INVESTIGATION OF THE NEUROMODULATOR POTENTIAL OF QUERCETIN DURING MEMORY DYSFUNCTION ASSOCIATED WITH DIABETES AND ALZHEIMER'S

Project report submitted in partial fulfillment of the requirement for the degree of Master of Technology

In

BIOTECHNOLOGY

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CERTIFICATE

This is to certify that the work titled **"Investigation of the Neuromodulator Potential of Quercetin during Memory Dysfunction Associated with Diabetes and Alzheimer's"** submitted by "RITIKA MAHAJAN" in partial fulfillment for the award of M. Tech (Biotechnology) of Jaypee University of Information Technology; Waknaghat has been carried out under my supervision. This work has not been submitted, partially or wholly, to any other University or institute for the award for this or any other degree or diploma.

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ABSTRACT

Diabetes mellitus is chronic metabolic disorder which is becoming epidemic globally. Impediments of diabetes are not only related to CNS but also cause neuropathology which may play important role in study of Alzheimer's disease. Several medications are there for the cure of diabetes but none of them is capable of halting, preventing or reversing neurological complications. Many experiments were performed to show link between diabetes and Alzheimer's. Effect of several flavonoids for the treatment of diabetes induced Alzheimer's were also observed and it was found that quercetin showed much effective activity and can cure Alzheimer's induced by diabetes. Present study was aimed to screen out natural molecules with potential to modulate neurological dysfunction related with type 2 diabetes mellitus and Alzheimer's disease through *in-vitro* and *in-vivo* experimentation and consisted of following broad objectives such as evaluate the effect of quercetin and rosiglitazone on type-2 diabetes, evaluate the effect of diabetes and drug treatment on hippocampal neuronal morphology using Golgi cox staining, screen herbal molecules for their potential to modulate Alzheimer's disease through *in-vitro* screening, To standardize intra-cerebral-hippocampal (ICH) injection in male Wistar rats, induce Alzheimer's disease in Wistar rats by injecting beta-amyloid (1-42) through ICH injection and evaluate the effect of amyloid-beta injection and quercetin treatment on memory dysfunction in the Alzheimer's model in male Wistar rats

Chapter 1

Introduction

1. INTRODUCTION

1.1 DIABETES

Diabetes Mellitus is a long-lasting metabolic disorder which is identified by consistently increase glucose levels in the blood, which can have most devastating effects on various organs of the body, amongst which brain is severely damaged. As per the research it has been found that there are two types of diabetes named Type 1 diabetes and Type 2 diabetes. The major cause of Type 1 diabetes is damage of beta cells which are found in pancreas. It is most common in childhood and adolescence. In this case insulin secretion gradually diminishes and person requires insulin injections for survival. The major contributing factors are genetic predisposition and environmental triggers. Type 2 diabetes mellitus, also known as non-insulin dependent diabetes mellitus (NIDDM), is triggered by increase glucose production in liver, Insulin resistance in the liver and muscles of skeleton, relative insulin deficiency and fat cells leads to over production of free fatty acids. Diabetes mellitus is described as group of chronic disorders, which is characterized by hyperglycemia and insulin resistance. The major contributing factors for diabetes mellitus are obesity, age, lack of physical activity, genetic predisposition etc. The major risk factors of Type 2 diabetes are unhealthy diet, increasing age, high blood pressure, overweight, physical inactivity, impaired glucose tolerance (IGT), ethnicity, poor nutrition during pregnancy, history of gestational diabetes (Fig 1). Symptoms of diabetes may vary from person to person. The major signs include frequent weight loss, excessive thirst, urination, increased hunger, tiredness, unclear vision, deficiency of interest, slow-healing wounds, burning sensation or deadness in the hands or feet, stomach pain and vomiting, frequent infections (Fig 2).

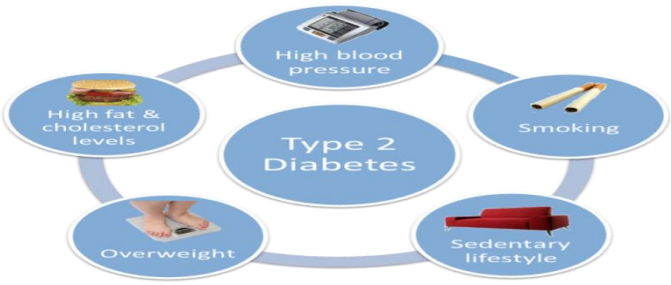


Fig 1: Threat factors for the progress of Diabetes Mellitus

Fig 2: Signs and symptoms Diabetes

According to many epidemiological studies it was found that 90% diabetes patients are affected by Type 2 diabetes. It was observed, patients suffering by type 2diabetes have greater chances of getting Alzheimer's disease, which is described as degeneration of neurons. The main organs of retention and emotions, hippocampus and amygdala were found to be shrunken in diabetes mellitus, which was very similar to the atrophy in AD [den Heijer et. al., 2003].

1.2 EPIDEMOLOGY

It was estimated that 415 million individuals had diabetes in the world in 2015 and the occurrence is increasing contineously, especially in lower belt countries. It was observed that India was found with 69.2 million people suffering with diabetes (8.7%) issued in 2015, and is projected to go up to 642 million in 2045 (Fig 3). It was found that the cost of the diabetes management is more than $645 billion a year globally, which includes

direct medical costs and the cost of reduced productivity. Diabetes is a elongated disorder that occurs more over when the pancreas are not able to produce enough insulin or when the body is not so efficient to produce insulin. Insulin is a peptide hormone that controls blood sugar which is responsible to give us energy that we need to survive. If the insulin does not enter the cells it may lead to hyperglcemia which may be harmful for person .

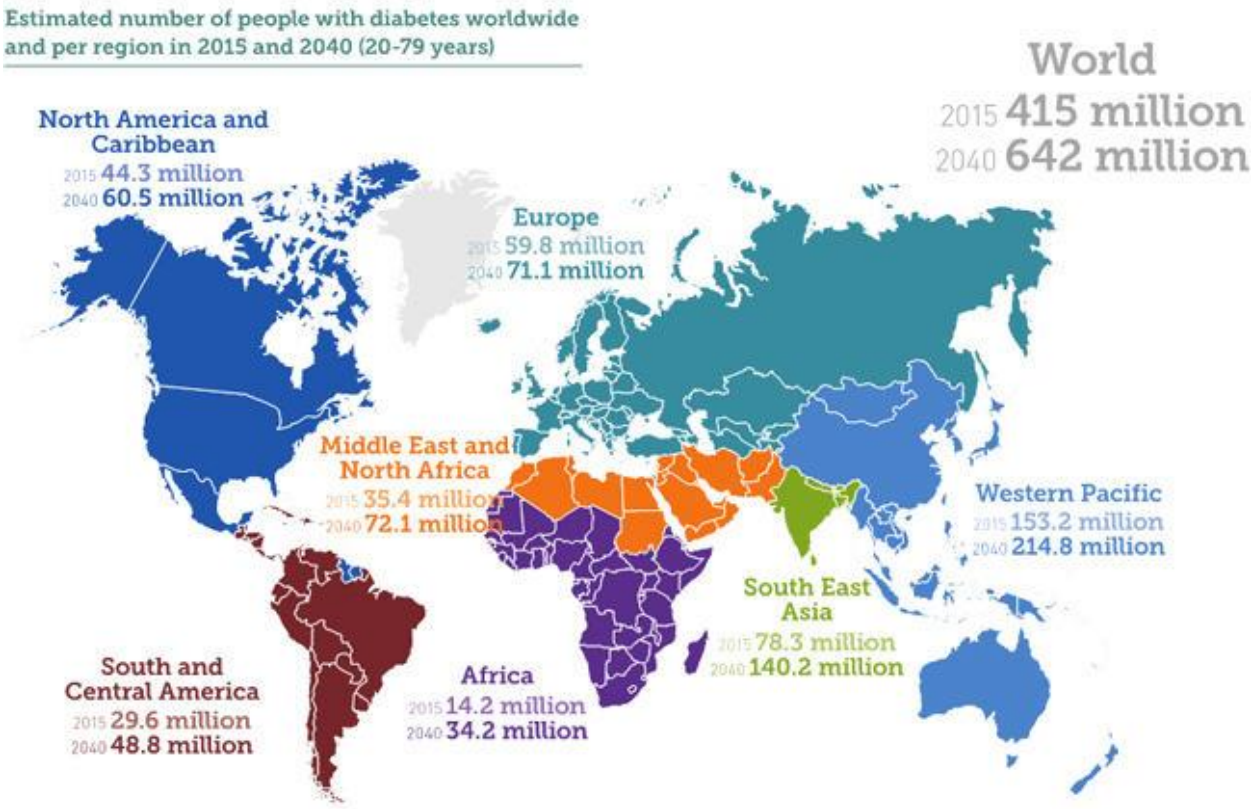


Fig 3: Global prevalence of diabetes and expected increase in near future

1.3 ALZHEIMER'S DISEASE

Among most common forms of mental disorder is Alzheimer's disease which is observed in elderly people. AD is characterized by impaired speech, poor co-ordination and diminished executive functions. The major symbols of AD are amyloid beta plaques, neurofibrillary tangles containing of hyperphosphorylated and aggregated tau protein, inflammation of neurons and degeneration of neurons. It is most common cause of age

related dementia as it accounts for as much as 50-60% of dementia cases especially in women [Basil et. al., 2012;WHO, 2004]

Production and deposition of beta amyloid peptide are the major factors responsible for Alzheimer's disease pathogenesis. Amyloid plaques are detected in the places among the nerve cells of the brain. They are made of largely indecipherable deposits of an superficially toxic peptide of protein, known as Amyloid- beta. This amyloid beta is said to be of 4KDa peptide, derived by the defective cleavage of APP. It is produced by the by consecutive proteolytic cleavage at the β and γ sites of amyloid precursor protein by β secretase and γ secretase respectively [Murphy et. al., 2010]

Neurofibrillary tangles, which is second major hallmark of Alzheimer's is said to be an irregular collection of abnormal protein threads originate inside nerve cells. These abnormal threads are called as tangles which are made up of tau protein. Microtubules support the healthy neurons which aid to transport neurotransmitter from the cell body down the axon. Tau contains phosphate molecules which attaches to microtubules for stabilization of it. But in AD, an abnormally high amount of additional phosphate molecules attach to tau. This results in hyperphosphorylation of tau protein, which may result towards tau disengagement by the microtubules and leads to formation of threads composed of tau protein. These thread like structures called as combined helical filaments, which get tangled with each other, results in twists inside the cell. These microtubules differentiate results in breaking the neuron's internal transport network. Due to this breakdown the ability and capacity of neurons to communicate with each other get damaged.

Worldwide prevalence of dementia, including Alzheimer's disease (Fig. 4), is projected at 24.3 million people and new cases occurring yearly with 4.6 million [Ferri CP et al. 2015]. According to the report of WHO out of 1.2 billion people whose age is about 60years estimated to be suffering from Alzheimer's and will exist till 2025 [World Health Organization, 2002.]. People with dementia becomes double every twenty years from 42.3 million in 2020 and 81.1 million in 2040. Highest rate (around 336%) of progress will be observed in India, China, South Asia, and western Pacific regions. About 24.3

million have dementia and 4.6 million incident or new cases are added yearly on the basis of 2001 global population. As per global scenario of Alzheimer's study conducted by World health organization and World Bank, dementia adds 4.1% of all disability-attuned life years (DALYs) (Fig 5) [WHO, 2004].

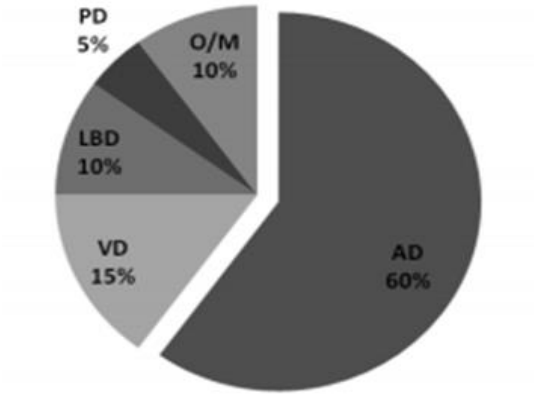


Fig.4: Approximate incidence of Alzheimer's disease in relation to different forms of dementia

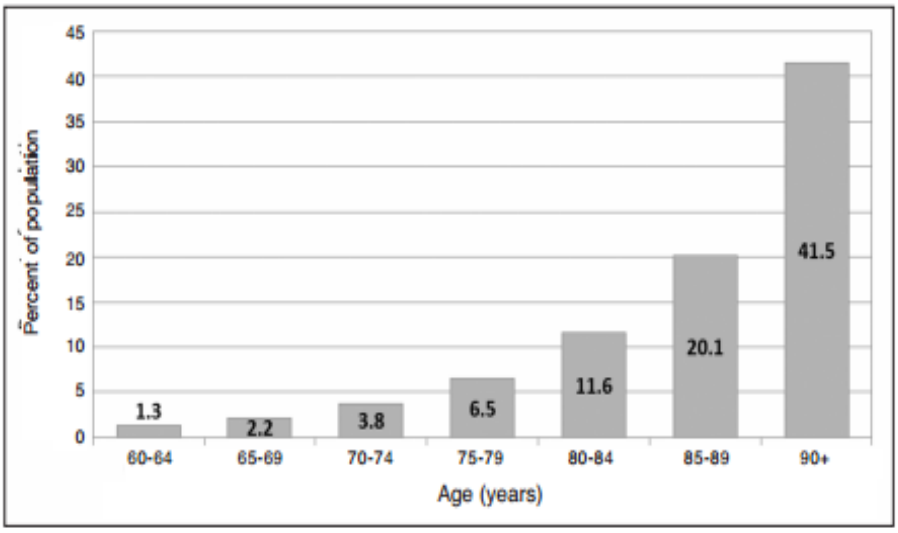


Fig. 5 Worldwide prevalence of Alzheimer's disease by age

The main threat features of Alzheimer's disease are:

- **Age.** Risk for Alzheimer's rises with the age. In most cases, it jerks growing up after the age of 65.
- **Gender.** It has been seen that women are more prone to get the disease as compared to men.
- **Family history.** It may be hereditary or genetic disorder.
- **Down syndrome.** The most common cause of Alzheimer's is chromosomal aberration on chromosome 21.
- **Other factors**. Increase in cholesterol levels and blood pressure may raise risk of AD.

1.4 EPIDEMOLOGY

Prevalence rates (PRs) of Alzheimer's have been studied from different regions of India and have been found to vary quite widely [Rajkumar et. al., 1997]. The possible reasons for this can be adoption of diverse methodology, multiethnicity, crucial criteria, and screening instruments, environmental and assorted factors. The occurrence of dementia vary from 3.39 to 0.84% in rural areas of North and South India, respectively [Chandra et. al., 1998]. Infrequent urban studies from various regions of India screening alike varying rates: such as 0.8-1.28% in East India [Banerjee et. al., 2008], 1.83% in North India [Raina et, al., 2010], 2.44 to 4.1% in West India [Vas et. al., 2001], and 3.6% in South India [Shaji et. al., 2005]. The differences may be accurate determining the multicultural, multiethnic, and environmental variances (Table 1).

1.5 AMYLOID BETA METABOLISM, CATABOLISM AND CLEARANCE

Amyloid precursor protein (APP) which is the initial point for beta amyloid plaques is actually a protein associated with cell membrane that acts as a barrier and encloses the cell. Specific enzymes cleave Amyloid precursor protein into distinct fragments in cell compartments, including the outermost cell membrane. Enzymes are called ά- secretase, β-secretase, and γ-secretase. This clearly depends on the enzyme which is involved and its site of cleaving

Table 1: Prevalence rates of dementia from different regions of India

Region	Study	Age/gender	Number of subjects	Prevalence rates (%)		Instruments used	Remarks
				All dementia	AD		
South India	Shaji *et al.*, 1996[6]	≥60	2067	3.39	1.31	Screening: MMSE and CAMDEX	Rural South Indian population in Kerala
		Male	965	2.8	0.73		
		Female	1102	3.54	1.81	Confirmation: Clinical and DSM IV	
	Rajkumar *et al.*, 1997[7]	≥60	750	3.5		Geriatric Mental State Examination	Rural South Indian population in Madras
	Shaji *et al.*, 2004[8]	>65		3.36			Urban South Indian population
North India	Chandra *et al.*, 1998[9]	≥55	5126	0.84		Screening: Hindi MMSE	Rural North Indian population
		≥65		1.36		Confirmation: CDR and DSM IV	
		Male		1.8	0.77		
		Female		1.25	0.46		
	Raina *et al.*, 2010[10]	>60	1856	1.83		MMSE and EASI	Migrated population in Jammu region of J and K
West India	Vas *et al.*, 2001[11]	>40	24,488	0.43	0.25	Sandoz clinical assessment geriatric scale and MMSE for screening. CDR and DSM IV for diagnosis	Urban western Indian population in Mumbai
		≥65		2.44	1.5		
		Male	11,875		0.2		
		Female	12,613		0.3		
	Saldanha *et al.*, 2010[12]	>65	2145	4.1		Community screening instrument	Urban population in Pune
East India	Das *et al.*, 2008[13]	60	5430	0.8	0.38 VaD: 0.33	BMSE and KCB-Kolkata cognitive battery	Urban Kolkata
	Banerjee *et al.*, 2008[14]	≥50	6129	0.62		Screening questionnaire for cognitive dysfunction, KCB-Kolkata cognitive battery	Urban Kolkata
		≥60	2720	0.1.28	0.34		

1.5.1 BENIGN AND HARMFUL PATHWAYS OF APP PROCESSING

Initially, the Alpha-secretase snips off the amyloid precursor protein molecule which has the capacity of becoming β-amyloid. The construction of the β-amyloid peptide abolishes and the capacity for formation of plaque. As the result of this cleavage sAPPα is generated and released, this has properties, such as encouraging growth and existence of neurons. The resultant fragments are free into the extra neuronal space, while the other bigger fragment still rests within the neuron. In harmful pathway, β-secretase enzyme first cleaves off the Amyloid precursor protein molecule at one end of the β-amyloid peptide, thus releasing soluble beta amyloid precursor protein from the cell. γ-secretase enzyme then cuts the resulting Amyloid precursor protein fragment, which is tethered in the neuron's membrane. β- Amyloid peptide on getting cleaved on both sides gets released into the space outside the neuron and sticks to other beta-amyloid peptides. Aggregates of proteins are formed. These small and soluble beta-amyloid peptide

aggregates are called oligomers. These oligomers can be of varying sizes and it can be that certain sizes of theirs might be accountable for reacting through receptors present on synapses and cells present in neighbours affect their ability to function.

1.5.2 BETA AMYLOID DEGRADATION AND CLEARENCE FROM BRAIN

Neprilysin (NEP) and insulin degrading enzyme (also known as insulysin; IDE),are two major enzymes which are responsible for most of degradation of amyloid beta (Iwata et. al., 2000). It is type II metalloprotease present in plasma membrane, which is accountable for the extracellular degradation of different sorts of peptides. IDE, which is also a metalloprotease, has been is active both intra- and extracellularly. IDE has coarsely greater affinity for insulin as compared to amyloid beta. Thus, insulin poses as an actual inhibitor of the IDE-dependent cleavage of amyloid beta, which is suspected to form the basis for a link between type II diabetes, and Alzheimer's. Both Neprilysin AND insulin dependant decreases in normal aging and in disease affected regions in case of AD [Maruyama M .et al 2005]. Apart from that a large evidence role for lysosomal degradation by enzymes such as cathepsin B is also shown. [Sun et. al., 2008].

A large amount of amyloid beta remains undegraded in spite of in spite of ample catabolism. If there is interference in this process it leads to accumulation of amyloid beta in brain. If the amyloid beta is soluble in nature it would exchange across the blood brain barrier by two main mechanisms that is the low-density lipoprotein receptor-related protein (LRP) on the abluminal (brain) side, and the receptor for advanced glycation end products (RAGE) on the luminal (blood) side. Total output of amyloid beta through the blood brain barrier; detect the amount of cerebral amyloid load [De Mattos et. al., 2002]. Many other co-morbid vascular abnormalities within the brain suffering from alzheimer's may occur due to disruptions in this mechanism [Zlokovic et. al., 2005].

1.6 INSULIN

Insulin secreted by the beta cells of pancreas is the peptide hormone which not only, maintains glucose level but also plays important role in insulin signaling. It facilitates glucose uptake by cells, protein and lipid metabolism, regulate carbohydrate and cell cycle control. Insulin resistance is one of the major causes of diabetes and is thus defined as the impaired sensitivity to insulin mediated glucose disposal. Insulin resistance, which is the major cause of diabetes mellitus, increases Advanced Glycation End- products (AGE), formation of reactive oxygen species by accelerating aging process results in oxidative stress [Smith et. al., 1995]. This oxidative stress leads to increase damage of DNA, reduced ATP production, mitochondrial dysfunction (Fig 6). Impairment of brain insulin signaling appears to be the core of neurodegeneration cascade in late onset of AD [Wands et. al., 2005] so the common link between prediabetes and late onset of AD is insulin resistance, which causes hyperinsulinemia [Tang et. al., 2013].

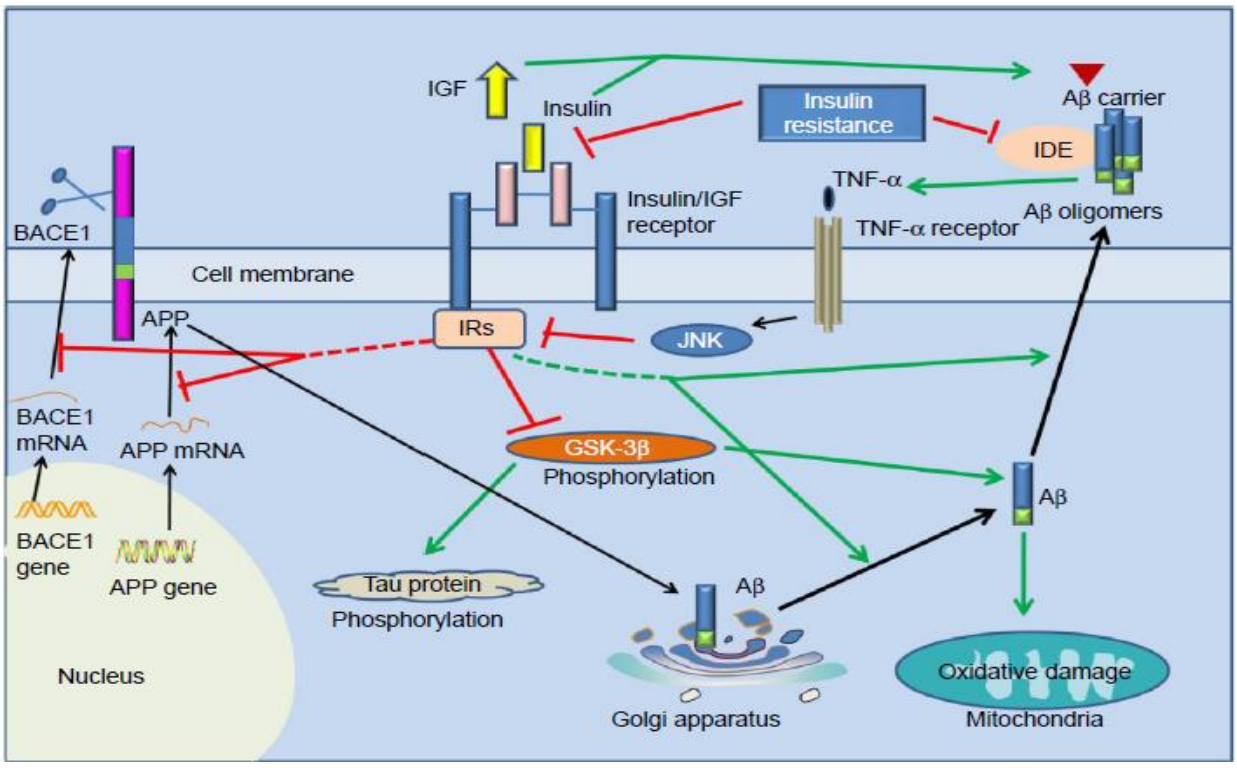


Fig: 6: Connecting linkage between Alzheimer's disease and Type 2 diabetes mellitus.

1.7 INTERLINK AMONG ALZHEIMER'S DISEASE AND DIABETES MELLITUS:

1.7.1 INSULIN PROCESSING AND RECEPTOR

Insulin receptors are present throughout the brain most probably in the olfactory bulb, cerebral cortex, hypothalamus, hippocampus and the amygdala. The highest deliberation of insulin receptor mRNA expression was found in the cerebellum and choroid plexus [Zhao et. al., 2009]. When the insulin binds to the insulin receptor it activates tyrosine kinases which results in autophosphorylation of tyrosine residues which initiates intracellular cascade (Fig 7). This signal transduction based on insulin controls the activity of several enzymes such as p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3-K) [White et. al., 1994], which stimulates glucose transport [Kim, 2012] and activates protein kinase-B (Akt/PKB) which inhibits apoptosis acts on glycogen synthase kinase-3 (GSK-3) [Lam et. al., 1994](Fig 8).

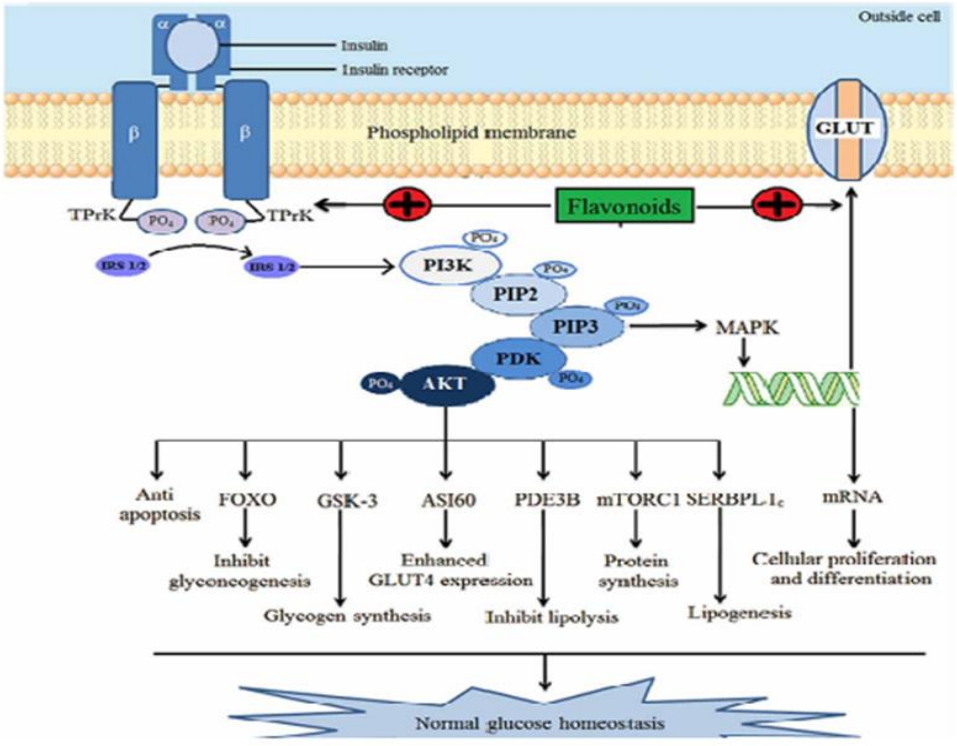


Fig 7: Schematic representation of insulin signaling and its potentiation by flavonoids for the management of diabetes

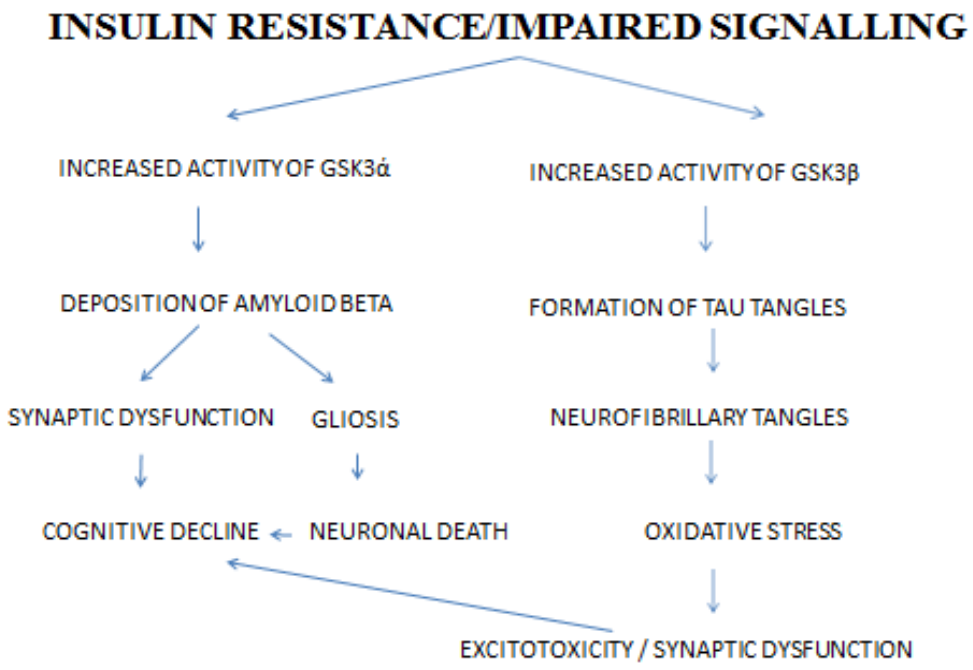


Fig 8: Insulin resistance, impaired signaling and development of AD

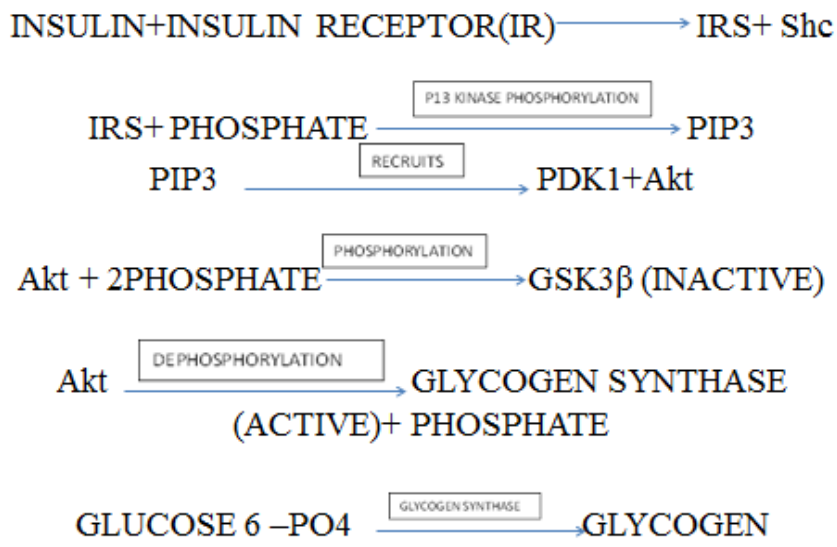


Fig 9: Insulin signaling pathway

This GSK-3 inactive upon phosphorylation by Akt, thereby dephosphorylating glycogen synthase (active form), this enzyme increases the rate of conversion of glucose 6- phosphate to glycogen [Dudek et. al., 1997]. There is a difference between insulin receptors present in periphery and brain based on their size, glycosylation and their insulin binding specificity (Fig 9) [Werner et. al., 1993]. Insulin receptor on chromosome 19 and IGF1R (human insulin like growth factor) on chromosome 15 are another receptors for the kinase family with different affinities [Smit et. al., 1998]. Insulin Growth Factor 2 Receptor, is structurally different from insulin receptor and Insulin Growth Factor 1Receptor [Werner et. al., 1993], binds to Insulin Growth Factor -I and Insulin Growth Factor -II (two different types of Insulin Growth Factor), but never binds to insulin [Rechler et. al., 1980].

1.7.2 ACETYLCHOLINE

From the recent research it has been found that there is correlation among blood sugar level, insulin resistance and cognitive dysfunction. Acetylcholine transferase enzyme (ChAT) leads to synthesis of acetylcholine communicated in insulin as well as in IGF-1 receptor cortical neurons of positive control. As the insulin/IGF-1 stimulates it enhances the expression of ChAT and so insulin/IGF-1 co-localization is reduced in Alzheimer's disease. As a result decrease insulin levels and insulin resistance may add to reduce in acetylcholine intensity which may show link between diabetes and Alzheimer's disease (Fig 10) [Kroner et. al., 2009].

1.7.3 AMYLOID BETA AND TAU

The amyloid generated in beta cells of pancreas results to β-cell dysfunction, type 2 diabetes and disturbance in glucose homeostasis. Patients affected by type 2 diabetes were detected with elevated amount of tangles and senile plaques. Amyloid beta is the result of cleavage of amyloid precursor protein by the secretases mainly alpha, beta and gamma. Accumulation of these amyloid beta leads to formation of senile plaques.

Interaction between amyloid beta and signaling pathways leads to hyper-phosphorylation of tau protein and results in formation of neurofibrillary tangles. This tau phosphorylation leads to activation of GSK3 which are regulated by P13K pathway which includes phosphorylation of glycogen synthase in glycogen biosynthesis. GSK3 is the major step in the formation of tangles so as to treat both diabetes causing Alzheimer's it may become as a target point (Fig 11).

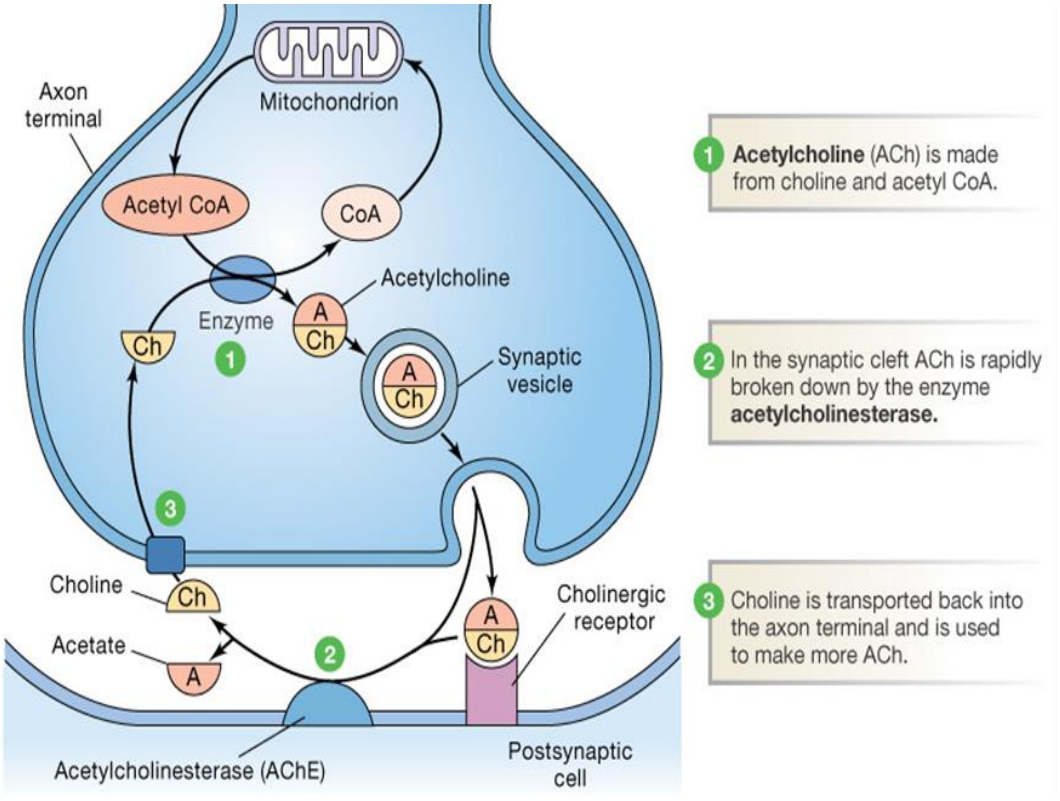


Fig 10: Synthesis and recycling of acetylcholine at the synapse

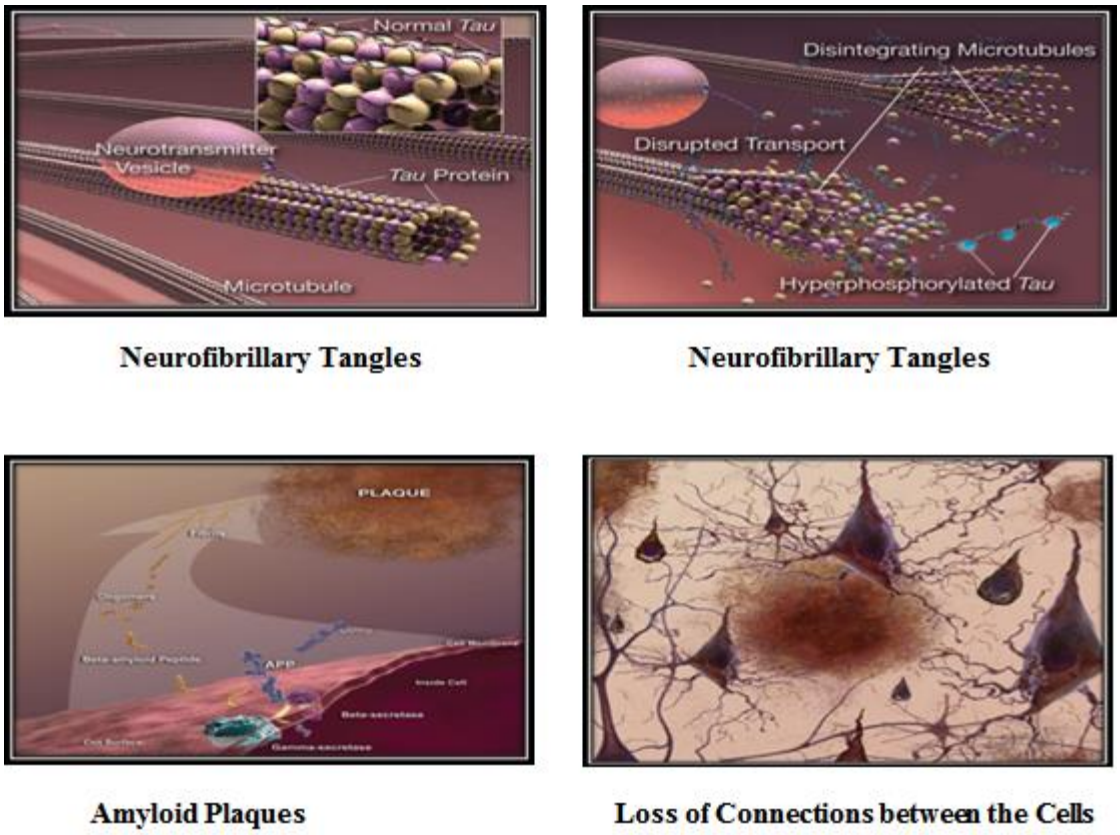


Fig 11: Neurofibrillary Tangles and Amyloid Plaques

1.7.4 INFLAMMATION

It was establish that increased stages of acute phase inflammatory products such as inflammatory mediator's interleukin-6, a-1-antichymotrypsin as well as C-reactive protein [Hak et. al., 2001] are allied with immunological disorders which results to insulin resistance. In Alzheimer's disease, patients the inflammatory products accumulate at different rate as compared with healthy people [Lue et. al., 2001]. Alzheimer's patients were detected with increased immunorectivity to interleukin-6 present in senile plaques of ventricular cerebrospinal fluid [Rosler et. al., 2001]. Peroxisome proliferator-activated receptor-g (PPARg) agonists, have anti-inflammatory effects belong to type of antidiabetic drugs that decreases insulin resistance (Fig 12) [Combs CK et al 2000].

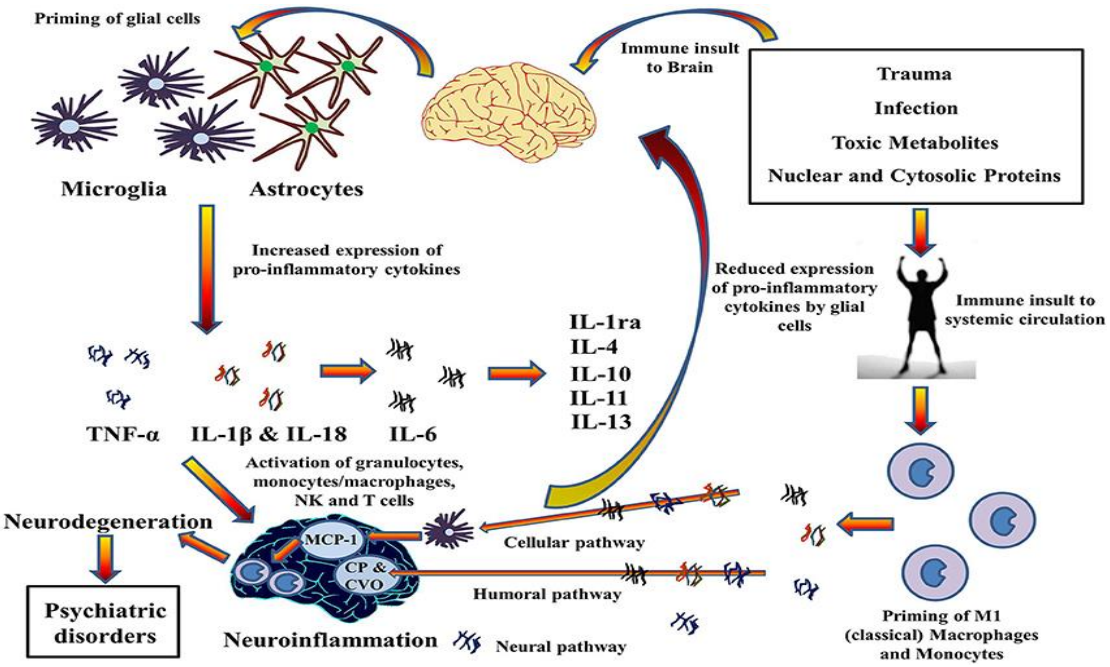


Fig 12: Cytokines theory of inflammation of neurons

1.7.5 MITOCHONDRIA DYSFUNCTION OXIDATIVE STRESS

Beta amyloid is potent mitochondrial poison, it inhibits key mitochondrial enzyme especially Cytochrome C oxidase. Beta amyloid is toxic material which leads toimpaired transport of electron, ATP production , oxygen consumption and membrane potential of mitochondria. Elevation of superoxide radical formation in mitochondria leads to oxidative stress, cytochrome C release and apoptosis. When ROS and RON interact with lipid membrane protein the mitochondrial membrane builder potential collapses which results in opening of permeability transition pore and leads to apoptosis. Dysfunctional mitochondrial release oxidizing free radicals leads to oxidative stress. Amyloid was found to generate ROS and RNS which leads to oxidative stress RAGE (receptor for advanced glycation end products) mediate amyloid beta pro-oxidant effects on neurons, microglia and cerebrovascular cells (Fig 13).

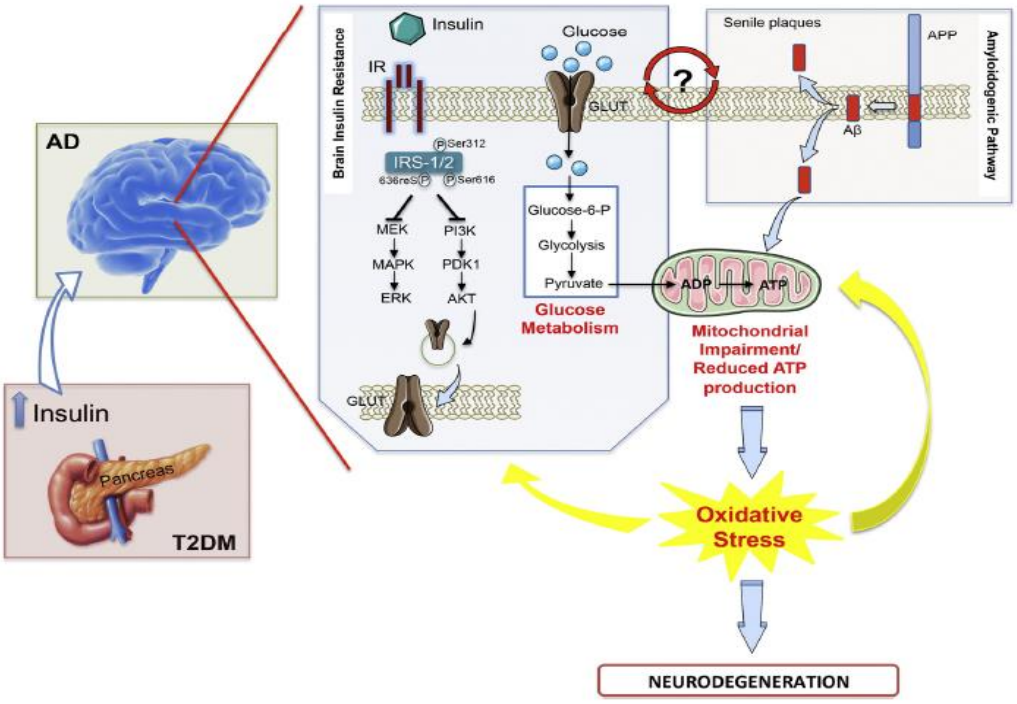


Fig 13: Effect of oxidative stress levels in Alzheimer disease brain

1.8 NEED FOR ALTERNATIVE THERAPEUTIC APPROACH FOR THE MANAGEMENT OF NEUROLOGICAL COMPLICATIONS

Reports for the clinical and experimental settings in the recent year clealy gives us an idea that neurological complications such as dementia and Alzheimer's are difficult to manage, especially when reinforced with type 2 diabetes mellitus. Almost all of the therapeutic drugs used in the management of these disorders have failed to provide any promising effect. To be more specific, despite of continuous drug treatments, severity these disorders continuously increases with time, suggesting that used drugs are ineffective in controlling progression of these disorders and does not reverse the damage that have already been set in. These drugs only provide symptomatic relief, besides possessing several side effects. Therefore need of the day is to screen some potential new drug molecules or adjuvant therapeutics strategy which not only control the progression of these disorders but also reverse the damage that have been already done in the body. One such approach is of natural molecules, which are comparatively safer and possesses

properties like antioxidants, anti-inflammatory etc., which shows a prime role in the development of neurodegenerative disorders. Quercetin, a chief bioflavonoid, is a well-known antioxidant molecule [Zhang et. al., 2011], possesses anti-inflammatory and anti-cancer properties [Joshi et. al., 2011] and is a key molecule in fighting several chronic neurodegenerative diseases. Quercetin and other nutraceuticals have become a great area of interest for neuroprotection now-a-days, based on their ability to counteract oxidative stress-mediated neurotoxicity and some additional potential mechanisms of neuroprotection. However, further targets for biological activity are to be expected, for example, signal transduction pathways, proteasome function, mitochondrial integrity, and so on.

Chapter 2

Literature Review

2. REVIEW OF LITERATURE

Alzheimer's disease is associated with progressive cognitive and memory loss and includes chronic degeneration of neurons present in the CNS. It is described by molecular hallmarks such beta amyloid peptide deposits in senile plaques extracellularly, cholinergic deficit, hyperphosphorylated neurofibrillary tangles made of tau protein intracellularly, extensive loss of neurons and synaptic variations in the hippocampus and cerebral cortex which are essential for cognitive and memory functions. According to the recent data it was found that worldwide 46.8 million people are suffering from dementia and this number will reach 131.5 million by 2030. There are several causes of Alzheimer's disease such as neurodegeneration, oxidative stress, inflammatory stress, decrease in glucose uptake, decrease in insulin signaling and alpha –beta-gamma secretase pathway.

AD has mostly been categorized as a heterogenous disorder thus implicating multiple abnormal signaling cascades to be involved in its pathogenesis. Insulin resistance known to disturb multiple different cascades of known significance to AD Production of cerebral insulin and insulin like growth factors led to severe impairments in metabolism of energy and utilization of glucose in AD. Insulin's ability to drag towards receptors was found to be comprimized in an AD brain. The decrease in processing of glucose is related to early stages of AD [Monte et. al., 2002].

Insulin is known to influence AβPP metabolism by accelerating its marketing to the plasma membrane from the place of generation that is trans-golgi network. Thus, insulin along with hyperinsulinaemia certifies the discharge of AβPP and the formation of amyloid beta. In patients with insulin resistance, hyperinsulinaemia may eventually lead to a reduction in Aβ degradation by insulin dependent enzyme, increasing its deposition in neuronal cells. AβPP-Aβ acts as a neurotoxin. Insulin also regulates tau phosphorylation. Insulin maintenances neuronal cytoskeletal functions with the help of phosphorylation of Akt , which is essential for cytoskeleton association and stabilization. Weakened insulin signaling can lead to tau hyperphosphorylation due to reticence of P13K/Akt and increased activation of glycogen synthase kinase-3β. The activation of Glycogen synthase kinase results to enhanced tau phosphorylation and the neurofibrillary

tangles formation. The impaired insulin receptor activation may be a direct result of decreased insulin levels in patients suffering with T2DM and Alzheimer's. Inhibition of insulin binding or dropping the affinity of insulin binding to its own receptor amyloid beta disturbs insulin signaling. [Rivera et. al., 2005]

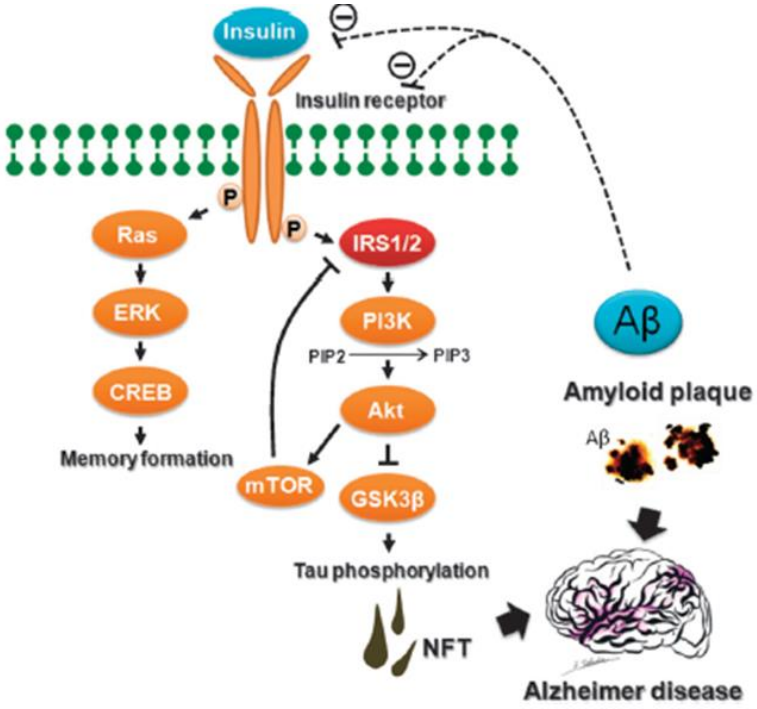


Fig 14: Insulin signaling, memory, and AD pathology

Character of insulin has also been implicated in the development of significant markers of AD pathology. β-N-acetylglucosamine mediated O-GlcNAcylation has been demosnstrated to inversely affect tau phosphorylation, which is one of the characterstic hallmark of AD. One of the major feature of Alzheimer is impaired glucose metabolism has been linked with downregulation of O-GlcNAcylationlead to hyperphosphorylation of tau. Glycogen synthase kinase remains inactivated due to insulin deficiency leading to hyperphosphorylation of tau protein. Duration of diabetes has been verified to positively correlate with neurotic plaques (Fig 14) [Takeda et. al., 2010].

Type 2 Diabetes Mellitus is a disease described by Insulin resistance and progressive β cell failure. The major cause of type 2 diabetes is said to be insulin resistance which may

be defined as the process in which pancreas are not able to produce effective amount of insulin or there is defect in insulin receptors due to which insulin does not bind and glucose remain in the blood. [Rodgers et. al., 2002]

Prevalance of diabetes and Alzheimers increases with age. There were many common features between Alzheimers and diabetes such as aging diseases, fat and higher cholesterol, cardiac risks and degenerative changes which shows link between diabetes and Alzheimer's. According to [Havrankova et. al., 1978] high appearance of insulin and its receptors in Alzheimer's brain is described by formation of neurofibrillary tangles due to hyperphosphorylation of tau protein and formation of senile plaques due to accumulation of amyloid beta. Both of them are involved in T2DM. Cell loss and degenerative change are other common features also involved in both diseases. T2DM is also a chronic metabolic disease that results from the careful destruction of pancreatic beta cells [Roche et. al., 2005].

Amyloid beta deposits are detected in pancreatic islets of patients suffering with diabetes. These amyloid beta deposists are made up of consist of islet amyloid polypeptide (IAPP) [Sun et. al., 2006]. Alzheimer's disease is subjected to insulin resistance and type 2 diabetes which is proved by considering pathogenic similarity and 90% structural similarity between amyloid precursor protein and islet amyloid polypeptide [Janson et. al., 2004]. Similarly, individuals suffering from T2DM will undergo with dementia more frequently [Ott et. al., 1999]. Diabetes mellitus has been found to not elevate the threat of incident AD in the Framingham cohort overall, but it has the potential to be a potent threat factor for AD in the absence of other known major AD risk factors [Beiser et. al., 2006].

A study led by Wen-Tung Wang et al. reports that the cerebral metabolic concerns of untreated hyperglycemia from the start to the chronic stage have been characterized in a rat model induced with streptozotocin. It was found that in acute state of hyperglycemia there was an increase in brain glucose level was accompanied by an increase in ketone bodies and osmolytes. Over the time of hyperglycemia they remained high as soon as the glucose levels were restored some neurological changes were seen which indicates the prolonged uncontrolled hyperglycemia in CNS [Wang et. al., 2012].

A study done by Wang and Yin et. al., rats were described by insulin deficiency along with polyphagia, polyuria, polydipsia and weight loss after STZ injection. Rats suffering from diabetes when compared with control rats were found to be suffering from synapse loss, amyloid beta aggregation and hippocampal atrophy. They were suffering from alzheimer's and their learning ability was decreased as compared to healthy rats. This evidently suggests that deviant absorption induced brain aging as characterized by Alzheimer's disease -like pathologies [Wang and Yin, 2014]

In Diabetes Mellitus, hyperglycemia persuades many side effects obtained by the activation of protein kinase C, activity of the polyol pathway, advanced glycation end-products generation , and higher activity of the hexosamine pathway [Springhorn et. al., 2012]. Mitohcondria is particularly affected by elevated glucose concentrations, which alter glucose metabolism, shape of mitochondria , Ca^{2+} homeostasis as well as generation of energy [Baptista et. al., 2013; Boudina et al., 2007]. Depolarization of mitochondrial membrane is due to hyperglycemia [Liu et. al., 2006; Russell et. al., 2002]. When the activity of mitochondria is impaired the energetics become abnormal [Fernyhough et. al., 2010; Reddy, 2009]. For evolution of disorders related to degeneration of neurons and aging mitochondria was found to play important role [Cardoso et. al., 2013].

Neuroimaging technology was found to be an effective tool to detect the relationship between alzheimer's and diabetes. To measure the energy metabolism of brain Positron emission tomography was used with the help of 18F-fluorodeoxyglucose (FDG) and 11C-Pittsbergh compound (PiB) [de Leon et. al., 1995]. To measure the brain metabolism in humans in vivo 1H-nuclear magnetic resonance spectroscopy was used [Pan et. al., 2001; Shulman et. al., 2004]. When related with age-matched controls, AD individuals showed glucose metabolism impairment in different regions such as posterior cingulate cortex, frontal areas and parieto-temporal lobe, during progression of disease [Minoshima et. al., 1997; Friedland et. al., 1983]. In contrast, the visual cortex and primary motor, basal ganglia nuclei and thalamic, cerebellum is less severely affected [Mosconi, 2005]. Hypometabolism occurred firstly in hippocampus and entorhinal

cortex, then to temporal, parietal, and posterior cingulate cortex. It may specify that the definite disease progression process occurs in Alzheimer's disease patients [Mosconi et. al., 2005].

Glucose metabolites is said to be multi-step process which is regulated by extracellular and intracellular factors [Kuzuya, 1990]. It may include glucose transportation and glucose metabolism. It was seen that insulin signaling pathway play an important role in regulating glucose trans-membrane transportation [Apelt et. al., 1999]. Balanced cellular glucose transportation also governed on the normal function of astrocytes [Duelli and Kuschinsky, 2001] and various glucose transporters present in brain [Choeiri et al., 2002; Duelli and Kuschinsky,
2001]. The study done by Steen et. al. (2005) observed that reduction of expression of insulin, insulin like growth factor leads to Alzheimer's disorder. This was correlated with many alterations in pathology like increased Glycogen synthase kinase-3b activity and Amyloid precursor protein mRNA level. Insulin transportation disruptions contribute to decrease in Cerebrospinal fluid insulin and Insulin Growth Factor-1 levels in AD related to continuous peripheral hyperinsulinemia [Bosco et al., 2011; de la Monte, 2009].

Furthermore, Simpson et al., (1994) also showed that the stages and the insulin-PI3K-Akt signaling activation components negatively correlated with the tau phosphorylation and positively with tau O-Glucoronide N Acylation, telling that impaired insulin-PI3KAkt signaling might be linked to neurodegeneration in Alzheimer's disease. The hippocampal dental gyrus has decreased GLUT1 and GLUT3 expression. It was found that the expression of GLUT3 was decreased in T2DM brain as compared to Alzheimer's brain. In addition, the decrease in O –Glucoronide N Acylation and elevation in tau phosphorylation were also detected, which is similar to the Alzheimer's brain [Liu et. al., 2009].

According to the reports of Chen et. al., (2013) glucose transporter dysfunction may be the common feature of type 2 diabetes and Alzheimer's disease. Interleukin- 1, Interleukin-6, Tumor Growth Factor -β found in AD brains by autopsy, and may play role

in AD progression [Lukiw and Bazan, 2010]. In addition, microglia and astrocytes have also participated in the inflammation in Alzheimer's disease. In the patients suffering from Alzheimer's Microglia groups present in Amyloid beta deposits was found [Chen et. al., 2013].

According to Guarner et. al. (2003) gut microbiota has been associated to insulin resistance via metabolic disorder. The gut microbiota has been linked to insulin resistance or T2D and obesity via metabolic disorder. Recent studies depend upon large-scale 16S rRNA gene sequencing, fluorescent in situ hybridization (FISH) and quantitative real time PCR shown a relationship between the composition of the microbiota of intestine and metabolic disordes like obesity and diabetes. According to Alam et. al., (2014) there is very important role of gut microbes in the development of obesity which is described as a type of chronic and low grade inflammation. Inflammation due to gut microbiota may be related to increased AGE formation, micro and macro vascular disease, insulin resistance, NFT tangles, activated astrocyte and microglia formation etc. It was observed that toll like receptors effects the level of IL-6, IL8 and IL-1β on stimulation of lipopolysaccharides. Thus it may affect our daily lives [Monte et. al., 2009]

According to study of Baptista et. al., [2013], it was observed that diabetes affect motor proteins like KIF1A, KIF5B and dynein responsible for axonal transport. QRT-PCR was used to assess mRNA expression of motor proteins and their protein stages by immunohistochemistry. The expression and immune response of Kinesin like protein1A and Kinesin like protein 5B were increased in diabetic patients there were no changes in dynein. There was increase in fluorescent accumulations due to increase in glucose concentration of Kinesin like protein 1A and synaptotagmin-1 and decreased KIF5B, SNAP-25 and synaptophysin immunoreactivity specifically in hippocampal axonal neurons. These changes suggest that anterograde axonal transport intervened by these kinesins may be impaired in hippocampal neurons results to changes in synaptic proteins contributing to changes in cognitive and memory and in hippocampal neurotransmission [Baptista et. al., 2013]

2.1 ONGOING TRIALS

2.1.1 PPARγ (Peroxisome Proliferator–Activated Receptor) agonists

This receptor shows major role in numerous processes which are complicated in the pathogenesis of both diabetes and alzheimer's containing inflammatory and metabolic processes, cell growth and differentiation [Berger et. al., 2002]. The role of Peroxisome Proliferator–Activated Receptor agonists was found from the course of drugs called Thiazolidinedione's. The stimulation of Peroxisome Proliferator–Activated Receptor agonist's action in response to changes in insulin leads to drop in serum glucose [Malinowski et. al., 2000]. These drugs improve neuronal calcium homeostasis in hippocampus, promote cholesterol homeostasis, improve insulin resistance and reduce cerebral inflammation by inhibiting tumor necrosis factor and IL-6 [Combs et. al. 2000]. This results in decrease in proliferation of amyloid beta peptide and thus improves cognition [Watson et. al. 2003]. Twelve studies were designed to explore the potential benefits of PPAR-gamma agonists, three of them were stopped early and one is presently ongoing. Seven of them found placebo vs. rosiglitazone 2, 4 and/or 8mg; one of them assessed cognitive efficacy of donepezil as compared with rosiglitazone and placebo; evaluated pioglitazone 15–30 mg vs. placebo. Use of thiazolidinedione's might confer some therapeutic use as an adjunctive agent in select patients in the pre-initial to initial stages of AD [Watson et. al. 2003].

2.1.2 INTRNASAL INSULIN

Insulin was found in the brain for short span of time through transport across olfactory and trigeminal perivascular channels and axonal pathways. The study of Reger et. al. [2006] was based on cognitive function and ApoE genotype found 45mins post administration of treatment agent. The people were treated with intranasal insulin (20 units) showed greater improvement in memory. Intranasal insulin had its different effects based on ApoE-e4 genotype. The studies declared preliminary which showed significant differences between placebo and intranasal insulin [Thorne et. al. 2004].

2.1.3 METFORMIN

An orally active biguanide named Metformin (N,N-dimethyllimidodicarbonimidic diamide) lowers blood glucose level by suppressing hepatic gluconeogenesis. Metformin is active biguanide which is responsible in increasing insulin sensitivity, peripheral oxidation of fatty acid uptake and declines gastrointestinal absorption of glucose. Current investigation is going on to see the result of metformin in patients with mild cognitive impairment. [Columbia University, Institute for the Study of Aging, National Institute on Aging 2010].

2.1.4 GLUCAGON-LIKE PEPTIDE-1 ANALOGUE

Glucagon-like peptide-1 is secreted by the gastrointestinal tract responsible for lowering blood glucose level by elevating insulin secretion through the proliferation of pancreatic β cell. It also contains neurotrophic properties and reduces the beta amyloid peptide in the hippocampus which is a obsessive marker of AD. Glucagon like peptide may also responsible for production of new nerve cells in brain of mouse [Luchsinger et. al., 2010]. The study which demonstrated the neurotrophic effect of GLP-1 analogue by reducing Aβ levels in the mice brain shows potential therapeutic effect of glucagon like peptide -1 analogue in Alzheimer's disease [Stephen et. al., 2010].

2.1.5 QUERCETIN

At low micromolar concentration, quercetin antagonizes cell toxicity induced by various oxidants and neurotoxic molecules believed to work by inducing oxidative stress. A recent study showed that quercetin glycosides (rutin, isoquercitrin) were capable of antagonizing changes in gene expression induced by 6-hydroxydopamine in PC12 cells [Magalingam et. al., 2015]. Nevertheless, a number of glucuronidated, methylated, and sulfated quercetin metabolites have been shown to have neuroprotective actions in vitro [Shirai et. al., 2006]. It was shown by a recent study that quercetin glycoside (isoquercetin, rutin) were capable of antagonizing changes in gene expression in PC12 cells induced by 6-hydroxydopamine [Magalingam et. al., 2015]. When administered in vivo quercetin exert neuroprotection and antagonize oxidative stress. A dosage of quercetin (0.5-50 mg/kg) was shown to protect rodents from neurotoxicity and oxidative

stress induced by various stress. [Ishisaka et. al., 2011]. Quercetin was found to provide protection against neurotoxicity of lead, tungsten and methylercury [Hu et. al., 2008]. Quercetin also antagonized cognitive impairment induced by feeding mice with a high fat diet [Xia et. al., 2015]. It also protect retina from apoptotic damage due to ischemia perfusion injury in rat model [Arikan et. al., 2015]. In an aged triple transgenic Alzheimer's disease model quercetin ameliorates Alzheimers and cognitive defects [Sabogal-Gu´aqueta et. al., 2015]. Research showed that an oral supplementation of quercetin along with fish oil enhances neuroprotection in rats exposed to 3-nitropropionic acid or chronically treated with the insecticide rotenone [Joseph et. al., 2013].

In rats chronic unpredictable stress (CUS) is widely used to study the neurological changes which occurs due to chronic stress leads to induce cognitive impairment, depression , insulin resistance and type 2 diabetes in animals [Patel et. al. 2016]. Due to chronic unpredictable stress hippocampal insulin signaling was disrupted and lead to decrease in expression of GLUT4 in hippocampus [Patel et al., 2016c]. It was further demonstrated that the high amount of quercetin in *Urtica dioica* extract may be responsible for neuromodulatory effect [Patel et al 2015]. Quercetin, a natural antioxidant was found to have many health benefits like anticancer activity, improve neuronal survival, anti-inflammatory effect, antiviral properties, cardiovascular protection , brain oxidative stress , insulin resistance etc. Quercetin has proved to improve memory performance in stressed animals. It also attenuated insulin resistance and upregulated insulin signaling in mice brain. The results of various experiments showed that intact hippocampal insulin signaling is required for proper cognitive functions and quercetin is said to be suitable for this. Quercetin not only alleviate pre-diabetics and insulin rate but also lowers serum cortisterone which may lower stress conditions. The inhibitory effect of quercetin was studied by (Shisheva and Shechter et. al., 2014). These findings are then checked from the evidence which says that the enhancement of GLUT4 expression in hippocampus may be associated with neurocognitive improvement. (Patel et al., 2007). Quercetin treated animals were seen with lower insulin receptors and increased GLUT4 expression in CA3 region of hippocampus of stressed animals. Quercetin treatment significantly reversed CUS mediated anxiety, depression, cognitive dysfunction and neuronal damage in the hippocampus, which may be attributed to its potential to

significantly reduce hippocampal oxidative stress markers, enhance antioxidant levels and alleviate inflammatory stress in the hippocampus.

Pharmacotherapy affords symptomatic relief and leads to cognitive renewal. Computational biology approaches act as consistent tools to select novel targets and therapeutic ligands. It includes Molecular Docking which is based on computer-assisted drug design and development. It is used in simulated screening of large libraries of compounds, and intends structural hypothesis of ligands bind with the target linked to lead optimization. There are three major parts of computer-aided drug design that are drug designs based on structure, drug design based on ligand, and approaches based on sequences. It mostly includes target identification, molecular docking, quantitative structure activity relationship and lead optimization. The major two components of Molecular docking are (1) a search algorithm (2) score function [Ou-Yang et. al., 2012]. The two major targets of AD are conventional and experimental. The conventional target includes acetylcholinesterase and N-Methyl- D-aspartate receptor. The experimental target involved beta-secretase, muscarinic and nicotinic ACh receptor and tau protein [Ou-Yang et. al., 2012].

"Effectiveness" of various antioxidants were studied based on several clinical trials. Human diet comprises of several phytochemicals which have special bioactivities. One of them described is Vitamin C which plays important role in reduction of Alzheimer's disease. Strong antioxidant and anticarcinogenic activities were reported for quercetin. So the protective effect of quercetin on hydroxyperoxide, which induces neurodegeneration, was evaluated by the preincubation of PC12 cells with vitamin C and quercetin before H2O2 treatment. The viability of the cells were found to be improved by quercetin as compared to vitamin C. It also suggested that quercetin is reliable to protect neuronal cells affected with oxidative stress from neurotoxicity. Efficacy of quercetin and vitamin C was evaluated through MTT assay. Deficiencies in nutrition play a major part in cognitive deficits, improving protective factors for memory deficits studied by [Ho Jin Heo and Chang Yong Lee*, 2004].

Good antioxidant properties help to scavenge the free radical species. Among them few are responsible for survival of cell and memory improvement by targeting amyloid

genesis and apoptosis pathway. Natural products are used alone or along with other neuroprotective compounds to improve memory and cognition in AD patients. It was found that culinary herbs and medicinal plants have healing power and are potentially valuable resource for drug discovery against AD. But there major disadvantages are poor bioavailability and low clinical efficacy. Then medicinal chemistry and new pharmaceutical technologies are used to prepare new formulation and design new compounds based on natural templates leads to develop new therapeutics against Alzheimer's disease [Ansari and Khodagholi, 2013].

Nutraceuticals are chemical substances which belong to class of natural dietary origin have neuroprotective effects. Several clinical trials humans showed effects of nutraceuticals against impaired cognition and dementia. Flavonoids, Vitamins and other Natural substances have been studied in Alzheimer's and they may be helpful for the maintenance of a good cognitive performance. The significant absence of well demonstrated studies there is no possibility for reference of nutraceuticals in dementia-related therapeutic [Sterner et. al., 2016]

Several phytochemicals were reviewed that have shown aids on many diseases and cognitive impairment. The major disadvantage of such a failure is lack of intervention which may include dosage kinetics, bioavailability and genetic related issues [http://lpi.oregonstate.edu/infocenter/cognition.html; Schulz, 2014]. Treatment based on phytochemicals for cognitive decline and depression can become a good option for clinical trials on humans due to their decrease toxicity and elevated bioavailability. Dietary intake of nutraceuticals can be for short or long period depends upon decrease in severity and incidence of neurodegenerative and other age-related diseases [Mecocci1 et. al., 2014].

A number of evidences are indicative of the neuroprotective nature of quercetin. For an instance, oral administration of quercetin (50 mg/kg body weight) in male Wistar rats (0.12–0.14 kg) was found to greatly reduce the elevated oxidative stress in the hippocampus of rats which were exposed to chronic forced swimming [Ishisaka et. al., 2011]. The study done by Sabogal-Guaqueta et. al., (25 mg/kg) of quercetin was injected intraperitonially every 48 hours for 3 months in triple transgenic (3xTg-AD) mice. On

providing quercetin treatment the cell density increased to in the subiculum and that at almost the same extent as that in Non Tg mice, which were treated with vehicle or quercetin. In transgenic mice quercetin proved to effective by reducing the amount of beta amyloid depositions and treat hyperphosphorylated tau protein in the hippocampus and amygdala .Astrocyte activation and microglial activation which are considered to be hallmarks of AD were decreased in quercetin treated 3xTg-AD mice [Sabogal-Guaqueta et. al., 2015]. Quercetin also provided neuroprotection against the neuronal injury induced by cadmium in frontal cortex of Sprague-Dawley rats [Unsal et. al., 2013]. The neurodegeneration caused by aluminium has been well documented. Apoptosis is the result of ROS generated by Aluminium lead to impaired antioxidant defense of cell and release of cytochrome c as of mitochondria to cytosol. Sharma et al., told that 10mg/kg body weight/day quercetin was administered to male albino rats (Wistar strain). Quercetin did not only decrease ROS production but also increased mitochondrial superoxide dismutase activity, it even prevented aluminium induced translocation of cytochrome-c, upregulated Bcl-2, downregulated Bax, p53, activated caspase 3 and reduced DNA fragmentation. Quercetin was also observed to block neurodegenerative changes induced by aluminum rats [Sharma et al., 2016]. Quercetin has also been found to protect against the neurodegeneration induced by metals such as lead, tungsten and methylmercury. It even greatly attenuates the neurotoxicity caused by polychlorinated biphenyls, such as of insecticide endosulfan and of MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine), in vivo. It also found to be neuroprotective in intracerebral hemorrhage models of rats [Costa et al., 2016].

Quercetin prevented the impairment of memory and the anxiogenic-like behavior, which was induced by STZ-diabetes. In also prevented the decrease in the NTPDase and increased the adenosine deaminase (ADA) activities in SN from cerebral cortex of STZ-diabetes. In STZ-diabetic mice quercetin was found to decrease AChE activity. Quercetin also prevented an increase in the malondialdehyde levels in all the brain structures [Maciel et. al., 2016].

Quercetin regulates eIF2a phosphorylation -ATF4 signaling through GADD34 induction, delaying deterioration of memory at the early stage of Alzheimer’s disease [Hayakawa et.

al., 2015]. The quercetin by activating macroautophagy and proteasomal degradation pathways, proved to prevent beta amyloid agregation and paralysis in *Caenorhabditis elegans* [Regitz et. al., 2014]. Quercetin (40 mg kg-1) reduced scattered senile plaques, alleviate Ab-induced mitochondrial dysfunction, and improve cognitive impairment in APPswe/PS1dE9 transgenic mice. The regulation of AMPK activity may be the chief mechanism by which quercetin affects AD phenotypes. Quercetin shows therapeutic promise in an Alzheimer transgenic mouse model, and it may act as potential therapeutic agent [Wang et. al., 2014]. Not just quercetin, but even its conjugates have been shown to exert neuroprotective effects in rats. Quercetin-3-O-glucuronide treatment has been well documented to significantly improve [Ho et. al., 2012].

The appearance of 3-O-β-glucuronide (Q3GA) has been observed in rat plasma after administering quercetin via oral route. Q3GA could thus be partly held responsible for elevating the antioxidant activity obtained through the intake dietary quercetin-rich foods [Moon et. al., 2001]. It has been well proven that Q3G is a major active component present the in plasma and in the tissue after the oral administration of quercetin or Q3G [Yang et. al., 2016]. But since quercetin is rapidly metabolized and is not sufficiently capable of crossing the blood-brain-barrier, an alternate strategy of administering quercetin had to be sought. For this purpose, liposomes were used for the nasal administration of quercetin in male Wistar rats which not only increase bioavailability as well as rapid absorption. Quercetin liposomes via nasal administration were found to significantly improve cognitive efficiency by attenuating the oxidative damage in hippocampus [Tong-un et. al., 2008].

Quercetin is considered to be therapeutically effective bioflavonoid because of its free radical iron chelating, quenching, and anti-inflammatory properties [Juurlink et. al., 1998]. It has been shown to strongly inhibit amyloid beta fibril formation, and protect HT22 murine neuroblastoma cells from Aβ (25-35) oxidative attack [Kim et. al., 2005]. In a study conducted by Gosslau A et al., Hsp68 was induced in C6 rat glioma cells by increased oxidative stress. Quercetin treatment has also proved to improve the viability of P19 cells against oxidative injury caused by hydrogen peroxide. Quercetin attenuated the generation of reactive oxygen species, hydrogen peroxide-induced nuclear condensation

was prevented, increased the caspase 3/7 activity and elevated the poly (APD-ribose) polymerase expression [Jembrek et al., 2012]. In vitro studies in neuronal cell lines and in primary neurons have shown that quercetin, at low micromolar concentrations, combat cellular toxicity induced by various oxidants (e.g., hydrogen peroxide, linoleic acid hydroperoxide) and other neurotoxic molecules which are believed to act by inducing oxidative stress (e.g., 6-hydroxydopamine and N-methyl-4phenyl-1,2,3,6-tetrahydropyridinium) [Mercer et. al., 2005]. A large number of methylated, glucuronidated and sulfated quercetin metabolites have shown to have neuroprotective actions in vitro proved by [Shirai et. al., 2006;Yeh et al., 2011]. It is found to be major flavonoid in coffee and play important role in inhibiting toxicity by glial cells in neuronal SH-SY5Y cells. It exhibited anti inflammatory as well as neuroprotective properties at 100 mg/mL concentration in both THP-1andU373 cells. Increased intracellular Glutathione stimulating hormone levels in SH-SY5Y cells under normal conditions shows quercetin have antioxidant properties. Reducing the release of pro inflammatory factors may help quercetin to protect the SH-SY5Y cells from glial cells. Ingredients of coffee also exert, antiinflamatory, antioxidative and neuroprotective effects by mechanisms such as by anti-caspase or anti-amyloid mechanisms [Lee et. al., 2016].

Chapter 3

Aim & Objectives

3.1 AIMS AND OBJECTIVE

Present study was aimed to screen out natural molecules with potential to modulate neurological dysfunction related with type 2 diabetes mellitus and Alzheimer's disease through *in-vitro* and *in-vivo* experimentation and consisted of following broad objectives.

- To evaluate the effect of quercetin and rosiglitazone on type-2 diabetes induced neuronal dysfunction in Swiss albino mice.
- To evaluate the effect of diabetes and drug treatment on hippocampal neuronal morphology using Golgi cox staining.
- To screen herbal molecules for their potential to modulate Alzheimer's disease through *in-vitro* screening.
- To standardize intra-cerebral-hippocampal (ICH) injection in male Wistar rats.
- To induce Alzheimer's disease in Wistar rats by injecting beta-amyloid (1-42) through ICH injection
- To evaluate the effect of amyloid-beta injection and quercetin treatment on memory dysfunction in the Alzheimer's model in male Wistar rats.

4. MATERIAL AND METHODS

4.1 MATERIALS

TABLE 2: List of Drugs, Chemicals and Natural Compounds

S. No.	CHEMICAL NAME	COMPANY
1	DTNB	Sigma
2	Butylcholinesterase enzyme	Sigma
3	Acetylcholinesterase enzyme	Sigma
4	Dimethyl sulfoxide	Sigma
5	Quercetin, Rutin, Rosiglitazone, Berberine, Piperine	Sigma- Aldrich
6	Bradford Reagent	Loba Chemi
7	Beta-Amyloid (1-42)	Sigma
8	Ketamine	Medindia
9	Xylazene	Medindia
10	Evans blue	HiMedia
11	Golgi cox solution	HiMedia

TABLE 3: List of Apparatus used in the current project

S. No.	APPARATUS	COMPANY
1	SDS-PAGE unit	Medox
2	Western blot unit	Medox
3	UV-Spectrophotometre	Labindia
4	Stereotaxic Apparatus	Kopf
5	Hamilton syringe	Sigma
6	Photoactometre	Medox

4.2 METHODOLOGY

4.2.1 To study the effect of Quercetin, Rutin, Berberine and Piperine on Butylcholineesterase activity and Acetylcholinesterase activity

Presence of excessive acetylcholinesterase (AChE) is commonly related with the development of β-amyloid plaques and neurofibrillary tangles (NFT) in the brain, and thus, is common cause factor for the development and progress of Alzheimer's disease. Cholinergic neuronal systems show an important role in cognitive functions. In AD brain, a significant deficiency of the neurotransmitter acetylcholine (ACh) and a decrease in activity of enzyme choline acetyltransferase (ChAT) has been detected. On hydrolysis the substrate analog reduces to acetate and thiocholine. In the presence of highly reactive dithiobisnitro- benzoate (DTNB) ion thiocholine generates a yellow color, which is vissible and can be quantitatively monitored by spectrophotometric absorption at 512nm[Gilman et. al., 2010].

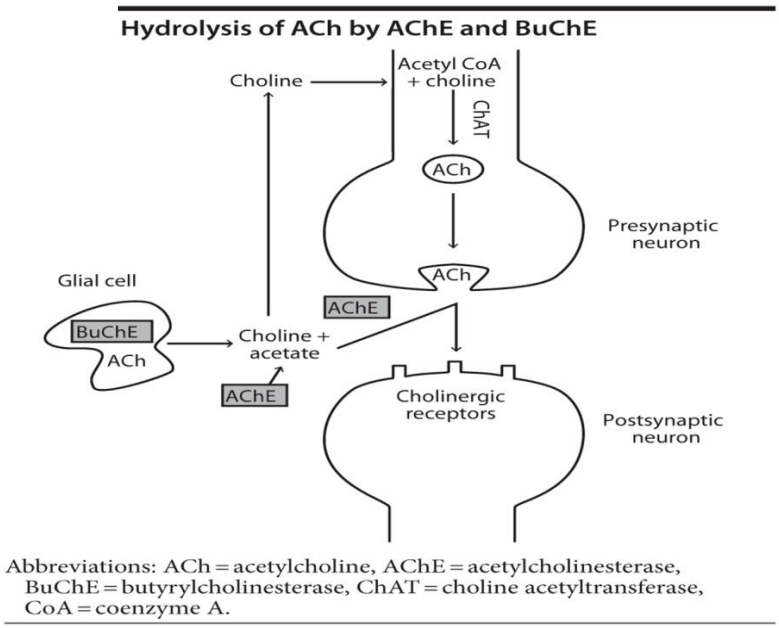


Fig 15: Acetylchoniesterase neurotransmission

A master mix solution comprising of 5,5-dithio-bis-(2-nitrobenzoic acid (DTNB), PBS Buffer and either acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) enzyme was prepared for different reaction volumes in the following concentrations (Table 4):-

Table 4: Composition of the reaction mixture used for the BChE inhibition assay

CONCENTRATION	PBS BUFFER (µl)	DTNB (µl)	BChE (µl)
1000	375	450	75
500	450	450	75
250	487.5	450	75
125	506.25	450	75
62.5	515.625	450	75

Then for every reaction an equal volume of the master mix was pipetted into 5 wells of the 96 flat bottom well plate. DMSO was taken as a control and was added to the first well containing the master mix and different drugs (Quercetin, Rutin, Berberine, Piperine) were respectively added to rest of the wells. The absorbance was taken at 512 nm wavelength. Butyrlythiocholine iodide was then added to all the five wells and absorbance was then recorded four times with a time interval of 60 seconds. Same protocol was followed for Acetylcholinesterase assay.

4.2.2 WESTERN BLOT

Amygdala is groups of nuclei almond shaped located deep in the temporal lobes of the brain. The right amygdala encourages negative emotions like fear and sadness and the left amygdala leads to pleasant or unhappiness emotions. Amygdala is larger in size in males as compared to females. It is basically related to connections, emotional learning, memory modulation, sexual orientation, social interaction, aggression, fear and anxiety. Cerebral cortex is the outermost covering of cerebrum of the brain. It is separated in two cortices by fissure called as hemispheres. The human cerebral cortex is 2-4millimeters (0.079-0.157) in thickness.

Western blot is the best technique used to differentiate and identify proteins. In this method under acidic conditions the red color of the dye gets converted to blue on binding to the protein being assayed. The red form of Coomassie dye firstly donates its free electron to the ionizable groups on the protein, which results in the disruption of the protein's native state. Then it is transmitted to a membrane (nitrocellulose membrane) producing a band for each protein. Incubation of membrane with labeled antibodies specific to the protein of interest. The antibody which remains unbound is washed off leaving only the bound antibody to the protein of interest. When antibodies bind to protein of interest on one band is seen. The thickness of the band determine the amount of protein present; thus applying a standard one can detect the amount of protein present in the sample [Thies et. al., 2013].

4.2.2.1 PROTEIN ESTIMATION USING BRADFORD ASSAY

The Bradford protein assay is used to measure the amount of total protein in a sample. The principle of this assay is that protein molecule bind to Coomassie dye results in a color change from brown to blue. Bradford assay is responsible to quantify the presence of the basic amino acid residues like, arginine ,lysine and histidine, which contributes to formation of the protein-dye complex [Bradford et. al., 1976].

PROCEDURE

In the following protocol 1.5 ml eppendorf was taken for the sample collection. The eppendorf was labeled for the collection of supernatant. The sample aliquots were taken from -80. They were thawed and centrifuged for 30 min at 10,000 rpm. Aliquots were put back in the ice bucket. The supernatant was then taken from it without resuspending it. 1200 µl Bradford reagent and 397 µl water were then added in the eppendorf. Protein sample was then added to the eppendorf. Mixing was done properly so as to dissolve all the contents properly. 300 µl from the mixture was taken and pipetted in duplicates in the 96 well plate. Absorbance was taken at 595 nm. As per the absorbance, the concentration of 30 µg was loaded for further experimentation for western blot studies.

4.2.2.2 SDS PAGE

Proteins separated by electrophoresis which is a commonly used method in which a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) used to denature the proteins. This method is known as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).Sodium dodecyl sulfate (SDS) is an amphipathic detergent. The stacking gel (pH: 6.8) with a large pore size, is placed onto the separation gel, the so called resolving gel (pH: 8.8) with smaller pores. In case of stacking gel all proteins move with same migration speed and develop a sharp stacking line. Movement into the small pore sized resolving gel results to a sieving effect which lead to separation of proteins based on molecular weight because small proteins move faster than larger molecules through the gel. Sodium dodecyl sulphate which denatures proteins by binding to the polypeptide backbone is an anionic detergent, which makes the protein molecule negatively charged. This negative charge distributes throughout the molecule yielding the same charge density per unit length. Removal of disulphide bridges in proteins is necessary for separation by size, the proteins are reduced by 2-mercaptoethanol. Hence in denaturing SDS-PAGE separations, migration is resolute not by intrinsic electrical charge of the polypeptide, but through molecular weight. [Murphy et. al., 2013].

PROCEDURE

Aliquots of protein samples were prepared by adding 6µl bromophenol blue dye to the amount of protein (given in table) in 1.5ml eppendorf. Resolving and stacking gel (10%) were prepared as per the Table 5 given below.

Table 4: Composition of recipe for 10% resolving gel and 4% stacking gel

	Resolving Gel (10%)	**Stacking Gel (4%)**
Water	4ml	3.4ml
TRIS-base	2.5ml(1.5M)	630µl(1M)
30% Acrylamide	3.3ml	830µl
10% SDS	100µl	50µl
10% APS	100µl	50µl
TEMED	6µl	5µl

Assembly was checked for leakage. Resolving gel was then poured followed by n-butanol (to reduce bubbles and vapours). Allowed gel formation without disturbing and washed 3-4times with autoclaved water. Marked the level of separating gel. Then 5ml stacking gel was added after removing the n-butanol. The comb was immediately inserted and kept undisturbed until the gel was formed. Marked the comb region and removed it cleanly after the gel formation. Added running buffer to the assembly. Loaded the sample and ladder (GAPDH) in the wells so formed. Gel was run at 100V till the dye got drained out of the gel.

4.2.2.3 SAMPLE PREPARATION

6 µl of loading dye was added to the eppendorf containing 30 µg protein. Samples were heated at 95 degree centigrade in boiling water bath for 5 mins to denature protein. It was then centrifuged for 10 sec at 800 rpm. The sample was loaded onto the SDS-PAGE gel. Gel was run at 100 V and 50 A current till loading dye almost reach the end of the gel.

4.2.2.4 TRANS BLOT

In trans blot method two filter pads, each of 4.5 × 6.5 cm were taken and kept soaked in transfer buffer for about half an hour. Gel was removed and the extra gel was cut. The gel was immersed in transfer buffer and equal sized nitrocellulose membrane was placed on top of it. Prepared for transblot process in transblot transfer apparatus. Cleaned it with methanol/isopropanol. Poured some transfer buffer on it. Placed first pad on it and added transfer buffer in small amount onto it. Placed equal size whatmann paper onto it prior to placing it in transfer buffer. The gel was placed on it and adjusted accordingly. Placed two soaked whatmann filter paper on it and poured a little more buffer onto it. Placed the lid of the apparatus. The current was set and gel was allowed to run for 1hr. After 1hr the lid was lifted to see transfer of spots/bands to membrane. Soaked the membrane in 3% BSA in PBS and rocked it for 2hrs. After 2hrs primary antibody was added to it and kept at 4 degree Celsius overnight. After overnight incubation it was kept on the rocker for 1 hour and then the primary antibody was removed and washes (3TIMES with PBS+2TIMES with PBST) were given to the membrane. Secondary antibody was then added to the membrane and kept on the rocker for 2hours. After that secondary antibody was removed and washes were given (5 times PBS+3times PBST). To visualize the

bands, DAB (500+9.5ml PBS), H_2O_2 (500) and CoCl2 (100) were added to the membrane. Washed with PBS and scanned the image of the gel.

4.3 Animal Model for Alzheimer's Disease (Amyloid Beta Induced Alzheimer's Disease)

Tau is an important microtubule associated neuronal protein which shows important role in maintaining the integrity of the microtubular filaments. Under normal conditions it was seen that nerve growth factor increases tau expression during the development of neurons. [Kimura et. al., 2014]. It maintains the stability of microtubules by promoting assembly of tubulin. [Davidowitz et. al., 2008]. In case of alzheimer's the tau protein is hyperphosphorylated and lead to formation of neurofibrillary tangles. Many evidances showed that amyloid beta can be processed outside or inside of the cells. Disruption of tau formation leads to production of amyloid beta and amyloid plaques. There are three major pathways which support the link between amyloid beta and tau leads to alzheimer's are: activation of tau kinases by amyloid beta inducing NFT formation by tau hyperphosphorylation , amyloid beta decreases degradation of tau by promoting proteasome dysfunction , Caspase-3 is activated by amyloid beta leads to truncation of tau and alter the tau aggregation leads to NFT (Fig. 16) [Blurton et. al., 2006].

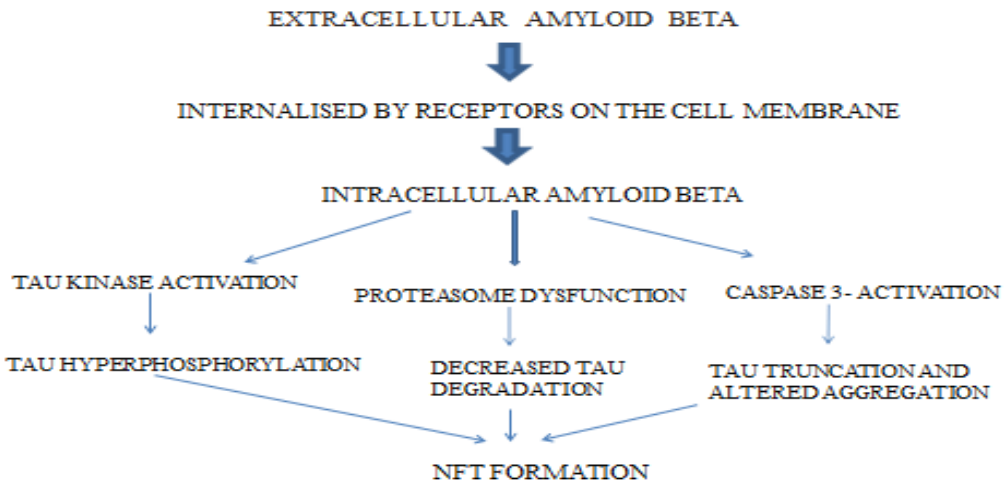
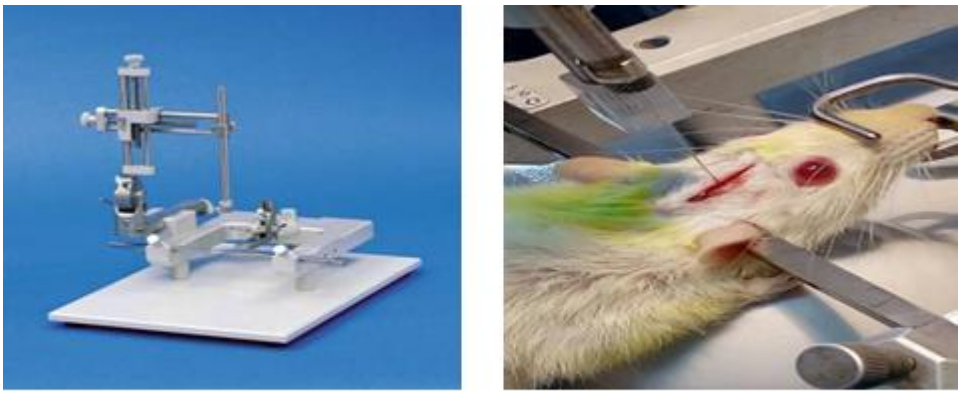


Fig 16: Schematic representation of the Amyloid beta induced Alzheimer's disease

Stereotaxis surgery is a martial form of surgical intervention which requires three-dimensional coordinate system to detect small targets inside the body and lead to ablation, lesion, implantation, biopsy, injection radiosurgery of the tissue etc. (Fig. 17).



Stereotaxic apparatus Stereotaxic surgery

Fig 17: Stereotaxic apparatus and stereotaxic surgery

4.3.1 STANDARDIZATION OF STEREOTAXIC SURGERY

Animal were anesthetized with 90 mg ketamine + 5 mg xylazene. Applied depilatory cream on the head to remove hair. Washed with PBS/Betadine. Inserted ear bars and fixed animal. Gave invasion and opened the skin above skull. Marked the coordinates: AP (-4.8mm), ML (±3.5mm) AND DV (3 mm).Drilled using needle. Marked the required distance on Hamilton syringe. Injected 5µL Evans blue in 5-10mins duration and kept syringe in place for 10 minutes. Applied dental cement and allowed the animal to stand for 30mins. The animal was sacrificed and the brain was isolated to observe the exact location of the ICH injection.

4.3.2 MEMORY EVALUATION

To evaluate the effect of amyloid ICH injection on memory performance in male Wistar rats and to evaluate the effect of quercetin treatment, animals were divided into 4 groups as follows. Group I: Control (no surgery was done), Group II: Sham operative (ICH

surgery + saline), Group III: Aβ control (ICH+ Aβ (1-42)), Group IV: Quercetin (ICH + Aβ (1-42)) + Quercetin).

Morris water maize was performed to evaluate the memory performance in rats. Briefly, apparatus consisted of a circular pool of 2 m diameter filled with water upto 50 cm height. Pool was divided into the 4 hypothetical quaderants, labelled as north, east, south and west. An escape platform of 5 cm × 5 cm was placed submerged 2 cm below the surface of the water such that it remained invisible to animals during trials. Entire experimentation was performed inside a room with low illumination. Learning was given for 4 days, during which animals were trained to find the hidden platform in a 60 sec experimentation session and transfer latency to find the hidden platform were recorded. On 7th day the intracerebrohippocampal (ICV) surgery was performed using stereotaxic apparatus and amyloid toxin was injected. On 14th day, 60 sec probe trial of the Morris Water Maize test was performed to evaluate the effect of amyloid injection and quercetin treatment on the memory index.

On 15th day learning trial of the passive avoidance step down latency was performed. Here, animals were placed on the wooden block and time taken by them to step down was recorded on day 1. As soon as the animlas touches the grid floor, a brief electric shock was given. Memory retension and retrieval was recorded 24 h after learning. Time taken by the animals to step down was recorded. The rats were sacrificed on 21st day and their hippocampus were isolated for protein expression analysis.

4.4 Effect of quercetin and Rosiglitazone on type 2 diabetes induced neuronal dysfunction in Swiss albino mice

As per many studies and research it has been revealed that there is an increased risk of alzheimer's in the diabetic patient as diabetes make neurons more vulnerable to tau and beta amyloid toxicity. Flavonoid like quercetin and rutin was found to be effective drug in preventing diabetes which is current treatment for curing memory dysfunction. Many epidemiological evidences showed direct relation between alzheimer's and diabetes. Alzheimer's disease (AD) is a chronic neurodegenerative disorder of the central nervous

system associated with progressive cognitive dysfunction and memory loss. Type 2 Diabetes and Alzheimer's disease have customarily been thought to be autonomous disorders. Hence, the results of recent epidemiology and science investigation have recommended possible relation and some common patho-physiological mechanisms. This was thus proved by performing Golgi staining which lead to neuroanatomical study as well as differentiate between healthy and weak neurons which may help to detect spike density [Cheng et. al., 2011].

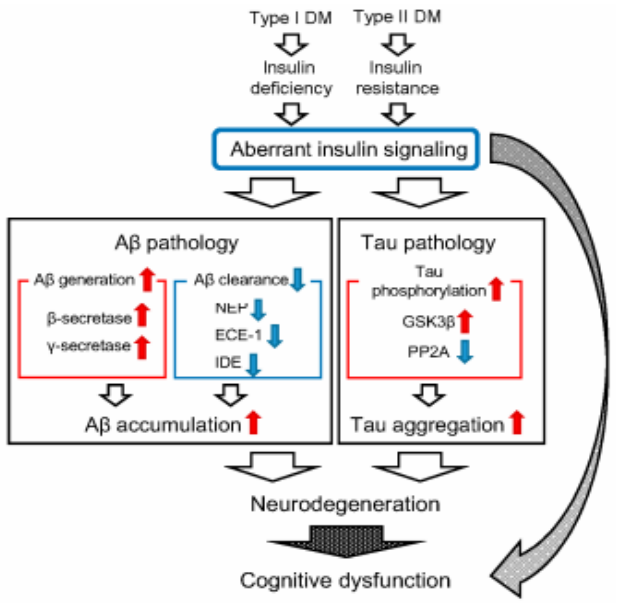


Fig 18: Link between diabetes and Alzheimer's

4.4.1 STEP DOWN LATENCY (Memory evaluation)

Step down latency was recorded as described above, with slight modifications. Briefly, mice were kept on a plastic platform in digital photoactometer. Time was noted for it to come down. As soon as it came down we took the reading. After 24hrs the experiment was performed again. After this a shock of 20mV was given to the mice as soon as it

came down. After 24hrs again the experiment was repeated. The time taken by the mice to come down was noted.

4.4.2 PROCEDURE OF GOLGI STAIN

Golgi cox staining is a method to study neuroanatomy and neuronal connections, differntiate healthy and weak neurons and detect spike density in brain. Hippocampal neuroanatomy i.e. neuronal integrity, morphology and spike density were assessed using golgi-cox staining technique. It is based on impregnation of neurons and glial cells in tissue blocks which are hardened by potassium dichromate then treated with silver nitrate.

PROCEDURE

Firstly, animals were sacrificed by cervical dislocation. The brain was dissected out and was washed with chilled water. It was then washed with golgi cox solution for 5 min. Blocks of tissue were then cut. These blocks were then placed in freshly prepared golgi cox solution in dark for 24 hrs at 37^{o}C. Sections of 200 µM thickness were then cut. Rinse them twice with distilled water for 5mins, dehydrated in 50% ethanol for 5mins. Then keep it in ammonium solution (3:1 NH3: H_2O) for 5-10mis. Rinsed twice with water for 5mins each. Kept it in sodium thiosulphate for 10mins in dark. Rinse twice with distilled water for 2mins. Dehydrated it twice (5-10mins each) in 70%, 80%, 95% ethyl alcohol and 99% 1-butanol, cleared in toluene and fixed in DPX on gelatinized slides.

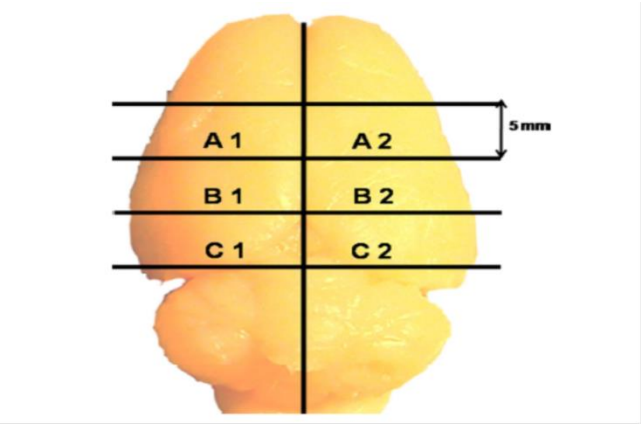


Fig 19: Preparation of the blocks of brain for Golgi-Cox staining

Chapter 5

Results & Discussion

5. RESULTS AND DISCUSSION

5.1 *In-vitro* Butylcholinesterase and Acetylcholinesterase inhibition assay

Results of the *in-vitro* Butylcholinesterase and Acetylcholinesterase inhibition assay are depicted in Fig. 20 and Table 6-7. Our results demonstrated that, for butylcholinesterase inhibition activity the IC50 value of Berberine vs Piperine, Quercetin vs Berberine, and Quercetin vs Piperine were found to be non-significantly different from each other, suggesting that difference between there IC50 value is nonsignificantly different. Further Quercetin vs Rutin, Rutin vs Berberine, and rutin vs piperine were found to be significantly different that means the difference between there IC50 value is much larger and so they are much different from each other and so there rate of reaction is measurable and comparable.

In the other case of acetylcholinesterase inhibition activity, IC50 value of Quercetin vs Rutin, Quercetin vs Berberine, Quercetin vs Piperine and Rutin vs Piperine were found to be non-significantly different that means difference between there IC50 value is much smaller and there rate of reaction is much closer. Results of Quercetin vs Berberine, Quercetin vs Pierine, Quercetin vs Rutin and Rutin vs Piperine were found to be significantly different that means the difference between there IC50 value is much larger and so they are much different from each other and so there rate of reaction is measurable and comparable.

From the above results it was concluded that potential of natural molecule to inhibit the activity of Butyl-cholinesterase in in following order: Quercetin > Piperine > Piperine > Rutin and for Acetylcholinesterase inhibition potential the order was found to be Piperine > Berberine > Rutin > Quercetin. Based on these findings, and our previous reports, we concluded that natural molecules are very potent in inhibiting the activity of butylcholinesterase and acetylcholinesterase enzyme activity at very low concentration, and thus, treating Alzheimer's or dementia with these molecules may result in the enhanced accumulation of acetylcholine in the synaptic cleft for better cholinergic

signaling and act as good neurotransmitter for the transmission of signals, thereby alleviating dementia.

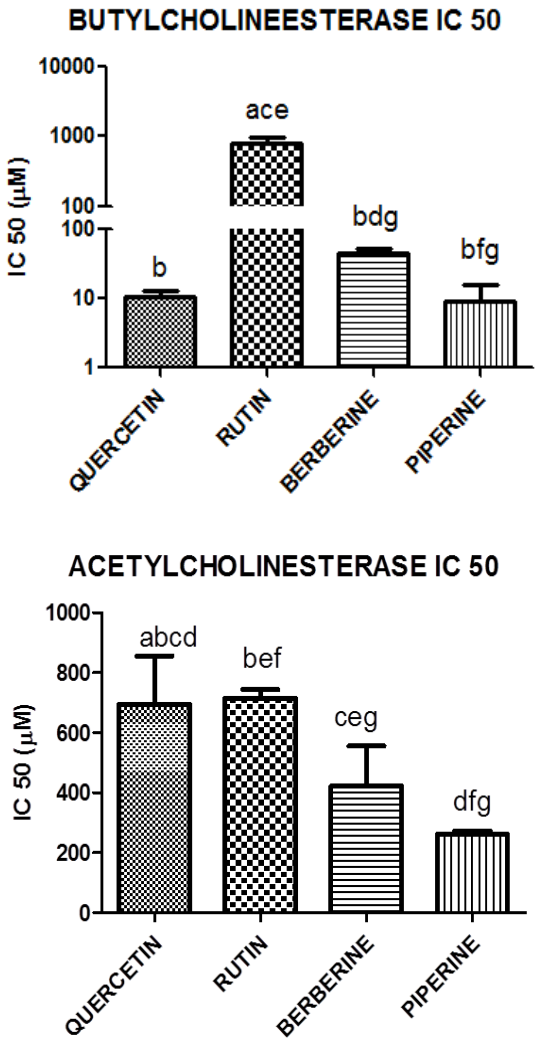


Fig 20: Inhibition of Butylcholinesterase and Acetylcholinesterase activity

The bars in fig. having same alphabetical denomination are non-significantly different from each other and bars having different alphabetical denomination are significantly different. These bars represent the IC50 values of the different drugs used i.e. of Quercetin, Rutin, Berberine and Piperine. IC50 is defined as that concentration of an inhibitor which is required to reduce the rate of an enzymatic reaction by 50%.

Table 5: Statistical representation of the results of *in-vitro* Butylcholinesterase inhibition

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
QUERCETIN vs RUTIN	-769.0	14.25	Yes	***	-1014 to -524.6
QUERCETIN vs BERBERINE	-32.89	0.6094	No	ns	-277.4 to 211.6
QUERCETIN vs PIPERINE	1.605	0.02973	No	ns	-242.9 to 246.1
RUTIN vs BERBERINE	736.2	13.64	Yes	***	491.7 to 980.6
RUTIN vs PIPERINE	770.6	14.28	Yes	***	526.2 to 1015
BERBERINE vs PIPERINE	34.50	0.6391	No	ns	-210.0 to 279.0

Table 6: Statistical representation of the results of *in-vitro* Acetylcholinesterase inhibition

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
QUERCETIN vs RUTIN	-21.26	0.2847	No	ns	-451.1 to 408.6
QUERCETIN vs BERBERINE	272.3	3.648	No	ns	-157.5 to 702.2
QUERCETIN vs PIPERINE	433.0	5.800	Yes	*	3.204 to 862.9
RUTIN vs BERBERINE	293.6	3.932	No	ns	-136.2 to 723.4
RUTIN vs PIPERINE	454.3	6.085	Yes	*	24.46 to 884.1
BERBERINE vs PIPERINE	160.7	2.152	No	ns	-269.1 to 590.5

5.2 BRADFORD ASSAY (Protein quantification)

Results of the Bradford assay are demonstrated in the Table 8 below. All the samples were evaluated and protein concentration was determined from the standard curve prepared using bovine serum albumin as a reference protein. Final concentration was made up to 30 µg/ 10µl and 30 µg or 10µl sample was loaded to evaluated protein expression in the hippocampus of animals.

TABLE 7: Results of the protein composition using Bradford Assay

SAMPLE	**Abs 1**	**Abs 2**	**Avg.**	**Slope (S)**	**AMT = S/Avg.**	**Conc. = 30/AMT**
CTRL-1	0.11	0.153	0.1315	0.0338	3.89053	10.14
CTRL+Q-1	0.125	0.132	0.1285	0.0338	3.80178	10.45
CTRL+ROSI-1	0.114	0.0982	0.1061	0.0338	3.13905	13.59
STZ-1	0.131	0.134	0.1325	0.0338	3.92012	10.03
STZ+Q-1	0.141	0.157	0.149	0.0338	4.40828	8.62
STZ+ROSI-1	0.13	0.163	0.1465	0.0338	4.33432	8.81
CTRL-2	0.275	0.249	0.262	0.0338	7.75148	4.39
CTRL+Q-2	0.258	0.279	0.2685	0.0338	7.94379	4.27
CTRL+ROSI-2	0.217	0.259	0.238	0.0338	7.04142	4.91
STZ-2	0.281	0.241	0.261	0.0338	7.72189	4.41
STZ+Q-2	0.318	0.349	0.3335	0.0338	9.86686	3.35
STZ+ROSI-2	0.342	0.398	0.37	0.0338	10.9467	2.99

5.3 SDS-PAGE WESTERN BLOT (Standardization)

Western blot using SDS-PAGE was standardized by evaluating the expression of house keeping protein, GAPDH, in the amygdala region of brain, which is known to play role in the memory formation and retrieval, along with association with other neurological functioning such as fear, depression etc. Results are demonstrated in Fig. 21 below.

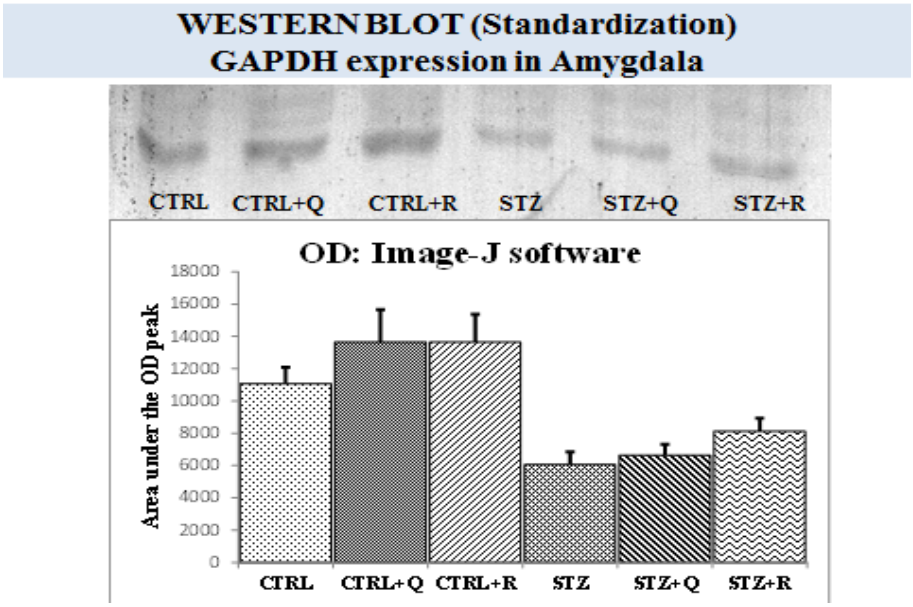
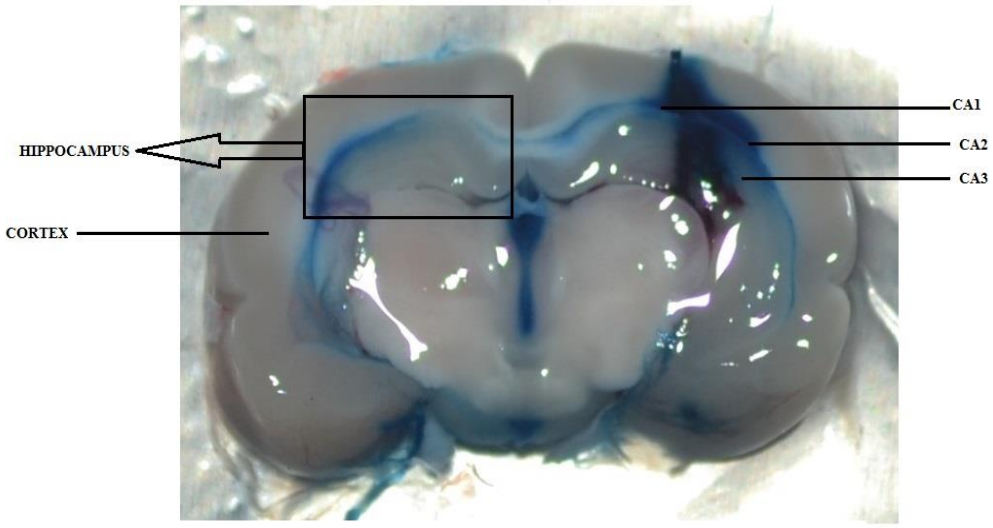


Fig 21: Standardization of Western blot using GAPDH expression in the amygdala region of the brain

As evident from the above figure, procedure for conducting Western blot for evaluating the expression of different proteins in the brain using SDS-PAGE have been successfully established. Above figure represents the developed bands of GAPDH in different group and graph represent the corresponding optical density (expression) calculated using Image-J software.

5.4 Standardization of stereotaxic surgery and ICH injection

Stereotaxic surgery and ICH injection was standardized and injection site was visualized by injecting Evans blue die into the hippocampus using Hamilton's syringe. Results are depicted in the Fig. 22. These images demonstrate the injection site and uniform spreading of Evan's blue dye throughout the hippocampus. We have successfully standardized Stereotaxic surgery and ICH injection site. This site was used further to inject amyloid beta to induce Alzheimer's like state.



Injection site depicted by Evan's blue dye

Stereotaxic surgery depicting Hamilton syringe injected into hippocampus (ICH)

Fig 22: Distribution of evans blue in hippocampus and stereotaxic surgery

It can be concluded from the above images that we have successfully indentified the coordinates for the ich injection. Evans blue dye is uniformly distributed throughout the hippocampus and needle marks clearly depict the location of injetion within the hippocampus.

5.5 Morris Water Maze (MWM)

MWM test was performed to test the spatial memory which contained a white circular pool filled with water at room temperature and had a submerged transparent platform kept below the water surface. The pool was divided into four different quadrants i.e. north, east, south and west. Each rat was allowed to swim for 1 min for four day. It can be concluded from the above graph that all the animals have intact learning ability and took similar time to find the hidden platform in MWM task. Results were non-significantly different before the Stereotaxic surgery (Fig. 22).

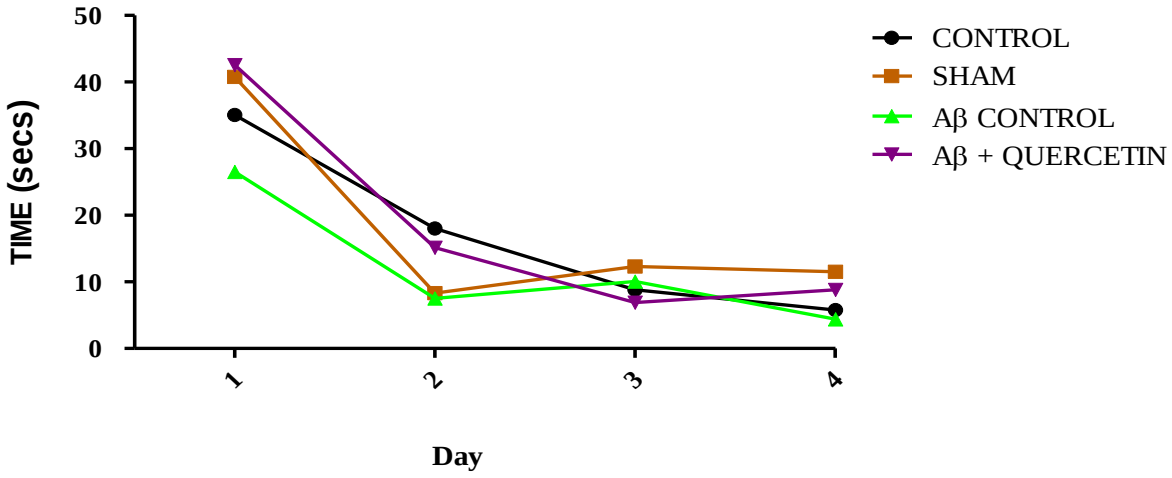


Fig. 23: Results of learning trial of MWM week before ICH injection of amyloid-beta (1-42)

Memory retention was evaluated in term of memory index 7 days after injecting amyloid beta into the hippocampus through stereotaxic surgery. Results are depicted in Fig. 23 below. It can be interpreted from the above Figure 23 that ICH injection of amyloid beta induced memory dysfunction as animals took significantly ($p<0.001$) more time to find hidden platform, when compared to control group. Time take by Sham operated animals was non-significantly different from control animals, indicating that surgical procedure did not interfered with the memory evaluation. Treating animals with Quercetin

significantly improved memory and time taken by these animals was significantly ($p<0.01$) lower than amyloid beta treated animals.

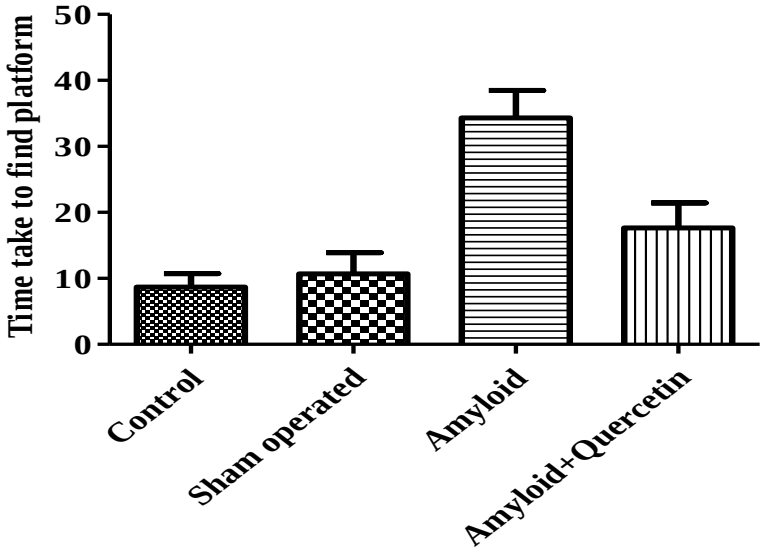


Fig 24: Effect of Amyloid beta (1-42) injection and quercetin treatment on memory performance in male Wistar rats.

From the above study it was observed that the evans blue is the effective dye for the identification of co-ordinates of hippocampus i.e AP, ML and DV so that it is easier to inject amyloid beta using stereotaxic apparatus. After injecting the amyloid beta the rats undergone spatial memory test called as MWM test. As a result of this test the rats were found to be suffering from impaired cognition. The rats were then treated with Quercetin and at the last day MWM test was performed again to see the effect of quercetin. It was found that Quercetin was effective in improving cognitive impairment and the rats were able to recognize the platform and reach there as soon as possible. In some studies it was also found that quercetin did not show perfect result and completely cure the memory but only can decrease the chances of impaired cognitive memory. So we can conclude that quercetin play important role in establishing good memory but cannot cure it completely.

5.6. Effect of diabetes on memory dysfunction and effect of Quercetin on it (Passive avoidance step down latency)

Previously in our lab, type II diabetes was induced in Swiss albino mice using intraperitoneal streptozotocin (STZ) (50 mg/kg) injection for 5 consecutive days in ice cold citrate buffer (pH 4.5) and quercetin treatment was provided for 8 weeks. After which effect of diabetes and quercetin treatment on memory dysfunction was evaluated using Passive avoidance step down task. Step down latency is the good parameter of learning and memory performance. In this the mice was shocked after they step down off the elevated platform during training on day 1, and their ability to remember the foot shock is evaluate 24 h after trial. Results are demonstrated in Fig. 24 below in terms of inflection ration calculated as: (time taken to step down on day 1 – time taken on day 2) / time taken on day 1. It was observed that diabetes induces severe memory dysfunction as the inflection ration was significantly ($p < 0.001$) lower than the control animals. Quercetin treatment improved memory performance and resulted in higher inflexion ratio. Rosiglitazone treatment also improved the diabetes impaired memory function, but the effect was not as prominent as observed in the Quercetin treated animals. These results suggest that quercetin may prove to be efficient molecule to counter diabetes associated memory dysfunction.

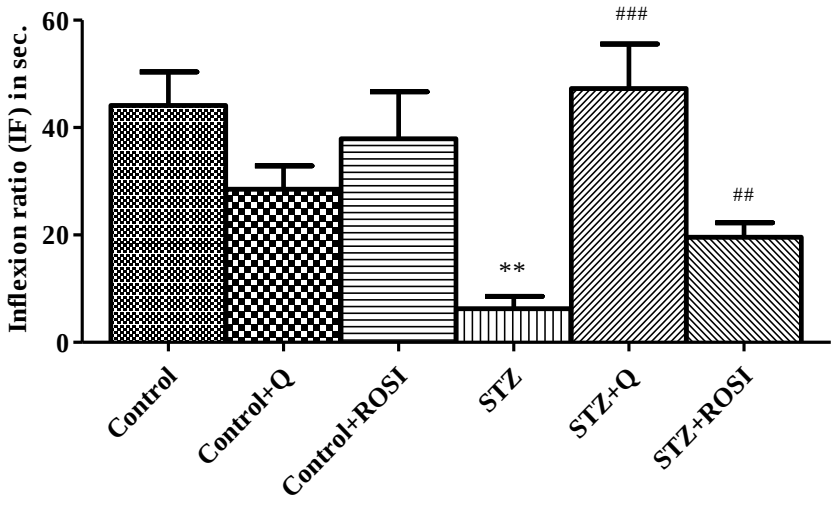


Fig. 25: Results of Passive avoidance step down task depicted in terms of Inflexion ratio

5.7 GOLGI COX (Neuronal Morphology 400X)

Neuronal morphology and neurodegeneration was evaluated using Golgi-Cox staining procedure. It was done to get an insight into the reason why diabetes results in memory dysfunction and effect of quercetin treatment on neuronal morphology was evaluated. Results are depicted in Fig. 25 below. Results of the Golgi-Cox staining revealed that diabetes induces severe neurodegeneration in the hippocampal neurons, especially in the CA3 region. Neurons in the CA3 region of the hippocampus were short, appeared severely damaged and showed fewer interneuron connections. Treating animals with quercetin and rutin resulted in improved neuronal morphology and rescued neurons from diabetes mediated degeneration. Neurons were long and prominent, possessed numerous interneuron connections and no signs of damage was observed.

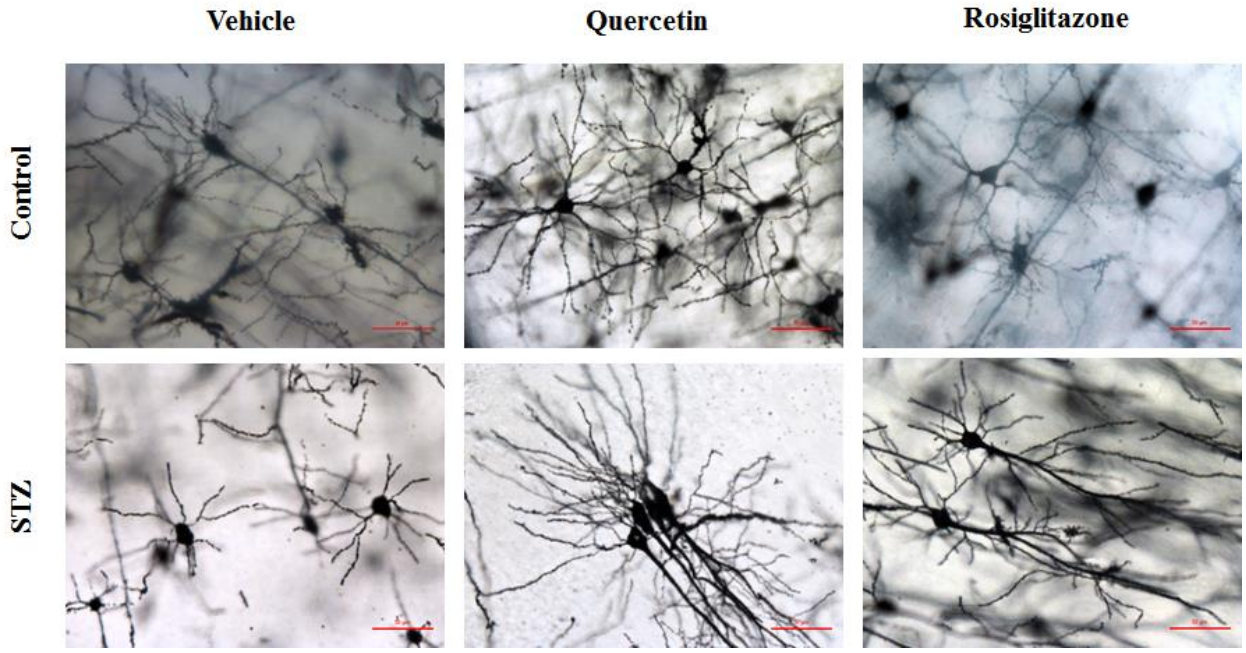


Fig. 26: Golgi-Cox Staining: Neuronal morphology (400X)

5.8 GOLGI COX (Spike density 1000X)

Results of the Golgi-Cox staining (1000 X magnification) revealed that subjecting animals to 8 week diabetes resulted in hippocampal neurodegeneration. Neurons in the CA3 region of the hippocampus were damaged and appeared fragmented. Further, diabetes resulted in lesser number of spike density and number of spikes were significantly reduced in STZ treated animals. Treating animals with quercetin, rutin and rosiglitazone improved neuronal morphology and synaptic plasticity. Neurons were long and prominent, besides, significantly greater number of spikes was observed in hippocampal neurons (Fig. 26).

It was concluded that step down latency proved to be a good method to check the impaired memory. Quercetin played important role in repairing the impaired memory and act as a good treatment tool for curing Alzheimer's. It was found that golgi cox staining is the best staining method which help to differenciate between the healthy and weak neurons. This may also help to determine spike densities which resemble a large number of healthy neurons may lead to good and effective memory.

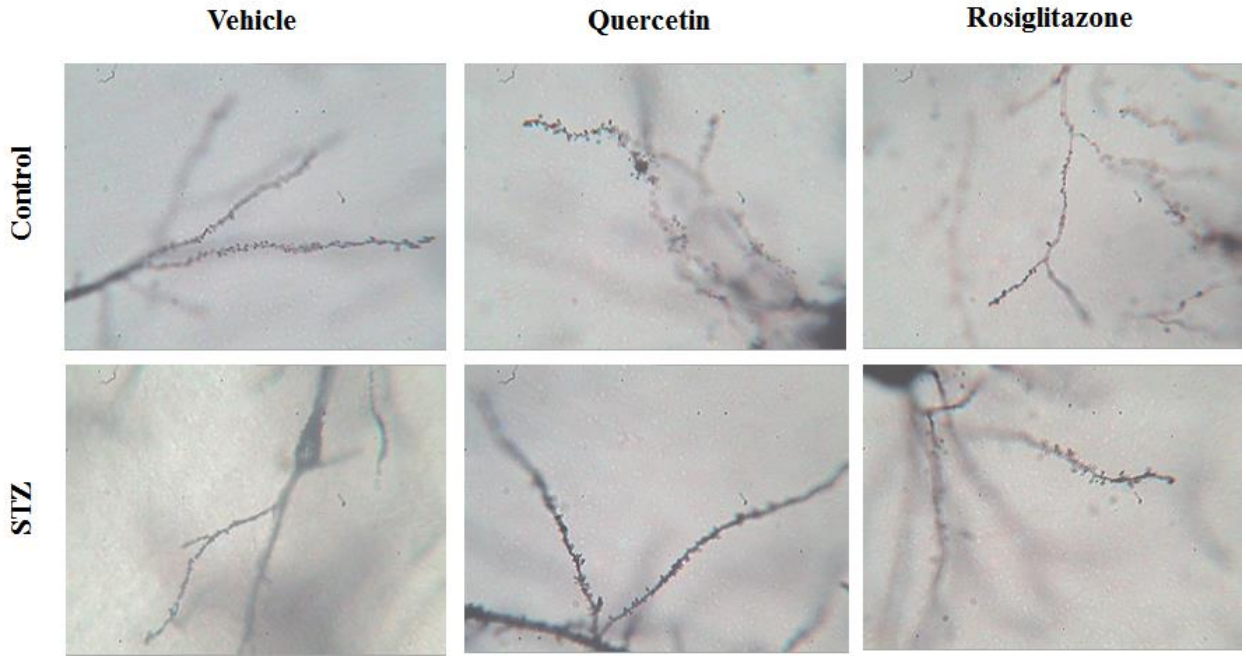


Fig 27: Golgi-Cox Staining: Spike density (1000X)

Chapter 6

Conclusion

6. CONCLUSION

In the present study we used *in-vitro* and *in-vivo* models to evaluate the effect of diabetes and Alzheimer's on enzyme activity, memory dysfunction and neuronal morphology, and evaluated whether or not natural compounds can alleviate these complications. Through *in-vitro* Butylcholinesterase and Acetylcholinesterase inhibition assay, we demonstrated that natural molecules possesses good potential to inhibit these enzyme. Since these enzymes plays a vital role in the cholinergic signaling, which is essential for learning and memory functioning as well as to maintain normal neuronal morphology, therefore, natural molecules, especially Quercetin, may prove very beneficial in improving cognitive dysfunction which are commonly associated with Alzheimer's disorder and neurological complications of diabetes. We further wished to evaluate the effect of Quercetin on memory dysfunction and wanted to look the role of neuronal morphology in the development of memory dysfunction, therefore we used two *in-vivo* animal models of memory dysfunction viz. Amyloid-beta (1-42) in male Wistar rats and type II diabetes model in Swiss albino mice. We standardized the stereotaxic surgical procedure for ICH injection of amyloid beta and successfully induced memory dysfunction in animals. We successfully induced type II diabetes in mice and demonstrated sever memory dysfunction in diabetic animals. Further, Quercetin efficiently improved memory performance in both the models, suggesting that it can be very essential molecule to cope up with memory dysfunction associated with Alzheimer's and Diabetes. Further, we wished to understand the potential mechanism which might play a crucial role in the development of memory dysfunction and thus evaluated the neurodegeneration and neuronal morphology. We observed that memory dysfunction in diabetic animals can be linked to severe neurodegeneration observed along with reduced spike density (which plays critical role in establishment of interneuron connections). Quercetin treatment improved neuronal morphology and spike density and neurons appeared to be healthy with many connections, which might have resulted in improved memory performance after Quercetin treatment. Our results suggest that Quercetin may find a clinical application in managing learning and memory dysfunction, however rigorous mechanistic studies still needs to be done.

Chapter 7

References

7. REFERENCE

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