Tests of biocompatibility of α-tocopherol with respect to the use as a stabilizer in ultrahigh molecular weight polyethylene for articulating surfaces in joint endoprostheses

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To inhibit the oxidation *in vivo* of hip-cups made of ultrahigh molecular weight polyethylene (UHMW-PE), the natural antioxidant α-tocopherol was added to the polymer. The added α-tocopherol may however undergo chemical transformations during manufacturing and sterilization by γ-irradiation of hip-cups which may differ from human metabolism. Therefore, the question of the biocompatibility of the respective transformation products was investigated on test samples, which were prepared under the same conditions as applied for the production and sterilization of hip-cups.

Thin plates ($25 \times 18 \times 2\,mm^3$) were fabricated out of test samples to investigate the cytotoxic activity according to EN 30993-5. In cytotoxicity testing, proliferation, mitochondrial activity and membrane integrity were not influenced by the material. In contrast, cell adhesion and cell spreading were diminished as shown with hemalum staining.

In order to investigate the genotoxicity, the α-tocopherol and its transformation products were extracted from test specimens by *n*-heptane at 185 °C under nitrogen atmosphere. Then the *n*-heptane was evaporated *in vacuo* and the remaining α-tocopherol and its transformation products were dissolved in DMSO. The genotoxicity of this extract was then tested by the Ames-test according to DIN UA 12 (1995), which showed no indication for genotoxic activity.

1. Introduction

Several preceding studies have shown that the *in vivo* oxidation of ultrahigh molecular weight polyethylene (UHMW-PE) used for articulating surfaces in joint-endoprostheses frequently caused failures of endoprostheses after 10 to 15 years due to molecular degradation and crystallization of the UHMW-PE followed by an enhanced formation of PE-debris [1–7]. To inhibit this oxidation, the stabilization of UHMW-PE with α-tocopherol (vitamine E), a natural antioxidant, was suggested by B. Dolezel and L. Adamirova [8]. In the human body, α-tocopherol acts as a scavenger of free radicals and protects the LDLs (low density lipoproteins) from being oxidized [9].

Previous examinations concerning the suitability of α-tocopherol as a stabilizer for UHMW-PE [10, 11] have proven α-tocopherol to have an impressive stabilization efficiency even compared to highly efficient technical antioxidants, which are however not permitted for applications *in vivo* due to their toxicity. These examinations also revealed that α-tocopherol partially degrades during sintering and sterilization by γ-irradiation [12], the standard processing steps for artificial hip-cups made of UHMW-PE. Fig. 1 shows HPLC-diagrams of UHMW-PE samples stabilized with α-tocopherol after different processing steps, where the α-tocopherol was re-extracted with *n*-heptane after each step. The formation of transformation products can reach up to 34% of the initial mass of α-tocopherol [12]. It may 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. The biocompatibility of these transformation products is still unknown and was hence investigated in this study.

When regarding the chemical structure of α-tocopherol (Fig. 2) with a phenolic group in para-position to the oxygen of the chroman ring [13], the stabilization

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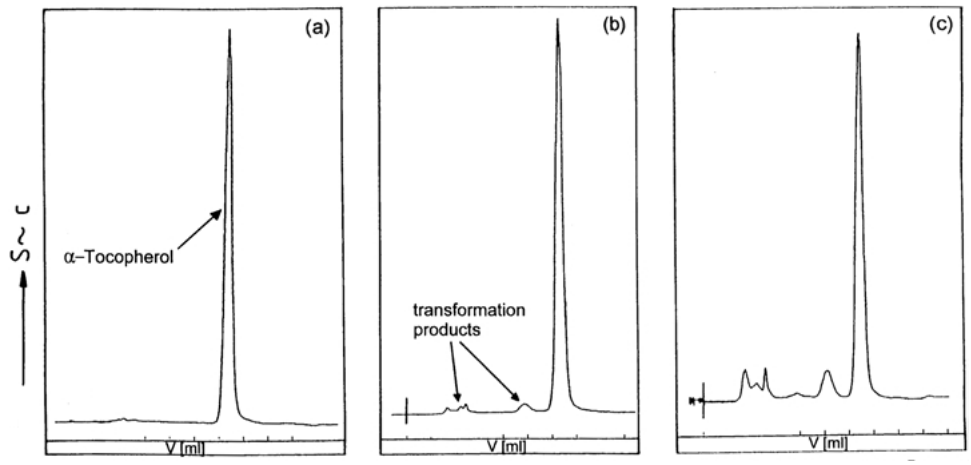

Figure 1 HPLC-data of α-tocopherol and its transformation products, extracted from UHMW-PE samples before processing (a), after sintering without special precautions to exclude oxygen (b) and after sterilization with γ-rays under nitrogen (c).

mechanism becomes quite obvious and is the same as with standard hindered phenols: the α-tocopherol reacts with radicals to give the stable tocopheroxyl radical (proton-transfer). The assumed final oxidation product *in vivo* is the α-tocopheryl quinone [14], while diastereoisomers of dimers and trimers as well as aldehydes were found in LDPE stabilized with α-tocopherol after several extrusion steps [15].

In this study, several screening tests concerning the biocompatibility of the transformation products of α-tocopherol during sintering and sterilization were carried out. Specimens made of UHMW-PE stabilized with 0.8% w/w α-tocopherol were prepared according to the standard processing methods of artificial hip-cups. For the cytotoxicity testing, thin plates were milled out of the UHMW-PE specimens while for the genotoxicity testing the α-tocopherol and its transformation products were re-extracted from the UHMW-PE specimens with *n*-heptane. The *n*-heptane was evaporated subsequently *in vacuo* at 45 °C and the test was carried out with the remaining concentrate.

The cytotoxicity was tested in accordance to EN 30993-5. Cell proliferation was determined via BrdU-labeling, mitochondrial activity via XTT-test, membrane integrity with the use of fluorescein diacetate and cell adhesion rate as well as cell morphology and cell spreading with hemalum staining. Genotoxicity was investigated in a bacterial reverse mutation test (Ames-test) with cultures of salmonella typhimurium bacteria cells.

2. Materials and methods

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

UHMW-PE was Hostalen GUR 1020 from Hoechst AG (now TICONA AG, Frankfurt/Main Germany), which fulfills the requirements of ISO 5834 Parts 1 and 2 (Implants for surgery – UHMW-PE powder and molded forms) and was obtained as a gift sample from the production site 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, thus 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). The ethanol was then evaporated in a vacuum-dryer at 50 °C for 6 h. Three different blends with 0.2%, 0.4% and 0.8% w/w α-tocopherol were prepared. These three samples of molding powder with α-tocopherol and neat UHMW-PE powder were each sintered to discs (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). Finally, all discs were washed and sterilized with γ-rays at 25 kGy in inert atmosphere by Sulzer Orthopedics Ltd, Winterthur, Switzerland, according to its standard procedure for artificial hip-cups.

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 ± 2% from the desired concentration.

For all toxicity testings, only samples with 0.8% α-tocopherol were used though 0.2% to 0.4% α-tocopherol are suggested as optimal concentration for UHMW-PE [10]. 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.

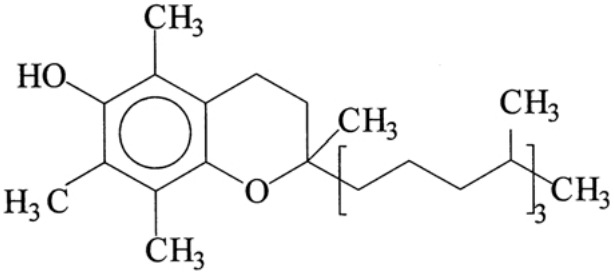

Figure 2 Chemical structure of α-tocopherol.

Thin plates ($25 \times 18 \times 2\,mm^3$) were fabricated out of the middle of the disk to investigate the cytotoxic activity according to EN 30993-5.

For the genotoxicity testing, a solution of the transformation products in DMSO was required. Thus, blocks with the dimension $150 \times 80 \times 50\,mm^3$ were cut out of the middle of the UHMW-PE-disk with 0.8% α-tocopherol and milled to small chips (thickness approx. 0.5 mm). These chips (1250 g) were filled in an autoclave vessel with *n*-heptane/*i*-propanol 995/5 v/v (which was also used as eluent in HPLC analysis) and the α-tocopherol as well as its transformation products were extracted from the UHMW-PE at 185 °C for 90 min under nitrogen atmosphere. After filtering off the PE, the *n*-heptane was evaporated *in vacuo* at 50 °C and the remaining α-tocopherol and its transformation products (approx. 20 ml) were dissolved in DMSO [12].

2.2. HPLC

Analytical HPLC was performed at room temperature with a flow rate of 1 ml/min using a normal-phase silicagel column (13 µm, Glass Works Kavalier, Votice, Czech Republic). The wavelength of the UV/Vis detector (ATI/Unicam UV/Vid.detector UV4-200) was set to 292 nm, the eluent was *n*-heptane/*i*-propanol 995/5 v/v.

2.3. Cytotoxic testings

All tests were carried out at the BMP, Labor für medizinische Materialprüfung GmbH, Aachen, according to EN 30993-5. The tests included a cell proliferation test (BrdU labeling), the XTT-test for mitochondrial activity, a membrane integrity test and a cell straining with hemalum for the detection of cell adhesion and changes of cell morphology. Thin plates ($25 \times 18 \times 2\,mm^3$) of stabilized UHMWPE specimens were used to investigate the cell cytotoxicity. As negative controls, a non-toxic material (''Biofolie'', Sartorius, Göttingen, Germany) was used for the direct tests (morphology, membrane integrity) and culturmedium RPMI 1640 (Bio Whittaker, 12-167-B, Verviers, Belgium) for the indirect tests (BrdU-, XTT-test). As positive controls we used PVC with toxic additives (Rehau, Rehau, Germany) for the direct tests and 10% ethanol for the indirect tests.

For the investigation of cell morphology and membrane integrity, 50 000 cells/ml of the L929 cell line (German Collection of Microorganisms and Cell Cultures, DSM ACC2, Braunschweig, Germany) were seeded on the probes and cultured for 24 h in RPMI 1640 medium supplemented with 10% fetal calf serum (BioWhittaker, 14–60 1F Lotnr. 5SB0013, Verviers, Belgium), penicillin/streptomycin solution (100 units/ml; 100 µg/ml, PAA, P11-010, Linz, Austria). Cells were maintained in a humidified atmosphere containing 7.5% CO_2 at 37 °C for 24 h. For the investigation of cell morphology, cells were fixed and stained with hemalum. Adhesive cells were counted and cell spreading as well as intracellular changes examined.

For the investigation of membrane integrity, cells were stained with fluorescein diacetate and ethidium bromide. Fluorescein diacetate can permeate into vital cells and colors the membrane green. Ethidium bromide invades into cells with damaged membranes and colors the nucleus of these cells red.

For indirect testing of cytoxicity, the plates were extracted in RPMI 1640 cell medium supplemented with 10% fetal calf serum for 72 h at 37 °C. Extraction medium of 1 ml was added to 3 cm^2 of the sample. Extraction medium was given to cells (30 000 cells/ml) for 24 h.

Cell proliferation was tested by detection of 5-bromo-2-deoxy-uridine (BrdU) incorporated into cellular DNA (Boehringer-Mannheim kit no. 1647229). Analysis was performed as recommended by the manufacturer.

Mitochondrial activity of the cells was tested by a colorimetric assay based on the cleavage of XTT to a soluble formazan salt (Boehringer Mannheim kit no. 1465015). This conversion occurs only in viable cells by active mitochondria. After an incubation period of 2 h, the formazan dye formed was quantitated using an ELISA reader (SLT, Spectra Shell, Crailsheim, Germany). Analysis was performed as recommended by the manufacturer.

2.4. Genotoxic testing – Ames-test

The bacterial reverse mutation test (Ames-test) was carried out by the Institut für Hygiene und Umweltmedizin, RWTH Aachen, according to DIN UA 12 (1995).

The extract of α-tocopherol and its transformation products dissolved in DMSO (see Section 2.1) was diluted to 4% aqueous DMSO with distilled water and filtered with a 0.2 µm sterile filter. Salmonella typhimurium TA 98 and TA 100, each with and without metabolic activation, were treated with the aqueous DMSO in the dilution steps from 1 : 1 to 1 : 16. Two sets of tests were carried out for each cell strain.

3. Results

3.1. Cytotoxic testing

Proliferation rate and mitochondrial activity of L929 cells was not influenced by the extraction medium from the stabilized UHMW-PE specimens, as shown in Fig. 3

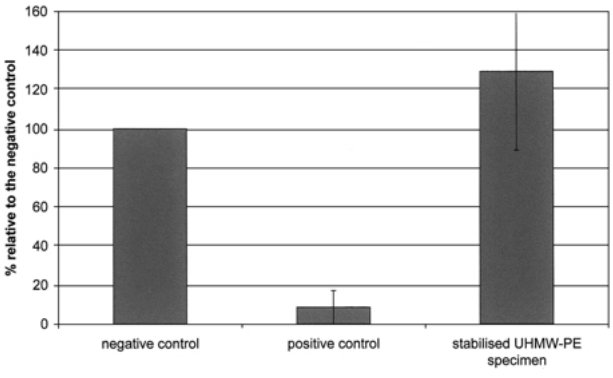

Figure 3 Proliferation activity of L929 cells after 24 h incubation with extraction medium of stabilized UHMW-PE specimens, negative control and positive control (10% ethanol), measured with BrdU-labeling.

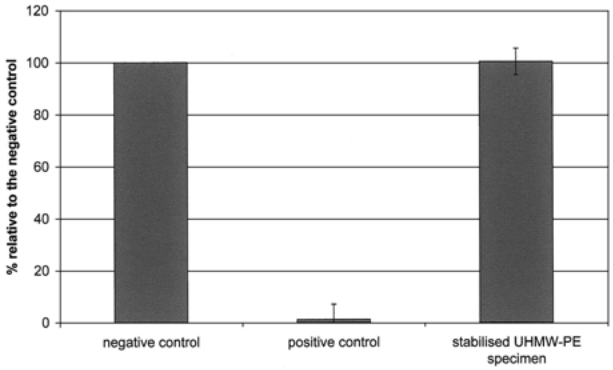

Figure 4 Mitochondrial activity of L929 cells after 24 h incubation with extraction medium of stabilized UHMW-PE specimens, negative control and positive control (10% ethanol), measured with XTT-test.

and Fig. 4. Membrane integrity of the cells seeded on the stabilized UHMW-PE specimens was also not influenced (not shown). There was no sign of cytotoxicity in these three tests.

However, as shown by direct hemalum staining of the cells 24 h after seeding on the stabilized UHMW-PE specimens, cell adhesion rate was diminished to 37% of the negative control. The cells did not spread in their typical way, as compared the negative control sample. Cells on the stabilized UHMW-PE specimens showed a rounded morphology and a smaller diameter (Fig. 5). There was no sign of direct cell toxicity.

3.2. Genotoxic testing – Ames-test

No genotoxic activity could be found in the bacterial reverse mutation test (DIN UA 12 1995). The induction rate (number of mutated cells treated with the test substance compared to the number of mutated cells of a negative control set) never exceeded 1.6. Substances are considered to be genotoxic at an induction rate of 2 or higher.

4. Conclusion

In this study the biocompatibility of transformation products of α-tocopherol used as a stabilizer for UHMWPE was investigated. These transformation products are formed during processing and probably differ from the oxidation products of α-tocopherol formed in human metabolism. Thus the nontoxicity of these substances must be proved before α-tocopherol can be used as a stabilizer for UHMW-PE-implants.

The cytotoxicity testing showed no evidence of a cytotoxic behavior of stabilized UHMW-PE specimens. Thus, the stabilization of UHMW-PE with α-tocopherol does not influence the known biocompatibility of UHMW-PE. However, adhesion rate and cell spreading were diminished. That means, that this material is biocompatible, but not bioactive. It should not be used as a material for implants, on which cells should adhere to build a strong contact between implant and body. But if UHMW-PE is used in orthopedic surgery, it has no direct contact to the surrounding bone, because it is either cemented or an inlay in a metal cup.

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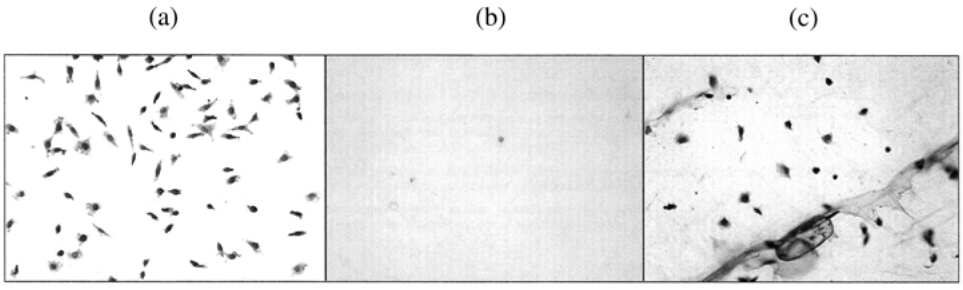

Figure 5 Hemalum staining of L929 cells after a 24 h incubation period on the negative control (a), positive control (b) and stabilized UHMW-PE specimens (c): Note the normal adhesion rate and cell spreading of the L929 cells seeded on the negative control. In contrast, there are no viable cells detectable on the positive control. On the stabilized UHMW-PE specimens, adhesion rate and cell spreading were diminished in comparison to the negative control. Cell showed a rounded morphology and a smaller diameter.

References

1. M. GOLDMAN, R. GRONSKY, R. RANGANATHAN R. and L. PRUITT, *Polym.* **37** (1996) 2909.
2. P. EYERER, *Kunststoffe* **77** (1987) 617.
3. F. J. BUCHANAN, B. SIM and S. DOWNES, *Plast., Rub. and Comp. Proc. and Appl.* **27** (1998) 148.
4. E. BRACH DEL PREYER, M. CROVA, L. COSTA, A. DALLERA, G. CAMINO and P. GALLINARO, *Biomaterials* **17** (1996) 873.
5. L. COSTA, M. P. LUDA, L. TROSSARELLI, E. M. BRACH DEL PREVER, M. CROVA and P. GALUNARO, *ibid.* **19** (1998) 659.
6. L. COSTA, M. P. LUDA, L. TROSSARELLI, E. M. BRACH DEL PREVER, M. CROVA and P. GALLINARO, *ibid.* **19** (1998) 1371.
7. M. GOLDMAN, M. LEE, R. GRONSKY and L. PRUITT, *J. Biomed. Mat. Res.* **37** (1997) 43.
8. B. DOLEZEL and L. ADAMIROVA, CS patent 221 403 (1981).
9. B. FREI, K. BRIVIBA and H. SIES in ''Natural Antioxidants in Human Health and Disease'' (Academic Press, San Diego, 1994) p. 107.
10. C. WOLF, T. KRIVEC, J. BLASSNIG, K. LEDERER and W. SCHNEIDER, *J. Mat. Sci.: Mat in Med.*, in print.
11. S. AL-MALAIKA, C. GOODWIN, S. ISSENHUTH and D. BURDICK, *Polym. Deg. Stab.* **64** (1999) 145.
12. C. WOLF in ''Tests of Biocompatibility of α-Tocopherol for the Use as a Stabilizer in Ultra-High Molecular Weight Polyethylene Cups of Hipendoprostheses'' (Diploma thesis, University of Leoben, department for Chemistry of Polymeric Materials, Leoben, 2000).
13. S. LAERMER in ''Proceedings of the ADDCON 95, Worldwide Additive and Polymer Conference'', Basel, April 1995.
14. R. CASANI in ''Kirk-Othmer, Encyclopedia of Chemical Technology Vol. 25, IV Edition'' (John Wiley & Sons, New York, 1997) p. 256.
15. S. AL-MALAIKA, H. ASHLEY and S. ISSENHUTH, *J. Polym. Sci. Part A: Polym. Chem.* **32** (1994) 3099.

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