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TLR7 在 1 型糖尿病大鼠肾缺血再灌注损伤中的作用研究 *

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摘要 目的:探讨 Toll 样受体 7(TLR7)介导的 MyD88/NF- κ B 信号通路在 1 型糖尿病大鼠肾缺血再灌注损伤中的作用。**方法:**雄性 SD 大鼠随机分为 3 组(n=6),糖尿病假手术组(DS),糖尿病缺血再灌注组(DIR),糖尿病缺血再灌注 + 氯喹预处理组(DIR+CQ)。采用腹腔注射链尿佐菌素 65 mg/kg 建立糖尿病模型,TLR7 抑制剂氯喹预处理于糖尿病模型成功后第 3 周 0.5%氯喹 40 mg/kg 进行腹腔注射,连续给药 7 天。于第四周采用双侧肾蒂夹闭 25 min,再灌注 48 h 建立肾缺血再灌注损伤模型。取大鼠肾脏 HE 染色观察大鼠病理学结果,血标本测定血尿素氮(BUN)和血肌酐(Scr)水平,ELISA 法检测白细胞介素 6(IL-6)和肿瘤坏死因子- α (TNF- α),TUNEL 法检测细胞凋亡,Western blot 检测 TLR7,MyD88 和 NF- κ B 蛋白表达。**结果:**与 DS 组相比,DIR 组肾小管肿胀,间质水肿,刷状缘丢失,空泡变性坏死,Paller 评分升高($P<0.01$)。与 DIR 组相比,氯喹预处理可以改善肾损伤($P=0.017$);与 DS 组相比,DIR 组 BUN,Scr,IL-6,TNF- α , 细胞凋亡指数 (Apoptosis %),TLR7,MyD88,NF- κ B 增高($P<0.05$);与 DIR 组相比,DIR+CQ 组 BUN,Scr,IL-6,TNF- α ,Apoptosis %,TLR7,MyD88,NF- κ B 降低($P<0.05$)。**结论:**TLR7 介导的 MyD88/NF- κ B 信号通路参与糖尿病肾缺血再灌注损伤,氯喹通过抑制 TLR7 表达,阻断 MyD88/NF- κ B 信号通路,降低炎症反应,从而减轻 1 型糖尿病大鼠肾缺血再灌注损伤。

关键词:TLR7;糖尿病;肾缺血再灌注损伤**中图分类号:**R-33; R587.2 **文献标识码:**A **文章编号:**1673-6273(2019)03-411-05The Effect of TLR7 on Renal Ischemia Reperfusion Injury
in Type 1 Diabetic Rats*HUANG Ya-yi, HUANG Ting, ZHAO Bo, WANG Hua-xin, XIAO Ye-da[△]

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ABSTRACT Objective: To investigate the role of MyD88/NF- κ B signaling pathway induced by TLR7 on renal ischemia reperfusion injury in Type 1 diabetic rats. **Methods:** The male SD rats, were randomly divided into three groups (n=6): diabetic sham group (group DS); diabetic ischemia reperfusion group (group DIR); diabetic ischemia reperfusion plus chloroquine pretreatment group (group DIR+CQ). The diabetic model was established by intraperitoneal injection of streptozotocin 65 mg/kg. Chloroquine, a TLR7 inhibitor, was pretreated by intraperitoneal injection of 0.5% chloroquine 40 mg/kg daily for 7 consecutive days at the third week after the diabetes model was successful. The renal ischemia reperfusion injury model was induced by occlusion of bilateral renal pedicle for 25 min and reperfusion for 48 h after the fourth week. The rats were sacrificed at 48h of reperfusion and the kidneys were removed for HE staining, the level of blood urea nitrogen (BUN) and blood serum creatinine (Scr), detection of interleukin - 6 (IL-6) and tumor necrosis factor α (TNF- α) by ELISA, cell apoptosis was detected by TUNEL and the expression of TLR7, MyD88 and NF- κ B protein by western blotting. **Results:** Compared with group DS, the renal tubules were swelling, interstitial edema, the brush border was losing and the cavitation was necrosis in group DIR, the Paller score was increased ($P<0.05$). After chloroquine preconditioning, the degree of renal injury was obviously relieved ($P=0.017$), compared with group DIR. Compared with group DS, BUN, Scr, IL-6, TNF- α , Apoptosis %, TLR7, MyD88 and NF- κ B were increased in group DIR ($P<0.05$). Compared with group DIR, BUN, Scr, IL-6, TNF- α , Apoptosis %, TLR7, MyD88 and NF- κ B were decreased in group DIR+CQ ($P<0.05$). **Conclusion:** MyD88/NF- κ B pathway induced by TLR7 is participated in Type 1 diabetic renal ischemia reperfusion injury. Chloroquine inhibits renal ischemia-reperfusion injury by inhibiting TLR7 expression, blocking MyD88/NF- κ B signaling pathway and reducing inflammatory reaction, thereby alleviating renal ischemia-reperfusion injury in type 1 diabetic rats.

Key words: TLR7; Diabetes; Renal ischemia reperfusion injury**Chinese Library Classification(CLC):** R-33; R587.2 **Document code:** A**Article ID:** 1673-6273(2019)03-411-05

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前言

糖尿病患者常伴有肾功能不全,且随着糖尿病病程的进展,肾功能不全的发生率呈逐年上升趋势,发生肾脏不良事件的几率亦急剧升高^[1]。糖尿病作为急性肾损伤的高危因素,具有高敏感性和预后差的特点^[2]。TLR7介导的MyD88/NF- κ B信号转导通路是炎症反应的重要信号通路之一^[3]。迄今为止,对于TLR7的研究主要集中在肝、肺、冠状动脉缺血再灌注及其相关疾病^[5-7]。然而,TLR7对糖尿病肾脏I/R的作用研究甚少。亦有研究表明Toll样受体(TLRs)是先天性免疫系统的重要组成部分^[8,9],在糖尿病的发病机制中起到触发作用^[10,11]。本研究拟评价TLR7介导的MyD88/NF- κ B信号通路在1型糖尿病大鼠肾缺血再灌注损伤中的作用。

1 材料与方法

1.1 材料与试剂

清洁级健康成年雄性SD大鼠,体重200 g~220 g,购自北京华阜康生物科技股份有限公司。肿瘤坏死因子 α (TNF- α)和白细胞介素6(IL-6)试剂盒购自伊莱瑞特生物科技股份有限公司。TUNEL试剂盒购自美国Roche公司。兔抗GAPDH和兔抗TLR7购自美国Novus Biologicals公司。兔抗MyD88购自英国Abcam公司。兔抗NF- κ B美国CST公司。氯喹购自美国Sigma公司。

1.2 方法

1.2.1 动物选择及分组 SD大鼠腹腔注射1%链脲佐菌素-柠檬酸盐缓冲液(Sigma公司,美国),剂量为65 mg/kg,3天后于尾静脉血检测血糖,血糖 ≥ 16.7 mmol/L为大鼠糖尿病模型制备成功,饲养周期为4周^[12]。取糖尿病模型制备成功的大鼠18只,采用随机数字表法分为3组(n=6):糖尿病假手术组(DS),糖尿病缺血再灌注组(DIR),糖尿病缺血再灌注+氯喹预处理组(DIR+CQ)。氯喹预处理于糖尿病模型成功后第3周0.5%氯喹40 mg/kg进行腹腔注射,连续给药7天。

1.2.2 大鼠肾缺血再灌注损伤模型的制备 在糖尿病大鼠模型造模成功后第四周,大鼠经腹腔注射2%戊巴比妥钠60 mg/kg,腹部正中切开显露肾脏,双侧肾脏肾蒂部位缓慢钝性分离,夹闭双侧肾蒂25 min后开放,肾脏由鲜红转为紫红表明阻断成功,肾脏由紫红恢复到鲜红提示再灌注成功。

1.2.3 指标测定 再灌注48 h后处死大鼠取肾组织,(1)HE染色观察大鼠病理学结果:制备石蜡切片,置于4%多聚甲醛溶液中固定24 h,HE染色,光镜下观察病理学结果。采用Paller法评估肾小管损伤,分数越高表示肾小管损伤程度越严重^[13]。(2)左心室取血标本检测血BUN和Scr水平。(3)取新鲜肾组织匀浆,取上清液,设立标准对照和空白对照,采用ELISA法检测IL-6和TNF- α 。(4)采用TUNEL试剂盒(Roche,美国)检测细胞Apoptosis%。肾脏石蜡切片常规处理,逐级脱蜡至水。制备TUNEL反应混合液后,处理组滴加50 μ L TUNEL反应混合液于样本上,经过孵育-洗涤-复染-脱水透明,晾干的切片可以在显微镜下观察或者采集图像。显微镜下组织切片上凋亡的细胞为棕褐色。以凋亡细胞数/视野细胞总数为凋亡指数进行数据统计。(5)采用Western blot法测定TLR7、MyD88和NF- κ B

蛋白指标。肾组织用裂解液提取蛋白,测定蛋白浓度。采用聚丙烯酰胺凝胶电泳分离目的蛋白。冰浴转膜后,脱脂奶粉室温封闭1-2 h。加入单克隆抗体TLR7 (1:500)、MyD88 (1:600)、NF- κ B (1:1000)4 $^{\circ}$ C孵育过夜。次日用HRP标记的二抗(抗兔,1:5000)孵育1 h后TBST清洗,显色曝光,以目的蛋白条带灰度值/GAPDH条带灰度值反映目的蛋白表达水平。

1.3 统计学处理

实验数据采用GraphPad Prism 6.0软件进行统计学分析。计量资料均采用均数 $\pm$ 标准误($\bar{x} \pm SE$)来表示,多组间比较运用单因素或双因素方差分析来进行,若 $P < 0.05$ 则差异具有统计学意义。

2 结果

2.1 肾脏组织病理学检测结果

HE染色光镜下观察:DIR组肾小管肿胀,间质水肿,刷状缘丢失,空泡变性坏死,表现出明显的肾组织病理损伤。DIR+CQ组的肾脏结构基本正常,与DIR组相比,肾组织病理评分显著下降($P=0.017$)(见图1)。

2.2 大鼠肾组织细胞凋亡情况

TUNEL法检测细胞凋亡,显示与DS组相比,DIR组细胞凋亡增多($P < 0.05$);与DIR组相比,DIR+CQ组细胞凋亡减少($P < 0.05$)。变化趋势与肾脏HE染色paller评分一致(见图2)。

2.3 大鼠肾脏功能及炎症因子检测

大鼠肾功能指标显示,与DS组相比,DIR组BUN和Scr增高($P < 0.05$);与DIR组相比,DIR+CQ组BUN和Scr降低($P < 0.05$)。肾脏组织炎症因子检测显示,与DS组相比,DIR组IL-6和TNF- α 增高($P < 0.05$);与DIR组相比,DIR+CQ组IL-6和TNF- α 降低($P < 0.05$)。见表1。

2.4 大鼠肾组织TLR7、MyD88和NF- κ B蛋白表达情况

Western blotting分析大鼠肾组织TLR7、MyD88及NF- κ B蛋白表达。与DS组相比,DIR组TLR7、MyD88、NF- κ B增高($P < 0.05$);与DIR组相比,DIR+CQ组TLR7、MyD88、NF- κ B降低($P < 0.05$)。见图3及表2。

3 讨论

急性肾缺血再灌注损伤是指肾脏组织缺血时和其后恢复血液灌注时器官功能未能恢复正常状态,甚至发生更为严重的组织器官损伤,如肾功能不全或肾功能衰竭。肾脏由于其组织结构和功能的特殊性,对缺血再灌注损伤尤为敏感。因此,临床上因缺血导致的急性肾功能不全或急性肾功能衰竭十分常见,如严重脱水、烧伤、失血性休克、弥散性血管内凝血、心肺复苏、心脏体外循环、肾脏移植术、心脏移植术和大血管手术等过程中,特别是多脏器功能衰竭的危重患者几乎都伴有急性肾衰竭的发生,而此后恢复灌注使肾功能损伤继续加重^[14]。临床上急性肾损伤(Acute kidney injury, AKI)延长住院周期,增加患者死亡率。值得关注的是急性肾损伤患者即使康复后也要面对较高的远期死亡率^[15]。在诱发AKI的众多病因中,缺血再灌注损伤依然是围术期AKI的主要病因^[16]。缺血再灌注损伤时短暂的阻断肾脏局部或总体血流供应后当血流供应恢复时,缺血肾组织将遭受持续严重损伤,其病理学特征表现为血管、肾小管

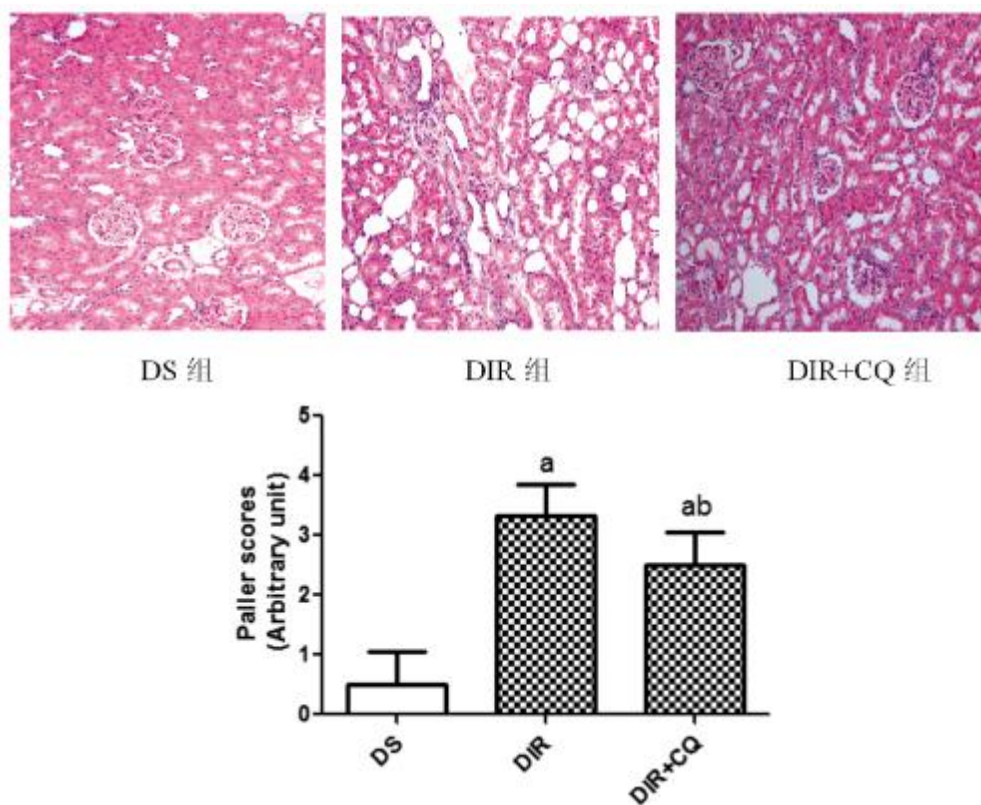


图1 肾小管 HE 染色 (n=6, × 200)

Fig. 1 The HE staining of Renal tubular (n=6, × 200)

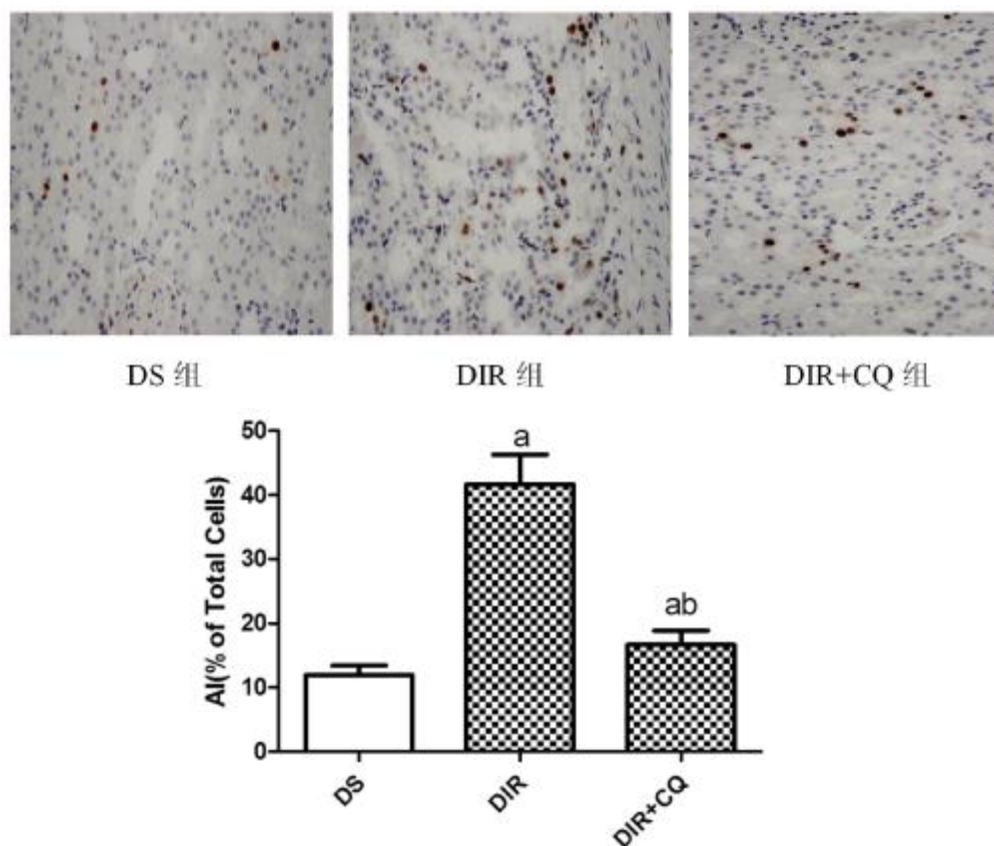
注:与 DS 组比较,^a $P<0.05$;与 DIR 组比较,^b $P<0.05$ 。Note: ^a $P<0.05$, compared with group DS; ^b $P<0.05$, compared with group DIR.

图2 肾脏细胞凋亡的比较 (n=6, × 200)

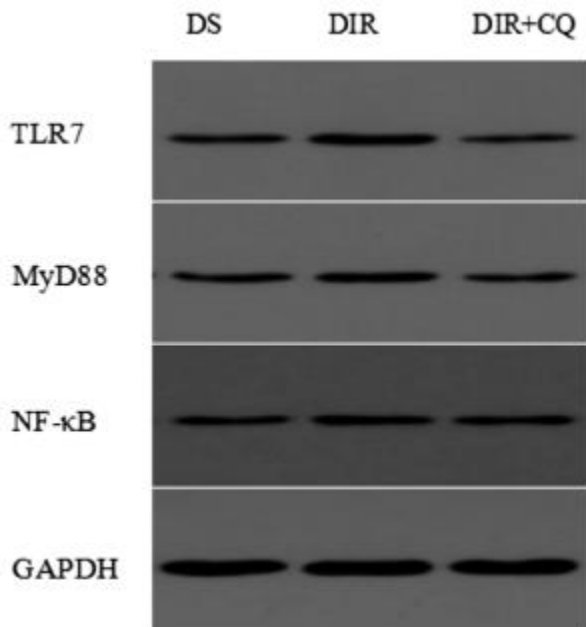
Fig. 2 The comparison of renal cell apoptosis (n=6, × 200)

Note: ^a $P<0.05$, compared with group DS; ^b $P<0.05$, compared with group DIR.

表 1 三组 BUN, Scr, IL-6, TNF- α 的比较 (n=6, $\bar{x} \pm s$)Table 1 The comparison of BUN, Scr, IL-6, TNF- α in three groups (n=6, $\bar{x} \pm s$)

	BUN	Scr	IL-6	TNF- α
DS	7.1620.941	34.264.891	53.775.367	59.874.783
DIR	23.602.727 ^a	112.38.739 ^a	112.19.388 ^a	117.07.562 ^a
DIR+CQ	14.341.364 ^{ab}	72.185.854 ^{ab}	74.456.219 ^b	80.775.563 ^{ab}

Note: ^a $P < 0.05$, compared with group DS; ^b $P < 0.05$, compared with group DIR.

图 3 各组肾组织 TLR7、MyD88 及 NF- κ B 蛋白表达Fig. 3 The expression of TLR7, MyD88 and NF- κ B proteins in renal tissues of each group (n=6)

内大量炎症因子侵袭^[17]。

根据国内外研究^[2], 1 型糖尿病肾病是青少年终末期肾病的主要类型。在肾功能不全的发生率方面, 糖尿病患者发生肾功能不全的发生率是非糖尿病患者的 17 倍, 并且随着糖尿病病程的进展, 肾功能不全的发生率呈逐年上升的趋势, 发生肾脏不良事件的机率急剧升高。Fernandez JG 等人的研究显示^[3], 如果糖尿病患者病程小于 5 年者, 糖尿病肾病的发生率为 7% 至 10%; 如果糖尿病患者病程处于 10 年至 20 年之间, 糖尿病肾病的发生率上升为 20% 至 35%; 如果糖尿病患者病程超过 20 年, 糖尿病肾病的发生率几乎为 100%。1 型糖尿病患者发病早、病程久, 40% 的 1 型糖尿病患者最后会进展成 T1DN^[18]。T1DN 目前的治疗多样化, 包括保护肾功能, 调整血糖水平, 维持合适肾灌注压, 改善高脂血症等, 这些措施都能延缓 T1DN

的进展^[19], 即使如此, 仍会有一部分糖尿病患者随着病情的发展最终发生慢性肾衰竭、终末期肾脏病和尿毒症等恶性不良并发症^[18]。T1DN 是糖尿病高致残率、致死率最重要的影响因素之一, 严重影响 T1DM 患者的生存质量^[20]。1 型糖尿病由于疾病病程长, 对全身多个脏器损伤持久, 尤其对肾脏的损伤严重, 疾病发展到晚期往往形成糖尿病肾病。因此, 糖尿病患者肾脏更为脆弱, 是最容易受到损伤波及的器官, 其临床表现往往早于其他组织器官, 常常导致急性肾损伤的发生。目前有研究认为, 糖尿病状态下机体处于高氧化应激和强炎症反应状态, 导致大量氧自由基和炎症因子的生成与释放, 从而诱发脏器产生更严重的缺血再灌注损伤。糖尿病肾病是糖尿病的主要并发症之一, 而高糖可诱发肾脏细胞的炎症反应。糖尿病患者发生肾脏不良事件几率较非糖尿病患者更高, 预后更差。因此糖尿病肾脏缺血再灌注损伤的机制及其防治是临床热点问题之一^[21]。

炎症反应是促进糖尿病病程进程的重要因素之一。当肾脏细胞受损后, 释放炎症介质, 并通过释放 TNF- α 、PDGF 等多种细胞因子及外基质进一步加剧局部组织的炎症反应, 从而参与糖尿病的发生发展^[22]。且糖尿病患者肾脏对缺血再灌注损伤的易损性增加, 引起肾血管和肾小管系统的复杂炎症反应过程, 其机制均涉及到: 炎性反应对 Toll 样受体的活化。TLR7 蛋白通过活化髓样分化因子 88 (MyD88) 依赖性途径从而激活核转录因子 (NF- κ B), 触发促炎性细胞因子和趋化因子的产生, 导致机体损伤^[23,24]。

Toll 样受体家族是近年来发现的在识别病原微生物、启动免疫防御反应中起重要作用的受体蛋白。MyD88 是万能受体, 它可触发除 TLR3 以外所有 TLR 对 NF- κ B 的激活。TLR7 正是通过依赖 MyD88 的信号通路活化免疫细胞发挥作用, 产生联级炎症反应, 释放大量炎症因子, 包括 TNF- α 、IL-2、IL-4、IL-6、CCL2/MCP-1、CXCL1, 和 CXCL10/IP-10 等^[25,26]。大量炎症因子释放进一步加重肾脏损伤^[27,28]。本研究结果表明: 缺血再灌注期间, TLR7 表达增加, 进而激活 MyD88/NF- κ B 信号通路, 导致 IL-6、TNF- α 表达增多, 导致炎症反应的爆发, 肾脏细胞凋亡增加。肾脏病理学改变显示糖尿病缺血再灌注组肾脏近曲小管多

表 2 三组 TLR7, MyD88, NF- κ B 的比较 (n=6, $\bar{x} \pm s$)Table 2 The comparison of TLR7, MyD88, NF- κ B in three groups (n=6, $\bar{x} \pm s$)

	TLR7	MyD88	NF- κ B
DS	0.3938 $\pm$ 0.023	0.3542 $\pm$ 0.037	0.3338 $\pm$ 0.027
DIR	0.6612 $\pm$ 0.082 ^a	0.6372 $\pm$ 0.069 ^a	0.7798 $\pm$ 0.083 ^a
DIR+CQ	0.4456 $\pm$ 0.037 ^b	0.4682 $\pm$ 0.034 ^{ab}	0.5762 $\pm$ 0.033 ^{ab}

Note: ^a $P < 0.05$, compared with group DS; ^b $P < 0.05$, compared with group DIR.

个上皮细胞肿胀伴坏死,管腔扩张,空泡形成,其中可见管型、充血,可见大量中性粒细胞、炎性细胞浸润,肾小球改变明显,肾脏损伤加重。从而证实 TLR7 通过活化 MyD88/NF- κ B 信号通路介导炎症级联反应的爆发导致机体失衡,引起肾脏功能严重受损。

氯喹作为一种常用的自噬抑制剂,近年发现其在治疗自身免疫性疾病,病毒感染性疾病,抗肿瘤方面具有重大的作用^[23]。近期研究发现,氯喹作为 TLR7 抑制剂,可通过调节 TLR7 信号通路调节免疫反应,减轻炎症反应^[29]。其机制可能为阻止细胞内由 TLR7 介导的 MyD88/NF- κ B 信号通路的转导,从而抑制炎症细胞因子的释放^[30],减轻炎症级联反应。本研究结果显示:氯喹预处理后,TLR7、MyD88 及 NF- κ B 蛋白表达均下降,同时炎症因子 IL-6、TNF- α 释放降低,肾脏细胞凋亡减少,肾脏病理损伤得到改善。这些结果表明,TLR7 加重糖尿病大鼠肾缺血再灌注损伤。本研究结果显示,1 型糖尿病大鼠肾急性缺血再灌注肾损伤中,TLR7 过度活化,通过 MyD88/NF- κ B 信号转导通路,诱发肾脏损伤,这也许是糖尿病加重肾缺血再灌注损伤机制之一。抑制 TLR7 信号转导通路,使 MyD88 和 NF- κ B 的蛋白表达下调,可减轻 1 型糖尿病大鼠肾急性缺血再灌注损伤。

综上所述,TLR7 介导的 MyD88/NF- κ B 信号通路参与了 1 型糖尿病大鼠肾缺血再灌注损伤中的作用。氯喹通过抑制 TLR7 蛋白表达,阻断 MyD88/NF- κ B 信号通路降低炎症反应,从而降低 1 型糖尿病大鼠肾缺血再灌注损伤。

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