

Utilization of longitudinal ultrasound measurements to quantify changes in knee joint synovial vascularity in a rabbit model of rheumatoid arthritis

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Research article

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Abstract

Objective: This study of rabbits with ovalbumin-induced arthritis (OIA), a model of rheumatoid arthritis (RA), examined the time course of changes in synovial neovascularization based on imaging from power Doppler ultrasound (PDUS) and contrast-enhanced ultrasound (CEUS).

Methods: 25 male New Zealand rabbits were in the OIA group and 5 were in the control group. Both rear knee joints of all rabbits were examined using conventional US and CEUS over 16 weeks. The knee synovia of OIA rabbits were sampled by US-guided biopsy, and the expression of CD31 and VEGF were determined by immunohistochemistry. The correlation of microvessel density (CD31 positivity) and VEGF at different times was analyzed using multimodal US.

Results: OIA rabbits had increased synovial expression of CD31 and VEGF from week 6 to 12 ($P < 0.01$). During the early stage of CEUS enhancement, dot enhancement was more common on weeks 6 and 8, and strip enhancement was more common on weeks 12 and 16 ($P < 0.05$). There were significant positive correlations of synovial CD31 and VEGF expression with PDI grade, CEUS grade, and peak intensity (PI) ($P < 0.05$ for all).

Conclusions: OIA rabbits mimicked early-stage RA at 4 to 8 weeks, middle-stage RA at 8 to 12 weeks, and late-stage RA at 12 to 16 weeks. PDI, CEUS, and PI, especially when combined with CD31 expression, accurately characterized the extent of synovial vascularization. Increased vascular morphology based on CEUS may have value for the early diagnosis of RA.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease with an autoimmune pathogenesis[1]. It mainly affects small joints of the hands and feet, but has many systemic manifestations. Angiogenesis, the growth of new blood vessels, is an essential component of the pathogenesis of RA, because it facilitates the invasion of inflammatory cells and an increase in local pain receptors, thus contributing to structural damage and pain. Recent studies evaluated neovascularization during RA and changes in synovial blood flow after treatment using noninvasive imaging methods[2–4], but these methods are limited in their ability to detect low-speed blood flow and microvascular changes.

Many studies have confirmed that power Doppler imaging (PDI) can detect pannus neovascularization[5, 6], and this technique is an important method used to assess the activity of RA and evaluate the effects of different therapies. Real-time gray-scale ultrasound (US) using the SonoVue contrast agent improves the ability to detect blood vessels as small as capillaries ($< 50 \mu\text{m}$)[7], and provides measurements of hemodynamic parameters that can be used for quantitative analysis. However, no previous studies have used US to determine longitudinal changes in vascularization during the progression of RA.

Microvascular density is defined as the average number of blood vessels per square millimeter of tissue area. The differentiated protein cluster 31 (CD31) is expressed on the surface of differentiated and

undifferentiated vascular endothelial cells, and is considered the gold standard for evaluating tumor neovascularization[8, 9] and is commonly used to evaluate angiogenesis during RA[10]. Vascular endothelial growth factor (VEGF) is an important pro-inflammatory factor that has a role in the pathogenesis of RA, and is also the most important and most powerful known pro-angiogenic factor[11, 12]. Rheumatologists have examined the role of VEGF in the angiogenesis of RA, and the effect of inhibition of VEGF as a potential therapy for RA.

The general pathological process of ovalbumin-induced arthritis (OIA) in rabbits is similar to that of RA in humans[13]. In this study, we used this rabbit OIA model with longitudinal PDI and contrast-enhanced US (CEUS) measurements to assess angiogenesis during different stages of modeling, including inflammation, pannus formation, and fibrosis. We characterized the vascular morphology of the synovium using CEUS during the early stages, and determined the correlation of measured values with pathological indexes of angiogenesis.

Materials And Methods

Ovalbumin-induced arthritis model

A total of 30 male New Zealand white rabbits (Songlian Experimental Animal Farm, Songjiang District, Shanghai, license No. SCXK [Shanghai] 2012-0011) were randomized to an OIA group (n = 25) or a sham-injected (control) group (n = 5). Previous studies have described the development of OIA in these rabbits[14–16], which includes basic sensitization and joint sensitization. The reagents were albumin from chicken egg white (A-5378, Sigma-Aldrich, Burlington, MA, USA) and Freund's complete adjuvant (F-5881, Sigma-Aldrich, Burlington, MA, USA). The sham-injected rabbits received saline alone. The Animal Ethics and Welfare Committee of Fujian Medical University provided approval of this study protocol (reference number: SYXK [min] 2016-0008), and this study was performed according to the guidelines of the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) [17].

Unenhanced PDUS

The experiments were performed over 16 weeks. Routine US assessments (including two-dimensional US and PDUS) were performed at weeks 6, 8, 12, and 16. Imaging of both rear knee joints was performed using a GE LOGIQ E9 machine (GE Healthcare, Waukesha, WI, USA) using a linear array probe (transmission frequency: 7 to 12 MHz). A single sonographer who had experience in musculoskeletal disease performed all US procedures. Prior to imaging, rabbits were placed in a supine position and then anesthetized with 1% pentobarbital sodium (3 mL/kg into the ear vein).

PDUS of synovial blood was quantified using the following scale: 0, no signal; 1, minimal signal; 2, moderate signal; 3, strong signal)[18–20].

CEUS

Following PDUS, knee joints were injected with the contrast agent (SonoVue, 0.1 mL/kg; Bracco SpA, Milan, Italy) and then examined by real-time CEUS. Scanning at 15 frames per s began shortly before injecting the SonoVue, while the operator maintained a stable transducer. The beam was focused at the level of or immediately below the synovial proliferation, and the beam gain was at the minimum. The left and right knee joints were examined using the same US parameters and digital videos were recorded. Two investigators independently quantified synovial enhancement offline using the time-signal intensity curve (TIC) analysis on the LOGIQ E9. According to the International Arthritis Contrast Ultrasound (IACUS) grading standard, synovial US contrast was semi-quantitatively divided into 3 grades (0, 1, or 2)[21]. Each of the highly enhanced synovial regions was defined as a region of interest (ROI). Most ROIs had diameters of approximately 2 to 3 mm. For each joint, 3 ROIs were selected for plotting the contrast enhancement TIC curves, and averages were calculated. Peak intensity (PI), the area under curve (AUC), and time-to-peak (TTP) were recorded.

Ultrasound-guided synovial biopsy

US-guided biopsy was performed in the OIA rabbits after US imaging. The biopsy sample was from the thickest synovial plane in the same recesses examined by CEUS. A coaxial outer needle and an 18-gauge Magnum Biopsy Needle (Bard Medical, Covington, GA) was used for sampling the joint capsules. The needle was followed in real-time using a longitudinal sonogram, and the operator guide the needle to the biopsy site. After the CEUS examination, control rabbits were euthanized by injecting an overdose of sodium pentobarbital, and their knee joints were collected for histological investigation.

H&E staining and immunohistochemistry

Specimens were fixed in 10% formaldehyde for 24 h, embedded in paraffin, and cut into 5- μ m sections for H&E staining and immunohistochemical detection of CD31 and VEGF. Staining for the anti-CD31 antibody (MAB-0031, Maxim, Fuzhou, China) used the labeled streptavidin-biotin method (Dako LSAB kit, Glostrup, Denmark). The microvascular density (MVD) of a joint was defined as the mean number of vessels in 3 microscopic fields (400 \times) that had areas in which the MVD was increased. Staining for the anti-VEGF-A antibody (ab-1316; Abcam Biotechnology, Cambridge, MA) also used the labeled streptavidin-biotin method (Dako LSAB kit, Glostrup, Denmark). VEGF is mainly located in the cell membrane and cytoplasm, and positive staining ranges from yellow to brown. Three areas with the highest positive staining were selected at low magnification (100 \times), and a VEGF score was then determined according to stain intensity and area of positive cells at high-power magnification (200 \times). The stain intensity was scored as 0 (no staining), 1 (< 10% of staining), 2 (10 to 50% staining), or 3 (> 50% staining). The mean VEGF score was calculated by multiplying the percentage of stained cells by the intensity of the staining. The mean of the three regional scores was selected. All results were analyzed by two experienced pathologists who were double blinded.

Statistical analysis

SPSS software (ver. 24.0, IBM, USA) was used for statistical analysis. Data are expressed as means \pm SDs. Fisher's exact test was used to compare the vascular morphology of the rabbit model at the early

stage of CEUS enhancement and during different weeks of animal modeling. The synovial expression levels of CD31 and VEGF in the OIA group at different stages were compared using a one-way repeated measurement variance analysis, and the Bonferroni method was used for two-way comparisons. Pearson correlation analysis and Spearman correlation analysis were used to assess correlations, as appropriate. A P value below 0.05 was considered significant.

Results

Establishment of the rabbit model of RA

After injection of ovalbumin, rabbits in the OIA group developed increasingly swollen knee joints and impaired functional ability over time. In contrast, rabbits in the control group had no obvious abnormalities. Four rabbits in the OIA group died of trauma caused by puncture wounds during biopsy (2 at week-6, 1 at week-11, and 1 at week-14).

Pathological results

H&E staining indicated only a small number of blood vessels in the synovial tissues of rabbits in the control group (Fig. 1A). In contrast, over time the knee synovial stroma of rabbits in the OIA group became thicker, had a greater number of blood vessels, and an increased size of the lumen (Fig. 1B to E).

Staining for CD31 indicated low expression in the control group (Fig. 1F). However, expression of CD31 in the OIA group increased gradually over time (Fig. 1G to J). Staining for VEGF was also low in the control group (Fig. 1K). However, as with CD31, the expression of VEGF in the OIA group increased over time (Fig. 1L to O).

Quantitative analysis of the expression of CD31 (a marker of MVD) and VEGF in the knee synovium of mice in the OIA group indicated statistically significant increases in both markers over time (Table 1).

Table 1
 Longitudinal changes in MVD and VEGF expression in the
 knee synovia of rabbits in the OIA group.

Week	Tissues (n)	MVD (mean ± SD)	VEGF (mean ± SD)
6	52	81 ± 34	1.6 ± 0.4
8	52	89 ± 38	1.9 ± 0.6
12	52	95 ± 41	2.2 ± 0.5
16	52	98 ± 45	2.4 ± 0.5
F value		28.19	6.08
P value		< 0.001	< 0.05

Unenhanced PDI

Unenhanced PDUS and CEUS indicated no detectable intra-articular signals in the knees of the control group (10 knee joints examined; Fig. 2A). However, some rabbits in the OIA group showed obvious synovial hyperplasia of the knee joints beginning on week-5, and all rabbits in the OIA group had bilateral synovial hyperplasia of varying degrees (grade 1 to 3) on week-6. The unenhanced PDI showed that the synovial blood flow of rabbits in the OIA group had PDI grades of 0 to 3 from week-6 to week-16 (Fig. 2B to E).

Due to the death of 4 rabbits in the OIA group during the course of the experiments, we evaluated 42 knees of 21 rabbits in the OIA group by conventional US at week-6, week-8, week-12, and week-16. Quantitation of the results indicated that the PDI grade of joints increased significantly over time for rabbits in the OIA group (Table 2).

Table 2

Longitudinal changes in the number of joints with different PDI grades for rabbits in the OIA group.

Week	Joints (n)	Grade 0 (n)	Grade 1 (n)	Grade 2 (n)	Grade 3 (n)	PDI grade (mean \pm SD)
6	42	3	18	15	6	1.57 \pm 0.83
8	42	0	4	28	10	2.14 \pm 0.57
12	42	0	3	25	14	2.26 \pm 0.59
16	42	0	1	26	15	2.33 \pm 0.53
F value						36.71
P value						< 0.001

CEUS imaging

Contrast-enhanced imaging data were unavailable for 1 rabbit due to incomplete anesthesia at week-8. Thus, we recorded longitudinal changes of CEUS of 40 knees of 20 rabbits in the OIA group. The synovia of these knee joints became increasingly pathological over time (Fig. 3A to E). All images were taken during the enhancement period (15 to 30 s after injection of the contrast agent). Quantitation of these results indicated that the CEUS grade increased significantly over time (Table 3).

Table 3

Longitudinal changes in the number of joints with different CEUS grades for rabbits in the OIA group.

Week	Joints (n)	Grade 0 (n)	Grade 1 (n)	Grade 2 (n)	CEUS Grade (mean \pm SD)
6	40	2	11	27	1.63 \pm 0.59
8	40	2	2	36	1.85 \pm 0.48
12	40	2	4	34	1.80 \pm 0.52
16	40	1	5	34	1.83 \pm 0.45
F value					17.63
P value					< 0.01

CEUS of the longitudinal changes in vascular morphology of the synovia

We also used TIC analysis with quantitative CEUS to analyze longitudinal changes in the knee joints (Figs. 4 and 5), Table 4 shows Longitudinal changes in CEUS-TIC quantitative indexes of knee joints in OIA group

Table 4

Longitudinal changes in CEUS-TIC quantitative indexes of knee joints in OIA group. P values are provided for different weeks (P^a), different tissues (P^b), and cross-effects between weeks and tissues (P^c).

Week	Joints (n)	PI (dB), mean \pm SD		TTP (s), mean \pm SD		AUC, mean \pm SD	
		Synovium	Periarticular tissue	Synovium	Periarticular tissue	Synovium	Periarticular tissue
6	40	-59.25 \pm 9.15	-60.57 \pm 5.89	9.56 \pm 3.05	9.67 \pm 3.86	553.5 \pm 315.7	168.3 \pm 48.6
8	40	-52.75 \pm 8.03	-58.69 \pm 4.93	9.41 \pm 2.97	9.46 \pm 4.05	568.4 \pm 345.7	168.9 \pm 49.0
12	40	-55.48 \pm 10.56	-59.18 \pm 5.11	10.1 \pm 3.10	9.39 \pm 4.23	938.9 \pm 613.5	172.5 \pm 51.5
16	40	-61.13 \pm 9.61	-61.06 \pm 5.81	9.86 \pm 2.88	9.49 \pm 4.18	768.4 \pm 415.1	171.7 \pm 48.7
F ^a value (P^a value)		6.14 (0.012)		0.995(0.325)		21.93(0.001)	
F ^b value (P^b value)		32.46(0.003)		2.519(0.121)		7.56 (0.011)	
F ^c value (P^c value)		5.54 (0.024)		1.119(0.253)		2.33(0.040)	

During the early stage of disease, CEUS indicated enhanced morphology of synovial vessels as "scattered dots" (no obvious strip vessels, multiple dots in the synovial area), a "single stripe" (one strip in the synovial area), and "multiple stripes" (2 or more strips in the synovial area).

At week-6 and week-8, scattered dot enhancement was more common than in early-stage disease. Over time, the development of strip vessels increased. At week-12 and week-16, single and multiple strips and twisted large synovial vessels accounted for the majority, and the pathology was more severe at week-16. The change from week 6 to week 16 was statistically significant ($P = 0.047$). Table 5 shows the quantitation of these CEUS results.

Table 5
 Longitudinal changes in the vascular morphology based on
 CEUS enhancement in the OIA group.

Week	Joints (n)	Punctiform ^a (n)	Single strip (n)	Multi strip (n)
6	40	25	11	4
8	40	20	14	6
12	40	16	17	7
16	40	13	17	10

We also examined the correlation of MVD (CD31) and VEGF expression in the synovium with different US parameters. The results indicated that MVD and VEGF expression had significant positive correlations with PDI, CEUS grade, and PI (Table 6).

Table 6
 Pearson correlation analysis of different
 US parameters with MVD (CD31
 positivity) and VEGF expression based on
 multimodal ultrasound and pathological
 indexes of the synovium in the OIA group
 on week-6-16.

	MVD (CD31)	VEGF
PDI grade	0.451^b	0.432^a
CEUS grade	0.586^b	0.557^b
AUC	0.198	0.201
TTP(s)	0.218	0.237
PI(dB)	0.579^b	0.587^b
a: $p < 0.05$; b: $p < 0.01$		

Discussion

The major findings of the present 16-week longitudinal US study of OIA rabbits were that the knee joints had gradual increases in synovial neovascularization, lumen size, and tissue disorganization, and that they eventually developed uneven and irregular lumens. Pannus formed by neovascularization, together with synovial hypertrophy, and there was infiltration of inflammatory cells and collagen fibers. Pannus

formation developed during week-8, but did not cause cartilage and bone destruction at that time. At week-12, the pannus was more obvious, and cartilage destruction was evident. At week-16, the vascular lumen was greatly enlarged, and the synovium was increasingly fibrotic.

Based on the extent of neovascularization and fibrosis in the pannus, some researchers classify the pathogenesis of RA as inflammatory, fibrous, or mixed and classify synovitis as progressing from inflammation, to pannus formation, and then fibrosis[22]. During the earlier stages of inflammation and pannus formation, the pannus is mainly inflammatory or mixed; then, due to the formation of many fibrous components, RA enters the fibrotic or sclerotic stage. During week-6, our OIA rabbits had obvious synovitis, but the proliferation of blood vessels was not yet apparent. Considering that this was the inflammatory stage, pannus formation followed at week-8 and week-12. At week-16, the fibrosis was obvious, and this corresponded to the fibrosis stage of RA. Therefore, in our OIA model, the first 8 weeks correspond to early-stage RA (inflammatory stage), weeks 8 to 12 correspond to middle-stage RA (pannus stage), and weeks 12 to 16 weeks correspond to late-stage RA (fibrosis stage). Another of our notable findings is that OIA rabbits had increasing expression of CD31 over time ($P < 0.001$). Because CD31 functions in angiogenesis, this indicated that the extent of synovial vascularization increased as RA progressed, with the greatest vascularization in late-stage disease. These results also suggest that vascularization index in OIA rabbits, which is based on pathological vascularization, reflects the different stages of RA.

Previous studies reported greater synovial CD31 expression in patients with RA and osteoarthritis (OA) than in healthy controls[23], and that expression was greater in RA than OA[24]. In agreement, intra-articular inhibition of local angiogenesis had a therapeutic effect on RA but no effect on OA[24]. Inhibition of angiogenesis, as an adjunct or even an independent treatment of RA, can prevent the transport of nutrients to the synovium, possibly leading to vascular degeneration and disease reversion. Thus, inhibition of angiogenesis in the synovium appears to be an effective method to inhibit the progression of RA.

Most previous clinical studies of the synovial expression of VEGF and CD31 in RA had a cross-sectional designs[24, 25], and no longitudinal studies have yet evaluated changes in the synovium during the different stages of RA. A novelty of the present study of OIA rabbits (as a model of RA) is that we used the minimally invasive technique of US-guided synovial biopsy to assess longitudinal pathological changes. Our results indicated increased VEGF expression during the early stage of pathogenesis, and that expression continued to increase over time. Moreover, VEGF expression had a strong positive correlation with MVD (CD31 positivity) and synovial pathological score. The pathogenesis in OIA rabbits is similar to the pathogenesis in RA, in that there is upregulation of synovial angiogenesis during early pathogenesis, and persistence of angiogenesis as pathogenesis proceeds. Our results therefore suggest that continued high expression of VEGF may be an important factor in the formation of pannus and the aggravation of synovitis. Therefore, early prevention of VEGF overexpression by use of a VEGF inhibitor may block the development of synovial angiogenesis and inflammation.

The results of the present study clearly showed that as disease progressed in OIA rabbits, the synovium developed more new blood vessels, and there was increased PDI grade, and increased disease score. These rabbits also had a positive correlation of MVD (CD31 positivity) with disease severity based on PDI grade. However, our observations at week-6 and week-8 (during the early stages of neovascularization), indicated that a low proportion of knees had high-grade PDI, and the correlation of PDI grade with MVD was weak. This suggests that PDI has limited sensitivity for detect neovascularization during early-stage disease. This is likely because PDI can only detect blood flow signals in vessels with diameters greater than 100 μm [7], and synovial neovascularization during early-stage disease is mainly in the capillaries. This interpretation is consistent with our pathological examinations of synovial tissues during early-stage disease. In the clinic, some patients with RA are negative in PDI evaluation soon after treatment, but experience inflammation relapse at some period after treatment. This may be related to the inability of PDI to detect microvascular changes, and result in missed diagnosis of patients with early-stage disease. Another limitation of PDI is that it is difficult to distinguish whether an increased blood flow signal in the synovium is due to an increase in the number of blood vessels or circulatory congestion.

We observed that during the early stage of US enhancement (5 to 15 s after injection of the contrast agent), most synovial vessels had "scattered dot" enhancement during week-6 and week-8, but "strip" or "multi strip" morphology was predominant during week-12 and week-16. In particular, there were significant differences in the early vascular morphology during different stages of enhancement ($P < 0.05$). This may be because there are mostly immature microvessels with small diameters during the early stages of modeling, but that over time the synovial blood vessels develop and become larger as the lumen increases in size. Our detailed longitudinal examinations of synovial tissues of OIA rabbits during different stages of disease confirm this interpretation. Our results thus suggest that early US enhancement of synovial vessels may provide valuable information for the early diagnosis of RA.

During early-stage RA, newly formed blood vessels in the synovium mainly consist of immature and dilated small blood vessels, and there are more blood vessels than in the normal synovium[26]. This reflects significant changes in the blood volume of the synovium. Therefore, blood volume can be used as a direct indicator of angiogenesis during RA. On the premise that the dose of the contrast agent, instrument parameters (frequency, mechanical index, etc.), and size of the ROI are fixed, the peak intensity (PI) reflects the blood volume in the synovial tissue of the designated area during a unit of time[27]. Therefore, the PI can be used as an index of synovial neovascularization. Our longitudinal analysis indicated the PI value of OIA rabbits increased from week-6 to week-8, possibly because the synovium was in the stage of pannus formation at week-8, at which time there was greater vascular density and more local blood perfusion. However, we also found that the PI value at week-16 was lower than at week-8, possibly because the synovium entered the stage of pannus fibrosis, in which there were increased fibrotic components relative to inflammatory pannus.

Interestingly, our analysis of OIA rabbits during week-6 and week-8 indicated that a small number of knee joints had PDI grades of 0. At this time, CEUS indicated blood flow in the synovium, and positive correlations of CEUS grade, PI (a CEUS-TIC quantitative index), and synovium MVD (CD31). At week-6 and

week-8, the correlation coefficients of CEUS grade and PI with synovial MVD (CD31) were also significantly greater than those of PDI grade with synovial MVD (CD31). This suggests that CEUS can detect synovium neovascularization during early-stage RA. CEUS is more sensitive than PDI, and CEUS semi-quantitative and quantitative analyses are particularly important. However, CEUS also has some limitations, in that it is invasive, expensive, time-consuming, and can only evaluate a single plane or cross-sectional perfusion of one joint at a time. Therefore, from the practical and economic point of view, PDI remains the first choice for evaluation of synovial blood flow in patients with suspected RA. Based on our results, CEUS may be recommended to evaluate the synovial microcirculation in patients whose PDI results are negative but are suspected of having early RA.

Conclusion

There were several important results of this longitudinal study, which used US measurements to quantify changes in knee joint synovial vascularity in an OIA rabbit model of RA. First, the OIA rabbit model successfully simulated the course of RA. First, our pathological analysis of the synovium suggested these rabbits developed early-stage disease (inflammation) at weeks 4 and 8, middle-stage disease (pannus formation) at weeks 8 and 12, and late-stage disease (fibrosis) at weeks 12 and 16. Second, OIA rabbits had increased expression of CD31 and VEGF during the early phase, increased pannus formation during the middle stage, and fibrosis remained relatively stable from weeks 12 to 16. Third, our quantitative parameters from PDI and CEUS indicated that PI was the best indicator of synovial vascularization, and that increased vascularization (based on CEUS) occurred early during pathogenesis. Thus, CEUS may have value for the early diagnosis of RA. Fourth, multimodal ultrasound, especially when combined with measurements of the synovial expression of CD31, provided an accurate evaluation of the extent of synovial vascularization in rabbits with OIA.

Abbreviations

OIA: ovalbumin-induced arthritis; RA: rheumatoid arthritis; US: ultrasound; PDUS: power Doppler ultrasound; CEUS: contrast-enhanced ultrasound; VEGF: Vascular endothelial growth factor ; PDI: power Doppler imaging; CD31: differentiated protein cluster 31; TIC: time-signal intensity curve; IACUS: International Arthritis Contrast Ultrasound; ROI: region of interest; PI: Peak intensity; AUC: area under curve; TTP: time-to-peak; MVD: microvascular density; OA: osteoarthritis

Declarations

Availability of data and materials

Please contact author for data request.

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Contributors

All authors were involved in the study conception and manuscript design, manuscript drafting and revising, and final approval of the submitted version.

Competing interests

None to declare.

Ethics approval

The Animal Ethics and Welfare Committee of Fujian Medical University approved the protocol of this study (reference number of SYXK [min] 2016-0008).

Consent for publication

Not applicable.

Provenance and peer review

Not commissioned; externally peer reviewed.

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Figures

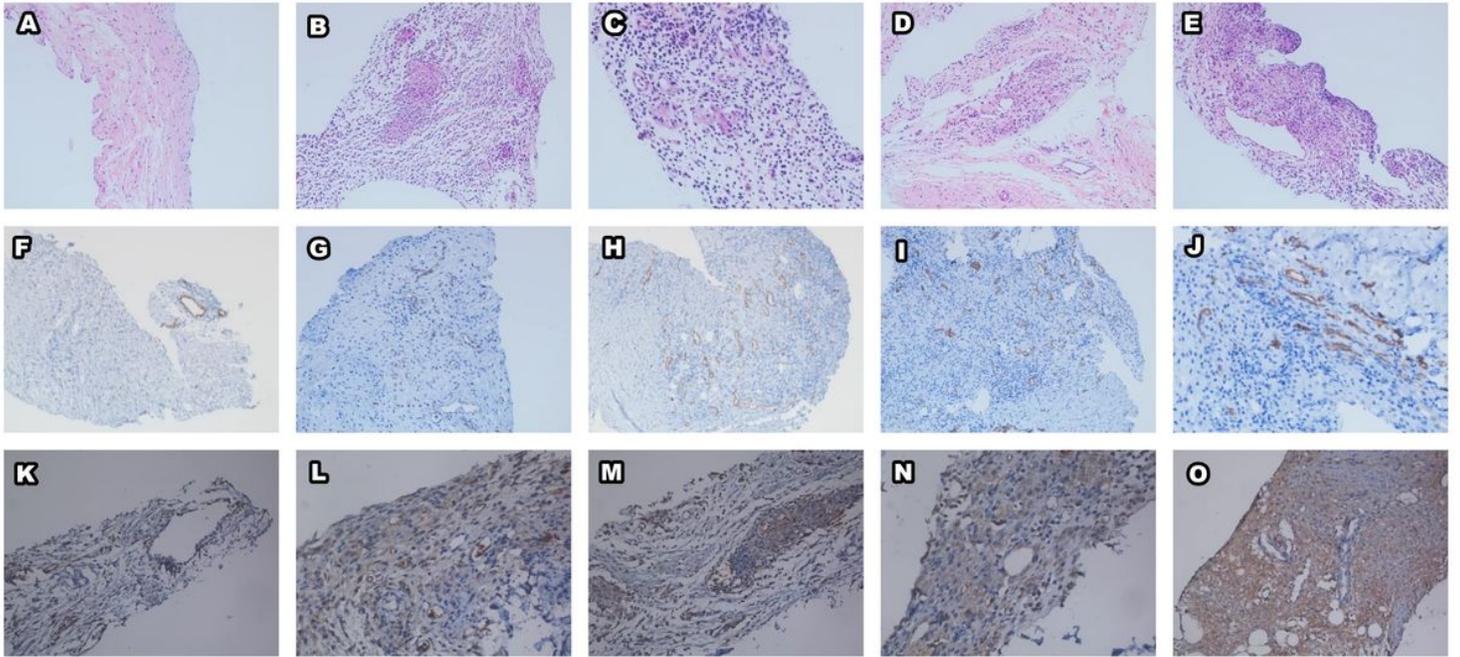


Figure 1

Longitudinal changes in H&E staining (top row) and immunohistochemical staining of CD31 (middle row) and VEGF-A (bottom row) in rabbit synovial tissues (200 \times). The control group was sampled on week-16, and samples were taken from the OIA group from week-6 to week-16. There were few blood vessels in the control group (A), but there were evident increases in vascularity and in size of the lumen in the OIA group (B to E). The OIA group also had increasing expression of CD31 (G to J) and VEGF (L to O) over time.

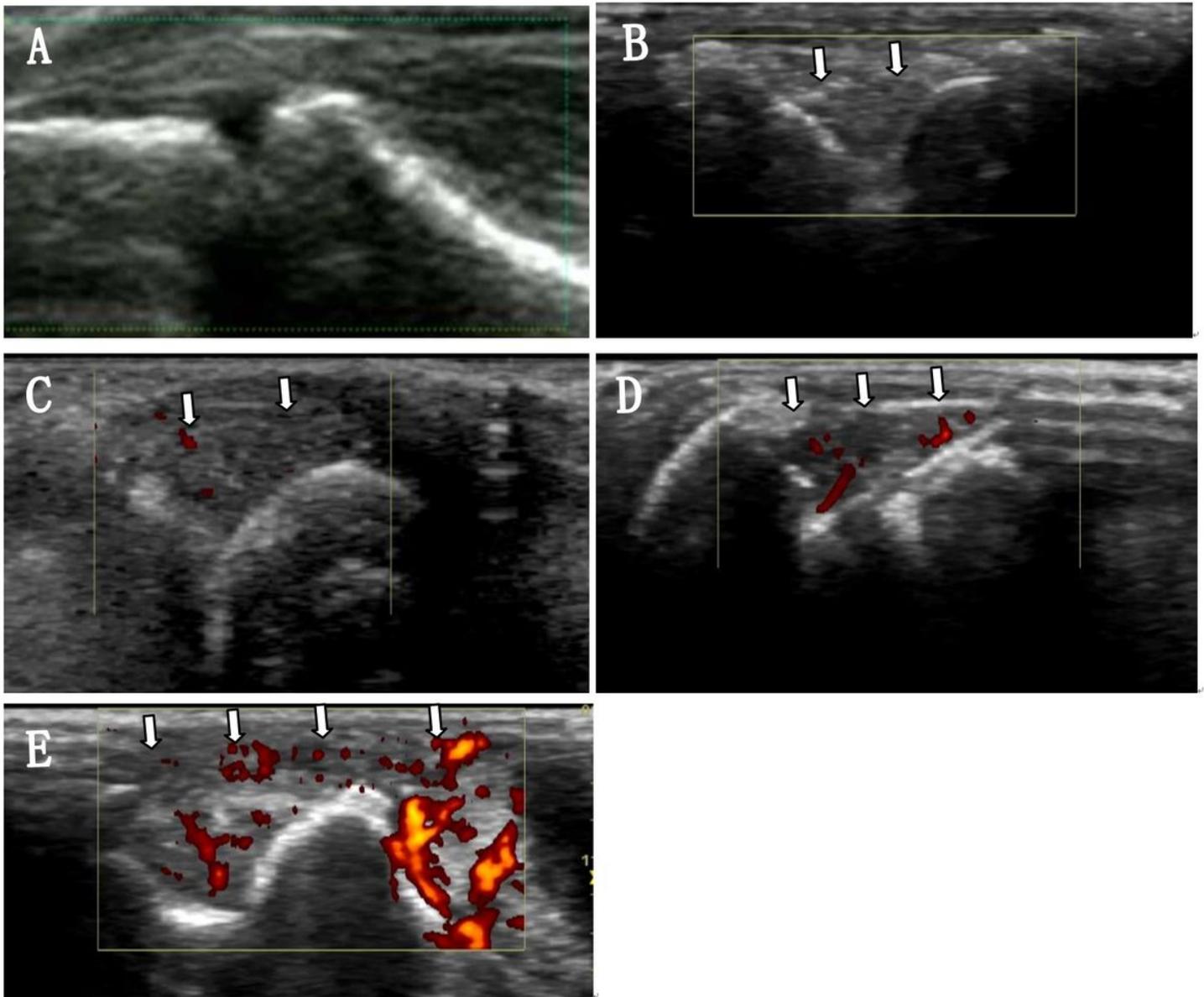


Figure 3

Representative PDI results in the control group (A) and longitudinal changes in the OIA group (B to E). (A) No synovial hyperplasia and no obvious blood flow in the control group. (B) PDI grade 0; no obvious blood flow (week-6). (C) PDI grade 1; scattered dots and small signals in the synovium (week-8). (D) PDI grade 2; increased blood flow in the hyperplastic synovium, with a thick strip and a tortuous course, but the positive area accounted for less than half of the total synovium; some destruction of bone cortex and formation of pannus (week-12). (E) PDI grade 3; abundant blood flow in the hyperplastic synovium, which was tree-like or reticular, tortuous, and disordered; the positive area accounted for more than half of synovium and the synovial blood flow extended to interrupt the bone cortex, suggesting increased pannus hyperplasia (week-16). Arrows indicate synovia.

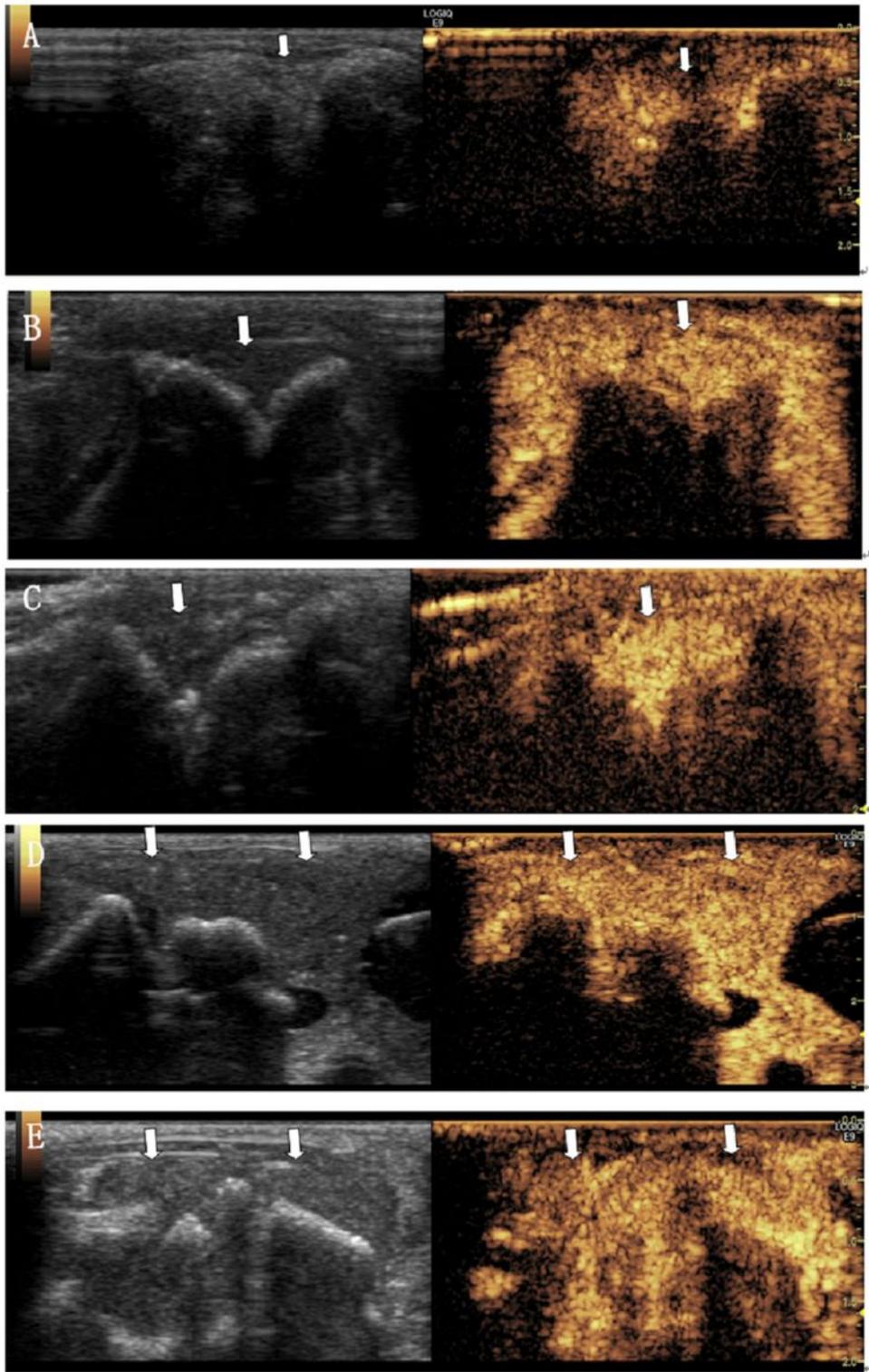


Figure 5

Representative CEUS results in the control group (A) and longitudinal changes in the OIA group (B to E). (A) Two-dimensional gray-scale (left) shows no obvious hyperplastic synovium, and the CEUS image (right) shows no enhancement in the synovial area, and that the contrast agent is evident in the surrounding tissue. (B) CEUS grade-1; the two-dimensional gray scale image shows a hyperplastic synovium and the CEUS image shows that the hyperplastic synovium was enhanced overall, with slightly

lower intensity than the surrounding tissue (week-6). (C) CEUS grade-2; the two-dimensional gray scale image shows a hyperplastic synovium and the CEUS image shows that the hyperplastic synovium was enhanced overall, with a stronger signal than the surrounding tissue (week-8). (D) CEUS grade-2; the two-dimensional gray-scale image shows a hyperplastic synovium, and the CEUS image shows that the hyperplastic synovium is uniformly enhanced, and its strength is greater than the tissues around the joint (week-12). (E) CEUS grade-2; the two-dimensional gray scale image shows a hyperplastic synovium and the CEUS image shows an inhomogeneous enhancement of the hyperplastic synovium, with a greater signal strength than the tissues around the joint (week-16). Arrows indicate synovia.

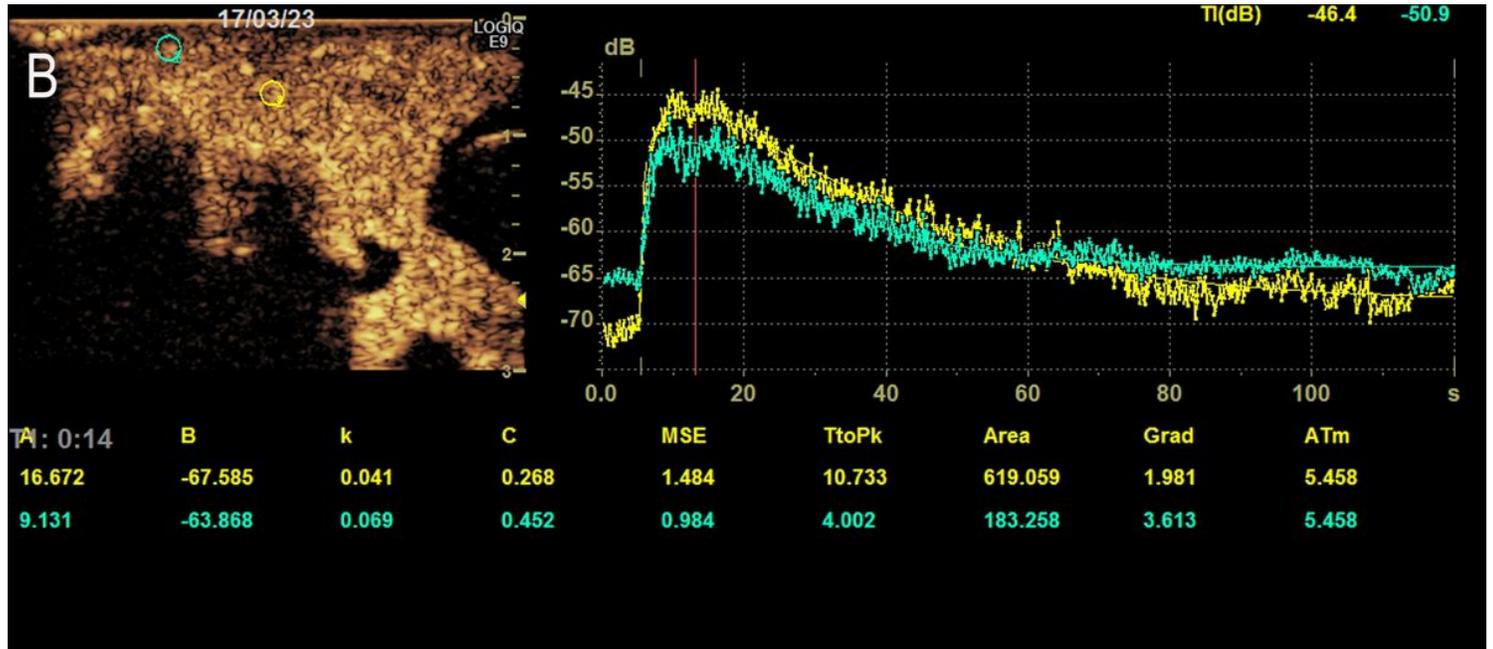


Figure 7

Representative quantitative CEUS obtained by TIC analysis (yellow: synovial ROI, green: periarticular tissue).

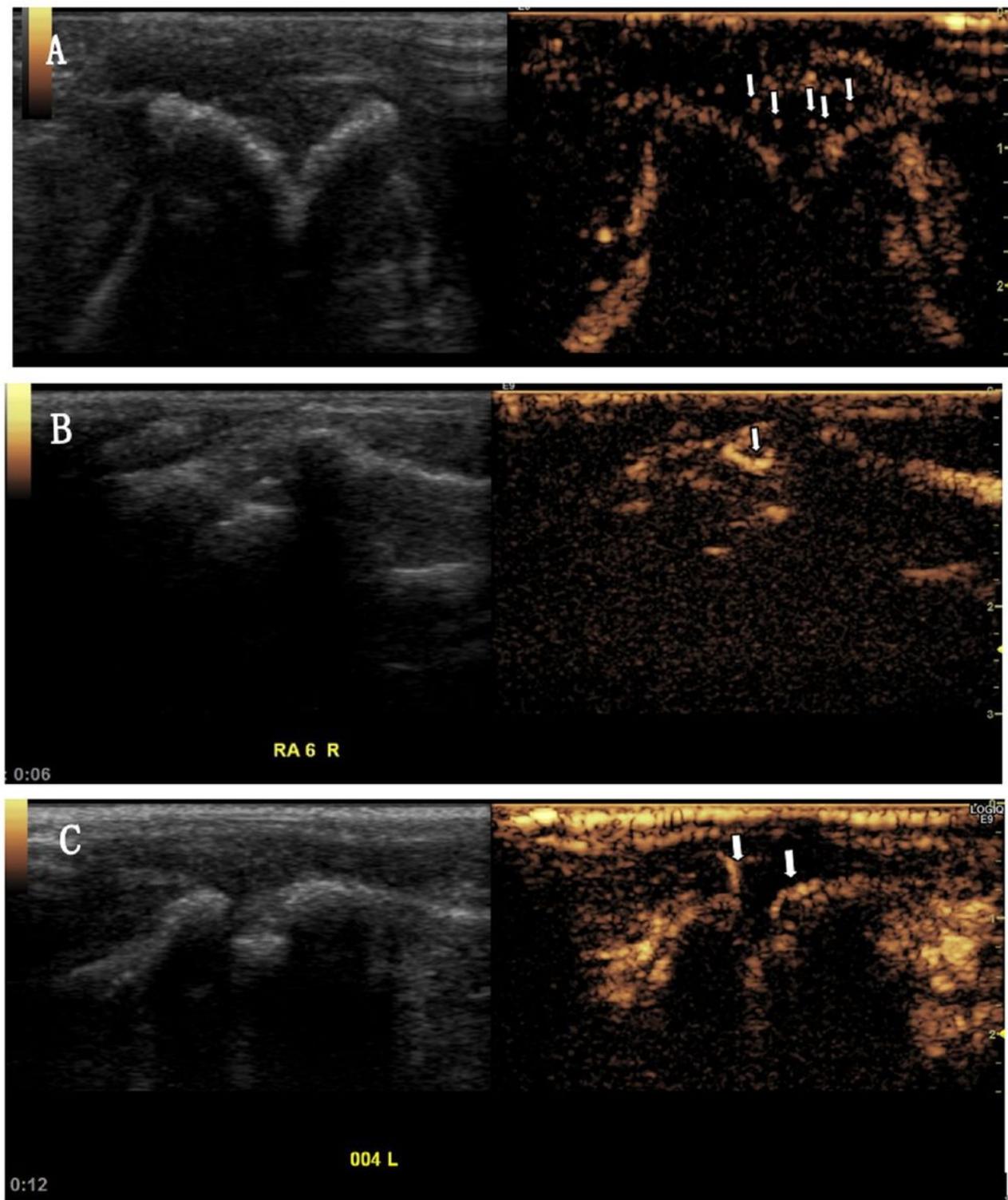


Figure 9

CEUS of vascular morphology of synovium during early-stage disease. (A) week-6; scattered dot like enhancement, (B) week-8; single enhancement, (C) week-12; multiple enhancement. Arrows indicate synovial vessels.