SCANNING ELECTRON MICROSCOPY EXAMINATION OF COLLAGEN NETWORK MORPHOLOGY AT THE CARTILAGE, LABRUM, AND BONE INTERFACES IN THE ACETABULUM

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Abstract

Sheep acetabula of approximately 1cm^3 in dimension consisting of cartilage, labrum, and bone were prepared for scanning electron microscopy. Examination of collagen architecture at the tissue interfaces fixed by conventional chemical, microwave enhanced chemical and cryo-fixation followed by freeze substitution were compared. Successful cryo-fracturing of dehydrated samples in liquid nitrogen cooled solvent was not possible. However, the specimens became sufficiently brittle when frozen in liquid nitrogen after critical point drying, and fracturing produced more consistent planes allowing imaging of the three interfaces. The labrum/cartilage interface was best preserved with cryo-fixation whereas the cartilage/bone interface was better visualized after chemical fixation due to an unidentified substance covering the collagen fibers after cryo-fixation. Finally, the labrum/bone interface was preserved similarly by both chemical and cryo-fixation. For a specimen containing three different tissues types of both hard and soft variety with mixed collagen fiber orientation, it is very demanding to find one fixation method which will enable detailed examination of the collagen fibers.

Key Words: Scanning electron microscopy, collagen, morphology, acetabular labrum, articular cartilage, subchondral bone.

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Introduction

The fibrocartilaginous acetabular labrum has a triangular shaped cross-section, and is attached to the bony margins of the acetabulum for 270° around the acetabular opening. It is contiguous with the transverse acetabular ligament, spanning the acetabular notch, forming a complete circle around the acetabulum. In addition to its bony attachment, the labrum is also attached to the acetabular articular cartilage forming a contiguous articular surface. Hence the labrum is attached to the articular cartilage, and both are attached to the underlying bone. At each of the interfaces, collagen fibers span across the interface transmitting shear and tensile traction forces.

Torn acetabular labra often occur in association with acetabular dysplasia (Dorrel and Catherall, 1986; Klaue *et al*., 1991). These tears cause severe debilitating pain and may contribute to degenerative changes in the acetabular articular cartilage. Klaue *et al*. (1991) hypothesized that in dysplastic hips with a shallow acetabulum, abnormally high shear forces may cause detachment of the labrum from the adjacent articular cartilage and bony acetabular rim. They suggested that the initial tear may begin at the articular cartilage labrum interface. Although this hypothesis is consistent with the often observed formation of subchondral bony ganglia or cysts, produced by a valvular pumping mechanism of joint fluid through this initial partial tear (Jayson and Dixon, 1970), labral tear mechanisms have not been demonstrated.

Examination of tissue morphology should provide insight into these tearing mechanisms. To the authors' knowledge, only two previous studies have used the scanning electron microscope (SEM) to examine the labrum. Although Shibutani (1988) investigated the three dimensional architecture of the labrum, he did not examine its interfaces with cartilage and bone. Later, Tanabe (1991) studied the aging of the labrum. Although some images showed labral collagen fibers extending into and intertwining with cartilage collagen fibers, the collagen fibers were difficult to examine because the cross-sections were produced by a razor blade and viewed only at low magnifications. Furthermore, they did not examine the labral attachment to bone which may be similar to the cartilage/bone interface where colla-
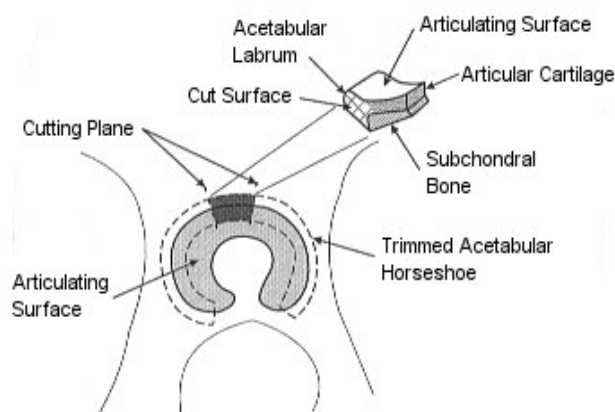
Figure 1. A schematic representation of the acetabulum and the specimen orientation prepared for SEM examination.

gen fibers penetrate directly into the calcified tissue.

With the advent of cryo-technology, SEM fixation techniques of large highly hydrated soft tissue have advanced considerably. Cartilage has been studied widely using various fixation techniques. Richards and Kääb (1996) compared conventional chemical, microwave enhanced chemical, and cryo-fixation for the SEM examination of tibial plateau articular cartilage. All three fixation methods, combined with low temperature freeze fracturing, provided cross-sections following the natural cleavage patterns, facilitating clear high magnification images of the 3-D collagen architecture.

In this study, these three fixation techniques were applied to the acetabulum and evaluated for the examination of the collagen fiber interaction at labrum/bone, labrum/cartilage, and cartilage/bone interfaces. Because of the similarity in tissue composition and size between the acetabulum and tibial plateau, it was anticipated that one of these techniques may adequately preserve the collagen fiber architecture at all three interfaces. Such images may then be used to quantify collagen fiber geometries (diameter, density, etc.) allowing calculation of relative interface strengths and further understanding of labral tear mechanisms.

Materials and Methods

Specimen dissection

Six sheep hips were harvested from three Swiss Mountain Sheep (Zucht Zahner, Truttikon, Switzerland), 4 to 6 years-old and 50 to 60 kg in weight. After dissection of soft tissue from the joint capsule, osteotomies were performed at the pubic and ischial rami, ischium, and subtrochanteric on the femur, allowing extraction of the intact joint. The joint was immersed in 0.1 mol l^{-1} piperazine-NN'-bis-2-

ethane sulphonic acid (PIPES) (Fluka, Buchs, Switzerland) pH 7.4 at 277 K and stored for not more than 12 h before further processing.

After capsular incision around the femoral neck base, the capsule was trimmed to the acetabular bone leaving only the undisturbed labrum. Unwanted bone, the acetabulum fossa, and pulvinar were trimmed from the acetabulum with an oscillating saw under PIPES buffer irrigation leaving only the labrum and articular cartilage on a thin shell of subchondral bone in the shape of a horseshoe. First, two parallel incisions approximately 2 mm apart were cut with a sharp scalpel at the division sites and the soft tissue was removed. Then an oscillating saw, under PIPES buffer irrigation, was used to cut radial specimens without disturbing the labrum and cartilage (Fig. 1). Finally each specimen was trimmed to average dimensions of 10x10x5 mm^3 and rinsed in PIPES buffer prior to SEM preparation. The specimens were randomly divided for either conventional chemical fixation, microwave enhanced chemical fixation or cryo-fixation such that specimens from various locations within the acetabulum were equally represented in each fixation group.

Specimen fixation

Conventionally fixed specimens were immersed in a primary fixative of 2.5% glutaraldehyde (Merck EM grade, Auer Bittman Soulie AG, Basel, Switzerland) /4% formaldehyde (Fluka) in 0.1 mol/l PIPES buffer pH 7.4 at 293 K for 30 min. Buffer rinsing was repeated twice to remove any unreacted aldehyde in the specimen. Postfixation was performed with 0.2% osmium tetroxide (Simec Trade AG, Zofingen, Switzerland) dissolved in 0.1 mol/l PIPES pH 6.8 at 293 K for 60 min and additional electron density (staining) was provided by 2% aqueous uranyl acetate (Fluka) for 60 min at the same temperature.

Microwave enhanced chemically fixed specimens (Richards and Kääb, 1996) were rinsed for 10 min in 0.1 mol/l PIPES buffer pH 7.4 at 293 K. Primary fixation was identical to the conventional method but was carried out in a normal microwave oven (M696, Miele AG, Spreitenbach, Switzerland) at 450 W (2.45 GHz ± 50 MHz) fitted with a field stirrer for 20 seconds or until the temperature reached 313 K (controlled by an automatic temperature probe immersed in 5 ml solution). A 300 ml beaker of water was placed in the microwave as a heat sink to absorb any heat production that might be localized in the specimen or fixative. The fixed specimen was then placed in 0.1 mol/l PIPES buffer at 277 K for 2x10 min to reduce the temperature of the specimen. Postfixation was carried out in a similar manner with both osmium tetroxide and uranyl acetate using the same concentrations as in the conventional fixation but irradiating the samples with microwaves for 20 seconds or until the desired temperature (313 K) was reached.
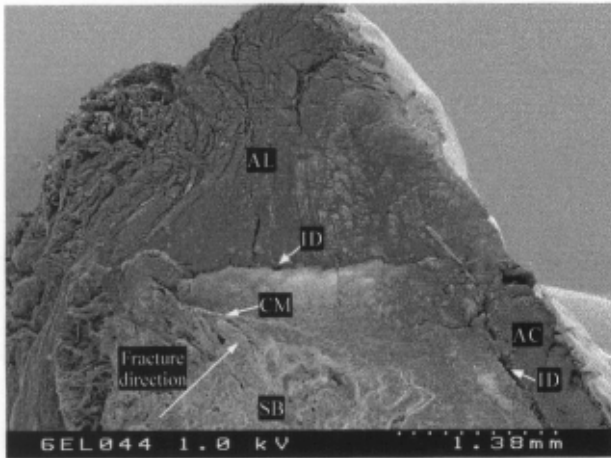
Dehydration was by immersion in a graded series of

Figure 2. A cross-section of the acetabulum freeze fractured before CPD. Chisel marks (CM) can be seen on the subchondral bone (SB). The acetabular labrum (AL) and articular cartilage (AC) have been compressed causing the tissues to be pulled away from the bone, damaging the interfaces (ID).

Figure 3. A cross-section of the acetabulum fixed using cryo fixation. The coarse fibers of the acetabular labrum (AL) can be distinguished clearly from the fine fibers of the articular cartilage (AC). The collagen fibers are arranged in a columnar fashion perpendicular to the tidemark (i.e., border with the underlying subchondral bone, SB). The labrum collagen fibers are of mixed orientation, but most of the larger bundles are oriented perpendicular to the tidemark. A highly electron dense (darker staining) superficial, and lower electron dense (lighter staining), deeper, regions can be seen in the articular cartilage.

ethanol (EM grade, Fluka) from 50-90%, for 15 min each at 293 K. Full solvent dehydration was achieved by immersing the samples in 100% ethanol three times for an incubation period of 15 min each at 293 K. Ethanol was exchanged for 1,1,2-trichlorotrifluoroethane (fluorisol) (Fluka) in ethanol:fluorisol ratios of 1:3, 1:1, 3:1 and finally in 100% fluorisol for 15 min each at 293 K.

Cryo-fixed specimens (Nötzli and Clark, 1994) were plunge frozen for 2 min in isopentane (Fluka), pre-cooled to 108 K. Freeze substitution was achieved serially in various methanol/acetone solutions (Humbel *et al.*, 1983; Steinbrecht and Müller, 1987). Initially the specimens were incubated in 10% acrolein (protein crosslinking) and 0.2% tannic acid (improves the preservation of surfaces by structural reinforcement of the collagen fibers) in a mixture of 59.8% acetone and 30% methanol at 193 K for 96 h (all chemicals from Fluka), followed by 5% of a 50% aqueous glutaraldehyde solution (protein crosslinking) in 10% methanol and 85% acetone at 253 K for 48 h; then finally 5% glutaraldehyde solution in 10% methanol and 85% acetone at 277 K for 24 h. All specimens were then rinsed in 100% ethanol at 293 K for 30 min to remove any unreacted fixative, and immersed in 1% osmium tetroxide dissolved in 100% ethanol for 2 h postfixation. In preparation for critical point drying, the specimens were rinsed and maintained in 100% acetone.

Critical point drying and freeze fracturing

Freeze fracturing, initiated at the subchondral bone and oriented perpendicular to the articulating surface, was used to expose radial cross-sections including the three interfaces. Both fracturing prior to and after critical point drying (CPD) were examined. Specimens fractured prior to CPD were taken from the acetone, fluorisol or ethanol, cooled with liquid nitrogen and fractured with a pre-cooled bone chisel. Liquid carbon dioxide, 2x30 min infiltration with an intermediate flush, was used as an exchange fluid for CPD. Specimens fractured after CPD were immersed in liquid nitrogen and fractured as described previously.

SEM examination

Fractured specimens were mounted on an aluminum stub and coated with 15 nm gold/palladium (Baltec MED 020, Balzers, Liechtenstein). The interfaces were examined with a Hitachi S-4100 field emission SEM (Rahn AG, Zurich, Switzerland) in secondary electron mode at 1 keV accelerating voltage and 10 mA emission current. SEM images were acquired digitally by a data acquisition system (Quartz PCI, Quartz Imaging Corporation, West Vancouver, Canada).

X-ray Microanalysis

Osmium and uranium were X-ray mapped (Oxford Link ISIS Pentafet Plus System with a Si(Li) detector, Oxford Instruments, High Wycombe, U.K.) from the cartilage
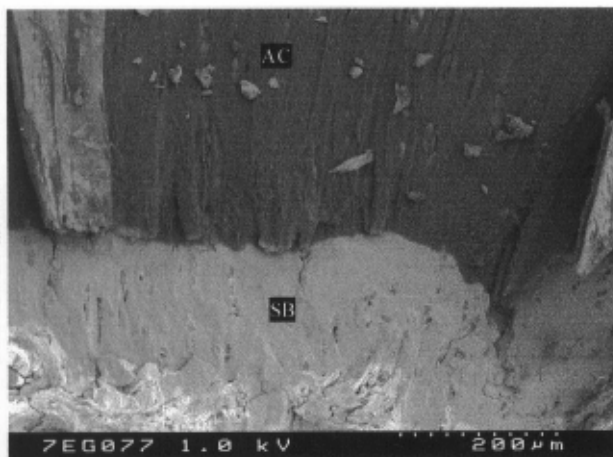
Figure 4. The cartilage/bone interface fixed by chemical fixation. Parallel columns of collagen fibers can be seen extending from the calcified zone (CZ) to the articulating
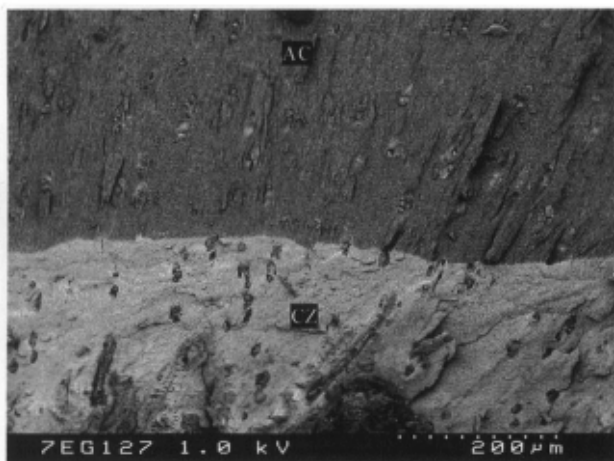

Figure 5. The articular cartilage/ bone interface after cryo-fixation. The fractured cartilage surface (AC) appears smooth and the columnar structure of the fibers is evident. CZ: calcified zone.

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articulating surface to the subchondral bone. The analysis was carried out in the 20 kV range for 10000 frames.

Results

Freeze fracturing after CPD produced more consistent clean fracture planes than did fracturing prior to CPD. Fracturing specimens before CPD was not possible because the specimens did not become sufficiently brittle, even after prolonged cooling (hours). In all specimens fractured prior to CPD, the interfaces were damaged with compression of the soft tissue and separation from the bone (Fig. 2).

In contrast, specimens fractured after CPD were brittle and the interfaces were clearly identifiable (Fig. 3). Even with fracturing after CPD, there were some artifacts produced. Although surfaces were smooth in appearance at low magnification (Fig. 4), at higher magnification, some fibers had been ripped away from the tissue during the fracturing procedure (Fig. 5). Nevertheless, these artifacts are easily recognizable and large artifact-free regions remain on the fractured surfaces.

At low magnification all three interfaces were clearly observed with all three fixation techniques (Fig. 3). The two soft tissues were clearly distinguishable from each other by tissue appearance. Labrum had a coarser appearance with thick collagen fibers oriented generally perpendicular to the bone contours. Cartilage had a smoother cross-sectional surface and the columnar structure of the thin collagen fibrils running perpendicular to the bone contour was discernible. Beneath both cartilage and labrum, there was a calcified zone (Collins, 1949), smoother fracture plane, which was contiguous with the underlying subchondral bone, rougher fracture plane. This calcified zone was thicker under the cartilage, but was still evident underneath the labrum. In all cryo-fixed specimens, the superficial one third (approximately) of the tissue thickness appeared more electron dense (darkly stained) than the deeper portions. The border between these regions was very sharp and no gradual transition could be observed even at higher magnification. X-ray mapping of the cartilage for osmium and uranium showed that there was a large signal in the dark area and a low signal in the light area with a definite sharp separation of the two areas in heavy metal distribution. Nevertheless, this apparent electron density (staining) difference was not evident in the areas of interest to this study, the three tissue interfaces, and did not interfere with our examination at higher magnifications. Finally, all microwave enhanced and conventional chemical fixation specimen appearances under the electron microscope were indistinguishable, even at higher magnification. Thus, the specimens from these two fixation techniques were pooled, and the technique will hence forth be referred to collectively as chemical fixation.

Although the articular cartilage/subchondral bone interface was preserved similarly with chemical and cryo-fixation at low magnification, differences became evident at moderate magnifications (Figs. 4 and 5). In both specimens, there was an underlying columnar architecture of the collagen fibers. However, the cryo-fixed specimens were smoother and the chemically fixed specimens had a fine reticular appearance. With both fixation methods at high magnification, collagen fibers were directly embedded in the calcified zone. In the cryo-fixed specimens, the collagen fibers were not seen as clearly due to a thin layer of unidentified substance covering them, perhaps giving the
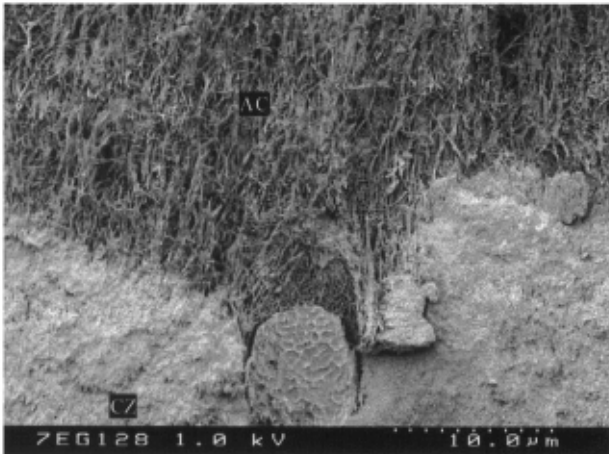
Figure 6. The interface between cartilage and bone fixed by cryo-fixation. Cartilage (AC) collagen fibers can be seen entering the calcified zone (CZ) in a parallel manner. However, the fibers are coated with an unidentified substance, obscuring identification of individual fibers.
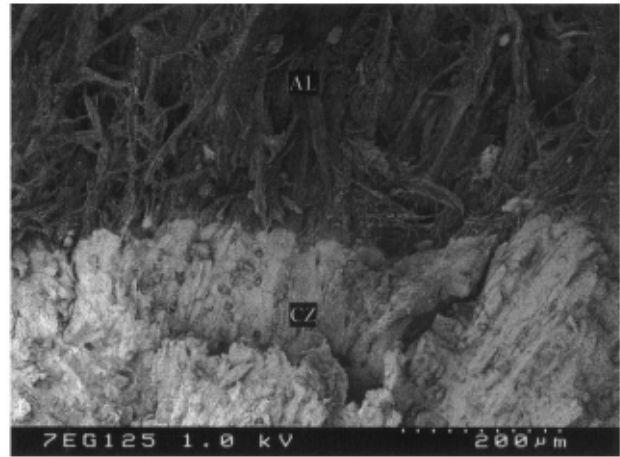

Figure 8. The labrum/bone interface fixed by cryo-fixation. Coarse fibers of the acetabular labrum (AL) of general orientation perpendicular to the tidemark can be seen entering the calcified zone (CZ). Some fibers have been
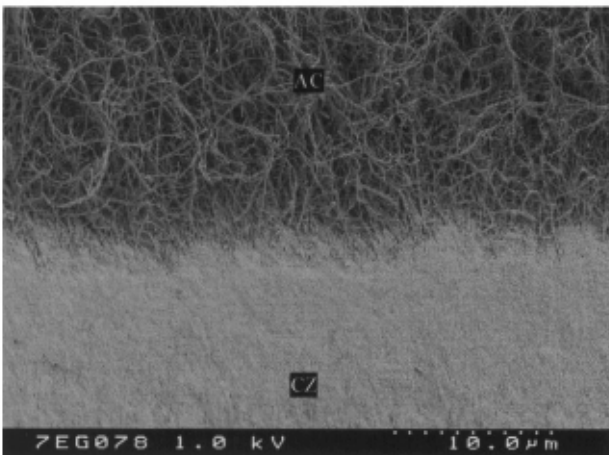

Figure 7. The interface between cartilage and bone fixed by chemical fixation. The mesh structure of the cartilage collagen fibers is clear. Although some fibers are orientated out of the plane, collagen fibers seen entering the calcified zone (CZ) are parallel within the fracture plane and oriented perpendicularly.

Figure 9. The interface between acetabular labrum (AL) and bone fixed by cryo-fixation. Parallel, partly mineralized thick collagen fibers can be seen entering the calcified zone (CZ). Some fibers are oriented randomly.

smoother appearance at lower magnifications (Fig. 6). In the chemical fixed specimens, individual collagen fibers were clear and distinct (Fig. 7). Although there were some fibers out of the fracture plane, perhaps as an artifact of fracturing, the fibers at the border of the calcified zone were generally within the fracture plane. Overall, collagen fibers at the cartilage/bone interface were best identified with chemical fixation.

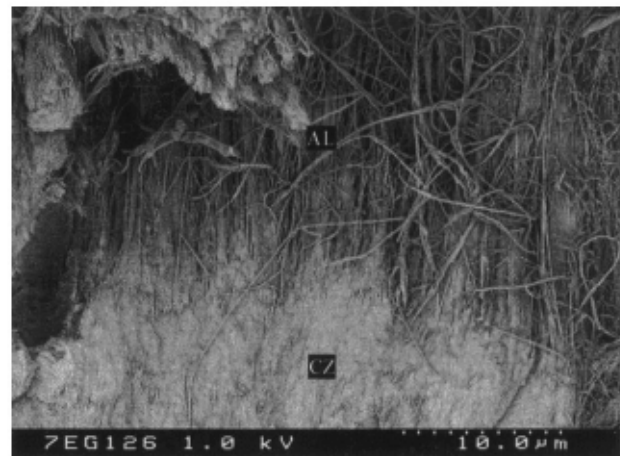Unlike the cartilage/bone interface, the acetabular la-brum/subchondral bone interface at moderate and high magnifications were similarly preserved with both cryo- and chemical fixation. At the interface between the labrum and bone, collagen fibers were more mixed in orientation, but were generally perpendicular to the calcified zone (Fig. 8). Again there were some fibers which had been pulled away, i.e., possible freeze fracturing artifacts. Although individual collagen fibers arranged in large bundles were difficult to identify at moderate magnification, at higher magnifications, parallel fibers much larger in diameter to cartilage fibers also embedded in a calcified zone were clearly evident (Fig. 9).
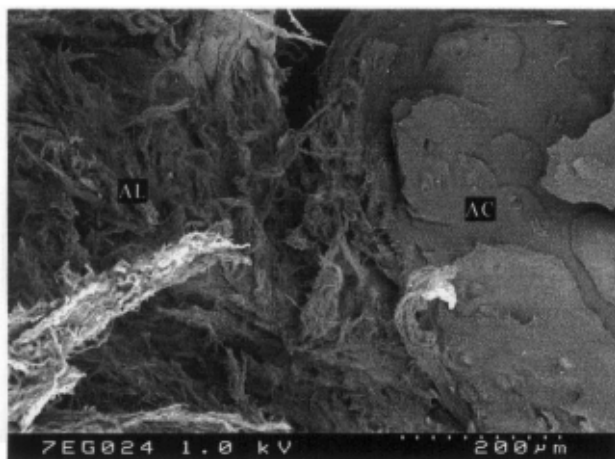
Figure 10. The labrum/cartilage interface fixed by chemical fixation. The acetabular labrum (AL) and articular cartilage (AC) are identifiable by their different tissue appearance. Cartilage has a smoother appearance than the coarser labrum.
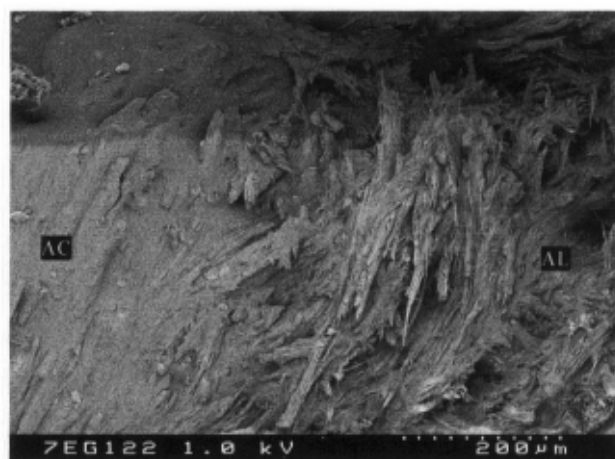

Figure 12. The labrum/cartilage interface fixed by cryo-fixation There is a definite visible interface between acetabular labrum (AL) and articular cartilage(AC) because of the differences in collagen fiber organization. The coarse fibers of the labrum can be seen intertwined with the fine
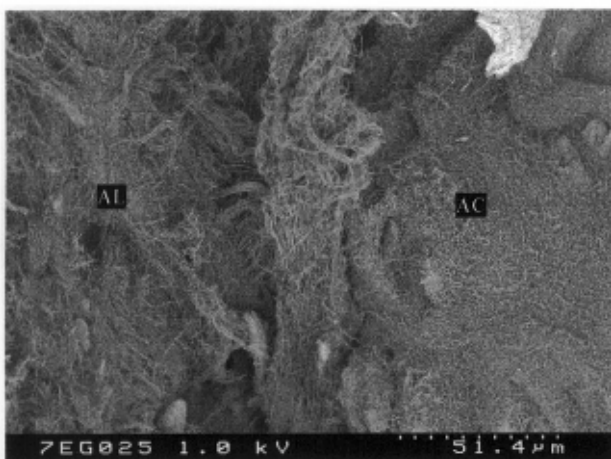

Figure 11. The interface between acetabular labrum (AL) and articular cartilage (AC) fixed by chemical fixation. The labrum and cartilage are not distinguishable in appearance.
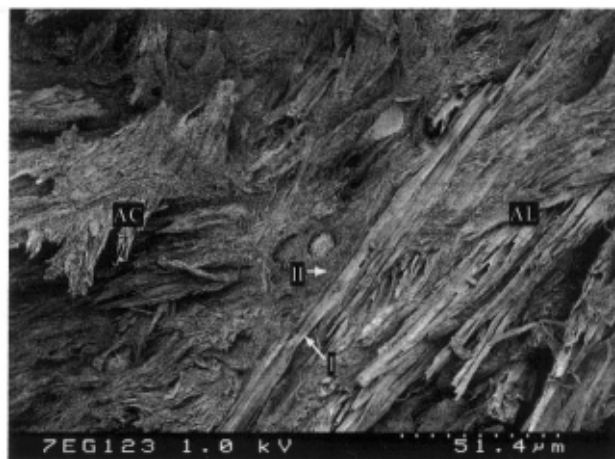

Figure 13. The interface between acetabular labrum (AL) and articular cartilage (AC) fixed by cryo-fixation. At higher magnification, it was evident that thick collagen fibers (I) from the acetabular labrum were intimately mixed in amongst the thinner articular cartilage collagen fibers (II).

Finally, imaging the acetabular labrum/cartilage interface at higher magnifications was only possible using cryo-fixation. Although cartilage and labrum were identifiable by their different tissue appearances with chemical fixation at moderate magnification (Fig. 10), at higher magnification, it was difficult to identify the exact interface (Fig. 11). In contrast, there was a definite visible interface between labrum and cartilage in the cryo-fixed specimens even at the highest magnifications. At moderate magnification the cartilage was smoother in appearance with chondrocytes embedded in the column oriented collagen fibers (Fig. 12). Labrum fibers were coarser and were turned towards the

cartilage. At high magnification, thick collagen fibers from the labrum were seen intertwined with the thinner collagen fibers of the cartilage (Fig. 13).

Discussion

In this study, conventional chemical, microwave enhanced chemical, and cryo-fixation for the SEM examination of the collagen fiber interaction at labrum/ bone, labrum/cartilage, and cartilage/bone interfaces of the acetabu-

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lum were evaluated. Preserving and imaging the three interfaces of the acetabulum was not possible with only one fixation method. Although tissue preservation was acceptable in all fixations at low magnification; at higher magnification, the collagen structure was not always distinct. The labrum/bone interface was preserved similarly by both chemical (conventional and microwave) and cryo-fixation. The labrum/cartilage interface was best preserved with cryo-fixation whereas chemical fixation provided the best cartilage/bone preservation. These differences are most likely due to differences between the interaction of fixation method and tissue extracellular matrix composition. Chemical fixation of cartilaginous tissues with aldehydes has been shown to cause extraction of the water-soluble proteoglycans (Engfeldt and Hjertquist, 1968; Thyberg *et al*., 1973). However, cryo-fixation preserves proteoglycans (Hunziker and Schenk, 1984) surrounding the collagen fibers. During freezing, there is a stabilizing mechanism causing a reduction in proteoglycan extraction when fixatives/solvents penetrate the specimen. The low temperature in which the fixation is carried out also decreases this extraction. This difference in proteoglycan preservation is believed to be the source of the different fixation results. At the cartilage/labrum interface with cryo-fixation, the proteoglycans provide support to the collagen fiber network, preserving it during fracturing. Whereas with chemical fixation, the aggressive extraction of the proteoglycans may cause artifacts causing the different collagen fibers of the labrum and cartilage to appear similar and making the interface difficult to identify. Conversely, at the cartilage/bone interface, presence of proteoglycans after cryo-fixation is detrimental. Here the collagen fibers are well supported by the calcified matrix, and the unidentified substance masking the collagen fibers is most likely the preserved proteoglycans. Finally, neither of these effects are significant at the labrum/bone interface. The collagen fibers of the labrum are also embedded in the calcified matrix, and significantly lower proteoglycan content of fibrocartilage compared to that in cartilage allows for collagen fiber identification (Koob and Vogel, 1987). Hence, both chemical and cryo-fixation provide good preservation of the collagen fibers at the labrum/bone interface.

In addition to the fixation method, the freeze fracturing procedure required modification. Fracturing parallel collagen fibers along the fibers is easily accomplished because the fracture plane follows a line of weakness in between the collagen fibers. This is suitable for the cartilage/bone interface as described by Richards and Kääb (1996). However, in the labrum, the collagen fibers are of mixed orientation with a large number of fibers running across the desired fracture plane (Shibutani, 1988). Fracturing was not possible when the specimens were cooled in solvents by liquid nitrogen even for a prolonged period (hours). The collagen fibers crossing the fracture plane maintained

enough toughness to resist fracturing indicating that no frozen solvent was present within the fibers. In contrast, fracturing after CPD was more successful. Since the specimens were exposed to the ambient environment for several hours after CPD, prior to fracturing, the collagen fibers may have absorbed some water. Immersion directly in liquid nitrogen would then have caused fiber embrittlement by freezing of this bound water allowing propagation of the fracture (personal communication Dr. P. Echlin). Hence, immersing critical point dried specimens in liquid nitrogen enabled a consistent fracture plane to be produced.

Although some modifications were made to the specimen preparation protocols of Richards and Kääb (1996), several artifacts could not be eliminated. Even after critical point drying, some collagen fibers ripped across the fracture plane and are evident in some of the images. Also an electron dense (darker staining) region was particularly evident in the articular cartilage. This region was X-ray mapped and was found to contain a higher concentration of osmium and uranium. Both the image and X-ray mapping showed a very sharp delineation of the region which was inconsistent with a diffusion front. Since there are no known tissue composition differences in cartilage with such a sharp transition, this was accepted as an unknown artifact. Fortunately, in both cases the artifacts were easily recognizable, and many large artifact-free regions of the specimen at the three interfaces were available for examination.

In general our low magnitude images of the labrum/cartilage and cartilage/bone interfaces are in agreement with images shown in previous reports. Shibutani (1988) showed that the labrum and cartilage had a different structure. A razor-cut radial section at low magnification showed cartilage with a smooth surface and labrum with a fibrous surface. We have also seen this structure difference. Tanabe (1991) showed images of the labrum/cartilage interface with labrum fiber bundles extending to the cartilage. These fibers were tightly connected to the cartilage fibers. These fibers could be seen also in our specimens, but the interaction was clearer due to the improvement in fixation and fracture methods. Unlike the labrum/ cartilage interface, collagen fibers of the cartilage/bone interface cross a tide mark, anchoring the tissue to the calcified zone (Boyde and Jones, 1983). This was seen in our specimens as well.

In conclusion, we have shown that the collagen architecture at the three interfaces of the acetabulum cannot be imaged with one fixation method. A combination of cryo- and chemical fixations must be employed where the cartilage/bone interface is best examined with chemical fixation, the labrum/cartilage interface with cryo-fixation, and the labrum/bone interface with either. Furthermore, fracturing after CPD produced more consistent surfaces with less ripping. Collagen morphology at the labrum/bone and cartilage/bone is very similar with collagen fibers directly

penetrating the calcified zone where the cartilage fibers are smaller in diameter. Attachment of the labrum to the cartilage is different in that thick labrum collagen fibers intertwine with thin cartilage collagen fibers. From these initial observations it would appear that there is a difference in the strength of these interfaces.

Acknowledgements

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Discussion with Reviewers

G.M. Roomans: Why were the joints left for up to 12 hours in buffer? Are you sure that this does not affect the architecture of the tissue? Also, how long did the dissection procedure take?

Authors: The removal procedure was in accordance to surgical techniques so that the surrounding tissues could be removed while keeping the joint capsule intact. The duration of the procedure was approximately 2 hours per hip. They were then placed in buffer to wash the tissues of blood before dissection. Further specimen dissection was carried out in buffer and took approximately 1 hour. Our previous experimental experience shows that in whole joint preparations of cartilage and bone, the period of 12 hours does not affect the collagen structures in cartilage (Kääb, 1998). Perhaps the reason for the minimal changes are that cartilage and labrum are anchored into bone which is stable, and that the cartilage is avascular requiring low oxygen tension for chondrocyte viability (Stoddart, 1997).

G.M. Roomans: The authors state that the cartilage/bone interface is "better" visualized after chemical fixation. While the interface is "easier" to see after chemical fixation, this may well be due, as the authors point out, to extraction artefact. Therefore, the image after cryofixation may actually represent the true situation and therefore be "better" from a scientific point of view.

Authors: We would like to emphasize that the procedure was developed in order to specifically visualize the collagen architecture in a natural state. However, in the natural state, the tissue is composed of a collagen network in which is embedded a finer proteoglycan network. In both chemical and cryofixation protocols, the natural state of proteoglycans is disturbed. In the latter, the proteoglycans are compressed by ice crystals and in the former, they are precipitated or even extracted from the tissue. Thus there is no "better" fixation protocol from a scientific point of view because neither provide a good method for observing the tissue in a "true" state. Hence for the purpose of observing

the collagen, structure it is advantageous to remove the masking effect of the proteoglycans, much like with enzymatic digestion, to provide a "better" view.

J. Wroblewski: How can the different preparation techniques described in the present study be improved/adopted to allow use of molecular or/and other markers for further identification of the different types of collagens and other components of the extracellular matrix in the acetabulum?
Authors: The techniques described were designed to improve morphological preservation of a mixed tissue type specimen with a highly fibrous structure. Because molecular and/or other markers were not envisaged for this purpose, it is difficult to respond with specific alterations without any further experimentation. However, there are a few significant issues which can be identified. Since the antigenic sites for antibody binding must be preserved for marking, usually small sample sizes are used such that the fixatives can penetrate the specimen completely. However, in order to capture the collagen architecture of a high osmotic potential heterogenous tissue, large samples must be used, because physical dissection may disrupt fiber architecture and release pre-tensioned fibers. Again to preserve antigenic sites, harsh protein crosslinking fixatives should be avoided. Thus a fixative other than acrolein should be used. Further-

more, osmium tetroxide and uranyl acetate should be omitted. However, it should be noted that this may decrease the contrast necessary for collagen fiber examination. Finally, the critical aspect of this technique was to fracture across the general orientation of collagen fibers in the labrum. We were able to accomplish this only by freeze fracturing after critical point drying. For labelling the observed fractured surface, another protocol would be necessary because critical point drying would probably destroy any antigenic sites. Alternatively, it is possible that the label could penetrate completely into the fracture plane before fracturing. However, this is highly unlikely since the permeability of cartilage and labrum are extremely low.

Additional References

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