In vitro cytotoxicity of E-glass fiber weave preimpregnated with novel biopolymer

M. VÄKIPARTA*, M. K. KOSKINEN
Turku Centre for Biomaterials, Institute of Dentistry, University of Turku, Itäinen Pitkäkatu 4B, FIN 20520 Turku, Finland
E-mail: marju.vakiparta@utu.fi

P. VALLITTU, T. NÄRHI, A. YLI-URPO
Department of Prosthetic Dentistry and Biomaterials Research, Institute of Dentistry, University of Turku, Lemminkäisenkatu 2, FIN 20520 Turku, Finland

The aim of this study was to investigate cytotoxicity of composition of E-glass fibers and novel biopolymer of poly(hydroxyproline). Growth and proliferation of the human gingival fibroblast cells on the surface of the materials was evaluated. The number of cells grown and proliferated on cell culture plastic was used as a control. Bi-directional fiber weaves were preimpregnated with poly(hydroxyproline). Cytotoxicities of the preimpregnated and non-impregnated materials were evaluated by the release of lactate dehydrogenase from the cells during the culture period of 24 h. The values of the lactate dehydrogenase activity of the materials' extracts showed non-toxicity for poly(hydroxyproline) preimpregnated E-glass fiber weaves. The growth of fibroblasts on the surface of the materials appeared normal after 11 days culture period; they looked healthy and normal in size and shape. The results of this study suggest that based on its' non-cytotoxicity the composition of E-glass fibers and poly(hydroxyproline) can further be evaluated as a material that is suitable for biomedical use.

1. Introduction

In recent years efforts have been made to reinforce non-resorbable polymers used in medicine and dentistry with fibers to improve their mechanical properties [1, 2]. Glass fibers are often used for reinforcing polymers because of their good adhesion to polymers via silane coupling agents [3]. Formation of so-called interpenetrating polymer network (IPN) layer (so-called sizing) between the matrix polymer and glass fibers was suggested to be further enhancing the adhesion between the polymer matrix and glass fibers [4]. The IPN structure was formed from linear polymer of the sizing, which is partially or totally dissolved by bi- or multifunctional acrylate monomers of the matrix [5]. Completion of polymerization formed IPN interphase between the glass fiber and cross-linked matrix, which increased the flexural strength of composite [4, 5].

Recent development in bioresorbable polymers has produced new types of linear polymers like biodegradable poly(hydroxyproline) [6]. Linear polymer chain structure of this polymer allows its use as a component for IPN structures and it was tested as sizing for glass fibers [4]. Fiber-reinforced composite containing poly(hydroxyproline) sizing may provide improved biocompatibility because of its poly(amino acid)-containing matrix. Amino acid based polymethyl methacrylate has also been investigated to be used as bone cement or in tissue repair applications to enhance their biocompatibility, and to improve the growth of tissue components into the matrix polymer [7]. Furthermore, the fiber-reinforced poly(hydroxyproline) containing materials can be used in various tissue engineering matrixes.

In this study, we describe the preimpregnating of the E-glass fiber weave with synthetic linear biopolymer of poly(4-hydroxy-L-proline). Our further aim was to examine possible cytotoxicity of the materials and to evaluate the proliferation rate of human gingival fibroblast cells on the surfaces of the experimental materials.

2. Materials and methods

2.1. Polymer

The experimental biopolymers, poly(trans-4-hydroxy-L-proline) ester (PE) and poly(trans-4-hydroxy-L-proline) amide (PA) were synthesized as described by Puska *et al.* [8, 9]. The polyester and polyamide of hydroxyproline were synthesized by melting condensation polymerization. Trans-4-hydroxy-L-proline was first converted into an ester by esterification. For synthesizing the PE, the monomer ester was benzylated and polymerized using catalyst (titanium acetyl acetonate). After polymerization

*Author to whom all correspondence should be addressed.

TABLE I Description of the test groups

Group	Description	Cytotoxicity	Cell proliferation assay
Control	Plain cell culture plate well	X	X
GF	E-glass fiber net in TFE[a]	X	X
GF-PA1	E-glass fiber net preimpregnated with PA1[b]	X	X
GF-PA2	E-glass fiber net preimpregnated with PA2[c]		X
GF-PE	E-glass fiber net preimpregnated with PE[d]	X	X

[a]Trifluoroethanol.
[b]Poly(trans-4-hydroxy-L-proline) amide, $M_w = 2200$ g/mol.
[c]Poly(trans-4-hydroxy-L-proline) amide, $M_w = 4400$ g/mol.
[d]Poly(trans-4-hydroxy-L-proline) ester.

the protection group of benzyl was removed and the pure PE was obtained. For synthesized the PA, the isolated and purified monomer were subjected to melt-polycondensation at elevated temperatures in vacuo. The molecular weight of the polymer was controlled by polymerization process. In this study the used molecular weight of the PE was 10 000 g/mol and PA 2200 g/mol (PA1) or 4400 g/mol (PA2). PE and PAs were solid after polymerization. For the use in preimpregnating a solution of PE and PAs was made by adding trifluoroethanol (TFE) as solvent so that polymer concentration was 50 mg/ml for PE and PA1. For PA2, the polymer concentration was only 34 mg/ml because of its lower solubility compared to PA1.

2.2. Preimpregnated glass fiber weaves

Bi-directional silanized E-glass fibers, i.e. fiber weaves (StickTech Ltd, Turku, Finland) (chemical composition: SiO_2 55%, CaO 22%, Al_2O_3 15%, B_2O_3 6%, MgO 0.5% and Fe + Na + K less than 1.0%, diameter of fibers: 6 μm) were preimpregnated in aseptic conditions with PE, PA1 or PA2 in solution of TFE or only with the TFE. The solvent TFE was evaporated in room temperature for 24 h from the preimpregnated and non-impregnated glass fiber weaves (dimensions of glass fiber weave specimen were 1 cm × 1 cm) before tested for cytotoxicity.

2.3. Cell culture

Human gingival fibroblast cells were used in this study. The cells were isolated as described by Larjava *et al.* [10]. Cells were explanted from biopsies of human gingiva obtained during surgical tooth extractions from healthy donors. Explanted fibroblast cells were routinely cultured in DMEM (D-5648, Sigma, Steinheim, Germany) supplemented with 10% (v/v) fetal calf serum (FCS; kibbutz Beit Haemek, Israel), penicillin–streptomycin (GibcoBRL) and fungizone (GibcoBRL). In cytotoxicity extractions and overall cytotoxicity tests 20 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES, H-4034, Sigma, Steinheim, Germany) was used as an additional buffering system. The medium was changed every other day and the cells were harvested at confluency. Cells were grown under standard cell culture conditions, + 37 °C in humidified atmosphere with 5% of CO_2.

2.4. Cytotoxicity

Three different materials were investigated (Table I). Six parallel samples were present in all experiments. Preimpregnated and non-impregnated glass fiber weaves were extracted in DMEM (4 ml) mentioned above for 48 h. Human gingival fibroblasts were obtained from confluent 250-ml cell culture bottle by detaching them with trypsin, resuspending and distributing this suspension evenly within wells of 6-well plate (Greiner bio-one Cellstar®, No. 657 160), same amount of cells per each well (cell number ca. 5×10^4–10×10^4). The cells were grown to confluency (ca. 10^6 cells per well). After that, the medium was removed and extracts from the experimental materials were added in, except for background measurements (negative control) fresh culture medium was added. In order to measure the maximal toxicity, the medium was changed for fresh culture medium and Triton X-100 (Triton X-100, T-8787, Sigma) in one plate at the concentration of 1% for Triton X-100 per well. Cytotoxicity of plain glass fiber weave with TFE was also measured to exclude the effect of solvent in the samples. Cells were then incubated with the extracts for 24 h. The solution volume in each sample was 1 ml.

The toxicity of extracts was evaluated spectrophotometrically (UV-visible spectrophotometer, UV-1601, Shimadzu) [11] by measuring released lactate dehydrogenase (LDH) from the cells.

2.5. Cell proliferation assay

Proliferation of human gingival fibroblasts was evaluated for preimpregnated and non-impregnated glass fiber weaves (Table I). Culturing was done in 6-well cell culture plates (Cellstar®) using 3 ml of DMEM per well. In total of 45 000 cells per well were seeded on the samples and counted after 1, 4, 5, 6 and 11 days. The cells were detached from the wells with trypsin (GibcoBRL) and number of cells was calculated in Bürcker chamber. The detachment of cells was ensured by phase contrast microscopic evaluation, which showed that only few cells were remaining in the wells. The number of cells grown on cell culture plastic was used as a control. The medium in each well was changed every other day to maintain the optimal growing conditions. For the microscopic evaluation the samples were washed once with phosphate buffered saline (PBS), fixed with absolute ethanol and stained with crystal violet 5% (w/v) in 20% methanol (Crystal Violet, C.1. 4255; Basic Violet 3, C-3886, Sigma). Finally, the stained samples were

washed with milli-Q water (Millipore Milli-Q academic, at 18 M Ωcm resistivity) to remove excess of the color. Samples were studied through phase contrast microscope (Olympus CK40).

2.6. Statistical evaluation

The statistical analysis was performed using SSPS software for Windows (Statistical Package for Social Science, SPSS Inc., version 11.0, Chicago, USA). The mean values of activity of lactate hydrogenase among the negative control, GF, GF-PE and GF-PA1 groups were compared by one-way ANOVA followed by Tukey's post hoc test.

3. Results

3.1. Cytotoxicity

The values of lactate dehydrogenase (LDH) activity resembling the amount of released LDH from each material are presented in Fig. 1. In the maximal toxicity measurement Triton X-100 has lysed fibroblast cells and the maximal amount of LDH was released, whereas in the negative control measurement the cells were allowed to grow under optimal conditions on plain cell culture plate. The LDH activities of preimpregnated and non-impregnated materials were lower than that of cells grown under optimal culture conditions (values of $p < 0.005$, < 0.07 and < 0.5 for GF–PE, GF–PA1 and GF, respectively). Of the preimpregnated materials GF–PE had lowest LDH activity.

3.2. Cell proliferation

In the beginning of culture period fibroblast proliferation (Fig. 2) seemed to be lower on the non-impregnated fiber weave and on all preimpregnated fiber weaves than on the plain cell culture well (control). After four days the cell number on the control was almost twice as high as on the non-impregnated or preimpregnated samples. In time the cell number on the materials GF–PA1 and GF–PA2 reached almost the control well level. The number of cells seeded on GF–PE was measured only for six days. At that time fewer cells were detected from GF–PE than on any other experimental materials. Observation with a phase contrast microscopy showed that after three days (Fig. 3(d): ten days) of culture the cells had a flattened appearance and a spindle-like shape (Fig. 3).

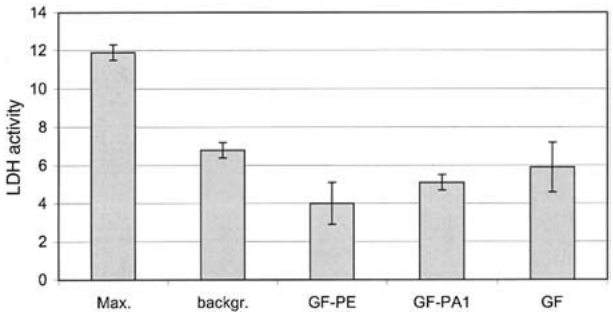

Figure 1 The values of LDH activity measured from the cells when treated with 48-h extracts.

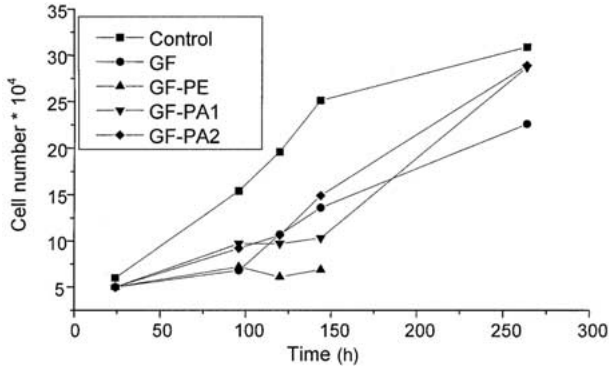

Figure 2 Cell grown measurements for PA/PE preimpregnated and non-impregnated E-glass fiber weave.

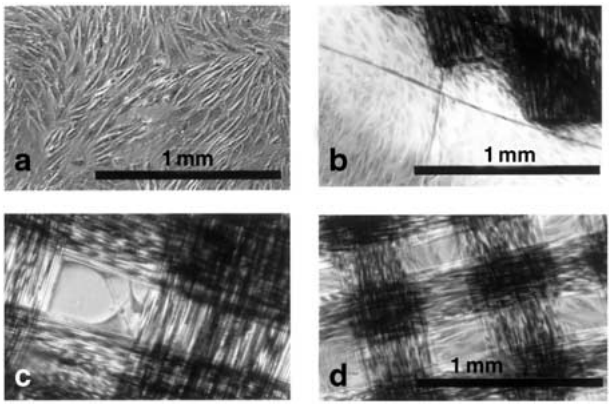

Figure 3 (a) Cells in bottom of the control well. (b) TFE treated E-glass fiber weave. (c) PA preimpregnated E-glass fiber weave attached by the cells. (d) PE preimpregnated E-glass fiber weave attached by the cells.

Trifluoroethanol treatment seemed to have no effect for the attachment of fibroblasts on E-glass fiber weave; fibroblasts attach equally well on both the GF (Fig. 3(b)) and the control surface (Fig. 3(a)). In fact, cells grew in close contact with the fiber weaves. Cells did attach and grew well on both the GF–PA1 and on the GF–PE weave (Fig. 3(c) and (d), respectively). On the bottom of each well the growth and proliferation of the cells was comparable to plain cell culture plastic. Their morphology looked also similar when compared with the cells grown in optimal conditions on a plain cell culture plastic (Fig. 3(a)).

4. Discussion

The poly(hydroxyproline) is new synthetic biopolymer, which is highly hydrophilic and dissolves easily in aqueous surrounding. This new polymer has good potential in several biomedical devices, which require fast dissolving component leaving interconnected pores or canals within the device.

In this study we investigated the cytotoxicity of the E-glass fiber weaves preimpregnated with poly(hydroxyproline). The growth of human gingival fibroblasts on preimpregnated versus non-impregnated E-glass fiber weaves was also evaluated. Non-impregnated silanized E-glass fiber weave showed no signs of cytotoxicity, as has been shown previously by the agar diffusion test [12].

There was no sign of cytotoxicity of the GF, GF–PA1 or GF–PE either. So conclusively, neither the PA nor the PE has toxic effects for human gingival fibroblasts. It can also be assumed that when the polymer dissolves from the biomedical device to the surrounding body fluid no toxic degradation products are released. It has to be noticed that the values of the LDH activity of the GF, GF–PA1 and GF–PE measured after 24 h were slightly below that of the control. This may be related with the differences in the proliferation rates of the cells towards negative control at point of cytotoxicity measurements. The differences among the samples were not statistically significant, but the trend was noticed that the values of LDH activity decreased from GF to GF–PE. Only statistically significant difference was found between the GF–PE and the negative control. During the first 48 h the cells grew better in optimal conditions on a cell culture plastic than on any of the experimental materials. This can be due to the weaker attachment of the cells to the surface of the experimental materials than to the cell culture plastic. Probably in time the cells will cover the whole material surface and eventually the degree of the surface coverage will be the same than in the control samples. However, preimpregnation of fiber weave with PA1 or PA2 may improve cell proliferation compared with non-impregnated fiber weave. Probably hydrophilic poly(hydroxyproline) makes the surface of the inert E-glass fiber weave more hydrophilic, which improves the cell attachment on the surface. These results indicate that poly(hydroxyproline) containing materials are suitable for the attachment and proliferation of human gingival fibroblasts. The accumulation of endotoxins was avoided by changing the medium reasonably often.

This study also revealed that poly(hydroxyproline) impregnation can improve cell attachment and support the growth of fibroblasts on E-glass fiber weaves in the early stages of healing. Additional studies are needed with non-adhesive cell culture plate to exclude the effect of optimal conditions created by the cell culture plate. Furthermore, behavior of impregnated materials has to be evaluated *in vivo*, before any final conclusions about their biological effects can be made.

5. Conclusions

No signs of cytotoxicity have been noticed in PA or PE preimpregnated E-glass fiber weaves. The cells seem to attach well on the surface of the preimpregnated and non-impregnated E-glass fiber weaves and the proliferation of the cells is comparable to the cells grown in optimal cell culture conditions.

Acknowledgment

The National Technology Agency (TEKES) is gratefully acknowledged for financial support. The authors belong in the bio- and nanopolymer research group, which forms a Center of Excellence (code number #77317) funded jointly by the Academy of Finland and the National Technology Agency.

References

1. W. PETTY, *J. Biomed. Mat. Res.* **14** (1980) 427.
2. S. SAHA and S. PAL, *J. Biomech.* **17** (1984) 467.
3. P. K. VALLITTU, *Quintessence Int.* **28** (1997) 39.
4. M. VÄKIPARTA, A. YLI-URPO and P. VALLITTU, *J. Mater. Sci. Mater. Med.* (submitted).
5. L. H. SPERLING, in ''Overview of IPNs. Interpenetrating Polymer Networks'', edited by D. Klempner, L. H. Sperling and L. A. Utracti, Advances in Chemistry Series: 239 (American Chemical Society, Washington DC, 1994) p. 4.
6. Y. LIM, Y. H. CHOI and J. PARK, *J. Am. Chem. Soc.* **121** (1999) 5633.
7. E. MÜH, J. ZIMMERMAN, U. KNESER, J. MARQUARDT, R. MÜLHAUPT and B. STARK, *Biomaterials* **23** (2002) 2849.
8. M. A. PUSKA and O. E. O. HORMI, *Polym. Preprints* **41** (2000) 1050.
9. M. A. PUSKA, A. K. KOKKARI, T. O. NÄRHI and P. K. VALLITTU, *Biomaterials* **24** (2003) 417.
10. H. LARJAVA, J. HEINO, T. KRUSIUS, E. VUORIO and M. TAMMI, *Biochem. J.* **256** (1988) 35.
11. C. KORZENIEWSKI and D. M. CALLEWAERT, *J. Immunol. Meth.* **64** (1983) 313.
12. P. K. VALLITTU and K. EKSTRAND, *J. Oral. Rehabil.* **26** (1999) 666.

Received 18 November 2002
and accepted 9 July 2003