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Research Article
Metformin: New Insights into Alzheimer Disease Protection

[1]Rania M. Khalil, [2]Abla Ebeid, [3]Hassan Fayed and [4]Sherien Abd-Elhady

[1]Department of Biochemistry, Faculty of Pharmacy, Delta University, Gamasa, Egypt
[2]Department of Clinical Pharmacy, Faculty of Pharmacy, Delta University, Gamasa, Egypt
[3]Department of Biochemistry, Faculty of Pharmacy, Horus University, Damietta, Egypt
[4]Department of Pharmacology and Therapeutic, Faculty of Pharmacy and Drug Manufacturing, Pharos University, Alexandria, Egypt

Abstract

Background and Objective: Alzheimer disease is a major problem continues to increase worldwide. Insulin resistance has recently been implicated in Alzheimer disease pathogenesis as it decreases insulin degrading enzyme and therefore induces the accumulation of amyloid beta. The aim of the study was to explore the effect of metformin in the brain of Alzheimer Disease (AD) rat model. **Materials and Methods:** Alzheimer disease was induced chemically in rats by adding 6 mg L^{-1} copper sulphate ($CuSO_4$) in drinking water. Metformin-treated group in which (150 mg/kg/day) metformin was given orally to Alzheimer induced rats for 28 days. Rat's behaviour was assessed by the Morris water maze test. Histopathology of the hippocampus of neurofibrillary tangles, amyloid beta and insulin-degrading enzyme levels were evaluated by enzyme-linked immunosorbent assay in hippocampus tissues. **Results:** The amyloid beta level was significantly reduced while insulin degrading enzyme level was significantly elevated in the metformin-treated group compared with Alzheimer disease rat model. **Conclusion:** It is concluded that the findings of the present study have proposed for the first time the potential effect of metformin in protecting against copper producing senile plaques that leads to Alzheimer disease.

Key words: Amyloid-β, copper sulfate, insulin-degrading enzyme, metformin, neurofibrillary tangles

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Corresponding Author: Rania M. Khalil, Department of Biochemistry, Faculty of Pharmacy, Delta University, Gamasa, Egypt Tel: 01007789860

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Alzheimer Disease (AD) is a neurodegenerative disorder affecting elderly people and continues to increase worldwide. The most pathological findings in the brain of AD patients are extracellular amyloid plaques, intracellular neurofibrillary tangles and loss of neurons. The AD patients are characterized by impaired memory and dementia[1,2]. The main component of the extracellular senile plaques is an amyloid beta peptide (Aβ) which is produced by sequential proteolytic cleavage of the Amyloid Precursor Protein (APP) via 2 enzymes: β-secretase and γ-secretase. These enzymes activity are elevated in AD brain leading to accumulation and deposition of Aβ[3]. On the other hand, tau protein is also synthesized in all neurons. Abnormal tau hyperphosphorylation leads to its accumulation forming neurofibrillary tangles (NFTs) and neuronal atrophy. It has been suggested that accumulation of Aβ stimulates tau hyperphosphorylation and NFTs formation. The aggregation of Aβ and hyperphosphorylated tau proteins is the major hallmarks of AD[4,5].

Insulin Degrading Enzyme (IDE) is involved in degradation of both insulin and Aβ. The brain is enhanced to uptake glucose by insulin which also increases the secretion of IDE for its degradation. Interestingly, insulin signalling increases the synaptic density and induces clearance of Aβ from the brain. Thus insulin resistance or impaired insulin signalling may result in decreased IDE and accumulation of Aβ.

Subsequently, it is not surprising that AD could be treated by insulin[6,7]. Insulin resistance and oxidative stress increase the production of Advanced Glycation End-products (AGEs) which are resulting from glycation of Aβ peptides. Several studies reported that AGEs is associated with oxidative stress and thus promote cellular damage. Moreover, measurement of AGEs level in blood and cerebrospinal fluid have been proposed for early detection of AD. Conversely, insulin regulates glucose uptake in the brain, promotes normal function of mitochondria and thus protects against oxidative stress, production of AGEs and neuron damage[8,9].

Failure of treatment of AD by targeting Aβ peptide and tau protein and the lack of understanding the etiology of AD suggesting further studies to explore other pathways involved in the neurodegeneration diseases. The relationship between AD and diabetes and the encouraging trials of treatment of AD by insulin recommend the potential effect of anti-diabetic drugs in managing AD[9].

The presence of insulin receptors in the brain including hippocampus indicated the importance of insulin signalling for normal brain function. Metformin sensitizes neuronal insulin receptors and improves the binding of insulin with its receptors. It has an anti-inflammatory and anti-oxidative effect. It has also been found that metformin has a neuroprotective effect among diabetic patients suffering from dementia[10]. Metformin is a crucial anti-diabetic drug however linking this effect in improving cognitive function in AD was not so far investigated. Our study aimed to explore the effect of metformin in the brain of AD rat model.

MATERIALS AND METHODS

Study area: The study was carried out at Department of Biochemistry, research lab from Jan, 2019-2020.

Supplement: Copper sulphate powder was purchased from El-Nasr Pharmaceutical Chemical Co., Egypt. Metformin hydrochloride powder was purchased from Sigma Chemical Co., USA. Rat Aβ and IDE ELISA kits were obtained from Bioneovan Co., Ltd., China.

Animals and experimental protocols: Thirty male Wister rats weighing (150-200 g) were obtained from the Faculty of Pharmacy, Pharos University in Alexandria, Alexandria, Egypt, acclimatized for one week and provided with free access to food and water. All animal's manipulations were performed in accordance with the guidelines from the Canadian Council on Animal Care (CCAC). Animals were randomized and divided into three groups, each contained 10 animals: Control group, rats received tap water, AD rat model, AD was induced in rats by drinking water contained 6 mg L^{-1} $CuSO_4$ for 60 days according to Awaad *et al.*[11] and metformin-treated group, rats supplied by drinking water contained 6 mg L^{-1} $CuSO_4$ for 60 days then metformin (150 mg/kg/day) was given orally and continued for another 28 days[12].

At day 29, rats were anaesthetized by diethyl ether then sacrificed. The hippocampi were carefully dissected and were divided into 2 portions. One portion was fixed in 10% formalin for histopathology. The second portion was kept at -80°C to estimate insulin degrading enzyme and amyloid β by enzyme-linked immunosorbent assay.

Assessment of cognitive deficits by Morris Water Maze (MWM) test: The MWM test is one of the most commonly used animal models to assess learning and memory. Briefly, an animal is being placed in a large circular pool (180 cm diameter×60 cm height) filled with water (22±2°C) to a depth of 40 cm and divided into four equal quadrants. A platform 15 cm in diameter was submerged approximately

2 cm beneath the water surface, in the centre of 1 of the 4 quadrants. The animal tendency to escape was accomplished by finding a hidden platform as the animal dislikes swimming. The water was made opaque by adding milk powder to hide the platform[13].

After the specified period of treatments, Each rat of the three studied groups was subjected to four consecutive training trials with four different starting positions (with an inter gap of 5 min) each day for four consecutive days in search for the hidden platform in the circular pool. A digital watch was used to record the average time taken by a rat to reach the platform. The 1st day was recorded as initial acquisition latency up to a maximum of 1 min. The 2nd, 3rd and 4th day escape latency time was taken as the index of acquisition or learning to locate the hidden platform in the water maze. To avoid rat exhaustion and confounding memory behaviour, different rats in each experimental group were subjected to the test.

Histopathology of brain hippocampus: Hippocampus samples were trimmed, passed with a serial passage of ethyl alcohol, cleared in xylene, embedded in paraffin and then sectioned at 5 µm slices (Microtome, Leica RM2155, Leica Inc., Nussloch, Germany). The sections were stained by Haematoxylin and Eosin (H&E). Histological analyses were done under light microscopy (Olympus Electron Microscope, Olympus, Japan).

Determination of hippocampus amyloid β (Aβ) and Insulin Degrading Enzyme (IDE) levels: Brain hippocampus tissue was homogenized in PBS (10 mg to 100 µL PBS). After that, homogenates were centrifuged for 20 min at 2000 rpm. Supernatants were collected immediately for Aβ and IDE measurement using rat Aβ and IDE ELISA kits, respectively by a microplate reader (Tecan, Infinite 200 PRO, Switzerland) at 450 nm. The Aβ and IDE levels were calculated according to the standard curve as described in their kits.

Statistical methods: Data were fed to the computer and analyzed by using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov, Shapiro and D'Agostino tests were used to verify the normality of distribution of variables. ANOVA was used to compare between more than two groups for normally distributed quantitative variables followed by *Post Hoc* test (Turkey) for pair wise comparison. Pearson coefficient was used to correlate between quantitative variables. Significance of the obtained results was judged at the 5% level[14].

RESULTS

Morris water maze test: Data presented in Table 1 assessed the cognitive function of rats in different groups by MWM test which revealed that the control rats progressively learned to locate the hidden platform in a significantly shorter time ($p<0.05$), throughout the 4 days. The metformin-treated group showed a significant decrease ($p<0.05$) in latency to reach the hidden platform in day 4 compared to day 1. However, the AD rat model showed a significant increase in time ($p<0.05$) to reach the hidden platform in days 3 and 4 compared to day 1. Moreover, the metformin-treated group showed significant improvement to reach the hidden platform when compared with AD rat model in days 2, 3 and 4 of training.

Histopathological findings: Histological staining by H&E revealed normal hippocampus tissue in the control group in which the pyramidal cells were arranged normally in 5-6 compact layers (Fig. 1a). Additionally, molecular layer shows

Table 1: Learning ability of rats in different groups by morris water maze test

Groups	Day 1	Day 2	Day 3	Day 4
Normal control	40.05±1.8	29.50±1.1$^{\#,a}$	17.05±7.0$^{\#,ab}$	08.38±2.0$^{\#*,abc}$
AD rat model	48.20±1.5	50.09±1.2	61.93±2.6^{ab}	67.26*±3.5^{abc}
Metformin treated group	44.22±1.4	39.01±1.6$^{\#}$	33.90±2.0$^{\#}$	29.00±1.9$^{\#,a}$

The learning ability of rats expressed in sec among 4 successive days assessed (n = 10), data are presented as Mean±SD. $^{a}p<0.05$ significant versus the 1st day within the same group, $^{b}p<0.05$ significant versus the 2nd day within the same group, $^{c}p<0.05$ significant versus the 3rd day within the same group, $^{\#}p<0.05$ significant versus AD rat model within the same day, *$p<0.05$ significant vs. metformin treated group within the same day

Table 2: Hippocampus concentration of Aβ and IDE in different groups measured by ELISA (n = 10)

Parameter	Control group	AD rat model	Metformin treated group
Level of Aβ (ng mL^{-1}) Mean±SD	00.10±0.02^{a}	00.13±0.01	00.11±0.01^{a}
Level of IDE (ng mL^{-1}) Mean±SD	40.09±3.6^{a}	33.08±1.01	39.04±4.01^{a}

Data are presented as Mean±SD, ^{a}Statistically significant at $p\leq0.05$ vs. AD rat model group

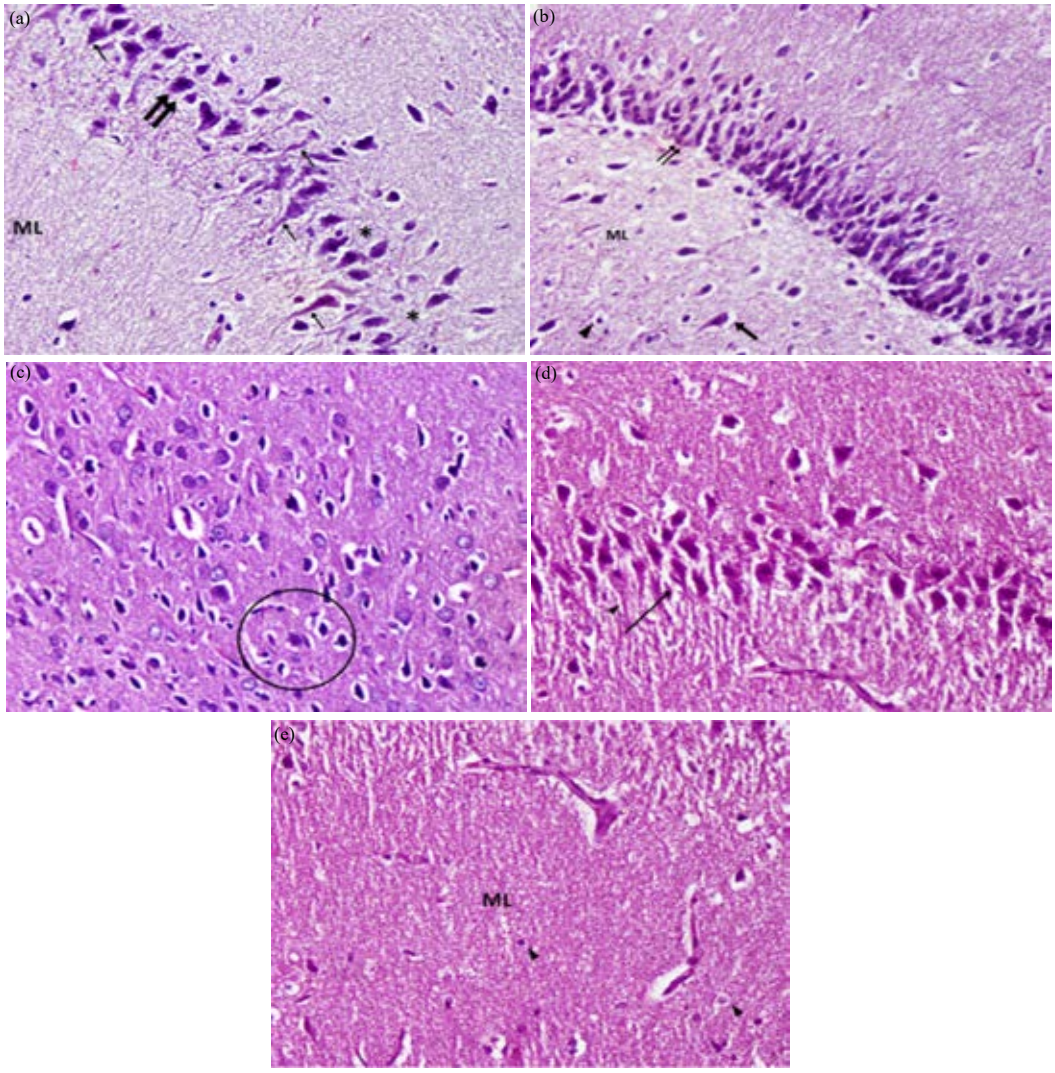

Fig. 1(a-e): Photomicrograph of hippocampus (H and E×400), (a) Control group, it shows 5-6 compact layers of normal pyramidal cells (double thin arrow). Molecular Layer (ML) shows many glial cells (arrow head) among neuronal processes as well as pyramidal cells (thick arrow), (b) Positive control group, Decreased thickness of pyramidal cell layer reaching 2 layers (double thin arrow). Some areas are devoid of cells. Notice neurofibrillary tangles in neuron cells (thick arrow). ML: Molecular layer, (c) Positive control group, Senile plaques in molecular layer (circle), (d) Metformin-treated group, Pyramidal cell layer (arrow) with few neurofibrillary tangles (arrow head) in neuron cells, (Metformin treated group) and (e) Metformin treated group, Molecular Layer (ML) with glial cells (arrow head)

many glial cells among neuronal processes as well as pyramidal cells. However, hippocampus histopathological damage was observed in the copper-induced AD group. The pyramidal cell layer thickness was decreased reached to 2 layers, with many areas devoid of cells and neurofibrillary tangles were noticed (Fig. 1b). Neurons with senile plaques in molecular layer were also observed (Fig. 1c). These abnormalities were attenuated by metformin treatment in which the cells had better morphology and more neuron cells were present compared to the untreated group. Also, glial cells were shown in the molecular layers. However, the pyramidal cell layer showed few neurofibrillary tangles in neuron cells of the metformin-treated group compared to the control group (Fig. 1d, e).

Effect of metformin on amyloid β (Aβ) concentration in hippocampus tissue: The level of Aβ in hippocampus tissues of AD rat model was significantly increased ($p<0.05$)

compared with the control group. There was a significant decrease ($p<0.05$) in Aβ level in the metformin-treated group compared to AD rat model (Table 2).

Effect of metformin on IDE concentration in hippocampus tissue: Insulin Degrading Enzyme (IDE) concentration was significantly decreased ($p<0.05$) in hippocampus tissues of AD rat model compared with the control group. While metformin-treated group showed significant ($p<0.05$) increase in IDE compared with AD rat model although the level of IDE did not reach to its level in the control group (Table 2).

DISCUSSION

Alzheimer Disease (AD) is a common form of dementia related to loss of memory and brain atrophy. Senile amyloid plaques, as well as NFTs containing hyperphosphorylated tau protein are histopathological findings in the brain of AD patients and indicating neurodegeneration[15]. It is noteworthy that hippocampus tissue damage appeared as decreasing in the pyramidal cell layer thickness and increasing of NFTs in neuronal cells together with the loss of neuron cells in some brain area in an AD rat model. These findings were in agreement with the previous study which revealed that the ingestion of a large amount of copper sulphate is one of AD risk and this problem appeared mostly in developed countries[16].

The administration of oral metformin showed an increase in the pyramidal cell and glial cell in the brain area as well as decrease quantity of NFTs inside the neuron cells. The present results were in agreement with the previous study which stated that other biguanides have therapeutic potential in AD treatment[17]. This is harmonized with other studies that showed the metformin effectiveness in attenuated tau phosphorylation by decreasing activity of tau kinase and increasing autophagy[18,19]. On the other hand, another study showed that the dephosphorylating tau protein by metformin treatment does not translate to decrease tau aggregation in the neurons which may exacerbate brain atrophy by promoting tau aggregation[20].

Indeed, the pathological changes that happened in AD rat model are assessed herein by the MWM test. Metformin improved the cognitive function in metformin-treated rats group. This coincides with the previous study that correlated the protection against memory impairment and the modulation of tau phosphorylation in scopolamine-induced memory deficit model to the administration of oral metformin. It also revealed the anti-inflammatory and anti-oxidative stress of metformin treatment[21]. Ahuja *et al.*[22] discovered that the possible effect of metformin to ameliorate cognitive deficit based on its therapeutic mechanisms. Collectively the beneficial mechanisms of metformin to attenuate cognitive disorders are; decrease the total tau protein that crosses rapidly the blood-brain barrier and activates AMPK enhancing cell metabolism and learning function, decreases AGEs, improves mitochondrial function and decreases oxidative stress together with increases neuron cell viability and density of synapse[22].

Actually, AD pathogenesis is initially triggered by the presence of extracellular Aβ proteins and its accumulation to form senile plaques which are found to produce oxidative stress and neurotoxicity in the brain[23]. The study showed a significant increase in the level of Aβ in AD rat model compared with the control group. The level of Aβ is significantly decreased by administration of oral metformin in the metformin-treated group compared with the untreated rats in the AD rat model. These results were in line with the previous study which reported that metformin reduces β-secretase which cleaves APP to produce Aβ thus decreases Aβ generation and accumulation[24].

Other study suggested that lysosomal autophagy process is altered in the brain of AD patients resulting in accumulation of neurotoxic Aβ proteins. Metformin increases autophagy via AMPK activation and induces Aβ clearance, however, this hypothesis is still controversial[25]. Moreover, metformin increases the efflux of Aβ across the BBB, therefore, protects neurons from the accumulation of neurotoxic senile plaques and attenuates brain atrophy[26]. In contrary another study showed that metformin increased the activity of γ-secretase in autophagosomes resulted in more Aβ generation. At this point, metformin may have expected opposite effects either delaying or enhancing neurodegeneration[27].

Evidence from recent studies showed the relation between AD and T2DM explained the common mechanisms involved in the pathology of both diseases. Insulin resistance is the most common cause of T2DM also exists in the brain of AD patients and exacerbates the accumulation of Aβ. Insulin resistance competitively inhibits IDE which is an important regulator of Aβ clearance[28]. Our finding revealed that the IDE level was significantly decreased in AD rat model compared with normal control and metformin-treated groups. These results were in agreement with other genetic researches which reported that the expression of IDE is reduced in late-onset AD brain and expressed its result as first documentation[29].

Moreover, genetic researches explained that AD is associated with variation of the IDE gene and the development of IDE activator could be of value in the effective treatment of AD[7,25]. Cell line studies reported that metformin sensitizes neuron cells to insulin and therefore prohibits AD pathology. However, knowledge about the effect of metformin in the CNS is relatively insufficient[28]. In the present study, the IDE level was significantly increased in the metformin-treated group compared with AD rat model. Metformin decreases insulin level in the brain and thus activates IDE to degrade Aβ. This therapeutic benefit of metformin in the treatment of AD encourages other trials to develop a successful treatment. Therefore, targeting brain insulin signalling may be promising in the treatment of AD. The present data raise the hope that metformin would have a neuroprotective effect through elevation of IDE level.

CONCLUSION

The current study proved that metformin as an insulin-sensitizing drug has a positive impact in increasing the level of IDE which enhances the clearance of Aβ, therefore improves the cognitive dysfunction associated with AD. However, more studies are needed to investigate molecular mechanism of metformin in the brain of AD patients.

SIGNIFICANCE STATEMENT

This study discovers the use metformin that can be beneficial for improvement of the cognitive dysfunction connected to Alzheimer disease. Correspondingly, the study will assist the researchers to find the serious extents of the influence of metformin in increasing the level of IDE which augments the clearance of Aβ that various researchers were not capable to sightsee. Thus, a coming eventual, further studies to examine molecular mechanism of metformin in the brain of Alzheimer patients may be arrived at.

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