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Research Article

PROLONGED AND UPGRADED ORAL $AlCl_3$ INDUCED TOXICITY ON THE FEMORAL DIAPHYSIS CELL COMPOSITION IN MALE RODENTS

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ABSTRACT: Aluminium contamination is often found in daily life unintentionally, including in drinking water and food appliances. Aluminium intoxication impairs the balance between reactive oxygen species (ROS) and antioxidant levels, which produces oxidative stress and apoptosis. In bone tissue, it has been reported in previous studies to alter mineral deposition and cellular composition. However, the effects of gradually increased dose and exposure periods have not been explored extensively. We examined later, using induced oral $AlCl_3$ on 27 male Wistar rodents aged 2-3 months. These animals were randomized equally into 3 groups, control (placebo), Al-1 (oral $AlCl_3$, 64 mg/kg of body weight/day for 67 days, then the dose was doubled to 128 mg/kg of body weight/day for 20 days) and Al-2 (oral $AlCl_3$, 128mg/kg of body weight/day for 67 days then the dose was doubled to 256mg/kg of body weight/day for 20 days). The means of osteocyte, osteoblast, osteoclast cell nuclei, and diaphysis trabecular density of the right femoral bone tissue were calculated from the longitudinal slices using Cell Sense, Adobe Photoshop, and ImageJ software after being stained with HE. Data were analyzed using Kruskal-Wallis or one-way ANOVA to seek significant differences with a significance level of $p<0.05$. In Al-2, osteocytes were significantly lower than other groups ($p=0.024$), whereas osteoclasts were significantly higher than Al-1 ($p=0.001$). There were no significant differences in the osteoblasts ($p=0.102$) and the trabecular bone density ($p=0.094$). In this study, upgraded and prolonged oral $AlCl_3$ induction caused impaired cellular components in the long bone diaphysis of male rodents' femur.

Key words: Aluminium toxicity, Health risk, Environmental pollution, Bone tissue, Male rats.

INTRODUCTION

Aluminum, the third most abundant element in nature, is a toxic element frequently identified in the environment (Rodriguez and Mandalunis 2018). Most cases of aluminum toxicity are acquired through the oral route, including from daily diets, drinking water, and various chemical drugs (Tietz *et al.* 2019, Willhite 2021). In recent years, the use of aluminum in food has also been found in many food additives (Tietz *et al.* 2019). Poisoning from inhalation of aluminium-containing gasses is rare but occasionally occurs in the aluminium processing industry (Hellstörm *et al.* 2006). Aluminium exposure can also enter the skin from applying cosmetics and body care products such as antiperspirants, toothpaste, and sunscreen (Tietz *et al.* 2019).

One of the highest accumulations of aluminium is in bone tissue. Aluminium will accumulate on the surface of the bone trabeculae and vascular channels before infiltrating the bone matrix, while it can also be deposited on the periosteal and endosteal surfaces (Rodriguez and Mandalunis 2018).

The levels of pro-oxidants and antioxidants in healthy people are balanced, preventing oxidative stress (Poprac *et al.* 2017). The accumulation of aluminium would disrupt this balance and increase the levels of reactive oxygen species (ROS) while altering the antioxidant levels resulting in oxidative stress and cell death (Yang *et al.* 2018). Oxidative stress can cause an imbalance between bone resorption and bone formation; it alters the bone remodeling process and has been reported to modify the osteoclasts and osteoblast activity (Bonaccorsi *et al.* 2018). Long-term administration of aluminium trichloride ($AlCl_3$, 0.126 mg/ml) in 3 days-old Wistar rat was reported to reduce the viability of the osteoblasts due to

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downregulation of the Wnt/â-catenin signaling pathway (Zhu *et al.* 2016). In another study by Song *et al.* (2020), rats were given $AlCl_3$ orally with a dose of 64/mg/day for 120 days. At this dosage level, $AlCl_3$ reduced bone mineral density in the lumbar spines, femoral metaphysis, and tibia (Song *et al.* 2020).

Furthermore, a study by Souza-Monteiro *et al.* (2021) reported that oral $AlCl_3$ intoxication, a dose of 8.3 mg/kg/day for 60 days, resulted in alveolar bone loss. Different dosages of $AlCl_3$ exposed to these animal models in these previous studies combined with different routes and duration might produce different alterations in bone metabolism, disrupting bone tissue morphology. According to the World Health Organization (WHO), the toxic dose for humans is approximately 1 mg/kg of body weight (Willhite 2021). In the current study, we would elucidate the outcome of different dosages and duration of oral-$AlCl_3$ exposures to the cellular composition in the femurs of male *Rattus norvegicus* rodents; to better understand the effect of aluminium intoxication on the diaphysis of the long bone.

MATERIALS AND METHODS

We applied the posttest-only controlled group design in this experimental study that has been approved by the committee of health research, Faculty of Medicine, Universitas Airlangga, Indonesia (No. 179/EC/KEPK/FKUA/2021).

Twenty-seven Wistar strains of male adult *Rattus norvegicus* rodents were randomized into 3 groups with equal numbers.

Control (placebo), Al-1 (oral $AlCl_3$, 64 mg/kg of body weight/day for 67 days, then the dose was doubled to 128 mg/kg of body weight/day for 20 days), Al-2 (oral $AlCl_3$, 128 mg/kg of body weight/day for 67 days then the dose was doubled to 256 mg/kg of body weight/day for 20 days). After these exposures, animals were sacrificed using an overdose of ketamine xylazine followed by decapitation; then, the harvest of the right femoral bone of each animal was done.

Tissue preparations

After being sacrificed, the rodent femur was taken. It was fixed in a 10% PBS formalin solution followed by a decalcification process using EDTA (ethylene diamine tetra acetic acid) solution for 2 weeks (Savista *et al.* 2019). The tissue was then initialized and longitudinally sliced with a thickness of 5μ. The bone tissue slices were then processed into histological preparations by HE routine staining (Kalanjati 2014). HE staining was chosen because it can show the cell nucleus well (Rimbun and Kalanjati 2019). Tissue slides were observed under a light microscope (Olympus, Japan).

Histopathology observation

All bone tissue slides were imaged from 10 fields of view using Cell Sense software (Olympus, Japan) with 200x and 400x magnification (Olympus Corporation 2009). The light setting on the microscope was made maximally to produce a white background. The diaphragm at 200x magnification was set to 2.65, while at 400x magnification, it was consistently set to 3.2. To calculate the density, trabecular bone was imaged using 200x magnification, then edited using Adobe Photoshop 2018 (USA) (Adobe Inc 2018). Bone trabecular area fraction (%) was measured using ImageJ 1.8.0. (NIH, USA) (Schneider *et al.* 2012). The average number of osteocytes, osteoblasts, and osteoclasts from all of the tissue slides were imaged using 400x magnification and then calculated using ImageJ 1.8.0. (NIH, USA). Further details are explained elsewhere by Wixey *et al.* (2019).

Statistical analysis

Data from 3 groups were tested using the Shapiro-Wilk normality test (Hanusz *et al.* 2016) and the Levene homogeneity test (Parra-Frutos 2013) to determine the distribution and homogeneity of the data set before comparison analysis using either one-way ANOVA followed by LSD posthoc test (parametric data set) or Kruskal-Wallis test followed by Mann-Whitney posthoc test (non-parametric data set); level of significance if $p<0.05$ (Mishra *et al.* 2019). A simple linear regression test was applied to find out any associations between the dependent and independent variables (IBM SPSS Statistics 17.0 (USA) (SPSS Inc 2008).

RESULTS AND DISCUSSION

In this study, osteoblasts in the control group had the highest average numbers (7.44±0.63), followed by Al-2 (6.56±0.65), and the least was in the Al-1 group (5.56±0.47). However, a significant difference was only shown between the control and Al-2 groups ($p=0.026$). The osteocyte average numbers, on the other hand, were the least in the Al-2 group (19.67±2.33), while in the Al-1 and control groups, were 24.00±1.40; 27.56±1.83, respectively ($p=0.024$) (Table 1 and Table 2).

Osteoblasts have an important role in bone formation. The presence of oxidative stress induced by $AlCl_3$ can reduce osteoblast proliferation through the Wnt/β-catenin signaling pathway. The targets of the Wnt/β-catenin signaling pathway are cyclin D1 and c-Myc, regulators of osteoblast proliferation (Huang *et al.* 2017). Aluminum

Table 1. Mean nucleus count and bone trabecular density of all subjects.

Dependent variable	Dose	Mean ± SE	SD	p Shapiro-Wilk	p Levene	p
Trabecular Density	Control	0.26 ± 0.01	0.03	0.0038*	0.009*	0.094 (Kruskal-Wallis)
	Al-1	0.25 ± 0.01	0.03	0.005*		
	Al-2	0.20 ± 0.03	0.08	0.570		
Osteocyte Cell Nuclei	Control	27.56 ± 1.83[a]	5.48	0.446	0.445	0.024* (one-way ANOVA)
	Al-1	24.00 ± 1.40	4.21	0.579		
	Al-2	19.67 ± 2.33	6.98	0.810		
Osteoblast Cell Nuclei	Control	7.44 ± 0.63	1.88	0.004*	0.816	0.102 (Kruskal-Wallis)
	Al-1	5.56 ± 0.47	1.42	0.246		
	Al-2	6.56 ± 0.65	1.94	0.273		
Osteoclast Cell Nuclei	Control	3.22 ± 0.43	1.30	0.080	0.996	0.001* (one-way ANOVA)
	Al-1	3.67 ± 0.41	1.22	0.286		
	Al-2	5.56 ±0.38[b]	1.13	0.248		

[a]Osteocyte cell nuclei in the control group were significantly higher than in Al-1 and Al-2 Groups (one-way ANOVA test). [b]Osteoclast cell nuclei in the Al-2 group were significantly higher than in Al-1 and control groups (one-way ANOVA test).

also can trigger apoptosis in osteoblast cells by increasing the concentration of intracellular Ca^{2+}, causing normal cell activity to be interrupted (Cao *et al.* 2016). *In vitro* studies indicated that $AlCl_3$ decreased blood levels of B-ALP (bone-specific alkaline phosphatase), PICP (procollagen I carboxyterminal propeptide), and BGP (bone γ carboxyglutamic acid protein) secreted by osteoblasts (Huang *et al.* 2017). Bone mineralization can be impeded by reducing B-ALP, PICP, and BGP (Yang *et al.* 2018). The differentiation of osteoblasts is closely linked to the production of osteocytes. If osteoblasts stop working, osteocyte proliferation is also inhibited (Crous and Abrahamse 2021). Apoptosis via the JNK (c-Jun N-terminal kinase) apoptotic pathway also decreased the number of osteoblasts (Yang *et al.* 2018). The average of osteoclasts was highest in the Al-2 group (5.56±0.38) when compared to the Al-1 (3.67±0.41) and control (3.22±0.43) groups. There were significant differences in osteoclast means between the Al-1 and Al-2 groups (p / 0.003) and between the Al-2 and control groups (p / 0.000) (Tables 1 and 2). In the process of bone resorption, osteoclasts play a critical role. The

Table 2. Analysis of differences on the trabecular density using Mann-Whitney and LSD post-*hoc* tests.

Testing	Trabecular Density (p Mann WhitneyTest	Nucleus Count (mean ± SE)		
		Osteocytes (p LSD Test)	Osteoblasts (p Mann-Whitney Test)	Osteoclasts (p LSD Test)
Control with Al-1 Gr	0.373	0.196	0.178	0.448
Control with Al-2 Gr	0.101	0.007[a]	0.026[b]	0.000[c]
Al-2 with Al-1 Gr	0.056	0.118	0.750	0.003[d]

[a]Osteocyte nucleus count between the control and Al-2 groups was significantly different. [b]Osteoblast nucleus count between the control and Al-2 groups was significantly different. [c]Osteoclast nucleus count between the control and Al-2 groups was significantly different. [d]Osteoclast nucleus count between Al-2 and Al-1 groups was significantly different.

hydrolytic enzymes and degradation products produced by osteoclasts, including TRACP-5B (tartrate-resistant acid phosphatase 5B) and CTX-1 (carboxy-terminal collagen crosslinks), can be used to measure the extent of bone resorption. TRACP-5b is secreted by osteoclasts and parallel with the levels of numbers and activity of these cells; while the CTX-1 in the serum reflects the levels of type-1 collagen hydrolysis from the bone damage (Chen *et al.* 2017, Dénarié *et al.* 2014, Xia *et al.* 2015); although here we did not pursue these indicators due to limitation of this study. $AlCl_3$ administration showed at least two different effects on the number of osteoclast cells. A low dose of $AlCl_3$ caused mild oxidative stress increasing the number of osteoclasts, which produced the resorption of damaged bone structures. When given in excessive doses, the effect is the polar opposite. As a result of high-oxidative stress, the number of osteoclasts will decrease due to the apoptosis of the osteoclast (Yang *et al.* 2018). The level of oxidative stress can be measured by the levels of antioxidant (*i.e.*, antioxidant glutathione), reactive oxygen species (*i.e.*, superoxide radical anion), and its product, such as lipid peroxidation showed by MDA (malondialdehyde) level (Tsikas 2017). Oxidative stress also elevates the RANKL (Receptor Activator of Nuclear Factor-Kappa B Ligand)/OPG (osteoprotegerin) ratio, where RANKL promotes osteoclastogenesis and OPG is a receptor capable of blocking RANKL and thus suppressing osteoclast activity via the Wnt/catenin pathway. The activity and number of osteoclasts will increase if OPG is reduced and RANKL is increased (Domazetovic *et al.* 2017). Another study reported Wnt role in fracture repair by the upregulation of, *i.e.*, Wnt5A, β-catenin, FZD (frizzled receptor), and other related genes, while Wnt related markers, *e.g.*, Wnt5B (Wnt family member 5B), LRP5 (low-density lipoprotein receptor-related protein 5), Dvl (disheveled), TCF1 (T cell factor-1) and PPARD (peroxisome proliferator-activated receptor delta) had also shown an increase (Houschyar *et al.* 2019). A study reported that after 24 hours of $AlCl_3$ exposure, the activity of Wnt3a and Dkk-1(dickkopf Wnt signaling pathway inhibitor-1) also β-catenin protein levels were depleted. In contrast, the application of external Wnt3a reversed the inhibitory effect of $AlCl_3$ on the osteoblastic differentiation and Wnt/β-catenin pathway (Cao *et al.* 2016).

The control group had the highest trabecular Density (0.26±0.01) when compared to the Al-1 (0.25±0.01) and Al-2 (0.20±0.03) groups; it showed that $AlCl_3$ induction here tended to decrease bone mineral density. Trabecular Density was calculated by a lower % of fraction area on the histopathology analysis at the diaphysis of the femoral bone (Schneider *et al.* 2012). Although the effect was not significant when analyzed semi-quantitatively using ImageJ, where the mean ± SD of the % fraction area of the diaphysis trabeculae width, shown as the grey-level differences between pixels of the images from each slide, amongst the three groups were not statistically significant (p=0.094).

In the previous study, $AlCl_3$ administration for 90 days lowers bone mineral density (BMD) and degrades trabeculae in the tibia and femur bones of 4-week-old male Wistar rats (Yang *et al.* 2018) and this might be due to the trabeculae of the bones accumulate most of the aluminum (Kruger *et al.* 2014). Because of an imbalance in the activity of osteoblasts and osteoclasts, the bone remodeling process is interrupted, resulting in a decrease in BMD in the bone tissue (Domazetovic *et al.* 2017). In this study, however, using Cell Sense software (Olympus Corporation 2009) and ImageJ 1.8.0 software (Schneider *et al.* 2012) as a method of evaluating the trabecular area, $AlCl_3$ accumulation caused no significant effect on the trabecular bone density. Research done by Hellström *et al.* (2006) showed that

Table 3. Relation analysis between variables using simple linear regression test.

Group	Cell Nucleus count	Trabecular Density		Osteoblasts	
		p	R^2	p	R^2
Control	Osteocyte	0.261	0.176	0.657	0.030
	Osteoblast	0.606	0.040	-	-
	Osteoclast	0.925	0.001	0.805	0.09
Al-1	Osteocyte	0.902	0.020	0.05[a]	0.445
	Osteoblast	0.660	0.029	-	-
	Osteoclast	0.866	0.004	0.049[b]	0.447
Al-2	Osteocyte	0.038[d]	0.0481	0.360	0.120
	Osteoblast	0.295	0.155	-	-
	Osteoclast	0.134	0.290	0.038[c]	0.484

[a,b]Osteoblast nucleus count in the Al-1 group significantly correlated with osteocyte nucleus count and osteoclast nucleus count. [c]Osteoblast nucleus count in the Al-2 group is significantly associated with osteoclast nucleus count. [d]Trabecular Density in the Al-2 group had a significant association with osteocyte nucleus count.

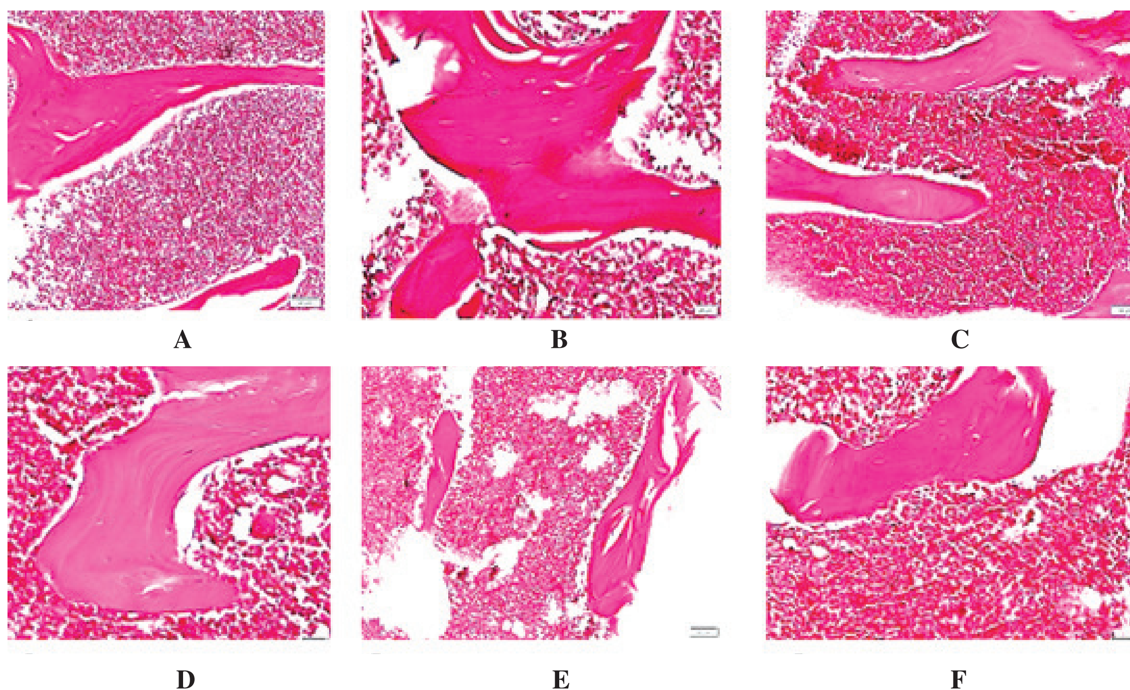

Fig. 1. Histology characterization (HE stain) of the rat femoral diaphysis.
(A-B) negative control (control group) (A : 200x, B : 400x). (C-D) lower-dose group (Al-1 group) (C: 200x, D : 400x). (E-F) higher-dose group (Al-2 group) (E : 200x, F : 400x).

there was no significant relationship between the amount of aluminium and the degree of BMD, BMC, and the size of the long bone of the middle-aged and older men and women of the proximal femur determined using DXA (dual-energy X-Ray absorptiometry) (Hellström *et al.* 2006). Another study done by Van Alstyne *et al.* (2006) reported that a 10% contained-$AlCl_3$ oral diet for 111 days showed no significant effect on the bone mineral density of the developing sheep (left the third metacarpal) determined by specific gravity procedures (Van Alstyne *et al.* 2006).

A simple linear regression was used to see a linear association between two variables where one variable was considered to affect other variables. In our study, the osteoblasts population was significantly related to osteoclasts numbers in the Al-1 and Al-2 groups (p=0.049; p=0.038, respectively). The osteoblasts either mature into osteocytes or experience apoptosis (Ru and Wang 2020). By converting mechanical strain into chemical signals, osteocytes help to regulate the activity of osteoblasts and osteoclasts (Tressguerres *et al.* 2020). Intercellular communication between osteoblasts and osteoclasts affects bone homeostasis. Eph receptors and ligands in osteoblasts play anti-osteoclastogenic and pro-osteoclastogenic roles in osteoclasts (Yuan *et al.* 2018). The RANKL-RANK (Receptor Activator of Nuclear Factor-Kappa B Ligand-Receptor Activator of nuclear Factor-KappaB) complex regulates osteoclast differentiation in osteoblasts. RANKL-RANK signal activity stimulates osteoclast differentiation, activates osteoclast maturity, promotes osteoclastogenesis, and prevents osteoclast apoptosis (Tressguerres *et al.* 2020). Osteoblasts secrete osteoprotegerin, which will inhibit the RANKL-RANK complex. RANKL-RANK complex will later inhibit osteoclastogenesis, disrupting bone resorption (Palumbo and Ferretti 2021). In a study by Yang *et al.* (2018), TRACP-positive osteoclasts increased in lower-dosage $AlCl_3$ exposure and decreased in the middle- and higher-dosage exposed groups. They also reported that the number of ALP-positive osteoblasts decreased (Yang *et al.* 2018).

We observed that the osteocyte means number significantly affected the bone density in the Al-2 group (p / 0.038) (Table 3). Osteocytes are vital for maintaining bone mass. Pathological diseases that produce osteocyte apoptosis will influence bone mineral density and bone resorption i.e.osteomalacia and osteoporosis cases (Yang *et al.* 2018). However, ones produced by prolonged and upgraded- Aluminum induced toxicity have yet largely been revealed, as shown in the current study. Osteocytes secrete growth differentiation factor-15 (GDF-15) in ischemia or other pathological situations, which might stimulate the process of osteoclastogenesis (Ying *et al.* 2020). Dead osteocytes release DAMPs (Damage-

associated molecular patterns) via a macrophage-inducible C-type lectin (Mincle). The activation of the Mincle causes osteoclastogenesis, which leads to an increase in bone loss (Andreev *et al.* 2020). Exposure to free radicals also causes osteoblasts' apoptosis as cells are later revealed to be osteocytes (Savista *et al.* 2019). As a result of this occurrence, bone remodeling occurs, resulting in a decrease in bone mineral density (Domazetovic *et al.* 2017). Due to limited resources, in this study, we only performed histopathology examination; further studies are necessitated to include biochemical parameters, *e.g.,* Wnt/ β-catenin or JNK- a pathway and bone mineral density measurement using dual-energy X-ray absorptiometry (DXA).

CONCLUSION

In our study, prolonged and upgraded $AlCl_3$ administration decreased the number of osteocytes while increasing the number of osteoclasts significantly, with the tendency to lower the trabecular diaphyseal density correlated to osteocyte alteration.

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