J Mater Sci: Mater Med (2007) 18:1247–1252
DOI 10.1007/s10856-006-0098-6

Biocompatibility of ultra-high molecular weight polyethylene (UHMW-PE) stabilized with α-tocopherol used for joint endoprostheses assessed in vitro

Christian Wolf · Klaus Lederer · Roswitha Pfragner · Konrad Schauenstein · Elisabeth Ingolic · Veronika Siegl

Received: 17 May 2005 / Accepted: 9 February 2006 / Published online: 3 February 2007

Abstract Adding the natural antioxidant α-tocopherol to ultra-high molecular weight polyethylene (UHMW-PE) can remarkably delay the oxidation of hip cups made thereof. However, α-tocopherol is likely to undergo different chemical transformations during manufacturing and sterilization of hip cups than in human metabolism. Therefore, the biocompatibility of the putative transformation products has to be investigated. In-vitro tests with L929 mice fibroblast-cells gave no evidence for cytotoxicity. To further ensure the biocompatibility, in-vitro tests with human cells were carried out in this study. Two different human cell lines, one adherent cell line, HF-SAR, and one suspension culture, GSJO, were tested on UHMW-PE-tablets (diameter: 15 mm; thickness: 2 mm; processed according to standard procedures for artificial hip-cups) with and without α-tocopherol with respect to cell viability, proliferation and morphology by means of cell counting, WSt-1 proliferation assay and scanning electron microscopy. Similar proliferation rates were found with both polyethylene samples. Further, we found intact morphology in light and electron microscopy on each substrate. The morphologic characteristics of skin fibroblasts were not changed by any material. Normal adherence and spreading of the fibroblasts was found on controls of glass, as well as on polystyrene and on stabilized and unstabilized polyethylene. The characteristic behaviour as suspension of the GSJO cells remained unchanged. The mitochondrial activity, as studied by WST-1 cell proliferation reagent, was identical on each substrate during the whole observation period of 7 days.

Christian Wolf—in partial fulfillment of a Ph.D. (Dr.mont.) thesis at the University of Leoben

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

C. Wolf
Polymer Competence Center Leoben GmbH (PCCL), Parkstraße 11, 8700 Leoben, Austria

E. Ingolic
Research Institute for Electron Microscopy and Finestructure Research, Technical University Graz, Steyrergasse 17, 8010 Graz, Austria

R. Pfragner · K. Schauenstein · V. Siegl
Department of Pathophysiology, Medical University Graz, Heinrichstrase 31, 8010 Graz, Austria

Introduction

Numerous papers describing the in-vivo-oxidation of ultra-high molecular weight polyethylene (UHMW-PE) used for articulating surfaces of joint-endoprostheses can be found in literature [1–18]. The oxidation causes molecular degradation and re-crystallization followed by an embrittlement and enhanced formation of PE-debris. Abrasive wear is considered the primary cause of failure in total hip endoprostheses, leading to peri-prostetic osteolysis, often limiting the lifetime to 10–15 years.

This oxidative degradation can be markedly delayed by adding the natural antioxidans α-tocopherol (vitamin E) to the UHMW-PE [19–21]. In the human

body, α-tocopherol acts as a scavenger of free radicals, and protects LDLs (low density lipoproteins) from being oxidized [22]. Although it could be shown that α-tocopherol possesses an impressive stabilization efficiency even compared to modern technical antioxidants, the vitamin is partially degraded during sintering and sterilization by γ-irradiation [19, 20, 23], the standard processing steps for artificial hip-cups made of UHMW-PE. The amount of transformation products can reach up to 34% of the initial mass of α-tocopherol [23]. It must be expected that α-tocopherol undergoes chemical changes during the sintering and sterilization process which are different from what happens in vivo and therefore different transformation products are to be expected. The assumed final main oxidation product in-vivo is the α-tocopherol quinone [24], while diastereoisomers of dimers and trimers as well as aldehydes were found in LDPE stabilized with α-tocopherol after several extrusion steps [21]. Obviously, the biocompatibility of these transformation products has to be ensured before any stabilization may be applied to commercial hip systems.

In a previous study [23] several screening tests concerning the biocompatibility of the transformation products of α-tocopherol were carried out in vitro with L929 mouse fibroblasts according to EN ISO 10993-5. The tests included a cell proliferation test (BrdU labelling), a XTT-test for mitochondrial activity, a membrane integrity test, a cell staining with hemalum for the detection of cell adhesion and changes of cell morphology and an Ames-test to investigate a possible genotoxic activity.

The tests did not reveal any evidence of a cytotoxic behaviour of stabilized UHMW-PE specimens. However, adhesion rate and cell spreading were slightly diminished, which means that this material is biocompatible, but not bioactive. Cells probably will not be able to adhere to build a strong contact between implant and tissues. In orthopaedic surgery, this is not relevant since the UHMW-PE has no direct contact to the surrounding bone. It is either cemented or applied as an inlay in a metal cup.

Nevertheless, we decided to carry out additional cytotoxicity-tests with human cells in order to further ensure the biocompatibility of UHMW-PE stabilized with α-tocopherol. Two human cell lines with different growth characteristics, HF-SAR, an adherent cell line, and GSJO, a suspension culture, were tested on UHMW-PE-tablets with and without α-tocopherol as to cell viability, proliferation and morphology.

Materials and methods

Preparation and processing of the UHMW-PE-specimens

UHMW-PE was Hostalen GUR 1020 from TICONA AG (Frankfurt/Main Germany), which fulfills 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 manufacturer in Oberhausen, Germany. DL-α-tocopherol was a gift sample of Hoffmann-La Roche (Grenzach-Wyhlen, Germany). α-Tocopherol is a brownish oil with a high viscosity, therefore it 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) to a concentration of 0.8% w/w. The ethanol was then evaporated in a vacuum-dryer at 50°C for 6 h [19].

For the tests, specimens in tablet form fitting into the culture wells with a diameter of 15 mm and a thickness of 2 mm were needed. The first tablets were milled out of disks, which were sintered from the UHMW-PE-powder (diameter = 600 mm, thickness = 60 mm) at 220°C and 35 bar for 7 h in an industrial facility. However, the cells accumulated in the milling grooves making it impossible to count the cells. Therefore, we directly sintered the powder in a 2mm thick mold with holes of 15 mm diameter for 105 min at 230°C under nitrogen atmosphere in a Collin 200 PV hydraulic press (Dr. Collin GmbH, Ebersberg, Germany). With this procedure we got tablets of the desired size with a smooth surface. The tablets were washed with ethanol and dried for 25 min at 60°C in an air oven. Finally, all tablets were washed and sterilized with γ-rays at 25 kGy in inert atmosphere by Centerpulse Ltd. (now Zimmer Inc., Winterthur, Switzerland) according to standard procedures for artificial hip-cups. In order to prevent floating, the tablets were fixed in the wells with silicone rings.

The biocompatibility of the different materials was tested by using methods described in detail earlier [25, 26]. Briefly, the morphology, proliferation, viability and cytotoxicity were analyzed in vitro. As in vitro models, two human cell lines were chosen for their differences in phenotype and proliferation, namely HF-SAR and GSJO.

Cell culture

The HF-SAR cell line was established from normal human skin of a 2 year old male (Personal communi-

cation). The cells grow adherent and have fibroblastic morphology. Cultivation was performed in Minimum Essential Medium Eagle (MEM-E, Biowhittaker, Vallensbaek Strand, Denmark) containing 10% fetal bovine serum (PAA Laboratories, Exton, PA, USA) without antibiotics. An inoculum of 1.0×10^5 cells/ml will multiply 2–3 fold in 7 days.

The GSJO cell line was established from a medullary thyroid carcinoma of a 68 year old male, according to detailed protocols [27]. The cells grew anchorage independently, forming suspensions of single cells and spheroid aggregates. Cultivation was performed in nutrient mixture Ham's F12 (Seromed, Göttingen, Germany) containing 10% fetal bovine serum (PAA Laboratories, Exton, PA, USA) without antibiotics. An inoculum of 1.0×10^5 cells/ml will multiply 3 fold after 3 days.

Both cell lines were mycoplasma-free, as tested by DAPI-staining (Sigma, Vienna, Austria).

Polyethylene-tablets with or without α-tocopherol were placed into microplates (24 wells, Sarstedt, Wiener Neudorf, Austria) and mounted in the wells with silicone rings. Control materials were round glass cover slips (Roth, Karlsruhe, Germany) and polystyrene—the standard substrate for cell cultures (Sarstedt, Wiener Neudorf, Austria). 0.5 ml suspensions of HF-SAR or GSJO cells were pipetted into each well of the microplates at a cell density of 2×10^5 cells/ml medium, and incubated at 37°C in a 5% CO_2 and 90% humidity incubator for 3, 4, 6 and 7 days.

Cell counting and determination of cell size

After incubation for 3, 4, 6, and 7 days, the cells from each well were analyzed in a Casy®-1 Cell Counter & Analyzer TTC (Schärfe System, Reutlingen, Germany). The combination of resistance measuring principle with pulse area analysis allows the quantitative and qualitative analysis of cells. From each well, 50 µl of cell suspension were taken and diluted 1:50 in counting fluid, mixed and singly suspended. From each well, the samples were measured 4 times. Five series of experiments were performed. The measurement data were performed by an integrated IBM-compativle computer in the Casy-1 TTC, graphical user interface Windows ™.

Cell numbers and cell size were determined.

Cytotoxicity evaluation by cell proliferation assay WST-1

After incubation for 3, 4, 6, and 7 days, the Cell Proliferation Reagent WST-1 (Roche Diagnostics, Vienna, Austria) was added to each well and further incubated. The number of viable cells was analyzed by the cleavage of tetrazolium salt WST-1, added to the culture medium. The amount of formazan dye formed directly correlates to the number of metabolically active cells analyzed, expressing the mitochondrial activity of the cells. The formazan produced was quantitated using a scanning multi-well spectrophotometer (ELISA plate reader) (Molecular Devices, Sunnyvale, CA, USA). The absorbance revealed directly corelates to the cell number.

Scanning electron microscopy (SEM)

In order to study the microstructures of cell membranes and the attachment to the substrate, the adherently growing HF-SAR fibroblasts were incubated on the different substrates for 3 days. After incubation, the cells were fixed in situ in 3% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, +4°C, washed , and postfixed on OsO_4 1% in 0.1M cacodylate buffer, critical point dried, and coated with Au-Pd. The morphology of the cells was examined by Leitz Scanning Microscope AMR 1600. Since GSJO cells grow as a suspension culture, the attachment-behavior was not examined.

Results

Cell counting and determination of cell size

The cell counts, comprising the viable and the dead cells of HF-SAR and GSJO cells showed minimal differences between stabilized and non-stabilized materials. A minor reduction of cell proliferation was caused by the silicone rings which were necessary to hold the tablets in the wells to prevent floating of the materials. The best proliferation was found in the control group, in which polystyrene—the most common substrate for cell cultivation—was used. No difference was found between the cell counts of adherent and suspension cells. (see Fig. 1). No effect was seen on the size of the cells (Fig. 2)

Cytotoxicity evaluation by cell proliferation assay WST-1

The mitochondrial activity of HF-SAR fibroblasts and of GSJO cells was determined after 3, 4, 6, and 7 days. HF-SAR and GSJO cells, grown on different substrates, showed the same growth characteristics. No

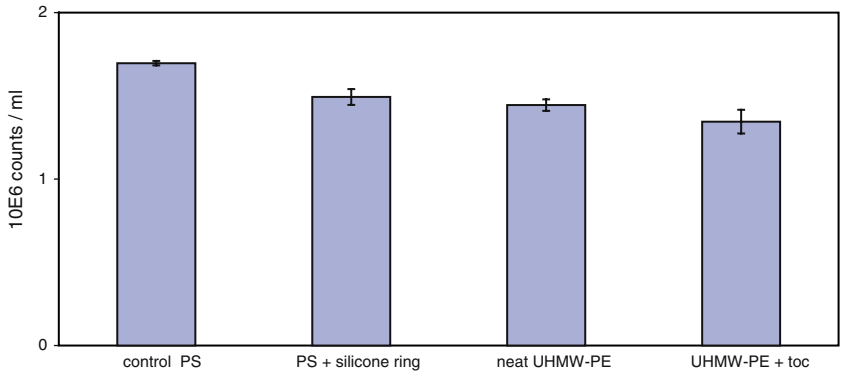

Fig. 1 Cell proliferation of GSJO cells on polystyrene, polystyrene with silicone ring, neat polyethylene and α-tocopherol-stabilized polyethylene after 3 days. The proliferation rates on polyethylene with or without α-tocopherol were equal

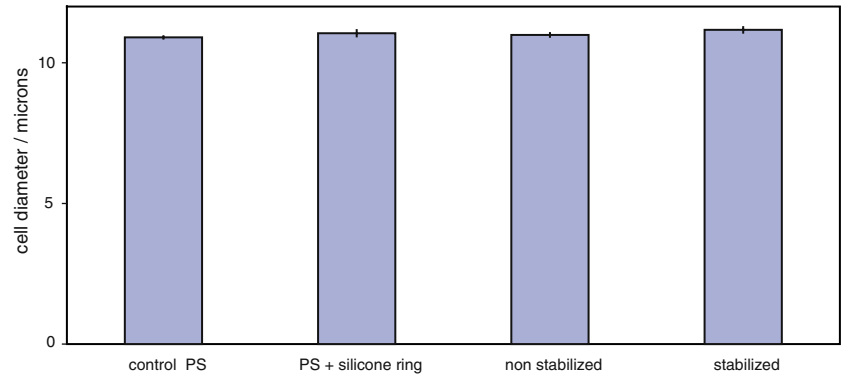

Fig. 2 Size of cells on polystyrene, polystyrene with silicone ring, neat polyethylene and α-tocopherol-stabilized polyethylene after 3 days. No cytotoxic effect on the size of the cells was found

impairment of the mitochondrial function or sign of cytotoxicity was found in either cell line (see Fig. 3).

Scanning electron microscopy (SEM)

The ultrastructure of cells was identical on each substrate. The fibroblasts formed monolayers which reached confluency after three days on each substrate. The characteristic morphology of spindle shaped cells was characteristic in all samples. In each group. Membrane integrity was not impaired. The attachment of the cells to the surface was normal and identical on each sample. No sign of cytotoxicity was found (see Fig. 4A, B).

Conclusion

In this study, the biocompatibility of α-tocopherol-stabilized polyethylene was compared with the known biocompatibility of polyethylene without stabilizer. We found similar proliferation rates with both samples. Slightly better growth qualities were seen on the control-substrates, i.e. on glass or especially on polystyrene, a substrate which is commonly used for cell culture vessels, which is not surprising. Further, we found intact morphology in light and electron microscopy on each substrate. The morphologic characteristics of skin fibroblasts were not changed by any material. Normal adherence and spreading of the

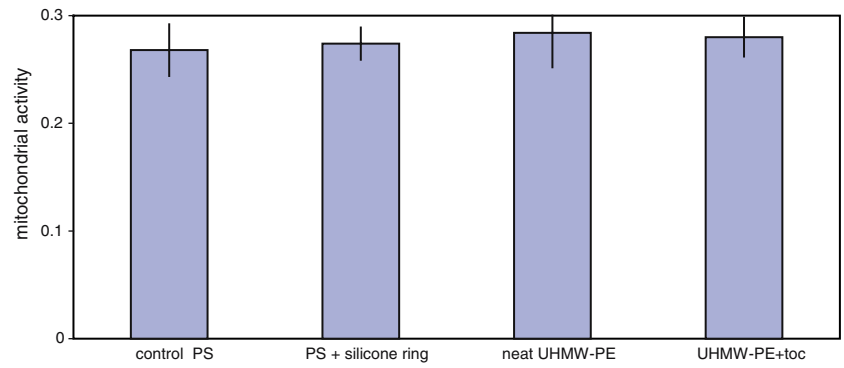

Fig. 3 WSt-1 assay of GSJO cells after 3 days. The assay is based on the cleavage of the tetrazolium salt WST-1 producing a soluble formazan salt. This conversion indicates the viable cells. The formazan is quantitated using an ELIZA reader. No cytotoxic effect was found

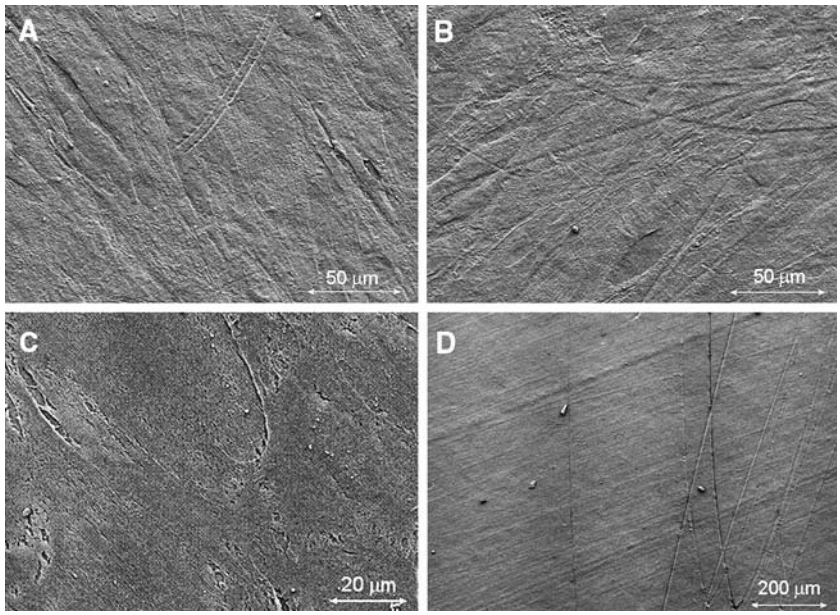

Fig. 4 HF-SAR human fibroblasts form confluent monolayers after 3 days on: neat polyethylene (**A**); α-tocopherol-stabilized polyethylene (**B**); Controls: Glass (**C**); α-tocopherol without cells (**D**). On each material, the morphology of the cells was normal, the cell membranes were not damaged. Scanning electron micrographs

fibroblasts was found on controls of glass, on polystyrene and on stabilized and unstabilized polyethylene. The characteristic formation of cell suspensions by the GSJO cells remained unchanged. The mitochondrial activity, as studied by WST-1 cell proliferation assay, was identical for each substrate during the whole observation period of 7 days.

Using selected methods for in vitro cytotoxicity assessment, we studied polyethylene and α-tocopherol stabilized polyethylene, and compared these materials with glass or polyethylene. We conclude from the present data that both materials, polyethylene and α-tocopherol stabilized polyethylene do not show any toxic effects to human cells, both adherent and non adherent. However, the in vitro results should be confirmed by in vivo testing to rule out possible interaction with other tissues, before the stabilized material can be considered for implants in orthopedic surgery.

Acknowledgments We like to thank Dr. Markus Windler and Dipl.Ing. W. Schneider of Zimmer Inc., Winterthur, Switzerland, for washing and sterilizing the specimens under inert atmosphere, Dr. Wolfgang Payer from TICONA AG, Oberhausen, Germany, for providing the Hostalen GUR 1020 as well as Dr. Ernst Wagner from Hoffmann-LaRoche AG, Grenzach-Wyhlen, Germany, for providing the α-tocopherol as gift samples.

References

1. M. GOLDMAN, R. GRONSKY, R. RANGANATHAN and L. PRUITT, *Polymer***37** (1996) 2909
2. P. EYERER, *Kunststoffe* **77** (1987) 617
3. F. J. BUCHANAN, B. SIM and S. DOWNES, *Plast. Rub. Comp. Proc. Appl.* **27** (1998) 148
4. E. S. GROOD, R. SHASTRI and C. N. HOPSON, *J. Biomed. Mat. Res.* **16** (1982) 399
5. J. A. DAVIDSON and G. SCHWARTZ, *J. Biomed. Mat. Res.: Appl. Biomat.* **21** (1987) 261
6. B. ZICAT, C. A. ENGH and E. GOKCEN, *J. Bone Joint Surg.* **77A** (1995) 432
7. M. GOLDMAN, R. GRONSKY, G. LONG and L. PRUITT, *Poly. Deg. Stab.* **62** (1998) 97
8. M. DENG and S. W. SHALABY, *J. Appl. Polym. Sci.* **58** (1995) 2111
9. J. L. HENRY, L. R. ASCENION and A. GARTON, *J. Polym. Sci.: Polym. Chem.* **30** (1992) 1693
10. E. BRACH DEL PREYER, M. CROVA, L. COSTA, A. DALLERA, G. CAMINO and P. GALLINARO, *Biomaterials* **17** (1996) 873
11. L. COSTA, M. P. LUDA, L. TROSSARELLI, E. M. BRACH DEL PREYER, M. CROVA and P. GALLINARO, *Biomaterials* **19** (1998) 659
12. L. COSTA, M. P. LUDA, L. TROSSARELLI, E. M. BRACH DEL PREYER, M. CROVA and P. GALLINARO, *Biomaterials* **19** (1998) 1371
13. M. GOLDMAN, M. LEE, R. GRONSKY and L. PRUITT, *J. Biomed. Mat. Res.* **37** (1997) 43
14. R. M. ROSE, E. V. GOLDFARB, E. ELLIS, A. N. CRUGNOLA, *J. Orthop. Res.* **2** (1984) 393
15. V. PREMNATH, A. BELLARE, E. W. MERILL, M. JASTY and W. H. HARRIS, *Polymers* **40** (1999) 2215
16. S. O. HAN, D. W. LEE and O. H. HAN, *Polym. Degrad. Stab.* **63** (1999) 237
17. A. SINGH, *Radiat. Phys. Chem.* **56** (1999) 375
18. B. YEOM, Y. J. YU, H. A. MCKELLOP and R. SALOVEY, *J. Polym. Sci. A: Polym. Chem.* **36** (1998) 329
19. C. WOLF, T. KRIVEC, J. BLASSNIG, K. LEDERER and W. SCHNEIDER, *J. Mat. Sci.: Mat. In Med.* **13** (2002) 185
20. S. AL-MALAIKA, C. GOODWIN, S. ISSENHUTH and D. BURDICK, *Polym. Deg. Stab.* **64** (1999) 145

21. S. AL-MALAIKA, H. ASHLEY and S. ISSENHUTH, *J. Polym. Sci. Part A: Polym. Chem.* **32** (1994) 3099
22. B. FREI, K. BRIVIBA and H.SIES, In ''Natural Antioxidants in Human Health and Disease'' (Academic Press, San Diego, 1994), p.107
23. C. WOLF, K. LEDERER and U. MÜLLER, *J. Mat Sci.: Mat. Med.* **13** (2002) 701
24. R. CASANI, In ''Kirk-Othmer, Encyclopedia of Chemical Technology vol. 25, IV Edition'' (John Wiley & Sons, New York, 1997), p. 256
25. B. RINNER, V. SIEGL, P PÜRSTNER, T. EFFERTH, B. BREM, H. GREGER and R. PFRAGNER, *Anticancer Res.* **24** (2004) 495
26. K. KACZIREK, M. SCHINDL, A. WEINHÄUSEL, C. SCHEUBA, C. PASSLER, G. PRAGER, M. RADERER, G. HAMILTON, M. MITTELBÖCK, V. SIEGL, R. PFRAGNER and B. NIEDERLE, *J. Clin. Endocrinol. Metab.* **89** (2004) 2397
27. R. PFRAGNER, A. BEHMEL, E. INGOLIC and G. H. WIRNSBERGER, Culture of Human Neuroendocrine Tumor Cells, In ''Culture of Human Tumor Cells'', edited by R. Pfragner and R. I. Freshney (Wiley-Liss, Hoboken, NJ, 2004), p. 373