

NEUROTOXIC EFFECT OF ENTERAL ALUMINIUM

A. BILKEI-GORZÓ

Pharmacological Department, Chinoin Pharmaceutical and Chemical Works Co. Ltd, H-1045 Budapest,
To u. 1-5, Hungary

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Abstract—Long Evans rats were treated for 90 days with water-soluble, insoluble or chelated aluminium compounds. The daily treatments given were as follows: controls, NaCl (100 mg/kg body weight) plus citric acid (30 mg/kg); AlCl₃ (30 or 100 mg/kg); Al(OH)₃ (100 mg/kg) plus citric acid (30 mg/kg); Al(OH)₃ (300 mg/kg). Their learning ability was determined in the labyrinth test at day 90, and the choline-acetyltransferase, acetylcholinesterase activity and aluminium content of the brains were measured. Soluble and chelated aluminium compounds seriously worsened the learning ability, and the aluminium content of the brain was elevated. Acetylcholinesterase activity increased and choline-acetyltransferase activity decreased, resulting in a diminished cholinergic activity, which is a characteristic of Alzheimer's disease.

INTRODUCTION

The connection between the aluminium content of the brain and various dementias has been pointed out (McDermott *et al.*, 1978; Perl and Brody, 1980). The occurrence of dementias is remarkably high in the Guam islands, where the aluminium content of the environment is also high (Perl *et al.*, 1982). The association between lifetime exposure to aluminium through the use of antiperspirants and antacids and Alzheimer's disease has been explored (Graves *et al.*, 1990). Workers exposed to aluminium have reduced memory ability and their mental and emotional balance is disturbed (Hosovski *et al.*, 1990). The importance of the aluminium entering the organism with food or drinking water has also been shown, and a positive correlation was demonstrated between the aluminium content of natural waters and the incidence of Alzheimer's disease (Martyn *et al.*, 1989).

A series of experiments has been carried out to establish changes in learning ability and behaviour during aluminium treatment (Petit *et al.*, 1980; Rabe *et al.*, 1982).

One of the most characteristic biochemical symptoms of Alzheimer's disease is the generally diminished function of the cholinergic system. 10 µM-aluminium decreases choline transport (King *et al.*, 1983); this concentration is not much higher than the physiological value. Aluminium elevates the activity of acetylcholinesterase at a range of 1–10 µM. However at higher concentrations it has a noncompetitive inhibitory effect on acetylcholinesterase (Marquis and Black, 1984). The activity of choline-acetyltransferase (CAT) is decreased by aluminium in brain tissue homogenate: this inhibition rapidly increased with concentrations up to 10 µM, but at higher con-

centrations the elevation became slower (Hetnarski *et al.*, 1980).

The aim of the present study was to evaluate whether enterally applied aluminium compounds induce symptoms in rats similar to those observed in Alzheimer's disease, and whether these symptoms can be provoked by insoluble and chelated aluminium compounds, which are common in the natural environment, or only by synthesized soluble aluminium compounds.

MATERIALS AND METHODS

Animals and treatment. Long Evans rats (2 months old) were obtained from LATI. They were kept under standard laboratory conditions (22 ± 2°C, 70% relative humidity) and housed in groups of 10 in plastic cages with stainless-steel covers fitted with food hoppers and water bottles. Drinking water was available *ad lib*. Each treatment group consisted of 10 males and 10 females. The treatments lasted for 90 days, and during the experiment the weight of the animals was measured and recorded daily. The substances were suspended in 1% carboxyl-methyl cellulose (1 ml/kg body weight) and the animals were treated orally once a day. Treatments were: (1) sodium chloride (100 mg/kg body weight) plus citric acid (30 mg/kg); (2) aluminium chloride (100 mg/kg); (3) aluminium chloride (30 mg/kg); (4) aluminium hydroxide (300 mg/kg); (5) aluminium hydroxide (100 mg/kg) plus citric acid (30 mg/kg).

We tested the effects of the soluble aluminium compounds with treatments (2) and (3), the effects of the insoluble compounds with treatment (4), the influence of the insoluble compound with chelating agent with treatment (5), and the first group was the control.

Learning ability test in a labyrinth. The trials were done at day 90 of the treatment in a 120 × 120-cm

Abbreviations: CAT = choline-acetyltransferase; DTNB = dithiobis-nitrobenzoate.

four-T-shaped labyrinth (Fig. 1) between 20.00 and 02.00 hr. There was a very low background noise level, and the room was illuminated with glimmer light during the experiment. The rats were not hyper-reactive to handling because of the daily treatment. Five male and five female rats per treatment group were tested. They were fasted for 48 hr and deprived of water for 24 hr before the experiment. The necessary degree of drive was determined in preliminary studies (with untreated rats). We finished the test within 8 hr because of the diurnal cycle of the rat. The reward was five drops of water in the goal-box. Rats could start after the door was drawn up, and they were replaced carefully in the start-box after drinking the five drops of water in the goal box. When the animals stood with fore-feet in the enclosed arm, we considered it as an error. The experiment was ended when the tested animal reached the goal-box without error on two consecutive occasions. There is only a 1/256 chance of a rat reaching the goal-box by chance on two successive times. In longer experiments the rats entered the closed arms of the labyrinth because their drive (thirst) decreased, and was exceeded by their exploratory drive. This may cause error when judging the rats' performance of the task. The maze was cleaned after each trial with 10% ethanol and dried. We measured the time that passed from drawing up the door to leaving the start-box, the time that passed from starting to finding the goal-box, and the number of attempts (runs) that were necessary for the rat to learn the labyrinth. For statistical evaluation, Student's unpaired *t*-test was used.

After the learning tests had been finished, the rats were killed by decapitation, the brains were removed and were cut into two equal parts. One part (alternately the left and right part) was used for the determination of the aluminium content, the other part for the determination of acetylcholinesterase and choline acetyltransferase activities.

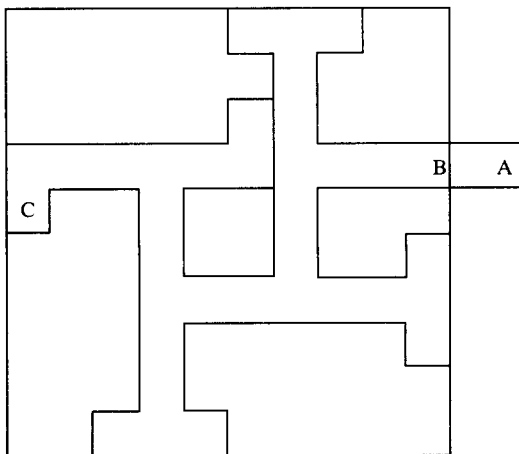


Fig. 1. Diagram of the four-T-shaped labyrinth used in the learning test. A = start-box; B = door; C = goal-box. The labyrinth measured 120 × 120 cm.

Determination of the aluminium content of the brains. The tissue samples were placed in a nitric acid-washed quartz cuvette. We added 65% nitric acid to the samples (100 mg sample/ml nitric acid). Heating the sample with the acid in an autoclave at 134°C yielded a transparent pale yellow liquid. The aluminium content was measured with a Labtam 8440 M emission spectrophotometer. The sample was vaporized into an argon plasma induced by a high frequency current (24.42 MHz), and the emission was measured at 237.312 nm. The results of the control and treated groups were compared using Student's unpaired *t*-test.

Acetylcholinesterase activity. The experiment was done according to Ellman's method (Ellman *et al.*, 1961). Briefly, acetylcholinesterase in the sample decomposes acetylcholine to thiocholine and acetic acid, and the thiocholine formed reacts with dithio-bis-nitrobenzoate (DTNB), and yields a yellow, photometrically measurable product. Solutions: phosphate buffer, 0.1 M, pH 8.0; acetylcholine iodide, 0.075 M (substrate); DTNB, 0.01 M (reagent).

The brains were homogenized in acetone at -30°C using a Universal MPW electric homogenizer and the suspension was stored at -25°C. This powder was solubilized before the enzyme assay at a concentration corresponding to 20 mg brain wet weight/ml buffer. The typical mixture contained 0.4 ml brain suspension, 2.6 ml buffer, 20 µl substrate and 100 µl reagent.

The reaction took place in a cuvette thermostatically controlled at 37°C, and the absorbance was measured with a Perkin Elmer-Hitachi 200 photometer at 412 nm (1-nm slit) and recorded continuously. The rates were calculated as follows according to Ellman *et al.* (1961): $R = 6.75 \times 10^{-6} dA$, where R = rate as mol substrate hydrolysed per min × mg protein, dA = change in absorbance per min. The protein content of the brain was determined according to Lowry (Lowry *et al.*, 1951). The results of the control and treated groups were compared using Student's unpaired *t*-test.

CAT activity of the brains. The brains of the animals treated for 90 days were suspended in cold (-30°C) acetone using a homogenizer and then strained, dried and frozen. The sample was resuspended in 50 mM-potassium phosphate buffer (pH 7.4), the homogenate was centrifuged (5000 *g*, 90 min, 4°C). The pH of the supernatant was adjusted to 4.5 with 50% acetic acid and stirred for 30 min. The solution was then centrifuged at 5000 *g* for 60 min. The pH of the supernatant was adjusted to 6.0 with 2 N-ammonium hydroxide. Solid ammonium sulphate was added to 65% saturation and stirred for 30 min. The precipitate was collected by centrifugation at 5000 *g* for 90 min, strained and frozen. The CAT activity was determined according to Hersch and Peet (1978) with slight modifications. Briefly, the activity of CAT could be determined by measuring

Table 1. Number of runs necessary to learn the labyrinth for rats treated with various aluminium compounds for 90 days

Treatment (daily dose)	Number of runs*	Significance†
Control	5.1 ± 2.88	—
AlCl ₃ (100 mg/kg)	17.1 ± 5.33	P < 0.001
AlCl ₃ (30 mg/kg)	14.1 ± 4.29	P < 0.001
Al(OH) ₃ (100 mg/kg) + citric acid (30 mg/kg)	16.0 ± 2.98	P < 0.001
Al(OH) ₃ (300 mg/kg)	13.2 ± 5.39	P < 0.01

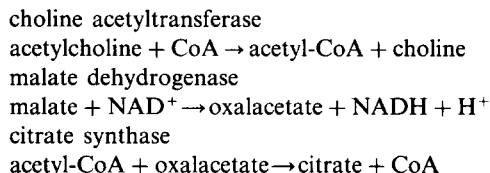
*Values are means ± SD for groups of 10 rats.
†In comparison with the controls. Student's unpaired *t*-test.

Table 2. Aluminium content of the brains of Long Evans rats treated orally with various aluminium compounds for 90 days

Treatment (daily dose)	Aluminium content (ppm)*	Significance†
Control	6.6 ± 3.01	—
AlCl ₃ (100 mg/kg)	22.2 ± 12.18	P < 0.01
AlCl ₃ (30 mg/kg)	16.1 ± 6.91	P < 0.01
Al(OH) ₃ (100 mg/kg) + citric acid (30 mg/kg)	18.0 ± 10.20	P < 0.05
Al(OH) ₃ (300 mg/kg)	11.0 ± 4.80	P < 0.05

*Values are means ± SD for groups of 10 rats.
†In comparison with the controls. Student's unpaired *t*-test.

the formation of NADH fluorometrically according to the scheme:



Before starting the trial we determined that aluminium has no effect on citrate synthase or on malate dehydrogenase. For measuring the effect of aluminium on CAT, the typical reaction mixture contained 50 mM-potassium phosphate buffer (pH 7.4), 0.25 M-sodium chloride, 0.25 mM-NAD, 1 mM-D,L-malate, 0.5 mM-mercaptoethanol, 10 mM-acetylcholine chloride, 0.01 mM-CoA, 5 U citrate synthase/ml, 5 U malate dehydrogenase/ml, and precipitate dissolved in phosphate buffer (0.1 N, pH 7.4). The protein content was determined with the Folin-Ciocalteu's phenol reagent (Lowry *et al.*, 1951).

The reaction was monitored with an OPTON spectrofluorometer equipped with a thermostatically controlled cell chamber which was maintained at 37°C. The increase in NADH fluorescence was followed (excitation at 340 nm, and emission at 460 nm) and recorded with a Perkin Elmer potentiometer. The reaction was started by adding CoA to the mixture. [Blanks without malate dehydrogenase were also included.]

RESULTS

The animals were weighed daily during the treatment in order to detect any toxic effect. There were no relevant differences in body weights or the general condition of the rats between the control and treated

groups. Drinking water and food intake did not differ between the groups, suggesting that the animals' motivation to drink and eat was not influenced by the treatments.

Learning test

Five males and five females were tested in each treatment groups. The starting time (i.e. from drawing up the door to the rats leaving the start-box), which negatively correlates with the level of motivation, the running time (passed from start to finding the goal), which depends on the motivation and the learning ability, and the number of tries that were necessary to learn the labyrinth were determined. There was no running order, we simply removed the door of the start-box. We supposed that rats had learned the labyrinth when they did not miss the correct way twice in a row. The environment was very similar during testing the different groups, and the behaviour of the rats (and therefore the nature of errors) was very similar across groups. All of the treated groups needed more repetition to learn the labyrinth than the control group (Table 1). The performance of the rats treated with 100 mg AlCl₃/kg was the worst. It is remarkable that aluminium in a chelated form had a strong effect in this trial.

It was not possible to compare temporal data because the number of trials differed widely between groups. There were no significant differences in start and trial times across groups as calculated by two-way ANOVA.

Aluminium content of the brains

The aluminium content of the brains of five males and five females was determined in each group (Table 2). The aluminium content was elevated in each treated group. The treatment with aluminium

Table 3. Acetylcholinesterase and choline-acetyltransferase (CAT) activities in the brains of rats treated orally with aluminium compounds for 90 days

Treatment (daily dose)	Acetylcholinesterase		CAT	
	Activity (nmol/min × mg protein)†	Potentialiation (%)	Activity (nmol/min × mg protein)†	Inhibition (%)
Control	20.37 ± 8.51	—	30.9 ± 16.9	—
AlCl ₃ (100 mg/kg)	37.20 ± 8.61**	83	13.0 ± 8.9*	58
AlCl ₃ (30 mg/kg)	27.25 ± 7.62	33	23.9 ± 11.3	23
Al(OH) ₃ (100 mg/kg) + citric acid (30 mg/kg)	33.73 ± 14.76*	65	26.2 ± 15.3	15
Al(OH) ₃ (300 mg/kg)	23.66 ± 7.01	16	32.6 ± 14.9	—

*Significantly different from the controls (Student's unpaired *t*-test; *P < 0.05; **P < 0.02).
†Values are means ± SD for groups of 10 rats.

chelate greatly elevated the amount of aluminium in the brain. The elevation was the highest in groups treated with soluble aluminium compounds.

Acetylcholinesterase and CAT activities

Enzyme activities were determined in the brain of five males and five females in every group. All the treatments elevated the acetylcholinesterase activity, and the increase was significant in two treatment groups (Table 3).

The CAT activity was lowered in each group except the $\text{Al}(\text{OH})_3$ -treated group (Table 3).

DISCUSSION

We found that the learning ability was diminished by the treatment with all of the aluminium compounds. A similar phenomenon has been described by Lipman *et al.* (1988), who used a shuttle-box and intracerebroventricular treatment. However, Connor *et al.* (1988) found a significant effect of aluminium only in the passive avoidance test and not in the labyrinth test. Aluminium applied intracerebroventricularly also causes motor and olfactory deficit, because of the unspecific toxic effect. A similar phenomenon was not detected when aluminium was given orally, because the intracranial aluminium concentration did not rise so sharply. We chose the labyrinth test instead of the shuttle-box or passive avoidance test because we think that solving this problem is a more natural task for a rat than the Skinner-type learning tests, and so it is appropriate for modelling the deterioration of the natural learning ability. We also considered that the labyrinth test is a good model of the deterioration of learning ability in Alzheimer's disease: patients with this disease could not solve problems that were previously solved routinely. We could not determine a progressive change in the learning ability with this arrangement, but the repetition of the task did not influence our experiment.

In our experiments aluminium hydroxide had some effect on the nervous functions, indicating that this strongly insoluble compound might be absorbed and exert its influence on the organism, as was shown by Cutrufo's EEG analysis (Cutrufo *et al.*, 1984).

An elevated activity of acetylcholinesterase in the brain of aluminium-treated rats was observed (this elevation was not relevant in the $\text{Al}(\text{OH})_3$ -treated group) and this corresponds to the results of an *in vitro* test (Marquis and Black, 1984). In the present study, the activity of CAT, partially purified from the brain, was lower in three of the treated groups (significant in one) in comparison with the controls, although the aluminium contents of the reaction mixtures were of similarly low levels in each group, because the aluminium content, which was initially at a high level in the treated groups, was removed during the purification of the enzyme. It has been pointed out (Atterwill, 1989) that aluminium directly inhibits the activity

of the pure enzyme. Presumably, the *in vivo* inhibitory activity of the metal consists of two additive elements: (1) a direct effect of the metal on the enzyme and (2) an indirect effect, based upon a decreased production of the enzyme and/or a loss of cholinergic cells.

We measured an elevated aluminium content in the brain of treated rats, so it is clear that both direct and indirect effects of aluminium affected the cholinergic system. Moreover, the activity of the enzyme that generates acetylcholine (CAT) was reduced and the activity of the enzyme that decomposes it (acetylcholinesterase) was elevated by aluminium, so it can be assumed that cholinergic activity is strongly diminished. The fact that insoluble and chelated aluminium, applied enterally, lowers the cholinergic function, supports the view that aluminium plays a causal role in the pathogenesis of Alzheimer's disease.

It is interesting to compare this result with the data of Szerdahelyi and Kása (1988) who reported that the aluminium content of the brain of rats treated with AF64A cholinotoxin was elevated. Our results indicate that aluminium may act as a cholinotoxin. It is probable that it is a self-induction process and it might be crucial in the pathogenesis of this disorder. It was shown in an *in vitro* trial that aluminium entering the brain has an effect on cholinergic enzymes (Miller and Levine, 1974), but there is no data to indicate how decreased cholinergic function can elevate the aluminium content. It is remarkable that the cholinergic system has a strong influence on the permeability of the blood-brain barrier (Eryurek *et al.*, 1990; Petrali *et al.*, 1991). According to our results, the aluminium content of the brain was elevated during the experiment. There was a negative correlation between the aluminium content and the CAT activity of the brain ($r = -0.750$), and a positive relation between the aluminium content and the acetylcholinesterase activity ($r = 0.552$) and between the aluminium content of the brains of the rats and the number of runs necessary to learn the labyrinth ($r = 0.857$). Water-soluble aluminium compounds have a stronger effect, and so not only the water-soluble salts, which are very rare in the natural environment, but also the chelated compounds were effective. This fact is remarkable, because it can be assumed that aluminium enters the organism mainly with food, and so the polyvalent organic anions found in plants may facilitate its absorption. This observation is in line with the phenomenon described by Hewitt *et al.* (1988), concerning the possible role of polyvalent anions in aluminium toxicity.

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