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Dietary aluminium and renal failure in the Koala (*Phascolarctos cinereus*)

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Summary. The study investigated the link between the potentially nephrotoxic levels of aluminium ingested in the natural diet of eucalypt leaves by koalas in the Adelaide Hills, South Australia, and the high incidence of renal failure in koalas within this habitat. Routine histology of kidney specimens revealed no pathologies at the light microscopic level and contrasted sharply with the clinical signs of renal failure. However staining with solochrome azurine and Perl's Prussian blue showed aluminium was present in some proximal convoluted tubules in all specimens. Aluminium was also found in bone samples. The presence of aluminium in bone and kidney tissues was confirmed using electron dispersive x-ray analysis with transmission and scanning electron microscopy. Ultrastructural changes, including a decrease in lysosomal numbers, were seen in proximal convoluted tubules and these changes were shown to coincide with the presence of aluminium. No aluminium was found in koalas that died from causes other than renal failure. It was concluded that renal failure in the koalas of the Adelaide Hills is characterised by the presence of aluminium in the kidneys and bone and it is probably related to the high levels of aluminium in their restricted diet of eucalypt leaves. However, it is not known if the presence of aluminium is the cause or effect of the renal failure. The study is the first account where aluminium ingested as part of the natural diet of mammals has been shown to accumulate in the animal and be implicated with nephrotoxicity.

Keywords: Aluminium, Koala, Nephrotoxicity, Histology, Ultrastructure

Introduction

A major cause of death in captive and wild koalas (*Phascolarctos cinereus*) in the Adelaide Hills, South Australia, is from severe renal dysfunctions, unassociated with infections. In 2000, five of 45 koalas (11%) were diagnosed with severe renal dysfunctions. In contrast, a mortality survey of koalas in New South Wales, eastern Australia, indicated less than 1% of deaths were due to renal failure not associated with cystitis or Chlamydia infection (Canfield, 1990). The demise of koalas with renal problems in the Adelaide Hills is characterised by lethargy, weight loss, production of large quantities of very dilute urine and an insatiable thirst. Blood chemistry shows elevated urea, cholesterol, and creatinine levels. Urine samples have a specific gravity (SG) <1.03 that contrasts to the normally concentrated urine. However, contradictory to clinical and biochemical characteristics, generally little or no pathological changes can be detected in the kidneys with routine light microscopic techniques.

Although the factors contributing to renal dysfunction in koalas of the Adelaide Hills have not been identified, an unpublished study by one of the authors (CL) implicated high levels of dietary aluminium as a possible cause. Dietary intake of aluminium at levels ≥ 5 mg per kg body weight per day are known to be toxic to rats (Somova et al., 1997). Koalas normally eat only eucalyptus leaves and analysis of leaves from eucalyptus plantations that provide food for the captive koala population, showed aluminium to be present in potentially nephrotoxic quantities (levels ≥ 5 mg per kg body weight per day) (Askew, 2001). These initial observations prompted an investigation into the presence of aluminium in bones and kidneys of koalas and to correlate the presence of aluminium in the kidneys with ultrastructural damage.

Ingested aluminium is almost entirely excreted in the faeces with less than 1% being absorbed across the digestive tract where it is coupled to transferrin and eliminated rapidly from the body by the kidneys (Jefferey et al., 1996; Habs et al., 1997). Absorption and subsequent elimination of aluminium from the body depend on the speciation and amount of the ingested aluminium compound as well as healthy kidneys (Alfrey et al., 1985; Martin, 1992). Excessive accumulation of aluminium in the body causes dysfunction in kidneys, bones and brain (Martin, 1992). In uremic patients

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aluminium absorption from the gut is increased (Alfrey, 1983) and in experimental uremic animals, aluminium accumulation in the kidneys is associated with impaired tubular function (Cacini and Yokel, 1988).

Materials and methods

Animals

Seventeen koalas from the Adelaide Hills formed the focus of the study. Sixteen animals, seven captive from Cleland Wildlife Park, and nine wild, were all emaciated, lethargic, showed insatiable thirst, and were voiding large volumes of dilute urine. Two wild animals also had physical injuries. The other koala, #13, a victim of a motor vehicle accident, was a large, robust male with multiple mandible fractures but no obvious signs of renal problems. This koala was used as a control in the study. In addition to these 17 koalas, two pouch young kidneys from Adelaide Hills' koalas were sampled from reference material in the Department of Anatomical Sciences, University of Adelaide. Data on biochemical analyses of plasma and urine were archived from veterinary records, Cleland Wildlife Park. Specimens from animals were collected and handled in accordance with the rules and regulations of the University of Adelaide Animal Ethics Committee, permit # S/3/96B. Animals were euthanased with lethal doses of pentobarbitone sodium (Lethabarb, Virbac, Peakhurst, NSW) after being assessed by Dr. Ian Hough, veterinary surgeon, Cleland Wildlife Park.

Kidney specimens

Kidneys from all koalas were used for light microscopic studies but specimens from only five adult animals were suitable for the electron microscopic examination of the kidneys. Kidneys were removed as soon as possible after death and specimens for light microscopic examination were fixed in 10% buffered formalin, processed and embedded in paraffin wax. For all animals, sections, 7 μm thick, were cut on a rotary microtome and stained with haematoxylin and eosin, solochrome azurine for aluminium (Powell et al., 1999), or Perl's Prussian blue for iron (Bancroft and Stevens, 1982). The last technique was used because iron can sometimes also be stained with solochrome azurine. The staining of sections was done in batches over a period of several months and to ensure the reproducibility of the techniques, kidney sections from koala #2 and #7 were selected as positive controls for solochrome azurine. Positive controls for Perl's Prussian Blue technique were paraffin sections of rat spleen.

General renal ultrastructure was investigated in five adult koalas - #8, #9, #12, #13 (control) and #14. Except for koala #13, these animals showed clinical signs of renal failure and aluminium was detected in their kidneys with solochrome azurine and Perl's Prussian Blue techniques. Koala #13 was the healthy male control in which no aluminium was found with light microscopic techniques. Small pieces (2-4 mm^3) were cut from the cortex and medulla of the kidneys, placed in 0.1 M phosphate buffered 3% paraformaldehyde, 3% glutaraldehyde fixative, pH 7.4, for 2-4 hours and some but not all specimens were also post-fixed in 1% OsO_4. The tissue samples were then dehydrated and embedded in TAAB® epoxy resin. For transmission electron microscopy, thick (1 μm) survey sections were cut with a glass knife on a Reichert Jung Ultracut microtome and stained with 0.025% toluidine blue in 0.5% sodium tetraborate. Areas of interest were identified from light microscopic examination and then thin sections of silver/gold interference colours (70-90 nm) were cut with a Diatome diamond knife (Diatome Ltd. Bienne, Switzerland), placed on copper (or appropriate) grids and stained with uranyl and lead citrate for electron microscopic viewing.

To correlate structures that stained for aluminium in light microscopy with structures showing cellular damage at the electron microscopy level, resin sections of kidney from koalas #9, #12, and #14 were used. For these animals, electron microscopy sections were compared qualitatively with survey sections and sequential 1 μm resin sections stained with solochrome azurine and Perl's Prussian blue staining techniques. Additionally, the quality of the preservation and images for koala #9 enabled a quantitative comparison of the number of lysosomes in aluminium-containing tubules and non aluminium-containing tubules to be done in this animal.

Aluminium-containing tubules in toluidine blue sections were identified from solochrome azurine sections and lysosomes counted in ten tubules with aluminium and ten without aluminium. The number of lysosomes per total area of tubules was calculated and data were analysed using unpaired t-tests. Electron microscopy confirmed the identity of lysosomes.

The co-location of aluminium and cellular damage in the kidney tubules was additionally evaluated in one koala (#9) using two electron microscopic techniques combined with X-ray microanalysis. Ultrathin sections, prepared from tissue that had not been post-fixed in osmium tetroxide, were mounted onto carbon-coated nylon grids and analysed using a Phillips CM200 electron microscope with electron dispersive x-ray analysis (EDX) attachment. Sequential, thick, resin sections were mounted onto glass slides and stained with solochrome azurine and Perls Prussian blue. These sections were photographed and the resulting montages were used as maps for the ultrastructural location of aluminium. The location of aluminium was also determined by mounting the remaining resin block onto a metal electron microscopy stub, cut surface upwards, and carbon coating (10 nm) the preparation prior to microprobe analysis with CAMECA SX51 electron microprobe.

Bone specimens

To assess the accumulation of aluminium in the koalas, samples of bone were tested for the presence of aluminium. Unfortunately, only two koalas were available for this part of the study. Femurs were removed from two koalas, #8 and #9, in whose kidneys aluminium had been detected. A small piece (12x2x2 mm) of bone from the distal metaphysis was fixed in buffered formalin, dehydrated over a two week period and embedded in TAAB® epoxy resin. Thick sections were cut with a glass knife and stained with toluidine blue, solochrome azurine and Perl's Prussian blue. The resin block was then mounted onto a metal stub, cut surface upwards, coated with a 10 nm carbon film, viewed with a Philips XL 20 scanning electron microscope, and aluminium detected with EDX attachment.

Results

Presence of aluminium in kidneys

Routine paraffin sections of kidney, stained with haematoxylin and eosin, showed normal mammalian renal structure. Renal corpuscles, some with a juxtaglomerular apparatus, proximal convoluted tubules (PCTs), distal convoluted tubules (DCTs), loops of Henle, and collecting tubules and ducts showed normal histological features in all koalas except #8, which showed obvious renal pathology at the light microscopic level. In this animal, there was focal medullary tubular damage with elongated crystals filling the lumen of the tubules and epithelial cells were absent. Further investigation showed the crystalline material stained positively with Pizzolato's silver peroxide technique (Bancroft and Stevens, 1982) for calcium oxalate. Other tubules, mainly thick segments of the loop of Henle had necrotic lining cells and were blocked with acidophilic protein material.

Aluminium was identified in the kidneys by light and electron microscopic techniques. With one exception, all kidneys from adult koalas in the Adelaide Hills showed positive staining for aluminium in some PCTs using solochrome azurine and Perl's Prussian blue techniques (Fig. 1). However aluminium was not widespread and areas stained blue with solochrome azurine formed approximately 1-5% of total renal cortical tissue. In some specimens, positive staining results with solochrome azurine and Perl's Prussian blue suggested the presence of iron or iron with aluminium. Staining with solochrome azurine was so intense in kidneys from koalas #2 and #7 that sections from these animals were subsequently used as positive controls for aluminium staining. The only adult koala in which aluminium was not present was a large, robust male (koala #13), which unlike the other koalas, was alert and in good physical condition apart from an incapacitating jaw injury. Kidney sections from pouch-young koalas were negatively stained with solochrome azurine and Perl's Prussian blue indicating an absence of aluminium and iron in these animals.

The presence of aluminium in one koala (koala #9) was also confirmed with EDX (Fig. 2a,b) and by mapping features of the electron microscopy section to an adjacent 'survey' section stained with solochrome

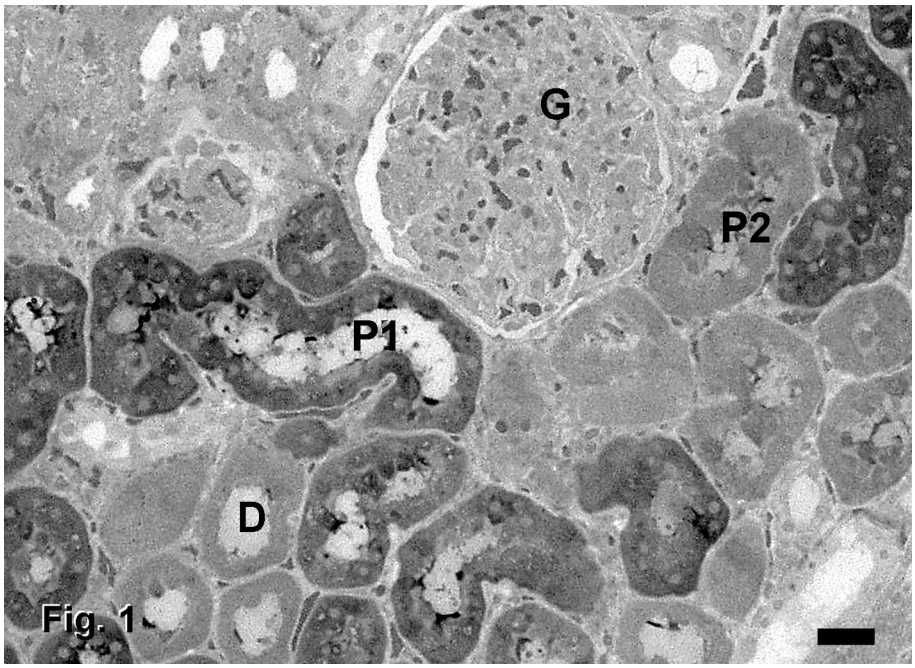


Fig.1. Light micrograph of resin section of koala (#9) kidney stained with solochrome azurine shows aluminium in some (P1), but not all (P2) PCTs. No aluminium was found in DCTs (D) or glomeruli (G). Bar: 35 μm.

azurine. For koala #9 aluminium was detected in seven of the 50 point analyses (14% of total) with the amount of aluminium at all seven points slightly above the detection limit of one part per thousand (i.e. 1000 parts per million). The peak shown in Figure 2a was mapped to a solochrome azurine positive (blue) tubule, while an adjacent negative (red) tubule showed no peak (Fig. 2b). Subsequent microprobe analysis with CAMECA SX51 electron microprobe confirmed the EDX results and quantified the level of aluminium present. No aluminium was present at 16 points (40%), which was consistent with the sporadic pattern of aluminium-containing PCTs observed with solochrome azurine. For the other 24 points (60%) where aluminium was detected, the mean aluminium concentration was 105±17 ppm with a range 10-270 ppm. No relationship for the collocation of aluminium with iron was recorded, while aluminium and calcium showed a weak, positive relationship (Table 1).

Table 1. Microprobe analysis of a solochrome azurine positive kidney section.

ELEMENT	CONCENTRATION (ppm)	RANGE (ppm)	% +ve POINTS	CORRELATION TO Al (r)	CORRELATION TO Fe (r)	CORRELATION TO Ca (r)
Al	105±17	10-270	60	1	-0.09	0.39
Fe	179±30	30-480	45	-0.09	1	0.05
Ca	114±16	10-270	48			

Microprobe analysis was done at 40 points along a 2 mm line, i.e. 40 points were sampled over approximately 35 tubules. Conc. is the mean (parts per million) of all points where the element was detected ± SE. Correlation of the three elements was taken at all 40 points.

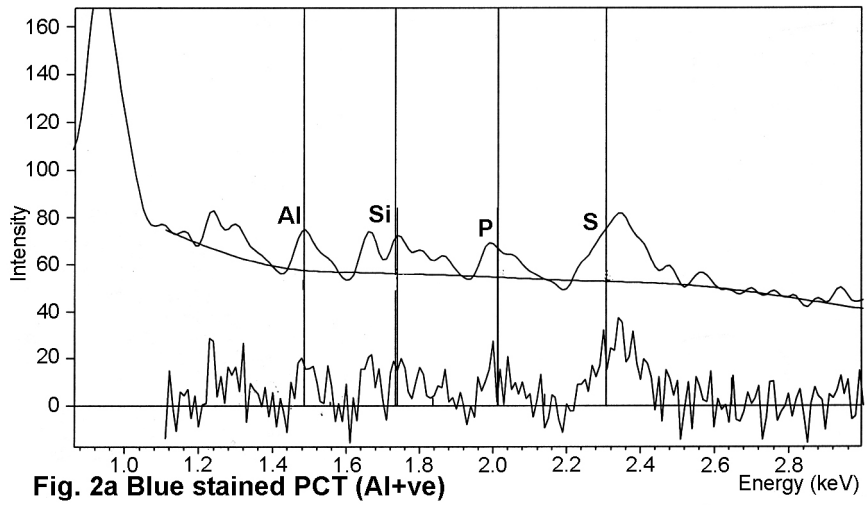


Fig. 2a Blue stained PCT (Al+ve)

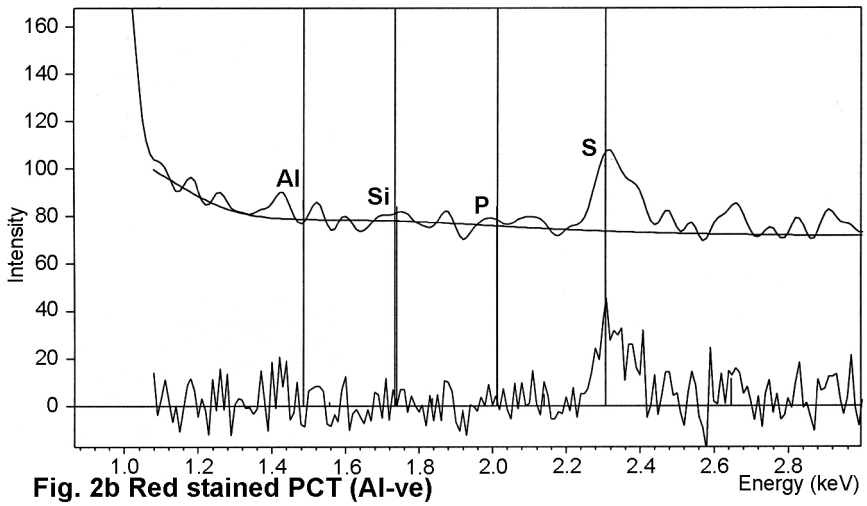
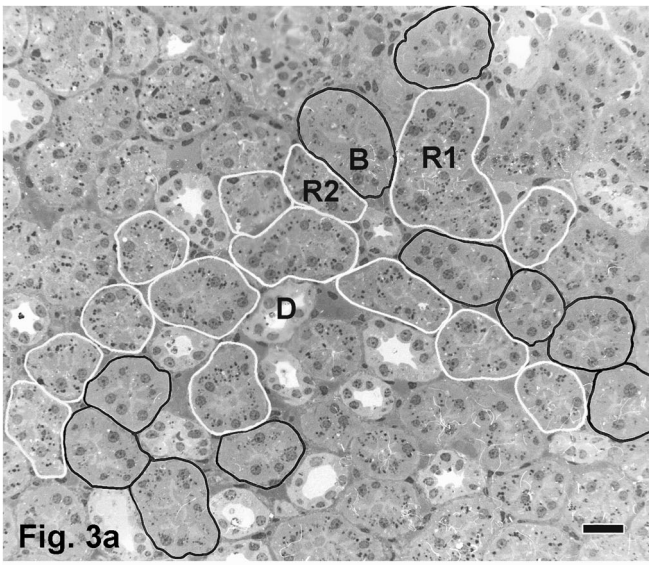


Fig. 2b Red stained PCT (Al-ve)

Fig. 2. Correlation between PCTs stained with azurine solochrome and the detection of aluminium with EDX. **a**. Shows aluminium present in PCT stained blue (positive) with azurine solochrome. **b**. Shows absence of aluminium in PCT stained red (negative) with azurine solochrome. In both figures, the lower reading shows the original spectrum while the upper reading shows the spectrum after background subtraction.

Correlation of aluminium presence in kidneys and cellular damage

Examination of routine paraffin sections revealed almost no pathologies. In fact, the normal light microscopic structure did not correlate with the abnormal biochemical results for blood and urine analyses or with the poor physical conditions of the animals. Generally, light microscopic examination showed normal glomeruli as well as normal cortical and medullary tubules. Basement membrane thickness was unremarkable for all renal structures at the light microscopic level and for glomeruli in electron microscopy. However, lysosomal density showed variation in the PCTs. These disparities were more pronounced in resin sections where tissue preservation was superior. For koala #9, the comparison of sequential sections stained with solochrome azurine and toluidine blue (Fig. 3a) showed the number of lysosomes per 10 μm^2 present in 10 aluminium-positive tubules was significantly lower compared with lysosomal numbers per 10 μm^2 in 10 aluminium-negative tubules. For aluminium-positive tubules, mean ± SD = 1.5±0.6 lysosomes per 10 μm^2; for aluminium-negative tubules, there were 3.1±0.3. Analysis with a two-sample unpaired t-test showed P <0.0001. Electron microscopy revealed fewer lysosomes and mitochondria for aluminium-positive tubules compared with aluminium-negative tubules in three of the four koalas (#9, #12, #14) from which sequential sections were correlated (Fig. 3b). The fourth koala, #13, the healthy, control animal, didn't have aluminium in its kidneys or variable ultrastructure of the PCTs.



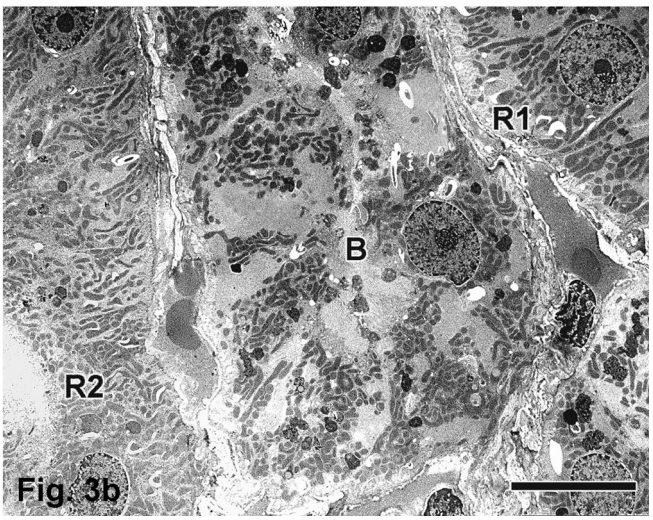


Fig. 3. a. Light micrograph of 1 mm, toluidine blue stained resin section of kidney from koala (#9) with renal failure. Sequential sections were stained with solochrome azurine and Perl's Prussian Blue to identify aluminium. The results were mapped onto the original section. Aluminium-positive PCTs, e.g. B, are outlined with black lines. They have significantly fewer lysosomes than aluminium-negative PCTs, e.g. R1 and R2, outlined with white lines. Bar: 35 μm. **b.** Electron micrograph of PCTs, identified in figure 3a as aluminium positive (B) and aluminium-negative (R1, R2). Intracellular damage is evident in cells of tubules with aluminium. Bar: 10 μm.

Presence of aluminium in bone

Aluminium but not iron was detected in the bone sections that were from koalas #8 and #9 and stained with solochrome azurine and Perl's Prussian blue. Aluminium was associated with the uncalcified osteoid border of bony trabeculae (Fig. 4). However with EDX analysis, aluminium was detected only at points of the line over the bone, indicating that aluminium was

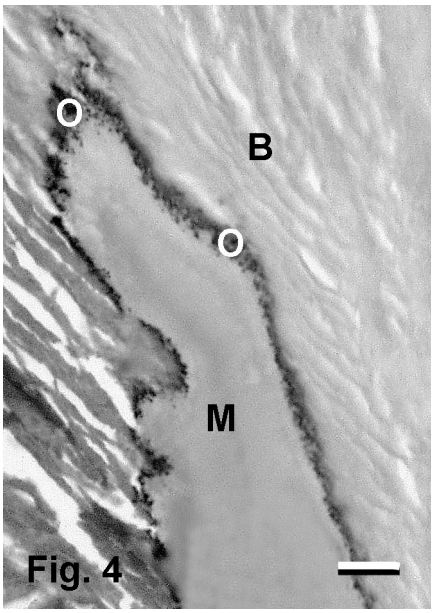


Fig. 4. Shows resin section of uncalcified bone from distal metaphysis of the femur (koala #9) stained with solochrome azurine. Aluminium is present in the osteoid layer (O) but not the calcified bone matrix (B) or bone marrow (M). With EDX, aluminium was detected in osteoid and bone matrix but not the bone marrow. Bar: 20 μm.

present within the mineralised bone matrix as well as the osteoid layer. No aluminium was detected in the bone marrow.

Biochemical analyses of koala plasma and Urine

Plasma samples from eight koalas in which aluminium was detected in their kidneys showed markedly high levels of urea and were similar to values characteristic of koalas diagnosed with renal failure (Table 2). Similarly, urine from the same eight koalas had a SG of 1.029±0.003 (mean ± SE) that was less than SG of 1.04 shown by koalas with renal failure. Urea levels in the positive control (koala #13) fell within the normal range for koalas in eastern Australia (Table 2) and urine SG was 1.04. Unfortunately insufficient data were available to determine creatinine values in the above eight koalas because blood samples were haemolysed.

Discussion

Dietary aluminium

Accurate estimations of the dietary intake of aluminium by koalas was not the focus of this study, but nevertheless it was important to establish that daily ingested amounts of aluminium per body weight were similar to levels that had been identified as nephrotoxic in rats (Somova et al., 1997). It seems unlikely that minimal levels for aluminium toxicity would be the same for the free living koala and experimental rat when the variability in digestive systems, diet, and environment are considered. The koala is herbivorous with a very long caecum and colon for hindgut fermentation; the rat is omnivorous with a simple digestive tract. Digesta makes a very slow passage (sometimes 30 days) through the digestive system of the koala (Hume, 1982), possibly increasing the opportunity for aluminium absorption compared with the rat. Finally the speciation of the aluminium given to experimental rats is known as well as some information on the effects of the digestive enzymes and secretions on aluminium speciation in the gut whereas no data are available for the koala.

Table 2. Plasma urea and urine SG. Shows plasma urea and urine SG in four groups of koalas, viz: reference group from eastern Australia, Cleland Wildlife Park koalas diagnosed with renal failure from 1995 to 2000, eight, aluminium-positive koalas from current study diagnosed with renal failure, and the aluminium-negative koala that was the healthy control in the current study. Values are presented as mean ± SE (n).

KOALA GROUP	UREA (mmol/L)	SPECIFIC GRAVITY
reference values[a]	0.2–6.6	1.06–1.14[b]
Renal Failure 95–00[c]	30±4 (11)	< 1.04[d]
Al +ve koalas	29±15 (8)	1.029±0.003 (8)
Al –ve koala	6.2 (1)	1.04 (1)

[a]: from Canfield et al, 1989; [b]: from Hemsley et al, 1998. For Adelaide Hills' koalas, the normal range is 1.04-1.14 (Ian Hough, Cleland Wildlife Park, personal communications); [c]: archived records of 11 koalas diagnosed with renal failure from 1995 to 2000; [d]: individual values unavailable.

Presence of aluminium in kidneys

Solochrome azurine has been shown to detect aluminium in tissues at levels as low as 20 mg/g (Kasa et al., 1995). The dye stains both aluminium and iron and its selectivity for aluminium is established by treating sequential tissue sections with Perl's Prussian blue technique to identify iron (Denton et al., 1984) or EDX analysis (Kasa et al., 1995). The presence of aluminium in the kidneys from Adelaide Hills' koalas was verified using these techniques. The use of kidney sections from koalas #2 and #7 as positive controls validated the negative staining of kidney specimens from the healthy adult male and the two pouch-young kidney specimens. The absence of aluminium in the pouch-young koalas from the Adelaide Hills supports the finding that aluminium is prevented from passing through mammary glandular cells and is absent in milk (Yokel, 1984).

The location of aluminium in the PCTs and the initial part of the thick descending loop of Henle is consistent with locations described in other studies (Chagnac et al., 1987; Liu et al., 1996; Somova et al., 1997). The average aluminium concentration of 105±17 ppm found in some PCTs was not accompanied by observable cellular damage at the light microscopic level. This contrasts to previous findings where, aluminium at 54 ppm in rat kidneys was associated with light microscopic damage (Liu et al., 1996). However the discrepancy probably arises from estimating aluminium levels by EDX point analysis in the current study and by spectrophotometry of kidney samples in the research by Liu and colleagues (1996). The latter method averages the concentration of aluminium in tissue samples and presumably there would be much higher levels of aluminium in PCTs compared with other renal components.

Iron was also detected in some PCTs and although tubular damage has been linked to free ferric ions (Alfrey and Hammond, 1990; Harris et al., 1995), the microprobe analysis indicated low correlation between the collocation of aluminium and iron.

Evaluation of aluminium presence in kidneys and cellular damage

Specimens from all koalas, except koala #8 showed no pathological renal damage in paraffin sections prepared for light microscopy. This lack of abnormal microstructure did not correlate with either the physical signs (insatiable thirst, polyuria) or the blood and urine biochemistry that indicated renal dysfunction. This lack of correlation has been previously noted (Dr Ian Hough,

personal communications) and suggests that unlike humans and rats, the koala requires almost 100% of its nephrons for adequate renal function and maybe has a much lower tolerance to physiological and biochemical challenges.

The variation in lysosomal density in the PCTs was similar to the pattern of solochrome azurine staining suggesting the presence of aluminium reduces lysosomal numbers and enzymatic activity. This observation supports previous studies where aluminium has been shown to reduce lysosomal enzyme activity both in the brain (Galle et al., 1980) and kidney (Chagnac et al., 1987). The current study also showed the presence of aluminium in the PCTs correlated with a decrease in organelles and inclusions in these cells and confirmed a lack of structural damage in the glomeruli and blood vessels (Liu and Nordberg, 1995).

Kidney sections from koala #8 were the only specimens to show obvious pathology at light microscopic level and the presence of oxalate crystals suggest that renal insufficiencies for this animal were due primarily to oxalic acid poisoning. Renal oxalosis has previously been described for koalas (Canfield and Dickens, 1982). For koala #8, it can be postulated that the increased body burden of aluminium was related to impaired renal function whereas in the other koalas, unknown factors must be directly or indirectly increasing the absorption of aluminium from the gut.

Presence of aluminium in bone

For humans, normal aluminium level in bone is negligible (2.2 ppm dry defatted tissue) (Alfrey et al., 1980) and this level is undetectable with solochrome azurine (Ellis et al., 1988). In experimental animals, aluminium concentration in bone has been shown to rise in response to increased levels of aluminium administered intravenously (Henry et al., 1984) and intraperitoneally (Robertson et al., 1983). From these studies it can be inferred that the aluminium, detected in koala bone, resulted from increased absorption from the gut coupled with decreased excretion by the kidneys. Increased absorption was probably linked to the high levels of aluminium ingested by these animals and decreased excretion by the kidney could have been related to the renal dysfunction. Furthermore, uremic conditions have been shown to promote aluminium absorption from the intestine (Alfrey et al., 1980).

Evaluation of aluminium presence in kidneys with plasma and urine analysis

Insufficient data were available to define a profile of plasma and urine analyses for koalas in which aluminium had been detected in the kidneys. Preliminary results indicate that these koalas have azotemia and dilute urine. Future studies by the authors will examine links between plasma and urine analyses, clinical data and the presence of aluminium in the kidneys.

In conclusion, the study demonstrated aluminium in the bones and kidneys of koalas that had renal failure and the presence of aluminium in PCTs correlated with cytoplasmic damage. However it is not known if the presence of aluminium is the cause or effect of the renal failure.

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