Sains Malaysiana 54(1)(2025): 255-263
http://doi.org/10.17576/jsm-2025-5401-20

Ameliorative Effects of Betanin in Mice with Trimethyltin-Induced Pancreatic and Hepatocytic Alterations

(Kesan Amelioratif Betanin pada Tikus dengan Perubahan Pankreas dan Hepatosit Terinduksi Trimethyltin)

WACHIRYAH THONG-ASA*, YANISA DETPAKDEE & KANLAYAWADEE SRISOONTHON

Animal Toxicology and Physiology Specialty Research Unit (ATPSRU), Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900 Thailand

Received: 28 May 2024/Accepted: 4 November 2024

ABSTRACT

The effects of TMT on metabolic alteration are on the rise, including obesity and diabetes. In the present study, we aimed to investigate the protective effect of betanin (Bet) against TMT-induced glycemic disturbance and pancreatic and hepatocytic alterations, expanding on TMT's and Bet's effects on metabolic diseases. Fifty male Institute of Cancer Research (ICR) mice were randomly divided into Sham-veh, TMT-L-veh, TMT-H-veh, TMT-L-Bet100, and TMT-H-Bet100 groups. A low dose (L) (1 mg/kg) and high dose (H) (2.6 mg/kg) of TMT were given via one-time intraperitoneal (i.p.) injection before intragastric gavage administration of treatments for 4 consecutive weeks. A weekly oral glucose tolerance test (OGTT) was conducted for glycemic control capacity evaluation with serum insulin assessment. Pancreatic and hepatic tissues were collected to analyze islet number and beta cell density, glycogen content, and histopathology. TMT exposure did not significantly change glycemic control capacity or serum insulin level ($p > 0.05$). TMT significantly reduced pancreatic beta cell density, and this was accompanied by a decrease in hepatic glycogen content and an increase in hepatosteatosis and inflammation ($p < 0.05$). Treatment with Bet significantly alleviated all these alterations ($p < 0.05$). Bet showed alleviative effects against TMT-induced pancreatic and hepatocytic alterations, including preventing pancreatic beta cell damage, maintaining the liver's glycogen content, anti-hepatosteatosis, and anti-inflammation.

Keywords: Betanin; glycemic control; hepatosteatosis; pancreatic beta cell; trimethyltin

ABSTRAK

Kesan TMT pada perubahan metabolik semakin meningkat, termasuk obesiti dan diabetes. Dalam kajian ini, kami berhasrat untuk mengkaji kesan perlindungan betanin (Bet) terhadap gangguan glisemik aruhan-TMT dan perubahan pankreas dan hepatosit, mengembangkan kesan TMT dan Bet pada penyakit metabolik. Lima puluh tikus jantan Institut Penyelidikan Kanser (ICR) secara rawak dibahagikan kepada kumpulan Sham-veh, TMT-L-veh, TMT-H-veh, TMT-L-Bet100 dan TMT-H-Bet100. Dos rendah (L) (1 mg/kg) dan dos tinggi (H) (2.6 mg/kg) TMT diberikan melalui satu suntikan intraperitoneal (i.p.) sebelum diberikan rawatan gavage intragastrik selama 4 minggu berturut-turut. Ujian toleransi glukosa oral secara mingguan (OGTT) telah dijalankan untuk penilaian kapasiti kawalan glisemik dengan penilaian insulin serum. Tisu pankreas dan hati dikumpul untuk analisis nombor kelompok dan ketumpatan sel beta, kandungan glikogen dan histopatologi. Pendedahan TMT tidak banyak mengubah kapasiti kawalan glisemik atau aras insulin serum ($p > 0.05$). TMT mengurangkan ketumpatan sel beta pankreas dengan ketara dan menurunkan kandungan glikogen hepatik dan peningkatan dalam hepatosteatosis dan keradangan ($p < 0.05$). Rawatan dengan Bet telah mengurangkan semua perubahan ini dengan ketara ($p < 0.05$). Bet menunjukkan kesan pengurangan terhadap perubahan pankreas dan hepatosit aruhan TMT, termasuk mencegah kerosakan sel beta pankreas, mengekalkan kandungan glikogen hati, anti-hepatosteatosis dan anti-keradangan.

Kata kunci: Betanin; kawalan glisemik; hepatosteatosis; sel beta pankreas; trimetiltin

INTRODUCTION

Organotin toxicity is one of the major risk factors for human diseases worldwide. It can be acquired from contaminating environmental chemicals and polyvinyl chloride plastic products. Organotin is used in pesticidal tins, antifoulant paints, agricultural pesticides, molluscicides, wood preservatives, and plastic heat stabilizers (Zhang et al. 2023). Trimethyl and triethyltin compounds are well absorbed in the gastrointestinal tract and are the most toxic organotin (Kimbrough 1976; Pagliarani, Nesci & Ventrella 2013). Human exposure to trimethyltin (TMT) is usually an occupational risk; therefore, high potency for neurotoxicity has been more widely studied and depicted. TMT intoxication induces seizures, aggression,

hyperactivity, and cognitive impairment (Geloso, Corvino & Michetti 2011). TMT induces neurodegeneration in specific brain areas (i.e., the hippocampus), leading to learning and memory deficits (Aschner & Aschner 1992). Pathophysiological characteristics are induced by TMT's relevance to some neurodegenerative diseases, such as temporal lobe epilepsy (Geloso, Corvino & Michetti 2011) and Alzheimer's disease (Ye et al. 2020).

Recently, TMT toxicity other than in the nervous system has been reported. Zhang et al. (2023) indicated that TMT can induce autophagy in a mouse pancreas and pancreatic tissue apoptosis and necrosis, leading to increased blood glucose and lipid levels in mice. This finding indicated TMT's alterative effect on glycemic and lipidemic control mechanisms and extended TMT's risk effect as a neurotoxin and metabolic disorder. It is well known that the alteration of glycemic control metabolism can lead to metabolic diseases, such as diabetes. Regulation of glucose homeostasis depends directly on hormonal manipulation of islet cells of the pancreas, followed by liver and insulin-sensitive organs. This coordination balances glucose utilization and synthesis (Regufe, Pinto & Perez 2020). Therefore, alteration of pancreatic islets, the liver, and insulin-sensitive organs can lead to misleading control of glucose homeostasis. The liver is an insulin-sensitive organ that is important in blood glucose homeostasis, including glycogenesis and glycogenolysis. It is the principal organ for the body's detoxification. However, high toxic exposure also induces liver damage. It has been reported that TMT's effect on liver toxicity includes oxidative stress-induced apoptosis, necroptosis, DNA damage, and immune dysfunction (Wang et al. 2022). Research on TMT's effect on the capacity for glycemic control metabolism and relevant organs is scarce. Therefore, studying TMT's effect on the capacity for glycemic control, according to pancreatic and hepatocytic alterations, is interesting.

Metabolic disease is a type of degenerative disease, and it is uncurable. Therefore, preventive therapy is the focus. A large number of consumers have a strong preference for 'functional foods' in improving their diet and maintaining their health (Chen et al. 2021). Maintenance of the body's metabolisms using dietary intervention may prolong cell function, increase cell viability, and protect against the attack of diseases (Thong-asa et al. 2019). Many of phytonutrients' bioactive compounds have been used as nurturing factors of degenerative disease, including cardiovascular and cerebrovascular diseases, cancer, diabetes, and chronic respiratory diseases (Shoaib et al. 2023). Betanin (Bet), a bioactive ingredient from beetroot, provides a variety of benefits, such as effective antioxidants; anti-inflammation; anti-cancer effects; and amelioration of neurodegeneration, cardiovascular, and cerebrovascular diseases as well as lung, liver, and kidney damage (Esatbeyoglu et al. 2014; Han et al. 2015; Silva et al. 2022; Thong-Asa, Jedsadavitayakol & Jutarattananon 2021; Thong-Asa et al. 2020). A recent report showed that Bet can positively regulate plasma glucose and insulin, indicating Bet's beneficial effects on glucose homeostasis in diabetic rats (Dhananjayan et al. 2017). Corroboration with evidence of organotin exposure is an occupational risk of metabolic disturbance and diseases (Tinkov et al. 2019). Therefore, in the present study, we investigated Bet's protective effect against TMT-induced glycemic disturbance and pancreatic and hepatocytic alterations, expanding on TMT's and Bet's effects on metabolic diseases.

MATERIALS AND METHODS

The Animal Ethics Committee, Faculty of Science, Kasetsart University, approved the experimental procedure (ID#ACKU66-SCI-027).

ANIMALS

Forty male Institute of Cancer Research (ICR) mice, 8 weeks old and weighing 40–50 grams, were obtained from the National Laboratory Animal Center, Mahidol University (Nakhon Pathom, Thailand). The mice were housed in individual cages at a controlled room temperature of 25 °C with a 12-hour light/12-hour dark cycle. A standard diet (mouse diet food No.082G) and reverse osmosis water were always allowed.

CHEMICALS AND REAGENTS

Bet, TMT, normal saline solution (NSS), phosphate-buffered saline (PBS), glucose, paraformaldehyde (PFA), hematoxylin & eosin (H&E), periodic acid Schiff (PAS), anti-insulin monoclonal antibody, diaminobenzidine (DAB), and citrate buffer were purchased from Chemical Express Co., Ltd. Bangkok, Thailand; Merck, Millipore, Darmstadt, Germany; and Agilent, CA, USA.

EXPERIMENTAL PROTOCOL

Mice were divided into five groups (n = 8 for each group): Sham-veh, TMT-L-veh, TMT-L-Bet100, TMT-H-veh, and TMT-H-Bet100. TMT 1 mg/kg and 2.6 mg/kg were selected and defined as low (L) and high (H) doses for one-time intraperitoneal (i.p.) injection (Liu et al. 2020; Thong-Asa et al. 2020). The vehicle (veh), NSS and Bet 100 mg/kg dissolved in NSS, was administered via intragastric gavage to the TMT-L-Bet100 and TMT-H-Bet100 groups (Thong-Asa et al. 2020). After 2-week acclimatization, an oral glucose tolerance test (OGTT) was conducted to assess all mice's baseline glycemic control capacity. After that one-time TMT injection was delivered, daily treatments for each group were administered and continued for 4 weeks (Figure 1(a)).

ORAL GLUCOSE TOLERANCE TEST

After 12 h of fasting, all mice were tested for a baseline blood glucose level and subjected to OGTT by gavage with glucose 2 g/kg of body weight. Blood was collected from the tail vein at 30, 60, 90, and 120 min after the glucose challenge to determine the blood glucose level using the glucometer (Sinocare-Safe-Accu2, blood glucose monitoring system). Five-time OGTT tests were defined as baseline (before treatments) and 1 (w1), 2 (w2), 3 (w3), and 4 (w4) weeks after treatments (Nagy & Einwallner 2018).

SERUM INSULIN DETERMINATION

Blood was collected from the tail vein and allowed to clot for 15-30 min at room temperature. The clot was removed by centrifuging at 1,000-2,000 g for 10 min in a refrigerated centrifuge, and the supernatant was collected as a serum. The serum insulin levels were measured using an enzyme immunoassay (Mouse ELISA kits, EZRMI-13K, Merck, Millipore).

HISTOLOGICAL ANALYSIS

Mice were euthanized by i.p. injection with 50 µl Zoletil 100 + X-Lazine (4:1). Cardiac perfusion with NSS, followed by 4% PFA, was done. Livers and pancreases were collected and stored in 4% PFA at 4 °C for 48 h before being processed, embedded in paraffin, and cut with a rotary microtome at a thickness of 5 µm.

PERIODIC ACID SCHIFF METHOD

Glycogen content in liver tissues was evaluated using the PAS method. Five slides (at 100-µm intervals) from each animal were selected, deparaffinized, hydrated in distilled water, and treated with periodic acid for 5 min followed by distilled water. They were incubated in Schiff's reagent for 15 min and washed in running tap water for 10 min. They were then rinsed in increasing concentrations of alcohol (70%, 80%, 95%, and 100%), cleared in xylene, and mounted as usual. Nonoverlapping images were captured at 200x magnification power in five regions around the central vein of each slide. Image analysis for the liver glycogen content was indicated by a PAS-positive area with diastase subtraction and represented as % PAS-positive area using NIH Image J (Thong-asa et al. 2019).

HEMATOXYLIN & EOSIN STAINING

Liver and pancreas tissue sections were deparaffinized and hydrated in distilled water. Then, the sections were stained in hematoxylin for 3-5 min, washed in running tap water for 5 min, dipped in acid alcohol for a few seconds, and then rinsed in running tap water. The sections were dipped in ammonia water until the sections turned blue and then in tap water. After that, they were counterstained in 1% eosin Y for 10 min, washed in tap water for 5 min, dehydrated in increasing concentrations of alcohols, cleared with xylene, and covered by a glass mounting. Liver tissues were scored for steatosis and inflammation following a nonalcoholic fatty liver disease guideline (Trovato et al. 2014). In addition, the pancreatic islet number and area were evaluated for the pancreas tissue. All image analyses used NIH Image J.

ANTI-INSULIN IMMUNOHISTOCHEMISTRY

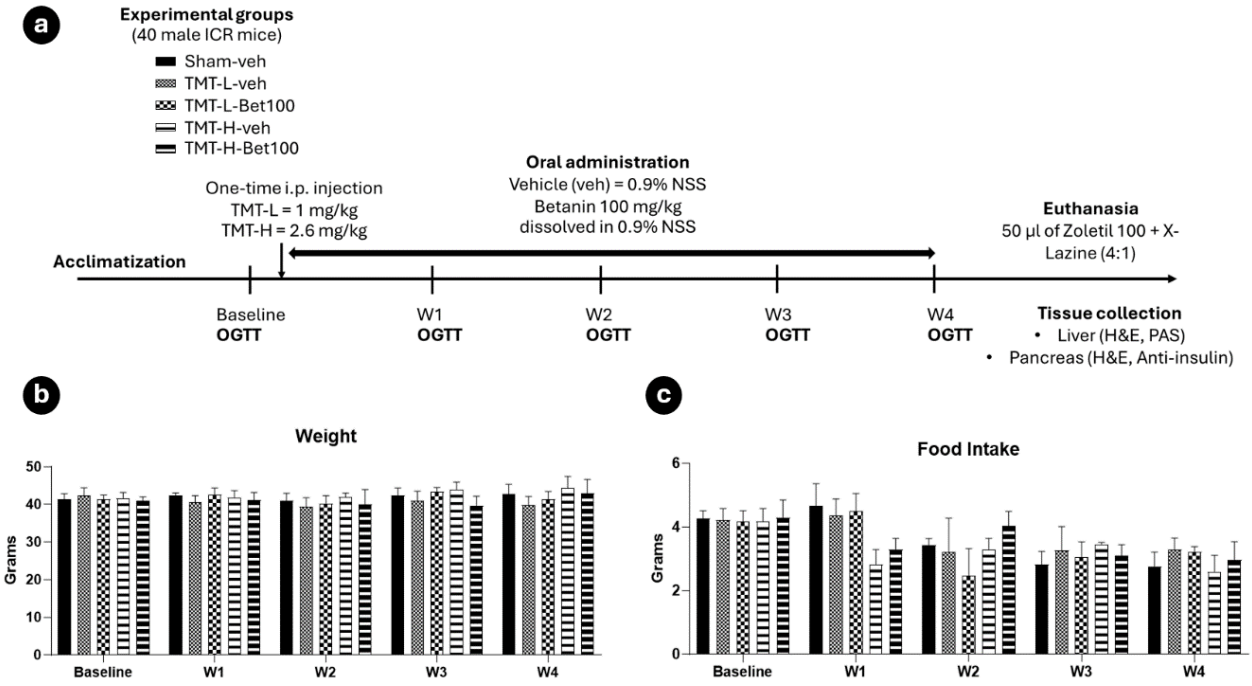
Anti-insulin immunohistochemistry was used to determine beta cells in pancreatic islets. Five pancreatic sections were selected from each mouse. They were kept in a hot-air oven overnight at 60 °C. Then, they were deparaffinized in xylene and hydrated in 100% and 95% ethanol and running tap water. Microwave-induced antigen retrieval was performed in a citrate buffer with a pH of 6 for 3 min at high power and 10 min at 30% power. After a cool-down period of 20 min, the sections were washed in running tap water for 5 min; incubated in 3% H_2O_2 in PBS for 10 min, followed by running tap water; and washed in a washing buffer. Next, brain sections were incubated in 10% NGS in PBS for 10 min at room temperature. After that, the sections were incubated for 24 h with the primary antibody at room temperature (rabbit anti-insulin monoclonal antibody [1:100): Ab181547]). Brain sections were washed in a washing buffer the next day and then incubated for 30 min at room temperature with a one-step polymer-HPR anti-mouse & rabbit system (Dako EnVision). After washing with a washing buffer, DAB was added for 5 min and washed with running tap water. The sections were dehydrated in 95% and 100% ethanol, cleared with xylene, and covered with a glass mounting. Pancreatic images were captured at 200x magnification power and analyzed for insulin-positive islet number and area using NIH Image J.

STATISTICAL ANALYSIS

All statistical analyses were conducted using GraphPad Prism 8.0.1. The normal distribution and variance homogeneity for each data group were checked using the Shapiro-Wilk normality and Levene's test, respectively. One-way analysis of variance was conducted, and Tukey's multiple comparisons were used for the post hoc test. The data were expressed as mean ± standard deviation. A p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The present study indicated that one-time TMT injection, either low-dose (1 mg/kg) or high-dose (2.6 mg/kg), with a long-lasting effect observation for 4 weeks showed no alterative effect on body weight (Figure 1(b)) or food intake (Figure 1(c)), indicated as statistically insignificant as compared to TMT-L-veh and TMT-H-veh to Sham-veh ($p > 0.05$). A tremor was present in some mice 6-48 h after



Veh =vehicle; TMT = trimethyltin; L = low dose; H = high dose; Bet = betanin; i.p. = intraperitoneal; NSS = normal saline; w = week; OGTT = oral glucose tolerance test; H&E = hematoxylin and eosin; PAS = periodic acid Schiff

FIGURE 1. Experimental protocol (a), histogram of animal's weight (b), and food intake (c)

TMT injection, and this symptom disappeared within a week. Mice undergoing high-dose TMT injection tend to reduce food intake in the first week and return to the average afterward. A previous report indicated the TMT toxic effects (dose range of 1.3-3 mg/kg) were weight loss, hyperexcitability, tremors, tonic-clonic seizures, posterior paresis, and death. However, these responses and toxic endpoints showed different rank orders among the strains, and we used the TMT chronic exposure procedure (Ekuta, Hikal & Matthews 1998). The present study included ICR mice and indicated that a transient tremor occurs only in some mice and is induced only by the high-dose TMT. We used a single dose and found that low and high doses of TMT exposure did not significantly affect body weight change, as exemplified in previous studies (Aramsirirujiwet et al. 2023; Choi et al. 2012; Thong-Asa et al. 2020).

In the present study, we investigated the effects of TMT on glycemic control capacity, serum insulin-level evaluation, and histological change in pancreatic islets and liver tissue. We found that glycemic control capacity indicated by OGTT during a 4-week experiment showed no difference in any groups ($p > 0.05$, Figure 2(a)-2(e)). No data on glycemic control capacity were indicated by OGTT in mice with TMT-induced toxicity, and our report indicated that a one-time TMT dose of 1-mg/kg and 2.6-mg/kg injections has a nonsignificant alteration effect on glycemic control capacity. These results were consistent with serum insulin levels that showed no difference in all groups (Figure 2(f)). A trend emerged in the first week that mice in high-dose TMT showed gradually reduced serum insulin, but it was not statistically significant ($p > 0.05$). A previous study indicated that chronic TMT exposure significantly increased pancreatic apoptosis and necrosis and significantly reduced serum insulin (Zhang et al. 2023). They used chronic TMT exposed in drinking water (0.01 mg/ML) for 18 consecutive days in C57Bl/6J mice. However, the present study used a single-dose TMT injection (1 and 2.6 mg/kg) with a 4-week follow-up in ICR mice and found no significant difference in the reduction of serum insulin levels. The different results of TMT's effect on serum insulin levels may include dose, duration, and mouse strain.

Histological assessment using H&E staining of pancreatic islets indicated no significant difference in the islet number (Figure 3(b)), islet area (Figure 3(c)), or islet density (Figure 3(d)) in any group ($p > 0.05$). High-dose TMT injection showed more aggregation among inflammatory cells than other groups (Figure 3(a)). Assessment of insulin-positive islets showed an interesting result, which was that low- and high-dose TMT injections significantly reduced the insulin-positive number ($p = 0.0241$ and $p = 0.0106$, respectively) and insulin-positive area ($p = 0.0012$ and $p = 0.0093$, respectively) of pancreatic islets when we compared TMT-L-veh and TMT-

H-veh to Sham-veh (Figure 3(e) and 3(f)). In addition, insulin-positive density evaluation indicated that the high-dose TMT injection significantly reduced the density of pancreatic beta cells ($p = 0.0488$) compared to Sham-veh (Figure 3(g)). Corroboration was found between glycemic control capacity and serum insulin level. Although we found a significant reduction in the number and area of insulin-positive islets in mice with low- and high-dose TMT exposure, it seems adequate for insulin production and maintaining glycemic control capacity. This result may be explained by a huge spectrum of functional islet responses and insulin phenotypes across mouse strains, and the ubiquitous C57Bl/6J mouse exhibiting the lowest secretory response (Yau et al. 2024). This may imply that the C57Bl/6J mouse exhibits more susceptibility than the ICR mouse.

We also evaluated the liver tissue pathology and glycogen storage capacity (Figure 4(a)). We found that TMT significantly reduced glycogen content in liver tissue when comparing TMT-L-veh ($p < 0.0001$) and TMT-H-veh ($p = 0.0018$) to Sham-veh (Figure 4(b)). The liver is an insulin-sensitive organ that is vital in regulating glucose homeostasis through communication between glucose utilization (glycolysis and glycogenesis) and gluconeogenesis (Dhananjayan et al. 2017). Our results indicated an inhibitory effect of TMT on glycogen content in hepatocytes. Data for TMT and liver glycogen in mice are rare. In recent research, organotin's effect on glycogen content was found only in the hepatopancreas of the bivalve, indicating the reduction in glycogen content as well (Sharma et al. 2023). TMT's effect on the reduction of the liver's glycogen may involve glycolysis and glycogenesis pathways, and further studies are required to elucidate this subject. We also observed hepatocytic pathological conditions and found that TMT's effect involved the activation of steatosis and inflammation. Hepatic steatosis significantly increased in the TMT-L-veh ($p = 0.0041$) and TMT-H-veh ($p < 0.0001$) groups, compared to the Sham-veh (Figure 4(c)) group. These indicated an alternative effect of TMT on glycemic and lipid metabolisms. A previous study indicated the link between the organotin compound (tributyltin) and hepatic steatosis (Zuo et al. 2011), but there has been no report in the case of TMT. Furthermore, increased hepatic steatosis can lead to further inflammation and hepatocytic damage. The present study indicated inflammatory activation of

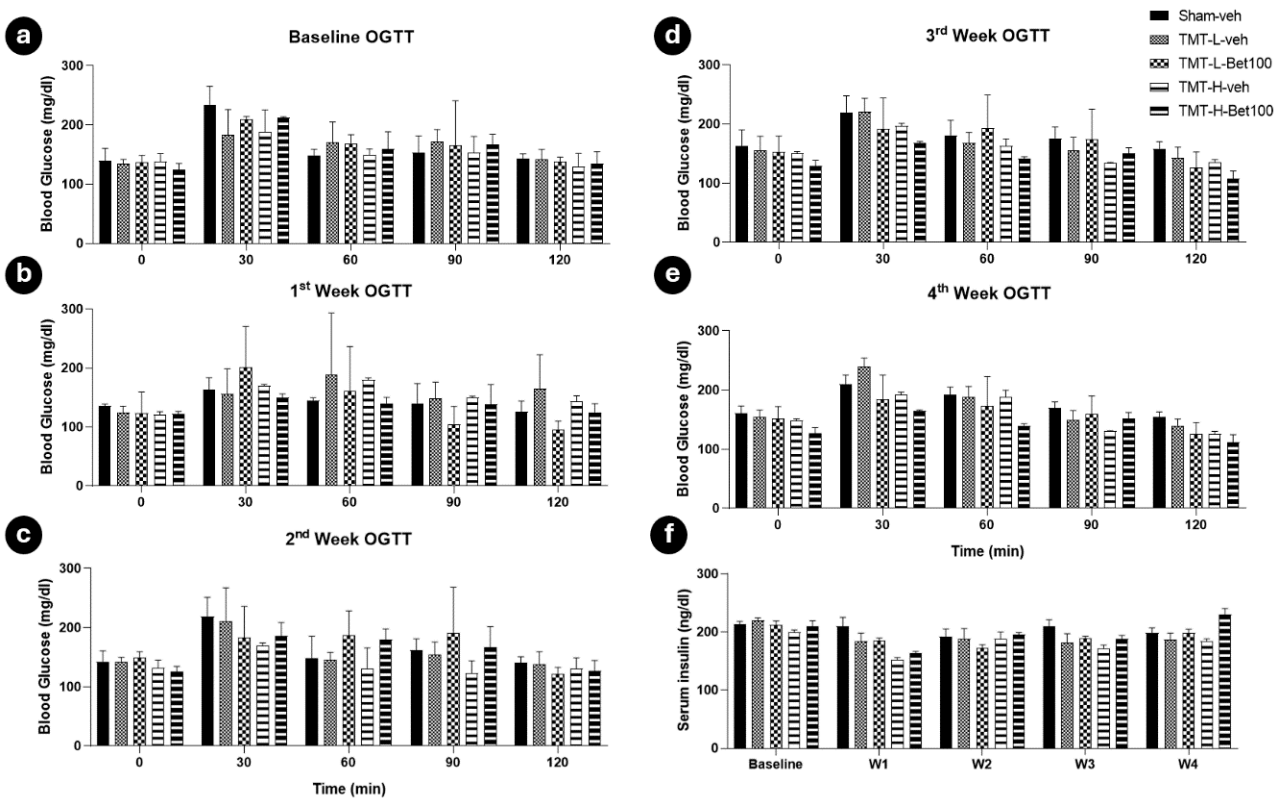
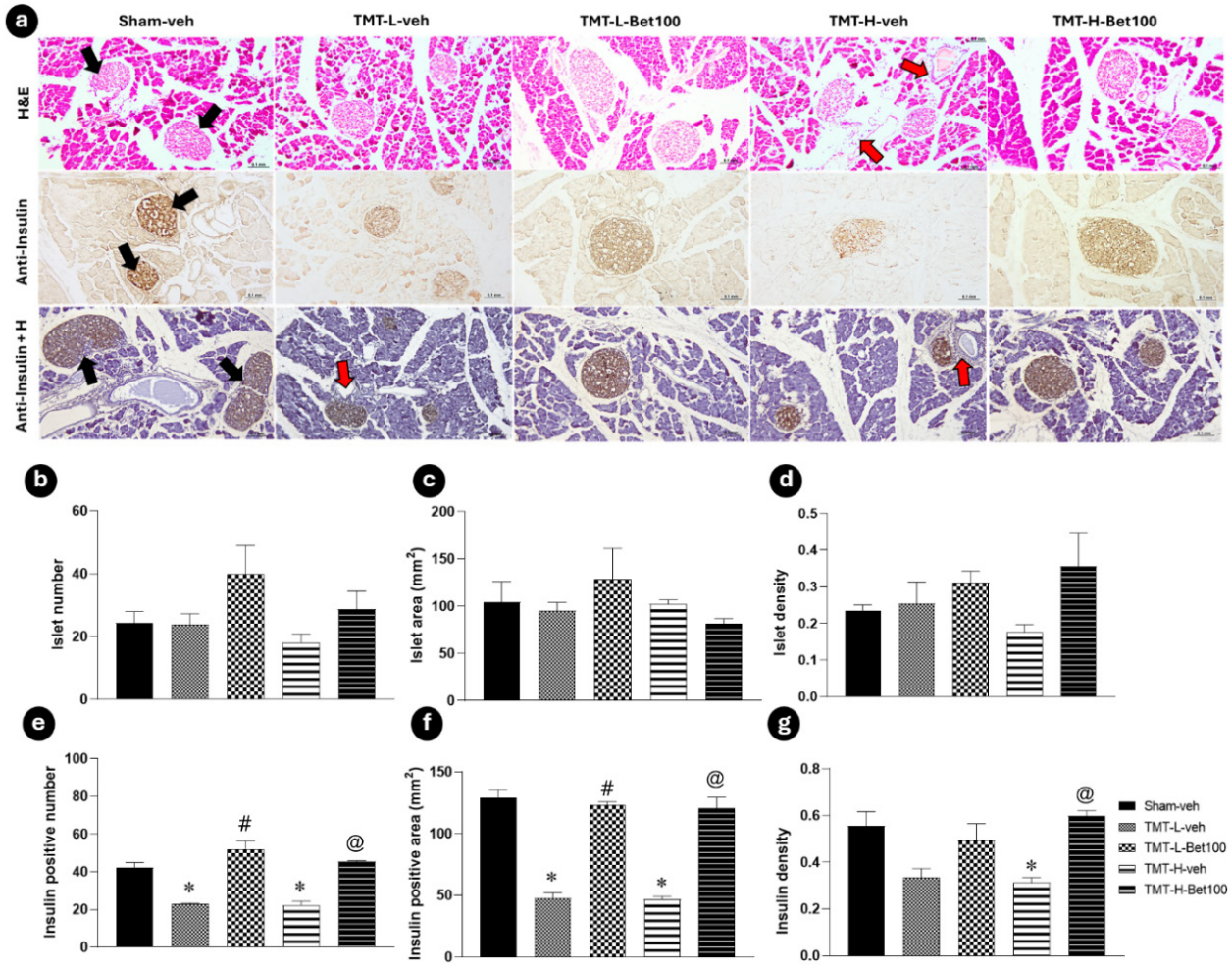


FIGURE 2. Glycemic control capacity test results from OGTT at baseline (a), the 1st week (b), 2nd week (c), 3rd week (d), 4th week (e), and serum insulin from baseline to the 4th week (f). Veh =vehicle; TMT = trimethyltin; L = low dose; H = high dose; Bet = betanin; w = week; OGTT = oral glucose tolerance test

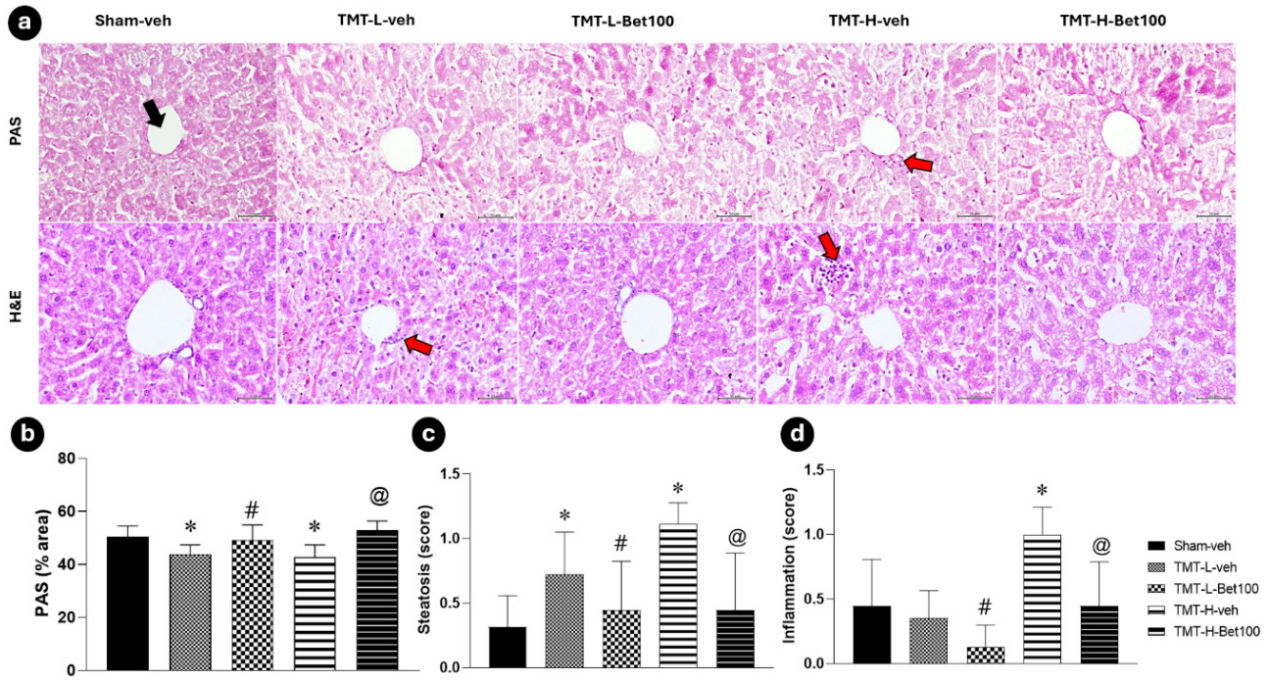


*indicates significant difference compared to Sham-veh, # indicates significant difference compared to TMT-L-veh, @ indicates significant difference compared to TMT-H-veh. H&E = hematoxylin and eosin; veh =vehicle; TMT = trimethyltin; L = low dose; H = high dose; Bet = betanin

FIGURE 3. Representative photomicrographs of pancreatic islets (a) staining with H&E, anti-insulin immunohistochemistry, and anti-insulin immunohistochemistry counterstained with hematoxylin, magnification power 200x. Black arrows indicate pancreatic islets and red arrows indicate inflammatory cell aggregation. Histograms indicate islet number (b), islet area (c), islet density (d), insulin-positive islet number (e), insulin-positive islet area (f), and insulin-positive density (g)

hepatic tissue and found a significant effect of TMT, especially with a high dose, in the induction of hepatic inflammatory cell aggregation. We found that TMT-H-veh had a significantly higher inflammatory score ($p = 0.0182$) than Sham-veh (Figure 4(d)). Taken together, our results depicted TMT's prominent effect on the decrease in the liver's glycogen content, activation of hepatosteatosis, and hepatic inflammation.

Bet 100 mg/kg treatment on a mouse with low- and high-dose TMT exposure protected the liver's tissue. Bet significantly increased the liver's glycogen content in TMT-L-Bet100 ($p = 0.0017$) and TMT-H-Bet100 ($p < 0.0001$), compared to their vehicle groups (Figure 4(b)). It has been reported that Bet can increase the activity of the glycolytic enzyme (glucokinase and pyruvate kinase), glucose-6-phosphate dehydrogenase, and decrease the activity of gluconeogenic enzymes (glucose-6-phosphatase and fructose-1,6-bisphosphatase), thereby increasing the glycogen content in the liver (Dhananjayan et al. 2017). Previous findings have also indicated that Bet is a potential therapeutic agent for hepatosteatosis, steatohepatitis, and diseases with similar pathophysiological characteristics and that it inhibits the inflammatory infiltration of the liver and necrosis death (Lugo-Radillo et al. 2020). The anti-



*indicates a significant difference compared to Sham-veh, # indicates a significant difference compared to TMT-L-veh, @ indicates a significant difference compared to TMT-H-veh. PAS = periodic acid Schiff; H&E = hematoxylin and eosin; veh =vehicle; TMT = trimethyltin; L = low dose; H = high dose; Bet = betanin

FIGURE 4. Representative photomicrographs of liver tissue (a) staining with PAS and H&E, magnification power 200x. Black arrows indicate the location of the central vein, and red arrows indicate inflammatory cell aggregation. Histograms show the glycogen content indicated by the percentage of PAS (b), liver cell steatosis score (c), and inflammation score (d)

inflammatory effect of Bet includes inhibiting inducible nitric oxide synthase expression and suppressing the proinflammatory nuclear factor kappa-B pathways (Silva et al. 2022). Bet was shown to inhibit the inflammatory enzymes, prominently cyclooxygenase 2 and subordinate cyclooxygenase 1 (Reddy, Alexander-Lindo & Nair 2005). Bet reduced the production of the inflammatory cytokines, TNF-α and IL-β, and increased levels of IL-10 have also been reported (Martinez et al. 2015). Therefore, the benefits of Bet's anti-hepatosteatosis and anti-inflammatory properties in the present study may include those indicated.

CONCLUSION

Exposure to TMT can cause significant damage to pancreatic beta cells. TMT toxicity also induced hepatocytic alterations, including decreased glycogen content, increased hepatosteatosis, and increased inflammatory infiltration. Bet's alleviative effect against TMT-induced pancreatic and hepatocytic alterations includes prevention of pancreatic beta cell damage, maintenance of the liver's glycogen content, anti-hepatosteatosis, and anti-inflammation. Therefore, Bet is useful as a pharmaceutical agent and dietary supplement.

ACKNOWLEDGMENTS

This research was supported by Research Financial Support for Project in Biology and Zoology, Department of Zoology and Undergraduate Research Matching Fund (URMF), Faculty of Science, Kasetsart University. WT: Conceptualization and Writing-original draft preparation. WT, YD, and KS: Methodology, Investigation, Data collection and analysis. All authors have read, revised, and agreed to the published version of this manuscript. The corresponding author can provide supplementary data upon a reasonable request. We certify no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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*Corresponding author; email: fsciwyth@ku.ac.th