ELSEVIER

Biomaterials 23 (2002) 2855–2861

Biomaterials

www.elsevier.com/locate/biomaterials

Impact of sterilization on the porous design and cell behavior in collagen sponges prepared for tissue engineering

Ernst Magnus Noah[a,*], Jingsong Chen[a], Xiangyang Jiao[a], Ingo Heschel[b], Nobert Pallua[a]

[a] *Department of Plastic, Hand-, and Burn Surgery, Technology University of Aachen, Pauwelsstr. 30, 52074 Aachen, Germany*
[b] *Helmholtz Institute for Biomedical Engineering, Technology University of Aachen, Aachen, Germany*

Received 31 May 2001

Abstract

This study investigates the impact of different sterilization processes on structural integrity and stability of collagen sponges designed for tissue engineering. Collagen sponges with uniform pore size (20 μm) were sterilized either with ethylene oxide (EO) or gamma irradiation (2.5 Mrad). Gamma-sterilized sponges showed a dramatic decrease of resistance against enzyme degradation and severe shrinkage after cell seeding. Collapsed porosity inhibited fibroblasts and barred completely the human umbilical vein endothelial cell ingrowth into the sponges. On the contrary, the porous structure and stability of EO-sterilized sponges remained almost unaltered. Fibroblasts and endothelial cells exhibited favorable proliferation and migration within sponges with normal morphology. Tubular formation by seeded endothelial cells occurred early in the first week. Therefore, we emphasize that the impact of sterilization of biomaterials is substantial and any new procedure has to be evaluated by correlating the impact of the procedure on the porous structure with cell proliferation behavior.

Keywords: Collagen sponges; Sterilization; Ethylene oxide; Gamma irradiation; Pore structure; Cell proliferation

1. Introduction

Collagen or collagen-based matrix is the most commonly used biomaterial in skin, connective tissue and peripheral nerve tissue engineering [1–3]. Besides its physiological properties as a natural extracellular matrix component, the microstructure and stability also play a crucial role in cell–cell and cell–matrix interaction. Collagen is available in gels, films or sheets and sponges. Collagen sponges differ in their porosity and stability. It is now well recognized that only a definite microstructure is suitable for each tissue to be engineered [4]. The appropriate porous structure maintains the shape and geometry of seeded cells, warrants a proper cell density and cell–cell contact, which are prerequisites for cells to function [4,5]. Meanwhile, as an active scaffold, biomaterials should be biodegradable to obviate long-term interference, but at a controlled rate to allow a prompt tissue regeneration [6].

Previously, a technical procedure was introduced to develop collagen sponges with a homogenous pore structure, which provided a tool to investigate the effect of porosity on cell behavior in collagen sponges [7]. The present study aims to identify an appropriate sterilization process for these special collagen sponges to achieve optimal maintenance of the structural integrity and stability.

Routinely used sterilization process for medical products, e.g. high pressure steam (autoclaving) and dry heat cannot be considered for heat- and water-sensitive biomaterials like collagen sponges. Currently the most widely utilized methods for collagen sponge sterilization are ethylene oxide (EO) gas infiltration and gamma irradiation. Earlier investigations focused on physical or chemical alterations after these two methods [3,8]. EO was claimed to alter the mechanical and physical properties of collagen slightly, but with a high risk of toxic residues [3,8]. Gamma irradiation, once introduced as the simplest and most effective way of sterilization without toxic substances, breaks chemical bonds, decreases tensile strength and modulus, thus affecting the exposed material fundamentally [9,10]. However, few studies referred to the effect of

*Corresponding author. Tel.: +49-241-808-9741; fax: +49-241-888-8448.
E-mail address: emnoah@aol.com (E.M. Noah).

0142-9612/02/$ - see front matter
PII: S0142-9612(01)00412-4

sterilization on cell behavior and cell–matrix interaction, which are key points related to tissue engineering.

In this study, the alteration of porosity and stability of collagen sponges was evaluated by resistance against enzyme degradation after different sterilization processes. Furthermore, differences in cell migration, proliferation and differentiation were also examined histologically and immunohistologically using human dermis fibroblasts and human umbilical vein endothelial cells (HUVECs).

2. Materials and methods

2.1. Collagen sponges preparation

Collagen sponges were prepared according to a directional freeze-drying procedure to achieve sponges with a uniform porous microstructure [7,11]. The basic material is a collagen suspension isolated from bovine skin which contains 1.8 wt% of type I collagen (Dr. Suwelack Skin & Health Care AG, GmbH, Billerbeck, Germany). In order to adjust the pore size of the collagen sponge, 1.5 wt% acetic acid was added to the basic material resulting in pH = 2.8. The suspension was then directionally solidified under thermally constant freezing conditions succeeded by a freeze-drying process. Thus, collagen sponges with a uniform microstructure and a defined pore size of 20 μm were prepared for this study.

2.2. Sterilization

2.2.1. Ethylene oxide

After preparation, the sponges were packed. The units were sterilized by exposing to a 100% ethylene oxide atmosphere at a relative humidity of 70% for 8 h at 55°C (Griffith Microscience, Zoetermeer, Netherland, now part of IBA, Belgium). After sterilization, the samples are aerated with warm air flow (40°C) at atmospheric pressure for 120 h to remove residual ethylene oxide, and stored in indicator bags, sealed and shipped back. By gas chromatography, a residual profile was evaluated and a minimal load of 7.69 μg/g EO was determined after 5 days.

2.2.2. Gamma sterilization

Randomized samples were chosen for gamma irradiation and they were packed and sealed in indicator bags and exposed to a nominal dose of 25 kGy (2.5 Mrad) (Beta-Gamma Service, Wiel, Germany).

2.2.3. Control

Non-sterilized sponges, manufactured by the same technique served as a control in the enzyme degradation evaluation.

2.3. Resistance against enzyme degradation

To study the in vitro degradation, five sponges of the three experimental groups (EO, gamma sterilization and control) were exposed to three enzymes separately ($n = 15$ per enzyme). Collagen sponges, 8 mg weight, 1 cm × 1 cm × 0.25 cm) were treated, respectively, in 3 ml pepsin (5 mg/ml in water, Boehringer Mannheim, Mannheim, Germany), collagenase/dispase (0.1 U collagenase + 0.8 U dispase/ml, Boehringer Mannheim, Mannheim, Germany), collagenase (210 U/ml, Sigma, Germany).

Specimens were incubated at 37°C and observed continuously during incubation. The incubation period that resulted in complete disappearance of collagen sponges was considered to be related to the resistance of the materials to enzyme degradation.

2.4. Fibroblasts and endothelial cells seeding

Collagen sponges were cut into 1 cm × 1 cm × 0.25 cm pieces in six-well plates (Becton Dickinson European HQ, Erembodegem-Aalst, Belgium) for cell seeding. Primary fibroblasts isolated from human dermis were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies GmbH, Karlruhe, Germany), the medium supplemented with 10% fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). Endothelial cells isolated from human umbilical vein were cultured in 1:1 mixed Iscove's modified DMEM and Ham's F12 (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 20% human serum and antibiotics (the same as in fibroblast medium). Passaged fibroblasts and HUVECs were seeded onto collagen sponges at a cell density of 7×10^5 cells/sponge/well in 150 μl medium. 4 h after seeding, 1.5 ml medium was added to each well. The small volume seeding medium and delayed addition of culture media were to ensure complete seeding onto sponges instead of the well plate. Cells were cultured in an incubator at 37°C under humid atmosphere in 5% CO_2. Culture medium was changed every 3 days.

2.5. Cell proliferation assay

At 0, 3, 7, 14 and 21 days, fibroblast proliferation in sponges was tested with MTT cell proliferation assay kit (Boehringer Mannheim, Mannheim, Germany). 150 μl MTT (tetrazolium salts) solution (5 mg/ml) was added to each well and incubated for 4 h. MTT can be cleaved into a formazan dye only by metabolic active cells. After incubation, the sponge was moved to a new tube and the converted formazan crystals were solubilized in 3 ml 100% isopropyl alcohol in 0.1 N HCl. Solubilized formazan dye was quantified using an ELISA spectrophotometer reader at the measurement wavelength of

570 nm and the reference wavelength of 690 nm. The absorbency directly correlates with the metabolic activity and thus correlates with the number of living cells.

2.6. Histology and immunohistochemistry

At 7, 14 and 21 days after seeding, sponges were fixed in 70% ethanol and prepared for paraffin section. 8 μm thick slides of every specimen was stained with hematoxylin and eosin. 6 μm thick deparaffined sections of fibroblast-seeded sponges were rehydrated with PBS and incubated with mouse anti-human vimentin antibody (1:10 dilution) in a moist chamber for 1 h. Then the sections were rinsed with PBS three times and incubated with a second antibody-peroxidase conjugated goat anti-mouse IgG (1:400 dilution) for 45 min. After extensive washing, the sections were exposed to peroxidase substrate DAB for 2 min, then rinsed and counterstained with hematoxylin. In order to identify endothelial cells, first antibody rabbit anti-human-Factor VIII (1:200 dilution) and second antibody swine anti-rabbit IgG (1:50 dilution) were used instead (antibodies and peroxidase substrate: DAKO Diagnostika GmbH, Hamburg, Germany).

2.7. Scanning electron microscopy

Sponges seeded with fibroblasts were cultured for 3 weeks and subsequently assigned for scanning electron microscopic (SEM) examination. Specimens were fixed for 2 h in a mixture of 4% glutaraldehyde and 2% formaldehyde in phosphate buffer (pH = 7.4) at room temperature. After thorough rinsing with 0.175 M phosphate buffer, they were immersed in 2% osmium tetroxide buffered with 0.2 M phosphate for 2 h, then dehydrated in acetone, subjected to critical point drying, sputter-coated with gold palladium and studied at high magnification.

2.8. Shrinkage and porosity measurements

The perimeters of each collagen sponge were traced on a transparent film macroscopically every day after cell seeding, and the porous microstructure of sponges was observed on H&E stained sections. The surface area and relative porosity were quantified by using a computerized image analysis system (Quantimed 600S, Leica, Cambridge, England). Under a microscope connected to a video camera at a magnification of 100×, the percent surface occupied by pores in a definite area was determined quantitatively on each section. The pore size was determined by direct measurement. Five measurements were made of each specimen at each time point after cell seeding. Data of porous surface percentage were statistically compared using the student's *t*-test.

3. Results

3.1. Resistance against enzyme degradation

In comparison with the EO-sterilized sponges and the unsterilized sponges, the degradation time of the gamma-sterilized sponges was significantly accelerated by each of the three enzymes. Compared with the unsterilized sponges, EO sterilization prolonged the degradation. This finding was repeatable for all tested enzymes (Table 1).

3.2. Shrinkage and porosity measurements after cell seeding

The gamma-sterilized sponges showed severe shrinkage of the surface area after cell seeding, whereas EO-sterilized sponges remained of the same size after seeding (Fig. 1). Measurements revealed an immediate loss of surface area of 28% with the cell seeding of the gamma-sterilized sponges. By day 21, sponges of this group showed just 39 percent of their original surface area.

Quantitative porosity analysis exhibited significant differences between EO- and gamma-sterilized sponges at all time periods observed (Table 2). The porous-surface-area percentage in EO-sterilized sponges remained almost unchanged until day 21. The pore size became slightly variable but still in the range of 18–28 μm. In contrast, the pore size in gamma-sterilized sponges decreased to 7–16 μm.

This demonstrates that the dramatic shrinkage of the surface area is evident due to the instability of the porous structure of the sponges after gamma sterilization.

3.3. Cell proliferation

During the first 3 days after seeding, fibroblasts showed inhibited proliferation in sponges sterilized with both methods (Fig. 2). Further, fibroblasts recovered in EO sterilized sponges and even exceeded the seeding

Table 1
Collagen resistance against enzyme degradation[a]

Collagen sponges	Pepsin (5 mg/ml)	Collagenase/ dispase (0.1 U C + 0.8 U D/ml)	Collagenase (210 U/ml)
EO-sterilized	180 ± 13.7	730 ± 27.2	114 ± 9.3
Gamma-sterilized	73 ± 8.1[b]	310 ± 22.8[b]	45 ± 6.4[b]
Unsterilized	157 ± 15.3	660 ± 52.9	80 ± 10

[a] Data represent time in minutes for complete degradation of the collagen sponges in enzymes.
[b] $p < 0.001$ compared with EO-sterilized sponges.

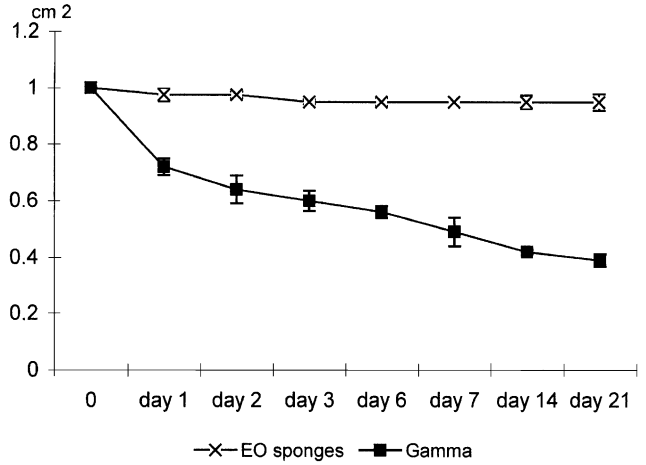

Fig. 1. Collagen surface area as a function of time after cell seeding; the data represent triplicated experiments.

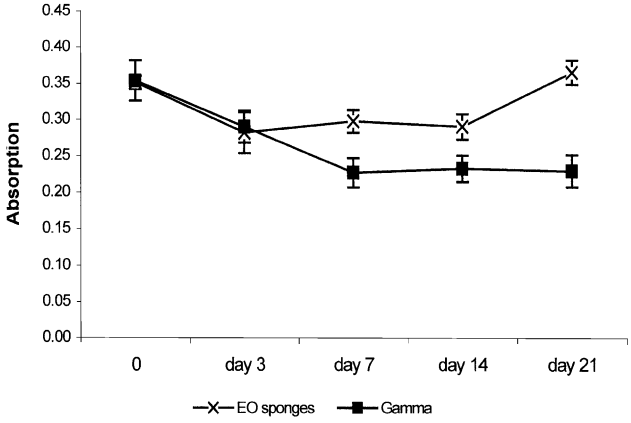

Fig. 2.

Table 2
Porosity of collagen sponges after cell seeding

Sponges	Surface		Percentage	Pore size (μm)
	Day 7	Day 14	Day 21	
EO-sterilized	77.74±3.49	79.17±4.01	70.92±3.36	18–28
Gamma-sterilized	55.94±3.33*	54.71±7.02[a]	39.18±2.57[a]	7–16

[a] $p<0.001$ compared with EO-sterilized sponges.

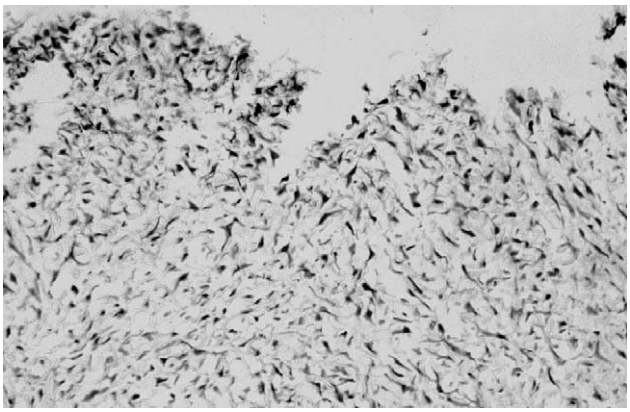

Fig. 3. 7 days after seeding, fibroblasts were found homogeneously in EO-sterilized collagen sponges; the cells maintained normal morphology; anti-vimentin staining, original magnification ×200.

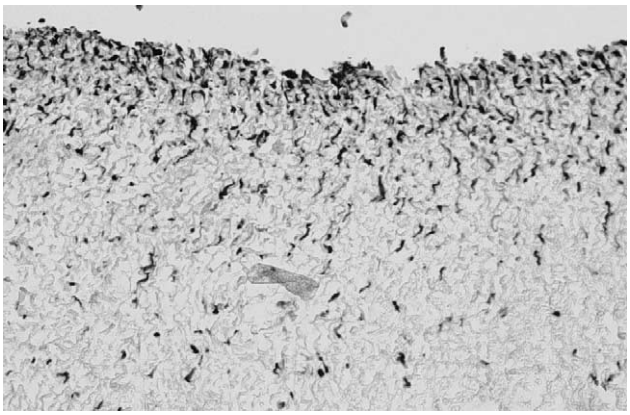

Fig. 4. 7 days after seeding, fibroblasts were hampered to migrate and densely populate the superficial layer of gamma sterilized sponges; the sponge was seen compacted and cells were twisted; anti-vimentin staining, original magnification ×200.

level at day 21. Fibroblasts seeded onto gamma-sterilized sponges decreased continuously until day 7 and then maintained a low level.

At day 7, on HE and anti-vementin stained sections, fibroblasts were found homogeneously in the upper half of the EO-sterilized sponges (Fig. 3). At day 14, fibroblasts were distributed throughout the entire sponge. In SEM, fibroblasts revealed a long spindle shape and were found spread over the pore surface. The microstructure of the sponges remained unaltered in comparison to unsterilized sponges (Fig. 5A).

In contrast, early at day 7, gamma-sterilized sponges were densely compressed with decreased pore size and twisted pore direction. The original microstructure was almost destroyed and not any more discernable. Fibroblasts were found densely populated on the surface of the sponges. The morphology was altered, and cells were shrunken and showed no extension (Fig. 4). Only a few cells reached the middle of sponges until day 14. 3 weeks after seeding, scattered fibroblasts were found under SEM twisted by thickened and compacted collagen fibers (Fig. 5B).

HUVECs, positively stained with anti-factor VIII antibody, were observed sparsely in the middle of EO-sterilized sponges at day 7 (Fig. 6), increased at day 14 and aggregated at day 21 to form lumen or tubular-like structures (Fig. 7). The tubular formation indicated the well differentiation of endothelial cells in collagen

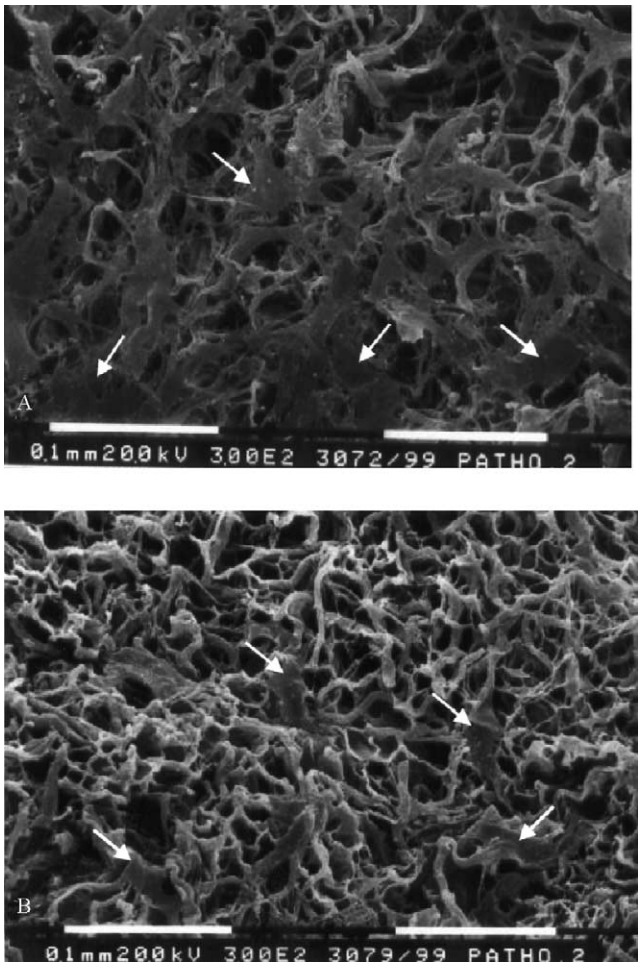

Fig. 5. At day 21 under SEM, fibroblasts (arrows) in EO-sterilized sponges: (A) with normal spindle morphology and in gamma sponges and (B) with twisted morphology; note the pore size and collagen fiber diameter; bar = 0.1 mm.

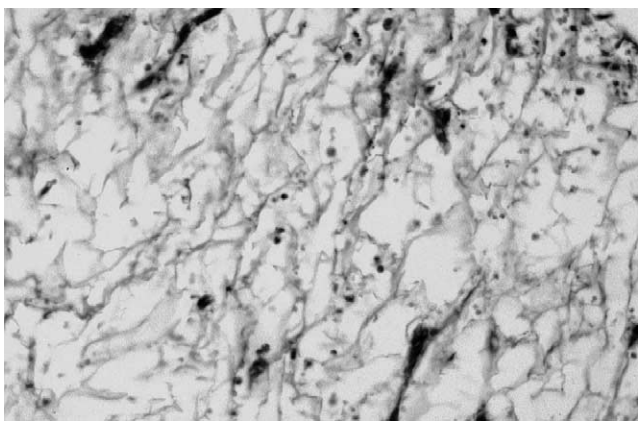

Fig. 6. At day 7, HUVECs scattered in the EO-sterilized sponges; anti-factor VIII staining, original magnification × 200.

matrix. In contrast, only a few cells were found on the seeding surface of gamma-sterilized sponges and almost no cell ingrowth occurred until day 21 after seeding (Fig. 8). Tubular structures were not seen.

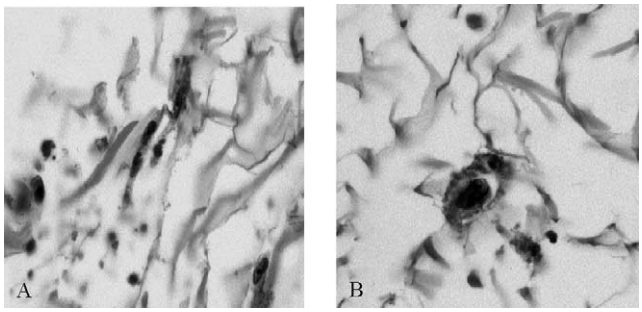

Fig. 7. At day 21, many tubular (A) or lumen-like (B) structures formed by HUVECs were found in EO-sterilized sponges; anti-factor VIII staining, original magnification × 400.

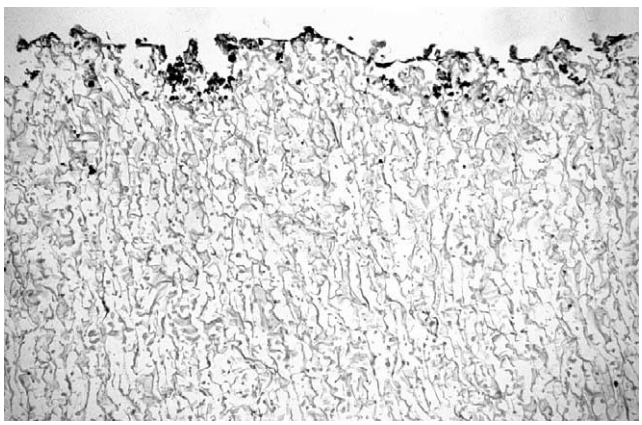

Fig. 8. At day 7, HUVECs were barred on the surface of gamma-sterilized sponges; no cell ingrowth occurred; anti-factor VIII staining, original magnification × 200.

4. Discussion

The present study focused on the possible alteration of porosity and stability of collagen sponges after EO or gamma sterilization with special emphasis on the influence on cell behavior in vitro.

Biomaterials designed for tissue engineering should be non-toxic, non-immunogenic and biologically able to facilitate cell proliferation and tissue regeneration. The biodegradation rate of biomaterials should be controlled to allow a continuous tissue reconstruction [6]. In addition, optimal materials should be porous with appropriate pore size suitable for cells to maintain their shape and function. In two-dimensional culture systems, a direct correlation of cell function and cell geometry was observed [12,13]. In three-dimensional sponge-like matrices with different pore sizes, seeded chondrocytes were also reported with different morphology, DNA synthesis and chondroitin sulphate production [4].

Collagen or collagen-based material is widely used for connective tissue engineering, because it is one of the major components of natural extracellular matrices. Among collagen-based materials, sponge-like matrices are most commonly exploited [14,15]. To study the

relationship between porous structure and function, it is necessary to develop collagen sponges with uniform porosity. However, various processing procedures with regard to solidification, cross-linking and sterilization, resulted in a variety of different physical, chemical and thus biological properties of the collagen. No objective evaluation was possible so far to compare the influencing factors [3].

A newly developed production process consisting of a directional solidification and freeze-drying was introduced previously [7,16,17]. Collagen suspension diluted with different concentration of acetic acid was frozen directionally at constant speed till $-80^{\circ}C$ and then freeze-dried into a sponge form. This kind of collagen sponge has a highly uniform pore size and direction.

In order to use collagen sponges, clinically appropriate sterilization of the material is necessary; anyhow, the mechanical properties of the material should not be altered. A variety of studies have shown that an unsuitable sterilization process can destroy the structure of collagen sponges, break chemical bonds and alter physical, chemical and biological properties substantially [3,8,10]. The most common methods for sterilization of collagen sponges are EO gas infiltration and gamma irradiation. EO was believed to alter the mechanical and physical properties of collagen slightly, but with a high risk of toxic residues [18,19]. A subsequent 2–3 weeks of aeration is crucial to reduce residual EO in order to comply with regulatory limit and to avoid cytotoxic effects [3,18,19]. In our series, we provided a residual evaluation of EO by gas chromatography in the sponges, which demonstrated a non-toxic level of EO after 5 days of degassing at $40^{\circ}C$ warm air.

Gamma irradiation was once introduced as the simplest and most effective way to sterilize biomaterials without toxic substances. However, it was found that this process breaks chemical bonds, and decreases tensile strength of collagen sponges moderately or even severely [9,10,20]. In addition to these known mechanical alterations by gamma sterilization, we evaluated the cell behavior in vitro.

In coincidence with other reports, collagen sponges sterilized with gamma irradiation showed severe shrinkage after cell seeding and became significantly unstable as indicated by a rapid enzyme degradation. The shrinkage reduced the surface area dramatically to 39% in three weeks. Planimetry revealed a reduction of the pore size between 20% and 65% showing a direct correlation of the surface reduction to the reduction of the pore size. The mechanisms of shrinkage are hypothesized to be the breakage of peptide bonds [9] and collapse of cross linking [10].

Shrinkage is not considered to be related to the biodegradation by seeded cells, as the MTT test revealed a higher proliferation in EO-sterilized sponges for seeded fibroblasts.

Further proliferation of the seeded cells was also shown to be influenced by this shrinkage. In gamma-sterilized sponges, only a few endothelial cells and fibroblasts were found to proliferate into the matrix. The reduced pore size to 7–16 μm seems to be unsuitable for directed proliferation of these cells. This supports the correlation of the structure of a matrix to endothelial and fibroblast cell proliferation as reported for chondrocytes [4]. A slight decrease of proliferation was also found in EO-sterilized sponges, which was assumed to be a normal adjustment of cells in a new matrix.

On the other hand, fibroblasts entrapped in densely compacted and twisted pores did not show the normal spindle shape. Changes in cell shape as well as unusual cell density can influence cell function. As seen in non-shrunken EO-sterilized sponges, fibroblasts were spread over the relatively flat surface of pore walls, distributed uniformly with normal spindle morphology. Endothelial cells were found at day 7 in the middle of the sponges, aggregated and well differentiated into tubular-like structures through day 7 to day 21, demonstrated by anti-Factor VIII staining.

In summary, this study shows that the impact of sterilization process on collagen sponges prepared for tissue engineering has to be considered and is non-negligible. EO can highly maintain collagen structural integrity and stability, while gamma irradiation is not recommended for collagen sterilization. Some new methods like formic acid (FA) [21], and dye-mediated photooxidation [22] were introduced recently, but still need further investigation. As shown in the present study, the investigation of these sterilization processes should also focus on the structural alterations and their impact on stability and cellular interaction.

Acknowledgements

This work was supported by a grant from the IZKF BIOMAT (BMBF-01KS9503/9). We wish to acknowledge the technical assistance of Mrs. M. Geiser-Letzel for her excellent assistance in immunohistochemistry.

References

[1] Sheridan RL, Tompkins RG. Skin substitutes in burns. Burns 1999;25:97–103.
[2] Doillon CJ, DeBlois C, Cote MF, Fournier N. Bioactive collagen sponges as a connective tissue substitute. Mater Sci Eng 1994;C2:43–9.
[3] Friess W. Collagen—biomaterial for drug delivery. Eur J Pharm Biopharm 1998;45:113–36.
[4] Nehrer S, Breinan HA, Ramappa A, Young G, Shortkroff S, Louie LK, Sledge CB, Yannas IV, Spector M. Matrix collagen type and pore size influence behaviour of seeded canine chondrocytes. Biomaterials 1997;18:769–76.

[5] Berthod F, Saintigny G, Chretien F, Hayek D, Collombel C, Damour O. Optimization of thickness, pore size and mechanical properties of a biomaterial designed for deep burn coverage. Clin Mater 1994;15:259–65.
[6] Langer R, Vacanti JP. Tissue engineering. Science 1993;260: 920–6.
[7] Schoof H, Bruns L, Apel J, Heschel I, Rau G. Einfluss des Einfriervorganges auf die Porenstruktur gefriergetrockneter Kollagenschwaemme. Ki-Luft und Kaeltetechnik 1998;34:247–52.
[8] Olde Damink LH, Dijkstra PJ, Van Luyn MJ, Van Wachem PB, Nieuwenhuis P, Feijen J. Influence of ethylene oxide gas treatment on the in vitro degradation behavior of dermal sheep collagen. J Biomed Mater Res 1995;29:149–55.
[9] Cheung DT, Perelman N, Tong D, Nimni ME. The effect of gamma-irradiation on collagen molecules, isolated alpha-chains, and crosslinked native fibers. J Biomed Mater Res 1990;24:581–9.
[10] Liu BC, Harrell R, Davis RH, Dresden MH, Spira M. The effect of gamma irradiation on injectable human amnion collagen. J Biomed Mater Res 1989;23:833–44.
[11] von Heimburg D, Zachariah S, Heschel I, Kuhling H, Schoof H, Hafemann B, Pallua N. Human preadipocytes seeded on freeze-dried collagen scaffolds investigated in vitro and in vivo. Biomaterials 2001;22:429–38.
[12] Singhvi R, Kumar A, Lopez GP, Stephanopoulos GN, Wang DI, Whitesides GM, Ingber DE. Engineering cell shape and function. Science 1994;264:696–8.
[13] Ingber DE, Prusty D, Sun Z, Betensky H, Wang N. Cell shape, cytoskeletal mechanics, and cell cycle control in angiogenesis. J Biomech 1995;28:1471–84.
[14] Miyata T, Taira T, Noishiki Y. Collagen engineering for biomaterial use. Clin Mater 1992;9:139–48.
[15] Pachence JM. Collagen-based devices for soft tissue repair. J Biomed Mater Res 1996;33:35–40.
[16] Heschel I, Rau G. Method for producing porous structures. International Patent Application PCT/DE 98/03403, November 19, 1997.
[17] Heschel I, Lückge C, Rödder M, Garbering C, Rau G. Possible applications of directional solidification techniques in cryobiology. In: Kittel P, editor. Advances of cryogenic engineering. New York: Plenum Press, 1996. p. 13–9.
[18] Vink P, Pleijsier K. Aeration of ethylene oxide-sterilized polymers. Biomaterials 1986;7:225–30.
[19] Matthews IP, Gibson C, Samuel AH. Enhancement of the kinetics of the aeration of ethylene oxide sterilized polymers using microwave radiation. J Biomed Mater Res 1989;23:143–56.
[20] Belkoff SM, Haut RC. Microstructurally based model analysis of gamma-irradiated tendon allografts. J Orthop Res 1992;10:461–4.
[21] Doillon CJ, Drouin R, Cote MF, Dallaire N, Pageau JF, Laroche G. Chemical inactivators as sterilization agents for bovine collagen materials. J Biomed Mater Res 1997;37:212–21.
[22] Moore MA, McIlroy BK, Phillips Jr RE. Nonaldehyde sterilization of biologic tissue for use in implantable medical devices. Asaio J 1997;43:23–30.