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· 基础研究 ·

体外氧糖剥夺培养条件促进人牙髓细胞内质网应激的研究

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【摘要】 目的 通过氧糖剥夺(oxygen-glucose deprivation, OGD)体外模拟细胞缺血缺氧, 观察人牙髓细胞(human dental pulp cells, hDPCs)内质网应激变化, 为缺血缺氧条件调控hDPCs的机制研究提供依据。**方法** 应用无糖DMEM培养液联合低氧培养(体积分数2% O₂)构建hDPCs OGD模型, 体外模拟hDPCs缺血缺氧, 设置对照组和实验组。对照组: 常规培养; 实验组: OGD处理0、2、4、8 h。MTT检测hDPCs OGD处理0、2、4、8 h后细胞存活率; qRT-PCR检测hDPCs内质网应激关键分子: 剪切X盒结合蛋白1(splicing x-box binding protein 1, sXBP1)、活化转录因子4(activating transcription factor 4, ATF4)、C/EBP同源蛋白(C/EBP homologous protein, chop)mRNA水平, Western blot检测内质网应激关键蛋白: 磷酸化蛋白激酶样内质网激酶(phosphorylated RNA-activated protein kinase-like ER-resident kinase, p-perk)、磷酸化真核起始因子-2α(phosphorylated eukaryotic initiation factor-2α, p-eIF2α)表达水平。**结果** 相较于OGD处理0 h, OGD培养hDPCs 2、4、8 h后, 死亡细胞增多, 细胞存活率显著下降($P < 0.05$)。与对照组相比, OGD处理4 h后, hDPCs内质网应激关键信号分子sXBP1、ATF4、CHOP mRNA表达水平升高; 内质网应激关键蛋白p-perk、p-eIF2α表达增加, 差异均有统计学意义($P < 0.05$)。**结论** hDPCs OGD培养条件下内质网应激水平明显升高。

【关键词】 人牙髓细胞; 氧糖剥夺; 内质网应激; 剪切X盒结合蛋白1; 活化转录因子4; C/EBP同源蛋白; 蛋白激酶样内质网激酶; 真核起始因子-2α

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Study of endoplasmic reticulum stress in human dental pulp cells induced by oxygen-glucose deprivation culture *in vitro* LI Lifan, ZHU Yaqin, JIANG Long. Department of General Dentistry, Shanghai Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University School of Medicine; National Center for Stomatology; National Clinical Research Center for Oral Disease; Shanghai key Laboratory of Stomatology, Shanghai 200011, China
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【Abstract】 Objective Oxygen-glucose deprivation (OGD) is used to mimic ischemia *in vitro* to observe whether endoplasmic reticulum (ER) stress is involved in human dental pulp cells (hDPCs) after OGD and to better understand the regulatory mechanism of hDPCs in ischemia. **Methods** hDPCs were cultured in glucose-free DMEM and hypoxia (volume fraction 2% O₂) to establish an hDPCs OGD model *in vitro*, which mimics hDPCs ischemia *in vitro*. hDPCs were divided into a control group (normal culture) and an experimental group (OGD 0 h, 2 h, 4 h and 8 h groups). After pretreatment with OGD for 0, 2, 4 and 8 h, hDPC viability was measured by methylthiazol tetrazolium (MTT) assay. qRT-

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PCR was used to detect the mRNA expression of ER stress markers [splicing x-box binding protein1 (sXBP1), activating transcription Factor 4 (ATF4) and C/EBP homologous protein (chop)]. Western blot was used to detect the protein expression of ER stress markers [phosphorylated RNA-activated protein kinase-like ER-resident kinase (p-perk) and phosphorylated eukaryotic initiation factor-2 α (p-eIF2 α)]. **Results** Compared with OGD 0 h group, cell viability of hDPCs decreased when exposed to OGD treatment for 2 h, 4 h and 8 h. Compared with the control group, mRNA expressions of ER stress makers (sXBP1, ATF4 and chop) and the protein expressions of ER stress protein markers (p-perk and p-eIF2 α) increased in OGD treatment cells after 4 h were higher in OGD cells. The differences were statistically significant ($P < 0.05$). **Conclusion** The results indicate that ER stress response is involved in hDPCs in OGD treatment.

【Key words】 human dental pulp cells; oxygen-glucose deprivation; endoplasmic reticulum stress; splicing x-box binding protein 1; activating transcription factor 4; C/EBP homologous protein; RNA-activated protein kinase-like ER-resident kinase; eukaryotic initiation factor-2 α

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内质网是细胞蛋白质合成加工的主要场所,多种因素如氧化应激、钙离子紊乱、细菌感染等刺激下均可影响细胞蛋白质的合成,破坏内质网的稳态。内质网通过减少蛋白质翻译、分子伴侣以及相关蛋白表达上调,促进错误折叠蛋白质降解等途径恢复稳态,以上变化统称内质网应激(endoplasmic reticulum stress, ERS)^[1]。内质网应激的作用是促进蛋白质降解以期恢复内质网的体内平衡。然而,如果没有实现内质网体内平衡,当细胞内的错构蛋白质超过内质网的处理极限时,促凋亡因子C/EBP同源蛋白(C/EBP homologous protein, chop)^[2]和caspase-12等^[3]介导的细胞凋亡途径就会被激活,引起细胞的死亡。缺血条件下,细胞生命活动所依赖的糖和氧气不足,易发生内质网应激,这在多项研究中得到了证实。体外氧糖剥离(oxygen-glucose deprivation, OGD)是目前国际上比较公认的体外模拟细胞缺血的手段^[4],研究显示,内质网应激可调节OGD导致的人SH-SY5Y细胞的死亡^[5]、小鼠骨细胞死亡^[6],并参与肾脏缺血再灌注损伤^[7]。牙髓由于其特殊的解剖结构,被低让性的硬组织所包绕,缺乏有效的侧枝循环,因此在受到外界刺激如外伤、充填操作以及局部药物应用等情况下,易发生缺血现象^[8-9]。目前对缺血条件下牙髓生物学行为了解并不多,内质网应激是否参与调控缺血条件下牙髓细胞的生命活动尚不清楚。因此,本研究通过无血清DMEM加上低氧(2% O₂)建立人牙髓细胞(human dental pulp cells, hDPCs)OGD模型来模拟牙髓细胞缺血^[10-12],检测其内质网应激变化情况及调控机制。

1 材料和方法

1.1 主要实验试剂和仪器

DMEM 高糖培养基、胎牛血清(Hyclone, 美国);0.25%胰蛋白酶(Gibico, 美国);噻唑蓝[3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide, MTT]、二甲基亚砜(dimethyl sulfoxide, DMSO)、 β -甘油磷酸钠、Trizol 总 RNA 提取试剂(Sigma, 美国);地塞米松(Sigma, 美国),维生素 C(Sigma, 美国);BCIP/NBT 碱性磷酸酶(alkaline phosphatase, ALP)显色试剂盒、RIPA 蛋白裂解液、BCA 蛋白浓度试剂盒(碧云天, 上海);RNA 逆转录试剂盒、Realtime PCR 试剂盒(Takara, 日本);兔抗鼠磷酸化 RNA 依赖的蛋白激酶样内质网激酶(phospho-PKR like ER kinase, p-perk)抗体(1:1 000 稀释)、兔抗鼠磷酸化活化真核起始因子 2 α (phospho-eukaryotic initiation factor, p-eIF2 α)抗体(1:1 000 稀释)、鼠抗人 β -actin 抗体(1:5 000 稀释)(Cell Signaling, 美国),鼠抗人基质细胞抗原-1(stromal cell antigen-1, STRO-1)抗体(1:1 000 稀释)(Abcam, 美国);酶标仪(Infinite, Tecan, 瑞士);流式细胞仪(Attune NxT, BD, 美国);倒置显微镜(IX70, 奥林巴斯, 日本)。

1.2 hDPCs 的培养与鉴定

本实验已经获得上海交通大学医学院附属第九人民医院伦理委员会的批准(审批号:SH9H-2020-T305-1)。取 14~28 岁健康个体因治疗需要拔除的健康恒牙,用无菌 PBS 冲洗。利用灭菌的高速涡轮开髓,取出牙髓组织,并用组织剪把牙髓组织剪成约 1.0 mm³ 大小的组织块,均匀铺在培

养皿底部,表覆细胞培养专用玻片,加入20%胎牛血清的DMEM培养液,于培养箱37℃,5%CO₂培养。每72h换1次培养液,待细胞长满后进行传代培养。

取第三代长势良好的细胞融合至70%~80%时,更换矿化诱导液培养(含有10mmol/Lβ甘油磷酸钠、50μg/L维生素C和0.1μmol/L地塞米松的DMEM培养液),培养7d后取出,PBS洗涤,4%多聚甲醛固定10min后进行ALP染色。收集第2代细胞制备单细胞悬液,PBS洗涤后离心,重悬获得细胞悬液后装入EP管中,加入STRO-1抗体避光孵育30min后,离心5min后去上清加入PBS重悬,使用流式细胞仪进行检测。

1.3 OGD模型建立及hDPCs形态观察、活性检测

取第三代牙髓细胞生长至80%~90%时,进行OGD处理。OGD处理前一天撤掉血清,将无糖培养基放入低氧培养箱(2%O₂)中30min,同时弃掉细胞原有的高糖培养基,更换为无糖培养基,进行低氧培养(作为OGD培养条件),常规培养的细胞作为对照。

OGD培养0、2、4、8h时,显微镜下观察细胞形态变化。

在96孔板中将hDPCs以每孔 5×10^4 个接种(每孔100μL),常规培养孵育24h,去除上清,每孔加入100μL无糖DMEM培养液后置于2%O₂培养。所有组别均设5个复孔。分别培养0、2、4、8h后,每孔加入20μL MTT溶液置于培养箱避光孵育4h后弃上清液,加入DMSO终止反应。常温充分震荡10min后,酶标仪选定490nm波长检测每孔吸光度值。

1.4 qRT-PCR

将OGD培养4h的hDPCs取出,加入Trizol获取细胞总RNA。每个样品的RNA反转录为cDNA,然后以SYBGreen为探针进行荧光定量PCR反应,检测内质网应激关键基因剪切型X盒结合蛋白1(spliced X-box binding protein 1, sXBP1)、激活转录因子4(activating transcription factor 4, ATF4)、chop mRNA表达。以管家基因GAPDH作为对照,具体引物序列参照文献^[13]详见表1。

1.5 Western blot 检测

取OGD培养4h的hDPCs,用预冷PBS冲洗细胞3次,加入RIPA裂解液冰上裂解收取上清液。用BCA法检测细胞样本总蛋白浓度。蛋白样品经聚丙烯酰胺凝胶电泳电泳分离,转至聚偏二氟乙

表1 qRT-PCR引物序列^[13]

Table 1 Sequences of the qRT-PCR primers^[13]

Genes	Sequence
GAPDH	F: 5'-CCTGCACCACCAACTGCTTA-3' R: 5'-GGCCATCCACAGTCTTCTGAG-3'
sXBP1	F: 5'-CTGAGTCCGAATCAGGTGCAG-3' R: 5'-ATCCATGGGGAGATGTTCTGG-3'
ATF4	F: 5'-GTTCTCCAGCGACAAGGCTA-3' R: 5'-ATCCTGCTTGCTGTTGTTGG-3'
chop	F: 5'-AGAACCAGGAAACGGAAACAGA-3' R: 5'-TCTCCTTCATGCGCTGCTTT-3'

sXBP1: spliced X-box binding protein 1; ATF4: activating transcription factor 4; chop: C/EBP homologous protein

烯膜膜上,脱脂奶粉封闭2h后,加入p-perk、p-eIF2α、β-actin抗体,4℃孵育过夜,二抗孵育1h,洗膜后电化学发光显色拍照。

1.6 统计学分析

采用SPSS18.0软件包对数据进行t检验,检验水准 $\alpha=0.05$ 。

2 结果

2.1 hDPCs的体外培养

原代培养1周左右,倒置显微镜中可见散在的牙髓细胞从各个组织块边缘爬出,呈梭形,形态类似于成纤维细胞;常规传代后,贴壁生长于整个培养皿底部(图1a、1b);流式细胞仪检测到牙髓细胞STRO-1抗体表达阳性(图1c);矿化诱导7d后,细胞ALP染色呈阳性(图1d);以上结果符合文献对于hDPCs特性的报道^[14]。

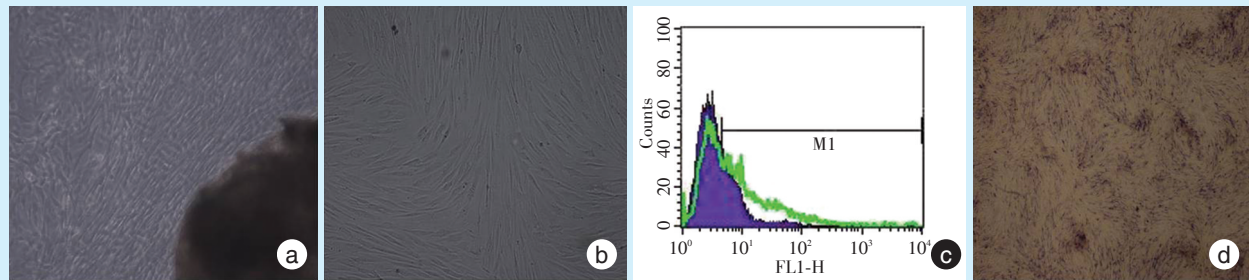
2.2 OGD培养对hDPCs细胞形态、活性的影响

显微镜下观察发现,对照组细胞呈梭形,胞体饱满,细胞连接紧密(图2a);OGD处理2h后细胞排列较稀疏,胞体变肿胀,可见少量死细胞(图2b),OGD处理4h后,细胞排列更加稀疏,细胞完整性遭到严重破坏,胞膜破裂,可见大量悬浮死细胞(图2c);OGD处理8h后细胞大量死亡,基本无细胞结构(图2d)。

MTT实验结果显示,与未经OGD处理的细胞相比,随着OGD处理时间增加,hDPCs吸光度值逐渐下降,细胞存活率下降,2h、4h、8h组与对照组(0h)差异均有统计学意义(图3)。由于OGD处理时间过长,细胞大部分发生死亡,因此将之后的OGD处理时间设为4h。

2.3 qRT-PCR 结果

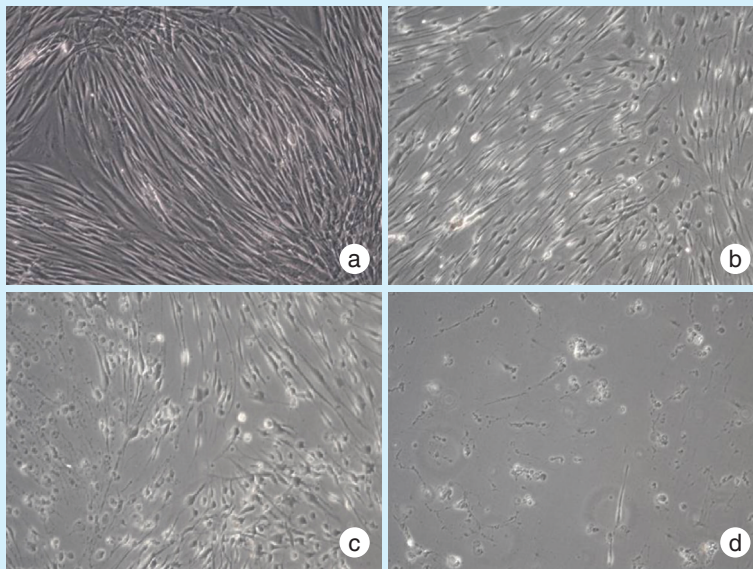
与常规培养细胞相比,OGD培养4h后,hDPCs



a: primary hDPCs crawled out from pulp tissues ($\times 100$); b: third passage hDPCs were spread all over dish ($\times 100$); c: hDPCs were positive for STRO-1 (40.12%); d: after 7 days of induction of mineralization, hDPCs were positive by ALP staining. hDPCs: human dental pulp cells; STRO-1: stromal cell antigen-1; ALP: alkaline phosphatase

Figure 1 Culture of hDPCs *in vitro*

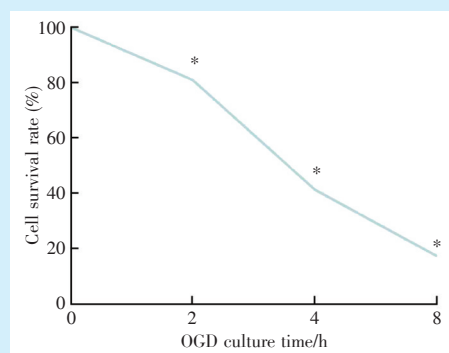
图1 人牙髓细胞的体外培养及鉴定



a: normal culture of cells for 8 h (control) ($\times 200$); b: OGD culture for 2 h ($\times 200$); c: OGD culture for 4 h ($\times 200$); d: OGD culture for 8 h ($\times 200$). With increasing of OGD treatment time, number of dead cells increased. hDPCs: human dental pulp cells; OGD: oxygen-glucose deprivation

Figure 2 Morphological changes in hDPCs after different OGD culture times

图2 氧糖剥夺不同时间下牙髓细胞形态变化



*: compared with OGD 0 h group, $P < 0.05$; hDPCs: human dental pulp cells; OGD: oxygen-glucose deprivation

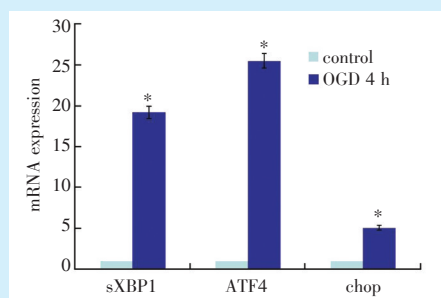
Figure 3 Detection of survival rate of hDPCs after different OGD culture times

图3 氧糖剥夺不同时间hDPCs细胞存活率检测

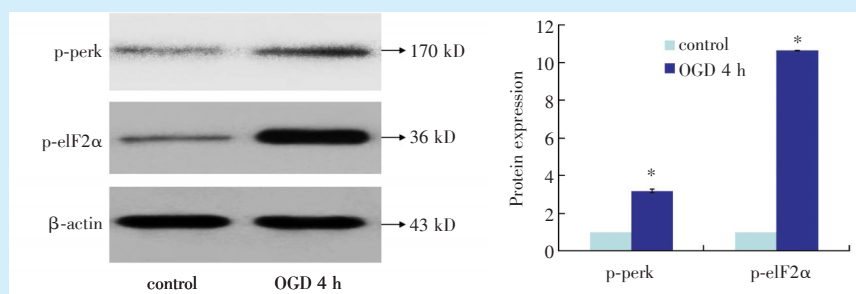
内质网应激关键分子 mRNA 表达明显增加:sXBP1 ($t=7.441$)、ATF4($t=6.953$)、chop($t=7.989$)的 mRNA 相对表达明显上调($P < 0.05$)(图4)。

2.4 Western blot 结果

OGD 处理 4 h 后, hDPCs p-perk($t=13.681$)和 p-eIF2 α ($t=42.907$)蛋白水平增加($P < 0.05$)(图5)。



*: compared with the control group, $P < 0.05$. sXBP1: spliced X-box binding protein 1; ATF4: activating transcription factor 4; chop: C/EBP homologous protein; OGD: oxygen-glucose deprivation
Figure 4 mRNA expression of endoplasmic reticulum stress markers detected by qRT-PCR 4 h after OGD culture
图4 qRT-PCR检测OGD培养4 h后hPDCs内质网应激关键信号分子mRNA表达



Expressions of p-perk and p-eIF2α increased in the OGD 4 h group compared with the control group. *: compared with the control group, $P < 0.05$; p-perk: phospho-PKR like ER kinase; p-eIF2α: phospho-eukaryotic initiation factor; OGD: oxygen-glucose deprivation; ERS: endoplasmic reticulum stress

Figure 5 Western blot for protein expression of ERS makers 4 h after OGD culture

图5 Western blot检测OGD培养4 h后hPDCs内质网应激关键信号分子蛋白表达

3 讨论

牙髓作为结缔组织,含有神经和血管,但由于其仅通过根尖孔与外界相通,因此在发生组织炎症、外伤、局部物理或化学药物刺激时很容易出现血供减少的现象。血供减少将导致细胞氧气和葡萄糖供应不足,限制了细胞能量产生所需的基本物质,造成细胞处于低能量状态,扰乱细胞正常活动,最终导致细胞的死亡^[15]。内质网应激则是细胞面对此类刺激产生的反应。细胞通过内质网应激减少未折叠或错构蛋白的堆积使内质网功能恢复稳态,从而调节细胞的生命活动^[16],为了证实在缺血缺氧条件下,内质网应激是否参与了调控牙髓细胞的生命活动。本研究首先在体外建立了OGD模型,模拟细胞的体外缺血^[4,10]。本研究实验结果显示,OGD处理的hPDCs,胞体肿胀变圆,排列疏松,漂浮的死细胞增多;较正常培养的细胞存活率下降,并呈现出明显的时间趋势。

同时,本研究从mRNA和蛋白水平两方面比较了OGD处理和正常培养的hPDCs内质网应激水平的变化。perk、ER跨膜蛋白肌醇酶1(inositol-re-

quiring enzyme type 1, IRE1)和ATF6这3个跨膜蛋白是细胞内质网应激的启动因子^[17-19]。细胞受到刺激后,内质网应激信号通路启动,可以激活各种转录因子,诱导下游目的基因的表达,磷酸化的IRE1能够诱发sXBP1的mRNA剪切成具有高度转录活性的sXBP1^[20]。同时perk蛋白的激活能够增加eIF2α的磷酸化从而导致转录因子ATF4的活化^[21]。如果细胞内质网应激活动持续发生而无法被缓解,则会进一步会激活信号通路的下游因子chop^[2]的表达,从而调控细胞的凋亡。在本研究中,OGD诱导的牙髓细胞ATF4、chop和sXBP1分子的mRNA表达明显升高;同时,磷酸化perk和eIF2α蛋白的表达明显增加。这些结果提示,hPDCs在OGD条件下内质网应激水平上调。

综上所述,hPDCs在OGD条件下细胞活性下降,并伴随内质网应激活动增强。在其他组织细胞的研究中发现,适度的内质网应激可以保护细胞,而持续严重的内质网应激活动则可以导致细胞死亡,同时会引起细胞内质网的扩张,并观察到细胞胞浆出现大量空泡^[22]。在机体脑缺血再灌注

损伤的研究中发现,内质网应激可以通过诱导 caspase12 激活^[23],引起一系列的级联反应,引发细胞的凋亡,从而加重机体细胞的损伤,然而 hPDCs 在 OGD 条件下,内质网应激的调控机制还不清楚。需要进一步的研究来阐明内质网应激与牙髓缺血的关系,为牙髓病治疗提供新的策略和方向。

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