Bone histomorphometry revisited

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ABSTRACT

Bone histomorphometry is defined as a quantitative evaluation of bone micro architecture, remodelling and metabolism. Bone metabolic assessment is based on a dynamic process, which provides data on bone matrix formation rate by incorporating a tetracycline compound. In the static evaluation, samples are stained and a semi-automatic technique is applied in order to obtain bone microarchitectural parameters such as trabecular area, perimeter and width. These parameters are in 2D, but they can be extrapolated into 3D, applying a stereological formula.

Histomorphometry can be applied to different areas; however, in recent decades it has been a relevant tool in monitoring the effect of drug administration in bone.

The main challenge for the future will be the development of noninvasive methods that can give similar information.

In the herein review paper we will discuss the general principles and main applications of bone histomorphometry.

Keywords: Histomorphometry; Bone; Bone biopsy; Bone metabolic diseases.

INTRODUCTION

Bone histomorphometry allows the quantitative study of bone microscopic organization and structure. In 1960, Harold Frost[1], an American orthopaedic surgeon, pioneered the study of metabolism, mass and structure of bone, boosting histomorphometry as a technique.

Parfitt in 1980[2, 3] stressed the importance of the study of bone microarchitecture and proposed a set of stereological techniques for its measurement. These techniques would later be used worldwide giving rise to systems of classification, rules and standardization of measurement units. Five years later, the Committee of the American Society of Bone and Mineral Research (ASBMR) decided to put together worldwide researchers to establish common terminology fostering the scientific evolution of the technique. Bone biopsy evaluation is performed in un-decalcified (mineralized) bone samples in order to obtain qualitative and quantitative data about the microarchitecture and metabolism of bone[4].

Bone, as all connective tissues, is composed of cells and an extracellular matrix mainly comprised of a collagen type I network impregnated with hydroxyapatite mineral crystals. The cortical bone is compact, enervated and vascularized. Cortical bone is present in the epiphyses of long bones and coats the bones of the body, giving rigidity and strength. The trabecular part of bone is formed by a network of trabeculae, which confers resistance to compression.

Bone is constantly remodelled by the dual action of two cell types, osteoclasts and osteoblasts. Osteoclasts resorb bone whereas osteoblasts form new bone in a process known as remodelling. This process is required for the development and maintenance of the bone tissue and its imbalance leads to bone diseases such as osteoporosis. Remodelling occurs at bone surface and its rate is higher in trabecular in comparison to cortical bone[5]. The analysis of bone microarchitecture is crucial for the detection of metabolic bone diseases, therefore histomorphometry can be used in several areas as a crucial tool in monitoring bone processes and the effect of drugs[6-10]. In this paper, the authors describe practical aspects of the histomorphometry technique, which may be useful as a guide for researchers who would like to take advantage of its possibilities.

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SAMPLE PREPARATION

BONE BIOPSY

The standard procedure involves the extration of the bone sample from the iliac crest following a systematized technique[1]. A small incision (two centimetres below and behind the anterior superior iliac spine) is made through the skin to achieve access to the external wall of the iliac crest by using a 5 to 8mm trephine,[11-13] which allows the removal of a cylindrical sample of bone (Figure 1). The sample is composed of an internal and external layers of cortical bone separated by an intermediate region of trabecular bone[1,14,15]. After the biopsy, the bone sample is processed in order to maintain its stiffness while it is being cut.

SAMPLE PROCESSING

Once the bone sample is collected, it is crucial to carry out the processing steps in order to prevent the deterioration of the tissue triggered by proteolytic enzymes and, at a later stage, to preserve its microarchitecture.

The different stages of processing have critical points concerning the preservation of microarchitecture. Thus, it is essential to optimize the contact time and temperature between the chemicals and the sample as insufficient fixation causes sample shrinkage, which subsequently leads structural distortions that can abolish histological evaluations.

Sample processing includes five steps: fixation, dehydration, clearing, impregnation and embedding[13]. Fixation is used to control the proteolytic processes and bacteria proliferation. This stage takes place in alcohol 70% for a minimum of 72 hours at 5ºC.

After fixation, the water in the tissue must be removed so that the sample can be placed in an impregnation solution, which is immiscible with water. This process is called dehydration and is achieved by increasing ethanol saturation from 96% to 100% over a period of 24 hours at 5ºC.

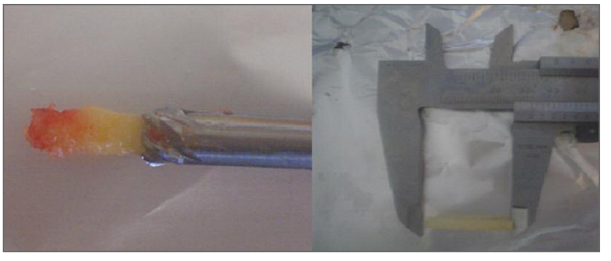

FIGURE 1. Bone biopsy showing the trephine of the drill with the cylindrical sample of bone already obtained

The next step is clearing in which the alcohol present in the bone tissue from the last step (dehydration) is replaced by an intermediate solution, Xylene for 24 hours at 5ºC, after which the sample becomes clear and transparent.

In the impregnation step the sample is embedded in methyl methacrylate for a minimum of 72 hours at -20ºC. This will provide rigidity to the sample without polymerization[11].

After the impregnation, the sample is embedded in methyl methacrylate at a constant temperature ranging from 5°C up to 10°C, depending on the methyl methacrylate used. High temperatures will lead to a quick polymerization, with the formation of CO_2 bubbles inside the sample container[16].

The methyl methacrylate solution should be poured over the bone sample in a glass mold, leaving a small space for air, so that the bottle does not break with the increasing temperature, during the reaction. If there is too much air inside the bottle it may take too long to polymerize. After polymerization, the mold is broken and the block with the sample inside is cut in a parallelepiped shape with a plaster cutter. However, other protocols do exists according to the sample characteristics, as it is possible to adapt the protocol by changing the duration of the sample processing steps and temperature conditions (for instance in the cases of samples that may be too thick or too fatty).

CUTTING

Cutting is performed in a microtome machine with a tungsten blade, capable of cutting calcified bone samples.

The block is placed in the microtome, orienting the sample with the cortical bone perpendicular to the edge of the blade. The cuts should be 5-10µm in thickness, according to the technique to be performed. For example, in the case of structural and static parameters the thickness must be approximately 5 -7µm, on the other hand in the case of dynamic parameters (namely tetracycline incorporation) the thickness required is 7-10µm, allowing a better intensity of tetracycline fluorescence.

The cut bone samples are then placed on glass slides that have already been wiped with alcohol 96%. Once on the glass slide, the bone sample should be stretched out with the aid of a brush to avoid sample wrinkling[16]. To keep the bone specimen stretched and attached to the slide, a polyethylene film is placed on its surface

followed by 48 hours of pressing at 55ºC. The cutting stage is then followed by staining.

Staining allows for the differentiation between structural components and cells within the tissue. There are several different stains available that might be used according to the objectives of the study.

STAINING

The histological staining is specific to target the desired structures[13]. Dyes used have affinity for specific structures allowing their visualization. There are different staining techniques available, such as toluidine blue, von Kossa, phosphatase acid, goldner trichrome, solochrome azurine and perl s method stain[13, 17]. In addition to selective staining, imunohistochemistry technique can also be used to allow antigen detection by using antibodies, providing additional information.

The toluidine blue staining uses a basic dye, which stains all the structures in different shades of blue. Toluidine blue stains mineralized bone as violet blue and the osteoid as pale blue. This staining also allows the visualization of cement lines, which correspond to different stages of bone growth[18].

The von Kossa technique is ideal when a highest contrast between calcified bone and osteoid is needed. Silver nitrate stains the calcified tissue (bone) black, while the trichrome will turn the non-calcified tissue (osteoid) into a red colour[17,19].

The acid phosphatase (a lysosomal enzyme present in bone cells) coloration allows the visualization of osteoclasts within bone tissue[17,20]. With this technique osteoclasts will stain red because they have a content rich in acid phosphatase, while the rest of the tissue will have a blue colour due to toluidine blue which is used as a contrast.

The Goldner Trichrome technique allows a highest contrast between osteoid and calcified bone, better for cellular detail. Light green stains the calcified tissue (bone) green, while the Orange G and Ponceau will turn the non-calcified tissue (osteoid) into a orange/ /red colour[21].

Solochrome azurine staining is ideal to identify the aluminium deposits commonly present in cement and mineralization lines[22]. This technique stains aluminium deposits with a violet/purple colour.

Perl s method stain allows the visualization of reactive ferric iron. The sections are treated with hydrochloric acid to release ferric ions reacting with potassium ferrocyanide to produce a blue compound[23,24].

TETRACYCLINE LABELLING

Tetracycline is fluorescent compound that has the ability to bind to mineralization fronts of amorphous minerals, labelling them with a yellow-green colour under fluorescent light, thus acting as a marker for bone formation and mineralization. Tetracycline should be taken (orally or parenterally) 21 days before bone biopsy. Two doses of this compound are usually taken with an interval of 10 days. This is to allow the identification of two distinct lines (usually synthesized within 3 days) that represent two phases of mineralization[7,11]. This technique allows the dynamic assessment of bone metabolism[1]. In order to perform tetracycline labelling and optimize its visualization, bone sections should be cut ranging from 7-10µm in thickness.

HISTOMORPHOMETRIC ANALYSIS

After processing and staining, the bone slides are visualized in a microscope equipped with a camera. Currently there are several softwares with specific tools that allow histomorphometric analysis. These softwares are optimized for quantitative microscopy and the measurements can be performed manually or automatically, in a semi-quantitative or quantitative setup[25-28]. If the sample does not have a homogeneous trabecular distribution, the error increases when measurements are performed automatically or semi-quantitatively. The evaluation is stoichiometric and

has a set of image analysis tools for the acquisition of results by the assignment of pixels. The sample is analyzed using a gray scale that corresponds to different tissue densities[29]. Within the selected areas, regions with bone debris should not be considered due to its interference with the quantification procedure (Figure 2).

The manual grid is useful to divide the sample in several fields with the same dimensions, allowing an easier quantification and analysis of the histomorphometric parameters (Figure 2). The selected area must be similar in all samples and the same parameters measured in order to standardize the analysis[30,31]. The total sample area analyzed will depend on the requirements of the study.

This technique performs the acquisition of images and data in two dimensions but it can provide measurable parameters for a three-dimensional evaluation that can be obtained using stereological formulae.

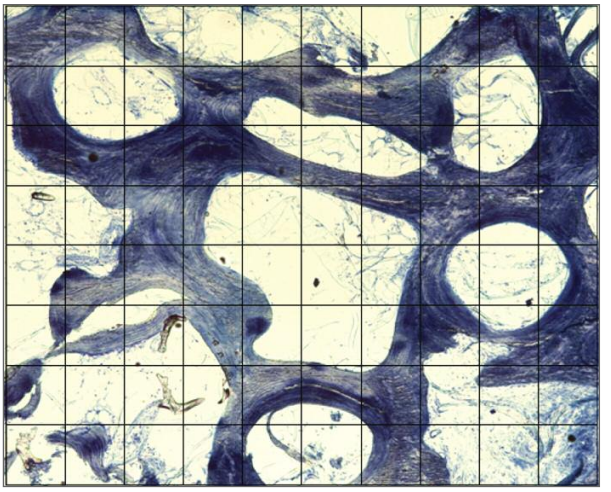

FIGURE 2. Trabecular bone sample with a special grid, which allows the direct measurement of parameters

HISTOMORPHOMETRIC PARAMETERS

Histomorphometric parameters are described according to the American Society for Bone and Mineral Research (ASBMR) nomenclature committee (Table I).

Several histomorphometric detailed parameters are listed below:

1. Bone area (2D, volume in 3D) is the percentage of occupied area by calcified bone in relation to the total area.
2. Trabecular width (2D, thickness in 3D), (Tb.Wi) = (2xB.Ar)/B.Pm, distance across individual trabeculae.
3. Trabecular number (Tb.N) = (B.Ar/T.Ar)/Tb.Wi, number of trabeculae that a line through a trabecular compartment would hit per millimetre of its length.
4. Trabecular separation (Tb.Sp) = (1/Tb.N) – Tb.Wi, distance between trabeculae.
5. Osteoid with (2D, osteoid thickness in 3D) is measured at four or eight equidistant points on seams longer than 600µm in length. A minimum of 20 seams per biopsy is measured on the same sections used for osteoid perimeter. All seams with a width of 3µm or more are included.
6. Osteoid perimeter (2D, osteoid surface in 3D) is the percentage of the bone surface occupied by osteoid in relation to the total bone perimeter.
7. Osteoid area (2D, osteoid volume in 3D) is the percentage of area by osteoid in relation to the total area.
8. Osteoblast perimeter (2D, osteoblast surface in 3D) is the percentage of the bone perimeter occupied by osteoblasts in relation to the total bone perimeter.
9. Mineralizing perimeter (2D, mineralizing surface in 3D) is assessed as the extent of bone perimeter that exhibits either single or double labels. Measurements are performed using fluorescence microscopy.
10. Mineral apposition rate is calculated as the mean distance between double labels divided by the time period between the administrations of the two labels.
11. Mineralization lag time is the interval between deposition and mineralization of matrix.
12. Bone formation rate is the amount of bone formed per year on a given bone surface.
13. Eroded perimeter (2D, eroded surface in 3D) is the percentage of bone perimeter occupied by eroded cavities.
14. Osteoclast perimeter (2D, osteoclast surface in 3D) is the percentage of bone perimeter covered by osteoclasts.

Histomorphometric measurements can be determined using a specific software which analyzes the microscopic images.

TABLE I. HISTOMORPHOMETRIC PARAMETERS

Parameters	Abbreviations
Strutural parameter	
Bone volume/Tissue volume (%)	BV/TV
Trabecular thickness (µm)	Tb.Th
Trabecular number (/mm)	Tb.N
Trabecular separation (mm)	Tb.Sp
Static formation parameter	
Osteoid Thickness (µm)	O.Th
Osteoid surface/bone surface (%)	OS/BS
Osteoid volume/bone volume (%)	OV/BV
Osteoblast surface/bone surface (%)	Ob.S/BS
Dynamic formation parameter	
Mineralizing surface/bone surface (%)	MS/BS
Mineral apposition rate (µm/d)	MAR
Mineralization lag time (d)	Mlt
Bone formation rate/bone surface ($\mu m^3 \times \mu m^{-2x} y^{-3}$)	BFR/BS
Static resorption parameter	
Osteoclast surface/bone surface (%)	Oc.S/BS
Eroded surface/bone surface (%)	ES/BS

BV: Bone volume; TV: Tissue volume; Tb: Trabecular; Th: Thickness; N: Number; Sp: Separation; O: Osteoid; OS: Osteoid surface; BS: Bone surface; OV: Osteoid Volume; Ob.S: Osteoblast surface; MS: Mineralizing surface; MAR: Mineralizing apposition rate; Mlt: Mineralizing lag time; BFR: Bone formation rate; Oc.S: Osteoclast surface; ES: Eroded surface

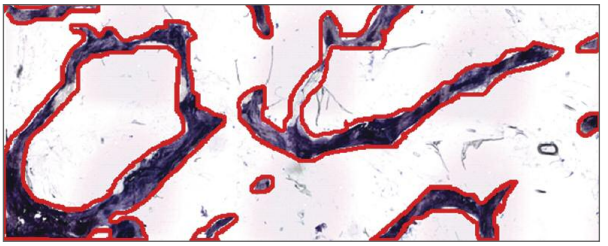

FIGURE 3. The trabecular area is obtained outlining the trabecula sample. This procedure is manual

FIGURE 4. Trabeculas are selected using the gray scale of the software, allowing the area quantification. This procedure is semi-automatic, because the investigator must decide what he considers not to be trabecula

Direct measures can be done manually, outlining the sample (Figure 3) or semi-automatically using a grey scale (Figure 4). Several measurements are indirect, requiring the directly values previously obtained.

HISTOMORPHOMETRIC APPLICATIONS

Histomorphometry evaluates *in vivo* bone metabolism and microarchitecture and can contribute to the diagnosis of bone-related diseases.

Regarding diagnostic application, histomorphometric analysis of bone michroarchitecture has been used for the detection of metabolic bone diseases such as osteomalacia[32], renal osteodistrophy[9,14,15,33-35] and osteoporosis[36]. Measurements of bone turnover, mineralization and volume, help to classify the different diseases since it reveals different histomorfometric profiles[33] (Table II).

Osteomalacia is a metabolic disease that involves abnormal mineralization of the bone matrix. Histomorphometric findings reveal an impairment of bone mineralization characterized by increased osteoid thickness, surface and volume. In severe cases, dynamic parameters are impossible to access, as the distance between double labels of tetracycline are undetectable, reflecting absence of mineralization[37].

TABLE II. HISTOMORPHOMETRIC FINDINGS IN DIFFERENT BONE CONDITIONS

Bone disease	Turn-over	Mineralization	Bone volume
Osteoporosis	High	Normal	Low
Osteomalacia	Low	Abnormal	Low
Adynamic bone	Low	Normal	Low
Osteitis fibrosa	High	Normal	High
Bone Paget disease	High	Abnormal	Normal

Parfitt *et al,* developed a mineralization index, based on several histomorphometric parameters. This is an important key tool to analyze bone biopsies for the diagnosis of different kinds of bone diseases such as renal osteodystrophy and osteomalacia[32].

Histomorphometry is also useful in other bone metabolic disorders such as osteoporosis, where it is used in randomized controlled trials to monitor the effect of new drugs in bone microarchitecture and bone remodelling[6,7].

In addition, histomorphometry measures some aspects of bone quality such as microarchitecture, texture of the osteoid matrix and the presence of changes in the mineralization process of both surface and interstitial layers[36].

HISTOMORPHOMETRY LIMITATIONS

One of the major limitations of histomorphometry is the invasive acquisition of the sample requiring bone biopsy[1]. A bone biopsy carries potential complications such as pain, hematoma, wound infection and, rarely, neuropathy[38]. Additionally, the information provided by the biopsy only gives a "photo"/time frame of a dynamic process.

Bone microarchitecture can be assessed by new non-invasive techniques, such as micro-CT, using 3-dimensional reconstruction. They have a strong potential for microstructural analysis, however, only provide structural information useful for the diagnosis of osteoporosis but inadequate for other metabolic bone diseases.

Histomorphometry *per se* is time consuming compared to the conventional histology technique. In ad-

dition, histomorphometry needs highly specialized technicians to prepare samples and to deal with specific equipment[1].

CONCLUSION

Bone histomorphometry is a commonly used technique in the field of medical sciences. It has been used not only as a diagnostic method in clinical practice (osteomalacia and renal ostheodistrophy) but also as an important tool in medical research.

The main challenge for the future of histomorphometry is to minimize tissue disruption in order to keep the structure close to the *in vivo* appearance.

Although micro-CT, has recently been proposed for the analysis of bone microarchitecture and microstructure, bone histomorphometry is still the gold standard for bone metabolic and mineralization evaluation.

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