·基础研究·

热量限制对老年小鼠骨骼肌卫星细胞增殖能力的影响

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【摘要】 **目的**　探讨热量限制对老龄小鼠骨骼肌卫星细胞增殖能力减退的延缓作用。**方法**　选取 12～13 月龄的 C57BL 雄性小鼠 12 只,按随机数字表法均分为实验组和对照组,每组 6 只,均单笼饲养。实验开始前,2 组小鼠均适应性饲养 2 周,计算平均进食量作为对照组投食量,约 75.09 kJ/d;实验组采用热量限制法投食,投食量为对照组的 60%,约 45.05 kJ/d。逐日投放饲料,每周称量体重一次,至实验第 15 周结束。分别比较 2 组小鼠实验前和实验第 15 周(实验后)的体重变化。实验第 15 周后分别取后肢骨骼肌制备单核细胞悬液,采用流式细胞术分选小鼠肌卫星细胞,并进行细胞周期测定;用免疫印迹法检测细胞周期蛋白(cyclin)A、cyclin D1、cyclin E 的表达,并进行统计学分析比较。**结果**　实验前,实验组和对照组小鼠体重分别为(30.7±0.4)g和(30.8±0.3)g,组间差异无统计学意义($P>0.05$);实验后,实验组小鼠的体重降至(19.5±0.4)g,较组内实验前明显降低($P<0.001$),且明显低于对照组小鼠的体重[(31.9±0.5)g],组间差异有统计学意义($P<0.001$)。与对照组[G_0/G_1 期(89.78±0.37)%;S 期(4.29±1.57)%]相比,实验组骨骼肌卫星细胞的 G_0/G_1 期比例[(62.18±0.42)%]明显下降($P<0.001$),S 期比例[(28.75±0.30)%]显著升高($P<0.001$);而且 cyclin A($P<0.001$)、cyclin E($P<0.01$)表达增强,cyclin D1 表达降低($P<0.01$)。**结论**　热量限制可以延缓老龄小鼠骨骼肌卫星细胞增殖能力的减退。

【关键词】 热量限制;　衰老;　肌卫星细胞;　增殖

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The effect of calorie restriction on the proliferation of satellite cells in elderly rats' skeletal muscles

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【**Abstract**】 **Objective**　To explore any effect of calorie restriction on the proliferation of satellite cells in the skeletal muscles of elderly rats. **Methods**　Twelve male C57BL rats aged 12 or 13 months were randomly divided into an experimental group and a control group, each of 6. The control group was fed 75.09 kJ/d as normal, while the experimental group was provided with 45.05 kJ/d (60% of normal). The intervention lasted for 15 weeks and each rat's weight was measured every week. After the intervention, limb muscle satellite cells were sorted by fluorescence-activated cell sorting after digestion, and the cell cycle was analyzed. Western blotting was used to assess the expression of cyclin A, D1 and E. **Results**　There was no significant difference in the average weight of the two groups before the experiment. After the 15 weeks the average weight of the experimental group had decreased significantly (to 19.5±0.4 g), and it was significantly lighter than that of the control group (31.9±0.5 g). The average percentage of the satellite cells in the G_0/G_1 phase had decreased significantly in the experimental group, but the percentage in the S phase had increased significantly. The expression of cyclin A and E was significantly greater in the experimental group compared with the control group, but the expression of cyclin D1 had decreased significantly. **Conclusion**　Caloric restriction can delay the proliferation of satellite cells in the skeletal muscles of elderly mice.

【**Key words**】 Calorie restriction;　Aging;　Muscle satellite cells;　Proliferation

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肌卫星细胞(muscle satellite cell)是骨骼肌组织内主要的干细胞成分,其功能是在机体疾病或受伤后增殖和再生,维持机体基本稳态[1],随着机体的衰老,肌卫星细胞的功能和活性也随之降低,表现为自我更新能力进行性减退;同时,干细胞增殖潜能逐渐降低,导致细胞增殖进程受损,分裂增殖失能[2-3]。

热量限制(calorie restriction)是在不引起营养不良的情况下,将每日摄入能量减少,通常可减少至正常

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饮食的 60%～80%。热量限制在细胞保护、机体修复及调节代谢方面均扮演了重要角色,被认为是一种有望延缓衰老,减轻年龄相关性疾病进程的干预手段[4]。本研究通过探讨热量限制对老年小鼠骨骼肌卫星细胞增殖能力的影响,以期为临床上应用热量限制法来防治老龄性肌肉衰老提供理论依据。

材料与方法

一、实验动物与材料

实验动物:选取 12～13 月龄的 C57BL/6 雄性小鼠 12 只,由苏州工业园区爱尔麦特科技有限公司提供。

实验材料:Collagenase/Dispase 酶(美国 Roche 公司);抗小鼠 CD45 藻红蛋白(美国 eBioscience 公司);抗小鼠 CD11b 藻红蛋白(美国 eBioscience 公司);抗小鼠 Sca-1 藻红蛋白(美国 eBioscience 公司);抗小鼠 CXCR4 别藻蓝蛋白(美国 eBioscience 公司);抗小鼠 CD29/β1-integrin 异硫氰酸荧光素(美国 eBioscience 公司);兔抗成肌分化(myogenic differentiation,MyoD)蛋白(英国 abcam 公司);羊抗兔藻红蛋白(天津三箭生物技术股份有限公司);细胞周期蛋白(cyclin)A 抗体(沈阳万类生物科技公司);cyclin D1 抗体(沈阳万类生物科技公司);cyclin E 抗体(沈阳万类生物科技公司);甘油醛-3-磷酸脱氢酶(glceraldehyde-3-phosphate dehydrogenase,GAPDH)抗体(美国 Bioworld 公司)。

二、实验方法及观测指标

1. 小鼠热量限制饮食方法:12～13 月龄的 C57BL 雄性小鼠 12 只,饲养于 12 h 光照与黑暗循环的(22±2)℃环境中,按随机数字表法分为实验组和对照组,每组 6 只,均单笼饲养。饲料采用华阜康生物 SPF 级大小鼠维持饲料,总热能 13.29 kJ/g(热量百分比:蛋白质 24.06%、脂肪 11.13%、碳水化合物 64.81%)。实验开始前,2 组小鼠均适应性饲养 2 周,计算平均进食量作为对照组投食量,约 75.09 kJ/d;实验组投食量为对照组的 60%,约 45.05 kJ/d。逐日投放饲料,每周称量体重 1 次,至第 15 周结束。分别比较 2 组小鼠实验前和实验第 15 周(实验后)的体重变化。

2. 原代骨骼肌卫星细胞的细胞周期测定方法:于实验第 15 周时,参考 Hashimoto[5] 及 Liu[6] 的方法,脱颈处死全部小鼠,并用 75%酒精浸泡消毒 5 min,无菌条件下尽可能分离小鼠双后肢肌肉,磷酸盐缓冲液(phosphate buffered solution,PBS)冲洗后,用眼科剪剪成肉糜;将肉糜与 1 mg/ml 的胶原酶或中性蛋白酶充分混合后,用 37 ℃水浴消化 1 h,消化过程中持续以 60～70 r/min 的频率振荡;加入含有 10%胎牛血清的细胞培养基(种类:Ham-F10),500 g/min 离心 5 min 后弃上清再次重悬,充分吹打均匀后分别过 100 μm 及 40 μm的细胞筛,得到骨骼肌单核细胞悬液。流式抗体4 ℃避光孵育 40 min,室温避光固定 40 min;加入细胞透膜液、核糖核酸酶(ribonuclease,RNase)A、4′,6-二脒基-2-苯基吲哚(4′, 6-diamidino-2-phenylindole,DAPI)染液共同孵育 1 h。行流式细胞仪检测,以 $CD45^-/CD11b^-/Scal\text{-}1^-/CXCR4^+/\beta1$-整联蛋白(integrin)$^+$方案标记出的为小鼠原代肌卫星细胞[7],测定小鼠肌肉卫星细胞群所处周期,即目标细胞周期。

3. MyoD 蛋白免疫荧光染色方法:分选出的卫星细胞培养后 95%乙醇固定 15 min;PBS 冲洗后 5%脱脂奶粉封闭 20 min;PBS 冲洗,加入兔抗 MyoD 蛋白(1∶100)4 ℃孵育过夜;PBS 洗去冲洗后加入羊抗兔藻红蛋白(1∶200)37 ℃孵育 1 h;PBS 漂洗,滴加DAPI 染液覆盖,染色 1 min 后荧光显微镜观察,随机取 5 个视野拍照。

4. 免疫印迹法检测细胞周期相关蛋白的相对表达量:小鼠胫前肌冰上剪碎,加细胞裂解液提取蛋白。用二辛可宁酸法检测总蛋白浓度,调整单孔上样量为 20 μg。经聚丙烯酰胺凝胶电泳分离,16 V 转膜 50 min,封闭 1 h,一抗(1∶1000 到 1∶8000)4 ℃避光翻转孵育过夜;洗膜 3 次,二抗(1∶8000)室温避光翻转孵育 1 h,再次洗膜后,电化学发光检测显色。使用 Quantity-one 软件进行半定量分析,采用目的条带光密度值与 GAPDH 条带的光密度值的比值来反映目的蛋白的相对表达量。

三、统计学方法

使用 GraphPad 6.0 版软件对所有数据进行统计学分析处理,数据以($\bar{x}\pm s$)表示,组间比较使用 t 检验,小鼠体重组内比较采用方差分析,$P<0.05$认为差异有统计学意义。

结　果

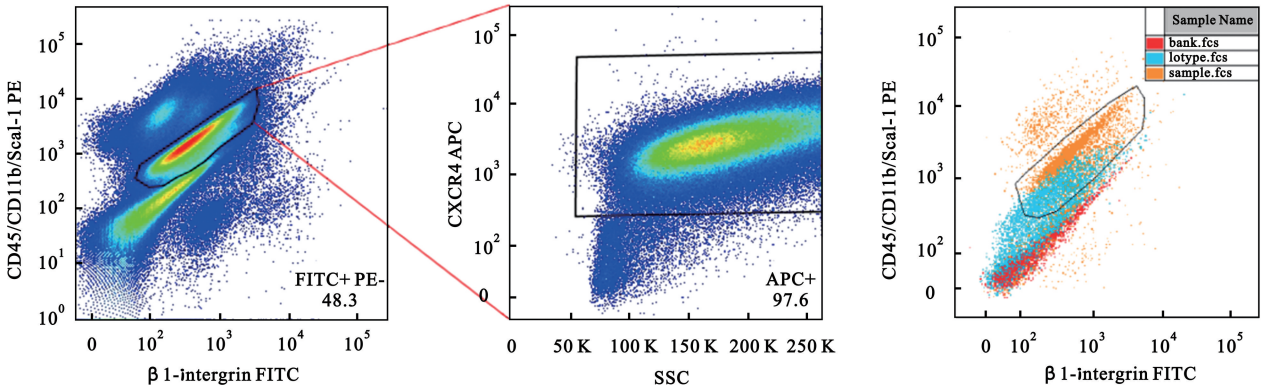
一、热量限制对小鼠体重的影响

实验前,实验组小鼠体重[(30.7±0.4)g]与对照组小鼠体重[(30.8±0.3)g]比较,组间差异无统计学意义($P>0.05$);实验第 15 周时,实验组小鼠的体重[(19.5±0.4)g]较对照组小鼠[(31.9±0.5)g]明显降低,差异均有统计学意义($P<0.001$)。对照组小鼠实验前体重与第 15 周体重差异无统计学意义($P>0.05$);实验组小鼠实验前体重与第 15 周体重差异有统计学意义($P<0.001$)。详见表 1。

表 1　各组小鼠实验前、后体重变化(g,$\bar{x}\pm s$)

组别	只数	实验前	实验第 15 周
对照组	6	30.8±0.3	31.9±0.5
实验组	6	30.7±0.4	19.5 ± 0.4^{ab}

注:与组内实验前比较,[a]$P<0.001$;与对照组同时间点比较,[b]$P<0.001$



注：左图红框内细胞群是表面标记为 $CD45^-/CD11b^-/Scal\text{-}1^-/\beta1$-整联蛋白$^+$的细胞；中图黑框内的细胞群是表面标记为 $CD45^-/CD11b^-/Scal\text{-}1^-/CXCR4^+/\beta1$-整联蛋白$^+$的卫星细胞；右图为藻红蛋白（PE）染色的空白对照、同型抗体对照及样品染色的重合图：红色为空白对照，蓝色为同型对照，黄色为样品染色

图 1　小鼠肌肉细胞流式分选图

二、骨骼肌卫星细胞分选

抗小鼠 CD45/CD11b/Scal-1 占据藻红蛋白（phycoerythrin，PE）通道，目的细胞阴性表达；抗小鼠 β1-整联蛋白（integrin）占据异硫氰酸荧光素（fluorescein isothiocyanate，FITC）通道，目的细胞呈阳性表达；抗小鼠 CXCR4 占据别藻蓝蛋白（allophycocyanin，APC）通道，目的细胞呈阳性表达。具体分群及同型抗体染色详见图 1。

三、细胞周期对比

原代卫星细胞周期图（图 2）显示，与对照组相比，实验组 G_0/G_1 期卫星细胞比例（图中深绿色部分）降低，S 期细胞比例（图中黄色部分）显著增高，且差异均有统计学意义（$P<0.001$），G_2/M 期细胞比例（图中浅蓝色部分）组间比较，差异无统计学意义（$P>0.05$）。详见表 2。

表 2　各组小鼠卫星细胞周期百分率比较

（%，$\bar{x}\pm s$）

组别	只数	G_0/G_1 期	S 期	G_2/M 期
对照组	3	89.78±0.37	4.29±1.57	6.11±1.13
实验组	3	62.18±0.42[a]	28.75±0.30[a]	8.56±1.34

注：与对照组比较，[a]$P<0.001$

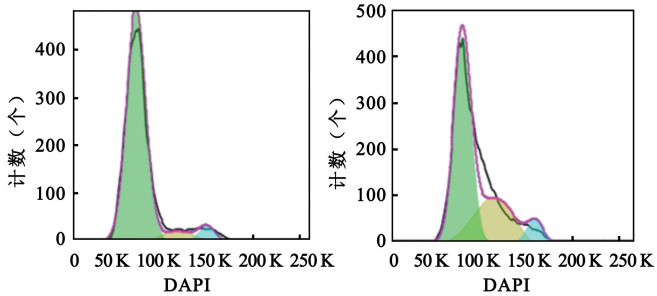
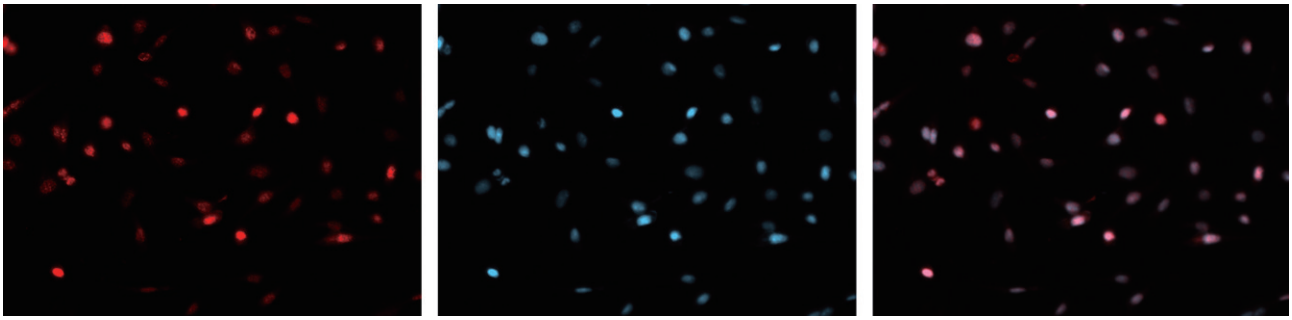


图 2　原代卫星细胞周期图

四、骨骼肌卫星细胞的鉴定

MyoD 蛋白为活化卫星细胞的标记蛋白，分选出的卫星细胞培养后行 MyoD 蛋白荧光染色，同时行 DAPI 染色细胞核。可见 95% 以上染色可重合，证明分选出的细胞为肌卫星细胞，超过 95% 以上的细胞双染均显色，可进一步确认分选出的细胞为肌卫星细胞。详见图 3。

五、周期相关蛋白的相对表达量与对照组相比，实验组 S 期蛋白 cyclin A（$P<0.001$）、G_1/S 期蛋白 cyclin E（$P<0.01$）表达增强（图 4），G_1 期蛋白 cyclin D1 表达降低，且差异有统计学意义（$P<0.01$），详见图 5。



MyoD 染色　　DAPI 染色　　重合图

图 3　原代卫星细胞 MyoD 鉴定

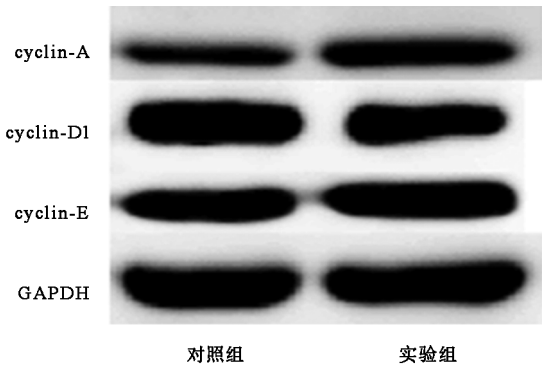
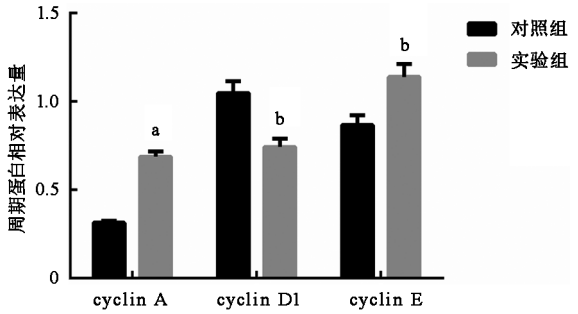


图 4　小鼠骨骼肌周期蛋白表达



注：与对照组比较，$^{a}P<0.001$；$^{b}P<0.01$

图 5　小鼠骨骼肌周期蛋白相对表达量

讨　论

骨骼肌卫星细胞存在 2 种亚型，分别是分化生成肌纤维的成肌祖细胞（myogenic progenitors）及自我更新保持卫星细胞池（satellite cell pool）相对恒定的卫星干细胞[8-9]。老龄化进程中，肌源性成肌祖细胞的比例增高，具有自我更新能力的卫星干细胞比例衰减，导致肌肉再生能力受损、卫星细胞储备缓慢耗竭[10]，从而造成肌纤维数目显著降低、肌肉质量及收缩能力明显下降，肌间脂肪沉积、慢性炎症和肌纤维化发生[11-12]。目前，在预防和改善年龄相关性肌肉功能退化及肢体失用方面，物理治疗有着重要作用[13]，这在本课题前期研究中也获得证实[14-15]。但物理治疗不当引起的继发损伤和部分老龄人行动受限影响了其应用的范围。因此，探索更多普适易行的延缓肌肉退行性变的方法十分必要。

热量限制作为一种可以广泛应用的干预措施，其预防老年相关疾病及延长健康寿命和生理寿命的作用在多种动物模型中得到了反复印证[16]。热量限制可产生多种效应，其中，调节线粒体代谢及减少氧化性损伤的效果已十分明确[17]。Cerletti 等[18]的研究证实热量限制可有效提高小鼠骨骼肌干细胞的活力和数量，并增加干细胞移植后的再生能力。本实验通过控制饮食进行热量限制，15 周后实验组小鼠体重明显降低（$P<0.001$）；通过流式细胞术分选出 2 组小鼠肌卫星细胞，研究结果显示，实验组小鼠卫星细胞处于活性细胞周期的比例远高于对照组小鼠（$P<0.001$），即实验组小鼠卫星细胞处于增殖期的比例远高于对照组小鼠，证明热量限制对老年小鼠肌卫星细胞增殖能力衰退的延缓效果明显；细胞增殖旺盛时 G_1/S 期蛋白 cyclin E 及 S 期蛋白 cyclin A 表达增强，细胞增殖缓慢时 G_1 期蛋白 cyclin D1 明显表达增强，亦说明细胞的有丝分裂过程离不开细胞周期相关蛋白的参与。本研究中，免疫印迹法检测结果显示，实验组 cyclin E（$P<0.01$）及 cyclin A（$P<0.001$）表达显著增强，cyclin D1（$P<0.01$）明显降低，说明实验组小鼠细胞增殖更旺盛，与卫星细胞周期结果一致。

综上所述，热量限制可有效延缓老年小鼠骨骼肌卫星细胞增殖能力的衰退。随着年龄的增长，肌卫星细胞增殖能力会逐渐减弱，而热量限制可以延缓甚至阻止这种趋势。这也许可为老龄性肌肉衰老的防治提供了一条新途径或新方法，其相关作用机制尚待进一步研究。

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·外刊撷英·

Curcumin and diabetic atherosclerosis

BACKGROUND AND OBJECTIVE Curcumin, a compound existing in turmeric, has been widely used as an anti-inflammatory treatment in traditional Chinese and Ayurvedic medicines. This animal study assessed the efficacy of L3, an analog of curcumin, compared to probucol, a traditional lipid lowering drug, for the prevention and treatment of the cardiovascular complications of diabetes.

METHODS Using a mouse model of diabetes, 93 mice were divided into eight groups, with seven of those groups undergoing diabetes induction. The mice were randomized to receive one of two doses of curucmin, one of two doses of probucol, or one of three doses of L3, while receiving a high cholesterol/fat diet. The animals were studied for changes in lipid panel results and antioxidant status, as well as reactive oxygen species (ROS) generation in the pancreas. The subjects were sacrificed after 16 weeks.

RESULTS After treatment with curcumin, plasma insulin levels returned to their normal levels. All three compounds were found to reduce blood lipids in a concentration dependent manner. All three compounds were also found to reduce the oxidative stress of liver and red blood cells. All treatment groups showed increased nitric oxide fluorescent intensities in the target tissues in a dose dependent manner. L3 was also found to decrease the production of ROS in the pancreas and lectin-like oxidized low-density lipoprotein receptor-1 expression in the aortic arch, as well as reduced fatty and atherosclerotic degeneration in the aortic arch, as compared with the control group.

CONCLUSION This animal study of diabetes found that L3, an analog of curcumin, can, through a number of mechanisms, inhibit diabetic atherosclerosis.

【摘自:Zheng B, Yang L, Wen C, et al. Curcumin analog l3 alleviates diabetic atherosclerosis by multiple effects. Eur J Pharmacol, 2016, 775(15): 22-34.】

Turmeric use for gingivitis

BACKGROUND AND OBJECTIVE It is estimated that gingivitis effects 80% of the population. Chlorhexidine is a broad-spectrum antiseptic, considered the gold standard for preventing and treating gingivitis. Curcumin, a polyphenol found in turmeric, has been found to have anti-inflammatory, antioxidant, antibacterial, antiviral and antifungal properties. This literature review compared the efficacy of turmeric with that of chlorhexidine for the prevention of gingivitis.

METHODS Medical databases were reviewed for studies concerning gingivitis which compared curcumin or turmeric, with chlorhexidine for the treatment of gingivitis.

RESULTS Data from five papers with a total of 290 participants were included in the analysis, with turmeric and chlorhexidine delivered as either a mouthwash or gel. The duration of all five studies was 21 days, with two studies including bacterial counts. All studies found that both turmeric and chlorhexidine improved scores on the gingival index (GI) and plaque index (PI). In two of the studies, chlorhexidine was found to be more effective in preventing plaque, with one of these finding that chlorhexidine was better at reducing inflammation.

CONCLUSION This literature review, focusing on the treatment of gingivitis, found that, overall, compared to the gold standard chlorhexidine, treatment with turmeric was equally effective for preventing and treating gingivitis.

【摘自:Stoyell KA, Mappus JL, Gandhi MA. Clinical efficacy of turmeric use in gingivitis: a comprehensive review. Complement Ther Clin Pract, 2016, 25: 13-17.】