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Hyaline cartilage surface study with an environmental scanning electron microscope. An experimental study

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Abstract To obtain images of the articular surface of fresh osteochondral grafts using an environmental scanning electron microscope (ESEM). To evaluate and compare the main morphological aspects of the chondral surface of the fresh grafts. To develop a validated classification system on the basis of the images obtained via the ESEM. The study was based on osteochondral fragments from the internal condyle of the knee joint of New Zealand rabbits, corresponding to fresh chondral surface. One hundred images were obtained via the ESEM and these were classified by two observers according to a category system. The Kappa index and the corresponding confidence interval (CI) were calculated. Of the samples analysed, 62–72% had an even surface. Among the samples with an uneven surface 17–22% had a hillocky appearance and 12–16% a knobbly appearance. As regards splits, these were not observed in 92–95% of the surfaces; 4–7% showed superficial splits and only 1% deep splits. In 78–82% of cases no lacunae in the surface were observed, while 17–20% showed filled lacunae and only 1–2% presented empty lacunae. The study demonstrates that the ESEM is useful for obtaining and classifying images of osteochondral grafts.

1 Introduction

Due to the characteristics of cartilaginous tissue, chondral lesions continue to pose a challenge for orthopaedic surgeons. Several surgical strategies are currently 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 transplant [1–9].

Various methods have been used to study the morphology of hyaline cartilage. One advantage of the environmental scanning electron microscope (ESEM) over conventional electron microscopy is that by examining samples directly, without the need for physical-chemical fixing techniques, it offers a more realistic view of morphology [10–14]. Moreover, its chamber does not require an absolute vacuum and thus it can be used to observe moist specimens [10, 15–17].

Samples do not need to be prepared in advance when using an ESEM due to the presence of an imaging gas inside the chamber. In scanning electron microscopy the interaction between the incoming beam of electrons and the sample results in various products being emitted. Of these products, secondary electrons (SE) are collect when a high resolution, topographical image is required. In ESEM the SE are accelerated towards the detector by an applied small positive bias. When the SE's have enough energy any collision with the imaging gas will result in the gas becoming ionized (positively charged) with an extra electron to be emitted. This reaction, as well as amplifying the SE signal, provides positively charged ions which drift to the negatively charged sample surface and reduce the charge build-up. [18–21].

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 vapour 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

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pressure gradient between the specimen and the microscope lens. Each compartment contains a pump system to generate the necessary vacuum [22].

The present study sought both to determine whether the environmental scanning electron microscope is a useful tool for evaluating the morphology and viability of fresh hyaline cartilage, and to develop a reference standard for assessing cartilage biopsies by means of this system.

2 Materials and methods

The present research involved an experimental, longitudinal and prospective study. Following the approach of Bailey [23], Weakley [10] and Williams [24] with respect to study animals for electron microscopy, we created a study group of fresh hyaline cartilage. These authors point out that the sampling of photographs in electron microscopy is considered to be statistically valid if they 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 [25] 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.

After sacrificing the animals the knees of their hind legs were dissected in sterile conditions. A sample of the internal femoral condyle was then taken using a trephine (fresh osteochondral grafts from the weight-bearing areas of the knee joint of New Zealand rabbits). Once the knee samples had been isolated they were then submerged in a sterile solution of 0.9% NaCl at room temperature until transfer to the centre where the electron microscope was housed. The physiological solution prevents the articular cartilage of the graft from dehydrating (due to contact with the air) prior to being examined.

Each cartilage disc was subsequently mounted on a stub in order to be studied under an environmental scanning electron microscope (ESEM^{TM} ElectroScan 2020 ESEM-FEG) at 276.65°K (3.5°C), with an accelerating voltage of 10 KV and 20 KV, and a chamber pressure of 5 Torr. Using the following pumpdown technique, water vapour 8 × (5–10 Torr), the chamber air was replaced by water vapour at the previously mentioned pressure.

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 with 100 images for each group. Two observers evaluated the images according to the following three broad categories: (A) evenness of surface; (B) presence of surface splits; and (C) lacunae in the surface, this being divided into two subcategories (Fig. 1) [18, 26].

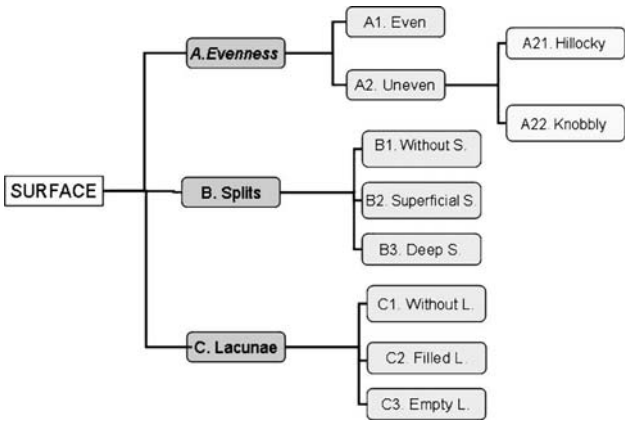

Fig. 1 Classification used according to the different categories: evenness, splits and lacunae

3 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 photographs were stored digitally in order to create a computerized data bank of images of the articular surface of fresh osteochondral grafts (Figs. 2, 3, and 4).

The results obtained regarding the evaluation and comparison of the main morphological aspects of the articular

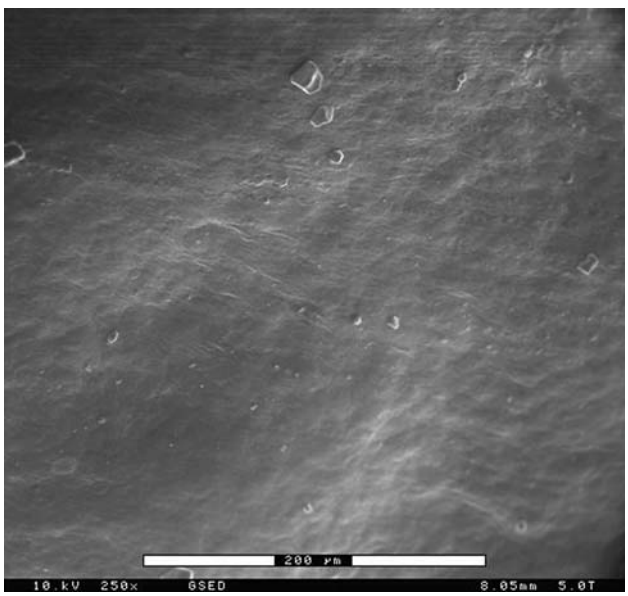

Fig. 2 Surface of fresh cartilage. A uniform area without splits or lacunae can be observed, there being some residues which may correspond to bone fragments or synovial fluid crystals

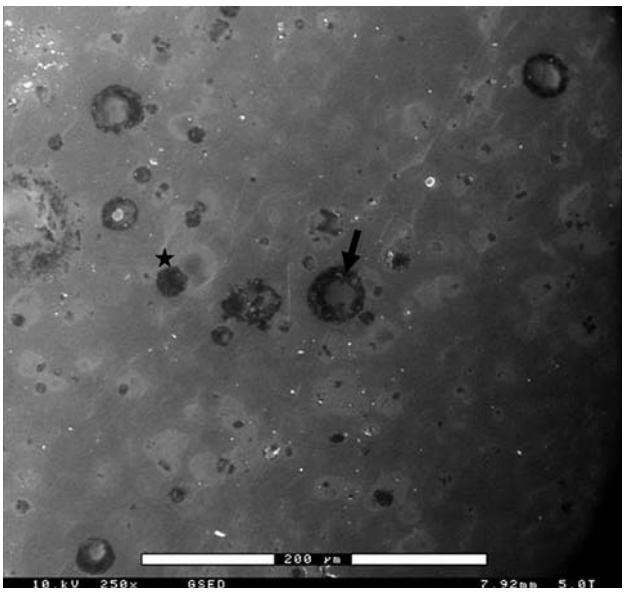

Fig. 3 Surface of fresh cartilage. The area shows no splits but there are some round lacunae filled with spherical bodies, which correspond to chondrocytes from the most superficial layer of tissue (*star*: unfilled; *arrow*: filled)

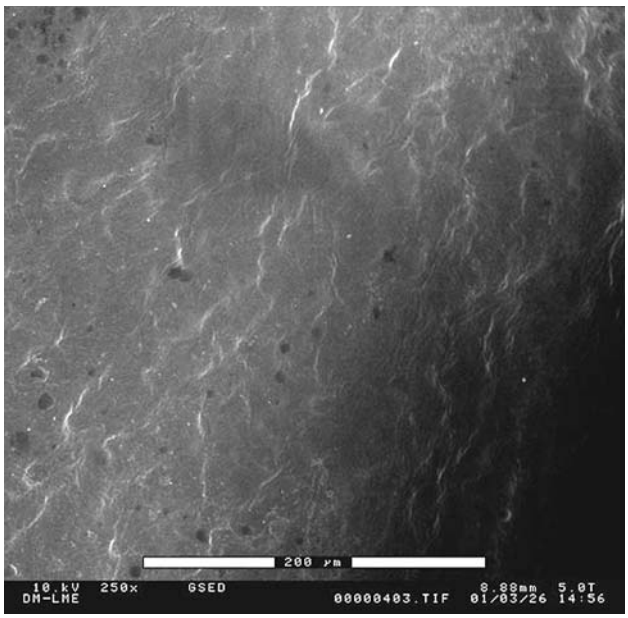

Fig. 4 Surface of fresh cartilage. A relatively uniform area can be observed, with a slightly hillocky appearance

surface of fresh osteochondral grafts are described below according to the different categories**.**

3.1 Evenness of surface

This category is sub-divided according to the most common and most easily identifiable topographic variations observed in the photographs studied: even surface and uneven surface. The latter sub-category includes surfaces with a hillocky or a knobbly appearance.

3.1.1 Even surface

This category refers to the nature of the surface relief. In the sample analysed 71% of the photographs showed an even surface.

3.1.2 Uneven surface

This is further sub-divided into:

3.1.2.1 Hillocky surface This category refers to the nature of the surface relief. In contrast to even surfaces the category of uneven surface seeks to describe imperfections, which generally take the form of either a hillocky or a knobbly appearance. A surface may show both a hillocky and a knobbly appearance, that is, the two types are not mutually exclusive. The presence of splits (fractures) or lacunae does not define a surface as uneven. Of the photographs studied the observers considered that 17% could be classified within this category.

3.1.2.2 Knobbly surface This category refers to the nature of the surface relief. The observers considered that 12% of the photographs taken could be classified within this category.

3.2 Surface splits

3.2.1 Surface without splits

92% of the studied photographs showed no splits. These observations provide support for our hypothesis that the physical-chemical fixing process creates artefact effects, and they are also consistent with the findings of Hong and Henderson [27], who argue that splits in the cartilaginous surface are atypical and, in some cases, are probably caused artificially during sample handling. Such splits must be taken into account as they may develop into lesions that would lead to the failure of an osteochondral graft following implantation into patients or experimental animals.

3.2.2 Surface with superficial splits

Of the photographs studied the observers considered that 4% could be classified within this category. We believe that the strong agreement observed in this category is mainly due to the low prevalence of this topographic feature in the images (fewer than 10% of the photographs analysed). The presence of superficial splits can be attributed to many factors such as uncommon topographic phenomena associated with the

cartilaginous surface or inadequate handling of the sample when extracting the specimen, this being a difficult procedure.

3.2.3 Surface with deep splits

Of the photographs studied the observers considered that 1% could be classified within this category. We believe it is of interest to establish the two categories of superficial and deep splits as these are aspects that can be observed on the surface of fresh cartilage, and not necessarily as the result of the physical-chemical fixing process used in conventional electron microscopy. Given the morphological characteristics of the splits we decided to reclaim this category that was originally proposed in 1983 by Jurvelin [28], despite it being a less common and minor topographic variation. (Fig. 5).

3.3 Lacunae

3.3.1 Surface with no lacunae

Of the photographs studied the observers considered that 78% of the images could be classified within this category. It should be emphasised that the superficial layer of articular cartilage is not renowned for being the area of tissue where the greatest number of cells are found.

3.3.2 Surface with filled lacunae

Of the photographs studied the observers considered that 17% of the images could be classified within this category. The content of the lacunae mainly comprised well-defined, spherical or smooth oval bodies, and no examples of other bodies such as bone particles were observed, in the hypothetical case that these might exist.

3.3.3 Surface with empty lacunae

Of the photographs studied the observers considered that 1% of the images could be classified within this category. Given the methodology used in the present study we believe that the occasional presence of lacunae identified as empty could either be due to limitations of the microscopy technique, which did not enable visualization inside the lacunae in question, or refer to lacunae that are genuinely empty as a result of some physiological process that has removed their content.

4 Discussion

Jurvelin et al. [28] developed a scale for evaluating the morphology of the articular surface by means of scanning electron microscopy and using patellas from New Zealand rabbits. However, the scale was not validated as they did not quantify the inter- and intra-observer variability. Furthermore, their scale was not designed to assess surface changes produced by different sample conservation procedures, and neither did it include any distinction or qualification regarding the artefacts that might be produced by the sample fixing method required by scanning electron microscopy.

Hong and Henderson [27] modified the scale proposed by Jurvelin et al. in order to study changes in the patellar cartilage surface following knee immobilization in rats, but

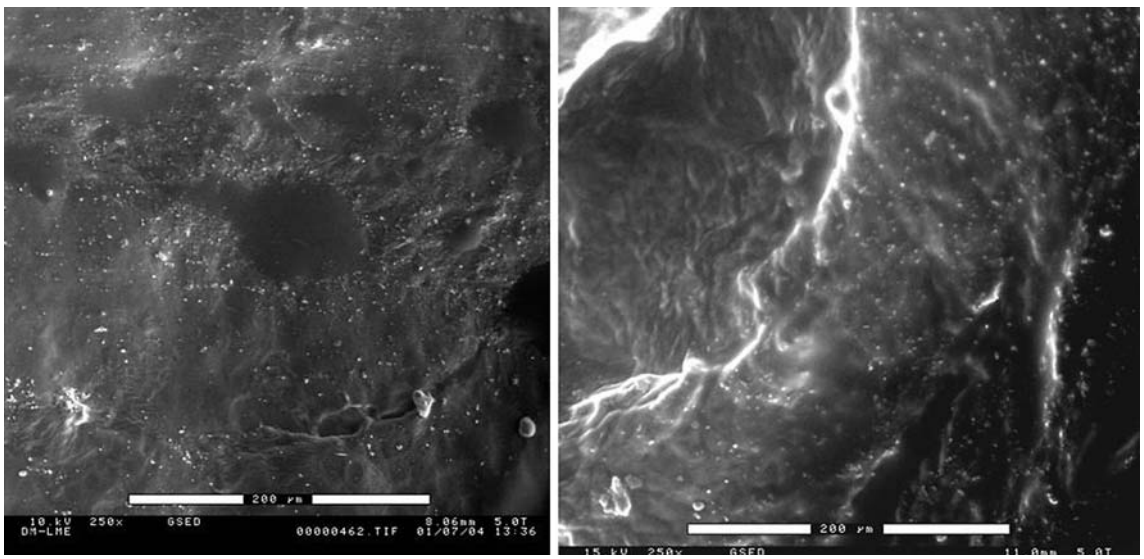

Fig. 5 Difference between a superficial split (*left*) and a deep split (*right*)

they also failed to validate their scale with respect to inter- and intra-observer variability.

O'Connor et al. [29] used conventional techniques of scanning electron microscopy at low temperature to describe dog femoral condyles following anterior cruciate ligament section. Although these studies describe changes that probably indicate early degeneration, they do not use a validated classification system and therefore the descriptions may be regarded as subjective. Neither is any mention made of the likely effect of low temperature or conventional fixing techniques when obtaining images via electron microscopy.

The ESEM enables a sample to be viewed directly whilst preserving its moistness and without distorting it. Gardner et al. examined the articular surface of fresh and frozen cartilage using a cryogenic scanning electron microscope. However, few reports describe use of the ESEM to study cartilaginous tissue [18, 26].

The images captured with the ESEM show an articular surface with very few defects, and those present have a knobbly or hillocky appearance; these hillocks are broader than they are high. Oval-shaped foramens with clean edges were also observed, and these most likely correspond to chondrocyte lacunae containing oval bodies with an even surface and round edges that do not touch the walls of the lacunae, and which could be examples of chondrocytes. Our observations have enabled us to verify that the irregularities detected are not the result of artefacts of the fixing process, as we did not use any such technique, and nor are they due to cold preservation methods. Therefore, we consider these images to be a reference standard for comparing similar samples subjected to cold preservation processes. Furthermore, the images enable us to confirm that the fresh articular surface of osteochondral grafts from the internal femoral condyle of New Zealand rabbits (albino females aged 10–12 months) is not completely even.

Studies of articular surface using conventional scanning electron microscopy have been criticised on both biological and physical grounds, since the preparations have to be dehydrated prior to being examined inside the vacuum of the sample chamber. The material must also be fixed with formalin or cold osmium tetroxide immediately after taking the sample, and is then left to air-dry. Alternatively, the sample may be cryofixed. Once dehydrated the specimen is coated with a thin film of electrically-conductive metal such as a gold/palladium alloy in order to prevent the surface becoming electrically charged during electron bombardment. Each of these preparative steps may produce artefacts. From the biological point of view the material examined is not strictly the same as the in vivo form as it has been deprived of physiological conditions since the point at which the animal was sacrificed, even though it is assumed that the avascular nature of cartilaginous tissue means that any changes which might occur will not do so quickly.

Another factor to consider is the reduced time required for sample handling and processing prior to observation with an ESEM, which in turn reduces the likelihood of artefacts being produced for these reasons.

The articular surface of fresh grafts often revealed rounded and/or elongated protuberances with an even surface, but we were unable to determine their origin or composition with the ESEM. Other authors have also reported such features using the conventional scanning electron microscope and have suggested, among other hypotheses, that they are due either to the extrusion of flattened chondrocytes or to the collapse of chondrocytes [30]. Another hypothesis could be based on the constitution of the layer of extracellular matrix overlying the chondrocyte, as transmission electron microscopy studies [31] of rat cartilage have shown this to be rich in densely-packed collagenous fibres which, in our view, could influence the formation of the observed protuberances and explain why they sometimes have an elongated form that is not consistent with the usual oval or spherical chondrocytes found on the articular surface. The images of small amorphous and whitish objects observed in some areas of the surface could reflect crystals or deposits from synovial fluid, or bone fragments (debris) formed during the trephine biopsy but which might then be removed from the field when the sample is washed with physiological solution. These particles of non-uniform distribution and size were first reported in 1980 by Bloebaum and Wilson [32], who used conventional scanning electron microscopy to study the morphology of rat cartilage. The authors concluded that the particles were large crystals of synovial fluid.

As regards the use of physiological solution to transport the fresh graft we believe this is important to prevent the sample from dehydrating due to atmospheric moisture or the surrounding air. It also serves to clean the surface of the osteochondral disc once the graft sample has been taken.

As mentioned previously the fresh osteochondral graft samples were taken from weight-bearing areas of the knee joint of New Zealand rabbits. Therefore, it would be interesting to determine whether the articular surface of fresh grafts from non-weight-bearing areas shows the same morphological characteristics, and whether or not the latter affect the biomechanics of the grafts. Fractures (splits) in the articular surface proved to be very uncommon in our fresh grafts, a finding which contrasts with the observations of Jurvelin et al. [28]. Splits may be caused for various reasons. The first of these is sample handling, as the cutting instrument and tissue section technique used may produce deformities that result in a loss of surface continuity. The second concerns exposure to physical-chemical conditions

(dehydration, critical point) which are important for observation under conventional electron microscopy. The fact that such procedures were not employed in our study may explain why we observed fewer fractures than did Jurvelin et al. It should also be noted that Hong and Henderson [27] and Jurvelin et al. [28] described predominantly fibrous surfaces, something which was not observed in the present study.

In conclusion, the environmental scanning electron microscope is a useful tool that enables photographic studies to be conducted of samples of fresh osteochondral graft surfaces, without the need for physical-chemical methods to fix the specimen or to expose it to an absolute vacuum in the sample chamber. Furthermore, the articular surface of fresh osteochondral grafts is not completely even and reveals various aspects that can be classified in a qualitative system, thus enabling them to be recognised and any changes to be detected in experimental and pathological conditions. These aspects constitute a reference standard that can be used to evaluate biopsies in this type of tissue.

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Conflict of interest statement All authors disclose any financial and personal relationships with other people ororganisations that could inappropriately influence (bias) our work. Including employment,consultancies, stock ownership, honoraria, paid expert testimony, patentapplications/registrations, and grants or other funding.

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