Effects of exposure to aluminum citrate in a model of induced alveolar bone loss in rats

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Aluminum (Al) is the most abundant metal in the earth's crust and is found naturally in the air, water, and soil. Al may pose a major threat to humans, causing diseases and bone disorders. Nevertheless, the effects of Al on alveolar bone are not reported in the literature. Here, we investigated the effects of sub-chronic intoxication with Al citrate in a model of alveolar bone loss, induced by ligature, in male *Wistar* rats. The animals were exposed to Al citrate (100 mg/kg/day), by gavage, for 30 days. We used 40 rats equally divided into four groups: Control; Experimental Periodontitis (EPeriodontitis); Al; Al+EPeriodontitis. After exposure period, we evaluated the concentration of Al in the blood and alveolar bone, as well as the Al measurement in alveolar bone. The results showed that the Al exposure promoted distribution of Al in blood serum and in the alveolar bone. The Al exposure *per si* results in alveolar bone loss as well as potentiates damages of periodontitis induced cases. These results suggest that levels aluminum in the blood and alveolar bone induce alveolar bone loss, besides present aggravating effect in the presence of induced periodontitis.

Keywords: Aluminum toxicity, Mining, Periodontitis

Aluminum (Al) is distributed over the earth crust and is widely used in natural processes and human activities[1,2]. Al is found in large concentrations in the soil and in the air around waste disposal sites, especially those of industries that use coal and Al mining companies[1-3]. The bioavailability of Al stems mainly from acid rain, which releases Al from the soil into fresh water, making it accessible to living organisms[4]. Such environmental availability in combination with its industrial use makes Al an important environmental pollutant, and its toxicity depends directly on its chemical form[1,3].

The impacts of Al intoxication on human health have been increasingly alarming in recent years[5,6]. Small amounts of Al are released into the environment from coal-fired power plants and incinerators, which can also contribute to the increased risk of exposure and intoxication[7,8]. Al can enter into the human body mainly in two ways: orally and through inhalation. Dermal absorption is negligible. The oral way is the main route of entry, accounting for 95% of Al entering into the human body, whereas the remaining

5% corresponds to inhalation[9,10]. However, with continued exposure through inhalation, Al gets deposited in the lungs, especially in individuals exposed to Al dust in the workplace.

Many studies have been conducted over the years on the effects of Al in the body[11-15]. Our previous studies showed that Al exposure induces damages in central nervous system (e.g. hippocampus) and salivary glands[16,17]. In fact, once in bloodstream it is known that this metal is absorbed and is excreted by the kidneys[9]. However, it can be accumulated, suggesting that after absorption, Al is distributed into the brain, liver, kidney cells and the bone, and can cause a decline in liver and kidney functions, a lack of motor coordination, abdominal cramps, headaches and seizures[15]. Similarly, it can also accumulate in the, muscles, kidneys and bone tissues[11,13,15].

Previous studies have claimed that Al can cause substantial bone loss[11-14] but no research has considered the effects of this metal on alveolar bone. Thus in the current study, we tried to analyze the effects of sub-chronic intoxication with Al citrate in rat alveolar bone, with or without a model of induced alveolar bone loss.

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Materials and Methods

Experimental animals

The sample of this study was composed of 40 male *Wistar* rats, weighing 250-300 g with 90 days old. The rats were maintained in collective plastic cages (5 animals per cage) with water and food *ad libitum*. The animals were maintained in a climate-controlled room (25ºC) with 12:12 h light/dark cycle (lights on 7:00 AM). The study was approved by the ethics committee of the Federal University of Pará and followed the guidelines suggested by the NIH Guide for the Care and Use of Laboratory Animals[18].

Experimental groups

The animals were divided into four groups with ten animals each: control (without ligature; gavage of distilled water [H_2O_{dist}]); Experimental Periodontitis - EPeriodontitis (with ligature; gavage of H_2O_{dist}); Al (without ligature; gavage of aluminum citrate); Al+EPeriodontitis (with ligature; gavage of aluminum citrate). The exposed groups received Al citrate ($C_6H_5AlO_7$) at a dose of 100 mg/kg by intragastric gavage for 30 days.

Induction of experimental periodontitis

On the 15th day of Al exposure, the animals underwent surgery for induction of periodontitis by ligature. The rats were anaesthetized with xylazine (90 mg/kg; i.p.) and ketamine (10 mg/kg; i.p.). The ligature (4.0 silk ligature) was subgingivally inserted on the second molar[19-21].

Blood sampling and Al measurement in plasma

At the end of the experimental period, the animals were anaesthetized with xylazine (90 mg/kg; i.p.) and ketamine (10 mg/kg; i.p.). Then, the blood was collected via orbital plexus puncture in the capillary hematocrit and stored in heparinized Eppendorf® tubes. To obtain the plasma, centrifugation was performed at 2500 rpm for 15 min. Determinations of the total concentration of Al in the digested samples were carried out using a Varian Spectra AA 240Z atomic absorption spectrometer (Mulgrave, Australia) equipped with a transverse Zeeman Effect background corrector and a longitudinal-heated graphite furnace atomizer.

Only control and Al-without Periodontitis were analyzed for Al deposition in bone and blood; it was not the objective of this research to analyze the effects of ligature-induced periodontitis on the Al deposition in these tissues.

Sample preparing and measurement of alveolar bone loss

After 15 days of insertion of ligature and 30 days from the beginning of the intoxication, the animals were anaesthetized and transcardially perfused with heparinized 0.9% saline solution followed by 4% paraformaldehyde in 0.2 M phosphate buffer. Then, disarticulation of the jaws was carried out to separate hemimandibles. The side with ligature and the left side with a stereomicroscope (Zeiss Stereomicroscope Discovery.V8), and the contralateral side was used to analyze the content of Al levels in the alveolar bone[22].

After dissection of the jaws, the hemimandibles were immersed in 8% sodium hypochlorite solution for 4 h. The samples were washed in running tap water and subsequently placed in an ultrasonic bath filled with distilled water for 10 min. To distinguish the cementoenamel junction, the hemimandibles were immersed in solution of 1% methylene blue for one minute and washed in running water to remove excess solution.

Alveolar bone loss was determined on the lingual surface of the second molars between the cementoenamel junction distance (CEJ) and the alveolar bone crest (ABC), measured at three equidistant locations: mesial (M), middle third (MT), and distal (D). To validate the measurements, one millimeter ruler was used to photograph the CEJ in the samples. The photographs were obtained on a 6.1. megapixel digital camera (CANON, Power shot A640, USA) coupled to a stereomicroscope (Stereo Discovery v8, Carl Zeiss, Germany) at 3.2X amplification for all experimental groups.

Investigation of the amount of Al in bone

For mapping the Al content in alveolar bone, samples were immersed and washed with 1% sodium hypochlorite solution for 10 min. After this step, the samples were placed in an ultrasonic bath for 3 min and subsequently analyzed by EDS (Energy Dispersive X-ray Spectrometry, EDX or EDS) with a scanning electron microscope (LEO-1430; Carl Zeiss, Germany). The results were expressed in weight% (wt.%) in terms of the mass fraction of Al in the sample.

Statistical analysis

After measuring the concentration of Al in serum, bone and the distance between the cemento-enamel junction and the alveolar bone crest, the values were entered into the software GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA). The Shapiro–Wilk test was used to test the normality of the data distribution.
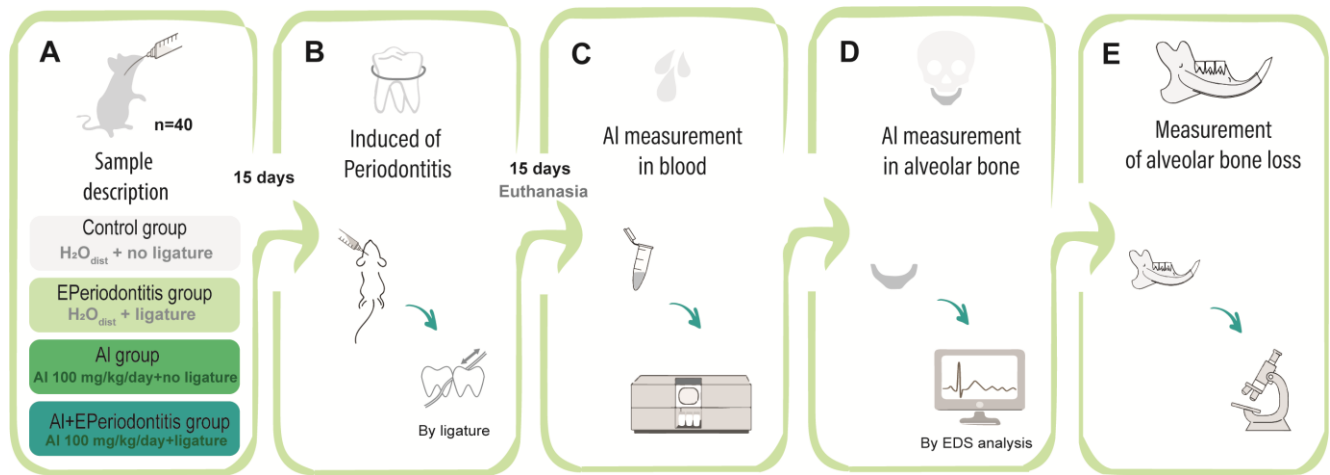
Fig. 1 — Study design. (A) The sample and model of exposure to aluminum (Al) citrate for 30 days by gavage. (B) On the 15th experimental day, was induced alveolar bone loss by ligature. After the exposure period to Al and experimental periodontitis, the animals were euthanized and samples were collected for measurement of Al levels in (C) blood and (D) alveolar bone by EDS analysis. (E) The alveolar bone loss was evaluation through the measurement between the cemento-enamel junction distance (CEJ) and the alveolar bone crest (ABC), measured at three equi-distant locations: mesial (M), middle third (MT), and distal (D).

For the measurement of Al in serum and bone, Student's t test for unpaired data was used. For measurements between the cemento-enamel junction and the alveolar bone crest, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for pairwise comparisons. A value of $P < 0.05$ was considered statistically significant for all analyses. The results were presented as mean ± S.E.M. All methodological procedures are summarized in Fig. 1.

Results

Sub-chronic exposure to aluminum citrate leads to Al levels increase in blood and alveolar bone of rats

The concentration Al in serum of the exposed animals (5.097 μg/L±652.6 μg/L) was significantly higher when compared to the control animals (3.171 μg/L±401.5 μg/L, p = 0.0457, Fig. 2A).

The analysis of the Al levels in alveolar bone was also confirmed by EDS, revealing a greater presence of Al in the exposed group (0.09±0.004%) compared to the control group (0.04±0.024%, p=0.001, Fig. 2B).

The Al exposure *per si* induces the alveolar bone loss in rats and in association with induced periodontitis, increases the alveolar bone loss in rats.

The measurements of alveolar bone loss in the in three areas, mesial (Control: 1.06±0.11; EPeriodontitis: 2.24±0.32; Al: 1.7±0.3; Al + EPeriodontites: 2.86±0.40), middle third (Control: 0.72±0.12; EPeriodontitis: 1.68±0.34; Al: 1.4±0.26; Al + EPeriodontites: 2.56±0.24) and distal (Control: 0.84±0.16; Periodontitis: 1.94±0.15;

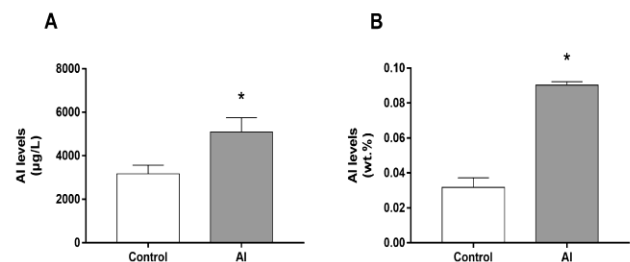
Fig. 2 — Effects of sub-chronic exposure to Al citrate (100 mg/kg/day) on the levels of in (A) serum (μg/L) and (B) alveolar bone (by EDS, weight% [wt.%]) of rats. [Results were expressed as mean ± standard deviation. * $P < 0.05$ Compared to control group (unpaired Student-t test)]

Al: 1.58±0.13; Al + EPeriodontites: 2.74±0.10), present statistical difference ($P < 0.05$) when compared control with experimental groups, as observed in Fig. 3.

The comparison between control and experimental periodontitis groups showed that the ligature was effective in inducing alveolar bone loss. Besides, the higher alveolar bone loss in the animals exposed to Al association to ligature, when compared to the experimental groups, indicate synergistic effect between both ($P < 0.001$).

Discussion

In this study, we demonstrated for the first time that Al citrate sub-chronical exposure citrate promotes levels Al in blood and also in the alveolar bone. This metal promotes alveolar bone loss in the absence or presence of periodontal disease induced by ligature.
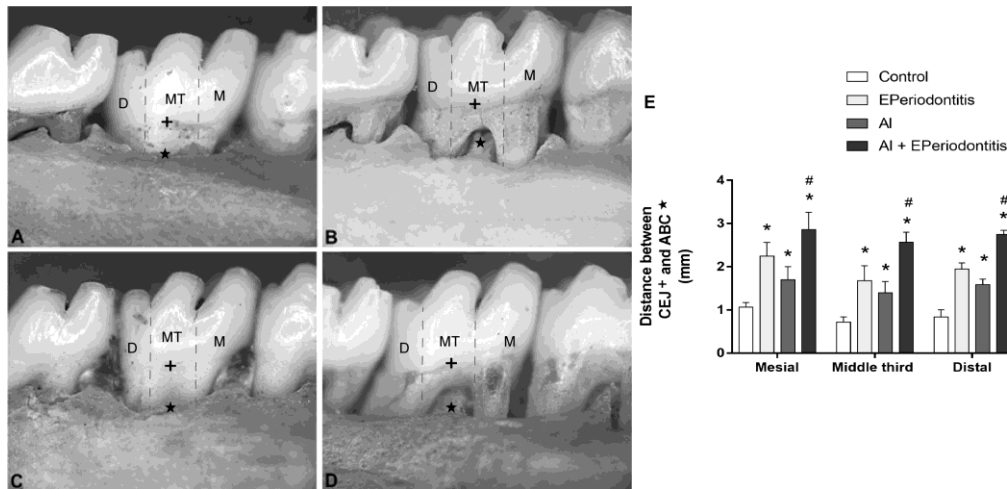
Fig 3 — Effects of sub-chronic exposure to Al citrate (100 mg/kg/day) on alveolar bone loss in rats. Representative photomicrographs of (A) Control group; (B) Experimental Periodontitis (EPeriodontontitis); (C) Al; (D) Al + EPeriodontitis. Results were expressed as mean ± standard deviation of distance between the cemento-enamel junction distance (CEJ) and the alveolar bone crest (ABC), measured at three equi-distant locations: mesial (M), middle third (MT), and distal (D). [*P <0.05 compared to control group; # P <0.001 compared to experimental groups (ANOVA with Tukey's post-test]

It is now well recognized that Al enters in the body from different sources. It is present in foods and beverages but other routes of penetration in the body have been identified: trans or percutaneous absorption (from vaccines and anti-sweating or antiperspirant products, *e.g.* alum stone) and locally released from metallic biomaterials. In the body, 95% of ingested Al is eliminated in the feces and the 5% remaining circulate in the blood mainly bound to transferrin and albumin[6].

The circulating Al can mainly be fixed in two preferential organs: brain and bone. In bone, the metal can bind to the phosphate groups of hydroxyapatite crystals (HA) of the calcified bone matrix, as it is also reported that the accumulation of Al in bone interferes with the metabolism of calcium (Ca) and phosphorus (P), the key elements needed for bone formation. From that, Al can cause of bone loss in laboratory animals and humans and high doses cause osteomalacia[6]. This could be the result of Al retention occurring through direct inhibitory actions of Al citrate on osteoblasts. In addition to the sub-chronic exposure, this metal interferes with the mineralization process and impairs osteoclast differentiation[12].

Al is one of the factors classically involved in the pathogenesis of low turnover of renal osteodystrophy (ROD)[23,24]. The main abnormalities of ROD encompass changes in turnover, mineralization, and bone volume[25]. Orofacial manifestations such as tooth mobility, dental spacing, and widening of the periodontal ligament are observed in ROD[26].

In our study, it was confirmed that Al citrate is able to accumulate in the blood, even when the intoxication period lasts only 30 days. This type of Al accumulation has been reported in other studies. Miu et al.,[27] found significant deposits of Al in the blood serum of *Wistar* rats intoxicated for six months at a dose of 0.85 mg/1000 g, where the concentration of Al in the blood was measured every two months, with metal build-up evident from the first measurement.

Furthermore, Ozturk and Ozdemir[28] showed that exposed of Wistar rats for three and six weeks to 16 mg/Kg (low doses) are enough to induced deposit of al in the blood. These studies corroborate our findings, because with higher doses but similar time periods, we found Al accumulation in the blood.

Several studies have demonstrated the aluminum accumulation in bone, like Kruger *et al*.,[29] that showed Al accumulation in the bones of patients on long-term parenteral nutrition. Added to this, Li *et al*.[30] showed that Wistar rats exposure to 100 mg/L of Al during 30, 60 or 90 days presented accumulation of Al in the bone. However, our findings are the first to demonstrate the deposit of the metal in alveolar bone. The control group presented Al in its matrix, which was expected, because this metal composed it naturally. Nevertheless, the presence of Al in the treatment group was significantly greater, suggesting that the toxicity of time and dose used caused Al deposits in the alveolar bone. Thus, the presence of this metal in the fabric caused bone loss in the absence or presence of periodontitis.

To simulate the clinical picture of periodontal disease, we used the ligature-induced periodontitis in rats as an experimental model where ligation acts as a mechanical trauma on the gingival area, reducing tissue integrity, allowing host–plaque interaction and bacterial plaque-formation[31].

In this paper, at first time, we demonstrated that Al caused alveolar bone loss in Al-without Periodontitis and increased this loss in Al-Periodontitis. Other studies, showed that Aluminum-induced decreased osteoblast surface, increased osteoid accumulation, and produced a cessation of mineralization (bone formation rate)[11,14,32,33]. Further, patients with stainable bone Al also have a higher volume of lamellar osteoid, a lower volume of woven osteoid, and significantly lower numbers of both osteoclasts and osteoblasts[14,34,35].

At the tissue level, accumulation of Al disturbs the mineralization process, decreasing the mineral apposition rate, increasing the lamellar osteoid surface, and decreasing the surface and volume of woven osteoid[34,36]. Al accumulation is not only associated with impaired mineralization but also loss of mass of spongy bone. The mechanisms of this phenomenon are related to a disproportionately larger effect of Al on bone formation than bone resorption[36].

Conclusion

From these findings, we can infer that after aluminum sub-chronical exposure induces aluminum (Al) levels in blood and also in the alveolar bone, causing alveolar bone loss in the presence or absence of periodontitis. In this way, the dose and the proposed time of intoxication by Al citrate have harmful effects on alveolar bone.

Conflict of interest

The authors have stated that they have no conflict of interest.

Acknowledgement

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