

CARTILAGE IMPLANT /

This invention relates to methods of culture of chondrocytes, and in particular to methods of preparation of chondrocyte cultures suitable for use in tissue grafting.

BACKGROUND OF THE INVENTION

Cartilage damage is a major manifestation of arthritis, in both inflammatory arthritic conditions such as rheumatoid arthritis and psoriatic arthritis and in degenerative arthritides such as osteoarthritis. Cartilage may also suffer damage as the result of injury, and damage to the meniscal cartilage of the knee is especially common in skiers, football players from all codes, basketball players and netball players. Damage to cartilage at other sites may also result from injury or disease, for example to the cartilage of the nasal septum or of the ear. In addition to cartilage damage caused by disease or injury, a cartilaginous structure may be absent or incomplete as a result of a congenital defect.

Because cartilage is a non-vascularised tissue, it heals very slowly after injury, and does not regenerate. Although there have been many attempts to utilise either cartilage cultured *in vitro* or isolated chondrocytes cultured under conditions adapted to favour development of a cartilage-like three-dimensional architecture as grafts to repair cartilage damage, their success has been limited by the very small size of the cartilage fragments which can be cultured, and of the cartilage-like cultured tissues which can be obtained using isolated chondrocytes.

US Patent No. 4642120 by Nevo and Itay, assigned to Ramot University Authority for Applied Research and Industrial Development Limited, describes gel compositions for repairing cartilage or bone defects, comprising embryonal chondrocytes or other cartilage cell precursors, together with fibrinogen, a protease inhibitor, and thrombin. The latter three components form a biological glue. US Patent No. 4846835 by Grande describes transplantation of chondrocytes seeded in a three-dimensional collagen matrix in order to promote healing of lesions in articular cartilage. US Patent No. 5041138 by Vacanti *et al.*, assigned to Massachusetts Institute of Technology and Children's Hospital, describes methods for preparing cartilaginous structures for implantation, comprising growing chondrocytes on a biodegradable and

biocompatible fibrous polymeric matrix. The polymer is optionally coated with a material such as basement membrane components, collagen, fibronectin, laminin, glycosaminoglycans and the like. The matrix can be cast or moulded into a desired shape. Similar techniques are also described in C Vacanti: "Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation" in *Plastic and Reconstructive Surgery*, 1991 88 753-759.

US Patent No.5512600 by Mikos *et al.*, assigned to Massachusetts Institute of Technology, describes a method for making a polymeric matrix suitable for culturing cells, including chondrocytes, comprising bonding non-woven fibres of a first polymer using a solution of a second polymer and heating to a temperature such that the first polymer but not the second polymer melts, and then dissolving the second polymer in order to remove it.

None of the methods hitherto available provides a cartilage implant which will survive and retain its three-dimensional form for prolonged periods *in vivo*. V. Ting *et al.*, "In Vitro Prefabrication of Human Cartilage Shapes Using Fibrin Glue and Human Chondrocytes" (*Annals of Plastic Surgery* 40 (4) 416, April 1998) stated that the size and shape of the constructs decreased rapidly:

"After 4 weeks culture *in vitro*, the volume of the construct decreased by approximately 75% and lost a significant degree of some features. --- After subcutaneous implantation of the construct for 4 weeks, further blunting of its features was seen along with reduction of its volume by approximately half, with a final volume of roughly 12% of its original volume at time of molding"

Thus there is a great need in the art for improved methods of producing a cartilage-derived tissue for grafting.

We have now surprisingly found that by using a three-dimensional support comprising a polymer core, suitably resorbable suture fibres coated with fibrin, a cartilage-like architecture of any desired three-dimensional shape can be obtained, with even distribution of chondrocytes, retention of chondrocyte morphology, increase in collagen content, and delayed degradation of the construct *in vitro* and *in vivo*. The polymer core provides support which enables prolonged retention of the shape of the

construct *in vivo*.

SUMMARY OF THE INVENTION

According to a first aspect, the invention provides a method of preparing a cartilage implant suitable for transplantation into a host, comprising the steps of:

- a) harvesting a tissue source of chondrocytes from a donor site;
- b) isolating chondrocytes from the tissue source;
- c) culturing the chondrocytes in the presence of a three-dimensional matrix of resorbable material which has been pre-coated by a tissue-compatible protein; and

- d) culturing the chondrocytes under conditions and for a time suitable to permit the chondrocytes to adhere to and grow into the matrix.

Preferably the chondrocytes are isolated by enzymatic digestion of the cartilage.

In an alternative aspect, embryonal chondrocytes, pre-chondrocytes or other chondrocyte precursors such as mesenchymal cells may be used in addition to or instead of chondrocytes. For example, International patent application No. PCT/US97/18862 (WO98/17791) by Advanced Tissue Sciences, Inc. describes the isolation of pre-chondrocytes from Wharton's jelly of the umbilical cord and their mitotic expansion in culture to produce cartilage tissue for therapeutic use. This has the advantage that the number of starting chondrocytes is not limited by the size of the tissue sample which can be taken from a donor site. Where chondrocyte precursors are used a preliminary incubation to permit expansion of cell members and cell differentiation may be required, and this may be effected as described in PCT/US97/18862.

Preferably the three-dimensional resorbable matrix is a resorbable suture material, such as polygalactin surgical suture material, or collagen-glycosaminoglycan suture material. The latter is marketed under the trade mark INTEGRA. Such suture materials are also known as absorbable. Any biologically-compatible sterilizable material which can be digested by body tissues and therefore will not permanently remain in the body may be used. Suitable materials are known to persons skilled in the art.

Preferably the tissue-compatible protein is fibrin; more preferably the

fibrin is freshly prepared, most preferably by the action of thrombin 500 on fibrinogen.

Preferably the matrix is coated with a tissue-compatible polymer prior to being coated with fibrin. Preferably the tissue-compatible polymer is either a polygalactin-poly-L-lactic acid (PGA-PLLA) co-polymer or is PLLA.

The period for which the chondrocytes are cultured in the presence of the matrix will depend on the size of the tissue defect to be repaired, but will usually be in the region of two to three weeks. Any suitable culture medium may be used, for example Dulbecco's modified Eagle's minimal essential medium, and will usually contain 5 to 10% serum. The serum is suitably foetal bovine serum. Where elective surgery to repair a cartilage defect is to be performed, it may be feasible to use the patient's own serum, and this is preferred where possible. Depending on the size of the matrix construct, serum-free medium may be sufficient to support chondrocyte growth, and may be used where appropriate.

The cartilage tissue or tissue used as the source of the chondrocytes or pre-chondrocytes or chondrocyte precursors may be autologous or heterologous, but is preferably autologous, in order to avoid complications caused by graft rejection. The tissue to be used as the chondrocyte source may also depend on the defect to be repaired, and may be ear cartilage, nasal septum, or meniscal or articular cartilage. It will be clearly understood that while it is preferable to use the same type of cartilage as that which is to be repaired, it is not essential to do so. No significant difference in the quality of the cartilage formed was observed when different tissue sources were used.

In a second aspect, the invention provides a matrix for chondrocyte culture, comprising a resorbable polymer material, optionally pre-coated with a tissue-compatible polymer, and coated with a tissue-compatible protein.

As described above, preferably the resorbable polymer is a resorbable suture material, such as polygalactin, and the tissue-compatible polymer is a co-polymer of PGA and PLLA, and the tissue-compatible protein is fibrin.

The matrix can be any desired shape, and for example may be in the form of an ear or a nose, or a part thereof. We have found that using the constructs and methods of the invention, the three-dimensional shape is retained *in vivo* for over 6 months following implantation.

According to a further aspect, the invention provides a method of preparing a matrix for chondrocyte culture, comprising the steps of:

- a) forming a three-dimensional matrix of a resorbable material in a mould of the desired three-dimensional shape;
- b) optionally coating the matrix with a tissue-compatible polymer; and
- c) coating the three-dimensional matrix with a tissue-compatible protein.

Preferably the resorbable material, the polymer and the protein are as defined above.

According to a fourth aspect, the invention provides a method of repairing a cartilage lesion, comprising the step of grafting the cartilage implant of the invention into a subject in need of such treatment.

The subject may be a human or an animal. The animal may be a domestic animal such as a horse, bovine, sheep or goat; a companion animal such as a dog or cat; or a zoo animal such as a felid, canid, bovine or ungulate.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a plaster mould A used to form a random tangle of PGA fibres into the desired three-dimensional shape.

Figure 2 illustrates the apparatus used for spraying fibrin on to the three-dimensional matrix.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of example only by reference to the following non-limiting examples, and to the figures.

Materials

Chemicals

Dulbecco's Modified Eagle's
Medium (DMEM)
Sodium bicarbonate

GIBCO	23700-040
GIBCO	895-1810IN

Penicillin	Sigma	p-3032
Streptomycin	Sigma	s-9137
Proline	Sigma	p-4655
Ascorbic acid	Sigma	a-4034
Non-essential amino acids	GIBCO	320-1140AG
Insulin	Sigma	I-6634
Transferrin	Sigma	T-1147
Foetal bovine serum	GIBCO	
Fibrinogen	Sigma	F-8630
Thrombin	Sigma	T-9549
Aprotinin	Boehringer Mannheim	981-532
Vicryl suture	Ethicon	W-9901
Compound of 50% PGA-50% PLLA		Medisorb 5050DL High I.V.
Methylene chloride		Sigma 17-3
Trypsin		GIBCO 840-7073IL
EDTA		Sigma E-5134
Collagenase type II		Sigma C-6885

The complete medium for growth of chondrocytes contained:

DMEM with glucose 4500 mg/L, glutamine 584 mg/L and HEPES 25mMol

Foetal bovine serum	10%
Penicillin	100 μ /cm ³
Streptomycin	100 μ g/cm ³
Non-essential amino acids	0.1 mMol
Proline	0.4 mMol
Ascorbic acid	50 mg/L

The collagen solution for digestion of cartilage contained:

Collagenase type II	3-3.85 mg/ml
DMEM	
Foetal bovine serum	5%

Example 1 Preparation of Chondrocytes

A sample of autologous ear cartilage 1 cm x 1 cm x 0.3 cm in size was taken from the donor site of a rabbit under sterile conditions, transferred immediately to the laboratory, where it was weighed and its size measured. The cartilage sample was washed several times with phosphate-buffered saline (PBS) pH 7.4 and excess connective tissue attached to the cartilage was trimmed off. The cartilage was diced into 1mm³ cubes, which were transferred into a 1.8 ml cryovial. Collagenase solution as described above (3.85 mg per ml, 10 ml collagenase per gram of tissue) was added, and the fragments were stirred at 40 rpm for 15 hours at 37°C. The fragments were then centrifuged at 1000 rpm for 5 minutes at room temperature to remove the collagenase. The pellet was resuspended with 1.5 ml 0.02% EDTA. After 10 minutes incubation, the pellets were washed twice with PBS, and then the fragments were triturated with a 10ml pipette to disperse the cells.

The isolated chondrocytes in culture medium were counted using a haemocytometer, and the cells were plated at a density of 1×10^4 per cm² in the complete medium described above. The culture was re-fed with 20 ml culture medium on a Monday-Wednesday-Friday schedule. The cells were routinely subcultured with trypsin-EDTA when the culture became pre-confluent.

Example 2 Preparation of Polymer Construct

Threads of polygalactin 910 (PGA) (0-0 vicryl suture material, W-9901, Ethicon Co., Somerville, N.J.) were unbraided. A random tangle (matrix) of PGA fibre was placed into a plaster mould, mould A, with the desired three-dimensional shape, as shown in Figure1. The mould comprises two complementary pieces, the convex piece fitting snugly into the dented (concave) piece. The space between the two pieces is in the shape desired in order to repair the defect. 0.2 ml 2% of a mixture of 50% PGA-50% poly-L-lactic acid(PLLA) in methylene chloride was added to form a

unit 1 cm x 1 cm x 0.2 cm in size. The methylene chloride was removed under vacuum, then the mixture was dried in air at room temperature. If desired, constructs can be prepared in batches, conveniently 5 to 6 at a time. The number per batch will depend on demand.

The 50% PGA - 50% PLLA mixture cross-links the PLA fibres to form a three-dimensional scaffold. 100% PLLA may alternatively be used.

Example 3 Preparation of Fibrin

Lyophilized fibrinogen was reconstituted with an aprotinin solution of 3,000 KIU/ml and kept for 2-3 hours in a water bath at a temperature of 37°C.

5 ml of 1 mM calcium chloride was transferred into a vial containing lyophilized Thrombin 500 (5 mg per vial), swirled briefly to dissolve the lyophilized material, and then kept at 37°C as a stock solution until used. Prior to use, 0.1 ml of the thrombin solution was diluted in 5 ml PBS to form the working solution.

Two syringes respectively holding the fibrinogen and thrombin solutions were placed into a two-syringe clip and armed with a "V"-shaped connector and a needle. The device is illustrated in Figure 2. This shows two syringes 1a and 1b with plunges 2 and 2b respectively. The syringes placed in a two-syringe clip 3 (for example a Duploject device), with a plunge driver 4, as indicated by the curved arrow. The V-shaped connector 5 leads the solutions from the two syringes into a single needle 6. The solutions were passed to two separate outlets. The solutions were ejected simultaneously from a separate nozzle just above each outlet by manual pressure on the syringe plungers, so that the thrombin and fibrinogen blended optimally in the needle as they were dispersed into overlapping spraying cones.

Equal volumes of the two sealant components were mixed and immediately applied to encapsulate the polymer construct from Example 2 in another plaster mould, Mould B, which had the same shape as, but was slightly bigger than, Mould A. The fibrin clots in 1 to 2 minutes. The fibrin was allowed to mature for 2 hours, and then the construct was removed from its mould. The polymer-fibrin construct was placed in a 35 mm petri dish and sterilised by irradiation with 2.5 million rads.

Example 4 Preparation of Chondrocyte-Polymer-Fibrin Constructs

For plating on to the constructs, chondrocytes were suspended in medium at a concentration of 5×10^7 per ml. Each construct was injected with 100 μ l of the cell suspension. The constructs were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in the complete medium described above in a glass bottle with stirring for 2 to 3 weeks to permit cell adhesion and growth into the polymer matrix. Before transplantation, the units were incubated in serum-free medium for one day and then washed three times in PBS prior to transplantation, to prevent stimulation of an immune reaction in the host.

Example 5

Implantation into a Host

The host rabbits were weighed and general anaesthesia was induced with 0.1 ml/kg promex 2 and 0.1 ml/kg ketapex intramuscularly. The rabbits were shaved on each side of the vertebra, and scrubbed dorsally with betadine. Subcutaneous pouches (2 cm x 2 cm) were created on each side of the vertebra, using sterile technique.

The constructs with cultured autologous chondrocytes were manually circled with a stainless steel ring as marker, then were inserted into the opposite pouches on the rabbits. The incisions were closed using 3-0 Ethilon sutures. The rabbits were sacrificed and the explants were removed for analysis on day 10, 60 and 141.

Example 6

Effect of Fibrin Glue Coating on Formation of New Cartilage

The effect of fibrin glue coating on chondrocyte growth and extracellular matrix production *in vitro* and *in vivo* was examined.

For the *in vitro* study, cultured human chondrocytes were inoculated into poly-galactin (PGA) or 50%PGA-50% poly-L-lactic acid (PLLA) with or without fibrin glue coating, and cultured for 3, 13 and 30 days. In the *in vivo* study, a total of 12 cell-polymer constructs were transplanted into three rabbits. Four polymer constructs with autologous chondrocytes per rabbit were PGA and 50% PGA/50% PLLA units, with or without fibrin glue coating. The rabbits were sacrificed on days 10, 60 and 141.

Over 30 days of *in vitro* culture, the average dry weight (DW) of the coated PGA constructs was 53.8% of the DW on day 3, while the average DW of the uncoated counterparts decreased to 19.6%. On day 30, the average collagen content

per coated PGA constructs was $1.00 \pm 0.09 \mu\text{g}$, about 1.37 times the average collagen content of the uncoated constructs ($n = 4$). For the sponges consisting of 50%PGA-50% PLLA, the average DW of the coated constructs decreased to 88.8% of the DW on day 3, while the uncoated counterparts decreased to 25.3%. The average collagen content per coated construct was $0.23 \pm 0.02 \mu\text{g}$, about 1.96 times the average collagen content of the uncoated counterparts.

In the *in vivo* study, the weight and size of the retrieved constructs revealed that solid, thick, new cartilage formed in each coated construct, while little or no cartilage formed in their uncoated counterparts. On day 60, the wet weight (WW) of the coated PGA construct was 666 mg, about 3.17 fold greater than that of the uncoated counterpart. On day 141, the WW of the coated 50% PGA-50% PLLA unit was 424 mg, 3.21-fold that of the uncoated counterpart. The histological appearance of the constructs presented compact and homogenous extracellular matrix (ECM) in the coated explants. In the peripheral layer of fibrin glue-coated constructs, the chondrocytes appeared spherical. Darker staining with toluidine blue indicated higher deposition of ECM. In the central area of the same construct, the chondrocytes appeared spindle-shaped, and less ECM was deposited.

We have found that the fibrin glue forms a coating on the chondrocyte-polymer constructs which assists in formation of new cartilage. This was demonstrated by an increase in collagen content, retention of chondrocyte morphology, and delay in degradation of the constructs, both *in vitro* and *in vivo*.

Without wishing to be bound by any proposed mechanism, we believe that the fibrin assists in retaining extracellular matrix in the constructs of the invention, and may also stimulate synthesis of extracellular matrix components by the chondrocytes.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.



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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS

1. A method of preparing a cartilage implant suitable for transplantation into a host, comprising the steps of:
 - a) harvesting a tissue source of chondrocytes from a donor site;
 - b) isolating chondrocytes from the tissue source;
 - c) culturing the chondrocytes in the presence of a three-dimensional matrix of resorbable material which has been pre-coated by a tissue-compatible protein; and
 - d) culturing the chondrocytes under conditions and for a time suitable to permit the chondrocytes to adhere to and grow into the matrix.
2. A method of preparing a cartilage implant suitable for transplantation into a host, comprising the steps of:
 - a) isolating embryonal chondrocytes, pre-chondrocytes or chondrocyte precursors from a tissue source thereof;
 - b) culturing the cells produced in step (a) for a time and under conditions suitable to permit said cells to multiply and differentiate to produce chondrocytes;
 - c) culturing the chondrocytes in the presence of a three-dimensional matrix of resorbable material which has been pre-coated by a tissue-compatible protein; and
 - d) culturing the chondrocytes under conditions and for a time suitable to permit the chondrocytes to adhere to and grow into the matrix.
3. A method according to claim 1 or claim 2, in which the three-dimensional resorbable matrix is a resorbable suture material.
4. A method according to claim 3, in which the resorbable suture material is polygalactin.
5. A method according to any one of claims 1 to 4, in which the tissue-

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compatible material is fibrin.

6. A method according to any one of claims 1 to 5, in which the matrix is coated with a tissue-compatible polymer prior to being coated with fibrin.

7. A method according to any one of claims 1 to 6, in which the tissue-compatible polymer is a polygalactin-poly-L-lactic acid copolymer or is poly-L-lactic acid.

8. A method according to any one of claims 1 to 7, in which the chondrocytes are derived from the host into which the implant is to be transplanted.

9. A method according to any one of claims 1 to 8, in which the chondrocytes are derived from Wharton's jelly.

10. A method according to any one of claims 1 to 8, in which the chondrocytes are derived from pre-chondrocytes or chondrocyte precursors.

11. A matrix for chondrocyte culture, comprising a resorbable polymer material, optionally pre-coated with a tissue-compatible polymer, and coated with a tissue-compatible protein.

12. A matrix according to claim 11, in which the resorbable polymer material is a resorbable suture material.

13. A matrix according to claim 12, in which the resorbable suture material is polygalactin.

14. A matrix according to any one of claims 11 to 13, in which the matrix is coated with a tissue-compatible polymer prior to being coated with fibrin.

15. A matrix according to any one of claims 11 to 14, in which the tissue compatible polymer is a polygalactin-poly-L-lactic acid copolymer or is poly-L-lactic acid.

16. A matrix according to any one of claims 11 to 15, in which the tissue-compatible protein is fibrin.

17. A method of preparing a matrix for chondrocyte culture, comprising the steps of:

- a) forming a three-dimensional matrix of a resorbable polymer material in a mould of the desired three-dimensional shape;
- b) optionally coating the matrix with a tissue-compatible polymer; and
- c) coating the three-dimensional matrix with a tissue-compatible protein.

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18. A method according to claim 17, in which the resorbable polymer material is a resorbable suture material.
19. A method according to claim 17 or claim 18, in which the resorbable suture material is polygalactin.
20. A method according to any one of claims 17 to 19, in which the matrix is coated with a tissue-compatible polymer prior to being coated with fibrin.
21. A cartilage implant, comprising a matrix according to any one of claims 11 to 16, and cultured chondrocytes adhered to and/or growing into the matrix.
22. A cartilage implant according to claim 21, prepared by a method according to any one of claims 1 to 11.
23. A cartilage implant according to Claim 21 or 22, in the shape of an ear or a nose.
24. A method of repairing a cartilage lesion, comprising the step of grafting a cartilage implant as defined in any one of Claim 21 to 23, into a subject in need of such treatment.
25. A method according to Claim 24, in which the subject is suffering from cartilage damage caused by inflammation.
26. A method according to Claim 24, in which the subject is suffering from cartilage damage caused by degenerative arthritis.
27. A method according to Claim 24, in which the subject is suffering from cartilage damage caused by trauma.
28. A method according to Claim 24, in which the subject is suffering from a congenital defect of cartilage.
29. A method according to any one of Claims 24 to 28, in which the subject is a non-human mammal.
30. A method according to any one of Claims 24 to 28, in which the subject is a human.

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CARTILAGE IMPLANT**ABSTRACT**

This invention relates to methods of culture of chondrocytes, and in particular to methods of preparation of chondrocyte cultures suitable for use in tissue grafting. The invention provides a matrix for chondrocyte culture, comprising a resorbable polymer material, optionally pre-coated with a tissue-compatible polymer, and coated with a tissue-compatible protein. Preferably the resorbable polymer material is a resorbable suture material. The invention also provides a cartilage implant, comprising a matrix according to the invention, and cultured chondrocytes adhered to and/or growing into the matrix, and methods of preparing cartilage implants, and methods of preparing cartilage lesions.

(FIGURE 1 IS SUGGESTED FOR PUBLICATION)

1/1

convex

dented

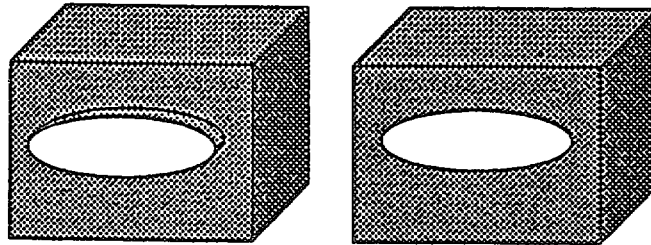


Fig. 1

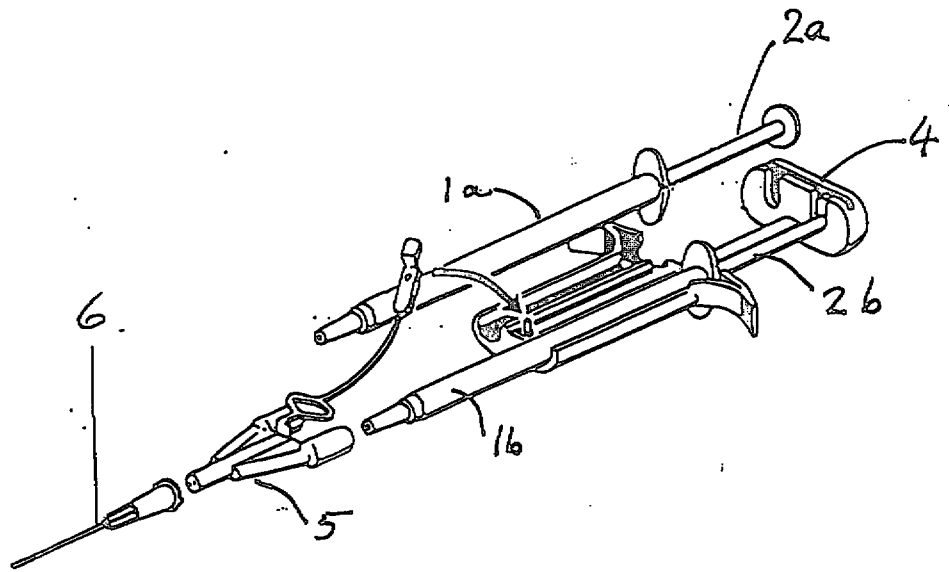
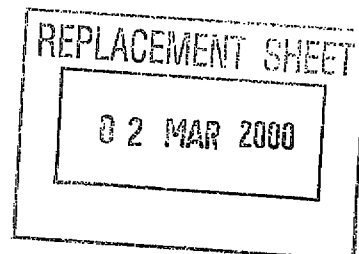
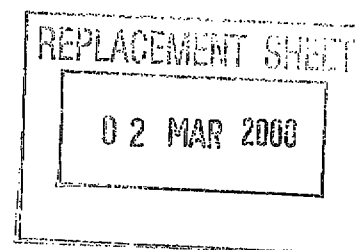


Fig. 2



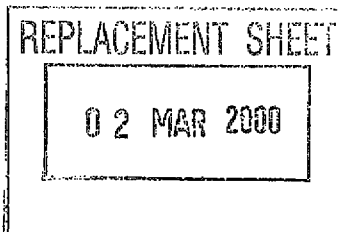
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 - b) isolating chondrocytes from the tissue source;
 - c) culturing the chondrocytes in the presence of a three-dimensional matrix of resorbable material which has been pre-coated by a tissue-compatible protein; and
 - d) culturing the chondrocytes under conditions and for a time suitable to permit the chondrocytes to adhere to and grow into the matrix.
2. A method of preparing a cartilage implant suitable for transplantation into a host, comprising the steps of:
 - a) isolating embryonal chondrocytes, pre-chondrocytes or chondrocyte precursors from a tissue source thereof;
 - b) culturing the cells produced in step (a) for a time and under conditions suitable to permit said cells to multiply and differentiate to produce chondrocytes;
 - c) culturing the chondrocytes in the presence of a three-dimensional matrix of resorbable material which has been pre-coated by a tissue-compatible protein; and
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3. A method according to claim 1 or claim 2, in which the three-dimensional resorbable matrix is a resorbable suture material.
4. A method according to claim 3, in which the resorbable suture material is polygalactin.
5. A method according to any one of claims 1 to 4, in which the tissue-



compatible material is fibrin.

6. A method according to any one of claims 1 to 5, in which the matrix is coated with a tissue-compatible polymer prior to being coated with fibrin.
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 - b) optionally coating the matrix with a tissue-compatible polymer; and
 - c) coating the three-dimensional matrix with a tissue-compatible protein.



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