

[DOI] 10.12016/j.issn.2096-1456.2023.10.003

· 基础研究 ·

LED红光上调MAPK信号促进炎症环境中人牙髓干细胞成骨/成牙本质分化

刘源^{1,2,3}, 惠以宁⁴, 姜冰^{2,3}, 郑艮子^{2,3}, 王瑶¹

1. 西南医科大学附属口腔医院预防保健科, 四川 泸州 (646000); 2. 西南医科大学口腔医学研究所, 四川 泸州 (646000); 3. 颌面修复重建和再生泸州市重点实验室, 四川 泸州 (646000); 4. 西南医科大学, 四川 泸州 (646000)

【摘要】 目的 探讨发光二极管(light-emitting diode, LED)红光对人牙髓干细胞(human dental pulp stem cells, hDPSCs)成骨/成牙本质分化的影响及机制。方法 本实验研究已通过单位伦理委员会审查批准。组织块酶消化法培养hDPSCs, 在0、1、5、10 $\mu\text{g}/\text{mL}$ 脂多糖(lipopolysaccharide, LPS)刺激下, CCK-8法检测hDPSCs增殖, 筛选LPS刺激浓度。设置CG组(矿化诱导)、LPS+CG组、LPS+CG+LED光照组(能量分别为2、4、6、8、10 J/cm^2)。第7天进行碱性磷酸酶(alkaline phosphatase, ALP)染色及活性测定, 实时荧光定量PCR检测ALP、成骨细胞特异性转录因子(osterix, OSX)、牙本质基质蛋白-1(dentin matrix protein-1, DMP-1)、牙本质涎磷蛋白(dentin sialophosphoprotein, DSPP)基因表达情况, 第21天进行茜素红染色及钙结节定量分析, 筛选最佳光照能量。设置LPS+CG组、LPS+CG+LED组(最佳能量), ELISA法检测第1、3、5、7天肿瘤坏死因子- α (tumor necrosis factor- α , TNF- α)及白介素-1 β (interleukin-1 β , IL-1 β)表达量。Western blot法检测细胞外调节蛋白激酶1/2(extracellular regulated protein kinases 1/2, ERK1/2)、c-Jun氨基末端激酶(c-Jun N-terminal kinase, JNK)、p38、细胞外调节蛋白激酶5(extracellular regulated protein kinases 5, ERK5)及磷酸化蛋白表达。分别阻断ERK1/2、JNK、ERK5、p38通路后, Western blot法检测第7天ALP、OSX、DMP-1、DSPP蛋白表达。结果 CCK-8结果显示10 $\mu\text{g}/\text{mL}$ LPS诱导下, 在第5、7天hDPSCs增殖低于0、1、5 $\mu\text{g}/\text{mL}$ LPS组($P < 0.05$), 后续选择10 $\mu\text{g}/\text{mL}$ 作为LPS刺激浓度。ALP染色及活性, ALP、OSX、DMP-1、DSPP的基因表达水平及钙结节定量结果显示LPS+CG+4 J/cm^2 组均高于其他处理组($P < 0.05$)。4 J/cm^2 LED红光促成骨/成牙本质分化能力最强, 作为后续实验光照能量。ELISA结果显示第5、7天, LPS+CG+LED组的TNF- α 与IL-1 β 的分泌表达量低于LPS+CG组($P < 0.05$)。Western blot结果显示4 J/cm^2 LED红光促进p-ERK1/2、p-p38、p-JNK、p-ERK5蛋白表达; 分别阻断通路后, LPS+CG+LED+U0126(抑制ERK1/2)、SP600125(抑制JNK)、BIX02189(抑制ERK5)组ALP、OSX、DMP-1、DSPP蛋白表达量低于LPS+CG+LED组($P < 0.001$), LPS+CG+LED+SB203580(抑制p38)组ALP、OSX、DMP-1蛋白表达量与LPS+CG+LED组比较无显著差异($P > 0.05$)。结论 LED红光促进炎症环境下hDPSCs成骨/成牙本质分化, 其作用机制可能为通过上调ERK1/2、JNK、ERK5信号减少炎症因子TNF- α 与IL-1 β 释放有关。

【关键词】 人牙髓干细胞; 脂多糖; 炎症环境; 发光二极管; 红光; 成骨/成牙本质分化; MAPK信号通路; 成骨细胞特异性转录因子; 牙本质基质蛋白-1; 牙本质涎磷蛋白

【中图分类号】 R78 **【文献标志码】** A **【文章编号】** 2096-1456(2023)10-0701-11

【引用著录格式】 刘源, 惠以宁, 姜冰, 等. LED红光上调MAPK信号促进炎症环境中人牙髓干细胞成骨/成牙本质分化[J]. 口腔疾病防治, 2023, 31(10): 701-711. doi:10.12016/j.issn.2096-1456.2023.10.003.

LED red light up-regulates MAPK signal to promote osteogenic/odontogenic differentiation of human dental pulp stem cells in an inflammatory environment LIU Yuan^{1,2,3}, HUI Yining⁴, JIANG Bing^{2,3}, ZHENG Genzi^{2,3},



微信公众号

【收稿日期】 2023-04-15; **【修回日期】** 2023-05-25

【基金项目】 四川省科技计划联合创新专项任务项目(2022YFS0634); 四川省科研课题计划项目(S21015); 西南医科大学校级课题项目(2021ZKMS013)

【作者简介】 刘源, 医师, 硕士研究生, Email: 516617861@qq.com

【通信作者】 王瑶, 教授, 博士, Email: 438730587@qq.com, Tel: 86-13882778989

WANG Yao¹. 1. Department of Preventive Health Care, the Affiliated Stomatological Hospital of Southwest Medical University, Luzhou 646000, China; 2. Institute of Stomatology, Southwest Medical University, Luzhou 646000, China; 3. Oral and Maxillofacial Reconstruction and Regeneration of Luzhou Key Laboratory, Luzhou 646000, China; 4. Southwest Medical University, Luzhou 646000, China

Corresponding author: WANG Yao, Email: 438730587@qq.com, Tel: 86-13882778989.

【Abstract】 Objective To study the effect of light-emitting diode (LED) red light on the osteogenic/odontogenic differentiation of human dental pulp stem cells (hDPSCs) and its mechanism were discussed. **Methods** This study has been reviewed and approved by the Ethics Committee. hDPSCs were cultured by tissue block enzyme digestion. The proliferative capacity of hDPSCs was detected by the CCK-8 at days 1, 3, 5 and 7 under stimulation with 0, 1, 5 and 10 $\mu\text{g/mL}$ lipopolysaccharide (LPS), and the LPS stimulatory concentration was screened. The CG group (mineralization induction), LPS+CG group, and LPS+CG+ (2, 4, 6, 8, and 10 J/cm^2) LED red light groups were set. On day 7, alkaline phosphatase (ALP) staining and ALP activity were determined. Relative expression levels of the ALP, osterix (OSX), dentin matrix protein-1 (DMP-1) and dentin sialophosphoprotein (DSPP) genes were measured by qRT-PCR. On day 21, alizarin red staining and calcium nodule quantitative determination were performed to screen the best light energy. The LPS+CG group and LPS+CG+LED group (optimal energy) were set up, and the secretion and expression levels of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) were detected by ELISAs on days 1, 3, 5 and 7. The relative expression levels of the extracellular regulated protein kinases 1/2 (ERK1/2), p38, c-Jun N-terminal kinase (JNK), and extracellular regulated protein kinases 5 (ERK5) proteins and their phosphorylated proteins in the MAPK signaling pathway were detected by Western blots. After the pathway was blocked, the relative expression levels of the ALP, OSX, DMP-1, and DSPP proteins after LED red light irradiation on day 7 were detected by Western blots. **Results** CCK-8 assays showed that the proliferation of hDPSCs induced by 10 $\mu\text{g/mL}$ LPS was lower than that of the 0, 1, and 5 $\mu\text{g/mL}$ groups on the 5th and 7th days ($P < 0.05$), and 10 $\mu\text{g/mL}$ was selected as the LPS stimulatory concentration in the follow-up experiment. ALP staining, ALP activity, gene expression levels of ALP, OSX, DMP-1 and DSPP and calcium nodule quantification in the LPS+CG+4 J/cm^2 group were higher than those in the other treatment groups ($P < 0.05$). 4 J/cm^2 LED red light had the strongest ability to promote osteogenic/odontogenic differentiation and was used as the LED light energy density in subsequent experiments. ELISA showed that the secretion and expression levels of TNF- α and IL-1 β in the LPS+CG+LED group were lower than those in the LPS+CG group on the 5th and 7th days ($P < 0.05$). Western blot analysis showed that 4 J/cm^2 LED red light promoted the expression levels of the p-ERK1/2, p-p38, p-JNK and p-ERK5 proteins. After the MAPK pathway was blocked, the expression levels of the ALP, OSX, DMP-1, and DSPP proteins in the LPS+CG+LED+U0126 (ERK1/2 inhibitor), SP600125 (JNK inhibitor), and BIX02189 (ERK5 inhibitor) groups were lower than those in the LPS+CG+LED group ($P < 0.001$). The protein expression levels of ALP, OSX and DMP-1 in the LPS+CG+LED+SB203580 (p38 inhibitor) group were not significantly different from those in the LPS+CG+LED group ($P > 0.05$). **Conclusion** In inflammatory conditions, LED red light promotes osteogenic/odontogenic differentiation of hDPSCs. This effect may be attributed to enhancement of the ERK1/2, JNK, and ERK5 signaling pathways, which reduces the production of the inflammatory cytokines TNF- α and IL-1 β .

【Key words】 human dental pulp stem cells; lipopolysaccharide; inflammatory environment; light-emitting diode; red light; osteogenic/odontogenic differentiation; MAPK signal pathway; osterix; dentin matrix protein-1; dentin sialophosphoprotein

J Prev Treat Stomatol Dis, 2023, 31(10): 701-711.

【Competing interests】 The authors declare no competing interests.

This study was supported by the grants from the Joint Innovation Plan of Sichuan Science and Technology Project (No. 2022YFS0634); the Sichuan Province Medical Research Project (No. S21015); the Scientific Project of Southwest Medical University (No. 2021ZKMS013).

人牙髓干细胞 (human dental pulp stem cells, hDPSCs) 是来源于人恒牙/乳牙牙髓的外胚层间充质干细胞, 适当诱导下具有良好的多向分化能力,

为组织再生提供细胞来源^[1]。研究发现革兰氏阴性菌脂多糖 (lipopolysaccharide, LPS) 可影响 hDPSCs 增殖, 低浓度 LPS 促进 hDPSCs 合成期细胞增多, 促

进其增殖、迁移;高浓度LPS可使hDPSCs DNA总量减少、蛋白合成降低、增殖能力降低、抑制碱性磷酸酶能力活性,减弱成骨向分化能力,严重可引起细胞死亡^[2-3]。因此,积极探索有利于LPS所致炎性环境下hDPSCs分化的细胞干预条件,有助于为骨再生和牙齿再生提供新的治疗思路。

发光二极管(light-emitting diode, LED)红光亮度高、方向性强、相干性好,可对研究对象施加照射后产生光化学效应,不会造成局部温度明显提高,并且不会对组织或细胞造成不可逆损伤^[4]。有实验研究表明,LED红光影响牙髓干细胞生长速率、克隆潜能以及成骨和软骨细胞分化^[5]。由此可见LED红光可对牙髓干细胞的分化产生影响,但LED红光对LPS所致炎性环境下hDPSCs成骨/成牙本质分化的影响鲜有报道。

细胞的分化受到信号通路和细胞因子的调控,有丝分裂原活化蛋白激酶(mitogen-activated protein kinases, MAPK)信号通路介导细胞外信号诱导的核反应,有4个亚族:细胞外调节蛋白激酶1/2(extracellular regulated protein kinases 1/2, ERK1/2)、c-Jun氨基末端激酶(c-Jun N-terminal kinase, JNK)、p38、细胞外调节蛋白激酶5(extracellular regulated protein kinases 5, ERK5),在调节细胞因子、黏附分子、趋化因子,调节系统性炎症反应中起着重要作用^[6]。本实验拟通过LPS构建体外炎性环境,探究LED红光调控hDPSCs成骨/成牙本质分化及作用机制,为牙和颌骨缺损修复机制提供实验依据。

1 材料和方法

本实验已通过西南医科大学附属口腔医院伦理审批(批号:20211119003)。

1.1 主要试剂和仪器

α -MEM(BI,以色列);优级胎牛血清(四季青生物,中国);0.25%胰酶细胞消化液(碧云天生物,中国);LPS(索莱宝,中国);BCIP/NBT碱性磷酸酯酶显色试剂盒(碧云天生物,中国);0.2%茜素红染液(索莱宝,中国);一抗(鼠抗人):CD34-APC、CD44-PE、CD45-FITC、CD73-PE、CD90-PE抗体(Ebioscience,美国);二抗(羊抗鼠):HRP-Goat anti Mouse(ASPEN,中国);一抗(兔抗人):GAPDH(ab181602, Abacam, 英国)、JNK、p-JNK(#9252, #4668, CST, 美国)、p38、p-p38(AF6456, #4511, Affbiotech, 美国)、ERK1/2、p-ERK1/2(ab184699,

ab201015, Abacam, 英国)、ERK5、p-ERK5(#3552, #3371, CST, 美国)、碱性磷酸酶(alkaline phosphatase, ALP)(ab133602, Abacam, 英国)、成骨细胞特异性转录因子(osterix, OSX)(DF7731, Affbiotech, 美国)、牙本质基质蛋白-1(dentin matrix protein-1, DMP-1)(PA5-120492, Thermofisher, 美国)、牙本质涎磷蛋白(dentin sialo phospho protein, DSPP)(DF14398, Affbiotech, 美国);二抗(羊抗兔):HRP-Goat anti Rabbit(AS1107, ASPEN, 中国);U0126(MCE, 美国);SB203580(MCE, 美国);SP600125(MCE, 美国);BIX02189(MCE, 美国);CCK-8试剂盒(APExBIO, 美国);BCA蛋白浓度测定试剂盒(增强型)(碧云天生物, 中国);碱性磷酸酶检测试剂盒(碧云天生物, 中国);SYBR Green PCR试剂盒(ELK, 中国);ELISA试剂盒(凡科维, 中国上海);SDS-PAGE凝胶制备试剂盒(碧云天生物, 中国)。LED红光灯(T6, 芮森, 中国);酶联免疫检测仪(Multiskan™ FC, Thermo, 美国);倒置相差荧光显微镜(DMi8, Leica, 德国);荧光定量PCR仪(QuantStudio6 Flex System, Life Technologies, 美国);SDS-PAGE电泳仪(165-8000, Bio rad, 美国)。

1.2 hDPSCs的分离、培养、鉴定

取西南医科大学附属口腔医院颌面外科拔除的新鲜健康的第三磨牙或因正畸治疗需要拔除的前磨牙,用含20%(v/v)青霉素-链霉素的PBS清洗至无明显血污,无菌条件下沿牙髓长轴劈开,拔髓针取出牙髓组织,PBS冲洗后眼科剪剪碎组织,I型胶原酶消化、终止、离心重悬后得到单细胞悬液。加入含15%(v/v)青霉素-链霉素FBS、1%(v/v)青霉素-链霉素的 α -MEM培养液,于37℃、5%CO₂细胞孵箱中培养,隔3d半换液。细胞长出后记为原代,当生长密度达80%~85%时,用胰酶消化传代,取生长状态好的P3代细胞进行后续实验。

取第3代细胞,适量PBS重悬细胞,各加入CD34-APC、CD44-PE、CD45-FITC、CD73-PE、CD90-PE抗体,避光条件下孵育30min,流式细胞仪检测表面抗原标志物。取第3代细胞以 2×10^4 个/mL密度接种于6孔板中,待细胞长至80%密度后开始成骨诱导和成脂诱导。诱导7d行ALP染色,21d行茜素红染色和油红O染色,并分别采集镜下图。

1.3 LPS浓度筛选

hDPSCs经0.25%胰酶消化,离心,终止后以 2×10^3 个/孔、200 μ L/孔均匀接种细胞至96孔板中,接种完毕后在接种有细胞的孔周围封闭一圈

PBS。37℃、5%CO₂孵育箱培养过夜后,次日更换为含不同浓度LPS(0、1、5、10 μg/mL)的普通培养基,每组设置3个复孔,隔2 d换液。分别于培养第1、3、5、7天同一时间使用CCK-8试剂盒检测细胞增殖活力,使用酶标仪测定在450 nm波长处OD值。

1.4 LED红光能量测定

根据课题组前期实验测量^[7],当LED红光发射灯距离高精度光功率计2 cm时,根据公式:光功率密度(W/cm²)=光总功率(W)/单位面积(cm²),测定光功率密度为66.7 mW/cm²,通过公式辐照曝光量(J/cm²)=光功率密度(W/cm²)×时间(s),计算得出辐照曝光量分别为2、4、6、8、10 J/cm²时,所需辐照时间为30、60、90、120、150 s。辐照第一天记为1 d,隔48 h辐照一次。

1.5 LED红光对炎症环境下hDPSCs成骨/成牙本质分化的影响

根据实验1.3结果,10 μg/mL为后续LPS刺激浓度。设置CG(矿化诱导)组、LPS+CG组及LPS+CG+不同能量(2、4、6、8、10 J/cm²)光照组;矿化诱导培养基为10 mmol/L β-甘油磷酸钠+50 μg/mL维生素C+10 mol/L地塞米松+10%(v/v)FBS+1%(v/v)青霉素-链霉素的α-MEM培养基。hDPSCs经0.25%胰酶消化、终止、离心后,加入完全培养基以2×10⁴个/mL密度接种至培养皿中,细胞融合至70%后按分组加入相应诱导液并进行光照。

1.5.1 ALP染色及活性测定 细胞处理第7天根据BCIP/NBT显色试剂盒说明书,细胞清洗、固定后进行碱性磷酸酶避光染色15 min,倒置显微镜下采集各组图像。处理第7天,细胞漂洗后每皿加入细胞裂解液,12 000 rpm离心5 min,收集总蛋白溶液。根据BCA试剂盒说明书,酶标仪测定波长为562 nm的各孔OD值,绘制蛋白标准曲线并计算各处理组蛋白浓度。根据碱性磷酸酶检测试剂盒说明书,96孔板设置空白孔,标准品孔以及样品孔并加入相应工作液,每孔设置2个副孔。轻吹混匀后37℃孵育10 min,酶标仪测定波长为450 nm的各孔OD值。根据公式计算各处理组ALP活性。

1.5.2 qRT-PCR检测hDPSCs成骨/成牙本质分化相关基因 细胞处理第7天,Trizol法提取细胞总RNA,将已提取的总RNA 65℃变性5 min,立即转入冰上操作。依次加入2 μL的5*RT Master Mix、RNA template, Nuclease-free Water补足至10 μL。溶解混匀后,以37℃,15 min;50℃,5 min;98℃,5 min;4℃程序进行反应反转录为cDNA。根据

SYBR Green PCR试剂盒说明书进行PCR扩增,反应程序为:95℃,3 min;95℃,5 s;56℃,10 s,72℃,25 s,循环40次;65℃,5 s;95℃,50 s。引物序列见表1。根据公式 $2^{-\Delta\Delta CT}$ 计算ALP、OSX、DMP-1、DSPP基因相对表达量。

表1 引物序列

Table 1 Primers sequence

Gene	Primers sequence	Length/bp
GAPDH	F: 5'-CATCATCCCTGCCTCTACTGG-3'	259
	R: 5'-GTGGGTGTCGCTGTTGAAGTC-3'	
ALP	F: 5'-GGACGATGGCTCTGATGACC-3'	250
	R: 5'-GGTTTCGCAGTACAGCTCCC-3'	
OSX	F: 5'-TTGATTAGGGGATCCCTAATAT-3'	307
	R: 5'-TTTGAATCTGATCCTAGAGAAAGC-3'	
DMP-1	F: 5'-TGTCTCAGGTGAGAAGAGGAATG-3'	185
	R: 5'-ACCCTTCCATTCTTCAGAATCC-3'	
DSPP	F: 5'-CATCTCCTAGCAAGATCAAATGTG-3'	190
	R: 5'-TGAATATGTAGAAAACTCTTCCC-3'	

ALP: alkaline phosphatase; OSX: osterix; DMP-1: dentin matrix protein-1; DSPP: dentin sialophosphoprotein

1.5.3 茜素红染色及钙结节定量测定 细胞处理第21天根据茜素红染色说明书,细胞清洗、固定后使用茜素红S染液染色5 min,倒置显微镜下采集各组图像。每组培养皿中加入1 mL氯化十六烷吡啶溶液,充分溶解,根据分组向96孔板中加入200 μL溶解液,每实验组设置2个复孔,调零孔加入200 μL氯化十六烷吡啶溶液。使用酶标仪测定在562 nm波长处OD值。根据公式计算各处理组钙结节。

1.6 ELISA法测定炎症细胞因子表达

根据实验1.5结果,4 J/cm²是LED红光调节10 μg/mL LPS所致炎症环境下成骨/成牙本质分化的最佳能量,后续选择4 J/cm²对细胞进行辐照。设置分组:LPS+CG组,LPS+CG+LED组。

取hDPSCs以2×10⁴个/mL、2 mL/皿接种于培养皿中,细胞融合至70%后按分组加入相应诱导液并进行LED红光辐照,记为实验第1天,每3 d更换培养基。分别于实验第1、3、5、7天无菌管收集培养基液体(包括已换液培养基),3 500 rpm×20 min离心,仔细收集上清液,吹打混匀后分别收集1 mL上清液。根据ELISA试剂盒说明书,96孔酶标包被板进行加样与加酶,每孔设置2个副孔。封板后37℃温育60 min,重复洗涤5次并拍干,加入显色剂后37℃避光显色15 min,终止反应后酶标仪上测定波长为450 nm OD值。根据公式计算各处理

组在1、3、5、7天细胞分泌肿瘤坏死因子- α (tumor necrosis factor- α , TNF- α)及白细胞介素-1 β (interleukin-1 β , IL-1 β)浓度。

1.7 Western blot测定MAPK信号通路相关蛋白表达

细胞处理第7天,提取各处理组细胞总蛋白。SDS-PAGE电泳将20 μ g蛋白转移到PVDF膜上。5%脱脂牛奶封闭1h,去除封闭剂后,4 $^{\circ}$ C加入一抗(p-ERK1/2、ERK1/2、p-p38、p38、p-JNK、JNK、p-ERK5、ERK5、GAPDH)过夜。TBST洗涤3次,加入稀释的二抗,室温孵育30 min。TBST洗涤4次后,配制ECL溶液进行化学发光,并通过AlphaEaseFC软件分析目标带的OD值。GAPDH作为参考蛋白,计算LPS+CG组与LPS+CG+LED组ERK1/2、p38、JNK、ERK5及磷酸化蛋白相对表达量。

设置LPS+CG组、LPS+CG+LED组、LPS+CG+LED+U0126(抑制ERK1/2)组、LPS+CG+LED+SB203580(抑制p38)组、LPS+CG+LED+SP600125(抑制JNK)组、LPS+CG+LED+BIX02189(抑制ERK5)组。细胞处理第7天,Western blot法检测各处理组ALP、OSX、DMP-1、DSPP蛋白相对表达量。

1.8 统计学分析

SPSS 27.0进行统计学分析,计量资料采用均数 \pm 标准差进行描述,两组间数据比较采用单因素方差分析, $P < 0.05$ 为差异具有统计学意义。使用GraphPad Prism 6绘图。

2 结果

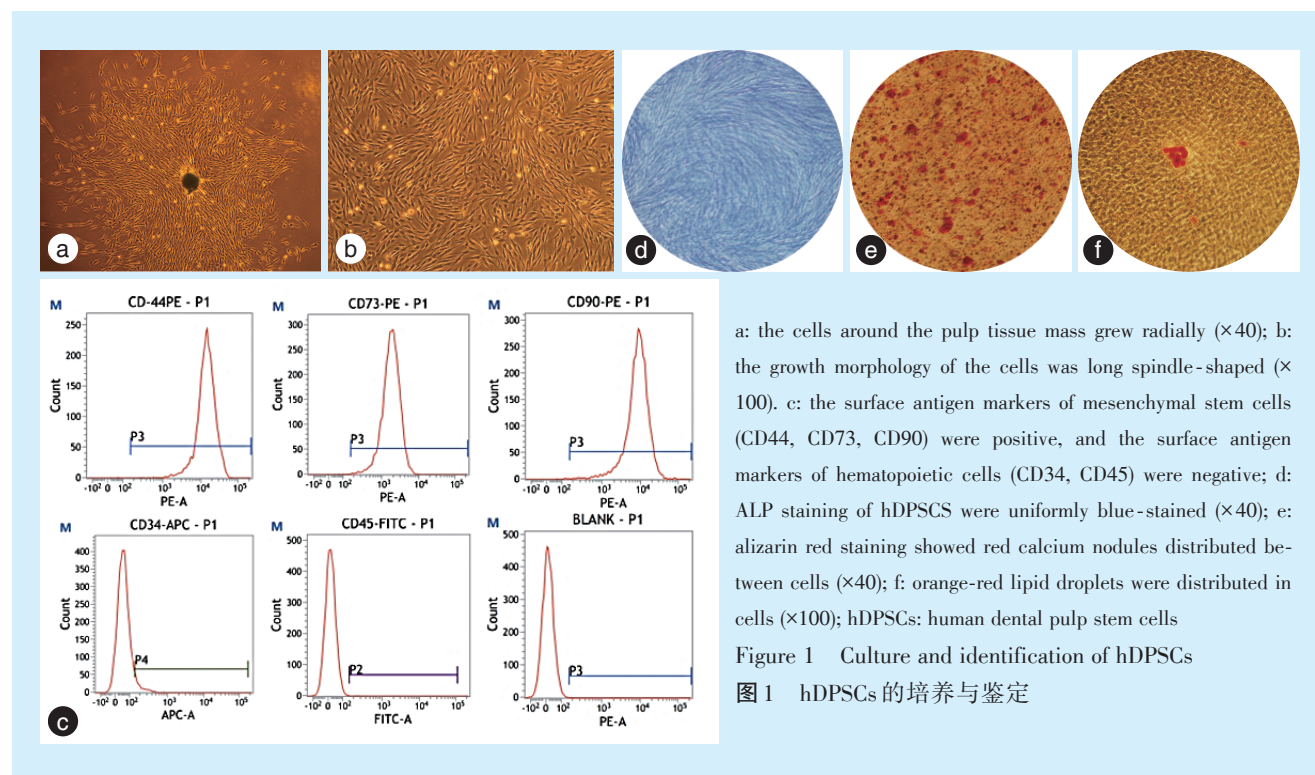
2.1 hDPSCs的培养与鉴定

牙髓组织培养第7天镜下可见组织块周围有细胞贴壁长出,从中央向四周放射状生长,细胞形态为梭形,类似成纤维细胞样。传代后可见细胞生长状态良好,形态均匀一致(图1a & 1b)。

第三代hDPSCs表面抗原检测结果为:间充质干细胞表面抗原标志物呈阳性表达:CD44、CD73、CD90的表达率依次为99.93%、93.75%、99.81%;造血系统来源细胞表面抗原标志物阴性表达:CD34、CD45的表达率依次为0.68%、0.09%(图1c)。成骨诱导7 d后,ALP染色镜下细胞呈蓝紫色(图1d)。成骨诱导21 d后,茜素红染色镜下见大小不一的红染钙结节分布于细胞之间(图1e)。成脂诱导21 d后,油红O染色示镜下细胞复层排列,橘红色脂滴出现在部分细胞胞浆内(图1f)。

2.2 不同浓度LPS溶液对hDPSCs增殖的影响

CCK-8示第1、3、5、7天,0 μ g/mL LPS组与1 μ g/mL LPS组增殖无显著差异($P = 0.894$, $P = 0.998$, $P = 0.965$, $P = 0.870$)。5 μ g/mL LPS组在第5天增殖低于0 μ g/mL LPS组、1 μ g/mL LPS组($P < 0.001$, $P < 0.001$)。10 μ g/mL LPS组在第5、7天增殖低于0 μ g/mL LPS组、1 μ g/mL LPS组、5 μ g/mL LPS组(第5天: $P < 0.001$, $P < 0.001$, $P = 0.009$, 第7天: $P < 0.001$, $P < 0.001$, $P = 0.002$)(图2)。由此



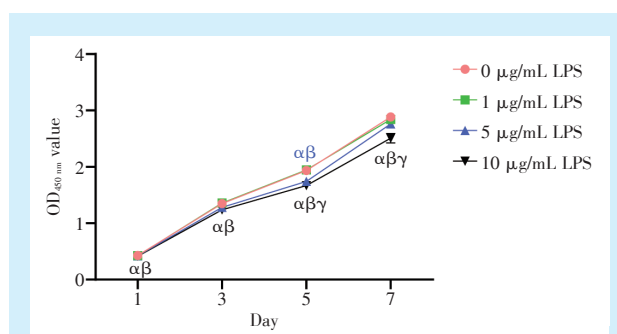
可见, LPS浓度为5、10 $\mu\text{g}/\text{mL}$ 时, 均可降低hDPSCs增殖能力, 但10 $\mu\text{g}/\text{mL}$ LPS增殖抑制作用强于5 $\mu\text{g}/\text{mL}$, 因此本实验选择10 $\mu\text{g}/\text{mL}$ 作为后续实验诱导炎症微环境的刺激浓度。

2.3 LED红光对炎症环境下hDPSCs成骨/成牙本质分化的影响

LED红光辐照hDPSCs 7 d后, ALP染色结果显示LPS+CG组较CG组稍浅, 光照组蓝染程度均高于LPS+CG组(图3a)。ALP定量结果显示LPS+CG组ALP活力低于成骨组($P = 0.007$), 光照组ALP活力均高于CG组与LPS+CG组(均 $P < 0.001$), LPS+CG+4 J/cm^2 组ALP活力高于其他光照组(均 $P < 0.001$)(图3b)。

第7天qRT-PCR检测矿化基因ALP、OSX、DMP-1、DSPP表达结果显示, CG组与LPS+CG组无显著差异($P = 0.968, P = 0.267, P = 0.902, P = 0.449$)。LPS+CG+2 J/cm^2 、4 J/cm^2 、6 J/cm^2 、8 J/cm^2 组均高于CG组与LPS+CG组($P < 0.05$)。LPS+CG+4 J/cm^2 组高于LPS+CG+2 J/cm^2 、6 J/cm^2 、8 J/cm^2 、10 J/cm^2 组(vs. LPS+CG+6 J/cm^2 , ALP: $P = 0.017$; OSX: $P = 0.001$; DMP-1: $P = 0.007$; DSPP: $P = 0.013$, 其余均 $P < 0.001$)。且LPS+CG+8 J/cm^2 组OSX、DMP-1、DSPP表达低于LPS+CG+6 J/cm^2 组($P = 0.016, P = 0.013, P < 0.001$), LPS+CG+10 J/cm^2 组ALP表达低于LPS+CG+8 J/cm^2 组($P = 0.004$)(图4)。

LED红光辐照21 d后, 茜素红染色结果显示CG组钙结节数目多于LPS+CG组, LPS+CG+4 J/cm^2 钙结节染色数目多且较大, 较其他LPS+CG+光照组钙结节染色深(图5a)。钙结节定量结果显示LPS+CG组钙结节定量少于CG组($P < 0.001$),



α: vs. 0 $\mu\text{g}/\text{mL}$ group, $P < 0.05$; β: vs. 1 $\mu\text{g}/\text{mL}$ group, $P < 0.05$; γ: vs. 5 $\mu\text{g}/\text{mL}$ group, $P < 0.05$; LPS: lipopolysaccharides

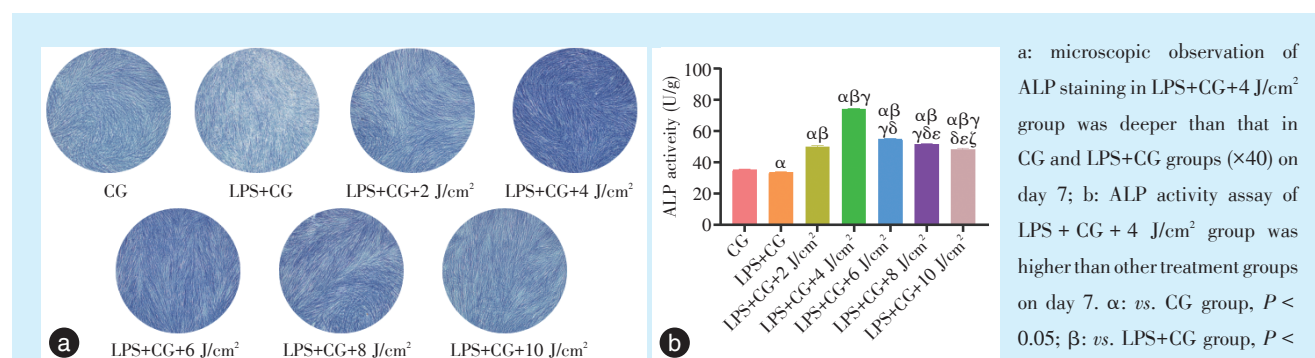
Figure 2 Effect of different concentrations of lipopolysaccharides on the proliferation of human dental pulp stem cells

图2 不同浓度脂多糖溶液对人牙髓干细胞增殖的影响

LPS+CG+光照组钙结节定量均多于LPS+CG组与CG组(均 $P < 0.001$), LPS+CG+4 J/cm^2 组钙结节定量多于其他LPS+CG+光照组(均 $P < 0.001$)(图5b)。

2.4 LED红光影响炎症环境下hDPSCs矿化时炎症因子的表达

ELISA结果显示, 细胞分泌TNF- α 与IL-1 β 的趋势均为先升后降。第1天, TNF- α 与IL-1 β 的分泌无明显差异($P = 0.887, P = 0.066$)。第3天, LPS+CG组的TNF- α 与IL-1 β 的分泌表达量均高于第1天($P < 0.001, P = 0.034$), LPS+CG+LED组的TNF- α 与IL-1 β 分泌量高于第1、5、7天($P < 0.001$), 且LPS+CG组的分泌量低于LPS+CG+LED组($P < 0.001$)。第5天, LPS+CG组的TNF- α 与IL-1 β 的分泌表达量高于第1、3、7天(均 $P < 0.001$), LPS+CG组的分泌量高于LPS+CG+LED组

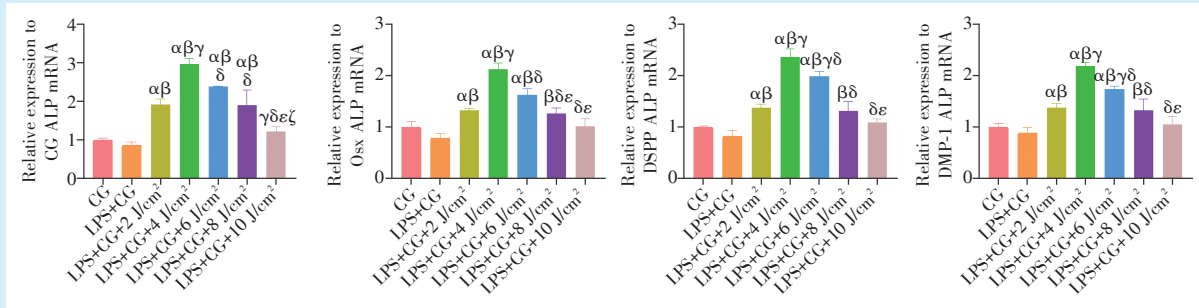


a: microscopic observation of ALP staining in LPS+CG+4 J/cm^2 group was deeper than that in CG and LPS+CG groups ($\times 40$) on day 7; b: ALP activity assay of LPS+CG+4 J/cm^2 group was higher than other treatment groups on day 7. α: vs. CG group, $P < 0.05$; β: vs. LPS+CG group, $P < 0.05$; γ: vs. LPS+CG+2 J/cm^2

group, $P < 0.05$; δ: vs. LPS+CG+4 J/cm^2 group, $P < 0.05$; ε: vs. LPS+CG+6 J/cm^2 group, $P < 0.05$; ζ: vs. LPS+CG+8 J/cm^2 group, $P < 0.05$. Treatment: LPS: 10 $\mu\text{g}/\text{mL}$ lipopolysaccharide; CG: mineralization induction

Figure 3 Effect of LED red light on the ALP activity of human dental pulp stem cells in inflammatory environment

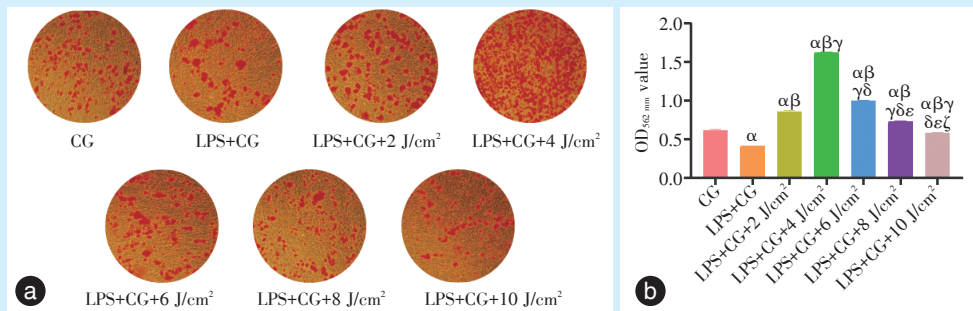
图3 LED红光对炎症环境下人牙髓干细胞ALP活性影响



These were the mRNA expression of mineralization related genes after 7 days of 4 J/cm² LED red light illumination. ALP: alkaline phosphatase; OSX: osterix; DMP-1: dentin matrix protein-1; DSPP: dentin sialophosphoprotein. α: vs. CG group, *P* < 0.05; β: vs. LPS+CG group, *P* < 0.05; γ: vs. LPS+CG+2 J/cm² group, *P* < 0.05; δ: vs. LPS+CG+4 J/cm² group, *P* < 0.05; ε: vs. LPS+CG+6 J/cm² group, *P* < 0.05; ζ: vs. LPS+CG+8 J/cm² group, *P* < 0.05. Treatment: LPS: 10 μg/mL lipopolysaccharide; CG: mineralization induction

Figure 4 Effect of LED red light on the expression of mineralization related genes in human dental pulp stem cells in inflammatory environment

图4 LED红光对炎症环境下人牙髓干细胞矿化相关基因表达的影响



a: microscopic observation of alizarin red staining after 21 days of 4 J/cm² LED red light illumination; b: quantification of calcium nodules after 21 days of 4 J/cm² LED red light illumination. α: vs. CG group, *P* < 0.05; β: vs. LPS+CG group, *P* < 0.05; γ: vs. LPS+CG+2 J/cm² group, *P* < 0.05; δ: vs. LPS+CG+4 J/cm² group, *P* < 0.05; ε: vs. LPS+CG+6 J/cm² group, *P* < 0.05; ζ: vs. LPS+CG+8 J/cm² group, *P* < 0.05. Treatment: LPS: 10 μg/mL lipopolysaccharide; CG: mineralization induction

Figure 5 Effect of LED red light on the formation of calcium nodules in osteogenic/odontogenic differentiation of human dental pulp stem cells in inflammatory environments

图5 LED红光对炎症环境下人牙髓干细胞成骨/成牙本质分化钙结节形成的影响

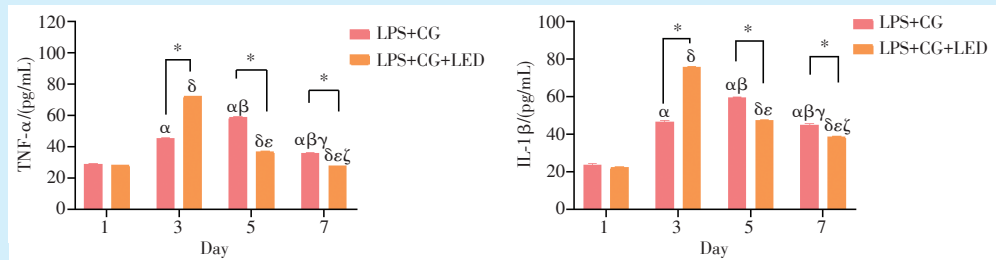
(*P* < 0.001)。第7天, LPS+CG组与LPS+CG+LED组的TNF-α与IL-1β的分泌表达量低于第5天(*P* < 0.001), 且LPS+CG+LED组的分泌表达量低于LPS+CG组(*P* = 0.002, *P* = 0.011)(图6)。

2.5 LED红光介导MAPK信号通路调控炎症环境下牙髓干细胞成骨/成牙本质分化

MAPK信号通路蛋白表达如图7, 第7天, LPS+CG+LED组的p-ERK1/2、p-p38、p-JNK的相对表达量高于LPS+CG组(*P* < 0.001), LPS+CG+LED组的p-ERK5相对表达量高于LPS+CG组(*P* = 0.003)。

未加MAPK通路阻滞剂时, LPS+CG组ALP、OSX、DMP-1、DSPP蛋白表达量均低于LPS+CG+

LED组(*P* < 0.001); 加入MAPK通路阻滞剂后, LPS+CG+LED+U0126(抑制ERK1/2)、LPS+CG+LED+SP600125(抑制JNK)、LPS+CG+LED+BIX02189(抑制ERK5)组ALP、OSX、DMP-1、DSPP蛋白表达量均低于LPS+CG+LED组(ALP: LPS+CG+LED vs. LPS+CG+LED+SP600125(抑制JNK), *P* = 0.004, 其余均*P* < 0.001); LPS+CG+LED+SB203580(抑制p38)组的ALP、OSX、DMP-1蛋白表达量与LPS+CG+LED组相比较无显著差异(*P* = 0.065, *P* = 0.193, *P* = 0.099), DSPP蛋白表达量低于LPS+CG+LED组(*P* < 0.001)(图8)。



Treatment: LPS: 10 $\mu\text{g}/\text{mL}$ lipopolysaccharide; CG: mineralization induction; LED: 4 J/cm^2 LED red light illumination. TNF- α : tumor necrosis factor- α ; IL-1 β : interleukin-1 β . α : vs. LPS+CG group on day 1, $P < 0.05$; β : vs. LPS+CG group on day 3, $P < 0.05$; γ : vs. LPS+CG group on day 5, $P < 0.05$; δ : vs. LPS+CG+LED group on day 1, $P < 0.05$; ϵ : vs. LPS+CG+LED group on day 3, $P < 0.05$; ζ : vs. LPS+CG+LED group on day 5, $P < 0.05$; *: $P < 0.05$

Figure 6 Effect of LED red light on the expression of inflammatory factors during mineralization of human dental pulp stem cells in inflammatory environment

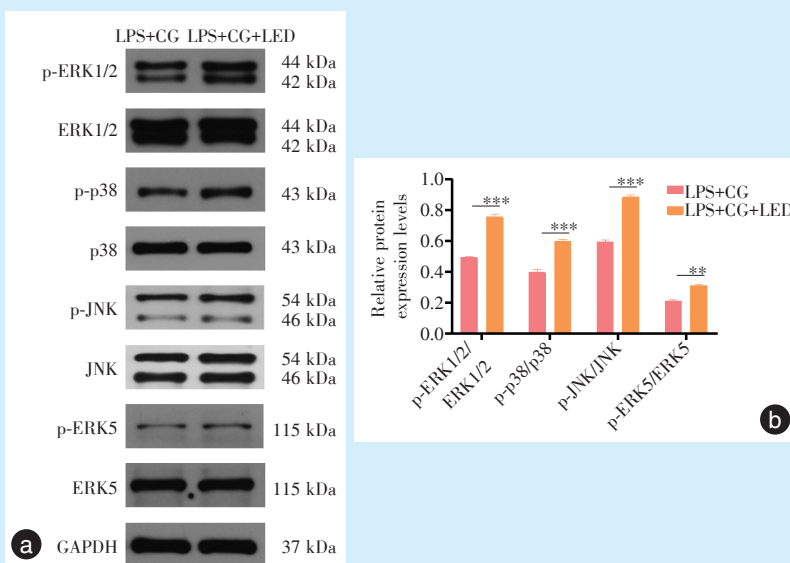
图6 LED红光对炎性环境下人牙髓干细胞矿化时炎症因子表达的影响

3 讨论

hDPSCs 来源于人牙髓组织,免疫原性低,具有高增殖能力,提供合适的外部诱导,在诱导因素胞外基质、细胞因子和物理因子等作用下,可以被定向诱导分化,是再生医学中具有前景的间充质干细胞^[8]。有研究报道,LED红光影响牙髓干细胞的细胞周期、线粒体膜电位和衰老^[9];5 J/cm^2 的LED红光促进高糖环境下牙周膜干细胞增殖和成骨分化,减轻氧化损伤^[10];2 J/cm^2 、4 J/cm^2 LED红光(850 nm, 80 mW/cm^2)显著提升乳牙牙髓干细胞活力、总蛋白产量、ALP活力以及ALP、Col-I基因表达^[11]。不同能量密度LED红光对间充质干细胞的增殖、分化有影响。低或不足的能量密度,未达到

生物刺激阈值,不会发生细胞反应;较高则可能破坏光感受器,导致生物调节效应降低^[12],合适的LED红光辐射剂量才能对间充质干细胞产生积极的生物学效应。

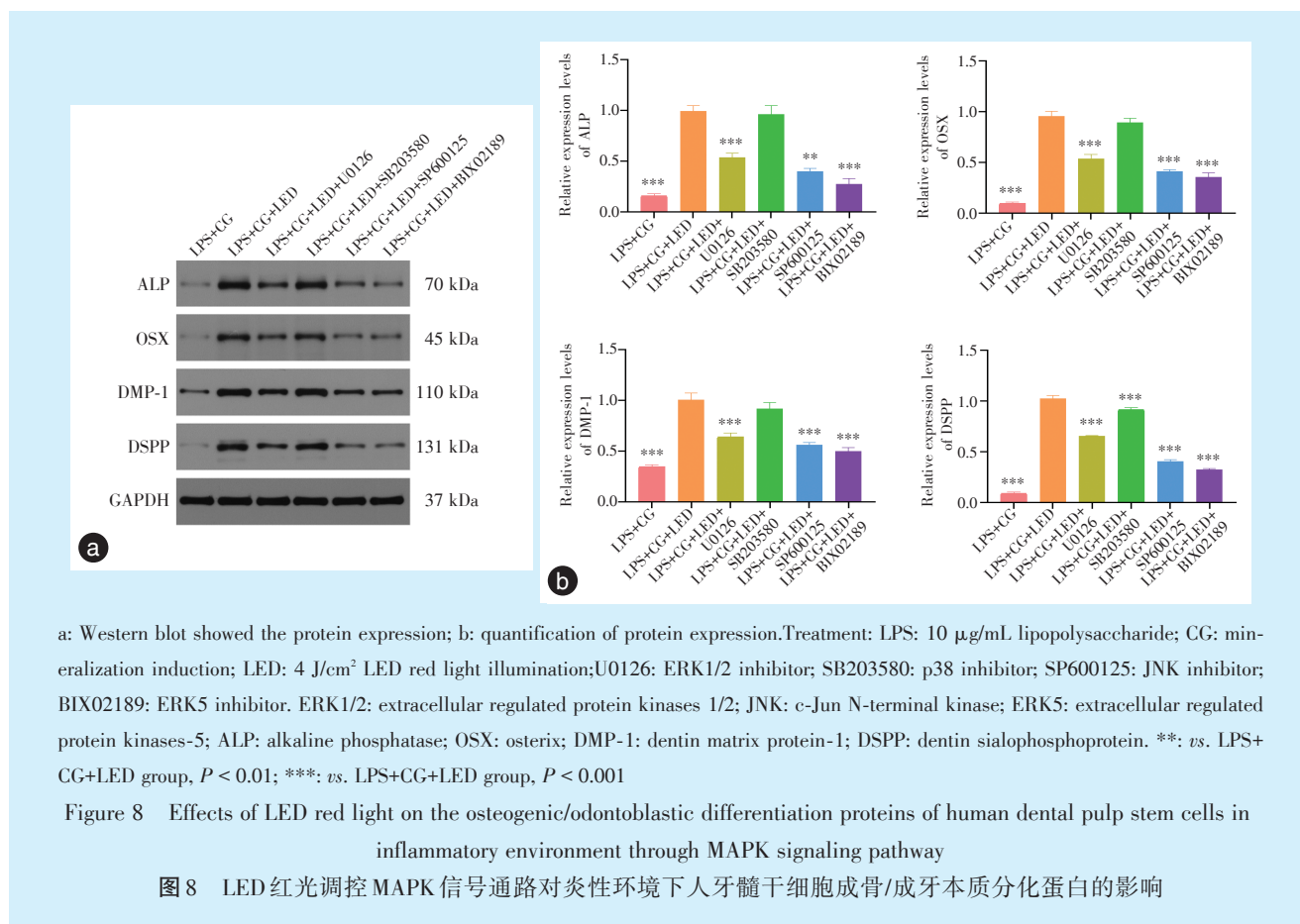
成骨/成牙本质分化是牙齿形成的基础,也是骨生成的关键步骤。在hDPSCs生长分化的炎性环境中,由于0、1 $\mu\text{g}/\text{mL}$ LPS的增殖速率无明显差异,5、10 $\mu\text{g}/\text{mL}$ 的增殖速率依次降低,与刘影等^[13]研究结果一致,因此选择10 $\mu\text{g}/\text{mL}$ LPS诱导炎症微环境。本实验中hDPSCs在10 $\mu\text{g}/\text{mL}$ LPS刺激下的矿化诱导比单纯矿化诱导7 d ALP染色稍浅及活力测定稍低,说明10 $\mu\text{g}/\text{mL}$ LPS对hDPSCs的成骨/成牙本质分化效应为抑制,与Sattari等^[2]报道的



a: Western blot showed the expression of MAPK signaling pathway protein after 7 days of 4 J/cm^2 LED red light illumination; b: quantification of protein expression 7 days of 4 J/cm^2 LED red light illumination. Treatment: LPS: 10 $\mu\text{g}/\text{mL}$ lipopolysaccharide; CG: mineralization induction; LED: 4 J/cm^2 LED red light illumination. MAPK: mitogen-activated protein kinases; ERK: extracellular regulated protein kinases; JNK: c-Jun N-terminal kinase. **: $P < 0.001$, ***: $P < 0.001$

Figure 7 Effect of LED red light on the expression of MAPK signaling pathway during mineralization of human dental pulp stem cells in inflammatory environment

图7 LED红光对炎性环境下人牙髓干细胞MAPK信号通路表达的影响



LPS对DPSCs的分化效应一致。LED红光辐照LPS致炎环境下的hDPSCs,比LPS矿化诱导下和单纯矿化诱导下ALP染色、茜素红染色更深,ALP活力和钙结节定量测定值更高,RT-PCR检测的成骨特异性基因ALP、OSX与成牙本质特异性基因DMP-1、DSPP相对表达量更高,说明LED红光正向调控炎症环境下的hDPSCs的成骨/成牙本质分化。其中,4 J/cm^2 能量密度LED红光辐照hDPSCs,ALP染色、ALP活力测定、RT-PCR、茜素红染色及钙结节定量测定结果最佳,说明4 J/cm^2 能量密度是LED红光上调10 $\mu\text{g}/\text{mL}$ LPS致炎环境下促成骨/成牙本质分化的最佳辐照能量。

有研究报道4 J/cm^2 LED红光上调牙周膜干细胞ALP、Runt相关转录因子2(Runt-related transcription factor 2, Runx2)、骨钙素(osteocalcin, OCN)、骨桥蛋白(osteopontin, OPN)、骨涎蛋白(bone sialoprotein, BSP)和血管内皮生长因子(vascular endothelial growth factor, VEGF)等基因和蛋白表达,促进干细胞成骨和成血管分化^[7,14]。还可以上调牙乳头干细胞的DMP-1、DSPP、BSP、ALP和Runx2基因和蛋白的表达,促进干细胞成牙本质和成骨分

化^[15-16]。本实验也发现4 J/cm^2 LED红光促成骨/成牙本质分化能力最强,随能量密度的增加,其成骨向分化能力呈依赖式下降,符合物理治疗中Arndt-Schulze法则:弱刺激引起生命活动,中等刺激促进生命活动直到达峰值,强烈刺激抑制生命活动直到产生负反应,最强刺激使生命活动停止^[17]。

研究报道LPS可以通过TLR4/MyD88/NF- κ B信号通路诱导牙髓干细胞分泌TNF- α 、IL-8、IL-12,引起牙髓损伤^[18]; Zhai等^[19]研究发现LPS刺激hDPSCs后,炎症介质IL-1 α 、IL-1 β 、IL-6、TNF- α 基因和蛋白表达升高。本实验中LPS作为炎症刺激源,处理hDPSCs后释放TNF- α 和IL-1 β 等炎症介质,培养液中炎症介质浓度升高则会引起hDPSCs增殖与分化的缓慢抑制,与前面报道一致。本研究示10 $\mu\text{g}/\text{mL}$ LPS成骨/成牙本质诱导培养下,非光照组TNF- α 和IL-1 β 分泌量在第5天达到高峰,第7天炎症反应得到缓解;4 J/cm^2 LED红光组炎症介质分泌在第3天达到高峰且高于非光照组,第5天炎症反应得到缓解,可能是由于4 J/cm^2 LED红光加速了炎症反应的进程。第5、7天时,4 J/cm^2 LED红光组TNF- α 和IL-1 β 分泌量低于非光照组。说明

4 J/cm² LED红光在促进炎症环境下成骨的同时,减少了炎症因子TNF- α 和IL-1 β 的释放,从而达到抗炎的目的,该结果与其他研究^[20]报道的LED红光光疗下调炎症因子与趋化因子的表达一致。

间充质干细胞成骨/成牙本质分化是一个由多种矿化基因调控、多种信号通路共同参与的一个复杂分化过程。MAPK信号通路有4个亚族:ERK1/2、JNK、p38与ERK5。其中ERK信号通路主要调控细胞生长和分化,p38与JNK信号通路的主要生理效应主要与炎症、细胞凋亡、应激反应有关^[21]。因此,物理刺激因素可能激活部分或全部MAPK亚族通路。本研究中LED红光促进MAPK信号通路蛋白p-ERK1/2、p-JNK、p-p38、p-ERK5表达增高,提示ERK1/2、JNK、p38、ERK5可能参与了LED红光促进炎症环境下hDPSCs成骨/成牙本质分化的过程。LED红光分别联合信号通路ERK1/2特异性阻滞剂U0126、JNK特异性阻滞剂SP600125以及ERK5特异性阻滞剂BIX02189干预hDPSCs时,ALP、OSX、DMP-1、DSPP矿化蛋白相对表达量低于LPS+CG+LED组,说明LED红光激活了ERK1/2、JNK、ERK5亚通路,也有可能MAPK阻滞剂加入后诱导干细胞凋亡,成骨/成牙本质分化向分化细胞数目减少,导致矿化蛋白部分表达下调有关^[22]。然而LED红光联合信号通路p38特异性阻滞剂SB203580干预hDPSCs时,LPS+CG+LED+SB203580组与LPS+CG+LED组矿化蛋白表达量无明显差异,可能是由于LED红光未激活p38信号通路,或者是因为SB203580有效抑制炎症因子(如IL-1 β 、TNF- α)诱导的部分信号转导^[23],逆转了炎症因子所导致的成骨抑制。因此LED红光可能通过激活MAPK信号通路中的ERK1/2、JNK、ERK5亚通路,来促进炎症环境下hDPSCs成骨/成牙本质分化。这些MAPK亚通路的激活与Yamauchi等^[24]研究LED红光所激活的通路一致。

综上,本研究探讨了能量密度为2~10 J/cm²的LED红光调控10 μ g/mL LPS致炎环境下hDPSCs成骨/成牙本质分化,其中4 J/cm²的LED红光促成骨/成牙本质分化效应最佳,且减少炎症因子分泌,同时ERK1/2、JNK、ERK5通路参与LED红光调控炎症微环境下hDPSCs成骨/成牙本质分化。不同的参数指标(光源、波长、能量密度、输出功率、照射频率、细胞与光源发射器之间的距离等)对干细胞具有不同的生物学效应,探索治疗的最佳参数有利于为牙髓干细胞的体外处理技术提供实验数

据支持。

【Author contributions】 Liu Y designed the study, performed the experiments and wrote the article. Hui YN, Jiang B, Zheng GZ designed the study, performed the experiments and analyzed the data. Wang Y designed the study and reviewed the article. All authors read and approved the final manuscript as submitted.

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(编辑 周春华, 曾曙光)



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