Veterinary Radiology & Ultrasound

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ORIGINAL RESEARCH OPEN ACCESS

Renal Perfusion in Healthy Horses: A Feasibility Study With Contrast-Enhanced Ultrasound

Diana Villa Verde Salazar[1] | Marília Alves Ferreira[1] | Pedro Henrique Salles Brito[1] | Ricardo Andrés Ramirez Uscategui[2] | Anna Carolina Mazeto Ercolin[1] | Gabriela Castro Lopes Evangelista[1] | Luiz Paulo Nogueira Aires[3] | Luciano Andrade Silva[1] | Marcus Antônio Rossi Feliciano[1]

[1]Departamento De Medicina Veterinária, Faculdade de Zootecnia e Engenharia De Alimentos, Universidade De São Paulo – FZEA/USP, Pirassununga, São Paulo, Brazil | [2]HVUT, Facultad De Medicina Veterinaria y Zootecnia, Universidad Del Tolima, Ibagué, Colombia | [3]Departamento De Clínica e Cirurgia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio De Mesquita Filho" – FCAV/Unesp, Jaboticabal, São Paulo, Brazil

Correspondence: Diana Villa Verde Salazar (dsalazar.vet@gmail.com)

Received: 29 January 2025 | **Revised:** 17 April 2026 | **Accepted:** 9 May 2026

Keywords: equine | microbubbles | senescence | urinary | vascular

ABSTRACT

This study assessed the feasibility of contrast-enhanced ultrasound (CEUS) for evaluating renal perfusion in horses and compared perfusion parameters between age groups. In a prospective study, eight healthy horses were divided into young (3–5 years, $n = 4$) and old (16–17 years, $n = 4$) groups. All animals underwent B-mode, Doppler ultrasonography, and CEUS of the right kidney after an intravenous bolus of SonoVue (0.02 mL/kg). Qualitative analysis assessed contrast kinetics, whereas quantitative parameters (peak intensity, time to peak [TP], area under the curve, and wash-out time) were obtained from time–intensity curves in the renal cortex and medulla. The technique was safely performed in all horses, and the 0.02 mL/kg dose proved optimal for achieving adequate renal enhancement and consistent quantitative measurements. Quantitative analysis revealed a significantly longer TP in the renal cortex of older horses compared to young ones ($p = 0.03$), suggesting an age-related reduction in perfusion velocity. No other parameters showed significant differences. CEUS proved to be a feasible and safe method for assessing renal perfusion in horses. The prolonged cortical TP in older animals indicates that CEUS may be a sensitive tool for detecting early, subclinical perfusion changes associated with aging, warranting further investigation in horses with renal disease.

1 | Introduction

The kidneys are vital organs for maintaining homeostasis, performing essential functions such as regulating water balance, eliminating metabolic waste, and controlling blood pressure, as well as performing endocrine functions, including the production of erythropoietin [1, 2]. Renal function is directly associated with blood flow, which corresponds to approximately 22% of the cardiac output, which is fundamental for maintaining the glomerular filtration rate [3].

Contrast-enhanced ultrasound (CEUS) is an imaging modality that assesses organ perfusion with special detail on tissue microvascularization. This exam uses contrasts made for gas microbubbles wrapped in a phospholipid layer. The main characteristics of these microbubbles are that they are smaller than a red blood cell, allowing them to pass through the smallest capillaries, are mostly eliminated by the lungs, which reduce the risks for patients with renal and hepatic impairment, and interact with the phospholipid layer to improve the stability of the molecule. This exam has been described for horses in a few reports of evaluation of placental–fetal relationship [4], tendons [5], and cornea [6]. It is an imaging technique that has some advantages over laboratory tests, allowing real-time assessment of the organ's microvascularization. It is shown to enable early detection of real lesions in mice [7], dogs [8], and cats [9]; however, to our knowledge, there are no reports yet of its applicability in horses.

Aging is known to cause significant changes in the kidneys, which are thought to be one of the organs most affected by the senescence process. Studies in humans have documented structural and functional changes associated with age, including loss of vascular tone, increased predisposition to vasoconstriction, reduced ability to adapt to systemic insults, and progressive loss of nephrons. These changes favor primary arterial disease, ischemia, inflammation, and the development of renal fibrosis, resulting in progressive impairment of renal function [10].

Although these changes have been widely described in humans, studies in horses are still scarce; the few available sources describe a higher incidence of kidney damage in older horses [11], suggesting that similar physiological and structural changes may occur in this species. Additionally, researchers pointed out that, in a hospital environment, acute kidney damage can reach between 14.8% [12] and 23% of horses [13].

The equine species has several conditions throughout its life that impact these organs and can lead to injuries that are difficult to monitor. One of the most common conditions is colic, which alters water–electrolyte balance, resulting in systemic alterations such as dehydration, endotoxemia, and septicemia [14]. In addition, some of these animals need to undergo emergency anesthetic and surgical procedures to correct displacement colic, torsions, incarcerations, and intestinal or gastric impactions that are unresponsive to clinical treatment, representing further insults to renal function. Acute kidney injury has already been described in this type of scenario [12, 15], in cases of ophidian accidents [16], and in foal disorders [17, 18]. Acute renal injuries are also a possibility in any type of acute condition in which there is major systemic decompensation related to the circulation and pressure of these animals.

Due to the importance of the kidneys for homeostasis and general health, this study aimed to demonstrate the feasibility of the CEUS technique for kidney assessment in horses, to establish normal values for CEUS evaluation in young and old horses, and to compare the CEUS variables between young and older horses.

2 | Materials and Methods

2.1 | Study Design and Animals

This study was approved by the Institutional Animal Care and Use Committee of the Faculty of Animal Science and Food Engineering (FZEA/USP) (protocol no. 4919090523).

This is a prospective feasibility study where a total of eight horses from the equine breeding sector of the Faculty of Animal Science and Food Engineering at the University of São Paulo were selected and divided into two experimental groups on the basis of age. One group consisted of four young animals ranging from 3 to 5 years old, and the other group consisted of four older animals ranging from 16 to 17 years old. The animals weighed 250–400 kg, and the volume of contrast was adjusted according to the body weight of each animal. As inclusion criteria, physical exam, urinalysis, complete blood count, and renal biochemical profile, as well as B-mode and Doppler imaging of the kidneys, were carried out beforehand to check the animals' health. Systolic blood pressure was not measured in this study. Animals presenting actual or historical renal alterations, dehydration, or medications in the last 15 days or infectious or inflammatory processes were excluded. Exclusion criteria were extended to animals with highly agitated behavior or excessive motion that could interfere with the image acquisition process.

2.2 | Diagnostic Imaging

All imaging tests were carried out using the MyLab Omega device (Esaote, Genova, Italy) and a convex electronic transducer with a frequency of 1–8 MHz. The right kidney was chosen for image acquisition because it was easier to localize using ultrasound. During image acquisition, the animals were restrained in a horse stock and had the right flank and right jugular region clipped. A 14G catheter was implanted in the jugular vein and secured with a no. 0 surgical nylon thread, under aseptic conditions. The horses were weighed and had their physical parameters rechecked (temperature, heart rate, respiratory rate, and aspect of mucous membranes). B-mode ultrasound was then carried out using acoustic gel. All adjustments were made to acquire the best image, as described below.

In B-mode ultrasound, renal structures were assessed in terms of topography, echotexture, echogenicity, size, and contours. Color Doppler ultrasound was then used to evaluate the architecture and integrity of the renal vasculature. Once the renal vascular structures had been identified, a pulsed Doppler was activated, and Doppler fluxometric analysis was performed, using a sampling window of 2–3 mm positioned in the central area of the interlobar arteries. Pulse repetition frequency was set to the lowest possible without generating image artifacts; Doppler gain was adjusted for better image quality according to each exam, and the wall filter was set to 50 Hz. The resistance index (RI) was obtained automatically by ultrasound as described in the literature [19–21].

2.3 | Contrast-Enhanced Ultrasound

The CEUS evaluation was performed following Doppler ultrasound, in the right kidney (due to the ease of evaluation), using contrast-specific software with a harmonic image secondary to the inverse pulse technique. Immediately after centralizing the image of the longest axis of the kidney in the longitudinal section on the screen, the transducer was held steadily in position, and the harmonic image was activated. Acoustic power, gain, depth, dynamic range, frequency, and focus were optimized before the evaluation, aiming to guarantee excellent image quality, and were kept constant throughout the experiment.

SonoVue was used as an ultrasound contrast (Bracco, São Paulo, Brazil), a sulfur hexafluoride-filled microbubble-based contrast media. To determine the adequate dosage to be used in this

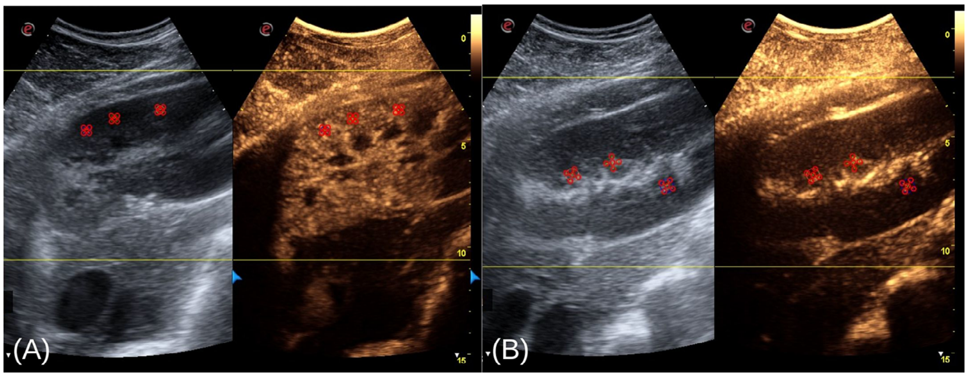

FIGURE 1 | Contrast-enhanced ultrasound of the right kidney in a horse, displayed in dual screen mode with B-mode images (left panels) and corresponding contrast-mode images (right panels). (A) Images acquired during the cortical phase, shortly after intravenous administration of an ultrasound contrast agent, with red crosshairs indicating regions of interests (ROIs) positioned within the renal cortex for quantitative perfusion analysis. (B) Images obtained during the medullary phase, with ROIs placed in the renal medulla to evaluate its perfusion.

study, the first animal was evaluated in a pre-pilot study with doses of 0.01, 0.02, and 0.03 mL/kg, consecutively. The time between each evaluation was established by the lack of visible contrast in the kidney and adjacent tissue. The 0.02 mL/kg contrast dose showed excellent-quality images, allowing clear visualization of the vascular flow and a clear analysis of the tissue perfusion pattern. Although the 0.03 mL/kg dose did not bring any additional benefits, it maintained good image quality. On the other hand, the 0.01 mL/kg dose resulted in images with poor intensity and definition, especially in regions with naturally lower perfusion, such as the renal medulla.

Then, in the main study, 0.02 mL/kg of contrast was injected as a bolus in approximately 1–2 s, and after a 20 mL contrast injection, 0.9% saline was injected as flushing. The moment of the application corresponded to T0. At this point, the video recording was initiated, and a time-lapse of at least 180 s was recorded for later analysis. The qualitative characteristics of the renal vasculature were assessed by the same operator in all exams at the moment of examination. The wash-in time, peak enhancement time, and wash-out time (WOT) were estimated, as described in the literature [22–24].

After the examination of the images, the video recordings were analyzed for quantitative parameters using the Q-pack software (Esaote, Genova, Italy). Regions of interests (ROIs) were established to automatically create time–intensity graphs. Analyses were carried out with two types of ROIs measuring 0.1 or 0.3 mm^2. For each ROI size, duplicate or triplicate measurements were randomly made at the same location of renal cortical and medullary regions as represented in Figure 1, avoiding regions of large vessels (very bright areas in the image) that could interfere with the quantitative evaluation. The Q-Pack software calculated each of these ROIs: The peak intensity (PI in %), the time to peak intensity (TP in s), the area under the curve (AUC), and the WOT (in s) from the time–intensity curves generated for renal cortical and medullary areas.

2.4 | Statistical Analysis

The analyses were carried out using R software (The R Foundation for Statistical Computing, Vienna, Austria). Initially, the repeated measurements of each ROI and the measurements of ROIs of each area (0.1 and 0.3 mm^2) were compared to determine the variation of the technique using the Friedman test with an arrangement to evaluate the two factors (ROI size and ROI repetitions). If this test showed similarity between ROI´s repeated measurements, the medians of the cortical and medullary ROI evaluation were used for subsequent evaluations. Later, the CEUS median measurements obtained for cortical and medullary evaluation were compared between younger and older horses using the Mann–Whitney *U* test. Finally, descriptive statistics, the median (interquartile range—IQR), the coefficient of variation, and the 95% confidence interval of the median were calculated to present the data results for each group, and the significance for all statistical tests was set at $p < 0.05$.

3 | Results

During data collection, no adverse reactions were observed in the horses studied, and the evaluations could be carried out successfully; only sudden movements brought some difficulties in the evaluation in very few cases. The right kidney could be evaluated in all horses of this study, without difficulties, between the 15th and 18th intercostal spaces. All the evaluated individuals presented normal echotexture, echogenicity, cortical/medullary ratio, and contours. The kidneys of the present study had a median (IQR) length of 13.3 (2.9) cm in young and 14.3 (1.0) cm in old horses, width of 5.6 (0.7) cm in young and 6.4 (0.5) cm in old horses, color Doppler evaluations were apparently normal in all horses, and the median (IQR) of RI was 0.5 (0.1) in young and 0.5 (0.1) in old horses.

3.1 | CEUS Qualitative Assessment

The qualitative evaluation of the images enabled the assessment of the renal microbubble transit, renal vascular filling, cortical and medullary contrast enhancement, and the subjective comparison among the age groups. Initially, contrast enhanced the renal artery, passing to the interlobar arteries, and enhanced the renal cortex in 19 (8.3) s in young horses and in 25 (13) s in old horses (qualitative wash-in time). Subsequently, there was a decrease in the cortical enhancement as the contrast reached the medullary

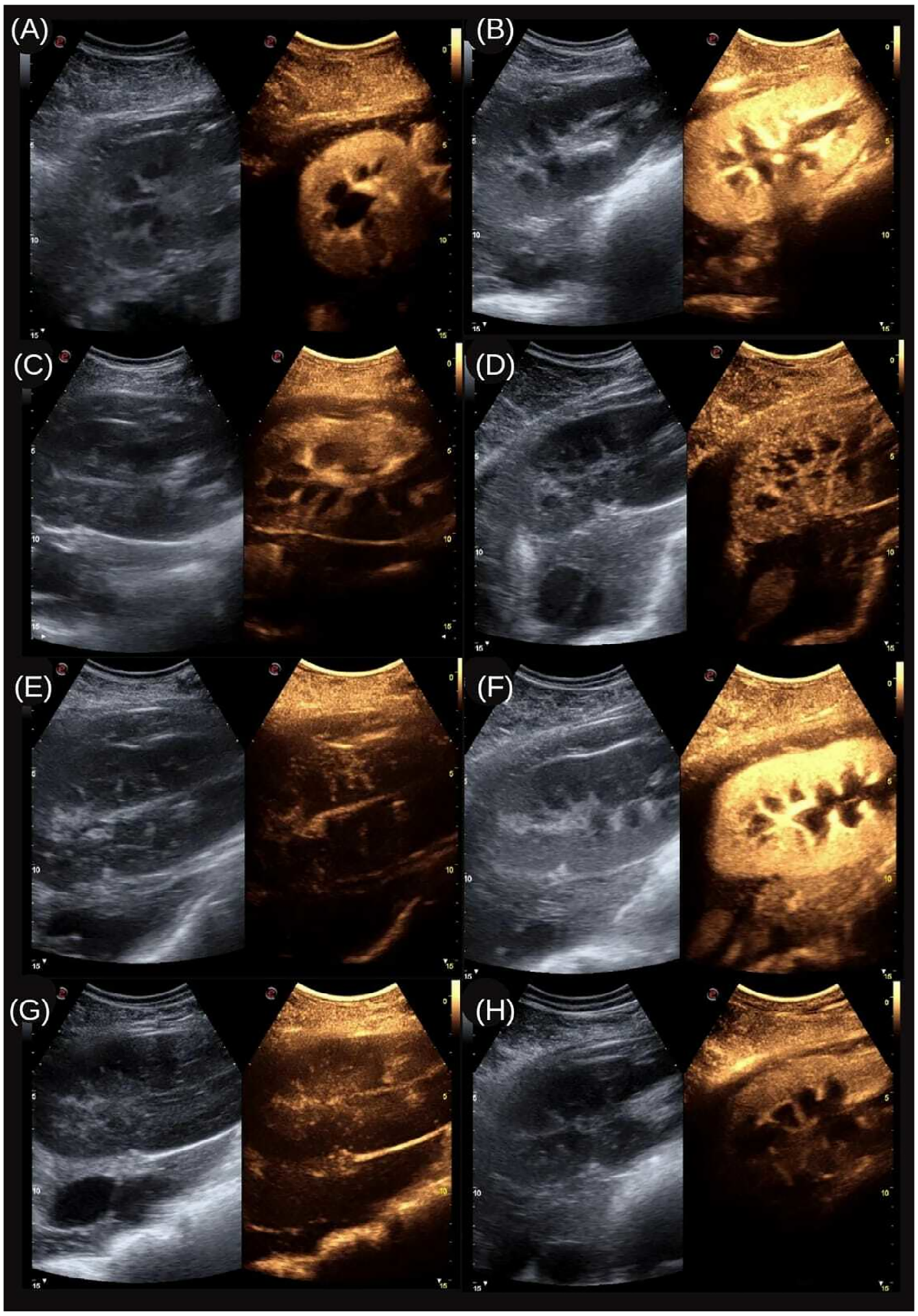

FIGURE 2 | B-mode and contrast-enhanced ultrasound images of the equine right kidney at the peak of contrast for the young and old groups. (A), (C), (E), and (G) correspond to animals of the old group, presenting 16, 16, 17, and 16 years, respectively. (B), (D), (F), and (H) correspond to animals of the young group, with 3, 3, 3, and 5 years, respectively. It is possible to identify a greater intensity peak in the younger group compared to the older animals, and there is a variation in intensity within the groups.

region. Contrast was less intense in the medullary region than in the renal cortex. The medullary wash-in time was 22 (4.8) s in young horses and 27 (11) s in old horses; finally, contrast leaves the medullary region of the kidney and disappears from the organ in approximately 3 min in all animals (Video S1).

Older animals in qualitative evaluation tend to present less intensity and slower perfusion when compared to the younger animals, as shown in Figure 2. Another interesting factor was related to tissue depth: the regions of the kidney in the near field of view showed better contrast intensity than the regions in the far field. There were also observed visual differences in the intensity of contrast uptake between animals of the same groups. For the younger group, Figure 2B,F presents higher visual cortical intensity than the kidneys in Figure 2D,H. For the older group, Figure 2A,C presents higher visual intensity of contrast at the renal cortex compared to Figure 2E,G. Despite these individual variations in overall enhancement, all anatomical ROIs remained

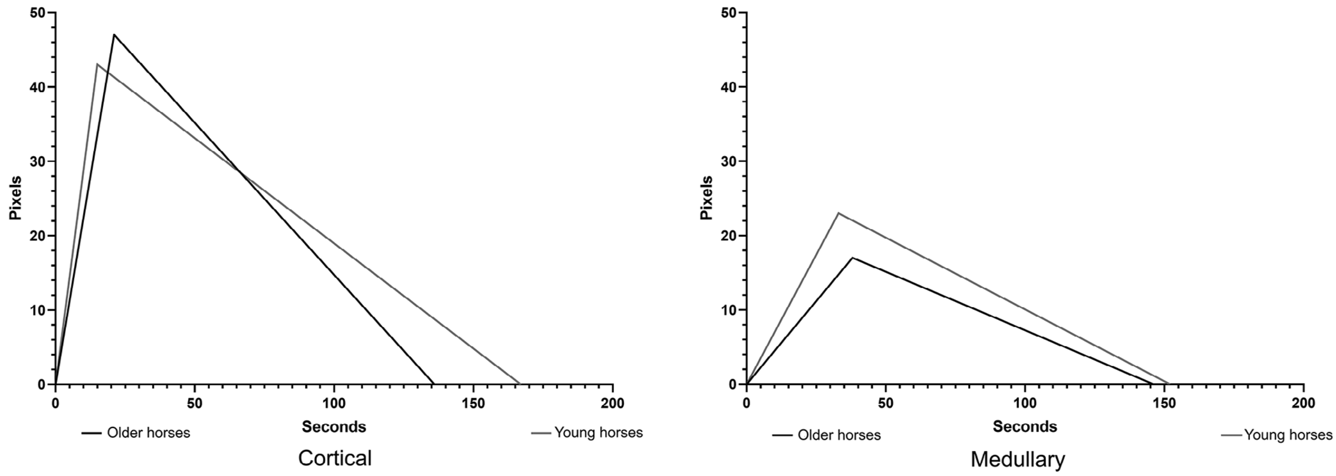

FIGURE 3 | Graphical representation of the time–intensity curve of the renal cortical (left) and medullary (right) ultrasound contrast study after contrast injection (time 0 in s) in young or older horses.

clearly identifiable. The cortical region enhances before the medullary region, and the cortical signal consistently exhibits higher intensity than the medulla in all individuals, allowing reliable differentiation between both regions.

3.2 | CEUS Quantitative Assessment

The perfusion indices were statistically similar between repeated measurements in each area and between the two ROI sizes evaluated ($p > 0.05$, Table A1). The perfusion time/intensity curve evaluated in this study was graphically represented in Figure 3. Cortical PI, AUC, and WOT resulted in similar values between young and old horses ($p > 0.05$), while the TP was longer in old animals ($p = 0.03$). In turn, medullary PI, TP, AUC, and WOT resulted in similar values between young and old horses ($p > 0.05$; Table 1).

Except for the WOT in both groups and TP in young animals, the perfusion indices showed wide variations in the animals studied (coefficient of variation >60%), which indicates a large difference in these parameters between individuals, and thus their central dispersion measures are also quite wide (Table 1).

4 | Discussion

In the present study, the use of commercial contrast agent proved to be possible and safe in both groups, corroborating the low-risk results found in previous studies in this species [4–6] and in retrospective studies in other species where the risk of anaphylactic reactions was very low (0.014%) [25–27], with severe adverse reactions or fatalities being described in 0.006% of cases, and even these were associated with the patients' medical circumstances and not with the contrast [28, 29].

Regarding the ultrasonographic positioning of the right kidney, between the 15th and 18th intercostal spaces [30, 31], and closer to the skin than the left kidney [31], our findings were consistent with the literature. Another characteristic that facilitated its consistent localization was the ventral support provided by the perirenal fat and adjacent organs such as the liver, pancreas, and cecum, which limits its movement [32]. The renal dimensions in the present study resulted in smaller mean values of width when compared to the literature [33]. This difference could be explained by the fact that the studied horses had a lower body weight than the animals described by Draper et al. [33]. There is no consensus about the resistivity index in horses, but some authors suggest that these animals have a lower resistivity index than what is described in other species [20]. The findings of the present study are similar to what is described in the literature [21].

When developing the technique, it was initially necessary to assess the dose required for the examination, which can vary according to the target organ and species. The result found indicated that increasing the contrast dose is not always the answer to improving image quality, as has already been described in the literature for ocular studies in horses [6]. Comparatively, the contrast dose for renal perfusion studies in dogs was 0.04 mL/kg [34] and in cats was 0.05 mL/kg [35].

Although there was no middle-aged group represented in this study, it was possible to characterize differences in the quantitative measurements of the renal perfusion between young and old horses. The tendency for older animals to show slower and lower perfusion, observed in the qualitative assessment, may indicate a reduction in physiological renal perfusion caused by aging and was confirmed by the prolongation of time to cortical peak. These findings were similar to those described for healthy cats in which there was a trend towards lower peak enhancement, wash-in time, and AUC for the renal cortex when comparing cats between 1 and 3 years of age with cats older than 10 years [35]. Similar findings have been described for cats with chronic kidney disease [9] and humans with diabetic nephropathy [36]. It is therefore considered a useful time parameter for detecting early perfusion changes related to chronic kidney disease; however, further investigations in the equine species are needed to identify the diagnostic capacity of the CEUS in various renal diseases.

The variation observed within animals of the same age may be the result of various factors such as blood pressure and individual reaction to contrast or movement, as already described [37]. The

TABLE 1 | Comparison table between the young and old groups for measurements made at the cortical and medullary regions of the kidney.

Variable	Group	Median	IQR	*p* value	CI95%	Variation coefficient (%)
Cortical region of the kidney						
PI	O	47.30	29.50	0.837	29–57	51
PI	Y	43.25	49.08	0.837	24–72	61
AUC	O	3358	6322	0.681	1307–7601	73
AUC	Y	3340	2712	0.681	1560–4230	59
TP	O	21.45*	35.07*	0.032	14–49	64
TP	Y	14.90*	7.350*	0.032	11–18	16
WOT	O	135.7	59.05	0.154	103–159	26
WOT	Y	167.3	87.10	0.154	101–187	27
Medullary region of the kidney						
PI	O	16.95	15.70	0.178	14–30	50
PI	Y	22.90	41.00	0.178	16–57	76
AUC	O	2631	2635	0.743	1900–5151	50
AUC	Y	2455	3251	0.743	1715–4475	74
TP	O	37.80	34.23	0.209	11–44	47
TP	Y	33.10	32.60	0.209	22–73	60
WOT	O	145.8	30.57	0.735	130–170	11
WOT	Y	151.6	44.23	0.735	132–175	17

Abbreviations: AUC, area under the curve; CI95%, confidence interval at 95% of the median; IQR, interquartile range; O, old; Y, young; PI, peak intensity (%); TP, time to peak (seconds); WOT, contrast wash-output time (seconds).
*Indicates significance in Mann-Witney U test (P<0,05) when compared between young and old horses.

coefficient of variation for different perfusion parameters varied from 16.5% to 191.7% in the medullary region and from 14.7% to 62.7% in the cortical region in a study with dogs [34]. Our findings agree with this statement, showing a high coefficient of variation for most of the perfusion parameters, for both cortex and medulla, among the studied individuals (>60%). WOT for young and older horses and TP for young horses were the perfusion parameters with a lower coefficient of variation, indicating that they would potentially be used as auxiliary diagnostic tools. These parameters were reported to have lower within-animal variability in a study with dogs [34].

Another factor observed in this study was that the difference in TP occurred only in the cortical region and was not observed in the medullary region. This indicates that age-related changes may be more present in the cortical region than in the medullary region, as described in humans [38]. Interestingly, the renal cortex receives more than 90% of the renal blood flow [39]. This region also contains the afferent and efferent arterioles, which are essential for nourishing the medullary region, which only receives 10% of the renal blood volume, and ensuring adequate blood volume and pressure for glomerular filtration [2]. Researchers debate the exact region where the ischemic damage initiates in the equine kidney [39]. For human patients, it is known that the kidneys are one of the organs that suffer most from the aging process due to their high metabolic rate and the high oxidative stress [40]. Elderly patients tend to show a reduction in functional reserve and a considerable decline in the capacity for adaptive homeostasis to external challenges, a process that occurs progressively throughout life [41]. The typical alterations described by medicine in elderly patients are glomerular sclerosis, reduction in glomerular volume, reduction in the hyalinized area of the afferent arterioles, and tubulointerstitial fibrosis [42]. All these factors translated into an alteration in the normal perfusion of the organ, which may be represented by changes in the contrast curve as found in other studies.

It is important to note that changes in function (detected by blood tests) and structural changes (detected by imaging tests) do not always occur concomitantly [43, 44]. This study, therefore, describes perfusion changes in older horses compared to younger animals. These changes were not accompanied by altered renal function (as these animals had normal blood tests, and no histopathologic exams were performed). By demonstrating the feasibility of this technique to evaluate equine kidney perfusion and showing a difference between the two studied groups, we therefore consider the potential of CEUS, principally TP and WOT, to be an auxiliary tool for the early detection of renal alterations in horses.

Some limitations of this study are mainly related to the number of animals assessed and not having a middle-aged group, which may have contributed to some variations between individuals of the same group. The unilateral assessment is also a factor to be considered, as comparisons of the same individual at different time points would have facilitated the identification of any structural alterations. Another limitation was the animal's motion during the examination, which may have influenced the quality of the graph generated, even with the image stabilization provided by the software used. The lack of multiple evaluators to determine interoperator variation is a relevant point for future studies. In addition, finally, the absence of blood pressure measurement before the exam and histopathological analysis is also a limitation in these studies and can be addressed in future studies.

In conclusion, this study evaluated the CEUS examination of the right kidney in young and old horses, paving the way for new studies, as we have shown that it is possible to perform the technique safely and have established quantitative parameters (PI, TP, AUC, and WOT) for future comparisons. Further studies are needed to introduce the technique into the routine care and assessment of this species, both in the characterization of lesions and in the assessment of renal impairment in the older animal population Video S1.

Author Contributions

Conception and design: Diana Villa Verde Salazar, Ricardo Andrés Ramirez Uscategui, Luiz Paulo Nogueira Aires, Luciano Andrade Silva, and Marcus Antônio Rossi Feliciano. **Drafting the article:** Diana Villa Verde Salazar, Anna Carolina Mazeto Ercolin, Gabriela Castro Lopes Evangelista, and Ricardo Andrés Ramirez Uscategui. **Final approval of the completed article:** Diana Villa Verde Salazar, Marília Alves Ferreira, Pedro Henrique Salles Brito, Ricardo Andrés Ramirez Uscategui, Anna Carolina Mazeto Ercolin, Gabriela Castro Lopes Evangelista, Luiz Paulo Nogueira Aires, Luciano Andrade Silva, and Marcus Antônio Rossi Feliciano. **Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved:** Diana Villa Verde Salazar, Marília Alves Ferreira, Pedro Henrique Salles Brito, Ricardo Andrés Ramirez Uscategui, Anna Carolina Mazeto Ercolin, Gabriela Castro Lopes Evangelista, Luiz Paulo Nogueira Aires, Luciano Andrade Silva, and Marcus Antônio Rossi Feliciano.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES)—Finance Code 001. We would like to thank RTS for providing the equipment necessary for this study.

Disclosure

This manuscript has not received prior publication and is not under consideration for publication elsewhere.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data supporting the results are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary File1: vru70188-sup-0001-VideoS1.mp4

Appendix

TABLE A1 | Friedman's test results.

Comparison/*p* value	PI	TP	AUC	WOT
ROIs size	0.2231	0.9897	0.6248	0.0832
ROIs repetitions	0.5637	0.5647	0.2893	0.7208

Abbreviations: AUC, area under the curve; PI, peak intensity; ROI, region of interest; TP, time to peak; WOT, contrast wash-output time (s).