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Effect of different alcohols on stratum corneum kallikrein 5 and phospholipase A_2 together with epidermal keratinocytes and skin irritation

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Abstract

OBJECTIVES: The aim of this exploratory study was to investigate the effect of ethanol, isopropanol and n-propanol on stratum corneum (SC) enzymes and keratinocytes *in vitro* together with their effects on skin condition and function.

METHODS: Activities of kallikrein 5 (KLK5) and phospholipase A2 (PLA2) as well as keratinocyte metabolic activity, interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) were measured *in vitro* in the presence and absence of the different alcohols. We also measured transepidermal water loss (TEWL), skin capacitance, visual dryness and visual redness on the volar forearms of 25 Caucasian women following application of the alcohols 20 and 100 times per day over a period of 14 days in a clinical study.

RESULTS: Reduced activities of KLK5 and PLA2 were observed in the presence of the alcohols. The greatest denaturing effect was always observed for n-propanol ($P < 0.001$), and in the case of PLA2, the effect of isopropanol was greater than ethanol ($P < 0.001$). Equally, ethanol had the mildest effects on keratinocyte metabolic activity and cytokine secretion ($P < 0.001$) and n-propanol always produced the most severe changes in normal and differentiated keratinocytes. These *in vitro* findings supported the clinical results where the major effects were on the induction of skin irritation (increased dropout rates) and ranked the intolerance of the different alcohols as follows: n-propanol > isopropanol > ethanol. At the high application frequencies, the effect of the different alcohols on transepidermal water loss (TEWL) and skin capacitance was similar, but at the low application frequencies, n-propanol had a significant effect on TEWL and capacitance values ($P < 0.05$). Equally, n-propanol and isopropanol produced significantly more skin redness at the low application frequencies.

CONCLUSIONS: Clearly, isopropanol and n-propanol caused significant SC and keratinocyte perturbation *in vitro* together with damage to skin condition and function *in vivo* whereas ethanol did not. As a result, we show that ethanol-based sanitizers are better tolerated by skin, particularly in high-use settings, than other alcohols and should be the active ingredient of choice.

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[Correction added on 18 October 2016, after first online publication: The sixth author, Dr. P Stapleton's last name was previously wrong and has been corrected in this current version]

Résumé

OBJECTIFS: Le but de cette étude exploratoire était d'étudier l'effet de l'éthanol, de l'isopropanol et du n-propanol sur les enzymes de la couche cornée (SC) et les kératinocytes *in vitro* ainsi que leurs effets sur l'état de la peau et la fonction cutanée.

MÉTHODES: Les activités de la kallikréine 5 (KLK5) et de la phospholipase A2 (PLA2), ainsi que l'activité métabolique des kératinocytes, l'interleukine-1α (IL-1α) et le facteur de nécrose tumorale-α (TNF) ont été mesurés *in vitro* en présence et en l'absence des différents alcools. Nous avons également mesuré la perte d'eau transépidermique (TEWL), la capacité de la peau, la sécheresse visuelle et une rougeur visuelle sur les avant-bras palmaires de 25 femmes Caucasiennes après l'application des alcools 20 et 100 fois par jour sur une période de 14 jours dans une étude clinique.

RÉSULTATS: La réduction des activités de KLK5 et PLA2 a été observée en présence des alcools. Le plus grand effet dénaturant a toujours été observé pour le n-propanol ($P < 0.001$) et dans le cas de la PLA2 l'effet de l'isopropanol est supérieure à l'éthanol ($P < 0.001$). De même, l'éthanol a eu les effets les plus douces sur l'activité métabolique des kératinocytes et la sécrétion de cytokines ($P < 0.001$) et le n-propanol produit toujours des changements les plus sévères dans les kératinocytes normaux et différenciés. Ces résultats *in vitro* ont confirmé les résultats cliniques où les effets majeurs étaient observés sur l'induction d'une irritation de la peau (augmentation des taux d'abandon par des panelistes) et ont classé l'intolérance aux différents alcools comme suit: n-propanol> isopropanol> éthanol. Aux hautes fréquences d'application, l'effet des différents alcools sur la perte d'eau transépidermique (TEWL) et de la capacité de la peau étaient similaires, mais pour les basses fréquences d'application, le n-propanol a eu un effet significatif sur la PIE et les valeurs de capacitance ($P < 0.05$). De même, le n-propanol et l'isopropanol produisent significativement plus de rougeur de la peau au niveau des basses fréquences d'application.

CONCLUSIONS: De toute évidence, l'isopropanol et le n-propanol causent une perturbation significative du SC et des kératinocytes *in vitro* et portent ainsi atteinte à la condition et la fonction cutanée *in vivo* que l'éthanol ne présente pas. En conséquence, nous montrons que les désinfectants à base d'éthanol sont mieux

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tolérés par la peau que les autres alcools, en particulier dans les paramètres d'utilisation élevés, et devraient préférer l'éthanol comme l'ingrédient actif de choix.

Introduction

Alcohol-based hand rubs (ABHRs) have a key role to play in mitigating the transmission of pathogens in healthcare settings [1]. Up to 100 applications of a product per day may be necessary to achieve full hand hygiene compliance (HHC) [2], and healthcare workers (HCWs) are at increased risk for occupational dermatitis because of such frequent washing and use of hand sanitizers [3]. The specific alcohol used in an ABHR may be n-propanol, isopropanol or ethanol depending on the country or region.

The ability of alcohols to denature proteins has been demonstrated, and their potency in this respect depends on alcohol chain length and hydrocarbon content together with their octanol–water partition coefficients [4–6]. Enzyme activities have also been shown to be reduced in the presence of ethanol. α-chymotrypsin and trypsin were reported to be completely inactivated by ethanol at 40% and 90% (v/v), respectively [7,8]. These effects may be relevant for stratum corneum (SC) enzymes that are involved in barrier function, profilaggrin processing or desquamation [9–11]. To our knowledge, the extent to which different alcohols may influence skin enzyme activities has not been examined.

Alcohols have also been shown to be toxic to epithelial cells *in vitro*. Enzymes and cytokines have been shown to be released with different alcohols [12–14]. However, again the relative effects of different alcohols on keratinocytes are largely unreported.

Ethanol is a known skin penetration enhancer that is thought to act by decreasing the molecular interaction between the polar head groups of the ceramides found in the skin and/or through interactions between their alkyl chains [15]. However, the direct evidence for this is limited especially for ethanol concentrations ≥75% [15,16]. Nevertheless, increased transepidermal water loss (TEWL) has been observed following ethanol application to subjects and this was attributed to extraction of lipids. Some researchers have observed changes in SC electrical resistance and conductivity at various ethanol concentrations as well as changes in SC lipid melting temperatures. The maximum effects were observed for ethanol: water (50 : 50) but neat ethanol was not examined independently [17]. More recently, it has been demonstrated that ethanol disrupted SC intercellular lipid structure, formed pores and promoted orthorhombic-(the most tightly packed lipid organization)to-hexagonal (a less tightly packed lipid organization) lipid phase transitions [18,19]. Also Thind *et al.* [20] confirmed that ethanol induces water-permeable defects in models of SC lipids *in vitro*. However, the effects of ethanol on SC lipids *in situ* have not been investigated in depth. Moghadem *et al.* [21] reported that ethanol produces very little change in the small- and wide-angle diffraction patterns of SC lipids. However, examining hairless mouse skin, Horita *et al.* [22] reported that ethanol modified the short but not the long periodicity phase of SC lipids. Changes to keratin structure have also been reported with increasing concentrations of ethanol. Interestingly, others report no effects on lipid fluidity at ethanol concentrations up to 70% using electron parametric resonance (EPR) spectroscopy [23]. Despite this, Kim *et al.* [24] have shown that alcohols fluidize liposomes composed of SC lipids, with propanol having more disruptive effects than ethanol. Again, there is only a limited amount of information available in the literature describing the effects of different alcohols on SC lipid phase behaviour, but clearly, these structures are disrupted.

The interaction of alcohols with skin *in vivo* is also poorly understood. Topical application of absolute ethanol to hairless mice has been shown to produce a mild and transient increase in epidermal mitotic rates and cellular damage comparable to that observed by tape-stripping of the SC [25,26]. Moreover, a whitening of the skin has been noted after solvent exposure which has been associated with changes in the structure and removal of skin lipids [27]. Nevertheless, as evaluated by a corneoxenometric assay, ethanol was the least aggressive solvent *ex vivo* [28]. Several studies have shown the negative effects of various propanol isomers on TEWL in soap or sodium lauryl sulphate (SLS) damaged skin whereas the use of a gel containing 78% ethanol and 5% isopropanol in normal subjects over an 8-day period had no effect [29–31].

Regarding the use of commercially available ABHRs on normal subjects using 20 applications on day 1 followed by 5 applications per day for the next 6 days, the products containing 75–80% alcohol were generally more drying compared with preparations with a lower alcohol content [32]. Moreover, use of emollients and humectants in the formulations has been shown to reduce skin problems [33–35]. However, there are no reported studies of the effects of these products at much higher frequencies of usage that are now advised for HHC.

The aim of this study was to understand the effects of various alcohols currently used in ABHRs on SC enzymes. To this end, the effects of alcohol exposure on the key SC desquamatory protease and profilaggrin-processing enzyme, kallikrein 5 (KLK5), and a lipid-processing enzyme, phospholipase A2 (PLA_2), were investigated [9–11]. In addition, measurements of membrane integrity and production of inflammatory cytokines by keratinocytes *in vitro* were conducted. Finally, the effects of high ABHR application frequencies, which are required for good hygiene compliance, were examined clinically. This was accomplished by applying 70% n-propanol, isopropanol and ethanol in a forearm controlled application treatment (FCAT) clinical study. Two application frequencies were selected (20 and 100 times per day), and the study was conducted

Table I Skin dryness and redness grading scale

	Skin grading scale	
Grade	**Redness**	**Dryness**
0.	No redness	Normal, healthy skin
1.	Barely detectable redness	Areas of powdering and/or washing. Some areas of small scales may be seen
2.	Slight redness	Definite generalized powderiness, early, cracking or some small lifting scales may be seen
3.	Moderate redness	Generalized small-to-medium-sized lifting scales, some erythema may by present
4.	Heavy or substantial redness	Large areas of scales and/or erythema
5.	Severe redness	Generalized large lifting scales, erythema and fissuring. Might see bleeding fissures
6.	Extreme redness	Generalized severe cracking and bleeding Large scales may be sloughing off

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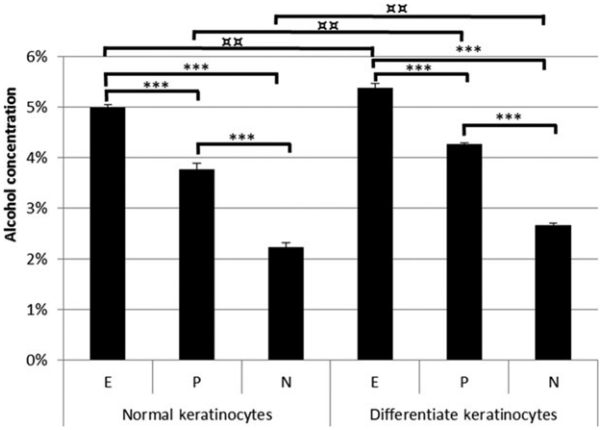

Figure 1 IC_{50} values for different alcoholic solutions for normal NHEK and differentiated NHEK. E = Ethanol, P = isopropanol and N = n-propanol (all concentrations are w/w in water). Ethanol exposure resulted in the highest IC_{50}, while n-propanol resulted in the lowest in both low- and high-calcium-treated NHEK. For both isopropanol and n-propanol, their $IC_{50'}$ values are significantly different to the IC_{50} value for ethanol (***$P < 0.001$, $n = 4$) and there is also a significant difference (¤¤$P < 0.01$, $n = 4$) between the low- and high-calcium-treated keratinocytes for each type of alcohol.

over 14 days. Skin was assessed by expert grading (dryness and redness) and with biophysical measurements (TEWL and skin capacitance). To the best of our knowledge, this is the first report of its kind to (i) evaluate the effects of different alcohols on skin condition when used at such high application frequencies and (ii) provide insights into their effects on key SC maturation enzymes.

Methods

Fluorometric-based detection of enzyme activities in the presence of alcohols

To measure the *in vitro* enzyme activities of PLA_2 and KLK5, the substrates PED6 (N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine) obtained from Life Technologies (Carlsbad, CA, USA) and Boc-Phe-Ser-Arg-AMC and Tos-Gly-Pro-Lys-AMC supplied by Bachem Distribution Services GmbH (Weil am Rhein, Germany), respectively, were used [36, 37]. The reaction buffer for PLA_2 consisted of 100 mmol L^{-1} of Tris-HCl pH 7.5, 200 mmol L^{-1} of NaCl, 2 mmol L^{-1} of ethylenediaminetetraacetic acid, 2 mmol L^{-1} of ethylene glycol-bis (β-aminoethylether)-N,N,N′N′-tetraacetic acid in HPLC grade water. To initiate the reaction, $CaCl_2$ was added at 10 mmol L^{-1}. To measure KLK5, 100 mmol L^{-1} of Tris-HCl (pH 8) was used. PLA_2 and kallikrein extracted from porcine pancreas (Sigma-Aldrich Ltd., Dorset, UK) were used at 6 mU and 12 mU, respectively. Substrates were dissolved in reaction buffer only (PED6) or DMSO (Boc-Phe-Ser-Arg-AMC) and were added to achieve final concentrations of 8 μmol L^{-1} (PED6) and 5 mmol L^{-1}. The enzyme solutions were incubated at 25% ethanol, isopropanol or n-propanol (Fisher Scientific, Loughborough, UK) at 37°C. At various time points, over a period of 180 minutes, the fluorescence intensity was detected with a PHERAstar Plus® spectrofluorometer (BMG Labtech, Aylesbury, UK). Excitation and emission wavelengths were 485 and 520 nm, and 350 and 460 nm (bandwidth 10 nm), for PLA_2 and KLK5 activities, respectively. Fluorescence intensity calibrations were determined with BODIPY® FL C_5 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic Acid; Life Technologies, Carlsbad, CA, USA), and 7-amino-4-methylcoumarin (Alfa Aesar, Heysham Lancashire), in the specific alcohol–buffer ratio. Enzyme activities for the various alcoholic solutions were determined after 180 min incubation at 37°C. Measurement of released AMC allows determination of enzyme activities.

Keratinocyte culture, MTT and TNF-α and IL-1α cytokine assays in the presence of alcohols

Neonatal human epidermal keratinocytes (NHEK; Life Technology, Grand Island, NY, USA) were cultured with keratinocyte growth medium (KGM, Medium 154: M-154-500 Life Technology with supplements S-001, Life Technologies) to make a final concentration of each supplement as bovine pituitary extract, 0.2% v/v; bovine insulin, 5 μg mL^{-1}; hydrocortisone, 0.18 μg mL^{-1}; bovine transferrin, 5 μg mL^{-1}; and human epidermal growth factor, 0.2 ng mL^{-1}. Keratinocyte differentiation was induced in KGM

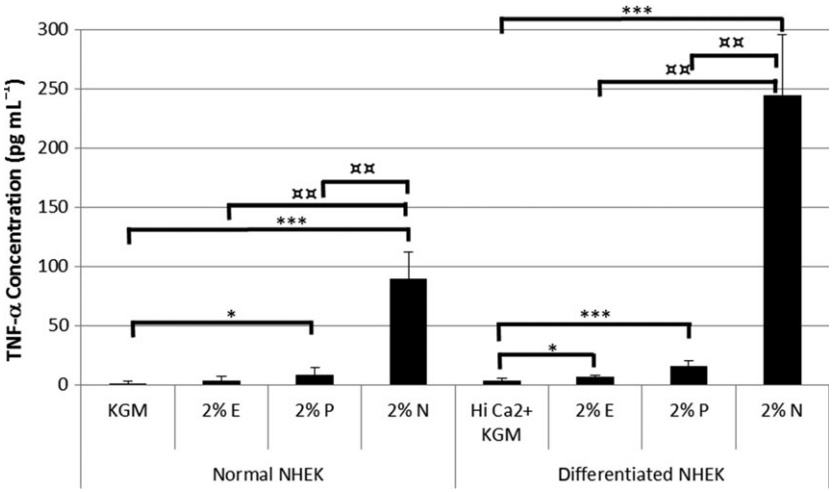

Figure 2 TNF-α concentration in NHEK culture medium after overnight treatment with 2% of the different alcohols for NHEK in low-calcium medium and high-calcium medium. E = Ethanol, P = isopropanol and N = n-propanol (all concentrations are w/w in water). TNF-α was highly induced in n-propanol-treated NHEK in both low-calcium and high-calcium medium (***$P < 0.001$ in both medium; $n = 4$). Isopropanol also significantly induced TNF-α from NHEK in both low-calcium and high-calcium medium (*$P < 0.05$ in low-calcium medium and *** $P < 0.001$ in high-calcium medium, $n = 4$), while ethanol induced the least levels of TNF-α (*$P < 0.05$, $n = 4$) in differentiated keratinocytes only). n-propanol induced significantly higher TNF-α compared with the other alcohols in both low-calcium and high-calcium medium ($P < 0.01$, $n = 4$).

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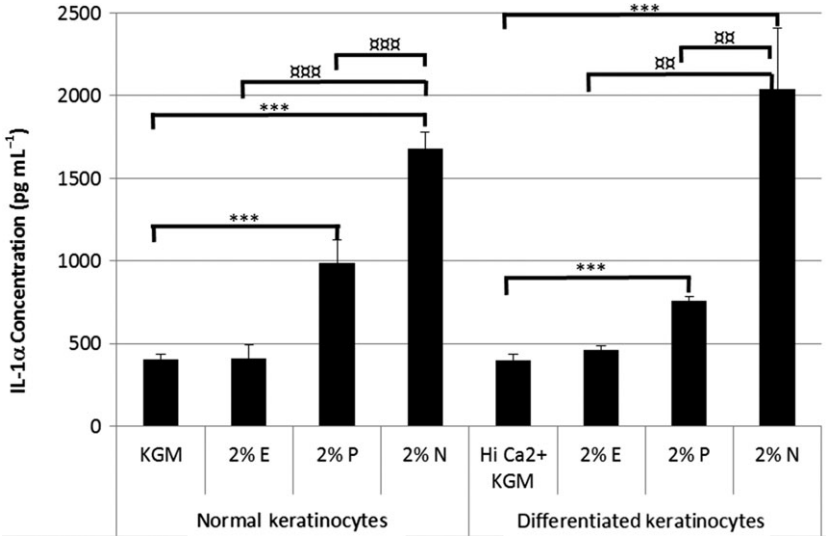

Figure 3 IL-1α concentration in NHEK culture medium after overnight treatment with 2% of different types of alcohols for NHEK in low-calcium medium and high-calcium medium. E = Ethanol, P = isopropanol and N = n-propanol (all concentration is w/w in water). Both n-propanol and isopropanol significantly induced IL-1α from NHEK in low-calcium medium and high-calcium medium (***$P < 0.0001$; $n = 4$), while n-propanol induced IL-1α levels to the greatest extent compared with the other alcohols in both low-calcium and high-calcium medium (¤¤¤$P < 0.01$, $n = 4$, ¤¤¤$P < 0.001$, $n = 4$). Ethanol did not significantly promote IL-1α expression in NHEK with either low-calcium or high-calcium medium ($P > 0.1$, $n = 4$).

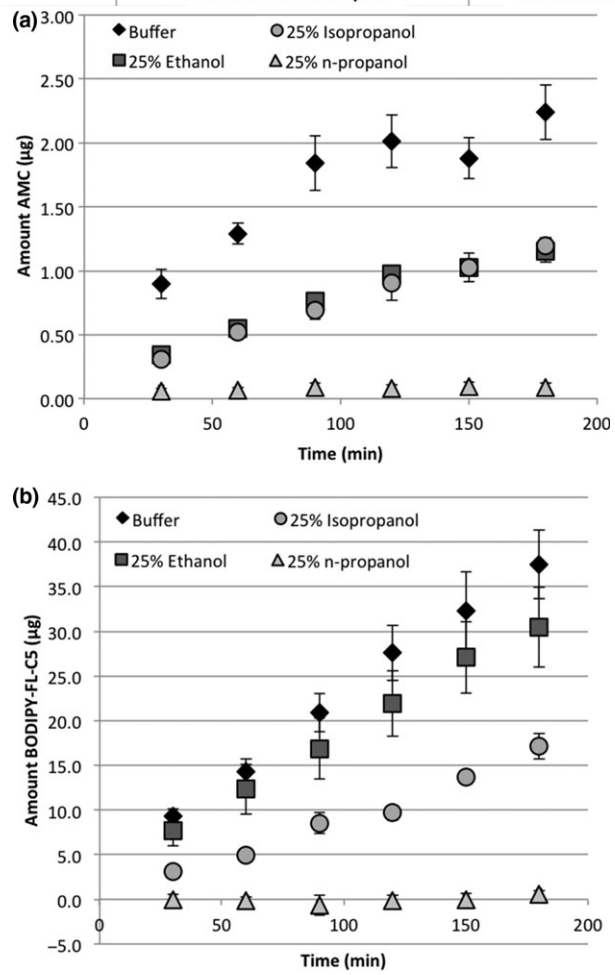

Figure 4 (a) Effects of alcohols on KLK5 activity. Ethanol and isopropanol are significantly different to n-propanol ($P < 0.001$). (b) Effect of alcohols on PLA_2 activity. Ethanol and isopropanol are significantly different to n-propanol ($P < 0.001$). Ethanol is significantly different to isopropanol ($P < 0.001$).

containing 1.3 mM $CaCl_2$ (Hi Ca^{2+} KGM), obtained from Thermo Fisher Scientific, Pittsburgh, PA, USA.

To determine the cellular toxicity of alcohols (ethanol, n-propanol and isopropanol) on NHEK, the MTT assay (Sigma-Aldrich, St. Louis, MO, USA) was used. NHEK were seeded into 96-well plates at a density of 10 000 cells in 200 µL of medium per well. After 48 h, the four replicate cell suspensions were incubated with varying concentrations of the alcoholic solution (0%, 0.1%, 0.5%, 1%, 2% up to 10% with 1% increments of 70% alcohol w/w in culture medium) in either KGM or Hi Ca^{2+} KGM overnight (16 h) at 37°C, 5% CO_2 and 95% humidity. Subsequently, the medium was aliquoted for ELISA and cells were supplemented with fresh phenol red-free DMEM (Life Technology) containing 0.5 mg mL^{-1} MTT solution and for 2 h at 37°C after which the medium was replaced with DMSO (100 µL per well, Fisher Scientific) to dissolve the formazan crystals, and the plate was incubated for 20 min at RT while shaking. The absorbance was read at 550 nm using a Synergy™ H1 microplate reader (Bio Tek, Winooski, VT, USA), and the half-maximal inhibitory concentrations (IC_{50}) were calculated. Cytokine assays were performed using a human IL-1α DuoSet ELISA development kit and a TNF-α DuoSet ELISA development kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Absorbance was read at 450 nm using a Synergy™ H1 microplate reader (Bio Tek, Winooski, VT, USA).

Forearm controlled application test (FCAT)

This study was an evaluator-blind, parallel-group FCAT (ethanol, isopropanol and n-propanol versus no treatment) single-centre study conducted on 25 Caucasian females aged 35–50 years with normal volar forearm skin. All subjects received all treatments. Subjects gave written informed consent, and the study was conducted in accordance with the Declaration of Helsinki guidelines. Subjects were excluded if they were pregnant, breastfeeding, peri/post-menopausal, had a known allergy or intolerance to any of the study materials or had participated in any other clinical trial in the prior 30 days. The study was conducted by North Cliff Consultants,

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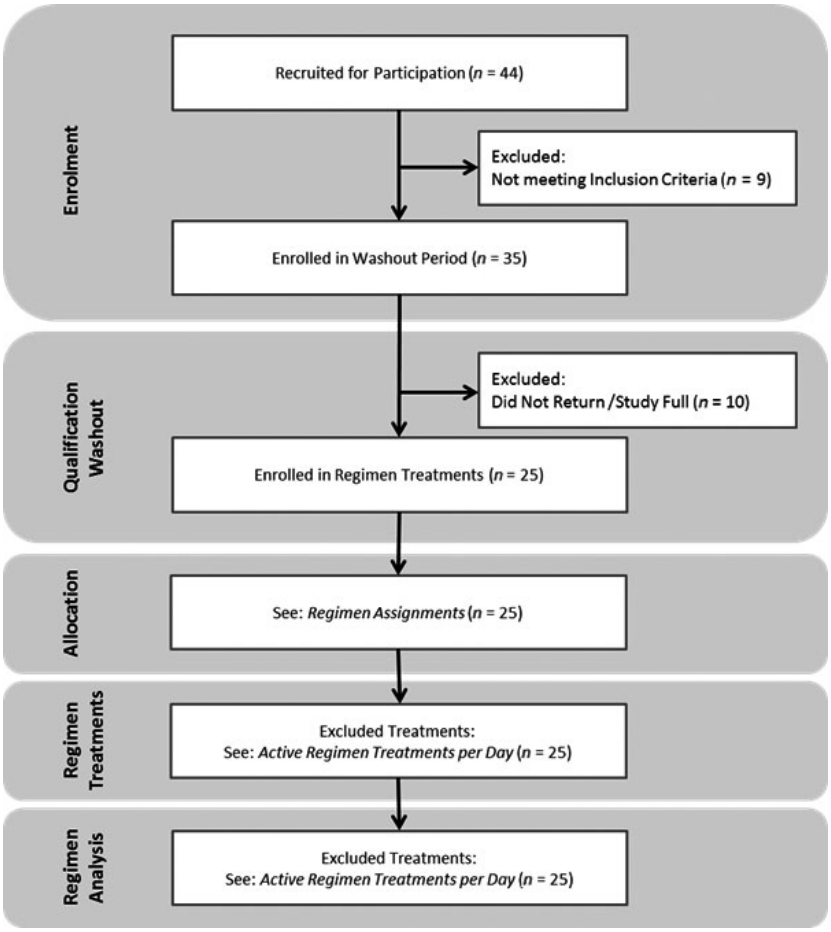

Figure 5 Flow diagram of participant's progress.

Inc, Cincinnati, USA, between 18th March and 8th April 2013, inclusive.

The study commenced with a 1-week washout period of the forearms using a commercially available gentle foam cleanser twice per day, avoiding use of any other products. To wash, subjects wet both hands and the opposing forearm and then dispensed one full pump of the soap into the hand; this was applied to the opposing forearm, and the forearm was gently washed in a back-and-forth motion using no more than five strokes. Lukewarm water was used for washing and rinsing which was performed again with a back-and-forth motion. Finally, subjects gently patted the forearms to dry the areas rather than rubbing with a towel to avoid any additional source of irritation or potential exfoliation.

Subjects acclimatized in an environmentally controlled room (70 ± 2°F, 40 ± 5% RH) for at least 30 min with their volar forearms exposed prior to any measurements or skin grading. Panellists exhibiting a visual redness or dryness score at any treatment site >3.0 on a 0–6 grading scale at baseline were excluded from participation (Table I). Four test sites (3 cm × 4 cm) were marked on the volar surface of each forearm using a Sharpie® from the wrist to the elbow. Upon arrival at the study site, subjects washed their forearms with Gojo regular clear and mild foam handwash (Gojo Inc, Cleveland, Ohio) and sat for at least 30 min with their volar forearms exposed to allow the arms to dry before a clinical investigator applied the treatments. Three ABHR systems containing 70% of the alcohol (ethanol, isopropanol and n-propanol) and water were used. Study supervisors applied 2 μL cm^{-2} of one test product to the centre of the appropriate treatment site using a positive-displacement pipette. The subjects rubbed the product using 20 circular rotations lasting approximately 10 sec on the site of their own skin while wearing finger cots. This was followed by the various test regimens and skin assessments conducted at regular intervals (2, 4, 7, 9, 11 and 14 days) over the 2-week evaluation period.

Panellists' forearms were marked to allow randomization of the regimens: three alcohol systems applied 20 times per day (standard frequency; SF); three alcohol systems applied 100 times per day (high frequency HF); and an untreated skin control. Following a total of 200 (SF) and 1000 (HF) individual alcohol system applications and corresponding skin measurements, data were tabulated and analysed. In addition to the test regimens, panellists' forearms were washed six times per day at scheduled intervals consistent with a minimal HCW daily washing routine as described above. A qualified skin grader then evaluated the treatment sites for visual dryness/redness, and if a site was graded as 5.0 or higher, the site was not treated further and that subject was considered as a dropout in the statistical analysis.

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SC capacitance was measured using a Corneometer CM825 (Courage & Khazaka Electronic, Cologne, Germany) and basal TEWL using an Aquaflux AF200 (Biox Systems, London, UK) following visual grading, on days 2, 4, 6, 7, 9, 11 and 12. Skin capacitance was expressed as the mean value of three recordings. TEWL was measured once. Due to the dropout rates, average TEWL and capacitance readings were computed across the whole of the treatment phase of the study. All procedures were conducted following published guidelines of the European Group on Efficacy Measurement of Cosmetics and Other Topical Products (EEMCO) [38–40].

Statistical methods

Analysis of variance (ANOVA) was used to assess the individual and interactive effects of alcohol type and application rate, and to compare with the untreated skin control. Chi-square analysis was also used to evaluate the rate of attrition by regimen due to skin condition meeting predetermined thresholds (i.e. a visual dryness/redness grade of 5.0 or higher). The software SPSS® Statistics (Version 22) package was used for the statistical analysis of the enzyme measurements. A one-way ANOVA test was performed based on the last time point (180 min) to assess differences in enzyme activities induced by the three alcohols. A probability of $P < 0.05$ was considered as statistically significant. Student's *t*-tests were used to compare keratinocyte marker analyses.

Results

Effects of the different alcohol solutions on keratinocytes

The IC_{50} values for different alcoholic solutions for normal NHEK and differentiated NHEK are shown in Fig. 1. Differences in the effects of the different alcohol solutions were observed between the different alcohols. Clearly, ethanol exposure resulted in the highest IC_{50} value, while n-propanol resulted in the lowest in both normal and differentiated NHEK. Ethanol was statistically superior to the other two alcohols ($P < 0.001$). All IC_{50} values were significantly different to controls ($P < 0.001$), and a higher IC_{50} value was obtained for each alcohol solution in the high-calcium-treated keratinocytes than the low-calcium-treated keratinocytes.

The effects of the different alcohols on the expression of TNF-α for low- and high-calcium-treated keratinocytes are shown in Fig. 2. As can be seen, n-propanol was the most irritating to the keratinocytes and greatly increased the expression of TNF-α compared with the other two alcohols ($P < 0.001$). Numerically, the effects of isopropanol were greater compared with ethanol and the secretion of TNF-α was greater for isopropanol compared with the medium control ($P < 0.05$ in low-calcium-medium-treated keratinocytes, $P < 0.001$ in high-calcium-medium-treated keratinocytes). Only ethanol was not significantly different to the medium control.

Also the effects of the different alcohols on the expression of IL-1α for low- and high-calcium-treated keratinocytes are shown in Fig. 3. Clearly, n-propanol was the most irritating to the keratinocytes and greatly increased the expression of IL-1α compared with the other alcohols ($P < 0.001$). Nevertheless, isopropanol was also more irritating to keratinocytes ($P < 0.001$) compared with ethanol. Ethanol treatment was not significantly different to medium control values.

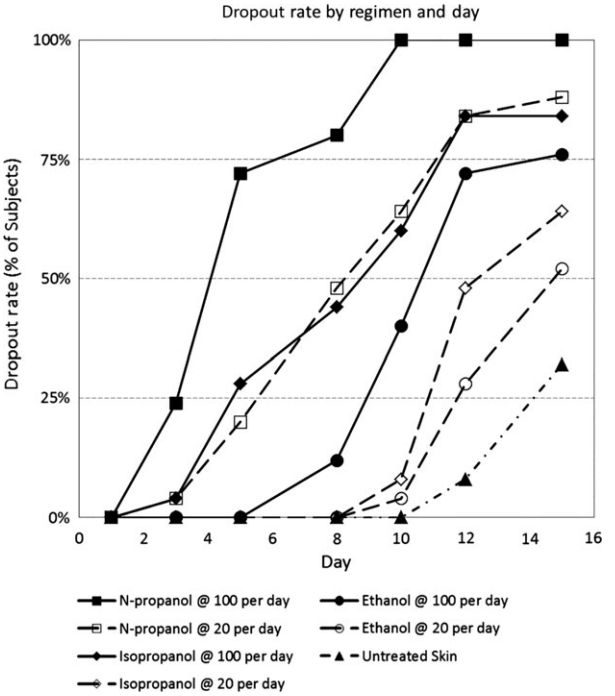

Figure 6 Effect of alcohol application frequency and type on dropout rates for skin irritation.

Effects of the different alcohol solutions on KLK5 and PLA_2 activities

The effects of the different alcohols (25%) on KLK5 and PLA_2 are shown in Fig. 4. Both of the enzymes were denatured to some extent by n-propanol compared with ethanol and isopropanol compared with buffer ($P < 0.001$). Moreover, isopropanol denatured PLA_2 to a greater extent than ethanol ($P < 0.001$). However, the effects of ethanol and isopropanol on KLK5 activity were similar.

Effects of the different alcohol solutions on skin in an FCAT

Of the 44 subjects who were screened, 35 were enrolled in the washout period and 25 (10 did not return for the study) were eligible for the study (Fig. 5). There were no significant differences in all test sites at baseline of the study (data not shown).

Overall comparisons

As shown in Fig. 6 and Table II, n-propanol treatment was associated with the highest dropout rates for skin redness. Its effects were manifest within the first days of the study where the high frequency (100 applications per day) resulted in the predetermined maximum visual redness score of 5.0 – 'severe redness' with several panellists. The dropout rate with n-propanol was the greatest, followed by isopropanol, and lowest dropout rates were observed for the ethanol regimen. By day 10, all treatments of n-propanol at 100 applications per day were stopped. Equally, more than 50% of the subjects stopped at the 20 per day application rate for

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Table II Number of active subjects in trial by regimen and day. Means with no letters in common are significantly different ($P < 0.05$)

	Day						
Regimen	**1**	**3**	**5**	**8**	**10**	**12**	**15**
N-propanol @ 100 per day	25	19	7	5 L	0 L	0 L	0 L
N-propanol @ 20 per day	25	24	20	13	9	4	3
Isopropanol @ 100 per day	25	24	18	14	10	4	4
Isopropanol @ 20 per day	25	25	25	25	23 H	13 H	9 H
Ethanol @ 100 per day	25	25	25	22	15	7	6
Ethanol @ 20 per day	25	25	25	25	24 H	18 H	12 H
Untreated Skin	25	25	25	25	25 H	23 H	17 H
Chi-square	0.00	0.53	5.56	16.77	29.51	79.33	65.31
P-value	1.00	1.00	0.59	0.02	0.00	0.00	0.00

P-values < 0.05 indicate significant differences in attrition among the regimens on days 8, 10, 12 and 15. Within a day, subject counts followed by 'L' indicate significantly lower than expected counts (i.e. significantly high attrition); subject counts followed by 'H' indicate significantly higher than expected counts (i.e. significantly low attrition). (Significance of subject counts was assessed using the cell chi-square >2.0 criterion).

n-propanol as well as that for isopropanol at 100 applications per day. For the ethanol-treated sites at 100 applications per day, more than 50% had been stopped at day 12. Equally, for the 20 times per day application rates for isopropanol and ethanol, less than 50% of sites had stopped application at day 15. However, numerically greater numbers did not dropout for ethanol compared with isopropanol.

Twenty applications per day comparisons

On average, the diminution in skin hydration and skin barrier function was significantly greater for n-propanol compared with other treatments (Fig. 7; $P < 0.05$). However, n-propanol and isopropanol produced significantly more skin redness compared with ethanol ($P < 0.05$). Equally, there was a trend of increasing skin dryness with these alcohols ($P = 0.1$; data not shown).

One hundred applications per day comparisons

The diminution in skin hydration and skin barrier function was similar for the different alcohols at this application frequency, with little difference in visual skin dryness (data not shown). Nevertheless, whereas there were no differences in the average redness between the alcohols, ethanol was not significantly different to the untreated control (Fig. 8; $P < 0.05$).

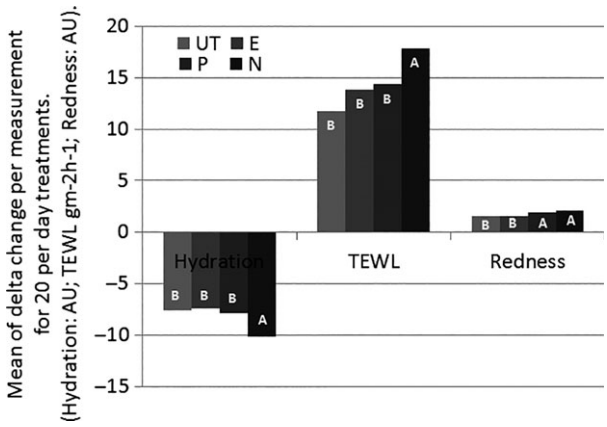

Figure 7 Average mean change in skin hydration, TEWL and skin redness after 20 applications of products per day over the whole course of the study. (UT = untreated; N = ethanol; P = isopropanol; N = n-propanol). Means with no letters in common are significantly different to each other ($P < 0.05$).

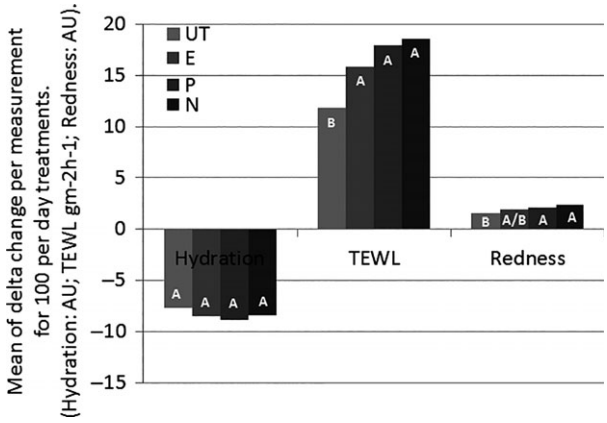

Figure 8 Average mean change in skin hydration, TEWL and skin redness after 100 applications of products per day over the whole course of the study (UT = untreated; N = ethanol; P = isopropanol; N = n-propanol). Means with no letters in common are significantly different to each other ($P < 0.05$).

Conclusions

Few subjects are impacted more by topical product usage than HCWs. As HHC and ABHRs play a significant role in strategies to reduce the threat of nosocomial infections [1], HCWs may be required to increase their use of ABHRs. As handwashing events ranging from <20 times per hour (58% compliance) to >60 per hour (37% compliance) [2] are being challenged as insufficient to meet recommended guidelines, hands may need to be sanitized up to 100 times per day to achieve 100% HHC [41]. Nevertheless, little is known about the effects of such frequent application of ABHRs on skin. Moreover, there is a paucity of data on the effects of different alcohols used in ABHRs on skin condition and function even at lower HHC levels (20 applications per day [42]). This study compares the effects of three different alcoholic formulations on

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skin, over 2 weeks, applied daily at standard application rates (20 times per day) and at a high frequency of application (100 times). In addition, *in vitro* testing was conducted to probe the potential effects of the individual alcohols at the molecular level.

Several groups have shown the denaturing effects of alcohols on haemoglobin, myoglobin, cytochrome C, trypsin and α-chymotrypsin [4–8]. Interestingly, the denaturant potential correlated with the log octanol–water (Log P) values of the different alcohols. We were interested in the inhibition of α-chymotrypsin and trypsin by ethanol as similar enzymes are present in the SC, namely the kallikreins [11]. We, therefore, investigated the effect of ethanol, isopropanol and n-propanol on KLK5 activity. The activity of KLK5 was significantly reduced in the presence of n-propanol compared with the other alcohols although all alcohols reduced activity relative to controls. PLA_2 is also one of the enzymes believed to be involved with SC barrier formation by degrading residual phospholipids to free fatty acids [9]. Interestingly, we observed even further discrimination between the alcohols on this enzyme with ethanol producing the least effect. We believe this is the first study showing such an effect with propanol isomers although ethanol has previously been shown to inhibit PLA_2 [43]. These results suggest that alcohols have the potential to inhibit SC enzyme activities in the order ethanol < isopropanol < n-propanol. Thus, ethanol should have the lowest effect on SC structure and function. In this respect, when evaluated by a corneoxenometric assay, ethanol was the least aggressive solvent followed by hexane [28].

Small amounts of alcohol also have the potential to influence keratinocyte behaviour. Ockentels *et al.* [13] showed that ethanol stimulated the release of interleukin-6 (IL-6) from keratinocytes whereas Neumes *et al.* [14] found increases in IL-6, IL-1α and TNF-α following ethanol treatment. McKarns *et al.* [12] have also shown that n-propanol has a greater effect than ethanol on the loss of rat liver epithelial cell membrane integrity as measured by the release of lactate dehydrogenase. Again, these studies suggest that n-propanol is more toxic to keratinocytes, consistent with our findings. The IC_{50} values for the MTT test in our studies were ~ 5% for ethanol, 4% for isopropanol and 2% for n-propanol, in both low- and high-calcium-treated keratinocytes. Similarly, negligible increases in TNF-α were observed for keratinocytes treated with ethanol and isopropanol compared with n-propanol. As for PLA_2, the effects of the alcohols on IL-1α secretion may be ranked as follows: n-propanol > isopropanol > ethanol. In fact, the ethanol treatments were not significantly different to the control buffer. These results again suggest that using ethanol *in vivo* should result in less irritation compared with the other alcohols evaluated here.

We observed changes in skin condition and function following application of various ABHRs over a 2-week period. For low rates of application, n-propanol was the harshest alcohol in terms of reducing skin hydration together with skin barrier function and ethanol was superior to the other alcohols in terms of not inducing skin dryness and redness. This is to be expected as *in vitro* studies demonstrated that n-propanol had the greatest denaturing effect on the barrier function, profilaggrin processing and desquamatory SC enzymes [9–11] and both isopropanol and n-propanol induced the greater amounts of cytokine secretion. At the higher frequency applications, this was not the case; however, skin redness did not differ between control and ethanol application. This was exemplified in the dropout rates where by day 10, all treatments of n-propanol at 100 applications per day were stopped. Equally, more than 50% of the subjects' treatments were stopped at the 20 per day application rate as well as that for isopropanol (100 applications daily). More than 50% of the ethanol-treated sites (100 applications daily) were no longer tested at day 12. Equally, both the 20 times per day application rates for isopropanol and ethanol had less than 50% of their sites stopped with ethanol having numerically greater numbers left. Thus, overall ethanol was shown to be the mildest alcohol tested clinically, consistent with the overall effects in the *in vitro* studies.

In conclusion, there is limited information on the relative effects of alcohols used in hand sanitizers on the skin barrier, SC enzymes and keratinocyte behaviour. However, this study demonstrates that ethanol offers advantages over isopropanol and n-propanol based on the findings of the *in vitro* and *in vivo* studies reported here. Clearly, isopropanol and n-propanol caused significant SC and keratinocyte perturbation *in vitro* together with damage to skin condition and function *in vivo* whereas ethanol did not. As a result, we show that ethanol-based sanitizers are better tolerated by skin, particularly in high-use settings, than other alcohols.

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