doi: 10.3978/j.issn.2095-6959.2020.05.001
View this article at: http://dx.doi.org/10.3978/j.issn.2095-6959.2020.05.001

·论著·

利拉鲁肽对高糖环境下体外培养的人髓核细胞凋亡的影响

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[摘　要]　**目的：**研究不同浓度的利拉鲁肽(liraglutide，LIR)对高糖环境下体外培养的人髓核细胞(nucleus pulposus cells，NPCs)凋亡的影响。**方法：**培养人髓核细胞株，第三代髓核细胞随机分为对照组(CON组，NPCM细胞培养液培养)、高糖组(HG组，0.2 mol/L高糖培养液培养)、利拉鲁肽10 nmol/L干预组(LIR10组，0.2 mol/L高糖+10 nmol/L利拉鲁肽)、利拉鲁肽100 nmol/L干预组(LIR100组)：0.2 mol/L高糖+100 nmol/L利拉鲁肽、利拉鲁肽1 000 nmol/L干预组(LIR1 000组)：0.2 mol/L高糖+1 000 nmol/L利拉鲁肽。各组细胞培养48 h，CCK-8对人髓核细胞的增殖活性进行定量分析，流式细胞术及ELISA检测细胞凋亡率，细胞内活性氧(ROS)检测评估氧化应激水平。采用SPSS 22.0软件进行统计学分析。**结果：**与正常对照组相比，高糖组细胞增殖活性明显减低，细胞内ROS生成及凋亡率明显增加(P<0.05)。利拉鲁肽(10，100，1000 nmol/L)的干预使细胞增殖活性较高糖组明显升高，ROS的水平及细胞凋亡率明显降低(P<0.05)。在浓度为100 nmol/L时，利拉鲁肽的促进细胞增殖，抑制氧化应激及抗凋亡作用最强，各组间差异有统计学意义(P<0.05)。**结论：**利拉鲁肽通过抗氧化应激抑制了高糖诱导的人髓核细胞凋亡，从而发挥保护作用。

[关键词]　利拉鲁肽；人髓核细胞；高糖；凋亡

Effect of liraglutide on apoptosis of human nucleus pulposus cells induced by high glucose

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Abstract　**Objective:** To evaluate the protective effect of liraglutide on apoptosis induced by high glucose in nucleus pulposus cells (NPCs). **Methods:** The third-generation NPCs were randomly categorized as follows: a control (CON) group (cultured in NPCM), a high glucose (HG) group (cultured in 0.2 mol/L high glucose concentration), a high glucose + liraglutide (HG+LIR) group [cultured in the high glucose (0.2 mol/L) medium

收稿日期 (Date of reception)：2019–07–02
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基金项目 (Foundation item)：国家自然科学基金 (31701238)。This work was supported by the National Natural Science Foundation of China (31701238).

containing various concentrations of liraglutide (10, 100, or 1 000 nmol/L)]. The cells were all cultured for 48 h, then cell viability was tested using cell counting kit-8; and the apoptosis rate were measured by flow cytometric analysis and ELISA; the intracellular reactive oxygen species (ROS) of the NPCs was measured using a ROS assay kit. All data were analyzed with SPSS software for Windows version 22.0. **Results:** Compared with the control group, cell viability decreased significantly and ROS levels, cell apoptosis rate increased significantly in the high glucose group (P<0.05). Our data demonstrated that liraglutide evidently increased cell proliferation activity and inhibited the apoptosis of NPCs induced by high glucose (P<0.05). Further analysis suggested that liraglutide suppressed ROS generation (P<0.05). The maximum effect was at 100 nmol/L of liraglutide, and the differences among different groups were statistically significant (P<0.05). **Conclusion:** Liraglutide could protect NPCs against high glucose-induced apoptosis by inhibiting oxidative stress.

Keywords apoptosis; liraglutide; nucleus pulposus cells; high glucose

慢性腰痛因其巨额的医疗支出引发经济压力已逐渐成为重要的社会经济问题[1]。椎间盘退变为慢性腰痛的主要病因，是一系列脊柱退行性疾病的病理基础[2-3]，但其确切的发病机制目前尚未明确。近年来国内外学者[4-5]指出：髓核细胞(nucleus pulposus cells，NPCs)的异常凋亡和加速衰老是参与椎间盘退变的两个主要细胞学进程。研究[6-7]表明：糖尿病是椎间盘退变的潜在病因。高糖会对椎间盘细胞的生物学产生负面影响，诱导椎间盘细胞衰老和凋亡[8-9]。本课题着眼于此，力求研究一种新的方法抑制高糖环境下椎间盘细胞的异常凋亡，从而有效干预糖尿病患者椎间盘退变进程。

胰高血糖素样肽-1(glucagon-like peptide-1，GLP-1)目前被认为是治疗2型糖尿病的一个强有力的选择[10]。除了有益于控制血糖，GLP-1还被报道在多种组织中发挥调节细胞增殖、分化和凋亡的功能[11-13]。利拉鲁肽(liraglutide，LIR)是一种生物合成的GLP-1类似物，与人GLP-1结构具有97%的同源性[14]。研究[15]表明：利拉鲁肽具有保护胰岛β细胞，减轻体重，改善骨质量，延缓动脉粥样硬化，改善心功能等作用。然而，关于GLP-1及其类似物在椎间盘退变中的作用仍然未知。本研究应用不同浓度的利拉鲁肽作用于高糖体外培养人髓核细胞48 h，观察各组人髓核细胞增殖、凋亡及氧化应激水平的变化，探讨利拉鲁肽对高糖环境下人髓核细胞的保护作用。

1 材料与方法

1.1 材料

人髓核细胞株购自美国ScienceCell研究实验室；倒置显微镜购自日本奥林巴斯公司；NPCM细胞培养液购自北京赛默飞世尔生物化学制品有限公司，胎牛血清培养液购自美国Hyclone公司；二甲基亚砜(DMSO)购自北京Solarbio公司；胰蛋白酶购自美国Sigma公司；CCK-8试剂盒购自美国Sigma-Alorich公司；ELISA法细胞调亡检测试剂盒购自德国Roche公司；Annexin V-FITC/PI双染试剂盒购自杭州联科生物技术有限公司；活性氧(ROS)试剂盒购自南京建成生物工程研究所。

1.2 方法

1.2.1 细胞培养

NPCM包含500 mL基础培养基，髓核细胞生长因子5，10 mL胚牛血清，及青霉素/链霉素溶液5 mL。细胞复苏后在含有5%的二氧化碳的37 ℃培养箱中培养。隔日更新培养基，去除残留的DMSO和未贴壁细胞。每2~3 d更换培养基。当NPCs达80%~90%的融合时，用0.25% (w/v)胰蛋白酶溶液消化传代，按1:3的比例分离培养。取生长良好的对数期细胞用于实验。

1.2.2 分组

第三代人髓核细胞随机分为以下5组：对照组(CON组，NPCM细胞培养液培养)、高糖组

(HG组，0.2 mol/L高糖培养液培养[16])、利拉鲁肽10 nmol/L干预组(LIR10组，0.2 mol/L高糖+10 nmol/L利拉鲁肽)、利拉鲁肽100 nmol/L干预组(LIR100组，0.2 mol/L高糖+100 nmol/L利拉鲁肽)、利拉鲁肽1000 nmol/L干预组(LIR1 000组，0.2 mol/L高糖+1 000 nmol/L利拉鲁肽)。用倒置相衬显微镜观察细胞形态，在相同的实验条件下，培养48 h[17]后检测各组细胞增殖活力及凋亡情况。

1.2.3 CCK-8 实验检测细胞增殖活力

CCK-8用于检测细胞增殖。人髓核细胞悬浮接种于96孔板，调整细胞密度为2×10^{5}细胞/100 μL孔。48 h后，每个实验孔中加入10 μL CCK-8溶液，排除孔中气泡，将培养板置于含有5% CO_2的37 ℃孵育箱中，孵育3 h。用酶标仪测定450 nm处的吸光度测量细胞增殖。

1.2.4 流式细胞仪 Annexin V-FITC 法检测细胞凋亡

采用Annexin V-FITC/PI凋亡检测试剂盒检测细胞凋亡。用0.25%的胰蛋白酶对NPCs消化，4 ℃离心后用PBS洗涤2次，弃上清，调节其终浓度为1×10^{6}个/mL。用双蒸水稀释5×Binding Buffer为1×工作液，取500 μL预冷1×Binding Buffer加入离心管重悬细胞。每管加入5 μL Annexin V-FITC，轻轻吹打混匀，避光条件下再加入10 μL PI，室温避光静置15 min。用流式细胞仪分析人髓核细胞的凋亡率。判断标准：横坐标是Annexin V-FITC，纵坐标是PI，左下象限代表正常存活细胞，右下象限代表凋亡早期细胞，右上象限代表凋亡晚期细胞，左上象限代表坏死细胞。

1.2.5 ELISA 法检测细胞调亡

高糖诱导细胞凋亡，应用ELISA细胞调亡试剂盒进行细胞凋亡检测。将人髓核细胞置于高糖、不同浓度的利格鲁肽溶液中48 h，室温裂解30 min，离心10 min，上清液中检测到细胞质核碎片中单链和寡链核小体的含量判断细胞凋亡程度。

1.2.6 ROS 检测

按照ROS检测试剂盒说明书检测髓核细胞的细胞内ROS水平。细胞用PBS清洗3次后用DCFH-DA孵育40 min，PBS清洗3次，用0.25%的胰蛋白酶消化并收集髓核细胞。每组10^{5}个细胞在荧光强度为490/585 nm波长处检测细胞内ROS的表达。

1.3 统计学处理

应用SPSS 22.0软件进行数据分析。数据结果以均数±标准差($\bar{x}$±s)表示。使用Student's t检验和post hoc单因素方差分析进行数据统计。实验均重复3次。P<0.05为差异有统计学意义。

2 结果

2.1 利拉鲁肽增加高糖诱导的人髓核细胞的增殖活性

CCK-8检测结果显示：高糖组细胞增殖活性明显低于对照组(P<0.05)。利拉鲁肽(10，100，1 000 nmol/L)干预组细胞增殖活性均较高糖组明显增加(P<0.05)，且利拉鲁肽100 nmol/L浓度组中作用最明显，各组间差异有统计学意义(P<0.05，图1)。

2.2 利拉鲁肽抑制高糖诱导的人髓核细胞凋亡

应用流式细胞术分析和细胞凋亡ELISA检测人髓核细胞的凋亡，结果显示：高糖(0.2 mol/L)导致凋亡率显著升高，应用利拉鲁肽可明显降低细胞凋亡(P<0.05，图2)。

2.3 利拉鲁肽降低高糖环境下人髓核细胞内 ROS 的生成

与正常对照组相比，高糖诱导细胞内ROS生成增加，利拉鲁肽(10，100，1 000 nmol/L)的干预显著降低了人髓核细胞内ROS的水平，各组差异有统计学意义(P<0.05，图3)。

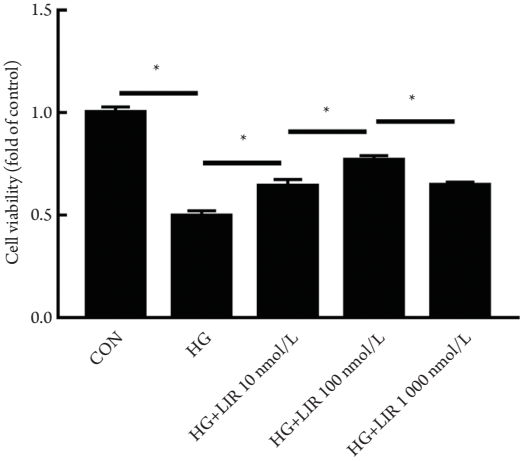

图1 利拉鲁肽对高糖环境下髓核细胞增殖活性的影响

Figure 1 Effect of liraglutide on high glucose-induced proliferation activity in nucleus pulposus cells

*P<0.05.

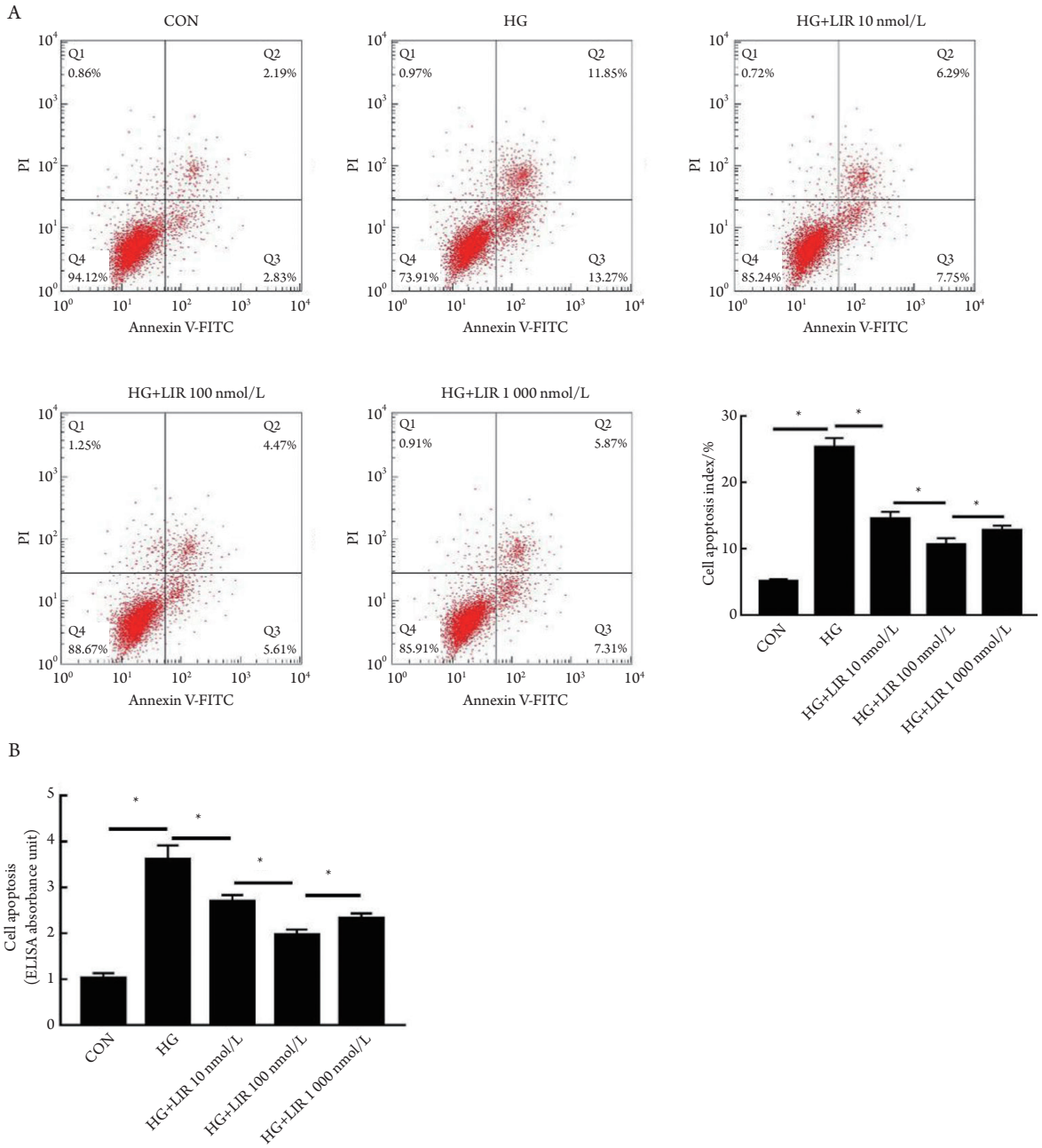

图2 利拉鲁肽对高糖诱导的髓核细胞凋亡的影响

Figure 2 Effect of liraglutide on high glucose-induced apoptosis in nucleus pulposus cells

细胞用高糖+不同浓度的利拉鲁肽(0，10，100或1 000 nmol/L)培养48 h。(A)应用流式细胞术，采用Annexin V-FITC/PI双染检测细胞凋亡。高糖组细胞的凋亡率明显高于对照组，利拉鲁肽的应用有效抑制了高糖诱导的髓核细胞凋亡。(B)应用ELISA法检测细胞凋亡，结果显示高糖增加了髓核细胞的凋亡，利拉鲁肽抑制了髓核细胞凋亡。*P<0.05。

Cells were exposed to high glucose and liraglutide (0, 10, 100, or 1 000 nmol/L) for 48 h. (A) Apoptosis was determined by annexin V-propidiumiodide (PI) double staining. The percentage of apoptotic cells by flow cytometry was higher for cells treated with high glucose than control group, which could be effectively decreased by the use of liraglutide. (B) Cell death detection ELISA showed that high glucose increased the apoptosis of nucleus pulposus cells and liraglutide suppressed it. *P<0.05.

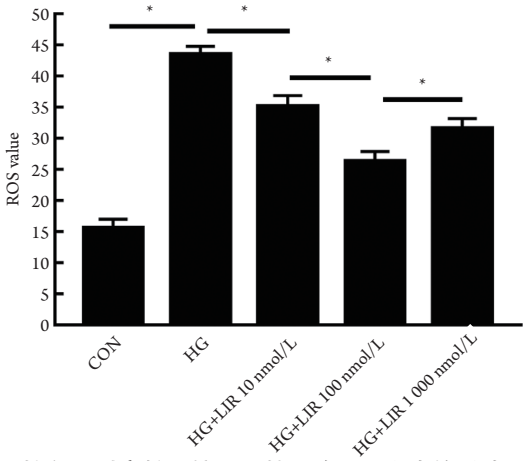

图3 利拉鲁肽对高糖环境下髓核细胞ROS生成的影响

Figure 3 Effect of liraglutide on high glucose-induced ROS generation in nucleus pulposus cells

高糖诱导髓核细胞内ROS生成增加，而利拉鲁肽的加入明显降低了细胞内ROS的生成。*P<0.05。

High glucose induced the increase of intracellular ROS rate, and the addition of liraglutide obviously decreased it. *P<0.05.

3 讨论

椎间盘退变是一种常见的疾病，给全世界的医疗保健系统带来巨大的社会经济负担[18]。多项临床研究发现糖尿病和椎间盘退变相关[7,19]。Jhawar等[19]研究指出：在与腰椎间盘突出症可能有关的危险因素中，糖尿病的相对危险度(1.52)高于高血压(1.25)、高血脂(1.26)、心肌梗死(1.13)和吸烟(1.10)。髓核细胞的凋亡增加在椎间盘退变过程中起到了重要作用[20]。基础研究[21]也证实：高糖可导致髓核细胞存活率下降，并诱导细胞凋亡。在高糖环境下，细胞产生过多的细胞内ROS，可诱导脂质过氧化，导致细胞膜完整性丧失，线粒体膜去极化，凋亡信号通路激活，包括caspase-9/3过表达，线粒体介导的凋亡通路的激活最终导致细胞凋亡[22-23]。在本研究中，与对照组相比，高糖组人髓核细胞ROS的生成增加，细胞凋亡率升高，这与之前的研究[22-23]结果一致。因此，抑制高糖诱导的髓核细胞ROS及凋亡可能是延缓椎间盘退变的潜在策略。

GLP-1是主要由空肠、回肠及结肠L细胞分泌的肠道激素[10]。除对血糖控制有益之外，GLP-1可在不同的组织中对细胞增殖、分化和凋亡的调节具有多种功能[24]。并且，它的抗凋亡作用已在多种细胞中被证实，如心肌细胞[25]、胰腺β-cells[26]和MC3T3-E1细胞[17]。然而，关于GLP-1在椎间盘退变中的作用罕有报道。利拉鲁肽是GLP-1的长效类似物，通过与GLP-1受体相结合发挥作用[27]。给予GLP-1可抑制葡萄糖刺激的诱导型一氧化氮合酶活性，降低活性氧代谢产物的衍生物水平[28]。利拉鲁肽也可抑制NF-κB信号激活和NADPH氧化酶，增加SOD-2和过氧化氢酶的水平[29]。

本研究发现：利拉鲁肽的干预显著降低了人髓核细胞内ROS的水平，部分缓解了高糖(0.2 mol/L)诱导的人髓核细胞凋亡。提示利拉鲁肽能可能通过抗氧化应激，从而抑制高糖诱导的人髓核细胞凋亡。利拉鲁肽在中等浓度(100 nmol/L)时效果最好，不依赖于剂量。可能的解释是较小浓度的利拉鲁肽刺激不能引起显著变化，而较高的浓度刺激反而引起相反效果。此外，由于GLP-1受体属于GPCR家族，接触激动剂后可能会发生脱敏或受体敏感性快速衰减[30]。有研究[17]显示利拉鲁肽可通过GLP-1R介导的cAMP/PKA和PI3K/AKT/GSK3β等途径抑制体外成骨细胞的凋亡。本研究未进一步验证利拉鲁肽在髓核细胞中发挥保护作用的具体信号通路，故结论仍有待进一步的研究验证。

综上所述，本研究提示利拉鲁肽可通过抗氧化应激有效抑制高糖诱导的人髓核细胞凋亡，这可能是一种潜在的延缓糖尿病患者椎间盘退变的治疗策略。

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本文引用： 姚明言, 舒民, 张靖, 李志红, 马金辉, 景园园. 利拉鲁肽对高糖环境下体外培养的人髓核细胞凋亡的影响[J]. 临床与病理杂志, 2020, 40(5): 1073-1078. doi: 10.3978/j.issn.2095-6959.2020.05.001

Cite this article as: YAO Mingyan, SHU Min, ZHANG Jing, LI Zhihong, MA Jinhui, JING Yuanyuan. Effect of liraglutide on apoptosis of human nucleus pulposus cells induced by high glucose[J]. Journal of Clinical and Pathological Research, 2020, 40(5): 1073-1078. doi: 10.3978/j.issn.2095-6959.2020.05.001