Research Article

Effect of Pyridoxine on toxicity of Aminoguanidine and its Neuroprotective effect in Aluminum Chloride induced Dementia in rats

Mamta F. Singh[1*], Shivani Bisht[1], Vinod Singh[2], Shradha Bisht[1]

[1]*Dept. of Pharmaceutical Sciences, SBSPGI, Balawala, Dehradun, 248161, Uttarakhand, India*

[2]*Dept. of Pharm. Sciences, Gurukul Kangri Vishva Vidhyalaya, Haridwar, Uttarakhand, India*

Abstract

Background: Nitric oxide synthase inhibitor, aminoguanidine is widely investigated for treating various pathological conditions including neurodegeneration but reported to produce dose dependent toxicities. The toxicities of aminoguanidine are mainly associated with deficiency of pyridoxine. **Objective:** The present study was aimed to evaluate the effect of co-administration of pyridoxine along with aminoguanidine on the toxicities and memory improving effect of aminoguanidine in demented rats. **Material and methods:** Dementia was induced in rats by aluminium chloride (17 mg/kg, p.o.) administered for a period of 30 days. The demented rats received pyridoxine (10 mg/kg, p.o.), aminoguanidine 300 mg/kg, p.o. and 150 mg/kg, p.o. alone and the combination of intermediate dose (150 mg/kg, p.o.) of aminoguanidine along with pyridoxine (10 mg/kg, p.o.) for a period of 30 days. Behavioral studies, biochemical assays (nitrates, calcium and LDH), estimations of acetyl cholinesterase and pro-oxidant- antioxidant assays (assay for lipid peroxidation and superoxide dismutase) were done to evaluate the effect of the treatment in dementia. **Results:** Aminoguanidine alone at higher dose level and the combination of intermediate dose of aminoguanidine with pyridoxine caused significant improvement (P<0.01) in the physical parameters of memory in morris water maze and radial arm maze model. Aminoguanidine in combination with pyridoxine significantly (P<0.001) decreased the level of acetylcholinesterase, LDH, nitrite, calcium and TBARS and also improved the level of antioxidant enzymes in demented rats. **Conclusion:** Results concludes that aminoguanidine at intermediate dose level with pyridoxine is effective in improving memory loss in the demented rats with no associated toxicity.

Keywords: Acetylcholinesterase, Aminoguanidine, Antioxidant, Nitric oxide synthase, Pro-oxidant

Introduction

Nitric oxide (NO) is an important signaling molecule in the nervous system. In the body, nitric oxide is produced during the metabolism of L-arginine by the enzyme nitric oxide synthase (NOS) and leads to the production of citrulline and nitric oxide. In the brain nitric oxide is recognized for its roles in synaptic plasticity, as a neurotransmitter and also involved in neurosecretion and neuroprotection (Steinert et al., 2010; Singh et al., 2012). It has been observed that overproduction of NO in the brain occurs due to nNOS activation by persistent stimulation of excitatory amino acid receptors mediating glutamate toxicity and/or due to iNOS induction by diverse stimuli, such as endotoxin or cytokines (Thatcher et al., 2005).

NO signalling contributes to various neurodegenerative diseases like alzheimers diseases through oxidative/ nitrosive stress and oxidative protein damage, enhanced nitrotyrosine immunoreactivity, inhibition of mitochondrial cytochrome c oxidase, activation of constitutive and the inducible isoforms of cyclooxygenase, and due to enhanced H_2O_2 production in amyloid β (Aβ) (Ridnour et al., 2004). In Alzheimer's disease, amyloid-beta increases the production of NO, which either attached to the apoptosis inducing protein called Drp1 and increases mitochondrial fission leading to increase in neuronal death or binds with superoxide anion to form peroxynitrite, which leads to the generation of free radicals and further causes cell death (Niwa, 2001). Studies indicates that in AD activation of iNOS increases Aβ deposition and tau protein phosphorylation, activation of apoptotic machinery, neuronal cell death and neurodegeneration (Nampoothiri et al., 2015).

Address for Corresponding Author:
Dr. Mamta F. Singh
Associate Professor, Department of Pharmacology
Sardar Bhagwan Singh Post Graduate Institute, Balawala, Dehradun, 248161, Uttarakhand, India
Ph: 8755352001
E Mail: mamta_fr2002@yahoo.co.in

Current treatments available for Alzheimer disease only provide temporary and modest improvement in cognitive impairment and are considered as symptomatic. Therefore there is a need to develop effective and novel medication by focusing on various alternative approaches. Knowledge of the endogenous phathophysiological actions of NO in the nervous system highlighted here raises the possibility of manipulating the NO system for therapeutic benefit and inhibitors of NO synthesis and NOS inhibitors can be evaluated for the treatment of neurodegenerative diseases like AD.

Aminoguanidine (AG) is a selective and competitive inhibitor of inducible nitric oxide synthase and is structurally similar to L-arginine. Various studies reports that AG in addition to decreasing the generation of NO also produces antioxidant effect by scavenging peroxinitrite radicals (Mansour et al., 2002). It is also reported that AG produces protective effect in inflammation and diabetic nephropathy but the use of aminoguanidine is associated with various kidney and liver toxicities, deficiency of vitamin B6 and has effect on biochemical pathways which are influenced by B6 (Okada et al., 1995). Life extension foundation forum in 2001 reported that the toxicities of AG are dose related and gets vanished on decreasing the dose.

Therefore the present investigation was undertaken to evaluate the effect of aminoguanidine along with pyridoxine (B6) in aluminium chloride induced dementia in rats. As the toxic effects of AG are dose dependent, therefore the present study was carried out with aminoguanidine alone at different dose levels and combination of intermediate dose of aminoguanidine along with pyridoxine to evaluate the effect of combination.

Materials and methods

Drugs and chemicals

Thiobarbituric acid, griess reagent, hydrogen peroxide, acetylthiocholine iodide, aminoguanidine, DTNB and pyridoxine hydrochloride were obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). $AlCl_3$ was obtained from Spectrochem Pvt. Limited, India. Diagnostic kits of Siemens were used for the estimation of various biochemical parameters.

Animals

Young male wistar rats with body wt of 200–250 g were used for the study. The animals were procured from Institutional animal house and were maintained under controlled temperature (23 ± 2^0C) and humidity ($50 \pm 5\%$) with 12:12 hour light and dark cycle. The animals were housed in sanitized standard polypropylene cages and animals have free access to food and water. The experimental protocol was approved by the Institutional Animal Ethical Committee, SBSPGI, Balawala, Dehradun (CPCSEA/IAEC/SBS/09/2012) and was carried out

in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Induction of dementia

Dementia was induced in animals by aluminium chloride (17 mg/kg) given orally for a period of 30 days (Krasovskii et al., 1979).

Aluminium chloride, aminoguanidine and pyridoxine were prepared freshly every day before administration. Animals were grouped into seven groups of six animals in each and received the following treatment for 30 days. The drugs were given as a suspension in distilled water using 1% CMC as suspending agent. Group 1 normal animals received 1% CMC in distilled water. Group II animals received $AlCl_3$ (17 mg/kg, p.o.). Group III Animals received $AlCl_3$ and aminoguanidine (higher dose, 300 mg/kg, p.o.). Group IV Animals received $AlCl_3$ and pyridoxine (10 mg/kg, p.o). Group V Animals received $AlCl_3$ along with the combination of intermediate dose of aminoguanidine (150 mg/kg, p.o.) and pyridoxine (10 mg/kg, p.o.). Group VI Animals received $AlCl_3$ and the intermediate dose of aminoguanidine (150 mg/kg, p.o.) alone. Group VII Animals received $AlCl_3$ and standard drug donezepil at a dose of 10 mg/kg, p.o.

Physical studies for memory were carried out before the treatment and at the end of treatment period. After physical studies, biochemical studies were carried out using animal serum and brain tissue.

Evaluation of memory

Drugs were administered 30 minutes before the trial on morris water maze and radial arm maze apparatus. The physical studies using radial arm maze and morris water maze apparatus were conducted in two parts. The probe trail was conducted from day 1 to day 5 of the treatment while retention trial was performed at the end of 30 days of the treatment.

Morris water maze apparatus (Morris, 1984)

Morris water maze test was used for the evaluation of spatial memory in rats. The maze consisted of a circular water tank (2 m in diameter and 75 cm in depth) filled with 50 cm deep water kept at 22^0C and made opaque by a non-toxic, water soluble dye. The tank was divided into four equal quadrants along the circumference of the pool. In one of the quadrant, a hidden platform (10 cm diameter) was kept, 2 cm below the surface of water.

The position of the hidden platform was kept constant throughout the study. The animals were then trained to locate this hidden platform. If the animal fails to find the

platform within 60 sec, it was gently guided to the platform and was allowed to stay there for 15 sec. Each animal had four acquisition trials per day for four consecutive days. Animals that failed to reach the platform within 20 sec on the day 4 were excluded from the study. On the day 5 (probe day), after 24 hours of last acquisition trial the hidden platform was removed and retention trial was conducted. The animals were allowed to swim for 60 sec. before the end of session. Retention trials were repeated on day 30 to all groups of animals to evaluate the memory consolidation. Time of the animals to reach hidden platform (escape latency), latency to find the target quadrant and percentage of time spent in target quadrant were measured during retention trials.

Radial arm maze apparatus

Radial arm maze is a popular and important tool for assessing spatial memory in animals. It was used as described by Olton (Olton et al., 1976). It consists of a central octagonal arena with 8 radial arms. The diameter of central arena was 34 cm. The arms were 86 cm in length, 10 cm in width and 24 cm in height. A food well of 2 cm in diameter and 0.5 cm deep was located 2 cm from the end of each arm in which reward pellets could be placed. At the beginning of the test single animals was placed in the center of the maze, facing the baited arm and allowed to explore the maze freely in the first day. On the day second, third and fourth, animals were placed in the center of the maze facing the baited arm and were given maximum six minutes to find the reward (food). If the animal found the food before the end of time then the watch was stopped and elapsed time was recorded.

Serum biochemical assays

After behavioral studies rats were anaesthetized with diethyl ether and the blood samples were collected from retro orbital plexus into micro centrifuge tubes. The blood samples were then centrifuged at 15,000 rpm (Remi C24 BL, VCEAL 7310, India) for 10 minutes and serum was obtained and preserved at -80^0C for analysis. Serum lactate dehydrogenase level was estimated by UV kinetic method (Stevens et al., 1983) and calcium was estimated by the method of Theodore S. Prokopow (Theodore, 1972) using diagnostic kits on a semiautomatic analyser (Transasia Erba Chem 5X, India).

Biochemical assays using rat brain

After collection of blood sample the animals were sacrificed by decapitation. An incision was made on the dorsal side of the skull to expose and remove the brain rapidly from each rat. A 10% w/v homogenate of samples were prepared in ice-cold 0.1M phosphate buffer (pH 7.4) using an Ultra-Turrax T25 homogenizer at a speed of 10,500 rpm at 4^0C and the mitochondrial supernatant was used for the biochemical estimations. Nitrite from the mitochondrial supernatant was estimated colorimetrically as an azo dye product of Griess

Reagent and the absorbance was measured at 546 nm (Guerra et al., 2005). Nitrite concentration was calculated by using the standard curve of sodium nitrite. The level of TBARS was estimated in brain mitochondrial fraction by the method of Slater and Sawyer (Slater et al., 1971) at 535 nm. Superoxide dismutase was estimated by the method of Misra and Fridovich (Mishra et al., 1971) and change in optical density /minute was measured at 480 nm against reagent blank. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit.

Estimation of acetylcholinesterase activity

Direct estimation of acetylcholine in tissue homogenate is difficult due to its short half life. Estimation of acetylcholinesterse activity provides an easy and valuable method for assessment of cholinergic functions. Acetylcholinesterase (AChE) activity was determined by the modified method of Ellman (Ellman et al., 1961). The acetylcholinesterase activity was measured by providing an artificial substrate acetylthiocholine. Thiocholine released due to the acetylthiocholine by AChE is allowed to react with the -SH reagent DTNB, which is reduced to thionitrobenzoic acid, a yellow colored anion with an absorption maxima at 412 nm. The concentration of thionitro benzoic acid detected using a UV spectrophotometer was then taken as a direct estimate of the AChE activity.

Statistical analysis

The statistical analysis was carried out using Graph Pad Prism 5.0 software. All values are presented as Mean ± SEM. Multiple comparisons between different groups were performed using Analysis of Variance (ANOVA) followed by Dunetts Multiple comparison Test. Difference level at P< 0.05 was considered statistically significant condition.

Results

Effect of aminoguanidine and pyridoxine on aluminium chloride induced spatial memory deficit in demented rats using morris water maze apparatus

In the present study administration of aluminium chloride for 30 days caused significant spatial memory impairment as observed in morris water maze test during the retention trial conducted on the 30th day (table 1). AlCl$_3$ significantly (P< 0.05) raised escape latency time (ELT) as shown in the table 1. During the probe trial on the 30th day, aluminum chloride treated rats were found to spend less time in the target quadrant (NW) than the control group. Demented animals treated with aminoguanidine 300 mg/kg, p.o. alone showed significant (P < 0.01) decrease in escape latency as

Table 1. Effect of aminoguanidine and pyridoxine alone and in combination on the escape latency in AlCl$_3$ induced demented rats by using morris water maze

Treatment groups (p.o.)	Escape latency time (secs) on Day 24	Escape latency time (secs) on Day 30th	Probe trial on the 30th day
Normal (Normal Saline, 1 ml/kg, p.o.)	31.00 ± 1.08	29.50 ± 0.64	24.50 ± 1.04
AlCl$_3$ (17 mg/kg, p.o.)	59.75 ± 2.09	66.50 ± 1.7	60.50 ± 1.55
AlCl$_3$+Aminoguanidine (300 mg/kg, p.o.)	40.00 ± 1.9 *	36.25 ± 1.93 **	31.00 ± 0.91 **
AlCl$_3$+Pyridoxine (10 mg/kg, p.o.)	49.25 ± 1.37	43.00 ± 1.44 *	37.00 ± 2.3 *
AlCl3 + AG (150 mg/kg, p.o.) + Pyridoxine (10 mg/kg, p.o.)	36.56 ± 0.91*	37.9 ± 0.65**	34.74 ± 1.32*
AlCl3 + Aminoguanidine (150 mg/kg, p.o.)	50.8 ± 1.3**	49.54 ± 0.91***	51.8 ± 0.91**
AlCl$_3$+Donezepil (1 mg/kg, p.o.)	31.00 ± 0.91**	20.25 ± 0.47 ***	22.50 ± 1.3***

The statistical significance of difference between means was calculated by ANNOVA followed by t-test for unpaired comparison. N= 6; Values are expressed as Mean ± SEM, *P< 0.05, **P< 0.01, ***P< 0.001. **Index:** AlCl$_3$ - Aluminium chloride, AG – Aminoguanidine

Table 2. Effect of aminoguanidine and pyridoxine alone and in combination on the average time elapsed in seconds in the radial arm maze test

Treatment groups (p.o.)	Day 2 (27th day of treaatment)	Day 3 (28th day of treatment)	Day 4 (29th day of treatment)	Day 5 (30th day of treatment)
Normal (Normal Saline, 1 ml/kg, p.o.)	129 ± 0.23	141.0± 1.08	124.78± 0.64	138.15 ±0.59
AlCl$_3$ (17 mg/kg,p.o.)	187.6 ± 1.8	169.68± 0.32	158.7± 2.06	201.6± 1.75
AlCl$_3$+Aminoguanidine (300 mg/kg, p.o.)	136.9± 0.79*	155.0±1.32	130.4±1.8*	153.3±0.8**
AlCl$_3$+Pyridoxine (10 mg/kg, p.o.)	178.0±1.6	168.50±1.54	149.65±0.77	177.5±1.54*
AlCl3 + AG (150 mg/kg, p.o.) + Pyridoxine (10 mg/kg, p.o.)	137.4± 0.76*	142.05±1.09*	138.05±0.65*	134.4±0.73**
AlCl3 + Aminoguanidine (150 mg/kg, p.o.)	164.6±1.45**	152.62±0.81*	148.03±1.06*	156.7± 1.92**
AlCl$_3$+Donezepil (1 mg/kg, p.o.)	133.0± 0.6**	138.36±0.66*	123.7±1.47**	129.07±0.73***

The statistical significance of difference between means was calculated by ANNOVA followed by t-test for unpaired comparison. N= 6; Values are expressed as Mean ± SEM, *P< 0.05, **P< 0.01, ***P< 0.001. Index: AlCl$_3$ - Aluminium chloride, AG – Aminoguanidine

well as spent more time in the target quadrant as compared to the demented animals. Treatment of the demented animals with the combination of intermediated dose of aminoguanidine along with pyridoxine also caused significant (P < 0.01) decrease in escape latency (36.56 ± 0.91 sec.) as compared to AlCl$_3$ treated (66.50 ± 1.7 sec) animals. However aminoguanidine alone at intermediate dose does not cause any significant decrease in the escape latency and also increased the time spent by the animal in the target quadrant on the 30th day. Standard drug donezepil also caused significant decrease in the escape latency in demented rats.

Effect of aminoguanidine alone and its combination with pyridoxine on the time elapsed in AlCl$_3$ induced memory deficit using radial arm maze apparatus

In radial arm maze test it is considered that as the time to find the bated arm decreases, learning and memory improves. As shown in the table 2, in AlCl$_3$ treated groups total time was higher than that of normal with an average of 186.6 sec. There was significant increase in the time elapsed (time to find the baited

arm) as compared to the normal animals. Administration of aminoguanidine alone at a dose of 300 mg/kg/day, p.o. to the demented animals for 30 days caused significant (P< 0.01) decrease in the elapsed time in the radial arm maze with an average of 20 % per day. Co-administration of pyridoxine along with intermediate dose of aminoguanidine caused equally significant (P< 0.01) decrease in the time needed to find the baited arm as the higher dose of aminoguanidine and hence indicated significant improvement in memory. Normal animals showed 50% errors while entering into the chamber. This percentage was increased in aluminium chloride treated animals and reduced in aminoguanidine and pyridoxine treated animals.

Effect of aminoguanidine alone and its combination with pyridoxine on the serum level of lactate dehydrogenase in the demented rats

The effect of the treatment on the level of LDH in the brain of demented rats is summarized in table 3. Results shows

Table 3. Effect of aminoguanidine and pyridoxine alone and in combination on the level of calcium, lactate dehydrogenase, nitrates and acetylcholinesterase in $AlCl_3$ induced demented rats

Treatment groups (p.o.)	Calcium (mg/dl)	LDH (U/L)	Nitrates (μ mol/L)	AchE (µmol/min/g tissue)
Normal (Normal Saline, 1 ml/kg, p.o.)	10.275 ± 0.52	112.1 ± 0.04	307.7 ± 0.57	15.72 ± 1.820
$AlCl_3$ (17 mg/kg, p.o.)	22.80 ± 0.06	345.2 ± 0.39	492.1 ± 0.07	53.690 ± 0.19
$AlCl_3$+Aminoguanidine (300 mg/kg, p.o.)	15.78 ±0.19*	138.4 ± 0.9 **	317.2 ±0.88**	40.555 ± 0.62*
$AlCl_3$+Pyridoxine (10 mg/kg, p.o.)	14.7 ± 0.08**	184.2 ±0.13**	472.0 ± 0.24	36.913 ± 0.55*
AlCl3 + AG (150 mg/kg, p.o.) + Pyridoxine (10 mg/kg, p.o.)	14.93 ±0.07**	140.0 ± 0.51*	328.9 ± 0.09*	41.17 ± 0.68*
AlCl3 + Aminoguanidine (150 mg/kg, p.o.)	19.5 ± 0.06*	263.1 ±.16***	416.0 ±0.04**	48.29 ± 0.80**
$AlCl_3$+Donezepil (1 mg/kg, p.o.)	21.10 ± 0.04*	310.1 ± 0.08	450.2 ± 0.03	20.95 ± 1.03**

The statistical significance of difference between means was calculated by ANNOVA followed by t-test for unpaired comparison. N= 6; Values are expressed as Mean ± SEM, *P< 0.05, **P< 0.01, ***P< 0.001. **Index:** $AlCl_3$ - Aluminium chloride, AG – Aminoguanidine

that in the $AlCl_3$ treated rats the level of LDH was significantly increased (345 IU/L) which indicates cell injury and neuronal degeneration by $AlCl_3$. The brain LDH level was significantly decreased (P < 0.05, 138 IU/L)) in the animal treated with aminoguanidine alone and pyridoxine alone (154 IU/L). However demented rats treated with the combination of intermediate dose of aminoguanidine along with pyridoxine showed significantly (P < 0.001) lowered levels of LDH (123.8 IU/L) as compared to the demented rats. However the intermediate dose alone does not have any significant effect on brain LDH level. Donepezil the standard drug for dementia did not have any effect on the level of LDH in demented rats.

Effect of aminoguanidine alone and its combination with pyridoxine on the serum level of nitrite in the demented rats

$AlCl_3$ caused significant upsurge (P < 0.01) in the level of serum nitrite after 30 days of administration in rats which indicates increased levels of NO (table 3). Administration of aminoguanidine as well as pyridoxine alone significantly (P < 0.05) reversed the elevation in serum nitrite level in demented rats. The intermediate dose of aminoguanidine alone does not have any significant effect on serum nitrite level whereas the combination of intermediate dose of aminoguanidine along with pyridoxine caused significant (P < 0.001) decrease in the serum level of nitrites in the demented rats. The standard drug donepezil caused less significant decrease in serum nitrite level in demented rats.

Effect of aminoguanidine alone and its combination with pyridoxine on the serum level of calcium in the demented rats

Calcium is one of the most important cation responsible for various brain functions. It is known that aluminium alters calcium flux and also increases intracellular calcium and oxygen free radicals level leading to neurodegeneration. Results of the present study indicates that administration of aluminium chloride leads to a significant increase (22.80 ± 0.06 mg/dl) as

compared to the calcium level (10.275 ± 0.5 mg/dl) in normal animals. Administration of aminoguanidine at a dose of 300 mg/kg alone caused moderate decrease (P < 0.05) in the blood level of calcium in $AlCl_3$ treated rats. Administration of combination of intermediate dose of aminoguanidine with pyridoxine and pyridoxine alone also caused moderate decrease (P < 0.05) in the blood level of calcium in $AlCl_3$ treated rats, whereas the intermediate dose alone does not have any significant (P < 0.5) effect on serum calcium level. The standard drug does not have any significant effect on brain calcium level.

As such aminoguanidine does not have any effect on blood calcium level. It may be due to the effect of the treatment on blood nitrite level which further decreases blood calcium level.

Effect of aminoguanidine alone and its combination with pyridoxine on the brain homogenate level of acetylcholinesterase in demented rats

Results indicates that 30 days of chronic exposure of $AlCl_3$ significantly (P < 0.01) increased the AChE activity in the frontal cortex and hippocampus of the treated rats as compared to the normal animals (table 3) indicating decrease in the level of acetylcholine. Aminoguanidine as such do not have any effect on the level of AchE in demented rats. In the present study administration of higher and intermediate doses of aminoguanidine alone does not have any effect on brain acetylcholinesterase level in demented rats. However pyridoxine alone and the combination of intermediate doses of aminoguanidine along with pyridoxine caused slight decrease (P < 0.5) in the activity of AchE in demented rats. The standard drug donepezil significantly lowered (20.95 ± 1.03 µmol/min/g tissue) the acetylcholinesterase level as compared to the $AlCl_3$ treated rats (53.690 ± 0.19 µmol/min/g tissues).

Table 4. Effect of aminoguanidine and pyridoxine alone and in combination on the brain homogenate level of oxidative stress and antioxidant enzymes in AlCl$_3$ induced demented rats

Treatment groups (p.o.)	Lipid peroxidation (nmol/L)	Superoxide dismutase (EU/dl)
Normal (Normal Saline, 1 ml/kg, p.o.)	41.40 ± 0.3342	252.9 ± 9.348
AlCl$_3$ (17 mg/kg in drinking water)	82.00 ± 2.858	75.37 ± 5.59
AlCl$_3$+Aminoguanidine (300 mg/kg, p.o.)	73.40 ± 2.108	97.57 ± 2.51
AlCl$_3$+Pyridoxine (10 mg/kg, p.o.)	51.87 ± 1.62**	193.2 ± 1.9 **
AlCl3 + AG (150 mg/kg, p.o.) + Pyridoxine (10 mg/kg, p.o.)	63.86 ± 1.680*	200.4 ± 1.28 *
AlCl3 + Aminoguanidine (150 mg/kg, p.o.)	75.40 ± 0.39**	89.1 ± 1.86**
AlCl$_3$+Donezepil (1 mg/kg, p.o.)	74.82 ± 1.632	83.0 ± 10.02

The statistical significance of difference between means was calculated by ANNOVA followed by t-test for unpaired comparison. N= 6; Values are expressed as Mean ± SEM, *P< 0.05, **P< 0.01, ***P< 0.001. Index: AlCl$_3$ - Aluminium chloride, AG – Aminoguanidine

Effect of aminoguanidine alone and its combination with pyridoxine on the level of thiobarbituric acid reactive substances (TBARS) and endogenous antioxidant enzymes level

Malondialdehyde content is considered as a marker of lipid peroxidation in various diseases. In the present study AlCl$_3$ caused significant increase (P < 0.01) in the level of TBARS (malondialdehyde) and decrease in the level of glutathione peroxidase and superoxide dismutase in the brain of demented rats indicating increase in oxidative stress (table 4). Demented rats treated with pyridoxine alone as well as with the combination of aminoguanidine and pyridoxine showed significant decrease (P < 0.01) in the malondialdehyde level. Treatment of demented animals with higher dose (300 mg/kg, p.o.) and intermediate dose (150 mg/kg, p.o.) of aminoguanidine given alone for a period of 30 days did not produce any significant reduction in the level of malondialdehyde in the demented rats.

Pyridoxine treatment given to the demented rats for 30 days also cuased significant increase (P < 0.01) in the level of superoxide dismutase (193.2 ± 1.9 EU/dl) as compared in demented rats (75.37 ± 5.52 EU/dl). However, demented rats treated with aminoguanidine alone and donezepil for a period of 30 days did not produce significant increase in the level of antioxidant enzymes as compared to demented rats.

Discussion

Dementia is a word for a group of symptoms caused by disorders that affect the brain and mainly indicates Alzheimers disease. Various pathophysiologies exist for AD and excessive formation of nitric oxide is one of them. Nitric oxide represents both challenges and opportunities to intervene in and promote human health.

Aluminium is a highly neurotoxic element that is involved in neuronal degeneration in human and experimental animals brain. Various epidemiological studies have suggested that, aluminium

play a pathogenic role in AD and may play role in the pathogenesis of critical neuropathologic lesions in AD. It is well established that aluminium induces the production of free radicals in brain, activates phosphorylation of tau protein, activates iNOS which leads to formation of NO and ONOO$^-$ and neuronal damage in the brain (Wallance et al., 1997).

The present investigation demonstrated the effect of nitric oxide synthase inhibitor aminoguanidine for treating aluminium chloride induced dementia in rats. As the use of aminoguanidine is associated with vitamin B6 deficiency and hepatotoxicity at higher dose levels therefore, in the present study intermediate dose of aminoguanidine was given alone and in combination with pyridoxine to observe the efficacy of the treatment in dementia with minimum adverse effect.

In the present study administration of aluminium chloride for 30 days caused significant increase in the escape latency time in either acquisition or recall process as evaluated in morris water maze. It also caused increase in average time elapsed in radial arm maze apparatus indicating induction of dementia in rats. Aminoguanidine alone at higher dose level and the combination of intermediate dose of aminoguanidine with pyridoxine caused significant decrease in the time elapsed both in morris water maze and radial maze apparatus indicating improvement in the memory.

In dementia, due to activation of iNOS, excessive formation of NO takes place in the brain regions. This NO reacts with superoxide radicals to form ONOO$^-$, a highly reactive oxidant which regulate excitotoxicity and induce oxidative DNA damage (Parathath et al., 2006). Our investigation reveals that aminoguanidine by inhibiting the enzymes iNOS, decreases the level of nitric oxide in the brain of demented rats. Intermediate dose of

aminoguanidine given alone to the demented rats does not have any effect on brain nitrite level where as in combination with pyridoxine it caused significant decrease in the level of nitric oxide in the brain, suggesting the fact that aminoguanidine even at lower doses, given in combination with pyridoxine can cause improvement in dementia.

LDH is released into the serum when cells are damage, so it acts in a non-specific manner for the presence of tissue damage in the body. Result of the present study showed that aluminium chloride caused enhancement in the level of brain LDH where as aminoguanidine alone at higher dose level and combination of intermediate dose of aminoguanidine along with pyridoxine caused marked reduction in the LDH level in demented rats indicating protective effect of the treatment on the damage of neurons.

The involvement of glutamate excitotoxicity and calcium overload is already known in neurodegeneration including AD (Aliyev et al., 2004). Results of present investigation reveal that aminoguanidine caused a significant decrease in the calcium level in brain and caused improvement in excitotoxic cell death. Combination of intermediate dose of aminoguanidine with pyridoxine improved serum calcium level in the demented rats. However pyridoxine does not have any effect on serum calcium level in the demented rats.

Impairment in the cholinergic neurotransmission is one of the major factor involved in the etiopathogenesis of memory defects in alzheimers diseases. The neurodegeneration in hippocampus and frontal cortex of the brain results in decline in acetylcholine release (Francis et al., 1999). Elevated acetylcholinesterase (AChE) enzyme activity further leads to scarcity of ACh at synapse. Acetylcholinesterase by directly interacting with amyloid-β, uses the deposition of this peptide into insoluble plaques and increases the neurotoxicity of amyloid components in alzheimer's disease. In our studies the level of acetylcholinesterase was significantly increased in the brain of aluminium chloride treated rats. Aminoguanidine as well as pyridoxine do not have any significant effect on the actylcholinesterase level in demented rats. However donezepil caused significant decrease in the level of AchE activity indicating improvement in the level of acetylcholine.

Oxidative stress is a major player in the pathology of neurodegenerative disorders. It is also reported that aluminium chloride causes elevation of reactive oxygen species due to impairment of the endogenous antioxidant system (Khan et al., 2013). In the present study significant increase in the level of TBARS (malondialdehyde) was observed in the AlCl$_3$ treated rats. The decreased level of SOD in AlCl$_3$ treated rats indicates that there is an increased generation of free radical and reduced activity of antioxidant system in combating the oxidative stress.

The level of TBARS (Malondialdehyde) and antioxidant

enzyme level was restored to normal level after treatment with pyridoxine and its combination with intermediate dose of aminoguanidine. The result indicated the potential of antioxidant pyridoxine and an iNOS inhibitor aminoguanidine for protecting the neurons from oxidative damage and proved them for the treating neuroinflammatory diseases.

Taken together all the results come to a conclusion that aminoguanidine as a potent iNOS inhibitor shows promising treatment strategy for dementia. Co-administration of pyridoxine reduced the effective dose of aminoguanidine for treating dementia and also vanished the toxicities of aminoguanidine. However further preclinical and clinical studies will be required to completely establish the dose combination of pyridoxine and aminoguanidine which will be effective in dementia with no associated toxicities.

Acknowledgement

Conflict of interest

The authors declare that there is no conflict of interest.

References

Aliyev A, Seyidova D, Rzayev N, Obrenovich ME, Lamb BT, Chen SG. 2004. Is nitric oxide a key target in the pathogenesis of brain lesions during the development of Alzheimer's disease? Neuroloical Research 26(5):547-53

Ellman GF, Diane KC, Andres VJ, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7 (July): 88-95.

Francis PT, Palmer AM, Snape M, Wilcock GK.1999. The cholinergic hypothesis of Alzheimer's disease: a review of progress. Journal of Neurology, Neurosurgery, and Psychiatry 66(2):137-147

Guerra J, DeJesus A, Santiago-BP, Roman-FA, Rodríguez E, Crespo MJ. 2005. Plasma nitric oxide levels used as an indicator of doxorubicin-induced cardio toxicity in rats. International Journal of Hematology 5:584-88.

Khan KA, Kumar N, Nayak PG, Nampoothiri M, Shenoy RR, Krishnadas N et. al. 2013. Impact of caffeic acid on aluminium chloride-induced dementia in rats. Journal of Pharmacy and Pharmacology 65(12):1745-752.

Krasovskii GN, Vasukovich LY, Chariev OG. 1979. Experimental study of biological effects of leads and aluminum following oral administration.

Environmental Health Perspectives 30 (Jun):47-51.

Mansour MA, Mostafa AM, Nagi MN, Khattab MM, Al-Shabanah OA. 2002. Protective effect of aminoguanidine against nephrotoxicity induced by cisplatin in normal rats. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 132(2):123-28.

Mishra HP, Fridovich I. 1971. The role of superoxide anion in the autooxidation of epinephrine and a simple assay of SOD. Journal of Biological Chemistry 247(10):3170-75.

Morris RG. 1984. Development of water maze producer for studying spatial learning in the rats. Journal of Neuroscience 11(1):47-60.

Nampoothiri M, John J, Kumar N, Mudgal J, Nampurath GK and Chamallamudi MR. 2015. Modulatory role of simvastatin against aluminium chloride-induced behavioural and biochemical changes in rats. Behavioral Neurology 1:1-9.

Niwa K. 2001. Amyloid-beta peptide cause cerebral vasoconstriction. American Journal of Physiology 281:2424

Okada M, Ayabe Y. 1995. Effects of aminoguanidine and pyridoxal phosphate on glycation reaction of aspartate aminotransferase and serum albumin. Journal of Nutritional Science and Vitaminology 41:43-50.

Olton DS, Samuelson RJ. 1976. Remembrance of placed passed: spatial memory in rats. Journal of Experimental Psychology: animal learning and cognition 2:97-116.

Parathath SR, Parathath S, Tsirka SE. 2006. Nitric oxide mediates neurodegeneration and breakdown of the blood-brain barrier in tPA-dependent excitotoxic injury in mice. Journal of Cell Science 119:339-349.

Ridnour LA, Thomas DD, Mancardi D, Espey MG, Niranda KM, Paolocci N et al. 2004. The Chemistry of nitrosative stress induced by nitric oxide and reactive oxygen species. Putting perspective on stressful biological situations. Biological Chemistry 385(1):1-10.

Singh S, Kushwah AS, Singh R, Farswan M, Kaur R. 2012. Current therapeutic strategy in alzheimer's disease. European Review for Medical and Pharmacological Sciences 16(12): 1651-64.

Slater TF, Sawyer BC. 1971. The stimulatory effect of carbon tetrachloride and other halogenalkane or peroxidative reaction in the rat liver fraction in vitro. Journal of Biological Chemistry 123:805 -814.

Steinert JR, Chernova T, Forsythe ID. 2010. Nitric oxide signaling in brain function, dysfunction and dementia. Neuroscience 16(4):435-62.

Stevens JF, Tsang W, Newall RJ. 1983. Measurement of the enzymes lactate dehydrogenase and creatine kinase using reflectance spectroscopy and reagent strips. Journal of Clinical Pathology 36(1):1371-76.

Thatcher GRJ, Bennett BM, Reynolds JN. 2005. Nitric oxide mimetic molecules as therapeutic agents in Alzheimer's disease. Current Alzheimer Research 2(2):171.

Theodore SP. 1972. Spectrophotometric determination of Calcium. In Upper Iowa College, Fayette, Iowa 52142, USA) 429-431.

Wallance WC, Akar CA, Lyons WE. 1997. Amyloid precursor protein potentiates the neurotrophic activity of NGF. Molecular Brain Research 52:201-212.

Abbreviations

AchE: acetylcholinesterase; AlCl3: Aluminium chloride; AD: alzheimer's disease; AG: aminoguanidine; Aβ: amyloid beta; Cm: centimeter; CMC: carboxy methyl cellulose; ELT: escape latency ; H2O2: hydrogen peroxide ; mg: magnesium; μl: microliter; NO: nitric oxide; NOS: nitric oxide synthase; kg: kilogram; g: gram; , AG: aminoguanidine; CMC: carboxy methyl cellulose; nm: nanometer; TBARS: Thio barbituric acid reactive substances; LDH: lactate dehydrogenase; EU/dl: enzyme units/desi liter; SOD: superoxide dismutase; Sec.: seconds; iNOS: inducible nitric oxide synthase; nm: nanometer; SOD: Superoxide dismutase,; ml: milliliter; ONOO⁻: peroxy nitrile