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Original Article

A novel model to assess lamellar signaling relevant to preferential weight bearing in the horse

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Highlights

- A novel model to study lamellar signaling events during preferential weight bearing is presented.
- Lamellar signaling events related to hypoxia and inflammation were assessed.
- Lamellar hypoxia inducible factor-1a was increased in the supporting limb relative to the contralateral hind limb.
- No differences in lamellar inflammatory signaling were present between the supporting limb and hind limbs.

Abstract

Supporting limb laminitis (SLL) is a devastating sequela to severe unilateral lameness in equine patients. The manifestation of SLL, which usually only affects one limb, is unpredictable and the etiology is unknown. A novel, non-painful preferential weight bearing model designed to mimic the effects of severe unilateral forelimb lameness was developed to assess lamellar signaling events in the SL. A custom v-shaped insert was attached to the shoe of one forelimb to prevent normal weight bearing and redistribute weight onto the SL. Testing of the insert using a custom scale platform built into the floor of stocks confirmed increased distribution of weight on the SL compared with the unloaded forelimb (UL) and the contralateral (CH) and ipsilateral (IH) hind limbs in six Standardbred horses. In a second part of the study, eight healthy Standardbred horses were fitted with the insert and tied with consistent monitoring and free access to hay and water for 48 h, after which the lamellae were harvested. Real-time qPCR was performed to assess lamellar mRNA concentrations of inflammatory genes and immunoblotting and immunofluorescence were performed to assess lamellar protein concentration and cellular localization of hypoxia-related proteins, respectively.

Lamellar mRNA concentrations of inflammatory signaling proteins did not differ between SL and either CH or IH samples. HIF-1 α concentrations were greater (P <0.05) in the SL compared to the CH. This work establishes an experimental model to study preferential weight bearing and initial results suggest that lamellar hypoxia may occur in the SL.

Keywords: Cell signaling; HIF-1 α; Hypoxia; Preferential weight bearing; Supporting limb laminitis

Introduction

Laminitis is a broad term used to describe injury to the digital lamellae occurring secondary to at least three diverse disease states: systemic sepsis, endocrinopathies (particularly involving insulin dysregulation), and severe lameness in one limb causing excessive weight bearing on the contralateral supporting limb (SL; Belknap and Parks, 2010). Over the last two decades, understanding of both sepsis-related laminitis and endocrinopathic laminitis has progressed, with much of the information emanating from experimental equine models of these types of laminitis (Garner et al., 1975; Asplin et al., 2007; Dyson et al., 2011; Leise et al., 2011; Risberg et al., 2014). However, there is minimal understanding of the pathophysiologic events occurring in SL laminitis (SLL), primarily due to the lack of a representative and humane animal model to study this type of laminitis.

SLL is a devastating sequela to chronic excessive loading of one limb which occurs when there is reduced weight-bearing on the contralateral limb due to pain (e.g. fracture, synovial sepsis), or the inability to support weight on the limb e.g. in traumatic neuropathies (van Eps et al., 2010). There is limited information published on the prevalence of SLL across such cases, although it is estimated that between 10 and 27% of horses with conditions involving non-weight-bearing lameness will develop SLL in the contralateral limb, depending on the study and underlying primary disease (Peloso et al., 1996; van Eps et al., 2010; Virgin et al., 2011). Several risk factors have been reported in clinical studies, including the weight and size of the horse, and the severity and duration of lameness (van Eps et al., 2010; Virgin et al., 2011). The development of SLL is unpredictable, both in regard to which horses will develop the disease and when it will become apparent, with the average time to onset of clinical signs (measured

from the time of the initial injury inducing primary non-weight bearing lameness) reported as days to weeks (Peloso et al., 1996; Richardson, 2008) with a mean of 14.5 days in one study (Wylie et al. 2015). SLL commonly leads to rapid and severe lamellar failure once signs are first noted, with subsequent distal displacement of the distal phalanx within the hoof capsule. Due to this catastrophic structural failure of the lamellae, mortality after development of SLL is high, with published estimates of at least 50-75% (Peloso et al., 1996; van Eps et al., 2010; Virgin et al., 2011).

Pathophysiologic events ranging from inflammation to hyperinsulinemia have been proposed to play a role in SLL, with minimal to no experimental data to support or refute the importance of these events. However, cadaver and short-term in vivo studies suggest a possible role of disrupted lamellar perfusion in SLL (Van Kraayenburg, 1982; van Eps et al., 2010; Sun et al., 2015), as opposed to endocrinopathic and sepsis-related laminitis (Burns et al., 2014; Pawlak et al., 2014; Risberg et al., 2014). Disrupted lamellar perfusion would likely create a hypoxic environment leading to altered cellular energy metabolism and possibly subsequent damage (Wylie et al., 2015). However, no equine model has been developed to study the effect of excessive weight bearing on lamellar physiology. The objectives of the current study were to develop an effective, humane experimental model of preferential weight bearing on one forelimb and to use this model to evaluate the effect of excessive weight bearing on lamellar regulation of a marker of tissue hypoxia, hypoxia-inducible factor-1 alpha (HIF-1α; Ho et al., 2006; Pawlak et al., 2014) and inflammatory markers shown to have increased expression in a model of sepsis-related laminitis (Leise et al. 2010). We hypothesized that cellular signaling consistent with

lamellar hypoxia would be present in lamellae of the SL of a horse that was unable to place normal weight on the contralateral limb for a 48 h period.

Materials and methods

Animal protocols

The experimental methods were approved by the Ohio State University (project number 2011A00000102; Approval date 22 September, 2011) and the University of Queensland Institutional Animal Care and Use Committees (SVS/098/15/GJCRF; Approval date 5 May, 2015). Two separate experiments were performed.

Preliminary altered weight bearing study

At the Australian Equine Laminitis Research Unit (The University of Queensland), six clinically normal mature geldings (five Standardbreds and one Thoroughbred; mean age 9.5 years, range 4-13; mean body mass 458 kg, range 440-480 kg) were restrained in stocks with a floor that consisted of a custom-built weighing-scale platform capable of recording individual load on all four limbs over time at a frequency of 20 Hz. Data was examined using commercial software (LabChart 7, AD Instruments). The shoe insert consisted of a V-shaped metal bar (V-insert) with the apex pointing towards the floor and each arm of the V secured to the branches of the steel shoe with bolts at points equidistant from the toe on both sides (Fig. 1); the height of the V-shaped bar at the apex was 7.5 cm from the ground surface of the point of the V to the bars of the horse's hoof. The construct necessitated overweighting of the contralateral forelimb due to the horse only being able to place the toe or the heel of the shod foot on the ground (Fig. 1), but never the entire sole. The insert did not contact the horse's hoof. Weigh-scale readings were

recorded for 1 h without intervention in each horse (control period) before the shoe insert was fitted to forelimb chosen by convenience sampling and shod with a custom shoe modified to fit the V-insert. Subsequently, weigh-scale data was recorded for a further 30 min once the V-insert was applied to the shoe (SL period). In two of the horses, the shoe insert was left on for a further 4 h before a second 30 min data recording was made (extended SL period). The mean body mass over the recording period for each limb was calculated and expressed as a percentage of the total body mass (Fig. 2). The horses were returned to the teaching herd after cessation of the weight bearing study.

Forty-eight hour insert application with lamellar harvest

At Ohio State University, eight healthy and clinically normal Standardbred horses aged 3-15 years old (mean age 9.85 years) and between 400 and 500kg body mass (mean 456 kg) were used. Lateromedial radiographs of both front feet were performed to determine if there was any evidence of chronic laminitis; horses were excluded if this was noted. The limb with the insert applied was designated 'unloaded limb' (UL), while the contralateral limb was labeled SL. The hind limbs were named in accordance with their position compared to the SL: 'contralateral hind' (CH; e.g. the right hind limb was designated CH if the left forelimb was the SL) and 'ipsilateral hind' (IH; Fig. 1).

For 48 h before V-insert placement, each horse was housed loose in a 12 x 12 m box stall, and was monitored hourly for general attitude and weight-bearing; physical parameters were recorded every 6 h. This schedule continued until termination of the experiment (48 h post-insert application). At the start of the experiment, the V-insert was attached to the shoe, and horses

were tied to minimize movement in a sparsely bedded stall. The horses had access to a full hay net and water bucket at all times. After 48 h, horses were administered detomidine (0.01 mg/kg IV; Dormosedan, Zoetis) and an IV overdose of barbiturate (80mg/kg; Euthasol; Virbac) to provide humane euthanasia in the stall.

Each of the SL, CH, and IH limbs were rapidly disarticulated at the metacarpophalangeal joint in a non-random order. Each foot was sectioned in a sagittal plane using a band saw in 3-5 cm sections before lamellar samples were sharply dissected away from keratinized hoof wall and underlying corium. Lamellar samples were either snap-frozen, or placed in optimal cutting temperature (OCT) medium and frozen on dry ice as previously described (Leise et al., 2012). Time from euthanasia to snap-freezing of samples was less than 20 min in all eight horses (deemed to be important when assessing markers of tissue hypoxia and preserving RNA quality in the samples). The UL lamellae were not included because: (1) the aim of the study was to compare lamellar signaling in a limb undergoing preferential weight bearing to limbs undergoing relatively normal weight bearing (i.e. not decreased weight bearing), and (2) time constraints of obtaining samples from all limbs (especially a limb which required shoe/insert removal) quickly to allow accurate assessment of lamellar hypoxia. Lamellar samples were stored at −80 °C.

Real-time quantitative polymerase chain reaction (RT-qPCR) procedure

Lamellar samples previously stored at -80 °C were pulverized using a custom dry-ice cooled Bessman tissue pulveriser to perform total RNA extraction (Absolutely RNA Miniprep, Agilent) with a DNAse step to degrade any genomic DNA. Poly (A) RNA (mRNA) was isolated from total RNA using Streptavidin magnetic beads (mRNA isolation kit, Roche). Four hundred

ng of mRNA was used in complementary (c) DNA synthesis via reverse-transcriptase (Retroscript, Ambion) PCR on a standard thermocycler. The cDNA was stored at -20 °C until ready for real-time PCR (RT-qPCR). Prior to RT-qPCR, external standards were created from equine specific primers (Appendix: Supplementary Table 1) as previously described (Leise et al. 2010).

Real-time quantitative PCR (RT-qPCR) was performed in the SYBR green fluorescent format using a Lightcycler 2.0 thermocycler (Roche) and quantified with external standards as previously described (Leise et al., 2011). Primers for hypoxia and oxygen-dependent cellular energy related genes including GLUT1, HIF-1α, NOS2, and PGK1 were examined. Primers for IL-1 β, IL-6, COX-2, ICAM-1, E-Selectin, ADAMTS4, MMP-2, MMP-9, and MMP-13 were used due to their documented regulation in experimental models of sepsis-related laminitis (Leise et al., 2011).

Several housekeeping genes, β-Actin, β2 microglobulin (β2M), and glyceraldyhyde-3-phosphate dehydrogenase (GAPDH) were assessed by geNorm (Ghent University) to identify the two that had the optimal score. Beta-Actin and GAPDH were identified by the geNorm algorithm as the best candidates to create a normalization factor. The average copy number was normalized, and fold change of each hind limb copy number over average SL copy number was calculated.

Immunoblotting

Protein was extracted from snap frozen lamellae that were first pulverized on dry ice,

then homogenized in M-Per lysis buffer, with the addition of 4M NaCl, protease and phosphatase inhibitors (Halt, Thermo Fisher Scientific), and PMSF as previously described (Leise et al., 2012). After 30 min of incubation on ice, the lysate was separated by centrifugation (18,000 *g* for 15 min at 4^{o} C) and the supernatant collected. Protein concentration was determined using Bradford reagent and a spectrophotometer. Samples were aliquoted and stored at −80 ^{o}C until immunoblotting. Lamellar protein concentrations of prolyl hydroxylase-2 (PHD2; Santa Cruz Biotechnology), the most ubiquitous regulator of HIF-1α concentrations (Berra et al., 2003; Fong and Takeda, 2008) and HIF-1α (Novus Biologicals), a commonly used marker of hypoxia were assessed. As previously described by Leise et al. (2011), protein (30μg) from individual horses was loaded on a 26 well Criterion SDS-PAGE gel (Bio-Rad), separated by electrophoresis, transferred onto a polyvinyldifluoridine (PVDF) membrane (Bio-Rad), blocked in 5% milk in Tris-buffered saline plus 0.1% Tween-20 (TBST) for 1 h at room temperature, then hybridized at 4 ^{o}C overnight in primary antibody (PHD2, 1:1000; HIF-1α 1:750), washed in TBST, incubated with the appropriate horseradish peroxidase (HRP)-linked secondary antibody (1:15000); and the chemiluminescent signal captured on Biomax light film (Carestream). After detection, the membranes were stripped for 15 min in a commercially available buffer (Restore, Pierce) and subsequently probed in the same manner against β-Actin as a loading control. Band intensities were calculated with ImageJ (NIH) and relative intensity was determined against β-Actin.

Hind limb and forelimb lamellar samples collected in a similar manner to that described for this study from control horses in a separate study on sepsis-related laminitis were used to perform a supplemental HIF-1α Western blot to ensure changes in protein concentration were not merely a normal physiologic difference between hind and forelimb weight bearing (Leise et al.,

2012).

Immunofluorescence

To visualize cellular localization of HIF-1α in lamellar tissues in the 48 h altered weight-bearing study, 10µm-thick frozen sections were made from lamellar tissue preserved in OCT and affixed to slides, fixed for 15 min in 4% formaldehyde, washed in phosphate buffered saline (PBS), and blocked for 1 h at room temperature in PBS containing 5% normal goat serum and 0.3% Triton X-100 (Sigma Aldrich). Sections were incubated at 4 ^{o}C overnight in a 1:100 dilution of primary antibody (Santa Cruz Biotechnology) in PBS containing 1% bovine serum albumin and 0.3% Triton X-100. After three washes, slides were incubated with a 1:200 dilution of a flourochrome-conjugated secondary antibody (Thermo Fisher Scientific) at room temperature for 1 h 30 min. The slides were then washed, air dried, cover- slipped with a mounting media containing 4, 6-diamidino-2-phenylindole (DAPI), cured overnight, and imaged on a DM IRE laser-assisted confocal microscope (Leica) equipped with digital imaging software.

Statistical analysis

Preliminary altered weight bearing study

Mean % body mass was compared between limbs for both the control (no V-insert) and the SL (V-insert applied) periods using one-way ANOVA for repeated measures, and between the control and SL periods for each limb using paired student's *t* tests.

RT-qPCR and immunoblotting data

Data were assessed via the D'Agostino-Pearson test for normality. Log and square

transformations were attempted to normalize non-normally distributed data. Further transformations were not performed if these did not achieve normal distribution, and the data were then assessed with non-parametric statistical tests. Normally distributed samples were assessed using a one-way repeated measures ANOVA with Dunnett's multiple comparisons post-test, and the non-normally distributed data were assessed using a Friedman's test with Dunn's multiple comparisons post-test. An α-error of 5% (P <0.05) was designated statistically significant. All statistics were performed using GraphPad Prism (GraphPad Software).

Results

All horses throughout the two parts of this study (preliminary and 48 h insert application) presented normal attitude, appetite and manure production.

Preliminary altered weight bearing study (pilot study)

Evaluation of model

The horses in the pilot study had a greater variation in weight-bearing on the IH than the CH after insert placement. On assessing the effect of the insert on weight bearing, horses in the control period of the preliminary study (no V-insert) bore 58% (standard deviation, SD 0.53%) of the weight in the forelimbs and 42% (SD 0.53%) in the hind limbs, consistent with previously published studies (Hood et al., 2001). Following the placement of the V-insert on the shoe of one forelimb, average weight borne was 43.2% (interquartile range [IQR] 34.5%-51.9%; SD 7.8 %) on the SL relative to 10.1% (IQR 2.0%-18.3%; SD 8.3%) on the UL limb. The CH bore 25.9% (IQR 22.3%-29.5%; SD 3.4 %), and 20.8% was borne on the IH (IQR 14.2%-27.4%; SD 6.3 %). This indicated that horses in the preliminary study bore 33% more weight on the SL than the UL

after V-insert attachment to one forelimb and 17% and 22% more on the SL than the CH and IH, respectively.

Forty-eight hour insert application with lamellar harvest

Evaluation of model

Physical examination parameters were not significantly different for the 48 h period after insert placement compared with the 48 h period before insert placement.

RT-qPCR procedure

There were no differences in lamellar mRNA concentrations of hypoxia/cellular energy-related genes GLUT1, HIF-1α, NOS2, PGK2, VEGF, or inflammatory genes ADAMTS4, COX-2, E-selectin, IL-1 β, IL-6, MMP-2, MMP-9, and MMP13 between SL, CH and IH samples (Appendix: Supplementary Table 2).

Immunoblotting

There was no difference in HIF-1α protein concentrations between fore and hind limbs in archived lamellar samples from control horses (n = 6) from a previous study (P = 0.23). A visible difference in band intensity was visualized on the immunoblot (Fig. 3). There was an increase in HIF-1α protein concentrations in the SL individual lamellar samples compared to the CH, but not the IH samples (P = 0.026, Table 1). PHD2 concentrations were not different between sample groups (P = 0.13; Supplementary Material 3).

Immunofluorescence

On immunofluorescence of lamellar samples in the SL, CH, and IH feet for HIF-1α, signal was localized primarily to the epidermal lamellae in all samples (Fig. 4). No stretching or elongation of lamellae or separation of secondary lamellae from primary was noted on the samples, as reported on histological samples in previous studies (Van Eps and Pollitt, 2009).

Discussion

Although previous studies of SLL have included cadaver studies and in vivo studies in which transient episodes of preferential weight bearing on one limb have been induced (Sun et al., 2015), this study is the first to establish an in vivo model for studying preferential weight bearing in which the horses undergo increased weight bearing on one forelimb relative to the other for an extended period of time. Overall, the model was well tolerated by the horses with no change in demeanor or physical exam parameters during the two-day time course. Although there are flaws in the protocol used which should be improved (discussed below), the significant increase in HIF-1α, a marker of hypoxia, in the SL (vs. CH), with no changes observed in markers of inflammation, indicates that the preferential weight bearing model is possibly of value in the study of SLL and that lamellar hypoxia may occur in the SL in horses undergoing preferential weight bearing.

HIF-1α is commonly used as a marker of hypoxia due to a well-documented increase in the protein in hypoxic cells/tissue, primarily due to decreased cellular oxygen resulting in decreased activity of prolyl hydroxylase-2 (PHD2; Fong and Takeda, 2008), the primary enzyme which hydroxylates proline residues on HIF-1α leading to proteasomal degradation of the protein (Forsythe et al., 1996; Huang et al., 1998; Semenza, 2011). Because HIF-1α activity is primarily

304 regulated at the post-translational level in hypoxia via proteosomal degradation, cellular/tissue
305 protein concentrations of HIF-1α can change with no corresponding change in mRNA
306 concentrations. However, HIF-1α can also be induced at the transcriptional level by
307 inflammatory signaling, as reported in an experimental model of sepsis-related laminitis (Pawlak
308 et al., 2014). Thus, lamellar inflammation, using markers of inflammatory gene expression
309 previously documented to increase in models of sepsis-related laminitis, was also investigated in
310 the present study. The significant increase in lamellar concentration of HIF-1α protein in the SL
311 compared to the hind limb, with no evidence of inflammatory signaling in the same samples,
312 indicates that increased lamellar HIF-1α protein concentration in the current study is most likely
313 due to hypoxia. Whereas lamellar HIF-1α concentrations appeared increased in the SL (vs. hind
314 limbs) in 6/8 horses (Fig. 3), two horses appeared to be non-responders, with higher HIF-1α
315 concentrations in other limbs. A similar incidence of non-responders is common in models of
316 sepsis-related laminitis (Leise et al., 2011), suggesting an inherent genetic variability in some
317 horses, possibly genetically resistant to endotoxemia/sepsis in an outbred population of horses
318 (Belknap and Black, 2012). It is possible that genetic variability played a role in the variability of
319 the data between horses in the current study, but it is also possible that the difference is due to
320 variability between horses regarding the preferential use of one or the other hindlimb for
321 increased support in this model. Lamellar concentrations of PHD2 were assessed due to the
322 possibility that changes in cellular PHD2 activity not related to hypoxia (i.e. decreased PHD2
323 concentrations via TGFβ-mediated inhibition of PDH2 expression (McMahon et al., 2006) may
324 affect HIF-1α protein concentrations. The lack of change in lamellar PHD2 concentrations
325 unaccompanied by change in concentrations of inflammatory signaling molecules investigated in
326 the present study further indicates that HIF-1α signaling was not affected by inflammation and

that the changes in lamellar HIF-1α concentrations were likely due to decreased cellular concentrations of oxygen (Berra et al., 2003; Fong and Takeda, 2008).

Weight distribution with the V-insert applied was assessed in the preliminary study at the University of Queensland, confirming greater weight bearing in the SL compared with either hind limb (Fig. 1), however the equipment to record weight distribution on each limb for the 48 h period was not available at Ohio State University, which is a limitation of the current study. If available, this methodology would have assisted in determining any effect of disparate weight bearing on the two hind limbs on the data obtained. The preliminary weight bearing study using the same custom shoe indicated that the shoe does not induce symmetric weight bearing loads on the hind limbs (more weight was consistently borne on the CH relative to the IH). However, both hind limbs supported much less weight than the SL after insert placement on the UL (Fig. 2). Another limitation of this study is its relatively short duration of 48 h. Although we were interested in establishing a humane model to determine the effect of preferential weight bearing on lamellar signaling and not attempting to induce laminitis, the lack of signs of laminitis in these horses at the time of termination of the study therefore does not establish that this model would inevitably lead to SLL.

Another concern is that, although this model provides a mechanical impetus for the horse to not place normal weight on one limb, for humane reasons, the study design dictated that horses were not sensing the pain that drives the preferential weight bearing that leads to SLL in most clinical cases. Horses in the current study still periodically shifted weight off of the SL, possibly due to sensory input from the foot (i.e. perhaps a similar sensation that humans feel of a

foot ‘falling asleep’ when weight is borne on one foot for extensive periods of time). In clinical cases, it is likely that the pain in the opposite limb overrides that sensation in the SL. Thus, it might have been better to also provide local anesthesia to the distal part of the SL during the protocol to allow consistent preferential weight bearing (and minimal movement) for the short periods for which the horse could be used (for humane reasons) in this model. In addition to causing preferential weight bearing, a recent study indicated that lack of limb movement may be more important than the actual weight bearing, thus accurate assessment of limb movement in future studies is important (Sun et al., 2015).

Conclusions

The current data provide preliminary evidence to support the hypothesis that lamellar hypoxia may occur during preferential weight bearing. However, further studies are required to more thoroughly assess both hypoxia-related signaling and other signaling associated with lamellar damage that could play a role in SLL. With improvements in study design, it is likely that the preferential weight bearing model with the V-insert can be used in the future to more accurately assess lamellar events leading to failure.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix: Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi: ...'

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Fig. 1. The V-shaped insert attached to a traditional steel shoe (a). With this insert, horses either kept the limb in mild flexion or in extension (b), but the insert prevented normal weight bearing. The limbs were designated as follows: the unloaded limb (UL) was the limb with the V-insert shoe. The contralateral forelimb was designated the supporting limb (SL). The hind limb contralateral to the SL was designated CH, and the hind limb ipsilateral to the SL was designated the IH (c).

Fig. 2. Mean percentage of body mass supported by each limb for 1 h without a V-insert (control period) and for 30 min following attachment of the V-insert. In the control period, the forelimbs bore significantly ($P < 0.05$) more weight than the hind limbs, but after V-insert placement, the supporting limb (SL) bore significantly more weight than the unloaded limb (UL) and the contralateral and ipsilateral hindlimbs (CH and IH, respectively). An asterisk indicates statistically significant difference vs. UL ($P < 0.05$).

Fig. 3. HIF-1α protein concentrations were assessed against the housekeeping gene β-actin via immunoblot. In pooled lamellar samples of supporting limb (SL), contralateral hindlimb (CH) and ipsilateral hindlimbs (IH), the SL sample presented greater intensity than either hindlimb sample (a). On graphical representation of HIF-1α immunoblot results of the individual samples from each limb, SL and hindlimb samples present distinct differences in relative intensity (b). The immunoblot of the samples from individual horses (c) illustrates the greater intensity of HIF-1 in all horses except Horses 6 and 7 (HIF-1α represented as 1 or 2 bands on immunoblots

510 depending on percentage of acrylamide in gel). An asterisk indicates significant difference vs.
511 SL ($P < 0.05$).
512
513 Fig. 4. Cellular localization of HIF-1α using immunofluorescence. Immunofluorescence for HIF-
514 1 α of the primary and secondary epidermal lamellae and surrounding dermis supporting limb
515 (SL; a) and contralateral limb (CL; b) samples. Note in both the lower magnification views and
516 higher magnification (insets), HIF-1 α protein (red signal) was primarily localized to epidermal
517 lamellar tissue (solid arrows), with little staining in the dermis (open arrows). No difference in
518 cellular localization is detectable between SL and CL samples. Blue stain indicates nuclear
519 material (4, 6-diamidino-2-phenylindole [DAPI] stain).
520

521 **Table 1** Protein concentrations of proteins/genes of interest in supporting limb (SL) samples
522 compared to samples from contralateral (CH) and ipsilateral (IH) hind limbs
523

Protein	SL	CH	IH	*P*
HIF-1α[a]	1.83 (1.07-2.59)	0.70* (0.42-0.98)	1.16 (0.84-1.49)	0.026
PHD2[b]	0.64 (0.50-0.70)	0.78 (0.67-0.97)	0.75 (0.57-0.82)	0.13

524 [a] Normally distributed data presented as mean (95% confidence interval of the mean)
525 [b] Non-normal distributed data presented as median (25%-75% interquartile range)
526 An asterisk indicates significant difference on post hoc testing vs. SL samples
527

528