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Biomechanical And Molecular Characteristics Of 'Hyperelastosis Cutis' In Quarter Horses

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BIOMECHANICAL AND MOLECULAR CHARACTERISTICS OF 'HYPERELASTOSIS CUTIS' IN QUARTER HORSES

By

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The biomechanical and molecular characteristics of equine hyperelastosis cutis (HC) are not fully known. This study sought to better characterize HC by analysis of ultimate tensile strength, modulus of elasticity, toughness, and thickness of skin from 23 affected and unaffected horses. In addition total soluble collagen and glycosaminoglycan concentrations of skin were analyzed from 26 affected and unaffected horses. Affected horses' skin proved to be significantly weaker at five of seven sample locations ($p \leq 0.05$). The modulus of elasticity proved to be significantly different at three of seven sample locations and toughness at two of seven locations ($p \leq 0.05$). No significant difference was proven to exist between HC affected and unaffected horses for skin thickness or total soluble collagen and GAG concentrations. Collectively this data demonstrates that HC animals' reduced skin tensile strength is not due to a deficit of either collagen or GAG, but likely a result of altered collagen micro-architecture.

DEDICATION

I would like to dedicate this project to my wife Emily, my parents Mark and Melinda, and the rest of my family and friends who have encouraged me to fulfill my dreams and desires.

ACKNOWLEDGEMENTS

I would like to thank the Faculty and Staff of the MSU-CVM AHC Equine Clinic and Necropsy for their patience and assistance during my sample collections, Dr. Sumalee Girvuangsawat for completing the statistical analysis, and Tom Thompson for providing me with great images I have used throughout this study. I would also like to thank Dr. Peter Ryan for his great advice and Dr. Steve Elder for all of his hard work and expert advice and assistance with this project. Without my 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. Ann Rashmir. I would like to especially thank her for giving me a chance to prove myself when many others would not. Without her great advice, vision, and patience with myself and this project I would not be where I am today. I hope that his project gives back to her at least a small portion of what she has given to me.

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CHAPTER I

INTRODUCTION

Equine hyperelastosis cutis, also known as hereditary equine regional dermal asthenia (HERDA) or Ehlers-Danlos Syndrome, is an autosomal recessive disease[1] characterized by loose, hyper-extensible, fragile skin. Clinically similar connective tissue dysplasias are seen in a wide array of species such as Ehlers-Danlos Syndrome (EDS) in humans,[2] cutaneous asthenia in dogs and cats,[3] and dermatosparaxis in cattle and sheep,[4] all of which exhibit decreased skin tensile strength.[2-4] In horses it presents predominately in Quarter Horses and horses of Quarter Horse lineage, with the majority of affected animals disseminating from cutting horse lines.[5-9]

Affected horses often develop severe lacerations, hematomas, and seromas from innocent trauma, frequently resulting in disfiguring scars. Due to their persistent wounds most horses can not be ridden or shown competitively and are humanely euthanized. Many horses affected with HC are not diagnosed until they begin training at approximately 1.5-2 years of age at which time the trauma of saddling leads to clinical presentation of characteristic HC wounds, but severely affected horses may develop signs shortly after birth.[9] Clinical presentation of HC consists primarily of 'stretchy' skin that appears to take longer than normal to return to its normal position when extended. The skin may also feel 'mushy' or 'doughy' to the touch and can be easily manipulated, especially along the dorsum of the horse where wounds typically occur.

Previous histological and ultrastructural studies of HC horses have found abnormalities present in tissues of affected animals to be inconsistent. The most common finding is that of loosely associated thin and shortened collagen fibres in the deep dermis separated by clear spaces, with fibrosis or granulation tissue present at the points of separation (Figure 1).[7-9]

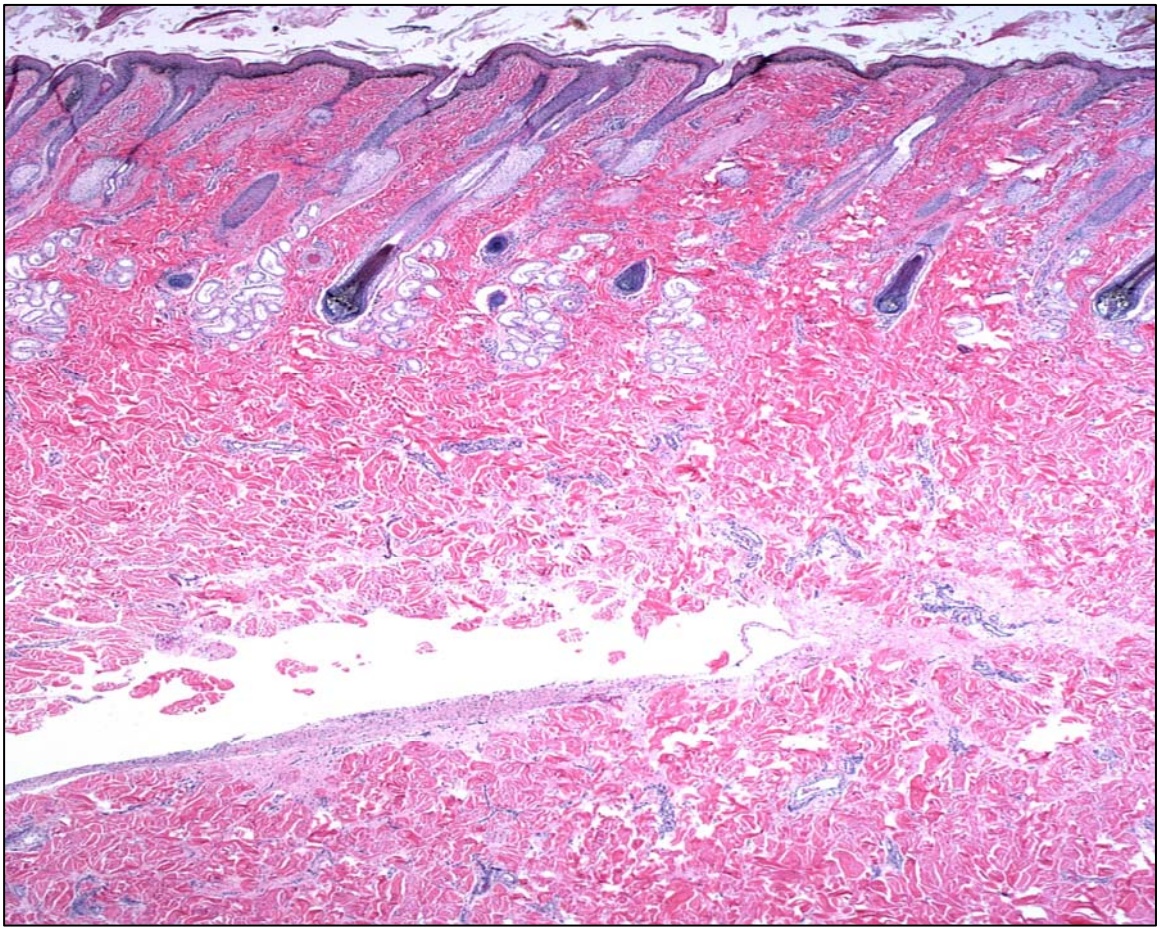

Figure 1

Zonal dermal separation throughout the deep dermis of HC affected skin with granulation tissue present through the bottom of the dermal cleft (H&E).

While scientists have reported statistically significant differences in collagen fiber arrangement, thickness, and overall assessment; none have done so for all three parameters in a single animal subject (Figure 2). A more recent study demonstrated histological comparison to be an inconsistent diagnostic feature, suggesting that severity of disease can vary among animals.[9] The difficulty in characterizing HC through histological analysis is further compounded by the normal periodicity of collagen fibres in HC affected skin.[8] Thinner feeling skin is often a reported clinical sign of HC and a slight decrease in skin thickness was noted in one study, but no statistically significant difference was observed.[7]

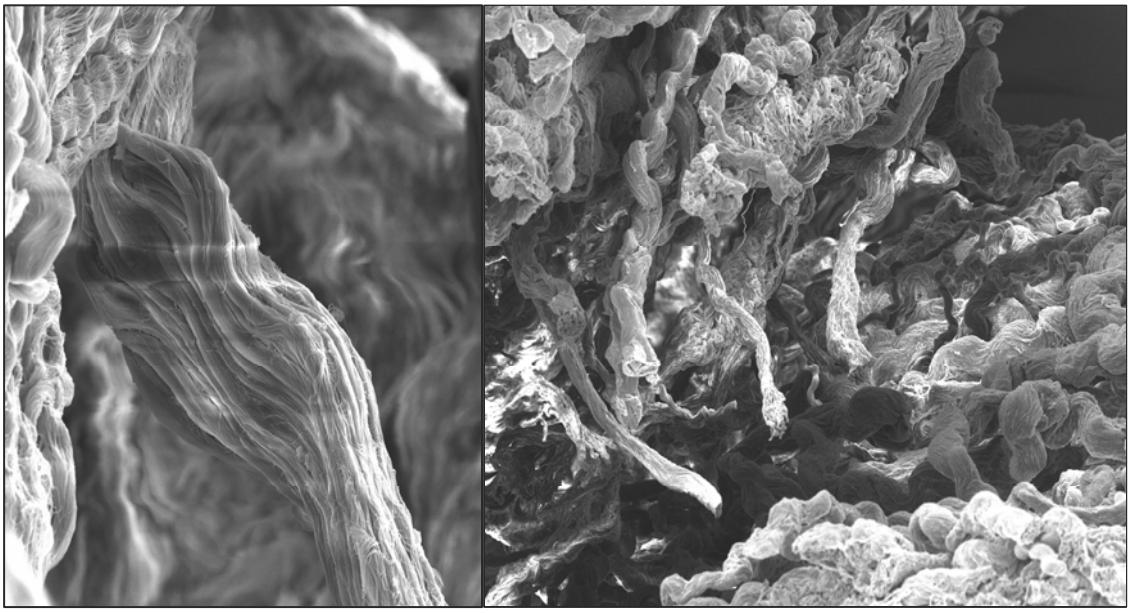

Figure 2

SEM of HC affected skin with a normal, tightly conserved, collagen bundle (Left). SEM of HC affected skin with disarranged collagen bundles with fragmentation present (Right).

Small leucine-rich proteoglycans (SLRPs) are theorized to regulate collagen fibrillogenesis, cartilage formation, bone mineralization, and many other regulatory functions of the body. Proteoglycans, including SLRPs, compose the core protein to which GAGs are attached in a bottle brush type structure and have been shown to induce symptoms clinically similar to EDS when altered in SLRP KO mice. In these studies SLRP KO mice exhibited a wide array of diseases resulting from abnormal collagen fibrillogenesis, including those similar to EDS.[10] A more recent study examining the SLRPs decorin and biglycan in human patients suffering from a disease belonging to the group of EDS disorders suggested that the actual concentration of SLRPs is not solely responsible for impaired collagen fibrillogenesis. Instead a mutation on the β4GalT-7 gene, resulting in altered decorin and biglycan glycosylation is now theorized as the underlying cause of irregular collagen fibrillogenesis.[11]

Most recently a homozygosity mapping approach was utilized to identify a missense mutation in horses suffering from HC. Sixty-four of the sixty-eight affected horses studied were found to carry a common haplotype across locus ECA1.[12] The horses carrying the characteristic haplotype were also found to share four homozygous DNA markers, two of which are predicted to cause a missense mutation of PPIB. The peptidyl-proplyl isomerase (PPI) family, of which PPIB is a member, offers further evidence promoting PPIB as a logical candidate gene for HC. PPIs have been theorized to be associated with protein folding of collagens which would make them a likely source of the histological characteristics of HC.

To date, research analyzing the biomechanical characteristics of HC affected and unaffected equine skin has yet to be presented in any peer-reviewed literature. The goal of this project was to further characterize the biomechanical characteristics of HC affected and unaffected skin so that HC may be better understood. In addition, molecular testing of the concentrations of total soluble collagen and GAG was conducted to determine whether the hyper-extensible nature of HC skin is due to a lack of collagen or GAG or because of their altered collagen micro-architecture. It is theorized that a substantial reduction in skin tensile strength will be observed in affected horses versus that of unaffected horses. It is also theorized that no difference in the concentrations of total soluble collagen and GAG will exist between affected and unaffected horses.

CHAPTER II

MATERIALS AND METHODS

Animals

The animals used in the biomechanical portion of this study were composed of 10 affected horses (experimental group) and 13 unaffected horses (control group). Affected horses ranged in age from 1-10 years (mean 3.7) while unaffected horses ranged in age from 11-24 years (mean 14.6). All affected animals were of the Quarter Horse breed and previously confirmed by clinical, pedigree, and DNA* analysis to be affected with HC. Biomechanical samples were large in nature, and seven samples were collected from each animal, making euthanasia necessary. Euthanasia and sample collection of affected animals was performed based on severity of disease, and as such most were euthanized at an early age. Unaffected horses consisted of 9 Quarter Horses, one Paint, one Paso Fino, one Thoroughbred, and one Percheron that were euthanized for terminal teaching procedures at the College of Veterinary Medicine (Mississippi State University) or conditions that did not affect the viability of their skin. HC status was confirmed to be unaffected by DNA analysis. All animals were humanely euthanized by a licensed veterinarian in accordance with AVMA guidelines with sample collection occurring

* HC status of experimental animals determined by DNA analysis (patent pending) by Dr. Nena Winand at Cornell University's College of Veterinary Medicine Molecular Genetics Laboratory.

immediately following euthanasia. All samples were stored at -80° C immediately following collection.

The animals used in the molecular portion of this study were composed of 13 affected horses and 13 unaffected horses. Horses ranged in age from 1-6 years of age (mean 2.4, 3). Skin samples from affected and unaffected horses from the biomechanical portion that met the predetermined age criteria of 1-6 years of age were also used in the molecular portion of the study. The remaining samples from unaffected horses were obtained by dermal punch biopsies from age-matched horses from the Mississippi Agricultural and Forestry Experiment Station research herd. All affected animals were Quarter Horses previously confirmed by clinical, pedigree, and DNA analysis to be affected with HC. Unaffected animals were composed of 11 Quarter Horses, one Paint, and one Tennessee Walking Horse. All samples were stored at -80° C immediately following collection. These studies were conducted with approval from the Mississippi State University Animal Care and Use Committee.

Tensile Strength

Skin tensile strength of horses was analyzed by collecting skin using a dumbbell-shaped template (Fig. 3) from seven separate sample locations. Sample locations were designated withers 1 (W1), withers 2 (W2), withers 3 (W3), withers 4 (W4), forelimb (FL), hind limb (HL), and abdominal (AB). Anatomically, samples were located beginning with W1 20 cm ventral of the withers with W2, W3, and W4 located approximately 20 cm caudally of W1, respectively. The FL and HL samples were located on the lateral side of the midpoint of the radius and tibia, respectively with the

abdominal (AB) sample located 20 cm cranial of the stifle joint with the animal in lateral recumbency (Fig. 4).

Figure 3

Dumbbell-shaped template for skin tensile strength analysis

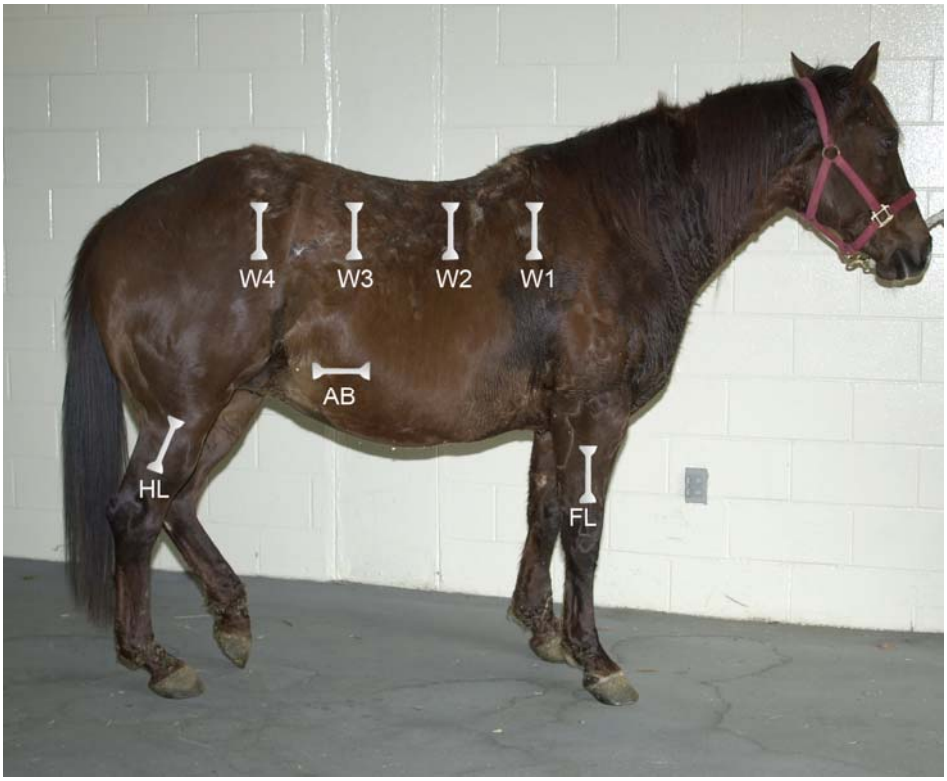

Figure 4

Anatomical sample locations for skin tensile strength analysis

Samples were transported to the biomechanical testing laboratory on ice for tensile strength analysis. Samples were individually thawed and equilibrated to room temperature immediately prior to testing. Sample thickness was then obtained with a Mach-1™ V500cs Micromechanical Test System (Biosyntech Inc., Laval, Quebec) immediately prior to tensile strength analysis. This was accomplished by a flat circular plate 42 mm in diameter coming into contact with the parallel base to establish a zero reference position. The actuator was then raised and a sample laid flat on the base so that the test portion was centrally located beneath the plate. The plate was then lowered at 25 µm/s until the sample provided 50 g of compressive resistance at which point the position of the actuator indicated the sample thickness under the applied stress of approximately 1 kPa.

Immediately following thickness measurement, ultimate tensile strength was determined using an Instron® 1011 Universal Testing Instrument (Instron®, Norwood, MA) with a 500 N load beam in conjunction with Instron® Series IX software to measure displacement (mm) and force (N) of the skin samples as they were pulled apart at a constant rate of 10 mm/min with a data recording rate of 10 points per second until failure was achieved or the load limit of 500 N was exceeded. Wire mesh was super-glued to the dumbbell ends of skin samples to prevent them from slipping out of the grips and the dumbbell ends were inserted in the clamps so that the test portion was of uniform width. Prior to the start of each test a tensile preload of 1 N was established at which point the gauge length (grip-to-grip distance) and width of each sample was measured with a digital micrometer.

Stress and strain were found by normalizing the force (N) and displacement (mm) data to the cross-sectional area (width x thickness) and gauge length respectively. Tensile strength was defined as the maximum tensile stress (N/mm^2). The modulus of elasticity (MPa), a measure of skin stiffness, was calculated as the best-fit line through the linear portion of the stress vs. strain curve for each sample. Energy to failure (J/mm^3) was calculated as the area under the stress vs. strain curve up to the point of failure; with failure defined as the point of ultimate tensile stress.

Collagen Assay

All assays were accomplished by using an additional skin sample collected immediately following euthanasia just adjacent to the W1 sample location or by dermal punch biopsy of normal horses. Total soluble collagen content of skin was measured using a Sircol™ Soluble Collagen Assay (Biocolor, Belfast, Ireland) in accordance with the manufacturer's instructions. In summary, a 4 mm dermal punch biopsy was used to obtain a uniform sample size from the previously collected skin samples and weighed to establish a basis for data normalization. Samples were digested in 1 mL of 0.5 M acetic acid containing 5 mg/mL pepsin. Samples were incubated for 48 hours at 4°C without mixing. Following incubation, 100 µL aliquots of each sample were collected and mixed with 1 mL of provided dye reagent for 30 minutes. Following 10 minutes of centrifugation at 10,000 x g, pellets were brought back into solution with aid of 1 mL of provided alkali reagent and a vortex mixer. Duplicate analyses from single tissue digestion solutions were performed. Optical absorbance at 540 nm was measured with a microplate spectrophotometer (Bio-Tek Instruments Inc, Winooski, VT). Total soluble

collagen content was then determined from a standard curve prepared from type I collagen (provided with kit) diluted in same solution in which skin samples were digested. Results are reported as micrograms of collagen per milligram of sample wet weight.

Glycosaminoglycan Assay

As in the collagen assay, samples were prepared from skin collected just adjacent to the W1 sample location immediately following euthanasia or by dermal punch biopsy of normal horses. Sulfated proteoglycans (PG) and glycosaminoglycans (GAG) were measured using a Blyscan™ Sulfated Glycosaminoglycan Assay (Biocolor, Belfast, Ireland) in accordance with the manufacturer's instructions. In summary, a 4 mm dermal punch biopsy was used to determine sample size from the previously collected skin samples and weighed to establish a basis for data normalization. Samples were digested in 1 mL of buffered sodium acetate (pH 6) with 10 µL/mL papain and 1 mg/mL cystine. Samples were incubated for a minimum of 48 hours in a 60°C water bath. Following incubation 100 µL of each sample was collected and mixed with 1 mL of provided dye reagent for 30 minutes. Following 10 minutes of centrifugation at 10,000 x g, pellets were brought back into solution with aid of 1 mL of provided alkali reagent and a vortex mixer. Duplicate analyses from single tissue digestion solutions were performed. Optical absorbance at 656 nm was measured with a microplate spectrophotometer (Bio-Tek Instruments Inc, Winooski, VT). GAG content was then determined from a standard curve prepared from chondroiton-4 sulfate (provided with kit) diluted in the same

solution in which skin samples were digested. Results are reported as micrograms (μg) of GAG per milligram (mg) of sample wet weight.

Statistics

Statistical analysis was performed using an independent sample t-test, analyzed with SAS/STAT® version 9.1 statistical software (SAS Institute Inc., Cary, NC).

CHAPTER III

RESULTS

Tensile Strength Analysis

Skin samples from the affected horses were significantly weaker at W1 ($p = 0.02$), W2 ($p = 0.01$), W3 ($p < 0.0001$), FL ($p = 0.01$), and HL ($p = 0.04$) when compared to the unaffected horses (Figure 5, Table 1). Upon statistical analysis the excessive difference in age between the affected and unaffected groups was a confounding factor, and age-matched controls are currently being collected to provide for a more accurate comparison. It is generally accepted that as an animal increases in age, a decrease in tensile strength is observed. This suggests that the difference in tensile strength between groups was not due to age as the affected horses, which were younger (mean 3.7), still had statistically weaker skin than the unaffected horses, which were older (mean 14.6). Interestingly, the FL, HL, and W1 samples were the strongest areas of skin in both affected and unaffected horses despite being the thinnest sections of skin. A slight trend of decreasing tensile strength was also evident for the sample locations W1-W3, with a dramatic rise in tensile strength in the most caudal skin samples (W4). There was no statistical significance between groups for skin samples from the W4 and AB site locations ($p = 0.09$ and $p = 0.85$ respectively).

The modulus of elasticity or skin stiffness was significantly different between affected and unaffected groups at the FL ($p=.006$), HL ($p<0.0001$), and AB ($p=.001$) sample locations (Table 2). The significant difference at these three sample locations located on the lower extremities of the horse are primarily due to the massive increase in the values for modulus of elasticity at these sample locations in unaffected horses (Figure 6). Although not statistically significant, the remaining sample locations were slightly lower for affected horses than that of unaffected horses, exhibiting a decreased ability in affected horses' skin to withstand force before returning to normal. HC affected horses exhibited a similar value for modulus of elasticity at all seven sample locations suggesting that although skin may appear normal in certain areas of the horses' body, that HC is present throughout.

Skin energy absorption, or amount of energy skin can absorb before failure, achieved statistically significant differences at W1 ($p = 0.05$), W2 ($p = 0.01$) and W3 ($p < 0.0001$) sample locations (Table 3). Although not statistically significant, the remaining sample locations for affected horses exhibited a lower energy to failure value than that of unaffected horses (Figure 7). Clinical presentation of wounds in HC horses is consistent with these findings, as wounds typically appear along the dorsal trunk of the horse, where the W1, W2, and W3 sample sites were located. Skin thickness on average was thinner for affected animals, but not statistically significant (Table 4). In both groups skin thickness increased from the W1-W4 sample locations, the HL and FL sample locations were thinner than the ventral sample locations, and the AB samples were slightly thinner than the skin from the lower limbs (Figure 8).

Collagen & Glycosaminoglycan Analysis

No statistically significant difference in total soluble collagen or GAG content was noted between affected and unaffected skin (Figure 9). An average of 0.5966 ±0.0737 µg of collagen per mg of wet weight tissue was observed for affected horses, while an average of 0.4466 ±0.0654 µg/mg was observed in unaffected horses (Table 5). For GAG an average of 0.7424 ±0.0502 µg of GAG per mg of frozen weight tissue was observed for affected horses, while an average of 0.7097 ±0.0429 µg/mg was observed in unaffected horses (Table 5). Horses used in both total soluble collagen and GAG assays were age-matched with no statistical difference noted in collagen or GAG concentrations with respect to age of the horse

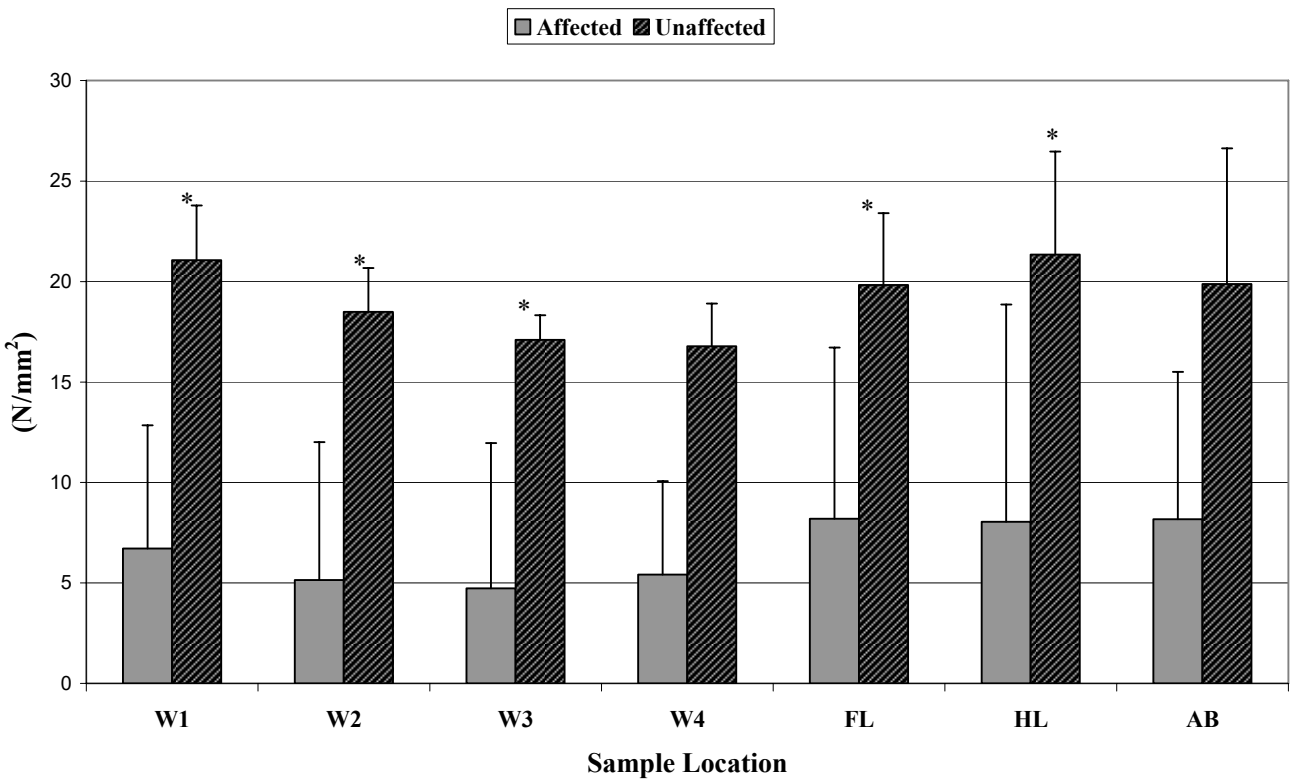

Figure 5

Ultimate skin tensile strength values from 10 affected and 13 unaffected horses. Statistically significant difference between groups for each site location denoted by * ($p \leq 0.05$).

Table 1

Ultimate Tensile Strength (N/mm^2)

Animal	Breed	Age	Sex	HC Status	W1*	W2*	W3*	W4	FL*	HL*	AB
A1	QH	1	F	Affected	7.33	2.14	3.86	6.61	11.65	13.48	11.55
A2	QH	1	F	Affected	6.00	4.00	4.99	7.31	10.07	3.45	5.73
A3	QH	1	M	Affected	4.40	7.48	5.89	7.62	8.40	8.26	2.62
A4	QH	2	F	Affected	6.83	7.10	6.46	†	7.44	5.54	†
A5	QH	2	F	Affected	6.21	7.35	4.90	4.73	5.78	5.80	9.62
A6	QH	3	M	Affected	2.71	1.35	5.37	†	4.17	1.19	5.64
A7	QH	4	F	Affected	5.02	5.81	2.68	2.20	12.29	†	†
A8	QH	5	F	Affected	7.60	4.23	4.19	†	12.34	5.76	4.56
A9	QH	9	M	Affected	12.92	5.13	5.74	4.04	1.66	17.12	22.98
A10	QH	10	F	Affected	8.11	6.83	3.21	†	8.18	11.84	2.74
B1	Perch	1	F	Unaffected	22.88	14.90	22.83	20.21	23.35	16.22	12.09
B2	QH	2	M	Unaffected	34.15	29.33	28.17	27.97	36.96	18.23	24.87
B3	QH	11	M	Unaffected	20.15	22.01	23.91	19.35	32.78	45.64	24.98
B4	QH	13	F	Unaffected	29.67	19.63	10.73	16.75	16.29	12.24	19.88
B5	QH	14	M	Unaffected	16.72	†	20.39	17.93	15.64	21.48	32.42
B6	QH	15	M	Unaffected	22.40	7.66	23.83	14.95	20.46	15.64	22.26
B7	QH	15	M	Unaffected	23.41	13.57	9.05	8.80	12.89	18.53	16.11
B8	Paint	17	F	Unaffected	12.09	22.78	3.99	17.30	11.41	12.43	29.36
B9	Paso	18	M	Unaffected	23.40	17.32	8.98	19.17	19.60	21.73	19.00
B10	QH	18	M	Unaffected	21.62	6.94	13.39	13.67	9.89	†	13.58
B11	TB	20	F	Unaffected	16.85	20.95	17.71	11.58	18.17	19.25	†
B12	QH	22	F	Unaffected	16.59	19.63	20.16	15.39	28.71	41.11	16.84
B13	QH	24	M	Unaffected	13.88	27.06	19.11	14.97	11.64	13.53	7.14
					6.71	5.14	4.73	5.42	8.20	8.05	8.18
				Affected Avg	±2.7	±2.1	±1.2	±2.1	±3.5	±5.1	±6.7
					21.06	12.48	17.09	16.77	19.83	21.34	19.88
				Unaffected Avg	±6.1	±6.8	±7.2	±4.6	±8.5	±10.8	±7.3

* Represents statistically significant difference between affected and unaffected groups for individual site.

† Represents unusable data due to samples slipping out of the grips.

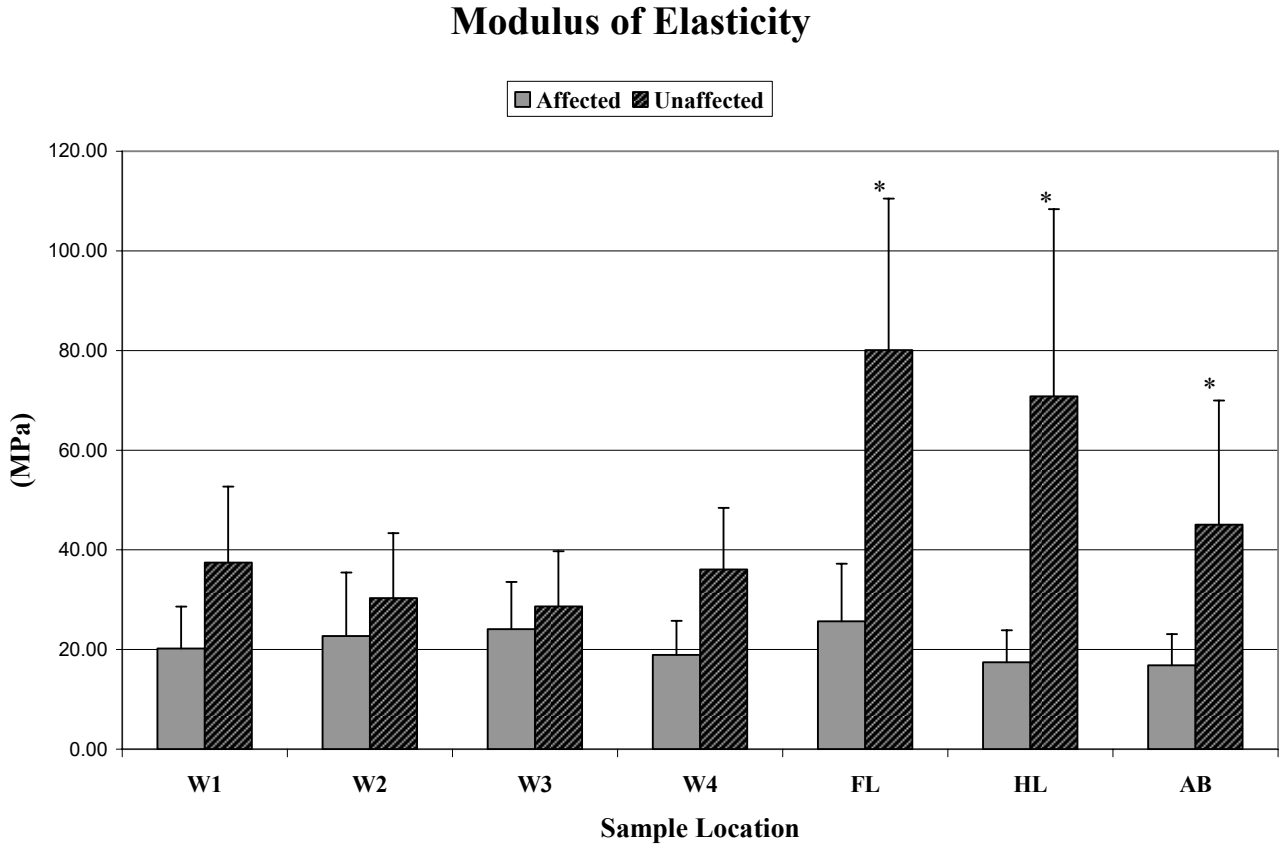

Figure 6

Modulus of elasticity average values from 10 affected and 13 unaffected horses. Statistically significant difference between groups for each site location denoted by * ($p \leq 0.05$).

Table 2

Modulus of Elasticity (MPa)

Animal	Breed	Age	Sex	HC Status	W1	W2	W3	W4	FL*	HL*	AB*
A1	QH	1	F	Affected	19.11	16.18	17.45	25.30	35.86	23.83	23.83
A2	QH	1	F	Affected	17.83	10.69	20.27	18.93	37.54	13.99	11.11
A3	QH	1	M	Affected	16.95	34.41	32.73	25.21	37.84	23.06	23.29
A4	QH	2	F	Affected	19.77	34.33	43.90	†	33.12	14.20	†
A5	QH	2	F	Affected	11.46	18.66	17.25	22.79	16.97	12.78	22.11
A6	QH	3	M	Affected	9.00	5.43	23.68	†	18.41	8.11	5.91
A7	QH	4	F	Affected	19.01	18.66	14.01	11.20	18.20	†	†
A8	QH	5	F	Affected	39.01	21.03	33.20	†	35.91	21.75	15.40
A9	QH	9	M	Affected	21.59	19.65	20.05	10.05	6.22	12.68	15.37
A10	QH	10	F	Affected	28.09	48.29	18.52	†	16.63	26.66	17.51
B1	Perch	1	F	Unaffected	27.89	21.86	36.02	32.38	58.65	45.60	68.30
B2	QH	2	M	Unaffected	35.77	38.00	31.28	45.06	90.36	81.83	70.64
B3	QH	11	M	Unaffected	42.83	53.44	44.79	56.01	157.73	153.63	80.35
B4	QH	13	F	Unaffected	41.40	39.62	32.48	29.33	68.85	57.56	60.97
B5	QH	14	M	Unaffected	44.08	†	24.04	46.72	54.80	50.37	27.50
B6	QH	15	M	Unaffected	14.31	20.69	21.39	39.60	90.06	52.85	12.08
B7	QH	15	M	Unaffected	42.66	22.42	22.44	31.36	59.13	69.88	47.66
B8	Paint	17	F	Unaffected	41.44	41.33	29.39	27.20	55.76	55.10	13.79
B9	Paso	18	M	Unaffected	65.30	48.98	48.50	57.13	89.22	36.06	63.73
B10	QH	18	M	Unaffected	62.13	21.83	18.38	34.00	57.20	†	53.55
B11	TB	20	F	Unaffected	25.55	17.68	36.58	30.06	80.85	53.10	†
B12	QH	22	F	Unaffected	19.78	20.86	15.96	25.69	119.63	140.83	30.58
B13	QH	24	M	Unaffected	23.58	17.43	11.52	14.16	58.82	52.94	11.51
					20.18	22.73	24.10	18.91	25.67	17.45	16.82
				Affected Avg	±8.4	±12.7	±9.4	±6.8	±11.5	±6.4	±6.2
					37.44	30.34	28.67	36.05	80.08	70.81	45.06
				Unaffected Avg	±15.2	±13.0	±11.0	±12.3	±30.4	±37.5	±24.9

* Represents statistically significant difference between affected and unaffected groups for individual site.

† Represents unusable data due to samples slipping out of the grips.

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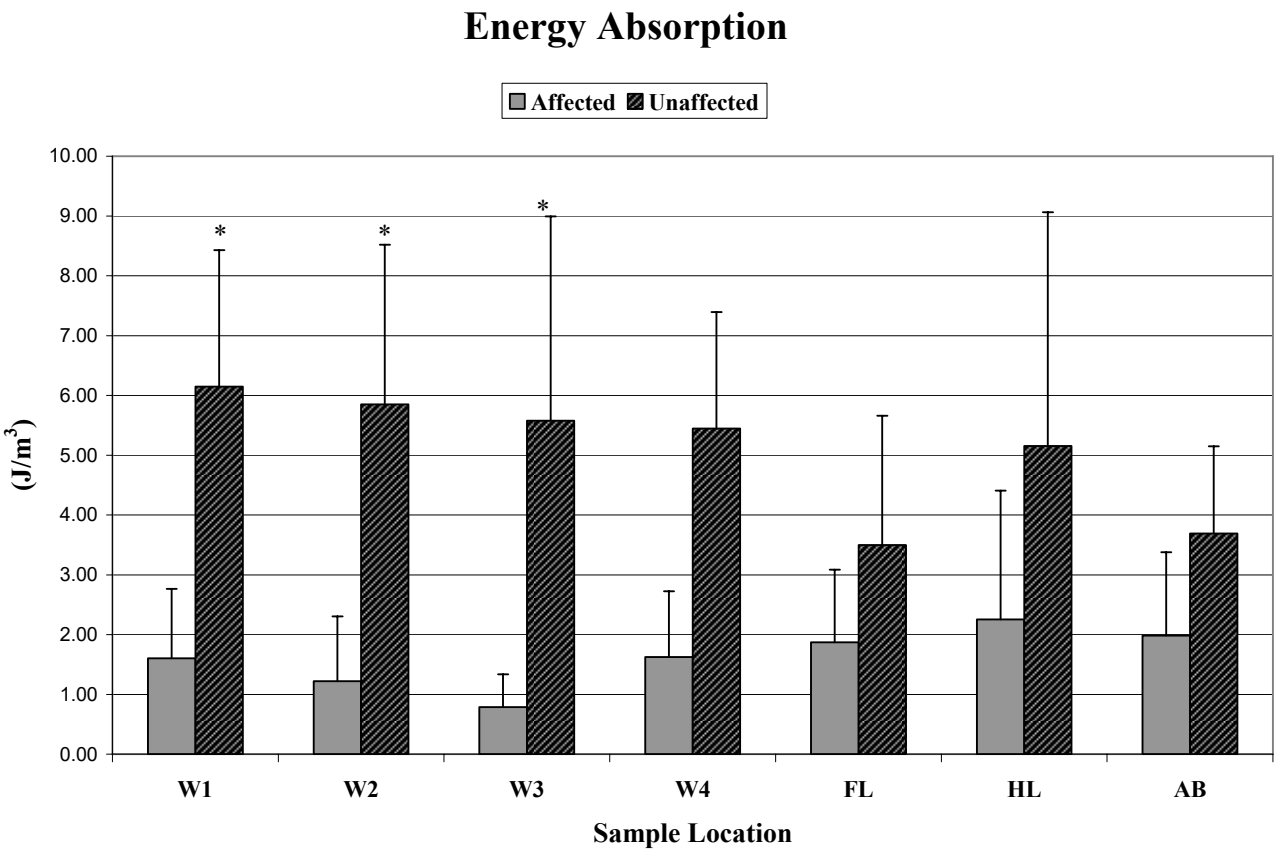

Figure 7

Energy absorption average values from 10 affected and 13 unaffected horses. Statistically significant difference between groups for each site location denoted by * ($p \leq 0.05$).

Table 3

Energy Absorption (J/m^3)

Animal	Breed	Age	Sex	HC Status	W1	W2*	W3*	W4	FL	HL	AB
A1	QH	1	F	Affected	1.49	0.15	0.77	3.40	1.98	4.11	2.45
A2	QH	1	F	Affected	1.95	1.00	0.52	2.07	2.13	0.40	1.91
A3	QH	1	M	Affected	0.61	0.87	0.73	1.98	1.46	2.14	0.17
A4	QH	2	F	Affected	2.43	0.84	0.68	†	1.06	1.52	†
A5	QH	2	F	Affected	1.78	3.40	1.87	1.08	1.58	1.41	3.26
A6	QH	3	M	Affected	0.48	0.20	0.78	†	0.52	0.09	2.16
A7	QH	4	F	Affected	0.81	0.99	0.34	0.28	4.39	†	†
A8	QH	5	F	Affected	0.88	1.12	0.28	†	3.07	0.93	1.20
A9	QH	9	M	Affected	4.38	2.95	1.63	0.93	0.28	6.99	4.30
A10	QH	10	F	Affected	1.21	0.66	0.30	†	2.23	2.68	0.42
B1	Perch	1	F	Unaffected	8.65	5.84	8.07	7.93	5.93	4.24	1.19
B2	QH	2	M	Unaffected	9.92	10.48	7.83	9.27	8.82	2.15	4.72
B3	QH	11	M	Unaffected	4.12	6.61	10.50	3.66	4.73	14.45	4.62
B4	QH	13	F	Unaffected	8.79	6.03	3.46	4.35	2.79	1.38	3.78
B5	QH	14	M	Unaffected	3.89	†	6.27	6.05	3.00	5.22	6.15
B6	QH	15	M	Unaffected	9.27	1.72	11.28	6.89	3.08	5.00	4.22
B7	QH	15	M	Unaffected	6.97	4.46	2.15	1.64	1.54	3.93	3.76
B8	Paint	17	F	Unaffected	3.78	5.71	0.30	5.61	1.97	1.86	4.93
B9	Paso	18	M	Unaffected	5.07	3.61	0.91	3.90	2.66	6.78	4.00
B10	QH	18	M	Unaffected	4.52	2.06	3.55	5.51	0.98	†	2.37
B11	TB	20	F	Unaffected	6.00	8.08	5.34	4.47	2.98	3.63	†
B12	QH	22	F	Unaffected	4.75	6.10	6.09	5.71	5.23	10.85	3.14
B13	QH	24	M	Unaffected	4.16	9.48	6.75	5.79	1.73	2.35	1.42
					1.60	1.22	0.79	1.62	1.87	2.25	1.98
				Affected Avg	±1.1	±1.0	±0.5	±1.1	±1.2	±2.1	±1.3
					6.14	5.85	5.58	5.44	3.50	5.15	3.69
				Unaffected Avg	±2.2	±2.6	±3.4	±1.9	±2.1	±3.9	±1.4

* Represents statistically significant difference between affected and unaffected groups for individual site.

† Represents unusable data due to samples slipping out of the grips.

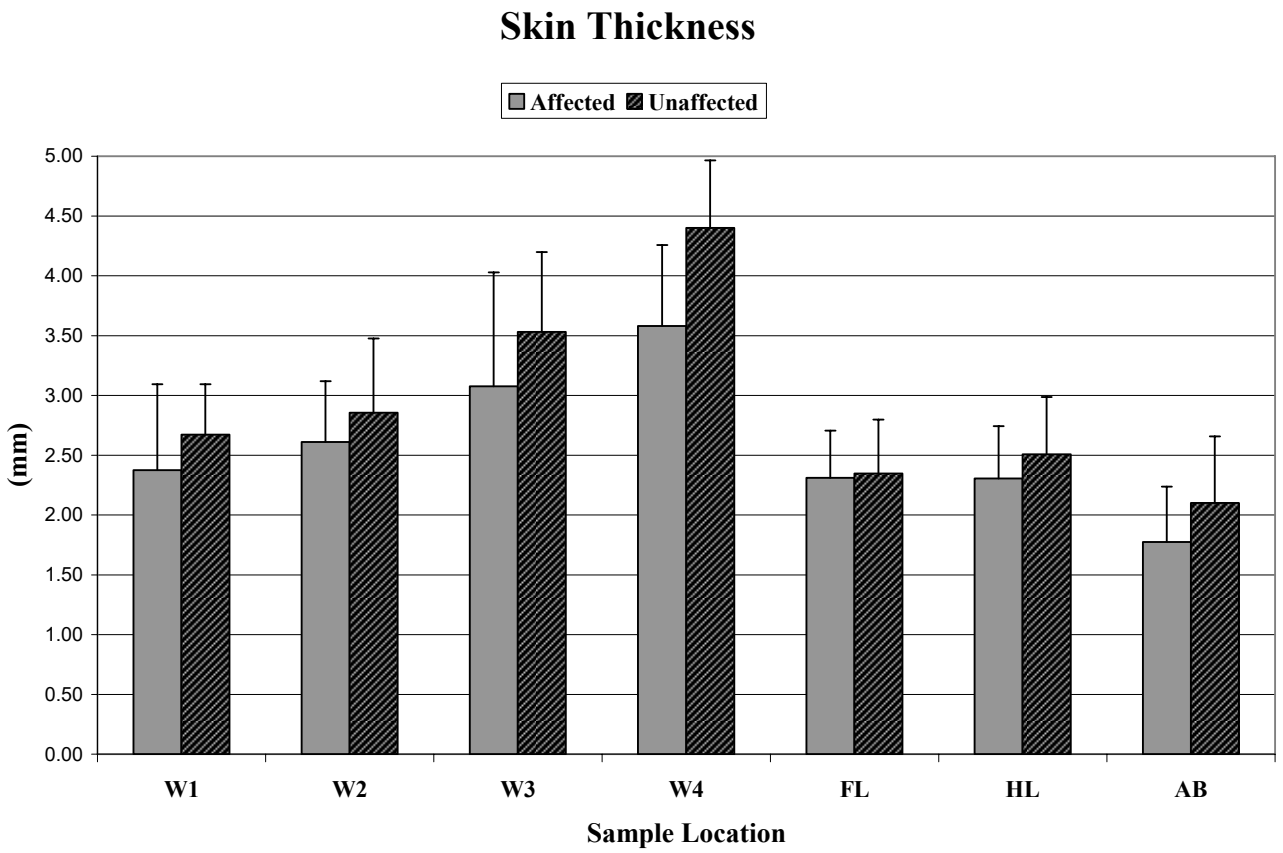

Figure 8

Skin thickness average values from 10 affected and 13 unaffected horses. Statistically significant difference between groups for each site location denoted by * ($p \leq 0.05$).

Table 4

Skin Thickness (mm)

Animal	Breed	Age	Sex	HC Status	W1	W2	W3	W4	FL	HL	AB
A1	QH	1	F	Affected	2.55	2.05	2.75	3.22	2.32	2.31	1.87
A2	QH	1	F	Affected	2.10	2.43	3.23	4.09	2.11	1.77	2.13
A3	QH	1	M	Affected	3.04	3.09	3.61	4.05	2.18	2.05	1.01
A4	QH	2	F	Affected	2.64	2.65	4.36	†	2.67	2.03	†
A5	QH	2	F	Affected	1.76	2.42	2.07	3.49	1.85	2.46	1.77
A6	QH	3	M	Affected	3.14	2.90	4.19	†	2.07	3.07	2.59
A7	QH	4	F	Affected	3.16	3.33	2.57	2.44	3.16	†	†
A8	QH	5	F	Affected	1.60	2.25	1.52	†	2.15	1.87	1.72
A9	QH	9	M	Affected	2.70	3.18	3.98	4.19	1.98	2.86	1.48
A10	QH	10	F	Affected	1.05	1.79	2.47	†	2.61	2.33	1.63
B1	Perch	1	F	Unaffected	3.14	2.95	3.70	4.55	2.50	2.80	1.62
B2	QH	2	M	Unaffected	2.56	2.61	3.60	4.55	2.80	2.15	2.29
B3	QH	11	M	Unaffected	3.01	3.54	4.09	4.11	2.20	2.20	1.61
B4	QH	13	F	Unaffected	1.92	2.29	3.34	4.14	1.83	1.77	1.30
B5	QH	14	M	Unaffected	2.89	†	3.71	4.71	3.11	2.97	1.83
B6	QH	15	M	Unaffected	2.90	3.66	4.40	5.75	2.25	2.75	2.65
B7	QH	15	M	Unaffected	2.84	3.18	4.34	4.82	2.45	2.65	2.02
B8	Paint	17	F	Unaffected	3.12	2.75	3.22	3.84	2.15	2.75	1.79
B9	Paso	18	M	Unaffected	2.83	3.81	1.82	3.81	2.83	3.03	2.39
B10	QH	18	M	Unaffected	2.65	2.95	3.89	4.59	2.45	3.12	3.12
B11	TB	20	F	Unaffected	1.77	1.74	2.95	3.49	1.55	1.94	†
B12	QH	22	F	Unaffected	2.65	2.34	3.34	4.34	1.76	1.77	1.77
B13	QH	24	M	Unaffected	2.45	2.45	3.49	4.50	2.62	2.70	2.83
				Affected Avg	2.37 ±0.7	2.61 ±0.5	3.08 ±0.9	3.58 ±0.6	2.31 ±0.3	2.31 ±0.4	1.78 ±0.4
				Unaffected Avg	2.67 ±0.4	2.86 ±0.6	3.53 ±0.6	4.40 ±0.5	2.35 ±0.4	2.51 ±0.4	2.10 ±0.5

* Represents statistically significant difference between affected and unaffected groups for individual site.

† Represents samples that could not be collected due to overall length of the animal or the presence of wounds or scars at the sample site.

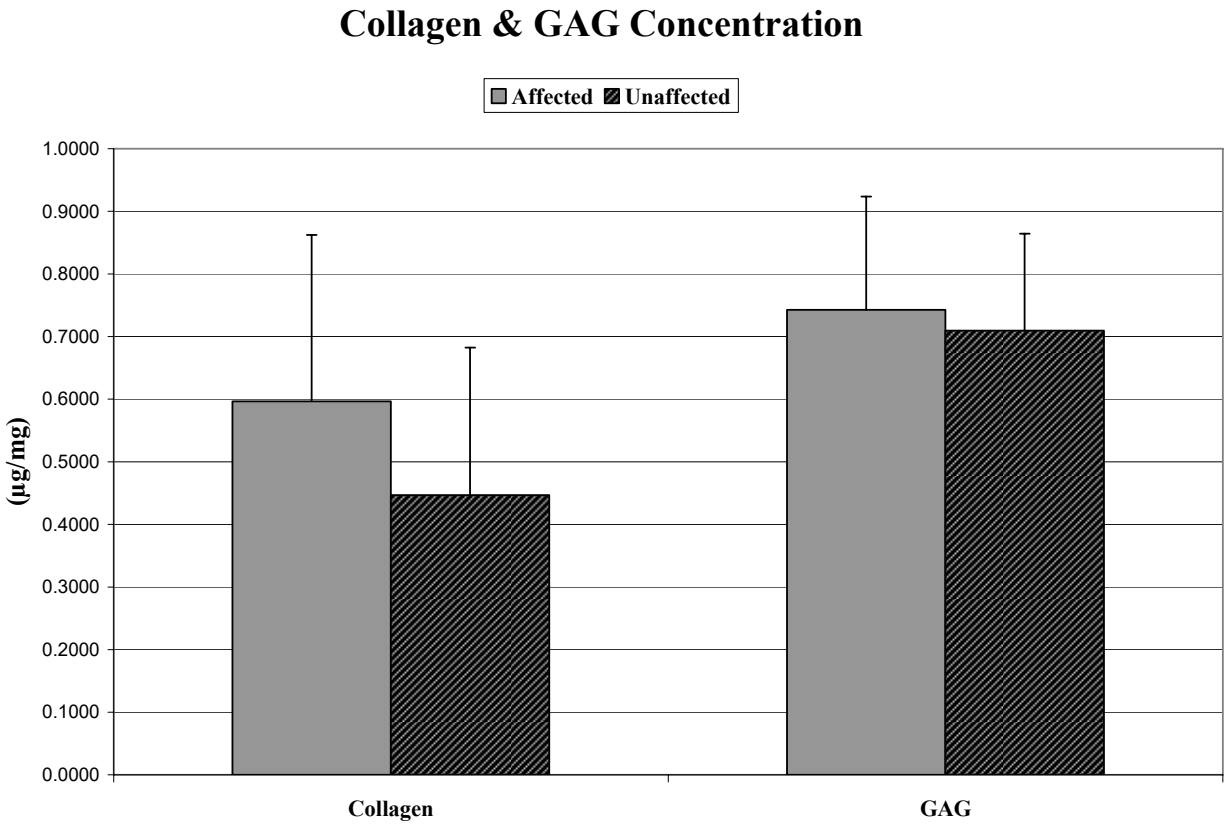

Figure 9

Total soluble collagen and glycosaminoglycan (GAG) concentration average values from 13 affected and 13 unaffected horses . Statistically significant difference between groups denoted by * ($p \leq 0.05$).

Table 5

Collagen/GAG Concentrations (µg/mg)

Animal	Breed	Age	Sex	HC Status	Collagen Concentration (µg/mg)	GAG Concentration (µg/mg)
A1	QH	1	F	Affected	0.8100	0.5937
A2	QH	1	F	Affected	0.2907	0.8181
A3	QH	1	M	Affected	0.4497	0.7365
A5	QH	1	M	Affected	1.1501	1.0651
A6	QH	1	M	Affected	0.5820	0.8609
A7	QH	2	F	Affected	0.7654	0.3986
A9	QH	2	F	Affected	0.2742	0.6448
A10	QH	2	M	Affected	0.5858	0.9298
A11	QH	2	M	Affected	0.7519	0.7750
A12	QH	3	M	Affected	0.5694	0.6425
A13	QH	4	F	Affected	0.2957	0.8956
A14	QH	5	F	Affected	0.8725	0.5208
A15	QH	6	F	Affected	0.3587	0.7709
B14	Paint	1	F	Unaffected	1.0497	0.8346
B15	QH	1	F	Unaffected	0.4008	1.0237
B16	QH	1	M	Unaffected	0.5265	0.7154
B17	QH	2	F	Unaffected	0.4478	0.7515
B18	QH	2	F	Unaffected	0.3552	0.6949
B2	QH	2	M	Unaffected	0.2605	0.8752
B19	QH	3	F	Unaffected	0.5075	0.6244
B20	QH	3	F	Unaffected	0.7989	0.6026
B21	QH	3	F	Unaffected	0.2594	0.8609
B22	QH	4	F	Unaffected	0.3154	0.5898
B23	TWH	5	M	Unaffected	0.3332	0.6411
B24	QH	6	M	Unaffected	0.2915	0.4933
B25	QH	6	F	Unaffected	0.2598	0.5186
				Affected Avg	0.5966 ±0.26	0.7425 ±0.18
				Unaffected Avg	0.4466 ±0.23	0.7097 ±0.15

Concentration of total soluble collagen and glycosaminoglycan (GAG) from affected and unaffected equine skin.

CHAPTER IV

DISCUSSION

Results from this study demonstrate a 2-3 fold reduction in tensile strength of HC horses for statistically significant sample locations. This data is consistent with recent studies in which a 4-8 fold reduction in skin tensile strength was observed in proteoglycan KO mice exhibiting EDS-like syndromes.[10,13-18] When tensile strength values for the unaffected group are examined individually a trend in decreasing tensile strength with increasing age is observed. Given this information it is likely a greater degree of difference would be observed had an appropriate set of age-matched samples been available, since the affected horses were much younger than the control horses. As such we are currently in the process of collecting and analyzing samples from young unaffected horses that are properly age-matched to our affected population.

As previously stated, histological studies have shown HC horses to have an irregular collagen micro-architecture compared to unaffected horses and this is most likely the underlying etiology of decreased skin tensile strength.[6-9] This scattered disruption of collagen fibres results in increased space between bundles with fibrosis or granulation tissue present at the points of separation, likely leading to the hyper-extensible and weak nature of HC horses' skin. Our results of decreased skin tensile strength in affected horses despite their young age combined with this current research increases the likelihood that decreased skin tensile strength in HC horses is not a

symptom of age, but a manifestation of biomechanical weakness due to altered collagen micro-architecture. Skin samples from the W4 site location were typically the thickest, which may account for the increased strength of affected skin from this area relative to other sites where unaffected and affected skin were compared. Samples taken from the AB site location were the most difficult due to irregular orientation of the skin when the animal is in lateral recumbency, possibly leading to poor sample consistency. HC affected horses also had noticeably hyper-extensible skin that was very difficult to remove in a consistent manner. The decreased biomechanical uniformity of the skin complicated sample harvesting, often becoming jagged or tearing during scalpel excision with multiple collections required to obtain a consistently sized sample. Furthermore, current literature and the clinical observation of HC affected horses sites few incidences of clinical lesions manifesting on the ventral surface of affected horses. This evidence lends credibility to the theory that HC manifests clinically at varying degrees of severity, regionally along the dorsum of the animal, possibly, in part due to increased environmental exposure (i.e. ultraviolet light, heat, trauma, etc.)

Samples used for biomechanical testing were large and required euthanasia of unaffected horses with an appropriate age-match to our young affected horses. As a result young horses with healthy skin were difficult to obtain from our clinical population of euthanized horses. Therefore our age-match was not ideal and resulted in an improper comparison between young and old horses. Furthermore, studies examining the effects of age on biomechanical properties of rat skin concluded that ultimate load, tensile strength, and breaking strength all sharply increase during maturation with a smaller decrease

observed following the end of maturation and beginning of senescence.[18] Our affected horses were much younger than our unaffected horses and should display a greater tensile strength value than that of our older unaffected horses. However, our data demonstrated a decrease in tensile strength amongst our young affected horses, supporting the theory that decreased tensile strength is most likely a result of pathology, not age.

Samples from unaffected horses typically 'failed' instantaneously by focal rapid shearing of the skin at a 45° angle, interestingly, the plane of max shear. However, samples from affected horses would typically stretch and shear during tensile strength analysis, and begin to fail at multiple sites prior to complete sample rupture. The appearance has been likened to stretching taffy or melting plastic. The lack of tightly conserved collagen bundling with an increased amount of space throughout the collagen matrix is most likely responsible for the numerous failure sites seen in HC samples. Furthermore, during tensile strength analysis affected horses' skin samples would routinely fail before reaching 100 N of force while unaffected horses' skin samples would routinely fail above 300 N with samples from the youngest unaffected horses exceeding the load limit of 500 N without failure.

Although not statistically significant, a trend of decreasing tensile strength was noted for W1-W3 sample locations. While the exact basis for this trend is unknown one possible theory is that environmental conditions such as ultraviolet light and heat stress may affect HC affected skin more than previously thought. While environmental influence in no way causes HC, it does appear to exacerbate the affected horse's skin over time. The W1, W2, and W3 sample locations are more exposed to environmental

conditions, such as ultraviolet light, leading to the possibility that environmental exposure may exacerbate HC. Investigations as to the affects of ultraviolet light on affected and unaffected fibroblast cells are currently being explored in our laboratory.

The modulus of elasticity was relatively the same at all seven sample locations for affected horses. Statistically significant differences between groups were present at the FL, HL, and AB sample locations primarily due to the massive increase in modulus of elasticity values for unaffected horses at these three sample locations. An increase in modulus could be expected at these sample locations as the skin on the lower extremities and abdomen needs to be able to withstand a greater amount of force than the skin located along the dorsal trunk of the horse. The similar modulus of elasticity for affected horses suggests that although HC may clinically present at varying degrees of severity, it is likely present all over the horses body and not just in areas presenting with lesions.

A decreased energy absorption was observed at all seven sample locations for affected horses with statistically significant differences between groups at the W1, W2, and W3 sample locations. Clinical presentation of HC wounds along the dorsal trunk of horses is consistent with these findings. The W1, W2, and W3 sample locations were able to withstand the least amount of energy before rupturing and as such would be expected to come from areas that wound easier. The samples from the dorsal trunk of affected horses exhibited the lowest energy absorption values, but only slightly lower than that of the samples from the lower extremities and abdomen. This data suggests the increased presence of wounds along the dorsal trunk of affected horses may be due to

some secondary problem, such as increased exposure to environmental conditions versus that of the lower extremities and abdomen.

No statistically significant difference in total soluble collagen was observed between affected and unaffected groups although the illustration in Figure 9 suggests otherwise. It has been reported in the clinically similar connective tissue disease, dermatosparaxis, that affected cats collagen is more soluble in on-denaturing solvents when skin samples were analyzed.[20] Previous attempts to quantitatively analyze collagen as a diagnostic tool in HC horses has suggested no difference in the presence of collagen between affected and unaffected horses.[6,8,9] However, had a larger sample size been analyzed in our study a statistically significant difference in total soluble collagen may have been observed. Based on these previous reports it is possible that HC affected horses do not possess more collagen than that of unaffected horses, but rather their loosely arranged collagen fibres are more soluble than the tightly arranged collagen fibres of unaffected horses.

Collagen and GAG findings in this study support the theory of irregular glycosylation of proteoglycans as an underlying cause of irregular collagen fibrillogenesis. As previously stated EDS-like symptoms can be induced experimentally by eliminating certain proteoglycans but no significant differences in GAG concentrations were noted in our study between affected and unaffected horses.[10, 13-18] Based on this data, it is possible that HC affected horses would not have a lower concentration of proteoglycans, but rather proteoglycans that were improperly glycosylated. A deficit or improperly glycosylated proteoglycans could possibly produce

similar symptoms. If irregular proteoglycans are an integral component of HC then the integrity of individual proteoglycans needs to be further examined in HC affected horses.

The delay in clinical presentation of HC can be very emotionally and financially demanding. Further characterization of HC will aid in understanding of why the disorder manifests itself in varying degrees of severity among horses. Now understanding the basis of HC horses hyper-extensible skin is likely due to altered collagen micro-architecture, and not a deficit of collagen or GAG, will aid in the future characterization and understanding of HC and possibly human EDS. While the reports of improperly glycosylated proteoglycans has yet to bet explored in HC affected horses, some areas of similarity have been characterized. The subtype of EDS to which HC is clinically similar, EDS Type VI, is often diagnosed by patients characteristic high urinary excretion of the collagen pyridinium crosslink deoxyprydinoline, resulting in a high ratio of deoxyprydinoline to pyridinoline.[21] This EDS Type VI characteristics strengthens the possible link between human EDS and equine HC as recently been reported that HC affected horses also exhibit an elevated deoxyprydinoline to pyridinoline ratio in their urine.[22]

Future directions for HC research based on the data presented here should focus on analyzing the skin tensile strength of properly age-matched horses and investigating further characteristics of HC, such as proper formation of proteoglycans and biomechanical analysis of animals that are carriers of HC. By understanding the etiology of HC researchers could break new grounds in EDS therapy using equine HC as an

animal model. Further research will better define the link between the two disorders and whether the horse is a suitable animal model for further study of the human disorders.

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