[redacted]

IN VITRO HUMAN ASSAY FOR EVALUATING IMMUNOGENIC AND SENSITIZATION POTENTIAL OF WCD PRODUCTS

[redacted]

Prepared by:

[redacted] PhD DABT
Cardno ChemRisk – Supervising Health Scientist

[redacted] PhD
Cardno ChemRisk – Managing Health Scientist

November 30, 2019

1. INTRODUCTION

Cardno ChemRisk was asked by WEN By Chaz Dean ("WCD") to conduct a comprehensive risk and safety assessment of the cosmetic product commonly known as WEN® by Chaz Dean Cleansing Conditioner (the "WEN Products"), and, specifically, whether the product causes hair loss and/or any other adverse dermal event, which evaluation was triggered by complaints and allegations that the WEN Products caused hair loss in a very small percentage of consumers. As part of that comprehensive risk and safety assessment, we performed an *in vitro* human assay for evaluating immunogenic and sensitization potential of the best-selling and most complained about of the WEN Products (Sweet Almond Mint).

To perform this test, we utilized an *in vitro* human assay skin explant test, the Skimune® assay, as an alternative approach to the use of animal models for immunogenic and skin sensitization testing (Ahmed et al. 2015). The Skimune® assay is a model of human *in vivo* immune responses and may be utilized to test adverse immune reactions to a compound or product of interest. The assay measures the ability of the test article to activate the human immune system in response to stimulation with the test article. This assay has been proven to be reproducible, reliable, and sensitive in differentiating between known sensitizers and known non-sensitizers (Ahmed et al. 2015). Specifically, the Skimune®, T cell proliferation, and interferon gamma (IFNγ) assays were used to evaluate the skin sensitization potential of the WEN Products.

2. BACKGROUND

The immune system protects the host from pathogens, toxic, or allergenic substances by producing cytokines or specialized cells that recognize and eliminate foreign, non-self substances (Chaplin, 2010). However, improper or excessive activation of the immune system may results in cytokine production and proliferation of activated T cells causing damage to tissue such as the skin (Ahmed et al. 2015).

3. METHODOLOGY

Test Articles

The examined test articles include WEN Sweet Almond Mint cleansing conditioner, Triton-X (Tx; negative control), and 2,4-Dinitrochlorobenzene (DNCB; positive control). Sweet Almond Mint cleansing conditioner was selected as it was the top selling of the WEN Products with reported adverse events. WEN Sweet Almond Mint cleansing conditioner was tested at 0.01% concentration, a concentration that showed >85% PBMC cell viability as determined by Trypan Blue Exclusion Test. Additionally, Triton-X was tested at 0.0001% and DNCB was tested at 0.1 µM.

Human Samples and Tissue Culture

This study was approved by the Local Research Ethics Committee, and all human samples were obtained from healthy volunteers (n=6) after informed consent. For the skin explant test, each volunteer donated 60 mL of peripheral blood and two 4-mm skin biopsies from the abdominal region. Peripheral blood mononuclear cells (PBMC) were prepared by density-gradient centrifugation using the Lymphoprep™ method, while $CD14^+$ cells were selected using MACS® technology (Miltenyl Biotec, Surrey, UK). Monocyte derived dendritic cells (MoDC) were generated as described by Kvistborg (Kvistborg et al. 2009), with some modification. Briefly, $CD14^+$ monocytes were cultured for 24 h with GM-CSF (50 ng/mL) and IL-4 (50 ng/mL), and then cultured with maturation stimuli (LPS 0.1 µg/mL, IL-6 10 ng/mL, IL-1β 10 ng/mL, TNF-α 10 ng/mL, PGE2 1 µM and 5 µg/mL) for a further 24 h. The $CD14^+$ fraction was collected and used as autologous lymphocytes in further tests. Cells were cultured in RP10 [RPMI 1640 (Gibco, UK) containing 100 IU/mL penicillin, 100 µg/mL streptomycine (Gibco, UK) and 2 mM L-glutamine (Gibco, UK) supplemented with 10% v/v heat inactivated fetal calf serum (Sera Lab)]. Skin biopsies were collected fresh in ex-vivo medium (X-Vivo™ 10, Lonza) and processed by washing in phosphate-buffered saline. Excess fat was removed before dissection and its use in the skin explant test.

[^{3}H]-Thymidine T Cell Proliferation Assay

The induction of a T cell proliferation response following co-culture of MoDCs exposed to the test articles with autologous T cells was measured. MoDCs were incubated with the test articles and then activated by autologous T cells for 5-7 days. Each sample was set up in triplet wells. A negative control (MoDC exposed to Triton-X and co-cultured with autologous lymphocytes) and a positive control (MoDC exposed to DNCB and co-cultured with autologous lymphocytes) were included in each assay. Untreated MoDC were co-cultured with autologous lymphocytes to determine the baseline proliferation (background). Supernatants were pooled together from triplicate wells and stored for cytokine analysis before {^{3}H]-Thymidine addition. A scintillation beta-counter was used to measure the radioactivity in DNA recovered from the cells as counts per minute (CPM) to determine the degree of cell division that occurred in response to the test articles.

INFɣ Assay

Interferon-ɣ levels were quantified in supernatants collected from the T cell proliferation assays using the MesoScale Discovery (MSD) platform following the manufacturer's instructions. A single sample per condition was analyzed (pooled from triplicate wells).

Skimune® Assay

The skin explant assay (n=6) in this setting consists of co-incubating MoDC which have been exposed to the test articles for 24 h with autologous lymphocytes followed by co-culture with autologous skin for 3 days. Skin explants were cultured with medium as a background control. Skin tests were performed in duplicate in each assay. Routine staining for histopathology was performed and skin was graded for histopathological damage (grades I-IV) using criteria which is very similar to that used and observed in clinic (Table 1). A test article was regarded as a strong positive if 75% or more of the tests were positive, moderately positive if 60-74% of the tests were

positive, and a weak positive if 41-59% of the tests were positive. A test article was regarded as a negative if ≤40% of the tests were positive (Table 1).

Statistical Analysis

For each test article, assays were performed using samples from 6 individual healthy volunteers. The data for the T cell proliferation responses to each individual test article is given as CPM. The mean data for the T cell proliferation assays are given as Stimulation Indices (SI), which is representative of the level of T cell proliferation in response to exposure to the test articles. To determine the SI, the test value was divided by the background value (autologous response) and calculated as an increase in T cell stimulation compared to the baseline control. A SI value of ≥3 was defined as the threshold for a significant positive response (Table 2). The data for IFNɣ cytokine secretion is given as levels (ng/mL) of IFNɣ in each sample. The mean data for IFNɣ cytokine secretion is given as a fold increase (FI). The FI is representative of the level of IFNɣ secretion by cells in response to the test article and was calculated in a similar manner to the SI. The FI threshold value of ≥3 was significant and considered positive (Table 2). Statistical analyses were carried out using 1-way ANOVA tests in STATA. A probability (p) of <0.05 was defined as statistically significant. A comparison of the test article response was made to the negative control (Tx) response, which indicates the level of change in T cell proliferation or IFNɣ levels.

4. RESULTS

[^{3}H]-Thymidine T Cell Proliferation Assay

The pooled T cell proliferation results for 0.01% WEN cleansing conditioner were not statistically significantly different from the negative control Tx group, and were statistically significantly different from the no treatment and positive control DNCB groups (Figure 1). The pooled T cell proliferation results for the positive control DNCB group were statistically significantly different from the no treatment control group, while the pooled T cell proliferation results for the negative control Tx group were not statistically significantly different from the no treatment control group (Figure 1).

The T cell proliferation SI ranged from 1.17 to 3.30 (mean 2.42) for the 0.01% WEN cleansing conditioner group. In comparison, the SI ranged from 0.82 to 2.74 (mean 1.65) for the negative control Tx group and 3.70 to 9.85 (mean 5.55) for the positive control DNCB group. An increase of ≥3 was considered a significant increase and a positive response. Two tests involving WEN cleansing conditioner at 0.01% showed a positive response, while the remaining tests did not show a positive response. The 0.01% WEN cleansing conditioner SI results were not statistically significantly different from either the negative control Tx or no treatment groups, but was statistically significantly different from the positive control DNCB group. SI results for the 6 independent experiments are shown in Figure 2.

IFNɣ Assay

[redacted]

The pooled IFNɣ results for 0.01% WEN cleansing conditioner were not statistically significantly different from the negative control Tx group and were statistically significantly different from the no treatment and positive control DNCB groups (Figure 3). The pooled IFNɣ results for the positive control DNCB group were statistically significantly different from the no treatment control group, while the pooled IFNɣ results for the negative control Tx group were not statistically significantly different from the no treatment control group (Figure 3).

The IFNɣ FI ranged from 1.17 to 3.94 (mean 2.12) for the 0.01% WEN cleansing conditioner group. In comparison, the FI ranged from 0.74 to 2.74 (mean 1.34) for the negative control Tx group and 3.19 to 6.47 (mean 4.42) for the positive control DNCB group. An increase of ≥3 was considered a significant increase and a positive response. One test involving WEN cleansing conditioner at 0.01% showed a positive response, while the remaining tests did not show a positive response. The 0.01% WEN cleansing conditioner FI results were not statistically significantly different from either the negative control Tx or no treatment groups, but was statistically significantly different from the positive control DNCB group. FI results for the 6 independent experiments are shown in Figure 4.

Skimune® Assay

The results from the Skimune® assay is summarized in Table 3. Representative images of skin biopsies from each treatment group are presented in Figure 5. The Skimune® assay showed a grade I negative response to WEN cleansing conditioner at 0.01% in 11 of 12 wells from 6 individual experiments.

One well in one experiment showed a grade II weak positive response to WEN cleansing conditioner at 0.01%. Both the negative control (Tx) and background control (medium) gave grade I negative responses in all experiments. Across all experiments, the positive control (DNCB) gave grade II weak positive responses in 4 wells and grade III positive responses in 7 wells (results were not available for one well).

Summary of results and immune prediction

The results from this study are summarized in Table 3. Treatment with 0.01% WEN cleansing conditioner resulted in 2 positive responses in 6 individual T cell proliferation experiments, 1 positive response in 6 individual IFNɣ secretion experiments, and 1 positive response in 6 individual Skimune® experiments. Treatment with negative control Tx and positive control DNCB resulted in all negative and positive responses, respectively, for all individual experiments. According to the criteria for immune response prediction (Table 2), treatment with 0.01% WEN cleansing conditioner is predicted to have a negative immune response. Treatment with negative control Tx and positive control DNCB is predicted to have negative and positive responses, respectively (Table 3).

5. CONCLUSION

The overall results of this study showed that treatment with 0.01% WEN cleansing conditioner resulted in a negative prediction for *in vitro* immune response while treatment with negative control Tx and positive control DNCB resulted in negative and positive responses, respectively. This means that the WEN Products are not immunogenic. In other words, the WEN Products would not be expected to induce an immune reaction or any disease brought on by an immune response.

6. REFERENCES

Ahmed, S.S., Wang, X.N., Fielding, M., Kerry, A., Dickinson, I., Munuswamy, R., Kimber, I. and Dickinson, A.M., 2016. An in vitro human skin test for assessing sensitization potential. Journal of Applied Toxicology, 36(5), pp.669-684.

Chaplin D. D. (2010). Overview of the immune response. *The Journal of allergy and clinical immunology*, *125*(2 Suppl 2), S3-23.

Table 1. Grading criteria for Skimune® assay

Grade	Observations	Response
I	Mild histopathological changes	Negative
II	Diffuse vacuolization of epidermis	Positive
III	Cleft formation between epidermis and dermis	Positive
IV	Complete separation of the epidermis from the dermis	Positive

Adapted from Ahmed et al. 2016, Sviland and Dickinson 1999, Svilabd et al. 1990, Lerner et al. 1974

Table 2. Criteria for immune response prediction

Assay	Criteria	Value	Predicted immune response
T cell proliferation	Stimulation index (SI)	≥ 3	Positive
		< 3	Negative
IFNγ secretion	Fold increase (FD)	≥ 3	Positive
		< 3	Negative
Skimune® assay	Grade II or more histological damage	> 75%	Positive
		60-74%	Moderately positive
		41-59%	Weakly positive
		< 40%	Negative

Table 3. Test article predicted immune response

Test Article	Skimune® positive response	T cell proliferation positive response	IFNγ secretion positive response	Predicted immune response
Triton-X (negative control)	0/12	0/6	0/6	Negative
DNCB (positive control)	11/11	6/6	6/6	Positive
0.01% WCD cleansing conditioner	1/12	2/6	1/6	Negative

Figure 1. Pooled T cell proliferation data

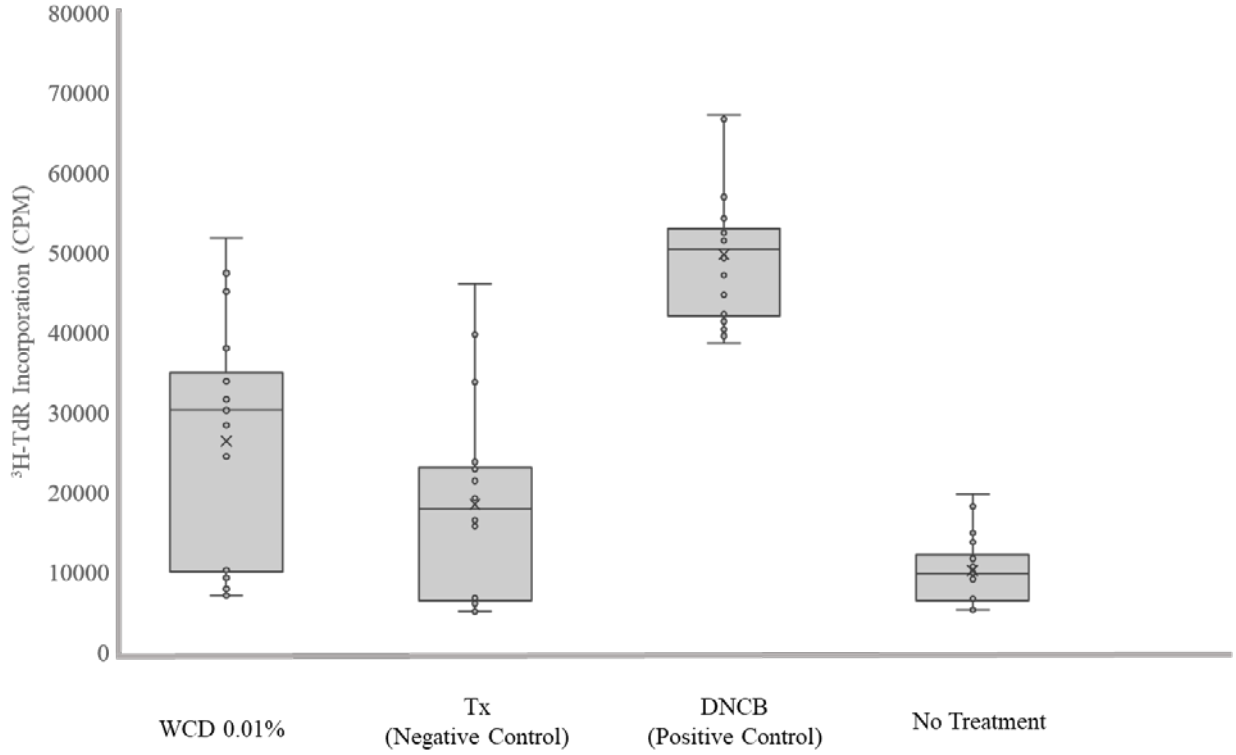


Figure 2. T Cell proliferation stimulation index (SI)

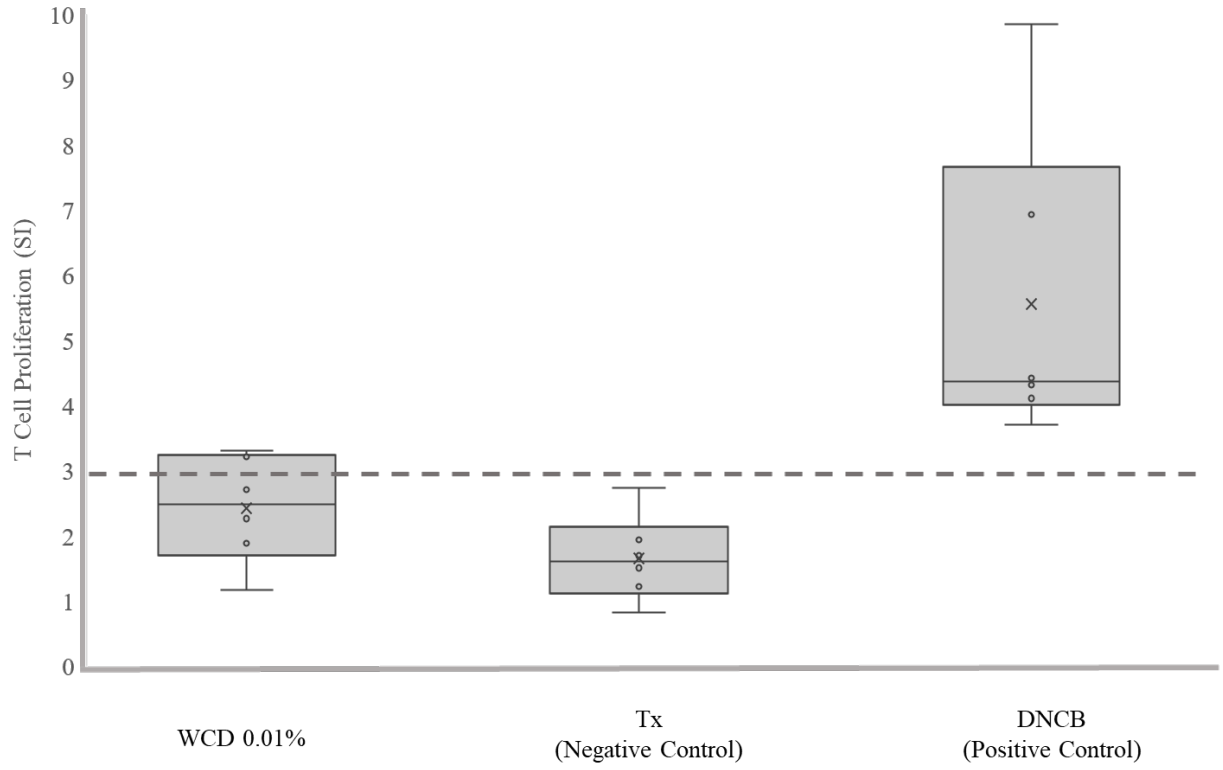


Figure 3. The pooled IFNɣ results

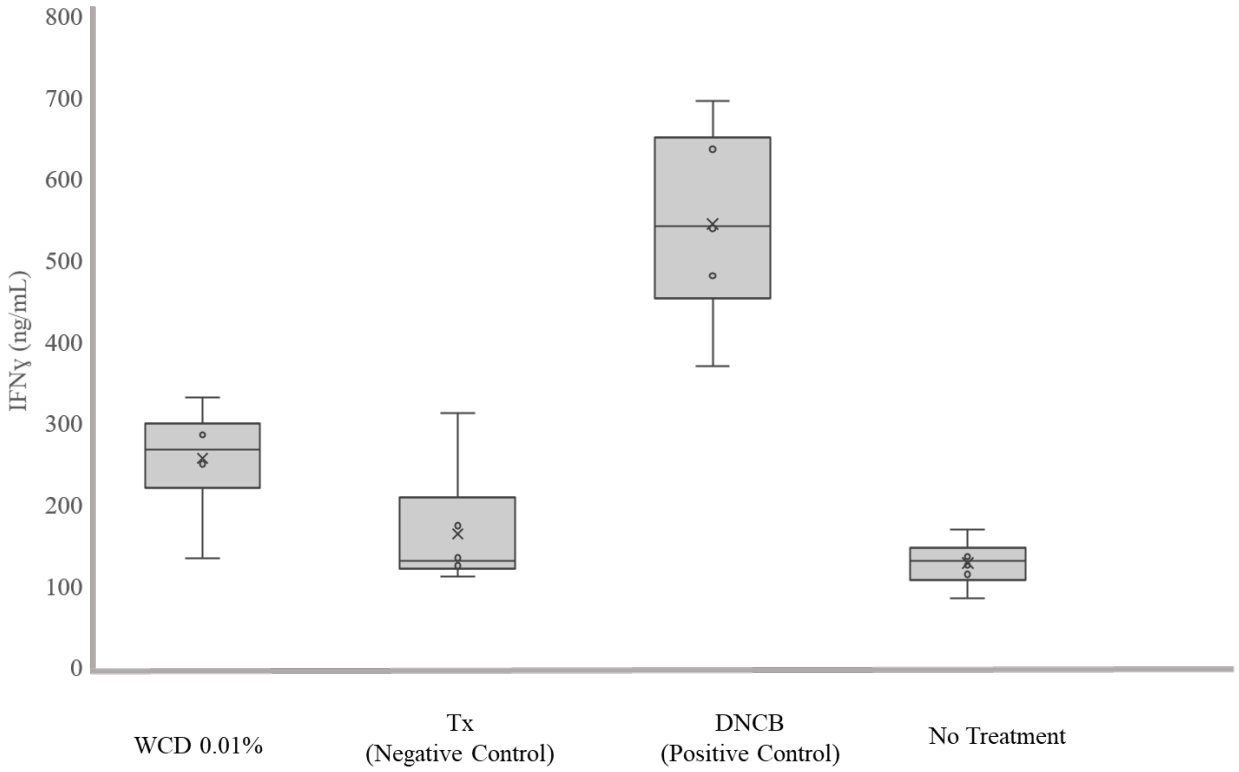


Figure 4. The pooled IFNɣ fold difference (FI)

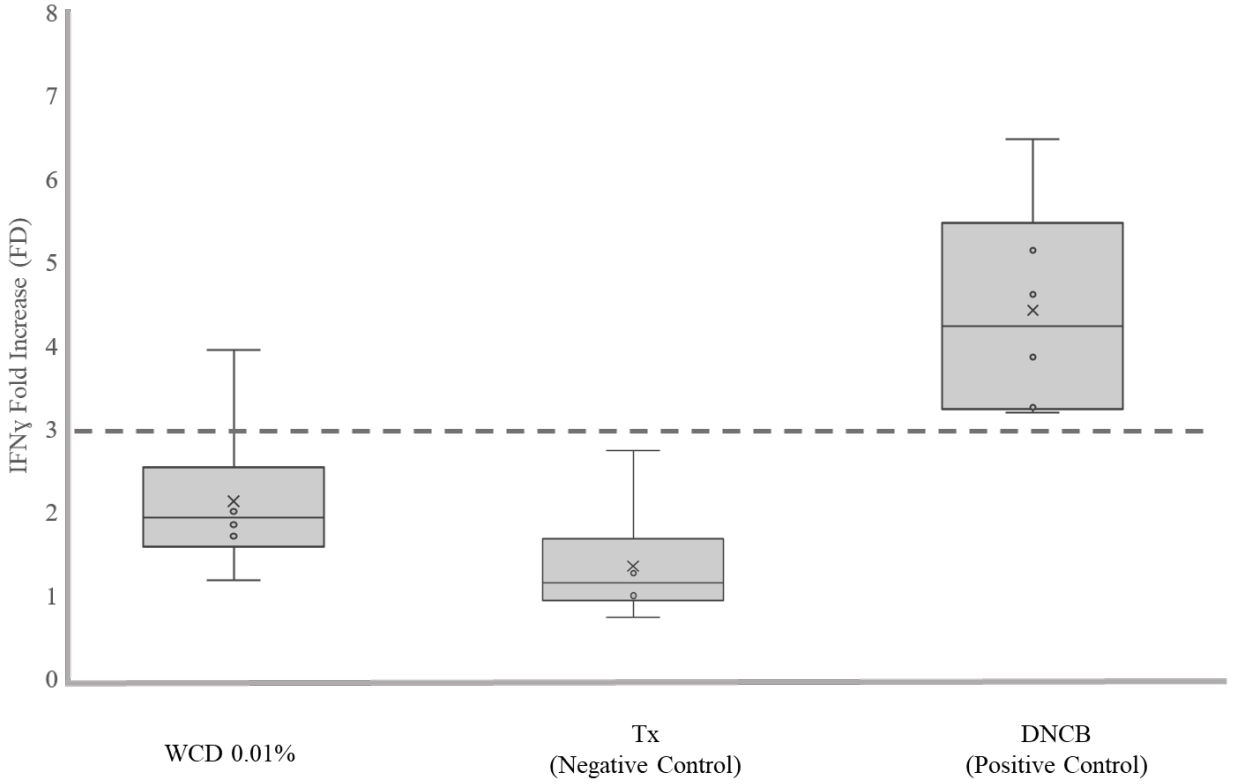
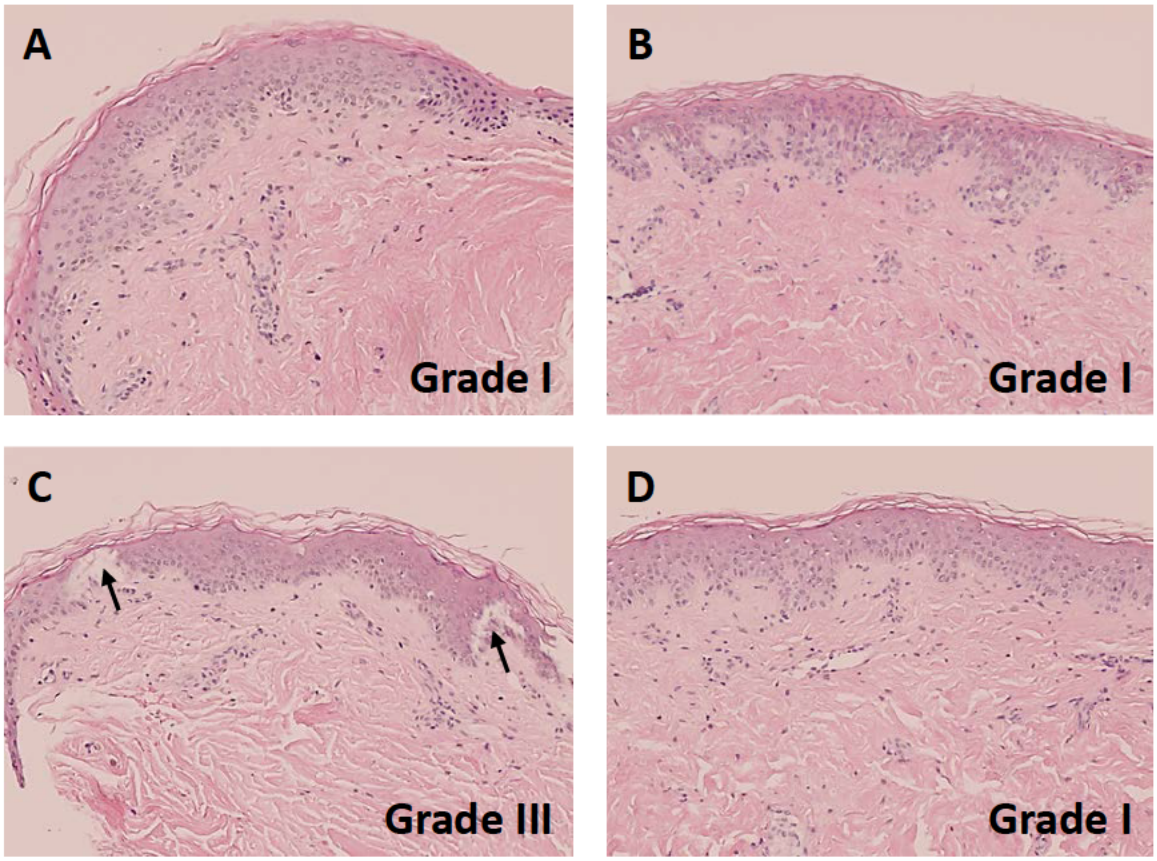


Figure 5. Skimune® assay histopathological analysis



Skin biopsies were co-cultured with autologous cells which have been previously exposed to test articles or controls for 24 hours. Skin explants were graded according to the Lerner clinical grading score; skin explants with < grade II score were negative and predicted to be non-sensitziers and ≥ grade II score were positive and predicted to be sensitizers. Representative skin explants for each treatment condition: **(A)** Media control; **(B)** Triton-X negative control; **(C)** DNCB positive control; and **(D)** 0.01% WCD cleansing conditioner. Black arrows indicate damage on the dermal/epidermal junction within the grading criteria.