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(54) **Titre : PREPARATION DE TISSU POUR UNE IMPLANTATION MENISCALE**
(54) **Title: PREPARATION OF TISSUE FOR MENISCAL IMPLANTATION**

(57) **Abrégé/Abstract:**
The present invention relates to a method of preparing a tissue matrix and its subsequent use in the replacement and/or repair of a damaged or defective meniscus. The invention also provides meniscal tissue that is substantially decellularised.

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(54) Title: PREPARATION OF TISSUE FOR MENISCAL IMPLANTATION

(57) Abstract: The present invention relates to a method of preparing a tissue matrix and its subsequent use in the replacement and/or repair of a damaged or defective meniscus. The invention also provides meniscal tissue that is substantially decellularised.

Preparation of Tissue for Meniscal Implantation

The present invention relates to a method of preparing a tissue matrix, especially a soft tissue matrix, for the replacement and/or repair of a damaged or defective meniscus. The invention also provides a meniscus tissue that is substantially decellularised for subsequent transplantation/implantation.

BACKGROUND

The human knee is an important and complex joint comprising three spatially inter-related bones (the femur, tibia and patella), ligaments and cartilaginous structures all of which interact to create a variety of motions. The surfaces of the knee bones within the joint are covered with articular cartilage. This important surface allows the bones to smoothly glide against each other without causing damage to the bone. The meniscus, a C-shaped cartilage cushion sits between the articular cartilage surfaces of the bone and acts as a shock absorber by distributing weight and so improves the overall stability of the knee joint. Each knee joint has a medial and lateral meniscus which is composed of fibrochondrocytes, proteoglycans and an extra-cellular matrix of collagen and elastin fibres. When menisci are damaged through injury, disease or inflammation arthritic changes can develop in the knee joint with consequent swelling, pain and/or loss of knee joint function in the affected individual. Whilst it is possible to repair a torn meniscus, a meniscus that is severely damaged or has an extensive tear may have to be removed.

Since joint cartilage in adults does not naturally regenerate to a significant degree once it is destroyed, damaged adult menisci have historically been treated by a variety of surgical interventions including removal and replacement with prosthetic devices. In older patients, a knee joint replacement is often the preferred option. However, for younger individuals (those under 50 or 55 years old) the alternative to replacing the entire joint is a meniscal transplant which uses either prosthetic menisci or donor tissue to replace the damaged meniscus.

A problem associated with the use of donor tissue for meniscal replacement is that the meniscus is a dense fibrocartilagenous tissue impregnated throughout with fibrochondrocytes which are the cells responsible for synthesis, maintenance and repair of the extracellular matrix. The human medial meniscus is approximately 4.5 mm in length and the lateral meniscus is approximately 3.5 mm in length, the thickness of each

range from 25 to 35 mm (porcine values are similar to those of human menisci.) Because the meniscus is a thick and dense tissue with cells located throughout and especially around the microvasculature it is extremely difficult to decellularise, especially at the central regions, which in turn means that it is difficult to prepare an immunologically inert or decellularised tissue for transplantation and so there is a risk of rejection. In other words there is low biocompatibility and a high risk of the host having an immunological reaction from either a heterograft or xenograft.

In order to provide an acellular biocompatible meniscal implant, artificial meniscal prostheses have been developed. However, a problem associated with an artificial meniscal prosthesis is that it not as robust as natural meniscal tissue and also it is deficient in the elastic properties of the natural menisci consequently such prostheses are not as effective at shock absorption as the natural material.

A method that could effectively decellularise donor meniscal tissue would offer immediate benefit to the treatment of individuals requiring a meniscal implant/transplant.

BRIEF SUMMARY OF THE DISCLOSURE

According to a first aspect of the invention there is provided a method of preparing donor meniscal tissue for subsequent implantation into a host comprising the steps of:

(i) ultrasonicating the tissue in a buffered solution;

(ii) freezing and thawing the tissue;

(iii) incubating the tissue in a hypotonic solution;

(iv) incubating the tissue in a hypotonic solution comprising an anionic detergent;

(v) repeating steps (iii) and (iv);

(vi) incubating the tissue in a solution comprising at least one nuclease enzyme; and

(vii) washing the tissue with an oxidising agent.

Preferably, step (ii) can be performed in advance of step (i), that is to say that the donor meniscal tissue can be subjected to a freeze/thaw procedure in advance of the ultrasonication procedure, the order of these two steps is not intended to limit the scope of the invention.

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", means "including but not limited to", and is not intended to (and does not) exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

In one embodiment of the invention the method comprises preparing a donor meniscal tissue for subsequent implantation into a host comprising the steps of:

(i) freezing and thawing the tissue;

(ii) incubating the tissue in a hypotonic solution;

(iii) incubating the tissue in a hypotonic solution comprising an anionic detergent;

(iv) repeating steps (ii) and (iii);

(v) incubating the tissue in a solution comprising at least one nuclease enzyme; and

(v) washing the tissue with an oxidising agent.

In this embodiment of the invention the step of ultrasonicating the tissue in a buffered solution is omitted. It has been found that the method of the present invention may be successfully accomplished without the step of ultrasonication however, it is believed that such a step allows for improved recellularisation on implantation once the meniscal tissue is decellularised. It is therefore an optional and preferred step in the invention but one that may be required to improve subsequent recellularisation and therefore in some instance will be performed as part of the method of the present invention.

It will be appreciated that the meniscus is obtainable by removing the whole or a portion of a medial or lateral meniscus from a knee joint of an allogeneic or xenogeneic donor

Preferably the recipient of the prepared meniscus is a human, alternatively the recipient may be any other species that requires a meniscal implant as a result of damage or degeneration of said tissue.

A xenograft or xeno-transplant is a transplant of tissue from a donor of one species to a recipient of another species. The terms heterograft and hetero-transplant are also sometimes used, while the term homograft or allograft refers to a same-species transplant.

In the present invention, where the recipient is a human, the donor is preferably a human or any other mammal that has a meniscus of approximately commensurate physiological properties such as thickness and strength.

Preferably the meniscal donor tissue is either human or porcine in origin.

As herein before stated decellularisation may be achieved in the absence of ultrasonication however when included in the method of the present invention, the ultrasonication step is carried out in phosphate buffered saline (PBS) or any other physiologically acceptable buffer solution.

Preferably, the ultrasonic energy is pulsed, a typical regime is 1, 2, 3, 4, 5, 6, 7 or 8 seconds on and 0.5, 1 or 2 seconds off, however it will be appreciated that the exact pulse timings is not intended to limit the scope of the invention.

Preferably, the ultrasonic power is between 100-700 Watts, and more preferably is about 400 Watts.

The ultrasonication step is preferably carried out for between 10-40 minutes, ideally for about 20 minutes and preferable is carried out at below room temperature. Ideally, ultrasonication is carried out on ice at about 4 °C.

The freeze/thaw process preferably comprises freezing the tissue at, for example between ¯10 to ¯80 °C, and typically at ¯20 °C for between 2-24 hours and subsequently defrosting the tissue for about 2, 3 or 4 hours until it reaches room temperature. This process is carried out at least once and preferably twice in the absence of a hypotonic

buffer and repeated again at least once and preferably twice when the tissue is immersed in the hypotonic buffer. It will be appreciated that the freeze/thaw in the presence and absence of a hypotonic buffer may be reversed and optionally alternated. A hypotonic solution is one in which the concentration of electrolyte is below that in cells. In this situation osmotic pressure leads to the migration of water into the cells, in an attempt to equalize the electrolyte concentration inside and outside the cell walls.

Preferably, the hypotonic buffer is 10 mM Tris solution at a pH of about 8.0 and includes approximately 0.1% (w/v) EDTA and aprotinin (at a concentration of approximately 10 $KIU.ml^{-1}$).

Preferably, the incubating step with a hypotonic solution comprises a two stage hypotonic wash at incrementally elevated temperatures. The first stage is incubation for about between 12-48 hours and typically about 24 hours at below room temperature but above freezing for example at around 4 °C and the second stage incubation which for about the same period at a temperature above room temperature for example at about 37 °C, a third stage (step (iv) in the embodiment where ultasonication is performed and step (iii) when it is omitted) is performed by incubating the tissue in a hypotonic solution additionally comprising an anionic detergent. The third wash step with the anionic detergent comprises incubation for between 1-3 days, and preferably for about 48 hours at a temperature above the second wash but below boiling for example at about 55 °C.

The temperatures and periods of incubation specified above in incubation steps herein before described exemplify an appropriate protocol for the methods of the present invention and are not intended to limit the scope of the invention.

Preferably the anionic detergent is sodium dodecyl sulphate (SDS). Preferably this is present in the hypotonic wash solution at a concentration in the range of 0.03-0.3% (v/v) and more preferably still is present at approximately 0.15% (v/v).

Preferably, the three stage incubation step of steps (iii) and (iv), when ultrasonication is performed and steps (ii) and (iii) when ultrasonication is omitted are repeated for a minimum of three cycles.

Preferably, in one embodiment of the invention following step (v) when ultrasonication is performed and step (iv) when ultrasonication is omitted of the repeated hypotonic

washing with and without an anionic detergent, the method further includes the step of washing the tissue in a buffer solution.

Preferably, the buffer is PBS. The wash process may comprise repeated incubations of between 1, 2 or 3 hours at a temperature of 40-60°C and ideally at about 55 °C. This step is preferably repeated a further 1, 2 or 3 times.

Preferably, the method includes an incubation (step (v) or step (vi) when an ultrasonication step is employed) with a solution comprising one or more nuclease enzymes.

The nuclease enzymes are used to digest any remaining nucleic matter which has been shown to act as sites for calcification.

A typical but non-limiting nuclease incubating solution 50 mM Tris solution pH7.5, 10 mM $MgCl_2$, bovine serum albumin (50 μg/ml) with RNase (1 U. I ml^{-1}) and DNase (50 $U.ml^{-1}$).

Tissue is preferably incubated for about 2, 3 or 4 hours typically at about 37 °C with the nuclease solution whilst being gently agitated.

Following incubation with the nuclease solution, the tissue is preferably then further incubated for about 12-48 hours and typically 24 hours at 30-50 °C and typically at about 37 °C in a hypertonic solution.

Preferably the hypertonic solution is Tris in solution (0.05 M) pH 7.6 plus 1.5 M NaCl and EDTA (0.1% w/v).

Preferably, the tissue is then washed in a buffer solution comprising PBS and a chelating agent for between 12-24 hours.

Preferably, the chelating agent is EDTA at a concentration of 0.1% (w/v).

Preferably, the oxidising agent of the final step of the methods of the present invention is peroxyacetic acid ($C_2H_4O_3$) also known as peracetic acid and commonly abbreviated to PAA.

Preferably, the concentration of PAA is in the range of 0.01 – 0.5 % v/v and more preferably still is about 0.1% PAA (v/v).

Preferably, the method further includes a multi-stage incubation wash in PBS at decreasing temperatures. A typical protocol for this final stage wash is a first wash with PBS for 12-48 hours and typically 24 hours at between 35-50 ºC and ideally at 45 ºC, a second incubation wash for a similar period at between 30-40 ºC and ideally at 37 ºC and a final incubation wash for a similar period at between 0-10 ºC and ideally at 4 ºC.

Preferably, the multi-stage incubation wash is repeated a further once, twice, third, fourth, fifth, sixth, seventh or eighth times.

It will be appreciated throughout the description of the methods of the present invention that the timings, temperatures and concentrations recited are given as examples only and are not intended to limit the scope of the invention.

Preferably, the method further includes the step of preserving the prepared tissue for subsequent use.

Such a step is illustrated by, for example, cryopreservation or deep freezing.

According to a further aspect of the invention there is provided a transplantation product produced by the methods of the present invention.

According to a yet further aspect of the invention there is provided meniscal tissue obtainable by the method of the present invention for use as a transplant tissue.

According to a yet further aspect of the invention there is provided use of meniscal tissue obtainable by the method of the present invention as a transplant tissue.

Preferably, the product produced by the present invention may be characterised by the absence (100%) or substantial absence (90%) of cells in the central area of the meniscal tissue.

The method of the present invention provides a means by which meniscal tissue can be prepared that is substantially devoid of cells such as fibrochondrocytes.

Preferably, the product produced by the present invention may be characterised by a genomic DNA (gDNA) content of between 0 to 20 ng/mg, more preferably by a gDNA content of 0-10 ng/mg and more preferably still be a gDNA content of 0-5 ng/mg.

The product of the present invention is therefore practically devoid of cells such as fibrochondrocytes and has a negligible if any gDNA content and as such is a most appropriate material for subsequent transplantation.

It is believed that the methods and products of the present invention advantageously provides natural meniscal tissue that is truly biocompatible with a host with minimal risk of rejection by the host by virtue of the improved method of decellularisation.

The methods of the present invention have been successfully employed to decellularise meniscal tissue from areas problematic to decellularisation in the outer and central areas of the meniscus and especially around areas of microvascularistion and deep within the centrally located tissue.

According to a yet further aspect of the invention there is provided a kit comprising the solutions as herein before described and optionally including a set of written instructions for use thereof.

According to a yet further aspect of the invention there is provided a method of treatment of an individual requiring a meniscal transplant comprising the steps of preparing a decellularised donor meniscal tissue according to the method of the first aspect of the invention and replacing the defective or damaged meniscus with the decellularised meniscus.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described by way of example only with reference to the following figures wherein:

Figure 1 shows a flow diagram of a typical step by step procedure according to the method of the present invention.

Figure 2 shows the DNA content and histology of the medial porcine meniscus after decellularisation using various protocols. Figure 2A shows fresh meniscus; Figure 2B shows the meniscus after a basic decellularisation procedure; Figure 2C shows the meniscus after decellularisation and ultrasonication; Figure 2D shows the meniscus after decellularisation at 55 ºC and ultrasonication; Figure 2E shows the meniscus after decellularisation at 55 ºC, ultrasonication and freeze thaw; Figure 2F shows the meniscus after decellularisation at 55 ºC (x 2), ultrasonication and freeze thaw; Figure 2G shows the meniscus after decellularisation at 55 ºC (x 3-4), ultrasonication, freeze thaw and PAA treatment.

Figure 3A shows a range of histology from fresh porcine meniscus and Figure 3B shows the histology from comparable areas following decellularisation according to the method of the present invention.

Figure 4 shows biomechanical data for fresh and decellularised meniscal tissue.

Figure 5 shows immunoperoxidase staining for alpha-gal in fresh and decellularised meniscal tissue

DETAILED DESCRIPTION

Materials and Methods

Preparation of Meniscal Tissue

Porcine menisci were obtained from a local abattoir within 24h of animal slaughter. The menisci were dissected from the knee joint by gently excising the knee capsule before cutting both the anterior and posterior cruciate ligaments to expose the meniscus. Incisions were then made perpendicular to the meniscal horn attachments to release the menisci. Excess tissue from the capsule and the meniscal attachments were then removed using scissors. The meniscus was then removed and washed in PBS (Oxoid) to remove excess blood. Samples were then stored at -40ºC on PBS moistened filter paper for future use.

Tissue/Histology Preparation

Tissue specimens (n = 3) were fixed in 10% (v/v) neutral buffered formalin for 48h and then dehydrated and embedded in paraffin wax. Serial sections of 6µm in thickness were

taken with 1 in 10 sections used. Standard haematoxylin and eosin (H&E) (Bios Europe Ltd, Skelmersdale, UK) staining was used to evaluate tissue histioarchitecture. Nucleic acids were stained using Hoechst dye (bis-benzimide H33258 pentahydrate; Molecular Probes, Eugene, OR). Monoclonal antibodies for α-Gal obtained from Alexis biochemicals, San Diego, USA.

Ultrasonication

Tissue samples (2cm width) were sutured to a aluminium gauze using 4-0 prolene™ sutures purchased from Southern Syringes Ltd. This was placed inside a 250ml glass beaker filled with ice cold PBS kept on ice. Various ultrasonication regimens (High Intensity ultrasonication processor, 600 Watts, model 601, Progen Scientific, Mexborough, South Yorkshire). were applied to the tissue samples via positioning the tissue sample directly under the probe with the PBS being changed after every treatment. On completion, the tissue was removed and subjected to remaining decellularisation procedure. The pulse regimen was 1 second on, 1 second off for 10mins before changing PBS and repeating.

Meniscal Indentation

Indentation apparatus was used to analyse the deformation under load of fresh and decellularised porcine menisci. The rig consisted of a shaft with a detachable 3mm, cylindrical, rigid, flat indenter at one end and the other end connected to a linear variable differential transformer (LVDT) for monitoring the displacement of the shaft. Weights were placed under the LVDT and the shaft movement was initiated by a manual release mechanism. The LVDT was calibrated using step heights and the calibration factor acquired. The LVDT had a resolution of 0.001 inches. The speed of the shaft was controlled by an oil-filled dashpot. Samples (n = 3) were firstly cut using a 6mm diameter cutter to remove cylindrical shaped plugs. A section measuring 3mm was removed from the centre of the original plugs. Samples were fixed to the base of the sample holder using double sided sticky tape (3M; Loughborough, UK) and a drop of cyanoacrylate glue. A load of approximately 2N was applied through a viscous dashpot. Samples were immersed in a PBS. Tests were run over 1h time periods. The LVDT allowed the generation of results in the format of time against voltage. Data was acquired using LabVIEW™ 8 (National instruments, Austin, U.S.A) and on application of the calibration factor, results were converted to time against deformation (mm).

Hydroxyproline assay.

Prior to performing the hydroxyproline assay, samples (n = 3) were lyophilized to a constant weight before being hydrolysed by incubation with 6M hydrochloric acid (HCL) for 4 h at 120˚C and neutralized using sodium hydroxide (NaOH). Standard calibrator solutions were made up using trans-4-hydroxy-L-proline (Sigma). Test solution (50 µl) was added to wells of a flat bottomed 96-well plate to which 100 µl of oxidizing solution (chloramine T hydrate; Sigma) was added and left for 5 min with gentle agitation. Ehrlich's reagent (100 µl) was then added to each well. The plate was then covered and incubated at 60˚C in a water bath for 45 min prior to the absorbance being read at 570 nm. The concentration of hydroxyproline was then determined by interpolation from a hydroxyproline standard curve.

Sulphated sugar assay.

Prior to performing the sulphated sugar assay, samples (n = 3) were lyophilized to a constant weight before enzymatically digesting the tissue in papain buffer (1 $mg.ml^{-1}$ papain, Sigma, in PBS at pH 6.0 with 5 mM cysteine-HCl, Sigma, and 5mM Na_2EDTA, VWR) for 48 h at 60˚C. Briefly, standard calibrator solutions were made up using chondroitin sulphate (Sigma). Standard or test solution (40 µl) were added to 250 µl of 1,9-dimethylene blue solution in wells of flat bottomed 96-well plates. The absorbance was then read at 525 nm after 1 min. The resultant concentration of sulphated sugars, representative of glycosaminoglycans (GAG) was then determined by interpolation from the standard curve.

Extraction and Analysis of gDNA presence

Genomic DNA (gDNA) was extracted using a DNA isolation kit for tissues (Roche Applied Sciences, Indianapolis, USA). Briefly, 200 mg of fresh and decellularized porcine meniscal tissue was digested using a Proteinase K solution (n=3). Following this, an RNase solution was applied to digest the RNA present within the samples. A protein precipitation solution was then added and samples were centrifuged (15,000 *g*, 20 min at 20ºC). Isopropanol (0.7 volumes, VWR) was then added to the pellet to recover any DNA present. The isolated DNA pellet was then washed with ice-cold 70% (v/v) ethanol and left to air dry prior to resuspension in tris-EDTA buffer (Sigma).

Qualitatively the presence of gDNA was analysed using an E-gel® PowerBase™ system (Invitrogen, Paisley, UK). A dry 2% (w/v) Agarose E-gel® (Invitrogen) was inserted into the

base prior to the addition of samples. Resuspended samples (4 µl) were prepared by adding loading buffer (2 µl, Invitrogen) to allow ease of sample loading. The total volume was then loaded into individual lanes of the E-gel® and then electophoresed. A 1 kb DNA ladder (Fermentas Inc, Sheriff Hutton, UK) was run in parallel to estimate the size of the DNA isolated. Staining with ethidium bromide allowed visual inspection on a Kodak Gel Logic™ 1500 system (Eastman Kodak Company, Harrow, UK). DNA was quantitated by measuring absorbance at 260-280nm in a Nanodrop™ spectrophotometer (Labtech Int, Ringmer, UK).

EXAMPLE 1

With reference to Figure 1 there is shown a typical flow chart of one embodiment of the method of the present invention. With regard to the protocol of Figure 1 the order of ultrasonication and freeze/thawing may be reversed, however as will be demonstrated hereinafter the steps of ultrasonication, freeze/thawing and treatment with PAA are essential in order to effect total decellularisation of meniscal tissue.

EXAMPLE 2

With reference to Figure 2A-G, there is shown a schematic representation of the meniscus as a right- angled triangle to approximate the overall cross sectional shape of the meniscus and a Hoechst stain showing the DNA content of cells which is an indication of cell density. In addition histology slides are provided to the right. Figures 2A-G shows the DNA content and histology of the medial porcine meniscus after decellularisation using various protocols.

Figure 2A shows fresh porcine medial meniscus and the presence of cells throughout the tissue with a uniform distribution of DNA content of cells or cell density from the outer to inner regions of the meniscus.

The protocol followed for decellularisation of porcine medial meniscus in Figure 2B is described in Booth, C et al "Tissue engineering of cardiac valve prostheses I: Development and histological characterisation of an accellular porcine scaffold" *The Journal of Heart Valve Disease* 11, pp.457-462, (2002). This process does not involve

ultrasonication or freeze/thawing but it does employ an SDS incubation step at room temperature. The results for DNA content following this procedure shows that cells have been removed from the meniscus peripheral area only and that cells are still present in the inner area. The protocol employed in Figure 2C is the protocol as described in Booth et al plus an additional ultrasonication step. The results show that although peripheral cells have been removed along with some from the inner area, cells still persist throughout most of the meniscus. Following the same protocol as that for Figure 2C but with the decellularisation anionic detergent (SDS) at an elevated temperature (55 °C) results (Figure 2D) show that although cells have been totally removed from a significant proportion of the meniscus they are still present in the centre of the meniscus even though at a reduced density. With regard to the protocol followed in Figure 2E, this shows the meniscus after decellularisation at 55 °C, ultrasonication and freeze/thaw. The results show that cells are found at a low density throughout the central area with a predomination of cell numbers localized around microvascularisation. In the protocol followed for Figure 2F, decellularisation at 55 °C (x 2), ultrasonication and freeze/thaw the results are even better, in so far as, there are very few cells remaining and that the cells have lysed and are randomly located within the center of the meniscus. Turning to the results of Figure 2G which employs the method of the present invention as depicted in Figure 1 of decellularisation at 55 °C (x 3), ultrasonication, freeze/thaw and PAA treatment, there is seen a complete removal of cells. The Hoescht stain shows a total absence of DNA due to complete cell removal.

The results obtained from the various decellularisation protocols show that using the method of the present invention complete decellularisation can be achieved which is not possible with any of the incomplete protocols tested or with other prior art methods.

EXAMPLE 3

With reference to Figure 3A there is shown cell distribution within fresh medial porcine meniscus. A cross-sectional area of the meniscus approximates to a right-angled triangle (see Figures 2A-G) and shows that the areas problematic to decellularisation include the outer and central areas of the meniscus, especially around microvascularisation deep within the centrally located tissue. As shown in Example 2A-E the areas which could be decellularised using an incomplete method according to the present invention were the superior and inferior peripheral meniscus and the inner meniscus. With reference to Figure 3B there is shown a comparable section of a porcine medial meniscus decellularised according to the method of the present invention wherein the meniscus, including the problematic outer and central areas, is completely

devoid of cells, in other words the meniscus is completely decellularised and provides a tissue that is suitable for transplantation into a host.

EXAMPLE 4

Figure 4 shows a graph of deformation against time for both fresh and decellularised menisci that has been decellularised according to the methods of the present invention. The data provides an illustration of a biomechanical test using indentation. The results show that decellularised meniscus has similar compressive biomechanical properties as compared to fresh tissue and therefore has commensurate physical properties and is suitable for implantation into a host.

EXAMPLE 5

The xenogenic Gal α 1-3 Gal β 1-4 Glc NAc-R or alpha-Gal epitope is known to be responsible for hyperacute rejection in xenotransplantation. In tissue engineering residual alpha-Gal epitope may induce severe inflammation in humans and may lead to graft failure. Figure 5A shows the positive immunoperoxidase staining for the presence of the alpha-Gal epitope using a monoclonal anti-alpha-Gal antibody in fresh porcine medial meniscus. When compared to a meniscus decellularised according to the methods of the present invention, Figure 5B shows an absence of the epitope by way of a negative stain. These results show that the meniscus prepared by the methods of the present invention is alpha-Gal epitope deficient and thus is suitable for implantation into a human host.

EXAMPLE 6

The concentration of hydroxyproline per mg of dry weight of the fresh porcine meniscal tissue was 143.3 (±23.29) $\mu g.mg^{-1}$. Following decellularization the concentration of hydroxyproline was found to be 123.96 (±36.3) $\mu g.mg^{-1}$. There was no significant difference in the hydroxyproline content of the fresh tissue compared to the decellularized tissue (ANOVA, $p > 0.05$).

EXAMPLE 7

The concentration of sulphated sugars per mg of dry weight of the fresh porcine

meniscal tissue was 30.3 (±3.9) $\mu g.mg^{-1}$. Following decellularization the concentration of sulphated sugars was found to be 12.3 (±1.6) $\mu g.mg^{-1}$, indicating a loss of 59.4%. There was a significant difference in the sulphated sugars content of the fresh tissue when compared to the decellularized tissue (ANOVA, $p < 0.05$), indicating loss of GAG's.

EXAMPLE 8

Decellularized meniscal tissue samples treated to extract genomic DNA (gDNA), loaded and electrophoresed on an agarose gel confirmed the absence of gDNA in comparison to the fresh meniscal tissue which displayed a clear band around 10, 000 base pairs (not shown). Results were quantitatively verified using spectrophotometry, in which a peak of absorbance between 260-280nm was seen for fresh tissue sample indicating the presence of gDNA. This peak corresponded to 40 (± 9.7) $ng.mg^{-1}$. A small peak was also recorded for decellularized tissue corresponding to 2 (± 0.5) $ng.mg^{-1}$.

In conclusion, the methods of the present invention show that it is possible to provide a completely decellularised and immunologically inert porcine or human donor meniscal tissue that retains its physical properties. Such menisci may be used for subsequent implantation into a host thus avoiding or minimising the likelihood of transplant rejection whilst providing the same strength and functional capabilities as that of a healthy meniscus.

CLAIMS:

1. A method of preparing donor meniscal tissue for subsequent implantation into a host comprising the steps of:

(i) freezing and thawing the tissue;

(ii) incubating the tissue in a hypotonic solution;

(iii) incubating the tissue in a hypotonic solution comprising an anionic detergent;

(iv) repeating steps (ii) and (iii);

(v) incubating the tissue in a solution comprising at least one nuclease enzyme; and

(vi) washing the tissue with an oxidising agent.

2. A method according to claim 1 further including the step of ultrasonicating the tissue in a buffered solution either before or after step (i).

3. A method according to claim 1 or claim 2 wherein a host or a recipient of the prepared meniscus is a human.

4. A method according to any one of claims 1-3 wherein the donor tissue is either human or porcine in origin.

5. A method according to any one of claims 1-4 wherein the freezing and thawing step comprises freezing the tissue at between $^{-}10$ to $^{-}80$ °C.

6. A method according to claim 5 wherein the tissue is frozen at $^{-}20$ °C.

7. A method according to any one of claims 1-6 wherein the freezing step is for between 2-24 hours and the subsequently thawing step is for 2, 3 or 4 hours until the tissue is defrosted.

8. A method according to any one of claims 1-7 wherein the freezing and thawing step process is carried out at least one or more times in the absence of a hypotonic buffer and repeated one or more times when the tissue is immersed in a hypotonic buffer solution.

9. A method according to claim 8 wherein the hypotonic buffer solution includes 10 mM Tris solution at a pH of 8.0 and includes EDTA and aprotinin.

10. A method according to any one of claims 1-9 wherein the incubating step with a hypotonic solution of step (ii) comprises a first and second stage hypotonic wash at incrementally elevated temperatures.

11. A method according to claim 10 wherein a first stage incubation is for between 12-48 hours at a temperature below that of room temperature but above that of freezing.

12. A method according to claim 11 wherein the first stage incubation period is for 24 hours at a temperature of 4 °C.

13. A method according to claim 10 wherein a second stage incubation is for between 12-48 hours at a temperature above that of room temperature.

14. A method according to claim 13 wherein the second stage incubation is for 24 hours at 37 °C.

15. A method according to any one of claims 1-14 wherein the third stage incubation step with the anionic detergent is for between 1-3 days and the incubation temperature is above that of the second stage incubation step.

16. A method according to claim 15 wherein the incubation period is for 48 hours at a temperature of 55 °C.

17. A method according to either claim 15 or 16, wherein the anionic detergent is sodium dodecyl sulphate (SDS).

18. A method according to claim 17 wherein the concentration of SDS is in the range of 0.03-0.3% (v/v).

19. A method according to claim 18 wherein the concentration of SDS is 0.15% (v/v).

20. A method according to any one of claims 10 to 19 wherein the incubation steps are repeated for a minimum of three cycles.

21. A method according to any one of claims 1 to 20 further including, after step (iv) a step of washing the tissue in a buffer solution comprising a chelating agent after the incubation step.

22. A method according to claim 21 wherein the buffer solution is PBS and the chelating agent is EDTA.

23. A method according to either claim 21 or 22 wherein the washing of the tissue comprises repeated incubations of between 1, 2 or 3 hours at a temperature of 40-60°C.

24. A method according to any one of claims 1-23 wherein the solution comprising nucleases is 50 mM Tris solution pH7.5, 10 mM $MgCl_2$, bovine serum albumin with RNase and DNase.

25. A method according to claim 24 wherein the tissue is incubated with the solution comprising nuclease for between 2, 3 or 4 hours at a temperature of 37 °C.

26. A method according to any one of claims 1-25 further comprising the step of incubating the tissue in a hypertonic solution with EDTA for between 12-48 hours at a temperature of about 37°C following the incubation with a solution comprising nucleases.

27. A method according to claim 26 wherein the tissue is incubated in a hypertonic solution and then washed in a buffer solution comprising PBS and EDTA for between 12-24 hours.

28. A method according to any one of claims 1-27 wherein the oxidizing agent in the step of washing the tissue with an oxidising agent is peroxyacetic acid ($C_2H_4O_3$).

29. A method according to claim 28 wherein the concentration of PAA is in the range of 0.01 – 0.5 % v/v.

30. A method according to claim 29 wherein the concentration of PAA is 0.1% (v/v).

31. A method according to any one of claims 1-30 further comprising the step of a multi-stage incubation wash in PBS at decreasing temperatures following the washing of the tissue in the presence of an oxidising agent.

32. A method according to claim 31 wherein a first wash is with PBS for between 12-48 hours at about 37°C, a second incubation wash is for between 12-48 hours at a lower temperature and a final incubation wash is for between 12-48 hours at between 0-10 °C.

33. A method according to either claim 31 or 32 wherein the multi-stage incubation wash is repeated a further once, twice, third, fourth, fifth, sixth, seventh or eighth times.

34. A method according to any one of claims 1-33 further including the step of preserving the prepared tissue for subsequent use.

35. A method according to claim 34 wherein the preserving step is either cryopreservation or deep freezing.

36. A method according to any one of claims 1 to 35 wherein an ultrasonication step is performed in advance of step (i) or after step (i).

37. A method according to claim 36 wherein the ultrasonication step is performed on the tissue when immersed in phosphate buffered saline (PBS) or any other physiologically acceptable buffer solution.

38. A method according to either claim 36 or 37 wherein ultrasonic energy is pulsed.

39. A method according to claim 38 wherein a pulse regime is 1, 2, 3, 4, 5, 6, 7 or 8 seconds on and 0.5, 1 or 2 seconds off.

40. A method according to any one of claims 36 to 39 wherein ultrasonic power is between 100-700 Watts.

41. A method according to claim 40 wherein the ultrasonic power is 400 Watts.

42. A method according to any one of claims 36 to 41 wherein the ultasonication step is carried out for between 10-40 minutes at below room temperature.

43. A method according to claim 42 wherein the ultrasonication step is carried out for 20 minutes at about 4 °C.

44. A meniscal transplantation product produced by the methods of any one of claims 1 to 43.

45. A product comprising meniscal tissue obtained by the method of any one of claims 1 to 43 for use as a transplant tissue.

46. Use of meniscal tissue obtained by the method of any one of claims 1 to 43 as a transplant tissue.

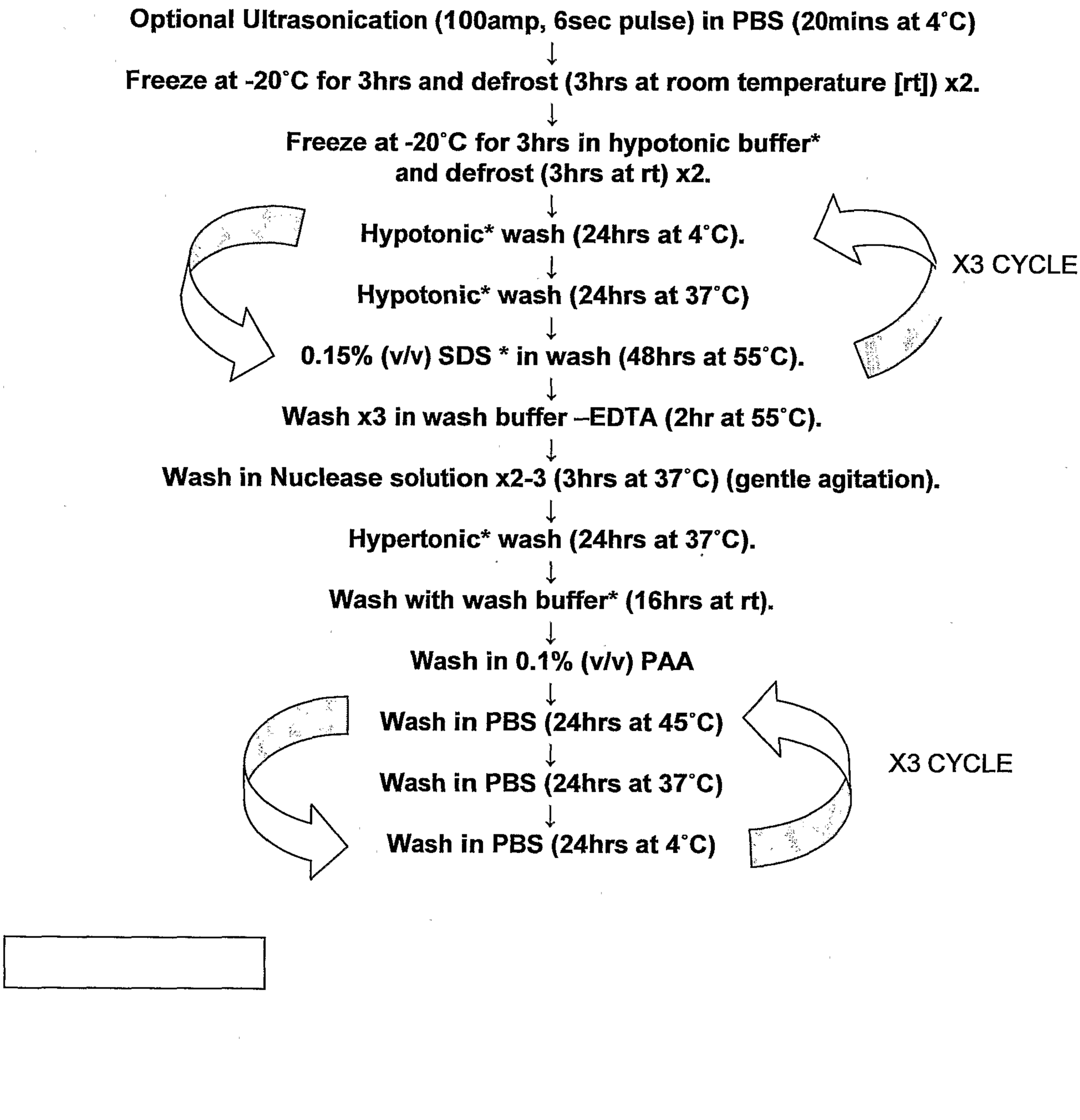
47. A meniscal product produced by the method of any one of claims 1 to 43 characterised by an absence (100%) or substantial absence (90%) of cells in the central area of the meniscal tissue.

48. A meniscal product according to claim 47 characterised by a genomic DNA (gDNA) content of between 0 to 20 ng/mg.

49. A meniscal product according to claim 48 wherein the gDNA content is between 0-10 ng/mg.

50. A meniscal product according to claim 48 wherein the gDNA content is between 0-5 ng/mg.

Figure 1


Optional Ultrasonication (100amp, 6sec pulse) in PBS (20mins at 4˚C)
Freeze at -20˚C for 3hrs and defrost (3hrs at room temperature [rt]) x2.
Freeze at -20˚C for 3hrs in hypotonic buffer*
and defrost (3hrs at rt) x2.
Hypotonic* wash (24hrs at 4˚C).
Hypotonic* wash (24hrs at 37˚C)
0.15% (v/v) SDS * in wash (48hrs at 55˚C).
X3 CYCLE
Wash x3 in wash buffer –EDTA (2hr at 55˚C).
Wash in Nuclease solution x2-3 (3hrs at 37˚C) (gentle agitation).
Hypertonic* wash (24hrs at 37˚C).
Wash with wash buffer* (16hrs at rt).
Wash in 0.1% (v/v) PAA
Wash in PBS (24hrs at 45˚C)
Wash in PBS (24hrs at 37˚C)
Wash in PBS (24hrs at 4˚C)
X3 CYCLE

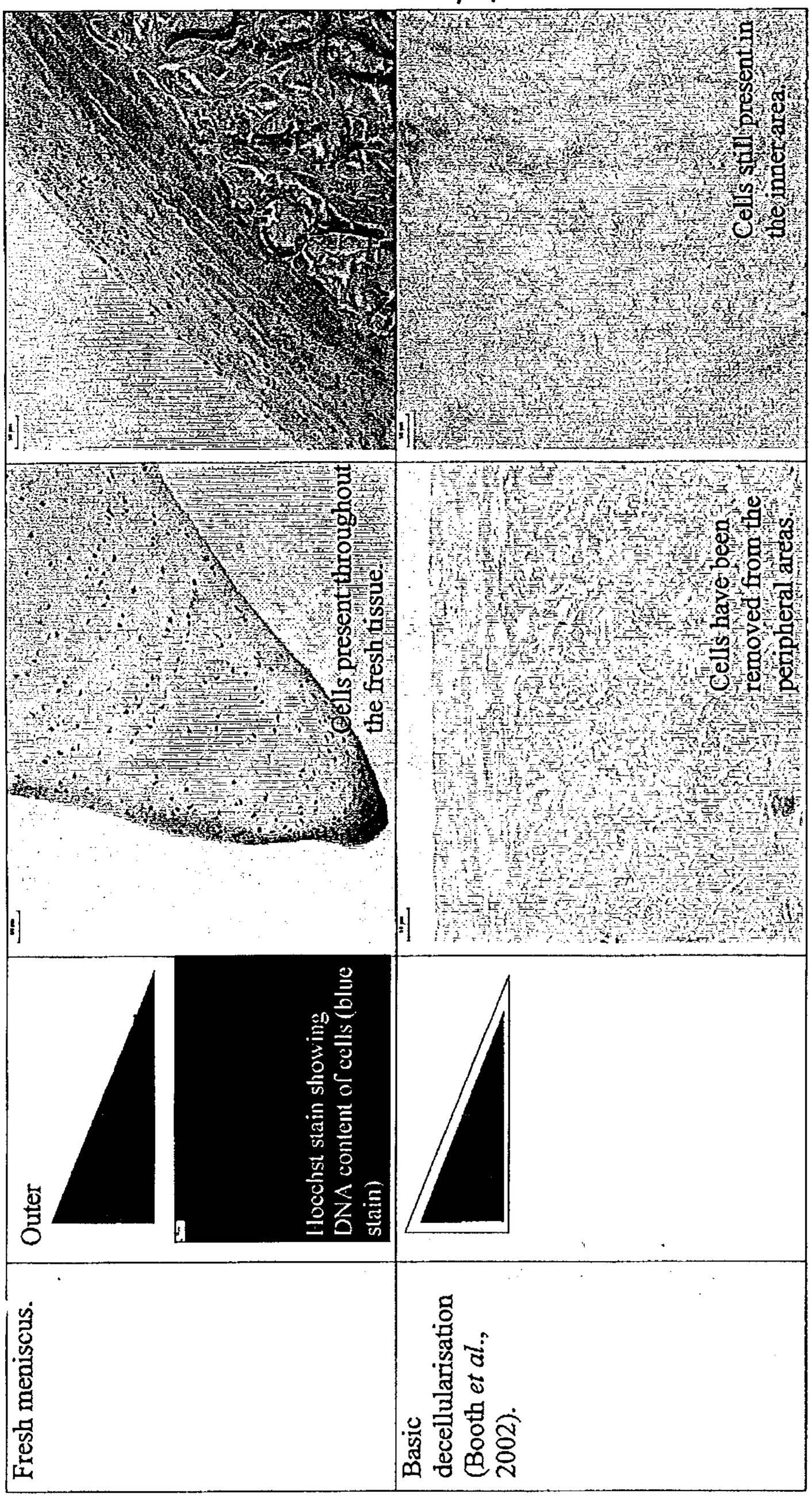

Cells still present in the inner area.
Cells present throughout the fresh tissue.
Cells have been removed from the peripheral areas.
Outer
Hoechst stain showing DNA content of cells (blue stain)
Fresh meniscus.
Basic decellularisation (Booth *et al.*, 2002).

Fig 2A

Fig 2B

Fig 2C

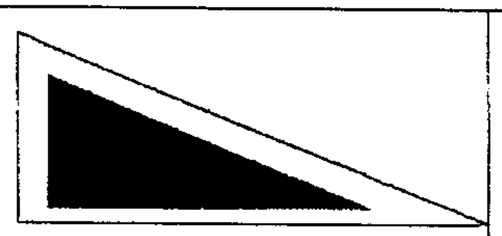
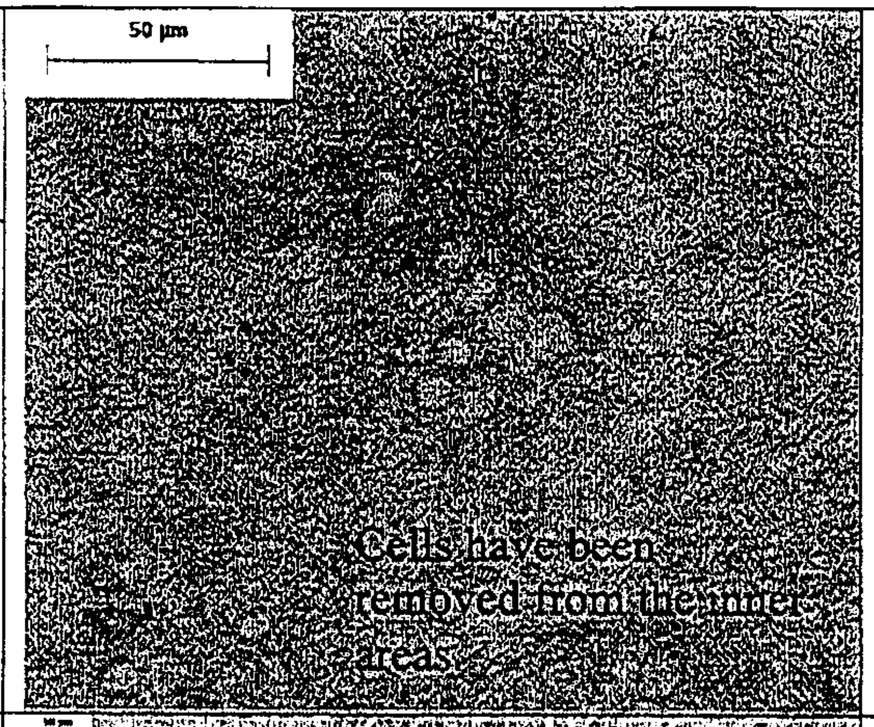
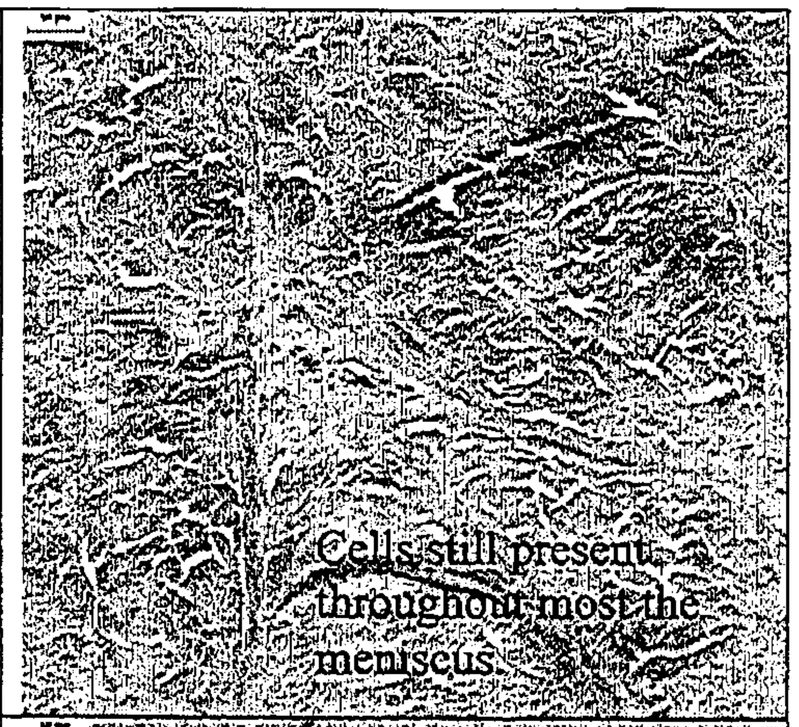
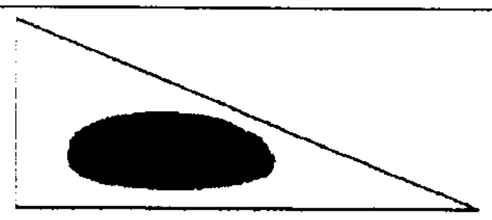
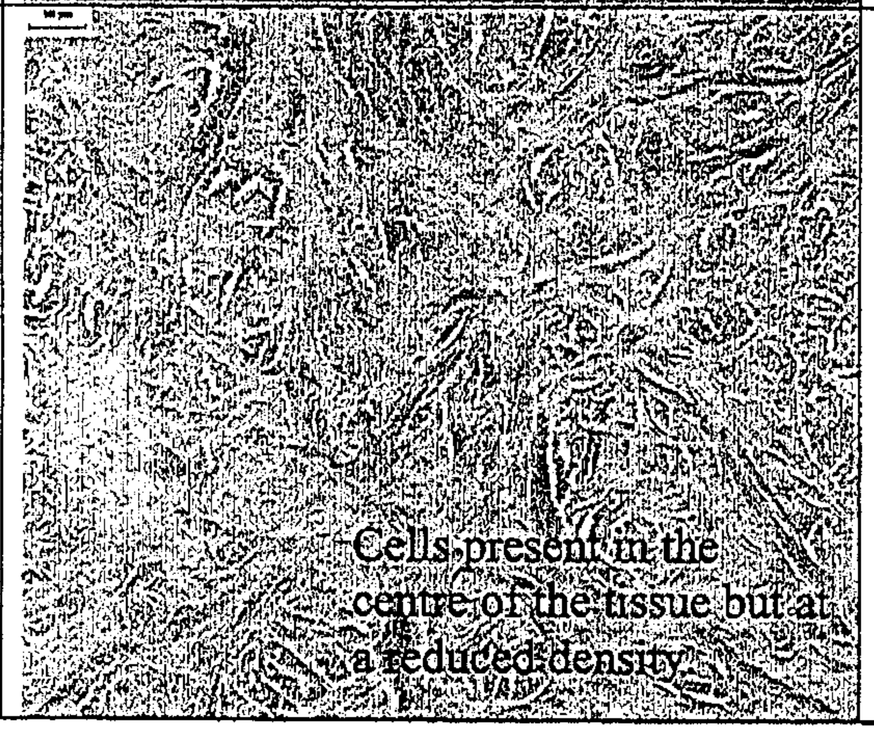
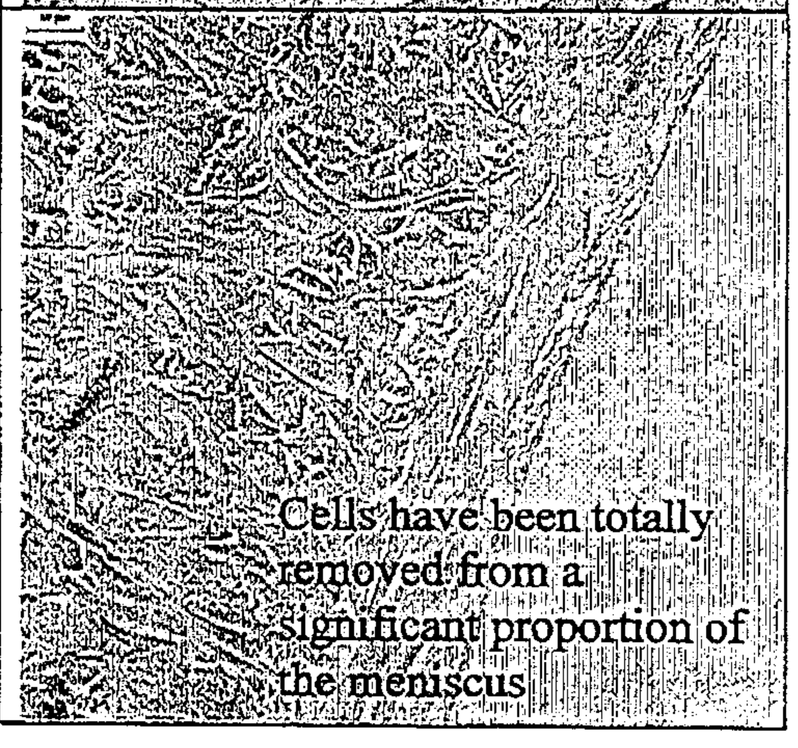
Basic decellularisation + ultrasonication.			
Decellularisation at 55°C + ultrasonication.			

Fig 2D

CA 02701175 2014-01-22

FIG 2E

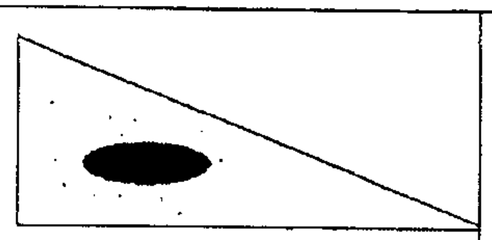
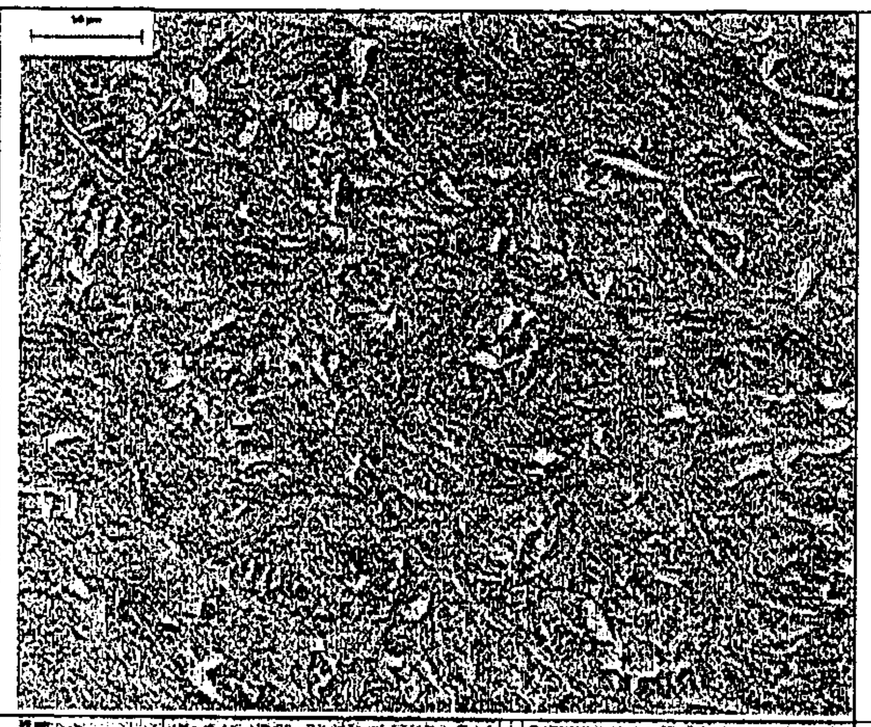
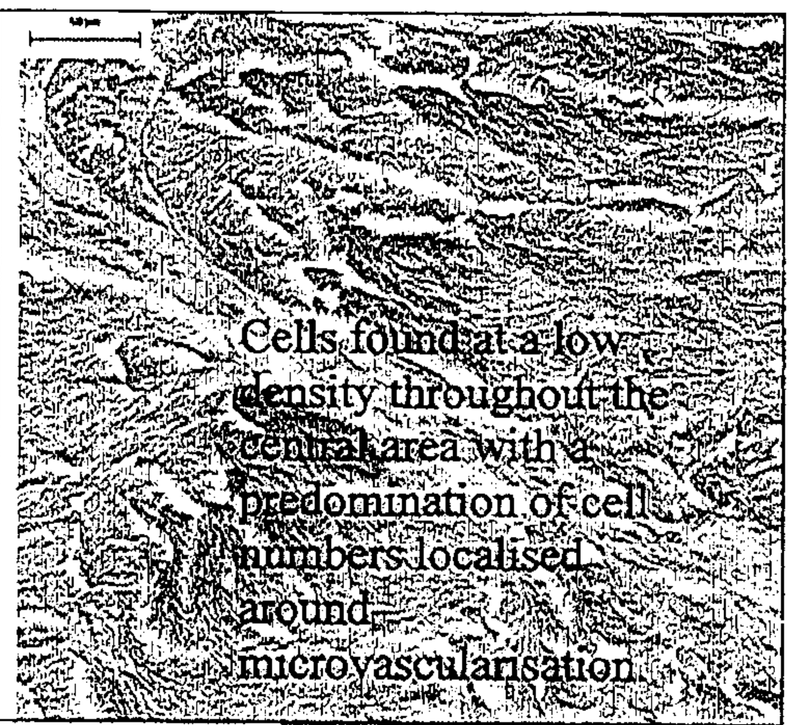
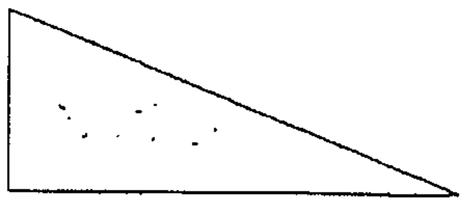
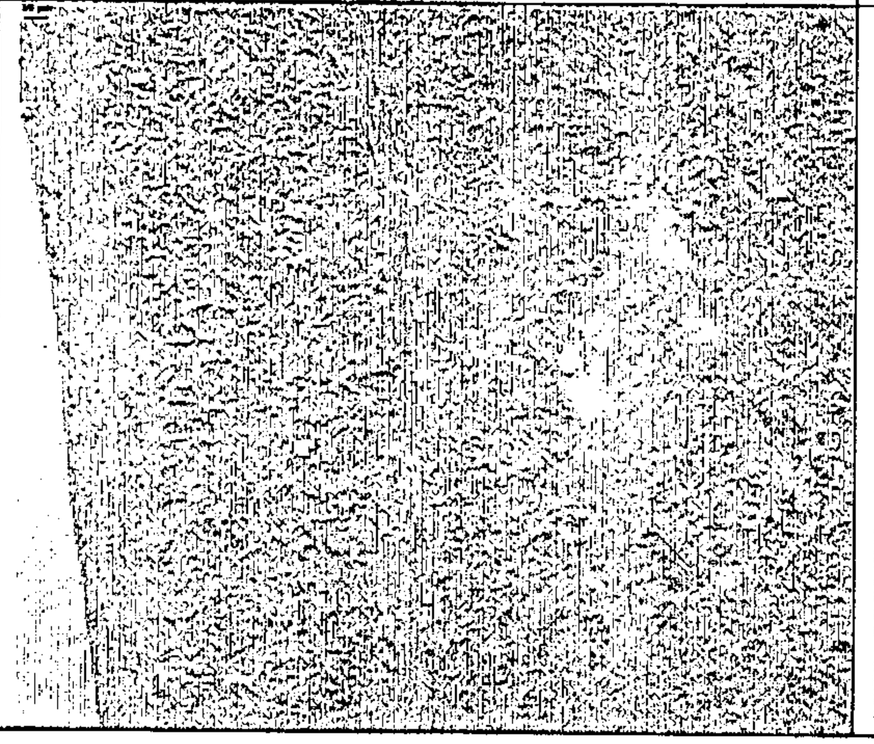
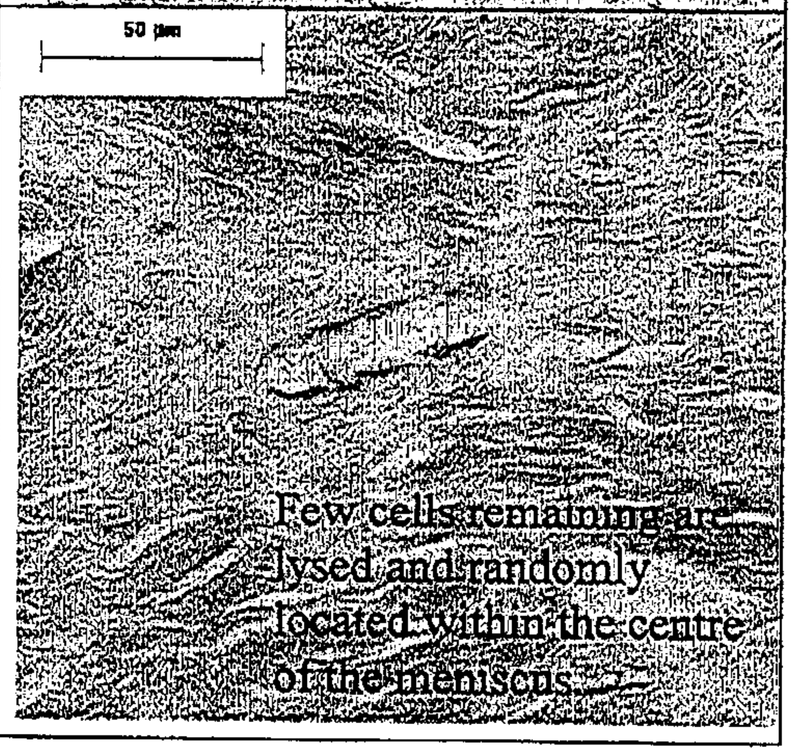
Decellularisation at 55°C + ultrasonication + freeze thaw.			 Cells found at a low density throughout the central area with a predomination of cell numbers localised around microvascularisation
Decellularisation at 55°C (x2) + ultrasonication + freeze thaw.			 Few cells remaining are lysed and randomly located within the centre of the meniscus

FIG 2F

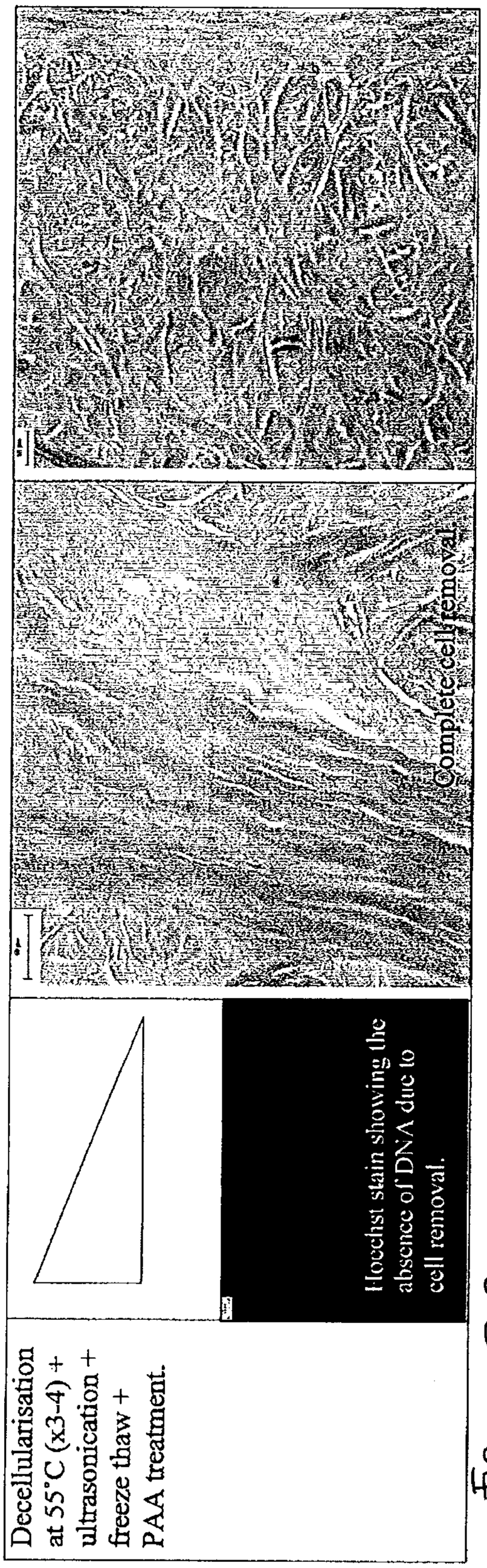

Complete cell removal.
Hoechst stain showing the absence of DNA due to cell removal.
Decellularisation at 55°C (x3-4) + ultrasonication + freeze thaw + PAA treatment.

Fig 2G

FIG 3A

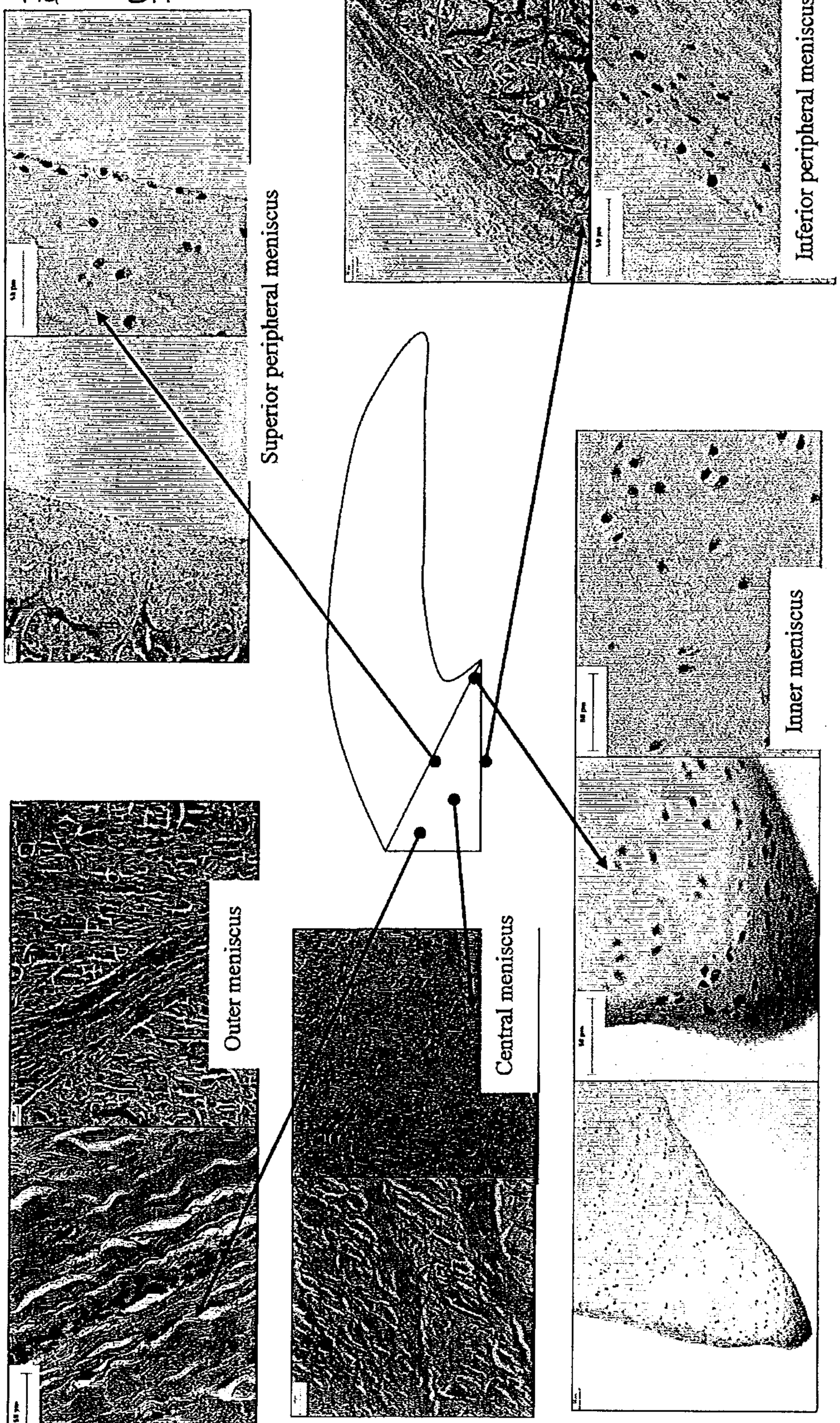

Inferior peripheral meniscus
Superior peripheral meniscus
Inner meniscus
Outer meniscus
Central meniscus

FIG 3B

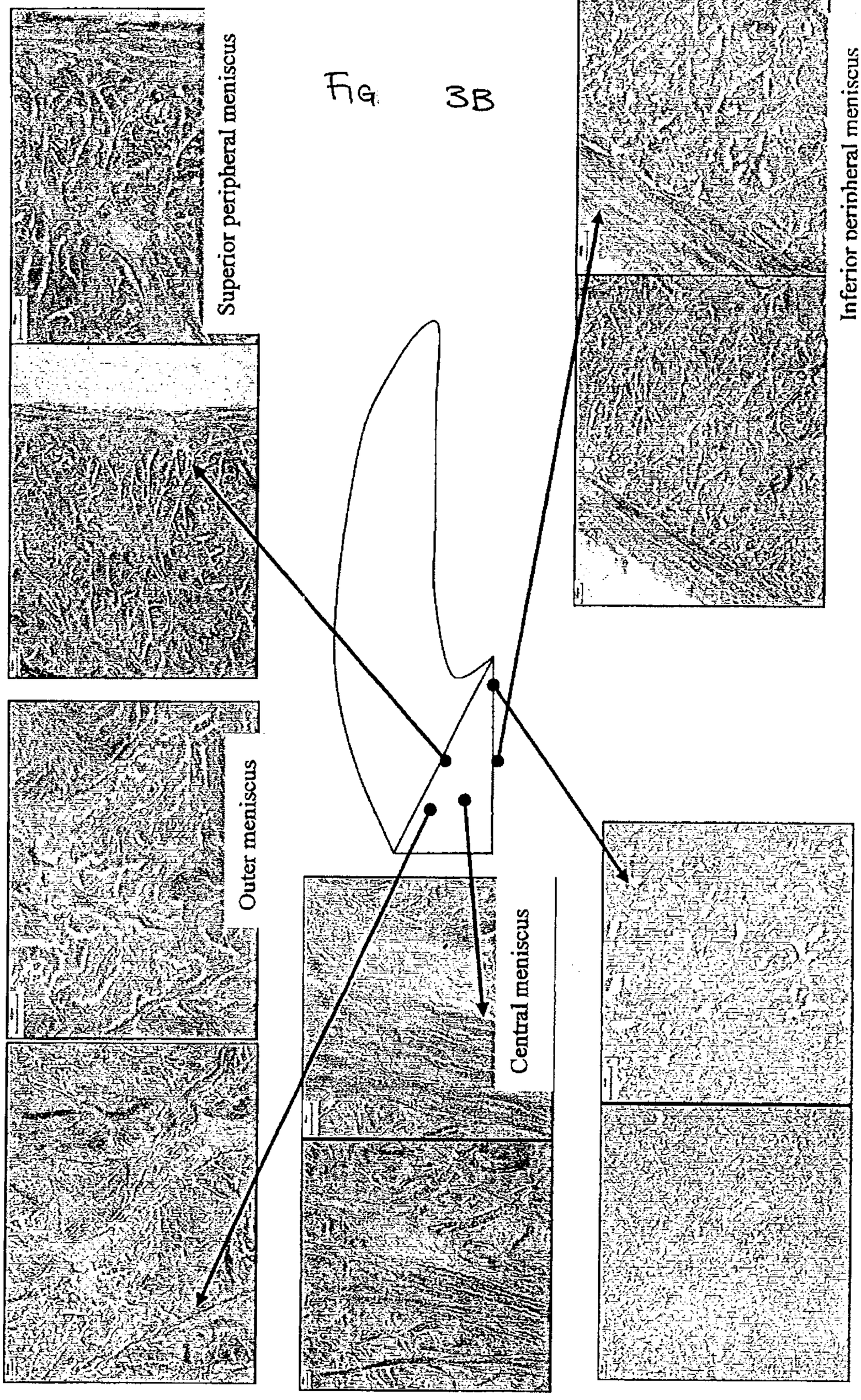
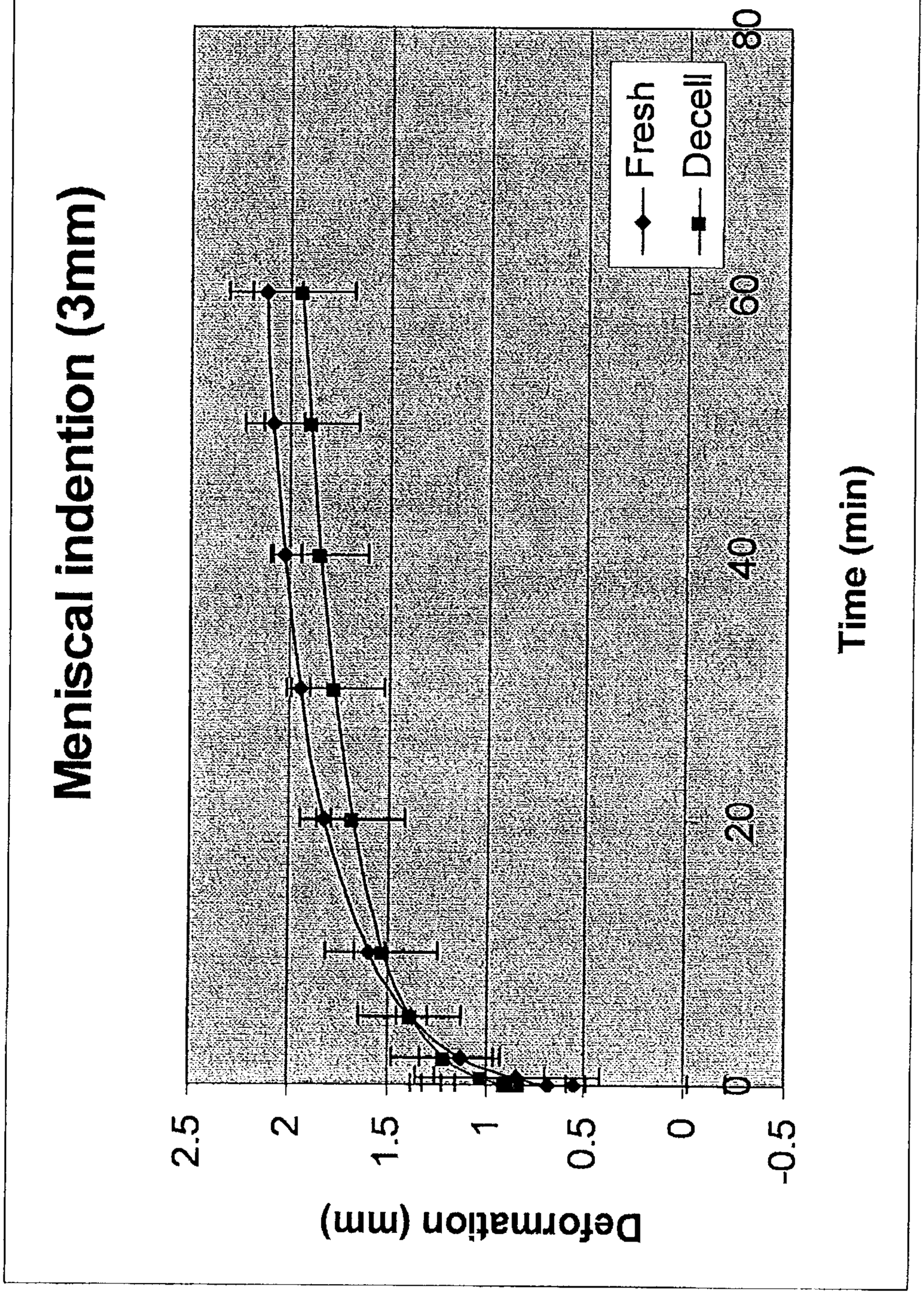

Superior peripheral meniscus
Inferior peripheral meniscus
Outer meniscus
Central meniscus
Inner meniscus

FIG 4


Meniscal indention (3mm)
Deformation (mm)
2.5
2
1.5
1
0.5
0
-0.5
0
20
40
60
80
Time (min)
Fresh
Decell

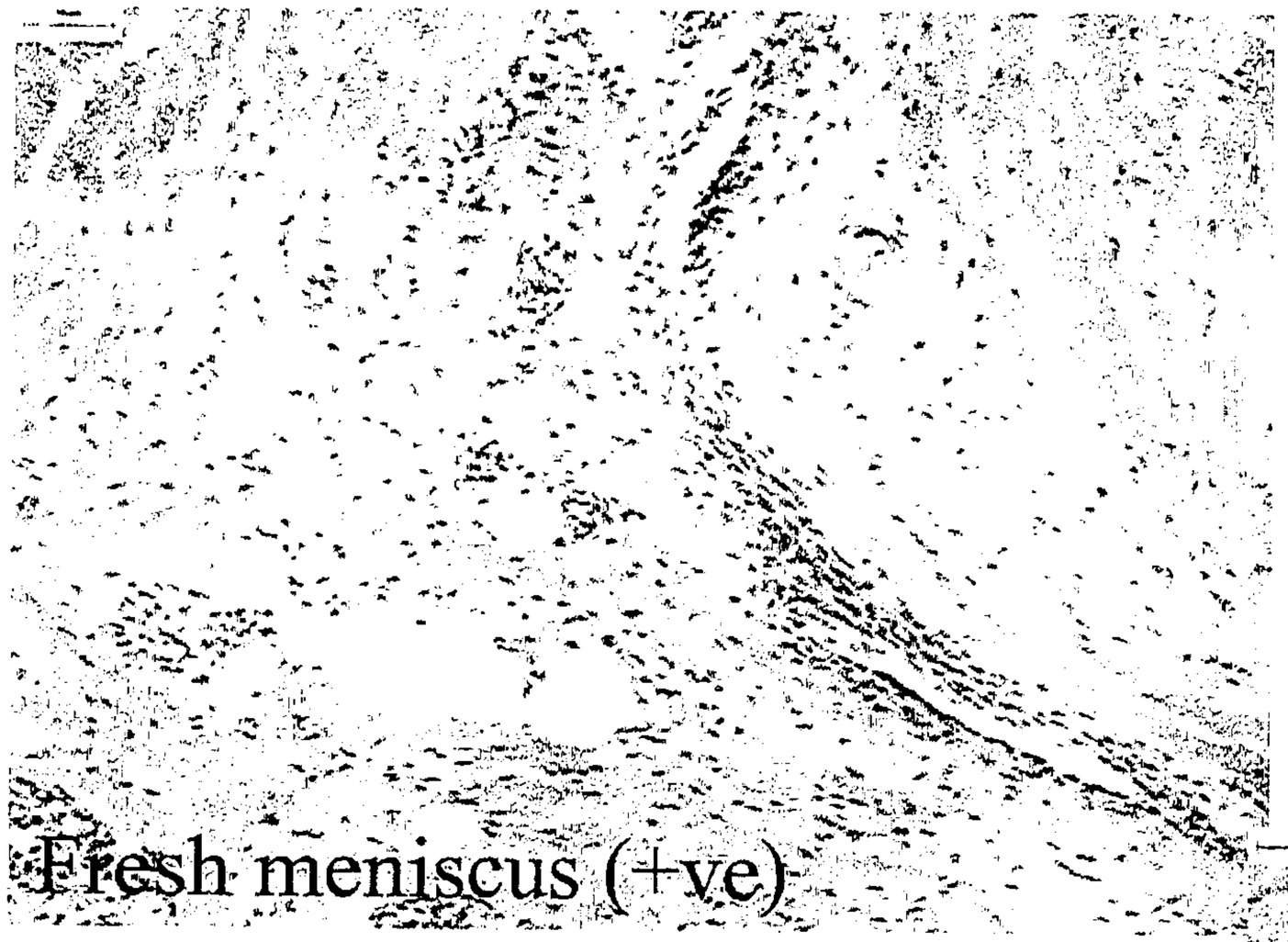


Fig 5A
Alpha Gal Fresh

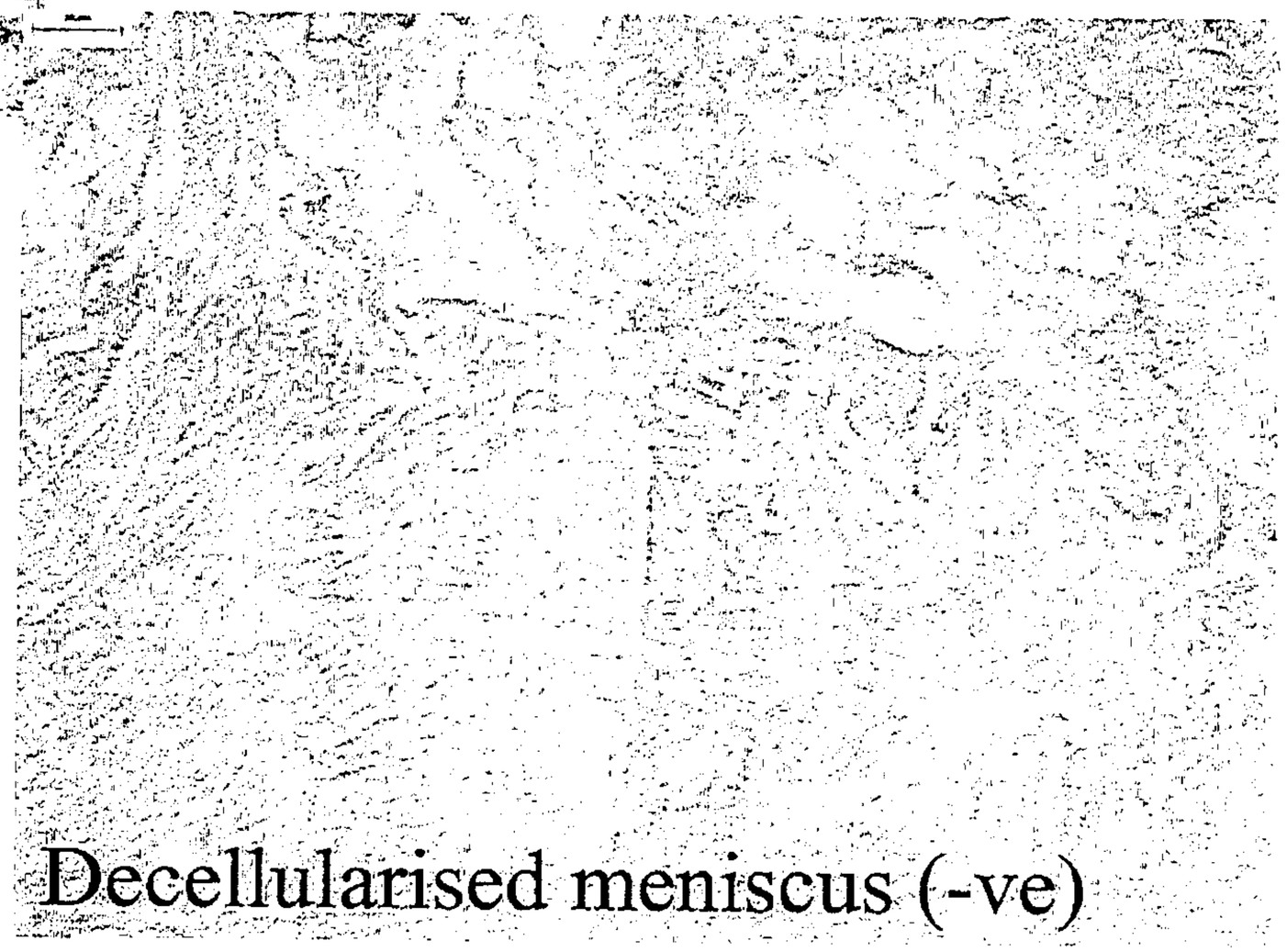


Fig 5B
Alpha Gal Decellularised