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· 基础研究 ·

# 甘氨酸引导羧甲基壳聚糖/无定形磷酸钙再矿化脱矿牙釉质表面的研究

郭宏磊, 张凯, 张旭

天津医科大学口腔医院牙周科, 天津(300070)

**【摘要】** 目的 探讨甘氨酸引导羧甲基壳聚糖(carboxymethyl chitosan, CMC)/无定形磷酸钙(amorphous calcium phosphate, ACP)对脱矿牙釉质表面的再矿化作用。方法 制备不同阶段再矿化液:①反应态 CMC/ACP (NaClO 处理的 CMC/ACP), ②反应态 CMC/ACP+甘氨酸;透射电子显微镜检测再矿化液颗粒形貌。选取 20 颗阻生齿制成釉质切片并脱矿,随机分为 A、B 两组,将反应态 CMC/ACP 涂抹于 A 组牙釉质表面;将加入甘氨酸的反应态 CMC/ACP 再矿化液涂抹于 B 组。扫描电子显微镜检测再矿化前后牙釉质表面形貌,纳米压痕实验检测牙釉质表面机械强度(包括压刻深度、硬度和弹性模量)。结果 透射电镜下观察,反应态 CMC/ACP 再矿化液颗粒光滑,粒径增加至 100~300 nm。反应态 CMC/ACP 加入甘氨酸后颗粒呈现出线状有序排列,15 min 后溶液内形成了微晶体,晶体长度约为 5~15  $\mu\text{m}$ 。A 组再矿化表现为颗粒状,且为杂乱的均质再矿化层,B 组脱矿牙釉质表面形成了较为均质的晶体形貌且排列有序,与天然牙釉质晶体形貌类似。B 组再矿化后压刻深度小于 A 组,也最接近天然牙釉质;B 组再矿化后的表面硬度和弹性模量与天然牙釉质差异无统计学意义。结论 通过在 NaClO 处理的 CMC/ACP 纳米颗粒中加入甘氨酸而形成的快速牙釉质再矿化模型,能够在釉质表面形成定向有序且快速的再矿化,再矿化的牙釉质表面的机械强度与天然牙釉质相近。

**【关键词】** 羟基磷灰石; 釉质龋; 再矿化; 羧甲基壳聚糖; 无定形磷酸钙; 次氯酸钠; 甘氨酸; 纳米压痕测试仪

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**Study on the remineralization of demineralized enamel surfaces with glycine-guided carboxymethyl chitosan/amorphous calcium phosphate** GUO Honglei, ZHANG Kai, ZHANG Xu. Department of Periodontology, Stomatological Hospital of Tianjin Medical University, Tianjin 300070, China

Corresponding author: ZHANG Xu, Email: zhangxu@tmu.edu.cn, Tel: 86-22-23332006

**【Abstract】 Objective** To explore effect on the remineralization of demineralized enamel surfaces with glycine-guided carboxymethyl chitosan (CMC)/amorphous calcium phosphate (ACP). **Methods** Remineralized solution at different stages were prepared: ①reactive CMC/ACP (CMC/ACP nanoparticles treated with NaClO), ②reactive CMC/ACP+glycine; transmission electron microscopy was used to detect the morphology of the remineralized solution particles. Twenty teeth were randomly divided into two groups: group A and group B. Reactive CMC/ACP was applied to the enamel surface of group A and group B was treated with reactive CMC/ACP remineralization solution containing glycine. Scanning electron microscopy was used to detect the enamel surface morphology before and after remineralization, and nanoindentation was used to detect the mechanical strength (including nanoindentation depth, hardness and elastic modulus) of the enamel surface. **Results** Under a transmission electron microscope, the particles in the reactive CMC/ACP remineralization solution were smooth, and the increase in particle size was approximately 100-300 nm. After the addition of

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**【作者简介】** 郭宏磊, 硕士, 主治医师, Email: hguo@tmu.edu.cn

**【通信作者】** 张旭, 教授, 博士, Email: zhangxu@tmu.edu.cn, Tel: 86-22-23332006



微信公众号

glycine, the particles in the reactive CMC/ACP remineralization solution particles showed a linear ordered arrangement, and microcrystals were formed in the solution 15 min later, with a crystal length of approximately 5-15  $\mu\text{m}$ . Remineralization in group A was granular and heterogeneous. In group B, the crystal morphology of the demineralized enamel was homogeneous and ordered, similar to that of natural enamel. The nanoindentation depth of group B after remineralization was smaller than that of group A, and it was closest to that of natural enamel, there was no significant difference between group B and natural enamel in terms of the hardness and elastic modulus of the enamel surface after remineralization. **Conclusion** CMC/ACP nanoparticles treated with NaClO can rapidly and specifically form directional and ordered remineralization on the enamel surface of a model of glycine-guided rapid remineralization of enamel caries. The surface structure of remineralized enamel is similar to that of natural enamel in terms of nanoindentation depth, hardness and elastic modulus.

**【Key words】** hydroxyapatite; enamel caries; remineralization; carboxymethyl chitosan; amorphous calcium phosphate; NaClO; glycine; nanoindenter

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**【Competing interests】** The authors declare no competing interests.

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早期龋坏主要表现为牙釉质表层的脱矿白垩斑而无明显龋洞形成,常见于正畸结束后的清洁不良托槽周围、酸蚀牙釉质表面以及易发生龋坏的窝沟处等<sup>[1]</sup>。其治疗方法主要包括了传统的充填治疗和非破坏性的再矿化治疗<sup>[2]</sup>。其中,再矿化治疗方法可最大程度保留牙体硬组织,减少对牙体硬组织的破坏<sup>[3]</sup>。但是目前该方法由于再矿化层较为不稳定,无法在微观上形成与天然牙釉质相近的羟基磷灰石有序排列的釉柱结构,因此导致了疗效不稳定,再矿化的效果在宏观上与天然牙釉质相差较大<sup>[4]</sup>。目前已有研究证实,壳聚糖类的衍生物,如磷酸化壳聚糖(phosphorylated chitosan, P-Chi)、羧甲基壳聚糖(carboxymethyl chitosan, CMC)均具有能够稳定钙磷离子,从而形成无定形磷酸钙(amorphous calcium phosphate, ACP)的功能<sup>[5]</sup>。此外,甘氨酸是一种简单氨基酸,对于羟基磷灰石具有一定的晶体排列作用<sup>[6]</sup>。因此,本研究结合CMC对钙、磷的稳定作用,并使用甘氨酸引导无定形磷酸钙,在脱矿牙釉质样本表面形成再矿化,探索牙釉质再矿化的新型矿化材料的体外方法及效果。

## 1 材料和方法

### 1.1 材料和设备

于天津医科大学口腔医院颌面外科完整拔除的尚未完全萌出的阻生齿20颗,本实验通过天津医科大学伦理委员会审查(审批号:TMUH-MEC20210216)。患者均知晓并签署知情同意,要

求牙体无缺损、无龋坏、无隐裂,釉质表面完整、无缺损及脱矿;CMC(海得贝生物工程有限公司,中国);磷酸氢二钾 $\text{K}_2\text{HPO}_4$ 、二水氯化钙 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 、甘氨酸、1%质量分数NaClO冲洗液(致远化学试剂有限公司,中国);37%磷酸凝胶(3M齿科材料,美国)。

低速金刚锯(蔚仪金相有限公司,中国);齿科低速手机(NSK,日本);磁力搅拌器(EMS-8C,博纳科技有限公司);透射电子显微镜(transmission electron microscopy, TEM)(jem-1230, JEOL, 日本);扫描电子显微镜(scanning electron microscope, SEM)(FE-SEM-5600 lv, JEOL, 日本);纳米压痕仪(Agilent nanoindenter G200, 安捷伦, 美国)。

### 1.2 方法

**1.2.1 再矿化液制备及TEM检测形貌** 375 mg CMC粉末缓慢加入100 mL双蒸水,以800 rpm的速度搅拌,然后再加入69.6 mg  $\text{K}_2\text{HPO}_4$ 。将117.6 mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 溶解于25 mL双蒸水中,将 $\text{CaCl}_2$ 溶液缓慢加入含有 $\text{K}_2\text{HPO}_4$ 的CMC中。最终配得的CMC/ACP溶液中Ca离子和P离子的摩尔浓度是4 mmol/L和8 mmol/L。4  $^\circ\text{C}$ 冷藏备用。取1 mL质量分数1%的NaClO溶液加入到20 mL CMC/ACP溶液,制得反应态CMC/ACP<sup>[7]</sup>。

110 kV电压下,TEM观察甘氨酸引导经NaClO处理的CMC/ACP溶液下的纳米颗粒的形态特点:CMC/ACP溶液;加入质量分数1% NaClO的CMC/ACP溶液;加入甘氨酸和质量分数1% NaClO的CMC/ACP溶液。

1.2.2 牙釉质脱矿样本的制备和再矿化实验 ①牙釉质脱矿样本的制备:20颗阻生齿,移除牙根,用金刚锯自轴面分割为面积约为 $4\text{ mm} \times 4\text{ mm} \times 0.5\text{ mm}$ (长 $\times$ 宽 $\times$ 厚)的2片釉质块并随机归组至A、B两组。超声荡洗10 min,然后用气枪吹干,37%磷酸涂布于釉质表面30 s后去除磷酸并吹干至釉质呈白垩色,用以模拟釉质龋脱矿表面。

②再矿化实验:将1.2.1中配置的反应态CMC/ACP涂抹于A组酸蚀后牙釉质表面,涂布10 min,涂布完成后置于模拟唾液中,并放置在摇床上。如此反复共计7 d。

取10 mL 1.2.1中配置的反应态CMC/ACP,加入30 mg甘氨酸固体粉末,搅拌后涂抹在B组牙釉质表面,后过程同A组相同。

③扫描电子显微镜检测再矿化前后牙表面形貌:从天然未经处理牙釉质、脱矿后牙釉质以及再矿化后各组样品中选取样品进行扫描电镜检测。样本检测前均使用乙醇梯度脱水和样品喷金处理<sup>[8]</sup>。

1.2.3 纳米压痕实验检测牙釉质表面机械强度 所有样品酸蚀处理前、酸蚀后以及7 d的再矿化完成后,均使用PBS缓冲液冲洗3次,使用纳米压痕测

试仪(nanoindenter)以100 mN力恒定载荷15 s进行釉质片表面压刻深度、硬度和弹性模量测试,以判断再矿化后牙釉质机械强度的变化<sup>[9]</sup>。

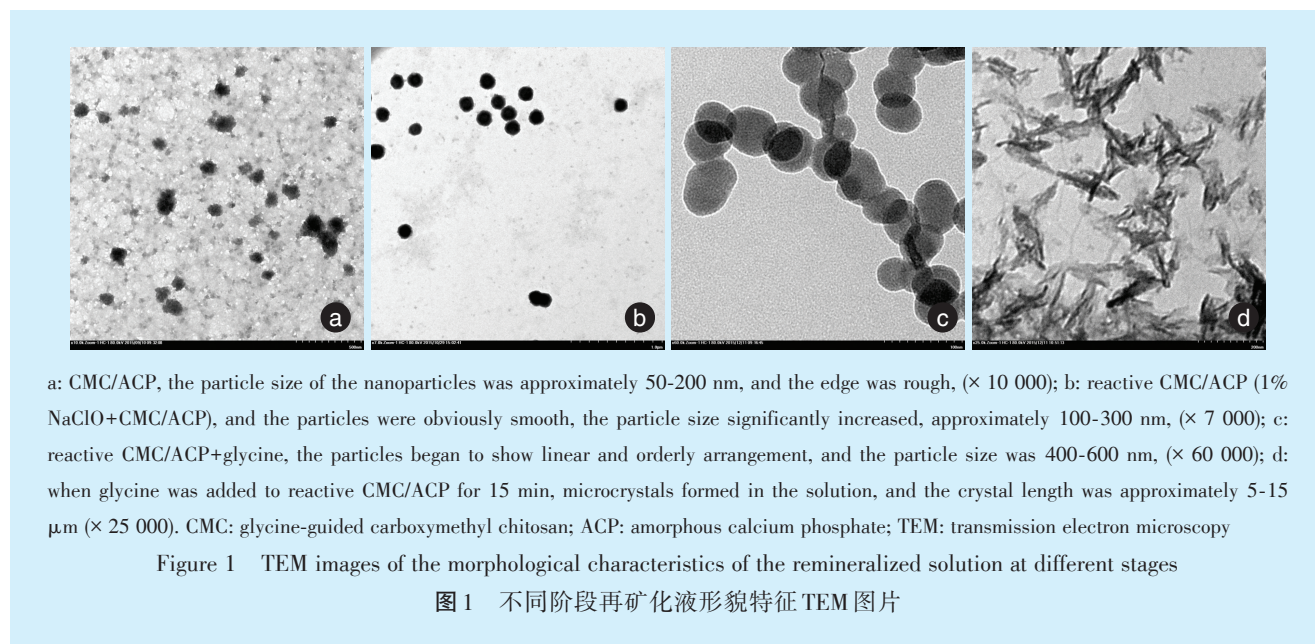
### 1.3 统计学分析

使用SPSS 15.0进行数据分析,酸蚀后、再矿化组以及天然牙釉质表面弹性模量和硬度值的比较使用one-way ANOVA,  $P < 0.05$ 为差异有统计学意义。

## 2 结果

### 2.1 TEM检测再矿化液形貌特征

如图1所示,图1a为CMC/ACP的透射电镜影像,表现为相对比较均质的纳米颗粒,纳米颗粒粒径约为50~200 nm,边缘较为粗糙。图1b为反应态CMC/ACP的透射电镜影像,可见颗粒明显光滑,颗粒粒径较前明显增加,约为100~300 nm。图1c为反应态CMC/ACP加入甘氨酸后的透射电镜影像,显示出颗粒开始呈现出线状有序排列,颗粒粒径400~600 nm。而图1d则为反应态CMC/ACP加入甘氨酸15 min时的表征,可见溶液内形成了微晶体,晶体长度约为5~15  $\mu\text{m}$ 。



### 2.2 牙釉质表面再矿化SEM结果

如图2a所示,天然牙釉质表面经37%磷酸酸蚀后呈现出典型酸蚀后的多孔鱼鳞状外观。A组再矿化结果典型代表如图2b所示,表现为颗粒状的再矿化外观,且再矿化结果为杂乱的再矿化层。B组再矿化形貌如图2c、2d所示,在脱

矿牙釉质表面形成了较为均质的晶体形貌,再矿化层晶体颗粒在釉质表面形成致密有序排列的釉质样层。微观上晶体形态与天然牙釉质晶体形貌类似。

### 2.3 再矿化前后牙表面机械强度

相同压刻力量作用下各组牙表面压刻深度如

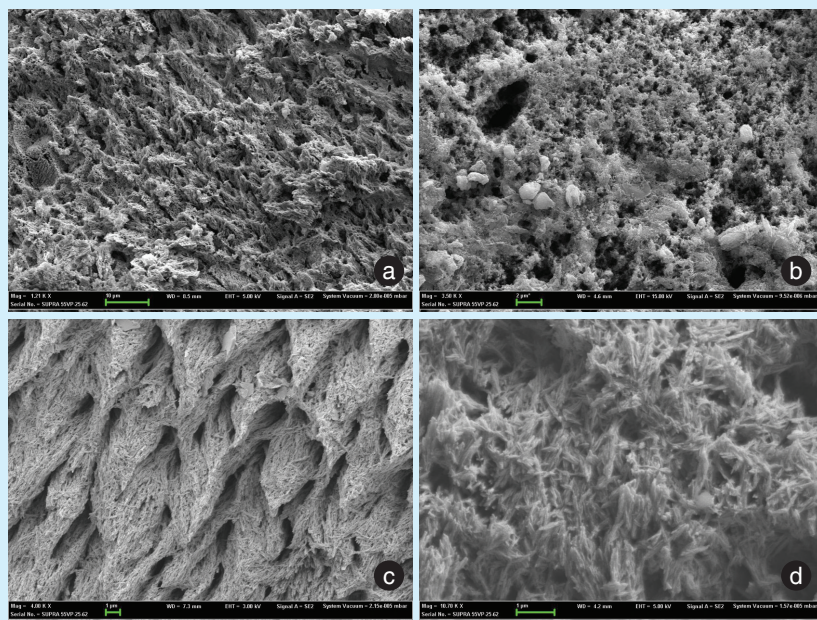


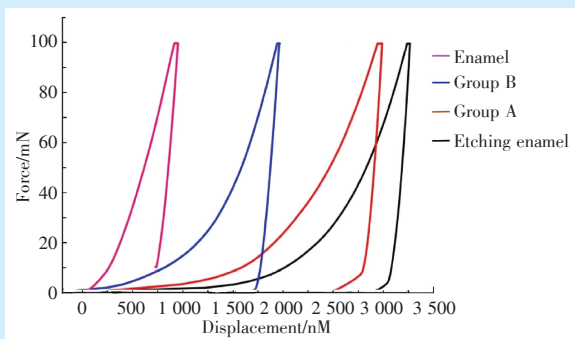
Figure 2 SEM images of enamel surface morphology before and after remineralization

图2 再矿化前后牙釉质表面形貌特征SEM图片

a: after acid etching, the natural tooth enamel showed a typical porous fish scale appearance, ( $\times 4\ 000$ ); b: after remineralization, group A (1% NaClO + CMC/ACP) showed a granular remineralization appearance with a disordered heterogeneous remineralization layer, ( $\times 3\ 500$ ); c: after remineralization, dense and orderly enamel-like layers were formed on the enamel surface in group B (1% NaClO + CMC/ACP+Glycine), ( $\times 1\ 200$ ); d: further magnification of the mineralized appearance of group B, hydroxyapatite crystals completely covered the enamel surface of the demineralized enamel with order arrangement ( $\times 10\ 700$ ). CMC: glycine-guided carboxymethyl chitosan; ACP: amorphous calcium phosphate; SEM: scanning electron microscope

图3所示。当压刻力量为100 mN时,天然牙釉质压刻深度为756 nm,天然牙釉质经37%磷酸酸蚀后牙表面压刻深度为3 280 nm,A组再矿化牙体表

面的压刻深度为2 780 nm,B组再矿化牙体表面压刻深度为1 570 nm。B组的再矿化压刻深度最为接近天然牙釉质。



The nanoindentation depth of group B after remineralization was the closest to that of natural enamel; group A: 1% NaClO + CMC/ACP; group B: 1% NaClO + CMC/ACP + glycine. CMC: glycine-guided carboxymethyl chitosan; ACP: amorphous calcium phosphate

Figure 3 Nanoindentation depth in each group

图3 各组压刻深度

根据纳米压痕实验所获得的牙釉质表面硬度和弹性模量值如表1所示,经计算可得B组再矿化

后的表面硬度和弹性模量与天然牙釉质差异无统计学意义。

表1 纳米压痕实验检测所得各组牙釉质表面硬度及弹性模量

Table 1 The hardness and elastic modulus of the enamel surfaces in the groups as determined by nanoindentation

Item	Acid-etched enamel/GPa	Group A/GPa	Group B/GPa	Natural enamel/GPa	$F$	$P$
Hardness	$0.30 \pm 0.12^b)$	$0.42 \pm 0.08^b)$	$0.88 \pm 0.21$	$1.05 \pm 0.12$	31.729	$< 0.001$
Elastic modulus	$35.70 \pm 0.10^b)$	$41.50 \pm 1.70^b)$	$61.70 \pm 1.70$	$52.10 \pm 10.70^b)$	22.359	$< 0.001$

1): compared with group B,  $P < 0.05$ ; there was no significant difference between group B and natural enamel in terms of the hardness and elastic modulus of the enamel surface after remineralization; group A: 1%NaClO+CMC/ACP; group B: 1%NaClO+CMC/ACP+glycine. CMC: glycine-guided carboxymethyl chitosan; ACP: amorphous calcium phosphate

### 3 讨论

成熟牙釉质的主要成分是含钙磷离子的羟基磷灰石晶体,羟基磷灰石属于六方晶系,大量羟基磷灰石晶体互相聚集排列形成纳米纤维。牙釉质的发育过程早期,首先形成羟基磷灰石纳米纤维的中央部分微晶,随后在釉原蛋白的调控下,晶体进一步排列聚集形成纤维束,纤维束在三维空间交叉排列形成釉柱和柱间质<sup>[10]</sup>,随后晶体逐渐成熟,有机物被成釉细胞吸收,无机物含量增高,组织硬度逐渐增大。

He等<sup>[11]</sup>研究发现,羧甲基壳聚糖可抑制钙离子和磷酸氢根间形成沉淀的功能,本实验使用CMC来形成钙磷的ACP状态,经TEM观察确认了其无定形状态,证实了He等<sup>[11]</sup>的观点。CMC为天然聚电解质,其中的大量羧甲基基团可以包裹钙、磷等金属离子,从而使磷酸氢根与金属离子隔绝<sup>[12]</sup>。本课题组前期实验已经证实,CMC对于ACP的稳定作用可以持续长达1周而难于发生即刻矿化,这一特点不利于未来临床椅旁转化和临床操作。Xiao等<sup>[13]</sup>认为质量分数1% NaClO通过氧化反应可以降解壳聚糖中的 $\beta$ -(1,4)糖苷键,分解CMC长链,使得ACP摆脱CMC的稳定作用。本实验通过在使用CMC/ACP前加入一定量的NaClO溶液来获得反应态ACP,该法模拟了天然牙釉质形成过程中釉原蛋白引导ACP排列,而釉原蛋白降解后ACP向羟基磷灰石发生转化这一生物特点<sup>[14-15]</sup>;并根据TEM观察印证了Xiao等<sup>[13]</sup>的观点。

获得反应态ACP后,如没有加任何引导条件,则容易获得没有正常结构的混乱羟基磷灰石沉淀结构,如图2b结构。本研究纳米压痕实验结果提示,仅使用经NaClO降解的CMC/ACP再矿化脱矿牙釉质表面,其再矿化机械性能与天然牙釉质差异具有统计学意义。釉质的高机械强度有赖于釉柱之间的排列有序,如果再矿化层的羟基磷灰石晶体能够有序排列,那么其机械性能将能够得到保障,从而再矿化的最终效果将与天然牙釉质的机械性能相接近。因此,课题组使用CMC作为仿生釉原蛋白,NaClO作为仿生蛋白酶降解CMC,甘氨酸作为仿生多肽作为对ACP形成有序羟基磷灰石的诱导因素,从而促进ACP向羟基磷灰石转化。这一序列来自于釉原蛋白的C端功能多肽CT-16序列。在甘氨酸多肽中,含有N端和C端的多肽在促进羟基磷灰石晶体形成的过程中,体现了促进颗粒自组装、排列形成链状结构以及随后形

成彼此平行磷灰石结构的作用,而去除了C端16个氨基酸的多肽则不具备此功能<sup>[16]</sup>。由此,本研究在NaClO释放反应态ACP后,加入了一定量的简单氨基酸—甘氨酸,使之作为对ACP形成有序羟基磷灰石的诱导因素。甘氨酸作为一种简单氨基酸,在加入再矿化液后,使得ACP的状态更加不稳定,促使其向羟基磷灰石发生转化<sup>[17]</sup>。结合已有研究,天然羟基磷灰石的 $\gamma$ 方向为其主轴(C-axis)的生长方向,笔者课题组认为甘氨酸的作用主要在于甘氨酸能够改变晶体之间界面能的各向异性<sup>[18]</sup>。根据对甘氨酸以及羟基磷灰石分子结构的计算机模拟可知,在甘氨酸缺失的情况下, $\gamma$ 方向的生长方向为长轴界面(001),而(001)的界面能量要低于(100)/(010)界面,而当加入甘氨酸后,这一关系则被逆转<sup>[19]</sup>。由此在甘氨酸存在的条件下,(001)界面更加不稳定,这就导致了最终羟基磷灰石晶体沿着(001)方向有序生长,这样可以保证溶液中的羟基磷灰石晶体向着低能量的方向进行有序生长,从而形成了较好的晶体结构。甘氨酸可特异性引导纳米颗粒从无序变为有序排列,从而形成细长的釉柱状晶体结构,这种结构与天然釉质类似<sup>[20]</sup>。微观上与天然牙釉质类似的晶体结构也对应着宏观上与天然牙釉质更加接近的机械强度。B组的再矿化外观、表面硬度和弹性模量都与天然牙釉质相近,因此可推断,加入甘氨酸、NaClO的CMC/ACP纳米颗粒引导的再矿化,晶体相对稳定且不易脱矿,抗机械摩擦的磨损率和抗撕脱性都与天然牙釉质有着相似的特性,后续实验还将对再矿化的牙釉质抗脱矿稳定性进行进一步的验证。

本研究通过在NaClO引导的CMC/ACP纳米颗粒中加入甘氨酸,形成快速牙釉质再矿化龋模型的方法,探讨再矿化液的成分对生物矿化的影响。甘氨酸既可以结合羟基磷灰石特异性连接序列<sup>[21]</sup>,也可以结合釉原蛋白中促进矿化的关键序列<sup>[22]</sup>,从而将活化的CMC/ACP矿化材料应用于脱矿牙釉质表面。TEM和SEM观察结果提示,CMC可使高浓度过饱和的ACP呈现无定形晶相结构,形成CMC/ACP纳米复合物,而NaClO又可快速降解CMC/ACP纳米复合物,使得纳米复合物的粒径增加,颗粒感更加明显;在加入甘氨酸引导后,反应态CMC/ACP(NaClO+CMC/ACP纳米复合物)可以在已脱矿的釉质表面形成定向有序的再矿化,再矿化的反应速度增加而且矿化的外貌具有特异

性,形成了均匀有序的羟基磷灰石结构。再矿化的牙釉质表面结构的硬度和弹性模量与天然牙釉质<sup>[23]</sup>相近。

综上,甘氨酸、NaClO 以及 CMC 联合促进 ACP 向羟基磷灰石晶体快速转化,并得到较好的再矿化效果,为探索相关的生物材料应用于临床早期釉质龋的再矿化治疗提供了基础。

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