Original article

CED
Clinical and Experimental Dermatology

Effect of the most common wound antiseptics on human skin fibroblasts

Manuel Rueda-Fernández,[1,2] Lucía Melguizo-Rodríguez,[1,2] Víctor J. Costela-Ruiz,[1,2] Elvira de Luna-Bertos,[1,2] Concepción Ruiz,[1,2,3] Javier Ramos-Torrecillas[1,2] and Rebeca Illescas-Montes[1,2]

[1]*Biomedical Group (BIO277), Department of Nursing, Faculty of Health Sciences, University of Granada, Granada, Spain;* [2]*Institute of Biosanitary Research (IbsGranada), Granada, Spain; and* [3]*Institute of Neuroscience, University of Granada, Centro de Investigación Biomédica (CIBM), Parque de Tecnológico de La Salud (PTS), Granada, Spain*

doi:10.1111/ced.15235

Abstract

Background. Antiseptics are used for the cleansing of acute or chronic wounds to eliminate micro-organisms from the wound bed. However, they have effects on the skin cells.

Aim. To determine the effects of hexetidine, povidone–iodine (PI), undecylenamidopropyl-betaine/polyhexanide (UBP), chlorhexidine, disodium eosin and hydrogen peroxide on human skin fibroblasts.

Methods. CCD-1064Sk cells were treated with hexetidine, PI, UBP, chlorhexidine, disodium eosin or hydrogen peroxide. Spectrophotometry was used to measure cell viability and flow cytometry was used to study apoptosis and necrosis after the treatment. *In vitro* wound scratch assays were performed to determine the gap closure.

Results. All antiseptics significantly reduced the viability of human skin fibroblasts compared with controls. The percentage wound closure was lower with hexetidine, PI and UBP. The scratch assay could not be measured after treatments with chlorhexidine, disodium eosin or hydrogen peroxide, owing to their cytotoxicity. The apoptosis/necrosis experiments evidenced a significant reduction in viable cells compared with controls. An increased percentage of apoptotic cells was observed after treatment with all antiseptics. Compared with controls, the percentage of necrotic cells was significantly increased with all antiseptics except for hexetidine.

Conclusion. The proliferation, migration and viability of human skin fibroblasts are reduced by treatment with hexetidine, PI, UBP, chlorhexidine, disodium eosin and hydrogen peroxide.

Introduction

Wounds, especially chronic wounds, frequently impair the quality of life of patients, increase healthcare costs and remain therapeutically challenging.[1] Wound healing is regulated by multiple cell, humoral and molecular processes, and alterations in the functioning of any of these can give rise to chronic wounds, defined as wounds that fail to heal within 3 months.[2]

Healing can be delayed by the presence of biofilm in the wound, i.e. microcolony aggregates enclosed in an extracellular polymeric substance (EPS) and distributed across the wound bed. This biofilm becomes entangled with fibroblasts or keratinocytes and the extracellular matrix, intercommunicating through quorum-sensing circuits via the respective receptors and signal molecules. The EPS provides an optimal environment for micro-organisms to evade the host immune response and the action of antibiotics.[3]

Correspondence: Dr Javier Ramos-Torrecillas, Department of Nursing, Faculty of Health Sciences, University of Granada, 60 Avenida de la Ilustración, 18016, Granada, Spain
E-mail: jrt@ugr.es

Accepted for publication 19 April 2022

Hence, cleansing of the wound may be necessary to clear this biofilm and other unwanted matter. In addition, antimicrobials are commonly applied directly to the wound to diminish or eliminate the bacterial load, with disinfectants, antiseptics or antibiotics used to kill, inhibit or reduce the number of micro-organisms.[4] Disinfectants act against virtually all micro-organisms, including spores; however, they can be toxic to tissues and are mainly used to sterilize inanimate surfaces. Antiseptics can be applied to intact skin and some open wounds, and are active against a wide spectrum of micro-organisms, including bacteria, fungi, viruses and protozoa, although they can also be frequently toxic to tissues.[5] Antibiotics are natural or synthetic compounds that can also inhibit or kill micro-organisms; they generally act against specific target microbials and therefore have a narrower spectrum of activity.[6] Routine topical antibiotics are not highly effective against biofilm, and due to their mode of application, they may in fact generate antibiotic resistance.[7]

Different pharmaceutical forms of topical antiseptics with a wide variety of active ingredients are applied as decontaminating agents to assist in chronic wound healing. Thus, considering the antibiofilm potential of these products, some authors emphasize the systematic use of antiseptics in uninfected wounds for wound-bed preparation.[8] However, there are limited data on different antiseptics and modes of application regarding the clinical efficacy, risk of systemic absorption or effect on the viability of tissue cell populations responsible for wound repair. Consequently, no consensus is available to guide healthcare professionals on the optimal selection and administration of antiseptics to avoid wound-healing impairment.[7,9]

The aim of this study was to analyse the effect of liquid antiseptic solutions of hexetidine, povidone–iodine (PI), undecylenamidopropyl-betaine/polyhexanide (UBP), chlorhexidine, disodium eosin and hydrogen peroxide on human skin fibroblasts *in vitro*.

Methods

Antiseptic solutions

Some of the best known and widely used liquid antiseptic formulations and dilutions were utilized in this study: 0.1% hexetidine (Oraldine®; Johnson & Johnson S.A. Madrid, Spain), 10% (v/v) PI (Betadine®; MEDA Manufacturing, Bordeaux, France), 0.1% (w/v) undecylenamidopropyl-betaine with 0.1% (w/v) polyhexanide (Prontosan®; B. Braun, Barcelona, Spain), 0.06% (v/v) chlorhexidine (Lacer S.A. Barcelona, Spain), 2% (w/v) disodium eosin (Farmalabor s.r.l. Canosa di Puglia, Italy) and 1.5% (v/v) hydrogen peroxide (Laboratorios Reig Jofré, Barcelona, Spain).[10] At least three independent experiments were conducted.

Cell culture

The CCD-1064Sk (CRL-2076™) human skin fibroblast cell line (American Type Culture Collection, Manassas, VA, USA) was used. Cells were cultured in DMEM (Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA) containing 1% glutamine and 2% HEPES (both Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK). The culture medium was free of antibiotics and fungicides to avoid possible synergic cytotoxic effects on the fibroblasts that would confound the results. Cultures were maintained at 37 °C in an atmosphere of 95% air humidity and 5% CO_2. Cells were separated from culture flasks using a solution of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (both Sigma), then washed and suspended in DMEM.

Cell viability assay

The cell viability study was performed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (Sigma). In brief, cells were cultured in 96-well plates at a concentration of 1×10^4 cells/mL and were synchronized for 24 h in DMEM supplemented with 2% FBS. The relevant antiseptic was added for a cell treatment of 1 min. Wells without antiseptic solution served as controls. After treatment, cells were added to DMEM without phenol red, but with MTT added, and incubated for 4 h. After incubation, the formazan crystals were dissolved by adding dimethyl sulfoxide, and the absorbance was measured at 570 nm using a spectrophotometer (Sunrise™; Tecan, Männedorf, Switzerland).

Wound healing assay

In vitro wound scratch assay was performed by culturing fibroblasts at a concentration of 5×10^3 cells/mL in 24-well plates. After verification of the confluence, a vertical and horizontal scratch was made using a p200 pipette tip. Cells were incubated for 1 min with the relevant antiseptic, using wells with culture medium but without antiseptic as controls. The migration pattern was examined at 0, 12 and 24 h. Cell migration areas were analysed using Motic Images Plus software (Motic China Group Co. Hong Kong). For each

well, four scratch measurements were taken. The percentage wound closure was calculated according to the formula:

$$\%\ \text{wound closure} = (W_0 - W_n)/W_0 \times 100\%,$$

where W_n is the gap width after a given time interval and W_0 is the width immediately after scratch formation.

Apoptosis and necrosis analysis

Apoptosis and necrosis were studied by culturing fibroblasts in six-well plates at a concentration of 5×10^5 cells/mL with synchronization, as described above. The relevant antiseptic solution was added for 1 min. Wells cultured without antiseptic served as controls. Cells were subsequently separated from the culture plates suspended in 300 µL PBS and labelled with annexin V and propidium iodide (Immunostep S L, Salamanca, Spain). Cells were analysed in an argon laser flow cytometer (FACS Vantage; BD Biosciences, San Jose, CA, USA) at a wavelength of 488 nm to determine the percentage of fluorescent cells. Percentages of annexin-positive (apoptotic) cells and propidium iodide-positive (necrotic) cells were calculated from counts of 2000–3000 cells.

Statistical analysis

Mean values with standard error of the mean (SEM) were calculated for all variables. Given the non-normal distribution of the data (Kolmogorov–Smirnov test), the Mann–Whitney *U*-test was used to compare data for each antiseptic compared with the control using *R* software (https://www.r-project.org). $P \leq 0.05$ was considered statistically significant.

Results

Cell viability assay

Treatment for 1 min with each antiseptic (hexetidine, PI, UBP, chlorhexidine, disodium eosin or hydrogen peroxide) exerted a significant inhibitory effect ($P < 0.01$) on fibroblast viability compared with untreated controls (Fig. 1).

Wound healing assay

The *in vitro* wound healing assay results (Figs 2 and 3) showed a significant reduction in percentage wound closure compared with controls at 12 h (3.44% ± 1.26 vs. 80.94% ± 3.93; $P < 0.001$) and 24 h (0.49% ± 2.92 vs. 100% ± 0.00; $P < 0.001$) after treatment with hexetidine. Cells treated with PI or UBP showed no migration capacity at 12 or 24 h post-treatment (0% ± 0.00 vs. 100% ± 0.00; $P < 0.001$), and also exhibited significantly reduced percentage wound closure compared with controls. This assay could not be conducted in fibroblasts treated with chlorhexidine, hydrogen peroxide or disodium eosin due to the cytotoxicity of the treatment.

Apoptosis and necrosis analysis

The flow cytometry results for annexin V- and propidium iodide-labelled fibroblast counts after 1 min of treatment with each antiseptic showed that the antiseptics significantly reduced ($P = 0.05$) the count of viable cells compared with controls (Fig. 4).

Treatments with hexetidine, PI, UBP, chlorhexidine and hydrogen peroxide significantly increased ($P \leq 0.05$) the percentage of cells in early and late apoptosis compared with controls, while treatment with disodium eosin significantly increased ($P < 0.05$) the percentage in late apoptosis alone.

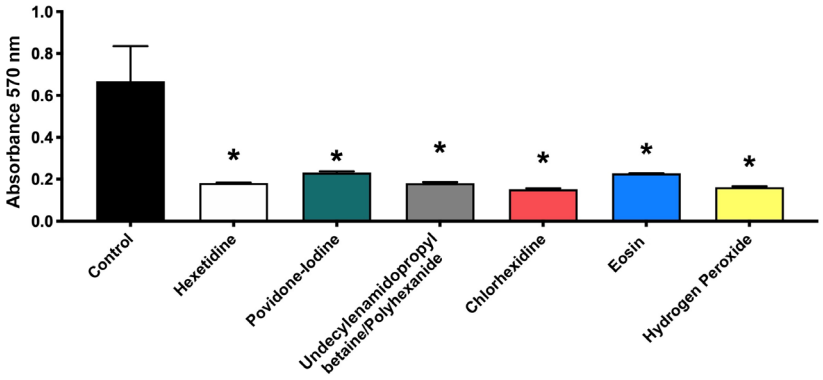

Figure 1 Cell viability of skin fibroblasts after 1 min of treatment with each antiseptic, $^*P \leq 0.05$.

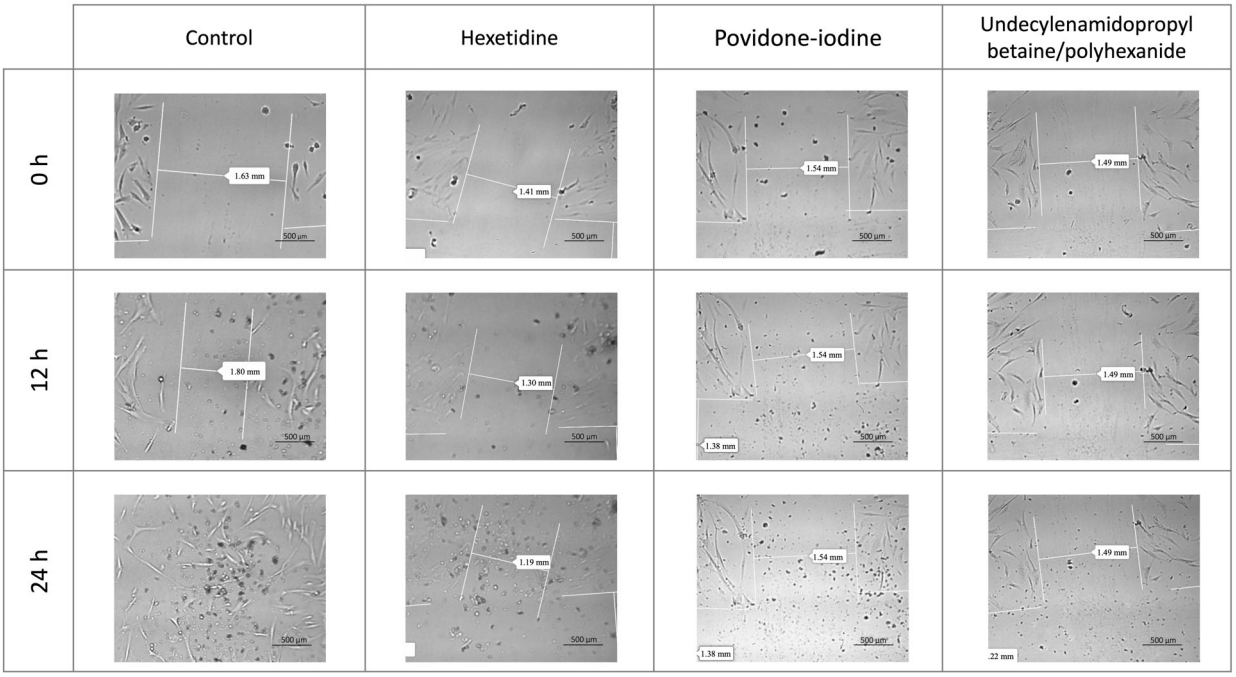

Figure 2 Time course of wound healing assay with control, hexetidine, povidone–iodine and undecylenamidopropyl-betaine/polyhexanide.

Treatments with PI, UBP, chlorhexidine, disodium eosin and hydrogen peroxide significantly increased ($P \leq 0.05$) the percentage of necrotic cells compared with controls.

Discussion

Antiseptics are increasingly being used on chronic wounds due to the rise in multidrug-resistant micro-organisms in biofilms. However, clinical guidelines for antiseptic use are limited to the treatment of wounds that are already infected or at risk of infection.[4] The ideal antiseptic for wound cleaning should combine maximum antimicrobial activity with minimum cytotoxicity. This *in vitro* study found that antiseptics have toxic effects on fibroblasts, which are cells that play a key role in wound healing. *In vitro* treatment with hexetidine, PI, UBP, chlorhexidine, disodium eosin or hydrogen peroxide significantly reduced the proliferative capacity of the fibroblasts, significantly increased their necrosis and apoptosis rates, and significantly diminished the percentage wound closure compared with controls.

Hexetidine is effective as a local antimicrobial and antifungal agent.[11] Compared with controls, treatment of cultured fibroblasts with hexetidine significantly reduced their proliferative capacity and significantly increased their apoptosis rate, although it did not increase their necrosis rate. We found only one previously published *in vitro* study on the effects of hexetidine on fibroblasts; Shakespeare *et al.*[12] treated 3 T3 fibroblasts with hexetidine (dilutions of 1 : 500 to 1 : 1000) and found a reduction in the proliferation capacity and viability of these cells. The present finding of a significant reduction in the *in vitro* percentage wound healing (migration capacity) of fibroblasts at 12 and 24 h may be explained by the effect of hexetidine on the proliferative capacity of these cells.

PI and UBP are commonly used to treat and prevent wound infection.[13,14] Compared with controls treatment with these agents significantly reduced the proliferative capacity of fibroblasts, significantly increased their apoptosis and necrosis rates, and significantly decreased the percentage wound closure at 12 and 24 h in the current study. These findings are in line with a study showing that treatment with PI at variable concentrations was found to exert a cytotoxic effect on human gingival fibroblast cultures through a dose-dependent inhibition of proliferation.[15] Likewise, Hirsch *et al.* reported a cytotoxic effect on human fibroblasts and keratinocytes *in vitro* after treatment with UBP or PI at similar concentrations to those we used in the present study.[16] The action of UBP and PI against multidrug-resistant micro-organisms is well documented, and these antiseptics are routinely applied to

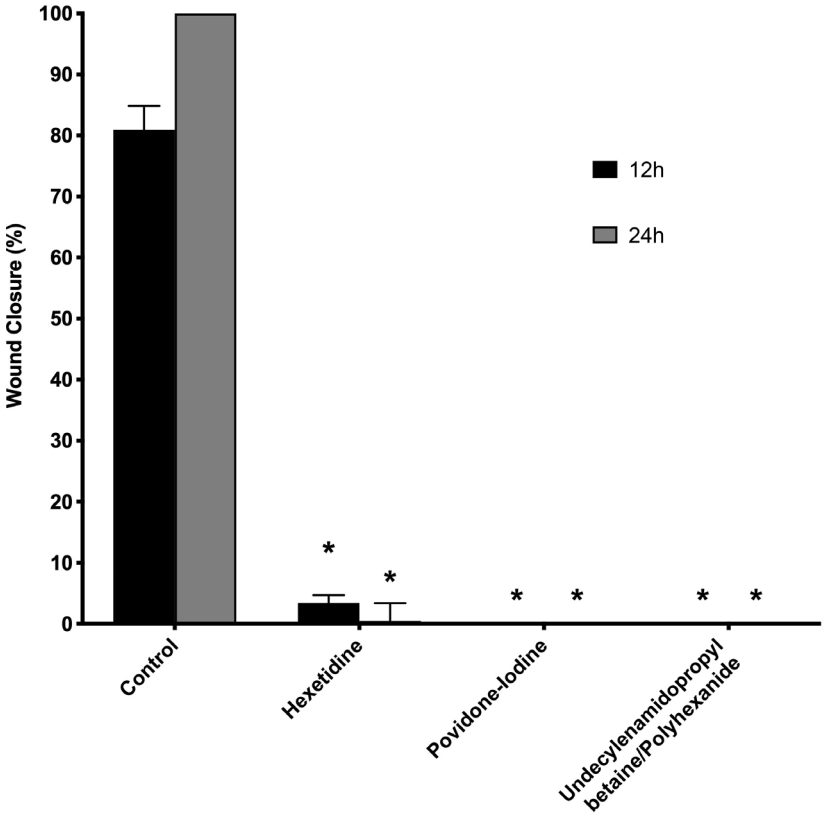

Figure 3 Percentage of *in vitro* wound closure of the measurable wells (control, hexetidine, povidone–iodine and undecylenamidopropyl-betaine/polyhexanide) at 12 and 24 h, $^{*}P \leq 0.05$.

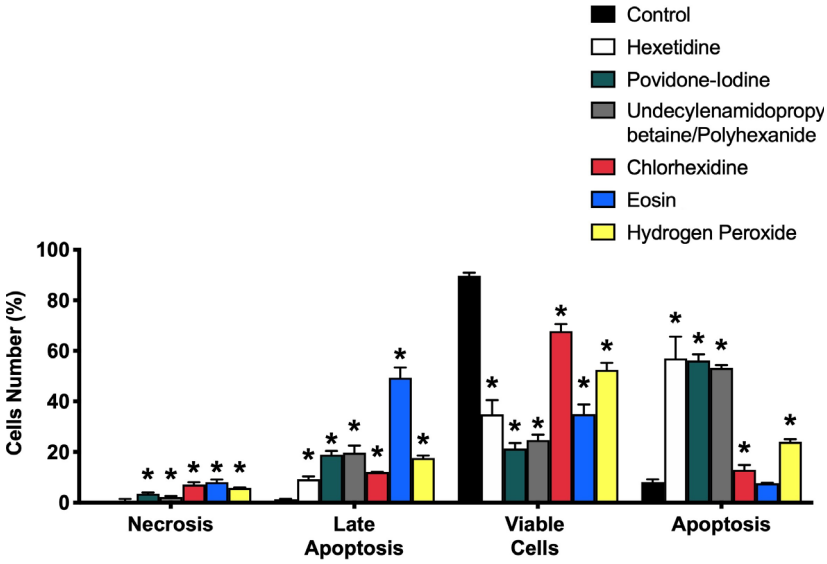

Figure 4 Percentage of apoptotic, necrotic and viable cells after 1 min of treatment with each antiseptic, $^{*}P \leq 0.05$.

treat long-term infected wounds with good outcomes. However, their preventive application in open wounds may be counterproductive due to their negative effects on fibroblasts, which may slow the healing process.[13]

Treatments with chlorhexidine, disodium eosin and hydrogen peroxide all reduced the proliferative capacity of fibroblasts and increased their necrosis rate. In fact, the cytotoxic effect of these antiseptics on the human skin fibroblasts meant the scratch assays could not be performed because there was no cell movement.

Chlorhexidine exerts prolonged antimicrobial activity and is frequently used for skin antisepsis

before surgery or directly applied to cleanse the wound bed.[17] In previous *in vitro* studies, Coelho *et al.*[18] and Sukumaran *et al.*[19] reported that treatment with chlorhexidine at varying concentrations for different time periods (1, 2, 4, 6, 8 and 10 min) had cytotoxic effects on gingival fibroblasts, reducing their viability at 24 h post-treatment.

The effectiveness of disodium eosin as an antibacterial agent is also well established, although there is little evidence about its activity against eukaryotic cell populations.[20] An *in vitro* study found that disodium eosin at different concentrations (0.002%–0.2%) reduced the proliferation of keratinocytes from skin biopsies and inhibited the release of healing-related inflammatory cytokines.[21] Disodium eosin is commonly used to treat napkin (diaper) dermatitis and psoriasis, among other skin conditions, but its application on open wounds is contraindicated.[22]

Hydrogen peroxide is widely used to prevent and treat local infectious processes and is known to have a toxic effect on tissues.[23] Hydrogen peroxide was found to induce dose-dependent apoptosis and necrosis in pulmonary alveolar epithelial cells and to inhibit wound healing in an *in vitro* model.[24] In another study, treatment of cultured human oral keratinocytes with hydrogen peroxide (at 0.01, 0.1, 1, 5, 10 or 100 nM) reduced their proliferation at concentrations above 5 mM and induced DNA damage and cytotoxicity, with a concentration-dependent increase in cell apoptosis.[25]

Base on these *in vitro* findings, the antiseptics under study can all be toxic for fibroblasts, which play a key role in wound healing.

Conclusions

Treatment for 1 min with hexetidine, PI, UBP, chlorhexidine, disodium eosin or hydrogen peroxide reduces the proliferation, migration and viability of human skin fibroblasts. All of these antiseptics are effective antimicrobial agents, but their utilization on wounds in active healing phase should not be recommended. It is necessary to develop novel antimicrobial treatments that do not damage epithelial cells and promote wound healing.

What's already known about this topic?

- Antiseptics are used in the cleansing of wounds as a preparation of the wound bed to eliminate micro-organisms.
- Many authors have questioned the use of antiseptics in wound cleansing, given the possible adverse effect on viable tissue.

What does this study add?

- The most common liquid antiseptics may impair the repair of open wounds, reducing the viability of skin fibroblasts.
- Hexetidine, PI and UBP would inhibit *in vitro* wound closure.
- Most of the wound antiseptics increase early and late apoptosis and necrosis of *in vitro* human skin fibroblasts.

Acknowledgement

This study was supported by the research group BIO277 (Junta de Andalucía) and Department of Nursing (University of Granada). Open access funding enabled and organized by ProjektDEAL.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This research received funding for open access charge from the University of Granada/CBUA.

Ethics statement

Ethics approval not applicable. The patient provided informed consent for publication of their case details and images.

Data availability

Datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

References

1 Olsson M, Järbrink K, Divakar U *et al.* The humanistic and economic burden of chronic wounds: a systematic review. *Wound Repair Regen* 2019; **27**: 114–25.
2 Martin P, Nunan R. Cellular and molecular mechanisms of repair in acute and chronic wound healing. *Br J Dermatol* 2015; **173**: 370–8.
3 Kadam S, Shai S, Shahane A *et al.* Recent advances in non-conventional antimicrobial approaches for chronic wound biofilms: have we found the 'chink in the armor'? *Biomedicines* 2019; **7**: 35.

4 World Union of Wound Healing Societies. Position document of World Union of Wound Healing Societies. Management of the biofilm. 2016. Available at: https://www.wuwhs.org/wp-content/uploads/2020/07/WUWHS_Biofilms_web.pdf (accessed 25 April 2022).
5 Punjataewakupt A, Napavichayanun S, Aramwit P. The downside of antimicrobial agents for wound healing. *Eur J Clin Microbiol Infect Dis* 2019; **38**: 39–54.
6 Dumville JC, Lipsky BA, Hoey C *et al*. Topical antimicrobial agents for treating foot ulcers in people with diabetes. *Cochrane Database Syst Rev* 2017; **6**: CD011038.
7 Norman G, Dumville JC, Mohapatra DP *et al*. Antibiotics and antiseptics for surgical wounds healing by secondary intention. *Cochrane Database Syst Rev* 2016; **3**: CD011712.
8 Bellingeri A, Falciani F, Traspedini P *et al*. Effect of a wound cleansing solution on wound bed preparation and inflammation in chronic wounds: a single-blind RCT. *J Wound Care* 2016; **25**: 160–8.
9 Norman G, Dumville JC, Moore ZEH *et al*. Antibiotics and antiseptics for pressure ulcers. *Cochrane Database Syst Rev* 2016; **4**: CD011586.
10 Wilkins RG, Unverdorben M. Wound cleaning and wound healing: a concise review. *Adv Skin Wound Care* 2013; **26**: 160–3.
11 Tirri BF. Antimicrobial topical agents used in the vagina. *Curr Probl Dermatol* 2011; **40**: 36–47.
12 Shakespeare V, Shakespeare PG, Evans BT. Effects of proprietary oral rinses containing chlorhexidine, hexetidine and benzydamine on the proliferation of human buccal epithelial cells in culture. *Arch Oral Biol* 1988; **33**: 881–5.
13 Bigliardi PL, Alsagoff SAL, El-Kafrawi HY *et al*. Povidone iodine in wound healing: a review of current concepts and practices. *Int J Surg* 2017; **44**: 260–8.
14 Horrocks A. Prontosan wound irrigation and gel: management of chronic wounds. *Br J Nurs* 2006; **15**: 1224–8.
15 Flemingson L, Emmadi P, Ambalavanan N *et al*. Effect of three commercial mouth rinses on cultured human gingival fibroblast: an in vitro study. *Indian J Dent Res* 2008; **19**: 29–35.
16 Hirsch T, Koerber A, Jacobsen F *et al*. Evaluation of toxic side effects of clinically used skin antiseptics in vitro. *J Surg Res* 2010; **164**: 344–50.
17 Privitera GP, Costa AL, Brusaferro S *et al*. Skin antisepsis with chlorhexidine versus iodine for the prevention of surgical site infection: a systematic review and meta-analysis. *Am J Infect Control* 2017; **45**: 180–9.
18 Coelho AS, Laranjo M, Gonçalves AC *et al*. Cytotoxic effects of a chlorhexidine mouthwash and of an enzymatic mouthwash on human gingival fibroblasts. *Odontology* 2020; **108**: 260–70.
19 Sukumaran SK, Vadakkekuttical RJ, Kanakath H. Comparative evaluation of the effect of curcumin and chlorhexidine on human fibroblast viability and migration: an in vitro study. *J Indian Soc Periodontol* 2020; **24**: 109–16.
20 Romano I, Ayadi F, Rizzello L *et al*. Controlled antiseptic/eosin release from chitosan-based hydrogel modified fibrous substrates. *Carbohydr Polym* 2015; **131**: 306–14.
21 Capriotti L, Didona B, Madonna S *et al*. Eosin treatment for psoriasis reduces skin leukocyte infiltration and secretion of inflammatory chemokines and angiogenic factors. *Eur J Dermatol* 2018; **28**: 457–66.
22 Tabolli S, Alessandroni L, Didona B *et al*. A randomized controlled trial to evaluate short-term treatment with eosin vs. topical steroids in psoriasis. *Clin Exp Dermatol* 2009; **34**: 304–8.
23 Urban MV, Rath T, Radtke C. Hydrogen peroxide (H2O2): a review of its use in surgery. *Wien Med Wochenschr* 2019; **169**: 222–5.
24 Geiser T, Ishigaki M, van Leer C *et al*. H_2O_2 inhibits alveolar epithelial wound repair in vitro by induction of apoptosis. *Am J Physiol Lung Cell Mol Physiol* 2004; **287**: L448–53.
25 Lin K-Y, Chung C-H, Ciou J-S *et al*. Molecular damage and responses of oral keratinocyte to hydrogen peroxide. *BMC Oral Health* 2019; **19**: 10.