Skin sensitization study of two hydroxypropyl methylcellulose components (Benecel® and E4M®) of an injectable bone substitute in guinea pigs

Y. AMOURIQ, X. BOURGES, P. WEISS, J. BOSCO, J.-M. BOULER, G. DACULSI
Centre de Recherche INSERM – EMI 99/03 sur les Matériaux d'Intérêt Biologique 1, Place Alexis Ricordeau 44 042 NANTES Cedex 1, France

Although initial results were promising for an injectable bone substitute (IBS) associating a hydroxypropyl methylcellulose (HPMC) polymer vector (Benecel®, 2 w/w %) with biphasic calcium phosphate (BCP), a sensitization reaction occurred probably related to the degree of polymer purity. In this context, Benecel® and another HPMC, E4M® were investigated in the present study. The expected composition of the polymers was confirmed by gas–liquid chromatography. Studies in the guinea pig showed that Benecel® has strong sensitization capacity and E4M® none. Benecel® manifests impurities (30 times more than E4M®) in individual fibers or rounded clumps that are apparently responsible for extreme sensitization. Purification by ultracentrifugation associated with 0.2 μm filtration can decrease sensitization capacity considerably, though with a slight loss of polymer concentration. Fourier transform infrared (FTIR) analysis showed that the impurities were largely cellulose derivatives. However, extraction by organic solvent, followed by FTIR studies and micro-X analysis, detected an oily substance containing carbon and silicon associated with the cellulose derivatives. E4M®, a polymer with no sensitization capacity, could replace Benecel® and improve results with IBS.

1. Introduction

Bone defects are frequently encountered in surgery today. Biphasic calcium phosphates (BCP), because of their biocompatibility and bioactivity, can be useful bone substitutes in these cases [1, 2]. Moreover, the use of an injectable bone substance (derived from a BCP) can improve and facilitate the performance of BCP [3].

Hydroxypropyl methylcellulose (HPMC) is a polymer cellulose derivative already used in ophthalmic applications [4]. HPMC in association with BCP constitutes an injectable bone substitute (IBS) now under development [5, 6]. BCP granules (60% hydroxyapatite, 40% β-tri calcium phosphate) are mixed with an HPMC aqueous solution (2 or 3 w/w %). The first polymer used for this purpose was Benecel® (2 w/w %) [7].

Although initial experimental studies of IBS gave good results [8, 9], several animals showed irritation (probably an allergic reaction) when IBS was used a second time. An unpublished industrial study [10] suggested a sensitization reaction, whereas cellulose derivatives are not known to cause allergic reactions [11].

The present study investigated the skin sensitization capacities of two-selected HPMC of pharmaceutical grade, Benecel® and E4M®, in the guinea pig. Our objective was to determine the degree of HPMC purity, its implications on sensitization and then to detect and characterize the impurities and consider how they could be eliminated.

2. Materials and methods

2.1. Material

HPMC is an odorless white powder that dissolves in water to form a gel. Viscosity depends on the molecular weight and concentration of the polymer. The two HPMC selected for this study were Benecel® MP 824 (Aqualon, Rueil-Malmaison, France, batch number UK 1538), used at 2% (w/w) in IBS, and E4M® (Methocel E4M Premium EP, Colorcon, Kent, England, batch number LB 230112N11) used at 3% (w/w) in IBS. Both polymer aqueous solutions had an apparent viscosity measured to 20 000 mPas (Visco-Star-L Fungilab; 1 rpm).

Benecel® was prepared at a 2% (w/w) concentration in aqueous dispersion using injectable water (Injectable Water, Renaudin Laboratory, France) and with magnetic stirring for three days at room temperature. E4M® was prepared in the same way at a 3% (w/w) concentration (viscosity equivalent to Benecel® 2% (w/w)).

For the skin sensitization test, the polymer was diluted to different concentrations [C]: [C1] = pure Benecel®

2% or E4M® 3% in aqueous solution; [C0.1] = 1 g of Benecel® 2% or E4M® 3% added to NaCl 0.9% or injectable water to obtain 10 ml of solution; and [C 0.*X*] = *X* g of Benecel® 2% or E4M® 3% added to NaCl 0.9% or injectable water to obtain 10 ml of solution.

2.2. Purification, determination of the polymer loss and insoluble part rate

HPMC was purified by centrifugation (4000 rpm for 1 h; 3500 G at the bottom of the tube; 2.OR, Heraeus, Hanau, Germany) and ultracentrifugation (20 000 rpm for 1 h; 46 500 G at the bottom of the tube; Beckman L7-67, Beckman Instruments, Palo Alto, California, USA). The HPMC at the bottom of the tube was considered to be the insoluble part.

HPMC at [C0.1] and [C0.2] concentrations was purified on a 0.2-µm-pore acetate cellulose filter (Minisart®, Sartorius AG, Goettingen, Germany) connected to a syringe (containing polymer) by a Luer-lock system.

As purification by filteration may decrease polymer concentration, polymer loss was determined by filtering ultracentrifuged Benecel® [C0.1] (dilution with injectable water) on Minisart®. Polymer before (three flasks, number 1, 2, 3) and after filteration (three flasks, number 4, 5, 6) was dehydrated in a drying-stove (Heraues, Hanau, Germany) at 50 °C for 15 h.

Using a Metler Toledo AT 261 scales (Metler, Viroflay, Switzerland), the weight of each flask and of each polymer before and after drying was measured, as well as the polymer concentration before and after filteration. The difference was analyzed statistically using Student's *t*-test.

Benecel® and E4M® 0.5% (w/w) were filtered on a 10-µm pore filter (FG Millex filter, Millipore, Bedford, MA, USA). The difference in weight between dry polymer before and after filteration for the same volume was determined according to an insoluble part rate (in relation to 10 µm filtration). Three measurements were performed for each polymer.

2.3. Chemicophysical characterization

HPMC was investigated by gas–liquid chromatography (GLC; Perkin Elmer Autosystem with an integrator 1020; Perkin Elmer, Norwalk, CT, USA) and Fourier transform infrared (FTIR) analysis on KBr (Magma-IR 550 Spectrometer, Nicolet, Paris, France).

For sample preparation, 20 mg of dry HPMC were measured in vials. Samples were taken up in 0.7 ml of methanolic HCl solution prepared by adding 0.6 ml acetyl chloride to 15 ml of methanol. The vials were sealed with silicon/aluminum stoppers. Methanolysis was conducted at 80 °C for 24 h, the methanolic HCl solution was removed under a nitrogen stream. The trimethylsilylation reagent prepared in the laboratory was composed of pyridine, hexamethyldisilazane and trimethylchlorosilane (9 : 3: 1, v/v/v). Zero point five millilitre of this reagent was introduced directly into the vials, sealed and heated at 80 °C for 2 h. The derivatized samples were rotary-evaporated at 50–60 °C, and the residue was immediately dissolved in 0.5 ml of dichloromethane. Gas capillary chromatography was performed with 1.5 µl of this solution.

The gas chromatograph (GC) was equipped with a 30 m × 0.25 mm (inner dimension) fused silica column (Simplicity-5, Supelco-France). The carrier gas was nitrogen at a flow rate of 1.2 ml/min. The injector and the FID detector were set to 220 °C and 300 °C respectively. Injection was performed with a splitting ratio of 50 : 1. Oven temperature was raised from 150 °C to 240 °C at 3 °C/min and then kept at 240 °C for 5 min.

The insoluble part of Benecel® resulting from centrifugation was dehydrated in a drying-stove (50 °C), and 2 mg were then mixed with 300 mg of KBr powder to make a pastille under 12 tons of pressure. FTIR analyses were performed in the 4000–400cm^{-1} range using a Nicolet Magna 550 FT-IR spectrometer (Nicolet, Paris, France). Thirty-two spectral scans at 4 cm^{-1} resolution were conducted for each sample, computer-averaged and apodized with a Happ-Genzel function to produce each FTIR spectrum.

Another insoluble part of the Benecel® resulting from ultracentrifugation was also dehydrated in the same conditions and then mixed with tetrahydrofurane (THF), an organic solvent, and filtered (0.2 µm filter). After evaporation of the major part of THF, a drop was placed on a KBr pastille, which was heated to evaporate all of the THF. This pastille was then studied by FTIR and Micro-X analysis (Hitachi S3200 N).

2.4. Microscopical evaluation

The insoluble part was mixed with distilled water and stained with methylene blue. The solution was filtered on an FG Mitex filter (Millipore, Bedford, MA, USA) with 10-µm pores and observed in light microscopy. The surface of the 0.2 µm filter (Minisart® used for ultracentrifuged Benecel® [C0.1] filteration) was investigated by scanning electron microscopy (SEM) after gold-palladium spotting.

2.5. Skin sensitization test

Young male Dunkin Hartley guinea pigs (weighing 350–400 g) were purchased from Charles River (Saint Aubin les Elboeuf, France) and acclimated for five days in an animal room maintained at 22 ± 2 °C. Two groups of 17 healthy animals were used, one for Benecel® (group 1) and one for E4M® (group 2). An identification number was tattooed on the ear of each guinea pig (Morin electric dermograph, Centravet, Dinan, France). Laboratory food (C15–50, Extralabo, Ets Pietrement, Provin, France) and tap water were provided *ad libitum* during the experimental period.

All test substances were placed in glass flasks (USP type I) and steam-sterilized at 121 °C for 20 min.

E4M® 3% and Benecel® 2% were diluted for intradermal injection with NaCl 0.9% placed in sterile glass vials (NaCl 0.9%, Renaudin Laboratory, France).

For intradermal sensitization, the selected concentration [C0.4] for Benecel® 2% and E4M® 3% was mixed with an equal volume of Freund's Complete Adjuvant (FCA, Sigma Chemical, St Louis, USA). For intradermal

challenge, the concentration used was [C0.1] for Benecel® 2% and E4M® 3%.

These concentrations were determined by preliminary tests on two animals for each polymer (concentrations from [C0.02] to [C1]). [C0.4] induced moderate erythema. [C0.1] is the highest concentration which induced no or very slight erythema.

The sensitization test method was based on the maximization test of Magnusson and Kligman [12–14]. Seventeen animals divided into three groups were used for each polymer: a test preparation group (10 animals), a control group (five animals), and two animals for preliminary tests. The hair on the back of each animal was clipped off and depilated with lukewarm wax in a 4×6 cm area four days before injection.

An equal-volume mixture of FCA and NaCl 0.9% was intradermally injected at a dose of 0.2 ml (0.1×2) into positions 1 and 1′ (Fig. 1), whereas HPMC [C0.4] (or NaCl 0.9% for the control group) was injected into positions 2 and 2′ and an equal-volume mixture of FCA and the test preparation (FCA and NaCl 0.9% for the control group) into positions 3 and 3′. Seven days later, an intradermal injection (0.1 ml) of HPMC [C0.4] was injected into positions 1, 2, 3, 1′, 2′ and 3′ (NaCl 0.9% for the control group).

Two weeks after the first intradermal injection, the hair on the abdomen of each animal was depilated with lukewarm wax. Four days later, 2×0.1 ml of polymer [C0.1] was injected into this area.

Other challenge injections were also performed (Table I).

Skin reactions were scored at 24 and 48 h according to the scheme given in Table II. Animals from the test group that showed a higher dermal reaction than the most serious reaction in control group animals were considered to have a sensitization reaction.

Sensitization was judged according to the ASTM norm [15], Table III.

Skin reactions in the control group (control group score) gave an idea of the irritant capacity of the substance compared to other test substances at the same concentration.

The control group score was determined by adding up the scores of the five animals in the control group at 24 and 48 h for erythema and oedema evaluation.

Hematoxylin-eosin staining was performed after 24 and 48 h on 5-μm-thick guinea pig dermal sections at injection level for light microscopy observations.

3. Results

3.1. Polymer loss and insoluble part rate

The concentration of ultracentrifuged and diluted Benecel® [C0.1] was $[C0.1_u]$:

HEAD

1. | 1′.
2. | 2′.
3. | 3′.

TAIL

Figure 1 Injection points on the guinea pig back.

Flask 1: $[C0.1_u] = 0.2029309\%$ (w/w)
Flask 2: $[C0.1_u] = 0.20453458\%$ (w/w)
Flask 3: $[C0.1_u] = 0.2042149\%$ (w/w)
$0.20178 < [C0.1_u] < 0.206002\%$ (w/w) ($p = 0.05$)
$0.19902 < [C0.1_u] < 0.208757\%$ (w/w) ($p = 0.01$)

The concentration of ultracentifuged polymer after filteration was $[C0.1_{u+f}]$:

Flask 4: $[C0.1_{u+f}] = 0.1977025\%$ (w/w)
Flask 5: $[C0.1_{u+f}] = 0.2012275\%$ (w/w)
Flask 6: $[C0.1_{u+f}] = 0.198257\%$ (w/w) (Fig. 2)
$0.19435 < [C0.1_{u+f}] < 0.2037713\%$ (w/w) ($p = 0.05$)
$0.18820 < [C0.1_{u+f}] = 0.209923\%$ (w/w) ($p = 0.01$).

Thus, polymer loss after filteration was less than 9.8% ($p = 0.02$).

The difference in polymer dry weight before and after filteration on the 10 μm filter was the same each time. The insoluble part represented about 3% for Benecel® and about 0.1% for E4M®.

3.2. GLC, FTIR and Micro-X analysis

The E4M® and Benecel® chromatograms were as expected. In particular, the substitution rates for methoxyls and hydroxypropyls groups were consistent with the manufacturer's analysis (Fig. 3).

The infrared spectrum for the insoluble part of Benecel® resulting from centrifugation corresponded to that for cellulose and Benecel®. The insoluble part of Benecel® resulting from centrifugation was also a cellulose derivative [16].

The infrared analysis of the insoluble part of Benecel® after ultracentrifugation and THF extraction did not identify the compounds. The low quantity did not permit any other characterization. The Micro-X analysis spectrum for the same insoluble part did not allow us to identify the viscous, oily substance containing carbon and silicon (Fig. 4).

3.3. Microscopical evaluation

Filtration (10 μm) and methylene blue staining identified much of the insoluble part of Benecel®, especially as long fibers (up to 500 μm).

The same fibers were apparent with SEM, but disappeared in observations of ultracentrifuged Benecel®. However, at a higher magnification, elongated or rounded clumps (about 5 μm) were observed on the 0.2 μm filter (Fig. 5).

E4M® showed nearly no insoluble part and especially no long fibers.

3.4. Sensitization test

Table IV indicates the results of the skin sensitization test.

For the sensitization reaction at 24 h in dermis at the Benecel® injection point, hematoxylineosin staining showed slack, edematous connective tissue with a low rate of cell infilteration. The epidermis was also oedematous in some areas.

TABLE I Challenge injection for the sensitization test

Test substance	[C]	Animal group	Day
Benecel® 2%	[C 0.1]	1	30
Benecel® 2% Ultracentrifuged	[C 0.1]	1	44
Insoluble Part Benecel® 2% (Obtained by ultracentrifugation)	[C 0.1]	1	51
Benecel® 2% ultracentrifuged and filtered (0.2 μm)	[C 0.1]	1	58
Benecel® 2% ultracentrifuged and filtered (0.2 μm)	[C 0.2]	1	65
E4M 3%	[C 0.1]	2	27
E4M 3%	[C 0.1]	2	35

TABLE II Scoring criteria for test reactions (erythema and oedema)

Score	Erythema: Erythema and Eschar	Oedema: Oedema formation
0	No erythema	No oedema
1	Very slight erythema (barely perceptible)	Very slight oedema (barely perceptible)
2	Well-defined erythema (pale red in color)	Slight oedema (edges of area well-defined by definite raising)
3	Moderate to severe erythema (red and area well-defined)	Moderate oedema (edges rasied approximately 1 mm)
4	Severe erythema (beet redness to slight eschar formation)	Severe oedema (edges raised more than 1 mm and extending beyond the exposure area)

TABLE III Sensitization capacity according to the percentage of sentitized animals

Percent of sensitized animals	Grade	Classification
0%	0	None
1–8%	I	Not different than controls
9–28%	II	Mild
29–64%	III	Moderate
65–80%	IV	Strong
81–100%	V	Extreme

At 48 h, dermal swelling had largely subsided, and the structure of the connective tissue was nearly normal. The rate of cell infilteration was high (much greater than at 24 h). Many empty vacuoles delimited by inflammatory cells (mainly monocytes) could be seen in the hypodermis, in accordance with a sensitization reaction. The empty vacuoles could correspond to polymer loss (Fig. 6).

4. Discussion

Hundreds of thousands of prosthesis (hip, knee, etc.) are implanted each year in the world, and surgery for these prosthesis is developing increasingly at the end of life. Bone defects occur frequently in such cases and complicate operations.

Autogenous and allogenic bone grafts can provide significantly better bone healing and volume.

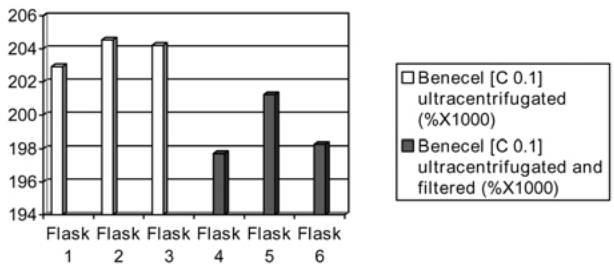

Figure 2 Concentration of ultracentrifugated Benecel® [C0.1] before and after filtration.

As a result of increased surgical time, morbidity, limited graft material for autogenous grafts, and contamination for allogenic grafts, there is a demand for synthetic bone substitutes. Biphasic porous calcium phosphates have already been used effectively as synthetic substitutes [17], and an injectable bone substitute would appear to provide greater facility and involve less invasive surgery [18].

The degree of purity [19] and the sensitization capacity of the polymer affect the performance of IBS. HPMC derivatives are not supposed to produce sensitization reactions and are already used in ophthalmic surgery.

An unpublished industrial study (1997) showed that an IBS associating BCP and Benecel® 2% has a strong sensitization capacity. As BCP bioactivity is well known, Benecel® is likely to be responsible for the sensitization response.

The present study tested two types of HPMC (Benecel® and E4M®), both of which showed the composition expected. The insoluble part of Benecel® was quite easy to identify since this polymer displayed many long fibers and rounded clumps. The insoluble part of E4M® was considerably smaller (30-fold less) and had no long fibers.

FTIR analysis showed that the insoluble part of

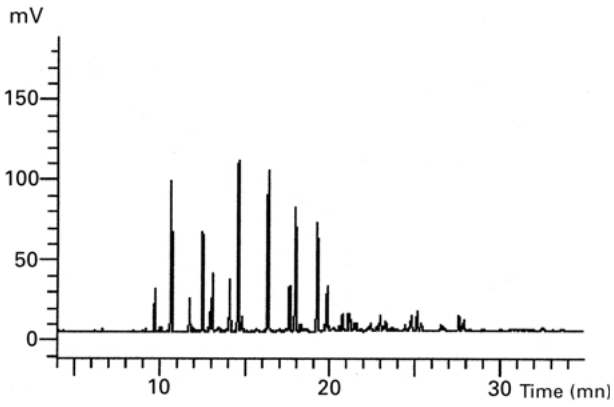

Figure 3 Benecel® chromatogram (GLC).

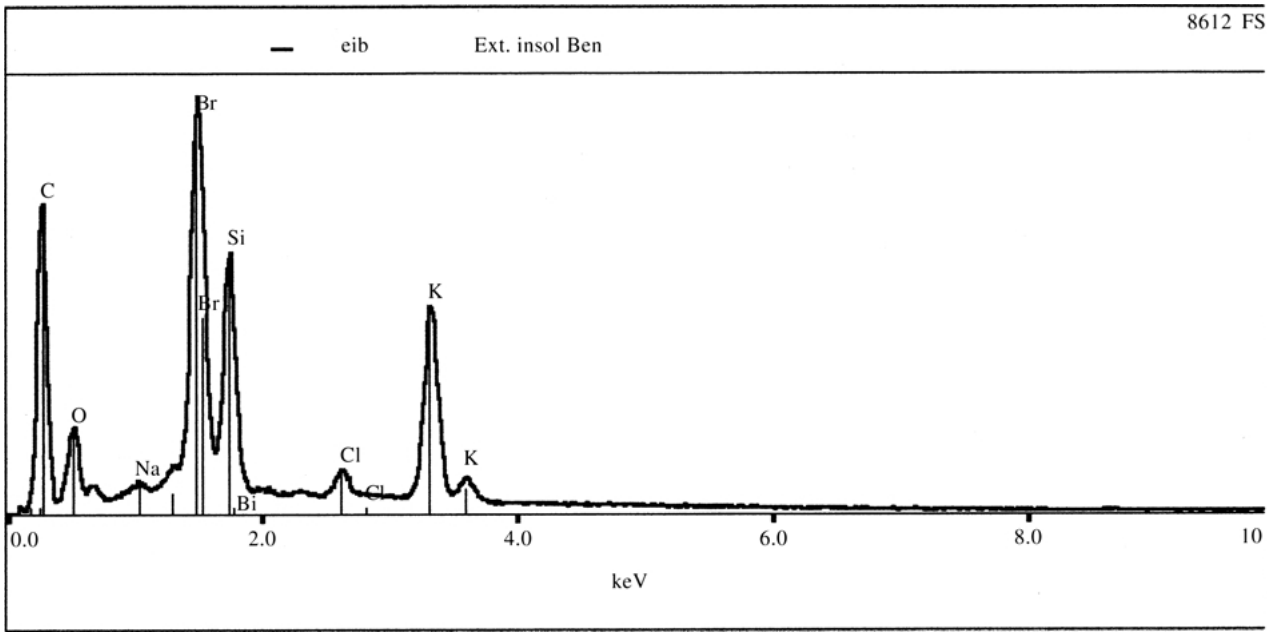

Figure 4 Insoluble part of Benecel® after THF extraction (Micro-X analysis).

Benecel® was a cellulose derivative. Extraction by organic solvent of the dried insoluble part indicated the presence of another substance (not a cellulose derivative) also likely to be responsible for the sensitization capacity of Benecel®. However, FTIR and X analysis did not allow us to determine the nature of this substance containing carbon, silicon.

Filteration on a 0.2 µm filter eliminated all insoluble parts. Polymer loss with this process appeared to be about 2–3% (less than 10%) when diluted 1 : 10 with injectable water. As the polymer is non-ionic, the results would surely have been the same for a polymer diluted with NaCl. After drying, NaCl weight was too great, as compared to polymer weight, to obtain a precise result, which led us to estimate polymer loss by dilution with injectable water. This type of filteration only works with a diluted substance when a syringe with a Luer-lock is used. As an undiluted substance requires too high a pressure for the filter, another system would be necessary.

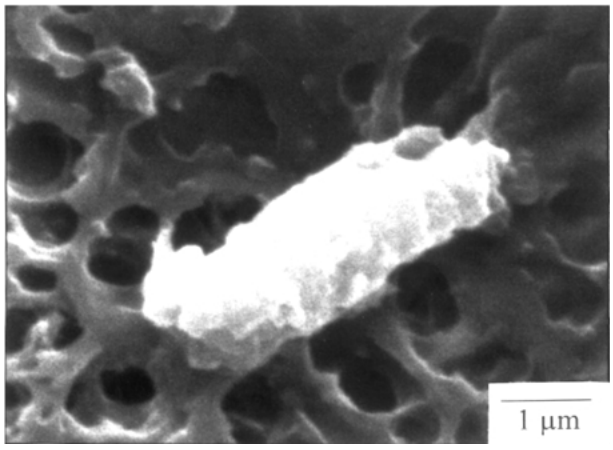

Figure 5 Insoluble part of Benecel® on 0.2 µm filter (SEM, X 10 000).

A sensitization test in the guinea pig using a maximized protocol is currently the reference method. When this test is negative, it is not absolutely certain that a substance has no sensitization capacity. Nonetheless, the guinea pig is the most sensitive species for allergic reactions. In fact, substances with high sensitization capacity in the guinea pig generally induce reactions in humans, whereas substances with slight sensitization capacity in the guinea pig may or may not induce reactions in humans [20]. The score of a tested substance in a control group gives a good idea of irritant capacity, even though it does not constitute a true irritation test.

In our study, Benecel® had high sensitization capacity when E4M® showed none. Histological results accorded with a sensitization reaction. With the same dilution, E4M® proved far less irritating for all animals tested, according to the oedema–erythema table. In particular, the control group showed lower scores.

The insoluble part of Benecel® displayed a very

TABLE IV Sensitization test results

Test substance	[C]	Score control group	Result + (%) test group	Sensitization capacity	Class
Benecel® 2%	[C0.1]	13	70–80	Strong	IV
Benecel® 2% Ultracentrifuged	[C0.1]	8	80	Strong	IV
Insoluble Part Benecel® 2% (Obtained by ultracentrifugation)	[C0.1]	25	89	Extreme	V
Benecel® 2% ultracentrifuged and filtered	[C0.1]	5	22	Mild	II
Benecel® 2% ultracentrifuged and filtered	[C0.2]	6	11–22	Mild	II
E4M 3%	[C0.1]	4	0	None	0
E4M 3%	[C0.1]	6	0	None	0

In the Benecel® group, one animal died at day 46.

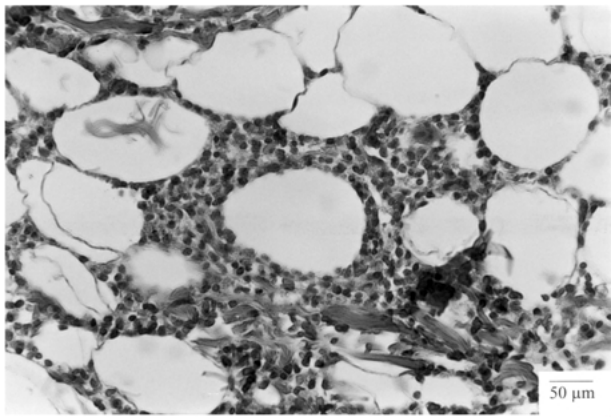

Figure 6 Connective tissue (guinea pig) 48 h after Benecel® injection.

high sensitization capacity and was more irritating. Ultracentrifugation associated with 0.2 µm filteration induced a slight decrease in Benecel® concentration, making its sensitization capacity mild. A second challenge injection with a double concentration allowed us to verify this result and compensate amply for polymer loss. This higher concentration was less irritating than Benecel® 2% [C0.1] (according to the oedema–erythema table). Purification of Benecel® (ultracentrifugation + 0.2 µm filteration) reduced irritation (in both groups of animals, the test preparation group and the control group) and sensitization capacity to mild levels. Test sensitization was not very precise for slight reactions. To be sure that sensitization capacity exists in such cases, another experiment should be performed with twice the number of animals. Purification of Benecel® by ultracentrifugation alone did not really change sensitization capacity (still strong), but irritation was slightly lower (possibly because of the suppression of fibers whose sharp-pointed ends can be irritating for cells).

HPMC has been used in human surgery without any apparent sensitization reactions. As HPMC is evidently not responsible for the sensitization capacity observed in Benecel® (though absent in E4M®), the problem relates to HPMC purification and industrial process.

5. Conclusion

E4M® and Benecel®, two pharmaceutical grade HPMC, show different degrees of purity. Though E4M® induces no sensitization reactions, Benecel® contains an insoluble part responsible for extreme sensitization reaction and irritation. The impurities in Benecel® are due to cellulose derivatives and an unidentified substance containing silicon and carbon. Purification by ultracentrifugation and 0.2 µm filteration can decrease irritation and sensitization capacity considerably, with only a slight loss of HPMC. This method is also applicable to other polymers.

The development of new biomaterials must take into account the degree of purity of the substances sold by manufacturers. As indicated in this study, two supposedly equivalent polymers of pharmaceutical grade displayed quite different biocompatibility.

The selection of a polymer vector for IBS is difficult because it should be satisfactory not only for physical and chemical properties (when mixed with BCP) but also for biological properties. The use of E4M® instead of Benecel® should improve results for the IBS. The IBS presents no risk of contamination (a synthetic substance), is easy to use (no mixing required) and decreases surgical time (injectable feature).

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