Ovariectomized rats’ femur treated with fibrates and statins. Assessment of pore-size distribution by $^1$H-NMR relaxometry

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Abstract
The effects of two wonder drugs, simvastatins and fenofibrates on the proximal part of the femoris of a series of ovariectomized and non-ovariectomized Wistar albino rats was estimated qualitatively and semi-quantitatively by the modern method of 1D $^1$H-NMR $T_2$-distribution. The 72 rats subjected to this study were divided in six groups and were sacrificed at two, four, six and eight weeks after ovariectomy and the proximal part of femoris was harvested. The CPMG (Carr–Purcell–Meiboom–Gill) echoes train curves were measured for the bones fully saturated with water during two months after two months of natural drying. These decays were analyzed by Laplace inversion and an average of normalized $T_2$-distributions was considered for all rat’s groups. The 1D averaged $T_2$-distributions present four peaks, which were associated with protons in four major environments, from which the free water protons are used as spy molecules to explore the boundaries of cavities. In the approximation of spherical pores, the averaged $T_2$-distributions were transformed in distributions of pores diameters. These were found in the range from 2 μm up to 2 mm. The relative amplitudes, widths and position of deconvoluted distributions of small, medium and large cavities are used for a qualitatively analysis of the effect of our lipid-lowering drugs. For a semi-quantitatively analysis, we chose the diameter $\mathcal{D}$ of proximal part of femoris’ trabecular cavities. We show that the positive or negative effects of treatments with simvastatins and fenofibrates are strongly dependent on the duration of treatment. Moreover, the treatment of healthy bone is generally counter-indicated.

Keywords: ovariectomized Wistar rats, osteoporosis, simvastatin, fenofibrates.

Introduction
Osteoporosis, an impairment of bone architecture resulting a bone thinning with direct effects on increased cortical porosity, bone fragility and fracture risk, is characterized as a reduction in bone mass [1]. There are two types of bone: (1) the cancellous bone, which have a lace-like structure of interconnected trabecular plates and bars surrounding marrow-filled cavities [2], and (2) the compact bone, which, in cross section, shows dense areas without cavities [2]. In osteoporosis, the trabecular bone is disrupted and in consequence, the cavities become larger while 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 [3]. The bone is usually considered as a composite material consisting of a mineralized with non-stoichiometric calcium apatite collagenous matrix. Bones contains water, for which a larger fraction occupies the spaces of the Haversian and lacuno-canalicular system while part of which is bound to collagen [4]. The bone is a porous structure with highly structured cavities from largest pores, followed by the vascular, lacunar-canalicular and collagen-apatite porosities [5]. There are many similarities between human and rat bone, therefore for pre-clinical osteoporosis studies; the rat models are frequently used. In order to obtain the equivalent of postmenopausal osteoporosis in women, an estrogen deficiency can be induced in rats by surgical ovariectomy [6].

A lipid hypothesis states that lipids and the products of their oxidation may contribute to the pathophysiology of osteoporosis [7]. Then, the drugs, which interact with lipids’ metabolism, may also affect the bone metabolism. Statins are hydroxyl-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors, with widely discussed pleiotropic effects [8]. The statins stimulate bone formation in vitro and in rodents and since this report [9], several studies have investigated by different means their action on both healthy and osteoporotic bone, as well as on the fracture healing process. Fibrates, by maintaining the bone mineral density and architecture at sham levels after ovariectomy have also been shown to lead to a positive effect on the rats’ bone [10].

In the treatment of primary osteoporosis, for the evaluation of medicinal products, 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 [11]. 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 morphological changes in bones can be assessed using many approaches like: the standard histological techniques, immunohistochemistry, confocal microscopy, micro-CT [12], scanning (SEM) and transmission (TEM) electron microscopy [13], cryo-porometry and thermo-porometry [14]. In bone, water is found in two forms: collagen-bound water and bulk water in the Haversian and lacuno-canalicular system [15]. The bone can be considered as a quasi-porous media [16], which
natively has a certain amount of water inside pores. In NMR, the water molecules can be used as spy molecules capable to explore the bone’s cavities. Thus, the bone water quantity can be measured non-invasively and non-destructively by one (1D) and two (2D) 1H-NMR transverse relaxometry. The 1H-CPMG (Carr–Purcell–Meiboom–Gill) pulse sequence is a modern NMR method, which correlated with Laplace inversion analysis lead to a relaxation T2 spectrum (called also Laplace spectrum). This will be used to determine the bone porosity and to assess the pore size distribution [17, 18]. There are just a few numbers of studies by NMR spectroscopy [19] or relaxometry [18, 20] on rats’ bones or rat optical nerve. There are many studies of human cortical bone based on 1H-NMR relaxometry in particular 1D T2 distribution [22–27] or 2D T1–T2 exchange maps [28, 29]. Laplace spectroscopy becomes in the last decade a standard method in the study of porous materials [30]. The study of various materials extends the Laplace algorithms from exponential [31] to non-exponential [32] kernels. The particular features of 2D Laplace analyses allow the identification and qualitative study of molecular exchange processes [33–35] and from here the pores’ interconnectivity. Otherwise, the magnetic resonance imaging (MRI), in particular T1-weighted images and T2-weighted with fat suppression, was used as an efficient method to highlights the presence of bone marrow edema (BME) caused by various diseases [34].

The aim of our study is to compare the effect of treatments with simvastatin or fenofibrate lipid-lowering drugs on both healthy and ovariectomy induced osteoporotic rat’s femoral bone, by evaluating, during eight weeks of observation, the changes in bone porosity, in particular the intratrabecular cavities diameter distribution. The NMR relaxometry measurement combined with Laplace analysis is used to obtain the Laplace spectra characteristic to the ovariectomized (OVX) and non-ovariectomized (NOVX) reference (W) rats or treated with fenofibrate (F) and simvastatin (S). Finally, for the studied series of rats, from the averaged T2 distributions we estimate the pores-size distributions function of evolution after ovariectomy.

Materials and Methods

For this study, we use a number of 72 Albino Wistar adult female rats. At the beginning of the observation (or the moment of ovariectomy), the average weight was 300 g and the age of rats was 16 months. This, in human women correspondent is 47-year-old. Half of these animals have been ovariectomized (label OVX) while half remained non-ovariectomized (label NOVX). The 72 rats were divided in six groups of 12 animals each as follows: (1) reference NOVX-W (no ovariectomy, no treatment); (2) reference OVX-W (ovariectomy, no treatment); (3) NOVX-F (no ovariectomy, treated with fenofibrate) and (4) OVX-F (ovariectomy, treated with fenofibrate); (5) NOVX-S (no ovariectomy, treated with simvastatin); (6) OVX-S (ovariectomy, treated with simvastatin). Inside of each primary group, we divided the animals in four subgroups of rats’ function of sacrifice time. The appurtenance of rats’ to each subgroup was not chosen from the beginning; the animals belonging to a specific group were randomly selected at the time of sacrifice.

Animal treatments and samples

For the treated groups of rats (three to six), the treatment started immediately after ovariectomy and continued until the animals were euthanized. Both simvastatin and fenofibrate were administered orally by gavage. The daily dose was 10 mg/kg for simvastatin and 10 mg/kg for fenofibrate. According to the drug monograph, higher doses in rats exert a pancreatic and liver carcinogenesis effects.

After ovariectomy at two, four, six, and eight weeks of observation, the animals from each group were sacrificed with an overdose of Ketamine and Xyline (8–10 mg/kg). From each rat, the right femoral bone was harvested and cut in three parts. The first section was made under the trochanter and the second one above the intercondylar fossa. It result three parts: (1) proximal part of femoris, which contain the femoral head, femoral neck and proximal diaphysis, (2) diaphysis, and (3) distal epiphysis. In order to remove the bone marrow, the femoral bone was kept in air a period of two months. Then, the dry bone was kept in formalin another two months before NMR measurements. The animal investigation has been approved by the Ethics Committee of the University of Medicine and Pharmacy of Tirgu Mureș, Romania, with the No. 29/26.06.2012.

1H-NMR methods

The 1H-NMR relaxation measurements was performed using the Bruker Minispec spectrometer equipped with a 10 mm probe-head, diameter large enough to accommodate the rats’ femoral parts without other adjustments. The Larmor frequency was 19.688 MHz and the temperature was maintained constant at 35°C. For the T2 spin–spin relaxation times measurements (by CPMG pulse sequence), the pulse length was 12.5 μs and 7000 echoes with an echo time of 0.4 ms were recorded. A recycle delay of 5 s ensures a good sample magnetization and 128 scans lead to a good signal to noise ratio. In order to find the T2 spin-spin relaxation times distributions, the CPMG curves were analyzed using a modern algorithm, which perform the Laplace inversion of the measured data. Some images were numerically processed as described before for the assessment of femoral bone osteoporosis. There we have shown that the treatment with simvastatins and fenofibrates has a negative effect on the dimensions of femoral diaphysis trabecular bone but present different effect on different particular sections of studied rat bones, i.e., proximal part of femoris, diaphysis or distal epiphysis.

Results

The typical bone architecture at the millimeter scale is presented in Figure 1 based on randomly selected histological images of a witness ovariectomized rats’ femurs. Figure 1a presents the trabecular bone structure and bone marrow, which fills, in a large proportion, the intertra trabecular cavities. The lower image (Figure 1b) shows the same section like the upper images but, in order to highlight the large cavities, the bone marrow was numerically removed.

The processed histological images (Figure 2, as randomly examples of parts of femoral bone belonging to witness, and treated with fenofibrates and simvastatins)
and NMR measurements on the same groups of rats’ femoral bones with bone marrow lead to the idea that, the most sensible part to the effects of osteoporosis of treated or non-treated animals is the proximal part of femoris. Therefore, in the present study, the focus is on this upper part of femur. By removing the bone marrow, we propose a new step in ovariectomy induced osteoporosis assessment, then the water can fill all bones’ cavities and a quantitative analysis can be performed on the de-fatened bone.

Figure 1 – Images based on randomly selected histological images of a witness ovariectomized femoral rat’s femurs (a) with bone marrow and (b) without bone marrow, which present the trabecular bone and large cavities.

Figure 2 – Images of sections in (a) reference; and treated with (b) fenofibrate and (c) simvastatin ovariectomized femoral rat’s femurs without bone marrow after eight weeks from ovariectomy and with pores filled with water. The pictures are based on randomly selected histological images where the cavities were filled numerically with blue to represent the water.

**1H-NMR measurements**

In Figure 3 top, the CPMG decays recorded for the same samples before removing the bone marrow (open-square for and non-ovariectomized rat and open-circle for an ovariectomized rat) and for the dry bone (lower triangle for non-ovariectomized rat and upper triangle for an ovariectomized rat) are compared. We observe a fast decay of the NMR signal of samples with bone marrow, indicating an origin into a more rigid protons reservoir. Large differences between CPMG curves recorded for the NOVX and OVX rats at same status of bone (with or without bone marrow) can be found for the bones with marrow, in the initial time regime (Figure 3b). Vice-versa, for the dry bone large differences between the NOVX and OVX CPMG decays are found at large measurement times (Figure 3a).

**Primary analysis of 1H-NMR measurements and association with bone hierarchically structure**

A better interpretation of the results can be obtained by analyzing the CPMG curves using the Laplace inversion algorithms. This assumes that curves decays multi-exponential and are characterized by specific parameters, *i.e.*, the spin–spin relaxation times, $T_2$. The $T_2$-distribution of the four curves presented in Figure 3 top are shown in Figure 3 bottom, for the reference non-ovariectomized rat (labeled with 13) in Figure 3c and for the reference ovariectomized rat (labeled with 02) in Figure 3d. Both rats were sacrificed after two weeks of observation (from ovariectomy). The $T_2$-distributions obtained for the same sample are directly compared in these figures.

The common factor of all distributions is that these curves present four peaks. These peaks were previously assigned to various pools (reservoirs) of $^1$H as follows: (1) to the intertrabecular cavities were assigned the peaks located at several hundreds of milliseconds (the largest $T_2$ values); (2) the protons located in the Haversian channels and transverse Volkmann canals were assigned to the peaks located at several tens of milliseconds (the main peak); (3) the peaks observed at several milliseconds $T_2$ values were associated with the NMR arising from protons located in pores which form the space between the osteocytes and lacunar-canaliculal wall and (4) the peaks observed at smallest $T_2$ values (several hundreds of microseconds) were associated with the NMR arising from protons from collagen or bound water to collagen.
Comparing the \(T_2\)-distributions from bone with and without bone marrow large differences can be found between Laplace spectrum obtained for non-ovariectomized (Figure 3c) and ovariectomized (Figure 3d) rats. The \(T_2\)-distribution of non-ovariectomized rat 13 measured for the proximal part of femoris with bone marrow present narrow peaks, which are well resolved. The \(T_2\)-distribution recorded for the same sample after drying and filling the pores for two months with water (Figure 2) is broader and the \(T_2\)-values are shifted, with a factor of 2–3 to larger values. These effects are predictable since a more rigid organic matter (the bone marrow) was replaced with liquid, leading to an increase of free (for water molecules self-diffusion) volume, then we observe an increase in the \(T_2\)-values and a broader distribution of pores. The \(T_2\)-distributions recorded for ovariectomized rat (labeled with 02) proximal part of femoris with and without bone marrow, are similar in respect of \(T_2\)-values and peaks widths. In the case of bone with marrow the \(T_2\)-distributions is a little better resolved.

![Image](a)

**Figure 3** – Comparison between the CPMG decays measured for water and bone marrow filled and water filled proximal part of femoris belonging to reference non-ovariectomized (NOVX – W: rat 13) and ovariectomized (OVX – W: rat 02) Wistar rats (a) full decays and (b) initial regime. For a better view, in Figure 3a, the decays are showing points from 20 to 20. Comparison between the \(T_2\) distributions obtained by 1D Laplace inversion analysis of CPMG decays presented in Figure 3 for water and bone marrow filled and water filled proximal part of femoris belonging to (c) witness non-ovariectomized (NOVX – W: rat 13) and (d) ovariectomized (OVX – W: rat 02) Wistar rats.

In Figure 4 (a–f) are presented the average \(T_2\)-distributions for all six study groups non-ovariectomized on the left side and ovariectomized on the right side for the reference (top), and treated with fenofibates (middle) and simvastatins (bottom) and for all four times of sacrifice (i.e., two, four, six, and eight weeks). These \(T_2\)-distributions were obtained for the defatted bone and then saturated with water (Figure 2) considering an average from distributions recorded for all animals belonging to the same group.

The four average \(T_2\)-distributions corresponding to the non-ovariectomized witness rats’ show that the bones of these animals are evolving during the eight weeks of observation (Figure 4a). This can be attributed to the natural growth of animals but also to the life style and physical activities. Compared with week two, the average \(T_2\)-distributions for the rest of the times becomes broader. Especially one can observe a decrease of the intensity of peaks belonging to middle cavities associated with water-protons located in the Haversian channels and transverse Volkmann canals. At the end of the observation time (week 8), the average \(T_2\)-distributions is more similar with the average \(T_2\)-distributions recorded at the beginning of the observation time (two weeks). A relative constant decay of water located at the level of Haversian channels and transverse Volkmann canals is observed from the average \(T_2\)-distributions recorded for the reference ovariectomized rats’ (Figure 4d). In time, these \(T_2\)-distributions show a reduction in the relative volume of middle cavities, and from increase resolution of Laplace spectra one can conclude that these are transformed into large pores (i.e., intertrabecular cavities) while new pores are formed in the space between the osteocytes and lacunar-canalicular walls.

We observe an interesting effect of average \(T_2\)-distributions recorded for the rats; treated with fibrates (Figure 4, b and e). Both groups, non-ovariectomized and ovariectomized rats at four week of observation present well resolved Laplace spectra. While at two weeks, the average \(T_2\)-distributions are broaden after two more weeks (a total of four weeks of treatment with fibrates), the peaks becomes narrow. For the NOVX rats, a large amount of water can be found in Haversian channels and transverse Volkmann canals but the volume of water located in middle cavities is reduced for OVX rats, for which, a large \(^1\text{H}\) reservoir can be found in the intertrabecular cavities. This effect is transient since, by continuing the treatment of animals with fenofibrates, the average \(T_2\)-distributions become again broaden. At eight weeks of observation, the total volume of Haversian channels and transverse Volkmann canals is relatively small. The peak corresponding to this medium cavities is almost absent from the average \(T_2\)-distributions recorded for ovariectomized rats treated with fenofibrate and sacrificed at eight weeks after ovariectomy.

The metamorphoses of rats’ proximal part of femoris bone, as response to the statins treatment, present a different behavior than the response to the treatment with fibrates (Figure 4, c and f). There are no spectacular changes in the average \(T_2\)-distributions. The evolution of
these distributions for the non-ovariectomized rats is more similar with the evolution of average $T_2$-distributions for the ovariectomized rats compares with the previous two cases (witness and treatment with fibrates). Nevertheless, comparing the average $T_2$-distributions recorded at eight weeks of observation one can conclude that statins can have a benefic effect on the rats which present osteoporosis (OVX-rats) but contrary can be contraindicated for the healthy rats (in this case the NOVX-rats).

Figure 4 – The average $T_2$-distributions for the (a) NOVX – W; (b) NOVX – F; (c) NOVX – S; (d) OVX – W; (e) OVX – F; (f) OVX – S rats groups sacrificed at two, four, six and eight weeks from ovariectomy (observation).

Cavities sizes distributions by $^1$H-NMR

From qualitative/observational analysis, one can go further to a semi-quantitative characterization of the processes undergo by the bone microscopically structures of proximal part of femoris of healthy or ovariectomized rats subjected to natural growth and/or treatment with statins or fibrates. For this semi-quantitative analysis, the bone has to be specially prepared, then to fulfill some conditions: (1) first, we have to eliminate from cavities of interest, as the intertrabecular cavities, the soft organic matter like the bone marrow and then (2) all cavities has to be saturated with water (see the blue color in Figure 2). The first condition was fulfilled by keeping the proximal part of femoris for two months in dry air then, at the end of this period, the bones were dried and the bone tissue can be treated as a porous media. The second condition was fulfilled by keeping the bone’s fragments for another two months in formalin. Then, the water molecules, under self-diffusion, can explore now the entire volume of cavities. In particular, from osteoporosis point of view, our interested will be focused on the time evolution of large pores, i.e., the intertrabecular bone cavities. The ovariectomy induces osteoporosis and effect of treatments can be evaluated by observing the changes, which occur in the characteristics of the peak located at large $T_2$-values from the average $T_2$-distributions measured for the all groups of rats. For that, we will analyze the dimensions of large pores, which, in the approximation of spherical pores, is only quasi-quantitatively. The variations in the local surface-to-volume ratio lead to local changes of the relaxation times according to the well-known relation,

$$\frac{1}{T_2^{\text{measured}}} = \frac{1}{T_2^{\text{free water}}} + \frac{S}{V} \rho$$

where $T_2^{\text{measured}}$ is the transverse relaxation time measured by NMR, $T_2^{\text{free water}} \approx 3 \text{s}$ is the transverse relaxation time of the bulk water, $\rho$ is the surface relaxation, $S$ is the surface and $V$ is the volume of a particular pore. If the trabecular cavities, i.e., the large pores, can be considered (with an acceptable approximation), as sphere, then the surface $S = 4\pi r^2$ and the volume $V = 4\pi r^3/3$ where $r$ is the pore radius. Introducing these relations and values, into an approximation of the spherical pores, the average diameter of a pore can be estimated using the measured transverse relaxation time $T_2^{\text{measured}}$ as,

$$\bar{r}_{\text{pore}} = \frac{18 \cdot \rho \cdot T_2^{\text{measured}}}{3 \cdot T_2^{\text{measured}}}$$

where we observe that for the determination of average pore diameter $\bar{r}_{\text{pore}}$ we have to know also the surface relaxation $\rho$. For that, we will perform some additional. On short first we have to estimate the surface relaxation $\rho$, considering the pores dimension from histological images measured before. Then, with the estimated value of $\rho$ we use the equation (2) for all the normalized average $T_2$-distributions to calculate the distribution of pores diameters.

Discussion

Cavities sizes distributions in rats’ proximal part of femoris

The pores diameter distributions are compared in Figure 4 (g–r) for all six groups of rats sacrificed at two
weeks of observation. Here, the average $T_2$-distributions [from Figure 4 (a–f), which is presented with light gray in Figure 4 (g–r)] measured for the non-ovariectomized (left) and ovariectomized (right) rats were converted using equation (2) into a distribution of pores’ diameters. Moreover, a deconvolution procedure was applied in Origin 8.1 to obtain (in the spherical pores limit), separately, the distributions of small (short dashed lines), medium (dashed lines) and large (continuous line) cavities diameters. The NMR signal arising from bound water has to be used in the mathematical procedure, which imply the deconvolution of distribution curve, but is clear that, from physical point of view, the equations (1) and (2) cannot be applied in this case. The bound water is fixed and cannot explore any pores; therefore, no values are associated in Figure 4 (g–r).

At two weeks of observation, the cavities limits for NOVX rats can be found between a minimum of $\sim 20\ \mu m$ (for all categories) up to a maximum of $\sim 300\ \mu m$ for reference (Figure 4g), $\sim 400\ \mu m$ for the rats treated with fibrates (Figure 4h) and $\sim 700\ \mu m$ for the rats treated with statins (Figure 4i). A larger distribution of pores diameter can be found for the OVX rats. Thus, the lower limit of pores is similar with the limit found in the case of NOVX rats of $\sim 20\ \mu m$ for all categories (witness and treated with fibrates and statins). The upper limit is found to be $\sim 3\ mm$ for witness (Figure 4j), $\sim 600\ \mu m$ for the rats treated with fibrates (Figure 4k) and $\sim 2\ mm$ for the rats treated with statins (Figure 4l). From this point of view seems that, in the early stage, the treatment with fibrates is able to reduce the effect of osteoporosis. From the point of view of the ration between the amplitudes of small, medium and large cavities distributions similarities with the curve recorded for the witness non-ovariectomized rats (Figure 4g) are found for distributions recorded for the ovariectomized rats treated with fibrates (Figure 4k) and for the non-ovariectomized rats treated with statins (Figure 4i). Differences to normal distribution (recorded for non-ovariectomized and non-treated rats – Figure 4g) are found for: (1) ovariectomized reference rats, which is an expected sign of an early osteoporosis; (2) ovariectomized rats and treated with statins, which show that, in the early stage, the treatment is not so efficacy and (3) for non-ovariectomized rats treated with fibrates, which is a warning signal, which must be interpreted in the sense that a treatment applied on healthy bone can lead to undesired effects.

Interesting distributions of cavities are recorded at the end of the observation times, i.e., week eight [Figure 4 (m–r)]. Compared with the beginning of the observation time (two weeks), the cavities size distributions recorded for the non-ovariectomized non-treated (NOVX-W) rats groups presents many similarities. Again, the peak corresponding to medium cavities is the highest peak (Figure 4m), smaller than the corresponding peak measured for the NOVX-W rats sacrificed at two weeks. For this distribution, one can remark the upper limit ($\sim 1\ mm$) for the average pore diameter estimated for the large cavities. A dramatic effect can be observed for the ovariectomized, untreated rats (Figure 4n). The amplitude and the integral area under the medium pores are very small, indicating a large reduction of volume of the Haversian channels and transverse Volkmann canals. As consequence, we observe an increase in the volume of small channels associated with the space between the osteocytes and lacunar-canalicular wall and especially the volume of inter trabecular bones. The range of the distribution of the large cavities is not increasing from week two (Figure 4j) to week eight (Figure 4p) but the relative amplitude does. This observation is consistent with our other NMR measurements [18, 36].

The effect of treatments with fenofibrates and simvastatins is also visible. From the overall shape and the relative peaks amplitudes, one can say that the most efficient way in maintaining the bone architecture is the treatment of osteoporotic bone with simvastatins (Figure 4r). Instead, the treatment of healthy bone with the same drug leads to a certain negative effects (Figure 4o). The same negative effect is observed for the treatment of non-ovariectomized rats with fenofibrates (Figure 4n). In fact, by comparing the relative amplitude of peaks corresponding to large and small proximal parts of femoris cavities, recorded for the treated rats, we can say that a long treatment with fibrates is more destructive than the treatment with statins. At the same conclusion we reach by observing the cavities’ medium sizes distributions, presented in Figure 4q for the ovariectomized rats treated with fibrates. The medium cavities volume, as observed from corresponding peak, becomes smaller. Moreover, grace to the deconvolution procedure one can observe a displacement of these medium cavities to smaller average diameters; therefore, we may assume also a destruction of spaces between the osteocytes and lacunar-canalicular.

The effect of long treatment with fenofibrates can be observed also in an extension of pores distribution.

**The effect of fenofibrate and simvastatin treatments on the rats’ proximal part of femoris**

The last step in our approach is to describe the osteoporotic femoral bone treated with fibrates and statins into a quantitative analysis. In the interpretation of such data, we have to be very careful and to remember that the distributions presented in Figure 4 (g–r) were obtained, from measured normalized $T_2$-distribution averaged for all rats from a subgroup of study, by applying a conversion procedure described by equation (2). However, this conversion is valid into an approximation of spherical pores, and is hard to imagine the Haversian channels and transverse Volkmann canals as perfect spherical pores. Therefore, although we have access to all values, which describes all three peaks (width, range, amplitude, integral area), our discussion will be limited to large pores [18, 37]. The parameter which will be considered for each large cavity will be the average diameter, $\bar{d}$. This is the diameter of large cavities with the most probable dimension. The variation of $\bar{d}$ function of eight weeks of observations are presented in Figure 5 (a–c), compared for non-ovariectomized and ovariectomized rats. The effect of osteoporosis on the healthy (NOVX) rats in clearly observed (Figure 5a) for the large cavities, i.e., the trabecular cavities of proximal part of femoris [18]. Here, the average diameter measured for trabecular cavities of OVX rats is, over the entire observation period, larger than the average diameter measured for NOVX rats.
One can observe also an increase of trabecular cavities for the witness non-ovariectomized rats from ~0.15 mm at two weeks to ~0.21 mm at eight weeks. This behavior can be explained by the fact that at 16 months, the age at the begging of experiment, the rats are not fully developed [38]. For the reference-ovariectomized rats, the increase of trabecular cavities is a combination of natural growth with the induced osteoporosis [18].

Figure 4 (continued) – The distribution of cavities diameters obtained from average T2 distributions grey line using equation (2) for the same group of rats sacrificed at two weeks (g) to (l) and eight weeks (m) to (r) from ovariectomy.

The difference between the treatments with fenofibrates and statins is visible from Figure 5 (b and c). While, in the case of treatment with fibrates (as in the case of untreated rats) during the entire time of observation, the average diameter of proximal part of femoris trabecular cavities is larger for ovariectomized rats (Figure 5b) than for non-ovariectomized rats, the treatment with statins is more efficacies. Here, starting with the week four, the average diameter of proximal part of femoris trabecular cavities measured for ovariectomized rats is, in fact, smaller (with a small quantity which usually falls into an experimental error limit) that the average diameter measured for non-ovariectomized rats (Figure 5c). Comparing our two lipid-lowering drugs, the effect in time is also very different for statins and fibrates. A large difference can be observed for average diameter, $d$ of proximal part of femoris trabecular cavities measured for ovariectomized rats compared with non-ovariectomized rats for groups treated with fenofibrates at four weeks (Figure 5b) and for groups treated simvastatins at two weeks (Figure 5c). Then in the case of treatment with fibrates, this difference is constantly reduced with time. A slight increase of the difference between the average diameters of proximal part of femoris trabecular cavities is observed...
in the case of treatment with simvastatins. This can be considered as a positive influence, which reduces the effect of ovariectomy-induced osteoporosis being in the favor of OVX rats. Another parameter, which can characterize the efficiency of the treatment with both drugs, is related to the time at which the maximum of average diameter of trabecular cavities occurs and correlated with this the remission time. In the case of treatment with fenofibrates, the maximum of proximal part of femoris trabecular cavities \( \bar{d} \) is obtained at week 6 and then for both, OVX and NOVX rats \( \bar{d} \) decays. More efficient is the treatment with simvastatin. This show a maximum for \( \bar{d} \) at two weeks from where, we observe a continuous decay also for OVX and NOVX rats. This result is in contradiction with the observation of Yao et al. [39], which reports that, the treatment with simvastatin at 120 days post-ovariectomy did not prevent nor restore the ovariectomy-induced bone loss in 3-month-old Sprague–Dawley adult female rats.

A better understanding of the effect and efficiency of studied lipid-lowering drugs as result from the observation of the average diameter \( \bar{d} \) of proximal part of femoris’ trabecular cavities can be obtain from the analysis of Figure 5 (d and e). Here, the average diameter \( \bar{d} \) is presented comparatively for reference rats and rats treated with fenofibrates and statins, function of eight weeks of observation.

For the non-ovariectomized rats, the effect of statins treatment during the first six weeks of observation has a negative effect on the proximal part of femoris by increasing the average diameter \( \bar{d} \) of intertrabecular cavities (Figure 5d). Only at the week 8 was observed a smaller value of \( \bar{d} \) measured for treated rats compared with the \( \bar{d} \) value measured for healthy rats. With the exception of week 6 (where the values are similar and inside the measurement error), the values measured for average diameter \( \bar{d} \) of intertrabecular cavities of rats treated with fenofibrates are smaller than the \( \bar{d} \) values obtained for rats treated with statins. Only after eight weeks of treatment, the average diameter of intertrabecular cavities of rats treated with fenofibrates and simvastatins lipid-lowering drugs is smaller than the \( \bar{d} \) of non-treated rats, in rest having a negative effect.

Figure 5 – The average diameters of large cavities from proximal part of femoris for (a) references; (b) treated with fibrates and (c) treated with statins of non-ovariectomized (NOVX) and ovariectomized (OVX) rats function of observation weeks (sacrificed weeks from ovariectomy). The dashed lines are drawn to guide the eyes. Comparison of the average diameters of large cavities from proximal part of femoris for reference rats and treated with fibrates and statins for (d) non-ovariectomized and (e) ovariectomized animal function of observation weeks (sacrificed weeks from ovariectomy).

The situation is different in the case of ovariectomy induced osteoporosis. With the exception of week 4, where the values of average diameter of intertrabecular cavities are the same in the experimental error limit for all rats, the treatment with fenofibrates and simvastatins drugs seems to have a positive effect, which is clearly observed for statins starting with week 6 and for fenofibrates with week 8.

In is well known that the changes in bone porosities influence the risk of fragility fractures in osteoporotic women [40] and the trabecular microarchitecture associated with fractures includes reductions in trabecular plate bone volume, number and connectivity and a more rod-like trabecular network [41]. Studies have shown that osteoporosis decreases the fracture risk by 30–40% compared to non-statin users [42]. In this recent study, Esposito et al. propose a model in which statins inhibit 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase leading to a blocking in the synthesis of mevalonate. Then, the synthesis of important intermediates like FPP (farnesyl pyrophosphate) isoprenoids and GGPP (geranyl-geranyl pyrophosphate) is reduced. The FPP and GGPP plays and important role in the activation of small G-proteins, which regulates the cellular activities. Thereby, the activation of BMP-2 by reduced FPP is favorable to osteoblast differentiation by the regulation of Runk2 gene. Finally, we may conclude that the observed benefic effect of statin treatment on the proximal part of femoris bone’s anabolism, can be associated with a mechanism where statins: (1) promote the osteoblast differentiation; (2) suppress the osteoblast apoptosis via Smad3 activation; (3) block the osteoclastogenesis through the increased expression of the estrogen receptors [42].
Conclusions

We found that the overall distributions of pores diameters lie in the range from 1 μm for all rats to several millimeters for ovariectomized rats. Then, from the evaluation of evolution over a period of eight weeks of observation (for non-ovariectomized rats or time from ovariectomy for ovariectomized rats) of small, medium and large pores, we found that the osteoporosis is clearly observed for the large pores, i.e., the trabecular cavities. It was shown that, in the case of Albino Wistar adult female rats with age 16–18 months, the simvastatin and fenofibrate lipid-lowering drugs treatment reduce the effects of ovariectomy-induced osteoporosis. This conclusion originates from the observation of reduction of the average diameter, $d$, associated with the intertrabecular cavities measured for proximal part of femoris of randomly selected specimens. This finding is in accord with our previous observation and results based on histological images and $^1$H-NMR measurements on the bone with marrow and which were obtained for the same animals. Finally, it is shown that the positive or negative effects of studied treatments are strongly dependent on the duration of treatment and on the bone itself, healthy or osteoporotic.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by CNCSIS–UEFISCDI, project number PN II–IDEI code 307/2011.

References


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Received: January 23, 2015

Accepted: August 4, 2015