# Elucidation of Neuroprotective Effect of Antidiabetic Drugs on Brain in Aβ-Induced Alzheimer's Disease Mouse Model

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**Dedicated to**

**My beloved parents**

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# LIST OF ACRONYMS

| | |
|---|---|
| AD | Alzheimer's disease (AD) |
| NFT | Neurofibrillary tangles |
| Aβ | Amyloid beta |
| SVZ | Subventricular zone |
| DG | Dentate gyrus |
| Tg | Transgenic |
| CBP | CREB binding protein |
| aPKC | Atypical protein kinase C |
| AMPK | AMP kinase |
| NPCs | Neural progenitor cells |
| SGZ | Subgranular zone |
| DCX | Doublecortin |
| IL-6 | Interleukin 6 |
| TNF-α | Tumor Necrosis Factor alpha |
| NeuN | Neuronal Nuclei |
| BDNF | Brain-derived neurotrophic factor |
| AICD | APP intracellular domain RNA |
| ChAT | Choline Acetyl transferase |
| Ach | Acetylcholine |
| CREB | CAMP-response element-binding |
| % | Percentage |
| MVM | Morris Water Maze |
| EPM | Elevated Plus Maze |
| PCR | Polymerase Chain Reaction |
| RT | Reverse Transcriptase |
| RT-PCR | Reverse Transcriptase Polymerase Chain Reaction |
| qRT-PCR | Qualitative Real Time Polymerase Chain Reaction |
| dNTPs | Deoxyribonucleotide Triphosphate |
| PFA | Para Formaldehyde |
| PBS | Phosphate Buffer Saline |
| ANOVA | Analysis of Variance |
| SEM | Standard Error Mean |

| | |
|---|---|
| RPM | Revolutions Per Minute |
| Δ | Delta |
| M | Micron |
| nm | Nanometer |
| µg | Microgram |
| mg | Milligram |
| gm | Gram |
| kg | Kilogram |
| µl | Microliter |
| sec | Second |
| min | Minute |
| hr | Hour |

# ABSTRACT

Alzheimer's disease (AD) the most common form of dementia is characterized by chronic neurodegeneration in the brain that results in a slow decline in memory and other cognitive skills accompanied with neuroinflammation. Evidences suggested that alterations in adult hippocampal neurogenesis may also contribute in cognitive deficits in AD. Recent studies have reported AD as a metabolic disease, primarily due to the aberrations in insulin. The present study elucidates the neuroprotective effect of metformin, a commonly prescribed antidiabetic in comparison to donepezil, an acetyl choline esterase inhibitor in amyloid beta (Aβ) -induced mouse model of neurodegeneration. The male Balb/c mice were divided into six groups (n=8 each group): Group 1(control), Group 2 (Aβ (1-40) -treated (1µg/µl)), Group 3 (donepezil-treated (15mg/kg)), Group 4 (metformin-treated (300mg/kg), Group 5 (Aβ (1-40) + donepezil (15mg/kg)), and Group 6 (Aβ (1µg/µl) + metformin (300mg/kg). Aβ (1µg/µl) was injected directly in hippocampus via stereotaxic surgery. After four hrs post-surgery, Metformin (300mg\kg) and Donepezil (15mg/kg) were administered consecutively for 15 days. Behavioral analysis was performed to analyze spatial memory, anxiety and depression like behavior through Morris water maze, elevated plus maze and novel object followed by histological and immunohistochemical analysis. Expression of neurogenesis (Ki67 and NeuN) and inflammatory markers (IL-6, TNF-α and GFAP) was quantified via qRT –PCR. The results demonstrated a significant cognitive decline and memory deficits in AD model. Interestingly, treatment with metformin normalized the expression levels of neurogenesis (Ki67 and NeuN) and inflammatory markers (IL-6, TNF-α and GFAP) along with improvement in the spatial memory. Moreover, metformin also increases the number of NeuN and Ki-67 cells in the hippocampus of Aβ-exposed animals. These results suggest that metformin-mediated adult hippocampal neurogenesis may have implications as a compensatory mechanism to overcome the burden of neurodegeneration. Furthermore, these significant effects of metformin on the expression of inflammatory markers make it a promising suppressor of inflammatory process in AD. These preliminary findings on the effects of metformin and donepezil provide a baseline data to strategize an alternate combination therapy to combat the deleterious effects of AD. However in depth analysis is still needed to explore the underlying mechanism of

action of metformin in mediating these important neurological processes involved in AD pathology.

## *Chapter 1*

# INTRODUCTION

The progressive neurodegenerative disorder, Alzheimer's disease is characterized by major pathological hallmarks which include the deposition of neurofibrillary tangles (NFT) and senile plaques (Kumar et al., 2016). It is marked by complex neuropathological, behavioral and biochemical consequences which results in neuronal loss in the brain areas especially hippocampus (Götz et al., 2008). These brain pathologies form the main cause of dementia that usually begins with the memory loss, apathy and depression (Kumar et al., 2016). Behavioral changes, confusion, impaired communication and eventually difficulty in walking, swallowing and speaking are late symptoms of AD (Alzheimer's Association 2011). The quantitative neurological correlation of dementia with AD can be associated with decreased synapse number (Selkoe et al., 2016). It is suggested that two-third of all dementia are caused by socio-economic burden that affect more than 3.6 million people and approximately this would become triple in number by 2050 (Alzheimer's Association 2011).

The senile plaques are usually comprised of Aβ peptides derived from amyloid precursor protein (APP) and aggregates of hyper-phosphorylated tau protein associated microtubules that form NFTs (Kumar et al., 2015). About 1% of Familial AD is accounted by mutations in three genes APP, presenilin 1 (PSEN1) and presenilin 2 (PSEN2). Proteolytic γ- secretase has a component PSEN1 that together with β-secretase forms Aβ (Sabbagh et al., 2013). Imbalance in various neurotransmitters level (acetylcholine, dopamine, serotonin and noradrenaline) has also been accounted in neurological, behavioral and psychological symptom of dementia (Lanari et al., 2006). In addition to genetic factors, environmental factors (neurotoxins, cytokines etc.) may also play an important role in the development and advancement of AD symptoms (Parihar et al., 2004).

Several controversies exist over whether neurogenesis is increased (Jin et al., 2004) or decreased in the pathogenesis of AD (Dong et al., 2009; Haughey et al., 2002). Factors involved in adult neurogenesis however, Wnt and Notch signaling play important roles in neurogenesis as observed from studies conducted on fetal brain (Lie et al., 2005). In the past the main focus was on activating cholinergic neurotransmission, anti-oxidants and calcium channel blockers to treat memory deficits in AD (Perry et al., 1978).

Reflecting a major paradigm shift, the focus has now shifted to an alternative approach; protecting selective neuronal populations and promoting synaptic formation and neurogenesis.

The role of inflammation as a causative factor or secondary process of AD has not been elucidated. However, several studies have identified the inflammatory pathways activated in AD through neuropathological analysis (Cameron and Landreth, 2010). The key mediator of inflammation in brain is microglia (resident macrophages of brain). These inflammatory macrophages have an ability to penetrate in various brain regions. Inflammation and oxidative stress may result from the release of pro-inflammatory chemical signals by these macrophages. Microglial activities are auto regulated by the expression of these pro-inflammatory signals. In AD, the inflammatory markers that are over-expressed include: TNF-α (tumor necrosis factor – alpha), IL-6 (Interleukin 6), GFAP (glial fibrillary acidic proteins) along with other cytokines. The neuronal processes in AD are affected by these inflammatory marker's chemical signals and results in oxidative stress and inflammation (Lee et al., 2010, Cameron and Landreth, 2010).

Modifications in Neuronal growth and function and interfering with neurotoxic processes are some of the approaches to overcome and protect the damaged neurons. The application of acetylcholine esterase (AChE) inhibitors, nerve growth factor, nonsteroidal inflammatory drugs, estrogen and compounds like antioxidants, neuronal calcium channel blockers and anti-apoptotic agents are some of the examples of drugs that have progressed to clinical domain (Parihar et al., 2004). Psychostimulant monotherapy is used conventionally as first-line treatment of various neurological disorders but they have also been employed in combination with other psychoactive substances (Quisenberry et al., 2013).

AD is an heterogenous disorder that employs several dysregulated signaling. Lower levels of AChE inhibitor during insulin deficiency substantiate an association between insulin resistance and dementia. Under this consideration anti-diabetic mediated therapy may be the potential approach to overcome the cognitive deficits and dementia (Ahmed et al., 2015). Considering the therapeutic application of antidiabetic drugs on cognitive decline, the present study aims to investigate the combined therapeutic effects of the antidiabetics and AChE inhibitor on AD associated consequences, specifically the neuronal death and memory impairment.

The objectives of present study include:

- To develop an Aβ-induced Alzheimer's disease mouse model.
- To elucidate the potential effects of Aβ on cognitive function in mice.
- To investigate the effects of Aβ, antidiabetic drug and AChE inhibitor i.e. Metformin and Donepezil, respectively on neurogenic and inflammatory markers.

# *Chapter 2*

# LITERATURE REVIEW

## 2.1 Alzheimer's Disease

During the 20th century, a significant increase in life expectancy resulted in increased number of old age people and increased risk of developing neurodegenerative disorders, AD referred as late-life mental failure and considered highly prevalent among other neurodegenerative diseases in human (Selkoe et al., 2001). Alois Alzheimer, who defined AD as clinicopathological syndrome in 1950s, didn't recognize that this disease was identical to common senile dementia. After the work of Blessed, Tomlinson, and Roth in 1960s, it was accepted that most common basis for developing AD was aging (Blessed et al., 1968). Fundamental features of disorder reported in the first Alzheimer's patient, Auguste D include gradual memory decline, cognitive deficit, and impaired behavior such as paranoia, delusion, confusion and language function decline. Pathogenesis of Alzheimer's is still not well defined even after several decades had been passed to the first description of AD features (Selkoe et al., 2001).

Brain lesions; neuritic plaques that were noticed in original Alzheimer's case were characterized as extracellularly deposited amyloid followed by neuronal injury, progressively seen in limbic and cortical system. Aβ, filamentous form of amyloid fibrils were found in such type of plaques (Dickson et al., 1997). This amyloid based neuritic plaques and NFTs are now categorized as the major hallmark for AD pathogenesis (Graeber et al., 1997).

Dementia the major manifestation of AD is characterized as progressive memory failure that gradually becomes more severe, and ultimately, debilitating. Individuals experiencing this disorder exemplify poor memory, difficulty in learning and problem solving, confusion, delusions (Goedert et al., 2006). The major neuropathological hallmark in AD pathology includes tau protein hyperphosphorylation with amyloid-β and senile plaques followed by neuronal death (Selkoe, 2001). Hippocampus is one of those brain areas that are affected early in this neuropathological disorder (Braak et al., 1993).

## 2.2 Adult Hippocampal Neurogenesis

The mammalian brain structure hippocampus is located below the medial temporal lobe and consists of CA (1, 2 and 3) fields including subiculum (Mu et al., 2011). Hippocampal circuitry has typically been characterized as a one-way, trisynaptic excitatory pathway (Li et al., 2009). The animal behavioral studies have suggested that the hippocampus plays a crucial role in learning and memory and it also depends on the functional and structural alteration (Squire et al., 1992; Bliss et al., 1973). De novo production of neurons has put forward the new possibility of plasticity that can nurture memory processes in adult dentate gyrus (DG). A previous study supported that, improvement in adult hippocampal neurogenesis promotes the spatial memory (Sahay et al., 2011).

Hippocampal dependent cognitive function is thought to be maintained necessarily by hippocampal neurogenesis (Deng et al., 2010). In adult hippocampal neurogenesis very few molecules central to AD had regulatory role. AD pathologies had a close association with aberration in neurogenesis of hippocampus as demonstrated by Rodriguez et al. (2008). In AD pathology hippocampus effect earlier so the memory deficits associated with hippocampal damage might be related with dysregulation of neurogenesis (Shors et al., 2001). Reduction in hippocampal neurogenesis has also been described in AD transgenic animal model (Mu et al., 2011). These investigations propose that aberrant neurogenesis has a key role in progression of AD. The association of Aβ toxicity with neurodegeneration has also seen in familial type mutation in AD (Haughey et al., 2002). Still, the most general view in the field is that AD related neuropathology deteriorates hippocampal neurogenesis and in consequence impairs cognition.

## 2.3 Role of Inflammation in Alzheimer's Disease

Neurodegeneration in AD is accompanied by "neuroinflammation". The pathological studies of the AD brain tissue indicate the activation of cellular pathways that in turn lead to inflammation (Cameron and Landreth, 2010). The inflammatory processes taking place in the neuronal environment involve certain factors including immune cells, chemical signals, and inflammatory markers that play key roles in bringing about these processes The immune cells in the brain i.e. microglia, second most abundant cells in the brain (first being neurons), act as an immune system unit for the CNS (Ferreira et al., 2014). These cells are also involved in synaptic

remodeling, identifying and removing pathogenic substances and brain tissue remodeling by secreting specific chemical signals [Rubio-Perez and Morillas-Ruiz, 2012].

Microglial cells, similar to macrophages in function, guard the systems within the brain in a "resting state" until they encounter a potential danger. Once they detect a possible danger, these cells get activated and release certain chemical signals, known as cytokines that further signal and activate other microglial cells. The release of these cytokines is usually short-lived until the danger is cleared. These activated microglial cells return to their resting state once the harmful substances have been removed from the system (Mandrekar-Colucci and Landreth, 2010, Lee et al., 2010).

In case of AD, Aβ protein's emergence and accumulation cause the activation of microglial cells. The Aβ proteins are recognized as potential harm by the microglial cells and are attacked by them. Aβ, unlike an infection, remains persistent whereas the microglial cells are over-activated and in turn overexpress pro-inflammatory cytokines (Zotova et al., 2010). This creates havoc in the brain tissue, and the microglial cells thus lead to neuroinflammation resulting in increased neuronal cell death i.e. neurodegeneration, a key feature of AD (Mawuenyega et al., 2010).

### 2.4 Influence of Insulin in Alzheimer's Disease

Previous studies suggested that insulin deficiency and insulin resistance had a crucial role in AD pathology as the insulin receptor loss was found at the initial stages of AD pathology which worsened with the progression of disease (de la Monte, 2012, de la Monta et al., 2005). The factors that lead towards the altered insulin signaling includes: oxidative stress, tau hyperphosphorylation, APP-Aβ deposition, and impaired glucose and energy metabolism (Steen et al., 2005). The decline in glucose processing and failure of insulin to bind to its receptor may coincide with early stages of AD (Iwangoff et al., 1980). Insulin signaling impairment may result from the consequences of Aβ pathology, impaired cholinergic system, tau hyperphosphorylation, pro-apoptotic and pro-inflammatory events. From above evidences researchers put forward the term type 3 diabetes for AD as it is considered as a brain-specific type of diabetes (Schubert et al., 2003). Linkage between AD pathology and insulin deficiency and resistance can be significant to hypothesis that antidiabetic drugs may contribute to overcome the cognitive impairment.

## 2.5 Therapeutic Strategies for Alzheimer's Disease

Yang et al. (2011) used a knock-out animal model to demonstrate NPC's depletion resulting from abnormal ApoE proliferation in DG. An abrupt increase in γ-secretase has been observed recently in lentiviral infected NPCs expressing short interfering RNA (siRNA) (Oddo et al., 2003). As per recent studies, regulation of transcriptional activity of various genes and NPC's proliferation can be influenced by APP. Soluble secreted APPs (sAPPs, mainly sAPPα) and APP intracellular domain (AICD) were two domains that influence neurogenesis via APP. Balance between α-secretase and β-secretase were crucial for determining the emergence and viability of Aβ proteins and sAPPα formation. Increased Aβ formation, decreased sAPPα and increased neurons demise has been observed by the disruption of this secretase balance (Zhou et al., 2008). sAPPα has neuroprotective effect in contrast to ACID via preventing hyperphosphorylation of tau and CDK5 over activation (Mckee et al., 2008). Imbalance in α-secretase and γ-secretase results in neurodegeneration in brain. Previous studies reported altered neurogenesis in AD model with imbalance GABAergic and glutamatergic neurotransmission but early GABAergic signaling inhibition or late facilitation of glutamatergic signaling can normalize neuronal development irrespective of high Aβ levels (Sun et al., 2017). These findings provide compelling evidence that improved cognition, and specifically improved memory, following social housing in AD mouse model, is linked to increased hippocampal BDNF expression and associated neurogenesis.

Acetylcholine esterase inhibitor is currently being used for the treatment of AD that increases the depleted levels of acetylcholine. Drugs that prevent abnormal excitation of neurons and that aggravate the NMDA-type glutamate receptors were approved by food and drug administration (FDA) (Lopez et al., 2009). The persuasive evident regarding preventing and reversal of disease by these agents were not reported. Lesser knowledge about adult hippocampal neurogenesis made it difficult to explore its consequences in AD which mostly effect in this disease. Evidence reported its crucial role in learning and memory, by origination of new born cells to improve cognitive deficits via modulating factors (Shors et al., 2001). Finally, with increase in age the adult hippocampal neurogenesis decline so it was not considered common event in old age individuals thus strengthen the purpose to preserve the brain repair mechanism (Van Praag et al., 2000).

## 2.6 Role of Anti-Diabetic Drugs in Alzheimer's Disease

Metformin first synthesized in 1920s; has been clinically approved for the treatment of diabetes in 1957. It is commonly prescribed for type II diabetes worldwide because of its long history of efficacy. It is found to ameliorate high blood sugar without stimulating insulin secretion or causing low blood sugar levels (Potts and Lim, 2012). The therapeutic mechanisms of metformin is not known, but it has been elucidated that metformin works by activating AMP-activated kinase (AMPK), which then reduces the production of glucose in the liver. In hepatocytes, metformin is involved in the activation of AMPK phosphorylates atypical protein kinase C (aPKC) (He et al., 2009). Cortical precursor differentiation mediated by CBP requires the phosphorylation of CBP by aPKC isoform ζ (aPKCζ). This was hypothesized that metformin might activate neurogenesis by activating aPKCs in NSCs. Wang et al. had demonstrated that metformin had an ability to improvise spatial memory (Wang et al., 2012). While the data reported above demonstrate only the hippocampal related neurogenesis mechanism, it might be possibly involved in other mechanism. Glucose uptake might be increased by AMPK activation in neurons showing the global effect of metformin on neuronal metabolism (Amato et al., 2011). Metformin mediated histone acetyltransferase CBP activation might facilitate the formation of memory by synaptic plasticity, similar to HDAC (histone deacetylase) inhibition by suberoylanilidehydroxamic acid (SAHA) (Wang et al., 2012).

## 2.7 Role of Acetylcholine Esterase Inhibitors in Alzheimer's Disease

Functional deficiencies in cholinergic systems include lowered expression of acetylcholine (ACh) and choline acetyltransferase (ChAT). In AD patients the FDA approved drug Donepezil (AChE inhibitor) was found to be very effective in amelioration of cognitive deficits (Fuchs and Gould, 2000). Despite the discovery of drug that slow down the symptoms of disorder no cure for AD has been found yet. Mild to moderate cases of AD can be treated with AChEI (donepezil, rivastigmine and galantamine). Attenuation of neuronal cell death and cognitive deficits followed by traumatic brain injury and enhancement of neurogenesis in hippocampus was shown by donepezil administration (Nibuya et al., 1996). Through CREB signaling cholinergic system may act on new born cells in hippocampus. Increased p-CREB cells were found with donepezil administration in DG (Kotani et al., 2008). After birth, in new born cells p-CREB cells were found expressed that were involved in cell

survival (Nakagawa et al., 2002 ; Nibuya et al., 1996). In cholinergic system, possibly CREB phosphorylation is directly enhanced by AChE inhibition or changes in BDNF levels in hippocampus may activates the CREB signaling (Zhao et al., 2003). Whereas in new born cell survival and CREB phosphorylation, BDNF is involved (Lee et al., 2002).

***Chapter 3***

# MATERIALS AND METHODS

## 3.1 Ethics Statement

All experiments were conducted in agreement with the decrees of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011). The protocols followed were permitted by the Internal Review Board (IRB), Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST).

## 3.2 Animals

Male Balb/c mice (n=48) of age group 2.5-3 months weighting 30-40 g are used in this study. All animals were housed in the animal house facility of ASAB, NUST. Mice were kept in cages at constant temperature (25±2 ºC) under natural light-dark cycle of (12-12 hrs.). Distilled water and standard diet fed has been given to animals.

## 3.3 Chemicals and Reagents

Aβ was obtained from Abcam (1-40), Metformin Hydrochloride (Glucophage) and Donepezil Hydrochloride (Donecept) was synthesized by Merck Sereno and ATCO Laboratories respectively. All chemicals utilized were purchased from Sigma-Aldrich (USA) unless indicated otherwise.

## 3.4 Study Design

A 16 days long plan was formulated to generate an AD like mouse model by injecting $A\beta_{1-40}$. Aβ (1µg/µl) was injected directly in hippocampus via stereotaxic surgery. Following which Donepezil hydrochloride and Metformin hydrochloride was given for 15 days to demonstrate the neuroprotective effect of antidiabetic and AChE inhibitor on neurogenesis in this mouse model. Behavioral analysis was done in the last five days, following which the animals were decapitated for gene expression, immunohistochemical and histological studies.

### 3.4.1 Animal Groups for Study

Into six groups animals were randomly divided and each group had 8 animals of age group 2.5-3 months. Details of all the groups are provided in Table 3.1.

**Table 3.1: Strategy of experimental groups used in study.**

| Sr No. | Groups | Treatment | Duration |
|---|---|---|---|
| 1. | Control group | Distilled water and feed | 16 Days |
| 2. | Aβ Group | 1µg/µl Aβ | Intrahippocampal injection |
| 3. | Donepezil Group(Kwon et al.,2014) | 15mg/kg Donepezil Hydrochloride | 15 Days |
| 4. | Metformin Group(Hwang et al., 2010) | 300mg/kg Metformin Hydrochloride | 15 Days |
| 5. | Aβ+ Donepezil Group | 1µg/µl Aβ 15mg/kg Donepezil Hydrochloride | Intrahippocampal injection 15 Days |
| 6. | Aβ+ Metformin Group | 1µg/µl Aβ 300mg/kg Metformin Hydrochloride | Intrahippocampal injection 15 Days |

### 3.4.2 Experimental study design

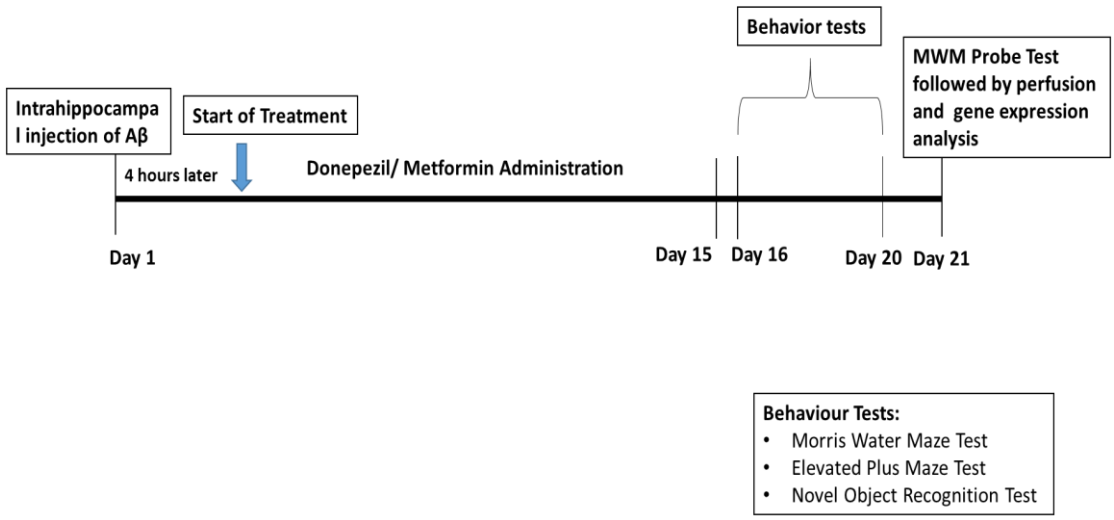


**Figure 3.1:** Experimental study design depicting the 20 days long plan for animal model development following oral administration of Metformin and Donepezil. After 15 days of treatment behavioral assessment was conducted. Post behavioral analysis animals were decapitated for histological and gene expression analysis.

## 3.5 Behavior Studies

### 3.5.1 Elevated Plus Maze

In animals, anxiety type: open places provoked anxiety in addition to height related anxiety was measured by Elevated plus maze test. The apparatus comprised of an elevated (50cm) maze consisting of 4 arms (30x5cm) making a plus sign shape. Two arms were surrounded by 20cm wall whereas the other two arms project out of the center without walls. Animal were positioned in the maze center and permit to move freely for about 5 min. Mice were observed for the number of entries and time spent in each arm by camera. The protocol was adopted from Arendash et al., (2004) with few modifications.

### 3.5.2 Novel Object Recognition (NOR) Task

The task performed to assess the learning and memory was novel object recognition. It was used to measure the short term memory by assessing mouse's capabilities to memorize and to check whether it has seen an object previously or not. The described protocol of NOR task by Ennaceur et al., (2005) was performed with few

modifications. The square glass chamber (40cmx40cmx40cm) is used to perform the novel object test. Mice are habituated for 5 min in the box to avoid bias based on intrinsic anxiety in new space. The NOR task comprises of two trials: in first acquisition trial two objects with different shapes but approximately same height and volume is placed equidistant from each other. Subjected mice were positioned in the center and enabled them to explore and familiarize themselves with the object for 10 min. After acquisition period (intra trial time of 20 min) test trial is performed. The whole situation is identical to acquisition trial but one of the objects is replaced with a new, novel object. During this time period mice are allowed to observe both of the objects for 10 min. Both the trials were documented by camera in order to calculate the time mice spend exploring each object. Sniffing and voluntarily touching the object is considered as interaction.

### 3.5.3 Morris Water Maize Test

The protocol described for Morris water maze test by Biessels et al., 1998 is carried out with few modifications. A circular pool with dimensions of 120×60 cm was filled with water (21°C± 2ºC) and added blue dye to make the water opaque. The pool has four quadrants and in the direction of North-West quadrant a transparent platform with the dimension of 13x32 cm was placed with its surface 1cm blow water. Five acquisition trials to each mouse were given for five consecutive days with distal cues on the pool walls so that animal can navigate from the release position. The animal was given 10 min intra trial interval before releasing from other directions. Mice which were released from one of the five directions were allowed to navigate around the pool for 90s. If mice locate the platform with in 90s than it was left there for 5s but if it fails to find the hidden platform with in 90s than it was guided to platform and allowed to sit for 20 s. Average escape latency of five days was calculated. The probe trial was conducted after 24 hrs in which platform was removed and mice were allowed to swim for 90s. In probe trial session no. of time spent in each quadrant and no. of platform crossings for each mouse was recorded.

## 3.6 Histological Examination of Brain Regional Tissues

### 3.6.1 Tissue Perfusion/Fixation

Herat perfusion protocol by Gage et al., (2012) was performed. Xylocaine (300µl/50g) was given intraperitoneally to anesthetize the mice. Sternotomy was done

to expose heart via midline incision. A needle was inserted to the depth of 5mm in left ventricle and incision in right atrium was made once a steady and slow flow rate of 5ml/min for normal saline was established. The normal saline was replaced with 100ml of 4% paraformaldehyde (PFA) solution after approximately 80ml of normal saline were allowed to flow through system. The isolated brain tissues were then places in 4% PFA at 4°C for 24 hrs. To dehydrate brain tissues different concentration of organic solvent (ethanol) were used. The following is the order of concentration used: 70% ethanol for 1 hr, 95% ethanol for 1 hr, and 100% ethanol for 1 hr. Xylene was swapped with ethanol for further hardening of brain. The brain was placed in xylene for 4 hrs before paraffin infiltration. Dehydrated brain was then placed in molten paraffin in incubator for 4 hrs at 60°C and allows solidifying at 4°C for block formation.

### 3.6.2 Haematoxylin and Eosin Staining

Haematoxylin-eosin staining was performed on brain tissues sections (5µ) of the control and treated groups. After deparaffinization the tissue was incubated in Mayer's haematoxylin solution for 8 mins followed by warm water washing for 10 mins, dipped in 95% ethanol and later counterstained with eosin for 30s.

### 3.6.3 Congo red staining

Congo red staining was performed for the visualizing amyloid protein plaques in the treated tissue sections. Congo red (1%) was mixed in 100ml distilled water and filtered. After deparaffinization, tissues were placed in ethanol and then in distilled water and later stained with Congo red for 25 mins followed by washing, several times for 7-8 mins. Tissues were then counterstained with haematoxylin for 2 mins followed by washing with distilled water. After drying in an incubator for 20-25mins tissues were cleared in xylene solution.

### 3.6.4 Immunohistochemistry

Poly lysine coated adhesive slides were used for fixing the tissue section (5µ). After rehydration in graded concentrations of ethanol, the retrieval of heat mediated antigen was done by incubating sections for 35 mins in sodium citrate buffer (pH 6). The sections were rinsed and incubated in 35% $H_2O_2$ and later in 5% bovine serum albumin (BSA) dissolved in phosphate buffer saline (PBS) for 10 mins (to avoid

nonspecific binding of antibodies). Overnight incubation of tissue section was done in BSA (0.1%) containing the rabbit monoclonal antibody for NeuN (1:100; ab177487) at4°C. The next day, sections were washed and incubated with HRP conjugate anti-rabbit IgG (ab97051) (1:100) at room temperature. After 1 hr of incubation, the sections were stained with 3, 3' diaminobenzidine ((0.025%) (DAB, ab50185)) solution for 10 mins. Cover slip was mounted after counterstaining the tissue sections with haematoxylin staining. The sections were visualized with OPTIKA B-150 LED microscope, (Italy) at 4X, 10X, and 40X resolutions. OPTIKA Vision Lite Version 2.12 software was used to capture the tissue images.

### 3.7 Brain Dissection & Hippocampus Isolation

Mouse was anesthetized using chloroform. Mouse neck was dislocated using surgical scissors, by having a cut at the posterior side of ears. A firm cut was made through the anterior part of the skull by having a small incision at caudal point. The parietal bone was then tilted and broken off by the use of covered narrow pattern forceps. The curved narrow pattern forceps slide under the anterior part of the brain to lift it out of skull. The isolated brain immediately transferred to petri dish containing chilled (PBS). Dissected hippocampus was placed in eppendorf tube and stored at -80°C till further use.

### 3.8 Gene Expression Analysis

#### 3.8.1 RNA Extraction

RNA extraction was performed using Trizol method as per manufacturer's instructions. Breifly, h the isolated hippocampus tissue was washed with 1X PBS. Trizol (1ml) was added to homogenize the hippocampus tissue using UP400S Ultrasonic Processor (Hielscher, Ultrasound Technology). To ensure the complete isolation of nucleoprotein complex, homogenized mixture was incubated at room temperature for 5mins. After incubation 0.2ml of chloroform per ml of Trizol was added to mixture. The sample turned milky after 15 s of vigorous shaking and then allowed to stand for 10mins at room temperature. At 4°C and 12000 rpm the sample was centrifuged (HERMLE Labortechnik GmbR Germany) for 15 mins. There phases of mixture was obtained after centrifugation from which a colorless aqueous phase on the top having RNA was cautiously removed and transferred to new labelled eppendorf. After addition of 0.5ml of isopropanol it was allowed to stand for 10 mins

at room temperature. Again centrifuge at 12000 rpm for 15 mins at 4°C . After second centrifugation, RNA pallet was observed at the side of the tube. The pallet was washed with ethanol (75%) after carefully discarding the supernatant. Following washing with ethanol (75%) RNA pallet was again centrifuged at 4°C for 5 mins at 7500 rcf. RNA was stored at -80°C till further use. RNA was removed from -80°C and thawed for Reverse Transcriptase (RT).

### 3.8.2 Quality of RNA

Prior to complementary DNA (cDNA) synthesis, the isolated RNA was run on 2% agarose gel to ensure the good quality of RNA as well as that isolated the RNA was not degraded. The gel containing RNA bands was visualized on Wealtech Dolphen Doc (S/N470883, Wealtech Biosciences Co., USA). The sharp bands of 18S and 28S RNA exhibits its adequate quality.

### 3.8.3 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The RNA (2µg) in 30µl PCR water using BioPhotometer plus (Eppendorf, Germany). The RT-PCR protocol includes: 4µl of 10 mM dNTP's, 4µl of 5mM oligo-dT, 1 µg template, 8 µl of RT buffer, 1 µl of RNaseoutTM Ribonuclease inhibitor, 2 µl of Revert aid RT and the required amount of PCR water to make the final volume up to 40 µl.

### 3.8.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) for Gene Expression Analysis:

The real time PCR was performed with HOT FIREPol® EvaGreen® qPCR Mix Plus in ABI Prism 7300 Sequence Detection System (Applied Biosystems, 7300). The PCR protocol consist of 1 µl of forward and reverse primers for the specific genes, 4 µl of HOT FIREPol® EvaGreen® qPCR Mix Plus, and the quantity of cDNA used was 1 µl template and reaction volume raise up to 20µl by adding DNase water. The condition set for thermo-cycling was: 95°C for 10mins, followed by 40 cycles for 15 s at 95°C, 1min at 60°C followed by 45s at 72°C followed by final dissociation step. To verify the quality of PCR product dissociation curve and gel electrophoresis were used. Each sample tested in duplicate and all values were normalized to β-Actin

values. The relative gene expression data was analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

**Table 3.8** List of primers used in this study:

| | Primer | Sequence |
|---|---|---|
| **B- Actin** | Forward | GCCTTCCTTCTTGGGTATGG |
| | Reverse | CAGCTCAGTAACAGTCCGC |
| **Ki67** | Forward | CTGCCTGCGAAGAGAGCATC |
| | Reverse | AGCTCCACTTCGCCTTTTGG |
| **NeuN** | Forward | GGCAATGGTGGGACTCAAAA |
| | Reverse | GGGACCCGCTCCTTCAAC |
| **IL-6** | Forward | CCTCTGGTCTTCTGGAGTACC |
| | Reverse | GCTGGAGTCACAGAAGGAGT |
| **TNF-α** | Forward | ATGAGCACAGAAAGCATGA |
| | Reverse | ACCACGCTCTTCTGTCTACT |
| **GFAP** | Forward | TGCAAGAGACAGAGGAGTGG |
| | Reverse | GCTCTAGGGACTCGTTCGTG |

### 3.9 Statistical analysis:

The results were analyzed through Graph Pad Prism version 5.01. One-Way ANOVA and Two-Way ANOVA followed by Bonferroni Multiple Comparison Test were used for analysis. Data is represented as mean ± Standard Error of the Mean (SEM). Results with $p$ value $<0.05$ were considered significant.

# *Chapter 4*

# RESULTS

## 4.1 Behavioral Analysis:

In order to assess the effects of antidiabetic drug and acetylcholine esterase inhibitor on learning and memory in male Balb/c mice, Morris water maze test and novel object recognition were performed. For the assessment of anxiety elevated plus maze was conducted.

### 4.1.1 Effect of Aβ, Metformin and Donepezil on cognitive functions:

To assess the influence of Metformin and Donepezil on cognitive deficits, Morris water maze test was employed. The effect of drugs on spatial memory can directly be assessed by average escape latency. The outcome of both drugs metformin and donepezil shows improvement in spatial memory and learning in comparison to controls. In Aβ-treated group the average escape latency depicts the decrease in spatial memory recorded over period of five days. Aβ+Metformin and Aβ+ Donepezil treated group demonstrated better results and depicted improved learning curve than Aβ-treated group. Day 5 escape latency was depicted in separate graph.

Reference memory was assessed by probe trial test. To check the exploration time, time in each quadrant was observed. The results demonstrates that Aβ-treated group spent relatively less time in target quadrant with significant decrease ($p<0.001$) in reference memory as compare to control. The Metformin and Donepezil treatment post Aβ exposure presented better spatial memory as compared to Aβ-treated group. Due to the cognitive deficit, the number of platform crossings was less for Aβ-treated group in comparison to control group, whereas the number of platform crossing was significantly ($p < 0.05$) increased after treatment with Metformin and Donepezil.

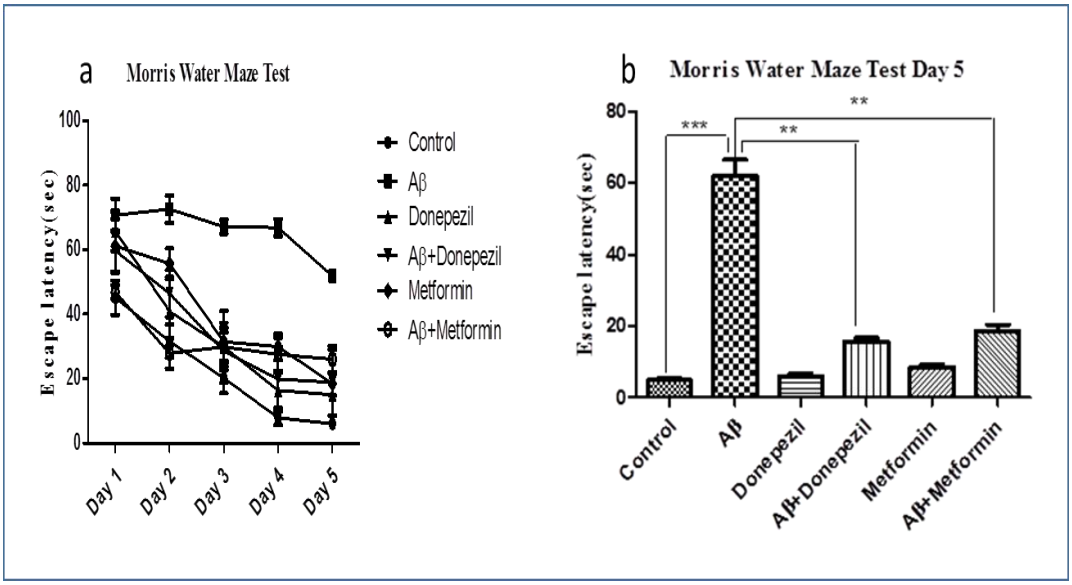


**Figure 4.1: Effect of Metformin and Donepezil on learning and memory using Morris water maze (MWM) test**: The graph demonstrates the escape latency (sec) to assess reference memory formation and learning among control, Aβ-treated, Aβ+ Metformin, Aβ+ Donepezil, Metformin and Donepezil-treated group. Aβ-treated mice demonstrated lower retention of spatial memory in comparison to control mice thereby finding platform much later than control mice. The data indicate that Aβ + Metformin and Aβ+Donepezil-treated mice find the platform relatively faster than Aβ-treated group. Error bars represent mean ± SEM

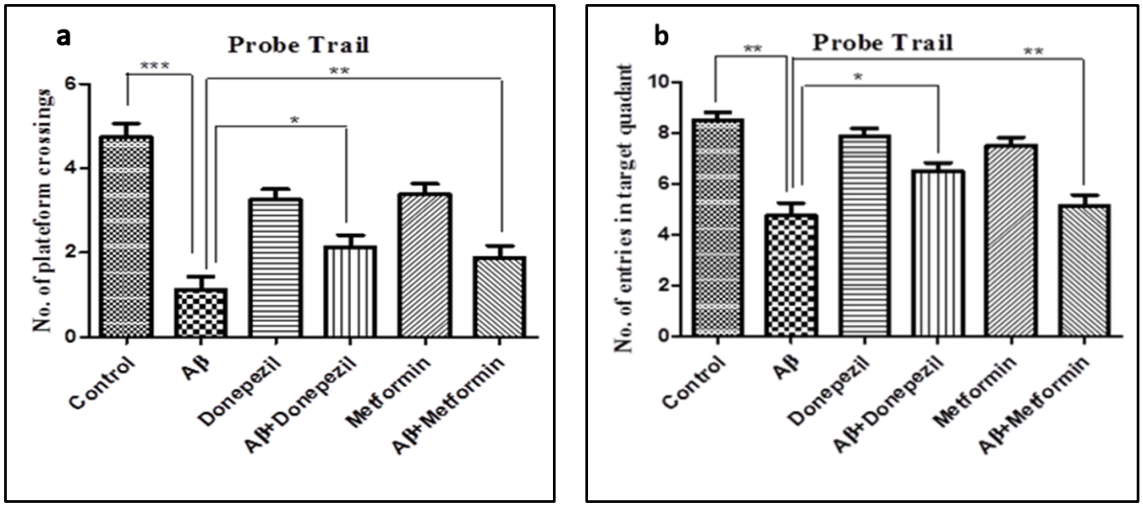


**Figure 4.2: Morris water maze: Probe trial**: The figure **(a)** depicts the number of platform crossings **(b)** No. of entries in target quadrant by Control, Aβ-treated group, Aβ+ Donepezil group, Aβ+Metformin, Metformin and Donepezil-treated group. The error bars represent mean ± SEM for One-Way ANOVA, followed by Bonferroni's multiple comparison test with $p < 0.05$ significance values.

### 4.1.2 Effect of Metformin and Donepezil on Recognition memory:

To assess the potential effect of Metformin and Donepezil on recognition memory and exploratory behavior by the exposure of different objects to subjected animal NOR task was conducted. This task consist of two sessions: first familiarized session that have two similar objects, second was test session that have one familiar object and one novel. The results from session one shows no significance but Aβ-treated group showed less interaction with both the objects (similar) as compared to control, whereas the interaction was improved after treating the Aβ-exposed group with metformin and donepezil. While session 2 results demonstrate that Aβ+Metformin and Aβ+Donepezil-treated groups shows significantly more interaction with novel object rather than familiar object (Figure 4.3)

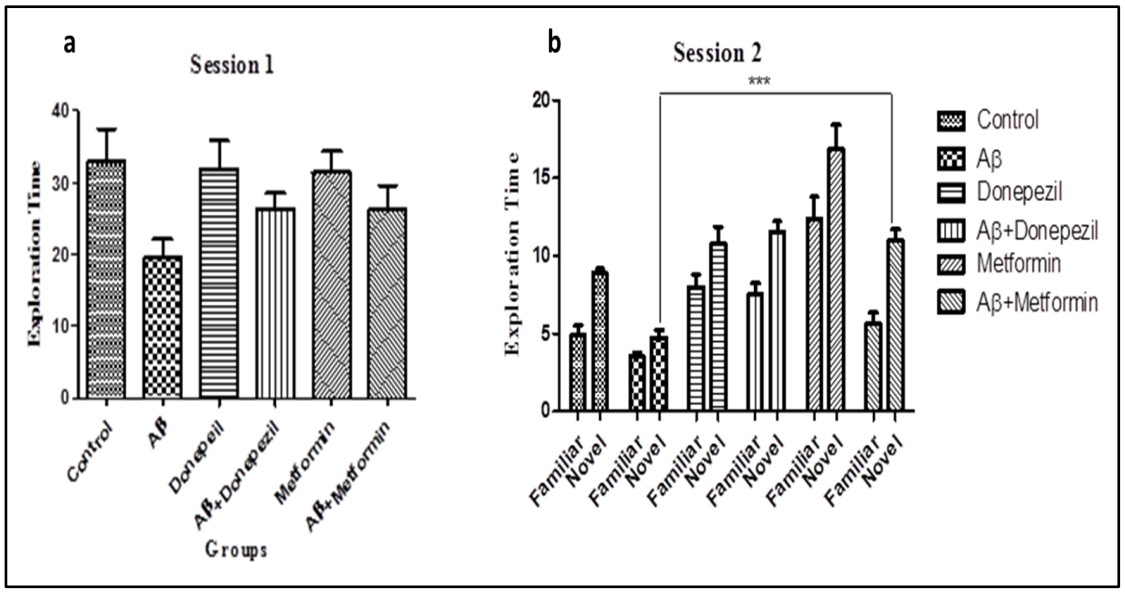


**Figure 4.3: Effect of Metformin and Donepezil on recognition-based memory and novelty**: **(a) Session 1**: to measure recognition memory. **(b) Session 2**: to measure novelty preference, by Control, Aβ-treated group, Aβ+ Metformin group, Aβ+Donepezil group, Metformin and Donepezil-treated group. Error bars represent mean ± SEM. $p < 0.05$ significance value.

### 4.1.3 Effect of Metformin and Donepezil on Anxiety and Activity:

Open spaces and height induced anxiety was evaluated by elevated plus maze test. In open arm Aβ-treated mice spent significantly less time ($p$<0.05) as compared to control depicting high level of anxiety. A significant improvement ($p$<0.001) was seen with Metformin and Donepezil treatment post Aβ exposure. Similarly there is a

significant difference in the number of entries to open arm of elevated plus maze (Figure 4.4).

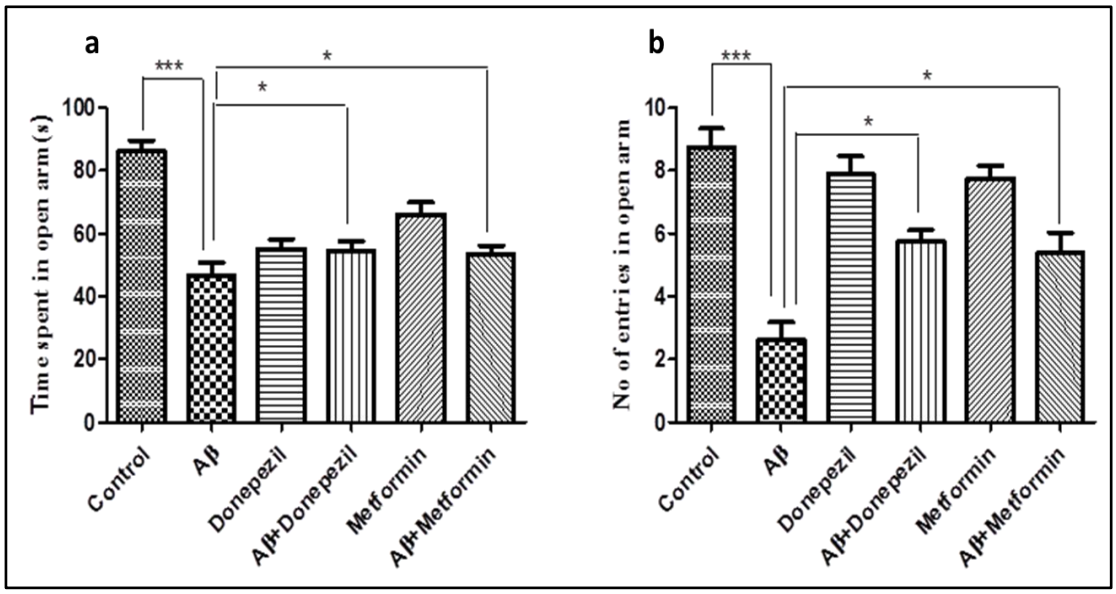


**Figure 4.4: Effect of Metformin and Donepezil on anxiety using elevated plus maze test: (a)** Time spent in the open arm. **(b)** Number of entries to open arm in elevated plus maze and Control, Aβ-treated group, Aβ+ Metformin group, Aβ+Donepezil group, Metformin and Donepezil treated group. The error bars represent mean ± SEM for one-way ANOVA, followed by Bonferroni's multiple comparison test. **= $p < 0.001$ significance value.

## 4.2 Histology of Brain

### 4.2.1 Haematoxylin and Eosin Staining

Neuronal degeneration was observed in H&E stained tissue sections. Dark neuronal pattern was observed in control whereas Aβ-treated tissue section shows less neuronal density in comparison to control. Metformin and donepezil treatment restore the cellular morphology and density after Aβ exposure as seen in Figure 4.5.

Quantitive representation of neurodegeneration was done by counting cell in the hippocampus. The neuronal density is significantly decreased ($p$<0.05) in the Aβ-treated group relative to control. An improvement ($p$<0.01) was observed in the cell number in the Aβ+metformin treated group and in Aβ+Donepezil treated group (Figure 4.6).

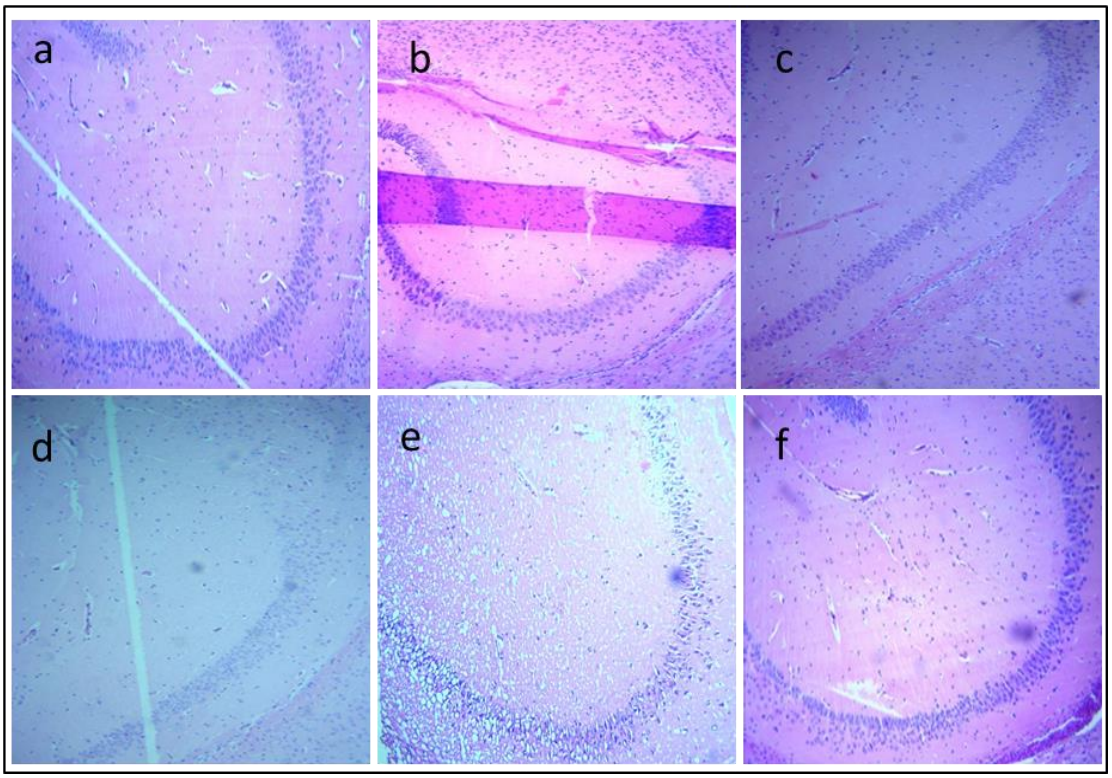


**Figure 4.5: H&E stained section of C- shaped Hippocampus: (**a) hippocampus of control group with pattern of dark neurons. (b) Donepezil treated group. (c) Metformin treated group. (d) Aβ-treated group depicts lesser neuronal density. (e) Aβ+Donepezil-treated group. (f) Aβ+Metformin-treated group. Magnifications at 10X.

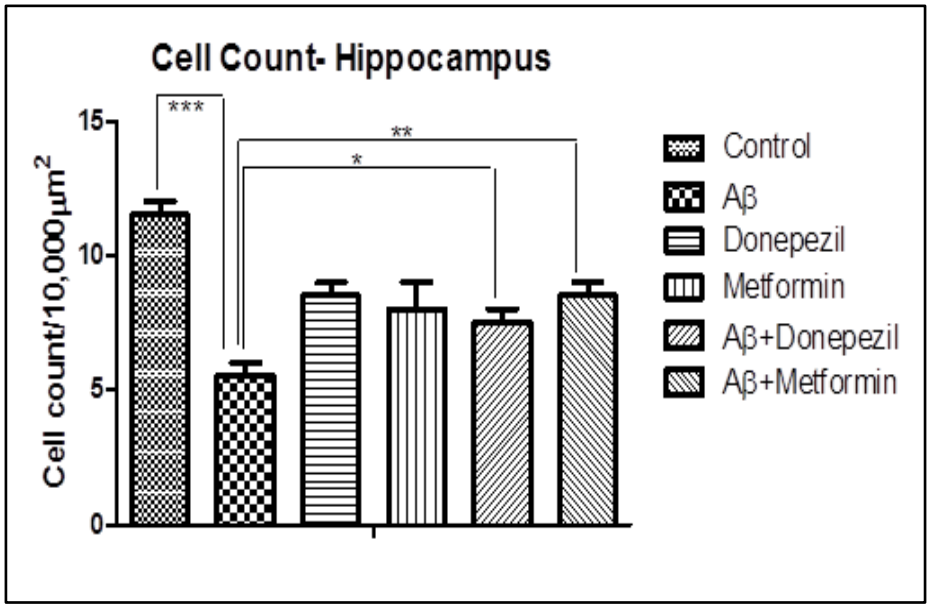


**Figure 4.6:** Histogram representing the cell number per 10,000 $\mu m^2$ in the hippocampus. The data were analyzed using One-Way ANOVA followed by Bonferroni Multiple Comparison Test and is shown as mean ± SEM.

### 4.2.2 Congo red staining:

Amyloid deposition was depicted by Congo red staining in tissue sections of hippocampus. No plaques were seen in control group. The Aβ-treated mice show the presence of plaques in the hippocampus that represent the neurodegeneration in brain. Whereas, in Aβ+Metformin and Aβ+Donepezil-treated groups no plaques were observed (Figure 4.7).

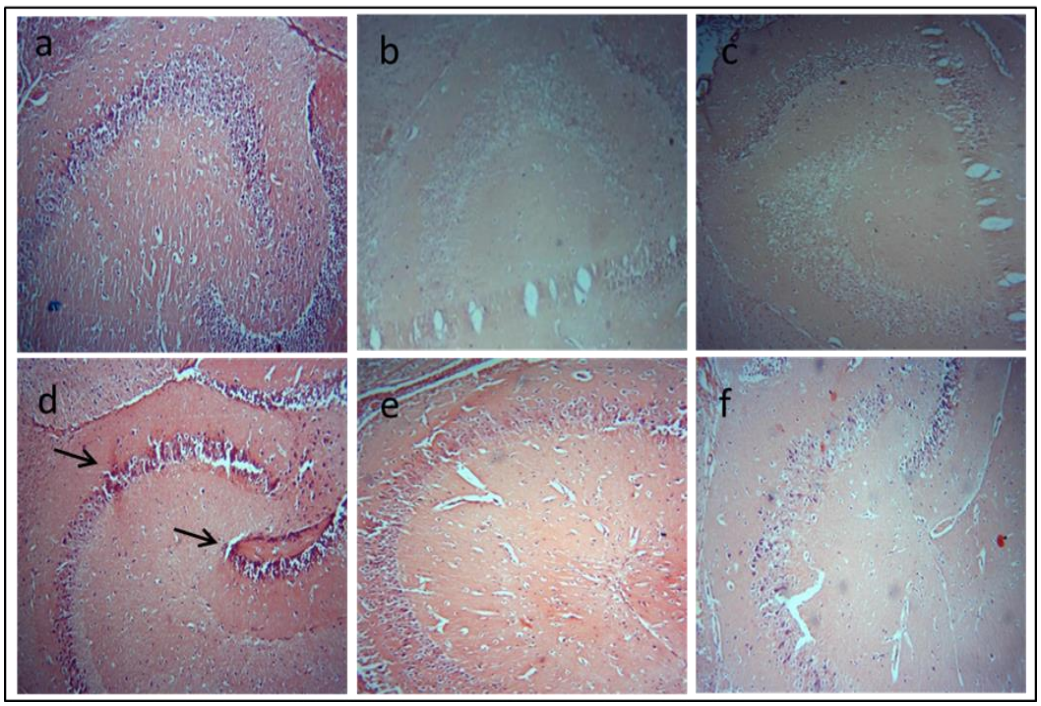


**Figure 4.7: Congo red stained sections of C- shaped hippocampus**: (a) Control group. (b) Donepezil-treated group. (c) Metformin-treated group. (d) Aβ-treated group. (e) Aβ+Donepezil group. (f) Aβ+Metformin group. Original Magnification 10X.

### 4.2.3 Immunohistochemical Evidence for Increased Neurogenesis in Hippocampus:

#### 4.2.3.1 Ki-67 immunoreactive neurons in Hippocampus:

Proliferative marker Ki-67 was used to study the neurodegenerative consequences in Aβ-treated mice and to evaluate the neuroprotective effect of Metformin and Donepezil administration. Hippocampal neurons were detected by immunoreactive Ki-67. No neuronal loss was observed in control. However considerable neuronal loss was detected in Aβ-treated group. Increased no of Ki-67 immunoreactive neurons was observed in both Aβ+Metformin and Aβ+Donepezil treated relative to Aβ-treated group. While no observable neuronal loss was seen in metformin and donepezil treated groups (Figure 4.8)

Quantitive representation of neurodegeneration was done by counting cell in the hippocampus. The neuronal density is significantly decreased ($p$<0.05) in the Aβ-treated group relative to control. An improvement ($p$<0.05) was observed in the cell number in the Aβ+metformin-treated group and in Aβ+Donepezil-treated group (Figure 4.9).

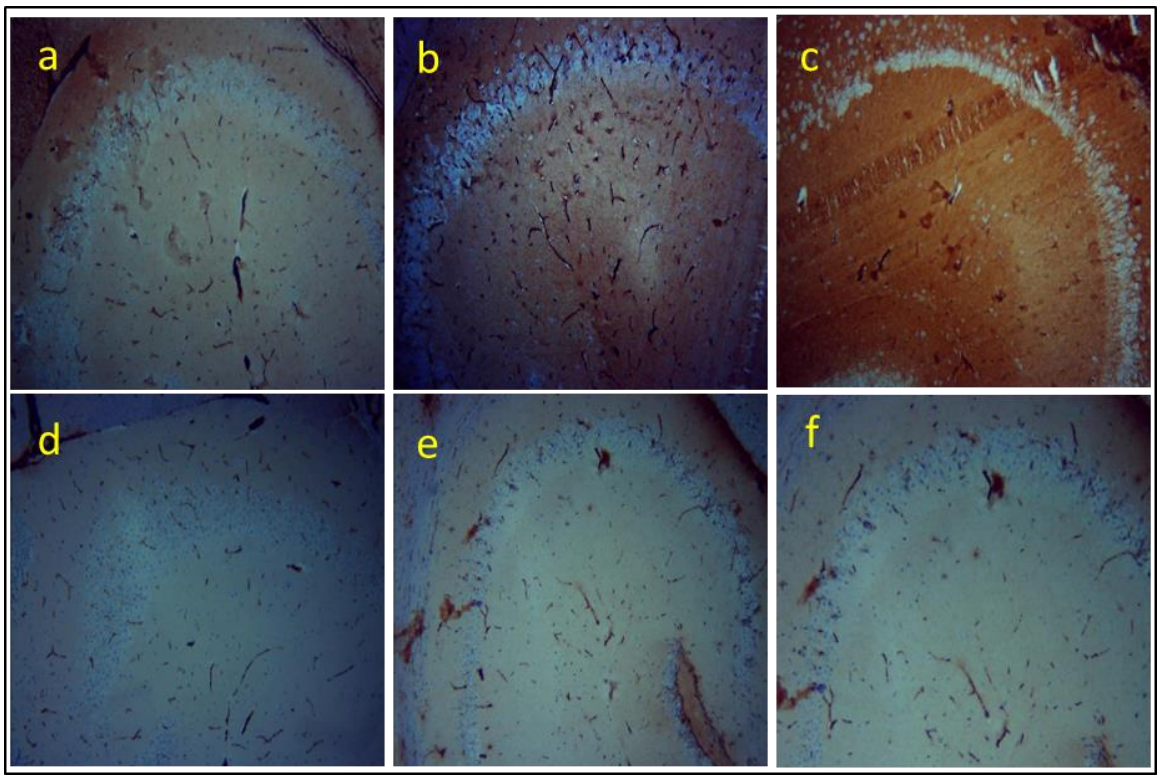


**Figure 4.8: Ki-67 neuronal Labeling of sections of C- shaped hippocampus: (a)** No neuronal loss was observed in Control. **(b)** Donepezil-treated group. **(c)** Metformin-treated group. **(d)** Substantial neuronal loss in Aβ-treated group. **(e)** Aβ+Donepezil. **(f)** Aβ+Metformin. Magnifications 10X.

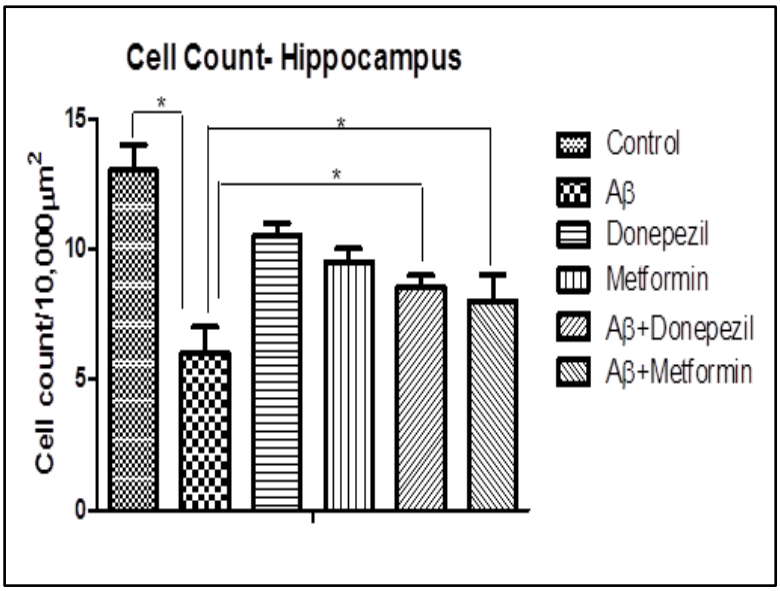


**Figure 4.9:** Histogram representing the cell number per 10,000 $\mu m^2$ in the hippocampus. The data were analyzed using One-Way ANOVA followed by Bonferroni Multiple Comparison Test and is shown as mean ± SEM.

### 4.2.3.2 NeuN immunoreactive neurons in hippocampus:

NeuN antibody was used to evaluate the effect of Aβ-treatment on neurogenesis in mice and to elucidate the neuroprotective effect of Metformin and Donepezil administration. Hippocampal neurons were detected by immunoreactive NeuN antibody. No neuronal loss was observed in control. However considerable neuronal loss was detected in Aβ-treated group. Increased no of NeuN immunoreactive neurons were observed in Aβ+Metformin and Aβ+Donepezil-treated relative to Aβ-treated group. While no observable neuronal loss was seen in metformin and donepezil-treated groups (Figure 4.10).

Quantitive representation of neurodegeneration was done by counting cell in the hippocampus. The neuronal density is significantly decreased ($p$<0.05) in the Aβ-treated group relative to control. An improvement ($p$<0.05) was observed in the cell number in the Aβ+metformin treated group and in Aβ+Donepezil treated group.

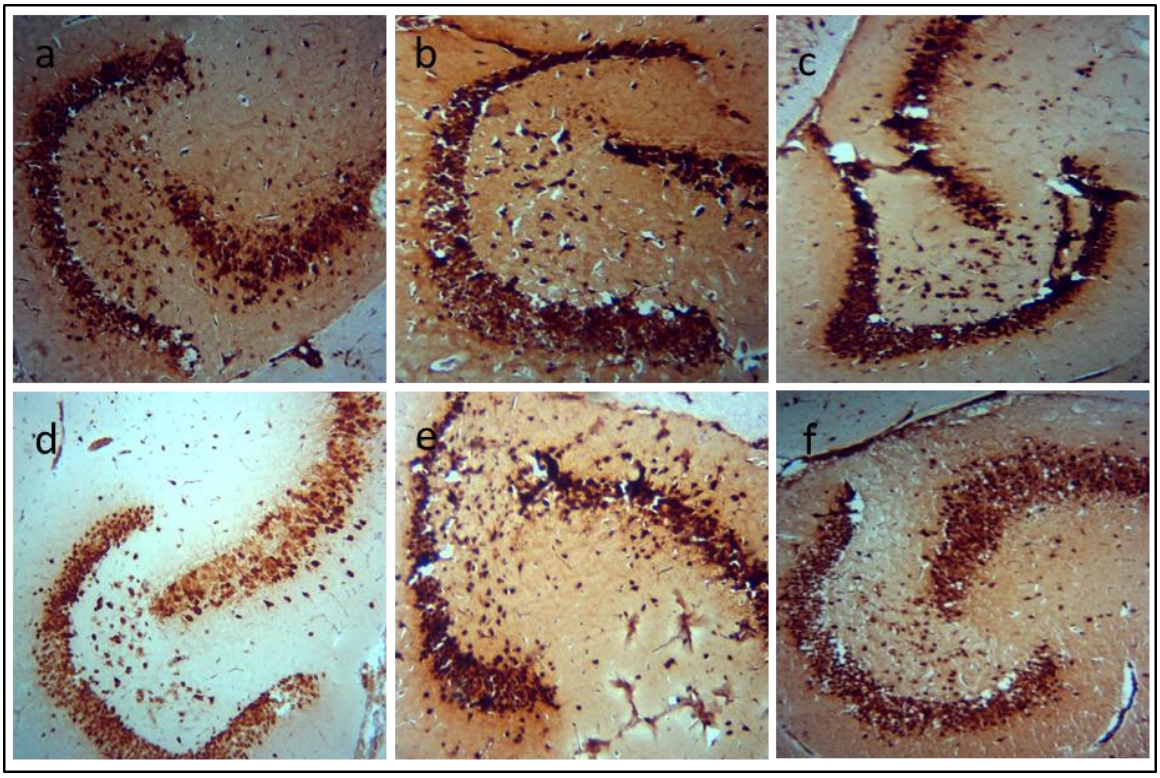


**Figure 4.10: NeuN labelled sections of C- shaped hippocampus: (a)** In Control no neuronal loss observed. **(b)** Donepezil-treated group. **(c)** Metformin-treated group. (d) Aβ-treated group shows less neuronal density. **(e)** Aβ+Donepezil-treated group. **(f)** Aβ+Metformin-treated group. Magnifications 10X.

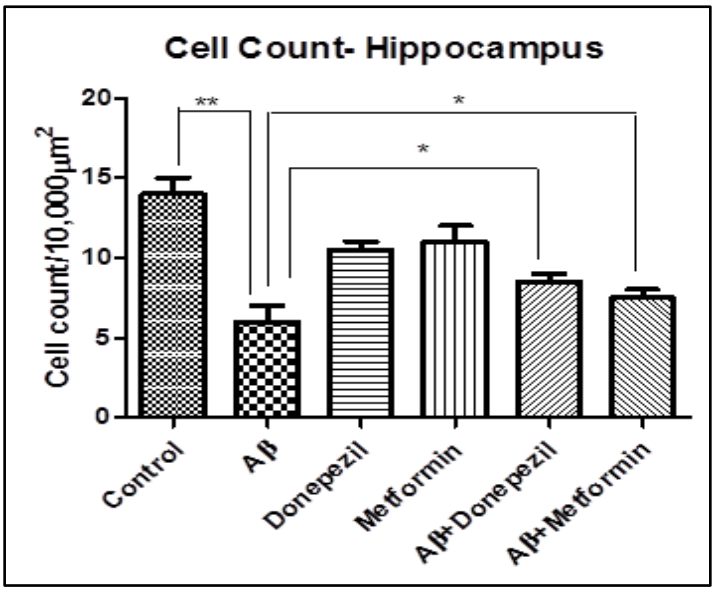


**Figure 4.11**: Histogram representing the cell number per 10,000 $\mu m^2$ in the hippocampus of the subjects. The data were analyzed using One-Way ANOVA followed by Bonferroni Multiple Comparison Test and is shown as mean ± SEM.

## 4.3 Transcriptional Analysis of Neurogenesis and Inflammatory markers:

### 4.3.1 Transcriptional Analysis of Ki67:

To observe the expression of neurogenesis quantitative real time PCR (qRT- PCR) was done. qRT-PCR results exhibit lower expression level of Ki67 in Aβ-treated group ($p$<0.001) in comparison to control group. While treatment with Metformin and Donepezil, post Aβ exposure depicts significant ($p$<0.001) increase expression level of Ki67. It is evident from the figure below that Aβ+metformin-treated group shows better results than Aβ-donepezil-treated group.

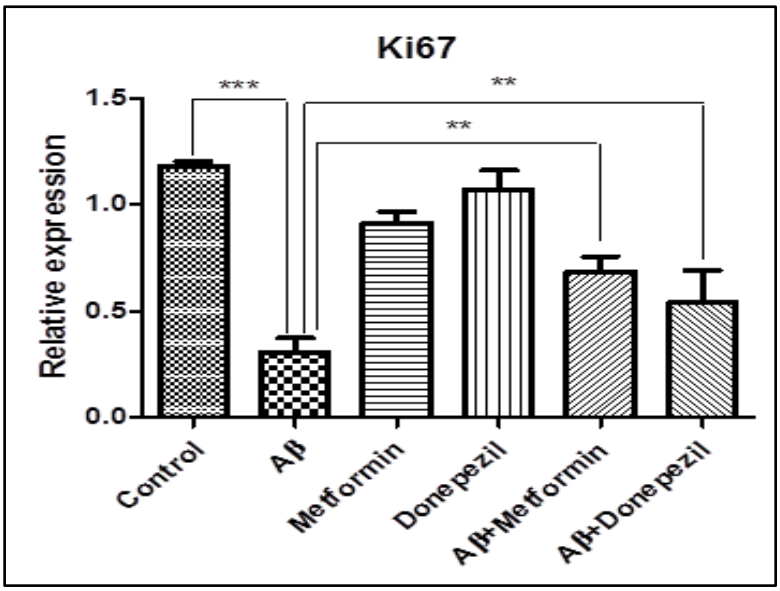


**Figure 4.12: Transcriptional analysis of Ki67:** Histogram representing transcriptional expression of Ki67 in control, Aβ-treated group, Aβ + Metformin-treated group, Aβ+Donepezil-treated group, Metformin and Donepezil treated group. The data is shown as mean ± SEM. $^{**}p<0.01$.

### 4.3.2 Transcriptional Analysis of NeuN:

In hippocampus, qRT-PCR results exhibit that the expression of NeuN was significantly decreased ($p<0.001$) in Aβ-treated group when compared to control group. The NeuN expression was significantly increased in Aβ+Metformin treated group than Aβ+Donepezil ($p<0.05$) treated group. While Aβ+Donepezil-treated group shows better results than Aβ-treated group. Both metformin and donepezil-treated group demonstrate increase expression level of NeuN as compared to Aβ-treated group.

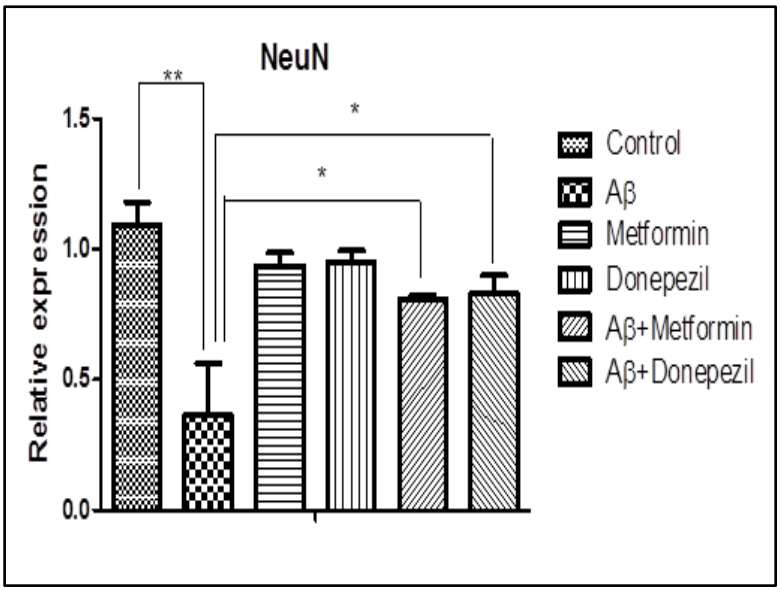


**Figure 4.13: Transcriptional analysis of NeuN:** Histogram representing transcriptional expression of NeuN in control, Aβ-treated group, Aβ + Metformin-treated group, Aβ + Donepezil-treated group, Metformin and Donepezil treated group. The data was analyzed by using One-Way ANOVA followed by Bonferroni Multiple Comparison Test. The data is shown as the mean ± SEM. To check the consistency samples were run in duplicates.

### 4.3.3 Transcriptional Analysis of Interleukin 6:

Expression analysis by qRT-PCR results demonstrated significantly increased ($p< 0.05$) expression level of IL-6 in Aβ group. In comparison to Aβ-treated IL-6 expression is significantly lower in control. However after Metformin and Donepezil administration, post Aβ exposure caused significant change in IL-6 gene expression. In comparison to Aβ-treated group, there was a significant lower IL-6 gene expression in Aβ+Metformin group. Aβ+Donepezil treated group also demonstrate a significant lower expression level of IL-6 as compared to Aβ group.

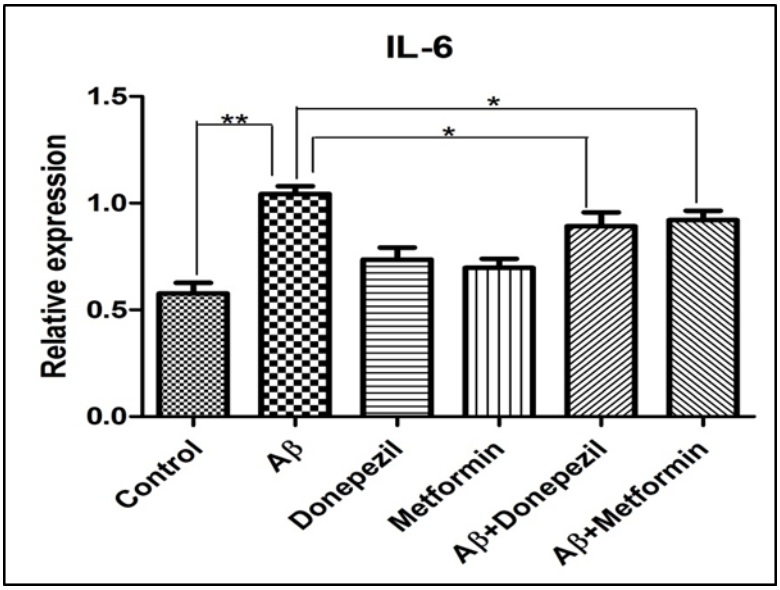


**Figure 4.14: Transcriptional analysis of IL-6:** Histogram representing the relative expression of IL-6 in control, Aβ-treated group, Metformin treated group, Donepezil treated group, Aβ+Metformin treated group and Aβ+Donepezil treated group. The data were analyzed using One-Way ANOVA followed by Bonferroni Multiple Comparison Test and is shown as mean ± SEM.

### 4.3.4 Transcriptional Analysis of Tumor Necrosis Factor Alpha:

Results analyzed by qRT-PCR demonstrate significant increase expression levels of TNF-α in Aβ-treated group in comparison to control. Whereas results of Metformin administration post Aβ exposure showed significant low expression level of TNF-α as compared to Aβ group. The Aβ+Donepezil treated group also depicts a significant decreased in TNF-α gene expression. Both Donepezil and Metformin treated group demonstrated decrease in the expression of TNF-α as compared to Aβ-treated group.

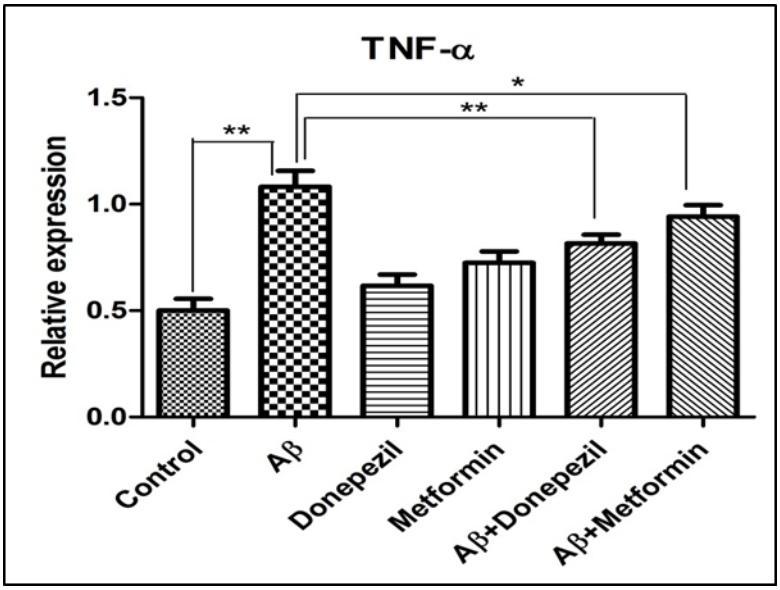


**Figure 4.15**: **Transcriptional analysis of Tumor Necrosis Factor alpha:** Histogram representing the relative transcriptional expression levels of TNF-α in control, Aβ-treated group, Metformin-treated group, Donepezil-treated group, Aβ+Metformin treated group and Aβ+Donepezil treated group. The data were analyzed using One-Way ANOVA followed by Bonferroni multiple comparisons test and is shown as mean ± SEM.

### 4.3.5 Transcriptional analysis of Glial Fibrillary Acidic Protein:

The expression level of GFAP analyzed by qRT-PCR were significantly increased ($p$<0.001) in Aβ-treated group as compared to control. After Metformin and Donepezil treatment to Aβ-treated group, results showed significantly low ($p$<0.05) expression levels in both groups.

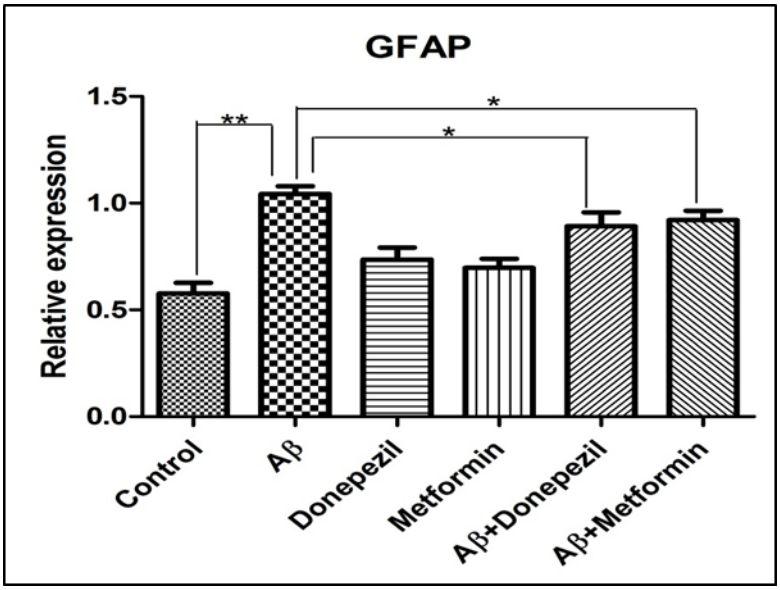


**FIGURE 4.16: Transcriptional analysis of GFAP**: Histogram representing relative transcriptional expression analysis of hippocampal GFAP in control, Aβ-treated group, Metformin-treated group, Donepezil-treated group, Aβ+Metformin-treated group and Aβ+Donepezil-treated group. The data were analyzed using One-Way ANOVA followed by Bonferroni multiple comparison test and is shown as mean ± SEM.

## *Chapter 5*

# DISCUSSION

Dementia most commonly caused by AD is the fourth most common cause of death. No definitive diagnostic and therapy is available for this debilitating neurodegenerative disorder (Glenner et al., 1984). The present study was undertaken with the aim to elucidate the neuroprotective effect of an anti-diabetic drug and acetylcholine esterase inhibitor on Aβ-induced neurodegeneration to comparatively assess the extent of improvement of AD associated symptoms by both these drugs.

In present study MWM test was performed to demonstrate the effect of exposure of Aβ, metformin and donepezil on spatial learning and memory. Throughout the course of learning phase, Aβ+Metformin and Aβ+Donepezil treated mice were successfully able to learn the location of hidden platform in comparison to Aβ-treated group that perform poorer during this test, as depicted by longer latency to reach the platform. These results are similar to those conducted previously on $AlCl_3$-induced neurodegeneration mouse model; Probe trial for testing memory on $6^{th}$ day also showed the similar findings as reported by Ahmed et al., (2017). These results demonstrate the potential of metformin to restore the spatial memory and learning that could be beneficial for symptomatic therapy for AD.

The observations recorded from the elevated plus maze test which is widely used to assess depression and anxiety (Daenen et al., 2003), showed significantly increase in Aβ- treated mice than in the control mice. The improvement in the anxiety levels showed by the Metformin-treated post Aβ exposure mice depicts the anti-anxiolytic potential of metformin. While similar effect was produced by the donepezil reflecting its ability to suppress the anxiety levels. However, how these two drugs specifically metformin; exerts its effects and what could be the exact molecular mechanism for its mode of action, still have to be explored further.

Novel object recognition task, conducted to assess the hippocampal mediated learning and memory formation provides further evidence on the potential of these two drugs. Rodents have the tendency to discriminate between similar and novel object and they are also able to interact more with novel object than similar object (Bevin and Besheer, 2006). Following Aβ exposure, mice exhibit decrease discrimination between similar and new objects during NOR task. Novelty seeking behavior was

reduced in Aβ-treated group. Aβ+Metformin and Aβ+Donepezil groups spent more time with novel object than familiar object that depicts their increase novelty seeking behavior. Improved preference for novel object in metformin treated groups can be attributed to its potential memory enhancing and neurogenic effects (Potts and Lim, 2012).

To further assess the effect of Aβ, metformin and donepezil on neurodegeneration and neuroprotection, histological and immunohistochemical analysis was performed. Neuronal degeneration was observed in Aβ-treated group. Histological alteration shown by H&E staining in Aβ-treated group was restored by metformin and donepezil post Aβ exposure. Congo red staining conform the formation of plaques in Aβ-treated group in comparison to control. While metformin and donepezil administration after Aβ treatment, cleared the plaque thus; explicating the significance of results.

Immunohistochemical results of Ki67 (proliferation) and NeuN (maturation) antibody labelling also shows promising results. As metformin and donepezil shows restoration of neuronal density post Aβ treatment validates its pro neurogenic potentials. These results in addition to previous data (Ahmed et al., 2015) indicate that metformin has a potential to rejuvenate the oxidative stress and morphological alteration associated with neurodegeneration and, promotes neurogenesis and enhances spatial memory formation in the adult mouse brain in a CBP-dependent fashion. The atypical PKC-CBP pathway is essential for neurogenesis and its precursors where metformin is considered to exert this action via activating this pathway (Wang et al., 2012) the reduce expression levels of Ki67 and NeuN in Aβ-treated group depicts the significant alteration in neurogenesis mechanism. While the restoration of these levels by metformin and donepezil provided the evidence of their neurogenic potential. The neurogenic effect of metformin could be explained by insulin - mediated Akt signaling pathway and consequent anti-apoptotic-associated protein synthesis that maintains cell viability so long so that they enter the late phase of neurogenesis; migration and maturation (Duarte et al., 2008; Dudek et al., 1997).

The neuroinflammation observed in the AD is due to the overexpression of these pro-inflammatory signals (Lee et al., 2010). These pro-inflammatory elements i.e. IL6, TNFα and GFAP when expressed, auto-regulate the microglial activities. The increase levels of IL6, TNFα and GFAP in Aβ-treated group and later decrease in

levels by metformin and donepezil shows the protective effects of these two drugs on inflammation.

# CONCLUSION

The present study has elucidated that Aβ-treated group depicts powerful detrimental effects consisting of diminished learning and memory and elevated anxiety. Treatment with metformin and donepezil demonstrated the positive impact on pathological hallmarks of AD. These preliminary findings on the effects of metformin and donepezil provide a baseline data to strategize an alternate combination therapy to combat the deleterious effects of AD. However in depth analysis is still required to explore the underlying mechanism of action of metformin in mediating these important neurological processes involved in AD pathology.

# *Chapter 6*

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