[DOI]10.12016/j.issn.2096-1456.补

·基础研究·

钙离子调控KLK4表达对成釉细胞生长的影响

刘晓静[1]，　高美丽[2]，　阮建平[3]
1. 榆林市第一医院口腔科，陕西 榆林(719000)；　2. 西安交通大学生命科学与技术学院，生物医学信息工程教育部重点实验室，陕西 西安(710049)；　3. 陕西省牙颌面疾病临床研究中心，西安交通大学口腔医院口腔预防保健科，陕西 西安(710004)

【摘要】　目的　探讨钙离子对成釉细胞株细胞(ameloblast-lineage cell，ALC)中激肽释放酶4(kallikrein-4，KLK4)表达及细胞生长的影响，为钙离子促进牙釉质正常矿化提供实验依据。**方法**　采用不同浓度$CaCl_2$(0、2.0、2.5、3.0、3.5 mmol/L)处理ALC 24 h、48 h，qRT-PCR和Western blot检测KLK4 mRNA和蛋白表达水平；CCK-8检测细胞相对活力；流式细胞术、Hoechst 33342染色检测钙离子对细胞周期和细胞凋亡的影响；Western blot检测葡萄糖调节蛋白78(glucose-regulated protein 78，GRP78)的蛋白表达水平。**结果**　与对照组(0 mmol/L $CaCl_2$)相比，2.5、3.0、3.5 mmol/L $CaCl_2$处理细胞24 h后，KLK4 mRNA表达上升($P<0.05$)，2.0、2.5、3.0、3.5 mmol/L $CaCl_2$处理细胞24 h后，KLK4蛋白表达上升($P<0.05$)；3.0、3.5 mmol/L $CaCl_2$处理细胞48 h后，KLK4 mRNA和蛋白表达上升($P<0.05$)。与对照组相比，2.0、2.5、3.0、3.5 mmol/L $CaCl_2$处理ALC细胞24 h、48 h后，细胞活力增加($P<0.05$)，其中2.5 mmol/L $CaCl_2$组中细胞活力最高。Hoechst 33342染色结果显示，3.0、3.5 mmol/L $CaCl_2$促使ALC细胞发生凋亡。流式细胞仪检测结果显示，与0、2.0、2.5、3.0 mmol/L $CaCl_2$组相比，3.5 mmol/L $CaCl_2$处理ALC细胞24 h后，G2/M期细胞比例增加，细胞凋亡率上升($P<0.05$)。3.0、3.5 mmol/L $CaCl_2$处理细胞24 h后，与对照组相比，GRP78蛋白表达下降($P<0.05$)；2.5 mmol/L $CaCl_2$处理细胞48 h后，与对照组相比，GRP78蛋白表达下降($P<0.05$)。**结论**　钙离子促进ALC中KLK4表达上升、细胞活力增加，但较高浓度的钙离子可使ALC的G2/M期阻滞，诱发ALC细胞凋亡，降低凋亡相关蛋白GRP78的表达。

【关键词】　成釉细胞；　成釉细胞株细胞；　钙离子；　激肽释放酶4；　细胞生长；　细胞活力；　细胞周期；　细胞凋亡；　葡萄糖调节蛋白78
【中图分类号】　R78　**【文献标志码】**　A　**【文章编号】**　2096-1456(2024)10-0762-10
【引用著录格式】　刘晓静，高美丽，阮建平. 钙离子调控KLK4表达对成釉细胞生长的影响[J]. 口腔疾病防治，2024，32(10)：762-　. doi:10.12016/j.issn.2096-1456.补.

微信公众号

Effect of calcium ion regulating KLK4 expression on the growth of ameloblast

LIU Xiaojing[1], GAO Meili[2], RUAN Jianping[3]　1. Department of Stomatology, the First Hospital of Yulin, Yulin 719000, China;　2. Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi′an Jiaotong University, Xi′an 710049, China; 3. Clinical Research Center of Shaanxi Province for Dental and Maxillofacial Diseases & Department of Preventive Dentistry, College of Stomatology, Xi'an Jiaotong University, Xi'an 710004, China
Corresponding author: RUAN Jianping, Email: ruanjp@xjtu.edu.cn, Tel: 86-13571960667

【Abstract】　Objective　To investigate the effect of calcium ions on the expression of kallikrein-4 (KLK4) and cell growth of ALC cells, and to provide an experimental basis for calcium ion nutrition promoting normal mineralization of enamel. **Methods**　ALC cells were treated with 0, 2.0, 2.5, 3.0, and 3.5 mmol/L $CaCl_2$ for 24 and 48 h. KLK4 expression was analyzed by qRT-PCR and Western blot analysis. The viability of ALC cells was determined by using CCK-8. AnnexinV-FITC/PI dual staining combined with flow cytometry and Hoechst 33342 staining were used to detect the ALC cell cycle and cell apoptosis. The protein expression level of glucose-regulated protein 78 (GRP78) was measured

【收稿日期】2024-03-10；　**【修回日期】**2024-06-07
【基金项目】陕西省重点研发计划项目(2023-YBSF-659)；榆林市科技计划项目(YF-2021-44)
【作者简介】刘晓静，副主任医师，博士，Email：809112483@qq.com
【通讯作者】阮建平，教授，博士，Email: ruanjp@xjtu.edu.cn，Tel: 86-13571960667

by Western blot analysis. **Results**　After 24 h of treatment with 2.5, 3.0, and 3.5 mmol/L $CaCl_2$, the expression of KLK4 mRNA was increased ($P < 0.05$), and after 24 h of treatment with 2.0, 2.5, 3.0, and 3.5 mmol/L $CaCl_2$, the expression of KLK4 protein was increased ($P < 0.05$). After 48 h of treatment with 3.0 mmol/L and 3.5 mmol/L $CaCl_2$, the expression of KLK4 mRNA and protein was increased ($P < 0.05$). Compared with the control group, the viability of ALC cells was increased after 24 and 48 h of treatment with 2.0, 2.5, 3.0, and 3.5 mmol/L $CaCl_2$ ($P < 0.05$), and the highest cell viability was observed with 2.5 mmol/L $CaCl_2$. Hoechst 33342 staining results showed that 3.0 mmol/L and 3.5 mmol/L $CaCl_2$ may promote apoptosis in ALC cells. Flow cytometry showed that the proportion of G2/M phase cells and the apoptosis rate increased after 3.5 mmol /L $CaCl_2$ treatment for 24 h ($P < 0.05$), compared with the 0, 2.0, 2.5, and 3.0 mmol/L $CaCl_2$ groups. After 24 h of treatment with 3.0 mmol/L and 3.5 mmol/L $CaCl_2$, the expression of GRP78 protein was reduced ($P < 0.05$), and after 48 h of treatment with 2.5 mmol/L $CaCl_2$, the expression of GRP78 protein was reduced ($P < 0.05$). **Conclusion**　Calcium ions can promote the increase of KLK4 expression and cell viability in ALC, but a higher concentration of calcium ions can block the G2/M phase of ALC cells, thus inducing apoptosis of ALC cells and reducing the expression of apoptosation-related protein GRP78.

【Key words】 ameloblast; ameloblast-lineage cell; calcium ion; kallikrein-4; cell growth; cell viability; cell cycle; cell apoptosis; glucose-regulated protein 78

J Prev Treat Stomatol Dis, 2024, 32(10): 00-00.

【Competing interests】 The authors declare no competing interests.

This study was supported by the grants from Shaanxi Province Key Research and Development Program (No. 2023-YBSF-659); Yulin Science and Technology Project (No. YF-2021-44).

成釉细胞通过合成、分泌、重吸收和降解釉基质蛋白引导釉基质矿化，因此成釉细胞在牙釉质的形成中起着重要的作用[1-2]。研究显示，当机体处于营养不良时易引起分泌期成釉细胞活动紊乱，导致釉质发育不全、氟牙症等[3-4]。钙是人体必需的营养元素，它参与釉质矿化、晶体形成及釉质成熟等[5-6]。在釉质成熟或矿化阶段，成釉细胞有机基质吸收过程中，随着晶体的宽度和厚度的增大，钙的需求相应增加[7-8]。成釉细胞将钙离子转运到细胞外，为其矿化提供所需的离子源，而钙库操纵的钙离子内流（store-operated calcium entry，SOCE）在成釉细胞分化中起着关键调节作用[9]。研究显示低钙成为基质相互作用分子1（stromal interaction molecule，STIM1）或钙离子释放激活钙通道蛋白1（calcium release-activated calcium channel protein 1，ORAI1）突变患者牙釉质缺陷的重要原因，STIM1导致的牙釉质缺陷是通过SOCE影响成釉细胞的分子昼夜节律时钟来实现的[10-11]。

研究表明，钙离子促进成釉细胞的分化及影响釉基质蛋白的分泌[12]。KLK4是一种糖基化的丝氨酸蛋白酶，由成熟期成釉细胞表达和分泌，是釉质矿化成熟的关键酶，其主要功能是在釉质成熟过程中清除釉基质蛋白和促进晶体彻底成熟。在小鼠中敲除KLK4后导致牙釉质硬度降低，蛋白质含量高于正常值[13-14]。Le等[15]研究发现氟诱发KLK4下调，与雄激素受体（androgen receptor，AR）和孕激素受体（progesterone receptor，PR）核转位减少相关。氟化钠处理小鼠成釉细胞株LS8细胞后，叉头转录因子O1（forkhead transcription factor，FoxO1）激活导致KLK4上调，FoxO1基因敲除降低了KLK4和MMP-20在mRNA水平上的表达，与氟化钠联合使用时，对KLK4的抑制作用增强，而成釉细胞内FoxO1缺失导致形成的釉质矿化程度降低[16]。细胞的生长和增殖需要适宜的细胞外钙环境[17-18]。GRP78在调节和维持内质网稳态中发挥着重要作用，并参与内质网中钙离子的调节[19-20]。因此，本研究采用不同浓度的钙离子作用于体外培养的成釉细胞株细胞（ameloblast-lineage cell，ALC），探究其对KLK4的表达及细胞生长的影响，为钙离子促进牙釉质正常矿化提供实验数据。

1　材料和方法

1.1　主要试剂与仪器

ALC由滨州医学院高玉光教授馈赠。$CaCl_2$（天津市天力化学试剂有限公司，中国）；高糖DMEM培养基、青链霉素双抗（SH30243.01、sv30010，Hyclone，美国）；胎牛血清（04-001-1ACS，BI，以色列）；胰蛋白酶、Hoechst 33342试剂盒（C0201、

C1027，上海碧云天生物技术有限公司，中国）；二甲基亚砜（D2650，Sigma，美国）；CCK-8试剂盒（BS350A，Biosharp，中国）；细胞凋亡检测试剂盒、细胞周期检测试剂盒（KGA105、KGA511，江苏凯基生物技术有限公司，中国）；BCA蛋白定量试剂盒（P0010S，上海碧云天生物技术有限公司，中国）；β-actin抗体（15204-1-AP，Proteintech，中国）；KLK4抗体、GRP78（ab231048、ab21685，Abcam，英国）；HRP标记山羊抗兔lgG抗体（bs-0295G-HRP，Bioss，中国）；ECL化学发光检测试剂盒（WBKLS0500，Millipore，美国）。

全波长酶标仪（Infinite M Nano，TECAN，瑞士）；荧光倒置显微镜（ECLIPSE Ti-S，Nikon，日本）；流式细胞仪（FACSVerse，BD，美国）；实时荧光定量PCR仪（CFX Connect，Bio-Rad，美国）；化学发光成像分析仪（ImageQuant LAS 4000，Cytiva，美国）。

1.2　实验方法

1.2.1　ALC培养　用含10%胎牛血清和1%青霉素/链霉素的高糖DMEM培养基，在37 ℃，5% CO_2浓度的培养箱中培养。待细胞融合到培养皿的80%时，0.25%胰酶消化细胞，传代培养。

1.2.2　qRT-PCR检测KLK4 mRNA表达　将ALC以3 × 105/mL密度接种于6孔板中，待细胞贴壁稳定后，吸弃旧培养液，每孔加入终浓度为0、2.0、2.5、3.0、3.5 mmol/L的含$CaCl_2$培养液2 mL，培养24 h、48 h后，Trizol法提取总RNA，按照逆转录试剂盒说明书操作将RNA逆转录为cDNA，qRT-PCR检测KLK4基因表达变化，以β-actin作为内参基因，采用$2^{-\Delta\Delta CT}$法计算目的基因的相对表达值，引物序列见表1。

表1　PCR引物序列

Table 1　PCR primer sequences

Primer	Sequence
KLK4-Forward	CCGGATCATACAAGGCCAGG
KLK4-Reverse	TGCGGATGCACCAAGACTC
β-actin-Forward	GGCTGTATTCCCCTCCATCG
β-actin-Reverse	CCAGTTGGTAACAATGCCATGT

1.2.3　Western blot检测KLK4和GRP78蛋白表达　细胞加药处理同1.2.2。培养24 h、48 h后，提取细胞总蛋白，BCA法进行蛋白定量。制备SDS-PAGE胶，蛋白等量上样，电泳，转膜，5%的脱脂牛奶封闭。分别加入一抗工作液KLK4（1∶500），GRP78（1∶1 000），4 ℃慢速摇床孵育过夜，洗膜3次，加入二抗工作液（1∶5 000），室温孵育2 h，将膜放入ECL显色液中，发光显影，采用Quantity One软件分析灰度值。

1.2.4　CCK-8检测细胞活力　将ALC以2 × 10^3个/孔的密度接种于96孔板中，待细胞贴壁稳定后，吸出旧培养液，每孔中加入终浓度为0、2.0、2.5、3.0、3.5 mmol/L的含$CaCl_2$培养液100 μL，培养24 h、48 h后，每孔加入10 μL CCK-8工作液，培养箱孵育4 h，酶标仪检测450 nm波长处吸光度值。

1.2.5　Hoechst 33342染色　细胞加药处理同1.2.2。培养24 h、48 h后，吸出培养液，加入Hoechst 33342染料，使其终浓度为5 μg/mL，37 ℃培养箱中避光染色15 min。吸弃染液，荧光显微镜下观察细胞核型变化，并拍照记录。

1.2.6　流式细胞仪检测细胞周期　细胞加药处理同1.2.2。培养24 h、48 h后，消化收集细胞到1.5 mL离心管中。1 000 rpm，离心10 min，弃上清液。重悬细胞于0.2 mL PBS中，加入0.8 mL 70%的预冷乙醇，震荡混匀，封口膜封口，4 ℃固定过夜。检测前，1 000 rpm，离心10 min，弃乙醇，PBS洗涤两次，离心，弃上清。每个离心管中加入RNA酶（50 μg/mL）和碘化丙啶PI染液（50 μg/mL）混合液500 μL，避光染色30 min。流式细胞仪检测细胞DNA含量变化，记录细胞周期各时相的比例。

1.2.7　流式细胞仪检测细胞凋亡　细胞加药处理同1.2.2。培养24 h、48 h后，消化收集细胞，4 ℃，1 000 rpm，离心5 min，弃上清，1 × Binding Buffer 500 μL重悬细胞沉淀，分别加入Annexin V-FITC和PI染液各5 μL，混合均匀，室温避光染色10 min，流式细胞仪检测细胞凋亡率。

1.3　统计学分析

采用Graphpad Prism 6.0软件进行统计分析。数据以均值±标准差表示，两组比较采用t检验；多组间比较采用单因素方差分析，组间两两比较采用LSD-t检验；采用双因素重复测量方差分析观察浓度和时间的交互效应。$P < 0.05$时差异具有统计学意义。

2　结　果

2.1　钙离子对ALC中KLK4表达的影响

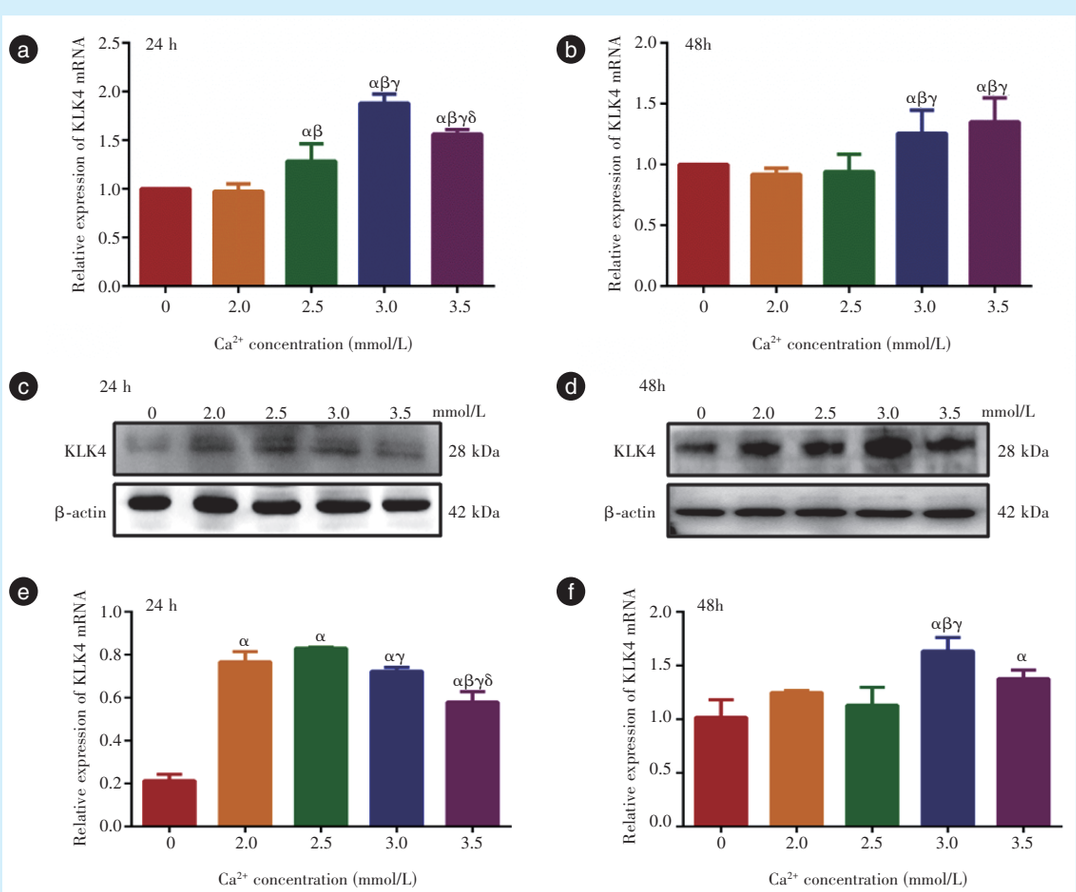
不同浓度$CaCl_2$处理ALC细胞24 h、48 h后，各组间KLK4 mRNA表达水平差异有统计学意义（$F = 45.44$，$P < 0.001$；$F = 5.976$，$P = 0.01$），组间两

两比较结果见图1。与对照组(0 mmol/L $CaCl_2$)相比，2.5、3.0、3.5 mmol/L $CaCl_2$处理细胞24 h后，KLK4 mRNA表达上升($P < 0.05$)。3.0、3.5 mmol/L $CaCl_2$处理细胞48 h后，KLK4 mRNA表达上升($P < 0.05$)。同一浓度$CaCl_2$处理ALC后，与24 h组相比，3.0 mmol/L $CaCl_2$处理细胞48 h后，KLK4 mRNA表达下降($t = 9.371$；$P = 0.011$)。双因素方差分析结果显示，浓度和时间存在交互效应($F = 5.676$，$P = 0.018$)。

不同浓度$CaCl_2$处理ALC细胞24 h、48 h后，各组间KLK4蛋白表达水平差异有统计学意义($F = 107.3$，$P < 0.001$；$F = 7.322$，$P = 0.255$)。组间两两比较结果见图1。与对照组(0 mmol/L $CaCl_2$)相比，2.0、2.5、3.0、3.5 mmol/L $CaCl_2$处理细胞24 h后，KLK4蛋白表达上升($P < 0.05$)。3.0、3.5 mmol/L $CaCl_2$处理细胞48 h后，KLK4蛋白表达上升($P < 0.05$)。同一浓度$CaCl_2$处理ALC后，与24 h组相比，0、2.0、3.0、3.5 mmol/L $CaCl_2$处理细胞48 h后，KLK4蛋白表达上升($P < 0.05$)。双因素方差分析结果显示，浓度和时间存在交互效应($F = 7.763$，$P = 0.036$)。

2.2　钙离子对ALC形态及细胞活力的影响

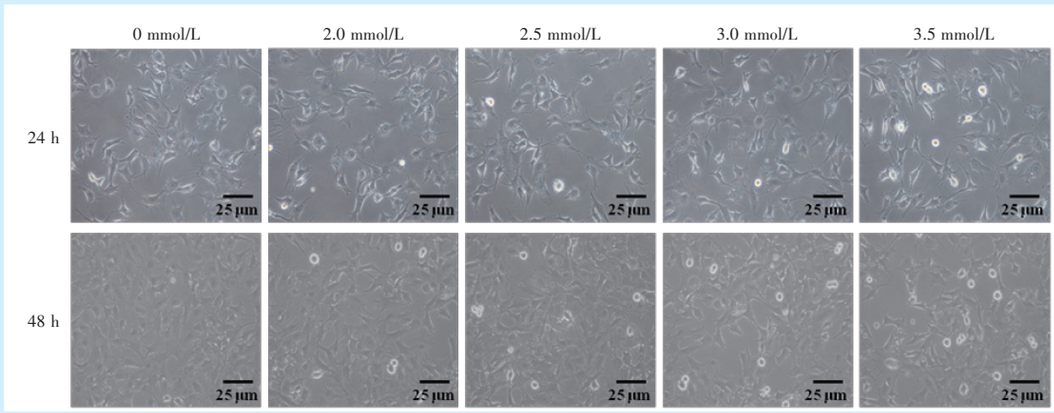
2.2.1　钙离子对ALC形态的影响　不同浓度$CaCl_2$处理ALC细胞24 h、48 h，细胞形态变化见图2。正常ALC呈“铺路石”状，椭圆形或多边形，成簇排列，彼此紧密相连。随着$CaCl_2$处理浓度的增加，ALC形态未见明显改变，但可见少量漂浮细胞，说明$CaCl_2$处理会影响ALC的黏附性。



KLK4 expression was detected by qRT-PCR and Western blot analysis of calcium ion treatment in ALC cells for 24 and 48 h. a-b: quantitative analysis of mRNA levels; c-f: representative photographs of the Western blot band and quantitative analysis of protein levels. α: *vs.* control group, $P < 0.05$; β: *vs.* 2.0 mmol/L $CaCl_2$ group, $P < 0.05$; γ: *vs.* 2.5 mmol/L $CaCl_2$ group, $P < 0.05$; δ: *vs.* 3.0 mmol/L $CaCl_2$ group, $P < 0.05$. Calcium ion from calcium chloride is indicated as Ca^{2+}

Figure 1　Effect of different concentrations of calcium ions on the KLK4 expression in ALC

图1　不同浓度钙离子处理对ALC中KLK4表达的影响



Cells morphology was observed by microscope after treatment with different concentrations of calcium ions for 24 h and 48 h. The cells in the 0 mmol/L $CaCl_2$ treatment group were elliptic or polygonal, arranged in clusters, and closely connected. No obvious changes cell morphology were observed, except that a small number of floating cells were found in the 2.0-3.5 mmol/L $CaCl_2$ treatment group. The magnification is 10× to 20× and the bar value is 25 μm

Figure 2　Effect of different concentrations of calcium ions on the morphology of ALC

图2　不同浓度钙离子处理对ALC形态的影响

2.2.2　钙离子对ALC相对活力的影响　不同浓度$CaCl_2$处理ALC细胞24 h后，各组间细胞相对活力差异有统计学意义（F = 3.149，P = 0.046），其中2.5 mmol/L $CaCl_2$组细胞活力最高，见表2。不同浓度$CaCl_2$处理ALC细胞48 h后，各组间细胞相对活力差异有统计学意义（F = 5.580，P = 0.006），其中2.5 mmol/L $CaCl_2$组细胞活力最高。同一浓度$CaCl_2$处理ALC，与24 h组相比，2.5 mmol/L $CaCl_2$处理细胞48 h后，细胞相对活力升高（t = 4.014；P = 0.028）。双因素方差分析结果显示，浓度和时间不存在交互效应（F = 1.964，P = 0.164）。

2.3　钙离子对ALC细胞周期的影响

不同浓度$CaCl_2$处理ALC细胞24 h后，在G2/M期，各组间细胞比例差异有统计学意义（F = 10.48，P = 0.001）。组间两两比较结果见图3，与0、2.0、2.5、3.0 mmol/L $CaCl_2$组相比，3.5 mmol/L $CaCl_2$组G2/M期细胞比例增加，差异具有统计学意义（P < 0.05）。不同浓度$CaCl_2$处理细胞48 h后，细胞各周期时相所占的比例未见明显变化（P > 0.05）。

2.4　钙离子对ALC细胞凋亡的影响

2.4.1　Hoechst 33342染色对细胞核的影响　采用不同浓度$CaCl_2$处理ALC后，Hoechst 33342染色观察钙离子对细胞凋亡的影响，见图4。3.0 mmol/L $CaCl_2$处理细胞24 h后，促使部分ALC细胞核出现亮蓝色碎块状染色，随着处理时间延长到48 h，3.5 mmol/L $CaCl_2$处理组ALC细胞核出现亮蓝色碎块状染色，表明3.0、3.5 mmol/L $CaCl_2$促使ALC发生细胞凋亡。

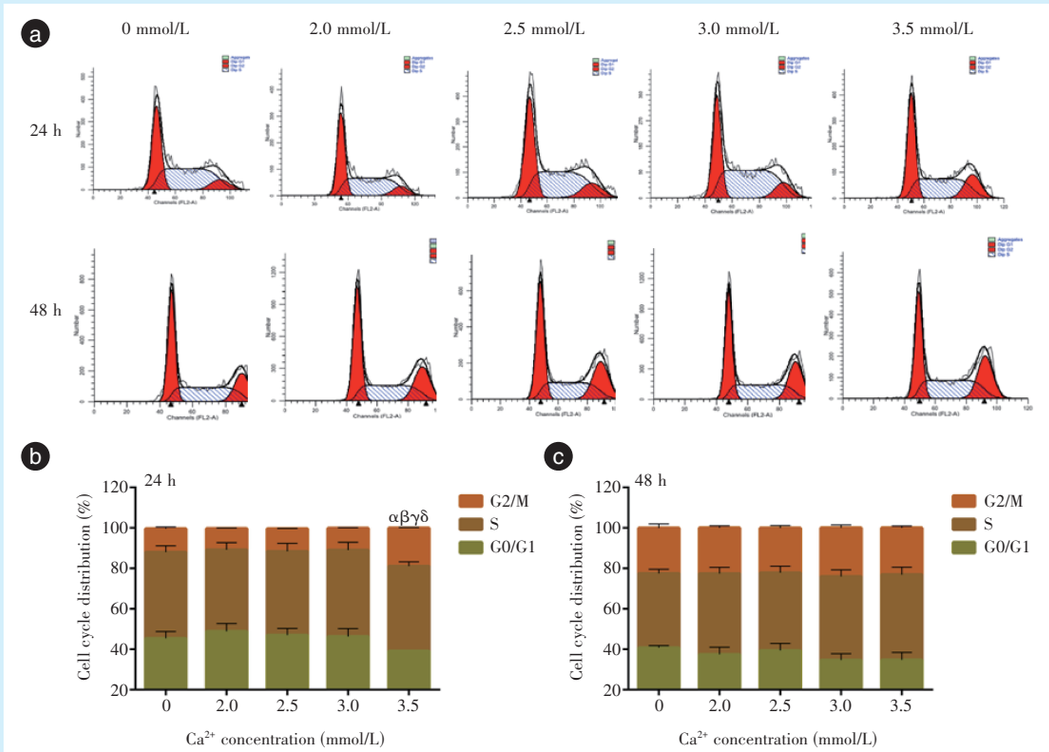
2.4.2　AnnexinV-FITC/PI双染流式细胞仪检测细胞凋亡　不同浓度$CaCl_2$处理ALC细胞24 h后，各组间细胞凋亡率差异有统计学意义（F = 10.26，P = 0.001）。组间两两比较结果见图5，与0、2.0、2.5、3.0 mmol/L $CaCl_2$组相比，3.5 mmol/L $CaCl_2$组细胞凋亡率升高（P < 0.05）。不同浓度$CaCl_2$处理ALC细胞48 h后，各组间细胞凋亡率差异无统计学意义（P > 0.05）。同一浓度$CaCl_2$处理ALC后，在24 h和48 h，细胞凋亡率差异无统计学意义（P > 0.05）。双因素方差分析结果显示，浓度和时间存在交互效应（F = 6.408，P = 0.013）。

表2　不同浓度钙离子处理对ALC相对活力的影响

Table 2　Effect of different concentrations of calcium ions on the relative viability of ALC

Groups	24 h	48 h	t	P
0 mmol/L $CaCl_2$	1.00 ± 0.00	1.00 ± 0.00	-	-
2.0 mmol/L $CaCl_2$	1.17 ± 0.16$^{\alpha}$	1.35 ± 0.08$^{\alpha}$	1.495	0.232
2.5 mmol/L $CaCl_2$	1.24 ± 0.08$^{\alpha}$	1.51 ± 0.10$^{\alpha}$	4.014	0.028
3.0 mmol/L $CaCl_2$	1.22 ± 0.10$^{\alpha}$	1.44 ± 0.34$^{\alpha}$	1.288	0.288
3.5 mmol/L $CaCl_2$	1.18 ± 0.11$^{\alpha}$	1.23 ± 0.10$^{\gamma}$	0.740	0.513
F	3.149	5.580		
P	0.046	0.006		

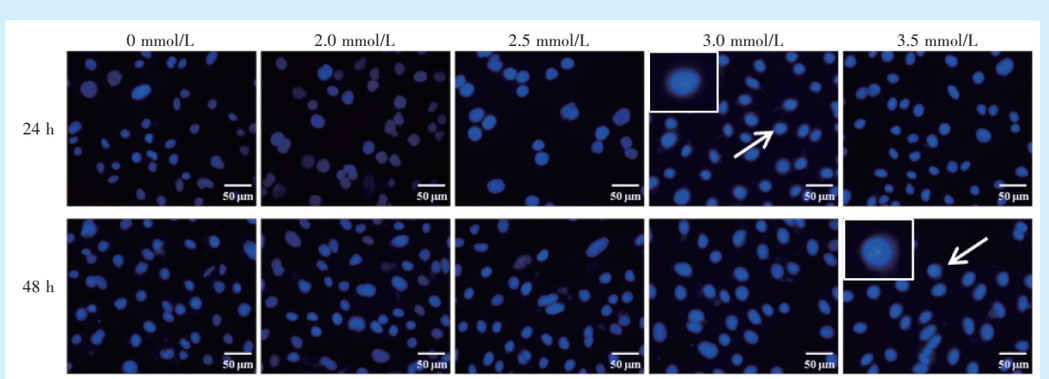
α: *vs.* control group (0 mmol/L $CaCl_2$), P < 0.05; γ: *vs.* 2.5 mmol/L $CaCl_2$ group, P < 0.05



The cell cycle distribution of ALC after 24 and 48 h was detected by flow cytometer. a: representative photographs of flow cytometer; b: compared with the 0-3.0 mmol/L $CaCl_2$ group, the G2/M phase cells increased after treatment with 3.5 mmol/L $CaCl_2$ for 24 h; c: there was no significant difference in the proportion of cell cycle phase among groups treated with different concentrations of $CaCl_2$ for 48 h. α: vs. control group, $P < 0.05$; β: *vs*. 2.0 mmol/L $CaCl_2$ group, $P < 0.05$; γ: vs. 2.5 mmol/L $CaCl_2$ group, $P < 0.05$; δ: *vs*. 3.0 mmol/L $CaCl_2$ group, $PP < 0.05$. Calcium ion from calcium chloride is indicated as Ca^{2+}

Figure 3　Effect of different concentrations of calcium ions on the cycle of ALC

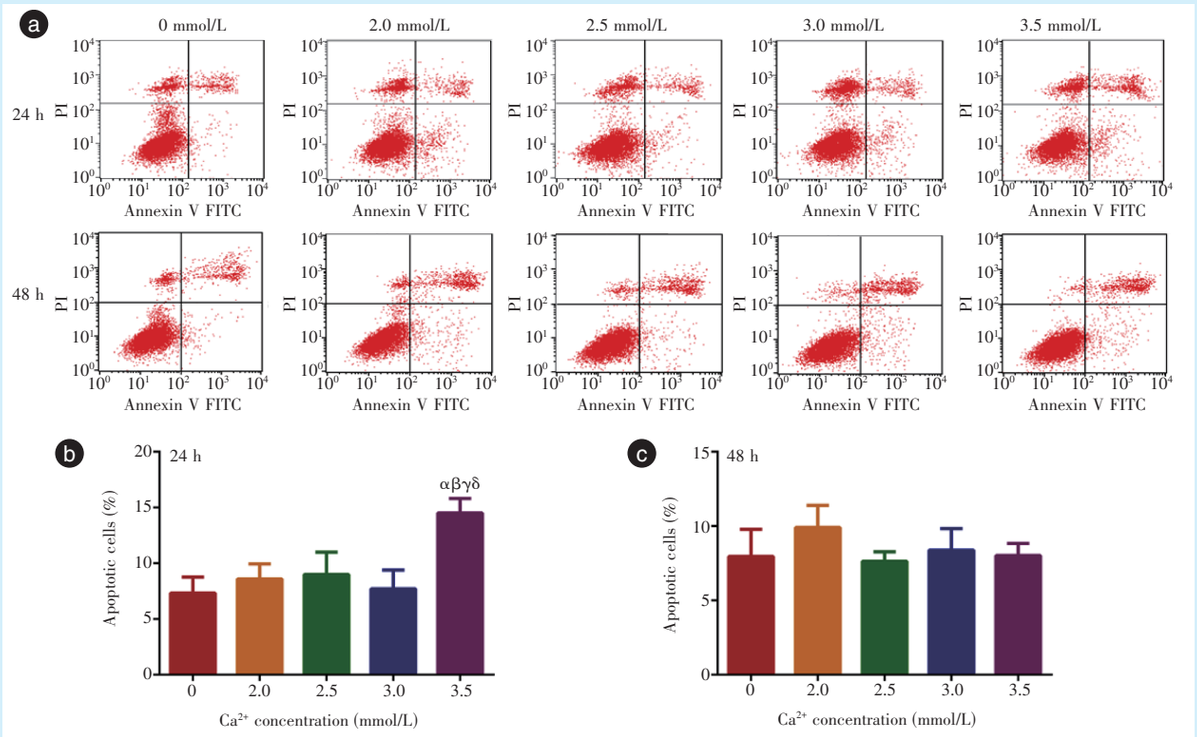
图3　不同浓度钙离子处理对ALC细胞周期分布的影响



ALC apoptosis was observed by Hoechst 33342 staining after treatment with different concentrations of calcium ions for 24 h and 48 h. After the cells were treated with 3.0 mmol/L $CaCl_2$ for 24 h, the nuclei were stained with bright blue fragments. After the cells were treated with 3.5 mmol/L $CaCl_2$ for 48 h, the nuclei were stained with bright blue fragments. The white arrow indicates the apoptotic nucleus. The magnification is 10× to 40× and the bar value is 50 μm

Figure 4　Effect of different concentrations of calcium ions on the apoptosis of ALC

图4　不同浓度钙离子处理对ALC细胞凋亡的影响



Apoptotic cells were detected by flow cytometer following calcium ion treatment in ALC for 24 and 48 h. a: representative photographs of the flow cytometer; b: after 24 h of treatment with 3.5 mmol/L $CaCl_2$, the cell apoptosis rate was increased compared with the 0-3.0 mmol/L $CaCl_2$ treatment group; c: there was no significant difference in cell apoptosis rate with different concentrations of $CaCl_2$ for 48 h. α: *vs.* control group, $P < 0.05$; β: *vs.* 2.0 mmol/L $CaCl_2$ group, $P < 0.05$; γ: *vs.* 2.5 mmol/L $CaCl_2$ group, $P < 0.05$; δ: *vs.* 3.0 mmol/L $CaCl_2$ group, $P < 0.05$. Calcium ion from calcium chloride is indicated as Ca^{2+}

Figure 5　Effect of different concentrations of calcium ions on the apoptosis of ALC by flow cytometer

图5　流式细胞仪检测不同浓度钙离子处理对ALC细胞凋亡的影响

2.5　钙离子对ALC中GRP78表达的影响

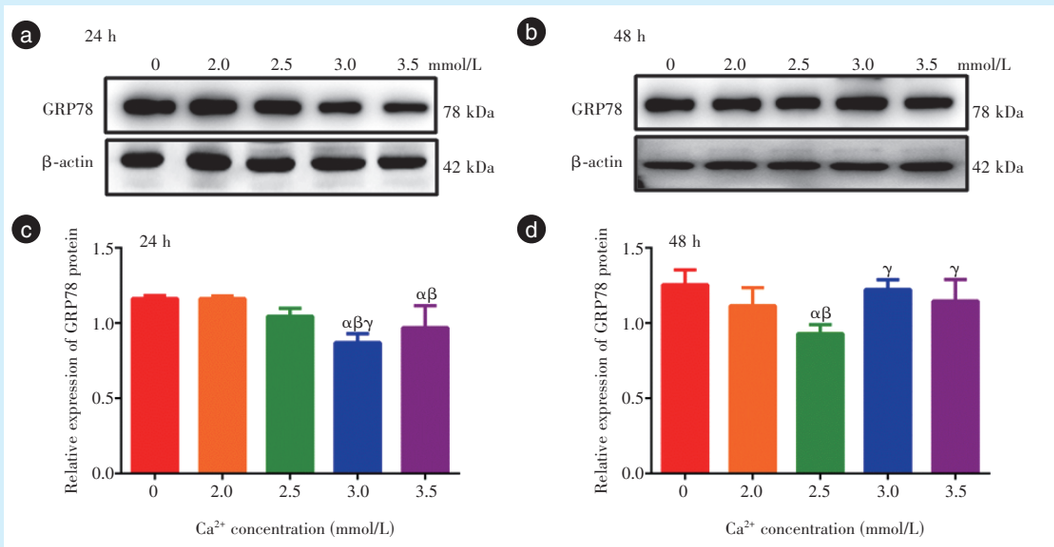
不同浓度$CaCl_2$处理ALC细胞24 h、48 h后，各组间GRP78蛋白表达水平差异有统计学意义（$F = 8.684$，$P = 0.003$；$F = 4.699$，$P = 0.022$）。组间两两比较结果见图6，与对照组相比，3.0、3.5 mmol/L $CaCl_2$处理细胞24 h后，GRP78蛋白表达下降（$P < 0.05$）。与对照组相比，2.5 mmol/L $CaCl_2$处理细胞48 h后，GRP78蛋白表达下降（$P < 0.05$）。

同一浓度$CaCl_2$处理ALC细胞24 h、48 h后，与24 h组相比，3.0 mmol/L $CaCl_2$处理细胞48 h后，GRP78蛋白表达上升（$t = 5.257$；$P = 0.034$）。双因素方差分析结果显示，浓度和时间存在交互效应（$F = 17.69$，$P = 0.001$）。

3　讨　论

KLK4是成熟期成釉细胞的标记物，如HAT-7球体中的成釉细胞标记物KLK4增加70倍[21]。钙离子处理LS8细胞后，除了2.0 mmol/L $CaCl_2$处理24 h组的KLK4蛋白表达没有明显变化外，其他浓度钙离子处理组的KLK4和釉成熟蛋白表达均显著增加，这表明钙离子可以上调LS8细胞中KLK4和釉成熟蛋白的表达[22]。本研究发现，$CaCl_2$处理ALC细胞24 h，KLK4表达水平显著升高，随着处理时间的延长（48 h），3.0、3.5 mmol/L $CaCl_2$处理使KLK4表达水平升高。本研究中KLK4 mRNA和蛋白表达的结果亦提示，3.0、3.5 mmol/L $CaCl_2$处理使ALC中KLK4过度表达。相反地，氟化物处理成釉细胞引起KLK4表达抑制时细胞生长也受到抑制，漂浮细胞增加，显示KLK4表达抑制降低细胞的生长[15]。KLK4的分泌促进牙釉质的矿化成熟，因而也可能促使ALC细胞成熟[23-24]，具体是何种效应还有待进一步研究。

为探索钙离子处理对KLK4调控的机制，本研究分析了不同浓度钙离子对ALC生长的影响，包



Protein expression of GRP78 was detected by Western blot analysis of calcium ion treatment in ALC for 24 and 48 h. a-b: representative photographs of Western blot band; c: after 24 h of treatment with 3.0 mmol/L $CaCl_2$, the expression of GRP78 protein was reduced compared with the 0-2.5 mmol/L $CaCl_2$ treatment group; after 24 h of treatment with 3.5 mmol/L $CaCl_2$, the expression of GRP78 protein was reduced compared with the 0-2.0 mmol/L $CaCl_2$ treatment group; d: after 48 h of treatment with 2.5 mmol/L $CaCl_2$, the expression of GRP78 protein was reduced compared with the 0-2.0 mmol/L $CaCl_2$ treatment group; after 48 h of treatment with 3.0-3.5 mmol/L $CaCl_2$, the expression of GRP78 protein was increased compared with the 2.5 mmol/L $CaCl_2$ treatment group. α: *vs.* control group, $P < 0.05$; β: *vs.* 2.0 mmol/L $CaCl_2$ group, $P < 0.05$; γ: *vs.* 2.5 mmol/L $CaCl_2$ group, $P < 0.05$. Calcium ion from calcium chloride is indicated as Ca^{2+}

Figure 6　Effect of different concentrations of calcium ions on the protein expression of GRP78 in ALC

图6　不同浓度钙离子处理对ALC中GRP78蛋白表达的影响

括对细胞形态、细胞活力、细胞周期、细胞凋亡及凋亡调控分子GRP78的影响。钙离子是一种多功能的第二信使，具有广泛的生理作用，包括肌肉收缩、细胞运动和囊泡转运，细胞生长或增殖[17, 25]。研究发现当MC3T3-E1细胞在不同的细胞外钙离子浓度（1.8 ~ 7.2 mmol/L）下培养30 min时，其增殖显著增加，显示细胞外钙离子促进MC3T3-E1细胞的增殖[26]。本研究中发现ALC细胞在不同浓度的$CaCl_2$处理后相对活力增加，但并非一直增加，其中2.5 mmol/L $CaCl_2$处理组细胞活力最高。然而，前期研究采用$CaCl_2$（0、2.0、2.5、3.0、3.5 mmol/L）处理小鼠成釉细胞株LS8细胞24 h、48 h，结果显示随着钙离子浓度的增加，LS8细胞的存活率略有下降[22]，说明钙离子对成釉细胞的影响还与细胞种类有关。

细胞周期的正常运转是细胞有序分化的必要保障[27]。本研究发现，3.5 mmol/L $CaCl_2$处理ALC细胞24 h后，G2/M期细胞比例增加，其他浓度$CaCl_2$处理细胞后细胞周期时相无明显变化，表明一定浓度的$CaCl_2$促进成釉细胞分裂的阻滞。前期研究对$CaCl_2$处理LS8细胞的研究发现，随着钙离子浓度的升高LS8细胞的S期细胞比例增加，G0/G1期细胞比例降低，G2/M期细胞比例降低，尤其在3.0、3.5 mmol/L钙离子浓度处理后降低更明显，这些发现表明，钙离子处理可刺激LS8细胞的S期阻滞[22]。研究揭示$CaCl_2$对不同种类成釉细胞的细胞周期的影响不同。

成釉细胞在釉质形成和釉质矿化中起着重要作用[28]。在釉质发育的分泌期、转化期和成熟期均可检测到成釉细胞凋亡。在釉质形成过程中，50%的成釉细胞在分泌后期和成熟后期发生细胞死亡。在成釉细胞有丝分裂过程中，凋亡可能通过消除子细胞的方式来调节细胞数量。在转化期，成釉细胞由高分泌期细胞转变为成熟期细胞并开始凋亡，在这一过程中，成釉细胞逐步退化，形成结构坚硬的釉质[29-31]。因此，成釉细胞凋亡将诱发釉质发育异常。本研究中发现$CaCl_2$处理ALC细胞24 h后，3.5 mmol/L $CaCl_2$促使ALC细胞凋亡，随着时间的延长，细胞对钙离子处理的适应性增强，引起的凋亡作用基本消失。当机体缺钙时补

充一定剂量的钙有利于改善体内钙平衡，但也要注意避免过量补充引发的一些负效应[32]。

钙离子是细胞存活/死亡过程中最重要的调节因子之一，能够激活或失活各种调节蛋白，如酶、转录因子或分子伴侣等[33]。GRP78是一种重要的分子伴侣，不仅参与蛋白质的正确折叠，还参与蛋白质在内质网膜上的转运，调节增殖、肿瘤进展、血管生成、自噬、化疗敏感性及细胞凋亡等[34-35]。在细胞损伤因素的刺激下，诱发内质网应激，GRP78活化后减轻内质网应激状态，减少细胞凋亡[36]。本研究中发现，2.5 mmol/L $CaCl_2$处理细胞48 h后GRP78蛋白表达显著降低，而细胞相对活力在此浓度处理组最高。3.5 mmol/L $CaCl_2$处理细胞24 h后GRP78表达显著降低，且明显引起ALC细胞凋亡，这揭示钙离子处理早期可能还有其他相关凋亡分子参与调控，随着作用时间的延长，其他效应分子对凋亡的调控效应更加明显，因此，不同浓度$CaCl_2$处理细胞48 h，细胞凋亡率无明显变化，而其他参与调控的凋亡分子还需进一步研究。

【Author contributions】 Liu XJ designed the study, performed experiments, analyzed the data and drafted the manuscript. Gao ML designed the study and performed the experiments. Ruan JP designed the study and revised the article. All authors read and approved the final manuscript as submitted.

参考文献

[1] Kurek M, Borowska B, Lubowiedzka-Gontarek B, et al. Disturbances in primary dental enamel in Polish autistic children [J]. Sci Rep, 2020, 10(1): 12751. doi: 10.1038/s41598-020-69642-3.

[2] Chun YP, Tan C, Villanueva O, et al. Overexpression of ameloblastin in secretory ameloblasts results in demarcated, hypomineralized opacities in enamel [J]. Front Physiol, 2024, 14: 1233391. doi: 10.3389/fphys.2023.1233391.

[3] Folayan MO, El Tantawi M, Oginni AB, et al. Malnutrition, enamel defects, and early childhood caries in preschool children in a suburban Nigeria population [J]. PLoS One, 2020, 15(7): e0232998. doi: 10.1371/journal.pone.0232998.

[4] Kim HY, Cooley V, Kim EJ, et al. Adult dental epithelial stem cell-derived organoids deposit hydroxylapatite biomineral [J]. Int J Oral Sci, 2023, 15(1): 55. doi: 10.1038/s41368-023-00257-w.

[5] Zhang X, Zhang Y, Li Y, et al. Restorative dental resin functionalized with calcium methacrylate with a hydroxyapatite remineralization capacity [J]. Materials(Basel), 2023, 16(19): 6497. doi: 10.3390/ma16196497.

[6] Mendonça LC, Rodrigues M, Bicalho AA, et al. Use of computerized microtomography, energy dispersive spectroscopy, scanning electron microscopy, and atomic force microscopy to monitor effects of adding calcium to bleaching gels [J]. Oper Dent, 2022, 47(3): e162-e173. doi: 10.2341/20-217-L.

[7] Costiniti V, Bomfim GH, Mitaishvili E, et al. Calcium transport in specialized dental epithelia and its modulation by fluoride [J]. Front Endocrinol (Lausanne), 2021, 12: 730913. doi: 10.3389/fendo.2021.730913.

[8] Ibrahim HF, Hassan GS. Qualitative and quantitative assessment of the potential effect of cigarette smoking on enamel of human smokers' teeth [J]. Arch Oral Biol, 2021, 121: 104953. doi: 10.1016/j.archoralbio.2020.104953.

[9] Costiniti V, Bomfim GHS, Neginskaya M, et al. Mitochondria modulate ameloblast Ca^{2+} signaling [J]. FASEB J, 2022, 36(2): e22169. doi: 10.1096/fj.202100602R.

[10] Said R, Lobanova L, Papagerakis S, et al. Calcium sets the clock in ameloblasts [J]. Front Physiol, 2020, 11: 920. doi: 10.3389/fphys.2020.00920.

[11] Souza Bomfim GH, Mitaishvili E, Schnetkamp PPM, et al. Na^+/Ca^{2+} exchange in enamel cells is dominated by the K^+-dependent NCKX exchanger [J]. J Gen Physiol, 2024, 156(1): e202313372. doi: 10.1085/jgp.202313372.

[12] Wald T, Verma A, Cooley V, et al. A suite of mouse reagents for studying amelogenesis [J]. bioRxiv, 2023. doi: 10.1101/2023.03.30.534992.

[13] Quan J, Liu Y, Ji L, et al. A novel DLX3 mutation causes tricho-dento-osseous syndrome with abnormal enamel structure and formation [J]. Arch Oral Biol, 2024, 157: 105849. doi: 10.1016/j.archoralbio.2023.105849.

[14] Chiba Y, Yoshizaki K, Sato H, et al. Deficiency of G protein-coupled receptor Gpr111/Adgrf2 causes enamel hypomineralization in mice by alteration of the expression of kallikrein-related peptidase 4 (Klk4) during pH cycling process [J]. FASEB J, 2023, 37(4): e22861. doi: 10.1096/fj.202202053R.

[15] Le MH, Nakano Y, Abduweli Uyghurturk D, et al. Fluoride alters Klk4 expression in maturation ameloblasts through androgen and progesterone receptor signaling [J]. Front Physiol, 2017, 8: 925. doi: 10.3389/fphys.2017.00925.

[16] Li J, Wang P, Gao J, et al. NaF reduces KLK4 gene expression by decreasing Foxo1 in LS8 cells [J]. Biol Trace Elem Res, 2018, 186(2): 498-504. doi: 10.1007/s12011-018-1325-y.

[17] Hua T, Robitaille M, Roberts-Thomson SJ, et al. The intersection between cysteine proteases, Ca^{2+} signalling and cancer cell apoptosis [J]. Biochim Biophys Acta Mol Cell Res, 2023, 1870(7): 119532. doi: 10.1016/j.bbamcr.2023.119532.

[18] Ahamad N, Sun Y, Singh BB. Increasing cytosolic Ca^{2+} levels restore cell proliferation and stem cell potency in aged MSCs [J]. Stem Cell Res, 2021, 56: 102560. doi: 10.1016/j. scr. 2021. 102560.

[19] Zhang Y, Zhang K, Ma L, et al. Fluoride induced endoplasmic reticulum stress and calcium overload in ameloblasts [J]. Arch Oral Biol, 2016, 69: 95-101. doi: 10.1016/j.archoralbio.2016.05.015.

[20] Li J, Yang K, Dai S, et al. ERS mediated by GRP-78/PERK/CHOP signaling is involved in fluoride-induced ameloblast apoptosis [J]. Biol Trace Elem Res, 2024, 202(3): 1103-1114. doi: 10.1007/

s12011-023-03746-5.

[21] Földes A, Sang-Ngoen T, Kádár K, et al. Three-dimensional culture of ameloblast-originated HAT-7 cells for functional modeling of defective tooth enamel formation [J]. Front Pharmacol, 2021, 12: 682654. doi: 10.3389/fphar.2021.682654.

[22] Gao J, Gao Z, Dang F, et al. Calcium promotes differentiation in ameloblast-like LS8 cells by downregulation of phosphatidylinositol 3 kinase/protein kinase B pathway [J]. Arch Oral Biol, 2020, 109: 104579. doi: 10.1016/j.archoralbio.2019.104579.

[23] Li Q, Guo Y, Yao M, et al. Methylation of Cdkn1c may be involved in the regulation of tooth development through cell cycle inhibition [J]. J Mol Histol, 2018, 49(5): 459-469. doi: 10.1007/s10735-018-9785-0.

[24] Chan B, Cheng IC, Rozita J, et al. Sodium/(calcium + potassium) exchanger NCKX4 optimizes KLK4 activity in the enamel matrix microenvironment to regulate ECM modeling [J]. Front Physiol, 2023, 14: 1116091. doi: 10.3389/fphys.2023.1116091.

[25] Patergnani S, Danese A, Bouhamida E, et al. Various aspects of calcium signaling in the regulation of apoptosis, autophagy, cell proliferation, and cancer [J]. Int J Mol Sci, 2020, 21(21): 8323. doi: 10.3390/ijms21218323.

[26] Tong J, Sun L, Zhu B, et al. Pulsed electromagnetic fields promote the proliferation and differentiation of osteoblasts by reinforcing intracellular calcium transients [J]. Bioelectromagnetics, 2017, 38(7): 541-549. doi: 10.1002/bem.22076.

[27] Park WH. Propyl gallate induces human pulmonary fibroblast cell death through the regulation of Bax and caspase-3 [J]. Ann Med, 2024, 56(1): 2319853. doi: 10.1080/07853890.2024.2319853.

[28] 朱蜜蜜, 高艳, 高玉光. Sirt1调控上皮细胞衰老的研究进展[J]. 口腔疾病防治, 2023, 31(2): 142-146. doi:10.12016/j.issn.2096-1456.2023.02.010.
Zhu MM, Gao Y, Gao YG. Research progress on the regulation of epithelial cell senescence by Sirt1[J]. J Prev Treat Stomato Dis, 2023, 31(2): 142-146.doi:10.12016/j.issn.2096-1456.2023.02.010.

[29] Zhu X, Ma Z, Xie F, et al. ASH2L, core subunit of H3K4 methylation complex, regulates amelogenesis [J]. J Dent Res, 2024, 103(1): 81-90. doi: 10.1177/00220345231207309.

[30] Alghadeer A, Hanson-Drury S, Patni AP, et al. Single-cell census of human tooth development enables generation of human enamel [J]. Dev Cell, 2023, 58(20): 2163-2180.e9. doi: 10.1016/j.devcel.2023.07.013.

[31] Mu H, Dong Z, Wang Y, et al. Odontogenesis-associated phosphoprotein (ODAPH) overexpression in ameloblasts disrupts enamel formation via inducing abnormal mineralization of enamel in secretory stage [J]. Calcif Tissue Int, 2022, 111(6): 611-621. doi: 10.1007/s00223-022-01023-6.

[32] Wang H, Zhang Y, Xin F, et al. Calcium-induced autonomic denervation in patients with post-operative atrial fibrillation [J]. J Am Coll Cardiol, 2021, 77(1): 57-67. doi: 10.1016/j.jacc.2020.10.049.

[33] Zhang IX, Herrmann A, Leon J, et al. ER stress increases expression of intracellular calcium channel RyR1 to modify Ca^{2+} homeostasis in pancreatic beta cells [J]. J Biol Chem, 2023, 299(8): 105065. doi: 10.1016/j.jbc.2023.105065.

[34] Gong Y, Zhang W, Yan P, et al. Pranoprofen inhibits endoplasmic reticulum stress-mediated apoptosis of chondrocytes [J]. Panminerva Med, 2020. doi: 10.23736/S0031-0808.20.03980-4.

[35] Zhang HX, Yuan J, Li RS. Thalidomide mitigates apoptosis via endoplasmic reticulum stress in diabetic nephropathy [J]. Endocr Metab Immune Disord Drug Targets, 2022, 22(7): 787-794. doi: 10.2174/1871530322666211230115743.

[36] Hernandez I, Cohen M. Linking cell-surface GRP78 to cancer: From basic research to clinical value of GRP78 antibodies [J]. Cancer Lett, 2022, 524: 1-14. doi: 10.1016/j.canlet.2021.10.004.

（编辑　罗燕鸿，邵龙泉）

官网