Cryopreserved and Frozen Hyaline Cartilage Imaged by Environmental Scanning Electron Microscope. An Experimental and Prospective Study

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ABSTRACT. ***Objective***. To obtain images of the articular surface of osteochondral grafts (fresh, frozen, and cryopreserved in RPMI) using an environmental scanning electron microscope (ESEM). To evaluate and compare the main morphological aspects of the chondral surface of the fresh, frozen, and cryopreserved grafts as visualized via ESEM.

Methods. The study was based on osteochondral fragments from the internal condyle of the knee joint of New Zealand rabbits, corresponding to the chondral surface from fresh, frozen, and cryopreserved samples. One hundred ESEM images were obtained from each group and then classified according to a validated system. The kappa index and the corresponding concordance index were calculated, and the groups were compared by Pearson's chi-squared test ($p < 0.05$).

Results. The articular surface of cryopreserved osteochondral grafts had fewer even surfaces and filled lacunae and a higher number of empty lacunae as compared to fresh samples; these differences correspond to images of cell membrane lesions that lead to destruction of the chondrocyte. Frozen grafts showed more hillocky and knobby surfaces than did fresh grafts; they also had a greater number of empty chondrocyte lacunae.

Conclusion. ESEM is useful for obtaining images of the surface of osteochondral grafts. When compared to fresh samples, cryopreservation in RPMI medium produces changes in the surface of hyaline cartilage, but to a lesser extent than those produced by freezing. (First Release July 1 2008; J Rheumatol 2008;35:1639–44)

Key Indexing Terms:
ELECTRON MICROSCOPY CARTILAGE CRYOPRESERVATION ARTHROPLASTY

Due to the characteristics of cartilaginous tissue, chondral lesions are currently the main challenge facing orthopedic surgeons. Several treatments are now available according to the degree of damage: these include arthroscopic debriding, microfracture surgery, mosaicplasty, autologous chondrocyte graft, and newer approaches such as the use of matrices and osteochondral allograft transplantation[1-3].

Environmental scanning electron microscope (ESEM) enables moist samples to be observed directly without prior preparation, thus avoiding dehydration and subsequent distortion of images obtained. This preservation of the initial degree of hydration makes it an important tool for observing tissue specimens. In a previous report we studied the articular surface of fresh and frozen samples[4].

The cryopreservation of various human tissues has achieved high viability indices, with many techniques being used successfully in heart valve allograft transplantation. As cryopreservation has a direct effect on the tissue surface[5-7], we believe that the morphological study of the graft surface will show a direct correlation with the degree of viability of the cryopreserved tissue[8-11].

The aim of our present study was to determine whether freezing or cryopreservation in RPMI medium was a suitable method for maintaining the survival of hyaline cartilage. Viability was assessed by using ESEM to observe any morphological changes produced in the articular surface of the osteocartilaginous tissue.

MATERIALS AND METHODS

We conducted an experimental, longitudinal, and prospective study. Following the approach of Bailey, Weakley, and Williams with respect to the study animals for electron microscopy (EM), we created one study group of fresh hyaline cartilage, another of frozen specimens, and a third of samples cryopreserved in RPMI[4]. The authors point out that the sampling of photomicrographs in EM is considered statistically valid if photos are taken at various magnifications (preferably at the smallest magnification possible), covering selected fields randomly or following a criterion that can easily be reproduced in all the samples. The formula of Jakstys[12] was used to calculate the minimum number of photographs required to produce statistically valid results: (5 animals per group) × (1 tissue block per animal) × (20 photographs per block) = 100 photographs per group.

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In order to generate a progressive series of images, 100 photographs at magnification 250× were taken of the articular surfaces for each study group.

After sacrifice the knees of animals' hind legs were dissected in sterile conditions. A sample of the internal femoral condyle was then taken using a trephine (osteochondral grafts from the weight-bearing areas of the knee joint of New Zealand rabbits).

Fresh hyaline cartilage group. Once the knee samples had been isolated, they were submerged in a sterile solution of 0.9% NaCl at room temperature until transfer to the center housing the EM. The physiological solution prevents the articular cartilage of the graft from dehydrating due to contact with the air prior to being examined.

Frozen hyaline cartilage group. The anatomical specimen was placed in a plastic bag, which was then sealed following the manual extraction of air. The bag was labelled and placed in a freezer chest at 233.15ºK (–40ºC) for the stipulated conservation time, which in the case of this study was 15 days. The thawing process for the osteochondral grafts involved 3 steps: (1) immersion of the bag containing the frozen graft in 0.9% NaCl solution at 313.15ºK (40ºC) for 3–4 min; (2) opening the bag and placing the graft in 0.9% NaCl solution at 313.15ºK (40ºC) for 3–4 min; and (3) placing the graft in 0.9% NaCl solution at room temperature for conservation and transportation[13].

Hyaline cartilage cryopreserved in RPMI group. The osteochondral graft for cryopreservation was submerged in RPMI medium without L-glutamine at 277.15ºK (4ºC) and for the minimum time required to enable processing, this being no longer than 2h after sacrifice. The anatomical specimens were then removed and placed in sterile plastic cryopreservation bags (Baxter-Cryocite® 750 ml); each bag contained 2 specimens, and any air bubbles were removed using a syringe. The bags were refrigerated at 277.15ºK (4ºC) for 30 min (pre-freezing stage) and subsequently in a controlled-rate freezer (Cryoson®), which enabled a controlled decrease in temperature of 1° (ºK or ºC) per minute until reaching 213.15ºK (–60ºC), followed by 3–4° (ºK or ºC) per minute down to 173.15ºK (–100ºC). The bags were then transferred to liquid nitrogen vats, where they were kept at 77.15ºK (–196ºC) for 15 days. The cryopreserved grafts were frozen according to the same protocol as that used for the frozen graft group.

Each cartilage disc was subsequently mounted on a stub in order to be studied under an ESEM™ (ElectroScan 2020 ESEM-FEG) at 276.65ºK (3.5ºC), with an accelerating voltage of 10 kV and 20 kV, and a chamber pressure of 10 Torr.

In order to generate a logical and comprehensible progression of information, photographs of the cartilage surface were taken for different areas of the sample, following a sinusoidal path. Articular surface morphology was classified using a system developed by our group using photographs of the surface of fresh osteochondral samples and following the methodology of Jurvelin as modified by Hong[14].

Samples do not need to be prepared in advance when using the ESEM, as it applies a gas (water vapor, argon, or nitrogen) between the electron detector and the specimen. This gas is ionized upon colliding with the secondary electrons, thus producing a dual effect. First, it amplifies the signal of the secondary electrons emitted by the sample (which are responsible for producing the image), and second, it prevents the sample from becoming electrostatically charged. By applying a potential difference between the detector and the specimen slide, the gas ions are attracted toward the specimen surface, acting in the same way as the gold or carbon coatings used in conventional EM[1].

In order to avoid atmospheric interference due to the lack of a vacuum the ESEM uses a secondary-electron detector that is able to operate in water vapor atmospheres of up to 10 Torr. The design of the microscope also includes what are known as pressure-limiting apertures, which separate different vacuum levels and thus create a gradient between the specimen and the microscope lens. These apertures are wide enough to allow the passage of electrons, but too narrow for gas molecules to pass from one compartment to another. Each compartment contains a pump system to generate the necessary vacuum[15].

Three broad categories of images were described: (A) evenness of surface; (B) presence of surface splits; and (C) presence of lacunae, divided into subcategories[4,16]. Images were classified using a table validated by our group and adapted to the ESEM according to the initial studies of Hong and Henderson[4,17](Figure 1).

RESULTS

Twenty ESEM photographs were taken for each of the cartilage samples; each of the images at a magnification of 250× covered 0.1172 mm^2 (total 100 images). The images were stored digitally to create a computerized data bank of images of the articular surface of both fresh and frozen osteochondral grafts (Figures 2-4).

A.1. Even surface. When comparing the groups of fresh versus frozen samples, the statistical tests rejected the null hypothesis, and thus it could be assumed that any differences were not due to chance. The results showed that the frozen samples presented changes in the cartilage surface that distinguished them from the even surface of fresh samples. We thus concluded that freezing introduces changes in the even surface of fresh osteochondral grafts. Significant, non-chance differences were also found when comparing fresh and cryopreserved samples, there being low concordance between the groups. This suggests that cryopreservation with RPMI also produces changes in the cartilage surface that distinguishes it from the surface of fresh grafts.

A.2. Uneven surface. *A.2.1. Hillocky surface*. When comparing this category between the groups of fresh and frozen grafts the distribution proved significant, and thus these differences would seem not to be due to chance. We therefore concluded that preservation by freezing increases the number of hillocks on the surface of fresh articular chondral grafts. Our view is that the hillocks observed correspond to elevations produced by the collagenous fibers packaged in the outer layers of the cartilaginous tissue and that jut out from a more superficial position of these layers (which would explain their presence in fresh grafts); alternatively, they could be due to a loss of tissue volume caused by expo-

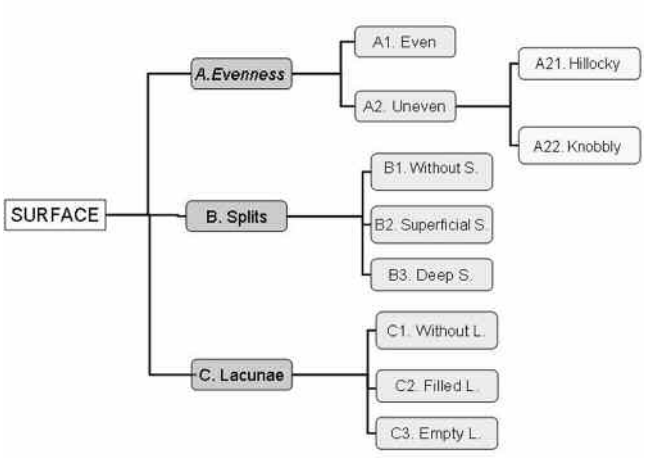

Figure 1. Classification used according to the different categories: evenness, splits, and lacunae.

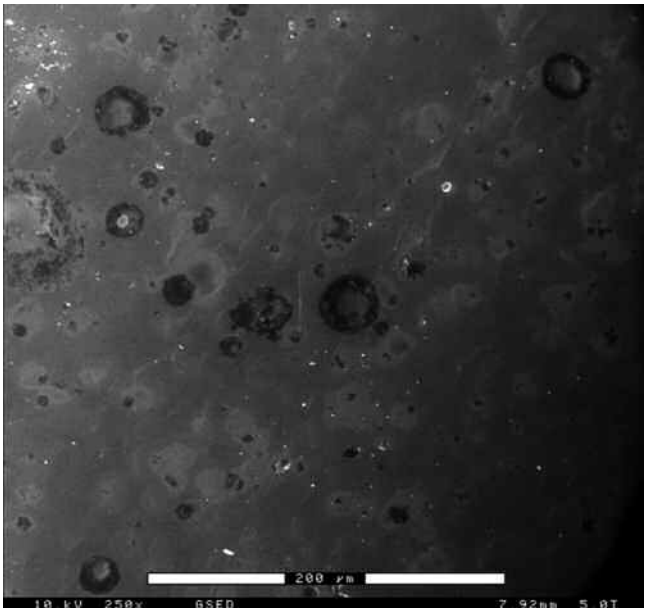

Figure 2. Surface of fresh cartilage. The area shows no splits, but there are some round lacunae filled with spherical bodies, which correspond to chondrocytes from the most superficial layer of tissue.

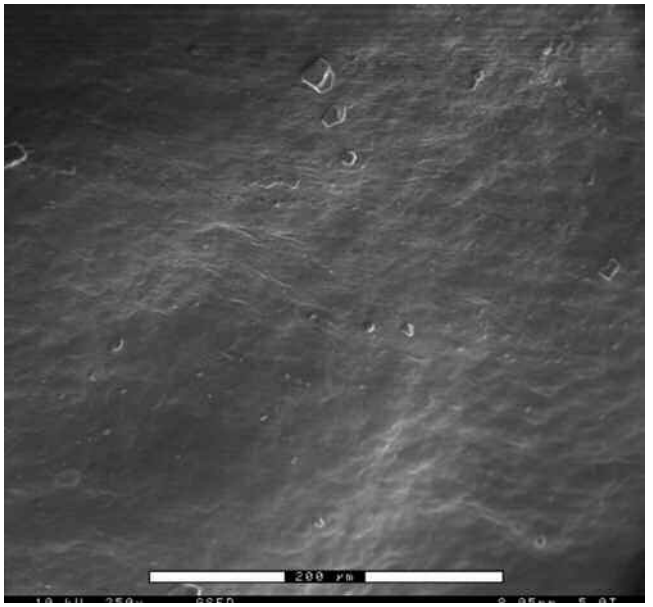

Figure 3. Surface of cryopreserved graft. The surface shows a uniform area with slightly hillocky, but rarely knobby appearance.

sure of the graft to a low temperature and low humidity environment (frozen storage).

The same significant result was found when comparing fresh with cryopreserved samples, and the concordance index was low. Therefore, we conclude that preservation with RPMI increases the number of hillocks on fresh articular surface. Once again, our view is that the hillocks observed correspond to elevations produced by the collagenous fibers packaged in the outer layers of the cartilaginous tissue and that jut out from a more superficial position of

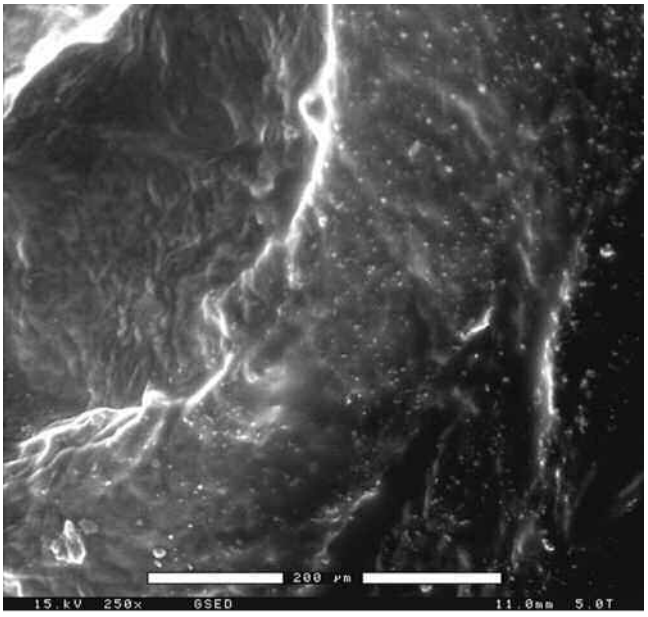

Figure 4. Surface of frozen graft. A non-uniform area with knobby features and without lacunae is observed. A superficial split is shown in the central area.

these layers (which would explain their presence in fresh grafts). Although the loss of volume due to humidity is partly counteracted by the cryopreservation medium, the osmotic effect of the solutes could exert a negative influence in this regard and account for the hillocky surface observed in cryopreserved grafts.

A.2.2. Knobby surface. For this category, significant differences were again observed when comparing the articular surface of fresh and frozen samples: exposing the fresh graft to a freezing process causes its surface to be significantly more knobby. We believe that this is due to a similar phenomenon to that described in the previous section, but in this case the observed knobby features are produced not by the densely packaged collagenous fibers but rather by the chondrocytes located in the most superficial layer of the tissue.

The comparison of fresh and cryopreserved samples revealed similar results, but with a moderate correlation index between the 2 groups (fresh vs cryopreserved in RPMI). Once again, we believe this is due to a similar phenomenon to that described for the previous category.

B. Surface splits. B.1. No splits. No comparison made for this category revealed significant differences between the experimental groups, the correlation indices being very high. This suggests that freezing does not in itself produce splits in fresh articular surface, and therefore these morphological events cannot be used as indicators to evaluate the preservation techniques used in this study. It is likely that this category would be useful in evaluating the procedure of extraction and subsequent handling of samples, and thus this topographic accident could be the subject of future research,

having ruled out that its occurrence is an artefact of the physical-chemical fixing processes used in EM.

B.2. Superficial splits. As with the previous category the comparisons between experimental groups revealed non-significant differences, and therefore we conclude that superficial splits are not directly related to the freezing process.

B.3. Deep splits. Once again the frequency distributions for the 2 groups studied showed no significant differences. Thus, we reiterate our view that surface splits are caused by effects other than freezing, for example, as a result of how the sample is obtained or handled.

C. Lacunae. C.1. Surface with no lacunae. As regards the presence of lacunae there were no significant differences between the articular surface of fresh and frozen grafts. The fresh group contained a large number of samples without lacunae and there was a good concordance index with respect to the cryopreserved group, thus suggesting that cryopreservation does not produce a great number of differences in this regard.

C.2. Filled lacunae. Analysis of the presence of chondrocytes inside the lacunae revealed a significant difference between the groups of fresh and frozen grafts. Post-freezing images of the articular surface showed no filled lacunae, and this could be related to a process of cell death caused by the adverse conditions of the external medium.

The same analysis for the fresh and cryopreserved groups also showed a reduction in the correlation index, this being moderate when comparing the 2 groups. This suggests that the cryopreservation procedure (with RPMI) reduces the number of chondrocytes visible on the articular surface, although the number remains much greater than that found among frozen samples.

C.3. Empty lacunae. Comparison of fresh and frozen grafts revealed significant differences, with the freezing procedure producing an increased number of empty lacunae. Exposing osteochondral tissue to a freezing process leads to the disappearance of chondrocytes, probably as a result of the low temperatures and by the same mechanism as described in the case of freezing; this illustrates that the freezing process used here is not completely effective in terms of maintaining the number of cells present in the fresh state.

Comparison of the fresh and cryopreserved groups revealed a low correlation index, and this suggests that cryopreservation with RPMI increases the number of empty lacunae and, therefore, of viable chondrocytes. However, once again the number of empty lacunae remained significantly lower than that found among frozen samples.

DISCUSSION

ESEM was used to take 100 photographs of the articular surface of fresh osteochondral grafts, as well as of grafts that had been preserved by freezing at 233.15ºK (–40ºC) or cryopreserved in RPMI medium at 77.15 ºK (–196ºC) for 15 days. No specimen was exposed to a physical or chemical fixing process, which might increase the likelihood of artefacts and thus lead to false observations regarding the morphology of the sample.

We chose to work with albino New Zealand rabbits as this animal is often used in cartilage studies[18-21]; further, its docility, size, and ease of handling make it ideal for laboratory work. The experimental animals were all aged 10-12 months, thus ensuring that the growth process did not interfere with the study variables.

RPMI medium was originally formulated in the Roswell Park Memorial Institute to be used in cryopreservation as a way of providing the nutrients and metabolites needed by tissues. Albumin is added to maintain the oncotic pressure and reduce the loss of solutes into the extracellular matrix during the drop in temperature[22]. Dimethylsulphoxide (DMSO) is the cryoprotective agent that penetrates the cell and intracellular matrix, producing an environment that favors the reduction in water content at temperatures that are low enough to reduce the harmful effect of the concentrated solutes and prevent the formation of intracellular crystals that could damage the cell membrane.

The images of fresh samples show an articular surface with very few defects, and those present have a knobby or hillocky appearance; these hillocks are broader than they are high. Oval-shaped foramens with clean edges were also observed: these may correspond to chondrocyte lacunae and in some cases they were filled with darker bodies that could be examples of chondrocytes. The irregularities detected are not the result of artefacts as no fixing procedures were used. We can also confirm that the fresh articular surface of the internal femoral condyle of New Zealand rabbits is not completely even.

The surface images of frozen grafts were of particular interest when compared with the images obtained from fresh samples, as the former showed both more and more severe topographic accidents. There was a higher proportion of irregularities, that is, hillocks and/or a knobby surface, although the 2 types of morphology did not necessarily coincide in the same region. However, it is very difficult to correlate the appearance of these differences with a specific factor and they may well have a multi-factor origin. One of the most important conditions is the dehydration of tissues upon being exposed to low temperatures in dry environments, as in the case of freezing; indeed, due to osmotic factors there may even be some cellular dehydration despite the tissue being immersed in cryopreservation medium. In some surface areas the protuberances were larger and more frequent than those observed in fresh samples. These protuberances are thought to be due to the protrusion of material from underlying chondrocytes, perhaps exacerbated by the various noxae to which the tissue was subjected.

Many oval foramens were also observed on the surface,

and these may correspond to chondrocyte lacunae. However, it should be noted that we were unable to detect the oval bodies identified as chondrocytes in the fresh grafts. This may be caused by a process of cell death due to osmotic stress, cold temperatures, or various factors simultaneously. The images of empty foramens are consistent with the descriptions reported by Gardner and McGillivray[23] for fresh cartilage surfaces observed under a scanning EM. As suggested by these authors, these foramens could be examples of lacunae that had previously housed chondrocytes, which then disappeared during the fixing process required by conventional EM.

Splits were also observed on the surface of frozen grafts. Evidently, the expansion of tissue fluids during the freezing process and the subsequent return to the natural state through reheating are key factors in the generation of surface disruptions.

It should be noted that in this group of grafts we did not observe the fibrous surfaces reported by Hong and Henderson[17] and Jurvelin, *et al*[14]. Our studies of the surface of frozen osteochondral grafts are the first in the field of cryobiology to show images in which the differences compared to fresh grafts cannot be attributed to the physical-chemical fixing methods required by conventional EM, this being possible thanks to the use of ESEM technology.

In some samples from the RPMI group the chondrocytes showed a small black spot, which may correspond to lesions in the plasma membrane. The plasma membrane plays a very important role in post-cryopreservation viability, as during the osmotic stress caused by reheating the cartilage the cell membrane has to enable the flow of fluids and electrolytes to continue at a rate that prevents the occurrence of cell death. Some cryoprotective agents such as DMSO increase the permeability of the cell membrane, through a mechanism that is not yet fully understood, and thus they reduce the potential damage caused during osmotic stress.

In the group of cryopreserved grafts we also obtained images that resembled those found in studies of freezing, and this could have various causes: insufficient exposure of the graft to the cryopreservation medium, suboptimal formulation of this medium (formulated mainly for vascular tissue), alteration in the tissue itself, or a combination of all three. As in the control group, images from cryopreserved grafts also showed hillocks and a knobby surface.

The results obtained for surface quality (evenness, hillocks, and knobby surface) showed that cryopreservation using RPMI medium produced changes in the surface of fresh chondral grafts, there being an increase in the number of hillocks and knobby features. These hillocks correspond to elevations produced by the collagenous fibers packaged in the outer layers of the cartilaginous tissue and that jut out from a more superficial position of these layers (which would explain their presence in fresh grafts). Although the loss of volume due to humidity is partly counteracted by the cryopreservation medium, the osmotic effect of the solutes could exert a negative influence in this regard and account for the hillocky surface observed in cryopreserved grafts. The presence of knobby surface areas is caused by a similar process, but in this case it would be due to the chondrocytes from the most superficial layers.

As regards the presence or absence of deep and/or superficial splits there were few differences between the groups, and those that were observed were not significant. Therefore, it can be concluded that cryopreservation does not produce changes in this aspect of the surface of chondral grafts.

In terms of the absence of lacunae very good correlation indices were obtained between the groups of fresh and cryopreserved samples. This was not the case, however, when evaluating the presence of filled lacunae, as there were fewer visible chondrocytes in the group of RPMI cryopreserved samples. Finally, the cryopreserved group also showed a greater number of empty lacunae, and therefore of nonviable chondrocytes, than did the fresh grafts.

In conclusion, the ESEM proved useful for obtaining images of the surface of osteochondral grafts, which could then be used to create an image bank for future studies. Both freezing and cryopreservation produced a decrease in the number of even surfaces and filled lacunae, as well as an increase in the number of empty lacunae, these changes being signs of chondrocyte destruction; however, these features were less marked among the group of cryopreserved samples. Therefore, on the basis of surface morphology studies it can be concluded that the use of RPMI cryopreservation medium leads to a higher degree of chondrocyte viability than is achieved through freezing methods.

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