ARUN PARASHAR

Ph. D.

2019

INVESTIGATION OF THE EFFECT OF RUTIN ON TYPE II DIABETES MELLITUS ASSOCIATED NEUROLOGICAL COMPLICATIONS

Thesis submitted in fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES

BY

ARUN PARASHAR

ENROLLMENT NO. 126751

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JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
WAKNAGHAT, DISTRICT SOLAN, H.P., INDIA

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Dedicated to my beloved Parents

Mr. T.R. Parashar & Mrs. Naro Devi Parashar

& Brother

Pankaj Parashar

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DECLARATION BY THE SCHOLAR

I, Arun Parashar, hereby declare that the work reported in the Ph.D. thesis entitled **"Investigation of the effect of rutin on type II diabetes mellitus associated neurological complications"** submitted at the **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Udayabanu Malairaman**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. thesis.

Arun Parashar

Department of Pharmacy

Jaypee University of Information Technology, Waknaghat, India

SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Investigation of the effect of rutin on type II diabetes mellitus associated neurological complications"**, submitted by **Arun Parashar** at the **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of his original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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Assistant Professor
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Arun Parashar

ABSTRACT

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by chronic, consistent and prolonged hyperglycemia. Diabetes is associated with neurological complications like neurodegeneration, cognitive decline, dementia and even disorders like Alzheimer's and Parkinson's. Our previous work elucidated a high quantity of rutin in *Urtica dioica* leaf extract, which showed pronounced neuroprotection against diabetes and depression. So, we aimed to explore the neuroprotective effects of rutin against type 2 diabetes (T2DM) mellitus associated neurological complications. We further aim to identify the role of insulin signaling in brain during T2DM and the effect of rutin treatment on it.

Our first objective was to study the neurological complications of chronic diabetes and the effect of rutin in it. Diabetes was induced using multiple low dose of streptozotocin (STZ), and treated with 100 mg/kg rutin for 2 months. STZ treatment led to significant hyperglycemia, glucose intolerance and hypoinsulinemia. Diabetic animals were observed to be depressed, anxious, and showed pronounced learning & memory deficits. These behavioral deficits were attributed to hippocampal neurodegeneration and impaired hippocampal insulin signaling. Rutin treatment improved hyperglycemia, glucose intolerance, attenuated hypoinsulinemia and behavioral dysfunction, and upregulated the hippocampal insulin signaling. In conclusion, rutin halts and ameliorates the progression of diabetes and associated neurological complications.

To better mimic the natural way of diabetes induction, our next objective was to develop an insulin resistant state, as observed in T2DM. For this we employed a 21 day chronic unpredicted stress (CUS) induced depression paradigm, along with rutin treatment (100 mg/kg; po; od). CUS induced pre-diabetes, insulin resistance (IR) and glucose intolerance along with similar behavioral dysfunctions. Molecular underpinnings included hippocampal neurodegeneration and IR. Rutin treatment improved glucose homeostasis, IR, behavioral dysfunctions and alleviated hippocampal neurodegeneration. Mechanistically, rutin modulated the hippocampal insulin signaling pathway by enhancing glucose transporter-4 (GLUT4) and insulin receptor (InR) expression, independent of insulin expression.

LIST OF ABBREVIATIONS

%	Percent
^{0}C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromole
ml	Milliliter
Aβ	Amyloid beta
AGE	Advanced Glycosylated End products
ALX	Alloxan
ANOVA	Analysis of variance
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
CA1	Cornu Ammonis 1
CA2	Cornu Ammonis 2
CA3	Cornu Ammonis 3
CNS	Central Nervous System
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CTCF	Corrected Total Cell Fluorescence
CTRL	Control
Control + R	Control + Rutin
CUS	Chronic Unpredicted Stress
CUS + R	Chronic Unpredicted Stress + Rutin
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DG	Dentate gyrus
DM	Diabetes Mellitus

DPP-IV	Dipeptidyl peptidase-4
EPM	Elevated Plus Maze
FBG	Fasting Blood Glucose
FITC	Fluorescein isothiocyanate
FS	Foot Shock
FST	Force Swim Test
FWD	Food and Water Deprivation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GD	Gestational Diabetes
GIP	Glucose-Dependent Insulinotropic Polypeptide
GLP-1	Glucagon-Like Peptide-1
GLUT	Glucose Transporter
GLUT2	Glucose Transporter 2
GLUT4	Glucose Transporter 4
HbA1c	Glycated Haemoglobin
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HPA	Hypothalamic-Pituitary-Adrenal axis
IAEC	Institute Animal Ethics Committee
IDE	Insulin Degrading Enzyme
InR	Insulin Receptor
i.p.	Intraperitoneal
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
MWM	Morris Water MAZE
NOR	Novel Object Recognition
OFT	Open Field Test
OGTT	Oral Glucose Tolerance Test

OL	Overnight Illumination
PAR	Poly ADP-Ribosylation
PASD	Passive Avoidance Step Down
PAST	Passive Avoidance Step Through
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline having 0.1% Tween-20
PKB	Protein Kinase B
p. o.	Oral route of drug administration
PPAR-γ	Peroxisome proliferator-activated receptor gamma
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
SPT	Sucrose Preference Test
STZ	Streptozotocin
STZ + R	Streptozotocin + Rutin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TC	Tilt Cage
TRITC	Tetramethylrhodamine
TST	Tail Suspension Test
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Diabetes mellitus (DM), commonly known as diabetes is a complex metabolic disorder characterized by prolonged and consistent hyperglycemia. Diabetes is mainly of two types: type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). T1DM is characterized by failure of the pancreas to produce insulin, therefore also known as Insulin-dependent diabetes mellitus (IDDM). T2DM is characterized by Insulin resistance (IR), a condition in which the ability of body cells to respond to insulin is compromised, in other words, Non-Insulin-dependent diabetes mellitus (NIDDM) [1].

Other than the most common and widely studied peripheral diabetic complication, such as cardiovascular, neuropathy, nephropathy, and retinopathy, there exists a significant CNS pathology too. Hyperglycemia causes various structural and functional abnormalities in the brain, including neurodegeneration, dementia, blood-brain barrier (BBB) disruption, organelle dysfunctioning (mitochondria, endoplasmic reticulum, cytoskeleton, and nucleus) etc [2].

Since glucose is the primary fuel for the brain, it is plausible to expect insulin to be an integral part of its homeostasis. But, for decades it was considered that glucose uptake in the brain is independent of insulin [3-5]. This notion has however been challenged by studies that reveal a more intricate role of insulin in the brain [6-8]. Additionally, new findings suggest a high concentration of insulin receptors (InR's) in various brain regions like hippocampus, cortex, hypothalamus, and olfactory bulb i.e. areas that modulate various aspects of behavior [6, 9]. Insulin mediates several different brain functions like neurotransmitter reuptake [10], glycogen metabolism [11], appetite control and satiety [12-14]. Also, locally synthesized insulin in the brain has been reported [15, 16]. The central prominence of diabetic complications can be estimated by the fact that Alzheimer's disease is now considered to be a type 3 form of diabetes [17]. Interestingly, Amyloid-β (Aβ), is cleared by insulin degrading enzyme (IDE), which means that during diabetes, impaired insulin signaling could be somehow associated with hippocampal accumulation of Aβ plaques leading to the development of Alzheimer's disease [18, 19].

Hippocampus, a brain regions involveds in learning & memory functions, and the prominent site for adult neurogenesis, is rich in InR's [20-22]. InR activation leads to synthesis and

translocation of glucose transporter (GLUT4), necessary for glucose uptake and hence maintaining energy homeostasis of the neuron. Studies suggest that diabetes is associated with reduced hippocampal neurogenesis [23-26]. Additionally, IR reduces glucose uptake, causing neuronal starvation and hence degeneration which ultimately translates to impaired neurobehavioral outcomes [27, 28]. Importance of central glucose homeostasis can be appreciated from the fact that although being about 2% of the body weight, brain utilizes approximately 60-70% of total body glucose [29]. This indicates that there exists a delicate relationship between diabetes, brain, and central insulin signaling, a critical research gap we address in our study.

Streptozotocin (STZ)-induced diabetes is a very cost-effective and expeditious technique that can be used in most strains of rodents. STZ is a broad-spectrum antibiotic that enters into the insulin-producing β cells of pancreatic islets via GLUT2 and causes DNA alkylation and eventual cell death [30-32]. The single high dose of STZ induces rapid and complete insulin deficiency resembling T1DM, while multiple lower doses cause limited injury to β-cells and can be used to develop T2DM [33].

The second part of our study comprises of inducing T2DM by a more natural way i.e. depression. Depression is a mood disorder, globally affecting more than 300 million people. Depression is characterized by agitation, restlessness and anger, irritability, social isolation, fatigue and lack of energy, hopeless and helpless feeling, worthless, self-hate, loss of interest or pleasure in activities that were once enjoyed, sleep-wake abnormalities, thoughts of death or suicide etc [34]. Depression is an aftermath of chronic and prolonged stress. Stress is a body's innate defense mechanism against day to day troubles. However, chronic and prolonged stress stems devastating effects on the body, especially brain. Mechanistic underlining comprises of consistently prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis, pumping excess of cortisol into the bloodstream [35-37]. HPA axis hyperactivity such as in chronic stress is known to induce neurological complications like neurodegeneration, reduced synaptic plasticity, and behavioral abnormalities like depression, anxiety, cognitive decline, dementia etc. [38, 39].

The chronic unpredictable stress model (CUS) is one of the best model to induce depressive-like behavior in rodents. CUS comprises of an array of unpredictable and mild stressors over a fixed period of time [40, 41]. CUS induces hyperglycemia and hypercortisolemia [42], thereby synergizing the neurodegenerative effects [43], translating to impaired

neurobehavioral outcomes such as memory impairment, depressive-like behavior and anxiety [44-47]. Hippocampus being rich in cortisol receptor becomes a direct target of cortisol toxicity during chronic stress.

Cortisol inhibits insulin secretion, and stimulates glucagon secretion, thereby inducing hyperglycaemic state [48, 49]. Stress-induced depression results in increased neurodegeneration and decreased hippocampal neurogenesis [50]. It has been demonstrated that glucocorticoid treatment induces neural cell cycle arrest [51] and apoptosis in neuronal progenitors and mature neurons [52]. Suppression of neurogenesis affects mood [53], fear conditioning, synaptic plasticity [54] and memory [55]. Additionally, chronic stress reduces insulin sensitivity and mediates IR (T2DM) [56]. Insulin-responsive GLUT4 is important in hippocampal glucose uptake [57] and is involved in learning & memory processes [58-60]. Further, InR in hippocampus and cortex is essential for neuronal plasticity and cognitive functioning. IR, such as observed during the depression, can thus reduce synaptic plasticity and alter neurobehavioral outcomes leading to complications like Alzheimer's disease [6]. Keeping these roles of insulin signaling in mind, we aimed to evaluate InR and its responsive GLUT4 in the hippocampus during chronic stress and diabetes-induced neurological complications.

In our previous reports, hydroalcoholic extract of *Urtica dioica* improved glucose intolerance and cognitive dysfunction of depressed [61] and diabetic mice [60]. HPLC analysis had revealed the high amount of rutin in *U. dioica* extract [62]. Therefore, we aimed to investigate the neuroprotective potential of rutin. We hypothesize that hippocampal insulin signaling might be critical in improving and/or reversing the neurobehavioral complications associated with diabetes.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Diabetes

Diabetes mellitus (DM), commonly referred to as diabetes is a complex metabolic disorder characterized by prolonged and consistent hyperglycemia. DM occurs when the pancreas can't produce insulin (type 1 diabetes), or when the body cells become insensitive to the insulin (type 2 diabetes). There are mainly three are types of diabetes viz Type 1, Type 2 and gestational diabetes.

A) Type 1 diabetes mellitus (T1DM)

It is characterized by failure of the pancreas to produce insulin, hence known as *Insulin-dependent diabetes mellitus (IDDM)*. Therefore, T1DM patients need insulin every day in order to control the blood glycemic levels.T1DM is often diagnosed in children, adolescents or young adults, therefore also known as juvenile diabetes. It currently affects 542,000 children with age up to 14 years. There is a 3% increase in the T1DM cases annually, which corresponds to almost 86,000 cases per year. T1DM is considered to be an autoimmune disorder attacking the β cells, but the exact underlying basis is not clear. T1DM is considered to have hereditary aspects. Some risk factors include a family history of diabetes, genetics, infections and other environmental influences. Symptoms of T1DM include feeling thirsty, hungry and tiredness, blurry vision, numbness or tingling in feet, weight loss, and excess urination. India is a second leading country in T1DM (Fig 2.1A) [1].

B) Type 2 diabetes mellitus (T2DM)

It is characterized by IR, a condition in which the ability of body cells to respond to insulin is compromised, in other words, *Non-Insulin-dependent diabetes mellitus (NIDDM)*. As the result of IR, cellular uptake of glucose is reduced leading to a glycemic build up in blood, also known as hyperglycemia. Some common symptoms of T2DM are polydipsia, polyuria, polyphagia, weight loss, fatigue and numbness in the extremities. Prolonged and chronic hyperglycemia leads to severe health complications like cardiac arrest, stroke, renal failure, amputation, blindness, and neuropathy, increasing the probability of premature death [63]. Approximately 90% of the diabetic cases belong to a T2DM category. Overweight and obesity, together with physical inactivity, are the strongest risk factor for T2DM. Other risk factors include genetic, metabolic, ethnicity, unhealthy diet, smoking etc. Once again India is the second leading country in T2DM too (Fig 2.1B) [1].

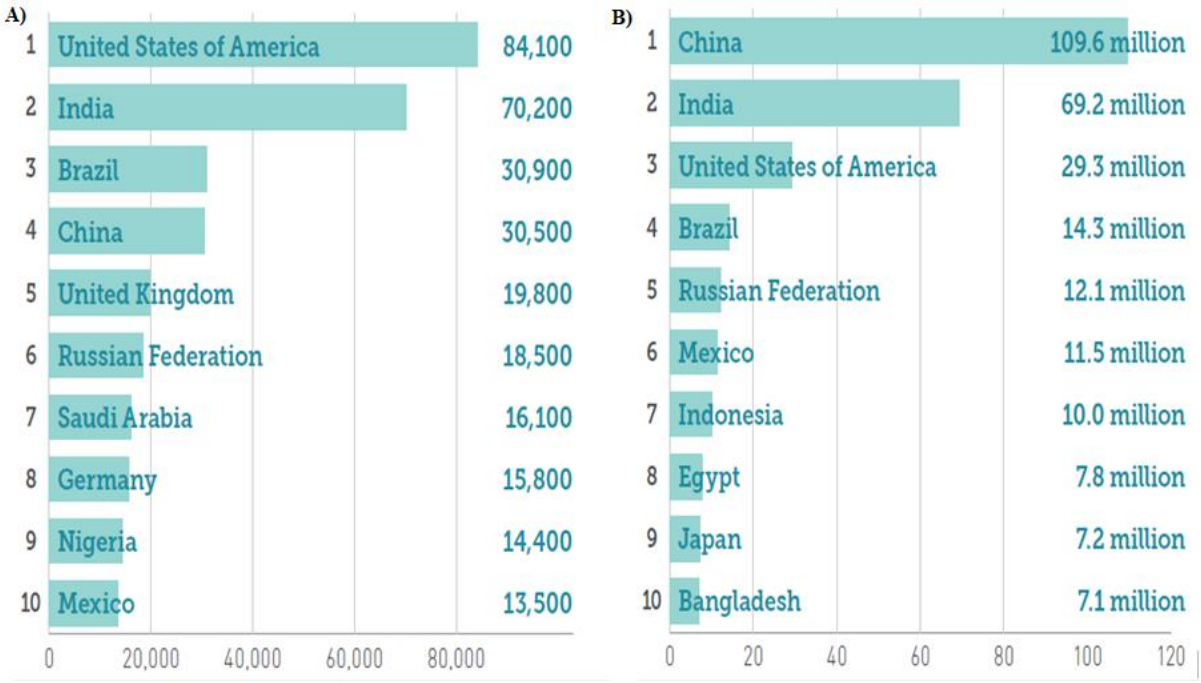


Figure 2.1: World's leading countries with **A)** type 1 diabetes (0-14 years), and **B)** type 2 diabetes [International Diabetes Federation Atlas, 2015]

C) Gestational Diabetes Mellitus (GDM)

GDM occurs during the third trimester of the pregnancy and is injurious to both mother and the fetus. GDM is managed by anti-diabetic drugs, and its symptoms usually disappear after pregnancy. GDM leads to increased risk for development of T2DM for both mother and child [1].

2.2 Diabetes: Global Facts

Diabetes currently affects approximately 415 million adults or 1 in 11, which is expected to rise to 642 million or 1 in 10 adults by 2040. Diabetes was responsible for 5 million deaths in 2015 which translated to 1 death in every 6 seconds. Additionally, diabetes affected approximately 20.9 million live births 2015 (1 in 7 births). Surprisingly, approximately 50% of the diabetics remain undiagnosed. Diabetes accounts for 673 billion USD in health expenditure in 2015 – 12% of global health expenditure. T1DM currently affects 542,000 children. Table 2.1 and figure 2.2 show the global impact of diabetes. Table 2.2 shows the Indian scenario of diabetes [1].

Table 2.1: Global epidemiology of diabetes. [International Diabetes Federation Atlas, 2015]

	2015	2040
Total world population	7.3 billion	9.0 billion
Adult population (20-79 years)	4.72 billion	6.16 billion
Child population (0-14 years)	1.92 billion	-
Diabetes (20-79 years)		
Global prevalence	8.8% (7.2-11.4%)	10.4% (8.5-13.5%)
Number of people with diabetes	415 million (340-536 million)	642 million (521-829 million)
Number of deaths due to diabetes	5.0 million	-
Health expenditure due to diabetes (20-79 years)		
Total health expenditure, R=2* 2015 USD	673 billion	802 billion
Hyperglycaemia in pregnancy (20-49 years)		
Proportion of live births affected	16.2%	-
Number of live births affected	20.9 million	-
Impaired glucose tolerance (20-79 years)		
Global prevalence	6.7% (4.5-12.1%)	7.8% (5.2-13.9%)
Number of people with impaired glucose tolerance	318 million (212.2-571.6 million)	481 million (317.1-855.7 million)
Type 1 diabetes (0-14 years)		
Number of children with type 1 diabetes	542,000	-
Number of newly diagnosed cases each year	86,000	-

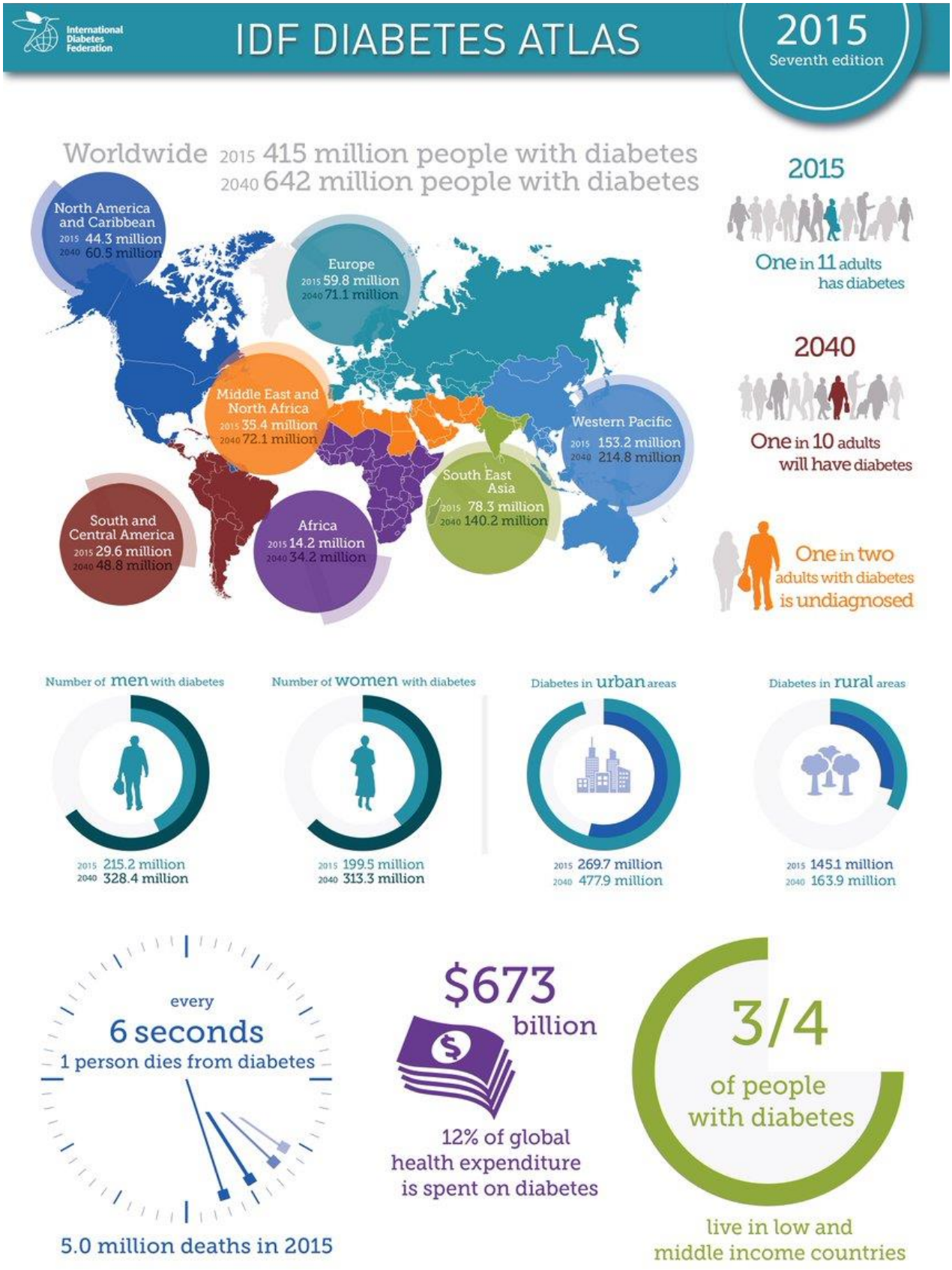


Figure 2.2: Global impact of diabetes [International Diabetes Federation Atlas, 2015].

Table 2.2: India at glance [International Diabetes Federation Atlas, 2015].

	2015	2040
Diabetes (20-79 years)		
Country prevalence	8.7% (7-10.6%)	10.9% (8.8-13.3%)
Age adjusted comparative prevalence	9.3% (7.6-11.4%)	10.1% (8.1-12.3%)
Number of people with diabetes	69 million (56-84 million)	123 million (99-150 million)
Number of people with undiagnosed diabetes	36 million (29-44 million)	64 million (51-78 million)
Proportion of undiagnosed cases	52.1	-
Number of deaths due to diabetes	1.0 million	-
Health expenditure due to diabetes (20-79 years)		
Total health expenditure, R=2* 2015 USD	6.5-11.0 billion	11.7-20.0 billion
Hyperglycaemia in pregnancy (20-49 years)		
Number of live births affected	5.9 million	-
Impaired glucose tolerance (20-79 years)		
Country prevalence	4.7% (2.3-6.7%)	5.5% (2.4-5.6%)
Number of people with impaired glucose tolerance	36 million (17.5-51.8 million)	63 million (29.08-82.8 million)
Type 1 diabetes (0-14 years)		
Number of children with type 1 diabetes	70,200	-

2.3 Anti-diabetic Drugs and Their Limitations

Currently used therapeutics for diabetes management are multiple targets oriented like stimulating insulin production, reducing glycogenolysis, improving insulin sensitivity, reducing carbohydrate absorption from GIT, increasing glucose excretion from urine. Other than insulin and its analogs, which is the only treatment for T1DM and in later stages of T2DM, table 2.3 mentions the list of currently used medication for diabetes along with their mechanism of action and adverse effects [64, 65]. Although efficient in controlling hyperglycemia current therapeutic approaches suffer from adverse effects such as hypoglycemia, cardiovascular complications, seizers, pre-eclampsia, eclampsia, congenital abnormalities, and even prenatal death (table 2.3). Additionally, anti-diabetic drugs have to be taken daily, throughout the life, which exerts a tremendous amount of financial and mental stress to the patients and their caretakers. Further, resistance develops against these drugs with time, especially insulin, and higher doses are eventually required, thereby escalating the adverse effects.

Since half of the cases in T2DM remain unnoticed, a large amount of damage has already been conferred to the body before a diagnosis is achieved. Current therapeutics manage to slow but fail to halt or reverse the progression of diabetic complications, especially CNS neuropathy. T2DM induced CNS neuropathy includes neurodegeneration, cognitive decline, learning and memory dysfunction, anxiety, depression, and neurological disorders like Parkinsonism and Alzheimer's. Hence, CNS complications of T2DM continues to be highly devastating by severely compromising the quality of life. Therefore, the need of the hour is to develop some alternative treatment strategies to make T2DM treatable.

Table 2.3: Currently used medications for diabetes along with their mechanism of action and adverse effects.

Class	Name	Mechanism of action	Adverse effects
Sulfonylureas	Gliclazide Glimepiride Glyburide	Stimulate the pancreas to produce more insulin	Hypoglycemia
Meglitinides	Nateglinide Repaglinide	Stimulate the pancreas to produce more insulin	Hypoglycemia
Biguanides	Metformin	Reduce the production of glucose by the liver	Diarrhoea, metallic aftertaste, nausea
Thiazolidinediones (TZD)	Pioglitazone Rosiglitazone	Increase insulin sensitivity of the body cells and reduce the production of glucose by the liver	Edema, weight gain *Pioglitazone :* increased risk of bladder cancer *Rosiglitazone :* Increased risk of heart attack
α-glucosidase inhibitor	Acarbose Miglitol Voglibose	Slow the absorption of carbohydrates (sugar) ingested	Bloating and flatulence
Dipeptidyl-peptidase-4 (DPP-4) inhibitors	Linagliptine Saxagliptine Sitagliptine Alogliptine	Intensify the effect of incretins, involved in the control of blood sugar	Pharyngitis, headache
Glucagon-like peptide-1 (GLP-1) agonist	Exenatide Liraglutide Dulaglutide	Mimic the effect of incretins	Nausea, diarrhea, vomiting
Sodium glucose co-transporter 2 (SGLT2) inhibitors	Canaglifozine Dapagliflozine Empagliflozine	Help eliminate glucose in the urine	Genital and urinary infections, more frequent urination

2.4 Diabetes, Brain and the Central Insulin Signalling

Diabetes inflicts severe damage to the CNS, commonly referred to as diabetes associated neurological complications. These primarily include neurodegeneration, learning and memory impairment, anxiety, depression, and neurological disorders like autism, Alzheimer's and Parkinsonism [66-69]. Currently, diabetes is being reviewed as a critical risk factor for the development of cognitive deficits, dementia, and depression [68-70]. T2DM associated central pathology is linked to the increased oxidative and inflammatory stress in the brain [46, 71]. T2DM causes various structural and functional abnormalities in the brain, including neurodegeneration, blood-brain barrier (BBB) disruption, organelle dysfunctioning (mitochondria, endoplasmic reticulum, cytoskeleton, and nucleus) etc. [2]. Figure 2.3 depicts the pathophysiology of diabetes associated neurological complications. Prolonged hyperglycemia aggravates the oxidative stress, microangiopathy, and endothelial dysfunction. With time, diabetes leads to secondary complications like, depression, aging, IR, insulin deficiency, hypoglycemia, hypertension, dyslipidemia etc., which accelerates the process of cognitive decline and dementia. [72, 73].

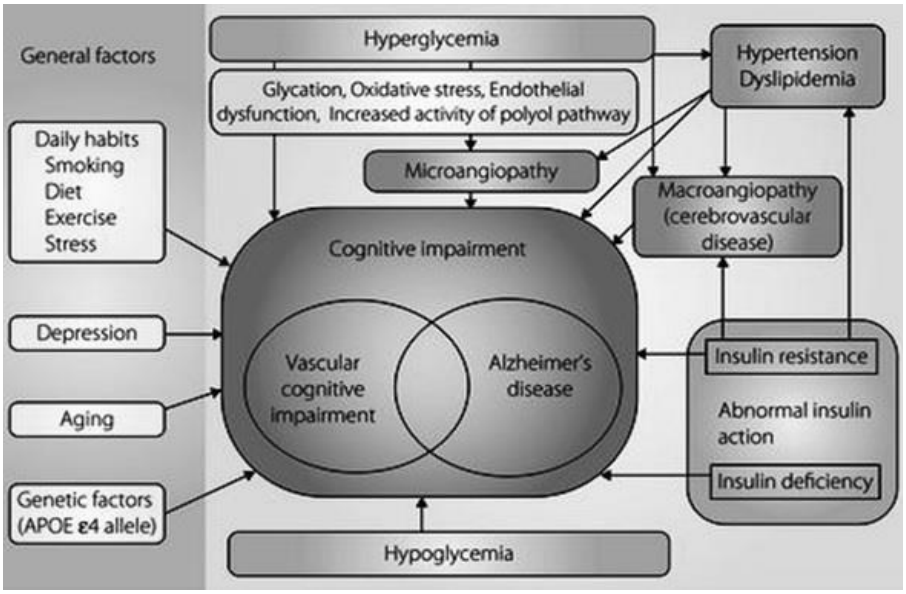


Figure 2.3: Pathophysiological pathways leading to the development of cognitive dysfunction from hyperglycemia, depression, and other factors [74].

Since glucose is the primary fuel for the brain, it is plausible to expect insulin to be an integral part of its homeostasis. But, for decades it was considered that glucose uptake in the brain is independent of insulin [3-5], which makes sense because brain being the master

organ needs to be prevented from hyperglycemia. Hence, insulin independence could be brain's innate defense mechanism. This notion has however been challenged by studies that reveal a more intricate role of insulin in the brain [6-8]. Recent findings suggest a high concentration of InR's in various brain regions like the hippocampus, cortex, hypothalamus and olfactory bulb that modulate various aspects of behavioral outcome [6, 9]. Insulin mediates several different brain functions like neurotransmitter reuptake [10], glycogen metabolism [11], appetite control and satiety [12-14]. Also, locally synthesized insulin in the brain has been reported [15, 16]. The central prominence of diabetic complications can be estimated by the fact that Alzheimer's disease is now considered to be a type 3 form of diabetes [17]. Interestingly, Amyloid-β (Aβ), is cleared by insulin degrading enzyme (IDE), which means that during diabetes, impaired insulin signaling could be somehow associated with hippocampal accumulation of Aβ plaques leading to the development of Alzheimer's disease [18, 19]. Hippocampus, as brains regions involved in learning & memory functions, and the prominent site for adult neurogenesis, is rich in InR's [20-22]. InR activation leads to synthesis and translocation of GLUT4 necessary for glucose uptake and hence maintaining energy homeostasis of the neuron. Further, studies suggest that diabetes was associated with reduced hippocampal neurogenesis [23-26]. Additionally, IR during T2DM lowers glucose uptake, causing neuronal starvation and hence degeneration which ultimately translates to impaired neurobehavioral outcomes [27, 28]. This indicates that there exists a delicate relationship between diabetes, brain, and central glucose homeostasis, a critical research gap we address in our study.

Approximately half the cases of T2DM remain undiagnosed [1], which is a serious matter because up till diagnosed, a great deal of damage has already been conferred to the body. Additionally, current therapeutics are designed for peripheral glucose clearance and no one accounts for the central glucose homeostasis, even-though brain (2% of the body weight) utilizes 60-70% of body's glucose [29]. This is why despite the glycemic control, progression of neurological complications remains unhalted. This progression might be slow, which is why it remains unnoticed and to this date understudied, and later on translates to cognitive decline, dementia, depression, anxiety, autism and neurological disorders like Alzheimer's and Parkinson's. So the research gap we address in our study is to understand the central glucose homeostasis via insulin signaling and how it may interfere with disease progression. With the constantly changing threat matrix of diabetic complications, there is a need to come

up with new therapeutic management strategies, and brain-targeted therapeutics could be the one to provide a better life for diabetic patients.

2.5 Depression-A Risk Factor for Diabetes

Depression is a common mental disorder characterized by diminished interest, depressed mood, feelings of guilt or worthlessness, reduced energy, self-hate, appetite and/or sleep disturbances, and mood swings of frustration, anger, and sadness. Chronic and frequent occurrences of these complications significantly impair an individual's ability of self-care and day to day activities. Depression may be a result of alcohol or drug abuse, medical complications, abnormal sleeping patterns, stressful life events, such as the death of someone close, divorce, loneliness (common in the elderly), relationship breakup etc. [34].

Depression is a major contributor to the global health burden and affects nearly everyone at some point in life. Depression affects approximately 350 million people globally. According to a WHO survey prevalence of depression varies from 1.6-26.3% [75]. Depression is the leading cause of suicide with almost 1 million deaths per year [76]. This is why depression is leading worldwide in terms of total years lost due to disability [76].

Neurological deficits and diabetes have a strong association with depression, in addition to mood, motor, autonomic, endocrine and sleep-wake abnormalities [69, 77, 78]. Prevalence of comorbid diabetes and depression is twice as compared to individual occurrence [70]. Both depression and diabetes are potential risk factors for each other and may share a common underlying pathology, such as increased HPA activity, bad dietary habits, sedentary lifestyle, and environmental and cultural risk factors. Together, diabetes and depression complicate, worsen and amplifying the clinical outcomes of each other [79]. Financially, the occurrence of diabetes and depression is an enormous burden on an individual and the health economies, without a guarantee on improvement in either disease or quality of life [80].

A meta-analysis study with diabetic subjects reported a 28% prevalence of depression in women, and 18% in men [69, 70]. Another study reported that about 45% of diabetics had undiagnosed depressive symptoms [81]. Further, glycated hemoglobin (HbA1c) levels showed high correlation with the clinical depression (Fig 2.4), suggesting a higher risk of the development of diabetes in depressive [69].

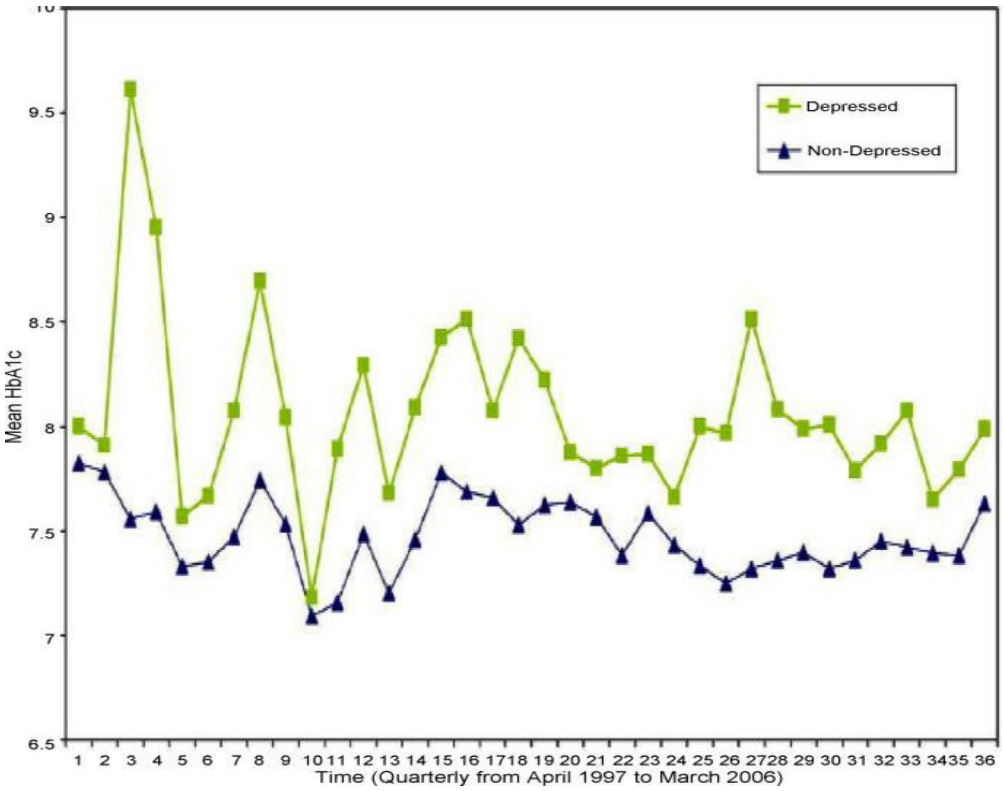


Figure 2.4: Relationship between HbA1c levels and depression [69]

The role of diabetes and depression in development of neurological impairments is well established. According to a study, approximately 45% of depressed and diabetic patients had cognitive deficits [82]. Literature suggests that there exists a bi-directional association between depression and diabetes [83, 84]. A meta-analysis study reported that depression led a 37 % increased risk of developing T2DM [85]. Similarly, baseline diabetes led to a 15% increased risk of developing depression [83].

The body undergoes many physiological alterations during the depression. Various tissues, hormones, neurotransmitters, and cytokines work together in order to rescue a stress response to maintain homeostasis [86, 87]. The most important anatomical structures of the brain involved are the hypothalamus, pituitary and the adrenal gland, constitute the HPA axis. Depression is associated with the hyperactivity of the HPA axis [36, 37], which results in increased cortisol levels and hence inflammation, oxidative stress, brain atrophy etc. Hippocampus being rich in cortisol receptors is more vulnerable to excitotoxic neurotransmitter such as glutamate during the depression, a direct neurodegenerative effect of cortisol. Hyperactivation of HPA axis induces neurodegenerative process, reduces neurogenesis and leads to cognitive dysfunction [35, 53]. Additionally, prolonged elevation

of cortisol inhibits insulin secretion, stimulates glucagon secretion, decreases body weight and induces T2DM like state [48, 49].

Clinically, depression is known to increase the risk of pre-diabetes and T2DM [84, 88-90]. It has been hypothesized that increased risk of T2DM in depressive patients is believed to be the result of increased counter-regulatory hormone mechanism, alterations in glucose transportation and increased inflammation [91]. These physiological changes are thought to contribute to IR and pancreatic β-cell dysfunction in T2DM. Further, patients with co-morbid depression and diabetes have higher hazard ratio (Fig 2.5).

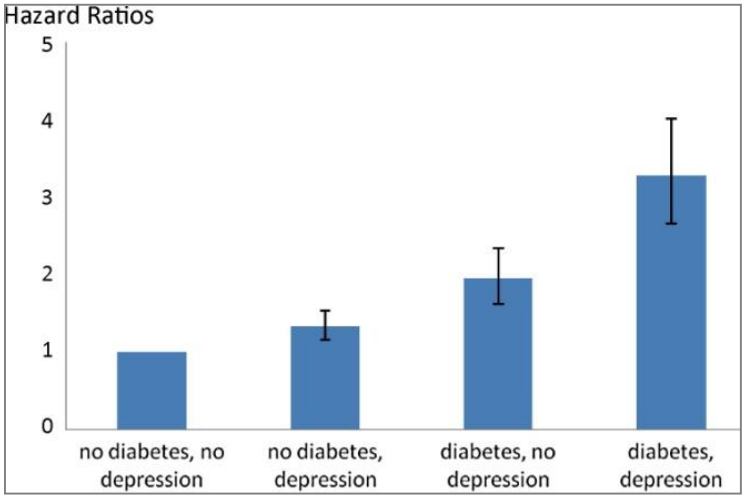


Figure 2.5: Effect of depression and/or diabetes on all-cause mortality [92].

In animal models, CUMS induced depressive phenotype induces significant hyperglycemia, glucose intolerance, hypercorticosteronemia, cognitive deficits, immunosuppression, and hypoinsulinemia [42, 78, 93].

Insulin acts as a growth factor in the brain by activating the dendritic sprouting, regeneration, and proliferation of stem cells, especially in the hippocampus. Impairment of insulin signaling in hippocampus might facilitate the development of Alzheimer's disease [94]. The relationship between mental illness and diabetes has been recognized for many years [79]. Diabetes was once described as “a consequence of prolonged sorrow” [95]. Nevertheless, it is a frequently ignored yet vital component of holistic diabetes care. Comorbid diabetes and depression is a challenging and under recognized clinical problem. Depressive symptoms affect up to one-third of people with diabetes and not only impair quality of life but also add to the difficulties experienced in diabetes self-management. It is incumbent on healthcare professionals to identify depression in people with diabetes when present and then treat this

rapidly and effectively in order to achieve the best clinical outcomes for these individuals. Most health services are poorly equipped to deal with comorbidity and, therefore, novel care pathways are needed to address this important public health problem.

2.6 Animal Models of Diabetes

Animal models have been extensively used in diabetes research. In the year 1889, Oskar Minkowski and Joseph von Mering removed the pancreas of a dog, leading to the development of diabetes mellitus. This experiment was extended by Frederick Banting and Charles Best by isolating insulin from the pancreas and administering to diabetic patients, thus paving a new era in diabetes treatment [Reviewed in [96]]. Most of the experimental work in diabetes is carried out in rodents [97, 98]. Table 2.4 enlists various animal models of diabetes research.

Table 2.4: Animal models for diabetes

S. No.	**Class**	**Animal models for T2DM**	
		Obese model	**Non-obese model**
1.	Genetic model	*ob/ob* mouse, *db/db* mouse, KK mouse, KK/A^y mouse, NZO mouse, TSOD mouse, M16 mouse Zucker fatty rat, ZDF rat, SH/N-cp rat, JCR/LA-cp rat, OLETF rat Obese rhesus monkey	Cohen diabetic rat, GK rat, Torri rat Non obese C57BL/6 mutant mouse, ALS/Lt mouse
2.	Diet induced T2DM	Sand rat, C57/BL 6J mouse, Spiny mouse	-----
3.	Chemical induced diabetes	GTG treated obese mouse	ALX induced diabetes STZ induced diabetes

4.	Surgical diabetic models	Ventromedial hypothalamus lesioned dietary obese diabetic rat	Partial pancreatectomized animals (rat, dog, pigs, dogs etc.)
5.	Transgenic/knock-out diabetic animals	β_3 receptor knockout mouse, Uncoupling protein (UCP1) knock-out mouse	Knock-out mice involving insulin, InR and its components of downstream cascade such as IRS1/2, GLUT4 etc., PPAY-γ knock-out mice, Glucokinase and GLUT2 gene knock-out mice, etc.

2.7 Streptozotocin (STZ) Induced Diabetes Model

STZ is an antibiotic derived from *Streptomyces achromogenes*. It's structurally similar to the glucosamine derivative of nitrosourea. It was back in 1963 when the diabetogenic property of STZ was identified in dogs and rats [99]. The molecular underlining of its diabetic effect is that it destroys the pancreatic β-cells [30, 100]. The deoxyglucose moiety is responsible for crossing cellular membrane, while nitrosourea moiety causes the destruction of pancreatic β-cells. STZ alkylates and breaks the DNA strands along with increase in the activity of poly-ADP-ribose synthetase, an enzyme responsible for depletion of nicotinamide adenine dinucleotide (NAD) in β-cells, resulting in energy deprivation and cellular death [30-32]. It has been reported that STZ induces both T1DM and T2DM [101, 102]. Experimental evidence suggests that high doses of STZ induce rapid and complete insulin deficiency resembling T1DM. However, multiple lower doses of STZ, which cause partial destruction of β-cells, can be used to produce type 2 diabetes [33]. In STZ treated mice, changes in spinal terminals of calcitonin gene-related peptide in sensory neurons were observed 4 weeks after diabetes and progressively worsened with time (6-7 weeks) [103]. With increasing duration of diabetes from 7 to 9 weeks, there is a loss in cutaneous C-fiber innervations [104] and

decrease in nerve conduction velocity as well as hypoalgesia [105, 106]. Besides, animals with STZ induced chronic diabetes showed depressive-like behavior and significant hypolocomotion with respect to control animals [107]. In another study, STZ induced diabetic animals showed cognitive dysfunction in a spatial version of the Morris water maze test. It has been suggested that STZ exacerbates cognitive ability in animals by down-regulating the expressions of BDNF and cAMP responsive element binding protein and by inducing hippocampus neuronal apoptosis [108].

2.8 Chronic Unpredictable Stress (CUS) Model of Depression

CUS also known as the chronic intermittent or variable stress model, is an extensively used rodent model to study depressive-like behavior. This model was developed by Paul Willner [40, 41]and it consists of the repeated exposure to an array of unpredictable stressors over a varying period of time (1.5-8 weeks). In human life, chronic exposure to unpredictable and uncontrollable stressors is often said to be a vital component in the development of depressive disorders [109, 110]. Similarly in rodents, chronic exposure severe stressors resulted in induction of marked depression as observed by reduced physical activity and non-acceptance of palatable rewards [41]. This non-acceptance and of a reward is known as anhedonia, a core clinical symptom of major depression. This paradigm of unpredictable micro stressors induce similar features of clinical depression in the rodents, making it a reliable model for depression [111]. The validity of this model was justified by the reversion of anhedonia by antidepressant drugs, mimicking the time course required for clinical effectiveness [112, 113].

Last few decades have witnessed a change in the endpoints of this model beyond reward salience. For example, CUS decreases the frequency of male sexual and aggressive behaviors, increases immobility in the forced swim and learned helplessness test, increases rapid eye movement sleep latency and reduces self-care [114].

Another important variable in this model is the time duration of CUS. Initially, the CUS paradigm was developed as an 8-week paradigm consisting of 3 weeks of initial stress exposure without any drug treatment followed by another 5 weeks of stress along with antidepressant therapy [113]. However, the most robust and reliable effects of CUS are observed on as early as 10 days and are present up till 3 weeks of CUS [111]. For example, 21 days of unpredictable mild foot shock stress induced significant glucose intolerance,

hyperglycemia, hypercorticosteronemia, immunosuppression, gastric ulcerations, cognitive deficits and depression in rats [42, 78].

Another critical parameter of this model is the precise post-CUS time interval to be considered for behavioral and/or molecular analysis. Usually, the examination is done on the last day of CUS to study the peak effect, because a longer rest period post-CUS exposure will start the recovery phase and diminished residual effects of stress [115, 116]. This recovery highlights the importance of consistency and critical time point standardization in experimental methodology. In addition, longer rest periods, such as 2 weeks post-CUS or longer, can be used to understand that how much long-lasting the effects of CUS and/or antidepressant therapy are present on specific outcome measures [111].

2.9 Rutin

Rutin, composed of quercetin and the disaccharide rutinose (rhamnose and glucose), is a flavonol glycoside widely distributed in plants (Fig 2.6). Its common name derives from *Ruta graveolens* a plant that contains high amounts of rutin, however other names such as rutoside, quercetin-3-O-rutinoside or sophorin have also been used.

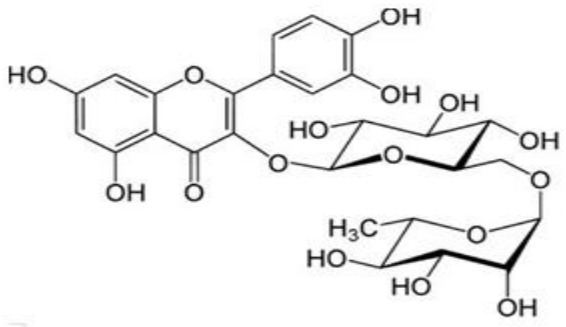


Figure 2.6: Structure of rutin

Rutin's biological role in plants relates mostly to its protection against UV-B radiation as the positive correlation between exposure to UV-B and the synthesis of rutin has been observed [117]. Interestingly, the leaves at the top of the plant contain more rutin than the lower leaves. It appears that factors such as geographic locations (high altitude) and even the position of leaves on the plant can determine the rutin content and thus the therapeutic efficacy of a plant.

Traditional and folk medicine have used rutin-rich plants for centuries in the form of beverages or foods. Today, due to its versatile properties, rutin has been found as a constituent in over 130 registered medicinal preparations [118]. It exhibits numerous significant benefits including anti-oxidant, anti-inflammatory, cardiovascular and neuroprotective effects, and anti-diabetic and anticancer activities [119, 120].

Rutin is found in many foods like tartary buckwheat seeds, asparagus, red pepper, apples, cherries, aronia berries and citrus fruits, among others and its abundance is characteristic for the inflorescence and leaves of many herbs such as rue, rosemary, dandelion or sage, and black and green tea are rich sources of rutin [121-124].

Neuroprotective Effects of Rutin

There have been numerous studies showing the benefits of rutin in supporting healthy brain and nervous tissue function. This is partially related to the fact that most of the neuropathology has been associated with oxidative stress and brain inflammation, followed by neurodegeneration and neuronal cell death. One example is epilepsy, which is a chronic disorder characterized by recurrent, unprovoked seizures [125]. In an animal model of seizures, rutin administration (intracerebroventricular injection) showed a dose-dependent reduction in number and severity of seizure onsets [126]. Also in chronic cerebral hypoperfusion (reduction in cerebral blood flow), which is a causative factor for the development of cognitive decline and dementia in the elderly, there was a marked improvement in cognitive function along with alleviation of oxidative, inflammatory and neuronal damage in rats supplemented with rutin [127].

Memory loss is a characteristic in patients with Alzheimer’s disease. It is believed that neurodegenerative progression is caused by extracellular Aβ plaque formation [128]. In test tube experiments, rutin has been shown to decrease Aβ aggregation and cytotoxicity along with attenuation of oxidative stress and inflammatory response. Moreover, oral rutin supplementation in animals resulted in a significant reduction in memory deficit as well as increased activation of antioxidant defense mechanisms and inhibition of brain inflammation [128, 129].

Parkinson's disease (PD) is another neurodegenerative condition that can be controlled by rutin. Symptoms of PD are because of the dopaminergic neurodegeneration in the substantia nigra that progressively impairs motor ability. In both *in vitro* and animal studies, rutin

pretreatment showed a significant protection against neurotoxic effects of oxidopamine (a substance used to destroy dopaminergic neurons) [130, 131]. Rutin significantly decreased the level of reactive oxygen species [132] and promoted survival mechanisms in neurons through down-regulation of the apoptotic genes (promoting cell death) and up-regulation of the anti-apoptotic genes [131, 132]. It was also found that rutin up-regulated the tyrosine hydroxylase (TH) gene, which is important in dopamine biosynthesis [131]. All of these findings indicate the need for further research and ignite hope for patients dealing with neurodegenerative conditions.

CHAPTER 3

OBJECTIVES

3. OBJECTIVES

Based on the neurological complications of diabetes, and neuroprotective effects of rutin as discussed previously, above mentioned literature, we divided our work broadly into two objectives discussed hereafter.

1. To study the effect of rutin on diabetes associated neurobehavioral outcomes using streptozotocin-induced diabetes model.
2. To study the effect of rutin on chronic unpredictable stress-induced depression as a risk factor for diabetes associated neurobehavioral outcomes.

CHAPTER 4

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Animals

Male Swiss albino mice (25-30 gm; 8-10 weeks old) were housed under a 12 h light/dark cycle (the lights were on from 7 a.m. to 7 p.m.) at 26 ± 2 °C, with access to food and water *ad libitum*. All animal experimentation was conducted in obedience with the Institutional Animal Ethical Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. All possible measures were taken to minimize the suffering of animals. Body weight and feed & water intake were measured consistently throughout the study.

4.2 Experimental Design of STZ Study

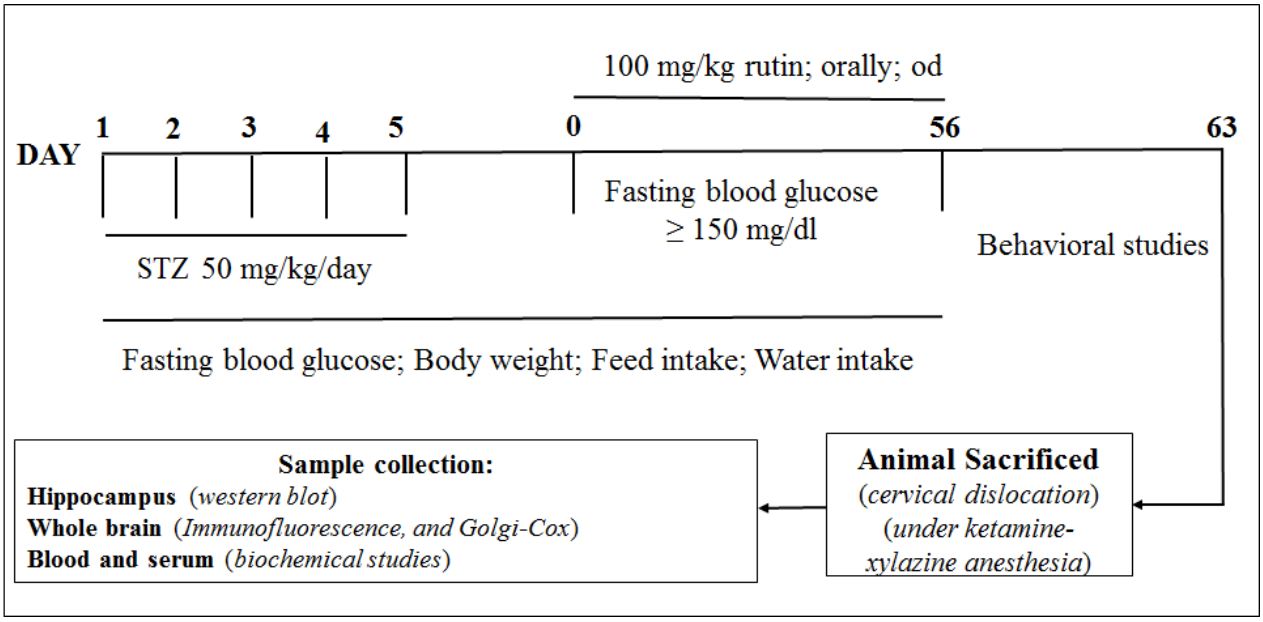


Figure 4.1: Experimental design for streptozotocin (STZ) induced diabetes study.

T2DM was induced using multiple low dose STZ model [33] (Fig 4.1). Animals were divided into following groups and treated as follows (n = 6-10):

Group I	Control; [administered vehicle (0.3 % carboxymethyl cellulose)]
Group II	Control + Rutin (R); (administered 100 mg/kg rutin in vehicle)
Group III	STZ; (administered vehicle)
Group IV	STZ + R; (administered 100 mg/kg rutin in vehicle)

Animals were injected with STZ (50 mg/kg, i.p.) for five successive days. The animals which showed the fasting blood glucose level ≥ 150 mg/dl were divided in group III and IV. Rutin was administered through oral gavage once daily till 56th day. After that, behavioral studies were conducted, following which the animals were euthanized; hippocampus was isolated, blood and serum were collected and stored for further studies.

4.3 Experimental Design of CUS Study

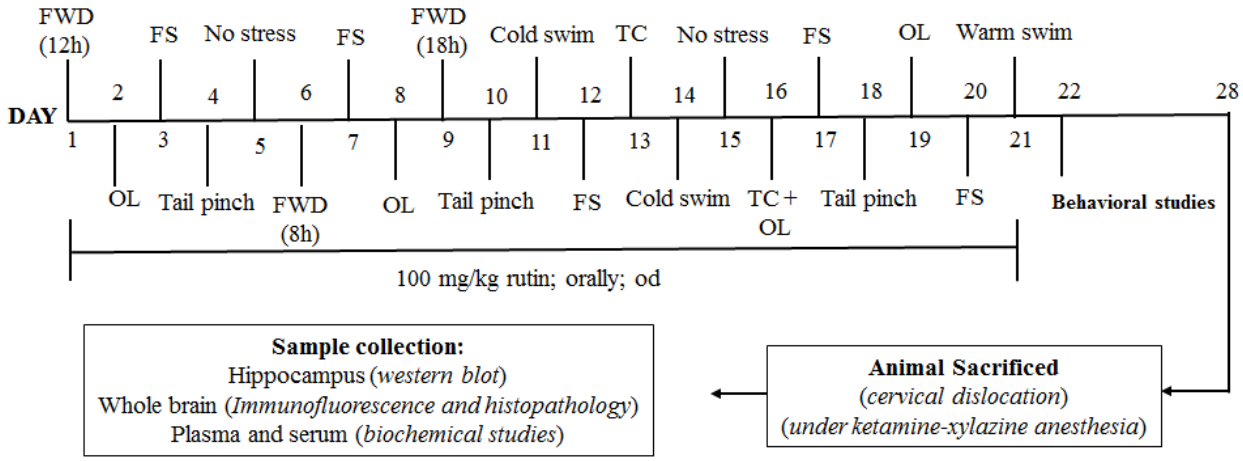


Figure 4.2: CUS procedure and experimental design. FWD: food and water deprivation (8–18 h); OL: overnight illumination; FS: foot shock (0.2 mA; 4 time in 2 min interval); tail pinch (5 min); cold swim: 3 min at 10 °C; TC: tilted cage at 45° and warm swim: 5 min 24 °C.

Animals were divided into groups and treated as follows (n = 6-8):

Group I	Control; [administered vehicle (0.3 % carboxymethyl cellulose)]
Group II	Control + Rutin (R); (administered 100 mg/kg rutin in vehicle)
Group III	Chronic Unpredicted Stress (CUS); (administered vehicle)
Group IV	CUS + R; (administered 100 mg/kg rutin in vehicle)

A 21-day CUS paradigm with some modifications (Fig 4.2) was imposed on the animals in group II and III [133, 134]. Stressors were given randomly at any time of the day, after which animals were subjected to behavioral profiling for depression, anxiety, locomotion, and cognition. After behavioral studies, the animals were euthanized; plasma and serum were collected for biochemical analysis. Immediately after cervical dislocation, the hippocampus was dissected and frozen at -80 °C for molecular studies.

4.4 Behavioral Analysis [135]

4.4.1 Locomotion and Muscle co-ordination

A) Open Field Test (OFT)

Animals were placed at the center of the open field apparatus [50 × 50 × 25 (h) cm] and were allowed to explore the arena for 10 minutes. Apparatus was cleaned with 70% ethyl alcohol between consecutive test sessions. The entire session was video-recorded and was analyzed later for a total number of line crossings.

B) Beam Walk

The beam walking apparatus consisted of two 60 cm metal beams with 6 and 12 mm width, suspended 1 m above a soft surface. The animals were trained on 12 mm beam 24 h before the test to enter a dark box parting from an inclined spot, having to cross the entire length of the bar to complete the task. The test was performed on 6 mm beam to evaluate the balance and the locomotor activity of the animals post CUS procedure. The time taken by the animal to cross the bar was counted (with a cut-off time of 1 min).

4.4.2 Depression

A) Forced swim test (FST)

The animals were subjected to a 6 min forced swim in a cylindrical tub (radius 24 cm and height 25 cm) filled with water (26±2 °C) to a height of 18 cm. The total immobility during the test was noted, and an animal was considered immobile whenever it remained floating passively without any movement in the limbs and its nose just above the water surface [136].

B) Tail suspension test (TST)

The animals were suspended 60 cm above the ground by an adhesive tape placed approximately 1 cm from the tip of the tail. The total immobility during the 6 min test was noted, and an animal considered immobile when it hung passively and completely motionless [137].

C) Sucrose preference test (SPT)

SPT was conducted as described previously [138]. Briefly, mice were habituated for 48 hours to 1% sucrose, and following a 4 hr deprivation, the preference for sucrose (1%) or water (identical bottles) was determined for 1 h. Sucrose habituation was performed during baseline but not during CUMS. Sucrose preference was determined regularly and calculated using formula.

Sucrose preference (%) = [Sucrose intake / (Sucrose intake + Water intake)] x 100

4.4.3 Anxiety

A) Elevated Plus Maze (EPM)

The EPM consisted of two open (30 × 5 cm) and two closed arms (30 × 5 cm, surrounded by 15 cm high walls). The apparatus was elevated 40 cm above the floor. Mice were placed in the center and allowed to explore the maze for 3 min. Total time spent by animals in the open and closed arm was calculated.

B) Open Field Test (OFT)

Animals were placed at the center of the open field apparatus [50 × 50 × 25 (h) cm] and were allowed to explore the arena for 10 minutes. Apparatus was cleaned with 70% ethyl alcohol between consecutive test sessions. Complete experiment was recorded and analyzed for total entries, and time spent in the central region of the apparatus.

4.4.4 Learning & Memory

A) Novel Object Recognition (NOR)

NOR paradigm was used to assess the recognition memory. Animals were placed in the center of the open field with an object A for 10 min. After 24 hrs, animals were placed in the open field, and 5 min later, two objects, object A (now familiar) and object B (novel), were placed diagonally in the chamber. Time spent exploring the objects was recorded for 10 mins, and discrimination index was evaluated [33].

B) Morris water maze (MWM) task

MWM task was used to assess the effect of diabetes, depression and rutin treatment on spatial memory. MWM consisted of a circular tank with 100 cm radius. The pool was filled with water at room temperature and divided in to four hypothetical quadrants with one of them having a hidden escape platform submerged 1 cm below water level. Each mouse was given habituation trial for 5 mins to familiarize with the maze without the platform. In training trials, each mouse was released at each of the quadrants facing the wall of tank and allowed to find the platform for 60sec, failing which it was guided to the platform at kept there for 5-10 sec for identifying the spatial cues associated with the platform. Such training trials were given twice day (morning/evening) for four days. Escape latency, time taken by the mouse to find the hidden platform, was recorded for each trial. Memory index was evaluated on the 5^{th} day, in absence of platform, by probe trial test, in which the number of crossings over the platform and time spent in platform quadrant was calculated over a period of 60 sec [139].

C) Passive avoidance step-through (PA-ST) task

PA-ST paradigm was employed to assess the effect of diabetes, depression and rutin treatment on short and long-term associative memory. Apparatus consisted of a box partitioned into light (10 cm × 14 cm × 16 cm) and dark (10 cm × 10 cm × 16 cm) chambers. The light compartment was painted white from inside and lighted up with 60 W bulb, kept 60 cm above the apparatus. Dark chamber was painted black and consisted of a metal grid floor to transfer an electric shock. The chambers were separated by a small guillotine door (5 × 5 cm) in a way that allowed free movement of animals. All mice were allowed to habituate with the compartments for 120 s. On day 1 of learning trial, step through latency was calculated by observing the time taken by mice to enter the dark chamber when placed in the light chamber. Once they entered the dark chamber, the door was shut and an electric shock (0.5 mA) for 2 sec was given. Short and long term memory retention was evaluated through step through latency on day 2 and day 7 respectively, in absence of shock [139].

D) Passive Avoidance Step-Down (PA-SD) Test

PA-SD is also used to assess long term and short term memory retrieval. The apparatus consisted of a wooden chamber (50 × 50 × 15 cm) with a metal grid floor to transfer an electric shock (0.5 mA) to the animals. Learning trials were given to animals by recording the step down latency, the time taken by mice to step down from a wooden platform to the grid floor, and delivering an electric shock as soon as all four paws touched the floor. Short and long term memory retention was assessed through step down latency on day 2 and day 7 respectively, in absence of shock, with 180 s as cut-off. Entire apparatus was cleaned with 70 % ethanol after every trial to eliminate all olfactory cues.

4.5 Biochemical Analysis

4.5.1 Estimation of blood glucose level

Blood glucose levels were measured using Accu-check (Roach Diagnostics) by tail sniping. In oral glucose tolerance test (OGTT), 12 h fasted mice were administered glucose (2 g/kg) body weight, and blood glucose levels were checked at 0, 15, 30, 60 and 120 min after glucose load.

4.5.2 Estimation of serum insulin level

Serum insulin levels were analysed using commercially available chemiluminescent immunoassay (AccuLite CLIA Microwells, Monobind Inc.).

4.5.3 Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)

Insulin resistance was calculated using HOMA2 calculator (The University of Oxford).

4.5.4 Estimation of Serum Cortisol levels

We used an HPLC-UV system to detect the serum cortisol levels at 250 nm. Mobile phase comprised of water:methanol (30:70) and dexamethasone was used as an internal standard [62].

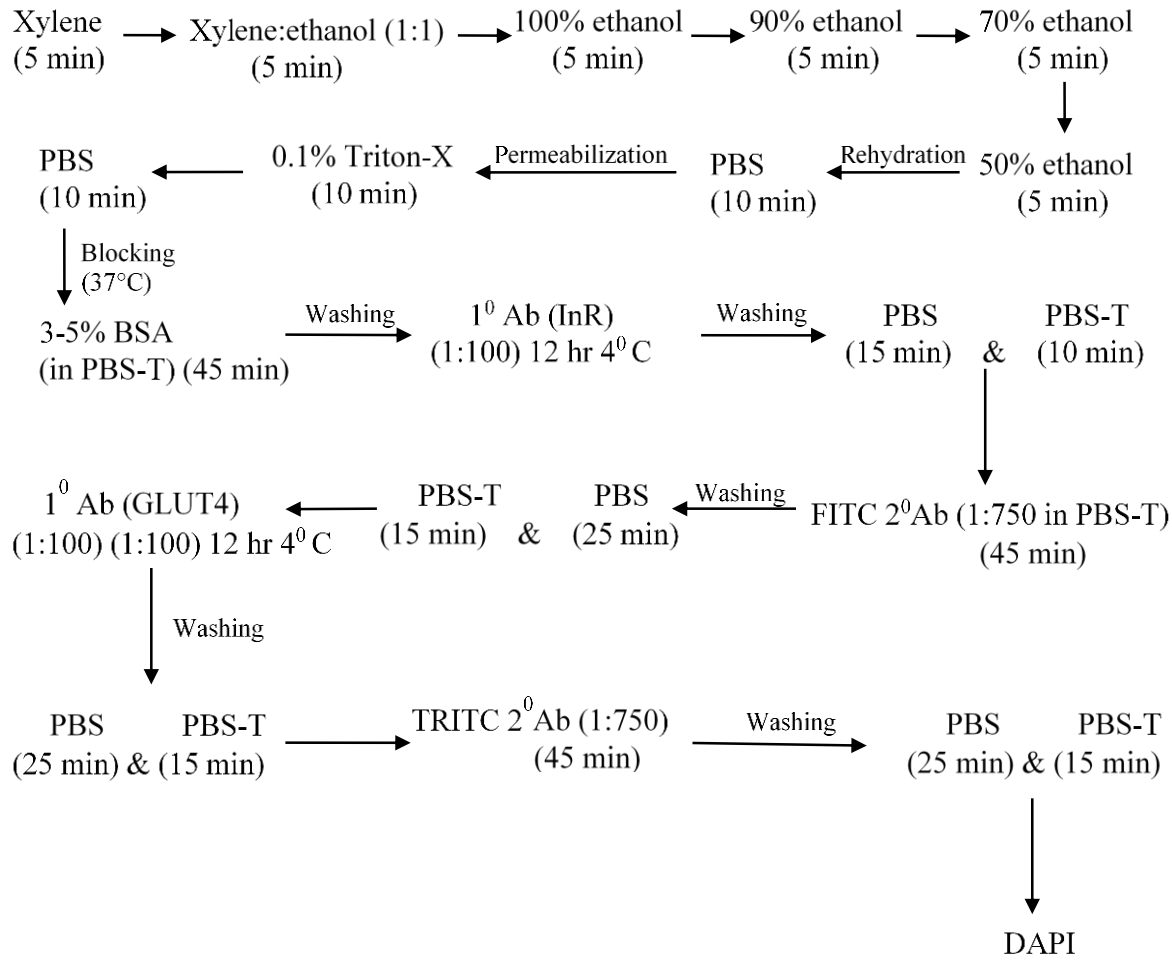
4.6 Molecular Analysis

4.6.1 Immunoblot

Immediately after the behavioral studies, mice were sacrificed by cervical dislocation. The hippocampus were removed at 4^0C, and, homogenized in RIPA buffer supplemented with proteinase inhibitor (Thermo Scientific™; Cat no.: 36978), centrifuged at 16,000 g for 30 min at 4 ºC, supernatant was separated and stored at -80 ºC until further use. Protein quantification was done using Bradford assay with bovine serum albumin (BSA) as a standard. 30 µg of total protein (pooled, n=3) from the stored supernatant was denatured with laemmli loading buffer at 95 °C for 10 min. Protein separation was done using 10 % SDS-PAGE and electroblotted to nitrocellulose membrane using semi-dry transblot. Blots were blocked with 3 % BSA (in PBS) at 37 °C for 1 hr, and then overnight at 4 °C with the respective primary antibodies (1:3000; prepared in PBS-T). Following antibodies were used: I) GAPDH (rabbit polyclonal IgG, SC-25778), II) insulin (rabbit polyclonal IgG, SC-9168), III) InR (rabbit polyclonal IgG, SC-711), and IV) GLUT4 (goat polyclonal IgG, SC-1606). After washing with PBS and PBS-T, the membranes were incubated with respective HRP conjugated sheep anti-rabbit / goat anti-mouse IgG (1:5000; prepared in PBS-T, Santa Cruz Biotechnology, Inc.) secondary antibodies for 2 h at 25 °C. The membranes were developed with 0.06 % 3,3'-diaminobenzidine tetrahydrochloride, 0.025 % CoCl2 in PBS and 0.01 % H2O2. The blot images were captured and band density was estimated using a densitometer (GS-800 Calibrated Densitometer, BioRad).

4.6.2 Immunofluorescence

Immediately after the behavioral studies, the mice were sacrificed using ketamine:xylazine (90:5 mg/kg) anaesthesia. Brains were fixed by double circulation technique, by infusing PBS followed by a mixture of 2 % formalin and 2 % glutaraldehyde solution. After fixation, brains were harvested, 5 μm sections were prepared using cryotome/microtome and fixed on glass slides, and stored at −20 °C until used for histopathology and immunofluorescence studies. Slides were brought to room temperature and then subjected to the following steps:



Images were captured using a fluorescence microscope (Nikon eclipse Ti fluorescence microscope) at 400X magnification [267]. InR and GLUT4 expression was calculated in the terms of total corrected total cell fluorescence (CTCF) intensity using imageJ software with the following sequation:

$$CTCF = \text{Integrated density} - (\text{Area of selected cell} \times \text{Mean background fluorescence})$$

4.6.3 Hematoxylin and eosin (H&E) staining

Neuronal morphology in the hippocampus was evaluated through H&E staining method. Sections were gradually deparaffinized by treating them with xylene (5 min), xylene: ethyl alcohol (1:1) (5 min), 100 % ethyl alcohol (10 min), 90 % ethyl alcohol (5 min), 70 % ethyl alcohol (5 min) and 50 % ethyl alcohol (5 min). The sections were rehydrated by PBS (2 × 5 min), followed by staining with hematoxylin and counterstaining with 1 % eosin for 15 min each at 37 °C. Sections were then gradually dehydrated by treating them with 50, 70, 90 and 100 % ethyl alcohol (5 min each), xylene: ethyl alcohol (1:1) (5 min) and xylene (5 min). Number of viable cells at one field in different regions of the hippocampus were observed under light microscope (Olympus **s**BX51TF microscope with DP70 color camera) at 400X.

4.6.4 Golgi-cox staining

Golgi-cox method is amongst the most reliable techniques for the neuroanatomical studies. This technique allows us to a wide range of neuronal parameters like neurodegeneration, neuronal integrity, dendritic arborization, interneuronal connections, synaptic connectivity and spine density. The purpose of using this technique was to understand the diabetes-associated neurodegenerative changes and the effect of rutin intervention in it [140]. A stock solution of 50mg/ml was prepared of potassium dichromate ($K_2Cr_2O_7$), potassium chromate (K_2CrO_4), and mercuric chloride ($HgCl_2$). Fresh golgi-cox staining solution was prepared by combining 20.8 ml $K_2Cr_2O_7$, 20.8 ml $HgCl_2$, 16.6 ml K_2CrO_4 and 41.6 ml ddH2O, and stored in dark for 48 hrs before use. Brains were isolated over ice post ketamine-xylazine anaesthesia, washed with ice-cold water and ice-cold golgi-cox solution. The block containing hippocampus was cut and dipped in golgi-cox solution at 37 ºC for 24 hrs. 200 µm thick sections were cut from the block and mounted on a glass slide. Sections were washed with **s**ddH_2O (2 × 5 min), followed by dehydration using 50 % ethanol and then dipped in ammonia: water solution (3:1) for 10 min in dark. Sections were **s**washed (ddH2O, 2 × 5 min) and immersed in 5 % sodium thiosulfate for 10 min in dark. Once again the sections were **s**washed (ddH2O, 2 × 5 min) and dehydrated gradually using 50, 70, 80, 95 and 100 % ethyl alcohol (5 min each), and xylene (5 min) and finally mounted with DPX. Hippocampal neurons were observed for neuronal morphology, inter-neuronal connections, neurodegeneration, dendritic arborization and spine density under light microscope (Olympus BX51TF microscope with DP70 color camera). Dendritic arborization (400X) and spine density (1000X) were calculated.

4.7 Statistics

Statistical significance of the data was determined by using GraphPad prism 6 software. Data were expressed as mean ± SD and the statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple comparison post hoc test at a significance level of * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). Two-way ANOVA with Bonferroni post hoc test was performed for all data to evaluate main effect and interaction at confidence level of $P < 0.05$. Data of the MWM were analyzed through repeated measures ANOVA. Further, eta-squared effect size (η^2) for ANOVA results and Cohen's d effect sizes estimates were performed.

CHAPTER 5

RESULTS

5. RESULTS

5.1 DIABETES STUDY

5.1.1 Physiological Parameters

1) Body Weight, Feed Intake & Water Intake

Body weight analysis, revealed insignificant STZ-rutin interaction [F (1, 20) = 1.36, $p > 0.05$ and $\eta^2 = 0.02$]. But, the main effect of STZ [F (1, 20) = 45.47, $p < 0.001$, $\eta^2 = 0.52$ and d= 3.6] and rutin [F (1, 20) = 20.32, $p < 0.001$, $\eta^2 = 0.23$ and d= 2.68] were significant (Fig 5.1A). In case of feed intake, our results revealed that STZ-rutin interaction [F (1, 12) = 18.65, $p < 0.001$ and $\eta^2 = 0.35$], main effect of STZ [F (1, 12) = 12.55, $p < 0.01$ and $\eta^2 = 0.23$ and d = 3.17] and main effect of rutin [F (1, 12) = 10.35, $p < 0.01$ and $\eta^2 = 0.19$ and d = 3.42] were significant (Fig 5.1B). For water intake our results revealed that STZ-rutin interaction [F (1, 12) = 3.44, $p < 0.001$ and $\eta^2 = 0.21$], main effect of STZ [F (1, 12) = 65.02, $p < 0.001$, $\eta^2 = 0.45$ and d= 6.04] and main effect rutin [F (1, 12) = 37.26, $p < 0.001$, $\eta^2 = 0.25$ and d= 4.92] were significant (Fig 5.1C). These results are in line with common symptoms of chronic diabetes, as diabetic animals had significant hyperglycemia, reduced body weight, polyphagia and polydipsia. Rutin was helpful in alleviating all of these parameters and hence controlling diabetes and associated complications.

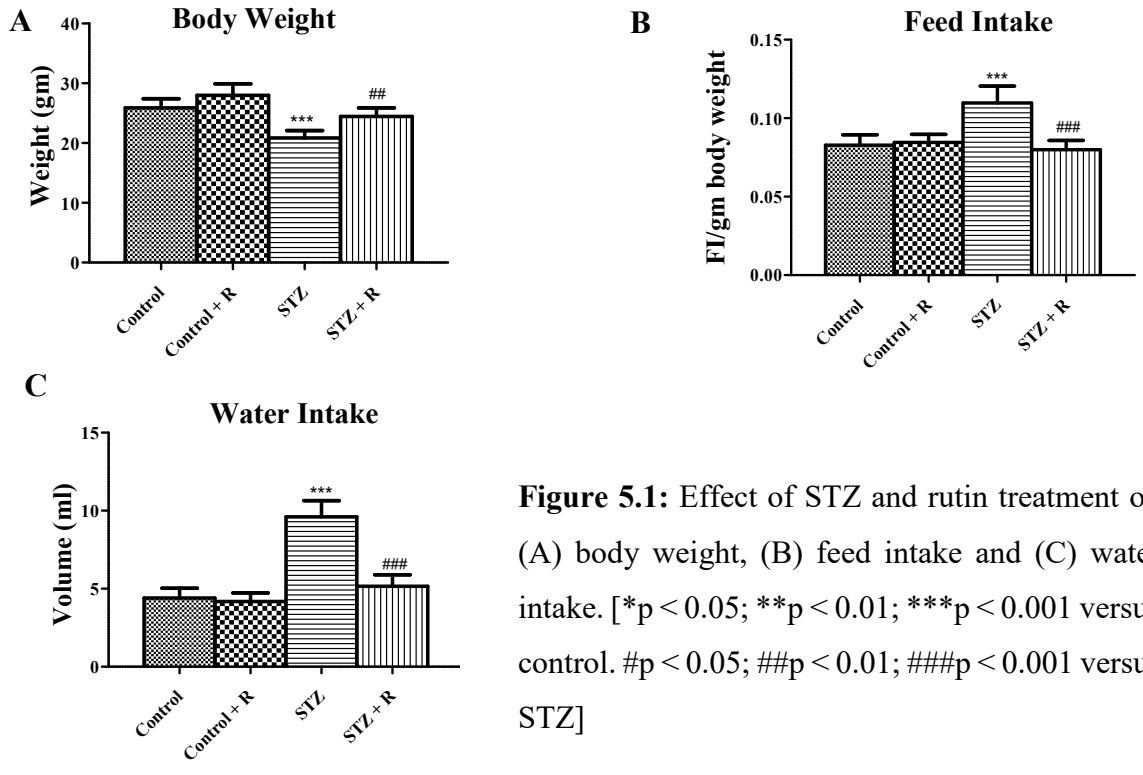


Figure 5.1: Effect of STZ and rutin treatment on (A) body weight, (B) feed intake and (C) water intake. [*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ versus STZ]

5.1.2 Biochemical Parameters

1) Fasting Blood Glucose (FBG)

FBG analysis revealed that STZ-rutin interaction [$F(1, 20) = 39.73$, $p < 0.001$ and $\eta^2 = 0.23$], main effect of STZ [$F(1, 20) = 76.66$, $p < 0.001$ and $\eta^2 = 0.45$ and $d = 4.71$] and the main effect of rutin [$F(1, 20) = 32.9$, $p < 0.001$ and $\eta^2 = 0.19$ and $d = 3.56$] were significant (Fig 5.2A).

2) Oral Glucose Tolerance Test (OGTT)

At 0 min, STZ-rutin interaction [$F(1, 20) = 29.9$, $p < 0.001$ and $\eta^2 = 0.21$], main effect of STZ [$F(1, 20) = 69.29$, $p < 0.001$ and $\eta^2 = 0.31$ and $d = 4.04$] and rutin [$F(1, 20) = 23.92$, $p < 0.0001$ and $\eta^2 = 0.16$ and $d = 3.02$] were observed to be significant. At 15 min, we observed a non-significant STZ-rutin interaction [$F(1, 20) = 3.42$, $p > 0.05$ and $\eta^2 = 0.02$], a significant main effect of STZ [$F(1, 20) = 73.18$, $p < 0.001$ and $\eta^2 = 0.51$ and $d = 3.3$] and a significant main effect of rutin [$F(1, 20) = 44.53$, $p < 0.001$ and $\eta^2 = 0.31$ and $d = 2.53$]. At 30 min, we observed a significant STZ-rutin interaction [$F(1, 20) = 32.82$, $p < 0.001$ and $\eta^2 = 0.2$], main effect of STZ [$F(1, 20) = 54.6$, $p < 0.001$ and $\eta^2 = 0.33$ and $d = 4.35$] and rutin [$F(1, 20) = 54.42$, $p < 0.001$ and $\eta^2 = 0.33$ and $d = 3.96$]. At 60 min, we observed a significant STZ-rutin interaction [$F(1, 20) = 33.39$, $p < 0.001$ and $\eta^2 = 0.27$], main effect of STZ [$F(1, 20) = 47.55$, $p < 0.001$ and $\eta^2 = 0.38$ and $d = 3.84$] and rutin [$F(1, 20) = 22.13$, $p < 0.001$ and $\eta^2 = 0.17$ and $d = 3.08$]. At 120 min of the OGTT, we observed a non-significant STZ-rutin interaction [$F(1, 20) = 31.83$, $p < 0.001$ and $\eta^2 = 0.27$], a significant main effect of STZ [$F(1, 20) = 38.12$, $p < 0.001$ and $\eta^2 = 0.32$ and $d = 3.43$] and rutin [$F(1, 20) = 27.8$, $p < 0.001$ and $\eta^2 = 0.23$ and $d = 3.16$] (Fig 5.2B)

3) Serum Insulin

For fasting serum insulin levels, STZ-rutin interaction [$F(1, 12) = 87.33$, $p < 0.001$ and $\eta^2 = 0.26$], main effect of STZ [$F(1, 12) = 177.9$, $p < 0.001$ and $\eta^2 = 0.53$ and $d = 8.6$] and the main effect of rutin [$F(1, 12) = 52.9$, $p < 0.001$ and $\eta^2 = 0.16$ and $d = 7.2$] were observed to be significant (Fig 5.2C).

Our results indicate that diabetic animals had significantly higher FBG levels and impaired glucose tolerance. They also had hypoinsulinemia, probably due to the multiple STZ injections. Rutin treatment improved the FBG levels, glucose tolerance and serum insulin levels, thereby exhibiting strong anti-diabetic activity.

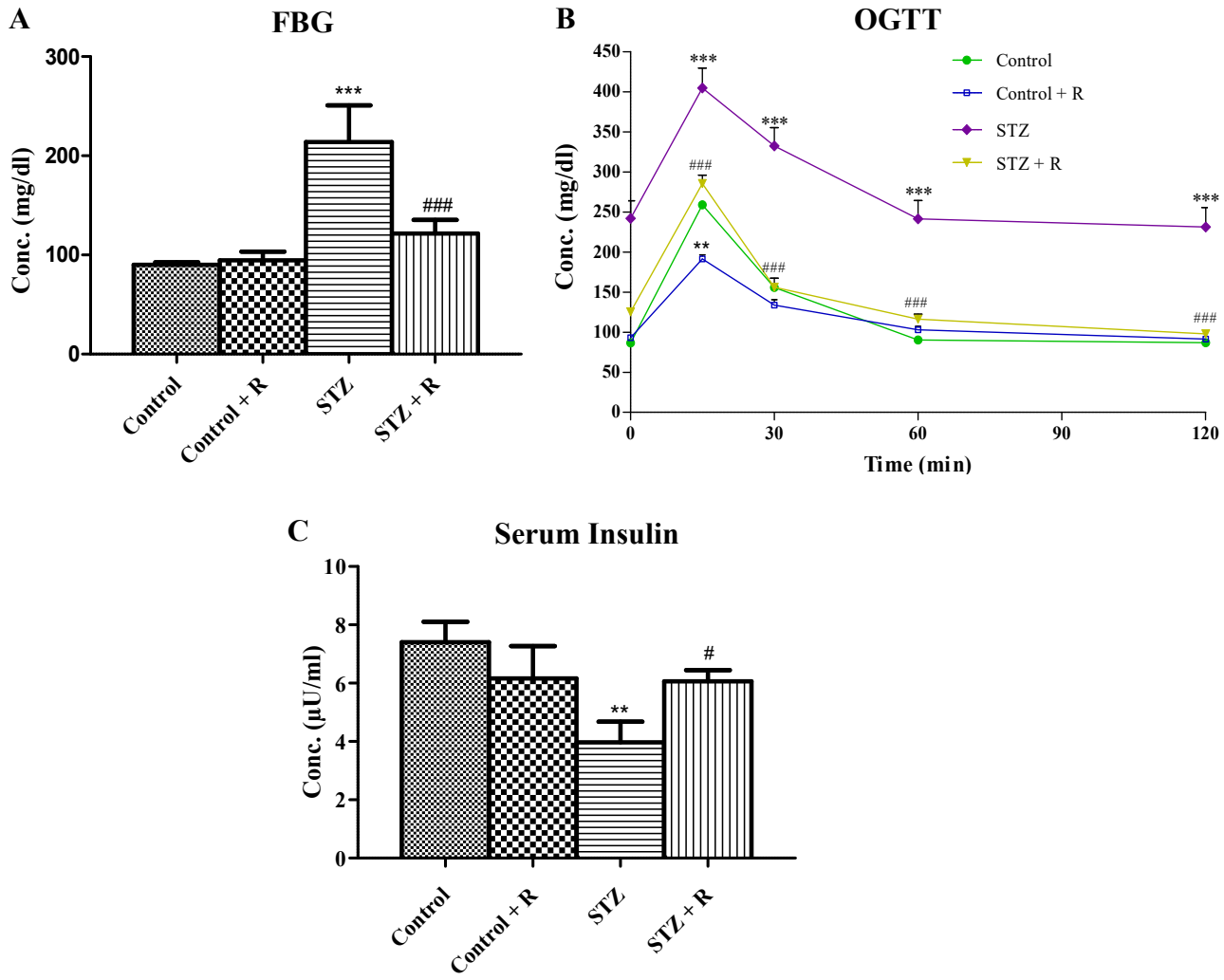


Figure 5.2: Effect of STZ and rutin treatment on A) fasting blood glucose (FBG), B) oral glucose tolerance test (OGTT) and C) serum insulin level. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ]

5.1.3 Locomotion & muscle coordination

1) Open Field Test (OFT)

Our results demonstrated a significant STZ-rutin interaction [$F(1, 20) = 4.44$, $p < 0.05$ and $\eta^2 = 0.12$], the main effect of STZ [$F(1, 20) = 4.53$, $p < 0.05$, $\eta^2 = 0.12$ and $d = 1.9$] and the main effect of rutin treatment [$F(1, 20) = 7.99$, $p < 0.01$, $\eta^2 = 0.22$ and $d = 2.27$] for the number of line crossings in the OFT (Fig 5.3A).

2) Beam Walk Test

For beam walk test (Fig 5.3B), our results revealed a significant STZ-rutin interaction [F (1, 20) = 15.14, $p < 0.001$ and $\eta^2 = 0.13$], the main effect of STZ [F (1, 20) = 15.14, $p < 0.001$ and $\eta^2 = 0.13$ and d = 2.18] and the main effect of rutin treatment [F (1, 20) = 68.38, $p < 0.001$, $\eta^2 = 0.57$ and d = 3.8].

Our results indicate that diabetes impairs locomotion and muscle coordination. Treating animals with rutin rescues these deficits as animal show improved locomotion in the OFT, and excellent muscle coordination in beam walk test.

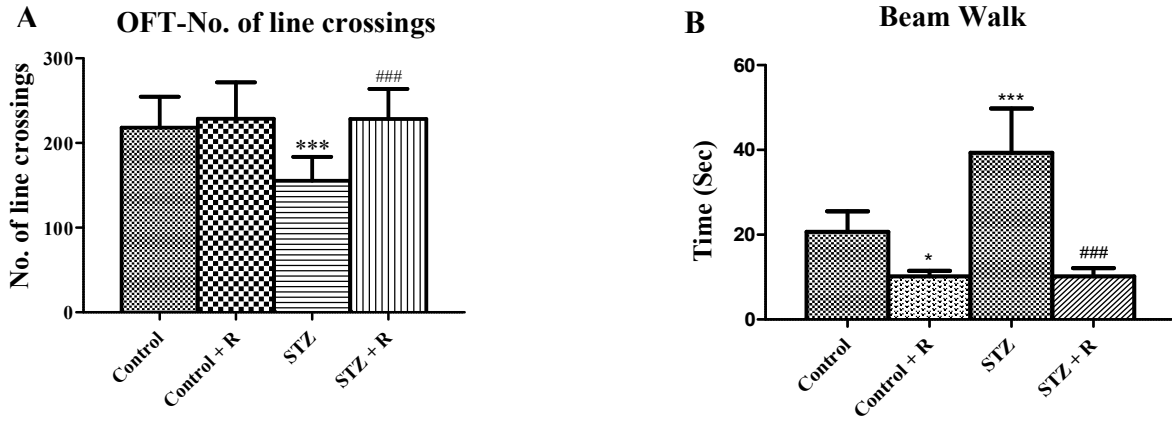


Figure 5.3: Effect of STZ and rutin treatment on locomotor & muscle coordination parameters. A) Open field-Number of line crossings, and B) beam walk-time taken to cross the beam. [*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. control. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ vs. STZ]

5.1.4 Anxiety

1) Elevated Plus Maze (EPM)

For EPM, our results revealed that STZ-rutin interaction [F (1, 20) = 20.75, $p > 0.001$ and $\eta^2 = 0.11$], the main effect of STZ [F (1, 20) = 129.8, $p < 0.001$, $\eta^2 = 0.7$ and d= 14.95] and the main effect of rutin treatment [F (1, 20) = 14.6, $p < 0.001$, $\eta^2 = 0.07$ and d= 8.2] were significant (Fig 5.4A).

2) Open Field Test (OFT)

For, time spent in center, our results revealed that STZ-rutin interaction [F (1, 20) = 19.58, $p < 0.001$ and $\eta^2 = 0.15$], the main effect of STZ [F (1, 20) = 51.83, $p < 0.001$, $\eta^2 = 0.4$ and d= 5.3] and the main effect of rutin treatment [F (1, 20) = 36.65, $p < 0.001$, $\eta^2 = 0.28$ and d= 4.8] were significant (Fig 5.4B). For, number of center entries, our results revealed that STZ-rutin

interaction [F (1, 20) = 21.15, $p < 0.001$ and $\eta^2 = 0.34$], the main effect of STZ [F (1, 20) = 24.1, $p < 0.001$, $\eta^2 = 0.24$ and d= 4.2] and the main effect of rutin treatment [F (1, 20) = 34.2, $p < 0.001$, $\eta^2 = 0.34$ and d = 6.4] were significant (Fig 5.4C). Our results reveal that diabetes led to increased anxiety like behavior and rutin treatment was effective in alleviating it.

Our results indicate that diabetes led to increased anxiety like behavior which was attenuated by rutin treatment as evident by increased time spent in the center of open field and open arm of EPM.

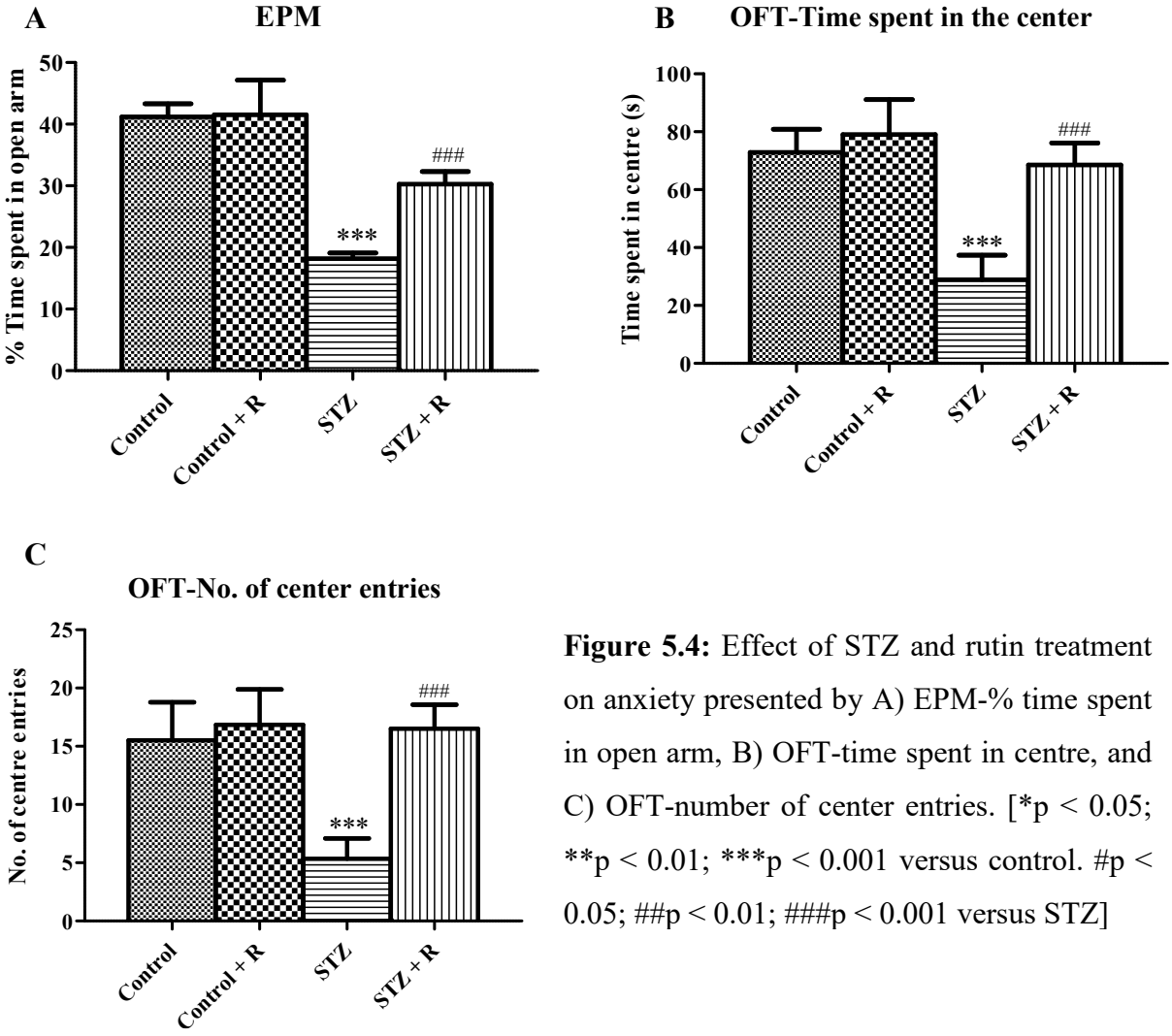


Figure 5.4: Effect of STZ and rutin treatment on anxiety presented by A) EPM-% time spent in open arm, B) OFT-time spent in centre, and C) OFT-number of center entries. [*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ versus STZ]

5.1.5 Depression

1) Sucrose Preference Test (SPT)

SPT was used to assess anhedonia like behavior. Our results revealed that STZ-rutin interaction [$F(1, 12) = 7.53$, $p < 0.05$ and $\eta^2 = 0.15$], main effect of STZ [$F(1, 12) = 10.7$ $p < 0.01$ and $\eta^2 = 0.21$ and $d = 2.5$] and main effect of rutin [$F(1, 12) = 20.15$, $p < 0.001$ and $\eta^2 = 0.4$ and $d = 3.7$] were significant (Fig 5.5A).

2) Tail Swim Test (TST)

In case of TST immobility time, our results revealed that STZ-rutin interaction [$F(1, 16) = 5.91$, $p < 0.05$ and $\eta^2 = 0.015$], main effect of STZ [$F(1, 16) = 36.22$. $p < 0.001$ and $\eta^2 = 0.09$ and $d = 3.4$] and main effect of rutin [$F(1, 16) = 334.61$, $p < 0.001$ and $\eta^2 = 0.85$ and $d = 9.9$] were significant (Fig 5.5B). Similarly, in case of TST number of upward turnings, our results revealed that STZ -rutin interaction [$F(1, 16) = 37.89$, $p < 0.001$ and $\eta^2 = 0.34$], main effect of STZ [$F(1, 16) = 25.19$, $p < 0.001$ and $\eta^2 = 0.22$ and $d = 5.53$] and main effect of rutin [$F(1, 16) = 33.37$, $p < 0.001$ and $\eta^2 = 0.29$ and $d = 5.78$] were significant (Fig 5.5C).

3) Forced Swim Test (FST)

For FST immobility time, our results revealed that STZ-rutin interaction [$F(1, 20) = 65.58$, $p < 0.001$ and $\eta^2 = 0.31$], main effect of STZ [$F(1, 20) = 64.73$, $p < 0.001$ and $\eta^2 = 0.31$ and $d = 5.28$] and main effect of rutin [$F(1, 20) = 58.12$, $p < 0.001$ and $\eta^2 = 0.27$ and $d = 4.97$] were significant (Fig 5.5D). Diabetic animals showed higher despair and reduced motivation and rutin proved to be an effective antidepressant.

Our results show that diabetes led to severe depressive like behavior with animals having increased despair and anhedonia as evaluated by TST, FST and SPT respectively. Rutin proved to be a potent anti-depressant by not only reducing the depressive behavior in diabetic animals but by increasing the threshold for depression in control animals.

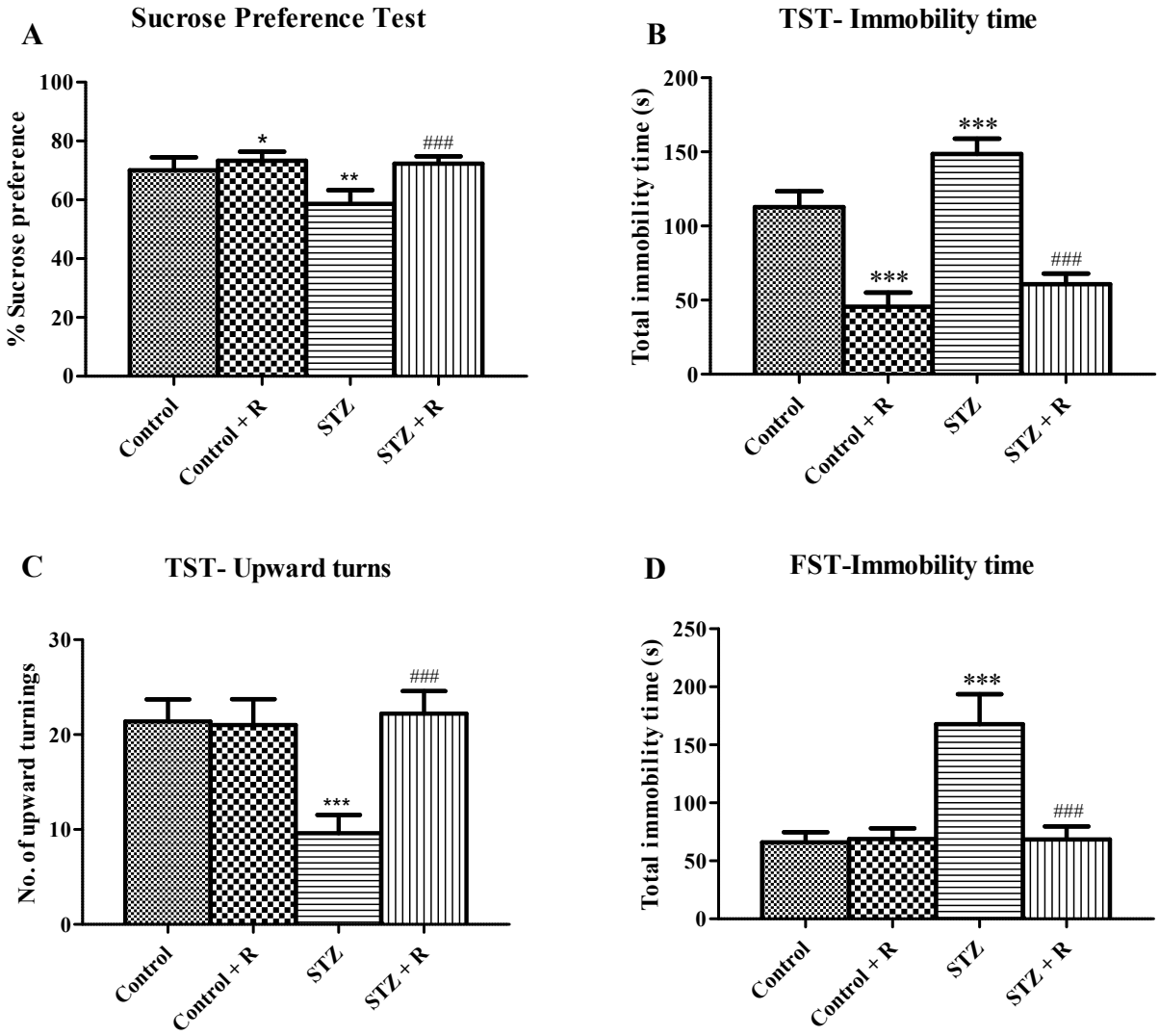


Figure 5.5: Effect of STZ and rutin treatment on depression presented by A) SPT-% sucrose preference, B) TST-immobility time, C) TST-number of upward turns, and D) FST-immobility time. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ]

5.1.6 Learning & Memory

1) Passive Avoidance-Step Down (PA-SD) Task

Short-term memory evaluated on day 1 revealed significant STZ-rutin interaction [F (1, 20) = 25.59, p < 0.001 and $\eta^2 = 0.3$], the main effect of STZ [F (1, 20) = 19.01, p < 0.001, $\eta^2 = 0.22$ and d = 2.77] and the main effect of rutin treatment [F (1, 20) = 19.45, p < 0.001, $\eta^2 = 0.23$ and d = 2.79]. Further, results of memory retention conducted on day 7 (long-term memory retention) of this study demonstrated significant interaction between STZ-rutin [F (1, 20) = 299.5, p < 0.001 and $\eta^2 = 0.25$], main effect of STZ [F (1, 20) = 277.17, p < 0.001, $\eta^2 = 0.23$ and d = 10.21] and rutin [F (1, 20) = 559.82, p < 0.001, $\eta^2 = 0.48$ and d = 29.54] (Fig 5.6A).

2) Novel Object Recognition (NOR) Test

For NOR test, discrimination index analysis revealed that STZ-rutin interaction [$F(1, 20) = 10.2$, $p < 0.01$ and $\eta^2 = 0.17$], the main effect of STZ [$F(1, 20) = 12.87$, $p < 0.001$, $\eta^2 = 0.21$ and $d = 2.54$] and the main effect rutin treatment [$F(1, 20) = 17.89$, $p < 0.001$, $\eta^2 = 0.29$ and $d = 2.76$] were significant (Fig 5.6B).

3) Morris Water Maze (MWM) Test

In MWM, learning was evaluated from day 1 to 4, known as the learning trials. For day 1, our results revealed an insignificant STZ-rutin interaction [$F(1, 20) = 2.43$, $p > 0.05$ and $\eta^2 = 0.04$] and the main effect of STZ [$F(1, 20) = 0.69$, $p > 0.05$, $\eta^2 = 0.01$ and $d = 0.44$]. The main effect of rutin [$F(1, 20) = 34.22$, $p < 0.001$, $\eta^2 = 0.59$ and $d = 3.05$] was observed to be significant. For day 2, our results revealed that STZ-rutin interaction [$F(1, 20) = 66.31$, $p < 0.001$ and $\eta^2 = 0.23$], the main effect of STZ [$F(1, 20) = 68.68$, $p < 0.001$, $\eta^2 = 0.24$ and $d = 5.05$], and the main effect of rutin [$F(1, 20) = 133.8$, $p < 0.001$, $\eta^2 = 0.46$ and $d = 6.29$] were significant. For day 3, our results revealed that STZ-rutin interaction [$F(1, 20) = 132.1$, $p < 0.001$ and $\eta^2 = 0.27$], the main effect of STZ [$F(1, 20) = 138.45$, $p < 0.001$, $\eta^2 = 0.28$ and $d = 8.26$], and the main effect of rutin [$F(1, 20) = 195.35$, $p < 0.001$, $\eta^2 = 0.4$ and $d = 9.29$] were significant. For day 4, our results revealed that STZ-rutin interaction [$F(1, 20) = 144.45$, $p < 0.001$ and $\eta^2 = 0.31$], the main effect of STZ [$F(1, 20) = 120.91$, $p < 0.001$, $\eta^2 = 0.26$ and $d = 7.47$], and the main effect of rutin [$F(1, 20) = 184.55$, $p < 0.001$, $\eta^2 = 0.39$ and $d = 8.5$] were significant (Fig 5.6C). Memory index was evaluated using number of platform crossings (probe trial) and time spent in the platform quadrant. For probe trial, our results reveal a significant STZ-rutin interaction [$F(1, 20) = 15.48$, $p < 0.001$ and $\eta^2 = 0.21$]. The main effect of STZ [$F(1, 20) = 18.17$, $p < 0.001$, $\eta^2 = 0.24$ and $d = 3.1$] and rutin treatment [$F(1, 20) = 21.07$, $p < 0.001$, $\eta^2 = 0.28$ and $d = 4.74$] were observed to be significant (Fig 5.6D). For time spent in platform quadrant, our results reveal that STZ-rutin interaction [$F(1, 20) = 8.57$, $p < 0.01$ and $\eta^2 = 0.15$], the main effect of STZ [$F(1, 20) = 2.07$, $p > 0.05$, $\eta^2 = 0.03$ and $d = 2.64$] and the main effect of rutin treatment [$F(1, 20) = 24.15$, $p < 0.001$, $\eta^2 = 0.44$ and $d = 3.75$] were significant (Fig 5.6E).

Our results demonstrated that diabetes impaired short-term as well as long-term memory retrieval in mice. Additionally, diabetes caused significant cognitive decline by deteriorating learning and memory abilities. Rutin treated diabetic animals prevented any such memory deficit and hence preventing cognitive decline (Fig 5.6).

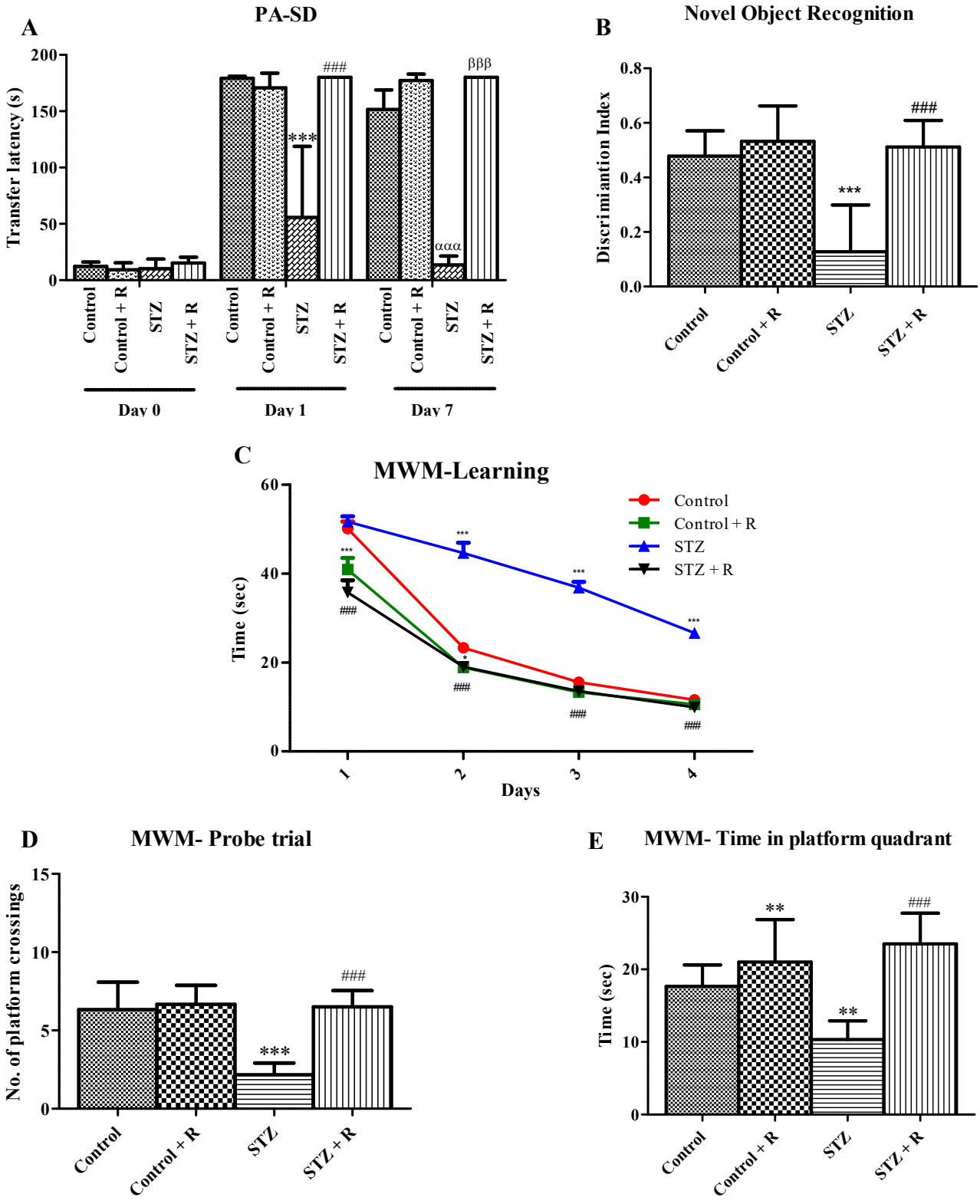


Figure 5.6: Learning and memory. A) PASD task, B) NOR-discrimination index, C) MWM-learning, D) MWM-probe trial and E) MWM-time spent in platform quadrant. [*p < 0.05; **p < 0.01; ***/ ααα p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###/ βββ p < 0.001 versus STZ].

5.1.7 Neurodegeneration

Neurodegeneration and neuronal morphology was evaluated by spine density (number of spines/10µm at 1000 X) and dendritic arborization (number of branches reaching 100 µm at 400 X) through Golgi-cox staining. For spine density, our results reveal that STZ-rutin interaction [F (1, 12) = 9.8, $p < 0.01$ and $\eta^2 = 0.23$], the main effect of STZ [F (1, 12) = 12.9, $p < 0.01$, $\eta^2 = 0.31$ and d= 3.43], and the main effect of rutin [F (1, 12) = 7.1, $p < 0.01$, $\eta^2 = 0.17$ and d= 3.74] were found to be significant (Fig 5.7B). For dendritic arborization, our results reveal that STZ-rutin interaction [F (1, 12) = 19.65, $p < 0.001$ and $\eta^2 = 0.22$], the main effect of STZ [F (1, 12) = 24.55, $p < 0.001$, $\eta^2 = 0.28$ and d= 5.4], and the main effect of rutin [F (1, 12) = 29.99, $p < 0.001$, $\eta^2 = 0.34$ and d= 5.6] were found to be significant (Fig 5.7C).

Our results show that diabetes inflicted severe hippocampal neurodegeneration, especially in the CA3 region. Rutin treatment rescued the neurons from this threat and their morphology appeared similar to control. In diabetes, neurons were short and shrunk, had less networking, and significantly lower spine density. Rutin treated neurons were healthy with extensive networking and significantly higher spine density, compared to untreated diabetic group (Fig 5.7A).

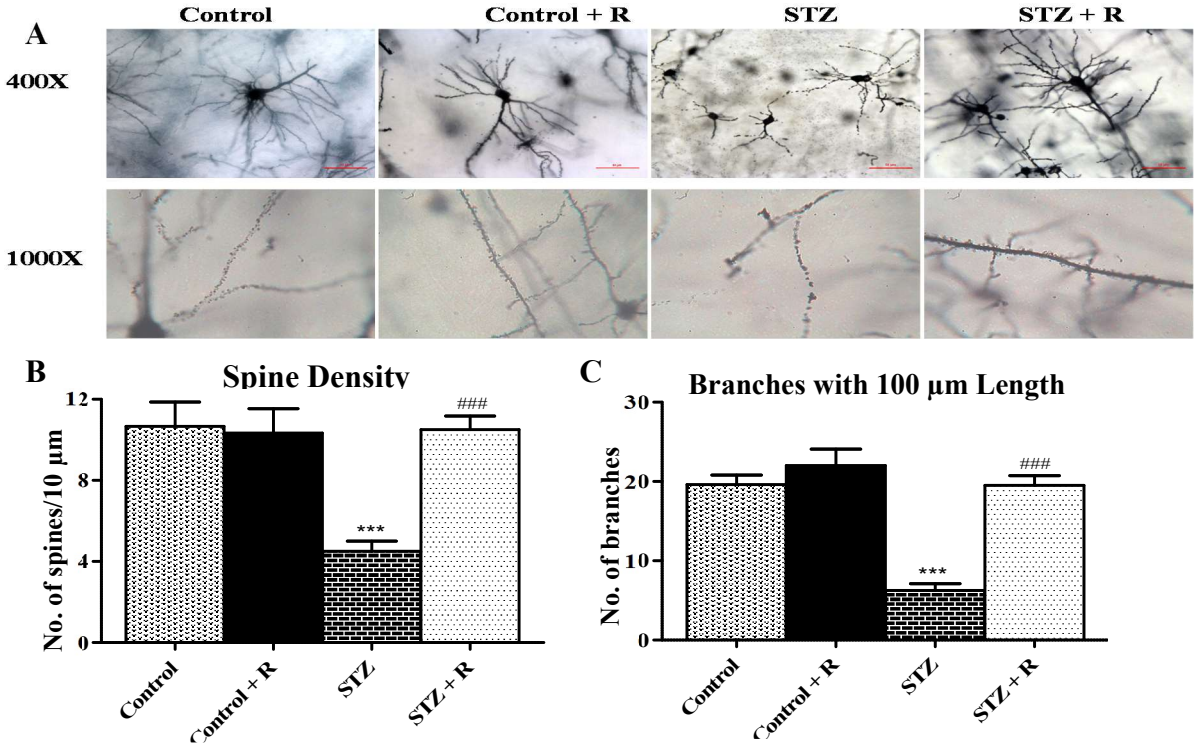


Figure 5.7: Effect of STZ and rutin treatment on hippocampal neurodegeneration. A) Golgi-cox stained neurons at 400 X and 1000 X, B) spine density, C) dendritic arborization. [*p < ***p < 0.001 versus control; ###p < 0.001 versus STZ]

5.1.8 Protein Expression

1) Western Blot

For insulin, we observed a significant STZ-rutin interaction [F (1, 12) = 22.41, $p < 0.001$ and $\eta^2 = 0.21$], main effect of STZ [F (1, 12) = 32.6, $p < 0.001$, η^2=0.29 and d= 7.19] and main effect of rutin [F (1, 12) = 42.04, $p < 0.001$, η^2= 0.38 and d= 5.68] (Fig 5.8B). For IR expression (Fig 5.8C), our results revealed an insignificant STZ-rutin interaction [F (1, 12) = 2.01, $p > 0.05$ and $\eta^2 = 0.01$]. Main effect of STZ [F (1, 12) = 10.55, $p < 0.01$, η^2=0.07 and d= 1.17] and rutin [F (1, 12) = 116.27, $p < 0.001$, η^2= 0.83 and d= 5.86] were found to be significant. For GLUT4 expression (Fig 5.8D), our results reveal significant STZ-rutin interaction [F (1, 12) = 4.95, $p < 0.05$ and $\eta^2 = 0.05$]. Main effect of STZ [F (1, 12) = 5.87, $p < 0.05$, η^2=0.065 and d= 0.11] and rutin [F (1, 12) = 67.39, $p < 0.001$, η^2= 0.74 and d= 2.27] were found to be significant. These results indicate that diabetes leads to reduced hippocampal insulin expression, although no change was observed in IR and GLUT4 expression. Rutin treatment not only increased insulin expression but also upregulated IR and GLUT 4 expression (Fig 5.8).

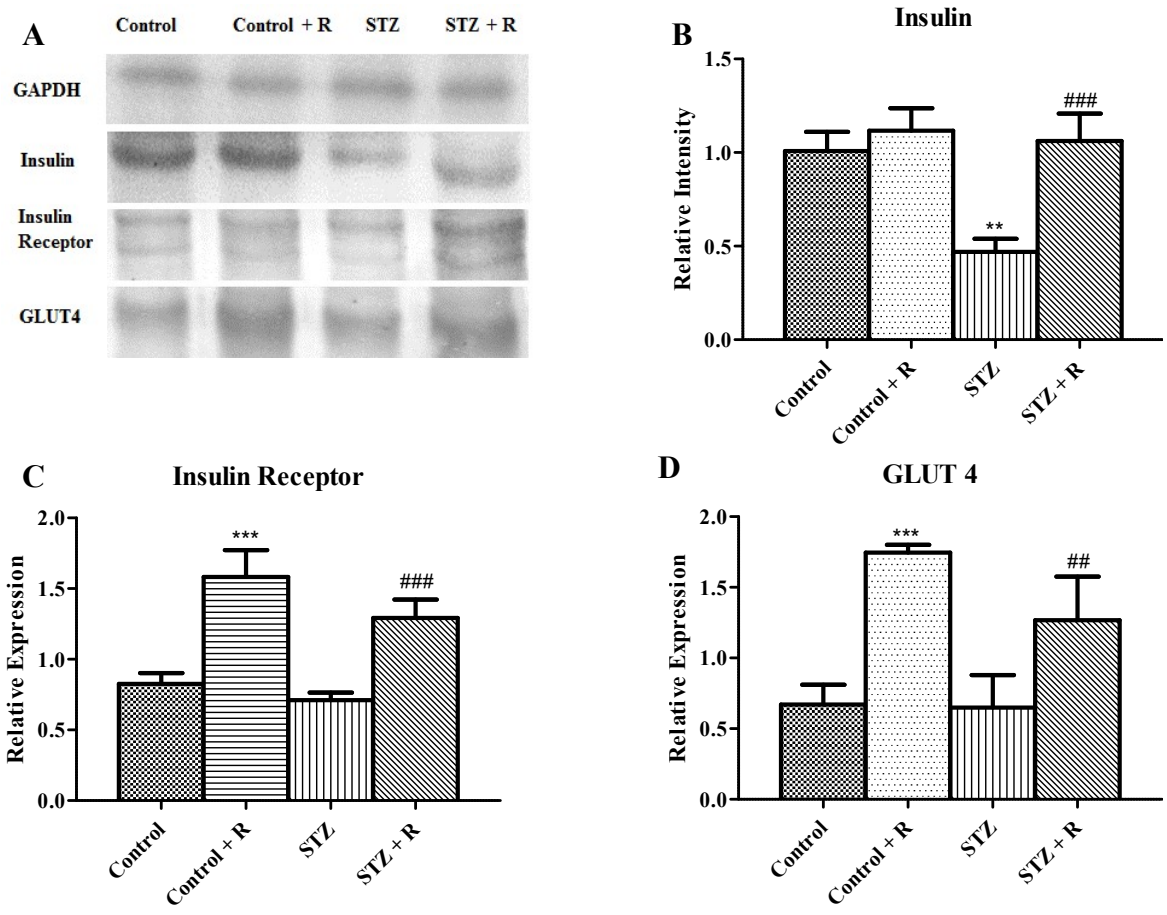


Figure 5.8: Effect of STZ and rutin treatment on A) the hippocampal immunoblot analysis, relative expression of B) insulin, C) insulin receptor, and D) GLUT4. [*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ versus STZ]

2) Immunofluorescence

Immunofluorescence was measured using corrected total cell fluorescence (CTCF) (Fig 5.9A). For hippocampal InR (Fig 5.9B), our results showed insignificant STZ-rutin interaction [F (1, 12) = 3.54, p > 0.07 and η^2 = 0.07] and the main effect of STZ [F (1, 12) = 3.78, p > 0.05, η^2 = 0.07 and d = 2.54]. The main effect of rutin [F (1, 12) = 29.16, p < 0.001, η^2 = 0.6 and d = 2.04] was observed to be significant. For GLUT4 (Fig 5.9C), STZ-rutin interaction [F (1, 12) = 0.008, p > 0.05 and η^2 = 8.68E-05], the main effect of STZ [F (1, 12) = 0.96, p > 0.05, η^2 = 0.01 and d = 0.53] were observed to be significant. The main effect of rutin [F (1, 12) = 79.139, p < 0.001, η^2 = 0.85 and d = 5.04] was found to be significant.

In these results we observed central IR like state with increased InR fluorescence without any change in GLUT4 fluorescence. Rutin treatment upregulated InR and GLUT4 in both control and diabetic state, suggesting a direct role in modulating central insulin signalling.

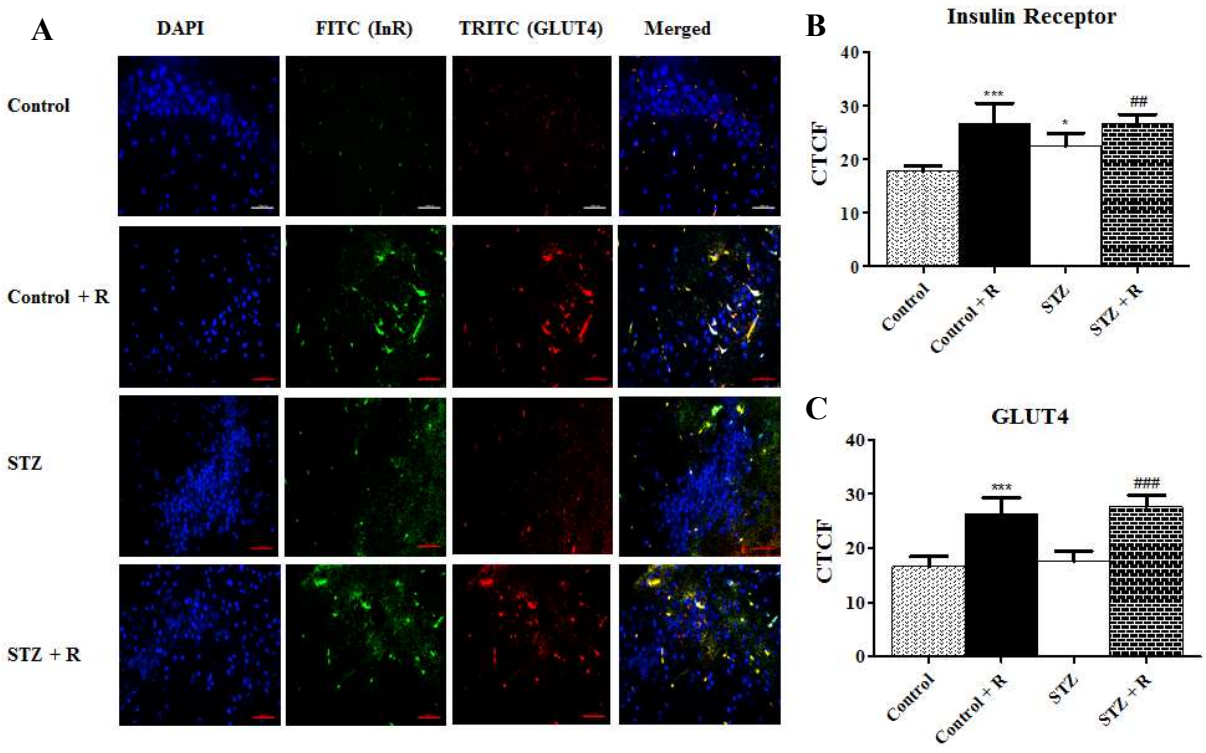


Figure 5.9: Effect of STZ and rutin on the expression of insulin receptor (InR) and glucose transporter 4 (GLUT4) in the hippocampal CA3 region. (A) Immunofluorescence images at 100 X magnification with DAPI (blue, nucleus), FITC (green, InR), and TRITC (red, GLUT4). Corrected total cell fluorescence (CTCF) of (B) InR, and (C) GLUT4. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ].

5.2 CUS STUDY

5.2.1 Physiological Parameters

1) Body Weight, Feed Intake & Water Intake

For body weight changes (Fig 5.10A), our results revealed a significant CUS-rutin interaction [$F(1, 16) = 46.5$, $p < 0.001$ and $\eta^2 = 0.39$], the main effect of CUS [$F(1, 16) = 12.6$, $p < 0.001$, $\eta^2 = 0.108$ and $d = 7.4$] and the main effect of rutin treatment [$F(1, 16) = 41.24$, $p < 0.001$, $\eta^2 = 0.354$ and $d = 6.01$]. For feed intake, our results reveal an insignificant CUS-rutin interaction [$F(1, 16) = 4.15$, $p > 0.05$ and $\eta^2 = 0.005$] and the main effect of CUS [$F(1, 16) = 0.66$, $p > 0.05$, $\eta^2 = 0.0008$ and $d = 2.05$]. However, a significant main effect of rutin treatment [$F(1, 16) = 783.8$, $p < 0.001$, $\eta^2 = 0.974$ and $d = 11.43$] was observed (Fig 5.10B). Results of water intake (Fig 5.10C) demonstrated an insignificant CUS-rutin interaction [$F(1, 12) = 5.56$, $p > 0.05$ and $\eta^2 = 0.168$], the main effect of CUS [$F(1, 12) = 1.51$, $p > 0.05$, $\eta^2 = 0.458$ and $d = 1.28$] and the main effect of rutin treatment [$F(1, 12) = 0.29$, $p > 0.05$, $\eta^2 = 0.0089$ and $d = 0.769$]. Overall CUS leads to a significant weight reduction, which is alleviated by rutin treatment. Further, rutin treatment increases feed intake in both groups, while the water intake shows insignificant change.

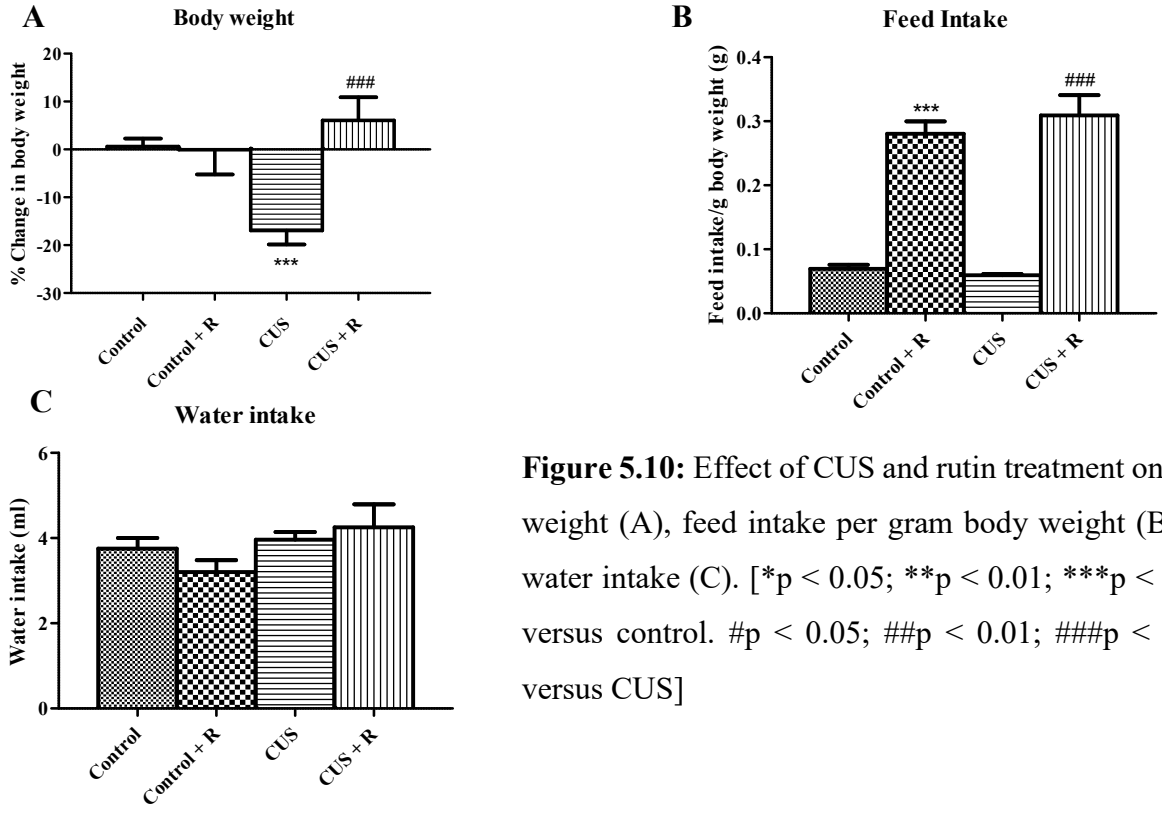


Figure 5.10: Effect of CUS and rutin treatment on body weight (A), feed intake per gram body weight (B) and water intake (C). [*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ versus CUS]

5.2.2 Locomotion & Muscle Coordination

1) Open Field Test

Our results demonstrated a significant CUS-rutin interaction [$F(1, 20) = 24.33$, $p < 0.001$ and $\eta^2 = 0.202$], the main effect of CUS [$F(1, 20) = 40.86$, $p < 0.001$, $\eta^2 = 0.34$ and $d = 6.19$] and the main effect of rutin treatment [$F(1, 20) = 35.1$, $p < 0.001$, $\eta^2 = 0.290$ and $d = 6.64$] for the number of line crossings in the OFT (Fig 5.11A).

2) Beam Walk Test

For beam walk test (Fig 5.11B), our results revealed a significant CUS-rutin interaction [$F(1, 16) = 10.17$, $p < 0.01$ and $\eta^2 = 0.102$], the main effect of CUS [$F(1, 16) = 3.02$, $p < 0.05$, $\eta^2 = 0.03$ and $d = 1.59$] and the main effect of rutin treatment [$F(1, 16) = 70.66$, $p < 0.001$, $\eta^2 = 0.71$ and $d = 5.3$].

Our results indicate that chronic stress impairs locomotion and muscle coordination. Treating animals with rutin rescues these deficits as animal show improved locomotion in the OFT, and excellent muscle coordination in beam walk test.

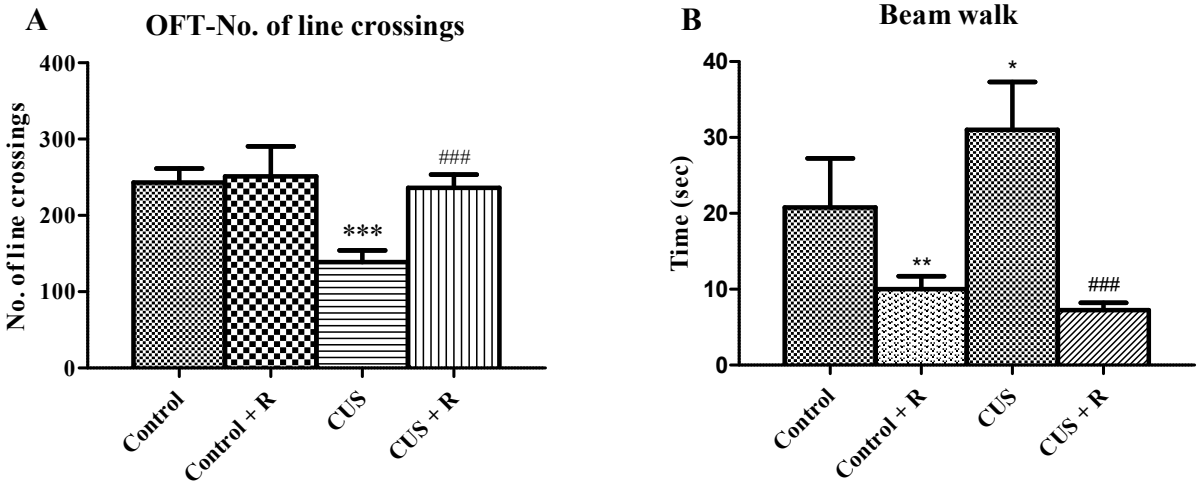


Figure 5.11: Effect of CUS and rutin treatment on locomotor & muscle coordination parameters A) open field test-Number of line crossings, and B) beam walk-time taken to cross the beam. [*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. control. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ vs. CUS]

5.2.3 Anxiety

1) Elevated Plus Maze (EPM)

Our results revealed an insignificant CUS-rutin interaction [F (1, 20) = 0.16, $p > 0.05$ and $\eta^2 = 0.003$] and the main effect of rutin treatment [F (1, 20) = 1.16, $p > 0.05$, $\eta^2 = 0.023$ and d = 1.02] in the EPM task. However, the main effect of CUS [F (1, 20) = 28.06, $p < 0.001$, $\eta^2 = 0.57$ and d = 3.29] was observed to be significant (Fig 5.12A).

2) Open Field Test (OFT)

Time spent and number of entries in the center of the OF were also used to evaluate anxiety levels in mice. For time spent in the center (Fig 5.12B), our results reveal significant CUS-rutin interaction [F (1, 20) = 22.67, $p < 0.001$ and $\eta^2 = 0.39$], the main effect of CUS [F (1, 20) = 8.9, $p < 0.001$, $\eta^2 = 0.015$ and d = 4.62] and the main effect of rutin treatment [F (1, 20) = 14.36, $p < 0.001$, $\eta^2 = 0.25$ and d = 2.72]. In case of the number of center entries (Fig 5.12C), our results reveal significant CUS-rutin interaction [F (1, 20) = 28.45. $p < 0.001$ and $\eta^2 = 0.32$], the main effect of CUS [F (1, 20) = 28.5, $p < 0.001$, $\eta^2 = 0.32$ and d = 3.65] and the main effect of rutin treatment [F (1, 20) = 10.73, $p < 0.001$, $\eta^2 = 0.122$ and d = 3.29]. These results suggest that chronic stress induces anxiety-like behavior in mice. Treating animals with rutin produces anxiolytic effect, and animals were observed to freely explore the OF and EPM.

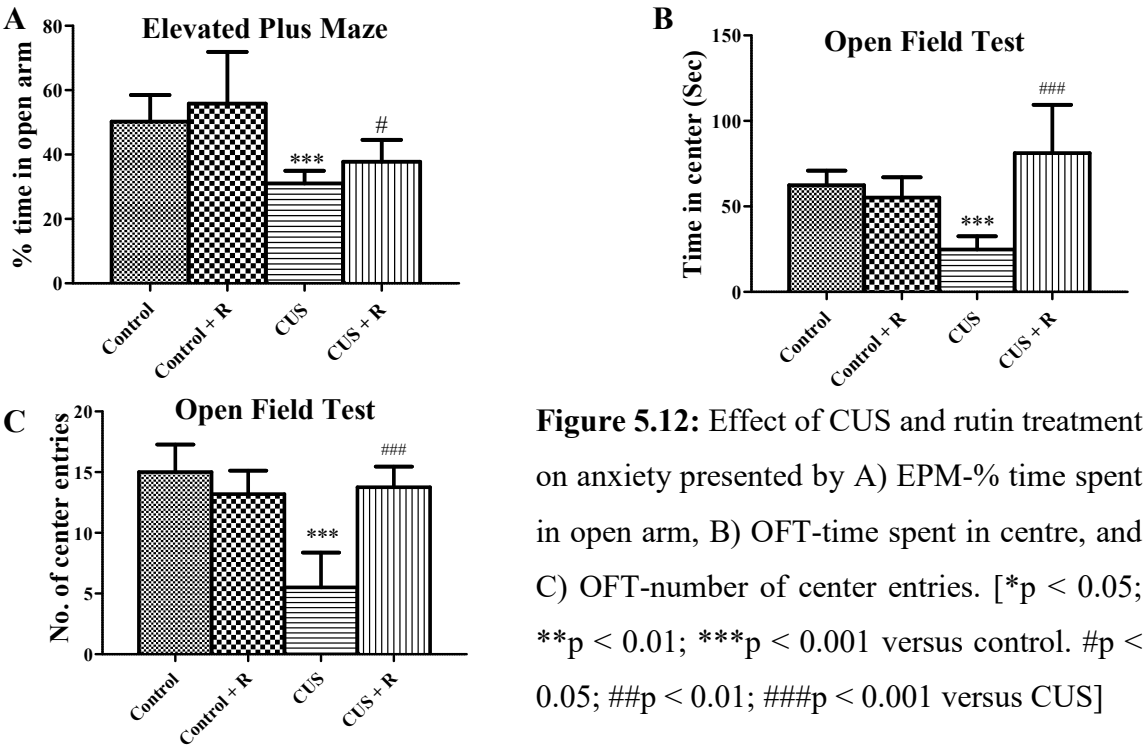


Figure 5.12: Effect of CUS and rutin treatment on anxiety presented by A) EPM-% time spent in open arm, B) OFT-time spent in centre, and C) OFT-number of center entries. [*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ versus CUS]

5.2.4 Depression

1) Sucrose Preference Test (SPT)

For SPT (Fig 5.13A), subjecting animals to 21 day CUS, resulted in significant CUS-rutin interaction [$F(1, 8) = 8.05$, $p < 0.05$ and $\eta^2 = 0.09$], the main effect of CUS [$F(1, 8) = 19.61$, $p < 0.01$, $\eta^2 = 0.22$ and $d = 4.42$] and the main effect of rutin treatment [$F(1, 8) = 53.77$, $p < 0.001$, $\eta^2 = 0.06$ and $d = 6.2$].

2) Tail Swim Test (TST)

In TST depressive behavior was evaluated in the form of immobility time and number of upward turns. In case of immobility time (Fig 5.13B), our results revealed a significant CUS-rutin interaction [$F(1, 16) = 6.78$, $p < 0.05$ and $\eta^2 = 0.056$], the main effect of CUS [$F(1, 16) = 1.46$, $p < 0.05$ and $\eta^2 = 0.012$ and $d = 1.46$] and the main effect of rutin [$F(1, 16) = 95.96$, $p < 0.001$ and $\eta^2 = 0.798$ and $d = 3.43$]. In case of number of upward turnings (Fig 5.13C), our results revealed significant CUS-rutin interaction [$F(1, 16) = 4.6$, $p < 0.05$ and $\eta^2 = 0.187$], the main effect of CUS [$F(1, 16) = 0.51$, $p < 0.05$ and $\eta^2 = 0.021$ and $d = 1.36$] and the main effect of rutin treatment [$F(1, 16) = 3.45$, $p < 0.05$ and $\eta^2 = 0.14$ and $d = 2.72$].

3) Forced Swim Test (FST)

Immobility time in FST (Fig 5.13D) revealed a significant CUS-rutin interaction [$F(1, 16) = 19.41$, $p < 0.001$ and $\eta^2 = 0.35$], the main effect of CUS [$F(1, 16) = 6.97$, $p < 0.01$ and $\eta^2 = 0.126$ and $d = 4.14$] and the main effect of rutin [$F(1, 16) = 5.43$, $p < 0.05$ and $\eta^2 = 0.17$ and $d = 1.6$].

Our results indicate that CUS-induced significant depression and despair in CUS subjected mice, as evident by reduced preference for sweetened water, increased immobility and reduced upward turns. Rutin not only proved to be an effective antidepressant for stressed animals, but also increased the threshold for depression in control animals.

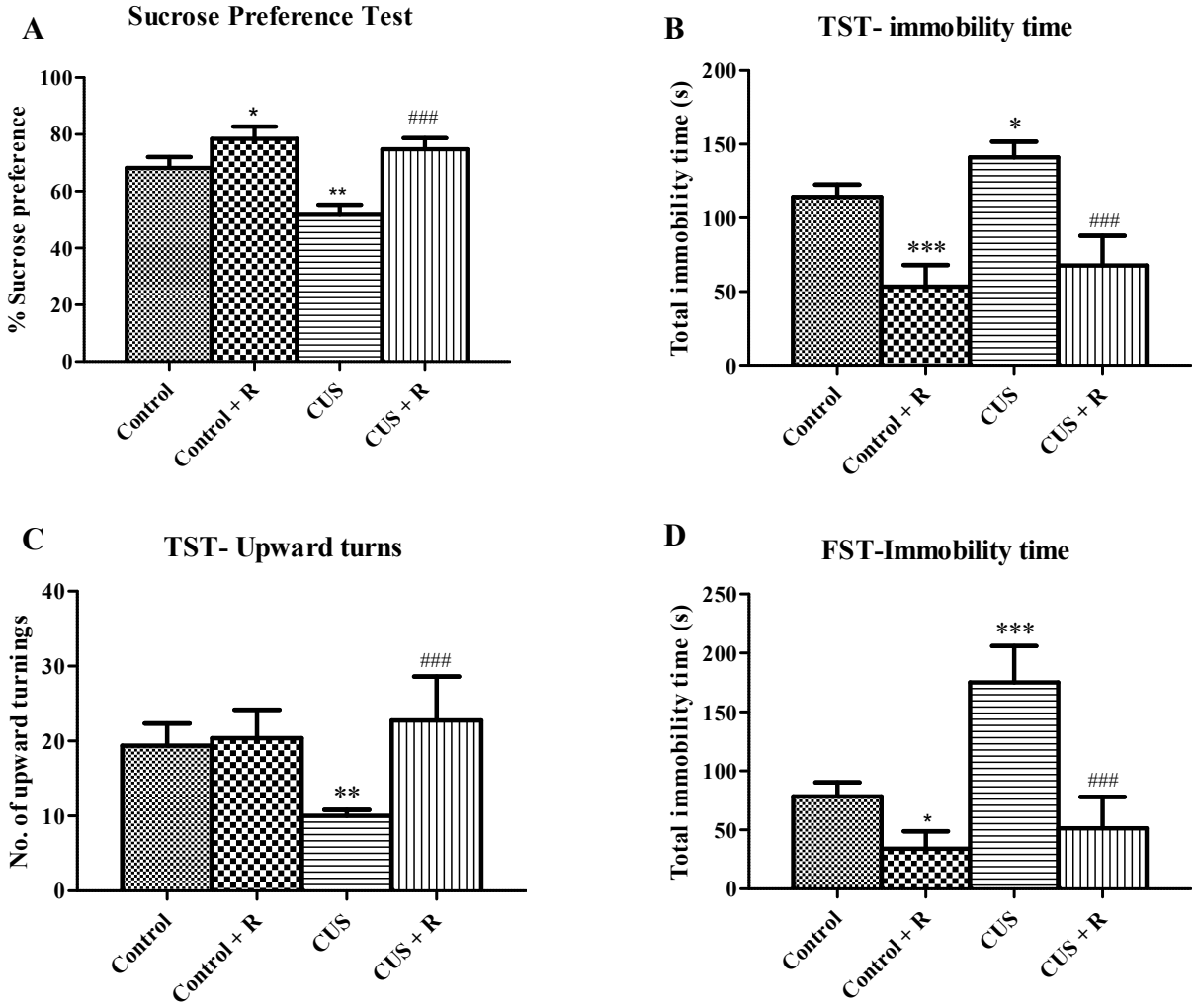


Figure 5.13: Effect of CUS and rutin treatment on depression presented by A) SPT-% sucrose preference, B) TST-immobility time, C) TST-number of upward turns, and D) FST-immobility time. [*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ versus CUS]

5.2.5 Learning & Memory

1) Passive Avoidance-Step through (PA-ST) Task

Short-term memory evaluated on day 1 revealed significant CUS-rutin interaction [$F(1, 16) = 50.62$, $p < 0.001$ and $\eta^2 = 0.41$], the main effect of CUS [$F(1, 16) = 23.15$, $p < 0.001$, $\eta^2 = 0.19$ and $d = 5.65$] and the main effect of rutin treatment [$F(1, 16) = 31.23$, $p < 0.001$, $\eta^2 = 0.26$ and $d = 6.43$]. Further, results of memory retention conducted on day 5 (long-term memory retention) of this study demonstrated significant interaction between CUS-rutin [$F(1, 16) = 97.65$, $p < 0.001$ and $\eta^2 = 0.23$], main effect of CUS [$F(1, 16) = 93.06$, $p < 0.001$, $\eta^2 = 0.22$ and $d = 6.54$] and rutin [$F(1, 16) = 217.7$, $p < 0.001$, $\eta^2 = 0.51$ and $d = 26.12$] (Fig 5.14A).

2) Passive Avoidance-Step Down (PA-SD) Task

Short-term memory evaluated on day 1 revealed significant CUS-rutin interaction [F (1, 16) = 14.33, $p < 0.001$ and $\eta^2 = 0.36$], the main effect of CUS [F (1, 16) = 4.695, $p < 0.05$, $\eta^2 = 0.11$ and d = 2.13] and the main effect of rutin treatment [F (1, 16) = 4.83, $p < 0.05$, $\eta^2 = 0.12$ and d = 2.32]. Further, results of memory retention conducted on day 5 (long-term memory retention) of this study demonstrated significant interaction between CUS-rutin [F (1, 16) = 93.57, $p < 0.001$ and $\eta^2 = 0.28$], main effect of CUS [F (1, 16) = 61.42, $p < 0.001$, $\eta^2 = 0.19$ and d = 8.59] and rutin [F (1, 16) = 153.9, $p < 0.001$, $\eta^2 = 0.47$ and d = 28.28] (Fig 5.14B).

Post hoc evaluation demonstrated that CUS impaired short-term as well as long-term memory retrieval in mice. Rutin treated CUS animals prevented any such memory deficit and hence preventing cognitive decline (Fig 5.14).

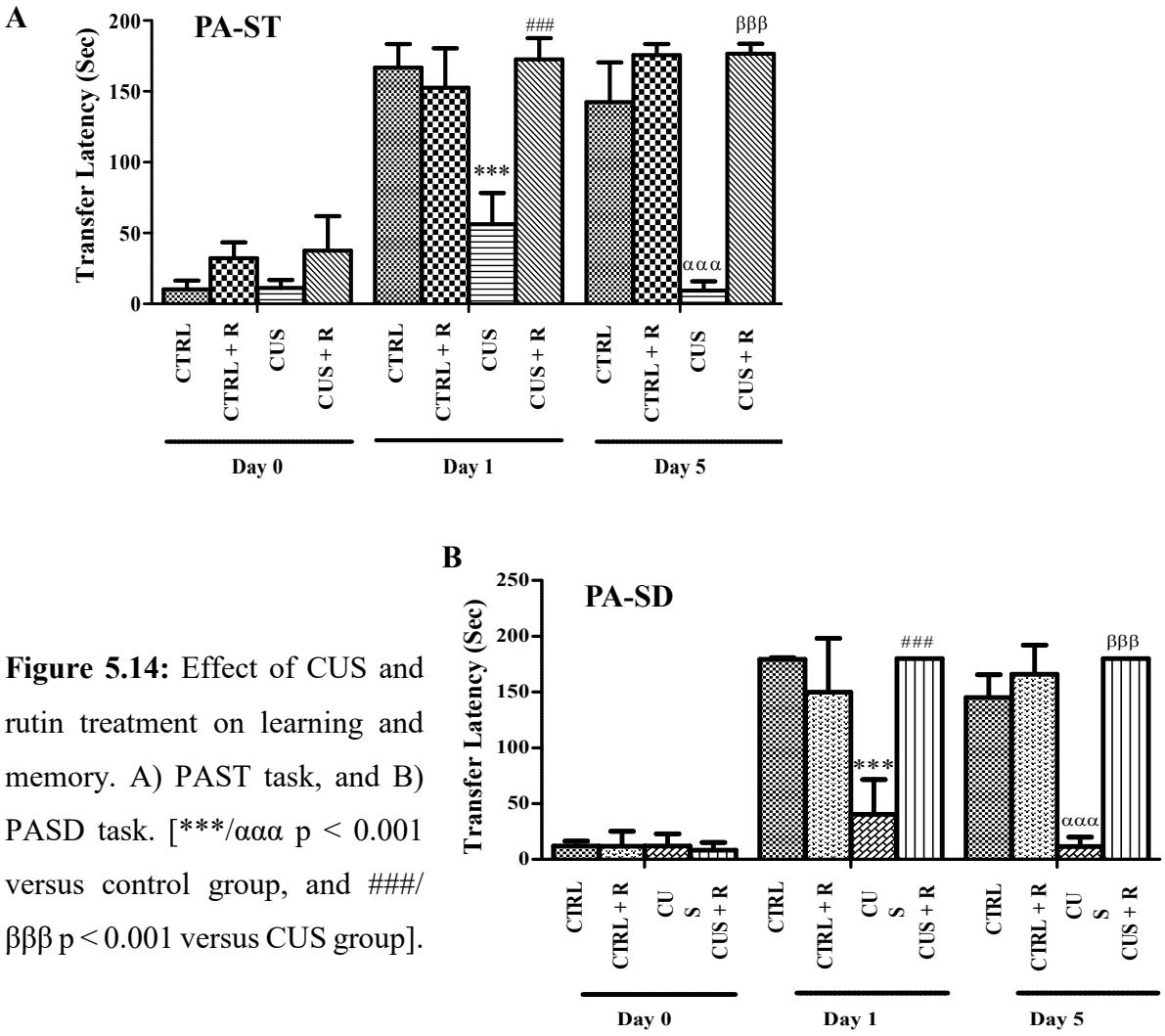


Figure 5.14: Effect of CUS and rutin treatment on learning and memory. A) PAST task, and B) PASD task. [***/ααα $p < 0.001$ versus control group, and ###/ βββ $p < 0.001$ versus CUS group].

3) Novel Object Recognition (NOR) Test

Memory was evaluated in terms of discrimination index (preference between novel and familiar objects). Our results revealed an insignificant CUS-rutin interaction [$F(1, 20) = 2.85$, $p > 0.05$ and $\eta^2 = 0.035$]. The main effect of CUS [$F(1, 20) = 33.18$, $p < 0.001$, $\eta^2 = 0.41$ and $d = 2.9$] and rutin treatment [$F(1, 20) = 25.16$, $p < 0.001$, $\eta^2 = 0.31$ and $d = 2.65$] were observed to be significant (Fig 5.15A).

4) Morris Water Maze (MWM) Test

Spatial memory was assessed using MWM test. Learning was evaluated from day 1 to 4, known as the learning trials. For day 1, our results revealed an insignificant CUS-rutin interaction [$F(1, 20) = 0.21$, $p > 0.05$ and $\eta^2 = 0.002$] and the main effect of CUS [$F(1, 20) = 0.02$, $p > 0.05$, $\eta^2 = 0.0002$ and $d = 0.24$]. The main effect of rutin [$F(1, 20) = 73.77$, $p < 0.001$, $\eta^2 = 0.784$ and $d = 3.51$] was observed to be significant. For day 2, our results revealed that CUS-rutin interaction [$F(1, 20) = 14.13$, $p < 0.001$ and $\eta^2 = 0.27$], the main effect of CUS [$F(1, 20) = 3.18$, $p < 0.0001$, $\eta^2 = 0.06$ and $d = 1.73$], and the main effect of rutin [$F(1, 20) = 13.8$, $p < 0.001$, $\eta^2 = 0.27$ and $d = 2.64$] were significant. For day 3, our results revealed that CUS-rutin interaction [$F(1, 20) = 7.6$, $p < 0.05$ and $\eta^2 = 0.25$], the main effect of CUS [$F(1, 20) = 18.45$, $p < 0.001$, $\eta^2 = 0.28$ and $d = 6.26$], and the main effect of rutin [$F(1, 20) = 21.83$, $p < 0.001$, $\eta^2 = 0.4$ and $d = 7.29$] were significant. For day 4, our results revealed that CUS-rutin interaction [$F(1, 20) = 35.21$, $p < 0.001$ and $\eta^2 = 0.25$], the main effect of CUS [$F(1, 20) = 45.91$ $p < 0.001$, $\eta^2 = 0.62$ and $d = 5.63$], and the main effect of rutin [$F(1, 20) = 65.32$ $p < 0.001$, $\eta^2 = 1.2$ and $d = 6.9$] were significant (Fig 5.15B).

Memory index was evaluated using number of platform crossings (probe trial) and time spent in the platform quadrant. For probe trial (Fig 5.15C), our results revealed a significant CUS-rutin interaction [$F(1, 16) = 8.29$, $p < 0.01$ and $\eta2 = 0.15$], main effect of CUS [$F(1, 16) = 22.09$, $p < 0.001$, $\eta2=0.39$ and $d= 3.3$] and main effect of rutin [$F(1, 16) = 10.14$, $p < 0.01$, $\eta2= 0.18$ and $d= 3.2$]. For time spent in platform quadrant, our results revealed an insignificant CUS-rutin interaction [$F(1, 16) = 2.95$, $p > 0.05$ and $\eta2 = 0.089$]. Main effect of CUS [$F(1, 16) = 1.59$, $p > 0.05$, $\eta2=0.05$ and $d= 1.61$] was insignificant too. Although the main effect of rutin [$F(1, 16) = 12.14$, $p < 0.01$, $\eta2= 0.38$ and $d= 2.17$] was significant (Fig 5.15D).

These findings suggest that chronic stress impairs memory, and that rutin treatment efficiently alleviates the memory dysfunction.

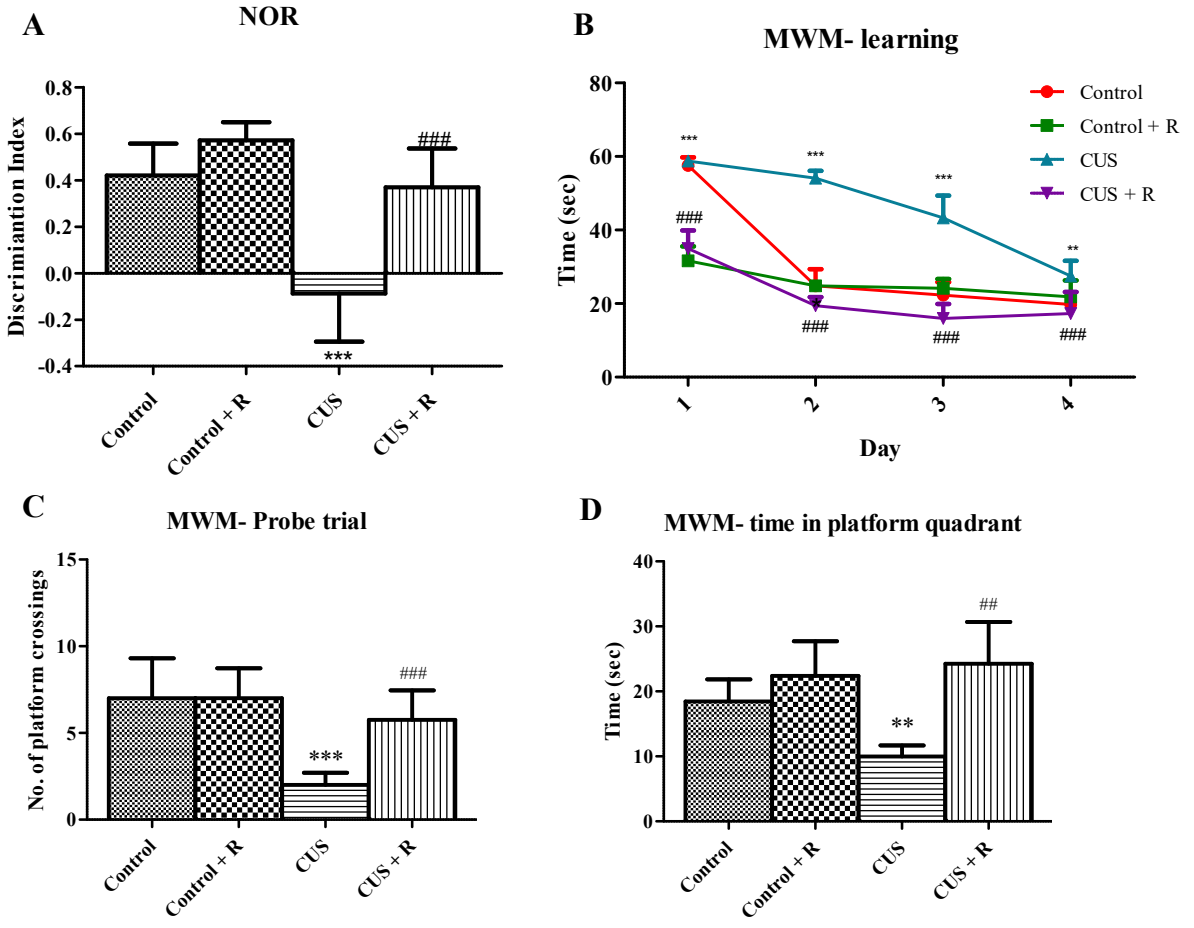


Figure 5.15: Learning and memory. A) NOR-discrimination index, B) MWM-learning, C) MWM-probe trial and D) MWM-time spent in platform quadrant. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]

5.2.6 Histopathological Evaluation (Hematoxylin & Eosin (H&E) Staining)

Integrity of the hippocampal neurons was determined by H&E staining of the 5 µm thick coronal hippocampal section. Neuronal damage was evaluated in CA1, CA2 and CA3 region of the hippocampus in terms of the number of viable neurons observed in one field of the microscope at 400 X magnification (Fig 5.16A). Our results revealed an insignificant CUS-rutin interaction [$F(1, 16) = 2.48$, $p > 0.05$ and $\eta^2 = 0.09$], the main effect of CUS [$F(1, 16) = 4.25$, $p > 0.05$, $\eta^2 = 0.16$ and $d = 2.18$] and the main effect of rutin treatment [$F(1, 16) = 3.53$, $p > 0.05$, $\eta^2 = 0.13$ and $d = 2.85$] in CA1 region (Fig 5.16B). Neuronal integrity analysis in the CA2 region revealed a significant CUS-rutin interaction [$F(1, 16) = 9.07$, $p < 0.01$ and $\eta^2 = 0.16$] and the main effect of rutin treatment [$F(1, 16) = 29.18$, $p < 0.001$, $\eta^2 = 0.53$ and $d = 3.37$]. However, the main effect of CUS [$F(1, 16) = 1.01$, $p > 0.05$, $\eta^2 = 0.018$ and $d = 1.16$] was insignificant (Fig 5.16C). A significant CUS-rutin interaction [$F(1, 16) = 67.76$, $p < 0.001$ and $\eta^2 = 0.42$], the main effect of CUS [$F(1, 16) = 16.5$, $p < 0.001$, $\eta^2 = 0.01$ and $d = 4.75$] and

the main effect of rutin treatment [F (1, 16) = 76.5, $p < 0.001$, $\eta^2 = 0.47$ and d = 7.01] was observed in the CA3 region of the hippocampus (Fig 5.16D). Although, majority of the hippocampus was intact, and did not show any significant damage, we observed that CUS-induced marked damage in the CA3 region. Rutin treatment alleviated this stress-mediated hippocampal neuronal loss.

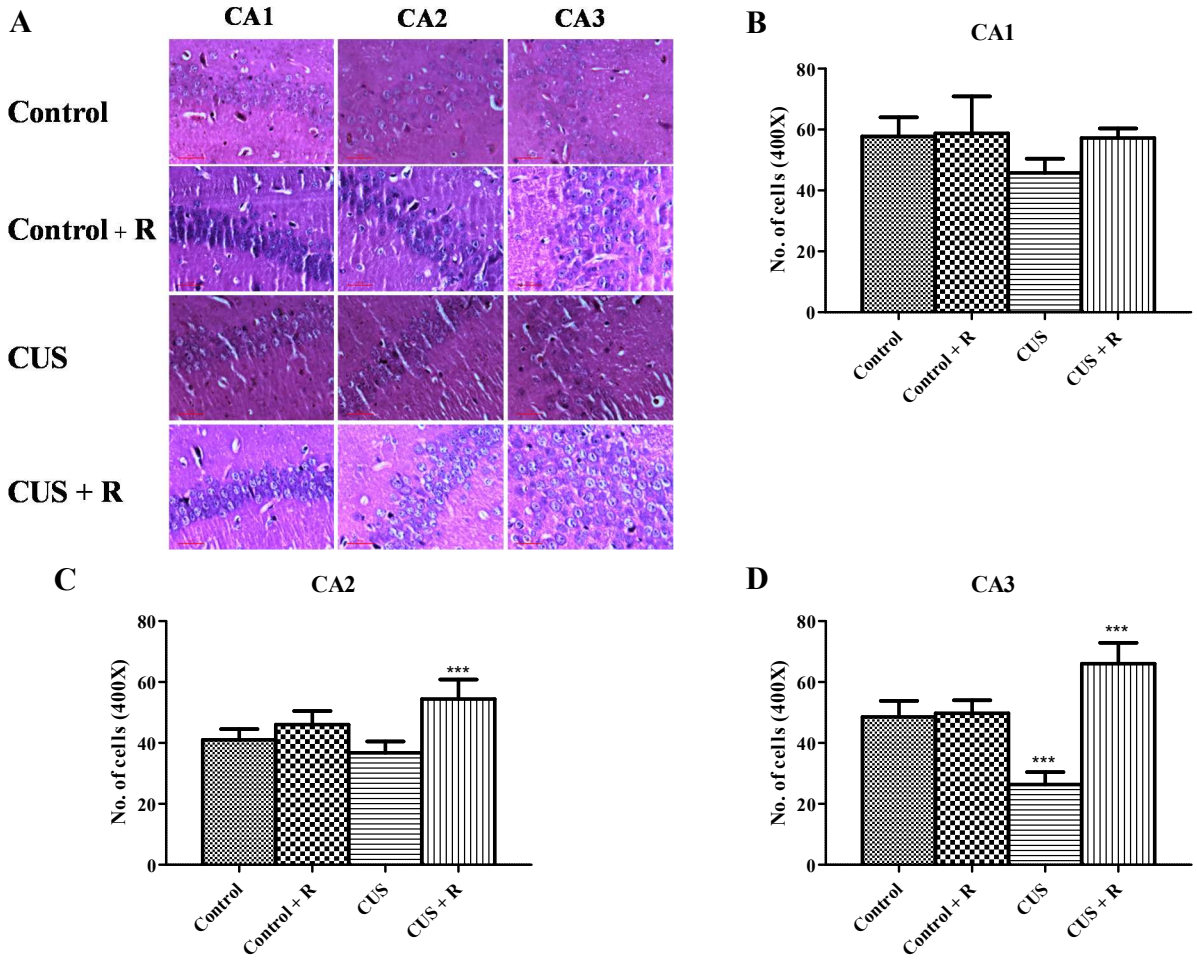


Figure 5.16: Effect of CUS and rutin treatment on hippocampal integrity. Images depict hematoxylin and eosin-stained sections (5 µm) of different regions of hippocampus, and the number of cells at 400X magnification. Results are depicted as mean ± SD (n = 4). [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]

5.2.7 Biochemical Parameters

1) Fasting blood glucose (FBG)

FBG revealed a significant CUS-rutin interaction [F (1, 20) = 11.66, $p < 0.01$ and $\eta^2 = 0.097$], main effect of CUS [F (1, 20) = 60.57, $p < 0.001$ and $\eta^2 = 0.5$ and d = 4.18] and main effect of rutin [F (1, 20) = 28.29, $p < 0.001$ and $\eta^2 = 0.23$ and d = 3.29]. These results indicate that CUS was associated with a significant increase in FBG leading to a development of a pre-diabetic

state in mice. Rutin treatment prevented the development of pre-diabetes and hence showed significant anti-diabetic effect (Fig 5.17A).

2) Oral Glucose Tolerance Test (OGTT)

A two way analysis of the data revealed a significant CUS-rutin interaction at 0 min [$F(1, 20) = 17.58$, $p < 0.001$ and $\eta^2 = 0.19$], main effect of CUS [$F(1, 20) = 23.62$, $p < 0.001$ and $\eta^2 = 0.26$ and $d = 3.93$] and rutin [$F(1, 20) = 27.47$, $p < 0.0001$ and $\eta^2 = 0.31$ and $d = 3.87$]. At 30 min of the OGTT we observed a non-significant CUS-rutin interaction [$F(1, 20) = 1.71$, $p > 0.05$ and $\eta^2 = 0.01$], a significant main effect of CUS [$F(1, 20) = 70.66$, $p < 0.001$ and $\eta^2 = 0.55$ and $d = 4.07$] and rutin [$F(1, 20) = 35.68$, $p < 0.001$ and $\eta^2 = 0.27$ and $d = 2.32$]. Two way analysis of OGTT at 60 min time revealed a significant CUS-rutin interaction [$F(1, 20) = 10.61$, $p < 0.01$ and $\eta^2 = 0.11$], main effect of CUS [$F(1, 20) = 35.55$, $p < 0.001$ and $\eta^2 = 0.39$ and $d = 3.76$] and rutin [$F(1, 20) = 24.53$, $p < 0.001$ and $\eta^2 = 0.27$ and $d = 2.56$]. At 120 min of the OGTT, we observed a non-significant CUS-rutin interaction [$F(1, 20) = 46.23$, $p < 0.001$ and $\eta^2 = 0.28$], a significant main effect of CUS [$F(1, 20) = 58.32$, $p < 0.001$ and $\eta^2 = 0.36$ and $d = 5.82$] and rutin [$F(1, 20) = 34.93$, $p < 0.001$ and $\eta^2 = 0.21$ and $d = 4.52$] (Fig 5.17B).

3) Serum Insulin level

Two-way ANOVA of the insulin levels revealed a significant CUS-rutin interaction [$F(1, 12) = 87.33$, $p < 0.001$ and $\eta^2 = 0.26$], a main effect of CUS [$F(1, 12) = 177.9$, $p < 0.001$, $\eta^2 = 0.53$ and $d = 8.6$], and rutin treatment [$F(1, 12) = 52.9$, $p < 0.001$, $\eta^2 = 0.16$ and $d = 7.2$]. Post hoc comparison revealed a significant increase in the serum levels of insulin in 21 day chronically stressed animals and treating animals with rutin resulted in a significant lowering in serum insulin levels, when compared to CUS (Fig 5.17C).

4) HOMA-IR index (Homeostatic Model of Assessment for Insulin Resistance)

Development of insulin resistance in the chronically stressed animals was evaluated in terms of HOMA-IR index. Results of the two-way ANOVA revealed a significant CUS-rutin interaction [$F(1, 12) = 86.52$, $p < 0.001$ and $\eta^2 = 0.22$], a main effect of CUS [$F(1, 12) = 215.8$, $p < 0.001$, $\eta^2 = 0.55$ and $d = 10.49$] and rutin treatment [$F(1, 12) = 71.4$, $p < 0.001$, $\eta^2 = 0.18$ and $d = 6.4$]. Post hoc comparison of the results of HOMA index suggest that CUS is associated with the development of significant insulin resistance in 21 days, when compared to CTRL. Treating stressed animals with rutin for 21 days significantly lowered the HOMA-IR index when compared to CUS, suggesting that rutin can efficiently improve insulin sensitivity in stressed animals (Fig 5.17D).

5) Serum Cortisol Level

Hypercortisolemia is the outcome of stress and we evaluated the effect of CUS and rutin treatment on serum cortisol levels. Results of the serum cortisol levels demonstrated a significant CUS-rutin interaction [$F(1, 12) = 121.7$, $p < 0.001$ and $\eta^2 = 0.16$], a main effect of CUS [$F(1, 12) = 519.2$, $p < 0.001$, $\eta^2 = 0.69$ and $d = 14.34$], and rutin treatment [$F(1, 12) = 95.88$, $p < 0.001$, $\eta^2 = 0.12$ and $d = 8.4$]. Post hoc analysis revealed that chronic stress significantly elevated serum cortisol levels when compared to control. Rutin efficiently lowered the stress levels and serum cortisol levels were found to be significantly lower than CUS (Fig 5.17E).

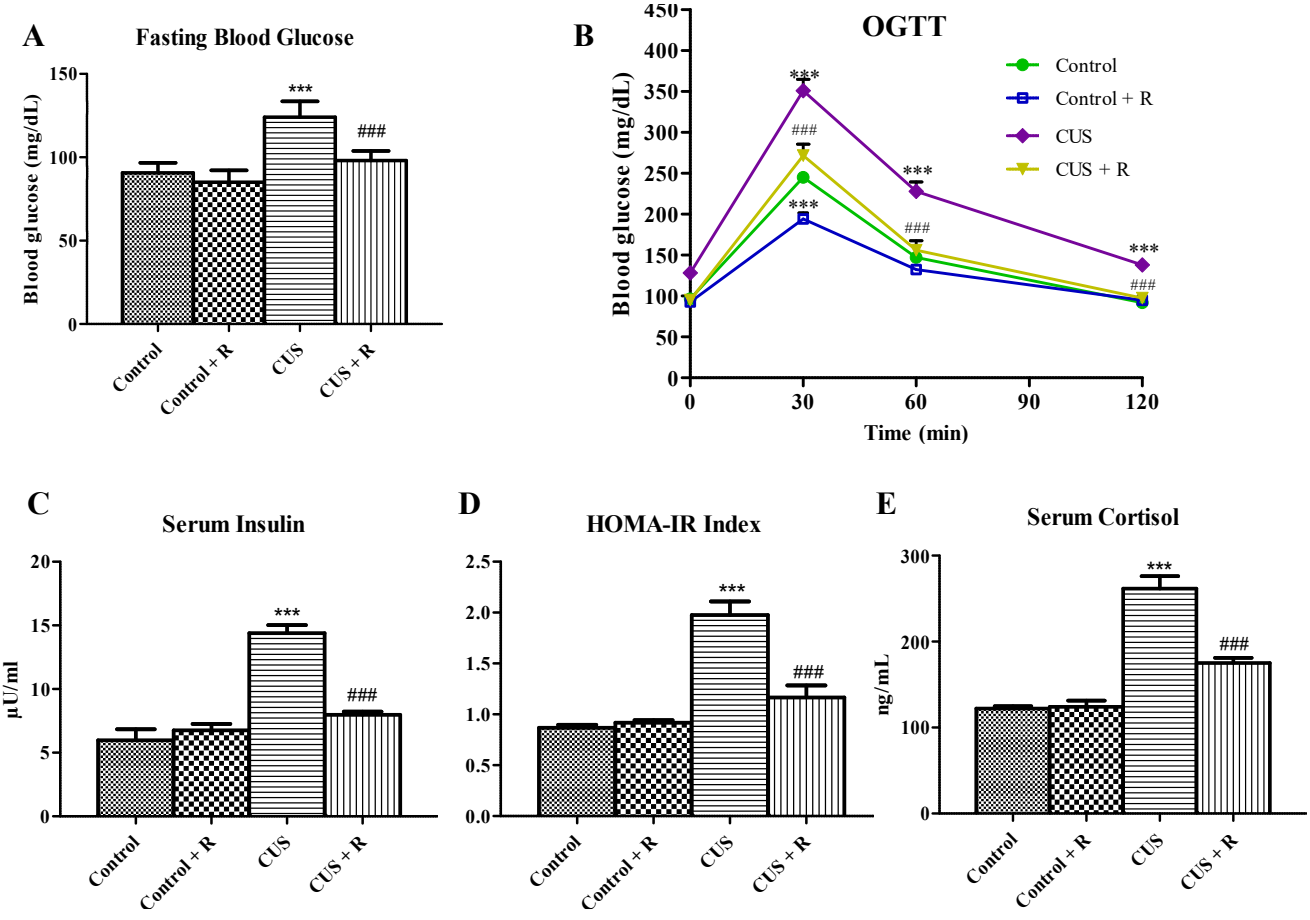


Figure 5.17: Effect of CUS and rutin treatment on A) fasting blood glucose, B) oral glucose tolerance test (OGTT), C) serum insulin levels, D) insulin resistance (HOMA-IR index), and E) serum cortisol levels (A). Results are depicted as mean ± SD (n = 4-6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test) and two-way ANOVA. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]

5.2.8 Protein expression

1) Western Blot

For hippocampal insulin expression (Fig 5.18B) our results showed insignificant CUS-rutin interaction [F (1, 12) = 0.48, p > 0.05 and η^2 = 0.04], main effect of CUS [F (1, 12) = 0.44, p > 0.05, η^2 = 0.03 and d = 0.02] and main effect of rutin [F (1, 12) = 0.31, p > 0.05, η^2 = 0.02 and d = 0.07]. For InR (Fig 5.18C), our results revealed a significant CUS-rutin interaction [F (1, 12) = 5.47, p < 0.05 and η^2 = 0.19], main effect of CUS [F (1, 12) = 9.38, p < 0.01, η^2 = 0.32 and d = 2.07] and main effect of rutin [F (1, 12) = 2.54, p < 0.05, η^2 = 0.09 and d = 1.54. In case of GLUT4 (Fig 5.18D), our results reveal significant CUS-rutin interaction [F (1, 12) = 9.95, p < 0.01 and η^2 = 0.11], main effect of CUS [F (1, 12) = 2.08, p < 0.05, η^2 = 0.024 and d = 3.18] and main effect of rutin [F (1, 12) = 64.24, p < 0.001, η^2 = 0.73 and d = 5.7]. Our results suggest that CUS led to the development of an InR state in the hippocampus with increased IR and decreased GLUT4 expression, even though no changes in insulin expression were observed. Rutin improved the insulin signaling by decreasing the IR but increasing the GLUT4 expression (Fig 5.18).

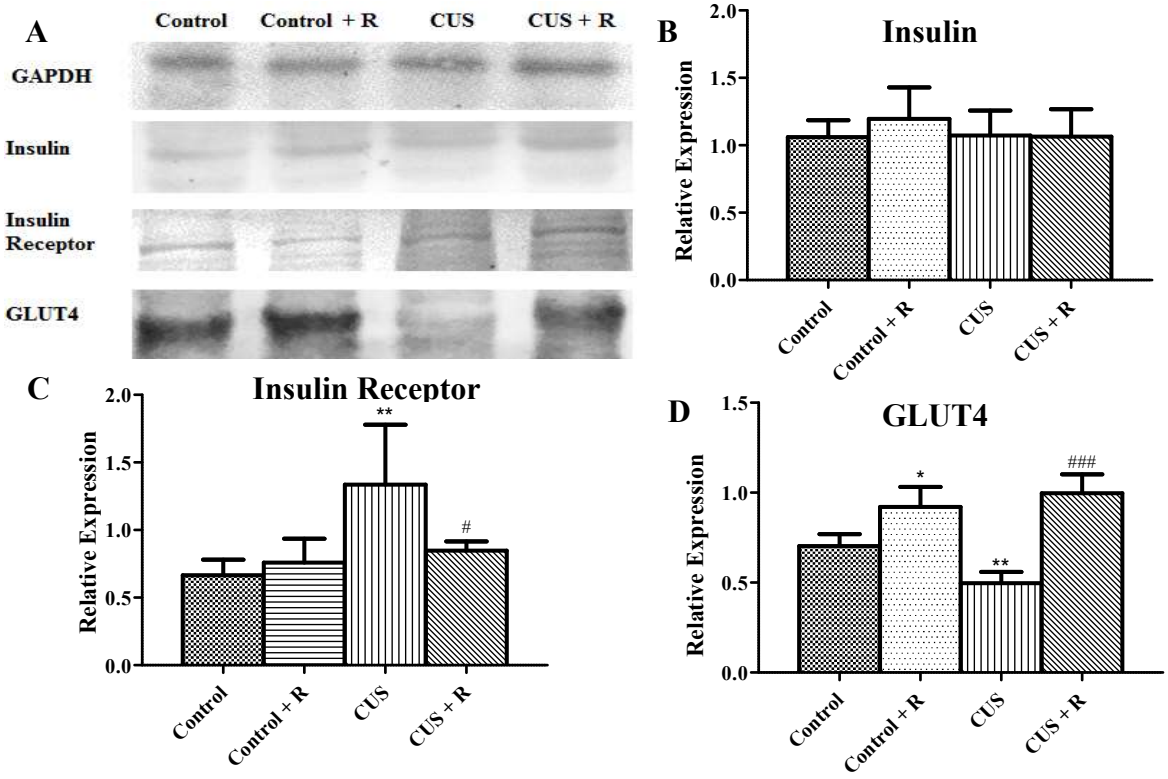


Figure 5.18: Effect of CUS and rutin treatment on A) the hippocampal immunoblot analysis, relative expression of B) insulin, C) insulin receptor, and D) GLUT4. Results are depicted as mean ± SD (n = 4). Statistical significance was determined using one-way ANOVA followed

by Dunnett post hoc test) and two-way ANOVA. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]

2) Immunofluorescence

Immunofluorescence was measured using corrected total cell fluorescence (CTCF) (Fig 5.19). For hippocampal InR (Fig 5.19B), our results showed insignificant CUS-rutin interaction [F (1, 12) = 1.11, p > 0.05 and η^2 = 0.02]. Although, the main effect of CUS [F (1, 12) = 17.45, p < 0.001, η^2 = 0.42 and d = 2.82] and the main effect of rutin [F (1, 12) = 10.3, p < 0.01, η^2 = 0.25 and d = 2.97] were observed to be significant. For GLUT4 (Fig 5.19C), CUS-rutin interaction [F (1, 12) = 20.39, p < 0.001 and η^2 = 0.14], the main effect of CUS [F (1, 12) = 4.75, p < 0.05, η^2 = 0.034 and d = 1.55] and the main effect of rutin [F (1, 12) = 101.9, p < 0.001, η^2 = 0.73 and d = 6.93] were observed to be significant. We observed central IR with increased InR and reduced GLUT4 fluorescence. Rutin treatment restored this anomaly by downregulating InR and upregulating GLUT4, suggesting a direct role in modulating central insulin signalling.

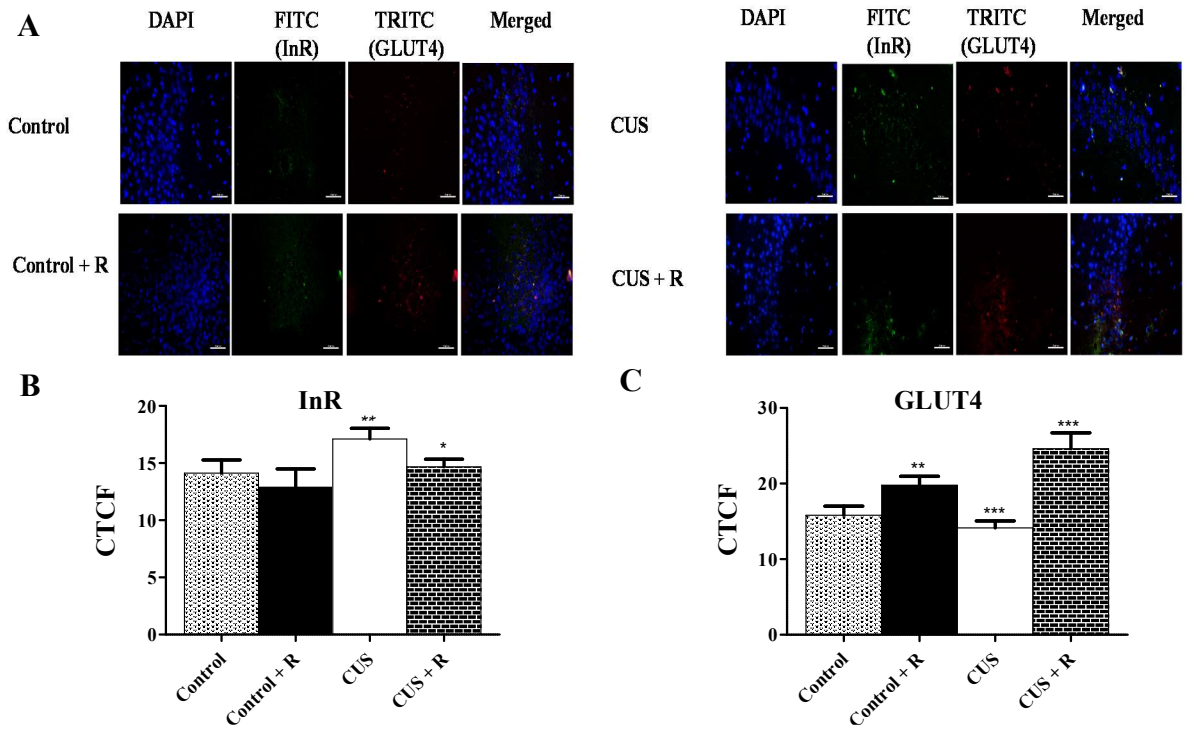


Figure 5.19: Effect of CUS and rutin treatment on the expression of insulin receptor (InR) and glucose transporter 4 (GLUT4) in the entire hippocampal CA3 region. (A) Immunofluorescence images at 400 X magnification with DAPI (blue, nucleus), FITC (green, InR), and TRITC (red, GLUT4). Corrected total cell fluorescence (CTCF) of (B) InR, and (C) GLUT4. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]

CHAPTER 6

DISCUSSION

6. DISCUSSION

Diabetes is currently one of the leading cause of global health burden. Besides being untreatable, its pathology like cardiovascular complications, nephropathy, and retinopathy eventually lead to premature death. Largely, considered as a peripheral disorder, recent times have witnessed an increased recognition of the central complications of diabetes. Diabetes is now being considered as a potential risk factor for neurological complications like depression, cognitive dysfunction, dementia and even Alzheimer's disease. In our previous reports, found that hydroalcoholic leaf extract of Urtica dioica efficiently improved diabetes mediated behavioral dysfunctions [59, 60, 62], and contained high amount of rutin [62]. Therefore, we evaluated the effect of rutin on long-standing diabetes mediated behavioral dysfunction.

We used the multiple low dose STZ induced diabetes model to better mimic the type 2 phenotype of diabetes rather than type 1. Physiological hallmarks of T2DM like polydipsia, polyphagia, and weight loss were observed in diabetic animals, which were prevented by rutin treatment (Fig 5.1). Biochemical analysis revealed hypoinsulinemia, increased FBG levels, and impaired glucose tolerance. Rutin treatment inhibited diabetes progression by increasing insulin levels, reducing FBG levels and improving glucose tolerance (Fig 5.2). Interestingly in OGTT, glucose levels in rutin treated control group was found to be lower than the control group, suggesting that rutin could prevent hyperglycemia in both diabetic and normal state.

Diabetes resulted in significant neurobehavioral complications, like impaired locomotion & muscle coordination, depression, anxiety, and learning & memory dysfunction. Locomotion & muscle coordination was impeded in diabetic animals as observed by a reduced number of line crossings in the OFT and increased time taken to cross a beam in the beam walk test (Fig 5.3). Diabetic induced anxiety, as suggested increased preference to the closed arm of EPM and hesitance to enter the central region of the open field (Fig 5.4). Diabetes induced significant depression in mice as suggested by the results of SPT, TST, and FST (Fig 5.5). Diabetes led to heightened despair as observed by higher immobility and reduced will to struggle in the TST and FST. Further, diabetes induced significant anhedonia, as suggested by reduced preference towards the sucrose water in the SPT. Further, persistent hyperglycemia led to cognitive deficits as assessed using learning & memory function tests (Fig 5.6). Diabetic animals showed impaired short -term and long term associative memory in PA-SD (Fig 5.6A). Diabetic animals showed impaired recognition memory during NOR test (Fig 5.6B). During MWM test we

observed that diabetic animals had learning impairment as they struggled to find the hidden platform (Fig 5.6C). And as expected they had significant working memory deficits during the MWM probe trial test (Fig 5.6D).

Rutin treated animals appeared healthy and didn't show any signs of behavioral deficits during the experimentation. Rutin treatment ameliorated the diabetes-induced impairment in locomotion & muscle coordination, as they had a higher number of line crossings in the OFT and took surprisingly less time to cross the beam in beam walk test as compared to vehicle-treated diabetic animals (Fig 5.3). Rutin attenuated the diabetes-induced anxiety, as observed by increased time spent exploring open arm of EPM and higher entries & time spent in the central region of the open field arena compared vehicle-treated diabetic animals (Fig 5.4). Rutin alleviated the diabetes mediated depression and anhedonia, as the animals showed increased struggling and reduced immobility during TST and FST and preferred sweetened water (Fig 5.5). Rutin treatment attenuated the diabetes-induced learning & memory deficits. Rutin treated animals were able to differentiate between novel and familiar object in NOR test, had improved short and long term memory retrieval during PA-SD, showed significantly improved learning in the MWM task, and exhibited excellent working memory during probe trial of MWM task (Fig 5.6).

Hippocampus is a critical region of the brain involved in the regulation of the behavioral functions. Hippocampal insult is involved in neurological complications like cognitive decline, depression, and anxiety [59]. Unscathed neurophysiological and neuroanatomical integrity in the hippocampus, is paramount for the intact neurobehavioral outcome. Since the hippocampus is highly sensitive to the hyperglycemia-induced neurodegenerative process [59], we performed the Golgi - Cox staining to visualize the hippocampal neuronal integrity (Fig 5.7). 8-weeks of consistent hyperglycemia resulted in significant neurodegeneration. Hippocampal neurons, especially in the CA3 region, of the diabetic animals appeared degenerated. Although the number of branches arising from soma were similar in all the groups, there a significant reduction in the number of branches of 100 μm length. Further, good spike density of the hippocampal neurons was also evaluated since it reflects the health of neuron and the new connections it can make. Diabetic hippocampi showed significantly reduced spike density (1000 X) and lower dendritic arborization (400 X). CA3 neuronal integrity is necessary for the intact neurobehavioral functions, and any damage is associated cognitive deficits [141, 142].

Rutin treatment showed potential neuroprotective effects by significantly increasing the dendritic arborization and spike density, and rescuing neurons from diabetic complications.

To understand the mechanistic viewpoints of diabetes and associated central complications, we explored neuronal insulin signaling pathway. The role of insulin in brain glucose uptake has always been a controversial issue. Even to this date, a large fraction of scientists believes that brain's glucose uptake in insulin-independent even though insulin has several other important roles like maintaining synaptic plasticity, neurotransmission, cognitive functioning, and satiety & appetite control. This differential role of insulin forms the basis of our research gap, which is why we aimed to investigate the expression of insulin signaling proteins (insulin, InR, and GLUT4), and whether or not they correlate to the central diabetic pathology.

Western blot analysis revealed that diabetes led to a significantly decreased insulin expression, but did not alter the InR and GLUT4 expression. Rutin treatment significantly upregulated the expression of insulin, InR, and GLUT4 in both control and diabetic groups (Fig 5.8). Similar results were obtained by immunofluorescence analysis of InR and GLUT4 in CA3 region of the hippocampus (Fig 5.9). The protein expression results might suggest that diabetic brain is independent of insulin signaling, as InR and GLUT4 levels didn't change, but it also has a beautiful interpretation that higher InR and GLUT4, such as observed in rutin treatment, can treat diabetes and its complications, exactly what rutin did. Upregulation of IR and GLUT4 expression independent of insulin in both control and the diabetic state could be the underlining of rutin's neuroprotective benefits.

Therefore we hypothesize that it is the time we focus on central therapeutics for diabetes management. With, IR being the major hurdle in diabetes, use of naturally occurring compounds like rutin, that circumvent the IR, could really change the dynamics of current diabetic therapeutics.

Since STZ induces diabetes by destroying the pancreatic β cells, therefore to use this model for T2DM is a debatable question. Therefore, to better mimic the natural induction of T2DM like state, we proceeded with CUS model in Swiss albino mice, which we have previously standardized in our lab to induce insulin resistance [61]. In line with our previous diabetes model, we evaluated the effect of CUS and rutin treatment on the development of hyperglycemia, insulin resistance, glucose intolerance and hippocampal insulin signaling along with the associated behavioral dysfunction.

CUS led to significant reduction in the body weight which was prevented by rutin treatment (Fig 5.10A). CUS didn't cause any significant change in feed intake, but rutin treatment in both control and CUS groups caused increased feed intake (Fig 5.10B). CUS and rutin treatment had no effect on water intake (Fig 5.10C).

CUS resulted in significant neurobehavioral complications, like impaired locomotion & muscle coordination, depression, anxiety, and learning & memory dysfunction. Locomotion & muscle coordination was impeded in stressed animals as observed by a reduced number of line crossings in the OFT and increased time taken to cross a beam in the beam walk test (Fig 5.11). CUS-induced anxiety, as suggested increased preference to the closed arm of EPM and hesitance to enter the central region of the open field (Fig 5.12). Stress-induced significant depression in mice as suggested by the results of SPT, TST, and FST (Fig 5.13). Stress led to heightened despair as observed by higher immobility and reduced will to struggle in the TST and FST. Further, CUS induced significant anhedonia, as suggested by reduced preference towards the sucrose water in the SPT. Further, chronic stress led to cognitive deficits as assessed using learning & memory function tests (Fig 5.14-15). Stressed animals showed impaired short -term and long term associative memory in PA-SD and PA-ST (Fig 5.14). Stressed animals showed impaired recognition memory during NOR test (Fig 5.15). During MWM test we observed that stressed animals had learning impairment as they struggled to find the hidden platform. And as expected they had significant working memory deficits during the MWM probe trial test.

Rutin treated animals appeared healthy and didn't show any signs of behavioral deficits during the experimentation. Rutin treatment ameliorated the CUS-induced impairment in locomotion & muscle coordination, as they had a higher number of line crossings in the OFT and took surprisingly less time to cross the beam in beam walk test as compared to vehicle-treated stressed animals (Fig 5.11). Rutin attenuated the CUS-induced anxiety, as observed by increased time spent exploring open arm of EPM and higher entries & time spent in the central region of the open field arena compared vehicle-treated stressed animals (Fig 5.12). Rutin alleviated the stress mediated depression and anhedonia, as the animals showed increased struggling and reduced immobility during TST and FST and preferred sweetened water (Fig 5.13). Rutin treatment attenuated the CUS-induced learning & memory deficits. Rutin treated animals were able to differentiate between novel and familiar object in NOR test, had improved short and long term memory retrieval during PA-SD, showed significantly improved learning

in the MWM task, and exhibited excellent working memory during probe trial of MWM task (Fig 5.14-15).

Hippocampal neurons are highly vulnerable to the hypercorticosteronemia-a hallmark of chronic stress. Hypercorticosteronemia damages neurons by disrupting synaptic plasticity [143, 144], reducing hippocampal volume [145] and impairing neuronal homeostasis [59], hence compromising the behavioral outcomes. Therefore, we examined the hippocampal neuronal integrity using hematoxylin & eosin staining for number of viable cells in one field at 400 X. CUS resulted in significant reduction in the number of viable cells in the CA3 region as compared to control. Rutin treatment significantly prevented this damage (Fig 5.16D). Rutin treatment also increased the viable neurons in CA2 region when compared with control and CUS group, indicating that rutin treatment could be involved in supporting neurogenesis (Fig 5.16C).

To validate the CUS model for diabetes, we evaluated the FBG levels, glucose tolerance, serum insulin, HOMA-IR index and serum cortisol levels. CUS resulted in significantly elevated FBG levels which fell under the category of pre-diabetic (Fig 5.17A). CUS also led to impaired glucose tolerance (Fig 5.17B). Further, stressed animals were having significantly higher serum insulin (Fig 5.17C) and cortisol levels (Fig 5.17E) when compared to control. Results of HOMA-IR index revealed that CUS led to the development of IR (Fig 5.17D). Rutin treatment alleviated pre-diabetes, and serum insulin and cortisol levels. It is probably why rutin also markedly alleviated IR and improved glucose tolerance (Fig 5.17). These findings suggest that CUS led to the development of T2DM like state and that rutin treatment reversed these complications.

Once again for the molecular underpinning of central diabetic complications, we evaluated expression analysis of insulin signaling (insulin, InR, and GLUT4) in the hippocampus (Fig 5.18). Expression analysis was performed using western blot (insulin, InR, and GLUT4) and immunofluorescence (InR and GLUT4 in CA3 region). In western blot, although expression of insulin showed no change in any of the groups (Fig 5.18B), CUS led to significantly increased InR (Fig 5.18C) and concomitantly reduced GLUT4 expression (Fig 5.18D) compared to control, suggesting the development of hippocampal IR like state. Rutin treatment downregulated the InR expression and upregulated GLUT4 in stressed animals thereby not only reversing IR but improving insulin sensitivity. Interestingly, rutin upregulated the GLUT4 expression in both control and CUS animals, suggesting a direct effect independent of InR

signaling (Fig 5.18). These results were reinforced by the corrected total cell fluorescence (CTCF) observed for InR and GLUT4 in CA3 region of the hippocampus (Fig 5.19). These findings suggest that rutin might have alleviated the CUS mediated neurobehavioral dysfunctions by modulating central IR and the neuronal insulin signaling pathway.

CHAPTER 7

CONCLUSION

7. CONCLUSION

Rationale behind selection of rutin was that in our previous work, we found that Urtica Dioica leaf extract had neuroprotective effects in diabetes and depression. The extract contained a high quantity of rutin in it. Therefore we selected rutin for its neuroprotective effects against diabetes induced neurological complications. To evaluate the effect of rutin on long standing diabetes, we used multiple low dose STZ induced diabetes model. After STZ injections, animals developed significant hyperglycemia, glucose intolerance and hypoinsulinemia. Rutin treatment showed potential anti-diabetic effects by significantly improving the glycemia, and glucose tolerance and increasing the insulin levels. We observed that diabetes led to polyphagia, polydipsia and reduced body weight. Rutin treatment efficiently rescued all of these complications in diabetic animals. Further we observed that diabetes led to impaired neurobehavioral outcomes like anxiety, depression and cognitive decline. Rutin treatment was effective in preventing these complications thereby serving its neuroprotective role efficiently. The neuroanatomical basis of the neurological complications of diabetes were associated with severe hippocampal neurodegeneration. Hippocampal neurons in diabetes had reduced dendritic arborization and spike density. Rutin treatment significantly prevented this damage and neurons appeared healthy. To understand the molecular basis of the central diabetic complications, we evaluated the insulin signalling in brain and whether or not it correlates with the disease pathology. We studied protein expression analysis of insulin, InR and GLUT4 via western blot (hippocampus), and InR and GLUT4 via immunofluorescence (in CA3 region of hippocampus). Our results revealed that the neuroprotective effects of rutin and its anti-diabetic potential could be attributed to its ability to up-regulate insulin, InR and GLUT4.

To better mimic the natural ways of diabetes induction, we employed the CUS led depression-induced diabetes model. CUS led to development of hyperglycemia (pre-diabetes), increased serum insulin, and insulin resistance (HOMA-IR index). Rutin treatment effectively prevented the hyperglycemia, reduce insulin levels and prevented the development of insulin resistance. CUS led to neurological complications like anxiety, depression and cognitive deficits, which didn't occur in rutin treated stressed animals. Rutin treatment also prevented the CUS induced hippocampal neurodegeneration and central insulin resistance. To conclude, rutin's anti-diabetic properties and neuroprotective effects against the central complications could be attributed to its ability to modulate hippocampal insulin signalling.

With significant advances in healthcare management and diabetes therapeutics, life expectancy in diabetic patients, continuous therapy, social stress and increased lifespan of the diabetic population have modified the spectrum of diabetic complications and associated morbidity. Therefore, besides traditional diabetic complications, a new set of unexpected complications have started to emerge which includes cancer, physical disability, cognitive dysfunction, depression etc. Diversification of diabetic complications and increased lifetime spent in the diabetic state has led to increased financial burden, intensification of disease and life monitoring quality system. Therefore we need to critically assess the current diabetic therapeutics, and search for suitable alternatives that could halt the diabetic progression as well as reverse the central complications that have already set in. With, insulin resistance being the major hurdle in diabetes, use of naturally occurring compounds like rutin, that circumvent the insulin resistance, could really change the dynamics of current diabetic therapeutics.

CHAPTER 8

REFERENCES

8. REFERENCES

[1] I. D. Atlas, "International Diabetes Federation, Brussels, 2015," ed, 2015.

[2] A. A. Sima, H. Kamiya, and Z. G. Li, "Insulin, C-peptide, hyperglycemia, and central nervous system complications in diabetes," *European journal of pharmacology,* vol. 490, pp. 187-197, 2004.

[3] H. Bachelard, "Cerebral metabolism and hypoglycaemia," *Hypoglycaemia. V. Marks and FC Rose, editors. Blackwell Scientific Publications, Oxford,* pp. 51-68, 1981.

[4] I. Cranston, P. Marsden, K. Matyka, M. Evans, J. Lomas, P. Sonksen*, et al.*, "Regional differences in cerebral blood flow and glucose utilization in diabetic man: the effect of insulin," *J Cereb Blood Flow Metab,* vol. 18, pp. 130-40, Feb 1998.

[5] S. G. Hasselbalch, G. M. Knudsen, C. Videbaek, L. H. Pinborg, J. F. Schmidt, S. Holm*, et al.*, "No effect of insulin on glucose blood-brain barrier transport and cerebral metabolism in humans," *Diabetes,* vol. 48, pp. 1915-21, Oct 1999.

[6] I. Arrieta-Cruz and R. Gutierrez-Juarez, "The Role of Insulin Resistance and Glucose Metabolism Dysregulation in the Development of Alzheimer s Disease," *Rev Invest Clin,* vol. 68, pp. 53-8, Mar-Apr 2016.

[7] J. C. Bruning, D. Gautam, D. J. Burks, J. Gillette, M. Schubert, P. C. Orban*, et al.*, "Role of brain insulin receptor in control of body weight and reproduction," *Science,* vol. 289, pp. 2122-5, Sep 22 2000.

[8] S. C. Woods, E. C. Lotter, L. D. McKay, and D. Porte, Jr., "Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons," *Nature,* vol. 282, pp. 503-5, Nov 29 1979.

[9] K. F. Neumann, L. Rojo, L. P. Navarrete, G. Farías, P. Reyes, and R. B. Maccioni, "Insulin resistance and Alzheimer's disease: molecular links & clinical implications," *Current Alzheimer Research,* vol. 5, pp. 438-447, 2008.

[10] D. P. Figlewicz, M. D. Brot, A. L. McCall, and P. Szot, "Diabetes causes differential changes in CNS noradrenergic and dopaminergic neurons in the rat: a molecular study," *Brain Res,* vol. 736, pp. 54-60, Oct 14 1996.

[11] I. Y. Choi and R. Gruetter, "In vivo 13C NMR assessment of brain glycogen concentration and turnover in the awake rat," *Neurochem Int,* vol. 43, pp. 317-22, Sep-Oct 2003.

[12] K. D. Niswender, C. D. Morrison, D. J. Clegg, R. Olson, D. G. Baskin, M. G. Myers, Jr., *et al.*, "Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia," *Diabetes,* vol. 52, pp. 227-31, Feb 2003.

[13] D. K. Sindelar, P. Mystkowski, D. J. Marsh, R. D. Palmiter, and M. W. Schwartz, "Attenuation of diabetic hyperphagia in neuropeptide Y--deficient mice," *Diabetes,* vol. 51, pp. 778-83, Mar 2002.

[14] A. J. Sipols, D. G. Baskin, and M. W. Schwartz, "Effect of intracerebroventricular insulin infusion on diabetic hyperphagia and hypothalamic neuropeptide gene expression," *Diabetes,* vol. 44, pp. 147-51, Feb 1995.

[15] N. P. Birch, D. L. Christie, and A. G. Renwick, "Proinsulin-like material in mouse foetal brain cell cultures," *FEBS letters,* vol. 168, pp. 299-302, 1984.

[16] S. U. Devaskar, S. J. Giddings, P. A. Rajakumar, L. R. Carnaghi, R. K. Menon, and D. S. Zahm, "Insulin gene expression and insulin synthesis in mammalian neuronal cells," *Journal of Biological Chemistry,* vol. 269, pp. 8445-8454, 1994.

[17] S. M. de la Monte and J. R. Wands, "Alzheimer's disease is type 3 diabetes-evidence reviewed," *J Diabetes Sci Technol,* vol. 2, pp. 1101-13, Nov 2008.

[18] R. Haque and A. Nazir, "Insulin-degrading enzyme: a link between Alzheimer's and type 2 diabetes mellitus," *CNS Neurol Disord Drug Targets,* vol. 13, pp. 259-64, Mar 2014.

[19] W. Q. Qiu and M. F. Folstein, "Insulin, insulin-degrading enzyme and amyloid-beta peptide in Alzheimer's disease: review and hypothesis," *Neurobiol Aging,* vol. 27, pp. 190-8, Feb 2006.

[20] G. A. Werther, A. Hogg, B. J. Oldfield, M. J. McKinley, R. Figdor, A. M. Allen*, et al.*, "Localization and characterization of insulin receptors in rat brain and pituitary gland using in vitro autoradiography and computerized densitometry," *Endocrinology,* vol. 121, pp. 1562-70, Oct 1987.

[21] J. M. Hill, M. A. Lesniak, C. B. Pert, and J. Roth, "Autoradiographic localization of insulin receptors in rat brain: prominence in olfactory and limbic areas," *Neuroscience,* vol. 17, pp. 1127-38, Apr 1986.

[22] M. Shimada, Y. Hirose, M. Nakanishi, H. Watanabe, S. Kawamoto, and M. Watanabe, "Autoradiographic studies on insulin receptor of the mouse hippocampus," *Recent Advances in Cellular and Molecular Biology,* vol. 4, pp. 271-278, 1992.

[23] W. J. Zhang, Y. F. Tan, J. Yue, M. Vranic, and J. Wojtowicz, "Impairment of hippocampal neurogenesis in streptozotocin-treated diabetic rats," *Acta Neurologica Scandinavica,* vol. 117, pp. 205-210, 2008.

[24] J. Beauquis, F. Saravia, J. Coulaud, P. Roig, M. Dardenne, F. Homo-Delarche*, et al.*, "Prominently decreased hippocampal neurogenesis in a spontaneous model of type 1 diabetes, the nonobese diabetic mouse," *Experimental neurology,* vol. 210, pp. 359-367, 2008.

[25] A. M. Stranahan, T. V. Arumugam, R. G. Cutler, K. Lee, J. M. Egan, and M. P. Mattson, "Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons," *Nature neuroscience,* vol. 11, pp. 309-317, 2008.

[26] B. T. Lang, Y. Yan, R. J. Dempsey, and R. Vemuganti, "Impaired neurogenesis in adult type-2 diabetic rats," *Brain research,* vol. 1258, pp. 25-33, 2009.

[27] J. A. Luchsinger, "Type 2 diabetes and cognitive impairment: linking mechanisms," *Journal of Alzheimer's Disease,* vol. 30, pp. S185-S198, 2012.

[28] C. T. Kodl and E. R. Seaquist, "Cognitive dysfunction and diabetes mellitus," *Endocrine reviews,* vol. 29, pp. 494-511, 2008.

[29] J. M. Berg, J. L. Tymoczko, and L. Stryer, "Biochemistry 5th edition, 2002," ed: WH Freeman. Available online through the NCBI Bookshelf: link.

[30] Y. Oztürk, V. M. Altan, and N. Yildizoğlu-Ari, "Effects of experimental diabetes and insulin on smooth muscle functions," *Pharmacological Reviews,* vol. 48, pp. 69-112, 1996.

[31] E. Ho, G. Chen, and T. M. Bray, "Alpha-Phenyl-< i> tert</i>-butylnitrone (PBN) inhibits NFκB activation offering protection against chemically induced diabetes," *Free Radical Biology and Medicine,* vol. 28, pp. 604-614, 2000.

[32] K. Juárez-Reyes, F. Brindis, O. N. Medina-Campos, J. Pedraza-Chaverri, R. Bye, E. Linares*, et al.*, "Hypoglycemic, antihyperglycemic, and antioxidant effects of the edible plant< i> Anoda cristata</i>," *Journal of ethnopharmacology,* vol. 161, pp. 36-45, 2015.

[33] Y. Ning, W. Zhen, Z. Fu, J. Jiang, D. Liu, L. Belardinelli*, et al.*, "Ranolazine increases β-cell survival and improves glucose homeostasis in low-dose streptozotocin-induced diabetes in mice," *Journal of Pharmacology and Experimental Therapeutics,* vol. 337, pp. 50-58, 2011.

[34] A. P. Association, *Diagnostic and statistical manual of mental disorders, (DSM-5®)*: American Psychiatric Pub, 2013.

[35] N. Sousa, J. J. Cerqueira, and O. F. Almeida, "Corticosteroid receptors and neuroplasticity," *Brain research reviews,* vol. 57, pp. 561-570, 2008.

[36] R. M. Sapolsky, L. C. Krey, and B. S. McEwen, "The Neuroendocrinology of Stress and Aging: The Glucocorticoid Cascade Hypothesis*," *Endocrine reviews,* vol. 7, pp. 284-301, 1986.

[37] C. M. Pariante and A. H. Miller, "Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment," *Biological psychiatry,* vol. 49, pp. 391-404, 2001.

[38] C. O. Bondi, G. Rodriguez, G. G. Gould, A. Frazer, and D. A. Morilak, "Chronic unpredictable stress induces a cognitive deficit and anxiety-like behavior in rats that is prevented by chronic antidepressant drug treatment," *Neuropsychopharmacology,* vol. 33, pp. 320-331, 2008.

[39] C. Rossetti, O. Halfon, and B. Boutrel, "Controversies about a common etiology for eating and mood disorders," 2014.

[40] R. C. Kessler, R. H. Price, and C. B. Wortman, "Social factors in psychopathology: Stress, social support, and coping processes," *Annual review of psychology,* vol. 36, pp. 531-572, 1985.

[41] R. J. Katz, "Animal model of depression: pharmacological sensitivity of a hedonic deficit," *Pharmacology Biochemistry and Behavior,* vol. 16, pp. 965-968, 1982.

[42] S. Bhattacharya and A. Muruganandam, "Adaptogenic activity of< i> Withania somnifera</i>: an experimental study using a rat model of chronic stress," *Pharmacology Biochemistry and Behavior,* vol. 75, pp. 547-555, 2003.

[43] W. Suwanjang, A. Y. Abramov, P. Govitrapong, and B. Chetsawang, "Melatonin attenuates dexamethasone toxicity-induced oxidative stress, calpain and caspase activation in human neuroblastoma SH-SY5Y cells," *The Journal of steroid biochemistry and molecular biology,* vol. 138, pp. 116-122, 2013.

[44] S. J. Lupien and M. Lepage, "Stress, memory, and the hippocampus: can't live with it, can't live without it," *Behav Brain Res,* vol. 127, pp. 137-58, Dec 14 2001.

[45] B. S. McEwen, "The neurobiology of stress: from serendipity to clinical relevance," *Brain research,* vol. 886, pp. 172-189, 2000.

[46] M. Alipour, I. Salehi, and F. G. Soufi, "Effect of exercise on diabetes-induced oxidative stress in the rat hippocampus," *Iranian Red Crescent medical journal,* vol. 14, p. 222, 2012.

[47] A. Kuhad and K. Chopra, "Curcumin attenuates diabetic encephalopathy in rats: behavioral and biochemical evidences," *European journal of pharmacology,* vol. 576, pp. 34-42, 2007.

[48] M. Ghaisas, V. Navghare, A. Takawale, V. Zope, M. Tanwar, and A. Deshpande, "Effect of< i> Tectona grandis</i> Linn. on dexamethasone-induced insulin resistance in mice," *Journal of ethnopharmacology,* vol. 122, pp. 304-307, 2009.

[49] R. Jatwa, H. S. Parmar, S. Panda, and A. Kar, "Amelioration of Corticosteroid-Induced Type 2 Diabetes Mellitus by Rosiglitazone Is Possibly Mediated through Stimulation of Thyroid Function and Inhibition of Tissue Lipid Peroxidation in Mice," *Basic & clinical pharmacology & toxicology,* vol. 101, pp. 177-180, 2007.

[50] S. Becker and J. M. Wojtowicz, "A model of hippocampal neurogenesis in memory and mood disorders," *Trends in cognitive sciences,* vol. 11, pp. 70-76, 2007.

[51] C. Crochemore, T. M. Michaelidis, D. Fischer, J.-P. Loeffler, and O. F. ALMEIDA, "Enhancement of p53 activity and inhibition of neural cell proliferation by glucocorticoid receptor activation," *The FASEB journal,* vol. 16, pp. 761-770, 2002.

[52] C. Crochemore, J. Lu, Y. Wu, Z. Liposits, N. Sousa, F. Holsboer*, et al.*, "Direct targeting of hippocampal neurons for apoptosis by glucocorticoids is reversible by mineralocorticoid receptor activation," *Molecular psychiatry,* vol. 10, pp. 790-798, 2005.

[53] C. Rossetti, O. Halfon, and B. Boutrel, "Controversies about a common etiology for eating and mood disorders," *Frontiers in psychology,* vol. 5, 2014.

[54] M. D. Saxe, F. Battaglia, J.-W. Wang, G. Malleret, D. J. David, J. E. Monckton*, et al.*, "Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus," *Proceedings of the National Academy of Sciences,* vol. 103, pp. 17501-17506, 2006.

[55] G. Winocur, J. M. Wojtowicz, M. Sekeres, J. S. Snyder, and S. Wang, "Inhibition of neurogenesis interferes with hippocampus-dependent memory function," *Hippocampus,* vol. 16, pp. 296-304, 2006.

[56] G. G. Piroli, C. A. Grillo, L. R. Reznikov, S. Adams, B. S. McEwen, M. J. Charron*, et al.*, "Corticosterone impairs insulin-stimulated translocation of GLUT4 in the rat hippocampus," *Neuroendocrinology,* vol. 85, pp. 71-80, 2007.

[57] Y. Emmanuel, L. E. Cochlin, D. J. Tyler, C. A. de Jager, A. D. Smith, and K. Clarke, "Human hippocampal energy metabolism is impaired during cognitive activity in a lipid infusion model of insulin resistance," *Brain Behav,* vol. 3, pp. 134-44, Mar 2013.

[58] C. A. Grillo, G. G. Piroli, R. M. Hendry, and L. P. Reagan, "Insulin-stimulated translocation of GLUT4 to the plasma membrane in rat hippocampus is PI3-kinase dependent," *Brain Res,* vol. 1296, pp. 35-45, Nov 03 2009.

[59] S. S. Patel, V. Mehta, H. Changotra, and M. Udayabanu, "Depression mediates impaired glucose tolerance and cognitive dysfunction: A neuromodulatory role of rosiglitazone," *Hormones and behavior,* vol. 78, pp. 200-210, 2016.

[60] S. S. Patel, S. Gupta, and M. Udayabanu, "Urtica dioica modulates hippocampal insulin signaling and recognition memory deficit in streptozotocin induced diabetic mice," *Metabolic brain disease,* vol. 31, pp. 601-611, 2016.

[61] V. Mehta, A. Parashar, A. Sharma, T. R. Singh, and M. Udayabanu, "Quercetin ameliorates chronic unpredicted stress-mediated memory dysfunction in male Swiss albino mice by attenuating insulin resistance and elevating hippocampal GLUT4 levels independent of insulin receptor expression," *Horm Behav,* vol. 89, pp. 13-22, Dec 23 2016.

[62] S. S. Patel, A. Parashar, and M. Udayabanu, "Urtica dioica leaves modulates muscarinic cholinergic system in the hippocampus of streptozotocin-induced diabetic mice," *Metabolic brain disease,* vol. 30, pp. 803-811, 2015.

[63] S. Louis, "Goodman & Gilman's pharmacological basis of therapeutics," 2011.

[64] A. J. Krentz and C. J. Bailey, "Oral antidiabetic agents," *Drugs,* vol. 65, pp. 385-411, 2005.

[65] S. E. Inzucchi, "Oral antihyperglycemic therapy for type 2 diabetes: scientific review," *Jama,* vol. 287, pp. 360-372, 2002.

[66] P. J. Lustman, L. S. Griffith, and R. E. Clouse, "Depression in adults with diabetes. Results of 5-yr follow-up study," *Diabetes Care,* vol. 11, pp. 605-12, Sep 1988.

[67] A. Nouwen, G. Nefs, I. Caramlau, M. Connock, K. Winkley, C. E. Lloyd, *et al.*, "Prevalence of depression in individuals with impaired glucose metabolism or undiagnosed diabetes: a systematic review and meta-analysis of the European Depression in Diabetes (EDID) Research Consortium," *Diabetes Care,* vol. 34, pp. 752-62, Mar 2011.

[68] S. B. Lupien, E. J. Bluhm, and D. N. Ishii, "Systemic insulin-like growth factor-I administration prevents cognitive impairment in diabetic rats, and brain IGF regulates learning/memory in normal adult rats," *J Neurosci Res,* vol. 74, pp. 512-23, Nov 15 2003.

[69] L. E. Egede and C. Ellis, "Diabetes and depression: global perspectives," *Diabetes Res Clin Pract,* vol. 87, pp. 302-12, Mar 2010.

[70] R. J. Anderson, K. E. Freedland, R. E. Clouse, and P. J. Lustman, "The prevalence of comorbid depression in adults with diabetes: a meta-analysis," *Diabetes Care,* vol. 24, pp. 1069-78, Jun 2001.

[71] G. Saravanan and P. Ponmurugan, "Antidiabetic effect of S-allylcysteine: effect on thyroid hormone and circulatory antioxidant system in experimental diabetic rats," *J Diabetes Complications,* vol. 26, pp. 280-5, Jul-Aug 2012.

[72] I. Komsuoglu Celikyurt, O. Mutlu, G. Ulak, E. Uyar, E. Bektas, F. Yildiz Akar, *et al.*, "Exenatide treatment exerts anxiolytic- and antidepressant-like effects and reverses neuropathy in a mouse model of type-2 diabetes," *Med Sci Monit Basic Res,* vol. 20, pp. 112-7, Jul 30 2014.

[73] L. Y. Jiang, S. S. Tang, X. Y. Wang, L. P. Liu, Y. Long, M. Hu, *et al.*, "PPARgamma agonist pioglitazone reverses memory impairment and biochemical changes in a mouse model of type 2 diabetes mellitus," *CNS Neurosci Ther,* vol. 18, pp. 659-66, Aug 2012.

[74] T. Kawamura, T. Umemura, and N. Hotta, "Cognitive impairment in diabetic patients: Can diabetic control prevent cognitive decline?," *Journal of diabetes investigation,* vol. 3, pp. 413-423, 2012.

[75] S. M. Bentley, G. L. Pagalilauan, and S. A. Simpson, "Major Depression," *Medical Clinics of North America,* vol. 98, pp. 981-1005, 2014.

[76] M. Marcus, M. T. Yasamy, M. van Ommeren, D. Chisholm, and S. Saxena, "Depression: A global public health concern," *WHO Department of Mental Health and Substance Abuse,* vol. 1, pp. 6-8, 2012.

[77] G. Dagytė, J. A. Den Boer, and A. Trentani, "The cholinergic system and depression," *Behavioural brain research,* vol. 221, pp. 574-582, 2011.

[78] Y.-H. Lin, A.-H. Liu, Y. Xu, L. Tie, H.-M. Yu, and X.-J. Li, "Effect of chronic unpredictable mild stress on brain–pancreas relative protein in rat brain and pancreas," *Behavioural brain research,* vol. 165, pp. 63-71, 2005.

[79] R. I. Holt and W. J. Katon, "Dialogue on Diabetes and Depression: Dealing with the double burden of co-morbidity," *J Affect Disord,* vol. 142 Suppl, pp. S1-3, Oct 2012.

[80] L. E. Egede, D. Zheng, and K. Simpson, "Comorbid depression is associated with increased health care use and expenditures in individuals with diabetes," *Diabetes Care,* vol. 25, pp. 464-70, Mar 2002.

[81] C. Li, E. S. Ford, G. Zhao, I. B. Ahluwalia, W. S. Pearson, and A. H. Mokdad, "Prevalence and correlates of undiagnosed depression among U.S. adults with diabetes: the Behavioral Risk Factor Surveillance System, 2006," *Diabetes Res Clin Pract,* vol. 83, pp. 268-79, Feb 2009.

[82] R. K. Solanki, V. Dubey, and D. Munshi, "Neurocognitive impairment and comorbid depression in patients of diabetes mellitus," *Int J Diabetes Dev Ctries,* vol. 29, pp. 133-8, Jul 2009.

[83] B. Mezuk, W. W. Eaton, S. Albrecht, and S. H. Golden, "Depression and type 2 diabetes over the lifespan: a meta-analysis," *Diabetes Care,* vol. 31, pp. 2383-90, Dec 2008.

[84] S. H. Golden, J. E. Williams, D. E. Ford, H.-C. Yeh, C. P. Sanford, F. J. Nieto*, et al.*, "Depressive symptoms and the risk of type 2 diabetes the atherosclerosis risk in communities study," *Diabetes Care,* vol. 27, pp. 429-435, 2004.

[85] M. J. Knol, J. W. Twisk, A. T. Beekman, R. J. Heine, F. J. Snoek, and F. Pouwer, "Depression as a risk factor for the onset of type 2 diabetes mellitus. A meta-analysis," *Diabetologia,* vol. 49, pp. 837-45, May 2006.

[86] E. J. Nestler, M. Barrot, R. J. DiLeone, A. J. Eisch, S. J. Gold, and L. M. Monteggia, "Neurobiology of depression," *Neuron,* vol. 34, pp. 13-25, 2002.

[87] V. Krishnan and E. J. Nestler, "The molecular neurobiology of depression," *Nature,* vol. 455, pp. 894-902, 2008.

[88] A. K. Eriksson, A. Ekbom, F. Granath, A. Hilding, S. Efendic, and C. G. Östenson, "Psychological distress and risk of pre-diabetes and Type 2 diabetes in a prospective study of Swedish middle-aged men and women," *Diabetic medicine,* vol. 25, pp. 834-842, 2008.

[89] N. Kawakami, N. Takatsuka, H. Shimizu, and H. Ishibashi, "Depressive symptoms and occurrence of type 2 diabetes among Japanese men," *Diabetes Care,* vol. 22, pp. 1071-1076, 1999.

[90] L. Palinkas, P. Lee, and E. Barrett-Connor, "A prospective study of type 2 diabetes and depressive symptoms in the elderly: the Rancho Bernardo Study," *Diabetic medicine,* vol. 21, pp. 1185-1191, 2004.

[91] D. L. Musselman, E. Betan, H. Larsen, and L. S. Phillips, "Relationship of depression to diabetes types 1 and 2: epidemiology, biology, and treatment," *Biological psychiatry,* vol. 54, pp. 317-329, 2003.

[92] L. E. Egede and C. Ellis, "Diabetes and depression: global perspectives," *Diabetes research and clinical practice,* vol. 87, pp. 302-312, 2010.

[93] Y. Pan, Y. Hong, Q.-Y. Zhang, and L.-D. Kong, "Impaired hypothalamic insulin signaling in CUMS rats: Restored by icariin and fluoxetine through inhibiting CRF system," *Psychoneuroendocrinology,* vol. 38, pp. 122-134, 2013.

[94] C. Hlscher, "Diabetes as a risk factor for Alzheimer's disease: insulin signalling impairment in the brain as an alternative model of Alzheimer's disease," *Biochemical Society Transactions,* vol. 39, p. 891, 2011.

[95] T. Willis, "1675. Pharmaceutice rationalis sive diabtriba de medicamentorum operantionibus in humano corpore," ed: MDCLXXV, Oxford.

[96] M. Karamanou, A. Protogerou, G. Tsoucalas, G. Androutsos, and E. Poulakou-Rebelakou, "Milestones in the history of diabetes mellitus: The main contributors," *World J Diabetes,* vol. 7, pp. 1-7, Jan 10 2016.

[97] P. Dey, M. R. Saha, S. R. Chowdhuri, A. Sen, M. P. Sarkar, B. Haldar*, et al.*, "Assessment of anti-diabetic activity of an ethnopharmacological plant< i> Nerium oleander</i> through alloxan induced diabetes in mice," *Journal of ethnopharmacology,* 2014.

[98] X.-g. Yao, X. Xu, G.-h. Wang, M. Lei, L. L. Quan, Y.-h. Cheng*, et al.*, "BBT improved glucose homeostasis by ameliorating β-cell dysfunction in type 2 diabetic mice," *Journal of Endocrinology,* pp. JOE-14-0721, 2015.

[99] N. Rakieten, M. Rakieten, and M. Nadkarni, "Studies on the diabetogenic action of streptozotocin (NSC-37917)," *Cancer chemotherapy reports. Part 1,* vol. 29, pp. 91-98, 1963.

[100] L. Srivastava, P. Bora, and S. Bhatt, "Diabetogenic action of streptozotocin," *Trends in Pharmacological Sciences,* vol. 3, pp. 376-378, 1982.

[101] P. Nain, V. Saini, S. Sharma, and J. Nain, "Antidiabetic and antioxidant potential of< i> Emblica officinalis</i> Gaertn. leaves extract in streptozotocin-induced type-2 diabetes mellitus (T2DM) rats," *Journal of ethnopharmacology,* vol. 142, pp. 65-71, 2012.

[102] J. Ventura-Sobrevilla, V. Boone-Villa, C. Aguilar, R. Román-Ramos, E. Vega-Avila, E. Campos-Sepúlveda*, et al.*, "Effect of varying dose and administration of streptozotocin on blood sugar in male CD1 mice," in *Proceedings of the Western Pharmacology Society*, 2011, pp. 5-9.

[103] S. Akkina, C. Patterson, and D. Wright, "GDNF rescues nonpeptidergic unmyelinated primary afferents in streptozotocin-treated diabetic mice," *Experimental neurology,* vol. 167, pp. 173-182, 2001.

[104] J. A. Christianson, J. M. Ryals, K. E. McCarson, and D. E. Wright, "Beneficial actions of neurotrophin treatment on diabetes-induced hypoalgesia in mice," *The Journal of Pain,* vol. 4, pp. 493-504, 2003.

[105] C. Demiot, M. Tartas, B. Fromy, P. Abraham, J. L. Saumet, and D. Sigaudo-Roussel, "Aldose reductase pathway inhibition improved vascular and C-fiber functions, allowing for pressure-induced vasodilation restoration during severe diabetic neuropathy," *Diabetes,* vol. 55, pp. 1478-1483, 2006.

[106] D. Sigaudo-Roussel, B. Fromy, and J. L. Saumet, "Diabetic neuropathy in animal models," *Drug Discovery Today: Disease Models,* vol. 4, pp. 39-44, 2007.

[107] S. A. El-Marasy, H. M. Abdallah, S. M. El-Shenawy, A. S. El-Khatib, O. A. El-Shabrawy, and S. A. Kenawy, "Anti-depressant effect of hesperidin in diabetic rats," *Canadian journal of physiology and pharmacology,* vol. 92, pp. 945-952, 2014.

[108] L.-y. Ma, D.-m. Zhang, Y. Tang, Y. Lu, Y. Zhang, Y. Gao*, et al.*, "Ghrelin-attenuated cognitive dysfunction in streptozotocin-induced diabetic rats," *Alzheimer Disease & Associated Disorders,* vol. 25, pp. 352-363, 2011.

[109] R. C. Kessler, "The effects of stressful life events on depression," 1997.

[110] K. Kendler, L. Karkowski, and C. Prescott, "The assessment of dependence in the study of stressful life events: validation using a twin design," *Psychological medicine,* vol. 29, pp. 1455-1460, 1999.

[111] M. N. Hill, K. G. Hellemans, P. Verma, B. B. Gorzalka, and J. Weinberg, "Neurobiology of chronic mild stress: parallels to major depression," *Neuroscience & Biobehavioral Reviews,* vol. 36, pp. 2085-2117, 2012.

[112] P. Willner, "Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS," *Neuropsychobiology,* vol. 52, pp. 90-110, 2005.

[113] P. Willner, A. Towell, D. Sampson, S. Sophokleous, and R. Muscat, "Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant," *Psychopharmacology,* vol. 93, pp. 358-364, 1987.

[114] P. Willner, "Animal models of depression: validity and applications," *Advances in biochemical psychopharmacology,* vol. 49, p. 19, 1995.

[115] S. Vancassel, S. Leman, L. Hanonick, S. Denis, J. Roger, M. Nollet, *et al.*, "n-3 polyunsaturated fatty acid supplementation reverses stress-induced modifications on brain monoamine levels in mice," *Journal of lipid research,* vol. 49, pp. 340-348, 2008.

[116] R. Häidkind, M. Eller, M. Harro, A. Kask, A. Rinken, L. Oreland, *et al.*, "Effects of partial locus coeruleus denervation and chronic mild stress on behaviour and monoamine neurochemistry in the rat," *European neuropsychopharmacology,* vol. 13, pp. 19-28, 2003.

[117] I. Kreft, N. Fabjan, and M. Germ, "Rutin in buckwheat: Protection of plants and its importance for the production of functional food," *Fagopyrum,* vol. 20, 2003.

[118] L. S. Chua, "A review on plant-based rutin extraction methods and its pharmacological activities," *J Ethnopharmacol,* vol. 150, pp. 805-17, Dec 12 2013.

[119] N. A. Al-Dhabi, M. V. Arasu, C. H. Park, and S. U. Park, "An up-to-date review of rutin and its biological and pharmacological activities," *EXCLI J,* vol. 14, pp. 59-63, 2015.

[120] A. A. Perk, I. Shatynska-Mytsyk, Y. C. Gercek, K. Boztas, M. Yazgan, S. Fayyaz, *et al.*, "Rutin mediated targeting of signaling machinery in cancer cells," *Cancer Cell Int,* vol. 14, p. 124, 2014.

[121] M. Atanassova and V. Bagdassarian, "Rutin content in plant products," *Journal of the University of Chemical Technology and Metallurgy,* vol. 44, pp. 201-203, 2009.

[122] S. Kreft, M. Knapp, and I. Kreft, "Extraction of rutin from buckwheat (Fagopyrum esculentumMoench) seeds and determination by capillary electrophoresis," *J Agric Food Chem,* vol. 47, pp. 4649-52, Nov 1999.

[123] R. Milašiene, K. Rawicka, O. Kornyšova, M. Ligor, A. Maruška, and B. Buszewska, "Evaluation of antioxidactivity of green and black tea (Camellia sinensis) and rooibos (Aspalathus linearis) tea extracts by means of hplc with reaction detector," *Ars Separatoria Acta,* pp. 27-33, 2007.

[124] E. Sofic, A. Copra-Janicijevic, M. Salihovic, I. Tahirovic, and G. Kroyer, *Screening of medicinal plant extracts for quercetin-3-rutinoside (rutin) in Bosnia and Herzegovina*: na, 2010.

[125] E. J. Shin, J. H. Jeong, Y. H. Chung, W. K. Kim, K. H. Ko, J. H. Bach, *et al.*, "Role of oxidative stress in epileptic seizures," *Neurochem Int,* vol. 59, pp. 122-37, Aug 2011.

[126] M. Nassiri-Asl, S. Shariati-Rad, and F. Zamansoltani, "Anticonvulsive effects of intracerebroventricular administration of rutin in rats," *Prog Neuropsychopharmacol Biol Psychiatry,* vol. 32, pp. 989-93, May 15 2008.

[127] J. Qu, Q. Zhou, Y. Du, W. Zhang, M. Bai, Z. Zhang, *et al.*, "Rutin protects against cognitive deficits and brain damage in rats with chronic cerebral hypoperfusion," *Br J Pharmacol,* vol. 171, pp. 3702-15, Aug 2014.

[128] P. X. Xu, S. W. Wang, X. L. Yu, Y. J. Su, T. Wang, W. W. Zhou, *et al.*, "Rutin improves spatial memory in Alzheimer's disease transgenic mice by reducing Abeta oligomer level and attenuating oxidative stress and neuroinflammation," *Behav Brain Res,* vol. 264, pp. 173-80, May 01 2014.

[129] J. Y. Choi, J. M. Lee, D. G. Lee, S. Cho, Y. H. Yoon, E. J. Cho, *et al.*, "The n-Butanol Fraction and Rutin from Tartary Buckwheat Improve Cognition and Memory in an In Vivo Model of Amyloid-beta-Induced Alzheimer's Disease," *J Med Food,* vol. 18, pp. 631-41, Jun 2015.

[130] M. M. Khan, S. S. Raza, H. Javed, A. Ahmad, A. Khan, F. Islam, *et al.*, "Rutin protects dopaminergic neurons from oxidative stress in an animal model of Parkinson's disease," *Neurotox Res,* vol. 22, pp. 1-15, Jul 2012.

[131] K. B. Magalingam, A. Radhakrishnan, P. Ramdas, and N. Haleagrahara, "Quercetin glycosides induced neuroprotection by changes in the gene expression in a cellular model of Parkinson's disease," *J Mol Neurosci,* vol. 55, pp. 609-17, Mar 2015.

[132] S. E. Park, K. Sapkota, J. H. Choi, M. K. Kim, Y. H. Kim, K. M. Kim, *et al.*, "Rutin from Dendropanax morbifera Leveille protects human dopaminergic cells against rotenone induced cell injury through inhibiting JNK and p38 MAPK signaling," *Neurochem Res,* vol. 39, pp. 707-18, Apr 2014.

[133] M. K. Bhutani, M. Bishnoi, and S. K. Kulkarni, "Anti-depressant like effect of curcumin and its combination with piperine in unpredictable chronic stress-induced behavioral, biochemical and neurochemical changes," *Pharmacology Biochemistry and Behavior,* vol. 92, pp. 39-43, 2009.

[134] R. J. Katz, "Animal models and human depressive disorders," *Neuroscience & Biobehavioral Reviews,* vol. 5, pp. 231-246, 1981.

[135] H. Vogel, *Drug discovery and evaluation: pharmacological assays*: Springer Science & Business Media, 2007.

[136] S. Kulkarni and A. K. Mehta, "Purine nucleoside—mediated immobility in mice: reversal by antidepressants," *Psychopharmacology,* vol. 85, pp. 460-463, 1985.

[137] L. Steru, R. Chermat, B. Thierry, and P. Simon, "The tail suspension test: a new method for screening antidepressants in mice," *Psychopharmacology,* vol. 85, pp. 367-370, 1985.

[138] M. Banasr and R. S. Duman, "Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors," *Biological psychiatry,* vol. 64, pp. 863-870, 2008.

[139] M. Udayabanu, D. Kumaran, and A. Katyal, "Free chelatable zinc modulates the cholinergic function during hypobaric hypoxia-induced neuronal damage: an in vivo study," *Neuroscience,* vol. 202, pp. 434-445, 2012.

[140] A. Ranjan and B. N. Mallick, "A modified method for consistent and reliable Golgi–Cox staining in significantly reduced time," *Frontiers in neurology,* vol. 1, p. 157, 2010.

[141] G. H. Maia, J. L. Quesado, J. I. Soares, J. M. do Carmo, P. A. Andrade, J. P. Andrade, *et al.*, "Loss of hippocampal neurons after kainate treatment correlates with behavioral deficits," *PLoS One,* vol. 9, p. e84722, 2014.

[142] W. Tang, J. Cheng, Z. Y. Wang, K. Y. Chen, Z. M. Han, Q. H. Wang, *et al.*, "The Synergistic Roles of the Chronic Prenatal and Offspring Stress Exposures in Impairing Offspring Learning and Memory," *J Alzheimers Dis,* vol. 53, pp. 221-36, Apr 23 2016.

[143] M. G. Stewart, H. A. Davies, C. Sandi, I. V. Kraev, V. V. Rogachevsky, C. J. Peddie, *et al.*, "Stress suppresses and learning induces plasticity in CA3 of rat hippocampus: a

three-dimensional ultrastructural study of thorny excrescences and their postsynaptic densities," *Neuroscience,* vol. 131, pp. 43-54, 2005.

[144] J. J. Kim and D. M. Diamond, "The stressed hippocampus, synaptic plasticity and lost memories," *Nat Rev Neurosci,* vol. 3, pp. 453-62, Jun 2002.

[145] B. S. McEwen, "Effects of adverse experiences for brain structure and function," *Biol Psychiatry,* vol. 48, pp. 721-31, Oct 15 2000.

CHAPTER 9

APPENDICES

9. APPENDICES

List of Publications

1. **Parashar A**, Mehta V, Malairaman U. Type 2 Diabetes Mellitus Is Associated with Social Recognition Memory Deficit and Altered Dopaminergic Neurotransmission in the Amygdala. *Annals of Neurosciences*. 2017;24(4):212-20.

2. **Parashar A**, Mehta V, Udayabanu M. Rutin alleviates chronic unpredictable stress-induced behavioral alterations and hippocampal damage in mice. *Neuroscience Letters*. 2017;656:65-71.

3. **Parashar A**, Udayabanu M. Gut microbiota: Implications in Parkinson's disease. *Parkinsonism & related disorders*. 2017;38:1–7

4. **Parashar A**, Udayabanu M. Gut microbiota regulates key modulators of social behavior. *European Neuropsychopharmacology*. 2016;26(1):78-91.

5. Sharma A, Mehta V, **Parashar A**, Malairaman U. Combinational effect of Paclitaxel and Clotrimazole on human breast cancer: Proof for synergistic interaction. *Synergy*. 2017;5:13-20.

6. Mehta V, **Parashar A**, Udayabanu M. Quercetin prevents chronic unpredictable stress induced behavioral dysfunction in mice by alleviating hippocampal oxidative and inflammatory stress. *Physiology & behavior*. 2017;171:69-78.

7. Mehta V, **Parashar A**, Sharma A, Singh TR, Udayabanu M. Quercetin ameliorates chronic unpredicted stress-mediated memory dysfunction in male Swiss albino mice by attenuating insulin resistance and elevating hippocampal GLUT4 levels independent of insulin receptor expression. *Hormones and behavior*. 2017;89:13-22.

8. Patel SS, **Parashar A**, Udayabanu M. Urtica dioica leaves modulates muscarinic cholinergic system in the hippocampus of streptozotocin-induced diabetic mice. *Metabolic brain disease*. 2015;30(3):803-11.

Conferences and Workshops

1. **Parashar A**, Mehta V, Sharma A, Udayabanu M, Rutin ameliorates chronic unpredictable stress-induced pre-diabetic state and cognitive dysfunction by preventing hippocampal damage and elevating hippocampal GLUT4 and insulin receptor expression levels. National conference On "Targeting Diabetes and Newer Strategies for Insulin Drug Delivery" held at L. R institute of Pharmacy, Solan (H.P).

2. **Parashar A,** Mehta V, Sharma A, Udayabanu M, Udayabanu Malairaman (2016). Screening of Herbal Molecules Against Type II Diabetes Mediated Neurological Complications. International conference on "Innovations in pharmaceutical sciences", held at Sri Aurobindo Institute of Pharmacy (SAIP), Indore (M.P) [27-28 February, 2016].

3. DBT sponsored workshop on "Statistical Techniques In Biological and Medical Sciences" (STBMS). [Jaypee University of Information Technology, Waknaghat, Himachal Pradesh, India: 13-18 June, 2016].

4. National Symposium on Computational System Biology NSCSB 2016. [Jaypee University of Information Technology, Waknaghat, Himachal Pradesh, India: 18-20 March, 2016]