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SKIN FROM HORSES WITH HEREDITARY EQUINE REGIONAL DERMAL ASTHENIA (HERDA) CONTAINS COLLAGEN CROSSLINKING PATTERNS THAT ARE ASSOCIATED WITH REDUCED TENSILE STRENGTH

By

Ashley Arwen Hill

A Thesis
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Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Science
in the Department of Clinical Sciences,College of Veterinary Medicine

Mississippi State, Mississippi

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Ashley Arwen Hill

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By

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Hereditary equine regional dermal asthenia (HERDA) is a recessive connective tissue disorder of Quarter Horse lineages. This study correlates previously identified decreases in skin tensile strength in HERDA with abnormal dermal collagen cross linking patterns that are also identified in urine from HERDA horses. Dermal collagen from HERDA horses has significantly less pyridinoline and significantly more deoxypyridinoline than control or carriers. Concentrations of hydroxylysine, the rate limiting substrate for these crosslinks were significantly lower in HERDA versus control and carriers. These characteristics of HERDA skin parallel humans with a similar syndrome of skin fragility, Ehlers Danlos Syndrome TypeVIA. This is the first biochemical evidence explaining the clinical skin fragility that characterizes HERDA and suggests that altered collagen lysine metabolism may be physiologically relevant to the clinical manifestation of HERDA. Evaluations of mature scars indicate that lesion and nonlesioned skin should not be viewed as biologically equivalent in HERDA investigations.

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DEDICATION

I would like to dedicate this project to my husband Rob, my parents Diane and Charles, and the rest of my family and friends who have encouraged me to pursue my dreams and make them a reality.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Marzia Pasquali and Liz Schwarz for inviting me into their lab at ARUP Laboratories and all of the expert advice and assistance they provided with this project. I would not have been able to complete this project without the help of Dr. Eleanor Cooke, Nisma Mujahid, Michael Tibbs, Jesse Grady, and Dr. Sumalee Girvuangsawat for completing the statistical analysis. Without the great committee members' assistance I would not have been able to complete this project.

Finally I would like to give a special thanks to Dr. Cyprianna Swiderski. Dr. Swiderski has given me more than I can ever truly thank her for by providing me with unending support and all of the little pushes I have needed to complete this project. I only hope that this experience has been as rewarding for her as it has been for me.

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CHAPTER I

INTRODUCTION

Hereditary equine regional dermal asthenia (HERDA), also known as hyperelastosis cutis (HC) is an autosomal recessive connective tissue disorder, first described in 1978, that affects Quarter Horses or horses of Quarter Horse lineage.[1,2] The skin of affected horses has been described as feeling 'doughy' or 'mushy' to the touch with areas that are poorly attached and hyperextensible, and other areas that are fragile and thin. Skin lesions, including subcutaneous hematomas and open wounds occur from normally innocuous contact, especially along the dorsum.[3] These wounds, which are slow to heal, result in disfiguring scars.[4] A horse affected by HERDA cannot be ridden or shown competitively and most are humanely euthanized. Prior to the availability of a DNA test in 2007 that identifies horses with HERDA, the condition was recognized when lesions developed in association with saddling, as the horses entered training around the age of 1.5-2 years. However, there appears to be variation in the severity of the disease with some horses developing signs shortly after birth.[5]

HERDA is an autosomal recessive connective tissue disorder with affected progeny (homozygotes) inheriting one copy of the genetic defect from the sire and the other copy from the dam.[2,6,7] Horses that inherit only one copy of the gene from either the sire or the dam never show clinical signs of HERDA and are considered to be 'carriers'. These asymptomatic heterozygotes are a source for the propagation of the HERDA trait. Although a point mutation in the peptidyl-prolyl isomerase B gene (*PPIB)*

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coding for cyclophilin B is associated with HERDA and is used to identify carrier and affected horses,[8] the method by which this mutation causes the clinical signs of HERDA is not yet documented. Tryon and colleagues have estimated the allelic frequency of the mutant PPIB allele to be 0.021 in the Quarter Horse population, with a disproportionate presence of cutting horses.[9] Horses consanguineous for the prolific sire Poco Bueno (AQHA 3044) have a 58x greater risk of developing HERDA when compared to other Quarter Horse lineages.[2] This is problematic because this bloodline is highly desirable in various equestrian disciplines, particularly cutting, and increases the chances of breeding two horses that carry the HERDA trait.[2] A number of obligate carriers have contributed disproportionately to the breeding population of Quarter Horses allowing the HERDA trait to propagate. Currently the carrier rate is estimated to be 28% in elite cutting horse lines.[10] Further, the large economic value of the affected bloodlines may prove for some a disincentive to responsibly identify carriers. Accordingly, in 2009 the AQHA mandated DNA testing to identify the mutant PPIB allele in all horses tracing to Poco Bueno or his dam Miss Taylor. Horses homozygous for the PPIB mutation cannot be registered with the AQHA.

The homozygosity mapping experiments that identified the PPIB mutation in horses with HERDA identified a common haplotype across the q arm of equine chromosome one (ECA1q) in sixty-four of the sixty-eight horses originally diagnosed as affected by HERDA.[11] Horses with this haplotype were also found to have four homozygous DNA markers in common, two of which code for a missense mutation of PPIB. The first mutation identified as an adenine to guanine at position 17 in the coding sequence (c.17A>G) and predicted to cause a glutamic acid to glycine substitution at position 6 (p.6E>G) in the signal sequence of the translated protein was not considered

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significant as it is common in the Quarter Horse breed. The putative identifying mutation for HERDA was a guanine to adenine mutation at position 115 (c.115G>A) in the coding sequence which is predicted to cause a glycine to arginine (p.39G>R) substitution at position 39 in the translated protein. The wild type c.115G in PPIB is highly conserved across all vertebrates.

Central to the purpose of investigations presented here is the fact that a causal association between the c.115G>A PPIB mutation and HERDA has not been established.[8] Problematic is the absence of these mutations in 4 of the 68 horses originally identified as the HERDA reference population. Of these four horses, one was dismissed as an inaccurate diagnosis with a justification that the horse had "begun training for performance events", one horse possessed the atypical genotype upon re-testing, and two horses were not available for follow up. However, clinical diagnosis of HERDA can be difficult in less severe cases.[4] In addition, the region of the genome identified as identical by descent on ECA1*q* could only be refined in fine structure mapping to 2.5 Mb with the available equine single nucleotide polymorphisms. Based upon sequence comparisons to primates, mouse, dog, and cow, this region of large linkage disequilibrium was reported to contain approximately 20 genes.

Rationale for the c.115G>A PPIB mutation as a putative causal mutation of HERDA is derived from the activities of the coded protein Cyclophilin B. Cyclophilin B has conventionally been viewed as the primary rate limiting peptidyl-prolyl cis trans isomerase in fibrillar collagen synthesis.[12] In this role, CyPB is thought to accelerate the folding of collagen by catalyzing the isomerization of peptidyl-proline bonds, making this protein a logical candidate gene for the histological characteristics of HERDA. More recently CyPB has been shown to form an intracellular collagen-modifying complex with

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cartilage associated protein (CRTAP) and prolyl-3-hydroxylase-1 (P3H1) which 3-hydroxylates proline at position 986 (P986) in the alpha1 chains of type I and type II collagen.[13,14] Mutations in any of these three genes have been shown to decrease P986 hydroxylation and cause severe or lethal autosomal recessive osteogenesis imperfect (OI).[13-16] OI is a disorder characterized by osteoporosis, bone fragility, and fractures that may be accompanied by bone deformity, tooth malformations, short stature, and shortened life span. PPIB mutation and total absence of CyPB have been shown to induce severe OI in both humans[13] and genetically altered mice[17], respectively. However, the presumed role of PPIB in P986 hydroxylation, and cis-trans isomerization of proline residues and associated collagen folding has been recently questioned by a homozygous start codon mutation of PPIB identified in human siblings that prevents translation.[18] The OI phenotype of these individuals lacks rhizomelia (shortening of the proximal portions of the limbs) and collagen from these individuals is both normally folded and has normal P986 hydroxylation suggesting that CyPB is not the exclusive peptidyl-prolyl cis-trans isomerase that catalyzes the rate-limiting step in collagen folding, and is not required for P986 hydroxylation as is currently thought. While the role of PPIB in collagen synthesis clearly requires clarification, it is relevant that bone fragility has not been demonstrated in HERDA horses which possess the c.115G>A PPIB mutation.

Despite its distinction from OI, HERDA does share clinical similarities with other diseases in the heterogeneous group of human inherited connective tissue disorders termed, Ehlers-Danlos syndrome (EDS), particularly those diseases characterized by loose, hyperextensible, and easily torn skin.[15,16,19] The human syndrome is genetically diverse and similar conditions have been described in domestic and laboratory animals

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including cattle, dogs, rabbits and cat.[1,6,20-24] Diseases in the EDS complex have been classified by genetic, clinical and biochemical characteristics.[15] EDS VI is an autosomal recessive disorder caused by defective hydroxylation of lysine residues on procollagen peptides.[25] EDS VI patients, therefore, have decreased hydroxylysine residues that are essential for forming covalent pyridinium crosslinks, specifically pyridinoline (PYD) and deoxypyridinoline (DPD) (also termed hydroxylyslpyridinoline and lyslpyridinoline, respectively), that give collagen its tensile strength.[8,26-28] Pyridinoline, the predominant pyridinium crosslink found in normal individuals, results from three hydroxylysine residues.[29] In contrast, deoxypyridinoline requires only two hydroxylysine residues and one lysine residue.[29] When compared to normal individuals, patients with EDS VI (Type A) have reduced total pyridinium crosslinks in skin, cultured skin fibroblasts, and urine with deoxypyridinoline levels far exceeding pyridinoline.[30,31] Consequently, the ratio of deoxypyridinoline:pyridinoline is markedly increased.

Clinical parallels between HERDA and the Ehlers-Danlos Syndrome (EDS) prompted our laboratory to investigate a possible role for defective hydroxylation of collagen lysine residues, which are critical to the formation of collagen pyridinium crosslinks, specifically in EDS VIA. The pyridinium crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPD), are the intermolecular bonds of collagen.[19] In normal individuals, PYD is the predominant type I collagen crosslink.[19] Our laboratory has demonstrated that the ratio of DPD to PYD in the urine of horses with HERDA is significantly elevated when compared to normal horses.[32] Furthermore, this assay is diagnostically valid for the identification of horses with HERDA. The mean ratio difference in the urine DPD:PYD ratio between affected and control horses was 2.48. The 95% CI for the difference between the means was as follows: 2.3, 2.67. In humans,

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similarly elevated urine DPD:PYD levels are diagnostic of human EDS Type VIA, which results from defective activity of collagen lysyl hydroxylase.[19]

Patients with Ehlers Danlos Syndrome Type VI fall into two categories. In Type VIA, the most common form, the biologic activity of the enzyme lysyl hydroxylase (LH)-1 which is coded for by the gene procollagen-lysine, 2-oxoglutarate 5-dioxygenase(PLOD)-1 is decreased.[33] To date more than 20 mutations causing EDS VIA have been characterized in the PLOD1 gene.[33-35] The second form of EDS VI, Type VIB, reflects a phenotypic classification based largely upon clinical parameters. These individuals have normal biologic activity of the enzyme LH using in vitro assays and normal urine DPD:PYD ratios.[36] One patient with EDS VIB had fewer hydroxylysine residues in collagen relative to the normal cohort.[8] The evolving identification of multiple LH isoenzymes (enzymes with similar activity and function coded by different genes or portions of genes), including LH1, LH2, its splice variants LH2a and LH2b, as well as LH3 may provide insight into the disparity in LH activity in Type A vs. Type B EDS VI. In contrast to EDS VIA, three EDS VIB patients have normal LH1 mRNA synthesis with decreases in one or both of the LH2 and LH3 isoenzymes.[36] Accordingly, in vitro assays of LH activity appear to be insensitive to decreases in the biologic activity of LH2 or LH3 and more reflective of LH1 activity, accounting for the normal LH activity in the face of clinical signs in EDS VIB patients. Though there is still debate, at this juncture a growing body of evidence suggests that the primary enzyme responsible for hydroxylation of lysine residues within the triple helices of the collagen molecule (that serve as substrates for pyridinium crosslink formation) is LH1. Furthermore, hydroxylation of the lysine residues on the ends of adjacent collagen molecules (ends of

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the collagen molecule are termed telopeptides) may be dependent upon the activity of other isoenzymes of LH.[37]

Our co-investigators recently demonstrated that the tensile strength of skin from HERDA horses is decreased relative to that of unaffected control horses.[38] This investigation evaluates the hypothesis that observed experimental and clinical evidence of skin fragility in HERDA result from alterations in the intermolecular crosslinks responsible for the tensile strength of dermal collagen, specifically pyridinoline (PYD) and deoxypyridinoline (DPD). Differences in these parameters associated with skin healing are also evaluated. The biochemistry of dermal collagen pyridinium crosslinking is assessed by comparing lysine, hydroxylysine, DPD and PYD concentrations as well as the DPD: PYD ratios in the skin of HERDA affected, normal, and carrier horses.

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CHAPTER II

MATERIALS AND METHODS

Animal Tissues:

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC). Skin samples for experiment #1 were aseptically obtained following sedation with detomidine (0.004 mg/kg) and butorphanol (0.004 mg/kg) from 21 horses including 6 horses with HERDA, 6 HERDA carriers, and 9 control horses lacking the HERDA trait. A portion of the study population in experiment #1 predated the availability of DNA testing. For these horses HERDA was confirmed based upon the presence of severe lesions in animals that were consanguineous for Poco Bueno within 8 generations. Severe dermatologic manifestations of the disease were characterized as subcutaneous hematomas, open wounds, and hypertrophic scars, associated with contact trauma. Both carrier and control horses lacked clinical manifestations of dermatologic disease. All carrier horses were positively identified as heterozygous for the PPIB mutation. HERDA was excluded from the control population by either breed selection (1 Arabian, 1 Tennessee Walking Horse, and 1 Thoroughbred), absence of implicated lineage (3 American Paint Horses) or absence of PPIB by DNA testing (6 horses of Quarter Horse descent). PPIB testing was performed courtesy of Dr. Nena Winand, Cornell University. HERDA affected individuals were Quarter Horses ranging from 19-48 months of age (mean: 34.8 months), consisting of 3 geldings, 2 stallions, and one female. HERDA carriers consisted of 4 Quarter Horses and 2 Quarter

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Horse/Thoroughbred crosses and ranged from 4-14 months (mean: 5.6 months), consisting of 1 gelding and 5 stallions. Control horses ranged from 30-288 months of age (mean: 123 months), consisting of 7 geldings, 2 stallions, and 1 female. The ratio of DPD : PYD in the urine of the control and HERDA affected group was also evaluated, and consistent with our prior findings, horses with HERDA had significantly higher ratios (>2.0).[2]

In experiment #2, a subset of horses from experiment #1 consisting of 3 HERDA affected horses and 3 control horses were also biopsied in regions of the skin that either contained lesions representative of HERDA, or evidence of scarring in the case of controls, respectively. Mature scars were selected in control horses for comparisons to mature scars in HERDA skin because both samples would be reflective of collagen biochemistry associated with skin healing. Three HERDA horses with lesion biopsies ranged in age from 19-43 months and included one stallion, one gelding and one female. Control horses with scarred skin included an American Paint Horse gelding, a Quarter Horse/Thoroughbred cross gelding, and a Quarter Horse female. The age range for scarred controls was 48-268 months.

Skin Hydrolysates:

Skin samples were incubated overnight in 4 ml of 2M NaBr solution to remove subcutaneous fat, epidermis, and hair. Once the dermis was isolated the samples were hydrolyzed at 150°C under vacuum for 16 hours in 6M HCl.[39] Portions of the resultant hydrolysates were analyzed to determine pyridinium cross-links and amino acid composition.

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Pyridinium Crosslink Quantification:

Extraction of the pyridinium cross-links, DPD and PYD, from the hydrolysates was performed according to established procedures.[30,31,39,40] Briefly, 500 ul of hydrolyzed dermis was fractionated on a freshly prepared cellulose column (2.5 ml of 5% CF-1 slurry in a solution of 7.3 M 1-butanol and 2.9 M glacial acetic acid in Nanopure water). Pyridinium crosslinks, PYD and DPD, were eluted with Nanopure water, lyophilized, reconstituted with 1% heptafluorobutyric acid and analyzed by reverse-phase high pressure liquid chromatography (HPLC) on a Waters HPLC System 625, equipped with a fluorescence detector with excitation at 297nm and emission at 395 nm. Pyridinium cross-links were separated on a Waters Nova-Pak C18 column (4µm; 15cm x 3.9mm) protected by a Waters Sentry Guard Column (Nova-Pak C18; 4µm; 2cm x 3.9mm).[39] Eluant A was 0.01M *n*-heptafluorobutyric acid (HFBA) in 100% water. Eluant B was 0.01M *n*-heptafluorobutyric acid in 100% acetonitrile. The column was equilibrated in 82% A and 18% B; the samples were eluted with the same isocratic gradient at a flow rate of 1 ml/min for 22 min. The column was then stripped with 100% B for 5 min and re-equilibrated with 82% A and 18% B before the next injection. PYD and DPD in skin hydrolysates were identified by comparison and co-elution with commercial reference standards.

Amino Acid Quantification:

Amino acid concentrations in the dermal hydrolysates were determined using a Biochrom 20 Plus amino acid analyzer. Briefly, 200 ul of dermal hydrolysate were dried under nitrogen, resuspended in 1 ml of HPLC grade water, lyophilized, and then resuspended in 200 ul of loading buffer (lithium citrate pH 2.2, Biochrom product # 80-2038-10). Samples were analyzed as a 1:22 and 1:100 dilution in a mixture containing 50

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ul of internal standard (500 µmol/l S-(2-aminoethyl)-L-Cysteine) and loading buffer. Collagen content of each sample was extrapolated from the measured hydroxyproline concentrations where the mass of collagen is seven and a half times the measured hydroxyproline mass and the molecular mass of hydroxyproline is 300,000.[29] PYD, DPD, hydroxylysine, and lysine concentrations were normalized to collagen content for intersample comparisons.

Statistical Analysis:

Statistical analyses were implemented in SAS® System for Windows, Version 9.1.3. Descriptive statistics for each variable (PYD, DPD, Total Crosslinks, DPD: PYD, Lysine, and Hydroxylysine) were determined using the UNIVARIATE procedure with normality assigned using the Kolmogorov-Smirnov statistic. Data were analyzed to assess the relative efficacy of the treatment (lesion, nonlesion, HERDA, carrier, or control) by using a non-parametric one way ANOVA (NPAR1WAY) procedure where the data were not normally distributed or of unequal variance to provide the nonparametric analysis. The general linear model (GLM) procedure was employed where the data were normally distributed to provide the analysis of variance. Post hoc analysis was performed using the least square (LS) means test to determine the differences between treatments when statistically significant differences ($p<0.05$) were identified by NPAR1WAY or GLM. This is the first paragraph of your text. Please note that paragraphs are indented automatically, so you can just hit the Enter key at the end of a paragraph and continue typing.

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CHAPTER III

RESULTS

Experiment 1 contrasted the pyridinium crosslink, lysine and hydroxylysine concentrations in the dermal collagen of 3 groups of horses: HERDA homozygotes, HERDA carriers, and control horses lacking the HERDA trait. Data are represented graphically for the concentrations of PYD, DPD, total pyridinium crosslinks, as well as the DPD:PYD ratio in Figures 1-4, respectively. Horses with HERDA had greater concentrations of DPD (x=99.4 $\pm$ 49.8 pmol/nmol collagen), and a higher DPD:PYD ratio (x=4.01 $\pm$ 0.68) in dermal collagen than carrier (DPD: 17.2$\pm$ 6.39 pmol/nmol collagen; DPD:PYD ratio x=0.36 $\pm$ 0.2) or control horses (DPD: x=36.1 $\pm$ 22.3; DPD:PYD: x=0.37 $\pm$ 0.05) and these differences were highly significant (DPD: p=0.001 for affected vs. carrier, p=0.007 for affected vs. control; DPD:PYD: p<0.0001 for affected versus normal or carrier). Conversely, horses with HERDA also demonstrated significantly lower concentrations of PYD (x=23.8 $\pm$ 9.32 pmol/nmol collagen) than carrier (PYD: x=60.1 $\pm$ 36.1 pmol/nmol collagen) or control horses (PYD: x=80 $\pm$ 30.7 pmol/nmol collagen). Despite the significant increase in DPD concentration and decrease in PYD concentration observed in the dermal collagen of HERDA horses, total pyridinium crosslink concentrations were not significantly different between groups (HERDA: x=123.2 $\pm$ 59 pmol/nmol collagen; Control x=101.7 $\pm$ 56 pmol/nmol collagen; Carrier: horses x=77.27 $\pm$ 37.3 pmol/nmol collagen). In contrast to the differences identified in comparisons to HERDA affected horses, PYD, DPD, and total pyridinium

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crosslink concentrations were not significantly different between carrier and control groups. The DPD:PYD ratio in carrier vs. control horses was also equivalent (carrier: x=0.36 $\pm$ 0.20 ; control: x=0.37 $\pm$ 0.05). These results indicate that despite equivalent total pyridinium crosslink concentrations in HERDA, carrier, and controls, horses with HERDA have greater concentrations of DPD, and lower concentrations of PYD which collectively contribute to a marked elevation in the DPD:PYD ratio. Whereas, PYD, DPD, total pyridinium crosslinks, and the DPD:PYD ratio do not differ between HERDA carriers and control horses.

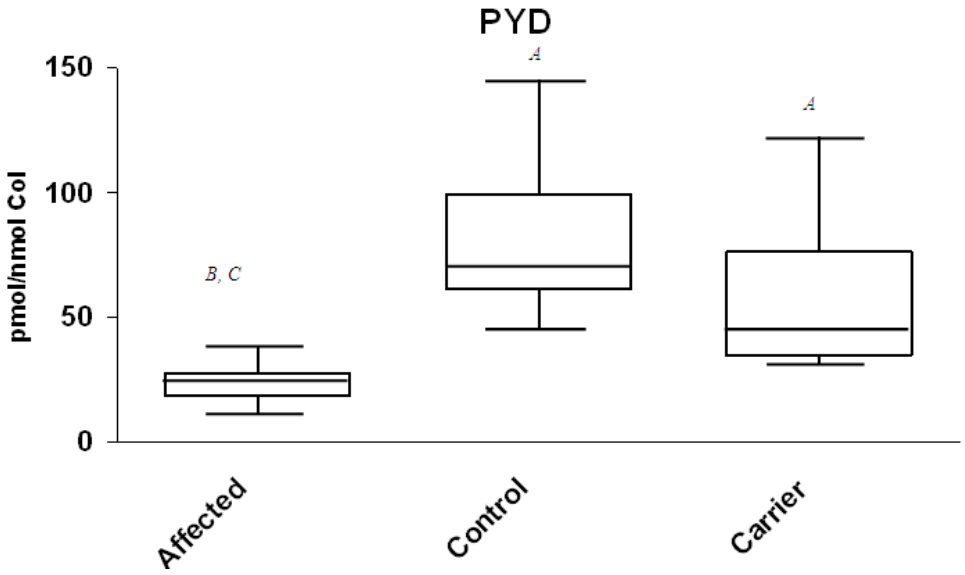

Figure 3.1 Pyridinoline concentrations (pmol/nmol collagen) from 6 HERDA affected, 6 HERDA carriers and 9 control horses. Statistically significant differences between groups are denoted by letters A (affected), B (control) , and C (carrier) above the box plots ($p \leq 0.05$).

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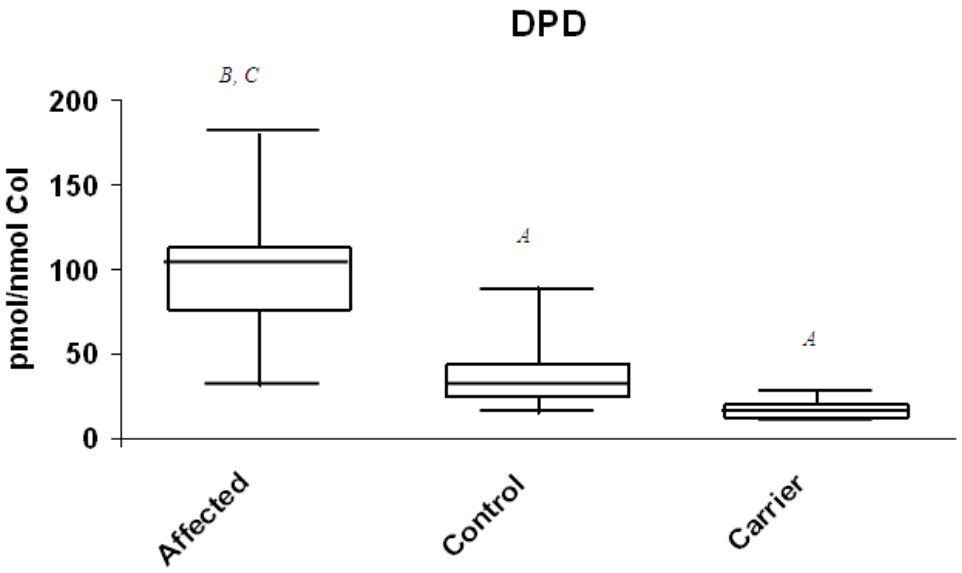

Figure 3.2 Deoxypyridinoline concentrations (pmol/nmol collagen) from 6 HERDA affected, 6 HERDA carriers and 9 control horses. Statistically significant differences between groups are denoted by letters A (affected), B (control) , and C (carrier) above the box plots ($p \leq 0.05$).

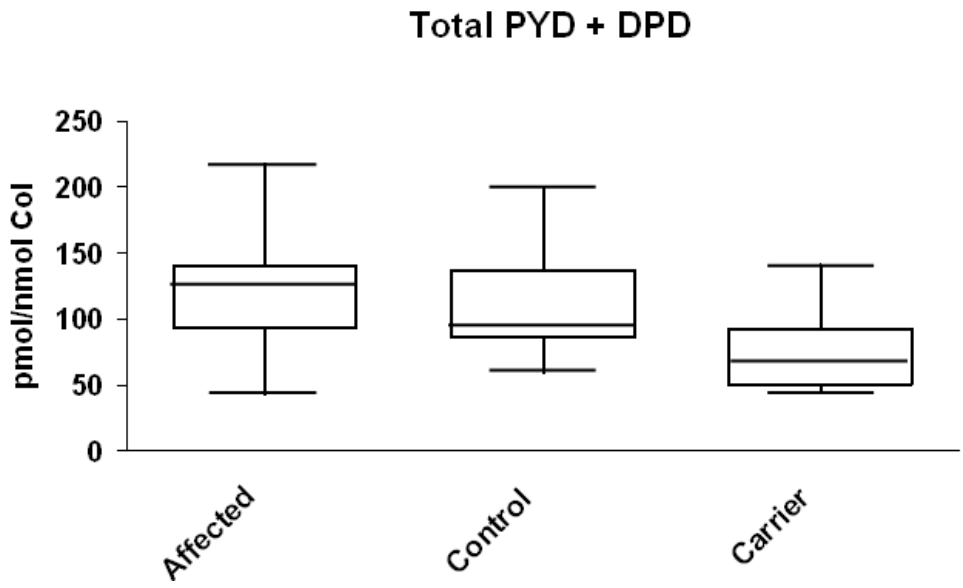

Figure 3.3 Total Pyridinium Cross-link concentrations (pmol/nmol collagen) from 6 HERDA affected, 6 HERDA carriers and 9 control horses. Statistically significant differences between groups are denoted by letters A (affected), B (control), and C (carrier) above the box plots ($p \leq 0.05$).

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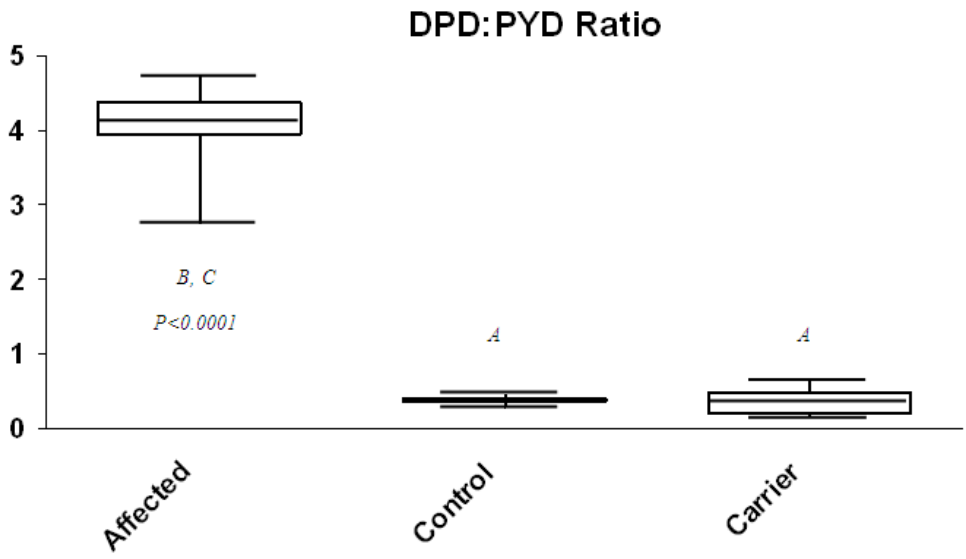

Figure 3.4 The ratio of Deoxypyridinoline to Pyridinoline from 6 HERDA affected, 6 HERDA carriers and 9 control horses. Statistically significant differences between groups are denoted by letters A (affected), B (control) , and C (carrier) above the box plots ($p \leq 0.05$).

Hydroxylysine, a key substrate for the formation of pyridinium crosslinks, is synthesized during post-translational modifications of lysine residues in procollagen by the action of lysyl hydroxylase-1. We identified a highly significant decrease in hydroxylysine concentrations of dermal collagen from HERDA horses (x=8.46 $\pm$ 0.96 nmol/nmol col) relative to that of carrier (hydroxylysine: 22.18 $\pm$ 2.36 nmol/nmol col, $p<0.0001$) or control horses (hydroxylysine: 19.01 $\pm$ 1.53 nmol/nmol collagen, $p<0.0001$). This data is represented graphically in Figure 5. Elevations in hydroxylysine concentrations of carrier relative to control horses were also significant ($p=0.0017$). Lysine concentrations in dermal collagen (Figure 6) of HERDA horses (x= 131.25 $\pm$ 15.8 nmol/nmol collagen) were significantly lower ($p=0.041$) than carrier horses (x= 152.57 $\pm$ 21.6 nmol/nmol collagen) and higher than controls (x= 118.44 $\pm$ 14.1 nmol/nmol collagen). Though the latter difference was not statistically significant, the higher lysine

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concentrations observed in carrier relative to control groups was highly significant (p=0.0009).

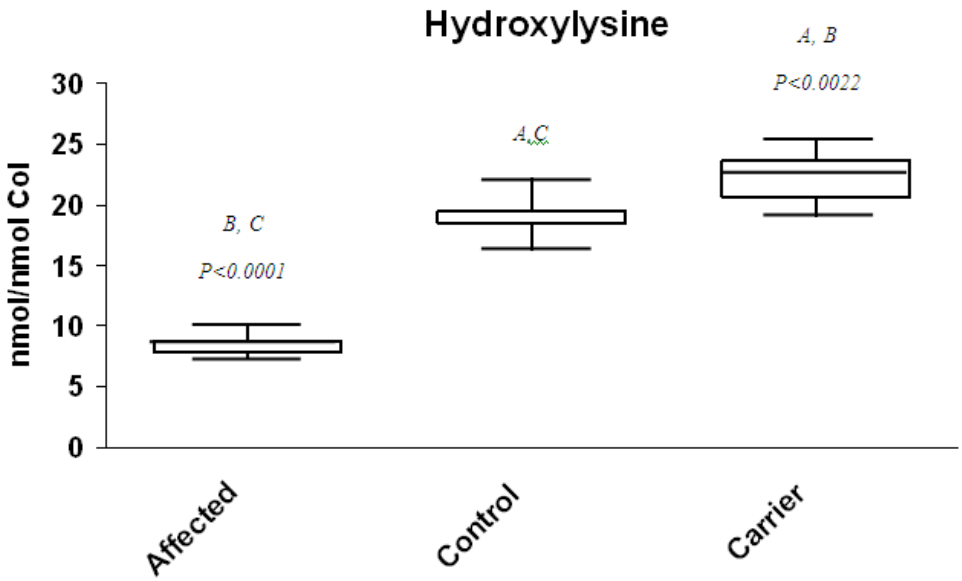

Figure 3.5 Hydroxylysine concentrations (nmol/nmol collagen) from 6 HERDA affected, 6 HERDA carriers and 9 control horses. Statistically significant differences between groups are denoted by letters A (affected), B (control) , and C (carrier) above the box plots ($p \leq 0.05$).

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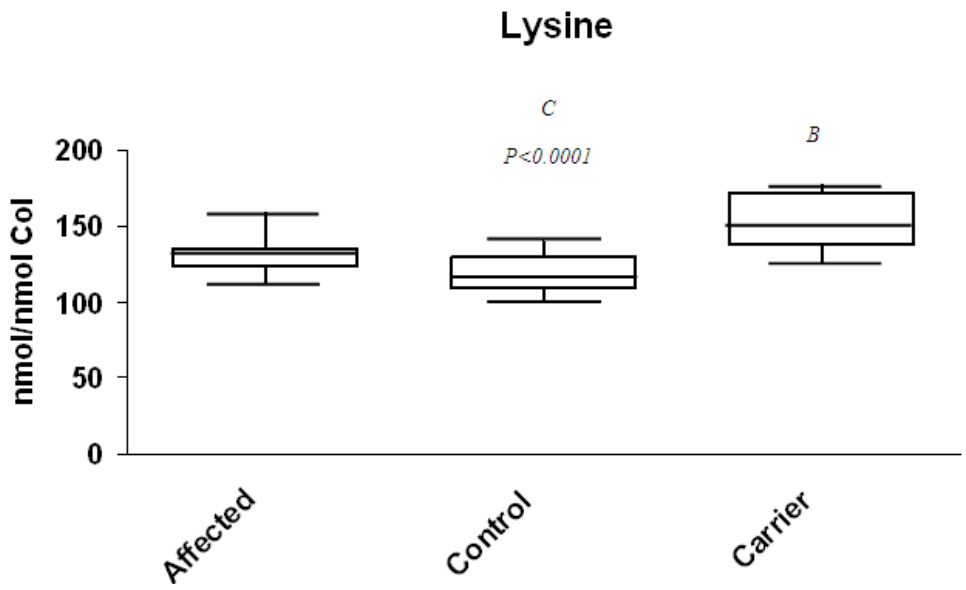

Figure 3.6 Lysine concentrations (nmol/nmol collagen) from 6 HERDA affected, 6 HERDA carriers and 9 control horses. Statistically significant differences between groups are denoted by letters A (affected), B (control) , and C (carrier) above the box plots ($p \leq 0.05$).

Experiment 2 contrasted the pyridinium crosslink, lysine, and hydroxylysine concentrations associated with healing of skin lesions by quantifying these variables in skin derived from two regions of the same horse (Figures 7-12). The first region, referred to as nonlesion (NL), appeared visually normal at biopsy. The second region, referred to as lesion (L) contained evidence of either a healed HERDA lesion for HERDA affected horses, or scarring in control horses. Nonlesion data from both HERDA affected and control horses was a subset of the data previously described and intergroup comparisons for the variables of PYD, DPD, total pyridinium crosslinks, DPD:PYD, lysine and hydroxylysine were in agreement with the previously identified results. Specifically relative to control horses, dermal collagen from HERDA horses had significantly greater concentrations of DPD, a highly significant increase in the DPD:PYD ratio, significantly lower concentrations PYD, and a highly significant decrease in hydroxylysine concentrations. Total pyridinium crosslink concentrations were not significantly different

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in the dermal collagen derived from nonlesioned skin of HERDA and control horses. However, unlike the parent group from which this subset of HERDA and control horses was derived, elevated lysine concentration in the dermal collagen of HERDA horses relative to controls reached statistical significance (p=0.0368).

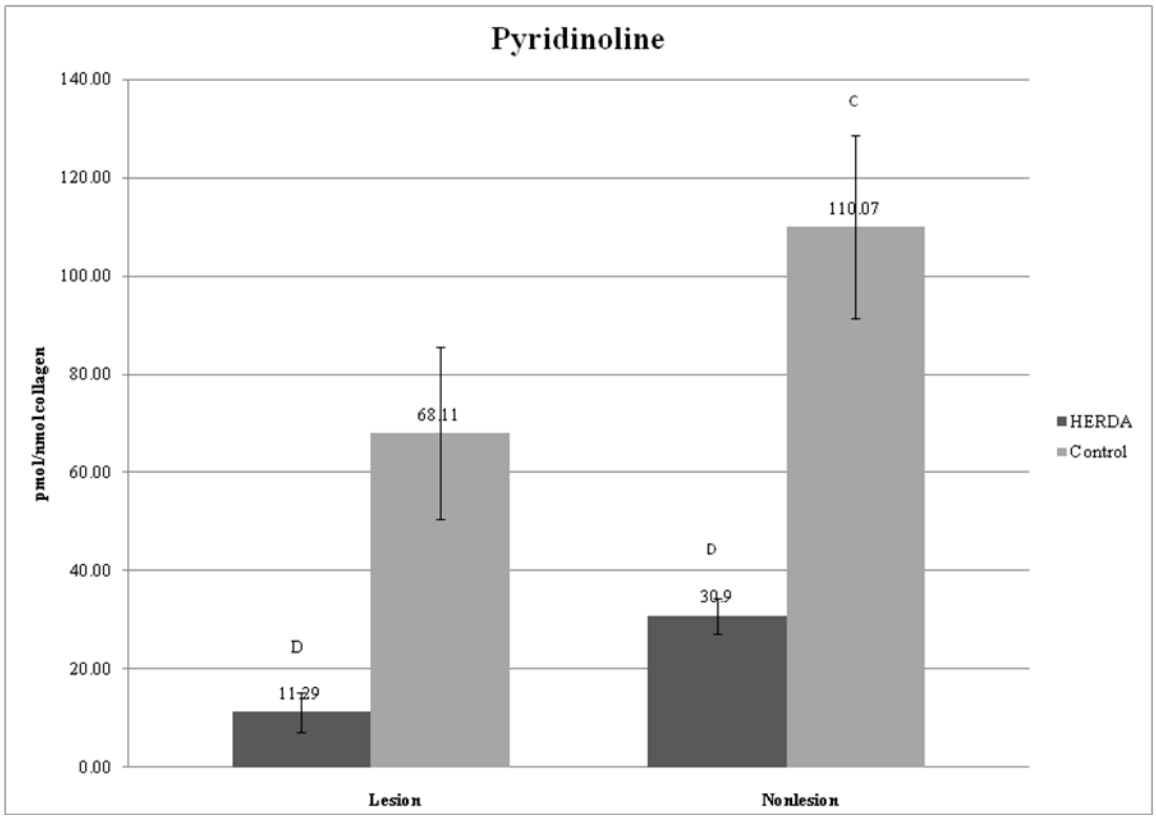

Figure 3.7 Average pyridinoline concentrations (pmol/nmol collagen) from lesion and nonlesion skin of HERDA affected horses and control horses. Error bars represent the standard error of the mean. Statistically significant difference between HERDA and control groups denoted by letters A (HERDA lesion), B (control lesion), C (HERDA nonlesion) and D (control nonlesion) above the bars ($p \leq 0.05$).

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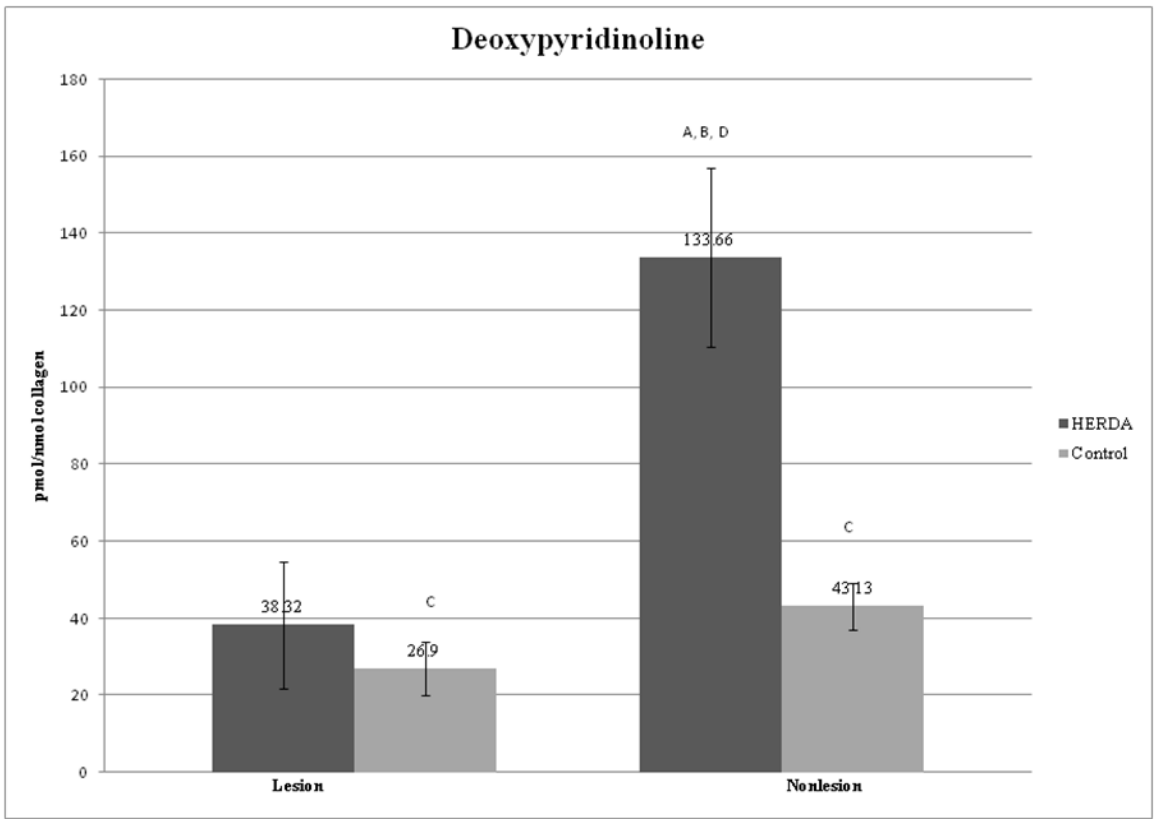

Figure 3.8 Average deoxypyridinoline concentrations (pmol/nmol collagen) from lesion and nonlesion skin of HERDA affected horses and control horses. Error bars represent the standard error of the mean. Statistically significant difference between HERDA and control groups denoted by letters A (HERDA lesion), B (control lesion), C (HERDA nonlesion) and D (control nonlesion) above the bars ($p \leq 0.05$).

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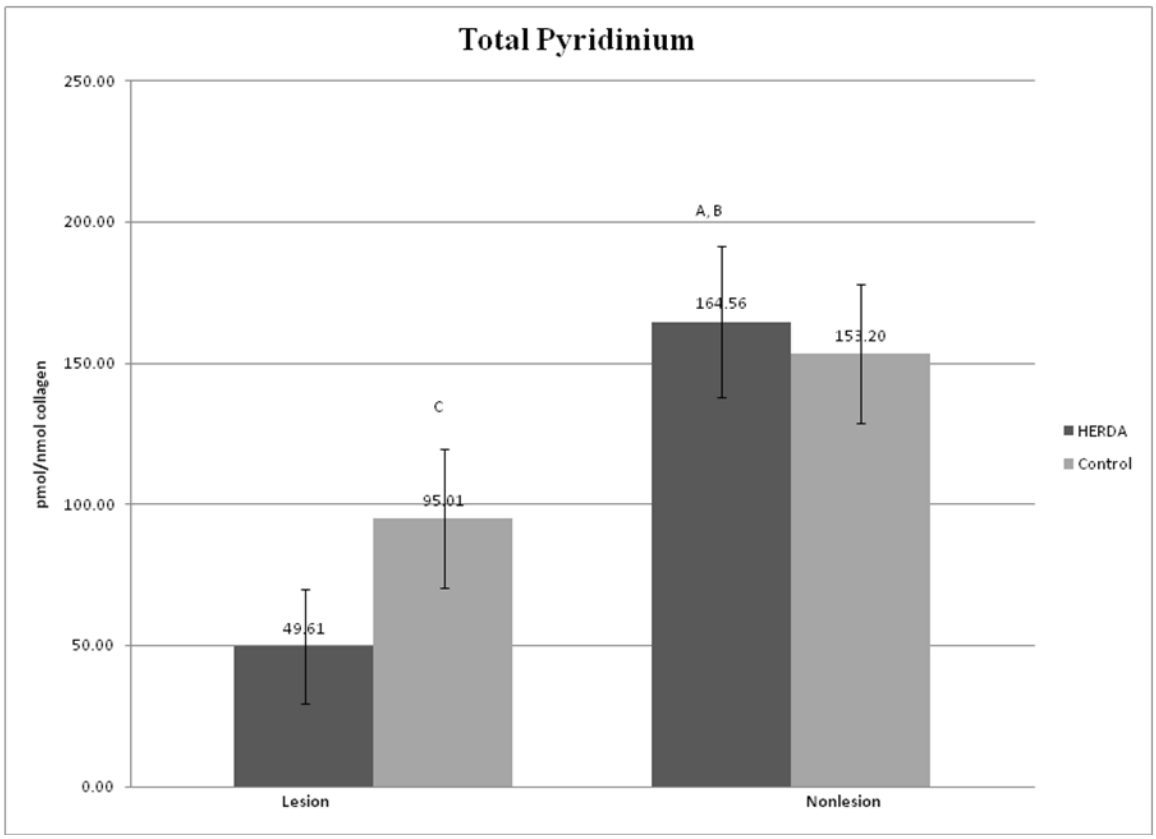

Figure 3.9 Average total pyridinium cross-link concentrations (pmol/nmol collagen) from lesion and nonlesion skin of HERDA affected horses and control horses. Error bars represent standard error of the mean. Statistically significant difference between HERDA and control groups denoted by letters A (HERDA lesion), B (control lesion), C (HERDA nonlesion) and D (control nonlesion) above the bars ($p \leq 0.05$).

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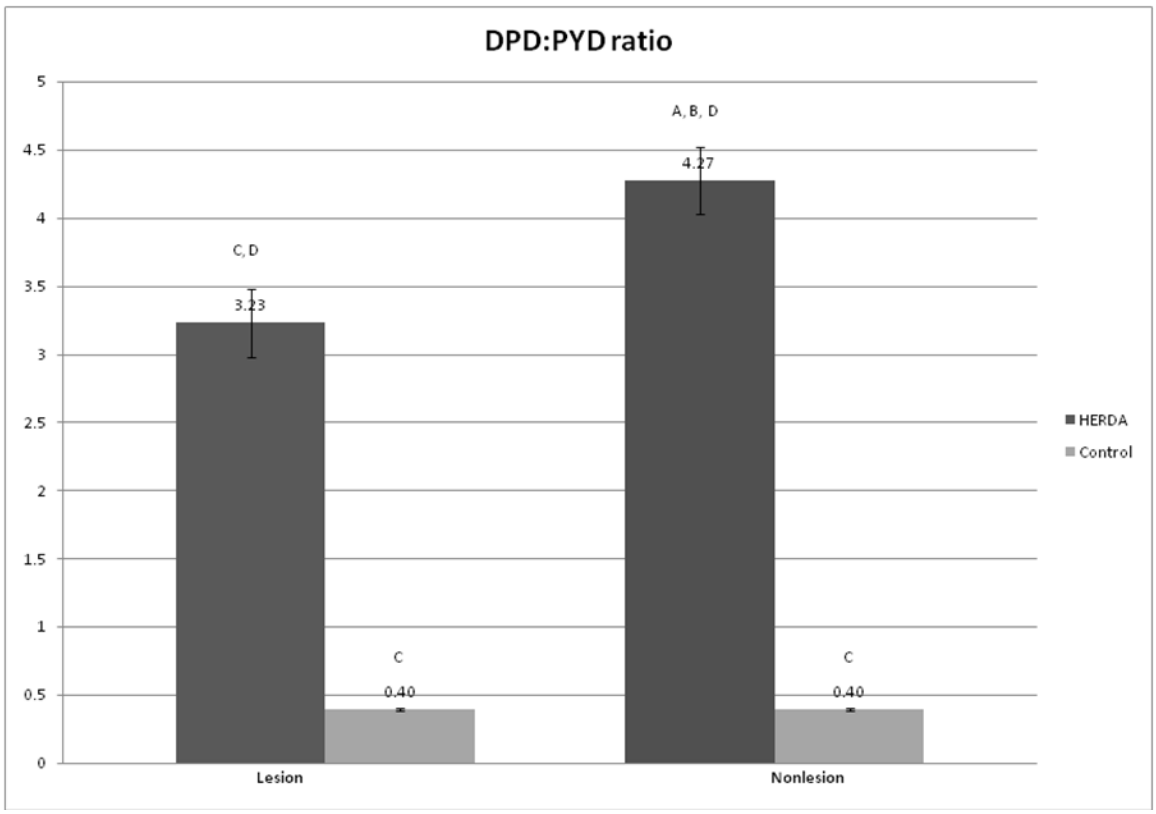

Figure 3.10 The average ratio of deoxypyridinoline to pyridinoline from lesion and nonlesion skin of HERDA affected horses and Control horses. Error bars represent the standard error of the mean. Statistically significant difference between HERDA and control groups denoted by letters A (HERDA lesion), B (control lesion), C (HERDA nonlesion) and D (control nonlesion) above the bars ($p \leq 0.05$).

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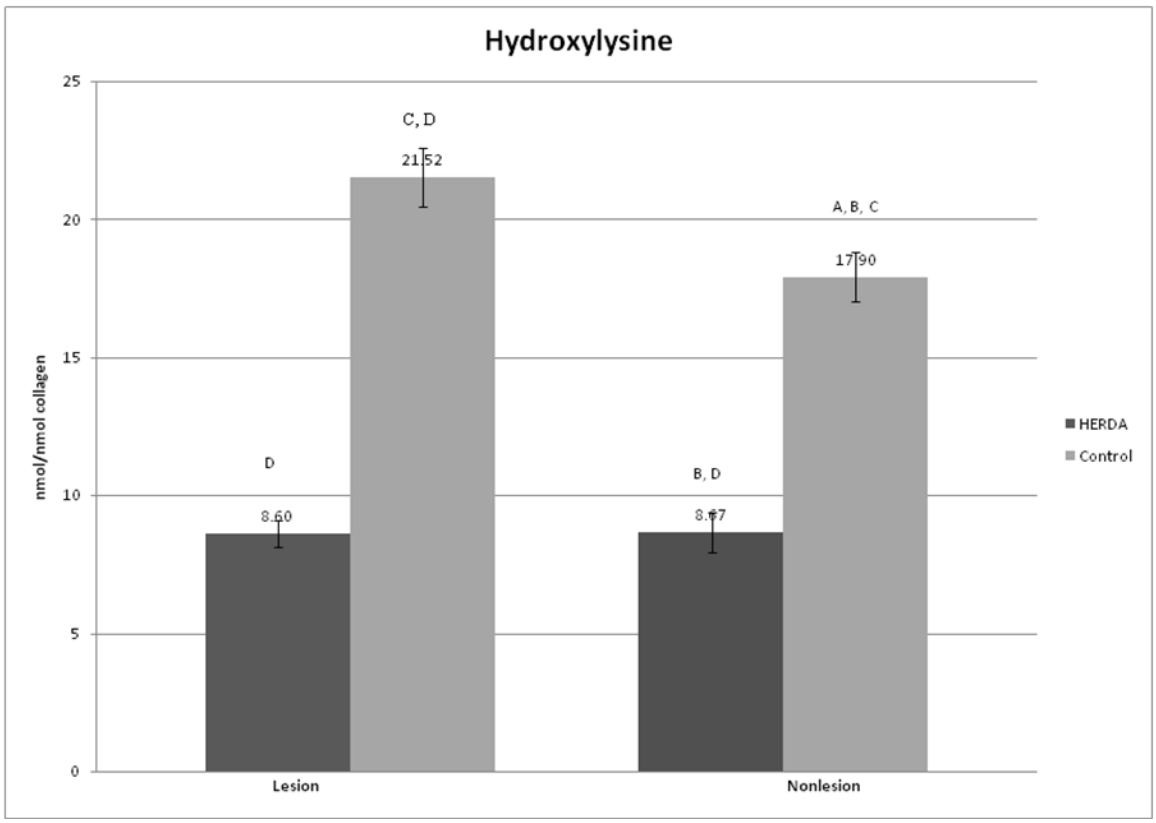

Figure 3.11 Average hydroxylysine concentration (nmol/nmol collagen) from lesion and nonlesion skin of HERDA affected horses and control horses. Error bars represent the standard error of the mean. Statistically significant difference between HERDA and control groups denoted by letters A (HERDA lesion), B (control lesion), C (HERDA nonlesion) and D (control nonlesion) above the bars ($p \leq 0.05$).

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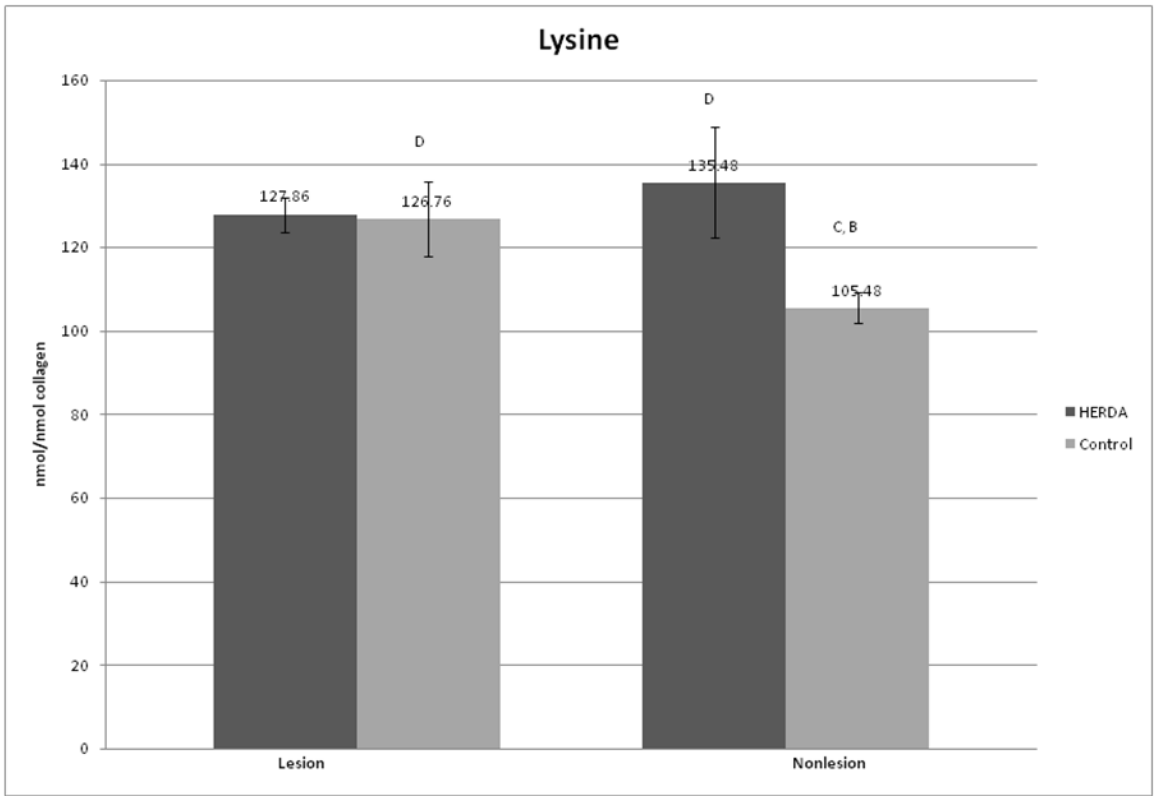

Figure 3.12 Average lysine concentrations (nmol/nmol collagen) from lesion and nonlesion skin of HERDA affected horses and control horses. Error bars represent the standard error of the mean. Statistically significant difference between HERDA and control groups denoted by letters A (HERDA lesion), B (control lesion), C (HERDA nonlesion) and D (control nonlesion) above the bars ($p \leq 0.05$).

Comparisons of dermal collagen derived from lesion and non lesion areas of control horses (Figures 7-12) indicate that healing of skin is associated with a decrease in PYD concentrations (NL: 110.0 7 $\pm$ 32.17 pmol/nmol collagen; L: 68.11 $\pm$ 30.26 pmol/nmol collagen) which approximated significance ($p=0.0525$) and a significant increase in hydroxylysine concentrations (NL: 17.9 $\pm$ 1.54 nmol/nmol collagen; L: 21.52 $\pm$ 1.81 nmol/nmol collagen, $p=0.0137$). Concentrations of DPD (NL: 43.13 $\pm$ 10.55 pmol/nmol collagen; L:26.9 $\pm$ 12.07 pmol/nmol collagen), total pyridinium crosslinks (NL: 153.2 $\pm$ 42.72 pmol/nmol collagen ; L: 95.01 $\pm$ 42.31 pmol/nmol collagen), lysine

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(NL: 105.48 ± 6.29 nmol/nmol collagen; L: 126.76 ± 15.59 nmol/nmol collagen), and DPD:PYD ratio (NL:0.40 ± 0.02; L: 0.40 ± 0.01) did not differ significantly between lesion and nonlesion skin of control horses. In contrast, comparisons of lesion and non lesion skin from HERDA horses identified a significant decrease in DPD concentrations (NL: 133.66 ± 40.28 pmol/nmol collagen; L: 38.32 ± 28.39 pmol/nmol collagen; p=0.002), an associated significant decrease in total pyridinium crosslink concentration (NL: 164.56 ± 46.43 pmol/nmol collagen; L: 49.61 ± 35.36 pmol/nmol collagen; p=0.0099), and significant decrease in DPD:PYD ratio (NL: 4.27 ± 0.42; L: 3.23 ± 0.44; p=0.003). PYD (NL: 30.9 ± 6.17 pmol/nmol collagen; L: 11.29 ± 6.97 pmol/nmol collagen), lysine (NL: 135.48 ± 23 nmol/nmol collagen; L: 127.86 ± 7.11 nmol/nmol collagen) and hydroxylysine concentrations (NL: 8.67 ± 1.25 nmol/nmol collagen; L: 8.60 ± 0.085 nmol/nmol collagen) were not different between lesion and nonlesion skin of HERDA horses.

Comparisons of dermal collagen from lesion skin of control horses and nonlesion HERDA skin (Figures 7-12) identified lower PYD concentrations in HERDA NL skin which approached significance (p=0.0782). Total pyridinium crosslink concentrations were not significantly different between control lesion and HERDA nonlesion dermal collagen (p=0.0765). Relative to control lesion skin, HERDA nonlesion skin demonstrated highly significant elevations in DPD concentration (p=0.001), DPD:PYD ratio (p<0.0001) and significantly lower hydroxylysine concentrations (p<0.0001). Lysine concentrations were not significantly different in control lesion versus HERDA nonlesion skin.

Comparisons of dermal collagen from nonlesion skin of control horses and lesion HERDA skin (Figures 7-12) identified in healed HERDA lesions a significantly lower

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concentration of PYD ($p=0.015$) and hydroxylysine ($p<0.0001$), and significantly higher DPD:PYD ratio ($p<0.0001$). Concentrations of DPD, total pyridinium crosslinks, and lysine were not significantly different between HERDA lesion and nonlesion control skin.

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CHAPTER IV

DISCUSSION

Results from this study demonstrate that despite equivalent total pyridinium crosslink concentrations in dermal collagen of HERDA, carrier, and control horses, horses with HERDA have significantly greater concentrations of DPD and significantly lower concentrations of PYD, which create a highly significant elevation in the DPD:PYD ratio relative to these parameters in carrier and control horses. In contrast, concentrations of PYD, DPD, total crosslinks, and DPD:PYD ratio were not significantly different between carrier and control groups. These findings in skin are consistent with earlier investigations of the urinary excretion of DPD and PYD in our laboratory, which demonstrated that relative to control or carrier horses, HERDA horses excrete significantly more DPD than PYD in their urine, and have an associated increase in urine DPD:PYD ratio ($p<0.0001$) that is diagnostic of HERDA.[32] Thus, we conclude that dermal collagen from horses with HERDA has an abnormal pattern of pyridinium crosslinking that is reflected in the urine of HERDA horses.

The pyridinium crosslinks, DPD and PYD are the covalent bonds responsible for the tensile strength of fibrillar collagen.[8,26-28] Hydroxylysine is a critical substrate for pyridinium crosslink formation and is synthesized during post-translational modifications of lysine residues by the action of lysyl hydroxylase-1 (LH1) (Figure 13).[19,33,37] The pyridinium crosslink DPD requires only two hydroxylysine residues for synthesis while PYD is derived from three hydroxylysine residues. Therefore, decreased LH1 activity

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would be expected to favor DPD production over PYD production by limiting the availability of hydroxylysine. Clinical relevance of LH1 activity in collagen disorders is evident in human EDS VIA, collagen LH1 deficiency, where signs of skin fragility and kyphoscoliosis predominate.[19] While skin fragility is characteristic of HERDA, we postulate that an absence of kyphoscoliosis in HERDA, despite pyridinium crosslinking that mimics kyphoscolotic EDS VIA could be explained by the quadruped orientation of the horse in which the head maintains the vertebral column in traction via the nuchal ligament. This contrasts sharply with human bipeds where inadequate soft tissue support for the spine coupled with the weight of the head and upright orientation allow kyphoscoliosis.

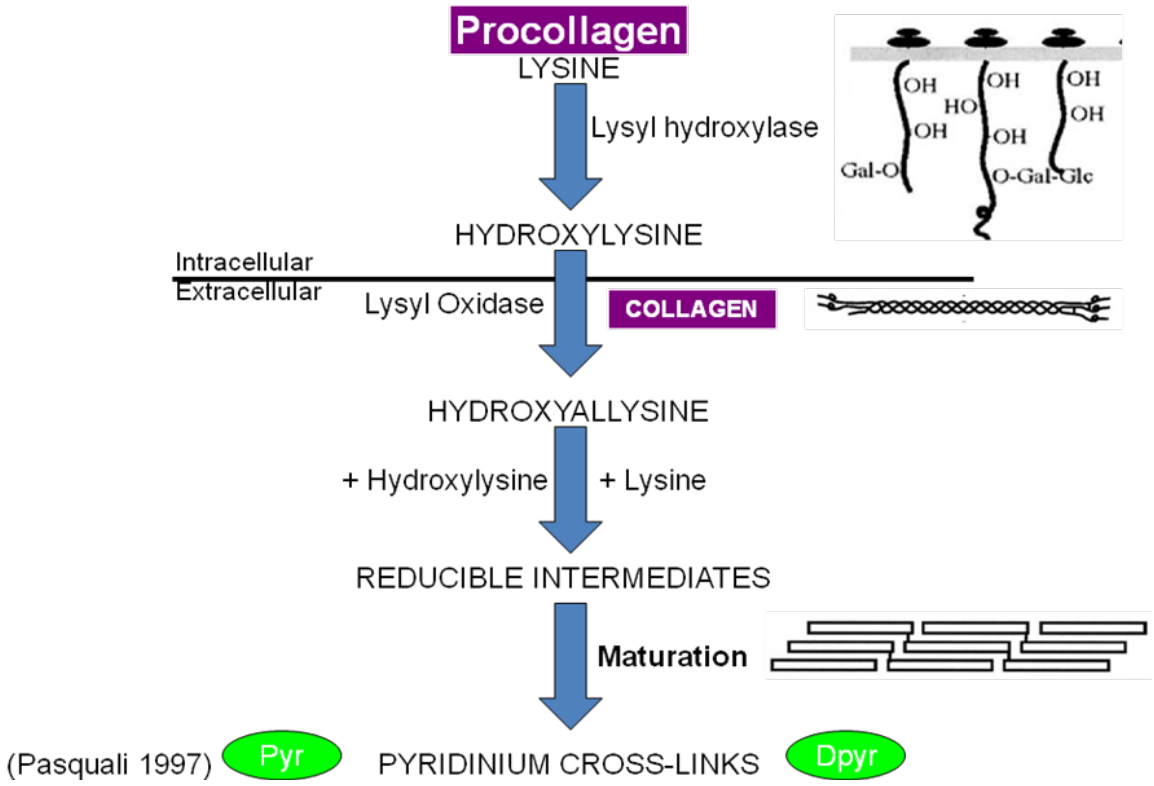

Figure 4.1 Simplified mechanism of collagen pyridinium cross-link formation.[19]

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Humans with EDS VIA associated skin fragility demonstrate significant elevations in DPD and an elevated DPD:PYD ratio which is both sensitive and specific for EDS VIA diagnosis.[19,41,42] By extension we propose that in HERDA the significant increase in DPD, which requires covalent bonds derived from two hydroxylysine residues, and decrease in PYD concentration which requires covalent bonds associated with three hydroxylysine residues, are causally associated with the syndrome of fragility and decreased tensile strength observed in skin from affected horses.

To investigate the potential that decreased lysine hydroxylation in HERDA horses could be responsible for increased DPD formation in the skin of HERDA horses, we quantified lysine and hydroxylysine concentrations in dermal collagen from carrier, affected, and control horses. Horses with HERDA had significantly lower hydroxylysine concentrations in dermal collagen relative to carrier ($p<0.0001$) or control horses ($p<0.0001$). These findings mirror findings in dermal collagen of EDS VIA patients, namely decreased hydroxylysine content, and an associated decreased pyridinoline and increased deoxypyridinoline concentration.[8,32,41,42]

To determine if higher lysine concentrations could be identified to support the theory that lysine hydroxylation was decreased in dermal collagen of HERDA, as well as to rule out the possibility that decreased hydroxylysine concentrations could reflect decreased lysine substrate, we quantified lysine concentrations in dermal collagen in HERDA, carrier, and control horses. We found that the mean lysine concentration of HERDA horses exceeded controls by 10% (though this difference was not statistically significant $p=0.16$). Curiously, lysine concentration of carriers was significantly higher than both HERDA and control horses. This suggests that lower hydroxylysine concentrations in HERDA horses relative to controls do not result from decreased lysine

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concentrations in HERDA dermal collagen. Accordingly, other factors capable of decreasing hydroxylysine formation should be investigated to determine the etiology of decreased hydroxylysine and deoxypyridinoline concentrations that characterize dermal collagen of HERDA horses. Further, the similarities between these biochemical characteristics of dermal collagen from HERDA and human EDS VIA supports a hypothesis that decreased hydroxylation of lysine residues on procollagen molecules and associated alterations in the profile of pyridinium crosslinks in mature collagen have a role in the etiopathogenesis of HERDA.

Since HERDA is an autosomal recessive trait, the finding that hydroxylysine and lysine concentrations of carriers were not intermediate to those of the control group was somewhat unexpected. We postulate that the significantly lower age of carrier horses in this study may confound this observation. Unfortunately HERDA carriers are asymptomatic and their usefulness coupled with the tendency for carriers to have elite bloodlines limits the availability of age matched carrier controls. The mean age of carrier horses was 5.6 months versus 35 months for HERDA and 123 months for control horses. Accordingly this is an observation that warrants further investigation.

Differences in collagen biochemistry between HERDA and normal horses prompted our interest in differential effects of healing on these parameters. In both HERDA and control horses, healing of skin was characterized by a decrease in DPD, PYD, and total crosslinks. However, the magnitude of the decrease was greater for all three parameters in HERDA horses, reaching statistical significance for all crosslink parameters in HERDA but not controls. In control horses the magnitude of the decrease in DPD and PYD, though not statistically significant, was equivalent (38%) and accordingly total pyridinium crosslinks also decreased by 38%. Thus, there was no

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change in the DPD:PYD ratio associated with healing in normal horses. By contrast, in HERDA the magnitude of the decrease in DPD (71%) exceeded that of PYD (63%), yielding a 70% decrease in total pyridinium crosslinks, and a 25% decrease in the DPD:PYD ratio in HERDA. Further, the DPD:PYD ratio associated with healing in HERDA horses decreased significantly, while that of controls was unchanged, indicating in normal but not HERDA horses the synthesis of DPD and PYD is equivalent during healing. These comparisons indicate that while DPD and PYD decrease in dermal collagen during healing in both normal and HERDA horses, the magnitude of the decrease in DPD and PYD concentrations in HERDA horses significantly exceeds that of normal horses. As pyridinium crosslinks provide the tensile strength of skin, we believe this difference in HERDA lesion pyridinium crosslinking contributes to repeated wounding and delayed healing of HERDA lesions, which we have observed in our HERDA research herd during a 6 year period.

Quantification of lysine and hydroxylysine concentrations associated with healing within groups demonstrated a significant (17%) increase in the average hydroxylysine concentration ($p=0.01$) and 20% increase in average lysine concentration in control horses which was not statistically significant ($p=0.1$). In HERDA, hydroxylysine concentrations were unchanged and a small (6%) but insignificant decrease in lysine concentration occurred in healed skin. Thus, while healing in normal horses was associated with a significant increase in hydroxylysine concentration that roughly parallels the increase in lysine concentration, hydroxylysine concentrations are unchanged in HERDA during healing. As the etiopathologic basis of HERDA is unknown, comparisons of lesion and nonlesion data within groups highlight that the biochemical composition of dermal collagen differs between healed skin of both HERDA

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and normal horses and indicates that lesion and nonlesioned skin cannot be viewed as biologically equivalent in investigations of HERDA.

Intergroup comparisons of the pyridinium crosslink and hydroxylysine concentrations of dermal collagen from lesions of control and HERDA horses demonstrate that dermal collagen from the lesions of HERDA horses have a significant decrease in the concentrations of PYD ($p=0.015$) and hydroxylysine ($p<0.0001$), as well as a highly significant increase in the DPD:PYD ratio ($p<0.0001$) relative to dermal collagen from lesions of control horses. Though not statistically significant, DPD concentrations were also higher in HERDA lesion relative to control lesion groups. These comparisons of lesions from HERDA and control horses parallel comparisons previously discussed for nonlesion skin from HERDA and control horses and indicate that in addition to the decrease in DPD, PYD and total crosslink synthesis that differentiates healing in HERDA and normal horses, there also remains in HERDA lesions relative to control lesions, a significant decrease in PYD and hydroxylysine concentration, as well as an increase in DPD concentrations. Accordingly, lesion and nonlesion skin of HERDA demonstrate a significant increase in DPD:PYD that is specific to HERDA but not normal skin. Particularly in light of the small sample size ($n=3$), these comparisons of dermal collagen from lesion and non lesion skin are consistent with our hypothesis that decreased lysine hydroxylation in dermal collagen from horses with HERDA favors the production of DPD over PYD.

Our findings indicate that in HERDA decreased hydroxylation of lysine residues in dermal collagen is associated with greater DPD and lower PYD crosslink formation during collagen synthesis. LH1 is the telopeptide hydroxylase responsible for the hydroxylation of lysine residues that serve as substrates in pyridinium crosslink

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formation and interactions between LH1 and PPIB have not been described. Accordingly a mechanism by which the PPIB mutation of HERDA could result in the combination of clinical skin fragility and the alterations in hydroxylysine and pyridinium crosslink profile demonstrated here is not known. Given the major role of LH1 in post-translational lysine hydroxylation, as well as the association of LH1 deficiency in human EDS VIA with skin fragility, decreased lysine hydroxylation, and an increased synthesis of DPD, it is plausible that our parallel findings of decreased lysine hydroxylation and a predominance of DPD in HERDA also reflect a decrease in collagen lysyl hydroxylase activity. Based upon our findings, further investigations into the defect in lysine hydroxylation that characterizes HERDA are warranted.

Three functions have been ascribed to Cyclophilin B (CyPB), the protein coded by PPIB. First, CyPB is considered the primary rate limiting peptidyl-prolyl cis trans isomerase in fibrillar collagen synthesis and is accordingly considered integral to proper collagen folding.[12] Second, CyPB is a molecular chaperone important in collagen synthesis, and third CyPB forms an intracellular collagen-modifying complex with cartilage associated protein (CRTAP) and prolyl-3-hydroxylase-1 (P3H1). This tri-molecular complex is responsible for the hydroxylation of proline at position 986 in the alpha chains of type I and type II collagen.[13,14]

Recent evidence suggests that hydroxylation of P986 is significant in ordered self assembly of collagen supermolecular structures.[43] Mutations in each of the components of the tri-molecular complex have been associated with decreased P986 hydroxylation and the clinical syndrome of autosomal recessive osteogenesis imperfecta (OI), which is characterized by diffuse abnormal fragility of bone and sometimes accompanied by sensorineural hearing loss, blue sclera, dentinogenesis imperfecta, and joint

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hypermobility.[13,14,44-46] Though some references to thinning of skin occur with human OI, overt skin abnormalities are not clinical hallmarks of human OI. In mice with CyPB deficiency associated OI, skin is hyperextensible, and has reduced stiffness, tensile strength, and P986 hydroxylation.[17] Unfortunately, neither the status of P986 hydroxylation of HERDA collagen nor the degree of lysine hydroxylation or pyridinium crosslink concentrations associated with PPIB mutation associated OI have been reported. However, mutations in LEPRE and CRTAP, the two other components of the tri-molecular P986 hydroxylation complex, cause excessive post-translational modification of collagen characterized by increased lysine hydroxylation.[47,48] This is presumed to reflect delayed collagen folding which allows increased post-translational modifications via LH1 and prolyl-4-hydroxylase. The increased lysine hydroxylation of collagen observed with LEPRE and CRTAP mutations is in stark contrast to our findings in horses with PPIB mutation and HERDA. It should be noted that in both mice and humans with PPIB mutations and recessive OI, excessive post translational modification of collagen has been documented but the nature of the overmodifications and specifically the presence of increased lysine hydroxylation have not been specifically reported.[13,17]

Both LH1 and P3H1 belong to the family of 2-oxoglutarate/iron dependent oxygenases, and share conserved residues in their active sites that are also present in prolyl 4-hydroxylase[49] (the primarily proline hydroxylase of fibrillar collagen), leading us to postulate that in addition to P3H1, Cyclophilin B could be important to the steric orientation of other members of 2-oxoglutarate iron dependent oxygenase family of enzymes, including LH1. Collectively these findings indicate that a possible role for interactions of PPIB and LH1 cannot be ruled out and quantification of lysine hydroxylation, and pyridinium crosslinking in human and mouse PPIB associated OI, as

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well as evaluation of P986 hydroxylation in HERDA would be key questions to elucidate whether such an interaction could explain the decreased lysine hydroxylation and pyridinium crosslink pattern we have identified in HERDA.

Moving beyond the traditional framework of CyPB function for interpreting our results, a recent New England Journal of Medicine publication by Barnes questions the dogma that CyPB is a critical cis-trans prolyl-isomerase that is necessary for both P986 hydroxylation and proper collagen folding. The study describes human siblings with a homozygous start codon mutation of PPIB that prevents translation.[18] The OI phenotype of these individuals lacks rhizomelia (shortening of the limbs) and despite one of these individuals being a 7 year old boy, skin of the proband was reportedly normal. Despite absence of CyPB activity in these individuals, their collagen was normally folded and had normal P986 hydroxylation. This study demonstrates that CyPB is not the exclusive peptidyl-prolyl cis-trans isomerase responsible for catalyzing the rate-limiting step in collagen folding and further that CyPB is not required for P986 hydroxylation. Unlike humans with PPIB mutation associated OI, bone fragility has not been described in horses with HERDA. Given the lack of skin lesions in humans devoid of CyPB activity ,and an inability to explain the reduced hydroxylysine and altered pyridinium crosslink pattern we have identified within the body of knowledge that exists for CyPB function, it is plausible that the PPIB mutation characterized in HERDA is identical by descent (and thus diagnostically valid) but not causal.

Collectively, our findings coupled with the absence of skin signs that would be analogous to HERDA lesions in syndromes associated with PPIB mutations in both mice and humans do not provide support for the c.115G>A PPIB mutation as a putative causal mutation of HERDA. Further, our results indicate that HERDA is associated with

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decreased lysine hydroxylation and an abnormal predominance of DPD, findings that parallel the biochemical characteristics of human LH1 deficiency, where skin fragility similar to HERDA is a clinical feature. Our results also document that HERDA is a valuable animal model of altered collagen metabolism that is particularly relevant to both human EDS and recessive osteogenesis imperfecta, based upon the respective association with altered pyridinium crosslink patterns, and mutation of the PPIB gene. Building upon the data presented here, future directions for HERDA research should focus on analyzing the metabolic pathways responsible for lysine hydroxylation in horses with HERDA and should investigate the systemic distribution of the observed pyridinium crosslink patterns. In light of conflicting clinical and biochemical phenotypes associated with absence of PPIB activity in humans and mouse models, elucidating the relationship between the HERDA PPIB mutation, changes in pyridinium crosslink formation (elevated DPD, decreased PYD), and decreased hydroxylysine concentrations that we observed in HERDA skin will provide valuable insight into the significance of the reported PPIB mutation in the HERDA phenotype and the more global role of PPIB in collagen metabolism. A link between the HERDA PPIB mutation and hydroxylation of lysine residues that contribute to pyridinium crosslink formation (and collagen tensile strength) would be an entirely novel finding in collagen biochemistry. This would not only refine the current understanding of the role of PPIB in collagen metabolism, but also has the potential to influence the diagnosis and future management of human collagen disorders.

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