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Azilsartan ameliorates aluminium chloride induced Alzheimer's disease like pathology

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease manifested with accumulation of neurotoxic proteins like beta-amyloid (Aβ) and hyperphosphorylated tau. Administration of angiotensin receptor blockers (ARBs) such as Telmisartan has demonstrated to generate significant memory improvement in AD. Azilsartan is an ARB with better bioavailability than Telmisartan. Hence, the present work evaluates the efficacy of Azilsartan against aluminium chloride ($AlCl_3$) induced AD. In the work, albino rats were divided into five groups (n=6). Group I served as control and received saline (10 ml/kg). Group-II was treated with $AlCl_3$ (100 mg/kg) for 42 days; Group-III and IV received Azilsartan (5 mg/kg) and Telmisartan (10 mg/kg) with $AlCl_3$ daily for 42 days. Y-maze, elevated plus maze and radial arm maze were used to evaluate memory functions. This was followed by biochemical and histological studies, along-with determination of Aβ content and anti-oxidant status. $AlCl_3$ was found to significantly ($p < 0.05$) reduce cognition and increased concentration of Aβ in a hippocampus with elevated lipid peroxidation levels. It also significantly ($p<0.05$) decreased superoxide dismutase and increased malondialdehyde content. However, brain histology showed presence of neurofibrillary tangles, neuronal dead cells, and pyknotic cells compared to normal group. Still, Azilsartan and Telmisartan significantly ($p<0.05$) reversed cognitive dysfunction, improved antioxidant status and decreased Aβ production. Thus we conclude that Azilsartan protects $AlCl_3$ induced AD-like pathology but, to a degree less than Telmisartan.

INTRODUCTION

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disease which occurs in a gradual manner and affects memory, thinking abilities and behaviour [1]. It is considered popularly as the common cause of dementia. AD is characterized by accumulation of extracellular senile plaques consisting of amyloid beta (Aβ) protein aggregation and phosphorylated tau proteins and neurofibrillary tangles in the cerebrocortices and hippocampal regions in the midbrain [2,3]. Other factors (inflammation, oxidative stress, genetics and ecological factors like aluminium toxicity) are also responsible for AD. Existing medications only provide symptomatic benefits and have no significant role in disease modification [4]. Hence, an alternative strategy of repurposing can be used to inhibit neurodegeneration process and other pathological complications associated with AD.

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Many studies have revealed that chronic administration of Aluminium (Al) results in AD-like symptoms, and aluminium (Al) is considered an environmental contributor to the pathogenesis of AD [5,6]. It has been shown that aluminium accumulates in the hippocampus of the rat brain (the site of memory and learning) and brings about cognitive impairment and dementia. Al also increases the permeability of the blood brain barrier to β-endorphin, which impairs memory and learning [7]. Moreover, Al intake is known to significantly decrease ACh content in mice brain. This effect may be one of the important mechanisms of the neurotoxicity for AD [8]. Al-induced neurotoxicity also develops oxidative stress by increasing the content of some metals (copper and iron). Al is mainly deposited in the hippocampus after chronic administration [9]. AD is one of the possible long-term effects of chronic Al exposure. The chronic application of Al induces accumulation of Aβ protein in cultured neurons of rat cerebral cortex and in neuroblastoma cells [10].

The renin angiotensin system (RAS) regulates blood volume, systemic vascular resistance and blood pressure [11]. It is also involved in AD pathogenesis. Inhibition of an over expressed RAS system is probably important in improving cognitive alteration associated with AD [12]. Angiotensin Receptor Blockers (ARBs) are prominent inhibitors of the RAS system and are used for the treatment of hypertension. They were reported to promote neurogenesis by inducing neurite outgrowth and decreasing neural damage [13]. Telmisartan, an angiotensin II type 1 receptor (AT1R) blocker (ARB), showed a preventive effect against amyloid beta protein extracellular deposition and endothelial dysfunction [14,15]. It also possesses protective effect against oxidative stress, inflammation and apoptosis in rat models of intracerebral haemorrhage [16]. Moreover, it was reported that Telmisartan administration had demonstrated improvement in cognitive alteration in an AD mouse model through its AT1R blockade effect, as well as by stimulation of the peroxisome proliferator-activated receptor-γ (PPAR-γ) [17,18].

Most research on ARBs against AD disease is limited to Telmisartan. Indeed, thorough literature survey has not reveal the activity of any other angiotensin receptor blocker against AD. Telmisartan has 42-58% bioavailability, whereas Azilsartan (another ARB) holds 60% bioavailability [19]. In this research, we investigated Azilsartan so to evaluate its efficacy against $AlCl_3$ induced Alzheimer's disease-like pathology. We chose Telmisartan as standard.

METHODOLOGY

Animals

Wistar albino rats of both sex (120-200 g) were raised from the Central Animal House Facility of School of Pharmaceutical Sciences, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar. All animals were kept under standard environmental conditions (25±3°C temperature, 45-55% relative humidity and light and dark cycle of 12 hr). Food and water were provided *ad libitum*. All the experimental protocols were approved by the Institutional Animal Ethical Committee of School of Pharmaceutical Sciences, Siksha O Anusandhan University (Approval No - IAEC/SPS/SOA/13/2020), and ethical guidelines were strictly followed during the experiments. These animals were acclimatized for 48 hours to adopt to the newer environment before the experimental work.

Chemicals

Azilsartan and Telmisartan were received as gift samples from Aurobindo (Telengana, India), Alkem (Sikkim, India), respectively. All the chemicals used for experimental purpose are of laboratory grade.

Experimental Design

All the rats were divided into four groups containing six in each. Group-I (Control) rats received saline (10 ml/kg) once daily for 42 days. Group II received $AlCl_3$ (100 mg/kg, p.o.) once daily for 42 days. Group III and IV received Azilsartan (5mg/kg p.o.) and Telmisartan (10 mg/kg p.o.), respectively, 1hr before the administration of $AlCl_3$ (100 mg/kg, p.o.) once daily for 42 days. Behavioural tests were done on day 1 and day 42. On day 43, animals were sacrificed for the biochemical estimations and histological studies.

Behavioural study

Y-maze

The Y-maze procedure was used to measure the spatial working memory through the spontaneous alternation of behaviour in rats. A Y-maze is formed of three equal arms settled at equal angles and named A, B, C. We placed animals at the edge of one arm and then they were permitted to freely walk between arms for 8 minutes. Arms were wiped well between each rat to remove any remaining odours. According to standard protocol, complete arm entry is counted when the rat totally enters its hind paws inside the maze arm. A consecutive entry on overlapping triplet combination pattern (CAB, ABC etc) in the three arms was defined as alternation. We recorded both number of alternations and total arm entries to obtain the percentage of spontaneous alternation behaviour (SAB) by using following equation [20].

$$\text{SAB (\%)} = \frac{\text{Number of alternations}}{\text{total arm entries-2}} \times 100$$

Radial arm maze

Number of correct responses was studied by applying the Radial Arm maze (INCO) procedure consisting of a maze with 8 arms and a central common area. Each rat was placed on the centre and allowed to enter the arms freely for 10 minutes. Entry into an arm which the rat had not visited previously was recorded as a correct response, whereas re-entry was counted as an error. The number of correct responses before committing the first error was calculated as the index of radial arm maze performance [21].

Elevated plus maze

In this maze, the time taken by the animal to enter a closed arm with all four limbs when placed at the end of one open arm facing away from central platform was recorded as the initial transfer latency. A 60 seconds cut off was set. The rat was then allowed to move freely in the maze regardless of open and closed arms for another 10 seconds. Twenty-four hours later, retention transfer latency testing was performed in the same way as in the acquisition trial. If the rat did not enter the enclosed arm within 60 seconds on the second trial, the transfer latency (day 1) was assigned 60 seconds [20]. The rats were again put into the elevated plus maze on day 42 to evaluate the transfer latency.

Biochemical estimations

After completion of all behavioural models, rats from each group were decapitated under anaesthesia with ketamine (87.5 mg/kg)/ xylazine (12.5 mg/kg) cocktail. The plasma and serum were then collected for biochemical estimations. The brain tissues were immediately removed and cleaned with cold saline over ice and were immediately stored in 10% formalin solution for further studies.

Enzymatic antioxidant assay

Preparation of Homogenates

For preparation of homogenates, 0.3 M of phosphate buffer at pH 7.4 was added 3 times to the weight of hippocampus and the brain tissues were homogenized using a homogenizer at oscillation frequency of 180-1800 per minute. The obtained homogenates were centrifuged at 10,000 rpm for 15 minutes at 4°C using a cooling centrifuge and finally the supernatant was collected and stored at -80°C until the assay to be performed.

Superoxide dismutase (SOD)

The SOD assay was done by using a JASCO (V-630) UV spectrophotometer. A blank was prepared by adding 0.5 ml of EDTA (1 mM) to 1.5 ml of Tris buffer (0.05 M), whereas 1 ml of Pyrogallol (0.2 mM) was added to the same blank preparation as control. The test preparation consisted of reagent blank and 50 µL of serum or brain homogenate in a separate test tube. Change in absorbance was recorded against blank at 420 nm. The percentage protection was calculated from the following equation [21].

$$\% \text{ Protection} = \frac{\text{A control} - \text{A test}}{\text{A control}} \times 100$$

The SOD content was determined by putting each % protection in the standard curve (Y= 56.53x-0.1198, $R^2 = 0.99$) and expressed as mmol/L (Prusty *et al*. 2017; Das *et al*. 2018).

Lipid peroxidation activity

Malondialdehyde (MDA), an end product of lipid peroxidation, was estimated by UV spectrophotometer. Blank solution was prepared by mixing 2 ml of Trichloro acetic acid (15%), 2 ml of Thiobarbituric acid (0.37%) and 2 ml of 0.25 N HCl. Test solutions were prepared by adding 100 µL of plasma or brain homogenate to the blank solution separately. The reaction mixture was heated for 60 minutes at 90 ^{0}C over water bath followed by gradual cooling and centrifuged at 3000 rpm for 15 minutes. Absorbance of supernatant was measured at 532 nm against a reagent blank. Extent of MDA content was determined from the standard curve and expressed as nmol/L [21,22].

Detection of amyloid-β content by ELISA test

A sandwich ELISA kit (Elabscience) was used for the determination of amyloid-β in the brain. The blood plasma and hippocampal piece of control, $AlCl_3$ and test drugs were taken and first homogenized in PBS at a ratio 1:9 using a homogenizer. After homogenization, the plasma and brain homogenates were centrifuged at 10000 rpm for 15 minutes and the supernatants were collected. For the estimation of amyloid-β, the standard solution was prepared using the serial dilution method to obtain a standard calibration curve. The protein concentration was subsequently assayed by applying the BCA method wherein about 100 µl of sample containing 250 µg of protein from soluble fraction of $A\beta_{1-42}$ were incubated in a microplate pre-coated with their corresponding antibodies for 90 minutes at 37°C. Afterwards, the microplate wells were washed with 350 µl of wash buffer solution and incubated with 100 µl of biotinylated detection antibody for 60 minutes at 37°C. Following this, the incubation samples were re-washed and HRP-labelled conjugate was added to each well and the microplate was incubated for 30 minutes. The samples were then washed again and incubated with a substrate reagent of 50 µl for 15 minutes at 37°C. Subsequently, 40 µl of stop solution was added to stop the reaction. Finally, the samples were preheated for some time at the same temperature, and optical density was measured by ELISA reader at a wavelength of 450 nm [23].

Histopathology

Fixed tissues were dehydrated in different mixtures of ethanol and water, followed by cleaning with xylene. The clean tissues were then embedded in paraffin and 5-6 µm thick sections were prepared. These was further stained with haematoxylin and eosin dyes, followed by mounting in DPX medium for microscopic observations [21].

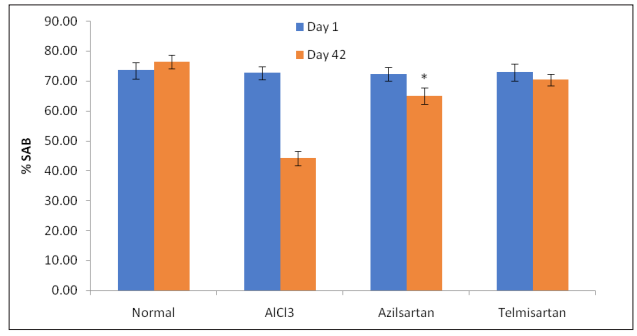
Statistical Analysis

The statistical analyses were performed by applying One-way ANOVA, then by Tukey's post hoc test. The statistical analysis of the experiment data was presented as mean ±SD. P-value and was established at 5% level of significance.

RESULTS

Y maze

A Y-maze (INCO, INDIA) was used to measure the spatial working memory through the spontaneous alternation of behaviour (SAB) in rats. Administration of $AlCl_3$ for 42 days significantly decreased % SAB ($p < 0.05$) when compared to control group. The group treated with Azilsartan (5 mg/kg) and Telmisartan (10 mg/kg) significantly increased the % SAB ($p < 0.05$) when compared with $AlCl_3$. There was no significant difference between % SAB of Telmisartan and Azilsartan (Figure 1).



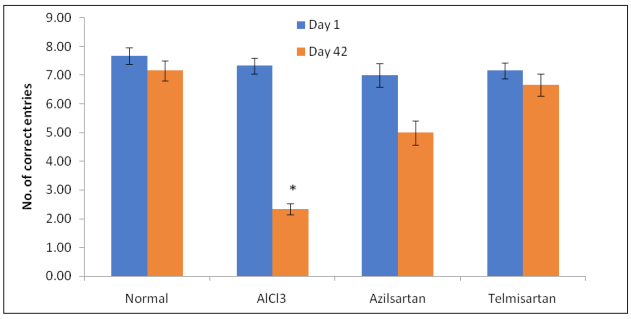
Values are presented as Mean ± SD (n=6) using one-way ANOVA, followed by Tukey's t-test. * $p<0.05$, Normal *vs*. $AlCl_3$, $AlCl_3$ *vs*. Azilsartan and $AlCl_3$ *vs*. Telmisartan; # $p<0.05$, Azilsartan *vs*. Telmisartan

Figure 1. Effect of Azilsartan on % spontaneous alternation behaviour (SAB) in $AlCl_3$ administered rats using Y-Maze

Radial arm maze

Administration of $AlCl_3$ for 42 days showed significant ($p<0.05$) decrease in number of correct responses as compared to control animals. Treatment groups (Azilsartan, Telmisartan) given together with $AlCl_3$ improved number

of correct responses significantly (p<0.05). Telmisartan showed significantly (p<0.05) more protective effect than Azilsartan (Figure 2).

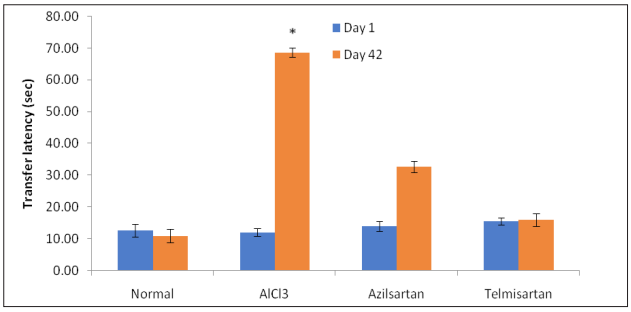


Values are presented as Mean ± SD (n=6) using one-way ANOVA followed by Tukey's t-test. * p <0.05, Normal *vs.* $AlCl_3$, $AlCl_3$ *vs.* Azilsartan and $AlCl_3$ *vs.* Telmisartan; # p <0.05, Azilsartan *vs.* Telmisartan

Figure 2. Effect of Azilsartan on number of correct responses in $AlCl_3$ administered rats using Radial arm maze

Elevated Plus Maze

Significant increase (p <0.05) in transfer latency was observed after treatment with $AlCl_3$ for 42 days as compared to control group. Group III and IV treated with Azilsartan (5 mg/kg) and Telmisartan (10 mg/kg), respectively, given together with $AlCl_3$ improved transfer latency significantly (p <0.05). Telmisartan (10 mg/kg) showed significantly (p <0.05) more protective effect than Azilsartan (5 mg/kg) (Figure 3).



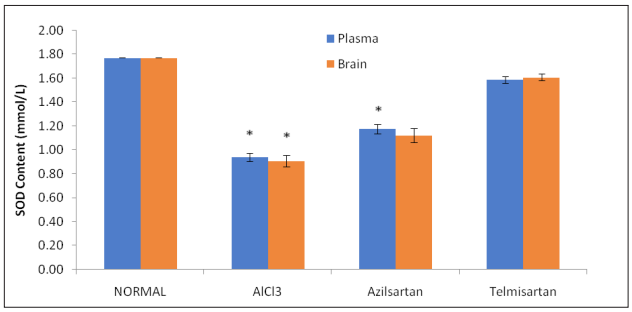
Values are presented as Mean ± SD (n=6) using one-way ANOVA followed by Tukey's t-test. * p<0.05, Normal *vs.* $AlCl_3$, $AlCl_3$ *vs.* Azilsartan and $AlCl_3$ *vs.* Telmisartan; # p<0.05, Azilsartan *vs.* Telmisartan

Figure 3. Effect of Azilsartan on transfer latency in $AlCl_3$ administered rats using Elevated Plus Maze

Enzymatic antioxidant status

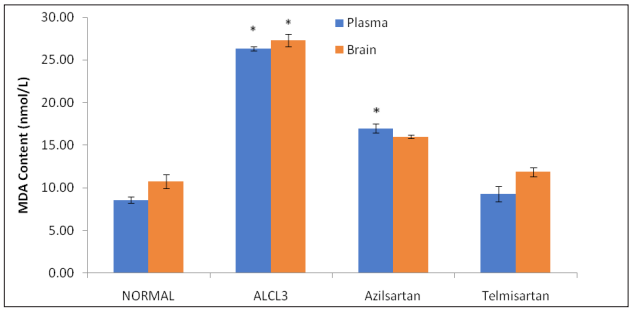
SOD and MDA

After 42 days of administration of $AlCl_3$, impaired antioxidant status was observed in group II animals i.e., decreased SOD level and increased MDA content as compared to control group. However, Telmisartan and Azilsartan showed significantly (p<0.05) increased SOD level (Figure 4) and decreased level of lipid peroxidation (Figure 5) as compared to $AlCl_3$ group. Telmisartan showed significantly (p<0.05) more protective effect than Azilsartan.



Values are presented as Mean ± SD (n=6) using one-way ANOVA followed by Tukey's t-test. * p<0.05, Normal *vs.* $AlCl_3$, $AlCl_3$ *vs.* Azilsartan and $AlCl_3$ *vs.* Telmisartan; # p<0.05, Azilsartan *vs.* Telmisartans

Figure 4. Effect of Azilsartan on superoxide dismutase (SOD) activity in $AlCl_3$ administered rats

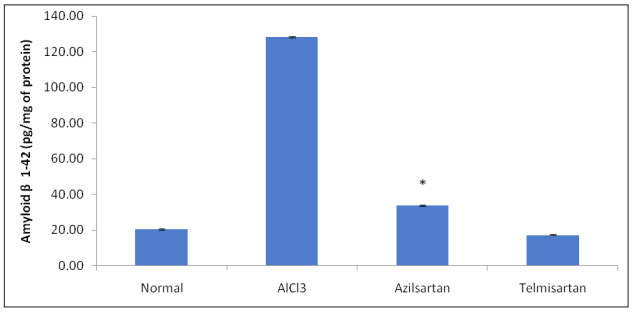


Values are presented as Mean ± SD (n=6) using one-way ANOVA followed by Tukey's t-test. * p<0.05, Normal *vs.* $AlCl_3$, $AlCl_3$ *vs.* Azilsartan and $AlCl_3$ *vs.* Telmisartan; # p<0.05, Azilsartan *vs.* Telmisartan

Figure 5. Effect of Azilsartan on Malondialdehyde (MDA) activity in $AlCl_3$ administered rats

Amyloid β estimation

After chronic administration of $AlCl_3$ for 42 days, we found that there was significant increase in the levels of Aβ 1-42. Both Azilsartan and Telmisartan produced significant (p<0.05) protective effect against $AlCl_3$ and decreased the amount of Aβ 1-42 produced in the brain cells. However, Telmisartan produced significantly more protection than Azilsartan (Figure 6).



Values are presented as Mean ± SD (n=6) using one-way ANOVA followed by Tukey's t-test. * p<0.05, Normal *vs.* $AlCl_3$, $AlCl_3$ *vs.* Azilsartan and $AlCl_3$ *vs.* Telmisartan; # p<0.05, Azilsartan *vs.* Telmisartan

Figure 6. Effect of Azilsartan on Amyloid β activity in $AlCl_3$ administered rats

Histopathology Study

Histological examination further confirmed the results of this study. The normal-control group showed normal appearance and organization of neuronal cells, whereas animals treated with $AlCl_3$ showed neurodegenerative changes. Accordingly, $AlCl_3$ treated rats displayed the

presence of neurofibrillary tangles, neuronal dead cells, and pyknotic cells when compared to the normal group. Both Azilsartan and Telmisartan showed protection from neurodegeneration (Figure 7).

ats

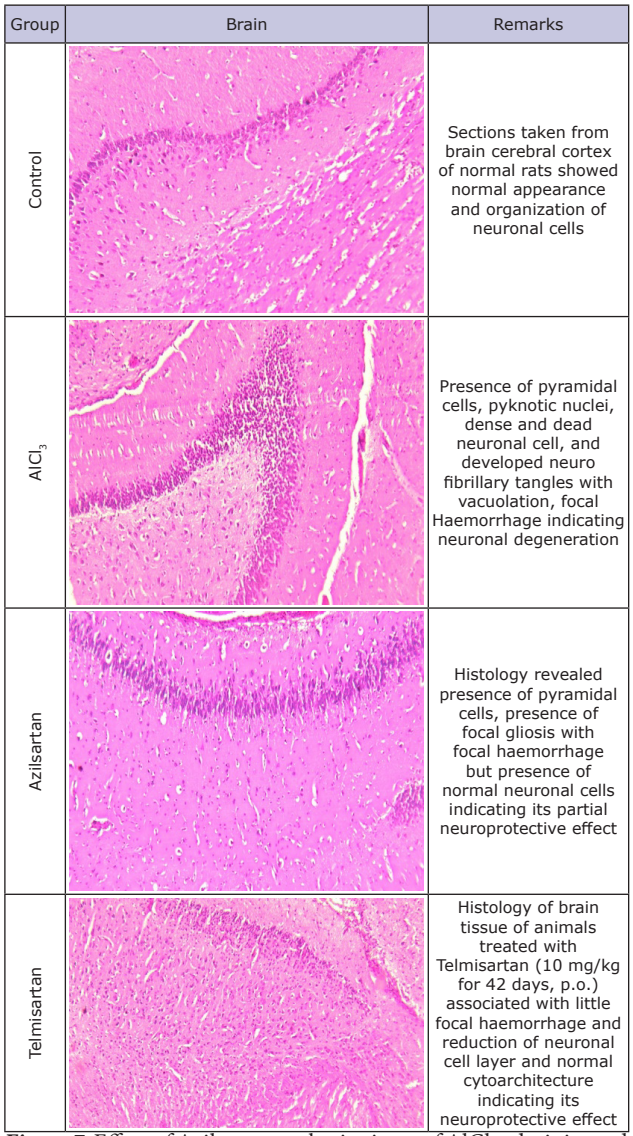


Figure 7. Effect of Azilsartan on brain tissue of $AlCl_3$ administered rats

Histology of normal rat brain showed normal appearance and organization of neuronal cells. However, $AlCl_3$ treated rat brain was associated with presence of pyramidal cells, pyknotic nuclei, dense and dead neuronal cell, and developed neuro fibrillary tangles with vacuolation, focal Haemorrhage indicating neuronal degeneration. Azilsartan treated rat brain tissue displayed the presence of pyramidal cells, focal gliosis with focal haemorrhage, but the presence of normal neuronal cells implies its partial neuroprotective effect. Sections taken from brain cerebral cortex of rats treated with Telmisartan (10 mg/kg for 42 days, p.o.) were associated with little focal haemorrhage and reduction of neuronal cell layer and normal cytoarchitecture - indicating its neuroprotective effect.

DISCUSSION

The drugs available today for Alzheimer's disease only control the symptoms of the disease and not the actual cause. Hence, there is a need to develop a therapeutic intervention with broad mechanism of action in combating AD [24]. The current study focuses on the effect of Azilsartan and Telmisartan on aluminum chloride induced AD-like pathology in a rat model. Various studies revealed that high aluminium level in brain can cause the pathological changes that resembles AD and these effects are mediated through several mechanisms such as oxidative stress, cholinergic dysfunction, Aβ deposition, inflammations, as well as other mechanisms which degrade memory and learning ability [25].

RAS in the brain may cause the death of neuronal cells, which then might lead to neurodegenerative disorders such as AD. There are two receptors for angiotensin II (Ang II): AT1 and AT2. The stimulation of the AT1 receptor causes oxidative stress and neuroinflammation, whereas that of the AT2 receptor has neuroprotective actions [19,26]. When AT1 receptors are blocked, neuroprotective action can be obtained. Treatment with AT1 receptor blockers is known to reduce anxiety and improve motor performance, spatial working memory and learning in aged rats [27]. It was also reported that blocking of AT1 receptor enhances the formation of Angiotensin IV, which has memory enhancing property [28].

ARBs were reported to possess positive cognitive actions [29]. Blockade of AT1 receptor reduces apoptosis, inflammation and oxidative stress [30]. According to previous research, Telmisartan administration protects against aluminum chloride induced AD-like pathological changes in rats. Moreover, it decreased hippocampal amyloid beta protein, phosphorylated tau protein. It also diminished levels of nuclear factor kappa-B, FAS ligand, tumor necrosis factor-alpha, malondialdehyde and acetylcholinesterase content [31]. Telmisartan protects oligodendrocytes and neuronal cells by decreasing inflammatory response in the brain through AT1 receptor blockade and peroxisome proliferator activated receptor-γ (PPAR-γ) stimulation [18,32,33]. Because of its known attributes, we used Telmisartan as standard in our study.

A centrally acting angiotensin converting enzyme (ACE) inhibitor, Perindopril administration brings about improvement in cognitive impairment [34]. In another study, a combination of Perindopril and Azilsartan showed efficacy against dementia [35]. Azilsartan also was found to effectively mitigate apoptosis and restore the dopaminergic content in a rat model of Parkinson's disease [36]. Therefore, we evaluated the efficacy of Azilsartan against aluminium chloride induced AD-like pathological changes in rats, and compared with that of Telmisartan.

In our study, $AlCl_3$ was administered for 42 days to develop AD-like symptoms. Cognitive function, antioxidant status and Aβ level was measured before and after drug administration. In the present study, animals treated with $AlCl_3$ demonstrated impairment in memory functions and decreased antioxidant status with elevation of Aβ. This is in agreement with earlier studies [37]. The cognitive

impairment by $AlCl_3$ was confirmed by significant decrease ($p< 0.05$) in percentage of spontaneous alternation behaviour (SAB) in the Y-maze, a significant decrease ($p< 0.05$) in number of correct responses in the radial maze and a significant ($p<0.05$) increase in transfer latency in the elevated plus maze [20]. Again, a decrease in SOD [20], elevation of MDA [20] and increased Aβ plaques [37] caused by aluminium chloride confirm the cognitive deficit.

Animals treated with Azilsartan and Telmisartan showed protection against $AlCl_3$ induced cognitive dysfunction as evidenced from significant ($p<0.05$) increase in SAB (Y-maze), increase in number of correct responses (radial arm maze) and decrease in the transfer latency (elevated plus maze). Moreover, recovery of SOD, MDA and Aβ levels in both brain homogenate and plasma were evident. The histological study also confirmed the neuroprotective effect of both Azilsartan and Telmisartan. Thus, like Telmisartan, Azilsartan significantly improved the memory and learning functions. These effects may be attributed to its anti-amyloid, antioxidant and neuroprotective actions. However, Telmisartan showed significantly more protective action than Azilsartan.

CONCLUSION

In our present study, we confirmed that Azilsartan and Telmisartan show neuroprotective effects (via antioxidant action and decrease in the production of Aβ in the hippocampal region) against $AlCl_3$ induced Alzheimer's disease in like pathological changes in rats. The improvement in memory functions was revealed through different behavioural models. Telmisartan, however, showed more protective effect than Azilsartan. Considering the fact that AD is dependent on multiple factors and involves several damaging processes such as oxidative stress, apoptosis and neuroinflammation, further comprehensive studies and clinical trials are required to fully establish the neuroprotective and memory enhancing effects of angiotensin receptor blockers.

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COMPETING INTEREST

The authors declare that there are no competing interests.

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