Anti-diabetic Effects of Compound K *versus* Metformin *versus* Compound K-Metformin Combination Therapy in Diabetic *db*/*db* Mice

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Compound K (CK) is a major intestinal metabolite of ginsenosides derived from ginseng radix. In our preliminary studies, CK has shown to exhibit anti-hyperglycemic effect through its insulin-secreting action, similar to that of insulin secretagogue sulfonylureas. Metformin, a biguanide, improves insulin resistance by reducing gluconeogenesis and enhancing peripheral glucose uptake, promoting reduction of the plasma glucose level. The aim of this study was to compare the anti-diabetic effects of CK and metformin due to differences in their mechanisms of action and also to investigate whether treatment of CK and metformin in combination show synergistic or additive effects compared to each drug alone. Seven week-old male *db*/*db* mice were treated for 8 weeks. CK was given at a dose of 10 mg/kg, metformin at 150 mg/kg and the same dosage of each drug was applied to CK plus metformin combination group. Significant improvements were observed in plasma glucose and insulin levels, homeostasis model assessment-insulin resistance (HOMA-IR) index and in hematoxylin and eosin-stained liver tissues in combination group. Although further studies to elucidate the benefits of co-administration of CK and metformin are needed, our findings may provide basis to the discovery of a new combination therapy on diabetes control in type 2 diabetics.

Key words compound K; metformin; combination therapy; *db/db* mice; anti-hyperglycemic effect

Diabetes mellitus is a potentially life-threatening disease affecting major populations worldwide. Epidemiological studies and clinical trials strongly support that hyperglycemia is the principal cause of microvascular (retinopathy, neuropathy, nephropathy) and macrovascular (heart disease, stroke, amputations) complications,[1] suggesting effective blood glucose control as the key to preventing or reversing diabetic complications and improving quality of life in patients with diabetes.[2,3] In order to control hyperglycemia, oral hypoglycemic agents such as, sulfonylureas, meglitinides, biguanides, thiazolidinediones, α-glucosidase inhibitors, glucagon like peptide-1 (GLP-1) analogues and dipeptidyl peptidase IV (DPP-IV) inhibitors are currently used. Having different classes of anti-diabetic drugs with their sites of action being different, accumulating evidences suggests that combination therapy using these agents may be highly effective in achieving and maintaining target blood glucose levels.[4—6]

The root of ginseng has been used for remedies in traditional Chinese medicine. The pharmacological properties of ginseng are mainly attributed to ginsenosides, which are the active components found in the extracts of different species of ginseng.[7] Numerous studies have been conducted and found the anti-diabetic effects of ginsenosides,[8—11] however, the active component responsible for this anti-diabetic action has yet been unknown. Compound K (CK), a final metabolite of protopanaxadiol ginsenosides,[12,13] is known to have anticancer, anti-pruritic, apoptotic and hepatoprotective effects.[14—18] Despite these various effects CK possesses, antidiabetic effect has not been studied as of this writing. In our preliminary studies with protopanaxadiols, CK enhanced insulin secretion presumably by acting directly on the pancreas.

Biguanides are used worldwide for the treatment of diabetes. With the liver being the site of action, metformin (MET) decreases a hepatic glucose production and increases muscle glucose uptake and disposal, thereby lowering hyperglycemia.[19,20] Therefore, CK and MET are both effective in lowering plasma glucose level *via* different mechanisms of action. In this study, we compared the efficacy of CK and MET in diabetic *db*/*db* mice. Furthermore, we assessed the effects of a combination of CK and MET to examine the possibility that this combination may be valuable for reducing hyperglycemia efficiently.

MATERIALS AND METHODS

Drugs and Chemicals CK used in this study was obtained from Central Research Center, Ilhwa Pharmaceutical Co. (Guri-Si, Korea) in the form of a dried powder. Metformin, diazoxide and nifedipine were purchased from Sigma (St. Louis, MO, U.S.A.). Mouse insulin enzyme immunoassay ELISA kit was purchased from Shibayagi (Gunma, Japan). Plasma triglyceride (TG), total cholesterol, HDL cholesterol (HDL-C) and hemoglobin Alc kits were purchased from Asan Pharmaceutical Co. (Seoul, Korea) and the mouse adiponectin ELISA kit was purchased from Adipogen (Seoul, Korea). Tween 80 was purchased from Yakuri Pure Chemicals (Kyoto, Japan).

Cell Culture and Insulin Secretion HIT-T15 cells were kindly obtained from Dr. K. S. Suh of the Kyung Hee Medical Center (Seoul, Korea). HIT-T15 cells (between passages 75—80) were cultured in RPMI 1640 media containing 11.1 mM glucose with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C, atmosphere 5% CO_2/O_2. Media was changed every 2 d and cells were subcultured every 5—6 d. HIT-T15 cells were seeded into 24-well plate at a density of 2×10^5 cells per well and grown for 24 h. The cells were washed twice and preincubated for 30 min in Krebs–Ringers Bicarbonate (KRB) buffer [115 mM NaCl, 4.7 mM KCl, 2.56 mM $CaCl_2$, 1.2 mM KH_2PO_4,

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1.2 mM $MgSO_4$, 20 mM $NaHCO_3$, 16 mM HEPES and 0.3% bovine serum albumin, pH 7.4]. Cells were then treated with KRB buffer containing 5 mM glucose with or without CK, and incubated for 1 h at 37 °C. After incubation, aliquots of the media were stored at −20 °C until insulin measurement. To explore how CK augments the glucose-stimulated insulin secretion, HIT-T15 cells were incubated for 1 h in KRB buffer containing either 0.5 mM diazoxide or 10 μM nifedipine in the absence or presence of CK (8 μM), and insulin concentration was measured.

Oral Glucose Tolerance Test (OGTT) in ICR Mice The ICR mice were fasted for 12 h prior to the experiment, and CK (12.5, 25 mg/kg) was administered orally 30 min prior to glucose challenge. Glucose (1.5 g/kg) was orally administered at 0 min, and the blood was withdrawn at 0, 30, 60 and 120 min after glucose administration. Plasma glucose and insulin levels were determined by the glucose oxidase method and mouse insulin ELISA kit, respectively.

Anti-diabetic Effects in *db*/*db* Mice. Animals Five-week-old male C57BL/KsJ *db*/*db* mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and were acclimatized in a room with 12–12 h light–dark cycle (8:00 A.M. to 8:00 P.M.), a temperature of 24±1 °C, and a humidity of 55±5%. Throughout the experimental period, animals were fed with standard rodent chow (LabDiets, U.S.A.) and water *ad libitum*. At seven-week-old, mice were randomly divided into four groups; diabetic control group (DC) and three treatment groups. Compound K was given at a dose of 10 mg/kg (CK), metformin at 150 mg/kg (MET) and the same dosage of each drug was applied to compound K plus metformin combination group (CK+MET). Body weight and blood glucose levels were measured every week. At the end of the study, blood was collected for plasma insulin, adiponectin and lipid level measurement. After sacrifice, liver was immediately removed and instantly soaked in liquid nitrogen and stored at −70 °C for morphological examination.

Hemoglobin A1c Using blood samples collected from *db*/*db* mice in the fasting state, percent HbA1c was measured with a Hemoglobin A1c kit according to the manufacturer's instructions.

Insulin and HOMA-IR Mouse insulin enzyme immunoassay ELISA kit was used to measure the plasma insulin concentration. Insulin resistance was determined by the homeostasis model assessment (HOMA) method by using the following equation: HOMA value for insulin resistance (HOMA-IR)=fasting insulin (μU/ml)×fasting glucose (mmol/l)/22.5.[21)]

Hepatic Histology Liver was removed and fixed in 10% neutral buffered formalin. The tissues were subsequently embedded in paraffin and sectioned with thickness of 5 μm using a microtome (Leica, Wetzlar, Germany). Tissue sections prepared onto aminosilane-treated slides were deparaffinized and rehydrated through graded alcohols to distilled water. Tissue sections were stained with hematoxylin–eosin and mounted with Canada balsam before analyzing under microscope (Olympus, Japan).

Statistical Analysis Data are expressed as mean values± S.D. and comparisons of data have been done by unpaired Student's *t*-test or ANOVA, as appropriate. Mean values were considered significantly different when $p<0.05$.

RESULTS

Effect of CK on Insulin Secretion To explore whether CK augments a glucose-stimulated insulin secretion, different concentrations of CK were treated to HIT-T15 cells. CK at the concentration range between 1 and 8 μM augmented a glucose-stimulated insulin secretion in a concentration dependent manner with the maximal response occurring at 8 μM (Fig. 1A). Next, to examine how CK enhances a glucose-stimulated insulin secretion, diazoxide (K^+ channel opener) and nifedipine (L-type of Ca^{+2} channel blocker) were used. Diazoxide (0.5 mM) blocked a glucose-induced insulin secretion from 31.9±2.7 to 15.4±1.2 μU/ml in HIT-T15 cells ($p<0.01$, Fig. 1B). In HIT-T15 cells supplemented with 5 mM glucose and 8 μM CK, diazoxide suppressed the insulin secretion to a level observed in 5 mM glucose with diazoxide ($p<0.001$). The addition of 10 μM nifedipine also reduced the insulin secretory effect of CK from 47.5±2.5 to 19.3±0.6 μU/ml ($p<0.001$, Fig. 1B), to a level observed in cells incubated with 5 mM glucose and nifedipine (18.5± 2.0 μU/ml).

OGTT in ICR Mice OGTT was performed to determine the effect of a single oral dose of CK on glucose tolerance and insulin secretion using the ICR mice (Fig. 1). Glucose challenge dramatically increased the blood glucose levels in control group mice, whereas CK-treated groups significantly prevented the blood glucose levels from rising, especially at 30 min after glucose load ($p<0.05$, Fig. 1C). When the area under the curve (*AUC*) was compared between groups, CK12.5 and CK25-treated groups (12.5, 25 mg/kg dose) showed 9% and 15% ($p<0.05$) reduction, respectively, compared to that of control group (inset at upper right corner of Fig. 1C). Plasma insulin level at 30 min after glucose load in control group was 31.9±0.8 μU/ml, whereas insulin levels in CK12.5 and CK25-treated groups were 35.8±1.8 ($p<0.05$) and 37.5±1.1 μU/ml ($p<0.01$), respectively, indicating that CK lowered the blood glucose levels by enhancing insulin secretion (Fig. 1D).

Effects of CK, MET and CK+MET on Metabolic Parameters Table 1 shows the effects of CK, MET and CK+MET on metabolic parameters in diabetic *db*/*db* mice treated for 8 weeks. Compared to DC, weight gain was decreased in both CK and MET groups; however, this decrease in weight gain was not observed in CK+MET combination group. All treatment groups showed increase in food intake, and water intake was increased in CK+MET group compared to DC. The HbA1c levels of treatment groups were all significantly lower than that of DC. With HbA1c level 5.9±0.2% in DC, CK showed 4.9±0.5% ($p<0.01$), 4.9±0.2% for MET ($p<0.01$) and 5.0±0.1% for CK+MET ($p<0.01$). The plasma adiponectin level was also determined at the end of the experiment. Although all treatment groups showed increase in the plasma adiponectin levels compared to DC, only CK group showed the significance. At the end of the experiment, mice were sacrificed and their liver and fat tissues were removed. When the epididymal fat was weighed, significant difference in fat weight/body weight (%) was observed in MET (5.4±0.4%, $p<0.01$) compared to DC (6.3±0.5%). The plasma lipid levels triglyceride, cholesterol, HDL-cholesterol and LDL-cholesterol levels were also determined; however, no significant differences were observed.

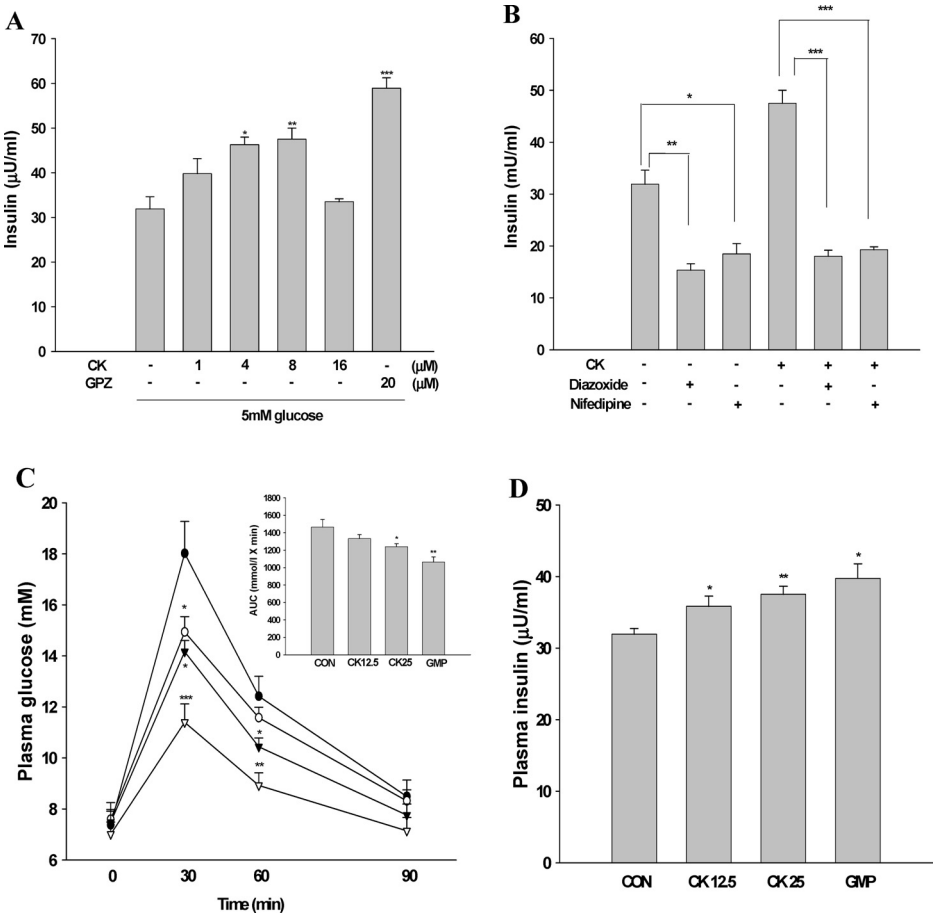

Fig. 1. Effect of CK on Insulin Secretion

Insulin concentration was measured in HIT-T15 cells treated with CK at different doses (A), and in the presence of diazoxide or nifedipine (B). Plasma glucose (C) and insulin levels at 30 min (D) were determined after oral glucose challenge (1.5 g/kg) after 12 h of food deprivation in ICR mice. Inset at the upper right corner of C indicates the area under the curve. Plasma glucose level was measured in control mice (●), mice with 12.5 mg/kg CK (○), mice with 25 mg/kg CK (▼) and mice with 2 mg/kg glimepiride (▽). Values are means±S.D. of six or seven mice. $*p<0.05$, $**p<0.01$, $***p<0.001$ compared to control.

Table 1. Effects of CK, MET and CK+MET on Metabolic Parameters

Parameter	DC	CK	MET	CK+MET
Weight gain (g)	26.1	25.1	23.7	27.3
Food intake (g/mouse)	219.8	234.5	233.3	233.0
Water intake (ml/mouse)	227.0	212.8	223.5	243.8
HbA1c (%)	5.9±0.2	4.9±0.5**	4.9±0.2**	5.0±0.1**
Plasma adiponectin	124.1±0.3	131.3±0.8*	138.6±23.1	128.7±17.5
Epididymal fat				
Weight (g)	3.5±0.5	3.4±0.4	2.9±0.3	3.4±0.4
Fat/body (%)	6.3±0.5	6.0±0.2	5.4±0.4**	6.0±0.6
Plasma lipid				
Triglyceride (mg/ml)	96.2±9.3	79.5±6.8	96.3±6.0	89.5±3.5
Cholesterol (mg/ml)	203.2±13.2	187.2±7.1	181.1±9.9	185.6±3.0
HDL-cholesterol (mg/ml)	67.8±11.1	61.8±5.4	74.8±2.1	75.6±5.6
LDL-cholesterol (mg/ml)	116.1±10.9	82.1±13.3	92.7±15.9	90.7±17.4

Values represent the mean±S.D. ($n=6$). $*p<0.05$, $**p<0.01$ *vs.* DC.

Effects of CK, MET and CK+MET on Plasma Glucose, Insulin and Insulin Resistance Index The effects of CK, MET and CK+MET on plasma glucose, insulin and HOMA value for insulin resistance in diabetic *db*/*db* mice are shown in Table 2. When the plasma glucose levels were measured at the end of the experiment, all treatment groups [CK; 7.5±0.6 mM ($p<0.01$), MET; 7.5±0.5 mM ($p<0.01$), CK+MET; 7.1±0.4 mM ($p<0.001$)] showed significantly decreased plasma glucose levels compared to DC (10.5±0.8 mM) with CK+MET treatment group being the most effective. The plasma insulin level was also detected. Compared to 405.9±27.5 μU/ml in DC, the plasma insulin concentrations of CK, MET and CK+MET were 309.6±44.7 μU/ml, 167.2±10.4 μU/ml ($p<0.05$) and 65.5±17.9 μU/ml ($p<0.05$), respectively. With the plasma glucose and insulin levels in each group, insulin resistance index was calculated

Table 2. Effect of CK, MET and CK+MET on Plasma Glucose, Insulin and HOMA-IR Index

	Plasma glucose (mM)		Insulin (μU/ml)	HOMA-IR
	Initial	Final		
DC	4.5±0.5	10.5±0.8	405.9±27.5	189.3±14.6
CK	4.4±0.6	7.5±0.6**	309.6±44.7	103.4±8.0***
MET	4.5±0.4	7.5±0.5**	167.2±10.4*	56.0±4.0***
CK+MET	4.4±0.5	7.1±0.4***	65.5±17.9*	20.7±1.7***

Values represent the mean±S.D. (n=6). Plasma glucose and insulin were analyzed in plasma samples obtained from blood of 12 h fasted mice. *p<0.05, **p<0.01, ***p<0.001 *vs.* DC.

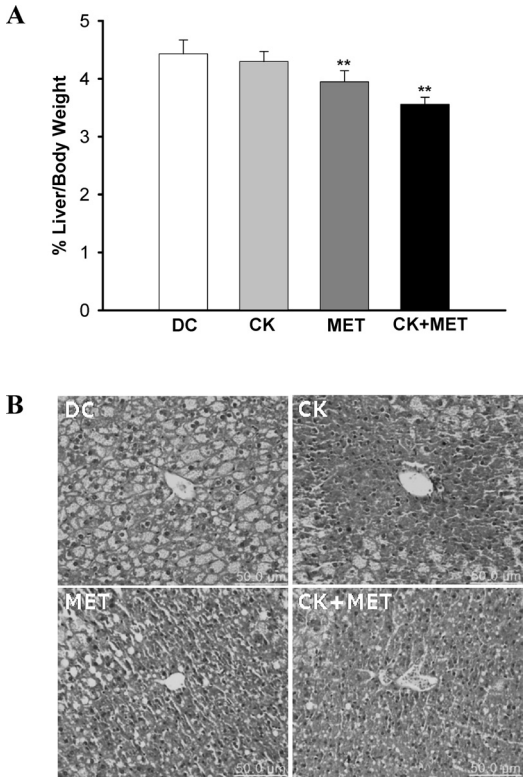

Fig. 2. Effect of CK on the Structure of Hepatocyte in *db*/*db* Mice

(A) Liver weight/body weight ratio. (B) Light microscopic observation of *db* mice stained with hematoxylin and eosin. Magnification of histological sections ×200. **p<0.01 compared to diabetic control.

using the equation presented in Materials and Methods. Due to the plasma glucose and insulin lowering effects of CK, MET and CK+MET, HOMA-IR indices of all treatment groups [CK; 103.4±8.0 (p<0.001), MET; 56.0±4.0 (p< 0.001), CK+MET; 20.7±1.7 (p<0.001)] were significantly reduced compared to DC, especially the CK+MET group showed the lowest insulin resistance index.

Effects of CK, MET and CK+MET on Liver/Body Weight Ratio and Hepatic Histology To examine the effects of CK, MET and CK+MET administration on the liver of diabetic *db*/*db* mice, the weight of liver tissue was measured and the liver weight/body weight (%) ratio was calculated. MET and CK+MET showed statistically significant differences when compared to the DC (p<0.01) (Fig. 2A). The hematoxylin and eosin-stained paraffin sections of liver tissues from DC, CK, MET and CK+MET groups are shown in Fig. 2B. Large lipid droplets are observed in the DC group. These lipid droplets are observed less in the treatment groups, especially in the CK+MET combination group.

DISCUSSION

Compound K (CK) is a final metabolite of protopanaxadiol ginsenosides. Although panax ginseng is known to have anti-diabetic activity, the active ingredient is not yet fully identified. In our preliminary studies, protopanaxadiol ginsenosides showed the insulin secretion-stimulating activity. Therefore, it would be interesting to know whether and how CK has an anti-diabetic activity. *In vitro* studies using HIT-T15 cells, CK enhanced the insulin secretion in a concentration dependent manner (Fig. 1A). This effect, however, was completely abolished in the presence of diazoxide or nifedipine (Fig. 1B). Insulin secretion-stimulating activity of a single oral CK administration was also confirmed in OGTT using ICR mice (Figs. 1C, D). From these studies, we may conclude that CK lowered the plasma glucose level by stimulating an insulin secretion and this action was presumably due to the blockade of ATP sensitive K^+ channel.

Metformin (MET), a biguanide that reduces hyperinsulinemia and improves hepatic insulin resistance,[22,23] is used as an oral anti-hyperglycemic agent to treat type 2 diabetics. Although metformin became available for diabetes in the 1950s the mechanism by which it improves insulin sensitivity remained unclear until finding that metformin activates AMP-activated protein kinase (AMPK)[24] and inhibits mitochondrial respiratory complex I,[25] mitochondrial permeability transition[26] and tyrosine phosphatase activity.[27]

The aim of this study was to firstly compare the efficacy of CK and MET and secondly to assess the effects of its combination in diabetic *db*/*db* mice. As shown in Tables 1 and 2, plasma glucose and hemoglobin A1c in CK (10 mg/kg) and MET (150 mg/kg) monotherapy groups were comparable. Plasma insulin level, on the other hand, was significantly low in the MET treated group, compared to CK, resulting in low HOMA-IR index. Furthermore, it is worth noticing that CK+MET combination therapy showed remarkably low plasma insulin level and HOMA-IR index. Action mechanism(s) for this marked reduction of plasma insulin level in combination group remains to be established. However, we also found that CK treatment ameliorated an insulin resistance through suppressions of endogenous glucose production (unpublished microarray and enzyme activities data) and lipogenesis in the liver (Fig. 2). In addition, CK has been shown to activate phosphorylation of AMPK in the HIT-T15 cells (unpublished result). With these observations, we speculate that CK+MET group could lower plasma insulin level and consequently HOMA-IR index more effectively than each drug alone.

Type 2 diabetes strikes 3—7% of adults in most westernized societies, and more than 160 million people worldwide. Rendered by an increasing obesity epidemic, the prevalence of type 2 diabetes is expected to more than double in the next 25 years creating a major healthcare challenge.[28] Insulin resistance is an early and sustained feature of type 2 diabetes.[29] When insulin concentrations are insufficient to compensate for insulin resistance, then hyperglycemia comes about. A therapeutic strategy to address both the hyperglycemia and the insulin resistance is, therefore, rational.

Recently, the importance of reducing not only fasting hyperglycemia, but also postprandial hyperglycemia, has been proposed. Postprandial hyperglycemia has shown to be an independent risk factor for the development of macrovascular complications.[30] Now, combining an insulin secretion enhancer with an insulin sensitizer is considered to increase effectiveness of hyperglycemia control. Given these circumstances, it is reasonable to consider the combination of an insulin secretion enhancer (compound K) and an insulin sensitizer (metformin) as a candidate to control hyperglycemia effectively.

Compared to monotherapy, CK+MET combination therapy was more effective in lowering plasma glucose level, plasma insulin concentration and HOMA-IR index. These results raise the possibility that CK+MET combination may be valuable for improving diabetic conditions efficiently. Combination treatment of CK and MET at different concentrations; for example, CK 20 mg/kg and MET 300 mg/kg, may also be taken into consideration for this may lead to finding the optimal dosage with the optimal effect.

Almost a quarter of adults in many industrialized countries have excessive fat accumulation in the liver.[31] Although the cause of fatty liver is not known, it is often associated with obesity and type 2 diabetes. Lin *et al.*[32] demonstrated that metformin improved fatty liver disease, reversing hepatomegaly, steatosis and aminotransferase abnormalities. We also examined the effects of CK, MET and CK+MET on fatty liver disease in *db*/*db* mice. After 8 weeks of treatment, the liver/body weight ratio was markedly reduced in CK+MET treated group, when compared to the CK or MET alone group (Fig. 2A). Hepatic histology also demonstrated that steatosis presented in the control mice has virtually disappeared from the livers of CK+MET treated mice (Fig. 2B). Therefore, addition of CK (*i.e.* CK+MET combination therapy) has shown to enhance the beneficial effect metformin has on the liver, suggesting the possibility of its use along with metformin on non-alcoholic fatty liver disease.

In summary, this comparative study shows that anti-hyperglycemic effects of CK and MET are comparable with doses of 10 and 150 mg/kg, respectively. In addition, the combination of CK and MET improved the plasma glucose and insulin levels, resulting in HOMA-IR index more efficiently, suggesting that a combination of CK and MET may be very useful in clinical practice for the effective improvement of hyperglycemia and insulin resistance.

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