Effect of prolonged water immersion on equine hoof epidermis in vitro

Ilka P. Wagner, DVM, and David M. Hood, DVM, PhD

Objective—To evaluate the effect of prolonged water exposure on tissue mass and solutes of outer and inner layers of the stratum medium, sole, frog, and the stratum medium (SMZA) zona alba layer of horses' hooves.

Specimen Population—10 hooves from 10 horses without foot abnormalities.

Procedures—Hoof wall tissue specimens were obtained and immersed for 10 days in distilled deionized water. Serial changes in mass were recorded during the immersion period. Subsequently, osmolarity and Na^+, K^+, Cl^-, and protein concentrations of the immersion solution were quantified.

Results—Fully cornified outer hoof wall, sole, and frog epidermal structures increased in mass, whereas the SMZA lost mass when immersed in water. All hoof structures had a variable loss of crystalloids during immersion, but none of the specimens lost proteins. The frog epidermis was distinct in that total solute lost during immersion could not be ascribed to Na^+, K^+, and Cl^-.

Conclusions and Clinical Relevance—Data support a 2-compartment model for the fully cornified outer stratum medium, frog, and sole that permits the exchange of crystalloids, but not proteins, across the cell membrane and infers that topical agents containing proteins cannot benefit the hoof. The unique osmotic behavior of the SMZA relative to other hoof structures suggests the hypothesis that it is composed of transitional epithelial cells. The solutes lost from frog epithelium are interpreted to reflect its unique lipid composition. (*Am J Vet Res* 2002;63:1140–1144)

The horse industry's awareness and concern regarding the potential influence of the environment on the material properties of the hoof is not new. One basis for this concern lies in the supposition that a low-elastic hoof wall decreases force dissipation within the foot, thereby increasing the forces transmitted through the limb and predisposing it to injury. Additionally, there is concern that horses subjected to alternating periods of wet and dry environments, such as high-dew mornings followed by dry afternoons or frequent washing, have altered composition or structure of the hoof, making it more susceptible to development of cracks.

Received Oct 5, 2001.
Accepted Mar 13, 2002.
From the Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, College Station, TX 77843 (Wagner, Hood); and the Hoof Diagnostic and Rehabilitation Clinic, PO Box 10381, College Station, TX 77842 (Hood).
Supported by The Hoof Project.
Address correspondence to Dr. Hood.

Responses to these concerns are represented by the development of management programs and numerous topical agents that are reputed to control hoof wall hydration. Results of empirical studies[1,2,a] of hoof wall hydration indicate that the percentage of relative hydration of the stratum medium, frog, and sole varies substantially and that regional variations exist within the stratum medium of the hoof wall. Results of other studies have substantiated that the material properties of the hoof components vary not only with specimen location[2-6] but also with hydration status[2,7] and have provided data that support the hypothesis that either over- or under hydration of the stratum medium of the wall affects its elastic modulus and fracture toughness.[2,8] Lastly, the ability of specific topical agents to control the rate of hoof wall dehydration has been evaluated[9]; results indicate that substantial differences between products are present.

The mechanisms by which the hoof regulates its hydration have not been widely studied. Conceptually, the physiology of hoof hydration can be separated into considerations of the forces responsible for the movement of water and the routes by which water moves through the wall. Intuitively, a hydrostatic pressure gradient, generated by the digit's circulation, can be hypothesized as a force that acts to drive water from the dermis into the hoof. An additional hydrostatic force is that engendered as the hoof wall keratinocytes are compressed mechanically during loading of the foot. Given that the hydrostatic pressures on the external surface of the foot are lower than that in the foot's internal regions, the direction of water movement in response to these hydrostatic forces is outward. Logically, osmotic forces also contribute to the movement of water through the hoof. Unlike the unidirectional hydrostatic pressure gradient, the direction of the osmotic gradient within the foot is conceptualized as being variable. Factors that affect the direction and magnitude of the osmotic gradient include concentration of the external environment and the relative status of dehydration of the keratinocytes and extracellular matrix of the hoof.

When considering the stratum corneum as a prototype, it is probable that the principle route of water movement is transcellular rather than extracellular, regardless of the force responsible.[10] In recent models, the stratum corneum is perceived as being a 2-compartment system consisting of a hydrophilic intracellular compartment that is embedded in a hydrophobic extracellular matrix.[11,12] In this model, the plasmalemma of the fully cornified keratinocytes, having undergone terminal cornification, is nonactive in regards to transport but is highly permeable to the passive movement of water. The intracellular crystalloids and pro-

teins serve to osmotically attract and hold water where the extracellular lipid matrix serves as the primary barrier to the movement of water.

If this model, developed to describe the barrier function of the stratum corium of soft epidermis, is accurate for hoof epidermis, then hoof epidermis should allow the passive movement of both water and crystalloids. The purpose of the study reported here was to better characterize the osmotic behavior of the stratum medium, sole, and frog of the hoof. Because differences have been detected for the hydration status and mechanical behavior of the outer stratum medium and the **stratum medium zona alba (SMZA)** layer, defined as the nonpigmented inner layer of the stratum medium lying just external to the laminar interface, these regions were tested individually.[3,4] Our hypothesis was that the epidermal cells and extracellular matrix of the hoof's outer stratum medium, SMZA, frog, and sole allow passive movement of water, proteins, and crystalloids secondary to concentration gradients.

Materials and Methods

The response of hoof epidermal tissues to prolonged immersion in water was characterized in vitro on hoof tissues of 10 hooves obtained at a local abattoir from 10 horses with healthy feet. Response to immersion was assessed in terms of changes in specimen mass and in terms of the quantity and nature of the solutes lost from hoof specimens. Hooves were considered normal if there were no obvious lesions on the external surface or at gross examination of a mid sagittal section of the foot. Each foot was rinsed and blotted dry to remove excess debris and subsequently sectioned with a band saw to obtain a 2-cm mid sagittal section. Four rectangular specimens (1 cm × 0.5 cm × 0.5 cm) were manually cut from each mid sagittal section. Specimens consisted of a section of the dorsal stratum medium that included the external surface of the wall, a section of the SMZA, a section of frog, and a section of the sole, for a total of 40 tissue sections. All specimens were obtained from the same relative position in each foot.

The initial mass of each specimen was determined by use of a high-resolution balance[b] within 1 hour of its removal from the foot, followed by immediate immersion in individual sterile tubes. Each tube contained 25 ml of distilled deionized water with an antimicrobial-antimycotic solution[c] containing penicillin (400 U/ml), streptomycin (0.4 mg/ml), and amphotericin B (1 µg/ml) for controlling bacterial growth. All specimens were immersed for 10 days at 24 C. Specimen mass was determined at 2-day intervals. The external surface of each specimen was manually blotted dry prior to mass determination, and the specimen was reimmersed in the distilled water. For each specimen, the time required for mass determination was < 15 seconds. Two sets of tubes containing either 25 ml of distilled, deionized water or 25 ml of water with the antimicrobial-antimycotic solution were prepared and used as controls.

Following the 10 days of immersion, the mass of each specimen was determined, the tissue specimens were discarded, and the immersion fluids were retained. Bacteriologic culture of fluids was performed on the soaking solutions to determine whether bacteria were present. Osmolarity was determined by use of freezing point depression with an osmometer[d] on a 5-ml aliquot of the fluid from each specimen and control tube. The osmometer had a repeatability of ± 2 mOsm/kg of water between 0 and 400 mOsm/kg of water. The remaining 20 ml of solution from each specimen tube was freeze-dried and reconstituted to a more concentrated 5-ml volume for solute identification and quantification. Solute characterization consisted of testing for the presence of protein with a modified Lowry test[13] and for the presence of Na^+, K^+, and Cl^-. Electrolyte concentrations were measured in a commercial laboratory by use of an atomic absorption flame technique (sensitivity of 0.5 ppm) for Na^+ and K^+ and an angiometric silver nitrate procedure (sensitivity of 5 ppm) for Cl^-.[14-16] Concentrations of protein and electrolytes reported for the fluids were mathematically corrected for changes in specimen volume.

Statistical analyses—Two sets of analyses were performed on the data. The first analysis was restricted to comparisons among the fully cornified outer stratum medium, frog, and sole. Absolute changes in specimen mass for the outer stratum medium, frog, and sole during the 10-day immersion period were analyzed by use of a repeated measures ANOVA. A Tukey test was used to identify differences in means if significant differences were detected. In addition, the percentage changes in masses of the outer stratum medium, frog, and sole were compared by use of a series of Mann-Whitney rank sum tests.

A paired *t* test was used to test whether the osmolarity or solute concentrations of the fluids collected at the end of the 10-day immersion period for each tissue type were different from that of the control fluid specimens. The osmolarity and solute concentration data for Na^+, K^+, Cl^-, and protein in the fluids after immersion were plotted and examined for distribution and variance. Osmolarity data from the fluid specimens collected after immersion were subjected to a log transformation and tested to determine whether differences were present among tissues by use of an ANOVA. A Tukey test was used to identify differences in means if significant differences were detected. A series of Mann-Whitney rank sum tests were used to determine whether the solute concentrations of the fluid specimens for the outer stratum medium, frog, and sole were different after immersion. Lastly, the osmolarity of the fluids after immersion were compared with the summated osmolarity attributable to Na^+, K^+, and Cl^- by use of a Mann-Whitney rank sum test.

The second set of analyses was restricted to comparison of data from the outer stratum medium and the SMZA. Mass data were evaluated for differences by use of a repeated measures ANOVA to determine whether the change in mass was significant over time, and a Mann-Whitney rank sum test was used to determine whether the percentage changes in the mass of the 2 tissues were different. A paired *t* test was used to test whether the osmolarity or solute concentrations of the fluids collected at the end of the 10-day immersion period for each tissue type were different from that of the control fluid specimens. The osmolarity and solute concentrations of the fluid specimens were evaluated for differences by use of a Wilcoxon signed rank analysis. A value of $P \leq 0.05$ was used to determine significance for all tests used in this study.

Results

The outer stratum medium, frog, and sole tissues all had a significant ($P < 0.001$) increase in mass during the immersion period, which was evident within 24 hours of immersion. Further changes in mass after this initial increase were not significant. By the tenth day of immersion, the mean ± SD change in percentage mass for the outer stratum medium and frog was 3.97 ± 0.6% and 35.198 ± 15.4%, respectively. These data were parametrically distributed but had large variances. Solar tissue mass increased by 4.09 ± 3.4% by the tenth day, but unlike the stratum medium and frog,

data were nonparametric in distribution. The percentage increase in mass of the frog tissue was significantly different from the stratum medium ($P < 0.001$) and sole ($P < 0.001$), but stratum medium and sole were not different from each other ($P = 0.098$).

Mean ± SD osmolarity of the control, stratum medium, frog, and sole fluid specimens were 3.75 ± 0.35, 12.30 ± 3.5, 46.2 ± 21.8, and 21.86 ± 12.4 mOsm/L, respectively. Data distribution was nonparametric, and significant differences in variance were detected. Compared with the osmolarity of the control fluid specimens, the osmolarity of specimen fluids was significantly ($P < 0.001$) increased. The osmolarity of the frog fluid specimens was greater ($P < 0.001$) than that of the fluids from all other tissue types.

No protein was detected in any of the control or postimmersion fluid specimens. Results of all bacteriologic cultures of fluids were negative. Control fluids contained 16.5 mg of Na^+/L, 0.7 mg of K^+/L, and 38.7 mg of CL^-/L. Mean ± SD of the Na^+ concentrations from the fluid specimens that contained stratum medium, frog, and sole were 173.80 ± 124.7, 180.00 ± 129.5, and 188.70 ± 131.7 mg/L, respectively. Fluid K^+ concentrations after immersion were 50.00 ± 24.2, 101.30 ± 154.9, and 135.47 ± 92.4 mg/L for the stratum medium, frog, and sole fluids, respectively. Fluid Cl^- concentrations were 158.44 ± 76.7, 207.73 ± 24.5, and 198.51 ± 191.4 mg/L for the stratum medium, frog, and sole tissues, respectively. The Na^+, K^+, and Cl^- concentrations of the fluids were all different from their respective concentrations in the control fluid specimens ($P < 0.001$). The only difference between values obtained before and after immersion in the electrolyte data was that of a higher K^+ concentration in the fluid specimens exposed to sole tissue.

The calculated osmolar contributions of the measured electrolytes were 13.38 ± 5.9 mOsm/L (outer stratum medium), 16.39 ± 13.4 mOsm/L (frog), and 17.43 ± 11.3 mOsm/L (sole). Comparison of these calculated osmolarities and the total measured osmolarities indicated that wall ($P = 0.587$) and sole ($P = 0.154$) were not different. The calculated osmolarity due to Na^+, K^+, and Cl^- was significantly lower ($P = 0.008$) than the total osmolarity measured for the frog.

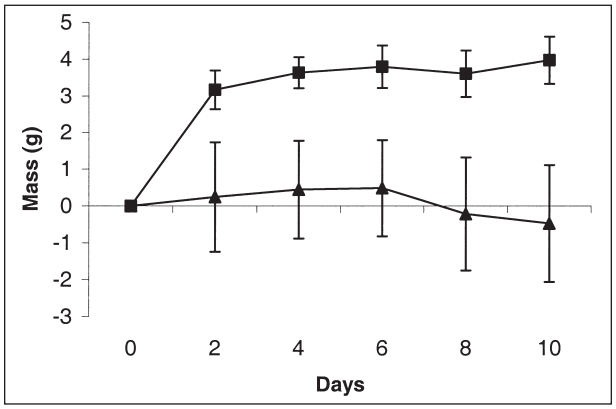

Figure 1—Changes in mass of the outer stratum medium (squares) and the zona alba layer of the stratum medium (triangles) of hoof tissues of 10 horses during a 10-day immersion period in water.

Compared with the outer stratum medium tissue, which had a net increase in mass, the SMZA decreased in mass in response to immersion (**Fig 1**). Four of the SMZA tissue specimens had a decrease in mass, and 6 had an increase in mass after 24 hours of immersion. Five of the 6 SMZA specimens that initially increased in mass had a subsequent decrease in mass. Whereas the outer stratum medium had a significant increase in mass by the first 24 hours, the SMZA specimens did not change significantly ($P = 0.007$) until the 10th day. Mean percentage change in mass of the SMZA was –0.49 ± 1.5%. The changes in the percentage mass of the outer stratum medium and SMZA tissues were significant ($P < 0.001$).

The osmolarity of the outer stratum medium fluid increased to 12.30 ± 3.5 mOsm/L and that of the SMZA increased to 7.60 ± 2.1 mOsm/L. Both of these were different from the osmolarity of the controls ($P < 0.001$) as well as from each other ($P = 0.002$). The Na^+ concentrations in the outer stratum medium and SMZA were 173.80 ± 124.72 and 173.68 ± 122 mg/L, respectively, and were not different ($P = 0.528$). Similarly, there was no difference in the Cl^- ($P = 0.097$) concentrations of the outer stratum medium (158.44 ± 44.0 mg/L) and the SMZA (131.42 ± 70.4 mg/L). The K^+ concentration of the outer stratum medium fluid (50.92 ± 24.2 mg/L) and that of the SMZA (31.82 ± 7.9 mg/L) were significantly different ($P = 0.041$).

Discussion

The recorded changes in total mass, percentage change in mass, or both for the outer stratum medium, frog, and sole indicated that all 3 types of hoof epithelia are capable of absorbing water. The uptake of water was rapid initially and then reached a plateau as the tissue became saturated. During the equilibrium phase, all hoof specimens lost nonsignificant amounts of mass. If it is presumed that tissues have undergone terminal cornification, it is logical that water movement is occurring principally in response to a preexisting osmotic-oncotic gradient across the epidermal plasmalemma. These data also infer that frog epithelium is distinct in its ability to hydrate, compared with either stratum medium or sole. Intuitively, the mechanism may relate to either an increased transmembrane osmotic gradient for the frog, or the water absorbed by the frog is occupying extracellular as well as intracellular sites.

The origin of the large SD associated with the percentage change in the mass of these tissues is most likely attributable to variations in environmental exposure prior to collection. Results of this study clearly support the view that both water and solutes can be gained or lost, secondary to physical conditions. It follows that if the horses used in this study had been exposed to a wet environment, their hooves would be capable of absorbing less water and eluting less electrolytes than horses chronically maintained in an arid environment. Given that the management history of the horses used as a source for the tissues was unknown, this must remain a speculation. The increased osmolarity and crystalloids observed in the fluids after immersion argues that prolonged

immersion causes loss of solute from the hoof. The epidermis is perceived as a 2-compartment system consisting of the extracellular lipid matrix and the intracellular proteinaceous matrix, which are separated by the terminally cornified plasmalemma. On the basis of the normal physiologic distribution of electrolytes, it is logical that Na^+ and Cl^- were principally eluted from the extracellular compartment and K^+ from the intracellular compartment. It is, however, impossible to identify the specific source of the electrolytes from these data.

The increased osmolarity and electrolyte concentrations of the postimmersion fluid specimens from different hoof epidermis tissues appear to generally agree with the changes in mass. Frog specimens gained most mass and contributed significantly more solute than did the outer stratum medium and sole. The specific electrolyte composition eluted from the hard epidermis was different from the other specimens, with significantly more K^+ being eluted from the sole than from the outer stratum medium or frog. The mechanism for this difference is unknown.

The absence of detectable protein in all of the postimmersion fluids infers that although the fully cornified cell membrane is relatively permeable to water and crystalloids, semipermeability to larger solutes is still present. The modified Lowry test was used in this study because it is be able to detect keratin proteins.[13] Most of the intracellular proteins in terminally cornified epithelial cells are thought to consist of relatively insoluble keratins and totally insoluble cell envelope proteins that are cross-linked and bound to both intracellular and plasmalemmal structures. Intuitively, smaller, more soluble proteins should be present and should exit the cell if the porous structure of the membrane does not restrict passage. That this did not occur suggests that the cornified membranes of the outer stratum medium, frog, and sole form a relatively rigid, protein-restrictive sieve. This allows the intracellular proteins to function oncotically to attract and hold water and thus assist in maintaining the hydration status of the hoof.

Comparison of the osmolarity of the postimmersion fluids with the summated osmolarity calculated from the crystalloids indicated that Na^+, K^+, and Cl^- contributed most of the solutes for the outer stratum medium and sole but not for the frog. This infers that solutes other than Na^+, K^+, and Cl^- were eluted from the frog in significant quantities. A circumstantial argument can be made that the additional solutes eluted from the frog are lipids. Histologically, frog epithelium is unique in that it is the only hoof epithelial tissue that is glandular.[e] Histochemically, the secretory products of these glands are compatible with neutral lipids. Intuitively, it is the presence of these lipids that contribute to the relative elasticity of the frog. By definition, neutral lipids are hydrophilic and can diffuse out of the frog after prolonged immersion in water. Because the postimmersion fluids were not evaluated for lipids, this hypothesis was not studied.

Comparisons of the outer stratum medium and the SMZA were completed as a separate analysis, because both of these layers are technically parts of the stratum medium. The SMZA was different from the outer stratum medium in that it decreased rather than increased in mass and more K^+ was eluted from the outer stratum medium. The mechanism underlying the loss of mass by the SMZA cannot be osmotic. Although the percentage hydration of the SMZA cells was high, which allowed a lower intracellular osmotic concentration, an inward osmotic gradient was still maintained because the immersion fluid was pure water. Thus, loss of tissue mass must have been be due to diffusion of solute from the extracellular matrix or the intracellular compartment, loss of solute from the intracellular compartment via an active transport process, or loss of cell integrity secondary to osmotic rupture of the cell. The latter 2 explanations are only possible if the plasmalemma of the SMZA cells was functionally intact and maintained a high degree of semipermeability. Because these properties are lost during terminal cornification, these are possible only if the cells of the SMZA were epithelial cells rather than being fully cornified.

Data from this study support the concept that the physiologic structures of the fully cornified epithelial cells of the outer stratum medium, frog, and sole are consistent with that of a 2-compartment model. This model would account for the exchange of crystalloids, but not proteins, with the environment in response to diffusion. This interpretation suggests that prolonged exposure of the equine hoof to water may result in changes in its electrolyte composition. The apparent inability of protein to diffuse out of the fully cornified hoof epithelia infers that applied topical agents containing proteins cannot move into the cells but would instead potentially serve as an oncotic force leading to further tissue dehydration. These data support the concept that the solutes eluted from the frog epithelium are different and may reflect its unique lipid composition. In addition, the osmotic behaviors of the outer stratum medium and the SMZA are distinct and this suggests the hypothesis that the cells of the SMZA have not undergone terminal cornification and thus may be transitional epithelial cells.

[a]Leach DH. *The structure and function of the equine hoof wall*. PhD dissertation, Department of Veterinary Anatomy, Western College of Veterinary Medicine, University of Saskatchewan, SK, Canada, 1980.
[b]Ohaus Precision Advanced Balance, Model No. GT140, Ohaus Corp, Florham Park, NJ.
[c]Sigma antibiotic/antimycotic solution (100X) No. A9909, Sigma-Aldrich Co, St Louis, Mo.
[d]Advanced Micro-osmometer, Model No. 3300, Advanced Instruments Inc, Norwood, Mass.
[e]Bolliger C. *The equine hoof: morphological and histochemical findings*. PhD dissertation, Veterinar-Anatomisches Institut der Universitat Zurich, Zurich, Switzerland, 1991.

References

1. Miyaki H, Ohnishi T, Yamamoto T, et al. Measurement of water contents of hoof wall, sole and frog in horses. *Exp Rep Equine Health Lab* 1974;11:15–20.

2. Hinterhofer C, Stanek C, Binder K. Elastic modulus of equine hoof horn, tested in wall specimens, sole specimens and frog specimens at varying levels of moisture. *Berl Munch Tierarztl Wochenschr* 1998;111:217–221.

3. Wagner IP, Hood DM, Hogan HA. Comparison of bending modulus and yield strength between outer stratum medium and stratum medium zona alba in equine hooves. *Am J Vet Res* 2001;62:745–751.

4. Leach DH, Zoerb GC. Mechanical properties of equine hoof wall tissue. *Am J Vet Res* 1983;44:2190–2192.

5. Douglas JE, Mittal C, Thomason JJ, et al. The modulus of elasticity of equine hoof wall: implications for the mechanical function of the hoof. *J Exp Biol* 1996;199:1829–1836.

6. Kasapi MA, Gosline JM. Strain-rate-dependent mechanical properties of the equine hoof wall. *J Exp Biol* 1996;199:1133–1146.

7. Bertram JEA, Gosling JM. Functional design of horse hoof keratin: the modulation of mechanical properties through hydration effects. *J Exp Biol* 1987;130:121–136.

8. Bertram JEA, Gosline JM. Fracture toughness design in horse hoof keratin. *J Exp Biol* 1986;125:29–47.

9. Robertson IP, Hood DM. The ability of commercial hoof wall products to maintain hydration of the equine hoof wall, in *Proceedings*. 42nd Annu Meet Am Assoc Equine Pract 1996;42:208–211.

10. Scheuplein RJ, Blank IH. Permeability of the skin. *Physiol Rev* 1971;51:702–741.

11. Elias PM. Epidermal lipids, barrier function, and desquamation. *J Invest Dermatol* 1983;80(suppl):44S–49S.

12. Schurer NY, Plewig G, Elias PM. Stratum corneum lipid function. *Dermatologica* 1991;183:77–94.

13. Grosenbaugh DA, Hood DM. Keratin and associated proteins of the equine hoof wall. *Am J Vet Res* 1992;53:1859–1863.

14. Sodium—method 3111B. In: Greeenberg AE, Clesceri LS, Eaton AD, eds. *Standard methods for the examination of water and wastewater*. 18th ed. Washington, DC: APHA, AWWA, WPCF, 1992;3–13.

15. Potassium—method 3500KD, FAA. In: Greeenberg AE, Clesceri LS, Eaton AD, eds. *Standard methods for the examination of water and wastewater*. 18th ed. Washington, DC: APHA, AWWA, WPCF, 1992;13–80.

16. Chloride—method Cl B. In: Greeenberg AE, Clesceri LS, Eaton AD, eds. *Standard methods for the examination of water and wastewater*. 18th ed. Washington, DC: APHA, AWWA, WPCF, 1992;4–49.