THE EFFECT OF TEMPERATURE ON THE TRANSDERMAL ABSORPTION OF ANTHRACENE IN HEALTHY INDIVIDUALS

A Thesis
by
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Abstract

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The respiratory routes of exposure to hazardous chemicals such polycyclic aromatic hydrocarbons among occupational groups such as coal tar workers, firefighters, and asphalt workers has been well studied; however, the contribution to dermal routes of exposure has remained understudied. Microdialysis (MD) is an intradermal sampling technique allowing bidirectional exchange of substances between the MD fiber and interstitial fluid, depending on concentration gradient and pressure. To determine if a noncarcinogenic PAH, anthracene, can be dermally absorbed and sampled via MD, multiple MD fibers were inserted into the ventral forearm and a 2.0% anthracene solution was applied over the sites. Dialysate from the MD fibers were sampled over 4 hours at a rate of 1 µL/min. The dialysate was measured using liquid chromatography and tandem mass spectrometry. Anthracene concentration in the dialysate samples was similar between the hot and thermoneutral sites ($P = 0.263$), with values of 2.9 ± 0.4 ppm and 3.5 ± 0.4 ppm respectively. Absolute SkBF (flux) was significantly higher at the heated versus the thermoneutral site ($P = 0.001$) with values of 35.7 ± 11.8 and 7.2 ± 1.0, respectively; however, values were not significantly different between sites when presented as

a percentage of maximum cutaneous vascular conductance (%CVC_{max}; P= 0.057) with values of 29.2 ± 8.3 and 8.6 ± 2.3, respectively. To our knowledge, this is the first protocol to examine dermal absorption of a PAH *in vivo* using MD.

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Chapter 1

Introduction

Approximately 345,600 career and 814,850 volunteer fire fighters in the USA dedicate their lives administering aid and suppressing fires (21). Previous researchers have observed greater cancer incidence among fire fighters including excess myeloma, non-Hodgkin's lymphoma, prostate and testicular cancer, compared to the general population (3, 14, 31, 41). Smoke from a structure burn contains a variety of organic materials resulting from the incomplete combustion of materials within the structure (24). These organic materials include many polycyclic aromatic hydrocarbons (PAHs), many of which have been identified as carcinogenic or potentially carcinogenic. During fire suppression, fire fighters wear turnout gear compliant to the National Fire Protection Association which includes a self-contained breathing apparatus (SCBA). The SCBA comprises a respirator with a full face mask that provides a skin and respiratory protection factor greater than 10,000, which should eliminate inhalation exposure to combustion products (3, 7). Despite this high level of protection, fire fighters do not appear to be completely protected from these hazardous chemicals including benzene and PAHs (17). Routes of exposure have been proposed as either re-breathing of PAHs when the apparatus is removed or dermal absorption during the fire suppression itself.

Respiratory routes of exposure have received more attention than dermal absorption of the hazardous chemicals from fire suppression. While the skin serves as a barrier between the body and the environment, some substances penetrate the skin with relative ease. The structure of the epidermis is a complex "brick and mortar" due to the stratum corneum. This structure acts as a barrier that very selectively prevents hydrophilic chemicals from passing through the

epidermis. Lipophilic compounds tend to permeate through the lipid matrix of the stratum corneum and into the interstitial fluid and eventually the blood stream (37, 43). One important consideration regarding the putative dermal absorption of PAH's in firefighters is the heat strain experienced during fire exposure and the associated increases in skin temperature (T_{sk}) and absorptive properties of the dermis. Several studies have examined the effects of temperature on dermal absorption of topically applied substances, focusing on maximizing absorption of pharmacologics. Increased skin temperature (T_{sk}) has been shown to increase permeability of various chemicals including lidocaine and fentanyl, with associated increases in skin blood flow (SkBf) further contributing to larger amounts of transdermal absorption and clearance of certain drugs versus thermoneutral conditions (20, 36-38, 44, 46, 63). Fire fighters are exposed to extremely high ambient temperatures as high as 200°C (59) during live fires and training exercises resulting in high cardiac output, SkBf and sweating rates to meet metabolic and thermoregulatory demands. Several studies have reported the presence of carcinogens on the skin surface of firefighters following fire exposure (2, 3, 12, 13), therefore increase T_{sk} and SkBf may exacerbate dermal absorption of carcinogens as a potential route of exposure.

Currently, the specific route of carcinogen exposure in firefighters is unclear when considering the use of protective clothing (12). PAH's or their metabolites have been identified in dermal swabs, blood, breath, and urine samples, yet, no studies have fully elucidated the mechanism of exposure. To further examine dermal absorption as a putative route of exposure, intradermal microdialysis may be employed. Microdialysis allows for a bidirectional exchange of substances between the microdialysis (MD) fiber and interstitial fluid, depending on concentration gradient and pressure. As an initial proof of concept, a non-carcinogenic PAH,

anthracene, will be applied to an area of the skin of healthy human subjects to determine; 1) PAHs can be sampled and measured via intradermal microdialysis and 2) PAH's are dermally absorbed, and 3) skin temperature alters dermal absorption of anthracene.

Problem Statement

The purpose of this study is to examine the effect of varying skin temperatures on the dermal absorption of anthracene in a healthy population (18-65 years old) via microdialysis sampling and diasylate analysis.

Aim 1: To determine the efficacy of microdialysis as a technique for interstitial fluid sampling and recovery of the lipophilic compound anthracene by conducting a two-phase pilot study to ensure an adequate flow rate for anthracene recovery from interstitial fluid.

Hypothesis: It is hypothesized that flow rates of 1uL/min will allow recovery of anthracene from the interstitial fluid, and that anthracene may be successfully measured via mass spectrometry.

A**im 2**: To determine the effect of skin temperature on dermal absorption of one non-carcinogenic PAH, anthracene, sampled via intradermal microdialysis.

Hypothesis: It is hypothesized that significantly higher concentrations of anthracene will be observed with increasing skin temperature due to enhanced transdermal absorption.

Summary

Fire fighters are exposed to many potentially hazardous chemicals during fire suppression including PAHs. Although there are strict standards of protection to limit exposure, evidence indicates the presence of hazardous chemicals on the skin surface and of metabolites in body fluids of fire fighters. With such strict protection standards, dermal absorption of these carcinogenic compounds may potentially be a route of exposure in fire fighters. Microdialysis is a technique that allows for the sampling of the interstitial fluid of the subcutaneous tissue. To assess the amount of absorption of such compounds, anthracene will be applied to the ventral forearm of healthy subjects and microdialysis will be utilized for sampling of the interstitial fluid. Fire fighters are exposed to high temperatures which has been shown to increase the amount of dermal absorption of some substances. In the present study, skin on the ventral forearm will be locally heated during the experimental phase of the protocol to determine the effect of temperature on absorption of the PAH anthracene. The protocol development to determine the amount of absorption of these lipophilic compounds will be used in future studies to assess absorption of these PAHs in fire fighters in the field during fire suppression.

Chapter 2

Review of Literature

2.1 Background

It has been established that fire fighters are exposed to a variety of different chemicals during fire suppression including polycyclic aromatic hydrocarbons (PAHs) (3, 7, 17, 26). These PAHs have been classified as either carcinogenic, probably carcinogenic, or possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC) (25). Fire fighters wear turnout gear and a self-contained breathing apparatus (SCBA) which should eliminate the inhalation exposure to these carcinogenic compounds. Nevertheless, some studies have found that fire fighters have elevated risk for multiple types of cancers (14, 26, 31, 55). Metabolites of carcinogens have been found in urine and blood, with respiratory rebreathing or dermal absorption as potential routes for exposure. Dermal carcinogen absorption has received limited attention compared to respiratory routes due to the challenges with measurement in vivo. Further, there are many factors affecting dermal absorption including the structure of the skin, the physical properties of the chemical, and many physiological responses. These factors determining dermal absorption will be explored in the following review.

2.1.1 Skin Anatomy and Physiology

The skin is the largest organ of the human body and provides the most protection from the external environment (32). This protection includes regulating which substances enter and exit the body through the skin. Skin is comprised of three layers: the epidermis, the dermis and the hypodermis. Functionally, the epidermis is impermeable to water and therefore serves to

conserve water in the body and prevent harmful water-soluble substances from diffusing through the skin. This remarkable ability to act as a barrier is attributed to the outer layer of the epidermis, the stratum corneum (43). Unlike many other structures, the stratum corneum consists of corneocytes interspersed throughout the extracellular matrix of lipids (40). With this milieu of lipid-infused extracellular matrix, the skin remains largely impenetrable to hydrophilic compounds but will allow for the diffusion and storage of lipophilic compounds. This structure prevents the loss of water from the body and act as a barrier to many topically applied drugs (16).

2.1.2 Factors Affecting Dermal Absorption

The permeability of the skin can be altered both chemically and physically with alterations in the skin structure allowing a chemical to pass more easily through the skin. Research has shown that many chemical enhancers will interact with the lipid matrix of the stratum corneum to enhance delivery. Solvents such as ethanol and methanol extract lipids from the stratum corneum, causing the corneocytes to expand or for gaps to occur between the cells which allows for greater absorption (16, 60). Iontophoresis and photomechanical waves (PW), or high amplitude pressure pulses generated during ablation by high-power lasers, have been shown to increase the rate of dermal drug delivery. While iontophoresis mainly acts on the charge of the chemical applied to the skin to add a driving force into the stratum corneum (27), PW have been shown to enhance transport of molecules through the stratum corneum by increasing its permeability (34).

Research indicates that the transdermal delivery of many chemicals is enhanced with elevated skin temperature (2, 20, 37, 44, 53, 54, 65). A body of research has demonstrated that

increased temperature, either locally and/or systemically, increases the delivery of drugs and other substances. Petersen and colleagues (38) locally clamped T_{sk} at 43°C in healthy male subjects during application of a nicotine patch on their arm. The locally elevated T_{sk} of 43°C resulted in enhanced nicotine uptake compared a thermoneutral T_{sk} temperature of 32°C (38). Human studies have indicated an increased penetration of harmful chemicals using heat application techniques such as sauna, heated water baths and exercise-induced hyperthermia (2, 18, 28, 33). The increase in T_{sk} elicits a restructuring of the lipid matrix resulting in greater absorption of some chemicals. A significantly greater plasma concentration of lidocaine was observed when locally heated lidocaine patches were applied to the skin of healthy volunteers for 2, 4, and 12 hours versus non-heated patch application (33). A drug study involving the absorption of a transdermal fentanyl delivery system (fentanyl TDS) found that application through a controlled heat-assisted drug Delivery (CHADD) patch (a patch that produces local heat for several hours to increase skin temperature) increased peak plasma concentration (46) versus no heating. Further, significantly increased peak plasma concentrations of testosterone (939 ng/dL) have been observed when a heat-generating patch of testosterone was applied to the skin of six healthy adults compared to those with a patch with no heat (635 ng/dL).

In more applied environments, when exposed to hot environments, systemic thermoregulatory responses are stimulated to dissipate heat through cutaneous vasodilation and sweating, likely altering absorption characteristics (9, 10). Firefighters can be exposed to environmental temperatures greater than 200°C (42) which significantly increases cutaneous vasodilation, cardiac output, and elicits high sweating rates in an attempt to reduce the increasing T_{core} and T_{sk}. During fire suppression, fire fighters experience significantly elevated

T_{sk}, T_{core} and cardiac output in response to extreme environmental temperatures, which could enhance the ability of the PAHs to penetrate through the skin and into circulation.

A further factor that may influence the microclimate of the skin and potentially dermal absorption is the presence of fluid on the skin surface. The human body primarily relies on heat dissipation through evaporation of sweat in hot environments and during exercise, with increasing sweating rate at higher workloads and more extreme temperatures. Firefighters experience higher rates of sweat loss during active duty (2950 ± 1034 mL of water in hot conditions vs. 1290 ± 525 mL in control conditions) (30), which may influence dermal properties, clothing and spreading of carcinogens over the skin surface. A body of research has documented a non-uniform pattern of sweating rates between different sites, making this a potential consideration for site sampling. Patterns of regional sweating rates in a study utilizing male athletes exercising at varied intensities (49) observed (a) sweat rates increased with increased exercise intensity in all regions of the body, (b), the posterior torso exhibited the greatest increase across all intensities (the only exception being the forehead), (c) and an increase in sweat rate from proximal to distal regions on the arms. Although overall sweat rates were significantly higher in males compared to females, similar regional sweat rate patterns were observed. Selection of sampling sites should therefore be carefully considered, recognizing potentially exposed skin sites, SkBf, and regional sweating rates.

2.3 Interstitial Sampling Via Intradermal Microdialysis

Microdialysis (MD) is an in vivo technique that allows for continuous sampling of the dermal extracellular space (22, 35, 39). The MD technique requires a microdialysis probe, microperfusion pumps, a perfusion fluid and a sampling device (39). The semipermeable

membrane is surrounded by a copper guide wire, with an exposed portion (~1cm) of the membrane placed intradermally in the extracellular space to sample the interstitial fluid (ISF). This allows for bidirectional flow between the perfusate and IF, and depending on the concentration gradient, can either deliver or sample chemicals in the IF (22). MD has been widely utilized to examine diverse research problems including sampling neurotransmitter release in the interstitial fluid to examine the pathophysiology of neurological disorders (23, 47). MD has also proven effective in delivering chemicals to the interstitial fluid to examine cell signaling pathways concerned with cutaneous microvascular function (12, 13, 48).

The recovery of a substance from the IF via MD is influenced by many factors including pore diameter of the fiber, perfusion rate, molecular size, and charge of the substance (15). Lower perfusion rates tend to yield more diffusion of compounds into the perfusate but lower the amount of sample collected. High perfusion rates allow for a higher sample amount but decrease the recovery of substances form the ISF. The ideal perfusion rate for adequate sample size and adequate sampling recovery was found to be 0.5-1.0 uL/min (15). The lower the molecular weight, the higher the recovery of the dialysate, therefore, a drug or chemical which will be topically delivered must be of a lower molecular weight. All these factors must be carefully considered when determining the suitability of intradermal MD for sampling or delivery.

2.5 Polycyclic Aromatic Hydrocarbons

PAHs are a result of the incomplete combustion of items found in houses and from the burning of the structures themselves. A variety of PAHs are also found in soil due to contamination from long-range transport (58), as well as the production of coal tar and asphalt

(61). There are a variety of these compounds and many of them have been classified as carcinogenic corresponding to exposure level and time. A meta-analysis observed a rising increase of developing cancers such as multiple myeloma, non-Hodgkin's' lymphoma, prostate and testicular cancer among petrochemical refinery workers and other occupations involving exposure to PAHs (31). A variety of PAHs such as benz[a]anthracene and benzo[a]pyrene have been shown to cause tumorigenesis in mice (4). Studies performed through NIOSH indicate the contamination of SCBAs with PAHs after only 25 minutes of use during fire suppression. The presence of these toxic chemicals has been found both on and underneath the protective turnout gear (62). Dermal absorption rates have been analyzed through skin swipes and areas of thicker skin appear to be more protective than areas of thinner skin (57).

To assess the ability to recover and analyze the presence of the PAHs through a route of dermal exposure, a chemical that is similar in structure to these carcinogenic compounds to which firefighters and other workers are exposed need to be considered. Anthracene is a nonmutagenic, noncarcinogenic PAH with a relatively low molecular weight that is present in the environment. While many PAHs are classified as either carcinogenic or possibly carcinogenic, anthracene itself is not considered by the NTP to be problematic in developing cancer in either animal or human models. Anthracene is not classified as carcinogenic and has a relatively low molecular weight which makes it a good candidate for the present study to test dermal absorption through the skin into MD fibers.

2.6 Summary

Despite personal protective equipment including turnout gear and SCBA, firefighters are exposed to highly carcinogenic compounds produced as byproducts of incomplete combustion.

Increases in ambient temperature, skin temperature, and skin blood flow have all been shown to increase delivery of many chemicals through the skin. To determine if transdermal absorption is a likely route of absorption of carcinogenic molecules, intradermal MD will be utilized to sample recovery of a topically applied non-carcinogenic PAH in the interstitial fluid at thermoneutral and warm local skin temperatures.

Chapter 3

Methods

3.1 Subjects

The subjects were recruited from Appalachian State University and the community of Boone through flier advertisements and email listserves. The subjects were healthy males and females aged 18-65 years old. Subjects were nonsmokers with no metabolic, cardiovascular or uncontrolled dermatological conditions. Females of reproductive age completed a pregnancy test and were excluded if positive.

3.2 Exclusion Criteria

Individuals with skin conditions including eczema, rashes, or disorders of pigmentation were excluded from the study as skin conditions that disrupt the normal structure of the dermis may alter the rate of absorption.

3.3 Experimental Techniques

Methods were developed for dermal delivery and recovery of anthracene (ANT). Intradermal microdialysis was utilized for the recovery of ANT from the interstitial fluid, following dermal application and detected via fluorescence and mass spectrometry (MS). Fluorescence was utilized in initial pilot studies simply to detect the presence of ANT in the

dialysate samples and MS was utilized by collaborators at North Carolina State University during the latter stages of pilot testing and experimental protocols for detection and quantification of ANT in the dialysate. These experimental techniques are outlined below. All procedures were approved by the Appalachian State University Institutional Review Board and the use of pharmacologics were approved by the Food and Drug Administration (FDA).

3.3.1 Intradermal Microdialysis

Subjects were instrumented with two intradermal microdialysis (MD) fibers (10-mm, 30-kDa cutoff membrane, MD 2000; Bioanalytical Systems, West Lafayette, IN) placed in the left ventral forearm using sterile technique. Insertion and exit points were marked on the skin, which was cleaned with alcohol and betadine (51). MD fibers were kept at least 2 cm apart to prevent interference between the local heating units or any cross-contamination. Prior to MD fiber placement, ice was applied to the MD sites for 5 minutes to temporarily anesthetize the skin. A beveled 25-gauge needle was inserted horizontally into the intradermal layers such that the entry and exit sites were roughly 1.5 cm apart. MD fibers were then threaded through the lumen of the guide needle, and the needle was removed leaving the MD fiber in place. The MD fibers were perfused with lactated Ringer's solution with 10% 2-hyroxypropyl-β-cyclodextrin (HBC). HBC increases the solubility of lipophilic drugs because of a complex created between the drug and the lipophilic cavity of the HBC. The HBC was mixed immediately before usage, dissolved in lactated Ringer solution and sterilized with syringe

microfilters (Acrodisc; Pall, Ann Arbor, MI). During the insertion trauma resolution period (~60 min), the solution was perfused through the MD fibers at a rate of 1 μL/min.

3.3.2. Fluorescence

For each of the pilot experiments involving fluorometry, an ANT standard curve was made for which sample values could be compared to and quantified. These calculations helped derive an estimate of the amount of ANT in any given sample and helps to reduce the baseline noise from the fluorometer.

A spreadsheet was created with wavelengths from the emission spectra and the intensity in counts per second for the 1 parts per billion (ppb), 10 ppb and 100 ppb anthracene standards. Using the 1 ppb spectra as a baseline, the 1 ppb intensity was subtracted from the 10 ppb and the 100 ppb to get a 9 and 99 ppb emission spectrum, respectively. The maximum and minimum fluorescence was found for the 9 and 99 ppb anthracene spectrum.

The averages of the peaks were subtracted out of the 9 ppb baseline and divided by the concentration (9 or 99 ppb) to give conversion factor of average fluorescence per ppb. This was then used to estimate the amount of ANT in a sample. The same formulas were used to subtract a solvent baseline from spectra from the dialysate samples. The conversion factors found above were then used to estimate the amount of ANT in the sample based on the amount of fluorescence.

3.3.3. Mass Spectrometry

Mass spectrometry (MS) is an analytical technique that is highly sensitive and versatile, which allow the detection of specific substances within a simple or complex matrix. Collaborators at North Carolina State University, under the guidance of Dr. Nelson Vinueza, developed a quantitative method via tandem mass spectrometry to measure anthracene from dialysate samples of this study (6, 19).

All experiments were performed on a Velos Pro linear ion trap mass spectrometer (Thermo Fisher Scientific) coupled to a Ultimate 3000 UHPLC system. Samples were introduced via autosampler and the injection volume was set to 10 μL. The mobile phase was made up by an isocratic elution of acetonitrile and water at a ratio of 9:1. The system was configured to bypass the column and the total run time for each sample was 5 minutes. Ionization was performed on positive mode via an atmospheric-pressure chemical ionization (APCI) source. Targeted tandem mass spectrometry (MS/MS) was utilized to select and fragment the analyte (anthracene) and internal standard (deuterated anthracene) ions. The matrix ions fragment differently than the analyte and internal standard ions, since they are a different class of compounds. It is straightforward then to monitor only the product-ions of the analyte and internal standard, which are typically formed by acetylene-loss ([M – C2H2]+•). Product-ion monitoring differentiates the analyte and internal standard from surrounding matrix ions, offering a simpler spectrum with higher sensitivity.

3.3.3.1 Preparation of Standard Curve

A calibration curve was established using an isotropic-labeled internal standard. Calibration solutions were prepared by collaborators at NC State by diluting stock solutions of anthracene in acetone. Detailed solutions for each calibration solution are shown in Table 1.

Table 1. Composition of calibration solution

Concentration: ppm	Internal standard: µL	Analyte solution: µL	Solvent: µL	Total volume: µL
10	200	10	790	1000
20	200	20	780	1000
50	200	50	750	1000
100	200	100	700	1000
150	200	150	650	1000
200	200	200	600	1000
QC-30	200	30	770	1000
QC-120	200	120	680	1000

The abundance ratio between peak m/z =152 (anthracene fragment) and m/z = 160 (deuterated anthracene fragment, internal standard) was recorded and a calibration curve (Figure 1) was established based on the relationship between concentration and abundance ratio.

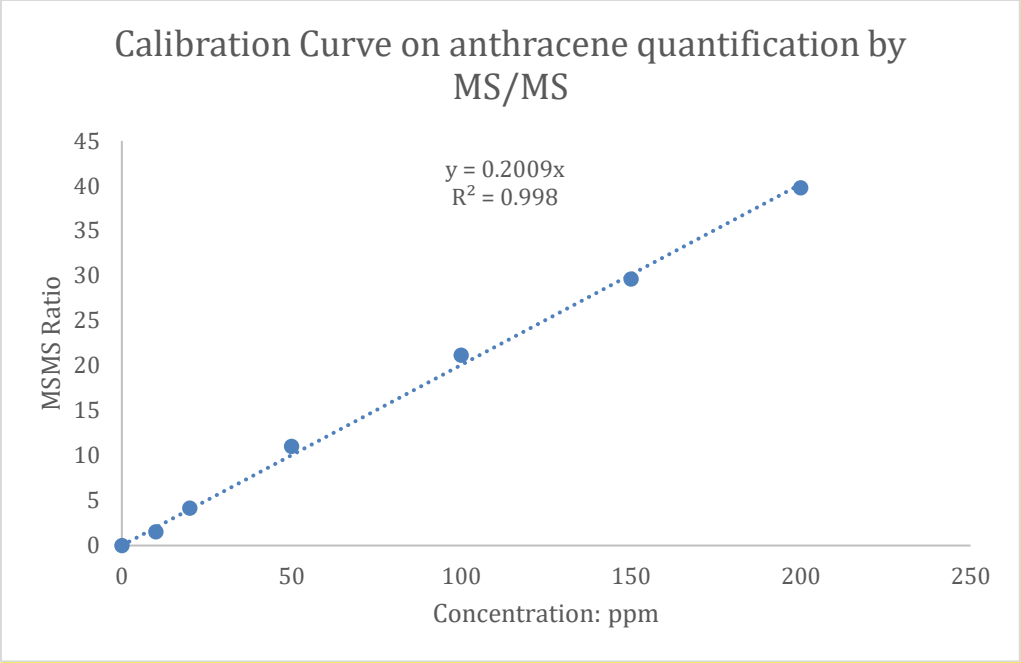

Figure 1 . Anthracene calibration curve from MS/MS with sample concentration 10-200 ppm.

3.3.3.2 Analysis of Human Test Samples

Following the development of the calibration curve, the dialysate samples from the interstitial fluid were analyzed. Samples were extracted and analyzed by tandem mass spectrometry. 300 µL of HPLC grade acetone was added to the sample tube. The tube was then stirred by a VWR vortex mixer for 15 seconds at 2200 rpm and centrifuged by a VWR minicentrifuge for 15 seconds at 8000 rpm. Then the supernatant was taken and transferred into a 2 mL HPLC vial and mixed with 200 µL of 24 ppm D10-anthracene solution. Then the extracted and prepared sample was analyzed by the same instrument conditions used to

establish the calibration curve. The abundance ratio obtained on each sample was then used to calculate anthracene concentration using the equation of the calibration curve. Samples S1 from all six test subjects were tested and the data were summarized in Table 2.

Table 2. Tandem mass spectrometry data from human test samples

Sample Name	Abundance		Ratio	ppm concentration
	m/z 152	m/z 160	152/160	
P2_S1_HOT	3404.6	5719.2	0.595	2.4
P2_S1_TN	6073.9	5376.0	1.130	4.6
P3_S1_HOT	6085.3	10421.7	0.584	2.4
P3_S1_TN	5411.9	8723.0	0.620	2.5
P4_S1_HOT	4726.9	10783.5	0.438	1.8
P4_S1_TN	7309.2	11565.4	0.632	2.6
P5_S1_HOT	6391.4	10347.2	0.618	2.5
P5_S1_TN	5968.7	9589.7	0.622	2.5
P6_S1_HOT	4850.9	5043.6	0.962	3.9
P6_S1_TN	5771.4	8064.6	0.716	2.9
P7_S1_HOT	5989.4	5429.3	1.103	4.5
P7_S1_TN	2507.0	1746.3	1.436	5.9

3.4 Pilot Testing

In vitro Testing

Vehicles for Delivery of Anthracene

Substances that are lipophilic in structure were required for the mixing of ANT and successful delivery through the skin. Extensive pilot work was conducted using different vehicle solutions to dissolve and deliver ANT into the skin. Further pilot work was required for recovery of ANT via MD which is a sampling technique that is frequently used for the delivery or recovery of hydrophilic substances. This presented a challenge to both deliver and

recover ANT from interstitial fluid into the MD fiber due to its lipophilicity. The pilot work conducted to achieve delivery and recovery of ANT via MD is outlined below.

3.4.1 Mineral Oil

Mineral oil (MO) is a clear liquid distilled from petroleum used in cosmetic products that has been reported to permeate into the stratum corneum. Due to this reported absorption and lipophilicity, a 0.1% and 0.2% solution of ANT in MO was achieved. Mixtures of 0.5% or higher were attempted, however, the ANT would not dissolve in concentrations greater than a 0.2% solution. MD fibers were submerged in petri dishes containing a 0.2% ANT in MO solution and equilibrated for 20 minutes to allow bidirectional exchange with the MD fiber (microperfusion pumps turned off). Dialysate samples were collected in a series of collection periods at a rate of 1 µL/min (Table 3) from 20-30 minutes, 40-70 minutes and 80-90 minutes following submersion. Between sample collection periods, pumps were turned off to allow equilibration between the ANT solution and MD fiber. Dialysate collections were then analyzed for the presence of ANT via fluorescence, as described in section 3.3.2.

Equilibrium	Collection	Equilibrium	Collection	Equilibrium	Collection	Equilibrium
20 min	10 min	10 min	30 min	20 min	10 min	20 min

Table 3. *In vitro* Testing Protocol

3.4.2 DMSO

Dimethyl-sulfoxide (DMSO) is a colorless liquid solvent that dissolves both polar and nonpolar compounds and has been utilized in previous MD studies as a vehicle solution (50). A solution of 0.2% and 0.5% ANT and 10% DMSO in MO was achieved. MD fibers were placed in petri dishes and collection was attempted as described above. Dialysate samples were not collected due to the 10% DMSO dissolving the MD membrane and preventing ANT recovery. DMSO was therefore not considered further for use in the study.

3.4.3. Vaseline

MO and DMSO proved unsuccessful as vehicle delivery solutions for ANT. Prior studies have developed fluorescent markers using anthracene and other PAHs in Vaseline, indicating that ANT can be adequately dissolved in Vaseline (29). A 1% mixture of ANT in Vaseline was achieved and the mixture was placed on a heating plate with a magnetic stir bar and heated to 75°C at 30 rpm to ensure adequate mixing. A MD fiber was placed in a petri dish with the 1% solution of ANT in Vaseline and with LR solution at a rate of 1 µL/min. Dialysate samples were collected at 2 hours, 6 hours, and 20 hours following initial submersion to determine recovery over time and to establish suitable equilibration periods. Between sample collection periods, pumps were turned off to establish equilibration. These dialysate samples were then measured using fluorescence as described in section 3.3.2. With some success in recovering ANT from the MD fiber with a 1% solution, the same protocol was attempted with

a 2% solution of ANT in Vaseline. However, the 2% of ANT did not dissolve fully into the Vaseline.

3.4.4. Aquaphor

The Vaseline proved successful as a vehicle delivery solution for ANT, however, no solution higher than 1% was achieved. As another option for vehicle delivery of ANT, a 2% solution of ANT in Aquaphor, an over-the-counter skin care ointment, was created and mixed on a heating plate at 75^{o} C at 30 rpm. A MD fiber was soaked in the 2% solution and dialysate collections, using the sample protocol as stated in the section of above, were examined via fluorescence described in section 3.3. 2. With detection of ANT with Aquaphor as a vehicle for delivery, Aquaphor was chosen as the vehicle for delivery and recovery of ANT.

3.4.5. Solution and Perfusion Rate for Anthracene Recovery

MD is a technique that is frequently utilized for the delivery or recovery of hydrophilic substances, the lipophilicity of the Aquaphor and ANT created challenges for successful recovery from the interstitial fluid. The perfusion rate of the microinfusion pumps determines the absolute and relative recovery of the dialysate, however higher perfusion rates create a back pressure that does not allow for substances in the interstitial fluid to freely move into the MD fiber. Pilot work using MO as the vehicle did not prove successful at a higher perfusion rate. Standard procedure for MD perfusion is typically 2 µL/min, but a rate of 1 µL/min was selected

based on pilot work with menthol from other labs (12). It was determined that an equilibration period (microinfusion pumps off) was necessary to permit bidirectional exchange between the interstitial fluid and the MD fiber. Two MD fibers were soaked in a 0.2% solution of ANT in MO as described above, with the perfusion rates set at 0.5 µL/min and 1 µL/min. The dialysate was collected occurring to table 3 and analyzed with fluorescence. This analysis determined that a perfusion rate of 1 µL/min was the optimal perfusion rate for detection and recovery.

3.5. *In Vivo* Testing

In conjunction with extensive *in vitro* pilot work to establish and detection of ANT using intradermal microdialysis, in vivo pilot work was simultaneously completed in the ventral forearm. The protocol utilized for *in vivo* testing is outlined in figure 3 below.

Fiber Insertion	Hyperemia	Anthracene Application	Dialysate Collection	Break	Dialysate Collection	Remove Fibers	Wash and Check Sites
20 min	45-60 min	15 min	1.5 hrs	2.5 hrs	1.5 hrs	15 min	10 min

Table 4. *In vivo* Testing Protocol.

3.5.1. Mineral Oil

To assess the delivery and recovery of ANT from the MD fibers *in vivo,* two MD fibers were inserted into the left ventral forearm. The 0.2% ANT solution in MO was applied directly to a 1 cm^2 area of skin directly over the ANT MD site (see figure 2). The protocol involving equilibration and dialysate sampling was conducting according to table 3 and then analyzed via fluorescence.

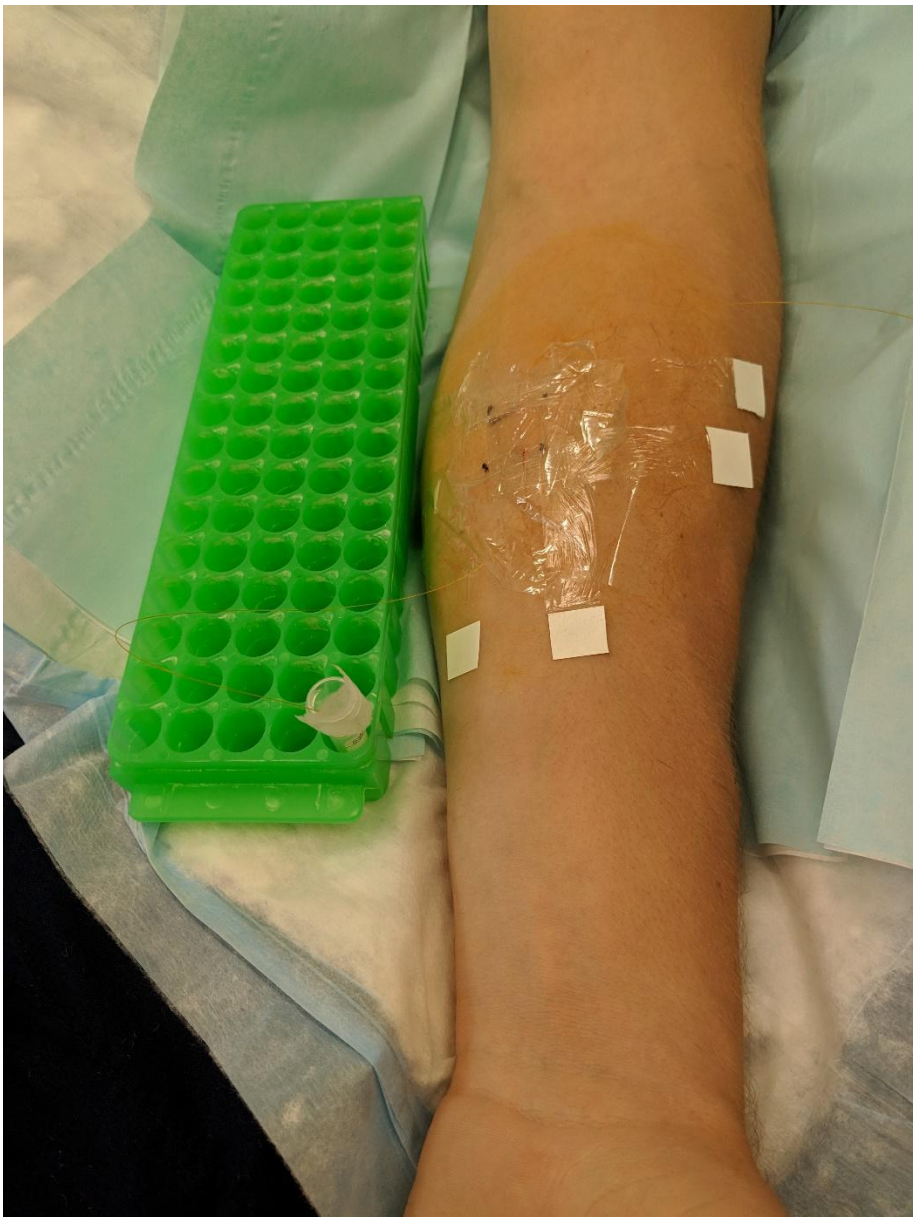

Figure 2. *In vivo* graphic showing a MD site in mineral oil applied to the 1 cm^2 and covered with plastic wrap to prevent evaporation.

3.5.2. Vaseline

Fluorescence analysis did not indicate the presence of ANT in the dialysate of the *in vivo* 2% ANT in MO solution. With *in* vitro success indicating the presence of ANT in the 1%

ANT in Vaseline solution, a 1% ANT solution in Vaseline was applied directly to the skin as described above. Dialysate samples were collected according to table 4 and then analyzed via fluorescence.

3.5.3 Aquaphor

Based on *in vitro* success with ANT recovery, a 2% ANT in Aquaphor solution was applied directly to the skin over MD sites using the protocols outlined in section 3.5.1. Dialysate samples were collected according to the protocol outlined in Table 5 and then analyzed via fluorescence. The fluorescence analysis indicated the presence of ANT starting ~3 hours after application on the skin, which informed the final protocol (there was no dialysate collection for the first 2½ hours) as indicated by the peaks at 398 nm and 420 nm in the fluorescence curves in figure 3.

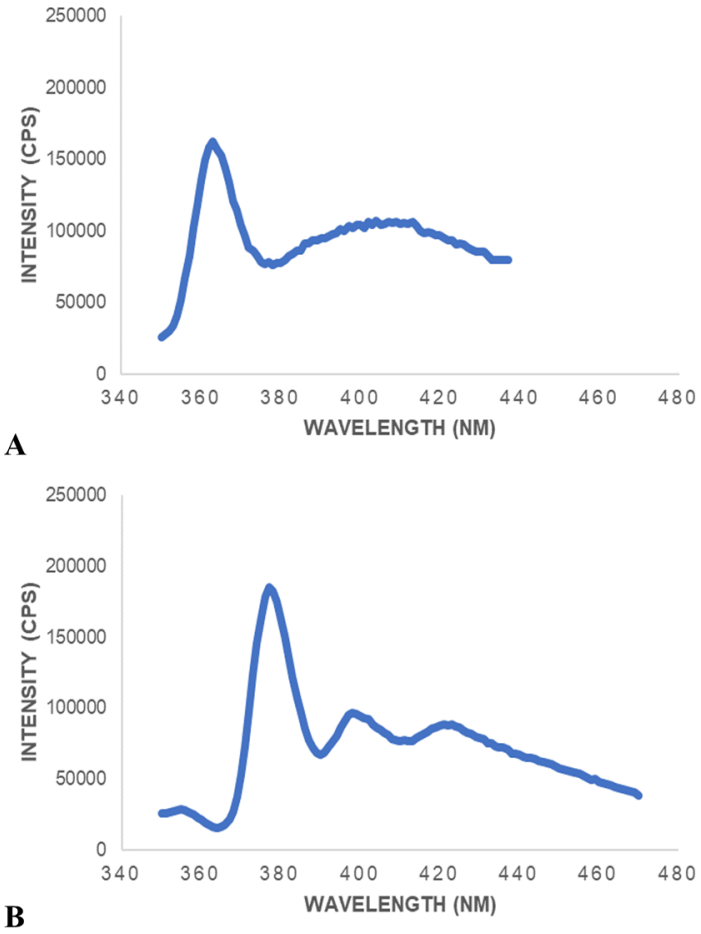

Figure 3. Representative traces of fluorescence patterns from dialysate collection. Traces indicate the absence of anthracene in the sample using mineral oil, dimethyl sulfoxide, or Vaseline as the vehicle (A) or the presence of anthracene utilizing Aquaphor as a vehicle (B) with peaks at 398 nm and 420 nm.

3.6. Experimental Protocols

All study procedures were approved by the institutional review board at Appalachian State University and conformed to the guidelines set forth in the Declaration of Helsinki. Written and verbal consent was voluntarily obtained from all subjects before participation in

the study. The experiment was conducted in a group of six young, healthy subjects who were free from dermatological conditions.

All experiments were conducted under thermoneutral conditions in the Thermal and Microvascular laboratory, Leon Levine Hall of Health Sciences. Subjects arrived at the lab in a fasting state (12-hour fast) for completion of an informed consent and an initial screening visit. All subjects completed a medical history and measurements of anthropometrics (height and weight), blood pressure, heart rate, and a blood panel. All female subjects had a negative urine pregnancy test for participation in the study. After screening, subjects attended the laboratory on a separate day for completion of the experimental testing. Subjects refrained from alcohol, caffeine, and intense exercise for 12 hours prior to the study. The 2% ANT in Aquaphor solution was mixed prior to the experiment whereby Aquaphor was heated at 70 °C for 5 minutes before ANT was added. The chemicals were mixed using a magnetic stir bar on a heating stirring plate for another 10 minutes before being weighed, with 0.2 g required for each site. The ANT solution was covered with foil and protected from sunlight until use. applied. Upon arrival to the laboratory MD insertion sites were marked on the left ventral forearm, sterile technique was utilized, and MD fibers were inserted as described in section 3.3.1. Two intradermal microdialysis fibers (30 kDa cutoff; MD 2000, LM-10, Bioanalytical Systems) were placed in the skin of the ventral surface of the forearm separated by at least 2 cm to prevent cross reactivity. Entry and exit points were covered with a small piece of tape to prevent ANT contamination beneath the skin. A 1 cm^2 template was used to create an isolated surface area of the skin for ANT cream placement. After fiber placement, 0.2 g of the 2% ANT in Aquaphor was applied to the skin over each MD fiber site. Subjects were then outfitted with

LH units (Moor Instruments, UK) and laser doppler probes to measure red blood cell flux (Moor Instruments)). After instrument implementation, a 10% HBC in lactated Ringer's solution was perfused through all sites at a rate of 1 µl/min (BASi Bee Hive controller and Baby Bee syringe drive). Heart rate and blood pressure were measured every 5 minutes throughout the protocol via brachial auscultation. A series of dialysate collections were performed using the outline as described in Table 3. All samples were collected, labeled and refrigerated before being sent to collaborators at North Carolina State University for analysis using MS.

T_{sk} was clamped at 33° C at both sites for 10 minutes and dialysate samples were collected during a thermoneutral baseline. One site (sites were randomized to counterbalance skin temperature site) was set to clamp T_{sk} at 43° C for a heating blood flow baseline. Both sites were clamped throughout the protocol. The experimental protocol was structured similarly to the outline in figure 3 in section 3.5. After ANT application, the MD fibers were perfused with a 10% 2 hydroxypropyl-beta-cyclodextrin in Lactated Ringer's solution for 90 minutes. A series of collections were performed starting 2 hours after the ANT application. After a 15-minute collection period, pumps were turned off for a 30-minute equilibration period. This procedure was followed for three sample collection periods. At the end of the collection period, the LH unit over the thermoneutral site was raised to 43° C and 56 mM sodium nitroprusside was perfused through the fibers at both MD sites at a rate of 4 µL/min to obtain maximal cutaneous vasodilation.

3.7. Data and Statistical Analysis

Red blood cell flux was digitized and recorded at 40 Hz for later offline analysis using Windaq software and Dataq data acquisition system (Windaq: Dataq Instruments, Arkon, OH). A five-minute average of LDF data was measured during each sample collection period and cutaneous vascular conductance (CVC; LDF/MAP) was calculated. Data for a percentage of CVC maximum (%CVC_{max}) were measured by averaging LDF values from a sixty second plateau obtained during 56 mM SNP perfusions and simultaneous 43° C heating ((CVC/CVC_{max}) *100). A two-tailed t-test was performed in SigmaPlot to determine significance ($\alpha = .05$; $n = 6$) and presented as mean +/- standard error. All descriptive subject characteristics were expressed as mean +/- standard error.

Chapter 4

Results

Subject characteristics for the six (5 male, 1 female) healthy subjects that completed the experimental protocol are presented in table 5.

Table 5. Subject characteristics.

	Mean	**Standard Error of the Mean**
Age	32.0	4.9
Height (cm)	162.7	10.7
Weight (kg)	91.3	17.7
RHR	64.2	5.3
SBP (mmHg)	130	4
DBP (mmHg)	74	3
Glucose (mg/dL)	93.8	4.7
HDL (mg/dL)	56.8	9.1
LDL (mg/dL)	118	10.1
Trigs (mg/dL)	141.7	26.0
TC (mg/dL)	186.3	18.0

Anthracene concentration (ppm) in the dialysate samples was similar between the hot and thermoneutral sites (P=0.263), with values of 2.9 ± 0.4 and 3.5 ± 0.4 respectively (Figure 4). Absolute red blood cell flux (LDF) and cutaneous vascular conductance (%CVC_{max}) are illustrated in figure 5. Absolute SkBF was significantly higher at the heated versus the thermoneutral site (P=0.001) with values of 35.7 ± 11.8 and 7.2 ± 1.0, respectively. Values were not significantly different between sites when presented as %CVCmax (P=0.057) with values of 29.2 ± 8.3 and 8.6 ± 2.3, respectively.

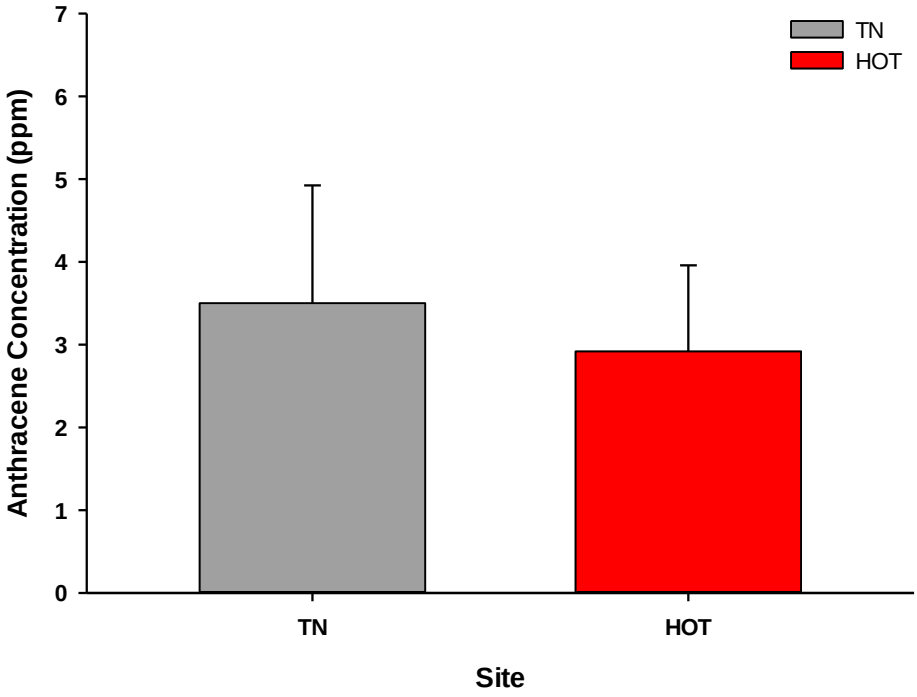

Figure 4. Anthracene concentration in dialysate samples expressed in parts per million (ppm) at the thermoneutral (TN) and hot sites collected between 2h30 min and 2h45 min following dermal anthracene application. No significant differences were observed between sites ($P>0.05$; $n = 6$).

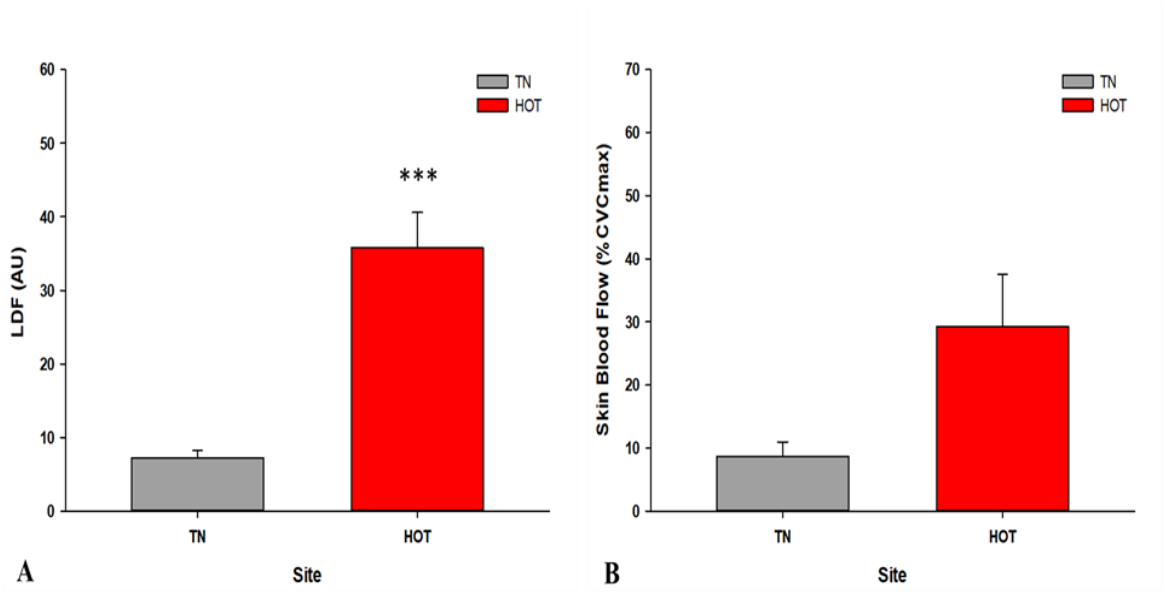

Figure 5. Skin blood flow expressed as (A) absolute LDF and (B) normalized to maximum vasodilation (%CVC) during the sample collection period. Maximum LDF was significantly higher in the heated site (P=0.001) 0.05; *n* = 6). *** P ≤ 0.001.

Chapter 5

Discussion

A major finding in this study was that microdialysis can be successfully used to recover the PAH anthracene following dermal application *in vivo*. This finding is methodologically important for the development of a protocol that allows for continuous sampling of dermal absorption with microdialysis, coupled with the measurement of anthracene with MS. While many studies have examined the deposition of PAHs on the skin of firefighters and other occupational groups, to our knowledge, this is the first protocol to examine dermal absorption of a PAH *in vivo* using MD. In accordance with our findings, studies have shown that small concentrations of these PAHs could be absorbed through the skin by assessing biological markers following environmental exposure, including fire suppression and charcoal grilling (64). Dermal tape stripping and dermal swabs have shown the presence of PAH particulates on the skin of workers exposed to these PAHs and urine analysis of PAH metabolites indicate a route of exposure of these PAHs, whether it be respiratory or dermal. Similarly, *in vivo* modeling studies used surface disappearance of dermally applied PAHs and 1-OH-pyrene excretion measurement to estimate differences in dermal PAH absorption (57). Recently, *ex vivo* human skin modeling found that benzo [a] pyrene was absorbed into the skin and its metabolites could be measured using high-performance liquid chromatography (8). While numerous studies have assessed the exposure to these toxic PAHs released during incomplete combustion with biological monitoring, including breath, urine, and dermal sampling, the intradermal sampling technique of MD allowed for the recovery of ANT directly from the interstitial fluid, directly demonstrating dermal absorption.

Anthracene was detected in both sample sites following 2 h 30 minutes of exposure in all subjects, but the amount of ANT recovered between the heated and thermoneutral sites was similar. This finding was contrary to our hypothesis since substantial evidence suggests there is greater dermal absorption of a wide range of transdermal products at higher temperatures. This has been observed in pharmacological studies aiming to improve dermal drug delivery, for example, the assessment of differences in serum concentrations of testosterone after the application of a heat-generating patch (45). Authors observed both maximum peak and area under the curve plasma testosterone was significantly higher in the group with the heated patch vs a thermoneutral control (46). Similarly, the effects of local heating on the systemic delivery of fentanyl from a CHADD patch has been assessed in multiple studies (20, 33, 46). The CHADD patch increased skin temperature to 41 ± 1°C and resulted in shortened peak serum fentanyl concentrations as well as increases in the total concentration of fentanyl (1, 46). Some literature remains inconsistent with the measurement of these transdermal drug delivery systems *in vivo* as many methods, such as exercise and increased ambient temperature, did not include a precise, controlled skin temperature measurement (2, 56). Nevertheless, much of the literature indicates that increased skin temperature from locally applied heat should increase the amount of anthracene recovered.

In the present study, one potential explanation for the similarity in ANT concentration between sites may be the significantly different skin blood flows. The LDF was significantly higher in the heated site which may have impacted the clearance of the interstitial fluid causing the anthracene to move away from the microdialysis membrane. In a study that investigated local blood flow on the recovery of sodium fluorescein via microdialysis *in vivo*, local vasodilation decreased the amount of fluorescein by approximately 50% (11). Recovery was

also increased at vasoconstricted sites suggesting that the recovery of a substance from a microdialysis probe will be directly impacted by the clearance of that substance. In conjunction with decreased recovery from increased clearance, the clearance rate of the substance is proportional to the blood flow around the MD pump (11). This may explain the similar concentration of anthracene recovered in the heated compared to the control site. Sampling via intradermal MD can be influenced by multiple factors and does not strictly measure dermal absorption directly. Recovery via MD does not fully reflect dermal absorption and therefore further work is needed to elucidate the effects of skin temperature on the dermal absorption of PAHs. Future studies should examine the effect of controlling skin blood flow beneath each MD fiber so that the effect of dermal absorption can be more fully elucidated without increased clearance as a confounding factor. Epinephrine has been observed to prolong the sensory blocking effects on anesthetics by slowing the clearance of these substances away from the site (5), and warrant further investigation.

Increases in dermal clearance due to higher blood flow may explain the similarity in the concentration of anthracene recovered from each site. Nevertheless, the successful recovery of a PAH such as anthracene through microdialysis has important practical applications. It is widely recognized that firefighters and other occupational groups such as coal tar workers and asphalt workers are exposed to these hazardous chemicals (7, 52). Respiratory routes of exposure are more commonly studied in the field, but deposition of PAHs on the clothing and skin of these workers are recognized. Currently, the overall contribution of dermal absorption to overall exposure is not known and may be underestimated. With the development of the present protocol, the amount of dermal absorption over time may be better assessed in the field during occupational exposure. Future studies could use this protocol to examine how dermal

PAH exposure contributes to the overall exposure of firefighters to carcinogens during fire suppression. Improving our understanding of dermal exposure and absorption may have greater relevance to consideration of the personal protective clothing worn by firefighters and other occupational groups during exposure to these harmful chemicals to ensure greater safety.

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Vita

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