Development of haemostatic decontaminants for treatment of wounds contaminated with chemical warfare agents. 3: Evaluation of *in vitro* topical decontamination efficacy using damaged skin

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Previous studies have demonstrated that haemostatic products with an absorptive mechanism of action retain their clotting efficiency in the presence of toxic materials and are effective in decontaminating chemical warfare (CW) agents when applied to normal, intact skin. The purpose of this *in vitro* study was to assess three candidate haemostatic products for effectiveness in the decontamination of superficially damaged porcine skin exposed to the radiolabelled CW agents, soman (GD), VX and sulphur mustard (HD). Controlled physical damage (removal of the upper $100\,\mu m$ skin layer) resulted in a significant enhancement of the dermal absorption of all three CW agents. Of the haemostatic products assessed, WoundStat™ was consistently the most effective, being equivalent in performance to a standard military decontaminant (fuller's earth). These data suggest that judicious application of haemostatic products to wounds contaminated with CW agents may be a viable option for the clinical management of casualties presenting with contaminated, haemorrhaging injuries. Further studies using a relevant animal model are required to confirm the potential clinical efficacy of WoundStat™ for treating wounds contaminated with CW agents. Copyright © 2017 John Wiley & Sons, Ltd.

Introduction

Over the last two decades, the development of haemostatic products for the rapid treatment of non-compressible, haemorrhaging wounds has received much attention (Acheson *et al.,* 2005; Alam *et al.,* 2004; Kozen *et al.,* 2008; Ling *et al.,* 2010; Pusateri *et al.,* 2003, 2006). In particular, haemostatic products have shown demonstrable efficacy in experimental models of moderate and severe battlefield-relevant haemorrhage (Arnaud *et al.,* 2009; Kheirabadi *et al.,* 2009; Li *et al.,* 2016; Littlejohn *et al.,* 2011). Correspondingly, a number of haemostatic products have been commercialized for military application in battlefield, pre-hospital and combat support hospitals (Cox *et al.,* 2009; Schreiber and Tieu, 2007; Wedmore *et al.,* 2006). Haemostatic products are also increasingly used clinically in civilian cases (Leonard *et al.,* 2016; Shanmugam and Robinson, 2009; te Grotenhuis *et al.,* 2016).

Haemostatic products (based on an absorptive mechanism of action) also have potential clinical application in sequestering toxic materials from within wounds. Previous studies have demonstrated that a number of products retain their clotting function in the presence of toxic chemicals (Hall *et al.,* 2015) and that, following topical application to undamaged skin, certain haemostats are highly effective in preventing the dermal absorption of chemical warfare (CW) agents (Dalton *et al.,* 2015). While normal skin can provide some protection against the ingress of xenobiotics, damage resulting from trauma associated with penetrating injury or any other insult that may compromise the stratum corneum (such as abrasions, excoriations or burns) could lead to enhanced local and systemic absorption (Chilcott *et al.,* 2002; Wu *et al.,* 2006). Thus, the purpose of this current work was to evaluate the effectiveness of commercially available haemostatic products for the decontamination of CW agents from superficially damaged skin. A secondary

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objective of this study was to quantify the effects of skin damage on the dermal absorption kinetics of CW agents.

Materials and methods

Haemostatic products and chemicals

Three haemostatic products identified from a previous study (Dalton *et al.*, 2015) were included in the present study as test decontaminants: QuikClot Advanced Clotting Sponge Plus® (Z-Medica Corporation, Wallingford, CT, USA); ProQR® (Biolife, Sarasota, FL, USA); and WoundStat™ (TraumaCure, Bethesda, MD, USA). The current in-service decontaminant fuller's earth (Sigma Chemical Co., Dorset, UK), was included as a positive control. The storage and use of CW agents was in full compliance with the Chemical Weapons Convention (1986). The CW agents (*S*-[2-(diisopropylamino)ethyl]-*O*-ethyl methylphosphonothioate (VX), *O*-Pinacolyl methylphosphonofluoridate (GD) and *bis*(2-chloroethyl) sulphide (HD), and their (^{14}C-)radiolabelled analogues were custom synthesized by TNO Defence, Security and Safety (Rijswijk, the Netherlands). The radiolabelled analogue was mixed with 5 g of undiluted agent to provide a stock solution with a nominal activity of ~1 mCi g^{-1} and stored for up to 2 months at 4°C. Aliquots of each stock solution were diluted with unlabelled CW agent immediately before each experiment to provide a working solution with a nominal activity of ~0.5 μCi μl^{-1}. Ultima Gold Liquid Scintillation Cocktail (LSC) was supplied by PerkinElmer, Buckinghamshire, UK, and Sigma.

Damaged skin preparation

The use of animals in this study was conducted in accordance with the Animals (Scientific Procedures) Act 1986. Full thickness skin was excised, post mortem, from the abdominal flank of male and female pigs (*Sus scrofa*, large white strain, six males, six females, weight range 20–30 kg). Skin samples were stored flat between aluminium foil sheets at −20°C until required, for a maximum of 6 months. When required, skin samples were thawed at 4°C and carefully clipped to remove excess hair. The upper 100 μm of epidermis was removed using a dermatome (Humeca Model D42; Eurosurgical Ltd., Guildford, UK) to produce controlled, physical damage to the skin barrier layer. The remaining skin was subsequently dermatomed to a thickness of 400 μm, cut into square sections (~3 × 3 cm) and mounted into static diffusion cells. Similar size 'undamaged' skin sections of 500 μm thickness (without removal of the upper 100 μm surface), were also prepared for comparison.

Diffusion cell assembly

Jacketed, Franz-type diffusion cells were purchased from PermeGear (Chicago, IL, USA). The prepared skin sections were placed flat between the (upper) donor and receptor (lower) chambers and clamped in place with the epidermal surface facing the donor chamber. Receptor chambers were filled with 50% (*v/v*) ethanol in deionized water solution ('receptor fluid'), so that the meniscus was level with the skin surface, and the volume of receptor fluid was recorded for each chamber. Each cell was placed in a Perspex™ clamp, which contained a magnetic stirrer to mix the receptor fluid in each individual cell via a Teflon™-coated iron bar placed within the receptor chamber. The receptor chamber jackets were heated by water supplied via a manifold and a circulating water heater and pump (Model GD120; Grant Instruments, Cambridge, UK). This enabled the skin temperature to be maintained at ~32°C (measured with infrared thermography; FLIR Model P640 camera; FLIR Instruments, West Malling, Kent, UK). Assembled diffusion cells were left to equilibrate for 16–24 h before starting the study.

Experimental procedure

The test groups included 'damaged, untreated', 'undamaged, untreated' and 'damaged, treated' skin. A finite dose (10 μl) of 14C–GD, 14C–HD or 14C–VX was applied to each skin section. For the 'damaged, treated' group, test products (200 mg) were applied to the exposure site of damaged skin sections 30 s after CW agent application. Six diffusion cells were used for each group. Samples of receptor fluid (250 μl) were withdrawn regularly from each diffusion cell over a 24 h period and transferred directly into 5 ml LSC for analysis using a PerkinElmer Tri-Carb Model 2810 TR scintillation counter. The receptor chambers were replenished following each sample by the addition of 250 μl fresh receptor chamber fluid. After 24 h exposure, the dosing chamber was removed and dismantled. The surface of the skin was swabbed using a dry cotton-wool swab and then placed in isopropanol (10 ml) in glass vials. The decontaminant (where applied) was collected and placed in 10 ml isopropanol. Samples of each solvent extract (250 μl) were diluted in 5 ml LSC for scintillation counting. The mass of radiolabel contained in each receptor fluid sample (per unit diffusion area, mg cm^{-2}) and extracted from swabs, skin and the test decontaminants was calculated in reference to calibration solutions containing known quantities of 14C–GD, 14C–HD or 14C–VX. The skin section was incubated in 20 ml Soluene-350 until fully dissolved, after which samples (250 μl) were diluted in LSC and analysed as above.

Data and statistical analyses

The endpoints analysed were (1) total amount of radiolabel in the receptor fluid at each time point; (2) maximum flux (J_{max}); and (3) mass of radiolabel quantified in each fraction (skin, swabs, receptor fluid, decontaminant). For direct comparisons between damaged and undamaged skin, chemical penetration data are presented as mg cm^{-3} to account for differences in skin thickness. Otherwise, chemical penetration data are presented as mg cm^{-2}. The maximum mean penetration rate (J_{max}) for each chemical (expressed as mg cm^{-2} h^{-1}) was estimated from the gradient of the slope for the linear region of the penetration profile. Statistical analyses of the data were performed using GraphPad Prism (GraphPad Software, Inc., version 6.0). Significance was defined as $P < 0.05$. J_{max} data were log transformed to fit the requirements for parametric analysis. Statistical differences in J_{max} data among groups were determined using a one-way ANOVA with *post-hoc* Dunnett's multiple comparisons test. First, J_{max} values for decontaminated groups were compared to the 'damaged, untreated' group. Second, comparisons in J_{max} values within the decontaminated groups were conducted between the test haemostats (QuikClot Advanced Clotting Sponge Plus®, ProQR® and WoundStat™) and the current in-service decontaminant, fuller's earth.

Comparisons of data between 'damaged, untreated' and 'undamaged, untreated' conditions were performed using the Mann–Whitney *U*-test. For the remaining data, statistical differences between three or more groups were determined by Kruskal–Wallis multiple comparisons with *post-hoc* Dunn's tests. Quantification of ^{14}C within each compartment (swab, skin and receptor fluid) was compared between the 'damaged, treated'

and 'damaged, untreated' groups. Quantification of ^{14}C within each decontaminant was compared between the test haemostats and the current in-service decontaminant, fuller's earth.

Results

Removal of skin barrier layer increases percutaneous penetration and alters dose distribution of radiolabelled chemical warfare agents

Penetration of all three chemicals (^{14}C–HD, ^{14}C–VX and ^{14}C–GD) was significantly greater through damaged skin compared to undamaged skin, with significantly greater J_{max} values (Fig. 1). Correspondingly, the enhanced penetration rate of radiolabelled CW agents across damaged skin relative to undamaged skin was accompanied by a significantly greater amount of radiolabel detected in the receptor fluid at 24 h post-exposure (Fig. 2).

Skin damage did not affect the amount of CW agents remaining on the skin surface 24 h post-exposure, as no significant difference was observed between the swabs for damaged or undamaged skin (Fig. 2). However, there were chemical-specific differences for the amount of radiolabel remaining within the skin. There was a significantly greater amount of ^{14}C–GD within the damaged skin samples relative to the undamaged skin samples (Fig. 2A). Conversely, the amount of ^{14}C–VX was significantly lower in the damaged skin samples than in the undamaged skin samples (Fig. 2C). No significant difference was observed between the amounts of ^{14}C–HD within damaged and undamaged skin (Fig. 2B).

Effectiveness of commercial haemostats as decontaminants is chemical-specific when CW agents are applied to damaged skin

All treatments significantly reduced the maximum rate of penetration of ^{14}C–GD across damaged skin (mg cm^{-2} h^{-1}) in comparison to 'damaged, untreated' skin (Fig. 3A). In addition, all haemostats (ProQR®, WoundStat™ and QuikClot ACS + ®) were as effective as fuller's earth, with no significant difference in J_{max} among the treatment groups.

Treatment of damaged skin with ProQR® resulted in a significantly higher proportion ($1.4 \pm 1.1\%$) of the dose being recovered from swabs of the skin compared to swabs from 'damaged, untreated' skin (Fig. 4). For the other groups, the proportion of the applied mass of ^{14}C–GD recovered from swabs of the skin did not differ significantly between the 'damaged, untreated' and the 'damaged, treated' groups. Fuller's earth was the only treatment to reduce significantly the proportion of the applied mass of ^{14}C–GD recovered from the skin in comparison to 'damaged, untreated' skin ($0.62 \pm 0.64\%$ and $9 \pm 3\%$ respectively). As indicated by the penetration profiles, the total proportion of ^{14}C–GD quantified within the receptor fluid was significantly lower for damaged skin treated with fuller's earth, ProQR® or WoundStat™ as compared to 'damaged, untreated' skin (Fig. 4). ProQR® and QuikClot ACS + ® retained a significantly lower proportion of ^{14}C ($55 \pm 10\%$ and $24 \pm 5\%$, respectively) than fuller's earth ($85 \pm 3\%$), whereas WoundStat™ did not differ significantly from fuller's earth in this respect.

The rate of penetration of ^{14}C–HD was significantly lower for skin treated with fuller's earth, WoundStat™ or QuikClot ACS + ® relative to 'damaged, untreated' skin (Fig. 3B). ProQR® was ineffective at reducing the rate of penetration, which was not significantly different from that in the 'damaged, untreated' control (Fig. 3B).
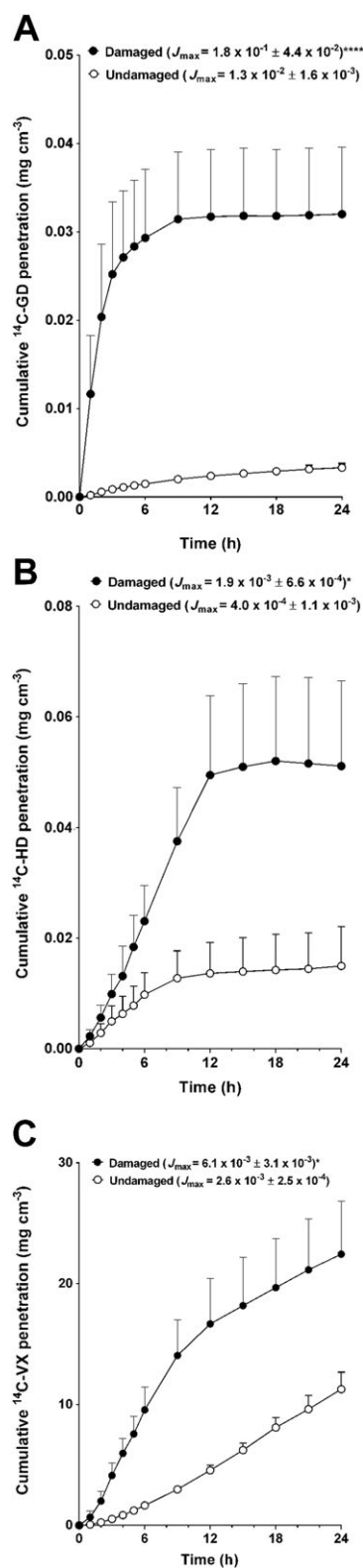

Figure 1. Cumulative penetration (mg cm^{-3}) of ^{14}C–labelled CW agents across 'damaged, untreated' and 'undamaged, untreated' porcine skin *in vitro*. Data points represent mean values (± standard deviation) for 'damaged, untreated' skin or 'undamaged, untreated' skin exposed to (A) ^{14}C–GD, (B) ^{14}C–HD and (C) ^{14}C–VX. Maximum penetration values (J_{max}) were calculated as mg cm^{-2} h^{-1} from the linear slope and asterisks indicate significant differences between the damaged group and the undamaged group (**$P < 0.01$; ****$P < 0.0001$).
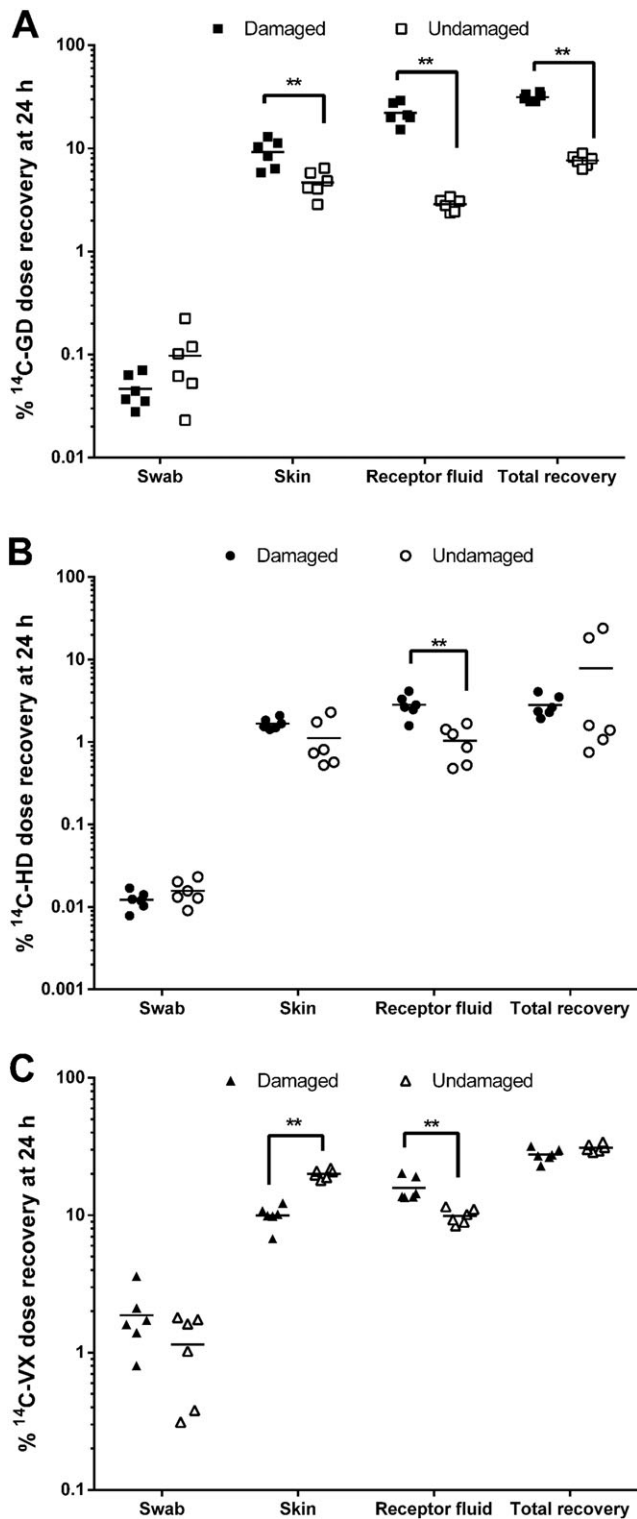
Figure 2. Quantification of radiolabel in swabs, skin, receptor fluid or decontaminant following 24 h exposure of 'damaged, untreated' and 'undamaged, untreated' *ex vivo* skin sections to CW agents (A) ^{14}C–GD, (B) ^{14}C–HD and (C) ^{14}C–VX. Individual data points are shown, with the central line indicating the mean value. Asterisks indicate significant differences between the 'damaged, untreated' group and 'undamaged, untreated' groups for swab, skin or receptor fluid (**$P < 0.01$).
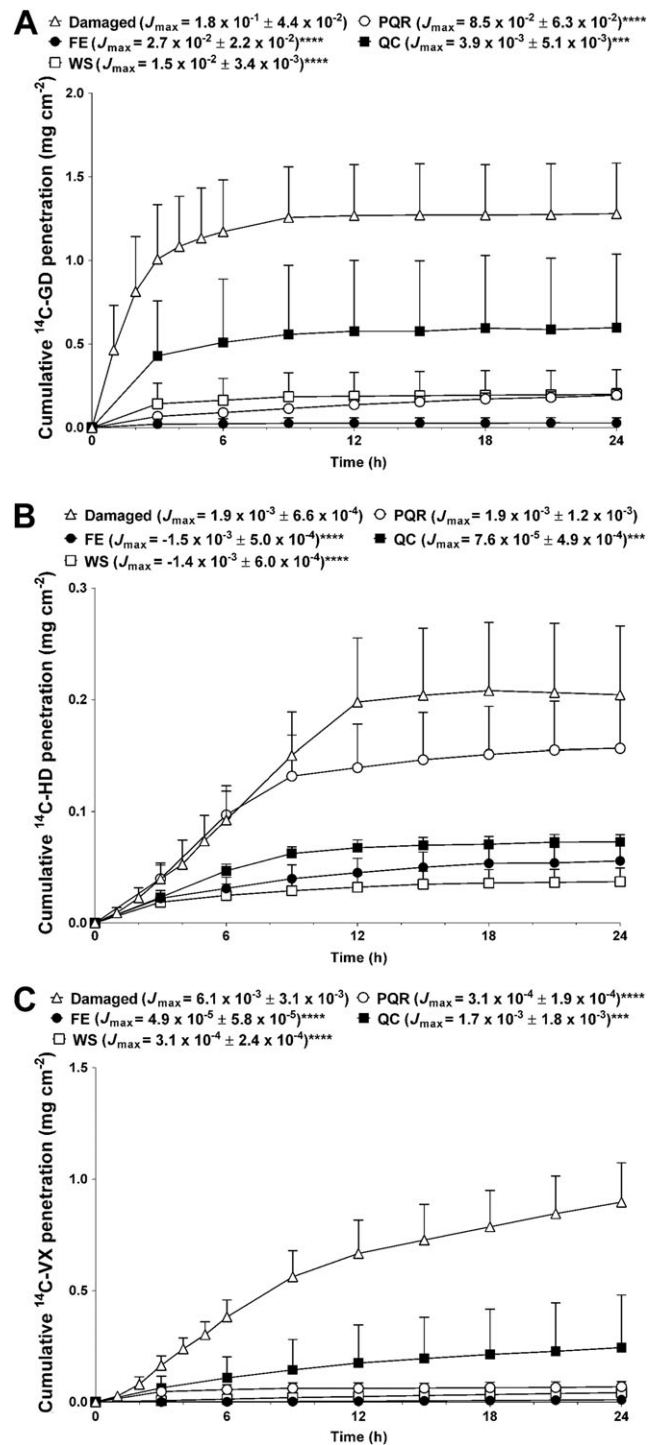

Figure 3. Cumulative penetration (mg cm^{-2}) of ^{14}C–labelled CW agents across 'damaged, untreated' and 'damaged, treated' porcine skin *in vitro*. Data points represent mean values (± standard deviation) for 'damaged, untreated' skin or damaged skin decontaminated with fuller's earth, ProQR®, WoundStat™ or QuikClot ACS + ® for each chemical contaminant: (A) ^{14}C–GD, (B) ^{14}C–HD and (C) ^{14}C–VX. Maximum penetration values (J_{max}) were calculated as mg cm^{-2} h^{-1} from the linear slope and asterisks indicate significant differences between the 'damaged, untreated' group and 'damaged, treated' groups (***$P < 0.001$; ****$P < 0.0001$). FE, fuller's earth; PQR, ProQR®; QC, QuikClot ACS + ®; WS, WoundStat™.**
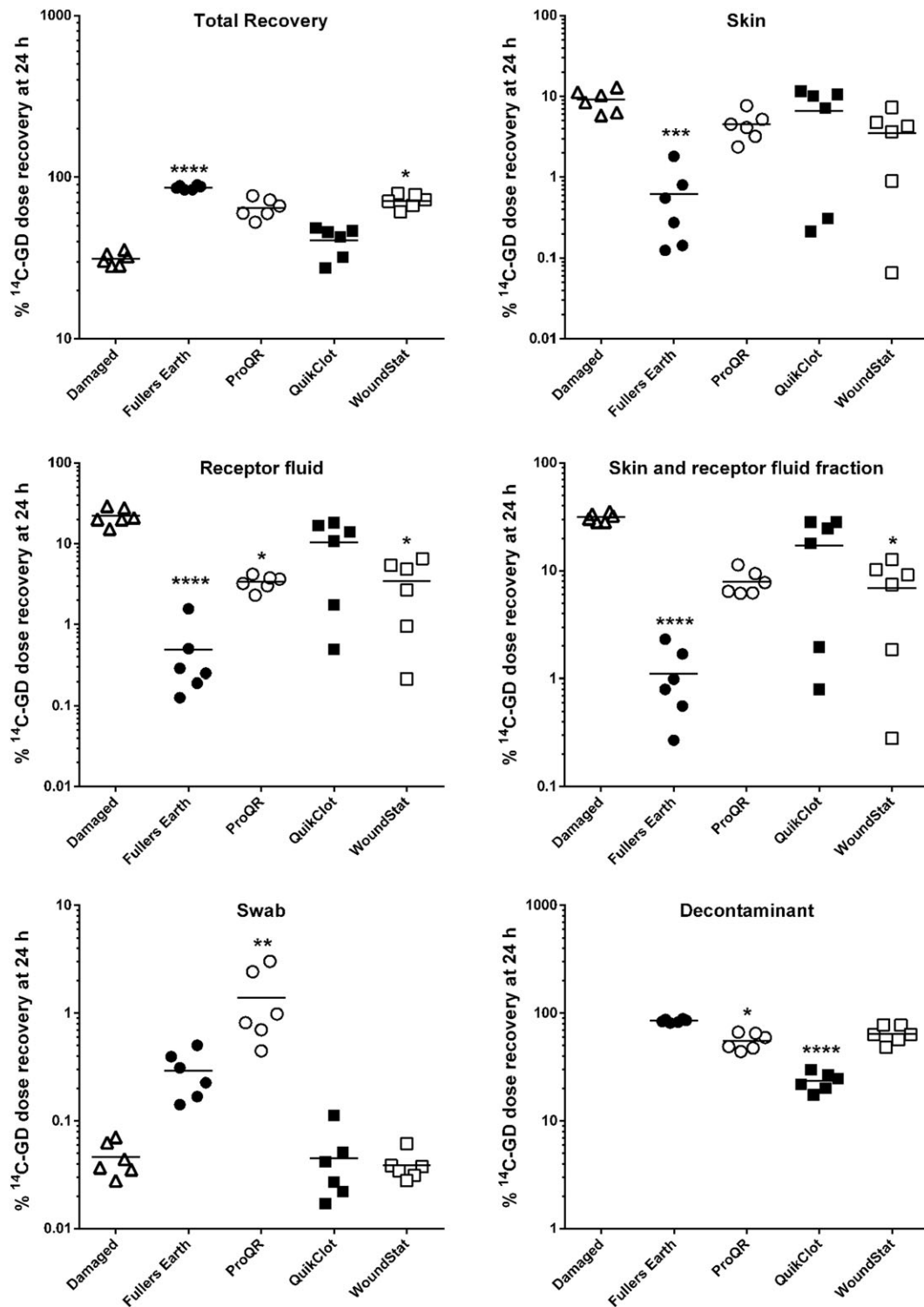
Figure 4. Quantification of radiolabel in swabs, skin, receptor fluid or decontaminant following 24 h exposure of 'damaged, untreated' and 'damaged, treated' *ex vivo* skin sections to ^{14}C–GD. Individual data points are shown, with the central line indicating the mean value. Asterisks indicate significant differences between the 'damaged, untreated' group and 'damaged, treated' groups (swab, skin and receptor fluid) or between fuller's earth and the test decontaminants (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

QuikClot ACS + ® was not as effective as fuller's earth in reducing the rate of penetration ($P = 0.0004$). However, WoundStat™ surpassed the effectiveness of the benchmark decontaminant, with a significantly lower rate of penetration compared to fuller's earth ($P = 0.0115$).

Treatment of damaged skin with fuller's earth or ProQR® resulted in a significantly higher proportion of the dose of ^{14}C–HD being recovered from swabs of the skin than for 'damaged, untreated' skin (Fig. 5). For the other groups, the proportion of the applied mass of ^{14}C–HD recovered from swabs of the skin was less
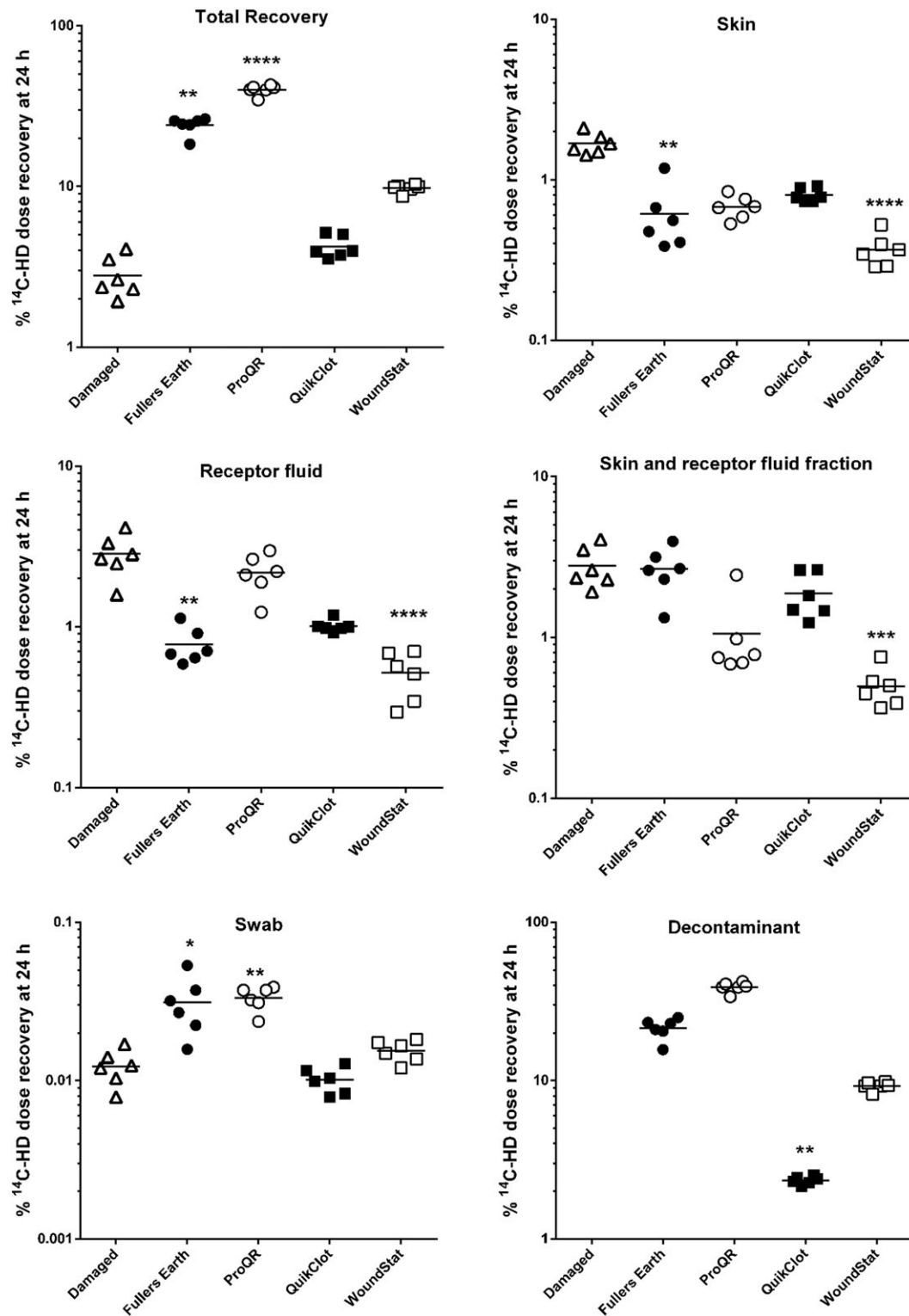
Figure 5. Quantification of radiolabel in swabs, skin, receptor fluid or decontaminant following 24 h exposure of 'damaged, untreated' and 'damaged, treated' *ex vivo* skin sections to ^{14}C–HD. Individual data points are shown, with the central line indicating the mean value. Asterisks indicate significant differences between the 'damaged, untreated' group and 'damaged, treated' groups (swab, skin and receptor fluid) or between fuller's earth and the test decontaminants (**$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$).

than 0.02% and did not differ significantly from that of the 'damaged, untreated' group (Fig. 5). Application of fuller's earth or WoundStat™ significantly reduced the proportion of the applied mass of ^{14}C–HD recovered from the skin in comparison to

'damaged, untreated' skin (Fig. 5). The total proportion of ^{14}C quantified within the receptor fluid was significantly lower for damaged skin treated with WoundStat™ or fuller's earth, compared to 'damaged, untreated' skin (Fig. 5). A significantly lower

proportion of ^{14}C was quantified from the QuikClot ACS + ® matrix in comparison to fuller's earth ($2 \pm 0.1\%$ and $21 \pm 3\%$, respectively), whereas ProQR® and WoundStat™ did not differ significantly from fuller's earth in this respect (Fig. 5).

All treatments significantly reduced the maximum rate of penetration of $^{14}C–VX$ across damaged skin (mg cm^{-2} h^{-1}) in comparison to 'damaged, untreated' skin (Fig. 3C). However, unlike $^{14}C–GD$, all haemostats, ProQR®, WoundStat™ and QuikClot ACS + ®, were significantly less effective than fuller's earth in reducing the maximum rate of penetration, with *P* values of 0.002, 0.02 and <0.0001, respectively.

Treatment of damaged skin with QuikClot ACS + ® and WoundStat™ resulted in a significantly lower proportion of the dose being recovered from swabs of the skin than for 'damaged, untreated' skin (<0.1% vs. $1.9 \pm 0.9\%$; Fig. 6). For the other groups, the proportion of the applied mass of $^{14}C–VX$ recovered from swabs of the skin did not differ significantly from that of the 'damaged, untreated' group (Fig. 6). The application of ProQR® and WoundStat™ significantly reduced the proportion of the applied mass of $^{14}C–VX$ recovered from the skin (<0.2%) in comparison to 'damaged, untreated' skin ($10 \pm 2\%$; Fig. 6). As previously indicated by the penetration profiles, the total proportion of ^{14}C quantified within the receptor fluid was significantly lower for damaged skin treated with fuller's earth, ProQR® or WoundStat™, in comparison to the 'damaged, untreated' group (Fig. 6). However, there was no significant difference in the amount of ^{14}C quantified within the receptor fluid between the 'damaged, untreated' group and the QuikClot ACS + ® treated group at 24 h post-exposure (Fig. 6). A significantly higher proportion of ^{14}C was quantified from the WoundStat™ matrix in comparison to fuller's earth, whereas ProQR® and QuikClot ACS + ® did not differ significantly from fuller's earth in this respect (Fig. 6).

Discussion

This study has demonstrated that superficial skin damage resulted in enhanced percutaneous penetration of radiolabelled CW agents ($^{14}C–GD$, $^{14}C–HD$ and $^{14}C–VX$) compared to undamaged porcine skin *in vitro*. Moreover, it identified a commercial haemostat (WoundStat™) capable of reducing $^{14}C–GD$, $^{14}C–HD$ and $^{14}C–VX$ penetration through damaged pig skin *in vitro* with an efficacy comparable to that of the in-service military decontaminant fuller's earth.

In vitro skin diffusion cells are a validated method for measuring percutaneous absorption of chemicals (Chilcott *et al.*, 2005a; OECD, 2004), including CW agents such as GD, HD and VX (Chilcott *et al.*, 2001; Dalton *et al.*, 2006, 2015; Vallet *et al.*, 2008; van Hooidonk *et al.*, 1980). Human skin is the gold standard model for *in vitro* percutaneous absorption studies. However, supplies of human tissue, as well as inter-individual variation in permeability, limit its use for high-throughput screening of test decontaminants. Porcine skin is generally considered a relevant model for human skin absorption, displaying similarities in biochemistry, histology and physiology (Chilcott *et al.*, 2001, 2005b; Dalton *et al.*, 2006; Freeman *et al.*, 2015). In addition, the use of a single animal donor for each chemical minimized inter-individual variation, aiding comparisons and highlighting differences between the treatments tested. However, any extrapolation of our observations to human skin should be made with caution, given the possibility of differences between human and porcine skin in the penetration of these chemicals (Dalton *et al.*, 2006). A further consideration is that the radiometric method used in the present study cannot differentiate between the original compound and products resulting from hydrolysis and/or metabolism (Munro *et al.*, 1999; Chilcott *et al.*, 2000; Creasy *et al.*, 2012). However, this analysis represents a conservative, 'worst-case' approach, as it assumes that all the recovered radiolabel is the original, toxic penetrant (Jokanović, 2009; Munro *et al.*, 1999).

While the effects of damage, such as needle punctures, abrasion or tape stripping, on general skin permeability have been assessed previously *in vitro* (Chilcott *et al.*, 2002; Davies *et al.*, 2015; Schlupp *et al.*, 2014; Wu *et al.*, 2006), to our knowledge this study is the first to use *in vitro* static diffusion cells to assess the penetration and decontamination of CW agents through damaged skin. Skin damage (removal of upper 100 μm layer) resulted in an increase in the amount of $^{14}C–HD$ and $^{14}C–GD$ detected in the receptor fluid compared to undamaged skin. Moreover, the maximum flux through damaged skin was greater compared with undamaged skin for all three CW agents. These observations are not surprising, given that the stratum corneum is considered the primary barrier to absorption (Scheuplein, 1976; Zhai and Maibach, 2002). The enhanced dermal absorption of CW agents would likely result in a more rapid onset of intoxication and/or more severe toxicity and thus emphasizes the importance of identifying an effective wound decontamination product.

WoundStat™ and fuller's earth retained a large proportion of the organophosphate contaminants (>65% dose recovery), thus limiting the dose available for absorption. However, the retention of $^{14}C–HD$ was lower for these decontaminants compared to the retention of organophosphates (~75% and 90% for fuller's earth and WoundStat™, respectively). Passive absorption is the primary mechanism through which haemostatic products stop bleeding and the same mechanism is responsible for the decontaminant properties of fuller's earth. In addition, the haemostats tested in this study have negative surface charges to facilitate coagulation through activation of factor XII. Therefore, the differences in retention between HD and organophosphates are probably attributable to differences in their physiochemical properties, with better absorption of more hydrophilic chemicals, i.e., VX and GD, compared to more lipophilic chemicals, such as HD. In addition, the total dose recovery was lower for HD than for the organophosphates. This was somewhat unexpected, given that GD is known to have a higher volatility than HD. However, permitting the test decontaminants to remain in place for the duration of the experiment may have prevented volatilization of the chemicals from the skin surface with increased retention of organophosphates, as discussed above.

Standard military doctrine dictates that skin decontamination should normally be instigated within 2 min of exposure. In the present study, decontamination was performed after 30 s to model the time between sustaining a significant haemorrhaging injury and subsequent application of a haemostatic product under battlefield conditions. As longer delays in treating a haemorrhaging wound could limit patient survival (Mabry *et al.*, 2000), a 30 s delay provides a more realistic exposure scenario for performing wound decontamination. A second deviation from established protocol was to leave the haemostatic product *in situ* for the duration of the study. Normally, decontamination products have only transient contact with body surfaces. However, with potentially long evacuation times (Alam *et al.*, 2003; Griffiths and Clasper, 2006; Mabry *et al.*, 2000; Spalding *et al.*, 1991), removal of wound dressings may be delayed in combat conditions; thus, the extended contact time used in this current study provides a more appropriate evaluation of product efficacy for wound decontamination.

In conclusion, the *in vitro* static diffusion cell model was found suitable for investigating chemical absorption through superficially damaged skin and has demonstrated that HD, GD and, to
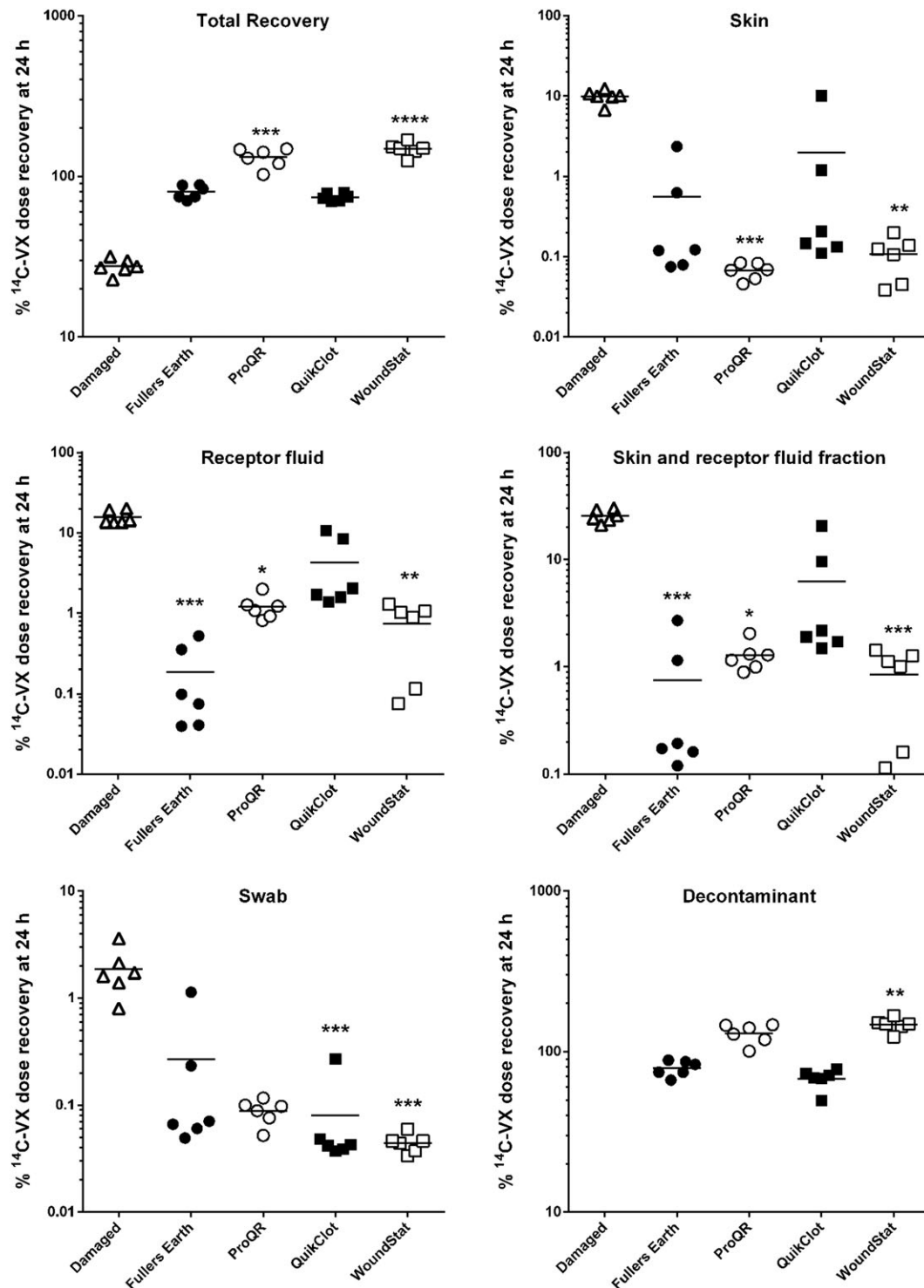
Figure 6. Quantification of radiolabel in swabs, skin, receptor fluid or decontaminant following 24 h exposure of 'damaged, untreated' and 'damaged, treated' *ex vivo* skin sections to ^{14}C–VX. Individual data points are shown, with the central line indicating the mean value. Asterisks indicate significant differences between the 'damaged, untreated' group and 'damaged, treated' groups (swab, skin and receptor fluid) or between fuller's earth and the test decontaminants (**$P < 0.01$; ***$P < 0.001$).

some extent, VX have enhanced toxicokinetic profiles. Utilization of this damaged skin model, in combination with other supporting data (Dalton *et al.,* 2015; Hall *et al.,* 2015), has facilitated the identification of haemostatic products that are ineffective as wound decontaminants. Overall, WoundStat™ was the only haemostatic formulation that demonstrated equivalence to the benchmark product (fuller's earth) in reducing the rate and extent of dermal absorption of all three CW agents, and is known to retain coagulation efficacy in the presence of CW agents (Hall *et al.,* 2015). Therefore, it is recommended that WoundStat™ should undergo further evaluation using an appropriate *in vivo* contaminated wound model.

Conflict of interest

The authors did not report any conflict of interest.

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