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Aluminium sulphate exposure increases oxidative stress and suppresses brain development in Ross broiler chicks

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Aluminium (Al) is known to have neurotoxic effects that can result in oxidative damage to a range of cellular biomolecules. These effects appear to be of significance in the developmental stages of the brain. We therefore investigated the oxidative and histopathological damage induced by Al during growth and development of the chick brain.

Material/Methods: We used a chick embryonic development model, with Al treatment of 500 μg Al sulphate in 0.1 ml saline injected into the egg air chambers at the beginning of their incubation period. The effects on chick-brain growth and development were then assessed at term (day 21). Determination of malondialdehyde and glutathione levels were used as relevant biological measures for increased oxidative stress in terms of lipid peroxidation and biochemical oxidative damage, respectively. Furthermore, we also monitored neuronal degeneration as estimated stereologically using the Cavalieri brain volume estimation tool.

Results: This Al treatment showed significantly increased MDA levels and decreased GSH levels, as indicators of increased biochemical oxidative damage. This was accompanied by significantly decreased brain volume, as a measure of neuronal degeneration during brain development in this chick embryonic development model.

Conclusions: Exposure to Al during chick embryonic development results in increased oxidative stress in the brain that is accompanied by neuronal degeneration.

key words: **aluminium • malondialdehyde • glutathione • oxidative stress • brain development • Cavalieri brain volume estimation**

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Background

Aluminium (Al) is ubiquitous in the environment and represents the third most common element in the Earth's crust and it generally exists in a combined state with other elements. The problem of Al contamination in our environment has been around for more than 25 years, despite which, it remains a neglected problem. In particular, Al is found in materials used in the pharmaceutical industry, and in manufactured foodstuffs, cosmetics and tap water. By overcoming the body barriers, Al can infiltrate into the blood and promote toxic effects in liver, bone and the central nervous system [1].

When pregnant mice are exposed to Al, the weights of the maternal spleen and liver increase, and their foetal top-to-heel lengths decrease [2], and again in pregnancy, the prooxidant effects of chronic Al exposure disrupt normal development in baby rats [3]. In rats exposed to Al, it has been shown that Al causes oxidative stress [4], and at high intraperitoneal doses Al causes important morphologic and ultrastructural damage to the rat kidney, liver and testis [5–7]. One reason for this might be the increased oxidative stress due to erythrocyte Al accumulation, with an induction of anaemia, potentially through the disruption of iron levels and alteration of iron homeostasis [8,9]. As another systemic problem, the skeletal system is one of the targets of Al toxicity, and Al intake can lead to osteomalacia [10].

However, one of the most sensitive targets of Al toxicity is the nervous system. Neurotoxic effects of Al have been indicated from various treatments with different salts of Al that have resulted in oxidative damage to a range of cellular biomolecules, such as lipids, proteins and nucleic acids [11]. This Al toxicity has been shown to affect plants, animals and human, with its effects seen as disturbances to various second-messenger signalling systems in cells, including phosphoinositide-derived signalling and Ca^{2+}-signalling pathways, and in the formation of lipid peroxides [11]. At the same time, although Al absorption from the diet is low in animals and human, Al can form complexes with citrate and transferrin, which can then cross the blood-brain barrier to affect the brain [11].

After systemic intravenous Al exposure in rats and rabbits, the extracellular Al concentrations were seen to increase primarily in the frontal cortex and hippocampus of the brain [12]. From further studies in rats, the neurochemical changes caused by Al in the brain depend on the duration of the exposure and are region-specific [13], with memory and learning disorders being reported [14,15]; in mice Al has been shown to cause oxidative stress in the brain [16]. When applied to cultured human brain cells, according to the time and concentration of the Al exposure, reductions in cell growth rates were seen [17].

In terms of the effects of Al on the functions of the brain, in the rat, it was reported that the impaired neural function caused by Al is related to its damage to intracellular Ca^{2+} homeostasis [18]. This has been suggested to be linked to disturbed K and Na currents, whereby Al damages the rat hippocampal CA1 neurons, promoting further damage to the central nervous system [19]. This appears to be mediated by changes in the amino-acid transmitters, with increases in glutamate and glutamine levels accompanied by a decrease in gamma-aminobutyric acid levels [20]. This is again focused on the hippocampus, with spongiform changes to the neurons seen, which appear to be an important mechanism in Al neurotoxicity [20]. As further proposed mechanisms of Al neurotoxicity in the rat, inhibition of acetylcholinesterase activity (enzyme rate; decreased V_{max}) has been reported in the brain [21], and disturbance of cellular communication through gap junctions in cultured foetal brain astrocytes [22]. Similarly, in isolated cerebellar granule cell neurons, Al neurotoxicity resulted in the formation of reactive oxygen species and elevated intracellular Ca^{2+} concentrations [23]. The resulting cell death here was also not related to apoptosis, as there was no activation of caspase-3 or increase in annexin-V binding.

Of particular relevance for the developmental aspects investigated in the present study, the use of the [^{26}Al] radioisotope have provided a tracer to demonstrate that following its subcutaneous injection in pregnant and lactating rats, considerable amounts were then found in the brain (and particularly the cell nucleus fraction) of both the mother and foetus [24]. Indeed, an earlier study investigating the effects of Al in the ontogenetic development of cholinergic and serotonin neurotransmitter receptors in the brain demonstrated that not only is the time of exposure to Al important for the effects seen, but also the timing of the exposure [25]. Thus, this study saw reduced muscarinic and serotonin receptor sensitivities with post-natal exposure, but also a paradoxical increase in 5-HT_{2c} receptor sensitivity in rats with prenatal exposure to high Al concentrations (3000 ppm Al sulphate in drinking water during pregnancy).

In the present study, we therefore investigated the oxidative and histopathological damage induced by Al during the development of the chick brain, as applied from the beginning of the incubation period of eggs of Ross broiler chicks.

Material and Methods

Animals

Thirty-five fertilized Ross broiler eggs (Abalıoğlu Holding, Izmir, Turkey) were divided into 3 study groups: the control group (with no treatment; n=10), the sham treatment group (n=12), and the active treatment group (n=13). All of the eggs were placed in an egg incubator (VGS, Veyisoğulları, Istanbul, Turkey), and on the first day of the incubation (day 1), the sham group had 0.1 ml saline and the Al-treated (active-treatment) group had 500 µg Al sulphate in 0.1 ml saline slowly injected into the air chambers; nothing was applied to the control group. At term (day 21), the eggs were opened and the live chicks (8/10, 8/12 and 8/13, respectively) were sacrificed under anaesthesia with 50 mg/kg ketamine and 5 mg/kg xylene (Merck, Germany). Their brains were then removed and divided into 2 parts, as the right and left hemispheres. The right hemispheres were used for the biochemical analyses, and the left hemispheres were used for stereological Cavalieri brain volume estimations.

During the study, all of the procedures were carried out in full accordance with the principles of "The Guide for the Care and Use of Laboratory Animals", on the protection of animals, and the study was approved by the Pamukkale University Experimental Animals Ethics Committee.

Biochemical analyses

Malondialdehyde measurements

The malondialdehyde (MDA) levels in the right hemisphere brain samples were determined using the method of Okhawa et al. (1979) [26]. Each half-brain sample was homogenized in 150 mM potassium chloride solution using 10 up-and-down strokes. The assays for MDA levels included: 0.4 ml brain homogenate, 1.5 ml 0.8% thiobarbituric acid, 1.5 ml 20% acetic acid (pH 3.5) and 0.2 ml 8.1% sodium dodecyl sulphate. These samples were mixed and incubated at 100°C for 1 h. The absorbance was then measured at 532 nm [26].

Glutathione measurements

Glutathione (GSH) estimations were carried out as described by Moron et al. (1979) [27], with some modifications. Briefly, after the homogenization of the samples as for the MDA measurements, 0.5 ml homogenate was mixed with 3.0 ml deproteinization solution (5.13 M NaCl, 0.2 M metaphosphoric acid, 6.8 mM EDTA in distilled water). Each sample was then centrifuged at 1000×*g* for 5 min, and 0.5 ml of the supernatants were added to 2.0 ml 300 mM Na_2HPO_4 and 0.5 ml 5,5'-dithiobis-(2-nitrobenzoic acid) reagent (DTNB; Ellman's reagent). The absorbance of the supernatants was then measured at 412 nm [27].

Stereological processes

After dissection and separation, the left chick-brain hemispheres were kept for 3 days in 10% formaldehyde. For brain-volume measurements, these brain hemispheres were rinsed in water and then dehydrated through a graduated ethanol series (70% to 100% ethanol). After being rinsed in xylene, the brain hemispheres were buried in paraffin. Systematically randomized coronal sections were taken from each brain block using a Leica RM-2125 microtome (Weltzlar, Germany), on the basis of 15 equally spaced 5 µm-thick sections. These were placed onto microscope slides and cresyl violet histological stain was applied.

The brain volume estimations were carried out according to the Cavalieri volume estimation formula: volume = (total point number) × (point invasion area) × (average section thickness). Points were counted on a monitor (SONY Trinitron KV-14LT1E TV, Japan) according to uniform random placing, using a stereomicroscope (Zeiss Stemi 200-C, Germany) and a camera (Canon Power Shot G-2, Tokyo, Japan) attachment over the point catheter sections with a systematic uniform random quality. To have an acceptable coefficient of error in the volume estimations, 15 sections were used for each sample and approximately 250 points were counted for each [28].

Statistical analysis

The data are given as means ± standard deviation, with box plot analyses given in the Figures. The data were evaluated for significant differences among the groups using the Mann-Whitney *U*-test and Kruskal-Wallis tests. P values were evaluated to a significance level of $P<0.05$. All of the statistics were analyzed using SPSS 11.0 software.

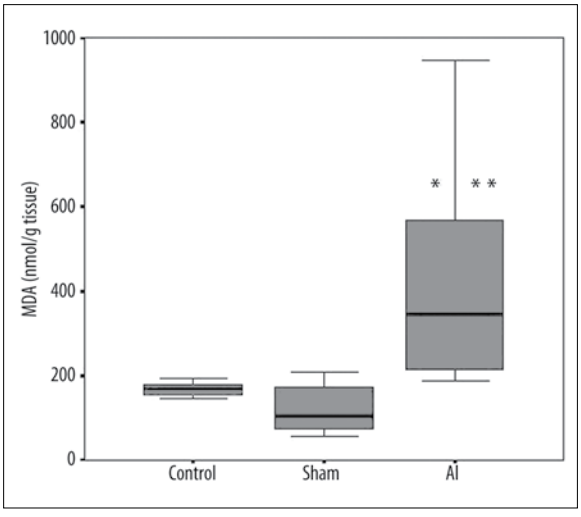

Figure 1. Box plot of chick-brain MDA levels as a measure of lipid peroxidation. The control and sham-treated chick-brain homogenate samples show similar MDA levels, while those for the Al-treated group are significantly increased compared to both control (* P=0.001) and sham (** P=0.015) treatments.

Results

The Al sulphate treatment of Ross broiler chicks from the first day of incubation resulted in increased MDA levels and decreased GSH levels, as indicators of increased biochemical oxidative damage.

For MDA, the mean (±SD) levels for the control, sham and Al treatments were 158.6 (±41.2), 142.8 (±107.2) and 423.7 (±276.5) nmol/g tissue, respectively. As illustrated in the box plot in Figure 1, this Al-induced 2.7-fold increase in MDA levels over the control treatment was significant when compared to both the control (P=0.001) and the sham (P=0.015) groups.

The mean (±SD) GSH levels seen for the control, sham and Al treatments were 293.6 (±51.4), 268.8 (±41.1) and 184.6 (±79.9) nmol/g tissue, respectively. Again, this 37% decrease in GSH levels over the control treatment was significant when compared to both the control (P=0.015) and the sham (P=0.038) groups (Figure 2).

In parallel, this Al sulphate treatment resulted in decreased brain volume as a measure of the brain development in these Ross broiler chicks. The mean (±SD) total brain volume estimations according to the Cavalieri volume formula (see Methods) for the control, sham and Al treatments were 10 354 (±1158), 10 068 (±900) and 8621 (±1407) μm^3. These thus demonstrated a significant 17% decrease in brain volume for the active-treatment group when compared to the control group (P=0.021), with this significance also maintained over the sham treatment (P=0.021) (Figure 3).

Of note, the comparisons between the control and sham treatment groups showed no significant differences for the MDA and GSH levels, and for the brain volumes (Figures 1–3). Similarly, there was no significant trend seen for the mortalities during the treatment period, indicating

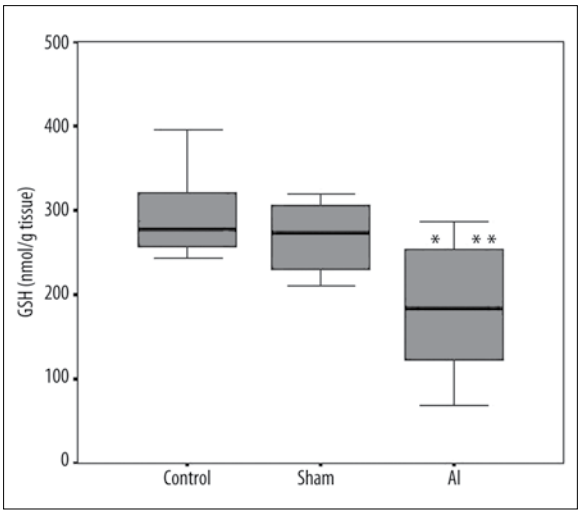

Figure 2. Box plot of chick-brain GSH levels as a measure of oxidative damage. The control and sham-treated chick-brain homogenate samples show similar GSH levels, while those for the Al-treated group are significantly decreased compared to both control (* P=0.015) and sham (** P=0.038) treatments.

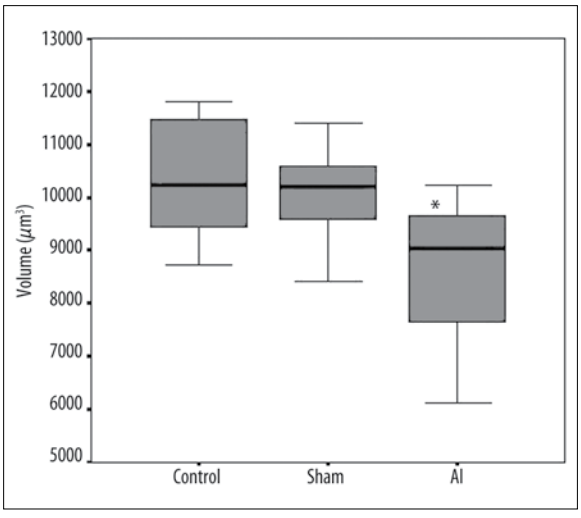

Figure 3. Box plot of chick-brain volume estimations according to the Cavalieri principle. The control and sham-treated chick brains show similar volumes, while those from the Al-treated group are significantly decreased compared to both control (* P=0.021) and sham (** P=0.021) treatments.

that the treatment with Al followed in the present study did not result in significant embryo death over the non-treated and sham-treated samples.

Discussion

In the present study we focused in particular on the neurotoxic effects of Al in terms of the growth and development of the brain, with the exposure of developing chicks to Al. Indeed, in an *in vitro* study of the toxic effects of Al on human embryonic cerebral neurocytes, increases in lipid peroxides were seen, which were indicative of the neurotoxic effects of Al being caused by lipid peroxidation and the resultant damage to the membranes [29]. Similarly, as indicated above, with the developing mouse brain, Al exposure of the foetus via Al treatment of the mother leads to inhibitory effects on post-partum development in general (seen as decreased weight and body length in the pups) and delayed neurobehavioral development [2]. From a biochemical viewpoint, in the developing rat exposed to Al from the treated mother through lactation, increased lipid peroxidation was seen in both the cerebrum and cerebellum of pup brains, which was accompanied by decreases in superoxide dismutase and catalase activities [3].

In the present study, for our biochemical measures of MDA and GSH, these have proven to be relevant biological measures for determination of lipid peroxidation and biochemical oxidative damage that can be caused by Al not only in mouse [16,30] and rabbit [31] models, but also mainly in rat models [3,14,24,25,32–41]. Furthermore, we have provided a more direct Al treatment to the developing brain by use of this chick embryonic development model. Indeed, this model has been shown previously to be useful for measured dosing of various teratogenic effects of some heavy metals [42], or specifically in some more recent studies comparing herbicide and heavy-metal effects [43,44]. Thus, although this model might not directly parallel the influence of these agents as environmental contaminants, it does provide for direct measured dosing of their absorption and monitoring of their effects on embryo development. At the same time, we also monitored neuronal degeneration as estimated stereologically using the Cavalieri brain volume estimation tool [28].

The increased lipid peroxidation and oxidative damage caused by Al treatment in the chick embryonic development model in the present study support conclusions from other various animal models. In particular, along with the present study, a number of studies have investigated the whole brain [3,4,16,30,36,39], while others have investigated more specific areas of the brain. Thus these effects of Al have been seen at the level of the cerebral cortex [31–35,37,38,41], hippocampus [14,31,33,35], cerebellum [32,34,38], medulla oblongata [32], hypothalamus [32] and brain stem [34].

Thus the biochemical data for Al treatment in this chick embryonic development model are in parallel with these indicators of increased lipid peroxidation and oxidative damage, although at this level it remains to be seen within which regions of the developing chick brain these neurotoxic effects might be focused.

Histopathologically, Al neurotoxicity appears to have been followed in fewer animal models. In the same rabbit study indicated above [31], the biochemical measures of MDA and GSH were accompanied by morpho-pathological examination of the cerebral cortex and hippocampus by light and electron microscopy. In both brain areas, atrophy and neuron apoptosis were seen to be accompanied by neurofibrillary degeneration, argyrophilic inclusion, Schwann cell degeneration, and nerve fibre demyelination. Similarly, histopathological examinations of the hippocampus in 2 rat models following Al treatment showed marked changes in general brain histology, as indicated by an increased number of vacuolated spaces [45], and more specifically, effects on the neuronal connectivity in the hippocampus [46].

With these Al effects seen on the hippocampus, a brain-damage model for the investigation of learning and memory functions was recently reported that was established via intragastric administration of elemental Al in adult rats [47]. Similarly, adult mice and rats treated with $AlCl_3$ plus D-galactose now represent a model of Alzheimer's disease due to the memory impairment and high amyloid beta-peptide levels found in the cerebral cortex and hippocampus [48].

Conclusions

Thus, various studies in the literature using some specific adult animal models support these neurotoxic effects of Al seen in the present study for this model of the developing chick brain.

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