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INVESTIGATING NEUROLOGICAL ABNORMALITIES IN DIABETIC PATIENTS: MEASURING NEUROHORMONES AND NEUROACTIVE MIRNAS IN BLOOD SAMPLES OF TYPE 2 DIABETES PATIENTS

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Abstract

Aim: Type 2 diabetes mellites (T2DM) is a chronic metabolic disorder associated with oxidative stress, increased inflammation, altered energy metabolism and neurological abnormalities. Therefore, this study aimed to clarify some neurological ambiguities in diabetes. By considering master regulatory role of miRNAs in biological process, we evaluated some neuroactive miRNAs (miR-125a, Let-7 miRNA, miR-181c, miR-504, miR-16) and neurohormones such as Gamma-aminobutyric acid (GABA), serotonin and dopamine in T2DM patients.

Methods: This study were performed on 30 T2DM patients and 30 non-diabetic controls. The level of GABA, serotonin, dopamine and biochemical parameters were determined by specific ELISA kit in blood serum samples. Also, the relative contents of the miRNAs were evaluated by the real-time quantitative polymerase chain reaction (RT-qPCR) analysis.

Results: The obtained results show that dopamine and serotonin increased in hyperglycemia condition possibly due to upregulation of miR-181c and miR-125a and down-regulation of miR-16. The mentioned changes in miRNAs network also could be considered as a cause of insulin resistance (IR). Reduced content of miR-16 could lead to reduced glucose uptake that was observed in diabetes. Circular concentration of GABA decreased also that could be considered as a reason for IR and decreased glucose uptake. GABA is an excitatory neurotransmitter and its reduction could be a possible cause for dementia related disease.

Conclusion: This study revealed the examined miRNAs plays essential role in oxidative stress, inflammation and IR in T2DM and have therapeutic potential. Based on neuroendocrine abnormalities in diabetes, exogenous hormones could be considered as therapeutic agents to control the metabolism rate and decrease the neurological side effects in T2DM.

Keywords: Neurological abnormalities; miR-16; Insulin resistance; Type 2 diabetes, miRNAs network; Non-enzymatic glycation

1. Introduction

Type 2 diabetes mellites (T2DM) is a multifactorial metabolic disease precipitated by a combination of oligo- and polygenic and other non-genetic risk factors including high caloric intake and sedentary lifestyle. The disease is characterized by peripheral insulin resistance, which is linked to hyperglycemia and hyperinsulinemia (Lindholm *et al.* 2006). At the cellular level, T2DM is associated with mitochondrial dysfunction, endoplasmic reticulum stress, increased inflammation, and altered energy metabolism (Lin & Beal 2006). Upregulation of glucose in circulation induces non-enzymatic glycation of intracellular and extracellular macromolecules that disrupts whole body homeostasis especially central and peripheral nervous system (CNS and PNS) (Yekta *et al.* 2022). According to our recent experiments, invitro hyperglycemia condition causes non-enzymatic glycation of acetylcholinesterase that affects kinetic behavior of enzyme and also changes its interaction with drugs (Yekta *et al.* 2022; Yekta *et al.* 2020). In vivo hyperglycemia condition also induces non-enzymatic glycation process on all of the enzymes, proteins and other macromolecules therefore, T2DM is a systemic disease that associated with different types of symptoms in the body in particular CNS due to high sensitivity (Association 2010). T2DM also leads to serious vascular complications that can affect several tissues, including the brain so risk of dementia-related disease such as Alzheimer increases in diabetic patients (Bauduceau *et al.* 2010; Biessels *et al.* 2006). Compared with the general population, people with T2DM have a 1.5-2.5 times increased risk of dementia, and at present one in ten to 15 cases of dementia can be attributed to T2DM (Bauduceau *et al.* 2010). Hyperinsulinemia also could be considered as a damaging condition due to crucial role of the insulin in regulation of the neurogenesis, dendritogenesis and neural cells metabolism (Jonik *et al.* 2022; Blázquez *et al.* 2014). While results of clinical studies support a link between T2DM and Alzheimer's and Parkinson's disease that is associated with changes in insulin, cholesterol, and amyloid metabolism in the brain (Talbot *et al.* 2012). This link could be neuroactive micro RNAs (miRNAs) and neurohormones which have essential role in glucose metabolism and insulin resistance.

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (CNS) and play essential role in balance between neuronal excitation and inhibition. Studies have demonstrated that oral GABA administration improved the circulation of glucose by rising plasma insulin and reducing plasma glucagon levels (Soltani *et al.* 2011). It is well documented that GABA deficiency is associated with several important neurological disorders such as Huntington's chorea, Parkinson's and Alzheimer's disease and other psychiatric disorders, like anxiety, depression, pain, panic, or mania (Gajcy *et al.* 2010). Dopamine is predominant catecholamine in the mammalian CNS that involved in locomotor activity and movement coordination, cognition, emotion, positive reinforcement, food intake, and endocrine homeostasis. in the central nervous system (Tavares *et al.* 2021). It is key regulator of glucose metabolism in CNS and energy balance (Asghar *et al.* 2011). Serotonin is a neuromodulator which have essential role in insulin and glucose metabolism (McGlashon *et al.* 2015). The role of brain serotonin is widely known in several physiological functions, including sleep, hunger, mood, memory, and learning management (McGlashon *et al.* 2015). Interestingly, neurohormones could regulate and also were affected by miRNA molecules as a class of small noncoding RNAs that negatively regulate gene expression by partially pairing to their target mRNAs, leading to translation repression and/or transcript degradation (Guay & Regazzi 2013). Therefore, miRNAs can regulate the response of target tissues to insulin and specific miRNAs involved in various aspects of glucose and lipid metabolism and also reciprocally regulate neurohumors circular content (Agbu & Carthew 2021). By considering the master regulatory role of miRNAs in glucose metabolism and insulin resistance, this study aimed to evaluate the circular contents of miR-125, miR-181c, miR-504, miR-16 and let-7 miRNA. Majority of them are involved in glucose metabolism, neural functions, and neurohormones biosynthesis and release that have not

been studied in T2DM yet. The possible effects of miRNAs on neurohormones circular concentration were studied through comparing the GABA, serotonin and dopamine levels in blood samples of T2DM patients and non-diabetic controls. This kind of researches could help to clarify some of the ambiguities in diabetes related neurological abnormalities and also explain the molecular events take place in high glucose toxicity.

2. Material and methods

2.1. Experimental design and participant

This study aimed to compare the miRNAs expression and also neurohormones concentration in blood serum of the patients suffering from T2DM and non-diabetic control individuals that were referred to the Specialized Center for Endocrinology and Diabetes in Baghdad (Iraq) between 1 October to 15 December 2022. For this purpose, 30 patients with T2DM disease and 30 non-diabetic individuals (as control group) were included in the study. Informed and written consent is obtained from patients. HbA1c > 6.5% was used as the diagnosis criteria for T2DM. HbA1c≥7% was defined as poorly controlled T2DM. For the serum lipid profile, hypercholesterolemia is defined as total cholesterol (TC) ≥ 200 mg/dl, hypertriglyceridemia when triglyceride (TG) is ≥150 mg/dl, high low-density lipoprotein (HDL-C) when the value is ≥ 130 mg/dl, and low High-density lipoprotein (LDL-C) as a value less than 40 mg/dl. Body mass index (BMI) was calculated as the weight in kilograms divided by height in meters squared (kg/m2). BMI was categorized into normal weight (<25 kg/m^2), Overweight (25-29.9 kg/m^2) and Obese (≥30 kg/m^2). Patients with liver deficiency, kidney disorder, thyroid disorder, acute coronary syndromes, Alzheimer disease, different cancers, patients taking vitamin supplements and people with family history of dementia were excluded from the study. Control group was selected among age- and sex-matched healthy peoples that had not been diagnosed with diabetes or prediabetes and don't suffer from heart disease, thyroid disorder and Alzheimer disease or other neurological disorders. In this study, 5 ml of blood samples were taken from the patients and centrifuged at 10000 rpm for 5 min to obtain blood serum samples and then stored at -20°C temperature before analysis. The study was approved by the health and ethics committee of the health center, and all the participants gave their informed consent in accordance with the Declaration of Helsinki. Relevant sociodemographic, clinical and laboratory data were obtained from the medical records of the patients including: age, gender, HbA1c, HDL-C, LDL-C, TC, TG, BMI, Body mass. This information was recorded on the data sheet. Anthropometric measurements were taken, including weight and height.

2.2. Estimation of biochemical parameters

The levels of the following biochemical were determined by ELISA Kit. Human ELISA kits were used for determination of GABA (Abcam, ab287792), Serotonin (Abcam, ab133053) and dopamine (Abcam, ab285238) according to the manufacture guideline in each case. All of the reagents and solution were placed on bench 30 min before test.

2.2.1. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

RT-qPCR was used to analyze the selected miRNA level including miR-125a, Let-7, miR-181c, miR-504, and miR-16. Total RNA in serum was extracted by using the RNA blood Kit and miRNA Purification Kit from Stem cell technology research center (STRC, Iran). The quality and quantity of RNA were monitored by a spectrophotometer. Then, the RNA was reverse transcribed to Complementary DNA (cDNA) using the miRcute Plus miRNA First-strand cDNA Kit according to the manufacturer's instructions. Finally, the RT-qPCR reaction was performed using the miRcute Plus miRNA qPCR (SYBR Green) Kit and specific primers (Table 1) on the ABI Step One Plus real-time PCR instrument. U6 was used as the internal reference gene, and the relative expression level of the studied miRNAs were calculated by the method of $2^{-\Delta\Delta Ct}$ (Yu *et al.* 2021).

2.3. Statistical analysis

Statistical assessment was done by using the analysis of variance (ANOVA) to determine statistical differences between results related to T2DM group and non-diabetic samples as control. Chi-square ($\chi 2$), logistic regression and t-test were applied to compare two experimental groups with each other and significant differences were showed in each plot by indication of star symbol according to the P value. All statistical analyses were performed with SPSS 26 statistical software.

3. Results

There is a significant accompaniment between glucose upregulation and dementia related disease specially Alzheimer disease that could be refer to hyperglycemia toxic effects on neural function, cognitive and mood (Bauduceau *et al.* 2010; Biessels *et al.* 2006). Therefore, this study aimed to clarify the possible molecular mechanism involved in hyperglycemia toxicity in miRNAs and neurohormones level due to their crucial role in neurogenesis, dendritogenesis, metabolism regulation and homeostasis of neurons. Therefore, 30 patients suffering from T2DM and 30 non-diabetic controls were selected. Demographic and anthropometric properties of patients and controls are shown in Table 2. Based on the results, BMI related to T2DM patients and control were calculated as 28.92±4.53 kg/m^2 and 27.81±3.57 kg/m^2 respectively that don't have significant difference according to statistical analysis (P>0.05). Cholesterol content in blood serum of the patients is 197.90±91.19 mg/dl versus 177.67±14.26 mg/dl in control subjects, P=0.205 confirmed cholesterol content is not significantly higher in T2DM. Our results revealed triglyceride (TG) content also is not deferent between both experimental groups (169.78±68.53 mg/dl versus 158.93±13.14 mg/dl in patient and control subjects, P=0.908). Circular content of insulin evaluated to be 21.98±10.44 µU/ml in patients versus 6.84±1.62 µU/ml in control subjects, P=0.0001 manifested insulin content in patient group is significantly more than control. Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) is a parameter to assessment insulin resistance in body. This parameter was 12.27±7.91 in T2DM patients versus 1.06±0.17 in control subjects, P=0.0001, higher amount of HOMA-IR showed insulin resistance in patient group. Essential elements of blood serum were measured in both experimental groups and results confirmed circular level of Zn and Fe raised and Cu content decreased in T2DM patients in comparison with non-diabetic control.

3.1. T2DM affects neurohormones level in circulation

Neurohormones are main regulators of body homeostasis and metabolism regulation that could be affected by hyperglycemia toxicity and insulin resistance conditions (Soltani *et al.* 2011; Asghar *et al.* 2011). According to the results (Fig 1A), cortisol concentration in blood samples of T2DM was estimated as 385.35±52.04 nmol/L while this parameter in control subjects was 178.04±35.35 nmol/L. Statistical analysis showed significant difference of cortisol content between control and T2DM (P<0.0001). According to the Fig. 1B, GABA concentration in blood serum of diabetic patients is 96.79±6.42 nmol/dL that is remarkably different from its content in non-diabetic samples (118.59±7.04 nmol/dL), P value estimated to be <0.0001). Our results showed dopamine level is 31.03±11.06 pmol/L in non-diabetic control blood samples that reduced to 18.18±3.97 pmol/L, that this moderate is significant according to multiple comparison of one-way ANOVA statistical analysis. As Fig 1D, patients with T2DM have more level of serotonin rather that non-diabetic control (42.82±17.84 ng/ml versus 24.74±4.62 ng/ml in patient and control subjects). Statistical analysis showed P value is less than 0.0001 so difference is significant.

3.2 High glucose toxicity changed the neuroactive miRNAs regulation

miRNAs are upstream regulators which could affect all of the process in living system in response to hyperglycemia and hyperinsulinemia toxicity such as biosynthesis and release of neurohormones, neurogenesis, neural metabolism and etc (Zhang *et al.* 2019). our results showed normalized expression of the Let-7 miRNA in patients suffering from T2DM is similar to the non-diabetic healthy control (Fig 2A). Therefore, upregulation of glucose and insulin could not change circular content on

Let-7 miRNA. While, Fig 2B shows that the relative expression of miR-181c increased in diabetic patients about 2-fold and this raise is significant according to the one-way ANOVA analysis ($P<0.001$). Similarly, miR-125a level increased in diabetic patients in comparison with non-diabetic healthy control ($P<0.001$) (Fig 2C). Fig 2D also manifested the normalized level of miRNA-504 in T2DM and control groups that resulted confirmed significant increased content of this miRNA as a result of diabetes ($P<0.001$). Finally, results demonstrated lower concentration of the miR-16 in T2DM circulation rather than blood serum of non-diabetic control ($P<0.001$) (Fig 2E).

4. Discussion

Neuropathy resulting from diabetes is estimated to affect 60-70% of people with diabetes depending on age, duration of diabetes, definition of neuropathy used, presence or absence of pain, and whether or not other causes of neuropathy are excluded (Galer *et al.* 2000). miRNAs are master regulators of cellular process that estimated to regulate the expression of more than 60 % of protein-coding genes, consequently controlling a number of biological and pathological processes (Deng & Guo 2019). In this research the possible mechanism of neurological abnormalities in diabetic patients were studied in the neuroendocrine and miRNAs level. Because, neurohormones play important role in glucose metabolism and insulin sensitivity (Soltani *et al.* 2011; McGlashon *et al.* 2015) that defected in T2DM patient. According to the results the patients suffering from T2DM showed high level of circular glucose concentration and also increased amounts of HOMA-IR which refer to insulin resistance and accompanied with dysregulation of neurohormones circular level. Insulin resistance means insulin receptor dysfunction in response to high amount of insulin hormone (Abdul-Ghani & DeFronzo 2010). The obtained results show that GABA was significantly decreased in diabetic patients while cortisol, dopamine and serotonin were significantly higher in T2DM patients compared to control subjects. Previous studies indicated that GABA triggers glucose uptake and has protective effects on insulin resistance (Soltani *et al.* 2011) so that GABA 's beneficial effects on diabetes, including the encouragement of replication, regeneration, defense of beta-cells from apoptosis, and an anti-inflammatory effect (Wang *et al.* 2017). Soltani et al., in 2011 indicated that GABA prevents inflammation of Langerhans islets (insulitis), and the development of systemic inflammatory cytokine (Soltani *et al.* 2011). It has also been reported that GABA in β-cells islets triggers the activation of PI3-K/Akt pathway and induces growth and survival (Wang *et al.* 2017). But it's not clear reduced amounts of GABA is cause of diabetes or a consequence. Dopamine is one of the major neurotransmitters in the brain which controls a variety of key functions such as locomotion, cognition, feeding behavior, energy homeostasis, motivation, memory, mood, learning, and hormone secretion (Asghar *et al.* 2011). Inhibition of the dopamine receptors by using neuroleptic drugs causes hyperinsulinemia in control cases (Sowell *et al.* 2002) that confirmed dopamine signaling pathway involvement in glucose and lipid metabolism. By considering insulin important role in neural homeostasis, dopamine reduction also associated with different range of neuropsychiatric abnormalities in diabetes patients (Pijl 2003). According to the previous results serotonin biosynthesis and release need to a set of enzymes that were produced in hindbrain and pancreatic β cells (GYLFE 1978). Therefore, both locally produced serotonin and serotonin present in the circulation might influence the function of pancreatic β cells. Paulmann and colleagues demonstrated that the intracellular concentration of serotonin correlates positively with insulin secretion rate and the extracellular serotonin might suppress insulin secretion (Paulmann *et al.* 2009). Ablation of the brain serotonin was confirmed to cause low insulin secretion in the mouse that led to hyperglycemia condition (McGlashon *et al.* 2015), providing the information that defective central serotonin functions may associate with the impairment of insulin signaling leading on the development of diabetes. Also, researches show that miRNA-16 deletion accompanied with insulin resistance (Lim *et al.* 2022). Interestingly, Lee et al. demonstrated a decrease in miR-16-5p expression in insulin-resistant skeletal muscle (Lee *et al.* 2016). Previous experiments revealed that miR-16 possibly involves in the occurrence and development of depression through targeting serotonin transporter gene and regulating its translational level (Shao *et al.* 2018). miR-16 has been reported to inhibit the

human dopamine D1 receptor expression also by targeting 3′UTR (Wu *et al.* 2020). Therefore, reduced miR-16 possibly regulates dopamine and serotonin synthesis and their responsible receptors that finally lead to insulin resistance that was observed in T2DM patients. The results showed increased amounts of miR-125a in T2DM, this miRNA is involved in the MAPK signaling pathway (Huber *et al.* 2002) and plays a critical role in insulin signaling (Fujishiro *et al.* 2003) so raised miR-125a could be contribute to insulin resistance. Interestingly, a set of miRNAs have recently been found to regulate adipose tissue biology (development and metabolism), insulin secretion and action, and therefore their imbalance may play a role in the development of obesity and related metabolic complications (Derghal *et al.* 2016). The obtained results in this research show that the relative expression of miRNA-181c and miRNA-504 were significantly higher in T2DM patients compared to non-diabetic control subjects ($P<0.001$). However, there is no significant difference in the relative expression of Let-7 miRNA in T2DM and control groups. Zhu et al. reported that global overexpression of Let-7g in mice resulted in growth retardation and impaired glucose tolerance (Zhu *et al.* 2011). However, in our results there is no significant difference in Let-7 expression in T2DM possibly due to long lasting disease, circular glucose level and genetic diversity. The miR-181 family (miR-181a, miR-181b, miR-181c, and miR-181d) is involved in regulating vascular inflammation and immunity (Sun *et al.* 2014). Specifically, miR-181c has been reported to play a significant role in inflammatory response by hampering CD4+ T cell activation (Xue *et al.* 2011). This miRNA also identified as an angiogenic regulator therefore its upregulation is responsible for diabetes-derived vascular complications (Solly *et al.* 2021). Overexpression of miR-181c also causes increase of both mitochondrial respiration and oxidative stress due to elevated mitochondrial cytochrome c oxidase subunit 2 (mt-COX2) (Das *et al.* 2012). Therefore, upregulation of miR-181c induces oxidative stress and neuroinflammation in neurodegenerative disease such as Parkinson's disease (Hutchison *et al.* 2013) and could be considered as neurological complexities in diabetes also.

4. Conclusion

Diabetes-derived neurological abnormalities are challenging problems that observed in the majority of diabetic patients and related animal models that we tried to elucidate some ambiguities by targeting the mutual relationship between miRNAs and neurohormones. Taken together, our results manifested the wide dysregulation in neurohormones levels such as reduction in GABA concentration and raise in cortisol, dopamine and serotonin. By considering important role of neurohormones in glucose metabolism and insulin resistance, neuroendocrine could be considered as an effective therapeutic target. Hormone replacement therapy could help to control the metabolism rate and also limit the neurological side effects in diabetic patients. This study also emphasizes the emerging role of miR-16, miR-181c, miR-504 and miR-125a in insulin resistance, reduced glucose uptake and neuroendocrine dysregulation and introduces them as potential therapeutic targets for T2DM and rescuing hyperglycemia-impaired neural damages.

Tables

Table 1. Sequence-specific primers which were used in RT-qPCR analysis.

miRNAs	Primer	Primer sequence	Mature miRNA sequence
miR-125a	Forward	5’ ACACTCCAGCTGGGTCCCTGAGACCCTTTAAC 3’	ucccugagacccuuuaaccuguga
	Reverse	5’ TGTCGTGGAGTCGGCAATTC 3’	
miR-81c	Forward	5′-ACACTCCAGCTGGGAACATTCAACCTG-3′	aacauucaaccugucggugagu
	Reverse	5′-TCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACTCACCG-3′	
miR-504	Forward	5′-CCAGCAAGACCCTGGTCTG-3′	agacccuggucugcacucuauc
	Reverse	5′-CAGAGCAGGGTCCGAGGTA-3′	
miR-16	Forward	TAGCAG CACGTAAATATTGGCG	uagcagcacguaaauauuggcg
	Reverse	TGCGTGTCGTG GAGTC	
Has-let-7	Forward	GCAGTGAGGTAGTAGGTTG	ugagguaggagguuguauaguu
	Reverse	CAGTTTTTTTTTTTTTTTCGCCAA	
U6	Forward	5’ CTCGCTTCGGCAGCACATATACTA 3’	
	Reverse	5’ ACGAATTTGCGTGTCATCCTTGC 3’	

Table 2. Biochemical parameters in control and patient groups

Parameter	Control	T2DM Patients	P-value
Age (Years)	55.63±6.94	60.22±14.04	0.097
Weight (kg)	76.85±11.24	80.53±14.21	0.006*
BMI (kg/cm2)	27.81±3.57	28.92±4.53	0.251
FBS (mg/l)	94.53±6.04	98.83±36.09	0.110
HbA1C (%)	4.17±0.30	8.60±2.20	0.0001*
Insulin (µU//ml)	6.84±1.62	21.98±10.44	0.0001*
HOMA-IR	1.06±0.17	12.27±7.91	0.0001*
Cholesterol (mg/dl)	177.67±14.26	197.90±91.19	0.205
Triglyceride (mg/dl)	158.93±13.14	169.78±68.53	0.908
HDL (mg/dl)	37.78±5.01	36.59±6.96	0.408
Zn (mg/dl)	12.35±2.05	14.72±1.37	0.0001*
Cu (mg/dl)	97.46±8.99	87.51±6.48	0.0001*
Fe (mg/dl)	67.33±16.02	101.50±14.58	0.0001*

Figure legends

Fig.1 Circular concentration of neurohormones in diabetic patients and non-diabetic controls. (A) Cortisol concentration of blood serum increased in T2DM rather than control. (B) GABA concentration in blood samples related to diabetic patients remarkably reduced rather than control. (C) Dopamine and (D) serotonin also increased in diabetic patients significantly. All data were expressed as mean ± SD. P value was showed on column in comparison of each data and P<0.0001 is significant.

Fig. 2 Neuroactive miRNAs evaluation by using RT-qPCR method. Data related to miRNAs count were normalized against U6 non-coding RNA and represented in column plot in each group. (A) reveals normalized count of the Let-7 miRNA, (B) shows normalized level of the miR-181c, (C) manifests normalized concentration of miR-125a, (D) reveals normalized content of the miR-504, and (E) shows normalized concentration of the miR-16. All data were expressed as mean ± SD. Significant differences indicated by star symbol above the column plots (P<0.05).

Fig. 3 Schematic illustration of studied parameters relationship. miRNAs are upstream regulators that cause oxidative stress, neuromodulation and insulin resistance that finally lead to neurological abnormalities in diabetic patients.

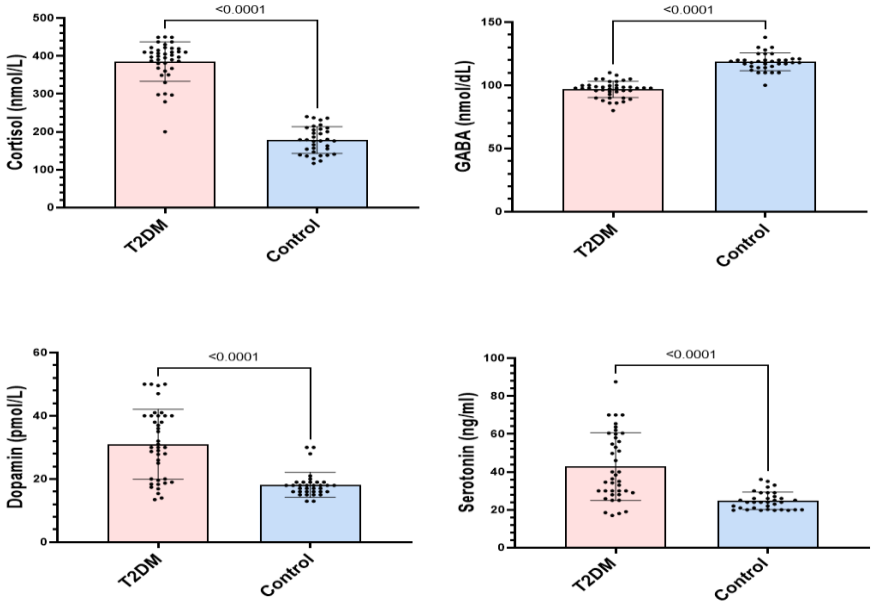

Fig. 1

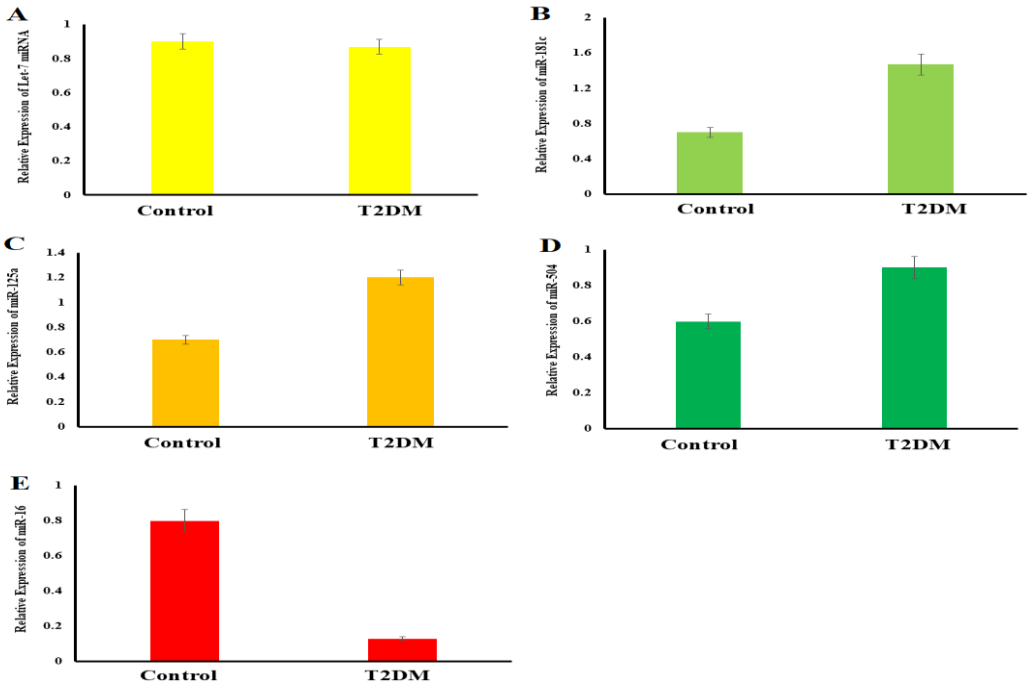

Fig. 2

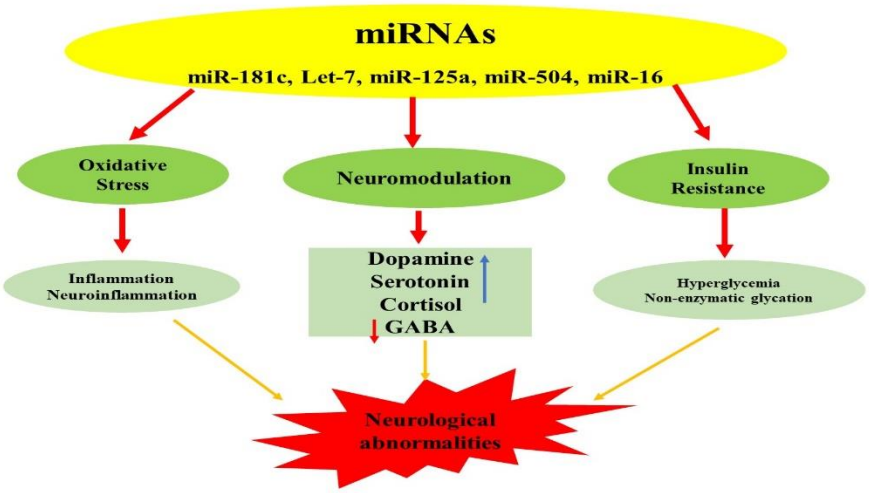

Fig. 3

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