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USE OF FLUORIDE FOR THE MEASUREMENT OF BONE REMODELLING IN RATS AND HUMAN BEINGS

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ABSTRACT: We examined a new method, based on the retention of fluoride (F), for estimating bone remodelling in rats and human beings. We tested the validity of the new methodology by comparing normal Sprague Dawley rats (Controls, n=20) to ovariectomized rats with high bone remodelling (OVX, n=20). We found that the mean±SD for both bone formation (*BF*) and bone resorption (*BR*) in the OVX rats were significantly higher (p<0.05, Student's t-test) than in the Controls (Controls *BF*=10.28±4.54, OVX *BF*=20.33±19.00; Controls *BR*=8.18±4.86, OVX *BR*=18.41±17.93 nmole F/min). The new method was also tested in human volunteers (n=10) and found not to have any side effects. Three of the advantages of the new technique, in comparison to the use of bone remodelling markers, were: (i) *BF* and *BR* can be measured simultaneously, (ii) the real rates of *BF* and *BR* are measured, and (iii) it is a low cost technique. The new method was validated and found to be useful for estimating bone remodelling in both rats and human beings.

Key words: Bone marker; Bone remodelling; Mathematical model; Pharmacokinetics; Rat.

INTRODUCTION

A previous methodology was developed in order to study bone remodelling in rats, by measuring the retention of fluoride, that involved an intravenous injection of fluoride, plasma fluoride measurements, and several urine fluoride determinations.[1] This technique correlates with bone formation and resorption markers and has some advantages in comparison to bone remodelling markers: bone formation and bone resorption can be measured simultaneously, the real rates of bone formation and resorption are measured, and it is a low cost technique. However, it has several disadvantages such as, anaesthesia, blood samples, multiple urinary samples collection, and intravenous fluoride dose. Consequently that technique was not useful for bone remodelling measurement in human beings, because intravenous injection of fluoride is not advisable in humans. However, as fluoride pharmacokinetics has been exhaustively studied in humans and rats,[2] a modification of the mentioned technique was done to avoid intravenous injection of fluoride and to overcome the mentioned disadvantages. The simplification done to the previous method results in several advantages such as: administration of an oral dose of fluoride, two urinary samples, the simultaneous measurement of both processes: bone formation (*BF*) and bone resorption (*BR*). As fluoride is a common component of water and foods, a correction for such ingestion has been introduced in the new model. Additionally, software has been designed to facilitate the calculation of *BF* and *BR*. These advantages allow this method to be used in human beings in contrast to the previously published method which was suitable for rats but not humans.

Mathematical model: The entire mathematical model developed and software for *BF* and *BR* calculation is available from http://hdl.handle.net/2133/10456.

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The model uses the following two equations to obtain the bone formation rate (*BF*) and the bone resorption rate (*BR*)**:**

$$BF = \frac{F_1 * V_1}{t_1} * \left(\frac{D}{\left(F_2 * V_2 - \frac{F_1 * V_1 * t_2}{t_1}\right)} - 1\right)$$ .....Equation 1

Where:

BF	=	bone formation rate (nmole/min)
F_1	=	urinary fluoride concentration (nmole/L) for urine collected during time t_1
V_1	=	urinary volume (L) of urine collected during time t_1
t_1	=	interval of time (min) during which V_1 is collected
F_2	=	urinary fluoride concentration (nmole/L) for urine collected during time t_2
V_2	=	urinary volume (L) of urine collected during time t_2
t_2	=	interval of time (min) during which V_2 is collected
D	=	oral dose of fluoride (nmole) administered at the end of t1 that determines the beginning of t_2

We advise a period of 24 hr for t_1 and t_2 in order to obtain reliable values of V_1, F_1 and V_2, F_2, respectively.

$$BR = \frac{F_1 * V_1}{t_1} * \frac{D}{\left(F_2 * V_2 - \frac{F_1 * V_1 * t_2}{t_1}\right)} - \frac{I}{t_2}$$ .....Equation 2

Where:

BR	=	bone resorption rate (nmole/min)
F_1	=	urinary fluoride concentration (nmole/L) for urine collected during time t_1
V_1	=	urinary volume (L) of urine collected during time t_1
t_1	=	interval of time (min) during which V_1 is collected
F_2	=	urinary fluoride concentration (nmole/L) for urine collected during time t_2
V_2	=	urinary volume (L) of urine collected during time t_2
t_2	=	interval of time (min) during which V_2 is collected
D	=	oral dose of fluoride (nmole) administered at the end of t1 that determines the beginning of t_2
I	=	oral dose of fluoride ingested in food and drinking water (nmole)

Example of BF and BR in rats:

1. A rat was housed in an individual metabolic cage, where water consumption and urine excretion were measured. Urine was collected and fluoride concentration determined. The values obtained were: t_1= 1530 minutes, V_1= 0.016 L, and F_1= 309113 nmole/L.

2. After t_1 a dose of fluoride was administered at a dosage of 20,000 nmole NaF/ 100 g body weight. The rat weighed 368 g and therefore D= 73,600 nmole.

3. After the dose the rat was housed again in the individual metabolic cage and the values obtained were: t_2= 1380 minutes, V_2= 0.017 L, and F_2= 1771203 nmole/L.

4. The I value was calculated using the fluoride concentration in the drinking water, so I= 634 nmole.

5. All the values obtained, were replaced into Equations 1 and 2, or in the software, and *BR* and *BF* obtained: *BR*= 8.81 nmole/min and *BF*= 6.04 nmole/min.

Procedure to obtain BR and BF of humans beings:

1. The patient must collect a 24 hour-urinary sample. So t_1= 1,440 minutes and the concentration of fluoride in urine is measured, F_1.

2. A known dose of fluoride is administered to the patient, which is a pharmacological dose (D). A dose of 4.7 mg is advised.

3. The patient must collect a 24 hour-urinary sample (t_2= 1,440 minutes) after the dose and fluoride urine concentration is measured (F_2).

The I value may be calculated by considering the food and the source of the drinking water consumed by the patient. This might be done with a questionnaire and the published data on the fluoride concentration in food. The developed software has a tool to estimate I which is available from: http://hdl.handle.net/2133/10456.

MATERIALS AND METHODS

Methodology validation: The validation of the methodology was performed in experimental models of rats with different bone remodeling. Bone formation (*BF*) and bone resorption (*BR*) were measured in two groups of Sprague Dawley rats with different bone remodelling: (i) Controls and (ii) ovariectomized rats (OVX), an accepted model of high bone remodelling status. Experiments were carried out in 70-day-old female Sprague Dawley rats, 20 rats per group, with an average body weight of 211±26 g. Rats were housed in collective cages with water and balanced food (Gepsa, Pilar, Córdoba, Argentina) *ad libitum*. During the experiments, rats were kept in a temperature-controlled environment of 23–25ºC, with 12hr–12hr light-dark cycles and filtered airflow at scheduled time intervals. Rats were treated according to the accepted international standards for animal care.[3] Ovariectomy produces an estrogen deficiency and is therefore an accepted model of postmenopausal osteoporosis characterized by high bone remodelling. Bilateral ovariectomy was performed to one group of rats and the success of the surgery was verified at the end of the experiment by comparing the decrease in weight of the uteri to that of the controls.[4] Urinary fluoride concentration was measured with an ion-selective electrode ORION 94-09.[5]

The *BF* and *BR* measurements were made 30 days after the surgery. In these experiments the values used were: t_1= 24 hr, t_2=24 hr, and D=20,000 nmole NaF/100 g body weight (bw). D=20,000 nmole NaF/100 g bw does not have deleterious effect on growth and other biological variables after 30 days of oral administration.[6]

Human beings application: The method was applied in 10 volunteer human beings, 5 women and 5 men. All were normal adults between 21 and 55 years old who had read and signed an informed consent form. In this test the values used were: t_1= 24 hr, t_2=24 hr, and D=250,000 nmole. The dose ($250{,}000 \times 19 \times 10^{-9}$ =4.75 mg) is nearly 500 times lower than the estimated lethal dose in human beings[7] and only 4 times higher than a normal daily intake of fluoride from fluoridated water as set by World Health Organization.

Statistical analysis and software development: R 3.2.3 software was used in the analysis of results.[8] Significant differences were considered to be present when $p<0.05$.

Software was used for the *BF* and *BR* calculations and is available from: http://hdl.handle.net/2133/10456.

Ethical approval: The ethical committee of the School of Medicine of Rosario National University approved this work.

RESULTS

Measurement of BF and BR in rats: BR and *BF* of normal rats (Controls) were compared to rats with augmented bone remodelling state, ovariectomized rats (OVX) (Table 1).

Table 1. Bone formation (*BF*) and bone resorption (*BR*) values (nmole/min) of rats with normal (controls) and high bone remodelling status (OVX). Data are expressed as mean±SD.

Group	*BF* (nmole/min)	*BR* (nmole/min)	n
Control	10.28±4.54	8.18±4.86	20
OVX	20.33±19.00 *	18.41±17.93 *	20

*Indicates the presence of a significant difference compared to the controls, using the Student's t-test, of $p<0.05$.

BF and *BR* values of the OVX group were higher than the control group as expected, Student's t-test, $p<0.05$.

Although values of *BF* and *BR* do not achieve the same values as in the previous work, the threshold set by the ROC curve done in the other work corresponds with both groups.

A significant correlation between *BF* and *BR* confirms the coupling between both processes as literature predicts, correlation coefficient: 0.97 (correlation test, $p<0.05$).

Measurement of BF and BR in human beings: The technique was performed in voluntary normal human beings and no side effects were reported, (Table 2).

Table 2. Bone formation (*BF*) and bone resorption (*BR*) values (nmole/min) volunteers human beings. Data are expressed as mean±SD.

Group	*BF* (nmole/min)	*BR* (nmole/min)	n
Men	157.65±121.7	165.22±127.8	5
Women	74.31±58.4	78.76±60.6	5

*Indicates the presence of a significant difference compared to the controls, using the Student's t-test, of $p<0.05$.

DISCUSSION

In conclusion, in this work we develop a simplified methodology to measure bone remodelling in rats based on a previously published technique.[1] The fluoride dose we use to measure bone remodelling is similar to the dose used for osteoporosis treatment.[9] Although, the dose used (4.5 mg) is higher than the dose (1.5 mg) contained in 1 L of drinking water with a fluoride level at the upper limit recommended by the WHO (1.5 mg/L), it is administered only once.[10,11]

It is important to highlight that, with the simplifications introduced in this method (an oral dose of fluoride and the collection of two 24-hour urine samples), the suitability of applying the method to human beings is immediately apparent because we eliminate the disadvantages of the previous one. There is no need to use anaesthesia, blood samples, or an intravenous dose of fluoride.

CONFLICT OF INTEREST STATEMENT

All authors have no conflicts of interest.

ETHICAL APPROVAL

All the procedures performed our studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All the applicable institutional and/or national guidelines for the care and use of animals were followed.

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