophagy activation, and autophagy receptors can mediate autophagy activation. For cells in a pathological diabetic state, a large amount of glucotoxicity harms cells by promoting intracellular accumulations of unfolded and misfolded proteins that thereby trigger the ERS response. In turn, ERS leads to the accumulation of a large amount of cytoplasmic Ca\(^{2+}\), which may activate CaMKKβ and its substrate CaMKK to eventually cause excessive autophagy. Meanwhile, calcium ions, ubiquitous second messengers that mediate various physiological functions, are known to activate AMPK through CaMKKβ [55]. AMPK is now recognized as a key cellular energy sensor that regulates several processes outside of lipid biosynthesis, including glycolysis, protein synthesis and autophagy [56]. At the cellular level, glucose withdrawal can deplete the cell of ATP, resulting in stimulation of AMPK, an upstream
activator of the core autophagic machinery [57]. In response to energy deprivation, AMPK then regulates autophagy induction by triggering signaling of upstream kinases that directly stimulate autophagy-associated proteins. Concurrently, AMPK indirectly regulates the autophagic machinery by releasing it from mTORC1-mediated repression [58]. Thus, the CaMKKβ/AMPK pathway mediates the interaction between ER stress and autophagy pathways to regulate protein synthesis, cell cycle and cell metabolic processes by phosphorylating downstream proteins that modulate cell growth, proliferation, apoptosis and autophagic processes. Therefore, we hypothesize that the CaMKKβ/AMPK pathway is a major target of JTTF action in preventing excessive autophagy of cells subjected to HG-induced injury. In this study, HG exposure increased expression of CaMKKβ and AMPK, indicating that the CaMKKβ/AMPK pathway is activated by HG exposure. Treatment with JTTF increased CaMKKβ and AMPK protein expression levels in β-cells damaged by prior HG exposure. Furthermore, AMPK activator AICAR blocked the effect of JTTF. Taken together, these results suggest that JTTF blocks CaMKKβ to activate the AMPK pathway in pancreatic islet cells injured by HG exposure.

Conclusion

In summary, the findings of this study show that HG activates ERS and excessive autophagy by inhibiting the CaMKKβ/AMPK pathway in pancreatic islet cells after injury due to HG exposure. By contrast, JTTF inhibits ERS and excessive autophagy through the CaMKKβ/AMPK pathway. These findings provide clues regarding the mechanism by which JTTF treatment alleviates T2DM, while also providing a theoretical basis for future JTTF use for the clinical treatment of T2DM. Nevertheless, more investigations are needed to elucidate detailed signaling pathways involved in JTTF-induced inhibition of ERS and excessive autophagy at different time points during HG-induced cellular injury.

Abbreviations

T2DM: Type 2 diabetes mellitus, JDTL: Jiedu Tonghuo Tiaogan Formula, TCM: Traditional Chinese medicine, H&E: Hematoxylin and eosin, DM: diabetes mellitus, GSIS: glucose-stimulated insulin secretion, URP: unfolded protein response, HPLC: high-performance liquid chromatography, FBG: fasting blood glucose.

Acknowledgments

We appreciate the financial support received from Shenzhen Science and Technology Innovation Program (grant number: JCY20190809110015528) and the National Natural Science Foundation of China (grant number: 81973813).

Authors’ contributions

QZ, CLP and LWS conceived and designed the experiments. QZ, CLP, WQJ and HW performed the research. CT, XHZ and NWZ analyzed the data. YW, NWZ and SNG wrote the paper. QZ, CLP and WQJ drafted the manuscript. QZ, CLP and LWS revised the manuscript. All authors gave the final approval and agreed to be accountable for all aspects of the work.

Funding

This study was supported by Shenzhen Science and Technology Innovation Program (grant number: JCY20190809110015528) and the National Natural Science Foundation of China (grant number:81973813).

Availability of data and materials

The raw data supporting the conclusions of this article will be made available by the authors,
without undue reservation, to any qualified researcher.

**Competing interests**

All authors declare no conflicts of interest.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

Not applicable.

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**Table 1** The Chinese herb drugs contained in Jiedu Tongluo Tiaogan Formula.

| Latin name | Chinese name | English name in Chinese pharmacopoeia (Ch.P.) | Plant part (s), processing | Weight (g) | Voucher numbers |
|------------|--------------|----------------------------------------------|-----------------------------|------------|----------------|
| Coptis chinensis Franch | Huanglian | Coptidis Rhizoma | Root | 15 | CM0715 |
| Radix Rhei Et Rhizome | Dahuang | Rhei Radix Et Rhizoma | Root | 9 | CM0728 |
| Astragalus propinquus Schischkin | Huangqi | Astragaliradix | Root | 15 | CM0774 |
| Salvia miltiorrhiza Bunge | Danshen | Salviae Miltiorrhizae Radix Et Rhizoma | Root and rhizome | 15 | CM0739 |
| Bupleuri Radix | Chaihu | Bupleuri Radix | Root | 10 | CM0727 |

**Table 2** Primer sequences for gene expression.

| Primer | Forward sequence (5’-3’) | Reverse sequence (5’-3’) |
|--------|--------------------------|--------------------------|
| INS1   | CCATCAGCAAGCAGGTCA        | CCACACACCAGTGAGAGGC       |
| INS2   | CCTGCTGGCCCTGCTTT         | GGCTGGGTAGTGGGTGGTCTTA    |
| PDX1   | TCCACCACACCTCCAGCTCA      | AATTCCTTTCCAGCTCCAG       |
| MafA   | ATCATCAGCTCCACCACCAT      | AGTCGAGTGACCTCCCTCTTT     |
| GAPDH  | TGGTATCGTGGAAGGACTCA      | CCAGTAGAGGCAGGATGAT       |
References

Uncategorized References

1. Rana JS, Khan SS, Lloyd-Jones DM, Sidney S: Changes in Mortality in Top 10 Causes of Death from 2011 to 2018. *J Gen Intern Med* 2020.

2. Deshpande AD, Harris-Hayes M, Schootman M: Epidemiology of diabetes and diabetes-related complications. *Phys Ther* 2008, 88(11):1254-1264.

3. Sun-Wang JL, Yarritu-Gallego A, Ivanova S, Zorzano A: The ubiquitin-proteasome system and autophagy: self-digestion for metabolic health. *Trends Endocrinol Metab* 2021.

4. Wang Z, York NW, Nichols CG, Remedi MS: Pancreatic beta cell dedifferentiation in diabetes and redifferentiation following insulin therapy. *Cell Metab* 2014, 19(5):872-882.

5. Wang Y, Xue J, Li Y, Zhou X, Qiao S, Han D: Telmisartan protects against high glucose/high lipid-induced apoptosis and insulin secretion by reducing the oxidative and ER stress. *Cell Biochem Funct* 2019, 37(3):161-168.

6. Lee D, Lee JS, Sezirahiga J, Kwon HC, Jang DS, Kang KS: Bioactive Phytochemicals Isolated from Akebia quinata Enhances Glucose-Stimulated Insulin Secretion by Inducing PDX-1. *Plants (Basel)* 2020, 9(9).

7. Lee D, Qi Y, Kim R, Song J, Kim H, Kim HY, Jang DS, Kang KS: Methyl Caffeate Isolated from the Flowers of Prunus persica (L.) Batsch Enhances Glucose-Stimulated Insulin Secretion. *Biomolecules* 2021, 11(2).

8. He S, Zhao J, Xu X, Cui X, Wang N, Han X, Guo Y, Liu Q: Uncovering the Molecular Mechanism of the Qiang-Xin 1 Formula on Sepsis-Induced Cardiac Dysfunction Based on Systems Pharmacology. *Oxid Med Cell Longev* 2020, 2020:3815185.

9. Wang Y, Yang H, Chen L, Jafari M, Tang J: Network-based modeling of herb combinations in traditional Chinese medicine. *Brief Bioinform* 2021.

10. Han X, Yang Y, Metwaly AM, Xue Y, Shi Y, Dou D: The Chinese herbal formulae (Yitangkang) exerts an antidiabetic effect through the regulation of substance metabolism and energy metabolism in type 2 diabetic rats. *J Ethnopharmacol* 2019, 239:111942.

11. Perreault L, Skyler JS, Rosenstock J: Novel therapies with precision mechanisms for type 2 diabetes mellitus. *Nat Rev Endocrinol* 2021, 17(6):364-377.

12. Yong J, Johnson JD, Arvan P, Han J, Kaufman RJ: Therapeutic opportunities for pancreatic beta-cell ER stress in diabetes mellitus. *Nat Rev Endocrinol* 2021, 17(8):455-467.

13. Hetz C, Chevet E, Harding HP: Targeting the unfolded protein response in disease. *Nat Rev Drug Discov* 2013, 12(9):703-719.

14. Almanza A, Carlesso A, Chinthu C, Creedican S, Doultsinos D, Leuzzi B, Luis A, McCarthy N, Montibeller L, More S et al: Endoplasmic reticulum stress signalling - from basic mechanisms to clinical applications. *FEBS J* 2019, 286(2):241-278.

15. Qu J, Zeng C, Zou T, Chen X, Yang X, Lin Z: Autophagy Induction by Trichodermic Acid Attenuates Endoplasmic Reticulum Stress-Mediated Apoptosis in Colon Cancer Cells. *Int J Mol Sci* 2021, 22(11).

16. Hu Y, Liu J, Yuan Y, Chen J, Cheng S, Wang H, Xu Y: Sodium butyrate mitigates type 2 diabetes by inhibiting PERK-CHOP pathway of endoplasmic reticulum stress. *Environ Toxicol Pharmacol* 2018, 64:112-121.

17. Xu C, Bailly-Maitre B, Reed JC: Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 2005, 115(10):2656-2664.
Cnop M, Ladriere L, Igoillo-Esteve M, Moura RF, Cunha DA: **Causes and cures for endoplasmic reticulum stress in lipotoxic beta-cell dysfunction.** *Diabetes Obes Metab* 2010, **12** Suppl 2:76-82.

Rivera JF, Costes S, Gurlo T, Glabe CG, Butler PC: **Autophagy defends pancreatic beta cells from human islet amyloid polypeptide-induced toxicity.** *J Clin Invest* 2014, **124**(8):3489-3500.

Yorimitsu T, Nair U, Yang Z, Kilonsky DJ: **Endoplasmic reticulum stress triggers autophagy.** *J Biol Chem* 2006, **281**(40):30299-30304.

Chino H, Mizushima N: **ER-Phagy: Quality Control and Turnover of Endoplasmic Reticulum.** *Trends Cell Biol* 2020, **30**(5):384-398.

Doherty J, Baehrecke EH: **Life, death and autophagy.** *Nat Cell Biol* 2018, **20**(10):1110-1117.

Bao Y, Pu Y, Yu X, Gregory BD, Srivastava R, Howell SH, Bassham DC: **IRE1B degrades RNAs encoding proteins that interfere with the induction of autophagy by ER stress in Arabidopsis thaliana.** *Autophagy* 2018, **14**(9):1562-1573.

Fernandes-da-Silva A, Miranda CS, Santana-Oliveira DA, Oliveira-Cordeiro B, Rangel-Azevedo C, Silva-Veiga FM, Martins FF, Souza-Mello V: **Endoplasmic reticulum stress as the basis of obesity and metabolic diseases: focus on adipose tissue, liver, and pancreas.** *Eur J Nutr* 2021.

Han JY, Li Q, Ma ZZ, Fan JY: **Effects and mechanisms of compound Chinese medicine and major ingredients on microcirculatory dysfunction and organ injury induced by ischemia/reperfusion.** *Pharmacol Ther* 2017, **177**:146-173.

Jia T, Wang YN, Feng Y, Wang C, Zhang D, Xu X: **Pharmac Activation of PKG2 Alleviates Diabetes-Induced Osteoblast Dysfunction by Suppressing PLCbeta1-Ca(2+)-Mediated Endoplasmic Reticulum Stress.** *Oxid Med Cell Longev* 2021, **2021**:552530.

Rocha M, Apostolova N, Diaz-Rua R, Muntane J, Victor VM: **Mitochondria and T2D: Role of Autophagy, ER Stress, and Inflammasome.** *Trends Endocrinol Metab* 2020, **31**(10):725-741.

Feng J, Chen X, Sun X, Wang F, Sun X: **Expression of endoplasmic reticulum stress markers GRP78 and CHOP induced by oxidative stress in blue light-mediated damage of A2E-containing retinal pigment epithelium cells.** *Ophthalmic Res* 2014, **52**(4):224-233.

Hu YX, Han XS, Jing Q: **Ca(2+) Ion and Autophagy.** *Adv Exp Med Biol* 2019, **1206**:151-166.

Dai SH, Chen T, Li X, Yue KY, Luo P, Yang LK, Zhu J, Wang YH, Fei Z, Jiang XF: **Sirt3 confers protection against neuronal ischemia by inducing autophagy: Involvement of the AMPK-mTOR pathway.** *Free Radic Biol Med* 2017, **108**:345-353.

Gomes A, Coelho P, Soares R, Costa R: **Human umbilical cord mesenchymal stem cells in type 2 diabetes mellitus: the emerging therapeutic approach.** *Cell Tissue Res* 2021.

Tahrani AA, Barnett AH, Bailey CJ: **Pharmacology and therapeutic implications of current drugs for type 2 diabetes mellitus.** *Nat Rev Endocrinol* 2016, **12**(10):566-592.

Zhang Y, Jiao Y, Tao Y, Li Z, Yu H, Han S, Yang Y: **Monobutyl phthalate can induce autophagy and metabolic disorders by activating the ire1a-xbp1 pathway in zebrafish liver.** *J Hazard Mater* 2021, **412**:125243.

Sullivan MA, Harcourt BE, Xu P, Forbes JM, Gilbert RG: **Impairment of Liver Glycogen Storage in the db/db Animal Model of Type 2 Diabetes: A Potential Target for Future Therapeutics?** *Curr Drug Targets* 2015, **16**(10):1088-1093.

Shrestha N, De Franco E, Arvan P, Cnop M: **Pathological beta-Cell Endoplasmic Reticulum...**
Stress in Type 2 Diabetes: Current Evidence. Front Endocrinol (Lausanne) 2021, 12:650158.

36. Hao Y, Shen S, Yin F, Zhang Y, Liu J: Unfolded protein response is involved in geniposide-regulating glucose-stimulated insulin secretion in INS-1 cells. Cell Biochem Funct 2019, 37(5):368-376.

37. Strzyz P: Unfolded protein response: Pro-survival clock sUPRe ssion. Nat Rev Mol Cell Biol 2018, 19(2):74-75.

38. Hu Y, Gao Y, Zhang M, Deng KY, Singh R, Tian Q, Gong Y, Pan Z, Liu Q, Boisclair YR et al: Endoplasmic Reticulum-Associated Degradation (ERAD) Has a Critical Role in Supporting Glucose-Stimulated Insulin Secretion in Pancreatic beta-Cells. Diabetes 2019, 68(4):733-746.

39. Ahumada-Castro U, Puebla-Huerta A, Cuevas-Espinoza V, Lovy A, Cesar Cardenas J: Keeping zombies alive: The ER-mitochondria Ca(2+) transfer in cellular senescence. Biochim Biophys Acta Mol Cell Res 2021:119099.

40. Elfiky AA, Baghdady AM, Ali SA, Ahmed MI: GRP78 targeting: Hitting two birds with a stone. Life Sci 2020, 260:118317.

41. Sivitz WI, Yorek MA: Mitochondrial dysfunction in diabetes: from molecular mechanisms to functional significance and therapeutic opportunities. Antioxid Redox Signal 2010, 12(4):537-577.

42. Wang CH, Wei YH: Role of mitochondrial dysfunction and dysregulation of Ca(2+) homeostasis in the pathophysiology of insulin resistance and type 2 diabetes. J Biomed Sci 2017, 24(1):70.

43. Maeda A, Shirao T, Shirasaya D, Yoshioka Y, Yamashita Y, Akagawa M, Ashida H: Piperine Promotes Glucose Uptake through ROS-Dependent Activation of the CAMKK/AMPK Signaling Pathway in Skeletal Muscle. Mol Nutr Food Res 2018, 62(11):e1800086.

44. Zhang Q, Feng Z, Lu J, Lu J, Guan S, Chen Y: Aflatoxin B1 inhibited autophagy flux by inducing lysosomal alkalization in HepG2 cells. Toxicol Mech Methods 2021, 31(6):450-456.

45. New J, Thomas SM: Autophagy-dependent secretion: mechanism, factors secreted, and disease implications. Autophagy 2019, 15(10):1682-1693.

46. Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A: Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death Differ 2009, 16(7):966-975.

47. Bachar-Wikstrom E, Wikstrom JD, Ariav Y, Tirosh B, Kaiser N, Cerasi E, Leibowitz G: Stimulation of autophagy improves endoplasmic reticulum stress-induced diabetes. Diabetes 2013, 62(4):1227-1237.

48. Zhu YN, Fan WJ, Zhang C, Guo F, Li W, Wang YF, Jiang ZS, Qu SL: Role of autophagy in advanced atherosclerosis (Review). Mol Med Rep 2017, 15(5):2903-2908.

49. Nussenzweig SC, Verma S, Finkel T: The role of autophagy in vascular biology. Circ Res 2015, 116(3):480-488.

50. Hassanpour M, Rahbarghazi R, Nouri M, Aghamohammadzadeh N, Safaei N, Ahmadi M: Role of autophagy in atherosclerosis: foe or friend? J Inflamm (Lond) 2019, 16:8.

51. Ravanant P, Srikanth IF, Talwar P: Autophagy: The spotlight for cellular stress responses. Life Sci 2017, 188:53-67.

52. Hurley JH, Young LN: Mechanisms of Autophagy Initiation. Annu Rev Biochem 2017, 86:225-244.

53. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, Lippincott-Schwartz J: Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell
54. Hamasaki M, Furuta N, Matsuda A, Nezu A, Yamamoto A, Fujita N, Oomori H, Noda T, Haraguchi T, Hiraoka Y et al: Autophagosomes form at ER-mitochondria contact sites. Nature 2013, 495(7441):389-393.

55. Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D: Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab 2005, 2(1):21-33.

56. Jeon SM: Regulation and function of AMPK in physiology and diseases. Exp Mol Med 2016, 48(7):e245.

57. Hardie DG, Ross FA, Hawley SA: AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol 2012, 13(4):251-262.

58. King KE, Losier TT, Russell RC: Regulation of Autophagy Enzymes by Nutrient Signaling. Trends Biochem Sci 2021, 46(8):687-700.