The Combinatorial Effect of Metformin and p38 MAPK Inhibitor on Blood Glycemic Parameters in Non-obese Hyperglycemic Rat

ผลของการได้รับยาเมทฟอร์มินร่วมกับตัวยับยั้ง p38 MAPK ต่อระดับน้ำตาลในเลือด หนูที่มีภาวะน้ำตาลในเลือดสูงแบบไม่อ้วน

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ABSTRACT

The combination of diabetic drugs could possibly cause hypoglycemic effect, which is one of the adverse drug effect in diabetes. This study aims to determine the combination of metformin, the first line drug for diabetes, and cardioprotective compound p38 MAPK inhibitor, SB203580. Since the synergistic cardioprotective, effect of these two drugs could provide more clinical usefulness in diabetic cardiomyopathy. However, the combination of metformin and p38 MAPK inhibitor on glycemic parameters is primarily need to be investigated. In this study, Goto-Kakizaki (GK) rats were divided into 3 group including metformin group, SB203580 group, and combination of metformin and SB203580 group. All animals were treated for 4 weeks. Then, blood glycemic parameters were determined. The results showed that treatment with SB203580 alone could not lower blood glucose and % HbA1c. In contrast. treatment with metformin alone could significantly lower blood glucose and % HbA1c, but not further reduced in combine treated group.

บทคัดย่อ

การได้รับยาเบาหวานร่วมกันมักส่งผลให้เกิดภาวะน้ำตาลในเลือดต่ำซึ่งเป็นอาการไม่พึงประสงค์จากการได้รับยารักษาเบาหวาน การศึกษานี้จึงมีจุดประสงค์ในการศึกษาผลของการได้รับยาร่วมกันระหว่างยาต้านเบาหวานชนิดเมทฟอร์มิน และตัวยับยั้งโปรตีน 38 (p38 MAPK inhibitor) ที่มีความสามารถในการป้องกันหัวใจจากภาวะหัวใจขาดเลือดซึ่งผลของยาทั้งสองชนิดน่าจะมีประโยชน์ต่อโรคหัวใจที่เกิดจากโรคเบาหวานได้ อย่างไรก็ตามผลของการได้รับยาร่วมกันระหว่างยาเมทฟอร์มินและสารประกอบตัวยับยั้งโปรตีน 38 ต่อระดับน้ำตาลในเลือดจำเป็นต้องศึกษาก่อนการใช้ยา ในการศึกษานี้แบ่งหนูเบาหวานชนิด Goto-Kakizaki (GK) rat เป็น 3 กลุ่ม ได้แก่ กลุ่มได้รับยาเมทฟอร์มิน, ตัวยับยั้งโปรตีน 38 ชนิดSB203580 และได้รับยาร่วมกันเป็นเวลา 4 สัปดาห์ ผลการศึกษาพบว่าหนูทดลองที่ได้รับ SB203580 ไม่สามารถลดระดับน้ำตาลในเลือดและ %HbA1c แต่หนูที่ได้รับเฉพาะยาเมทฟอร์มินมีระดับในเลือดและ% HbA1c ลดลงอย่างมีนัยสำคัญ แต่อย่างไรก็ตามไม่มีการส่งเสริมการลดลงของระดับน้ำตาลในเลือดและ HbA1c ในกลุ่มที่ได้รับยาร่วมกัน

Keywords: Diabetes mellitus, Hypoglycemic effect, Drug combination

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Introduction

Type 2 diabetes(T2DM) is a major type of diabetes, which account for 90-95% of all diabetic cases (American Diabetes, 2010). T2DM is initiated by insulin resistance with progressive loss of insulin action (DeFronzo et al., 2015). Insulin resistance is a malfunction of insulin receptor signaling lead to a reduction of glucose uptake, by glucose transporter (GLUT) 4. This results in an increasing in blood glucose level or hyperglycemia. The hyperglycemia could cause both of microvascular and macrovascular complications such as cardiovascular disease (especially ischemic heart disease), retinopathy, atherosclerosis, and nephropathy (Papatheodorou, Papanas, Banach, Papazoglou, & Edmonds, 2016) . Many studies suggested that diabetes is apparently associated with obesity, particularly an increase in adipocyte and plasma free fatty acid has been shown to contribute to insulin resistance, and finally T2DM (DeFronzo et al., 2015). In contrary, several previous studies reported that T2DM also could be found in non-obese patients, who have BMI lower than 25 kg/m^2(Barma, Ranabir, Prasad, & Singh, 2011; Kashima, Inoue, Matsumoto, & Akimoto, 2015). It has been reported that non-obese T2DM or lean T2DM have higher prevalence in Asian countries, where the average prevalence of lean T2DM was about 60% among T2DM patients (Ramachandran, Snehalatha, & Ma, 2014). More importantly, one of quarter of global death from diabetes was found in this region (Kashima et al., 2015; Tang et al., 2016) Therefore, lean T2DM are also need to be concerned and further studies for a better understanding of pathophysiology, as well as drug response.

Metformin, a biguanide anti-diabetic drug, is a first-line drug for diabetes treatment, recommended by the American Diabetes Association (ADA) and European Association of the Study of Diabetes (EASD) (Inzucchi et al., 2012). Metformin can decrease blood glucose level, decrease glycogenesis in the liver and increase insulin sensitivity for up-taking glucose into the target cells (Rojas & Gomes, 2013). Moreover, it has been reported that metformin could limit myocardial infarct size and cardiac remodeling in animal models of myocardial infarction, which metformin could provide cardioprotective effect, especially in patients suffering from myocardial ischemia (El Messaoudi, Rongen, de Boer, & Riksen, 2011). In addition, clinical study also reported that metformin gave 36% lower risk of all-cause of mortality and 39% lower risk of myocardial infarction (El Messaoudi et al., 2011).

p38 Mitogen Activate Protein Kinase (MAPK) is a family of serine/threonine protein kinases that plays an important role in cardiac cellular stresses. In particular, p38 MAPK can be activated during insulin resistance, as well as diabetic models and during myocardial ischemia/reperfusion (Kumphune, Chattipakorn, & Chattipakorn, 2012). These could aggravate lethal cardiac injury. In addition, the inhibition p38-MAPK activity by using inhibitors has been shown to reduce myocardial injury and infarction (Kumphune et al., 2012). Therefore, the inhibition of p38-MAPK by using pharmacological inhibitors may possibly be benefit to reduce myocardial ischemia in diabetes. Moreover, it has been reported that p38-MAPK inhibitors could improve left ventricular functions (Greer, Ware, & Lefer, 2006; McGaffin et al., 2008; Thakker et al., 2006), endothelial function (Bell, 2003), and reduced cardiac inflammation in insulin resistance or diabetic models (Eguchi et al., 2008). These findings point out the usefulness of p38-MAPK inhibition in diabetes. Therefore, it could be very interested to study if the combine treatment of metformin and p38-MAPK inhibitor (SB203580) may be provided beneficial effects in diabetic heart with myocardial ischemia/reperfusion injury. However, the information about inhibition of p38 MAPK in ischemia/reperfusion injury

in diabetes has never been studied. The previous study showed that inhibition of p38 MAPK could improve glucose uptake by increasing translocation of GLUT1 and GLUT4 that play role in glucose uptake into the cell therefore leading to reduce blood glucose level (Carlson & Rondinone). So, the synergistic effect of those two drugs in lowering blood glucose level could possibly cause adverse effect on hypoglycemia, which limit the clinical usefulness, which is needed to be primarily investigated.

Objectives of the study

The aim of this study was to investigate the combinatorial effect of metformin and p38 MAPK (SB203580) inhibitor on blood glycemic parameters in non-obese hyperglycemic rat.

Methodology

1. **Animal model**

Ten adult male Wistar rat and 40 adult male non-obese diabetic model Goto-Kakizaki (GK) rats (5-week age) were purchased from Nomura Siam International, Bangkok, Thailand. All animals were maintained under environmentally control condition (22 ± 1 °C, 12 h light/12h dark cycle) at Center for Animal Research, Naresuan University, Phitsanulok, Thailand until 14 weeks old. All protocols used in this study were approved by the committee of Center for Animal Research, Naresuan University (NU-AE581023). Animals were housed until age of 16 weeks, then the drug administration were performed for 4 weeks, until age of 20-weeks old.

2. **Treatment protocol**

Animals were divided into 5 groups as following;

Group 1 Control group = Wistar rat (n =10)

Group 2 Diabetic group = GK rat (n =10)

Group 3 Metformin group = GK rat treated with 15 mg/kg metformin, twice daily via oral gavage (n =10)

Group 4 p38 inhibitor group = GK rat treated with 2 mg/kg SB203580 via intraperitoneal injection, every 2 days (n =10)

Group 5 Combination group = GK treated with 15 mg/kg metformin, twice daily via oral gavage and 2 mg/kg SB203580 via intraperitoneal injection, every 2 days (n =10)

At the end of drug treatment protocol, all animals were euthanatized by intraperitoneal injection of 100 mg/kg.BW. pentobarbital. The blood samples were collected from tail vein for determining the glycemic parameters.

3. **Confirmation of diabetic phenotype**

The confirmation of diabetic phenotype was performed by determining blood glycemic parameters, based on the diagnosis criteria of diabetes in human, including fasting blood glucose (FBG), percentage of glycated hemoglobin (%HbA1c), and oral glucose tolerance test (OGTT). For measuring fasting blood glucose, blood sample was collected from tail vein and measured by SDGlucoNavii® Glucose meter. The percentage of HbA1c was performed by CLOVER A1c™ from tail vein blood sample. The oral glucose tolerance test(OGTT) was performed by serial blood collection from tail vein at different times including 0, 30, 60, 90, 120 min, after feeding the animal

model with 75 mg glucose solution via oral gavage. Blood glucose was measured by SDGlucoNavii® Glucose meter. The criteria for confirming the diabetic condition, in experimental rats in this study, was based on any of diagnostic criteria in human from ADA criteria, including FBG level of 126 mg/dL or higher, or %HbA1c of 6.5 % or higher, or a 2-hour plasma glucose level of 200 mg/dL or higher during OGTT.

4. Determination of blood glycemic parameters after drug treatment

After animal models were treated with metformin, or SB203580, or combination of metformin and SB203580 for 4 weeks, plasma was collected from all animal in each group. The glycemic parameters were performed again, which include fasting blood glucose level, % HbA1c. In addition, plasma insulin level was also measured by rat insulin ELISA kit (MERCK®) for determining the effect of drug treatment on insulin level. Briefly, 10 μl of plasma and 80 μl of detection antibody, conjugated with horseradish peroxidase, were added to 96-well plates pre-coated with capture antibody for 2 hrs at room temperature. TMB substrate was added to reaction for 30 min at room temperature. The reactions were terminated by addition of stop solution before measuring absorbance at 450 nm. The concentration of insulin was calculated from standard curve produced by serial diluted standard insulin protein.

5. Statistical analysis

All values were expressed as mean ±S.E.M. All comparison was analyzed by ANOVA, followed when appropriate by the Tukey-Kramer test. The statistical test was performed using commercially available software (GraphPad Prism version 5). A *p*-value less than 0.05 was considered as statistical significant.

Results

The results in this study were divided into 2 parts including 1) confirmation of lean T2DM model 2) effect of metformin, SB203580, and metformin in combination of SB203580 on glycemic parameters and plasma insulin.

1. Confirmation of lean T2DM model

1.1 Bodyweight of animal models before treatment

The body weight of Wistar rat (Control) and Diabetic rat (GK rat) was determined during week 7 and week 15. The results showed that the bodyweight of Wistar rat (Control) was 178 ± 4.0 g at 7 weeks old and 421.7 ± 15.1 g at 15 weeks old. The growth rate was analyzed by linear regression analysis and found that the linear equation of Wistar rat growth rate was y = 30.438X -34.862. In diabetic model (GK rat), the bodyweight at 7 and 15 weeks old was 140.6 ± 1.6 g and 297.6 ± 2.0 g, respectively. The linear equation of GK rat growth rate was y = 19.625X + 3.175. The results showed that mean bodyweight of Wistar rat were higher than that of GK rat. In addition, the slope of linear equation of Wistar rat was greater than that of GK rat (Figure 1).

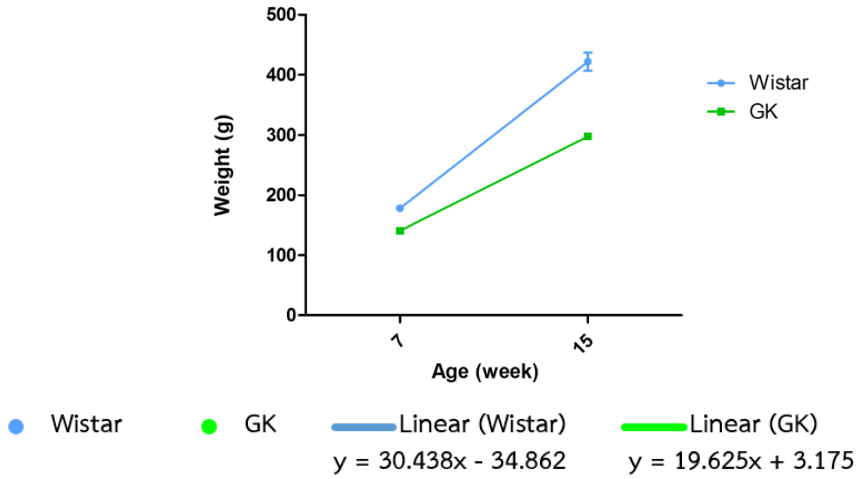

Figure 1 The growth rate of animal models at 7 and 15 weeks old

1.2 The fasting blood glucose level and percentage of HbA1c before treatment

The fasting blood glucose level, percentage of HbA1c and oral glucose tolerance test (OGTT) were performed at week 15 of age (before drug administration) in each desired groups. According to there are no official diagnosis criteria of diabetes in laboratory animal, the diabetic-like phenotype in this study was performed by using the diagnosis criteria of diabetes in human from American Diabetes Association (ADA) guideline 2017 (DeFronzo et al., 2015).

The fasting blood glucose level was 79.10 ± 3.707 mg/dl, 168.3 ± 12.03 mg/dl, 167.4 ± 12.57 mg/dl, 160.9 ± 3.707 mg/dl and 178.4 ± 8.916 mg/dl in control group, diabetes group, metformin group, SB203580 group and combinatorial group, respectively. The fasting blood glucose level in diabetes group, metformin group, SB203580 group and combination group were higher than 126 mg/dl, which was greater than ADA diagnostic criteria for diabetes (Figure 2A).

The percentage of HbA1c before treatment was 4.12 ± 0.03%, 6.31 ± 0.21 %, 6.93 ± 0.16 %, 6.50 ± 0.16 %, and 6.66 ± 0.22 % , in control group, diabetes group, metformin group, SB203580 group and combination group, respectively. The result showed that percentage of HbA1c in metformin group, SB203580 group and combination group, were higher than the ADA diagnostic criteria at 6.5% (Fig. 2B).

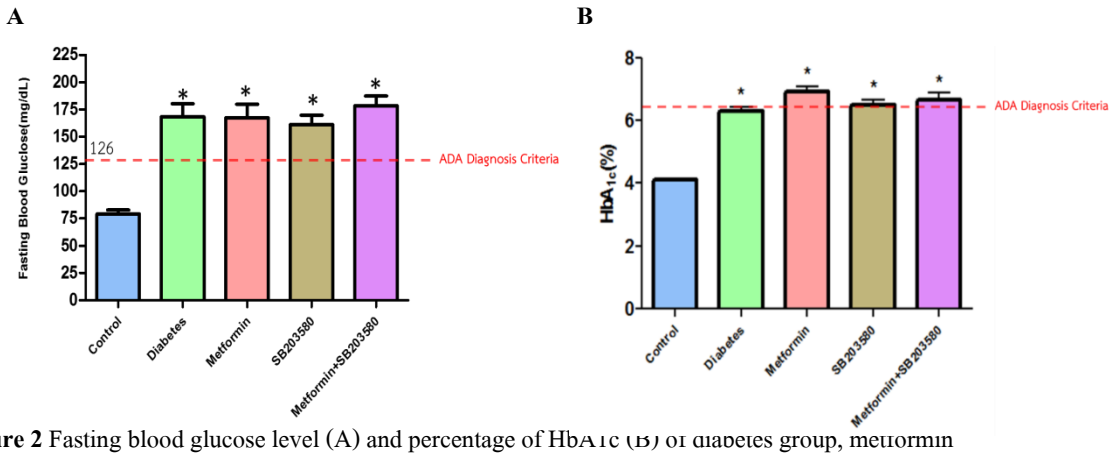

Figure 2 Fasting blood glucose level (A) and percentage of HbA1c (B) of diabetes group, metformin group, SB203580 group and combinatorial group before treatment compared with control group (*p<0.05). The red dash line represents the cut off value according to the ADA diagnostic criteria.

The results from oral glucose tolerance test (OGTT) showed that after treatment with 75 g glucose, the blood glucose was increased after 30 min to 2 hrs. There was not significantly different of blood glucose at each time point in diabetic group, metformin group, SB203580 group, and combination group (Figure 3). The blood glucose level 2 hrs after treated with 75 g glucose of each group was 114.1 ± 5.86 mg/dl, 275.2 ± 13.44 mg/dl, 286 ± 12.22 mg/dl, ±283.0 ± 14.43 mg/dl, and 281.0 ± 14.46 mg/dl in control group, diabetes group, metformin group, SB203580 group and combination group, respectively. The results showed that blood glucose level in diabetes group, metformin group, SB203580 group and combination group were greater than 200 mg/dl, which is the ADA diagnostic criteria (Figure 4).

The results of plasma glycemic parameter including fasting blood glucose level, percentage of HbA1c ,and oral glucose tolerance test (OGTT) in diabetes group, metformin group, SB 203580 group and combination group before treatment was higher than the criteria for diagnosis hyperglycemic condition, which sufficient to provide the confirmation of diabetic-like phenotype in GK rat.

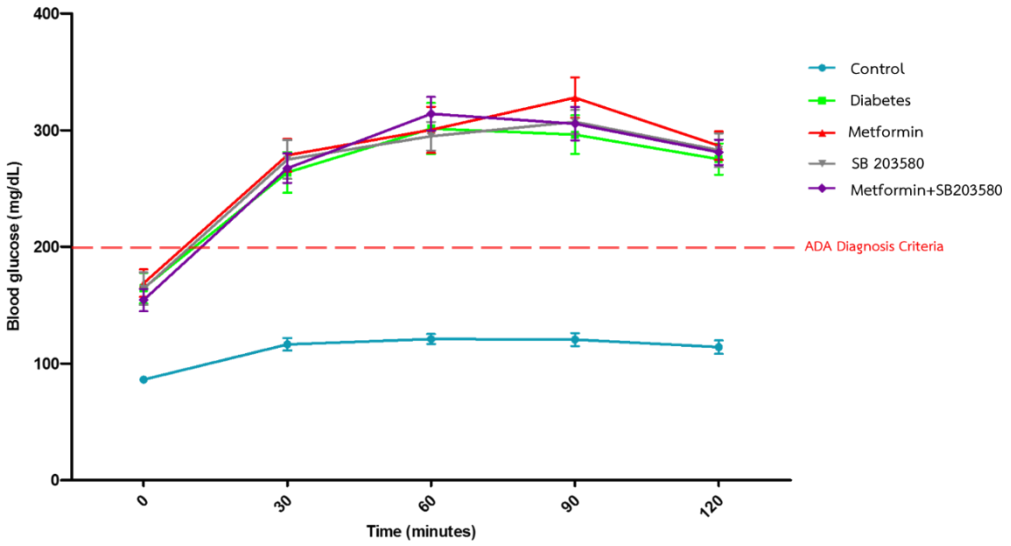

Figure 3 Blood glucose level after treated with 75 g glucose for 2 hrs in each group of animal models before treatment

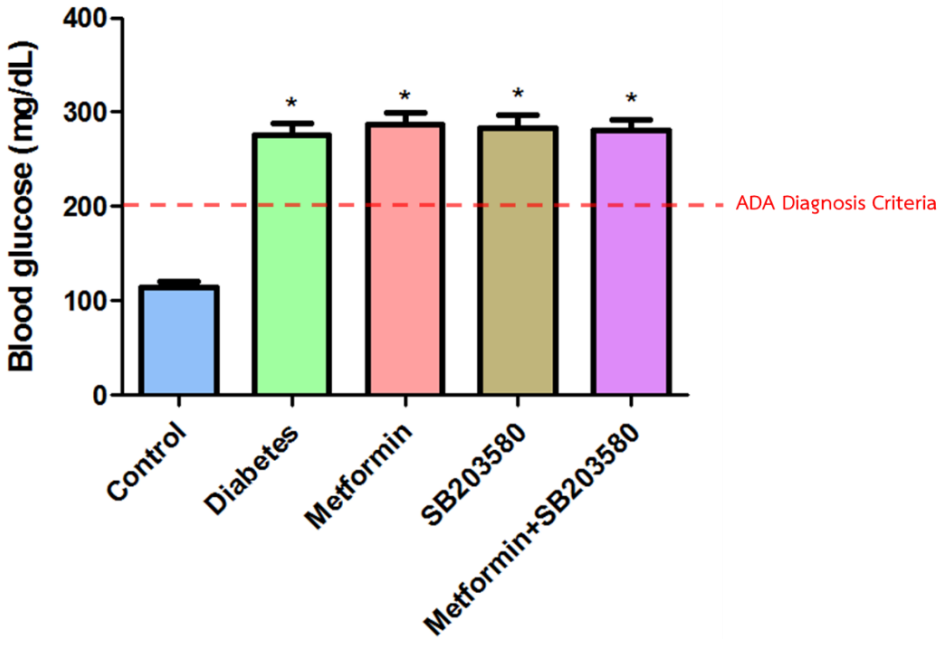

Figure 4 Blood glucose level after treated with 75 g glucose for 2 hrs in each group of animal models before treatment (*p<0.05)

2. Effect of metformin, SB203580, and metformin in combination of SB203580 on glycemic parameters and plasma insulin

2.1 Effect of drug treatment on bodyweight

The bodyweight of Wistar rat (control group) as well as all drug treated groups was measured. The results were analyzed for determining the growth rate. The results showed that the body weight of Wistar rat (control group) was 421.7 ± 15.1 g at 15 weeks old and 457.7 ± 15.6 g at 21 weeks old. The growth rate was analyzed by linear regression analysis and found that the linear equation of Wistar rat growth rate was $Y = 6X + 33.17$. In metformin group after treated with metformin for 4 weeks old, bodyweight was 318.5 ± 3.7 g at 21 weeks old, with the linear equation of growth rate $Y = 2.35X + 226.75$. In SB203580 treated group, the bodyweight was 319.5 ± 5.407 g, with the linear equation of growth rate $Y = 4.2X + 231.3$. Finally, the body weight of rat treated with both of metformin and SB203580 was 315.5 ± 4.241 g, with the linear equation of growth rate $Y = 2.7667X + 257.4$. The result showed that Wistar rat had bodyweight and slope value of linear equation greater than other groups. In addition, the results also showed that there were no significant difference in body weight and growth rate among diabetes and drug treated group (Figure 5).

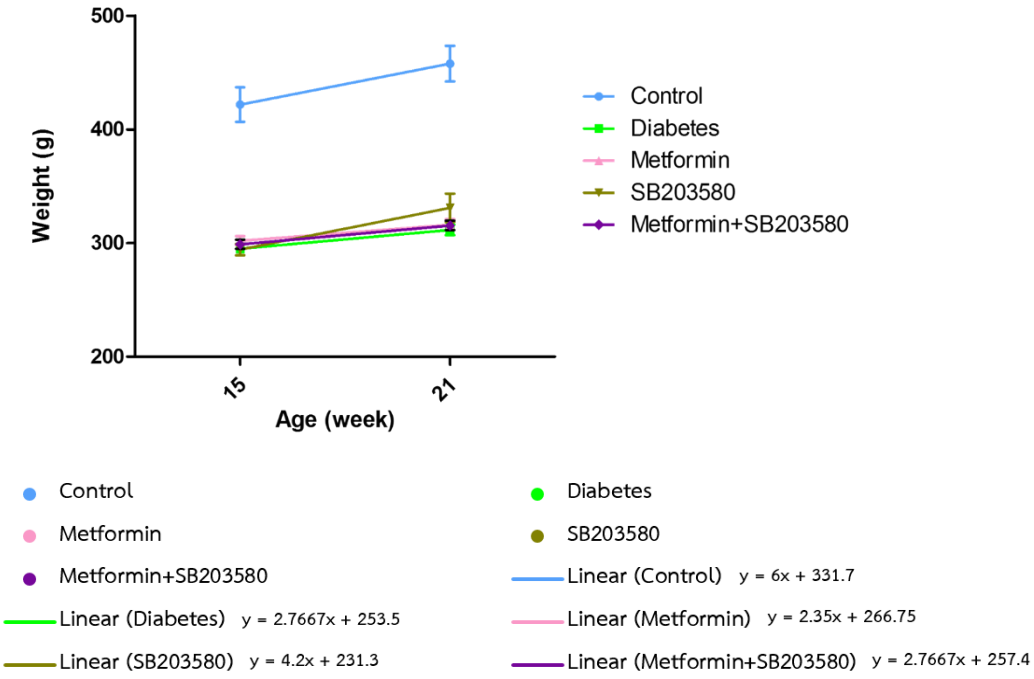

Figure 5 The growth rate of animal models at 15 and 21 weeks old

2.2 The fasting blood glucose level and percentage of HbA1c after drug treatment

Animals in each group were treated with desired drug as mentioned in material and method. The comparison of fasting blood glucose (FBS) as well as percentage of HbA1c level before and after 4-weeks treatment was performed in each drug treated group. The results showed that treatment with metformin was significantly

decreased FBS from 167.4 ± 4.412 mg/dl to 122.3 ± 4.412 mg/dl (p = 0.0017) (Figure 6A). In SB203580 group, the results showed that FBS level was not significantly decreased when compared to pre-treatment group (p = 0.0747) (Figure 6A). Furthermore, FBS level in animal treated with metformin in combination with SB203580 was also significantly decreased, when compared to untreated group (178.4 ± 8.916 mg/dL vs 124.3 ± 4.305 mg/dL (p < 0.0001) (Figure 6A). However, analysis between drug treated groups showed that there were no further decreased of FBS in animal treated with metformin in combination with SB203580, when compared to metformin treated group or SB203580 treated group.

The results of HbA1c level were also similar to the findings in FBS. Treatment with metformin was significantly decreased %HbA1c from 6.930 ± 0.1660% to 5.189 ± 0.1620% (p<0.0001) (Figure 6B), whereas SB203580 treatment could not significantly decreased %HbA1c when compared to post-SB203580 treatment group (p = 0.0634) (Figure 6B). Furthermore, combination of metformin and SB203580 could significantly decreased %HbA1c, when compared to untreated group (6.456 ± 0.0929% vs 5.030 ± 0.0943% (p < 0.0001) (Figure 6B), but did not further decreased when compared to metformin treated group or SB203580 treated group.

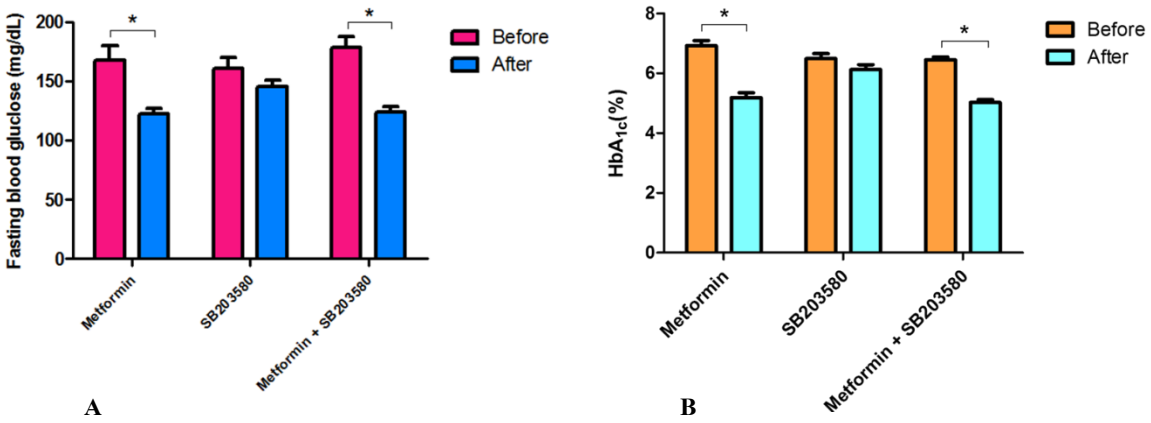

Figure 6 Fasting blood glucose level (A) and percentage of HbA1c (B) after treatment for 4 weeks in each group (*p<0.05)

Plasma insulin level after treatment for 4 weeks in each group

Plasma insulin level was measured in all plasma samples and we analyzed by comparing between control group, diabetic group, and drug treated groups. Plasma insulin level in diabetes was significantly lower than control group (9.233 ± 2.019 ng/ml vs 18.07 ± 6.156 ng/ml, p<0.05). Treatment with metformin could significantly reduce plasma insulin level (3.210 ± 4.231 ng/ml). The plasma insulin was increased in SB203580 treated group (11.73 ± 3.075 ng.ml), which significantly higher than that of metformin treated group and drug combine group. There were no further decreased of plasma insulin in animal treated with metformin in combination with SB203580, when compared to metformin treated group (Figure 7).

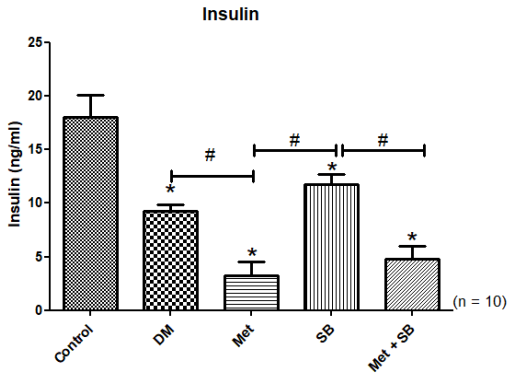

Figure 7 Plasma insulin level treatment for 4 weeks in each group (*p<0.05 vs control) (#p<0.05 among treated groups)

Discussion and Conclusions

In this study, spontaneous type 2 diabetes Goto-kakizaki rat (GK rat) was used as animal model. The glycemic parameters at 15 weeks old was performed to diagnosis diabetic-like model, by using diagnostic criteria in human announced by American Diabetes Association(ADA) guideline 2017 (DeFronzo et al., 2015). The findings showed that all glycemic parameters, including FBS, %HbA1c, and OGTT, provided the evidence that all GK rat in this study exhibit diabetic-like phenotype. Another major finding of this study was that in the combination of metformin and SB203580 could significantly reduce plasma glycemic parameters, when compared to diabetes group. However, the combination of metformin and SB203580 could not further decreased FBS level, %HbA1c, and plasma insulin, when compared to metformin treated group. These results suggested that SB203580 could not interfere the glucose lowering effect of metformin, and could be unlikely to cause adverse effect of hypoglycemic, when the combination of these 2 drugs is given.

The previous studies showed that metformin could decrease blood glucose and plasma insulin level by inhibiting respiratory chain complex 1, hence decreased hepatic gluconeogenesis, and increase insulin sensitivity (Giannarelli, Aragona, Coppelli, & Del Prato, 2003; Viollet et al., 2012). The improvement in insulin sensitivity by metformin also lead to a reduction of plasma insulin level as can also be clearly seen from this study. The effect of p38 MAPK inhibition on plasma glucose level is still controversial. Although some previous study reported that SB203580 could decrease blood glucose level by increase glucose transporter expression, and lead to enhance glucose uptake into the cell (Carlson & Rondinone), however, there were many studies showed the failure of p38 MAPK inhibition on glucose uptake, and therefore, lead to inability to reduce plasma glucose. For example, Sweeney G et al. reported that pretreatment of SB203580 in 3T3-L1 adipocyte and L6 myotubes could significantly reduce glucose uptake (Sweeney et al., 1999). In addition, C. N. Antonescu and colleges reported, p38 MAPK inhibitor could decrease insulin-stimulated glucose uptake in L6 myotubes, and had no effect GLUT4 translocation (Antonescu et al., 2005). The results from our study also showed that treatment of SB203580 could not significantly reduce blood glucose, % HbA1C, and insulin. These results suggested the inability of p38 MAPK inhibition on blood glucose reduction.

There are some limitations in this study. The potential of combining between metformin and SB203580 was only assessed in term of plasma glycemic control. Drug interaction between metformin and SB203580 possibly cause many systemic complications such as vasodilation, increase cardiovascular disease and renal failure (May & Schindler, 2016). Therefore, drug toxicity in various vital organs, the heart, brain, kidney, liver, etc. need to be intensively investigated.

In conclusion, the combinatorial effect of metformin and p38 MAPK inhibitor could not further reduce plasma glycemic parameters and plasma insulin level in non-obese type 2 diabetes model. The results suggest that SB203580 could not interfere the glucose lowering effect of metformin. Therefore, combination between metformin and SB203580 could possibly provide therapeutic benefits of these 2 drugs, with less likely to have adverse effect of hypoglycemia.

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