Cytotoxic evaluation of hair relaxers and straighteners actives in human-derived skin cells

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Abstract

Most hair relaxers or straighteners used for Afro-textured hair have a pH above 11, which according to international occupational health and safety guidelines is corrosive to the skin. Further, relaxers are associated with 3-6 times increased risk of alopecia. More so, the skin gets into contact with these hair straightening products during application resulting in skin irritation. The use of animals as test subjects to determine the potential risk and safety margin of these products is standard practice, however, with the European Union (EU) ban on the use of animals for testing, animal testing needs to be replaced by newer in vitro laboratory technologies.

This study used previously, and newer established *in vitro* cell-based technologies, and human-skin derived cells as laboratory models to evaluate relaxer-induced cytotoxicity. These technologies include endpoint (MTT, CCK-8 and flow cytometry) and real-time assays (real-time cell analysis (RTCA) and extracellular flux analysis).

Cellular toxicity was evident following treatment of keratinocytes and fibroblasts with acceptable concentrations of thioglycolic acid (TGA, $HSCH_2COOH$), ammonium bisulphite (NH_4HSO_3), lithium hydroxide (LiOH) and sodium hydroxide (NaOH) based relaxers. Real-time assays showed significant reductions ($P<0.001$) in cell index and ATP production following treatment of keratinocytes with as little as one-tenth of the acceptable concentrations. Increased apoptosis (47.9%, 58.0%, 76.7% and 80.3%) also occurred in cells after treatment with TGA, NH_4HSO_3, NaOH and LiOH, respectively.

The use of skin cells in conjunction with advanced cell-based technologies could serve as alternatives to laboratory animals for accessing the toxicity margin of hair relaxers, straighteners and other cosmetics.

1. Introduction

The practice of hair straightening with relaxers is common among females with afro-textured hair.[1] In South Africa, approximately 60 % of afro-textured adult women and 80 % of girls wear relaxed or chemically straightened hair.[2-4] The afro-textured hair is tightly coiled: knotted and has a flattened cross-sectional appearance[5]. The primary mechanism of action of relaxers in hair straightening is the breaking of the disulphide bonds of the hair[6] . The reduction in the disulphide bond in hair is brought about by the thiols, sulphites, alkalis, and other reducing agents found in relaxers.[7] This hair straightening process, however, weakens the hair shaft.[8] Also, there is an association of relaxer with changes in the amino acid contents of the hair, especially cysteine, which ultimately results in fragile or damaged hair and alopecia.[9]

Chemical hair straighteners are classified based on pH and mode of action: Alkaline hair straighteners (or hair relaxers) [6], which produce the most permanent straightening[10] and are the preferred choice of straighteners. The active compound in this type of relaxers includes sodium hydroxide (NaOH), lithium hydroxide (LiOH), potassium hydroxide (KOH), calcium hydroxide $Ca(OH)_2$ or guanidine carbonate $((CH_5N_3)_2H_2CO_3)$ or a combination of the latter two compounds.[11] The pH of this group of hair straighteners ranges from 12 to 14[1][10, 12, 13]. The second group of hair straighteners are the least used perm relaxers. This class of hair straighteners are based on thioglycolic acid (TGA or $HSCH_2COOH$) or the derivative; ammonium thioglycolate $(NH_4^+HSCH_2CO_2^-)$ with pH in the range of 7 to 9.5 [14] The reaction with hair involves a reduction of disulphide bonds, followed by molecular shifting produced by stretching the hair on rollers, and by re-oxidation using neutralizing solutions based on sodium bromate $(NaBrO_3)$ or hydrogen peroxide (H_2O_2).[6, 15, 16] The third group of hair straighteners are the low pH hair relaxers which are based on sulphite (SO_3) or bisulphite (HSO_3) [6, 17]. The pH range for these products is 4 – 6, and at this pH, broad cystine cleavage by sulphite occurs. This group of relaxers exists as inorganic salts e.g. Sodium sulphite (Na_2SO_3), ammonium sulphite $((NH4)_2SO_3)$, sodium bisulphite $(NaHSO_3)$

[18]. The washing of the sulphite-treated hair with water slowly reverses the straightening reaction, thus rebuilding disulphide bonds, and, the rate of cystine reformation increases with increasing pH [6].

Alkaline relaxers elicit the most damage to hair due to the high pH [6]. A product with pH above 11 is considered dangerous and corrosive [10, 19]. Similarly, the damage by reduction-oxidation in perm relaxers cause a decrease of about 5 – 20 % in wet tensile properties of the hair [20].

Another major problem resulting from the treatment of hair with relaxers/straighteners is the contact with the eyes and skin causing irritation or damage. For decades, the Draize irritation test (using rabbits) was the gold standard for evaluating the toxicity of cosmetics and other potentially hazardous materials [21, 22]. However, with the complete ban of animals as models for cosmetic safety testing by the European Union (EU) [23], there is a need for *in vitro* non-animal substitutes. Alternative testing models may include *ex vivo* skin models [24, 25], full-thickness human skin equivalent models [26, 27], human reconstructed epidermis (RHE) [28, 29] and human immortalised keratinocytes (HaCaTs). HaCaTs is used widely as an *in vitro* monolayer models for cosmetic safety testing [30, 31]. There is very little data on the harmful effects of hair relaxers and straighteners on the skin, partly due to the lack of a well-developed and suitable skin model to test these products. This challenge coupled with the fact that information on the cellular effects of these products for predicting toxicity are almost non-existent, perhaps due to the scarcity of high throughput cell-based assay technologies to measure certain cellular events. Thus, this study seeks to determine the cytotoxic effect of relaxers and straighteners on human-skin derived cells using established and recently developed cell-based assay technologies.

2   Materials and Methods

2.1   Preparation of relaxer/straightener actives

The following are the actives used in this study; TGA, NH_4HSO_3, LiOH and NaOH. The concentrations of active ingredients of relaxers were prepared as described in Table 1. These concentrations are based on the approved maximum concentrations by the European Union (EU) legislation on chemicals in food and cosmetics of 2009 and the South African Department of Health (SADH) Regulation Gazette of 2017 on chemical composition in cosmetics [14, 23]. The pHs of the active's solution were adjusted to the recommended values with a pH meter (METTLER TOLEDO, US).

2.2   Cells and culture conditions

2.2.1   Keratinocytes

Human immortalised keratinocyte cell lines (HaCaTs) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 100 U/ml Penicillin/ 100 µg/ml Streptomycin (Sigma, SA) at 37 °C, 5 % CO_2 and 95 relative humidity (SHEL LAB, US).

2.2.2   Fibroblasts

Normal human fibroblasts (NHFs) derived from skin were isolated from discarded skin tissue from elective surgery at Groote Schuur Hospital (Cape Town, South Africa) after obtaining due patient consent and with approval from the University of Cape Town Health Research Ethics Committee (HREC REF: R023/2019). Briefly, the skin tissues were transported in 10 ml DMEM supplemented with 100 U/ml Penicillin/100 µg/ml Streptomycin upon collection from the hospital. Excess adipose tissue was removed aseptically from the tissue, which was cut into small pieces and placed in 35 mm tissue culture dishes. Microscope glass coverslips were used to hold the tissues in place and

incubated at 37 °C until fibroblast explants formed confluent monolayers. Resultant fibroblasts were cultured in complete DMEM, harvested, and stored in -80 °C and then liquid nitrogen until further use.

2.3   Cell viability assays

2.3.1   3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability assay, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to evaluate the cytotoxic effects of relaxer/straightener actives on human skin cells (keratinocytes and fibroblasts) monolayers. Briefly, $1x10^4$ cells/well were seeded in a 96-well plate to a final volume of 100 µl for 24 hours at 37 °C. Followed by treatment with different concentration of actives for 20 minutes. This treatment duration is in line with the standard relaxer application time on the hair, to attain visible straightening effect. Cells were washed twice with culture medium, thereafter 0.5 mg/ml of the MTT solution was added to each well. The plates were incubated for 3 hours at 37 °C. Absorbance measurements were taken at 595 nm using a microplate reader (Varioskan Lux; Thermofisher Scientific).

2.3.2   Cell energy phenotyping

Cell energy phenotype following actives treatment was determined using the Agilent Seahorse XFe96 Extracellular Flux (XF) Analyzer (Agilent Technologies, USA). About $4x10^4$ cells/well were seeded into 96-well extracellular flux cell culture plate to a final volume of 100 µl and incubated at 37 °C for 24 hours. Twenty-four hours prior the experiment, the cartridges were equilibrated in a non-CO_2 incubator at 37 °C. Cells were treated with active ingredients for 20 minutes. Thereafter, culture medium was changed to assay medium (XF base medium supplemented with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose) and cells incubated in a non-CO_2 incubator at 37 °C for 1 hour. Stressor compounds (100 µM of both Oligomycin and FCCP mix) were added to the cells

according to the manufacturer's instructions (XF Cell Energy Phenotype Test kit, Agilent). The cartridges and utility plate were loaded into the XF analyser for calibration, and the utility plate later replaced by the extracellular flux cell culture plate with cells and loaded into the XF analyser. The experiment ran for 2 hours.

2.4 Real-time Relaxer-induce cytotoxicity (RIC) assays

2.4.1 Real-time cell analyses assay (RTCA)

The xCELLigence^{TM} real-time cell analysis (RTCA) system (ACEA Biosciences, Inc. San Diego, CA, USA) was employed to assess cellular impedance (CI, a measure of cell viability) after treatment of skin cells with hair relaxer actives. Cells ($4x10^4$ cells/well) were cultured in special 16-well culture plates (E-plates, ACEA Biosciences, Inc. San Diego, CA, USA).

Prior to cell seeding, background signals resulting from culture media impedance (80 µl per well) was subtracted. Cells ($4x10^4$ cells/well) were seeded into 16-well E-plates in 100 µl culture media per well in duplicates to give a final volume of 180 µl and cultured for 24 hours. Hair relaxer actives were added in 1:10 dilution ratio (i.e., 20 µl/well) varying concentrations. Control wells (i.e., cells alone without treatment) was sent up along with the experiments making the total reaction volume 200 µl. The CI was measured at intervals of 1 hour to observe and evaluate anti-proliferative or cytotoxic effects of the relaxer actives for a duration of 72 hours post-treatment.

2.4.2 Mito stress test assay

Using the Agilent Seahorse XFe96 Extracellular Flux Analyser, mitochondrial stress following treatment of skin cells with relaxer actives was evaluated in real-time. Cells ($4 x10^4$ cells/ well) were seeded into 96-well extracellular flux cell culture plate in 100 µl of cell culture medium and incubated at 37 °C for 24 hours. Twenty-four hours before the experiment, the cartridges were equilibrated in a non-CO_2 incubator at 37°C. Thereafter, culture medium was then changed to assay

medium (XF base medium supplemented with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose) and cells incubated in a non-CO_2 incubator at 37 °C for 1 hour. Relaxer actives, 100 µM of Oligomycin, FCCP and Retinone A were added to the cartridge ports A-D respectively according to the manufacturer's instructions (XF Mito stress Test kit, Agilent). The cartridges and utility plate were loaded into the XF analyser for calibration, and the utility plate later replaced by the extracellular flux cell culture plate with cells and loaded into the XF analyser. The experiment ran for 2 hours.

2.4.3   Glycolysis stress test assay

Real-time glycolysis stress test following treatment of skin cells with relaxer actives was evaluated using the Agilent Seahorse XFe96 Extracellular Flux Analyser as well. Cells were seeded as before, and cartridges equilibrated. Culture medium was then changed to assay medium (XF base medium supplemented with 1mM pyruvate) and cells incubated in a non-CO_2 incubator as before. Relaxer actives, glucose, Oligomycin, and 2-DG were added to the cartridge ports A-D respectively according to the manufacturer's instructions (XF Glyco stress Test kit, Agilent). The experiment ran for 2 hours.

2.5   End-point relaxer-induced cytotoxicity (RIC) recovery assays

2.5.1   The 3-day recovery assay

A 3-day recovery of keratinocytes post relaxer active treatment was evaluated by means of cell counting kit-8 (CCK-8) proliferation assay (Sigma Aldrich). HaCaTs ($1x10^4$ cells /well) were cultured in a 96-well plate for 24 hours at 37°C, followed by treatment with relaxer actives for 20 min. Cells were washed twice with culture medium, thereafter 100 µl/well of fresh complete medium was added. At day 3, 10 µl of the CCK-8 solution was added to each well. The plate was incubated for 3 hours at 37 °C and absorbance read at 450 nm.

2.5.2   The 7-day recovery assay

Briefly, $1x10^5$ cells/well were cultured in a 24-well plate for 24 hours at 37 °C. Followed by treatment with relaxer actives for 20 min. Cells were washed twice with culture medium, thereafter fresh complete medium was added into each well. Cells were then cultured for a further 7 days, to allow for recovery. For cell counts, medium was removed, and cells were lifted off the plates and washed once with phosphate buffered saline (PBS). The cell suspension was then run through the BD (Becton-Dickinson) Accuri C6 Plus Sampler flow cytometer (BD Pharmigen, San Diego, CA, USA). Events were recorded per µl and analysed using the BD CSampler Plus Software.

2.5.3   Analysis of the mode of cell death from RIC

Analysis of the mode of cell death by early apoptosis or late apoptosis was determined by Flow cytometry using the FITC Annexin V Apoptosis Detection Kit I (FITC-AV, BD 51-66211E) and propidium iodide (PI, BD 51-65874X) staining solution respectively, and as per manufacturer's protocol. HaCaTs ($5x10^5$ cells/well) were cultured in a 6-well plate for 24 hours at 37°C followed by treatment with actives for 20 min. Cells were washed twice with culture medium; thereafter fresh complete medium was added into each well. Cells were then cultured for a further 24 or 48 hours. At each time point, cells were harvested, washed twice with PBS and stained with both Annexin V and PI dyes. The cell-dye mixture was vortexed briefly and incubated at ambient temperature for 15 minutes in the dark, then 400 µl of binding buffer was added to the stained cells. Stained cells were analysed using flow cytometer equipped with a 488 nm blue laser. Both AV and PI were excited by the blue laser and detected by a 533/30 filter and a 585/40, 670 lower band pass (LP) respectively. Ten thousand events were acquired and analysed using FlowJo v9.4.7 (Tree Star, In.). After compensation was performed using singly stained compensation controls with the two fluorochromes employed (i.e., FITC-AV and PI) and cells were gated on scale (using size and granularity parameters). Untreated stained controls to FITC and PI were used to assigned gates and were

designated as FITC-AV$^-$ and PI$^-$ double negative while cells undergoing early apoptosis was referred to as FITC-AV$^+$PI$^-$ and late apoptosis/necrosis, FITC-AV$^+$PI$^+$.

2.7. Statistical analysis

All experiments were performed in triplicates with each experiment repeated thrice. The mean and standard error of mean were calculated, and statistical significance was tested using two-way analysis of variance with repeated measures (*Two-way RM ANOVA*), and *Unpaired t test*. Statistically significance for all conditions was considered at $p < 0.05$. All statistical analyses were performed using GraphPad Prism v6 software program (San Diego, CA, USA).

3.   Results

3.1.   pH measurements of active ingredients

The pH of the hair relaxer actives was measured, following dilutions to attain specific concentrations. TGA had an average pH of 2, whereas that of NH_4HSO_3 was pH 4, both exhibiting acidic pHs. On the other hand, the hydroxide-based actives (LiOH and NaOH) were at pH 12 and 13 respectively, indicating a basic pH (Table 1).

Table 1. pH of the various concentrations of hair relaxers and straighteners. Thioglycolic acid [TGA ($HSCH_2COOH$)], ammonium bisulphite (NH_4HSO_3), lithium hydroxide (LiOH) and sodium hydroxide (NaOH). Active concentrations was made up according to the industry concentration, half industry, tenth industry and twentieth industry concentration.

	TGA				NH_4HSO_3				LiOH				NaOH			
%	8	4	0.8	0.4	6	3	0.6	0.3	4	2	0.4	0.2	2	1	0.2	0.1
pH	1.93	2	2.26	2.37	3.47	4.06	4.13	4.16	11.44	11.04	12.23	12.07	13.54	13.56	12.36	12.59
Average pH of **TGA**			**2.14**	Average pH **of** NH_4HSO_3			**4.00**	Average pH **of LiOH**			**11.70**	Average pH **of NaOH**			**13.01**

3.2.   Relaxer-induced cytotoxicity on keratinocytes and fibroblasts

Both human immortalised skin keratinocytes and normal human dermal fibroblasts were used to evaluate the relaxer-induced cytotoxicity following treatment with relaxer actives by use of MTT assays. First, cells were exposed to the EU and SADH recommended concentrations of the relaxer actives. Post-treatment, both HaCaTs and NHFs treated with TGA exhibited the cell viabilities higher than the untreated controls. Treatment of HaCaTs remained unchanged while that of NHF increased by > 100 % (as indicated by an increase in absorbance at 595 nm compared to the

untreated cells, Fig. 1a and b). Severe reductions (>5-fold, HaCaTs and > 2-fold, NHFs in comparison to untreated controls) in the cell viabilities were, however, observed in cells treated with NH_4HSO_3, LiOH and NaOH respectively (Figs. 1a and b).

Having determined the cytotoxicity profiles of the actives at these concentrations, we then determined the dose dependent effect of relaxers actives at maximum, half, one-tenth, and one-twentieth of the approved concentrations. Here, there was a slight increase in the cell viability ($\leq$2-fold) of HaCaTs post-treatment with 8% and 4% TGA. In contrast, we observed a slight decrease ($\leq$2-fold) in cell viability with 0.8 % TGA, whereas 0.4 % TGA resulted in a 2-fold reduction in cell viability ($p < 0.01$, Fig. A1a). NHFs, on the other hand, were more resistant to the cytotoxic effects of relaxers at all concentrations of TGA, as seen by a significant increase in cell viability (Fig. 1e).

Treatment with NH_4HSO_3 (6 % and 3 %) resulted in about 6-fold reduction in cell viability of HaCaTs, whereas NHFs were more susceptible at all concentrations tested (Figs. A1b and f).

Hydroxide-based actives (LiOH and NaOH) were cytotoxic to both epidermal and dermal skin cells at all the concentrations, resulting in significant reduction in cell viability (approximately 5-fold overall cell viability for HaCaTs and 2-fold cell viability in NHFs in Figs A1c-d and g-h). Based on the estimated overall IC_{50} of all the relaxer actives used in this experiment, we decided to continue subsequent experiments at one-tenth of the approved maximum concentrations.
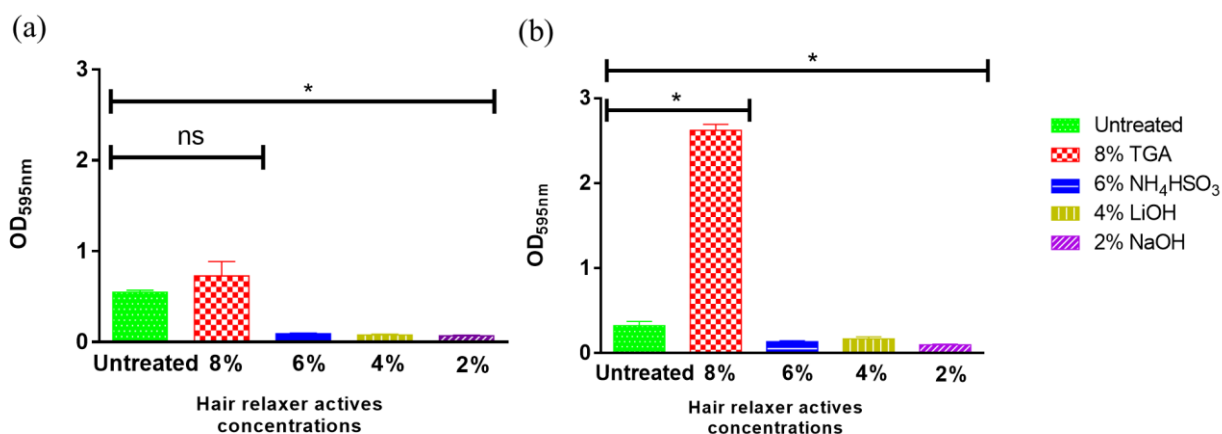
Figure 1. Evaluation of relaxer-induced cytotoxicity. Treatment of HaCaTs (A) and NHFs (B) with hair relaxer actives at approved maximum concentrations. Cells were cultured in 96-well plates overnight, treated for 20 mins with actives and cell viability accessed by MTT assay. Bars indicate means and whiskers indicate standard error of means: ns= not-statistically significant and * = p≤ 0.05 indicates statistical significance. HaCaTs = Human immortalised keratinocytes; NHFs = Normal human skin fibroblasts.

3.3. Evaluation of RIC in Real-time via RTCA and Extracellular Flux analyses

3.3.1. Evaluation of RIC in Real-time using RTCA

A real-time cell monitoring tool was used to evaluate the relaxer-induced cytotoxic effects on both HaCaTs and NHFs. Both cell types showed a similar profile (Figs. 2a and b), in that there was marked increase in cellular impedance (cell index; CI, indicated by black arrows) at 1-hour post-treatment with TGA (CI of 5 compared to 3; untreated). (Figs. 2a and b). Furthermore, HaCaTs showed a non-significant decrease in cell index 5 hours later with TGA, whereas fibroblasts showed a significant reduction at this time point. Both skin cells exhibited a further significant reduction in CI at 24- and 48-hour time-points (Figs. 2a and b).
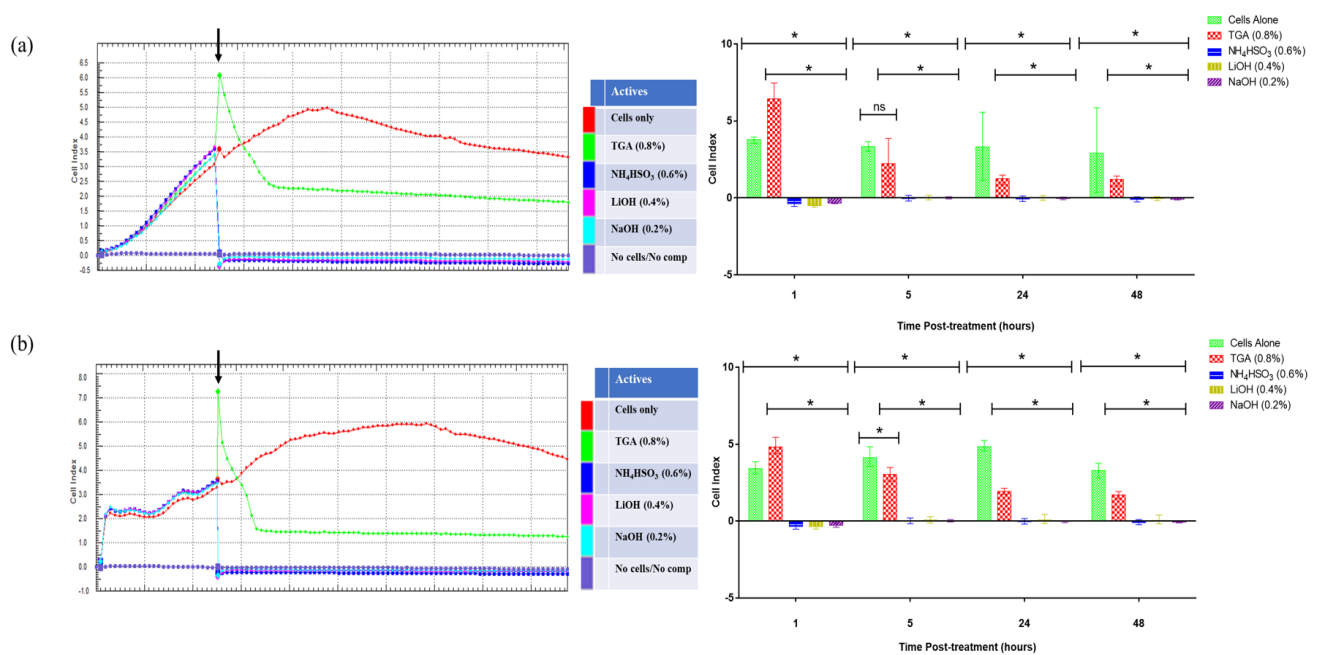
Figure 2. Evaluation of relaxer-induced cytotoxicity by Real-time cell analysis (RTCA). Keratinocytes (A) and fibroblasts (B) were plated onto E-plates, treated with tenth industry concentrations and cellular impedance (at 1-hour intervals) overtime recorded. Bars indicate means and whiskers standard error of means; ns= not-statistically significant and * = p<0.05, significant.

3.3.2. Evaluation of RIC in Real-time using Extracellular Flux analyses

Glycolytic stress and Mitochondrial stress tests were performed in real-time on HaCaTs post-treatment with relaxer actives revealed that treatment with all relaxers resulted in a significant (p≤0.05) reduction in both glycolysis and ATP production (Fig. 3a and b respectively).
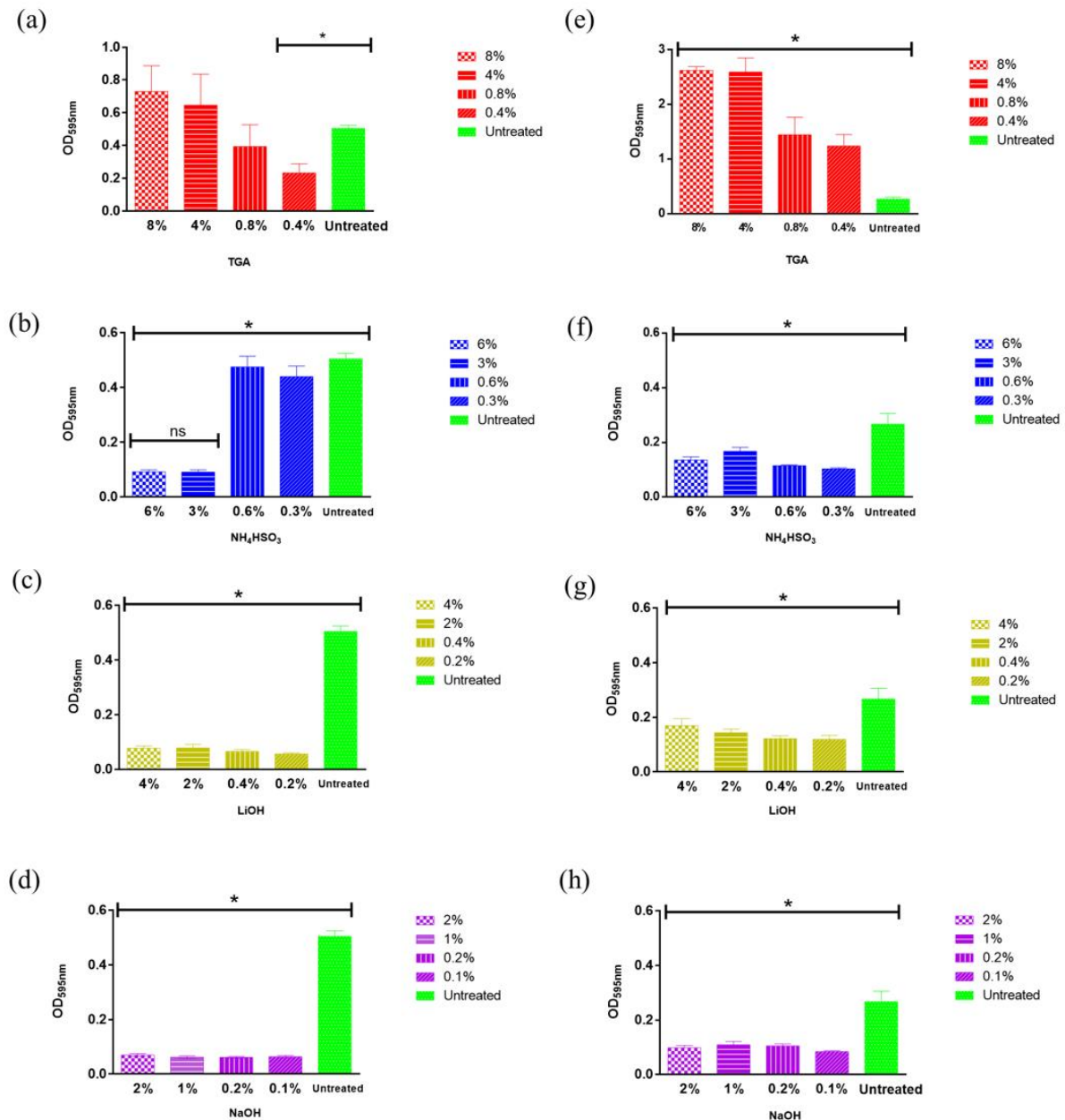
Figure 3. Extracellular flux analysis of HaCaTs in real-time. Cells were plated in 96-well extracellular flux cell culture plates and treated with one-tenth of the approved concentration of relaxer/straightener actives. Glycolytic stress and Mitochondrial stress assay kits were used to measure both glycolysis (A) and ATP production (B), respectively. Error bars indicate standard error of means. * = p≤0.05 indicates statistical significance HaCaTs = Human immortalised keratinocytes.

3.4.   Evaluation of recovery of keratinocytes from RIC

Cell counting kit-8 (CCK-8) proliferation assay was utilised to determine the ability of epidermal cells (i.e., HaCaTs) to recover from the cytotoxic effects of relaxer actives 72 hours (short-term) and 7 days (long-term) post-treatment; and by means of counting the number of events/µl via flow cytometry. Here we show that there were no signs of cell recovery at these two time points as judged by the > 8 and 9-fold decrease in cell viability in both the CCK8 and flow cytometry experiments respectively (Figs. A2a-b).

3.5.   Evaluation of mode of cell death

Annexin V and Propidium iodide staining was used to evaluate the mode of keratinocyte cell death (early and late apoptosis respectively) following exposure to relaxer actives. As shown in Figure 4a-b, all the actives resulted in a significantly lower population of live cells (less than 10 % of the population in all actives). Also, the results suggest that the fraction of the cell population in early apoptotic increased with the different class of hair relaxer/straightener actives, i.e., TGA, NH_4HSO_3, NaOH and LiOH (47.9%, 58.0%, 76.7% and 80.3% respectively) as compared to untreated cells (10.9%). We also observed that the fraction of cells in late apoptosis was minimal (< 20 %) across all treatment groups as well as the untreated group.
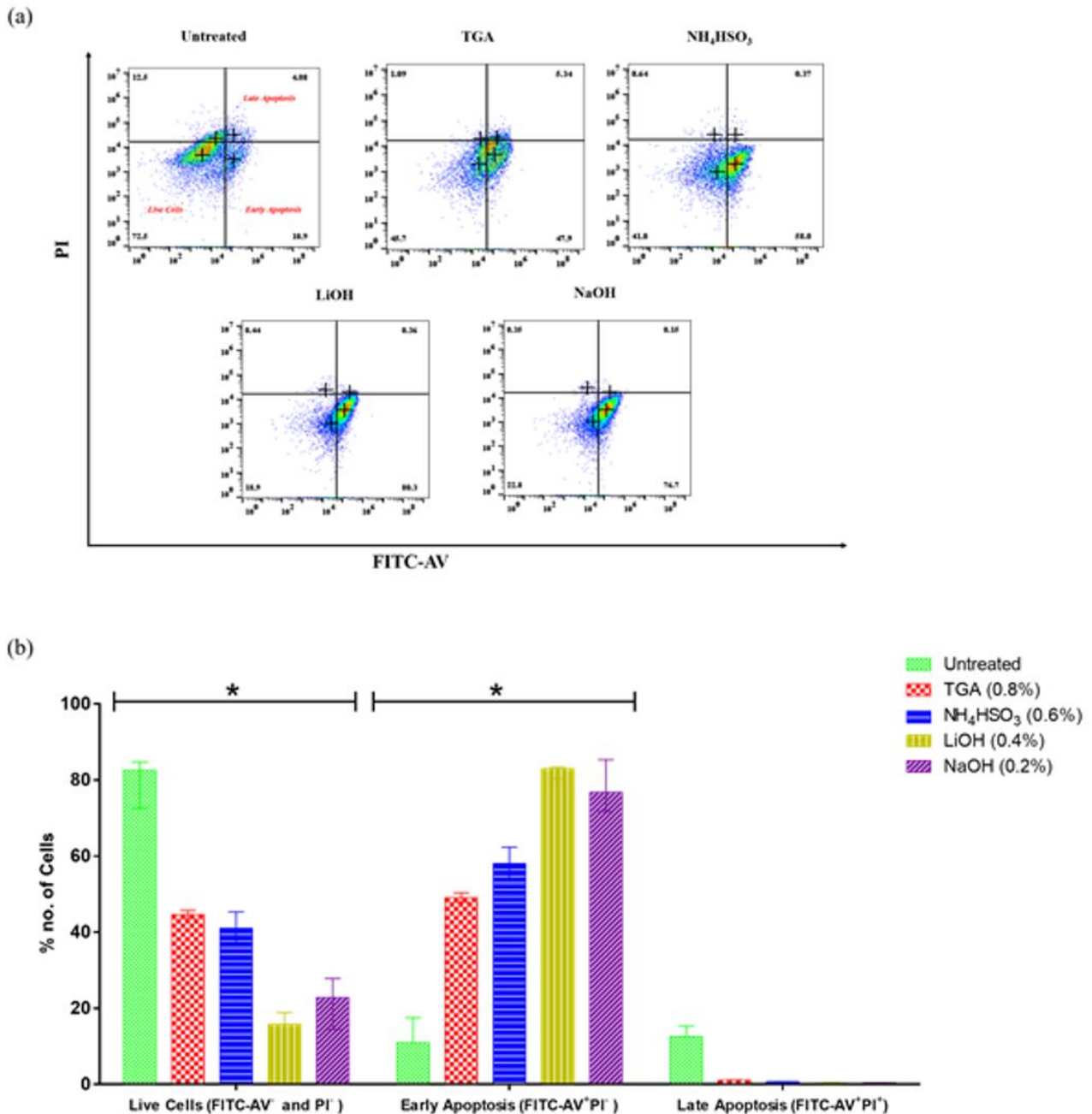
Figure 4. Mode of cell death. HaCaTs were seeded and cultured overnight, followed by treatment with a tenth of approved of maximum concentration of actives. Annexin V (FITC-AV) and Propidium iodide (PI) were used to stain the cells. Events were acquired by the flow cytometer and analysed by using the FlowJo software v9.4.7. (a). Gated live, early apoptosis and late apoptosis populations (b). * = p≤0.05 indicated statistical significance and was tested using Two-way RM ANOVA. HaCaTs = Human immortalised keratinocytes.

4. Discussion

In this study, we used an alternative non-animal *in vitro* monolayer of immortalised skin keratinocytes (HaCaTs) in cosmetic testing [30-33], as well as normal human dermal fibroblasts (NHFs) to evaluate relaxer-induced cytotoxicity. A widely accepted method for end-point cytotoxicity evaluation; MTT, as well as the 'new generation' WST-8 (CCK-8) based assays were employed in this study. Furthermore, potential metabolic perturbations were determined via extracellular flux analysis. We used the same latter technique to decipher pH-dependant metabolic disturbances in real-time, alongside the RTCA for analysis of potential anti-proliferation effects of relaxer actives. Following cytotoxic effects of relaxer actives on both epidermal and dermal cells, it was pertinent to explore potential cellular recovery; both short (72 hours) and long-term (7 days) by means of proliferation assays (CCK-8 and flow cytometry). We also evaluated the mode of cell death via Annexin V and propidium iodide.

We observed a pH dependant cytotoxic effect of relaxer or straightener actives; TGA with the lowest pH being less cytotoxic, whereas NaOH at pH 13 was the most cytotoxic on both epidermal and dermal skin cells. However, keratinocytes were shown to be more susceptible to higher pH relaxer actives, wherein a 5-fold reduction in cell viability was evident. This was in contrast with fibroblasts, where relaxers resulted in 2-fold reduction in cell viability. Such differences may be attributed to the varying pH tolerance, which in turn impacts on cellular functions. Kruse et al., recently showed that keratinocytes proliferate within 6.5-8.5 pH ranges, whereas fibroblasts had a wider tolerance (pH 6.5-11.5) [34]. Studies have also indicated differential pH-dependant cellular phenotypes of keratinocytes over a range of pH; low pH allows for differentiation, mid-range for proliferation, whereas high pH results in a migratory phenotype [34, 35].

This study was designed in a way RIC was quantifiable by both real-time (RTCA and extracellular flux analysis), and an end-point assay (CCK-8 and flow cytometry). The former provided an insight

into the potential real-time cellular perturbations such as proliferation and/or cytotoxicity and metabolic changes resulting from such RIC respectively.

Cellular impedance, as a marker of cell viability and proliferation, can be measured and recorded with an arbitrary unit; cell index (CI,). In this study we saw a rapid surge of cellular impedance within an hour of exposure of both keratinocytes and fibroblasts to TGA, which then significantly decreased with time up to 48 hours. In the mitochondria, thioglycolate can be a substrate for acetyl-CoA synthase, which in turn results in accumulation of 2-mercaptoacetyl-CoA. The accumulation of 2-mercaptoacetyl-CoA then results in the inactivation of ketogenesis enzymes, thereby inhibiting fatty acid breakdown [36]. Thus, the inhibition of fatty acid metabolism may allow cells to effectively utilise available glucose in the medium as the source of energy, hence the acute increase in cellular index following treatment with TGA. Furthermore, Lai et al., demonstrated that TGA alone did not affect cell cycle,[31] but only in combination with UV-B, thereby possibly supporting our observations.

The use of extracellular flux analyses afforded us a real-time evaluation of cellular bioenergetics post-treatment with relaxers. Here, we evaluated the RIC effects via glycolysis and mitochondrial stress tests, using extracellular flux analysis. Both glycolysis, as well as ATP production rates were significantly reduced post-treatment. Interestingly, we observed a pH-dependant reduction in ATP production following mitochondrial stress test of epidermal cells. The possible resultant inactivation of ketogenesis (alternative energy source), may also be attributed to the slightly higher ATP production compared to other actives [36]. On the contrary, the lowest ATP synthesis may result from mitochondrial damage due to increase in free radicals (OH^-) produced by the sodium hydroxide-based relaxer. Also, the water-soluble properties of NaOH can free up OH- groups, thereby resulting in mitochondrial perturbations. This latter phenomenon will need further investigation via more extracellular flux methods.

Having evaluated relaxer-induced cytotoxicity in real-time, it was pertinent to explore the end-point assays, with the aim of further elucidating possible recovery (short/long-term). For this reason, we determined the RIC recovery at 3 days and 7 days post-treatment via CCK-8 and flow cytometry respectively. Treatment with all relaxer and straightener actives showed no recovery at all time-points, which was evident even post-TGA treatment, wherein we had showed increases in cell viability via MTT and RTCA. This indeed alludes to the speculation that relaxers, both permanent and temporary may cause irreversible cellular perturbations *in vitro.*

Stemming from in the lack of cell recovery post relaxer active treatment, we found it pivotal to establish the mode of cell death via well-known markers of early and late apoptosis; Annexin V and propidium iodide. Here we show direct relationship of early apoptosis population of keratinocytes with increasing pH of relaxer actives (that is the higher the pH of relaxer, the higher the frequency of Annexin V^+ only cells (early apoptosis). This observation was coupled by the inverse correlation between the frequency of the late apoptosis (Annexin V^+/PI^+) cell population and relaxer pH. As mentioned previously [36], high pH sodium hydroxide readily frees up its OH- groups, thereby posing possible mitochondrial dysfunction, potentially leading to apoptosis.

5. Conclusions

We showed the cytotoxic effects of relaxer/straightener actives on human-skin derived cells using established and recently developed cell-based assay technologies. Thus, the use of skin cells in conjunction with advanced cell-based technologies could serve as alternatives to laboratory animals for accessing the toxicity margin of hair relaxers, straighteners, and other cosmetics.

Study Limitations: Lack of stratified keratinocyte layers, coupled with the dermal matrices did not allow us to fully appreciate the possible damaging effects of these relaxer/straightener actives. Such include epidermal thinning, penetration to the dermal layer, as well as cell differentiation. Also, the pH of the TGA (pH 2) active is not reflective of the TGA-based relaxers/straighteners (pH 6-7.5).

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the University of Cape Town Health Research Ethics Committee (HREC REF: R023/2019, 16/06/2019).

Informed Consent Statement

Informed consent was obtained from the patients whose discarded skin tissues were used in obtaining human skin-derived fibroblasts.

Data Availability Statement

All the data related to this study are available upon reasonable request from the corresponding author.

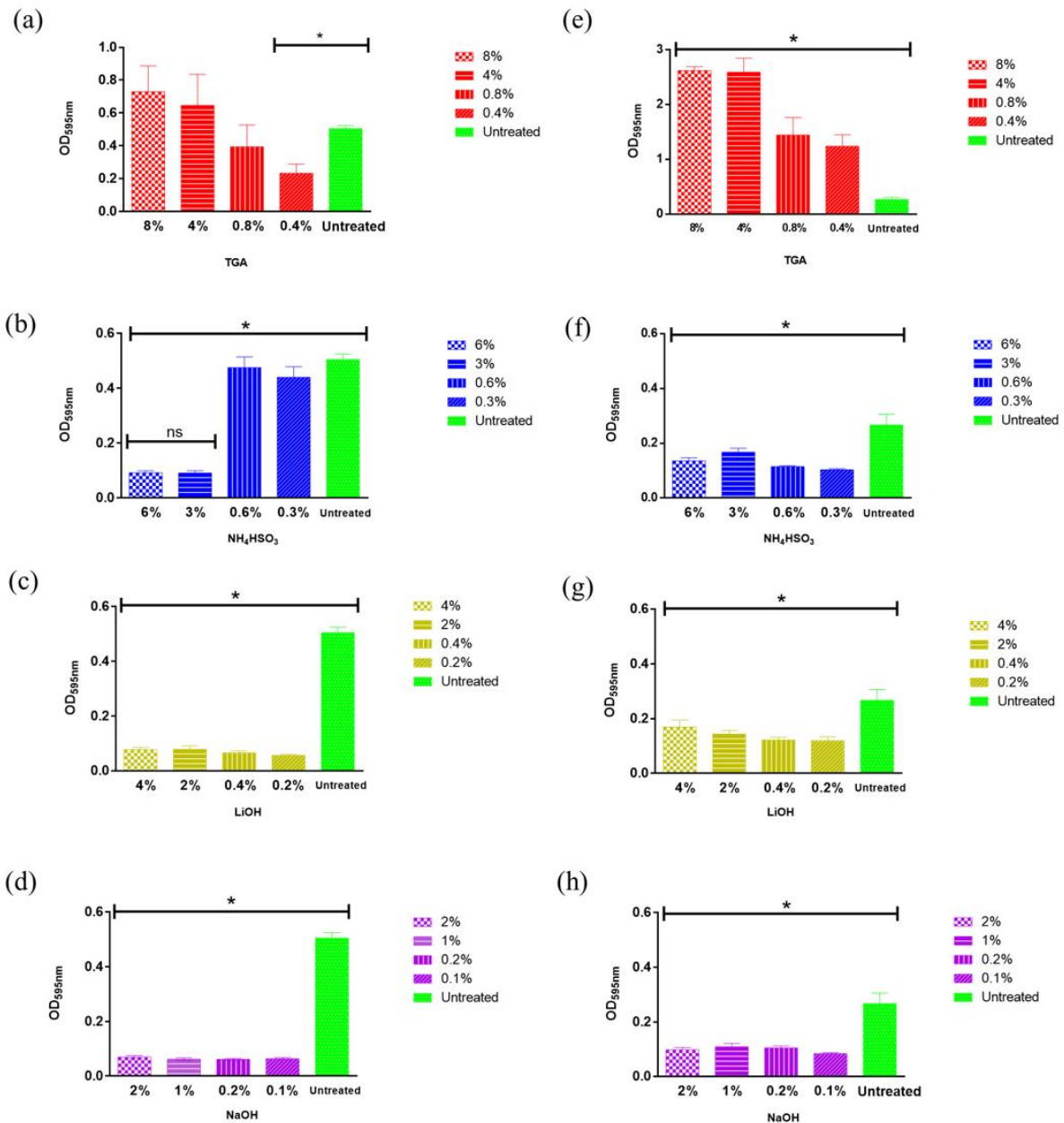**Conflict of Interest**: The authors have none to declare.

Appendix

Figure A1. Evaluation of a dose-dependent relaxer-induced cytotoxicity on HaCaTs (A-D) and NHFs (E-H). Cells were cultured in 96-well plates overnight, treated for 20 mins with relaxer actives at different concentrations and MTT cytotoxicity assay was performed. Error bars indicate standard error of means; * = p≤ 0.05 indicates statistical significance. HaCaTs = Human immortalised keratinocytes; NHFs = Normal human skin fibroblasts.
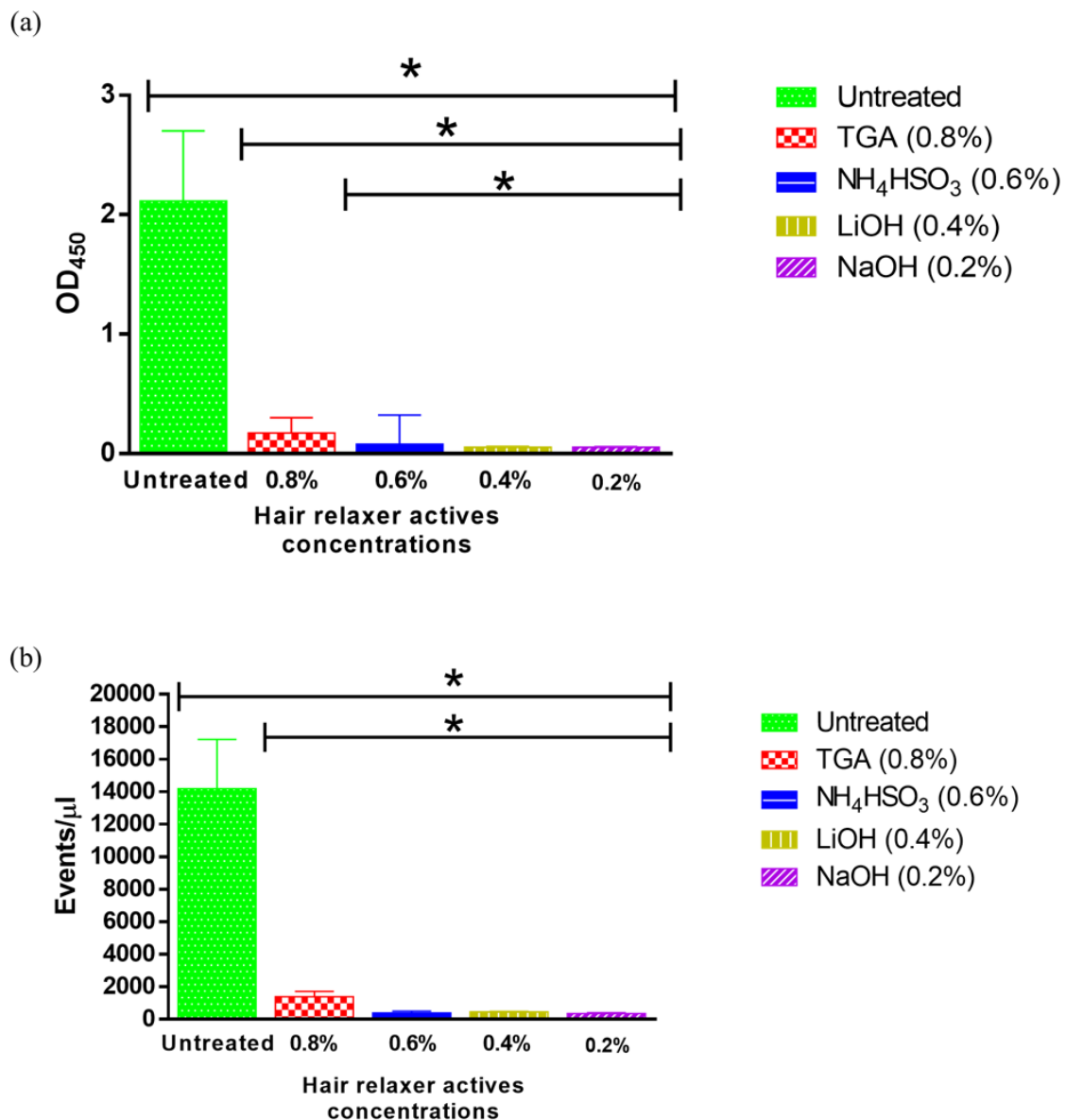
(a)

(b)

Figure A2.   Recovery from relaxer-induced cytotoxicity. HaCaTs were plated and cultured overnight, followed by treatment with a tenth of the maximum approved concentration of actives. Cellular recovery was evaluated 3 days and 7 days post-treatment by means of CCK-8 (a) and flow cytometry (b) respectively. Error bars indicate standard error of means; * = $p \leq 0.05$ indicates statistical significance. HaCaTs = Human immortalised keratinocytes.

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