低氧对血清剥夺后山羊颞下颌关节盘细胞凋亡和自噬的影响

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摘要 该研究体外分离山羊颞下颌关节盘(temporomandibular joint disc, TMJ disc)细胞并培养至p2代。通过HE(Hematoxylin-Eosin)染色、Hoechst 33258和丹磺酰戊二胺(dansylcadaverine, MDC)荧光染色观察血清剥夺后细胞的形态学变化以及是否存在自噬和凋亡。随后, 通过流式细胞术和实时荧光定量聚合酶链反应(Real-time PCR)分别检测血清剥夺0 h、12 h、24 h、36 h、48 h和60 h后细胞的凋亡率和自噬水平的变化以及加入自噬抑制剂3-MA(3-methyladenine)后的凋亡变化。检测给予血清(10% FBS)或血清剥夺(0% FBS)的细胞在常氧(21% O_2)或低氧(2% O_2)条件下的凋亡和自噬的变化。结果发现: 血清剥夺后, 凋亡率随时间的延长逐渐上升, 自噬率先上升后下降; 当血清剥夺诱导的自噬被3-MA抑制后, 细胞的凋亡率明显上升。自噬能够抑制血清剥夺引起的细胞凋亡, 说明自噬在细胞的存活中有很重要的作用。与常氧培养的细胞相比, 低氧条件下细胞的凋亡率和自噬率均下降。低氧通过降低细胞的过度自噬减少长期血清剥夺引起的细胞凋亡, 比常氧更有利于细胞的存活。

关键词 颞下颌关节盘; 自噬; 凋亡; 血清剥夺; 低氧

Effects of Hypoxia on the Apoptosis and Autophagy of the Goat Temporomandibular Joint Disc Cells after Serum Deprivation

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Abstract In this work, they isolated and cultured temporomandibular joint disc (TMJ disc) cells of goat to p2 generation *in vitro*. The morphological changes of cells were observed by Hematoxylin-Eosin (HE) staining, Hoechst 33258 and dansylcadaverine (MDC) fluorescence staining were used to observe whether autophagy or apoptosis existed after the serum deprivation. After 0 h, 12 h, 24 h, 36 h, 48 h and 60 h of serum deprivation, apoptosis rate and autophagy rate of cells were detected by flow cytometry and Real-time PCR, respectively. Changes of apoptosis was observed after applying 3-methyladenine (3-MA), an autophagy inhibitor. The changes of apoptosis and autophagy with serum (10% FBS) or serum deprivation (0% FBS) were detected under the conditions

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of oxygen (21% O_2) or hypoxia (2% O_2). The results showed that the rate of apoptosis increased gradually with the prolongation of the time, and the autophagy first increased and then decreased after the serum deprivation. When the autophagy induced by serum deprivation was inhibited by 3-MA, the rate of apoptosis of cells increased obviously. Autophagy could inhibit the apoptosis induced by serum deprivation, which showed that autophagy played an important role in cell survival. Compared with normoxia-cultured cells, the apoptosis rate and autophagy rate of the cells decreased under hypoxia condition. Hypoxia reduced the apoptosis of cells caused by long-term serum deprivation by reducing excessive autophagy, which was more conducive to cell survival than normoxia.

Keywords temporomandibular joint disc; autophagy; apoptosis; serum deprivation; hypoxia

TMJ关节盘(temporomandibular joint disc, TMJ disc)位于颞骨关节面和髁突之间, 将关节腔分为完全不交通的关节上腔和关节下腔, 关节盘具有较强的抗压和抗摩擦力, 主要功能有: 协调颞骨和髁突关节面的形态、大小差异; 分散负荷、缓解咀嚼压力; 是下颌进行正常三维运动的重要结构基础[1]。颞下颌关节盘的损伤引起关节炎等关节的退行性改变, 出现关节紊乱症状, 影响患者的生活质量[2-3]。

TMJ关节盘是无血管组织, 它的营养来自于关节盘周围滑液以及盘后区的血管的梯度扩散[4-7], 处于低血低氧的环境。关节盘受力甚至受损的情况下, 这种特殊的营养供应方式使关节盘营养进一步受限[2,5,7]。

自噬是细胞在面对低氧、感染、营养缺乏等有害刺激时的一种自我保护反应, 通过降解损坏的细胞器或者大分子物质获取能量以维持细胞的稳态[8-10]。自噬与许多退变性疾病的发生发展有关, 如: 帕金森病、阿尔茨海默病等神经退行性疾病; 骨性关节炎、风湿性关节炎等软骨退行性疾病[11]。颞下颌关节退行性改变作为退行性疾病的一种, 出现自噬现象[12]。同时, 病变的关节盘细胞也出现明显的凋亡[13-14]。因为共享基因, 自噬和凋亡常相伴发生[15], 但是在颞下颌关节盘细胞内凋亡和自噬水平如何、血清剥夺是否能够诱导凋亡和自噬的发生、低氧环境对血清剥夺后颞下颌关节盘细胞自噬和凋亡的影响如何并不清楚。本实验模拟生理缺血缺氧条件, 初步探索山羊颞下颌关节盘细胞凋亡和自噬的发生情况以及二者之间的关系。

1 材料与方法

1.1 主要试剂

丹磺酰戊二胺(dansylcadaverine, MDC)、吖啶橙(acridine orange, AO)、自噬抑制剂3-MA(3-methyladenine)购自Sigma公司; Annexin V-FITC凋亡检测试剂盒购自BD Pharmingen公司; HE染色试剂盒(Hematoxylin-Eosin/HE Staining Kit)、Hoechst 33258购自索莱宝公司。

1.2 山羊颞下颌关节盘细胞的分离和培养

自屠宰厂购买新鲜的3~6月龄山羊头, 清洗干净, 75%酒精浸泡30 min。无菌条件下整块取出双侧TMJ关节盘, 清除关节盘周围韧带及附着的肌肉组织, PBS充分冲洗3次后用眼科剪将其剪碎至约1 mm^3大小, 在37 ℃、90 r/min恒温水浴摇床上用0.2% I型胶原酶消化15 h左右, 离心收集细胞。完全培养基(含10% FBS的DMEM/F12)重悬细胞并接种于培养瓶中, 放入37 ℃、5% CO_2的培养箱中培养, 2~3天换液1次, 待细胞融合率达90%, 0.25%胰酶消化传代, 本研究主要使用传代后的第二代细胞。

1.3 实验分组及干预

常氧(21% O_2)下, 血清给与组(10% FBS, 0 h)作为对照组, 血清剥夺组(0% FBS)分别处理细胞12 h、24 h、36 h、48 h、60 h。

低氧(2% O_2)下血清给与(10% FBS)或血清剥夺(0% FBS) 36 h, 常氧(21% O_2)下的血清给与(10% FBS)或血清剥夺(0% FBS) 36 h作为对照组。

1.4 HE染色观察细胞形态学变化

将关节盘细胞以1×10^4每孔的密度接种于6孔板中的盖玻片上, 待细胞完全贴壁后给予不同刺激, 取出盖玻片。PBS轻轻漂洗2次, 4%多聚甲醛常温固定5~10 min, 苏木精染色15 min, 自来水冲洗返蓝, 分化液分化30 s, 自来水蓝化数分钟, 蒸馏水洗。经50%、70%、80%、90%梯度乙醇溶液脱水各1 min, 入伊红染液1~3 min, 入100%乙醇2次, 各1 min, 二甲苯透明1 min, 中性树胶封片, 光学显微镜下观察。实验方法参考试剂说明书。

1.5 荧光染料检测细胞凋亡和自噬

1.5.1 Hoechst 33258检测细胞凋亡 将关节盘

细胞以1×10⁴/孔的密度接种于6孔板中, 待细胞完全贴壁后给予不同刺激, 去除培养液, 加0.5 μg/mL Hoechst 33258染色液, 充分覆盖待染样品, 37 ℃培养15~20 min, PBS冲洗细胞2次, 直接在荧光显微镜下观察(激发波长350 nm, 发射波长460 nm)。实验方法参考试剂说明书。

1.5.2 MDC检测细胞自噬[16] 将关节盘细胞以1×10⁴/孔的密度接种于6孔板中, 待细胞完全贴壁后给予不同刺激, 去除培养液, 加50 μmol/L MDC染色液, 充分覆盖待染样品, 37 ℃培养25~30 min, PBS冲洗细胞3次, 直接在荧光显微镜下观察(激发波长335 nm, 发射波长518 nm)。

1.6 MTT检测细胞的生长曲线

山羊TMJ关节盘细胞以每孔4 000个细胞的密度接种于无菌96孔板中, 每孔加200 μL细胞悬液。培养细胞约24 h后换液(分3组: 10% FBS组、0% FBS组、DMEM/F12组, 每组设5个复孔), 分别于0 h、12 h、24 h、36 h、48 h、60 h测MTT。终止实验前4 h每孔加5 mg/mL MTT液20 μL, 继续孵育4 h后吸弃上清液, 避光条件下每孔加入DMSO 150 μL, 震荡器上震荡10 min, 酶标仪检测(波长490 nm)。

1.7 流式细胞仪检测细胞凋亡率和自噬率

1.7.1 流式细胞仪检测细胞凋亡率 将关节盘细胞以1×10⁵/孔的密度接种于6孔板, 细胞完全贴壁后血清剥夺进行干预。加入自噬抑制剂3-MA后, 分别于0 h、12 h、24 h、36 h、48 h、60 h各时间点用冷PBS冲洗2遍, 胰酶消化离心, PBS重悬离心2次, 弃上清, 加1×BD缓冲液100 μL重悬细胞。分别加入5 μL Annexin V和5 μL PI, 常温染色15 min, 加入400 μL 1×BD缓冲液, 半小时内通过流式细胞仪检测细胞凋亡率。实验方法参考试剂说明书。

1.7.2 流式细胞仪检测细胞自噬率[17] 将关节盘细胞以1×10⁵/孔的密度接种于6孔板, 细胞完全贴壁后血清剥夺进行干预, 分别于0 h、12 h、24 h、36 h、48 h、60 h各时间点用冷PBS冲洗2遍, 胰酶消化离心, PBS重悬离心2次, 弃上清, 加入PBS缓冲液100 μL重悬细胞, 加入5 μL AO染色液, 常温染色10 min, 加入400 μL PBS缓冲液, 半小时内通过流式细胞仪检测细胞自噬率。

1.8 Real-time PCR检测细胞自噬相关基因的表达

将关节盘细胞以1×10⁵/孔的密度接种于6孔板, 细胞完全贴壁后血清剥夺进行干预, 分别于0 h、12 h、24 h、36 h、48 h、60 h各时间点加Trizol、氯仿、异丙醇等提取总RNA。取1 μL提取的RNA经超微量分光光度计检测D_{260}/D_{280}的比值在1.8~2.0, 提示RNA质量较好。总RNA用gDNA Eraser处理后, 采用逆转录试剂盒PrimeScript™ RT reagent Kit with gDNA Eraser将纯化的RNA逆转录合成cDNA。以cDNA作为模板进行Real-time PCR扩增。Real-time PCR采用TaKaRa公司的SYBR Premix Ex Taq II试剂盒, 在Agilent Mx3000P Real-time PCR仪上进行, 分别扩增*Beclin 1*、*LC3-II*和内参*GAPDH*基因。*Beclin 1*上游引物: 5′-GGC TGA GAG ACT GGA TCA GG-3′, 下游引物: 5′-CTG CGT CTG GGC ATA ACG-3′; *LC3-II*上游引物: 5′-GAG AAG CAG CTT CCT GTT CTG G-3′, 下游引物: 5′-GTG TCC GTT CAC CAA CAG GAA G-3′; *GAPDH*上游引物: 5′-AGG GCT GCT TTT AAC TCT GGT-3′, 下游引物: 5′-CCC CAC TTG ATT TTG GAG GGA-3′。计算基因的相对表达量$F=2^{-\Delta\Delta Ct}$。

1.9 数据统计

应用SPSS 24.0统计软件分析, 实验数据以mean±S.E.M.形式表示, 多组数据使用单因素方差分析(ANOVA), 各组间数据差异比较采用LSD检验, 两组数据间使用配对*t*检验。$P<0.05$为差异有统计学意义。

2 结果

2.1 血清剥夺后山羊颞下颌关节盘细胞的形态学表现

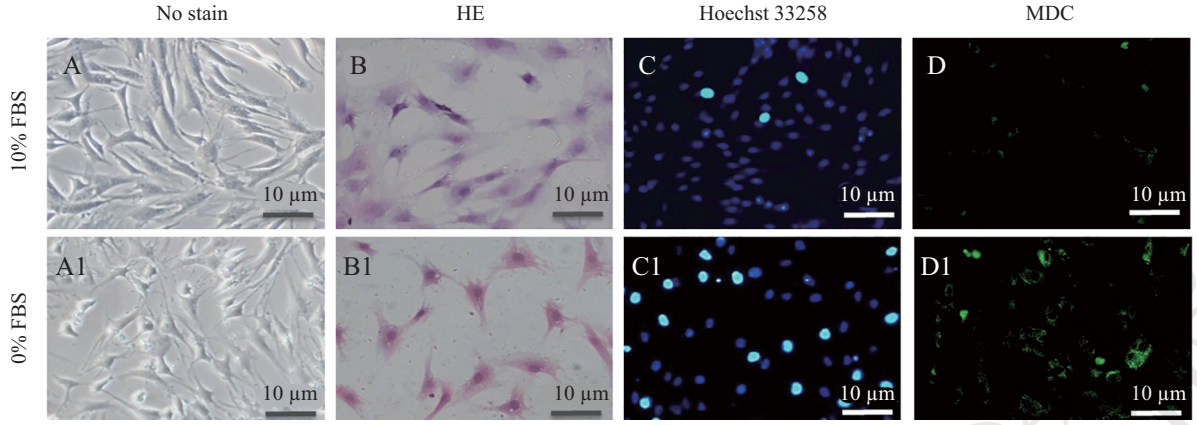
光学显微镜下可见, 与血清给与组相比, 血清剥夺后细胞由长梭形变为卵圆多边形。荧光显微镜下观察到血清剥夺组的细胞经Hoechst 33258染色后, 出现明显的亮蓝色, 说明血清剥夺诱导细胞发生凋亡。血清剥夺后的颞下颌关节盘细胞经MDC染色后, 荧光显微镜下可观察到明显的自噬现象(图1)。

2.2 血清剥夺后山羊颞下颌关节盘细胞的生长变化

MTT结果显示, 血清剥夺后细胞呈现负增长的趋势, 随着时间的延长, 细胞的存活率逐渐下降(图2)。

2.3 血清剥夺诱导山羊颞下颌关节盘细胞发生凋亡和自噬

Annexin-V/PI染色的流式结果显示, 血清剥夺24 h后细胞出现明显的凋亡现象, 随着时间的延长, 细胞的凋亡率逐渐升高, 而细胞的存活率逐渐下降,



A~D依次为血清给与条件下关节盘细胞的未染色、HE染色、Hoechst 33258荧光染色、MDC荧光染色图; A1~D1依次为血清剥夺条件下关节盘细胞的未染色、HE染色、Hoechst 33258荧光染色、MDC荧光染色图。

A-D were observed with serum: no staining, HE staining, Hoechst 33258 fluorescence staining, MDC fluorescence staining; A1-D1 were observed under the condition of serum deprivation: no staining, HE staining, Hoechst 33258 fluorescent staining, MDC fluorescence staining.

图1 血清剥夺前后山羊TMJ关节盘细胞的显微观察

Fig.1 Microscopic observation of TMJ disc cells of goat before and after serum deprivation

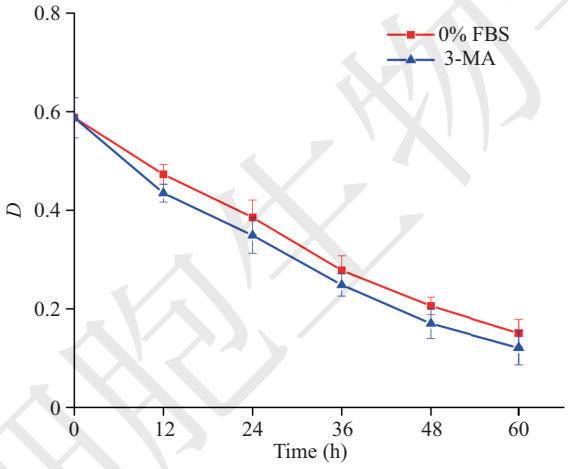


图2 血清剥夺前后山羊TMJ关节盘细胞的生长曲线

Fig.2 Growth curve of TMJ disc cells in goat before and after serum deprivation

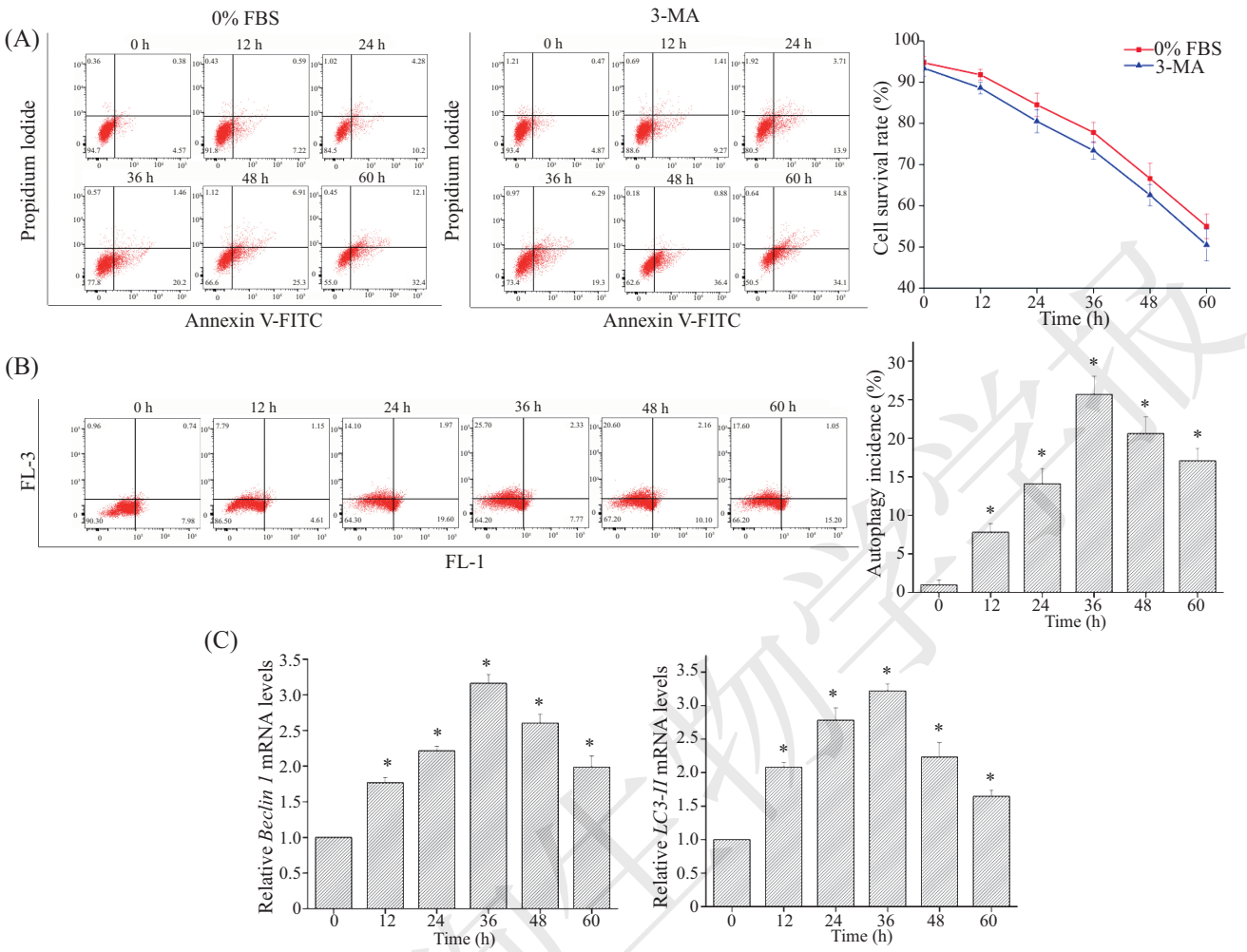
自噬抑制剂3-MA的加入使细胞的存活率进一步下降(图3A), 这与MTT检测结果的变化趋势一致。AO染色流式结果发现, 血清剥夺12 h后细胞即可发生明显自噬, 36 h达峰值, 随后细胞的自噬率呈下降趋势(图3B); Real-time PCR检测自噬相关基因*Beclin 1*和*LC3-II*的表达水平, 细胞自噬水平的变化趋势跟流式结果一致(图3C)。

2.4 低氧培养有利于细胞的存活

Annexin-V/PI染色的流式结果显示, 血清剥夺后低氧培养的细胞存活率高于常氧下细胞的存活率, 加入自噬抑制剂3-MA后, 常氧和低氧培养的细胞存活率均下调(图4A)。AO染色的流式结果发现, 无论是给与血清还是血清剥夺, 与常氧培养的细胞相比, 低氧下细胞的自噬率明显降低(图4B)。Real-time PCR检测自噬相关基因*Beclin 1*和*LC3-II*的表达水平, 结果跟流式结果一致(图4C)。

3 讨论

TMJ关节盘是无血管组织, 主要营养来源于其周围滑液和血管的梯度供应, 处于低血低氧环境中, 病理条件下, 细胞的营养供应进一步受限[2,4-7]。本实验采用2%的氧浓度, 因为本课题组前期研究发现, 2%的氧浓度更适合山羊TMJ关节盘细胞的扩增[18]。*Beclin 1*和*LC3-II*作为自噬相关标记物, 在软骨的退行性改变的发生过程中明显增加, 说明自噬与关节退行性改变密切相关[19-20]。凋亡作为程序性细胞死



A: 流式细胞术检测血清剥夺0 h、12 h、24 h、36 h、48 h、60 h后细胞的凋亡率及存活率; B: 流式细胞术检测血清剥夺0 h、12 h、24 h、36 h、48 h、60 h后细胞的自噬率; C: Real-time PCR检测血清剥夺0 h、12 h、24 h、36 h、48 h、60 h后自噬相关基因*Beclin 1*和*LC3-II*的表达水平。数据表示为均数±标准误。*P<0.05, 与0 h组比较。

A: the apoptosis rate of cells with serum were detected by flow cytometry, as well as serum deprivation at 0 h, 12 h, 24 h, 36 h, 48 h and 60 h; B: the autophagy rate of cells with serum were detected by flow cytometry, as well as serum deprivation at 0 h, 12 h, 24 h, 36 h, 48 h and 60 h; C: the expression levels of autophagy-related genes *Beclin 1* and *LC3-II* with serum deprivation were detected by Real-time PCR, as well as serum deprivation at 12 h, 24 h, 36 h, 48 h and 60 h. Values were presented as mean±S.E.M.. *P<0.05 compared with 0 h group.

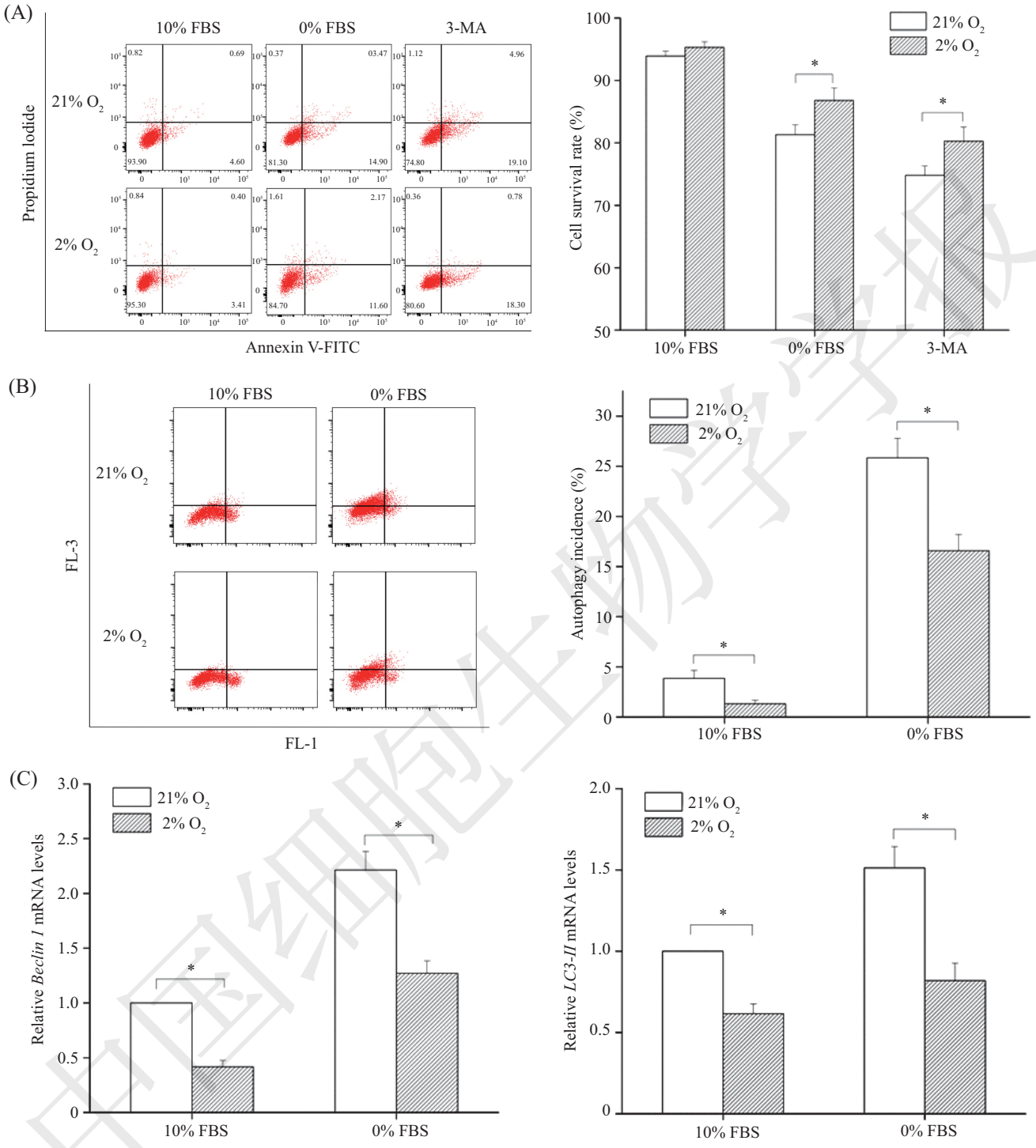
图3 血清剥夺前后山羊TMJ关节盘细胞凋亡和自噬的变化

Fig.3 Changes of apoptosis and autophagy of TMJ disc cells in goat before and after serum deprivation

亡的另一种方式, 可以跟自噬被同一刺激诱发, 两种死亡方式在分子水平可能存在相同的信号通路, 二者相互影响[13-14,15,21-22]。自噬抑制剂3-MA通过下调自噬相关标记物的表达增加细胞凋亡率, 说明自噬对细胞的存活有重要意义[23-25]。

本研究结果表明, 无血清培养的山羊TMJ关节盘细胞出现明显的自噬和凋亡现象, 抑制自噬后凋亡水平明显上升, 说明血清剥夺过程中两种细胞死亡方式同时存在。这与椎间盘细胞在无血清培养条件下的变化趋势一致[26]。低氧条件下细胞的凋亡率和自噬率均下降, 自噬抑制后细胞的凋亡率上升, 说明低氧有利于细胞存活。Cisewski等[5]的研究发现, 低氧条件下TMJ关节盘细胞ATP的产生及基质的合成均下降, 但与常氧条件下培养的细胞相比, 低氧下TMJ关节盘细胞的存活率增加。Fan等[27]通过基因*PADI4*的敲除抑制低氧条件下成纤维样细胞的自噬, 引起细胞的凋亡, 说明低氧条件下细胞的凋亡也可能受自噬调节, 椎间盘细胞研究中这一过程的发生与线粒体损坏产生过量的氧自由基有关[16]。

我们发现, 血清剥夺能够诱导细胞发生凋亡和自噬, 而低氧有利于细胞的存活, 但相关分子机制还不清楚。颞下颌关节是负重关节, 承受的载荷比较



A: 流式细胞术检测常氧和低氧条件下血清剥夺前后细胞的凋亡率及存活率; B: 流式细胞术检测常氧和低氧条件下血清剥夺前后细胞的自噬率; C: Real-time PCR检测常氧和低氧条件下血清剥夺前后自噬相关自噬相关基因*Beclin 1*和*LC3-II*的表达水平。数据表示为均数±标准误, **P*<0.05。

A: the apoptosis rate of cells before and after serum deprivation under normoxia and hypoxia was detected by flow cytometry; B: the autophagy rate of cells before and after serum deprivation under normoxia and hypoxia was detected by flow cytometry; C: Real-time PCR was used to detect the expression of autophagy-related genes *Beclin 1* and *LC3-II* before and after serum deprivation under normoxia and hypoxia. Values were presented as mean±S.E.M., **P*<0.05.

图4 低氧条件下山羊TMJ关节盘细胞自噬和凋亡的变化

Fig.4 Changes of autophagy and apoptosis of TMJ disc cells in goat under hypoxic conditions

复杂, 比如拉应力、压应力、剪切力或者三者的混合[28-29]。力学因素对TMJ关节盘细胞凋亡和自噬的影响以及二者之间的关系与机理尚无实验证明。

本研究通过Hoechst 33258、MDC荧光染色观察凋亡和自噬的发生, 流式检测凋亡率和自噬率, 以及Real-time PCR检测自噬相关基因*Beclin 1*和*LC3-II*的mRNA的水平, 主要目的在于初步证明山羊颞下颌关节盘细胞中是否存在自噬和凋亡现象, 为探索血清剥夺及低氧对于自噬和凋亡的影响奠定基础。今后将通过扫描电镜对培养细胞进行自噬小体的切片观察并结合下游产物的蛋白水平分析, 进行自噬、低氧和血清剥夺相关关系的机理研究, 在蛋白水平揭示颞下颌关节盘细胞发生自噬和凋亡的变化规律。

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