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· 基础研究 ·

释氧光动力疗法对根管内粪肠球菌清除效果的体外研究

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【摘要】目的 探讨光敏剂亚甲基蓝(methylene blue, MB)释氧配方光动力疗法(photodynamic therapy, PDT)对根管内粪肠球菌的清除效果。**方法** 分为MB组和释氧MB组, MB组采用50 μmol/L MB溶液, 释氧MB组采用50 μmol/L MB混合在释氧乳液中, 利用N-乙酰-L-色氨酸酰胺(N-acetyl-L-tryptophan amide, NATA)及1,3-二苯基异苯并呋喃(1, 3-diphenylisobenzofuran, DPBF)分别检测MB组、释氧MB组的氧化活性及单线态氧的产生, 分析释氧光敏剂配方的释氧能力。收集单根管前牙, 构建离体牙的粪肠球菌根管感染模型后, 扫描电镜观察粪肠球菌在根管中的分布。将粪肠球菌根管感染离体牙分为三组: 常规根管治疗组、MB-PDT治疗组、释氧MB-PDT治疗组, 每组10个, 获取根管内细菌样本后, 于体外培养24 h计算菌落数(CFU/mL), 分析PDT对根管内粪肠球菌的清除率。将余下粪肠球菌根管感染离体牙分为三组, 每组5个样本, 分别获取根上、根中、根下三段牙本质小管中1 000 μm深度的细菌样本, 体外培养24 h计算菌落数(CFU/mL), 分析各组根管不同部位牙本质小管粪肠球菌清除率。**结果** 释氧MB组的氧化活性及单线态氧的产生能力均强于MB组。扫描电镜可见粪肠球菌分布于牙本质小管, 表明体外根管感染模型构建成功。释氧MB-PDT治疗组粪肠球菌清除率高于常规根管治疗组和MB-PDT治疗组($P < 0.05$)。释氧PDT明显改善了根下段牙本质小管的杀菌效果, 粪肠球菌清除率优于另外两组($P < 0.05$)。**结论** 利用释氧MB进行PDT可以获得更好的根管杀菌效果, 并且可以显著提高根下段牙本质深层的粪肠球菌清除效果。

【关键词】 光动力疗法; 释氧光敏剂; 亚甲基蓝; 活性氧; 单线态氧; 根管消毒; 牙髓感染; 粪肠球菌; 离体牙



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An *in vitro* study on the clearance effect of oxygen-releasing photodynamic therapy on *Enterococcus faecalis* in the root canal YANG Guofeng¹, CHEN Weiting². 1. Shanghai Stomatological Hospital, Shanghai Key Laboratory of Craniomaxillofacial Development and Diseases, Fudan University, Shanghai 200001, China; 2. Shanghai Xuhui District Dental Center, Shanghai 200032, China

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【Abstract】 Objective To investigate the clearance effect of photodynamic therapy (PDT) with an oxygen-releasing photosensitizer methylene blue (MB) formula on *Enterococcus faecalis* in the root canal. **Methods** Groups were divided into the MB group and oxygen release MB group. Samples in the MB group were mixed with 50 μmol/L MB solution, and samples in the oxygen release MB group were mixed with 50 μmol/L MB in oxygen release emulsion. N-acetyl-L-tryptophan amide (NATA) and 1,3-diphenylisobenzofuran (DPBF) were used to detect the oxidative activity and singlet oxygen generation of different formulas of MB (MB group, oxygen-releasing MB group), and the oxygen-releasing capaci-

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ty of the oxygen-releasing photosensitizer formula was analyzed. The single root canal anterior teeth were collected, and the *Enterococcus faecalis* root canal infection model of isolated teeth was constructed. The distribution of *Enterococcus faecalis* in the root canal was observed by scanning electron microscopy. The isolated teeth with *Enterococcus faecalis* root canal infection were divided into three groups: the conventional root canal therapy group, MB-PDT therapy group, and oxygen-releasing MB-PDT therapy group, with 10 teeth in each group. Bacterial samples in the root canals of each group were obtained, the number of colonies was analyzed(CFU/mL) after 24 h of *in vitro* culture, and the clearance rate of *Enterococcus faecalis* in the root canal by photodynamic therapy was evaluated. The remaining isolated teeth with *Enterococcus faecalis* root canal infection were divided into 3 groups, with 5 samples in each group. Bacterial samples at a depth of 1 000 μm in the dentin tubules of the upper root, middle root and lower root segment were obtained and cultured *in vitro* for 24 h for calculations. The number of colonies (CFU/mL) was analyzed, and the clearance rate of *Enterococcus faecalis* in the dentin tubules of different segments of the root canals in each group was analyzed. **Results** The oxidation activity and singlet oxygen generation capacity in the oxygen-releasing MB group were stronger than those in the MB group. Scanning electron microscope detection showed that *Enterococcus faecalis* was distributed in dentinal tubules, indicating that the model of root canal infection *in vitro* was successfully constructed. The clearance rate of *Enterococcus faecalis* in the oxygen-releasing MB-PDT therapy group was higher than that in the conventional therapy group and the MB-PDT therapy group ($P < 0.05$). Oxygen-releasing PDT significantly improved the bactericidal effect in the lower dentinal tubules, and the clearance rate of *Enterococcus faecalis* was better than in the other two groups ($P < 0.05$). **Conclusion** Photodynamic therapy with the oxygen-releasing photosensitizer MB can achieve a better root canal bactericidal effect and can significantly improve the clearance effect of *Enterococcus faecalis* in the deep dentin of the lower root segment.

[Key words] photodynamic therapy; oxygen-releasing photosensitizer; methylene blue; reactive oxygen; singlet oxygen; root canal disinfection; dental pulp infection; *Enterococcus faecalis*; isolated tooth

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[Competing interests] The authors declare no competing interests.

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根管内的持续性感染未得到消除是根管治疗失败的主要原因,主要由粪肠球菌等顽固性致病菌引起^[1-2]。单独使用机械仪器预备无法彻底清理根管系统,光动力疗法(photodynamic therapy, PDT)可杀灭根管内细菌,在根管消毒、辅助控制根管感染中效果较好^[3]。PDT在根管内发挥杀菌效果,需要激光、光敏剂、氧三个重要因素共同作用,即光敏剂在根管内均匀扩散后,通过激光一定波长的照射,在氧气的参与下,产生活性氧和单线态氧,从而起到杀菌作用^[4]。受自身解剖结构影响,根管内部及牙本质小管深处不可避免存在氧不足的情况,影响了PDT治疗效果^[4-6]。常用光敏剂亚甲基蓝(methylene blue, MB)的光照波长为660 nm,对粪肠球菌杀菌效果较好,对MB配方进行改性后可进一步提高PDT杀菌能力,但目前对释氧配方光敏剂的研究仍较少^[5-7]。本研究拟在前期研究的基础上,通过构建单根管离体前牙粪肠球菌根管感染

模型,观察释氧MB配方的PDT对根管内粪肠球菌的杀菌效果,为临床应用提供参考。

1 材料和方法

1.1 主要仪器和材料

粪肠球菌标准菌株(ATCC29212, ATCC, 美国),脑-心浸液(brain heart infusion, BHI)(海博,中国),戊二醛溶液(上海生工,中国),PBS(上海生工,中国),1%次氯酸钠(朗力生物,中国),生理盐水冲洗液(双鹤,中国),MB(Sigma, 美国),全氟十氢化萘(Sigma, 美国),H₂O₂(遂成,中国),Triton-X100(上海生工,中国),N-乙酰基-L-色氨酸酰胺(N-acetyl-L-tryptophan amide, NATA)(Sigma, 美国),1,3-二苯基异苯并呋喃(1,3-diphenylisobenzofuran, DPBF)(Sigma, 美国)。

扫描电镜(S3400, 日立,日本),ProTaper机用马达和锉(登士柏,瑞士),光动力口腔治疗仪



(HHL-1 000, 呵威, 加拿大), 液相色谱仪(LC-20A, 岛津, 日本), 分光光度计(UV-2600, 岛津, 日本)。

1.2 不同配方 MB 的光氧化活性和单线态氧产生速率测定

1.2.1 分组 参照文献所述^[8], 分为 MB 组和释氧 MB 组, MB 组采用 50 μmol/L MB 溶液, 释氧 MB 组采用 50 μmol/L MB 混合在释氧乳液中(全氟十氟化萘:H₂O₂:Triton-X100=75:24.5:0.5)^[7]。

1.2.2 光氧化活性测定 浓度为 10 μmol/L 的荧光测定模型底物 NATA 与不同测试配方中的 50 μmol/L MB 混合(总体积 3 mL)。采用 660 nm 波长, 30 mW 功率辐照测试溶液诱导氧化剂的产生, 激光尽量接近测试液体表面而不接触。利用液相色谱仪测量 NATA 浓度在 290 nm 波长随着辐照时间增加的下降速率, NATA 浓度的下降速率代表了 MB 或释氧 MB 的光氧化活性。检测间隔为 5 min, 检测 20 min。所有实验重复 3 次。

1.2.3 单线态氧产生速率测量 浓度为 100 μmol/L 的单线态氧清除剂 DPBF 与不同测试配方中的 50 μmol/L MB 混合(总体积 3 mL)。利用光度计测定 DPBF 浓度在 420 nm 波长随着辐照时间增加而降低的速率, 分析不同配方 MB 单线态氧产生的速率, 检测间隔为 5 min, 检测 20 min。所有实验重复 3 次。

1.3 粪肠球菌根管感染模型的构建

粪肠球菌的根管感染模型构建参考以往文献报道^[9], 具体如下。获取上海市口腔医院口腔颌面外科临床拔除的 63 个根尖发育完成、单根管离体牙(审批号:沪口防伦审[2020]014 号), 将牙根表面清理干净, 修整牙体, 保留牙根 9 mm 的长度。采用 ProTaper 镍钛机械进行常规根管预备, 根管冲洗液交替冲洗根管, 冲出碎屑。离体牙根清理完成后, 121 °C 高温高压消毒 20 min, 杀灭牙根内微生物。随后, 将灭菌后的离体牙根, 置于粪肠球菌 BHI 肉汤中进行培养 3 周, 细菌浓度为 10⁸ CFU/mL, 每 3 d 更换 1 次培养基。培养 3 周后, 取其中 3 个样本, 采用高速涡轮手机沿牙体长轴纵向磨除沟槽, 用骨凿沿沟槽将牙体纵向劈开, 用 2.5% 戊二醛固定后, 脱水、干燥、喷金, 随后采用扫描电子显微镜观察根管内粪肠球菌的分布, 确认根管感染模型构建成功。

1.4 光动力疗法对根管内粪肠球菌的清除效果

取粪肠球菌根管感染的离体牙根 30 个, 随机分为常规根管治疗组、MB-PDT 治疗组和释氧 MB-PDT 治疗组, 每组 10 个离体牙根。同时另取 10 个

离体牙根不做任何处理, 获取根管内样本作为细菌测定基线。常规根管治疗组采用 1% 次氯酸钠溶液常规处理根管 1 min; MB-PDT 治疗组采用 1% 次氯酸钠溶液处理根管后, 注入 50 μmol/L MB 溶液静置 1 min, 采用光动力治疗仪照射 1 min(660 nm, 30 mW), 重复此步骤 1 次; 释氧 MB-PDT 治疗组采用 1% 次氯酸钠溶液处理根管后, 注入 50 μmol/L MB 释氧乳化溶液静置 1 min, 采用 660 nm 激光照射 1 min, 重复此步骤 1 次。

利用无菌纸尖在根管内取样, 静置 60 s 后, 放入盛有 1 mL 无菌水的无菌离心管中并充分振荡 10 s。将细菌样本在 BHI 肉汤培养基中 37 °C 下培养。样本在孵育 24 h 后, 分别检测每个培养皿上菌落形成情况并进行细菌计数。将未处理样本的细菌数作为计算基线, 按下列公式计算各组细菌的清除率:

$$\text{细菌清除率}(\%) = (M-N)/M \times 100\%$$

其中 M 为基线细菌数, N 为处理后的细菌数。实验及计算方法参考文献[9]。

1.5 光动力疗法对不同根管部位牙本质小管内粪肠球菌的清除效果

取粪肠球菌根管感染的离体牙根 15 个, 随机分为常规根管治疗组、MB-PDT 治疗组和释氧 MB-PDT 治疗组, 每组 5 个离体牙根, 同时另取 5 个离体牙根不做任何处理, 获取根管内不同部位牙本质小管内样本作为细菌测定基线。将牙根沿牙体长轴纵向劈开, 将牙根三等份分为根上、根中、根下三段, 采用小球钻(直径 1 mm)在根上、根中、根下三段牙根部位的中心部位获取深度 1 000 μm 的牙本质碎屑, 为保证每个部位收集的牙本质碎屑尽可能保持一致, 应在球钻头部刚好完全没入根管内壁时停止取样, 同时为了避免产热过多, 应该间断钻磨, 随后用无菌水将碎屑转移到离心管中并充分振荡 10 s。

每组根管内处理方法及细菌培养计数方法同 1.4 所述。实验及计算方法参考文献[9]。

1.6 统计学方法

采用 SPSS 20.0 软件(IBM, 美国)进行数据分析, 数据用 $\bar{x} \pm s$ 表示, 多组间比较采用方差分析, 两两比较采用 t 检验, 检验水准 $\alpha = 0.05$ 。

2 结 果

2.1 各组光氧化活性和单线态氧产生速率

NATA 浓度的下降速率代表了光氧化活性。 $10 \text{ min}(t = 3.570, P = 0.023)$ 、 $15 \text{ min}(t = 3.507, P =$

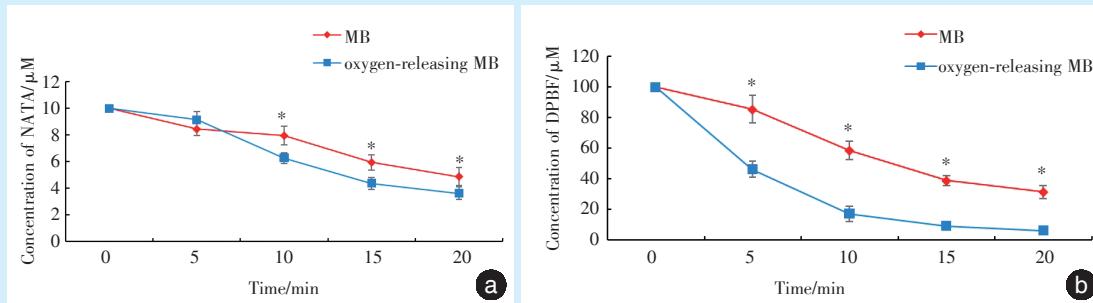
0.025)、20 min ($t = 2.904, P = 0.044$)时,释氧 MB 组 NATA 浓度的下降速率高于 MB 组下降速率($P < 0.05$)(图 1a)。

DPBF 浓度的下降速率代表了单线态氧产生的速率。5 min ($t = 6.453, P = 0.003$)、10 min ($t = 9.166, P = 0.001$)、15 min ($t = 15.600, P < 0.001$)、20 min ($t = 10.180, P = 0.001$)时,释氧 MB 组中

DPBF 的下降速率高于 MB 组中下降速率($P < 0.05$)(图 1b)。

2.2 粪肠球菌感染根管模型的建立

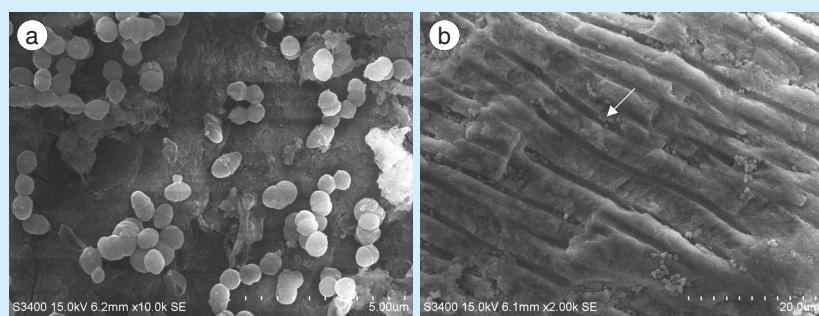
经过 3 周的培养,采用扫描电子显微镜观察根管壁表面,可以见到根管表面大量圆形的粪肠球菌附着,并侵入到牙本质小管内部,说明根管感染模型成功建立(图 2)。



a: the decreasing rate of NATA in oxygen-releasing MB group was higher than that in MB group ($P < 0.05$); b: the decreasing rate of DPBF in oxygen-releasing MB group was higher than that in MB group ($P < 0.05$); $n = 3$, *: $P < 0.05$. MB: methylene blue; NATA: N-acetyl-L-tryptophan amide; DPBF: 1, 3-diphenylisobenzofuran

Figure 1 Singlet photooxidation activity and oxygen generation capacity determination in MB group and oxygen-releasing MB group

图 1 MB 组和释氧 MB 组光氧化活性和单态氧产生能力测定



a: a large number of round *Enterococcus faecalis* on the surface of the root canal can be seen ($\times 10000$); b: a large number of *Enterococcus faecalis* invading the dentin tubules ($\times 2000$) (arrow: *Enterococcus faecalis* in the dentin tubules)

Figure 2 Scanning electron microscopic observation of root canals infected by *Enterococcus faecalis*

图 2 扫描电镜观察粪肠球菌感染的根管

2.3 各组根管内粪肠球菌清除率

常规根管治疗组根管内粪肠球菌清除率为($90.43 \pm 1.43\%$),MB-PDT治疗组为($95.95 \pm 0.77\%$),释氧 MB-PDT 治疗组为($98.57 \pm 1.19\%$),三组根管内粪肠球菌的清除率差异具有统计学意义($F=16.5, P < 0.05$),释氧 MB-PDT 组粪肠球菌清除率高于 MB-PDT 治疗组和常规根管治疗组,MB-PDT 治疗组高于常规根管治疗组,差异均具有统计学意义($P < 0.05$)。

2.4 不同根管部位牙本质小管内粪肠球菌清除率

三组根管不同部位(根上、根中、根下段)牙本

质小管内粪肠球菌清除率如表 1 所示。根上段,三组清除率差异无统计学意义($F = 33.7, P > 0.05$);根中段,释氧 MB-PDT 组清除率高于常规根管治疗组,差异具有统计学意义($P < 0.05$);根下段,MB-PDT 治疗组清除率高于常规根管治疗组,释氧 MB-PDT 组高于常规根管治疗组和 MB-PDT 治疗组,差异均有统计学意义($P < 0.05$)。

3 讨 论

粪肠球菌是一种革兰氏阳性兼性厌氧菌,在根管内可形成生物膜,在牙本质小管内的潜行深



表1 不同根管部位牙本质小管内粪肠球菌清除率测定

Table 1 Determination of the clearance rate of *Enterococcus faecalis* in dentin tubules of different sections $\bar{x} \pm s, \%$

Group	n	Clearance rate of <i>Enterococcus faecalis</i>		
		Upper	Middle	Lower
Root canal therapy	5	97.47 ± 1.04	92.48 ± 2.83	85.44 ± 1.43
MB-PDT therapy	5	99.39 ± 0.87	95.73 ± 1.95	91.94 ± 2.47 ^a
Oxygen-releasing MB-PDT therapy	5	98.67 ± 1.18	98.96 ± 0.78 ^a	97.87 ± 1.14 ^{a,b}

a: compared with root canal therapy group, $P < 0.05$; b: compared with MB-PDT therapy group, $P < 0.05$. MB: methylene blue. PDT: photodynamic therapy

度可达1 000 μm,能够在较为恶劣的条件下生存,可以耐受根管内pH值、温度和氧张力的巨大变化,具有较强的耐药性^[10]。由于根管结构的复杂性以及粪肠球菌的特点,常规根管治疗方法仍然存在一定的局限性,无法完全清除根管内的粪肠球菌感染^[5]。PDT作为一种新型的根管消毒方法,具有诸多优点^[11]:可以杀伤病原微生物而不对正常的人体细胞产生影响;没有激光治疗产热过多损伤牙周组织的问题;光谱杀菌,无耐药性;杀菌能力较为安全高效(无次氯酸钠的毒性)等。但是根管内氧不足的特点大大影响了PDT的根管消毒能力。改善根管内的氧环境,可以更好提高PDT在根管消毒中的作用。研究发现,利用氧载体混合H₂O₂释氧的配方,提高了根管消毒效果^[7,12]。本研究采用了离体牙粪肠球菌根管感染模型,利用此配方进一步研究了PDT在根管消毒中的作用。

本研究中,释氧MB组中的氧化活性及单线态氧的产生能力均强于MB组,提示该配方进一步改善了反应中的内在氧环境,对后续PDT治疗中进一步提高杀灭微生物的效果具有重要意义,与前期研究结果一致^[7]。研究发现,PDT与1%次氯酸钠联用可以起到较好的效果^[6,13],同时重复两次PDT治疗的效果要明显优于单次PDT治疗的效果,杀菌率可以有效提高到95%左右^[14]。因而,本研究在应用1%次氯酸钠根管消毒的基础上,采用了两次PDT治疗进行研究。结果显示,MB-PDT治疗组的粪肠球菌清除率(95.95 ± 0.77)%高于常规根管治疗组,释氧MB-PDT治疗组的粪肠球菌的清除率(98.57 ± 1.19)%得到进一步提高;表明改善根管内氧环境后,提高了PDT治疗对根管内粪肠球菌的杀菌能力。

粪肠球菌可以潜伏至牙本质小管内1 000 μm的深度,常规根管消毒采用的次氯酸钠等消毒溶液的渗透能力无法达到此深度^[15],同时因为根管结构形态的复杂性(如根下段根管分歧、侧枝等),进一步影响了根管消毒效果^[16]。PDT治疗中,MB

溶液能更好渗透进入牙本质小管深层^[7],以及自由氧在根管内不同位置充分释放均是其发挥消毒作用的关键因素。因而本研究进一步研究了释氧MB应用于PDT治疗在根上、根中、根下不同部位的清除效果差异。结果显示,各组根上段粪肠球菌清除率差异无统计学意义;在根中段,释氧MB-PDT组的粪肠球菌清除率高于常规根管治疗组,但与MB-PDT治疗组无明显差异;在根下段,MB-PDT治疗组的粪肠球菌清除率(91.94 ± 2.47)%高于常规根管治疗组(85.44 ± 1.43)%,释氧MB-PDT组粪肠球菌清除率(97.87 ± 1.14)%高于MB-PDT治疗组。提示释氧PDT主要改善了根下段内氧环境,并进一步提高了PDT对根管内粪肠球菌的辅助清除效果。

综上,PDT可以辅助根管治疗,提高常规根管治疗中根管内粪肠球菌清除率,释氧PDT通过提高根管内自由氧的含量,可进一步提高PDT的杀菌能力,且对于根下段部分效果更加明显。

[Author contributions] Yang GF designed the study, performed the experiments, analyzed the data, and wrote the article. Chen WT assisted in the writing of the article. All authors read and approved the final manuscript as submitted.

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