Inhibitory effect of low-intensity pulsed ultrasound on the fibrosis of the infrapatellar fat pad through the regulation of HIF-1α in a carrageenan-induced knee osteoarthritis rat model

TAKASHI KITAGAWA[1,2], HIROHISA KAWAHATA[1,3], MOTOKUNI AOKI[1,3,4] and SHINTAROU KUDO[1,3,4]

[1]Graduate School of Health Sciences, Morinomiya University of Medical Sciences, Suminoe-ku, Osaka 559-8611;
[2]Department of Rehabilitation, Higashiosaka Hospital, Joto-ku, Osaka 536-0005;
[3]Inclusive Medical Sciences Research Institute and [4]Department of Physical Therapy,
Morinomiya University of Medical Sciences, Suminoe-ku, Osaka 559-8611, Japan

Received February 21, 2022; Accepted July 7, 2022

DOI: 10.3892/br.2022.1562

Abstract. Fibrotic changes in the infrapatellar fat pad (IFP) are involved in the pathogenesis of knee osteoarthritis (KOA). HIF-1α is a transcription factor that is activated during hypoxia and is suggested to play a role in fibrosis in various organs. However, its participation in the fibrotic changes in IFP remains unclear. Therefore, we investigated the role of HIF-1α in IFP fibrosis using a carrageenan-induced KOA rat model and evaluated the potential of low-intensity pulsed ultrasound (LIPUS) as a novel treatment for KOA. A rat model was prepared by intra-articular injection of 0.5% carrageenan (50 μl) using 8-week-old male Wistar rats. Fibrosis of the IFP was evaluated histologically by hematoxylin and eosin and Sirius Red staining at 1 and 2 weeks after intra-articular injection. The mRNA expression levels of HIF-1α and fibrosis-related molecules, CTGF and VEGF, were analyzed using reverse transcription-quantitative PCR, and the DNA binding activity of HIF-1α was assessed using a binding assay. In addition, the effect of irradiation with LIPUS on the fibrosis of IFP was verified. Histological studies demonstrated a significant increase in the fibrosis of IFP 1 and 2 weeks after intra-articular injection of carrageenan, accompanied by overexpression of CTGF and VEGF, which was followed by upregulation of transcriptional activation of HIF-1α. Moreover, intervention with LIPUS for 2 weeks after injection of carrageenan attenuated fibrosis of IFP, accompanied by a significant reduction in the transcriptional activation of HIF-1α and decreased the gene expression levels of HIF-1α, CTGF, and VEGF. The present study demonstrated that activation of HIF-1α promoted fibrosis of IFP in carrageenan-induced arthritis in rats and that intervention with LIPUS decreased the activity of HIF-1α and inhibited fibrosis. These results suggest that LIPUS may serve as a novel approach for the treatment of KOA, through its modulation of HIF-1α.

Correspondence to: Professor Shintarou Kudo, Graduate School of Health Sciences, Morinomiya University of Medical Sciences, 1-26-16, Nanko-kita, Suminoe-ku, Osaka 559-8611, Japan
E-mail: shintarou.iimt@gmail.com

Key words: infrapatellar fat pad, knee osteoarthritis, fibrosis, HIF-1α, low-intensity pulsed ultrasound

Introduction

Knee osteoarthritis (KOA) is a chronic disease characterized by degenerative changes in the articular cartilage, structural changes in the subchondral bone, and secondary synovitis. Patients with KOA have a limited range of motion, experience joint pain, and exhibit gait disturbances, and serious KOA interferes with daily life (1). Although radiographic findings do not necessarily correlate with the symptoms of KOA (2), and a previous report suggested that degenerative and structural changes in synovitis and bone marrow lesions are important factors that cause joint pain and impair the quality of life (3). In a clinical study using MRI, Hill *et al* (4) reported that synovitis in KOA correlates with pain severity, and more recently, it was reported that in addition to the synovial tissue, fibrotic changes in the infrapatellar fat pad (IFP) are also involved in the pathogenesis and mechanism of joint pain in KOA (5).

The IFP is the fatty tissue that fills the space in the knee joint surrounded by the patellar tendon, proximal tibia, and femoral condyle at the inferior border of the patella (6). The IFP is in contact with the superior edge of the patella, the medial patellar retinaculum, and the lateral patellar retinaculum, and plays an important role in the smooth movement of knee joints (7). Furthermore, the IFP is histologically a collagen-rich fibrous adipose tissue sequential to synovial tissue and is innervated by branches of the posterior tibial nerve, which are distributed by nerve fibers with free nerve endings (8). It also acts as a buffer against joint loading by adjusting the contact pressure on the knee joints during joint movement (9).

Furthermore, the IFP is known to secrete several growth factors and cytokines and is reported to affect the metabolism of articular cartilage and synovial tissue. Distel *et al* (10) demonstrated that the expression of proinflammatory cytokines and IL-6 expression in the IFP and subcutaneous adipose

tissue were elevated in obese patients, suggesting that cytokine secretion from the IFP may contribute to the degeneration of articular cartilage. It has also been reported that lymphocyte infiltration, angiogenesis (11), and an increasing number of cells expressing monocyte chemoattractant protein-1 (MCP-1) and IL-6 were observed in the IFP of KOA patients. Thus, the IFP is an important tissue in the progression of KOA from the perspective of induction of inflammation in knee joints. Moreover, the IFP is reported to be involved in the fibrosis of synovial tissues (12), and changes in the mechanical properties of IFP by fibrosis reduce the buffering capacity against joint loading (13,14). Therefore, fibrosis of the IFP is a major cause of functional impairment and pain in KOA patients. However, the mechanism underlying IFP fibrosis remains unclear.

In general, tissue fibrosis is observed as a physiological event with the deposition of extracellular matrix components such as collagen (15). Cirrhosis of the liver, renal disease, and pulmonary fibrosis are known to involve severe fibrosis, which is hypothesized to be a response to chronic inflammation (16,17). Hypoxia is also considered to be strongly involved in fibrosis in various adipose tissues *in vivo* (18). Studies on obese patients have demonstrated that an increase in the amount of adipose tissue results in hypoxic conditions in the tissue by delayed angiogenesis, which activates the transcription factor, HIF-1α, and causes fibrosis in adipose tissues (19). Thus, HIF-1α may also be a key molecule in the fibrosis of IFP in the pathogenesis of KOA. Regarding the relationship between hypoxia and KOA, it was reported that HIF-1α in articular cartilage and synovial fluid is activated and is correlated with the severity of KOA (20). In addition, Sotobayashi *et al* (21) demonstrated that the transcriptional activity of HIF-1α in the synovial tissue was increased in a contracture model of joint immobilization, resulting in synovial tissue fibrosis. These results strongly suggest that the increased expression and activation of HIF-1α are important factors that promote fibrosis in IFP in the pathogenesis of KOA.

In this study, we focused on HIF-1α and investigated the direct involvement of HIF-1α in the mechanism of fibrosis of IFP accompanied by the progression of KOA using an animal model. In addition, to examine a useful and practical management approach for KOA, the inhibitory effect of low-intensity pulsed ultrasound (LIPUS) on HIF-1α activation and fibrosis in IFP was evaluated, as it has been reported that LIPUS attenuates the fibrosis of synovial tissues followed by reduced activation of HIF-1α in a joint contracture model (23,24).

Materials and methods

KOA model. A KOA model was established and LIPUS intervention was performed on male Wistar rats weighing 300-400 g, obtained from Japan SLC. Animals were anesthetized with 1.5% isoflurane mixed with oxygen using inhalation anesthesia and were maintained on the same concentration of anesthetic throughout the entirety of the procedure. Animals were then subjected to 0.5% carrageenan injection into the bilateral knees (25). Animals were sacrificed by intraperitoneal administration of an overdose of 1-1.5 ml pentobarbitone (150-200 mg/kg), which amounted to 64.8 mg/ml pentobarbitone. Death was confirmed by checking for cardiac arrest, after which the animal was observed for ~5 min. After ensuring that there were no signs of recovery, tissues were harvested from the animals.

The unilateral knee joints of the rats were treated with LIPUS (BR Sonic-Pro, Ito Co., Ltd.). LIPUS was set at a frequency of 3 MHz and an output power of 120 mW/cm^2, based on a previous study, and was performed for 15 min a day, 4 times a week, every other day (23).

The samples were isolated and used for reverse transcription-quantitative (RT-q)PCR, ELISA, and histological analysis.

In addition, the IFP was harvested along with the synovial tissues after dissection at the inferior pole of the patella and flipped with the patellar tendon (26).

Ethics statement. All animal procedures were approved by the Ethics Committee for Animal Experiments of the Morinomiya University of Medical Sciences (approval no. 2019A001) and performed in accordance with our institutional guidelines. The animal procedures were also performed in compliance with the law (no. 105) and notification (no. 6) of the Japanese government and conducted in accordance with the guidelines of the National Research Council. All surgeries were performed under anesthesia, and all efforts were made to minimize suffering. Signs of significant distress in animals, such as joint infection, behavioral restriction due to excessive pain, and avoidance behavior were considered humane endpoints requiring immediate intervention. However, there were no cases requiring euthanasia due to observation of a humane endpoint and, therefore, animals were euthanized only at the end of the experimental period.

Histological and immunohistochemical studies. The excised joint was decalcified with Morse's solution as previously described (27) and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4˚C. Then, the excised joint was processed for routine paraffin embedding. Tissue cross-sections (5 μm) were rehydrated and stained with immersion in hematoxylin and eosin (HE) solution for 3-5 min at room temperature (28).

Toluidine blue staining and collagen staining were also performed to evaluate the progression of osteoarthritis (OA) caused by carrageenan and for measuring the OASRI score (29). For toluidine blue staining, tissues were immersed in the Toluidine blue solution for 15 min at room temperature. For total collagen staining, sections were rehydrated and incubated using a Picrosirius Red Stain Kit for 2 h at room temperature according to the manufacturer's protocol (SR; Polysciences, Inc.), which stains collagen I and III. The stained area was measured using ImageJ (National Institutes of Health. Version 1.48) (21).

For immunohistochemical (IHC) staining, the anti-RM-4 antibody (cat. no. KT014, Medical Chemistry Pharmaceutical Inc.) was used to analyze the infiltration of macrophages in the IFP. IHC staining was performed using the high polymer HISTOFINE simple stain mouse MAX-PO (Nichirei Bioscience Inc.) method, as described previously (21). Briefly, 5 μm-thick sections were deparaffinized, rehydrated before blocking endogenous peroxidase activity with 3% hydrogen peroxide, and preincubated with 1.5% blocking reagent (Roche Applied Science) in TBS at room temperature for 1 h. Diluted

primary antibodies (1:500) were then applied to the sections, and these sections were further incubated at room temperature for 1 h. Following this, the sections were rinsed twice with TBS for 5 min each and incubated with HISTOFINE simple stain mouse MAX-PO (rat) (Nichirei Bioscience Inc.) for 30 min at room temperature. Peroxidase activity was visualized by treatment with 0.05% diaminobenzidine containing 0.3% hydrogen peroxide. The sections were rinsed in water, dehydrated, cleared, and mounted.

RT-qPCR. Total RNA was extracted from the knee capsule, excluding the cartilage and meniscus. Excised tissues were homogenized in cold PBS and centrifuged at 20,000 x g for 15 min at 4˚C. Total RNA was extracted from the tissue samples using ISOGEN II (Nippon Gene) and resuspended in PBS, and its purity was assessed by spectrophotometry. Only samples with an A260/A280 ratio in the range of 1.8-2.0 were used. cDNA was synthesized using an iScript cDNA synthesis kit according to the manufacturer's protocol (Bio-Rad Laboratories, Inc.). Amplification reactions were performed using SsoFast EvaGreen Supermix (Bio-Rad Laboratories), with 100 μm of each primer and 1 μg cDNA in a final volume of 20 μl. Amplification reactions were performed in a MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.), with the following amplification protocol: Initial denaturation of 1 min at 95˚C; followed by 40 cycles of 1 sec at 95˚C and 5 sec at 61-65˚C, with each primer. The expression levels of hypoxanthine-guanine phosphoribosyltransferase (*HRPT*) as a housekeeping gene were used as the internal control, and the comparative Cq method ($2^{-\Delta\Delta Cq}$) was used to quantify the gene expression levels (30). Oligonucleotides were synthesized using Gene Design Inc. (Ibaragi, Osaka, Japan). The primer sequences used were obtained from previous studies (21,22,27,31) and are listed in Table I.

ELISA. Synovial tissue including IFP was removed from the knee joint. Proteins were extracted from the knee IFP. Extraction was performed using a Nuclear Extract Kit (Active Motif) according to the manufacturer's protocol. The binding activity of HIF-1α in the homogenized tissue was measured using an HIF-1α transcription factor assay kit, according to the manufacturer's instructions (cat. no. ab133104, Abcam).

Statistical analysis. All data are presented as the mean ± SEM. The distribution of the data was analyzed first and subsequently analyzed using a one-way ANOVA followed by a Tukey-Kramer post-hoc test or a Kruskal-Wallis followed by a Steel-Dwass post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Histological analysis of fibrosis in IFP. Histological analysis revealed that intra-articular administration of carrageenan resulted in significant progress in the pathogenesis of OA, OARSI score: Saline, 0.6±0.55; Car 1 week, 1.8±0.84 (P<0.05 vs. Saline); and Car 2 weeks, 2.4±0.55 (P<0.01 vs. Saline). HE, SR, and IHC staining for macrophages showed cellular infiltration and fibrotic tissue proliferation in the adipose tissue of IFP at 1 and 2 weeks after intra-articular injection of carrageenan, and cellular infiltration and fibrosis were higher after 2 weeks compared with after 1 week (Fig. 1A).

Table I. Sequences of the primers.

Name	Sequence, 5'-3'	(Refs.)
CTGF		(21,22)
Forward	CACCCGGGTTACCAATGACAA	
Reverse	AGCCCGGTAGGTCTTCACACTG	
VEGF		(21)
Forward	GCAATGATGAAGCCCTGGAG	
Reverse	GGTGAGGTTTGATCCGCATG	
HIF-1α		(25)
Forward	ACCGTGCCCCTACTATGTCG	
Reverse	GCCTTGTATGGGAGCATTAACTT	
HRPT		(25)
Forward	TGTTTGTGTCATCAGCGAAAGTG	
Reverse	ATTCAACTTGCCGCTGTCTTTTA	

SR staining, which is specific for type I and III collagen, demonstrated that significant collagen fibril proliferation was observed after both 1 and 2 weeks (middle-left and middle-right panels in Fig. 1A). The area stained was significantly increased 1 and 2 weeks after treatment with carrageenan compared with that of the saline group. Furthermore, the stained area was greater after 2 weeks of treatment with carrageenan compared with after 1 week (Fig. 1B).

Gene expression of fibrosis-related molecules and HIF-1α in the IFP. We quantitatively analyzed the mRNA expression levels of CTGF and VEGF, well-known major factors involved in the mechanisms of fibrosis (32-34), and found that their expression was significantly increased 1 week after intra-articular injection of carrageenan compared with the expression after saline injection and remained high after 2 weeks (Fig. 2A and B). Moreover, the mRNA expression levels of HIF-1α, which regulates and induces the expression of CTGF and VEGF at the genetic level, were higher 1 and 2 weeks after intra-articular injection of carrageenan compared with that after saline injection (Fig. 2C). Furthermore, the transcriptional activity was significantly increased 2 weeks after the intra-articular injection of carrageenan compared with that after saline injection (saline, 0.558±0.028; carrageenan, 0.597±0.020; P<0.05).

Inhibitory effect of LIPUS on fibrosis in IFP. We also examined the inhibitory effect of LIPUS on fibrosis in IFP. Histological analysis showed that the area stained by SR was significantly decreased in IFP treated with LIPUS for 2 weeks after intra-articular injection of carrageenan (Car + LIPUS group) compared with that of IFP without LIPUS after injection (Car group) (inside the square box of left panels in Fig. 3A and B), indicating that synovial fibrosis was attenuated by LIPUS intervention. The HIF-1α mRNA levels were significantly lower in the Car + LIPUS group in both 1 and 2-week time points compared with that in the Car group (Fig. 4A). The

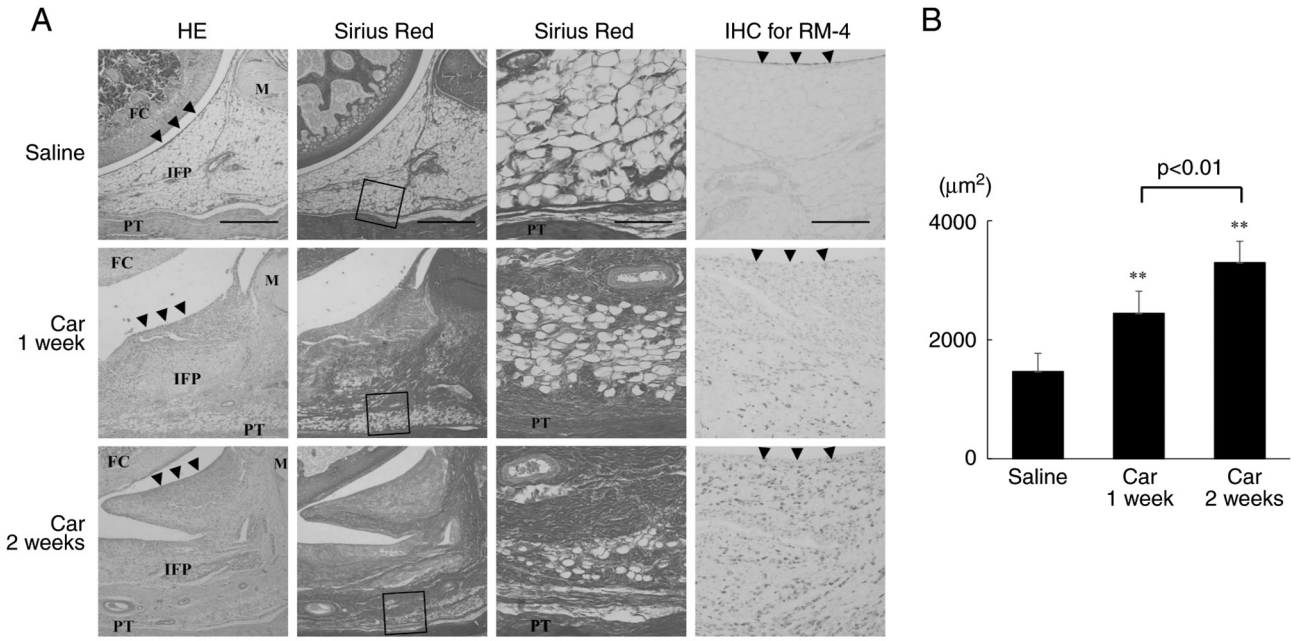

Figure 1. Histological analysis of the IFP. (A) HE staining (left panels), SR staining (middle-left and middle-right panels) for analysis of collagen in the fibrosis of the IFP, and IHC staining for RM-4 (right panels). Left panel: magnification, x40; scale bar, 500 μm. Middle-left panel: magnification, x40; scale bar, 500 μm. Middle-right panel: magnification, x200; scale bar, 100 μm. Right panel, magnification, x100; scale bar, 200 μm. Arrowheads, synovial membrane. (B) Quantitative analysis of the volume of collagen in the synovium measured using ImageJ. **P<0.01 vs. saline. n=5 per group. Data are presented as the mean ± SEM. HE, hematoxylin and eosin staining; SR, Sirius Red staining; IHC, immunohistochemical staining; IFP, infrapatellar fat pad; FC, femoral condyle; M, meniscus; PT, patella tendon; Saline, a rat knee injected only with saline; Car 1 week, a rat knee injected 1 week after intra-articular injection of carrageenan; Car 2 week, a rat knee injected 2 weeks after intra-articular injection of carrageenan.

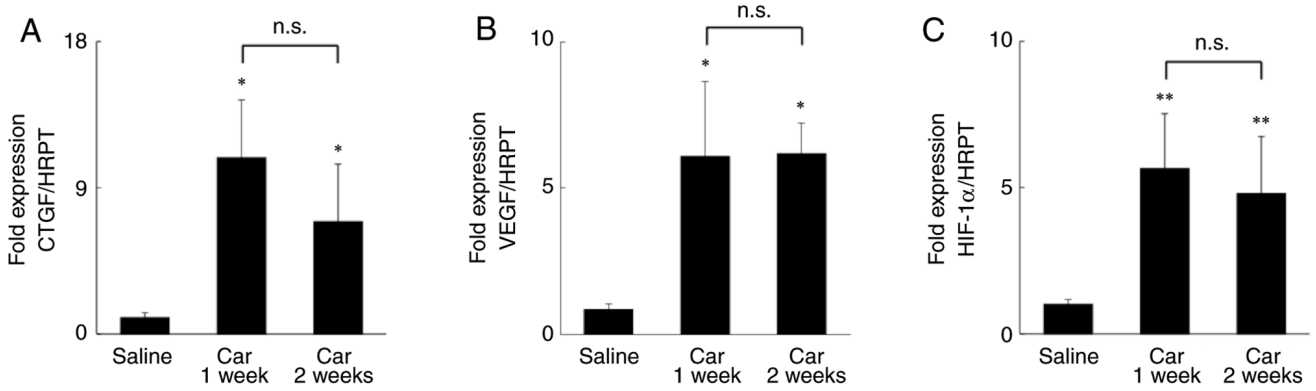

Figure 2. mRNA expression levels of CTGF, VEGF, and HIF-1α in the IFP of a rat knee. Relative mRNA expressions of (A) CTGF, (B) VEGF, and (C) HIF-1α. *P<0.05, **P<0.01 vs. saline. n=5 per group. Data are presented as the mean ± SEM. CTGF, connective tissue growth factor; VEGF, vascular endothelial growth factor; Saline, a rat knee injected only with saline; Car 1 week, a rat knee injected 1 week after intra-articular injection of carrageenan; Car 2 week, a rat knee injected 2 weeks after intra-articular injection of carrageenan.

HIF-1α binding activity was lower in the Car + LIPUS group compared with the Car group 1 week after intra-articular injection of carrageenan (Car group, 0.531±0.012; Car + LIPUS group, 0.513±0.011; P<0.05). In addition, intervention with LIPUS resulted in the inhibition of the expression of CTGF and VEGF 1 and 2 weeks after injection of carrageenan (Fig. 4B and C).

Discussion

The IFP in patients with KOA has been reported to show hypo-intensity on MRI scans, which is indicative of fibrosis (35,36), and fibrotic IFP has been hypothesized to be correlated with the symptoms of KOA and articular cartilage damage (36). Fibrosis of IFP has a major impact on the pathogenesis of KOA and controlling the fibrosis of IFP may be a novel avenue for the management of KOA, in addition to conventional drug-based and physical therapy.

Pathological fibrosis induced by inflammation is widely observed in various diseases such as liver cirrhosis, nephrosclerosis, and pulmonary fibrosis and is caused by the activation of myofibroblasts, which play a major role in tissue repair and induce the production and accumulation of extracellular matrix components such as collagen (16). TGF-β and various inflammatory cytokines secreted by innate immune cells such as infiltrated macrophages are considered

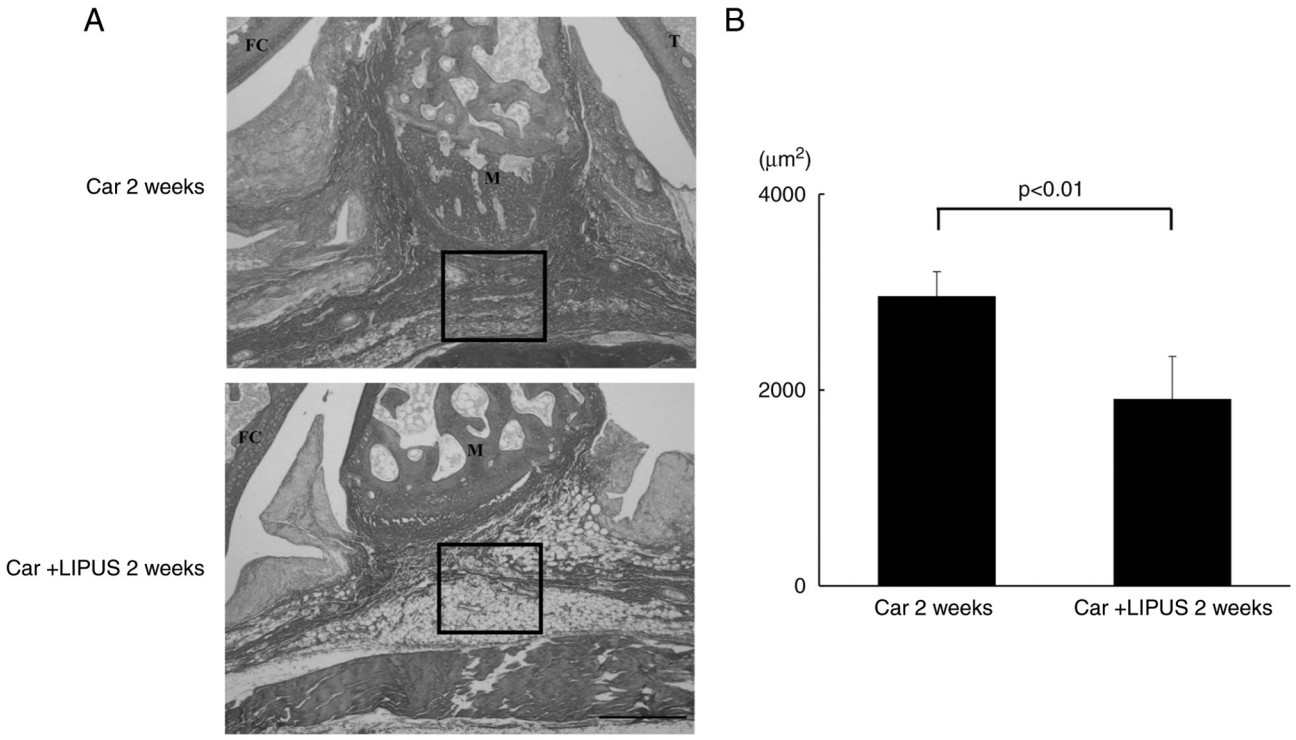

Figure 3. Histological analysis of the effect of LIPUS on the fibrosis of the IFP assessed by SR staining. (A) SR staining. magnification, x200; scale bar, 100 μm. Upper panel, Car 2 weeks. Lower panel. (B) Quantitative analysis of the volume of collagen in the synovium measured using ImageJ. n=5 per group. Data are presented as the mean ± SEM. LIPUS, low-intensity pulsed ultrasound; IFP, infrapatellar fat pad; SR, Sirius Red; FC, femoral condyle; M, meniscus; T, tibia; Car 2 week, a rat knee injected 2 weeks after intra-articular injection of carrageenan; Car + LIPUS 2 weeks, Car + LIPUS 2 weeks, a rat knee treated with LIPUS 2 weeks after injection of carrageenan.

to activate myofibroblasts and promote the production of collagen (37). The same mechanisms occur in the IFP, which include inflammatory cell infiltration followed by fibrosis, in carrageenan-induced animal models of KOA, and in other OA-induced models using ACLT or monoiodoacetate (38-41). Furthermore, it has been demonstrated that macrophages migrate into hypoxic tissues to secrete inflammatory cytokines (42), and HIF-1α, which is a transcription factor that is activated in response to hypoxic conditions, is intricately involved in macrophage migration and its functions (43). These findings suggest that a hypoxic environment that induces macrophage infiltration via activation of HIF-1α may contribute to fibrosis in IFP. In the present study, significant macrophage infiltration was observed in the IFP of the rat KOA model immediately after carrageenan administration, suggesting the presence of a hypoxic environment in the IFP (Fig. 1A). In addition, the results demonstrated that the expression and activity of HIF-1α increased after 1 week in the IFP.

Importantly, pathological fibrosis is primarily caused by fibrosis-associated growth factors such as CTGF and VEGF, whose expression is regulated by the transactivation of HIF-1α (32-34). Sotobayashi *et al* (21) demonstrated that HIF-1α activation induced by joint immobilization promoted the expression of CTGF and VEGF, resulting in the development of synovial tissue fibrosis that leads to joint contracture, followed by immobilization and disuse. In addition, they also provided important results indicating that inhibition of HIF-1α activity by decoy oligonucleotides attenuated fibrosis of synovial tissue through suppression of the expression of CTGF and VEGF. Yabe *et al* (44) showed that the expression of HIF-1 was increased in a joint contracture animal model. These results strongly suggest that the increased expression and activation of HIF-1α shown in the present study are involved in the mechanisms of fibrosis in IFP through upregulation of CTGF and VEGF. In fact, the present study demonstrated not only increased gene expression and activation of HIF-1α, but also induced expression of CTGF and VEGF, followed by accumulation of type I collagen. Therefore, it is hypothesized that HIF-1α is involved in the fibrosis of IFP in KOA and that regulation of HIF-1α may serve as a novel therapeutic strategy for the management of KOA.

Interestingly, in this study, LIPUS was applied to decrease the expression of HIF-1α based on our previous study (23), and intervention with LIPUS inhibited the gene expression levels of fibrosis-related factors such as CTGF and VEGF, which were suppressed through attenuation of both gene expression and HIF-1α activation. These results are also supported by recent studies reporting similar findings regarding the effect of LIPUS on the activation of HIF-1α and its detailed mechanisms (23). miR-31-5p, which regulates the cytoskeleton, was identified as a mechanosensitive miRNA following LIPUS stimulation, and LIPUS prevented long-term hypoxia-induced myocardial fibrosis by regulating the HIF-1α/DNA methyltransferase 3α signaling pathway through the mechanosensitive protein TRAAK (45,46). These reports suggest that the unique stimulation of microscopic vibrations by LIPUS directly affects the regulation of transcriptional activation of HIF-1α via induction of mechanosensitive molecules. Moreover, LIPUS treatment successfully decreased the fibrotic lesions in the IFP in the rat model of KOA, suggesting that LIPUS is an effective device

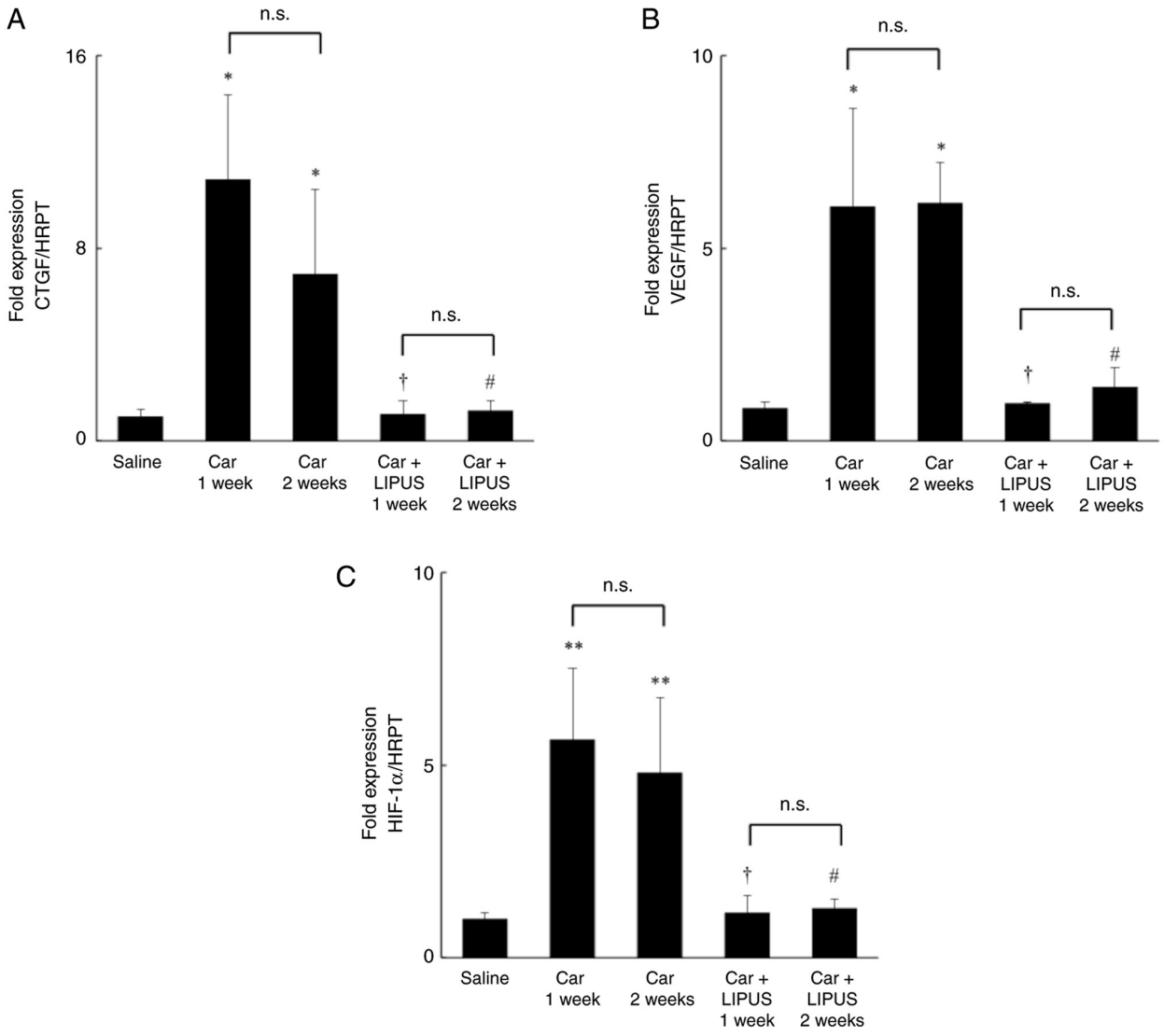

Figure 4. Effect of LIPUS on the mRNA expression of HIF-1α, CTGF, and VEGF in the IFP of a rat knee. Relative mRNA expressions levels of (A) CTGF, (B) VEGF, (C) HIF-1α. Car 1 week, 2 weeks, a rat knee injected at 1 or 2 weeks after intra-articular carrageenan injection. *P<0.05, **P<0.01 vs. saline; †P<0.05 vs. Car 1 week; $^{\#}$P<0.05 vs. Car 2 weeks. Data are presented as the mean ± SEM. LIPUS, low-intensity pulsed ultrasound; IFP, infrapatellar fat pad; CTGF, connective tissue growth factor; VEGF, vascular endothelial growth factor; Saline, a rat knee injected with saline only; Car 1 week, a rat knee injected 1 week after intra-articular injection of carrageenan; Car 2 week, a rat knee injected 2 weeks after intra-articular injection of carrageenan; Car + LIPUS 2 weeks, Car + LIPUS 1 week, a rat knee treated with LIPUS 1 week after injection of carrageenan; Car + LIPUS 2 weeks, Car + LIPUS 2 weeks, a rat knee treated with LIPUS 2 weeks after injection of carrageenan.

to regulate HIF-1α and attenuate the fibrotic process of IFP in KOA. In clinical settings, it has been reported that LIPUS treatment for KOA relieves joint pain, joint swelling, and a reduction in the limitation of joint range of motion (47), and our findings suggest that this phenomenon is mediated by the antifibrotic effects of LIPUS via the regulation of CTGF, VEGF, and HIF-1α in the IFP.

The present study aimed to clarify the involvement of HIF-1α in the development of fibrosis of the IFP in KOA. The IFP was harvested to investigate gene expression levels and the transcriptional activity of HIF-1α and related molecules.

Although the present study demonstrated that HIF-1α participated in the development of fibrosis in the IFP in KOA, there are several limitations. One of these is the technical limitation on the collection of IFP samples. The synovial tissue could not be completely removed from the IFP because of the continuous histological connection of the IFP with its surrounding tissues. Therefore, the gene expression and transcriptional activity may include some synovial tissue responses. Additionally, although the involvement of HIF-1α in the development of fibrosis of IFP was investigated, degeneration of the articular cartilage, another major factor in the pathogenesis of KOA, was not fully evaluated in this study. In addition, the effects of LIPUS on HIF-1α activation and degeneration of articular cartilage should be investigated in the future. Mechanical stress has also been suggested to be involved in the pathogenesis of KOA. However, given that carrageenan was used to induce synovitis in this study, the relationship between HIF-1α and fibrosis of IFP in OA models due to joint instability, such as destabilization of the medial meniscus or resection of the ligament, requires further investigation. Finally, the effect of LIPUS on fibrosis of IFP was evaluated only 2 weeks after LIPUS intervention; lack of data at 1 week is a limitation of the present study.

In conclusion, the present study using a rat animal model of KOA demonstrated that activation of HIF-1α, which is considered to be induced by hypoxic conditions in the IFP, promoted fibrosis of IFP, followed by upregulation of fibrosis-related molecules, CTGF and VEGF. Notably, intervention with LIPUS resulted in attenuation of fibrotic changes in the IFP through reduction of HIF-1α activity. Our findings reveal a novel therapeutic strategy for the treatment of KOA, focusing on HIF-1α.

Acknowledgements

We would like to thank Mr Hiroshi Kawanami (Research Assistant, Graduate School of Health Sciences, Morinomiya University of Medical Sciences) for the excellent technical assistance he provided.

Funding

This work was supported by the Japanese Non-surgical Orthopedics Society (JNOS) grant (grant no. JNOS202002).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TK, HK, MA and SK contributed to the conception and design of the study, performed the experiments, and contributed to the acquisition, analysis, and interpretation of data. TK, HK, MA and SK drafted the manuscript, and revised it critically for important intellectual content. HK and MA verified all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Morinomiya University of Medical Sciences (grant no. 2019A001).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sellam J and Berenbaum F: The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nat Rev Rheumatol 6: 625-635, 2010.
2. Dieppe PA and Lohmander LS: Pathogenesis and management of pain in osteoarthritis. Lancet 365: 965-973, 2005.
3. Hunter DJ and Bierma-Zeinstra S: Osteoarthritis. Lancet 393: 1745-1759, 2019.
4. Hill CL, Gale DG, Chaisson CE, Skinner K, Kazis L, Gale ME and Felson DT: Knee effusions, popliteal cysts, and synovial thickening: Association with knee pain in osteoarthritis. J Rheumatol 28: 1330-1337, 2001.
5. Belluzzi E, Stocco E, Pozzuoli A, Granzotto M, Porzionato A, Vettor R, De Caro R, Ruggieri P, Ramonda R, Rossato M, *et al*: Contribution of infrapatellar fat pad and synovial membrane to knee osteoarthritis pain. Biomed Res Int 2019: 6390182, 2019.
6. Leese J and Davies DC: An investigation of the anatomy of the infrapatellar fat pad and its possible involvement in anterior pain syndrome: A cadaveric study. J Anat 237: 20-28, 2020.
7. Macchi V, Picardi EEE, Fontanella CG, Porzionato A, Stecco C, Tortorella C, Favero M, Natali A and De Caro R: The characteristics of the lobular arrangement indicate the dynamic role played by the infrapatellar fat pad in knee kinematics. J Anat 235: 80-87, 2019.
8. Macchi V, Stocco E, Stecco C, Belluzzi E, Favero M, Porzionato A and De Caro R: The infrapatellar fat pad and the synovial membrane: An anatomo-functional unit. J Anat 233: 146-154, 2018.
9. Nakanishi S, Morimoto R, Kitano M, Kawanishi K, Tanaka A and Kudo S: Difference in movement between superficial and deep parts of the infrapatellar fat pad during knee extension. J Funct Morphol Kinesiol 6: 68, 2021.
10. Distel E, Cadoudal T, Durant S, Poignard A, Chevalier X and Benelli C: The infrapatellar fat pad in knee osteoarthritis: An important source of interleukin-6 and its soluble receptor. Arthritis Rheum 60: 3374-3377, 2009.
11. Favero M, El-Hadi H, Belluzzi E, Granzotto M, Porzionato A, Sarasin G, Rambaldo A, Iacobellis C, Cigolotti A, Fontanella CG, *et al*: Infrapatellar fat pad features in osteoarthritis: A histopathological and molecular study. Rheumatology (Oxford) 56: 1784-1793, 2017.
12. Bastiaansen-Jenniskens YM, Wei W, Feijt C, Waarsing JH, Verhaar JA, Zuurmond AM, Hanemaaijer R, Stoop R and van Osch GJ: Stimulation of fibrotic processes by the infrapatellar fat pad in cultured synoviocytes from patients with osteoarthritis: A possible role for prostaglandin f2α. Arthritis Rheum 65: 2070-2080, 2013.
13. Maculé F, Sastre S, Lasurt S, Sala P, Segur JM and Mallofré C: Hoffa's fat pad resection in total knee arthroplasty. Acta Orthop Belg 71: 714-717, 2005.
14. Fontanella CG, Macchi V, Carniel EL, Frigo A, Porzionato A, Picardi EEE, Favero M, Ruggieri P, de Caro R and Natali AN: Biomechanical behavior of Hoffa's fat pad in healthy and osteoarthritic conditions: Histological and mechanical investigations. Australas Phys Eng Sci Med 41: 657-667, 2018.
15. Henderson NC, Rieder F and Wynn TA: Fibrosis: From mechanisms to medicines. Nature 587: 555-566, 2020.
16. Wynn TA and Ramalingam TR: Mechanisms of fibrosis: Therapeutic translation for fibrotic disease. Nat Med 18: 1028-1040, 2012.
17. Wynn TA: Cellular and molecular mechanisms of fibrosis. J Pathol 214: 199-210, 2008.
18. Sun K, Tordjman J, Clément K and Scherer PE: Fibrosis and adipose tissue dysfunction. Cell Metab 18: 470-477, 2013.
19. Halberg N, Khan T, Trujillo ME, Wernstedt-Asterholm I, Attie AD, Sherwani S, Wang ZV, Landskroner-Eiger S, Dineen S, Magalang UJ, *et al*: Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. Mol Cell Biol 29: 4467-4483, 2009.
20. Qing L, Lei P, Liu H, Xie J, Wang L, Wen T and Hu Y: Expression of hypoxia-inducible factor-1α in synovial fluid and articular cartilage is associated with disease severity in knee osteoarthritis. Exp Ther Med 13: 63-68, 2017.
21. Sotobayashi D, Kawahata H, Anada N, Ogihara T, Morishita R and Aoki M: Therapeutic effect of intra-articular injection of ribbon-type decoy oligonucleotides for hypoxia inducible factor-1 on joint contracture in an immobilized knee animal model. J Gene Med 18: 180-192, 2016.
22. Ohtomo S, Nangaku M, Izuhara Y, Takizawa S, de Strihou CV and Miyata T: Cobalt ameliorates renal injury in an obese, hypertensive type 2 diabetes rat model. Nephrol Dial Transplant 23: 1166-1172, 2008.
23. Sotobayashi D and Kawahata H: Beneficial effect of low-intensity pulsed ultrasound on progression of joint contracture. J Judo Ther 27: 125-132, 2019 (In Japanese).
24. Itaya N, Yabe Y, Hagiwara Y, Kanazawa K, Koide M, Sekiguchi T, Yoshida S, Sogi Y, Yano T, Tsuchiya M, *et al*: Effects of low-intensity pulsed ultrasound for preventing joint stiffness in immobilized knee model in rats. Ultrasound Med Biol 44: 1244-1256, 2018.
25. Hansra P, Moran EL, Fornasier VL and Bogoch ER: Carrageenan-induced arthritis in the rat. Inflammation 24: 141-155, 2000.

26. Clockaerts S, Bastiaansen-Jenniskens YM, Runhaar J, Van Osch GJ, Van Offel JF, Verhaar JA, De Clerck LS and Somville J: The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: A narrative review. Osteoarthritis Cartilage 18: 876-882, 2010.
27. Wang W, Liu Y, Yang C, Qi X, Li S, Liu C and Li X: Mesoporous bioactive glass combined with graphene oxide scaffolds for bone repair. Int J Biol Sci 15: 2156-2169, 2019.
28. Zarella MD, Breen DE, Plagov A and Garcia FU: An optimized color transformation for the analysis of digital images of hematoxylin & eosin stained slides. J Pathol Inform 6: 33, 2015.
29. Aigner T, Cook JL, Gerwin N, Glasson SS, Laverty S, Little CB, McIlwraith W and Kraus VB: Histopathology atlas of animal model systems-overview of guiding principles. Osteoarthritis Cartilage 18 (Suppl 3): S2-S6, 2010.
30. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
31. Seol D, Choe H, Zheng H, Jang K, Ramakrishnan PS, Lim TH and Martin JA: Selection of reference genes for normalization of quantitative real-time PCR in organ culture of the rat and rabbit intervertebral disc. BMC Res Notes 4: 162, 2011.
32. Baumann B, Hayashida T, Liang X and Schnaper HW: Hypoxia-inducible factor-1α promotes glomerulosclerosis and regulates COL1A2 expression through interactions with Smad3. Kidney Int 90: 797-808, 2016.
33. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD and Semenza GL: Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16: 4604-4613, 1996.
34. Higgins DF, Biju MP, Akai Y, Wutz A, Johnson RS and Haase VH: Hypoxic induction of Ctgf is directly mediated by Hif-1. Am J Physiol Renal Physiol 287: F1223-F1232, 2004.
35. Yoon KH, Tak DH, Ko TS, Park SE, Nam J and Lee SH: Association of fibrosis in the infrapatellar fat pad and degenerative cartilage change of patellofemoral joint after anterior cruciate ligament reconstruction. Knee 24: 310-318, 2017.
36. Han W, Aitken D, Zhu Z, Halliday A, Wang X, Antony B, Cicuttini F, Jones G and Ding C: Hypointense signals in the infrapatellar fat pad assessed by magnetic resonance imaging are associated with knee symptoms and structure in older adults: A cohort study. Arthritis Res Ther 18: 234, 2016.
37. Ding N, Wei B, Fu X, Wang C and Wu Y: Natural products that target the NLRP3 inflammasome to treat fibrosis. Front Pharmacol 11: 591393, 2020.
38. Takahashi I, Matsuzaki T, Kuroki H and Hoso M: Induction of osteoarthritis by injecting monosodium iodoacetate into the patellofemoral joint of an experimental rat model. PLoS One 13: e0196625, 2018.
39. Inomata K, Tsuji K, Onuma H, Hoshino T, Udo M, Akiyama M, Nakagawa Y, Katagiri H, Miyatake K, Sekiya I, *et al*: Time course analyses of structural changes in the infrapatellar fat pad and synovial membrane during inflammation-induced persistent pain development in rat knee joint. BMC Musculoskelet Disord 20: 8, 2019.
40. Ekundi-Valentim E, Santos KT, Camargo EA, Denadai-Souza A, Teixeira SA, Zanoni CI, Grant AD, Wallace J, Muscará MN and Costa SK: Differing effects of exogenous and endogenous hydrogen sulphide in carrageenan-induced knee joint synovitis in the rat. Br J Pharmacol 159: 1463-1474, 2010.
41. Ashraf S, Mapp PI, Shahtaheri SM and Walsh DA: Effects of carrageenan induced synovitis on joint damage and pain in a rat model of knee osteoarthritis. Osteoarthritis Cartilage 26: 1369-1378, 2018.
42. Egners A, Erdem M and Cramer T: The response of macrophages and neutrophils to hypoxia in the context of cancer and other inflammatory diseases. Mediators Inflamm 2016: 2053646, 2016.
43. Semba H, Takeda N, Isagawa T, Sugiura Y, Honda K, Wake M, Miyazawa H, Yamaguchi Y, Miura M, Jenkins DM, *et al*: HIF-1α-PDK1 axis-induced active glycolysis plays an essential role in macrophage migratory capacity. Nat Commun 7: 11635, 2016.
44. Yabe Y, Hagiwara Y, Suda H, Ando A, Onoda Y, Tsuchiya M, Hatori K and Itoi E: Joint immobilization induced hypoxic and inflammatory conditions in rat knee joints. Connect Tissue Res 54: 210-217, 2013.
45. Zhao K, Weng L, Xu T, Yang C, Zhang J, Ni G, Guo X, Tu J, Zhang D, Sun W and Kong X: Low-intensity pulsed ultrasound prevents prolonged hypoxia-induced cardiac fibrosis through HIF-1α/DNMT3a pathway via a TRAAK-dependent manner. Clin Exp Pharmacol Physiol 48: 1500-1514, 2021.
46. Costa V, Carina V, Conigliaro A, Raimondi L, De Luca A, Bellavia D, Salamanna F, Setti S, Alessandro R, Fini M and Giavaresi G: miR-31-5p is a LIPUS-mechanosensitive MicroRNA that targets HIF-1α signaling and cytoskeletal proteins. Int J Mol Sci 20: 1569, 2019.
47. Yang PF, Li D, Zhang SM, Wu Q, Tang J, Huang LK, Liu W, Xu XD and Chen SR: Efficacy of ultrasound in the treatment of osteoarthritis of the knee. Orthop Surg 3: 181-187, 2011.