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DIMENSIONAL CHANGES OF ARTICULAR CARTILAGE DURING IMMERSION-FREEZING AND FREEZE-SUBSTITUTION FOR SCANNING ELECTRON MICROSCOPY

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Abstract

In this study, the dimensional changes of articular cartilage during cryofixation (immersion-freezing) followed by freeze-substitution and critical-point-drying or tertiary-butyl alcohol drying were compared with conventional fixation techniques. The cryotechnique resulted in 11% shrinkage of the articular cartilage. During conventional chemical fixation, the sample shrank by 28%. No difference in shrinkage could be observed between the two drying methods. Morphologic examination by scanning electron microscopy (SEM) showed no difference between the rabbit tibial plateau cartilage of small excised samples and whole knee joints fixed by the cryotechnique. The collagen structure was unaffected, but the chondrocytes showed formation of segregation artifacts (ice crystal damage) compared to conventional fixation.

For the study of articular cartilage with SEM, cryofixation followed by freeze-substitution is superior to conventional fixation methods with respect to specimen shrinkage, but ice crystal artifacts will be introduced into the ultrastructure. It allows immediate preservation of large samples with now damage to collagen structure.

Key Words: Articular cartilage, shrinkage, immersion-freezing, freeze-substitution, critical point drying, scanning electron microscopy, collagen structure.

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Introduction

The study of biological tissue by scanning electron microscopy (SEM) requires that specific preparation techniques are applied. For imaging under high vacuum the tissue must be dried, usually following water substitution (dehydration), with an organic solvent. Such manipulations can lead to the introduction of artifacts such as specimen shrinkage [11, 12, 14, 40]. Shrinkage can influence the morphologic appearance of a sample, and hence dimensional changes can disturb the positional relationship of structures. Therefore, one parameter that can be used to validate a specific fixation method is to measure the dimensional changes of a tissue during the various fixation steps.

In general, slight tissue shrinkage [13] or a small amount of swelling is induced by fixation in buffered glutaraldehyde, followed by a water wash [22]. It is well established that chemical fixation for biological specimens followed by dehydration in organic solvents almost always results in its shrinkage by the time all the water has been removed [14, 40]. All solvents used as dehydration fluids cause tissue to shrink [40].

General tissue shrinkage can not be avoided when drying. Boyde *et al.* [13] demonstrated that, for both plant and animal tissue, shrinkage of the original volume of up to 60% occurred during critical-point-drying (CPD), 15% during freeze-drying and 80% during air-drying. These findings were based on measurements of volume or area changes during drying procedures. There is general agreement that freeze-drying, whereby the water is converted into ice and then sublimed away at low temperature and high vacuum, is best for dimensional preservation [9, 10, 61].

Many of these shrinkage measurements have been done on cultured mammalian cells. However, individual tissues show different shrinkage behaviors, probably reflecting the properties of their extracellular components [40]. Articular cartilage is usually prepared using conventional aldehyde fixation techniques for morphologic studies with the SEM [5, 6, 19, 21, 23, 27, 28, 37, 41, 45, 57].

The dimensional changes occurring during processing of cartilage have been documented by Boyde and Jones [12]. They observed that adult femoral head cartilage shrank 25%

in ethanol, up to 30% during CPD and up to 39% two days after CPD. For routine histological preparation, Gilmore and Palfrey [29] described a reduction of cartilage thickness in human femoral condylar articular cartilage of 50%.

Another method of preserving articular cartilage for morphologic studies is cryofixation at high or ambient pressure followed by freeze-substitution [26, 33, 34, 55]. The ice in the frozen tissue is removed by means of an organic solvent at a temperature higher than that at which the specimen was frozen. The actual chemical fixation occurs during the substitution phase. In general, tissue shrinkage associated with cryofixation is thought to be slight [44], but has not been quantified.

In 1994 Nötzli and Clark [48] introduced cryo-fixation at ambient pressure followed by freeze-substitution into the study of the collagen architecture of whole rabbit knee joints under mechanical load. The present study was undertaken to quantify the effectiveness of this cryofixation method for articular cartilage in terms of shrinkage behavior during the different stages of preservation. An additional goal was to evaluate the tertiary-butanol drying method. Further, we were interested in the final product of the cryotechnique. The collagen matrix morphology of small samples of rabbit tibial plateau cartilage and whole rabbit knee joints fixed by the cryotechnique was compared to conventional chemical fixation. The evaluation of cryotechnique quality for whole rabbit knee joints was of interest because immediate fixation of such large samples is necessary to assess the relationship of different joint components (tibial and femoral cartilage, menisci) when the whole joint is mechanically loaded.

Materials and Methods

Sample preparation

Full thickness samples of articular cartilage were obtained from adult porcine metacarpals and adult rabbit tibial plateaus. Vertical knife cuts were used to remove 4x3 mm^2 squares of the cartilage, 1 to 2 mm in thickness, which included the overlying articular cartilage and a layer of underlying bone (Fig. 1). To include cartilage from distinct areas, twenty cubes of porcine cartilage were taken from the central region of the cartilage and 12 from the periphery. Twelve cubes were taken from the rabbit tibial plateau. During preparation the cartilage was carefully prevented from drying by the use of pH 7.2 buffered Ringer's solution [18]. The fixation process started not later than 20 minutes post mortem. To assess the fixation quality in large specimens, five whole knee joints from adult white New Zealand rabbits were used for cryofixation (without shrinkage measurement).

Cryofixation and freeze-substitution

Half the cubes in each group were frozen by immersion for 60 seconds in isopentane slush, precooled with liquid nitrogen to below 113K. The whole knee joints were immersed for 4 minutes. Samples were then removed and fixed by freeze-substitution as described below. The initial fixative was a solution of 10% acrolein and 0.2% tannic acid in a mixture of 30% methanol and 58.8% acetone held at 193 K for 7 days. Specimens were rinsed in acetone twice at 193 K for 20 min. each and then transferred into a second fixation solution of 5% glutaraldehyde (from a 50% aqueous solution) in a mixture of 10% methanol and 85% acetone at 250 K for 4 days. The temperature was then slowly increased to 277 K and samples remained at that temperature for 24 hours. The samples were then washed with 100% ethanol and slowly brought to room temperature. The samples were rinsed again twice each time for 30 min. in 100% ethanol prior to secondary fixation in 1% osmium tetroxide for 120 min at 277 K.

Conventional fixation

To compare the dimensional changes obtained with a familiar, conventional, aqueous fixation method, additional samples were fixed by conventional fixation. Samples were rinsed for 10 min. in 0.1 mol l^{-1} piperazine-NN´-bis-2-ethane sulphonic acid (PIPES) buffer pH 7.4 at 293 K. Primary fixation was in 2.5% glutaraldehyde with 4% paraformaldehyde in PIPES pH 7.4 at 293 K for 2 hours. Samples were rinsed twice for 10 min. each in PIPES pH 7.4 before secondary fixation in 0.2% osmium tetroxide in PIPES pH 6.8 at 293 K for 60 min. They were then rinsed twice for 10 min. each in 0.1 mol l^{-1} PIPES pH 6.8 at 293 K and stained with 2% aqueous uranyl acetate for 60 min. at 293 K. Each fixed sample was dehydrated using graded ethanol solutions of 50%, 60%, 70%, 80%, 90%, 100%, 100% for 15 min. each respectively.

Critical-point-drying, *t*-butyl alcohol drying and SEM imaging

Half the samples from each fixation group were dried with a Polaron E3000 critical point drier (Agar Scientific, Stansted, U.K.), using CO_2 as a transitional fluid after a final wash with absolute ethanol. Specimens were flushed twice and allowed to equilibrate twice for 40 min. The other half of the samples were vacuum dried following the technique of Inoué and Osatake [36]. Briefly, the post-fixed specimens were ethanol rinsed, transferred into tertiary butanol and rinsed twice for 20 min. The glass container with the specimen was placed in the refrigerator (277 K), where the *t*-butyl alcohol was frozen within a few minutes. The container was transferred into a vacuum evaporator, connected to a water pump and the specimens left there for sublimation for 6 hours.

For morphologic evaluation, specimens were coated with 8 nm of gold in a Baltec MED 020 unit (Baltec, Balzers, Liechtenstein) and examined with a Hitachi S-4100 field emission SEM (Hitachi, Tokyo, Japan). It was operated in secondary electron detection mode at an acceleration voltage of 1-2 kV [38] and an emission current of 10 µA.

Dimensional changes

Measurements of the two-dimensional changes in size (x- and y-axes) which occurred during the different stages of

sample preservation were performed as area measurements (mm^2) taking the initial area as a reference [10, 12]. The dimensions were measured while the sample was immersed in the medium to prevent drying effects. The front face of the sample (Fig. 1) was viewed under a light microscope and examined at a magnification of x32. The contours of the cartilage were traced with a cursor on a digitizing table (Kontron Electronics, Munich, Germany). A mirror allowed the projection of the cursor onto the sample in the field of view. The areas were measured and recorded with a computer program. The samples were checked for bending in the z-axis not quantitatively but microscopically when examined under the light microscope.

For each sample four measurements were made at equivalent stages of the preparation process to limit statistically the error caused by resolution limitations and operator error by inexact tracing of the contours. All fresh specimens were measured prior to any treatment. Those fixed by freeze-substitution were measured after freeze-fixation and after drying. Those fixed by the conventional technique were measured after fixation, after dehydration in 100% ethanol and after drying by the critical point or sublimation methods. All specimens were again measured five days after drying.

Results

Dimensional changes

There was no significant difference at all in the average shrinkage behavior between porcine metacarpal cartilage and rabbit tibial plateau cartilage. Also the shrinkage of central load bearing parts and peripheral parts of the joint was similar. No bending of the blocks in the third dimension could be observed during either method of fixation.

There was a significant difference in the shrinkage behavior of articular cartilage prepared by cryofixation followed by freeze-substitution and that of samples fixed by conventional techniques (Fig. 2). With conventional fixation the cartilage shrank by 4% during fixation (including uranyl acetate staining) and by an additional 6% during dehydration in 100% ethanol. For the equivalent steps in the cryotechnique, the articular cartilage changed its dimensions by only 2%. Specimens prepared by cryotechnique were necessarily held at low temperatures during fixation and dehydration in organic solvents, consequently measurements could not be made between those stages. The greatest shrinkage for both methods of fixation occurred during CPD, during which the cryopreserved samples shrank by 9%. The dimensions of conventionally fixed samples were reduced twice as much during the same process, shrinking by 18%. Neither group showed signs of additional sample shrinkage five days after CPD.

The average cryopreserved sample, after all the steps of preservation, retained 89% of its initial size, whereas those conventional fixed retained 72%. No differences were detected

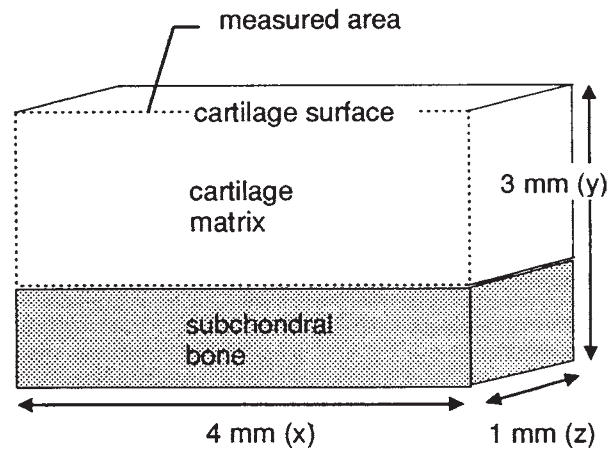


Figure 1. The area of the cartilage (front face contour; without bone; interrupted line) was measured during the different steps of fixation.

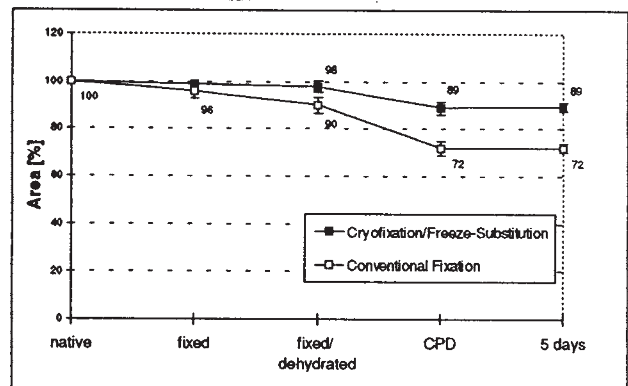


Figure 2. Shrinkage of articular cartilage. The relative dimensional changes are shown in area %. The final difference between the cryomethod and conventional fixation is 17%. The error bars indicate the SD.

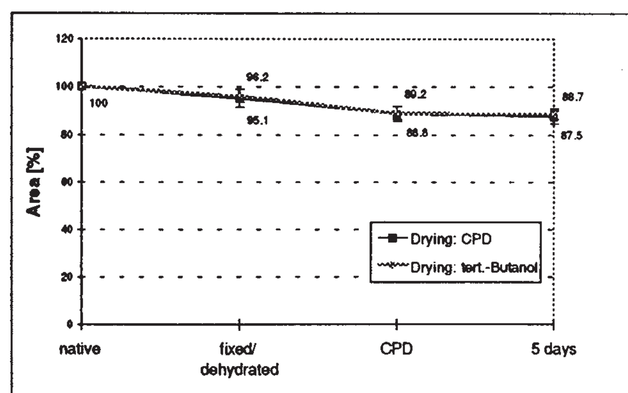


Figure 3. Shrinkage of cryofixed and freeze-substituted cartilage during drying. There is no difference between critical-point-drying and the *t*-butyl alcohol drying method. The error bars indicate the SD.

in the shrinkage behavior of cartilage when dried using either the critical point or the *t*-butyl alcohol method (Fig. 3). Also no additional change could be observed five days after *t*-butyl alcohol drying.

Morphologic results

The SEM images showed different results depending on the fixation technique. The conventionally fixed chondrocytes showed less shrinkage from the walls of the lacunae (Fig. 6) while those fixed by cryotechnique showed a characteristic spongy pattern (segregation artifacts), presumably due to ice crystal damage (Figs. 7, 8). Chondrocytes also showed ice crystal damage in the most superficial layer (Fig. 5). Nonetheless, the contours of the cryofixed chondrocyte lacunae were maintained; specifically the pattern of the surrounding collagen matrix fibers was intact and comparable to conventionally fixed tissue (Figs. 6, 7, 8).

The collagen fibers of conventionally fixed samples (Figs. 4, 6) showed no difference in comparison to cryo-fixed cartilage collagen fibers. Both methods showed the collagen fibers running parallel and perpendicular from the calcified cartilage towards the surface. In the intermediate (or transitional) zone they turn in order to build the tangential zone at the surface [4]. The morphologic quality of the large samples fixed by cryo-technique was comparable to that of the small samples (Fig. 7, 8). The collagen fibers were intact, while chondrocytes were destroyed in both but maintained the contours of the surrounding collagen matrix.

Discussion

This study compared the dimensional changes in articular cartilage prepared for SEM by a conventional technique to those in material fixed by immersion-freezing and freeze-substitution. Cartilage shrank with the use of cryotechniques by a total of 11% compared to 28% during conventional fixation. Use of sublimation-drying showed no advantage over CPD and no further shrinkage was observed five days after drying.

The histological effects of shrinkage in articular cartilage attendant on cryotechniques and conventional chemical fixation are well documented [19, 26, 33, 37, 49]. In these morphologic studies, chondrocyte shrinkage is the focus of interest. Specimen shrinkage in cartilage for SEM preparation has been quantified only by Boyde and Jones [12], who found a volume reduction of 25% to 30% with conventional chemical fixation techniques. Our observations are quite similar.

We used immersion-cryofixation followed by freeze-substitution to preserve articular cartilage for morpho-logic study with SEM because it is a simple and effective method for immediate preservation of large samples (whole rabbit knee joints). The protocol tested here is based upon our own experience and published recommendations for cryofixation techniques. Isopentane precooled in LN_2 has relatively rapid cooling rates [52].

Propane provides better cooling rates but is very flammable, especially in large volumes [51, 53]. Freeze-substitution to dissolve the ice from a frozen specimen is best performed between -193 K and -183 K [2, 47, 55, 59], followed by slowly increasing the temperature. A mixture of organic liquids such as acetone and methanol seems to be best to substitute water at these temperatures [31, 55]. Methanol can also be used as a substitute in the presence of high amounts of water [31, 55]. Additional crosslinking can be achieved by adding acrolein and glutaraldehyde [16, 55, 60].

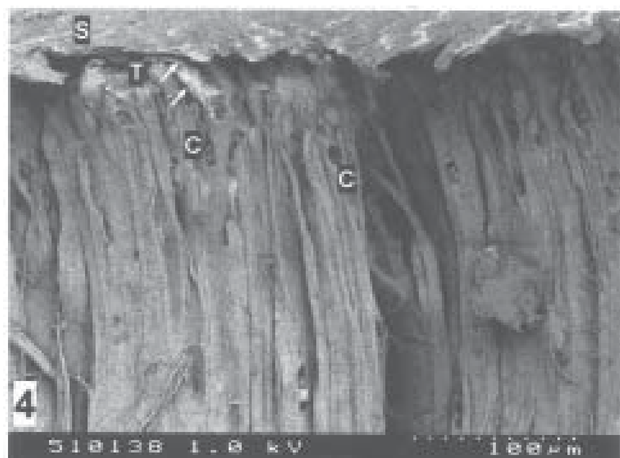
In our experiments we kept the cartilage attached to the subchondral bone to avoid deformation of the sample, which if it had been allowed to happen, would have made the measurement of the dimensional changes almost impossible. Furthermore, the shrinkage was only measured in two dimensions. Area measurements are described as being appropriate to quantify the dimensional changes of tissue during preservation [10, 12]. Shrinkage studies with other tissues describe equal changes in all three axes [3]. Also, when in articular cartilage different layers show different shrinkage behavior [12], changes in the third dimension are considered to influence the area shrinkage results only to a minor degree. The value of such comparative studies of fixation techniques will not be influenced by this factor in any case.

Water and proteoglycan concentrations vary in articular cartilage by depth and with location on the joint surface [17, 43]. This heterogeneity could cause different shrinkage behavior in different regions. Nuehring *et al.* [49] observed this in a histological study of conventionally fixed physeal cartilage. We compared samples from different topographical regions and did not find significantly different shrinkage, perhaps because our measurement techniques were not sensitive enough. The shrinkage behavior in the different cartilage layers was not investigated here.

Unfortunately, for technical reasons we could not measure the dimensions of frozen cubes prior to fixation. It is possible that dimensional changes during the actual freezing process damage the matrix, but we saw no direct evidence of that. Since fixation and dehydration occur simultaneously, we cannot know which process causes the shrinkage.

It has been reported before that preservation of tissue by cryotechniques results in less shrinkage than when conventional chemical fixation is applied. This has been related to the belief that freeze-substitution is a more gentle process [30]. Nevertheless, collapse phenomena or only slight morphological signs of shrinkage have been described after high pressure-freezing of growth plate cartilage, but no quantification was reported in these cases and the analysis was subjective [26, 33, 56].

Why the shrinkage during cryopreservation appears to be much less in cartilage tissue is an open question. In other tissues, the cessation of enzymatic activity at low



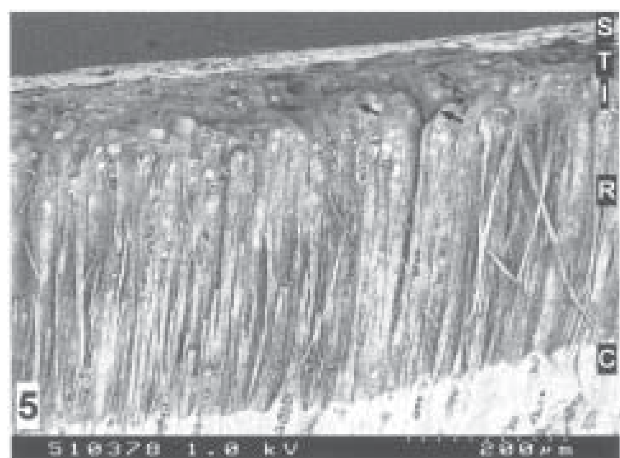
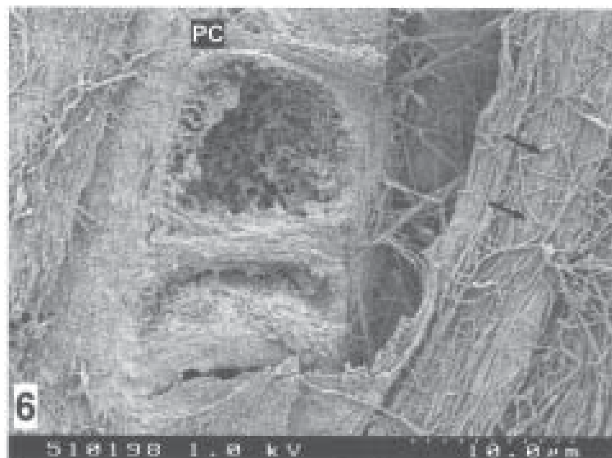


Figure 4. Conventional immersion-fixation (small sample). Collagen fibers bending (arrow) towards the surface (S: surface layer) to form a thin tangential layer (T). Multiple chondrocytes-lacunae (C) in the intermediate and radial zone.

Figure 5. Cryofixation and freeze-substitution (whole knee joint). The parallel running collagen fibers can be seen from subchondral bone to surface (S). The fibers are broken in the intermediate/transitional (I) zone (arrow). Tangential (T) radial (R) and calcified zone (C).

Figure 6. (*right column*) Conventional immersion-fixation (small sample). Fracture (with possible sectioning artifact) through two chondrocytes. The chondrocytes are surrounded by its pericellular matrix (PC). The collagen is running vertically parallel (arrow).

Figure 7. (*right column*) Cryofixed and freeze-substituted cartilage (small sample, calcified/radial zone). The chondrocyte ultrastructure shows a characteristic sponge pattern (arrow). The contours of the cryofixed chondrocyte lacunae are maintained (arrow). Collagen fibers can be identified running parallel (arrow).



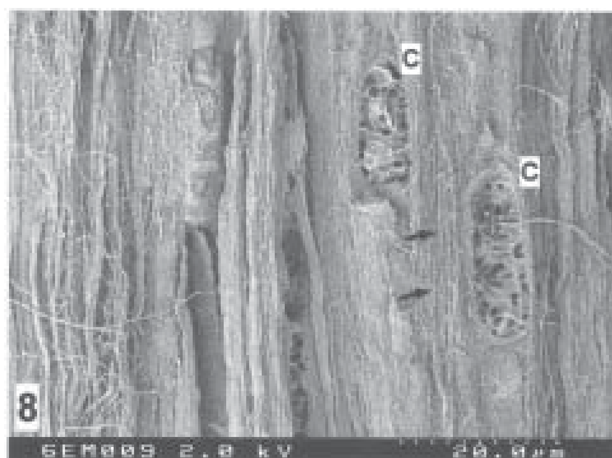


Figure 8. Cryofixed and freeze-substituted cartilage (whole knee joint, radial zone). Comparable to Fig. 7. Chondroctyes (C) laying within parallel running collagen fibers (arrow).

temperature must be considered, but is not a likely factor in cartilage. Water extraction is, however, a great concern in a tissue which contains 60-80% water [1, 46]. Bridgman and Reese [16] suggested that freeze-substitution preserves tissue volume if the organic solvents do not extract water as efficiently as conventional means. This however would not explain why there is less shrinkage during CPD when any residual water would be removed. We believe that three factors could work to minimize shrinkage with freeze-substitution: aldehyde cross-linking of the matrix collagen molecules is more complete leading to a more rigid framework. Water does not leave the cartilage prior to this fixation because most of the water is bound to some degree, which we assume makes it different to cell water [24]. Proteoglycans are not extracted because, unlike in aqueous fixatives, they are insoluble in the organic solvents [32, 33].

Two methods of drying were analyzed in this study. Sublimation with *t*-butyl alcohol as described by Inoué and Osatake [36] showed the same amount of dimensional change of the cartilage samples as those dried by CPD. We tested *t*-butyl alcohol because it is simple to use and because it avoids the high temperature required to pass the critical point for CO_2. Also, different effects have been described for different drying agents. For example, Lodin *et al.* [42] and Boyde [8] found that *t*-butyl alcohol caused shrinkage to tissue. However, we were unable to find any differences in articular cartilage.

CPD is widely used in drying for SEM-studies [15, 20, 37, 39, 45, 50, 57]. It gives good preservation of structures, however, the associated dimensional shrinkage has not been avoided. In fact, in the whole process of sample fixation and preparation for SEM, CPD has been the main factor producing shrinkage [12]. In the drying methods tested here, the freeze-substituted samples showed 50% less shrinkage during CPD than occurred with conventionally fixed material.

Boyde *et al.* found 9 % additional shrinkage in cartilage within 2 days after CPD due to incomplete extraction of the intermediate fluid [7, 12, 14]. In our study there was no additional shrinkage within 5 days after drying. It is most likely that the prolonged periods in organic solvents used here removed more unbound water and were responsible for the absence of late (post drying) shrinkage.

Morphological examination by SEM showed differences between conventionally fixed and cryofixed specimens. Tissue processing in a near-native condition by using low temperatures can only be achieved, if water is frozen in its liquid state (true vitrification). When ice crystals begin to form, as in this case caused by freezing at ambient pressure and big sample size, tissue components become segregated into phases of pure water and of concentrated biological residues with consequent formation of segregation artifacts [35, 56]. The cryofixed condrocytes show this characteristic pattern clearly. However, their general contours (lacunae) were maintained. The conventionally fixed chondrocytes do not show these characteristic changes. No major signs of shrinkage could be observed in either conventional or cryofixed samples. Immersion-freezing at 113 K is described to introduce no ice crystal artifacts within a zone of 10-30 µm thickness on the sample surface [52, 56]. However, chondrocytes also showed ice crystal damage, means segregation artifacts within this zone. The presence of such artifacts within this maximally 30 µm thick zone, which are introduced during primary freezing [52], can be explained by the low thermal conductivity of this tissue containing large amounts of water [1, 46, 58].

The large samples show the same morphology (chondrocytes and collagen fibers) as the small samples, though such large samples were not treated optimally in the sense of cryotechnique. This may also be explained by the high water content of the tissue, which allows an easy exchange of substances and by the anatomy of the rabbit knee joint, where the fixative can penetrate well through the joint interspace. The collagen structure was well preserved by cryofixation at the magnification used. Structure analysis at higher magnification was beyond the scope of this study. For studying cartilage ultrastructure of chondrocytes and proteoglycans powerful methods are available e.g. high-pressure freezing [25, 33, 35]. The morphological findings in collagen structure correspond well to the findings of other authors [19, 21, 23, 37, 45, 50, 54, 57].

Conclusions

In this study the shrinkage of articular cartilage during preservation by a cryotechnique was measured and compared to the results of conventional fixation technique. Using immersion-cryofixation at ambient pressure followed by freeze-substitution, the total amount of cartilage shrinkage was 11%, 17% less than that obtained during conventional fixation. Morphological examination revealed an unaffected collagen structure and a well preserved pericellular collagen matrix, but only chondrocytes indicated the formation of segregation artifacts due to ice crystal damage. This low temperature technique allows immediate preservation of large samples (whole rabbit knee joints) with now damage to collagen structure.

Acknowledgments

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Discussion with Reviewers

Reviewer I: Aldehyde fixation in aqueous solution had long been recognized as harmful for articular surfaces. Also surface irregularities are much more sensitive and reliable markers for cartilage preservation than area measurements.
Authors: We do not agree. In the current study the matrix collagen structure was evaluated and not the articular surface. All fixation alters the structure of tissue. The aldehydic fixation is widely and successfully used for articular cartilage fixation and its modification of ultrastructure is well known too. If one decides to use fixatives, there is not much choice but to use aldehydes. In general, surface irregularities such as pits and humps are a morphological marker for cartilage preservation. They are probably artifacts of the drying procedure and not of glutaraldehyde fixation itself. It is not the fixative itself that causes the damage but rather the tissue which is not stiffened enough to survive CPD without some alteration.

There are powerful techniques to preserve articular cartilage (e.g. high-pressure freezing conventional fixation, microwave enhanced fixation) but each of the methods has advantages and disadvantages and therefore one has to choose the method best suited to the specific research goal. The low temperature technique which was evaluated here is not optimal in the sense of cryo-technique but is the only method currently available which allows immediate preservation of large samples with good preservation of the matrix collagen structure in the low and middle ranges of magnification. The harm of aldehydes is an important question if, for example, the ultrastructure of molecules and proteo-glycans is of interest.

Reviewer I: Cartilage specimens attached to subchondral bone are quite bulky and problems arising from inhomo-genous and/or impeded diffusion may consequently arise.
Authors: We agree to some extent. Leaving the sub-chondral bone attached makes the cartilage more bulky and could lead to incomplete diffusion. However, incomplete fixation should be - as a consequence of leaving the subchondral bone - limited to the calcified layer and radial zone close to the subchondral bone. We did not observe any artifacts related to these zones. Furthermore, an important strength of freeze-fixation is its ability to penetrate very large specimens. We found that all fixatives penetrate much further into the tissue perhaps because the fixative does not react as rapidly. Certainly the organic solvents diffuse into tissue faster than an aqueous solution. Freeze-substitution seems better at penetration and dehydration which explains the lower shrinkage. Removing the subchondral bone leads to significant artifacts e.g. in the surface [62, 63, 66]. Also our experience shows the cartilage can deform without stabilizing subchondral bone and lead to artifacts in the overall collagen fiber structure.

Reviewer I: The troubles in preservation arise when extraction and loss of soluble proteoglycans, glycoproteins and small molecular weight species which are not or may be not be fixed by glutaraldehyde occur.
Authors: Fixatives work in several ways. For example, conventional proteoglycan stains precipitate proteoglycans, so that it does not dissolve and elute. Freeze-substitution does the same for these soluble proteo-glycans and small molecular weight species. We precipitate them by substituting the water with organic solvents in which these molecules are not soluble. We fix the collagen so that it will be very stiff and not change shape; we are less worried about its solubility and elution. Others worry that fixatives extract proteoglycans. We believe they do, but only in aqueous media. Proteoglycans are not soluble in methanol or acetone so freeze-substitution does not extract them. The substances which are not fixed by aldehydes are precipitated i.e., rendered insoluble by the fixation medium.

Reviewer I: No matter how efficiently organic solvents extract water, the residual water which may not have been removed, would evaporate as instantaneously as the vacuum in the column is built.
Authors: Who can say how much water remains in specimens? However, there should be very little residual water in the specimen if the protocol is followed correctly. Most of the water in cartilage is bound to some degree. We assume this makes it different from cell water. Water that is not bound can be substituted, water that is bound is not functionally substituted by the organic solvent, since it cannot „hydrate" the proteo-glycans. But this is why it is so important to fix the collagen matrix before the water is removed. Water bound to the proteoglycans puts tension on the collagen molecules indirectly. Perhaps there is also water that is bound to the collagen molecules directly and when that is removed, the collagen shape changes slightly.

L. Edelmann: Did you try to reduce the shrinkage of conventionally fixed or freeze-substituted material during CPD by using higher concentrations or no chemical fixatives?
Authors: This might be an interesting question but was beyond the scope of this study. The concentration of our fixatives is widely used for the preservation of articular cartilage. Higher or lower concentrations of fixations might lead to minimized shrinkage but can on the other side lead to morphological artifacts as well. Use of fixatives is necessary for the stabilization and preservation of the sample. In earlier series we tried different concentrations, but the protocol used in this study was far superior to those used in other trials with regard to artifacts and morphological quality.

R. Wroblewski: Did you try to use freeze-drying instead of freeze-substitution?

L. Edelmann: Small shrinkage values can be obtained when following optimal freeze-drying protocols. Did you try freeze-drying?

Authors: The goal of this study was to compare a widely used fixation technique and a low temperature method as used for the study of articular cartilage under load in terms of specimen shrinkage. There are preservation methods available other than those we used, e.g. high-pressure freezing and freeze-drying. Freeze-drying is known to introduce less shrinkage than CPD. Morphological studies based on freeze-drying have been performed [64, 65]. Freeze-drying is a technically demanding method and will of course introduce its own specific morphological artifacts as well.

During freeze-drying the ice is removed by low temperature vacuum sublimation from outside to inside. If melting or evaporating takes place before freeze-drying is complete, what is difficult to control for large samples, shrinkage of the tissue may occur [7]. With freeze-substitution the sample is maintained at the same temperature which leads to better temperature control. Shrinkage during freeze-drying is lower compared to conventional fixation but there will be only a minimal difference compared with the shrinkage of freeze-substi-tuted samples.

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