

# Protective effects of glycerol and xylitol in keratinocytes exposed to hyperosmotic stress

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**Purpose:** Our goal was to study whether glycerol and xylitol provide protection against osmotic stress in keratinocytes.

**Methods:** The experiments were performed on HaCaT keratinocytes. Hyperosmotic stress was induced by the addition of sorbitol (450, 500 and 600 mOsm). Both polyols were applied at two different concentrations (glycerol: 0.027% and 0.27%, xylitol: 0.045% and 0.45%). Cellular viability and cytotoxicity were assessed, intracellular Ca<sup>2+</sup> concentration was measured, and the RNA expression of inflammatory cytokines was determined by means of PCR. Differences among groups were analyzed with one-way ANOVA and Holm-Sidak post-hoc test. When the normality test failed, Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn’s method for pairwise multiple comparison was performed.

**Results:** The higher concentrations of the polyols were effective. Glycerol ameliorated the cellular viability while xylitol prevented the rapid Ca<sup>2+</sup> signal. Both polyols suppressed the expression of IL-1 $\alpha$  but only glycerol decreased the expression of IL-1 $\beta$  and NFAT5.

**Conclusions:** Glycerol and xylitol protect keratinocytes against osmotic stress. Despite their similar chemical structure, the effect of these polyols displayed differences. Hence, joint application of glycerol and xylitol may be a useful therapeutic approach for different skin disorders.

**Keywords:** hyperosmotic stress, glycerol, xylitol, intracellular calcium concentration

## Introduction

Local hyperosmotic condition is revealed to be associated with several inflammatory disorders (eg, corneal inflammation in dry eyes syndrome, inflammatory bowel disease, etc.).<sup>1</sup> Presumably, a local hyperosmotic challenge may contribute to the development of irritant contact dermatitis (ICD). ICD is a non-immunologic, non-specific inflammatory skin disease induced by physical, mechanical or chemical factors and accompanied by impaired barrier function. This results in increased skin permeability and transepidermal water loss (TEWL).<sup>2</sup> Water evaporation can lead to a higher osmotic pressure in the superficial layer of the skin. Furthermore, hyperosmolarity induces various intracellular responses in different types of cells and reduces cellular viability.<sup>3–7</sup> It has been described that a hyperosmotic stimulus elevates intracellular calcium (Ca<sup>2+</sup>) concentration in HaCaT keratinocytes.<sup>6,8</sup> Although its exact molecular mechanism has not yet been revealed, it has been shown that Ca<sup>2+</sup> is derived from both intra- and extracellular compartments.<sup>6,8</sup>

Keratinocytes are known to produce pro-inflammatory cytokines when exposed to osmotic stress.<sup>9</sup> At mRNA level, expression of tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin 1-beta (IL-1 $\beta$ ), IL-8,<sup>3</sup> IL-6<sup>4</sup> and nuclear factor of activated

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40 T cells 5 (NFAT5)<sup>5</sup> was found to be higher in epithelial  
cells. In normal human epidermal keratinocytes, elevated  
mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 was  
observed.<sup>9</sup>

45 NFAT5 is a principal transcription factor involved in  
water homeostasis during normal cornification<sup>10</sup> and acti-  
vated by hyperosmotic stress;<sup>5</sup> however, its regulation by  
Ca<sup>2+</sup> signals is equivocal.<sup>11–13</sup> Local hyperosmotic stress of  
the skin activates NFAT5 in macrophages<sup>1</sup> thereby intensify-  
ing the electrolyte clearance via lymphatic vessels.<sup>14</sup>

50 Under experimental conditions, different methods are  
used to induce hyperosmotic stress. Sodium chloride is  
usually used for this aim<sup>3–5</sup> and non-ionic organic agents  
such as sorbitol<sup>9</sup> and sucrose<sup>4</sup> can also be applied on  
keratinocyte cultures.

55 Glycerol and xylitol have well-known beneficial effects  
on the skin<sup>15–18</sup> but their role as osmolytes has not yet been  
fully clarified. It has been revealed that glycerol composes  
the principal osmolyte system of several bacterial species<sup>19</sup>  
while xylitol inhibits inflammatory cytokine expression.<sup>20</sup>

60 Previously, we have shown the anti-inflammatory and anti-  
irritant effects of glycerol and xylitol.<sup>16–18</sup> Other in vitro  
experiments have revealed that glycerol suppresses human  
leukocyte antigen-DR (HLA-DR) mRNA level and xylitol  
upregulates filaggrin mRNA expression.<sup>21</sup>

65 The aim of the present study was to investigate  
whether glycerol and xylitol provide protection against  
hyperosmotic stress in vitro. Their effects on cellular vi-  
ability and cytotoxicity, intracellular Ca<sup>2+</sup> concentration,  
expression of pro-inflammatory cytokines and cellular vi-  
70 ability were studied.

## Materials and methods

### Cell culture

75 HaCaT cells kindly provided by Dr N. E. Fusenig (Heidelberg,  
Germany) were cultured in Dulbecco's modified Eagle's me-  
dium containing 4.5 g/L glucose, 1.8 mM Ca<sup>2+</sup> (DMEM-HG)  
supplemented with 10% fetal bovine serum (FBS), 2 mM  
L-glutamine, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin,  
0.25  $\mu$ g/mL amphotericin B and grown at 37°C in a humidified  
atmosphere containing 5% CO<sub>2</sub>. Three to four days after plat-  
80 ing, cells were collected from semi-confluent cultures by 5–10  
mins trypsinization (0.25% trypsin-EDTA solution). The pro-  
teolitic digestion was stopped by FBS and the cells were sedi-  
mented (10 mins, 4°C, 1,500 g) and resuspended in the  
medium for further use. Cellular viability was determined by

the trypan blue exclusion test. The experiments were per- 85  
formed on cultures, where trypan blue was excluded from  
95% of the cells.

### Preparation of the treating media

For osmolarity measurement, Model 5600 Vapro® 90  
Vapor Pressure Osmometer (Dieren, The Netherlands)  
was used. The basal osmolarity of the serum-free  
DMEM-HG medium was 338 mOsm. Media contained  
3 or 30 mM polyols, respectively, which was equivalent  
to 0.027 or 0.27 w/w% glycerol and 0.045 or 0.45 w/w  
% xylitol.<sup>21</sup> The osmolarity of these solutions was also 95  
measured. The final osmolarity of 450, 500 and 600  
mOsm was reached by the addition of the appropriate  
amount of 1.83M sorbitol stock solution. Serum-free  
DMEM-HG media containing only glycerol, xylitol or  
sorbitol in concentrations mentioned above, respectively, 100  
were also applied.

### Cellular viability and cytotoxicity

Cells were seeded into 96-well culture plates at  
a density of 10<sup>4</sup> cells/well in DMEM-HG supplemented  
with the appropriate agents detailed above, and serum- 105  
starved 24 hrs before any treatments. For 60 mins, cells  
were incubated with or without 0.27% glycerol or  
0.45% xylitol in serum-free DMEM-HG, followed by  
incubation with 450, 500 or 600 mOsm culture medium  
with or without 0.27% glycerol or 0.45% xylitol for 110  
24 hrs. Experiments were carried out in triplicates, and  
data are presented as the means of three experiments.

For the cell viability assay, 0.5% MTT 115  
(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-  
mide) solution was added to the cells at 37°C for 4 hrs,  
and then the formazan crystals were solubilized and the  
optical density (OD) was measured at 540 nm by  
a Multiscan Ex spectrophotometer (Thermo Labsystems,  
Beverly, Massachusetts, USA) and Ascent Software  
(Thermo Fisher Scientific, Waltham, Massachusetts, 120  
USA). The absorbance rate was compared to untreated  
samples and represented as percentage rate of living cells.

Cytotoxicity was assessed by using the Cytotoxicity 125  
Detection Kit PLUS (Roche Diagnostics, Risch,  
Switzerland) according to the manufacturer's instructions.  
Briefly, released lactate dehydrogenase (LDH) was quan-  
tified from the supernatant by a colorimetric method and  
optical density was measured at 492 nm. Measured values

were corrected with the background values of the cell-free culture medium. Results are presented of % in cytotoxicity, where lysed cells correspond to 100% cytotoxicity.

### Determination of changes in $[Ca^{2+}]_i$

HaCaT keratinocytes were plated onto 13 mm diameter uncoated sterile coverslips (VWR, Radnor, Pennsylvania, USA) and were left to attach and proliferate for 24 hrs. Coverslips with the attached cells were transferred and incubated in Tyrode's solution (144 mM NaCl, 0.4 mM  $NaH_2PO_4$ , 4 mM KCl, 0.53 mM  $MgSO_4$ , 1.8 mM  $CaCl_2$ , 5.5 mM glucose and 5 mM HEPES, pH=7.4). HaCaT cells were loaded by incubation for 20 mins with the acetoxymethyl ester (AM) form of a single wavelength calcium-sensitive fluorescent dye (Fluo-4, Molecular Probes Inc., Eugene, Oregon, USA, 5  $\mu$ M from a stock of 1 mM in DMSO +20% pluronic acid Pluronic F-127 Sigma-Aldrich, Saint Louis, Missouri, USA and 6.25 nM/mL Probenecid, Molecular Probes Inc., Eugene, Oregon, USA) at room temperature in dark. After incubation period, the cells were washed in indicator-free Tyrode's solution to remove any dye. The technique for intracellular calcium detection was based on established procedures described earlier.<sup>22</sup> Subsequently, cells were incubated for 30 mins with 0.027% or 0.27% glycerol and 0.045% or 0.45% xylitol<sup>21</sup> in Tyrode solution, respectively.

Optical measurements were performed using a Zeiss Axiovert 100 microscope (Zeiss, Oberkochen, Germany) equipped with a xenon lamp and used in epifluorescent mode at 100x magnification. The coverslips were placed into a low volume imaging chamber (Warner Instruments, Hamden, Connecticut, USA) (37°C) at the microscope stage and cells were superfused with Tyrode alone for at least 10 mins (control period). Hyperosmotic stimulus was added to the cells in rapid perfusion (2–3  $\mu$ L/sec) of 450 mOsm sorbitol, followed by the addition of 30 nmol/mL A23187 ionophor in Tyrode. Cells in the 75×75  $\mu$ m frame were illuminated at 485 nm and the emitted light was recorded at 535 nm. Images of relative fluorescence intensity of a cell group (3–5 cell/group) were collected in 8–10 independent experiments per treatment group with the Mintron 7266pd Color CCD Camera (New Taipei City, Taiwan). Raw data were analyzed with WinWCP V4.5.0 (Glasgow, Scotland) and Clampfit 10.6 (San Jose, California, USA) softwares.

Calibration was performed by the  $[Ca^{2+}]_i = K_d (F - F_{min}) / (F_{max} - F)$  method<sup>23</sup> where  $[Ca^{2+}]_i$  is the intracellular  $Ca^{2+}$  concentration,  $K_d$  is the dissociation constant (345 nM),  $F$  is the fluorescence intensity evoked with the

hyperosmotic stimulus,  $F_{min}$  values were developed from corrected fluorescence intensity before osmotic stress and  $F_{max}$  values were derived from the maximal fluorescent response after the addition of the ionophor solution. Background correction was calculated with the autofluorescence of unloaded cells and the decrease of fluorescence intensity in untreated cells caused by bleaching or dye efflux. For representative figures,  $\Delta Ca^{2+} = \Delta F / F = (F - F_{rest}) / F_{rest}$  was calculated.<sup>23</sup>

### Real-time polymerase chain reaction (RT-PCR)

Cells were seeded into 6-well plates at a density of  $2 \times 10^5$  cells/mL and serum-starved overnight. Cells were pre-incubated for 60 mins with 0.27% glycerol or 0.45% xylitol, followed by incubation with 450 mOsm sorbitol for 2 and 6 hrs.<sup>3–5</sup> Untreated or sorbitol-treated cells served as negative or positive controls, respectively. Total RNA was isolated using TRIzol reagent (Sigma-Aldrich, Saint Louis, Missouri, USA) according to the manufacturer's instructions. To avoid DNA contamination, DNase treatment was performed and intron-spanning assays were used. cDNA was synthesized from 1  $\mu$ g total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA). Real-time RT-PCR experiments were carried out with the Universal Probe Library system (Roche Diagnostics, Risch, Switzerland) using TaqMan probe-based assays and a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The applied primers are listed in Table 1. The expression of each gene was normalized to the 18S rRNA gene, and relative mRNA levels were calculated by the  $\Delta\Delta C_t$  method, compared to untreated, time-matched control samples.

### Statistical analysis

Data analysis was performed with SigmaStat for Windows 11.0 software (Jandel Scientific, San Rafael, California, USA). Differences among groups were analyzed with one-way ANOVA and Holm-Sidak post-hoc test and data are presented as mean (m) values with standard deviation (SD). When the normality test failed, Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison was performed. In such cases, median values (M) with 25th and 75th percentiles (25p and 75p, respectively) are given.  $P < 0.05$  was considered statistically significant.

**Table 1** Known sequences of primer pairs and probes used for RT-PCR

	Sense	Antisense
TNF- $\alpha$	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA
IL-1 $\alpha$	AACCAGTGCTGCTGAAGGA	TTCTTAGTGCCGTGAGTTTCC
IL-1 $\beta$	AAAGCTTGGTGATGTCTGGTC	AAAGGACATGGAGAACACCACT
IL-6	CAGGAGCCCAGCTATGAACT	GAAGGCAGCAGGCAACAC
NFAT5	TCAGACAAGCGGTGGTGA	AGGGAGCTGAAGAAGCATCA
IL-8	TaqMan® Gene Expression Assay, Thermo Scientific	

## Results

### Glycerol of 0.27% but not xylitol ameliorated the viability of HaCaT cells exposed to hyperosmotic stress

225 During a period of 24 hrs, osmotic stresses of 450 and 500 mOsm did not influence cellular viability, and the average cytotoxicity values were negligible. However, 600 mOsm sorbitol significantly reduced viability. The reduction was also considerable in the additional polyol-treated groups, but glycerol treatment resulted in significantly higher viability as compared to the positive control (DMEM-HG +600 mOsm sorbitol) group. 0.45% xylitol failed to improve the survival of the cells. The average cytotoxicity value of the 0.27% glycerol +600 mOsm sorbitol-treated group was somewhat lower but did not differ significantly from that of its control (DMEM-HG+600 mOsm sorbitol)

group. Viability and cytotoxicity in groups exposed to 0.27% glycerol and 0.45% xylitol alone did not differ significantly from that of their matched DMEM-HG control group (Figure 1A, B).

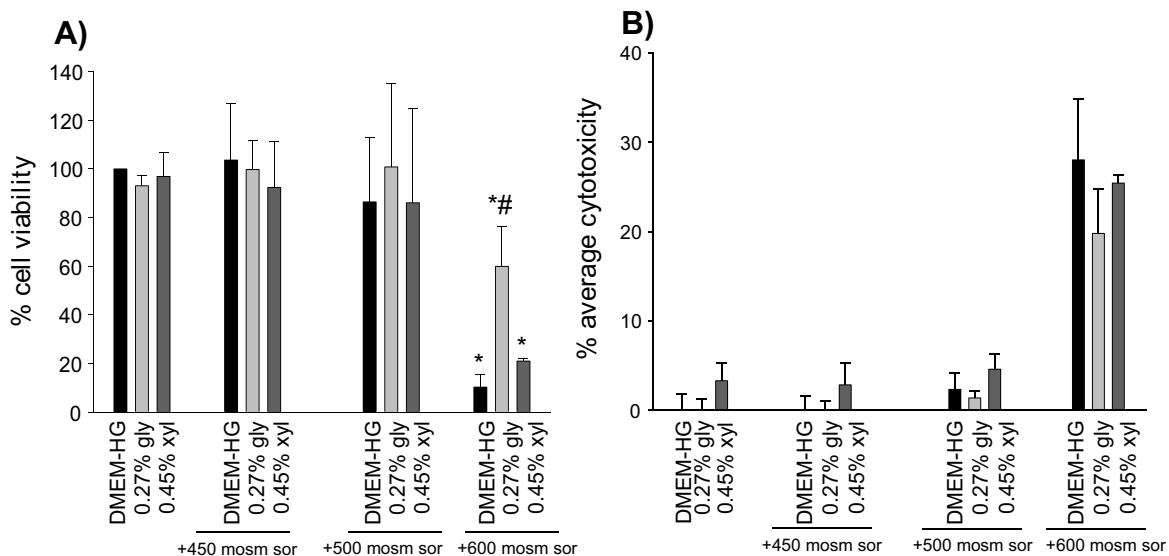
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### Xylitol of 0.45% provides protection against the hyperosmotic stimulus-induced increase in intracellular Ca<sup>2+</sup> concentration

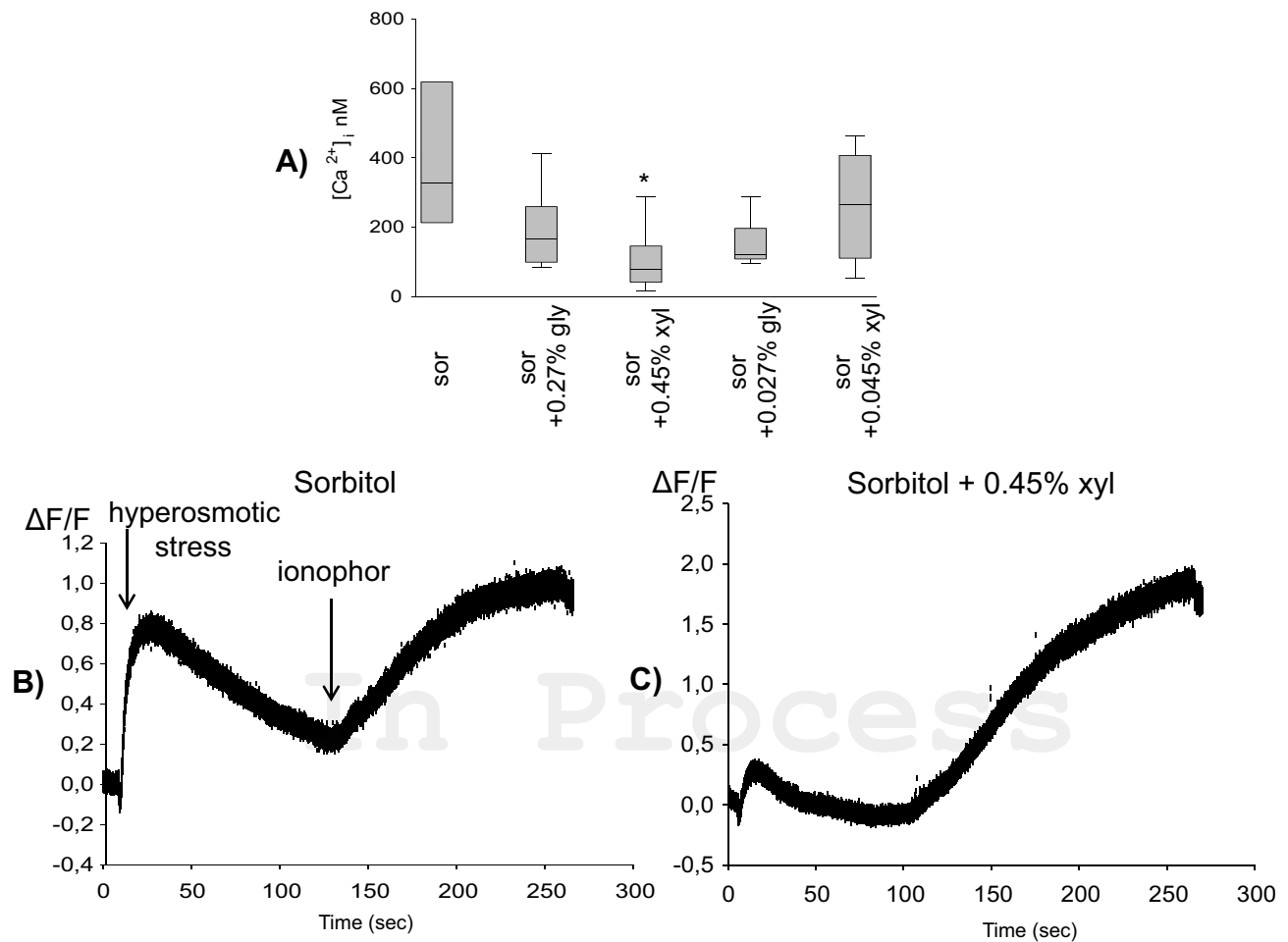
Hyperosmotic stress induced by 450 mOsm sorbitol was accompanied by a short elevation of intracellular Ca<sup>2+</sup> concentration. This elevation (M=328.6 nM, 25p=232.0, 75p=602.4) was prevented neither by glycerol nor the lower concentration of xylitol, but it was suppressed by the higher concentration (0.45%) of xylitol (M=78.76 nM, 25p=45.92, 75p=140.72) (Figure 2A). Figure 2 also shows

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**Figure 1** (A) The effects of glycerol and xylitol on cellular viability in hyperosmotic stress. (B) The effects of polyols in osmotic stress-induced cytotoxicity. Statistical analysis was performed with one-way ANOVA and Holm-Sidak post-hoc test (mean±SD). \**p*<0.05 vs DMEM-HG., ##*p*<0.05 vs DMEM-HG +600 mOsm sor, *n*=3. **Abbreviations:** DMEM-HG, Dulbecco's modified eagle's medium containing 4.5 g/L glucose; sor, sorbitol; gly, glycerol; xyl, xylitol.



**Figure 2 (A)** Xylitol protected against the hyperosmotic stimulus-induced increase in intracellular  $Ca^{2+}$  concentration. Statistical analysis was performed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method (median, 25th and 75th percentile),  $*p < 0.05$  vs sor,  $n = 8-10$ . **(B and C)** A representative curve of the relative fluorescence on 450 mOsm sorbitol **(B)** and additional xylitol-treated group **(C)**.

**Abbreviations:** sor, sorbitol; gly, glycerol; xyl, xylitol.

two representative curves of this response from the sorbitol-treated (Figure 2B) and the sorbitol and 0.45% xylitol-treated groups (Figure 2C).

## 255 Polyols prevented the elevation in the mRNA expression of inflammatory cytokines and NFAT5 induced by osmotic stress

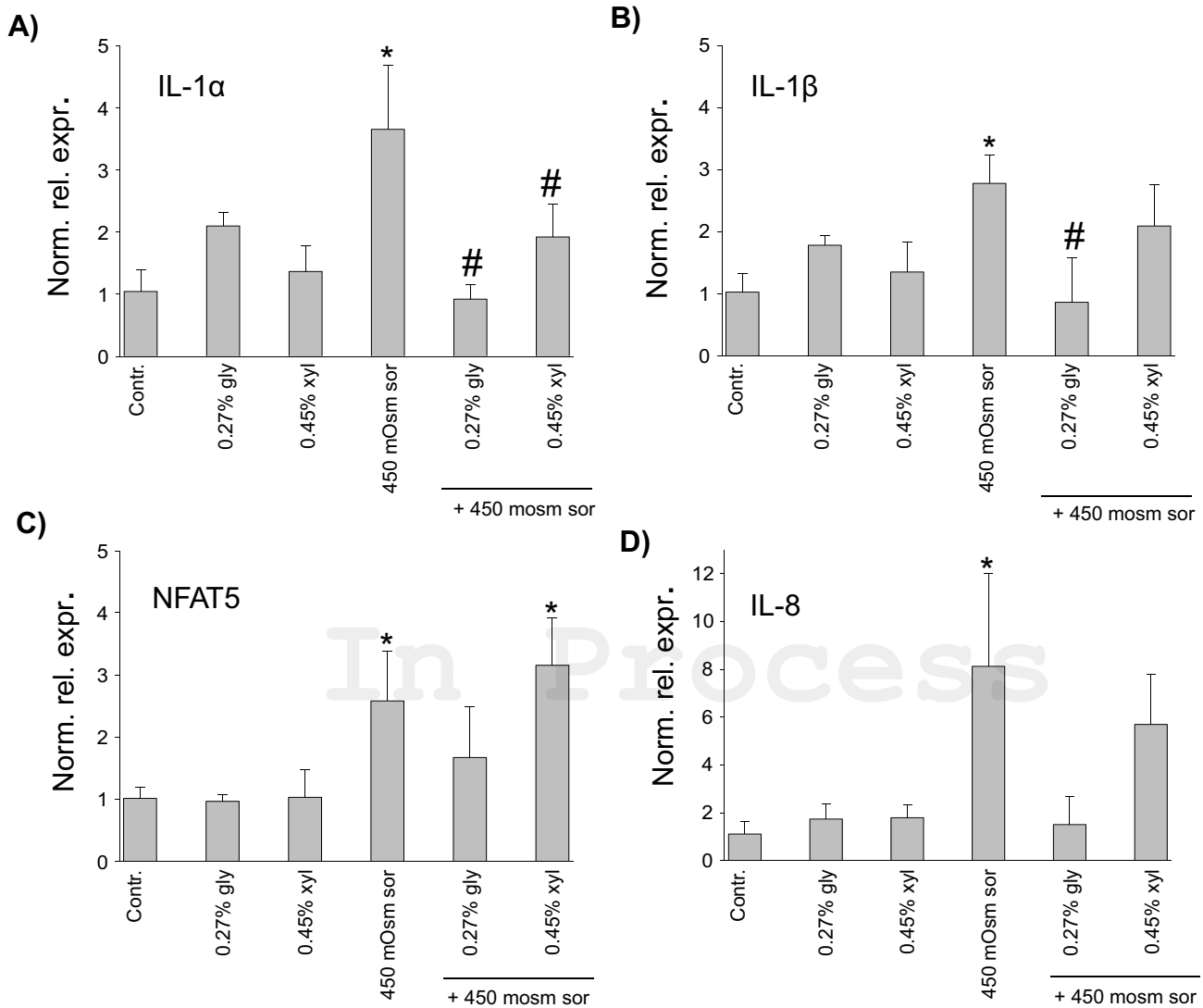
260 The mRNA expression of inflammatory cytokines (TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8) and the osmosis-related NFAT5 was measured after 2 and 6 hrs hyperosmotic stress. After 6 hrs, no significant changes were detected (data not presented). Following 2 hrs treatment, the cytokine levels of glycerol or xylitol-treated groups were similar to that of the untreated control group. 450 mOsm sorbitol induced considerable increase in IL-1 $\alpha$ , IL-1 $\beta$ ,

IL-8 and NFAT5 expression. Both 0.27% glycerol and 0.45% xylitol prevented the elevation in the expression of IL-1 $\alpha$  (Figure 3A). Furthermore, both 0.27% glycerol and 0.45% xylitol led to considerably lower expression of IL-8, and the expression levels did not differ significantly from those of untreated control group (Figure 3D). As concerns IL-1 $\beta$  and NFAT5, only 0.27% glycerol diminished considerably their expression (Figure 3B, C). No significant difference was found among the six study groups in TNF- $\alpha$  and IL-6 expression (data not shown).

## Discussion

ICD is a frequent occupational disorder<sup>24</sup> which is characterized by impaired barrier function leading to increased TEWL. Water evaporation exposes keratinocytes to a condition of high osmotic pressure.<sup>9</sup> In our previous animal experiments, sodium lauryl sulfate (SLS)-induced skin irritation resulted





**Figure 3** In vitro effects of polyols on (A) IL-1 $\alpha$ , (B) IL-1 $\beta$ , (C) NFAT5 and (D) IL-8 expression. The expression of each gene was normalized to the 18S rRNA gene and relative mRNA levels were calculated by the  $\Delta\Delta C_t$  method, compared to the untreated, time-matched control samples. Statistical analysis was performed with one-way ANOVA and Holm-Sidak post-hoc test (mean + SD), \* $p < 0.05$  vs Contr., # $p < 0.05$  vs 450 mOsm sor,  $n = 3$  (a-c) and Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method (median, 25th and 75th percentile), \* $p < 0.05$  vs Contr,  $n = 3$  (d) Despite the difference in the statistical tests, mean + SD values are shown in all subfigures for uniform presentation. **Abbreviations:** Norm. rel. expr, normalized relative expression; Contr, control; sor, sorbitol; gly, glycerol; xyl, xylitol.

in the reduction of epidermal thickness<sup>17</sup> that can be considered as an indirect evidence for osmotic stress. Thus, the present study aimed at the investigation of osmotic challenge potentially accompanying ICD. In response to hyperosmotic stress, organic osmolytes are accumulated by the cells. In the skin, betaine, myoinositol and taurine are important osmolytes and the expression of their transporters (betaine/GABA transporter, sodium/myoinositol transporter and taurine transporter) is induced by osmotic stress.<sup>25</sup>

The anti-irritant and anti-inflammatory effects of glycerol and xylitol have already been demonstrated in animal experiments.<sup>17</sup> Joint application of glycerol and xylitol

increases skin hydration, decreases TEWL, improves biomechanical properties of the skin and induces a higher filaggrin production in the epidermis after 2 weeks of application.<sup>18</sup> However, their cellular mechanism of action has not been revealed in details. We assumed that these polyols may act as organic osmolytes and therefore may have a role in osmoregulation.

It has been described that the effects of polyols may depend on the applied concentration.<sup>17,26</sup> Hence, two different concentrations were tested in our in vivo studies<sup>16,17</sup> and also two different concentrations were chosen for the present experiments, based on previous in vitro results.<sup>21</sup> According

to the results of the effect of polyols on  $\text{Ca}^{2+}$  concentration in our preliminary experiments, only the higher polyol concentrations were applied in the measurement of cellular viability, cytotoxicity and cytokine expression.

In order to induce osmotic stress, instead of the ionic sodium chloride and the detergent SLS, sorbitol was chosen, which is a metabolically inactive, inert agent. Sorbitol is also a polyol osmolyte such as glycerol and xylitol; however, it has no known protective effect in inflammatory skin conditions, and it is used to induce osmotic stress.<sup>8</sup>

Osmotic challenge influences cellular viability in an osmolarity- and time-dependent manner.<sup>4-7</sup> Such effect is also characteristic of ICD: severe irritation of the skin (24 hrs exposure to SLS) can induce tissue necrosis *in vivo*<sup>27</sup> but milder irritant challenges do not lead to necrosis.<sup>28</sup> In our experiments, 24 hrs exposure to 450 and 500 mOsm sorbitol did not influence cellular viability and cytotoxicity, but 600 mOsm resulted in a significant decrease in viability as compared to untreated control cells.

However, instead of 600 mOsm, 450 mOsm osmotic stress was applied to measure intracellular calcium concentration, in order to examine the protective effects of polyols, with the elimination of cell death. According to our pilot study, 450 mOsm hyperosmotic stimulus with sorbitol was sufficient to induce a short elevation of intracellular  $\text{Ca}^{2+}$  with a kinetics similar to that of measured by Dascalu et al who applied 500 mOsm sucrose.<sup>6</sup> Although the exact molecular mechanism is still not fully clarified, it has been described that dihydropyridine-sensitive  $\text{Ca}^{2+}$  channels are not affected.<sup>6</sup> The transient receptor potential vanilloid 1b (TRPV1b) non-selective cation channel, which is expressed also by HaCaT cells,<sup>29</sup> can be induced by cell shrinking in hypertonicity<sup>30</sup> and therefore may have a potential role in  $\text{Ca}^{2+}$  response. In osmotic stress, increased inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) synthesis were observed, as well.<sup>31</sup> TRPV1 can also be activated by  $\text{Ca}^{2+}$ <sup>32</sup> and DAG.<sup>33</sup> Moreover, IP3 receptors may contribute to  $\text{Ca}^{2+}$  influx, too.<sup>34</sup>

Based on literature data,<sup>21</sup> the applied polyols alone did not influence intracellular  $\text{Ca}^{2+}$  concentration. According to our results, xylitol prevented the elevation of intracellular  $\text{Ca}^{2+}$  concentration induced by the hyperosmotic sorbitol solution while glycerol did not influence this parameter. Further investigations are necessary to reveal the reason for this difference and the exact mechanism via which xylitol inhibits  $\text{Ca}^{2+}$  signal.

In addition to the rapid  $\text{Ca}^{2+}$  response, the applied osmotic challenge has longer effects, as well. 2 hrs of exposure to 450 mOsm sorbitol increased the expression

of IL-1 $\alpha$ , IL-1 $\beta$  and IL-8 in HaCaT cells. It has been described that cytokine expression elevates in keratinocytes when 500 mOsm of osmotic stimulus is present for 6 hrs.<sup>9</sup> Cytokine production may originate from the  $\text{Ca}^{2+}$  response.<sup>35</sup> However, in a recent study, pro-inflammatory responses evoked with ATP- or bradykinin-induced elevation of the intracellular  $\text{Ca}^{2+}$  concentration could not be suppressed by glycerol or xylitol. Moreover, none of these polyols influenced the subcellular translocation of the  $\text{Ca}^{2+}$ -dependent protein kinase C (PKC) $\alpha$ . However, xylitol but not glycerol translocated the  $\text{Ca}^{2+}$ -independent PKC $\delta$ .<sup>36</sup> TRPV1 activation can also lead to enhanced cytokine production via direct or indirect NF- $\kappa$ B activation.<sup>37</sup> Independently from the  $\text{Ca}^{2+}$  response, hyperosmotic stress also activates the transcription factor NFAT5<sup>5</sup> which regulates TNF- $\alpha$ <sup>38</sup> and can bind the promoter of IL-1 and IL-6.<sup>1</sup> Thus, elevated expression of NFAT5 might have contributed to the increased cytokine expression in the present study. Our previous *in vivo* investigations have already shown the anti-inflammatory effect of glycerol and xylitol.<sup>16-18</sup> However, differences can be found as compared to the present *in vitro* results. Glycerol and xylitol decreased the mRNA expression of IL-1 $\beta$  and TNF- $\alpha$ , but had no effect on the IL-1 $\alpha$  levels in a murine model of ICD.<sup>17</sup> Several factors may explain the beneficial effects of polyols on cytokine expression. In addition to the prevention of  $\text{Ca}^{2+}$  signal and the inhibition of NFAT5 expression, glycerol and xylitol may affect the inflammatory process via stabilizing protein structure. As chaperon osmolytes, these polyols are able to enhance protein folding, thereby assisting in the development of the final structure essential for the optimal enzyme function, and they promote protein-protein and protein-DNA interactions.<sup>39</sup>

Our findings indicate that glycerol provides protection not only against acute inflammation but also against a more serious damage which occurs after a relatively longer time-span. The protective effect of glycerol was found to appear after a 24 hrs exposure to osmotic stress when aquaporin-3 (AQP-3) gene expression shows a peak.<sup>40</sup> If AQP-3 expression increases at protein level, as well, it provides an enhanced intracellular transport of glycerol. This theory is supported by our observation that cellular viability was ameliorated only when glycerol was continuously available (data not shown).

## Conclusion

The effects of glycerol and xylitol were tested under hyperosmotic condition as an *in vitro* model of osmotic

**Table 2** The chemical structure, known and novel properties of glycerol and xylitol

	Glycerol	Xylitol
Chemical structure	$\begin{array}{c} \text{CH}_2\text{OH} \\   \\ \text{H}-\text{C}-\text{OH} \\   \\ \text{CH}_2\text{OH} \end{array}$	$\begin{array}{c} \text{CH}_2\text{OH} \\   \\ \text{H}-\text{C}-\text{OH} \\   \\ \text{HO}-\text{C}-\text{H} \\   \\ \text{H}-\text{C}-\text{OH} \\   \\ \text{CH}_2\text{OH} \end{array}$
Known properties	<i>in vivo</i> anti-inflammatory and anti-irritant effects <sup>16–18</sup>	
	Composition of the bacterial osmolyte system <sup>19</sup>	Inhibition of TNF- $\alpha$ and IL-1 $\beta$ expression <i>in vitro</i> <sup>20</sup>
	HLA-DR mRNA suppression <sup>21</sup>	Filaggrin mRNA upregulation <sup>21</sup>
Novel <i>in vitro</i> features in HaCaT keratinocytes exposed to hyperosmotic stress	Suppression of IL-1 $\alpha$	
	Amelioration of cell viability	Prevention of rapid intracellular Ca <sup>2+</sup> signal
	Decrease in the expression of IL-1 $\beta$ and NFAT5	

stress accompanying ICD and other xerotic skin diseases. The applied polyols supported cell viability, prevented hyperosmosis-induced Ca<sup>2+</sup> signal and the expression of inflammatory cytokines. Despite their similar chemical structure, the effect of these polyols displayed differences (Table 2). Hence, joint application of glycerol and xylitol may be a useful therapeutic approach for different skin disorders.

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## Disclosure

The authors report no conflicts of interest in this work.

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