"TO STUDY THE EFFECT OF EPALRESTAT, AN ALDOSE REDUCTASE INHIBITOR ON MEMORY AND LEARNING IN DIABETIC MALE WISTAR RATS"

Thesis submitted to

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH

(BELAGAVI)

(Deemed-to-be-University)

[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

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Placed in Category 'A' by MHRD (GoI)

For the award of the degree of
Doctor of Philosophy
In the Faculty of Medicine
(Pharmacology)

By

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ACKNOWLEDGEMENT

At this moment of accomplishment I wish to express my sincere acknowledgements to all those who have contributed to this thesis work and supported me in one way or the other during this amazing journey.

It's my pleasure to express deep sense of gratitude and heartfelt thanks to my Supervisors, Dr. S. S. Torgal, Professor - Department of Pharmacology, J. N. Medical College, Belagavi, for his guidance and co-operation not only during my study but also during my entire course. His simplicity, discipline, caring attitude, inquisitive outlook will be cherished in all works of my life. During the most difficult times of my thesis writing, he gave me the support and freedom I needed to move on. I appreciate all his contributions of timing and ideas to make my Ph.D. experience productive and stimulating.

I would like to acknowledge the kind support of Dr. Sanjay Kumar Mishra, Associate Professor and Scientist - KAHER's Dr. Prabhakar Kore Basic Science Research Centre, Belagavi for his inestimable guidance, valuable suggestions and constant encouragement during this doctoral research. It was under his guidance that I developed a focus, and gained interest in research work. Apart from guiding me, he has unwearyingly been a continuous source of moral support and advice to me. This work would have been impossible without his constant guidance. He was instrumental in providing the cooperation, timely advice and shared his knowledge for the smooth progress of the study.

Here I take this privilege and

pleasure to acknowledge Dr. V. D. Patil, Registrar, KLE Academy of Higher Education and Research, and Dr. (Mrs.) N. S. Mahantashetti, Principal and Dean, J. N. Medical College, Belagavi, for permitting me to pursue my studies and providing all the necessary facilities to carry out this research work.

I owe my warmest and humble thanks to Dr. Daksha Dixit - Director of Academic Affairs, KLE Academy of Higher Education and Research for her untiring support and co-operation. I would like to express my heartfelt appreciation to Dr. Ramesh Chavan, Professor and Head, Department of Pathology, J. N. Medical College, Belagavi for his invaluable guidelines and support to complete histopathology study.

I express my sincere gratitude to Dr. P. A. Patil, Professor and Head, Department of Pharmacology, USM KLE International Medical Program, Belagavi for his kind guidance and support.

It's my pleasure to express my deepest gratitude to Dr. A. P. Hogade, Professor and Head, Department of Pharmacology, J. N. Medical College, Belagavi for his support and encouragement.

I would also like to thank Dr. Sunil S. Jalapure, Deputy Director of KAHER's Dr. Prabhakar Kore Basic Science Research Centre, Belagavi for providing me the necessary infrastructure and timely assistance to carry out my research studies.

I sincerely thank to Dr. Jyoti M. Nagamoti, Professor - Department of Microbiology for her constant support towards animal procurement. I also thank to Mr. S. B. Desai, officer in charge, Central Animal House for support during animal experiments. My sincere thanks to Dr. Harpreet Kaur, Asst. Professor - Department of Physiology, J. N. Medical College, Belagavi for her kind co-operation in plagiarism check.

I convey my special thanks to Dr. S. S. Tyagi, Professor and Head - Department of Biostatistics, J. N. Medical College, Belagavi for his support to complete statistical analysis. I also acknowledge support of Dr. Nayana Hashilkar, Dr. Rekha Nayaka, Dr. Urmila Kagal, Dr. Anupama M. G., Dr. Netravathi A. B., Dr. Sapna Patil, and Dr. Jyoti Benni for their

support during the research work. I sincerely thanks to Dr. Gaurav Shukla, Department of Pharmacology, J. N. Medical College, Belagavi for his direct and indirect support from the day one of animal study to data analysis.

If not for the prayers, blessings, love and affection from my friends, my work would never have been complete. I take this opportunity to genuinely acknowledge my seniors and colleagues, who guided and supported me from the early days of my research. Mr. Sushant Shengule, Ms. Damita Cota, Dr. Mahejabeen, Dr. Avinash, Dr. Somaling, Dr. Vidya, Dr. Shujauddin, Dr. Ranjita, Mrs. Nisha, Dr. Suneel Dodamani, Ms. Dhanashree, Dr. Kimi, Dr. Prathamesh, Dr. Neha, Dr. Aarti, Dr. Savithasree, and Dr. Amitha, have provided a friendly environment in my working place and lent a helping hand to me in many ways during this study.

I also express my thanks to the technical staff members Mrs. Madhumathi, Ms. Renuka and Mrs. Swati who helped me throughout the study. I extend my thanks to non-teaching staff Mr. Dayanand, Mr. Sanjay, Mr. Veerangouda Patil and Mr. Kempanna.

I wholeheartedly thank my family for being my inspiration and for their blessings, guidance, motivation and support throughout my life.

Utmost of all, I praise the God, the Almighty for providing me this opportunity and granting me the capability, indispensable to proceed with and complete this work successfully.

Lastly, I would like to thank all the people, who are somehow, were involved in this research work and provided me with the necessary help and made it possible for me to write this thesis.

ABSTRACT

Background:

Type-2 diabetes mellitus (T2DM) is known to be connected with cognitive impairment (CI). Evidence from various studies revealed that oxidative stress and neuro-inflammatory contributes are linked to neurodegeneration. Epalrestat [EPS; aldose reductase inhibitor (ARI)] is commonly prescribed medicine for diabetic peripheral neuropathy. It is evident that drugs with a rhodanine structure having antioxidant and anti-inflammatory characters. Thus, it is suggestive that EPS can be potential drugs for treatment of CI induces by DM. However, there is no study reports regarding the effect of EPS like compound on memory and learning in diabetes. EPS may produce possible neuroprotection in experimental rats. Aggregation of tau and Aβ-amyloid proteins were seen in Alzheimers disease (AD), both are toxic to neurons and EPS is found to have the potential for clearing tau protein. Because of this pleiotropic effect it could be hypothesized that it may produce the beneficial effects for the management of neurodegenerative disease and thus improve learning and memory associated with DM. Therefore, In view of paucity of information the proposed study is designed with following objectives:

1. To estimate the effect of EPS on memory and learning in high fat diet (HFD) and streptozotocin [STZ] induced diabetic rats.
2. To estimate the effect of EPS on 'tau protein' level in brain tissue.
3. To estimate the effect of EPS on gene expression level
4. To estimate the effect of EPS on oxidative stress markers

Methodology: The experimental animals were distributed into six different groups (n=10): NC; DC; Diabetic animals along with EPS administration (54, 27 and 13.5 mg/kg); and Diabetic animals with 1 mg/kg donepezil (Donep) treatment. Memory impairment was

induced by HFD and single dose of STZ (35 mg/kg, i.p.). After confirmation of diabetes, animals were treated with EPS (54, 27 and 13.5 mg/kg, p.o) and donep (1 mg/kg, p.o) for 28 days. On completion of treatment schedule, analysis of memory and learning deficit was performed using behavioral paradigms: Elevated Plus Maze (EPM), Morris Water Maze (MWM) and Passive Avoidance (PA) test followed by estimation of oxidative stress markers: Catalase (CAT) and Reduced glutathione (GSH). The proinflammatory cytokines such as Interleukin-6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-α), were estimated with the help of RT PCR. Whereas, rat tau protein was analysed using ELISA kit. The histopathological examination of rat hippocampus was performed to detect neuronal apoptosis.

Results: EPS and donep treatment indicated significant improvement in cognitive impairment in diabetic rats by markedly decreased EL to reach a hidden platform and enhanced time spent in particular quadrant using MWM test, reduced TL in EPM, and also there is a significant enhanced TL using passive avoidance test were noted. Memory-enhancing potential of EPS administration (54, 27 and 13.5, mg/kg) was comparable with the DC group. The EPS (54, 27 mg/kg) and donep treatment significant enhanced catalase ($p<0.001$, $p<0.01$, $p<0.001$) and glutathione levels ($p<0.001$, $p<0.01$, $p<0.001$) when campare with DC Rats. On the other hand, EPS at 27 mg and 54 mg/kg displayed decreased levels of TAU protein whereas, no significant changes were noted with donep treatment. Similarly, EPS treatments presented reduced gene expression levels of TNF-α and IL-6. Histopathological observations displayed, epalrestat could attenuate neuron apoptosis, clumped processes, vacuolations, disorganization and thinning of the layers.

Conclusion: Present study demonstrated that EPS and donep treatment resulted in decreased level of tau protein and histopathological changes in rat hippocampus by their

antioxidant and anti-inflammatory potential. The study result suggested that EPS and donep could be potential candidate for improvement of cognitive impairment in diabetic rats and can be deliberated as potential drug candidate in neurodegenerative disease management.

Keywords: type-2 diabetes, cognitive impairment, epalrestat, donepezil, oxidative stress, cytokine, TAU-protein.

LIST OF ABBREVIATIONS

ARI	:	Aldose reductase inhibitor
Ach	:	Acetyl choline
AChE-I	:	Acetylcholinesterase inhibitor
AD	:	Alzheimer's disease
ADA	:	American diabetes association
AGEs	:	Advanced glycation end products
ANOVA	:	Analysis of variance
APP	:	Amyloid precursor protein
ATP	:	Adenosine triphosphate
Aβ	:	Amyloid-beta
BDNF	:	Brain derived neurotrophic factor
BW	:	Body weight
CA	:	Cornus ammonis
CAT	:	Catalase
CI	:	Cognitive impairment
CNS	:	Central nervous system
CPCSEA	:	Committee for the Purpose of Control and Supervision of Experiments on animals
CREB	:	cAMP response-element binding protein
CVD	:	Cardiovascular disease
DA	:	Dopamine
DC	:	Diabetic control
DM	:	Diabetes mellitus
DN	:	Diabetic neuropathy

DNA	:	Deoxyribonucleic acid
DONEP	:	Donepezil
DPN	:	Diabetic peripheral neuropathy
DPP-4	:	Dipeptidyl peptidase-4
DTNB	:	5,5- dithiobis 2-nitrobenzoic acid
dl	:	Deciliter
EA	:	Enclosed arm
ELISA	:	Enzyme –linked immune sorbent assay
EL	:	Escape latency
ELT	:	Escape latency time
EPM	:	Elevated plus maze
EPS	:	Epalrestat
FDA	:	Food and drug administration
FPG	:	Fasting plasma glucose
FTD	:	Frontotemporal dementia
GABA	:	Gamma amino butyric acid
GAPDH	:	Glyceraldehyde 3-Phosphate dehydrogenase
GFR	:	Glomerular filtration rate
GLUT	:	Glucose transporter
GIP	:	Gastric Inhibitory Peptide
GSH	:	Glutathione
g	:	Gram
H&E	:	Haematoxylin & Eosin
HFD	:	High fat diet
HIC	:	High-income countries
HO	:	Heme oxygenase

IDDM	:	Insulin dependent diabetes mellitus
IL-6	:	Interleukin-6
IL-1β	:	Interleukin- 1 beta
IAEC	:	Institutional animal ethics committee
I.P	:	Intraperitoneal
I.V	:	Intravenous
kg	:	Kilogram
kU/L	:	Kilounit per liter
LBD	:	Lewy body dementia
LMIC	:	Low and middle-income countries
LTP	:	long term potentiation
MAPK	:	Mitogen activated protein kinase
MAO	:	Monoamine oxidase
MCI	:	Mild cognitive impairment
MT	:	Microtubule
MWM	:	Morris water maze
mL	:	Milliliter
mg	:	Milligram
mmol/L	:	Millimoles Per Litre
mmol/ml	:	Millimole per milliliter
mg/dl	:	Milligram Per Deciliter
min.	:	Minute
NIDDM	:	Non-Insulin Dependent Diabetes Mellitus
N	:	Number
ng/L	:	Nanogram per litre
NADPH	:	Nicotinamide adenine dinucleotide phosphate

NC	:	Normal control
NE	:	Norepinephrine
NFTs	:	Neurofibrillary tangles
NSAIDs	:	Nonsteroidal anti-inflammatory drugs
Nrf2	:	Nuclear factor erythroid 2-related factor 2
NMDA	:	N-methyl-D-aspartate
NO	:	Nitric Oxide
Nrf2	:	Nuclear factor erythroid 2-related factor 2
nm	:	Nanometer
ng/ml	:	Nanograms per milliliter
OA	:	Open arm
OD	:	Optical density
OGTT	:	Oral glucose tolerance test
PA	:	Passive avoidance
PAA	:	Passive avoidance apparatus
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PCOS	:	Polycystic ovarian syndrome
PFC	:	Prefrontal cortex
Q	:	Quadrants
Qg	:	Goal quadrant
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
RT-PCR	:	Real time polymerase chain reaction
secs.	:	Seconds
S	:	Seconds

SEM	:	Standard error of mean
SOD	:	Superoxide dismutase
SSRI	:	Selective serotonin reuptake inhibitors
STL	:	Step through latency
STZ	:	Streptozotocin
TCA	:	Tri chloro acetic acid
T2DM	:	Type-2 diabetes mellitus
T1DM	:	Type-1 diabetes mellitus
TNF-α	:	Tumour necrosis factor-α
TL	:	Transfer latency
VD	:	Vascular dementia
WHO	:	World health organization
5-HT	:	5-hydroxytryptamine
µl	:	Microlitre

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1. INTRODUCTION

1.1 Background

Dementia is described as a group of symptoms associated with decline in memory, deterioration in cognitive and communication abilities with affect the activities of daily living. Most commonly affected cognitive function in dementia is episodic memory. It has been observed that 10% of individuals aging >70 years and about 20–40% aging >85 years have clinically identifiable memory impairment. The cognitive functions are affected in dementia condition in addition to memory loss including judgment, language, calculation, visuospatial, praxis and problem-solving abilities. Neuropsychiatric and social deficits are also established in many dementia syndromes, manifesting as depression, anxiety, insomnia, sleep disturbances, apathy, hallucinations, delusions, compulsions, agitation or disinhibition [1].

'World Alzheimer Report 2015' estimated that globally dementia prevalence in 2015 is 46.8 million people and this estimate could be double at every two decades, leading to approximately 74.7 million in 2030 which would further reach 131.5 million by 2050. These figures are 12-13% higher than previous estimates reported by 'World Alzheimer Report 2009'. According to the 2015 estimates, East Asia region is the main region with the maximum people (9.8 million) suffering from dementia followed by Western European (7.4 million), Southern Asia (5.1 million) and Northern America (4.8 million) region. While, the number vary from one country to another in which over a million people live with dementia. Such countries are - China, US, India, Japan, Brazil, Germany, Russia, Italy, Indonesia and France having 9.5, 4.2, 4.1, 3.1,1.6,1.6, 1.3, 1.2, 1.2 and 1.2 million peoples resepctively [2].

Various aetiologies of neuronal death have been hypothesized like inflammation, oxidative stress, amyloid cascade including tau protein, vascular, cholesterol, dyslipidemia, diabetes and abnormal insulin signalling etc. which are being explored for dementia treatment and most of these pathologies were considered irreversible as dead neurons could neither be regenerated nor replaced [3]. Amnesia condition is commonly present as a symptom in dementia. The common causes of neurodegenerative brain diseases are like Alzheimers, vascular dementia (VD), levy body dementia (LBD) and Frontotemporal dementia (FTD) [4].

Alzheimers Disease (AD) is a common form of dementia and is estimated to be of 50-75% of clinical cases [5]. In AD, there is a typical deposition of insoluble amyloid and neurofibrillary tangles in brain interfering with normal brain cells functioning. Acetylcholine (Ach), a neurotransmitter has a major contribution in learning and memory. Its deficiency is well established in AD [6][7]. In United States, it was estimated that $226 billion has been spent in 2015 for the treatment of < 50 % of people suffering from AD [8].

DM is characterized by hyperglycaemia over a prolonged period due to malfunctioning of insulin secretion, action or both [9]. W.H.O reported that an around 422 million adults to be diabetic in 2014, while it was almost 108 million in 1980. The prevalence of DM has almost doubled from 4.7% to 8.5% among adults. The reason is change in life style increasing the risk factors. Prevalence of diabetes has been raised faster in low and middle income countries (LMICs) as compared to high-income countries (HIC) over the past decade [10].

Diabetics may affect on memory, rapid information processing, cognitive elasticity and psychomotor ability [11]. The association of DM with CNS increases the risk of

memory impairment and AD [12][13]. Several studies suggest that, dysfunctions observed in structure and function of central nervous system (CNS) and end organ damage associated with diabetes occurs by several mechanisms [14-17]. High-calorie diets in animals are reported to affect the arrangement and role of brain hippocampus [18-21].

Oxidative stress is vital factor in pathophysiology of AD and T2DM. Dysfunction of mitochondrial Aβ and glial cells activation in the brain of AD patient contribute to oxidative stress by producing reactive oxygen species (ROS), resulting in production of inflammatory cytokines. Consequently, many recent studies conducted and found that compounds with antioxidant properties can be used for preventing the advancement of AD and T2DM [22-24]. In an animal study, improvement of memory impairment was observed in experimental mice of AD. Whereas, decrease levels of cytokine IL-1β and IL-6 in brain tissue and enhanced superoxide dismutase levels and glutathione /glutathione disulfide ratio were perceived after flavonoid (rutin) treatment [25]. Similarly, a study in 2012 presented that in neuroblastoma cells, rutin treatment cause decreased Aβ cytotoxicity and aggregation with decline in oxidative stress, which prevent mitochondrial destruction and production of microglial cytokines [26]. Another study by Niture in 2014 observed that oral administration of rutin and cytokines i.e IL-6 and TNF-α re-established the antioxidant status in liver of T2DM rats [27]. Whereas, In transgenic AD mice, quercetin treatment produce neuroprotective effects by improving memory and learning deficiency, scattered senile plaques and dysfunction of mitochondria by restoring of ROS levels as well as adenosine triphosphate levels [28**].**

Oxidative stress and inflammation increases risk of CI and dementia. In this direction, a study was performed and observed that oral treatment of resveratrol to diabetic rats resulted in enhance level of oxidative stress markers. While reduced hyperglycemia was observed with decreased cytokine levels (TNF-α and IL-6) and polymorph nuclear cell-mediated nuclear factor kappa-B (NF-κB) function [29]. These findings suggests for further scientific research in developing more efficacious drugs against amnesia.

Currently, drugs in clinical use are mostly for improving symptoms of cognitive disorders mainly affected in the elderly populations. Pharmacotherapy is employed for prevention and treatment of amnesia in neurodegenerative diseases. It is aimed at protecting from excitotoxicity (N-Methyl-D-aspartate antagonists) or supplementing neuronal transmission of viable neurons (Acetyl cholinesterase inhibitors) or selectively improving efficiency of higher telencephalic integrative activities (nootropics – piracetam). However, these drugs are unable to arrest the disease pathology and their significant adverse effects have limited their use [30] [31] Therefore, more efficacious remedies are needed for the treatment. Drugs such as – statins [32], NSAIDs [33], metformin [34], omega 3 fatty acids [35], etc are reported to be beneficial in amnesia. Whereas, oxidative stress, hyperglycaemia, inflammatory cytokines (IL-6 and TNF-α) and tau protein hyper phosphorylation have been stated to cause CI. Thus it could be hypothesized that drugs that control hyperglycaemia may also help in managing CI.

Epalrestat (EPS), is an aldose reductase inhibitor (ARI). EPS being established for anti-inflammatory and antioxidants property is generally prescribed for Diabetic Neuropathy (DN). Intracellular sorbitol accumulation, which causes diabetic

complications during hyperglycemia, is reduced by EPS [36]. It also increases the intracellular GSH levels, important in avoiding oxidative injury in rat Schwann cells [37]. Whereas, another study conducted by Ohmura et al 2009 results found that treatment with EPS reduced, erythrocytes lipid hydroperoxides levels in diabetics [38]. A study has reported that EPS upregulates Heme oxygenase (HO)-1 having antioxidant and anti-inflammatory properties. SOD and catalase (CAT) activate nuclear factor erythroid 2-related factor 2 (Nrf2) and suggest that EPS may prevent many neurological diseases [39]. Hence, it can be concluded that anti-oxidant and anti-inflammatory properties of EPS can be beneficial for the treatment of CI induced by HFD and low dose of STZ in T2DM rats. However, till date there are no studies regarding the effects of EPS on CI.

In view of scarcity of information the present study is aimed to explore the treatment effects of EPS on memory and learning in diabetes male Wistar rats by preventing memory impairment and reducing oxidative stress, inflammation and tau protein.

1.2 LITERATURE REVIEW

1.2.1 Diabetes mellitus (DM): Global Health Havoc

DM, presents with prolonged hyperglycemia accompanied with impairment in carbohydrates, proteins and lipids metabolism [40-42]. W.H.O estimated earlier that around 422 million people were diabetic in 2014, whereas in 1980 it was 108 million. The prevalence of DM in adults became double from 4.7% to 8.5% since 1980 and it has amplified quicker in low and middle revenue countries (LMICs) compared to HIC in last decade [10][43]. The prevalence of DM in India is more than the average worldwide [44]. Largest number of diabetics is reported in India after China, which may be because of the large population in the country. In 2013, 65.1 million people were suffering from diabetes and estimated to 109 million by 2035 [45-48].

There are two major form of DM:

i. Type- 1 is instigated by lack of insulin discharge by pancreatic beta cells (IDDM).
ii. Type- 2 is instigated insulin resistance (NIDDM).

This reduced sensitivity to insulin is caused by several factors as presented in Table 1. Insulin resistance reduces efficient absorption and utilization of glucose from the food by the cells, except in brain [49]. This results in enhanced glucose concentration and cells starts to utilize more of fats and proteins for their energy needs.

1.2.2 Causes of Diabetes Mellitus

1.2.2. A T1DM

T1DM is initiated by a lack of insulin production by pancreas beta cells. It is an autoimmune ailment and body's own immune system destroys the beta cells. It is seen

in all age groups. [50][51]. Several features like genetic susceptibility, food and certain viruses may contribute to the disease [52-54].

Table 1: Insulin resistance – causes [49]

Sl. No	Causes
1.	Obesity/overweight
2.	Excess glucorticoids
3.	Excess growth hormone
4.	Polycystic ovary disease
5.	Pregnancy, gestational diabetes
6.	Lipodystrophy
7.	Autoantibodies to the receptors of insulin
8.	Mutations of receptors for insulin
9.	Mutations that cause genetic obesity
10.	Mutations of the peroxisome proliferators' activator receptor γ (PPAR γ)
11.	Hemochromatosis

1.2.2. B T2DM

Genetics: Some genes are described to cause maturity-onset diabetes of the young. Genetics can contribute for both diabetes types [55].

Family history: As documented by American Diabetes Association, a person whose both parent have T1DM has a 10 to 25% chance of developing the illness. While whose both parent have T2DM, it is 50%.

Lifestyle: factors such as obesity, stress, deprived diet, increased consumption of sweets and lack of exercises are the major contributors.

Table 2: The clinical presentation of Diabetes Mellitus in patients

Features	T1DM	T2DM
Age of onset	Usually less than 20 years	Usually greater than 30 years
Body mass	Low (wasted) to normal	Obese
Plasma insulin	Low or absent	Normal to high initially
Plasma glucagon	High, can be suppressed	High, resistant to suppression
Plasma glucose	increased	increased
Insulin sensitivity	Normal	Reduced
Therapy	Insulin	Weight loss, thiazolidinediones, metformin, sulfonylureas, insulin

Weight and body type: Overweight and obesity adds risk of T2DM and gestational diabetes. Visceral fat may cause insulin resistance.

Physical activity: Lack of consistent exercise is the key reason of obesity and diabetes.

Diet: Whether diet influences progress of diabetes is controversial. Studies report suggests that heavy consumption of simple sugars rises threat of DM and low glycemic index foods reduces this risk.

Other diseases: Medical illnesses like high blood pressure, hyperlipidemia, polycystic ovarian syndrome (PCOS), asthma and sleep apnea comprise been linked to T2DM [56][57].

Hormones: Hormones may influence DM in different ways; e.g. stress hormones such as cortisol may cause fluctuating glucose levels in T2DM.

1.2.3 Epidemiology and Etiology of T1DM

As per study reports in 2014, an estimated 387 million people are diabetic worldwide [58]; among which 5% and 10% have T1DM [59]. It is usually diagnosed at the age of 4 to 5 years, or in adolescence [60][61]. Three sorts of autoantibodies identified to cause T1DM are: Islet cell cytoplasmic antibodies, Islet cell surface antibodies and Specific antigenic targets of islet cells like those against glutamic acid decarboxylase [62].

1.2.4 Pathogenesis of T1DM

T1DM Pathogenesis is associated with destruction of insulin-producing pancreatic β-cells [63].

1.2.5 Epidemiology and Etiology of T2DM

T2DM is the major form of DM and accounting for 90% diabetic cases [64]. The rise of prevalence is predicted more in developing countries. In these countries, people aged 40 to 60 years are affected most, in contrast to older people as in developed countries [65]. This could be because of the change in lifestyle causing high food intake with lesser physical activity resulting in increasing prevalence of overweight [66] [67].

The incidence of DM rises with age, generally related cases are detected after 40 years. [68]. It’s major responsible different factors are genetic, lifestyle changes and stress [69].

1.2.6 T2DM Pathogenesis

As per normal physiological aspects, a synchronized feedback mechanism of insulin sensitivity of tissues and its secretion, maintains the glucose concentrations in blood [70], whereas in T2DM, insulin secretion is disturbed due to impaired pancreatic β-cell dysfunction (Figure 1) [61].

T2DM is affected in 1 to 2% of Caucasians [71], higher in Pima Indians [72] and Arabs [73] and 50% in South India, indicating that genes contribute more than environmental factors [74][75].

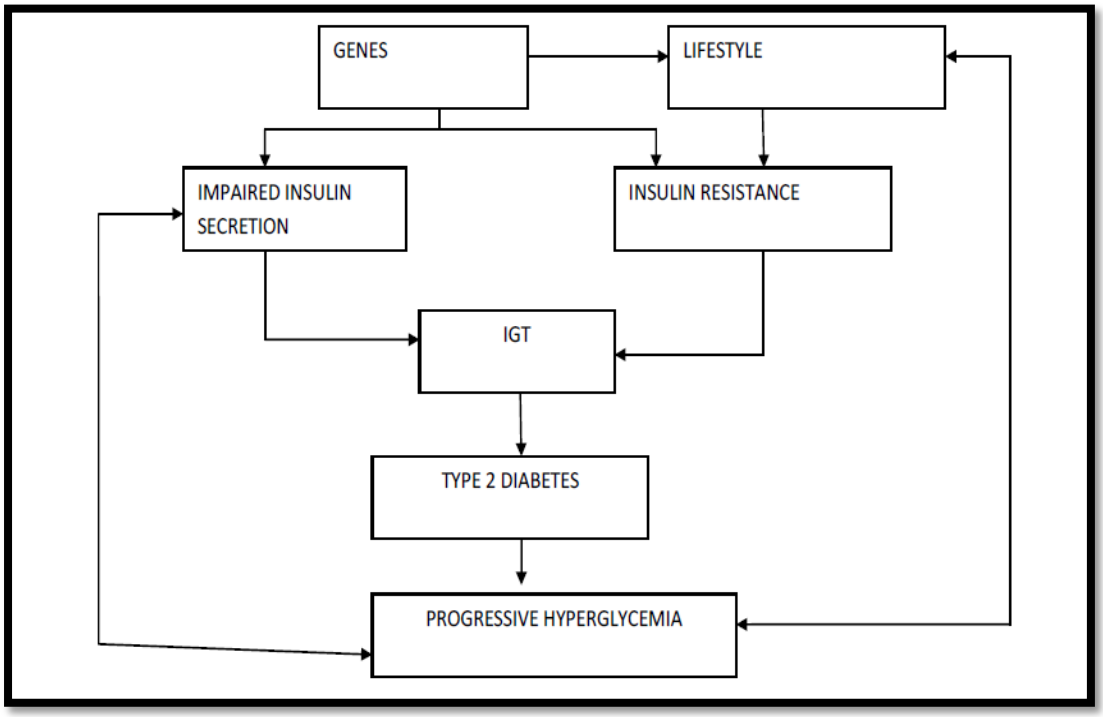


Figure 1: T2DM Pathogenesis

Pancreatic abnormalities in islet secretory cells in T2DM are noted. Insulin secretion defects may result in glucose resistance and amino acid hypersensitivity for insulin release. Beta cells may reduce to half in volume and number and alpha cells are increased causing hyperglucagonemia. The islets exhibit hyalinization and amyloid

deposition containing amylin (a secretory peptide), whose importance in T2DM pathogenesis is not well elucidated [76-79]. In type-2 diabetic patient high levels of amylin was seen, in obese patient the insulin resistant as well as impaired glucose tolerance in patient were observed [80][81].

Beta cells and amyloid deposit contact in T2DM has been seen through electron microscopy [82]. The insulin receptor gene on chromosome 19 encodes the protein having alpha and beta subunits. Mutations in this gene are linked to T2DM and resistance to type A insulin [83][84]. Insulin resistance alone cannot cause overt glucose intolerance, but in obese people it may impair insulin action. As insulin resistance is also seen in non-diabetic obese individuals, it could be a secondary event in T2DM. Defect in insulin secretion result in hyperglycemic condition and insulin resistance [85].

Insulin resistance in T2DM is not totally clear, it may involve reduced insulin receptor number [86], condensed tyrosine kinase activity [87-89] or abnormalities distal to the receptor relating glucose transporter (GLUT) proteins [90]. Genetic susceptibility to T2DM may be influenced by GLUT2 and GLUT4 genes expressed in liver, pancreatic beta cells, skeletal muscle and adipocytes [91].

Obesity has genetic and environmental causes which have effect on the T2DM improvement [92-97]. The progress from obesity to T2DM is resultant of succession of pathophysiological events [98]:

a) Increase in adipose tissue mass, causing increased oxidation of lipids.
b) Insulin resistance blocking the glycogen cycle.
c) Unused glycogen prevents further glucose storage leading to T2DM.
d) Complete β-cell collapse.

Nutrient consumption with increased fat and decreased carbohydrate content leads to hyperinsulinemia of obesity. Dietary fibres may improve T2DM [99].

Environmental factors and T2DM pathogenesis

Obesity, aging, alcoholism, smoking etc are the common risk factors of T2DM. Even mild obesity will have 4 to 5 fold increase in risk of developing diabetes, if led by the enhancement of visceral fat [69]. Several factors contribute for upregulation of visceral fat such as stress, unhealthy diet and lifestyle, endocrine disorders, lowered energy consumption, genetic factors, aging etc.

1.2.7 Pathophysiology of T2DM (NIDDM)

The elements of NIDDM can be divided into four groups:

i) Those with normal glucose tolerance.

ii) Chemical diabetes

iii) DM with fasting plasma glucose - FPG less than 140 mg/dl.

iv) DM in association with FPG greater than 140 mg/dl.

Despite having high levels of plasma insulin, those with poor glucose tolerance will have hyperglycemia, indicating insulin resistance. As this impaired glucose tolerance progresses to DM, the level of insulin declines [61]. Pathophysiology of T2DM is described in Figure 2.

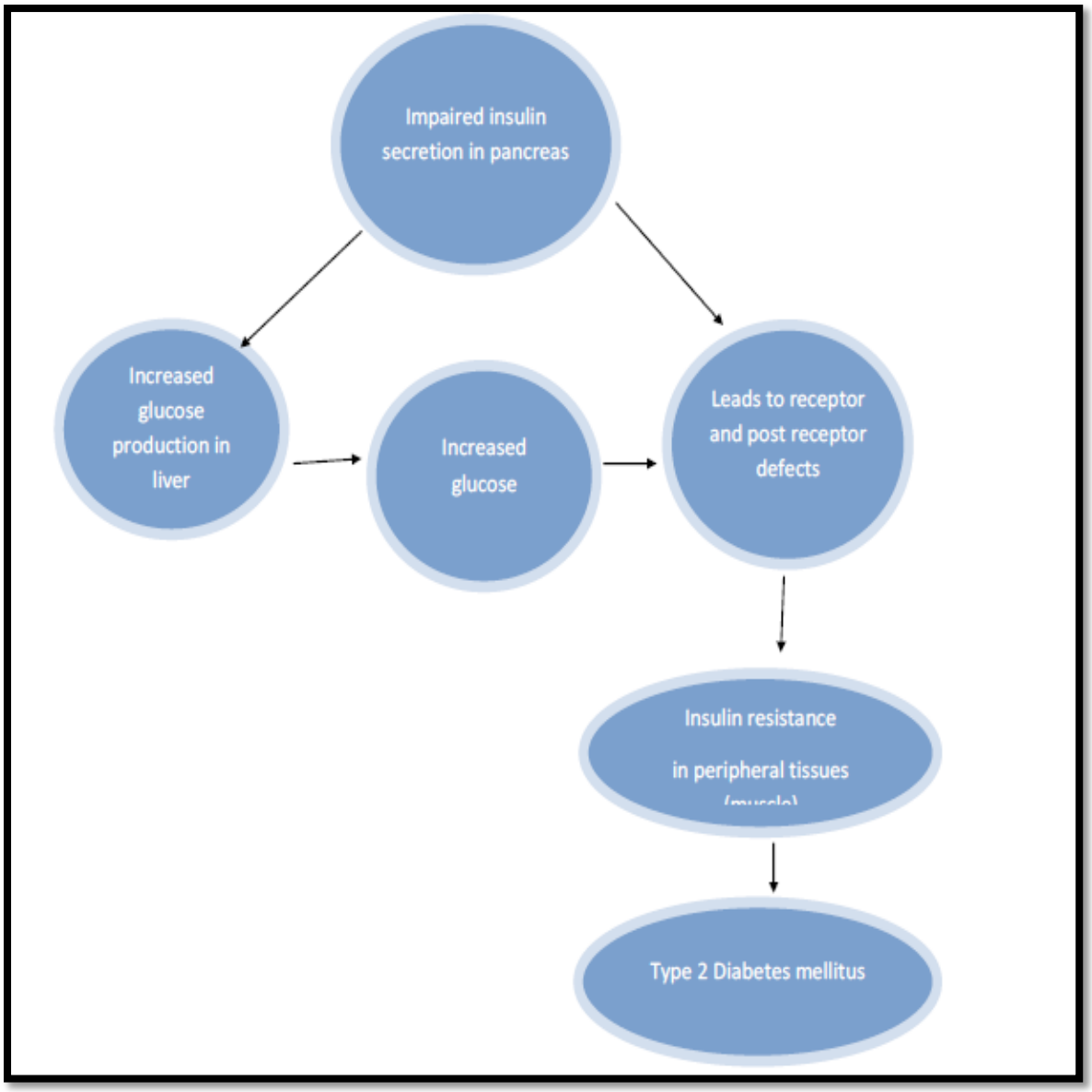


Figure 2: Pathophysiology of T2DM

1.2.8 DM Signs and Symptoms

A. **Symptoms of T1DM**

Irregular urination and thirst, extreme hunger, unusual weight loss, excessive fatigue and irritability.

B. **Symptoms of T2DM**

Frequent urination and thirst, increased hunger, unexplained weight gain, irritability and fatigue, blurred Vision.

C. **Warning Signs of Diabetes**

Decelerated wound healing, fungal infections of skin and urinary tract infections.

1.2.9 Diagnosis of DM

Screening/ diagnosis of DM are tabulated in table no.3 [100-105]. Several tests are available to detect glucose levels for its diagnosis. Random plasma test, FPG test [105], OGTT [106], Glycated proteins, Glycated haemoglobin [107], Fructosamine test are the most common tests used for the purpose.

Table 3: Screening and ADA guidelines for T2DM

Criteria:

* **Overweight and obese children**
  - o BMI >85th percentile for age and sex
  - o Weight for height >85th percentile
  - o Weight >120% of ideal for height

Plus any two of the following risk factors:

a. Family history of T2DM in first or second-degree relative

b. Race/Ethnicity

* Native-American
* Latino
* African-American
* Asian American
* Pacific Islander

c. Signs of insulin resistance or conditions associated with insulin resistance

* Acanthosis nigricans
* Hypertension
* Dyslipidemia
* PCOS
* Small for gestational age birth weight

d. Maternal history of diabetes or GDM during the child's gestation

Age of initiation: age 10 years or at onset of puberty, if puberty occurs at a younger age

Frequency: every 3 years

Preferred Test: FPG plasma glucose

1.2.10 Complications of DM

DM presents with a number of complications ranging from diabetic ketoacidosis due to hyperglycemia and coma due to hypoglycemia (Figure 3). It may cause microvascular disease, macrovascular disease, neuropathy, cardiovascular diseases (CVDs) and cerebrovascular disease. Other complications of diabetes are depression [108], dementia [109] and sexual dysfunction [110-112].

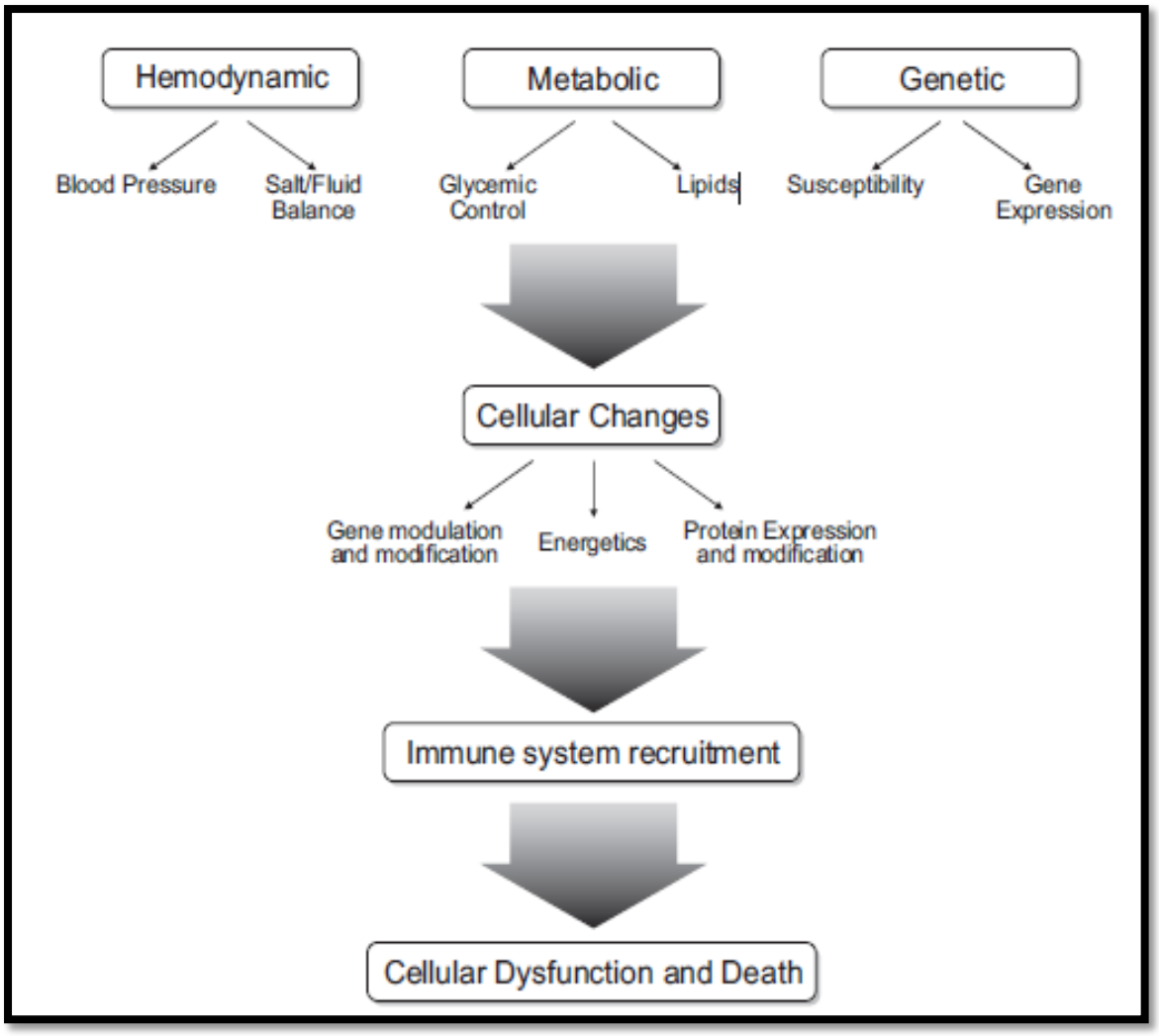


Figure 3: Complications of diabetic mellitus

Diabetic nephropathy is the main reason of renal failure in western societies [113], with increase in proteinuria and reduction in glomerular filtration rate (GFR) [114-116]. Diabetic retinopathy results in blindness in adults having 20 to 74 years of age [117-119]. While, Diabetic Neuropathy (DN) causes poor sensory and motor actions in limbs and diabetic foot ulcers. T2DM is documented for its association with increased risk of coronary heart disease, also has a chance of myocardial infarction in diabetic patients (Figure 4) [131-136].

1.2.11 Treatment of DM [137]

A. Drug Treatment for DM

B. Non Pharmacological Management of DM

A. **Drug Treatment for DM**

T1DM requires insulin administration as it occurs by insulin deficiency whereas T2DM caused due to insulin resistance by cells and is managed by drugs which lowers glucose levels in blood by different mechanisms. Treatment strategy consists of increasing the insulin secretion or insulin sensitisation of target organs. This includes administration of insulin, insulin sensitizers, Alpha-Glycosidase Inhibitors, Peptide Analogs, Glucagon-Like Peptide (GLP) Analogs and Agonists, Gastric Inhibitory Peptide (GIP) Analogs DPP-4 Inhibitors Amylin Analogues etc.

Enhanced concentration of aldose reductase (AR) in hyperglycemia condition of DN cases results in increased sorbitol level.Which leads to cellular injury and decreased myoionositol in peripheral nerves and thereby leading to reduced activity of Na+/K+-ATPase, which is essential for nerve conduction [120-127].

Among the various ARIs, quercetin, puerarin, rutin, baicalein, ellagic acid, chlorogenic acid, epigallocatechin-gallate, naringin, eugenol, baicalin, and curcumin were reported to be effective in DN [128]. Studies have established that EPS can postpone the development of DN and can improve the associated symptoms, without side effect [129]. From clinical trials it proved reported that dose dependednt administration of EPS improved motor and sensory nerve conduction [130].

B. **Non pharmacological management of DM**

i) Life style changes to Control DM

Proper dietary and lifestyle practices, regular montitoring of health conditions and maintaining of glucose levels can control the comorbidities which may develop later in T2DM.

ii) Exercise

Helps to prevent diabetes by losing weight and reducing blood glucose levels. It makes our body tissues more sensitive to insulin.

iii) Diet

Rich fibre foods helps to prevent diabetes.

1.2.12 Development of Memory

A. Introduction

Memory is the method of maintaining information overtime. Memory process and its evolution have a complicated physiology. The regions of brain are highly coordinated, integrated and distributed to one another. It is intriguing to know that it was a case of 'memory Impairment' which had shed light on neuroanatomy of memory and its functioning. The aspects of memory are stemmed from a lifelong and post-mortem study of an interesting case of a man named Henry Molaison (H.M), although animal models and other examples substantiated it [138][139].

Memory is a unique treasure sculpted over a lifetime of experiences and knowledge which is required for the proper functioning of life. The functioning or stages of memory involves encoding of sensory information, its storage in the form of a code and retrieval of this coded information [140].

B. Memory: Neuroanatomy and Neurophysiology

i) Memory can be divided into short term memory and long term memory: Short term memory denotes to the holding of information in the conscious state for a short time. Long-term memory denotes to memory which are removed from conscious state but can be retrieved after longer time [141]. There are different sites for formation of short term memories and storing them for a longer use. Memory repetitive sensory stimulations lead to formation of permanent memory in the form of long term memory.

ii) Hippocampus and adjoining areas are concerned with converting short term memory into long term memory (Figure 4): Neurophysiologic studies and brain lesioning studies on animals reported that structures of medial temporal lobe involved in memory consolidation [138][139][142]. It consists of structures like hippocampus and its underlying cortices/perihippocampal cortices (entorhinal, perirhinal, parahippocampal areas) [143]. The other areas involved in memory storage are the diencephalon [144], the amygdala (memory for emotionally disturbing or aversive experiences) [145] and the pre-frontal cortex (for working memory) [146]. The hippocampus temporarily stores acquired information which is very vulnerable to interferences. This information could be made permanent by transferring it to other brain structures (such as cortical regions) where it is reasonably immune to interferences. Long term memory formation involves long term potentiation (LTP) which produces changes which strengthens the connection between neurons for long time (Figure 6) [147].

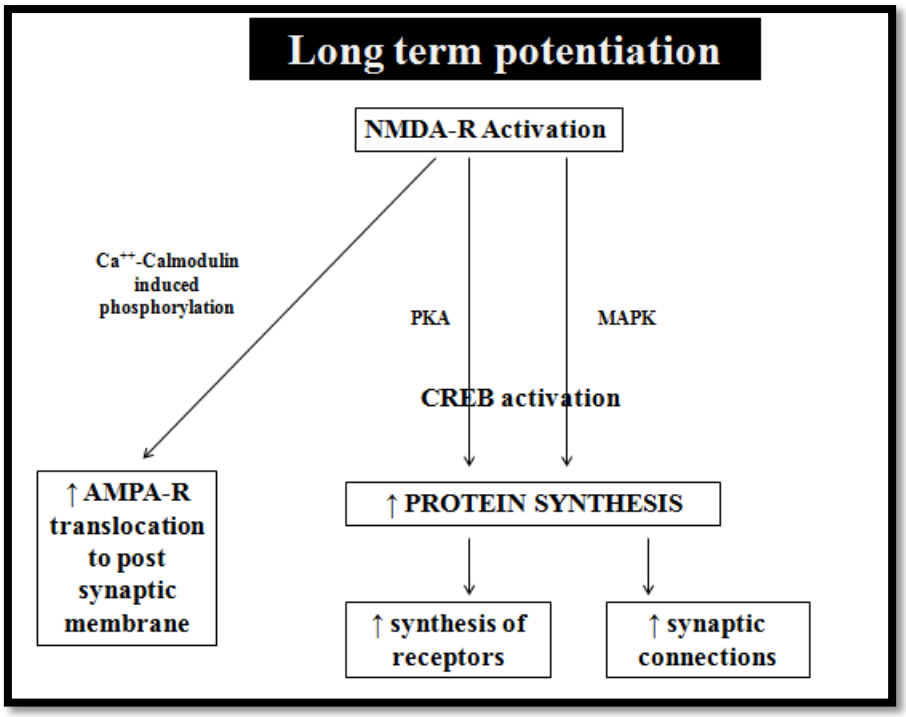


Figure 4: Process of LTP in Long Term Memory Formation

It produces lasting changes in the strength of the connection between neurons and involves: [147][148].

- **Strengthening of pre-existing connections** - by translocation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA receptors) to post-synaptic membrane.
  - NMDA receptor (NMDA-R) activation by glutamate results in Ca^{++}-calmodulin-dependent phosphorylation of pre-existing AMPA glutamate receptor and translocation to post synaptic membrane.
  - Arachidonic acid and Nitric Oxide (NO), retrograde messengers, are also released which acts pre-synaptically to sustain synaptic activity.
- **Formation of new synaptic connections** - This requires both translation and transcription

- This result following protein kinase A (PKA-a cAMP-dependent protein kinase) and mitogen activated protein kinase (MAPK) activation which further activates the transcription factor cAMP response-element binding protein (CREB).
- CREB controls a transcription cascade which yields structural changes in synapse [149]

iii) The process of long term memory- Long term memory has two major forms – declarative and non-declarative (Figure 5) [144][145][150][151].

C. Neurochemistry of Cognition

The complexity of neural networking in cognitive function leads to difficulty in identifying specific neuronal substrates, especially those which can offer pharmacological goals for cognitive dysfunction [152]. Still, the search is on to identify specific neurochemical targets, to which drugs can be used to prevent or treat cognitive dysfunction [153].

Neurotransmitters which primarily influence cognition are dopamine (DA), ACh, norepinephrine (NE) and 5-hydroxytryptamine (5-HT). There are some neuromodulators which directly and indirectly affect cognitive processes like cytokines, neurotrophic factors (BDNF-Brain derived neurotrophic factor), histamine, glutamate, calcium, neuropeptides etc.

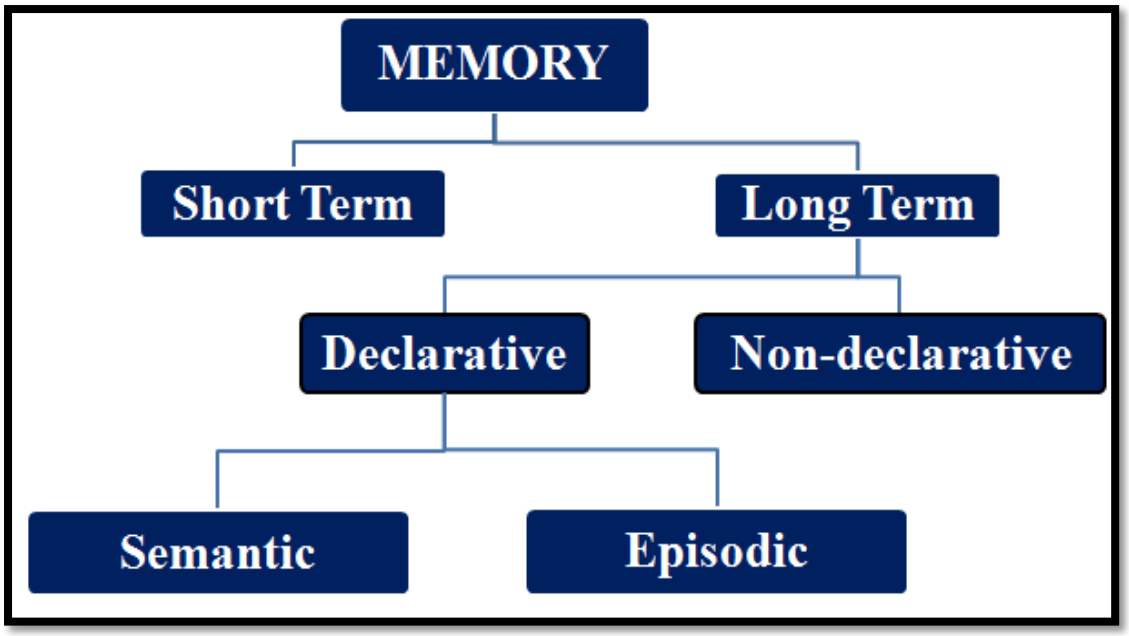


Figure 5: Diagram showing types of memory

The three systems which were identified to have the major role include the NMDA-glutamate system, the cholinergic system and the GABAergic system [152][154].

The hippocampus and PFC obtain an affluent cholinergic input and also innervated by serotonergic, [155] dopaminergic, noradrenergic and histaminergic [156] neurons. These along with the amygdala are innervated by GABAergic interneurons and they communicate via glutamatergic neurons.

Molecular manipulations that upregulates the function of NMDA receptors or increased pre-synaptic glutamate release have been previously reported to enhance learning and memory [148]. Chamberlain et al documented the role of NE as a key neurotransmitter for functioning of working memory [156]. Dopaminergic receptors are located in PFC [146] hippocampus [153][157] which are linked to the cognitive dysfunction seen in psychiatric diseases. The promising effect of selective serotonin reuptake inhibitors (SSRI) in neurodegenerative process of mild cognitive impairment (MCI) has established the role of serotonergic system in cognition. SSRIs in various

studies have promoted neurogenesis, by demonstrating neurotrophic activity [158]. Histaminergic system indirectly affects cognition as H_3 receptor agonists have shown to decrease cholinergic neurotransmission and thereby impairing cognition [155][159].

These molecular targets potentially constitute a vast repertoire of mechanisms to which drugs could be developed for countering CI [152].

1.2.13 Disorders of Memory

Memory disorders may range from simple forgetting, pure memory loss, to age associated CI (AACI) to dementia.

Amnestic Syndrome and Amnesia

Amnesia is a condition in which memory and learning process are more affected than other cognitive functions such as intelligence, perception and language capabilities in an otherwise alert and responsive patient. The syndrome can be caused in several ways: head injuries [160], vascular accidents, infections, substance abuse or degenerative neurological disease processes (e.g. AD) [161][162].

The term dementia derives from the Latin *demens* ("*de*": private, "*mens*": mind, intelligence, judgment—"without a mind") [163]. Dementia is described as "any psychological impairment or worldwide cognitive decline in an earlier unimpaired person" and is categorized by the progressive decline in cognitive function, intellectual, emotional, and behavioral skills, interference in daily life acitivity of its sufferers; caused due to any disease and/or brain damage [163][164].

1.2.14 Causes of Amnesia

Amnesia commonly presents as a symptom in dementia. The common causes are neurodegenerative brain diseases like AD, VD, LBD and FTD [4]. Some less common causes are infections, tumours, hypothyroidism, subdural haemorrhage, hydrocephalus, DM, dyslipidaemia, toxins or deficiencies of vitamins. A number of psychiatric disorders are also associated with CI like depression, learning disabilities, age-related cognitive decline and mental retardation [164].

Neurodegenerative brain diseases although being the more common cause of amnesia as a symptom have very limited treatment modalities against it [164]. The main common reason of neurodegenerative brain diseases is still known to be AD. The diagnostic criteria of AD include three stages. The first stage is that of asymptomatic amyloidosis which is preclinical, the second is MCI that is predementia phase of amyloidosis along with some neurodegeneration and the third final stage is of dementia which is amyloidosis with neurodegeneration plus cognitive decline [165].

The formation of toxic amyloid plaques from peptides due to the abnormal processing of amyloid precursor protein (APP) into toxic A-beta peptides forms the basis for the initiation of AD. It increases various inflammatory processes, further creating NFT by tau proteins hyperphosphorylation by the same amyloid cascade. This leads to neuronal dysfunction, neuronal death and regional progression of Alzheimer's symptoms mentioned above [165].

VD is the 2nd common type of age associated dementia [166]. A number of mechanisms can cause dementia including oxidation, inflammation, disease induced neurotoxicity and genetic vulnerability. VD, as the name suggests results from ischemic or haemorrhagic brain injury owing to cerebrovascular disease. The drop in

blood flow to the brain might be because of direct occlusion from a blood clot or secondary to chronic illnesses such as hypertension, diabetes, and dyslipidaemia [167].

1.2.15 Pharmacotherapy of Amnesia

Amnesia can exhibit separately or as a feature of cognitive disorders of different dementias. Pharmacotherapy of the same is presently limited to either shielding from excitotoxicity (NMDA antagonists) or supplementing neuronal transmission of viable neurons (Acetylcholinesterase inhibitors- AChE-I) or specifically enhance efficiency of higher telencephalic integrative exercises (nootropics – piracetam) [30][31]. Currently, no appropriate drug is reported which could modify the disease pathology [168]. Majority of approved drugs against CI are aimed at restoring the cholinergic neurotransmission [154]. The major hurdles restricting development of such drugs are [158]:

- Multimodal pathway of memory processing
- Presence of blood–brain barrier (BBB),
- Existence of efflux transporters (eg: P –glycoprotein).
- Dogma – that neurons cannot be generated

❖ **Pathophysiologic Hypothesis Aiding Drug Development**

A) Cholinergic Hypothesis –signs of AD is initiated by decreased Ach in synaptic cleft due to loss of cholinergic neurons. Thus, by increasing ACh in the cleft should recover the memory and cognition impairment [168] Drugs with anticholinesterase activity like donepezil (donep), rivastigmine and galantamine have been accepted by US-FDA [168] but the improvements have been modest [153].

B) Abnormal Protein Folding – Seen mainly with AD and characterised by amyloid plaques development and hyper phosphorylated tau which are themselves neurotoxic [30].

C) Excitotoxicity – Phenomenon seen with sustained stimulation of excitatory amino acids (eg. glutamate) causing intracellular Ca^{++} overload. This overload causes neuronal death by activating proteases; recruiting free radicals, oxidants and causing lipid peroxidation [169]. So, drugs are used to prevent this excitotoxicity like NMDA antagonists [30], calcium channel antagonists and protease inhibitors.

D) Inflammatory condition and Oxidative Stress –Various factors cause accumulation of oxidative stress like ageing, [170] excitotoxicity, protein misfolding, [171] hypoxia, mitochondrial dysfunction etc. It is both the cause and inflammation effect which lead to neuronal loss [30]. Various pharmacological agents are being developed against this oxidative stress [170] (free radical scavengers, anti-inflammatory drugs, drugs reversing vascular dysfunction, etc).

E) Oxidative Stress and AD: There are evidences showing that brain in AD patients experience oxidative stress during the disease development. There will be an imbalance in radical production of ROS and antioxidative defence, both having major roles in the process of age-related neurodegeneration and cognitive decline [172].

1.2.16 Pathophysiological Mechanism

Diabetes-related complications not only affect insulin dependent organs like muscle and adipose tissue but also extended upto the other vital organs i.e., kidney, liver, eyes and brain, which are directly affected by the glucose toxicity in DM. Many epidemiological studies provide evidence that T2DM may cause memory problems and CI [173][174]. Impaired glucose metabolism, insulin resistance, increased β-

amyloid formation, oxidative stress, pro-inflammatory mediators and the existence of advanced glycated end products (AGEs) may affect the neuronal survival and functioning [175].

A. Effect of Diabetes on Brain

Diabetes associated hyperglycemia induces drastic changes in vascular and neuronal cells, as well as documented in both animal models and diabetics. The changes at both cellular and functional level following diabetes are pleotypic in nature as glucose and its metabolites is well-known to affect several cellular pathways. As glucose is the prime source of energy in brain and its consistent functioning is reliant on the continuous supply of glucose [176][177], so any alteration in glucose content or supply would possibly affect the cerebral functions [178][179]. The well-recognized diabetic impediments in CNS are listed as: impaired cognition [180-182] dementia [183-186], altered learning and memory processes [187-189] and AD [190-192].

B. Alteration in Brain and glucose metabolism in AD

Numerous studies have report that T2DM is a key danger factor for AD [193]. Substantial deterioration in cerebral glucose usage is observed in AD. It suggests that the irregularities in oxidative and energy metabolism caused by metabolic disturbances in glycolysis [194][195]. Fukuyama et al. conducted PET studies to study decresed cerebral glucose metabolism. Among AD pateints oxygen consumption, glucose usage and local blood flow were lower in the frontal, parietal and temporal regions. This may be because of irregular glucose metabolism in parietotemporal region [196].

C. Link between insulin resistance and AD

Luchsinger et al. stated that hyperinsulinemia augmented risk for AD [197]. Also, it is known that high levels of insulin and its resistance are linked to risk of AD [198][199]. Although interplay between diabetes and AD remains debatable [200], the abnormalities in insulin metabolism, are believed to facilitate AD, through their influence on the metabolism of Aβ, oxidative stress, tau hyperphosphorylation and inflammation (Fig. 6).

D. Insulin resistance and TAU Phosphorylation

AD causes neuronal death either by protein modifications, oxidative stress, inflammation, dysregulated immunity, neurotoxic agents. AD is defined by Aβ pathology and tau pathology [NFTs]. There is cumulative evidence that when tau proteins adopt pathological forms, they can cause cell death [201][202].Tau proteins accumulate with tubulin to stabilize microtubules (MT) and vesicular transport. NFTs are hyperphosphorylated and cumulative form of tau proteins. Once it is hyperphosphorylated, tau becomes insoluble and lacks affinity for MTs, leading to neurodegeneration [203].

E. Insulin resistance and oxidative stress level

The increased oxidative stress and the activation of signaling pathways are linked to AD [204][205]. Oxidative stress damages the proteins [206][207], lipids [208] and nucleic acids (DNA, RNA) [209-211].

F. Insulin Resistance and Inflammatory mediators

According to the reports, inflammation intermediates the progress of AD. In brains of AD, presence of Aβ plaque and neurofibrillary tangle, trigger astrocytes and microglia

to release inflammatory mediators – cytokines / chemokines resulting neuroinflammation [212-215]. In fact, in AD, neurons themselves produce inflammatory cytokines like, IL-1β, IL-6, TNF-α and complement proteins [216-218].

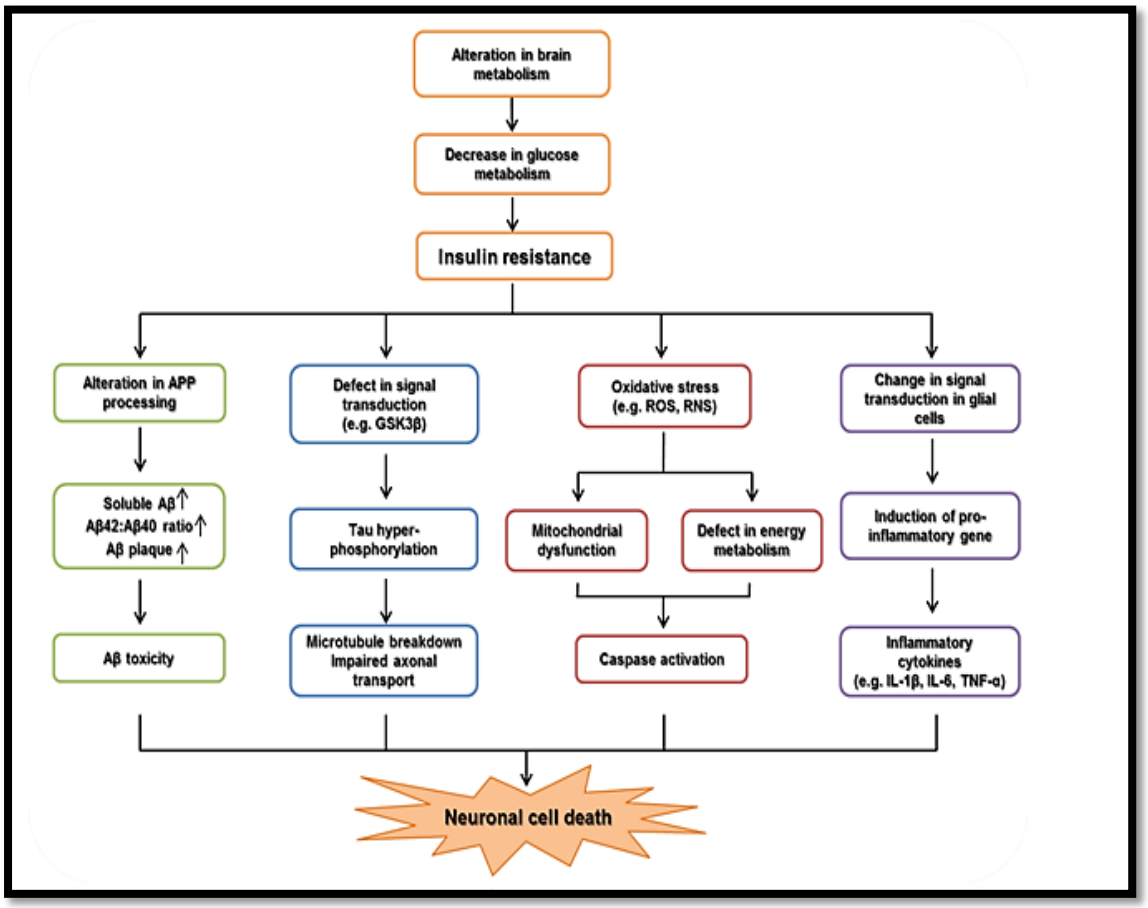


Figure 6: Graphic representation of molecular paths linking insulin resistance and AD [219].

G. Mechanism and Consequent Effect of Cellular Damage Following Diabetes in Brain

In diabetes, increased glucose level counter with oxygen and generates a series of oxidative radicals. The state of oxidative stress caused by increased free radicals, formation of AGEs, augment the protein kinase C activation and glucose shuntin, resulting in cell death [220][221].Studies on STZ-induced rodent model characterized the diabetic encephalopathy in cerebral cortex, cerebellum and hypothalamus. Diabetes trigger various morphological alterations in brain cells like amplified area of myelinated neurons, disarray of myelin sheath, perivascular and mitochondrial swelling [222].

Hippocampus is more susceptible to diabetes. Previous studies conducted on spontaneous model of autoimmune T1D and STZ-induced model depicted the deleterious effect of diabetes. Diabetes dramatically decreases the cell proliferation [223-225], alters the neurogenesis [226-228] and causes neuronal apoptosis in hippocampus [226][227][229] and also damags the dendrites and synaptic structures of CA3 neurons [230-232]. Diabetes also reduces the dendritic growth of newly formed neurons [233][188]. Diabetic condition also affects the synaptic plasticity [234][235]and associated learning memory [236][224]225]. These affects on hippocampus provides an explanation for CI.

1.2.17 Memory Disorders: Drug Development Strategies

Several drug development strategies in memory disorders are documented; most of them are in investigational stages.

- Drugs Aimed at Increasing Neurotransmission like Cholinesterase Inhibitors [237][238].

- Drugs Acting on Nicotinic Receptors [239][240]
- Combined Cholinesterase Inhibitors and Monoamine Oxidase (MAO) Inhibitors [168]
- Drugs Aimed at Neuroprotection
- Drugs Against Excitotoxicity [35]
- Antioxidants and anti-inflammatory mediators [241][242]
- Apoptosis inhibitors
- Drugs Improving Cerebral Metabolism [31]
- Drugs Improving Vascular Dysfunction
- Immunotherapy
- β secretase inhibitors
- Drugs Enhancing Memory Formation
- Nootropic drugs
- Drugs Recruiting Neural Stem Cells and Promoting Neurogenesis

1.2.18 Drugs Used in the Present Study:

DN is very common in DM patients [243][130]. Epalrestat (EPS) is easily absorbed into the neuron and it is an enzyme inhibitor in dose dependedent manner [36][244][245]. EPS was approved in Japan in 1992 for easing of neuropathy symptoms, abnormality of vibration sense and irregular changes in heart beat associated with DPN. Side effects are nausea and vomiting and increase in certain liver enzymes [130][36].

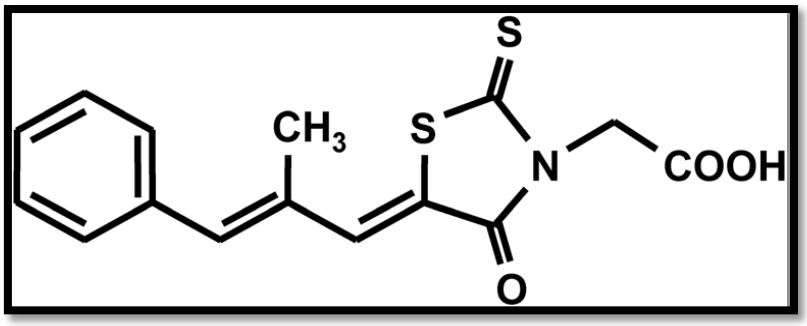


Figure 7: EPS Chemical Structure

Evidence

EPS treated diabetic rats displayed improvement in nerve morphological abnormalities [246]. In another dose dependent study EPS improved the effects of DN like upper limb spontaneous pain, motor nerve conduction [247]. It can control diabetic cardiovascular autonomic neuropathy in early or mild cases [248]. EPS proves to have the potential to prevent many neurological diseases [249]. As per our recent published findings experimental diabetic rats administered with EPS proved that it could act as a beneficial agent for CI prevention and disease management [250][251].

[A] Link Between Study Drugs and Disease

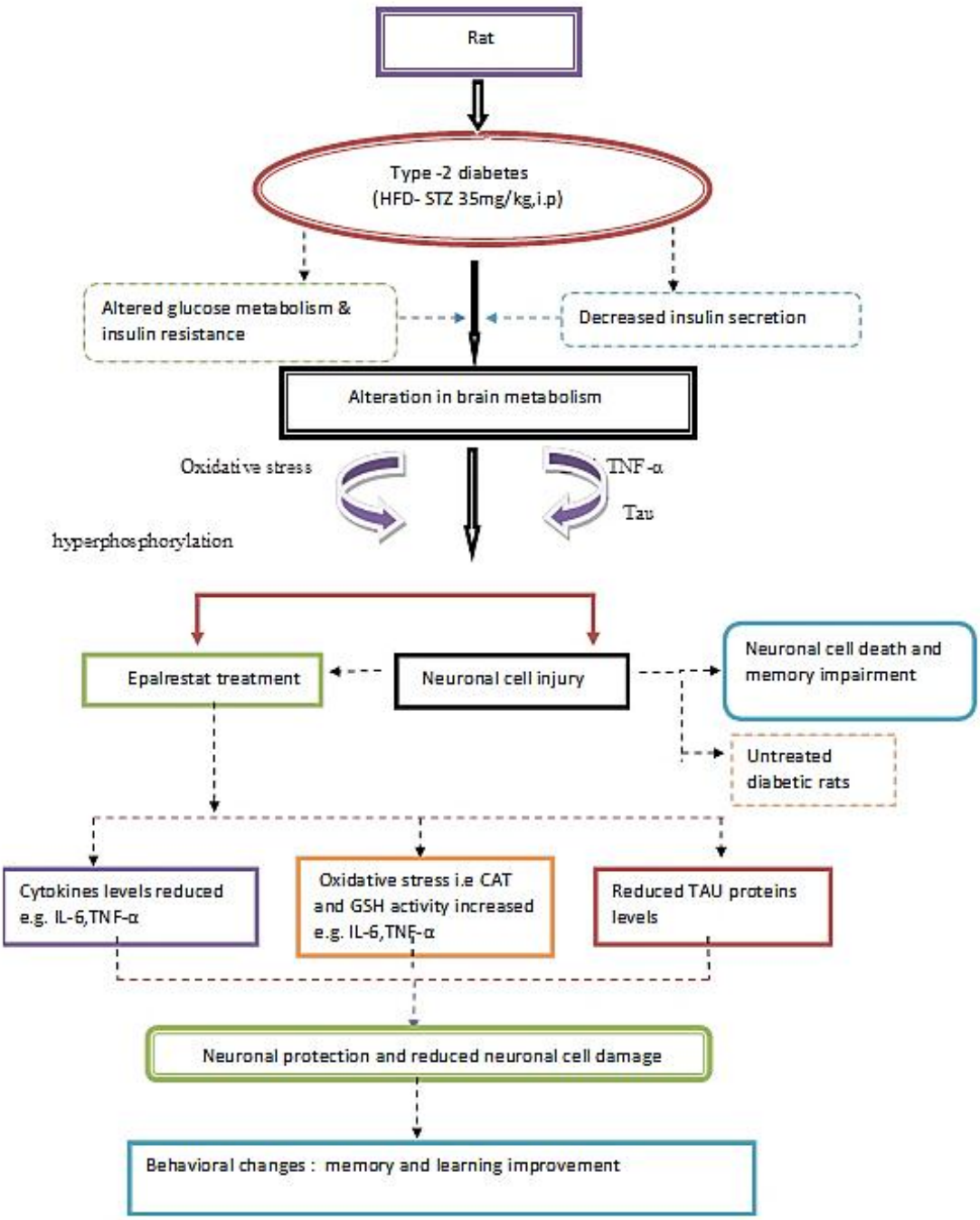


Figure 8: Showing Pathogenesis of Neuronal Cell Death

i) T2DM: During diabetes increased level of glucose counters with oxygen and generates a series of oxidative radicals. The state of oxidative stress caused by these free radicals increase the formation of AGEs, enhance the protein kinase C activation and glucose shunt in the hexosamine pathway that could ultimately damage the cell constituents and resulted in cell death.

ii) Hyperphosphorylation of tau: Tau is a normal constituent of neurons. On hyper phosphorylation it causes unstability in the cytoskeletal structure of neurons leading to neuronal death.

iii) Inflammation and Oxidants: These are the cause and result of pathologic processes leading to neuronal death.

[B] Link between EPS and CI

Augmented oxidative damage was seen in the brains of experimental hyperglycemic rats [252][253].Oxidative stress increases cytokines and ILs, leading to pathogenesis of AD. Inflammation in T2DM comprise a role in the susceptibility of T2DM patients to AD and in the progression of T2DM in AD patients [254][255].

i) Role of EPS in Oxidative Stress:

Hyperglycemia activates the polyol pathway and nicotinamide adenine dinucleotide phosphate (NADPH) mediated reduction of glucose to sorbitol [256]. As NADPH is invoved in detoxification of ROS and hydrogen-peroxide, a large drain on the NADPH pool could negotiate the ability of the cell to protect from oxidative stress. NADPH is also required for GSH reductase to regenerate GSH [257-261]. Furthermore, the polyol pathway activity contributes to nonenzymatic glycation/ glycoxidation [262-266]. A number of investigators recommended that

hyperglycemia-induced GSH depletion take place because of glycation or reduced expression of key enzymes of GSH biosynthesis, γ-glutamyl cysteine synthetase and glycation of GSH reductase [267][268]. Thus, it is know that inhibition of polyol pathway reduced oxidative stress. Whereas lipid hydroperoxides levels in erythrocytes of diabetic patients were significantly reduced by EPS.

ii) Role of EPS in inflammation:

A Study has documented that HO-1 is a target for neuroprotection and neuroinflammation in neurodegenerative diseases [269]. Phytochemicals can upregulate HO-1 expression *via* the Nrf2 pathway [270]. EPS upregulates HO-1, SOD and CAT by activating Nrf2 and results shown that it can prevent several neurological disorders[39]. The anti-inflammatory and anti-oxidant properties of EPS can be beneficial in managing CI. However, there is lack of literature regarding the effect of EPS on CI in animal models. Therefore, in view of scarcity of information the present study was planned to evaluate EPS effects on memory and learning in diabetic male Wistar rats.

1.2.19 Screening Methods for Assessment of Learning and Memory:

The process of memory formation is allocated into three general stages [149].

Stage 1 - Acquisition trial: Involves the initial learning process of a new experience.

Stage 2 - Storage of this learning into a short-term memory which is transient and labile.

Stage 3 - Consolidation of this memory into a long-term memory.

Behavioural Models for the Evaluation of Learning and Memory Processes can be Classified into: [271]

A. **Exteroceptive Aversive Stimuli Models –** External stimuli are used

B. **Interoceptive Aversive Stimuli Models –** Internal stimuli are used

Table 4: Brief synopsis of methods used in assessment of Learning and Memory

Models	**Parameters measured**	**Advantages**	**Limitations**
Behavioural Animal Models [271]			
A. Exteroceptive Aversive Stimuli Models			
Behaviour in mazes			
Morris water maze (MWM) [272]	**Learning** • Escape latency time (ELT) • Escape path length **Spatial Memory** • Index of retrieval • No. of crossings **Working Memory**	1) High face, predictive validity and sensitivity 2) Open field test 3) Natural motivation 4) Rodents are natural swimmers 5) Decreases possible olfactory or visual bias.	Risk of hypothermia

<table>
<tr><td colspan="4">Land mazes</td></tr>
<tr><td>Elevated plus maze (EPM) [273]</td><td rowspan="3">Mainly Assessment of Working Memory and Spatial Discrimination

• No.of correct entries
• No.ofincorrect entries</td><td rowspan="3">No risk of hypothermia
High face, predictive validity</td><td rowspan="3">1. Motivation needs to be given
2. No natural tendency
3. Olfactory cues</td></tr>
<tr><td>Barnes maze</td></tr>
<tr><td>Radial arm maze</td></tr>
<tr><td>Y Maze</td><td colspan="3"></td></tr>
<tr><td>PA</td><td colspan="3">Inhibition of Learnt Behaviour by an Aversive Stimulus</td></tr>
<tr><td>Step down avoidance</td><td rowspan="3">Memory of Learnt Task
• Step down latency
• Step through latency (STL)</td><td rowspan="3">High face, predictive validity and sensitivity</td><td rowspan="3">Highly aversive stimuli</td></tr>
<tr><td>Step through avoidance</td></tr>
<tr><td>Uphill avoidance</td></tr>
<tr><td>Active avoidance</td><td colspan="3">Animals have to predict the onset of aversive stimuli by other visual or audio clues</td></tr>
<tr><td>Shuttle box avoidance</td><td rowspan="2">Memory
% of conditioned reflex retained over a no. of tasks done</td><td rowspan="2">High face, predictive validity and sensitivity</td><td rowspan="2">Highly aversive stimuli</td></tr>
<tr><td>Pole jumping apparatus</td></tr>
</table>

B. Interoceptive Aversive Stimuli Models			
Electroshock induced amnesia	Electric Shock Induces Retrograde Amnesia of Learnt Task.	1. No conditioning or motivation required 2. Less of observer bias	1. Highly aversive stimuli 2. No assessment of working memory possible
Hypoxic Stress-induced Learning Deficits	Electric Shock Induces Retrograde Amnesia of Learnt Task.		
Pharmacological Methods of Inducing Amnesia			
Agents Used	**Principle**		
Scopolamine Induced amnesia	Amnesia is induced by inhibition of cholinergic transmission		
Diazepam induced amnesia	Amnesia is induced by GABAergic inhibition and oxidative stress		
Streptozocin induced amnesia	Amnesia is induced by restricting glucose metabolism by neurons		
Colchicine induced amnesia	Induces neuronal death by inhibition of MTs		
Sodium nitrite induced amnesia	Induces chemical hypoxia in brain		

1.2.20 Experimental Animal Models of DM

Several animal studies have been reported to study the DM and related consequences more precisely. These models are not just providing evidence regarding pathogenesis of the disease but also helping to a large extent in developing therapeutic preventive strategies to combat the diabetic and associated complications. These animal models were developed using chemical, genetic, surgical (pancreatectomy) and other techniques.

Chemicals used to induce the diabetic in animal models are aimed to selectively destroy the pancreatic beta cells partially or completely and retain the hyperglycemic state for a sustainable duration without causing unwanted conditions other than diabetes. Studies over the past 70 years considered, alloxan and Streptozotocin (STZ) as the most used chemical substances for developing successful animal models for both T1DM and T2DM [274-276]. The universal proclaim regarding alterations in pancreatic β- cells as well as in the animal physiology following alloxan [277][278] and STZ [279-281] induced diabetes made them a well-accepted diabetogenic drug even today. The metabolic disturbances in rats caused by these diabetogenic drugs [282] and their mechanisms of cytotoxic action on the pancreas are thoroughly illustrated by various studies [277][283][284].STZ and alloxan induce diabetics at specific dose depending upon the animal species and nutritional status of the animal [285][286].

Intriguingly, besides having similar diabetogenic action, STZ is widely opted over alloxan for developing the animal model for diabetes [287][288][281]. STZ is not only capable of inducing an irreversible diabetes [289][290][281] but also has a low mortality rates in comparison to alloxan [291-294]

1.2.21 A Brief Account on STZ: A Diabetogenic Drug

In 1963, Rakieten and his co-workers appealed that STZ is a diabetogenic drug [279]. It is a naturally occurring broad-spectrum, cytotoxic chemical isolated from a mould *streptomycetes achromogenes.* STZ is popularly used to induce both insulin-dependent and NIDDM.

Mechanism of Action

STZ induces diabetes in mammals via inhibiting insulin production in pancreas by destroying beta cells.

STZ selectively accumulates in the pancreatic beta cells through low affinity GLUT-2 transporters [295]. The DNA alkylating methylnitrosourea moiety (especially at o^6 position of guanine) of STZ causes DNA fragmentation [296][297][283] along with a chain of damaging events like protein methylation [296][298] NO generation that inhibits aconite activity [299] and reduced level of cellular NAD+ and ATP that conclusively causes necrotic cell death and hamper the insulin biosynthesis and secretion [300][301]. Furthermore, the diabetogenic toxicity of STZ is also accompanied and accelerated by the resultant oxidative stress caused due to a low level generation of ROS, counting superoxide and hydroxyl radical generation from hydrogen peroxide dismutation during hypoxanthine metabolism following enhanced ATP dephosphorylation (Figure 9 ‘a’ and ‘b’).

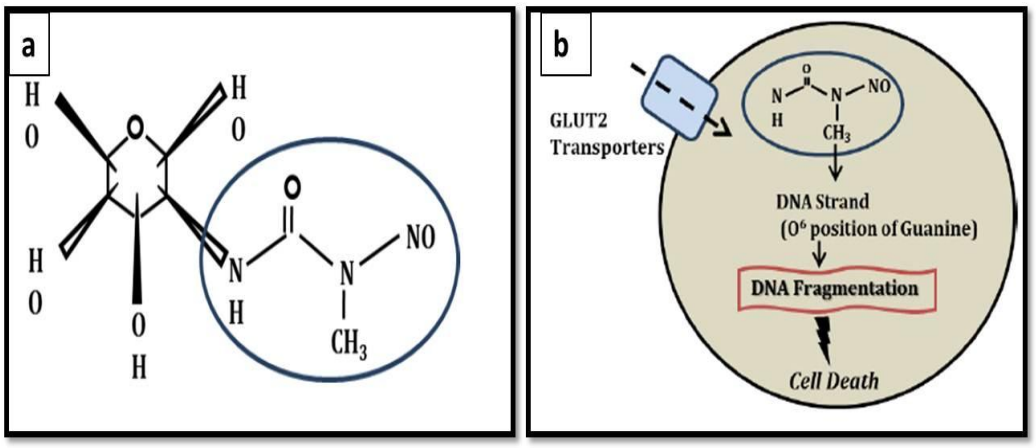


Figure 9a: Cytotoxic methylnitrosourea moiety (N-methyl-N-N-Nitrosourea) attached to the glucose (2-deoxyglucose) molecule; b: STZ (through i.p. or i.v. injection) action in β cells

1.3 Aim and Objectives

Aim

The objective of this research project is to study the Effect of Epalrestat, An Aldose Reductase Inhibitor on Memory and Learning in Diabetic Male Wistar Rats.

Study objectives

1. To evaluate the effect of epalrestat on memory and learning in High Fat Diet and streptozotocin [STZ] induced diabetic rats.
2. To evaluate the effect of epalrestat on tau protein level in brain hippocampus.
3. To evaluate the effect of epalrestat on gene expression level.
4. To study the effect of epalrestat on oxidative stress markers.

2. METHODS AND MATERIALS

2.1 Animals

Wistar rats (male; 150 - 200 g) were procured and kept for the animal study at central animal house facility of Jawaharlal Nehru Medical College, Belagavi, Karnataka. The experimental rats were familiarised to day-night cycle (12:12 hr) for seven days, prior to study. Three rats were kept in each polypropylene cage and were maintained at constant room temperature (22^{o}-25^{o}C). Standard chow pellet (Amrut Brand, Mahrashtra-India) was given with water *ad libitum*. The study design and protocol was approved by Institutional Animal Ethical Committee (IAEC No.: 7/D; dated 18.05.2016) and animals were maintained as per CPCSEA guidelines: Committee for the Purpose of Control and Supervision of Experiments on Animals throughout the study duration.

2.2 Drugs and solutions

EPS suspension in 1% gum acacia, STZ in citrate buffer (pH 4.4) and Donep HCl in distilled water was freshly prepared every time before administration. Per oral treatment of EPS (13.5, 27, 54 mg/kg) and Donep (1 mg/kg) was identified as per literature and human dose calculation [31][36][302-305]. The clinically human equivalent doses of drugs were calculated to rat equivalent doses by conversion table [306].

2.2.1 List of common chemicals and ELISA Kits

S.N.	Particulars	Makes / Source
1	Epalrestat	Micro Labs Ltd., India
2	Streptozotocin(STZ)	Enzo Life Sciences, UK
3.	Donep HCL	Alkem Laboratories Ltd., India
4.	Glucometer/Strips (Accu Check)	Roche Diagnostics India Pvt. Ltd. Mumbai
5.	Standard Chow Pellet	Amrut Brand,Maharashtra, India
6.	High Fat Diet	VRK Nutritional Solutions, Maharashtra, India
7.	Rat TAU ELISA Kits	Genxbio Health Sciences Pvt. Ltd. India

2.3 Experimental Diabetes Induction with High Fat Diet and STZ

T2DM was induced in rats according to prior reports with slight amendments [307][308]. Rats were fed with HFD for 14 days followed by single dose of STZ 35 mg/kg injection via intraperitoneal route (IP). All STZ injected rats were given with 5% of glucose instead of water for 24 hr to reduce hypoglycemic shock associated mortality. Tail vein puncture procedure was performed after 48 hr for blood withdrawal to measure glucose levels. Rats with FPG levels above 200 mg/kg were accounted as diabetic. Body weight (BW) and blood glucose levels were recordeded initially and upon experiment completion. Day of diabetes confirmation was considered as ‘day one’ of diabetic condition. The composition of HFD and study grouping is given in Table 5 and Table 6.

Table 5: High Fat Diet composition

Ingredients	Diet (g/kg)
Powdered NPD	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and Minerals Mix	60
DL-Methionine	03
Yeast Powder	01
Sodium Chloride	01

Table 6: Grouping of Experimental Animals

Group 1	Normal control (NC)	Vehicle only [1ml/kg/day]
Group 2	Diabetic control (DC)	STZ [35 mg/kg]
Group 3	Standard control	STZ + Donep [1mg/kg/day]
Group 4	Test–1	STZ + EPS [13.5mg/kg/day
Group 5	Test–2	STZ + EPS [27 mg/kg/day]
Group 6	Test–3	STZ + EPS [54 mg/kg/day]

STZ (35 mg /kg; IP) was injected in animal group 2 to 6 only and rats in respective treatment groups were administered with test/standard drug.

2.4 Treatment Schedule

After confirmation of Diabetes, rats were treated with drugs - EPS [13.5, 27, 54 mg/kg/day] and Donep [1 mg/kg/day] in respective groups. Whereas, rats in normal control group were administered with distilled water daily [1 ml/kg/day] for 28 days. Behavioural tests were performed using EPM, MWM and PAT method in from day

29 to 38. Though, test and standard drug treatment was continued as per experimental design.

Study Parameters:

- ❖ Rat FPG level was measured using glucometer strips
- ❖ Behavioral tests: MWM, EPM and PAT
- ❖ Biochemical estimations:
    - Estimation of oxidative stress markers [CAT and GSH] in plasma samples.
    - Analysis of inflammatory cytokines [TNFα and IL-6] in hippocampus tissues.
    - Tau protein evaluation of rat hippocampus by ELISA kit method
- ❖ Histopathological examinations

Blood samples were collected and brain structures were removed for separation of hippocampus to process biochemical and histopathological examinations. On completion of experimental procedures, all animals were processed for cervical dislocation.

A. Behavioral Tests: [271-273][309-311]

i) Morris Water Maze [MWM]

The standard reported procedure was adopted for MWM test and is briefly mentioned below:

Day 1 training – Rats were habituated by means of the task and trials were not counted. The adjustment by the rats were confirmed when they learnt that there is an escape route from this aversive stimulus i.e. water.

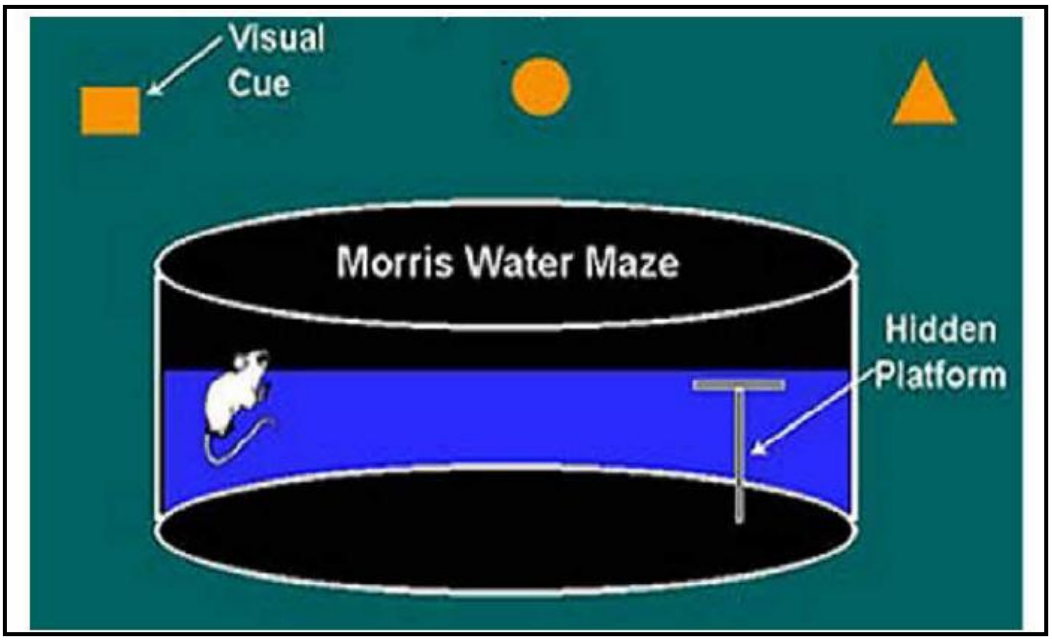


Figure 10: Morris Water Maze Apparatus

Day 2 to 5 - Acquisition trial: Control and drug treated (on each day for four days) rats were released into water facing towards the wall in one of the quadrants (Q), subjected to 4 trials /day for four days with 5 mins interval, with the subsequent trial occurring after finishing the ongoing trial with all the 10 rats for that same quadrant. Throughout consecutive trials and succeeding days, initial points was changed every time as following (Table 7).

Table 7: Order of release of rats in specific quadrants

Days	1st trial	2nd trial	3rd trial	4th trial
1	Q1	Q2	Q3	Qg
2	Q2	Q3	Qg	Q1
3	Q3	Qg	Q1	Q2
4	Qg	Q1	Q2	Q3

Initially, the rats were skilled to locate the hidden platform by 'hit and trial' method. They learnt the allotted place of hidden platform by using distal cues. Numerous distal cues were assigned to the study rats under stringent observance to their equal fixity during all the trial days. Whereas, the place for the investigator was fixed according to the distal cues. Further, rats were permitted to escape to the platform and stay for 20 sec. (to generate a spatial memory of the hidden platform with the aid of distal cues). The moment in time required to run away to the platform, Escape latency time (ELT) was recorded and was compared to different groups. If the rats were unable to locate the platform within 2 min, then manually guide to the assigned place and retained for 20 sec. Rats failing in the task during successive trials for 2 consecutive study days, were left out from study.

Day 6 - Retrieval trial: On sixth day of the study, platform from goal quadrant was removed and rats were evaluated for time spends in previous goal quadrant. The time consumed in the earlier goal quadrant was compared among control, diabetes induced and drug treated groups.

ii) Elevated Plus Maze [EPM]

Figure 11: Elevated Plus Maze Apparatus

The maze consists of 4 arms. The apparatus was kept in bright light room and was above the ground level (50 cm).

Anxiety Protocol

The rats were placed at middle display place of the apparatus with heads oriented towards an OA. The rat entry frequency to OAs and EAs were scored and times spent in the OAs were noted for 5 mins. The frequency of rat entry into the OAs of the maze apparatus and the spent time in those arms were anxiety measures. The number of EA entries measures locomotor activity. Entry is defined by all paws (four) entering arm and an arm exit by two paws leaving an arm.

Learning Protocol

EA was divided into two parts by drawing a line. On days 1 and 2, the time utilized for each rat to cross the EA line (transfer latency-TL) was noted. The rats were initially placed on OA ending and allowed to learn for 90 sec. The rats were expected to have their 4 paws across the EA line; if fails to do so within time limit, were manually placed beyond the line and TL was noted as 90 sec. After crossing the line, the rats were allowed to explore the apparatus for 30 sec. Learning was demarcated as reduced TL on day 2 as compared to day 1.

iii) Passive Avoidance Apparatus (PAA)

Figure 12: Passive Avoidance Apparatus

Associative learning and memory were evaluated by two compartment apparatus i.e PAA. Individual animals were positioned in an illuminated cubicle facing away and at the utmost distance from the entrance to the dark chamber. On first day, rats were allowed for 5 min. to discover both the partitions. 2nd day, time utilized by rats to reach in the dark section for first time was noted down and immediately another two learning periods were made. At 3rd trial ending, once the rat entered into dark area, door of the section was closed and three inescapable foot shocks (50V, 50 Hz, 1 sec)

were given, resulting in back of animals to home cage. On third Day (after 24 hr.), each study rat was kept once more for PAT as described earlier. The latency time required by rat to back into dark section was measured.

B. Biochemical estimations:

i) Measurement of blood glucose levels and body weight

ACCU - Check glucose meter was used to measure blood glucose levels in rats. Body weight and FPG levels were measured before and after the animal experiment.

ii) Estimation of CAT activity

According to Goth (1991), the CAT activity was measured in rat plasma samples. Briefly, estimation steps are mentioned below [312].

200 uL of plasma sample was incubated with 1 mL of substrate at 37 °C for 60 sec.

↓

Reaction was stopped with the help of 1 mL of stop solution. Resulting in yellow complex which was measured at 405 nm using spectrophotometer

↓

Blank 1 : (1.0 mL substrate + 1.0 mL stop solution + 0.2 mL plasma)

Blank 2: (1.0 mL substrate + 1.0 mL stop solution + 0.2 mL buffer)

Blank 3 :(1.0 mL buffer + 1.0 mL stop solution + 0.2 mL buffer)

Calculation:

$$\text{Serum catalase activity (kU/L)} = \frac{\text{A (sample)} - \text{A (blank 1)}}{\text{A (blank 2)} - \text{A (blank 3)}} \times 271$$

The assay result was expressed as k U/L, where k is the first-order rate constant.

iii) Estimation of reduced GSH activity

As per earlier documented method by Beulter et al. (1963), the plasma GSH content was measured in rat plasma samples. Follwing mentioned procedure steps were adopted for the estimation [313].

Plasma sample was mixed with Tri Chloro Acetic Acid (TCA) in 1:1 ratio and was centrifuged at 1000x*g* for 10 min. at 4 °C.

Supernatant (0.5 mL) was added with 200 uL of 0.3 M disodium hydrogen phosphate

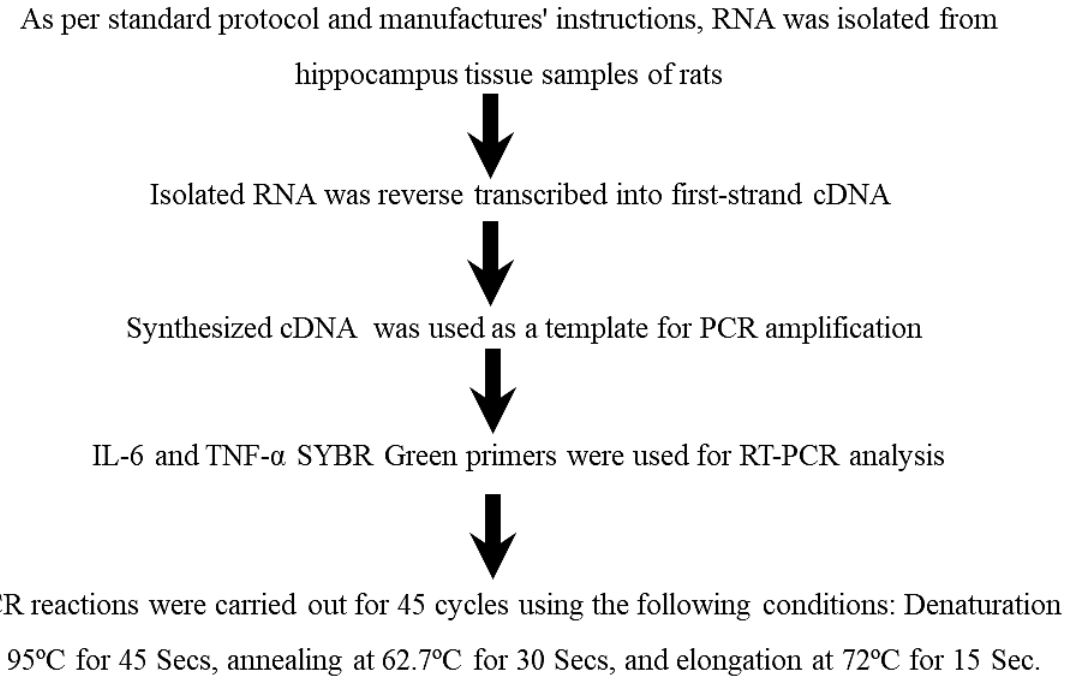
Freshly prepared 0.25 mL of 0.001 M 5, 5 - dithiobis 2 - nitrobenzoic acid (DTNB) was dissolved in 1% (w/v) citric acid and OD was measured at 412 nm.

Standard curve was plotted with the help of 10 - 100 µM of reduced GSH form and was designated as nanomoles of reduced GSH of protein. OD was measured at 412 nm using spectrophotometer

iv) Evaluation of cytokine levels by RT-PCR method

Inflammatory markers (cytokines) in rat hippocampus tissue samples were determined analysed for TNF-α and IL-6 levels in RT-PCR (ABI) using SYBR Green primers. The comparitive expression levels of the mark genes were calculated as a ratio to the GAPDH (housekeeping gene). Amplified PCR product specificity was analysed by melting curve analysis. All experiments were performed in triplicate. The relative quantification analysis was signified in the form of relative expression to normal group

(delta delta Ct) [314]. Following steps were adopted according to manufacturer instructions:



Primer sequences used in RT-PCR assays in hippocampus tissues

Primers	Sequences (5′ 3′)	Annealing temperature (°C)
IL-6	Forward AACTCCATCTGCCCTTCAGGAACA Reverse AAGGCAGTGGCTGTCAACAACATC	62.7
IL-1β	Forward AGCAGCTTTCGACAGTGAGGAGAA Reverse TCTCCACAGCCACAATGAGTGACA	62.7
MCP-1	Forward TGCTGTCTCAGCCAGATGCAGTTA Reverse TACAGCTTCTTTGGGACACCTGCT	62.7
TNF-α	Forward AGAACAGCAACTCCAGAACACCCT Reverse TGCCAGTTCCACATCTCGGATCAT	62.7

v) Tau protein level estimation using ELISA Kit

Tau proteins levels were determined by ELISA kit in hippocampus samples. Tissue samples were homogenized in PBS (pH 7.4) and homogenates was centrifuged at

3000x*g* for 20 mins. at 4°C. The Supernatant was used as analyze in ELISA kit using following steps.

Note- important point for assay procedure:

- All reagents/buffers and samples were kept at room temperature prior to use.
- The standards and samples were processed in duplicates.
- Removable 8-well strips were used as appropriate for experiment. The number of stripes required was determined by number of samples to be tested including standards for the calculation.
- As per standard and sample concentrations and corresponding OD values, linear regression equation of the standard curve was calculated.

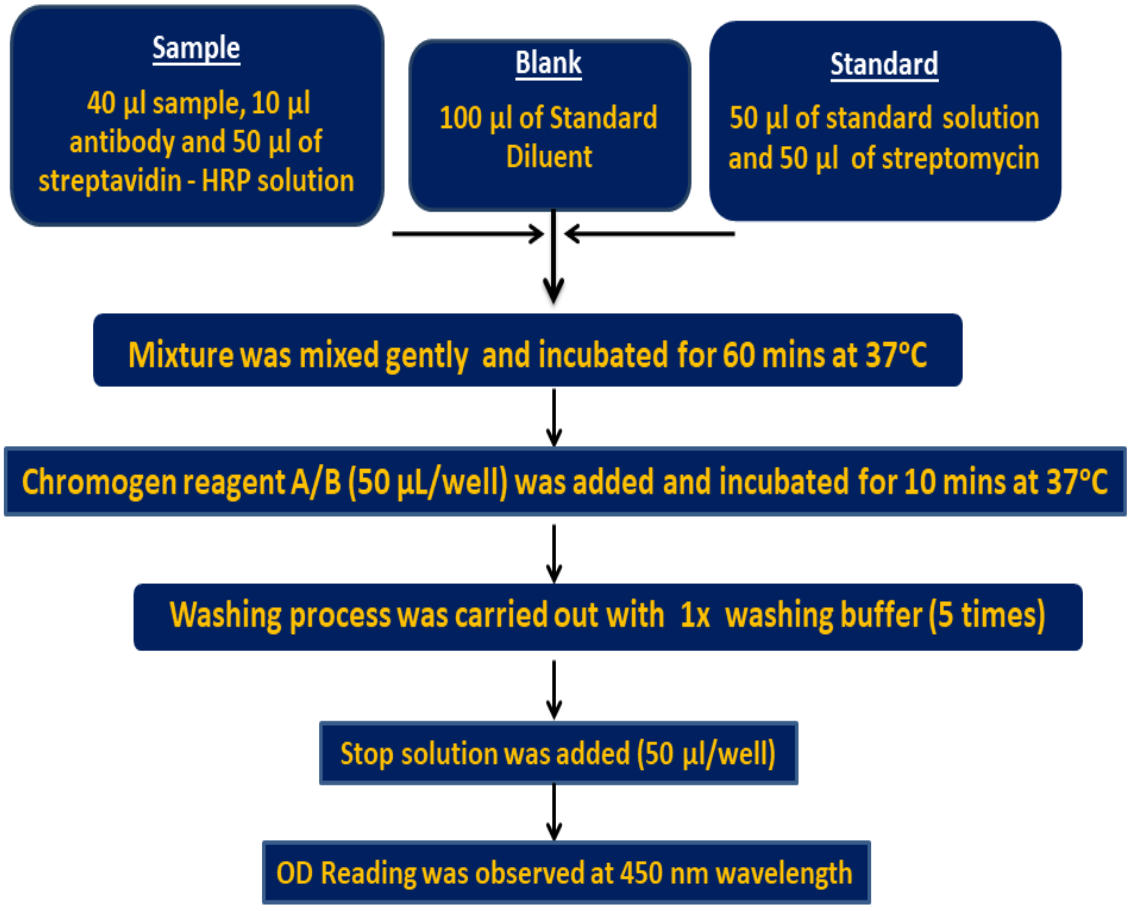


Table 8: Reagent preparation

S. N.	Reagent	Blank (/well)	Std. (/Well)	Sample (/Well)
1	Standard Diluent	100 µl	--	--
2	Standard Solutions	--	50 µl	--
3	Sample (serum, plasma etc)	--	--	40 µl
4	Biotin abeled Anti-TAU Antibody	--	--	10 µl
5	Streptavidin - HRP solution	--	50 µL	50 µl
6	Chromogen Reagent A	50 µL	50 µL	50 µL
7	Chromogen Reagent B	50 µL	50 µL	50 µL
8	Stop Solution	50 µL	50 µL	50 µL
Total		**250 µL**	**250 µL**	**250 µL**

C. Brain and Pancreas [Histopathology]:

Pancreatic and hippocampus tissue samples were fixed in formalin (10%; in neutral buffered) and was dehydreated by treatment with ethanol and water (gradient of mixture). The resulted samples were washed using xylene solution and was set in paraffin. Tissues were sections (5 µm thickness) and stained with H and E followed by observation under light microscope [315][316].

3. STATISTICAL ANALYSIS

Graph Pad Prism 5.00 Software [San Diego, USA] was used for statistical analysis. The study findings were presented as Mean ± Standard Error of Mean (SEM). One-way ANOVA method was used for analysis and group comparisons were carried out with Dunnett's tests. $P < 0.05$ was considered as statistically significant.

4. RESULTS

In the current study, neuroprotective effect of EPS was carried out using HFD and STZ induced diabetes in Male Wistar rats. The paradigm used was the MWM, PA, EPM test and biochemical parameters. There was no mortality in any group.

4.1 Behavioral Parameters

4.1.1 Effect of Epalrestat treatment on TL in elevated plus maze test

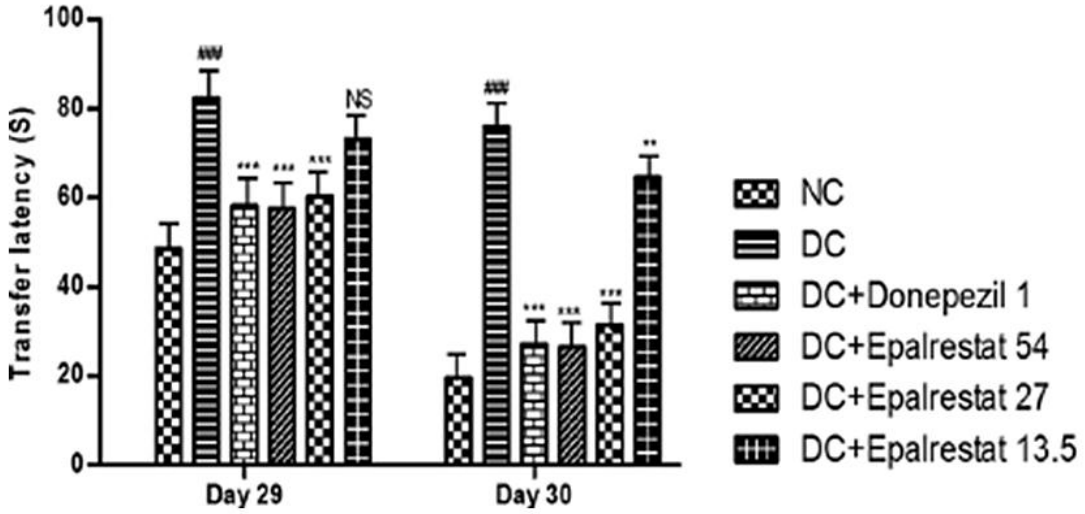


Figure 13: Effect of EPS treatment on TL in HFD - STZ induced diabetic rats for evaluation of memory impairment: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds SEM: Standard error of mean, n=10, Data was expressed in mean ± SEM, statistical analysis was carried by one-way ANOVA followed by Dunnett's test. Significance at $^{**}p<0.01$, $^{***}p<0.001$, ns: Not significant vs. DC group and $^{###}p<0.001$ vs. NC group.

The effect of Donep and EPS treatments in diabetic rats for evaluation of mean transfer latencies using EPM test are presented in Figure 13. Statistical analysis revealed that chronic treatment with Donep 1 mg/kg and EPS (57, 27 mg/kg) had significant effect on the transfer latencies on day 29 as compared with DC ($p<0.001$).

On the day 30, HFD-STZ induced TL was reduced when compared with findings of day 29. This clearly indicates the learning behaviour of animals on the day 30, whereas there was no difference in TL tested on day 29 and 30 in DC animals. While, Donep (cholinesterase inhibitor) showed a significant reversal of HFD-STZ induced deficits. Although, Donep and EPS treated animals (57, 27 and 13.5 mg/Kg) resulted in significant decreased TL values when compared with DC on day 30.

4.1.2 Effect of Epalrestat treatment on Passive avoidance test

Treatment	Time taken to enter small compartment (S)				Day 33
	Day 31	Day 32			
	Trial 1	Trial 2	Trial 3	Trial 4	TL
NC	55.50±6.77	36.10±5.42	27.90±5.31	18.40±3.18	153.5±15.93
DC	114.4±5.86	99.89±6.85$^{###}$	73.33±6.27$^{###}$	66.89±6.48$^{###}$	34.78±3.97$^{###}$
DC+ Donep 1	99.56±3.31	55.56±5.40***	35.22±4.58**	23.33±2.90***	150.6±17.86***
DC+EPS 54	101.5±2.63	52.70±8.40***	33.50±7.89***	19.10±3.31***	141.6±13.49***
DC+EPS 27	107.7±17.62	66.10±8.47 **	56.20±9.13 ^{ns}	39.70±5.86***	99.20±12.56**
DC+EPS 13.5	124.9±3.52	100.2±4.98 ^{ns}	68.33±4.12^{ns}	57.00±4.87^{ns}	54.89±6.52 ^{ns}

Table 9: Effect of EPS treatment on HFD - STZ induced diabetic rats for evaluation of memory impairment: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at $^{**}p<0.01$, $^{***}p<0.001$, ns: Not significant vs. DC group and $^{###}p<0.001$ vs. NC group.

4.1.3 Effects of Epalrestat treatment on TL in Passive Avoidance Test

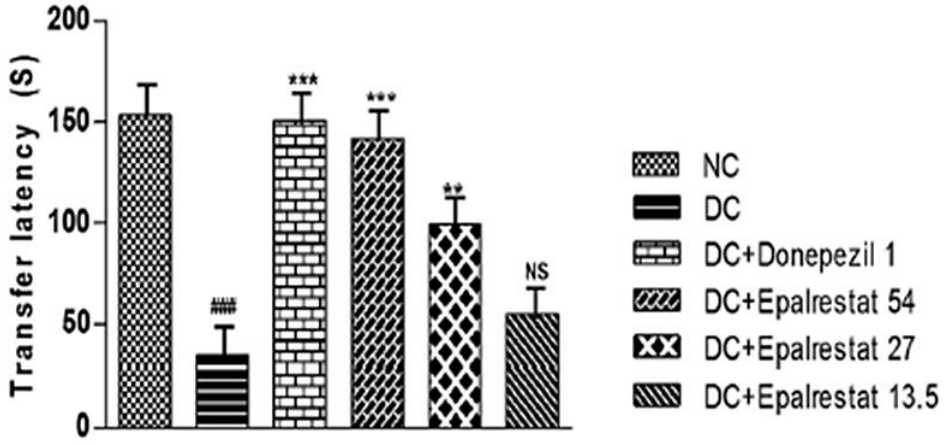


Figure 14: Effect of EPS treatment on TL in HFD - STZ induced diabetic rats for evaluation of memory impairment: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at $^{**}p<0.01$, $^{***}p<0.001$, ns: Not significant vs. DC group and $^{\#\#\#}p<0.001$ vs NC group.

In the current study, we used PA task to assess short-term/long-term memory to assess the outcome of EPS. Memory performance was correlated with the latency to escape from the light compartment; the better the recollection, the greater the latency.

Acquisition trial:

In first trial (prior to electrical shock), no remarkable variation in the STLs among the treatments and DC group of rats was observed (Table 9). This finding confirmed similarity with exploratory performance of various group of rats in the dark compartment. However, there was notable variation between the different experimental groups in terms of trial numbers to acquisition criterion ($P < 0.001$, $P<0.01$) (Fig. 14 and Table 9) were observed. Specifically, the trial numbers to acquisition in Donep ($P < 0.001$, $P<0.01$) and EPS treated group ($P < 0.001$, $P<0.01$)

were significantly less than DC group. Consistent with cognitive deficit incidences, the trial numbers to acquisition in DC group was significantly higher than NC group ($P < 0.001$).

Retention:

In the retention test which was conducted 24 h after the aversive stimuli (foot shock), retention of memory was significantly decreased in DC group as compared with NC group. Whereas significant enhanced TLs were observed in the animals treated with Donep and EPS (54, 27 mg/kg; $P<0.001$, $P<0.01$) as compared with DC group.

4.1.4 Effect of Epalrestat treatment on spatial memory using MWM Test

Treatment	Time taken to reach target platform (s)				Probe test: time spent in target quadrant (s)
	Day 34	Day 35	Day36	Day37	Day 38
NC	45.80±7.79	36.80±5.68	30.23±5.25	17.63±2.78	62.90±2.90
DC	85.00±2.79$^{\#\#}$	82.22±6.05$^{\#\#}$	72.81±12.49$^{\#}$	63.25±10.58$^{\#\#}$	22.44±1.28$^{\#\#\#}$
DC+Donep 1	50.53±10.08*	43.28±9.74*	34.86±12.07*	27.50±7.18*	56.11±1.91***
DC+EPS 54	48.58±9.48*	40.45±10.31*	31.33±7.17*	29.30±5.89*	59.50±2.80***
DC+EPS 27	57.40±9.85^{ns}	52.75±10.88^{ns}	38.35±9.18^{ns}	35.73±8.40^{ns}	35.70±1.46***
DC+EPS 13.5	82.69±3.39^{ns}	79.56±7.64^{ns}	70.44±9.38^{ns}	53.44±7.61^{ns}	26.11±1.70^{ns}

Table 10: Effect of EPS treatment on TL in HFD - STZ induced diabetic rats for evaluation of memory impairment: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at $^{*}p<0.05$, $^{**}p<0.01^{***}$, $p<0.001$, ns: Not significant vs DC, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs NC.

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4.1.5 Effect of EPS treatment on Probe Test using Morris Water Maze Test

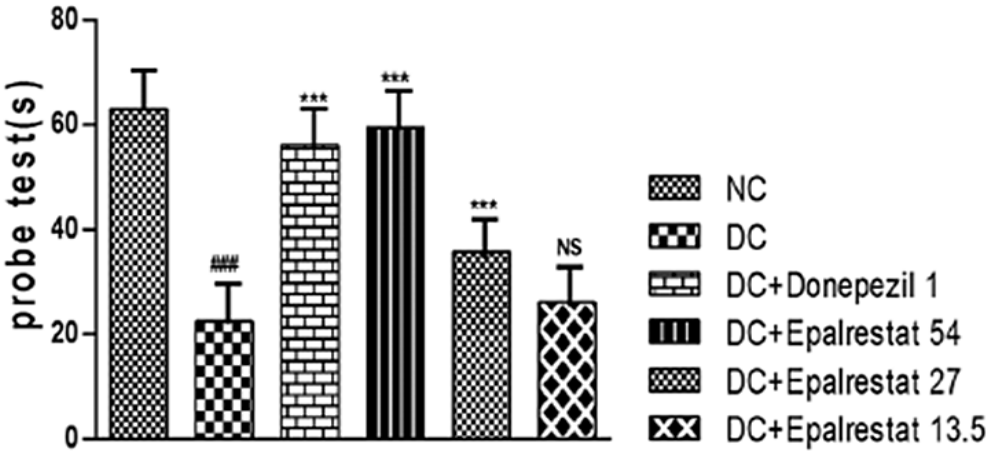


Figure 15 : Effect of EPS treatment (Probe test in MWM task) in HFD - STZ induced diabetic rats for evaluation of memory impairment: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at ***p<0.001, ns: Not significant vs. DC group& ### p<0.001 vs. NC group.

The memory impairment was assessed using MWM task. The mean ELT was measured to assess spatial memory in the experimental rats. The treatment with Donep and EPS during training session, significantly influenced the EL (Table 10 and Fig. 15). Further, statistical analysis revealed that Donep and higher dose of EPS (54 mg/Kg) significantly reduced (P<0.05) EL over the course of the training sessions when compared to the DC group. In the spatial probe test on day 38, the time consumed inside the target quadrant by the DC group was decreased as compared to NC group (P<0.001) (Fig.15). Moreover, animals treated with EPS (27 and 54 mg/kg) resulted in enhanced time spent within the target quadrant as compared to DC groups (P<0.001). These effects of EPS were similar to that shown by Donep treatment (P<0.001).

4.2. Biochemical estimations:

4.2.1 Effect of Epalrestat treatment on blood glucose levels

Treatment	Glucose mg/dl	
	Onset of study	End of study
NC	96.50 ± 1.48	100.2 ± 3.16
DC	98.11 ± 4.69	456.4 ± 11.44***
DC+ Donep 1	100.8 ± 4.78	411.6 ± 14.72***
DC+ EPS 54	97.20 ± 3.83	396.1 ± 20.85***
DC+ EPS 27	100.2 ± 3.99	404.4 ± 17.43***
DC+ EPS 13.5	99.89 ± 4.67	413.7 ± 10.94***

Table 11: Effect of EPS treatment on blood glucose levels in HFD- STZ induced diabetic rats for evaluation of memory impairment: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, SEM: Standard error of mean, n=10, Data expressed in mean ± SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at *** $p<0.001$ vs NC group.

Effects of EPS treatment on rat body weight and blood glucose levels

The rat blood glucose levels at the commencement of experiment had no changes were observed amongst animal groups. However, blood glucose level in DC rats was significantly higher as compared with normal rats at the end of experiment. There was no remarkable variation in BW among the groups at the commencement of the study. However, the DC rats had significant reduced BW as compared with NC rats at the end. EPS and Donep treatment leads to no significant changes in blood glucose levels and BW when compared with DC rats (data not shown).

4.2.2 Effect of Epalrestat treatment on CAT activity

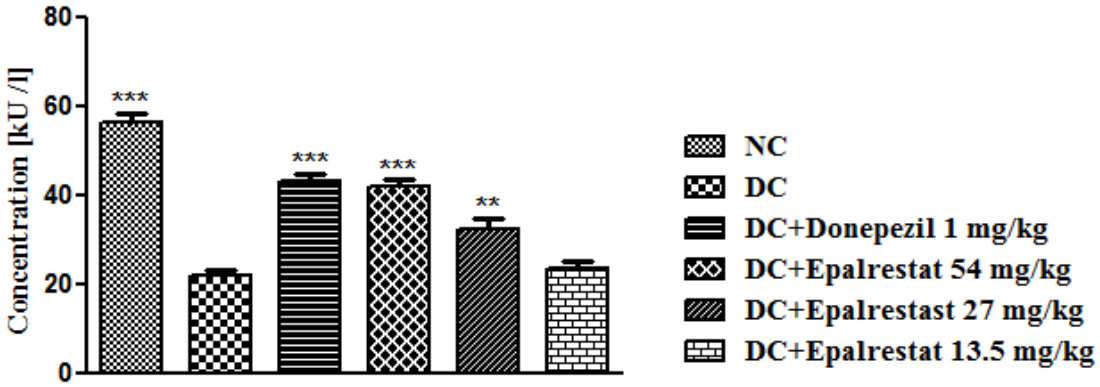


Figure 16: Effect of EPS treatment on calatase activity in HFD-STZ induced diabetic rats for evaluation of memory impairment: Data expressed in mean ± SEM (n=10), statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at $^{**}p<0.01$, $^{***}p<0.001$ comparison with DC rats.

4.2.3 Effect of Epalrestat treatment on GSH activity

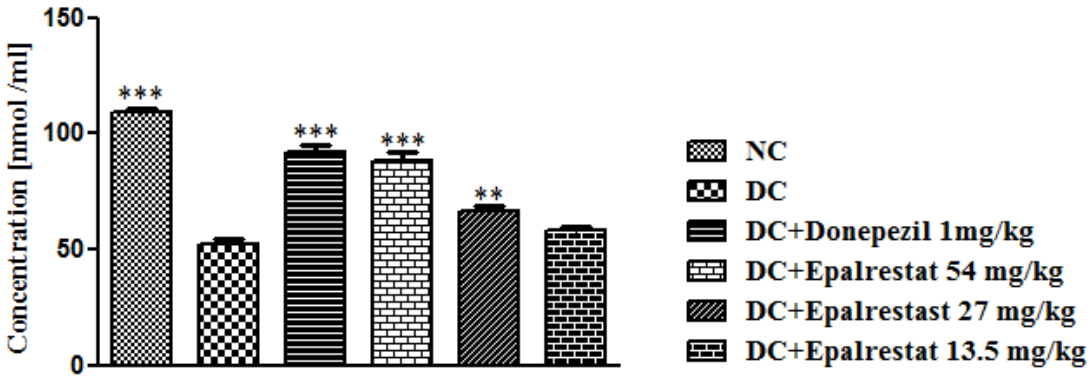


Figure 17: Effect of EPS treatment on GSH activity in HFD- STZ induced diabetic rats for evaluation of memory impairment: Data expressed in mean ±SEM (n=10), statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at **p<0.01, ***p<0.001 comparison with DC rats.

The CAT and GSH activity was showed in Fig. 16 and Fig. 17, which was significantly higher in NC rats ($p<0.001$) as compared with DC rats and also these levels were significantly higher in diabetic rats treated with EPS 54, 27 mg/kg ($p<0.001$, $p<0.01$) and Donep 1 mg/kg ($p<0.001$). However, no significant change was observed in diabetic rats treated with lower dose of EPS (13.5 mg/kg).

4.2.4 Effect of Epalrestat treatment on IL-6 gene expression levels

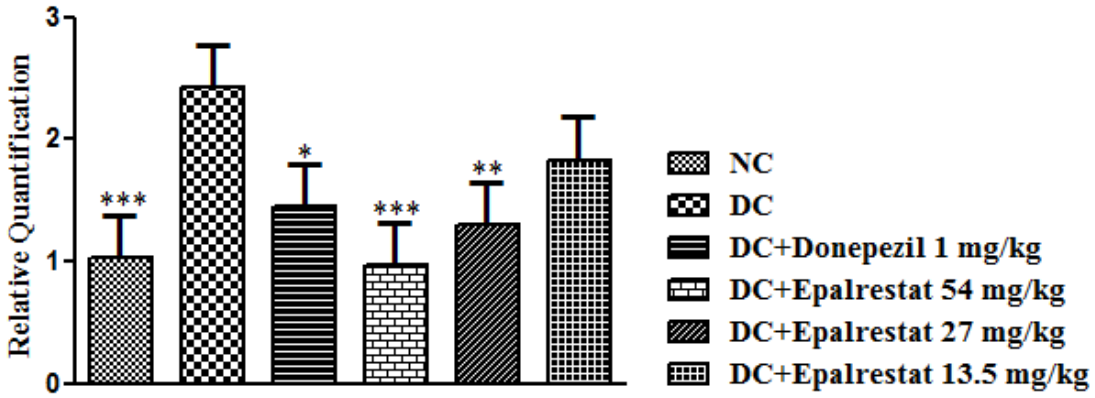


Figure 18: Effect of EPS treatment on IL-6 activity in HFD-STZ induced diabetic rats for evaluation of memory impairment: Data expressed in mean ± SEM (n=10), statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ comparison with DC rats.

4.2.5 Effect of Epalrestat treatment on TNF-α gene expression levels

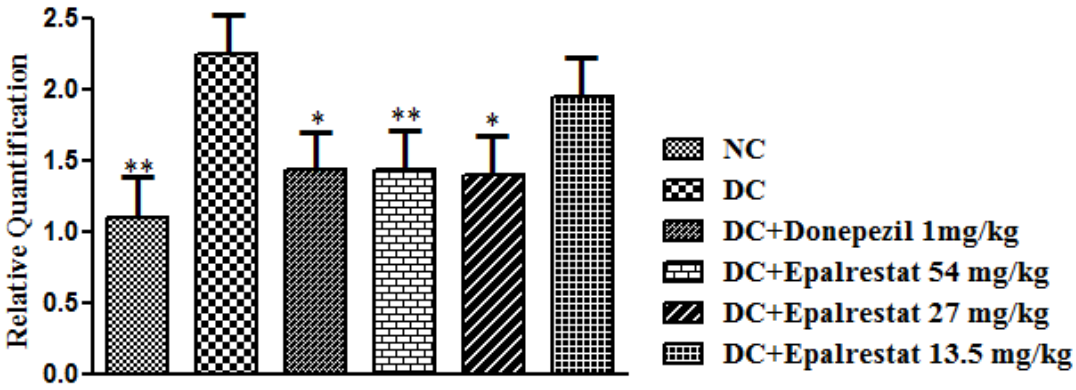


Figure 19: Effect of EPS treatment on TNF-α activity in HFD-STZ induced diabetic rats for evaluation of memory impairment: DC: Data expressed in mean±SEM (n=10), statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at *p<0.05, **p<0.01 comparison with DC rats.

The IL-6 and TNF-α levels are shown in (Fig. 18 and Fig. 19) and their levels were significantly enhanced (p<0.001) in the DC rats as compared with the NC rats while these levels were significantly lowered in diabetic rats treated with EPS 54, 27 mg/kg (p<0.01 and p<0.05) and Donep 1 mg/kg (p<0.05).

4.2.6 Effect of Epalrestat treatment on rat TAU proteins levels

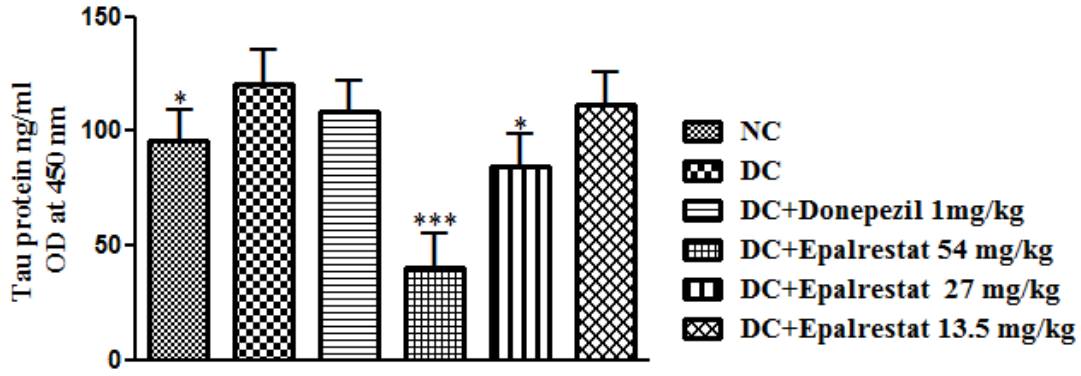


Figure 20: Effect of EPS treatments on analysis of TAU proteins levels in HFD-STZ induced diabetic rats for evaluation of memory impairment: Data expressed in mean ± SEM (n=10), statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at *p<0.05, ***p<0.001 comparison with DC rats.

The concentration levels of total rat TAU protein was showed in Fig.20. TAU protein levels were significantly enhanced ($p<0.001$) in DC rats as compared with treatment groups. The EPS 54 and 27 mg/kg treated diabetic rats were observed with significant lowered levels of TAU protein ($p<0.001$ and $p<0.05$) respectively. Whereas, no significant variations was observed in low dose of EPS 13.5 mg/kg and Donep 1 mg/kg treated DC rats.

4.2.7 Effect of Epalrestat treatment on Pancreas [Histopathology]

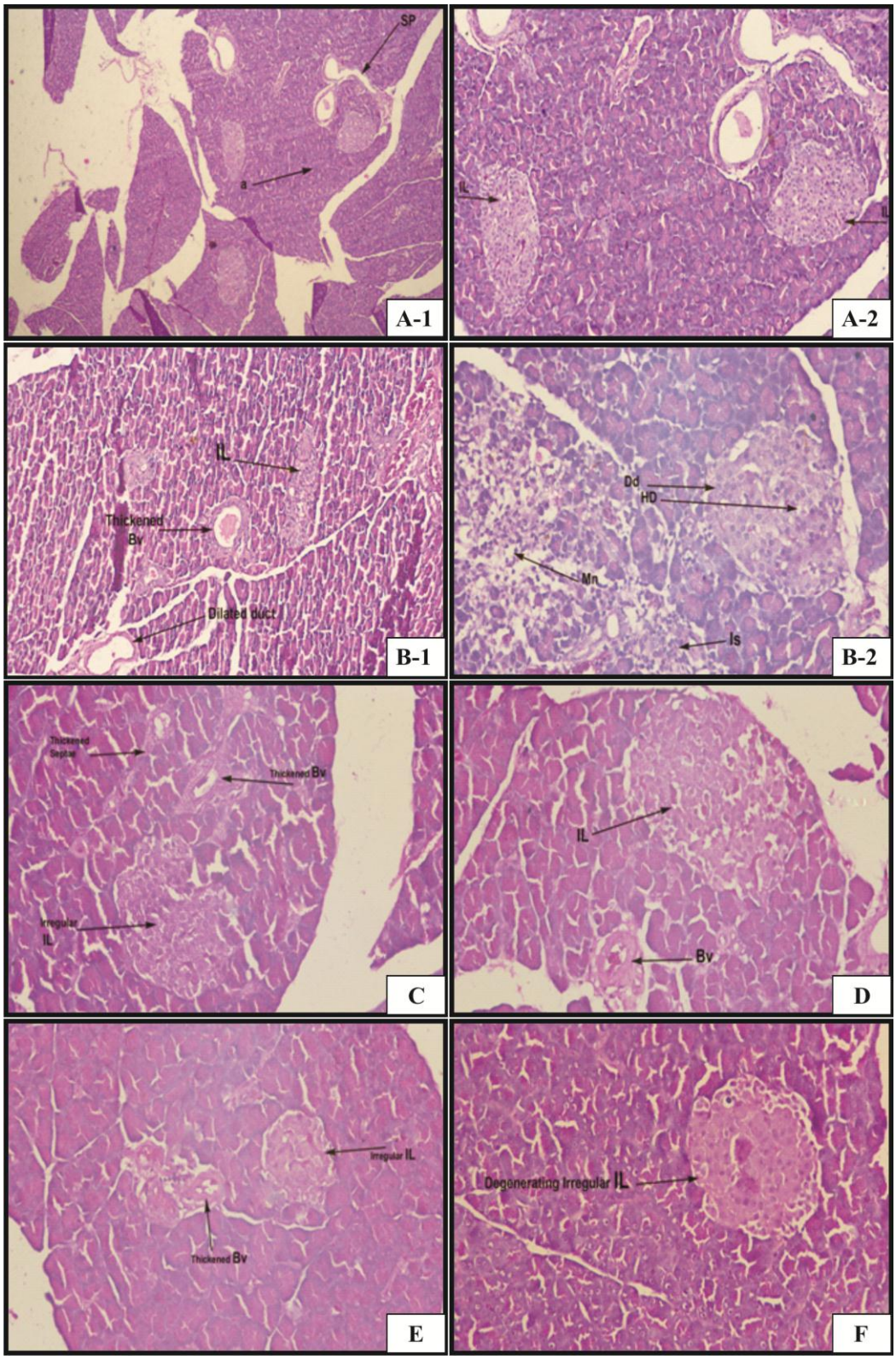


Figure 21: Effect of EPS treatment on histoarchitecture of pancreas on HFD-STZ induced diabetic rats for evaluation of memory impairment: (fig.21A) NC shows normal islets of Langerhans (IL), delicate septa (SP), Acini (a). (fig.21B) DC shows shrinkage both in size and number of islets of Langerhans (IL), thickened blood vessels (Bv), dilated duct, hydropic degeneration and degranulation (Dd) of islets cells, mononuclear (Mn), interstitial stroma (Is). Histopathology findings of (Fig.21C) Donep1mg/kg, (Fig.21D) EPS-54mg/kg, (Fig.21E) EPS- 27mg/kg, (Fig.21F) EPS-13.5mg/kg treatment showed similar effects when compared to the DC group. (magnification A-1,B-1(100x) and A-2,B-2,C,D,E,F:400x).

Histopathological examination of the Pancreas

Staining (H and E) displayed no markable deviations in pancreatic histology in NC rats (Fig. 21A). In contrast, DC rats displayed injured, declined number, shrunken and decreased diameter of pancreatic β-cell (Fig. 21B). Likewise, administration of Donep (1 mg/kg), EPS (54, 27, 13.5 mg/kg) revealed similar impact as observed in DC rats (Fig. 21 C, D, E, F).

4.2.8 Effect of Epalrestat treatment on Hippocampus [Histopathology]

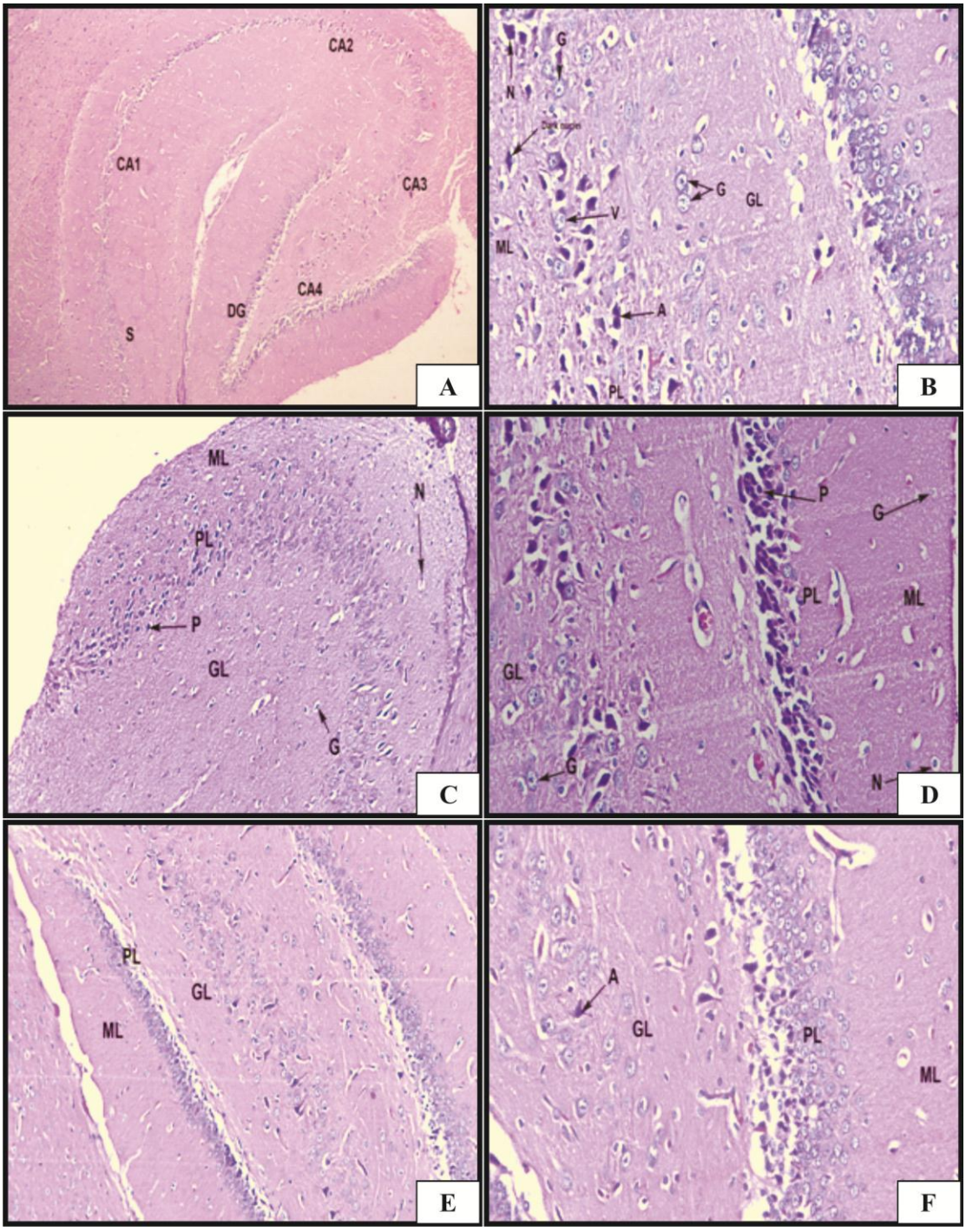


Figure 22: Effect of EPS treatment on histoarchitecture of hippocampus on HFD-STZ induced diabetic rats for evaluation of memory impairment: NC (Fig.22A) shows the Cornus ammonis CA (Fig.22B) DC shows pyramidal layer (PL) marked darkened nuclei with Vacuolation (v). Apoptotic cells (A), granular layer (GL), glial cells (G). Molecular layer (ML) shows marked enlargement of the neurons (N) and glial cells (G) with few apoptotic cells (A). (Fig.22C, D, E and F) shows DC group treated with Donep and EPS (54 and 27mg/kg) treatment shows normal size glial cells (G) and neurons in the molecular layer (ML). The pyramidal layer (PL) is of normal thickness with normal size pyramidal cells (P) there is decrease in Vacuolation (V) in small pyramidal layer (PL), Granular layer (GL) the Glial cells (G) are of normal size with less number of Vacuolation (V). (Magnification: Fig. 22A-40x and B, C, D, E, F 400x)

Histopathological examination of the Hippocampus

Rats in NC group displayed normal Cornus ammonis (CA) which is a hippocampus structure showed in (Fig. 22A). The neurons are separated into 4 layers i.e CA1-CA4 Whereas, rats in DC group showed in (Fig.22B) which displayed disorganization of PL vacuolation with clear dark nuclei and clumping. Apoptotic cells are seen in PL. The granular layer (GL) shows some cell reduce with raise glial cells. Enlargement of neurons, glial cells and low amount of apoptotic cells were observed in molecular layer (ML). Whereas, Donep 1 mg/kg treatment in diabetic rats (Fig. 22C) and EPS 54 and 27 mg/kg (Fig. 22D, E) displayed normal size of glial cells and neurons in the ML. Histopathologically restoring is seen in Donep and EPS treated rats whereas, no changes were observed in DC rats and diabetic rats adminsitered with of EPS 13.5 mg/kg (Fig. 22F).

5. DISCUSSION

In this study, we evaluated the potential neuroprotective outcome of EPS on oxidative stress, tau protein and neuroinflammation in diabetic induced memory and learning impairment in wistar rats.

Both insulin-dependent and non insulin dependent DM can be induced by STZ. In present study STZ was used, because it is a common experimental diabetic model and provides appropriate illustration of hyperglycemia mediated prolonged oxidative stress [317][318].

Discussion: Behavioural parameters:

T2DM rats exhibited noticeable memory impairment and was confirmed with behavioral parameters like EPM, PA and MWM task. Concomitant treatment with EPS resulted positive effects as compared to diabetic rats. Behavioural fluctuations in experimental animals were also noted.

Earlier studies has indicated that HFD and low dose of STZ induces T2DM [307][308]. Results of this study revealed similar effect as well as diabetic rat indicates spatial memory and learning deficits in MWM. This was confirmed by EPM and PA task results, which was associated with avoided and reversed impairment by EPS treatment in comparison with diabetic rats. We used MWM task to test spatial memory by observing the EL to reach a hidden platform. The diabetic rats were affected more when compared to NC rats, confirmed with previous reports [319].

Further, rats treated with Donep and higher dose of EPS learned the platform location faster than diabetes control rats and these findings were persistent throughout the

trials. Additionally, enhanced time spent in goal quadrant in experimental rats treated with Donep and higher dose of EPS were also revealed in the similar effects suggesting their ability to swim (motor activity) was unaffected by diabetic condition.

In PA test, a significant enhanced TLT was noted as compared to DC (STZ treated) and directs for successful learning and memory function in EPS treated rats. Whereas, STZ treated rats failed to demonstrate an increase in TLT in retention trial. As per earlier findings Donep administrations prior to trials in STZ induced rats attenuated the memory impairment from 2^{nd} trial onwards [320]. In similar manner, treatment with EPS also attenuated the memory impairment from 2^{nd} trials onwards. These results confirm previous findings that have shown CI in STZ-induced diabetic rats associated with hippocampal dysfunction [12][317][321].

EPM, PA and MWM test were used to analyse learning and memory. We evaluated the EPS treatment orally for 4 weeks towards improvement in learning and memory. All the study doses of EPS enhanced the learning and memory as directed by the decreased TL in comparision with diabetic rats. Decreased latency time in all repeated trials using MWM test indicated learning and memory function, whereas decreased TL in EPM test and increase TL during retention trial in PA test showed improvement of memory.

Previous study report advocates that decreased TL time on 2^{nd} day as compared to 1^{st} day in EPM test indicates retention of memory [273][322]. In similar way, rats treated with donep and EPS were observed with significant decreased TL on 30^{th} day trial as compared to 29^{th} day trial, suggesting memory retention in treated rats. Considering such findings, several diabetic studies has correlated cognitive scarcity with possible

mechanisms including hyperglycaemia induced neuronal damage, dyslipidemia, amyloidopathy, taupathy etc [323][324].

In diabetes, oxidative stress plays a vital role in memory impairment [325][326]. EPS treatment increases the intracellular GSH which is essential for oxidative stress management of endothelial cells, inhibiting numerous vascular diseases [37].These interpretations suggest for significant antioxidant activity of EPS treatment against STZ induced oxidative stress [38]. In addition, there are many justifications that polyol pathway induces gains in oxidative stress.

Neuroinflammatory mediators and markers of oxidative stress are capable in causing cognitive alterations via several mechanisms that could possibly affect the neuronal activity and cell existence. Several studies were conducted earlier to conceptualize the possible cause and link between neuroinflammation, oxidative stress, behaviour and CIs [326-328]. Following this, EPS has been documented previously for up-regulation of HO-1, dismutase and CAT by activating Nrf 2 and suggested its beneficial effect on numerous neurological conditions [39]. Interestingly, EPS treatment (an in-vitro study) has been documented for up regulation of HO-1 on rat Schwann cells and human neuroblastoma cells, suggesting EPS potential to prevent neurological diseases.

Discussion: Biochemical estimation:

EPS treatment neutralised behavioral and biochemical changes in diabetic rats. The study results indiacted decreased BWs in untreated diabetic rats when compared with NC. Such observation is supported with earlier study reports [308][309][329]. While, no remarkable change in BW and blood glucose level was observed upon treatment with EPS and Donep [37][39][38].

T2DM induced rats displayed up-regulated IL-6, TNF-α and tau proteins levels and decreased CAT and GSH levels, resulting in memory impairment. This was supported by histopathological examinations in pancreas and hippocampus tissue samples. EPS treatment improved the cognitive function without altering blood glucose levels [251]. This might be because of antioxidant, anti-inflammatory potential of EPS, which is documented to up-regulate HO-1 concentration and reduced oxidative stress.

Oxidative stress in T2DM condition may also cause insulin resistance and β-cell dysfunction [252]. Even, it may result in brain damage as reported by few experimental models [253]. Oxidative stress provokes inflammatory cytokines leading to inflammation, known to have significance in T2DM which may promote progression of T2DM in AD [254][255].

GSH are important for maintaining cellular redox potential [330]. Schwann cells treated with EPS have resulted in enhanced intracellular GSH concentration, which may prevent from oxidative stress mediated diseases [37]. Rats treated with EPS and Donep reduced TNF-α and IL-6 to control levels and enhanced CAT and GSH levels.

The histopathological examination of pancreas in NC rats displayed normal pancreatic β-cell whereas, untreated diabetic rats were observed with injured pancreas, decreased

and shrunken islets cells numbers. These results have similar opinion as documented previously [331]

Hippocampus involved in many aspects of learning, memory and navigation. Initial studies on hippocampus of diabetic rats are reported with cell loss of large PL, V and disorganization of all the layer [332][333]. These conditions were markedly improved by Donep and EPS treatment.

Neurological disorders such as AD is associated with gathering of neurotoxic proteins i.e. tau and Amyloid-beta (Aβ) plaques [334]. EPS has the potential for enhanced clearance of tau protein, which might be because of rhodanine ring structure present in EPS. It was further tested on neuronal cell models of tau aggregation and revealed appreciable findings. This supports of present study hypothesis that EPS can enhanced clearance of tau protein [334][335]. However, there are no peer reviewed reports available regarding the effect of EPS on memory and learning in diabetes.

Thus, the present study reports the protective outcome of EPS on CI induced by HFD and STZ in T2DM rats. This may be possibly due to alterations in IL-6 and TNF-α cytokines, CAT, GSH, TAU protein levels.

6. SUMMARY

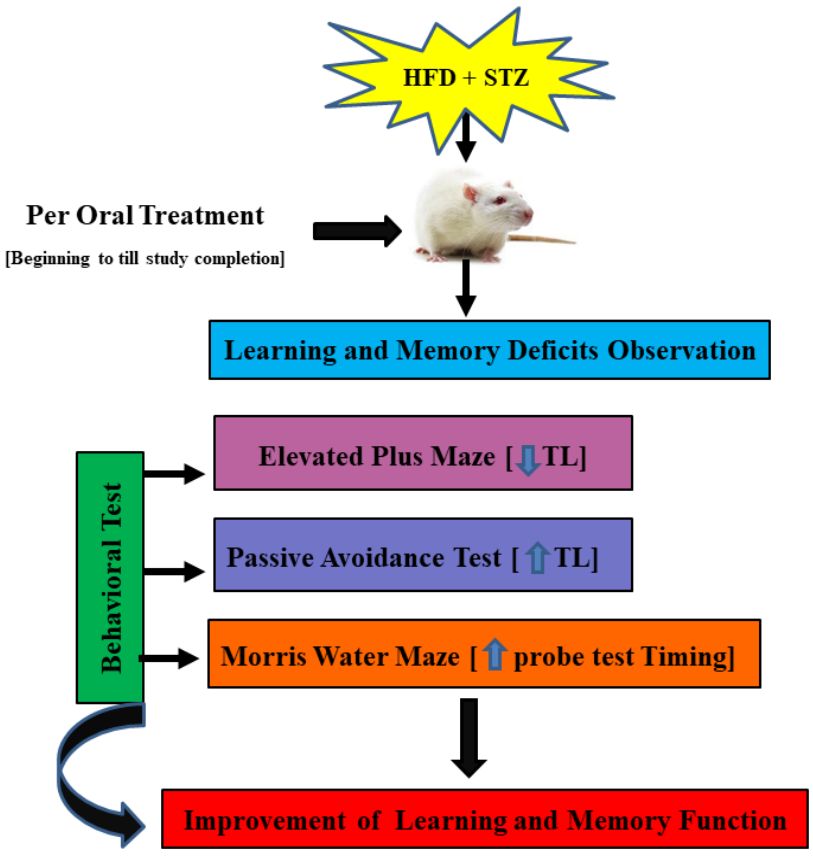
The present thesis **"Effect of Epalrestat, An Aldose Reductase Inhibitor on Memory and Learning in Diabetic Male Wistar Rats"** was carried out on High Fat Diet - STZ induced Type-2 diabetic rats. Previous studies have demonstrated that HFD in combination with low dose of STZ induces type-2 diabetes and is a common experimental diabetic model which provides a relevant example of chronic oxidative stress, Inflammation due to hyperglycemia. T2DM rats exhibited noticeable memory impairment that was confirmed with behavioral parameters like EPM, PA test and MWM task. Concomitant treatment with EPS shown positive effects in comparison with diabetic rats and the behavioural changes were observed in diabetic treated rats.

Oxidative stress and inflammation is considered to play a fundamental role in development of memory impairment in diabetes. The present study indicated the significant increased levels of pro-inflammatory cytokines, TNF-α and IL-6 as well as decreased levels of oxidative stress markers like CAT and GSH in the untreated diabetic rats. Whereas, EPS treatment rescued this effect by lowering TNF-α and IL-6 levels and enhanced CAT and GSH levels.

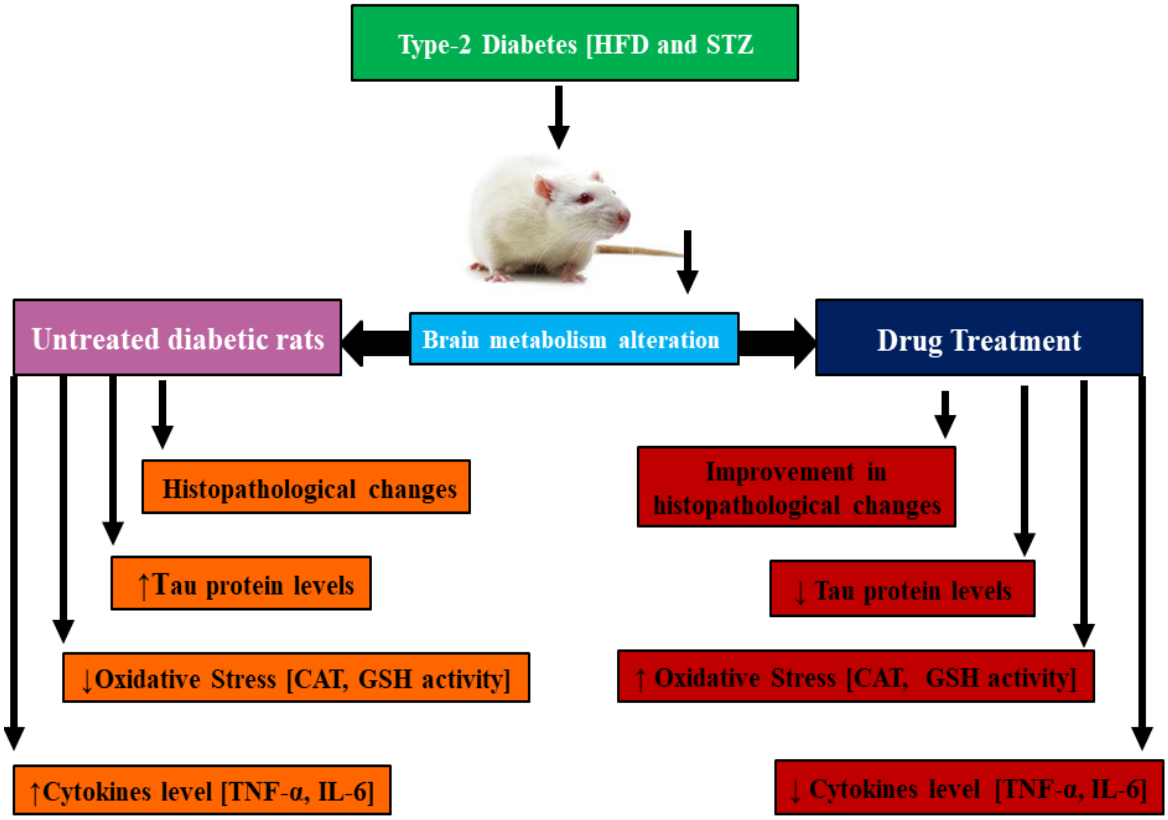
Alzheimer disease is characterized by pathological aggregation of two proteins, tau and Aβ-amyloid, both of which are considered to be toxic to neurons. Epalrestat is the only aldose reductase inhibitors which have rhodanine based structure. Rhodanine containing compounds had been investigated for activity on tau aggregation inhibition. This is in direct support of this study hypothesized that rhodanine ring structure containing compound like epalrestat has the potential for enhanced clearance of tau protein, one of the chief pathological features of Alzheimer's disease.In our study we observed that treatment with EPS decreased tau protein levels in diabetic

rats. Epalrestat is also known to posses pleiotropic effects like anti-inflammatory, antioxidants effects etc. The anti-inflammatory and anti-oxidant properties of epalrestat can be beneficial in the management of neurodegenerative disease by improving the memory and learning in animal model. However, there is no study reports regarding the effect of epalrestat like compound on memory and learning in diabetes. Therefore, there is a need of scientific research to develop the efficacious and safer drugs to fight against the dementia and also particularly to evaluate the mechanism of action on neuroprotection and neurogenesis. Epalrestat may produce possible neuroprotection in animal models.

Graphical abstract: Behavioral Parameters



Graphical abstract: Biochemical Analysis and Hislogical Observations


Type-2 Diabetes [HFD and STZ
Untreated diabetic rats
Brain metabolism alteration
Drug Treatment
Histopathological changes
↑Tau protein levels
↓Oxidative Stress [CAT, GSH activity]
↑Cytokines level [TNF-α, IL-6]
Improvement in histopathological changes
↓ Tau protein levels
↑ Oxidative Stress [CAT, GSH activity]
↓ Cytokines level [TNF-α, IL-6]

7. CONCLUSION

To summarize, baased on our results shows a positive association of EPS treatment enhanced learning and memory activity. It was more significant at the dose of 27 and 54 mg/kg. Suggesting the potential benefits of EPS treatment improved learning and memory as indicated by decreased EL to reach a hidden platform and increased time spent in goal quadrant using MWM task, reduced TL in EPM and also there is a significant increase in the transfer latencies using PA test were observed in behavioral test. Memory-enhancing activity of EPS was comparable with DC group.

The treatment with EPS significantly increased CAT and GSH activities in plasma as compared to DC group. Furthermore, EPS treatment significantly reduced tau proteins levels. The proinflammatory cytokine levels like TNF-α and IL-6 was decreased with EPS treatment and Oxidative stress like CAT and GSH activity enhanced with EPS treatment in diabetic rats. Histopathology study of hippocampus indicated that EPS could attenuate apoptosis, vacuolations and clumping processes.

In conclusion, the present study revealed that diabetic rats treated with EPS could enhanced CI and act as a beneficial agent for management of CI in diabetes. However, further extensive studies are needed to establish its accurate mechanism of action for potent and efficacious agent in the treatment of memory deficit.

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9. ANNEXURES

ANNEXURE I: ANIMAL ETHICAL COMMITTEE APPROVAL LETTER:

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Jawaharlal Nehru Medical College, Belgaum

MDC / AH / _____ Date : 18-5-2016.

CERTIFICATE

This is to certify that the M.Sc / M.D / Ph.D / Research - Project entitled

"Effect of Epalrestat, on aldose reductase inhibitor on Memory and Learning in Diabetic Male Wistar rats"

Submitted by Ms / Dr. Shruti Jaiswal., Dept. of Pharmacology, JNMC.,

has been approved by the Institutional Animal Ethical Committee meeting held on 14-5-2016. vide Resolution No. 7/D.

Rats : 60 Male

Signatures :
&Name

Member Secretary
IAEC-JNMC

CPCSEA NOMINEE 14/5/2016
IAEC-JNMC

ANNEXURES II: PUBLICATIONS:

Sr.No.	Article Title	Journal	IF
1	Neuroprotective effect of epalrestat mediated through oxidative stress markers, cytokines and TAU protein levels in diabetic rats.	Life Sciences 2018; 207:364-371	2.70
2	Neuroprotective effect of epalrestat on memory impairment in streptozotocin – induced type-2 diabetic rats using different behavioral models.	Asian Journal of Pharmaceutical & Clinaical Research 2018;11:411-415	

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 1, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

NEUROPROTECTIVE EFFECT OF EPALRESTAT ON MEMORY IMPAIRMENT IN STREPTOZOTOCIN-INDUCED TYPE-2 DIABETIC RATS USING DIFFERENT BEHAVIORAL MODELS

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Received: 25 October 2017, Revised and Accepted: 23 November 2017

ABSTRACT

Objective: The present study was designed to evaluate the protective effects of epalrestat (EPS) on memory and learning in type-2 diabetes.

Methods: Sixty percent high-fat diet for 2 weeks and a single dose of streptozotocin (35 mg/kg, ip) was used to induce memory impairment in rats. Once the diabetes is confirmed, test drug (EPS - 13.5, 27, and 54 mg/kg, oral) and donepezil (1 mg/kg, oral) were administered to different groups of rats for 4 weeks followed by an assessment of memory and learning deficit using behavioral paradigms: Elevated plus maze (EPM), Morris water maze (MWM), and passive avoidance test.

Results: EPS and donepezil showed significant improvement in learning and memory of rats, as indicated by markedly decreased escape latency to reach a hidden platform and increased time spent in target quadrant using MWM task, reduced transfer latency in EPM, and also there is a significant increase in the transfer latencies using passive avoidance test were noted. Memory-enhancing activity of EPS (13.5, 27, and 54 mg/kg) was comparable with the diabetic control group.

Conclusion: The study findings suggest that memory-enhancing effect of EPS may be mediated by its antioxidant and anti-inflammatory activities. This recommends the potential effect of EPS therapy as a useful memory restorative agent in the treatment of neurodegenerative disease seen in type-2 diabetes rat.

Keywords: Type-2 diabetes, Epalrestat, Donepezil, Learning and memory, Morris water maze, Passive avoidance, Elevated plus maze, High-fat diet.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i1.23313

INTRODUCTION

Diabetes mellitus (DM) is the most common endocrine disorder characterized by increased blood glucose levels, resulting from defective insulin secretion, resistance to insulin action, or both. DM is often associated with severe complications, and there is an increasing appreciation that cognitive function declines in DM [1-3]. Chronic hyperglycemia is associated with disturbance of the blood sugar level which leads to nerve cells damage in the brain causing cognitive impairment [4].

Animal studies have shown that high-calorie diets impair the structure and function of the hippocampus, a brain region critical for learning and memory [5,6]. In animal models, both type-1 and type-2 diabetic animals are reported to induce severe memory deficits [7,8]. The increased oxidative stress in diabetes produces oxidative damage in many regions of rat brain including hippocampus.

Oxidative stress and harmful free radicals play an important role in the development of memory impairment. Free radicals and reactive oxygen substances generated by living cells as a result of physiological and biochemical processes and accumulation of these free radicals in the body can cause oxidative damage to lipids, proteins, and DNA, which leads to diabetes and neurodegenerative diseases [9,10].

Epalrestat (EPS) (5-[(1*Z*,2*E*)-2-methyl-3-phenyl propenylidene]-4-oxo–2–thioxo-3-thiazolidine acetic acid; EPS) is an inhibitor of aldose reductase, well proven to have beneficial effects for the treatment of diabetic neuropathy and is easily absorbed by neural tissue and inhibits aldose reductase with minimum adverse effects [11]. It is reported for anti-inflammatory and antioxidant effects using rat Schwann cell and human neuroblastoma cell line [12-14]. Therefore, the anti-inflammatory and anti-oxidant properties of EPS can be beneficial in the management of neurodegenerative disease by improving the memory and learning in an animal model. However, there are no study reports regarding the effect of EPS such as compound on memory and learning in diabetes. In the present study, the effect of EPS was evaluated experimentally with regard to learning and memory in rats.

METHODS

Animals

Male Wistar rats (150–200 g) were procured from the central animal house facility of Jawaharlal Nehru Medical College, Belagavi. The rats were acclimatized to 12:12 h light-dark cycle for 7 days, before an animal study. They were maintained at constant room temperature (22°C–25°C) and on standard chow pellet (Amrut Brand) with water *ad libitum*. The animals were housed in polypropylene cages with 3 animals per cage. The study was approved by the Institutional Animal Ethics Committee constituted as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (Resolution No.: 7/D; dated 18.05.2016).

Drugs and solutions

EPS (Micro Labs Ltd., India), streptozotocin (STZ) (Enzo Life Sciences, UK), donepezil hydrochloride (Alkem Laboratories Ltd., India), and Glucometer/strips (Accu Chek: Roche Diagnostics India Pvt., Ltd., Mumbai) were used. EPS was suspended in 1% gum acacia, whereas,

STZ and donepezil hydrochloride were dissolved in citrate buffer (pH 4.4) and distilled water, respectively. Drug solutions/suspensions were prepared fresh before administration. EPS at doses of 57, 27, and 13.5 mg/kg, p.o. and donepezil hydrochloride1 mg/kg, p.o. were selected on the basis of previous literature and as per human dose [11,15,16-18]. The clinically equivalent human doses of drugs were converted into rat equivalent doses by the conversion table devised by Paget and Barnes [19].

Experimental induction of diabetes

Type-2 diabetes was induced in rats by previously described methods [20,21]. The rats were fed with a high-fat diet (HFD) (VRK Nutritional Solutions, Pune, India) for the initial period of 2 weeks followed by low dose of STZ (35 mg/kg; i.p) was administered. All diabetic rats were given 5% glucose solution to prevent from hypoglycemic shock, wherea, normal control (NC) rats were administered with vehicle (1 ml/kg; p.o.). The fasting blood glucose was measured 48 h after STZ injection. The rats injected with STZ showing fasting blood glucose > 200 mg/kg noted as diabetic and used for further study. The HFD composition is given in Table 1.

Treatment schedule

The confirmation day of diabetes was taken as day 1 of diabetic condition. A total of 60 rats (10 normal; 50 HFD-STZ induced diabetic rats) were used and divided into six groups (n=10) and received the following treatment:

Group I: NC received known volume of vehicle (1 ml/kg, p.o)
Group II: Diabetic control (DC) - vehicle only (1 ml/kg, p.o)
Group III: Diabetic rats + Donepezil hydrochloride (1 mg/kg, p.o)
Group IV: Diabetic rats + EPS (57 mg/kg, p.o)
Group V: Diabetic rats + EPS (27 mg/kg, p.o)
Group VI: Diabetic rats + EPS (13.5 mg/kg, p.o).

All the drugs were administered orally for 4 weeks. Behavioral tests were carried out at the end of the treatment.

Assessment of cognitive function [7,22,23]

Elevated plus maze (EPM)

The test was used to evaluate spatial long-term memory. The maze has two open and two enclosed arm (OA and EA), and it was elevated 25 cm above the ground. The behavioral tests were conducted in a quiet room illuminated by a dim light.

Learning protocol

EA divided into two equal parts. Each individual rat was kept at the end of the OA on day 1 and initial transfer latency (TL) was recorded. To become habituated with the maze, the animals were allowed to explore the plus maze for 90 s after reaching the EA. On the 2nd day, 24 h after the first exposure, TL was again noted. A long latency period to reach EA indicates poor retention compared with significantly shorter latencies

Passive avoidance test (step through test)

The passive avoidance apparatus have two chambers, light and dark compartment with grid floor 50 × 50 cm and 35 cm high walls, separated by a wall with a guillotine door 6 × 6 cm. One of the two chambers was illuminated with 100 V bulb placed at 150 cm height, and the other was dark. The test was conducted on 3 consecutive days at the same time of the day. On day 1 (trial 1) and day 2 (trial 2, 3, and 4), acquisition trial was conducted, and individual rat was kept in illuminated chamber of the apparatus. At the end of the 3rd trial as soon as rat entered the dark compartment, it received an electric shock on the feet (50 V, 50 Hz, 1 s) through the stainless steel grid floor. The time when rat entered in the dark chamber was noted as step-through latency (STL). Retention was tested after 24 h, and STL was recorded. Cutoff time allotted was 300 s. Increase in the STL was considered as an index of improvement of memory.

Morris water maze test (MWM)/spatial discrimination

During spatial discrimination, the hidden platform was kept at 1.5 cm below the water level changing the area of the pool from that used during cue discrimination training. We added milk to make the pool water opaque, in which platform shown invisible. The platform has fixed in one place. Rats had trained to four consecutive trials each day for 4 consecutive days. Each trial was given 120 s. Rats were allowed to start swimming in each trial from one of the four locations (north, south, east, and west); the choice of the location was random for each rat and each trial. The rat should escape to the platform within 120 s, and if that did not occur, we guided them gently toward the hidden platform where they remained for 10 s. Probe trial conducted on the 5th day in which platform was removed from the swimming pool and allowed the rat to swim freely for 120 s, and time spent in target location was noted as a function of memory.

Statistical analysis

The study results were expressed as mean±standard error of mean. Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. $p<0.05$ was considered as statistically significant.

RESULTS

Blood glucose levels

At the onset of the study, all experimental animals had equivalent blood glucose levels (Table 2). At the conclusion of the experiment, glucose concentrations were highly significantly elevated in donepezil 1mg/kg and EPS-treated animals relative to those in the NC.

Effect of EPS on performance of EPM

The effect of donepezil and EPS treatments in diabetic rats on mean transfer latencies in the EPM test is shown in Fig. 1. Statistical analysis revealed that chronic treatment with donepezil 1 mg/kg and EPS (57, 27 mg/kg) had a significant effect on the transfer latencies on day 1 (day 29) as compared with DC ($p<0.001$).On the day 2 (day 30), HFD-STZ induced TL was drastically decreased when compared to the day 1. This clearly indicates the learning behavior of animals on the day 2, whereas there was no difference in TL tested on day 1 and 2 in DC animals. On the other hand, cholinesterase inhibitor, donepezil showed a significant reversal of HFD-STZ induced deficits. However,

Table 1: Composition of HFD

Ingredients	Diet (g/kg)
Powdered NPD	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and minerals mix	60
DL-methionine	03
Yeast powder	01
Sodium chloride	01

HFD: High-fat diet, NPD: Normal pellet diet

Table 2: Blood glucose levels (means±SEM) in the six groups of rats at the onset and at the end of the experiment

Treatment	Glucose mg/dl	
	Onset of the study	End of the study
NC	96.50±1.48	100.2±3.16
DC	98.11±4.69	456.4±11.44***
DC+Donepezil 1	100.8±4.78	411.6±14.72***
DC+EPS 54	97.20±3.83	396.1±20.85***
DC+EPS 27	100.2±3.99	404.4±17.43***
DC+EPS 13.5	99.89±4.67	413.7±10.94***

DC: Diabetic control, NC: Normal control, EPS: Epalrestat, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at ***p<0.001 versus NC group

donepezil and EPS-treated animals (57, 27, and 13.5 mg/Kg) showed significant decreased TL when compared to DC on day 2.

Effect of EPS treatment on TL (passive avoidance test)

In the present study, we used passive avoidance task to assess short-term/long-term memory for evaluating the effect of EPS. Memory performance was correlated with the latency to escape from the light compartment, the better the recollection, and the greater the latency.

Acquisition

There was no significant difference in the STLs among the treatments and DC group of rats in the first acquisition trial (before receiving the electrical shock) (Table 3). This observation designates that the exploratory behavior of the different groups of rats in the dark did not differ. However, significant differences were observed among the different experimental groups with respect to the number of trials to acquisition criterion ($p<0.001$, $p<0.01$) (Fig. 2 and Table 3). Specifically, the number of trials to acquisition in donepezil-treated group ($p<0.001$, $p<0.01$) and EPS-treated group ($p<0.001$, $p<0.01$) were significantly less than DC group. Consistent with the presence of a cognitive deficit, the number of trials to acquisition in DC group was significantly greater than NC group ($p<0.001$).

Retention

In the retention test which was conducted 24 h after the aversive stimuli (foot shock), retention of memory was significantly decreased in DC group ($p<0.001$) as compared to NC group, whereas significantly enhanced TLs were observed in the animals treated with donepezil and EPS (54, 27 mg/kg; $p<0.001$, $p<0.01$) in comparison to DC group.

Effects of EPS treatment on spatial memory deficits in the MWM tasks

The memory impairment was assessed using MWM task. The mean escape latency time was measured to assess spatial memory in the experimental rats. The treatment with donepezil and EPS during training session significantly influenced the escape latency (Table 4 and Fig. 3). Further, statistical analysis revealed that donepezil and higher dose of EPS (54 mg/Kg) significantly reduced ($P<0.05$) escape latency over the course of the training sessions when compared to the DC group. In the spatial probe test, performed on day 5 (day 38), the time spent within the target quadrant by the DC group was decreased as compared to those of the NC group ($p<0.001$) (Fig. 3). Moreover, animals treated with EPS (27 and 54 mg/kg) resulted in enhanced time spent within the target quadrant as compared to DC groups ($p<0.001$). These effects of EPS were similar to that shown by donepezil treatment ($p<0.001$).

DISCUSSION

The study was designed and evaluated for the effect of EPS in diabetes-induced memory impairment: Memory and learning in rats. Type-2 diabetic rats exhibited marked impairment in memory that was revealed with behavioral parameters: EPM, passive avoidance test, and MWM task. Concomitant treatment with EPS, an aldose reductase inhibitor, responds the behavioral changes induced by diabetes.

Earlier studies directed that HFD in combination with a low dose of STZ induces type-2 diabetes [21]. Results of the present study showed the similar effect as well as diabetic rat indicates spatial memory and learning deficits in MWM task.

This was substantiated by the study results in the EPM and passive avoidance task, which was associated with avoided and reversed impairment by EPS treatment in rats. In the present study, we used MWM task to test spatial memory by observing the escape latency to reach a hidden platform. The diabetic rats were severely impaired as compared with NC rats, confirming earlier findings [24]. Furthermore, rats treated with donepezil and higher dose of EPS learned the platform location faster than diabetes control rats, and these findings were persistent throughout the trials. In addition, enhanced time

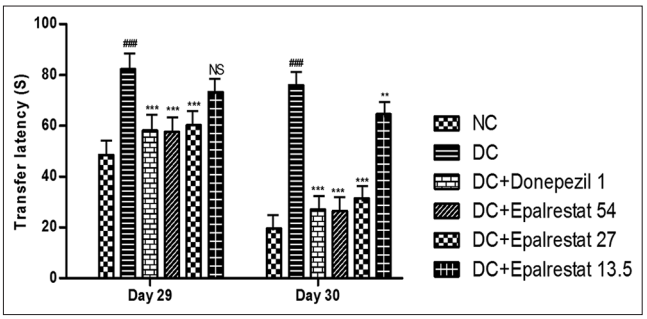

Fig. 1: Effect of EPS on transfer latency in high-fat diet streptozotocin-induced memory impairment on elevated plus maze: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at **p<0.01, ***p<0.001, ns: Not significant versus DC group and ###p<0.001 versus NC group

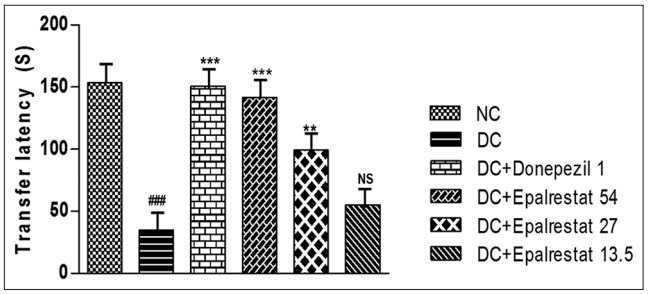

Fig. 2: Effect of EPS on transfer latency in high-fat diet streptozotocin-induced memory impairment on passive avoidance test: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at **p<0.01, ***p<0.001, ns: Not significant versus DC group and ###p<0.001 versus NC group

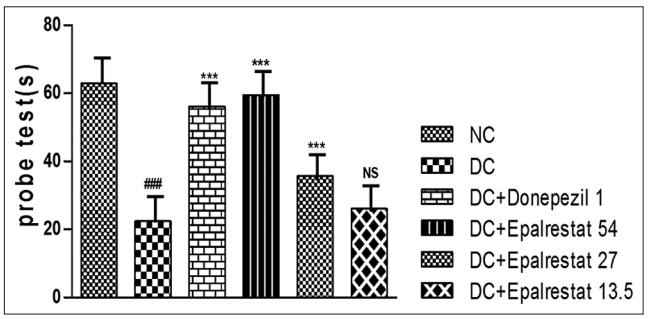

Fig. 3: Effect of EPS on probe test in Morris water maze task in high-fat diet streptozotocin-induced memory impairment rats: DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at ***p<0.001, ns: Not significant versus DC group and ###p<0.001 versus NC group

spent in target quadrant in experimental rats treated with donepezil and higher dose of EPS was also revealed in the similar fashion suggesting their motor performance (ability to swim) was unaffected by diabetic condition. In a passive avoidance test, a significant increase in TL time (TLT) as compared with DC (STZ treated) directs successful learning and memory function in EPS-treated rats, whereas STZ-treated rats failed to demonstrate an increase in TLT in retention

Table 3: Effect of EPS on HFD–STZ-induced memory impairment in the passive avoidance test

Treatment	Time taken to enter small compartment (s)				Day 33
	Day 31	Day 32			
	Trial 1	Trial 2	Trial 3	Trial 4	TL
NC	55.50±6.77	36.10±5.42	27.90±5.31	18.40±3.18	153.5±15.93
DC	114.4±5.86	99.89±6.85$^{###}$	73.33±6.27$^{###}$	66.89±6.48$^{###}$	34.78±3.97$^{###}$
DC+Donepezil 1	99.56±3.31	55.56±5.40***	35.22±4.58**	23.33±2.90***	150.6±17.86***
DC+EPS 54	101.5±2.63	52.70±8.40***	33.50±7.89***	19.10±3.31***	141.6±13.49***
DC+EPS 27	107.7±17.62	66.10±8.47**	56.20±9.13^{ns}	39.70±5.86***	99.20±12.56**
DC+EPS 13.5	124.9±3.52	100.2±4.98^{ns}	68.33±4.12^{ns}	57.00±4.87^{ns}	54.89±6.52^{ns}

DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by oneway ANOVA followed by Dunnett's test. Significance at **p<0.01, ***p<0.00, ns: Not significant versus DC group and $^{###}$p<0.001 versus NC group, HFD: High-fat diet, STZ: Streptozotocin, TL: Transfer latency

Table 4: Effect of EPS on spatial memory in MWM task in HFD–STZ induced memory impairment rats

Treatment	Time taken to reach target platform (s)				Probe test: Time spent in target quadrant (s)
	Day 34	Day 35	Day 36	Day 37	Day 38
NC	45.80±7.79	36.80±5.68	30.23±5.25	17.63±2.78	62.90±2.90
DC	85.00±2.79$^{##}$	82.22±6.05$^{##}$	72.81±12.49$^{#}$	63.25±10.58$^{##}$	22.44±1.28$^{###}$
DC+Donepezil 1	50.53±10.08*	43.28±9.74*	34.86±12.07*	27.50±7.18*	56.11±1.91***
DC+EPS 54	48.58±9.48*	40.45±10.31*	31.33±7.17*	29.30±5.89*	59.50±2.80***
DC+EPS 27	57.40±9.85^{ns}	52.75±10.88^{ns}	38.35±9.18^{ns}	35.73±8.40^{ns}	35.70±1.46***
DC+EPS 13.5	82.69±3.39^{ns}	79.56±7.64^{ns}	70.44±9.38^{ns}	53.44±7.61^{ns}	26.11±1.70^{ns}

DC: Diabetic control, NC: Normal control, EPS: Epalrestat, S: Seconds, SEM: Standard error of mean, n=10, Data expressed in mean±SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at *p<0.05, **p<0.01, ***p<0.001, ns: Not significant versus DC. #p<0.05, $^{##}$p<0.01, $^{###}$p<0.001 versus NC. HFD: High-fat diet, STZ: Streptozotocin, MWM: Morris water maze

trial. As per earlier findings, donepezil administrations before trials in STZ-induced rats attenuated the memory impairment from 2nd trial onward [25]. In similar manner, treatment with EPS also attenuated the memory impairment from 2nd trial onward. These results confirm the previous study findings that have shown cognitive impairment in STZ-induced diabetic rats, which is associated with hippocampal dysfunction [26-28].

In our study, the EPM, passive avoidance, and MWM were used for the assessment of learning and memory. We evaluated the EPS treatment orally for 4 weeks improved learning and memory. All the doses of EPS improved the memory, as reflected by the diminished TL compared with diabetic rat, and decreased latency time in all repeated trials in MWM indicates learning and memory function, whereas decreased TL in EPM and increased TL during retention trial in PAT test showed improvement of memory.

Previous studies reported that decreased TLT in the 2nd day compared to 1st day in EPM indicated retention of memory [29,30]. The treatment with donepezil and EPS in experimental rats revealed that significant decreased TL on 2nd day trial (30th day) as compared to 1st day trial (29th day). This finding indicated memory retention in treated rats. In several studies, these cognitive deficits in diabetes were correlated for probable mechanisms such as hyperglycemia-induced end-organ neuronal damage, dyslipidemia, amyloidopathy, and tauopathy [31,32].

In the present study, EPS treatment significantly improved learning and memory impairment in HFD–STZ-induced diabetic rats using behavioral parameters. On the other hand, EPS treatment had no effect on blood glucose levels during the study, suggesting that there is no antidiabetic effect of EPS in experimental rats. This is also supported by earlier reports for blood glucose levels on EPS treatment [18]. Oxidative stress is considered to play a fundamental role in the development of memory impairment in diabetes [33,34]. EPS increases the intracellular levels of glutathione (GSH) which plays a crucial role in protecting endothelial cells from oxidative stress, thereby preventing several vascular diseases caused by oxidative stress [13]. These observations suggest that EPS have significant antioxidant activity against STZ-induced oxidative stress [14].

STZ-induced diabetes was used in the present study because it is a well-known model of experimental diabetes and provides a good and relevant example of chronic oxidative stress due to hyperglycemia [26,35]. In addition, there are several potential explanations for polyol pathway-induced increase in oxidative stress. Hyperglycemia activates the polyol pathway, and reduction of glucose to sorbitol through aldose reductase may lead to NADPH consumption [36]. As NADPH is used in several critical reductive metabolic steps, a large drain on the NADPH pool could compromise the ability of the cell to protect itself from oxidative stress. NADPH is also required for GSH reductase to regenerate GSH [37-40]. Considering the importance of oxidative stress in the pathophysiology of diabetic state and development of cognitive impairment, reduction of oxidative stress by EPS may produce a beneficial effect on diabetic-induced cognitive impairment.

Neuroinflammatory mediators and oxidative stress markers are capable in causing cognitive alterations through several mechanisms that could possibly affect the neuronal properties and cell survival. Several studies were conducted earlier to conceptualize the possible cause and link between neuroinflammation, oxidative stress, behavior, and cognitive impairments [34,41-43].

According to the previous findings, heme oxygenase (HO)-1 has potent antioxidant and anti-inflammatory functions; however, EPS upregulates HO-1, dismutase, and catalase by activating Nrf2 and suggests that EPS has the beneficial effect on improvement of several neurological disorders [12].

Interestingly, a recent *in vitro* study reported that EPS treatment on rat Schwann cells and human neuroblastoma cell line upregulates HO-1 suggesting the potential of EPS to prevent neurological diseases. Therefore, a pilot study was conducted by us to analyze behavioral effects in EPS-treated rats using STZ-HFD induced model.

CONCLUSION

Based on our results obtained in the present study, the EPS has shown enhanced learning and memory activity. In particular, it was more significant at the dose of 27 and 57 mg/kg. However, further extensive studies are needed to establish its exact mechanism of action for potent and efficacious agent in the treatment of memory deficit.

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(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

Life Sciences 207 (2018) 364–371

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Neuroprotective effect of epalrestat mediated through oxidative stress markers, cytokines and TAU protein levels in diabetic rats

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ARTICLE INFO

Keywords:
Type-2 diabetes
Cognitive impairment
Epalrestat
Oxidative stress
Cytokine
TAU protein

ABSTRACT

Aims: Type-2 diabetes mellitus (DM) is associated with cognitive impairment. Increasing evidence establishes that neuro-inflammatory and oxidative stress condition plays a main role in the development of neurodegeneration. Epalrestat, an aldose reductase inhibitor is commonly prescribed for the treatment of diabetic peripheral neuropathy. Its beneficial effects for antioxidant, anti-inflammatory potential and being rhodanine structure containing compound suggests possible role for treatment of DM associated with cognitive dysfunction.

Main methods: In the present study, we evaluated the effect of epalrestat (54, 27, 13.5 mg/kg, p.o.) and donepezil (1 mg/kg, p.o.) on Tau protein levels, oxidative stress and inflammatory markers in high fat diet (HFD) and Streptozotocin (STZ; 35 mg/kg, i.p.) induced cognitive impairment in diabetic rats.

Key findings: The epalrestat - 54, 27 mg/kg p.o. and donepezil treatment significantly increased CAT ($p < 0.001$, $p < 0.01$, $p < 0.001$) and GSH ($p < 0.001$, $p < 0.01$, $p < 0.001$) activities respectively as compared to diabetic control rats. In addition, similar dose of epalrestat treatment indicated considerably lowered TAU protein levels ($p < 0.001$, $p < 0.05$) while no significant effect was noted with donepezil. These treatments significantly decreased gene expression of TNF-α (1.6, 1.6, 1.7 fold change) and IL-6 (2.5, 1.9, 1.7 fold change). Histopathological examination indicated that epalrestat could attenuate apoptosis of neurons, vacuolations and clumped processes, disorganization and thinning of all the layers.

Significance: Our findings suggest that diabetic rats treated with epalrestat could ameliorate the cognition deficits and might act as a beneficial agent for prevention and treatment of cognitive impairment in diabetes.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by elevated blood glucose levels. Globally 366 million people were estimated to have diabetes as per record 2011 and this is predicted to rise to 522 million by 2030 [1]. Diabetes can be divided into two main categories: type-1 and type-2 diabetes with type-2 accounting for approximately 90% of diabetes cases. In type-2 diabetes, the first stage is peripheral insulin resistance, where the sensitivity of tissues to insulin decreases [2].

DM is associated with several comorbid complications including structural and functional dysfunctions in CNS and end organ damage via many diverse mechanisms. Diabetic patients are more prone to Alzheimer's disease and other dementias [3–6]. Cognitive deficits are well documented in both type-1 and type-2 DM [7–10]. Diabetes lead to abnormalities, structural, neurochemical changes associated with neuronal damage, oxidative stress, cell death and loss of information processing [9, 11]. Earlier studies also demonstrated that consumption of high energy diet is a risk factor for the development of neurological disorders via free radicals mediated oxidative stress [12].

Free radicals like reactive oxygen species generated by living cells as a result of physiological and biochemical processes and accumulation of these free radicals in the body can cause oxidative damage to biomolecules that may leads to diabetes and other neurodegenerative diseases [13, 14]. Further, a microtubule-binding protein (TAU protein) that stabilizes neuronal microtubules under normal physiological conditions may undergo modifications in certain pathological situations mainly through phosphorylation and result in generation of aberrant aggregates that are toxic to neurons. This process occurs in a number of neurological disorders collectively known as tauopathies, the most commonly recognized characteristics of Alzheimer's disease [15]. Increased tau phosphorylation has been reported in diabetic animal brains [16–20].We speculate that tau protein cleavage in diabetic conditions (especially in type-2 diabetes) may be a crucial linkage for the increased incidence of neurological disorders. Therefore, the present study was planned to evidence this possibility in type-2 diabetic rat

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https://doi.org/10.1016/j.lfs.2018.06.021
Received 10 March 2018; Received in revised form 19 June 2018; Accepted 20 June 2018
Available online 21 June 2018

model. As per previous reports TNF-α and IL-6 are the major cytokines produced by adipose tissue known to be higher levels among diabetes cases [21]. Additionally, as per earlier findings an increased level of cytokines such as IL-6 and TNF-α in diabetes leads to neurological disorders [22, 23].

Epalrestat (EPS) (5-[(1*Z*, 2*E*)-2-methyl-3-phenyl propenylidene]-4-oxo-2-thioxo-3-thiazolidine acetic acid) is an aldose reductase inhibitor (ARI) which is approved in Japan for the treatment of diabetic neuropathy and known to have minimum side effects [24]. It is known to have anti-oxidant, anti-inflammatory properties. Study by Sekar et al. [25] indicated that hyperglycemia is associated with low levels of GSH which leads to tissue damage attributed to oxidative stress in human. In other reports by Sato et al. [26] and Yama et al. [27] verified the enhanced glutathione (GSH) levels upon treatment with EPS. Since GSH is essential for prevention in oxidative stress injury. Therefore, there is a need of further research for efficacious and safer drugs to fight against cognitive impairment and also particularly to evaluate their mechanism of action on neuroprotection of neurogenesis. Hence, the purpose of the study was to assess the neuroprotective effect of epalrestat treatment in HFD-STZ induced diabetic rats.

2. Materials and methods

2.1. Animals

The adult male Wistar rats aged 8–10 weeks (150–200 g) were procured from animal house facility at J. N. Medical College, Belagavi, India. The animals were maintained under standard laboratory conditions, 12:12 h light/dark cycle throughout the experiment. Animals had free access of water and standard laboratory feed ad libitum prior to the dietary manipulation. The animal study was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and was approved by the Institutional Animal Ethics Committee (Resolution No.: 7/D; dated 18.05.2016) of J. N. Medical College, Belagavi, India.

2.2. Drugs and solutions

High Fat Diet (HFD) (VRK Nutritional Solutions, Pune, India), Epalrestat (Micro Labs Ltd., India), Streptozotocin (STZ) (Enzo Life Sciences, UK), Donepezil hydrochloride (Alkem Laboratories Ltd., India) and Glucometer and strips (Accu check: Roche Diagnostics India Pvt. Ltd. Mumbai) were used. Epalrestat was suspended in 1% gum acacia. Whereas, STZ and donepezil hydrochloride was dissolved in citrate buffer (pH 4.4) and distilled water respectively. Drug solutions/suspensions were prepared fresh. Doses of Epalrestat (54, 27 and 13.5 mg/kg, p.o.) and donepezil hydrochloride (1 mg/kg, p.o.) were selected on the basis of previous report and as per human dose [28–32].The clinically equivalent human doses of drugs were converted into rat equivalent doses by the conversion table as per Paget and Barnes [33]. All other common laboratory chemicals were of analytical grade.

2.3. Experimental induction of diabetes

Type-2 diabetes was induced in rats according to previous reports with minor modifications [34, 35]. In brief, rats were feed with a HFD for 2 weeks followed by low dose (35 mg/kg) of STZ intraperitoneal injection. STZ treated rats received 5% of glucose instead of water for 24 h after diabetic induction in order to reduce hypoglycemic shock related mortality. Blood samples were collected from the tail vein 48 h after STZ to measure glucose levels. Animals with fasting blood glucose levels above 200 mg/kg were considered diabetic rats and used for further study. The body weight and glucose blood levels were measured at onset and end of the study. The compositions of HFD were given in Table 1.

Table 1
Composition of high fat diet [HFD].

Ingredients	Diet (g/kg)
Powdered Normal Pellet diet (NPD)	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and minerals mix	60
DL-methionine	03
Yeast powder	01
Sodium chloride	01

2.4. Treatment schedule

The confirmation day of diabetes was considered as day one of diabetic condition. Total of 60 rats (10 normal; 50 HFD-STZ induced diabetic rats) were used and divided into six groups ($n = 10$) and received following treatment:

Group I: Normal control (NC) received known volume of vehicle (1 ml/kg, p.o.).
Group II: Diabetic control (DC) - vehicle only (1 ml/kg, p.o.).
Group III: Diabetic rats + Donepezil (1 mg/kg, p.o.).
Group IV: Diabetic rats + Epalrestat (57 mg/kg, p.o.).
Group V: Diabetic rats + Epalrestat (27 mg/kg, p.o.).
Group VI: Diabetic rats + Epalrestat (13.5 mg/kg, p.o.).

All the drugs were administered orally for the period of 4 weeks. Our previous findings have shown that epalrestat treatment significantly improved memory deficit in diabetic rats [36].

2.5. Biochemical assays

At the end of the treatment schedule, blood samples were collected from tail vein and rats were sacrificed by decapitation. Simultaneously pancreas as well as brain structures were removed and separated into hippocampus for histopathology and biochemical evaluation. The samples were stored at −80 °C until processed for biochemical estimations.

2.6. Evaluation of blood glucose levels and body weight

Blood glucose levels in experimental rats were measured using ACCU - Check glucose meter on lateral vein. Body weight and fasting blood glucose levels were measured before and at the completion of the experiment.

2.7. Estimation of catalase (CAT) activity

The plasma CAT activity was measured as per previously described method by Goth [37]. Briefly, 0.2 mL of samples was incubated in 1.0 mL substrate (65 μmol per ml hydrogen peroxide in 60 mmol/l sodium-potassium phosphate buffer; pH 7.0) at 37 °C for 60 s. Sample catalase activity is linear up to 100 kU/l. The samples were diluted with the phosphate buffer (2 to 10 fold) and the assay was repeated if the catalase activity exceeded 100 kU/l. The enzymatic reaction was terminated with 1.0 ml of 32.4 mmol/l ammonium molybdate and the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm on the spectrophotometer (Shimadzu Corporation, Japan) against the blank containing all the components except the enzyme. The result is expressed in kU/l, where k is the first-order rate constant.

2.8. Estimation of reduced glutathione (GSH) activity

The glutathione (GSH) content in plasma were estimated using

method of Beulter et al. [38]. The plasma was mixed with trichloroacetic acid (TCA) (10% w/v) in 1:1 ratio. The tubes were centrifuges at 1000 ×*g* for 10 min. at 4 °C. 0.5 ml of supernatant obtained was mixed with 2 ml of 0.3 M disodium hydrogen phosphate. Then 0.25 ml of 0.001 M freshly prepared 5,5-dithiobis-2-nitrobenzoic acid (DTNB) dissolved in 1% w/v citric acid and absorbance was measured spectrometrically at 412 nm. A standard curve was plotted using 10–100 μM of reduced form of GSH and expressed as nanomoles of reduced GSH of protein.

2.9. Estimation of TNF-α and IL-6 levels in rat hippocampus by RT-PCR methods

The levels of proinflammatory cytokines TNF-α and IL-6 in hippocampus tissue of control and experimental groups of rats were determined by RT-PCR. In brief, Total RNA was isolated using the TRI reagent (Sigma Aldrich), according to the manufacturer's instructions. Total RNA (2 mg) was reverse transcribed into first-strand cDNA (ABI) following the manufacturer's procedure. The synthesized cDNA (50 ng/ml) was used as a template for polymerase chain reaction (PCR) amplification. Real-time PCR was performed using step one Real-time PCR system (ABI). PCR was carried out for 45 cycles using the following conditions: denaturation at 95 °C for 45 s, annealing at 62.7 °C for 30 s, and elongation at 72 °C for 15 s. The relative expression levels of the target genes were calculated as a ratio to the housekeeping gene GAPDH. All the samples were in triplicate and each time no template control was done during plate run. Melting curve analysis was performed to assess the specificity of the amplified PCR products. A dissociation curve analysis of all primers showed a single peak. The SYBR Green primers were used for Real-time PCR studies. All relative quantification analysis was represented in the form of relative expression to the normal group (delta delta Ct) [39].

2.10. Estimation of TAU proteins by ELISA method

Rat TAU proteins in brain hippocampus tissue were determined using ELISA kit (Genxbio Health Sciences Pvt. Ltd. Greater Noida, India. Catalog no: GXBR191213. Hippocampus tissues were dissected and homogenized in PBS (pH 7.4). Homogenates was centrifuged at 3000 rpm for 20 min at 4 °C. The clear extracts obtained were taken for quantitative measurement of TAU proteins by sandwich ELISA method as per manufacturer's instructions. The sensitivity of the assay was 0.52 ng/l and the detection range was 1–400 ng/l.

2.11. Histopathology of brain and pancreas

Pancreatic tissue samples were fixed in 10% neutral buffered formalin and then dehydrated by successively passing through a gradient of mixtures of ethyl alcohol and water. The samples were rinsed by xylene and embedded in paraffin. Pancreatic sections (5 μm thickness) were cut, stained with hematoxylin and eosin dye (H and E), and examined under light microscopy while brain samples were preserved in 10% formalin for histopathological examination [40, 41].

2.12. Statistical analysis

All the results were expressed as Mean ± Standard Error of Mean (SEM). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison tests using GraphPad Prism 5.00 Software [San Diego, USA]. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Effects of epalrestat treatment on blood glucose and body weight in HFD-STZ induced cognitive impairment in rat

In the present study, body weight and blood glucose levels were determined at the onset and at the end of the experiment. As observed from our own previous report [36] the blood glucose levels at the onset of the study had no significant differences among the groups. On the other hand, the blood glucose levels for the diabetic control rats were significantly increased as compared to the normal control rats at the end of the experiment. In relation to body weight, no significant differences among the groups were observed at the onset of the experiment. However, the diabetic control rats were observed with significant reduced body weight as compared to normal control rats at the end of the experiment. Whereas, epalrestat and donepezil treatment leads to no significant changes in blood glucose levels as well as in body weight when compared with diabetic control rats (data not shown).

3.2. Effects of epalrestat treatment on CAT and GSH activity in HFD-STZ induced cognitive impairment in rat plasma

As shown in Fig. 1 and Fig. 2 CAT and GSH activity, which is significantly increased in normal control rats ($p < 0.001$) as compared to diabetic control rats and also these levels were significantly increased in diabetic rats treated with epalrestat 54, 27 mg/kg ($p < 0.001$, $p < 0.01$) and donepezil 1 mg/kg ($p < 0.001$). However, no significant change was observed in diabetic rats treated with lower dose of epalrestat (13.5 mg/kg).

3.3. Effects of epalrestat treatment on gene expression level: IL-6 and TNF-α in HFD-STZ induced cognitive impairment in rat hippocampus

As shown in (Fig. 3 and Fig. 4) IL-6 and TNF-α levels in hippocampus tissue was significantly increased ($p < 0.001$) in the diabetic control rats as compared to the normal control rats while these levels were significantly reduced in diabetic rats treated with epalrestat 54, 27 mg/kg ($p < 0.001$, $p < 0.01$ and $p < 0.05$) and donepezil 1 mg/kg ($p < 0.05$) treatment.

3.4. Effect of epalrestat treatments on analysis of total rat TAU proteins

In the present study we observed the expression of total rat TAU protein level in hippocampus tissues of rats as showed in Fig. 5. TAU protein levels were significantly increased ($p < 0.001$) in diabetic control rats as compared to treatment groups. The epalrestat 54 and 27 mg/kg treated diabetic rats were observed with significant decreased levels of TAU protein ($p < 0.001$ and $p < 0.05$) respectively. Whereas, no significant change was observed in low dose of epalrestat 13.5 mg/kg and donepezil 1 mg/kg treated diabetic control rats.

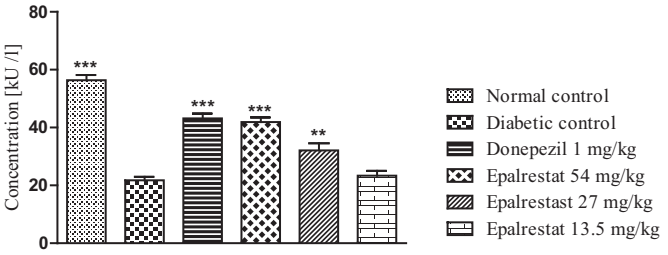

Fig. 1. Effect of epalrestat treatment on catalase (CAT) activity in high fat diet-streptozotocin induced cognitive impairment. Data expressed in mean ± SEM ($n = 10$), statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at $^{**}p < 0.01$, $^{***}p < 0.001$ comparison with diabetic control rats.

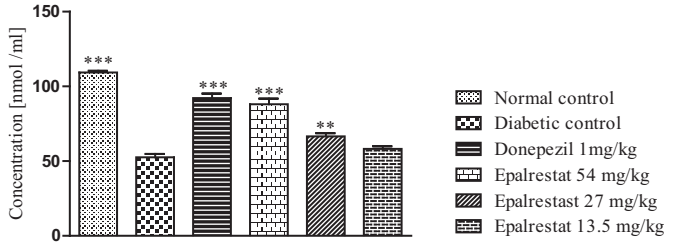

Fig. 2. Effect of epalrestat treatment on glutathione (GSH) activity in high fat diet- streptozotocin induced cognitive impairment. Data expressed in mean ± SEM ($n = 10$), statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at $^{**}p < 0.01$, $^{***}p < 0.001$ comparison with diabetic control rats.

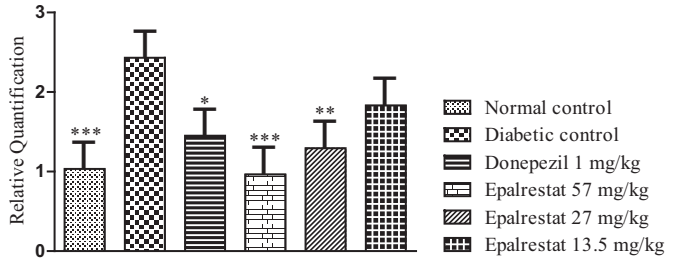

Fig. 3. Effect of epalrestat treatment on interleukin-6 (IL-6) activity in high fat diet- streptozotocin induced cognitive impairment. Data expressed in mean ± SEM ($n = 10$), statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at.$^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ comparison with diabetic control rats.

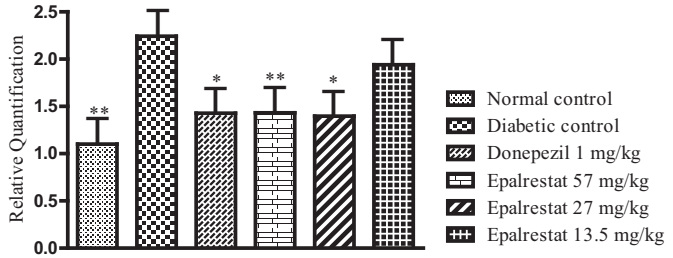

Fig. 4. Effect of epalrestat treatment on tumor necrosis factor-α (TNF-α) in high fat diet- streptozotocin induced cognitive impairment. Data expressed in mean ± SEM, statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at $^{*}p<0.05$, $^{**}p < 0.01$ comparison with diabetic control rats.

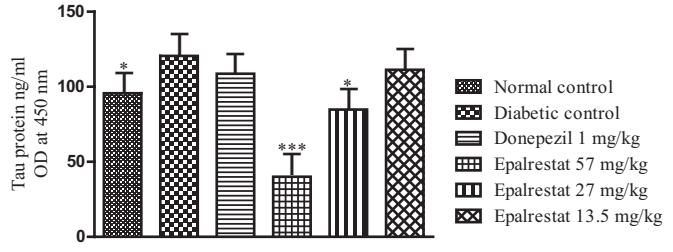

Fig. 5. Effect of epalrestat treatments on analysis of TAU proteins levels in high fat diet- streptozotocin induced cognitive impairment. Data expressed in mean ± SEM (n = 10), statistical analysis by one-way ANOVA followed by Dunnett's test. Significance at $^{*}p<0.05$, $^{***}p < 0.001$ comparison with diabetic control rats.

3.5. Histopathological examination of the pancreas

Tissue sections staining (H and E) observed no distinguished changes in pancreatic histology in normal control rats (Fig. 6A). In contrast, diabetic control rats were observed with injury of the pancreas, decreased number of islets cells and reduced diameter of the pancreatic islets (Fig. 6B). Additionally, shrunken islets were also observed in diabetic control rats as compared to normal control rats. Likewise, administration of donepezil 1 mg/kg, epalrestat (54, 27, 13.5 mg/kg) revealed similar effects as observed in diabetic control rats (Fig. 6C, D, E, F).

3.6. Histopathological examination of the hippocampus

Rats in normal control group are showing Cornus ammonis (CA), which is a structure of the hippocampus in the inferomedial part of the temporal lobe (Fig. 7A). The main neuron layer has been traditionally divided into four layers: CA1–CA4. Results of the present study in diabetic control rats (Fig. 7B) shows disorganization of pyramidal layer (PL) marked darkened nuclei with Vacuolation (v) and clumping (c) of processes which is mainly seen in small pyramidal layer (PL). Apoptotic cells (A) are seen in pyramidal layer (PL).The granular layer (GL) shows some cell loss with increase glial cells (G). Enlargement of neurons (N), glial cells (G) and few apoptotic cells (A) were seen in molecular layer (ML). Whereas, diabetic rats treated with donepezil 1 mg/kg (Fig. 5C) and Epalrestat 54 and 27 mg/kg (Fig. 7D, E) shows normal size of glial cells (G) and neurons in the Molecular layer (ML). The pyramidal layer (PL) is of normal thickness with normal size pyramidal cells (P). There is decrease clumping (C) of processes and Vacuolation (V) in the small pyramidal layer (PL). In the Granular layer (GL) the Glial cells (G) are of normal size with less number of Vacuolation (V). Histopathologically regenerating activity is seen in donepezil and epalrestat treated rats whereas, no changes were observed in diabetic control rats and diabetic rats treated with low dose of epalrestat 13.5 mg/kg (Fig.7F).

4. Discussion

The present study evaluated the neuroprotective effect of epalrestat on HFD - low dose of STZ induced cognitive impairment in male Wistar rats. The study finding recommends that type-2 diabetic rats exhibited marked impairment in memory that could be associated with increased IL-6 and TNF-α and TAU proteins levels. In addition, decreased CAT and GSH levels in diabetic rats and histopathological changes in pancreas as well as brain tissue (hippocampus).

EPS treatment which is a, aldose reductase inhibitor counteracts the physical, behavioral and biochemical changes in diabetes conditions. In the present study, HFD and single low dose of STZ was used for the induction of type-2 diabetes. Learning and memory ability of experimental rats were evaluated using different behavioral models to understand the effect of Epalrestat treatment on cognitive deficits in high fat diet-streptozotocin induced type-2 diabetes [36]. Earlier studies also reported that HFD and low dose of STZ were used for the induction of type-2 diabetes [35, 42, 43]. The present study demonstrated that decreased body weights were observed in untreated diabetic rat comparison with normal control rats. These observations are similar findings of earlier studies, which showed that STZ induced diabetic rats decreased body weight [35, 44–46].

Recent studies indicated that treatment with epalrestat had no effect on blood glucose levels whereas, improvement of memory and learning were observed in diabetic rats [36]. These findings suggest that antioxidant, anti-inflammatory property of epalrestat may be responsible for improvement of memory impairment. However, results of the present study also showed that, there is no significant change were observed in blood glucose levels as well as body weight in diabetic rats treated with epalrestat and donepezil treatment comparison with diabetic control rats. In addition, epalrestat which are reported to possess

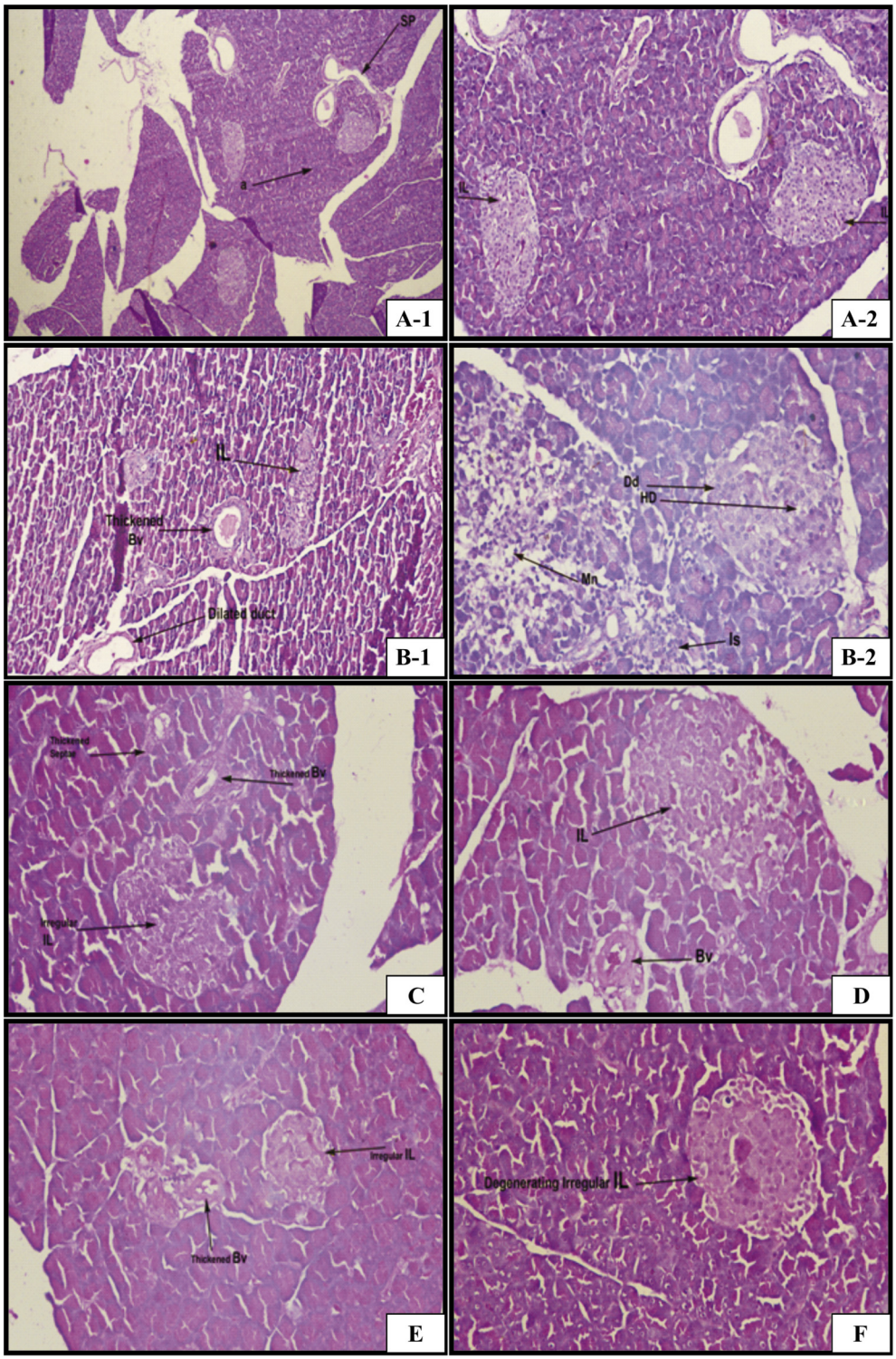

Fig. 6. Effect of epalrestat treatment on histoarchitecture of pancreas on high fat diet – streptozotocin induced cognitive impairment in rats: (Fig.6A) Normal control shows normal islets of Langerhans (IL), delicate septa (SP), Acini (a). (Fig.6B) Diabetic control shows shrinkage both in size and number of islets of Langerhans (IL), thickened blood vessels (Bv), dilated duct, hydropic degeneration and degranulation (Dd) of islets cells, mononuclear (Mn), interstitial stroma (Is). Histopathology findings of (Fig.6C) donepezil 1 mg/kg, (Fig.6D) Epalrestat-54 mg/kg, (Fig.6E) Epalrestat-27 mg/kg, (Fig.6F) Epalrestat-13.5 mg/kg treatment showed similar effects when compared to the diabetic control group (magnification A-1, B-1: 100× and A-2, B-2, C, D, E, F: 400×).

anti-inflammatory and antioxidant property. Moreover, upregulation of heme oxygenase (HO)-1 by EPS may prevent the development and progression of disorders caused by oxidative stress [26, 27, 47].

Oxidative stress is one of the most common pathogenic factors leading to insulin resistance and β-cell dysfunction in type-2 diabetes [48]. Increased oxidative damage was observed in the brains of experimentally induced hyperglycemic rats [49]. Oxidative stress provokes inflammation and subsequently increases inflammatory mediators such as cytokines and interleukins, which is attributing to biochemical disorders. On the other hand, inflammation is recognized as an important pathophysiological finding in type-2 diabetes that may have a role in the susceptibility of type-2 diabetes patients to neurological disorders and in the progression of type-2 diabetes in Alzheimer's disease [50, 51]. GSH, the most important antioxidant, plays an important role in maintaining the cellular redox state [52]. Treatment of Schwann cells with EPS caused a dramatic increase in intracellular GSH levels, which may prevent the development and progression of disorders caused by oxidative stress [26].

The present study indicated the significant increased levels of pro-inflammatory cytokines, TNF-α and IL-6 as well as decreased levels of

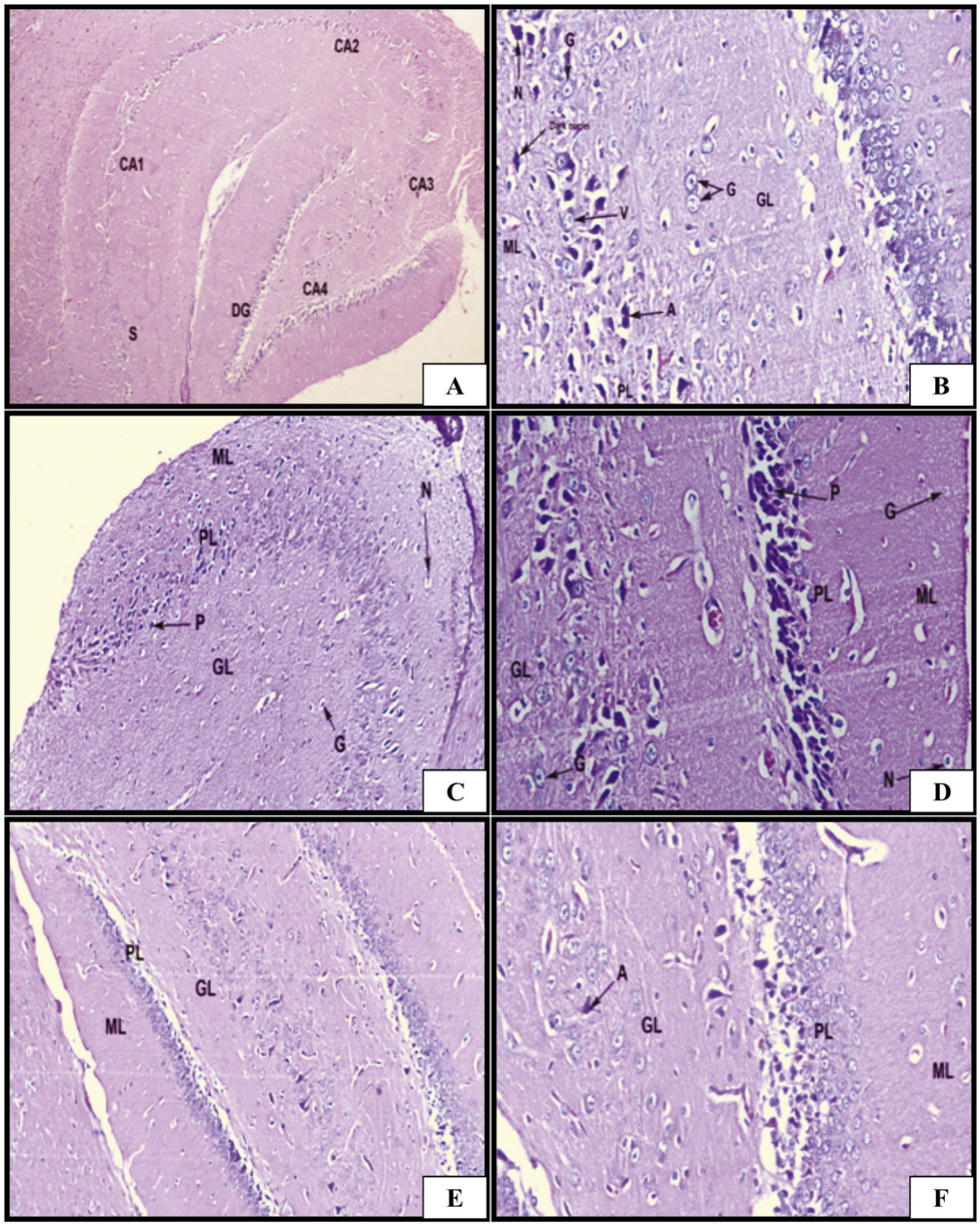

Fig. 7. Effect of Epalrestat treatment on rat hippocampus of high fat diet – streptozotocin induced cognitive impairment in rats: Normal control (Fig. 7A) shows the Cornus ammonis CA. (Fig. 7B) diabetic control shows pyramidal layer (PL) marked darkened nuclei with Vacuolation (v). Apoptotic cells (A), granular layer (GL), glial cells (G). Molecular layer (ML) shows marked enlargement of the neurons (N) and glial cells (G) with few apoptotic cells (A). (Fig. 7C, D, E and F) shows diabetic Control group treated with donepezil and epalrestat (54 and 27 mg/kg) treatment shows normal size glial cells (G) and neurons in the molecular layer (ML). The pyramidal layer (PL) is of normal thickness with normal size pyramidal cells (P) there is decrease in Vacuolation (V) in small pyramidal layer (PL), Granular layer (GL) the Glial cells (G) are of normal size with less number of Vacuolation (V). (Magnification: Fig. 7A - 40× and B, C, D, E, F 400×).

oxidative stress markers (CAT and GSH) in the untreated diabetic rats. Whereas, epalrestat and donepezil treatment rescued this effect by lowering TNF-α and IL-6 to control levels and enhancing levels of CAT and GSH. Microscopic observation of the pancreatic β-cells in normal control rats was typical whereas untreated diabetic rats showed injury of the pancreas, decreased number of islets cells and diameter of the pancreatic islets and also islets were shrunken in diabetic control rats as compared to the normal control rats.

These results agree with findings of Alese et al. [53]. Whereas, diabetic rat treated with donepezil and EPS treatment shows no significant changes and histopathology findings are similar to the diabetic control group. Hippocampus is mainly responsible for the collection, consolidation and retrieval of various aspects of learning, memory and spatial navigation. Earlier findings of diabetic rats hippocampus revealed the presence of cell loss of large pyramidal layer, Vacuolation and disorganization of all the layer, many apoptotic large cells [54, 55]. As seen in our study there is markedly improved by donepezil (1 mg/kg) and epalrestat (54 and 27 mg/kg) therapy and no much difference were observed in low dose of epalrestat (13.5 mg/kg).

Neurological disorders associated with accumulation of two major proteins i.e. TAU and Amyloid-beta plaques both are considered to be toxic to neurons [56]. Rhodanine ring structure containing compounds like epalrestat has the potential for enhanced clearance of TAU protein, one of the chief pathological features of Alzheimer's disease. Rhodanine ring structure containing compounds were tested for its tau aggregation inhibition activity and results indicated that rhodanine compounds are more effective on inhibitors of tau aggregation among the compounds tested. It was further tested on neuronal cell models of tau aggregation and revealed appreciable findings [56, 57]. This is in direct support of this study hypothesized that rhodanine ring structure containing compound like epalrestat has the potential for enhanced clearance of tau protein, one of the chief pathological features of Alzheimer's disease.

We have previously demonstrated that epalrestat treatment produced significant improvement in learning and memory deficit in diabetic rats (36). However, the mechanism by which epalrestat produced a significant improvement in functional recovery is not known. Therefore, the present study was carried out to elucidate the mechanism of neuroprotection by which epalrestat improves learning and memory deficit.

In conclusion, our study findings suggest that epalrestat and

donepezil by way of their antioxidant and anti-inflammatory potential resulted in decreased level of TAU protein and histopathological changes in hippocampus thereby conferred protection against HFD-STZ induced cognitive impairment in rats. Further, study suggests that both epalrestat and donepezil are effective in preventing diabetic complications in neurodegenerative disease. Thus, epalrestat may be considered as potential candidate in the management of neurodegenerative disease in diabetic rat, pending further investigations to trace out the exact mechanistic pathways.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgment

We would like to thank Department of Pharmacology, J. N. Medical College - KAHER, and BSRC - KAHER, Belagavi for providing the research facility to carry out the research work. We thank Dr. Ramesh Chavan - Pathology Department, J. N. Medical College; Dr. Gaurav; Dr. Avinash; Dr. Somaling and Ms. Damita for their technical assistance for histopathological and biochemical evaluation.

HFD: High-fat diet, NPD: Normal pellet diet.

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"TO STUDY THE EFFECT OF EPALRESTAT, AN ALDOSE REDUCTASE INHIBITOR ON MEMORY AND LEARNING IN DIABETIC MALE WISTAR RATS"

An Errata submitted to

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

Accredited **'A' Grade by NAAC (2nd Cycle)** Placed in Category 'A' by MHRD (GoI)

[Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

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In the Faculty of Medicine

(Pharmacology)

By

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November 2019

Errata submission of the PhD thesis:

Observations	Clarifications / Corrected as	Corrected thesis copy P. N.
1. Abbreviations should be in a alphabetical order	As per kind suggestion, the Abbreviations have been corrected in an alphabetical order and incorporated in thesis.	List of Abbreviations Page Nos: XIV to XVIII
2. Methodology section is week. Processing of animal samples and detailed procedures are missing in many sections. It should also include the flow chart of study design and treatment schedule.	To avoid the plagiarism percentage [self publications and others] in methodology section, the detailed processing of animal samples and detailed procedures were avoided as it has been already published in publications on this thesis as well as previously reported in literature. Although respective references were cited at appropriate places. Further, sample processing and stepwise parameter procedures for individual parameters are mentioned in their respective sections for clarity. As per suggestion and more clarity, type of samples undertaken for individual parameters are now mentioned in study parameter section and incorporated in the thesis. **Corrected as:** - Estimation of oxidative stress markers [CAT and GSH] in plasma samples. - Analysis of inflammatory cytokines [TNFα & IL-6] in hippocampus tissues Flow Chart of study design: the details of animals, drugs and solutions, experimental diabetes induction with HFD and STZ, treatment schedule and study parameters are written under specific headings and the flow chart / graphical abstract of the performed study [Behavioral parameters and Biochemical investigations] have been already given in the summary section of the thesis i. e. Chapter 6: Summary, Page No. 78 & 79. In addition, the link between test drug and disease is shown in introduction chapter on page no. 33. Hence repetition was avoided in thesis.	Page No. 46, study parameters, Biochemical estimations, Point 1 and 2

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3. Objective 2 of the thesis to estimate the tau protein levels has not been achieved correctly. why the levels of these marker checked instead of gene expression?	Objective 2 of the thesis for tau protein estimation was performed and mentioned. It was not clear due to missing of page No. 53 of the thesis having details of the estimation process. We apologise for the mistake and thank to the reviewer. The results of the protein level are mentioned at page no. 67. Further, in addition to gene expression analysis we also performed tau protein estimation as neurological disorders are associated with accumulation of two major proteins i.e. TAU and Amyloid-beta plaques and both are considered to be toxic to neurons. Tau proteins accumulate with tubulin to stabilize microtubule (MT). Neurofibrillary tangles (NFTs) are hyperphosphorylated and cumulative form of tau proteins. Once it is hyperphosphorylated, tau becomes insoluble and lacks affinity for MTs, leading to neurodegeneration. Epalrestat has the potential for enhanced clearance of TAU protein, one of the chief pathological features of Alzheimer's disease. This is in direct support of this study hypothesized that rhodanine ring structure containing compound like epalrestat has the potential for enhanced clearance of tau protein.	Page No. 53 is incorporated in the thesis as was missing.
4. Objective 3 has not been achieved	Objective 3 for estimation of gene expression levels is achieved and details are mentioned on page no. 52 and 53 of the thesis. Due to missing page no. 53 it was not understanble. We apologise for such mistake and page no. 53 has been incorporated. The IL-6 and TNF-α levels are shown in Fig. 18 and Fig. 19 at page no. 65 and 66 respectively.	Page No. 53 is incorporated in the thesis as was missing.

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5. Page 52, method for the evaluation of cytokine levels by RT-PCR is incorrect.	As mentioned in above points, due to missing of Page No. 53 it was not clear. The said page is incorporated in thesis having method details by RT-PCR.	Page No. 53 is incorporated in the thesis as was missing.
6. Page 53 is missing in the thesis. Care should be taken while compiling the thesis.	We apologise for missing Page No 53 in the thesis. As per kind suggestion, correction has been made.	Page No: 53 is incorporated in thesis as was missing.
7. Page 54 partly ELISA protocol but not for RT- PCR method.	As mentioned above, we are apologizing for missing Page No 53. The said page has been incorporated having details of ELISA protocol.	Page No: 53 have been incorporated.
8. Details regarding primer sequence selected for PCR and kit details for ELISA used for the study need to be highlighted. Melting curve analysis for the makers used in PCR need to be shown. Purity of the RNA isolated also needs to be confirmed by running a blot. These should be included in the revised thesis	The details having primer sequence selected for RT PCR and ELISA kit details are given on Page No. 53 and this page was missing. Now it is incorporated in thesis. Meting curve analysis was performed to assess the specificity of the amplified PCR products and the quality of RNA isolation was analysed using Nanodrop i.e. 260/280 ratio [more than 1.8]. Article published in Life sciences, 2018	Material and Methods, Page No: 53 have been incorporated.

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9. [4.1] section is for behavioral parameter not the physical parameters	Correction has been made as Behavioral parameters	- Table of Contents, Page No: XX - Results, Page No. 57
10. Coding and sequence of animal grouping is not consistent throughout the thesis.	We apologise for the mistake. As per kind suggestion, we have corrected the coding and sequence of animal grouping in result section for uniformity with material and methods chapter.	Results, Page No: 63 - 67
11. Why Antioxidant enzyme levels were estimated in plasma, instead of different part of the brain, as that would have been more relevant to correlate oxidative stress with memory loss.	As per reported studies, the antioxidant levels in such studies are performed by both the methods. In this study, we estimated in plasma samples [page no. 46, 63, 64] and results are discussed at Page No: 74.	-
12. The DC group should have been compared with NC instead of comparing all the groups with DC groups.	We have compared DC rats with NC as well as with DC rats having treatments with EPS / Donepezil and significance has been shown in all related figures and tables.	-

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13. What is the rationale behind performing one way ANOVA for the analysis of the data?	Since animal groups were more than two i.e. 6 groups. Therefore as per data analysis recommendation and previous literature one way ANOVA for the analysis of the data was performed. Details are given in Chapter 3: Statistical Analysis, Page No: 314	-
14. Why the blood glucose levels were checked only at the end of the study? Establishing the diabetes condition in animals before starting the memory related experiments is required to certain that memory impairment is due to diabetes.	We have checked blood glucose levels at onset of the animal study and end of the study as well. The results are mentioned in Table No. 11, Page No. 62, Biochemical estimations.	-

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15. Quantum of the work is insufficient, the more detailed mechanistic study should have been planned.	The submitted study in thesis was performed as per approved research topic "Effect of Epalrestat, An aldose Reductase Inhibitor on Memory and Learning in Diabetic Male Wistar Rats. The in-vivo study was performed into two major sections: **A) Behavioral studies:** includes passive avoidance test to examine long-term memory test, morris water maize test for hippocampal function assessment, and elevated plus maize (EPM) - to evaluate rodent anxiety- related behaviors. **B) Cognitive impairment study induced by HFD and STZ** After behavioural tests, treatment was continued and blood samples were collected and all animals were sacrificed for brain structure removal towards separation of hippocampus for biochemical estimations and histopathological examination. Following biochemical estimations were carried out - • Estimation of oxidative stress parameters [Catalase, Glutathione] in plasma samples • Estimation of inflammatory mediators using RT-PCR method [Tumor Necrosis Factor - α (TNFα), Interleukin – 6 (IL-6)] in hippocampus tissues • Estimation of tau protein by ELISA in Hippocampus tissues Histopathological examinations: to observe the changes. The study was published in two international journal – - Life Sciences, 2018 [IF 3.448] - Asian Journal of Pharmaceutical and Clinical Research, 2018 [indexed in Index Copernicus, Google scholar]	

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