Title: Bone mass recovery of osteopenic-vitamin D insufficient rats from strontium ranelate treatment: Does the response depend on vitamin D nutritional status or on source of vitamin D (D2 vs. D3)?

Article Type: Full Paper

Keywords: strontium ranelate; vitamin D; rats; bone markers

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Abstract: It was investigated if vitamin D (vitD) status or source (D2 vs. D3) interferes with bone mass recovery from strontium ranelate (SrRa) treatment of rats with vitD insufficiency and established osteopenia. Osteopenic and vitD insufficient rats were divided in groups to complete a 105-day period. 1st experiment: they were fed diets that only varied in vitD (100 vs. 0 IU%) and/or SrRa (0 vs. 900mg/kg/day) content. A SHAM group received vit D throughout the experience. 2nd experiment: rats were divided to receive vitD2 or vitD3 by diet and SrRa by gavages in a fasting state. Two SHAM groups received vitD2 or vitD3 throughout the study. Results: Levels of 25OHD were reduced in groups lacking dietary vitD (p<0.001). Independently of vitD status or source, SrRa did not affect body weight gain or bone alkaline phosphatase levels; osteocalcin and C-terminal telopeptide of type I collagen levels were reduced (p<0.05) and bone Sr content was increased (p<0.0001), although no improvement in biomechanical parameters was observed; total skeleton bone mineral content and proximal tibial bone mineral density were increased (p<0.05); there was a reduction in the trabecular number and, an increase in the trabecular surface and bone volume without reaching SHAM levels. Conclusion: This is the first study which examined SrRa effects in an osteopenic vitamin D-insufficient experimental model. Under our experimental condition, SrRa increased bone Sr content independently of vitD status or source, however no evidence of an anabolic or anti-fracture effect was found and only a slight decrease in some bone resorption parameters were observed.

Response to Reviewers: Reviewer #1:

 Comment:
1. Adding clearly the number of rats in total and in each group under study. These should be listed in page 4.

Response:

In agreement with the reviewer the number of rats in total and in each group under study was added in material and methods.
Pag 4:

1st. paragraph: "A total of eighty rats….."
2nd. Paragraph: "Subsequently rats (n=8) were…."
Last paragraph: "The 32 OVX…."

Comment:
2. The significant statistic results should be put at each table. This may facilitate the understanding of the readers.

Response:
In agreement with reviewers 1 and 2 the significant statistic results were changed and added to the table.

Comment:
3. How about the effect of Sr compound to the cancellous bone? This seemed not well described.

Response:

The results related to cancellous bone were described as follows:
1.- By histology of the middle third of the tibia: bone volume and the number of trabeculae of decalcified sections
2.- By densitometry of the proximal tibia BMD.
The results related to cortical bone were described by the biomechanical properties.

Reviewer #2:
Comment
1. There are several small grammatical errors seen especially in the section of abstract and the title of this paper should be revised by a native English speaker.

Response:

In agreement with the reviewer the title and the abstract were seen by a native English speaker.
Title: "Bone mass recovery of osteopenic-vitamin D insufficient rats from strontium ranelate treatment: Does the response depend on vitamin D nutritional status or on source of vitamin D (D2 vs. D3)? "
Abstract: "It was investigated if vitamin D (vitD) status or source (D2 vs. D3) interferes with bone mass recovery from strontium ranelate (SrRa) treatment of rats with vitD insufficiency and established osteopenia. Osteopenic and vitD insufficient rats were divided in groups to complete a 105-day period. 1st experiment: they were fed diets that only varied in vitD (100 vs. 0 IU%) and/or SrRa (0 vs. 900mg/kg/day) content. A SHAM group received vit D throughout the experience. 2nd experiment: rats were divided to receive vitD2 or vitD3 by diet and SrRa by gavages in a fasting state. Two SHAM groups received vitD2 or vitD3 throughout the study. Results: Levels of 25OHD were reduced in groups lacking dietary vitD (p<0.001). Independently of vitD status or source, SrRa did not affect body weight

gain or bone alkaline phosphatase levels; osteocalcin and C-terminal telopeptide of type I collagen levels were reduced (p<0.05) and bone Sr content was increased (p<0.0001), although no improvement in biomechanical parameters was observed; total skeleton bone mineral content and proximal tibial bone mineral density were increased (p<0.05); there was a reduction in the trabecular number and, an increase in the trabecular surface and bone volume without reaching SHAM levels. Conclusion: This is the first study which examined SrRa effects in an osteopenic vitamin D-insufficient experimental model. Under our experimental condition, SrRa increased bone Sr content independently of vitD status or source, however no evidence of an anabolic or anti-fracture effect was found and only a slight decrease in some bone resorption parameters were observed"

Comment

2. page 4 and 5; In experiment 1 and experiment 2. Why did the authors change the way of feeding SrRa from diet to gavage? Did the authors find that the animals did not take the accurate dose of SrRa through foods in the first experiment?

Response:
We conducted two experiments in order to deal with the different issues mentioned in the literature related to SrRa treatment but in different models from our model which is closer to clinical reality.
In the first experiment, the SrRa was mixed in the diet. This is the commonly used form in most experiments and we are sure that the animal took the accurate dose of SrRa.
In the second experiment, we changed the way of supplying SrRa to resemble that which is used in clinical practice: in "a fasting state" by dissolving the granulated drug in water and away from food.

Comment

3. page 4 ;experiment 1: Why did the authors use vitamin D2 instead of D3 in the first experiment? If the authors had intended to examine the effects of vitamin D, they should have examined the effects of vitamin D2 and D3 in the experiment 1.

Response:
The first experiment was something "like a general study", to compare the effect of SrRa in hipovitaminosis vs. normal vitamin D status. We used D2 instead D3 in the first experiment because according to the American Institute of Nutrition (1993 modified in 1997) the optimal rodent diet should contain 100UIVitamin D, without specifying the source of vit D. In our nutritional experiments, we have been regularly D2.
In the second experiment, we explored the possible differences between D2 and D3 because according to several authors D3 is more effective than D2 to rise and to maintain the levels of 25OHD

Comment:

4. page 7 line 14 to 16: In terms of body weight, more detailed explanation is also provided in the next paragraph. The authors should avoid redundant writings.

Response:
In agreement with the reviewer we deleted redundant writings related to body weight:

Page 7: "Results"
Food intake and survival:
SrRa treatment mixed in the diet or by gavages was well-tolerated and safe and did not have effects on animal survival and food consumption.
Experiment 1:
Body weight:
The mean BW for all groups of rats at t=0, t=60 and t=105 are show in table 1. There were no significant differences in BW at baseline (t=0). As expected, the four OVX gained higher BW compared

to the SHAM group (p<0.0001) both, at t=60 and t=105. In addition, as SrRa treatment and/or vitamin D status did not affect BW changes, there were not differences in BW among the four OVX groups. "…..

Comment:
5. Tables: The authors tried to show statistical significance with different English letters, but I cannot identify where the statistical differences are. Please use other methods to show in tables where the significant differences exist.

Response
In agreement the reviewer the statistical differences were change by signs.

Bone mass recovery of osteopenic-vitamin D insufficient rats from strontium ranelate treatment: Does the response depend on vitamin D nutritional status or on source of vitamin D (D2 vs. D3)?

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Running title: Strontium ranelate effect on osteopenic-vitamin D insuficient rats

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Key words: Strontium Ranelate – osteopenic rats – vitamin D

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ABSTRACT:

It was **investigated** if vitamin D (vitD) status or source (D2 vs. D3) interferes with bone mass recovery from strontium ranelate (SrRa) treatment of rats with vitD insufficiency and established osteopenia. Osteopenic and vitD insufficient rats were divided in groups to complete a 105-day period. 1st experiment: they **were** fed diets that only varied in vitD (100 vs. 0 IU%) and/or SrRa (0 vs. 900mg/kg/day) content. A SHAM group received vit D throughout the experience. 2nd experiment: rats were divided to receive vitD2 or vitD3 **by diet** and SrRa **by gavages** in a fasting state. Two SHAM groups received vitD2 or vitD3 throughout the study. Results: Levels of 25OHD were reduced in groups lacking **dietary** vitD (p<0.001). Independently of vitD status or source, SrRa did not affect body weight gain or bone alkaline phosphatase levels; osteocalcin and C-terminal telopeptide of type I collagen **levels** were reduced (p<0.05) and bone Sr content was increased (p<0.0001), although no improvement in biomechanical parameters **was** observed; total skeleton bone mineral content and proximal **tibial** bone mineral density were increased (p<0.05); there was a reduction in **the** trabecular number and, an increase in **the** trabecular surface and bone volume without reaching SHAM levels. Conclusion: This is the first study which examined SrRa effects in an osteopenic vitamin D-insufficient experimental model. Under our experimental condition, SrRa increased bone Sr content independently of vitD status or source, however no evidence of an anabolic or anti-fracture effect was found and only a slight **decrease** in some bone resorption **parameters were** observed.

INTRODUCCION

In-vitro studies indicate that strontium ranelate (SrRa) acts as an antiresortive drug by reducing osteoclast activity (1) and by increasing the apoptosis in isolated rabbit osteoclasts (2). There is some evidence that SrRa could also induce positive effects on bone formation (3,4. In rat calvaria organ culture, SrRa enhanced the replication of preosteoblastic cells, and, consequently, the activity of functional cells and bone matrix synthesis. Short-term studies in intact rats have shown similar effects measured by histomorphometry. These rats presented increased bone formation indices, number of trabeculae and amount of osteoid with decreased bone resorption parameters (5,6). However, long-term effects of SrRa on bone parameters in ovariectomized (OVX) rats have been controversial. Ammann et al (7) and Bain S et al (8-9) found that the administration of SrRa immediately after the procedure increased bone resistance to fracture by increasing bone mass and improving bony architecture and stiffness. Conversely, Fuchs RK et al (10) demonstrated that SrRa administered in the same conditions did not stimulate an anabolic bone response, and failed to improve the bone biomechanical properties of OVX rats.

Estrogen withdrawl induces an increment in bone remodelling which lead to trabecular bone loss (11,12). In this regard, the OVX rat is the most commonly used model to study bone loss associated to estrogen deficiency (11). We previously found that the OVX Wistar rat lose approximately 20% of their total body bone mineral content (BMC) becoming osteopenic 40 days after surgery (13). According to our knowledge, there are no experimental reports addressing the effect of SrRa treatment on bone formation and bone resorption parameters of osteopenic animals. Therefore, the present report assessed the bone recovery of OVX rats with established osteopenia by SrRa treatment. Since vitamin D (vit D) status (i.e. normal or insufficient) affects bone mass recovery with bisphosphonate therapy in osteopenic OVX rats (13) we also evaluated the role of vit D status and/or the type of vit treatment (D2 vs. D3) of these osteopenic animals given affected SrRa either on the diet or by gave in a fasting state.

MATERIAL AND METHODS:

Animals:

Three-month-old virgin female Wistar rats (300-350 g) were obtained from our laboratory (General and Oral biochemistry Department, School of Dentistry, Buenos Aires University). The animals were housed at room temperature (21± 1ºC) with 55% ±10% relative humidity under 12-hour light/dark cycles. Food and deionised water were supplied "ad libitum" throughout the experiments. Body weight (BW) and food consumption were assessed in the morning three times per week. Rats were maintained in keeping with the National Institutes of Health Guide for the Care and Use of Laboratory. The protocols for the present studies were approved by the Buenos Aires University Institutional Review Board.

A total of eighty rats were randomly assigned to undergo bilateral OVX or a SHAM operation by a dorsal approach. Surgery was performed under anesthesia (0.1 mg/100 g body weight (BW) ketamine hydrochloride and 0.1 mg/100 g bw acepromazine maleate) (Holliday Scott SA, Buenos Aires, Argentina). Ovariectomy was confirmed at necropsy by the uterine horn atrophy.

Experiment 1:

The first 15 days post surgery, the **32** OVX rats were fed a rodent commercial diet (Granave SA, Bs. As. Argentina) followed by an additional 45-day period of feeding a semisynthetic diet that cover all nutritional recommendations according to the American Institute of Nutrition (AIN) (14) but free of vitamin D (0 IU%). According to our previous study, in such conditions OVX rats become osteopenic and vitamin D insufficient (13). Subsequently rats **(n=8)** were divided into four experimental groups, each given specific diets and treatments for 45 additional days in order to complete a 105-day period, as described below:

Vitamin D insufficiency:

OVX-D: OVX rats continued feeding the AIN diet without vit D.

OVX-D+SrRa: OVX rats continued feeding the AIN diet without vit D which was mixed with 900mg/kg/day of SrRa (Protelos 2 g, Servier, Argentine). According to previous studies, this dose of SrRa has been shown to prevent OVX-induced bone loss and to improve bone resistance (6, 14).

Vitamin D sufficiency:

OVX+D2: rats were fed the AIN diet containing 100IU% vitamin D2

OVX+D2+SrRa: rats were fed the AIN diet containing 100IU% vitamin D2 mixed with SrRa as mentioned above.

Control group:

Normal age related changes were determined in SHAM rats. These animals were kept as controls to be compared with the experimental groups of the same age. After the 15-days of rodent diet they received the AIN 93 diet containing vitamin D2 till the end of the experiment, as the experimental groups.

Experimet 2:

This experiment was designed to evaluate the potential differences between D2 and D3 and the manner utilized to administer SrRa.

The **32** OVX rats underwent the procedure ante mentioned during the first 60 days of experience to become osteopenic and vitamin D insufficient. Then, they were divided into

four groups of 8 animals each and treated as follow for 45 additional days in order to complete a 105-day period:

OVX+D2: rats were fed the AIN 93 diet containing 100IU% of vitamin D2.

OVX+D3: rats were fed the AIN 93 diet containing 100IU% of vitamin D3

OVX+D2+SrRa: rats were fed the AIN 93 diet containing 100IU% of vitamin D2 and received 900 mg/kg/day of SrRa by gavages in a fasting state.

OVX+D3+SrRa: rats were fed the AIN 93 diet containing 100IU% of vitamin D3 and received 900 mg/kg/day of SrRa by gavages in a fasting state.

Control SHAM rats:

SHAM rats were divided in two groups. After the 15-days of rodent diet, one of the groups was fed the AIN 93 diet containing vitamin D2 and the other the AIN 93 diet containing vitamin D3 till the end of the experience.

Biochemical determinations

Fasting blood samples were collected at baseline ($t = 0$), at treatment onset ($t = 60$) and at the end of the experimental period ($t = 105$). Levels of calcium (sCa), phosphate (sP), 25hydroxyvitamin D (25OHD), bone alkaline phosphatase (bALP), osteocalcine (BGP) and C-terminal telopeptide of type I collagen (CTX) were determined in the serum samples as previously described (13). In brief, sCa (mg/dl) was measured by atomic absorption spectrophotometry (AAS) employing Lanthanum Chloride as interference suppressor. The sP (mg/dl) was measured by colorimetric methods. Serum 25OHD was assayed by a competitive protein binding method (Diasorin, Minnesota) with an intra-assay coefficient variation (CV) of 9%. The b-ALP was measured using a colorimetric method (Boehringer Mannheim, Germany) after bone enzyme isoform precipitation with wheat-germ lectin. The BGP was measured by immunoassay (ELISA) (Rat-osteocalcine Osteometer BioTech, Herlev, Denmark), with a 6% intra-assay variation coefficient (CV). The CTX (ng/ml) was assessed employing an ELISA method (Rat-laps. Osteometer, BioTech, Herlev, Denmark), with a 6% intra-assay CV.

The left femur was removed at the end of the experiment and the Sr content determined. The femur was dehydrated at 100ºC for 24hs, weighed and calcinated at 600ºC till constant weight. Dry bone ashes were weighed and dissolved in 6N HCl and Sr content was measured by AAS employing potassium nitrate as ionization suppressor.

DXA measurements

Total skeleton bone mineral density (teBMD) and bone mineral content (teBMC) were determined "in vivo" under light anaesthesia using a total body scanner with software designed specifically for small animals (DPX Alpha 8034, Small Animal Softer, Lunar

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Radiation Corp., Madison, WI) at t=0, t=60 and t=105. The precision of the software was assessed by measuring one rat five times after repositioning between scans both on the same and on different days (13). The coefficient of variation (CV) was 0.9% for teBMD and 3.0% for teBMC. The different sub-areas were analyzed on the image of the animal on the screen, using a ROI for each segment. The BMD CV for the different studied areas was: 1.8% for lumbar spine (LS) and 3.5% for the proximal tibia (PT). All analyses were carried out by the same technician in order to minimize inter-observer variation.

Bone mechanical testing:

Between the different steps of preparation, each left tibia was kept immersed in physiological solution to avoid drying of the bone that could affect the biomechanical properties. The tibia was placed in the material testing machine on two supports separated by a distance of 20 mm, and the load was applied on the middle of the shaft, thus performing a three-point bending test. The mechanical resistance to failure was tested using a servo controlled electromechanical system (Instron 1114; Instron Corp., High Wycombe, UK) with the actuator displaced at 2 mm/minute. The load/displacement curve was recorded. Maximal load (expressed in N), stiffness (slope of the elastic part of the curve, expressed in N/mm), and energy (total energy absorbed, expressed in N.mm) were determined (15,16).

The yield point, indicating the transition between the elastic and plastic phases of the deformation, was determined on the load-deformation curve from the first series of experiments to calculate the elastic energy (area under the elastic part of the curve, expressed in N.mm) and the plastic energy (area under the plastic part of the curve, expressed in N.mm). All biomechanical evaluations were performed in a blind manner.

Histological determinations

At the time of sacrifice, the right tibia was removed and fixed by immersion in buffered formalin for 48 h, decalcified in 10% ethylene-diamine tetraacetic acid (EDTA) (pH 7) during 25 days and embedded in paraffin. Two 8- to 10-µm-thick longitudinally oriented sections of subchondral bone were obtained at the level of the middle third, including primary and secondary spongiosa. The sections were microphotographed (AXIOSKOP, Carl Zeiss) to perform histomorphometric measurements on the central area of the metaphyseal bone displayed on the digitalized image. The following static histomorphometric parameters were measured according to Parfitt et at (17): Bone volume fraction (BV/TV) (%): the percentage of cancellous bone within the total measured area; osteoblast surface (Ob.S/BS) (%): the fraction of trabecular bone surface covered with osteoblasts; osteoclast number (Oc.N/B.Ar): the number of osteoclasts in the total studied area; trabecular number (Tb.N, 1/mm); thickness (Tb.Th, um) and spacing (Tb.Sp, um)

To evaluate the presence of osteoid, the right femur was fixed in 10% phosphate-buffered formaldehyde. After dehydration, undecalcified bone samples were embedded in methyl methacrylate and longitudinal sections (5–7 um) were cut employing a Polycut microtome (Reichert Jung, Heidelberg, Germany). Bone sections were stained using the modified Masson's Trichrome staining technique (18).

Statistical methods

Results were expressed as mean ± standard error (SE). Differences between mean values in untreated, treated and control groups were evaluated using ANOVA for normal distribution and Kruskal-Wallis test for non parametric analysis (BV/TV). A Dunnett´s test was used as "a posteriori test" to find the differences among groups. Similarly, histological data were analyzed using a single time point factorial analysis. Statistical analyses were performed using SPSS for Windows 11.0 (SPSS, Inc., Chicago, IL). A value of P below 0.05 ($P < 0.05$) was considered significant.

RESULTS

Food intake and survival:

SrRa treatment mixed in the diet or by gavages was well-tolerated and safe and did not have effects on animal survival and food consumption.

Experiment 1:

Body weight:
The mean BW for all groups of rats at t=0, t=60 and t=105 are show in table 1. **There were no significant differences in BW at baseline (t=0). As expected,** the four OVX gained higher BW compared to the SHAM group (p<0.0001) both, at t=60 and t=105. In addition, as SrRa treatment and/or vitamin D status did not affect BW changes, there were not differences in BW among the four OVX groups.

Serum biochemical determinations:
The sCa and sP at t=60 and t=105 and 25OHD, BGP and CTX levels at t=105 are shown in table 2. There were no differences in the levels of these serum parameters at t=0 (data not shown). As expected, at the end of the experience, the levels of 25OHD were significantly reduced in the OVX group fed the vit D free diet (p<0.001). At t=60 the OVX groups had higher sCa and lower sP levels compared to SHAM group (p<0.05 and p<0.0001, respectively) without significant differences among them. At t=105 and independently of vit D status, sCa levels were similar in SrRa treated or untreated groups (p<0.01). Conversely, sP

levels were higher in SrRa treated vs. untreated rats but only reached significance in the Vit D insufficient group (p<0.01).

At t=105, the serum BGP and CTX levels of both OVX untreated groups were significantly higher as compared to SHAM levels (p<0.001 for both markers), independently of vitamin D status; without differences between them. The serum levels of BGP of OVX-SrRa-treated rats received or not Vit D were similar to those detected in the SHAM animals and were significantly lower compared to their correspondent OVX-untreated group (p<0.01). The serum CTX levels of OVX-SrRa treated rats given Vit D did not show significantly lower levels as compared to those of SHAM rats; but reached significance compared to their correspondent OVX-untreated groups (p<0.01). Regarding OVX-SrRa treated group deficient in Vit D, the CTX levels were significantly higher than the SHAM group (p<0.01) without differences compared to it OVX-untreated group (table 2).

The longitudinal serum b-ALP levels at different times of SrRa treatment are shown in Figure 1. Independently of vit D status, ovariectomy and SrRa treatment did not affect serum b-ALP levels throughout the study. A significant reduction in the levels of b-ALP was observed in all groups without differences among then at t=105.

Bone parameters:

The femur Sr content, the biomechanical and histological studied parameters, and the changes between t=105 and t=60 in teBMC and PTBMD are shown in table 3. OVX-SrRa treated groups, independently of vit D status, had a significantly higher femur Sr content than SHAM and OVX-SrRa untreated groups (p<0.0001).

The ovariectomy reduced the maximum load with a significant impact in OVX rats who did not receive dietary vit D (p<0.05). Independently of vit D status, the stiffness and the energy biomechanical parameters of OVX rats were significantly reduced compared to SHAM group (p<0.05). The SrRa treatment failed to modify these three biomechanical parameters which remained lower than in the SHAM group (p<0.05) (table 3).

Independently of vit D status, the ovariectomy reduced teBMC between t=105 and t=60 compared to SHAM group (p<0.05) reaching significance in the OVX-D group. Both OVX groups also had significantly reduced the PTBMD compared to SHAM group (p<0.05). Independently of Vit D status, the SrRa treatment has significantly increased these two densitometric parameters (p<0.05).

The histological findings of the OVX groups showed a reduction in BV/TV (p<0.0001) (figure 2) and in the number of trabeculae (figure 3 and table 3) compared to SHAM rats with a greater effect in the OVX-D group. SrRa treatment, independently of vitamin D status, improved the BV/TV (p<0.05) and trabeculae number as compared to OVX-SrRa untreated groups there was a tendency to be higher in the vit D-treated group, though the values were significantly lower than those of SHAM group (p<0.001). The increase of Ob.S/BS observed in OVX receiving vit D as compared to SHAM rats (p<0.05) was not significantly reduced by

SrRa treatment. In addition, no changes were observed in OVX-D SrRa-treated or untreated groups compared to SHAM rats. Ovariectomy significantly increased the Tb.Th vs. SHAM group (p<0.001), without differences detected by vit D status (table 3); the Tb.Sp (p<0.0001) was also significantly higher in OVX-D vs. OVX+D group (p<0.01). After 45 days of treatment, the SrRa treatment failed to improve these histological parameters in the OVX groups receiving or not vit D. Additionally, independently of vit D status, Oc.N/B.TA was significantly increased by ovariectomy and SrRa treatment reduced such increment only in the group receiving vit D (p<0.05). None of the studied groups exhibited osteoid (data not shown).

Experiment 2:

Body weight:

The mean BW for studied groups at t=0, 60 and 105 are shown in table 4. There were no significant differences in BW at baseline (t=0). As expected, at the beginning of treatment (t=60) and independently of the source of vit D, the OVX groups had higher BW compared to their SHAM group (p<0.0001); the SrRa treatment did not affect BW changes, consequently at the end of experiment (t=105) the four studied OVX groups had higher BW than their SHAM group (p<0.0001) without differences among them.

Serum biochemical determinations:

The sCa, 25OHD and BGP levels at t=60 and t= 105 and CTX levels at t=105 are shown in table 5. At baseline there were not differences in serum levels of these studied parameters (data not shown). Independently of the source of vit D, the sCa levels of SHAM groups were significantly lower compared to their OVX-treated or untreated groups (p<0.05), and without significant differences among them both, at t=60 and at t=105. As expected, 25OHD levels were significantly reduced at t=60, independently of vit D source, estrogenic status and SrRa treatment. In addition they were similar in all the studied groups at t=105.

The levels of BGP were significantly higher in OVX groups than in their correspondent vit D-SHAM group at t=60 (p<0.05). Independently of vit D type, SrRa-treatment reduced the BGP and CTX levels of the OVX-untreated groups (p<0.05) to the levels observed in their correspondent vit D-SHAM group and without differences between them (table 5).

Bone parameters:

The femur Sr content and the biomechanical studied parameters are depicted in table 6. Independently of vit D source, the OVX-SrRa treated groups had a significantly higher tibia Sr content than SHAM and OVX-SrRa untreated groups (p<0.0001). The OVX reduced (p<0.05) and the SrRa treatment failed to improve the stiffness and energy biomechanical parameters compared to SHAM group (p<0.05); only an slightly non significant increment in the maximal load was observed (table 6).

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The changes between t=105 and t=60 in teBMC, PTBMD and LSBMD are shown in table 6. Independently of vit D source, the ovariectomy significantly reduced such densitometric parameters compared to SHAM groups (p<0.01) whereas the SrRa exposure significantly increased these values above those of SHAM groups (p<0.01).

Independently of vit D source, the histological findings in OVX groups compared to their correspondent SHAM group (table 7) showed: a significant reduction in BV/TV (p<0.0001) and in the number of trabeculae. In contrast there was a significant increment in the trabecular separation and osteoclast number (p<0.05) without changes in trabecular thickness and osteoblast surface compared to their correspondent SHAM group. Independently of the vit D status, SrRa treated groups showed a significantly higher BV/TV and trabeculae number and a significantly lower Tb.Sp than their correspondent OVX untreated groups although without reaching SHAM values (p<0.05). Moreover, SrRa treatment significantly reduced the osteoblast surface compared to both, their correspondent OVX and SHAM groups (p<0.05) while the osteoclast surface was significantly reduced compared to their correspondent OVX group (p<0.05) reaching the values observed in their correspondent SHAM group (table 7).

DISCUSSION:

The results of the present study showed that, under our experimental conditions, SrRa treatment of OVX rats with established osteopenia failed to demonstrate any improvement on bone formation markers, trabecular bone mass and biomechanical parameters during a period of 45 days which represents approximately 3 years in the humans (19). Indeed, there were no detectable beneficial effects of a large dose of SrRa on bone formation and only a slight effect on bone resorption activity was observed. The lack of effects occurred independently of vit D status of the rats; source of vit D and type of SrRa treatment supplied (diet vs. gavage).

The SrRa (S12911) is a salt composed of an organic moiety (ranelic acid) which is able to bind two stable Sr atoms and increases the Sr bioavailability. Clinically, this drug is an orally administered agent that has been used for the treatment of osteoporosis (20,21). SrRa has been studied in various rodent models including intact animals, and those subjected to immobilization or OVX induced osteoporosis. Some of these studies suggested that SrRa acts both, as an antiresortive and as an anabolic agent (7,8,16). However, data supporting these dual-acting effects are limited. Conversely, several animals models "in vivo" showed that SrRa inhibited bone resorption without suppressing bone formation (10,22,23).

Sr is an alkaline earth metal chemically close to Ca; bone is the major target where it binds to the hydroxyapatite "in vivo" inducing metabolic effects which appear to depend on the used dose. At low dose, Sr reduces bone resorption and could increase bone formation leading to an increment in bone mass (24). In this regard, Sr is mainly adsorbed on the bone

surface and only a small amount is incorporated into bone by ionic substitution (25-28). However, in treated animals, even with large doses over a long period of time, the total amount of Sr in bone is always very low as compared to Ca. Only a small percent of the bone Ca content can be replaced, theoretically a maximum of 1 Ca atom out of 10 can be substituted by Sr (26).

Sr consumed in food or as a pharmacological treatment is taken up primarily by bone in a way directly related to Sr plasma levels and depending on length of treatment, gender and Ca intake (28, 29). It is important to point out that in the present study the rats were exposure to 900mgRaSr/kg/day supplied both, by gavage in a fasting status or diet; this dose is higher than the 625mg/kg/day used in other experiments (30) in which there was an increase in the serum Sr levels to values effective enough to prevent bone loss in OVX rats (31). In the present study, independently of vit D status or the source of vit D supplied, both types of SrRa treatment to OVX-osteopenic rats fed the AIN 93 recommended Ca intake (14) during approximately two remodeling cycles, increased about 5x the bone Sr content. However, SrRa treatment only induced a modest improvement in bone volume when compared to the respective OVX-untreated group. This effect was not a consequence of the high administration of Sr which could reduce the amount of Ca in bone and can cause hypocalcemia (23) because no changes in sCa and sP compared to control animals were observed. We assume that such improvement in bone volume was a consequence of the reduction in bone resorption as shown by biochemical and histological indices.

Previous experimental studies showed that SrRa treatment not only decreased bone resorption indices but also increased biochemical markers and histological parameters of bone formation (5,9,16,24,28,31,32). The results of the present report showed that SrRa exposure, independently of vit D status or the source of vit D, failed to increase the two studied bone formation markers. Indeed, both types of SrRa treatment did not induce any change in the longitudinal evaluation throughout the studied period of the bone formation marker b-ALP and it reduced the increment in the bone formation marker BGP induced by OVX. Regarding bone resorption, the increment on CTX levels induced by OVX was dependent on vit D status but independent of the vit D source or SrRa treatment because only diminished in rats receiving dietary vit D. The histological findings also showed that SrRa exposure failed to increase the osteoblast surface in either vit D states or sources. This finding, in conjunction with the reduction in trabecular separation and the increment in trabecular number, suggest that Sr was incorporated in the previously formed bone. As a result, the bone volume of OVX osteopenic rats treated with SrRa was only slightly affected. These findings are in agreement with a previous report in osteopenic ovariectomized rats treated with 150 or 450 mgSrRa/kg rat/day orally (33).The effects on bone resorption and bone formation parameters by exposure to 900 mgSrRa/kg/da during a 45-day period indicated that this element was able to prevent ovariectomy-induced bone loss without affecting bone formation.

Several studies have suggested that SrRa is associated with large increases in BMD. However, much of this increment is a physical effect caused by increased attenuation of X-rays due to the accumulation of Sr in bone tissue. Sr has a higher atomic number compared with Ca (87.62 vs. 40.08). This effect result in an overestimation of both BMC and BMD by ~10% applied to all the DXA clinical studies (34, 35). In the present report, the changes in total skeleton bone mass or in proximal tibia density by SrRa treatment remained positive after applying the reduction of the 10%. Since there are no standard correction factors in the literature for DXA animal studies this percentage was used taken into account the clinical studies. The data confirmed the deposition of Sr into bone, although such increment in densitometrical values was not translated into significant improvements in the mechanical properties of bone. Indeed, using a 3-point bending test performed at the level of the midshaft tibia, which mainly contains cortical bone, showed that SrRa exposure, independently of vit D status, failed to improve any of these three studied bone mechanical properties. Since bone strength is an important factor regarding anti-fracture efficacy, under our experimental conditions, SrRa treatment failed to improve bone resistance to fracture of OVX-osteopenic rats. Our results are in agreement with a previous study on OVX rats treated with lower doses of SrRa (28) and with a recent investigation in a model of immobilisations-induced bone loss (36).

Conclusion: Under the present experimental condition, the SrRa exposure to a dose of 900 mg/kg/day during a 45-day period and independently of vitamin D status or type of treatment increased Sr content in bone; however, no evidence of an anabolic or anti-fracture effect was found. Only a slight decrease in bone resorption parameters was noted. This is the first study examining the effect of SrRa in osteopenic vitamin D-insufficient rats and needs to be supported by further animal experiments increasing the time of exposition to the drug.

Acknowledgment:

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	BW (g) t=0	BW (g) t=60	BW(g) t=105
SHAM	286±9	351±9 **	355±13 **
OVX+D	291±6	378±7 *,**	396±9 *,**,#
OVX+D+SrRa	297±10	387±11 *,**	402±17 *,**
OVX-D	298±9	392±17 *,**	412±18 *,**,#
OVX-D+SrRa	297±8	394±7 *,**	419±8 *,**,#

(*): p<0.05 compared to SHAM
(**): p<0.04 compared to baseline
(#): p<0.05 compared to t=60   Table 1

	SHAM	OVX+D	OVX+D+SrRa	OVX-D	OVX-D+SrRa
Ca (mg/dl) t=60	8.8±0.4	9.4±0.4 *	9.5±0.3 *	9.6±0.3 *	9.5±0.2 *
Ca (mg/dl) t=105	9.7±0.5 ##	9.6±0.5	9.4±0.1	9.8±0.4	9.6±0.5
P (mg/dl) t=60	7.8±0.9	5.3±1.2 *	5.9±1.2 *	4.9±0.4 *	4.8±0.9 *
P (mg/dl) t=105	7.2±0.9	6.5±1.2	7.1±1.8	5.8±1.6 #	7.5±1.0 ##
25OHD(ng/dl) t=105	40.5±3.5	45.5±6.8	36.8±1.5 *	10.3±0.8 *□	10.5±1.8 *□
BGP (ng/ml) t=105	99.4±10.6	132.2±8.3 *	77.9±8.6	161.9±5.5 *	81.8±9.7
CTX (ug(ml) t=105	18.6±7.1	40.8±2.3 *	27.5±2.2	45.5±3.7 *	44.2±6.1 *

(*): $p<0.05$, compared to SHAM group
(**) $p<0.05$ compared to OVX-D+SrRa
(#): $p<0.05$ compared to OVX-D+SrRa group
(□): $p<0.05$ compared to the OVX+D groups
(##): $p<0.05$ compared to t=60

Table 2

table 3

	SHAM	OVX+D	OVX+D+SrRa	OVX-D	OVX-D+SrRa
Femur Sr content (ppm)	10.7±1.0	12.7±2.8	508.2±88.1 **	26.3±10.7	479.8±83.9 **
Max. Load (N)	90.2±8.0	84.3±8.6	86.4±2.6	72.0±12.8 * #	74.8±11.2 * #
Stiffness (N/mm)	1945±290	1614±195*	1650±284 *	1587±81 *	1500±192 *
Energy (mJ)	42.2±6.3	25.9±7.5*	21.2±6.1*	20.2±4.7 *	24.8±12.3 *
Λ TeBMC (t=105 - t=60) (mgr)	0,3±0,1	-0,4±0,3 *	1,1±0,4 *,**	-1.2±0.2 *	1,1±0,3*,**
Λ PTBMD (t=105 - t=60) (mgr/cm²)	8,7±2.2	3,9±2,0 *	17,4±4,2 *,**	0.0±2.8 *	11,3±6,2 *,**
Ob.S/BS	33.6±8.2.	52.8±4.6 *	37.8±5.8	29.4±12.6 * #	31.3±6.1
Oc.N/BS (·/mm)	0.39±0.05	0.57±0.19*	0.56±0.22 *	0.56±0.27 *	0.60±0.26 *
Tb. Th (um)	0.048±0.004	0.053±0.004*	0.057±0.003*	0.056±0.004 *	0.052±0.002*
Tb.N (1/mm)	4.02±0.61	0.64±0.11 *	1.09±0.36 *,**	0.45±0.19 *	0.73±0.26 *
Tb.Sp (um)	0.24±0.03	1.81±0.18*	1.13±0.15 *	3.42±0.35 * #	2.75±0.45 * #

(*): $p < 0.05$ compare to SHAM

(**): $p < 0.05$ compared to Sr-untreated groups

(#): $p < 0.05$ compared to the +D group

Table 3

table 4
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	BW (g) t=0	BW (g) t=60	BW(g) t=105
SHAM + D3	286±14	284±34.	301±55 #
SHAM + D2	283±18	286±25	306±38 #
OVX + D3	288±57	338±42 * #	343±53 * #
OVX+ D2	281±16	348±14 * #	356±25 * #
OVX+D3+RaSr	282±36	359±29 * #	341±32 * #
OVX+D2+RaSr	282±31	329±47 * #	334±73 * #

Table 4

(*): p<0.05 compared to SHAM
(#): p<0.05 compared to t=0

	SHAM + D3	SHAM + D2	OVX+D3	OVX+D2	OVX+D3+RaSr	OVX+D2+RaSr
Ca (mg/dl) t =60	8.6±0.5	8.5±0.8	9.2±04 *	9.4±0.4 *	9.5±0.5 *	9.6±0.4 *
Ca (mg/dl) t =105	9.0±0.9	9.0±0.5	9.4±0.5 *	9.6±0.5 *	9.6±0.6 *	9.2±0.5 #
25OHD(ng/dl) t =60	10.6±0.7	10.2±1	10.6±1.7	10.9±1.3	11.1±1.1	9.9±1.8
25OHD(ng/dl) t =105	30.9±1.1	36.3±5.3	31.8±3.4	32.2±3.0	36.7±3.6	31.7±5.9
BGP t=60	64.5±6.7	59.7±7.2	137.6±28.2 *	142.3±21.5 *	108.7±19.6 *	113.3±19.2 *
BGP t =105	67.7±8.6	68.6±4.1	122.6±27.3 *	128.2±26.1 *	79.4±11.1 **	77.3±10.7 **
CTX t =105	21.7±8.6	29.3±2.7	44.2±3.1 *	44.8±3.3 *	33.8±4.5 **	34.2±5.2 **

Table 5

(*): p<0.05 compared to SHAM
(#): p<0.05 compared to t=60
(**): p<0.05 compared to SrRa-untreated groups

table 6
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	SHAM + D3	SHAM + D2	OVX+D3	OVX+D2	OVX+D3+RaSr	OVX+D2+RaSr
Femur Sr content (ppm)	6.2±0.1	5.6±0.4	4.6±0.5	6.7±2.8	404.5±88.3 **	303.1±76.1**
Max. Load (N)	91 ±12	89±17	71±6 *	74±9 *	82±16	78±9
Stiffness (N/mm)	1936 ±19	1922 ±28	1737 ±16*	1694±29 *	1745 ±43 *	1698 ±20 *
Energy (mJ)	40.6 ±3.3	38.7 ±6.7	29.9 ±2.2 *	28.9±3.5 *	31 ±3.4 *	28.4 ±3.4 *
ATeBMC(mgr) (T=105-T60)	1.0 ± 0.1	0.9±0.3	-1.2 ±0.1 *	-1.6±0.4 *	2.3 ±0.5 *, **	2.4 ±0.6 *, **
APTBMD (mgr/cm^2) (T=105-T60)	8.1 ± 1.3	10.4 ±1.2	0.8 ±0.2 *	1.4±1.0 *	28.7 ±2.6 *, **	23.1 ±2.1 *, **
ALS BMD (mgr/cm^2) (T=105-T60)	5.0±0.9	4.3±0.9	-3.5±0.9 *	-4.2±0.5 *	6.6±0.7 *, **	6.8±0.6 *, **

(*): $p<0.05$ compared to SHAM

Table 6

(**): $p<0.05$ compared to SrRa-untreated groups
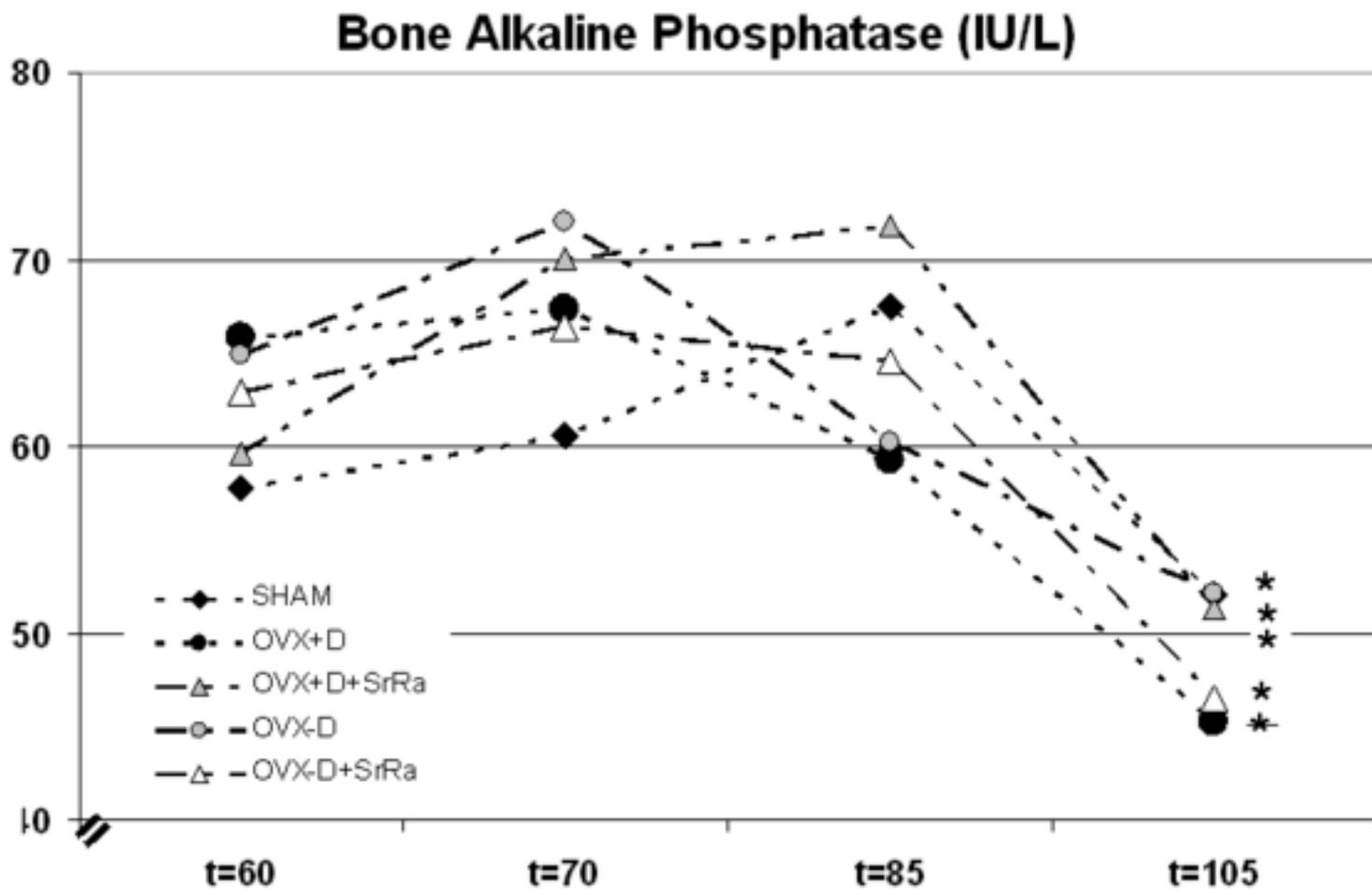
table 7
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	SHAM + D3	SHAM + D2	OVX+D3	OVX+D2	OVX+D3+RaSr	OVX+D2+RaSr
BV/TV (%)	24.3±10.8	35.4±9.4	2.9±1.7 *	3.1±1.6 *	4.4±2.3 *, **	5.3±2.5 *, **
Ob.S/BS	43.4±8.5	39.1±8.8	54.1±5.4	52.8±9.2	20.4±2.0 *, **	19.4±3.7 *, **
Oc.N/BS (·/mm)	0.41±0.12	0.38±0.09	0.66±0.26 *	0.67±0.38 *	0.37±0.18	0.41±0.21
Tb. Th (um)	0.042±0.006	0.050±0.005	0.043±0.006	0.053±0.004	0.042±0.022	0.046±0.001
Tb.N (1/mm)	4.34±1.67	5.26±1.91	0.76±0.26 *	0.64±0.11 *	1.18±0.88 *, **	1.32±0.61 *, **
Tb.Sp (um)	0.23±0.06	0.19±0.04	1.30±0.26 *	1.81±0.18 *	0.88±0.30 *, **	0.83±0.16 *, **

Table 7

(*): $p < 0.05$ compared to SHAM

(**): $p < 0.05$ compared to SrRa-untreated groups

Figure 1

(*) P<0.05 compared to t=60, t=70 and t=85
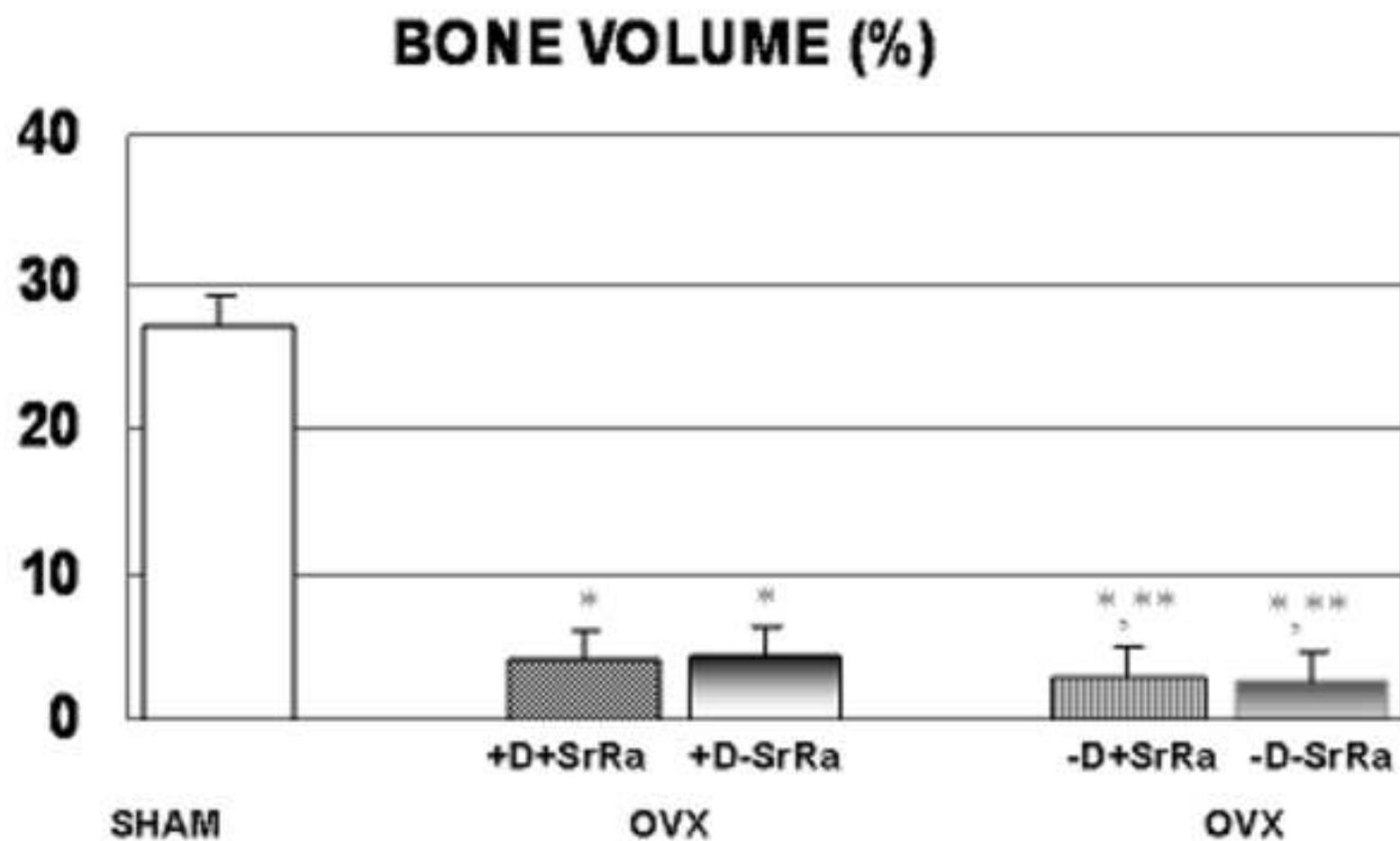
Figure 2

Legends:

Table 1:

Body weight (g) throughout the 1st. experience. Values are given as mean±SEM. t=0: baseline; t=60: beginning of treatment; t=105: end of the experience.

Table 2:

Mean±SEM values of serum Calcium (Ca) and phosphorus (P) levels at t=60 and at t=105 and 25hydroxyvitamin D (25OHD), osteocalcine (BGP) and C-terminal telopeptide of type I collagen at t=105.

Table 3:

Mean±SEM values of Tibia Sr content (ppm), Maximal Load (N), Stiffness (N/mm), Energy (mJ), changes in total skeleton bone mineral content (teBMD) and proximal tibia bone mineral density (PTBMD) between t=105 and t=60 [ΛTeBMC (t=105 - t=60)] (mgr) and [ΛPTDMD (t=105 - t=60), respectively] (mgr/cm2), and histological parameters obtained at the level of the middle third: Osteoblast surface [Ob.S/BS (%)], total osteoclast number [Oc.N/BS (·/mm)], thickness [Tb. Th (um)], trabecular number [Tb.N (1/mm)] and spacing [Tb.Sp (um)].

Table 4:

Body weight (g) throughout the 2nd. experience. Values are given as mean±SEM. t=0: baseline; t=60: beginning of treatment; t=105: end of the experience.

Table 5:

Mean±SEM values of serum Calcium (Ca), 25hydroxyvitamin D (25OHD) and osteocalcine (BGP) levels at t=60 and at t=105 and C-terminal telopeptide of type I collagen at t=105.

Table 6:

Mean±SEM values of Tibia Sr content (ppm), Maximal Load (N), Stiffness (N/mm), Energy (mJ), changes in total skeleton bone mineral content (teBMD), proximal tibia and lumbar spine bone mineral density (PT and LS BMD, respectively) between t=105

and t=60 [ΛTeBMC (t=105 - t=60)] (mgr); [ΛPTDMD (t=105 - t=60)] (mgr/cm2) and [ΛLSDMD (t=105 - t=60)], respectively.

Table 7:

Mean±SEM values of histological parameters obtained at the level of the middle third: Bone volume [BV/TV(%)], Osteoblast surface [Ob.S/BS (%)], total osteoclast number [Oc.N/BS (·/mm)], thickness [Tb. Th (um)], trabecular number [Tb.N (1/mm)] and spacing [Tb.Sp (um)].

Figure 1:

Mean value of bone alkaline phosphatase (IU/L) throughout the 1st. experience.

Figure 2:

Bone volume (%) a obtained at the level of the middle third after dead (t=105).

Figure 3:

Decalcified sections histology of the proximal third of the tibia in all studied groups at the end of the experiment. Hematoxylin-eosin staining technique shows trabeculae stained in red X50. Note the decrease in the number and the lack of connectiviy of trabeculae in all OVX groups, independently of vitamin D status.