Article

The use of mouse ribs in organ culture improves the *in vitro* bone resorption assay

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Summary

This study investigated *in vitro* bone resorption determining the calcium release in ribs, long bone and calvaria prelabelled with ^{45}Ca from 17 day mouse fetuses, both in the absence and in the presence of specific stimuli, such as parathyroid hormone and calcitonin. 10^{-7} M rat parathyroid hormone (1-34) (rPTH (1-34)) stimulated bone resorption (evaluated through the ratio treated ribs/control ribs) in 93% of the organ cultures, while lower success rate was obtained using calvaria and long bone from the same animals. In the absence of test substances, no differences were observed in paired ribs from the same fetus, while corresponding ribs from different fetuses showed considerable differences. Within every single hemithorax, bone resorption varies according to the rib position either in control ribs, or in those ones treated with rPTH (1-34). In the presence of rPTH (1-34), bone resorption showed to be dose-dependent producing a maximal response at 10^{-6} M, a minimal response at 10^{-8} M and a half-maximal response at $5x10^{-8}$ M. Salmon calcitonin (sCT) had no effect upon basal resorption, while it acted as a potent inhibiting agent in PTH response. The conspicuous number of samples which can be obtained from a relatively low number of mice, the reproducibility of results, together with the hormone sensitivity make the fetal mouse rib model an excellent tool for evaluating bone resorption *in vitro*.

KEY WORDS: bone resorption, calcitonin, calcium release, parathyroid hormone, ribs

Introduction

Several *in vitro* models have been developed to evaluate the bone remodeling process both in normal as well as in pathological conditions (1,2). Usually, the effects of hormones and other substances on bone resorption are quantified by analyzing the mobilization of minerals from bones, either following the release of ^{45}Ca from prelabeled bones (3-6), or by determining the release of stable calcium and inorganic phosphate (7). Four main experimental systems are currently used in different laboratories: 1) fetal rat long bones (radius and ulna) in stationary cultures (3); 2) calvarial bones from mouse (5,8) and rat fetuses (9) in stationary cultures, from mouse newborns both in stationary cultures (10-13) and in roller tubes (14), and from adult rats (15); 3) neonatal mouse vertebrae (16) and 4) bone fragments incubated with isolated osteoclasts (17).
However, interpretation of the results obtained has been hampered by the considerable variability of calcium release observed in control cultures. This phenomenon may reflect differences in the rate of bone resorption in the animals at the time of dissection, possibly caused by variable hormone concentrations retained by the explants (11). In a similar fashion, other, as yet unidentified factors could critically influence the variability of the basal resorptive activity. In order to reduce the error due to biological variability in spontaneous mineral mobilization, it would be necessary to increase the number of samples analyzed and this could be achieved using either more fetuses or more bones from the same fetus (i.e. vertebrae).
All these considerations prompted us to use ^{45}Ca prelabeled ribs from 17 day old mouse fetuses as a model to evaluate the *in vitro* resorptive activity of bone organ cultures.

Materials and methods

Fetal mouse rib assay

Pregnant Swiss CD-1 mice on the 15th day of gestation (Charles River, Calco, Italy) were injected with 40 µCi of $^{45}CaCl_2$ (Hamersham, Arlington Heights, IL, USA). The mothers were killed on the 17th day of gestation and the fetuses were removed with a binocular dissecting microscope, the thoraxes were divided into two parts and individual ribs were separated and divided into right and left group, the former used as control group, the latter as treated group. Each of the two groups was composed by 12 individual ribs, which were identified by a serial number ranging 1 to 12. After the mineralized shafts of the ribs were dissected free of surrounding tissue and cartilage, each of the paired ribs was incubated in single wells of 48-well plate (Costar, Cambridge, MA, USA) using Coon's modified HAM F12 medium with 5% calf serum (Gibco Brl, Gaithersburg, MD, USA) for 24 h at 37°C in humidified atmosphere of 5% CO_2 and 95% air in order to allow the exchange of loosely complexed ^{45}Ca. Paired ribs were then transferred into fresh medium with and without test substances [rPTH (1-34) and sCT (Bachem, Bubendorf, Switzerland)] for 72 h. At the end of the incubation time the media were analysed for ^{45}Ca concentration. Bones were dissolved in 300 µl of 2 N HCl for 4 h at 90°C and ^{45}Ca was evaluated. The radioactivity in the medium and bone extracts was counted separately by a liquid scintillation counter using INSTA-GEL scintillation fluid (Packard, Groningen, The Netherlands). Results were expressed as the treated/control ratio and calculated using the following formulae:

$$^{45}\text{Ca release (\%) from a single rib} = \frac{^{45}\text{Ca in medium}}{^{45}\text{Ca in medium} + ^{45}\text{Ca in bone}} \times 100$$

A treated (T) to control (C) ratio was calculated and used as an index of stimulation, a ratio greater than 1.08 representing an increase of bone resorption, a ratio smaller than 0.92 representing an inhibition of bone resorption. A range of bone resorption of ±0.08 has been chosen; this choice is based both on the statistical error, due to the total counts, and to a "casual systematic error", due to the manipulation procedures in the sample preparation. Assuming the same total counts for every ^{45}Ca determination, the statistical

$$\text{error is (at 1 SD): } \frac{\Delta\left(\frac{T}{C}\right)}{\frac{T}{C}} \approx 2x \frac{\Delta\,(\text{total count})}{\text{total count}} = 2x \frac{\sqrt{\text{total count}}}{\text{total count}}$$

Taking the equivalent of 2 SD we obtain 2x1%=2%. For the so called "systematic error" due to the manipulations in the sample preparation, a factor equal to 4 has been arbitrarily assumed. This gives 4x2%= 8%.
Every T/C ratio was calculated using ^{45}Ca releases (%) from ribs with the same serial number. Ribs with the same serial number were defined as corresponding ribs.

$$\text{T/C ratio} = \frac{^{45}\text{Ca release (\%) from the treated rib}}{^{45}\text{Ca release (\%) from the control rib}}$$

The mean T/C ratio for each experimental group of 12 pairs of corresponding ribs was calculated as the mean of the T/C ratios of the single pairs of corresponding ribs.
In the fetal mouse rib model the observations are represented by not independent sample, naturally paired samples (i.e. pairs of corresponding ribs). So the comparisons were always carried out between pairs of data, reducing partly some of the sources of biological variability and allowing a more exact comparison. Statistical analysis was tested by paired Student's t-test of the differences between treated and corresponding control rib.

Fetal mouse long bone assay

Bone resorption was measured as previously described (3), with some modifications. Briefly, pregnant mice were injected on 15th day of gestation with 40 µCi of $^{45}CaCl_2$. 48 hours later the shafts of the long bones were dissected out. Later, the long bones were treated in the same experimental conditions ad described in the fetal mouse rib assay. The paired long bones were used as control and as treated sample, respectively.

Fetal mouse calvaria assay

The bone-resorbing activity was measured as previously described (5), with some modifications. Briefly, pregnant mice were injected on 15th day with 40 µCi of $^{45}CaCl_2$. 48 hours later the calvaria were excised and divided into paired halves. Later, the half-calvaria were treated in the same experimental conditions as described in the fetal mouse rib assay. The paired half-calvaria were used as control and as treated sample, respectively.
All animal experimentations here described were conducted in accordance with the highest standards of humane animal care.

Results

The effect of 10^{-7} M rPTH (1-34) on bone resorption, measured as release of ^{45}Ca in treated compared to control bones, was evaluated in the fetal mouse rib, calvaria and long bone models as reported in Fig. 1. Results are organized in three groups: a) PTH-stimulated bone resorption greater than 8% of controls (T/C>1.08); b) PTH-stimulated bone resorption between -8% and +8% above control (0.92 <T/C <1.08); and c) PTH-stimulated bone resorption less than 8% below control (T/C < 0.92).The results showed that 47% of calvaria, 42% of humeri, 47% of ulnae, 40% of radii, 47% of femurs, 58% of tibiae show a T/C ratio >1.08. Conversely, when the effect of 10-7 M rPTH(1-34) was evaluated in the fetal mouse rib assay, 93% of ribs presented a T/C ratio >1.08 (Fig. 1).

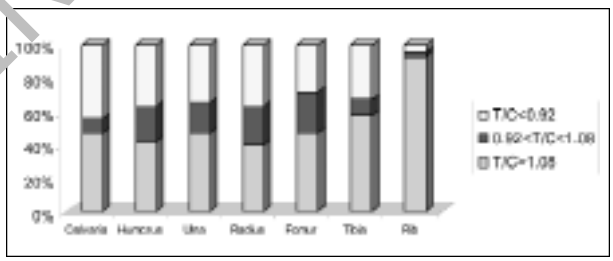

Figure 1 - Effect of 1 µM rPTH(1-34) on bone resorption in fetal mouse calvaria long bone and ribs.

Table I - Variability of basal values related to hemithorax origin.

	Total comparisons	Comparisons with no significant differences	Comparisons with significant differences 0.01<p<0.05	p<0.01
Comparisons between hemithoraxes from a same fetus[a]	20	20	–	–
Comparisons between hemithoraxes of different fetuses from a same pregnant mouse[b]	232	189	34	9
Comparisons between hemithoraxes of different fetuses from different pregnant mice[c]	528	326	67	135

The fetuses were obtained from 3 mothers: 6 fetuses from mother number 1, 6 fetuses from mother number 2, 8 fetuses from mother number 3. Every single comparison was carried out comparing of two hemithoraxes those ribs having the same serial number. Student's *t*-test for paired samples was used to evaluate the paired ribs' significant differences.
[a] Every hemithorax in one fetus was compared with the other one from the same fetus.
[b] Every hemithorax in one fetus was compared with every hemithorax from all the other fetuses from the same mother.
[c] Every hemithorax in one fetus was compared with every hemithorax from all the fetuses from the other mothers.

The release of ^{45}Ca in the absence of tested substances in paired ribs of the same fetus did not show significant differences. In contrast, significant differences were observed in ^{45}Ca release from corresponding ribs from different fetuses, either from the same or different mothers (Table I).
When basal bone resorption was compared in ribs from a single hemithorax, significant differences were found with respect to the position of the rib (Fig. 2 A and B). Similarly, when the effects of two different doses of rPTH(1-34) were evaluated among ribs from a single hemithorax, the differences in bone resorption as a function of rib position were even more evident (Fig. 2 A and B).

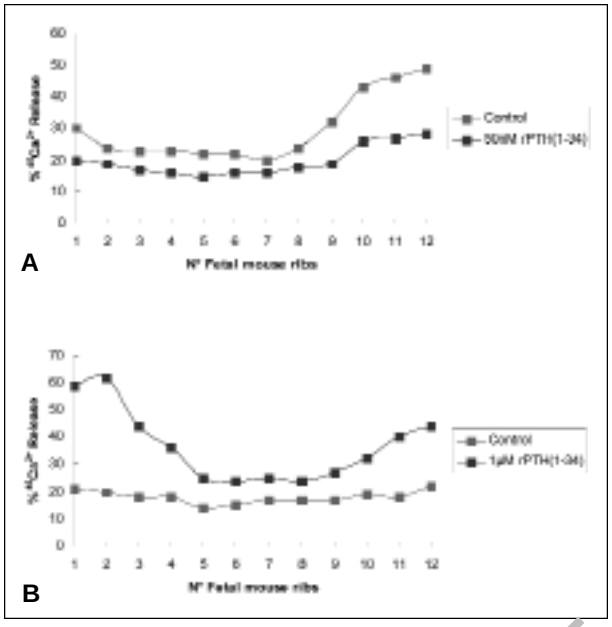

Figure 2 - ^{45}Ca percent release as function of serial number of the ribs in a control hemithorax and ^{45}Ca percent release as function of serial number of the ribs in a hemithorax after stimulus with $5x10^{-8}$ M rPTH(1-34) (A) and with 10^{-6} M rPTH(1-34) (B).

The effects of different concentrations of rPTH(1-34) on bone resorption of fetal mouse ribs were evaluated in paired ribs from 9 fetuses from the same mother. A dose dependent relationship of bone resorption was demonstrated, with maximal response at 10^{-6} M PTH, minimal detectable response at 10-9 M, and half-maximal response at $5x10^{-8}$ M (Fig. 3). Similar results were obtained with ribs of fetuses derived from 5 different mothers.
In fetal mouse ribs sCT had no effect on basal bone resorption at any of the doses tested from 10^{-11} M to 10^{-5} M. However, a potent inhibitory effect on PTH-stimulated bone resorption was evident at sCT concentrations between 10^{-10} M and 10^{-5} M (Fig. 4).

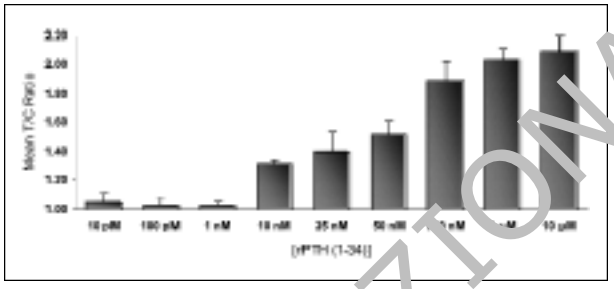

Figure 3 - Effect of different concentration of rPTH (1-34) on bone resorption of fetal mouse ribs.

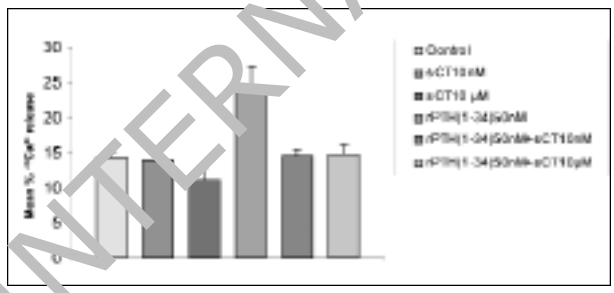

Figure 4 - Effect of 10 nM and 10 μM sCT on rPTH (1-34) stimulated bone resorption.

Discussion

In order to compare the results from the models described in the existing literature, with the one we describe in the present report, ^{45}Ca release from fetal mouse long bones, fetal mouse calvaria and fetal mouse ribs in fetuses from different mothers were compared.
The choice of fetuses instead of newborn mice is due to the fact that the sterile removal of ribs can be carried out only in fe-

Table II - Effect of 10^{-6} M rPTH(1-34) on bone resorption in fetal mouse calvaria, long bones and ribs.

Bone	Total cases	Cases with T/C>1.08	% Total	Cases with T/C>0.92 T/C<1.08	% Total	Cases with T/C<0.92	% Total
Calvaria	43	20	47	4	9	19	44
Humerus	43	18	42	9	21	16	37
Ulna	43	20	47	8	18	15	35
Radius	43	17	40	10	23	16	37
Femur	43	20	47	11	25	12	28
Tibia	43	25	58	4	9	14	33
Rib	276	257	93	8	3	11	4

Calvaria and long bones were prepared from 43 fetuses. Ribs were prepared from 23 fetuses.

Table III - Effects of different concentrations of rPTH(1-34) on bone resorption of fetal mouse ribs.

Concentration	Mean T/C Ratio	Significance
10^{-5} M	2.09	P<0.01
10^{-6} M	2.03	P<0.01
10^{-7} M	1.88	P<0.01
$5x10^{-8}$ M	1.51	P<0.01
$2.5x10^{-8}$ M	1.40	P<0.01
10^{-8} M	1.31	P<0.02
10^{-9} M	1.13	P<0.05
10^{-10} M	1.03	N.S.
10^{-11} M	1.05	N.S.

A dose-response curve from a representative experiment. Each mean T/C ratio was calculated as the mean of the T/C ratios of the 12 pairs of corresponding ribs. The significance was calculated using Student's *t*-test for paired samples.

Table IV - Effect of 10^{-8} M and 10^{-5} M sCT on rPTH(1-34) stimulated bone resorption.

Treatment	Mean % Release	Mean T/C Ratio	Significance
Control	14.39	0.97	N.S.
sCT 10^{-8} M	13.95		
Control	11.83	0.95	N.S.
sCT 10^{-5} M	11.27		
rPTH(1-34) $5x10^{-8}$ M	24.3	0.6	P<0.01
rPTH(1-34) $5x10^{-8}$ M + sCT 10^{-8} M	14.5		
rPTH(1-34) $5x10^{-8}$ M	28.41	0.64	P<0.01
rPTH(1-34) $5x10^{-8}$ M + sCT 10^{-5} M	18.07		

The mean % release was evaluated as mean of ^{45}Ca percent release of 12 ribs of a hemithorax. Each mean T/C ratio was calculated as the mean of the T/C ratios of the 12 pairs of corresponding ribs. The significance was calculated using Student's *t*-test for paired samples.

tuses. In fact, the vicinity of the ribs to the bronchial tree can cause contamination of the ribs removed from newborn mice.

PTH is a powerful stimulus of bone resorption (3,18). In our study, more than 90% of the ribs we processed showed a positive response to PTH stimulation, while this percentage lowered when the other bone models were analyzed. Our results put in evidence that ribs are a highly responsive model to evaluate the processes involved in bone resorption.

^{45}Ca release in control ribs, and after stimulation with 10^{-7} M rPTH (1-34), was evaluated as function of the fetus age. The best results were obtained using 17 day old fetuses (data not showed).

Comparing the ^{45}Ca release basal values obtained in the two hemithoraxes from the same fetus with those recorded with hemithoraxes from different fetuses of the same mother, the biological variability increased. This variability became drammatically higher when comparison was made between hemithoraxes explanted from fetuses of different mothers. These results may be probably due to the evident different embryo growth degree, despite the same duration of their gestation. To minimize the biological variability, in our experiments we always calculated T/C ratios using corresponding bones from the same fetus.

When bone resorption was stimulated by PTH, the group composed by ribs from 1 through 3 and that from 10 through 12 proved to be more responsive than that from 4 through 9. The significant differences taken in PTH response as to the rib position were probably due to the different degree of bone remodelling among the different ribs.

In our model the least concentration of PTH able to produce a significant increase in bone resorption was 10^{-9} M. This sensitivity to PTH was of the same magnitude order of those obtained with other experimental models (4,11,16,18).

In mouse calvarial bone resorption assay, a potent inhibitory effect of sCT on basal level of ^{45}Ca release has been demonstrated (3,12,15). In our experiments sCT failed to inhibit basal level of bone resorption, but it was very effective in inhibiting the effects of PTH , in accordance with results in other models (3, 16, 19).

It is important to note that from a technical viewpoint rib explants do not involve more difficulties than those encountered in the long bone organ culture models.

In conclusion, the model we propose represents a real improvement in the *in vitro* bone resorption assay when compared with those previously used, making possible to reduce the number of animal sacrificed and to obtain more reproducible data.

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