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VIABILITY OF CULTURED PRIMARY HUMAN SKIN CELLS TREATED WITH 1, 6-HEXAMETHYLENE DIISOCYANATE MONOMER AND ITS OLIGOMER ISOCYANURATE

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A technical report submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in Public Health in the Department of Environmental Sciences and Engineering in the Gillings School of Global Public Health.

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ABSTRACT

Kaitlyn Phillips

Viability of Cultured Primary Human Skin Cells Treated With

1, 6-Hexamethylene Diisocyanate Monomer and Its Oligomer Isocyanurate

(Under the direction of Dr. Leena Nylander-French)

The diisocyanate monomer 1,6-hexamethylene diisocyanate (HDI) and its oligomer HDI isocyanurate are components in sprayed polyurethane coatings. Exposure via the lungs and skin can lead to allergic sensitization and asthma. Research on these compounds has focused on effects of exposure on respiratory and immune cells and variation in gene expression, pathway activation, and mechanisms influencing toxic response. Here, we focused on the toxic effects of HDI monomer and HDI isocyanurate on three types of cultured primary human skin cells, namely keratinocytes, melanocytes, and fibroblasts. To determine the cell-type specific toxicity, we used a luminescent ATP-viability assay. The dose-response data indicated that sensitivity to death varied among the different skin cell types and death by necrosis. The observed variations in toxicity between the HDI monomer and HDI isocyanurate as well as between the cell types may have important implications for developing an adverse effect, for regulatory limits, and for worker safety.

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LIST OF ABBREVIATIONS

ACGIH	American Conference of Governmental Industrial Hygienists
ANOVA	Analysis of variance
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FLG; FLG	Filaggrin gene; filaggrin protein
HDI	1,6-Hexamethylene diisocyanate
LC_{50}	50% of the lethal concentration
MDI	Methylene diphenyl diisocyanate
NCO	Nitrogen carbon oxygen
NEAA	Non-essential amino acids
NIOSH	National Institute for Occupational Safety and Health
OEL	Occupational exposure limit
OSHA	Occupational Safety and Health Administration
PEL	Permissible exposure limit
PPE	Personal protective equipment
REL	Recommended exposure limit
RLU	Relative light units
STEL	Short-term exposure limit
TDI	Toluene diisocyanate
TLV	Threshold limit value
TWA	Time weighted average
Th1	Type 1 T helper cell
Th2	Type 2 T helper cell
ex/em	Excitation wavelength in nanometers/emission wavelength in nanometers
rATP	ribonucleotide adenosine triphosphate

INTRODUCTION

Isocyanate Chemical Structures and Characteristics

Isocyanates are a group of small reactive chemicals (with N = C = O functional groups) that are used in polyurethane products such as foams and coatings [1,2]. Classification of individual compounds depends on the number of NCO-groups present; diisocyanate monomers such as HDI are comprised of two NCO-groups, and poly-isocyanates such as HDI isocyanurate are comprised of multiple NCO-groups (Figure 1) [2]. Isocyanates react with compounds containing active hydrogen atoms (i.e., polyols or amines) to form polyurethane or other complex polymeric products [2–4]. Figure 2 illustrates the formation of the urethane bond.

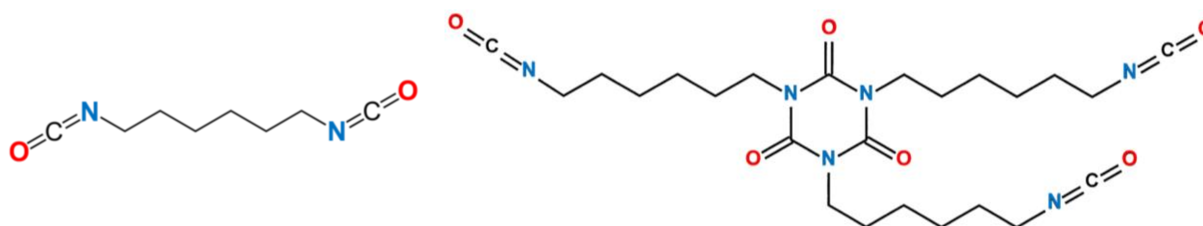

Figure 1: The structures of 1,6-hexamethylene diisocyanate (HDI) (left) and HDI isocyanurate (right).

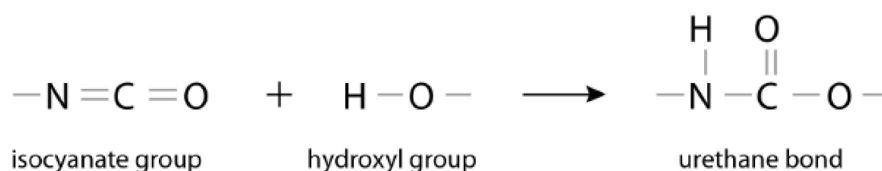

Figure 2: Formation of the urethane bond (courtesy of Tosoh Asia Pte. Ltd).

Isocyanates in the Workplace

The use and production of isocyanates doubled between 2004 and 2014 and is predicted to continue to increase at an estimated rate of 5% a year [5,6]. Isocyanates used in the automotive-repair industry are primarily aliphatic isocyanates, particularly HDI [7]. Aromatic isocyanates include toluene diisocyanate (TDI) and methylene diphenyl diisocyanate (MDI) and are mainly used for foam production, elastomers, and coatings[8]. Isocyanates are also found in specialty glues and paints, so there is a potential for non-occupational exposure in general population [9].

Polyurethane paints commonly used in the automotive industry contain monomeric and polymeric species of HDI [7]. Polymeric diisocyanates like HDI isocyanurate are less volatile than smaller diisocyanates like HDI monomer, and may as a result remain on the skin surface longer and potentially elicit stronger toxic, adverse responses than the monomer [10]. Exposure to isocyanates can damage or irritate skin and mucous membranes, or cause allergic sensitization [11].

Regulatory Standards

There are fewer regulatory standards for poly-isocyanates than for monomeric isocyanates, despite the fact that poly-isocyanates are the major source of isocyanate exposure in workplaces [2]. Current NIOSH documentation regarding HDI monomer exposure shows a National Institute for Occupational Safety and Health (NIOSH) time-weighted average (TWA) recommended exposure limit (REL) of 0.005 ppm for an 8-h work day, and an Occupational Safety and Health Administration (OSHA) 10-minute ceiling permissible exposure limit (PEL) of 0.020 ppm. This means that an employee can be exposed to 0.020 ppm HDI monomer, and no higher, for a maximum period of 10 minutes during an 8-h work day [12].

The American Conference of Governmental Industrial Hygienists (ACGIH) has set an 8-h threshold limit value (TLV) of 34 mg/m^3 for HDI monomer and the state of Oregon has set an

TWA occupational exposure limit (OEL) of 500 mg/m^3 for HDI monomer [13,14]. These levels were set in an effort to prevent sensitization, but they are not necessarily sufficient to protect workers who have already been sensitized. Bayer offers some guidelines about HDI poly-isocyanates, but nothing specifically for HDI isocyanurate. Bayer recommends a TWA of 500 μg/m^3 for HDI poly-isocyanates during an 8-h work day [15].

Exposure Routes

Exposure may occur via inhalation or skin exposure. Factors such as personal protective equipment (PPE) used, number of exposure periods per day, and duration of exposure are common causes for exposure variability [16].

Inhalation Exposure

Inhalation has been considered the primary route of exposure to isocyanates, and as such, research and regulation have focused heavily on inhalation exposures [2]. When breathing-zone concentrations of isocyanates for auto-shop workers were assessed, the geometric mean of HDI isocyanurate exposure was observed to be higher than any of the other analytes [17]. Additional analysis of the personal breathing-zone samples in spray-painters indicated that there was significant variation in composition of isocyanates among the samples collected, demonstrating that current short-term exposure limits (STEL) may not accurately reflect exposures that occur in the workplace [18]. Respirators with a protection factor of 25 or greater are required to protect the workers whose personal breathing zones were monitored, and the most commonly used half-face respirators (with a protection factor of 10) do not suffice [19]. In general, occupational exposures to airborne isocyanate have been reduced through improved engineering controls, safety, and the use of less volatile isocyanates like poly-isocyanates [20]. Despite this, isocyanate-induced asthma

continues to occur even in work settings where measured respiratory exposures are non-detectable, but where there is an opportunity for skin exposure to occur [18–22].

Skin Exposure

Skin exposure may occur when airborne vapor, aerosols, and/or particles contact the skin via deposition on unprotected skin, or may result from unintended penetration of protective equipment [7,21,23–25]. In a recent review, Redlich *et al* concluded that skin exposure takes place frequently despite the use of PPE [26]. There is considerable variation in PPE usage between workers [27]. A majority of workers (69%) reported always using gloves, but there was a large amount of variation between type of glove used (*i.e*., latex or nitrile) [27,28]. Fent *et al*., in a study of automotive spray-painters, observed that skin concentrations of isocyanates were significantly higher in painters who did not wear coveralls or gloves [29]. HDI isocyanurate was the primary species measured in the skin, regardless of PPE, with a 95% detection rate. Analyte-specific breathing-zone concentration and paint time were the most significant factors contributing to skin exposure [29].

Previous studies have demonstrated that HDI isocyanurate penetrates skin better than HDI monomer and that HDI isocyanurate makes up a greater percentage of exposure than the HDI monomer [10,30]. Therefore, automotive spray-painters may be exposed to high doses of HDI isocyanurate via skin exposure. Results from animal and human studies have indicated that less than 20% of isocyanates will leave the skin through evaporation [10,31]. From this, we can conclude that skin exposure to isocyanates is significant even when accounting for evaporation. Using an equation from Walker *et al.* and worker data, it was estimated that it would take between 6 and 40 minutes of exposure at levels present within this specific auto-body shop to achieve a total body burden equivalent to the ACGIH TLV for HDI monomer inhalation exposure [10,32]. Skin

exposure could comprise the bulk of exposure for workers exposed to isocyanates, but skin exposure was not accounted for when the major regulatory limits were developed and set.

Health Effects

The NIOSH publication, *Preventing Asthma and Death from Diisocyanate Exposure,* lists potential health effects of isocyanate exposure as contact dermatitis, skin and respiratory tract irritation, immune sensitization, occupational asthma, and, occasionally, hypersensitivity pneumonitis [33]. Occupational asthma is the most common health outcome as a result of isocyanate sensitization, and death from severe asthma as a result of sensitization has been reported [25,33–35]. The estimated prevalence of occupational asthma in workers exposed to isocyanate is 1% - 20% [33,36]. Contact dermatitis has also been linked to sensitization. Typically, sensitization will take place over months or years; however, as few as one or two exposures can be sufficient. After sensitization has taken place, a very small exposure can be enough to trigger a dramatic and potentially deadly response [11,12,37].

The NCO-group can react with nucleophiles that are commonly in carrier proteins like blood albumin. Because of its abundance in blood, albumin is the main protein carrier of isocyanates *in vivo* [38–40]. Several other peptides and proteins found in cells, serum, and skin have also been observed to bind with isocyanates [38,39]. HDI monomer has been observed to react with glutathione *in vitro* to form a conjugate [41]. The significance of isocyanate reactivity with proteins is that it can act as a hapten when covalently bound to a protein or macromolecule and, subsequently, elicit an immune response [38,39]. Haptenization is important for immune recognition and the development of allergy [41].

Wisnewski *et al.* observed that sub-cytotoxic concentrations of HDI monomer formed micelles on the surface of *in-vitro* human airway epithelial cells that appeared to be taken up by

the cells over several hours post-exposure [42]. They also documented susceptibility of airway epithelial cell proteins to isocyanate conjugation[42]. Verstraelen *et al.* investigated alterations in gene expression of *in vitro* human alveolar epithelial cells after exposure to a variety of sensitizing chemicals, including HDI monomer[43]. They observed enhanced gene expression of proteins associated with immune response and function, but not specifically with respiratory sensitization. HDI is a known respiratory sensitizer, but the specific role of airway epithelial cells is unclear [41,44]. Airway epithelial cells may play a role in the human respiratory immune response. Human and animal studies indicate potential gene profiles and biological pathways that are activated during exposure, as well as the disease progression of respiratory sensitization, but additional research is required to understand the role epithelial cells play in the respiratory immune response [3,40,41,43–47]

Sensitization in Animal Studies

Animal studies have provided evidence that skin exposure can provide a route for respiratory and allergic sensitization [20,26,31,48–51]. Studies have shown that skin may be a more effective sensitization route than inhalation exposure [45,49] and result in airway sensitization when followed by a subsequent inhalation exposure [25,45,47,51]. Several animal studies have shown that only one or two skin exposures at low isocyanate concentrations could induce sensitization [48,49,51].

Cell Studies

Genes and pathways influencing sensitization and other health effects have been investigated in *in vitro* cell-culture studies. A gene that may contribute to increased exposure dose and development of adverse health effects is the epidermally expressed filaggrin gene (*FLG*). Loss of function mutations in the filaggrin gene can cause atopic dermatitis, which

contributes to skin barrier function [52]. Filaggrin loss of function mutations have been significantly associated with atopic disease, asthma, and allergies [52,53]. Additionally, disruptions such as atopic dermatitis in the skin barrier predispose the skin to being penetrated by external chemicals and pathogens [54]. Atopic dermatitis is one of the most common chronic inflammatory skin diseases, and its overall prevalence is increasing [55]. It is estimated to affect over 15% of children and between 2-10% of adults in industrialized countries [55]. Kabashima *et al.*, when investigating the pathogenesis of atopic dermatitis, observed that while a single hapten exposure provoked a T helper type 1 cell (Th1) response, repeated exposure shifted the response towards T helper type 2 cell (Th2)-dominated responses [54]. Barrier dysfunction may predispose the skin to Th2 conditions due to the activation of keratinocytes, which favor a Th2 response [54]. Th2 cytokines will decrease filaggrin expression by keratinocytes, suggesting that Th2 conditions will lead to further barrier dysfunction [54]. De Benedetto *et al.* came to a slightly different conclusion [53]. They reported that in individuals who already had atopic dermatitis, initial exposure to an allergen induced a Th2 response via the exposed keratinocytes; which was amplified with future exposures [53]. Disruptions in the barrier function of the skin may predispose an individual to receiving a higher than average dose from an average exposure. This chain of events, namely exposure, followed by an atopic inflammatory response, and resulting disruptions in the skin barrier leads to the development of a positive feedback loop leading to more disruption and more exposure. This amplified exposure to haptens leads to the development of an immune response, which may result in isocyanate induced asthma.

There have been several studies investigating the role of DNA methylation status in response to isocyanate exposure [22,56]. Ouyang *et al*. concluded that there were several genetic associations with individuals who were either protected or especially susceptible to sensitization

[56]. Subjects with isocyanate-induced asthma had higher levels of IFN-gamma promoter methylation, but the ultimate role of increased methylation was unclear. Nylander-French *et al*. suggested that DNA methylation may affect gene expression of proteins involved in HDI mass transport, permeation, and metabolism [22]. They also suggested that methylation may mediate individual responses by modifying levels of exposure and cellular responses [22].

GOAL AND SPECIFIC AIMS

In this study, my goal was to investigate differences in toxicity and mechanism of cell death caused by HDI monomer and its oligomer, HDI isocyanurate, exposure in cultured normal human skin cells; specifically, keratinocytes, fibroblasts, and melanocytes. Previous studies have shown that HDI monomer, oligomers, and other isocyanates can have a variety of effects on cultured cells, cancer cell lines, or animal models [7,25,38–41,43,48,49,57–59]. However, only in a few of the studies cell type-specific toxicity in cultured primary human cells were investigated or the mechanism of cell death identified [40,42,43,50,59].

Cancer cell lines have many mutations that may cause them to respond to insult differently than normal cells [60]. As a result, experiments using cancer cell lines are not as relevant to human exposures. Cultured primary human skin cells are more challenging to isolate and have a limited lifespan, but generally respond similarly as the same cells *in vivo*. It is important to use primary human skin cells when performing toxicology studies because they because they are more relevant to human exposures.

This study was designed to:

1. Quantify LC_{50} values for HDI monomer and HDI isocyanurate in cultured human skin cells.
2. Investigate the mechanism of cell death in cells exposed to HDI monomer and HDI isocyanurate using assays that measure the kinetics of cell death and caspase 3/7 activity.

METHODS

Human Primary Skin Cell Culture Conditions

Primary skin cells were isolated from neonatal foreskin obtained from the University of North Carolina Memorial Hospital, Chapel Hill, NC. Unidentified tissues are considered medical waste and thus were exempt from University of North Carolina Institutional Review Board's approval (IRB exemption Study #10-1251). Keratinocytes and melanocytes were isolated from the epidermis and fibroblasts from the dermis of four to seven individuals using a method similar to Basic Protocol 1 in "Isolation, Culture, and Transfection of Melanocytes" by Godwin *et al* [61]. Fibroblasts isolated from neonatal foreskin were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% Cosmic calf serum (Hyclone GE Healthcare Life Sciences, Logan Utah), 1X NEAA (non-essential amino acids; Gibco), and Glutamax™ (Gibco). Melanocytes and keratinocytes were cultured in DermaLife Basal Medium (Lifeline Cell Technology, Frederick, MD) supplemented with LifeFactors DermaLife M or LifeFactors DermaLife K (Lifeline Cell Technology) growth factors, respectively. GlutaMax™ was substituted for the provided L-glutamine in LifeFactors M and K. The condition of the cultures was assessed daily or every other day and cell cultures were split (1:3 to 1:5) whenever they reached 70% confluence. Cells were passaged in this fashion until they began to show signs of differentiation or senescence. For optimal culture growth, cells were allowed to recover for 24 h from thawing or passaging before use in an experiment or medium was changed 18 – 24 h before plating for an experiment. See Table 1 for a description of specific cells used.

Table 1: The cell types used in the study. The number of experiments performed on a given cell type is indicated by parentheses. The notation for specific cell strain is as follows: the first letter, K, M, or F, corresponds to keratinocytes, melanocytes, and fibroblasts. The three-digit number identifies skin samples.

HDI Monomer			**HDI Isocyanurate**		
Keratinocytes	Melanocytes	Fibroblasts	Keratinocytes	Melanocytes	Fibroblasts
K051 (n=4)	M051 (n=5)	F051 (n=8)	K051 (n=4)	M051 (n=4)	F051 (n=4)
K065 (n=5)	M065 (n=6)	F065 (n=4)	K065 (n=4)	M065 (n=4)	F065 (n=5)
K066 (n=4)	M066 (n=5)	F066 (n=5)	K066 (n=3)	M066 (n=4)	F066 (n=5)
K070 (n=5)	M070 (n=6)	F070 (n=7)	K070 (n=4)	M070 (n=4)	F070 (n=4)
				M092 (n=1)	
				M093 (n=1)	
				M098 (n=1)	

Isocyanate Treatment of Primary Skin Cells

Logarithmically growing cells (~70% confluent) were plated in black 96-well tissue culture plates (Greiner Bio-one, Germany) at a concentration of approximately 7,000 cells per well for fibroblasts and 12,000 – 15,000 cells per well for keratinocytes and melanocytes. After plating, cells were allowed to re-attach and recover overnight prior to treatment. Cells were rinsed with 200 µL of cell-appropriate basal medium (Gibco or Lifeline Cell Technology) and then exposed to 200 µL of dilutions of either HDI monomer or HDI isocyanurate. The isocyanates were initially dissolved and diluted in dimethysulfoxide (DMSO; Sigma-Aldrich, St. Louis MO) and then serially diluted in DMSO and then basal medium (final concentration 0.05% DMSO). Basal medium was used instead of supplemented medium for treatment in order to prevent the isocyanate to react with the protein and serum supplements before contact with cells.

Because of its reactivity to air and water, isocyanate dilutions were prepared immediately before treatment in glass vials with Teflon™ cap liners. The concentration of the stock HDI monomer solution in DMSO was 6 M (1.51 g isocyanate in 0.5 mL DMSO); and 0.1 M for the stock HDI isocyanurate solution (0.2 g isocyanurate in 4 mL DMSO). The stock HDI monomer or HDI isocyanurate solution was then serially diluted into DMSO, producing concentrations ranging from 0.06 M to 6 M HDI monomer in DMSO or 0.001 M to 0.1 M HDI isocyanurate in DMSO. These solutions were then diluted 1:1000 into 2 mL of basal media so that the final DMSO concentration was 0.05%. This produced a working concentration range of 30µM to 3000 µM HDI monomer or 20 µM to 0.5 µM HDI isocyanurate in basal media. Specifically, cells were exposed to (1) 30 µM, 100 µM, 200 µM, 300 µM, 600 µM, 1200 µM, 2400 µM, or 3000 µM of HDI monomer or (2) 0.5 µM, 1 µM, 2 µM, 5 µM, 10 µM, or 20 µM of HDI isocyanurate. A 4-h exposure period was used to simulate a worker's average daily exposure period. At the end of the 4-h exposure, the exposure media was removed, 200 µL of supplemented growth media was added into each well, and cells were cultured overnight (18 h) to allow for cellular recovery to occur.

Cell Viability

Approximately 18 h after the exposure, cell viability was measured using the Promega CellTiter Glo 2.0 ATP luminescence assay according to manufacturer's instructions (Promega, Madison, WI) [62]. This assay, after adding the single reagent, results in cell lysis and then generates a luminescent signal that is proportional to the amount of ATP present. The amount of ATP present is proportional to the number of cells present in culture. We included an 18-h recovery period post treatment so that the amount of ATP present was more proportional to the amount of living cells, as the treatment may have inhibited ATP production but not killed some

cells. Because the half-life of the assay reagent is greater than five hours, its stability helps avoid errors that may be present in other methodologies used to measure ATP [62]. We chose to use Promega CellTiter Glo 2.0 instead of the common MTT assay for several reasons. The MTT assay uses MTT tetrazolium compound to measure the number of viable cells, and has been a widely-accepted method since the 1980's. The MTT compound is directly added to cells in culture and incubated for 1 to 4 h. Viable cells will reduce tetrazolium into a precipitate that accumulates inside the cells. A second reagent is added to lyse the cells and solubilize the precipitate. The absorbance is measured to estimate viable cell number. However, the MTT assay is less sensitive than fluorescent or luminescent methods for measuring viable cell number, and many chemical compounds are known to interfere with this assay [63]. The MTT reagent is also cytotoxic, so longer incubation times that would otherwise enhance sensitivity are limited. The detection sensitivity also varies considerably between different cell types and the metabolic activity of the cells. Results from an MTT assay will not produce as accurate a dose-response curve as would results from a more sensitive assay, such as CellTiter Glo.

Cells were rinsed once with the appropriate basal medium and then assayed using 100 µL of basal medium and 100 µL of CellTiter Glo 2.0 luminescence reagent. The plates were then shaken for two minutes and incubated for ten minutes inside the plate reader at room temperature (~ 22°C) prior to reading with the GloMax microplate reader (Promega). Luminescence was measured in RLU (relative light units). Black 96-well plates were used to minimize spill-over effect of luminescence from the adjacent wells during reading. A standard curve of rATP (ribonucleotide adenosine triphosphate) dissolved in basal medium was performed to determine the linear range of the luminescence (0 – 6 µM rATP). Previously frozen aliquots of diluted rATP were further diluted with the appropriate basal media and mixed with an equal volume of

the CellTiter Glo 2.0 luminescence reagent in the 96-well plate. The standard curve included samples of 0, 0.3, 1, 3, 6, and 100 µM rATP. A graph of the linear values (0 – 3 µM rATP) was then produced within Microsoft Excel, and the r-squared value of a linear trend line for the data was determined to ensure the standard curve was linear. All r-squared values were greater than 0.9, with values ranging from 0.95 to 0.99 most common.

The average RLU value from triplicate samples was recorded and background RLU value from the wells containing only basal medium (no cells) were subtracted from all triplicate sample RLU averages. Viability of the isocyanate-treated cells was compared to the 0.05% DMSO control. To calculate the LC_{50} (concentration that kills 50% of cells), the percent viability was plotted against log-transformed (base 10) exposure concentrations and the 50% decrease in viability was determined from the linear portion of the curve using the linear regression equation of the line. Standard error was determined by calculating the standard deviation for all LC_{50} values for one cell type, and dividing the standard deviation by the square root of the number of experiments. All statistical analyses were performed using R version 3.3.0. P-values comparing differences in responses to the test chemical between cell types were produced using paired t-tests. We used ANOVA tests to investigate differences in responses between the three cell types to HDI monomer, differences in responses between the three cell types to HDI isocyanurate, and differences in responses between cells used within a specific cell type such as keratinocytes (i.e. comparing K051, K065, K066, and K070 responses to HDI isocyanurate) (see Table 1).

Cell Death

The toxicity assay CellTox Green Assay (Promega) was used to measure the kinetics of cell death. This assay measures cell death by changes in membrane integrity by detecting a fluorescent DNA intercalator binding to DNA released from dead cells. Since the fluorescent

complex is stable over time, cell death can be quantified cumulatively [64]. Cells were exposed to isocyanates and the fluorescent complex concurrently and the kinetics of cell death followed over time. Cells were plated in a black 96-well plate at a concentration of 15,000 cells (keratinocytes and melanocytes) per well and left to attach and recover overnight. HDI monomer and HDI isocyanurate dilutions were prepared according the same dilution protocols outlined above, and CellTox Green reagent was diluted into the cell medium at a concentration of 1:1000. Cells lysed with supplied CellTox Green Lysis Solution acted as 100% toxicity control. Cells were rinsed with 200 µL basal medium per well immediately before treatment. Then, 100 µL of solution with both diluted isocyanates (prepared as before) and fluorescent reagent (specific to CellTox Green) was added to each well and fluorescence was measured every 15-30 min for three hours. Plates were read at 485 excitation wave-length in nanometers (ex) /520 emission wavelength in nanometers (em) using a BioTek Cytation 3 microplate reader after shaking for one minute at 700 rpm. Cultures were returned to incubator between the readings.

To investigate the mechanism of cell death, we performed the ApoTox Glo Assay (Promega). This is a 3-in-1 assay that measures both live and dead cells concurrently using fluorescent substrates. Cell viability is measured via a protease detected within intact viable cells. Dead cells are quantified by detection of a dead cell-specific protease activity released into the medium. Apoptosis is measured by quantification of caspase 3/7 activities in lysed cells using luminescent substrates. The resulting luminescent signal is proportional to the amount of caspase activity [65].

There were two reagents prepared for the ApoTox Glo Assay, the viability/cytotoxicity combined reagent solution and the Caspase-Glo® 3/7 reagent. The viability/cytotoxicity reagent is prepared by mixing the GF-AFC substrate and bis-AAF-R110 substrate into 2.0 mL of assay

buffer. This was used immediately or stored at 4°C and used within seven days as per manufacturer instructions. The Caspase-Glo® 3/7 reagent is the reagent measuring apoptosis. The Caspase-Glo® 3/7 reagent was prepared by transferring the contents of the Caspase-Glo® 3/7 buffer bottle into the amber bottle containing Caspase-Glo® 3/7 substrate. The Caspase-Glo® 3/7 reagent was mixed by swirling or inverting the contents until the substrate is thoroughly dissolved to form the Caspase-Glo® 3/7 reagent (~20 seconds). The Caspase-Glo® 3/7 was used immediately or stored at 4°C and used within seven days as per manufacturer instructions.

Cells were prepared for the assay by plating at 17,000 cells per well in a 96-well plate. The test chemical concentrations used were 300 µM and 800 µM HDI monomer and 5 µM and 10 µM HDI isocyanurate. These doses were used because they represent the middle and upper range of the LC_{50} values calculated previously. The necrosis control was 50 µM ionomycin in basal media and the apoptosis control was 10 µM staurosporine in basal media. Cells treated with basal media only and cells treated with basal media plus DMSO were positive controls. Cells were treated for 4 h.

After the treatment period, 20 µL viability/cytotoxicity reagent was added to each well, briefly mixed by orbital shaking (300 – 500 rpm for 30 seconds) and incubated at 37°C for 45 min. Fluorescence was measured at 400Ex/505Em (viability) and 485Ex/520Em (cytotoxicity) with the BioTek Cytation 3 microplate reader.

To measure the presence of apoptosis, 100 µL of Caspase-Glo® 3/7 reagent was added to each well and briefly mixed by orbital shaking (300 – 500 rpm for 30 seconds). Plates were incubated for 95 min at room temperature and luminescence was measured with the integration time set between 0.5 – 1 second with the BioTek Cytation 3 Plate reader.

Statistical Analyses

Standard deviation and standard error were calculated to compare LC_{50} values. Paired t-tests and ANOVA tests were performed to compare cell viability between the different cell types exposed to isocyanates and to investigate differences between individual donors of keratinocytes, melanocytes, or fibroblasts. All statistical analyses were performed using R version 3.3.0. Graphs were produced within Microsoft Excel or R version 3.3.0.

RESULTS

Cell Viability

A rATP standard curve was prepared with each Celltiter Glo 2.0 assay performed. This was done to ensure the reagent was performing consistently between experiments, and to determine the linear range of the assay. The optimal number of cells per well was determined by comparing RLU values to the standard curve to ensure it was in linear range. The standard curve included concentrations of 0, 0.3, 1, 3, 6, and 100 µM rATP. A graph of the linear values (0 – 3 µM rATP) was then produced within Microsoft Excel, and the r-squared value of a linear trend line for the data was determined to ensure the standard curve was linear. All r-squared values were greater than 0.9, with values ranging from 0.95 to 0.99 most common.

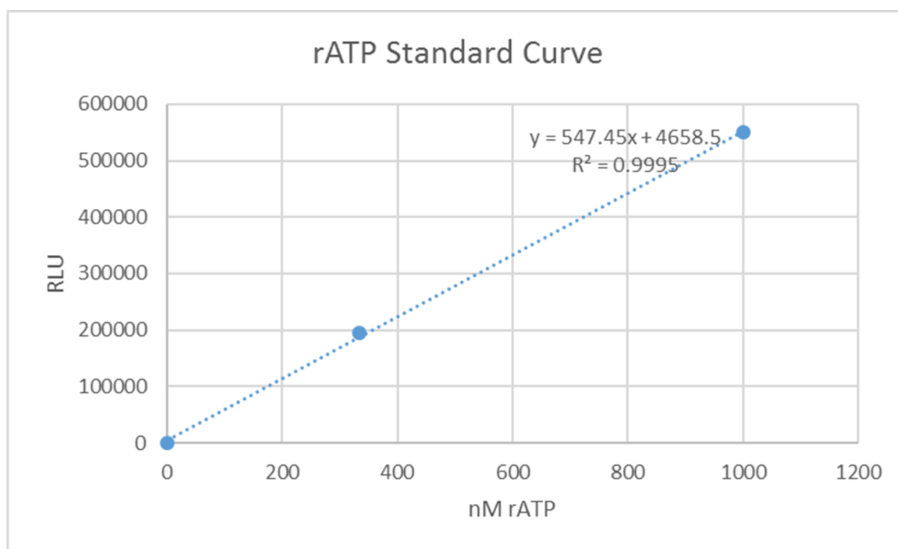

Figure 3: A representative rATP standard curve.

We used the CellTiter Glo 2.0 results to calculate LC_{50} values, since this assay had the most sensitive and reproducible dose response curve. In CellTiter Glo 2.0, cells are treated and then allowed to recover in supplemented media for 18 hours before being rinsed and being exposed to the reagent in basal media [62]. Then, the reagent in CellTiter Glo 2.0 lyses the remaining living cells and reacts with the ATP that was inside those cells. This could give inaccurate readings if cells had stopped producing ATP but not died. We attempted to minimize this effect by allowing cells to recover for 18 hours between treatment and measurements.

We observed several key differences when investigating cell viability. HDI isocyanurate was 20 – 100 times more toxic than HDI monomer ($p < 0.01$), depending upon cell type (Figure 4). We also observed a statistically significant difference in cell viability between cell types in response to HDI monomer exposure ($p \leq 0.02$) (Figure 4). Fibroblasts were significantly more susceptible to HDI isocyanurate toxicity than keratinocytes ($p < 0.006$) or melanocytes ($p < 0.003$) (Figure 5). There was no significant difference between the fibroblast and melanocyte response to HDI isocyanurate ($p = 0.8$) (Figure 5).

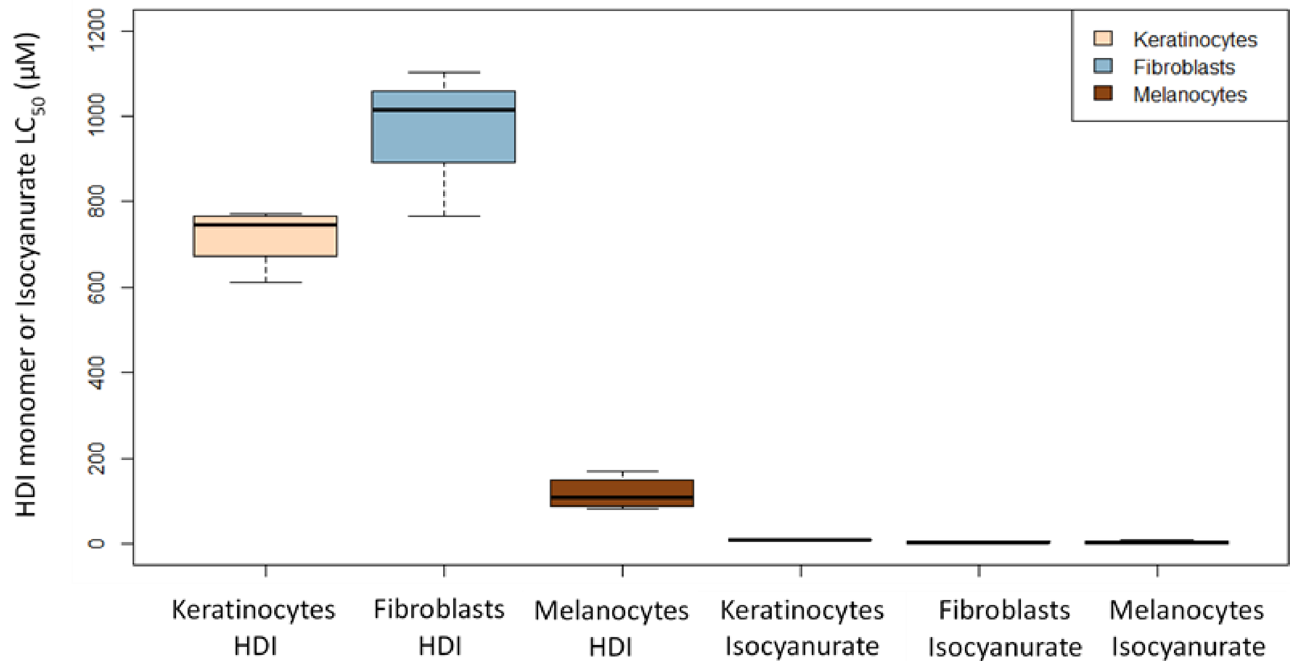

Figure 4: The average LC_{50} levels for cells exposed to HDI monomer or HDI isocyanurate (n = 4-7 per cell type; see Table 1).

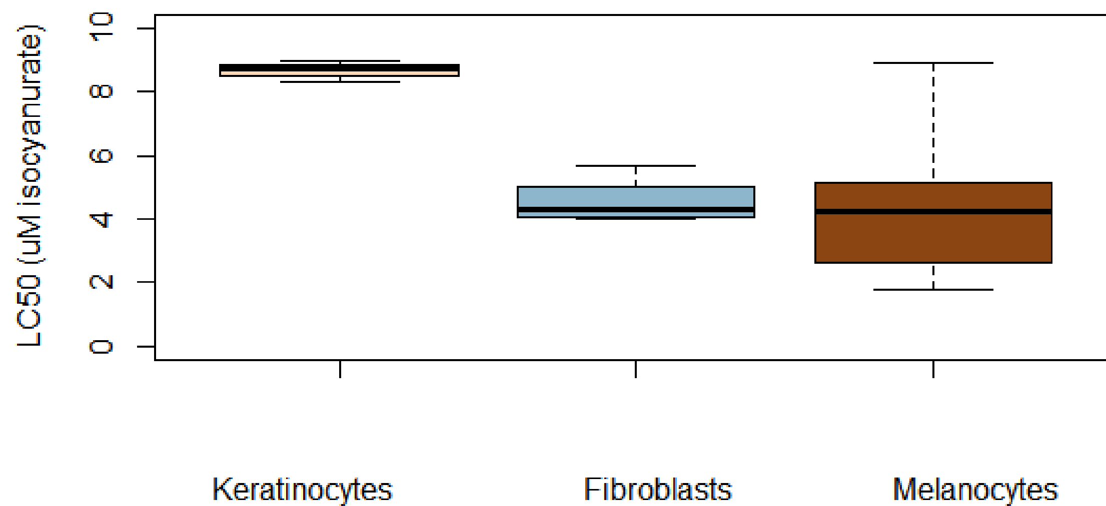

Figure 5: The average LC_{50} levels for the different cell types when exposed to HDI isocyanurate (n = 4-7 per cell type; see Table 1).

ANOVA was used to compare cell viability between the different cell types exposed to HDI monomer or HDI isocyanurate. A highly significant difference ($p<0.001$) was observed between the cell types when exposed to either of these compounds. These results support the observed significant difference in LC_{50} values between the different cell types when exposed to either of these compounds. Melanocytes were the most sensitive cell type to HDI monomer exposure. Keratinocytes were the least sensitive cell type when exposed to HDI isocyanurate while fibroblasts and melanocytes had approximately similar response.

In addition, ANOVA tests were performed to investigate differences between individual donors of keratinocytes, melanocytes, or fibroblasts. However, no significant differences were observed between individual donors for any of the cell types; further confirming our observation that there was no significant difference in toxicity between individual donors' primary cultured human skin cells (keratinocytes, melanocytes, or fibroblasts).

A second cell viability assay was performed with the ApoTox Glo assay. The viability results were confirmatory of the results observed from the CellTiter Glo 2.0 assay (data not shown).

Cell Death

We utilized the CellTox Green Assay (Promega) to investigate the kinetics of cell death. Rapid cell death within one hour of exposure to HDI monomer or HDI isocyanurate indicates death by necrosis as opposed to apoptosis that generally requires more time and the presence of caspases 3/7 [66] (Fig. 6). The control group exhibited a small amount of cell death because the cells were treated in 0.05% DMSO in basal media. The presence of DMSO and the absence of supplements in the media caused some cell death. In the CellTiter Glo 2.0 experiments, we observed a clear dose response indicating that higher doses of isocyanate produced more cell

death. The dose-response curve obtained by the CellTox Green Assay shows a trend for dose response but falls off at the highest dose. We interpret these results to indicate that at high doses, the isocyanates inhibit the assay to some degree. This experiment demonstrates that necrotic cell death by isocyanate exposure occurs rapidly and within the first hour of exposure.

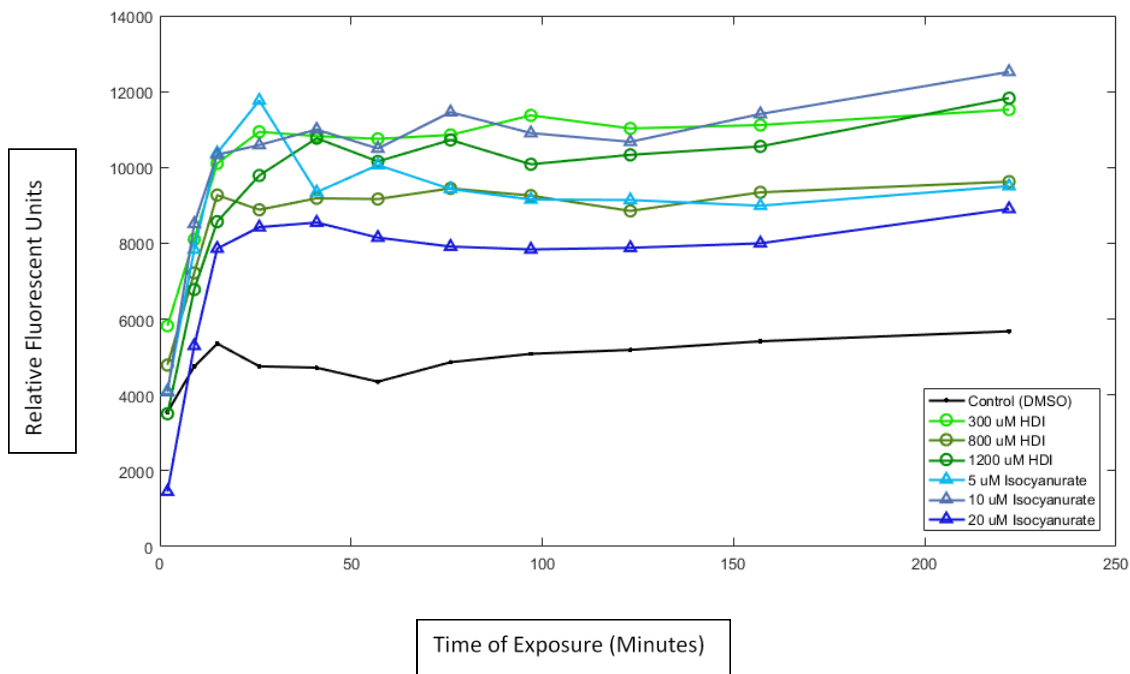

Figure 6: Time course of cell death as measured using the CellTox Green cytotoxicity assay performed on primary human keratinocytes, K075.

To confirm that isocyanate induced death was necrotic and not caused by apoptosis, the luminescent ApoTox Glo assay was used to measure apoptotic caspase 3/7 activities. Figure 7 shows that neither the HDI monomer nor HDI isocyanurate induced the expression of caspases 3/7. Only the apoptosis control (10 µM staurosporine) produced luminescence in all of the caspase 3/7 detection experiments. This indicates that apoptosis was not the mechanism of cell

death in isocyanate-treated cells. The experiment conducted with fibroblasts yielded similar results (F070, n = 1) (data not shown).

The ApoTox Glo assay also included a fluorescent cell death assay that measured the activity of a protease released from dead cells. This cytotoxicity assay did not work with our compounds. HDI monomer exposure did not result in toxicity levels that were significantly different than the positive controls of basal media or DMSO in basal media. We can conclude that HDI monomer appears to interfere with the cytotoxicity protease reaction. HDI isocyanurate exposure, interestingly, did produce expected levels of cytotoxicity. These results demonstrate the importance of including proper controls in order to determine if the test compound interferes with proper function of the assay.

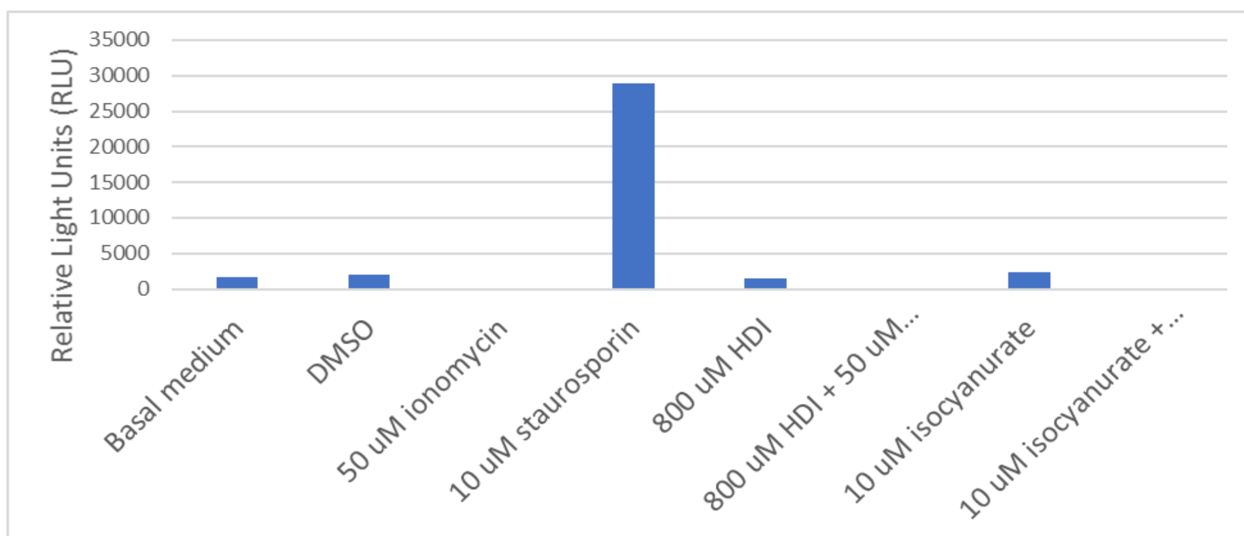

Figure 7: Results from a representative ApoTox Glo Assay performed on primary human keratinocytes, K075 (n = 3).

DISCUSSION

In this study, we investigated differences in toxicity and mechanism of cell death caused by HDI monomer and oligomer exposure in normal human skin cells; specifically, keratinocytes, fibroblasts, and melanocytes. We quantified LC_{50} values for HDI monomer and HDI isocyanurate in cultured human skin cells, and further investigated the mechanism of cell death.

Assay Comparisons

We observed differences in the dose response between the luminescent CellTiter Glo 2.0 assay, the fluorescent CellTox Green assay and the two fluorescent viability/cytotoxicity assays in the ApoTox Glo kit. The four assays are designed to measure cell viability and death using different approaches, which can influence the results. The most sensitive assay was the luminescent CellTiter Glo 2.0 assay, which measures ATP content of live cells. In general, luminescent assays have a greater dynamic signal range than fluorescent and pigmented assays and thus are more sensitive. We found the data from this assay very reproducible. However, despite the fact that this assay uses an enhanced luciferase, it is still limited by the half-life of the enzyme, which limits the timing of the assay. The CellTox Green assay was designed to measure the kinetics of cell death because its substrate is stabile over long periods of time. It uses a fluorescent DNA intercalator that binds to DNA released from dead cells. This assay clearly showed that cells die rapidly in the presence of isocyanates, but we observed a limited dose response with highest doses yielding lower fluorescence. We hypothesize that the isocyanates at higher doses may interfere with either the binding of the intercalator to the DNA or inhibited the

unwinding of the DNA to make it available to the intercalator. Isocyanates are known to preferentially bind amines on proteins that act as a sink for these compounds *in vivo* (e.g., serum albumin). The ApoTox Glo kit contains fluorescent reagents for measuring proteases inside of living intact cells as well as proteases released from lysed dead cells. Since isocyanates bind proteins readily, we believe that they inhibited the dead cell protease activity in the ApoTox Glo cytotoxicity assay. The fact that the viability assay in this same kit worked well confirms our hypothesis that the live cell protease was protected from isocyanate binding by the cell membrane. Thus, the type of a viability/cytotoxicity assay used should be thoroughly vetted before use.

We have shown that isocyanates kill cells rapidly by cell necrosis (Fig 6) and confirmed this by the absence of caspase 3/7 activity (Fig 7). Verstraelen *et al* suggested the method of death within immortalized human bronchial cells was apoptosis, based on gene activity and signaling pathways. There are several factors that could explain this. They used a cancer line of respiratory cells, which may respond differently to isocyanates than primary skin cells. They measured selective gene markers and canonical signaling pathways, including the gene *CASP9* and several pathways involving the CASP9 protein, including an antigen presentation pathway and an apoptosis signaling pathway. They theorized that *CASP9* may have some biological relevance relating to respiratory sensitization[43]. We measured caspase 3/7 activities, which are effector caspases at the terminal end of the apoptotic pathway and are more specific for apoptosis. Additionally and more importantly, they exposed cancer cells to a variety of different chemical combinations of sensitizing, irritant, and non-sensitizing chemicals which in combination could affect the type of response. They also used supplemented medium that could

have affected the effective isocyanate dose due to serum proteins acting as a sink for isocyanate binding.

Differences between Isocyanates

In this study, we observed a large difference in cellular toxicity between HDI monomer and HDI isocyanurate exposure. In order to theorize why we observed this difference, we must consider the many complex chemical and physical differences between these compound that could have factored into the results. Reactivity, lipid solubility, and deposition site are all thought to influence health outcomes [67,68]. Poly-isocyanates have additional reactive NCO-groups, but the inherent reactivity of the NCO-group alone does not sufficiently describe the observed differences [2,40,42]. Bello *et al.* theorized that properties of the protein that becomes attached to the NCO-group may have an effect on cell permeability, which may contribute to variations in reactivity and toxicity [2].

Different isocyanate monomers and poly-isocyanates cause similar health outcomes, typically immune sensitization and asthma. Immunologic cross-reactivity has been observed between different isocyanates, which suggests commonality in the mechanism of sensitization [69–71,58]. This may be due to the same protein becoming attached to the NCO-group, or simply due to the NCO-group itself [2]. Data on health effects relating to poly-isocyanates such as HDI isocyanurate are more limited, but sufficient to demonstrate that these compounds can cause similar health outcomes as isocyanate monomers. Chemical-induced asthma and hypersensitivity pneumonitis have been observed as a result of poly-isocyanate exposure [20,25,26,34].

Some animal studies have indicated that HDI monomer may be better able to induce allergic airway inflammation than poly-isocyanate forms like HDI isocyanurate[47,49,58,57] . In animal studies, both forms of HDI have produced similar levels of antibody titers indicating that

the allergenic response may be very similar [47,68]. However, Pauluhn did not observe a response in animals sensitized with HDI isocyanurate when later exposed to conjugates; but he acknowledged that this may have been related to inappropriately produced conjugates [47]. In this study, we observed much lower LC_{50} values associated with HDI isocyanurate exposure than HDI monomer. We cannot directly compare the LC_{50} values calculated here to regulatory standards for exposure, such as TLV. However, we can consider our results in context with the published peer-reviewed scientific literature. HDI isocyanurate may sensitize humans and animals using the same mechanism as HDI monomer, and HDI isocyanurate penetrates the skin faster [10]. Taken together, evidence exists that HDI isocyanurate may have a large impact on overall exposure that is not being accounted for in current regulatory standards.

Factors Influencing Susceptibility to Isocyanate Toxicity

Structure of the Skin

The structure of the skin itself has significant effects on penetration of the compounds, and ultimately on toxicity. As individual isolated cell types were used in this study, we must consider how the cell types may behave or how the exposure may be modified by the overall structure of the skin.

We observed differences in susceptibility for isocyanate toxicity between the cell types we tested. It is well known that different skin cell types communicate with each other, and this influences cell activity. The top layer of the skin is the cornified layer or stratum corneum, made up of dead keratinocytes and lipids, which forms the major protective barrier against water loss and foreign substances (see Figure 8). Below is the viable epidermis, containing mostly keratinocytes and melanocytes (at the basement membrane) as well as Langerhans cells, which are important in immune responses. The dermis, located beneath the viable epidermis, contains

fibroblasts, hair follicles, blood vessels, and many other elements embedded in a collagen matrix [25,53,72].

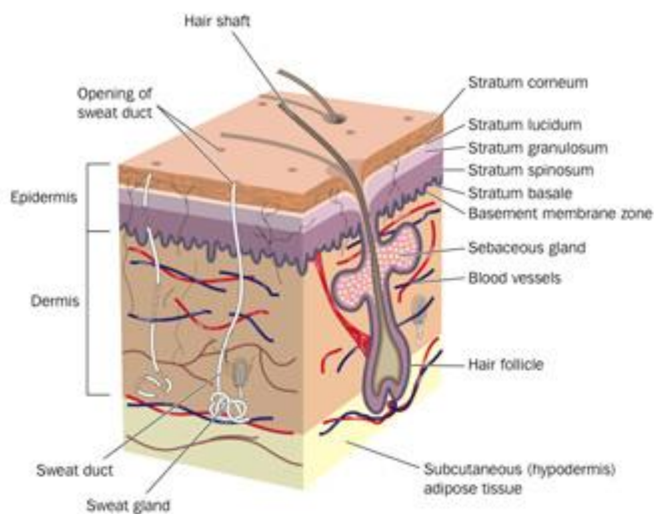

Figure 8: Structure of skin. Courtesy of CliniMed UK [72]

There are also differences in skin between different areas of the body. The thickness of the non-hydrated stratum corneum is between 10 and 50 μm in most areas of the body, but it may be up to 10 times as thick on areas like the palms of the hands or soles of the feet [73]. The rate of absorption of chemicals through the various areas of the skin generally follows the following order, from the fastest to the slowest: scrotal, forehead, armpit, scalp, back, abdomen, palm, and undersurface of foot [74]. Despite large variability in permeability and skin structure, current regulations for skin exposure do not address the differing doses that may be absorbed by different areas of the body.

Cell Types

Figures 4 and 5 illustrate the large difference observed in toxicity between the different cell types. The cell types used in this the study were keratinocytes, melanocytes, and fibroblasts

isolated from neonatal foreskins. Keratinocytes are localized in the stratum corneum, the top or most external part of the skin and comprise 90% of the cells within the epidermis [73]. In the experiments performed here, keratinocytes were the least susceptible to isocyanate toxicity of tested skin cells types. Because of the location, abundance, and relative resistance to toxicity, keratinocytes are the most important cells used here regarding the development of an immune response. In other studies, keratinocytes were observed to be relatively resilient, potentially because of higher enzymatic activities [75–77]. Differing activity levels may play a role in the cell response to xenobiotic exposure and, thus, warrant further investigation. Of the three cell types used here, melanocytes are the least abundant within the skin and were the least resistant to the isocyanate toxicity. Other research suggests that melanocytes may be the most vulnerable skin cell, potentially because of low antioxidant enzyme activity or high cellular proliferation with low relative efficiency of DNA repair processes [78,75,77,76]. As isocyanates do not cause cell death through oxidation or DNA damage, these observations are not directly applicable within this study. However, it is interesting that melanocytes appear to be an especially vulnerable cell in many settings.

Skin Penetration

Many factors influence skin absorption. Key factors include lipid solubility, size of molecules, concentration, co-exposures, skin integrity, hair follicles, and clothing [79]. Generally, more lipophilic substances are better absorbed and smaller molecular weight haptens and allergens can better penetrate the cornified layer [25]. Thomasen *et al.* established that HDI isocyanurate and HDI monomer can penetrate the skin without being metabolized [10]. They investigated penetration patterns of HDI monomer, polymeric HDI, and two clearcoat paints containing HDI monomer and HDI isocyanurate. Both monomeric and polymeric HDI were

readily absorbed through the excised full thickness human skin [10]. HDI isocyanurate was observed to have the shortest absorption time regardless of paint formulation. Compounds tested were neat or in ethyl acetate (EA) solution, because EA is a common solvent used for isocyanates. EA was observed to enhance skin penetration. Polyurethanes used in occupational settings are frequently mixed with reducers, which could have a similar enhancing effect.

In the second set of experiments described by Thomasen *et al*, penetration patterns for both slow-drying and fast-drying clearcoat were investigated [10]. Both formulations contain a larger proportion of HDI poly-isocyanates than HDI monomer. Slow-drying clearcoat contains significantly more HDI monomer and HDI biuret, another HDI trimer, than fast-drying clearcoat. Evaporative losses were minimized through the use of a plastic lid that covered the skin samples. Slow-drying clearcoat was observed to penetrate the skin more quickly than fast-drying clear coat. These results are supported by Bello *et al.* 2006, which investigated the residence time of isocyanates on hairless guinea pig skin *in vitro* and saw similar patterns regarding rapid penetration and minimal evaporation [31]. Observations indicating that HDI isocyanurate is absorbed either just as well or faster than HDI monomer suggests that even a short exposure could result in a considerable body burden [10,29,30]. Here, we observed a higher toxicity associated with polymeric HDI. The results observed in this study suggest that even a short exposure to HDI isocyanurate could result in a significant body burden and, thus, a higher cytotoxicity. This has concerning implications for worker health and future development of limit values merits further investigation.

Inter-individual Variability

There are many factors that may impact inter-individual variability and susceptibility to toxicity. Filaggrin is an important protein in the structure of the skin. It facilitates terminal

differentiation of keratinocytes, which contributes to the formation of the cornified layer [52]. Null mutations in *FLG* as well as inflammation in the skin can cause changes in FLG expression leading to atopic dermatitis. Atopic dermatitis compromises the skin barrier function and thus increases individual susceptibility to environmental exposures. Individual genetic and epigenetic alterations can influence immune responses. The immune response is modified by keratinocytes, which act as an important first line of defense. DNA methylation may also play a role in individual differences, and may increase susceptibility to HDI[22] . DNA methylation may affect gene expression of proteins involved in isocyanate mass transport, permeation, and metabolism and thereby mediate individual responses [22]. These genetic factors can have a significant impact on susceptibility, and our current safety limits may not protect all workers.

In a review published by the World Health Organization, Byford reports that the barrier properties of the skin may be influenced by species of animal, age, sex, and race, anatomical site, skin condition, temperature and blood flow rate, and hydration [73]. When considering inter-individual variability, age, sex, and race as well as hydration status are relevant. In this study, we only used cells from male donors due to the availability of foreskin samples. Given that most isocyanate exposed workers are male, the use of cells from male donors in this study is appropriate [80]. Age may also influence susceptibility. Matsuo *et al* 2004 investigated the responses of fibroblasts from old and young donors to oxidative stress [81]. They observed that fibroblasts from old donors were more resilient against oxidative stress, and theorized that this was due to an increase in glutathione peroxidase activity [81]. All cells used in this study were isolated from neonatal foreskin, and therefore from the youngest possible donors. These cells may have relatively low enzymatic activity and thus be more susceptible to toxicity. No significant differences relating to race have been reported in previous studies or were observed in

the experiments performed in this study (data not shown)[73,76]. Hydration status is an environmental condition that can drastically alter the barrier function of the skin. Decreased hydration status may be the probable cause for increase in absorption [73]. The humidity of the environment as well as individual differences in hydration may influence variations in susceptibility. We used isolated skin cells in this study and, therefore, exposure conditions were not modified by hydration status or humidity of the environment. However, the hydration status of the skin appears to be very important when considering occupational skin exposures and penetration of xenobiotics through the skin.

Monitoring exposure levels and setting limits specific to skin exposure are needed to protect workers from potential adverse health effects like contact dermatitis or occupational asthma. These data suggest that special attention should be paid towards preventing skin exposure to HDI isocyanurate.

Limitations of the Study

This study used monocultures of skin cell types, in which tissue-specific interactions do not occur. This study also did not use immune cells such as Langerhans cells, so we could not assess the effect of immunologic response within the skin. Additionally, it was not possible to compare the doses used in this study to the skin exposure levels experienced by workers exposed to isocyanates because no methodology is available to accurately measure skin penetration in an occupational setting.

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PRACTICUM REPORT

The Effect of Intermittent Noise Stress on Ozone-Induced Cardiovascular Dysfunction in Wistar-Kyoto Rats.

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Previous studies have established that acute exposure to air pollution increases the risk of cardiovascular dysfunction. Intrinsic factors are likely the most important determinants of how the body responds to an exposure. But data also suggests that non-environmental stressors like noise, which is a common urban public health problem, can modify and in fact worsen the response. Noise can cause obvious psychological disturbances typical of non-specific stress, but also changes that can increase the number of cardiovascular disease related mortalities. Therefore, we hypothesized that short-term exposure to noise would worsen the cardiovascular response to ozone.

Male Wistar-Kyoto rats were implanted with radiotelemeters for the measurement of heart rate (HR), blood pressure (BP) and electrocardiogram (ECG) and exposed to intermittent noise (85-90 dB) for one week; after which they were exposed to either ozone (0.8 ppm) or filtered air. Left ventricular functional responses to dobutamine were measured using a Millar probe as well as arrhythmic sensitivity to aconitine in a separate set of untelemetered rats 24 hours after exposure.

HR and BP decreased in all telemetered animals during ozone exposure; noise caused HR to increase. Noise caused BP to decrease, both during noise exposures and post-exposure. Noise

and ozone had a significant interaction on ventricular tachycardia and ventricular fibrillation (see Figure 1). Baseline left ventricular pressure (LVP) was significantly higher in animals exposed to both noise and ozone when compared to no noise; furthermore those animals had the least amount of change in LVP, dP/dT max and min with increasing doses of dobutamine. These animals also had a higher arrhythmic sensitivity to aconitine. In conclusion, these results suggest that noise alters the cardiovascular response to ozone exposure. Thus, non-environmental stressors may be playing an important role in modifying the response to air pollution and may in fact increase the risk in people with underlying cardiovascular disease.

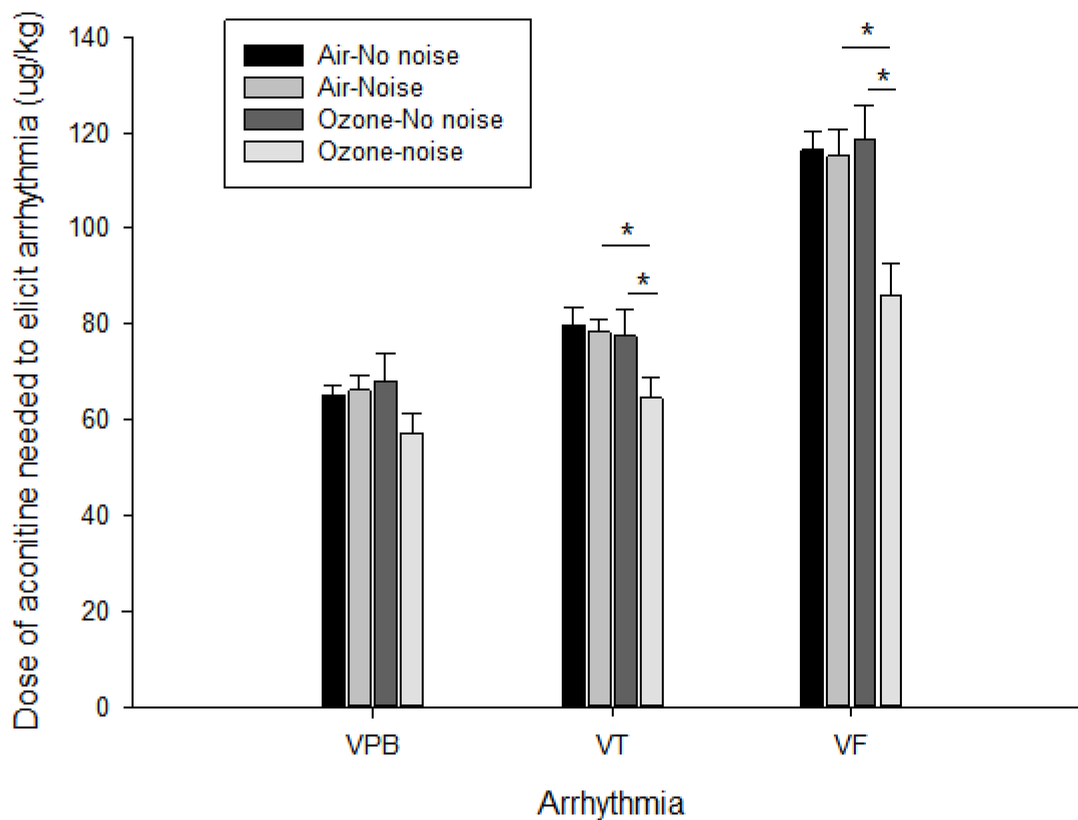

Figure 9. Ventricular Premature Beat, Ventricular Tachycardia, and Ventricular Fibrillation in Rats. Courtesy of Mehdi Hazari.