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Animal experiments with ultra-high molecular weight polyethylene (UHMW-PE) stabilised with α-tocopherol used for articulating surfaces in joint endoprostheses

C. Wolf · K. Lederer · H. Bergmeister · U. Losert · P. Böck

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Abstract Numerous investigations proved the impressive suitability of α-tocopherol as a stabilizer for ultra-high molecular weight polyethylene (UHMW-PE) used for endoprostheses. Regarding the biocompatibility of this new biomaterial, in-vitro celltoxicity tests gave no hint for a cyto- or genotoxic activity. In this study, animal experiments are carried out to further ensure the biocompatibility of this biomaterial.

Thin UHMW-PE-films ($20 \times 6 \times 0.23$ mm^3) were implanted subcutaneously into rats. Morphology and reactivity of surrounding connective tissue against either pure UHMW-PE material or UHMW-PE containing α-tocopherol were studied at timed intervals (2 weeks, 3 month, and 6 month after operation) in 3 groups of animals, each group comprising 10 animals. Parallel to these tests, material changes were investigated in these 3 groups of 10 animals at the same intervals after operation with the help of infrared spectroscopy (FTIR). Within the implantation time, no noteworthy oxidative degradation could be observed. The amount of lost α-tocopherol due to diffusion is low enough to ensure a lifetime stabilisation of the UHMW-PE.

The implants were all well tolerated and definitely encapsulated already 2 weeks after operation. Presence or absence of α-tocopherol in the implants did not evince morphological differences. Therefore, negative consequences were not manifest in the presence of α-tocopherol.

1 Introduction

In-vivo-oxidation of ultra-high molecular weight polyethylene (UHMW-PE) used for articulating surfaces in joint-endoprostheses can cause failures of endoprostheses after 10 to 15 years due to molecular degradation and recrystallization of the UHMW-PE followed by an embrittlement of the material [1–7]. This oxidation can be delayed decisively by adding the natural antioxidant α-tocopherol (vitamin E) to the UHMW-PE [9, 10]. In the human body, α-tocopherol acts as a scavenger of free radicals and protects the LDLs (low density lipoproteins) from being oxidised [8]. Although α-tocopherol proved itself to be an impressive antioxidant for UHMW-PE, it partially degrades during sintering and sterilization by γ-irradiation [9, 11], the standard processing steps for artificial hip-cups made of UHMW-PE. It must be expected that α-tocopherol undergoes different chemical changes during the sintering and sterilization process compared to human metabolism and therefore different transformation products are likely to be formed [13, 14]. Several biocompatibility-tests have already been carried out with animal as well as human cells, none of them showing evidence for a cyto- or genotoxic behaviour of the material

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C. Wolf
Department of Chemistry of Polymeric Materials, University of Leoben, 8700 Leoben, Austria, and Polymer Competence Center Leoben GmbH (PCCL), Parkstraße 11, 8700 Leoben, Austria, www.pccl.at
e-mail: christian.wolf@unileoben.ac.at

K. Lederer (✉)
Department of Chemistry of Polymeric Materials, University of Leoben, 8700 Leoben, Austria
e-mail: polychem@unileoben.ac.at

H. Bergmeister · U. Losert
Institute of Biomedical Research, University of Vienna, General Hospital Vienna, Vienna, Austria

P. Böck
Department of Histology and Embryology, University of Veterinary Medicine Vienna, Vienna, Austria

[11, 12]. The next step for admission to clinical studies are these animal experiments.

Stabilised as well as unstabilised UHMW-PE films were subcutaneously implanted into rats. Morphology and reactivity of surrounding connective tissue were studied at timed intervals (2 weeks, 3 month, and 6 month after operation). All specimens were examined regarding oxidative degradation with the help of FTIR spectroscopy.

2 Materials and methods

2.1 Preparation and processing of the stabilized UHMW-PE-specimens

UHMW-PE was Hostalen GUR 1020 (LOT No.: B15331047) from Hoechst AG (now TICONA AG, Frankfurt/Main Germany), which fulfils the requirements of ISO 5834 Part 1 and 2 (Implants for surgery—UHMW-PE powder and moulded forms) and was obtained as a gift sample from the production site in Oberhausen, Germany. DL-α-tocopherol (LOT-No.: 606876) was a gift sample of Hoffmann-La Roche (Grenzach-Wyhlen, Germany). α-Tocopherol, a brownish oil with a high viscosity, was dissolved in ethanol (with a concentration of 50 g/l) and mixed into the UHMW-PE-powder drop by drop in a screw-cone mixer (Nauta-Vrieco, type: 03 VB-1). The ethanol was then evaporated in a vacuum-dryer at 50°C for 6 h [15, 16]. For the animal experiments, samples with 0.8% α-tocopherol were prepared although 0.2 to 0.4% α-tocopherol are suggested as optimal concentration for UHMW-PE [9]. The higher concentration was chosen in order to simulate the "worst case" and to ensure the validity of the results in an extended concentration range.

The stabilised and neat UHMW-PE powder was sintered to disks (diameter = 600 mm, thickness = 60 mm), at 220°C and 35 bar for 7 h in an industrial facility usually used for the production of running surfaces of skis at Isosport GmbH (Eisenstadt, Austria).

HPLC studies were carried out to determine the homogeneity of the α-tocopherol distribution. The α-tocopherol concentration of test samples, each taken from different locations of the disk, differed only by $\pm 2\%$ from the desired concentration.

For implantation, films ($20 \times 6 \times 0.23$ mm^3) were fabricated out of the middle of the disks. The edges of all films were rounded to prevent an inflammation of the surrounding tissue. Finally, all films were washed and sterilized with γ-rays at 25 kGy in inert atmosphere by Zimmer, Inc. (former Centerpulse Ldt., Winterthur, Switzerland) according to the standard procedure for artificial hip-cups.

2.2 Fourier transform infrared spectroscopy (FTIR)

The degree of oxidation of the UHMW-PE films was investigated with the help of FTIR spectroscopy. A Perkin Elmer® AutoImage FTIR-microscope connected to a Perkin Elmer® Spectrum One spectrometer was used to measure the carbonyl number (CO-number) of five points on each film according to DIN 53383 (aperture size: 100×100 μm^2, Resolution: 1 cm^{-1}, 4 scans per spectrum). The CO-number is the ratio of the absorbance at 1718 ± 15 cm^{-1} (carbonyl group) to the absorbance at 2020 ± 20 cm^{-1} (C-H vibration).

Mapscans were carried out scanning the whole area of a specimen with a grid distance of 200×200 μm^2. The peak area instead of the height was used to calculate the CO-number in the mapscan. All spectra were collected in transmission.

The α-tocopherol concentration was determined by the ratio of the area of the α-tocopherol-peak at 1265 cm^{-1} to the area of the PE-peak at 2020 cm^{-1}.

2.3 Animal experiments

All animals received humane care in compliance with the principles of laboratory animal care formulated by the Institute of Laboratory Animal Resources and the Guide For The Care and Use of Laboratory Animals prepared by the U.S. National Academy of Sciences and published by the U.S. National Institutes of Health (Pub. No. 86-23, revised 1985, Bethesda, Maryland, USA). The studies were carried out on 3 months old, female Sprague Dawley rats. Anesthesia was induced with ketamine (Ketanest®, Parke-Davis, Vienna, 100 mg/kg) and xylazine (Rompun®, Bayer AG, Leverkusen, 5 mg/kg). Two dorsal, subcutanous pouches were made on each rat bilaterally by incision and dissection. After insertion of the specimens (neat UHMW-PE on the left and UHMW-PE with α-tocopherol on the contralateral side) the skin was closed by silk sutures.

Morphology and reactivity of surrounding connective tissue against either neat UHMW-PE material or UHMW-PE containing α-tocopherol were studied at timed intervals (2 weeks, 3 month, and 6 month after operation) in 3 groups of animals, each group comprising 10 animals. Parallel to these tests, material changes were investigated in 3 groups of 10 animals at the same intervals after operation (in fact 60 rats were used, 30 for histological investigations, 30 for material testing, in groups of 10 animals for 3 time intervals).

Animals were sacrificed after 2 weeks, 3 month and 6 month with an overdose of thiopentone. Skin and adhering subcutaneous connective tissue were removed together with the implants left *in situ*. Specimens were immersion-fixed in 5% neutral formalin (0.2 M phosphate buffer, pH 7.2) for 24 h, reduced in size and routinely embedded into paraffin wax by means of a Tissue-Tek 2000 (Miles Laboratories,

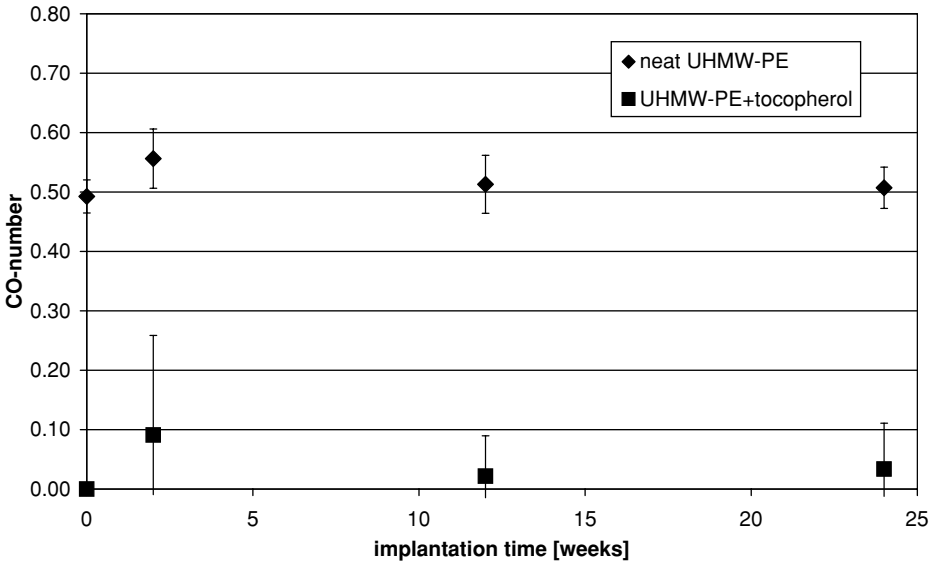

Fig. 1 CO-number of UHMW-PE films with and without α-tocopherol versus implantation time (2 weeks, 3 months and 6 months after implantation, the points represent the mean values ($n = 50$), the bars represent the standard deviations).

Mishawaka, Ind) embedding device. Microtome sections were prepared perpendicular to the surface and by that perpendicular to the implanted films of synthetic material. Serial sections were mounted and routinely stained with Haematoxylin, Eosin and van Gieson connective tissue stain to show collagen bundles with acid fuchsin, and Weigerts resorcinolfuchsine to identify elastic fibers (all staining procedures according to Romeis [17]). Macrophages were identified by immunostaining for lysozyme (rabbit polyclonal anti-lysozyme from Neo-Markers, Fremont, CA; dilution 1:150), myofibroblasts by immunoreactivity for actin (mouse monoclonal anti-muscle actin from Dako, Glostrup, DK; clon HHF 35, dilution 1:400) and vimentin (mouse monoclonal anti-vimentin from Dako, Glostrup, DK; clon V 9, dilution 1:200); unlabeled primary antibodies were identified by means of the Dako Envision^{TM} staining system.

3 Results

3.1 FTIR spectroscopy

Each UHMW-PE film was investigated after implantation concerning its degree of oxidation by measuring the CO-number (see 2.2, above). Figure 1 shows the mean value of the CO-number of the 10 samples per group versus the implantation time. As expected, no oxidation of the material could be detected during 6 month in-vivo. However, Fig. 1 clearly reveals the pre-damaging of the unstabilised UHMW-PE due to sintering and sterilisation with γ-rays. Stabilisation with α-tocopherol inhibits this degradation during processing.

Close examination of the spectra revealed the presence of a small second oxidation peak at 1747 cm^{-1} (ester group, in addition to the ketone peak at 1718 cm^{-1}) in approx. 2 samples of each group of 10, which was independent of implantation time and stabilisation respectively. This peak did not seem to be distributed homogeneously throughout the samples, therefore a mapscan was carried out scanning the whole area of one sample. Figure 2 shows the mapscan of the CO-number of a stabilised specimen which was implanted for 2 weeks. As described in 2.2., the area of both oxidation peaks, 1718 cm^{-1} and 1747 cm^{-1} respectively, was used to calculate the CO-number instead of the height of the highest peak according to DIN 53383. In Fig. 2, a weak local oxidation of the material can be seen. No reliable explanation can be given for this fact; since this oxidation is independent of the implantation time and already present after 2 weeks, it may possibly be caused by some sort of inflammation reaction shortly after implantation.

Figure 3 shows the loss of α-tocopherol during the implantation time in-vivo. The α-tocopherol concentration drops from 0.73% to 0.55% within 6 month, a small decrease with regard to an average film thickness of 0.23 mm. In fact, the animal experiments could be considered as a sorption experiment according to J. Crank and G. S. Park [18] for calculating the diffusion coefficient. Evaluation of the data yields an diffusion coefficient of $4.02 * 10^{-13}$ cm^2/s (at a body temperature of rats of 36.5–37.9°C). Assuming a hip cup to be a two dimensional infinite plate with a thickness of 8 mm, the loss of α-tocopherol within 10 years amounts to 3%. These findings clearly show that enough α-tocopherol remains in the UHMW-PE to ensure an adequate lifetime stabilisation.

3.2 Animal experiments

3.2.1 General observations

The implants were generally positioned just beneath the panniculus carnosus; they were well tolerated in all animals without any sign of inflammation. The epidermis was seen in most of the sections, thus allowing one to discern between

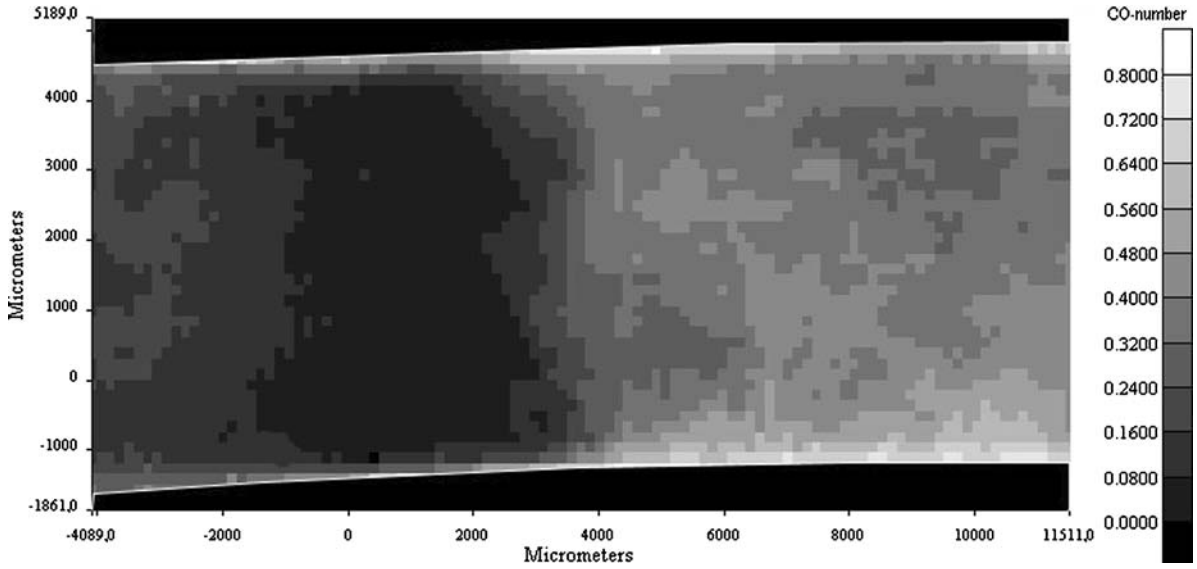

Fig. 2 Mapscan of the CO-number of a stabilised specimen which was implanted for 2 weeks (scanned area: 15.6 × 6 mm^2, specimen dimension: 20 × 6 mm^2).

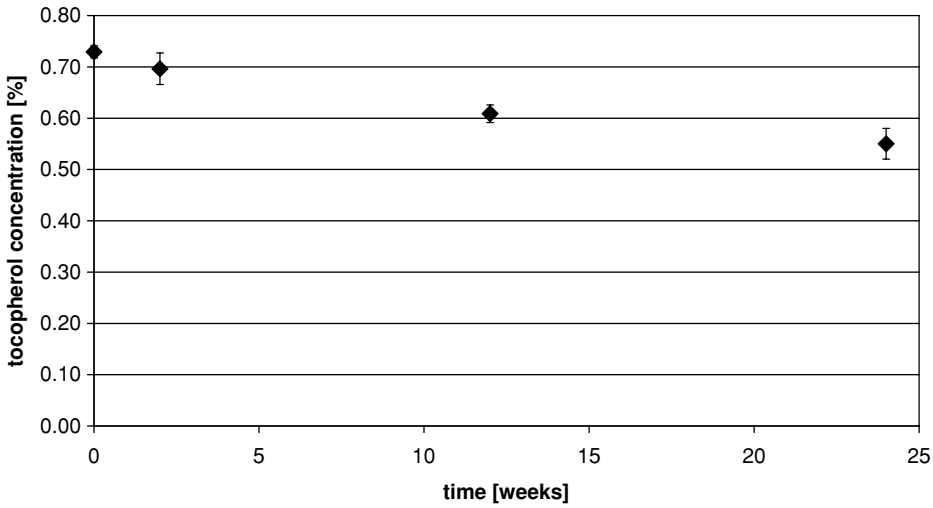

Fig. 3 Drop of the α-tocopherol concentration as a function of implantation time (the points represent the mean values ($n = 15$), the bars represent the standard deviations).

superficial and deep surface of the implants. There were no remnants due to surgery, such as fibrin or free erythrocytes, even not in the short survival group (2 weeks). A well defined capsule of coarse connective tissue free of elastic fibers was developed already after 2 weeks of implantation. Dimension and composition of this capsule did not change during the time period studied (2 weeks up to 6 month), its general organisation is schematically shown in Fig. 4.

The connective tissue capsule was attenuated along the edges of the implants and showed continuously increasing thickness over the flat surfaces of the implants. This increase in thickness was less distinct along the small sides of the rectangular profiles but was pronounced over the wide surfaces. Remarkably, the connective tissue capsules on the superficial surface of the implants (towards the epidermis) were regularly thicker than on the opposite (deep) surface. The

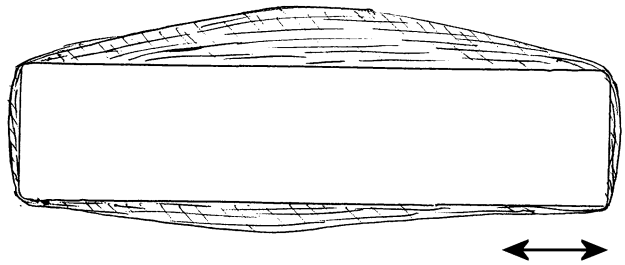

Fig. 4 The connective tissue capsule of implants, schematic drawing, bar represents 0.3 mm.

thickness of the connective tissue capsule on the superficial surface measured up to 200 μm.

The smooth-surfaced implants showed hyalin structure without any morphological signs of corrosion. The implants were not connected to the surrounding tissue. Therefore, and

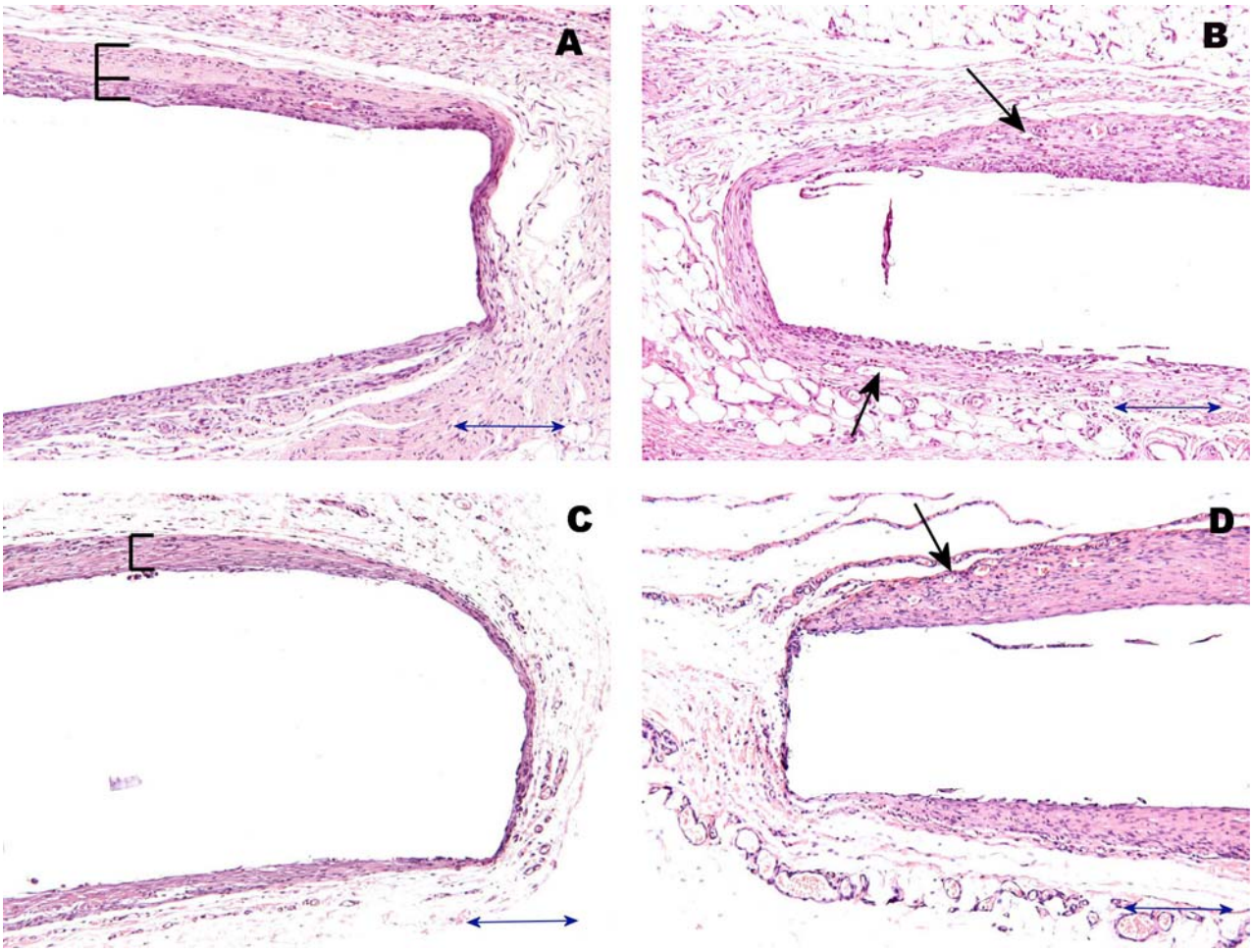

Fig. 5 The connective tissue capsules of subcutaneous implants, 2 weeks (Figs. 5A and B) and 6 month (Figs. 5C and D) after operation. Comparison of UHMW-PE without (Figs. 5A and C) and with α-tocopherol (Figs. 5B and D). Haematoxylin & Eosin staining, bar represents 200 μm. (A) The connective tissue capsule of implants consists of two layers (brackets), the inner one is rich in cells, the outer one fibrous. (B) Lines of macrophages at the surface of implants detach from the connective tissue capsule when implantation material is lost from the sections. Arrows point to capillaries at the capsule surface. (C) After 6 month of implantation, the outer fibrous layer of the capsule remains prominent (bracket) whilst the inner layer is reduced. (D) Numerous capillary profiles (arrows) at the capsular surface after 6 month of implantation. Note also lines of detached macrophages.

because of the marked difference in consistency between implant material and connective tissue, the implants were frequently lost from the sections during histological processing.

The connective tissue capsule covering the implants consisted of two layers (Fig. 5A):

(1) an inner cell-rich layer next to the implants surface clearly stood out against
(2) an outer fibrous layer that bordered against loosely organized subcutaneous connective tissue.

Each of these two layers could be further subdivided:

Subdivision of the inner layer was obtained by discerning between an innermost stratum of macrophages (making contact to the implants), and an outer stratum of fibroblasts and macrophages within a network of delicate collagen fibrils devoid of capillaries. Macrophages of the inner stratum form a simple squamous, cuboid, or even columnar or pseudostratified epitheloid layer of lysozyme-positive cells. The macrophages do not firmly adhere to the implant, to each other, or to outside connective tissue. Consequently, a share of these cells was easily dislocated (Figs. 5B and D) or even lost during preparation. The tendency to form multinucleated giant cells was not pronounced. The remaining second stratum of the inner layer was also cell-rich, comprising macrophages and fibroblasts. This stratum also included delicate collagen fibrils, whilst coarse bundles of collagen, capillaries and granulocytes were missing (Fig. 5A).

Subdivision of the outer layer was indicated by discerning an inner stratum of coarse collagen bundles free of capillaries with only rare fibrocytes, from an outer coarse but vascularized stratum. The inner stratum of coarse collagen fibers represented the mechanically resistant cover of the implant. Varying thickness of this stratum caused the varying thickness of the entire connective tissue envelope of the implants.

Table 1 Connective tissue capsule, mean cell number per 0.01 mm^2, ($n = 5$)

Inner layer, 0–35 μm		Outer layer, 35–70 μm
	14 days after implantation	
38.0	without α-tocopherol	27.4
30.8	with α-tocopherol	25.5
	3 month after implantation	
25.1	without α-tocopherol	18.6
24.7	with α-tocopherol	14.2

The inner stratum was thin at the implants edges; it became the thicker the wider the surface areas of the implants to be covered. Coarse collagen bundles stained with acid fuchsin preparations, most probably indicating type I collagen. Collagen bundles ran parallel to the implants surface, elastic fibers were missing. Interposed connective tissue cells were scarce. Most of these fibroblasts stained for actin, indicating their nature as myofibroblasts. Tissue components and organization of the inner and outer stratum corresponded besides to two additional constituents of the outer stratum, capillary loops and free granulocytes (Fig. 5B).

Loosely organized adventitial tissue connected the connective tissue capsule of implants with subcutis and cutis. This adventitial tissue was well vascularized and devoid of elastic fibers and fat cells.

3.2.2 Time course of experiments and comparison of implants without and with α-tocopherol

Composition and dimension of connective tissue capsules of all implants were in accordance with the general description given above already 14 days after implantation, the shortest time interval studied (Figs. 5A and B). Later on, i.e. 3 and 6 month after implantation, composition and dimension of the connective tissue capsules had not significantly changed (Figs. 5C and D). Hence, it was morphologically impossible to diagnose implantation time, as it was impossible clearly to discern between implants consisting of material without (controls) or with α-tocopherol (cf. Figs. 5A and C with Figs. 5B and D, respectively). It should be emphasized that connective tissue capsules of implants with α-tocopherol were well vascularized (Figs. 5B and D) and the amount of collagen fibers in the capsules increased with time as cellularity decreased (Figs. 5C and D). We did not perform detailed morphometrical analysis but mean values of cell counting are shown (Table 1). Results support morphologically observed differences between inner and outer layer of the connective tissue capsule, decreasing cellularity with implantation time and reduced cellularity in case of implants with α-tocopherol.

4 Conclusion

Implants had been well tolerated and were definitely encapsulated already 2 weeks after operation. Presence or absence of α-tocopherol in the implants did not evince morphological differences. Therefore, negative consequences were not manifest in the presence of α-tocopherol.

No biological degradation of implanted material could be observed in 6 month implantation time.

Where the connective tissue capsules bordered against the implants, the capsules closely resembled a synovial membrane: a simple or pseudostratified layer of macrophages with intermingled fibroblasts (corresponding to synovialocytes type A and B) rested on connective tissue (corresponding to the fibrous layer of a synovial membrane). After removal of the implant, a cavity was left that resembled a synovial bursa. It remains to prove whether or not such an artificial bursa will persist after removal of the implant.

Up to now, α-tocopherol has remarkably proved its suitability as a stabilizer for UHMW-PE in numerous investigations. Neither the in-vitro toxicity tests nor the animal experiments show an evidence for a toxic behaviour of UHMW-PE stabilised with α-tocopherol. In cooperation with Zimmer, Inc. (Winterthur, Switzerland), clinical studies of Vitasul® (tradename of Centerpulse for UHMW-PE stabilised with α-tocopherol) are in preparation.

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