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Superabsorbent polymer-containing wound dressings have a beneficial effect on wound healing by reducing PMN elastase concentration and inhibiting microbial growth

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Abstract A comprehensive in vitro approach was used to assess the effects of superabsorbent polymer (SAP) containing wound dressings in treatment of non-healing wounds. A slight negative effect on HaCaT cells was noted in vitro which is most likely due to the Ca^{2+} deprivation of the medium by binding to the SAP. It could be shown that SAP wound dressings are able to bind considerable amounts of elastase reducing enzyme activity significantly. Furthermore, SAP's inhibit the formation of free radicals. The SAP-containing wound dressings tested also exhibited a significant to strong antimicrobial activity effectively impeding the growth of gram-negative and gram-positive bacteria as well as yeasts. In conclusion, in vitro data confirm the positive effect of SAP wound dressings observed in vivo and suggest that they should be specifically useful for wound cleansing.

Abbreviations

ECM	Extracellular matrix
IL-8	Interleukin-8
MMPs	Matrix metalloproteases
PMN granulocytes	Polymorphonuclear granulocytes
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAP	Superabsorbent polymer
TIMPs	Tissue inhibitor of matrix metalloproteases

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1 Introduction

Current concepts of modern wound management are focused on a moist wound environment, which is considered to promote optimal wound healing. Today, a variety of occlusive dressings for the treatment of chronic wounds is available in different forms, including films, foams, and gels, as well as of diverse materials such as alginates, polyurethane, hyaluronic acid, or collagen. Not all of them are able to handle the excess amount of exudate of highly exuding chronic wounds. Hence, wound dressings containing superabsorbent polymers (SAP) have been developed. SAP's are able to absorb a multiple amount of water of their own dry weight and are mainly used as an absorbent for water and aqueous solutions in diapers, adult incontinence products, feminine hygiene products, and similar applications [1]. In wound dressings, where they are preswollen with Ringer's solution before application, they can also provide moisture to the wound [2]. SAP's are mainly prepared from acrylic acid (Fig. 1) and a cross-linker by solution or suspension polymerization [1]. The resulting polyacrylate superabsorbers have a high density of ionic charges which accounts for the hydroactive properties [1] and the protein binding capacity [2, 3]. Polyacrylate containing wound dressings have been shown to be particularly effective in the cleansing phase where the proteolytic activity is high and leading to tissue breakdown [2–4]. Furthermore, Eming et al. have found that polyacrylate superabsorbers can inhibit MMP activity in vitro and ex vivo [2]. However, chronic, non-healing wounds are not only characterized by elevated levels of proteases such as matrix metalloproteinases (MMPs) [5, 6] but also by excessive amounts of PMN-derived elastase [7, 8] as well as high concentrations of free radicals [10]. These inflammatory mediators shift the balance of synthesis and

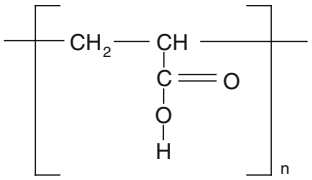

Fig. 1 Chemical structure of poly(acrylic acid). It contains an ionizable group on each repeat unit (–COOH). Typically the hydrogen is replaced by a sodium ion

degradation towards tissue destruction. Hence, the aim of this study was to investigate the binding capacity of two SAP-containing wound dressings (dressing A: Vliwasorb®, dressing B: Zetuvit® plus; Fig. 2) for elastase as well as their antioxidative potential in vitro. Previously, a comprehensive approach to characterize wound dressings in vitro has been proposed including cell culture based evaluation of biocompatibility and testing of antimicrobial activity in addition to analyzing the binding capacity for inflammatory mediators [11]. Biocompatibility has become the central request for the medical application of materials and devices. Thus, extracts of the wound dressings have been prepared according to the DIN EN ISO 10993-12 and their influence on cell proliferation was tested using HaCaT keratinocytes. Furthermore, chronic wounds are often colonized by different kinds of microorganisms, the most prominent being *Staphylococcus aureus* and *Pseudomonas aeruginosa* [12]. Previous studies showed that a hydrogel dressing with a SAP-core traps bacteria efficiently and reduces the number of viable germs [4, 13]. For this reason the antimicrobial activity of the SAP-containing wound dressings was tested against *S. aureus*, *P. aeruginosa*, *Klebsiella pn.*, and *Candida albicans* in accordance to the JIS L 1902:2002.

2 Materials and methods

2.1 Materials

The tested SAP-containing dressings (Fig. 2) were obtained from the manufacturers; dressing A, Vliwasorb®, consisting of sodium polyacrylate (CAS 9003-04-7) imbedded in cellulose, was allocated by Lohmann & Rauscher GmbH and Co. KG (Rengsdorf, Germany) and dressing B, Zetuvit® plus, composed of polyacrylate superabsorber particles [2] blended with soft cellulose fluff, was aquired from Paul Hartmann AG (Heidenheim, Germany).

Human PMN elastase standard (1 µg/ml) was taken from the Enzyme immunoassays (ELISA) purchased from Milenia Biotech (Gießen, Germany). Elastase from pig pancreas (PP elastase) was used from the EnzChek Elastase Assay Kit (Invitrogen GmbH, Darmstadt, Germany).

The human HaCaT keratinocytes were a gift from Prof. Dr. N.E. Fusenig, DKFZ, Germany. PBS (phosphate buffer saline), DMEM (Dulbecco's modified Eagle medium), Pen/Strep/Fungizone (antibiotic–antimycotic solution), FCS (fetal calf serum) and Trypsin–EDTA for cell culture were

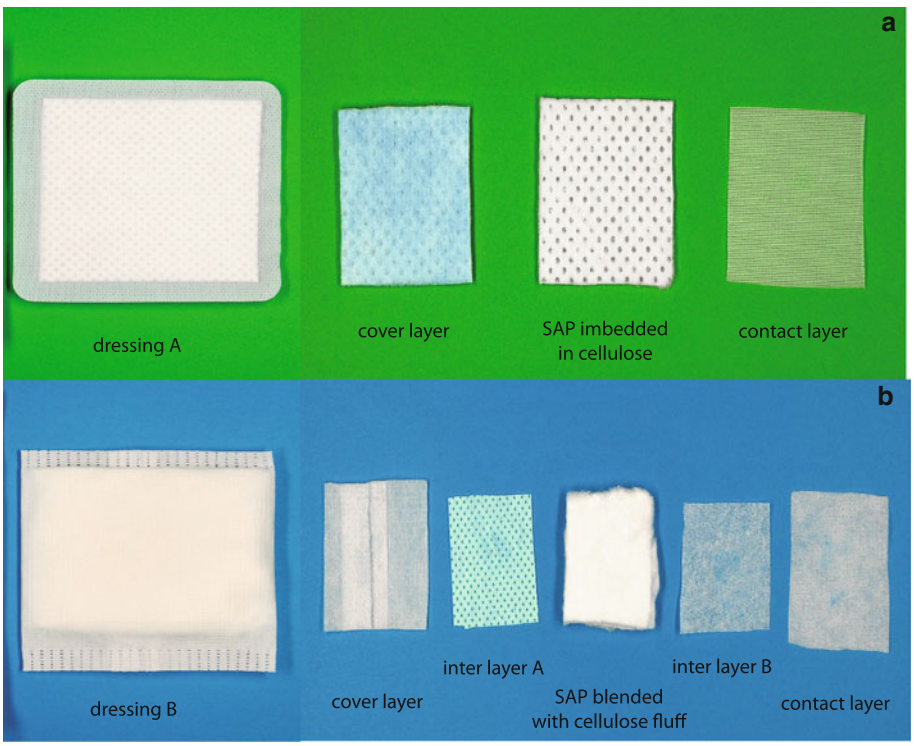

Fig. 2 Composition and assembly of the SAP-containing wound dressings A (**a**) and B (**b**). Dressing A consists of sodium polyacrylate imbedded in cellulose, an elastic wound contact layer which prevents adherence to the wound bed, and a water-impermeable cover layer. Dressing B is composed of polyacrylate blended with soft cellulose fluff. Its contact layer and inter layer B are made of pressed cellulose fibers which allow the exudate to quickly pass into the absorbent core while the surface of the contact layer has a hydrophobic finish to prevent adherence to the wound. The interlayer A of dressing B is non-woven, water-repellent, and air-permeable which is thought to protect against contamination

obtained from PromoCell (Germany). Triton-X 100 was purchased from Merck (Germany).

Staphylococcus aureus ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 4352, and *Candida albicans* ATCC 10231 were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). For cultivation of bacteria and yeast, special peptone and "lab-lemco" powder for preparation of caso-bouillon and Sabouraud-liquid-medium, respectively, were purchased from Oxoid (U.K.). 0.9% NaCl solution was obtained from Fresenius Kabi Deutschland GmbH (Germany) and Tween 20 was purchased from Roth (Germany).

2.2 Functional investigations

The lyophilized protein standards were reconstituted as recommended in the manufacturers' instructions. For further experiments the reconstituted PMN elastase standard was diluted to a concentration of 250 ng/ml. A 100 U/ml stock solution of PP elastase (purity > 95%, specific activity 20–22 U/mg) was prepared in distilled water. For experiments, the stock solution was diluted to 0.1 U/ml in the reaction buffer (0.1 M Tris-HCl, pH 8.0, containing 0.2 mM sodium azide and 0.5% bovine serum albumin). Samples of the polyacrylate wound dressings were cut using 8 mm punch biopsies (Stiefel Laboratorium GmbH, Germany) corresponding to 0.5 cm^2. The samples were placed into 24-well cell culture plates (Greiner, Germany). Each specimen was taken in a final volume of 1 ml of protease solution. Samples were incubated up to 24 h at 37°C on a plate mixer (THERMOstar, BMG Labtech GmbH, Germany). After incubation, supernatants were collected, immediately frozen and stored at −20°C until testing. Subsequently, bond protein was eluted from the individual wound dressing samples by shaking in 1 ml PBS for 1 h at 37°C. PP elastase activity in both, the supernatant and the eluates was determined using the EnzChek Elastase Assay Kit (Invitrogen GmbH, Darmstadt, Germany). The assay was run as recommended in the instructions. Briefly, 50 µl; reaction buffer and 50 µl DQ elastin substrate (elastin from bovine neck filament; with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-s-indacene-3-propionic acid; 100 µg/ml) were added followed by the injection of 100 µl sample solution. The fluorescence was measured continuously for 1 h, at room temperature (excitation wavelength: 495 nm, emission wavelength: 538 nm), using a fluorescence plate reader (FLUOstar Galaxy, BMG Labtech GmbH, Offenburg, Germany). To test whether elastase bound to the SAP was still active or not, the respective wound dressing samples were incubated with the PP elastase substrate DQ elastin (100 µl) in reaction buffer (900 µl) for 1 h. Afterwards, fluorescence was measured using a fluorescence plate reader as described (excitation wavelength: 495 nm, emission wavelength: 538 nm). For determination of the PMN elastase concentration the specific ELISA was purchased from Milenia biotec (Gießen, Germany) and run as recommended by the manufacturer. Optical density was measured at 450 nm with a reference measurement at 620 nm using a plate photometer (BMG Labtech GmbH, Germany). Subsequently, the PMN elastase concentrations were evaluated according to a 4-parameter-fit with lin-log coordinates for optical density (linear scale) and concentration (logarithmic scale).

The capability of the wound dressings tested to scavenge free radicals was assessed using the chemiluminescent ABEL® Antioxidant Test Kits specific for peroxynitrite anion and free radicals like superoxide anion containing Pholasin®. Both test kits were purchased from Knight Scientific Limited (UK). The assays were run as previously described [14]. In brief, to each sample of wound dressing (2, 3, 4, and 5 mg, respectively) the assay solutions were added. After injection of the solution generating free radicals, the measurement of luminescence was carried out using the LUMIstar Galaxy plate reader (BMG Labtech GmbH, Germany). A control without sample was run with each assay. Antioxidant activity delays the appearance of the luminescence peak (peroxynitrite assay) and lowers its light intensity (peroxynitrite assay and superoxide assay, respectively). The antioxidant capacity of a sample was expressed as percent reduction of peak luminescence as follows: [(peak, control)−(peak, sample)] × 100/(peak, control).

2.3 Biocompatibility assay

The wound dressing extracts were prepared according to the standard used for evaluation of textile cytotoxicity (DIN norm EN ISO 10993-12). Briefly, 1 g of each SAP-containing wound dressing was incubated in 50 ml of culture medium (DMEM) in Erlenmeyer flasks (Greiner, Germany) at 37°C for 24 h under shaking (ThermoBath, GFL, Germany). Samples were prepared under sterile conditions and it was taken extra care to avoid cross-contamination. Afterwards, wound dressing extracts were filtered over gauze by centrifugation at 1000 rpm to remove any insoluble material residues. This filtrate was then sterilized by passage through a 0.2 µm filter and distinguished as original extract (100%). Human HaCaT keratinocytes were cultured in DMEM supplemented with 1% antibiotic–antimycotic solution (10,000 U/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin) and 10% fetal bovine serum. The cells were cultured for 5–7 days in 75 cm^2 cell culture flasks (Greiner, Germany) at 37°C and in humidified atmosphere containing 5% CO_2 atmosphere. For experiments, the cells were harvested

through trypsin–EDTA treatment, seeded into 96-well microtiter plates (Greiner, Germany) at a density of 40,000 cells/cm^2. After 48 h, the culture medium was replaced by either fresh DMEM (negative control) or wound dressing extract in DMEM (100%) and serial dilutions of the original extract (75, 50, 25, 10, and 1%), respectively. Cells were then further incubated for 1, 24, and 48 h. The determination of cell proliferation was carried out on the basis of a luminometric ATP assay (ATPlite, Perkin Elmer Inc., Waltham, MA, U.S.) according to the manufacturer's recommendations. In brief, lysis solution was added to the wells containing treated or control cells. Subsequently, substrate solution (luciferase/D-luciferin) was added to each well. After incubation, the luminescence was measured using a microplate luminometer (LUMIstar Galaxy, BMG Labtech, Germany). ATP concentrations were calculated on the basis of a standard curve. Cell number per ml was calculated on the basis of an ATP-cell number-standard curve. Furthermore, the cell culture supernatant was collected and stored at −20°C until analysis. The specific enzyme immunoassays for the quantitative measurement of human IL-8 in the cell culture supernatants were purchased from Milenia Biotech (Gießen, Germany). The assays were run as recommended in the instructions. Optical density was measured at 450 nm with a reference measurement at 620 nm. Subsequently the interleukin concentrations were evaluated according to a 4-parameter-fit with lin-log coordinates for optical density (linear scale) and concentration (logarithmic scale).

2.4 Testing of antimicrobial activity

The determination of antimicrobial activity was performed according to the Japanese industrial standard (JIS L 1902: 2002, "Testing method for antibacterial activity of textiles"). Following test microbes have been used: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 4352, and *Candida albicans* ATCC 10231. Appropriate culture medium was inoculated with the test microbes and cultivated for 24 h at 37°C under aerobic conditions. For experiments, 400 mg samples of the SAP-containing wound dressings were incubated with each test microbe (200 µl) for 6 and 24 h at 37°C under aerobic conditions. Samples of polyester material were used as control; they are known not to inhibit microbial growth. For germ number determination the incubated samples were extracted in 10 ml 0.9% NaCl solution with Tween 20. Serial dilutions were plated on Columbia agar plates (BioMerieux, France), incubated for 24 h at 37°C and colonies counted afterwards. The cfu/ml (colony forming units per milliliter) and the total microbial count of the samples (in cfu) were calculated. The growth reduction compared to the starting value was determined using the following equation and rated according to the JIS L 1902: 2002.

$$\text{Growth reduction } [\log \text{cfu}] = \log\left({}^{24\text{h}}\text{MW } [\text{cfu}]_{\text{control(polyester)}}\right) - \log\left({}^{24\text{h}}\text{MW } [\text{cfu}]_{\text{sample}}\right)$$

Rating: no antimicrobial activity $= < 0.5$ log microbial growth reduction

Slight antimicrobial activity $= 0.5 - 1$ log microbial growth reduction

Significant antimicrobial activity $= > 1 - \leq 3$ log microbial growth reduction

Strong antimicrobial activity $= > 3$ log microbial growth reduction

2.5 Statistical analysis

All values cited are expressed as means ± SE (standard error). One-way analysis of variance was carried out to determine statistical significances (Microsoft® Excel 2000). Differences were considered statistically significant at a level of $P < 0.05$.

3 Results and discussion

3.1 Functional investigations

PMN elastase is a serin protease that serves in the degradation of invading pathogens and cell debris during the normal healing process. However, excessive elastase concentrations lead to the damage of healthy tissue [6–8]. As long as the protease concentration is abounding in the chronic wound, the healing success remains limited [15]. Both SAP-containing wound dressings achieved a highly significant reduction of elastase activity in the test solution (Fig. 3a). It could be shown that this reduction in elastase activity is due to the binding of the protease to the wound dressing samples (Fig. 3b). Most notably was the fast kinetic of the attachment of the protein to the polyacrylate, upon contact the enzyme concentration in solution was

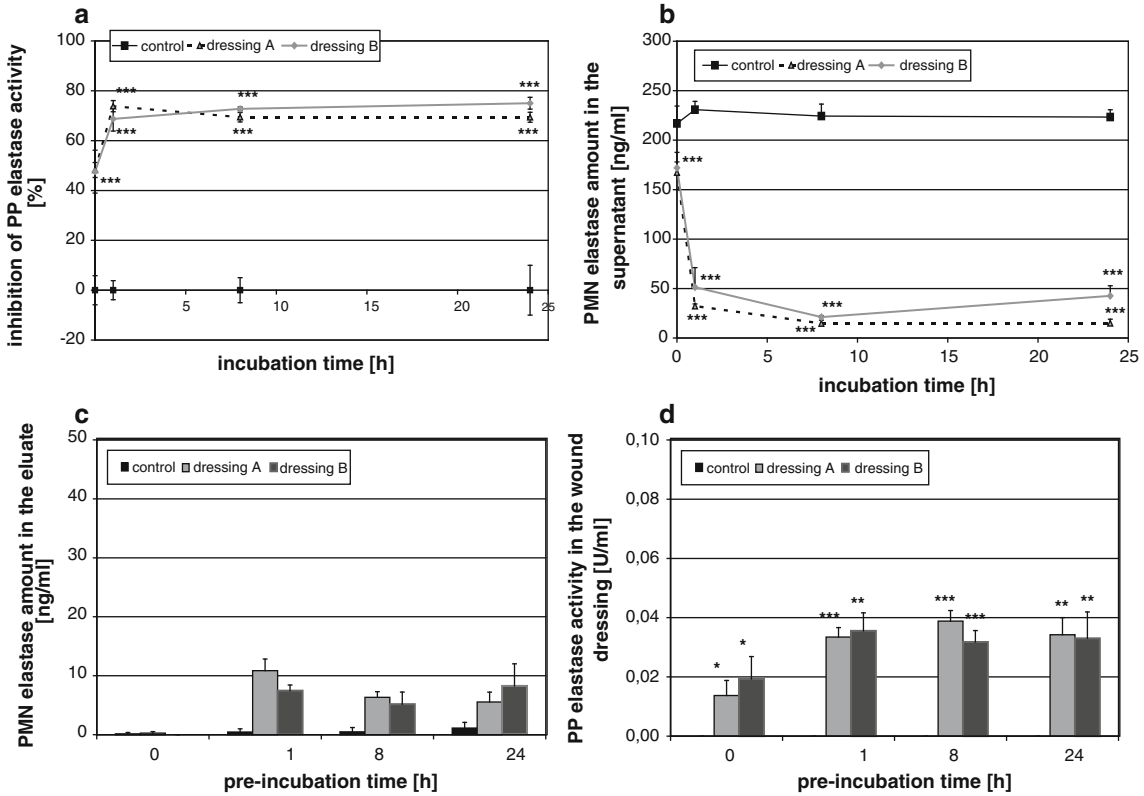

Fig. 3 SAP-containing wound dressings reduce the elastase activity in a defined enzyme solution (**a**). Both dressings with polyacrylate superabsorber are able to bind significant amounts of PMN elastase during incubation for 24 h at 37°C (**b**). Subsequently, only marginal amounts of protease could be eluted from the samples (**c**). However, elastase was not inactivated by binding to the SAP; a significant substrate turnover could be noted when the dressings were incubated with the PP elastase substrate DQ elastin (**d**). A control without addition of wound dressing samples was included in the binding assay. *Asterisks* indicate significant deviations from the control at the respective incubation time (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

decreased. Moreover, the binding of elastase seems irreversible under the test conditions; only marginal amounts of elastase could subsequently be eluted from the material (Fig. 3c). Superabsorbent polymers are cosslinked networks of flexible polymer chains with a high density of carboxylate groups, typically joined with sodium ions [1]. Upon contact with water the sodium detaches and goes in solution, leaving the charged carboxyl group behind. As these groups are all negatively charged they repel each other causing the polymer to unwind and absorb more water. The hydrogen in water is bound to polyacrylate due to electrostatic forces but it can be easily replaced by other molecules with positively charged groups like proteins which are then attached instead [3]. Elastase is known to be bound to other polymers, such as oxidized regenerated cellulose (ORC), collagen or alginate, by electrostatic interactions [11, 14]. It seems likely that SAP bind and remove elastase from the test solution by the same mechanism. Eming et al. observed in their experiments both, a significant reduction of MMP concentration as well as an indirect effect of SAP on the MMP activity by binding essential Ca^{2+} and Zn^{2+} ions [2]. These findings could be confirmed in the experiments performed with MMP-2 (data not shown). However, wound dressing samples that were incubated with elastase substrate after incubation and subsequently to the elution process showed a significant substrate turnover (Fig. 3d). Hence, elastase is neither directly nor indirectly inhibited by the SAP-containing wound dressings in contrast to the matrix metalloproteinases. The clinically beneficial effect is rather achieved by reducing the overall concentration of the protease.

The increased liberation of reactive oxygen and nitrogen species, such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and nitric oxide ($NO^{\bullet}$), in chronic wounds has been shown to thoroughly impair the healing process. While granulocytes and macrophages are able to produce ROS and RNS as a defense mechanism against invading micro organisms, the excessive overproduction of these species causes severe cellular damage [9, 10]. Consequently, the scavenging of free radicals has been proven beneficial for wound healing [16]. The tested SAP-containing wound dressings showed a significant antioxidant potential. As illustrated in Fig. 4, the scavenging capacity for free radicals is concentration dependent but equally effective for

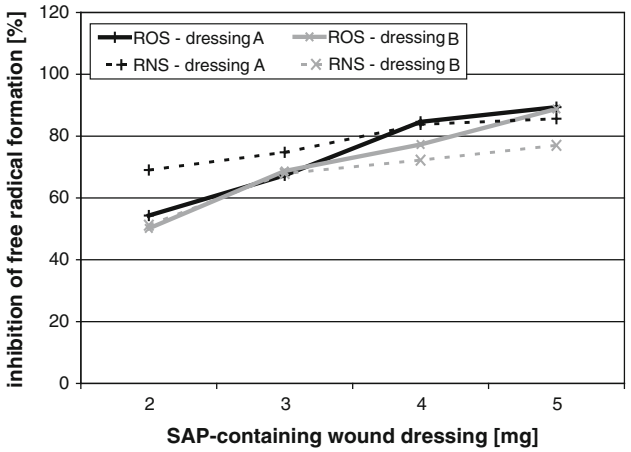

Fig. 4 Scavenging of ROS and RNS by the SAP-containing wound dressings dressing A and dressing B. The antioxidant effect was found to be dose-dependent

reactive oxygen and nitrogen species (ROS/RNS). The observed antioxidant capacity can be attributed to the carboxyl groups of the polyacrylate.

3.2 Biocompatibility assay

Figure 5 shows the results of the biocompatibility study performed according to the DIN norm EN ISO 10993-12. Treatment of human HaCaT keratinocytes with the pure extracts (100%) of dressing A (Fig. 5a) as well as dressing B (Fig. 5b) had a slight toxic effect on the cells. In accordance, a significant increase in the IL-8 release could be observed for both wound dressings (dressing A: Fig. 5c, dressing B: Fig. 5d). This could be due to residual acrylic acid monomers present in the SAP's used for the dressing manufacture [17]. It is known that non-polymerized acrylic acid exhibits cytotoxic effects and triggers inflammatory reactions [18]. The extract dilutions had no cytotoxic effects on the cells. However, an antiproliferative influence on the HaCaT keratinocytes could be noted in the experiments (Fig. 5a, b). It is unlikely that this is due to the acrylic acid monomers as it was not accompanied with a significant inflammatory reaction (Fig. 5c, b). Supposably this effect is caused by the deprivation of Ca^{2+} from the cell culture medium by the SAP. Polyacrylate is able to bind Ca^{2+} ions [2], which then are absent from the

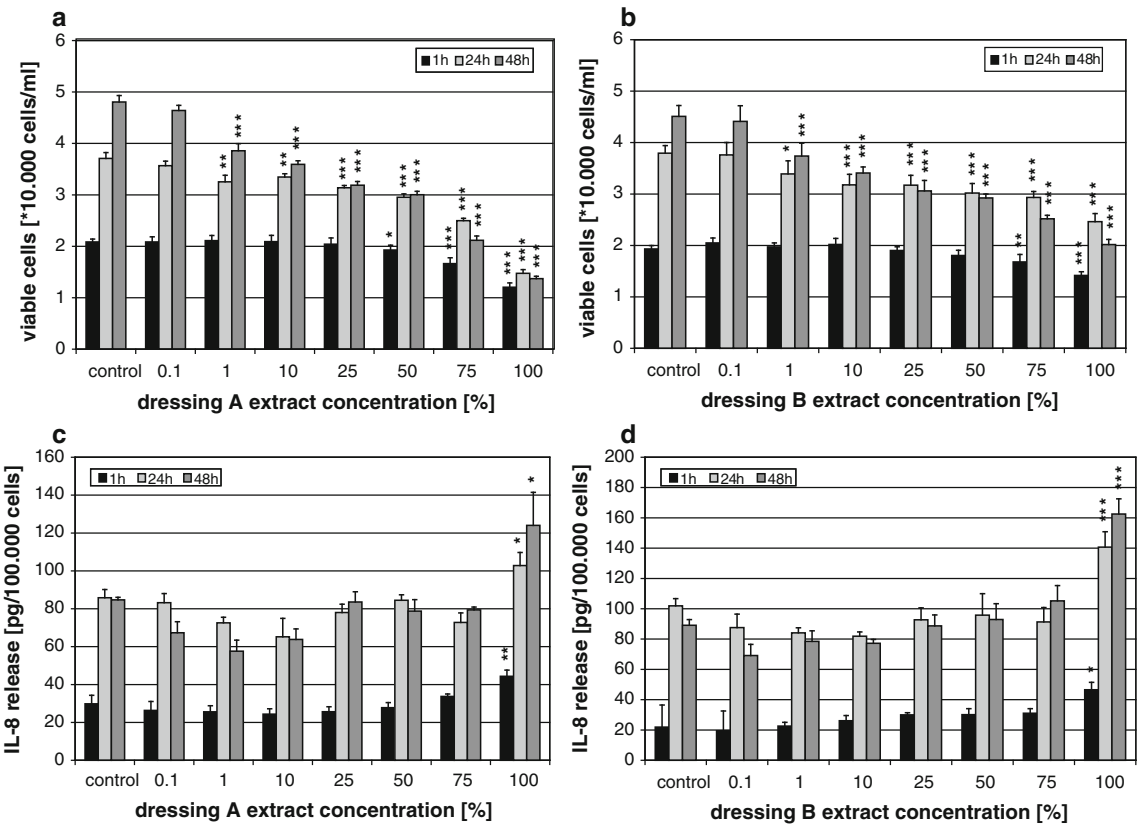

Fig. 5 Biocompatibility assessment using the keratinocyte HaCaT cell line showed cytotoxic effect of both, dressing A (**a**) and dressing B (**b**), original extracts (100%). This effect was associated with a distinct inflammatory reaction indicated by a significant release of IL-8 from the HaCaT cells under the influence of 100% extract of dressing A (**c**) and dressing B (**d**). Diluted extracts did not exhibit a toxic effect but had an antiproliferative influence on the HaCaT cells in vitro, most likely due to the deprivation of Ca^{2+} in the medium by SAP

medium. However, HaCaT keratinocyte proliferation is strictly dependent on high Ca^{2+} concentrations [19, 20]. Hence, lack of Ca^{2+} diminishes HaCaT cell proliferation but does not affect cell viability or cause an inflammatory reaction.

3.3 Antimicrobial activity

Results of the antimicrobial activity test according to the Japanese industrial standard (JIS L 1902:2002, "Testing method for antibacterial activity of textiles") are summarized in Fig. 6. Dressing A showed a strong reduction of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* growth and a significant inhibition of *Staphylococcus aureus* and *Candida albicans* (Fig. 6a). Dressing B achieved a similar result (Fig. 6b). The polyacrylate superabsorber adheres and retains the bacteria hindering their progeny. Hence, bacteria are removed with each dressing change, lowering the bacterial burden and establishing a physiological milieu [4]. The differences in the antimicrobial activity against the different micro-organisms are most likely due to the diverse structures of the cell wall of the microbes. The gram-negative species are far more susceptible to the water deprivation caused by the polyacrylate superabsorber than the gram-positive *Staphylococcus aureus* or the yeast *Candida albicans* (Prof. Kramer, personal communication). Hence, the SAP-containing wound dressings achieve a higher log reduction against gram-negative bacteria strains. Nonetheless, the in vitro data confirm the positive effect of SAP-containing wound dressings on the bio-burden observed under clinical conditions.

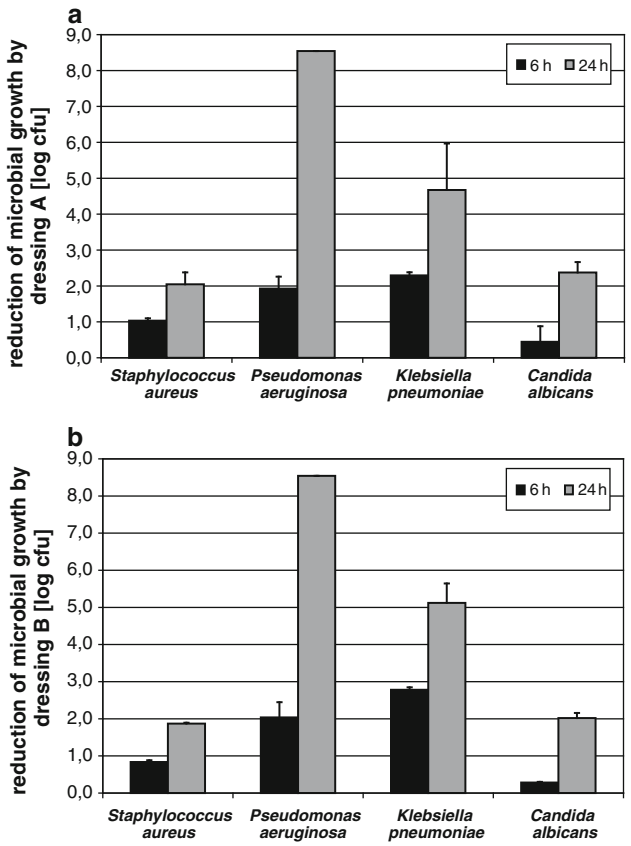

Fig. 6 Reduction of microbial growth on a log scale by dressing A (**a**) and dressing B (**b**) tested according to the Japanese industrial standard (JIS L 1902:2002, "Testing method for antibacterial activity of textiles"). The *graphs* show the reduction of the microbial growth in (log cfu) compared to an untreated control

4 Conclusions

In conclusion, superabsorbent polymers (SAP) based on polyacrylate are an interesting class of synthetic polymers. These synthetic materials are mostly cheaper and easier to mass produce than their natural counterparts. They can also be effectively shaped and fabricated into desired forms with well-defined properties [21]. Thus, there is an interest for using wound dressings of synthetic origin with beneficial effects on wound healing, like exudate up-take, provision of moist conditions, binding of inflammatory mediators and reduction of the bio-burden. For this part, SAP-containing wound dressings present a valuable and unique family of wound management products. First, they can be easily manufactured. Second, they possess a high absorption capacity being able to soak up a multiple amount of fluid of their own dry weight while providing moisture to the wound bed at the same time [2]. Hence, they are thought to be particularly effective in the cleansing phase during the treatment of chronic wounds where the proteolytic activity is high and tissue destructions occurs [2]. Using in vitro methods, it could be shown that SAP not only reduce MMP activity [2] but also exhibit a significant binding capacity for elastase as well as a distinct antioxidant potential. The clinically observed disruption of biofilms and the removal of bacteria from the wound bed [4] could be confirmed in vitro showing a significant to strong reduction of micro-organisms such as *Staphylcoccus aureus*, *Candida albicans*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. No significant difference could be observed between the two SAP-containing wound dressings in vitro. Therefore, it can be concluded that all effects observed are mediated by the SAP contained in the dressings. Clinical studies have to show whether there are differences between the wound dressings tested during daily practice, concerning e.g., handling, patient acceptance or cost effectiveness.

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