

## 细胞骨架在流体剪切力诱导成骨细胞 COX-2 基因和蛋白表达中的作用

王常德<sup>1,2</sup>, 孜里哈·买赛尔<sup>3</sup>, 康鹏德<sup>2</sup>, 裴福兴<sup>2</sup>

1. 西北民族大学医学院附属医院, 甘肃 兰州 730030; 2. 四川大学华西医院骨科, 四川 成都 610041; 3. 新疆伊宁市人民医院, 新疆 伊宁 835000

**【摘要】目的:**探讨细胞骨架在流体剪切力(FSS)诱导成骨细胞COX-2基因和蛋白表达中的作用。**方法:**将第3代小鼠原代成骨细胞分为4组:对照组、细胞松弛素D(CD)组、FSS组、FSS+CD组。FSS组和FSS+CD组分别施加12 dyne/cm<sup>2</sup>的FSS 60 min, FSS组只加FSS, 而FSS+CD组在加入CD预处理60 min后施加FSS 60 min; 对照组不做任何处理; CD组仅加入CD。应用实时荧光定量PCR、蛋白质印迹法和激光共聚焦显微镜检测COX-2 mRNA和蛋白的表达以及肌动蛋白细胞骨架的变化, 并对结果进行双因素方差分析。**结果:**FSS组与其他3组相比, COX-2的mRNA和蛋白表达显著增加( $P < 0.05$ ); CD组与对照组及FSS+CD组相比, COX-2的mRNA和蛋白的表达水平无显著差异( $P > 0.05$ ); FSS+CD组与FSS组相比, COX-2的mRNA和蛋白的表达水平显著降低( $P < 0.05$ )。**结论:**应用FSS能使成骨细胞骨架发生重排、增多、增密且与流体方向保持一致。细胞骨架微丝的完整性与COX-2基因和蛋白表达密切相关, 而CD对COX-2基因和蛋白的表达基本没有影响。

**【关键词】**成骨细胞; COX-2基因; 流体剪切力; 细胞骨架

**【中图分类号】**R318.01

**【文献标志码】**A

**【文章编号】**1005-202X(2017)07-0743-05

## Cytoskeleton integrity in the expression of COX-2 gene and protein in osteoblasts induced by fluid shear stress

WANG Changde<sup>1,2</sup>, MAISAI'ER Ziliha<sup>3</sup>, KANG Pengde<sup>2</sup>, PEI Fuxing<sup>2</sup>

1. Affiliated Hospital, Northwest University for Nationalities, Lanzhou 730030, China; 2. Department of Orthopedic Surgery, West China Hospital, Sichuan University, Chengdu 610041, China; 3. Yi'ning People's Hospital, Yi'ning 835000, China

**Abstract: Objective** To investigate the role of cytoskeleton integrity in the expression of osteoblasts COX-2 gene induced by fluid shear stress (FSS). **Methods** The third generation of mouse primary osteoblasts was divided into 4 groups, namely control group, cytochalasin D (CD) group, FSS group, FSS+CD group. The FSS of 12 dyne/cm<sup>2</sup> was applied for 60 min in FSS+CD group and FSS+CD group, respectively. Only FSS was added in FSS group, while FSS was applied for 60 min in FSS group after pretreatment with CD for 60 min; no treatment for the control group; only CD was added in CD group. The real-time fluorescence quantitative PCR, western blot and confocal laser scanning microscopy were used to detect the expression of COX-2 mRNA and protein and to observe the change of F-actin cytoskeleton. The obtained results were analyzed with two-way analysis of variance. **Results** The expression levels of COX-2 mRNA and protein were significantly increased in FSS group, compared with those in the other 3 groups ( $P < 0.05$ ). No significant differences were found in the expression levels of COX-2 protein and mRNA between CD group and control group or FSS+CD group ( $P > 0.05$ ). The expression levels of COX-2 mRNA and protein were significantly decreased in FSS+CD group, compared with that in FSS group ( $P < 0.05$ ). **Conclusion** The application of FSS can rearrange and increase the cytoskeleton, and increase the density of cytoskeleton, keeping consistent with the direction of the fluid. The integrity of cytoskeleton microfilament is closely related to the expressions of COX-2 gene and protein, while CD had trivial effect on the expression of COX-2 gene and protein.

**Keywords:** osteoblasts; COX-2 gene; fluid shear stress; cytoskeleton

**【收稿日期】**2017-03-08

**【基金项目】**中央高校基本科研业务费(31920140093)

**【作者简介】**王常德, 博士, 副主任医师, 研究方向: 关节外科, 生物力学, E-mail: wangchangde76@163.com

前言

骨结构的完整性与骨量的保持是在动态骨重建过程中进行的,在此过程中机械应力起着非常重要的作用。有研究显示在骨塑形期间增加机械应力负载能明显促进骨形成,抑制骨吸收<sup>[1-2]</sup>。然而,当生理机械负荷不存在时(如:卧床休息、术后关节固定或暴露于失重环境等),骨的吸收会显著增加。骨组织是多孔的系统,流体通过骨组织多孔系统时产生流体剪切力(Fluid Shear Stress, FSS),并促进成骨细胞产生生物化学信号,继而将这些信号转入细胞内促进骨组织改建,但其具体的细胞机制至今仍然不清楚。Mu等<sup>[3]</sup>研究认为机械应力和生物化学信号能调节成骨细胞的增殖与分化,并促进成骨相关基因(I型胶原、骨桥蛋白、碱性磷酸酶和骨钙素)的表达。利用细胞松弛素D(Cytochalasin D, CD)打断肌动蛋白细胞骨架微丝结构后,成骨相关基因c-fos和骨桥蛋白的表达显著下降<sup>[4]</sup>。本研究采用CD打断肌动蛋白细胞骨架,同时施加12 dyne/cm<sup>2</sup>的FSS,观察肌动蛋白细胞骨架重组与COX-2基因和蛋白表达之间的关系,探索二者之间可能的机制。

1 材料与方法

1.1 材料

昆明小鼠(新生1~3 d内)(四川大学华西医学实验动物中心提供);L-DMEM培养基(GIBCO,美国);Triton X-100(GIBCO,美国);特级胎牛血清(GIBCO,美国);Trizol试剂盒(Invitrogen,美国);FITC-Phalloidin(Sigma,美国);CD(Sigma,美国);碘化丙啶(Sigma,美国);COX-2抗体(Santa cruz,美国);Rhodamine标记的羊抗兔抗体(Chemicon,美国);7500全自动荧光定量PCR仪(ABI,美国);Axiovert 200倒置荧光显微镜(Zeiss,德国);LEICA TCS SP2激光共聚焦显微镜(Leica公司,德国)。

1.2 方法

取新生1~3 d内的雄性昆明乳鼠10只,脱颈处死后,浸泡在75%酒精中15 min,在无菌操作台内取出颅盖骨,去除骨表面肌肉、筋膜,无菌磷酸盐缓冲溶液(Phosphate Buffer Saline, PBS)冲洗3次,用眼科剪剪碎颅盖骨,成稀泥样改变,将组织碎块均匀地铺散在25 mL培养瓶底壁上,每个培养瓶底放置20块左右,然后翻转培养瓶,将粘有组织块的瓶底向上,加入含20%胎牛血清的L-DMEM培养基5 mL,置于37℃、5%CO<sub>2</sub>培养箱中培养4 h之后缓慢翻转培养瓶,置培养箱中继续培养。约第7天,成骨细胞从骨组织块下爬出;第14天细胞增殖加快,待细胞生长融合达80%,进行消化传代。

1.3 成骨细胞的鉴定

取第3代细胞爬片,用PBS清洗2次,并用4%多聚甲醛固定,进行碱性磷酸酶(Alkaline Phosphatase, ALP)染色和茜红素钙结节染色。

1.4 细胞应力加载和样本制备

细胞融合至80%时,取出培养皿内的盖玻片,放入平行板流体小室,安装导管及微型蠕动泵后移入37℃、5%CO<sub>2</sub>培养箱内,给FSS组、FSS+CD组施加12 dyne/cm<sup>2</sup> FSS 60 min;给CD组和FSS+CD组内分别加入CD(终浓度25 μmol/L)培养6 h,对FSS+CD组施加FSS 60 min。然后弃细胞培养液,PBS冲洗2遍;4%多聚甲醛固定30 min,使用PBS轻洗2遍;0.2% TritonX-100 常温下渗透10 min,PBS再洗2遍;1%的牛血清白蛋白覆盖室温作用10 min(不洗),在5 μg/mL(含1%DMSO 0.01 mol/L PBS稀释)FITC-phalloidin室温下染色40 min,PBS清洗两遍后,加入5 μg/mL的PI,室温染色40 min,缓冲甘油封片,激光共聚焦显微镜观察拍照。上述操作过程均需避光。

1.5 实时荧光定量PCR(Real-time Fluorescence Quantitative PCR, RT-qPCR)检测

1.5.1 cDNA合成 使用美国普洛麦格试剂盒提取细胞总RNA,逆转录反应合成cDNA。

1.5.2 RT-qPCR反应 使用美国普洛麦格公司GoTaq®Probe qPCR Master MixA6101进行RT-qPCR反应的测定,结果如表1和表2所示。

表1 COX-2、β-action引物设计  
Tab.1 Primer design of COX-2 and beta-action

Primer name	Primer sequence
COX-2	5'-CAGCAAATCCTTGCTGTTC-3'
	5'-TGGGCAAAGAATGCAACATC-3'
β-action	5'-TCAGGAGGAGCAATGATCTTG-3'
	5'-TCCTCCCTGGAGAAGAGCTA-3'

表2 RT-qPCR反应体系  
Tab.2 RT-qPCR reaction system

Group	Concentration	Volume/μL
Nuclease-free water		7.2
Upstream primer	0.2 μmol/L	0.4
Downstream primer	0.2 μmol/L	0.4
GoTaq PCR Master Mix, 2×	1×	10.0
cDNA		2.0

RT-qPCR:Real-time fluorescence quantitative PCR

## 1.6 细胞骨架免疫荧光染色

分别对4组细胞样本给予相应处理后,弃培养液,使用PBS轻洗2次;然后采用45%多聚甲醛固定15 min,0.2%Triton X-100透化细胞30 min,山羊血清封闭30 min,以消除非特异性染色,并加入FITC-phalloidin 4℃湿盒孵育1 h,PBS轻洗10 min×3次,加入4',6-二脒基-2-苯基吲哚(4',6-diamidino-2-phenylindole, DAPI),室温下避光孵育10 min;最后加入5 μL封片剂于载玻片上,避免产生气泡,封固后上激光共聚焦显微镜观察,并用AxioVision 4.1图片处理软件分析图片。

## 1.7 统计学方法

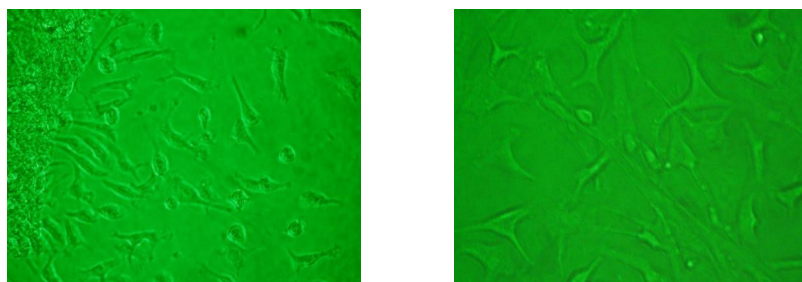
数据全部用均数±标准差表示,使用SPSS 21.0统计软件对COX-2的mRNA和蛋白表达水平进行双

因素方差分析, $P<0.05$ 具有统计学差异。

# 2 结果

## 2.1 原代成骨细胞鉴定

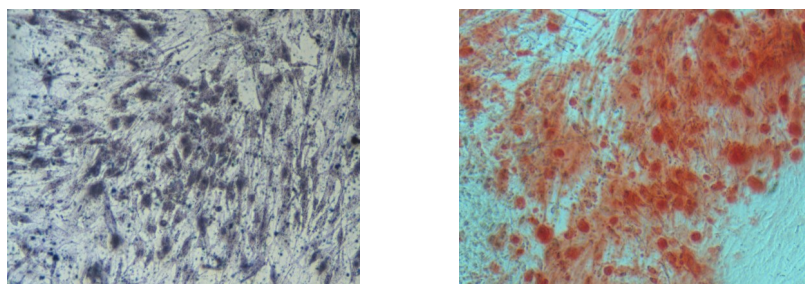
倒置相差显微镜下见第3代成骨细胞细胞形态不规则,多呈短梭形、三角形或多角形,有较多突起,部分细长突起常跨越细胞与远处的细胞突起相连接;胞核呈圆形或椭圆形,居中或偏于一侧,轮廓清晰,可见1~3个核仁。细胞80%融合时,多呈梭形或立方形,排列紧密(图1)。随着培养时间的延长,成骨细胞可呈重叠生长。ALP染色结果显示,细胞质内有大量蓝色颗粒状物质,部分分散在细胞周围;茜素红钙结节染色显示,细胞汇合后呈多层重叠生长,14 d左右出现大量钙结节,茜素红染色呈红色的颗粒样物(图2)。



a: Growth of primary cell culture at the 10th day (×20) b: Third generation cells (polygons, triangles) (×40)

图1 原代细胞培养

Fig.1 Primary cell culture



a: Alkaline phosphatase staining results b: Alizarin red calcium nodules staining results

In Fig.2a, a large number of blue particles were observed around the cytoplasm and cells; in Fig.2b, a large number of red calcium nodules formed, with different sizes and shapes.

图2 成骨细胞鉴定

Fig.2 Osteoblast identification

## 2.2 CD处理对FSS诱导的肌动蛋白骨架的影响

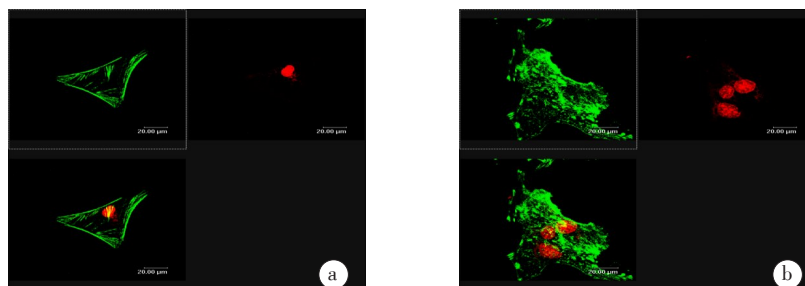
给第3代成骨细胞施加12 dyne/cm<sup>2</sup> FSS 60 min后发现,在对照组,F-肌动蛋白微丝稀疏,且随机分布,细胞形态规整(图3a);在CD组,F-肌动蛋白微丝大部分断裂明显,细胞形态不规整(图3b);在FSS组,F-肌动蛋白微丝增多、增密,F-肌动蛋白微丝沿细

胞长轴与FSS方向一致(图4a);在FSS+CD组,肌动蛋白微丝部分断裂,细胞形态保持规整(图4b)。

## 2.3 使用细胞骨架微丝阻断剂CD对COX-2的mRNA表达的影响

与对照组相比,CD组的COX-2的mRNA表达水平变化不明显( $P>0.05$ );FSS组与其他3组相比,FSS组的

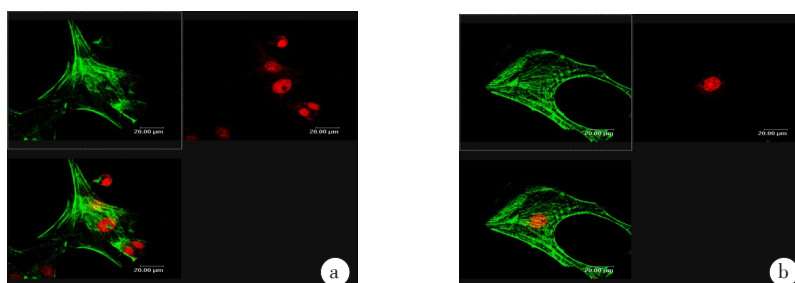




a was the results in control group, showing F-actin microfilament sparse, random distribution; b was the results in cytochalasin D (CD) group, in which most of the microfilaments were broken and the cell morphology was irregular.

图3 对照组及CD组的检测结果

Fig.3 Detected results in control group and CD group

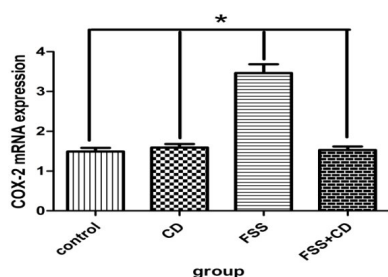


a was the results in fluid shear stress (FSS) group, showing increased and denser microfilament, and the long axis of the cell was consistent with the FSS direction; b was the results in FSS+CD group, in which part of microfilaments were broken, while the morphology of cells remained intact.

图4 FSS组及FSS+CD组的检测结果

Fig.4 Detected results in FSS group and FSS+CD group

COX-2的mRNA表达升高最明显( $P<0.05$ );与对照组及CD组相比,FSS+CD组的COX-2的mRNA表达不明显( $P>0.05$ )。各组COX-2 mRNA表达见图5。



\*: FSS group was compared with the other three groups,  $P<0.05$ .

图5 各组COX-2 mRNA表达量

Fig.5 Expression of COX-2 mRNA in control, CD, FSS, FSS+CD

groups

## 2.4 使用细胞骨架微丝阻断剂CD对的COX-2蛋白表达的影响

与对照组相比,CD组的COX-2的蛋白表达水平略有升高( $P>0.05$ );FSS组与其他3组相比,FSS组的COX-2的蛋白表达升高最明显( $P<0.05$ );与CD组相比,FSS+CD组的COX-2的蛋白表达变化不明显( $P>$

0.05)。各组COX-2蛋白表达见图6和图7。

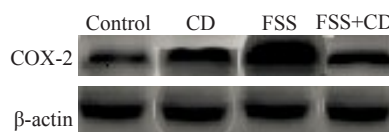
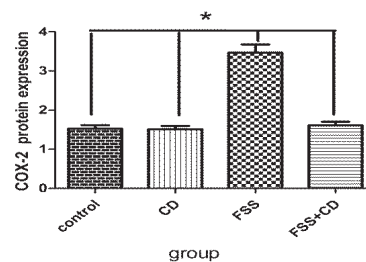


图6 各组COX-2蛋白的表达

Fig.6 Expression of COX-2 protein in control, CD, FSS, FSS+CD groups



\*: FSS group was compared with the other 3 groups,  $P<0.05$ .

图7 COX-2蛋白相对表达量

Fig.7 Relative expression of COX-2 protein in control, CD, FSS, FSS+CD groups

## 3 讨论

骨细胞通过各种途径感受和转导机械应力<sup>[5]</sup>。而

基质-整合素-细胞骨架途径就是其中最重要的途径之一,在骨的力传导中整合素直接连接骨细胞与骨基质,机械刺激从骨基质通过细胞骨架转入到细胞核,激活相关信号通路,促进成骨相关靶基因的表达。细胞骨架本身由肌动蛋白、微管和中间丝组成<sup>[6]</sup>。细胞骨架连接着机械感应系统的所有组件,在骨细胞机械力转导过程中肌动蛋白纤维起着至关重要的作用<sup>[7]</sup>。Delaine-Smith等<sup>[8]</sup>发现初级纤毛是一种基于微管的天线状扩展,在力学转导过程中的作用也不容忽视。机械应力刺激作用于成骨细胞,通过成骨细胞膜上分子感受器将机械信号传递到细胞骨架进而散布到细胞的各个部位,促进成骨相关靶基因的激活和抑制<sup>[9-10]</sup>。而本研究的主要目的是探索利用CD打断肌动蛋白细胞骨架对COX-2基因与蛋白表达的影响。通过CD打断肌动蛋白细胞骨架,再施加合适的FSS,利用激光共聚焦显微镜、RT-qPCR和蛋白质印迹法Western blot等技术能观察到肌动蛋白细胞骨架在FSS作用下发生重排、增密、增多,且与流体方向一致。其中,RT-qPCR检测显示,在FSS作用下COX-2 mRNA表达显著升高,而在加入CD处理1 h后下降明显,在单纯的CD作用下COX-2 mRNA的表达与对照组几乎无明显差别;Western blot检测到的COX-2蛋白表达与RT-qPCR结果相一致。本研究结果表明,肌动蛋白细胞骨架的完整性与COX-2基因和蛋白的表达密切相关。有研究发现对MC3T3-E1细胞施加12 dyn/cm<sup>2</sup> FSS,COX-2蛋白表达在15和45 min达到高峰<sup>[11]</sup>。这与我们的实验结果相一致。Pavalko等<sup>[12]</sup>研究发现,使用FSS给成骨细胞加载1 h后发现COX-2蛋白表达明显依赖于细胞骨架的完整性。但在时隔几年后该课题组的研究结果却截然相反,这可能与应力加载时间、CD浓度等有关。也有研究显示,在小鸡胚胎成骨细胞中加入CD后,应力诱导的骨桥蛋白表达明显受到抑制,这表明机械应力的调节作用依赖于完整的肌动蛋白细胞骨架<sup>[13]</sup>。还有学者发现使用细胞松弛素B打断肌动蛋白细胞骨架微丝能明显阻止FSS诱导的PGE2表达<sup>[14]</sup>。然而,FSS诱导的一氧化氮和钙离子升高是不依赖于完整的细胞骨架网络<sup>[15]</sup>。本研究结果显示,FSS介导的COX-2 mRNA和蛋白的表达与肌动蛋白细胞骨架完整性密切相关。因此,我们认为成骨细胞骨架在FSS诱导的COX-2 mRNA和蛋白的表达中起着关键作用。分析其可能的原因是整体或部分细胞骨架在力-化学信号转导过程中起着转换器的作用,调节力学信号向生物化学信号之间的转换<sup>[16-17]</sup>。因此,FSS介导的成骨细胞COX-2基因和蛋白的表达需要完整的细胞骨架作为前提。理解这些细胞机制可以促进科研工作者和医务人员更好地改进组织工程骨植入物的设计,更有效地促进骨科相关疾病的治疗。同时,本研究将为进一步研究FSS在成骨细胞中的信号转导机制提

供理论基础,也为临床治疗骨质疏松性疾病、骨缺血坏死性疾病提供理论依据。

## 【参考文献】

- [1] AISHA M D, NOR-ASHIKIN M N, SHARANIZA A B, et al. Orbital fluid shear stress promotes osteoblast metabolism, proliferation and alkaline phosphates activity *in vitro* [J]. *Exp Cell Res*, 2015, 337(1): 87-93.
- [2] 苏佳灿,任可,张春才.应力对骨折愈合的影响及其作用机制[J].第二军医大学学报,2001,22(10):988-990.  
SU J C, REN K, ZHANG C C. The effect of stress on fracture healing and its mechanism of action [J]. *Academic Journal of Second Military Medical University*, 2001, 22(10): 988-990.
- [3] MU W, WANG X, ZHANG X, et al. Fluid shear stress upregulates E-Tmod41 *via* miR-23b-3p and contributes to F-actin cytoskeleton remodeling during erythropoiesis [J]. *PLoS One*, 2015, 10(8): e0136607.
- [4] PAVALKO F M, CHEN N X, TURNER C H, et al. Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton-integrin interactions [J]. *Am J Physiol*, 1998, 275(6 Pt 1): C1591-C1601.
- [5] FREUND J B, GOETZ J G, HILL K L, et al. Fluid flows and forces in development: functions, features and biophysical principles [J]. *Development*, 2012, 139(7): 1229-1245.
- [6] MANIOTIS A J, CHEN C S, INGBER D E. Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure [J]. *Proc Natl Acad Sci USA*, 1997, 94(3): 849-854.
- [7] KLEIN-NULEND J, BAKKER A D, BACABAC R G, et al. Mechanosensation and transduction in osteocytes [J]. *Bone*, 2013, 54(2): 182-190.
- [8] DELAINE-SMITH R M, SITTICHOKECHAIWUT A, REILLY G C. Primary cilia respond to fluid shear stress and mediate flow-induced calcium deposition in osteoblasts [J]. *Faseb J*, 2014, 28(1): 430-439.
- [9] YANG Z, TAN S, SHEN Y, et al. Inhibition of FSS-induced actin cytoskeleton reorganization by silencing LIMK2 gene increases the mechanosensitivity of primary osteoblasts [J]. *Bone*, 2015, 74(5): 182-190.
- [10] GONG X, FAN Y, ZHANG Y, et al. Inserted rest period resensitizes MC3T3-E1 cells to fluid shear stress in a time-dependent manner *via* F-actin-regulated mechanosensitive channel(s) [J]. *Biosci Biotechnol Biochem*, 2014, 78(4): 565-573.
- [11] XIANG Y H, SHAO M F, SONG Y, et al. Effect of cytoskeleton reorganization inhibition on the activation of extracellular signal-regulated kinase in osteoblasts by fluid shear stress [J]. *Chin J Stomatol*, 2012, 47(11): 680-683.
- [12] PAVALKO F M, BURRIDGE K. Disruption of the actin cytoskeleton after microinjection of proteolytic fragments of  $\alpha$ -actinin [J]. *J Cell Biol*, 1991, 114: 481-491.
- [13] SU Y P, CHEN C N, CHANG H I, et al. Low shear stress attenuates COX-2 expression induced by resistin in human osteoarthritic chondrocytes [J]. *J Cell Physiol*, 2017, 232(6): 1448-1457.
- [14] JIANG J, ZHAO L G, TENG Y J, et al. ERK5 signalling pathway is essential for fluid shear stress-induced COX-2 gene expression in MC3T3-E1 osteoblast [J]. *Mol Cell Biochem*, 2015, 406(1/2): 237-243.
- [15] TANG M, PENG Z, MAI Z, et al. Fluid shear stress stimulates osteogenic differentiation of human periodontal ligament cells *via* the extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase signalling pathways [J]. *J Periodontol*, 2014, 85(12): 1806-1813.
- [16] LIU Y S, LIU Y A, HUANG C J, et al. Mechanosensitive TRPM7 mediates shear stress and modulates osteogenic differentiation of mesenchymal stromal cells through osterix pathway [J]. *Sci Rep*, 2015, 5: 16522.
- [17] GONG X, YANG W, WANG L, et al. Prostaglandin E2 modulates F-actin stress fiber in FSS-stimulated MC3T3-E1 cells in a PKA-dependent manner [J]. *Acta Biochim Biophys Sin*, 2014, 46(1): 40-47.

(编辑:谭斯允)