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ASSESSMENT OF BIOCHEMICAL MARKERS OF CONNECTIVE TISSUE METABOLISM IN HORSES WITH LAMINITIS

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Summary. This study aimed to evaluate biochemical changes in urine associated with mineral and connective tissue metabolism in horses diagnosed with laminitis, and to identify potential markers for early diagnosis and monitoring of this condition. Twenty horses participated in the study: 10 clinically healthy (control group) and 10 animals with laminitis. A clinical examination was conducted, assessing behavior, posture, response to palpation, and physiological indicators. Concentrations of calcium (Ca), inorganic phosphorus (P), uronic acids, hydroxyproline, and creatinine were determined in urine samples. To eliminate the influence of variations in urine output, the ratio of each indicator to the creatinine level was calculated. Animals with laminitis exhibited the following characteristic clinical signs: weight transfer to the hind limbs; shortened gait; lameness; increased pulsation of the palmar arteries; pain; and localized temperature increase in the hoof area. Biochemical analysis of the urine revealed significant increases in calcium (+31.1%), phosphorus (+78.8%), uronic acids (+49.4%), and hydroxyproline (+50.6%) levels ($p < 0.001$). The Ca/P ratio decreased by 24% ($p < 0.001$). Relative indicators also increased: Ca/creatinine (30.2%), P/creatinine (75.0%), uronic acids/creatinine (47.4%), and hydroxyproline/creatinine (50.0%). The obtained data showed that laminitis in horses is accompanied by local and systemic metabolic disorders. Urine markers and their creatinine ratios can be used as additional, sensitive indicators of laminitis severity and treatment effectiveness

Keywords: horses, urine, calcium, phosphorus, hydroxyproline, uronic acids, creatinine

Introduction. Metabolic disorders of lipid metabolism in horses are often observed alongside internal diseases and can act as a marker of disease as well as an independent risk factor for complications (Bertin, 2023; Durham et al., 2019). These disorders accompany endocrine dysfunction, hepatogenic pathologies, and chronic inflammatory processes, and also occur in a state of negative energy balance (Perez-Ecija et al., 2021).

Laminitis is one of the most common and clinically significant manifestations of metabolic disorders in horses (Morgan, Keen, and McGowan, 2015). It is one of the most complex orthopedic pathologies in horses, involving damage to the laminar apparatus of the hoof and leading to significant functional disorders, prolonged lameness, and loss of working capacity. It is characterized by inflammation and degenerative changes in the connective tissue structures that attach the hoof horn to the distal phalanx, creating a risk of irreversible anatomical deformities (De Laat et al., 2019; Luomala, 2022). Key risk factors for laminitis include carbohydrate metabolism disorders, insulin resistance, obesity, systemic inflammatory conditions, and technological disorders in the feeding and housing of animals (De Laat et al., 2016). The mechanisms of laminitis development are multifactorial, including vascular, endocrine, and immunoinflammatory components, as well as an imbalance in the matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), which leads to the destruction of the basal membrane of the hoof apparatus (Clutterbuck et al., 2010).

Early diagnosis is particularly challenging because clinical manifestations typically emerge when significant tissue damage has already occurred. Therefore, it is important to study molecular and biochemical markers that would enable metabolic disorders of connective tissue to be detected in time, even before clinical symptoms develop. Promising biomarkers include extracellular matrix degradation, particularly focal adhesion proteins (talin 1, vinculin, and cadherin 13), glycoproteins (α2-macroglobulin), and heat shock proteins (HSP90), as well as components of the complement and blood coagulation systems (Campolo et al., 2020; Espinosa-López et al., 2025). Despite significant progress in studying the pathophysiology of laminitis, many questions remain regarding the mechanisms of extracellular matrix damage and the role of systemic metabolic disorders in developing this pathology. Further research in this area is necessary to improve early diagnosis and develop effective approaches to the prevention and treatment of laminitis (Serteyn et al., 2024).

In clinical practice, assessing the lipid profile (including triglycerides, cholesterol, free fatty acids, HDL, LDL, and VLDL lipoproteins) is becoming increasingly important. However, as Zemek et al. (2024) have noted, even in animals with obvious clinical abnormalities, deviations from reference values are not always recorded. This highlights the need for greater attention to be paid to interpreting such indicators in combination with clinical symptoms.

Hyperlipidemia can develop in ponies and miniature horse breeds even with moderate stress or energy deficiency, causing particular clinical concern (De Laat et al., 2016).

Disorders of lipid metabolism in horses and ponies commonly accompany internal diseases, particularly metabolic syndrome, endocrinopathies, and liver pathologies. At the same time, hypertriglyceridemia can complicate the underlying disease and have independent clinical significance. Some animals, especially ponies, quickly develop dyslipidemic changes in response to stress or energy deficiency that remain unnoticed without timely diagnosis (Bertin, 2023).

Although methods for assessing lipid metabolism in horses are available, they are not widely used in veterinary practice. The absence of systematic reference values and diagnostic algorithms, as well as the limited number of studies, makes the clinical interpretation of results difficult (Zemek, Kemp and Bertin, 2024). This is why studying lipid metabolism in normal and pathological conditions is relevant and has practical significance for improving diagnostic approaches.

This study **aimed** to evaluate biochemical changes in urine associated with mineral and connective tissue metabolism in horses diagnosed with laminitis, and to identify potential markers for early diagnosis and monitoring of this condition.

Materials and methods. The study was conducted on two groups of horses: Group I comprised clinically healthy animals (n = 10), while Group II comprised animals with a confirmed diagnosis of laminitis (n = 10). All animals underwent a clinical examination, including an assessment of their general condition and measurement of basic physiological parameters, as well as an examination involving palpation, percussion, and auscultation of organs and systems. A comprehensive diagnosis of laminitis and associated metabolic disorders was made based on anamnestic data, characteristic clinical changes, and laboratory test results, by the recommended criteria (Geor, 2009).

The selection of biochemical indicators for study was based on the pathogenetic mechanisms of laminitis. Determining calcium and phosphorus concentrations in urine, as well as their ratio (Ca/P), enabled us to evaluate disorders of mineral metabolism, which are crucial for bone and connective tissue remodeling. Uronic acid and hydroxyproline are sensitive markers of extracellular matrix and collagen degradation, while creatinine levels reflect kidney function, enabling other indicators to be adjusted for concentration capacity.

Urine samples were collected during physiological urination in dry sterile containers. After collection, the biomaterial was immediately delivered to the laboratory for biochemical analysis. The following indicators were determined in urine: hydroxyproline, which was assessed using a spectrophotometric method after acid hydrolysis. This method is based on the formation of a colored complex involving chloramine T and para-dimethylaminobenzaldehyde. This allowed for a quantitative assessment of the hydroxyproline content at a wavelength of 540 nm. This method has been adapted for use in veterinary practices and is recommended in studies of collagen metabolism (McIlwraith et al., 2018). The level of glycosaminoglycans in biological fluids was quantitatively assessed using a modern modification of the classic carbazole method, which allowed for the determination of uronic acids via photometry (Zhang et al., 2016).

The calcium concentration was determined using a photometric method with an arsenazo III reagent. At a pH of 6.5, the reagent forms a colored complex with calcium, enabling an accurate measurement of calcium content in urine (Morozenko and Leontieva, 2016).

Inorganic phosphorus was studied using a photometric method based on the formation of a phosphomolybdenum complex, which in the presence of a reducing agent (ascorbic acid) acquires an intense blue color with a maximum absorption at a wavelength of 700–720 nm (Morozenko and Leontieva, 2016; Baylor, Chandler and Marshall, 1982).

Creatinine concentration was determined by the photometric method using the classic Jaffe reaction with a standard set of reagents produced by Reagent PJSC (Ukraine), which is widely used in veterinary laboratory diagnostics (Morozenko and Leontieva, 2016).

To improve the accuracy of the interpretation of the results, the ratio of the studied substances to the creatinine concentration was calculated: calcium/creatinine, phosphorus/creatinine, uronic acids/creatinine, and hydroxyproline/creatinine, which reduced the influence of daily diuresis variability and is widely recognized in connective tissue metabolism studies.

The research was conducted following the recommendations of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and under Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic bioethical principles (Simmonds, 2017). Under the current procedure, the research program was reviewed and approved by the Bioethics Committee of the State Biotechnological University (Kharkiv, Ukraine).

The results were processed by methods of variation statistics. To compare mean values Student's *t*-test was used (Van Emden, 2019).

Results and discussion. A clinical examination revealed a significant difference in behavior between horses diagnosed with laminitis and those that were clinically healthy. Healthy horses exhibited active behavior and a symmetrical posture, as well as a natural gait with an even distribution of body weight across all limbs. Their pulse and respiratory rates, body temperature, hoof condition, and response to palpation were all within physiological limits (Wylie et al., 2016). In contrast, horses with laminitis exhibited characteristic clinical symptoms caused by pain and inflammation in the hoof area. During the examination, an altered posture was observed: the front limbs were extended forwards and the hind limbs were tucked under the torso. This is a typical reaction designed to reduce the

load on the affected areas. Limited motor activity, lameness, and a cautious, tense gait were observed, which worsened when changing direction. In some cases, forced immobility was observed (Pollitt, 2010; Van Eps, Collins and Pollitt, 2010). Palpation revealed an increase in local hoof wall temperature, pronounced pain in the coronet area, and, when pressure was applied to the sole. The pulsation of the palmar artery was intensified, further indicating the development of an inflammatory process (Belknap and Geor, 2012). Some animals showed general depression, an increased body temperature (up to 38.9–39.4 °C), tachycardia (up to 52–58 beats/min), and rapid breathing (up to 24–28 breaths/min), indicating a systemic response to inflammation. These indicators remained stable in healthy horses: body temperature was within the range of 37.5–38.2 °C; heart rate was 28–40 beats/min; and respiratory rate was 10–14 breaths/min. Due to severe pain, percussion examination was limited, but in acceptable cases, a change in sound conductivity in the sole area was recorded. This indirectly indicated soft tissue edema or displacement of the coffin bone (Orsini, 2014).

Urine analysis revealed changes in the metabolic status of horses with laminitis, showing significant biochemical parameter deviations compared to clinically healthy animals (Table 1).

Table 1 — Results of biochemical studies of horse urine

Indicator	Animal group		Δ
	I clinically healthy (n = 10)	II with laminitis (n = 10)	
Ca, mg/l	136.8 ± 3.0	179.4 ± 2.5	+42.6***
P, mg/l	27.4 ± 1.3	49.0 ± 2.9	+21.6***
Ca/P	5.0 ± 0.2	3.8 ± 0.2	−1.2***
Uronic acids, mg/l	8.3 ± 0.4	12.4 ± 0.6	+4.1***
Hydroxyproline, mg/l	31.4 ± 1.4	47.3 ± 2.1	+15.9***
Creatinine, mmol/l	8.7 ± 0.3	8.7 ± 0.3	0.0

Note: *** — $p < 0.001$.

Biochemical analysis of the urine of horses with clinically confirmed laminitis revealed systemic changes in their metabolic status, which differed significantly from the parameters observed in healthy animals. The obtained data indicated active disruption of mineral and connective tissue metabolism, likely to be both localized to the hoof and systemic. The concentration of calcium in the urine of animals with laminitis was significantly higher (by 31.1%) than in clinically healthy horses ($p < 0.001$). This hypercalciuria suggests a reduction in tubular calcium reabsorption efficiency or an increase in filtration due to endocrine changes associated with the inflammatory process. It is known that, in response to chronic inflammation (including laminitis), osteolysis mechanisms are activated and parathyroid hormone levels increase. This stimulates the mobilization of calcium from bone tissue and enhances its excretion by the kidneys (Alexander, Fuster and Dimke, 2022; Etemadi et al., 2023; Messa et al., 2023). Additionally, at the local level, the destruction of calcium-containing structures in affected tissues may further contribute to increased calcium levels in biological fluids.

At the same time, a significant increase in inorganic phosphorus levels was observed, rising by 78.8% ($p < 0.001$). This phosphaturia may be due to the inhibition of phosphate reabsorption in the proximal tubules and hormonal imbalances, particularly increased FGF23 activity and decreased calcitonin sensitivity (Diniz, 2013; Tsuboi et al., 2020). Excessive phosphorus excretion in urine during laminitis reflects profound disturbances in mineral homeostasis, which are often associated with signs of secondary hyperparathyroidism.

These changes, taken together, contributed to a shift in the Ca/P ratio in urine. In the control group, the ratio was 5.0 ± 0.2; in the sick animals, it was 3.8 ± 0.2 (a difference of −24.0%; $p < 0.001$). A decrease in this ratio is considered a more sensitive indicator of mineral metabolism disorders than absolute values of individual macronutrients. It may result from changes in hydroxyapatite crystal formation, decreased osteogenesis, and bone tissue resorption. Similar dynamics are also associated with the dysfunction of vitamin D-dependent regulation of phosphorus-calcium metabolism (Hans and Levine, 2024).

Additionally, a substantial 49.4% ($p < 0.001$) increase in uronic acid content was observed in the urine of horses with laminitis, indicating the activation of glycosaminoglycan degradation processes in the intercellular substance. Uronic acids are components of sulphated mucopolysaccharides, and excessive excretion of these acids indicates the destruction of the structural matrix of connective tissue. This matrix plays a key role in the formation and maintenance of the functional state of the hoof horn (Williams, Anastasopoulou and Sapra, 2024).

Hydroxyproline, a stable fragment of degraded collagen, was also significantly elevated in the urine of animals with laminitis, increasing by 50.6% ($p < 0.001$). This confirmed the presence of active collagen breakdown in hoof tissues, consistent with the morphological changes described in chronic laminitis, particularly lamellar layer disintegration and ground substance edema (Kaneko, Harvey and Bruss, 2008; Torres Sánchez et al., 2024). Therefore, uronic acids and hydroxyproline can be considered early biochemical markers of connective tissue destruction in this condition.

Creatinine levels remained stable in both groups (8.7 ± 0.3 mmol/l; $p > 0.05$), indicating no significant impairment of renal filtration function. This enabled us to interpret changes in other parameters as reflecting systemic inflammation and metabolic disorders rather than nephropathy (Kamińska et al., 2020).

To reduce the impact of variations in daily urine volume, animal hydration status, and glomerular filtration rate, relative indices were used, i.e., the ratios of key biochemical markers to creatinine levels (Table 2). This approach is widely used in clinical biochemistry because, as a stable end product of muscle metabolism that is filtered exclusively by the glomeruli and not reabsorbed in the renal tubules, creatinine serves as a reliable internal standard (Onwuka et al., 2021).

Table 2 — Ratio of biochemical indicators in urine to creatinine level

Indicator	Animal group		Δ
	I clinically healthy (n = 10)	II with laminitis (n = 10)	
Ca/creatinine	15.9 ± 0.5	20.7 ± 0.7	+4.8***
P/creatinine	3.2 ± 0.2	5.6 ± 0.3	+2.4***
Uronic acids/ creatinine	0.97 ± 0.07	1.43 ± 0.06	+0.46*
Hydroxyproline/ creatinine	3.6 ± 0.1	5.4 ± 0.2	+1.8***

Notes. *** — $p < 0.05$; *** — $p < 0.001$.

Our study found that in horses with laminitis, the calcium-to-creatinine ratio (Ca/creatinine) increased significantly, rising from an average of 15.9 ± 0.5 in the healthy group to 20.7 ± 0.7 (an increase of 30.2%; $p < 0.001$). This indicates increased calcium excretion relative to baseline renal filtration function, and could result from impaired endocrine control of calcium homeostasis (in particular, parathyroid hormone activation), as well as osteolysis due to chronic inflammation.

A similar pattern was observed for the P/creatinine ratio. Its level in sick animals was 5.6 ± 0.3, which is 75% higher than in healthy horses (3.2 ± 0.2; $p < 0.001$). This phosphaturia, accompanied by a preserved filtration function, also indicates the activation of tissue demineralization processes that accompany laminitis (Kanepa et al., 2024).

The uronic acid/creatinine and hydroxyproline/ creatinine ratios were particularly valuable for diagnosis. In animals with laminitis, the uronic acid/creatinine ratio increased to 1.43 ± 0.06, compared to 0.97 ± 0.07 in the control group (+47.4%; $p < 0.05$). The hydroxyproline/ creatinine ratio increased to 5.4 ± 0.2, compared to 3.6 ± 0.1 in healthy animals (+50.0%; $p < 0.001$). These changes indicated intense connective tissue degradation and intercellular matrix catabolism activation, particularly of glycosaminoglycans and collagen. The obtained indicators reflected deep degenerative-inflammatory processes in hoof tissue accompanied by the destruction of the basal lamina, the disorganization of the lamellar structure, and the formation of fibrosis (Galantino-Homer and Brooks, 2020; Paes, Carino and Aguiar, 2024; Belknap and Geor, 2016; Pollitt, 2019).

Calculating the ratio of the concentrations of the studied markers to the amount of creatinine in the urine reduced the influence of physiological variations, ensuring a more accurate interpretation of the results. This approach provides a more accurate and reproducible assessment of calcium, phosphorus, uronic acid, and hydroxyproline excretion levels since creatinine is filtered exclusively in the glomeruli, not reabsorbed in the tubules, and is a stable marker of kidney function and urine concentration capacity (Kamińska et al., 2020; Onwuka et al., 2021).

Using relative indicators avoids errors associated with accidental dilution or concentration of urine. This method is widely recognized in human medicine for monitoring metabolic disorders, bone tissue disorders, fibrosis, and osteolysis (Kanepa et al., 2024; Williams, Anastasopoulou and Sapra, 2024). While these methods are not yet routine in veterinary medicine, they have high potential for implementation in diagnostic practices, especially for cases involving systemic connective tissue and kidney damage, as well as mineral metabolism disorders.

Thus, the results of the study confirmed that horses with laminitis experience profound and complex systemic disorders that affect mineral and connective tissue metabolism. These disorders manifested as a significant increase in the absolute concentrations of calcium, phosphorus, uronic acids, and hydroxyproline in urine, accompanied by a corresponding increase in their ratios to creatinine. The increase in these markers' relative values allowed for a more objective assessment of pathological processes, regardless of physiological fluctuations in urine volume or the kidneys' functional state.

The detected changes indicated not only local damage to the hoof tissue, including degradation of the extracellular matrix and destruction of the basement membrane and lamellar architecture, but also a systemic metabolic imbalance resulting from chronic inflammation. Disruption of mineral homeostasis and breakdown of connective tissue components in laminitis are not isolated, local reactions; rather, they are a complex, multi-level pathology affecting the skeletal, endocrine, and extracellular systems (McGrath et al., 2024, Belknap and Geor, 2016; Pollitt, 2019; Torres Sánchez et al., 2024).

The obtained indicators can serve as sensitive additional diagnostic markers for evaluating the activity and severity of laminitis, detecting systemic complications early, and monitoring the effectiveness of therapeutic and preventive interventions. Using them in conjunction with clinical, morphological, and instrumental methods can significantly enhance the informative value of a comprehensive diagnosis and monitoring of laminitis in horses.

The detected changes can be used as additional laboratory markers to comprehensively diagnose and monitor laminitis (Paes, Carino and Aguiar, 2024; Patterson Rosa et al., 2020; Belknap and Geor, 2016;

Pollitt, 2019), which indicates a systemic metabolic imbalance (Hans and Levine, 2024).

Markers such as uronic acids and hydroxyproline provide information about the extent of degradation of the connective tissue component, as confirmed by foreign and domestic studies (Torres Sánchez et al., 2024; Xenoulis, Steiner and Monnet, 2023).

Conclusions. 1. A clinical examination of horses with laminitis revealed the following symptoms: a forced posture with unloading of the front limbs; a cautious and shortened gait; lameness, which was especially noticeable when turning; local hyperthermia of the hooves; pain in the coronet area; and increased pulsation of the palmar arteries. Some animals showed systemic manifestations, such as hyperthermia, tachycardia, and polypnea. These signs confirmed the development of an acute pain syndrome and an inflammatory response, which are characteristic of the clinical course of laminitis.

2. Biochemical analysis of urine in horses with laminitis revealed a significant increase in calcium (+31.1%), inorganic phosphorus (+78.8%), uronic acids (+49.4%), and hydroxyproline (+50.6%) compared to clinically healthy animals ($p < 0.001$), indicating impaired mineral and connective tissue metabolism.

3. The Ca/P ratio in the urine decreased by 24% ($p < 0.001$), which indicates an imbalance between calcium and phosphorus, impaired mineralization processes, and increased bone resorption.

4. Calculating the ratios of biomarkers to creatinine concentration in urine (Ca/creatinine, P/creatinine, uronic acids/creatinine, and hydroxyproline/creatinine) provided a more accurate interpretation of the results. This method reduced the influence of individual variations in urine output and allowed for an objective assessment of the degree of metabolic disorders in laminitis.

5. An increase in the relative values of uronic acids and hydroxyproline to creatinine indicates the activation of glycosaminoglycan and collagen degradation in the extracellular matrix structures of hoof tissue. This confirms the development of a destructive inflammatory process in connective tissue.

6. The obtained biochemical urine parameters can be used as additional laboratory markers for the comprehensive diagnosis of laminitis. They can also be used to stratify the severity of the disease, detect systemic complications early, and monitor the effectiveness of therapeutic measures.

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