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In vitro evaluation of biocompatibility of different wound dressing materials

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The *in vitro* biocompatibility of newly developed wound dressings consisting of different chitosan salts (chitosan lactate, glutamate and chloride) and a chitosan derivative (methylpyrrolidinone chitosan) was compared with three commercially available wound dressings made of collagen, calciumalginate, and gelatin, by evaluation in a fibroblast cell culture system. Three experimental models which reflect relevant stages of wound healing were used, and the significant influence of the experimental setting on the results was demonstrated. Collagen and methylpyrrolidinone chitosan were the most compatible materials under the investigated test conditions. Chitosan chloride and glutamate were the least compatible substances. The results indicated that wound dressings made of chitosan lactate and methylpyrrolidinone chitosan as well as the three commercially available dressings are well tolerated.

1. Introduction

During the complex event of wound healing, fibroblasts are the first cell population to appear in the wound after the end of the inflammatory phase [1]. Cytocompatibility with this cell population is a fundamental aspect of the overall judgment of a wound dressing material. Some main requirements that have to be met by wound dressings are listed below [2]:

- maintenance of a sufficient humidity
- absorption of exudative fluids and toxins
- gaseous exchange
- thermal isolation
- protection from infection
- absence of toxic compounds
- possibility of dressing change without irritation

Chitosan is a cationic glucosamine composed of 1–4 β-linked glucosamine and N-acetylglucosamine residues. The polysaccharide has been investigated for many interesting applications in the pharmaceutical and medical field such as contact lenses and sutures [3]. In pharmaceutical technology it has been used as a carrier material for the sustained release of drugs [4–7] and as a tablet binder [8–10]. It has been shown that the material and some derivatives of chitosan are capable of accelerating wound healing [11–13]. Another property of chitosan which makes it interesting for use as a wound dressing is its antimicrobial activity against bacteria and fungi (e.g. strains of E. coli, Klebsiella, Enterobacter, Proteus, Pseudomonas and Candida) [14, 15]. In the present study we compare four different chitosan fleeces with three commercially available dressing materials: a collagen fleece for the treatment of burns, a gelatin sponge used as a haemostyptic in dentistry and for the coverage of burns, and a calcium alginate fibre pad for the management of ulcers and burns.

To evaluate the influence of these materials on the viability of fibroblasts a direct contact *in vitro* assay using the activity of mitochondrial enzymes as indicator reaction measured by MTT-test [16] was developed. Three different experimental settings were employed:

A. seeding and growth of the cells on the materials;
B. addition of the materials during the early logarithmic growth phase to a cell culture system;
C. addition of the materials to confluent cells during the plateau phase.

Settings A and B serve as models for fibroblast migration into injured tissue and proliferation in the wound. Setting C evaluates the influence of the materials on newly formed granulation tissue.

2. Materials and methods

2.1. Cell line

Swiss 3T3 fibroblasts were a gift from SCIOS NOVA Inc., Mountain View, California, USA. A pool of cells of passage 4 was stored under standard conditions (at −189 °C under liquid nitrogen) in aliquots of 1 ml containing 1.4 Mio. cells. Dimethylsulfoxide was used as cryoprotectant.

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2.2. Culture medium

Dulbecco's modified Eagles Medium without sodiumpyruvate but with 4500 mg/ml glucose (Gibco, Paisley, Scotland) supplemented with 2 mM glutamine (Gibco, Paisley, Scotland), 50 U/ml penicillin and 50 µg/ml streptomycin (Biochrom KG, Berlin, Germany).

2.3. Cell culture conditions

Prior to an experimental run, cells were subcultured as follows: new cultures were initiated by seeding the cells from one stock vial into a 25 cm^3 tissue culture flask (Greiner, Frickenhausen, Germany) in 5 ml of growth medium. After 4 h of incubation at 37 °C in a humidified atmosphere containing 10% CO_2 the medium was changed in order to remove cytotoxic residues of DMSO. Cells were passaged at their late logarithmic growth phase (3 days later) and were seeded into 75 cm^3 tissue culture flasks at a density of 40 000 cells/ml in 15 ml of growth medium. Viability of cells was confirmed by microscopic phase contrast observation of the subcultures and by trypan blue (Biochrom KG, Berlin, Germany) exclusion test.

2.4. Preparation of the samples

Methylpyrrolidinon chitosan (MP-chitosan) was provided by Professor R. Muzzarelli, Faculty of Medicine, University of Ancona, Italy. Chitosan CTA-3 was purchased from Katakura Chikkarin Ltd, Tokyo, Japan. Chitosan glutamate Pronova MV (Poly-D-Glucosaminehydroglutamate) was obtained from Protan A/S, Drammen, Norway. Lactic acid and hydrochloric acid were of analytical grade (E. Merck, Darmstadt, Germany).

Chitosan fleeces were prepared by freeze drying as follows: solutions of methylpyrrolidinon chitosan or chitosan glutamate in demineralized water and CTA-3 either in 0.6% lactic acid or in 0.25% hydrochloric acid were prepared under aseptic conditions by stirring overnight with a magnetic stirrer at 200 rpm. The acid concentrations used with CTA-3 were the minimal amounts necessary for the solubilization of chitosan. The solutions were poured into petridishes (diameter 50 mm, Greiner, Frickenhausen, Germany), cooled down to − 30 °C, and freeze dried at 20 °C for 18 h to yield a final pressure of 0.267 Pa in a freeze drier LN2 (Edwards Kniese & Co., Marburg, Germany).

Lyostypt^R (B. Braun Melsungen AG, Melsungen, Germany) a collagen fleece, Sorbalgon^R (P. Hartmann AG, Heidenheim, Germany), a non-woven pad made of calcium alginate fibres, and Gelita^R (B. Braun Melsungen AG, Melsungen, Germany) a gelatin sponge were used in the commercially available form.

All materials were cut into small pieces of 4 × 4 mm size under aseptic conditions.

2.5. Test conditions

2.5.1. Cell growth on the samples

The test samples were immersed in 24-well cell culture plates (Costar, Bodenheim, Germany) using one well per sample and three wells for one type of material. Each well was filled with 0.5 ml of growth medium in order to soak the specimens with medium. The plates were incubated at 37 °C, 10% CO_2 in a humidified atmosphere. After 3h incubation the cells were seeded on each specimen at a density of 48 000 cells/ml in aliquots of 0.5 ml to reach a final cell content of 24 000 cells per well. At day 1, 2, 3, 4, 7, 9, and 14 an MTT-test was performed, as described below.

2.5.2. Cells in their logarithmic growth phase

Cells were seeded into 24-well cell culture plates in aliquots of 1 ml/well at a density of 24 000 cells per well and were incubated at 37 °C, 10% CO_2 in a humidified atmosphere. After the cells had become adherent (after 4h), the samples were added to separate wells using three wells for one type of material. The MTT-test was performed on days 1, 2, 3, 4, 7, and 9.

2.5.3. Confluent cells

20 000 cells were seeded into 24-well cell culture plates in 1 ml of growth medium. During the following 6 days cells were grown to confluency under the same conditions as described above. The samples were added and on day 1, 2, 3, 6, and 14 MTT-tests were performed.

Wells without test samples served as controls.

2.6. MTT-test [16]

At a time 4 h prior to the end of the desired incubation time, 150 µl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetraolium-bromide (MTT, Sigma Chemicals, Deisenhofen, Germany) solution (4 mg/ml in phosphate-buffered saline) were added to each well and the plates were incubated at 37 °C, 10% CO_2 and 96% humidity. At the end of the incubation time the medium was removed under vacuum, and the formazan crystals were dissolved in DMSO for photometric measurement (E. Merck, Darmstadt, Germany) by vibrating on a plate shaker. The solutions of each well were pipetted in three aliquots of 150 µl into a 96-well microtiter plate (Nunc, Wiesbaden-Biebrich, Germany), and the optical densities were read at 540 nm using an ELISA Reader (Easy Reader EAR 400 ATC, SCT-Lab Instruments, Crailsheim, Germany). The reference wavelength was 650 nm.

3. Results and discussion

3.1. Compatibility during growth on the samples

Methylpyrrolidinone chitosan and Lyostypt^R showed similar growth curves with OD-values (optical densities) 0.2 lower than the controls without test samples. Chitosan Lactate, Sorbalgon^R and Gelita^R had a growth delay but afterwards reached the OD-values of methylpyrrolidinone chitosan and Lyostypt. Cells grew slowly on chitosan glutamate. This material

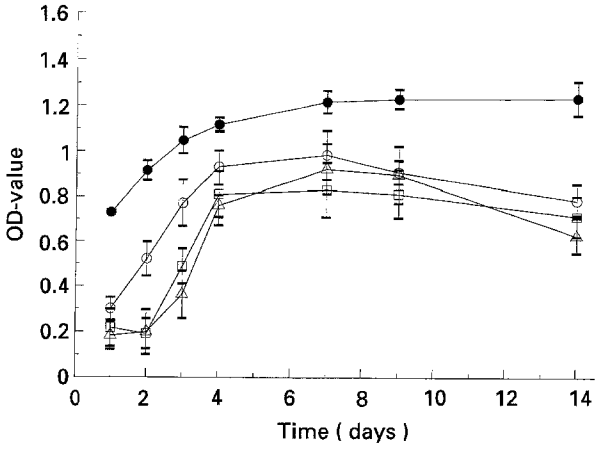

Figure 1 Compatibility of commercially available wound dressings with Swiss 3T3 fibroblasts during cell growth on the samples: Controls –●–, Lyostypt –○–, Sorbalgon –△–, Gelita –□–.

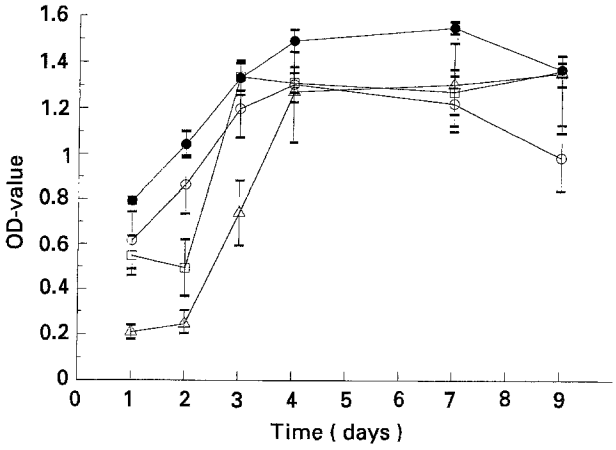

Figure 3 Compatibility of commercially available wound dressings with Swiss 3T3 fibroblasts in their logarithmic growth phase: Controls –●–, Lyostypt –○–, Sorbalgon –△–, Gelita –□–.

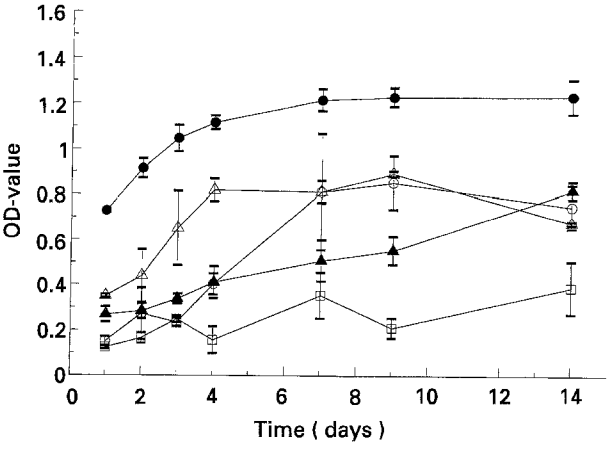

Figure 2 Compatibility of chitosan fleeces with Swiss 3T3 fibroblasts during cell growth on the samples: Controls –●–, MP-chitosan –△–, Chitosan lactate –○–, Chitosan chloride –□–, Chitosan glutamate –▲–.

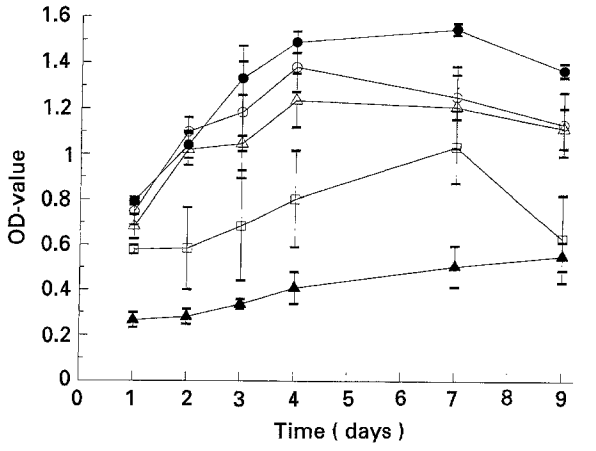

Figure 4 Compatibility of chitosan fleeces with Swiss 3T3 fibroblasts in their logarithmic growth phase: Controls –●–, MP-chitosan –○–, Chitosan lactate –△–, Chitosan chloride –□–, Chitosan glutamate –▲.

yielded OD-values of chitosan lactate and methylpyrrolidinone chitosan on day 14. Chitosan chloride caused a distinct growth delay which lasted until the end of the observation time (Figs 1 and 2). In summary, the following rank order in biocompatibility was obtained:

Methylpyrrolidinone chitosan = Lyostypt^R > chitosan lactate = Gelita^R = Sorbalgon^R > chitosan glutamate > chitosan chloride

3.2. Compatibility during the logarithmic growth phase

Methylpyrrolidinone chitosan and Lyotypt^R showed nearly the same growth curves as the controls. Until the second observation day chitosan lactate had OD-values similar to the controls. From the third day on, cell growth was reduced compared to the controls. After a growth delay of one day, Gelita^R reached the OD-values of the controls. Sorbalgon^R also showed a 1 day delay but the following proliferation period was not as marked as with Gelita^R. Finally, Sorbalgon^R also reached the OD-values of control cultures. Chitosan chloride and, particularly, chitosan glutamate caused a growth delay during the whole observation time (Figs 3 and 4). Material testing during the logarithmic growth phase, therefore, resulted in the following biocompatibility rank:

Methylpyrrolidinone chitosan = Lyostypt^R > chitosan lactate > Gelita^R > Sorbalgon^R > chitosan chloride > chitosan glutamate

3.3. Compatibility with confluent cells

As shown in Figs 5 and 6, none of the investigated wound dressings reached the OD-values of the control curves. Chitosan lactate and -chloride showed markedly lower OD-values than all other materials. The commercially available wound dressings and methylpyrrolidinone chitosan were close to the control and were therefore considered to be biocompatible in this setting. The cytocompatibility of chitosan glutamate was between those groups. In conclusion, the resulting biocompatibility rank was:

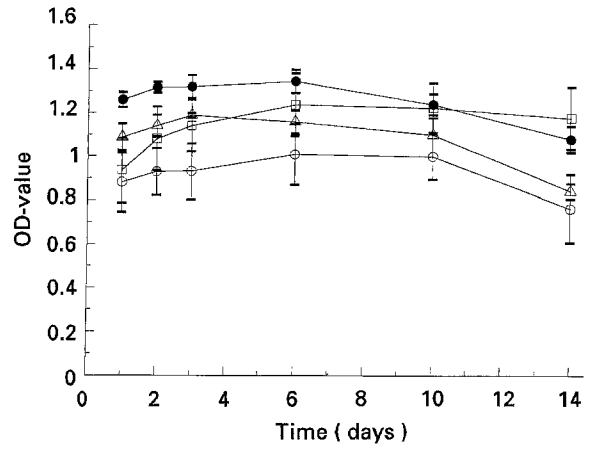

Figure 5 Compatibility of commercially available wound dressings with confluent Swiss 3T3 fibroblasts: Controls –●–, Lyostypt –○–, Sorbalgon –△–, Gelita –□–.

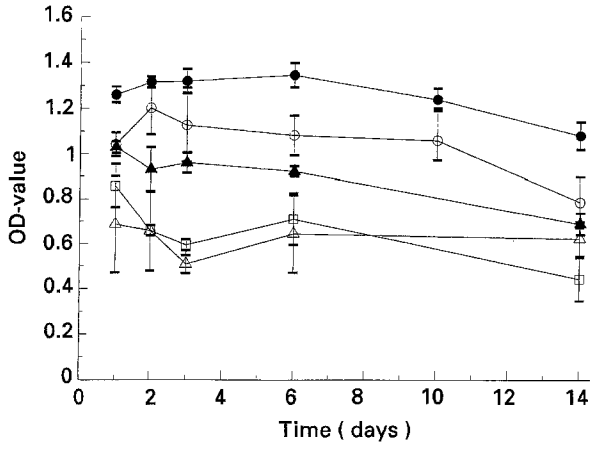

Figure 6 Compatibility of chitosan fleeces with confluent Swiss 3T3 fibroblasts: Controls –●–, MP-chitosan –○–, Chitosan lactate –△–, Chitosan chloride –□–, Chitosan glutamate –▲–.

Methylpyrrolidinone chitosan = Lyostypt^R = Sorbalgon^R = Gelita^R > chitosan glutamate > chitosan lactate = chitosan chloride

The different investigative conditions led to differing information about the biocompatibility of the materials. Confluent cells (setting C) were least sensitive to the samples and showed no distinct differences between the materials. Cells in the logarithmic growth phase (setting B) turned out to be most sensitive to different dressing compositions and therefore this was deemed to be the most suitable test system.

Considering the results of the three methods together, Lyostypt^R and methylpyrrolidinone chitosan showed the best biocompatibility, whereas chitosan chloride and -glutamate had lowest compatibility in all three tests. Gelita^R, Sorbalgon^R and chitosan lactate were of intermediate compatibility (Table I).

As indicated by the results described above, the acids in chitosan salts seem to significantly influence biocompatibility. Completely avoidance of acids by using a suitable water-soluble chitosan derivative such as methylpyrrolidinon chitosan results in a very cytocompatible material.

Further development effort in the area of chitosen-based wound dressings should therefore be directed to the development and evaluation of even more compatible salts: lactate is definitely less irritating than chloride and glutamate. Other salts might even be superior to lactate. On the other hand, the alternative of complete elimination of anionic counterparts, as in the case of methylpyrrolidinone chitosan, seems to be the most intriguing way to produce a highly compatible water-soluble chitosan.

Methylpyrrolidinone chitosan and chitosan lactate also posses excellent wound healing promoting and antimicrobial properties. Both are promising candidates for the production of new wound dressings. Consequently, they deserve further evaluation.

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TABLE I Judgement on biocompatibility of the investigated materials. The figures 1–6 a correspond to the biocompatibility rank obtained with the three methods (a)–(c)

	(a) Growth on the samples	(b) Logarithmic growth phase	(c) Confluent cells	Total of methods (a)–(c)
MP-chitosan	1	1	1	3
Chitosan glutamate	3	6	2	11
Chitosan lactate	2	2	3	7
Chitosan chloride	4	5	3	12
Sorbalgon	2	3	1	6
Lyostypt	1	1	1	3
Gelita	2	4	1	7

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