

# 长链非编码RNA ZEB1-AS1通过HMGB1/TLR-4信号轴加剧大鼠脑缺血/再灌注损伤

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**摘要:**目的 探讨长链非编码RNA ZEB1-AS1在脑缺血/再灌注损伤(CI/RI)中的作用及其机制。方法 通过脑中动脉闭塞构建雄性SD大鼠局灶性CI/RI模型,实时定量PCR和Western blot分别检测ZEB1-AS1和HMGB1的时序表达并确定了CI/RI最佳时间(2 h/24 h)。将大鼠分为Sham组、CI/RI组、CI/RI+si-NC组、CI/RI+si-ZEB1-AS1组、CI/RI+OE-NC组、CI/RI+OE-ZEB1-AS1组。向CI/RI大鼠侧脑室注射ZEB1-AS1沉默/过表达载体及对照载体后,通过神经功能缺损评分和转盘实验分析认知功能;干湿重法分析脑水含量;Western blot检测白蛋白水平以评价血脑屏障完整性;ELISA法分析脑脊液和血清IL-1 $\beta$ 和TNF- $\alpha$ 水平。分别用FJC和TUNEL法检测CI/RI大鼠皮层中神经元丢失和细胞凋亡情况,Western blot分析HMGB1和TLR-4水平。此外,体外建立SH-SY5Y细胞氧-糖剥夺/复氧模型来模拟缺血/再灌注微环境,TUNEL法、Hoechst 33258染色、流式细胞术检测ZEB1-AS1沉默对神经元凋亡的影响;Western blot分析HMGB1和TLR-4水平。结果 qPCR和Western blot结果显示,ZEB1-AS1和HMGB1在CI/RI大鼠脑组织中的表达随着再灌注时间的延长升高(24 h达到峰值),而后逐渐降低。神经功能缺损评分和转盘试验结果显示,与OE-NC组相比,ZEB1-AS1过表达加重了CI/RI大鼠认知功能受损( $P<0.05$ );而si-ZEB1-AS1组大鼠的认知功能则好于si-NC组( $P<0.01$ )。OE-ZEB1-AS1组大鼠的脑水含量高于OE-NC组( $P<0.05$ );而与si-NC组相比,si-ZEB1-AS1组大鼠的脑水含量减少( $P<0.01$ )。血脑屏障渗漏检测数据提示,si-ZEB1-AS1组大鼠脑脊液中白蛋白的含量低于si-NC组( $P<0.01$ );OE-ZEB1-AS1组大鼠脑脊液中的白蛋白含量则高于OE-NC组( $P<0.05$ )。ELISA结果显示,si-ZEB1-AS1组大鼠脑脊液和血清IL-1 $\beta$ 和TNF- $\alpha$ 水平低于si-NC组( $P<0.01$ );而OE-ZEB1-AS1组大鼠脑脊液和血清IL-1 $\beta$ 和TNF- $\alpha$ 含量高于OE-NC组( $P<0.01$ )。OE-ZEB1-AS1组大鼠皮层FJC阳性神经元数量高于OE-NC组( $P<0.01$ );与此相反,si-NC组相比,ZEB1-AS1敲减则降低了FJC阳性神经元数量( $P<0.01$ )。Western blot结果显示,与OE-NC组相比,ZEB1-AS1过表达促进了HMGB1和TLR-4水平( $P$ 均 $<0.01$ );而ZEB1-AS1敲减则产生相反的结果( $P<0.01$ )。细胞水平上,与si-NC组相比,ZEB1-AS1敲减降低了TUNEL阳性细胞数( $P<0.01$ );流式细胞术检测结果进一步证实该现象。此外,Western blot结果进一步证实,ZEB1-AS1敲减下调了HMGB1和TLR-4水平( $P$ 均 $<0.01$ )。结论 长链非编码RNA ZEB1-AS1通过HMGB1/TLR-4信号轴加剧脑缺血/再灌注损伤。

**关键词:**脑缺血/再灌注损伤;长链非编码RNA ZEB1-AS1;高迁移率族蛋白1;Toll样受体4

## Long noncoding RNA ZEB1-AS1 aggravates cerebral ischemia/reperfusion injury in rats through the HMGB1/TLR-4 signaling axis

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**Abstract: Objective** To investigate the role of long non-coding RNA ZEB1-AS1 in cerebral ischemia/reperfusion injury (CI/RI). **Methods** We detected the temporal changes of ZEB1-AS1 and HMGB1 expression using qPCR and Western blotting in SD rats following CI/RI induced by middle cerebral artery occlusion (MCAO). The rat models of CI/RI were subjected to injections of vectors for ZEB1-AS1 overexpression or knockdown into the lateral ventricle, and the changes in cognitive function, brain water content, blood-brain barrier integrity, and IL-1 $\beta$  and TNF- $\alpha$  levels in the cerebrospinal fluid (CSF) and serum were observed. Neuronal loss and cell apoptosis in the cortex of the rat models were detected by FJC and TUNEL methods, and HMGB1 and TLR-4 expressions were analyzed with Western blotting. We also examined the effects of ZEB1-AS1 knockdown on apoptosis and expressions of HMGB1 and TLR-4 in SH-SY5Y cells with oxygen-glucose deprivation/reoxygenation (OGD/R). **Results** In CI/RI rats, the expressions of ZEB1-AS1 and HMGB1 in the brain tissue increased progressively with the extension of reperfusion time, reaching the peak levels at 24 h followed by a gradual decline. ZEB1-AS1 overexpression significantly aggravated cognitive impairment and increased brain water content, albumin content in the CSF, and IL-1 $\beta$  and TNF- $\alpha$  levels in the CSF and serum in CI/RI rats ( $P<0.05$ ), while ZEB1-AS1 knockdown produced the opposite effects ( $P<0.05$  or  $0.01$ ). ZEB1-AS1 overexpression obviously increased the number of FJC-positive neurons in the cortex and enhanced the expressions of HMGB1 and TLR-4 in the rat models ( $P<0.01$ ); ZEB1-AS1 knockdown significantly reduced the number of FJC-positive neurons and lowered HMGB1 and TLR-4 expressions ( $P<0.01$ ). In SH-SY5Y cells with OGD/R, ZEB1-AS1 knockdown significantly suppressed cell apoptosis and lowered the expressions of HMGB1 and TLR-4 ( $P<0.01$ ). **Conclusion** ZEB1-AS1 overexpression aggravates CI/RI in rats through the HMGB1/TLR-4 signaling axis.

**Keywords:** cerebral ischemia/reperfusion injury; lncRNA ZEB1-AS1; HMGB1; Toll-like receptor-4

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缺血性脑卒中是最常见的脑血管疾病之一,常导致患者残疾甚至死亡<sup>[1,2]</sup>。尽管静脉溶栓、抗炎和神经保护等治疗取得了一定的临床疗效,但缺血性卒中患者因神经元快速损伤,引发严重的神经功能障碍<sup>[3,4]</sup>。因此,亟需寻找具有高特异性和敏感性的理想生物标志物,用以改善缺血性中风的诊断、治疗和预后。

作为核DNA结合蛋白,高迁移率族蛋白1(HMGB1)从坏死和濒死的神经细胞中释放,导致缺血性脑卒中中的小胶质细胞活化和炎症因子的表达,并激活Toll样受体,从而促进炎症反应的激活<sup>[5,6]</sup>。随后,由HMGB1/TLR-4触发的炎症信号使核因子 $\kappa$ B核易位<sup>[7]</sup>。Toll样受体是一种模式识别受体,在大脑的神经元、小胶质细胞和星形胶质细胞中表达,参与炎症介质的释放<sup>[8]</sup>。研究表明,TLR-4缺陷/基因敲除小鼠在缺血性损伤后有轻微的损伤和较少的炎症反应<sup>[9,10]</sup>。

长链非编码RNAs(lncRNAs)在表观遗传、转录、RNA加工和翻译等多水平上参与了基因表达的调控<sup>[11,12]</sup>。lncRNA的缺陷、突变和异常表达与包括缺血性脑卒中在内的多种复杂疾病有关<sup>[13,14]</sup>。研究证实,lncRNA ZEB1-AS1是肿瘤相关的重要调节因子<sup>[15]</sup>,其在包括胃癌<sup>[16]</sup>、乳腺癌<sup>[17]</sup>、结肠直肠癌<sup>[18,19]</sup>、肝细胞癌<sup>[20-22]</sup>、脑瘤<sup>[23-25]</sup>等多种肿瘤中高表达,并通过不同的机制参与了上述肿瘤的发展。此外,ZEB1-AS1在非肿瘤疾病中也发挥重要作用。如ZEB1-AS1高表达促进了糖尿病肾病的纤维化<sup>[26]</sup>;且其在糖尿病的肺组织中下调<sup>[27]</sup>。最新研究证实,ZEB1-AS1参与了血管内皮细胞的氧化损伤<sup>[28-30]</sup>。

然而,ZEB1-AS1是否参与缺血性脑卒中以及其可能的分子机制尚未见报道。鉴于此,本研究首先检测了ZEB1-AS1在大鼠CI/RI模型脑组织和SH-SY5Y细胞氧-糖剥夺/复氧(OGD/R)模型中的表达,然后分析ZEB1-AS1在CI/RI模型中的生物学作用。最后,针对ZEB1-AS1的潜在分子机制进行评价,从而为缺血性脑卒中的诊治提供新的潜在靶点。

## 1 材料和方法

### 1.1 动物和神经细胞系

36只SPF级雄性SD大鼠(上海斯莱克,合格证号:20170005034832),周龄为4~5周。SH-SY5Y细胞系(广州赛库生物科技)。本研究获得了皖南医学院动物伦理委员会的批准(LLSC-2021-024),并严格按照《赫尔辛基宣言》的标准严格执行。

### 1.2 主要试剂

胎牛血清(FBS)、DMEM培养基、M-MLV反转录酶、pcDNA3.1(+)、体内转染试剂Entranster™-in vivo(英格恩生物公司)、脱氧葡萄糖Hanks缓冲液(碧云天)、PVDF膜、青霉素/链霉素溶液、TBST缓冲液、ECL试剂、Annexin V-FITC/PI、TTC染液、福尔马林、TNF- $\alpha$ 和

IL-1 $\beta$ 检测试剂盒(生工生物),抗HMGB1、TLR4、GAPDH和白蛋白一抗以及HRP偶联的二抗(CST)、Trizol、Lipofectamine 2000试剂(Invitrogen),SYBR Green Real-Time PCR Master Mixes (Agilent),QuickChange Lightning试剂盒(Stratagene),TUNEL检测试剂盒、Hoechst 33258染液(Abcam),引物及重组腺病毒(携带GFP报告基因)由GenePharma(上海)合成或构建。

### 1.3 大鼠脑立体定向注射

实验分组:假手术(Sham)组、脑缺血再灌注(CI/RI)组、CI/RI+siNC组、CI/RI+siZEB1-AS1组、CI/RI+OE-NC组、CI/RI+OE-ZEB1-AS1组,6只/组。将大鼠麻醉后,固定于立体定向仪。用Entranster™-in vivo转染试剂分别与5  $\mu$ L siNC、siZEB1-AS1、OE-NC和OE-ZEB1-AS1(各 $10^9$  U/mL)载体混合。然后注入侧脑室(前囟后0.2 mm,中线外侧1.0 mm和大脑表面下方1.5 mm)。24 h后,对大鼠进行MCAO。

### 1.4 大鼠脑缺血/再灌注损伤(CI/RI)大鼠模型的建立

参照改良的Zea-Longa栓线法制备脑中动脉闭塞(MCAO)大鼠模型<sup>[22]</sup>。实验前大鼠禁食12 h,禁水6 h,自主呼吸下用1.5%异氟醚的30% O<sub>2</sub>/68.5% NO气体麻醉大鼠,行颈正中切口,游离大鼠颈总动脉、颈外动脉和颈内动脉,用动脉夹轻轻夹住在距其交叉处1 cm处,用眼科镊挑起动脉,分离颈外动脉主干,在其交叉远心端处结扎,分离颈内动脉后用动脉夹轻轻夹闭。用眼科剪在颈外动脉远心端和固定线中点处剪小口,用眼科镊将A5级线栓缓慢插入颈内动脉中,直至插到线栓黑头处,即可停止进栓,2 h后,去除丝线以恢复血液再灌注24 h,用激光多普勒血流仪确认局部缺血和再灌注情况,再灌注72 h后处死大鼠。除MCAO外,Sham组大鼠进行相同的手术。所有动物研究均经皖南医学院动物护理委员会批准,并严格按照皖南医学院动物护理和使用委员会指南和《赫尔辛基宣言》的规定进行操作。

### 1.5 氧糖剥夺/复氧(OGD/R)细胞模型的建立与载体转染

实验分组:对照(NC)组、OGD/R组、OGD/R + si-NC组以及OGD/R + siZEB1-AS1组,每组3个生物学重复。SH-SY5Y细胞在含10% FBS、2 mmol/L谷氨酰胺、青霉素(100 U/mL)/链霉素(100  $\mu$ g/mL)的DMEM培养基在37  $^{\circ}$ C、5% CO<sub>2</sub>的加湿培养箱培养。待细胞融合度达80%后,用Lipofectamine 2000™转染试剂按常规流程将si-NC和si-ZEB1-AS1转染SH-SY5Y细胞,以进行ZEB1-AS1干扰。继续培养24 h后,将细胞置于无葡萄糖的DMEM中在含1% O<sub>2</sub>、94% N<sub>2</sub>和5% CO<sub>2</sub>的环境下对细胞进行氧糖剥夺(OGD)。然后,在常氧条件(37  $^{\circ}$ C、5% CO<sub>2</sub>)下,将细胞置于DMEM完全培养基在37  $^{\circ}$ C下孵育24 h。对照组则不进行OGD处理,并保持在常氧条件下的DMEM中培养。

### 1.6 RNA提取和qRT-PCR

用Trizol™试剂分别提取组织和细胞总RNA。2 μg RNA为模板,用M-MLV逆转录酶和Oligo(dT)引物合成cDNA。用SYBR Green Real-Time PCR Master Mixes进行PCR扩增ZEB1-AS1,并以GAPDH为内参。

PCR扩增在7900 H T Fast RealTime PCR仪上进行。PCR反应条件为:94 ℃ 5 min;94 ℃ 20 s,58 ℃ 45 s,72 ℃ 1 min,35个循环。PCR扩增数据以 $2^{-\Delta\Delta Ct}$ 进行定量。引物序列(表1)。

表1 qRT-PCR引物序列

Tab.1 qRT-PCR primer sequences used in this study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ZEB1-AS1	5'-GAGAGGCTAGAAGTTCCGCT-3'	5'-ACAAGCACCGTGTGGGTATT-3'
GAPDH	5'-TGTGAACGGATTGGCCGTA-3'	5'-GGTCTCGCTCCTGGAAGATG-3'

### 1.7 Western blot

脑组织和细胞用含蛋白酶抑制剂的RIPA裂解缓冲液提取总蛋白。用12.5% SDS-PAGE凝胶电泳分离样品后,移至PVDF膜上。然后将膜用5%脱脂牛奶的TBST缓冲液室温封闭1 h。膜与包括抗HMGB1(1:1000)、抗-TLR-4(1:1000)、抗白蛋白(1:1000)和抗-GAPDH(1:1000)的一抗在4 ℃下孵育过夜。TBST洗膜后,用辣根过氧化物酶偶联的二抗室温下孵育1 h。ECL试剂盒可视化蛋白条带。用ImageJ软件读取各组条带的灰度值,与各组的内参GAPDH进行比较后,各组数据再与Sham组进行比较,获得绘图数据。

4%多聚甲醛(w/v)固定脑切片10 min,用PBS洗片,多聚甲醛加醋酸后4 ℃固定切片5 min,PBS洗片。依次加入平衡缓冲液和TdT,37 ℃反应1 h。拍片并计算TUNEL阳性细胞百分比。

### 1.8 脑水肿和血脑屏障测定

大鼠引颈处死,取出两侧半球,以脑组织干重/湿重法<sup>[31]</sup>测定对侧基底神经节、同侧基底神经节(Ipsi-BG)、对侧皮质、同侧皮质(Ipsi-CX)和小脑的脑水肿程度。血脑屏障(BBB)评估则用Western blot检测白蛋白水平。

针对Hoechst 33258染色评估细胞凋亡。根据说明书用多聚甲醛固定细胞玻片15 min。PBS洗涤玻片后,室温下与Hoechst 33258染色液(1 mg/mL)一起孵育5 min。吸除Hoechst 33258染色液后,玻片用PBS冲洗。荧光显微镜(激发波长350 nm,发射波长460 nm)拍片并计算凋亡细胞百分比。

### 1.9 Fluoro-Jade C(FJC)染色

FJC法是确定神经元退化的经典方法。脑组织切片脱蜡、脱水后,0.06% KMnO<sub>4</sub>孵育10 min,去离子水冲洗30 min。随后用0.1%乙酸浸片20 min,37 ℃干燥,二甲苯洗涤后用DPX固定。荧光显微镜下计数FJC阳性细胞百分比。

此外,本研究也用FITC-Annexin V和碘化丙啶双染按照流式细胞检测试剂盒说明书检测细胞凋亡。细胞用预冷1×PBS(4 ℃)洗涤1次,加入300 μL的1×结合缓冲液重悬;加入5 μL的Annexin V-FITC混匀后,室温避光孵育15 min;上机前加入5 μL的PI染色5 min。随后补加200 μL的1×结合缓冲液。通过流式细胞仪和CellQuest Pro软件评估细胞凋亡率。

### 1.10 Nissl染色

Nissl染色用于评估脑组织中神经元的丢失<sup>[32]</sup>。脑切片脱蜡并用蒸馏水水合后,将各组大鼠的脑切片与0.5%甲苯胺蓝在37 ℃下孵育30 min。随后,将脑切片用梯度酒精脱水并用二甲苯澄清,并将脑切片用中性树脂封片。在光学显微镜下计数颞皮质中的尼氏染色阳性神经元。每个脑切片至少获得3张显微照片。死细胞不包括在Nissl阳性神经元计数范围内。

### 1.13 炎症因子TNF-α和IL-1β水平的检测

大鼠麻醉后,固定于定向仪,头颈部剃毛,用剪刀暴露枕骨大孔,用1 mL一次性注射器抽取脑脊液;并进行摘眼球采血。脑脊液和血液分别用1500 g和3000 r/min离心30 min,收集上清。按照TNF-α和IL-1β的ELISA试剂盒操作说明书进行含量测定。

### 1.11 神经功能评定

按照文献[33, 34]采用神经功能缺损评分对神经功能状态进行评分。

### 1.14 统计学分析

用SPSS 20.0软件进行数据分析,定量资料以均数±标准差表示,两组间比较用非配对Student's *t*检验,多组间比较采用单因素方差分析。*P*<0.05为差异具有统计学意义。每个实验重复3次。

### 1.12 细胞凋亡检测

用TUNEL测定试剂盒测定细胞凋亡。室温下,用

## 2 结果

### 2.1 CI/RI模型大鼠脑中ZEB1-AS1和HMGB1上调

各组大鼠脑血流检测结果显示,CI/RI组大鼠已出现严重的脑中动脉闭塞,即CI/RI造模成功(图1A)。与Sham组相比,CI/RI组中ZEB1-AS1的表达自再灌注3 h开始持续升高,并于再灌注24 h达到最高(*P*<0.01,图1B)。

Western blot结果也显示,与Sham组相比, HMGB1的表达趋势与ZEB1-AS1的表达趋势一致(图1C)。

神经行为评分结果显示, CI/RI组大鼠的评分低于Sham组( $P<0.01$ )。但用si-ZEB1-AS1处理的CI/RI大鼠其神经功能评分高于CI/RI+si-NC组大鼠( $P<0.01$ )。然而,与CI/RI+OE-NC组相比, CI/RI+OE-ZEB1-AS1

组大鼠的认知功能更差( $P<0.05$ ,图1D),且低于CI/RI组( $P<0.05$ )。此外,轮转实验结果显示, CI/RI大鼠的轮转时间短于Sham组( $P<0.05$ )。CI/RI+OE-ZEB1-AS1组大鼠轮转时间也少于CI/RI+OE-NC( $P<0.05$ ,图1E)。但CI/RI+si-ZEB1-AS1组大鼠轮转时间则长于CI/RI+si-NC组大鼠( $P<0.01$ ,图1E)。

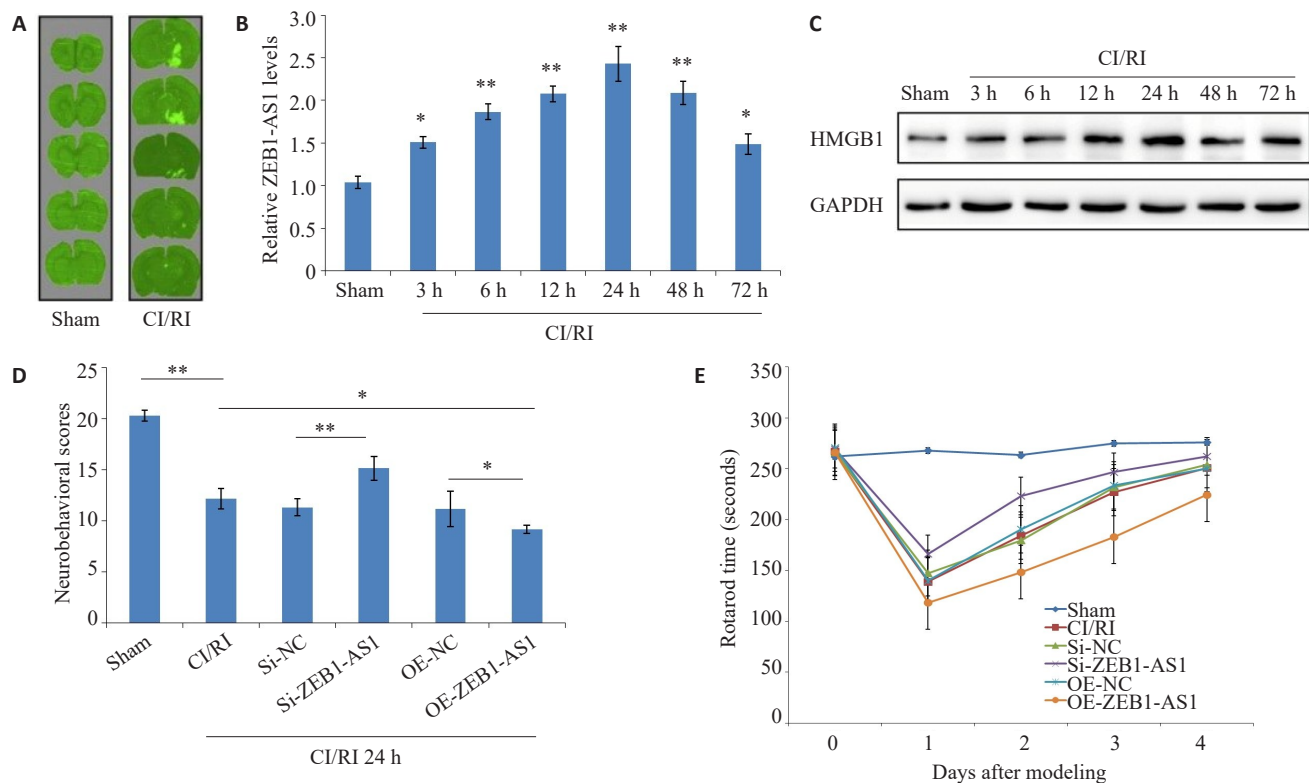


图1 大鼠CI/RI模型中ZEB1-AS1和HMGB1表达及神经行为评分

Fig.1 Expression of ZEB1-AS1 and HMGB1 and neurobehavioral scores of rat models of CI/RI. A: Representative cerebral blood flow images of the rat model. B, C: Expression of ZEB1-AS1 (B) and HMGB1 (C) after CI/RI in rats. D: Neurobehavioral test results of CI/RI rats treated with different plasmids. E: Roulette test results of CI/RI rats treated with different plasmids. \* $P<0.05$ , \*\* $P<0.01$ ,  $n=6$ .

## 2.2 ZEB1-AS1对CI/RI模型大鼠血脑屏障及炎症因子的影响

CI/RI引发大鼠BBB的通透性增加,导致白蛋白渗漏。Western blot结果显示:与Sham组相比, CI/RI组大鼠脑组织中白蛋白水平升高( $P<0.01$ )。而si-ZEB1-AS1组中的白蛋白水平则低于si-NC组( $P<0.01$ ),相反,与OE-NC组相比, ZEB1-AS1过表达(OE-ZEB1-AS1)则增加了白蛋白水平( $P<0.05$ ,图2A,B)。另外, ZEB1-AS1过表达其BBB渗漏更严重( $P<0.05$ )。

为评估ZEB1-AS1对CI/RI模型脑水肿的影响,我们检查了不同脑区的脑含水量。结果显示, ZEB1-AS1的过表达加重了CI/RI诱导的Ipsi-BG和Ipsi-CX区的脑水肿( $P<0.01$ ,图2C);相反, ZEB1-AS1的敲低则抑制了Ipsi-BG和Ipsi-CX区的水肿( $P<0.01$ )。但抑制或过

表达ZEB1-AS1不影响其他脑区的含水量。此外, ZEB1-AS1过表达组大鼠脑水肿程度高于CI/RI组大鼠( $P<0.01$ )。

ELISA结果显示, CI/RI+si-ZEB1-AS1组大鼠脑脊液中TNF- $\alpha$ 和IL- $\beta$ 的水平低于CI/RI+si-NC组( $P<0.01$ );相反,与CI/RI+OE-NC组大鼠相比, ZEB1-AS1过表达促进了TNF- $\alpha$ 和IL- $\beta$ 的分泌( $P$ 均 $<0.01$ )。血清TNF- $\alpha$ 和IL- $\beta$ 也表现出相同的趋势;且ZEB1-AS1过表达导致其炎症介质水平高于CI/RI组( $P$ 均 $<0.01$ )。

## 2.3 ZEB1-AS1过表达促进了CI/RI模型大鼠神经元变性及细胞凋亡

CI/RI组大鼠FJC阳性神经元多于Sham组( $P<0.01$ ,图3A~B)。同样, CI/RI+OE-ZEB1-AS1组FJC阳性的神经元也多于CI/RI+OE-NC组( $P<0.01$ ),而与CI/

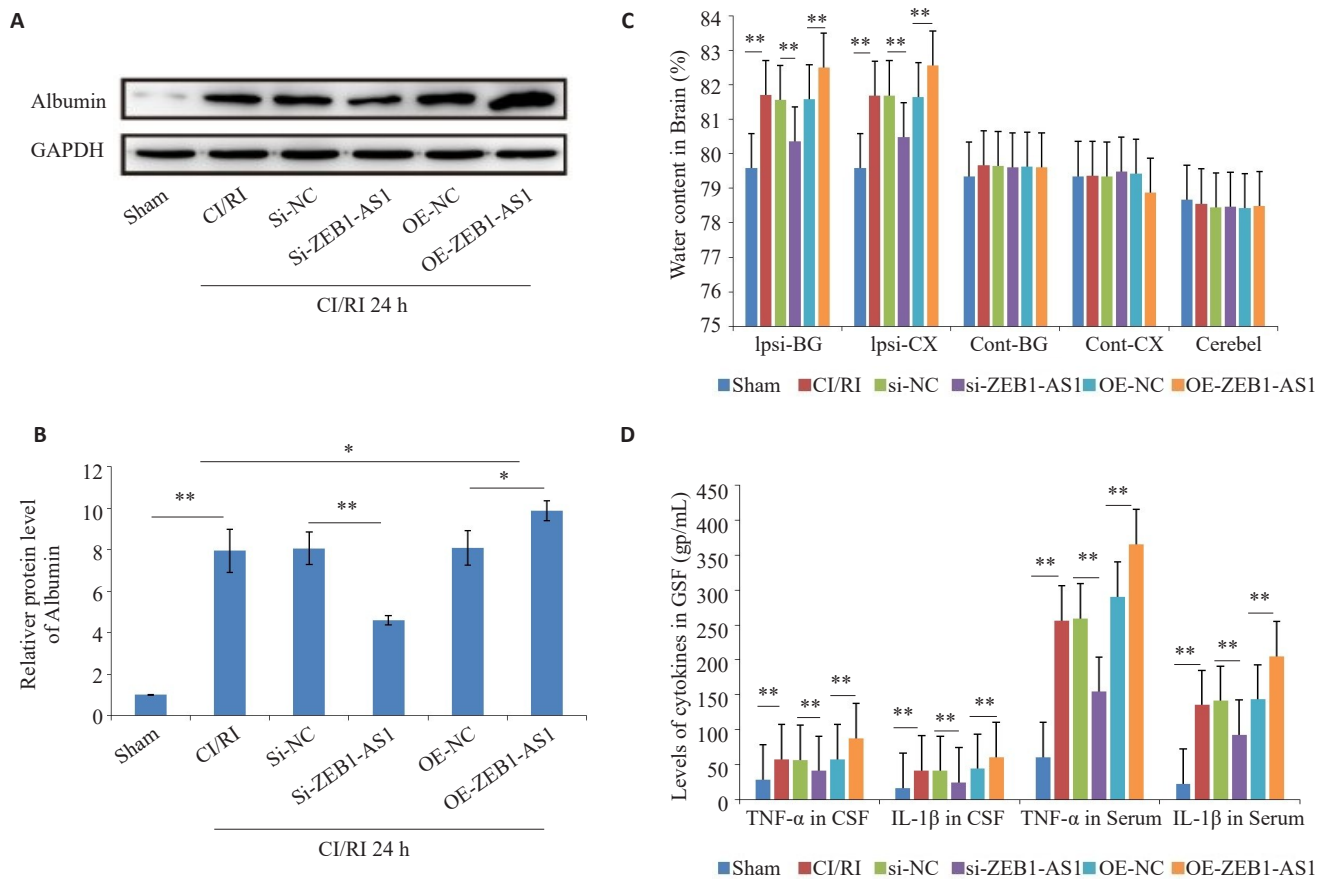


图2 ZEB1-AS1的表达对血脑屏障、脑水肿及炎症因子的影响

Fig.2 Effects of ZEB1-AS1 expression on blood-brain barrier, cerebral edema and inflammatory factors. **A, B**: Western blotting of albumin levels and quantitative analysis of the results. **C**: Effect of ZEB1-AS1 expression on cerebral edema in the Cont-BG, Ipsi-BG, Cont-CX, Ipsi-CX, and cerebellum. **D**: Effect of ZEB1-AS1 expression on TNF- $\alpha$  and IL- $\beta$  levels in the cerebrospinal fluid and serum. \* $P < 0.05$ , \*\* $P < 0.01$ ,  $n = 6$ .

RI+si-NC组相比,CI/RI+si-ZEB1-AS1组FJC阳性的神经元数量少于CI/RI+si-NC组( $P < 0.01$ )。此外,与CI/RI组相比,CI/RI+OE-NC和CI/RI+si-NC组FJC阳性的神经元数量未见明显改变( $P > 0.05$ )。

尼氏染色结果显示,CI/RI组大鼠海马CA1区存活神经元的数量少于Sham组( $P < 0.01$ );同样,与CI/RI + OE-NC组相比,ZEB1-AS1过表达使存活的神经元数量也下降( $P < 0.01$ )。相反,ZEB1-AS1过表达则神经元数量多于CI/RI+si-NC组( $P < 0.01$ ,图3C,D)。此外,与CI/RI组、CI/RI+OE-NC组以及CI/RI+si-NC组间存活神经元数目差异无统计学意义( $P > 0.05$ )。

TUNEL染色显示,与Sham组相比,CI/RI诱导了更多的神经元凋亡( $P < 0.01$ );且ZEB1-AS1过表达促进了CI/RI诱导的神经元凋亡( $P < 0.01$ )。但与CI/RI+si-NC组相比,抑制ZEB1-AS1的表达则降低了神经元的凋亡( $P < 0.01$ ,图3E~F)。

此外,ZEB1-AS1过表达其诱导的神经元丢失、存活神经元数目以及神经元凋亡数目与CI/RI组的差异存在统计学意义( $P$ 均 $< 0.01$ ,图3B、D、F)。

#### 2.4 ZEB1-AS1过表达促进了脑组织HMGB1和TLR-4

的表达水平

为检测ZEB1-AS1在CI/RI进程中的潜在机制,分析CI/RI模型大鼠脑组织中HMGB1和TLR-4的表达水平。结果显示(图4):相对于Sham组,CI/RI组的HMGB1和TLR-4水平均增加( $P < 0.01$ ),而CI/RI+si-ZEB1-AS1组HMGB1和TLR-4蛋白水平较CI/RI+si-NC组下降( $P < 0.01$ )。过表达ZEB1-AS1后,CI/RI+OE-ZEB1-AS1组中的HMGB1和TLR-4蛋白表达较CI/RI+OE-NC组升高( $P < 0.01$ )。此外,HMGB1和TLR-4水平在CI/RI组、CI/RI+OE-NC组以及CI/RI+si-NC组间未见明显变化( $P > 0.05$ )。

#### 2.5 ZEB1-AS1对OGD/R模型细胞的细胞凋亡及HMGB1和TLR-4表达的影响

TUNEL结果显示(图5A~B),OGD/R组细胞凋亡的数量多于NC组( $P < 0.01$ ),OGD/R+si-NC与OGD组相比凋亡细胞数量未见明显变化,而经OGD/R+si-ZEB1-AS1组比OGD/R+si-NC组凋亡细胞数量下降( $P < 0.01$ )。流式细胞分析进一步证实了上述结果(图5C~D)。

Western blot结果显示(图5E~F),较正常组而言,

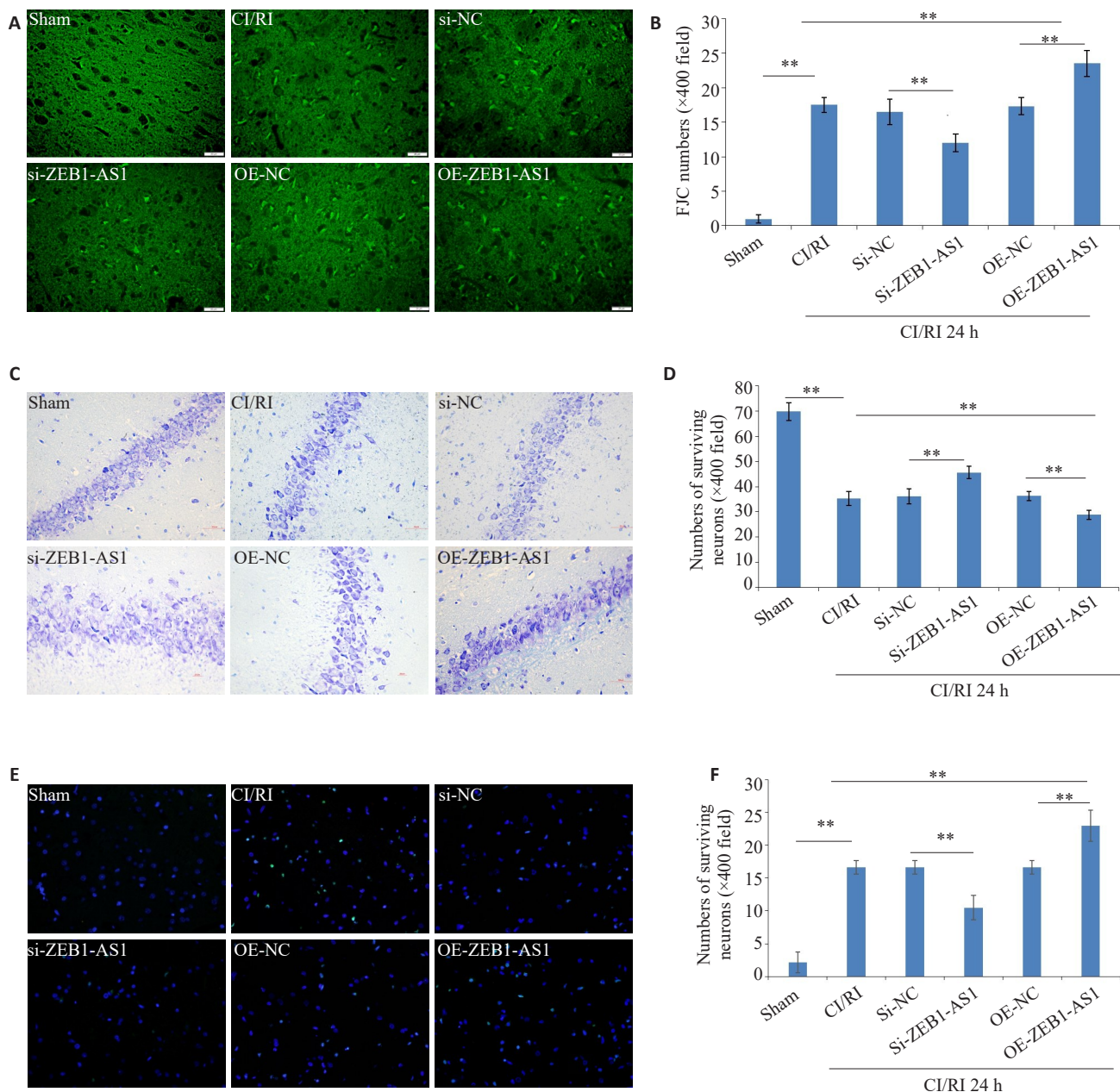


图3 ZEB1-AS1 表达对CI/RI诱导的神经元变性和凋亡的影响

Fig.3 Effects of ZEB-1-AS1 expression on CI/RI-induced neuronal degeneration and apoptosis (Original magnification: ×400). **A, B:** FJC staining for detecting degeneration of cortical neurons in CI/RI rats with different treatments (arrows indicate FJC-positive neurons) and quantification FJC-positive neurons (×400). **C, D:** Nissl staining of neurons in the hippocampal CA1 region (arrow indicates Nissl-positive neurons) and quantification Nissl-positive neurons (×400). **E, F:** TUNEL staining of the neurons in the brain tissue (×400) and quantitative analysis of TUNEL-positive cells. \*\**P*<0.01, *n*=6.

OGD/R组中HMGB1和TLR-4蛋白水平增加(*P*<0.01), 而OGD/R+si-ZEB1-AS1组中HMGB1和TLR-4水平低于OGD/R+si-NC组(*P*<0.01)。但OGD/R+si-NC组HMGB1和TLR-4蛋白水平较OGD/R组未见明显变化(*P*>0.05)。

### 3 讨论

CI/RI是缺血性脑卒中的重要病理生理过程,与神经元凋亡等有关。研究证实,ZEB1-AS1在ox-LDL诱导的内皮细胞损伤中起作用<sup>[28]</sup>,进一步发现是通过miR-

590-5p/HDAC9轴实现的<sup>[29]</sup>。lncRNAs在缺血性中风中发挥重要的作用<sup>[33, 35, 36]</sup>。然而,关于ZEB1-AS1在CI/RI中的生物学功能尚有待阐明。本研究结果显示,ZEB1-AS1在CI/RI大鼠受损脑组织中显著上调。为了进一步探索ZEB1-AS1在CI/RI中的生物学作用,我们检测了ZEB1-AS1沉默/过表达对CI/RI大鼠认知功能、神经元数量、脑水肿、血脑屏障等的影响。结果显示,ZEB1-AS1过表达严重损害了CI/RI大鼠的认知功能,且活性神经元数量明显减少;此外,模型大鼠的脑水肿加重和血脑屏障渗漏。而ZEB1-AS1沉默则使模型大

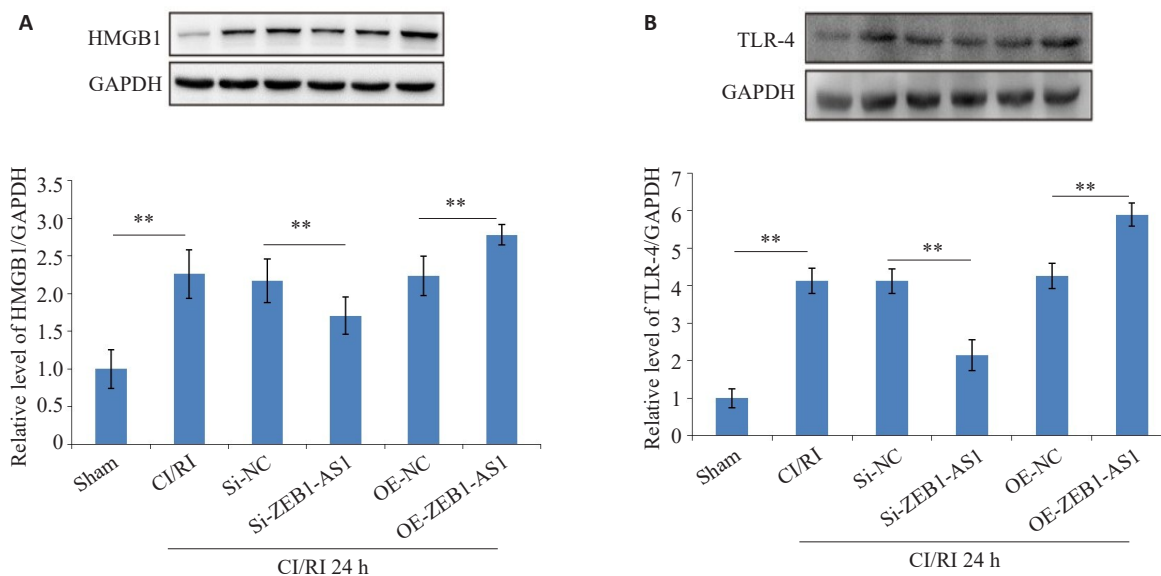


图4 ZEB1-AS1对CI/RI模型大鼠脑组织中HMGB1和TLR-4表达的影响

Fig.4 Effects of ZEB1-AS1 on expression of HMGB1 and TLR-4 in CI/RI model rat brain. A: Changes of HMGB1 protein in CI/RI treatment groups; B: Changes of TLR-4 in CI/RI groups. \*\*P<0.01, n=6.

鼠的认知功能的恢复、活性神经元丢失减轻、脑水肿缓解以及血脑屏障渗漏减轻。TUNEL检测结果进一步提示,ZEB1-AS1沉默降低了神经元凋亡。有研究发现,lncRNA CAMK2D相关转录物2(C2dat2)上调促进了CI/RI诱导的神经元凋亡<sup>[37]</sup>。

原发性脑缺血性损伤诱发的炎症反应(如TNF-α和IL-1β)是继发性脑损伤的关键机制<sup>[38,39]</sup>。本研究数据表明,ZEB1-AS1过表达促进了CI/RI大鼠脑脊液和血清TNF-α和IL-1β水平,反之,则抑制了脑脊液和血清TNF-α和IL-1β的表达。说明ZEB1-AS1的表达通过释放炎症因子促进了I/R的继发性脑损伤。该结果与Hua等<sup>[30]</sup>的结果类似,该研究发现敲减ZEB1-AS1消除了ox-LDL诱发的TNF-α和IL-1β水平。提示ZEB1-AS1是促进CI/RI进程中炎症应答的重要分子。

ZEB1-AS1的表达异常通过多种机制调节细胞的增殖和凋亡。如ZEB1-AS1通过促进转录因子ZEB1的表达通过上皮间质转换促进了肺纤维化<sup>[40]</sup>,ZEB1-AS1的表达升高抑制了p53表达促进的糖尿病肾病的肾纤维化<sup>[26]</sup>。有研究报道,ZEB1-AS1通过miR-942/HMGB1信号促进了ox-LDL诱导的HcT-AEC细胞损伤和THP1细胞的炎症应答<sup>[30]</sup>。大量研究证实,HMGB1是I/R损伤活化的星形胶质细胞分泌的一种细胞因子样介质,参与了缺血性脑神经元损伤和神经炎症<sup>[41]</sup>。HMGB1中存在TLR-4特定结合的功能结构域,二者的结合并受CI/RI应激反应的正向调节<sup>[42,43]</sup>。本研究数据显示,ZEB1-AS1抑制导致HMGB1和TLR-4表达下降。由此推测,在CI/RI进程中,ZEB1-AS1的表达升高可能通过促进HMGB1和TLR-4的表达而导致脑功能损害。

但该机制中是否存在其他中间分子如microRNAs或RNA结合蛋白等有待阐明。

综上所述,ZEB1-AS1/HMGB1/TLR-4信号在CI/RI进程中发挥重要的促进作用,有望为CI/RI损伤治疗提供新的潜在靶点。

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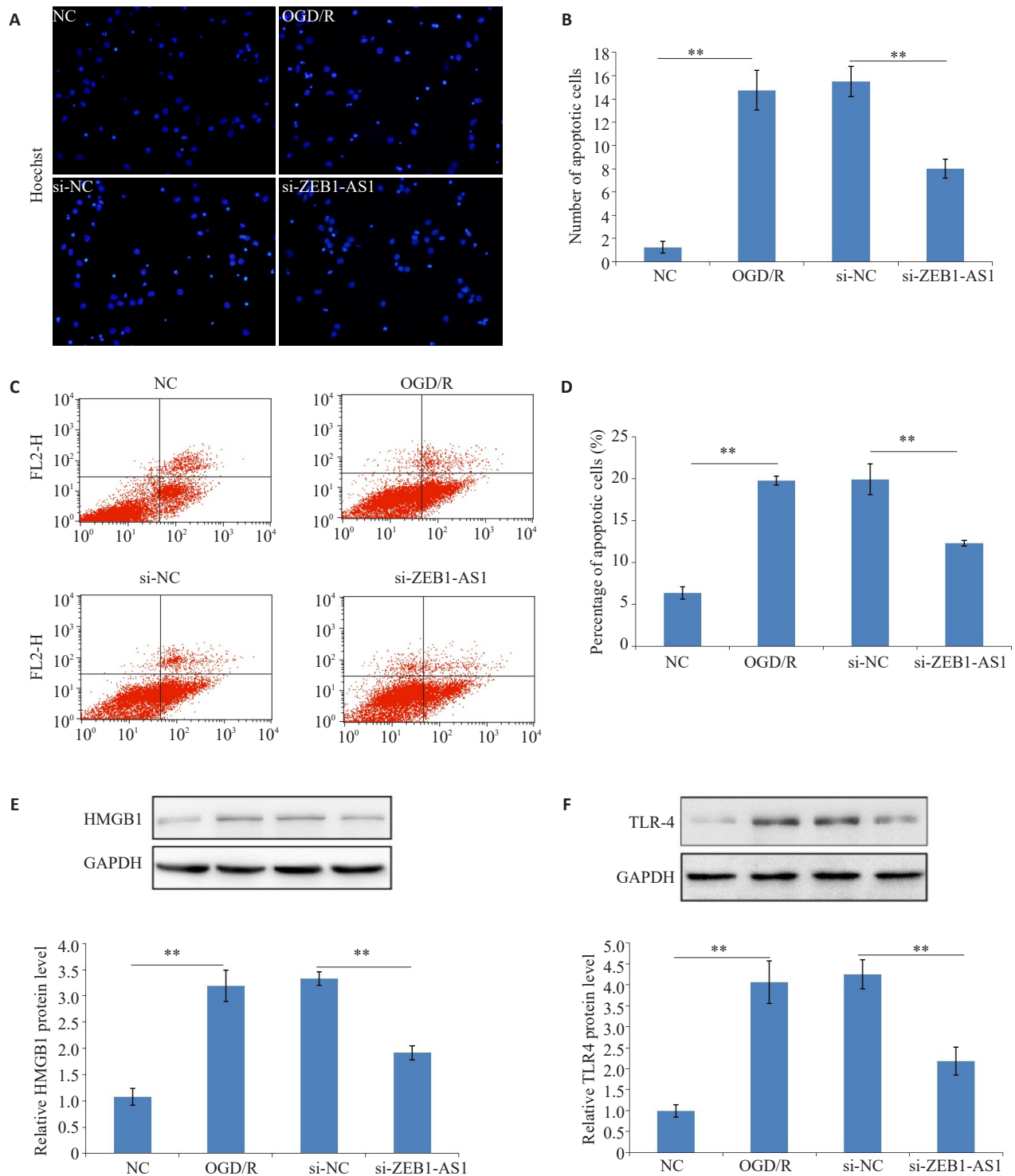


图5 OGD/R处理的SH-SY5Y细胞中细胞凋亡、HMGB1和TLR-4蛋白的水平改变

Fig.5 Apoptosis and expressions of HMGB1 and TLR-4 proteins in OGD/R-treated SH-SY5Y cells. **A, B:** TUNEL staining of apoptotic cells (arrow) in OGD/R-treated SH-SY5Y cells and quantitative analysis of the apoptotic cells ( $\times 400$ ). **C, D:** Annexin-V/PI staining of apoptosis cells in OGD/R-treated cells analyzed by flow cytometry and quantitative analysis of the apoptotic cells. **E:** Changes of HMGB1 protein in OGD/R-treated SH-SY5Y cells. **F:** Changes of TLR-4 protein in OGD/R-treated SH-SY5Y cells.  $**P < 0.01, n = 6$ .

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