

Determination of insulin resistance, β -cell function and insulin sensitivity using modified homeostatic model assessment in diabetic rats treated with melatonin

Short Title: Determination of insulin resistance using HOMA

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Abstract

The study aimed to determine insulin resistance, beta cell function and insulin sensitivity of diabetic rats treated with melatonin by employing a structural mathematical/computer model (Homeostatic Model Assessment). Alloxan-fructose-induced type 2 diabetic rat model was created by a single-dose of alloxan (150mg/kg, i.p.) given to 14-days fructose solution (20% w/v) pre-treated (in drinking water) rats. Blood glucose level was assessed three days post induction for hyperglycemia, and rats with fasting blood glucose (FBG) levels greater than 200 mg/dL were considered diabetic. Rats were randomly grouped into four (n=6) and treated as control, melatonin, diabetic untreated and diabetic treated groups respectively. Melatonin (10mg/kg, p.o.) was administered daily for 15 days following diabetic induction. Treatment of diabetic rats with melatonin significantly ($P < 0.05$) reduced the FBG, C-peptide and insulin resistance with increased insulin sensitivity level when compared with diabetic untreated rats. However, no changes were observed in the insulin and HOMA-%B groups. As evidenced from the positive improvements in beta cell function, insulin sensitivity, and decreased insulin resistance; treating type 2 diabetes with a pharmacological dose of melatonin is an important way to boost the body's antioxidant defense system and subsequently improve anti-hyperglycemic conditions by blocking mechanisms that lead to hyperglycemia. These findings suggest that early and intensive treatment of insulin resistance is the best strategy to delay the emergence of long-term complications from type 2 diabetes, slow down the disease's progression, and maybe prevent its onset. The implementation of straightforward and reliable diagnostic techniques, such as the HOMA model, is necessary for the early detection of insulin resistance and beta-cell dysfunction.

Keywords: Type 2 diabetes; insulin resistance and sensitivity; β -cell functions; HOMA; melatonin

1. Introduction

Diabetes, a chronic endocrine disease is characterized by persistent hyperglycemia and associated with abnormalities of carbohydrate, lipid, and protein metabolism (1). According to the classification of the American Diabetes Association, it is divided into different types according to the underlying cause, with type 1 and type 2 diabetes being the two main types and most frequent. Type 1 diabetes mellitus (T1DM) is mainly observed in children and adolescents and accounts for less than 10% of all diabetic cases and generally caused by an autoimmune reaction to antigens of pancreatic β -cells, leading to impaired insulin production. Type 2 diabetes mellitus (T2DM) is more common among adults, accounting for more than 90% of the diabetes cases globally and is characterized by defects in insulin secretion and inefficiency of insulin action (2).

Melatonin, a tryptophan-derived endocrine hormone mainly synthesized by the pineal gland and locally by numerous other tissues, is known to be an anti-inflammatory agent and a powerful antioxidant (3).

Studies have shown that melatonin may be associated with diabetes mellitus due to a functional inter-relationship between melatonin and insulin, a key hormone that regulates glucose levels. For instance, melatonin has been found to influence insulin secretion both in vivo and in vitro, and night-time melatonin levels are related to night-time insulin concentrations in patients with diabetes (3).

A decreased level of melatonin secretion is independently associated with the increased risk of type 2 diabetes developing in a patient. Hence, it is recently reported that variants of melatonin membrane receptors and low secretion of melatonin aggravate the risk of developing type 2 diabetes (4), suggesting that melatonin may be involved in the regulation of glucose homeostasis. Emerging body of evidence shows that melatonin reduces diabetic complications by ameliorating oxidative damage (5).

It is commonly known that obesity and T2DM are characterized by insulin resistance and beta-cell dysfunction, but it can be difficult to pinpoint exactly who has this condition. Beta-cell dysfunction arises when beta-cells are unable to compensate for the insulin resistance, whereas insulin resistance is characterized by a decrease in insulin mediated glucose elimination in insulin-sensitive tissue and an increase in hepatic glucose synthesis (6,7). The relationship between beta cell malfunction and

insulin resistance that initiates the aetiology of diabetes is not well understood, despite their intrinsic complexity. It is likely that these two pathogenic states work in concert to worsen diabetes by influencing one another. Thus, glucose homeostasis will be maintained by maintaining beta cell function, insulin signalling in beta cells, and insulin signalling in tissues that receive glucose.

It may be possible to evaluate risk-stratify, and monitor the therapy of diabetes by measuring both of these markers at the time of T2DM diagnosis. Research-based "gold standard" stimulatory techniques including the hyperinsulinemic euglycemic clamp, the hyperglycemic clamp, the intravenous glucose tolerance test, and the C-peptide to glucose ratio are not feasible for normal use since they are inconvenient, time-consuming, and expensive (6).

"Homeostatic" models, like the homeostasis model assessment (HOMA) test created in 1985 by David Matthews *et al.* (8) are a straightforward surrogate index and test that mathematically models the fasting plasma glucose and insulin concentrations and provides an estimate of an individual's level of beta-cell function (HOMA%B) and degree of insulin sensitivity (HOMA%S) (8). According to Song *et al.* (9), HOMA-IR is the inverse of HOMA%S. An updated HOMA model (HOMA2) was published by Levy *et al.* (10) and it takes into account changes in hepatic and peripheral glucose resistance, such as the reduction in the suppression of hepatic glucose output (by hyperglycemia), increases in the insulin secretion curve for plasma glucose concentrations above 10 mmol/L, and the effects of circulating proinsulin. These early insulin-based models do, however, have major drawbacks. First off, because insulin secretion is pulsatile, using a single sample for insulin assessment is restricted. Additionally, the liver extracts around 50% of the insulin released by beta cells, leading to a significant variance in insulin levels between assays (11). The equimolarly secreted C-peptide is not removed by the liver and other organs, and the half-life of C-peptide in blood is significantly longer than that of insulin (10-30 min vs. 4 min), making the measurement of C-peptide a more accurate representation of beta-cell insulin secretion compared to the measurement of insulin itself (12). The HOMA Calculator, a computer program that could quickly and easily access the HOMA2 model, was later launched in 2004.

Insulin resistance contributes to the pathophysiology of diabetes and is a hallmark of obesity, metabolic syndrome, and cardiovascular diseases. The homeostatic model assessment is a known method for quantifying beta cell function, and since no single

test allows beta-cell function to be assessed with accuracy and specificity comparable to those of insulin sensitivity. Therefore, quantifying insulin sensitivity and resistance in humans and animal models is of great importance for epidemiological studies, clinical and basic science investigations, and eventual use in clinical practice by employing a structural mathematical/computer model (HOMA) to determine parameters of insulin resistance and beta-cell function in diabetes for optimum results, thus a reason for this study.

2. Materials and Methods

2.1. Drugs and Chemicals

We bought melatonin, fructose, and alloxan powder from Sigma-Aldrich (Sigma-Aldrich Cooperation, USA). Every other reagent utilized was of high quality and analytical grade.

2.2. Experimental Animals

Twenty four male Wistar rats (*Rattus norvegicus albinus*) weighing between 150-250g procured from the Department of Physiology Animal House, University of Calabar, Cross-River State were used for this study. The rats were acclimatized for 14 days prior to the study, housed in plastic cages with wood-chip bedding changed daily and fed on normal rat chow and water. The animals were exposed to 12 hour' light and 12 hour' dark cycles photoperiod and kept under hygienic conditions.

2.3. Induction and Assessment of Experimental Diabetes Mellitus

Alloxan-fructose-induced type 2 diabetic rat model was created by a single-dose of alloxan (150mg/kg, i.p.) given to 14-days fructose solution (20% w/v) pre-treated (in drinking water) rats (13). Blood glucose level was assessed three days post induction for hyperglycaemia using glucose meter (GlucoDr. Auto ®), and rats with fasting blood glucose levels greater than 200 mg/dL were considered diabetic. Following the induction of diabetes, rats were then returned to normal chow and water *ad libitum*.

2.4. Experimental Design

Rats were randomly grouped into four (n=6) and treated as control, melatonin, diabetic untreated and diabetic treated groups respectively. Melatonin (10mg/kg, p.o.) was administered daily for 15 days following diabetic induction.

2.5. Measurement of Blood Glucose Levels

Blood glucose levels were measured from the fresh blood samples collected from the tail vein of fasting rats by pricking. Glucose levels were determined using blood glucose meter (GlucoDr. Auto ®) that uses test strips to assess a glucose oxidoreductase mediated dye reaction, in accordance with the manufacturer's instructions.

2.6. Sample Collection

All the animals were humanely sacrificed on the last day of experimental period. The overnight fasted rats were anesthetized with ketamine/xylazine (0.1 mL/100 g.bw i.p.). Blood samples were then obtained by retro-orbital bleeding and poured into plain tubes. Sera was obtained by centrifugation at 3000 rpm for 15 min, carefully separated, and stored at -20°C until biochemical assessment.

2.7. Hormonal Assay of Serum Insulin and C-peptide

Fasting Insulin and C-peptide levels were determined using rat insulin Enzyme Linked Immuno Sorbent Assay (ELISA) kit and commercially available C-peptide enzyme immunoassay (EIA, Sigma-Aldrich) respectively according to the manufacturer's protocol.

2.8. Homeostatic Model Assessment (HOMA)

Insulin Resistance (IR), Beta Cell Function (% β) and insulin sensitivity (%S) were determined using HOMA. Data gotten from Fasting C-Peptide and Fasting blood glucose at the end of the experimental period were used to calculate HOMA-IR, HOMA-% β and HOMA-%S using HOMA2 Calculator (v.2.2.3) downloadable at <http://www.dtu.ox.ac.uk/>

2.9. Statistical Analysis

The mean \pm standard error of the mean (SEM) was used to express all the data. ANOVA was used to compare means, and Tukey multiple comparisons and

Student's t-test were used to assess differences in means. . Results were analyzed using Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). A statistical significance level of $p < 0.05$ was applied.

3. Results

The results of all the experiments carried out are shown in tables 1 and figures 1 to 6 below. All the values given are expressed as mean \pm SEM. Superscripted a, b, c or with asterisks in the table and bar charts indicate values that are significantly different from the control, melatonin or diabetic groups of all the variables measured.

3.1. Effect of melatonin on fasting blood glucose, insulin and C-peptide of diabetic rats

The effect of melatonin on fasting blood glucose (FBG), insulin and fasting C-peptide (FCP) are shown in figures 1 to 3 below. Values of FBG were: control- 105.00 ± 4.074 , melatonin- 106.00 ± 5.206 , diabetic- 377.80 ± 14.800 , and Diab+Mel- 226.80 ± 16.760 . Blood glucose level significantly increased ($p < 0.01$) in the diabetic group when compared to the control and melatonin groups. However, supplementation with melatonin significantly reduced the glucose level in the treatment group when compared with the diabetic group as presented in fig. 1, and still significantly higher in the supplemented group when compared with control ($p < 0.01$).

As shown in fig. 2, the mean values of insulin were 13.86 ± 0.470 , 13.40 ± 0.401 , 16.50 ± 0.692 and 15.33 ± 0.276 for control, melatonin, diabetic and diabetic treated groups respectively. There was a significant increase ($p < 0.05$) in the insulin levels of the diabetic group when compared to the control and the melatonin groups. However, no significant differences were observed between the diabetic-treated and diabetic group, as the treated group was still increased when compared to the control and melatonin groups.

The mean values of C-peptide were 3.19 ± 0.336 , 0.79 ± 0.106 , 7.04 ± 0.463 and 3.00 ± 0.315 for control, melatonin, diabetic and diabetic treated groups respectively (fig. 3). There was a significant decrease ($p < 0.05$) in the melatonin group when

compared to the control group. There was a significant increase in the C-peptide levels of diabetic rats when compared to the control and melatonin groups. However, treatment with melatonin significantly ($p < 0.01$) reduced the C-peptide to almost the level of the control group.

3.2. Homeostatic Model Assessment of insulin resistance (HOMA-IR), beta cell function (HOMA-%B) and insulin sensitivity (HOMA-%S) in diabetic rats treated with melatonin.

HOMA Assessment of insulin resistance, beta cell function and insulin sensitivity in diabetic rats treated with melatonin is presented in table 1.

There was a significant increase in the HOMA-IR index of the diabetic (20.24 ± 3.163) group when compared to control (2.45 ± 0.279) and melatonin (0.61 ± 0.080) groups. However, following treatment, there was a significant decrease ($p < 0.01$) in the melatonin treated group (3.13 ± 0.415) when compared to the diabetic group (fig. 4).

As presented in fig. 5, there was a significant decrease in the beta cell function of the diabetic group when compared to the control group. There was also a significant decrease in the melatonin only and diabetic treated groups when compared with the control. However, melatonin supplementation had no effect on the beta cell function when compared with the diabetic group.

Also, insulin sensitivity of diabetic rats (5.48 ± 0.891) was significantly decreased ($p < 0.05$) when compared to control (42.54 ± 3.912) and melatonin only groups (173.30 ± 19.390). However, treatment with melatonin significantly ($p < 0.05$) increased its sensitivity when compared with the diabetic group as shown in fig 6.

Table 1: HOMA assessment of insulin resistance (HOMA-IR), beta cell function (HOMA-%B) and sensitivity (HOMA-%S) in fructose-alloxan-induced diabetic rats treated with melatonin.

Variables	GROUPS			
	Control	Melatonin	Diabetic	Diab+Mel
FBG (mg/dL)	105.00±4.074	106.00±5.206	377.80±14.800 ^{a*,b*}	226.80±16.760 ^{a*,b*,c}
Insulin (µIU/mL)	13.86±0.470	13.40±0.401	16.50±0.692 ^{a,b}	15.33±0.276 ^{a,b}
C-Peptide (ng/mL)	3.19±0.336	0.79±0.106 ^a	7.04±0.463 ^{a,b}	3.00±0.315 ^c
HOMA-IR	2.45±0.279	0.61±0.080	20.24±3.163 ^{a*,b*}	3.13±0.415 ^{c*}
HOMA-%B	129.20±9.923	48.56±5.690 ^{a*}	47.60±3.916 ^{a*}	34.70±2.956 ^{a*c}
HOMA-%S	42.54±3.912	173.30±19.390 ^{a*}	5.48±0.891 ^{a,b*}	34.34±4.453 ^c

Values are expressed as mean ± SEM (n=5; a: p<0.05 vs. Ctrl; b: p<0.05 vs. Mel; c: p<0.05 vs. Diab; a*: p<0.01 vs. ctrl; b*: p<0.01 vs. Mel; c*: p<0.01 vs. Diab)

Fasting blood glucose (FBG), Homeostatic Model Assessment of insulin resistance (HOMA-IR), beta cell function (HOMA-%B) and insulin sensitivity (HOMA-%S)

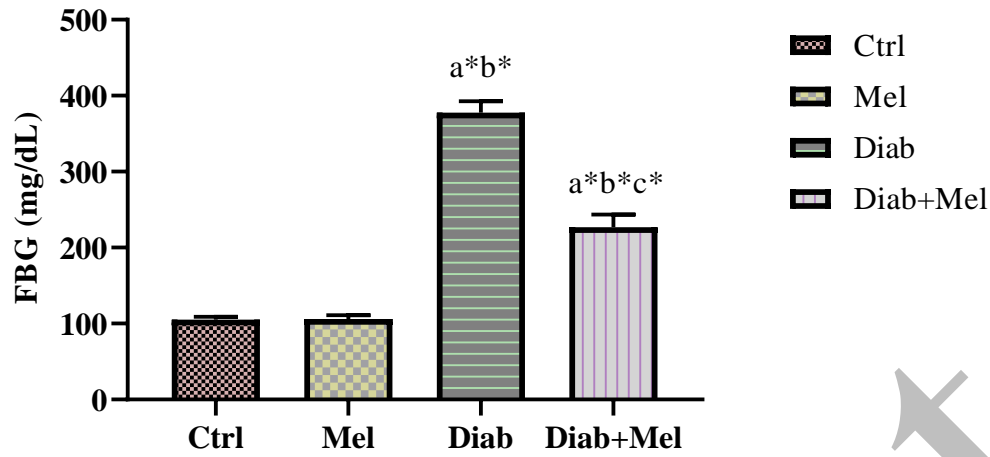


Fig. 1: Effect of melatonin on fasting blood glucose level of the alloxan-fructose-induced diabetic rats. (n=5; a*: p<0.01 vs. ctrl; b*: p<0.01 vs. Mel; c*: p<0.01 vs. Diab)

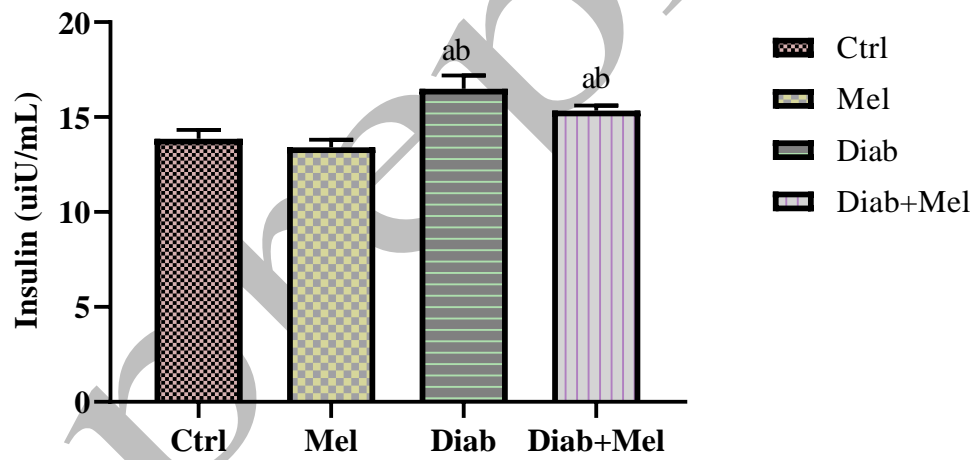


Fig. 2: Effect of melatonin on fasting insulin level of the alloxan-fructose-induced diabetic rats (n=5; a: p<0.05 vs. Ctrl; b: p<0.05 vs. Mel).

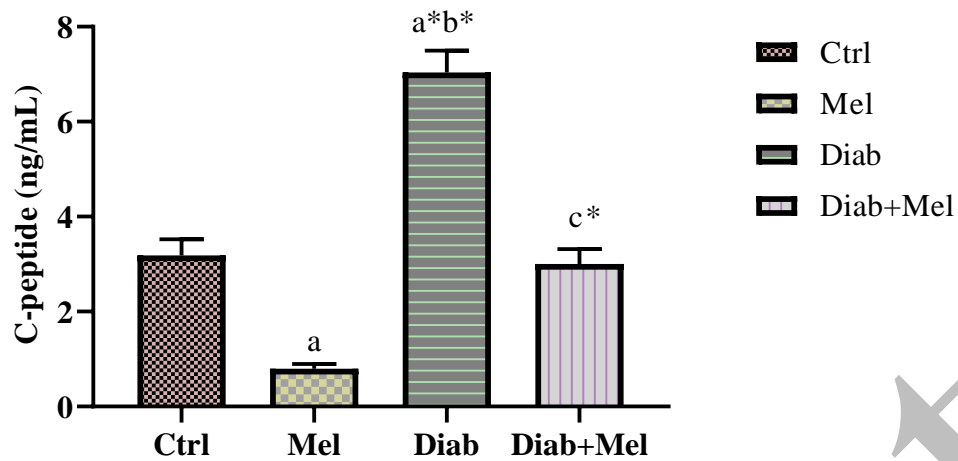


Fig. 3: Effect of melatonin on C-peptide levels of the alloxan-fructose-induced diabetic rats (n=5; a: $p < 0.05$ vs. Ctrl; a*: $p < 0.01$ vs. ctrl; b*: $p < 0.01$ vs. Mel; c*: $p < 0.01$ vs. Diab)

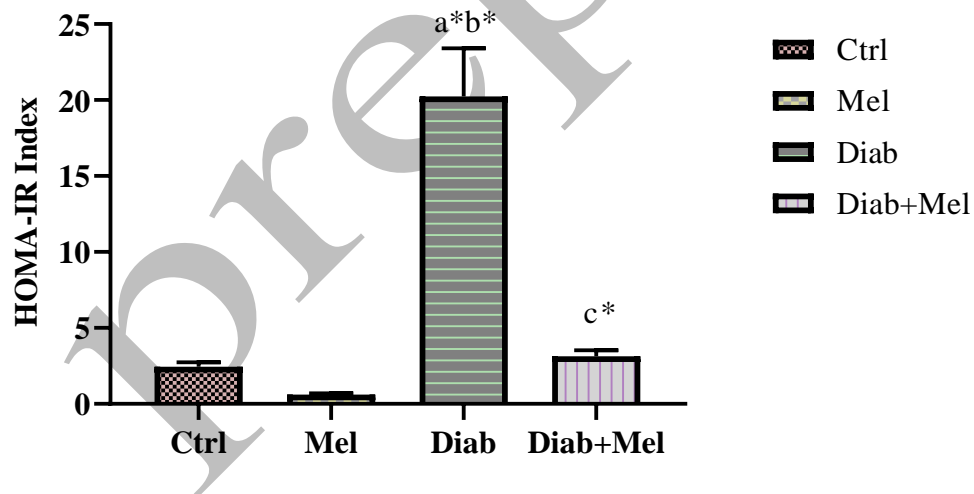


Fig. 4: Effect of melatonin on HOMA-IR index of the alloxan-fructose-induced diabetic rats. (n=5; a*: $p < 0.01$ vs. ctrl; b*: $p < 0.01$ vs. Mel; c*: $p < 0.01$ vs. Diab)

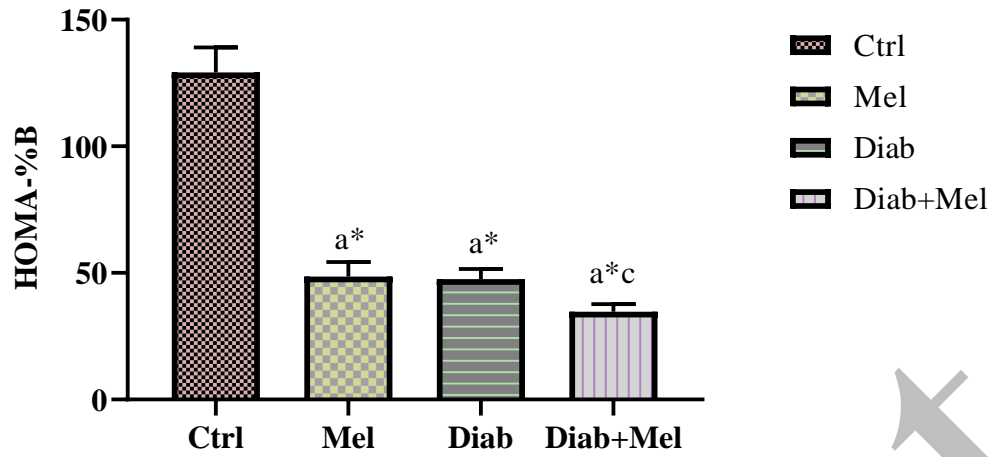


Fig. 5: Effect of melatonin on beta cell function (HOMA-%B) of the alloxan-fructose-induced diabetic rats. (n=5; a*: p<0.01 vs. ctrl; c: p<0.05 vs. Diab)

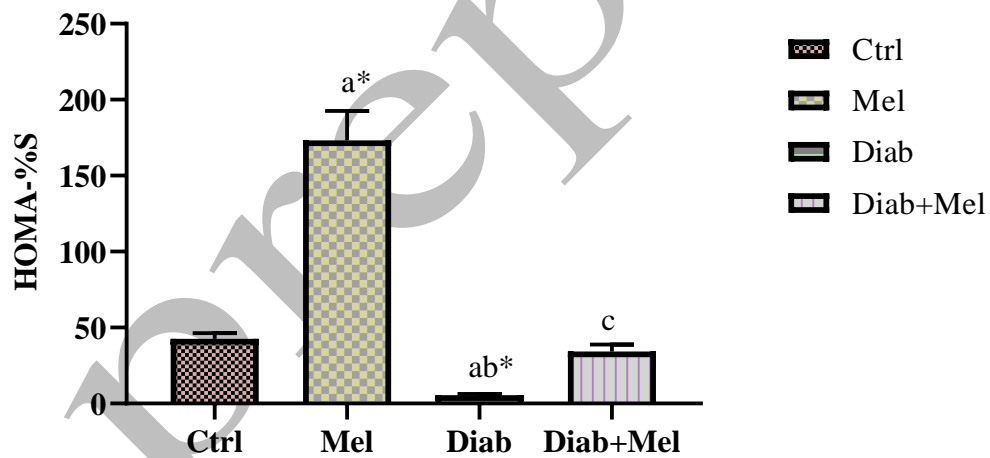


Fig. 6: Effect of melatonin on insulin sensitivity (HOMA-%S) of the alloxan-fructose-induced diabetic rats (n=5; a: p<0.05 vs. Ctrl; c: p<0.05 vs. Diab; a*: p<0.01 vs. ctrl; b*: p<0.01 vs. Mel)

4. Discussion

Diabetes is due to either the pancreas not producing enough insulin, or the cells of the body not responding properly to the insulin produced. The development of type 2 diabetes involves two primary factors: insulin resistance (IR) and malfunction of the pancreatic beta cells. In contrast to insulin resistance, which is a condition in which a particular concentration of insulin has a biological effect that is less than predicted, beta-cell dysfunction is the inability of the beta cells to produce the optimal insulin concentration required to maintain glucose homeostasis. Pathophysiologically, prolonged IR will ultimately lead to beta-cell failure when the beta cells' maximum amount of insulin production is insufficient to overcome IR (14).

In this study, an alloxan-fructose-induced type 2 diabetic rat exhibited considerable elevation in the levels of fasting blood glucose, insulin, C-peptide and insulin resistance (HOMA-IR). However, a decreased levels of beta cell function (HOMA-%B) and insulin sensitivity (HOMA-%S) were recorded. Supplementation with melatonin however, reversed these conditions except for insulin where no differences were observed between the diabetic and melatonin treated rats.

According to reports of earlier studies by Fabiyi-Edebor and Fasanmade (13), which reported that intraperitoneal administration of alloxan to fructose-fed rats in drinking water produced experimental Type 2 diabetes. In this study, diabetic rats demonstrated a significant increase in fasting blood glucose and insulin resistance, indicating the development of Type 2 diabetes and is consistent with earlier reports of many researchers (13,15) in different models of diabetic animals.

Previous experiments on animals showed a decreased level of insulin in diabetic state (16,17). However, Xuyan *et al.* (15) reported an elevated level of insulin in the HFD-STZ-induced diabetic rats. This is in tandem with our findings that the insulin level increased in alloxan-fructose diabetes.

In our study, insulin sensitivity (HOMA-%S) and beta cell function (HOMA-%B) were highly decreased, while insulin resistance (HOMA-IR) was elevated in the alloxanized fructose-fed rats. Abdulwahab *et al.* (17) and Fabiyi-Edebor and Fasanmade (13), also reported a high HOMA-IR level in diabetic rats, indicating the development of insulin resistance. The increased severity of insulin resistance may be due to the hyperglycemic plateau induced by alloxan (13).

The observed elevation of the C-peptide in the alloxanized-fructose rats is consistent with reports in the literature, that C-peptide levels are associated with diabetes type and duration of disease (18).

The potential of melatonin to attenuate hyperglycemia is demonstrated by the way that its supplementation greatly inhibited the rise in fasting blood glucose levels in diabetic rats as observed in our study. The current data agree with several other research conducted previously on animals in different models of diabetes (15,19). The present findings therefore, indicate that melatonin can exert anti-hyperglycemic and insulin-sensitizing effects. Also, exogenous melatonin administered before an evening test meal improved glucose tolerance and insulin sensitivity.

The effect of melatonin administration on the levels of insulin has conflicting results. For instance, treatment with melatonin significantly reduced the serum level of insulin in HFD-STZ-type 2 diabetic rats (15), while Lo *et al.* (16) reported that that melatonin administration increased insulin level. However, according to earlier report by Prunet-Marcassus *et al.* (20), melatonin had no effect on plasma insulin level. This agrees with our finding which reported no effect of melatonin on insulin levels.

Meanwhile, She *et al.* (21) reported that melatonin administration reduced insulin resistance and elevated glucose intake in 3 T3-L1 adipocyte. This agrees with our observation that melatonin reduced insulin resistance (HOMA-IR) to near normal level in the control group. However, Lo *et al.* (16) reported that melatonin did not improve the HOMA-IR in the melatonin-treated groups.

Insulin is a key hormone that regulates circulating glucose levels and cellular metabolism after food intake in many tissues (4). According to Shen *et al.* (22), it is generally accepted that insulin resistance leads to failure of response to insulin, eventually causing impairments in metabolic functions. Insulin resistance has been associated with a pro-inflammatory state increasing the risk for complications in T2DM (3). In early or progressing stage of T2DM, insulin resistance exists persistently and the amount of β -cell mass becomes inadequate with rapidly increased plasma glucose levels. The observed increase in insulin level with concomitant increase in the glucose level in this study may be as a result of insulin resistance and reduced insulin sensitivity. Thus, even with the high level of insulin, the hyperglycemic condition characterizing diabetes still persisted, because the cells are not sensitive to the circulating insulin.

Type 2 diabetes is primarily characterized by insulin resistance (IR), which is abnormal insulin action, and in order to meet the increased demand to maintain near normoglycemia, pancreatic β -cells produce more insulin, which leads to hyperinsulinemia as a sign of IR.

Pancreatic β -cells release equimolar quantities of C-peptide (a byproduct of proinsulin co-secreted with insulin) in addition to producing more insulin, which also led to the increased C-peptide as seen in this study. C-peptide is a more reliable indicator of endogenous insulin secretion and β -cell function than insulin. While insulin is mainly extracted by the liver, C-peptide is mainly metabolized by kidneys subjected to negligible first-pass metabolism by the liver and therefore, serves as a surrogate for endogenous insulin secretion.

Melatonin levels are down-regulated in metabolic disorders such as diabetes with IR, and activation of melatonin signaling delays disease progression. Melatonin has been reported to reduce hyperglycemia, improve insulin resistance, and protects pancreatic β -cells (23). Earlier, it was reported that it protects pancreatic β -cells from STZ-induced cellular oxidative injury and the development of T2DM. This is achieved by enhancing glucose tolerance, lowering hyperinsulinemia, improving insulin sensitivity, and restoration of the activity of islet β -cells. These findings agree with the ability of melatonin to reverse the reduction of glucose transporter 4 (GLUT4) gene expression, glucose intolerance and insulin resistance, and augmented gluconeogenesis in melatonin-deficient pinealectomized rats.

The possible mechanism by which melatonin exerts its effects could be mainly attributed to insulinogenic activity that stimulates insulin secretion, regenerate β -cells or even protect the remaining β -cells through its strong antioxidant properties (24). The effect of melatonin has also been attributed to the direct scavenging of free radicals, up-regulation of endogenous antioxidants, and inhibition of pro-oxidant enzymes.

Additionally, two melatonin receptors (MT1 and MT2) have been linked in separate studies to insulin secretion and the development of T2DM by a number of signaling pathways, but a thorough explanation of the underlying molecular mechanism to rationally and precisely explain this association has not yet been elucidated.

According to Peschke *et al.* (25), human pancreatic tissue and rat islets both poses these receptors. Recent studies have shown that the pancreatic β -cell's putative signaling pathway is regulated by these melatonin membrane receptors. Melatonin

affects pancreatic beta-cells and insulin release through intracellular cyclic adenosine monophosphate (cAMP), cyclic guanine monophosphate (cGMP), and inositol triphosphate (IP₃) signal transduction pathways, which contributes to the maintenance of glucose homeostasis. Additionally, melatonin modulation of cAMP results in decreased gluconeogenesis and increased glycolysis, lowering blood glucose levels.

In conclusion, this study gives strong affirmation of the useful impact of melatonin on alloxan-fructose-induced type 2 diabetes mellitus. Treating T2DM with a pharmacological dose of melatonin is a key strategy to strengthening the body's antioxidant defense system and consequently improving anti-hyperglycemic conditions by blocking mechanisms that lead to hyperglycemia, as clearly evidenced from the favorable improvements in the beta cell function, insulin sensitivity and reduced insulin resistance. According to these results, the best way to prevent the onset of the disease, slow down its course, and as well as potentially delay the emergence of long-term complications from type 2 diabetes is to begin early and aggressively treating insulin resistance. For the early identification of insulin resistance and beta-cell dysfunction, this calls for the adoption of simple and trustworthy diagnostic methods like the HOMA model.

Ethical Statement

This study was performed with the animals cared for and humanely treated in accordance with the Guide for the care and use of laboratory animals. Experimental protocols were approved by the Faculty of Basic Medical Sciences Animal Research Ethics Committee, University of Calabar (FAREC-FBMS), with the approval number **230PHY3523**; and in line with International guiding principles for biomedical research involving animals.

Acknowledgements

The authors are grateful to Mr. Sylvester Ibekailo, Mr. Kenneth Oparaji, and Mr. Liberty Okoro, the chief technologist of the Department of Physiology, for providing technical support.

Conflict of interest

The authors declare no conflict of interest

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

Funding

Nil

Author contributions

ARN, DEI, CSO and UAI contributed to the design and conducted the study, collection, analysis, interpretation of data, and wrote the manuscript. ARN, CSA, SAB and ABA contributed to the experimental design and critically analyzed the manuscript. ARN, CSA, SAB, ABA, DEI, CSO, OOU and UAI participated in the manuscript preparation. All authors read and approved the final manuscript.

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