Original Article
In vivo and in vitro characteristic of HIF-1α and relative genes in ischemic femoral head necrosis

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Abstract: Background: Legg-Calvé-Perthes Disease (Perthes' disease) is a childhood hip disorder initiated by ischemic necrosis of the growing femoral head. So far, the etiology and pathogenesis of Perthes' disease is poorly understood. Materials and methods: Avascular osteonecrosis rat model was established to mimic the pathophysiological changes of femoral head necrosis. The chondrocytes of newborn Sprague-Dawley rats were isolated and cultured in hypoxic and normoxic condition. The expression characteristic of the hypoxia-inducible factor-1 alpha (HIF-1α) was evaluated both in vivo and in vitro models. Vascular endothelial growth factor (VEGF) and apoptotic genes in chondrocytes treated with normoxia and hypoxia were also studied. Results: HIF-1α expression increased greatly after ischemic operation and kept at relative high level in the arthromeningitis stage and declined in the stages of osteonecrosis and reconstruction. The HIF-1α mRNA levels of chondrocytes incubated at hypoxia were significantly higher than the cells treated with normoxia at 24 and 72 hours. Hypoxia inhibited VEGF expression; chondrocytes could oppose this inhibition manifested by the increasing of VEGF mRNA level after 72 hours hypoxia. The expression of apoptotic genes, Casp3, Casp8 and Casp9, elevated in chondrocytes after hypoxia with time differences. Conclusion: Hypoxia might be an etiological factor for femoral head necrosis, HIF-1α, VEGF as well as apoptotic genes participated the pathophysiological process of ischemic osteonecrosis.

Keywords: Legg-Calvé-Perthes Disease, ischemic osteonecrosis, hypoxia-inducible factor-1 alpha (HIF-1α), vascular endothelial growth factor (VEGF), apoptotic genes

Introduction

Legg-Calvé-Perthes Disease (Perthes' disease) is a childhood hip disorder initiated by ischemic necrosis of the growing femoral head [1, 2]. The name originated from American, French and German orthopedic surgeons: Arthur Legg; Jacques Calvé and Georg Perthes [3]. The annual incidence of Perthes' disease among children under age 15 years ranged from 0.2 per 100,000 to 19.1 per 100,000 [3]. The clinical pathophysiological procedure underwent 3 to 5 years including stages of arthromeningitis; osteonecrosis and collapse; disintegrating and reconstruction [4]. Unfortunately, current clinical treatment on Perthes' disease is limited and the outcome is poor [5].

The etiology of Perthes' disease is not known, children with Perthes' disease have delayed bone age, disproportionate growth, and a mildly shortened stature [6]. Perthes' disease may be idiopathic, or it may result from a slipped capital femoral epiphysis, trauma, steroid use, sickle-cell crisis, toxic sinusitis, or congenital dislocation of the hip [7]. In short, current clinicians believe that different etiological factors lead to the disruption of blood flow to the femoral head, then all patients share similar pathophysiological procedure of ischemic necrosis of the femoral head, and the outcome varied by individual prognostic factors [8].

The clinical research on Perthes' disease was impeded by its occult onset, complex changes in disease course, and the rare and dispersive case [9]. A large number of evidences suggested that disruption of blood flow to the femoral head including arterial occlusion is the main risk factor for Perthes's disease [10]. Also, animal experiments have demonstrated that femoral neck blood supply damaged by operation will lead femoral head necrosis in goat, rat and pig [11]. Thus, animal models were employed

widely in Perthe's disease research instead of clinical patients.

Osteogenesis and angiogenesis are two important and tightly coupled physiological processes during bone development and regeneration [12]. The hypoxia-inducible factor-1 alpha (HIF-1α) pathway has been identified as a key component in this process which participates and controls the mesenchymal cells in the developing stroma and angiogenic signals to recruit new blood vessels into bone [13]. HIF-1 is a DNA binding protein complex contains at least two basic helix-loop-helix PAS-domain (bHLH-PAS) proteins, HIF-1α and aryl hydrocarbon nuclear receptor translocator (ARNT) [14]. Current knowledge show that HIF-1α is the dominant host responser to various hypoxic physiological conditions and will be activated and accumulates rapidly in the host cells [15]. Studies demonstrated that overexpression of HIF-1 in mature osteoblasts profoundly increases angiogenesis and osteogenesis via the action of vascular endothelial growth factor (VEGF) on the endothelial cells [16-18]. Study in porcine ischemic femoral heads model showed that HIF-1α activates Sox9 expression and enhances Sox9-mediated transcriptional activity which suggested a chondroprotective role of HIF-1α [19]. Chondrocytes in the superficial layer of the acute ischemic epiphyseal cartilage showed HIF-1alpha activation and VEGF up-regulation with subsequent revascularization occurring in the cartilage [20].

Above literatures suggested temporal and spatial correlations between HIF-1α and the physiological process of Perthes' disease, while, the repertoire in the chondrocytes of ischemic femoral head remain to be further evaluated. In this study, femoral head necrosis rat model was established and the possible roles of HIF-1α, VEGF as well as apoptosis were assessed primarily.

Materials and methods

Ethical issue

All animal experiments in this report were conducted in accordance with internationally recognized guidelines for animal experiments (Animal Research: Reporting in vivo Experiments guidelines) and were approved by the Animal Ethics Committee of Children's Hospital of Fudan University.

Animal experiment

6-week-old Sprague Dawley rats were used in animal experiments. Of vascular deprivation-induced femoral head necrosis group, the blood supply for femoral head was damaged by vasotomy around the neck of femur as reported previously [21]. For the control, sham operation group, right hip was exposed by operation and blood supply for femoral head was not damaged. There are 5 rats in each group, both the experimental and control groups included 6 groups, the rats in these groups will be followed up on day 1, 5, 10, 15, 30 and 60, respectively. The pathophysiological changes in femoral head will be checked by the naked eye, H&E staining and immunohistochemistry.

Histopathology and immunohistochemistry

Arthromeningitis, osteonecrosis and collapse, disintegrating and reconstruction in femoral head were verified by histopathology. Hematoxylin and eosin (H&E) staining was used. The femoral heads were removed and inflated with 4% paraformaldehyde. Then the tissues were embedded in paraffin wax and cut into sections (5 μm thick), which were stained using a standard H&E protocol. To study the level of HIF-1α in the femoral heads of mice, slides were dewaxed, rehydrated and processed for antigen retrieval. Endogenous peroxidase was quenched with 0.03% hydrogen peroxide for 30 min, and nonspecific reaction was blocked with 10% skimmed milk for 30 min at room temperature, sections were then incubated with antibody against HIF-1α (Abcam, Cambridge, MA), at 4°C for 2 hours. After washing with phosphate buffered saline buffer for 3 times, the slides were incubated with horseradish peroxidase-labeled secondary antibody (VECTOR LABORATORIES, Burlingame, CA) at room temperature for 20 min. the HIF-1α expression and localization were visualized by diaminobenzidine (VECTOR LABORATORIES, Burlingame, CA) reaction for 5 min.

Primary cell culture and hypoxia treatment

To study the pathophysiological changes and the role of chondrocyte in hypoxia, the chondrocytes of newborn Sprague Dawley rats were isolated and cultured following the protocol of Gosset et al [22]. Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) with high glucose, supplemented with 10% fetal bovine serum, 100 U of
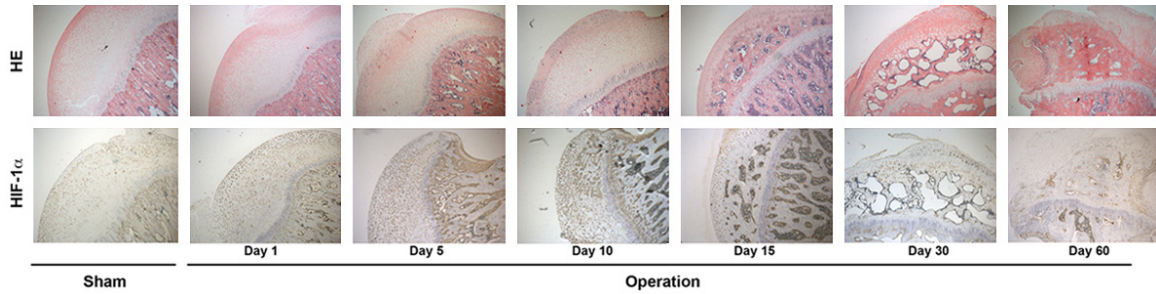
Figure 1. Histopathology assessment on caput femoris. Upper, Hematoxylin and eosin (H&E) staining was performed on the avascular osteonecrosis rat model mimicked the pathophysiological changes of femoral head necrosis; Lower, immunohistochemical staining using anti-HIF-1α antibody.

Table 1. Expression of VEGF and HIF-1

Time course (hours)	VEGF		HIF-1α	
	Normoxia	Hypoxia	Normoxia	Hypoxia
0	1.00 (0.82~1.22)*	0.00 (0.00~0.00)	1.00 (0.83~1.21)	0.43 (0.36~0.52)
8	0.15 (0.13~0.19)	0.06 (0.05~0.08)	0.39 (0.31~0.49)	0.75 (0.63~0.89)
24	0.94 (0.80~1.11)*	0.16 (0.13~0.19)	0.55 (0.46~0.66)	8.36 (5.23~13.37)*
72	0.05 (0.04~0.06)	0.20 (0.17~0.24)*	0.06 (0.05~0.07)	6.09 (5.11~7.25)*

The genes expression levels were calcaulted by the $2^{-\Delta\Delta CT}$ method. Data were presented as $2^{-\Delta\Delta CT}$ and range. *indicated significance (p < 0.05) when compared with normoxia or hypoxia. VEGFA, vascular endothelial growth factor A; HIF-1, hypoxia-inducible factor-1.

penicillin, and 100 μg of streptomycin per ml of medium. For hypoxic treatment, cells were exposed to 10% CO_2 and 0.5% O_2 balanced with nitrogen in a Sanyo three gas incubator (Sanyo, Japan).

Gene expression analysis

To study the gene expression profile in pathophysiological procedure of Perthes' disease model, real-time reverse transcript PCR analysis was used. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed to cDNA using the Two-Step RT-PCR Clone Kit (Biovisualab, Shanghai, China) according to the manufacturer's protocol. The genes and their corresponding primers were: ACTB: (Forward: 5'CGTAAAGA-CCTCTATGCCAACA3'; Reverse: 5'GGAGGAGC-AATGATCTTGATCT3'). Cycs: (Forward: 5'CACA-GATGCCAACAAGAACAAAGG3'; Reverse: 5'AG-GGATGTACTTTTTGGGATTTTCC3'). Casp9: (Forward: 5'GCGACATGATCGAGGATATTCAGC3'; Reverse: 5'TGCCTCCCTCGAGTCTCAAGATC3'). Casp8: (Forward: 5'ACGTCTGGGCAACGAAGAACT-G3'; Reverse: 5'GCACCAGGACATCATTGATGGAC-3'). Casp3: (Forward: 5'TGTATGCTTACTCTACCG-CACCCG3'; Reverse: 5'GCGCAAAGTGACTGGAT-GAACC3'). HIF-1: (Forward: 5'AGCTTCTGTTATG-AGGCTCACCATC3'; Reverse: 5'TCTTCAATGTCA-

AGATCACCAGCAC3'). VEGF: (Forward: 5'AAAT-CCTGGAGCGTTCACTGTGAG3'; Reverse: 5'AAC-ATTTACACGTCTGCGGATCTTG3').

Quantitative PCR was performed using a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA) and the GREAT Real-Time SYBR PCR Kit (Biovisualab, Shanghai, China) following the manufacturer's instructions. The relative mRNA expression level was calculated using the $2^{-\Delta\Delta CT}$ method with the CT values normalized using β-actin as an internal control [23, 24].

Statistical analyses

An analysis of variance (ANOVA) was used to determine the differences between all groups. Pairs of groups were compared using Student's t-test. *P*-values < 0.05 were considered to indicate statistical significance.

Results

Avascular osteonecrosis rat model mimicked the pathophysiological changes of femoral head necrosis

As showed in **Figure 1**, the femoral head of rat displayed pathophysiological changes of femo-
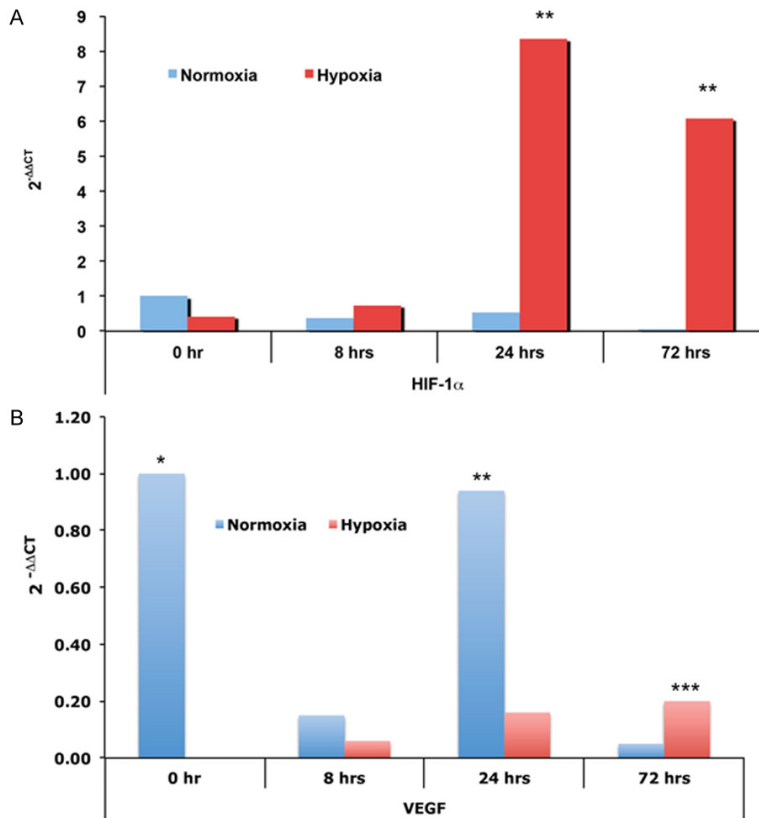
Figure 2. Expression of HIF-1α and VEGF. Chondrocytes were isolated from newborn rat, cultured in either normoxic or hypoxic incubators. The expression of HIF-1α and VEGF were checked using qRT-PCR. A. Expression of HIF-1α, *indicated significance (P < 0.05) when compared with control, the $2^{-\Delta\Delta CT}$ and ranges were showed in **Table 1**. B. Expression of VEGF, *indicated significance (P < 0.05) when compared with normoxia or hypoxia groups, the $2^{-\Delta\Delta CT}$ and ranges were showed in **Table 1**.

ral head necrosis, on day 5 and day 10, cartilage layer is hypertrophic and loose which represented the arthromeningitis; on day 15 and day 30, femoral head showed osteonecrosis and collapse; on day 60, femoral head displayed disintegrating and reconstruction (**Figure 1**, upper), in conclusion, histopathological assessment showed that avascular osteonecrosis rat model mimicked the pathophysiological course of femoral head necrosis.

HIF-1α expression in the femoral head following ischemic injury

Since HIF-1α is the dominant response factor to ischemic injury, HIF-1α expression in the femoral head following ischemic injury was evaluated. As showed in **Figure 1**, the expression of HIF-1α increased greatly after operation and kept at relative high expression level from day 1 to day 10, which suggests that HIF-1α

expression was activated in the pathophysiological process of arthromeningitis (**Figure 1**, bottom). On day 15 and day 30, immunohistochemical assessment showed that the HIF-1α expression fall back to relative low level, which suggests that HIF-1α expression declined in the pathophysiological process of osteonecrosis and collapse (**Figure 1**, bottom). As expected, on day 60, HIF-1α expression also kept at low level in the pathophysiological process of disintegrating and reconstruction (**Figure 1**, lower).

HIF-1α expression in the chondrocytes

Above in vivo data showed the expression characteristics of HIF-1α, which is HIF-1α expression elevated in femoral head at early stage of ischemic injury. Current biotechnology is hard to know the cellular and molecular pathophysiological change in detail, while, early cellular and molecular pathophysiological change is important to the development and prognosis of pathophysiology. To learn the HIF-1α expression in chondrocytes at early stage of hypoxia, the chondrocytes were isolated. The chondrocytes were then cultured in hypoxic and normoxic incubators, HIF-1α mRNA level was determined. As showed in **Table 1**, the $2^{-\Delta\Delta CT}$ of HIF-1α in normoxic and hypoxic conditions at 0, 8, 24 and 72 hours were 1.00 (0.83~1.21), 0.39 (0.31~0.49), 0.55 (0.46~0.66) and 0.06 (0.05~0.07); and 0.43 (0.36~0.52), 0.75 (0.63~0.89), 8.36 (5.23~13.37) and 6.09 (5.11~7.25), respectively. The HIF-1α mRNA levels of chondrocytes incubated at hypoxia were significantly higher than the cells treated with normoxia at 24 and 72 hours (**Figure 2A**).

VEGF expression in the chondrocytes

Since angiogenesis and osteogenesis are tightly coupled during bone development and regen-

Table 2. Expression of apoptotic genes

Time course (hours)	Condition	Genes			
		CYCS	Casp3	Casp8	Casp9
0	Normoxia	1.00 (0.58~1.73)	1.00 (0.83~1.20)	1.00 (0.83~1.22)	1.00 (0.82~1.22)
	Hypoxia	0.22 (0.17~0.27)	0.15 (0.12~0.17)	0.33 (0.27~0.40)	0.03 (0.03~0.04)*
8	Normoxia	0.36 (0.31~0.42)	0.29 (0.25~0.34)	0.35 (0.28~0.44)	0.22 (0.19~0.26)
	Hypoxia	0.20 (0.17~0.23)	0.46 (0.39~0.54)	0.21 (0.18~0.24)	0.03 (0.03~0.04)
24	Normoxia	0.38 (0.31~0.46)	1.12 (0.95~1.31)	0.32 (0.27~0.37)	0.62 (0.53~0.74)
	Hypoxia	0.41 (0.36~0.47)	6.15 (4.08~9.26)*	4.43 (3.92~5.00)*	0.63 (0.52~0.76)
72	Normoxia	0.25 (0.18~0.36)	0.29 (0.25~0.34)	0.31 (0.26~0.38)	0.10 (0.08~0.13)
	Hypoxia	1.02 (0.86~1.21)	2.47 (2.14~2.85)*	3.10 (2.52~3.81)*	1.42 (1.16~1.75)*

The genes expression levels were calcaulted by the $2^{-\Delta\Delta CT}$ method. Data were presented as $2^{-\Delta\Delta CT}$ and range. *indicated significance ($p < 0.05$) when compared with normoxia or hypoxia. CYCS, Cytochrome C, Somatic; Casp, caspase.

eration and VEGF is a well-characterized proangiogenic factor [12], the expression of VEGF was then studied by real-time RT-PCR analysis, as showed in **Table 1**, the $2^{-\Delta\Delta CT}$ of VEGF in normoxic and hypoxic conditions at 0, 8, 24 and 72 hours were 1.00 (0.82~1.22), 0.15 (0.13~0.19), 0.94 (0.80~1.11) and 0.05 (0.04~0.06); and 0.00 (0.00~0.00), 0.06 (0.05~0.08), 0.16 (0.13~0.19) and 0.20 (0.17~0.24), respectively. The VEGF mRNA levels were difficult to understand, chondrocytes in normoxic condition showed irregular VEGF mRNA levels, VEGF mRNA levels of chondrocytes in hypoxic condition showed increasing tendency (**Table 1**; **Figure 2B**). At the initial stage (0 hour) and 24 hours incubation, VEGF levels of normoxia are significantly higher in the chondrocytes incubated in normoxic condition than that in hypoxic condition (**Table 1**; **Figure 2B**). At 72 hours, VEGF level increased and is significantly higher in the chondrocytes incubated in hypoxic condition than that in normoxic condition (**Table 1**; **Figure 2B**).

Apoptotic genes expression

The apoptosis was presumed to participate in the ischemic injury in many diseases, to study whether and how the apoptosis involved in ischemic osteonecrosis, the expression of CYCS, Casp3, Casp8 and Casp9 in chondrocytes in hypoxic and normoxic conditions were also studied. As showed in **Table 2**, all genes displayed expression fluctuation along with time course, although CYCS gene displayed expression difference between normoxia and hypoxia at all time points, there is no significance was observed. Of Casp3 and Casp8, the

mRNA levels in the chondrocytes incubated in hypoxic condition were significantly higher than that in normoxic condition at 24 and 72 hours, which might suggest that Casp3 and Casp8 involved the early response of ischemic osteonecrosis (**Table 2**). Of Casp9, its mRNA level was significantly lower in hypoxic chondrocytes than that in normoxic chondrocytes at 0 hour, conversely, its mRNA level was significantly higher in hypoxic chondrocytes than that in normoxic chondrocytes at 72 hour (**Table 2**).

Discussion

Perthes' disease is a big challenge to children's health, while, the etiology and pathogenesis remain unclear [25], however, some studies insight into the pathogenesis of a femoral head deformity after ischemic necrosis have been gained [25]. In this study, we established an avascular osteonecrosis model in rat, histopathological assessment showed that rat model represented the pathophysiological course of femoral head necrosis, the rat model showed arthromeningitis, osteonecrosis and collapse, disintegrating and reconstruction within 60 days observation. In vivo data showed that HIF-1α expression elevated in femoral head at early stage of ischemic injury and kept at low level in the pathophysiological process of disintegrating and reconstruction. Although HIF-1α is known to be elevated during hypoxia through a regulated proteolytic process [12], the dynamic change of HIF-1α in whole pathophysiological process of avascular osteonecrosis model is rare. Our in vitro experiments showed HIF-1α expression in chondrocytes elevated significantly 24 hours after hypoxic

treatment, and HIF-1α level kept higher compare to normoxic incubated chondrocytes up to 72 hours. Although HIF-1α level elevated in culture cells in hypoxic treatment was also reported by other researches [26, 27], there are two main differences between our study and other reports, previous experiments are mainly performed in cell line strains, in our study, the chondrocytes were primarily isolated from newborn rat; pre-existing reports showed that the HIF-1α will increased quickly within hour when triggered by hypoxia [26-28], our data showed that the HIF-1α increased a bit of a lag which might represented the characteristic of isolated chondrocytes. The VEGF could be released by hypertrophic chondrocytes and plays a critical role in angiogenesis [29], our data showed that chondrocytes did produce VEGF, while hypoxia restrained the expression of VEGF and the restraint was broken through to a certain extent along with the hypoxic time increasing, which suggests that ischemic injury also inhibits VEGF producing in chondrocytes and hence contributed to the pathogenesis of ischemic femoral head necrosis. Further apoptosis relative genes analysis showed that Casp3, Casp8 and Casp9 participated in the hypoxic injury of chondrocytes.

Hydroxylation of HIF-1α requires molecular oxygen, and the hydroxylation is inhibited by hypoxia [12]. Under the condition of hypoxia, the HIF-1α accumulates in the cytoplasm and then translocates to the nucleus, where it dimerizes with the HIF-1β. The dimer of HIF-1α and HIF-1β then binds to a highly conserved hypoxia-response element within promoters of a cluster of hypoxia-responsive genes [30]. Genes containing functional hypoxia-response element encode proteins involved in angiogenesis including VEGF and endothelin-1 [31]. In this study, our immunohistochemical staining using anti-HIF-1α antibody showed that the HIF-1α concentrated in the nucleus of the chondrocytes in cartilage (**Figure 1**), which suggested that HIF-1α pathway was activated and played its function in early stage of avascular osteonecrosis. Also, our following VEGF data showed that hypoxia inhibited VEGF expression, chondrocytes could oppose this inhibition manifested by the increasing of VEGF mRNA level after 72 hours hypoxia.

Many studies demonstrated that HIF-1α protein keeps as very lower level in cells at normoxia condition [12, 18-20], our in vivo data also confirmed this phenomenon, HIF-1α protein is relative lower in the tissue without ischemic injury and in the stages of osteonecrosis and reconstruction in rat with avascular osteonecrosis.

This is a primary report focused on the HIF-1α, VEGF and apoptotic genes in mimicking femoral head necrosis, the expressions of VEGF and apoptotic genes were only examined in mRNA level, no protein data of these genes were provided. We are devoting ourselves to complete current research. In conclusion, our primary data suggested that hypoxia might be a main etiological factor for ischemic femoral head necrosis and HIF-1α, VEGF as well as apoptotic genes participated in the pathophysiological process of ischemic osteonecrosis temporally and spatially.

Disclosure of conflict of interest

None.

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