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The effect of gamma-ray and ethylene oxide sterilization on collagen-based wound-repair materials

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The effects of 2.5, 5.0, 7.5 and 10 Mrad gamma-irradiation and ethylene oxide sterilization on collagen-coated vicryl mesh, on collagen film and vicryl mesh were investigated. No cytotoxic effects were observed towards either L929 cells or human fibroblasts with any of the treatments, even at the highest doses of irradiation. Although the rate of biodegradation and tissue reaction towards the test materials appeared to be relatively unaffected by ethylene oxide, the rate of breakdown in the lumbar muscles of laboratory rats was considerably increased by irradiation. Dose-dependent irradiation damage to the vicryl mesh was confirmed *in vitro* using viscometry and by an increase in the rate of hydrolysis in phosphate-buffered saline (PBS) at 37 °C. Tensiometric studies on irradiated collagen films showed a dose-dependent reduction in both the break load and elongation before breaking. A small reduction in the break load and stretch to breaking was also observed following treatment with ethylene oxide. The findings presented favour ethylene oxide as a method of sterilization of both the composite membrane and its individual components. However, gamma-irradiation produced no toxic effects or adverse tissue reaction under the conditions described, and is more convenient to use. Hence, provided that the efficacy of the membrane would not be compromised by the higher rate of degradation, it could also be considered for sterilization of the material.

1. Introduction

Collagen has many desirable features that make it a widely acceptable choice as a biomaterial for a large number of surgical applications. Indeed, because of its biodegradability, haemostatic properties, non-cytotoxicity, low antigenicity, ability to support cellular growth and high tensile strength [1], this fibrous protein has already found considerable use in clinical medicine over the past few years [2–19]. Such applications include injectable collagen for augmentation of tissue defects [3, 6], haemostasis [7, 8], burn and wound dressings [9–11], hernia repair [12, 13], bioprosthetic heart valves [14], vascular grafts [15, 16], a drug-delivery system [1, 17], ocular surfaces [18] and nerve regeneration [19].

Recently, a collagen film reinforced with a water-soluble mesh (vicryl, Polyglactin 910; Ethicon Inc., Somerville, New Jersey, USA) has been developed for urinary tract surgery [20–22], and this material has now been used in several other clinical applications [23–26]. When such a composite material is to be considered for routine use in a number of surgical areas, clearly it must be supplied as a sterile product. Additionally, when the methods that are currently available for the sterilization of commercial medical products, namely heat, ethylene oxide gas and gamma-irradiation, can affect one or more of the individual components, then a great deal of consideration must be given to choosing a method that will finally be used to sterilize the material, and which will not compromise the efficacy of the product.

Because of thermal damage in the presence of moisture caused to vicryl [27] as well as the denaturation temperature of collagen, autoclaving or

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heat-sterilizing the composite membrane cannot be considered.

In a rabbit model of urinary tract repair [22] it was shown that the effect of gamma-irradiation did not adversely influence the efficacy of the collagen–vicryl membrane when compared with non-irradiated material, even though *in vivo* studies using rabbit ear-chambers [28] demonstrated that the irradiated membrane was absorbed more rapidly than either material that had been disinfected by immersion in ethanol or sterilized by exposure to ethylene oxide gas. Furthermore, in a clinical study of patients in whom the material was used to repair fistulae of the urinary tract [29] it was observed that in some cases the irradiated membrane was broken down too rapidly at the operative site, especially in the presence of infection, resulting in leakage before healing could take place.

The work presented in this paper therefore examined in more detail the effect of both gamma-irradiation and ethylene oxide gas on the collagen–vicryl composite membrane, vicryl mesh and collagen film. Materials were irradiated with 2.5, 5.0, 7.5 and 10.0 Mrad gamma-irradiation or subjected to an ethylene oxide sterilizing cycle. Cytotoxicity was assessed using both established mouse fibroblast and primary human cell lines. Following exposure to irradiation, vicryl mesh and collagen-coated vicryl mesh were incubated in PBS over a period of 28 days, and the decrease in tensile strength was observed. Irradiation damage to vicryl mesh was determined viscometrically. Irradiated collagen film was also examined tensiometrically. The collagen-coated vicryl mesh was implanted into the lumbar muscles of laboratory rats in order to investigate any overall changes in the biodegradation rate and tissue reaction that may have resulted from irradiation or ethylene oxide treatment.

2. Materials and methods

2.1. Preparation of the collagen–vicryl composite membrane

Collagen–vicryl membranes were prepared from style 9 and style 12 vicryl mesh and 0.3% (w/v) acid-swollen collagen slurry by methods described previously [28, 30]. Style 9 mesh is knitted from vicryl filaments (Ethicon Inc., Somerville, New Jersey, USA) with triangular holes 0.61 mm (base width)× 0.66 mm (base-to-apex), whereas style 12 mesh contains larger hexagonal holes (3.44 mm long axis × 2 mm short axis [28]).

Samples were sterilized with 2.5, 5.0, 7.5 or 10.0 Mrad gamma-irradiation from a ^{60}Co source or by exposure to ethylene oxide gas under a Department of Health approved sterilizing cycle for medical products (Ethicon Ltd, Edinburgh, UK). Samples were prehumidified for a minimum of 48 h at 21–24 °C and at 75–85% relative humidity (R.H.). The membrane, suitably packaged, was then placed into the sterilizer and allowed to stand at 8.6×10^4 Pa pressure for 15 min. After rehumidification to 70–90% R.H. ethylene oxide gas was injected at $1.5–1.6 \times 10^5$ Pa pressure for 8 h at 27–32 °C. After this period the pressure was reduced to 8.6×10^4 Pa for a minimum of 1 h, and the samples were transferred to a vacuum drying chamber on heated shelves at 45–50 °C. The pressure was reduced to 10^4 Pa (this step required 12 h), after which the pressure was brought up to atmospheric pressure with nitrogen and the pieces of membrane were sealed. Residual levels of ethylene oxide were assessed by gas chromatography and were below the permitted maximum of 25 p.p.m. for vicryl mesh (Ethicon, Edinburgh, UK).

2.2. Preparation of collagen film

Collagen film was prepared from a 0.3% (w/v) acid-swollen collagen slurry that had been homogenized at 4 °C for 90 s in a Waring blender before air-drying according to the method of Gorham *et al.* [31]. Such films were typically 20–25 μm thick.

2.3. Cytotoxicity studies

All test materials were initially screened in cell culture via the direct contact method in which a medical adhesive was used to attach a 1 cm square of the test material to the surface of 60 mm Petri dishes [32]. Samples tested included collagen film, collagen-coated vicryl mesh and vicryl mesh (styles 9 and 12). All test materials were treated with up to 10 Mrad gamma-irradiation or ethylene oxide sterilization as described above. The test procedure included both positive (cytotoxic) controls [doped poly(vinyl chloride); Ethicon, UK] and negative (non-cytotoxic, medical adhesive or polyethylene) controls. L929 mouse fibroblast cells [32] and a primary culture of human fibroblasts derived from foreskin biopsies were employed in the study. Both cell types were grown and maintained in Eagle's minimum essential medium (E-MEM) supplemented with 7.5% foetal calf serum before use. In each test 5 ml E-MEM containing a 1 ml suspension of 1×10^4 cells ml^{-1} prepared from a trypsinized confluent monolayer were added to each test-plate and incubated as described previously [32]. Cells were monitored regularly for signs of cell attachment and growth, as well as any inhibitory effects and cytotoxicity assessed after 4 days [32].

In addition to the direct-contact method, an extraction procedure was carried out in which both L929 and human fibroblast cell lines were grown in media that had been pre-incubated with irradiated and ethylene oxide-sterilized materials as follows. Extracts were prepared by incubating 1 cm squares of the same test materials used in the direct-contact method in 10 ml extraction medium (E-MEM) at ambient temperature for 4 or 10 days. Both positive and negative controls were included in the procedure. After the appropriate extraction period, the pH of each extract was adjusted to 7.2–7.4 with sterile 0.1 M HCl or 0.1 M NaOH.

Stock cultures of both L929 and human foreskin fibroblasts were propagated in E-MEM growth medium and maintained in sterile 25 cm^2 tissue culture flasks (Costar, NBL Biologicals) at 37 °C in 5% CO_2–95% air. Immediately before use a cell suspension was prepared from a trypsinized confluent monolayer so that 150 μl contained between 500 and

1000 cells. Aliquots (150 µl) were transferred into each well of a 96-well plate and incubated for 24 h at 37 °C in 5% CO_2–95% air, after which 100 µl aliquots of the extracts from the test materials prepared as described above were added. Sixteen wells were used for each individual extract, and incubation was carried out under the same conditions used for cell growth before assessing any cytotoxic effect. Following the incubation period, each well was examined microscopically and photographed, after which the culture medium was aspirated from each well. Cells were then counted using a methylene blue staining technique [33, 34]. The cell layer was then fixed with 100 µl methanol for 30 min. The fixative was subsequently removed from each plate and a 75 µl portion of filtered 0.5% (w/v) methylene blue solution in 0.1 M sodium borate buffer, pH 8.5, was added to each well and the whole allowed to stand at ambient temperature for 30 min. Excess dye was removed by serially dipping the plates into four tanks of the borate buffer. After drying at 37 °C, 100 µl 1:1 solution of ethanol and 0.1 M HCl was added to each well. The plates were shaken gently and the absorbance measured at 620 nm for each well using a microplate reader (Titertek Multiscan; Flow Laboratories, Irvine, UK). The instrument was adjusted to zero on the first well, which contained elution solvent only.

2.4. Implantation into the lumbar muscles of laboratory rats

Collagen-coated vicryl mesh (style 9) which had been either irradiated at 2.5 Mrad, sterilized by exposure to ethylene oxide gas or disinfected by immersion in methanol was implanted into the lumbar muscles of Sprague–Dawley rats using the methods described previously [31, 33]. Rats were killed at 3, 7, 14, 21, 28, 35, 49 and 70 days and the appropriate sections processed for routine histology [31, 33].

2.5. Hydrolysis of irradiated collagen-coated and uncoated vicryl mesh in PBS

In order to test the effects of irradiation on the rate of solubilization on both vicryl mesh (styles 9 and 12) and collagen-coated vicryl mesh, 4 cm × 1 cm portions of each material were incubated in PBS at 37 °C for up to 28 days. Sodium azide (0.05%) was included as a bacteriocide. Test samples were removed at regular intervals and stretched to breaking point using an Instron tensiometer as described in [30].

2.6. Tensile strength testing of collagen film

Portions of collagen film 1 cm wide which had been either sterilized by ethylene oxide or subjected to 2.5, 5.0, 7.5 or 10.0 Mrad gamma-irradiation, together with control untreated film, were presoaked in PBS at ambient temperature. The force required to break the sample and the amount of elongation before breaking were subsequently determined using an Instron tensiometer under the conditions described in [30].

2.7. Analysis of irradiated vicryl mesh by viscometry

The test procedure was essentially that used by the manufacturers of vicryl (Ethicon Inc., Somerville, New Jersey, USA) to assess any chain scission in the final product. Portions of vicryl mesh (style 9, 5 mg) which had been subjected to up to 10 Mrad gamma-irradiation were accurately weighed and dissolved in 5 ml hexafluoroisopropanol. Non-irradiated samples were also included as controls. Pure solvent (5 ml) was added to a clean Cannon–Fenske size 75 viscometer which was subsequently rinsed and dried. A 5 ml test sample was then added and the viscometer was suspended in a 25 °C water bath for 20 min. The liquid level was pushed above the upper timing mark using dry filtered nitrogen and allowed to return to its initial position. The solvent level was again pushed above the upper timing mark and the time taken for it to run between the two timing marks recorded. A viscosity value (NiH) for each sample relative to the pure solvent was determined for each sample using

$$\mathrm{NiH} = \frac{\text{solution flow time/solvent flow time}}{\text{sample concentration in g(100 ml)}^{-1}}$$

3. Results

3.1. Cytotoxicity studies

Under all test conditions employed, no immediately obvious cytotoxic effects were observed either with gamma-irradiation or with ethylene oxide sterilization. Fig. 1 shows that cell attachment and growth occurs readily on irradiated materials with the direct contact method. Identical results were obtained following ethylene oxide sterilization of the collagen. No differences could be seen in any of the samples with increasing doses of irradiation, and all test materials resembled the control membrane in this respect. The effect of a positive cytotoxic control can readily be seen in Fig. 1b: in all cases both human fibroblasts and mouse L929 cells could be seen attaching and growing under the plate and across the vicryl and coated vicryl mesh, even at the highest doses of gamma-irradiation (Fig. 1). Although the cells growing on the material surface (Fig. 1c) did appear rounded, this effect was also observed on the control, non-irradiated collagen film. Additionally, earlier work [35, 36] showed that although cell proliferation and growth readily occur on collagen films prepared in this way, changes in cell morphology such as rounding have been observed.

The results of the extraction studies using L929 cells and human fibroblasts are presented in Tables I and II, which clearly demonstrate that, even following 10 days extraction, no cytotoxic effects were observed in any of the test materials. Indeed, at both times the lowest value recorded for the methylene blue test with human fibroblasts was 84% of the control, and in many cases the value obtained exceeded 100%. When a known cytotoxic material was included in the test a value of only 45% (at 4 days extraction) and 27% after 10 days was observed for human fibroblasts; the corresponding values for the mouse cells were 69 and 13%, respectively.

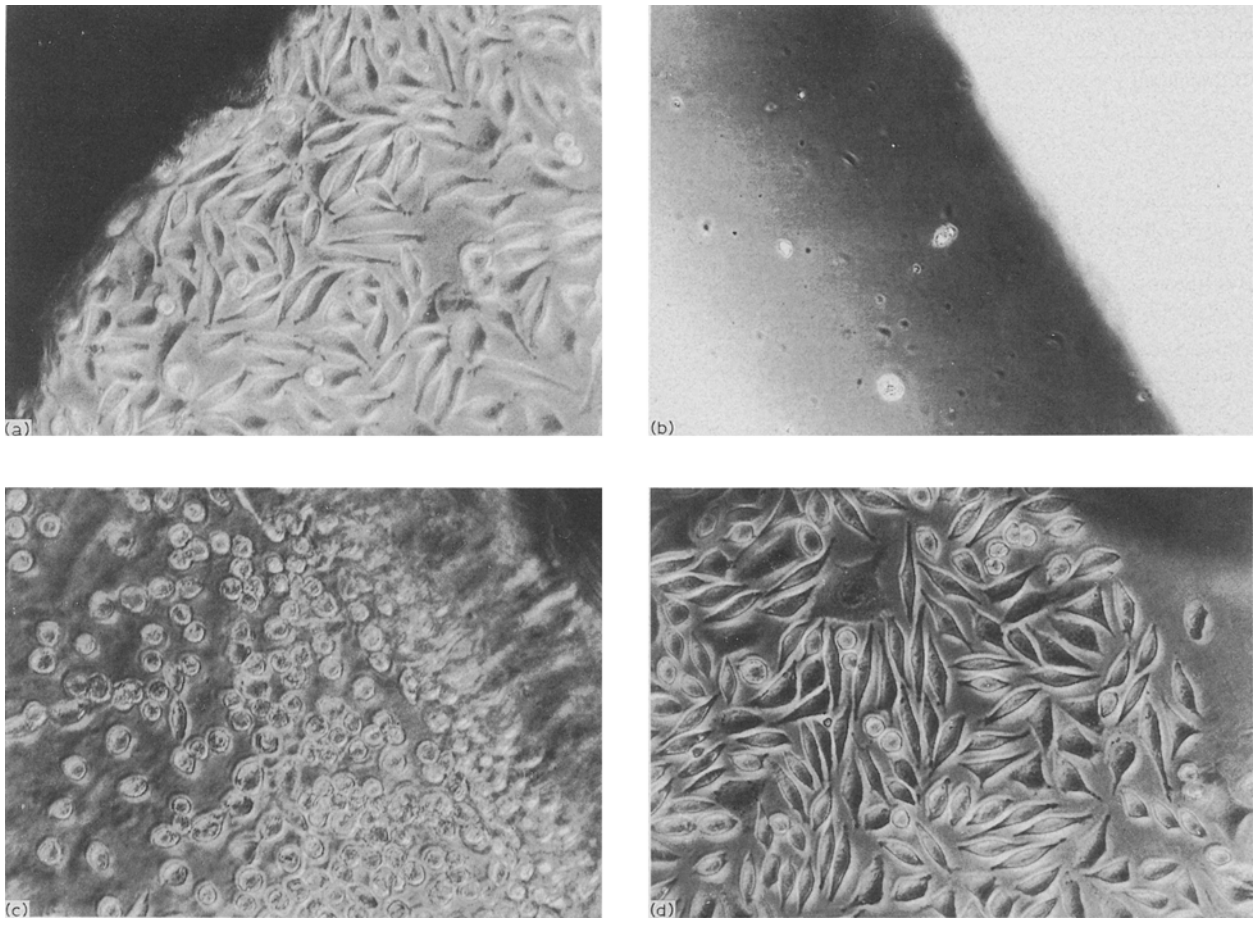

Figure 1 (a) Medical adhesive (negative control), normal healthy fibroblasts are seen migrating towards the interface and attaching along its edge. A cytocompatible response is observed (non-irradiated; × 135). (b) PVC disc (positive control), a severe growth inhibition of human fibroblasts is recorded. Only lysed dead cells are evident, showing a toxic response (non-irradiated; × 135). (c) Collagen/vicryl (style 12) composite membrane after 10 Mrad gamma-irradiation. A concentrated cell attachment is seen with L929 cells, showing a compatible response (× 135). (d) Collagen/vicryl (style 12) composite membrane that has been treated with 10 Mrad gamma-irradiation. Healthy fibroblast growth and attachment is observed across the polystyrene culture vessel surface immediately below the mesh (× 135).

3.2. Implantation into the lumbar muscles of laboratory rats

From the histology obtained it is evident that, following irradiation, both the collagen and vicryl components of the composite membrane were being absorbed more rapidly than those of the non-irradiated control material, whereas the ethylene oxide-sterilized sample showed a similar absorption profile to that of the control.

Fig. 2a–d shows control and gamma-sterilized material at 3 days. In the control sample the vicryl component can be seen as a row of vicryl filaments completely surrounded by collagen film. The material excited a polymorphic reaction with some fibrin and macrophages present. The ends of the collagen layer had started to "ribbon out", showing the start of the absorption process. With the irradiated material, however, the collagen coating the vicryl filaments could not be seen as a distinct layer, but was more diffuse and interspersed with fibrin, macrophages and neutrophils, although the last of these were not very numerous. There was also some cellular infiltration between the vicryl filaments by macrophages and neutrophils.

The difference between irradiated and non-irradiated material was also obvious at 7 days, when the absorption of the collagen was considerably more advanced for the gamma-sterilized material. Also, with the irradiated sample the vicryl filaments were becoming infiltrated by cells with evidence of absorption which was not seen at this time with the control sample.

At 14 days (data not shown) further infiltration between vicryl filaments by cells and on-going absorption of the vicryl component was seen with the irradiated material. The membrane appeared to be surrounded by granulation tissue with a population of macrophages, fibroblasts and some polymorphonuclear leucocytes. The collagen had almost disappeared in the case of the irradiated membrane at this stage, but was still visible as a diffuse layer in the control sample in which the vicryl component was also starting to become infiltrated by cells. At 21 days a pattern similar to that at 14 days could be seen with absorption progressing. Giant cells were notably present at this stage.

At 28 days (data not shown) both collagen and vicryl still remained in the control rats, although absorption was well under way. A capsule of collagen surrounded a cellular reaction zone. With the irradiated membrane at this time a thin collagen capsule

TABLE I Results of collagen/vicryl extraction test using mouse fibroblasts (results are expressed as % proliferation following 72 h growth in extraction medium)

Test material	4 day extraction			10 day extraction		
	Mean absorbance at 620 nm	Standard deviation (σ_{n-1})	Proliferation (%)	Mean absorbance at 620 nm	Standard deviation (σ_{n-1})	Proliferation (%)
Medical adhesive (negative)	1.825	0.023	100.0	1.780	0.027	100.0
PVC (positive)	1.259	0.041	69.0	0.231	0.035	13.0
L929 Mouse cells	1.798	0.028	98.5	1.800	0.032	101.0
Ethylene oxide						
Collagen film	1.742	0.047	95.4	1.655	0.041	95.2
V mesh, style 9	1.628	0.043	94.4	1.659	0.076	92.2
V mesh, style 12	1.742	0.040	95.4	1.623	0.039	91.2
C/V9	1.646	0.038	91.4	1.623	0.087	91.2
C/V12	1.834	0.013	100.5	1.534	0.012	86.2
Gamma-irradiated						
Coll. (control)	1.816	0.086	99.5	1.658	0.025	93.2
Coll., 2.5 Mrad	1.945	0.015	106.6	1.641	0.036	92.2
Coll., 5.0 Mrad	2.000	0.027	109.6	1.587	0.029	89.2
Coll., 7.5 Mrad	2.019	0.020	110.7	1.552	0.018	87.2
Coll., 10.0 Mrad	1.927	0.024	105.6	1.569	0.016	88.2
V mesh 9 (control)	1.927	0.024	109.6	1.641	0.032	92.2
V mesh 9, 2.5 Mrad	1.853	0.034	101.5	1.729	0.017	97.2
V mesh 9, 5.0 Mrad	1.927	0.040	105.6	1.784	0.056	100.2
V mesh 9, 7.5 Mrad	1.779	0.040	97.5	1.748	0.025	98.2
V mesh 9, 10.0 Mrad	1.908	0.070	104.6	1.784	0.027	100.2
V mesh 12 (control)	1.779	0.036	97.5	1.748	0.007	98.2
V mesh 12, 2.5 Mrad	1.908	0.011	104.6	1.729	0.019	97.2
V mesh 12, 5.0 Mrad	1.964	0.011	107.6	1.712	0.011	96.2
V mesh 12, 7.5 Mrad	1.889	0.004	103.6	1.676	0.015	94.2
V mesh 12, 10.0 Mrad	1.927	0.002	105.6	1.480	0.002	83.2
C/V9 (control)	1.834	0.017	100.5	1.534	0.033	86.2
C/V9, 2.5 Mrad	1.889	0.006	103.6	1.569	0.020	88.2
C/V9, 5.0 Mrad	1.705	0.049	93.4	1.587	0.040	89.2
C/V9, 7.5 Mrad	1.853	0.002	101.5	1.676	0.014	94.2
C/V9, 10.0 Mrad	1.797	0.009	98.5	1.480	0.002	83.2
C/V12 (control)	1.778	0.011	97.5	1.729	0.003	97.2
C/V12, 2.5 Mrad	1.778	0.011	97.5	1.748	0.003	98.2
C/V12, 5.0 Mrad	1.778	0.048	97.5	1.712	0.006	96.2
C/V12, 7.5 Mrad	1.742	0.032	95.4	1.760	0.011	99.2
C/V12, 10.0 Mrad	1.760	0.030	96.5	1.694	0.002	95.2

Calculated values are expressed as a percentage of the negative control following a 4 and 10 day extraction period. Each value represents the mean of 16 determinations. PVC, poly(vinyl chloride), positive control disc; V mesh, vicryl mesh; Coll., collagen; C/V9, collagen-coated mesh, style 9; C/V12, collagen coated mesh, style 12.

surrounded a zone of macrophages, giant cells and fibroblasts with absorbing vicryl filaments. Some collagen deposition was noted around the remaining pockets of vicryl.

At 35 days (Fig. 2e–h) all that could be seen of the irradiated membrane was variable-sized residues of vicryl filaments at different stages of absorption surrounded mainly by macrophages in a thin collagen capsule. The vicryl had almost disappeared at 49 days and there was no trace of irradiated material at 70 days. The control sample at 35 days still showed evidence of implant collagen cells with vicryl filaments noticeably absorbing (Fig. 2). At 49 days some sections showed completely absorbed control material, leaving only hypercellular areas to mark the implant site. In other sections, however, both collagen coating and vicryl filaments could be seen surrounded by macrophages and giant cells in a host–collagen capsule. By 70 days, as for the irradiated sample, all traces of the implant had disappeared.

3.3. Incubation of test materials in PBS

Figs 3 and 4 show the effect of 2.5, 5.0, 7.5 and 10.0 Mrad gamma-irradiation on style 9 vicryl mesh, and on collagen-coated style 9 vicryl mesh. Similar results (not shown) were obtained for the style 12 material. From the results obtained, it can be seen that most damage to the material occurs with the first 2.5 Mrad irradiation. Subsequent doses of irradiation still produced a destructive, although less dramatic, effect.

3.4. Viscometry of vicryl mesh

Fig. 5 shows the effect of increasing doses of irradiation on style 9 vicryl mesh. The results show a

TABLE II Results of collagen/vicryl extraction test using human fibroblasts (results are expressed as % proliferation following 72 h growth in extraction medium)

Test material	4 day extraction			10 day extraction		
	Mean absorbance at 620 nm	Standard deviation, σ_{n-1}	Mean proliferation at 620 nm (%)	Mean absorbance at 620 nm	Standard deviation, σ_{n-1}	Mean proliferation at 620 nm (%)
PE (negative)	0.387	0.010	100.0	0.396	0.021	100.0
PVC (positive)	0.175	0.110	45.2	0.109	0.187	27.7
Human fibroblasts	0.385	0.020	99.5	0.395	0.025	99.8
Ethylene oxide						
Collagen film	0.351	0.011	90.8	0.369	0.024	93.2
V mesh, style 9	0.350	0.013	90.6	0.358	0.026	90.6
V mesh, style 12	0.367	0.020	94.9	0.357	0.014	90.1
C/V9	0.382	0.027	98.8	0.360	0.035	91.0
C/V12	0.398	0.025	102.8	0.360	0.029	91.0
Gamma-irradiated						
Coll. (control)	0.356	0.009	91.9	0.400	0.023	101.0
Coll., 2.5 Mrad	0.344	0.006	89.0	0.377	0.026	95.2
V mesh 9 (control)	0.344	0.016	89.0	0.341	0.025	86.1
V mesh 9, 2.5 Mrad	0.343	0.019	88.5	0.336	0.017	84.7
V mesh 9, 10.0 Mrad	0.331	0.013	85.6	0.344	0.015	86.9
V mesh 12 (control)	0.359	0.017	93.0	0.390	0.017	98.5
V mesh 12, 2.5 Mrad	0.349	0.040	90.3	0.388	0.025	97.9
C/V9 (control)	0.359	0.010	92.7	0.402	0.017	101.5
C/V9, 2.5 Mrad	0.387	0.060	100.0	0.414	0.019	104.8
C/V9, 10.0 Mrad	0.366	0.018	94.6	0.408	0.021	103.3
C/V12 (control)	0.352	0.012	90.9	0.353	0.021	88.1
C/V12, 2.5 Mrad	0.338	0.007	87.3	0.359	0.027	90.8

Calculated values are expressed as a percentage of the polyethylene (PE) negative control following a 4 and 10 day extraction period. Each value represents the mean of 16 determinations. PVC, poly(vinyl chloride), positive control disc; V mesh, vicryl mesh; C/V9, collagen-coated mesh, style 9; C/V12, collagen-coated mesh, style 12.

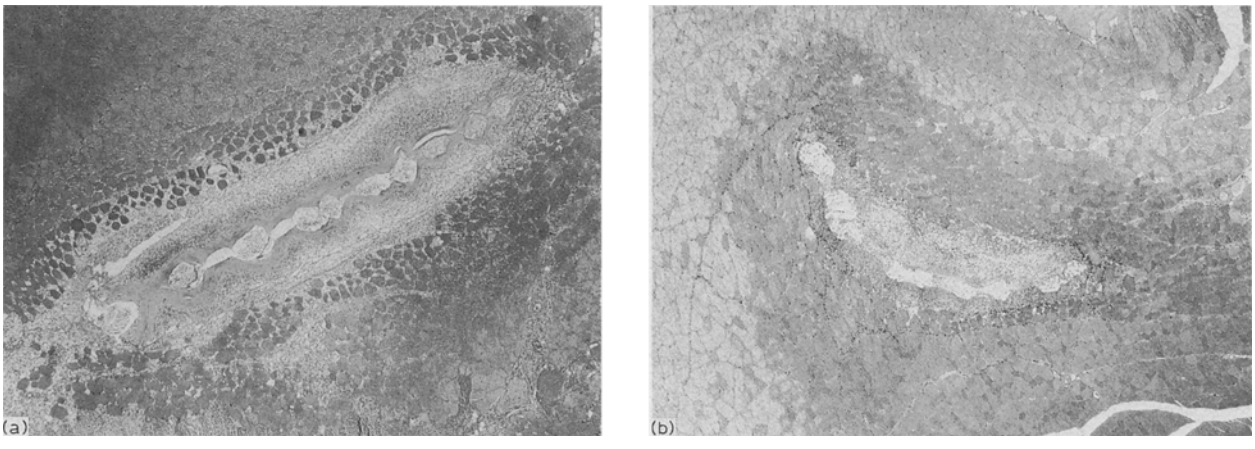

Figure 2 (a) Non-irradiated collagen-coated vicryl mesh (style 9), 3 days post-implantation. Both collagen film and vicryl components are still intact. The material has excited a polymorphic reaction, with some fibrin and macrophages present. The ends of the collagen layer have started to "ribbon out", showing the start of the absorption process (HPS trichrome, × 4.5). (b) Collagen-coated vicryl mesh (style 9) subjected to 2.5 Mrad ^{60}Co irradiation, 3 days post-implantation. The collagen coating the vicryl filaments cannot be seen as a distinct layer, but is more diffuse and interspersed with fibrin, macrophages and neutrophils. There is also some cellular infiltration between the vicryl filaments by macrophages and neutrophils. (one pass; HPS trichrome, × 4.5). (c) Non-irradiated collagen/vicryl at 3 days post-implantation, showing a higher magnification of a section of (a). The end of the collagen layer is separating into thin laminae, infiltrated by both polymorphonuclear cells and mononuclear types (HPS trichrome, × 45). (d) Irradiated collagen/vicryl at 3 days post-implantation showing a higher magnification of a section of (b). The collagen is generally infiltrated by a mixed population of cells including a number of polymorphonuclear leucocytes. The degree of cellular infiltration is much greater than that shown for the non-irradiated product at the same time (HPS trichrome, × 22.5). (e) Non-irradiated collagen-coated vicryl mesh after 35 days implantation. Although somewhat degraded, the collagen layer can still be clearly seen (HPS trichrome, × 11). (f) Non-irradiated collagen-coated vicryl mesh after 35 days implantation, showing a higher magnification of a section of (e). The space within the vicryl filament bundle has been infiltrated by fibroblasts and mononuclear cells, probably macrophages. A similar population surrounds the outer margin of the remaining intact collagen layer (HPS trichrome, × 45). (g) 2.5 Mrad irradiated collagen-coated vicryl mesh at 35 days post-implantation. There is nothing that can be identified with certainty as the original collagen layer, and many of the vicryl filaments are losing their individual identities (HPS trichrome, × 11). (h) Irradiated collagen-coated vicryl mesh, showing a higher magnification of a section of (g). The layer peripheral to the filaments of vicryl that remain is densely populated with mononuclear cells, but no recognizable remnants of the collagen layer that occupied this area can be seen (HPS trichrome, × 45).

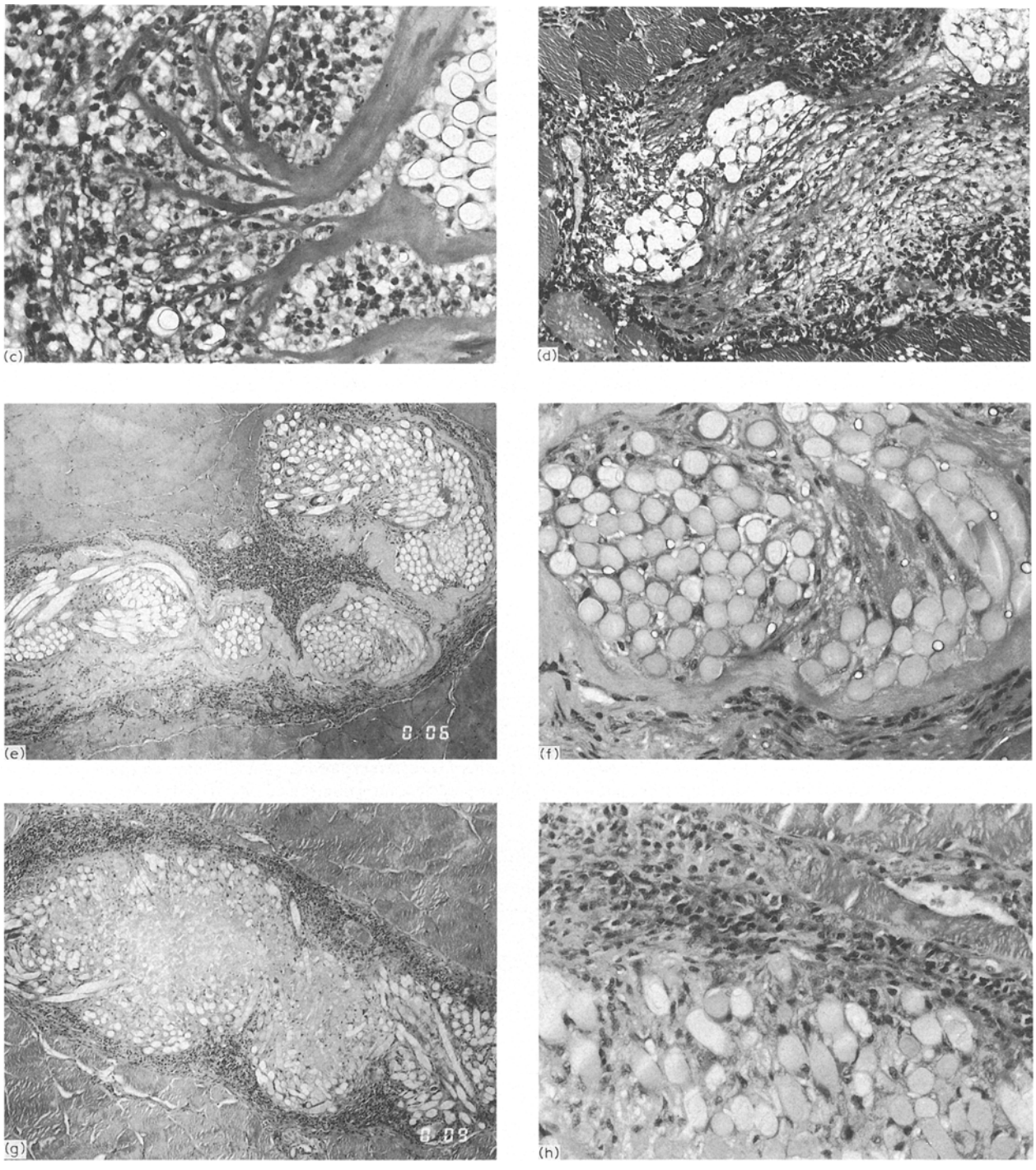

Figure 2c–h Continued

decrease in viscosity (and hence molecular weight) following irradiation.

3.5. Tensile strength testing of collagen film

Table III shows the effect of increasing doses of gamma-irradiation on both the tensile strength and the percentage elongation of the material at breaking of dry irradiated collagen film, and a dose-dependent decrease in both parameters can be observed. Ethylene oxide (Table III) also produced some decrease in the tensile strength and elasticity of the collagen film, which was shown to be significant using Student's *t*-test for unpaired values. As shown in Table III, although the differences observed at 2.5 Mrad gamma-irradiation were not significant, a significant difference was observed at the higher doses.

4. Discussion

Both *in vitro* and *in vivo* results clearly show that increasing doses of gamma-irradiation cause increasing damage to the collagen and vicryl components of the composite membrane. Ethylene oxide, however, appears to have relatively little effect.

The effects of irradiation on collagen have been the subject of several reports [37–42]. The effect of irradiation is dependent, in particular, on the water content of the specimen [37–39]. In an early investigation [37] it was shown that strips of ox-hide when

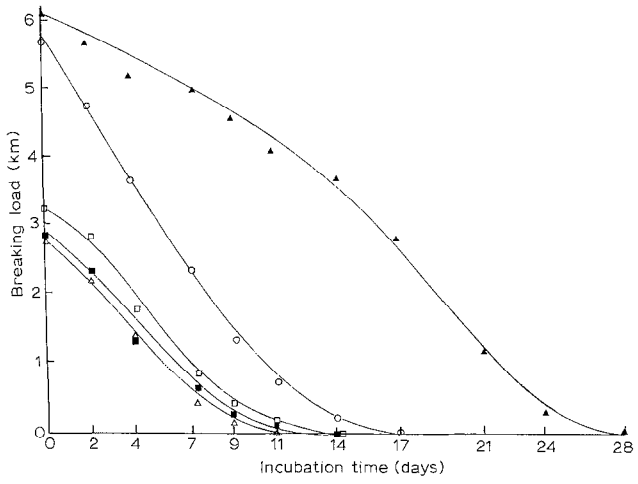

Figure 3 Effect of ^{60}Co irradiation on the rate of solubilization of style 9 vicryl mesh at 37 °C in PBS. (▲) Control, non-irradiated, (○) 2.5 Mrad, (□) 5 Mrad, (■) 7.5 Mrad and (△) 10 Mrad.

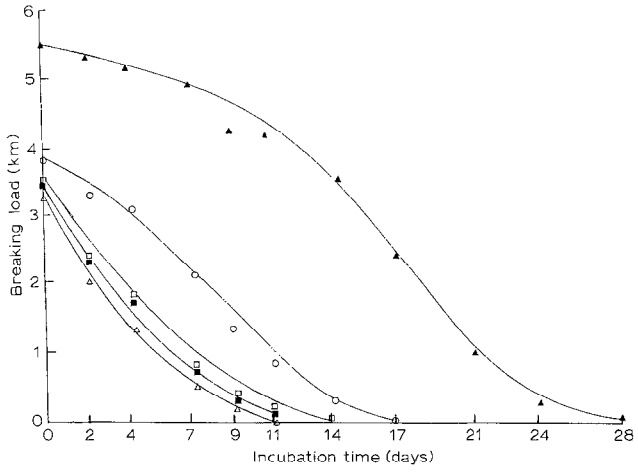

Figure 4 Effect of ^{60}Co irradiation on the rate of solubilization of collagen-coated vicryl mesh (style 9) at 37 °C in PBS. (▲) Control, non-irradiated, (○) 2.5 Mrad, (□) 5 Mrad, (■) 7.5 Mrad and (△) 10 Mrad.

irradiated at 5 and 50 Mrad levels retained 68 and 35% of their original strength at 18% moisture level, respectively. At 50 Mrad samples irradiated in the "dry" state (5% moisture) underwent a considerable increase in solubility, whereas relatively little effect was seen in the "wet" state (80% moisture). The shrinkage temperature of the dry-irradiated ox-hide could not be measured as the sample almost immediately disintegrated upon contact with water at 25 °C, whereas the wet-irradiated material did not appear to shrink even in boiling water. Upon irradiation in air at 18% R.H. the shrinkage temperature decreased by nearly 20 °C at 5 Mrad, and at 50 Mrad it was shown by those workers [37] that the collagen disintegrated and dissolved at 26 °C. At 59 Mrad and at 5 and 80% moisture, loss of crystallinity as shown by X-ray diffraction was observed, particularly at the high moisture level.

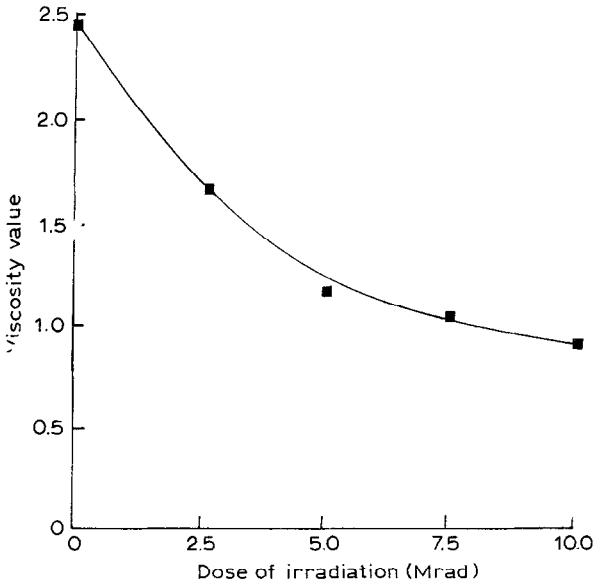

Figure 5 Effect of gamma-irradiation on the viscosity of style 9 vicryl mesh.

In another study [38] it was shown that doses of irradiation in excess of 10 Mrad caused a gradual destruction of the fibril banding pattern which finally disappeared at 40 Mrad. The effects of irradiation on the fibrils were compared with those of other physical and chemical treatments such as hydrogen-bond breaking (urea) and chemical cross-linking agents (formaldehyde). This comparison indicated that the loss of banding pattern was due primarily to swelling of the fibrils, resulting from disorganization of the collagen macromolecule and the subsequent ingress of water. Evidence was obtained [38] that intermolecular cross-links were produced when the fibres were irradiated in the presence of water. In contrast, irradiation in the dry state resulted in fragmentation of the macromolecule.

TABLE III Break load and elongation of gamma-irradiated and ethylene oxide-sterilized collagen films

Sample	Break load, mean	Standard deviation, σ_{n-1}	*P*-value	Elongation, mean (%)	Standard deviation, σ_{n-1}	*P*-value
Control film	399.5	63.5		87.1	7.8	
2.5 Mrad	356.1	82.3	0.199	83.7	13.8	5.08
5.0 Mrad	206.4	53.7	0.001	56.5	9.6	0.00
7.5 Mrad	178.2	60.4	0.00	52.5	13.7	0.00
10.0 Mrad	135.0	45.3	0.00	50.3	12.1	0.00
Ethylene oxide-sterilized	314.6	97.3	0.0196	104.0	19.7	0.0196

Each value represents the mean of 10–12 measurements. Standard deviations are shown as well as the *P*-values obtained from Student's *t*-test for unpaired variables, comparing each treatment to the control film. A radiation dose-dependent reduction of tensile strength and percentage elongation to breaking is indicated. Both parameters have also been somewhat reduced by sterilization with ethylene oxide.

The effect of irradiation on collagen was reviewed by Bailey and Tromans [39]. One of the most important factors that influenced the fate of collagen following irradiation was the effect of moisture. In the wet state a cross-linking reaction was the most predominant feature. The degree of cross-linking obtained was dose-dependent and resulted in an increase in the shrinkage temperature and decrease in the solubility of the collagen. Conversely, when collagen was irradiated in the dry state, scission of the peptide alpha-chains predominated, resulting in a decrease or loss of the shrinkage temperature and an increase in the solubility. Also, upon irradiation a loss of banding pattern, particularly in the wet state, was observed.

These reports are consistent with our findings. The collagen–vicryl composite membrane, because of the water-sensitive nature of the vicryl, was irradiated in the dry state. Our results showed a decrease in the breaking strength of the collagen film and an increase in its rate of absorption following implantation *in vivo*, both being indicative of damage to the collagen, resulting from gamma-irradiation.

More recently, Liu *et al.* [41] investigated the effects of gamma-irradiation on pepsin-extracted injectable human amnion collagen. Material was subjected to 0.25–2.5 Mrad gamma-irradiation in PBS at ambient temperature. The solubility in PBS at 75 °C was reduced from 100% for the non-irradiated material to 16% for the 2.5 Mrad irradiated sample. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis demonstrated also that chain scission was occurring with the presence of peptide fragments smaller than native alpha-chains. Irradiation caused a slight increase in the resistance to bacterial collagenase, whereas it became more sensitive to the action of trypsin, the latter being dose-dependent. An increase in the fibril diameter was observed by electron microscopy following irradiation, but no destruction of the banding pattern was observed at 2.5 Mrad. No differences in the amino-acid composition were noted under the conditions of the experiments. The authors reported that little damage had been caused to the collagen.

Cheung *et al.* [42], however, reported that doses of gamma-irradiation in excess of 1 Mrad can damage collagen, both untreated and chemically cross-linked. They showed an increased resistance to digestion by pronase following irradiation, whereas sodium dodecyl sulphate–polyacrylamide gel electrophoresis showed a dose-dependent proportion of lower-molecular weight components, indicating scission had occurred of the alpha-chains. However, gel filtration also revealed the presence of some higher-molecular weight component, showing a cross-linking effect of irradiation.

The effect of ethylene oxide on collagen is less well understood, although it does react with lysine and hydroxylysine residues to some extent [1]. Consistency of treatment by this method is therefore important.

Ethylene oxide is regularly employed in the sterilization of vicryl [43]. However, gamma-irradiation produces a dose-dependent chain scission of this polyester. Indeed, controlled levels of irradiation (7.5 Mrad) have recently been used to produce a rapidly absorbing suture material from vicryl threads (Vicryl-Rapide; Ethnor SA, Paris).

The results reported in this study clearly show that there is no cytotoxic effect, even upon prolonged extraction of the composite membrane or its individual collagen and vicryl components, towards either mouse L929 cells or a primary human fibroblast cell line, either with ethylene oxide sterilization or up to 10 Mrad gamma-irradiation.

Furthermore, following implantation into the lumbar muscle of rats, no adverse tissue reaction was observed with ethylene oxide-treated material or with membrane sterilized with 2.5 Mrad gamma-irradiation. However, although the rates of biodegradation of both collagen and vicryl were severely affected by 2.5 Mrad gamma-irradiation *in vivo* (higher doses of irradiation were not employed in this particular experiment), ethylene oxide produced little effect on the absorption time of the membrane. From these studies it may therefore be concluded that, although both methods of sterilization appear to be "safe", in that no obvious cytotoxic effects or adverse tissue reaction occur, the absorption time is considerably shortened by exposure to radiation. This finding has since been confirmed in a clinical investigation [29].

Three *in vitro* experiments clearly demonstrate the effect of irradiation of the materials under the conditions employed. The rate of hydrolysis of the vicryl mesh at 37 °C in PBS was severely affected by 2.5 Mrad gamma-irradiation. Increasing doses of irradiation still produced an increasing but proportionally smaller effect. In this particular experiment little difference was observed between the coated and uncoated meshes. Although the vicryl mesh is by far the stronger component [30], this result is predictable but does indicate that the collagen has had little or no protective effect on the vicryl during the irradiation process. The chain scission effect of increasing doses of irradiation on vicryl mesh could also be seen in the viscometric experiments in which, again, irradiation caused a dose-dependent decrease in the viscosity of vicryl solutions. These latter results are compatible with those obtained showing a decrease in the tensile strength during prolonged incubation in PBS at 37 °C.

Physical testing of collagen film following irradiation (Table III) showed a dose-dependent decrease in both the tensile strength and the percentage elongation before breaking of the material. A decrease in the latter parameter would be consistent with some cross-linking occurring even though peptide bond scission and degradation of the collagen is predominant following irradiation in the dry state [37, 39], as shown by a decrease in the tensile strength of the collagen and by its higher rate of absorption *in vivo*. This latter finding was also demonstrated by Bird *et al.* [28] in a rabbit ear-chamber model. Ethylene oxide (Table III) did produce some reduction in the tensile strength or elasticity of the collagen but, unlike irradiation, had little effect on the rate of absorption *in vivo* in the

lumbar muscles of rats. Again, this is consistent with the ear-chamber findings of Bird *et al.* [28].

In conclusion, the results presented here show that gamma-irradiation doses of 2.5 Mrad or more produce a degradative effect on both the collagen and vicryl membrane, resulting in an increased rate of absorption *in vivo*, whereas ethylene oxide has little effect. Neither method of sterilization produced any cytotoxic or tissue reaction over what was found with the control components. Hence, from our findings both irradiation and ethylene oxide appear to be safe methods that can be used to sterilize the composite membrane or its individual components. However, gamma-irradiation, although more convenient, does lead to accelerated absorption *in vitro* and *in vivo*.

References

1. M. CHVAPIL, R. L. KRONENTHAL and W. VAN WINKLE, JR, in "International Review of Connective Tissue Research", Vol. 6, edited by D. A. Hall and D. S. Jackson (Academic Press, London, 1973) 1, p. 1.
2. A. HUC, *J. Amer. Leather Chem. Assoc.* **80** (1985) 105.
3. P. J. KLOPPER, *Eur. Surg. Res.* **18** (1986) 218.
4. B. B. PHARISS, *J. Amer. Leather Chem. Assoc.* **75** (1980) 474.
5. F. DeLUSTRO, S. T. SMITH, J. SUNDSMO, G. SALEM, S. KINCAID and L. ELLINGSWORTH, *Plast. Reconstruct. Surg.* **79** (1987) 581.
6. A. M. KLIGMAN and R. C. ARMSTRONG, *J. Dermatol. Surg. Oncol.* **12** (1986) 351.
7. M. D. STEIN, L. M. SALKIN, A. L. FREEDMAN and V. GLUSHKO, *J. Periodont.* **56** (1985) 35.
8. J. H. C. VORMOOLEN, J. RINGERS, G. T. A. M. BOTS, A. VAN DER HEIDE and J. HERMANS, *Neurosurgery* **20** (1987) 701.
9. M. CHVAPIL, *J. Biomed. Mater. Res.* **16** (1982) 245.
10. I. V. YANNAS and J. F. BURKE, *ibid.* **14** (1980) 65.
11. B. A. PRUITT and N. S. LEVINE, *Arch. Surg.* **119** (1984) 312.
12. R. T. J. HOLL-ALLEN, *J. R. Coll. Surg. Edinb.* **29** (1984) 151.
13. *Idem, ibid.* **29** (1984) 154.
14. T. ISHIHARA, V. J. FERANS, M. JONES, S. W. BOYCE and W. C. ROBERTS, *J. Thorac. Cardiovasc. Surg.* **81** (1981) 747.
15. P. MENASCHE, P. FLAUD, A. HUC and A. PIWNICA, *Lift Support Syst.* **2** (1984) 233.
16. C. P. WEINBERG and E. BELL, *Science* **231** (1986) 397.
17. J. J. REIDY, B. M. GEBHARDT and H. E. KAUFMAN, *Cornea* **93** (1990) 196.
18. H. S. GEGGEL, J. FRIEND and R. A. THOFT, *Invest. Ophtholmol. Vis. Sci.* **26** (1985) 901.
19. W. COLIN and R. B. DONOFF, *J. Dent. Res.* **63** (1984) 987.
20. R. MOHAMMED, M. J. MONSOUR, S. D. GORHAM, D. A. FRENCH and R. SCOTT, *Urol. Res.* **15** (1987) 239.
21. M. J. MONSOUR, R. MOHAMMED, S. D. GORHAM, D. A. FRENCH and R. SCOTT, *ibid.* **15** (1987) 235.
22. R. SCOTT, R. MOHAMMED, S. D. GORHAM, D. A. FRENCH, M. J. MONSOUR, A. SHIVAS and T. HYLAND, *Brit. J. Urol.* **62** (1988) 26.
23. D. GOSSOT, M. CELERIER and E. SARFATI, *Presse Méd.* **17** (1988) 30.
24. R. CARACHI, A. AZMY, S. GORHAM, J. REID and D. A. FRENCH, *Brit. J. Surg.* **76** (1989) 496.
25. J. M. SIKSIK, P. H. CUGNENC, P. FRILEUX, P. GALLIX, D. CALISE and M. ARSCA, *Ann. Chir.* **43** (1984) 236.
26. G. AUDRY, C. GRAPIN, A. BELLOVARD and M. GRUNER, *Chir. Rediatr.* **34** (1990) 47.
27. D. A. FRENCH, Private communication.
28. I. N. BIRD, C. A. SILVER, S. D. GORHAM and D. A. FRENCH, *J. Mater. Sci. Mater. Med.* **21** (1991) 36.
29. R. SCOTT, S. D. GORHAM, M. AITCHESON, S. P. BRAMWELL, M. J. SPEAKMAN and R. N. NEDDNGS, *Brit. J. Urol.* **68** (1991) 421.
30. S. D. GORHAM, M. J. MONSOUR and R. SCOTT, *Urol. Res.* **15** (1987) 53.
31. S. D. GORHAM, T. P. HYLAND, D. A. FRENCH and M. J. WILLINS, *Biomaterials* **11** (1990) 113.
32. S. SRIVASTAVA, S. D. GORHAM and J. M. COURTNEY, *ibid.* **11** (1990) 133.
33. M. H. OLIVER, N. K. HARRISON, J. E. BISHOP, P. J. COLE and G. J. LAURENT, *J. Cell Sci.* **92** (1989) 513.
34. B. PELLETIER, F. DHAINAUT, A. PAULY and J.-P. ZAHND, *J. Biochem. Biophys. Methods* **16** (1988) 63.
35. S. SRIVASTAVA, S. D. GORHAM, D. A. FRENCH, A. A. SHIVAS and J. M. COURTNEY, *Biomaterials* **11** (1990) 162.
36. S. SRIVASTAVA, PhD thesis, Bioengineering Unit, University of Strathclyde (1988).
37. J. H. BOWES and J. A. MOSS, *Radiat. Res.* **16** (1962) 211.
38. A. J. BAILEY and W. J. TROMANS, *ibid.* **23** (1964) 145.
39. A. J. BAILEY, in "International Review of Connective Tissue Research", Vol. 4, edited by D. A. Hall and D. S. Jackson (Academic Press, London, 1968) p. 233.
40. G. G. MILLER, J. M. KENNING and D. T. DAWSON, *Radiat. Res.* **115** (1988) 515.
41. B. LIU, R. HARRELL, R. H. DAVIS, M. H. DRESDEN and M. SPIRA, *J. Biomed. Mater. Res.* **23** (1989) 833.
42. D. T. CHEUNG, N. PERELMAN, D. TONG and M. E. NIMNI, *ibid.* **24** (1990) 581.
43. P. H. CRAIG, J. A. WILLIAMS, K. W. DAVIS, A. D. MAGOUN, A. J. LEVY, S. BOGDANSKY and J. P. JONES, *Surg. Gynecol. Obstet.* **41** (1975) 1.

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