doi: 10.13241/j.cnki.pmb.2017.07.009

血糖波动对 2 型糖尿病大鼠心肌组织形态的影响及机制研究

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摘要 目的：观察血糖波动对 2 型糖尿病大鼠心肌组织形态的影响并探讨其可能机制。**方法：**选取 45 只雄性 Sprague-Dawley(SD)大鼠，按随机数字法分成正常对照组(CON 组)和糖尿病组(DM 组)。DM 组给予 4 周高糖高脂饲料喂养后，予小剂量链脲佐菌素 35 mg/Kg 腹腔注射建立 2 型糖尿病大鼠模型。将成模大鼠按随机数字法分成糖尿病对照组(CDM 组)和波动血糖组(FDM 组)。FDM 组大鼠每天定时皮下注射短效胰岛素并错时给予葡萄糖，建立糖尿病血糖波动模型，CON 组、CDM 组予等量生理盐水皮下注射。每周测血糖值 2 天，4 次 / 天，动态观测各组大鼠外形、进食量、饮水量等变化。造模成功 12 周后取腹主动脉血测定糖化血红蛋白(HbAlc)，取大鼠心脏组织行 Masson 染色观察心肌纤维化水平，免疫印记法分别检测心肌组织 AKT、p-AKT 蛋白的表达，免疫组织化学法分析各组大鼠心肌细胞中 Caspase-3 蛋白的表达。**结果：**①与 CON 组相比，FDM 组、CDM 组大鼠 HbA1c、血糖变异系数(CV)水平升高，差异具有统计学意义(P<0.05)；与 CDM 组相比，FDM 组 HbA1c 水平差异无统计学意义(P>0.05)，CV 值进一步升高，差异具有统计学意义(P<0.05)。②与 CON 组相比，CDM 组、FDM 组大鼠心肌组织形心肌纤维化水平升高，差异具有统计学意义(P<0.05)；与 CDM 组相比，FDM 组心肌组织心肌纤维化水平进一步升高，差异具有统计学意义(P<0.05)。③与 CON 组相比，FDM 组、CDM 组大鼠心肌 p-AKT 蛋白表达水平均减少，差异具有统计学意义(P<0.05)；与 CDM 组相比，FDM 组 p-AKT 蛋白表达水平均进一步减少，差异具有统计学意义(P<0.05)。而三组大鼠心肌组织 AKT 蛋白表达水平差异无统计学意义(P>0.05)。④与 CON 组相比，FDM 组、CDM 组大鼠心肌 Caspase-3 蛋白表达水平均升高，差异具有统计学意义(P<0.05)；与 CDM 组相比，FDM 组 Caspase-3 蛋白表达水平进一步升高，差异具有统计学意义(P<0.05)。**结论：**血糖波动可加重 2 型糖尿病大鼠的心肌纤维化，其机制可能与抑制 AKT 活化有关。

关键词：糖尿病；血糖波动；糖尿病心肌病；AKT；Caspase-3

中图分类号：R-33；R587.1 **文献标识码：**A **文章编号：**1673-6273(2017)07-1237-05

A Study on the Effects and Mechanisms of Blood Glucose Fluctuation on Myocardium of type 2 Diabetic Rats

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ABSTRACT Objective: To investigate the effects of blood glucose fluctuation on myocardium in rats and explore the related mechanisms. **Methods:** Forty-five male Sprague Dawley rats were firstly randomly divided into the diabetes mellitus group (DM group) and the control group (CON group). The CON group was fed a standard diet, and the DM group was fed with a high sugar and fat diet for 4 weeks. Then, the DM group received single intraperitoneal (i.p.) injections of streptozotocin at 35 mg/kg. Three days later, blood samples from the tail vein were collected and measured. The diabetic rats exhibited elevated blood glucose levels (≥ 16.7 mmol/L) lasting 3 days, confirming diabetes. Then the diabetic rats were randomly divided into continuous high blood glucose group (CDM group) and fluctuating blood glucose group (FDM group). FDM group was induced by daily subcutaneous insulin and glucose feeding at different time points. 12 weeks later, aorta blood was collected to measure glycosylated hemoglobin A1c (HbA1C). Myocardial fibrosis was measured by Masson's trichrome staining. The levels of Akt and p-Akt expressions were determined by western blot. The levels of Caspase-3 were determined by immunohistochemistry. **Results:** ① The HbA1c level was significantly increased in the CDM and FDM groups compared with the CON group (both P<0.05), and no significant difference was found between CDM and FDM groups (P>0.05). The CV value of blood glucose in FDM and CDM groups were increased compared with the CON group (both P<0.05), which was significantly higher in the FDM group compared with the CDM group (P<0.05). ② Masson's trichrome staining showed that the percentage area of fibrosis in the CDM group were higher than that of the CON group (P<0.05), but the ratio in FDM group was

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（收稿日期：2016-05-20 接受日期：2016-06-16）

significantly higher than that of the CDM group (P<0.05). ③ The expression levels of AKT showed no significant difference between three groups (P>0.05). However, the expression levels of p-AKT were significantly decreased in the CDM and FDM groups compared with the CON group (P<0.05), and levels in the FDM group was significantly lower than of the CDM group (P<0.05). ④ The expression levels of Caspase-3 were significantly increased in the CDM and FDM groups compared with the CON group (P<0.05), which was significantly higher in the FDM group than that in the CDM group (P<0.05). **Conclusion:** Blood glucose fluctuation could aggravate the fibrosis in heart, and which was possibly related to the inhibition of AKT activity.

Key words: Diabetes mellitus; Blood glucose fluctuation; Diabetic cardiomyopathy; AKT; Caspase-3

Chinese Library Classification(CLC): R-33; R587.1 Document code: A

Article ID: 1673-6273(2017)07-1237-05

前言

糖尿病性心肌病 (diabetic cardiomyopathy,DCM) 于 1972 年由 Rubler 等[1]首次提出,作为糖尿病(diabetes mellitus,DM)独立的并发症已被肯定[2]。DCM 是以心肌功能障碍及病理性心肌细胞损伤为特点,表现为心肌肥大、纤维化、凋亡以及坏死[3]。而由多种因素触发的心肌细胞凋亡与坏死在 DCM 病情进展中发挥着重要作用[4]。近年国内外研究发现波动性血糖的危害可能超过持续性高血糖[5-7]。研究表明血糖波动可能与糖尿病心血管风险发生有关[8],波动性高血糖较持续性高血糖更能增加心血管内皮细胞凋亡[9-11]。Saito S 等[12]研究亦发现血糖波动可增加细胞凋亡,促进心肌细胞死亡及纤维化,加重心肌的损害。但到目前为止,血糖波动加重 DCM 病情进展的具体机制尚不完全明确。AKT 是一种重要的抗凋亡因子,通过降低细胞凋亡相关蛋白发挥心脏保护作用, 高血糖抑制 AKT 信号通路是触发心肌凋亡及坏死的关键环节[13-15]。本研究通过建立血糖波动糖尿病大鼠模型,观察正常血糖、持续性高血糖和波动性血糖对心肌纤维化改变及心肌组织 AKT、p-AKT 蛋白表达水平的影响,旨在初步探讨血糖波动对 DCM 进展的影响及其可能机制。

1 材料和方法

1.1 材料

1.1.1 实验动物 6~8 周龄清洁级健康、雄性 Sprague-Dawlay (SD)大鼠 45 只,体重 150~180 g(徐州医科大学 SPF 级实验动物中心提供,许可证号:SCXK(沪)2008-0016)。

1.1.2 主要仪器和试剂 全自动糖化血红蛋白仪、电泳仪、酶标仪、凝胶成像系统、水平电泳槽及 Odyssey 激光成像系统购自美国 BIO-RAD 公司, 光学显微镜购自日本 OLYMPUS 公司,OLYMPAS 显微镜(显微摄像)购自日本奥林巴斯公司。链脲佐菌素(STZ)购自美国 Sigma 公司,HBAlc 检测试剂盒购自美国 BIO-RAD 公司,Masson 染色试剂盒购自南京建成科技有限公司, 免疫组化 SABC 试剂盒购自武汉博士生物工程有限公司, 兔抗大鼠 Caspase-3 多克隆抗体 (一抗)、兔抗大鼠 AKT1+2+3 多克隆抗体(一抗)及兔抗大鼠 p-AKT(Ser473)多克隆抗体(一抗)购自北京博奥森生物工程有限公司。

1.2 方法

1.2.1 2 型糖尿病大鼠模型的建立方法 大鼠自由饮水,在温度为 21℃ ~27℃ ,相对湿度 40%~70%,保持通风干燥、环境安静,12 h 光照周期,垫料干燥的饲养环境中适应性喂养 1 周。随机取 10 只作为正常组 (CON 组), 其余 35 只为糖尿病组(DM 组),CON 组喂予普通饲料,DM 组喂予高糖高脂饲料。4 周后,DM 组大鼠禁食 12 h,用现配的柠檬酸缓冲液(0.1 mmol/L,pH 4.2)溶解 STZ 后,配成 1%浓度,按 35 mg/kg STZ 腹腔注射诱导 2 型糖尿病,CON 组大鼠一次性腹腔注射相同体积新鲜配制的柠檬酸盐缓冲液。注射 STZ 72 h 后取大鼠尾静脉血,用血糖仪测定随机血糖值,(最大血糖值为 33.3 mmol/L, 更高的血糖值仪器显示为 High, 这种情况血糖值被当作为 33.3 mmol/L),连续检测 3 天,随机血糖水平≥ 16.7 mmol/L 为造模成功,该过程中 1 只死亡,34 只造模成功。

1.2.2 血糖波动糖尿病大鼠模型的建立方法 将 34 只糖尿病造模成功的大鼠按随机数字表法分成两组:持续高血糖组(CDM 组)17 只和波动血糖组(FDM 组)17 只。FDM 组,每日定时皮下注射 1 次普通胰岛素 12~23 U, 并错时喂予高糖水,根据血糖调整胰岛素用量,使一天中血糖最大值≥ 20 mmol/L、血糖最小值≤ 10 mmol/L。

1.2.3 标本的采集 糖尿病大鼠模型建立成功后,每周测血糖值 2 天,每天 4 次(10:00、12:00、14:00、16:00),以 CV 值(CV= 标准差 / 均数)作为血糖波动的统计学指标。干预 12 周后,用 10%水合氯醛按 3~3.5 mL/kg 腹腔麻醉,取腹主动脉血测定糖化血红蛋白,取心尖部组织 -80℃ 保存,其余部分置于 4%多聚甲醛溶液中固定。

1.2.4 Masson 染色 按照 Masson 染色试剂盒步骤进行染色。染色结果采用 IDA-2000 高清晰度数码显微图像分析系统进行自动分析,计算心肌组织纤维化相对面积,每组按随机数字法选取 3 张切片,每张切片随机选取 5 个 400 倍视野,测定心肌组织蓝染区面积与整个视野面积的比值。

1.2.5 免疫印记法检测心肌 AKT、p-AKT 蛋白的表达 采用 Image-J 图像分析软件扫描, 以目的蛋白条带灰度值与 β-actin 条带灰度值的比值来反映目的蛋白表达水平。

1.2.6 免疫组织化学法检测心肌 Caspase-3 蛋白的表达 按照免疫组化 SABC 试剂盒步骤进行染色。应用 Image-ProPlus6.0 图像分析系统进行图片分析, 每组按随机数字法选取 3 张切片,每张切片随机选取 5 个 400 倍视野,测定其阳性染色的光密度平均值(即累积光密度 IOD/ 面积 A)。

1.3 统计学处理

采用 SPSS 13.0 统计软件进行数据处理, 计量资料数据以均数± 标准差($\bar{x}$± s)表示,多组间比较先行方差齐性分析,若方差齐则采用单因素方差分析,组间有别则多组间的两两比较采用 q 检验,两组间计数资料差异比较采用 x^2 检验,以 P<0.05 表示差异有统计学意义。

2 结果

2.1 各组大鼠的一般情况比较

实验过程中 CDM 组死亡 3 例,FDM 组死亡 5 例,CON 组无死亡。糖尿病大鼠明显多尿、多饮、消瘦、活动减少、毛发污秽无泽,其中 FDM 组糖尿病表现更加明显,同时伴有嗜睡、反应迟钝等症状。CON 组大鼠饮水量、食量、尿量均正常,反应敏捷、活动频繁、精神振奋,毛发白亮有光泽。

2.2 各组大鼠生化指标检测结果的比较

2.2.1 血糖 CON 组大鼠在整个实验过程中血糖稳定,处于正常范围内;CDM 组大鼠血糖处于持续稳定的高糖状态;FDM 组大鼠血糖则波动性较大。我们采用血糖变异系数(即血糖 CV 值)= 血糖标准差 / 血糖平均值表示血糖波动性,结果显示与 CON 组相比,CDM 组、FDM 组 CV 值均升高(P<0.05),与 CDM 组相比,FDM 组血糖 CV 值进一步升高,差异具有统计学意义(P<0.05)。(表 1)。

表 1 三组大鼠 CV 值的比较(%,$\bar{x}$± s)

Table 1 Comparison of the CV value of rats between three groups(%,$\bar{x}$± s)

Group	N	CV value
CON	10	15.30± 1.02
CDM	14	21.66± 1.59[a,b]
FDM	12	62.40± 4.47[a,b]

Note: compared with CON group, [a]P<0.05; compared with CDM group, [b]P<0.05.

2.2.2 糖化血红蛋白(HbA1c) 实验第 16 周,取大鼠腹主动脉血检测 HbA1c,结果显示与 CON 组相比,CDM 组、FDM 组大鼠 HbA1c 水平均升高,差异具有统计学意义(P<0.05),与 CDM 组相比,FDM 组 HbA1c 水平差异无统计学意义(P>0.05)(表 2)。

表 2 三组大鼠 HbAlc 值的比较(%,x± s)

Table 2 Comparison of the HbA1c of rats between three groups(%,x± s)

Group	N	HbA1c
CON	10	5.12± 0.73
CDM	14	18.02± 1.23[a]
FDM	12	17.89± 1.96[a]

Note: compared with CON group, [a]P<0.05.

2.3 各组大鼠心脏组织形态的比较

Masson 染色结果可见蓝染部分为胶原阳性染色,代表纤维化,胶原染色面积越大,提示纤维化越明显。光镜(× 400)下见 CON 组心脏组织病理改变不明显,细胞形态正常、排列整齐、紧密,心肌组织中几乎无蓝色染色;与 CON 组相比,CDM 组心肌细胞间基质成分增多,蓝染面积增加;与 CON 组相比,FDM 组心肌细胞间隙增宽,蓝染面积明显增多,心肌纤维化更加明显。与 CON 组相比,CDM 组、FDM 组纤维胶原染色相对面积增多,差异具有统计学意义(P<0.05),其中 FDM 组较 CDM 组进一步增多,差异具有统计学意义(P<0.05)。(图 1、2)

2.4 各组大鼠心脏组织 AKT、p-AKT 表达的比较

与 CON 组相比,CDM 组和 FDM 组 p-AKT 蛋白表达减少,差异具有统计学意义(P<0.05),FDM 组蛋白表达较 CDM 组进一步减少,差异具有统计学意义(P<0.05);而三组间 AKT 蛋白表达量差异均无统计学意义(P>0.05)。(图 3、4)

2.5 各组大鼠心脏组织 Caspase-3 表达的比较

光镜下观察到细胞内棕黄色颗粒为蛋白阳性表达,蓝染颗粒为细胞核,进一步行灰度值分析显示与 CON 组相比,CDM 组和 FDM 组 Caspase-3 蛋白表达增加,差异具有统计学意义(P<0.05),FDM 组蛋白表达较 CDM 组进一步增加,差异具有统计学意义(P<0.05)。(图 5、6)。

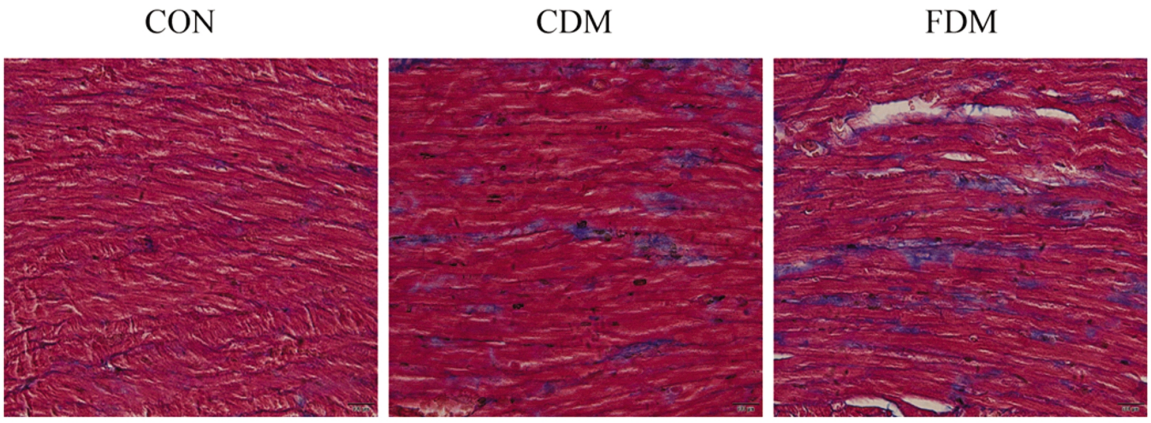

图 1 三组大鼠心肌组织纤维化面积的比较(Masson 染色,× 400)

Fig. 1 Comparison of fibrosis area of rat cardiac tissue between three groups (Masson stain)

3 讨论

2010 年美国糖尿病协会 (American Diabetes Association, ADA)将 HbA1c 作为诊断糖尿病的标准之一写入 ADA 糖尿病诊疗指南[16]。然而,HbA1c 作为长期血糖控制指标仅反映糖尿病患者 2~3 个月内平均血糖水平,却不会随着短期血糖波动的变化而发生明显改变[17]。血糖波动(glucose fluctuation,GF)又被称为血糖变异性(glucose variability,GV)或血糖稳定性(glucose stability,GS)等,是指血糖水平在峰值和谷值之间漂移的非稳定状态[18],血糖变异系数即血糖 CV 值是反映血糖离散程度的统计学指标,可反映血糖的波动情况。本研究结果显示 FDM 组大鼠 CV 值更高,HbA1c 水平与 CDM 组持平,提示

FDM 组大鼠血糖水平具有更大波动性，且血糖波动在短期内不会显著影响 HbA1c 水平的变化[17]。

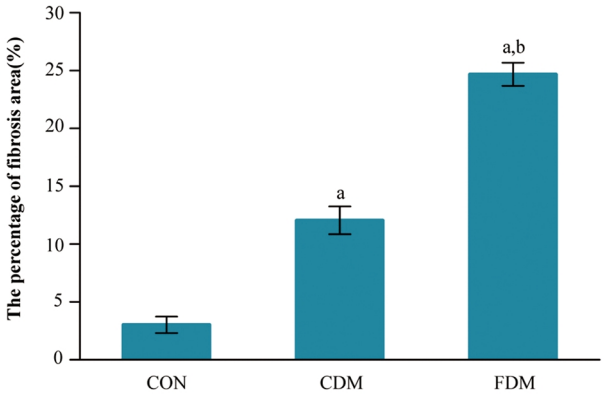

图 2 三组大鼠心肌组织纤维化面积百分比的比较(%)

Fig. 2 Comparison of the percentage of fibrosis area of rats' cardiac tissue between three groups

Note: Data were expressed as ± SD. aP<0.05, compared with CON; bP<0.05, compared with CDM group.

糖尿病心肌病(DCM)是糖尿病的慢性并发症之一，主要以心肌细胞排列紊乱、细胞间隙增宽和心肌细胞凋亡与坏死增加及纤维化为病理特征[19]。由于心肌细胞缺乏自我再生能力，坏死后可由纤维组织替代，从而影响心肌正常功能，因此这一特征具有致命性损害[20-22]。在本实验中，Masson 染色显示糖尿病大鼠心肌组织结构较正常组异常，与 DCM 病理学特征类似，

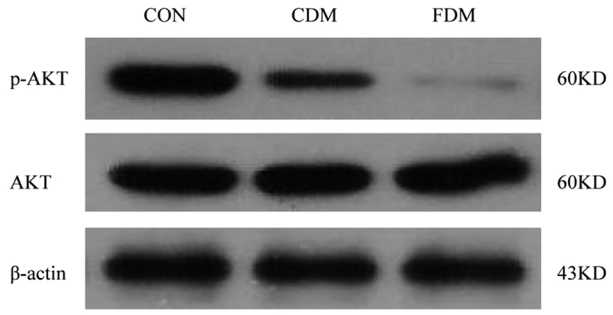

图 3 各组大鼠心脏组织 p-AKT、AKT 蛋白的免疫印迹分析

Fig. 3 Western blotting analysis of p-AKT, AKT protein expressions in cardiac tissue of rats between three groups

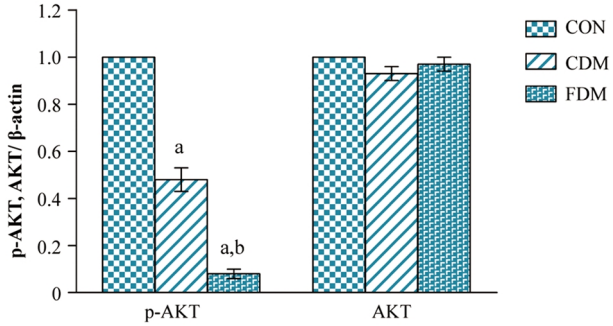

图 4 各组大鼠心脏组织 p-AKT、AKT 蛋白表达的比较

Fig. 4 Comparison of the p-AKT, AKT protein expressions in cardiac tissue of rats between three groups

Note: Data were expressed as $\bar{x}$± SD. aP<0.05, compared with CON; bP<0.05, compared with CDM group.

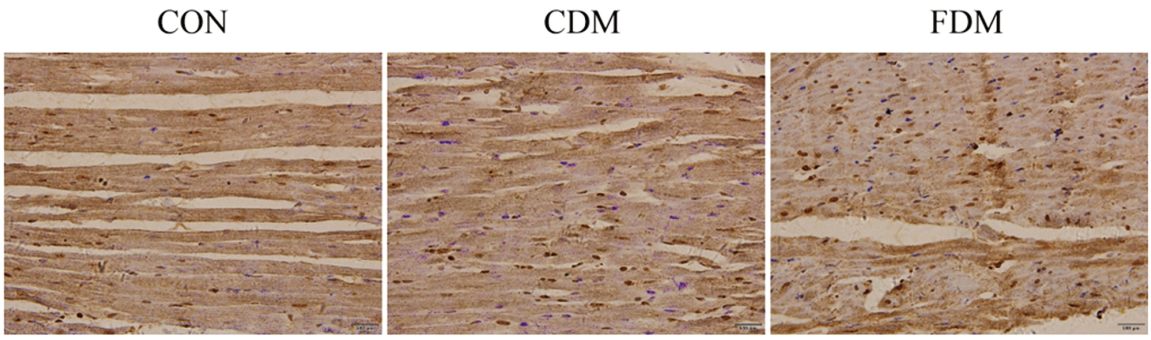

图 5 三组大鼠心肌细胞 Caspase-3 蛋白表达的比较(免疫组化染色，× 400)

Fig. 5 Comparison of Caspase-3 protein expression in rat cardiac cell between three groups (Immunohistochemistry)

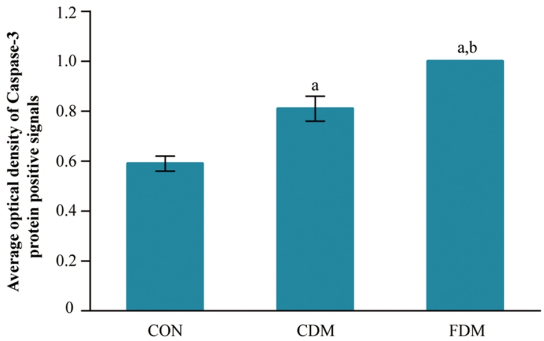

图 6 三组大鼠心肌细胞 Caspase-3 蛋白表达的比较

Fig. 6 Comparison of Caspase-3 protein expression in rat cardiac cell between three groups

Note: Data were expressed as $\bar{x}$± SD. aP<0.05, compared with CON; bP<0.05, compared with CDM group.

其中 FDM 组更加明显，CDM 组大鼠心肌纤维化水平较 CON 组升高，而与 CDM 组相比，FDM 组具有更高的纤维化水平，提示血糖波动可加重糖尿病时心肌纤维化。血糖波动通过多方面机制促进 DCM 的进展。杨玉芝等[23]临床研究发现血糖波动可能通过影响结缔组织生长因子水平参与 DCM 的心肌纤维化。亦有相关基础研究证实间歇性高血糖可增加氧化应激水平，加剧细胞凋亡，促进心肌细胞死亡，加重糖尿病心肌损害[24]。

慢性持续性高血糖是引起糖尿病慢性并发症的关键启动因素[25]，可引起心脏组织氧化应激水平明显升高[26,27]，进而提高激活通过多条信号通路诱导心肌细胞凋亡，AKT/GSK-3β 信号转导通路为其中最重要的信号传导途径之一[28-30]。此外，Ma H 等[31]研究表明糖尿病时心肌细胞凋亡可能与高血糖诱导的 AKT 信号通路变化有关。既往的研究表明与持续性高血糖相比，间歇性高血糖更能增加氧化应激水平，从而加剧细胞凋亡[23]。

因此，我们推测血糖波动增加可能通过影响 AKT 信号通路诱导心肌损伤。

蛋白激酶 B (AKT)通路是细胞内最经典的生存通路，在细胞的生存及抗凋亡中起到重要的作用[32,33]。AKT 是该通路中最重要的靶酶，具有两个磷酸化位点即 Ser473 和 Thr308[34]，受到上游的磷脂酰肌醇 3 磷酸激酶 (phosphatidylinositol 3-kinase, PI3K)活化后，将进一步磷酸化下游的相关蛋白因子。Caspase-3 是 AKT 下游重要的凋亡蛋白，AKT 磷酸化水平减低可促进 Caspase-3 表达增加，进一步促进细胞的凋亡[35]。本实验通过免疫印记法分别对三组大鼠心肌组织中 AKT、p-AKT (Ser473)蛋白进行半定量分析，结果显示 AKT 蛋白在三组大鼠心肌中表达水平基本相似 (P>0.05)。与 CON 组相比，CDM 组心肌 p-AKT(Ser473)蛋白表达水平减少(P<0.05)；与 CDM 组相比，FDM 组心肌 p-AKT (Ser473) 蛋白表达水平进一步减少(P<0.05)。通过免疫组化法检测各组大鼠心肌细胞的 Caspase-3 蛋白表达结果发现其在 CON 组、CDM 组、FDM 组中的表达量呈依次递增趋势(P<0.05)。以上结果提示与持续高血糖相比，血糖波动可进一步减少 AKT 的磷酸化表达，抑制 AKT 活性，进而增加凋亡蛋白 Caspase-3 的表达。因此，血糖波动可加重糖尿病心肌纤维化，其机制可能与抑制 AKT 活性有关，但是否依赖于 AKT 通路的活化仍有待于进一步的研究证实。

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