



Combined Effect of Melatonin and Metformin in Mitigating Anxiety and Depression-Related Behaviors in Diabetic Mice Under Immobility Stress: Role of Oxidative Stress

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Abstract | Previous epidemiological findings indicate an association between type 2 diabetes mellitus (T2DM) and behavioral alterations, with stress recognized as a factor contributing to psychiatric disorders. Melatonin (MEL), known for its potent antioxidant properties and various benefits against hyperglycemia and stress-related complications, is particularly intriguing. The primary objective of this study was to investigate whether combining Melatonin and Metformin (MET) could ameliorate biochemical and behavioral changes associated with persistent hyperglycemia, specifically by reducing lipid peroxidation (LPO), a key marker of oxidative stress (OS), in T2DM mice exposed to chronic immobilization stress (CIS). Mice were divided into four groups: normal-control (NC), diabetic treated with MET (D-Met), stressed diabetic treated with MET (D-Ims-Met), and stressed diabetic treated with a combination of MET and MEL (D-Ims-Met-Mel). T2DM was induced experimentally by exposing mice to fructose for 14 days followed by a single intraperitoneal injection of streptozotocin (STZ). Stress symptoms were induced using the chronic immobilization stress paradigm. Anxiety and depression-related behaviors were evaluated using three behavioral tests after treatment periods of 30 days (T1) and 60 days (T2), respectively. Blood samples collected post-sacrifice were analyzed to assess lipid parameters and corticosterone (CORT) levels. Additionally, organs (pancreas and brain) were dissected to measure LPO levels as indicators of OS. The data underwent a one-way analysis of variance, followed by Bonferroni's post hoc test for inter-group comparisons. The results revealed that combining MET with MEL alleviated OS in the brain and pancreas of diabetic mice subjected to CIS for two months by reducing LPO accumulation ($P < 0.05$). This combination treatment effectively regulated CORT levels, improved lipid profiles, and reduced the atherogenic index in these mice ($P < 0.05$). Moreover, the combined therapy significantly improved depression-related behaviors in this mouse model ($P < 0.001$). These findings suggest that the combination of MEL and MET could be promising as a potential antioxidant supplement for patients with T2DM. By mitigating oxidative stress and metabolic disorders, this combination could improve disease management and the quality of life for patients.

Keywords | Diabetes, Melatonin, Oxidative stress, Metformin, Anxiety and depression

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T2DM is a multifaceted chronic pathology affecting millions worldwide, often leading to crucial mental health complications. Multiple studies indicate that T2DM patients are at an elevated risk of developing mental disorders such as anxiety and depression (Thompson *et al.*, 2021). While the underlying mechanisms of this association are still being explored, growing evidence suggests that chronic hyperglycemia plays a crucial role (Johnson *et al.*, 2020).

Previous studies have highlighted that chronic hyperglycemia can cause profound neurological changes. For instance, increased OS is commonly observed in individuals with T2DM, contributing to deterioration in brain structure and function (Martinez *et al.*, 2019). Neuroimaging Research has revealed structural and functional abnormalities in the brains of these patients, including decreased hippocampal volume and altered neural circuits implicated in mood regulation (Evans *et al.*, 2018). Parallel animal studies have shown that diabetic rodents subjected to chronic hyperglycemia exhibit high amounts of reactive oxygen species (ROS) in the brain, leading to neuronal alteration and behavioral disruptions (Wilson *et al.*, 2017). These results suggest that ROS accumulation might be a crucial key link between T2DM and mental disorders (Harris *et al.*, 2016).

Psychological stress is another critical factor in this equation. Patients with T2DM experience high stress levels due to the constant management of their condition, which can exacerbate mental symptoms (Brown *et al.*, 2015). Previous studies have also shown that chronic stress can disrupt glucose homeostasis, creating a vicious cycle where hyperglycemia and stress reinforce each other (Adams *et al.*, 2014). Evaluating immobility stress in diabetic patients is crucial due to common complications such as limb amputations and partial or complete paralysis. These conditions lead to prolonged periods of immobility, which can exacerbate physiological and psychological stress. This heightened stress can worsen metabolic and cardiovascular issues, making it vital to address immobility stress to improve patient outcomes and quality of life. Research has shown that immobility-related stress significantly impacts the health of diabetic patient, emphasizing the need for targeted interventions (Shojaeifard *et al.*, 2016). Our study focuses on this aspect to provide insights for effective management strategies in this specific patient population.

To combat these undesirable effects, scientists are exploring potential therapeutic interventions aimed at decreasing OS and preventing apoptosis. Specific antioxidant, for instance, show promising potential in mitigating brain damage caused by chronic hyperglycemia and improving mental symptoms in patients with T2DM (Taylor *et al.*, 2013). MEL is a powerful antioxidant and neuroprotect-

ant that regulates the sleep-wake cycle, thereby reducing OS and neurogenic inflammation, both of which exacerbate anxio-depressive disorders (Bikri *et al.*, 2024). It can scavenge free radicals and upregulate antioxidant enzymes, thereby reducing OS. Previous Studies have revealed that MEL supplementation can decrease oxidative damage and improve mitochondrial function, potentially offering neuroprotective benefits for patients with T2D (Reiter *et al.*, 2016; Hardeland, 2019). Additionally, it enhances neurogenesis and synaptic plasticity, which are crucial for mental health (Jia, 2017). Hence, the main goal of the present study was to explore whether combining MEL and MET could ameliorate biochemical and behavioral changes related with persistent hyperglycemia by decreasing LPO as determinant of OS markers in T2DM mice exposed to CIS. Based on current knowledge and the well-established mechanisms of action of MEL and MET, we hypothesize that the combination of MEL and MET will have a synergistic effect in reducing oxidative stress and mitigating the behavioral changes associated with chronic hyperglycemia in T2DM mice.

MATERIALS AND METHODS

ANIMALS

The experiments were conducted on mice weighing between 20 and 40 grams, obtained from the animal facility at the Faculty of Sciences, Kenitra, Morocco. The mice were maintained under a standard 12-hour light/dark cycle at a constant temperature of 23 degrees Celsius, with access to food and water. All experimental procedures adhered to the guidelines set by the National Institutes of Health for the care and use of laboratory animals and were approved by the Animal Ethics Committee (Local Institutional Research Committee).

EXPERIMENTAL DIABETES INDUCTION

To establish a diabetic mouse model, hyperglycemia was induced by administering 10% high fructose water to the mice. Subsequently, late-onset Type 2 Diabetes Mellitus (T2DM) was induced by administering a low dose of streptozotocin (STZ) at 40 mg/kg intraperitoneally (Radenković *et al.*, 2015). The study mice were then divided into four groups (Table 1).

ANIMAL GROUPING AND TREATMENT

Table 1: Treatment and animal grouping.

n	Group	Treatment
7	NC	0.9% NaCl + DMSO
7	Diabetic group (D-Met)	MET + DMSO
7	Diabetic group exposed to immobility stress (D-Ims-Met)	MET + DMSO
7	Diabetic group exposed to immobility stress (D-Ims-Met-Mel)	MET + MEL + DMSO

OPEN FIELD TEST (OFT): The Open Field Test (OFT) was used to assess anxiety. The wooden apparatus, measuring 40 × 40 cm, featured a 30 cm high enclosure and was positioned under strong illumination. The OFT was divided into 25 squares, consisting of 9 central squares and 16 peripheral squares. At the start of the 10-minute test, each animal was placed in the center of the apparatus and allowed to explore freely. The measured parameters included the time spent in the central squares (TSCS), which indicates anxiety, and the number of returns to the central squares (NRCS) (Carter and Shieh, 2015).

ELEVATED PLUS MAZE TEST (EPM): The Elevated Plus Maze (EPM) test was used to evaluate anxiety-related behavior. During the 10-minute test, each animal was placed at the center of the EPM, facing one of the enclosed arms, and allowed to explore the area freely. Anxiety-related measures included the time spent in the open arms and the duration of stretching in the closed arms (Komada *et al.*, 2008).

FORCED SWIM TEST (FST): To assess depressive-like behavior, we used the Forced Swim Test (FST). In this test, each mouse was placed in a glass cylinder filled with 15 cm of water maintained at a temperature of $23 \pm 2^\circ\text{C}$ (Adem Can *et al.*, 2012). Over a 5-minute period, the mice were made to swim, and the time spent immobile was measured (de Moraes *et al.*, 2018).

BIOCHEMICAL ANALYSES

MEASUREMENT OF PLASMA CORTICOSTERONE: ELISA kits were used to evaluate plasma CORT levels in mice according to the manufacturer's guidelines. Absorbance was measured at 450 nm using a microplate reader, and the CORT concentration in the plasma was reported in units of ng/ml.

ANALYSIS OF LIPID PARAMETERS

Following the behavioral tests, conducted over a period of 24 hours, all animals were anesthetized using chloral hydrate. Blood was collected and centrifuged at 4°C at 4000 rpm for 10 minutes. The collected plasma was utilized for spectrophotometric lipid analysis, measuring total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), and total triglycerides. Enzymatic diagnostic kits from DiaSys (System Diagnostic GmbH, Germany) were employed according to the manufacturer's instructions. The concentrations of very low-density lipoprotein cholesterol (VLDL-c), the atherogenic index of plasma (AIP), and cardiovascular risk indices 1 and 2 were calculated using the protocols described by Bikri *et al.* (2022).

PREPARATION OF HOMOGENATES

The brain was dissected and processed separately. The tissues were homogenized using a Dounce homogenizer in

an ice-cold lysis buffer. The resulting homogenates were centrifuged for 15 minutes at 14,000 g and subsequently stored at -80°C (Bikri *et al.*, 2021a). For the pancreas, the preparation followed the procedure outlined by Erejuwa *et al.* (2010). In brief, a ten percent (w/v) tissue homogenate was created in Tris-HCl using an ice-chilled homogenizer vessel. The suspended mixture was then centrifuged at $1000 \times g$ for 10 minutes at 4°C in a refrigerated centrifuge.

The brain was dissected and processed separately. Brain tissues were homogenized in an ice-cold lysis buffer using a Dounce homogenizer. The resulting homogenates were centrifuged at 14,000 g for 15 minutes and subsequently stored at -80°C . The pancreas was prepared following the protocol by Erejuwa *et al.* (2010). In brief, a 10% (w/v) tissue homogenate was created in Tris-HCl using an ice-chilled homogenizer vessel. This mixture was then centrifuged at $1000 \times g$ for 10 minutes at 4°C in a refrigerated centrifuge.

EVALUATION OF MARKERS FOR OS: LPO ASSAY

To assess the OS marker, a LPO assay was conducted by measuring thiobarbituric acid reactive substances (TBARS) levels, analyzing the formation of lipid peroxides in brain homogenates (Draper and Hadley, 1990). A 2000 μL mixture, consisting of 1000 μL of 10% trichloroacetic acid and 1000 μL of 0.67% thiobarbituric acid, was added to the samples. This mixture was heated for 15 minutes in a water bath at 90°C . Subsequently, butanol (2:1 v/v) was added to the solution, and the mixture was centrifuged at 8000 g for 5 minutes (Freitas, 2004). Similarly, for pancreatic tissue, TBARS levels were measured by combining the tissue homogenates with 1000 μL of 10% trichloroacetic acid and heating the solution for one hour in a water bath (Quintanilha *et al.*, 1982).

DATA ANALYSIS

The data underwent a one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test for inter-group comparisons. Statistical significance was set at $p < 0.05$ for all analyses. Mean values are presented with their corresponding standard deviations (SD).

RESULTS AND DISCUSSION

FBG AND BODY WEIGHT CHANGE

Figure 1 illustrates the changes in FBG and BW across different groups. At T1 (72 hours post-STZ injection), significant differences in FBG levels were observed among the groups (Figure 1A). Three days after STZ injection, all mice administered this diabetogenic agent exhibited a statistically significant increase in FBG levels compared to the normal control (NC) mice.

At T2 (sacrifice day), the results indicated a significant difference in FBG levels between the D-Met group ($P=0.000$)

and the D-Ims Met group ($P=0.029$) compared to the NC group. Additionally, chronic treatment with MET, as well as MET combined with MEL, significantly decreased FBG levels compared to the diabetic group not exposed to immobility stress ($P=0.001$ for both).

The BW results are presented in Figure 1B. These findings revealed no significant changes between the evaluated groups at any time points: T1 (before STZ injection), T2 (after 1 month of treatment), and T3 (sacrifice day).

CORT LEVELS EVALUATION

Figure 2 illustrates the CORT levels for each group. The D-Ims-Met group exhibited a significant increase in plasma CORT levels after 2 months of treatment compared to the NC group ($P=0.000$). However, the diabetic group exposed to stress and treated with a combination of MET and MEL did not show a significant difference in CORT levels ($P=0.093$).

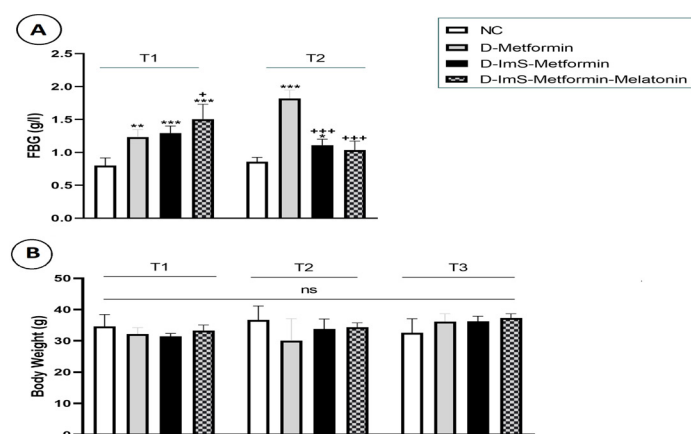


Figure 1: Changes in FBG (A) and body weight (BW) (B). Data are expressed to mean $\pm$ SDSD ($n=7$). * $p<0.05$, ** $p<0.001$, *** $p<0.01$ versus NC group. + $p<0.05$ ++ $p<0.01$ versus D-Met. @ $p<0.05$ @@ $p<0.01$ @@@ $p<0.001$ versus D-Ims-Met.

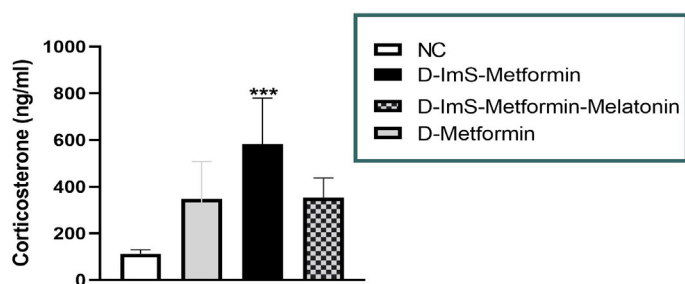


Figure 2: Changes in CORT level. Data are expressed to mean $\pm$ SDSD ($n=7$); * $p<0.05$, ** $p<0.001$; *** $p<0.01$ versus NC group. + $p<0.05$ ++ $p<0.01$ versus D-Met. @ $p<0.05$ @@ $p<0.01$ @@@ $p<0.001$ versus D-Ims-Met.

ANXIETY AND DEPRESSIVE RELATED-BEHAVIORS

As depicted in Figure 3, the analysis of anxiety in hyperglycemic mice treated with a combination of MET and MEL and exposed to stress, based on the Elevated Plus Maze

(EPM) test, revealed a significant reduction in the percentage of entries into the open arms (Figure 3A) after 8 weeks of treatment (T2) compared to the NC group ($P=0.025$). Additionally, the percentage of time spent in the open arms by the group exposed to immobility stress and treated with MET and the combination of MET and MEL decreased compared to the NC group at T1 ($P=0.013$ and $P=0.031$, respectively). At T2, the group exposed to stress and treated with the combination of MET and MEL (D-Ims-Met-Mel) also showed a significant decrease in the percentage of time spent in the open arms compared to the NC group ($P=0.019$).

Regarding the Open Field Test (OFT) results, at T1, the stressed group receiving combined treatment exhibited fewer returns to the central squares compared to the D-Met group. Conversely, at T2, the D-Met mice showed fewer returns to the central squares compared to the NC group. Additionally, there was a significant decrease in the percentage of time spent in the central squares for both the D-Met group and the D-Ims-Met-Mel group compared to the NC group at T1.

The immobility time results, summarized in Figure 3F, show a significant increase in immobility time for the D-Met mice compared to the NC group, and a significant decrease in immobility time for the D-Ims-Met-Mel group compared to the D-Met group after one month of treatment (T1). At T2, the D-Met mice exhibited increased immobility time compared to the NC group, while the D-Ims-Met group and the group treated with both agents showed a significant decrease in immobility time compared to the D-Met group ($P=0.040$ and $P=0.000$, respectively).

To evaluate the potency and efficacy of the treatments on various physiological functions in STZ-diabetic mice subjected to immobility stress, lipid and cardiovascular parameters were analyzed (Figure 4). After 2 months of STZ injection, the D-Met group exhibited significantly higher levels of total cholesterol, triglycerides, VLDL-C, and AIP compared to the NC group ($P=0.020$, $P=0.000$, $P=0.000$, $P=0.009$, respectively). In contrast, the D-Ims-Met and D-Ims-Met-Mel groups showed a notable decrease in triglycerides, VLDL-C, and AIP levels compared to the D-Met group. Additionally, cholesterol levels were significantly decreased in the D-Ims-Met-Mel group compared to the D-Met group.

The levels of the OS marker malondialdehyde (MDA) in the pancreas and brain of both normal and diabetic mice, exposed or not to stress, were evaluated, with the findings presented in Figure 5. At the end of the treatment period, results revealed a significant increase in MDA concentrations in the brains of D-Met mice and D-Ims-Met mice compared to the NC group ($P=0.033$ and $P=0.000$, respectively).

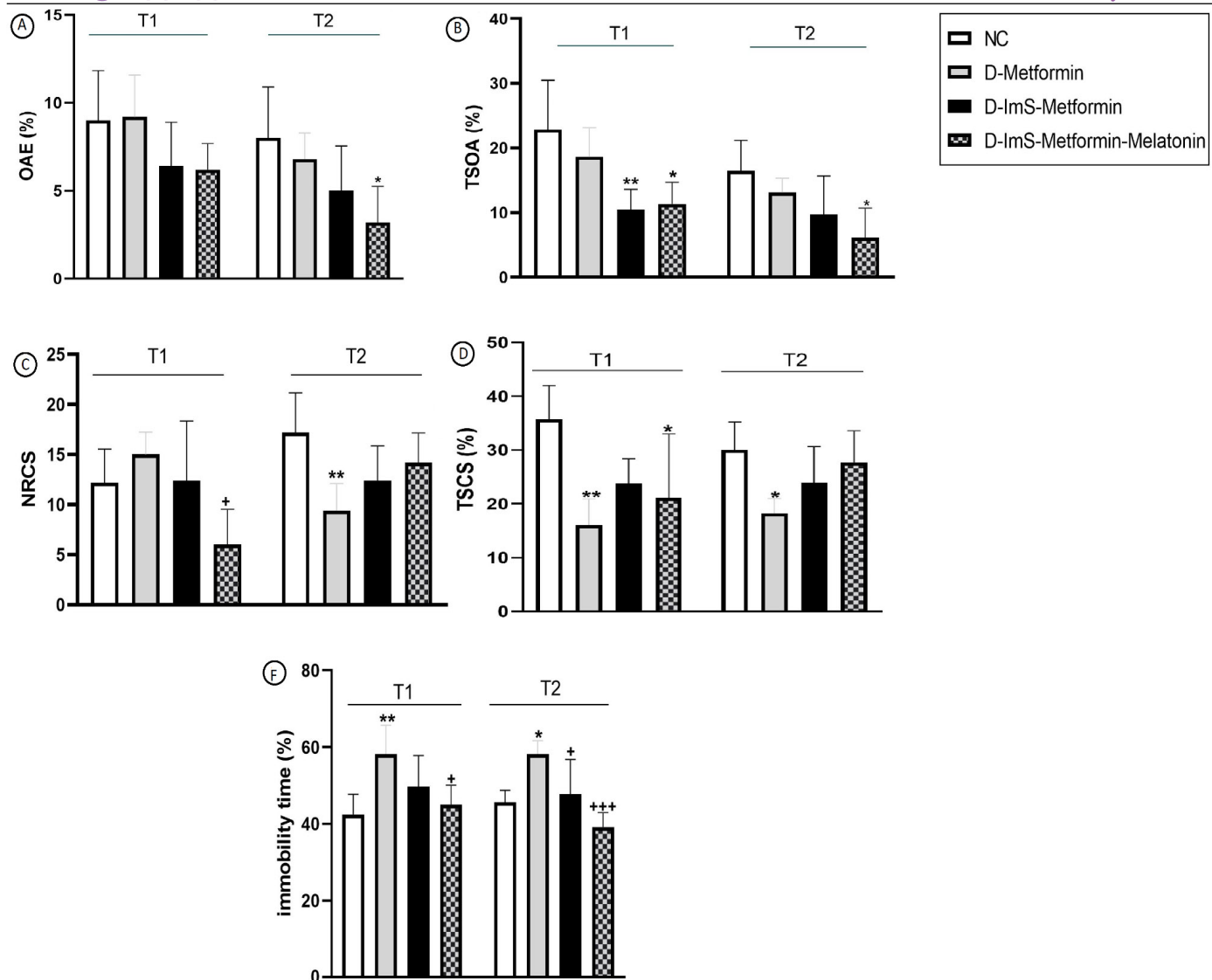


Figure 3: Effects of MET and MEL phenolic in STZ diabetic mice exposed or not to immobility stress on anxiety and depressive-like behavior. Data are expressed to mean $\pm$ SD (n=7). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.01$ versus NC group. + $p < 0.05$ ++ $p < 0.01$ versus D-Met. @ $p < 0.05$ @@ $p < 0.01$ @@@ $p < 0.001$ versus D-ImS-Met; T1: after 1 month of treatment; T2: after 2 months of treatment. *

(Figure 5A). A significant decrease was also noted in the brains of D-ImS-Met-Mel mice compared to D-ImS-Met mice.

Furthermore, remarkably higher levels of MDA in the pancreas (Figure 5B) were observed in diabetic mice, both exposed and not exposed to stress, treated with MET compared to the NC group ($P=0.017$ and $P=0.000$, respectively). Compared to the D-ImS-Met group, treatment with MEL induced a significant reduction ($P=0.041$) in MDA levels in the stressed diabetic group treated with the combination of MET and MEL.

T2DM is a metabolic burden characterized by insulin resistance and persistent hyperglycemia (Galicja-Garcia, 2020). Although significant strides have been made in understanding the pathophysiology of T2DM and chronic stress, as well as their crucial complications, effective therapeutic options remain limited. It is, therefore, reasonable

to explore the potential of antioxidant supplementation in preventing these complications. Earlier research has shown that compounds with strong antioxidant properties can safeguard animals from the adverse effects of chronic hyperglycemia and restraint stress-induced pathology (Bikri et al., 2022; Noor et al., 2023). Hence, the main goal of the present study was to explore whether combining MEL and MET could enhance biochemical and behavioural changes, related with persistent hyperglycemia, by decreasing LPO as determinant of OS markers in T2DM mice exposed to CIS.

The results of the present study revealed that STZ injection following 14 days of fructose exposure remarkably increases FBG and CORT levels in diabetic mice. This effect was observed both in D-Met mice and in diabetic mice treated with MET and subjected to immobilization stress for 8 weeks. In contrast, no significant differences in BW were observed between the groups at different time points. In this context, a previous study demonstrated that

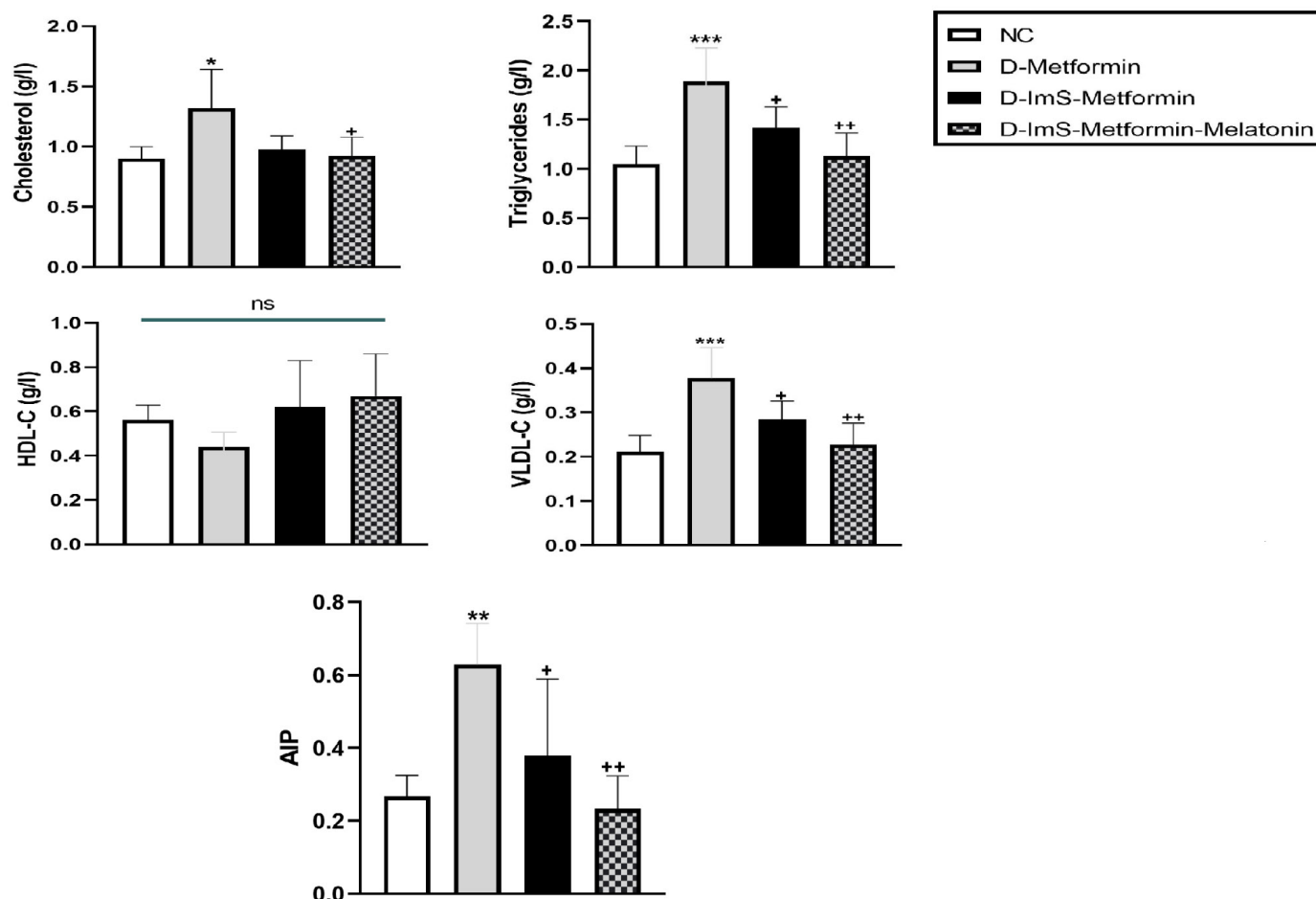


Figure 4: Biochemical analysis; Data are expressed to mean $\pm$ SDSD (n=7); * p <0.05, ** p <0.001; *** p <0.01 versus NC group. + p <0.05 ++ p <0.01 versus D-Met. @ p <0.05 @@ p <0.01 @@@ p <0.001 versus D-ImS-Met.

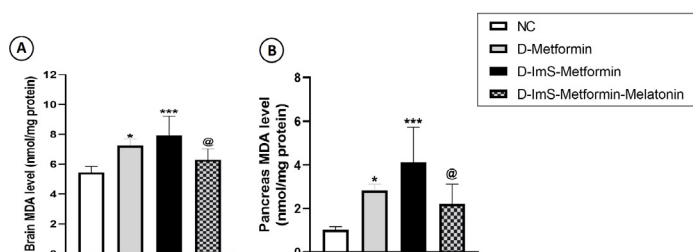


Figure 5: Malondialdehyde levels in brain (A) and Pancreas (B). Data are expressed to mean $\pm$ SDSD (n=7). * p <0.05, ** p <0.001, *** p <0.01 versus NC groups. + p <0.05 ++ p <0.01 versus D-Met. @ p <0.05 @@ p <0.01 @@@ p <0.001 versus D-ImS-Met.

immobilization stress over a period of 2 weeks induced hyperglycemia in animal models (Aghajanyan *et al.*, 2017). This result is significant as it provides a significant explanation for our experimental findings. Chronic stress is known to alter various physiological parameters, including glucose metabolism (Sharma *et al.*, 2022). Numerous studies indicate that elevated levels of stress hormones can impair the function of insulin-producing beta cells, leading to a decrease in insulin production (Wong *et al.*, 2019). Moreover, the effect of immobilization stress on hyperglycemia was studied using a mouse model. After being exposed to

immobilization stress for 16 hours per day for 2 consecutive days, the mice exhibited adrenal gland hypertrophy and elevated serum levels of glucose and CORT, while insulin secretion remained unchanged (Kasuga *et al.*, 1999). These results suggest that the elevation of blood glucose was likely due to the stimulation of the hypothalamic-pituitary-adrenal axis by stress. When animals are subjected to prolonged immobilization stress, there is an elevation in CORT levels (Qin *et al.*, 2011), a stress hormone, which can lead to increased blood glucose levels. This stress-induced hyperglycemia may be an evolutionary defense mechanism, allowing the body to quickly mobilize energy in response to a perceived threat. Accordingly, our observations of elevated glucose levels in stressed animals using the immobilization paradigm for 8 weeks are consistent with this hypothesis and support the notion that chronic stress can have profound and lasting metabolic effects. In the current study, 8 weeks of treatment with MET, in combination with MEL, significantly decreased FBG levels and restored CORT levels in diabetic mice exposed to CIS. These findings suggest a beneficial effect of this combination on glycemic regulation and stress response. Based on a systematic review, MEL plays a crucial role in ameliorating glycemic control by increasing insulin sensitivity and decreasing

FBG levels (Doosti-Irani, 2018). Another study revealed that MEL supplementation effectively decreases glycemic variability in patients with T2DM (Martorina and Tavares, 2023). In addition, an experimental study revealed that MEL alleviates stress in rats subjected to CIS (Gomaa *et al.*, 2017). This effect is partly due to MEL's ability to enhance the central release of oxytocin and monoamines in the frontal cortex. These findings support the hypothesis that antioxidant supplementation, particularly MEL, recognized as a potent antioxidant, could be a promising strategy to improve biochemical alterations associated with chronic hyperglycemia in diabetic mice subjected to CIS.

Previous experimental studies have shown that prolonged exposure to hyperglycemia and stress, either separately or together, disrupts lipid metabolism, resulting in dyslipidemia (Habib, 2015; Bikri *et al.*, 2022). This is marked by increased levels of cholesterol, triglycerides, and LDL-cholesterol, which are key factors in the development of coronary heart disease and atherosclerosis. Dyslipidemia plays a significant role in the development of heart disease in diabetic patients, an association highlighted by assessing cardiovascular risk markers such as VLDL, triglycerides, and AIP (Du and Qin, 2023; Bikri *et al.*, 2021b). In the current study, two months post-STZ injection, diabetic mice treated with MET revealed a marked elevation in total cholesterol, triglycerides, and VLDL-C levels compared to normo-glycemic mice. In addition, diabetic mice subjected to CIM for the same duration exhibited significantly increased levels of VLDL-C, triglycerides, and AIP. In this context, a recent experimental study revealed that chronic stress significantly affects lipid parameters and alters cardiovascular markers in hyperglycemic rats compared to non-stressed hyperglycemic rats (Bikri *et al.*, 2022). The findings of this study indicate that chronic stress exacerbates lipid abnormalities, notably by increasing levels of total cholesterol, triglycerides, and LDL. Furthermore, cardiovascular risk markers, AIP, cardiovascular risk index I and cardiovascular risk index II, were also higher in chronically stressed rats. Chronic stress conditions, like those related with long-term illness, lead to elevated corticosteroid generation, which can provoke insulin resistance (Yaribeygi *et al.*, 2022). This resistance fosters triglyceride synthesis and hinders the clearance of both HDL and LDL cholesterol (Ormazabal *et al.*, 2018). These results suggest that stress management could be a significant element in controlling cardiovascular complications associated with hyperglycemia. It has been shown that consuming antioxidant molecules can help prevent cardiovascular diseases by decreasing blood lipid levels (Zhou *et al.*, 2021). In the present study, an 8-week treatment of stressed diabetic mice with a combination of MET and MEL significantly improved their blood lipid profiles and regulated cardiovascular risk markers. The conclusions of multiple studies suggest significant potential for MEL in preventing cardiovascular

diseases. A recent systematic review and meta-analyses have revealed that MEL supplementation could improve lipid parameters, thereby offering protection against cardiovascular diseases (Loloei *et al.*, 2019). Additionally, other research has shed light on the complex mechanisms through which MEL acts on cholesterol-dependent atherogenesis. It appears that MEL has the ability to directly regulate cholesterol concentration while also protecting tissues from damage caused by oxidized lipoproteins (Karolczak and Watala, 2019). These findings underscore the importance of considering MEL as a potential element in cardiovascular complications prevention associated with chronic hypoglycemia.

Persisting hyperglycemia associated with disrupted lipid metabolism has been well demonstrated to significantly enhance OS markers in both the CNS and the pancreas (Caturano, 2023; El Aboubi, 2023). This is primarily due to the alteration of antioxidant defenses and/or an increase in the production of endogenous ROS (Reis *et al.*, 2006). OS occurs when there is an imbalance between the generation of ROS and the body's ability to detoxify these reactive intermediates or repair the resulting damage. Chronic accumulation of ROS has been identified as a significant contributor to the development of diabetes complications (Volpe *et al.*, 2023). In this study, after two months of treatment, the Fructose-STZ-induced T2D model revealed a significant increase in LPO levels in both the pancreas and the brain. In this context, several experimental studies showed that prolonged hyperglycemia increased remarkably the production of MDA as indicated by increased LPO levels in brain regions (El Aboubi, 2023; Bikri *et al.*, 2021b). LPO, a process where free radicals damage cell membranes by stealing electrons from lipids, serves as a marker of OS and can lead to cell dysfunction and death. Additionally, previous studies have shown that chronic hyperglycemia elevates LPO products in various brain regions such as the prefrontal cortex and hippocampus, as well as in the pancreas (Obafemi *et al.*, 2020; Bikri *et al.*, 2022). These results underscore the harmful effects of prolonged hyperglycemia on neural and pancreatic tissues, highlighting the crucial importance of managing blood glucose levels to prevent OS damage and its related complications. In the current study, subjecting these diabetic mice to immobilization stress for two months led to a heightened production of LPO, in both the brain and pancreas. Various studies have shown that chronic stress alters the antioxidant system, leading to an increased generation of ROS. By disrupting the balance between ROS and antioxidant molecules, chronic stress contributes to a state of OS. This OS is involved in the development of multiple pathologies (Juszczyk, 2021). In this line, a previous investigation found that subjecting rats to repeated chronic mild stress led to a notable increase in MDA levels in both the pancreas and brain (Bikri *et al.*, 2022). The administration of MET treatment

combined with MEL significantly provided protection for Fructose-STZ-induced diabetic mice subjected to CIS for 8 weeks against OS products, as compared to diabetic mice exposed to CIS and treated solely with MET. Our findings suggest that MEL supplementation may mitigate damage to the pancreas and brain in stressed diabetic mice by reducing OS. The ability of MEL to protect cells against OS likely arises from its dual action of enhancing the activity of antioxidant defense mechanisms and scavenging free radicals (Argun *et al.*, 2014). Moreover, MEL can activate membrane receptors, thereby stimulating the generation of a range of antioxidant enzymes through various signaling pathways (Shiu *et al.*, 2010).

The significant relation between OS and hyperglycemia in the brain regions plays a determinant role in the onset of psychiatric symptoms (Bikri *et al.*, 2022). Previous experimental studies have shown that chronic hyperglycemia can exacerbate neuronal OS, leading to neurochemical dysregulation and synaptic alteration related to anxiety and depression (Johnson *et al.*, 2019; Smith *et al.*, 2020). It has been revealed that chronic hyperglycemia induces an excessive generation of ROS, thereby increasing OS and causing neuronal alteration directly correlated with anxiety and depressive behaviors (Lee *et al.*, 2021). Additionally, chronic stress, particularly when combined with hyperglycemia, can exacerbate oxidative damage in the CNS, leading to behavioral changes (Bikri *et al.*, 2021b). Hence, the present study assessed the effects of MET and MEL on anxiety/Depression-related behaviors in diabetic mice exposed to CIS. In this line, an experimental study found that the group under CIS treated with MEL exhibited reduced anxiety-related behavior, higher levels of noradrenaline, oxytocin and serotonin in the brain, and improved histopathological structure, compared to the chronically stressed group (Gomaa *et al.*, 2017). Furthermore, immobility time, as an indicator of depressive symptom, significantly elevated in diabetic mice treated with MET alone but decreased in the stressed diabetic group treated with both MET and MEL after one month. After two months, the combination treatment continued to show beneficial effects, with a significant decrease in immobility time compared to the MET-treated group. MET, well-known for its antidiabetic effects, ameliorates insulin sensitivity and decreases systemic OS and inflammation (El Aboubi, 2023; Cameron *et al.*, 2016). MEL is a powerful antioxidant and neuroprotectant that regulates the sleep-wake cycle, thereby reducing OS and neurogenic inflammation, both of which exacerbate anxio-depressive disorders (Jiang *et al.*, 2020; Bikri *et al.*, 2024). Additionally, it enhances neurogenesis and synaptic plasticity, which are crucial for mental health (Jia, 2017). Together, these agents potentiate their beneficial effects, offering increased protection against neuropsychiatric alterations induced by diabetes and CIS, compared to MET alone, which, while

effective metabolically, does not directly address the specific neuroprotective mechanisms of MEL (Sharma *et al.*, 2019). Our study has some important limitations that deserve recognition. Firstly, the experimentation was conducted exclusively on male mice, which limits our ability to explore the effects of sexual dimorphism. This restriction may affect the generalizability of the results to both sexes, and future research should include female subjects to assess potential sex differences and improve the generalization of the conclusions. Additionally, we used only corticosterone as a biomarker to assess stress. Although corticosterone is a well-established indicator of stress, the absence of other stress markers limits the scope of our assessment. Including additional biomarkers would have provided a more comprehensive understanding of the mechanisms of oxidative stress and its effects on mouse physiology. These limitations should be considered when interpreting our results and guiding future research in this area.

CONCLUSION AND RECOMMENDATIONS

The Results of the current study revealed that combining MET with MEL can alleviate OS in the brain and pancreas of diabetic mice subjected to CIS for 2 months by decreasing LPO accumulation. This combination effectively maintains CORT levels, improves lipid profiles, and decreases the atherosclerosis index in these mice. Additionally, the combined treatment remarkably improves anxiety and depression-related behavior in this mouse model. These findings suggest that the combined use of MET and MEL could be a promising therapeutic strategy for managing OS-related complications in T2D patients, particularly those experiencing chronic immobility stress. By improving both metabolic and psychological outcomes, this combination therapy has the potential to enhance the overall quality of life for T2D patients. Future clinical trials should explore the efficacy and safety of this combined treatment in human subjects to validate its benefits and optimize its use in clinical practice.

NOVELTY STATEMENT

This study introduces a novel therapeutic approach by demonstrating that the combination of Metformin and Melatonin effectively mitigates oxidative stress in diabetic mice under chronic immobilization stress. The dual treatment not only reduces lipid peroxidation and maintains corticosterone balance but also improves metabolic health and alleviates anxiety and depression-like behaviors.

AUTHOR'S CONTRIBUTIONS

All authors contributed equally to this research.

The authors have declared no conflict of interest.

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