Advanced glycation endproducts in horses with insulin-induced laminitis

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Abstract

Advanced glycation endproducts (AGEs) have been implicated in the pathogenesis of cancer, inflammatory conditions and diabetic complications. An interaction of AGEs with their receptor (RAGE) results in increased release of pro-inflammatory cytokines and reactive oxygen species (ROS), causing damage to susceptible tissues. Laminitis, a debilitating foot condition of horses, occurs in association with endocrine dysfunction and the potential involvement of AGE and RAGE in the pathogenesis of the disease has not been previously investigated. Glucose transport in lamellar tissue is thought to be largely insulin-independent (GLUT-1), which may make the lamellae susceptible to protein glycosylation and oxidative stress during periods of increased glucose metabolism. Archived lamellar tissue from horses with insulin-induced laminitis (n = 4), normal control horses (n = 4) and horses in the developmental stages (6 h, 12 h and 24 h) of the disease (n = 12) was assessed for AGE accumulation and the presence of oxidative protein damage and cellular lipid peroxidation. The equine-specific RAGE gene was identified in lamellar tissue, sequenced and is now available on GenBank. Lamellar glucose transporter (GLUT-1 and GLUT-4) gene expression was assessed quantitatively with qRT-PCR in laminitic and control horses and horses in the mid-developmental time-point (24 h) of the disease. Significant AGE accumulation had occurred by the onset of insulin-induced laminitis (48 h) but not at earlier time-points, or in control horses. Evidence of oxidative stress was not found in any group. The equine-specific RAGE gene was not expressed differently in treated and control animals, nor was the insulin-dependent glucose transporter GLUT-4. However, the glucose transporter GLUT-1 was increased in lamellar tissue in the developmental stages of insulin-induced laminitis compared to control horses and the

insulin-independent nature of the lamellae may facilitate AGE formation. However, due to the lack of AGE accumulation during disease development and a failure to detect an increase in ROS or upregulation of RAGE, it appears unlikely that oxidative stress and protein glycosylation play a central role in the pathogenesis of acute, insulin-induced laminitis.

*Keywords***:** Equine; Glucose; AGE; RAGE; GLUT-1; Oxidative stress

List of abbreviations:

AGE	advanced glycation endproduct
ECM	extracellular matrix
GLUT	facilitative glucose transporter
MDA	malondialdehyde
RAGE	receptor for advanced glycation endproduct
ROS	reactive oxygen species

1. Introduction

The study of protein glycosylation and the formation of advanced glycation endproducts (AGEs) has become an increasingly important area of research since recognition of their role in the progression of diabetic complications (Brownlee, 1992a). AGEs are formed by an irreversible non-enzymatic reaction (the Maillard reaction) between carbohydrates and proteins (or lipids or nucleic acids) containing a free amino group (Singh et al., 2001). AGEs accumulate in tissues as a consequence of aging and during several pathological processes that include hyperglycaemia, cancer and inflammation (Brownlee, 1992b; Ramasamy et al., 2005; Takino et al., 2010). The concentration of AGEs in a tissue depends, in part, on the availability of carbohydrate (usually glucose) and thus glycation is enhanced in diabetic patients (Yamagishi and Matsui, 2010). However, AGEs can form during euglycaemia, especially in cells that are freely permeable to glucose (Schmidt et al., 1995).

AGEs are capable of modulating cell function by binding with multiple cell surface binding sites, of which the best characterised is the signal transduction receptor known as RAGE (Bucciarelli et al., 2002). An interaction between AGE and RAGE results in the release of pro-inflammatory cytokines and oxidative stress, thrombosis and over-expression of various proteins in the extra-cellular matrix (ECM) such as collagen and laminin (Brownlee, 1992a; Gawlowski et al., 2009; Sick et al., 2010; Simm et al., 2004). In diabetic humans, basement membrane pathology results in renal, vascular and retinal complications that increase morbidity and mortality rates associated with the disease (Giugliano et al., 1996; Yamagishi and Matsui, 2010, 2011).

While hyperglycaemia is seldom reported in horses and the development of diabetes mellitus is rare (Johnson et al., 2005), insulin resistance seems to be increasingly common (Geor and Frank, 2009). One complication of altered insulin and glucose metabolism in horses and ponies is the development of laminitis, a foot disease that can result in lameness and loss of function (Jeffcott et al., 1986; Johnson et al., 2004). The pathogenesis of endocrinopathic laminitis is only partially understood and potential mechanisms for the disease include vascular perturbations to the digit, ECM degradation, glucotoxicity, inflammation and oxidative stress (de Laat et al., 2010b; Geor and Frank, 2009).

Although laminitis occurring in conjunction with hyperinsulinaemia has received attention, less is known about hyperglycaemia and abnormal glucose metabolism in this species. Hoof lamellar tissue contains both insulin-dependent (GLUT-4) and insulin-independent (GLUT-1) glucose transporters (Asplin et al., 2011; Mobasheri et al., 2004). Strong mRNA expression of GLUT-1 was present in the lamellae of ponies with insulin-induced laminitis and normal controls (Asplin et al., 2011), which may indicate that overexposure of lamellar cells to glucose is feasible, even during euglycaemia.

Clinical and histopathological laminitis has been induced experimentally in horses and ponies with a euglycaemic, hyperinsulinaemic clamp (Asplin et al., 2007; de Laat et al., 2010a), permitting study of the developmental and acute stages of hyperinsulinaemic laminitis. Other experimentally-induced and naturally-occurring forms of laminitis have been linked with excessive production of pro-inflammatory

cytokines, supporting a role for inflammation in the disease pathogenesis (Belknap et al., 2007; Treiber et al., 2009). Reactive oxygen species (ROS) can be released from damaged inflammatory cells during disease states such as diabetes, and cause tissue damage (Siekmeier et al., 2007). However, whether pro-inflammatory cytokines and oxidative stress play a significant role in endocrinopathic laminitis pathophysiology is unclear. To our knowledge, determination of whether AGEs accumulate in the lamellar tissue of horses with hyperinsulinaemia has not yet been investigated. ROS have been studied previously in horses, but not in those with experimentally-induced hyperinsulinaemic laminitis. Analysis of AGEs and ROS concentrations in lamellar tissue from horses in the developmental phase of insulin-induced laminitis will determine whether these inflammatory processes occur prior to the onset of clinical signs at 48 h, thus investigating their role in disease pathogenesis.

The study hypothesis was that advanced glycation and oxidative stress are increased in the developmental and acute stages of insulin-induced laminitis and contribute to disease pathogenesis. The aims of the current study were to quantify lamellar glucose transporter (GLUT-1 and GLUT-4) gene expression, and determine if protein glycosylation and oxidative lipid and protein damage occur in lamellar tissue, during the developmental (6 h, 12 h, 24 h) and acute (48 h) stages of insulin-induced laminitis, and compare this to normal horses. Identification of the equine-specific RAGE gene has not, to our knowledge, been previously reported in horses. RAGE is present on many cell types including endothelial cells, keratinocytes and monocytes (Brett et al., 1993; Lohwasser et al., 2005), and these cells are present in equine lamellae (Faleiros et al., 2011). A secondary aim of the study was to confirm the

presence of the equine-specific RAGE gene in lamellar tissue and submit the sequence to GenBank.

2. Materials and Methods

2.1. Samples

The experimental protocol was approved by the Animal Ethics Committee of The University of Queensland which ensured compliance with the *Animal Welfare Act of Queensland (2001)* and the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition 2004).* Archived hoof lamellar tissue from Standardbred horses treated with a euglycaemic hyperinsulinaemic clamp (n = 16), or a balanced electrolyte solution (n = 4), was used in the current study (de Laat et al., 2010a; de Laat et al., 2011). Horses in the treatment group received a combined insulin (Humulin-R (Eli Lily, Australia), 6 mIU/kg bwt/min) and glucose (50% dextrose (Baxter, Australia), variable rate to maintain euglycaemia) i.v. infusion for 6 h (n = 4), 12 h (n = 4), 24 h (n = 4), or until the onset of Obel grade 2 (Obel, 1948) laminitis (48 h; n = 4). Control horses were treated for 48 h. Age, bodyweight and body condition score was similar for each group of horses (Table 1). Blood samples (10 mL) were taken from the left jugular vein during the experiment to measure blood glucose (mmol/L) and serum insulin (μIU/mL) concentration (Table 1). Blood glucose was measured immediately with a glucometer (Accucheck-Go, Roche, Switzerland) validated for equine blood (ρ_c = 0.96) and serum was stored at -80°C until analysed for insulin concentration with a validated (McGowan et al., 2008) radioimmunoassay (Coat-a-count, Siemens Healthcare Diagnostics, IL, U.S.A.).

Samples of lamellar tissue from all horses were examined for markers of oxidative damage, whereas only the 24 h, 48 h and control groups were analysed for RAGE, GLUT-1 and GLUT-4 gene expression.

2.2. Oxidative protein damage and lipid peroxidation

Protein was extracted from 100 mg of frozen hoof lamellar tissue by homogenisation as described previously (de Laat et al., 2011). Total protein concentration was assayed in triplicate (intra-assay CV = 4.1%) using the bicinchoninic acid method (Pierce, IL, USA) and measured on a NanoDrop (Thermo Scientific, DE, U.S.A.) spectrophotometer (562 nm). Protein samples from each horse were diluted (1 x phosphate buffered saline) to a final concentration of 10 µg/mL and contained a detergent (Triton X-100) concentration of < 0.001% to prevent interference with the protein coating of the ELISA plate.

Commercial ELISA kits (Oxiselect, Cell Biolabs, CA, and U.S.A.) were used according to the manufacturer's instructions for the detection and quantification of AGE- and malondialdehyde (MDA)-protein adducts and protein carbonyls, against known bovine serum albumin (BSA) conjugated standards, in all samples. Commercial ELISAs have been used previously to measure ROS in horses (McFarlane and Cribb, 2005; Treiber et al., 2009). Although an AGE-adduct ELISA has not been used previously in horses to our knowledge, AGE structure is consistent regardless of the protein source and the antibody kits are thus suitable for use with any species (Cell Biolabs, CA, U.S.A.). Absorbance was measured on a microplate reader (Titertek Multiskan Plus MKII, Titertek-Berthold, AL, U.S.A.) at 450 nm.

2.3. RNA extraction and cDNA synthesis

Frozen lamellar tissue (100 mg) was pulverised in a pre-chilled heavy-duty foil envelope on a cold metal block and total RNA was extracted using 1mL of Trizol reagent (Invitrogen, Australia) according to the manufacturer's instructions. Each sample was visualised on a 2% agarose gel with SybrSafe stain (Invitrogen, Australia) to confirm RNA integrity, and RNA concentration was determined by UV spectrophotometry. Prior to cDNA synthesis, RNA samples were treated with RNAse-free DNAse I (Invitrogen, Australia) to eliminate possible genomic DNA contamination. For each sample, 1µg of total RNA was reverse-transcribed to cDNA using the Reverse Transcription System (Promega, WI, U.S.A.) and stored at -80°C until analysis.

2.4. Cloning and sequencing of the equine-specific RAGE gene

Oligonucleotide primer sets for the RAGE gene were designed within the most homologous region based on multiple alignments of the bovine, murine and human sequences and synthesized by Sigma Aldrich, Australia. PCR products of the predicted size (409 bp) were cloned into the pGEM-T easy cloning vector (Promega, WI, U.S.A.) and positive colonies were sequenced using an M13 universal sequencing primer and a Big Dye Terminator Cycle Sequencing Kit 3.1 (ABI Prism, Applied Biosystems, Australia). The nucleotide sequence was resolved on an automated DNA sequencer (Applied Biosystems 373A). Programs from the Australian National Genomic Information Services (ANGIS) were used to analyse the resulting DNA

sequences. The equine-specific RAGE gene sequence is now available on GenBank; accession number: BankIt1492591JQ004799.

2.5. qRT-PCR and PCR analyses

The qRT-PCR assays for relative quantification of GLUT-1, GLUT-4, and RAGE in lamellar tissue samples were performed using SensiMix 2 x SYBR Green Master Mix (Bioline, Australia). The PCR primer sets for each target gene and for two house-keeping genes (Table 2) were designed from equine-specific sequences (GenBank accession no: GLUT-1, DQ139875, GLUT-4, AF531753, β2-microglobulin, X69083 and GAPDH, AF157626) using primer design software (Primer3 v 0.4.0, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Sigma Aldrich, Australia. Two different house-keeping genes have been used to investigate other forms of laminitis (Coyne et al., 2009; Visser, 2008) so both were analysed in the current study in order to select the most stable house-keeping gene. Analysis of the amplification efficiency ($E = 10^{[-1/slope]}$) of the target and house-keeping genes was determined on serial dilutions of cDNA by qRT-PCR, with each primer set having efficiencies within 10% of 2, which permits use of the $2^{-\Delta CT}$ equation (Schmittgen and Livak, 2008). The specificity of all primers used in this study was checked via a Blast search (Altschul et al., 1997) and the results were confirmed by detection of a single band of the expected PCR product size following 2% agarose gel electrophoresis (data not shown).

PCR reactions were set-up in 100-well genediscs (Qiagen, Australia) using a Corbett CAS-1200 robot (Qiagen, Australia) and run on a Corbett Rotor-Gene 6000 series

(Qiagen, Australia). The exact primer concentrations and PCR conditions were determined during initial optimisation runs. Following optimisation experiments, assays of 10 µL reaction volumes were prepared comprising 2 µL optimally diluted cDNA, 5 µL of SensiMix 2 x SYBR Green Master Mix (Bioline, Australia), 1 µL H_20 and 2 µL of forward and reverse primers at varying concentrations as determined by optimisation experiments (Table 2). The cycling conditions were as follows: 95°C for 10 min, followed by 45 cycles of 15 s at 95°C, 15 s at 58 °C and 30 s at 72°C. Specificity of the amplified products was checked immediately after the PCR with melting curve analysis and sequencing of the PCR products. All samples were amplified on the same disc for every primer pair to ensure equal amplification conditions. No-template controls using water instead of cDNA templates were included for each gene as negative controls. Samples were amplified in duplicate and the results recorded as cycle threshold (C_T) values of background-subtracted, qPCR fluorescence kinetics. For each sample, C_T values for each target gene were averaged and relative gene expression was calculated for target genes against the reference gene β2-microglobulin, using the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008).

2.6. Statistical analyses

Serum insulin, blood glucose, AGE and ROS concentrations and gene expression were compared between groups using Kruskal-Wallis ANOVA on ranks. Pair-wise comparisons were made with Tukey's post-hoc test. All data are expressed as mean ± se or median (range) and significance was accepted at $p < 0.05$. Statistical analyses were performed using R, version 7.2.7.

3. Results

3.1. Oxidative protein damage and lipid peroxidation

The concentration of AGEs (ng/mL) in the lamellae of control horses and in treated horses during the developmental phases of laminitis (6 h, 12 h, 24 h) was negligible. A significant accumulation of AGEs had occurred by the acute phase (48 h) of laminitis (Table 3). Lamellar concentration of the lipid peroxidation product MDA (pmol/mg) did not differ between groups (Table 3). However, the lamellar protein carbonyl (nmol/mg) tissue concentration was decreased ($p < 0.05$) late in the developmental phase (24 h) of laminitis compared to the other time-points (Table 3).

3.2. Gene expression

As expected, the equine-specific RAGE gene sequence showed very high homology (86 – 89%) to all identified species due to the highly conserved nature of the region used to design the primers. β2-microglobulin was determined to be the most stable house-keeping gene. Both GLUT-1 and GLUT-4 were found to be present in lamellar tissue (Table 4). Relative gene expression of GLUT-1 was increased in the group treated with insulin for 24 h compared to the control group whereas GLUT-4 and RAGE gene expression did not differ between the groups (Table 4).

4. Discussion

The accumulation of AGEs in the lamellar tissue of horses after just 48 h of hyperinsulinaemia is a significant finding of this study. To our knowledge, this is the first study to examine the concentration of protein glycosylation by-products in equine hoof lamellae. The finding of negligible amounts of AGEs in normal lamellae is not surprising given the young age of the horses studied, and shows that AGEs do not appear to be routinely present in the lamellae of young horses. Recent evidence has shown that AGEs can be generated more rapidly than previously thought and are potential early arrivals at sites of injury and trauma (Ramasamy et al., 2005). This premise is supported in the current study by the finding of significant AGE accumulation in the lamellae of horses with Obel grade 2 (Obel, 1948) lameness following 48 h of hyperinsulinaemia. The AGE concentrations in lamellar tissue, in this acute stage of laminitis, were modest and further accumulation of AGEs may have occurred if the study had been prolonged.

AGE generation leads to increased release of ROS and inflammation, which initiates an ongoing cycle of AGE accumulation and oxidative stress (Ramasamy et al., 2010). The failure to detect an increase in oxidative protein damage (protein carbonyl) and cellular lipid peroxidation (MDA) as a consequence of the insulin and glucose infusion in this study may be due to the acute time-course of the experiment. However, a lack of oxidative stress has also been reported in more chronic forms of endocrinopathic laminitis (Keen et al., 2004; Treiber et al., 2009). Neither oxidants (MDA, 3-nitrotyrosine) nor anti-oxidants (glutathione, glutathione peroxidase and superoxide dismutase) were found to be increased in blood samples from ponies that had previously suffered recurrent, pasture-associated laminitis when compared to ponies that had never experienced laminitis (Treiber et al., 2009). Likewise, studies on

ponies with equine Cushing's disease, which is a condition consistently associated with an increased risk for laminitis (McGowan et al., 2004), found no difference in MDA and glutathione peroxidase levels in peripheral blood samples between affected and normal animals (Keen et al., 2004).

Other experimental models of laminitis (alimentary and inflammatory) also have not been associated with increased lamellar lipid peroxidation or protein carbonylation (Burns et al., 2011). However, researchers have found that the equine digital lamellae may be particularly susceptible to injury induced by ROS, due to a lack of the anti-oxidant, superoxide dismutase (Loftus et al., 2007). Further studies have demonstrated that the addition of superoxide dismutase blocks AGE production in cultured bovine endothelial cells exposed to hyperglycaemia (Nishikawa et al., 2000). Anti-oxidant markers were not examined in this study and analysis of their potential role in the suppression of oxidative stress in insulin-induced laminitis is warranted.

The identification and sequencing of the equine-specific RAGE gene in the lamellar tissue of horses is a novel finding of this study. The lack of RAGE upregulation in the current study may be a consequence of the euglycaemic status of the subjects or alternatively, may simply be related to the acute time-course of the study. Increased expression of an enzyme that degrades AGEs, glyoxalase 1, has been shown to prevent increases in RAGE expression and reduce ROS production (Yao and Brownlee, 2010), and if present in the horses in the current study may have contributed to the lack of upregulation of RAGE and ROS. Analysis of glyoxalase 1 would be required to further investigate this.

Three previous studies using different techniques agree that the insulin-independent glucose transporter, GLUT-1, is expressed in the lamellae of healthy horses and ponies (Asplin et al., 2011; Mobasheri et al., 2004; Wattle and Pollitt, 2004). While Mobasheri *et al.* (2004) reported that immunolocalisation of insulin-dependent GLUT-4 was strong in normal lamellar keratinocytes, the opposite was found by Wattle and Pollitt (2004). GLUT-4 lamellar mRNA expression was also not found to be prevalent in healthy horses and ponies using conventional PCR (Asplin et al., 2011). Quantitative RT-PCR results from the current study showed that both GLUT-1 and GLUT-4 were present in the lamellar tissue of control horses.

Conventional PCR has shown that lamellar GLUT-1 mRNA expression was strong in ponies with insulin-induced laminitis and equine Cushing's disease, while GLUT-4 expression was weak (Asplin et al., 2011). In chronic laminitis, lamellar immunolocalisation of GLUT-1 and GLUT-4 was decreased (Mobasheri et al., 2004). In the current study, gene expression of GLUT-4 remained unchanged between groups, whereas GLUT-1 was upregulated in the lamellar tissue of horses treated for 24 h. Failure of GLUT-1 expression to remain significantly elevated at the onset of laminitis (48 h) may suggest that GLUT-1 expression decreased as the disease progressed, similar to the findings of Mobasheri *et al.* (2004). Alternatively, the small samples sizes may account for an apparent increase in GLUT-1 expression at 48 h not reaching statistical significance. A reliance on GLUT-1 facilitative glucose transport may suggest that glucose saturation of lamellar keratinocytes can occur during increased glucose delivery. The upregulation of lamellar GLUT-1 gene expression in the developmental stages of insulin-induced laminitis may further predispose this tissue to glucose saturation. Lamellar AGE accumulation in the horses treated with

insulin and glucose for 48 h may be enabled by the largely insulin-independent nature of glucose transport in this tissue.

In human diabetic patients, AGEs result in the over-production of laminin and collagen, causing thickening and reduced elasticity of the ECM, which can lead to complications such as nephropathy (Dronavalli et al., 2008; Mendez et al., 2010). This occurs by a receptor-independent pathway (Wendt et al., 2003). Widening of the basement membrane has been reported following ultrastructural analysis of insulin-induced laminitis in two ponies (n = 5) and AGEs may contribute to this ECM pathology (Nourian et al., 2009). However, studies of alimentary carbohydrate overload laminitis have reported a loss of structural components of the ECM (collagen type IV and laminin-5) in the developmental and acute (48 h) phase of the disease (Visser and Pollitt, 2011), which suggests that different pathogenic mechanisms may occur in hyperinsulinaemic laminitis. Temporal analysis of ECM components in insulin-induced laminitis is required.

However, while AGE accumulation occurred in the treated horses concurrently with the development of major lamellar pathology, such as ECM dysfunction, some lamellar pathological processes, including morphological changes and cellular proliferation, have been observed earlier (12 h to 24 h) in the developmental phase (de Laat *et al.*, unpublished data). Thus, histopathological changes precede the onset of clinical disease and AGE accumulation (24 to 48 h). Furthermore, RAGE upregulation did not occur during the development or onset of laminitis. Thus, it is unlikely that AGE-RAGE interaction plays a significant role in the pathogenesis of acute hyperinsulinaemic laminitis. However, AGEs may contribute to lamellar

pathology by receptor-independent pathways. AGE involvement in chronic forms of the disease is also possible, as further generation of AGEs may occur as the disease progresses. Lamellar AGE concentration in chronic disease requires investigation.

In the present study, advanced glycation of lamellar tissue occurred within 48 h in horses treated with insulin and glucose. Although protein glycosylation may contribute to lamellar ECM pathology, and potentially complicate the disease in the longer term, it appears unlikely to be the sole aetiopathogenic factor in hyperinsulinaemic laminitis. Based on the current results, further investigations of AGEs, RAGE and the role of ROS in lamellar disease in horses are warranted.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., Lipman, D.J., 1997, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

Asplin, K.E., Curlewis, J.D., McGowan, C.M., Pollitt, C.C., Sillence, M.N., 2011, Glucose transport in the equine hoof. Equine Veterinary Journal 43, 196-201.

Asplin, K.E., Sillence, M.N., Pollitt, C.C., McGowan, C.M., 2007, Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies. The Veterinary Journal 174, 530-535.

Belknap, J.K., Giguere, S., Pettigrew, A., Cochran, A.M., Van Eps, A.W., Pollitt, C.C., 2007, Lamellar pro-inflammatory cytokine expression patterns in laminitis at the developmental stage and at the onset of lameness: innate vs. adaptive immune response. Equine Veterinary Journal 39, 42-47.

Brett, J., Schmidt, A.M., Yan, S.D., Zou, Y.S., Weidman, E., Pinsky, D., Nowygrod, R., Neeper, M., Przysiecki, C., Shaw, A., Migheli, A., Stern, D., 1993, Survey of the distribution of a newly characterized receptor for advanced glycation end-products in tissues. Am. J. Pathol. 143, 1699-1712.

Brownlee, M., 1992a, Glycation products and the pathogenesis of diabetic complications. Diabetes Care 15, 1835-1843.

Brownlee, M., 1992b, Nonenzymatic glycosylation of macromolecules. Prospects of pharmacologic modulation. Diabetes 41 Suppl 2, 57-60.

Bucciarelli, L.G., Wendt, T., Rong, L., Lalla, E., Hofmann, M.A., Goova, M.T., Taguchi, A., Yan, S.F., Yan, S.D., Stern, D.M., Schmidt, A.M., 2002, RAGE is a multiligand receptor of the immunoglobulin superfamily: implications for homeostasis and chronic disease. Cell Mol Life Sci 59, 1117-1128.

Burns, T.A., Westerman, T., Nuovo, G.J., Watts, M.R., Pettigrew, A., Yin, C., Belknap, J.K., 2011, Role of Oxidative Tissue Injury in the Pathophysiology of Experimentally Induced Equine Laminitis: A Comparison of 2 Models. Journal of Veterinary Internal Medicine 25, 540-548.

Coyne, M.J., Cousin, H., Loftus, J.P., Johnson, P.J., Belknap, J.K., Gradil, C.M., Black, S.J., Alfandari, D., 2009, Cloning and expression of ADAM-related metalloproteases in equine laminitis. Veterinary Immunology and Immunopathology 129, 231-241.

de Laat, M.A., Kyaw-Tanner, M.T., Nourian, A.R., McGowan, C.M., Sillence, M.N., Pollitt, C.C., 2011, The developmental and acute phases of insulin-induced laminitis involve minimal metalloproteinase activity. Veterinary Immunology and Immunopathology 140, 275-281.

de Laat, M.A., McGowan, C.M., Sillence, M.N., Pollitt, C.C., 2010a, Equine laminitis: Induced by 48 h hyperinsulinaemia in Standardbred horses. Equine Veterinary Journal 42, 129-135.

de Laat, M.A., McGowan, C.M., Sillence, M.N., Pollitt, C.C., 2010b, Hyperinsulinemic laminitis. Vet Clin North Am Equine Pract 26, 257-264.

Dronavalli, S., Duka, I., Bakris, G.L., 2008, The pathogenesis of diabetic nephropathy. Nat Clin Pract End Met 4, 444-452.

Faleiros, R.R., Nuovo, G.J., Flechtner, A.D., Belknap, J.K., 2011, Presence of mononuclear cells in normal and affected laminae from the black walnut extract model of laminitis. Equine Veterinary Journal 43, 45-53.

Gawlowski, T., Stratmann, B., Ruetter, R., Buenting, C.E., Menart, B., Weiss, J., Vlassara, H., Koschinsky, T., Tschoepe, D., 2009, Advanced glycation end products strongly activate platelets. Eur. J. Nutr. 48, 475-481.

Geor, R., Frank, N., 2009, Metabolic syndrome--From human organ disease to laminar failure in equids. Veterinary Immunology and Immunopathology 129, 151-154.

Giugliano, D., Ceriello, A., Paolisso, G., 1996, Oxidative stress and diabetic vascular complications. Diabetes Care 19, 257-267.

Jeffcott, L.B., Field, J.R., McLean, J.G., Odea, K., 1986, Glucose-tolerance and insulin sensitivity in ponies and Standard-bred horses. Equine Veterinary Journal 18, 97-101.

Johnson, P.J., Messer, N.T., Slight, S.H., Wiedmeyer, C., Buff, P., Ganjam, V.K., 2004, Endocrinopathic laminitis in the horse. Clinical Techniques in Equine Practice 3, 45-56.

Johnson, P.J., Scotty, N.C., Wiedmeyer, C., Messer, N.T., Kreeger, J.M., 2005, Diabetes mellitus in a domesticated Spanish mustang. J Am Vet Med Assoc 226, 584-588, 542.

Keen, J.A., McLaren, M., Chandler, K.J., McGorum, B.C., 2004, Biochemical indices of vascular function, glucose metabolism and oxidative stress in horses with equine Cushing's disease. Equine Veterinary Journal 36, 226-229.

Loftus, J.P., Belknap, J.K., Stankiewicz, K.M., Black, S.J., 2007, Laminar xanthine oxidase, superoxide dismutase and catalase activities in the prodromal stage of black-walnut induced equine laminitis. Equine Veterinary Journal 39, 48-53.

Lohwasser, C., Neureiter, D., Weigle, B., Kirchner, T., Schuppan, D., 2005, The Receptor for Advanced Glycation End Products Is Highly Expressed in the Skin and Upregulated by Advanced Glycation End Products and Tumor Necrosis Factor-Alpha. J Invest Dermatol 126, 291-299.

McFarlane, D., Cribb, A.E., 2005, Systemic and pituitary pars intermedia antioxidant capacity associated with pars intermedia oxidative stress and dysfunction in horses. American Journal of Veterinary Research 66, 2065-2072.

McGowan, C.M., Frost, R., Pfeiffer, D.U., Neiger, R., 2004, Serum insulin concentrations in horses with equine Cushing's syndrome: Response to a cortisol inhibitor and prognostic value. Equine Veterinary Journal 36, 295-298.

McGowan, T.W., Geor, R., Evans, H., Sillence, M., Munn, K., McGowan, C.M., 2008, Comparison of 4 assays for serum insulin analysis in the horse. Journal of Veterinary Internal Medicine 22, 115.

Mendez, J.D., Xie, J.L., Aguilar-Hernandez, M., Mendez-Valenzuela, V., 2010, Molecular susceptibility to glycation and its implication in diabetes mellitus and related diseases. Mol. Cell. Biochem. 344, 185-193.

Mobasheri, A., Critchlow, K., Clegg, P.D., Carter, S.D., Canessa, C.M., 2004, Chronic equine laminitis is characterised by loss of GLUT1, GLUT4 and ENaC positive laminar keratinocytes. Equine Veterinary Journal 36, 248-254.

Nishikawa, T., Edelstein, D., Du, X.L., Yamagishi, S., Matsumura, T., Kaneda, Y., Yorek, M.A., Beebe, D., Oates, P.J., Hammes, H.P., Giardino, I., Brownlee, M., 2000, Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404, 787-790.

Nourian, A.R., Asplin, K.E., McGowan, C.M., Sillence, M.N., Pollitt, C.C., 2009, Equine laminitis: Ultrastructural lesions detected in ponies following hyperinsulinaemia. Equine Veterinary Journal 41, 671-677.

Obel, N., 1948, Studies on the Histopathology of Acute Laminitis. Dissertation: Almqvist and Wiksells Boktryckeri A.B., Uppsala, Sweden.

Ramasamy, R., Vannucci, S.J., Yan, S.S., Herold, K., Yan, S.F., Schmidt, A.M., 2005, Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. Glycobiology 15, 16R-28R.

Ramasamy, R., Yan, S.F., Schmidt, A.M., 2010, Advanced glycation endproducts: from precursors to RAGE: round and round we go. Amino Acids Oct 19, Epub ahead of print.

Schmidt, A.M., Yan, S.D., Stern, D.M., 1995, The dark side of glucose. Nat Med 1, 1002-1004.

Schmittgen, T.D., Livak, K.J., 2008, Analyzing real-time PCR data by the comparative C-T method. Nature Protocols 3, 1101-1108.

Sick, E., Brehin, S., André, P., Coupin, G., Landry, Y., Takeda, K., Gies, J., 2010, Research paper: Advanced glycation end products (AGEs) activate mast cells. British Journal of Pharmacology 161, 442-455.

Siekmeier, R., Steffen, C., Marz, W., 2007, Role of oxidants and antioxidants in atherosclerosis: results of in vitro and in vivo investigations. J Cardiovasc Pharmacol Ther 12, 265-282.

Simm, A., Caßelmann, C., Schubert, A., Hofmann, S., Reimann, A., Silber, R.E., 2004, Age associated changes of AGE-receptor expression: RAGE upregulation is associated with human heart dysfunction. Experimental Gerontology 39, 407-413.

Singh, R., Barden, A., Mori, T., Beilin, L., 2001, Advanced glycation end-products: a review. Diabetologia 44, 129-146.

Takino, J., Yamagishi, S., Takeuchi, M., 2010, Cancer malignancy is enhanced by glyceraldehyde-derived advanced glycation end-products. J Oncol 2010, 739852.

Treiber, K., Carter, R., Gay, L., Williams, C., Geor, R., 2009, Inflammatory and redox status of ponies with a history of pasture-associated laminitis. Veterinary Immunology and Immunopathology 129, 216-220.

Visser, M., 2008. Investigation of proteolysis of the basement membrane during the development of equine laminitis. The University of Queensland, St Lucia, Brisbane.

Visser, M.B., Pollitt, C.C., 2011, Immunohistochemical Distribution of Laminin-332 and Collagen Type IV in the Basement Membrane of Normal Horses and Horses with Induced Laminitis. Journal of Comparative Pathology In Press, Corrected Proof.

Wattle, O., Pollitt, C.C., 2004, Lamellar metabolism. Clinical Techniques in Equine Practice 3, 22-33.

Wendt, T., Tanji, N., Guo, H.C., Hudson, B.I., Bierhaus, A., Ramasamy, R., Arnold, B., Nawroth, P.P., Yan, S.F., D'Agati, V., Schmidt, M., 2003, Glucose, glycation, and RAGE: Implications for amplification of cellular dysfunction in diabetic nephropathy. J. Am. Soc. Nephrol. 14, 1383-1395.

Yamagishi, S., Matsui, T., 2010, Advanced glycation end products, oxidative stress and diabetic nephropathy. Oxid Med Cell Longev 3, 101-108.

Yamagishi, S., Matsui, T., 2011, Advanced glycation end products (AGEs), oxidative stress and diabetic retinopathy. Curr Pharm Biotechnol 12, 362-368.

Yao, D., Brownlee, M., 2010, Hyperglycemia-induced reactive oxygen species increase expression of the receptor for advanced glycation end products (RAGE) and RAGE ligands. Diabetes 59, 249-255.

Tables

Table 1: Mean ± se age, bodyweight, body condition score (BCS) and median (range) serum insulin (µIU/mL) and blood glucose (mmol/L) concentration for five groups of Standardbred horses treated with either a glucose and insulin infusion for 6 h (*n* = 4), 12 h (*n* = 4), 24 h (*n* = 4), 48 h (*n* = 4) or with an electrolyte solution for 48 h (*n* = 4).

Parameters	***Control***	***6 h***	***12 h***	***24 h***	***48 h***
Age (years)	5.4 ± 0.6	9.0 ± 2.0	5.8 ± 1.8	7.3 ± 2.2	5.8 ± 1.3
Bodyweight (kg)	432 ± 14	428 ± 18	463 ± 40	429 ± 16	427 ± 34
BCS (1 – 9)	4.3 ± 0.25	4.5 ± 0.6	5 ± 0.4	4.5 ± 0.3	4.6 ± 0.25
Serum Insulin (µIU/mL)	9.4 (8.7 - 11.5) *	884 (498 - 1165)	1093 (883 - 1269)	918 (852 - 1123)	1080 (1028 - 1201)
Blood Glucose (mmol/L)	5.8 (5.4 - 6.4)	4.1 (3.5 - 4.3)	3.8 (3.5 - 4.4)	5.0 (4.0 - 5.9)	4.6 (4.0 - 5.6)

Key: * $p < 0.05$ compared with all other groups (Tukey's test)

Table 2: Primer sequences and concentrations (nM) for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) gene expression of the receptor for advanced glycation endproducts (RAGE) and glucose transporters (GLUT-1 and GLUT-4) in lamellar tissue from normal horses (*n* = 4) and horses in the developmental (24 h; *n* = 4) and acute (48 h; *n* = 4) stages of insulin-induced laminitis.

Gene	***Forward primer (5'-3')***	***Reverse primer (5'-3')***	***Amplicon size (bp)***	***Primer conc. (nM)***
β2-Microglobulin	ACCCAGCAGAGAATGGAAAGC	CATCTTCTCTCCATTCTTTAGCAAATC	101	600
GAPDH	GAT TGT CAG CAA TGC CTC CT	AAG CAG GGA TGA TGT TCT GG	193	500

RAGE	TTC TGA CTT GCT CCC ACT TTG TT	GTC TGC TTC TCT CTG ACC TTA TCC A	90	600
GLUT-1	CTTTGGCCGGCGGAAT	AAGGACTTGCCCAGTTTTGAGA	91	500
GLUT-4	TGCTATGGGTCCCTACGTCTTC	GAAACCCGAGGCCGGA	95	500

Table 3: Lamellar concentration of AGEs, MDA, and protein carbonyl (median, range) in five groups of Standardbred horses treated with either a glucose and insulin infusion for 6 h (*n* = 4), 12 h (*n* = 4), 24 h (*n* = 4), 48 h (*n* = 4) or with an electrolyte solution for 48 h (*n* = 4).

Parameters	***Control***	***6 h***	***12 h***	***24 h***	***48 h***
AGE (ng/mL)	1 (0.6 - 8.5)	4 (2.4 - 6.3)	4 (2.4 – 5.7)	2 (1.6 – 2.7)	596 (178 – 1020) *
MDA (pmol/mg)	0.31 (0.02 - 0.6)	0.07 (0 - 0.6)	0.15 (0.06 - 0.3)	0.56 (0.3 - 0.8)	0.9 (0.5 - 1)
Protein carbonyl (nmol/mg)	1.9 (1.4 - 2.3)	2.4 (2.1 - 3.6)	1.8 (1.3 - 3.6)	0.2 (0.01 - 0.3) *	1.5 (1.3 - 1.6)

Key: * $p < 0.05$ compared with all other groups (Tukey's test)

Table 4: GLUT-1, GLUT-4 and RAGE gene expression was analysed by qRT-PCR in horses treated with insulin and glucose for 24 h (*n* = 4), 48 h (*n* = 4) and control (*n* = 4) horses treated with an electrolyte solution. Median (range) relative gene expression of target genes to the reference gene β2-Microglobulin ($2^{-\Delta CT}$) is shown.

Subjects	***GLUT-1***	***GLUT-4***	***RAGE***
Control	1.02 (0.47 – 1.40)	5.32 (0.68 – 13.5)	0.05 (0.003 – 0.19)
Insulin 24h	6.92 (3.09 – 15.3)*	0.8 (0.58 - 6.06)	0.004 (0.0007 – 0.16)
Insulin 48h	3.65 (2.65 – 9.15)	4.39 (2.32 – 6.35)	0.01 (0.005 – 0.02)

Key: * Increased ($p > 0.05$) expression of the GLUT-1 gene compared to the other groups.