THE EFFECTS OF ORGANIC AND HARSH CLEANERS ON *ANOLIS CAROLINENSIS* INTEGUMENT

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A Report of a Senior Study

by

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Spring, 2017

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ABSTRACT

Household bleach has been used in the home for cleaning hard surfaces since the 18th century and has caused a number of injuries over time. This study further investigates how detrimental this caustic substance can be to the epidermis of *Anolis carolinensis* in comparison to organically branded products claiming to be safer. Bleach cause significantly reduced cell width (p=0.001) and number (p=0.009) on stratum corneum, whereas the organic cleaner showed no difference. This study illustrates that *Anolis carolinensis* are an appropriate model organism to examine the effects of certain substances on the integumentary system and how the skin recovers could lead to valuable insight in the fields of dermatology and stem cell research.

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CHAPTER I

INTRODUCTION

EVOLUTION OF AMNIOTE INTEGUMENT

The function of skin varies among different groups of vertebrates. In mammals, skin reduces water loss, provides protection against abrasive action and microorganisms, and generally acts as a permeable barrier to the environment (WHO Guidelines 2009). In amphibians and reptiles, skin can be an ornamental sensory mechanism serving as a way of communication and locomotion. Terrestrial amphibians use their skin as an area of gas exchange and contain osteoderms to decrease evaporative water loss in some species (Duellman and Trueb 1994). Osteoderms are also present in some reptiles but serve as a fortification of the scales for defense rather than gas exchange. Cellular renewal is a further characteristic of vertebrate skin and serves to maintain the integrity of structure and its various functions.

Early reptiles had to adapt to the vast difference of life on land versus life in the water. A major challenge facing Reptilia was the production of a barrier in the skin to limit desiccation by evaporative water loss. There was also a need to provide protection from UV radiation and provide mechanical protection from the rough terrain (Chang *et al*. 2009). Patterns of ossified units suggest that Paleozoic amphibians possessed a scaled integument, thus, it seems probable that modern vertebrate integuments are derived secondarily from ancestral conditions of integument that were in some sense ‘scaled’ (Lillywhite 2006).

The two principle layers of skin present in all vertebrates is the dermis and epidermis. The dermis is the deeper layer of connective tissues containing both blood vessels and nerves. The outermost layer is known as the epidermis which is composed of up to seven sub-layers of closely packed cells called strata (Figure 1). The epidermis also expresses a variety of proteins and other molecules that perform various tasks such as: ultra-violet (UV) absorption, homeostasis, immune response, etc. (Lee *et al.* 2006). Whereas there is no vascularization in the epidermis, it obtains nutrients through diffusion of substances to and from the capillaries in the dermis that are juxtaposed to these cells.

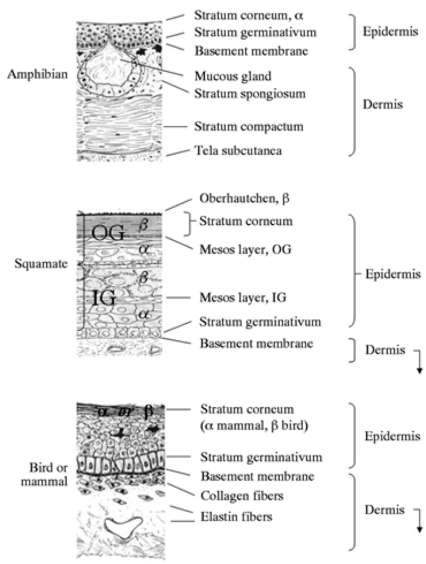

Figure 1: Variation of integument layers among Amphibia, Reptilia, and Mammalia. Taken from Water Relations of tetrapod integument (Lillywhite 2006).

The most superficial layer of the epidermis is the stratum corneum which is a multilayer stratum of flat, polyhedral-shaped, non-nucleated cells called corneocytes which are composed of insoluble bundles of keratins surrounded by a cell envelope. Formation of a stratum corneum over the body surfaces evolved in adult amphibians about 350 million years ago. The stratum corneum functions primarily as a permeability barrier varying in texture and shape. Current knowledge of the this layer's function has come from studies of the epidermal responses to perturbation of the skin barrier such as: (i) extraction of skin lipids with nonpolar solvents; (ii) physical stripping of the stratum corneum using adhesive tape; and (iii) chemically-induced irritation. All such experimental manipulations lead to a transient decrease of the skin barrier efficacy as determined by transepidermal water loss (WHO Guidelines 2009).

During ecdysis (shedding of the skin exhibited by Reptilia), the stratum corneum is shed and replaced by new cells from the stratum germinativum (Porter 1972). Mitotic activity takes place in this basal layer, which proliferates cells that are eventually lost from the animal's surface. All cell types that are present in the epidermis are generated from precursors in the basal layer. Localized cellular proliferation and differentiation give rise to appendages such as claws, glands, hair or feathers, which together with patterned folds or scales, contribute to integumentary form (Lillywhite 2006).

Keratin is a prevalent structural feature of integument and is formed in the epidermis of all living vertebrates, traced back 450 million years ago to epidermal cells of lampreys. Subsequent modification of keratin expression engendered a variety of integuments, allowing for different species to occupy different niches. For example, cyclostomes, most osteichthyans, lissanmphibians and many larval or adult amphibians are protected principally by mucus, and thus the body is covered largely by non-keratinizing epidermis that suffices for aquatic life (Maderson and Alibardi, 2000). Additional mechanical support is provided by a cytoskeleton and a terminal web of keratin filaments in superficial cells.

Amphibians are exceptional among tetrapods in having very little keratin and a thin stratum corneum (Lillywhite 2006). Although keratin was presumably present in basal amphibians, neither extensive keratinization nor synthesis of β-type keratins characterizes the skin of modern amphibian lineages. Therefore, amphibians prevent desiccation through secretion of a superficial aqueous film, which must be replenished, or by shielding the stratum corneum with superficial lipids (Lillywhite 2006).

The skin of amphibians is glandular and produces three principal categories of secretions: mucus, various toxins, and lipids. Mucus plays various roles in the biology of integument and is especially effective in relation to lubrication and keeping the skin hydrated and moist. Those amphibians without a mucus film either avoid drying conditions or absorb water from the substrate around them. Therefore, the importance of mucus secretions in protecting exposed epidermal surfaces from dehydration suggests a fundamental dichotomy of skin organization and water balance (Lillywhite and Maderson, 1988).

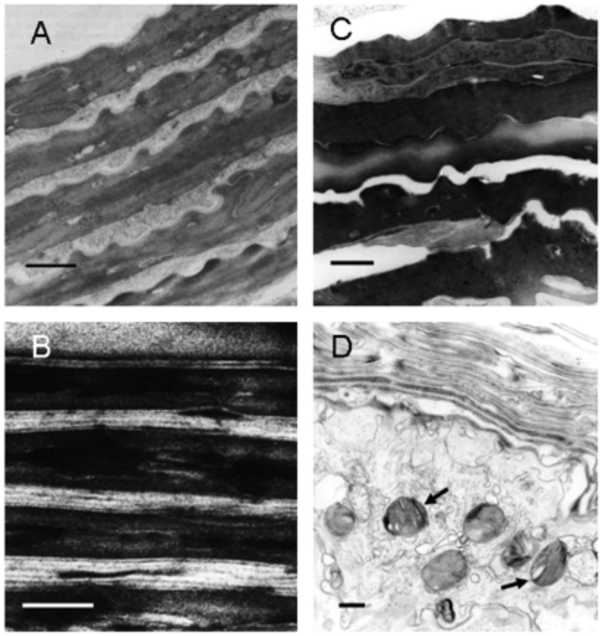

Figure 2: Electron Micrograph showing details of stratum corneum and permeability barrier of terrestrial vertebrates. (A) Section through a portion of cocoon of a burrowing hylid frog, *Pternohyla fodiens*. (B) Section through mesos layer of snake epidermis (*Natrix natrix)*. Laminated lipids occur between darker bands of keratin layers. (C) Section through stratum corneum of human skin. Lipids occur between distinct layers of keratin. (D) Section through epidermis of canary, showing nucleated layers as well as stratum corneum (top). As presented by Lillywhite 2006.

In contrast, reptiles have a thick scale layer made up of β-keratins and other complex lipids. Three typical reptile scales exist today: overlapping, tuberculate and elongated (frill), with overlapping scales being the most common. In some species, the scales are modified into sharp spines to dissuade predators and some are fortified with internal bony plates called osteoderms (Halliday and Adler 2000). But scales do not necessarily provide strength of structure, nor, as commonly misunderstood, do they provide an effective waterproofing function (Roberts and Lillywhite, 1980).

In Sauropsid amniotes (the ancestors of reptiles and birds), a β-keratinized layer formed above the α-keratinized layer and became the major constituents of scales and feathers, providing mechanical support as well as a way to uptake solar energy (Porter 1972). β-keratins have a small molecular weight of about 10-25 kD and exhibit unique arrangements of pleated sheets (Shames *et al*. 1989). Molecular studies suggest that β-keratins in reptiles and birds occupy a functional role analogous to that of mammalian keratin associated proteins (mKAPs, Rogers, 2004). Furthermore, the proteins so far indicated as β-keratins seem to represent the reptilian equivalent of the keratin-associated or matrix proteins present in mammalian hairs, claws, and horns.

The main components of mammalian hair are cysteine-rich type I and type II keratins also known as hard α-keratins or "hair keratins". Eckhard et al. 2008 determined that the genome of chickens contained one type II hair keratin-like gene and the *Anolis carolinensis* (green anole lizard) contained two type I and four type II hair keratin-like genes (expressed most strongly in the digits). This study also suggests that cysteine-rich α-keratins are not restricted to mammals and the evolution of mammalian hair involved the co-option of pre-existing structural proteins.

A comparison of the half-cysteine and glycine content of vertebrate α and Ø keratins suggests that the α and Ø proteins of reptiles may be related to the soft α-keratins of mammals and amphibians (Jean Wyld and Alan Brush 1978). The hard keratin (claws, scales, feathers, etc.) probably represents a uniquely derived group of proteins dissimilar to that of vertebrate keratins. The X-ray diffraction pattern of reptiles is very similar to that obtained from the avian hard keratins, leading to the conclusion that the framework of the filaments was also composed of β-sheets, same as the avian keratins (Fraser and Parry 1996). It has been proposed that the feather β-keratin subfamily (claws, feathers, feather-like, and scale) evolved from the scale β-keratin subfamily through a deletion event followed by gene duplication, whereas others suggest that the feather genes are basal to the avian scale genes (Greenwold and Sawyer 2010). Total or near absence of scales is a derived character in many amphibians, mammals and birds (Maderson and Alibardi, 2000).

In Therapsid amniotes, the ancestors of mammals, scales were lost and the α-keratinized layer was strengthened by mammalian-type HRP (histidine-rich protein) rather than by β-keratins (Wu et al. 2004). α-keratin molecules show a helical arrangement and form polymers. They exist in the epidermis of all vertebrates and have a molecular weight of about 40-70 kDa. It is the presence of HRPs in the stratum corneum of mammals that provides a barrier to water loss. HRP is also known as filaggrin which functions as a keratin matrix protein within the epidermis. It is a highly polar protein while being poor in nonpolar amino acids (Lynley and Dale 1983).

Table 1: Comparison of fibrous protein structures and properties as taken from Principles of Biochemistry (http://www.bioinfo.org.cn/book/biochemistry/chapt07/sim1.htm)

Structure	Characteristics	Examples of occurrence
α Helix, cross-linked by disulfide bonds	Tough, insoluble protective structures of varying hardness and flexibility	α-Keratin of hair, feathers, and nails
β Conformation	Soft, flexible filaments	Fibroin of silk
Collagen triple helix	High tensile strength, without stretch	Collagen of tendons, bone matrix
Elastin chains cross-linked by desmosine and lysinonorleucine	Two-way stretch with elasticity	Elastin of ligaments

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The replacement of β-keratins with HRP may have allowed for the skin to be more flexible, but because of its polar properties the epidermis has lost some of its ability to maintain water balance as efficiently as one with β-keratins. The loss of β-keratins may also have to do with a change of predation and habitat. Reptiles are slower and more susceptible to predation than mammals thus they require a thicker skin to act as a shield against attackers while mammals are more equipped to actively defend themselves (e.g. claws, teeth, etc.). Reptilia also tend to live in drier climates, such as deserts, which means a lot of water would be lost to the air. Therefore, to reduce water loss reptilian skin must be thick and rigid.

Using reptiles as the model of human skin, this study hopes to determine that reptile integument behaves similarly if not the same when exposed to external pollutants or irritants. *Anolis carolinensis* (Green Anole) was chosen as a model for understanding tail anatomy and regeneration in lizards for three main reasons. First, it is a suitable taxon for developing and maintaining captive breeding populations, and colonies of healthy animals can be developed and maintained relatively easily (Lovern et al., 2004). *A. carolinensis* is a small, arboreal iguanid lizard commonly found on some Caribbean islands and in the Southeastern United States, with northern most extent being North Carolina and Tennessee. Green anoles form hierarchies in which the subordinates become browner in coloration and the dominant becomes a bright green (Greenburg *et al.* 1984). While the body and claws share similar amino acids with bird claws and feathers, the head and tail contain similar amino acids also contained in mammalian hair, making them a good candidate for human skin cell comparison.

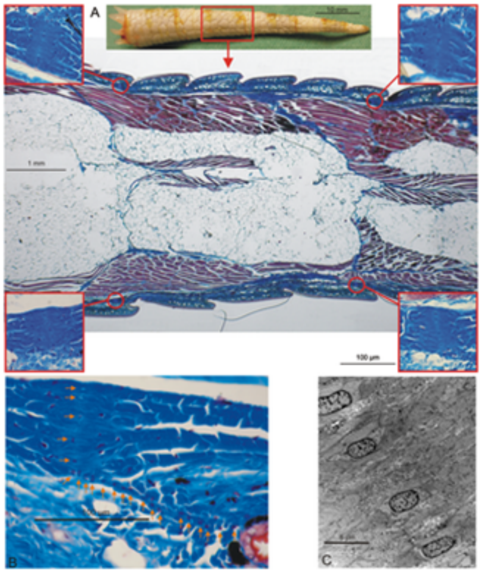

Figure 3: Transverse section of *A. carolinensis* tail as shown in Kristian W. et al 2012 (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0051803)

A daily tasks of janitorial staff is to clean schools, churches, large industries using typically bleach-based cleaners due to the high success of killing microbes. Bleaches based on sodium hypochlorite (NaOCl) are most widely used even in households to disinfect hard surfaces and bleach laundry (Racioppi et al. 2002). Bleach is cheap and easily obtainable, making it a better option for most when cleaning. It is known to be effective against a broad range of pathogens: gram-positive and gram-negative bacteria, fungi, spores and viruses including human immunodeficiency virus (HIV), however it causes acute inflammation followed by necrosis when it comes in contact with all tissue except heavily keratinized epithelium (Mehdipour et al. 2007).

In recent years, there has been a growing demand for safe alternatives to these harsh cleaners, especially in the home setting. This has brought up brands like Honest Company and Simple Green that promise to make their products without potentially health-compromising chemicals or compounds. One of the ingredients in the Honest Company's All Surface Cleaner is Phenoxyethanol which was found as the cause of contact dermatitis in Lovell *et al.* 1984. However, it also contain Citrus grandis oils that have benefits of free radical scavenging (Tsai *et al.* 2007). While these cleaners may be promoted by environmentally savvy industries, this study hopes to determine that these cleaners are in fact safer than bleach by studying the composition of the skin after longer-term exposure.

CHAPTER II

METHODOLGY

TREATMENT AND COLLECTION OF *A. CAROLINENSIS* INTEGUMENT

Animals and Exposure

Fourteen small, unsexed Green Anoles (*Anolis carolinensis*) were purchased from Carolina Biological Supply Company (Item# 147232). Each anole was put into a separate container with holes drilled into the sides and tops. They were randomly split into three groups: control, organic, and bleach. Five anoles served as the control, five as the organic and four as bleach.

The Anoles were kept in a light controlled room on a cycle of twelve hours of light and twelve hours of darkness. Each tank was kept on a tall shelf in order to reduce animal stress and each tank was equipped with plastic plants and a water bowl. Each Anole was fed a diet of 2-3 crickets every two to three days and water was sprayed into each tank daily for consumption and humidity control. Two heat lamps were fixed a safe distance away (12in.) from each enclosure to ensure that one side was well warmed while leaving the other side cool to thermoregulation. These lamps were turned on in the morning and turned off in the evening.

Carefully handling the Anoles by holding them firmly by the torso, the five controls were treated by dipping the tails into a 100 mL beaker of water for 30 seconds to simulate the same stress the organic and bleach might experience. Five Anoles were treated with Honest Co. MultiSurface Cleaner, 26 fl. oz. (Walmart Model#1607027) by dipping their tails into a beaker of 100mL of cleaner for 30 seconds (see Table 2 for active ingredients). The last four were treated with bleach using this dipping method, filling a beaker with 20 mL of bleach

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(Great Value Cleaning Bleach Walmart # 553565758, see Table 2 for ingredients) and filling the rest with water to 100 mL. All applications were done daily for three weeks. At the end of week three each Anole was subjected to a tail clip of 2cm from the tip of the tail, pressing firmly against wounds that bled, and placed immediately into Bouin's fixative in a Falcon tube enough to cover all the tissue. Any premature deaths were immediately subjected to tail clips and fixed in Bouin's before being disposed of properly. Animal husbandry and treatments were approved by Maryville College IACUC approval number 201716 (see appendix 1).

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Endpoints Measured

The clipped tail was placed in Bouin's fixative to prevent decay of the tissue. After one week, the tissue was transferred to 70-75% alcohol to clear out excess fixative. Then the tissue was dehydrated using the procedures listed in Humasin (2016). After dehydration, tissues were infiltrated with paraffin wax under a vacuum for several hours in varying waxes before being embedded in a wax block. Once the wax was dry, excess wax was cut away from the preserved tissue and then the tissue was placed on a microtome to be sectioned as desired. These ribbons were then floated on a warm water bath with a pinch of gelatin in the bath and then they were mounted on slides. These slides were allowed to dry before staining. The staining process is a hematoxylin and eosin staining process discussed in Humasin (2016).

Data Analysis

Once the sections were stained, the prepared slides were viewed under a microscope. The thickness, width, size and shape of the cells were examined among the control, the

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organic treatment and the bleach treatment. These slides were then also compared to a known slide of human epidermis provided by the Maryville College Animal Physiology and Anatomy lab. Any notable changes were marked and photographed.

A t-test assuming equal variance was performed to show whether or not there was significance between the control group vs. organic group, control group vs. bleach group, and organic group vs. bleach group in terms of number of cells in the width of the stratum corneum.

CHAPTER III

RESULTS

QUALITATIVE AND QUANTITATIVE REVIEW OF A. CAROLINENSIS TAIL HISTOLOGY

Quantitative

Measurements of the stratum corneum, which appeared as a dark purple band near the outer edge of the cell, determined that the control, organic and bleach groups had average cell number of three, two and one cells across, respectively (see Figure 1). Cell number was significantly lower in the bleach group compared to the controls (p=0.009). However, there was no significant change between the organic group and the bleach group (p=0.175). Likewise, there was no statistically significant difference between the control and the organic group (p=0.067).

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The average cell width of the stratum corneum was also measured with control groups averaging at 0.55µm, organic groups at 0.57µm, and bleach groups at 0.23µm (see Figure 2). Cell width was significantly less in the bleach tails compared to controls ($p<0.001$), while there was no significant variation between control and organic (p=0.356).

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Qualitative

There were two Anoles, a control and an organic, that died during the experimentation process due to stress and did not receive the full three week exposure. For this reason the results from these two individuals were removed from the rest of the data.

There does appear to be a slight thinning of the Oberhauchen layer, shown in light pink outside of the stratum corneum that may be a result of increased shedding to relieve skin cell damage or agitation. Furthermore, there is the appearance of a thin black layer beneath the stratum corneum of the organic and bleach groups--worse so in the bleach groups. The composition of this mass is unknown and would require further investigation to determine its composition and its formation.

While the organic group varied slightly from the control in thickness and composition, there is an abundance of damage to the dermis in the bleach groups where there are obvious signs of thinning to 1 cell across (see Figure 3C). There is also a more prominent Oberhauchen layer in some areas and areas where it is nearly nonexistent. There also appears to be less structural integrity within the tail itself. Most individuals in the bleach samples had blackened tails which confirms the atrophy of the cells within the tail.

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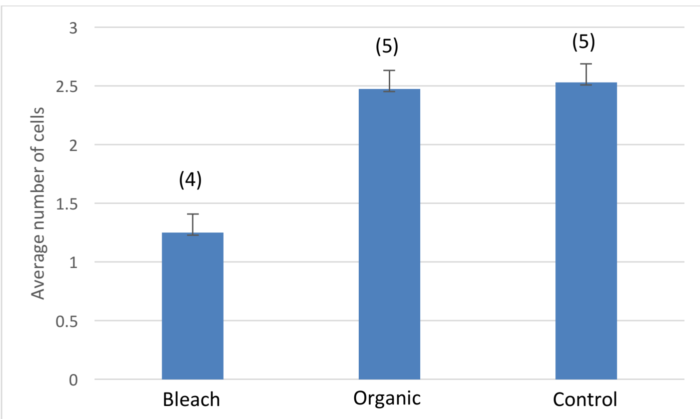

Figure 4 (FIX IN TEXT): Comparison of average cell number+1 SE in stratum corneum after exposure to generic bleach, Honest Company Multipurpose cleaner (organic), and water (control).

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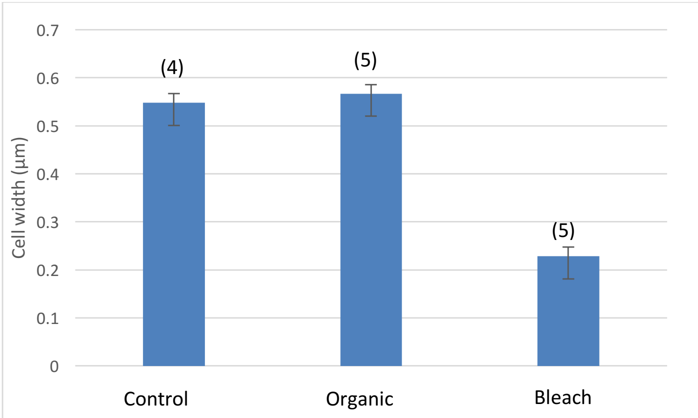

Figure 5 (FIX IN TEXT): Comparison of average cell width after exposure to generic bleach, Honest Company Multipurpose Cleaner (organic), and water (control) for 3 weeks.

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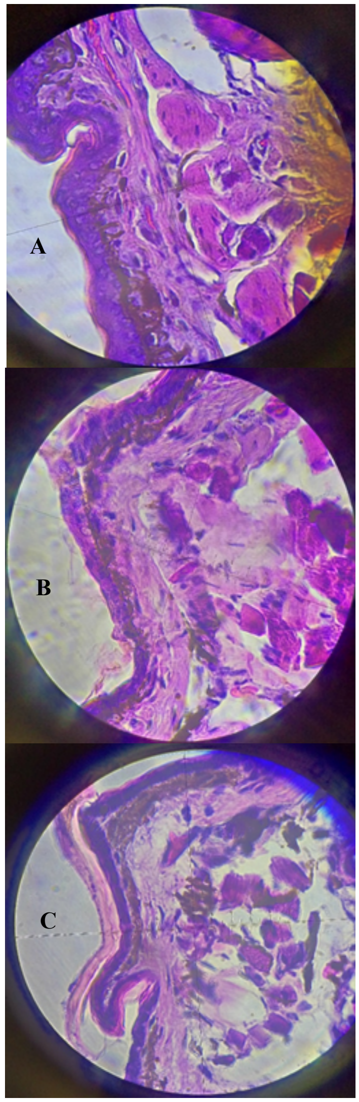

Figure 6 (FIX IN TEXT): *Anolis carolinensis* tail sample stratum corneum after three weeks exposure to water (control, A), Honest Company MultiPurpose Cleaner (organic, B), and Bleach (C) under 400x magnification

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CHAPTER IV

DISCUSSION

ANALYSIS OF *A. CAROLINENSIS* AS A HUMAN INTEGUMENT MODEL

The results of this study show that household bleach does have an effect on the structural integrity of the epidermis, specifically the stratum corneum, while there is little effect of organic cleaners on these cells. Thus, buying less caustic cleaning products would save the consumer the potential of doing one's self harm or potential others during disposal of said products.

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Without a doubt, the integumentary system remains a vital aspect of all creatures in which it plays roles from immune defense and physical protection to locomotion and sensory communication. When exposed to certain substances this structure and its functions begin to diminish, but in many cases the skin can heal and regenerate itself.

BETTER TRANSITION NEEDED…Studies done on skin regeneration have begun to focus on the use of human umbilical cord Wharton's jelly–derived mesenchymal stem cells (hUC-MSCs) in the recovery of deep skin injury. In this study a middorsal, full thickness incisional wound was performed on mice and new regeneration of Langerhans cells, sebaceous glands, sweat glands and epidermis could be seen (Zhang et al. 2012). Similarly, Kishi et al. 2012 found that fetal regeneration may be caused by reduced expression of TGF-β1 and higher levels of hyaluronan in the extracellular matrix, allowing for wounds to heal without inflammatory responses, granulation proliferation, and scar formation as is observed in adults. Therefore skin injuries passed the gestation period remain a growing concern especially in the area of fully body burns.

The exposure of the skin to even minor levels of bleach has shown just how sensitive the skin truly is. Hypochlorite, a component of bleach, was determined to exert profound cytotoxic effects on fibroblasts--which play a role in wound healing--as low as 0.05% of concentration. After four hours of exposure and a concentration less than 0.01% the mitochondria's survival diminished from 71% to 10% (Racioppi et al. 1994). Sodium hypochlorite (NaOCl) has a pH of 11-12.5 which causes injury primarily by oxidation of proteins (Mehdipour et al. 2007). It is likely that since bleach is intended to kill bacteria that it could compromise the healthy bacteria living on the skin upon contact and perhaps in serious cases it could affect the Langerhans cells of the epidermis that alert the immune system to invading viruses and bacteria.

The stratum corneum provides one of the key factors to regulate cutaneous sensitization and by understanding this barrier is important for understanding self-defense mechanisms because of its close functional link to skin-associated innate an adaptive immunity. Accumulating evidence also shows that enhanced cutaneous sensitization is one of the major causes of many allergic disorders including atopic dermatitis, asthma, food allergy and anaphylaxis (Matsui et al. 2015). While reptile stratum corneum possesses a highly impermeable barrier based on stiff epidermis, mammalian stratum corneum has evolved to be highly moistened, resulting in the acquisition of a soft epidermis and thus leaving our skin susceptible to infections and/or allergy with a complicated crosstalk between innate and adaptive immune systems.

The Langerhans cells are located in the stratum spinosum beneath the stratum granulosum which rests under the stratum corneum. These cells are thought to be involved in induction of antigen-specific TH2 responses as well as maintenance of peripheral tolerance. In the resting state the tips of the Langerhans cell dendrites are aimed at the apical side of the stratum germinativum. Once activated, they extend their dendrites through the tight junction beneath the stratum corneum where external antigens can be absorbed (see Figure ???). Furthermore, the function and dysfunction of the stratum corneum determines the nature and quality of cutaneous sensitization.

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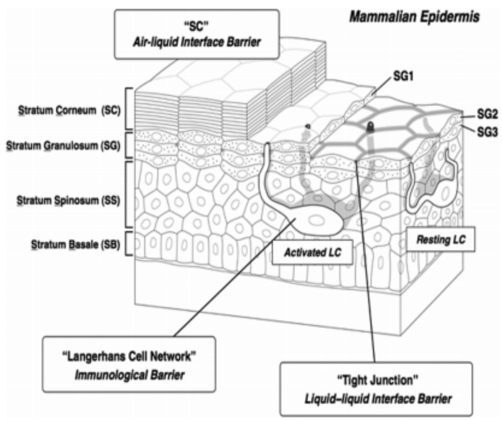

Figure ????: Structure of mammalian epidermis and its three barrier elements as taken from Matsui et. al 2015.

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Although recent research has told a great deal about the regenerative abilities and factors of lizards' tails, there has been little discussion about the importance of their integument system as a model for human integument. The present study confirms it is possible for *Anolis carolinensis* to serve as a model for humans due to the similarities of the

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stratum corneum; however, it is still uncertain what the exact mechanism of skin regeneration is and to what extent it can be modified. A future study of the anole tails is warranted to analyze the recovery process after the exposure to environmental irritants.

What is already known about anole skin regeneration on the tail after autotomy (tail loss) is that remnants of the torn original integument collapse over the site of loss and, within days, a wound epithelium begins to form. There is also an alignment or chain of cell nuclei that localize in the dermis creating a cellular zipper that may facilitate controlled rupturing of the dermis during autotomy (Gilbert et al. 2015). Some lizards are also capable of what is known as regional integumentary loss. Regional integumentary loss seems to be associated with a discrete zone of structural weakness within the stratum compactum of the dermis that are not located in the fractural plane (the area of breakage between scales that forms during autotomy). Not much is truly known about the mechanisms.

There also appears to be little histology analysis of reptilian tail tissue specifically investigating skin and its layers, and many questions remain. What are the mechanisms of skin regeneration in lizards and is the mechanism similar if not the same in humans? If they are different, can these mechanisms be mimicked in humans? With a focus on burn recovery, could lizards be used to study the effects of burns and how the regenerative process proceeds or is inhibited? Understanding these principles of the integument would not only provide insight into the anatomy and physiology of lizards but also serve as a valuable complement to the fields of dermatology and potential regenerative medicine.

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APPENDICES

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