Summary

Reason for performing the study: To elucidate the relationship between mechanical behavior and microscopic morphology of the corio-epidermal junction (CEJ) of equine hooves under testing conditions.

Objectives: To determine the mechanical parameters and the two-dimensional length density of profiles of secondary lamellae of the CEJ in the dermal region (L_A(*SL, dermal*)) and to assess possible correlations.

Methods: Specimens of the CEJ were taken from the front, quarter and heel parts from three equine hooves, (n=25) and exposed to a uniaxial tensile test until rupture to obtain Young's moduli of elasticity, ultimate stress, and strain. The neighboring specimens to those used for biomechanical experiment were processed histologically to quantify the L_A(*SL, dermal*) using stereological grids.

Results: The estimated L_A(*SL, dermal*) was 0.022±0.006 μm^{-1} (mean±SD). Young's modulus of elasticity in the small deformation region was 0.31±0.04 MPa, and Young's modulus of elasticity in the linear region was 7.58±1.59 MPa. The ultimate stress was 2.09±0.96 MPa, and the ultimate strain was 0.59±0.25. The mechanical Young's modulus of elasticity in the region of small deformations has a moderate correlation with the L_A(*SL, dermal*).

Conclusions: As with most soft biological tissues, the CEJ has a nonlinear mechanical behavior. Within the range of small deformations, which correspond to physiological loading of the CEJ, a higher L_A(*SL, dermal*) is correlated with a higher resistance of the CEJ against high stresses transmitted from the distal phalanx to the hoof wall.

Potential relevance: The condition of the CEJ apparatus may be easily quantified as the length density of the profiles of secondary dermal lamellae. Quantification of L_A(*SL, dermal*) provides a simple tool that could be used e.g., for comparing the proneness of the various parts of the CEJ to initial stages of laminitis.

Introduction

The corio-epidermal junction (CEJ) of equine hoof wall, or the lamellar interconnection between connective tissue of the dermis and the keratinized stratified squamous epithelium, has to transfer and withstand high loading between third phalanx, hoof wall, and environment [1]. From 550 to 600 primary epidermal and dermal lamellae each bearing 100 to 200 secondary lamellae interlock with each other [2, 3]. The exceptionally large surface of this junction dissipates high local stresses from the distal phalanx and ensures even energy transfer during peak loading of the equine foot [3]. The complex of desmosomes between epidermal cells, hemidesmosomes connecting dermal cells to the basement membrane of the CEJ, anchoring fibers connecting basement membrane, and collagen fibers of the dermis and collagen fibers entering the distal phalanx as Sharpey's fibers provide stiffness and flexibility to this particular structure [2, 3, 4, 5].

It is well known that the morphology of the lamellae of the CEJ differs depending on the age of the horse, hoof wall region, and hoof geometry [2, 6, 7]. However, to our knowledge, the correlation of quantitative parameters of this lamellar structure with the mechanical properties of the CEJ has not yet been studied.

In our study, we focus on the relationship between the two-dimensional (2D) length density of secondary CEJ lamellae and Young's moduli of elasticity, ultimate stress, and ultimate strain. Young's modulus of elasticity or stiffness is measured in N/m^2 (Pa) and is defined as the slope of the stress-strain curve. Stress corresponds to the force acting on an area of a deformable body, while strain is the ratio of the length change caused by the stress to the original dimension of the object. In most biological materials, the stress-strain curve is nonlinear and can be divided into five parts [8, 9], as shown in Fig. 1A.

For our experiment, the mechanical behavior and surface of the CEJ were assessed for different regions of the equine hoof wall. The specific aims of the study were as follows:

(i) to test whether the overall hematoxylin-eosin (HE) stain is sufficient for quantitative assessment of the surface of the CEJ

(ii) to quantitatively describe the length density of the microscopic profiles of secondary lamellae in the dermal region in different parts of the hoof with presumably different relative CEJ surfaces given by laminae spacing [2, 6, 7]

(iii) to elucidate how the complex structure of the CEJ correlates with its mechanical behavior, any possible correlations between Young's modulus of elasticity in the small deformation region, Young's modulus of elasticity in the large deformation (linear) region, ultimate stress, ultimate strain, and the 2D length density of secondary dermal lamellae shall be analyzed.

Materials and Methods

Specimen collection and preparation

The 25 specimens of CEJs used for mechanical measurements were taken from three equine hooves. Nine specimens were collected from one adult horse, and 16 specimens from two foals, eight specimens per hoof. The legal and ethical requirements have been met with regards to the humane treatment of animals described in this study. Because the collecting of hooves and the mechanical experiments were performed in distant facilities, it was necessary to use a preservation process. In our earlier study [10], we found that neither the mechanical properties nor the morphological features necessary for the assessment of CEJ length density were changed by the deep freezing conservation (-20°C) utilized for preservation before cutting and measuring the specimens. Therefore, the hooves used in the present study were also kept frozen before further processing and measurements commenced. Tissue blocks specimens (approximately 20 mm in length, 8 mm in thickness and 10 mm in width) were cut from the frontal part, quarter parts and heels of the hooves. We used the sampling strategy published in [10] and [11] to retrieve samples with distinctly different mechanical and morphological properties [2, 6, 7]. Each specimen contained a part of the coffin bone and/or the hoof cartilage, the CEJ, and the wall horn. Another 25 specimens adjacent to those used for mechanical testing were cut from the hooves for histological processing. From these specimens, the bone and wall horn were removed. The remaining tissue blocks were fixed in buffered formalin according to Lillie [12]. Afterwards, the specimens subjected to mechanical examination were histologically processed to assess the morphology of the rupture line.

Histological processing and quantitative morphology

For preparation of histological sections, the specimens were dehydrated in graded ethanol solution and embedded in paraffin. All tissue blocks were cut transversally to the lamellae of the CEJ into 5-µm-thick sections, mounted on slides and stained with HE.

One section per specimen was used for stereological quantification. The 2D length density of the secondary lamellae of the CEJ and the length of the basement membrane profile per surface of tissue profile in the section (Fig. 1B) were assessed. As described in [9], only the deep, dermal half of the CEJ was taken into account (L_A(*SL, dermal*)) because this part is known to be affected first in laminitis [13, 14] and therefore is of special interest. A net of circular arcs was superposed on the micrograph of the section of the CEJ in a random manner. The number of intersections between the arcs and the structure of interest, namely the basement membrane, was counted (Fig. 1B) [15]. The resultant estimation of 2D length density was calculated by:

$$L_A(SL,\ dermal) = \left(\frac{\pi}{2} \cdot \sum Q_i\right) \Big/ \left((l/p) \cdot \sum P_i\right)$$

where *l/p* is the length of a circular arc of the test system, ΣQ_i is the number of intersections between the system of circular arcs and the basement membrane of secondary lamellae in the dermal region and ΣP_i is the total number of circular arcs in the reference region. The units of L_A(*SL, dermal*) are µm/µm^2=µm^{-1}. A total of 25 sections and 50 image fields (two per each section) representing a reference area of 19,1 mm^2 per all samples were examined. The total number of intersections between the testing probe of circular arcs and basement membrane was 2,219 for all samples. For this evaluation, the Ellipse[2] software was used.

Comparing the staining methods and analyzing the variability within series of sections

For comparing the routine HE staining with immunohistochemistry, another ten pairs of histological sections were taken from five randomly selected tissue samples. Each pair consisted of one section stained with HE and a consecutive section with immunohistochemical detection of the basement membrane. The immunohistochemical processing was as follows: the sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.6% H_2O_2 in methanol. The sections were pretreated with 0.1% protease from Streptomyces griseus[3] in phosphate-buffered salt

105 solution (PBS) for 20 min at room temperature. Nonspecific binding activity was blocked with normal
106 goat serum[4] in PBS at room temperature. The sections were incubated overnight with a polyclonal
107 rabbit anti-rat laminin antibody[5] (dilution 1:750) at 4°C. Products of the immunoreaction were detected
108 using the anti-rabbit PowerVision Kit[6] and visualized with diaminobenzidine[7] in 0.03% H_2O_2 in Tris-
109 buffered saline (pH 7.4). All sections were counterstained with Mayer's hematoxylin. From each pair of
110 sections, two micrographs were taken, with the first micrograph representing the HE stained section
111 and the second representing the corresponding part of corio-epidermal junction stained with laminin
112 antibody. Both micrographs were then aligned (registered) with the software Imagreg[8] to verify that the
113 corresponding structures would be evaluated and that the mutual shift and rotation of the paired
114 sections would be minimized. The registered pairs of sections then underwent quantitative assessment
115 with the same magnification and the same settings of stereological sets of circular arcs as all the other
116 samples, in which we estimated the surface density of the CEJ. The intersections between the profile
117 of the basement membrane and the circular arcs were counted in both staining methods (Fig. 1C, D)
118 and then compared using the Wilcoxon matched pairs test.

119 To assess the sampling error caused by the variability of adjacent histological sections stained with HE
120 and used for quantitative assessment of the surface of the CEJ, the following procedure was
121 employed: from ten serial (consecutive) histological sections, we took ten micrographs with the 20×
122 objective. The series of micrographs was focused on corresponding parts of the sections such that
123 they mapped the differences caused by the three-dimensional nature of the dermal lamellae. The
124 series of micrographs was then aligned with the software Imagreg, as described above. We then
125 counted the number of intersections between the stereological system of circular arcs and the profile
126 of the basement membrane using the same settings as in the rest of the study. The variation in the
127 number of intersections counted in the serial sections was assessed using the coefficient of error (CE)
128 calculated with the quadratic approximation formula of Matheron, modified for use in a stereological
129 context [16]. The resulting value was 0.041, which quantified the sampling error in our study.

130 Mechanical measurements

131 The specimens underwent the uniaxial tension test until tissue rupture using the traction machine
132 Zwick/Roell[9] at room temperature. The clamping of individual specimens was performed so that only

the CEJ was exposed to loading; the bone and the wall horn of the hoof were held in the clamps of the traction machine. First, the specimens were preconditioned using 50 cycles with linearly increasing and decreasing elongation up to 18% of the initial length. After this preconditioning, a linear increase in loading was applied until CEJ rupture. The loading velocity was 500 mm/min corresponding to the gallop of horses [11]. The applied forces and measured deformations were recorded during the testing and used for further evaluation.

Young's modulus of elasticity was determined for small (E_1, strain up to 10% depending on the stress-strain curve shape) as well as large (E_2, strain between 20 and 40% depending on the stress-strain curve shape) deformation. The stress was defined as the measured force divided by initial cross-sectional area (thickness × width) of the tissue specimen. The strain was defined as the elongation of the specimen divided by initial height of the CEJ lamellae, or the distance between the clamps of the traction machine. Ultimate stress and ultimate strain were determined at the point of CEJ rupture. "details to be provided on acceptance" software [17] was used to determine all mechanical parameters.

Statistical analysis

The normality of the L_A(*SL, dermal*), Young's moduli and ultimate stress and strain were tested by Shapiro-Wilk's W-test. Because some data sets did not pass the test for normality, non-parametric methods were also used. In the Results section, all data are given as the mean ± standard deviation. The correlation between mechanical parameters and the L_A(*SL, dermal*) was analyzed using the Spearman correlation coefficient.

Results

When comparing the HE staining and the immunohistochemical detection of laminin, we found no significant differences between the paired values based on sections grouped by staining method ($p=0.140$). Therefore, the HE-stained sections could be used for further morphological analysis.

Stress-strain curves showed similar shapes in all tested tissue samples; the stress-strain curves were nonlinear with visible tissue stiffening (Fig. 1E). During initial preconditioning the unloading stress-strain curve remained below the loading stress-strain curve. After approximately 10 more cycles of

loading and unloading, the loading and unloading stress-strain curves followed the loading and unloading stress-strain curves of previous cycles. This state was defined as an end-point of preconditioning, after which the tensile test with linearly increasing loading until tissue rupture could be started.

A small deformation region with low Young's modulus of elasticity (E_1=0.31±0.04 MPa) and a linear region with higher Young's modulus of elasticity (E_2=7.58±1.59 MPa) can be observed on the stress-strain curve (for example, see Fig. 1E).

The ultimate stress when rupture of the CEJ occurred was 2.09±0.96 MPa, and the ultimate strain was 0.59±0.25. Microscopical analysis of histological sections showed that the line of rupture crossed the dermal and epidermal lamellae approximately in the middle of the CEJ (Fig. 1F). Only in a few cases could detachment of epidermal lamellae from their dermal counterpart be observed.

The mean stereological estimated L_A(*SL, dermal*) of all samples was 0.022±0.006 μm^{-1}. Correlations between mechanical parameters and L_A(*SL, dermal*) are given in Table 1.

Discussion

The results of mechanical testing confirmed that the CEJ of the equine hoof as a complex belongs to viscoelastic materials. The mechanical response of such materials is a combination of pure elasticity, where the material, after deformation caused by loading, returns to its original shape after unloading, and viscosity, i.e., the resistance of a fluid to deformation. The viscoelastic behavior is linked to viscoelastic phenomena, such as stress relaxation, creep, and hysteresis [8]. The hysteresis was demonstrated in our study during preconditioning. The unloading stress-strain curve did not follow the loading curve exactly but remained below the loading curve meaning that energy dissipation did occur.

The end-point of preconditioning was set to the time point when loading and unloading stress-strain curves followed the loading and unloading stress-strain curves of previous cycles. Under such conditions, the tissues of CEJ were assumed to have the same mechanical properties as in the hoof of the living horse [8]. After preconditioning, the tensile test with linearly increasing loading until tissue rupture could be started.

187 Stress-strain curves of the CEJ had a typical shape for biological materials (Fig. 1A). The curves were
188 non-linear, showing a small deformation region, stiffening, and a linear region (Fig. 1E). The small
189 deformation region was characterized by a low gradient, i.e., low modulus of elasticity (E_1
190 approximately 0.3 MPa). In the linear region, the modulus of elasticity (E_2 approximately 7.5 MPa) was
191 considerably higher, representing the stiffening of the CEJ tissues with increasing strain. This behavior
192 has been described before for soft biological materials, such as tendons, skin, ligaments, muscles, and
193 arteries [18, 19, 20]. In general, the stiffening of biomaterials can be explained on different levels.
194 Collagen crimping, i.e., the wavy structure of unloaded collagen fibers, appears to play an important
195 role in the CEJ as in all organs containing collagen. Stretching collagen first straightens the crimp. In
196 this initial phase of loading, the material properties of collagen only marginally influence the modulus of
197 elasticity of tissue. After stretching, collagen fibers with their high modulus of elasticity (1-2.5 GPa for
198 collagen in rat-tail tendon [21], in comparison to 0.1 MPa for cells [21, 22]) begin to contribute to the
199 total mechanical response of the tissue [8, 23]. In addition, strain stiffening of cytoskeletal networks, as
200 described for micro- and intermediate filaments [24 and references therein], can be expected to play
201 an important role in the CEJ of the equine hoof, especially regarding the living epidermal cell layers
202 forming the secondary epidermal lamellae. The same applies for hemidesmosomes linking basal
203 epidermal cells to the basement membrane of the CEJ [25]. Strain stiffening and the elastic response
204 of extra- as well as intracellular matter depend on the biochemical milieu, including ion concentration,
205 hydrogen bonding, and hydration forces [24, 26]. Comparatively small changes of this internal milieu,
206 e.g., in subclinical phases of laminitis, might thus considerably affect the mechanical properties of the
207 CEJ.

208 It must be noted that mechanical testing of the CEJ as performed for this study neither mimics the
209 physiological load of this structure nor the changes during laminitis. In healthy adult horses,
210 orientation, spacing, and curvature of CEJ lamellae are optimized for uniform stress redistribution and
211 load transmission from the digital phalanx to hoof wall [2, 6, 7] and thus describe trajectories along the
212 main direction of forces acting in the respective regions of the hoof. Mechanical implications of this
213 three-dimensional arrangement are not taken into account in simple mechanical testing when force is
214 applied perpendicular to CEJ lamellae. However, the mechanical test illustrates the exceptionally firm
215 interconnection of epidermal lamellae, basement membrane, and dermal lamellae.

The obtained ultimate stress of the equine CEJ at 2.09 MPa is in agreement with values obtained for the hooves of beef bulls 2.27-4.87 MPa [27]. The somewhat higher ultimate stress obtained for bulls could be related to the adaptation of the CEJ to the higher weight of the animals (527 kg in contrast to maximally 400 kg of the horses used in our study) and more probably to the different loading velocities used during the CEJ measurements in our study versus those in [27]. The velocity was only 30 mm/min for bulls (to reach the quasi-static conditions that allow the viscoelastic tissues within the sample to continuously adjust to the changing loading) in contrast to the 500 mm/min (horse's gallop) used in our case. Because the tissue behaves like viscoelastic material, as our results demonstrate, the resultant mechanical values depend highly on the loading velocity because of the viscosity. However, the order of magnitude of ultimate stress for both tissues is the same.

As already described in a previous study on microcracks induced within the CEJ during ultimate strength testing [28], the rupture of the tissue on application of critical tensile force is not in the dermal or epidermal region (Fig. 1F). Neither the basement membrane nor the tissue surrounding it is the weakest link. In most samples, the rupture line crosses the middle of primary epidermal and dermal lamellae. This demonstrates that there is no difference between the mechanical properties of the basement membrane and surrounding tissue when they are integrated into the CEJ in the case of ultimate stresses and strains. Interestingly, when the hooves of healthy cattle are tested in the same way as described in our study, disruption occurs primarily at the corio-epidermal junction [27], which is similar to the damage of the equine CEJ due to laminitis [13]. This could be a consequence of the simpler CEJ of ruminants with only primary epidermal and dermal lamellae in contrast to the primary and secondary lamellae of the equine CEJ. The increase in the CEJ surface by the formation of secondary lamellae thus appears to contribute to the increased mechanical resistance of this structure. The significant correlation between the CEJ length density and Young's modulus of elasticity in the small deformation region found in our study supports this hypothesis. Under physiological conditions, the individual parts of the equine digit are exposed mostly to small deformations [29]. Increasing L_A(*SL, dermal*) increases the ability of the CEJ to resist loading. Note that the length density is a relative parameter, while the absolute surface area of the CEJ could give different results.

We observed different correlations in small and large deformation regions. In the small deformation region, we found a significant negative correlation between Young's modulus of elasticity and ultimate

stress as well as between Young's modulus of elasticity and ultimate strain. In contrast, in the large deformation region, we observed a positive correlation between ultimate stress and ultimate strain as well as between ultimate stress and Young's modulus of elasticity. Moreover, we discovered no relationship between CEJ length density and Young's modulus of elasticity in the large deformation region, ultimate stress and ultimate strain. Therefore, it appears that the structures that are responsible for the behavior of the CEJ at the large loading are not its secondary lamellae, but some other components of the CEJ. In contrast, the secondary dermal lamellae can be regarded as responsible for the mechanical behavior of the CEJ at small (physiological) deformations and strongly influencing its stiffness at small loading.

The total mechanical properties of the tissue sample result from a series of constituents linked together, i.e., the bone, periosteum, and connective tissue of the dermis; the CEJ; the epithelium; and the horned wall of the hoof [2]. Our results suggest that within the physiological loading range, the morphology of the CEJ explains a substantial part of its mechanical behavior. The most important finding in our study is that the condition of the CEJ apparatus may be easily quantified as the length density of the profiles of secondary dermal lamellae. Quantification of L_A(SL, dermal) provides a simple tool that could be used for comparing the proneness of the various parts of the CEJ to the initial stages of laminitis. This quantitative parameter can be easily assessed with routinely used HE staining, which yields results comparable to those based on specific immunohistochemical detection of the basement membrane. Our results suggest that the low L_A(SL, dermal) might be related to the locally specific vulnerability of the CEJ when comparing various parts of hooves in the proximo-distal and medio-lateral directions. Confirmation of the latter hypothesis would require another study with additional hooves. However, in this study we have been able to demonstrate the quantitative relationship between CEJ morphology and biomechanical properties.

Acknowledgments

Manufacturers' addresses:

[1] Sigma-Aldrich, Vienna, Austria.

[2] ViDiTo, Kosice, Slovac Republic.

[3] Sigma, Vienna, Austria.

[4] DakoCytomation, Glostrup, Denmark.

[5] DakoCytomation, Glostrup, Denmark.

[6] Immunovision Technologies, Daly City, CA, USA.

[7] Sigma-Aldrich, Vienna, Austria.

[8] Jiří Janáček, The Academy of Sciences of the Czech Republic, Prague, Czech Republic.

[9] Zwick/Roell, Ulm, Germany.

Figure legend:

Fig. 1: A – A nonlinear stress-strain curve characteristic of soft biological materials: I – the toe curve region with low stiffness, II – the heel region of the curve with stiffening of the tissue, III – the linear region with high stiffness, IV – the region before tissue rupture with initialization of the rupture at the end of this region, V – the region of rupturing of individual components of the tissue. B – The stereological assessment of the two-dimensional length density of the secondary lamellae of the corio-epidermal junction (CEJ): the basement membrane is marked by a red line; the intersections between basement membrane and the superposed circular net are marked yellow. Bar=100 μm. C-D – Comparison of stereological assessment of CEJ length density in hematoxylin-eosin stained sections (C) and corresponding sections with immunohistochemical detection of laminin of the basement membrane (D). The intersection between the basement membrane of the epidermis and the testing grid are marked yellow. The results of the quantitative analysis are the same, independent of the

staining method used. Bars=10 0 µm. E – Example of the non-linear stress-strain curve of the CEJ; note the small deformation region (red line) with low stiffness and the linear region (green line) with higher stiffness. F – An example of rupture of the CEJ after mechanical testing: the line of rupture crossed dermal and epidermal lamellae approximately in the middle of the CEJ. Bar=1000 µm.

Table legend:

Table 1: Spearman rank order correlations (R) between mechanical parameters and length density of the corio-epidermal junction of the equine hoof (CEJ). E_1, Young's modulus of elasticity in the small deformation region; E_2, Young's modulus of elasticity in the linear region; L_A(*SL, dermal*), two-dimensional length density of secondary lamellae of CEJ. Bolded correlations are significant at $p<0.05$. Autocorrelations and repeating values are replaced by '–'.

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