Bone Histomorphometry

—Practice and Technique—

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Introduction

Histomorphic examination of an abnormal tissue is often essential for precise diagnosis and indeed the biopsy has become an integral part of the practice of medicine. With bone biopsy has been a routine procedure for identification of localized bone lesions for decades, it is less often used in evaluating metabolic bone disease.

Instrumentation

For biopsy of the ilium it is essential that a oritable instrument is used. The standard orthopedic biopsy using a hammer and chisel or equivalent instruments causes considerable postoperative pain and is useless for quantitative histology. The Jamshidi needle is satisfactory for measurement. There are several types of bone biopsy trephines that are now commercially available and they are essentially based on the instrument used for a bone marrow biopsy. The trephines are of two general categories ; iliac creast and transiliac.

Iliac crest trephines are obviously limited in size by the thickness of the ilium. They have an internal diameter of 3 to 5 mm. Specimens obtained via the crest consist mainly of trabecular bone ; the small amount of cortical bone at the upper end is too close to ligaments and muscle attachments to be useful. There is usually some distortion of trabecular architecture because of the rotary to and fro motion of the instrument. Preservation of trabecular architecture is usually better with an electrically driven trephine. Transiliac trephines are of larger internal diameter(6 to 10 mm) and the biopsy core contains both cortical(external and internal) and trabecular bone of adequate quantity and good quality. There is essentially no distortion of bone architecture since it is a cylindrical plug of bone drilled out of the ilium in toto. However, since the biopsy site has to be accurately located vertically as well as horizontally, there is greater sample variation than for crest biopsy.

Procedure for Obtaining Bone Biopsy

Irrespective of the approach used, iliac bone biopsy can safely be performed in out-patients and neither hospitalization nor operating room time is required. It is essential to demonstrate a normal coagulation profile (normal prothombin and partial thromboplastin time and platelet count) prior to the procedure. This is particularly important in hemodialized patients or in those with hematogenous malignancies. In patients on maintenance hemodialysis, biospy should be performed on an interdialysis day and the postbiopsy dialysis should be delayed by one day. After an overnight fast, the patient is premedicated prior to starting the procedure. The skin over the appropriate biopsy site is then prepared taking the usual antiseptic precautions.

1. Iliac Crest Biopsy

With the superior approach the patient lies in the supine position and the anterior iliac crest area is prepared ; 10 to 15 ml of 1% lidocaine

hydrochloride is used to anesthetize the skin and subcutaneous tissue. A 1- to 2-cm long incision is made directly over the iliac crest border, about 2 to 3 cm posterior to the anterior superior iliac spine. The periosteum is exposed, anesthetized, and incised in a similar fashion. The outer guide of the instrument is firmly applied (vertically) over the exposed bone. Holding the device the plane of the body of ilium, the inner trephine is slowly advanced with a rotary motion applying a moderate pressure. The depth is determined by the instrument size and is usually 1 to 2 cm long. The specimen is freed by fracturing its base with a gentle rocking motion and the instrument is withdrawn with specimen held in the trephine. Techinically this is the easiest of the bone biopsy procedures, but the specimen quantity and quality are not always satisfactory.

2. Transiliac Bone Biopsy

This is slightly more invasive than through the crest, but with proper anatomic orientation, the procedure can safetly be done in 30 to 60 min. During this lateral approach the patient assumes a supine lateral position with semiflexion at both hip and knee. A triangualr area is outlined with the anterior superior iliac spine as a guide. Roughly it is a 5 cm isolateral inverted triangle, the base of which is formed by the iliac crest border. This goometric outlining is important to minimize the site variation between sequential biopsies. More local anesthetic is needed because of the bulk of subcutaneous and muscle tissue in this location. A 2-to 3 cm long incision is made, and the glistening fascia lata is exposed. The fascia itself is then incised along its fiber direction, and the dissection is continued until the scalpel torches the periosteum and bone underneath. The patient will feel slight pain at this point. Scraping of the periosteum after further local anesthesia is helpful in minimizing the discomfort. With the index finger, the surface of the bone is freed of its muscle attachments, and the plane of the body of the ilium is ascertained. The instrument is introduced and the outer sleeve is fixed of the bone perpendicular to its plane. The procedure is then completed with the inner drilling trephine by rotary (90 to 180° clockwise and anticlockwise) motion while applying steady but gentle pressure. Although there is a quard to prevent excessive advance of the trephine, it is necessary to feel and distinguish the differences in resistance of the outer cortex, the intervening trabecular bone, and the inner cortex to avoid injury to the internal organs. After the trephine has pierced the inner cortex it is rotated 360° 2 of 3 times to achieve separation of the specimen from the inner muscle attachments. The trephine is withdrawn with circular, clockwise motions and the outer sleeve is removed. Incised fascia lata must be sutured with interrupted absorbable chromic material to avoid herniation of the underlying muscle. The wound is then closed in two layers. A pressure dressing is then applied.

Complications

Sinde these two types of bone biopsy procedures are invasive, local hematoma and wound infection are the two most frequent conplications and then to occur more commonly in patients on maintenance hemodialysis. Hematoma is more common with the transiliac approach and less with iliac crest approach. Transient neuropathy due to severing or entrapment of one of the cutaneous branches of the femoral nerve may cause hyperesthesia at the biopsy site. This usually lasts about 2 to 3 weeks, but occasionally the patient may feel local paresthesia for up to 4 to 6 months. Fracture of the pelvis has been reported in two patients with osteomalacia.

There are two complications that are specific to transiliac bone biopsy. First, biopsy specimens may dislodge from the trephine during withdrawal. Second, the curring end of the trephine (a 2-cm long piece)may separate from the shaft after penetrating the outer cortex of ilium.

Pain during and after the bone biopsy proce-

dure is an expected complication. The pain is slightly more with the transiliac than with the iliac crest approach. It is usually mild in the majority of patients and does not last for more than 2 to 3 days. It only a minority of patients (less than 1 %) does the pain last more than 7 days.

Processing of Undecalcified Bone Specimens for Bone Histomorphometry

The reasons for avoiding decalcification are obvious and numerous ; most important is the conservation of the differences between calcified and uncalcified bone (osteoid) in order to be able to diagnose and measure abnormalities in the bone formation and/or bone mineralization processes. Other reasons also have to be kept in mind ; the ability to make dynamic measurements of the meneralization rate through fluorescent labels and optimal conservation of the cell structure and minimal shrinkage of the bone marrow to avoid artifacts at the bone-bone marrow interface are among the most important. Undoubtedly, bone histomorphometry cannot be performed satisfactorily on decalcified specimens.

General Comments on the Methods Described

1. Taking the Biopsy

The trephine should be a minimum internal diameter of 6mm or, for bitter results 8mm. It is critical that the biopsy needle teeth always be perfectly sharp. It not, it will be impossible to go through the bone without pushing. This results in compression and fracture of the specimen, markedly compromising the histomorphometry. When taking the biopsy, the physician has to be strictly instructed not to push through the bone, but, instead, to turn very gently back and forth. The same precautions apply when pushing the biopsy out of the needle.

2. Fixation

Fixation has to be immediate and fixative solution should be cold (4°C). Of all the fixatives I have used, 70% ethanol has proven to be the best for bone, I believe that the main advantage of this procedure is that dehydrations is carried out at the same time as fixation and therefore avoids the overnight washing in water that has to be performed with formalin. This usually results in swelling of tissues, followed by shrinking during dehydration, and often leads to more retraction at the bone marrow-bone interface.

Fixation-Dehydration Procedures

1) Place biopsy immediately in 70% ethanol
2) Vacuum for 1 hour and store in 4°C for 24 hours
3) Place biopsy in 95% ethanol and vacuum for 1 hour and store in refrigerator for 24 hours.
4) Place biopsy in 100% ethanol and vacuum for 1 hour and store in refrigerator for 24 hours. Repeat this step 6 times.

3. Prepare Methyl Methacrylate Solution

1) Uninhibited Methyl Methacrylate

Use glass column (4.5cm Intermal diameter X 63cm length). Fill the column to 47cm with dry alumina. Fill remainder of column with methyl mehtacrylate and collect uninhibited methyl methacrylate and store 4°C.

2) Distearate PEG

To melt one whole bottle of PEG 600(100gm) in the oven. Simultaneously melt 42.86gm of PEG6000 in a glass bottle in the oven. Poured the melted PEG600 into the melted PEG6000 and stirred. Poured mixture onto aluminum foil. Allowed mixture to harden and store at room temperature in the bottle.

3) Make Methyl Methacrylate Solution

54ml uninhibited methyl methacrylate
5gm PEG
2.5ml dibutylphthalate

Dissolve with gentle heat. Measure volume. Add Benzoyl peroxide according to volume.

4. Place Fixed Bniopsy Specimen in Methyl Methacrylate (M & A) Solution and Vacuum for 1 Hour. To Store in Refrigerator for 48 Hours.

5. Place the Biopsy Specimen in Another M & A Solution and Vacuum for 1 Hour and to Store in Refrigerator for 48 Hours.

6. Embedding the Specimen.

Place the biopsy specimen in 5ml of Methyl methacrylate solution and add 2 drops of JB-4 embedding kit component B. Vacuum for 10 min. in the flow of nitrogen gas. And let the specimen to polymerize to 24 hours in room temperature 24 to 28°C.

Embedding of the specimen after fixation has to be performed according to the following goals

1) The bone specimen must be infiltrated and not only surrounded by the embedding material ; the infiltration procedure must therefore be carried out slowly for best results.

2) The embedding material should ideally be as hard as the calcified bone itself to avoid vibration and fractures during sectioning.

7. Sectioning

The microtome has to have enough power for the size of the specimen to be sectioned. The Jung K sliding microtome is best but the Jung Autocut is very sufficient for small pieces. The knives have to be of the HK-2 profile ; the angle of the HK-1 is too small, resulting in frequent damage to the edge during sectioning and the angle of the HK-3 is too wide, resulting in increased shattering due to the marked change in direction that the section has to take to go over the knife. The thickness of the section which is best for good cellular detail is 4 to 5 μm. 8um is a bit too thick for fluorescence, on the other hand, 8 um is too thin for a good contrtast and better results are obtained between 10 and 15um. Most important for quality of the morphology is the flatness of the sections at the end of the procedure.

8. Staining

The goal one wants to achieve when staining sections of undecalcified bone is to make clear and reliable distinction between calcified bone and osteoid tissue and, at the same time, to get as much cellular detail as possible, especially for those cells which are to completely dissolve theplastic embedding medium the most reliable stain for the distinction between osteoid and calcified bone is the Von Kossa silver impregnation. The most commonly used stain in bone laboratories is the Goldner modification of the Masson trichrome. In our laboratory, we have selected toluidine blue at acid pH as the stain giving us the most satisfactory lamellar pattern under the polarizing filter. We also selected acid solochrome azuring stain for aluminum detection. Finally we mounted unstained section to see fluorescent labelling.

Bone Histology and Histomorphometry

1. Indications for Bone Biopsy

Bone biopsy in the clinical setting are follows ; suspected osteomalacia, diagnostic classification of renal osteodystrophy, osteopenia in young individuals(younger than 50 years of age), osteopenia in individuals with abnormal calcium metabolism, hereditary childhood bone diseases that present problems in terms of classification, and evaluation of treatment in certain disesaes (e.g. osteomalacia, hypophosphatasia).

2. Criteria for Good Quality Bone Biopsy Samples and Sections

The good quality bone biopsy samples show intact and present both cortices, uncompressed and untwisted sample and adequate material for complete analysis. The good sections shows intact trabecular structure (e.g. no cracks or fractures), closely aligned marrow with trabecular

surfaces, appropriate staining, and intact tetracycline labeling.

Tetracycline Labeling

The following attributes are required of an optimal bone label for this purpose : 1) nontoxic, 2) inexpensive, 3) simple, 4) widely available, 5) stable, 6) detectable, 7) labels new bone, and 8) useable as a tissue-time marker.

Tetracycline incorporates itself into new bone at the time it is formed, and it remains in situ as long as the crystal remains intact both in vivo and in vitro. It is easily detected by fluorescence under excitation by blue light at 360-mm wavelength ; it is cheap, nontoxic, simple, and widely available. The drug accumulates at the plane of initial calaification (the calcification front, zone of demarcation) during the time it is present in the circulation. It accumulates in the form of a band at this plane and if the circulating levels are maintained for a period of the time, the width of the band can function as a tissue-time marker.

Democlocycline fluoresces with an orange color and all the remainder fluoresce with a light lemon color. We uses tetracycline hydrochloride (250mg four times daily) or democlocycline(150mg four times daily). The tetracycline must be taken on an empty stomach. No dairy products or calcium supplements should be taken for one hour before and after the tetracycline. The minimum schedule is 2 days of label, 0 days free, 2 days of label then 5 days before biopsy (2-10-2 : 5).

Histomorphometric Indices

Bone histomorphic parameters can be divided into two groups, one is static parameter using light microscopic image, the other is dynamic parameter using teracycline labeling. Static parameter provides information on the amount of bone present and proportion of bone surface engaged in a particular phase of remodeling activity. Dynamic parameter yield information of the rate of cell-mediated processes involved in remodeling process.

1. Static Parameters

1) Osteoid Volume (OV/BV %)

Fraction of a given volume of bone tissue (mineralized bone+osteoid) that is osteoid.

2) Osteoid surface(OS/BS %)

Fraction of the entire trabecular surface that is covered by osteoid seams.

3) Ostoeid Thickness(O, th, μm)

Average width of osteoid seams.

4) Cancellous Bone Volume (C-BV/TV %)

Fraction of a given volume of whole cancellous bone tissue (bone+marrow) that consists of mineralized and nonmineralized bone.

5) Eroded Surface (ES/BS %)

Fraction of the entire trabecular surface that is occupied by resorption bags (Howship's lacunae)

2. Dynamic Parameters

1) Mineralizing Surface (MS/BS %)

Measure of proportion of bone suface of which new mineralized bone was being deposited at the time of labeling. Fraction of the trabecular surface being double teracycline labeling plus one half of the single labeling surface.

2) Mineral Apposition Rate(MAR/μm/day)

Measure linear rate of production of calcified bone matrix of osteoblasts. Calculated by dividing average distance between first and second tetracycline labels by the time interval.

3) Bone Formation Rate

(BFR/BS, μm3/μm2/day)

Volume of mineralized bone made per unit surface of trabecular bone per year. Calculated by multiplying mineralizing surface by mineral apposition rate.