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A longitudinal study of rats chronically exposed to aluminum at human dietary levels

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Abstract

According to the World Health Organization, oral ingestion of aluminum additives is the main form of aluminum exposure for the general public. Aluminum salts are added to a range of commercially-prepared foods and beverages: to clarify drinking water, make salt free-pouring, color snack/dessert foods, and make baked goods rise. In the present study, six Wistar rats chronically consumed aluminum from 16 months of age to the conclusion of their lifespan (averaging 29.8 months) in an amount (1.5 mg/kg bodyweight) equivalent to the high end of the total aluminum range ingested daily by humans living in contemporary urban society. The rats were memory-trained in a continuous rewarded alternation T-maze task and tested weekly from 5 months of age onwards. This longitudinal study compared their mean memory performances over 15 consecutive weeks during middle age (12–23 months) and old age (≥24 months). Four out of six rats continued to perform the memory task in old age without significant deficit. The remaining two obtained significantly lower mean memory scores in old age than in middle age and exhibited soft signs associated with dementia. Their hippocampal neurons stained for aluminum, showing some but not all features of aluminum accumulation that occur in human hippocampal neurons. In view of evidenced linkages of aluminum with β-amyloid plaque and neurofibrillary tangle formation in humans with Alzheimer's disease, the findings suggest this protocol is worth testing in larger groups of animals.

Keywords: Aluminum; Aging; Rats; Animal model; Dementia; Alzheimer's disease

Recent findings relate cerebral aluminum to the formation of β-amyloid [8] and neurofibrillary tangles (NFTs) [25] in brains of humans with Alzheimer's disease (AD), rekindling interest in aluminum exposure as a potential cause of AD in susceptible individuals. Experimentally, aluminum enhances the size and number of amyloid plaques that form in cortical tissue of transgenic rodents engineered to express a mutant human gene for amyloid precursor protein [19].

Orally ingested aluminum additives currently comprise the main form of aluminum exposure for the general public [28]. Most Americans (representative of humans living in contemporary urban society) ingest 0.01–1.4 mg total aluminum/kg body weight/day, based on the weight of an average 70 kg human [12]. "Total aluminum" as defined here is the amount of aluminum consumed from foods, beverages (including water), and aluminum additives. Up to 10 mg (0.14 mg/kg) aluminum/day is ingested from freshly-prepared natural sources, mainly fresh fruit, vegetables and meat. Taking into account data from a US food additives survey, Greger estimates approximately 50% of Americans also ingest up to 24 mg aluminum/day (i.e., <0.34 mg/kg bodyweight), 45% between 24 and 95 mg aluminum/day (0.34–1.36 mg/kg bodyweight), and 5% more than 95 mg aluminum/day (>1.36 mg/kg bodyweight) as additives in commercially-processed foods and beverages [3,12]. For example, ready-to-eat pancakes contain up to 180 mg aluminum/serving [21].

Almost all experimental studies investigating the effects of aluminum on animals have utilized aluminum exposure levels much higher than those to which humans are chronically exposed. As toxic metals cause different clinical pictures depending on the subjects' age, health, dose levels, route, and other conditions of metal exposure [13], we questioned whether rodents that chronically ingested aluminum at the high end of the range for total aluminum in the human diet would develop any characteristic effect of AD in old age, despite the expected absence of plaques and tangles. As a step towards answering this question, we carried out a longitudinal study of a small group of rats chronically fed aluminum in equivalent amounts.

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This study was carried out in compliance with ethics approval from the Commonwealth Scientific and Industrial Research Organisation's Animal Care and Ethics Committee at Prospect, NSW, Australia in accordance with the Animal Welfare Act, 1992 (Australia). Six outbred male Wistar rats were obtained as weanlings from the University of Technology Sydney Animal Breeding Facility (Gore Hill, NSW). Our animal house had large uncovered windows in one wall, allowing exposure to natural light and dark cycles. Three rats were housed in each cage that had a solid bottom and a high stainless steel mesh top. As their companions died in old age, the rats were housed in pairs. From age 20 months, they were given monthly dental treatment to maintain their lower incisors at an appropriate length.

Until 4 months of age, the rats were fed a diet formulated for growing and breeding rodents and given Sydney tap water, both provided *ad libitum*. No attempt was made to control the amount of aluminum the animals consumed during their main growth period. At 5 months of age when the rats had achieved most of their maximum length, we began to monitor their aluminum intake. We fed them twice weekly a low-protein/low-fat rodent maintenance diet at 140 g/animal/week, with Sydney tap water provided *ad libitum*. The diet included vitamins and minerals and had an aluminum content of 9 ppm (Gordon's Specialty Stockfeeds, Yanderra NSW, Australia), We recorded rat bodyweights weekly, and the amounts of water they drank both when housed in their home cages and, on several occasions, when housed individually in metabolism cages.

Beginning at age 16 months and thereafter, the water given to the rats contained additional aluminum supplied for convenience as aluminum chloride ($AlCl_3{\cdot}6H_2O$, ICN Biomedicals Inc., Irvine, CA) in ultrapure water (Milli-Q Plus; Millipore Corporation, Billerica, MA) at 20 mg aluminum/L. Food aliquots and water samples were assayed for aluminum by the Trace Metals Laboratory (Pacific Laboratory Medical Services, Royal North Shore Hospital, Sydney, Australia).

At 5 months of age, the rats were trained to perform a continuous rewarded alternation T-maze task that depends on the dorsal hippocampus [1] and has both working memory and reference memory components. Then, the rats were tested weekly by the same person, blind to their previous scores, for the rest of their lives. The longest-lived rat performed the task for 102 weeks. Scores and times were recorded for each testing session. Each score was based on the percentage of correct choices the rats made out of 10 trials. Initially, both T-maze arms were baited with rewards so the first run from the stem into an arm did not contribute to the score. Thereafter, alternate arms were baited. Replaced in the T-maze stem between each run, a rat had to alternate between right (R) and left (L) arms for 10 trials to score 100% (e.g. (R)LRLRLRLRLR), subtracting 10% per error. Approximately 12 h of memory tests were recorded on videotape.

The rats continued their weekly testing routine until they eventually began to show marked physical deterioration, and were then euthanized with Nembutal. Their brain was rapidly removed, fixed in 10% neutral-buffered formalin, paraffin-embedded, sectioned, and hippocampal sections were processed with the Walton stain [24], a sensitive bright field/fluorescence method for aluminum. For comparison, brains from three 6-month (young) euthanized rats were processed in the same way. With this technique, sections are initially stained with 0.5% phloxine B, then 5% phosphotungstic acid, followed by differentiation with 95% ethanol, and counterstaining. Photomicrography was carried out with a Leica research microscope with Kohler illumination (Leica Instruments, Wetzlar, Germany).

Paired *t*-tests were used to analyze the longitudinal memory performances (SigmaStat, Systat Software Inc., Richmond CA). Scores over 15 consecutive weeks beginning at the onset of aluminum treatment in middle age were compared with scores from 15 consecutive weeks preceding and including the animal's last test. Δ = mean1 (performance in middle age) − mean2 (performance in old age).

The amount of pelleted food given to the rats at their twice-weekly feedings since 5 months of age was sufficient to maintain their bodies at a healthy adult weight of 500 ± 50 g for most of their lifespan. On average, they ingested a mean load of 0.36 mg aluminum/kg bodyweight/day from their food rations. With this protocol, the rats had glossy coats and retained a sleek appearance well into old age.

At 5 months, the rats drank 0.055 L water/day on average. This amount steadily decreased over time until leveling off during their middle age. Throughout middle age and old age, the rats drank an average of 0.029 L each day. Sydney tap water generally contains less than 0.01 mg aluminum/L as it is clarified with iron salts instead of alum [23]. Thus, during the period between 5 and 16 months, the rats obtained less than 0.001 mg aluminum/kg bodyweight/day from their drinking water and their total aluminum level averaged 0.36 mg/kg/day.

From 16 months onwards, the rats drank water with a mean aluminum load of 1.16 mg aluminum/kg each day, raising their total aluminum intake from food and water to 1.52 mg aluminum/kg/day, equivalent in amount (mg/kg bodyweight basis) to the high end of the human dietary range for total aluminum. Since the animals finished eating their food rations on the day before they were re-fed, they could be observed drinking water without feeding and they remained motivated for their weekly memory tests given just prior to their end-of-week feeding session.

In middle age, Rats 6 and 8 had lower mean memory scores than all other rats (Table 1). Rat 6's lower scores in middle age gradually improved ($p < 0.001$), almost catching up in old age to scores obtained by Rats 4 and 10. Rats 2, 4, 10 maintained their mean memory scores at 70–90% accuracy in old age, without significant change from their scores in middle age. Conversely, Rats 0 and 8 had significantly lower mean memory scores in old age than in middle age ($p < 0.01$ and < 0.001, Table 1). Although the movement of all rats appeared spontaneous and unimpaired in old age, some moved more slowly, walking instead of running, and taking approximately 28% longer to perform the maze task than when younger ($p < 0.05$).

From 28.5 months onwards, Rat 0 made a substantial number of errors without favoring either maze arm. Rat 8's longitudinal memory scores showed a downturn at 23 months and by 25 months the downward trend was well defined. Increasingly, Rat 8 exhibited marked bias for turning into the right maze arm

Table 1
Comparison of the rats' mean memory scores in middle age (mean1; $n1 = 15$ tests) and old age (mean2; $n2 = 15$ tests)

Rat ID	Middle age scores mean1 (%)	$n1$	Old age scores mean2 (%)	$n2$	Difference	95% confidence interval		Significance level
0	83.33	15	62.00	15	21.33	9.3337	33.3329	*
2	76.67	15	69.33	15	7.33	−7.9792	22.6383	NS
4	84.00	15	87.33	15	[−3.33]	−16.516	9.8493	NS
6	56.00	15	82.67	15	[−26.67]	−39.8493	−13.484	[**]
8	62.00	15	38.00	15	24.00	6.7653	41.2347	**
10	85.33	15	85.33	15	0.00	−13.2379	13.2379	NS

For significance levels: *, $p < 0.01$; **, $p < 0.001$; NS, not significant. Unbracketed asterisks indicate statistically significant declines in scores; the bracketed asterisk and difference indicate increase in score.

instead of alternating to make a correct choice. This change is illustrated in the accompanying Quicktime movies entitled "Rat 8 in middle age.mov" and "Rat 8 in old age.mov". In his last 10 test sessions, Rat 8 made 82% of turns into the right arm. In addition to these errors indicative of impaired working memory, both rats made errors indicative of impaired reference memory. On some runs, they stopped short of the reward location to hunt for the reward in the wrong place, as evident in the latter Quicktime movie. Both rats lived well beyond the age their memory scores began to decline: Rat 0 lived to 32.0 months and Rat 8 to 30.8 months. The mean life span for all rats was 29.8 ± 0.7 months (mean ± S.E.M.) with a range of 27.0–32.0 months.

Concurrent with their deficits in working memory and reference memory, Rats 0 and 8 exhibited soft signs associated with dementia: repetitive behavior (excessive preening), indecision, and inability to concentrate on the task at hand. For example, instead of immediately choosing an arm and moving down it, as previously, they tended to move a few steps towards one arm, then reverse 180° towards the other, and then turn 90° to walk backwards into the stem, rearing on their hind legs to peer over the edge of the maze, or interrupting their maze task to preen themselves. When, at this stage, the rats were put into metabolism cages overnight for urine collection, the bottles of Rats 0 and 8, unlike the others, contained as much water after the rats' confinement as before, suggesting that Rats 0 and 8 were unable to recognize their water bottles in the less familiar position.

Neurons in brain sections from the aged rats and young rats showed difference when processed with the Walton stain for aluminum. Hippocampal neurons in brain sections from the 6-month rats were entirely aluminum-negative (Fig. 1A). In contrast, hippocampal neurons from all aged rat brain sections we examined showed varying extents of aluminum accumulation. In most neurons, aluminum was confined to the nucleolus; their morphology was otherwise normal at optical microscopy magnifications (Fig. 1B). Other hippocampal neurons stained more extensively for aluminum: either throughout the nucleoplasm (Fig. 1C) or both the nucleoplasm and cytoplasm, selectively displaying cytopathological features including shrinkage. Brains from Rats 0 and 8 had abundant neurons at these higher stages of aluminum accumulation, as did brains from some cognition-intact aged rats. However, we were unable to identify, in any rat brain sections examined, neurons with nuclear aluminum confined to the nucleolus with discrete aluminum-rich cytoplasmic regions as described in human brain [25].

Thus, we have described a protocol and small longitudinal study in a rat model that simulates exposure to the maximum amount of total dietary aluminum some humans chronically ingest. As rats are normally prandial drinkers [11], and aluminum is more readily absorbed on an empty stomach [5], we utilized a twice-weekly feeding protocol so the rats would drink some water on an empty stomach as humans often do. As a matter of husbandry, their lower incisors required routine clipping to maintain them at an appropriate length. If left unattended, the lower incisors of old rats can grow up into their nasal cavity, interfering with breathing and eating.

Although we monitored the rats' total aluminum intake from 5 months onwards, the additive aluminum treatment commenced when the rats were 16 months old. This allowed ample time for normal neural development so that any cognitive change, which might occur, would be due to neurodegeneration instead of impaired neural development. The rats' exposure to additive aluminum was 11–16 months, depending on longevity. Given that rats age about 35 times faster than humans [7], this exposure was approximately 32–47 years in human age equivalence. In

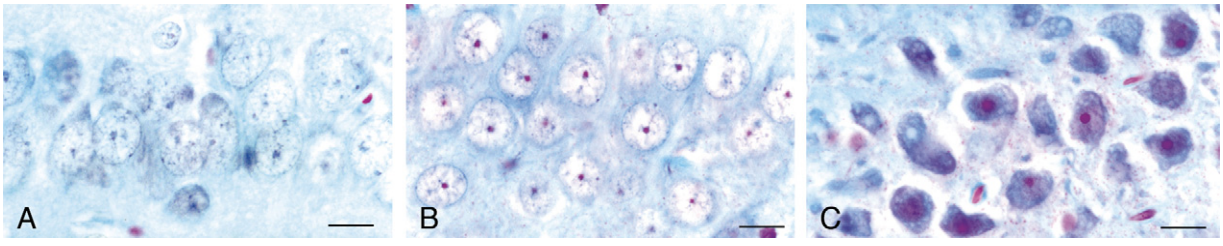

Fig. 1. Aluminum staining in sectioned rat hippocampal neurons. (A) Neurons from a 6-month rat lack evidence of aluminum staining. (B) Aged rat neurons with aluminum stain confined to the nucleolus. (C) Neurons from Rat 0 exhibit aluminum staining throughout the nucleoplasm. Scale bars = 10 μm.

view of the results, we now consider that the aluminum addition could begin earlier when rats are, say, 8–12 months old. This would extend the length of chronic treatment, allow more time for brain aluminum accumulation and, possibly, increase the percentage of affected animals.

We faced the choice of maintaining the rats in solitary confinement for their entire life to enable precise measurements of their food and water intake or measuring the average food and water consumption for each cage of animals. On the one hand, the six animals were of similar size (500 ± 50 g) and none drank excessive amounts of water on occasions when housed individually in metabolism cages. On the other hand, long-term social isolation is an animal welfare issue, it can alter neuronal morphology [4] and might impair the rats' memory task performance. Hence, we chose to cage the animals with companions.

The amount of food the rats ingested was sufficient to maintain their bodies at a healthy adult weight, thereby preventing over-eating and obesity in old age. In the present study, the condition of the fully-grown rats' bodies was probably comparable to that of humans able to maintain a healthy weight into old age. We note that the weight control of this protocol is much different from the 50% food restriction some investigators have given to rats post-weaning to slow their body growth, delay puberty, significantly alter the aging process, and extend life span [16].

All rats in the study proved they were able to successfully alternate prior to commencing the additive aluminum treatment, including the two rats that subsequently had significantly lower mean memory scores during old age than in middle age. The memory task scores of Rats 0 and 8 showed decline when they were approximately equivalent to the human ages of 83 and 67 years, respectively. Most Wistar rats have a natural bias for right turns, both at the individual and population levels [2]. Training for the continuous rewarded alternation task apparently requires the rats to suppress this natural inclination to prefer one arm more than the other. In old age, Rat 8's inherent right-biased laterality replaced the cognitive activity needed to make correct choices in the rewarded alternation task.

Despite having the same aluminum treatment, Rats 0 and 8 were behaviorally-affected whereas others were not, possibly indicating Rats 0 and 8 absorbed more aluminum and were thus more susceptible to aluminum toxicity. It is known that a small amount of aluminum can enter the brain from a minute oral dose simulating alum-treated drinking water, with more aluminum uptake into some brains than others from a standardized oral dose [5,26]. Also, some humans (including Alzheimer patients) absorb more aluminum from a standardized oral dose than others [6,17]. Relatively efficient aluminum absorption is probably influenced by genes since humans with Down's syndrome absorb six times more aluminum from a standardized oral dose than age-matched controls [18]. Moreover, some inbred mouse strains absorb and accumulate more aluminum in their brains than other inbred strains under uniform exposure conditions [9].

We further considered whether Rats 0 and 8 might have had a motor problem that contributed to incorrect choices by inducing delays. However, close observation, supplemented by review of videotape records, showed that delays between choices were unlikely to be associated with difficulty in moving. Rather, they resulted from preening and other apparently effortless movements that did not contribute to their scores.

This is the first application of the Walton stain to an animal study and we determined that rat neurons stain as for human neurons with one major difference. The aged human hippocampus contains neurons either with: (1) aluminum confined to the nucleolus; (2) nuclear aluminum in the nucleolus and throughout the nucleoplasm; (3) nuclear aluminum in the nucleolus and nucleoplasm as well as throughout the cytoplasm; or (4) nuclear aluminum confined to the nucleolus with discrete aluminum-rich regions in the cytoplasm. The latter neurons are those in which NFTs form [25]. All aged rat brain sections we examined had neurons comparable to those described above as (1–3) but lacked the neurons described as (4) that involve NFT formation. This may be a clue as to why AD-type NFTs do not develop in rat brains.

Experiments have shown the Walton stain specifically discloses aluminum bound to organic molecules in cellular and sub-cellular structures [24]; in some, aluminum has been confirmed with spectrometry [25]. In the present study, the Walton stain revealed that hippocampal neurons from all aged rats had varying degrees of nuclear aluminum accumulation whereas hippocampal neurons from the young rats lacked visible indication of aluminum. These histological results agree with instrumental analyses that have consistently shown higher aluminum levels in brain tissue of older than younger individuals [15,14,22], supporting the concept that aluminum accumulates in the brain over time.

Brains from Rats 0 and 8 contained many neurons at higher stages of aluminum accumulation but a larger study would be necessary to determine whether similarly-affected rats have either significantly more aluminum-positive neurons than others that remain cognition-intact or significantly more aluminum-positive neurons in specific brain regions critical for memory-processing. Nevertheless, the present finding that aged rat neurons contain aluminum after chronic exposure to the high end of the human range for dietary aluminum indicates that the Walton stain could be valuable in future studies to determine whether there is an aluminum dose level sufficiently low to prevent aluminum accumulation in the brain.

Over the past decade, a number of reports have described impaired acquisition of spatial learning in otherwise apparently healthy aged rats without identifiable neuropathology or hippocampal neuronal loss (e.g. [10,20]). The criterion used to identify those cognitively impaired rats was a significantly longer latency on a Morris water maze task over that of young controls, similar to the poor performance of Rat 6 in middle age. Major differences between those cognitively-impaired water maze-tested rats and Rats 0 and 8 of our study were: (1) the former took longer to learn their maze task while the latter lost their ability to correctly perform the maze task they previously performed with competence; and (2) the former had normal hippocampal neuron morphology whereas the latter exhibited hippocampal pathology. These differences together with the soft signs described above suggest that Rats 0 and 8 exhibited a disease state; namely, an animal form of dementia. Webster's

Medical Dictionary [27] defines dementia as "a condition of deteriorated mentality that is characterized by marked decline from the individual's former intellectual level." These findings suggest this protocol is worth repeating in studies with larger groups of rats, rabbits, and/or other short-lived species exposed to additive aluminum from an earlier age.

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Appendix A. Supplementary data

The Quicktime movies associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2006.08.093.

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