AN EFFECTIVE WAY OF ASSESSING CROSSLINKS OF COLLAGENOUS PROTEINS IN BIOMATERIALS AND TISSUES.

S. Gunasekaran, B.R. Constantz, M. Monjazeb, J. Skedros† and R.D.Bloebaum†

Norian Corporation,
Mountain View, CA-94043

Introduction:

Cross-linking of collagenous implants plays a significant role in increasing their physical strength and resistance to biodegradation, as well as attenuating the immune response. The level of crosslinking in collagen fibres varies in different tissues relative to their function and their mechanical property requirements. Therefore, it is necessary to study those differences in natural tissues in order to develop an equivalent model for a biomaterial.

It is well understood that most of the cross-links of collagen are due to the availability of free amino-groups on the surface of the molecules. However, there is no simple reliable biochemical method presently available to assess the extent of the interaction of different crosslinking agents with the e-amino groups of the protein[1,2]. A modified Formol titration technique[3] seems to be an effective method for determining the relative amounts of available amino-groups on the surface of the proteins. This test is time consuming and has problems. The primary aim of this investigation is to develop a more practical method to test the level of amino-groups available for aldehyde cross-linking in collagen or gelatin. When the molecule is fully cross-linked by an aldehyde, all possible reactive amino sites of the protein are occupied for the cross-linking. The remaining unreacted aldehyde-groups are an indirect measure of cross-linking using the rosalinine dye of the Schiff reagent.

Our secondary goal is to assess the variation in the relative levels of protein cross-linking between two areas of a bone that are under different stress conditions[4]. Mule deer calcaneus bone is an excellent model to study the chemical differences between the compressive and the tensile portions of a given bone. The superior portion of the bone is always under compression while the inferior section is in tension. We attempted to study the chemical changes occurring to organic (mainly collagen) and inorganic (hydroxyapatite) components of calcaneus bone.

Methods:

Estimation of Protein Cross-linking by Schiff reagent test through Amino-group Determination:

In brief, 150 mg of acetone precipitated protein was powderized and soaked in 10 ml of 0.05 M Borate buffer (pH 9.0) containing 30 µg of formaldehyde. After 16 hrs of incubation at 37°C, the sample mixture was centrifuged and 50 µl of supernatant was added to 5 ml distilled water and 50 µl of Schiff reagent (1% rosalinine). Following 20 min incubation at 37°C, the color developed was measured at 540 nm. Higher the optical density reflects lesser free amino groups in the protein (ie. the test sample is more cross-linked). For every experimental run, a standard curve for aldehyde estimation was made.

During the validation of the method, different amounts of gelatin (0-300 mg) was tested and the results are reported. The main precautions taken in the methodology are:
Plastic containers were avoided all the time. Screw capped glass vials were used to avoid any air-borne contamination. Never used ethanol during the incubation, which interferer the results. Borate buffer was not used during the rosalinine dye incubation.

Effect of bone mechanics on protein cross-links:

Seven Mule deer calcaneus bones were used. Age or sex of the animal were not controlled because each bone has served as its own control. Each specimen was carefully cleaned of muscle and connective tissues. The bone was sectioned into superior (compressive stress) and inferior (tensile stress) portions and ground to approximately 500 micron size particles. Each ground sample was first analyzed by X-ray diffraction (XRD) and Fourier Transform Infrared (FTIR). The samples were then demineralized and the proteins (mainly collagen) were characterized for amino acid composition. Subsequently, using the above protocol the relative amounts of protein cross-linking were estimated from the level of reactive amino groups available in the proteins.

FIGURE:1

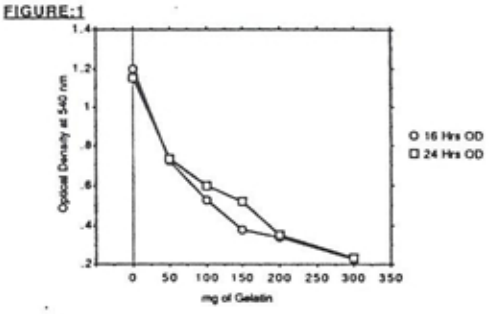
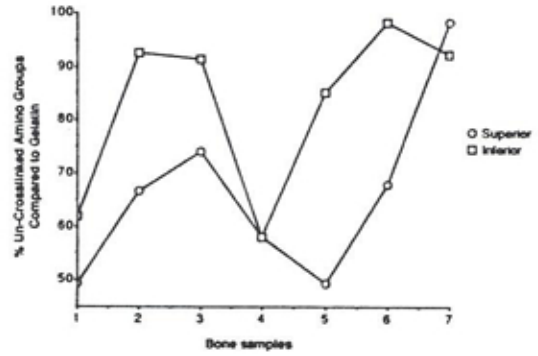


FIGURE:2
Cross-link differences within the Calcaneus Bone



Results and Discussions:

Figure1 indicates that higher amounts of protein (gelatin) consumed higher quantities of formaldehyde which is reflected in lesser values of OD. Under optimal conditions 150 mg of gelatin reacted with approximately 8.4 µg of formaldehyde. The reaction was totally completed at 16 hours. The method also found to respond well to purified collagen samples.

Calcaneous bone portions showed no significant difference between the XRD patterns or the amino acid analyses . However, the FTIR data showed certain differences in the amide peaks of the spectra. Highly significant changes were noticed in the relative amounts of collagen cross-linking between the two bone sections. Five out of seven samples had an average of 24% higher cross-linking of collagen in the superior (compressive stress) portion compared to samples from the inferior (tensile stress) segment of the bone. We also noticed that one of the samples showed an increase (6%) in cross-linking at the inferior side compared to the superior (Fig.2). We attribute this anomaly to lack of skeletal maturity since the animal ages were unknown.

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†Div. Orthopedic Surgery, University of Utah Med Center, Salt Lake City, UT-84148