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Three-dimensional labeling of newly formed bone using synchrotron radiation barium K-edge subtraction imaging

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Abstract

Bone is a dynamic tissue which exhibits complex patterns of growth as well as continuous internal turnover (*i.e.*, remodeling). Tracking such changes can be challenging and thus a high resolution imaging-based tracer would provide a powerful new perspective on bone tissue dynamics. This is, particularly so if such a tracer can be detected in 3D. Previously, strontium has been demonstrated to be an effective tracer which can be detected by synchrotron-based dual energy K-Edge Subtraction (KES) imaging in either 2D or 3D. The use of strontium is, however, limited to very small sample thicknesses due to its low K-edge energy (16.105 keV) and thus is not suitable for *in vivo* application. Here we establish proof-of-principle for the use of barium as an alternative tracer with a higher K-edge energy (37.441 keV), albeit for *ex vivo* imaging at the moment, which enables application in larger specimens and has the potential to be developed for *in vivo* imaging of preclinical animal models. New bone formation within growing rats in 2D and 3D was demonstrated at the Biomedical Imaging and Therapy bending magnet (BMIT-BM) beamline of the Canadian Light Source synchrotron. Comparative X-ray fluorescence imaging confirmed those patterns of uptake detected by KES. This initial work provides a platform for the further development of this tracer and its exploration of applications for *in vivo* development.

Keywords

Barium; Growth; Bone; K-edge Subtraction; Functional Imaging; Synchrotron; XRF

Introduction

Bone in a living system is in a state of constant flux that depends on the health of the individual. Bone remodeling (*i.e.*, bone turnover) is a coupled process of bone resorption and bone formation through which the mechanical and chemical properties of bone is kept in

balance. Disorders of bone remodeling occur when this homeostasis is disturbed and the rates of bone resorption and bone formation differ (Feng, McDonald 2011). Bone disorders are detected by various imaging modalities, either directly by imaging the function of the bone or indirectly (most cases) through detection of the resulting pathological anatomical changes. Direct assessment of bone remodeling is performed through administration of radioactive tracers to assess physiological aspects of bone health, with ^{99m}Tc -Methyl Diphosphonate (^{99m}Tc -MDP) being the most commonly used radiotracer, followed by ^{18}F Fluorine (^{18}F). While nuclear medicine is an invaluable diagnostic tool, the use of radioactive materials could be seen as a limitation because of the challenges involved with nuclear materials handling, storage, waste management, and selection of the ideal radiotracer. For example, the use of ^{18}F in imaging bone diseases is limited by its short half-life of 110 minutes, whereas in the case of ^{85}Sr Strontium (Sr) the long half-life is an issue ($T_{1/2} = 64.8$ days). Therefore, it would be advantageous if a non-invasive and non-radioactive tracking method was also available for pre-clinical research purposes.

Current functional imaging techniques (e.g., Single Photon Emission Tomography (SPECT), Positron Emission Tomography (PET)), while being very sensitive and efficient in most clinical diagnostic applications, have limited resolution (e.g., mm-scale) – a key limitation for preclinical research involving small animals where micron-scale microarchitecture is frequently targeted. Pre-clinical studies are required to understand the mechanisms of disease progression, as well as for development of novel diagnostic and therapeutic compounds. Identifying the 3D spatial distribution of trace elements over time in bone mineral will create new opportunities for tracking bone changes. Sr, a bone-seeking element similar to calcium (Ca), was an element of interest to Cooper and colleagues because of the association between Sr and stronger bone, as well as the potential of Sr to be used as a bone tracer (Cooper et al. 2012). They identified the spatial distribution of Sr in the bone of an osteoporosis rat model using a dual energy K-edge subtraction imaging (KES) technique and subsequently applied this approach to an osteoarthritis model in the rat (Panahifar, Cooper & Doschak 2015). Based upon these studies, it became clear that Sr, with its low K-edge energy (16.105 keV K-edge), is only suitable as a tracer within very small bone specimens (90% absorption by 2.4 mm of bone) and is unsuitable for *in vivo* application due to the associated high absorbed radiation dose. The absorbed radiation dose is defined not only by the radiation delivered during scans but rather the absorbed percentage in the tissue. The absorption is higher in lower X-ray energies, that is the case for Sr. In fact, that is the reason why in clinical diagnostic imaging lower energy X-rays are filtered, to reduce unnecessary radiation dose to skin and superficial tissues. To overcome this limitation the current study explores barium (Ba) as an alternative tracer. Ba, like Sr, is an alkaline earth metal which incorporates into the hydroxyapatite lattice of bone. The higher K-edge energy of Ba (37.441 keV K-edge) significantly increases its penetrating power (90% absorption by 20.7 mm of bone) and accordingly lowers the absorbed radiation dose. A water-soluble form of Ba is necessary for its *in vivo* delivery to bone, however, this is challenging as soluble Ba salts are known for their toxicity. In fact, barium carbonate (BaCO_3) is used as a rodenticide (Harrison, Woodville 1948, Naheed, Khan 1989). On the other hand, barium chloride (BaCl_2), while also water-soluble and potentially toxic, has been previously tested for safe levels of use (Dietz et al. 1992). Therefore, according to the literature tolerable low doses of

BaCl₂ were used in this study (see Discussion). A soluble stable isotope of Ba is a potential alternative tracer, especially for longitudinal *in vivo* studies to track bone changes in preclinical disease models over time, and the current study sought to establish proof-of-principle for the detection of Ba within growing rat bones when dosed at levels below known toxic doses. Our secondary objective was to confirm that the uptake of Ba is indeed targeted to new bone formation.

Materials and Methods

Chemicals

Barium chloride in anhydrous form (99.9% purity, MW 208.23 g/mol) was purchased from Sigma-Aldrich (Oakville, Ontario).

Animals

12 male Sprague-Dawley weanling rats (24-28 days old) were included in the study (Charles River, Quebec, Canada). Weanlings were chosen due to their fast growth rates, which increases until day 60 when it then slowly declines (Mercer, Haijazi & Hidvegi 1993). The following protocol was approved by the University of Saskatchewan Animal Research Ethics Board, Animal Use Protocol # 20110124.

Treatment

After one week of acclimatization animals were given different concentrations of soluble Ba (BaCl₂) in their drinking water. The Ba dosage and period of dosage time were varied with the goal of determining the detectable limits when imaged at 05B1-1 beamline of the Biomedical Imaging and Therapy (BMIT) beamline complex at Canadian Light Source (CLS). Drinking water for four rats was supplemented with 0.005 M BaCl₂ (1041 mg/L BaCl₂ ~ 1000 ppm BaCl₂ equivalent to 0.68 g/L Ba²⁺). Two rats were euthanized after 14 days and the other two were euthanized after 28 days of treatment. The drinking water for a second set of four rats was supplemented with 0.01 M BaCl₂ (~2000 ppm BaCl₂ or 1.37 g/L Ba²⁺). This concentration was chosen based on the findings of Dietz and colleagues (Dietz et al. 1992), as well as the National Toxicology Program (National Toxicology Program 1994). Similar to the first set, two rats were euthanized after 14 days and the other two were euthanized after 28 days. Two control rats were euthanized after 14 days and the second pair of rats was euthanized after 28 days. Prior to euthanization with CO₂, the rats were sedated with isoflurane and the cardiac puncture technique was used to recover blood for toxicological studies. Long bones were dissected for imaging at BMIT.

Ba K-edge Subtraction Imaging

BMIT beam line has a Si (2, 2, 0) double crystal Bragg monochromator with a beam size of 240 mm × 7 mm (width and height, respectively). Dissected limbs were secured on a plastic holder for imaging at BMIT. Projection images for KES were collected using the Hamamatsu Flat panel detector (C9252DK-14) with a 200 µm pixel size as well as a 100 µm pixel size at above and below the 37.44 keV K-edge of Ba, followed by subtraction of the two images to obtain the map of Ba distribution in the bones. When acquiring projections a phantom of a known concentration of Ba was placed next to the sample and scanned in the

same manner for later verification of the measured Ba concentrations by the KES method. For flat field correction at each energy 10 projections were collected with no object in the beam (*i.e.*, Flats) and 10 projections with no beam (*i.e.*, Darks). Radiation dose was estimated for the 200 μm resolution images by including thermoluminescent dosimeters (TLD) beside the sample during the scans. The analysis was completed by Mirion Technologies (Irvine, CA).

For CT experiment, 600 projections were acquired over 360 degrees for each of the two energies, using Hamamatsu C4742-95-12HR X-ray camera (22.4 μm). The flat projections were collected at the beginning to also use them for correction of ring current decay. For that reason, it was ensured that at least a few pixels on each side of the field of view are not occupied by the sample. The reconstruction process produces a planar linear attenuation map of the object, $\mu(x, y)$, from X-ray projections. In dual energy KES CT, for simplicity it can be assumed that density of the object material (ρ_M) is homogeneous, thus;

$$\mu(x, y) = \frac{\mu}{\rho_C} \rho_C(x, y) + \frac{\mu}{\rho_M} \rho_M(x, y) \quad (1)$$

Where ρ_C is the density of a contrast element and $\frac{\mu}{\rho_{M, C}}$ is the mass attenuation coefficient for the object material and contrast element, respectively. Because there are two imaging energies in KES (Table 1), then the above K-edge ($\mu_H(x, y)$) and below K-edge ($\mu_L(x, y)$) CT reconstructions can be written as:

$$\mu_L(x, y) = \frac{\mu}{\rho_C}(E_L) \rho_C(x, y) + \frac{\mu}{\rho_M}(E_L) \rho_M(x, y) \quad (2)$$

$$\mu_H(x, y) = \frac{\mu}{\rho_C}(E_H) \rho_C(x, y) + \frac{\mu}{\rho_M}(E_H) \rho_M(x, y) \quad (3)$$

Where E_L is the photon energy below the edge and E_H is the photon energy above the edge. Therefore, the densities of the contrast element and object material are:

$$\rho_C(x, y) = \frac{\frac{\mu(E_L)}{\rho_M} \mu_H(x, y) - \frac{\mu(E_H)}{\rho_M} \mu_L(x, y)}{\frac{\mu(E_L)}{\rho_M} \frac{\mu(E_H)}{\rho_C} - \frac{\mu(E_H)}{\rho_M} \frac{\mu(E_L)}{\rho_C}} \quad (4)$$

$$\rho_M(x, y) = \frac{\frac{\mu(E_H)}{\rho_C} \mu_L(x, y) - \frac{\mu(E_L)}{\rho_C} \mu_H(x, y)}{\frac{\mu(E_L)}{\rho_M} \frac{\mu(E_H)}{\rho_C} - \frac{\mu(E_H)}{\rho_M} \frac{\mu(E_L)}{\rho_C}} \quad (5)$$

Complete details on the reconstruction algorithm can be found elsewhere (Cooper et al. 2012).

The reconstruction of the CT data sets was performed in NRecon software v.1.6.1 (Bruker micro-CT). 3D rendering, registration and subtraction of the CT data sets were performed in Amira v.6.0.0, and image analysis for projection data was performed in ImageJ v.1.49d.

X-ray Fluorescence Mapping

X-ray fluorescence mapping was carried out at the Stanford Synchrotron Radiation Lightsource (SSRL) with the 3 GeV SPEAR3 storage ring operating at 500 mA on beamline 2-3, employing a 1.3 Tesla bend magnet source. A Si (111) double-crystal monochromator was employed with Kirkpatrick-Baez microfocusing optics (~2 µm beam spot), with the incident X-ray energy set to 13.450 KeV. The incident intensity was monitored using an N₂-filled ion chamber. Samples were oriented at 45° to the incident beam and raster scanned using Newport stages, with a silicon-drift Vortex detector positioned at 90° to the incident beam. Data collection was performed in continuous collection mode with an approximate dwell time of 500 ms per point using a 20 µm step size. Data was processed using the Microprobe Analysis Kit, v.1.01 [ref Webb SM (2004) The MicroAnalysis Toolkit: X-ray Fluorescence Image Processing Software, v1365, pp196-199].

Toxicology

The rats were weighed weekly throughout the study and the consumption of food and water was monitored. Kidney function was determined by testing the plasma colorimetrically for the urea concentration following the manufacturer's protocol (QuantiChrom™ Urea Assay Kit, BioAssay Systems, CA).

Results

Projections were acquired for the excised limbs with an average absorbed radiation dose of 3.81 mGy per projection (for 200 µm resolution). All treatment protocols resulted in sufficient concentration levels of Ba in bones for detection by the KES technique. Ba was predominantly incorporated in the new bone of rats laid down during the rapid growth period (*e.g.*, in the growth plates of the long bones). By increasing the duration of the treatment more Ba was deposited in the bones due to the longer bioavailability of the elemental compound. Similarly, increasing the Ba dose had a direct effect on the concentration of Ba being incorporated in the bones, as expected. The highest Ba concentration in the hind paws after calibration against the phantom was measured as 0.98 mg/cm² (4-week 0.01 M dose), 0.52 mg/cm² (4-week 0.005 M dose), 0.57 mg/cm² (2-week 0.01 M dose), 0.27 mg/cm² (2-week 0.005 M dose) (Figure 1).

Within the long bones, Ba was mostly detected in the metaphyses (adjacent to growth plates) of bones where the natural elongation process occurs and the epiphyses whose trabecular bone undergoes rapid remodeling (Figure 2). The highest Ba concentration was measured adjacent to the growth plate of femur and tibia as 1.24 mg/cm^2 (4-week 0.01 M dose). This value was 0.28 mg/cm^2 for animals dosed 4 weeks with the 0.005 M Ba solution. Other than in the skeleton, a significant amount of Ba was also detected in the gastrointestinal tract as its biological excretion route (data not shown).

In order to validate our KES images, XRF maps of Ba were also obtained. Ba L_{α} fluorescence was corrected for scatter and Ca K_{α} fluorescence. XRF data revealed that Ba was predominantly deposited on the trabecular surfaces of the bone that could be as a result of bone growth or active bone turnover, which is more rapid in the trabecular bone than cortical bone (Figure 3C, red arrows). Moreover, considerable concentration of Ba was detected in the periosteal layer of cortical bone (Figure 3C, red arrowheads), indicating appositional growth and elongation process of long bones in the region beneath the growth plate. Minute levels of Ba were also detected in the bones of control rats, likely through dietary intake (Figure 3A). In general, the XRF results confirmed the findings of the KES imaging (Figure 4).

Toxicology

All animals tolerated the treatments well and similar to the literature no overt toxicity related to Ba was noticed at the trialed doses (*e.g.*, rats gained weight, groomed normally and behaved normally). The Ba intake was estimated based on the water consumption volume during the study, assuming rats in each cage drank equally (Table 2). While it became apparent that water intake decreased for the rats receiving BaCl_2 , (Table 2) the results of the urea assay indicated that the kidneys were functioning within a normal range for rat weanlings (Won et al. 2015, Katayama et al. 2010). Moreover, the plasma urea levels for rats given Ba in their drinking water did not differ from 2 weeks to 4 weeks (Table 3).

Discussion

Bone elemental composition is mainly comprised of Ca and P, but several other elements such as Zn, Sr, Pb, U, *etc.*, are also known to fit in the structure of the hydroxyapatite (Pemmer et al. 2013, Bourgeois et al. 2015), some with biological role, and some without. Among those, Sr and Ba due to their resemblance to Ca could potentially be employed as tracers for Ca, if non-toxic doses of them could provide sufficient signal-to-noise ratio. Sr has a safe biological profile and its distribution in bone has previously been studied using several X-ray techniques such as EPMA (Electron Probe Micro Analysis) (Panahifar, Maksymowych & Doschak 2012, Wu et al. 2013), XRF (Pemmer et al. 2013), KES (Cooper et al. 2012, Panahifar, Cooper & Doschak 2015), *etc.* However, 3D imaging of Sr distribution in bone is somewhat limited as the only currently known method for 3D mapping of non-radioactive Sr is through the KES method, which itself is limited by the small penetrability of 16.105 keV X-rays (*i.e.*, Sr K-edge) in the bone. Therefore, we aimed to evaluate the possibility of employing Ba as a better candidate (K-edge 37.441 keV) for imaging bone turnover at the non-toxic low doses. Imaging studies have utilized Ba for

decades but mainly in the form of barium sulfate (BaSO_4) for gastro-intestinal imaging, after oral or enema administration (Barrs 2006). BaSO_4 in pre-clinical research has also been used for imaging micro-vascularization in bones of adult rats and mice using desktop micro-CT and synchrotron micro-CT imaging (Fei et al. 2010, Roche et al. 2012). The compound BaSO_4 is advantageous because it is insoluble and therefore toxicity is not an issue, unless administered in high doses, as was identified when the death of rats occurred after the intragastric administration of BaSO_4 when the dose reached 25% to 40% of body weight (Boyd, Abel 1966). Radioactive isotopes of barium in the form of ^{131}Ba and $^{135\text{m}}\text{Ba}$ has also been developed and employed for imaging bone but they never gained popularity due to the complexities with their production. For instance, ^{131}Ba decays by 6 γ -rays ($E_\gamma = 124\text{--}496$ keV), but the main limiting factor for its production with a nuclear reactor is the 0.1% natural abundance of ^{130}Ba that is required as the bombardment target, which means production of high specific activity of ^{131}Ba requires costly enrichment of the ^{130}Ba (Spencer, Lange & Treves 1971). In order to avoid the complications of dealing with radioactive materials, in this study we decided to use non-radioactive Ba as a tracer of bone turnover, to be detected by the synchrotron KES technique.

Ba is not required for normal physiological processes and its presence in bone tissue is often the result of dietary exposure. An analysis of Ba in bone was included in a study on the metabolic behavior of Ba and Sr in rats (Bligh, Taylor 1963). Rats were dosed with radioactive $^{140}\text{BaCl}_2$ and $^{85}\text{SrCl}_2$ and tissues were assayed for radioactivity after euthanization at intervals over a period of 80 days. After examination of the femur, it was noted that the highest concentration was found in the distal ends and the lowest concentration in the diaphysis. Ba at the low experimented dose in our study was successfully detected by XRF and KES in all trialed doses and administration durations. It was shown that in this rapidly growing animal model Ba in the bone was primarily incorporated in the mineralizing regions, particularly in the growth plates of long bones, indicating natural elongation process. Moreover, it was detected on the trabecular surfaces of epiphyses, as well as in the endosteal and periosteal layers of cortical bone, indicating appositional growth.

While Ba is a good candidate for imaging bone turnover in terms of its adequate K-edge energy (37.441 keV) as well as its primary accumulation in the bone, the major drawback with using soluble Ba compounds in clinical use is its cardiac and kidney toxicity. Cardiotoxicity of Ba is due to the blockade of potassium channels by Ba^{2+} ions in the heart (National Toxicology Program 1994). The highest Ba intake dose by rat weanlings (350–400 g at endpoint) in this study was approximately 15.93 mg/day elemental Ba for 4 weeks. Dallas and Williams summarized the studies that address the toxic effects of Ba on cardiovascular system and the kidney (Dallas, Williams 2001). It has been reported that short-term oral exposure (1–10 days) to BaCl_2 doses up to 209 mg/kg (~ 138 mg/kg Ba) produces no significant toxic effects (Borzelleca, Condie & Egle 1988). This is at least 2.5–3.5 times higher than the highest Ba intake by rats in our study (assuming rats weigh 300–400 g). Tardiff and colleagues added up to 250 ppm of BaCl_2 into the drinking water of young adult rats for 4, 8, and 13 weeks (the water intake for the highest dosed group was slightly less) and interestingly identified that increasing the dose but not the duration of treatment results in higher Ba concentrations in bone as its primary accumulation site, as

well as in liver, skeletal muscle, heart (Tardiff, Robinson & Ulmer 1980). Earlier studies determined that the no-effect level was 2000 ppm $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ in the drinking water of B6C3F1 mice and Fischer 344/N rats, exposed to Ba for 92 days (Dietz et al. 1992). This result was similar to the findings by the National Toxicology Program that found after a 15 day study with rats, no chemical-related deaths or evidence of toxicity was noted, even when the concentrations went as high as 2000 ppm, which resulted in daily consumption of 110 mg Ba/kg/day by rats (National Toxicology Program 1994). In agreement with the literature, in this proof-of-principle study we did not notice any signs of toxicity in rats. A slight decrease in water consumption after week two was noticed in Ba groups, which could be a behavioral response to the change in taste of the water with Ba added in it (Cory-Slechta, Weiss 1981). Commonly, nephrotoxicity is accompanied with an increase in water consumption. Supporting a lack of toxicity, the plasma urea test showed no change in urea levels in rats, indicating no renal damage at the trialed doses and durations. In this study we only focused on Ba distribution in bone, however, it has been reported in the literature that in non-skeletal tissues, Ba also accumulates in the heart, eye, skeletal muscle, kidney, and liver, though much less than in bone and teeth, although at much lower concentrations (McCauley, Washington 1983).

The presented methodology and information on the role Ba in imaging of bone turnover can potentially lead to new strategies regarding the study of bone remodeling disorders in pre-clinical animal models (Feng, McDonald 2011). This is significant as currently the only method for imaging the function of the bone is through nuclear medicine. Nuclear medicine is a very sensitive technique with adequate resolution provided for clinical applications (5-10 mm). Although the recent advancements in micro-PET and micro-SPECT technology have made resolutions of 1-2 mm feasible, however, more often higher resolution imaging data is necessary due to the smaller size of the organs and pathological lesions in small animals. The proposed methodology here, while using non-toxic levels of Ba and no radioactive isotopes, produces images of high resolution (micron level) that provide information on the structure of bone due to the absorption of X-rays as well as the function of bone due to the Ba tracer concentrating in the mineralizing and remodeling events. The resolution of the images is mainly dependent on the detector capabilities and currently could be as high as 1-2 μm at the CLS. The KES imaging can be performed using double crystal Bragg monochromator such as the system that was used in this study or by using a single crystal bent Laue monochromator (Suortti et al. 1993). The double crystal monochromator is more sensitive because it does not block the area around the 'edge' and also prevents geometrical errors that occur when using a bent crystal (Zhu et al. 2014). However, it is not suitable for *in vivo* imaging due to the need for two separate scans. On the other hand, bent Laue crystal provides both scanning energies in one shot and thus images can be subtracted simultaneously, but because it uses a curved crystal it causes some geometrical errors, especially at the interface of bone and soft tissues. Therefore, in this study we chose to use a double crystal monochromator since we were evaluating *ex vivo* samples. However, for *in vivo* KES imaging a simultaneous subtraction method must be used. The main limitation of the KES set up in this study was the need to hold the sample perfectly motionless during the two sets of imaging at below and above the K-edge of Ba. The slightest movement during or between the imaging will appear as a false positive Ba signal when the two data sets are

subtracted to produce Ba map, although it can be minimized by application of 3D registration algorithms for CT data. The KES is a non-invasive and non-destructive imaging method has the advantage of keeping the samples intact for further analysis for histological correlation or other purposes.

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Figure 1. KES projections

Top row: the KES projections show the distribution of Ba in the hind paws of the rats dosed with different durations and doses of BaCl_2 . Note that most of the Ba is deposited in the epiphyses of the bones. Bottom row: the plain radiography of samples are included as anatomical reference. (Resolution: $100\mu\text{m}$)

Figure 2. KES projections

The panel shows Ba distribution in the femur and tibia (hind limbs) of rats. Note the deposition of Ba in growth plates. (Resolution: 200 μm)

Figure 3. XRF Maps of bone sections (femur)

A) Control; B) 0.005 M BaCl₂, 2 weeks; C) 0.01 M BaCl₂, 2 weeks; D) 0.01 M BaCl₂, 4 weeks. Note the deposition of Ba in the trabecular surfaces (red arrows) and cortical regions (red arrowheads).

Figure 4. Synchrotron micro-CT KES

A transverse view of the diaphysis of a rat femur dosed with 0.01 M BaCl₂ for 2 weeks showing the spatial (3D) distribution of Ba in the bone. Note the high Ba content in the forming trabeculae (red arrowheads) as well as in the endosteal and periosteal layers of the cortical bone. The XRF data shows the same uptake pattern (Resolution: XRF = 20 μm, KES = 22.4 μm). *Note that the XRF and KES data are not from the same animal, but from the same treatment group.

Table 1

Mass attenuation coefficients used in the calculations to extract Ba concentrations. Ba values from National Institute of Standards and Technology (NIST; <http://www.nist.gov/pml/data/>). The compact bone value is derived from International Commission on Radiation Units (ICRU).

	Energy (keV)	Barium μ/ρ (cm ² /g)	Compact Bone μ/ρ (cm ² /g)
High	37.54	28.879	0.584
Low	37.34	5.518	0.590

Table 2
Average water and Ba consumption by rats (values are calculated for elemental Ba not BaCl₂)

Rat pairs	2-week group (per cage)	4-week group (per cage)
Control	192.5 ml per week	233.75 ml per week
0.005 M BaCl ₂	168 ml per week (~8.24 mg Ba/day/rat *)	218.75 ml per week (~10.72 mg Ba/day/rat)
0.01 M BaCl ₂	150 ml per week (~14.69 mg Ba/day/rat)	162.5 ml per week (~15.93 mg Ba/day/rat)

* Assuming rats in cages drank equally.

Table 3
Plasma urea concentration

Rat pairs	Urea 2-week (mg/dL)	Urea 4-week (mg/dL)
Control	46.7	46.4
0.005 M BaCl ₂	48.34	48.03
0.01 M BaCl ₂	51.74	49.81

Conversions: Blood Urea Nitrogen (BUN) (mg/L) = Urea (mg/dL)/2.14 1 mg/dL urea = 0.167 mmol/L