The novel treatment of SGLT2 inhibition works on type 2 diabetes mellitus

(**Please only read abstract, introduction and discussion.)**

Abstract

SGLT2 inhibition is the novel treatment for treating diabetes, which is more efficient than regular treatments such as metformin etc. The study was to determine the effects of SGLT2 inhibition on glucose tolerance and on nephropathy, kidney disease and glucose transporter expression. Moreover compared with treatment metformin and co-therapy (metformin + EMPA combination treatment). After 20 weeks treatment for 6 mice groups, the study were collected results for metabolic caging, glomerular filtration rate (GFR), oral glucose tolerance test (OGTT), area under glucose curve (AUGC), Albumin standard assay, Western blot, and Periodic Acid Schiff's (PAS) staining. The results were analysed by one-way ANOVA in Tukey's multiple comparison tests, which had been concluded that co-administration of metformin and EMPA was more efficient on treating hyperglycemia than mono-therapy of EMPA, and EMPA treating on diabetic mice might help attenuate hyperglycemia depended kidney imprison, glomerular basement membrane thicken and also hyperglycemia independent increased GFR.

Introduction

Type 2 diabetes mellitus (T2DM) is becoming the fastest growing disease in worldwide, paralleling the overweight and obesity epidemic [20]. Diabetes estimated to affect 11 million people in Australia and 9 million patients affected by Type 2 diabetes [14]. Diabetes is characterized by a combination of deficiency in insulin secretion and sensitivity. The important features of T2DM are progressive pancreatic β cell failure and non-insulin dependent mellitus, which different with Type 1 diabetes mellitus that is associated with the immune system and insulin dependency [18]. Insulin resistance is the most common feature in a T2DM patient. It is defined as losing cellular signalling in response to the hormone insulin. Most of insulin resistance is present in skeletal muscle, liver and adipose tissue [6]. There are several diabetes complications that might arise due to glucotoxicity, including microvascular diseases such as neuropathy, nephropathy and retinopathy, and macrovascular disease such as cardiovascular, cerebral and peripheral vascular disease [4].

Diabetic nephropathy would be leading result in the end of stage renal disease (ESRD) and chronic kidney disease [5][15]. The mechanisms cause the diabetic nephropathy are complex and various. The briefly description is that the early hemodynamic result in the glomerular hyperperfusion and hypertension which lead hyperfiltration of kidney and followed by increasing leakage of albumin from the glomerular capillaries and alter the structure of the kidney such as thickening of glomerular and tubular basement membrane, progressive proteinuria, renal interstitial fibrosis, focal glomerulosclerosis and podocytes injury and loss [2][10]. Therefore the increasing amount of albumin in urine excretion would be the biomarker of diabetic nephropathy [16][19]. Moreover, the development of diabetic nephropathy reduce glomerular filtration rate (GFR), which decline the kidney function and parallel with arterial hypertension, and increase cardiovascular risk [17]. Nevertheless, only 20%-40% of all diabetic patients are affected by diabetic nephropathy, although the reason about why not all patients developed diabetic nephropathy is not clear [2] [5]. Currently, the most common treatment for diabetic nephropathy is RAS blockade but these therapies only slow progression of the disease [3].

Therapies for T2DM focus on three ways that agents work toward improving glycemic control. First is increasing insulin section (insulin secretagogues), second is increasing insulin action (insulin sensitizers), third is decreasing insulin need (inhibitors of glucose reabsorption)[20]. Currently, the most common treatment for T2DM is metformin, sulphonylureas and exogenous

insulin therapy. Glibenclamide is the most popular sulfonylurea used in treatment of T2DM, and it is the oral agent as the first line of the therapy. The agent binds to a regulatory protein, called SU receptor on pancreatic β cells, and results in closure of ATP-dependent potassium channels, leading to membrane depolarization and influx of calcium through voltage calcium gate this leads to continuous insulin secretion [20]. An adverse effect of this treatment is the risk of hypoglycemia since insulin continues to be secreted from the pancreas [20]. Additionally, metformin is also the agent frequently used in first line of treatment of T2DM. The action of metformin is suppression of gluconeogenesis in the liver to decrease hyperglycemia. Some clinic reports suggest there is improvement in cardiovascular morbidity and mortality with metformin treatment [11]. However, metformin is not efficient drug during later stages of the diabetes since the gastrointestinal upset and depends on residual β-cell function. Therefore, recently, other therapies have been considered for the control of blood glucose.

Sodium-glucose co-transporters (SGLTs) allow for reabsorption of glucose in the kidney. SGLT2 is high capacity and low-affinity transporter found in apical side of proximal convoluted tubule cell (PTC) and coupled with glucose transporter (GLUT2) that located in basolateral side of the cell (**Figure 1**). SGLT2 is responsible for about 90% of glucose reabsorption into the blood. The mechanism of SGLT2 is transport of glucose against a concentration gradient into the PTC and passive reabsorption into the plasma by GLUT2 transporters, some studies found high SGLT2 expression of the diabetic patients than healthy people [4][15].

Inhibition of reabsorption of glucose is a novel therapeutic strategy to reduce the plasma glucose level. The inhibition involves lowering the renal glucose reabsorption threshold by blocking the glucose transporter. The mechanism of SGLT2 inhibition is independent of insulin secretion and there is reduced risk for hypoglycemia, making it an attractive therapeutic option. There are several SGLT2 inhibitors have been usually used in Australia, Dapagliflozin and Canagliflozin [21]. However, Empagliflozin (EMPA) was used in the study, which is a potent oral SGLT2 inhibitor, under development by Boehringer-Ingelheim [15]. Using HEK293 cells (a human embryonic kidney cell line), SGLT2 had been overexpressed and EMPA significantly blocked ^{14}C alpha methyl glucose uptake [8]. It means that EMPA cause a suppression of the signal factor expression that induced by high glucose, and this signal factor could induce renal inflammation and fibronectin, it suggests that SGLT2 inhibitor might be useful in limiting

glucose induced renal inflammation, which means the inhibitor might protect the proximal tubular cells from glycotoxicity [15].

Blockade of SGLT2 reduces plasma glucose level but increases the urinary glucose excretion, which might result in glycosuria with deleterious effects on kidney function and more water excretion and dehydration in the long term [4]. Furthermore, blockade of this co-transporter may elevate tubuloglomerular feedback since sodium delivery to the macula densa is increased, which causes a drop in glomerular filtration rate (GFR) and constrict the afferent arteriole [10][22]. While SGLT2 inhibitors have been found as effective anti-diabetic agents in animal models and humans without kidney disease, the effects on initiation and progression of renal injury in animal models of diabetic nephropathy have not been fully determined [10][15].

The aims of this study were to determine how EMPA affects glucose tolerance and renal function in a mouse model of type 2 diabetes with nephropathy, renal imprison and its effects on renal glucose transporter expression. This is in comparison with untreated mice (vehicle) and those administered with the common anti-diabetes therapy, metformin. It was hypothesized that EMPA would reduce the plasma glucose level and increase the glucose excretion in urine, but alter the way glucose is transported in the kidneys and influence glucose transporter and other kidney protein such as kidney injury molecule (KIM)-1 expression, which may have adverse effects for kidney function.

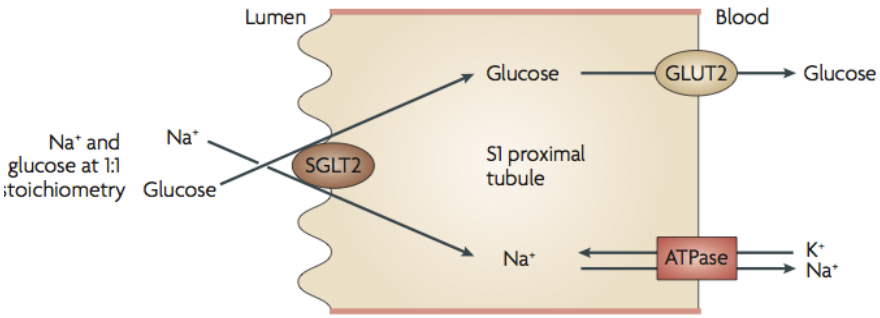


Figure 1. SGLT2 mediates glucose reabsorption in the kidney

Method

All experiments were approved by The University of Queensland Animal Ethics Committee (#462/12). Db/m mice and db/db mice were bred and housed in plastic cages with stainless steel wire lids in an environmentally controlled room at temperature 20-22°C, relative humidity 45-55%, 12h light/dark cycle (PACE Facility, The University of Queensland). All mice had access to standard mice chow. 6 groups mice were tested in this study, db/m control, db/m EMPA, db/db control, db/db EMPA, db/db metformin, db/db metformin+EMPA cotherapy, and each group contained 5 mice. A pre treatment blood sample was taken by tail tipping. Mice received oral treatment by daily gavage each afternoon from 10-20 weeks of age (EMPA: 10mg/kg/day, metformin: 250mg/kg/day, cotherapy: dose as per single therapy).

Metabolic cage measurements

Mice were weighed and placed individually in metabolic cages for 24hr at 12-13 weeks and 16-17 weeks of age to measure the food intake, water intake and urine volume. Before 24hr collection at both ages, mice were trained with a 3hr session and two days later, a 6hr session to minimise stress and associated behavioral changes. Measurements began 2 days following the acclimatization period. Food and water were weighed and mice were allowed *ad libitum* access throughout the experiment. Urine was collected in removable clean containers. After 24hr, food and water intake, as well as urine volume were recorded. Blood was collected by tail tipping, centrifuged at 3,000 rpm for 10 minutes and plasma stored in eppendorf tubes at -20°C for next biochemical analysis with urine samples.

Glomerular Filtration Rate (GFR)

The glomerular filtration rate was estimated using a miniaturized device placed on the mice back for measurement of transcutaneous decay of intravenously injected FITC-labeled sinsitrin. Measurements were performed for about 1hr and software was used to determine the half-life and calculate GFR as per manufacturers instructions [18] (**Figure 2**).

Oral Glucose Tolerance Test (OGTT)

Mice were fasted for 6 hours before an oral glucose tolerance test (OGTT). Mice were weighed to determine the volume of glucose solution to gavage. Glucose solution (2g/kg; 50% w/v glucose solution, Phebra, Lane Cove, Australia) was immediately administered directly into the stomach of mice via a 1ml syringe

attached to a 20 gauge 38mm long flexible plastic gavage needle with a 1.6mm diameter ball end (Walker Scientific, add city, Western Australia, Australia) in restrained mice [1]. Blood glucose was determined prior to glucose gavage, and at 5, 15, 30, 60 and 120 min post-gavage by tail tipping. Mice were unrestrained during the test and housed in their usual group boxes apart from being restrained during the single gavage and during blood collection.

Cull

Cull was performed on all mice. Blood glucose was determined by tail tipping, and blood sample collected into 1.5ml eppendorf tube with EDTA. Mice were injected with sodium pentobarbital (intraperitoneal; 32.5mg/ml diluted stock; multiply mouse weight by 3.08 for 100mg/kg). Reflexes were checked and once the mouse was under, a midline incision was performed. Urine was collected using 1ml syringe with 27gauge needle into bladder. Urine was stored in labeled 1.5ml eppendorf tube and frozen for later tests. The left and right kidneys were excised and weighed separately,, sliced into coronal sections and a piece fixed in 10% NBF solution for later processing. Kidney cortex was sliced away and immediately snap frozen in liquid nitrogen and placed in -80°C for later Western blot test.

Plasma membrane protein extraction, BCA Protein Assay and Western Blot

A plasma membrane protein extraction kit was used to separate cytosol and plasma membrane protein of frozen kidney cortical tissue (Biovision, Catalog #K268-50). Briefly, tissues were lysed in Homogenization Buffer and Protease Inhibitor Cocktail (10ul to 5ml buffer) and centrifuged at 700xg for 10 minutes at 4°C. The supernatant was centrifuged at 10000xg for 30 minutes at 4°C. The supernatant was the cytosol fraction. To purify plasma membrane protein, a lower phase and upper phase solution were added to the pellet and kit instructions followed. After the protein was collected, a BCA Protein Assay was performed to determine the protein concentration. 50ug of protein was mixed with Laemmli buffer/2-ME containing mercaptoethanol and heated at 95°C for 5 minutes to denature the protein. Sample and protein standard were then loaded into Bi-rad pre-cast 10% Tris-HCl gels, filled with running buffer from 10X bio-rad stock and then run the gels at 200V for 30minutes. Gels were then transferred onto PVDF membranes in transfer buffer that contained 1x Tris/Glycine (Bio-rad Cat# 162-0260) at 350mA for 1 hr. Membranes were blocked with 5% skim milk for 1hr gently rolling at room temperature. 1M TBS-T (Tris Buffered Saline with 0.1% Tween) was used to wash the membrane for 10

minutes. Membrane were incubated overnight at 4°C with the following primary antibodies: GLUT4 rabbit anti–mouse used at 1:300 (Abcam), SGLT2 rabbit anti–mouse used at 1:400 (Abcam), Kidney Injury Molecule-1 (KIM-1) rabbit anti-mouse used at 1:1000 (Abcam) and loading control β-actin rabbit anti-mouse used at 1:2000 (Sigma), diluted in 1M TBS-T containing 0.05% skim milk. Membranes were washed with 1M TBS-T 3 x 10 minutes and incubated with IR-secondary antibody for 1 hr at room temperature. Membranes were washed in 1M TBS-T 3 x 10 minutes and visualized using the Li-cor infrared (IR) fluorescent detection.

Periodic Acid Schiff's Staining

Periodic Acid Schiff's stain was used to assess the degree of kidney damage. PAS staining gave the pink image after performing, and the blue dots indicated nuclei and pink was the cytosolic fibers. Slides were dewaxed in xylene first and then rehydrated in 100% EtOH, 95% EtOH, 70% EtOH and distilled water. Slides were placed in 1% periodic acid for 5 minutes, washed briefly, and placed into Schiff's reagent for 15 minutes. Slides were washed and placed in Mayer's Haematoxylin for 5 minutes, then washed and placed in Scott's tap water briefly. Finally, slide were dehydrated from 70%, 95% to 100% EtOH. They were then cleared using xylene and coverslipped using DPX. Sections were observation was a light microscope (Nikon Brightfield) and representative images taken at x20 magnification.

Statistical Analyses

Results were given as mean ± SEM (standard error of the mean) as indicated. For comparisons, one-way ANOVA in Tukey's multiple comparison tests. Statistic and data analysis was performed using GraphPadPrism Software Version 6c (GraphPad Software, Inc.).

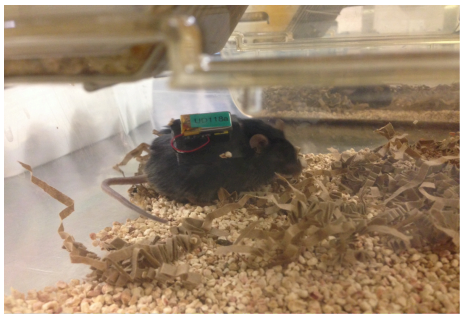


Figure 2. Mice with miniaturized device to detect the transcutaneous decay of intravenously injected FITC-labeled sinsitrin.

Results

Metabolic caging

-Water intake

Water intake during metabolic caging 1 is presented in **Figure 3**. There were no significant different between all 6 groups (P>0.05). In metabolic caging 2, the mean values for water intake were shown in **Figure 4**. All db/m mice drank less water than db/db mice (p<0.05). Empagliflozin treatment in db/db mice resulted in a significant reduction in water intake compared with db/db Control (p<0.05).

-Food intake

The mean values of food intake in metabolic caging 1 are presented in **Figure 5**. There were no differences between each group (p>0.05). **Figure 6** shows the mean value of food consumption during metabolic caging 2. There were no significant differences between each group (p>0.05).

-Urine volume

Figure 7 displays the results of urine volume recorded during metabolic caging 1 and it shows that the healthy mice (db/m) had much lower urine volume than diabetic mice (db/db) (p<0.05), and there was a trend that the drug treatments of diabetic mice decreased the urine excretion volume compared with db/db control. Furthermore, in **Figure 8**, it shows the urine volume collected in metabolic caging 2 was higher in all diabetic mice groups than healthy mice groups (p<0.0001).

Table 1. Mice number in metabolic cages for each group.

	db/m water control	**db/m EMPA**	**db/db hydroxyl control**	**db/db EMPA**	**db/db metformin**	**db/db metformin+EMPA cotherapy**
Metabolic cage 1	N=4	N=4	N=5	N=5	N=5	N=5
Metabolic cage 2	N=5	N=5	N=5	N=5	N=5	N=5

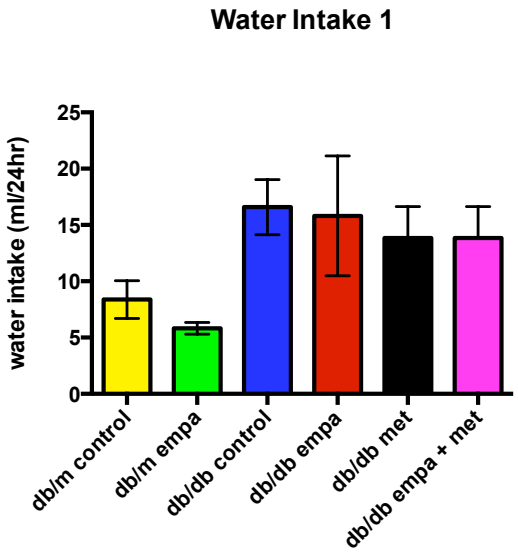


Figure 3. H_2O intake 1. H_2O intake recorded in metabolic caging at 12-13 weeks of age (n=4 for db/m control and db/m EMPA, n=5 for rest of the groups). Data presented mean=±SEM. No significant differences between each group (p>0.05).

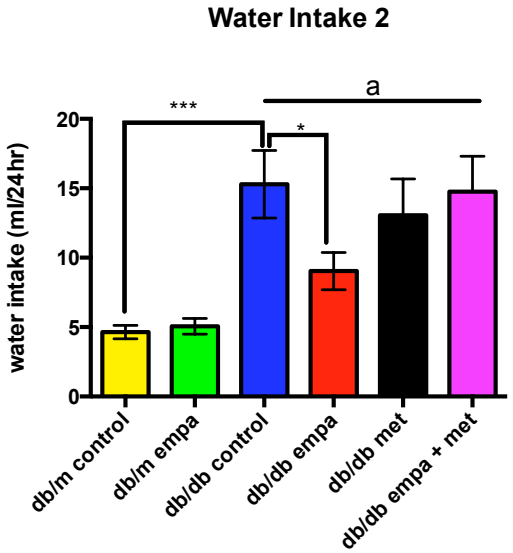


Figure 4. H_2O intake 2. H_2O intake recorded in metabolic caging at 16-17 weeks of age (n=4 for db/m control, n=5 for rest of the groups). Data presented mean=±SEM, one star: p<0.05, a: p<0.05 vs db/m groups.

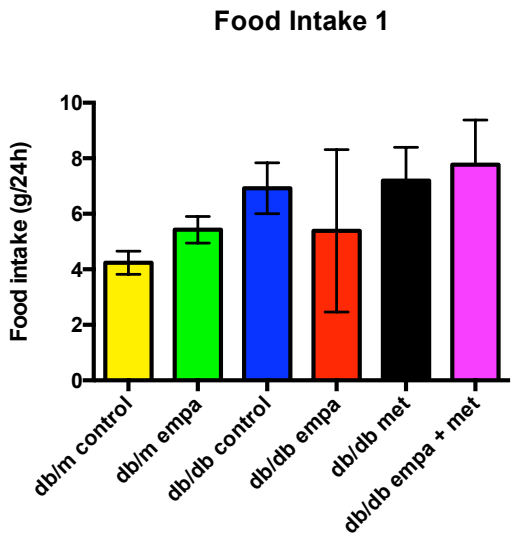


Figure 5. Food intake 1. Food intake during in metabolic caging at 12-13 weeks of age. (n=4 for db/m control and db/m EMPA, n=5 for rest of the groups). Data presented mean=±SEM. No significant differences between each group (p>0.05).

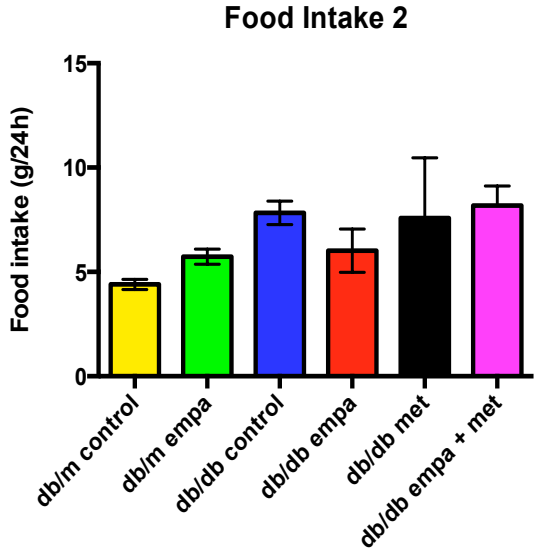


Figure 6. Food intake 2. Food intake during metabolic caging 2 at 16-17 weeks of age (n=4 for db/m control and n=5 for rest of the groups). Data presented mean=±SEM. No significant differences between each group (p>0.05).

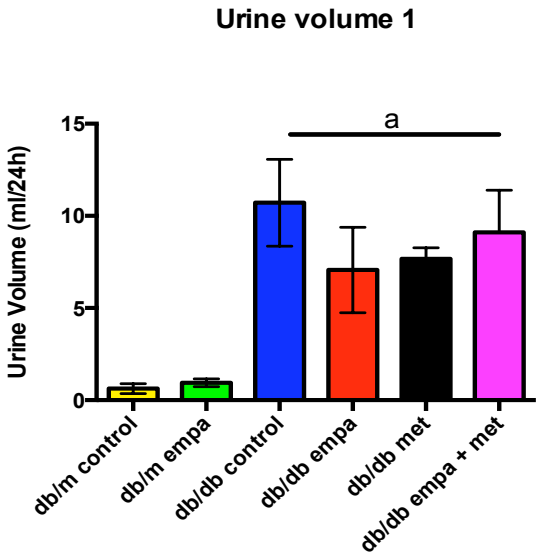


Figure 7. Urine volume 1. Urine volume recorded during metabolic caging 1 at 12-13 weeks of age (n=4 for db/m control and db/m EMPA, n=5 for rest of the groups). Data presented mean=±SEM, a: p<0.05 vs db/m groups.

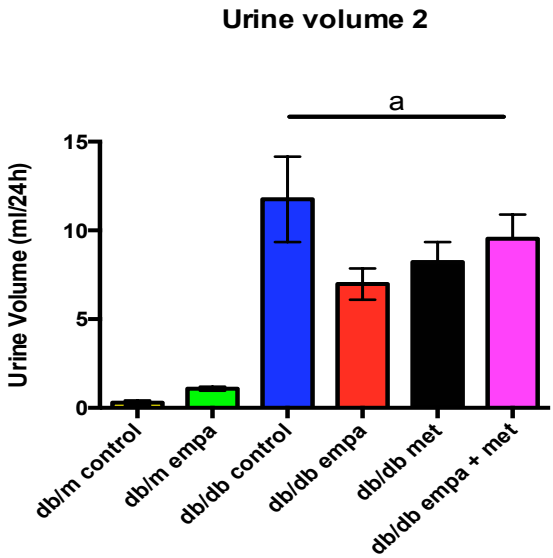


Figure 8. Urine volume 2. Urine volume collected during metabolic caging 2 at 16-17 weeks of age (n=4 for db/m control and n=5 for rest of the groups). Data presented mean=±SEM, a: p<0.5 vs db/m groups.

Body Weight and Fasting Glucose

Diabetic mice had a significant increase in body weight compared with healthy mice at all ages ($p<0.0001$, **Figure 9A-C**). However, treatments did not change the body weight in db/db mice.

In Figure **10A-C**, the fasting blood glucose of mice at week 11, week 15 and week 20 of age is presented. The db/db mice groups had significant high fasting glucose value than db/m mice groups. The treatment of EMPA in db/db mice was remarkably efficient at reducing blood glucose levels at all ages compared with db/db control ($p<0.01$). In the meantime, co-therapy was the second efficient treatment that decreased the glucose level ($p<0.01$ vs. db/db control).

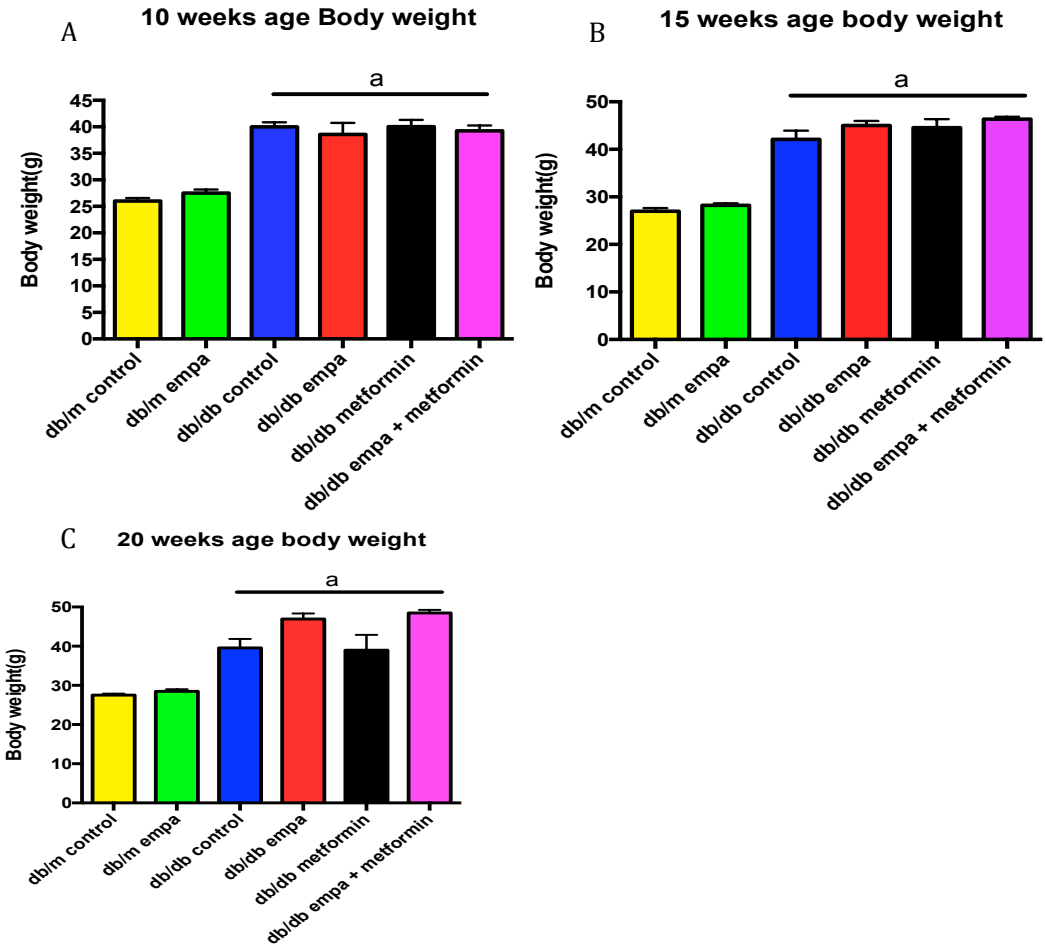


Figure 9. Body weight of mice. (A) Mice at age of 10 weeks (B) 14 weeks (C) 17 weeks (n=5 for each group). Data presented mean=±SEM, a: $p<0.05$ vs db/m groups.

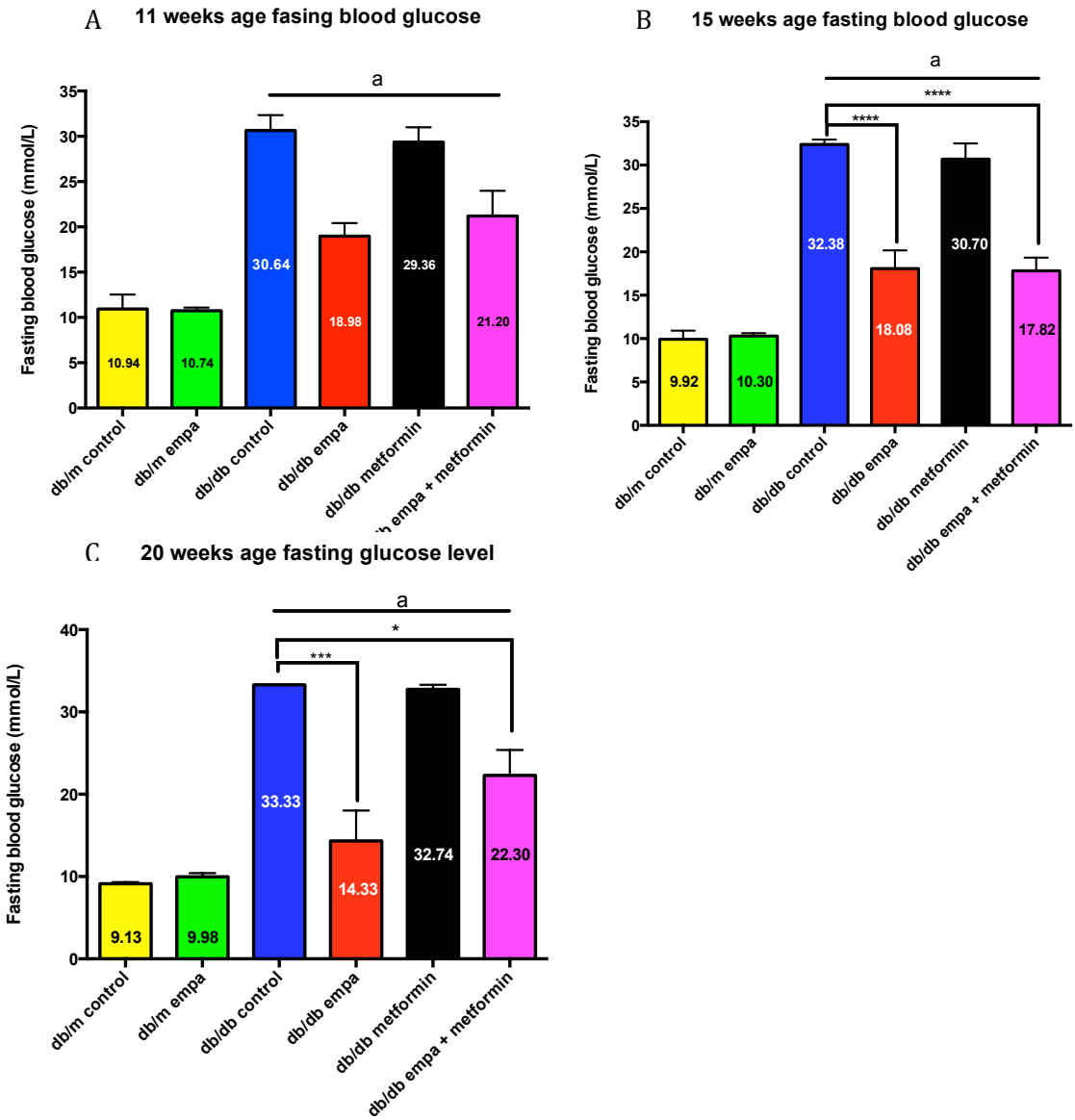


Figure 10 A-C. Fasting blood glucose of mice. (A) Mice at age of 11 weeks (B) 15 weeks, (C) 20 weeks. (n=5 per group) Data presented mean=±SEM, a: $p<0.05$ vs db/m groups, one star: $p<0.05$, two stars: $p<0.001$, three stars: $p<0.0001$, four stars: $p<0.00001$.

Glomerular Filtration Rate (GFR)

The glomerular filtration rate (GFR) is presented in **Figure 11**. It was clear to see that db/db control group had the highest value of GFR (mean=1383ul/min/100g) than db/m mice and the three therapies in db/db mice (EMPA 1016 ul/min/100g, metformin 835.3ul/min/100g, co-therapy 948.2ul/min/100g) but the only significant difference by one-way ANOVA was between db/db control and db/db metformin ($p<0.05$)

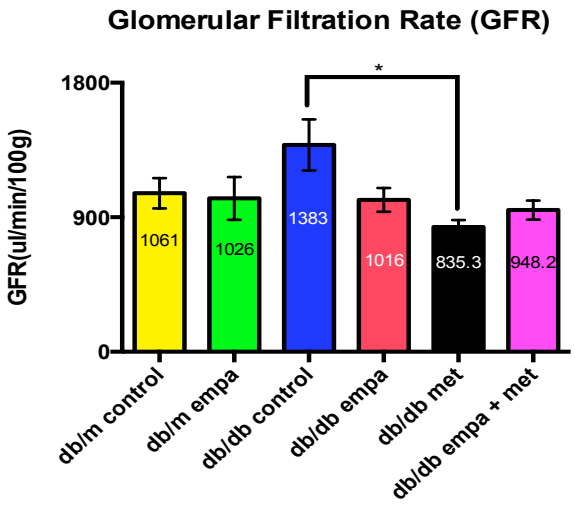


Figure 11. Glomerular Filtration Rate (GFR). (n=4 for db/m control and n=5 for rest of the groups). Data presented mean=±SEM, one star: $p<0.05$..

Oral Glucose Tolerance Test (OGTT)

Oral glucose tolerance test (OGTT) was presented in **Figure 12**. At 0 minutes, prior to glucose gavage, db/m mice had lower fasting blood glucose compared with db/db mice ($p<0.05$) expect the db/db co-therapy group. After gavage of glucose solution, all db/m mice had significantly lower blood glucose compared to db/db mice at all time points ($p<0.05$). Moreover, at 0 minutes, db/db EMPA and db/db co-therapy groups had lower blood glucose compared to db/db control ($p<0.05$). At 5 minutes after gavage, only db/db co-therapy had reduced blood glucose compared to db/db control group ($p<0.05$). Furthermore, at 120 minutes, the only significant difference to db/db control group was db/db co-therapy with lower blood glucose ($p<0.05$).

Figure 13 indicates the area under glucose curve (AUGC) (n=5 per group). Diabetic mice had higher AUGC than healthy mice ($p<0.05$). The therapies did not have a significant effect on mice for AUGC ($p>0.05$).

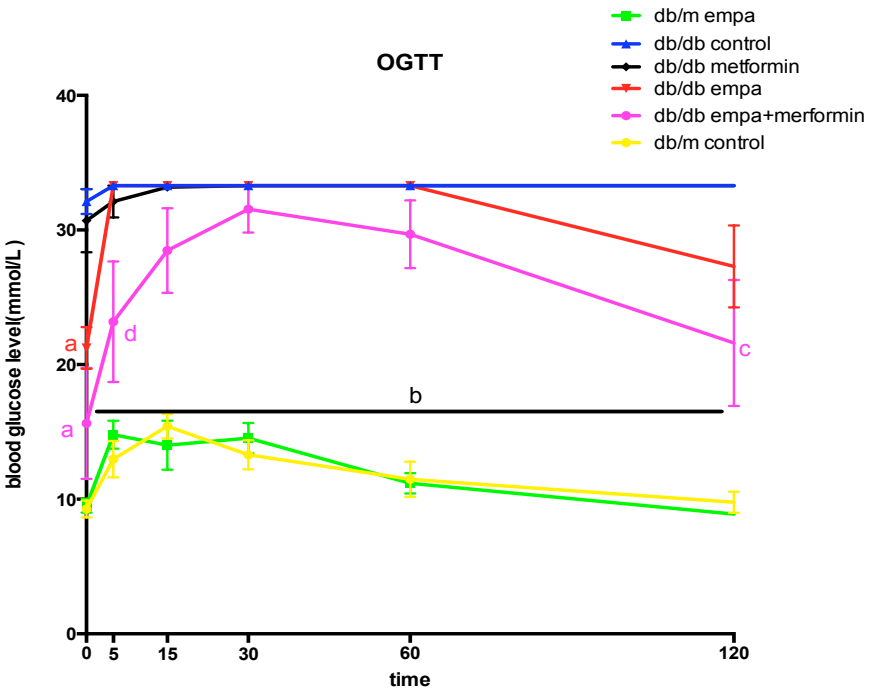


Figure 12. Oral glucose tolerance test. (n=5 for each group). Data presented mean=±SEM, a: p<0.05 vs db/db control, b: p<0.05 vs all db/db groups c: p<0.05 vs db/db control, d: p<0.05 vs db/db control.

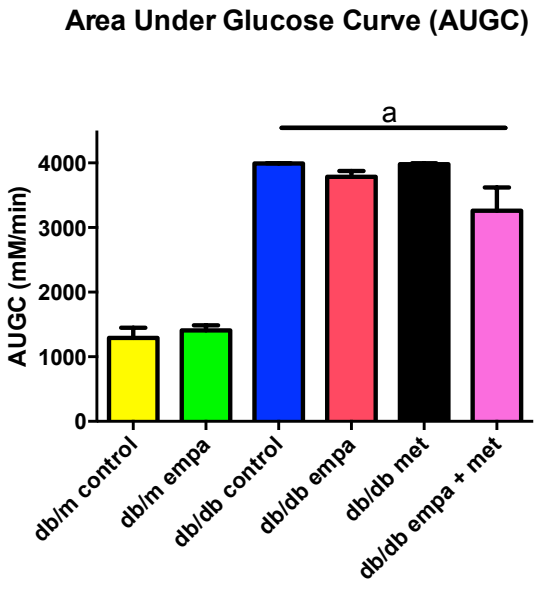


Figure 13. Area under glucose curve (AUGC). (n=5 for each group). Data presented mean=±SEM, a: p<0.001 vs db/m groups

Cull Data

There were a total of 24 mice culled in this study. **Table 2** below shows the mean body weight, blood glucose, weight of left kidney, right kidney and total kidney, and the total kidney to body weight ratio (×1000). db/m mice had lower body weight and blood glucose levels than db/db control mice ($p<0.05$). Furthermore, the body weights of treatment groups were higher than control groups ($p<0.05$). Moreover, db/db EMPA and db/db co-therapy had lowest ratio of kidney and body weight compare to db/m groups and db/db control ($p<0.05$).

Table 2. Body and organ weights and blood glucose levels at cull. (n=4-5/group) Data presented mean±SEM. a: $p<0.05$ vs db/db groups. b : $p<0.001$ vs db/db groups. c: $p<0.05$ vs db/m control. d: $p<0.05$ vs db/m EMPA. e: $p<0.05$ vs db/db control.

Mouse #	Db/m control	Db/m EMPA	Db/db control	Db/db EMPA	Db/db metformin	Db/db EMPA+met
Body weight (g)	27.50± 0.42[a]	28.76 ±0.41[a]	37.90± 3.63	46.20 ±2.05	38.70 ±3.89	48.14 ±0.94
Blood glucose	9.13 ±0.19[b]	9.52 ±0.32[b]	33.30	22.70 ±6.81	32.74 ±0.56	22.14 ±3.27
Left kidney (g)	0.17 ±0.01	0.17	0.18	0.18 ±0.01	0.19 ±0.02	0.19 ±0.01
Right kidney (g)	0.17 ±0.01	0.18 ±0.01	0.21 ±0.01	0.20 ±0.01	0.19 ±0.05	0.21
Total kidney (g)	0.34 ±0.02	0.34 ±0.01	0.39 ±0.01	0.38 ±0.02	0.39 ±0.04	0.40 ±0.01

Albumin standard assay

The albumin assay is presented in **Figure 14**. There was a significant increase in albumin excretion rate in all db/db mice compared to db/m mice at both ages during metabolic caging ($p<0.05$), but no significant effect of treatment.

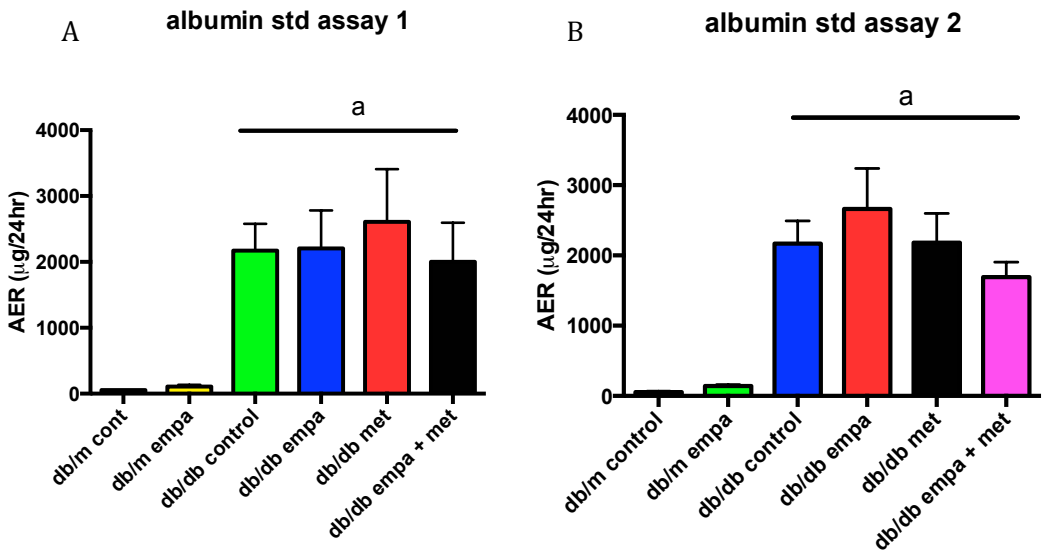


Figure 14. A & B Albumin excretion rate. Age of A) 12-13 weeks and B) 16-17 weeks. Data presented mean±SEM, (A: n=1 db/m control, n=4 db/m EMPA, db/db EMPA, n=5 rest of the groups; B: all groups n=5), a: $p<0.05$ vs db/m mice.

Western blot

The results of Western blot are shown below. **Figure 15** shows GLUT4 protein expression. The bands were not very clear, but it showed that bands for the membrane fraction were all around 50KDa (expected size of GLUT4), and metformin, co-therapy treatment for db/db mice had darker bands than other groups. Additionally, cytosol protein did not give any clear results. **Figure 16** gave the results of KIM-1. It indicates that all bands are about 50KDa, and db/m EMPA and db/db control had greater protein expression.

The results of SGLT2 are shown in **Figure 17**, there were two bands for each groups, and all around 50 KDa. In the figure, it showed membrane bands in metformin group and co-therapy group had higher values of protein than other groups, but they were loaded onto a different gel so it is difficult to compare accurately at this stage. Moreover, the cytosol bands in all the groups were not clear.

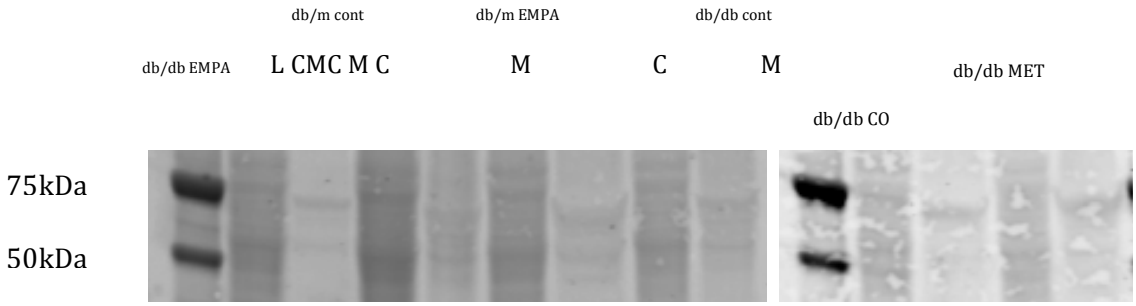


Figure 15. Glucose transporter 4 (GLUT4). L=protein ladder, C=cytosol fraction, m=membrane fraction.

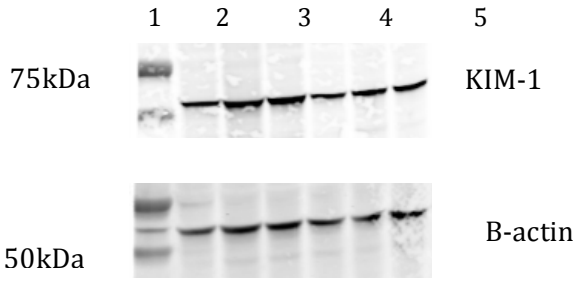


Figure 16. Kidney injury molecule (KIM)-1 expression in cytosol fractions. Lane 1=ladder, lane 2=db/m control, lane 3=db/m EMPA, lane 4= db/db control, land 5= db/db EMPA, lane 6= db/db metformin, lane 7=db/db EMPA+metformin.

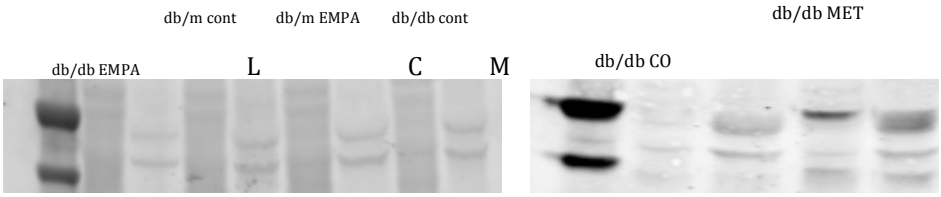
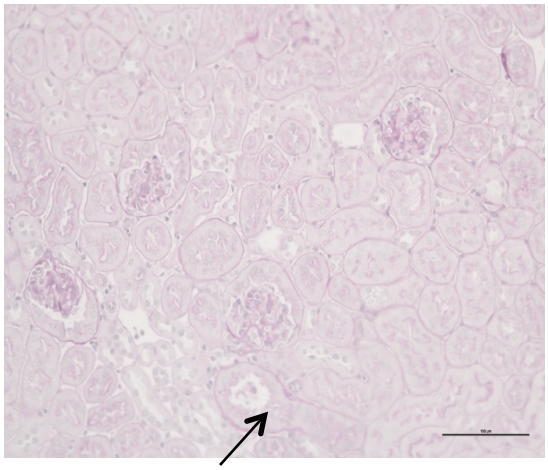


Figure 17. SGLT2. L=protein ladder, C=cytosol fraction, m=membrane fraction.

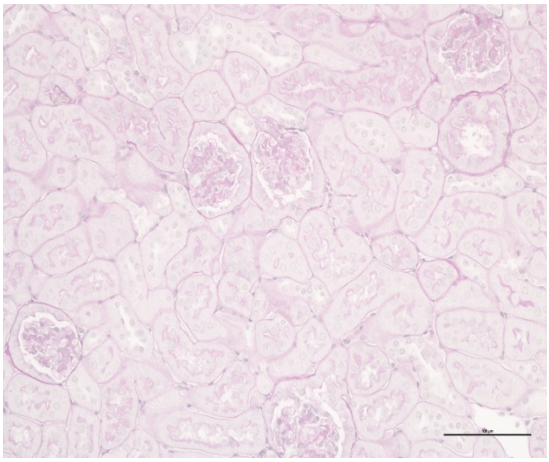
Kidney histology

PAS of fixed kidney tissue is presented below (**Figure 18 a, b, c, d**). The blue dots indicated the nucleus and the pink indicates collagen fibre. In db/db control, there was greater degree of glomerular fibrosis than db/m groups, determined by more intense pink staining and basement membrane thickening. Moreover, there was evidence for tubular damage in these mice. Empagliflozin treatment in the db/db group improved the degree of kidney damage.

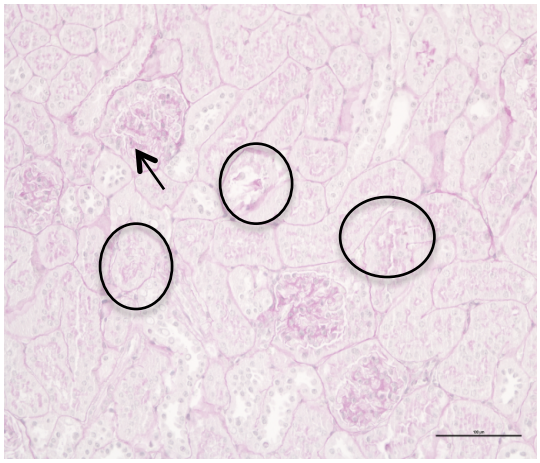
13-142 db/m control (a)



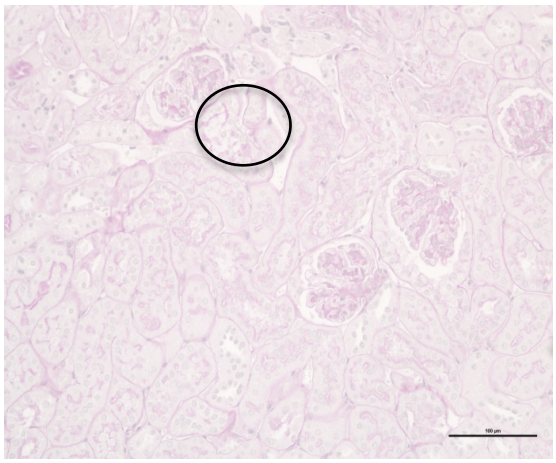
13-165 db/m empa (b)



13-171 dbdb control (c)



13-175 dbdb empa (d)



Scale = 100µm

Figure 18 a-d. PAS staining of each group in scale 100µm. Arrow indicated glomerular basement membrane thickening, circle indicates tubular damage.

Discussion

The aims of this study were to determine the effects of SGLT2 inhibition on renal function in T2DM mice and also on glucose transporter expression in kidney. The findings showed that after EMPA treatment, the glomerular basement membrane was thinner than db/db control based on PAS staining result and also KIM-1 in western blot results did not have high expression than other group, which means it might help to protect the kidney cell.

SGLT2 inhibitors reduced blood glucose level in type 2 diabetes mellitus. The fasting glucose levels were reduced after treating with EMPA and co-therapy (EMPA + metformin) in diabetic mice, but no difference was seen between these two treatments, which mean it was unclear to discover which treatment was more efficient.

However, according to oral glucose tolerance test (OGTT), co-therapy for diabetic mice had the highest efficacy for glucose clearance in blood. Oral glucose tolerance test (OGTT) was considered to be more sensitive than the test for fasting blood glucose level. The OGTT allows for an estimation of pancreatic β-cell secretory function and insulin sensitivity, in the mean time, the plasma insulin levels will be quantified which will help to indication of βcell secretory function and sensitivity more accurate. They help in the diagnosis of T2DM and evaluation of severity or progression of the disease. The co-administration of metformin and EMPA enhances the effects of treatment, which elicits the biological or medicinal response in a tissue system, animal or human [13]. It might be a reasonable idea that co-therapy treatment for diabetic mice is more efficient than mono-treatment, which was supported by the OGTT results in this study. Whereas, the metformin mono-therapy did not give any significant effect on treating hyperglycemia in the weekly fasting blood glucose test or oral glucose tolerance test. Although metformin works in the liver to inhibit gluconeogenesis to treat hyperglycemia and remit T2DM, most patients need further treatments in order to maintain normal or near-normal glycaemia [12]. Therefore, co-administration of EMPA and metformin was the most efficient treatment for glucose tolerance than mono-therapies.

Glomerular filtration rate (GFR) is a measure of kidney filtration function or kidney function. There was a trend for db/db control to hyperfilter compared to db/m groups, but not significant. All the treatments for diabetic mice reduced GFR compared with the db/db control, but the only significant difference was in the metformin group. The reasons of hyperfiltration in diabetes mice are caused

by more glucose and sodium co-transport into proximal tubular cell, there is the reduction in Na^+ delivery to macula densa, which reduce Na-Cl-K concentration at macula densa, and through tubuloglomerular feedback which possible decrease the hydrostatic pressure in Bowman space and increase the GFR. The hyperfiltration is dependent on Na^+ concentration at macula densa, which is independent on hyperglycemia [22]. Therefore the decreasing of GFR in EMPA group and co-therapy group were might because the reabsorption of glucose and Na^+ reduced via SGLT2 inhibition, it might help to lower the diabetic-induced high GFR. Otherwise, the GFR decreased in the metformin group, since metformin was not an efficient treatment for diabetic in the study, which might be not able to defence kidney disease induced by hyperglycemia such as glomerulosclerosis, nephropathy etc. Furthermore, metformin did not improve blood glucose level in the study, the maximum detection limit of the glucometer device was reached to 33.3mmol/L, which read all 'high', the plasma glucose elisa would might show the plasma glucose was lower in metformin group in the future detection, and which might be enough of a decrease in blood glucose drop the GFR.

However, the protein expression of kidney injury molecule (KIM) -1 was greater in db/m EMPA and db/db control, which means kidney injury might be present in db/m EMPA and db/db control group. As hyperglycemia is known to damage kidney cells and thicken the glomerular basement membrane which same as observed from the PAS stains results [9], it might explain why KIM-1 protein expression was raised in db/db control group. After treating EMPA in diabetic mice, the KIM-1 protein expression was normal, which mean EMPA might help reduce kidney injury, which cause by hyperglycemia. Some studies demonstrate that SGLT2 inhibition could maintain in patients with severe renal impairment [7][22] On the other hand, EMPA in healthy group mice was showed that KIM-1 protein expression was increased might because blocking SGLT2 inhibition disturbed normal glucose reabsorption. Since more sodium might be delivered to macula densa and elevate tubuloglomerular feedback which may induce constriction of the afferent arteriole, leading to reduced blood flow into the kidney, which might cause the injury of kidney [10]. Furthermore, glucosuria is a potential risk for urinary tract and genital infection, which may have raised markers of kidney injury in db/m EMPA group [3][4].

The detection of kidney glucose transporter expression was also preformed by Western blot; glucose transporter type 4 (GLUT4) and SGLT2 were tested individually. However, the results of GLUT4 were not clear and it was difficult to

analyse the results, it might because high protein concentration loaded into gel, which distressed the band moving. Whereas the results of SGLT2 showed that metformin treatment and co-therapy treatment increased its protein expression than other groups. The reason metformin treatment was associated with high SLGT2 expression in the study was unclear and requires further investigation.

In order to test kidney pathology in the study, PAS staining was performed. The representative images show db/m control mouse had less intense pink staining than other groups, which compared with db/db control that the thick glomerular basement membrane correlated with the KIM-1 Western blot result. Also, more tubular damage existed in db/db control than db/db EMPA, which means EMPA treatment in diabetic mice may help ease the kidney damage induced by hyperglycemia and might protect the glomeruli and tubules. The probable explanation was that EMPA helped to lower hyperglycemia, which decreased the GFR to normal level after treatment. This prevented a high perfusion pressure induced by hyperglycemia through the kidney. In addition, it indicates that EMPA attenuated the glomerular size, and molecular markers of kidney growth, inflammation [22]; therefore EMPA might help protect the kidney cells in diabetic mice.

Future Directions

In the future discover, analyse the plasma insulin level to help detect the β-cell function and sensitivity, which might be give more ideas about GFR declined in metformin group. Moreover, it needs more repeat experiment for western blot to give stronger evidence and also do more PAS staining for kidney histology. The albumin excretion in urine also need to be determined in the future, which could help to testing the pathological of kidney disease.

Conclusion

In conclusion, SGLT2 inhibition by EMPA could help strongly decrease blood glucose level in diabetes mice, which it was the consequence of decreasing renal glucose reabsorption. Otherwise, co-administration of metformin and EMPA was the most effective than mono-therapy EMPA. Moreover, treating with EMPA in diabetic mice might attenuate kidney impairment, glomerular basement membrane thicken and inflammation, which depended on blood glucose level and also remitted hyperfiltration in glomerular that independent on hyperglycemia.

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