辛酸钠对骨骼肌细胞缺糖缺氧/再灌注损伤的保护作用

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摘 要 该研究探索了辛酸钠对骨骼肌细胞缺糖缺氧/再灌注(OGD/Rep)损伤的保护作用。利用锥虫蓝染色法测定6种不同浓度的辛酸钠培养液对正常培养骨骼肌细胞24 h存活率的影响。随后采用缺糖缺氧后复糖复氧的方法构建骨骼肌细胞OGD/Rep损伤模型, 将细胞随机分为对照组、OGD/Rep组、0.25 mmol/L辛酸钠组和0.50 mmol/L辛酸钠组。CCK8法测定各组细胞活性, 检测各组细胞中乳酸脱氢酶(LDH)、超氧化物($\cdot O_2^-$)以及超氧化物歧化酶(SOD)水平; JC-1荧光探针测定各组细胞线粒体膜电位水平; TUNEL染色法检测各组细胞凋亡数目, Western blot测定各组细胞Bax、Bcl-2、Mfn-2、Drp-1蛋白表达水平。结果表明, 6种浓度辛酸钠处理组中, 0.25 mmol/L辛酸钠组、0.50 mmol/L辛酸钠组与对照组细胞存活率差异无统计学意义(P>0.05)。与对照组相比, OGD/Rep组细胞活性降低, 细胞LDH水平升高, 细胞中$\cdot O_2^-$产生量增加, SOD活性降低, 线粒体膜电位降低以及细胞凋亡数目增多(P<0.05)。与OGD/Rep组相比, 0.25 mmol/L辛酸钠组、0.50 mmol/L辛酸钠组细胞活性均增强, 细胞LDH水平下降(P<0.05); 0.25 mmol/L辛酸钠组$\cdot O_2^-$水平有下降趋势, 但差异无统计学意义(P>0.05); 0.25 mmol/L辛酸钠组SOD活性增强(P<0.05)。0.25 mmol/L和0.50 mmol/L辛酸钠组与OGD/Rep组相比线粒体膜电位增加, 细胞凋亡数目减少。与对照组相比, OGD/Rep组Bcl-2/Bax蛋白值降低, Drp-1和Mfn-2蛋白表达量降低; 而与OGD/Rep组相比, 0.25 mmol/L、0.50 mmol/L辛酸钠组Bcl-2/Bax蛋白值升高, Drp-1和Mfn-2蛋白表达量增加。研究表明, 早期应用辛酸钠可减轻OGD/Rep引起的骨骼肌细胞损伤, 抑制细胞的过氧化状态并减少细胞凋亡, 其机制可能与辛酸钠调节线粒体结构动态平衡相关。

关键词 辛酸钠; 骨骼肌细胞; 缺糖缺氧/再灌注

Protective Effect of Sodium Octanoate on Oxygen and Glucose Deprivation/Reperfusion Injury of Skeletal Muscle Cells

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Abstract This work was to investigate the protective effect of sodium octanoate on OGD/Rep (oxygen and glucose deprivation/reperfusion) injury of skeletal muscle cells. A trypan-blue assay was used to determine the effects of six different

收稿日期: 2021-09-10 接受日期: 2021-10-26
国家自然科学基金(批准号: 82072126)资助的课题
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Received: September 10, 2021 Accepted: October 26, 2021
This work was supported by the National Natural Science Foundation of China (Grant No.82072126)
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concentrations of sodium octanoate on the 24 h survival rate of skeletal muscle cells. The OGD/Rep model of skeletal muscle cells was constructed by the compound sugar and reoxygenation method after glucose and oxygen deprivation. The cells were randomly divided into the control group, the OGD/Rep group, the 0.25 mmol/L sodium octanoate group and the 0.50 mmol/L sodium octanoate group. Cell activity was determined by CCK8 assay, and the levels of LDH (lactate dehydrogenase), $\cdot O_2^-$ (superoxide) and SOD (superoxide dismutase) in each group were detected. The mitochondrial membrane potential was measured using the fluorescent probe JC-1. Apoptotic cells were detected by TUNEL assay. Western blot was used to detect the expression of Bax, Bcl-2, Mfn-2 and Drp-1 proteins. Among the groups treated with six concentrations of sodium octanoate, only the 0.25 mmol/L sodium octanoate group and 0.50 mmol/L sodium octanoate group had no statistically significant difference in cell survival rate with the control group (P>0.05). Compared with the control group, OGD/Rep group showed lower cell viability, higher LDH level, higher $\cdot O_2^-$ production, lower SOD activity, lower mitochondrial membrane potential and more apoptotic cells (P<0.05). Compared with the OGD/Rep group, the cell activity of the 0.25 mmol/L and 0.50 mmol/L sodium octanoate groups were increased, and the LDH release decreased (P<0.05); the $\cdot O_2^-$ of the 0.25 mmol/L sodium octanoate group showed a trend towards decrease but the difference was not significant statistically (P>0.05); SOD activity increased in 0.25 mmol/L sodium octanoate group (P<0.05). Compared with OGD/Rep group, the mitochondrial membrane potential increased and the number of apoptotic cells decreased in 0.25 mmol/L and 0.50 mmol/L sodium octanoate groups. Compared with the control group, the ratio of Bcl-2/Bax protein and the expression of Drp-1 and Mfn-2 proteins decreased in OGD/Rep group, while the ratio of Bcl-2/Bax protein and the expression of Drp-1 and Mfn-2 proteins increased in 0.25 mmol/L and 0.50 mmol/L sodium octanoate groups compared with OGD/Rep group. This study suggests that the early application of sodium octanoate can reduce the OGD/Rep-induced injury of skeletal muscle cells, reduce the peroxidation damage and reduce the apoptosis of skeletal muscle cells. The mechanism may be related to the regulation of sodium octanoate on the dynamic balance of mitochondrial structure.

Keywords sodium octanoate; skeletal muscle cells; oxygen and glucose deprivation/reperfusion

骨骼肌缺血再灌注损伤(ischemia-reperfusion injury, IRI)是临床常见的症状，多见于肢体动脉损伤/栓塞、骨筋膜室综合征、挤压综合征等疾病，主动脉球囊阻断、心脏或主动脉手术也可导致骨骼肌的IRI[1-4]。严重的骨骼肌IRI可引起患者肌肉坏死、挛缩、肾衰竭，甚至死亡[5-6]。目前，临床上确切有效的骨骼肌IRI干预策略还不足，包括药物、低温、高压氧、缺血预处理及后处理等措施均处于不同程度的探索阶段[1-2]。IRI引起的损伤与组织能量供应不足有关，而补充ATP则有助于减轻缺血引起的骨骼肌损伤[7-11]。多项研究表明，n-6和n-3等多不饱和脂肪酸对心、脑、肠、肝脏的IRI有保护作用，其作用机制可能与减少氧自由基产生、减轻炎症与氧化应激反应相关[12-15]。与长链脂肪酸相比，中链脂肪酸在体内具有代谢优势，辛酸钠作为一种中链脂肪酸在骨骼肌IRI中的作用尚未见报道。本实验采用骨骼肌细胞缺糖缺氧/再灌注(oxygen and glucose deprivation/reperfusion, OGD/Rep)损伤模型模拟骨骼肌IRI，探索辛酸钠在骨骼肌细胞OGD/Rep损伤中的作用，为其进一步的临床应用提供实验依据。

1 材料与方法

1.1 实验试剂

DMEM高糖培养基、DMEM无糖培养基购自美国Gibco公司；胰酶细胞消化液、青–链霉素溶液购自合肥白鲨生物科技有限公司；胎牛血清购自以色列BioInd公司；细胞裂解液、5× 蛋白上样缓冲液、BCA蛋白浓度测定试剂盒、乳酸脱氢酶(LDH)细胞毒性检测试剂盒、线粒体膜电位检测试剂盒、超氧化物($\cdot O_2^-$)检测试剂盒、总超氧化物歧化酶(SOD)活性检测试剂盒、CCK8试剂盒均购自上海碧云天生物技术有限公司；脱脂奶粉、SDS-PAGE彩色凝胶配制试剂盒购自上海生工生物工程有限公司；TUNEL检测试剂盒购自上海七海复泰生物科技有限公司；辛酸钠购自美国Sigma公司；0.4%锥虫蓝购自浙江吉诺生物医药技术有限公司；β-tubulin抗体、Bax抗体、线粒体动力学相关蛋白-1(dynamin-related protein-1, Drp-1)抗体、线粒体融合蛋白-2(motofusin-2, Mfn-2)抗体均购自美国CST公司；Bcl-2抗体购自英国Abcam公司。

1.2 骨骼肌细胞的诱导分化及培养

将C2C12小鼠成肌细胞(中国科学院分子细胞科学卓越创新中心)置于37 ℃、5% CO_2常氧细胞培养箱中培养, 用含10%胎牛血清及1%青–链霉素的高糖DMEM培养基进行常规细胞培养及传代。待细胞扩增至一定数目后将培养基换为含2%马血清和1%青–链霉素的高糖DMEM培养基进行7~14天的诱导分化以获得骨骼肌细胞, 镜下观察骨骼肌细胞融合至70%~80%时进行实验。

1.3 骨骼肌细胞OGD/Rep模型的建立以及分组

检测不同浓度辛酸钠对正常培养骨骼肌细胞的影响, 于常氧培养箱中分别使用含0.25 mmol/L、0.50 mmol/L、1 mmol/L、2 mmol/L、4 mmol/L和8 mmol/L 6个不同浓度辛酸钠的高糖培养基对骨骼肌细胞进行24 h培养, 以筛选最佳实验浓度。将骨骼肌细胞在低氧工作站(氧浓度为0.4%)中使用无糖DMEM培养基培养4 h, 随后更换高糖DMEM培养基并将其放入常氧培养箱(氧浓度为21%)继续培养4 h以建立骨骼肌细胞OGD/Rep模型。然后将细胞随机分为对照组、OGD/Rep模型组、0.25 mmol/L辛酸钠组和0.50 mmol/L辛酸钠组。对照组细胞在常氧培养箱中使用高糖DMEM培养基正常培养8 h。辛酸钠组细胞在低氧工作站中使用含不同浓度辛酸钠的无糖培养基培养4 h, 随后将培养基更换为高糖培养基并于常氧培养箱中继续培养4 h。

1.4 检测方法及指标

1.4.1 *锥虫蓝检测细胞存活率* 将细胞在6孔板中进行培养, 待细胞密度达70%~80%后换用不同浓度辛酸钠培养基, 再放至常氧培养箱中培养24 h。胰酶消化液收集细胞后用0.4%锥虫蓝染色, 镜下检测细胞存活率。

1.4.2 *CCK8检测细胞活性* 96孔板培养细胞, 待细胞密度达70%~80%后进行分组实验, 然后向每孔加入10 μL CCK8溶液, 再放入37 ℃常氧培养箱孵育4 h。用酶标仪测定波长450 nm处的吸光度(*D*)值。

1.4.3 *酶标仪检测细胞LDH活性、·O_2^-水平及SOD活性* 96孔板培养细胞, 待细胞密度达70%~80%后进行分组实验。每孔加60 μL LDH检测工作液, 混匀后室温避光孵育30 min, 酶标仪测定波长490 nm处的吸光度值。检测·O_2^-水平时, 每孔加入200 μL超氧化物检测工作液, 37 ℃孵育3 min, 酶标仪在波长450 nm处测定吸光度值。检测SOD需要用6孔板培养细胞, 待细胞密度达70%~80%时进行分组实验, 用胰蛋白酶及细胞裂解液制作各组全细胞裂解液, 加入反应工作液后于37 ℃孵育30 min, 用酶标仪在波长450 nm处测定吸光度值, SOD活性与吸光度值呈负相关。

1.4.4 *线粒体膜电位检测* 用6孔板培养细胞, 待细胞密度达70%~80%后进行分组实验。每孔去除培养基后用PBS洗涤, 加入1 mL细胞培养基和1 mL JC-1染色工作液, 于37 ℃孵育20 min后用染色缓冲液洗涤2次, 加入2 mL细胞培养液, 于荧光显微镜下观察。

1.4.5 *TUNEL检测* 用6孔板进行细胞培养, 待细胞密度达70%~80%后进行分组实验。用多聚甲醛和PBS分别进行细胞固定和水化, 用蛋白酶K增加样品通透性, 加入标记反应液在37 ℃孵育90 min, PBS洗涤后滴加PI染色液, 黑暗中于37 ℃孵育5 min, 用去离子水洗涤。封片后于荧光显微镜下观察。

1.4.6 *Western blot检测β-tubulin、Bax、Bcl-2、Mfn-2、Drp-1蛋白表达量* 对各组细胞中Bax、Bcl-2、Drp-1、Mfn-2蛋白水平进行检测。使用细胞裂解液裂解细胞后进行BCA蛋白浓度测定。95 ℃加热8 min进行蛋白变性, 按照每孔20 μg的蛋白量上样, 采用10%分离胶进行SDS-PAGE凝胶电泳(程序: 80 V 30 min; 110 V 40~60 min)。使用PVDF膜进行低温湿转(程序: 250 mA 90 min)。用5%脱脂奶粉于37 ℃封闭1 h。TBST洗膜10 min。一抗于4 ℃孵育过夜, TBST洗膜(10 min, 3次), 二抗室温孵育2 h。用TBST洗膜(10 min, 3次)后进行ECL显影。一抗按1:1 000配制, 二抗按1:2 500配制。

1.5 统计学分析

利用Graphpad Prism 8软件进行统计, 计量资料以均值±标准差($\bar{x}\pm s$)表示, 多组间比较采用单因素方差分析, 组间比较用配对样品*t*检验。以$P<0.05$为差异有统计学意义。

2 结果

2.1 不同浓度辛酸钠对骨骼肌细胞存活率的影响

不同浓度辛酸钠培养基培养骨骼肌细胞24 h后, 用锥虫蓝染色测定骨骼肌细胞存活率。结果表明1 mmol/L、2 mmol/L、4 mmol/L、8 mmol/L的辛酸钠均使骨骼肌细胞存活率降低($P<0.05$)。而0.25 mmol/L和0.50 mmol/L辛酸钠组与对照组相比细胞存活率差异无统计学意义($P>0.05$)(图1B)。

2.2 辛酸钠对OGD/Rep损伤的骨骼肌细胞活性的影响

与对照组相比, OGD/Rep模型组细胞活性明显

降低(P<0.05)。而与OGD/Rep组相比，0.25 mmol/L辛酸钠组与0.50 mmol/L辛酸钠组细胞活性明显增强(P<0.05)(图2)。

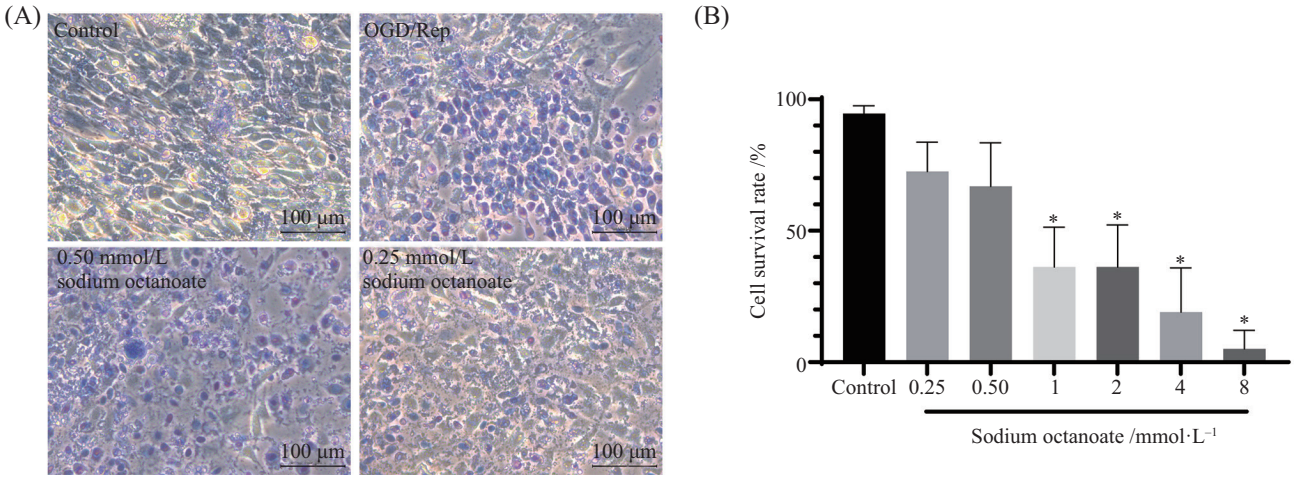
2.3 辛酸钠对OGD/Rep损伤的骨骼肌细胞LDH、$\cdot O_2^-$、SOD水平的影响

与对照组相比，OGD/Rep组LDH释放量及$\cdot O_2^-$产生量增加，SOD活性降低(P<0.05)。而与OGD/Rep组相比，0.50 mmol/L辛酸钠组LDH释放量减少(P<0.05)，而$\cdot O_2^-$、SOD水平与OGD/Rep组差异无统计学意义(P>0.05)。0.25 mmol/L辛酸钠组与OGD/Rep组相比，LDH释放量减少，SOD活性增强(P<0.05)，而$\cdot O_2^-$产生量在两组间差异无统计学意义(P>0.05)(图3)。

2.4 辛酸钠对OGD/Rep损伤的骨骼肌细胞线粒体膜电位的影响

线粒体功能与线粒体膜电位密切相关，为此，我们应用了JC-1，一种广泛用于检测线粒体膜电位的理想荧光探针，以确定辛酸钠对骨骼肌细胞OGD/Rep后线粒体膜电位的影响。

如图4所示，与对照组相比，骨骼肌细胞OGD/Rep后线粒体膜电位降低；而与OGD/Rep组相比，0.25 mmol/L辛酸钠组和0.50 mmol/L辛酸钠组线粒体膜电位增加。

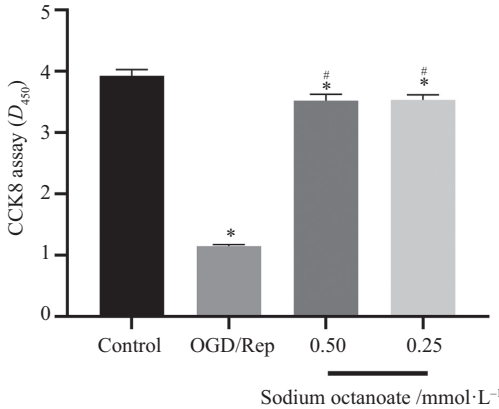


A: 光学显微镜下观察各组细胞锥虫蓝染色; B: 各组骨骼肌细胞存活率; *P<0.05, 与Control组相比。

A: trypan blue staining of cells in each group was observed under microscope; B: the survival rate of skeletal muscle cells in each group. *P<0.05 compared with Control group.

图1 不同浓度辛酸钠对骨骼肌细胞存活率的影响

Fig.1 Effects of different concentrations of sodium octanoate on the survival rate of skeletal muscle cells

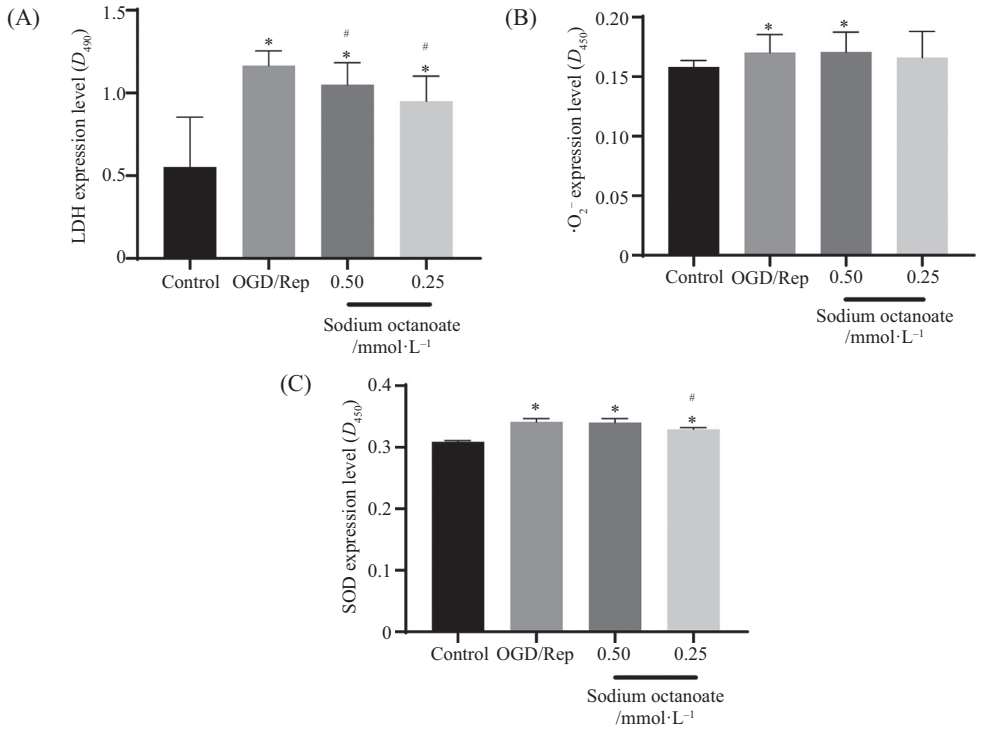


CCK8检测各组细胞活性; OGD/Rep: 缺糖缺氧/再灌注; *P<0.05, 与对照组相比; #P<0.05, 与OGD/Rep组相比。

The cell viability of each group was detected by CCK8 test; OGD/Rep: oxygen and glucose deprivation/reperfusion; *P<0.05 compared with Control group; #P<0.05 compared with OGD/Rep group.

图2 辛酸钠对骨骼肌OGD/Rep损伤后细胞活性的影响

Fig.2 Effects of sodium octanoate on cell activity after skeletal muscle OGD/Rep injury

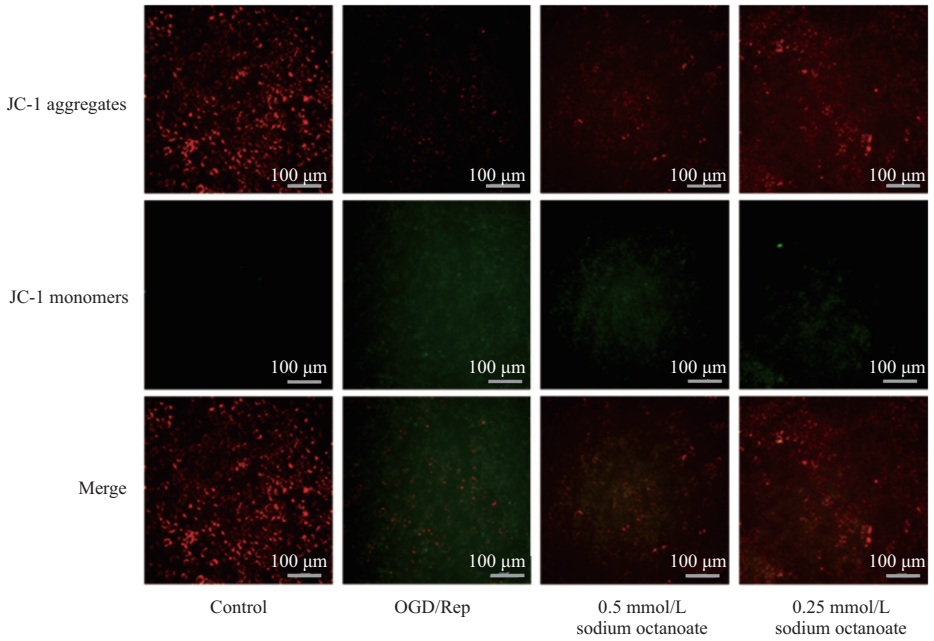


A~C: 酶标仪检测各组细胞LDH、$\cdot O_2^-$、SOD水平; OGD/Rep为缺糖缺氧/再灌注; *P<0.05, 与对照组相比; #P<0.05, 与OGD/Rep组相比。

A-C: detection of LDH, $\cdot O_2^-$, SOD levels in each group by microplate reader; OGD/Rep: oxygen and glucose deprivation/reperfusion; *P<0.05 compared with Control group; #P<0.05 compared with OGD/Rep group.

图3 辛酸钠对骨骼肌细胞OGD/Rep损伤后LDH、$\cdot O_2^-$、SOD水平的影响

Fig.3 Effects of sodium octanoate on LDH, $\cdot O_2^-$, SOD levels of skeletal muscle cells after OGD/Rep injury

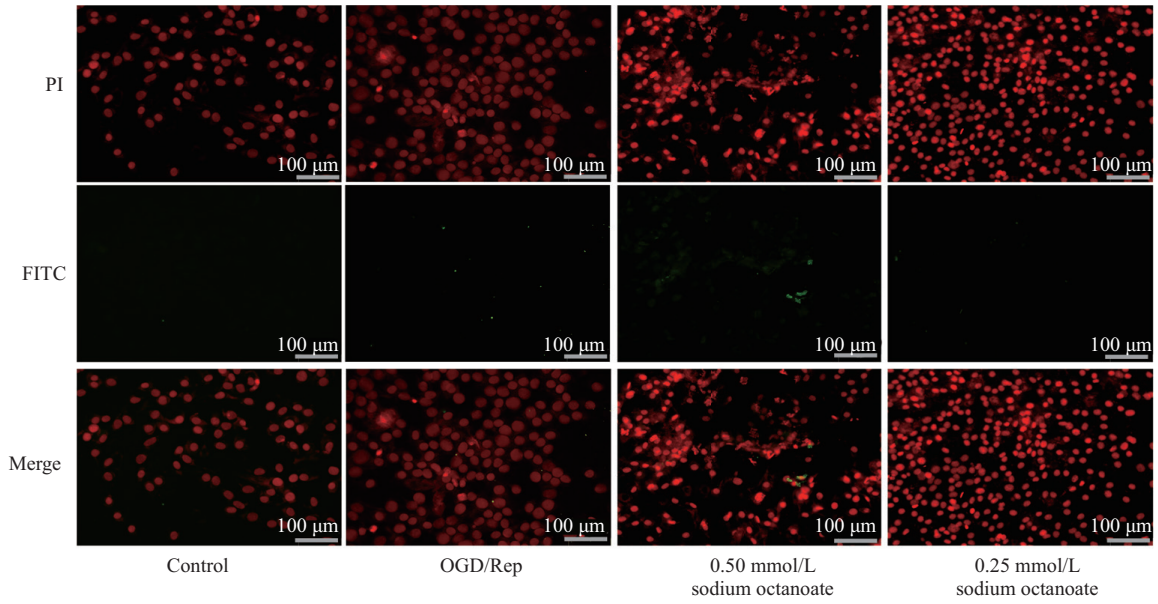


JC-1荧光探针检测各组细胞线粒体膜电位水平; OGD/Rep: 缺糖缺氧/再灌注; JC-1 aggregates指较高的线粒体膜电位时JC-1聚集呈红色, 而JC-1 monomers指较低的线粒体膜电位时JC-1单体状态呈绿色。

Detection of mitochondrial membrane potential in each group by JC-1 fluorescence probe; OGD/Rep: oxygen and glucose deprivation/reperfusion; JC-1 aggregates refer to the red aggregation of JC-1 at higher mitochondrial membrane potential, while JC-1 monomers refer to green JC-1 monomer state at lower mitochondrial membrane potential.

图4 辛酸钠对骨骼肌细胞OGD/Rep损伤后线粒体膜电位的影响

Fig.4 Effects of sodium octanoate on mitochondrial membrane potential after OGD/Rep injury in skeletal muscle cells



TUNEL检测各组细胞凋亡数目; OGD/Rep: 缺糖缺氧/再灌注; PI染料标记所有细胞的细胞核, FITC标记凋亡细胞的细胞核。
The number of apoptotic cells in each group was detected by TUNEL; OGD/Rep: oxygen and glucose deprivation/reperfusion; the nuclei of all cells were labeled with PI dye and the nuclei of apoptotic cells were labeled with FITC.

图5 辛酸钠对骨骼肌细胞OGD/Rep损伤后细胞凋亡的影响

Fig.5 Effect of sodium octanoate on apoptosis of skeletal muscle cells after OGD/Rep injury

2.5 辛酸钠对OGD/Rep损伤的骨骼肌细胞凋亡的影响

如图5所示, 对照组细胞凋亡细胞数目较少。与对照组相比, OGD/Rep组凋亡细胞数目增多, 而与OGD/Rep组相比, 0.25 mmol/L辛酸钠组和0.50 mmol/L辛酸钠组细胞凋亡数目均减少。

2.6 辛酸钠对OGD/Rep损伤的骨骼肌细胞Bax、Bcl-2、Mfn-2、Drp-1蛋白表达水平的影响

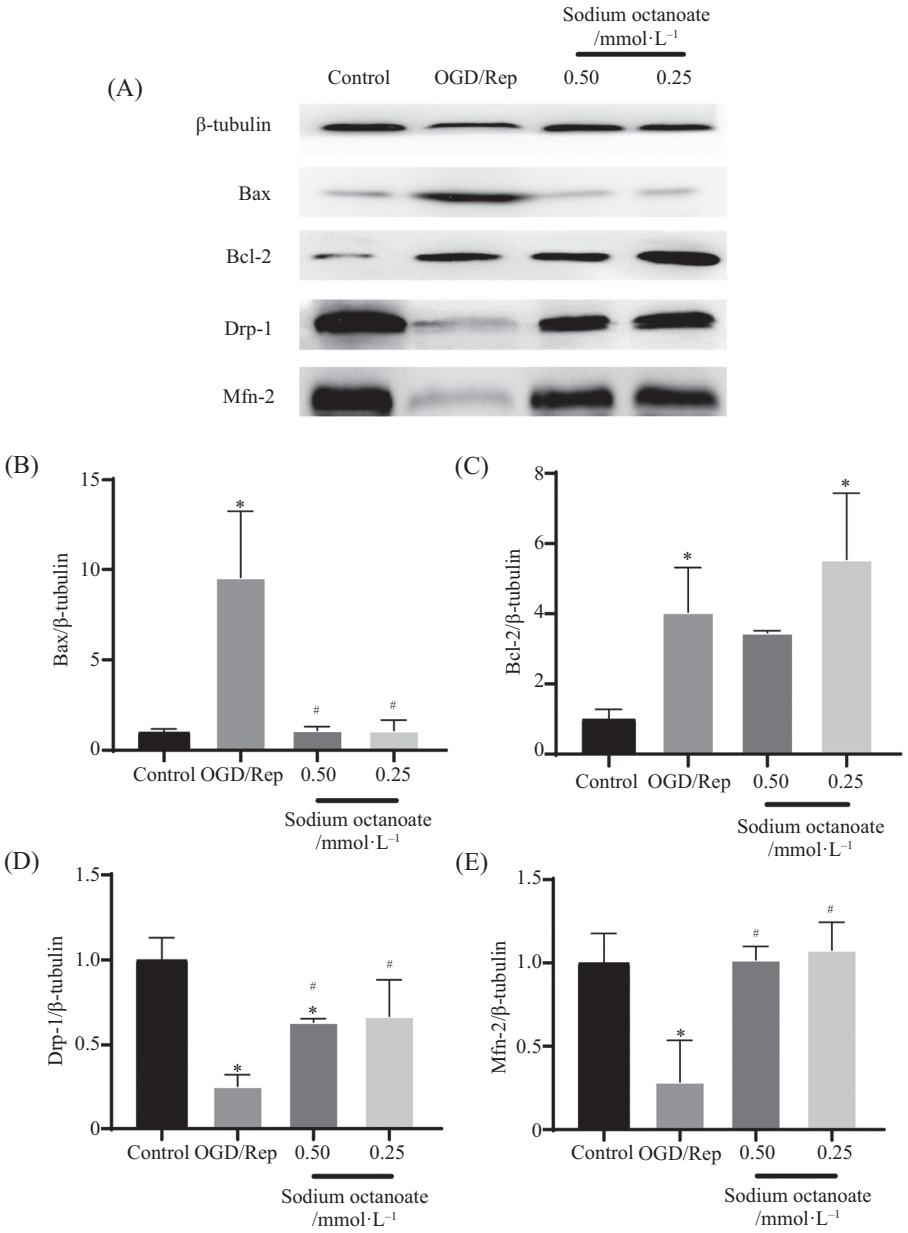
如图6所示, 与对照组相比, OGD/Rep组Bax、Bcl-2蛋白表达量增加, 而Mfn-2、Drp-1蛋白表达量均降低; 与OGD/Rep组相比, 0.25 mmol/L辛酸钠组和辛酸钠0.50 mmol/L辛酸钠组Bax蛋白表达量降低, 而Bcl-2、Mfn-2、Drp-1蛋白表达量增加。

3 讨论

骨骼肌细胞OGD/Rep是构建体外骨骼肌IRI模型的常用方法[16-18]。本研究通过骨骼肌细胞OGD/Rep模型, 验证辛酸钠对骨骼肌细胞OGD/Rep后的保护作用, 发现在缺血早期补充辛酸钠可增强骨骼肌细胞OGD/Rep后的细胞活性, 增强SOD酶活性, 减轻过氧化损伤, 抑制线粒体膜电位的降低以及细胞凋亡, 其作用途径可能与辛酸钠调节线粒体的结构与功能相关。

骨骼肌IRI在临床上较为常见, 情况严重时可导致患者截肢、多器官功能衰竭甚至死亡。患者骨骼肌缺血会引起组织能量供应不足, ATP耗竭之后, 骨骼肌细胞线粒体膜电位平衡会紊乱[7-9]。ATP代谢产生的ADP被异化为次黄嘌呤和黄嘌呤, 机体在代谢次黄嘌呤和黄嘌呤时会产生活性氧(reactive oxygen species, ROS), 继而导致细胞膜损伤和通透性增加[19-21]。同时, 骨骼肌细胞ATP供应不足可导致细胞膜钠–钾-ATP酶通道关闭, 诱发细胞肿胀[6]。ROS在骨骼肌再灌注损伤中发挥重要作用, 次黄嘌呤代谢产生的$\cdot O_2^-$、H_2O_2等氧自由基可诱发局部和全身炎症反应[22]。不仅如此, 损伤的线粒体复合体在再灌注早期代谢中可产生更多的$\cdot O_2^-$[22]。与此同时, 缺血后产生的大量ROS又以线粒体为靶点进行攻击, 损伤细胞结构和酶功能。骨骼肌IRI机制非常复杂, 除了以上氧自由基损伤机制外, 还有血管内皮细胞和中性粒细胞的相互作用、钙超载、线粒体能量代谢障碍等。本次研究发现, 骨骼肌细胞OGD/Rep损伤导致细胞活性降低, 超氧化物产生量增加, SOD酶活性降低, 细胞膜结构完整性降低, LDH释放量增加, 与前文研究[16-18]一致。

线粒体是参与细胞能量代谢的主要细胞器。本研究发现骨骼肌细胞OGD/Rep损伤线粒体, 导致线粒体膜电位降低, Bcl-2/Bax值降低, 线粒体动力学蛋



A~E: Western blot检测各组细胞中Bax、Bcl-2、Mfn-2、Drp-1蛋白的表达水平; *P<0.05, 与对照组相比; #P<0.05, 与OGD/Rep组相比。

A-E: Western blot was used to detect the protein expression levels of Bax, Bcl-2, Mfn-2 and Drp-1 in each group; *P<0.05 compared with Control group; #P<0.05 compared with OGD/Rep group.

图6 辛酸钠对骨骼肌细胞OGD/Rep损伤后Bax、Bcl-2、Mfn-2、Drp-1蛋白表达量的影响

Fig.6 Effects of sodium octanoate on the protein expression of Bax, Bcl-2, Mfn-2 and Drp-1 in skeletal muscle cells after OGD/Rep injury

白表达量降低。线粒体形态结构处于高度动态变化中, 其分裂和融合需要Drp-1、线粒体分裂蛋白1(fission protein 1, Fis1)和Mfn-2的调节, 以适应细胞在各种应激状态下的不同能量代谢需求。Fis1和Drp-1蛋白介导线粒体分裂, 而Mfn-1和Mfn-2蛋白介导线粒体融合[23-25]。研究发现心肌IRI后线粒体动态平衡被打破, Drp-1蛋白表达上调, 使线粒体倾向于分裂, 导致线粒体碎片化、片段化[23,26]。本研究中骨骼肌细胞OGD/Rep后线粒体动力学蛋白表达量均降低, 与前文研究不一致。此差异可能与骨骼肌细胞损伤程度及OGD/Rep后不同阶段细胞代谢能力不同有关。因此, 本研究发现在骨骼肌细胞OGD/Rep早期应用辛酸钠可促进线粒体动力学蛋白表达, 调节线粒体动态平衡, 促进线粒体功能的恢复。

综上所述, 在缺血早期应用辛酸钠对骨骼肌细胞的OGD/Rep损伤有保护作用, 可抑制细胞的过氧化状态并减少细胞凋亡, 其机制可能与辛酸钠调节线粒体结构动态平衡相关。

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