Contrast-enhanced ultrasonography to assess blood perfusion of skeletal muscles in normal dogs

Juyeon OH¹, Sunghoon JEON¹ and Jihye CHOI¹*

¹Department of Veterinary Medical Imaging, College of Veterinary Medicine, Chonnam National University, Gwangju 500–757, South Korea

ABSTRACT. This study evaluated perfusion of skeletal muscle using contrast enhanced ultrasonography in humerus, radius, femur and tibia (Received 26 June 2014/Accepted 17 February 2015/Published online in J-STAGE 6 March 2015)

Bone fracture can be healed through direct or indirect process according to the stability and the gap between the fragments [7, 10]. Although some characteristics including the bone fracture, decrease of the gap between fragments, callus formation and fixation devices can be evaluated on radiography, neovascularization and blood perfusion in the fracture site, which are one of the most important factors for fracture healing, can’t be estimated [19]. Because the bone changes follow after blood supply in fracture healing, we hypothesized that perfusion of surrounding soft tissues can be used as an earlier indicator of hemodynamic changes during bone healing. Several previous studies discussing power Doppler, dynamic contrast-enhanced magnetic resonance imaging and scintigraphy have reported that evaluation of the neovascularization can be helpful for predicting whether the condition is toward normal fracture healing, delayed union or nonunion [4, 6, 33, 36, 37]. Contrast enhanced ultrasonography (CEUS) can detect blood flow with low volume and slow velocity in smaller vessels through harmonic signals from a microbubble contrast agent in real time after injecting the contrast medium [15]. Dynamic blood perfusion can be visually evaluated based on the bright signal formed by contrast agent at a low mechanical index. In addition, blood velocity and volume can be assessed quantitatively based on the time–intensity curve (TIC) generated from CEUS.

In veterinary medicine, the quantitative assessments using the TIC on CEUS have been applied to various organs, including the canine liver [3, 23–25], spleen [26], lymph nodes [40], adrenal gland [28, 29], kidney [8, 13, 39], pancreas [14, 32] and superficial tumors [27]. Neovascularization is one of the most important factors for fracture healing. Therefore, microcirculation in skeletal muscles can be used as an early indicator of hemodynamic changes during fracture healing. However, no study has applied CEUS to skeletal muscles in veterinary medicine. Although applying CEUS to musculoskeletal regions is just beginning even in humans, CEUS has been used to assess perfusion of skeletal muscle in various conditions including acute muscle injury, peripheral arterial disease, diabetes mellitus with peripheral arterial disease and ischemia and also in osseous and osseocutaneous free flaps [1, 2, 5, 9, 11–16, 18, 21, 22, 30, 31].

This study was performed to establish the protocol of contrast enhanced ultrasonography for the surrounding soft tissue of thoracic and pelvic limbs as a preliminary study for estimating perfusion in skeletal muscles of fracture cases. In the quantitative parameters of CEUS, the peak intensity (PI) and area under the curve (AUC) represented for blood volume, and the time to peak intensity (TP) represented for blood velocity.

MATERIALS AND METHODS

Eight 2–3-year-old male beagles (mean body weight; 10.51 ± 1.48 kg, range; 9.7–11.5 kg) were used. All dogs were clinically healthy based on a physical examination, complete blood counts, biochemistry, urinalysis, thoracic and abdominal radiography, and abdominal ultrasonography. There were unremarkable findings on lateral and cranio-caudal radiographs of thoracic and pelvic limbs. The dogs were housed individually and were provided commercial food and water ad libitum.

After a 24 hr fast, the dogs were given intramuscular in-
jections of a combination of 1.5 mg/kg zolazepam/tiletamine (Zoletil, Virbac, Carros, France) and 0.03 mg/kg medetomidine (Domitor, Orion Corp., Espoo, Finland). Blood pressure was recorded before and after anesthesia.

Conventional B-mode ultrasonography and CEUS were conducted in left humerus, radius, femur and tibia using the same 10 MHz linear probe and ultrasound equipment (ProSound Alpha 7, Aloka, Tokyo, Japan). The dog was positioned in a right lateral recumbency, and each examining limb was extended toward the operator and fastened. A conventional B-mode examination was performed to localize the scanning region for CEUS. The transducer was placed over the lateral plane from the shoulder joint to the humeral diaphysis proximally covered by the triceps muscle; the lateral plane from the proximal radial metaphysis to the diaphysis proximally covered by the extensor digitorum communis muscle and distally by the abductor pollicis longus muscle; the lateral plane from the stifle joint to the femoral diaphysis proximally covered by the tensor fasciae latae muscle and distally by the vastus lateralis muscle; or the lateral plane from the stifle joint to tibial diaphysis proximally covered by the tibialis cranialis, respectively. The longitudinal image of each bone surface including muscle was displayed on a monitor, with positioning so that the proximal metaphysis was on the right and the diaphysis was on the left. Color Doppler ultrasonography was performed to evaluate the presence of vessels. Second higher harmonics were performed in the extended pure harmonic detection mode for CEUS. The transmitted energy was reduced to a magnitude of 7% with a 0.07 mechanical index. Intrusion depth was set to 2.0–3.5 cm to visualize the muscle according to the appendicular regions. The pulse repetition frequency was set to 15 Hz with a 61% gain value. Contrast medium (SonoVue, Bracco, Milan, Italy) was administered by bolus injection into the cephalic vein via a three way stopcock no.2, and a 20 gauge intravenous catheter. A 0.5 ml (2.5 mg) or 1 ml (5 mg) of contrast medium was injected through a 1 ml syringe into each dog, immediately followed by a bolus injection of 5 ml saline. At the same time, dynamic sequences were stored for up to 110 sec by keeping the transducer in the same location. CEUS was completed in all dogs within 2 hr after opening the contrast medium bottle, and the contrast medium was agitated before injection into other dogs. A single appendicular region was examined in each dog on the same day, and the other regions were examined at 2-day intervals. All CEUS was performed by one examiner (OJY), and quantitative and qualitative evaluations of all CEUS were performed three times by two reviewers (OJY, JSH) blinded to the scanned region. All dynamic cine loops were evaluated using the installed software (SOP-ALPHA7-14, Aloka), which displayed the acoustic intensity according to time. Region of interest (ROI) in an arc shape of 1.1 × 2.9 mm was manually defined over the muscle to acquire the TIC. Motion correction was manually performed frame by frame. Quantitative corresponding parameters of the TIC, including PI, AUC and TP, were obtained from ROI. PI was measured with the greatest acoustic intensity. TP was defined as the duration from the start of contrast agent injection to PI. AUC was calculated as the area of the TIC using integration.

The protocol used in this study was approved by the Animal Care and Use Committee of Chonnam National University, and the animals were cared for in accordance with the Guidelines for Animal Experiments of Chonnam National University.

A statistical analysis was performed using the SPSS statistical program (SPSS Statistics Version 21, IBM Corp., Armonk, NY, U.S.A.). Analysis of reproducibility between reviewers was performed using the intra-class correlation coefficient test (ICC). ICC values were evaluated by the criteria: poor reliability, <0.4; fair to good, 0.4–0.75; and excellent, >0.75 [35]. Repeated-measures analysis of variance was used to investigate the intra- and inter-reproducibility coefficients of the TIC parameters including PI, TP and AUC and inter-reproducibility coefficients of vascularity score among four appendicular regions in normal dogs. A P<0.05 was considered significant for all analyses. Data are reported as mean ± standard deviation.

RESULTS

The mean blood pressure in eight dogs was 145.5 ± 7.52 mmHg before anesthesia and 126.38 ± 5.90 mmHg after anesthesia.Transient increase of vascular intensity and then disappearance was easily observed from all skeletal muscles after injecting the contrast medium (Fig. 1). The blood perfusion of skeletal muscle was reliably assessed using CEUS.

A total of 1,711 frames of CEUS were obtained from ROI over 110 sec, and a typical parabolic shape to the TIC was generated from muscle area (Fig. 2). The mean values of PI, AUC and TP measured from muscle were not significantly different among the four appendicular regions (Table 1). No significant difference in mean peak intensities, areas under the curve or TP was observed within each skeletal muscle of the four regions, according to the dosage of contrast medium. In quantitative analysis, the mean intra- and inter-reproducibility coefficients of PI, AUC and TP are represented in Table 2.

DISCUSSION

Assessing blood perfusion is meaningful to predict prognosis or monitor progression of fracture healing. The imaging modality has to be sensitive enough to detect low perfusion for an accurate assessment of blood flow in muscle, because normal skeletal muscle perfusion is very low at rest [41]. The ultrasonic contrast medium, in principle, stays strictly intravascularly, and each single microbubble of contrast medium can be detected using CEUS, independent of velocity and volume of blood flow [5]. The blood volume measured with CEUS has a positive correlation with histologic capillarization [41]. Thus, CEUS has the capacity to assess skeletal muscle perfusion accurately not only at rest under normal conditions but also in fracture healing. A second-generation contrast agent was selected in this study, because this agent coated with inert gas is more stable allowing
longer persistence in the blood stream than first-generation agents coated with air. The size of sulfur hexafluoride-filled microbubbles is uniform in this contrast medium, and this agent has prolonged and excellent stability with a vial time of 6 hr and a half time of 6 min in peripheral blood [27]. In contrast to the first-generation contrast agents which become disrupted and release a large amount of acoustic emission at a high mechanical index, low mechanical index imaging using second-generation agents minimizes the microbubble burst to enhance the contrast effect and produces a nonlinear oscillation which provides for harmonic frequencies for a longer duration [27]. These non-destructive contrast agents allow for continuous real-time imaging and quantitative evaluation of blood perfusion by TIC analysis.

The mean values of PI and AUC represent the blood volume, and TP determines blood velocity [12]. PI and AUC in skeletal muscle tended to be higher in the humerus and femur than those in the radius and tibia, but no significant difference was observed. TP in the thoracic limbs tended to be more rapid (about 4 min) than that in the pelvic limbs; however, no significant difference among the 4 regions was observed. Thus, blood volume and velocity in skeletal muscle of the humerus. Transient increase of vascular intensity (arrows) and then disappearance was easily observed from all skeletal muscles after injecting the contrast medium.

Fig. 1. Contrast enhanced ultrasonography on harmonic detection mode before (A) and after (B) contrast agent injection at the skeletal muscle of the humerus. Transient increase of vascular intensity (arrows) and then disappearance was easily observed from all skeletal muscles after injecting the contrast medium.

Fig. 2. Conventional ultrasonography on color Doppler mode (A) and contrast enhanced ultrasonography on harmonic detection mode (B) of radius. Region of interest placed over the muscle on contrast enhanced ultrasonography and time-intensity curves (C). The muscle area had a typical parabolic curve, which consisted of inflow, outflow and peak intensity.
vascularization could provide early prognostic information. Therefore, quantitative TIC analysis of skeletal muscular vascularity with high reproducibility. There are some limitations in this study. First, this study was performed under general anesthesia, and blood volume and velocity may be different from those of patients without anesthesia [38]. The pharmacological effects of anesthetics itself can influence the TIC values [38]. In a previous study, the vasodilation effect of anesthetics altered local blood pressure and heart rate, and TP in liver was about 11.6 sec faster in dogs without anesthesia than for those under anesthesia [23]. Second, it was difficult to assess blood perfusion accurately in some muscular areas containing fascia, ligaments and tendons, because they show hyperintensity. Although there were some limitations, CEUS is expected to be an excellent modality for evaluating changes of blood perfusion in skeletal muscle. This study provided quantitative reference value of muscular perfusion including blood volume and velocity in normal dogs. A 0.5 ml aliquot contrast medium is suggested as a practical dose for assessing the skeletal muscle. The CEUS protocol for evaluating the blood perfusion of skeletal muscles can be used as basic information for further studies evaluating the blood perfusion of skeletal muscles in various pathologic states including bone fracture using CEUS.

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| Table 1. Mean values of time-intensity curve parameters in appendicular muscles after injection of 0.5 ml or 1 ml contrast agent |
|-----------------------------|-------------------|-------------------|-------------------|
|                            | PI (level)        | AUC               | TP (msec)         |
|                            | 0.5 ml            | 1 ml              | 0.5 ml            | 1 ml              | 0.5 ml            | 1 ml              | 0.5 ml            | 1 ml              |
| Humerus                    | 28.84 ± 9.69      | 22.62 ± 4.20      | 1,642.39 ± 643.23 | 1,474.06 ± 372.80 | 40,763 ± 10,274   | 38,576 ± 10,983   |
| Radius                     | 19.86 ± 8.92      | 16.15 ± 4.44      | 1,402.64 ± 851.85 | 838.91 ± 366.67   | 37,649 ± 5,976    | 43,101 ± 11,463   |
| Femur                      | 23.25 ± 14.36     | 28.31 ± 10.37     | 1,786.42 ± 1,279.55 | 2,041.50 ± 769.36 | 43,969 ± 5,976    | 38,576 ± 8,281    |
| Tibia                      | 16.40 ± 6.18      | 18.50 ± 1.60      | 1,143.29 ± 475.52 | 1,309.10 ± 280.76 | 42,890 ± 10,373   | 45,770 ± 6,707    |

Data are mean and standard deviation.

| Table 2. Mean intra- and inter-reproducibility coefficients of peak intensity, area under the curve and time to peak intensity of the soft tissue in each appendicular bone region using 0.5 ml or 1 ml contrast agent in appendicular muscles |
|-----------------------------|-------------------|-------------------|-------------------|
|                            | Intra-reproducibility coefficients | Inter-reproducibility coefficients | |
|                            | PI                | TP               | AUC               | PI                | TP               | AUC               |
|                            | 0.5 ml            | 1 ml              | 0.5 ml            | 1 ml              | 0.5 ml            | 1 ml              |
| Humerus                    | 0.759             | 0.827             | 0.835             | 0.871             | 0.864             | 0.806             |
| Radius                     | 0.906             | 0.808             | 0.832             | 0.755             | 0.952             | 0.860             |
| Femur                      | 0.924             | 0.970             | 0.931             | 0.933             | 0.678             | 0.927             |
| Tibia                      | 0.742             | 0.744             | 0.801             | 0.899             | 0.751             | 0.856             |

|                            | Intra-reproducibility coefficients | Inter-reproducibility coefficients | |
|                            | PI                | TP               | AUC               | PI                | TP               | AUC               |
|                            | 0.5 ml            | 1 ml              | 0.5 ml            | 1 ml              | 0.5 ml            | 1 ml              |
| Humerus                    | 0.859             | 0.972             | 0.935             | 0.935             | 0.984             | 0.906             |
| Radius                     | 0.906             | 0.998             | 0.942             | 0.765             | 0.962             | 0.860             |
| Femur                      | 0.924             | 1.000             | 0.971             | 0.86              | 0.678             | 0.927             |
| Tibia                      | 0.772             | 0.774             | 0.841             | 0.891             | 0.885             | |
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