Effects of long-term extracorporeal blood perfusion of the distal portion of isolated equine forelimbs on metabolic variables and morphology of laminar tissue

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Objective—To establish an ex vivo model of blood perfusion in the distal portion of isolated equine forelimbs that closely represents the in vivo situation in the laminar tissue of the hoof.

Sample Population—18 forelimbs collected from 9 healthy adult horses following slaughter at a licensed abattoir.

Procedures—The distal portion of isolated equine forelimbs from 9 horses were perfused under physiologic conditions over a period of 6, 8, and 10 hours with autologous blood. To determine cell viability in perfused tissues, indicators for metabolism (lactate generation and glucose and oxygen consumption) as well as indicators for cell damage (potassium concentration and lactate dehydrogenase activity) were examined at 1-hour intervals from samples of the perfusate. Weight gain in the forelimb was used to determine the edema index. After perfusion, light and electron microscopic examinations of laminar tissue specimens were performed.

Results—During hemoperfusion of the isolated forelimbs, mean ± SD glucose consumption was 197.4 ± 65.1 mg/h, lactate generation was 1.84 ± 0.79 mmol/h, and oxygen consumption was 6.4 ± 10⁻⁶ – 8.9 ± 10⁻⁵ mL•g⁻¹•min⁻¹. Neither an efflux of potassium into the perfusate nor a relevant increase of the lactate dehydrogenase activity was detected, indicating low amounts of cellular damage in the perfused tissues. Weight gain of forelimbs was 1.02 ± 0.95%. Histologic and ultrastructural appearance of the laminar tissue revealed no signs of tissue damage.

Conclusions and Clinical Relevance—Isolated equine limbs were perfused under physiologic conditions over a period of ≤ 10 hours without structural damage to the laminar tissue. (Am J Vet Res 2009;70:669–677)

Acute laminitis is a severely debilitating and painful disease of the lamellar tissue of the equine digit. For affected horses, the disease often represents a life-threatening or career-ending situation; therefore, more effective means of prevention and treatment are necessary. Despite extensive research, precise mechanisms and triggers underlying laminitis in horses remain unidentified, and further investigations are required to develop adequate methods for prevention and treatment.

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Abbreviations

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<td>IQR</td>
<td>Interquartile range</td>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>sO₂</td>
<td>Oxygen saturation</td>
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To date, the preferred method to investigate the pathogenesis of acute laminitis is the induction of this disease in healthy horses by nasogastric administration of an aqueous extract of black walnut or carbohydrate overload. Horses are often euthanatized after clinical signs of acute laminitis are obvious, and consequently, these in vivo experiments are associated with pain and stress for study horses. Recently, in vitro studies were performed by use of cultured equine lamellar hoof explants or isolated vascular preparations from digital or laminar vessels. However, the disadvantage of the use of explants is that examination of the influence and the interaction of different factors such as changes in vascular permeability or in circulation of the dermis caused by vasoconstriction or vasodilatation is not possible. Also mechanical testing of explant tissues is fraught with difficulties. In vascular preparations, the direct effect of different mediators on the vascular
tone of vessels can be examined, but unlike the in vivo situation, vessels are exposed to the mediator from the luminal and the adventitial surface. Additionally, the interaction between blood flow and the vascular endothelium that is regulating the flow rate by local mediator production is neglected.

In contrast to cell- or tissue-based investigations, an isolated perfused limb allows examination of more complex interactions. Studies on extracorporeal perfusion of isolated equine or bovine limbs were first performed over a period of 3.5 to 5 hours with electrolyte solutions or with a perfusate containing washed RBCs. However, the lack of neutrophils and platelets limits investigation of the pathogenesis of laminitis where activation of these cells is thought to play an important role. Furthermore, the perfusion time of these studies is too short to investigate the effect of specific laminitis triggers on the laminar tissue, as a minimum of 8 hours is reported to be necessary for the development of clinical signs of laminitis after administration of a black walnut extract.

The purpose of the study reported here was to establish an ex vivo model of blood perfusion in the distal portion of isolated equine forelimbs in which cell viability in the equine digit is preserved for up to 10 hours. As equine forelimbs from slaughtered horses were used, this isolated limb model may reduce or even replace the need for in vivo experiments.

Materials and Methods

Animals—The distal portion of both forelimbs and 4 to 7 L of blood from 9 adult horses of various breeds slaughtered at a licensed abattoir were used in the study. The group of horses included 5 mares, 2 stallions, and 2 geldings that were 9 to 20 years old (mean age, 12.9 ± 3.2 years old); the body weight of the horses was 350 to 500 kg (mean weight, 454.4 ± 44.8 kg). Horses with clinical signs of laminitis or other hoof diseases were excluded. Horses were euthanized by use of a penetrating captive bolt followed by exsanguination and after death underwent the routine processing.

Preparation of forelimbs and perfusate—During exsanguination of the horses, 4 to 7 L of autologous mixed arterial-venous blood was openly collected in glass bottles. Anticoagulation was performed with heparin (5,000 U/L).

Within 3 to 5 minutes after exsanguination, both forelimbs were disarticulated at the level of the middle carpal joint and placed on ice for surface cooling. Directly afterwards, the cut end of a medical grade polyvinyl chloride tubing (3-mm internal diameter) was introduced into the median artery, while the radial artery and the palmar branch of the median artery were ligated. To remove remaining blood and to decrease the time of warm ischemia, the median artery was immediately flushed with 500 mL of ice-cold oxygenated physiologic salt solution that contained glucose and albumin.

On the basis of the composition of equine plasma, the flushing solution contained sodium (136 mmol/L), potassium (3.6 mmol/L), chloride (118 mmol/L), calcium (1.96 mmol/L), bicarbonate (25.2 mmol/L), phosphate (0.72 mmol/L), glucose (86 mg/dL), and albumin (6.5 g/dL). Final osmolality was 290 mOsm/L, and colloid osmotic pressure was 19.6 mm Hg. To prevent clot formation and obstruction of blood vessels, the flushing solution was supplemented with heparin (3,000 U/L).

For transport to the laboratory, isolated forelimbs and collected blood were stored in an insulated box filled with ice. Mean ± SD transportation time was 123.8 ± 8.6 minutes. In a pilot study, histologic examinations of laminar tissue specimens collected from 9 forelimbs directly after transport revealed no substantial morphologic changes as the result of transportation time.

After transport, autologous plasma was separated from approximately 3 to 5 L of the collected blood of each horse. A mixture of autologous blood and plasma in a 3:2 ratio was used as the perfusate.

Perfusion system—Extracorporeal perfusion of the equine forelimbs was performed with 600 mL of the perfusate in a recirculating perfusion system (Figure 1). Perfusions of the distal portion of isolated equine forelimbs are referred to as forelimb perfusions. The total perfusion time was 6 hours (n = 3), 8 hours (3), and 10 hours (3). To prevent hemolysis as a result of mechanical and biochemical influences of the extracorporeal perfusion system, the perfusate was exchanged every 2 hours. The interval between exchanges was designated as a perfusion interval. Because of glucose consumption by perfused tissues, which was determined in a pilot study, an addition of 25 mg of glucose/dL was necessary after the first hour of each perfusion interval.

Directly after transport, the median artery of the right forelimb was connected with the perfusate reservoir via a tubing system passing through a variable speed peristaltic pump. To avoid edema formation as a result of high resistance of the vessels in the cold forelimb, an equilibration period of 30 minutes with slowly increasing blood flow (beginning with 20 mL/min) was performed. After the equilibration period, the blood flow rate was set at 70 to 100 mL/min (depending on forelimb weight, the rate of blood flow was approx 12 mL·kg⁻¹·min⁻¹). This blood flow rate was chosen because the physiologic rate of digital arterial blood flow in vivo is reported as 60 to 109 mL/min. The aim was to obtain physiologic arterial blood pressures of 85 to 125 mm Hg. For direct arterial pressure measurements, the tubing in the median artery was also...
Blood flow and arterial pressure were recorded every 10 minutes.

For oxygenation of the blood, the perfusate was first directed through thin-walled gas permeable silicone tubing (2-mm internal diameter and 0.23-mm wall thickness) coiled inside an oxygen chamber that was filled with room air. To remove microaggregates from the perfusate, the oxygenated blood was then directed through a transfusion filter with a pore size of 200 µm. Immediately before being directed into the median artery, the perfusate was warmed to 35°C because the physiologic temperature in the hoof is reported as 27.7°C to 34.8°C. For accurate assessments of changes in the weight of the forelimb during the perfusion, the isolated forelimb was placed on electronic scales during the entire perfusion time.

The left forelimb of each horse was stored at room temperature (approx 23°C) for 6 hours (n = 9), 8 hours (3), or 10 hours (3). Tissue specimens from nonperfused forelimbs were served as controls for histologic examination.

**Determination of cell viability in perfused forelimbs**—Arterial and venous blood samples (1 mL) were collected in the beginning of each perfusion interval and subsequently at 1-hour intervals. Immediately after sample collection, blood gas analysis (including determination of PO₃, PCO₂, SO₂, electrolyte concentrations, pH, Hct, and hemoglobin concentration) was performed. Oxygen content of the arterial and venous blood samples (mL of O₂/L) was calculated as product of hemoglobin concentration (g/L), SO₂ (%), and a factor of 1.34. The oxygen consumption (mL·g⁻¹·min⁻¹) was calculated as product of the rate of the blood flow (mL·g⁻¹·min⁻¹) and the arterial-venous difference of the oxygen content (mL of O₂/mL).

The glucose, lactate, and protein concentrations and LDH activity of the perfusate were determined prior to the start of each perfusion interval, after an hour, and at the end of the perfusion interval. The glucose concentration of the perfusate was additionally determined before and after each supplementation with glucose. For these analyses, 5-mL samples from the perfusion reservoir were centrifuged at 3,000 × g for 5 minutes. The plasma was immediately separated, and the protein concentration was measured by an optical refractometer. For the determination of glucose and lactate concentrations and LDH activity, plasma was stored at −18°C for 48 to 72 hours, and then thawed and analyzed in an International Organization for Standardization 9001:2000 certified clinical laboratory. On the basis of these data, the glucose consumption, lactate generation, and increase of LDH activity per hour were calculated. The extent of edema formation during forelimb perfusion was estimated by calculating the increase in tissue weight.

**Control perfusions**—To determine which changes were induced by the perfusion system itself, additional perfusions of the tubing system without connecting the forelimb were performed for 1 hour (n = 9). Subsequently, perfusions of the tubing system alone are referred to as control perfusions. In the beginning and at the end of control perfusions, arterial and venous blood samples were collected (the venous blood sample was collected at the end of the tubing system) and blood gas analysis was performed. Additionally, glucose and lactate concentrations and LDH activity in the perfusate were determined prior to the start and at the end of each 1-hour control perfusion. The degree of mechanical hemolysis caused by the perfusion system was estimated by determining changes in LDH activity and potassium concentration in the perfusate during control perfusion. To estimate the role of blood cell (ie, RBCs, WBCs, and platelets) metabolism within the tubing and the reservoir of the perfusion system, indicators for metabolism such as glucose consumption, lactate generation, and the arterial-venous difference of the oxygen content were also calculated for the control perfusions and compared with the results of forelimb perfusions. For each variable, the difference between forelimb perfusion and control perfusion was calculated and presented as net value for the metabolism of the tissues within perfused forelimbs.

**Light and transmission electron microscopy**—To estimate the degree of cell injury and ultrastructural changes caused by perfusion of isolated forelimbs, histologic examination of laminar tissue specimens was performed after each perfusion. To remove remaining blood and to minimize time of warm ischemia before tissue specimen collection, the median artery of each perfused forelimb was first flushed with 100 mL of cold (4°C) physiologic salt solution. Directly afterwards, a full-thickness segment of the dorsal aspect of the hoof was isolated by use of a band saw. Blocks of laminar tissue (approx 15 × 10 mm) were obtained by sharp dissection from the proximal and distal aspect of the dorsal portion of the hoof wall. Specimens were cut in halves and fixed in formaldehyde (4%) for light microscopy or glutaraldehyde (2.5%) for transmission electron microscopy. After fixation, specimens were embedded in paraffin or Epon, respectively. Sections for light microscopy were stained with H&E and the Periodic acid–Schiff method by use of a standard histlogic technique. Additionally, type IV collagen and laminin immunostaining were performed. For transmission electron microscopy, semithin sections (1 µm) stained with methylene blue-azure II were used to obtain an overview. Finally, ultrathin sections (90 nm) were made and contrasted with uranyl acetate and lead citrate. Specimens collected in the same way from the nonperfused left forelimb of each horse were used as controls for morphologic studies. As hypoxic and posthypoxic cell damage include pyknosis of nuclei and formation of vacuoles in the membrane containing organelles, physiologic appearance of these organelles indicated viability of the perfused laminar tissue.

**Statistical analysis**—Data analyses were performed by use of a commercial software program. All data were evaluated for normal distribution by use of the Kolmogorov-Smirnov test. Descriptive statistics such as mean ± SD and 95% confidence intervals were used to summarize results for normally distributed data. Non-normally distributed data are reported as median and IQR. Comparison of the data from blood gas analyses
of arterial and venous blood samples was performed by use of a 2-tailed paired t test for normally distributed data or the Wilcoxon test for nonnormally distributed data. Arterial blood pressure, oxygen and glucose consumption, lactate generation, increase of LDH activity, and plasma protein concentration were analyzed by use of a repeated-measures ANOVA. Significant differences over time identified by an ANOVA were isolated by use of the Bonferroni post hoc test for multiple comparisons. Glucose consumption, lactate generation, and increase in LDH activity during the first hour of forelimb perfusion (time point 1) were regarded as baseline values. Comparison of the baseline values of these variables with 1-hour control perfusion was performed by use of a 2-tailed unpaired t test. Comparison of LDH activity and potassium concentration in the beginning and at the end of control perfusions was performed by use of a 2-tailed paired t test. Values of P < 0.05 were considered significant.

Results

Hemodynamic variables—For controlled reperfusion of the distal portion of isolated equine forelimbs, the blood flow was slowly increased beginning at 20 mL/min. The resulting mean arterial pressure at the beginning of forelimb perfusion was 53.6 ± 13.9 mm Hg (n = 9). After an equilibration period of 30 minutes, isolated equine forelimbs were perfused with a median blood flow of 70.0 mL/min (IQR, 2.5 mL/min) and a mean arterial pressure of 117.7 ± 40.4 mm Hg (n = 414 measurements from 9 perfused forelimbs). Directly after the exchange of the perfusate and additionally after withdrawing arterial blood samples from the circulation, arterial pressure increased for a short time before returning to baseline values, but after the equilibration period, differences in arterial pressure over time were not significant (P = 0.101).

Cell viability variables of perfused tissues—During forelimb perfusion, mean glucose consumption was 197.4 ± 65.1 mg/h (n = 72 values from 9 perfused forelimbs). In the course of perfusion, glucose consumption increased (Figure 2), but compared with the baseline value (time point 1), no significant (P = 0.085) differences were identified over time. During control perfusion, the mean glucose consumption (80.7 ± 22.7 mg/h; n = 9) in the perfusate was significantly (P = 0.008) lower than during forelimb perfusion. The resulting mean net glucose consumption of the tissues within perfused forelimbs was 116.7 mg/h.

The mean lactate generation during forelimb perfusion was 1.84 ± 0.79 mmol/h (n = 72 values from 9 perfused forelimbs). In the course of perfusion, a variation of lactate generation with lower values in the second hour of each perfusion interval was detectable. Additionally, lactate generation gradually increased overall (Figure 2), but compared with the baseline value (time point 1), no significant (P = 0.624) differences were identified over time. During control perfusion, the mean lactate generation (0.79 ± 0.25 mmol/h; n = 9) was significantly (P = 0.009) lower than during forelimb perfusion. The resulting mean net lactate generation of the tissues within perfused forelimbs was 1.05 mmol/h.

The mean LDH activity increase during forelimb perfusion was 12.0 ± 14.7 U/h (n = 72 values from 9 perfused forelimbs). In the course of perfusion, an increase in LDH activity was measured in the perfusate (Figure 2), but no significant (P = 0.105) differences were identified over time. During control perfusion, a significant (P = 0.028) increase of the LDH activity was measured from 390 ± 107 U/L at 0 minutes to 418 ± 96 U/L after 60 minutes. The increase in mean LDH activity of 17.1 ± 19.2 U/h (n = 9) was not significantly (P = 0.066) different from the increase in LDH activity during forelimb perfusion.

The mean plasma protein concentration during forelimb perfusion was 7.66 ± 0.99 g/dL (n = 108 samples from 9 perfused forelimbs) with no significant (P = 0.803) differences over time. At the start of the forelimb perfusion, the mean weight of the distal portion of isolated equine forelimbs was 5,971 ± 1,595 g. The mean increase in tissue weight during forelimb perfusion was 1.02 ± 0.95% (n = 9).

Blood gas analysis—During forelimb perfusion, the median Hct of the perfusate was 30% (IQR, 6%) and the median hemoglobin concentration was 9.7 g/dL (IQR, 2.0 g/dL; n = 216 samples from 9 perfused forelimbs). During control perfusion, the median Hct...
was also 30% (IQR, 6%) and the median hemoglobin concentration was 9.7 g/dL (IQR, 2.0 g/dL).

During forelimb perfusion, $\text{S}_2$ and $\text{P}_2$ of arterial blood samples were significantly ($P < 0.001$) greater than that of venous blood samples (arterial-venous difference for $\text{S}_2$ was 2.9% and for $\text{P}_2$ was 22.7 mm Hg; Figure 3). The $\text{P}_2$ of the arterial blood samples was significantly ($P < 0.001$) lower, compared with the venous blood samples (arterial-venous difference was −2.7 mm Hg). The mean arterial-venous difference for the oxygen content of the perfusate was $0.76 \pm 7.37 \text{ mL of O}_2/\text{L (n = 108 values from 9 perfused forelimbs)}). The resulting mean oxygen consumption during forelimb perfusion was $6.4 \times 10^{-6} \pm 8.9 \times 10^{-5} \text{ mL} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ (n = 108 values from 9 perfused forelimbs) with no significant ($P = 0.527$) differences over time. The $\text{P}_2$ and potassium concentration of the arterial blood samples were significantly greater, compared with that of venous blood samples (arterial-venous difference for $\text{P}_2$ was 0.03 and for potassium was 0.07 mmol/L; $P < 0.001$ and $P = 0.002$, respectively), whereas the concentrations of sodium, chloride, and calcium in the arterial and venous blood samples were not significantly different ($P = 0.369$, $P = 0.351$, and $P = 0.206$, respectively). During control perfusion, the $\text{pH}$ of the arterial blood samples was significantly ($P < 0.001$) greater (arterial-venous difference for $\text{pH}$ was 0.02) and the $\text{P}_2$ of the arterial blood samples was significantly ($P < 0.001$) lower (arterial-venous difference was −1.7 mm Hg), compared with that of the venous blood samples, but the arterial-venous differences of both variables were lower than during forelimb perfusion. Contrary to forelimb perfusion, the potassium concentration as well as $\text{S}_2$ and $\text{P}_2$ of the arterial and venous samples of control perfusions were not significantly different ($P = 0.681$, $P = 0.206$, and $P = 0.613$, respectively). The potassium concentration in the beginning and at the end of control perfusions was not significantly different ($P = 0.272$) either. Similar to forelimb perfusion, the concentrations of sodium, chloride, and calcium in the arterial and venous blood samples of control perfusions were not significantly different ($P = 0.594$, $P = 0.347$, and $P = 0.053$, respectively). The mean arterial-venous difference for the oxygen content during control-perfusion was $0.68 \pm 4.08 \text{ mL of O}_2/\text{L (n = 9). The resulting mean net oxygen consumption of the tissues within perfused forelimbs was } 9.4 \times 10^{-7} \text{ mL} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$. 

**Light microscopy**—After 6 to 10 hours of perfusion, the epidermal basal
cells had a relatively homogenous cytoplasm and euchromatic nuclei (Figure 4). In the dermal laminar tissue, leucocytes were only sporadically detectable. No evidence of disintegration of the dermal collagen fibrils was observed. In light microscopic examination of sections stained by the Periodic acid–Schiff method as well as by use of type IV collagen and laminin immunohistologic stains, the basement membrane was visible as a continuous line closely adherent to the epidermal basal cells.

The laminar tissue of nonperfused equine forelimbs stored for 6 to 10 hours at room temperature had histologic changes characterized by swelling and vacuolization of the cytoplasm as well as karyolysis and pyknosis of the nuclei of the epidermal basal cells (Figure 4). In the dermal laminar tissue, loss of alignment of the collagen fibrils was obvious. In many areas, the basement membrane had lifted away from the underlying basal cells. At these locations, the basement membrane appeared thinner than in adjacent areas.
Transmission electron microscopy—After 6 to 10 hours of perfusion, the dermal-epidermal junction as well as the dermal and epidermal cells of the laminar tissue had a normal appearance (Figure 5). The basement membrane was closely attached to the basal cell plasmalemma via numerous hemidesmosomes. A large number of anchoring filaments was detectable between the dense plaque of the hemidesmosomes and the lamina densa of the basement membrane. Fibrocytes, endothelial cells, and the epidermal basal cells contained intact intracellular organelles (nuclei, mitochondria, Golgi apparatus, and rough endoplasmic reticulum).

The laminar tissue of nonperfused equine forelimbs stored for 6 to 10 hours at room temperature was characterized by marked changes of the cellular structures such as swelling of mitochondria with dilated cristae, enlargement of the intermembrane space, and rarefaction of the mitochondrial matrix, as well as vacuolization of perinuclear space, rough endoplasmic reticulum, and other membrane-containing intracellular organelles (Figure 3). In some areas, the basement membrane had an irregular thickness and appeared indistinct and partly detached from the basal cells. In these areas, the hemidesmosomes and anchoring filaments were only vaguely visible.

Discussion
Isolated organ models have become gradually more relevant in strategies to reduce and replace animal experiments. In other studies, the perfusion apparatus for such ex vivo experiments (eg, the system used recently for the perfusion of porcine and bovine forelimbs) is complicated and expensive because of an integrated dialysis system. Compared with this equipment, the present model of the isolated blood-perfused equine forelimb is a quite simple preparation that can be realized with relatively low costs.

Despite the simple perfusion technique used, it was possible to obtain a perfusion in the present ex vivo model that successfully mimics in vivo perfusion of the equine digit. Hemodynamic variables like arterial blood pressure and blood flow in the perfused forelimbs as well as Hct and hemoglobin concentration of the perfusate were within physiologic ranges over the entire perfusion period of ≤10 hours. The oxygenation of the arterial blood inside the oxygenation chamber was also adequate and resulted in a physiologic arterial PO\textsubscript{2}.

During forelimb perfusion, changes of the reported variables represent the sum of changes caused by metabolism within perfused forelimbs as well as changes that occurred in the perfusion system as the result of blood cell metabolism and hemolysis in the tubing system. Performing control perfusions of the tubing system alone was therefore intended to aid in the interpretation of results of perfused forelimbs and to allow the calculation of net values of oxygen consumption, glucose consumption, and lactate generation within the perfused tissues. Such control perfusions have not been performed in other studies on perfusion of organs.

As oxygen consumption as well as glucose consumption and lactate generation are considered to be indicators of cell metabolism, these variables allow assessment of cell viability of the isolated perfused forelimbs. The significant arterial-venous differences of sO\textsubscript{2} and PO\textsubscript{2} during forelimb perfusion and the nonsignificant arterial-venous differences of both variables during control perfusion as well as the net oxygen consumption of $9.4 \times 10^{-7}$ mmol/kg/min can be regarded as evidence that a considerable amount of oxygen was used up by the perfused tissues within the forelimbs. The constant amount of the oxygen consumption with no significant differences over time is also an indication for a good-quality oxygenation of the tissue and the viability of cells.

During forelimb perfusions, a significantly greater decrease in glucose concentration in the perfusate was found, compared with that of control perfusions. The difference between the glucose consumption during forelimb perfusion and the decrease in glucose as the result of blood cell metabolism during control perfusion was regarded as mean net glucose consumption of the tissues within the isolated perfused forelimbs (116.7 mg/h). Because the distal portion of the forelimb of horses does not include tissues with glycogen storage capacity such as striated muscle, this net glucose consumption of the isolated perfused forelimbs reflects glucose metabolism (glycolysis and oxidation) within the perfused tissues. A considerable amount of the net glucose consumption may be attributed to glucose use by keratinocytes because these cells have a high requirement for glucose for maintenance of adhesion between the epidermal basal cells and the basement membrane within the laminar tissue of the hoof.

Similar to the glucose consumption, the amount of lactate generation during forelimb perfusion was significantly greater than the increase in lactate as the result of blood cell metabolism during control perfusion. The relatively high net lactate generation in the tissue of the isolated perfused equine forelimbs (1.05 mmol/h) is consistent with in vivo conditions because at a physiologic glucose concentration and under normal oxygenated conditions, a large part of the glucose that is taken up by the living cells in the hoof of horses at rest is metabolized to lactate. As lactate is a product of anaerobic glycolysis, the low sO\textsubscript{2} of the perfusate in the beginning of each perfusion interval may be an explanation for the variation in lactate generation during perfusion intervals. Despite the increase in lactate during the perfusion of the equine forelimbs, the pH of the arterial and venous blood samples remained within physiologic ranges.

Other important variables for the evaluation of cell viability in tissues within the isolated perfused equine forelimbs are the potassium concentration and the LDH activity in the perfusate because an increase of these variables is regarded as an indicator of cell damage as the result of tissue hypoxia or hemolysis. To estimate the degree of hemolysis within the perfusion system, both variables were determined in the beginning and at the end of control perfusions. The significant increase of the LDH activity during the control perfusion can be regarded as an indicator for mechanical destruction of blood cells caused by the extracorporeal perfusion system, but with an increase in LDH activi-
ity of only 17.1 ± 19.2 U/h, hemolysis was considered mild. The potassium concentration was not significantly increased during control perfusion, indicating also only low amounts of blood cell damage in the form of hemolysis.

To estimate the degree of cellular damage within the perfused tissues that may be caused by an insufficient oxygen supply during perfusion or by ischemia-reperfusion injury, LDH activity and potassium concentration were also determined during forelimb perfusion. One of the first signs of hypoxia or ischemia-reperfusion-mediated generation of reactive oxygen species is an increased degradation of the plasma membrane sodium-potassium pump. As a consequence of disrupted osmotic equilibrium, intracellular potassium is lost, whereas sodium and water move into the cell leading to cellular edema. For the evaluation of cell viability in isolated perfused tissues, a potassium efflux of ≤ 7% is considered acceptable. In the ex vivo preparation of the present study of the isolated perfused equine forelimb, there is a small arterial-venous decrease of the extracellular potassium concentration during forelimb perfusion that can be explained by an uptake of potassium into cells. This potassium uptake against the transcellular potassium gradient can be regarded as evidence for an intact function of the sodium-potassium pump and, consequently, as indication for the viability of the cells within perfused forelimbs. Additionally, the increase of the net LDH activity within the tissues of perfused forelimbs is negligible because the LDH activity increase during forelimb perfusion was even smaller than the LDH activity increase as the result of hemolysis during control perfusion. The insignificant net LDH activity increase can also be regarded as indication for the lack of cellular damage within the perfused tissues.

Another variable to evaluate the success of the perfusion of an isolated organ or tissue is the extent of edema formation in the perfused tissues, which can be estimated by calculating the weight gain of the perfused organ. By use of weight gain as an edema index for different isolated perfused organs and tissues (eg, the bovine uterus, the equine skin flap, and the porcine ear, which were perfused for 4 to 8 hours), an increase of the tissue weight of 6% to 20% was considered acceptable. In the ex vivo preparation of the present study, the mean weight gain was only approximately 1%. From this low increase in tissue weight, it can be concluded that the extent of the edema formation was negligible.

Cell viability of laminar tissue within the isolated equine forelimbs after hemoperfusion of 6 to 10 hours was confirmed by histologic and ultrastructural findings. Inadequate perfusion would result in tissue hypoxia and energy depletion that causes cell damage and cell death. Another cause of cell damage could be the generation of oxygen-derived free radicals as the result of ischemia-reperfusion injury, which could be expected after the transportation time of approximately 2 hours. However, the laminar tissue of the isolated perfused equine forelimbs had no signs of a structural cellular damage. Especially the ultrastructural appearance of mitochondria, nucleus, and rough endoplasmic reticulum can be regarded as indicators for the cellular viability of the laminar tissue because hypoxia as well as oxidative stress would quickly cause structural alterations within these organelles.

Possible limitations of the hemoperfused limb model may include the necessity of anticoagulation of the perfusate with heparin. This model will therefore not be suitable for the study of pathologic changes caused by the formation of blood clots. Additionally, the exchange of the perfusate every 2 hours (which was necessary to minimize hemolysis caused by the influences of the perfusion system) is problematic in blood cell-mediated pathologic processes that take longer than 2 hours.

For the use of the isolated perfused equine digit as a model to study laminitis, the total length of the perfusion of 10 hours should be sufficient to induce the initial pathologic changes in the laminar tissue because in black walnut–induced laminitis, a substantial reduction in circulating leukocytes and an immigration of the activated neutrophils and monocytes in the laminar tissue is evident within 3 to 4 hours after oral administration of the black walnut extract, and the first clinical signs are obvious at 8 hours.

By use of the isolated perfused equine digit as a model to study laminitis, the distal portion of the forelimbs obtained from healthy horses may be perfused under conditions that are thought to be involved in the pathogenesis of laminitis (eg, changes of hemodynamic variables to imitate ischemia and ischemia-reperfusion injury or application of specific substances within the perfusate like lipopolysaccharide) by comparing the results with perfusion under physiologic conditions. However, before this model can be used for hemodynamic studies, the reactivity of the vessels within perfused forelimbs on vasoconstrictor and vasodilator agents during the entire perfusion time has to be proven in future experiments. Other perspectives for the use of the presented model may be in the study of pharmacologic processes (eg, the effect of application of specific receptor agonists, antagonists, or enzyme inhibitors on laminar tissue).

References


