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# EVALUATION OF THE USE OF TANNIC ACID IN PREPARATION OF THE RABBIT KNEE MENISCUS FOR SCANNING ELECTRON MICROSCOPY

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## Abstract

The femoral faces of medial and lateral menisci of the rabbit knee were structurally reinforced with tannic acid and studied using the scanning electron microscope. Significant improvement in preservation of the meniscal surfaces, as compared to previous studies, was brought about by the structural reinforcement technique. Menisci, although detached from tibial plateaux, retained well their shape, dimensions, and microarchitecture. Differences in surface morphology between medial and lateral menisci and between longitudinal sectors on menisci were recorded. Appearances of surface irregularities were reduced to a very low level, and the possibility that this minimum exists *in situ* is forwarded.

**Key Words**: Meniscus, scanning electron microscopy, tannic acid, structural reinforcement.

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## Introduction

The menisci of the synovial joint in the mammalian knee are directly involved in the biomechanics and in some of the arthropathies of the knee. However, rigorous definition of their biomechanical function(s) has not yet been achieved (Bullough *et al.*, 1970; Maroudas, 1979; Ghadially *et al.*, 1983; Nakano *et al.*, 1986; Proctor *et al.*, 1989; Fithian *et al.*, 1990). The morphology of their surfaces has gained only little attention, although studying meniscal surfaces might add useful information for the assessment of meniscal function in health and disease. The close similarity in fine structure between the rabbit knee meniscus and that of the healthy uninjured human meniscus has been noted (Ghadially, 1983, p. 107). This renders the rabbit meniscus a preferred experimental model for clinical studies.

The morphological properties of meniscal surfaces have been the subject of light microscopy (LM) and transmission electron microscopy (TEM) studies (Meachim, 1976; Ghadially, 1983; Ghadially *et al.*, 1978, 1983; Heatly, 1980; Merkel, 1980). Unfortunately, LM and TEM studies can only make use of sectioned tissues and do not enable one to visualize the surface of a whole specimen. This drawback is exemplified by the fact that in histological and ultrathin sections, meniscal surfaces appear either smooth or wavy to markedly undulated, depending on the site and plane of section.

Surfaces of menisci have been studied in the scanning electron microscope (SEM), but only at low resolution (Inoue *et al.*, 1971; Cameron and Macnab, 1972, 1973; Moshurchak, 1978; Merkel, 1980; Ghadially, 1983). In these studies, "surface irregularities" (undulations, pits, and humps, generally referred to under this generic name), which were hypothesized to have resulted from insufficient preservation, were consistently discerned (Ghadially, 1983, p. 107). Moreover, dried menisci, unless attached to tibial plateaux, curled and lost their shape following preparation. Consequently, their examination in the SEM became very difficult (Ghadially, 1983, pp. 134-137).

When menisci were divided into longitudinal sectors, sectoral differences in microanatomical and biomechanical properties (Adams and Ho, 1987; Arnoczky *et*

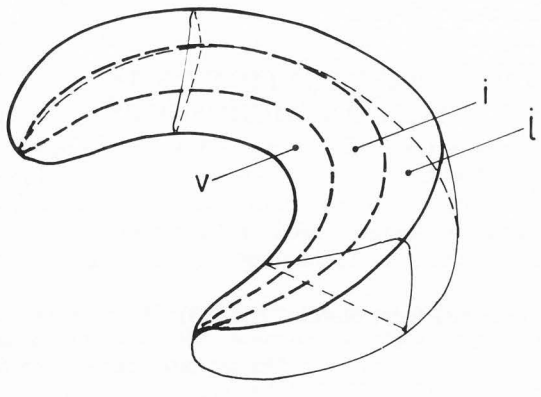


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**Figure 1.** Schematic drawing of the longitudinal sectors on menisci; J: juxtasynovial; i: intermediate; v: visceral.

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*al.*, 1988; Proctor *et al.*, 1989; Fithian *et al.*, 1990), in biochemical composition (Adams and Ho, 1987; Fithian *et al.*, 1990; McDevitt and Weber, 1990), and in ultrastructural organization (Fithian *et al.*, 1990) were revealed. This clearly indicates the relevance and necessity of studying the meniscal sectors in the SEM. Such a study might enable one to assess whether a correlation between surface morphology and the above parameters can be made.

We sought to learn whether a structurally reinforcing preparation method that has already been shown to improve preparation of articular and synovial surfaces (Levanon and Stein, 1991, 1992) might serve to preserve the meniscal surfaces as well and provide us with a new answer to an old question: do irregularities on meniscal surfaces exist *in vivo* or are they an artifact of preparation? We fixed rabbit menisci by a modification of the GTGO method (Gamliel *et al.*, 1983; Levanon and Stein, 1991, 1992), which serves to structurally reinforce a tissue with tannic acid, divided the menisci into longitudinal sectors, and studied each sector in the SEM.

## Materials and Methods

Seven albino rabbits (weighing about 1.8 kg, either sex, local strain) were killed by an injection of an overdose of sodium pentobarbital into the ear vein. The stifle joints were surgically exposed by parapatellar incision and rigorously rinsed by phosphate-buffered saline (PBS) in order to keep joint cavity apposing surfaces wet and to remove possible residual synovial fluid (Wysocki and Brinkhous, 1972; Moshurchak and Ghadially, 1978). Medial and lateral menisci were liberated from adjoining parameniscal fibrofat and synovial linings, rinsed in PBS, and immediately put into a standard fixative solution containing 2.5% glutaraldehyde in PBS (375 mOsm, final pH 7.4). Fixation was carried out for 3 to 5 hours at room temperature and followed overnight at 4°C. The specimens were washed thoroughly with PBS to remove excess glutaraldehyde, incubated in a solution of 2% tannic acid and 2% guanidine-HCl in PBS for 1 hour (Gamliel *et al.*, 1983; Levanon and Stein, 1991, 1992), and postfixed in 1% $OsO_4$ in PBS for 1 hour. They were dehydrated in ascending ethanols, rinsed in hexamethyldisalazane as an intermediate fluid that allows air drying rather than critical point drying (Nation, 1983: Levanon and Stein, 1992), air dried, and kept in a desiccator until used. Menisci were mounted on copper stubs with silver conductive paint, with the femoral face [the concave face, clearly distinguishable from the flatter tibial face (Ghadially, 1983)] laid upwards. They were coated with gold in a Polaron coating unit (E-5000) to provide an 18-nm-thick coating and examined in a JEOL T-300 SEM operated at 25 kV and 0° tilt relative to the stub holder. Dried menisci could be stored for 9 months without any sign of deformation.

Fourteen medial and 14 lateral menisci were included in the present study. A specimen was mounted on a stub as a whole and longitudinally divided into three sectors: juxtasynovial (peripheral), intermediate, and visceral (inner), according to Fithian *et al.* (1990) and Proctor *et al.* (1989). These sectors ran parallel to the circumference of the meniscus (Fig. 1), and each was systematically accounted for and all its appearances recorded. Micrographs were taken in at least three standard magnifications so that surface details could be compared.

## Results

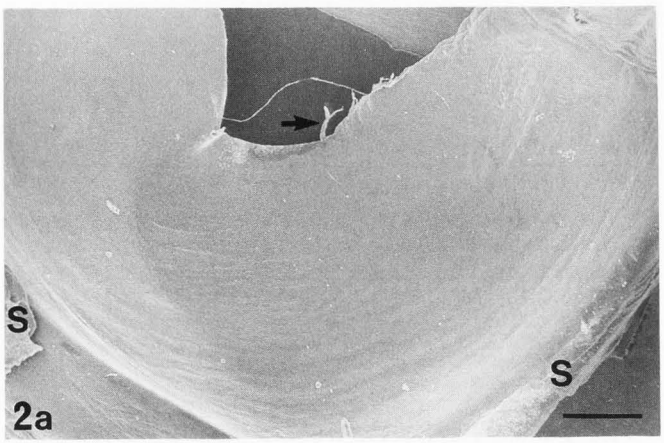
For ease of comparison, whole menisci (Fig. 2) and juxtasynovial strip, medium meniscus (Fig. 3) are presented on one plate; transitional areas are presented on one plate (Figs. 4 and 5); and lateral meniscus is presented on one plate (Figs. 6 and 7)

### General appearance of the tissue

Meniscal specimens retained their shape and dimensions, as judged by examination in the binocular, from the fresh stage throughout the preparation procedure. No curling, cracking, or other indications of adverse effects of the preparation were noted. Occasionally, small tears and chondrophytes on the central third of the visceral sector were seen [cf. Ghadially (1983) and Cameron and Macnab (1973)].

Lateral menisci were flatter than medial menisci (see Table 1), and the transition between juxtasynovial and intermediate sectors looked steeper in the medial menisci and more moderate in the lateral ones (Figs. 1, 2, 3d, 4c, and 6).

On the juxtasynovial sectors of both menisci, long undulations of various thicknesses, made out of furrows and crests that were arranged in parallel, ran along the specimens' margins (Figs. 3, 4, and 6). Undulations on medial menisci looked bolder, at least partly because their contours were oriented in profile to the observer, while those on the lateral menisci, which were oriented *en face*, appeared much flatter, to the measure that they were, in places, difficult to discern. Undulations on a medial meniscus created an area that was much too convoluted to allow reliable detection of contours of pits



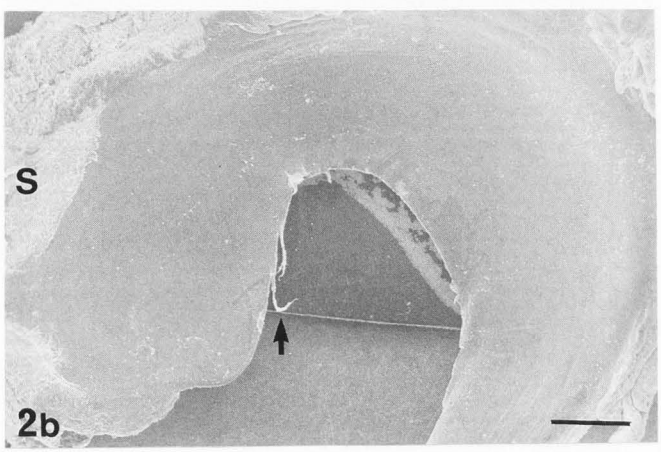




**Figure 2 (above)**. Whole menisci shown at low magnification: **a**: medial meniscus; **b**: lateral meniscus. Note chondrophytes (arrows) and residues of synovial linings (S). Bars = 1.35 mm.

**Figure 3 (at right)**. Juxtasynovial strip, medial meniscus. Bold undulations are prominent. Note fine surface coat, whose details are made discernible under high resolution. Reliable identification of pits and humps in Figure **3c** cannot be made, due to intensely corrugated surface. Note residue of synovial lining (S) and a tear in the visceral margin (arrow). In Figure **3d**, empty and solid arrowheads, respectively, point to the fields on which Figures 3 (a-c) and 4 were recorded. Bars = 1 $\mu$m (a); 10 $\mu$m (b); 100 $\mu$m (c); 1 mm (d).

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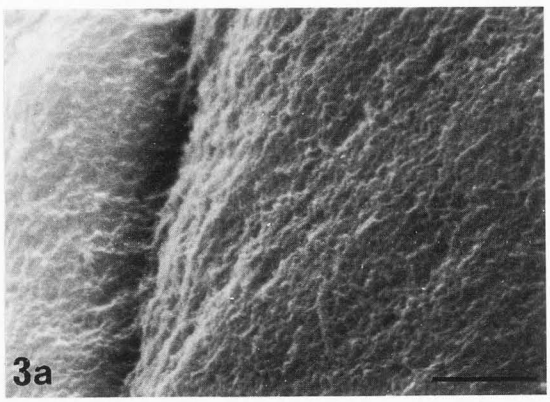
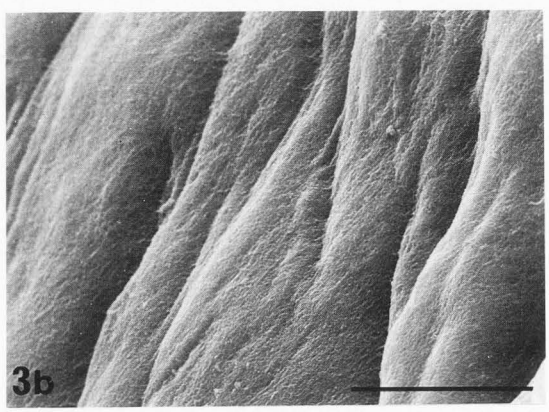
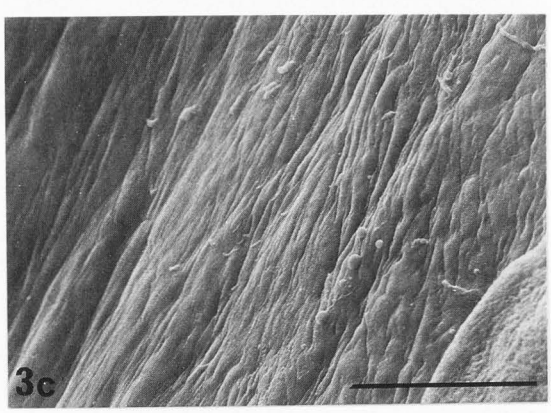
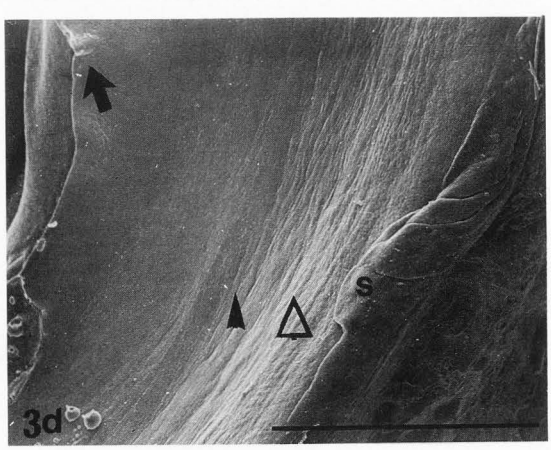
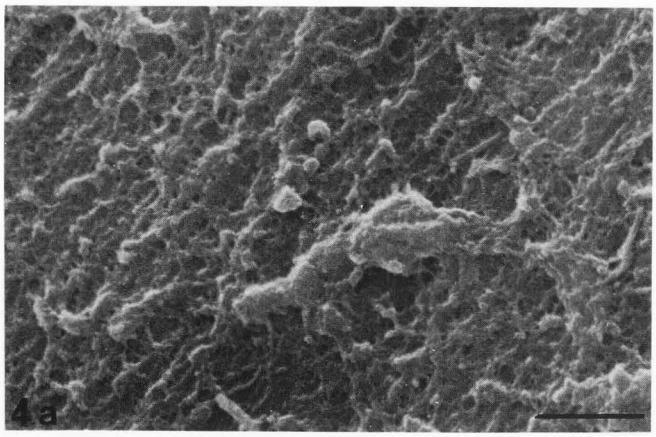
and humps (Fig. 3b). Undulations became gradually more shallow and their orientation more *en face* on the transition area towards the intermediate strip, thus rendering pits and humps discernible (Fig. 4c). Contours of the latter were spindle-shaped and ran parallel to the crests. However, at the lowest magnification (Fig. 3d) pits and humps were "levelled off", and the general impression was that of a smooth area. Pits and humps, albeit clearly discernible on the medial menisci, were not detectable on juxtasynovial sectors or elsewhere on the

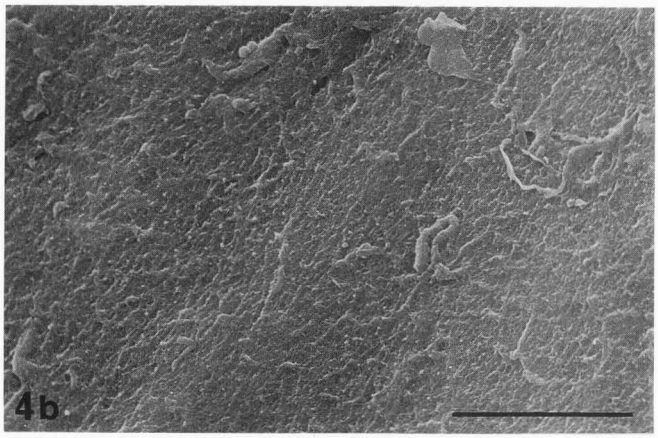


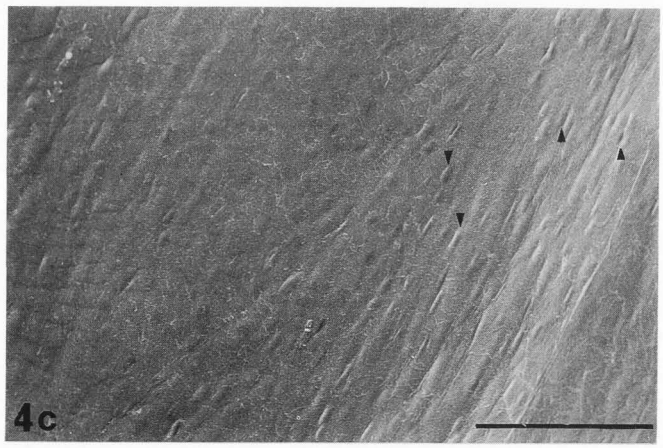



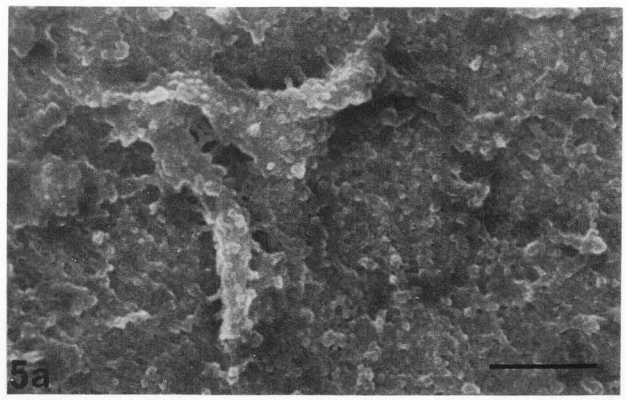



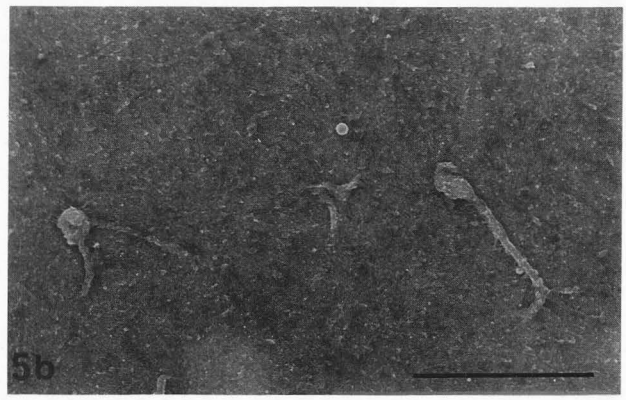





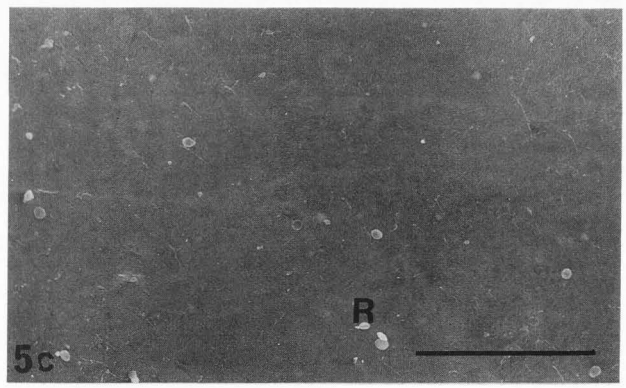
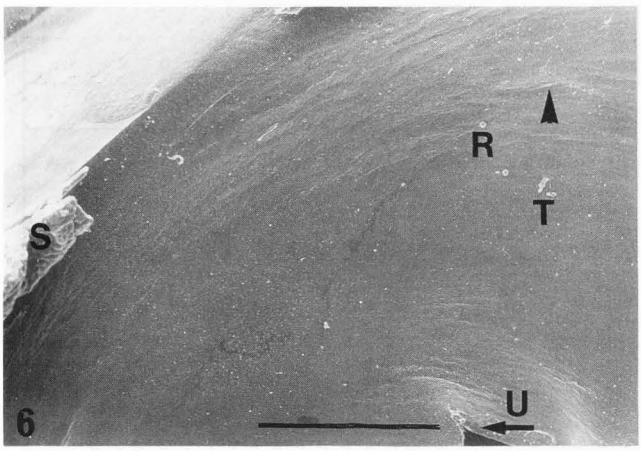
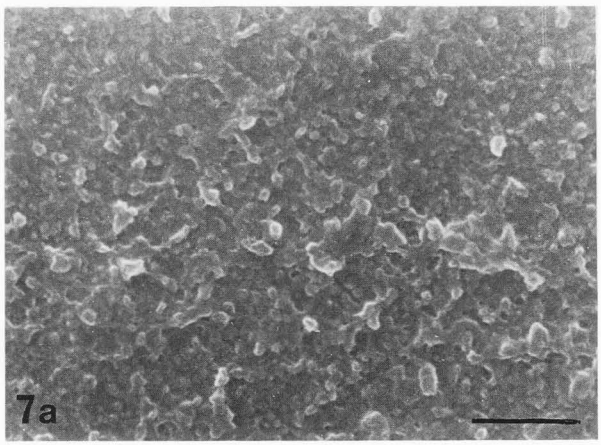





**Figure 4.** Transitional area between intermediate strip and juxtasynovial strip, medial meniscus. Note surface coat with acellular processes. Moderate undulations allow detection of randomly oriented pits (upward arrowheads) and humps (downward arrowheads). Bars = 1 $\mu$m (a); 10 $\mu$m (b); 100 $\mu$m (c).



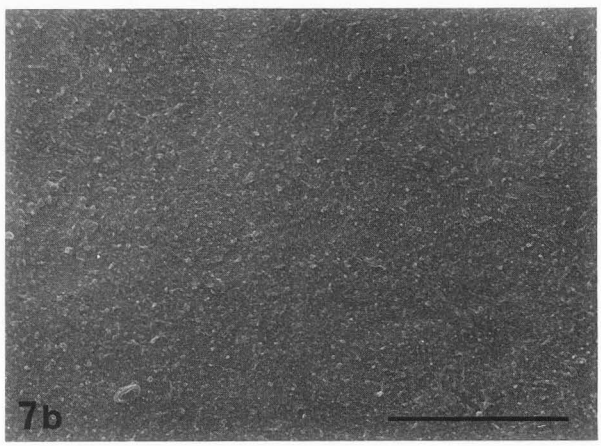




**Figure 5 (at left)**. Transitional area between intermediate strip and visceral strip, lateral meniscus. Note low-profile surface processes under high magnification (**a** and **b**), but perfectly smooth surface under low magnification (**c** and **d**). The latter is beset, in places, with erythrocytes (R, in Fig. 5c) and tags of tissue (T). In Figure 5d, note residue of synovial lining (S) and a chondrophyte (arrow); a curved arrow points to the field on which Figure 5 (a-c) was recorded; and empty arrowhead points to the field on which Figure 7 was recorded, on the same meniscus. Bars = 1 $\mu$m (a); 10 $\mu$m (b); 100 $\mu$m (c); 1 mm (d).

**Figure 6 (above)**. Lateral meniscus. Note undulations (U) adjacent to and extended from a visceral tear (arrow), juxtasynovial undulations (arrowhead), and residue of synovial lining (S). Erythrocytes (R) and tags of tissue (T) are occasionally found. The intermediate strip is devoid of surface irregularities. Bar = 1 mm.

**Figure 7 (at right)**. Visceral strip, lateral meniscus. Note uniform, finely organized surface extracellular matrix, whose details are made discernible under high resolution. No bolder details were present on this strip. Bars = 1 $\mu$m (a); 10 $\mu$m (b).

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lateral menisci (compare Figs. 4c and 5c). Intermediate strips on medial specimens were mostly devoid of pits and humps.

The intact, untorn parts of the visceral strips in all the specimens were invariably smooth without any surface irregularities (Fig. 5). Tears (Fig. 3) and chondrophytes (Fig. 2a) were present on seven out of the 14 menisci (either type). When a tear on a visceral strip occurred, it was accompanied, in places (Fig. 6), by slight undulations. The latter could carry elongated pits and humps, such as those in Figure 4c. Pits and humps anywhere looked shallower and less distinct than reported in previous publications (Ghadially, 1983, p. 136; Moshurchak and Ghadially, 1978). It is noteworthy that they were present only on undulated areas; when undulations were absent, no pits or humps were discernible.

**High-resolution appearance of the surface medial menisci**

Bold crests (see above) were coated by fine amorphous interfibrillary matrix whose texture could be designated as intermediate between granular and filamentous (Fig. 3). In the juxtasynovial sector, generally no distinct trend towards either the filamentous or the granular type could be discerned (Fig. 3a). Only rarely were organized filament- or particle-shaped structures seen (not shown).

Figures 4a and 4b were recorded from the transition area between the juxtasynovial and the intermediate sectors. The surface of the intermediate strips was coarse and porous, similar to that in Figure 3a, but in addition, the intermediate strips carried surface processes. The latter varied in size and shape, were of a fairly homogenous density (Fig. 4b), and were deposited at random orientation.

**Lateral menisci**

Figure 5 shows a field of processes sparser than those shown in Figure 4 (medial meniscus). This appearance was often recorded in medial menisci also, and is shown here only as an example of the variability in density, dimensions, and shape of surface processes that existed in intermediate sectors on both meniscal types.

**Table 1.** Surface morphological appearances of the different sectors on medial and lateral menisci.

| | Juxtasynovial | | Intermediate | | Visceral | |
|---|---|---|---|---|---|---|
| | Medial | Lateral | Medial | Lateral | Medial | Lateral |
| **Low magnification observations** | | | | | | |
| Undulations | Bold | Flat | Absent | Absent | Occasionally, but only in conjunction with tears | |
| Pits and humps | Difficult to discern due to corrugated area, but probably present | Absent | Absent | Absent | Only on undulations accompanying tears | Absent |
| **High magnification observations** | | | | | | |
| Surface coat | Made of fine, amorphous interfibrillary matrix; no acellular processes | | Coarse and porous, with acellular processes at random orientation | | With fine, short and homogeneously shaped granular acellular processes | |
| Cells or cellular processes | Absent | | Absent | | Absent | |
| Fibrillation | None | | None | | None | |

Total lack of surface irregularities in intermediate and uninjured visceral strips was recorded everywhere.

Figure 7 represents visceral strips in both menisci. The surface consisted of fine, short granular processes that formed a homogenous arena without any bolder details. No fibrillation, i.e., appearance of collagen fibers denuded from interfibrillary matrix, was noted anywhere.

In none of the specimens under study were cells or cellular processes exposed to the surface detected. Although longitudinal division produced strips that were distinct in their gross and fine morphology, cross division of menisci produced sectors that did not differ morphologically.

## Discussion

Although menisci were resected and processed detached from their tibial plateaux, their shape and dimensions were preserved in excellent condition. This is in contrast to results reported by Ghadially (1983, pp. 133-136), who announced that when menisci were processed after having been separated from tibiae, they suffered severe shrinkage, curling, and distortion. In addition, fibrillation was completely absent in the present study, in contrast to previous reports (Ghadially, 1983, p. 104; Ghadially *et al.*, 1983). This bears further witness to improved preservation.

Tears and chondrophytes have previously been reported as common findings in human and animal menisci (Cameron and Macnab, 1972; Merkel, 1980; Ghadially, 1983). According to Cameron and Macnab (1972, 1973), they appear almost exclusively on the central third of the visceral strip, as found in the present study as well, and result from biomechanical stresses during life, rather than from damage during resection.

### Preparation and preservation of the tissue

In a previous article (Levanon and Stein, 1991) we described in detail a technique of structural reinforcement of articular cartilages from the rabbit knee, for the SEM. In the present study, the same technique proved very useful in enhancing the preservation of the meniscal fibrocartilage as well.

The preservation requirements of the meniscus are dictated by the material properties of its matrix and will be best understood in this context. The matrix of the meniscus is considered to be composed of two phases, a solid phase, which accounts for as little as 26% of the meniscal wet weight and a fluid phase (74% wet weight) (Fithian *et al*, 1990). The solid phase consists mostly of collagen, proteoglycans (PG), and other insoluble macromolecules, and its behavior is that of a porous composite. The fluid phase, which consists of water and low-molecular-weight species, is removed during preparation for the SEM, yielding a most delicate meshwork of sparse sieve macromolecules. This vulnerable assemblage is liable to collapse, due to adverse effects of the scanning beam (Ghadially, 1983, pp. 80-102). It has been reported that such a collapse is reflected in curling and distortion of the whole meniscal structure (Merkel, 1980) or, in less severe cases, produces surface imprints of underlying structures such as collagen bundles (as undulations) and cells (as pits and humps) (Meachim, 1976).

In the present study, surface irregularities were limited, in the medial meniscus, to the juxtasynovial sector and to areas near visceral tears. In the lateral meniscus, juxtasynovial undulations were even less discernible. Pits and humps were present on undulated areas only, and did not appear on juxtasynovial strips on lateral menisci. Compared with earlier reports, this is a significant reduction in surface irregularities and can safely be taken as bearing witness to improvement in tissue preservation due to the effect of tannic acid.

The question should be raised, are juxtasynovial irregularities (and visceral undulations near tears) an artifact of preparation that cannot ultimately be avoided even by using tannic acid, or do they exist, at least to some extent, *in situ*?

To the naked eye, freshly obtained unfixed menisci appear to bear few undulations on the juxtasynovial strip (Ghadially, 1983, p. 104). This appearance (which has nothing to do with preparation conditions), under the lowest resolution in use, might bear witness to additional adjacent and thinner undulations whose presence could be disclosed only under higher resolution. Independent reports collectively indicate that absence of undulations on the intermediate and visceral strips is a direct result of higher PG content, which forms a thicker surface coat that supports and conceals fibrillary contours on these strips (Ghadially, 1983, p. 107; Cameron and Macnab, 1972; Moshurchak and Ghadially, 1978; Adams and Ho, 1987).

The observations of Engfeldt and Hjertquist (1968) and Bloebaum and Wilson (1980) showed that structural reinforcement of cartilaginous tissues is vital for the prevention of leakage of tissue PG into the medium and the production of cartilage specimens without surface irregularities. A visceral tear is a cut through the collagen meshwork, whose integrity has been shown to be primarily responsible for the structural integrity of cartilage (Pottenger *et al.*, 1982). Such a cut results in dissociation of PG from collagen fibers and leads to eventual collapse of the latter, which under the SEM would be reflected in surface irregularities. Tannic acid is known to interact strongly with collagen fibers (Simionescu and Simionescu, 1976), and we hypothesize that this interaction endows the intact menisci with structural reinforcement and subserves to effectively preserve meniscal surfaces. On the other hand, tannic acid seems not to suffice in preventing the appearance of visceral undulations near tears, possibly due to leakage of PG that had already occurred before resection. However, salient and abundant surface irregularities, reported in previous studies, might be explainable as resulting from the loss of some tissue component(s) other than PG, as well. Retention of these unknown compounds might be brought about by their interaction with tannic acid, which can well serve to prevent their extraction during postfixation and/or dehydration (Kageyama *et al.*, 1985). This, in turn, might result in stabilizing the lax sieve macromolecules against the deleterious effects of the scanning beam.

The precise chemical nature of the effect of tannic acid has not been elucidated. Tannic acid has been reported to act as a mordant which avidly binds *in situ* not only to collagen fibers (Simionescu and Simionescu, 1976; Kageyama *et al.*, 1985), but to cartilaginous composite polysaccharides (Kageyama *et al.*, 1985) and to phosphatidylcholine in several tissues, including articular cartilage (Kalina, 1988; Schrijvers *et al.*, 1989; Hills, 1989, 1990).

**Fine morphology of the surface**

Amorphous or finely textured interfibrillary matrix was found spread all over the three strips, leaving no appearance of cellular structures exposed on the surface (Figs. 3a and 4a). Differences in fine morphology among the three strips could be discerned. The juxtasynovial strips, on both types of menisci, were characterized by fine, uniformly amorphous surface coat whose details could be made discernible only at high magnification (Figs. 3a and 5a). In the visceral strips of both types of menisci, interfibrillary matrix with small granules homogeneously coated the surface (Figs. 7a and 7b). In the intermediate strips, surface processes evolving from and mounded on an amorphous extracellular matrix, beset in places by particles, were common. Only in the latter strip, on both types of menisci, were surface processes, of various dimensions and densities, recorded (Figs. 4 and 5). They were randomly oriented, irrespective of the long axis of the strip. This pattern is reminiscent of that recorded in articular hyaline cartilage; however, here these differences were much more moderate than those recorded in condyles and patellas (Levanon and Stein, 1991).

Ghadially (1983, p. 106) examined the surfaces of six defined meniscal sectors in the TEM, but did not find any ultrastructural differences between them. We were able to characterize delicate and clearly reiterative differences between the longitudinal sectors, due to the option offered by the SEM of screening the whole surface of a given structurally reinforced specimen.

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## Discussion with Reviewers

**K. Draenert**: Please comment on possible artifacts caused by $OsO_4$.

**Authors**: We are not aware of an article that presents, in a systematic and controlled way, compelling evidence for induction of tissue artifacts by $OsO_4$ in the SEM. Hunziker (1991, Additional References) claimed a detrimental effect of osmium on PG precipitates, even when a cationic dye had been initially introduced into the primary aldehyde fixative, but he did not present any experimental details in support of this statement. Although originally Hunziker's statement was made in the context of a TEM study, we think that it might apply to SEM specimens as well. However, the effect of tannic acid (whose mechanism of action in the preservation of glycosaminoglycans is completely different from that of cationic dyes) was not included in his study. This might leave Hunziker's declaration inapplicable to the present study. At first sight, the fact that the morphological features of each of the various sectors (in both meniscal types) were so highly reiterative strongly suggests that

these features were not mere artifacts caused by $OsO_4$, but rather present something inherent in and typical to the meniscal surfaces. In other words, $OsO_4$, in the course of fixation, might bring about some changes in the tissue surface details, the high reproducibility of which, however, seems to imply that these changes (had they occurred) stand for endogenous and genuine character(s) of the tissue's sectors and should therefore be regarded as significant.

**K. Hodde**: You discuss the possibility that you are describing artifacts and what is your conclusion in that regard? Apart from the undulations visible at inspection of fresh tissue, the furrows, crests, pits and humps all might be the result of specimen bulk shrinkage, especially when you describe that pits and humps do not occur without undulations, and that slight undulations always accompany tears in otherwise invariably smooth visceral surfaces.
**Authors**: The possibility of artifacts is mentioned in the **Introduction** in relation to previous studies. Extensive analysis of the present results shows unambiguously that a significant reduction in surface irregularities has been achieved through the introduction of tannic acid to the protocol. A plausible mechanism of action has been suggested in the **Discussion**, which is based on its action as a mordant that binds to tissue components and enhances their retention *in situ*. The much fewer and less conspicuous surface irregularities that still appeared in certain locations in the present study have been explained on the basis that they occurred in sites from which tissue substances had leaked out before resection, during life (see text for actual references). See also above answer to Dr. K. Draenert.

**K. Hodde**: How do you know that there are no solidified remnants of the synovial fluid coating the surface of the menisci?
**Authors**: Extensive rinsing with PBS was previously reported to effectively remove any soluble residues of synovial fluid from cartilaginous and synovial linings (see **Materials and Methods**, and references cited in Levanon and Stein, 1991, 1992). Synovial fluid residues are not expected to solidify on these surfaces following rinsing with PBS.

**K. Draenert**: The authors should be more careful with respect to the interpretation of the granular and filamentous structures which are produced by the $OsO_4$ fixation.
**Authors**: No interpretation for granular and filamentous structures was suggested. However, their reiteration was straightforward and therefore is being referred to as significant. See also answer to first question above.

**P. M. Frederik**: In the discussion on the action of tannic acid during fixation, the authors suggest that an improved retention of tissue components may result in a smooth(er) appearance of the menisci. Have the authors also considered the possibility that a smooth appearance is the result of the (secondary) deposition of material brought about by tannic acid [e.g., phospholipids from the synovial fluid; see also Schrijvers *et al.* (1989) on the formation of P-lipid deposits]?
**Authors**: We agree with the reviewer that tannic acid might exert its effect, at least partly, through interaction with tissue phospholipids (see **Discussion**) and by improving the retention of the latter on the surface and subsurface of the tissue. However, we think that, at present, too little is known of the biochemistry of the (tannic acid-treated) meniscal surfaces to allow further discussion of the possibility forwarded by the reviewer. Hills (1989, 1990) examined thin sections of scrapings from tannic acid-treated articular cartilages and found oligo- and multilamellar structures, partly deposited on the surface and partly embedded in (or evolving from) various depths of the tissue. However, Hills did not report on a confluent coating of these multilamellar structures on the cartilaginous surfaces (as seems to be required by the reviewer's suggestion), nor did he show that they originated from the synovial fluid. The existence of lipids in the meniscal extracellular matrix, on the other hand, has been amply established (Ghadially, 1983). But again, the incertitude as to their origin, as well as the insufficient knowledge of their quantitative contribution to the uppermost meniscal surface coat, clearly seems to leave the area open, at present, to any assumption on the question of where Hills' tannic acid-induced multilamellar structures came from, or to other attempted correlation between biochemical and microscopical data. See also the extensive discussion in Levanon and Stein (1991).

**K. Draenert**: In cartilage fixation, loss of substances will start within the first 10 minutes.
**Authors**: We agree with the reviewer that loss of substance(s) from within the surface sieve network of the specimens under study takes place soon after suspension in the fixative solution. In an ancillary study (not reported here), we examined a primary fixative that contained both glutaraldehyde and tannic acid, in order to see whether this combination might enhance preservation of the tissue surface. That protocol did not bring about any further reduction in surface irregularities or any other beneficial effects beyond those of the present protocol, which we therefore decided to adopt. We did not quick-freeze the specimens in liquid nitrogen. Freeze-drying does not seem a method of choice, since it involves drying the specimens without avoiding the interface between the gaseous and the solid (frozen) faces. As is well known, passage of the tissue via this interface is the factor directly responsible for uncontrollable damage and distortion of surface details (see Cohen, 1977, Additional References).

**K. Draenert**: It is very important to know the exact details of different steps of dehydration.
**Authors**: Dehydration with ascending ethanol concentrations was conducted as follows: 50% (20 minutes),

75% (20 minutes), 96% (2 x 20 minutes), and 100% (2 x 20 minutes), exactly as described by Gamliel *et al.* (1983) (see **Materials and Methods**).

**K. Draenert**: Do you think that a perfusion method will further enhance the preservation with your tannic acid?
**Authors**: We do not think that fixation by vascular perfusion would prove significantly advantageous in preparation of meniscal (and articular) cartilages for EM for two reasons:

First, perfusion is best done on tissues rich in vascular arborization, such as brain or digestive tract, thus gaining quick and effective distribution of the fixative throughout the tissue. Menisci harbor some blood vessels, but only in the juxtasynovial third, while articular cartilages are completely devoid of any vascularization. Both cartilages are supplied with nutrients from the synovial linings via the synovial fluid, which bathes all the joint cavity apposing surfaces. For glutaraldehyde, which in itself is an extremely slow diffusing agent, this is quite a long trek to go. Thus, the advantage of quick fixation soon after death of the animal is lost, with the concomitant possible decay of structural details.

Second, introducing fixative solution into the synovial fluid, which contains all the serum proteins, will most probably precipitate a significant fraction of the latter on meniscal (and other joint apposing) surfaces, thus producing a thick artifactual layer that would hamper visualization of surface details. The second argument also renders articular perfusion impractical.

**K. Hodde**: Did you modify the Gamliel and/or Nation procedures? If so, what differences did you see?
**Authors**: We used the Gamliel method, our only modification being the replacement of freon by hexamethyldisalazane, as described in **Materials and Methods**.

**K. Hodde**: Do you think there is less shrinkage with those procedures than with ethanol critical point drying (CPD)?
**Authors**: A comparative study of the shrinking properties of the ethanol-critical point drying protocol versus those of the present one was not aimed at in this study. However, the main importance of the present study lies in the structural reinforcement that is bestowed upon the specimens and which might well include some protection against shrinkage. Obviously, this last point deserves to be shown morphometrically in order to clinch the issue.

**K. Hodde**: Have you considered reflected light intravital microscopy to inform you on the actual *in-vivo* situation?
**Authors**: Reflected light intravital microscopy seems of little advantage, since it is not based upon increased resolution, the latter being the key factor in this instance.

**K. Hodde**: Could you correlate the surface features you have seen, assuming those are not artifacts, with any of the microscopical-anatomical, biomechanical, biochemical and ultrastructural-organizational differences you found in the literature?
**Authors**: As explained in detail in the **Introduction**, the purpose of this article was to work out a system of improved preservation properties for menisci, in the SEM. This, in turn, might provide a good ground for further elucidation of the structure and function relationships in meniscal surfaces. We did not attempt to correlate the present findings with previous analyses, since we believe that such a correlation should best be done concomitantly in a concerted experimental system, which to our best knowledge has not been reported yet.

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