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HMGB1 对大鼠糖尿病足溃疡炎症的影响及机制分析 *

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摘要 目的：分析高迁移率族蛋白 1(High mobility group protein 1，HMGB1)对大鼠糖尿病足溃疡炎症的影响及机制。**方法：**经腹腔注射链脲佐菌素建立糖尿病大鼠模型，并在大鼠的双后足背部做一 3 mm× 7 mm 的全层矩形皮肤缺损，接种质控菌株。将建模成功的大鼠随机分成：模型组(等量生理盐水)、低剂量组(12.5 μg·kg^{-1}·d^{-1})、中剂量组(25 μg·kg^{-1}·d^{-1})和高剂量组(50 μg·kg^{-1}·d^{-1})，每组 6 只，每天单次腹腔注射，连续给药 14 d。另取 6 只大鼠作为空白组(等量生理盐水)。于规定时间对各组大鼠创面愈合率、微血管密度 (Microvascular density，MVD)、组织中血管内皮生长因子 (Vascular endothelial growth factor，VEGF)、CD45、Toll 样受体 4(Toll like receptor 4，TLR4)、核因子 κB (Nuclear factor kappa-B，NF-κB)、白介素 -1β(Interleukin-1β，IL-1β)、白介素 -6(Interleukin-6，IL-6)、IL-8(Interleukin-8，IL-8)和肿瘤坏死因子 -α(Tumor necrosis factor-α，TNF-α)mRNA 或蛋白表达量进行检测。**结果：**与对照组相比，模型组大鼠创面治愈率和微血管密度均较低，VEGF、CD45、TLR4、NF-κB、IL-1β、IL-6、IL-8 和 TNF-α mRNA 或蛋白表达量均较高(P<0.05)。与模型组相比，三个剂量组大鼠创面治愈率和微血管密度均明显降低(P<0.05)，VEGF、CD45、TLR4、NF-κB、IL-1β、IL-6、IL-8 和 TNF-α mRNA 或蛋白表达量均较高(P<0.05)。且随着 HMGB1 注射剂量增大，创面愈合率、血管生成明显减少(P<0.05)，炎症因子表达量显著升高 (P<0.05)。**结论：**HMGB1 对大鼠糖尿病足溃疡炎症有促进作用，机制可能与 HMGB1/TLR4/NF-κB 信号通路相关。

关键词：高迁移率族蛋白 B1；糖尿病足；炎症；微血管密度

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Effect and Mechanism of HMGB1 on the Inflammation of Diabetic Foot Ulcer in Rats*

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ABSTRACT Objective: To analyze the effect and mechanism of High mobility group protein 1(HMGB1) on the inflammation of diabetic foot ulcer in rats. **Methods:** The diabetic rat model was established by intraperitoneal injection of streptozotocin, and a full-thickness rectangular skin defect of 3 mm× 7 mm was made on the back of two hind feet of the rat, and inoculation of quality control strains as successful model of foot ulcer. The rats were randomly divided into three groups: the model group (equal amount of normal saline), the low dose group (12.5 μg·kg^{-1}·d^{-1}), the medium dose group (25 μg·kg^{-1}·d^{-1}) and the high dose group (50 μg·kg^{-1}·d^{-1}). Each group consisted of six rats, which were injected intraperitoneally once a day for 14 days. Another 6 rats were taken as the blank group. The wound healing rate, microvascular density (MVD), vascular endothelial growth factor (VEGF), CD45, toll like receptor 4 (TLR4), nuclear factor kappa B (NF-κB), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8(IL-8) and tumor necrosis factor-α(TNF-α) mRNA or protein expression were detected. **Results:** Compared with the control group, the wound healing rate and microvessel density of the model group were lower, the expression of VEGF, CD45, TLR4, NF-κB, IL-1 β, IL-6, IL-8 and TNF-α mRNA or protein were higher (P<0.05). Compared with the model group, the wound healing rate and MVD of the three dose groups were lower, the expression of VEGF, CD45, TLR4, NF-κB, IL-1 β, IL-6, IL-8 and TNF-α mRNA or protein were higher (P<0.05). Compared with the model group, the wound healing rate and MVD of the three dose groups were significantly reduced (P<0.05), the expression levels of VEGF, CD45, TLR4, NF-κB, IL-1β, IL-6, IL-8 and TNF-α mRNA or protein were higher (P<0.05). With the increase of HMGB1 injection dose, the wound healing

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rate and angiogenesis decreased significantly (P<0.05), and the expression of inflammatory factors increased significantly (P<0.05). **Conclusion:** HMGB1 could promote the inflammation of diabetic foot ulcer in rats, and the mechanism may be related to HMGB1/TLR4/NF-κB signal pathway.

Key words: HMGB1; Diabetic foot; Inflammation; Microvascular density

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前言

糖尿病(Diabetes Mellitus,DM)是一组以慢性高血糖为特征的代谢性疾病[1,2]。国际糖尿病联盟(International Diabetes Federation,IDF)2019年发布数据显示全球约有4.63亿20~79岁成人患糖尿病(11个人中有1个为糖尿病患者);预计到2030年,糖尿病患者会达到5.784亿;预计到2045年,糖尿病患者会达到7.002亿。我国糖尿病患者人数居于全球首位,美国列于第三位,与西方相比,我国糖尿病流行形式更为严峻[3,4]。长期的高糖血症会导致大量并发症的出现,如心血管疾病[5]、肾脏疾病[6]、肝脏疾病[7]、视网膜病变[8,9]等。

糖尿病足(Diabetic Foot,DF)是指糖尿病患者在长期高血糖的基础上出现的不同程度的神经、末梢血管病变,引起的下肢肢体的溃疡、感染和(或)深层组织的破坏,对患者造成极大的痛苦,严重影响其生活质量[10-12]。统计结果显示糖尿病患者中约15 %会出现足部溃疡,其中需要截肢的概率为15 %~20 %[13,14]。感染、慢性炎症、免疫应答、糖尿病血管病变、血流动力学异常是引起DF的关键因素[15-17]。近年来,炎性反应在DF患者的发病机制中受到越来越多的重视。

高迁移率族蛋白1(High mobility group protein 1,HMGB1)为HMGB家族的一员,是一种高度保守的核蛋白,广泛分布在哺乳动物细胞中[18]。研究表明HMGB1是一种重要的炎性介质,当机体受缺血、缺氧、炎症等刺激时,中性粒细胞、单核巨噬细胞、免疫细胞等可主动分泌HMGB1,或通过坏死细胞的被动释放转移至细胞外,在炎症的放大及维持中起着至关重要的作用,为晚期炎症反应的标志物[19]。HMGB1可引起机体全身或局部炎性反应综合症,主要与Toll样受体4和下游炎性因子相关[20]。本研究就HMGB1对大鼠糖尿病足溃疡炎症的影响及机制进行分析,结果如下。

1 材料和方法

1.1 主要试剂和仪器

HMGB1、链脲佐菌素、多聚甲醛购自美国Sigma-Aldrich公司;枸橼酸钠缓冲液、兔抗鼠CD31单克隆抗体、兔抗鼠VEGF单克隆抗体和兔抗鼠CD45单克隆抗体购自赛默飞世尔科技(中国)有限公司;葡萄糖购自青岛海博生物;水合氯醛购自上海阿拉丁生化科技股份有限公司;TLR4、NF-κB、IL-1β、IL-6、IL-8和TNF-α ELISA试剂盒购自艾博抗(上海)贸易有限公司;OLYMPUS倒置相差显微镜购自日本奥林巴斯公司;实时荧光定量PCR仪器购自赛默飞世尔科技(中国)有限公司。质控菌株金黄色葡萄球菌-ATCC25923,菌悬液浓度1× 10^8/mL,兰州大学第一医院病原微生物室提供。

1.2 实验动物

SPF级雄性SD大鼠,8~10周龄36只,体重210~230 g,购自甘肃中医药大学实验动物中心。

1.3 模型建立及实验动物分组

将24只大鼠禁食水12 h后,经腹腔注射链脲佐菌素(STZ)55 mg/kg (用新鲜配制的0.1 mol/L、pH值为4.2的枸橼酸钠缓冲液配制,现用现配),诱导建立糖尿病大鼠模型。一周后,所有大鼠提前禁食水12 h后,按照2 g/kg标准灌胃葡萄糖溶液(200 g/L),2 h后采尾缘静脉血测定血糖值,将2次血糖≥ 16.7 mmol/L为糖尿病大鼠模型建立成功。各组大鼠采用10 %的水合氯醛按0.3 mL/kg腹腔注射麻醉后,在大鼠的双后足背部做一3 mm× 7 mm的全层矩形皮肤缺损,创口注射质控菌株视为足部溃疡造模成功,并记为第0天[21,22]。

将建模成功的大鼠随机分成:模型组、低剂量组(125 μg·kg^{-1}·d^{-1})、中剂量组(25 μg·kg^{-1}·d^{-1})和高剂量组(50 μg·kg^{-1}·d^{-1}),每组6只,每天单次腹腔注射,连续给药14 d。另取6只大鼠作为空白组,模型组和空白组每天单次腹腔注射等量生理盐水。

1.4 观察指标

1.4.1 创面愈合率 分别于给药后1 d、3 d、7 d、14 d对大鼠足背创面拍照,采用Image-Pro Plus 6.0软件计算其面积,并按照公式计算创面愈合率。创面愈合率=(原始创面面积 - 当前测量面积)/ 原始创面面积× 100 %[23]。

1.4.2 免疫组化检测MVD 取血后,采用颈椎脱臼法处死大鼠,收集足背部创伤组织标本,将其从中部分为两部分,取其中一半组织标本浸入10 %的中性多聚甲醛中固定,经脱蜡、封闭后,于切片上滴加兔抗鼠CD31单克隆抗体对血管内皮细胞进行染色标记,以见到染色浅黄色至深棕色为阳性细胞。取10个高倍镜(× 400)视野下血管数目的均值[24]。

1.4.3 免疫印迹法检测VEGF和CD45表达 取部分组织在液氮中研磨至粉末状,并放入单班制裂解液缓冲液中裂解,裂解液采用免疫印迹法进行检测。拍的照片用Quantity One图像分析软件对图像进行灰度分子,得到的灰度值与对应β-actin灰度值的比值为该蛋白质的相对表达量[25]。

1.4.4 RT-PCR检测组织中mRNA的表达 按照说明书将组织中总RNA经RNAiso Plus抽提后,取1 μg的总RNA反转录成cRNA,采用20 μL反应体系进行PCR反应。对组织中TLR4、NF-κB、IL-1β、IL-6、IL-8和TNF-α mRNA的表达量进行检测。

1.4.5 ELISA法检测组织中细胞因子检测 剩余组织,按照TLR4、NF-κB、IL-1β、IL-6、IL-8、TNF-α试剂盒操作说明书进行检测。

1.5 统计学方法

应用SPSS 23.0软件,计量资料以($\bar{x}\pm s$)表示,多组间比较采用方差分析,两组间比较采用SNK-q检验,P<0.05为差异

有统计学意义。

2 结果

2.1 五组创面愈合率对比

与对照组相比，模型组各时间点大鼠的创面治愈率均较低($P<0.05$)。与模型组相比，腹腔注射 HMGB1 后 1 d、3 d、7 d 和 14 d，三个剂量组大鼠创面治愈率均较低 ($P<0.05$)，且随着 HMGB1 注射剂量增大，大鼠创面愈合率降低，见表 1。

表 1 创面愈合率检测结果(n=6，$\bar{x}\pm s$)

Table 1 Test results of wound healing rate (n=6, $\bar{x}\pm s$)

Time	Control group	Model group	Low dose group	Middle dose group	High dose group	F	*P*
1 d	13.22± 1.82	10.92± 1.19	10.30± 0.92	9.13± 0.91	7.60± 0.92	18.090	＜0.05
3 d	48.93± 1.82	38.98± 1.40	33.95± 1.76	29.10± 1.65	23.72± 1.14	225.641	＜0.05
7 d	80.88± 2.83	61.92± 2.03	55.98± 2.21	47.38± 2.51	38.68± 1.92	284.371	＜0.05
14 d	92.50± 2.15	78.48± 1.47	67.92± 2.49	56.95± 2.33	48.45± 2.99	331.204	＜0.05

2.2 五组 MVD 对比

三个剂量组 MVD 高于模型组，模型组 MVD 显著高于对照组，且高剂量组 > 中剂量组 > 低剂量组($P<0.05$)，见表 2。五组 MVD 的免疫组化图蓝色点状颗粒代表细胞核，棕黄色颗粒代表新生血管。对照组分布少量的 MVD；模型组和低剂量组 MVD 的分布较对照组少量增多；中剂量组 MVD 分布多于模型组和低剂量组，且染色较模型组和低剂量组加深；高剂量组 MVD 显著增多、染色深，见图 1。

表 2 MVD 检测结果(n=6，$\bar{x}\pm s$)

Table 2 MVD test results (n=6, $\bar{x}\pm s$)

Result	Control group	Model group	Low dose group	Middle dose group	High dose group	F	*P*
MVD(a)	9.00± 0.89	11.33± 0.82	12.33± 0.52	13.67± 1.37	18.50± 1.52	63.446	＜0.05

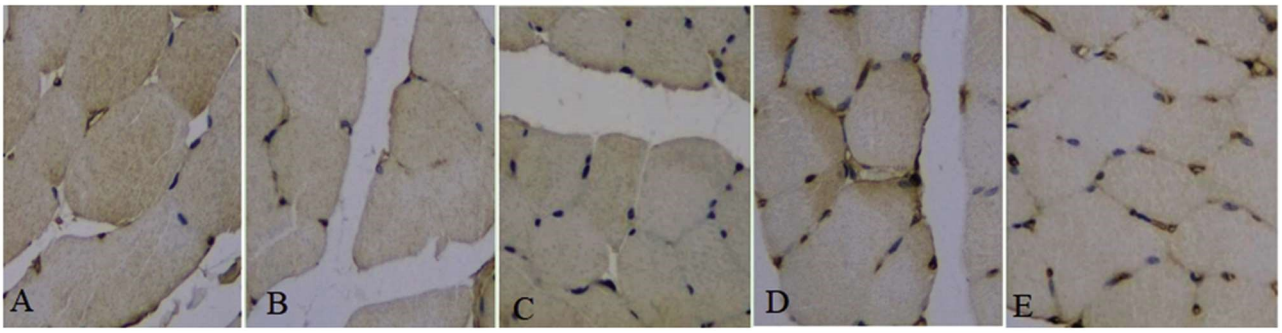

图 1 五组组织中 MVD 免疫组化检测图(× 400)

(A 表示对照组，B 表示模型组，C 表示低剂量组，D 表示中剂量组，E 表示高剂量组)

Fig.1 MVD immunohistochemical detection chart in five groups of tissues (× 400)

(A represents the control group, B represents the model group, C represents the low-dose group, D represents the middle-dose group, E represents the high-dose group)

2.3 五组 VEGF 和 CD45 表达对比

三个剂量组 VEGF 和 CD45 的表达量均显著高于模型组，模型组 VEGF 和 CD45 的表达量也显著高于对照组，且高剂量组 > 中剂量组 > 低剂量组 ($P<0.05$)，见表 3。五组 VEGF 和 CD45 的表达量的免疫印迹图梭形或环形物为 VEGF 和 CD45 蛋白染色。对照组有极少量的 VEGF 和 CD45 的蛋白表达；模型组和低剂量组 VEGF 和 CD45 蛋白表达较对照组增多；中剂量组较对剂量组显著增多；高剂量组 VEGF 和 CD45 表达较中剂量组增多，见图 2。

表 3 组织中 VEGF 和 CD45 检测结果(n=6，$\bar{x}\pm s$)

Table 3 VEGF and CD45 test results in tissues(n=6, $\bar{x}\pm s$)

Result	Control group	Model group	Low dose group	Middle dose group	High dose group	F	*P*
VEGF/β-actin	0.64± 0.06	1.02± 0.09	1.49± 0.08	1.72± 0.12	2.09± 0.20	137.660	＜0.05
CD45/β-actin	0.29± 0.04	0.42± 0.04	0.56± 0.05	1.08± 0.08	0.76± 0.08	152.207	＜0.05

2.4 五组组织中 mRNA 及蛋白的表达对比

三个剂量组 TLR4、NF-κB、IL-1β、IL-6、IL-8 和 TNF-α mRNA 和蛋白中的表达量均显著高于模型组，模型组 TLR4、NF-κB、IL-1β、IL-6、IL-8 和 TNF-α mRNA 和蛋白中的表达量也显

著高于对照组，且高剂量组 > 中剂量组 > 低剂量组($P<0.05$)，见表 4 和表 5。

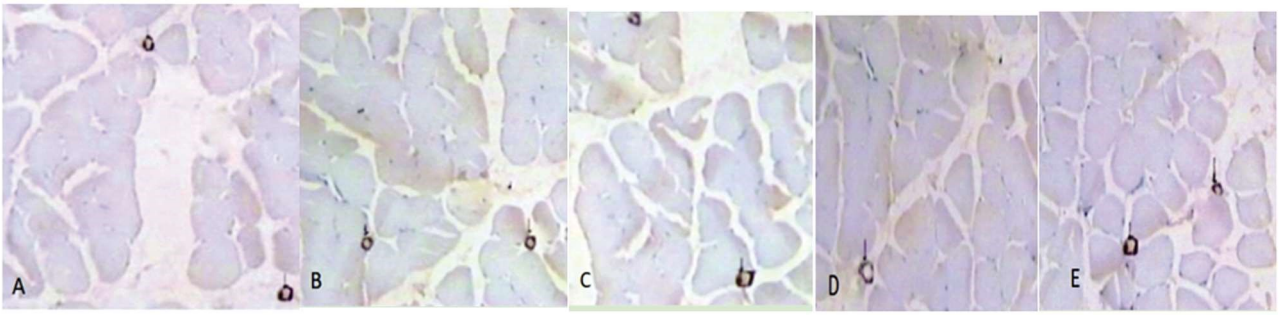

图 2 五组组织中 VEGF 和 CD45 免疫印迹检测图（A 表示对照组，B 表示模型组，C 表示低剂量组，D 表示中剂量组，E 表示高剂量组）

Fig.2 FDetection of VEGF and CD45 in five groups of tissues (A represents the control group, B represents the model group, C represents the low-dose group, D represents the middle-dose group, E represents the high-dose group)

表 4 组织中细胞因子 mRNA 表达量检测结果(n=6，$\bar{x}\pm s$)

Table 4 Detection results of cytokine mRNA expression in tissues (n=6, $\bar{x}\pm s$)

Result	Control group	Model group	Low dose group	Middle dose group	High dose group	F	*P*
TLR4	2.25± 0.23	2.83± 0.29	3.50± 0.18	3.85± 0.35	4.75± 0.46	54.677	＜0.05
NF-κB	2.85± 0.60	3.83± 0.61	3.85± 0.23	4.72± 0.34	5.60± 0.54	27.381	＜0.05
IL-1β	2.93± 0.47	3.90± 0.32	4.00± 0.08	5.98± 0.38	7.82± 0.51	132.367	＜0.05
IL-6	1.72± 0.34	2.90± 0.42	2.95± 0.23	4.35± 0.49	6.20± 1.15	46.213	＜0.05
IL-8	3.35± 0.43	3.78± 0.66	5.07± 0.54	5.50± 0.36	6.88± 0.42	49.602	＜0.05
TNF-α	8.15± 0.77	8.67± 0.56	9.57± 0.96	11.18± 0.86	12.07± 0.59	28.369	＜0.05

表 5 组织中细胞因子蛋白表达量检测结果(n=6，$\bar{x}\pm s$，ng/g)

Table 5 Detection results of cytokine protein expression in tissues (n=6, $\bar{x}\pm s$, ng/g)

Result	Control group	Model group	Low dose group	Middle dose group	High dose group	F	*P*
TLR4	11.70± 1.62	14.52± 1.86	17.30± 1.07	26.80± 1.28	39.58± 1.14	380.321	＜0.05
NF-κB	3.70± 1.03	6.03± 1.31	9.33± 1.21	13.85± 1.30	20.03± 1.62	148.771	＜0.05
IL-1β	32.52± 1.71	43.05± 2.01	53.13± 2.24	73.73± 2.44	83.67± 2.02	613.793	＜0.05
IL-6	17.12± 1.13	20.70± 0.78	21.15± 0.91	30.13± 0.84	42.03± 1.51	531.574	＜0.05
IL-8	12.28± 1.14	15.42± 0.71	17.10± 0.85	23.33± 1.20	26.62± 1.01	209.202	＜0.05
TNF-α	17.92± 0.79	20.05± 0.74	22.70± 0.99	31.37± 0.80	35.23± 1.42	350.157	＜0.05

3 讨论

糖尿病组溃疡是糖尿病最常见的并发症之一，该并发症可引起足部溃疡、下肢截止等，严重影响患者的生活质量。据统计，15 %的糖尿病患者会出现糖尿病足部溃疡 (DFU)，DFU 可导致患者感染、坏疽、截止，如治疗不及时，甚至可导致死亡[13]。目前糖尿病足溃疡的治疗主要清创和预防感染为主，具体的作用机制尚未完全明确，故导致有效治疗手段缺乏。影响 DFU 愈合的因素复杂，既有糖尿病周围神经病变、周围血管病变，感染因素，也有全身营养状况不良、创面局部因素等影响创面修复过程。正常伤口的愈合过程包括出血和凝固，急性炎性反应、细胞迁移、增殖和分化、血管生成、细胞外基质及上皮形成，需要多个细胞因子和趋化因子信号的引导、维护和相互协调。DFU 伤口的愈合过程与正常伤口愈合不同，存在过程缺陷和延迟。

HMGB1 是一类广泛分布与高等真核生物细胞核内重要的染色体结合蛋白，辅助细胞核多种功能。HMGB1 作为一种重要的炎性介质和致炎细胞因子，可引起机体全身或局部炎性反应综合症，具有启动和维持炎症反应的作用。当机体受缺血、缺氧、炎症等刺激时，HMGB1 能由坏死细胞、感染细胞分泌至细胞外，作为一种信号分子，促进细胞增殖分化并向损伤组织迁移的作用。同时，HMGB1 能刺激单核细胞、中性粒细胞等产生 IL-6、IL-8、TNF-α 等多种促炎细胞因子的表达，又可在多种趋化因子的作用下，促进 NF-κB 活化，进行启动后续炎症反应，进一步加重机体的病理损伤[19]。HMGB1 最主要的受体包括外源性配体 TLRs，如 TLR2、TLR4、TLR9，和内源性配体 RAGE。TLRs 的成员是机体天然免疫系统中的基础组成成分，表达与多种细胞尤其是天然免疫细胞的膜表面或细胞内。TLRs 是一类模式识别受体，可识别病原体相关分子模式(PAMPs)和损伤相关分子模式(DAMPs)，在防御和应对病原体入侵中发挥着重要作用。被释放到细胞外的 HMGBl 主要与

TLR2、TLR4 结合，通过髓样分化因子 MYD88 依赖或非依赖途径，促进细胞质中的 NF-κB 活化并进入细胞核，诱导炎性因子如 TNF-α、IL-6、IL-1β、IL-8 及趋化因子的释放，进而引起炎症反应发生及放大[26-28]。

VEGF 是血管发生、血管生成、淋巴管生成及血管通透性的重要调节器。外周血管狭窄或者闭塞导致缺血一直被认为是 DFU 的主要原因。DF 患者局部血液循环不佳易引起局部组织缺氧，VEGF 在缺氧环境下可与内皮细胞膜上的 VEGF 受体结合，激活蛋白激酶，从而促进内皮细胞增生。VEGF 也可促进新生血管的生成，诱导细胞因子的释放，促进创面愈合。HMGB1 作为一种促新生血管生成的细胞因子，具有直接或间接促进血管生成的作用[29,30]。研发发现 HMGB1 可活化局势细胞促其分泌血管生成因子如 VEGF，且在体外三维血管模型中，HMGB1 可直接诱导凝胶中培养的血管内皮细胞生长。另外，HMGB1 可直接活化干细胞如内皮组细胞和造血干细胞，动员其迁移至受损的组织局部，促进创伤组织的血管形成[31]。

本研究就 HMGB1 对大鼠糖尿病足溃疡的炎症的影响及机制进行了研究分子，证明 HMGB1 可促进大鼠溃疡部位的炎症反应，其机制可能与 HMGB1/TLR4/NF-κB 信号通路相关，为糖尿病足溃疡临床治疗提供了理论依据。

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