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Assessment of femoral bone osteoporosis in rats treated with simvastatin or fenofibrate

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Abstract: Background: The effects of two lipid-lowering drugs, simvastatin and fenofibrate on osteoporosis in the femurs of healthy and ovariectomized female rats were investigated quantitatively by histological images and ^{1}H NMR relaxometry. Methodology: The intertrabecular cavities percentage was estimated from numerically cleaned histological images which initially present both the trabecular bone architecture and bone marrow filling the intertrabecular pores. The eight weeks evolution of intertrabecular cavities percentage measured from histological images of randomly selected sections in ovariectomized rats femoral diaphysis is similar with the evolution of transverse relaxation time centre of gravity $T_{2,CG}$, a selected parameter to quantify the changes in intertrabecular cavities dimensions from one-dimensional (1D) transverse relaxation time T_2 distributions. Results: The analysis of histological images recorded for non-ovariectomized rats show that the treatment with simvastatin and fenofibrate has a negative effect on the dimension of the femoral diaphysis trabecular bone. The analysis of 1D T_2 distributions show that the simvastatin and fenofibrate lipid-lowering drugs produce different effects on different particular sections of studied rat bones, i.e. proximal part of *femoris*, diaphysis or distal epiphysis. Conclusions: Finally, it is shown that the effects of the treatments are strongly dependent on the duration of treatment.

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1 Introduction

Osteoporosis is characterized as a reduction in bone mass and an impairment of bone architecture resulting a bone thinning with direct effects on increased cortical porosity, bone fragility and fracture risk. Macroscopically, there are two types of bone: compact and cancellous. Whereas the compact bone is dense, the cancellous bone is a lace-like structure of interconnected trabecular plates and bars surrounding marrow-filled cavities [1]. In osteoporosis, the cavities become larger and trabecular bone is disrupted. At the same time, the cancellous bone becomes thinner and its porosity increases. As these changes occur and the bone mineral density decreases, the water density in the bone increases [2].

The „lipid hypothesis“ states that lipids and the products of their oxidation may contribute to the pathophysiology of osteoporosis [3]. Thus, drugs interracting with lipid metabolism may also affect the bone metabolism. Statins are hydroxymethylglurarl-CoA reductase inhibitors, with widely discussed pleiotropic effects [4]. It has since been reported [5] that statins stimulate bone formation in vitro and in rodents. Several studies have investigated by different means their action on both healthy and osteoporotic bone, as well as on the fracture healing process, with controversial results. Fibrates, peroxisone proliferator-activated receptor alpha agonists, have also been shown to exert a positive effect on the bone, by maintaining the bone mineral density and architecture at sham levels after ovariectomy in rats [6].

For the evaluation of medicinal products in the treatment of primary osteoporosis, the European Medicines Agency recommends the use of the ovariectomized rat model and one of the three main techniques for assessing the osteoporosis is bone histology [7]. At the same time, the porosity of a general porous media can be evaluated

directly by specific methods, like microscopic images or indirectly by the study of dynamics of various fluids absorbed in such materials. The bone can be considered as a porous medium [8] which natively has a certain amount of water inside pores. Therefore, the water molecules can be used as *spy elements* which explore the cavities. In bone, water is found in two forms: collagen-bound water and bulk water in the Haversian and lacuno-canalicular system [9]. The quantity of water in bone can be measured noninvasively and non distructively by one (1D) and two (2D) ^{1}H NMR transverse relaxometry. The ^{1}H CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence is a NMR method which correlated with Laplace inversion analysis lead to a relaxation T_2 spectrum that can be used to determine the porosity and to assess the pore size distribution in bone [10]. There are just a few studies by NMR spectroscopy [11] or relaxometry [12] on rats' bones or rat optical nerve and frog sciatic nerve [13], but there are many studies of human cortical bone by ^{1}H NMR relaxometry in particular 1D T_2 distribution [14-19] or 2D T_2–T_2 exchange maps [20,21].

The aim of our study is to compare the effect of simvastatin or fenofibrate treatment on both healthy and osteoporotic rat femoral bone, by evaluating the changes in bone porosity and in particular the intratabecular cavities during eight weeks of observation. The methods are based on the quantitative analysis of histological images which imply the bone destruction and ^{1}H NMR relaxometry which is a non-destructive method. The effects on proximal part of *femoris*, diaphysis and distal epiphysis are evaluated separately.

2 Experimental Procedures

2.1 Study groups

The study protocol and surgical procedures have been approved by the Ethics Committee of the University of Medicine and Pharmacy Tirgu Mures, Romania with the number 29/26.06.2012. A number of 72 Albino Wistar adult female rats, aged 16-18 months, weighing on average 300 g, were used for this study. In half of the animals a bilateral ovariectomy was carried out in order to induce osteoporosis. For the surgical intervention the animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xiline (10 mg/kg). The rats were divided into 6 groups of 12 animals as follows: i) witness NOVX (no ovariectomy, no treatment); ii) witness OVX (ovariectomy, no treatment); iii) NOVX-S (no ovariectomy, treated with simvastatin); iv) OVX-S (ovariectomy, treated with simvastatin); v) NOVX-F (no ovariectomy, treated with fenofibrate) and vi) OVX-F (ovariectomy, treated with fenofibrate) [22].

2.2 Treatment

Both simvastatin and fenofibrate were administered orally by gavage. For the treated groups (3 to 6) the treatment started immediately after ovariectomy of rats from groups 2, 4 and 6 and continued until the animals were euthanized. The daily dose was 10 mg/kg for simvastatin as first described by Mundy *et al.* [5] or 10 mg/kg for fenofibrate as higher doses in rats have been shown to exert pancreatic and liver carcinogenesis effects according to the drug monograph.

2.3 Samples preparation

At 2, 4, 6 and 8 weeks post-ovariectomy, 3 random animals from each group were euthanized with an intraperitoneal injection of ketamine. The right femur was sampled and preserved in 10% formalin until the NMR measurements were made. Just before NMR measurement, the surface formalin was wiped out with an absorbent paper and two sections were made: under the trochanter and above the intercondylar fossa. We obtained three parts of each femur: (1) the proximal part containing the femoral head, femoral neck and proximal diaphysis, (2) the diaphysis and (3) the distal epiphysis. Then the right femurus of each rat was decalcified and paraffin-embedded. Hematoxylin-eosine stained histological images of randomly selected samples of diaphysis were obtained and examined with a Nikon Eclipse 600 microscope.

2.4 NMR measurements

Proton NMR measurements were performed using the BRUKER MINISPEC mq20 spectrometer working at 19.7 MHz and 35°C. The T_2 relaxation times distributions were obtained by performing the Laplace inversion of ^{1}H CPMG (Carr-Purcell-Meiboom-Gill) decays [8-12]. The CPMG pulse sequences with an echo time of 0.4 ms had 7000 echoes and, in order to ensure a good signal to noise ratio, an accumulation of 64 scans with a recycle delay of 5 s was performed.

2.5 Data processing

For a better evaluation of bone cavities from histological images, the bone marrow was numerically removed using CorelDraw™ 11 software. In this form, the images seen as a map of quasi-binary information are used for the

quantitative determination of the percentage of trabeculas and intertrabecular cavities. For a quantitative evaluation of trabecular bone content the histological images without bone marrow were saved in bitmap format which was read as a matrix into a custom made C++ numeric program. This numeric program analyzed the matrix into a binary mode and calculated the percentage of trabecular bone content or vice-versa the percentage of intertrabecular cavities. This numeric analysis was performed for both ovariectomized and non-ovariectomized samples for control or treated rats.

The analysis of NMR data is based on the interpretation of T_2 relaxation times distributions obtained by 1D Laplace inversion of the measured CPMG echoes train decays. This method is defined as an ill conditioned problem, but in the last decade a series of numeric procedures were developed [23, 24] and are successfully used for Laplace analysis of NMR properties of various samples [25-30]. Here it was shown that despite the uncertain which is specific to Laplace analysis related to the absolute width of the obtained distributions it is perfectly alright to perform a comparative analysis if the measurement and analysis conditions are identical for a series of similar samples, condition which is fulfilled in our case.

3 Results

3.1 Histological findings

Examples of histological images are presented in Fig. 1 for some rats belonging to the control groups of non-ovariectomized animals non-treated (witness) or treated with fenofibrate or simvastatine and sacrificed after eight weeks of observation (treatment). The upper histological images present the trabecular bone structure and bone marrow which fills in a large proportion the intertrabecular cavities. The lower images show the same section like the upper images but the bone marrow was numerically removed.

It was seen that at 2 and 4 weeks after the beginning of the treatment, NOVX-W, NOVX-S and NOVX-F groups had similar histological structure. Small differences start to be observed with week 6, where the trabecular bone in NOVX-W group was thicker than in NOVX-S and NOVX-F [22]. At week 8, NOXV-W (Fig. 1a) had thicker bone trabeculas than both the other two NOVX groups which received the treatment (Fig. 1b and 1c).

The time evolution of intertrabecular cavities (and/or the trabecular bone macroscopic architecture) can be evaluated from histological images without bone marrow of arbitrary selected sections in rat femoral diaphysis recorded during eight weeks of observation post ovariectomy. Some of such images are presented in Fig. 2 for all 4 time moments of sacrifice and belonging to all three groups: witness (OVX-W) un-treated, and treated with simvastatin (OVX-S) and fenofibrate (OVX-F). From Fig. 2 at a visual inspection is obvious that the samples belonging to OVX-W groups appears to have the thinnest trabeculas and largest intertrabecular pores which become connected at 6 and 8 week of observation. Among the treated groups the OVX-S seems to have the better connected trabecular bone structure at all evaluation times.

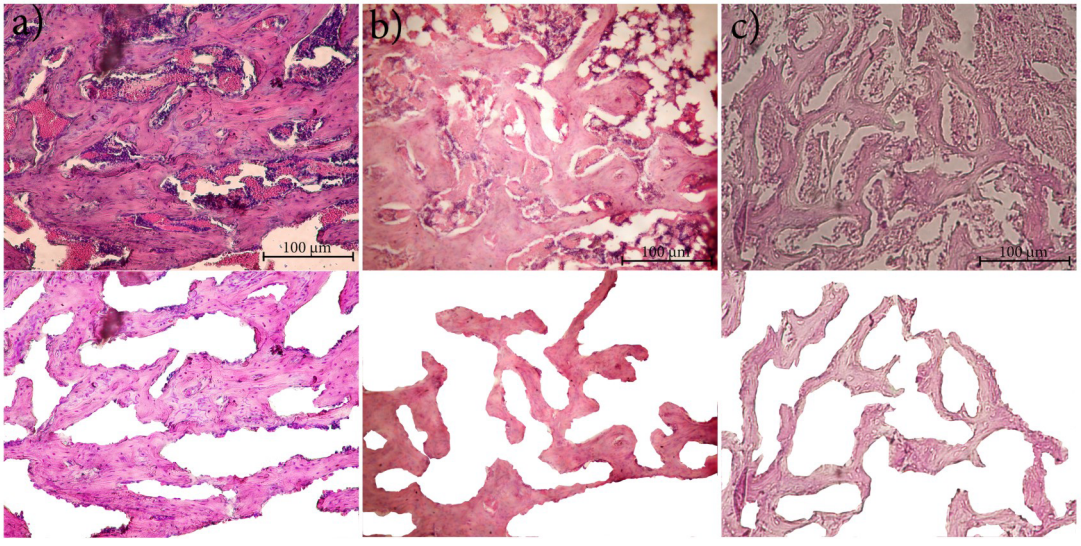

Fig. 1 Histological images of randomly selected sections of rat femoral diaphysis a) witness; or treated with b) fenofibrate and c) simvastatin with bone marrow (upper figures) and without bone marrow (lower figures) of NOVX rats.

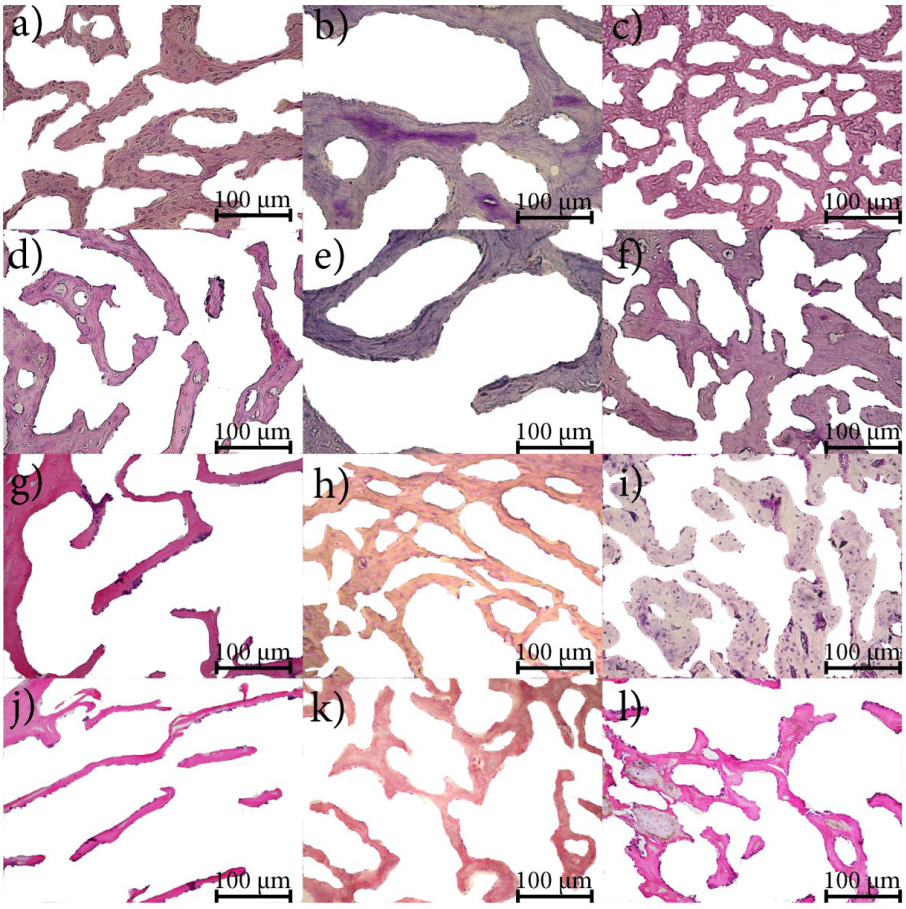

Fig. 2 Histological images of randomly selected sections of femoral diaphysis for non-treated (W-first column), fenofibrate treated (F-second column) or simvastatin treated (S-third column) rats without bone marrow recorded at 2 (a, b, c), 4 (d, e, f) 6 (g, h, i) and 8 (j, k, l) weeks of observation after ovariectomy.

The calculated percentages of intertrabecular cavities for the NOVX groups are presented in Fig. 3a compared for the witness and treated with simvastatins and fenofibrates. It can be seen that the percentage of intertrabecular cavity in the NOVX (untreated) group decreases with time (grey bars). At 4, 6 and 8 weeks after ovariectomy the percentage of intertrabecular cavities in to the NOVX-S was larger than in the NOVX-W group. A larger percentage of intertrabecular cavities are observed also in the case of NOVX-F at 4 and 6 weeks compared with the control groups but lower than for samples from rats treated with simvastatins. At 8 weeks, the percentage of intertrabecular cavities in treated rats (within experimental error) was ~ 67 %, whereas in the control group where the intertrabecular cavities were ~42 %.

Ovariectomized animals differ in that: i) induced osteoporosis by ovariectomy leads to an increase of intertrabecular cavities percentage and ii) treatment with simvastatin (OVX-S) or fenofibrate (OVX-F) resulted in smaller percentages of intertrabecular cavities than the OVX-W (Fig. 3b). The single exception is found for OVX-F data obtained at 4 weeks where the percentage of intertrabecular cavities measured in this case (~71 %) was larger than the percentage measured for control group (~ 66 %).

3.2 Normalized T_2 distributions

To compare the NMR data with histological images the analysis was focused on NMR signal originating from the water pools corresponding to large pores like the intertrabecular cavities. Figure 4 presents the normalized T_2 distributions measured for the samples harvested from proximal part of *femoris* of ovariectomized rats sacrificed at two (Fig. 4a) and eight (Fig. 4b) weeks after ovariectomy. Each figure presents a comparison between T_2 distributions of untreated groups (OVX-W) and treated groups with fenofibrate (OVX-F) and simvastatin (OVX-S). All T_2 distributions presented in Fig. 4 are the average of distributions [16] obtained for individual samples of rats belonging to each group.

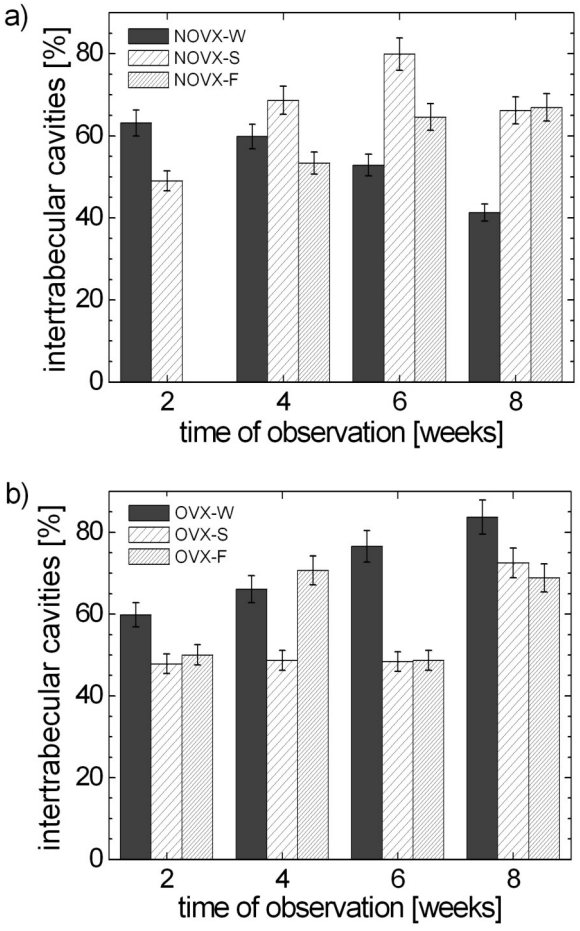

Fig. 3 Bar plots of intertrabecular cavities percentage development during eight weeks of observation for a) non-ovariectomized rats or b) ovariectomized rats given (W) no treatment or treated with fenofibrate (F) or simvastatin (S) evaluated from histological images like those shown in Fig. 2.

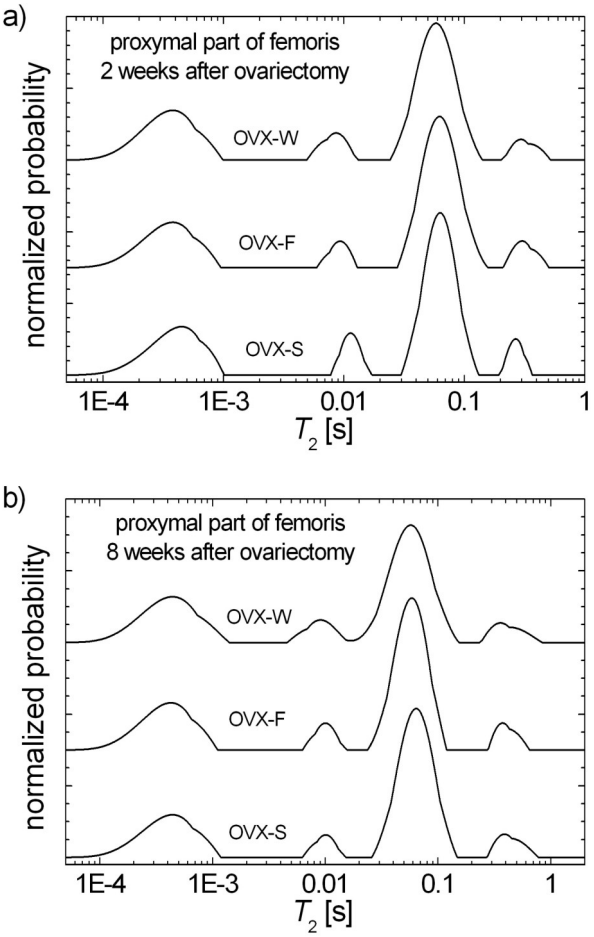

Fig. 4 The average 1D distribution of ^{1}H transverse relaxation time T_2 obtained for ovariectomized rats from groups of non-treated (W), fenofibrate treated (F) or simvastatin treated (S) and sacrificed at a) two or b) eight weeks after ovariectomy.

In order to assign the peaks to different porosities, several measurements and analysis were performed (private communications, the results exceed the purpose of this report and will be published elsewhere). The hierarchical structure of porous femur found in the agreement with the distribution the four peaks in the measured T_2 distributions can be described as: i) the peaks located at several hundreds of milliseconds (the largest T_2 values*)* correspond to intertrebecular cavities; ii) the peaks located at several tens of milliseconds (the main peak) correspond to the protons located in the Haversian channels and transverse Volkmann canals; iii) the peaks observed at several milliseconds T_2 values correspond to the NMR arising from protons located in pores which form the space between the osteocytes and lacunar-canalicular wall and iv) the peaks observed at smallest T_2 values (several hundreds of microseconds) correspond to the NMR arising from protons from collagen or bound water to collagen. As expected, the bone marrow would affect dramatically the integral area and widths of peaks located at T_2 values larger than 1 ms but would have a negligible influence on the peaks maximum position (the T_2 value at which occur the maximum). Likewise, the bone marrow would not influence in any way the peaks located under 1 ms (private communications, not shown here). At a visual inspection (see Fig. 4), the differences between T_2 distributions recorded for untreated and treated rats at two or eight weeks were not very large. Therefore a quantitatively analysis was performed on all T_2 distributions, which include the T_2 *centre of gravity*, integral area and log-width for all four peaks. Moreover, in order to be directly compared with the histological data, we selected only the peaks associated with the intertrabecular cavities. For asymmetrical peaks, as those characterized by the largest T_2 values the maximum peaks did not reliably represent the distribution. Instead of the T_2 values at which the maximum occurred, the T_2 *centre of gravity* parameter was preferred. If we denote with $f(T_2)$

the normalized T_2 distribution, then the T_2 centre of gravity parameter can be calculated as $T_{2,CG} = \int\{f(T_2)\ T_2\}/\int\{f(T_2)\}$.

In Figure 5, the time evolution $T_{2,CG}$ centre of gravity corresponding to intertrabecular cavities are presented for the ovariectomized control group (OVX-W) and treated (OVX-S and OVX-F) groups during the eight weeks after ovariectomy.

Bone can be considered a quasi-porous medium [8], therefore T_2 values may be interpreted in terms of bulk and surface relaxation. In brief, the largest pores will have a largest T_2 values. In Fig. 5a the $T_{2,CG}$ centre of gravity are represented for the femoral diaphysis. The values of $T_{2,CG}$ measured for the untreated rats increases with time which corresponds to an increase in the intertrabecular cavities sizes. Moreover, with the exception of week 2, the values of $T_{2,CG}$ measured in both the simvastatin (OVX-S) and fenofibrate (OVX-F) treatment groups are lower than the $T_{2,CG}$ values determined for the untreated rats. Even if the pore sizes cannot be directly translated into percentage of intertrabecular cavities, the NMR results are in agreement with the data observed from histological images (see Fig. 3b).

For the untreated rats the effect of ovariectomy is osteoporosis, as observed in the proximal part of *femoris* where the dimension of the intertrabecular cavities is more reduced than in the diaphysis. In fact (within the experimental error limit), the $T_{2,CG}$ values can be considered constant (see Fig. 5b). The evolution of $T_{2,CG}$ value, measured for distal epiphysis of OVX-W group post ovariectomy is not linear, presenting a maximum at week 4 and then decaying dramatically at week 8 (see Fig. 5c). Within experimental error limits, and with a small deviation in week 6, we may consider that the treatment of ovariectomized rats with simvastatin has no influence on the size of intertrebacular located at the level of distal epiphysis.

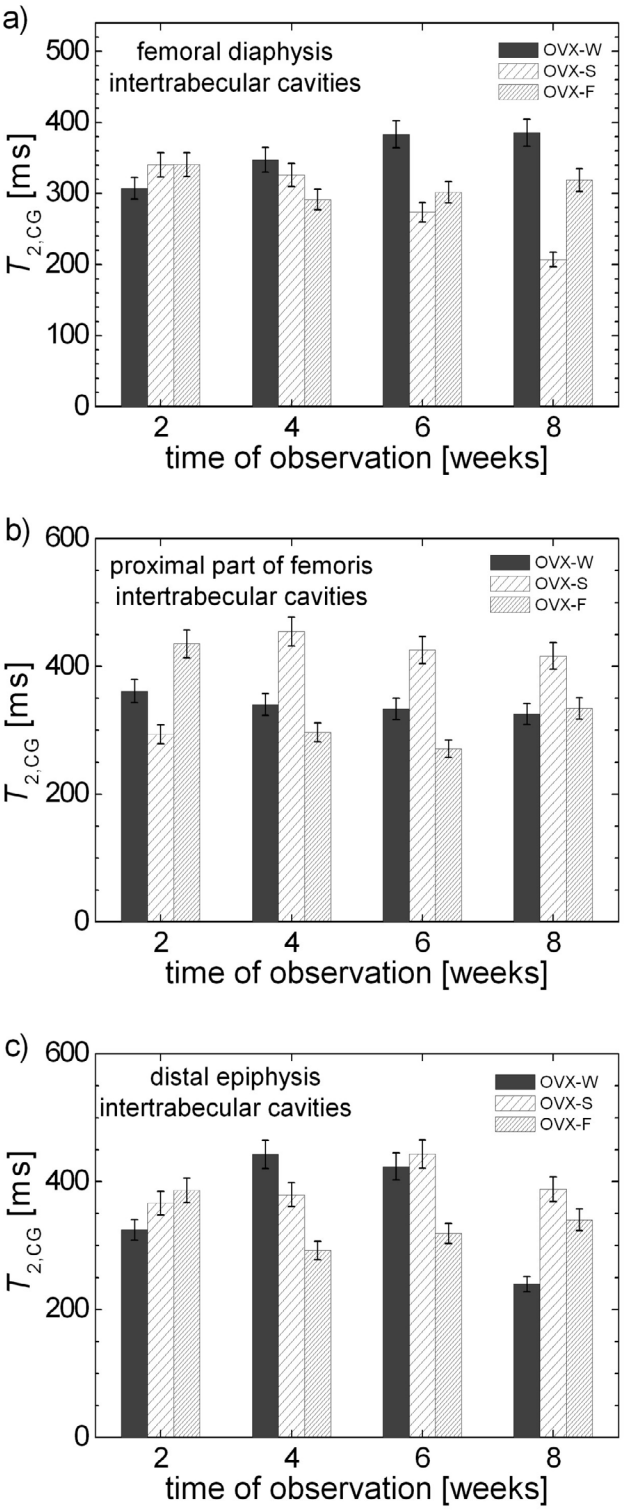

Fig. 5 Bar plots of centre of gravity of $T_{2,CG}$ centre of gravity corresponding to intertrabecular cavities for ovariectomized rats, either non-treated (W), fenofibrate treated (F) or simvastatin treated (S), evaluated from 1D T_2 distributions like those shown in Fig. 2 corresponding to a) femoral diaphysis; b) proximal part of *femoris* and c) distal epiphysis.

4 Discussions

4.1 The effect of lipid-lowering drugs evaluated from histological images

In this study, the histological aspects of trabecular bone in the distal epiphysis were compared in osteoporotic and healthy bone under simvastatin and fenofibrate treatment. This is a clear indication that the simvastatins and fenofibrates have an action pathway which interferes with the natural growth of bone. Unfortunately, the action of these so called wonder drugs [31] has a negative influence, in the sense of the increase of the bone

intertrabecular porosity if these drugs are administrated to healthy animals.

But from a comparative analysis (see the similar values obtained for OVX-S and OVX-F at 2, 6, and 8 weeks) we may attribute this difference to sample selection or inter-subject variation, rather than the effect of fenofibrate. Contrary with the findings of Yao *et al.* [32] which reports that simvastatin does not prevent or restore ovariectomy-induced bone loss in 3-month-old Sprague Dawley adult female rats 120 days post ovariectomy, our results demonstrate that in Albino Wistar adult female rats aged 16-18 months, simvastatin did reduce the effects of ovariectomy induced osteoporosis, indicated by reduced percentage of intertrabecular cavities observed from randomly selected specimens.

The limitation of the histological examination is that only a small part of the bone can be examined and the analysis of a complex three dimensional structure is made with a two dimensional technique [33]. A more complete analysis can be performed using other experimental techniques like the NMR relaxometry.

4.2 The effect of lipid-lowering drugs evaluated from T_2 distributions

One of the most powerful methods used for the study of the state of water in bone is based on the 1D transverse relaxation spectra also known as the T_2 distributions. The common feature of our T_2 distributions is the presence of four peaks which can be associated with different pools (or ^{1}H reservoirs). There are few reports on the association of peaks from T_2 distributions measured on rats' femurs. Horch *et al.* [17, 18] showed that, for human cortical bone, the T_2 values under millisecond correspond to bound water (primary to collagen) while the T_2 values larger than 1 ms (up to 1 s) correspond to pore water and lipids (bone marrow). Moreover, the majority of studies on human cortical bone are focused on the association of ultra-short T_2 values to collagen methylene protons (150 ms ≤ T_2 ≤ 1 ms), collagen-bound water protons (50 ms ≤ T_2 ≤ 1 ms) water protons in pores in lipid protons (1 ms ≤ T_2 ≤ 1 s) [16]. Differences with these reported data are expected to occur since in our case we have a rat femurs measured separately for proximal part of *femoris*, diaphysis and distal epiphysis which contain both trabecular as well as cortical bone.

As mentioned before, the average normalized T_2 distributions, like those presented in Fig. 4 present similar features and the differences, due to the treatment (or lack of treatment), are not so large as to allow a clear interpretation by visual inspection. In the short and medium term (up to 6 weeks) the simvastatin and fenofibrate treatments seem to have an opposite effect. Simvastatin (OVX-S) starts at 2 weeks with a $T_{2,CG}$ value lower than the $T_{2,CG}$ value corresponding to untreated animals (OVX-W) and then this value becomes larger. This can be an indication of a negative effect of simvastatin treatment observed as an increase in the size of intertrabecular cavities located at the level of proximal part of *femoris*. The treatment with fenofibrate seems to have a positive effect (the $T_{2,CG}$ value measured for the OVX-F groups to be lower than the $T_{2,CG}$ value measured for the OVX-W group) in the medium term (4 and 6 weeks) as can be seen from Fig. 5b. In the case of proximal part of *femoris* and distal epiphysis, treatment with fenofibrate has a positive effect only in the medium term (4 and 6 weeks after ovariectomy).

The changes in bone porosities influence the risk of fragility fractures in osteoporotic women [34] and the trabecular microarchitecture associated with fractures includes reductions in trabecular plate bone volume, number and connectivity and a more rod-like trabecular network [35]. Studies have shown that osteoporosis decreases the fracture risk by 30-40% compared to non-statin users [36]. However, data in the literature remains insufficient and controversial for several reasons, including differences in dose regimen and duration, allowing a significant space for bias.

5 Conclusions

In healthy rats, both simvastatin and fenofibrate treatment showed a negative effect on the trabecular bone located at the level of femoral diaphysis. These results are consistent with other studies which concluded that to a certain extent, statins inhibit bone resorption and promote bone formation, but have no significant effect on bone mineral density in healthy rats [37, 38]. In osteoporotic bone, studied by analysis of histological images and ^{1}H NMR relaxometry, both treatments showed a positive effect by increasing the percentage of femoral diaphysis trabecular bone. In this way the analysis of ^{1}H T_2 distributions has been shown to be a valuable tool for the characterization of intertrabecular cavities in osteoporotic rats. Moreover, the NMR data shows different effects of treatment with simvastatin or fenofibrate is dependent on the femoral section, which are probably due to action of different biological mechanisms specific to each location at the level of femurs, therefore opening the possibility for further investigations.

Conflict of interest: Authors declare nothing to disclose.

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