AFM AND NANOINDENTATION CHARACTERIZATION OF HUMAN LAMELLAR BONE PREPARED BY MICROTOME SECTIONING AND MECHANICAL POLISHING TECHNIQUE

INTRODUCTION: The lamellar structure of osteonal bone has been extensively studied by scanning electronic microscopy (SEM), polarized light microscopy (PLM), and transmission electronic microscopy (TEM). Previous works show that these lamellae are results of various orientation of collagen matrix or structural difference among them. Under these assumptions we can imagine that these lamellae with structural/component difference or orientation/organization difference may respond differently to external physical forces. A very typical case to apply the physical force is in the process of sample preparation. The aim of sample preparation is very simple: to remove excessive materials and to expose the surface of interest. How will the lamellar surface look like under different preparation method, for instance, microtomy section using diamond knife and mechanical polishing using hard alumina particles? How will the lamellar structure react to these forces? If we zoom in and look at the details of these processes from a tribological view, we may find out that cutting involves a higher shear rate and exerts a shear stress upon materials ahead of the knife. Grinding is a process of plastic deformation with hard abrasive particles removing small amount of surface materials. The structural/orientation difference will be clues revealing the specimen- preparation- dependent surface features. Our studies investigated the surface of the lamellar bone by atomic force microscopy (AFM) and nanoindentation. We want to characterize the surface features by measuring the roughness and elastic modulus of thick and thin lamellae of the bone samples prepared by two different methods.

METHODS: A bone sample was obtained from the mid-shaft of one human femur (52 years old, male; cause of death: sudden heart attack). Two sections were cut from the mid-diaphysis of the bone with a diamond saw (Buehler ISOMET 1000, Buehler, Lake Bluff, Illinois) along the longitudinal direction. Each section was then cut transversely into two pieces, with the two mating faces representing the surfaces on which nanoindentation testing was performed. One piece was prepared by microtome sectioning while mating face was prepared by mechanical polishing. Bone samples were embedded in low viscosity epoxy resin (Buehler ep-thin, Buehler, Lake Bluff, Illinois) and cured for about one night in the laminar hood. Samples prepared by mechanical polishing were ground using carbide paper (Carbimet paper discs, Buehler) in ascending grit order. After grinding, the sample was washed and polished using 0.05µm alumina suspension (Gamma Micropolish #3, Buehler, Lake Bluff, Illinois) on micro-cloths (TEXMET®, Buehler, Lake Bluff, Illinois). Samples prepared by microtome sectioning were cut by diamond knife (Leica Ultracut UCT ultramicrotome). Both the polished and microtomed samples were sonicated in distilled water to remove debris on the sample surface.

AFM (NanoScope IIIa/Dimension 3100, Digital Instruments, San Barbara, California) images were taken using Tapping mode at the appropriate setpoint, scanning rate, and feedback parameters. Regions of typical osteonal appearance of the cross-sectioned surfaces from samples are selected of interest. The magnification scales are chosen at 50x50 µm and 20x20 µm. At these scale levels surface features are easily distinguishable between the thick and thin lamellae in each group. Before the roughness analysis all the images are subjected to the same order of noise reduction such as flattening and planefit. Each measurement is randomly selected from regions of thick or thin lamellae using a region of interest (ROI) of 1x1 µm dimension. At this dimension, ROI can be accommodated completely into the thick or thin lamellae region. The mean roughness of each box area is calculated using:

$$R_s = \frac{1}{L_x L_y} \int_{0}^{L_y} \int_{0}^{L_x} |f(x, y)| dxdy$$

where \(f(x, y)\) is the surface relative the center plane and \(L_x\) and \(L_y\) are the dimensions of the surface. Higher magnification (1x1 µm) phase images were obtained on individual thick and thin lamellar regions on both groups to compare the differences.

Nanoindentation was performed using the TriboIndenter (Hysitron, Inc., Minneapolis, MN) on cross-sectioned osteon of mechanically polished sample under a constant load of 1500 µN. Indentation modulus and hardness of each lamella was determined using the Oliver-Pharr method on the unloading curve of stiffness data. One-way ANOVA was used to see if any differences exist among the means of groups of roughness data. The Bonferroni method was used for post hoc pairwise multiple comparisons. Independent t-test was used to test the difference of indentation modulus and hardness of thick and thin lamellae of mechanically polished samples.

RESULTS: AFM images of the lamellation structure around an osteon of microtomy and polished samples were compared at different scales. Figure 1 revealed a clear lamellar structure around a Haversian canal. The thick and thin lamellae are seen to be alternatively light and dark in appearance. The thick lamellae are about 5µm wide in the radial direction from the center of the Haversian canal. The thickness of thin lamellae is much shorter, about 1µm in length. Figure 2 illustrates the lamellar structure of an osteon after diamond cutting. Thick and thin lamellar structures are not so distinct or seem to be more flat than those of Figure 1. More details can be observed on the lamellar regions at higher magnification.

DISCUSSIONS: Combination of results from AFM and roughness measurement demonstrates the materials in thick lamellae are more resistant to mechanical stress than thin lamellae during polishing. The roughness of thick lamellae and thin lamellae are significantly different after mechanical polishing, but are not significantly different upon microtome sectioning. The wearing out process as well as the cutting process on the lamellar bone surface are both very intriguing; especially since the structure of lamellar bone is hierarchical and complex.