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研究论文

# 肝细胞生长因子对氧糖剥夺 / 再灌注神经元的保护作用

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**摘 要：**本文旨在探讨肝细胞生长因子(hepatocyte growth factor, HGF)对神经元氧糖剥夺 / 再灌注损伤的影响。取原代培养 12 d 的 Sprague-Dawley 大鼠大脑皮层神经元，无糖、无氧(95% $N_2$ + 5% $CO_2$)孵育 2 h 后，换含 25 mmol/L 葡萄糖的培养液、常氧培养 0~24 h，以 MTT 比色法检测细胞活力、乳酸脱氢酶(lactate dehydrogenase, LDH)漏出率作为细胞损伤指标，建立体外氧糖剥夺 / 再灌注损伤细胞模型；用流式细胞仪和 Hoechst 33258 染色分析细胞凋亡率；用 RT-PCR 和 Western blot 分别检测大鼠脑皮层神经元 HGF 受体 c-Met mRNA 和蛋白的表达。于氧糖剥夺 2 h/ 再灌注 24 h 处理前 2 h，加入不同终浓度(5~120 ng/mL)的 HGF，观察 HGF 对皮层神经元的影响。结果显示，c-Met 表达于皮层神经元，氧糖剥夺 2 h/ 再灌注 24 h 后，c-Met mRNA 和蛋白表达均显著上调，神经元细胞活力明显降低，LDH 漏出率和细胞凋亡率显著增高。HGF 预处理明显促进氧糖剥夺 / 再灌注损伤神经元的存活，降低 LDH 漏出率，最大效应剂量为 80 ng/mL。流式细胞术和 Hoechst 33258 染色结果均显示，HGF (80 ng/mL)显著降低氧糖剥夺 / 再灌注神经元的细胞凋亡率。此外，c-Met 抑制剂 SU11274 (5 μmol/L)完全阻断 HGF 的神经保护作用。结果表明，HGF 对皮层神经元氧糖剥夺 / 再灌注损伤具有直接的保护作用，呈一定的剂量依赖关系，并能有效对抗神经元凋亡。

**关键词：**肝细胞生长因子；神经元；氧；葡萄糖；再灌注
**中图分类号：**R338

# Protection of hepatocyte growth factor on neurons subjected to oxygen-glucose deprivation/reperfusion

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**Abstract:** The present study was conducted to investigate the effect of hepatocyte growth factor (HGF) on cortical neurons exposed to oxygen-glucose deprivation/reperfusion (OGD/R). Primary cultured cerebral cortical neurons were prepared from Sprague-Dawley rats. The cells were used for experiments after culture for 12 d *in vitro*. To initiate OGD/R, the culture medium was replaced by glucose-free medium, and cells were transferred to a humidified incubation chamber flushed by a gas mixture of 95% $N_2$ and 5% $CO_2$ at 37 °C for 2 h. Following this treatment, neurons were fed with glucose-supplemented (25 mmol/L) medium, and returned to the incubator under normoxic condition for 0-24 h. The cell viability was assessed by MTT assay, and cell injury was evaluated by lactate dehydrogenase (LDH) leakage rate. The percentage of apoptotic cells was analyzed by flow cytometry and Hoechst 33258 staining. The expressions of c-Met mRNA and protein were detected by RT-PCR and Western blot analysis, respectively. Oxygen-glucose deprivation for 2 h decreased the cell viability and increased LDH leakage rate in cultured cerebral cortical neurons. The cell viability declined and LDH leakage rate increased with the reperfusion time going on (0-24 h). To explore the influence of HGF on neurons under oxygen-glucose deprivation for 2 h/reperfusion for 24 h ($OGD_2/R_{24}$) condition, the cultures were pretreated with HGF at different concentrations (5-120 ng/mL) 2 h prior to $OGD_2/R_{24}$. The results showed that $OGD_2/R_{24}$ treatment significantly decreased the cell viability, increased LDH leakage rate and the percentage of apopototic cells. Pretreatment with HGF at 5 ng/mL and 10 ng/mL did not

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affect the decrease in cell viability resulting from $OGD_2/R_{24}$. In the presence of 20 ng/mL HGF, the increase in cell viability in cortical neurons exposed to $OGD_2/R_{24}$ began to appear, and 80 ng/mL of HGF exhibited the maximal effect. HGF at 5, 10 and 20 ng/mL did not affect the increase in LDH leakage rate in cortical neurons exposed to $OGD_2/R_{24}$. In the presence of 40 ng/mL HGF, the decrease in LDH leakage rate in cortical neurons subjected to $OGD_2/R_{24}$ began to appear, and 80 ng/mL of HGF displayed the maximal effect. In addition, HGF at 80 ng/mL significantly attenuated cell apoptosis resulting from $OGD_2/R_{24}$. As detected by semi-quantitative RT-PCR and Western blot analysis, c-Met mRNA and protein were expressed in cerebral cortical neurons cultured for 12 d *in vitro*. c-Met mRNA and protein expressions in cortical neurons exposed to $OGD_2/R_{24}$ were significantly upregulated and were not affected by pretreatment of HGF at 80 ng/mL. Treatment with c-Met inhibitor SU11274 (5 μmol/L) completely eliminated HGF-mediated protection of cortical neurons subjected to $OGD_2/R_{24}$. The results suggest that HGF directly protects cortical neurons against OGD/R-induced cell injury in a dose-dependent manner, and HGF has a potent anti-apoptotic action on neurons exposed to OGD/R.

**Key words:** hepatocyte growth factor; neurons; oxygen; glucose; reperfusion

肝细胞生长因子(hepatocyte growth factor, HGF)又称为离散因子(scatter factor, SF)，最初被认为是肝细胞强有力的促有丝分裂原。现已知HGF是一种多功能的细胞因子，不仅能促进多种细胞的增殖、迁移、存活，还具有促血管形成的作用[1-3]。原癌基因编码的产物——c-Met是目前唯一已知的HGF受体。HGF与跨膜受体c-Met结合后，通过位于受体C-末端的多功能位点激活细胞内的信号分子[4,5]，产生多种生物学功能。

HGF及其受体c-Met在发育和成年哺乳动物脑的不同区域都有表达，并在中枢神经系统中发挥着各种生物学效应[[6-8]]。体外实验表明，HGF显著促进新生小鼠皮层神经元树突的生长及分支[9]，促进丘脑神经元轴突的生长，且具有化学引诱物的特性[10]。体内实验表明，外源性HGF可减轻脑缺血引起的学习和记忆障碍，减小梗死灶的体积[1,11]。

HGF是对抗脑缺血损伤强有力的保护因素。然而，在脑缺血损伤中，HGF是否通过直接作用于神经元来产生神经保护效应尚未明确，因为在体内HGF减轻脑缺血损伤至少部分与其促血管形成作用有关[1,12,13]。因此，我们采用离体实验模型研究HGF对神经元是否具有直接保护作用，以消除体内各种因素干扰。

## 1 材料与方法

**1.1 大脑皮层神经元的分离培养** 取孕18 d左右的Sprague-Dawley大鼠胎鼠(湘雅医学院实验动物中心)，无菌条件下分离出大脑皮层组织，胰酶37 ℃消化10 min。分离细胞重悬于添加B-27的neurobasal培养基(Gibco公司，美国)，种植在预先用多聚-*D*-赖氨酸(Sigma公司，美国)包被过的多孔培养板或皿中。置37 ℃、5% $CO_2$、饱和湿度培养箱内(Forma Scientific公司，美国)培养，培养12 d的细胞用于实验。

**1.2 氧糖剥夺/再灌注** 氧糖剥夺：细胞培养液换为预先通以无氧混合气体(95% $N_2$ + 5% $CO_2$) 30 min的无糖培养液，然后将细胞立即置于通有无氧混合气体(95% $N_2$ + 5% $CO_2$)的密闭室内[14,15]，37 ℃、饱和湿度条件下培养2 h。氧糖剥夺2 h，记为$OGD_2$。再灌注：在达到设定的氧糖剥夺时间后，换含25 mmol/L葡萄糖的培养液，37 ℃、5% $CO_2$、饱和湿度培养箱内培养。氧糖剥夺2 h后，再灌注0 h、6 h、12 h、24 h，分别记为$OGD_2/R_0$、$OGD_2/R_6$、$OGD_2/R_{12}$、$OGD_2/R_{24}$。

**1.3 药物处理** 在氧糖剥夺处理前2 h，加入人重组HGF (R&D systems，美国)，终浓度分别为5、10、20、40、80、120 ng/mL。SU11274 (Sigma公司，美国)的终浓度为5 μmol/L。

**1.4 MTT比色法检测细胞活力** 加入MTT (终浓度为0.5 mg/mL)，于37 ℃孵育1 h，弃上清，加入二甲基亚砜，置酶标仪(BioTek公司，美国)540 nm处测定OD值。以对照组细胞活力为100%，实验组细胞活力(%)= 实验组OD值/对照组OD值×100%。

**1.5 乳酸脱氢酶(lactate dehydrogenase, LDH)漏出率的测定** 收集细胞孵育液，全自动生化分析仪(Hitachi公司，日本)测定LDH活性，此为孵育液LDH活性。神经元用1% Triton X-100处理30 min后收集细胞裂解液，测定LDH活性，此为细胞LDH活性。LDH漏出率(%)= 孵育液LDH活性/(孵育液LDH活性 + 细胞LDH活性)×100%。

**1.6 细胞凋亡的检测**

**1.6.1 流式细胞术** 收集细胞，加入冰预冷的70%乙醇于4 ℃固定24 h，调整细胞密度为$1.0\times10^6$

个/mL，取 1 mL 细胞悬液，用 PBS 洗 3 次，细胞重悬于含 RNase A 的 PI 染液中，37 ℃孵育 30 min，以流式细胞仪(Coulter 公司，美国)分析细胞凋亡率[16]。

**1.6.2 Hoechst 33258 染色** 待测细胞弃培养液，3% 多聚甲醛固定 10 min，弃固定液，PBS 漂洗后，加入 1 μg/mL Hoechst 33258 (Invitrogen 公司，美国)，室温孵育 10 min，荧光显微镜(Olympus 公司，日本)下观察并计数。凋亡细胞显示核浓染、碎裂等典型的形态学改变，同一处理随机选择至少 10 个观察视野，以凋亡阳性细胞数所占细胞总数的百分比为细胞凋亡率(%)。

**1.7 RT-PCR 检测 *c-Met* mRNA 的表达** 常规方法提取各组总 RNA，以 RT-PCR 法检测 *c-Met* mRNA 的表达。*c-Met* 正向引物：5'-TCTCGGAGCCAC-AAACTA-3'，反向引物 5'-TGAATAATCGGGA-GGGTA-3'，产物大小 445 bp。*β-actin* 正向引物：5'-TCACCAACTGGGACGATA-3'，反向引物：5'-GTCTTTACGGATGTCAACG-3'，产物大小 650 bp。按照 RevertAid™ First Strand cDNA Synthesis Kit (Fermentas 公司，立陶宛)说明进行逆转录反应：70 ℃ 5 min，37 ℃ 5 min，42 ℃ 60 min，70 ℃ 10 min。PCR 反应条件：预变性 94 ℃ 3 min；变性 94 ℃ 30 s，退火 56 ℃ (*c-Met*)、58 ℃ (*β-actin*) 40 s，延伸 72 ℃ 1 min，28 个循环；再延伸 72 ℃ 10 min。取 PCR 产物进行 1.8% 琼脂糖凝胶电泳，凝胶成像分析系统扫描。

**1.8 Western blot 检测 c-Met 蛋白的表达** 参照 Zhu 等[17]的方法，提取蛋白。取制备的蛋白样品，SDS-聚丙烯酰胺凝胶电泳后，转移至 PVDF 膜 (Millipore 公司，美国)上。于含 3% 牛血清白蛋白的 TBST (含 0.05% Tween-20 的 TBS)中室温封闭 1 h，加入兔抗 Met 多克隆抗体(Santa Cruz 公司，美国)，4 ℃孵育过夜。TBST 漂洗 3 次，加入辣根过氧化物酶标记羊抗兔 IgG 二抗(Santa Cruz 公司，美国)，室温孵育 1 h。TBST 漂洗 3 次，用 ECL Western blot 试剂盒(Pierce 公司，美国)检测。

**1.9 统计分析** 用 SPSS 统计软件分析实验数据，结果以 mean±SD 表示，组间比较用方差分析，$P<0.05$ 为有统计学意义。

## 2 结果

### 2.1 氧糖剥夺/再灌注对皮层神经元的影响

#### 2.1.1 氧糖剥夺/再灌注对皮层神经元细胞活力的影响

以 25 mmol/L 葡萄糖、常氧条件下培养的神经元为对照，其细胞活力在较长时间内无明显变化。氧糖剥夺条件下培养 2 h 的神经元($OGD_2/R_0$ 组)细胞活力降低为 69.08% (与对照组比较，$P<0.05$)。并随再灌注时间延长，细胞活力呈降低趋势。再灌注 24 h ($OGD_2/R_{24}$ 组)细胞活力仅为 40.56% (图 1)。

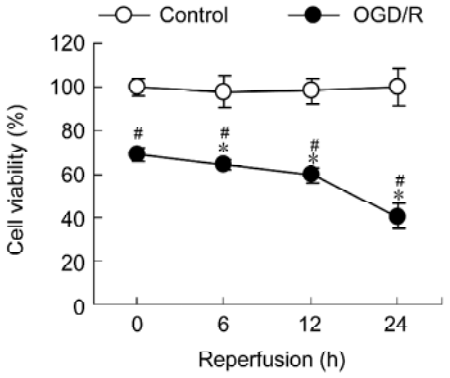


图 1. 氧糖剥夺/再灌注对皮层神经元细胞活力的影响

Fig.1. Effect of oxygen-glucose deprivation/reperfusion on cell viability in cortical neurons. $^{*}P<0.05$ *vs* $OGD_2/R_0$ group, $^{\#}P<0.05$ *vs* control.

#### 2.1.2 氧糖剥夺/再灌注对皮层神经元 LDH 漏出率的影响

以 25 mmol/L 葡萄糖、常氧条件下培养的神经元为对照，其 LDH 漏出率很低，在较长时间内无明显变化。氧糖剥夺条件下培养 2 h 的神经元($OGD_2/R_0$ 组) LDH 漏出率升高到 13.11% (与对照组比较，$P<0.05$)。从再灌注 12 h ($OGD_2/R_{12}$ 组)开始 LDH 漏出率高于 $OGD_2/R_0$ 组($P<0.05$)。再灌注 24 h ($OGD_2/R_{24}$ 组) LDH 漏出率升至 21.62% (图 2)。

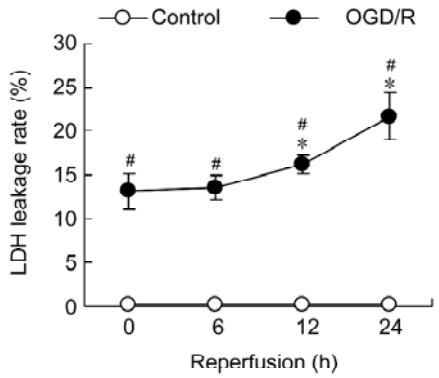


图 2. 氧糖剥夺/再灌注对皮层神经元 LDH 漏出率的影响

Fig. 2. Effect of oxygen-glucose deprivation/reperfusion on LDH leakage rate in cortical neurons. $^{*}P<0.05$ *vs* $OGD_2/R_0$ group, $^{\#}P<0.05$ *vs* control.

### 2.2 HGF 对神经元氧糖剥夺 / 再灌注损伤的影响

#### 2.2.1 HGF 对神经元细胞活力的影响

于氧糖剥夺处理前 2 h 加入不同终浓度(5~120 ng/mL)的 HGF，MTT 比色法测定 HGF 对 $OGD_2/R_{24}$ 神经元细胞活力的影响(图 3)。终浓度为 5 ng/mL、10 ng/mL 的 HGF 对神经元细胞活力无明显影响。当 HGF 终浓度达 20 ng/mL 时，HGF 开始显示出保护效应(与 $OGD_2/R_{24}$ 组比较，$P$<0.05)。随 HGF 剂量增加，细胞活力呈上升趋势，在 80 ng/mL 时达高峰，神经元细胞活力增加到 82.23%。

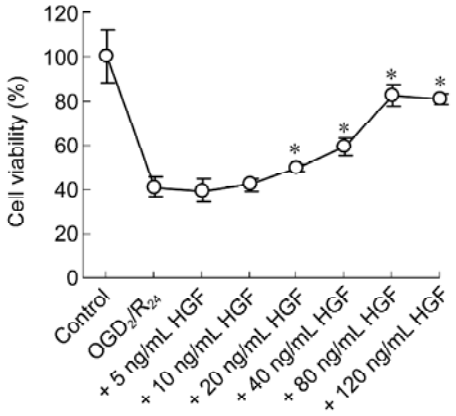


图 3. 不同浓度 HGF 对 $OGD_2/R_{24}$ 神经元细胞活力的影响
Fig. 3. Effects of HGF at different concentrations on cell viability in cortical neurons exposed to oxygen-glucose deprivation for 2 h/reperfusion for 24 h ($OGD_2/R_{24}$). $^*P$<0.05 *vs* $OGD_2/R_{24}$ group.

c-Met 抑制剂 SU11274 (5 μmol/L)完全阻断 HGF (80 ng/mL)的促细胞存活效应($P$<0.05)(图 4*A*)。

#### 2.2.2 HGF 对神经元 LDH 漏出率的影响

于氧糖剥夺处理前 2 h 加入不同终浓度(5~120 ng/mL)的 HGF，检测 LDH 漏出率的变化(图 5)。5 ng/mL、10 ng/mL、20 ng/mL HGF 对 $OGD_2/R_{24}$ 神经元的 LDH 漏出率均无明显影响。当 HGF 终浓度达 40 ng/mL 时，$OGD_2/R_{24}$ 神经元的 LDH 漏出率开始降低(与 $OGD_2/R_{24}$ 组比较，$P$<0.05)，并随 HGF 剂量增加，LDH 漏出率呈下降趋势。HGF 最大效应剂量为 80 ng/mL，神经元 LDH 漏出率降低为 8.34%。

SU11274 (5 μmol/L)完全抑制 HGF (80 ng/mL)的保护作用，神经元 LDH 漏出率显著增高($P$<0.05)(图 4*B*)。

#### 2.2.3 HGF 对神经元细胞凋亡率的影响

于氧糖剥夺处理前 2 h 加入终浓度为 80 ng/mL 的 HGF，流式细胞仪分析 HGF 对 $OGD_2/R_{24}$ 神经元细胞凋亡率的影响。以 25 mmol/L 葡萄糖、常氧条件下培养的神经元为对照，其细胞凋亡率仅为 2.26%。$OGD_2/R_{24}$ 神经元细胞凋亡率高达 37.8%。

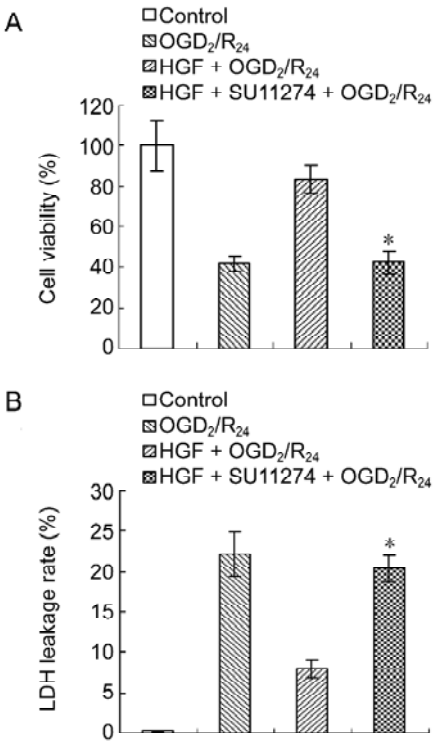


图 4. c-Met 抑制剂 SU11274 对 HGF 神经保护作用的影响
Fig. 4. Effects of c-Met inhibitor SU11274 on cell viability (*A*) and LDH leakage rate (*B*) in cortical neurons exposed to oxygen-glucose deprivation for 2 h/reperfusion for 24 h ($OGD_2/R_{24}$) in the presence of HGF (80 ng/mL). $^*P$<0.05 *vs* HGF + $OGD_2/R_{24}$ group.

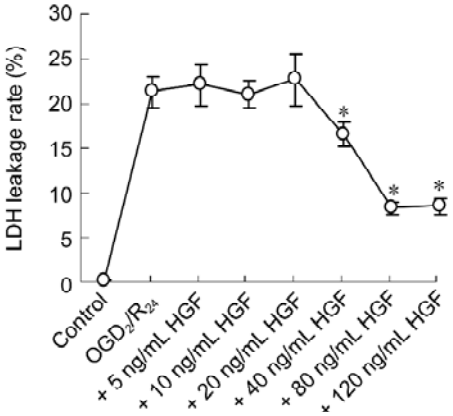


图 5. 不同浓度 HGF 对 $OGD_2/R_{24}$ 神经元 LDH 漏出率的影响
Fig. 5. Effects of HGF at different concentrations on LDH leakage rate in cortical neurons exposed to oxygen-glucose deprivation for 2 h/reperfusion for 24 h ($OGD_2/R_{24}$). $^*P$<0.05 *vs* $OGD_2/R_{24}$ group.

用终浓度为 80 ng/mL 的 HGF 预处理，细胞凋亡率降为 9.41% (图 6)。

用 Hoechst 33258 染色方法检测 80 ng/mL HGF 对 $OGD_2/R_{24}$ 神经元细胞凋亡率的影响，结果显示，细胞凋亡率明显降低(图 7)。

### 2.3 c-Met 的表达

#### 2.3.1 *c-Met* mRNA 的表达

以 25 mmol/L 葡萄糖、常氧条件下培养的神经元为对照。RT-PCR 的结果显示，$OGD_2/R_{24}$ 显著上调 *c-Met* mRNA 表达(与对照组比较，$P<0.05$)。80 ng/mL HGF 预处理对 $OGD_2/R_{24}$ 神经元 *c-Met* mRNA 的表达无明显影响(图 8)。

#### 2.3.2 c-Met 蛋白的表达

以 25 mmol/L 葡萄糖、常氧条件下培养的神经元为对照。Western blot 结果显示，$OGD_2/R_{24}$ 显著上调 c-Met 蛋白的表达(与对照组比较，$P<0.05$)，80 ng/mL HGF 预处理对 $OGD_2/R_{24}$ 神经元 c-Met 蛋白的表达无明显影响(图 9)。

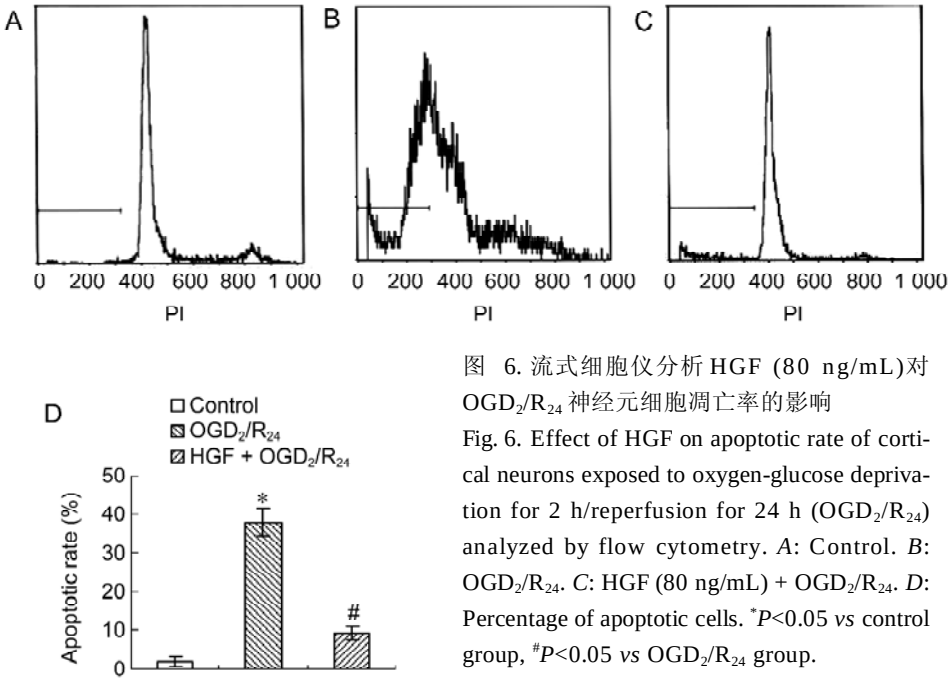


图 6. 流式细胞仪分析 HGF (80 ng/mL)对 $OGD_2/R_{24}$ 神经元细胞凋亡率的影响

Fig. 6. Effect of HGF on apoptotic rate of cortical neurons exposed to oxygen-glucose deprivation for 2 h/reperfusion for 24 h ($OGD_2/R_{24}$) analyzed by flow cytometry. *A*: Control. *B*: $OGD_2/R_{24}$. *C*: HGF (80 ng/mL) + $OGD_2/R_{24}$. *D*: Percentage of apoptotic cells. $^*P<0.05$ *vs* control group, $^\#P<0.05$ *vs* $OGD_2/R_{24}$ group.

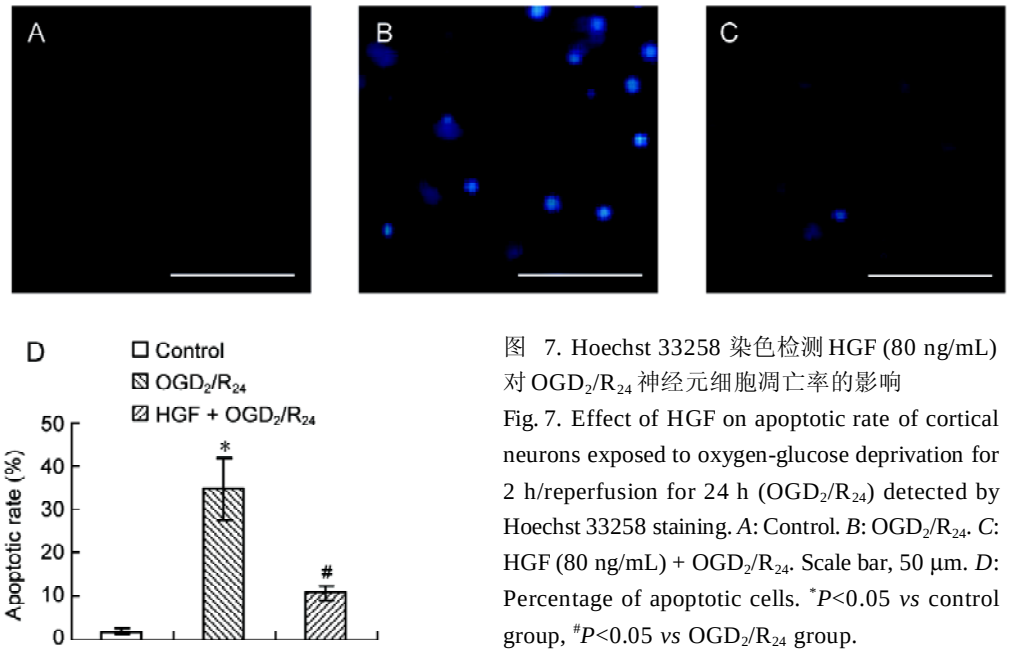


图 7. Hoechst 33258 染色检测 HGF (80 ng/mL)对 $OGD_2/R_{24}$ 神经元细胞凋亡率的影响

Fig. 7. Effect of HGF on apoptotic rate of cortical neurons exposed to oxygen-glucose deprivation for 2 h/reperfusion for 24 h ($OGD_2/R_{24}$) detected by Hoechst 33258 staining. *A*: Control. *B*: $OGD_2/R_{24}$. *C*: HGF (80 ng/mL) + $OGD_2/R_{24}$. Scale bar, 50 μm. *D*: Percentage of apoptotic cells. $^*P<0.05$ *vs* control group, $^\#P<0.05$ *vs* $OGD_2/R_{24}$ group.

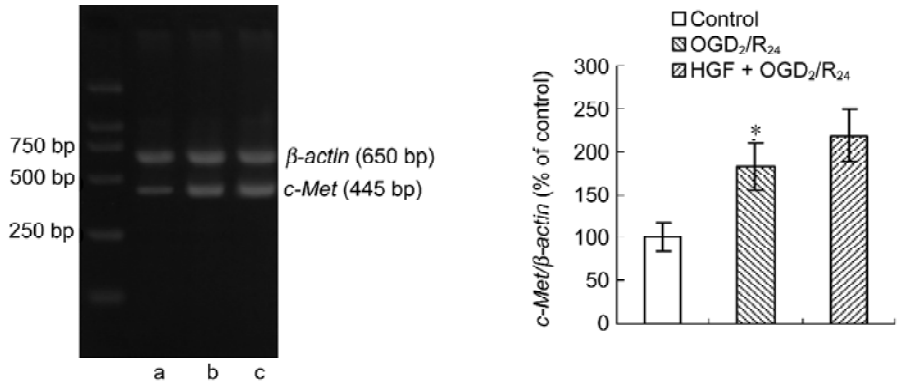


图 8. *c-Met* mRNA 在大脑皮层神经元的表达

Fig. 8. Expression of *c-Met* mRNA in cortical neurons. a: Control; b: $OGD_2/R_{24}$; c: HGF (80 ng/mL) + $OGD_2/R_{24}$. $^*P<0.05$ *vs* control group.

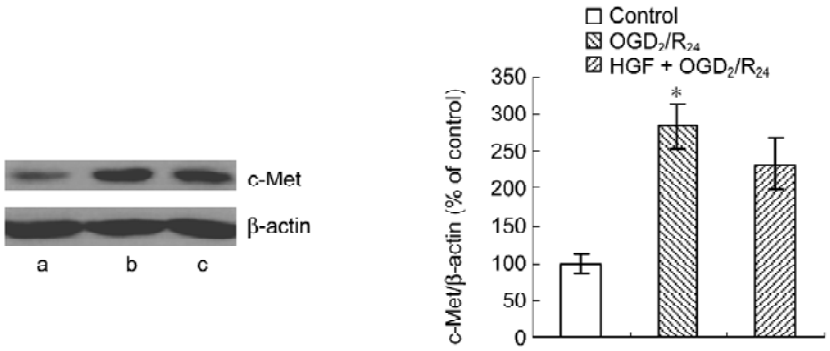


图 9. c-Met 蛋白在大脑皮层神经元的表达

Fig. 9. Expression of c-Met protein in cortical neurons. a: Control; b: $OGD_2/R_{24}$; c: HGF (80 ng/mL) + $OGD_2/R_{24}$. $^*P<0.05$ *vs* control group.

## 3 讨论

HGF 及其受体 c-Met 在脑不同区域的神经元都有表达，如海马 CA1 区、大脑皮层及小脑颗粒细胞层等[6,7,18]。本实验用 RT-PCR 和 Western blot 在体外培养的大脑皮层神经元检测到 c-Met mRNA 和蛋白的表达。Nagayama 等[19]通过建立小鼠大脑中动脉阻塞模型研究发现，脑缺血能上调 HGF 和 c-Met 的表达。我们的结果显示，$OGD_2/R_{24}$ 神经元 c-Met mRNA 和蛋白表达显著增多，而 HGF 对 $OGD_2/R_{24}$ 神经元 c-Met mRNA 和蛋白表达均无明显影响。

氧、糖缺乏是脑缺血产生损伤的两个基本因素，脑细胞的损害在本质上主要是脑组织氧、糖缺乏而引发的一系列事件的结果。本实验用体外氧糖剥夺/再灌注模型来模拟体内缺血/再灌注损伤。结果显示，大鼠脑皮层神经元氧糖剥夺处理 2 h 后，随着再灌注时间延长(0~24 h)，细胞活力呈降低趋势，LDH 漏出率呈增高趋势，神经元损伤加重。接着，我们研究了不同浓度 HGF (5~120 ng/mL)对 $OGD_2/R_{24}$ 神经元的影响。结果显示，HGF 增强细胞活力的效应从 20 ng/mL 开始出现，在 80 ng/mL 达高峰。而降低 LDH 漏出率的效应从 40 ng/mL 开始出现，最大效应剂量也是 80 ng/mL。提示 HGF 对神经元氧糖剥夺/再灌注损伤具有保护作用，并呈一定的剂量依赖关系。c-Met 抑制剂 SU11274 (5 μmol/L)完全阻断 HGF 的保护作用，说明 HGF 的神经保护效应依赖于 c-Met。

大量体内及体外研究证实，氧糖剥夺可诱发神经细胞凋亡的发生，其机制可能与钙超载、NMDA 受体、一氧化氮及自由基等多种因素有关[20-22]。本实验用流式细胞术和 Hoechst 33258 染色法分析了 HGF (80 ng/mL)对氧糖剥夺/再灌注损伤神经元细胞凋亡率的影响，结果均显示 HGF 能有效减少神经细胞凋亡。有研究表明，HGF 对多种细胞发挥抗凋亡作用。如 HGF 能保护原代培养的肝细胞对抗低氧/复氧引起的细胞凋亡[23]、减少游离脂肪酸诱导的大鼠胰岛细胞凋亡[24]、抑制血清缺失导致的人脐静脉内

皮细胞凋亡，且该效应呈一定的剂量依赖关系[25]。我们的结果显示，HGF 对氧糖剥夺 / 再灌注损伤神经元有强大的抗凋亡效应。因此，HGF 的神经保护作用至少部分与抑制细胞凋亡有关。

神经元是神经系统的基本功能单位，是脑缺血损伤保护的核心所在。本研究表明，在体外培养的大脑皮层神经元存在 HGF 受体——c-Met 的表达，氧糖剥夺 / 再灌注上调 c-Met 表达。HGF 对体外培养的皮层神经元氧糖剥夺/再灌注损伤具有直接的保护作用，并呈一定的剂量依赖关系，为临床应用 HGF 治疗脑缺血损伤提供实验依据，并为采取有效安全的脑缺血防治策略提供新的线索。

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