

Sustained antidiabetic effects of a berberine-containing Chinese herbal medicine through regulation of hepatic gene expression

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Running title: Antidiabetic effects of TCM through regulation of gene expression

Word count: 3950 (text), 194 (abstract)

4 figures and 2 tables

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Abbreviations: Akt, Protein Kinase B; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CCO, cytochrome c oxidase; CYP7a1 (cytochrome P450, family 7, subfamily a, polypeptide 1; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IGFBP1, insulin-like growth factor binding protein 1; ITT, insulin tolerance test; OGTT, oral glucose tolerance test; SREBP, sterol regulatory element binding protein; ZDF, Zucker diabetic fatty rat.

Abstract

Diabetes and obesity are complex diseases associated with insulin resistance and fatty liver. The latter is characterised by dysregulation of the Akt, AMPK and IGF-1 pathways and expression of microRNAs. In China, multi-component Traditional Chinese Medicine (TCM) has been used to treat diabetes for centuries. In this study, we used a 3-herb, berberine-containing TCM to treat male Zucker diabetic fatty rats. TCM showed sustained glucose-lowering effects for 1 week after a single-dose treatment. Two-week treatment attenuated insulin resistance with disappearance of fatty degeneration and hepatocyte regeneration lasting for 1 month post-treatment. These beneficial effects persisted for 1 year after 1-month treatment. Two-week treatment with TCM was associated with activation of AMPK, Akt and IGFBP1 pathways with downregulation of miR29-b and expression of a gene network implicated in cell cycle, intermediary and NADPH metabolism with normalization of CYP7a1 and IGFBP1 expression. These concerted changes in miRNA, RNA and proteins may explain the sustained effects of TCM in favor of cell survival, increased glucose uptake and lipid oxidation/catabolism with improved insulin sensitivity and liver regeneration. These novel findings suggest that multi-component TCM may be a useful tool to unravel genome regulation and expression in complex diseases.

Keywords: Liver; Traditional Chinese medicine; berberine; diabetes; insulin resistance; AMP-activated protein kinase; microRNA; microarray; Zucker diabetic fatty rat

INTRODUCTION

Diabetes and metabolic syndrome are complex chronic diseases. For millennia, traditional medicines and natural products have been used for therapeutic and nutritional purposes (1). Metformin is the mainstay treatment in the control (2) and prevention (3) of diabetes and associated co-morbidities (4). It was synthesized based on the blood-glucose-lowering effect of isoamylene guanidine in *Galega officinalis* (5). Metformin exhibits multiple therapeutic effects including improved insulin sensitivity through activation of AMP-activated protein kinase (AMPK) (6). *Coptis chinensis* Franch is a popular herb used by Traditional Chinese Medicine (TCM) practitioners to treat diabetes containing berberine with metformin-like actions including dual effects on AMPK (7) and mitochondrial respiratory chain complex I (8).

Recent studies have uncovered regulatory mechanisms of DNA translation and expression in complex diseases such as diabetes and obesity (9). MicroRNAs (miRNAs) are small non-coding tissue-specific RNAs which bind to regulatory sites of target mRNA resulting in either degradation and/or translational repression with decreased protein synthesis (10). These non-coding RNAs may play important pathophysiological roles in regulation of energy metabolism (11-13). Overexpression of hepatic miRNAs has been reported in diabetic obese rodents and insulin-resistant adipocytes (14-16). Natural compounds might modulate these epigenetic phenomena to change expression of gene networks (9). Given their pluripotent effects, we hypothesize that multi-component TCM may alter gene expression to influence multiple phenotypic traits in diabetes and obesity. We used validated animal models (Zucker diabetic fatty (ZDF) rats) and molecular tools (microarrays, miRNAs and Western blot) to investigate the antidiabetic effects of a berberine-containing TCM and its regulation of hepatic gene expression.

MATERIALS AND METHODS

The study was approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. The animal experiments were conducted in accordance to the Animals (Control of Experiments) Ordinance of the Department of Health of the Hong Kong SAR Government.

Plant materials and sample preparation

Based on literature search, *Coptis chinensis* Franch, *Astragalus membranaceus* and

Lonicera japonica are popular herbs used in diabetes for thousands of years. One of the most popular formulae, referred to as JCU (Supplementary figure 1), contains *Rhizoma coptidis* (JCU-1), *Radix astragali* (JCU-2) and *Flos loniceræ* (JCU-3) at a ratio of 1 : 1.5 : 6. JCU-1, known as *huanglian* in Chinese *pinyin*, is the dried root and stem of *Coptis chinensis* Franch; JCU-2, known as *huangqi* in Chinese *pinyin*, is the dried root of *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao; and JCU-3, known as *jinyinhua* in Chinese *pinyin*, is the dried flower of *Lonicera japonica* Thunb. All three herbs were authenticated with high performance liquid chromatography (HPLC) analysis using phytochemical markers (17), which demonstrated 3.6% berberine in JCU-1, 0.040% astragaloside IV in JCU-2, and 1.5% chlorogenic acid and 0.10% luteolin in JCU-3.

According to Chinese pharmacopeia, the raw ingredients of JCU include 60g of JCU-1, 90g of JCU-2, and 360g JCU-3. We used validated methods of aqueous extraction to reduce the volume of JCU-2 and JCU-3 (SIPO patent no: 200510097793.4 200610137944.9 200610016490.X). The final formula was made up of 60g of crude JCU-1 in fine powder, 60g of crude JCU-3 in fine powder admixed with 29.73g of aqueous extracts from 90g of crude JCU-2 and 135.15g of aqueous extracts from 300g of crude JCU-3.

Chromatographic system

The HPLC analysis was performed on a serial system (Agilent 1100, Agilent Technologies, Santa Clara, CA) with the detector of Agilent G1365D MWD using validated methods in terms of linearity, limits of detection, quantification, reproducibility and recovery. Using chemical references from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China) with 98% of purity, these assays were confirmed to be accurate, reproducible and sensitive.

Briefly, samples were separated on a reverse-phase analytical column (Zorbax® XDB-C8, 4.6×150mm, 5µm; Agilent Technologies). The mobile phase was acetonitrile and 0.1% aqueous acetic acid, and the flow rate was 0.1ml/min. Chemical profiles of the composite formula JCU and its water extract were analysed by HPLC. The berberine concentration was 0.42% in JCU powder and 1.39% in JCU-1 water extract.

Zucker diabetic fatty rats

Five parental pairs of Zucker rats were obtained from the Monash University Animal Services (Melbourne, Australia) and transferred to the Laboratory Animal Services Centre at the Chinese University of Hong Kong. The Zucker diabetic fatty (ZDF) rats inherit obesity through a simple recessive gene (*fa*) of leptin receptor defect (18). The animals had free

access to water and were fed a standard laboratory rat diet (5001 Rodent Diet, LabDiet, St Louis, MO) containing 12% of energy from fat (4.5% fat by weight), 60% of energy from carbohydrate and 28% of energy from protein (23% protein by weight).

Study design and intervention

Single dose-experiment

We conducted 3 sets of animal experiments to study the effects of JCU on blood glucose levels. In a single-dose study (Supplementary figure 2A), 9 male ZDF rats (4-month old) were given orally a single dose of JCU (4.0g/kg) and observed up to 1 week post-treatment. Fasting and 2-hour blood glucose levels during oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were measured on days 0, 1 and 7.

2-week treatment

Thirteen male ZDF rats (10-month old) orally received either JCU (4.0g/kg, N=7) or vehicle (water) (10ml/kg, N=6) for 2 weeks and observed up to 2 months post-treatment. OGTT, ITT and blood tests for renal, liver and lipid parameters were performed at week 0 and 2 with repeat OGTT and ITT at 1 month and 2 month post-treatment when the animals were sacrificed for histological examination of liver and pancreatic tissues (Supplementary figure 2B). Western blotting, microarray and quantitative PCR analysis were applied to the liver tissues to examine expression profiles.

1 month treatment

Twelve male ZDF rats (7 month-old) were matched for body weight (range, 430–470g) and treated with either JCU (4.0g/kg, N=7) or vehicle (10ml/kg, N=5) followed by a 12-month post-treatment observation period. OGTT, ITT and biochemical tests including fasting insulin were performed at month 0 and month 1. During the post-treatment period, OGTT was performed monthly for 12 months followed by sacrifice of animals for histological examination of liver tissues (Supplementary figure 2C).

Laboratory assays

Serum insulin concentrations were measured using rat insulin ELISA kits (Merckodia, Sweden). Blood glucose during OGTT (glucose powder, 2.5g/kg) and ITT (0.5U/kg, i.p.) were measured by a blood glucose meter (Onetouch Ultra, LifeScan, Milpitas, CA). Lipids, liver and renal function parameters were measured using methods previously reported (19).

Histopathological examination and immunofluorescence microscopy

Tissue specimens from pancreas and liver were obtained and fixed in neutral formaldehyde and embedded in paraffin. For immunofluorescence microscopy, pancreas

slides were stained with mouse anti-insulin antibody (1:100 dilution, Zymed, South San Francisco, CA) and counterstained with DAPI.

Western blot of hepatic signalling kinases

Fresh liver proteins were obtained from male ZDF rats at 2 month after 2-week treatment with JCU (N=7) or vehicle (N=6). As previously reported (20), Western blot was performed to detect signaling molecules using the following primary antibodies (dilution, 1:1,000; Cell Signaling Technology, Danvers, MA): AMP-activated protein kinase α (AMPK α), phosphorylated AMPK (pAMPK), acetyl-CoA carboxylase (ACC), pACC, Akt, pAkt, HMG-CoA reductase (HMGCR), sterol regulatory element binding protein 1 (SREBP1), SREBP2, cytochrome c oxidase (CCO), and β -actin. Signals were then quantitated by densitometry and corrected for the β -actin signal.

MicroRNA expression detected by quantitative real-time PCR (RT-PCR)

Fresh liver specimens were collected from the ZDF rats at 2 month after 2-week treatment with either JCU (N=7) or vehicle (N=6). The miRNA samples from liver specimens were extracted using mirVanaTM miRNA Isolation Kit (Applied Biosystems Inc, CA) and thereafter reverse transcribed to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems Inc). The miR-U87 was used as internal quantitative control. The relative expression levels of miRNAs in the vehicle-treated ZDF rats were normalized as 1.

Total mRNA microarray assay

Fresh liver specimens were obtained from male wild type Zucker normal rats (N=3) and ZDF rats at 2 month after 2-week treatment with either JCU (N=3) or vehicle (N=3). Total RNA from rats' livers was extracted using RNeasy Mini Kit with DNase digestion (Qiagen, Valencia, CA). Equal amount of total RNA prepared from each animal within the same treatment group was pooled for analysis. The total RNA sample was sent to the Li Ka Shing Institute of Health Sciences Core Laboratory (The Chinese University of Hong Kong) for reverse transcription, labelling, microarray hybridization, washing, and scanning using the Affymetrix GeneChip[®] Gene 1.0 ST Array System. GeneSpring GX2 10.0 software was used to reconstruct global normalization and gene network with ≥ 1.5 fold change as cut-off point for differential expression.

The mRNA sample was thereafter estimated via quantitative real-time PCR using TaqMan20 \times Universal PCR Master Mix (Applied Biosystems Inc, CA). For mRNA amplification, primers were synthesized by Invitrogen (Invitrogen, Cergy-Pontoise, France) and listed as follows: (1) insulin-like growth factor binding protein 1 (IGFBP1), forward 5'-

GAAGCTTTTCTCATCTCCATACATGT-3'; reverse 5'-AAGGCCCTACCTCAGAC TGA-3', and (2) cytochrome P450 family 7 (CYP7a1), forward 5'-ATGACACGCTCTCCACCTTTG A-3'; reverse, 5'-AGCTCTTGGCCAGCACTCTGT-3'. Relative mRNA expression was quantified by the comparative Ct method and expressed as $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data are expressed as mean±standard deviation. SPSS (Statistics Package for the Social Sciences 10.0.7 for Windows, 2000, SPSS, Chicago, IL) was used to perform statistical analysis. Student's *t*-test and Paired *t* test were used to detect between-group and within-group difference, respectively. A 2-tailed *p* value < 0.05 was statistically significant.

RESULTS

Quality control of the berberine-containing JCU by HPLC

Since decoction is the traditional method of preparing TCM, bioactive compounds are more likely to reside in water extracts. HPLC analysis indicated that the 5- and 16-month old preparations of JCU water extracts had almost identical chemical peaks with comparable berberine concentration of 11.86% and 12.83%, respectively (Supplementary figure 1C). These data suggest that there was little chemical decomposition in the JCU formula during a 1.5 year study period using berberine as a reference.

Validation of the Zucker diabetic fatty rat model

The *fa/fa* genetic defect for the leptin receptor (OBR) was detected by PCR (Supplementary figure 3A). Genotyping of 35 obese and 548 lean rats, aged between 4 and 12 weeks, confirmed that the obese animals were all homozygous for the mutation (*fa/fa*), whereas the lean rats were either heterozygous (*fa/FA*, *lean*) or homozygous wild type (*FA/FA*, *normal*). Compared with male Zucker normal and lean rats, male Zucker fatty rats exhibited overt obesity (Supplementary figure 3B-C), glucose intolerance and insulin resistance from 12 weeks onwards (Supplementary figure 3D-E), and developed overt diabetes from 18 weeks onwards (Supplementary figure 3F). In this study, normal blood glucose was defined as a fasting blood glucose < 5.6 mmol/L and 2-hour OGTT blood glucose < 7.8 mmol/L. Impaired fasting glycemia was defined as a fasting blood glucose of 6.1-6.9 mmol/L; impaired glucose tolerance, 2-hour OGTT blood glucose of 7.8-11.0 mmol/L; and diabetes, a 2-hour post-load glucose ≥ 11.0 mmol/l during OGTT. We used male Zucker fatty rats of at least 20-week old with diabetes and insulin resistance in all experiments.

We validated these animals by treating 8 male 20-week old ZDF rats with metformin (single dose, 50mg/kg) for one week (Supplementary figure 3G) which reduced post-treatment blood glucose levels during OGTT (paired t-test, all $p \leq 0.021$). The area under curve (AUC) of blood glucose fell from 25.5 ± 6.0 mmol/l to 17.2 ± 1.7 mmol/l ($p = 0.036$), while body weight increased from 447.8 ± 41.3 g to 454.4 ± 39.2 g (paired t-test, $p = 0.004$).

Sustained glucose-lowering effects after JCU treatment for 1-day, 2-weeks and 1-month

In the single-dose study, JCU treatment improved glucose tolerance [Fig. 1a (A-B)] and insulin sensitivity [Fig. 1a (C-D)] in 9 male ZDF rats, with reduced AUC for blood glucose during OGTT (Fig. 1a, B) and ITT (Fig. 1a, D). The blood-glucose-lowering effects persisted for at least 1 week after the single-dose intervention was discontinued (Fig. 1a, B-D). During 1-week post-treatment period, 8 of 9 rats treated with a single dose of JCU had 25% sustained reduction of AUC blood glucose during OGTT (paired 2-tails t -test $p < 0.001$).

Prolonged treatment with JCU for 2 weeks (Fig. 1b) and 1 month (Fig. 3) confirmed these sustained beneficial effects. During 2-week treatment, significant blood glucose-lowering effect was observed 2 hours after the oral gavage of JCU and sustained during an extended 3-hour OGTT [Fig. 1b (A-B)] and 2-hour ITT [Fig. 1b (C-D)]. The reduction in AUC of blood glucose during OGTT and ITT persisted for 2 months after stopping 2-week treatment [(Fig. 1b (E- F))].

During 1-month treatment period, blood glucose levels were lower in JCU-treated ZDF rats during OGTT and ITT than vehicle-treated animals (Figure 1c, Supplementary table 1). After treatment discontinuation, fasting and 2-hour blood glucose levels during OGTT were monitored up to 390 days monthly (Fig. 1c). Fasting [Fig. 1c, A)] and 2-hour [(Fig. 1c, B)] blood glucose levels were lower in the JCU-treated than vehicle-treated group throughout the observation period. By day 360, 3 of 5 control rats and 1 of 7 rats in the JCU-treated group died. In the surviving rats, all 6 JCU-treated animals remained non-diabetic with a fasting blood glucose < 7 mmol/l and 2-hour blood glucose < 11 mmol/l during OGTT, compared to only 1 of the 2 surviving rats in the vehicle-treated group without diabetes.

Effects of JCU treatment on liver and renal function and intermediary metabolism

After 2-week treatment, blood triglyceride decreased in the JCU-treated rats and increased in the vehicle-treated rats with significant between-group difference. Blood creatinine and urea levels tended to decrease in the JCU-treated rats, albeit not significant. In the 1-month experiment, these trend differences persisted but did not reach statistical significance. In both the 2-week and 1-month experiments, treatment with JCU significantly

decreased the liver enzyme, alanine aminotransferase (ALT) levels, by approximately 50% (Table 1). After 1-month treatment, despite a lower fasting blood glucose, fasting serum insulin concentrations were similar between the JCU-treated (43.9 ± 22.3 pmol/l) and control (37.8 ± 9.1 pmol/l) groups ($p=0.600$).

Effects of JCU treatment on liver and islet histology

Figures 4A-D show the histopathological changes of liver specimens obtained at 2 (Fig. 2A-B) and 12 months (Fig. 2C-D) after discontinuation of 2-week and 1-month treatment respectively. In ZDF rats treated with JCU for 2 weeks (Fig. 2A) and 1 month (Fig. 2C), there was normalization of liver tissues. In the vehicle-treated group for 2 weeks (Fig. 2B) and 1 month (Fig. 2D), there were foci of fatty change and diffuse hepatocyte ballooning degeneration.

Figures 2E-H show the histopathological features on HE staining (Fig. 2E-F) and immunostaining of pancreatic islets identified by insulin (Fig. 2G-H) in animals sacrificed at 12 months after 1 month treatment. Both vehicle-treated and JCU-treated animals showed similar islet cytoarchitecture (Fig. 2E-F) and insulin reactivity (Fig. 2G-H).

Sustained activation of AMPK and Akt in the liver after 2 weeks of JCU treatment

After 2-week treatment, rats were sacrificed at 2 month for protein studies of the liver sections. Compared with the vehicle-treated rats, JCU-treated animals showed upregulated hepatic expression levels of pAMPK and pAkt (Fig. 3a, A) with increased pAMPK:AMPK ratio and pAkt:AKT ratio compatible with persistent activation of AMPK and insulin signalling pathway, despite treatment discontinuation for 2 months. The JCU treatment also stimulated expression level of the mitochondrial CCO (Fig. 3a, B) and repressed the protein expression levels of pACC, HMGCR, and SREBP1, consistent with activation of hepatic AMPK signalling pathway and a switch from ATP consumption (e.g. fatty acid and cholesterol synthesis) to ATP production (e.g. fatty acid and glucose oxidation) pathways.

Sustained suppression of hepatic miRNA expression after 2 weeks of JCU treatment

We examined a panel of 7 miRNA markers implicated in hepatic insulin action (13) in the JCU- or vehicle-treated rats at 2 month after 2-week experiment. Compared with the vehicle-treated rats, the JCU-treated animals had decreased hepatic expression levels of 6 miRNA markers (miR-1, miR-21, miR-29a, miR-29b, miR-122, miR-150) reaching significance for miR-29b with 50% reduction and a 1.5-fold increase in miR-29c expression (Fig. 3b).

Sustained global gene expression in the liver after 2 weeks of JCU treatment

We applied a microarray assay which detected 27,342 hepatic genes to total RNA samples extracted from fresh liver specimens of wild type Zucker normal rats (N=3) and ZDF rats sacrificed at 2 month after 2-week treatment with either JCU (N=3) or vehicle (N=3). Compared with wild type normal rats, vehicle-treated animals demonstrated a 1.5-fold or greater change in 313 genes and a 2-fold or greater change in 76 genes. In the 313 genes, 247 genes were annotated and among them, 49 genes were related to lipid or glucose metabolisms (lipid, 39 genes; fatty acid, 14 genes; cholesterol, 10 genes; bile acid, 5 genes; and glucose, 4 genes). Analysis using a standard DAVID tool (<http://david.abcc.ncifcrf.gov>) and Chi-square tests demonstrated that p-values of the different interested terms (Supplementary table 2a) and the union of all genes annotated by these terms (Supplementary table 2b) related to lipid metabolism were less than 0.0001. Compared to vehicle, JCU treatment corrected 33 (10.5%) of 313 genes with over 1.5-fold change in expression and 6 (7.9%) of 76 genes with over 2-fold change in expression. Eight of 33 corrected hepatic genes were related to gluconeogenesis and/or lipid metabolism (Table 2).

Gene network analysis

Twenty-six annotated genes of these 33 genes were subjected to the GeneSpring GX 10.0 software for gene network reconstruction and biological pathway analysis which suggested a potential central regulating role of IGFBP1 and CYP7a1 (Supplementary figure 4A). The former plays a pivotal role in cell cycle while the latter is the rate-limiting enzyme in hepatic efflux of cholesterol through conversion to bile acids. Quantitative real-time PCR confirmed that the JCU treatment significantly upregulated hepatic expression of IGFBP1 (10.9-fold) and CYP7a1 (3.8-fold) compared with vehicle (Supplementary figure 4B).

We performed enrichment analysis by examining existing annotations of the 33 genes using the DAVID tool which showed that 5 genes (Cyp7a1, G6pdx, Me1, Rdh2, Por) were associated with the term of Nicotinamide adenine dinucleotide phosphate (NADP) (Benjamini adjusted $p=0.0025$). The latter is an essential substrate for fatty acid synthesis and other pathways including cytochrome P450 related pathways. Amongst these 5 genes, 4 were related to lipid and/or glucose metabolism while Por interacts with different P450 enzymes to complete the electron transfer chain in energy production, resulting in reduced fatty acid synthesis, amelioration of fatty liver and improved liver function.

DISCUSSION

Using validated animal models and molecular tools, we reported for the first time the sustained antidiabetic effects of a berberine-containing TCM in ZDF rats through complex

regulation of genome expression. The sustained antidiabetic effects of JCU included improvement of hyperglycemia, amelioration of insulin resistance, normalization of elevated liver enzyme and hepatocyte ballooning degeneration. These sustained metabolic and histopathological changes after treatment discontinuation were associated with changes in gene and protein expression implicated in energy metabolism and cell cycle, in part mediated via changes in miRNA (Fig. 4). On a miRNA level, JCU down-regulated miR29-b which may target Akt and Btg2 to respectively influence glucose uptake and CYP7a1 transcription, with increased glucose uptake and lipid catabolism. On a protein level, JCU activated AMPK and Akt with reduced expression of ACC, SREBP1 and HMGCR and increased expression of mitochondrial CCO, favoring lipid oxidation. On an mRNA level, JCU corrected a panel of gene expression to favor lipid catabolism, lipid oxidation and cell survival, with IGFBP1 and CYP7a1 playing possible linking roles.

Rationale for using a multipronged strategy to study effects of TCM

In this experiment, we used metformin to validate the ZDF rat model but did not use it as a comparative agent in the mechanistic study since during the 12-month period after 1-month treatment, only JCU but not metformin (50 mg/kg body weight) showed sustained favourable effects (data not shown). These observations were in line with TCM clinical practice where treatment is usually given for 2 weeks rather than a prolonged period. In our pilot studies (data not shown), the multi-component formula of JCU stimulated glucose uptake in rat skeletal muscle L6 cells and in lowered blood glucose levels in Zucker diabetic fatty rats more effectively than the single compound of berberine. Other authors have also reported molecular mechanisms underlying the synergistic effects of TCM formula using cell cultures and animal studies (21). Based on these principles, we treated the animal with JCU for 2 weeks and examined the sustained blood-glucose lowering effects using a multi-pronged strategy.

Pluripotent effects of TCM on energy metabolism and cell cycle

The sustained, coordinated and multi-layered changes in genome and protein expression by JCU treatment suggested a re-setting of energy homeostasis with predominant energy dissipation and catabolism with improved cell survival. These genomic changes were consistent with the phenotypic changes including weight reduction, improved insulin sensitivity, amelioration of liver fat accumulation and hepatocyte degeneration with increased cellular regeneration. To our best knowledge, such complex beneficial effects have not been reported with any conventional western medicine containing a single chemical except for

thiazolidinediones with beneficial effects on gluco-lipotoxicity, durability of glycemic control (22) and cancer growth (23).

Apart from animal studies which have demonstrated anti-diabetic (24) and antihypertensive effects of TCM (25), human studies have also reported sustained therapeutic effects of TCM and acupuncture in patients with irritable bowel syndrome (26), low back pain (27) and cancer associated with chemotherapy-induced hepatotoxicity (28). Despite these clinical benefits, there have been few comprehensive studies which examined the molecular mechanisms underlying the pluripotent effects of natural medicine.

MicroRNA and gene networks

Several lines of evidence suggested that the biological effects of plant medicines might be mediated through alteration of gene expression. Based on this premise, we discovered that 2-week treatment with JCU changed the expression levels of several miRNAs, with repression of upregulated hepatic expression of miR29-b. Several studies have reported association of insulin resistance with upregulation of miR29 and consequent repression of insulin-stimulated glucose uptake (14). Since the rat miR29-b had 272 predicted target genes, including Akt, changes in this single miRNA might have diverse biological effects (29). On data mining (micrRNA.org), we discovered that one of these targets might be Btg2, which is a transcription co-regulator. Interestingly, Btg2 was also implied to regulate transcription of CYP7a1, a key enzyme for conversion from cholesterol to bile acids, which was also upregulated by JCU. Thus, apart from modulating Akt expression, miR29-b might interact with Btg2 to increase CYP7a1 expression to promote cholesterol catabolism (supplementary figure 4A).

Effects of JCU on fatty liver

We further discovered that 33 genes, mainly implicated in energy metabolism and cell cycle were changed by JCU treatment. Gene network analysis suggested the linking roles of IGFBP1 and CYP7a1 in a molecular network of genes that regulate cholesterol catabolism, bile acid biosynthesis and cell cycle. These inferred gene-gene networks are compatible with changes in hepatic genes implicated in gluconeogenesis, lipogenesis, cytochrome P450 pathway and glucose utilization in diabetic ZDF rats with pre-diabetes, diabetes, and late-stage diabetes (30).

The insulin-IGF1-IGFBP pathway constitutes a complex network to modulate energy metabolism and cellular growth (31), in part mediated through the PI3K-Akt-mammalian target of rapamycin (mTOR) pathway (32-34). In agreement with other reports (35), our

vehicle-treated ZDF rats had reduced hepatic IGFBP1 mRNA which was normalized after 2-week treatment with JCU. Other workers have reported spontaneous apoptosis in livers of IGFBP1-deficient mice (36). In humans, low fasting serum IGFBP1 concentration was associated with increased liver fat and reduced hepatic insulin sensitivity, independent of obesity (37). In human hepatocytes, endoplasmic reticulum (ER) stress induced IGFBP1 expression as an adaptive response (38). Collectively, these data suggest that JCU treatment increased IGFBP1 expression, possibly through increased insulin signaling, and in concert with other genes, to promote liver repair from damage due to fatty degeneration.

Limitations and future investigations

In this study, fasting serum insulin concentrations were similar between the JCU-treated and control groups after one-month treatment. Besides, immuno-histological examination demonstrated similar islet size, shape and number of islets with similar insulin reactive cells between the two groups. While our study concentrated mainly on hepatic mechanism, since we did not measure glucose-stimulated insulin levels, we could not exclude possible effects of JCU on insulin secretion.

The reduction in SREBP1c protein level might be correlated to the inhibition from AMPK activation since AMPK is a regulator of SREBP1. In mice, activation of SREBP-1 and inhibition of hepatic AMPK accounted for ethanol-induced fatty liver (39; 40) while hepatic activation of AMPK and suppression of SREBP-1 protected against hepatic steatosis, hyperlipidemia and accelerated atherosclerosis in diet-induced insulin resistance (41). In this study, the joint inhibition of SREBP-1 and activation of Akt and AMPK may be particularly relevant to the sustained anti-diabetic actions of the TCM formula.

Due to small numbers of ZDF rats in each group, we were not able to demonstrate a beneficial effect of JCU on plasma triglyceride level despite its sustained blood glucose lowering effects (Table 1). Although Western blot assays showed altered protein levels of ACC, HMGCR and COO with JCU treatment, this was not accompanied by significant changes in gene expression. While altered protein expression level might have occurred post-transcription, the small number of samples and large variability of mRNA expression might reduce the study power to detect differences between expression for each gene. Alternatively, use of p-value cut-off for differential expression and gene network analysis may help identify differentially expressed genes with statistical significance but do not have a large expression fold change.

Our study also showed that long-term treatment with JCU might prevent body weight gain in ZDF rats (Supplementary table 1). In db/db mice, berberine treatment reduced body

weight and improved triglyceride level (7). Given the importance of abnormal fat metabolism in insulin resistance, it will be useful to further explore the effects of berberine-containing JCU on fat mass and metabolism.

CONCLUSIONS

Using scientifically validated animal models and *state-of-the-art* methodologies, we have confirmed the pluripotent effects of a long-tested TCM in altering gene expression, in part through changes in miRNA, to explain its sustained beneficial effects on glucose metabolism, fatty liver and cellular repair.

CONTRIBUTIONS

Hai-Lu Zhao, designed experiment, analysed data, wrote manuscript; Yi Sui, generated and analysed data, wrote manuscript; Chun-Feng Qiao, designed experiment, generated data; Kevin Y Yip, analysed data; Ross KK Leung, analysed data; Stephen KW Tsui, contributed to discussion; Harriet KT Wong, generated data; Xun Zhu, generated data; Jennifer J Siu, generated data; Lan He, generated data; Jing Guan, generated data; Li-Zhong Liu, generated data; Heung-Man Lee, analysed data; Hong-Xi Xu, designed experiment, generated data; Peter CY Tong, designed experiment; Juliana CN Chan, designed experiment, contributed to discussion, edited manuscript.

ACKNOWLEDGEMENTS

We are grateful to Dr. Tony James and Mr. Lik Wong Lam at the CUHK Laboratory Animal Services Centre for their assistance in breeding the Zucker rats. We thank Professor Wai-Yee Chan, Director, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong for his invaluable advice. Dr. Hai-Lu Zhao takes full responsibility for the manuscript and its originality. The work was partially supported by a grant from the Hong Kong Jockey Club Charities Trust (JCICM -P2-05 (CUHK)), Hong Kong Foundation for Research and Development in Diabetes, CUHK Focused Investment and Liao Wun Yuk Diabetes Memorial Research Fund. The funding body did not have any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CONFLICT OF INTERESTS

None.

REFERENCES

1. Capasso L: 5300 years ago, the Ice Man used natural laxatives and antibiotics. *Lancet* 352:1864, 1998
2. Tang HS: [Study on relative problems of acupuncture and moxibustion for treatment of simple obesity]. *Zhongguo Zhen Jiu* 28:522-524, 2008
3. Salpeter SR, Buckley NS, Kahn JA, Salpeter EE: Meta-analysis: metformin treatment in persons at risk for diabetes mellitus. *Am J Med* 121:149-157 e142, 2008
4. Selvin E, Bolen S, Yeh HC, Wiley C, Wilson LM, Marinopoulos SS, Feldman L, Vassy J, Wilson R, Bass EB, Brancati FL: Cardiovascular outcomes in trials of oral diabetes medications: a systematic review. *Arch Intern Med* 168:2070-2080, 2008
5. Hundal RS, Inzucchi SE: Metformin: new understandings, new uses. *Drugs* 63:1879-1894, 2003
6. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE: Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108:1167-1174, 2001
7. Lee YS, Kim WS, Kim KH, Yoon MJ, Cho HJ, Shen Y, Ye JM, Lee CH, Oh WK, Kim CT, Hohnen-Behrens C, Gosby A, Kraegen EW, James DE, Kim JB: Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states. *Diabetes* 55:2256-2264, 2006
8. Turner N, Li JY, Gosby A, To SW, Cheng Z, Miyoshi H, Taketo MM, Cooney GJ, Kraegen EW, James DE, Hu LH, Li J, Ye JM: Berberine and its more biologically available derivative, dihydroberberine, inhibit mitochondrial respiratory complex I: a mechanism for the action of berberine to activate AMP-activated protein kinase and improve insulin action. *Diabetes* 57:1414-1418, 2008
9. Lawless MW, O'Byrne KJ, Gray SG: Histone deacetylase inhibitors target diabetes via chromatin remodeling or as chemical chaperones? *Curr Diabetes Rev* 5:201-209, 2009
10. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281-297, 2004
11. Krutzfeldt J, Stoffel M: MicroRNAs: a new class of regulatory genes affecting metabolism. *Cell Metab* 4:9-12, 2006
12. Lynn FC: Meta-regulation: microRNA regulation of glucose and lipid metabolism. *Trends Endocrinol Metab* 20:452-459, 2009
13. Tang X, Tang G, Ozcan S: Role of microRNAs in diabetes. *Biochim Biophys Acta* 1779:697-701, 2008
14. He A, Zhu L, Gupta N, Chang Y, Fang F: Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. *Mol Endocrinol* 21:2785-2794, 2007
15. Herrera BM, Lockstone HE, Taylor JM, Ria M, Barrett A, Collins S, Kaisaki P, Argoud K, Fernandez C, Travers ME, Grew JP, Randall JC, Gloyn AL, Gauguier D, McCarthy MI, Lindgren CM: Global microRNA expression profiles in insulin target tissues in a spontaneous rat model of type 2 diabetes. *Diabetologia* 53:1099-1109, 2010
16. Zhao E, Keller MP, Rabaglia ME, Oler AT, Stapleton DS, Schueler KL, Neto EC, Moon JY, Wang P, Wang IM, Lum PY, Ivanovska I, Cleary M, Greenawalt D, Tsang J, Choi YJ, Kleinhanz R, Shang J, Zhou YP, Howard AD, Zhang BB, Kendziorski C, Thornberry NA, Yandell BS, Schadt EE, Attie AD: Obesity and genetics regulate microRNAs in islets, liver, and adipose of diabetic mice. *Mamm Genome* 20:476-485, 2009
17. Song JZ, Yiu HH, Qiao CF, Han QB, Xu HX: Chemical comparison and classification of Radix Astragali by determination of isoflavonoids and astragalosides. *J Pharm Biomed Anal* 47:399-406, 2008

18. Zucker LM, Antoniades HN: Insulin and obesity in the Zucker genetically obese rat "fatty". *Endocrinology* 90:1320-1330, 1972
19. Sui Y, Zhao HL, Ma RC, Ho CS, Kong AP, Lai FM, Ng HK, Rowlands DK, Chan JC, Tong PC: Pancreatic islet beta-cell deficit and glucose intolerance in rats with uninephrectomy. *Cell Mol Life Sci* 64:3119-3128, 2007
20. Yang X, Zhao H, Sui Y, Ma RC, So WY, Ko GT, Kong AP, Ozaki R, Yeung CY, Xu G, Tong PC, Chan JC: Additive interaction between the renin-angiotensin system and lipid metabolism for cancer in type 2 diabetes. *Diabetes* 58:1518-1525, 2009
21. Wang L, Zhou GB, Liu P, Song JH, Liang Y, Yan XJ, Xu F, Wang BS, Mao JH, Shen ZX, Chen SJ, Chen Z: Dissection of mechanisms of Chinese medicinal formula Realgar-Indigo naturalis as an effective treatment for promyelocytic leukemia. *Proc Natl Acad Sci U S A* 105:4826-4831, 2008
22. Kahn SE, Haffner SM, Heise MA, Herman WH, Holman RR, Jones NP, Kravitz BG, Lachin JM, O'Neill MC, Zinman B, Viberti G: Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *N Engl J Med* 355:2427-2443, 2006
23. Monami M, Lamanna C, Marchionni N, Mannucci E: Rosiglitazone and risk of cancer: a meta-analysis of randomized clinical trials. *Diabetes Care* 31:1455-1460, 2008
24. Shapira MY, Appelbaum EY, Hirshberg B, Mizrahi Y, Bar-On H, Ziv E: A sustained, non-insulin related, hypoglycaemic effect of electroacupuncture in diabetic *Psammomys obesus*. *Diabetologia* 43:809-813, 2000
25. Shih HC, Lee TH, Chen SC, Li CY, Shibuya T: Anti-hypertension effects of traditional Chinese medicine ju-ling-tang on renal hypertensive rats. *Am J Chin Med* 33:913-921, 2005
26. Bensoussan A, Talley NJ, Hing M, Menzies R, Guo A, Ngu M: Treatment of irritable bowel syndrome with Chinese herbal medicine: a randomized controlled trial. *JAMA* 280:1585-1589, 1998
27. Hsieh LL, Kuo CH, Lee LH, Yen AM, Chien KL, Chen TH: Treatment of low back pain by acupressure and physical therapy: randomised controlled trial. *BMJ* 332:696-700, 2006
28. Liu ML, Chien LY, Tai CJ, Lin KC: Effectiveness of Traditional Chinese Medicine for Liver Protection and Chemotherapy Completion among Cancer Patients. *Evid Based Complement Alternat Med*, 2009
29. <http://www.microrna.org/microrna/home.do> accessed on 13 May 2010. .
30. Suh YH, Kim Y, Bang JH, Choi KS, Lee JW, Kim WH, Oh TJ, An S, Jung MH: Analysis of gene expression profiles in insulin-sensitive tissues from pre-diabetic and diabetic Zucker diabetic fatty rats. *J Mol Endocrinol* 34:299-315, 2005
31. Lee PD, Giudice LC, Conover CA, Powell DR: Insulin-like growth factor binding protein-1: recent findings and new directions. *Proc Soc Exp Biol Med* 216:319-357, 1997
32. Cichy SB, Uddin S, Danilkovich A, Guo S, Klippel A, Unterman TG: Protein kinase B/Akt mediates effects of insulin on hepatic insulin-like growth factor-binding protein-1 gene expression through a conserved insulin response sequence. *J Biol Chem* 273:6482-6487, 1998
33. Finlay D, Ruiz-Alcaraz AJ, Lipina C, Perrier S, Sutherland C: A temporal switch in the insulin-signalling pathway that regulates hepatic IGF-binding protein-1 gene expression. *J Mol Endocrinol* 37:227-237, 2006
34. Patel S, Lochhead PA, Rena G, Fumagalli S, Pende M, Kozma SC, Thomas G, Sutherland C: Insulin regulation of insulin-like growth factor-binding protein-1 gene expression is dependent on the mammalian target of rapamycin, but independent of ribosomal S6 kinase activity. *J Biol Chem* 277:9889-9895, 2002
35. Dubuis JM, Deal CL, Van Vliet G: The growth hormone (GH)-independent growth of the obese Zucker rat is not due to increased levels of GH receptor messenger RNA in the liver. *Mol Cell Endocrinol* 113:215-223, 1995

36. Leu JI, George DL: Hepatic IGFBP1 is a prosurvival factor that binds to BAK, protects the liver from apoptosis, and antagonizes the proapoptotic actions of p53 at mitochondria. *Genes Dev* 21:3095-3109, 2007
37. Kotronen A, Lewitt M, Hall K, Brismar K, Yki-Jarvinen H: Insulin-like growth factor binding protein 1 as a novel specific marker of hepatic insulin sensitivity. *J Clin Endocrinol Metab* 93:4867-4872, 2008
38. Marchand A, Tomkiewicz C, Magne L, Barouki R, Garlatti M: Endoplasmic reticulum stress induction of insulin-like growth factor-binding protein-1 involves ATF4. *J Biol Chem* 281:19124-19133, 2006
39. Hu M, Wang F, Li X, Rogers CQ, Liang X, Finck BN, Mitra MS, Zhang R, Mitchell DA, You M: Regulation of hepatic lipin-1 by ethanol: Role of AMPK-SREBP-1 signaling. *Hepatology*, 2011
40. You M, Matsumoto M, Pacold CM, Cho WK, Crabb DW: The role of AMP-activated protein kinase in the action of ethanol in the liver. *Gastroenterology* 127:1798-1808, 2004
41. Li Y, Xu S, Mihaylova MM, Zheng B, Hou X, Jiang B, Park O, Luo Z, Lefai E, Shyy JY, Gao B, Wierzbicki M, Verbeuren TJ, Shaw RJ, Cohen RA, Zang M: AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell Metab* 13:376-388, 2011

Table 1. Changes in lipid profiles, renal and liver function tests in Zucker *fa/fa* rat after 2-week and 1-month treatment with either Chinese medicine (JCU) or vehicle.

	JCU (4g/kg)				Vehicle (water 10 ml/kg)				P value (between groups)		
	Baseline	End	Difference	P*	Baseline	End	Difference	P*	Baseline	End	Difference
2-Week treatment											
Number of ZDF rats		7				6					
ALT (U/L)	164.7±25.7	86.8±17.9	77.6±77.2	0.029	150.3±33.1	138.0±39.8	-12.3±35.0	0.740	0.739	0.013	0.036
Total cholesterol (mmol/L)	3.2±1.0	3.6±1.1	-0.5±1.5	<0.001	3.5±0.9	3.4±0.6	-0.1±0.7	0.099	0.421	0.680	0.476
Triglyceride (mmol/L)	4.6±2.0	4.7±2.8	-1.2±3.7	0.313	4.4±1.4	7.9±2.0	3.5±2.5	0.797	0.773	0.019	0.007
HDL-cholesterol (mmol/L)	2.7±0.9	2.9±1.0	-0.6±1.3	0.001	2.9±0.7	2.7±0.6	-0.2±0.6	0.149	0.569	0.570	0.509
Creatinine (µmol/L)	27.6±6.6	18.4±3.8	14.3±12.4	0.645	23.0±2.9	14.8±2.7	-8.2±4.8	0.230	0.082	0.052	0.206
Urea (mmol/L)	7.2±1.1	6.6±0.9	-2.4±3.5	0.181	7.2±1.1	7.4±0.5	0.3±1.1	0.845	0.926	0.044	0.053
1-Month treatment											
Number of ZDF rats		7				5					
ALT (U/L)	157.2±34.2	67.0±32.9	83.2±9.6	<0.001	107.8±27.0	101.8±14.7	-0.8±7.2	0.643	0.232	0.036	0.017
Total cholesterol (mmol/L)	2.6±1.2	2.9±0.7	0.3±0.5	0.296	1.9±0.7	2.5±0.3	0.6±0.7	0.134	0.295	0.355	0.408
Triglyceride (mmol/L)	3.4±2.4	4.1±1.6	0.7±2.3	0.529	2.4±1.5	3.4±0.8	1.0±1.8	0.287	0.450	0.414	0.818
HDL-cholesterol (mmol/L)	1.7±0.5	2.2±0.5	0.7±0.6	0.110	1.4±0.5	2.1±0.1	0.7±0.6	0.092	0.423	0.750	0.991
Creatinine (µmol/L)	39.8±5.7	29.8±5.9	-8.7±8.1	0.042	30.2±6.3	29.4±4.0	-0.8±5.4	0.759	0.049	0.903	0.122
Urea (mmol/L)	9.0±2.3	5.7±0.5	-3.2±2.8	0.044	8.3±2.5	6.4±0.8	-1.9±3.1	0.236	0.695	0.188	0.545

Data are Mean±SD. *p: p value of paired t test within-group.

HDL, high density lipoprotein; ALT, alanine transferase.

Table 2. Hepatic genes whose expression intensity was changed by 1.5-fold or more between 1) wild type Zucker normal rats (ZN) and Zucker *fa/fa* rats treated with vehicle (ZDF-V) and 2) JCU-treated (ZDF-JCU) and vehicle-treated Zucker *fa/fa* rat.

Gene symbol	Gene description	ZN vs ZDF-V	ZDF-JCU vs ZDF-V
Btg2	B-cell translocation gene 2, anti-proliferative	2.1 ↑	3.0 ↑
Cml4	Camello-like 4	4.4 ↑	1.5 ↑
Ccrn4l	CCR4 carbon catabolite repression 4-like B	3.9 ↑	1.9 ↑
Coq10b	Coenzyme Q10 homolog B (<i>S. cerevisiae</i>)	2.1 ↑	1.6 ↑
Cyp7a1* #	Cytochrome P450, family 7, subfamily a, polypeptide 1	3.0 ↑	1.9 ↑
Dbp	D site albumin promoter binding protein	2.4 ↑	1.6 ↑
Dusp1	Dual specificity phosphatase 1	1.8 ↑	2.2 ↑
Gcnt2	Glucosaminyl (N-acetyl) transferase 2	1.8 ↑	1.8 ↑
G6pdx*	Glucose-6-phosphate dehydrogenase X-linked	3.5 ↓	1.9 ↓
Igfbp1* #	Insulin-like growth factor binding protein 1	5.3 ↑	8.3 ↑
Jun	Jun	1.6 ↑	2.3 ↑
LOC313220*	Similar to bile acid Coenzyme A	5.0 ↑	2.0 ↑
Me1*	Malic enzyme 1	2.8 ↓	2.1 ↓
Npas2	Neuronal PAS domain protein 2 (predicted)	1.5 ↓	1.5 ↓
Olr1448	Olfactory receptor 1448 (predicted)	1.9 ↑	1.5 ↑
Por	P450 (cytochrome) oxidoreductase	2.0 ↑	1.6 ↑
Pnpla3*	Patatin-like phospholipase domain containing 3	14.3 ↓	2.1 ↓
Rdh2*	Retinol dehydrogenase 2	3.0 ↑	1.5 ↑
RGD1562060	Similar to short chain dehydrogenase reductase 9	3.4 ↓	1.8 ↓
Rhbdd2	Rhomboid domain containing 2	2.2 ↑	1.5 ↑
Sds*	Serine dehydratase	4.2 ↑	2.3 ↑
Snf1lk	SNF1-like kinase	1.6 ↑	1.9 ↑
Slc25a25	Solute carrier family 25	1.7 ↑	2.4 ↑
Tysnd1	Trypsin domain containing 1	1.5 ↑	1.6 ↑
Upp2	Uridine phosphorylase 2 (predicted)	2.9 ↑	1.6 ↑
Wee1	Wee 1 homolog	1.8 ↑	1.6 ↑

Note: Genes without annotation are not listed in this table.

*Genes related to glucose and/or lipid metabolic process.

Hepatic expression of CPY7a1 and IGFBP1 were validated by quantitative real-time PCR.

FIGURE LEGENDS

Fig 1a. Blood glucose levels of male ZDF rats during study with single dose treatment.

Male ZDF rats (n=9, age= 20.3±2.0 weeks, body weight= 463.9±29.8 g) were orally administered with a single dose of JCU. Blood glucose levels during OGTT (glucose 2.5 g/kg) and ITT (insulin 0.5 U/kg, i.p.) and their AUC were monitored on days 0 (baseline), 1 (JCU treatment) and 7 after the single dose. Data are mean±SD. * P<0.05.

- A. Blood glucose levels during OGTT before (solid line) and after (dash line) the single dose treatment.
- B. AUC derived from blood glucose levels during OGTT conducted on day 0 (baseline), day 1 (JCU) and day 7 (FU1w) after discontinuing JCU.
- C. Blood glucose levels during ITT before (solid line) and after (dash line) the single dose treatment.
- D. AUC derived from blood glucose levels during ITT conducted on day 0 (baseline), day 1 (JCU) and 7 (FU1w) after discontinuing JCU.

Fig 1b. Blood glucose levels of male ZDF rats during study with 2-week treatment.

A total of 13 male ZDF rats were treated with JCU (n=7, body weight=520.0±38.2 g, age=10.1±0.4 months; dash line) or vehicle (n=6, body weight=532.5±26.6 g, age=9.9±0.4 months; solid line) for 2 weeks followed by a 2-month observation period post treatment.

Data are mean±SD. * P<0.05.

- A. OGTT (glucose 2.5 g/kg) was performed in male ZDF rats orally treated with JCU (dash line) or vehicle (solid line) on day 1 after treatment. JCU was orally administered 120 minutes before the oral glucose challenge.
- B. AUC derived from blood glucose levels during OGTT performed on day 1 after treatment.
- C. ITT (insulin 0.5 U/kg) was performed in male ZDF rats orally treated with JCU (dash line) or vehicle (solid line) on day 1 after treatment.
- D. AUC derived from blood glucose levels during ITT performed on day 1 after treatment.
- E. AUC derived from blood glucose levels of OGTT performed during the 2-week treatment period followed by a 2-month period after discontinuation of treatment with JCU (dash line) or vehicle (solid line).

- F. AUC derived from blood glucose levels of ITT performed during the 2-week treatment period followed by a 2-month period after discontinuation of treatment with JCU (dash line) or vehicle (solid line).

Fig 1c. Blood glucose levels of male ZDF rats in the study with 1-month treatment.

Seven male ZDF rats were treated with JCU (dash line) and 5 treated with water (solid line). After discontinuing the 1-month treatment, fasting (panel A) and 2-hour blood glucose levels after challenge with oral glucose 2.5 g/kg (panel B) were measured monthly for 12 months. Data are mean±SD. * P<0.05.

Fig 2. Light microscopy of histopathological changes in liver and pancreatic islet.

Liver specimens of male ZDF rats were obtained at the end of 2 months after stopping the 2-week treatment (panel A, B) and that of 1 year after stopping the 1-month treatment (panel C, D) with either JCU or vehicle. Tissue sections (4 µm) were stained with haematoxylin and eosin (HE). Light microscopic examination revealed normalized liver histological structure after JCU (panel A, C) but diffuse ballooning degeneration after vehicle (panel B, D). Pancreas specimens of male ZDF rats were obtained at the end of a 1 year observation period after discontinuing the 1-month treatment with either JCU or vehicle. The specimens were stained with haematoxylin and eosin (HE; panel E, F; light microscopy) or insulin (green; panel G, H; immunofluorescence microscopy). The rats treated with JCU (panel E, G) or vehicle (panel F, H) exhibited similar pancreas cytoarchitecture (panel E, F) and insulin staining (panel G, H). Original magnification ×200.

Fig 3a. Expression of hepatic signaling enzymes detected by Western blot.

Fresh liver specimens were obtained from the male ZDF rats at the end of the 2-month follow up period after discontinuing the 2-week treatment with JCU (n=7, close bar) or vehicle (n=6, open bar). Total protein samples were isolated from the fresh liver specimens and then probed with primary antibodies by Western blotting.

- A. Relative expression levels of AMP-activated protein kinase (AMPK), phosphorylated AMPK (pAMPK), AKT, pAKT, acetyl-CoA carboxylase (ACC) and pACC.
- B. Relative expression levels of HMG-coenzyme A reductase (HMGCR), sterol regulatory element binding protein (SREBP) 1, SREBP2, and cytochrome c oxidase (CCO).

Fig 3b. Relative expression levels of miRNA markers in liver.

The miRNA samples were isolated from fresh liver specimens collected at the end of 2 month observation after discontinuing the 2-week treatment with JCU (open bar) or vehicle (close bar). Expression levels of the 7 miRNA markers were detected by reverse transcript and quantitative real-time PCR. Data are mean±SD. * P<0.05.

Fig 4. Possible mechanisms for the sustained and pluripotent effects of JCU in ZDF rats.

The pluripotent effects of 2-week treatment with a berberine-containing 3-herb formula on expression of miRNA, RNA and proteins in liver cells of Zucker fatty rats sacrificed 2 months post-treatment which included:

- 1) increased expression of AMPK with reduced lipid synthesis and increased lipid oxidation
- 2) increased Akt expression possibly via repression of miR-29b with increased IGFBP1 expression and increased insulin signalling resulting in enhanced glucose uptake
- 3) increased CYP7a1 expression possibly via increased Btg2 (a co-regulator of transcription) expression through repression of miR-29b with increased cholesterol conversion to bile acid
- 4) increased expression of a gene network implicated in cell cycle either directly or through expression of IGFBP1
- 5) increased expression of gene networks implicated in NADPH utilization resulting in lipid oxidation either directly or indirectly through expression of CYP7a1

These concerted and multilayered changes in genome expression is expected to attenuate insulin resistance, improve intermediary metabolism, ameliorate liver fat accumulation, reduce hepatocyte degeneration and promote cellular regeneration.