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·基础研究·

高糖通过氧化应激及线粒体DNA释放加重牙龈成纤维细胞的炎症反应

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【摘要】 目的　探讨高糖（high glucose，HG）环境是否加重牙龈卟啉单胞菌（*Porphyromonas gingivalis*，*P.g*）脂多糖（lipopolysaccharide，LPS）引起的人牙龈成纤维细胞（human gingival fibroblasts，HGFs）炎症反应及其机制，为糖尿病加剧牙周炎的机制提供依据。**方法**　将HGFs分为4组，分别为对照组（基础培养基）、LPS组（加入5 μg/mL *P.g*-LPS培养24 h）、HG组（加入25 mmol/L葡萄糖培养24 h）、HG+LPS组（加入25 mmol/L葡萄糖+5 μg/mL *P.g*-LPS培养24 h）。在以上处理的培养基中培养24 h后取细胞进行实验。用2’，7’-二氯二氢荧光素二乙酸酯（2，7-dichlorodihydrofluorescein diacetate，DCFH-DA）和MitoSOX Red染色分别检测细胞内活性氧（reactive oxygen species，ROS）和线粒体活性氧（mitochondria reactive oxygen species，mtROS），并通过共聚焦荧光显微镜分析荧光强度和直接测量细胞悬液的荧光强度。使用免疫荧光法检测HGFs的线粒体DNA（mitochondrial DNA，mtDNA）含量变化。通过实时荧光定量PCR检测胞浆及细胞上清中mtDNA含量。蛋白质免疫印迹检测环鸟苷酸-腺苷酸合成酶（cyclic GMP-AMP synthase，cGAS）-干扰素刺激因子（stimulator of interferon genes，STING）通路相关蛋白的表达，并通过实时定量PCR检测肿瘤坏死因子α（tumor necrosis factor-α，TNF-α）、白细胞介素1β（interleukin-1β，IL-1β）、白细胞介素6（interleukin-6，IL-6）的mRNA的表达水平。**结果**　与对照组相比，LPS组和HG组中ROS与mtROS均显著上升，且在HG+LPS组中升高更加显著，呈协同增强效应（ROS：$F = 396.5$，$P < 0.001$；mtROS：$F = 29.38$，$P < 0.001$，CI<1）。胞浆mtDNA含量在LPS组显著升高，HG+LPS组升高更显著（$F = 27.85$，$P < 0.001$）。上清液mtDNA在LPS组和HG组中均显著升高，HG+LPS组升高更加显著（$F = 15.26$，$P < 0.001$）。cGAS-STING通路中的p-STING、p-TBK1、p-P65在LPS组和HG组中有不同程度激活，在HG+LPS组中激活程度最高（p-STING：$F = 52.67$，$P < 0.001$；p-TBK1：$F = 15.67$，$P = 0.001$；p-P65：$F = 9.83$，$P = 0.005$），p-IRF3在各组间无显著差异（$P = 0.072$）。促炎细胞因子TNF-α在HG+LPS组中显著高于对照组（$F = 15.05$，$P < 0.001$），IL-1β在LPS组、HG组中均上升，HG+LPS组上升更显著（$F = 30.98$，$P < 0.001$），IL-6在各组间无显著差异（$P = 0.847$）。**结论**　高糖与LPS协同加剧氧化应激，其机制为mtDNA释放增加，激活cGAS-STING通路，导致HGFs炎症反应加重。

【关键词】 人牙龈成纤维细胞；　高糖；　牙龈卟啉单胞菌；　脂多糖；　氧化应激；　线粒体DNA；　线粒体活性氧；　环鸟苷酸-腺苷酸合成酶；　干扰素刺激因子；　炎症；　细胞因子
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High glucose exacerbates the inflammatory response in gingival fibroblasts through oxidative stress and mitochondrial DNA release　GENG Yiran, ZANG Xiaoying, LIU Jia, LUAN Qingxian. Department of Periodontology, Peking University School and Hospital of Stomatology & National Center of Stomatology & National Clinical Research

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【Abstract】 Objective To investigate if high glucose (HG) exacerbates *Porphyromonas gingivalis* (*P.g*) lipopolysaccharide (LPS)-induced inflammatory response in human gingival fibroblasts (HGFs) and to explore the underlying mechanisms. To provide a basis for the mechanism of diabetes aggravating periodontitis. **Methods** HGFs were divided into four groups: the control group (basal medium), the LPS group (treated with 5 μg/mL *P.g*-LPS for 24 h), the HG group (treated with 25 mmol/L glucose for 24 h), and the HG+LPS group (treated with 25 mmol/L glucose + 5 μg/mL *P.g*-LPS for 24 h). After culturing for 24 h in the respective media, the cells were harvested for experiments. Intracellular reactive oxygen species (ROS) and mitochondrial reactive oxygen species (mtROS) were detected using 2′, 7′ - dichlorodihydrofluorescein diacetate (DCFH-DA) and MitoSOX Red staining, respectively. Fluorescence intensity was analyzed by confocal fluorescence microscopy and directly measured in cell suspension. Immunofluorescence was used to detect changes in mitochondrial DNA (mtDNA) content of HGFs. Real-time fluorescence quantitative PCR was used to detect the content of mtDNA in cytoplasm and cell supernatant. Protein expression of the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway was assessed by western blot, while mRNA expression levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) were detected by PCR. **Results** Compared to the control group, both the LPS group and the HG group exhibited a significant increase in ROS and mtROS, with a more pronounced elevation in the HG+LPS group, demonstrating a synergistic effect (ROS: $F = 396.5$, $P < 0.001$; mtROS: $F = 29.38$, $P < 0.001$, CI < 1). The cytoplasmic mtDNA content was significantly elevated in the LPS group, with a more marked increase in the HG+LPS group ($F = 27.85$, $P < 0.001$). The supernatant mtDNA levels were significantly higher in both the LPS and HG groups, with a more pronounced elevation in the HG+LPS group ($F = 15.26$, $P < 0.001$). The phosphorylated proteins p-STING, p-TBK1, and p-P65 in the cGAS-STING pathway showed varying degrees of activation in the LPS and HG groups, reaching the highest levels in the HG+LPS group (p-STING: $F = 52.67$, $P < 0.001$; p-TBK1: $F = 15.67$, $P = 0.001$; p-P65: $F = 9.83$, $P = 0.005$), while p-IRF3 showed no significant differences among the groups ($P = 0.072$). Pro-inflammatory cytokine TNF-α was significantly higher in the HG+LPS group compared to the control group ($F = 15.05$, $P < 0.001$), and IL-1β increased in both the LPS and HG groups, with a more pronounced rise in the HG+LPS group ($F = 30.98$, $P < 0.001$). IL-6 showed no significant differences among the groups ($P = 0.847$). **Conclusion** High glucose and LPS act synergistically to enhance oxidative stress, accompanied by increased mtDNA release, which activates the cGAS-STING pathway, thereby amplifying the inflammatory response in HGFs.

【Key words】 human gingival fibroblasts; high glucose; *Porphyromonas gingivalis*; lipopolysaccharide; oxidative stress; mitochondrial DNA; mitochondria reactive oxygen species; cyclic GMP-AMP synthase; stimulator of interferon genes; inflammation; cytokines
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牙周炎是一种由细菌引起、免疫炎症反应介导的炎症性、破坏性疾病，已被列为糖尿病的第六大并发症。大量研究证实，糖尿病是牙周炎发展的重要危险因素，二者之间存在相互促进作用[1]。目前普遍认为，糖尿病通过高糖环境、晚期糖基化终末产物（advanced glycation end-products，AGEs）积累、氧化应激等多重机制，导致核因子κB（nuclear factor kappa-B，NF-κB）的激活和炎症细胞因子的表达增加，扰乱骨代谢平衡；或改变口腔微生物群，使其更具致病性，从而加剧牙周炎症与骨吸收[2-3]。但糖尿病对牙周组织在细胞水平的具体调控机制尚未完全阐明。

氧化应激被认为在糖尿病相关牙周破坏中扮演关键角色。研究表明，糖尿病可引起活性氧（reactive oxygen species，ROS）过量生成，使其在牙周组织中显著积累[4-5]，进而诱导线粒体功能障碍与

线粒体DNA（mitochondrial DNA，mtDNA）损伤[6-7]。mtDNA释放至胞质，可作为损伤相关分子模式引发广泛的炎症反应[8]。环鸟苷酸-腺苷酸合成酶（cyclic GMP-AMP synthase，cGAS）是一种胞质DNA传感器，可识别并结合mtDNA[9]，催化生成第二信使环鸟苷酸-腺苷酸（cyclic GMP-AMP，cGAMP），进而激活干扰素刺激因子（stimulator of interferon genes，STING）[10]，诱导干扰素调节因子3（interferon regulatory factor 3，IRF3）和NF-κB通路的活化[11]，促进Ⅰ型干扰素及肿瘤坏死因子α（tumor necrosis factor alpha，TNF-α）、白细胞介素1β（interleukin-1β，IL-1β）、白细胞介素6（interleukin-6，IL-6）等多种促炎细胞因子的表达[12]。cGAS-STING通路在多种糖尿病并发症中已有报道[13-15]，然而其在糖尿病微环境下的牙周炎发病机制中的作用，尤其是对牙龈成纤维细胞炎症表型的调控尚未阐明。

人牙龈成纤维细胞（human gingival fibroblasts，HGFs）是牙周组织中最主要的细胞类型之一，不仅在维持组织结构和功能中发挥核心作用[16-17]，也是牙周免疫炎症反应的重要参与者[18]。本课题组既往研究发现，牙周炎患者的HGFs中ROS增高引起mtDNA的释放。在健康HGFs中，来自牙龈卟啉单胞菌（*Porphyromonas gingivalis*，*P. g*）的脂多糖（lipopolysaccharide，LPS）可以上调ROS水平并调节线粒体功能，促进mtDNA的释放[19]。

本研究通过构建高糖联合LPS刺激的HGFs体外模型，拟探讨高糖是否协同加重LPS诱导HGFs的氧化应激与mtDNA释放，并进一步分析cGAS-STING通路激活及其在炎症放大中的作用，为阐明糖尿病加剧牙周炎的机制提供新的实验依据，也为未来靶向干预提供理论基础。

1　材料和方法

1.1　主要试剂和仪器

DMEM培养基（C11995500，Gibco，美国）；*P.g*-LPS（ATCCs 33277，Invivogen，法国）；IG9000，D-葡萄糖溶液（1M 无菌）（IG9000，Solarbio，中国）；2′，7′-二氯二氢荧光素二乙酸酯（2，7-dichlorodihydrofluorescein diacetate，DCFH-DA）（D6883-50MG，Sigma-Aldrich，美国）；MitoSOX Red（M36008，Invitrogen，美国）；Hoechst 33342（IH0070，索莱宝，中国）；Mito-Tracker Red CMXRos（C1035，碧云天，中国）；PicoGreen dsDNA Reagent 双链DNA检测试剂（MF0784，懋康生物，中国）；HEPES（pH7.4）（PB180325，普诺赛，中国）；Digitonin（HY-N4000，MCE，美国）；QiAamp DNAmini 50T 试剂盒（51304，Qiagen，德国）；THUNDERBIRD Next SYBR qPCR Mix（QPS-201，Toyobo，日本）；BCA 蛋白定量试剂盒（P0010，碧云天，中国）；一步法10%PAGE凝胶制备试剂盒（PG213，雅酶，中国）；蛋白marker（PR1910，Solarbio，中国）；STING（D2P2F）Rabbit mAb（13647S，Cell Signaling Technology，美国）；Phospho-STING（Ser366）（E9A9K）Rabbit mAb（50907S，Cell Signaling Technology，美国）；IRF-3（D83B9）Rabbit mAb（4302S，Cell Signaling Technology，美国）；Phospho-IRF-3（Ser396）（D6O1M）Rabbit mAb（29047S，Cell Signaling Technology，美国）；TBK1/NAK（E8I3G）Rabbit mAb（38066S，Cell Signaling Technology，美国）；Phospho-TBK1/NAK（Ser172）（D52C2）XP® Rabbit mAb（5483S，Cell Signaling Technology，美国）；NF-κB p65（D14E12）XP® Rabbit mAb（8242S，Cell Signaling Technology，美国）；Phospho-NF-κB p65（Ser536）（93H1）Rabbit mAb（3033S，Cell Signaling Technology，美国）；Goat Anti-Rabbit IgG，HRP Conjugated（CW0103S，江苏康为世纪，中国）；ECL 发光液（P1050，普利莱，中国）；SteadyPure 快速RNA提取试剂盒（AG21023，湖南艾科瑞生物，中国）；qPCR引物（上海生工，中国）；线粒体基因NADH dehydrogenase 1（ND1）质粒（上海生工，中国）。激光共聚焦显微镜（TCS-SP8 STED 3X，Leica，德国）；多功能微孔板检测系统（BioTek Synergy Neo2，Agilent Technologies，美国）；实时荧光定量PCR仪（QuantStudio 1，ThermoFisher，美国）；多功能酶标仪（SpectraMax M4，美谷分子，中国）；电泳仪（Powerpac HC，Bio-Rad，美国）；电子压片成像仪（Touch Imager，E-blot，中国）；核酸分析仪（Nanodrop 8000，Thermo，美国）。

1.2　细胞培养及实验分组

原代HGFs商业化获得（CP-H240，Procell，武汉，中国），并在6代内使用。使用DMEM培养基培养，将HGFs随机分为4组，每组6孔：①对照组，培养基不作任何处理；②LPS组，培养基中加入5 μg/mL *P.g*-LPS；③高糖组（HG组），培养基中加入25 mmol/L无菌葡萄糖；④高糖合并LPS组（HG+LPS组），培养基中加入25 mmol/L葡萄糖和5 μg/mL *P. g*-LPS。在以上处理的培养基中培养24 h后取细胞进行实验。

1.3　细胞内ROS和线粒体ROS染色

用10 μmol/L的DCFH-DA和5 μmol/L的MitoSOX Red分别在DMEM培养基中孵育30 min，分别用于检测HGFs胞内ROS和线粒体活性氧（mitochondria reactive oxygen species，mtROS）。使用共聚焦荧光显微镜（63x油镜）观察DCFH-DA（激发光488 nm，发射光535 nm）或MitoSOX Red（激发光510 nm，发射光580 nm），在相同参数下随机拍摄每组各6张图像。用ImageJ分析图像中HGFs荧光强度。将HGFs接种于六孔板，上述染料处理后，使用胰蛋白酶将细胞消化后获得细胞悬液。室温下以1 000 rpm离心3 min，弃上清，加入1 mL PBS轻柔吹打重悬。重复3次。使用多功能微孔板检测系统分别在488 nm和510 nm直接测量细胞悬液的荧光强度。

1.4　免疫荧光法检测HGFs的mtDNA含量变化

用Hoechst、Mitotracker和PicoGreen dsDNA分别依次在DMEM培养基中孵育20min，用于标记HGFs细胞核、线粒体和胞内DNA。在共聚焦荧光显微镜（63x油镜）中观察，在相同参数下每组随机选取10个完整细胞拍摄。使用ImageJ排除核DNA干扰后，计算黄色共染面积占红色线粒体面积的比例，用以表示线粒体中mtDNA含量变化。

1.5　HGFs的mtDNA提取及定量检测

根据West等[20]建立的方法进行细胞质液提取。通过QIAamp DNA Mini Kit从HGFs细胞质液和上清液中分离提取DNA。通过实时荧光定量PCR检测线粒体基因ND1的拷贝数，每组设置5个样本，并根据ND1质粒的标准曲线来分析ND1含量，用以评估mtDNA水平。数据归一化后进行比较。引物序列见表1。

1.6　蛋白质免疫印迹法检测HGFs的cGAS-STING通路相关蛋白表达情况

采用预冷的RIPA裂解液提取HGFs中的总蛋白，并使用BCA试剂盒进行蛋白定量，每组3个样本。取10 μg蛋白样品与上样缓冲液混合，经SDS-PAGE电泳分离后，转印至PVDF膜。用5%脱脂牛奶封闭后，将膜与下列一抗在4°C下孵育过夜：STING、p-STING、IRF3、p-IRF3、TBK1、p-TBK1、p65及p-p65。洗膜后，采用羊抗兔二抗进行孵育，并使用ECL发光液显影。

1.7　实时定量PCR检测HGFs促炎细胞因子的mRNA水平

使用RNeasy Mini Kit从HGFs中分离总RNA。使用PrimeScript RT Master Mix进行逆转录。采用2xSYBR Green和各基因引物进行实时荧光定量PCR，每组5个样本，重复3次，并通过两步PCR扩增标准程序。以β-actin作为内参基因，$2^{-\Delta\Delta Ct}$法测定TNF-α、IL-1β、IL-6 mRNA的相对水平，归一化后进行比较。各引物序列见表1。

表1　实时荧光定量PCR引物序列

Table 1　Primer sequences for Real time fluorescence quantitative PCR

Gene	Forward	Reverse
ND1	5′-CACACTAGCAGAGACCAACCGAAC-3′	5′-CGGCTATGAAGAATAGGGCGAAGG-3′
TNF-α	5′-GTAGCCCATGTTGTAGCAAA-3′	5′-CCTGGGAGTAGATGAGGTACA-3′
IL-1β	5′- TTCTTCGACACATGGGATAACG -3′	5′- TGGAGAACACCACTTGTTGCT -3′
IL-6	5′-CAGGAGAAGATTCCAAAGAT-3′	5′-CTCTTGTAACATGTCTCCTT-3′
β-actin	5′- TGGCACCCAGCACAATGAA -3′	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′

ND1: NADH dehydrogenase 1; TNF-α: tumor necrosis factor alpha; IL-1β: interleukin-1β; IL-6: interleukin-6

1.8　统计学方法

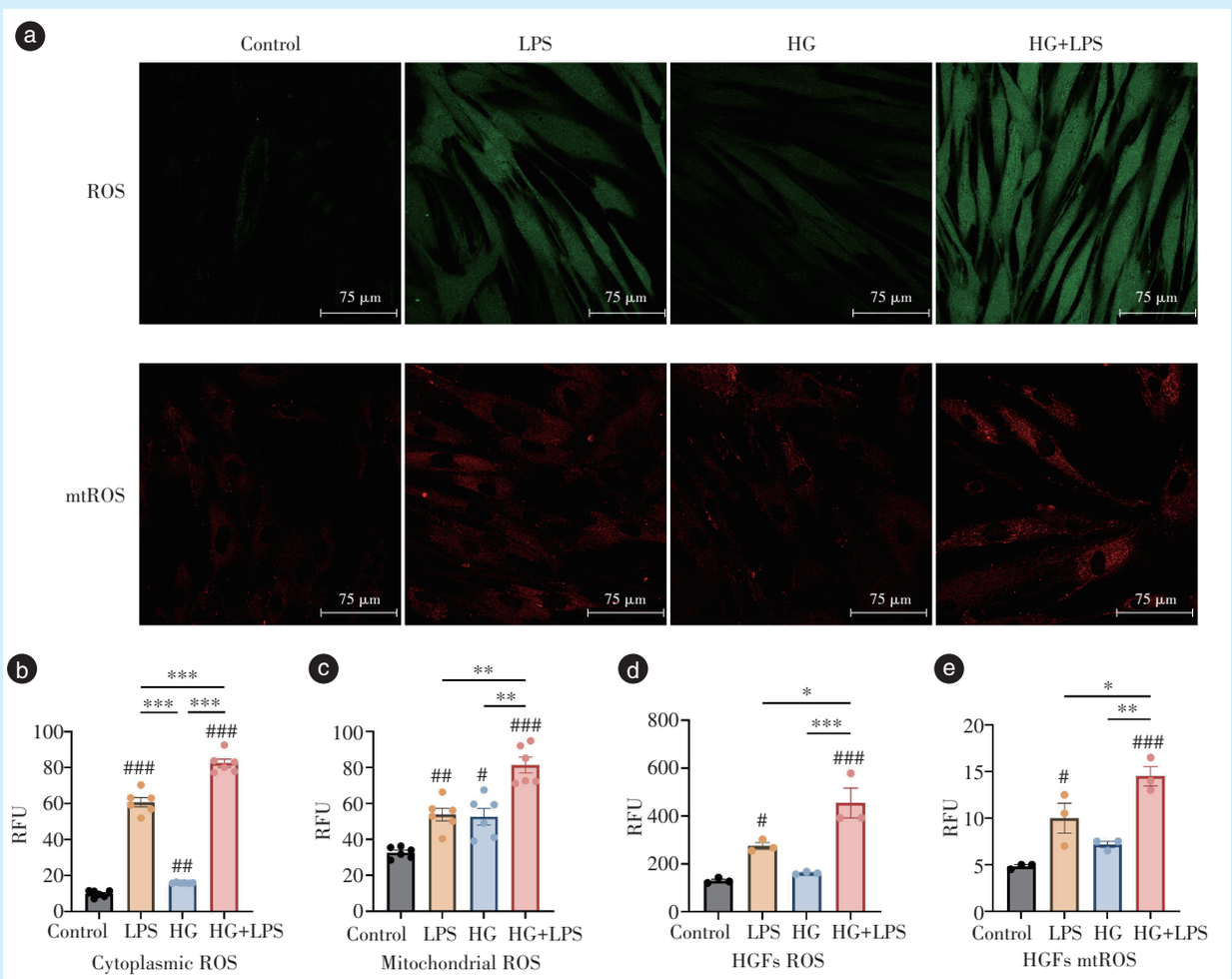
通过GraphPad Prism 10进行统计分析。符合正态分布的数据，多组间比较采用单因素方差分析，经Brown-Forsythe方差齐性检验，若方差齐性，采用Tukey事后检验；若方差不齐，采用Dunnett′s T3进行两两比较。对于非正态分布数据通过Kruskal-Wallis秩和检验进行分析。P <0.05为差异有统计学意义。为了定量分析高糖和LPS之间的协同效应，根据Chou-Talalay方法，假设剂量效应关系是线性的，并计算协同指数（combination index，CI）[21]，CI < 1表示协同作用，CI = 1表示相加作用，CI > 1表示拮抗作用。

2　结　果

2.1　高糖环境加重*P.g*-LPS引起的HGFs中ROS和mtROS水平升高

共聚焦显微镜观察发现，在HGFs中给予*P.g*-LPS和高糖刺激后，细胞内ROS（绿色）和mtROS

（红色）荧光强度显著升高，且HG+LPS组升高更为显著（ROS：F = 396.5，P < 0.001，CI=0.78；mtROS：F = 29.38，P < 0.001，CI=0.84）。这表明LPS和高糖环境均会诱导HGFs的ROS和mtROS增加，从而增加细胞的氧化应激（图1a & 1b）。对HGFs消化离心后的重悬液进行荧光强度测定结果表明，虽然HG组较对照组ROS和mtROS荧光强度升高并不显著（ROS：P =0.895；mtROS：P=0.387），但LPS组荧光强度显著升高，HG+LPS组进一步升高，差异具有统计学意义（ROS：F = 21.01，P < 0.001，CI=0.54；mtROS：F = 18.19，P < 0.001，CI=0.78）（图1c-1e），以上结果与共聚焦显微镜所观察到的结果基本相符，高糖和LPS在增强氧化应激方面存在协同作用（CI<1）。



a: HGFs were incubated with DCFH-DA to indicate intracellular ROS levels (green) and MitoSOX Red to indicate mtROS levels (red); Scale bar = 75 μm. b & c: fluorescence intensity of ROS and mtROS was quantified using ImageJ. n = 6. d & e: fluorescence intensity of cell suspensions was directly measured using a multimode microplate reader at excitation wavelengths of 488 nm (ROS) and 510 nm (mtROS). n = 3. *: P < 0.05, **: P < 0.01, ***: P < 0.001; #: compared to the control group, P < 0.05; ###: compared to the control group, P < 0.001. Control group: no treatment; LPS group: treated with 5 μg/mL *P.g*-LPS; HG group: treated with 25 mmol/L glucose; HG+LPS group: co-treated with 5 μg/mL *P.g*-LPS and 25 mmol/L glucose. HGFs: human gingival fluorescent fibroblasts; DCFH-DA: 2, 7-dichlorodihydrofluorescein diacetate; ROS: reactive oxygen species; mtROS: mitochondrial reactive oxygen species; *P.g: Porphyromonas gingivalis;* LPS: lipopolysaccharide; RFU: relative fluorescence unit

Figure 1　High-glucose environment exacerbates the elevation of ROS and mtROS levels in HGFs induced by *P.g*-LPS

图1　高糖环境加重*P.g*-LPS引起的HGFs中ROS和mtROS水平升高

2.2　高糖环境增强*P.g*-LPS引起的HGFs中mtDNA的释放

通过免疫荧光，对胞内的DNA和线粒体进行染色，其中共染的黄色部分指示线粒体内的mtDNA（图2a）。线粒体内mtDNA荧光表达在LPS组和HG组中均有显著下降，在HG+LPS组中下降

更为显著（F = 26.19，P < 0.001）（图2b），说明LPS和高糖均会引起线粒体中的mtDNA释放，但二者之间为拮抗作用（CI=1.45）。通过PCR检测发现，LPS会引起HGFs胞浆中mtDNA含量升高，在HG+LPS组进一步升高，结果有统计学差异（F = 27.85，P < 0.001），高糖和LPS存在强烈协同作用（CI=0.23）（图2c）。在上清液中，除了LPS组和HG+LPS组的增加，本研究还发现单纯高糖刺激也会促进HGFs的mtDNA释放至细胞外，结果有统计学差异（F = 15.26，P < 0.001）（图2d），高糖和LPS在其中为相加作用（CI=1.03）。

2.3　高糖环境增强*P.g*-LPS引起的HGFs中cGAS-STING信号通路的激活

在高糖和LPS刺激24 h后，对HGFs中cGAS-STING信号通路相关蛋白含量进行检测（图3a）。LPS组、HG组、HG+LPS组磷酸化的STING（phosphorylated STING，p-STING）和下游磷酸化的TBK1（phosphorylated TBK1，p-TBK1）的表达水平较Control组显著增加，且HG+LPS组增加更加显著，差异具有统计学意义（p-STING：F = 52.67，P<0.001；p-TBK1：F = 15.67，P=0.001）（图3b & 3c），高糖和LPS在p-STING中存在协同作用（CI=0.74），在p-TBK1中为相加作用（CI=1.03）。此外P65、IRF3的磷酸化有不同程度增强。其中，磷酸化P65（phosphorylated P65，p-P65）在LPS组和HG+LPS组中显著升高，在HG组略升高（F = 9.83，P=0.005）（图3d），高糖和LPS表现为轻微的拮抗作用（CI=1.19）。p-IRF3在四组间无显著差异（P=0.072），只有HG+LPS组略升高（P=0.053）（图3e）。说明高糖和LPS激活了HGFs中cGAS-STING通路，引起STING及下游TBK1、P65相关炎症反应增强，高糖和LPS共同刺激下，通路激活进一步增强。

2.4　高糖环境加重*P.g*-LPS引起的HGFs细胞炎症反应

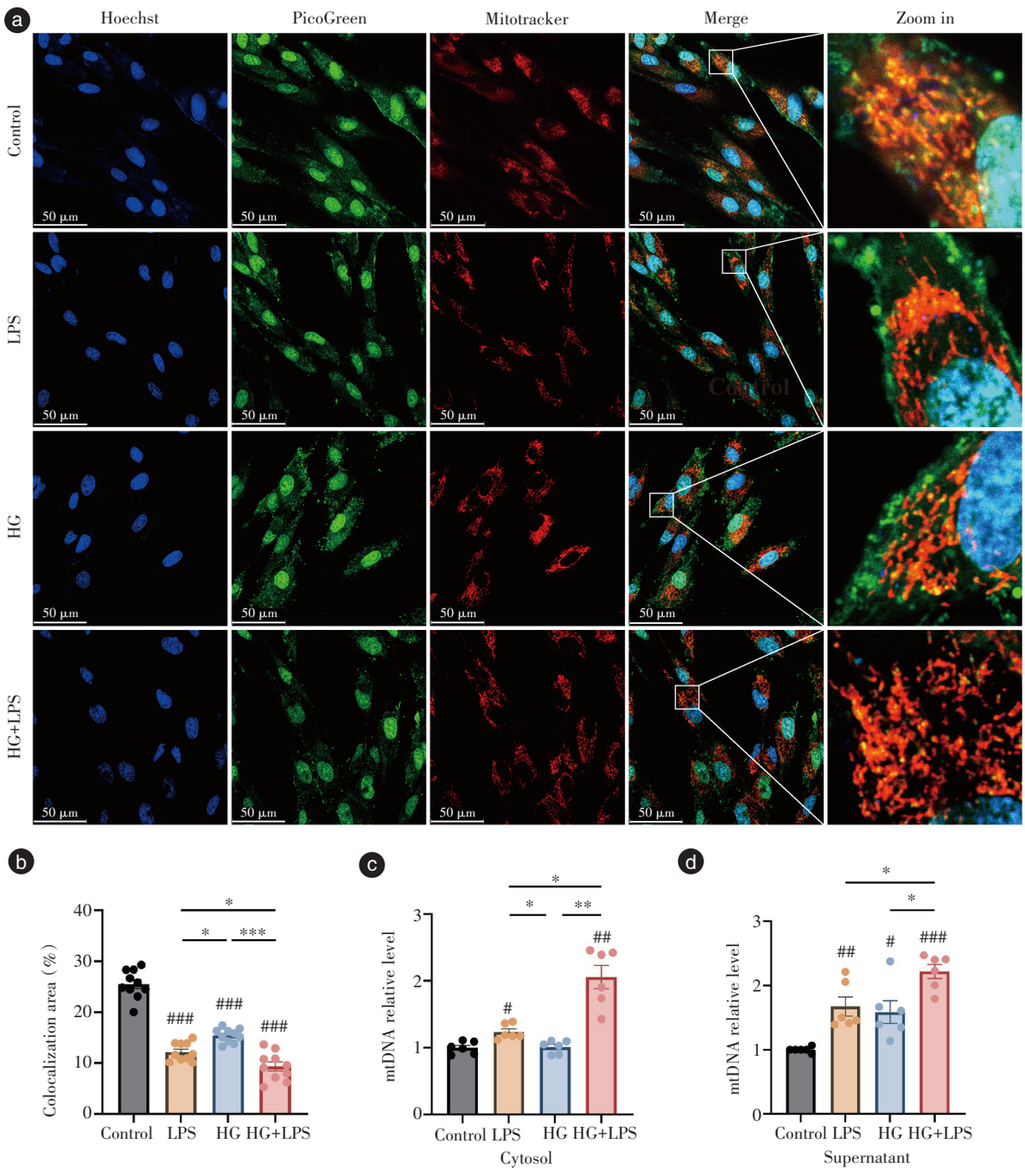
实时定量PCR结果显示，促炎细胞因子IL-1β的mRNA水平在LPS组和HG组均有显著升高，在HG+LPS组进一步协同升高（F = 30.98，P < 0.001，CI=0.86），这与mtDNA释放的增加呈相似趋势。TNF-α的mRNA水平在HG+LPS组中表达较对照组显著升高（F = 15.05，P<0.001），高糖与LPS在该过程中表现出强烈的协同作用（CI = 0.21）。此外，4组间IL-6的mRNA水平也未观察到显著差异（P=0.847）（图4）。

3　讨　论

本研究旨在深入探讨高糖环境与*P.g*-LPS联合刺激下HGFs的炎症放大机制。其中*P.g*-LPS浓度参考既往体外实验有关HGFs线粒体功能的相关研究[19]，高糖浓度参考既往有关糖尿病状态下颌面部疾病的相关体外研究中普遍使用的葡萄糖浓度[5, 22-23]。本研究结果表明，高糖环境和*P.g*-LPS双重刺激可协同诱导线粒体氧化应激，伴随mtDNA释放至胞质和胞外，激活cGAS-STING通路，驱动NF-κB活化及下游促炎细胞因子的表达升高。这为阐明糖尿病加剧牙周炎的机制提供了细胞层面的新视角。

线粒体功能障碍是牙周炎发病机制的一个重要组成部分[24-25]，本课题组既往已经在牙周炎患者HGFs及LPS刺激的健康HGFs中发现了线粒体结构功能异常以及mtDNA释放现象，并证实其由ROS介导的线粒体通透性转换孔开放所触发[19, 26-27]。既往研究也表明牙周炎患者存在mtDNA突变和缺失[28]。与之相似的，高血糖诱导的氧化应激可以通过线粒体功能障碍破坏细胞修复机制[6, 29]，大量糖尿病并发症研究也发现心血管[30]、肾脏[31]、视网膜[32]等细胞的线粒体结构功能受损。有证据表明糖尿病牙周炎大鼠牙龈中存在线粒体功能障碍[33]。本研究结果从细胞学角度佐证这一观点。本研究发现，高糖环境显著加剧了*P.g*-LPS诱导的HGFs氧化应激水平，并使mtDNA释放现象更为显著。这表明持续的高血糖代谢紊乱与局部的感染协同作用，共同放大并延续了由mtDNA介导的免疫炎症反应，从而从细胞层面阐释了糖尿病患者牙周组织破坏更为严重的潜在原因。

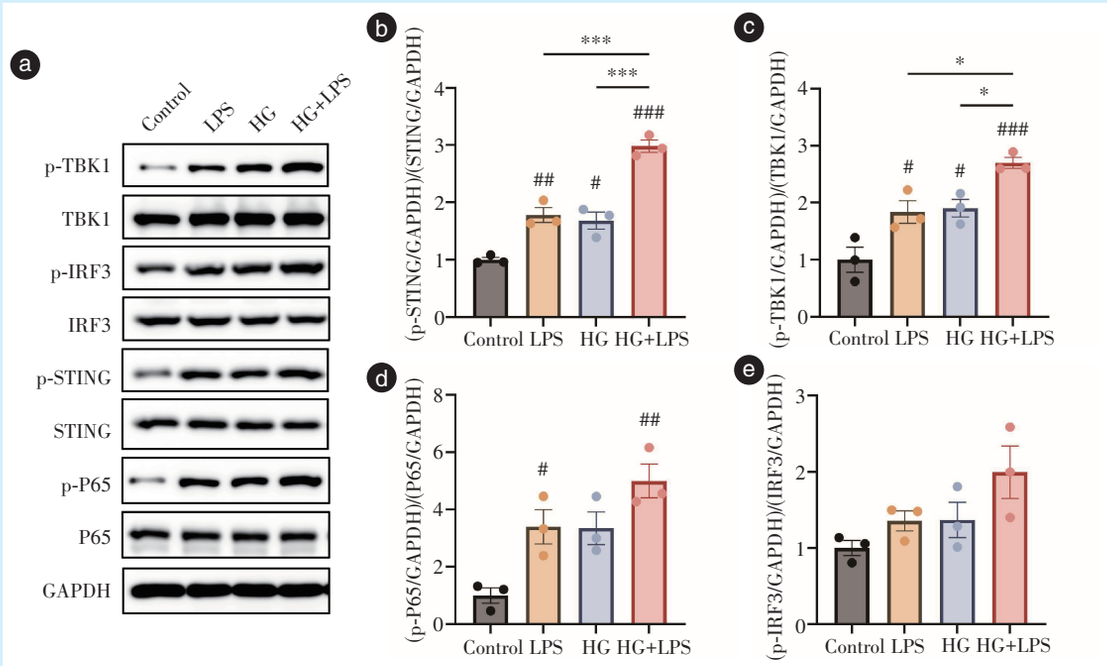
mtDNA具有与微生物相似的DNA序列，正常情况下分布于线粒体中，当mtDNA释放出线粒体，将被视为外源性异物[34]。已有的研究认为mtDNA作为损伤相关模式分子可以与先天性免疫系统的模式识别受体相结合，激活多种炎症通路[8]。其中，cGAS-STING通路近年来被认为是感知内源性危险信号（如mtDNA）的核心传感器[9]，在糖尿病相关并发症中也得到了广泛的研究[14-15]。有研究表明，在心肌细胞中高糖可导致发生线粒体氧化损伤，并通过激活cGAS-STING通路加剧炎症反应[35]；外源性提取的mtDNA也足以直接激活该细胞中的cGAS-STING信号及其下游炎症反应[36]，支持mtDNA作为内源性危险信号直接参与免疫激活



a: representative immunofluorescence images of HGFs showing the co-localization of PicoGreen (green) and Mitotracker (red). Blue = Hoechst; Yellow = Merge. Scale bar = 50 μm. b: the ratio of yellow fluorescence signal intensity to red signal intensity was calculated in randomly selected intact cells from each group to indicate mtDNA within mitochondria. n = 10. c & d: HGFs were stimulated with HG and LPS for 24 hours. mtDNA levels in the cytosol and supernatant were measured and normalized for statistical analysis. n = 6.*: P<0.05, **: P<0.01, ***: P<0.001; #: compared to the control group, P<0.05; ##: compared to the control group, P<0.01, ###: compared to the control group, P<0.001. Control group: no treatment; LPS group: treated with 5 μg/mL *P.g*-LPS; HG group: treated with 25 mmol/L glucose; HG+LPS group: co-treated with 5 μg/mL *P.g*-LPS and 25 mmol/L glucose. HGFs: human gingival fluorescent fibroblasts; LPS: lipopolysaccharide; *P.g: Porphyromonas gingivalis;* mtDNA: mitochondrial DNA

Figure 2　High-glucose environment enhances the release of mtDNA in HGFs induced by *P.g*-LPS

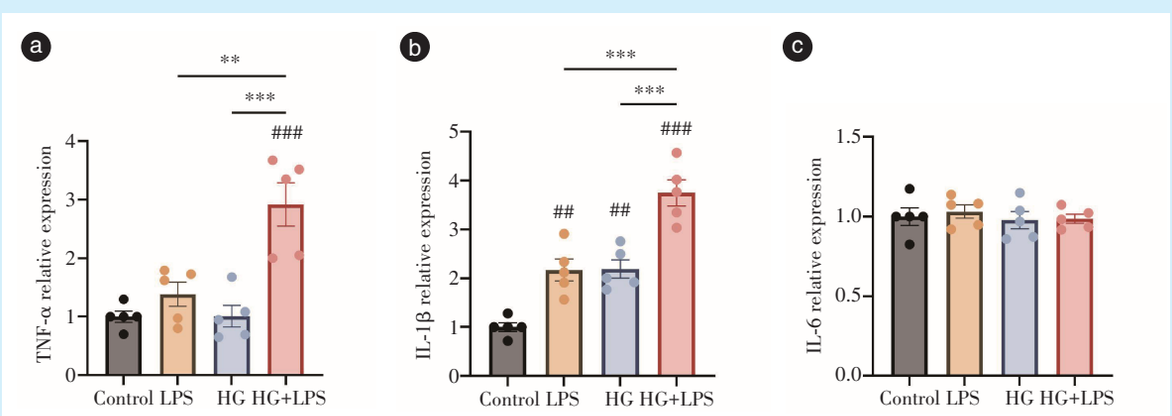
图2　高糖环境增强 *P.g*-LPS引起的HGFs中mtDNA的释放



a: representative Western blot images showing protein expression levels of key components in the cGAS-STING pathway in HGFs. b-e: quantitative analysis of p-STING, p-TBK1, p-P65 and p-IRF3 protein levels normalized to GAPDH. n = 3. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$; #: compared to the control group, $P < 0.05$; ##: compared to the control group, $P<0.01$, ###: compared to the control group, $P < 0.001$. Control group: no treatment; LPS group: treated with 5 μg/mL *P.g*-LPS; HG group: treated with 25 mmol/L glucose; HG+LPS group: co-treated with 5 μg/mL *P.g*-LPS and 25 mmol/L glucose. HGFs: human gingival fluorescent fibroblasts; LPS: lipopolysaccharide; p-STING: phosphorylated stimulator of interferon genes; p-TBK1: phosphorylated TANK-binding kinase 1; p-P65: phosphorylated NF-κB P65; p-IRF3: phosphorylated interferon regulatory factor 3; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; cGAS: cyclic GMP-AMP synthase; STING: stimulator of interferon genes

Figure 3　High-glucose environment enhances the activation of cGAS STING signaling pathway in HGFs induced by *P.g*-LPS

图 3　高糖环境增强 *P.g*-LPS 引起的 HGFs 中 cGAS-STING 信号通路的激活



a-c: relative mRNA expression levels of (a) TNF-α, (b) IL-1β, and (c) IL-6 in HGFs after challenge with high glucose and LPS. Gene expression was determined by qPCR and normalized to β-actin. n = 5.*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$; #: compared to the control group, $P < 0.05$; ##: compared to the control group, $P<0.01$, ###: compared to the control group, $P < 0.001$. Control group: no treatment; LPS group: treated with 5 μg/mL *P.g*-LPS; HG group: treated with 25 mmol/L glucose; HG+LPS group: co-treated with 5 μg/mL *P.g*-LPS and 25 mmol/L glucose. HGFs: human gingival fluorescent fibroblasts; LPS: lipopolysaccharide; *P.g: Porphyromonas gingivalis;* TNF-α: tumor necrosis factor alpha; IL-1β: interleukin-1β; IL-6: interleukin-6; β-actin: beta-actin.

Figure 4　High-glucose environment exacerbates the inflammatory response of HGFs cells induced by *P.g*-LPS

图 4　高糖环境加重 *P.g*-LPS 引起的 HGFs 细胞炎症反应

的观点。在牙周组织细胞中，高糖同样可调控类似通路。既往研究显示，高糖通过激活NF-κB信号上调牙周膜细胞中核因子κ-B配体受体致活剂（receptor activator ofnuclear factor kappa-B ligand，RANKL）表达，促进牙周炎导致的骨吸收[22]；近期还有研究发现，高糖炎症微环境可导致牙周膜干细胞发生线粒体功能障碍，并伴随cGAS-STING-TBK1-P65轴激活，进而放大炎症应答[5]。本研究中发现，在高糖和LPS的双重刺激下，mtDNA的释放增加，可能导致cGAS-STING通路被过度激活，引起下游TBK1和P65磷酸化增强，驱动了NF-κB介导的促炎细胞因子TNF-α和IL-1β的分泌，增强局部炎症反应。这些证据共同表明，高糖可能通过诱导线粒体损伤与mtDNA释放，进而激活cGAS-STING与NF-κB通路并驱动炎症级联反应。该信号轴在不同细胞中均有发现，暗示其在糖尿病相关牙周微环境中可能广泛参与炎症调控；其中，mtDNA-cGAS-STING通路尤其可能作为上游事件，在一定程度上放大了牙周组织的炎症应答。

本研究采用CI模型量化了高糖与LPS在不同层面的相互作用。结果显示，两者的协同效应具有明显的通路特异性和层级性。在诱导氧化应激这一上游事件中，观察到稳定的协同作用（CI < 1）。然而，在mtDNA免疫荧光共定位和下游特定信号蛋白的磷酸化水平上，则出现了拮抗效应（CI > 1）。关于免疫荧光，本研究染料无法标记破裂的线粒体[37]，这会在共染比例分析中产生误差。关于通路蛋白的激活情况，笔者推测这可能是由于本研究使用的单一刺激浓度已近乎饱和，无法产生叠加效应。这些发现共同表明，高糖与LPS的相互作用形成了一个复杂的调控网络，而非简单的线性叠加，其最终对牙周炎症的作用，是这些协同与拮抗过程整合后的结果，未来的研究可以采用多剂量梯度和非线性模型将进一步精确量化其协同效应。

本研究结果显示，高糖与LPS刺激可显著激活HGFs中cGAS-STING通路关键节点p-STING及其下游信号分子p-TBK1与p-P65，而p-IRF3的活化略有升高却未达统计学意义。该现象提示，在牙周炎病理背景下，cGAS-STING信号可能更倾向于通过NF-κB轴驱动炎症反应[5, 22, 38]，IRF3介导的干扰素通路激活相对有限。值得注意的是，尽管NF-κB通路被激活，其下游因子IL-6的转录水平并未同步升高。这一信号与表型解耦的可能原因有：NF-κB的充分活化需要其他转录因子的协同作用，在缺乏共刺激信号的情况下，IL-6的转录响应可能受限[39]；同时，高糖或炎症微环境可能通过转录后调控机制影响IL-6 mRNA的稳定性，从而削弱其表达积累。在糖尿病合并牙周炎动物模型中观察到的IL-6升高可能源于更复杂的体内微环境[40]，其中AGEs的积累会通过晚期糖基化终末产物受体（receptor for advanced glycation end-products，RAGE）信号通路促进IL-6生成[41]。此外，在完整的牙周组织中，上皮细胞及浸润的免疫细胞也可能是IL-6的来源[42-43]，这进一步解释了单一类型HGFs在体外培养条件下与体内组织反应之间的差异。

本研究还存在一定的局限性。研究聚焦于体外细胞水平，虽然能够精确控制变量并阐明机制，但无法完全模拟体内复杂的免疫细胞—间质细胞互作环境，并且目前的结果尚未进行干预实验（例如使用STING抑制剂或ROS清除剂[44-45]）。未来计划在细胞模型中通过血糖波动模型更好地模拟体内环境，并完善mtDNA-cGAS-STING-炎症的因果链，随后在动物模型中验证该通路的关键作用。

综上所述，本研究基于课题组前期对牙周炎中mtDNA释放现象的发现，进一步在糖尿病牙周炎背景下揭示高糖环境与LPS协同加剧氧化应激，并伴随mtDNA释放增加，激活cGAS-STING通路，从而显著放大HGFs的炎症反应。该过程提示，氧化应激与mtDNA释放共同参与了高糖环境与LPS加剧牙周局部炎症的分子机制。为理解糖尿病加剧牙周炎的作用机制提供了新的视角，并为靶向治疗策略提供了理论依据。

【Author contributions】 Geng YR designed the study, performed experiments, analyzed the data and drafted the manuscript. Zang XY performed the experiments. Liu J and Luan QX designed the study and revised the article. All authors read and approved the final manuscript as submitted.

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