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Uranium deposition in bones of Wistar rats associated with skeleton development

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HIGHLIGHTS

- Uranium deposited in bones increases faster in younger animals saturating in older.
- *U* data were fitted by a sigmoid curve, suggesting that it mimics calcium metabolism.
- Bone mineral density indicates that even minute *U* could induce death of bone cells.

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ABSTRACT

Sixty female Wistar rats were submitted to a daily intake of ration doped with uranium from weaning to adulthood. Uranium in bone was quantified by the SSNTD (solid state nuclear track detection) technique, and bone mineral density (BMD) analysis performed. Uranium concentration as a function of age exhibited a sharp rise during the first week of the experiment and a drastic drop of 70% in the following weeks. Data interpretation indicates that uranium mimics calcium. Results from BMD suggest that radiation emitted by the incorporated Uranium could induce death of bone cells.

1. Introduction

Uranium is heavily present in rock phosphate, which is used as a source of phosphorus in the making of fertilizers and livestock feed supplements, as dicalcium phosphate (DCP). The prolonged utilization of such fertilizers leads to absorption of substantial amounts of uranium by plants, contributing to the increase of this element in the human diet (Yamazaki and Geraldo, 2003). Furthermore, DCP is extensively used in broilers diet, another important consumption item by humans (Sebastian et al., 1996; Lima et al., 1995). It is important to note, however, that DCP can present concentrations of uranium as high as 200 ppm (Arruda-Neto et al., 1997). Also, insoluble forms of uranium are greatly solubilized during digestion giving rise to uranyl compounds, particularly uranyl nitrate.

Health hazards generated by uranium are of two categories: toxicological and radiobiological. It is well documented from toxicity studies that the kidney is the target organ and that chronic ingestion of uranium may cause kidney lesions and malfunction

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(Zamora et al., 1998). This element also causes damage to the microvasculature of the liver and induces hepatitis (Alpen, 1990).

However, the radiobiological issue is a matter of much greater concern, since (1) uranium has three natural isotopes which are long-life α-emitters, and (2) the uranyl radical $^{++}UO_2$ produced in the gastrointestinal tract seems to mimic ^{++}Ca. Additionally, uranium belongs to the category of "bone-seeker radionuclides", and about 80% of incorporated uranium is accumulated in the skeleton (ICRP 69, 1995).

In an earlier experiment performed at this Laboratory with Beagle dogs, uranium mixed with food was administered to the animals. It was observed that this element accumulates similar amounts in both mineral bone and marrow (Arruda-Neto et al., 2004b). Thus, doses from α-emitters are imparted to the entire bone marrow volume and, consequently, primitive hematopoietic stem cells, concentrated in the central marrow (Lord, 1990), are subject to radioactive burdens as intense as those in mineralized bone. Thus, possible radiobiological risks need to be taken into account, even for small amounts of uranium. Regarding consequences to humans, children are of much greater concern because of the higher absorption rate of essential elements in their growing skeleton (Tandon et al., 1998).

This circumstance motivated the need to measure the content of uranium in the bones of Wistar rats, following chronic ingestion, starting in the postweaning phase and at intervals of three days. This data could prove to be very useful for extrapolations to humans, particularly children, since the vast majority of studies has been conducted with adult animals following single administration of acute dosages (Tandon et al., 1998; Ubios et al., 1998) as far as prolonged intake (Arruda-Neto et al., 2001). In fact, with an ad-hoc multiple compartment model (Garcia et al., 1999) it would be possible to estimate the content of uranium transferred to organs and milk by using uranium accumulation data in bones as input.

The experiment described in this study is part of an ongoing comprehensive project on teratogeny, dealing particularly with trans-placental biokinetics of uranium, a circumstance motivating the desire to use female animals also in this study.

2. Materials and methods

2.1. Animals

Sixty female Wistar rats, 21 days old (so far breastfed) were separated into 8 groups with the following composition: (a) a first group with 3 animals (control group); (b) 6 groups having 8 animals each, with 5 submitted to Uranium treatment (treated animals) and 3 as control; and (c) one group having 9 animals, with 5 submitted to Uranium treatment and 4 as control. Natural Uranium was administered as Uranyl nitrate. Naturally occurring uranium is composed of three major isotopes, uranium-238 (99.275% natural abundance), uranium-235 (0.72%), and uranium-234 (0.006%). This isotopic composition is routinely verified in our Laboratory by means of neutron activation spectroscopy analysis—see details in Arruda-Neto et al. (2004b).

The animals were maintained with temperature and ventilation under control with light cycle of 12 h/12 h, fed with controlled amounts of ration, and water "ad libitum".

Uranyl nitrate, homogeneously mixed into the ration at a concentration of 50 ppm (parts per million), was administered to the treated animals. These animals were sacrificed at the ages of 25, 29, 33, 36, 43 and 50 days. The animals in the control group were fed with noncontaminated ration, and were sacrificed at the ages of 22, 25, 29, 33, 36, 40, 43 and 50 days, respectively. The animals were clinically checked throughout experiment duration, and their weight and amount of ration ingested verified daily.

2.1.1. Euthanasia and necropsy

The animals were anesthetized with ketamine at a dose of 80 mg/kg associated with xylazine in a dose of 10 mg/kg intraperitoneally. While they were still alive, blood was collected via intracardiac puncture with 3 ml syringes, using 30×7 needles in the fourth left lateral intercostal space. About 2 ml of blood was collected and stored in collection tubes with and without heparin. The animals died of hypovolemia; autopsy followed by external and cavity inspection was then performed. Organs were removed and stored in 10% formalin. Femora were dismembered and stored under freezing conditions for posterior processing (next paragraph).

2.2. Quantification of uranium in femora

To determine *U* concentrations in bone samples of Wistar rats the SSNTD (solid state nuclear track detection) technique was employed using a polycarbonate plastic named PCLIGHT (1 mm thickness), produced by Policarbonatos do Brasil S/A (Yamazaki and Geraldo, 2003).

Initially, the bones were stored in an incubator at 70 °C for three hours to obtain dry weight. These samples were placed in a muffle furnace for one hour for the carrying out of calcination, reaching a maximum temperature of 800 °C. Following this, each bone was macerated under a 250 W lamp, intended to minimize absorption of water from the environment, and were weighed again to establish the weight after calcination in a muffle furnace.

The calcinated samples were oven-dried at 90 °C until attaining a constant weight ($\pm$ 1%). About 40 mg dry weight from each bone powder was treated with acqua-regia solution (1HNO_3: 3HCl), at a temperature around 115 °C, for approximately 1 hour, in order to eliminate organic compounds and to obtain a homogeneous solution. The resulting residue was diluted to a total volume of 10 mL. Aliquots of 10 μL were deposited on plastic detectors (area of 1.2 cm^2) followed by 5 μL of a Cyastat detergent solution (5%, Cytec Industrias). Evaporation of water and volatile compounds were accomplished by exposing the set to an infrared lamp (250 W) at a temperature around 75 °C. The Cyastat detergent solution works as an electrostatic neutralizer reducing the droplet surface tension and making it possible to obtain deposits with better homogeneity. The deposits were covered with an extremely thin (about 20 μg/cm^2) collodion film (isoamile acetate plus elastic collodion 1:1 in volume) in order to prevent contamination and water absorption from room humidity.

A standard solution having a precisely known uranium content was prepared in the same way as described above for bone samples, to be used as neutron flux monitor during irradiations. A solution of the reference bone sample IAEA-A12 was also prepared, so as to check the analytical accuracy of the method.

Plastic films corresponding to bone samples from three animals were sandwiched between two *U* standard samples, and this set was accommodated inside an aluminum *rabbit* (22 mm diameter by 70 mm height), a container usually employed for irradiations at IPEN-IEA-R1 (3.5 MW) pool type research nuclear reactor—over 20 *rabbits* were used. With the *U* standards in each *rabbit* a much better neutron flux monitoring was achieved.

The *rabbits* were placed near the reactor core for irradiation at the position EIRA 24B, where the estimated thermal neutron flux was 1.2×10^{13} n/cm^2 s. The irradiation time employed for all samples was about 3 min.

After irradiations, chemical etching of the plastic detectors was carried out at approximately 60 °C, for a period of 65 min, in a NaOH (6 N) solution. For these chemical etching conditions the fission tracks produced in the detectors presented the best visibility condition when observed under a conventional optical microscope. The fission tracks were counted by scanning the entire

area of the deposit with a system consisting of a binocular optical microscope, at a magnification of 400 ×, together with a video camera and a PC computer. Sample aliquots from each animal were deposited on 6 plastic films which, after irradiation, provided from 200 (younger animals) to 600 fission tracks. Considering that each group consisted of 5 animals, final results of this study correspond to 1000 (younger animals) to 3000 Uranium fission events.

2.3. Bone mineral density

Dissected animal bones were maintained in salt solution (NaCL 0.9%) at −20 °C. Bone mineral density (BMD) analysis of total femora (epiphysis and diaphysis) was performed with a Densitometer (Hologic Inc., Discovery Model, MA, USA) using double X-ray emission sources (DXA). Data analysis was performed with the *Small Animal* software (High Resolution mode), provided by the supplier. Results were expressed as g/cm^2 with an instrumental uncertainty of 1.9% for total femora.

3. Results and discussion

3.1. Data statistical analysis

3.1.1. Definition of U concentration

This work analyzes bones of Wistar rats as a function of their age (t, in days), taking bone samples of 5 animals per age. The Uranium concentration (C_i) in the ith sample ($i=1, 2 \ldots n=5$) is defined as

$$C_i = \frac{m_i(U)}{m_i(\text{bone})} (\mu g/g), \tag{1}$$

where m_i(bone) is the bone sample mass and $m_i(U)$ its U mass content.

Bone samples were irradiated with neutrons (see above) attached to U standard samples, that is, U samples with precisely measured masses [$m_i(U{-}s)$]. Since irradiation and counting geometries are the same, the following relationship holds,

$$\frac{m_i(U)}{m(U{-}s)} = \frac{N_i(U)}{N(U{-}s)} \tag{2}$$

where $N_i(U)$ and $N(U{-}s)$ are the total number of fission tracks from the bone sample U content and from the U standard sample, respectively. Thus,

$$m_i(U) = \frac{N_i(U)}{N(U{-}s)} m(U{-}s) \tag{3}$$

Substituting Eq. (3) in Eq. (1),

$$C_i = \left[\frac{N_i(U)}{N(U{-}s)}\right] \times \left[\frac{m(U{-}s)}{m_i(\text{bone})}\right] \tag{4}$$

3.1.2. Standard deviation of C_i—σ_{Ci}

The standard deviation (σ_{Ci}) of C_i is obtained by a conventional expression (Caria, 2000) derived from Eq. (4),

$$\left[\frac{\sigma_{C_i}}{C_i}\right]^2 = \left[\frac{\sigma_{m(U-s)}}{m(U{-}s)}\right]^2 + \left[\frac{\sigma_{m_i}}{m_i(\text{bone})}\right]^2 + \left[\frac{\sigma_{N_i}}{N_i(U)}\right]^2 + \left[\frac{\sigma_{N(U-s)}}{N(U{-}s)}\right]^2 \tag{5}$$

Generically speaking, the quantity σ_x/x express the *relative experimental uncertainty* associated with the determination of x, while its standard deviation (σ_x) results from a combination of all systematic and statistical *errors*.

We observe that the fission tracks counting uncertainties in $N_i(U)$ and $N(U{-}s)$ are small (1.2% to 2%) because uncertainties arising from detection efficiency were cancelled out in the ratio $N_i(U)/N(U{-}s)$ (Eq. (4)). The masses of the bone samples and U standard samples were determined down to 4–6% [m_i(bone)] and 2–3% [$m(U{-}s)$] of accuracy, respectively. With all these data in Eq. (5), it was estimated that the *experimental uncertainties* σ_{C_i}/C_i for the Uranium concentrations (all bone samples) ranged from 5% to 8%.

3.1.3. Averaging and dispersion

Averaging the U concentration in the bones of animals at an age of t days provides the mean value

$$\overline{C}(t) = \frac{\sum_{i=1}^{n} C_i}{n}, \quad (n=5) \tag{6}$$

where the ***dispersion*** (δ) of this mean value is defined as

$$\delta^2 = \sum_{i=1}^{n} \frac{(C_i - \overline{C})^2}{n}, \quad (n=5) \tag{7}$$

The comparison between the *dispersion of the mean* (δ) and each of the *i*th *standard deviation* (σ_{C_i}) provides an important check of the internal consistency calculation. In fact,

(1) if $\sigma_{C_i} \approx \delta$, it is possible conclude that standard deviations were realistically estimated, and that no statistically significant data fluctuation could be observed.
(2) if $\sigma_{C_i} \ll \delta$, three possibilities come out: data fluctuation, underestimation of standard deviations or both.
(3) if $\sigma_{C_i} \gg \delta$, the only possible conclusion is that standard deviations were quite overestimated.

Results here obtained show that nearly all calculated *standard deviations* are similar to the corresponding *dispersion of the mean*, except for one data (metabolic in nature) fluctuation noted at $t=25$ days, as discussed below.

Finally, it should be emphasized that each data point shown in Figs. 1 and 2 corresponds to an averaging of samples taken from 5 animals per age. Since only averaging is our data handling procedure, it suffices to calculate the external standard deviation of the averaged values (see above). This simply is a conventional parametric statistics in the normal model (Caria, 2000).

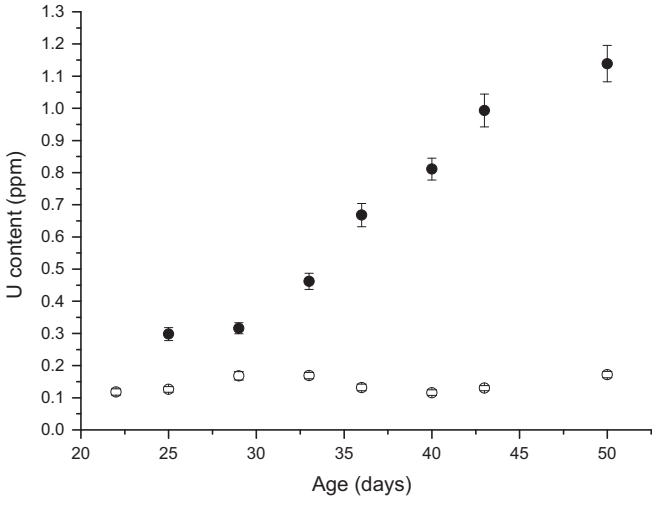

Fig. 1. Full circles: concentration of uranium in the femora of Wistar rats doped with uranyl nitrate expressed in parts per million (ppm), which is equivalent to μg/g, as function of the age. Open circles: the same as before for the control animals (fed with commercially available ration). See text for more details.

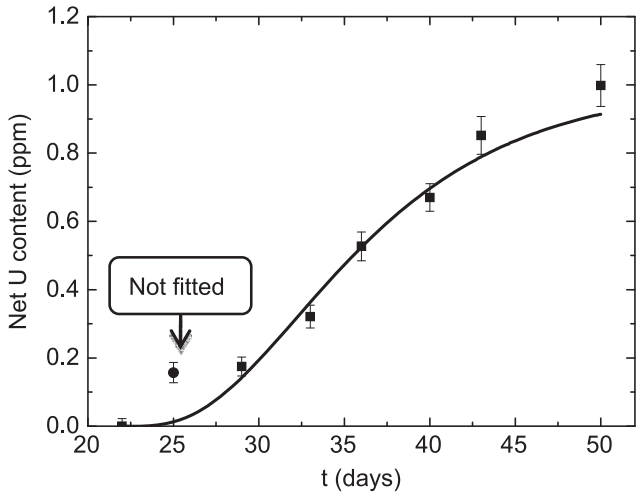

Fig. 2. Data points: net content of *U* in femora after subtraction of a background (see Fig. 1 and text for details). Full curve: fitting of Eq. (1) to the data points.

3.2. Uranium accumulation in bones

3.2.1. The amount of ingested uranium issue

The animals did not show weight loss. Weight gain in all animals during the whole period of the experiment was compatible with the normal development of Wistar rats. There were, however, minor transitory treatment effects ($P<0.05$) in body weight change; these were attributed to the normal fluctuations in weight within any group of animals and were not considered biologically significant or *U*-induced. These observations indicate that different animals in a group consumed nearly the same amount of food.

This circumstance is additionally reinforced by the fact that the *standard deviations* of Uranium concentrations in individual animals of a group were similar to the corresponding *dispersion of the mean*, demonstrating that no statistically significant data fluctuation could be observed (see Section 3.1. above).

We would like to emphasize that in this experiment, and in several other similar experiments so far conducted at our Laboratory with rats, Beagle dogs and Cobb broilers (Arruda-Neto et al., 2001, 2004a, 2004b, 2005), the animals were submitted to a long-lasting daily diet with uranium, initiated immediately after weaning and lasting till maturity. Therefore, contrary to experiments where animals are submitted to single doses, the measured quantities of Uranium in organs correspond to Uranium absorption rates integrated in a long period of time. Thus, possible fluctuations driven by metabolism and/or by food ingestion differences are smoothed out in time, while in single-dose experiments data smoothing is achieved by using a larger number of animals. This peculiarity of experiments carried out a this Laboratory can be assessed by a mere visual inspection of Uranium concentrations plotted as a function of time, where after a prolonged feeding period the amount of transferred radioactive material saturates. In this sense, the number of animals we have been using, in this and previous studies, proved to be adequate.

3.2.2. Concentration of uranium measured in femora

The results for the concentration of uranium measured in the animals femora were obtained as micrograms of uranium per gram of bone ashes (μg/g, or, U-ppm), and presented in Fig. 1 as a function of the animal age. These results correspond to the average of measurements obtained from five animals of the same age, and their uncertainties (standard deviations) all range between 4% and 11%. The average result for animals not submitted to the uranium diet, 0.14 ± 0.02 μg/g (ppm), is one order of magnitude lower than those for the older doped animals.

It is noted, on the one hand, that uranium concentrations in bones of the control animals are quite low, indicating that the commercially available ration used would contain very low levels of uranium too. This circumstance was further verified by direct measurement of uranium concentrations in ration samples, providing an average of (0.28 ± 0.03) ppm. On the other hand, for animals diet ration doped with 50 ppm of uranium, the concentration of this contaminant in their femora seems to increase faster in the early stages of the animal life then saturating in adult animals (Fig. 1). In fact, in an earlier experiment using Beagle dogs our group also found that the concentration of uranium in bones increases sharply with age in the early stages of the animal's life (Arruda-Neto et al., 2004b).

3.3. Age dependence of uranium accumulation

Results for the doped animals, presented in Fig. 1, have been reproduced in Fig. 2 after subtraction of a background consisting of the average *U* concentration measured in control animals (0.14 ± 0.02 ppm). Considering the likely possibility that uranium mimics calcium, as suggested elsewhere two decades ago (Priest and Van De Vyverm, 1990), the data points in Fig. 2 should exhibit a sigmoid-like trend, as in skeleton growing curves. In this sense, these data were fitted with a sigmoid function as given by West et al. (2001),

$$C(t)=C_{\infty}[1-e^{-\lambda(t-22)}]^4 \tag{8}$$

where C_{∞} is the *asymptotic concentration* (for high longevity animals) and λ is the fitting parameter.

However, the data point at $t=25$ d was excluded from the fitted data set. It probably constitutes a metabolic fluctuation driven by *U* intake immediately after the animals weaning phase. It is very likely that in this phase young animals are often in positive uranium balance (between uptake and excretion) due to a build up of uranium in the growing skeleton (Tandon et al., 1998). As a result, an excellent fitting of the sigmoid (Eq. (8)) to the experimental data was achieved, providing $C_{\infty}=1.00\pm0.02$ ppm and $\lambda=0.136\pm0.004$ d^{-1}.

The inflexion point (t_{infl}) is a root of the second derivative of $C(t)$; in other words, it is a solution of

$$d^2C(t)/dt^2=0 \tag{9}$$

providing (since $\lambda=0.136$)

$$t_{infl}=\ln 4/\lambda+22=32.2 \text{ d}.$$

The fact that *U* concentration data is fitted by a sigmoid indicates that *U* incorporation in the skeleton is similar to the growing process, where $t_{infl}=32.2$ d separates the anabolic from the catabolic Uranium accumulation phases. Regarding these two growing phases, in Fig. 3, the ratio M_C/M_D as a function of the animals' age was plotted, where M_C and M_D represent the weights of the calcinated and dry bone samples, respectively. Interestingly, it is noted that the average content of non volatile compounds in femora is 27% and 50% of the dry weight of animals younger and older than t_{infl}, respectively.

Regarding the alleged uranium mimicry of calcium, as discussed above, we note that the age dependence of environmental uranium concentration in the skeleton was investigated by Larivière et al. (2007) using human vertebrae bone, and for ages up to 65 years. Quite revealing, they found out that the profile of uranium was comparable to the calcium *turnover* rate (that is, the exchange rate of calcium in bone). This correlation between calcium *turnover* rate and uranium concentration in bones strongly suggests that uranium deposition is driven, at least in part, by this

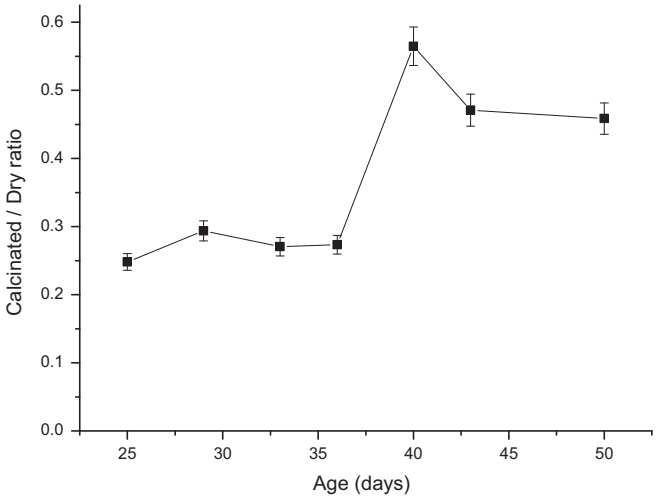

Fig. 3. Ratio of calcinated to dry weights as a function of the animals' age. The lines are only to guide the eyes.

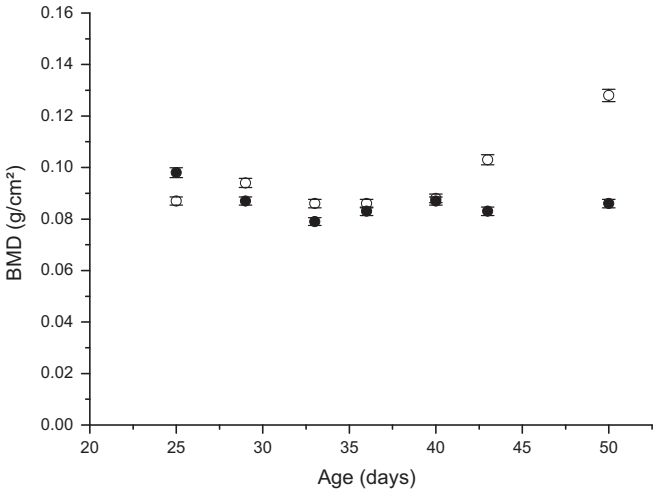

Fig. 4. Full circles: bone mineral density (BMD) measured in treated animals. Open circles: the same as before for control animals.

biological process (in this sense, Fig. 3 in Larivière et al. (2007) is very pedagogical).

3.4. Bone mineral density—Possible U deleterious effect

Results obtained from the analysis of bone mineral density (BMD) revealed that in animals younger than 40 days no significant changes in the BMD were verified in comparison with control animals (Fig. 4). On the other hand, in two groups of older animals, 43 and 50 days old, treated with *U* during a period of 21 and 28 days, BMD decreased by 20% and 33% (standard deviation smaller than 2%), respectively. This could be an indication that the *U* deleterious effect on bone should only be observed in prolonged contamination or in animals with bone formation already completed.

Results obtained from the control group exhibited a discrete BMD increase for older animals, an expected consequence of the animal growth. For the treated older animals, on the contrary, an average decrease of 40% was observed. As argued below, these results suggest that the radiation emitted by *U* could induce death of bone cells, thereby decreasing their population.

In fact, Fukuda et al. (2006) have carried out recently an experiment with Wistar rats intramuscularly injected with depleted Uranium. Depleted Uranium (DU) was used because the goal of that work was the study of Uranium toxicity only. Comparatively to natural Uranium, the radioactivity of DU is quite small. The bone mineral density (BMD) was measured in tibia by peripheral quantitative computed tomography (pQCT). Changes in total BMD and trabecular BMD that could be induced by DU were not observed, thus indicating that the heavy metal toxicity of Uranium is not effective in this regard (BMD changes). On the other hand, it was found that DU inhibits bone formation or calcification and accelerates bone resorption.

4. Conclusions

1. The concentration of Uranium in the animals' femora increases faster in the early stages of the animal life then saturating in adult animals.
2. The concentration of Uranium as a function of the animals' age was fitted by a sigmoid curve, another compelling evidence that uranium mimics calcium metabolism.
3. The inflexion of the fitted sigmoid curve indicates that transition from the anabolic to catabolic uranium accumulation phases occurs when animals are approximately 32 days old.
4. The results from BMD suggest that the radiation emitted by the incorporated *U* could induce death of bone cells, thus decreasing their population.

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