

ORIGINAL ARTICLE

Quantitative assessment of spinal cord perfusion by using contrast-enhanced ultrasound in a porcine model with acute spinal cord contusion

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Objectives: To quantify spinal cord perfusion by using contrast-enhanced ultrasound (CEUS) in a porcine model with acute spinal cord injury.

Methods: Microcirculatory changes of acute spinal cord injury were shown by CEUS in a porcine model with spinal cord contusion at three selected time points, coupling with conventional ultrasound (US) and Color Doppler US (CDFI). Time-intensity curves and perfusion parameters were also obtained by autotracking contrast quantification (ACQ) software in the epicenter of contusion site, adjacent region and distant region, respectively. Neurologic and histologic examinations were used to confirm the severity of injury.

Results: Conventional US revealed the spinal cord was hypoechoic and homogeneous, whereas the dura mater, pia mater and cerebral aqueduct were hyperechoic. On CDFI intramedullary blood vessels were displayed as segmental and columnar. It was homogeneous on CEUS. After spinal cord contusion, the injured region on gray scale US was hyperechoic. CDFI demonstrated intramedullary blood vessels of adjacent region had increased and dilated during the observation period. On CEUS the epicenter of contusion site was hypoperfusion, whereas its adjacent region was hyperperfusion compared with the distant region. Quantitative analysis showed that peak intensity decreased in epicenters of contusion but increased in adjacent regions significantly at all time points ($P < 0.05$). Evaluation of neurological function for post-contusion demonstrated significantly deterioration in comparison before injury ($P < 0.05$).

Conclusions: CEUS is a practical technique that provides overall views for evaluating microcirculatory pattern in spinal cord injury. Quantitative analysis shows the efficacy in assessment of perfusion changes after spinal cord injury.

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INTRODUCTION

A better understanding of spinal cord microcirculation is essential to further research in spinal cord injury. Although some methods, such as washout of radioactive indicators,¹ implanted hydrogen electrode,² the trapping of labeled microspheres,³ laser-Doppler flowmetry,^{2,4} closed spinal window⁵ and magnetic resonance imaging (MRI),⁶ have made significant contributions to the study of spinal cord blood flow (SCBF), these techniques have some shortcomings. The radioactive microsphere trapping method requires the killing of an animal and harvesting of tissue for the radioactivity counting. Thus, it cannot provide the real time information, and it usually needs many animals to get adequate data for analysis. A tiny delay in tissue harvesting will lead to a significant error for the mandatory timing. Although the laser-Doppler technique is easy to use, non-invasive and recorded on-line in the assessment of microcirculatory changes of the brain and spinal cord, the major disadvantages are that the blood flow recorded is limited in a small tissue volume, the probe position on detecting point is hardly to consistently inter animals and the method is very

sensitive to artifacts.⁷ In addition, the main drawback of closed spinal window with light microscopy or even with image-intensified high-speed video system ($1000 \text{ frames s}^{-1}$) is the depth of tissue penetration and so, this approach is restricted to observe the microcirculation in the spinal pia mater but not in the parenchyma of spinal cord.^{5,7} A typical non-invasive approach of MRI to measure perfusion is arterial spin labeling (ASL). The basic principle of ASL is that blood is magnetically labeled and serves as an intrinsic contrast agent. Because no contrast agent is needed, ASL techniques are completely non-invasive. For SCBF quantification ASL as the alternative method for absolute quantitative perfusion measurement might suffer from too low sensitivity. Recently, Weber *et al.*⁸ have presented a first report of quantitative SCBF measurements in mice by MRI with a pulsed ASL technique based on a presaturated flow-sensitive alternating inversion recovery (presat-FAIR) EPI sequence. However, MRI is not a continuous real-time perfusion.

US has recently benefited from the introduction of contrast agents.⁹ These agents consist of microspheres that enhance ultrasonic reflection signals and modify acoustic properties of regions of

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interest. The second generation echo-contrast agent SonoVue (Bracco, Milan, Italy) consists of microbubbles stabilized by phospholipids and filled with sulphur hexafluoride (SF₆). It has a mean diameter of 2.5 µm and is a blood pool imaging agent. Therefore, contrast-enhanced ultrasound (CEUS) has made microvascular imaging possible.^{10,11} In comparison with those methods mentioned above, CEUS have a lot of advantages, including low costs, better availability, subject (patient) comfort, and no exclusion of patients or animals with certain indwelling metals (internal fixation) and bedside measurement, for example, for intraoperative animals or patients. What's more, CEUS enables the simultaneous measurement of tissue perfusion (microcirculation) in real time.

Scientists have long used animal spinal cord contusion models to study the pathophysiology of spinal cord injury.¹² Contusion models had an important role in the discovery of progressive secondary tissue damage and most therapies that have gone to human clinical trial were first validated in spinal cord contusion models. There is a linear relationship between force and displacement and that both of these factors have a role in determining the anatomical and functional deficits after spinal cord contusion injury.¹³

The purposes of this study were to evaluate the role of CEUS in microcirculation of acute spinal cord contusion, and to investigate its perfusion characteristics of CEUS.

MATERIALS AND METHODS

Study design

Six juvenile, domestic female pigs, 3 months of age and weighing 15–16 kg, were subjected for this experiment. Spinal cord contusions of the animals were produced by a modified spinal cord impactor system. CEUS was performed to monitor microcirculatory changes pre and post spinal cord contusions. All procedures were approved by the Ethics Committee and the Animal Experimentation Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University, and conducted in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals.

Anesthesia and perioperative management

After pretreatment with 15 mg kg⁻¹ ketamine and 0.03 mg kg⁻¹ atropine given intramuscularly to induce the anesthesia, animals were further anesthetized with intravenous Thiameylal (1.5 mg kg⁻¹) administered slowly. Intramuscular buprenorphine (0.1 mg kg⁻¹) was used as an analgesic. The antibiotic Ceftiofur (3 mg kg⁻¹) was given intramuscularly to prevent bacterial infection.

Porcine model of spinal cord contusion

After a deep level of anesthesia, the pig was placed prone on the table and a midline dorsal incision was made in the skin from T₈ level to T₁₃. The muscles around T₈ to T₁₃ were incised and retracted on both sides of the vertebral column to expose spinous processes and laminae. Total laminectomy of T₉ to T₁₂ was performed with preservation of the dura. Spinous processes of T₈ and T₁₃ were reserved for clamping with the modified spinal cord impactor system. The thoracic spinal cord contusion model was induced with a modified weight-drop impactor (50 g weight, dropped from a height of 15 cm).

US imaging protocol, contrast agent and injection technique

First of all, before contusion, conventional gray-scale US and Color Doppler US (CDFI) were performed on the exposed thoracic cord. Real-time CEUS using low mechanical index (0.25) started as soon as the bolus injection of contrast agent was performed. An intravenous bolus injection of 2.0 ml of SonoVue was used, followed by a flush of 10 ml of saline. Images and clips lasting for 70 s were stored on hard disk for online analysis.

Then, the thoracic spinal cord of the animal was injured by a modified spinal impactor system (50 g weight, dropped from a height of 15 cm). The impactor was immediately removed from the injured spinal cord. The US examination procedures were performed again. Three time points were

selected to demonstrate the microperfusion after spinal cord contusion: 0 min (instantly), 30 min and 120 min.

CEUS was performed using Acuson Sequoia 512 (Siemens, Mountain View, CA, USA), equipped with 15L8w-S transducer, frequency 7.0–10.0 MHz, and contrast pulse sequencing software: ACQ. All US examinations were performed by one radiologist (Xi Lin, with 5 years of experience in CEUS).

Quantitative analysis in CEUS

ACQ software was applied to derive the clips. Three regions of interest were set in epicenters of contusion site in injured thoracic cord, adjacent regions and distant regions, which were ~1.5 cm far from the injured site. Time-intensity curves and perfusion parameters: peak intensity (PI) were obtained by ACQ software from regions of interest. Images obtained with the sequences were analyzed by one radiologist (An-hua Li, with 5 years of experience in CEUS).

Postoperative management

After incision was closed, the pig was placed in a separate pen as soon as it was alert. Food and water were provided starting on the first postoperative day (POD). Analgesic treatment (buprenorphine 0.1 mg kg⁻¹) was maintained for all 7 PODs.

Neurological evaluation

All animals survived the operative procedure and completed the full observation period of 7 PODs. To confirm the severity of the neurological deficit after spinal cord contusion, neurological examination, using the Tarlov score,¹⁴ was employed on POD 2 and POD 7 simultaneously by two investigators. The modified Tarlov Scoring was a four-point scale that depicts no locomotion as 0 points and a normal gait as 4 points. After assessment of the Tarlov score on POD 7, the animals were killed. Histological observation was performed to show the severity of injury to the spinal cord. The hematoxylin and eosin stained sections were transfer to digital images under microscopy. Then the percentage of lesion tissue area compared with the whole cord cross-sectional area was measured by using Adobe Photoshop CS3 (Adobe, San Jose, CA, USA).

Data analysis

All values obtained were expressed as the mean ± s.d. One-way ANOVA test was performed in comparison of PI after contusion by G*power software (Düsseldorf, Germany). A *P* < 0.05 was considered to be statistically significant.

RESULTS

CEUS and ACQ analysis

Precontusion. Conventional US revealed the subdural space and the spinal cord in sagittal plane to be hypoechoic and homogeneous, whereas the dura mater, pia mater and cerebral aqueduct were hyperechoic (Figure 1a). On CDFI in sagittal plane intramedullary blood vessels were divided into dorsal and ventral parts with reverse blood flow direction by cerebral aqueduct. They were displayed as segmental and columnar (Figure 1b). After contrast agent administration, the enhanced characteristic of the spinal cord was homogeneous (Figure 4a).

Postcontusion. Conventional gray scale US demonstrated that the injured region was hyperechoic. There was a corresponding grow in the area of hyperechoicity during the observation period (Figures 2a and b). The injured spinal cord was swelling. The architecture distortions of the dura mater, pia mater and cerebral aqueduct were also displayed. CDFI demonstrated intramedullary blood vessels of adjacent region had increased and dilated during the observation period (Figures 2c and 3).

On CEUS the enhanced characteristic of contused spinal cord was heterogeneous (Figures 2d and 4b–d). The epicenter of contusion site which was hyperechoic region on gray scale US was hypoperfusion, while its adjacent region was hyperperfusion and the distant spinal

cord was moderately enhanced on CEUS (Figure 4). In comparison of distant regions, quantitative analysis showed that PI decreased in epicenters of contusion but increased in adjacent regions significantly at all time points ($P < 0.05$) (Figure 5).

Functional evaluation

Figure 6 displayed the neurological function recovery of the animals. None of the six animals recovered from the protocol with normal neurological function, with a Tarlov score of 4 on the morning after surgery. All animals emerged a Tarlov score from 0–2 on POD 2, and remained a same range on POD 7, which was significantly deteriorated in comparison of normal function ($P < 0.05$). Compared with the

POD 2, no significant recovery was observed during the 7 days after contusion ($P > 0.05$).

Histological examination

Histology imaging confirmed the degree of contusion (Figure 7). Serious damage such as hemorrhaging or cavity formation was observed on hematoxylin and eosin stain sections.

DISCUSSION

In this study, CEUS provided advantages in assessment on micro-circulatory changes within spinal cord in a pig model. Hyperfusion was shown in adjacent region and hypofusion in the epicenter of contusion on CEUS, as compared with distant region. The objective observation was confirmed by ACQ analysis. Previously, SCBF was evaluated by hydrogen clearance method, radioactive tracer micro-sphere technique or laser-Doppler flowmetry technique in literatures. Laser Doppler was a popular real-time technique for SCBF. However, it could only measure SCBF within superficial tissues in large animals because of its limited detecting ability, which was only up to a 3-mm depth of spinal cord.¹⁵ The method was also too sensitive in artifacts.⁴ Moreover, it could not provide an overall view of spinal cord microperfusion. In contrast, this study suggested that CEUS was easy to be performed. It provided advantages of real-time microperfusion, an overall view and non-radiation. Therefore, it was useful for evaluating microperfusion of spinal cord.

To date, a comparison between CEUS and perfusion MRI in spinal cord is lacking. A typical non-invasive MRI approach to measure perfusion is ASL. The basic principle of ASL is that blood is magnetically labeled and serves as an intrinsic contrast agent. Because no contrast agent is needed, ASL techniques are completely non-invasive. For SCBF quantification, ASL as the alternative method for absolute quantitative perfusion measurement might suffer from too low sensitivity. Recently, Duhamel *et al.*⁶ have presented a first report of quantitative SCBF measurements in mice by MRI with a pulsed ASL technique based on a presaturated flow-sensitive alternating inversion recovery (presat-FAIR) EPI sequence. In comparison with those methods, such as laser-Doppler flowmetry or MRI, CEUS have a lot of advantages, including low costs, better availability, subject (patient) comfort, and no exclusion of patients or animals with certain indwelling metals (internal fixation) and bedside measurement, for example, for intraoperative animals or patients. What's more, compared with MRI, CEUS enable the simultaneous measurement of tissue perfusion in real time.

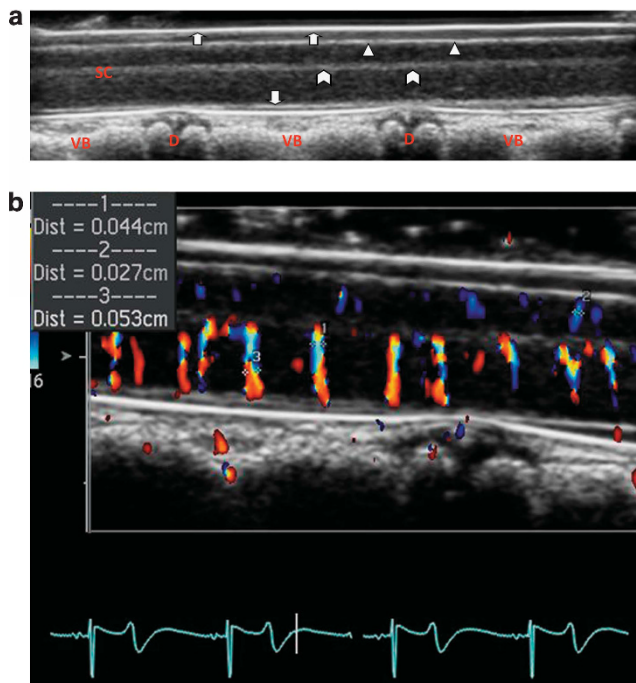


Figure 1 Sonography of uninjured spinal cord (longitudinal view): (a) Gray scale and (b) CDFI of uninjured spinal cord. SC, spinal cord; VB, vertebrae body; D, disc. \uparrow \downarrow indicate dura mater of spinal cord; Δ indicates spinal pia mater; \square indicates cerebral aqueduct.

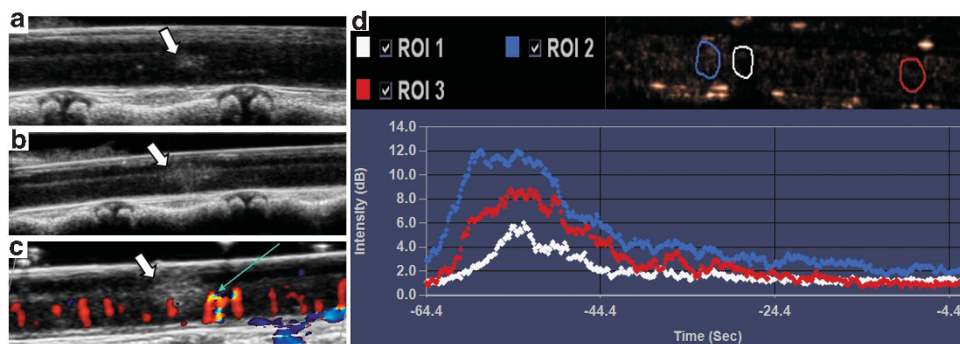


Figure 2 (a, b) Gray-scale sonography of spinal cord immediately post injury demonstrates increases in lesion area in 1 min. (c) CDFI demonstrated the dilated intramedullary blood vessels of injured region (arrow). (d) Representative CEUS and ACQ analysis showed the pattern of ischemic areas in contusion site after SonoVue infusion and enhancement in adjacent region. \square indicated contusive lesion, long arrow indicated dilated vessels in adjacent region. ROI (regions of interest) 1, ROI 2, and ROI 3 represented the epicenter of contusion site, adjacent region and distant region, respectively.

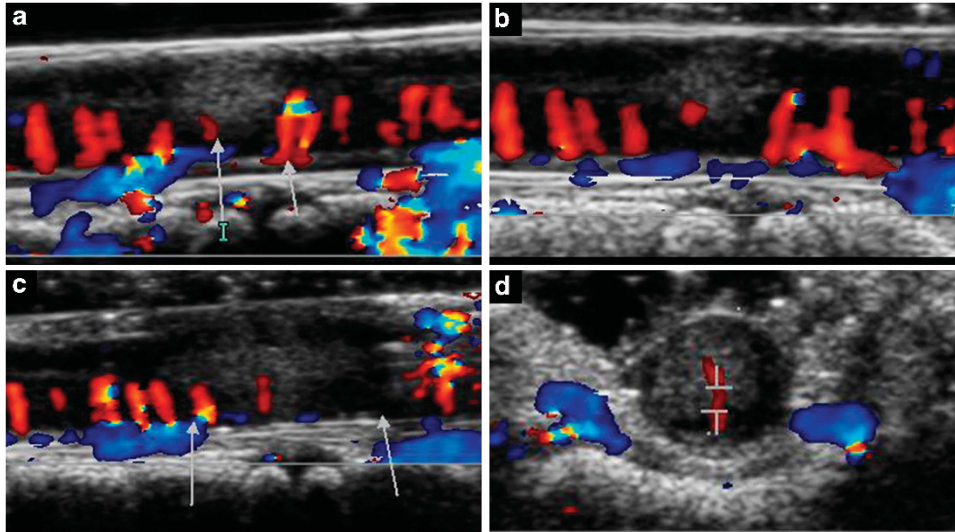


Figure 3 CDFI of contused spinal cord during time course. (a) 0 min (instantly after contusion); (b) 30 min after contusion; (c) longitudinal view of the contusive cord at 120 min after injury and (d) axial view of the contusive cord at 120 min after injury.

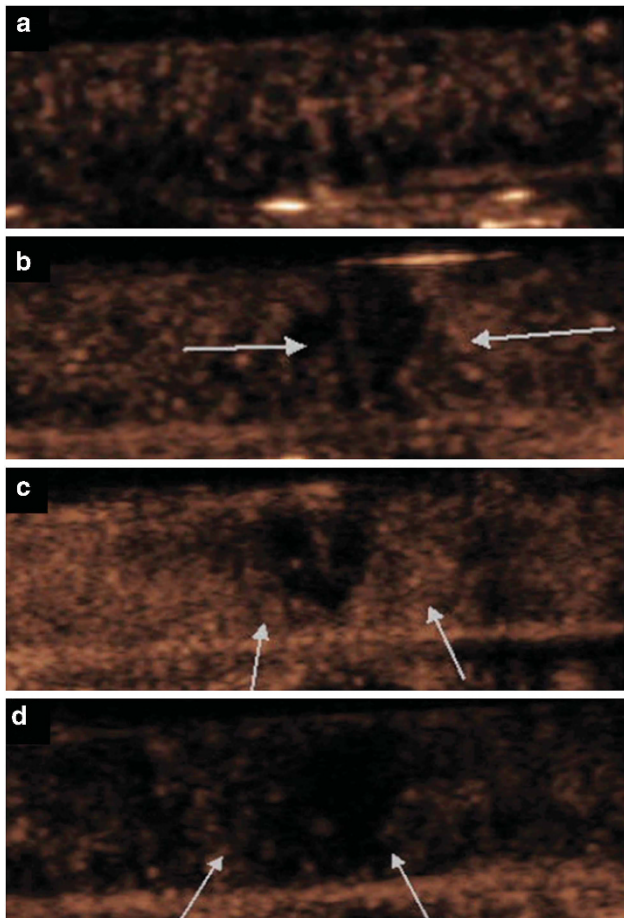


Figure 4 Representative CEUS pictures of the spinal cord: (a) uninjured spinal cord; (b) 0 min after contusion; (c) 30 min after contusion; (d) 120 min after contusion.

On gray scale US the epicenter was hyperechoic. There was a corresponding grow in the area of hyperechoicity during the experimental session. The latter finding suggested secondary injury. Less

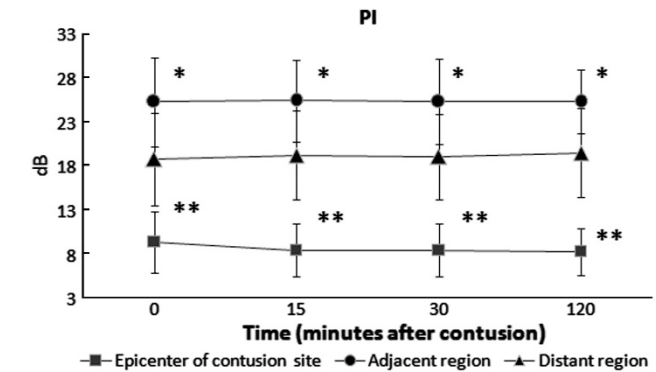


Figure 5 Diagram showed the PI was markedly decreased in the epicenter of contusion but increased significantly in the adjacent region, as compared with distant region. * $P < 0.05$, ** $P < 0.01$.

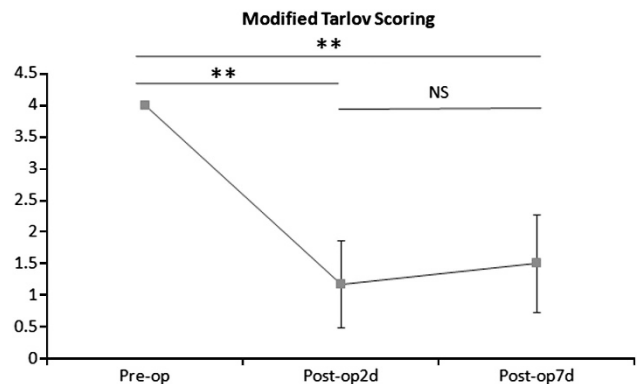


Figure 6 Neurological function of the animals evaluated by modified Tarlov scoring scale. Deterioration in neurological function was significantly emerged and no obviously recovery was observed during 7 days after contusion. * $P < 0.05$, ** $P < 0.01$; NS: no statistical significant. A full color version of this figure is available at the *Spinal Cord* journal online.

blood flow was observed in epicenter on CDFI. Using CEUS, its enhancement pattern was hyperechoic and quantitative analysis showed that PI of the epicenter was significantly lower than adjacent

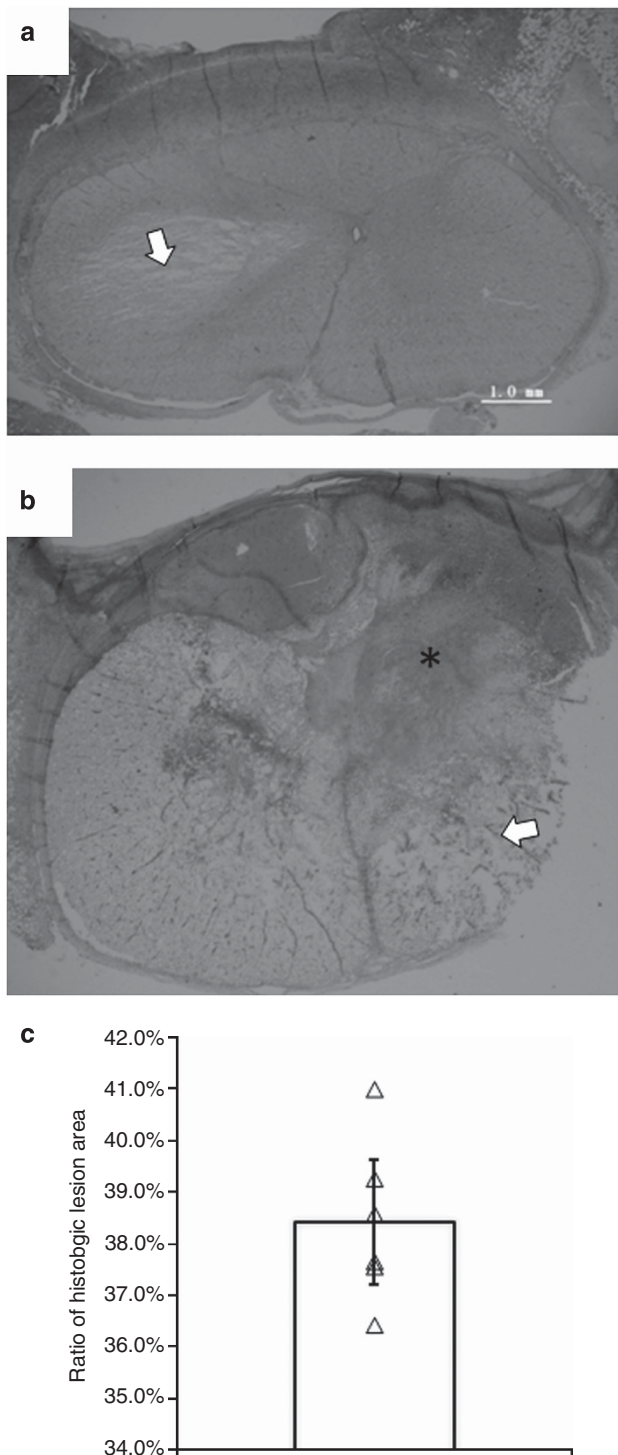


Figure 7 (a, b) Photomicrograph of representative hematoxylin and eosin-stained spinal cord cross-section, showing degrees of tissue damage in response to the injury severity. Magnification bars are indicated. (c) Ratio of histological lesion area of spinal cord. \square indicate necrosis and cyst formation or micro cysts of injured spinal cord; * indicate hemorrhage of injured spinal cord. A full color version of this figure is available at the *Spinal Cord* journal online.

and distant regions throughout this period ($P < 0.05$). This result in general was consistent with what was previously reported.¹⁶ The most significant difference from previous studies was the perfusion

in adjacent regions. A majority of studies reported significant post-traumatic hypoperfusion in adjacent segments of spinal cord.^{17–19} Likewise, a number of clinical and experimental studies had shown less cerebral blood flow in and around contused brain parenchyma.^{20,21} However, this study showed that on CDFI and CEUS there was a correspondingly significant hypervascularity and hyperperfusion in the adjacent region, which at least lasted 120 min. Quantitative analysis confirmed this observation. These findings provided more information on changes of perfusion in spinal cord injury.

Acute spinal cord injury resulted in the initial physical disruption of structures in the spinal cord and in the generation of secondary events that collectively injure intact and neighboring tissue. Microcirculatory events had a key role in both primary and secondary injury mechanisms. Early hypoperfusion showed obviously on CEUS and by quantitative analysis in contusion site. One possible explanation was that the initial impact disrupted the blood supplies within the spinal cord and resulted in local infarction caused by hypoxia and ischemia.²² Moreover, hypoperfusion frequently indicated vascular damage from immediate vasospasm of superficial vessels to intraparenchymal hemorrhage and the formation of thromboses.^{21,23} Hyperperfusion in pericontusion area were ascribed to an increasing volume of blood flow, dilation of microvessels, loss of autoregulation or opening of an arteriovenous shunt. In a moderate controlled cortical impact injury model, mean density and diameter of cortical microvessels were significantly reduced and increased, respectively, in the contusion margin at the initial time points.²⁴ In our study, there was significant difference in vessels dilation on CDFI and hyperperfusion on CEUS in adjacent region. These vivid and real-time features by CDFI and CEUS provided strong evidence that vessel dilation in pericontusional region was a response to the mechanical insult and metabolic demand. The latter was due to acute increasing in oxygen consumption²⁵ and glucose metabolism after trauma.²⁶

There were several limitations in this study. First of all, because of the attenuation of bone, CEUS could not be performed in spinal cord canal as it interfered with the integrity of spinal lamina and it could not be used after closed incision. Special probe is needed for spinal cord in the future. Second, it was small sample and there were lack of graded models on spinal cord injury in this study.

Microbubble contrast agents are currently implemented in a variety of both clinical and preclinical US imaging studies.²⁷ The therapeutic and diagnostic capabilities of these contrast agents are ongoing and show potential clinical prospects.²⁸ Most human spinal cord injuries result from fracture and dislocation of the spinal column. Spinal cord contusion models have had a significant contribution to the field.¹² Standardized procedure could help in confirmation of the severity of contusive spinal cord. The neurological results and the histological imaging from our study indicated that all pigs received moderate–severe contusion. Therefore, it is possible to evaluate the efficacy of this technique in clinical applications, including traumatic or iatrogenic spinal cord injury. What's more, it is deserved to investigate CEUS thoroughly in combination with neuroprotective or therapeutic genes or drugs for spinal cord injury in the future.

CONCLUSIONS

CEUS is a practical technique that provides overall views for evaluating microcirculatory pattern in spinal cord injury. Quantification analysis shows the efficacy in assessment of perfusion changes after spinal cord injury.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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