BJMS
Borneo Journal of Medical Sciences

ORIGINAL ARTICLE

Metformin Preserves Function and Histology of Liver in Type 2 Diabetic Rat Model

Wan Amir Nizam Wan Ahmad[1], Nor Asiah Muhamad Nor[2], Nor Hidayah Abu Bakar[3], Liza Noordin[4]*

[1] Biomedicine Programme, School of Health Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
[2] Faculty of Health Science, Universiti Sultan Zainal Abidin, Gong Badak Campus, 21300, Kuala Terengganu, Malaysia
[3] Faculty of Medicine, Universiti Sultan Zainal Abidin, Medical Campus, 20400, Kuala Terengganu, Malaysia
[4] Department of Physiology, School of Medical Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia

*Corresponding author's email: lizakck@usm.my

Received: 18 August 2023

Accepted: 2 February 2024

Published: 1 May 2024

DOI: https://doi.org/10.51200/bjms.v18i2.5061

Keywords: *diabetes mellitus, metformin, lipid profile, liver disease, animal model*

ABSTRACT

Diabetes mellitus (DM) causes significant morbidity and mortality worldwide. Metformin is considered first-line oral therapy for type 2 DM, together with lifestyle modification. The objective of this study was to evaluate the protective effects of metformin on the liver in the type 2 DM rat (T2DR) model. The rats were fed a high-fat diet (HFD) to become obese, followed by a single low dose of streptozotocin (STZ) at 35 mg/kg intraperitoneally to induce T2DR. Twenty-eight male Sprague Dawley (SD) rats were divided into four groups equally (n=7): Control, Obese (obese rats), T2DR (Untreated T2DR), or Met-T2DR (T2DR on oral metformin at 250 mg/kg/day for six weeks). Weekly levels of fasting blood glucose (FBG) were measured. Rats were euthanised, and liver function tests and lipid profiles were measured. The histology of the liver was examined using haematoxylin and eosin staining. The met-T2DR group demonstrated a significant decrease in FBG levels beginning in week 3 and preserved liver function and histology, and lipid profile comparable to control. The effect of metformin in lowering blood glucose was demonstrated, thus controlling diabetes and preventing liver complications. The mechanism of the hepatoprotective effect could be linked to glycaemic control and lipid metabolism.

INTRODUCTION

Diabetes mellitus (DM) has emerged as a global major public concern, with a substantial risk of morbidity and mortality (Tang et al., 2024). Diabetes was estimated to affect 8.8% of adults aged 20 to 79 in 2015, with a predicted increase to 10.4% by 2040. Its prevalence in Southeast Asia is expected to gradually increase from 11.3 percent in 2019 to 12.6 percent in 2045 (Amirudin et al., 2021). Numerous complications that involved macrovascular and microvascular changes have been attributed to DM. Type 2 DM (T2DM) is characterised by progressive loss of β-cell function and mass (Sayyed Kassem et al., 2023) associated with insulin resistance in muscle (Den Hartogh et al., 2023) and adipose tissue (Ahmed et al., 2021). It was previously referred to as 'non-insulin-dependent diabetes', and it accounts for 90–95% of all diabetes cases. T2DM alters insulin resistance and lipid metabolism, which leads to pancreatic β-cell dysfunction (Skovso, 2014).

Proper treatment is necessary to prevent complications by delaying the progression of the disease. Continuous medical care with risk-reduction strategies for various factors is critical in the management of diabetes. The primary goal of DM treatment, which improves the quality of life, are to prevent or delay complications. It is acknowledged that a non-pharmacological approach, lifestyle changes, and the use of pharmacological agents are required in the treatment. Metformin, an oral hypoglycaemic guanide drug, is a first-line treatment for T2DM, particularly in overweight and obese people with a good safety profile (Chandra et al., 2019; Pinyopornpanish et al., 2021). This medicine was used to treat T2DM in the late 1950s, and it is still the drug of choice used by nearly 150 million people. Furthermore, because of its low cost, great efficacy, and weight-loss benefits, this drug has been the first-line oral hypoglycaemic medication for many years (Raqib et al., 2022). The main actions of metformin include suppressing hepatic gluconeogenesis, improving uptake of glucose, and increasing insulin sensitivity (Shaw, 2013; Rena et al., 2017; Baker et al., 2021). The effects of metformin among diabetic patients have been inconsistent, however, several evidence demonstrated the beneficial effects of metformin in previous studies. For example, metformin improved lipid profiles, decreased body weight, decreased hyperinsulinemia, improved endothelial function (Nasri and Rafieian-Kopaei, 2014), and decreased oxidative stress (Abdel-Moneim et al., 2022).

Obesity contributes to the development of T2DM (Lang et al., 2019), whereby a body mass index (BMI) of more than 25 kg/m2 is considered a risk (ElSayed et al., 2023). Diabetic dyslipidaemia in T2DM is associated with lipid metabolism abnormalities such as decreased high-density lipoprotein cholesterol (HDL-C) levels, increased triglycerides (TG), and low-density lipoprotein-cholesterol (LDL-C) (Wu and Parhofer, 2014). Furthermore, insulin resistance has been attributed to the link between the T2DM and development of nonalcoholic fatty liver disease (NAFLD), which may lead to nonalcoholic steatohepatitis, liver fibrosis, and cirrhosis (Pinyopornpanish et al., 2021; Kosmalski et al., 2022). NAFLD, a consequence of lipid acquisition exceeding lipid disposal (Ipsen et al., 2018), is described as fat accumulation in the hepatocytes in patients without excessive alcohol consumption (Marusic et al., 2021). Although the pathogenesis of NAFLD is poorly understood, dysregulation of lipid delivery, hepatic lipid uptake, and oxidation have been linked to promoting lipid deposition in the liver (Marusic et al., 2021). Meanwhile, insulin resistance which is described physiologically as the inability of some tissues to respond to normal insulin levels can cause NAFLD due to impairment of insulin signalling pathways such as insulin receptor substrates (IRSs) and phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway (Chao et al., 2019). A previous study demonstrated that 12 to 24 weeks of treatment with metformin significantly decreased the BMI, liver enzymes,

liver fat content, and haemoglobin A1c (HbA1c) and improved insulin resistance in NAFLD patients with T2DM (Feng et al., 2017; Zhang et al., 2017; Tian et al., 2018). On the basis of this, the aim of this study was to evaluate the protective effects of metformin on liver function and structure in a type 2 DM rat (TD2R) model.

MATERIALS AND METHODS

Chemicals and reagents Metformin and streptozotocin were purchased from Hovid Bhd, Malaysia, and Sigma Aldrich, Germany respectively. The rat pellet was purchased from Altromin, Germany. Haematoxylin, eosin, and formaldehyde were purchased from Leica Biosystem, USA. Other chemicals were of analytical grade.

Animals

This study included twenty-eight (28) male Sprague-Dawley rats (N=28) weighing 200-250 g and aged 8-10 weeks. Rats were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM), Malaysia. The animal procedure was approved by the Institutional Animal Care and Use Committee (IACUC), USM (Ref: USM/ISCUC/2017/9110 (886)). Animals were housed for a week prior to the intervention in a standard polypropylene cage under controlled conditions (23 ± 1°C, 60-70 percent humidity, and 12-hour light-dark cycle) with access to a standard pellet diet and drinking water ad libitum.

Experimental design

The experimental design is shown in Figure 1. Animals were divided into two groups; Control (n=7) and Obese (n=21). The control group was given a standard Altromin diet, while the obese group received a self-prepared high-fat diet (HFD 32% fat) to induce obesity. The standard diet was Altromin pellet (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) that was composed of soy, wheat and corn with approximately 24% protein, 64%

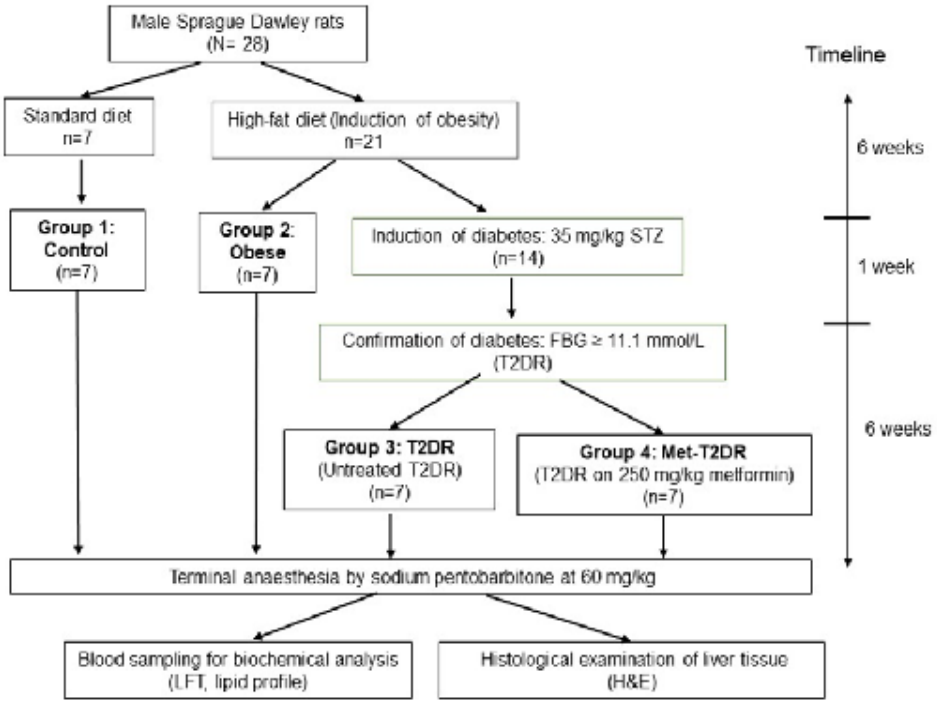

Figure 1: The flow chart of the study. T2DR: Untreated type 2 diabetes mellitus rat, Met-T2DR, T2DR treated with 250 mg/kg/day of metformin, STZ: streptozotocin, LFT: liver function test, FBG: fasting blood glucose, H&E: haematoxylin & eosin.

carbohydrates and 12% fat in terms of caloric content (Zakaria et al., 2022) while for the preparation of HFD, 32 g of ghee (saturated fat from an animal), 300 mg calcium, and 100 IU of vitamin D3 per 100 g of standard rat pellet were mixed well to become dough-like consistency (Noordin et al., 2022). Obese rat has a BMI of more than 0.68 g/cm2 (Nouvelli et al., 2007). BMI was calculated as follows: body weight (g) / length2 (cm2).

Diabetes mellitus was induced in obese rats using streptozotocin (STZ) at 35 mg/kg (i.p) (Noordin et al., 2021). One week following STZ induction, levels of fasting blood glucose (FBG) were measured. FBG levels of more than 11.1 mmol/L were considered diabetes (Cai et al., 2020), and were assigned as T2DR. T2DR were divided into two groups: untreated or treated with metformin at 250 mg/kg orally for six weeks. The dose of 250 mg/kg was based on previous studies that demonstrated a significant decrease in plasma levels of glucose (Roxo et al., 2019; Shen et al., 2020; Noordin et al., 2022). The study groups include Control, Obese, T2DR (Untreated-type 2 diabetes mellitus rat), and Met-T2DR (T2DR treated with 250 mg/kg/day metformin), with seven rats in each group.

The rats from the control group were given a standard diet, while the other rats were given HFD throughout the study. FBG levels were measured weekly by tail pricking in the dorsal vein after an overnight fast (8 hours) using the portable Accu-Check Advantage glucometer. Rats were euthanised at the end of the study by sodium pentobarbitone at 60 mg/kg (i.p). Blood was withdrawn via cardiac puncture for biochemical analysis, and the liver was isolated for histological examination.

Measurement of biochemical parameters

Liver function test, including liver enzymes (aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), and lipid profile, including total cholesterol, triglycerides (TG), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) were measured in the serum. Blood samples were kept in nonheparinised tubes, left to clot at room temperature, and centrifuged at 1,100 x g for 15 min. Blood samples were sent to B.P. Clinical Lab Sdn. Bhd., Kota Bharu, Kelantan, Malaysia.

Histopathology assessment

Liver tissue was dissected out, rinsed with phosphate buffer solution, and fixed in 10% formalin. The specimen was then embedded in paraffin and sections at a thickness of 2-3 µm. The specimens were put on glass slides and left on a hot plate (HI1220; Leica Microsystems). Then, they were deparaffinised with xylene and dehydrated by a descending ethanol series. After staining with Haematoxylin and Eosin, the sections were dehydrated by an ascending ethanol series followed by xylene.

Statistical Analysis

Data are presented as the mean (Standard Deviation; SD). Results were analysed by one-way analysis of variance (ANOVA) followed by the post hoc Tukey's test and two-way repeated measure ANOVA. Graph Pad Prism software version 9 for Windows (GraphPad, San Diego, CA) was used for analysis. A p-value of < 0.05 was considered significant.

RESULTS

Metformin decreases FBG levels in T2DR

Pre-induction FBG was not significantly different between the groups. One-week post-streptozotocin (STZ) injection (Week 0), FBG levels were higher in T2DR significantly compared to the control group (Table 1). Throughout the six weeks, the FBG levels in the T2DR group remained significantly higher compared to the control ($p<0.001$) and obese ($p<0.001$) groups. Treatment with metformin significantly reduced the FBG levels as compared to T2DR, beginning in week 3 ($p<0.05$). The reduction of FBG with metformin

was comparable to the control group

Table 1: Weekly levels of fasting blood glucose in the experimental groups.

Group	Control	Obese	T2DR	Met-T2DR
Day 0	5.10 (0.49)c	5.62 (0.26)c	17.23 (0.65)	17.80 (1.88)
Week 1	4.69 (0.47)c	5.51 (0.30)c	21.24 (2.78)	17.46 (4.21)
Week 2	4.40 (0.42)c	4.84 (0.58)c	20.56 (3.38)	17.64 (6.98)
Week 3	4.59 (0.29)c	5.49 (0.34)c	23.06 (2.19)	13.34 (6.49)a
Week 4	4.12 (0.22)c	5.29 (0.45)c	22.17 (2.56)	11.64 (6.33)b
Week 5	4.32 (0.30)c	5.50 (0.34)c	22.31 (1.69)	8.98 (4.55)c
Week 6	4.35 (0.39)c	5.69 (0.52)c	22.43 (1.30)	7.53 (3.42)c

Data were analysed by using two-way repeated measures ANOVA. ap<0.05, bp<0.01 and cp<0.001 when compared to T2DR. T2DR, Untreated-T2DR; Met-T2DR, T2DR treated with metformin.

Metformin preserves liver function parameters and lipid profile in T2DR

The liver enzymes increased significantly in the T2DR group compared to the control; including AST, ALT, and ALP. The metformin group had significantly lower levels of all the liver enzymes compared to the T2DR. Meanwhile, TC and TG were significantly higher in the T2DR group compared to the control and obese groups. LDL-C level was significantly higher in the T2DR group compared to the control group. Treatment with metformin significantly reduced TC, TG, and LDL-C levels compared to the T2DR group. However, no significant differences were observed in HDL-C levels between the groups (Table 2). There were also no significant differences in total protein, albumin, globulin, and total bilirubin between the groups.

Table 2: Levels of liver function test parameters and lipid profile in the experimental groups.

Groups	Control	Obese	T2DR	Met-T2DR
Liver function tests				
Total protein (g/L)	66.89 (3.40)	73.00 (8.02)	67.75 (2.56)	68.56 (10.08)
Albumin (g/L)	27.44 (1.67)	29.75 (1.99)	27.13 (0.90)	28.44 (1.81)
Globulin (g/L)	39.44 (5.03)	43.25 (6.50)	40.63 (8.86)	40.11 (9.33)
A/G ratio (g/L)	0.70 (0.09)	0.70 (0.80)	0.69 (0.12)	0.77 (0.12)
Total bilirubin (µmol/L)	1.70 (0.01)	1.71 (0.01)	1.70 (0.01)	1.70 (0.00)
AST (U/L)	123.60 (23.60)a	152.0 (18.19)	173.70 (53.70)	114.0 (25.97)a
ALT (U/L)	68.56 (9.06)b	73.86 (19.45)a	112.00 (37.26)	70.86 (10.84)b
ALP (U/L)	269.00 (44.86)c	454.50 (96.78)c	1539.0 (176.2)	351.0 (275.7)c
Lipid profiles				
Total cholesterol (mmol/L)	1.60 (0.12)c	2.10 (0.24)a	2.88 (0.40)	1.75 (0.20)c
Triglycerides (mmol/L)	1.10 (0.39)b	2.50 (0.94)a	6.40 (3.06)	1.02 (0.22)c
LDL-C (mmol/L)	0.58 (0.28)b	1.53 (2.00)	3.68 (1.83)	1.00 (0.23)a
HDL-C (mmol/L)	0.44 (0.05)	0.60 (0.22)	0.60 (0.07)	0.48 ± 0.10

Data were analysed by using one-way ANOVA. ap< 0.05, bp< 0.01 and cp< 0.001 when compared to T2DR. T2DR, Untreated-T2DR; Met-T2DR, T2DR treated with metformin.

Metformin preserves normal histology of the liver

The histology of the liver is shown in Figure 2. The control rat showed a normal hepatic lobule that consists of polygonal hepatocytes, a regular nucleus, and cytoplasm (Figure 2A). In the obese (Figure 2B) and T2DR (Figure 2C) groups, there were fatty changes seen, which were characterised by the presence of macrovesicular and microvesicular steatosis. The lipid accumulates in the hepatocytes as vacuoles, which have a clear appearance. In addition, ballooning of hepatocytes was seen in the obese and T2DR groups. In the metformin group, the liver tissue was preserved, similar to the control group

DISCUSSION

The present study was successful in developing an animal model of T2DR. The combination of HFD and a low dose of STZ causes minimal dysfunction of the pancreatic β-cell. This method was chosen in this study to develop the T2DM model similar to earlier studies (Fang et al., 2019; Noordin et al., 2021). A low dose of STZ causes mild impairment of insulin secretion, like in the late-stage T2DM. This model exhibits long-lasting and stable hyperglycaemia, making it a widely research tool in the DM study (Guo et al., 2018). T2DM is a metabolic disorder that affects insulin resistance, the function of pancreatic beta-

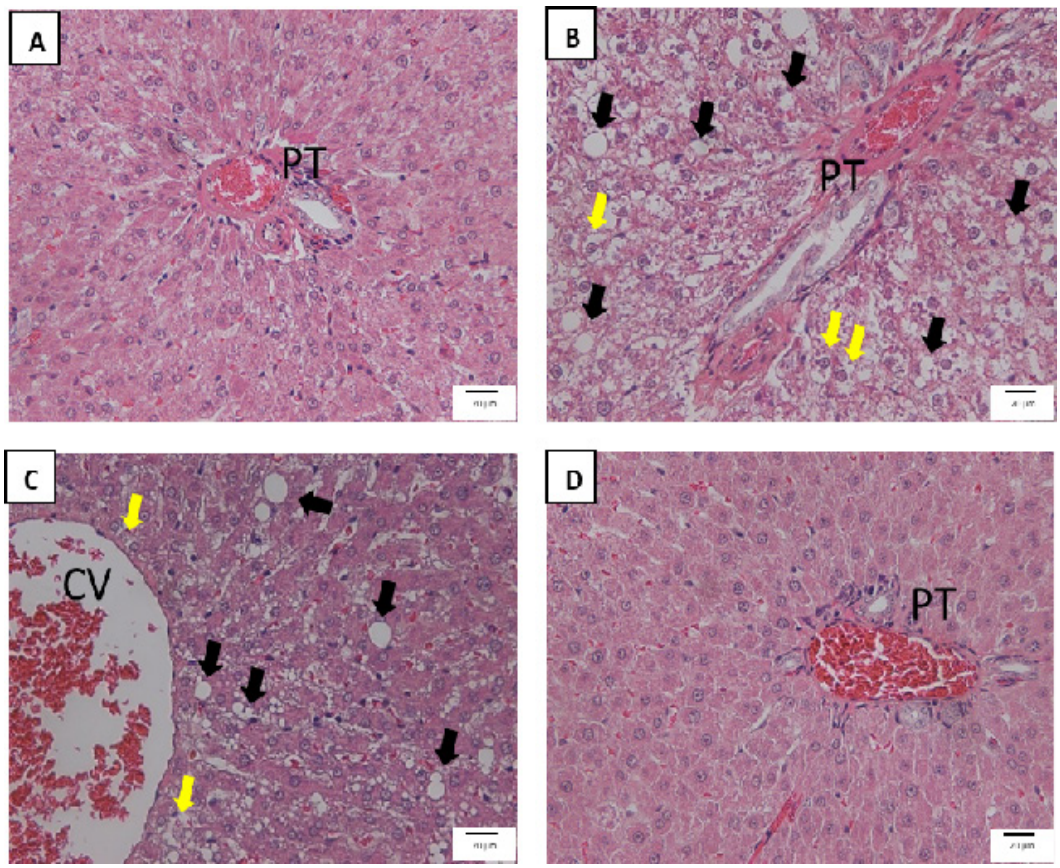

Figure 2: Histopathological sections of liver with Haematoxylin and Eosin staining in all groups. Control (A), Obese (B), T2DR (C), and Met-T2DR (D). Control group shows normal appearance of liver tissue with no pathological changes. Obese and T2DR groups show the presence of lipid droplets while the liver tissue in Met-T2DR group is preserved. T2DR: Untreated-type 2 diabetes mellitus rat, Met-T2DR: T2DR treated with 250 mg/kg/day of metformin. Portal triad (PT), Central vein (CV), Lipid droplets are marked using black arrows, and ballooning of hepatocytes are marked using yellow arrows. Magnification X400, scale bar=20µm.

cell, and lipid metabolism (Skovso, 2014). Obese animals develop insulin resistance, exhibit metabolic characteristics, and develop a disease progression similar to that seen in T2DM patients (Fang et al., 2019). Changes in the biochemical and histology of the liver in diabetic animal models simulate chronic liver disease in humans (Lucchesi et al., 2015).

We also evaluated the potential liver-protective effects of metformin. Metformin is a widely used drug for treating T2DM due to its glucose-lowering effects, safe, and relatively low cost (LaMoia and Shulman, 2021). We found that the levels of FBG remained consistently high throughout the experimental period compared to the control and obese groups. It was demonstrated that metformin significantly reduced FBG levels beginning in week 3.

The liver is the primary site of metformin action, and suppression of hepatic glucose production by metformin is widely accepted for lowering glucose levels in the blood (Horakova et al., 2019; LaMoia and Shulman, 2021). Metformin increases adenosine monophosphate-activated protein kinase activity in the liver and reduces hepatic gluconeogenesis and lipogenesis. Besides lowering blood glucose, metformin also increased peripheral insulin sensitivity (Kristensen et al., 2014), increased muscle metabolic insulin sensitivity (Jahn et al., 2022), and inhibited transepithelial glucose transport in the intestine (Horakova et al., 2019). Most recently, a study on diabetic patients showed that the gut is the primary site of metformin, whereby this drug increases the basolateral intestinal glucose uptake that results in hypoglycaemia in the portal vein and subsequent reduction of glucose production in the liver (Tobar et al., 2023). Metformin is also effective when combined with other glucose-lowering agents; for example, the combination of metformin and glipizide, a second-generation of sulfonylureas, was more potent than a single therapy of metformin in improving glycaemic control and ameliorating oxidative stress (Baker et al., 2021; Abdel-Moneim et al., 2022).

Liver disease has been reported as one of the causes of death in DM patients (Gopal et al., 2014). T2DM is linked to alterations in liver function and lipid profile (Khadke et al., 2019). ALT and AST are recommended as standard indicators of biomarkers and liver function for predicting toxicity. In this study, we observed increased liver enzymes, AST, ALT, and ALP in the T2DR group, indicating an impairment of liver function in this group. In diabetics, elevated ALT and AST levels are related to non-alcoholic fatty liver disease (Mykhalchyshyn et al., 2015). The elevated levels of these transaminases in this study could be attributed to parenchymal liver cells damage (Gopalakrishnan et al., 2020). Treatment with metformin, interestingly, preserved normal liver function, implying that the drug has protective effects on the liver.

Obesity is the leading cause of T2DM, and it contributes to high triglyceride levels and insulin resistance (Gheibi et al., 2017). Insulin resistance and T2DM are associated with lipid abnormalities such as an increase in TC, TG, and LDL-C, as well as a decrease in HDL-C levels (Rosenblit, 2016; Zhang et al., 2017), which was consistent with our study. We also demonstrated that metformin had a significant decrease in TC, TG, and LDL-C compared to the T2DR group, indicating the anti-hyperlipidaemic activity of metformin.

NAFLD is the primary cause of fatty change worldwide, which can be caused by diabetes, obesity, or metabolic syndrome. NAFLD is characterised by fat accumulation in hepatocytes (Firneisz, 2014). Liver changes range from fatty degeneration of liver cells, which comprises of macrovesicular or microvesicular triglycerides accumulation, to steatohepatitis in the advanced stage (Aly and Kleiner, 2011). NAFLD is attributed as the consequence of excessive TG in the cytoplasm of hepatocytes (Benedict and Zhang, 2017),

which may support the presence of fatty change in the T2DR group in this study. Lipotoxicity, inflammation and oxidative stress play a crucial role in the progression of NAFLD (Yang et al., 2019). Metformin preserved the normal histology of the liver, which may be related to lower levels of TC, TG, and LDL-C.

CONCLUSION

Our animal model of T2DR induced by a combination of HFD and a low dose of STZ exhibits hyperglycaemia, liver dysfunction, dyslipidaemia, and liver steatosis. Metformin treatments significantly attenuated the pathological features of T2DM, by lowering blood glucose, protecting liver functions, regulating lipid metabolism, and retaining normal morphology of the liver. These effects could be due to its anti-hyperglycaemic and hypolipidaemic effects, which need further research to elucidate its exact mechanism of action. However, there is a potential limitation to our study whereby we did not evaluate the direct influence of metformin on insulin signalling pathways, which is considered essential for future research.

CONFLICT OF INTEREST

No conflicts of interest.

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Agriculture's Herbal Research Grant Scheme (HRGS) for the financial support (project: NH1015S020/USM 304.PPSK.6150169.K123). The authors also acknowledge the facilities and technical assistance of all staff from the Animal and Research Centre (ARASC), Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia.

REFERENCES

Aly, F. Z., & Kleiner, D. (2011). Update on fatty liver disease and steatohepatitis. Advances in Anatomic Pathology, 18(4), 294. https://doi: 10.1097/PAP.0b013e318220f59

Amirudin, N., Aimanan, S. N. M., Kassim, R., & Panting, A. J. (2021). The importance of health information seeking among diabetes patients in Malaysia: A preliminary observation. Malaysian Journal of Social Sciences and Humanities (MJSSH), 6(12), 205-212. https://doi: 10.47405/mjssh.v6i12.1203

Baker, C., Retzik-Stahr, C., Singh, V., Plomondon, R., Anderson, V., & Rasouli, N. (2021). Should metformin remain the first-line therapy for treatment of type 2 diabetes?. Therapeutic Advances in Endocrinology and Metabolism, 12, 2042018820980225. https://doi: 10.1177/2042018820980225

Benedict, M., & Zhang, X. (2017). Non-alcoholic fatty liver disease: An expanded review. World Journal of Hepatology, 9(16), 715. https://doi: 10.4254/wjh.v9.i16.715

Cai, W. D., Ding, Z. C., Wang, Y. Y., Yang, Y., Zhang, H. N., & Yan, J. K. (2020). Hypoglycemic benefit and potential mechanism of a polysaccharide from Hericium erinaceus in streptozotoxin-induced diabetic rats. Process Biochemistry, 88, 180-188.

Chandra, K., Singh, P., Dwivedi, S., & Jain, S. K. (2019). Diabetes Mellitus and Oxidative Stress: A Co-relative and Therapeutic Approach. Journal of Clinical & Diagnostic Research, 13(5). https://doi: 10.7860/JCDR/2019/40628.12878

Chao, H.W., Chao, S.W., Lin, H., Ku, H.C., & Cheng, C.F. (2019). Homeostasis of Glucose and Lipid in Non-Alcoholic Fatty Liver Disease. International Journal of Molecular Sciences, 20(2), 298. https://doi.org/10.3390/ijms20020298

Den Hartogh, D. J., Vlavcheski, F., & Tsiani, E. (2023). Muscle Cell Insulin Resistance Is Attenuated by Rosmarinic Acid: Elucidating the Mechanisms Involved. International Journal of Molecular Sciences, 24(6), 5094.

ElSayed, N. A., Aleppo, G., Aroda, V. R., Bannuru, R. R., Brown, F. M., Bruemmer, D., ... & Gabbay, R. A. (2023). Addendum. 2. Classification and Diagnosis of Diabetes: Standards of Care in Diabetes—2023. Diabetes Care 2023; 46 (Suppl. 1): S19–S40. Diabetes Care, dc23ad08. https://doi.org/10.2337/dc23-S002

Fang, J. Y., Lin, C. H., Huang, T. H., & Chuang, S. Y. (2019). In vivo rodent models of type 2 diabetes and their usefulness for evaluating flavonoid bioactivity. Nutrients, 11(3), 530. https://doi: 10.3390/nu11030530

Feng, W., Gao, C., Bi, Y., Wu, M., Li, P., Shen, S., Chen, W., Yin, T. and Zhu, D. (2017), Randomized trial comparing the effects of gliclazide,

liraglutide, and metformin on diabetes with non-alcoholic fatty liver disease. Journal of Diabetes, 9: 800-809. https://doi.org/10.1111/1753-0407.12555

Firneisz, G. (2014). Non-alcoholic fatty liver disease and type 2 diabetes mellitus: the liver disease of our age?. World Journal of Gastroenterology: WJG, 20(27), 9072. https://doi:10.3748/wjg.v20.i27.9072

Gheibi, S., Kashfi, K., & Ghasemi, A. (2017). A practical guide for induction of type-2 diabetes in rat: Incorporating a high-fat diet and streptozotocin. Biomedicine & Pharmacotherapy, 95, 605-613. https://doi: 10.1016/j.biopha.2017.08.098

Gopal, V., Mandal, V., Tangjang, S., & Mandal, S. C. (2014). Serum biochemical, histopathology and SEM analyses of the effects of the Indian traditional herb Wattakaka volubilis leaf extract on wistar male rats. Journal of Pharmacopuncture, 17(1), 13. https://doi: 10.3831/KPI.2014.17.002

Gopalakrishnan, R., Elumalai, N., & Alagirisamy, R. (2020). Effect of polyherbal drug on oxidative stress and insulin resistance in high-fat diet-induced type 2 diabetic rats. All Life, 13(1), 310-320. https://doi.org/10.1080/26895293.2020.1776772

Guo, X. X., Wang, Y., Wang, K., Ji, B. P., & Zhou, F. (2018). Stability of a type 2 diabetes rat model induced by high-fat diet feeding with low-dose streptozotocin injection. Journal of Zhejiang University. Science. B, 19(7), 559. https://doi: 10.1631/jzus.B1700254

Horakova, O., Kroupova, P., Bardova, K., Buresova, J., Janovska, P., Kopecky, J., & Rossmeisl, M. (2019). Metformin acutely lowers blood glucose levels by inhibition of intestinal glucose transport. Scientific Reports, 9(1), 6156. https://doi: 10.1038/s41598-019-42531-0

Ipsen, D. H., Lykkesfeldt, J., & Tveden-Nyborg, P. (2018). Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. Cellular and Molecular Life Sciences, 75, 3313-3327.

Jahn, L. A., Hartline, L., Liu, Z., & Barrett, E. J. (2022). Metformin improves skeletal muscle microvascular insulin resistance in metabolic syndrome. American Journal of Physiology-Endocrinology and Metabolism, 322(2), E173-E180. https://doi: 10.1152/ajpendo.00287.2021

Khadke, S. P., Kuvalekar, A. A., Harsulkar, A. M., & Mantri, N. (2019). High energy intake induced overexpression of transcription factors and its regulatory genes involved in acceleration of hepatic lipogenesis: A rat model for type 2 diabetes. Biomedicines, 7(4), 76. https://doi: 10.3390/biomedicines7040076

Kosmalski, M., Ziółkowska, S., Czarny, P., Szemraj, J., & Pietras, T. (2022). The coexistence of nonalcoholic fatty liver disease and type 2 diabetes mellitus. Journal of Clinical Medicine, 11(5), 1375. https://doi: 10.3390/jcm11051375

Kristensen, J. M., Treebak, J. T., Schjerling, P., Goodyear, L., & Wojtaszewski, J. F. (2014). Two weeks of metformin treatment induces AMPK-dependent enhancement of insulin-stimulated glucose uptake in mouse soleus muscle. American Journal of Physiology-Endocrinology and Metabolism, 306(10), E1099-E1109. https://doi.org/10.1152/ajpendo.00417.2013

LaMoia, T. E., & Shulman, G. I. (2021). Cellular and molecular mechanisms of metformin action. Endocrine Reviews, 42(1), 77-96. https://doi.org/10.1210/endrev/bnaa023

Lang, P., Hasselwander, S., Li, H., & Xia, N. (2019). Effects of different diets used in diet-induced obesity models on insulin resistance and vascular dysfunction in C57BL/6 mice. Scientific Reports, 9 (1): 19556. https://doi.org/10.1038/s41598-019-55987-x

Lucchesi, A. N., Cassettari, L. L., & Spadella, C. T. (2015). Alloxan-induced diabetes causes morphological and ultrastructural changes in rat liver that resemble the natural history of chronic fatty liver disease in humans. Journal of Diabetes Research, 2015. https://doi: 10.1155/2015/494578

Marusic, M., Paic, M., Knobloch, M., & Liberati Prso, A. M. (2021). NAFLD, insulin resistance, and diabetes mellitus type 2. Canadian Journal of Gastroenterology and Hepatology, 2021.

Mykhalchyshyn, G., Kobyliak, N., & Bodnar, P. (2015). Diagnostic accuracy of acyl-ghrelin and it association with non-alcoholic fatty liver disease in type 2 diabetic patients. Journal of Diabetes & Metabolic Disorders, 14, 1-7. https://doi: 10.1186/s40200-015-0170-1

Nasri, H., & Rafieian-Kopaei, M. (2014). Metformin: current knowledge. Journal of Research in Medical Sciences: the official journal of Isfahan University of Medical Sciences, 19(7), 658.

Noordin, L., Nor, N. A. M., Bakar, N. H. A., & Ahmad, W. A. N. W. (2021). Metabolic and pancreatic derangement in type 2 diabetic rat. IIUM Medical Journal Malaysia, 20(4). https://doi.org/10.31436/imjm.v20i4.1917

Noordin, L., Wan Ahmad, W. A. N., Muhamad Nor, N. A., Abu Bakar, N. H., & Ugusman, A. (2022). Etlingera elatior flower aqueous extract protects against oxidative stress-induced nephropathy in a rat model of type 2 diabetes. Evidence-Based Complementary and Alternative Medicine, 2022.

Novelli, E. L. B., Diniz, Y. S., Galhardi, C. M., Ebaid, G. M. X., Rodrigues, H. G., Mani, F., ... & Novelli Filho, J. L. V. B. (2007). Anthropometrical parameters and markers of obesity in rats. Laboratory Animals, 41(1), 111-119. https://doi: 10.1258/002367707779399518

Pinyopornpanish, K., Leerapun, A., Pinyopornpanish, K., & Chattipakorn, N. (2021). Effects of metformin on hepatic steatosis in adults with nonalcoholic fatty liver disease and diabetes: insights from the cellular to patient levels. Gut and liver, 15(6), 827.

Raqib, T. M., Polus, R. K., & Mohammad, N. S. (2022). Prevalence of Vitamin B12 Deficiency in Patients with type 2 Diabetes Mellitus on Metformin. Diyala Journal of Medicine, 23(1), 22-32.

Rena, G., Hardie, D. G., & Pearson, E. R. 2017. The mechanisms of action of metformin. Diabetologia; 60 (9): 1577–8. https://doi: 10.1007/s00125-017-4342-z

Rosenblit, P. D. (2016). Common medications used by patients with type 2 diabetes mellitus: what are their effects on the lipid profile?. Cardiovascular Diabetology, 15(1), 95. https://doi.org/10.1186/s12933-016-0412-7

Roxo, D. F., Arcaro, C. A., Gutierres, V. O., Costa, M. C., Oliveira, J. O., Lima, T. F. O., ... & Baviera, A. M. (2019). Curcumin combined with metformin decreases glycemia and dyslipidemia, and increases paraoxonase activity in diabetic rats. Diabetology & Metabolic Syndrome, 11(1), 1-8.

Sayyed Kassem, L., Rajpal, A., Barreiro, M. V., & Ismail-Beigi, F. (2023). Beta-cell function in type 2 diabetes (T2DM): Can it be preserved or enhanced?. Journal of Diabetes, 15(10), 817-837.

Shaw, R. J. (2013). Metformin trims fats to restore insulin sensitivity. Nature Medicine, 19(12), 1570-1572. https://doi.org/10.1038/nm.3414

Shen, X., Wang, L., Zhou, N., Gai, S., Liu, X., & Zhang, S. (2020). Beneficial effects of combination therapy of phloretin and metformin in streptozotocin-induced diabetic rats and improved insulin sensitivity in vitro. Food & Function, 11(1), 392-403.

Skovso, S. (2014). Modeling type 2 diabetes in rats using high fat diet and streptozotocin. Journal of Diabetes Investigation, 5(4), 349-358.

Tang, Y., Feng, X., Liu, N., Zhou, Y., Wang, Y., Chen, Z., & Liu, Y. (2024). Relationship between systemic immune inflammation index and mortality among US adults with different diabetic status: Evidence from NHANES 1999-2018. Experimental Gerontology, 185, 112350.

Tian, F., Zheng, Z., Zhang, D., He, S., & Shen, J. (2018). Efficacy of liraglutide in treating type 2 diabetes mellitus complicated with non-alcoholic fatty liver disease. Bioscience Reports, 38(6), BSR20181304.

Tobar, N., Rocha, G. Z., Santos, A., Guadagnini, D., Assalin, H. B., Camargo, J. A., ... & Saad, M. J. (2023). Metformin acts in the gut and induces gut-liver crosstalk. Proceedings of the National Academy of Sciences, 120(4), e2211933120. https://doi.org/10.1073/pnas.2211933120

Wu, L., & Parhofer, K. G. (2014). Diabetic dyslipidemia. Metabolism, 63(12), 1469-1479. https://doi: 10.1016/j.metabol.2014.08.010

Yang, J., Fernández-Galilea, M., Martínez-Fernández, L., González-Muniesa, P., Pérez-Chávez, A., Martínez, J. A., & Moreno-Aliaga, M. J. (2019). Oxidative stress and non-alcoholic fatty liver disease: effects of omega-3 fatty acid supplementation. Nutrients, 11(4), 872. https://doi: 10.3390/nu11040872

Zakaria, Z., Othman, Z. A., Suleiman, J. B., Mustaffa, K. M. F., Jalil, N. A. C., Ghazali, W. S. W., ... & Kamaruzaman, K. A. (2022). Therapeutic Effects of Heterotrigona itama (Stingless Bee) Bee Bread in Improving Hepatic Lipid Metabolism through the Activation of the Keap1/Nrf2 Signaling Pathway in an Obese Rat Model. Antioxidants, 11(11), 2190.

Zhang, R., Cheng, K., Xu, S., Li, S., Zhou, Y., Zhou, S., ... & Zhou, Y. (2017). Metformin and diammonium glycyrrhizinate enteric-coated capsule versus metformin alone versus diammonium glycyrrhizinate enteric-coated capsule alone in patients with nonalcoholic fatty liver disease and type 2 diabetes mellitus. Gastroenterology Research and Practice, 2017. https://doi: 10.1155/2017/8491742