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Mitigating Ammonia-Triggered Inflammation in Human Skin Model: Modulatory Effects of a Purified Water Wipe Formulation

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Abstract

Irritant contact dermatitis is an inflammatory skin condition caused by exposure of the skin to substances or the environment that damages the skin barrier. Irritant contact dermatitis presents on the skin as a rash accompanied by redness, itching, burning sensation, blisters and cracking of the skin. In babies, it is more commonly known as diaper dermatitis or diaper rash but can also present in older children and adults with incontinence issues. General measures to prevent irritant contact dermatitis in babies includes frequent nappy changes to keep the baby's skin as dry as possible, regular cleaning to remove urine and feces to maintain a healthy skin pH level and the use of products with minimal chemical irritants (wipes, diapers, barrier creams etc.). This study evaluates the effectiveness of a water-based wipe liquid (WaterWipes®) to reduce urine-induced inflammation in a 3D in vitro human skin model.

Keywords: irritant dermatitis; diaper rash; cytokines; inflammation; in vitro skin model

1. Introduction

Skin irritation due to prolonged contact with urine and feces, is caused by the direct contact of ammonia, urea and other irritants in the bodily fluids. Urine and feces combine to form ammonium hydroxide which raises the pH level of the skin. Factor in bile salts and enzymes in the feces can also breakdown the stratum corneum, along with urine creating a wet skin surface for the growth of microbes such as *Candida albicans*, the use of diapers can provide the perfect environment for irritant contact dermatitis (ICD) [1–5].

ICD is often exacerbated by physical friction from diapers and other incontinence products [6]. In particular, premature babies are more likely to be susceptible to ICD due to their thinner skin (2-3 times thinner than full-term babies) [7]. More frequent diaper changes and the use of product with fewer chemical ingredients are recommended to reduce the likelihood of ICD [9–11].

Studies have shown that the use of wet wipes with minimal ingredients are more effective in cleaning and reducing erythema in baby diaper cleaning outcomes when compared to traditional cloth and water use [9]. Wet wipes designed for use on baby skin tend to be formed of a base material impregnated with mild cleaning agent, preservatives and pH buffering agents, as raised pH on the skin can worsen the symptoms [12]. The wipes presented in this study are composed of a 100% viscose non-woven spun lace material impregnated with ultra-pure water (99.9%) and 0.1% fruit seed extract, plus a trace of benzalkonium chloride as the preservative. This composition makes the wipes suitable for use on sensitive or irritated skin, in line with recommendations from the European roundtable meeting that wet wipes designed for use on baby skin should be formulated with ingredients shown to not cause irritation and be suitable for long-term use [8,10].

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The aim of this study was to assess the impact of the WaterWipes® liquid on pro-inflammatory markers in vitro using a 3D human skin model exposed to the ammonia in synthetic urine, and in vivo on skin erythema.

2. Materials and Methods

2.1. Test Items

Four test items were used in this study: WaterWipes®, a competitor product, tap water and soapy water. A negative control group (no urine irritation, no treatment) and an untreated control group (urine irritation, no treatment) were also included.

2.2. 3D In Vitro Human Skin Equivalent

Primary adult human dermal fibroblasts were embedded into a fibrin matrix to produce dermal equivalents (DEs). The DEs were cultured to allow the fibroblasts to remodel the matrix. Primary neonatal human keratinocytes were applied to the DE surface and cultured under liquid for 48 hours. Labskin was cultured at the air liquid interface (ALI) until a stratified epidermis was formed. Incubation conditions for all cultures were 37 ± 2 °C in 5 ± 1% (v/v) CO_2 at ≥95% Relative Humidity (RH).

2.3. Synthetic Urine Composition

Synthetic urine was made by combining urea, sodium chloride, ammonium chloride, sodium sulphite, sodium dibasic, creatine, ammonium hydroxide and distilled water. Once the components had fully dissolved, the solution was filter sterilised through 0.2µM vacuum filter. Aliquots were taken from the filtrate for contamination and sterility checks using solid agar medium.

2.4. Treatment Protocol

Quintuplicate Labskin-S replicates for each test item group were used. All Labskin-S models were exposed to 11µL of synthetic urine for 6 hours (±30 minutes) except for the negative control group. The surface of the treated Labskin-S models was then washed with twice with 500µL of the test items, or in the case of the untreated control group, not washed at all. The surface of the Labskin-S models was not blotted dry to mimic real-world exposure to the test items. All Labskin-S models were incubated at 37 ± 2 °C in 5 ± 1% (v/v) CO2 at ≥95% RH for a further 18 hours ±1 hour. The undernatent media from each model was collected and sampled for the presence of pro-inflammatory markers and the tissue from each model subjected to a cell viability assay using MTT.

Protocol outline is shown in Figure 1.

2.5. Cell viability using MTT

The MTT assay is a widely used test that measures cell metabolic activity to determine cell viability or proliferation. It uses a reagent called 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT that reduces to Formazan, resulting in a colour change (yellow to purple) that can be read using a multi-plate spectrophotometer.

The undernatent media was replaced with 1mL of 1mg/mL MTT prepared in Labskin maintenance medium. The Labskin models were incubated at 37 ± 2 °C in 5 ± 1% (v/v) CO2 at ≥95% RH for 3.5 hours ±5 minutes. The Formazan was collected from the Labskin models using isopropanol and the absorbance measured at 540nm.

2.6. Pro-Inflammatory Markers

The collected undernatent media was analysed for the presence of pro-inflammatory cytokines, using a human Il-1α ELISA (R&D Systems) and a human pro-inflammatory 10-plex panel (Meso Scale

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Discovery). The cytokines on the 10-plex panel were IL-1α/ IL-1β, IL-2, IL-4, IL-6, IL-13, IL-10, IL-12P70, TNF-α, and IFN-γ.

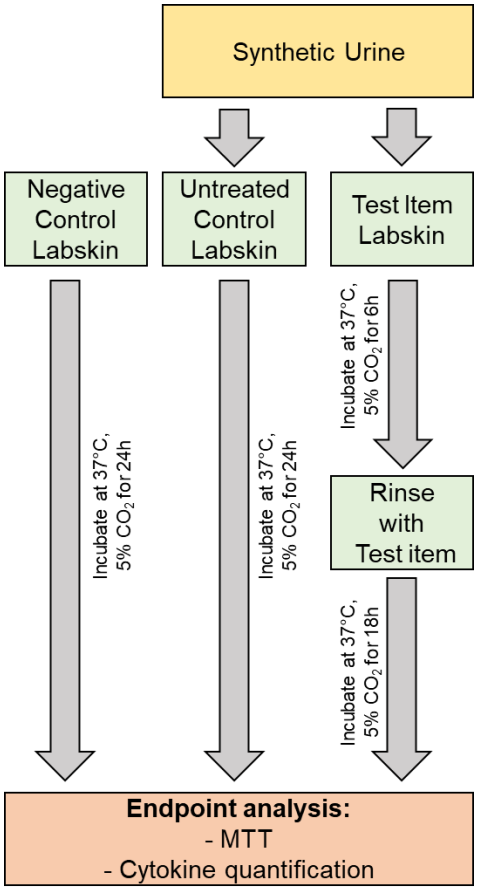

Figure 1. Experimental design for cell viability and inflammation assays.

3.3. Results

3.1. Cell Viability

All absorbance readings were within the limits of detection of the assay and the plate reader. Raw absorbance values are shown in Figure 2.

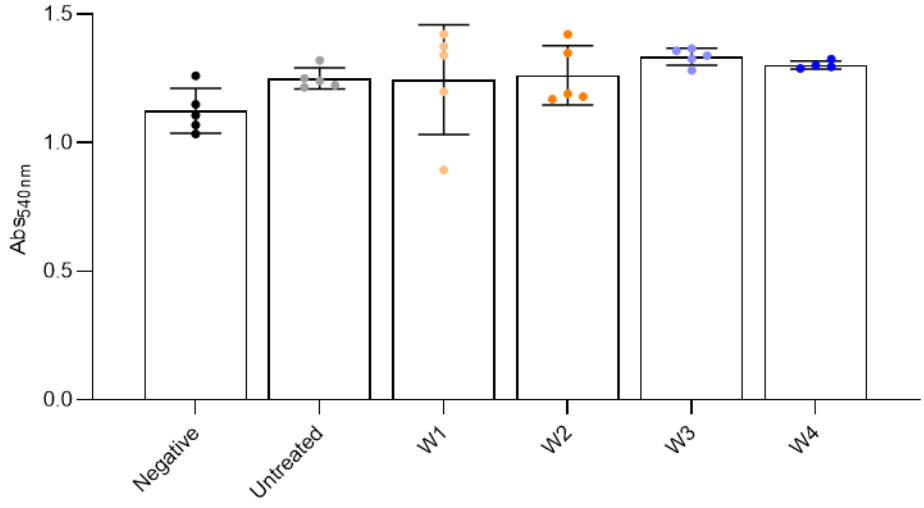

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Figure 2. Absorbance values at 540nm of formazan extracted from Labskin[1.1] following the MTT assay. Data points represent individual Labskin[1.1] units (n=5). Graph displays mean ± SD.

Table 1. List of cytokines (and their roles) analyzed in the undernatant using the multiplex and conventional ELISA.

Cytokine	Role
IFN-γ	Acts in a proinflammatory manner to recruit and activate macrophages as part of response to injury or infection.
IL-1α	Acts in a proinflammatory manner to further perpetuate an inflammatory response. Released in response to mechanical, chemical or microbial damage.
IL-1β	
IL-2	A cytokine that directs T-cell expansion and differentiation into Th1 and Th2 cells.
IL-4	Help differentiate helper T cells, primarily into Th2 cells. These cytokines are known to be involved in promotion of allergic inflammation.
IL-13	
IL-6	A pleiotropic cytokine with numerous biological activities. It plays a central role in acute inflammation and induces release of proinflammatory cytokines from keratinocytes.
IL-12p70	A bioactive form of IL-12 cytokine. It is a proinflammatory cytokine that helps to differentiate helper T cells, primarily into Th1 cells.
IL-10	A potent anti-inflammatory cytokine that strongly inhibits the production of proinflammatory cytokines.
TNF-α	Acts in a proinflammatory manner and is used as a general marker for inflammation. This cytokine in combination with IL-4 and IL-13 can have a suppressing effect on formation of certain barrier proteins such as keratin or filaggrin.

The percentage cell viability for each test item was then calculated as a percentage of the negative control cell viability (Figure 3). All values were above 100%, indicating that there was no cell death/ loss of cell viability after exposure to the synthetic urine and treatment with the test items.

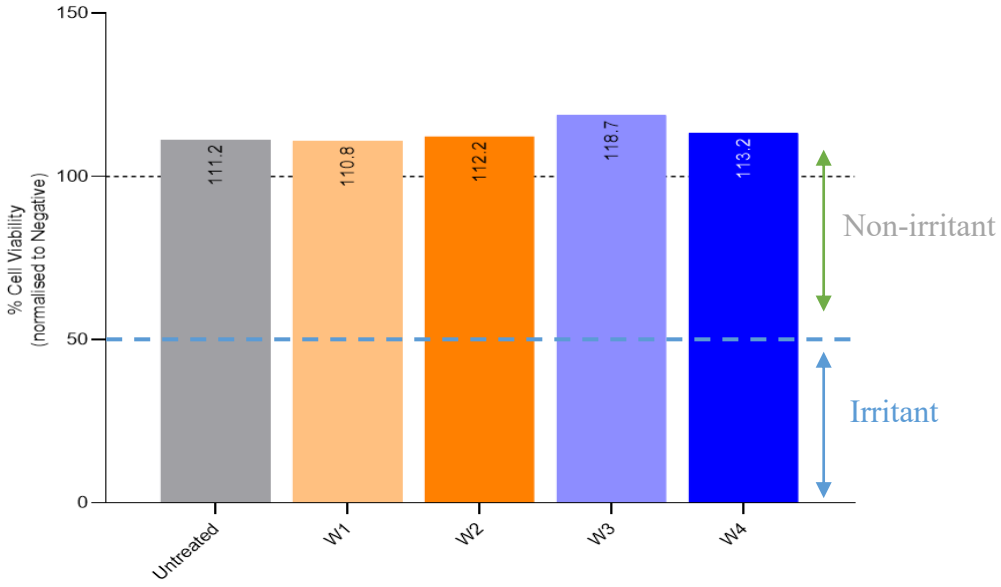

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Figure 3. Percentage cell viability calculated from the average absorbance readings and normalised to the negative control (Negative). The black dotted line represents 100% cell viability (negative control), and the magenta dotted line represents 50% cell viability, the threshold of irritant classification.

3.2. Proinflammatory Cytokine Release

Figures 4–6 below show the graphed data for the pro-inflammatory cytokine release. One-way ANOVA tests were performed on the data to assess statistical significance.

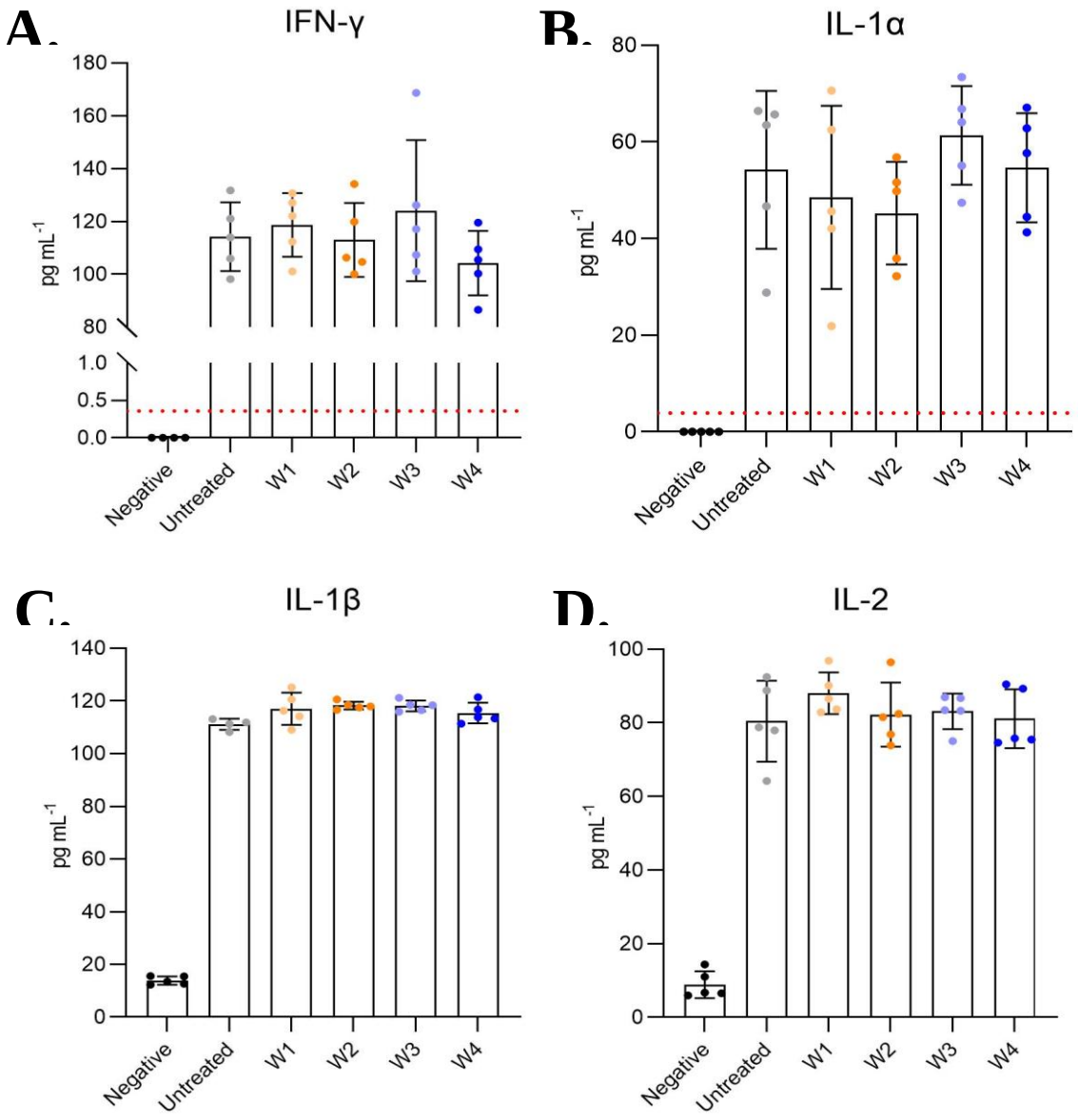

Figure 4. Cytokine quantification results. Data points represent individual Labskin units with outliers removed (n=4-5). Graph displays mean + SD. Upper Limit of quantification is not displayed, as none of the data points have crossed this threshold. Black dotted line represents the upper limit of quantification. Red dotted line represents the Lower limit of quantification (LLOQ) on graphs where data has crossed this threshold. A. IFY-γ. B. IL-1α. C. IL-1β. D. IL-2. Statistically significant differences are not shown on graphs for clarity.

Interferon gamma (IFN-γ) is a pro-inflammatory cytokine activated during injury or infection. Its release activates macrophage white blood cells to migrate to the site of injury or infection to assist with removal of microbes or dead cells and assist with tissue repair. Figure 4 panel A shows the data for the IFN-γ release. All test items showed statistically significant upregulation compared to the

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negative (no synthetic urine, no test item) control. When compared to the untreated control (synthetic urine, no test item) there was no statistically significant release of IFN-γ with any of the test items ($p>0.05$).

Interleukin 1 alpha (IL-1α) is a pro-inflammatory cytokine that is often seen as the 'first responder' in inflammatory cascades. It operates as a secreted cytokine and an intracellular cytokine and is involved in infection, inflammation, fever, wound healing and cellular damage. Figure 4 panel B shows the data for IL-1α release. All test items showed statistically significant upregulation compared to the negative (no synthetic urine, no test item) control. When compared to the untreated control (synthetic urine, no test item) there was no statistically significant release of IL-1α with any of the test items.

Interleukin 1 beta (IL-1β) is a pro-inflammatory cytokine produced by white blood cells (monocytes, macrophages) and is involved in infections, inflammatory conditions, temperature regulation and tissue damage. Figure 4 panel C shows that there was a statistically significant increase in the IL-1β release caused by the untreated control when compared to the negative control ($p=<0.0001$), indicating that the synthetic urine did induce a cytokine release. Test items W2 and W3 triggered a statistically significant increase in IL-1β release when compared to the untreated control ($p>0.0359$ and $p>0.0422$ respectively). There was no statistically significant increase in IL-1β release for W1 or W4 when compared to the untreated control

Interleukin 2 (IL-2) is a pro-inflammatory cytokine involved in the regulation of T-cell and NK cell responses. It plays a critical role in infection by allowing the identification of 'self' and 'foreign' cells by the immune system. Figure 4 panel D shows that there was a statistically significant increase in the IL-2 release caused by the untreated control when compared to the negative control ($p=<0.0001$), indicating that the synthetic urine did induce a cytokine release. When compared to the untreated control (synthetic urine, no test item) there was no statistically significant release of IL-2 with any of the test items.

Interleukin-4 (IL-4) is an anti-inflammatory cytokine that inhibits the release of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Figure 5 panel A shows that there was a statistically significant increase in the IL-4 release caused by the untreated control when compared to the negative control ($p=<0.0001$), indicating that the synthetic urine did induce a cytokine release. There was a statistically significant increase in the release of IL-4 with the W1 treatment compared to the untreated control and the W4 test item group ($p=0.0004$ and $p=0.0044$ respectively). There was no statistically significant increase in IL-4 release for W2 and W3 when compared to the untreated control or W4 test item.

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that is released in response to infection and injury and helps to recruit white blood cells to fight infection and inflammation. It also acts as a protein signal or myokine during exercise to decrease inflammation and intramuscular stress and injury. Figure 5 panel B shows that there was a statistically significant increase in the IL-6 release caused by the untreated control when compared to the negative control ($p=<0.0001$), indicating that the synthetic urine did induce a cytokine release. All test items showed statistically significant upregulation compared to the negative (no synthetic urine, no test item) control. When compared to the untreated control (synthetic urine, no test item) there was no statistically significant release of IL-6 with any of the test items.

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine that is produced by immune cells to dampen down an immune response and prevent tissue damage due to inflammation. Figure 5 panel C shows the data for IL-10 release. There was a statistically significant increase in the IL-10 release caused by the untreated control when compared to the negative control ($p=<0.0001$), indicating that the synthetic urine did induce a cytokine release. There was a statistically significant decrease in the amount of IL-10 released with W2, W3 and W4 compared to the untreated control ($p=0.0009$, $p=0.0046$ and $p<0.0001$respectively), but not for W1. There was a statistically significant increase in the release of IL-10 for W1 compared to W4 ($p>0.5$).

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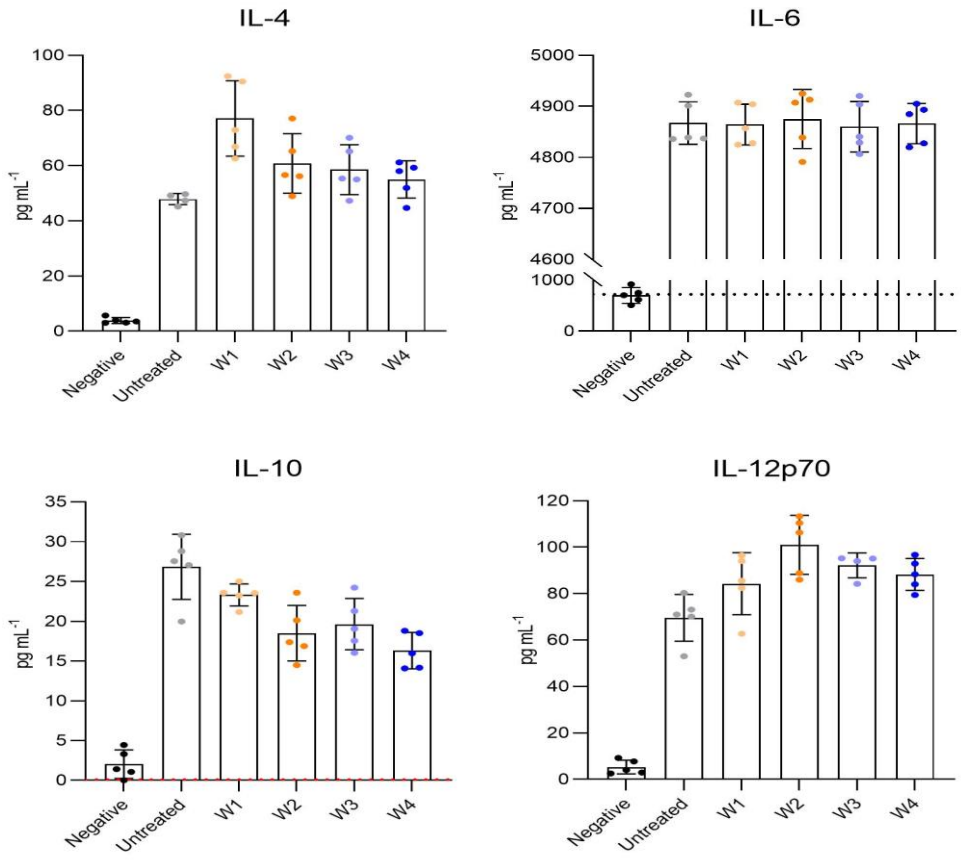

Figure 5. Cytokine quantification results. Data points represent individual Labskin units with outliers removed (n=4-5). Graph displays mean + SD. Black dotted line represents the upper limit of quantification on graphs where data has crossed this threshold. Lower limit of quantification (LLOQ) is not shown as none of the data sets have crossed this threshold. A. IL-4. B. IL-6. C. IL-10. D. IL-12p70. Statistically significant differences are not shown on graphs for clarity.

Interleukin 12p70 (IL-12p70) is a pro-inflammatory cytokine produced by activated macrophage and dendritic cells to prevent infections and inflammation. Figure 5 panel D shows the data for IL-12p70 release. There was a statistically significant increase in the IL-12p70 release caused by the untreated control when compared to the negative control (p=<0.0001), indicating that the synthetic urine did induce a cytokine release. There was a statistically significant increase in the amount of IL-10 released with W2, W3 and W4 compared to the untreated control (p=0.002, p=0.0135 and p=0.0379 respectively), but not for W1.

Interleukin 13 (IL-13) is a pleiotropic protein involved in inflammatory and infection responses, in particular, in atopic dermatitis. It can promote pro-inflammatory responses but can also have anti-inflammatory effects by inhibiting the release of other pro-inflammatory cytokines such as IL-1β, IL-8 and TNF-α. Figure 6 Panel A shows the data for IL-13 release and that there was a statistically significant increase in the IL-13 release caused by the untreated control when compared to the negative control (p=<0.0001), indicating that the synthetic urine did induce a cytokine release. All test items showed statistically significant upregulation compared to the negative (no synthetic urine, no test item) control.

When compared to the untreated control (synthetic urine, no test item) there was no statistically significant release of IL-13 with any of the test items.

Tumor Necrosis Factor Alpha (TNF-α) is upregulated when the immune system is fighting infection or disease, and is often upregulated in autoimmune diseases such as psoriasis, Crohn's disease or rheumatoid arthritis. Figure 6 panel B shows that there was a statistically significant increase in the TNF-α release caused by the untreated control when compared to the negative control

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(p=<0.0001), indicating that the synthetic urine did induce a cytokine release. All test items showed statistically significant upregulation compared to the negative (no synthetic urine, no test item) control. When compared to the untreated control (synthetic urine, no test item) there was no statistically significant release of TNF-α with any of the test items.

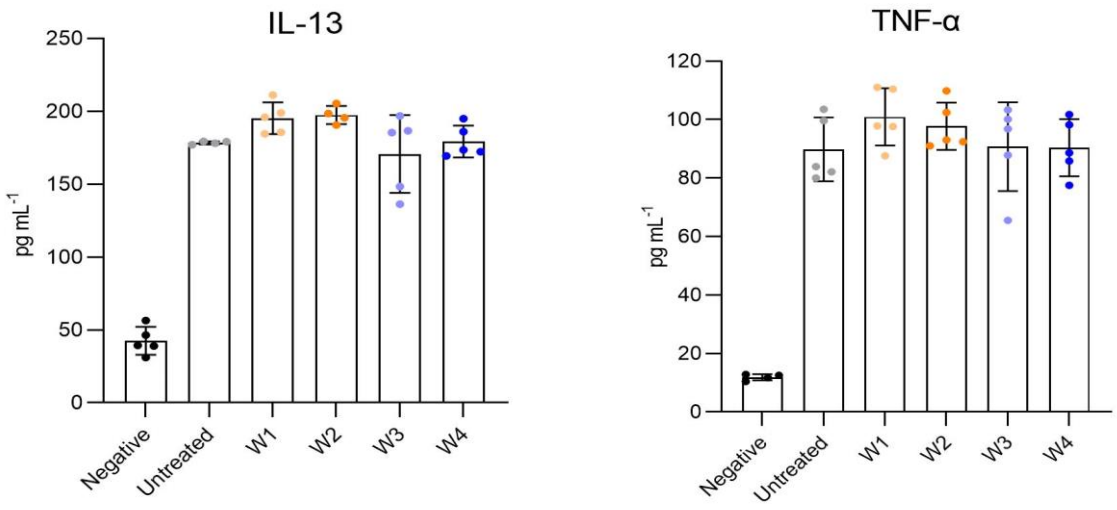

Figure 6. Cytokine quantification results. Data points represent individual Labskin units with outliers removed (n=4-5). Graph displays mean + SD. Black dotted line represents the upper limit of quantification on graphs where data has crossed this threshold. Lower limit of quantification (LLOQ) is not shown as none of the data sets have crossed this threshold. A. IL-13. B. TNF-α. Statistically significant differences are not shown on graphs for clarity.

4. Discussion

The cell viability data from the MTT assay showed that the application of the four test items did not cause necrosis or damage to the Labskin-S models treated with the synthetic urine. This is because this was only a single application of the synthetic urine to the surface of the Labskin and not prolonged exposure with physical aggravation. However, this lack of tissue damage allowed the investigation of the cytokines involved in the response to the test items without having to discount responses due to necrosis or apoptosis due to tissue damage.

The application of the synthetic urine to the surface of the Labskin-S model was able to induce the release of cytokines involved in inflammation. All ten cytokines investigated in this study were elevated with the application of the synthetic urine in the untreated control compared to the negative (no urine, no treatment) control. W2 and W3 test items both shows a statistically significant increase in the release of IL-1β and IL-12p70 cytokines, both promoters of inflammation, while both test items also have a statistically significant reduction in IL-10 release, an anti-inflammatory cytokine. This indicates that both test items are not having an anti-inflammatory effect, but may in fact, be exacerbating inflammation. Item W2 was a baby wipe product containing 17 ingredients and readily available in supermarkets. Item W3 was a baby wash containing 14 ingredients.

W4 test item has a significant increase in the pro-inflammatory cytokine IL-12p70 release, and a significant decrease in IL-10 release. This indicates that this is not as inflammatory as W2 and W3. This item was tap water.

W1 shows a statistically significant increase in IL-4 and IL-10 anti-inflammatory cytokines and no statistically significant release of any of the pro-inflammatory cytokines, indicating that this test item is reducing the inflammatory response. This item is a baby wipe made with minimal ingredients containing just 2 ingredients. It was interesting that of the 3 cleaning products, W1, W2, W3, the product with the least number of ingredients showed an anti-inflammatory type of reaction. Anti-

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inflammatory cytokines suppress or resolve inflammation once the threat is controlled, preventing excessive tissue damage. This is an important concept in the role of diaper dermatitis as it is beneficial to the body to have contact with products which will reduce immune cell activation and help promote tissue repair and healing.

Given that infant skin is structurally more vulnerable, especially in the diaper area, because it has a thinner stratum corneum, a higher trans epidermal water loss (TEWL), the lipid organization in baby skin is immature, and baby skin has reduced resilience to friction and irritants, all mean that once inflammation begins, it escalates more quickly and causes more barrier disruption than in adult skin(13).

The diaper area of babies and older adults with incontinence is daily affected by moisture, friction, urine, feces, and pH shifts resulting in rapid cytokine signalling and the release of pro-inflammatory cytokines (e.g., IL-1α, IL-1β). Inflammatory cells then activate and the skin barrier starts to breakdown leading to more inflammation and more breakdown and an ongoing cycle if not interrupted.

Anti-inflammatory cosmetic products with ingredients such as zinc oxide and other cleansing materials which can help down-regulate pro-inflammatory mediators will be of benefit to incontinence affected skin. Products such as W1 may help calm erythema, reduce discomfort, and generally prevent escalation. This supports the evidence of 2 pieces of earlier published work.

The BaSICS (Baby Skin Integrity Comparison Survey) study from 2021 concluded that in a large study of babies aged from birth to eight weeks, who had been randomly allocated different brands of wipe, was the first research to demonstrate that wipe formulation is related to incidence of clinically significant irritant diaper dermatitis [11]. In that study, babies who were cleaned with the cosmetic wipe with fewest ingredients had significantly fewer days of rash. In the hospital setting, babies in the neonatal unit for a variety of reasons have higher incidence of diaper dermatitis than the general baby population. Studies have recommended cleaning with diaper wipes, containing the least number of irritants and fragrances, as being more beneficial than using cotton wool with water [13–15]. The Utah study showed that both preterm and full-term babies tolerated the novel wipes well and along with other skin care guidelines, the diaper wipes might contribute to the decreased diaper dermatitis [12]. This work brings the in vivo and in vitro work together to support the data.

5. Conclusions

The skin exposed to incontinence in adults and the diaper area in babies is a closed, occluded, warm, alkaline, friction-rich microenvironment. Inflammation in this area spreads faster and is harder to resolve without intervention. Anti-inflammatory products break the inflammatory cascade, restore homeostasis and protect against ongoing irritant exposure. W1 shows an elevation of release of IL-4 and IL-10 cytokines, both of which play an anti-inflammatory role in down-regulating or blocking the release of other pro-inflammatory cytokines. This indicates that W1 could be playing a role in dampening down the inflammatory response triggered by exposure of the Labskin models to synthetic urine. In comparison with the Untreated control, test item W1 resulted in fewer statistically significant increases in cytokine release than the treatments “W2” and “W3”. This may indicate a reduction in the irritation potential of using “W1” for cleansing of baby skin during diaper changes to prevent irritant diaper dermatitis. This result agrees with data previously published [11,12] that babies cleansed with wipes with fewer ingredients, such as the WaterWipes® products were less likely to display symptoms of diaper dermatitis, such as inflamed skin, than babies cleansed with wipes containing a greater number of ingredients.

Author Contributions: Conceptualization, JS, BC, NK, and EG.; methodology, JS, NK, BC, and EG,; investigation and data analysis NK and BC,; writing—original draft preparation, JS,BC and NK.; writing—review and editing, JS,BC,NK, EG, COC,; funding acquisition, JS and EG. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Authors JS and EG are employed by the company WaterWipes UC. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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