上海交通大学

Shanghai Jiaotong University

博士学位论文

Netrin-1调控ADSCs增殖、迁移及修复糖尿病周围神经血管病变的研究

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上海交通大学

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Netrin-1 Regulates Proliferation, Migration and Repair of Diabetic Peripheral Neurovascular Diseases

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摘要

目的：探明糖尿病周围神经血管病变（DPNV）对神经轴突导向因子 Netrin-1 蛋白的影响，探索 Netrin-1 在调控脂肪干细胞（ADSCs）增殖、迁移及修复 DPNV 中的角色。阐明 Netrin-1 对 ADSCs 的调节作用与具体分子机制。为探索 DPNV 的临床防治的新治疗方案提供理论基础与依据。

方法：①获取临床糖尿病与非糖尿病患者的下肢缺血肌肉组织以及外周血血清，通过免疫组化、免疫荧光、Western Blot、ELISA 等方法分析缺血组织及血液中 Netrin-1 与炎症因子的表达水平、小血管密度以及 Netrin-1 与血管内皮细胞的共定位情况，通过统计学分析阐明 Netrin-1 的表达水平与 DPNV 的临床相关性。②获取 C57/BL 小鼠脂肪组织，分离并培养 ADSCs，成功建立基因转染体系使 ADSCs 过表达绿色荧光蛋白（GFP）及 Netrin-1（N-ADSCs）。通过 CCK-8、Western Blot、流式、Transwell 等检测 N-ADSCs 与 ADSCs 在高糖环境下的增殖、迁移、粘附、向内皮细胞分化等能力之间的差异。③构建 2 型糖尿病（T2DM）小鼠（db/db）后肢失神经模型，体内移植 N-ADSCs 与 ADSCs，并应用激光多普勒观察血流灌注，通过免疫荧光和免疫组化，评估 ADSCs 在体内存活、迁移、分化和促血管新生，治疗 DPNV 的效率。④应用 Western Blot 探讨 Netrin-1 介导的 ADSCs 增殖，迁移，粘附，分化，促血管新生能力和细胞凋亡的信号通路，应用 ELISA 检测 Netrin-1 介导的 ADSCs 旁分泌多种细胞因子与生长因子。明确其具体分子机制。

结果：①糖尿病患者下肢缺血组织及外周血血清中 Netrin-1 表达水平显著下降，外周血血清中 IL-6、IL-1β、MCP-1 等炎症因子的表达明显上升,糖尿病患者下肢缺血肌肉组织中血管密度显著下降，并且 Netrin-1 与血管内皮细胞存在共定位情况；②成功从 C57/BL 小鼠脂肪组织中获取足量 ADSCs，明确最佳转染时间与最佳感染复数（MOI），建立 NTN-1 通过腺病毒转染 ADSCs 的过表达 Netrin-1 的转染体系。N-ADSCs 在高糖环境下表现出显著升高的增殖、迁移、粘附、向内皮细胞分化及抗凋亡的能力。③体内移植 N-ADSCs 的后肢失神经 T2DM 小鼠表现出显著升高的激光多普勒血流灌注指数和新生小血管数量，免疫荧光显示 N-ADSCs 的存活数量及分化为内皮细胞的数量显著增多。④Western Blot 和

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ELISA 的结果显示 Netrin-1 可能通过上调 AKT/PI3K/eNOS/P-38/NF-κB 信号通路以及促进 ADSCs 旁分泌多种细胞因子及生长因子从而调节 ADSCs 增殖、迁移、粘附、分化以及促血管新生，治疗 DPNV。

结论：DPNV 使下肢缺血组织及血液中 Netrin-1 水平显著降低。利用腺病毒基因转染体系使 ADSCs 过表达 Netrin-1 可提升 ADSCs 在高糖环境下的增殖、迁移、粘附、分化及抗凋亡能力，Netrin-1 可能通过上调 AKT/PI3K/eNOS/P-38/NF-κB 信号通路以及促进 ADSCs 旁分泌多种细胞因子及生长因子的机制调节 ADSCs 体内存活、迁移及促进血管新生，提升 ADSCs 体内移植修复 DPNV 的治疗效率。本项研究的结果为 DPNV 临床防治的新治疗方案提供了理论基础与依据。

关键词：糖尿病周围神经血管病变，Netrin-1，脂肪干细胞，细胞移植，血管新生

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ABSTRACT

Objective: To determine the effect of diabetic peripheral neurovascular disease (DPNV) on the axon guidance factor Netrin-1 protein, and to explore the role of Netrin-1 in regulating adipose-derived stem cells (ADSCs) of their proliferation, migration and repairment of DPNV. To elucidate the specific regulatory molecular mechanism of Netrin-1 on ADSCs, which will provide theoretical basis for exploring new options for clinical prevention and treatment of DPNV.

Methods: ① Ischemic hindlimb muscle tissue and peripheral blood serum from diabetic and non-diabetic patients were obtained. The expression of Netrin-1 and inflammatory factors in diabetic patients were analyzed by immunohistochemistry, immunofluorescence, Western Blot and ELISA, as well as small vessel density, and colocalization of Netrin-1 and endothelial cells. Statistical analysis of the clinical correlation between the expression of Netrin-1 and DPNV in diabetic high glucose environment was also performed. ② Adipose tissue was obtained from C57/BL mice, ADSCs were isolated and cultured, and a gene transfection system was successfully established to make ADSCs overexpress green fluorescent protein (GFP) and Netrin-1 (N-ADSCs). CCK-8, Western Blot, flow cytometry, Transwell, etc. were performed to detect the differences in the proliferation, migration, adhesion, and differentiation into endothelial cells of N-ADSCs and ADSCs in a high glucose environment. ③ A type 2 diabetes mellitus (T2DM) mice (db/db) lower extremity denervation model was constructed, N-ADSCs and ADSCs were transplantated in vivo, and blood perfusion was observed by laser Doppler, immunofluorescence and immunohistochemistry were performed to evaluate ADSCs of the survival, migration, differentiation, angiogenesis and treatment efficiency of DPNV in vivo. ④ Western Blot was performed to explore the Netrin-1-mediated signaling pathways of proliferation, migration, adhesion, differentiation, angiogenesis and apoptosis of ADSCs, ELISA was applied to detect Netrin-1-mediated paracrine secretion of ADSCs, revealing the underlying molecular mechanisms.

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Results: ① The expression levels of Netrin-1 in the ischemic tissues and peripheral blood serum of diabetic patients were significantly increased, while the expression of nflammatory cytokines such as IL-6, IL-1β and MCP-1 was decreased in peripheral blood serum. The vascular density in the lower extremity muscle tissue was decreased, and Netrin-1 was found co-localized with endothelial cells; ② Sufficient amount of ADSCs were successfully obtained from adipose tissue of C57/BL mice, the optimal time and the multiplicity of infection(MOI) were determined, and an adenovirus transfection system was established. N-ADSCs exhibit significantly improved proliferation, migration, adhesion, differentiation into endothelial cells, and anti-apoptosis ability in high glucose environments. ③ In vivo denervated T2DM mice transplanted with N-ADSCs demonstrated significantly increased laser Doppler perfusion index and number of new small blood vessels. Immunofluorescence showed that the number of ADSCs survived and the number of differentiated endothelial cells significantly increased. ④ The results of Western Blot and ELISA demonstrated that Netrin-1 may regulate the proliferation, migration, adhesion, differentiation of ADSCs and promote angiogenesis in diabetic neurovascular diseases by up-regulating the AKT/PI3K/eNOS/P-38/NF-κB signaling pathway and promoting the paracrine of multiple factors of ADSCs to treat DPNV.

Conclusion: DPNV causes a significant decrease in Netrin-1 levels in blood and ischemic tissue of the lower extremities. Overexpression of Netrin-1 by ADSCs can enhance the proliferation, migration, adhesion and differentiation of ADSCs by using a gene transfection system. Netrin-1 may up-regulate the AKT/PI3K/eNOS/P-38/NF-κB signaling pathway and promote the ADSCs paracrine of multiple factors to enhance the survival of ADSCs in vivo and promote angiogenesis, thus improving the therapeutic efficiency of ADSCs transplantation in DPNV. The results of this study provide a theoretical basis for a new option for clinical prevention and treatment of DPNV.

Keywords: diabetic peripheral neurovascular disease, Netrin-1, adipose-derived stem cells, cell transplantation, angiogenesis

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目 录

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符号说明

英文缩写	英文名称	中文名称
DPNV	Diabetic peripheral neurovascular disease	糖尿病周围神经血管病变
T1DM	Type 1 diabetes mellitus	1 型糖尿病
T2DM	Type 2 diabetes mellitus	2 型糖尿病
GDM	Gestational diabetes mellitus	妊娠期糖尿病
DKA	diabetic ketoacidosis	糖尿病酮症酸中毒
ADSC	Adipose-derived stem cell	脂肪干细胞
VEGF	Green fluorescent ptotein	绿色荧光蛋白
CCK-8	Cell counting kit-8	细胞计数试剂盒
ERK	Extracellular signal-regulated kinase	细胞外信号调节激酶
WB	Western Blot	蛋白质印迹法
RT-PCR	Reverse transcription polymerase chain reaction	反转录酶-聚合酶链锁反应
ELISA	Enzyme-linked immunosorbent assay	酶联免疫吸附试验

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英文缩写	英文名称	中文名称
LEA	Lower extremity amputations	下肢截肢
ESRD	end-stage renal disease	终末期肾病
HHS	Hyperglycemia hypertonic state	高血糖高渗状态
CVD	Cardiovascular diseases	心血管疾病
DR	Diabetic retinopathy	糖尿病视网膜病变
DN	Diabetic neuropathy	糖尿病神经病变
AGEs	Advanced glycation end products	晚期糖基化终末产物
CLI	Critical limb ischemia	下肢严重缺血
iPSCs	Induced pluripotent stem cells	诱导多能干细胞
eNOS	Endothelial nitric oxide synthase	内皮型一氧化氮合酶
Akt	Protein kinase B	蛋白激酶 B
NF-κB	Nuclear transcription factor kappa-B	细胞核转录因子κB

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英文缩写	英文名称	中文名称
p38 MAPK	Mitogen-activated protein kinase 38	丝裂原活化蛋白激酶
DCC	Deleted in colorectal cancer	结肠癌缺失基因
FSC	Fetal stem cell	胎儿干细胞
ASC	Adult stem cell	成人干细胞
HSC	Hematopoietic stem cell	造血干细胞
MSC	Mesenchymal stem cell	间充质干细胞
NSC	Neural stem cell	神经干细胞
AKI	Acute kidney injury	急性肾损伤
IFG	Impaired fasting glucose	空腹血糖受损
IL-6	Interleukin-6	白细胞介素-6
IL-1β	Interleukin-1β	白细胞介素-1β
MCP-1	Monocyte chemotactic protein 1	单核细胞趋化蛋白
FAK	Focal Adhesion Kinase	局部粘着斑激酶

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英文缩写	英文名称	中文名称
HDL	High density lipoprotein	高密度脂蛋白
PI3K	Phosphoinositide 3-kinase (pi3k) enzyme	磷脂酰肌醇-3-羟激酶
ECM	Extracellular matrix	细胞外基质
HbA1c	Glycosylated hemoglobin	糖化血红蛋白
JNK	Jun N-terminal kinase	应激活化蛋白激酶
TNF-α	Tumor necrosis factor-α	肿瘤坏死因子-α
EPC	Endothelial progenitor cells	内皮祖细胞
BMSC	Bone mesenchymal Stem cells	骨髓基质干细胞
ESC	Embryonic stem cell	胚胎干细胞
VEGF	Vascular endothelial growth factor recepto	血管内皮生长因子受体
RNA	Ribonucleic acid	核糖核酸
AST	Aspartate transaminase	谷草转氨酶
ALT	Alanine transaminase	谷丙转氨酶

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英文缩写	英文名称	中文名称
PBS	Phosphate buffer saline	磷酸缓冲盐溶液
eGFR	Glomerular filtration rate	肾小球滤过率
TG	Triglyceride	甘油三酯
OD	Optical density	吸光度
FBS	Fetal bovine serum	胎牛血清
TGF-β	Transforming growth factor	转化生长因子-β
IGF	Insulin like growth Factor	胰岛素样生长因子
BSA	Bovine serum albumin	牛血清白蛋白
FITC	Fluorescein Isothiocyanate	异硫氰酸荧光素
DAPI	4-6-diamidino-2-phenylindole	4，6-联脒-2-苯基吲哚
EDTA	Ehylene diamine tetraacetic acid	乙二胺四乙酸
MOI	Multiplicity of infection	感染复数
DMEM	Dulbecco’s modified eagle medium	DMEM 培养液

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英文缩写	英文名称	中文名称
ROS	Reactive oxygen species	活性氧
DNA	Deoxyribonucleic acid	脱氧核糖核酸
PARP	Poly-ADP-ribose polymerase	多聚二磷酸腺苷核糖聚合酶
GAPDH	Reduced glyceraldehyde-phosphate dehydrogenase	磷酸甘油醛脱氢酶
PKC	Protein kinase C	蛋白激酶 C
NADPH	Nicotinamide Adenine Dinucleotide Phosphate	还原型烟酰胺腺嘌呤二核苷酸磷酸
NO	Nitric oxide	一氧化氮
ONOO-	Peroxynitrite anion	过氧亚硝基阴离子
ET-1	Endothelial vasopeptide-1	内皮缩血管肽-1
MCP-1	Monocyte chemoattractant protein-1	促炎基因单核细胞化学引诱物蛋白-1
VCAM-1	Vascular cell adhesion molecule-1	血管细胞粘附分子-1

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英文缩写	英文名称	中文名称
HLA	Human leukocyte antigen	人类白细胞抗原
EP	Eppendorf	EP 管
SD	Standard deviation	标准差
mL	Millilitre	毫升
mg	Milligram	毫克
TBST	Tris-Buffered-Saline with tween	洗膜缓冲液
min	Minute	分钟
h	Hour	小时
μg	Microgram	微克
COX-2	Cyclooxygenase-2	环氧合酶-2
PGIS	Prostacyclin synthase	前列环素合酶
TXA_2	Thromboxane A2	血栓素 A2
ICAM-1	Intracellular adhesion molecule-1	细胞内粘附分子-1

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英文缩写	英文名称	中文名称
PET	Positron emission tomography	正电子发射断层扫描
μL	Microliter	微升
μm	Micrometer	微米

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绪论

糖尿病（DM）是一种全球共患的非传染性疾病。其本质为一种血糖水平持续升高的全身性代谢紊乱[1,2]。糖尿病通常分为1型糖尿病（T1DM）、2型糖尿病（T2DM）、妊娠期糖尿病（GDM），以及其他特定类型糖尿病。其中2型糖尿病是最常见的形式。糖尿病具有多种发病机制，其具体发病机制可能是胰岛素分泌受损或由于靶器官组织的胰岛素抵抗和/或胰腺β细胞的广泛损坏[1,3]。

在过去的几十年里，全球糖尿病的发病率和流行率都表现出显著的升高。随着越来越多的人形成了不健康的生活方式与饮食习惯，未来的发病率预计将会持续升高。根据国际糖尿病联盟和世界卫生组织的统计，全球糖尿病患者的人数从1980 年的 1.53 亿飙升至 2017 年的 4.25 亿。预计到 2045 年，全世界将有 6.29 亿人患有糖尿病[4，5]。其中，发展中国家由于经济条件的改善、生活水平的提高以及习得发达国家的不良生活习惯，糖尿病增长速度尤为显著。而作为发展中国家的代表的中国，在过去 30 年里，中国糖尿病患者从 1980 年的不足全人口的 1%飙升至 2013 年的 10.9%。目前中国已有超过 1.21 亿人患有糖尿病，糖尿病人数位居世界第一[6-9]。与此同时，国际糖尿病联盟估计全球有多达 2.12 亿的糖尿病患者尚未检出糖尿病。因此，糖尿病的实际发病率甚至更高[10,11]。

糖尿病的主要危害在于持续升高的血糖对于靶器官靶组织的破坏，如不加以干预，最终会引起多种的糖尿病并发症。其具体的发病机制包括某些遗传和表观遗传修饰、营养因素和久坐生活方式等[12]。糖尿病的急性并发症包括如糖尿病酮症酸中毒(DKA)、高血糖高渗状态(HHS)、乳酸酸中毒和低血糖，它们是糖尿病发病率和死亡率高的重要原因，也大大增加了糖尿病的护理难度与成本[13,14]。而慢性并发症主要是由糖尿病引起的血管病变。包括：（1）糖尿病大血管并发症，主要是心血管疾病（CVD），包括冠心病、中风和周围动脉血管疾病（PAD），其主要原因是糖尿病引起的动脉粥样硬化导致的一系列大血管病变。（2）糖尿病微血管并发症：包括终末期肾病(ESRD)、糖尿病视网膜病变（DR）、糖尿病神经病变(DN)、以及糖尿病导致的下肢截肢(LEA)。糖尿病微血管并发症的发病机制可能包括：产生晚期糖基化终产物(AGEs)、创造促炎微环境、诱导氧化应激等[15-17]。

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近年来，随着临床医生对于糖尿病的认知进一步加深，以及患者健康意识和依从性的提高，由于多种降糖药物如胰岛素、二甲双胍等的普及以及抗血小板、降脂的药物如阿司匹林、他汀类药物的应用，虽然糖尿病患者比起非糖尿病患者的 CVD 的发病率依然高出 2-4 倍，但可喜的是，糖尿病患者 CVD 的患病率以及 CVD 相关的死亡率在过去 20 年内正在持续下降[18-22]。然而，因糖尿病微血管病变引起的总截肢事件不但没有明显下降，反而在有些国家报道有上升趋势[23,24]。究其原因，盖因其具有发病率高、出现早、起病隐匿、病程长、难治愈的特点，相当一部分患者因为糖尿病周围神经病变导致对疼痛不敏感，进而无法及早发现由糖尿病周围血管病变导致下肢缺血而引起的间歇性跛行及静息痛等典型动脉血管狭窄闭塞的症状，因此常常在出现严重血管并发症时才会就医[25-28]。而其血管病变合并神经病变的复杂性，往往导致病情进一步恶化，迁延不愈，相当一部分患者进展为糖尿病足[29-31]。临床上现行的治疗方式，如药物保守治疗、血管重建、经皮腔内血管成形术等，虽然能不同程度地改善患者的病情和预后，但远期疗效并不理想，且在临床的应用具有一定局限性，对于失去手术和介入治疗机会的下肢严重缺血（CLI）的糖尿病患者，往往以截肢告终，部分患者甚至有生命危险[32-35]。而这不但给患者带来巨大痛苦，同时为医药卫生带来沉重的财政负担。因此，解决糖尿病周围神经血管病变（DPNV），恢复周围神经血管的功能，避免截肢甚至死亡的结局，成为了亟待解决的难题。

近年来，干细胞移植技术及再生医学不断飞速发展，大量研究证实了干细胞移植能够促进缺血肢体侧枝血管形成[36-40]，改善和恢复患肢血流，提高患者的生活质量，达到治疗肢体缺血的目的，干细胞治疗严重肢体缺血已展示了较好的临床应用前景，成为当前研究的热点[41-45]。目前，各种干细胞已被用于组织工程和再生医学，干细胞主要分为三大类:胚胎干细胞(ESCs)、胎儿干细胞(FSCs)和成人干细胞(ASCs)。胚胎来源的干细胞包括胎儿组织细胞、脐带血细胞、胎盘和羊水细胞、造血干细胞(HSCs)和间充质干细胞(MSCs)。成人干细胞可分为骨髓基质干细胞(BMSCs)、肌卫星细胞、神经干细胞(NSCs)、造血干细胞(HSCs)和脂肪来源干细胞(ADSCs)[46,47]。ESCs 能够自我更新并分化为体内任何类型的细胞，但由于伦理及政治考虑，很难将 ESCs 应用于临床研究和实践。诱导的多能干细胞（iPSCs）是一种经过基因重组的体细胞，具有 ESCs 的特性，然而

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iPSCs 较低的诱导效率以及获取方法的复杂性、昂贵的成本，使其很难大量获取和应用[48,49]。临床及实验中目前常采用成人干细胞主要包括 BMSCs、HSCs 及内皮祖细胞（EPCs），并取得了一定的治疗效果[50-52]，但临床上采集外周血或骨髓干细胞风险较大，患者体验较差，且患者多为老年人，往往合并多种基础疾病，其 BMSCs 和外周血中 HSCs 及 EPCs 不仅数量减少，而且增殖、迁移、分化及成血管能力均显著降低，较难满足临床的广泛应用[53]。而近年来成为热点的 ADSCs 可见于任何类型的白色脂肪组织，包括皮下脂肪和网膜脂肪[54]，人类拥有丰富的皮下脂肪组织，尤其是糖尿病患者常常合并肥胖，临床上通过安全无副作用的吸脂术可以很容易地获取大量脂肪组织[55,56]。研究发现，脂肪组织中含有大量的 ADSCs，能够分化为脂肪细胞、成骨细胞、软骨细胞、心肌细胞、神经细胞等，具有较强的多向分化潜能，在特定诱导条件下可分化为内皮细胞及平滑肌细胞，且 ADSCs 能够分泌多种促血管生成因子，自体或者异体移植后可促进新生血管的形成、改善小鼠缺血下肢的血流[36,57]。研究发现，与自体骨髓和外周血干细胞相比。脂肪组织的获取比骨髓便宜得多，侵入性更小，数量更多，同时具有比 BMSCs 更高的干细胞增殖率，并且超过 25 代仍然保留干细胞表型和间充质多能性[58-63]。因此，脂肪组织是一种丰富的、实用的、有吸引力的自体细胞替代供体组织来源。而脂肪组织来源的 ADSCs 很有希望成为治疗 DPNV 的合适选择。

近年来国内外学者在干细胞移植治疗糖尿病患者下肢缺血方面开展了大量的研究工作，部分研究已应用到临床试验中，移植后糖尿病患者下肢缺血症状及客观指标虽然得到一定程度的改善，但其远期疗效仍欠佳[36,64-70]。同时，Kočí Z 等人发现糖尿病下肢缺血患者自体分离提取的 ADSCs 较正常组的增殖能力以及旁分泌能力均大幅降低，凋亡水平上升[71]。Ja Hea Gu 等人从糖尿病患者体内脂肪提取到的糖尿病 ADSCs 在低氧条件下产生血管内皮生长因子(VEGF)和诱导细胞增殖的能力表现出明显的受损与不足[72]。大量动物实验与研究中亦发现糖尿病小鼠 ADSCs 的增殖能力受损，成血管能力受损甚至丧失[73-76]。Rennert RC 等人运用单细胞分析等技术发现，糖尿病微环境会在原位改变 ADSCs 的生态位，选择性地耗竭表达血管新生因子相关基因的细胞亚群，从而使糖尿病 ADSCs 在体外和体内实验中表现出受损的血管新生能力[77]。以上研究表明，糖尿病患者

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体内的持续高糖环境降低了体内移植的 ADSCs 在靶病变部位的存活、迁移以及成血管能力，使得 ADSCs 对 DPNV 的治疗效果大打折扣，无法满足临床的需要，而其机制可能与高级糖基化终产物、产生促炎微环境和诱导氧化应激相关，但具体机制目前尚未完全明确[15-17]。因此，如何促进 ADSCs 在糖尿病周围血管损伤部位的存活、分化及促进失神经支配后糖尿病血管的新生，对防治 DPNV 病变的发生、发展具有重要的意义。

研究发现，DPNV 是一种混合性病变，伴有血管，神经和组织损伤[28,78-80]。既往的研究往往只关注单一因素在 DPNV 治疗中的作用和意义，没有将神经、血管作为一个整体的功能单位进行研究，且忽略神经因素在调控血管新生中的重要作用，因此疗效受限[26,81-83]。此外，神经和血管表现出类似的复杂分支和生长模式，这些神经和血管遵循相同的迁移路径，到达相同靶器官或部位。血管生成和轴突生长之间的相似性表明它们可能受一些共同的信号分子的调节[84]。Jones 和 Li 的研究表明，一些信号分子可以调节神经和血管发育过程[85]，尤其是最初被认为影响神经轴突生长导向调节器的 4 对配体和受体，现大部分研究证实其也同时参与血管的生成[86-88]。Netrin-1 是第一个确定的神经轴突导向因子，Netrin-1 和 G-netrin 与层粘连蛋白 γ 链具有同源性，该肽由一个氨基末端区域 VI 的近 600 个残基组成，连接着重复的 3 个层粘连型表皮生长因子(V-1、V-2、V-3)和一个羧基末端区域[89]。另一项研究发现，Netrin-1 不仅参与神经系统的功能活动，还参与血管系统的功能活动。Ding 等人证明 Netrin-1 不仅促进中枢神经系统的神经元迁移和分泌，而且还调节非神经组织中内皮细胞和干细胞的存活，粘附，迁移，增殖和分化，并抑制他们的细胞凋亡[90,91]。Wilson 等人系统地研究斑马鱼和哺乳动物，证实 Netrins 可以诱导血管生成。Netrin-1 通过与 UNC5H 受体结合激活 Src/FAK/paxillin 相关信号通路，促进血管内皮细胞粘附，迁移和增殖，形成新的毛细血管网络，该功能可以通过抑制斑马鱼 NTN-1 mRNA 而被阻断[92]。Lu 等人还发现 Netrins 可刺激哺乳动物的血管生成并加速缺血组织的血管生成。该过程依赖于 Netrin-1 受体 DCC 来调节 ERK/eNOS 信号通路[93]。Brunet 等人证明 Netrin-1，Netrin-4 和 VEGF 促进血管生成，但 Netrin-1 在促进内皮细胞分化和神经损伤恢复的双重作用方面具有更大的优势[94]。此外，Netrin-1 不但参与神经生长和血管生成;它还可以增强人体血管和淋巴系统不同

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阶段内皮细胞的有丝分裂，迁移和粘附[95]。因此，我们认为通过基因转染 ADSCs 过表达 Netrin-1 可以改善 ADSCs 在高血糖条件下的活力，迁移和向血管内皮细胞分化，促进 DPNV 血管新生。

关于兼具促进神经生长和血管新生双重作用的 Netrin-1 的基础研究在如火如荼进行的同时，临床上对 Netrin-1 的关注也与日俱增，随着临床研究的增多与深入，Netrin-1 与多种炎症性疾病的临床相关性也日渐明了。Mulero P 等通过对临床 90 例多发性硬化症患者及 30 例对照组血液标本的研究发现，多发性硬化症患者，尤其是在复发的患者中，血清中 Netrin-1 含量显著低于健康对照组。提示 Netrin-1 可能作为多发性硬化症处于炎症活跃期的生物标志物[96]。Reeves WB 等发现患者肾 Netrin-1 的表达在肾缺血反应中增加，推测其可能是一种限制组织损伤的体内平衡措施。小鼠肾缺血引起的急性肾损伤（AKI）模型显示肾小管 Netrin-1 表达和尿液中 Netrin-1 水平均升高[97]。这一观察结果在一项前瞻性的临床试验中得到了验证，Ramesh G 等证实心血管手术后的患者尿液中 Netrin-1 水平升高与 AKI 的发生具有显著的相关性，提示 Netrin-1 可以作为急性肾损伤的生物标志物[98]。在心肌缺血再灌注方面，许多细胞和动物实验表明 Netrin-1 在心肌缺血再灌注中发挥保护心肌的重要作用，未来可能作为一个心肌缺血再灌注的非手术的治疗靶点[99-103]。同时诸多研究发现，在糖尿病及糖尿病并发症中，Netrin-1 也具有作为生物标志物的潜能。Jung 等人最近的一项临床研究表明 Netrin-1 可能是早期检测空腹血糖受损(IFG)或 T2DM 的一种新的生物标志物。他们发现与对照组相比，IFG 或 T2DM 患者的血清 Netrin-1 水平显著升高，血清 Netrin-1 与空腹血糖、糖化血红蛋白(HbA1c)、HOMA 法胰岛素抵抗指数（HOMA-IR）、谷草转氨酶（AST）、丙氨酸转氨酶（ALT）呈显著正相关，Netrin-1 与高密度脂蛋白（HDL）胆固醇、肾小球滤过率（eGFR）呈显著负相关。此外，血清 Netrin-1 与 IFG 或 T2DM 的存在具有独立相关性[104]。与此相反，Liu 等人对 56 名人体受试者进行了临床研究，其中 30 名新患 2 型糖尿病患者被分配到治疗组，其余的被分配到对照组，以评估 Netrin-1 在糖尿病患者中的影响程度。他们发现糖尿病患者的 Netrin-1 水平明显低于健康对照组。另外，Netrin-1 的程度与胰岛素抵抗及血糖(空腹及餐后)、空腹胰岛素、甘油三酯（TG）、血红蛋白 A1c 水平的稳态模型评价呈负相关[105]。上述两项临床研究在 Netrin-1 水平与 DM 临

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床相关性的发现是相互矛盾的，因此需要进一步的高质量研究来确定两者之间的实际关系。在糖尿病并发症方面，J. Liu 等关于 DR 的研究共纳入糖尿病患者 18 例，其中 10 例为 DR 患者，8 例为无 DR 患者。DR 患者玻璃体内 Netrin-1 和 VEGF 水平明显高于对照组[106]。K.Miloudi 等通过对糖尿病黄斑水肿的患者和对照组无糖尿病黄斑水肿患者的比较发现，糖尿病黄斑水肿患者玻璃体内 Netrin-1 显著升高 8 倍[107]。近年来的诸多研究均表明 Netrin-1 在糖尿病视网膜病变中可能是一种全新的生物标志物和潜在的治疗靶点。在糖尿病外周神经病变方面，Netrin-1 由于具有持久的化学吸引能力，可富集轴突延伸，在损伤后的成人神经系统中高表达。Dun 和 Parkinson 的实验表明，Netrin-1 在维持施万细胞增殖，周围神经再生和迁移方面起着至关重要的作用。因此，为了刺激受损的外周神经的恢复和可用的恢复，靶向 Netrin-1 信号通路将是一种新的治疗策略[108,109]。这些数据还表明，Netrin-1 可能是受损外周神经中施万细胞的内源营养因子[109]。因此，对于同样属于糖尿病并发症的糖尿病外周神经病变，Netrin-1 可能也是一个潜在的生物标志物与治疗靶点。

Netrin-1 的相关基础和临床研究正在如火如荼地进行，然而，目前尚未有任何临相关研究证实 Netrin-1 与 DPNV 之间的临床相关性，以及 Netrin-1 对 ADSCs 体内移植治疗 DPNV 是否具有调节作用。但是，结合既往的文献研究结果以及我们的前期工作，我们认为，Netrin-1 与 DPNV 之间具有一定的临床相关性，或许是 DPNV 潜在的生物标志物。而且，通过基因转染使 ADSCs 过表达 Netrin-1，能够改善 ADSCs 体内移植后，在糖尿病高血糖条件下的增殖、迁移和向血管内皮细胞分化的能力，从而促进 DPNV 的血管新生。

因此，本项研究拟首先获取临床糖尿病与非糖尿病患者的下肢缺血肌肉组织以及外周血单核细胞，通过免疫组化、免疫荧光、WB、PCR 等分析糖尿病患者病变组织中 Netrin-1 及炎症因子的表达水平、小血管密度、巨噬细胞数量以及 Netrin-1 与内皮细胞的共定位情况，并统计分析明确糖尿病高血糖环境下 Netrin-1 的表达与 DPNV 的临床相关性。其次在细胞实验方面，获取 C57/BL 小鼠脂肪组织，分离并培养 ADSCs，建立稳定的基因转染体系使 ADSCs 过表达绿色荧光蛋白（GFP）及 Netrin-1（N-ADSCs）。通过 CCK-8、WB、流式、Transwell 等检测 N-ADSCs 与 ADSCs 在高糖环境下的增殖、迁移、粘附、向内皮细胞分化等能力

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之间的差异。体内实验方面，拟构建 T2DM 小鼠（db/db）下肢失神经模型，体内移植 N-ADSCs 与 ADSCs，并应用激光多普勒观察血流灌注，通过免疫荧光和免疫组化，评估 ADSCs 在体内存活、迁移、分化和促血管新生的效率。最后，应用 Western Blot 探讨 Netrin-1 介导的 ADSCs 增殖，迁移，粘附，分化，促血管生成能力和细胞凋亡的信号通路，应用 ELISA 检测 Netrin-1 介导的 ADSCs 旁分泌多种因子。明确 Netirn-1 调控 ADSCs 增殖、迁移以及治疗糖尿病周围血管神经病变的具体分子机制。

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第一章 Netrin-1 与 DPNV 临床相关性的研究

1. 引言

随着人均寿命的延长、人民生活水平的提高以及西方生活和饮食习惯的普及，糖尿病已成为危害人民健康的一大慢性病[1-3]。糖尿病的危害不仅在于持续的高血糖状态给其他疾病如动脉粥样硬化、高血压、癌症以及感染性疾病提供了发展的温床，成为诸多重大疾病的独立危险因素，同时它所造成的诸多并发症也不断威胁着人民的健康[12-17]。在糖尿病的诸多并发症中，糖尿病周围神经血管病变（DPNV）由于是血管与神经以及周围组织损伤的混合性病变，导致起病隐匿，病程长、病情迁延不愈，最终往往以糖尿病足的溃疡及下肢远端缺血坏死告终。据统计，糖尿病患者一生中罹患足部溃疡的风险可能高达 25%。糖尿病患者下肢截肢率是非糖尿病患者的 15 倍，1 型或 2 型糖尿病患者足部溃疡的年发病率为 1.9%至 2.2%[110-112]，发展为糖尿病足溃疡的患者，轻者截趾，重者截肢，更有甚者，由于肢端坏死组织的毒素大量吸收，导致肝肾等全身多器官功能衰竭而死亡[113-115]。临床上虽然有药物治疗、血管重建术、经皮腔内血管成形术（球囊扩张与支架植入）等治疗方法，但存在单纯血糖控制无法移植 DPNV 进展、重建血管内血栓形成、支架内再闭塞等问题，远期疗效并不理想[116-119]。因此，DPNV 为我国医疗卫生系统带来了巨大的负担。

DPNV 的主要元凶是糖尿病代谢紊乱引起的持续性高血糖[120]。越来越多研究发现，炎症在高血糖对周围神经细胞及血管内皮细胞的破坏中起到了至关重要的作用。高血糖对周围神经细胞的损伤机制主要是由于高血糖导致细胞内活性氧（ROS）线粒体产生增加。ROS 导致核 DNA 中的链断裂，进而激活多聚二磷酸腺苷核糖聚合酶（PARP）。然后 PARP 修饰了磷酸甘油醛脱氢酶（GAPDH），从而减少其活性。最后，降低的 GAPDH 活性激活多元醇途径，增加细胞内 AGEs 形成，激活蛋白激酶 C（PKC）和随后的核转录因子-κB（NF-κB），并激活己糖胺通路。而这四种机制又反过来通过如内质网应激、p38 丝裂原活化蛋白激酶（p38 MAPK）活化等促炎症反应产生更多的 ROS，从而构成了一种自我强化的疾病机制[121]。而高血糖引起的血管内皮细胞损伤机制也与氧化应激有关为：

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细胞内高浓度的葡萄糖导致 PKC 活化，然后激活由还原型烟酰胺腺嘌呤二核苷酸磷酸（NADPH）氧化酶和 $p66^{Shc}$ 衔接蛋白，产生大量的 ROS。剧增的氧化应激使一氧化氮（NO）快速失活，导致负责蛋白质亚硝基化的促氧化剂过氧亚硝基阴离子（$ONOO^-$）大量产生。同时 PKC 激活相关酶活性，从而增强内皮型一氧化氮合酶（eNOS）解偶联并导致自由基的进一步积累。另一方面，高血糖症降低 eNOS 活性，减弱 Ser1177 的活化磷酸化。同时高血糖诱导的 PKC 活化导致内皮缩血管肽-1（ET-1）的合成增加，诱导血管收缩和血小板聚集。超氧阴离子的积累还通过激活 NF-κB 信号传导来促进促炎基因单核细胞化学引诱物蛋白-1（MCP-1），血管细胞粘附分子-1（VCAM-1）和细胞内粘附分子-1（ICAM-1）的上调。这些事件导致单核细胞粘附，迁移和血细胞渗出，在内皮下层形成泡沫细胞。来自泡沫细胞的炎症因子使血管处于炎症状态以及平滑肌细胞增殖，从而加速动脉粥样硬化过程。糖尿病中的内皮功能障碍还源于通过上调环氧合酶-2（COX-2）和通过增加的亚硝基化使前列环素合酶（PGIS）失活来增加血栓素 A2（TXA_2）的合成。此外，ROS 增加葡萄糖代谢物甲基乙二醛的合成，导致 AGE/RAGE 信号传导和促氧化剂己糖胺和多元醇途径的激活[122-129]。因此，高血糖主要通过引发神经细胞和血管内皮细胞进入一系列愈演愈烈的炎症状态，从而破坏它们的功能，最终引发 DPNV，以及糖尿病足溃疡坏死的结局。同时 Papanas N 等研究发现，糖尿病持续高血糖所产生的低水平的促炎症状态，使得甚至在糖尿病前期和葡萄糖耐量受损的情况下也会发生神经病和血管功能障碍[130]。以上对于机制的研究更加凸显了高血糖促进炎症机制的重要性以及早期干预的必要性。

近年来，Netrin-1 在多种炎症性疾病中的诊断及治疗价值不断被挖掘出来。研究发现，Netrin-1 能够抑制炎症，保护正常细胞[131-135]。许多急慢性炎症疾病中，都伴随有 Netrin-1 表达的上升或下降[96-105]。这可能与机体内 Netrin-1 抑制炎症细胞迁移，抑制炎症细胞因子和趋化因子的产生，通过抑制 NF-κB 活化调控 COX-2 的表达，促进巨噬细胞向抗炎的 M2 样表型分化，以及通过 MAPKs、ERKs、p38 等信号转导途径调节炎症反应等机制相关[136-137]。同时，Netrin-1 被报道能够降低心肌缺血再灌注后的炎症反应，抑制神经细胞及血管内皮细胞的氧化应激与炎症，抗细胞凋亡[100-107]。但 Netrin-1 的表达是否与炎症

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介导的 DPNV 有临床相关性尚未见诸报道，Netrin-1 在 DPNV 的发生发展中的具体作用机制也并不明确。因此，本研究拟获取临床糖尿病与非糖尿病患者的下肢缺血肌肉组织以及外周血血清，通过免疫组化、免疫荧光、Western Blot、ELISA 等分析糖尿病患者病变组织及血液中 Netrin-1 与炎症因子的表达水平、小血管密度以及 Netrin-1 与内皮细胞的共定位情况，并统计分析明确糖尿病高糖环境下 Netrin-1 的表达与 DPNV 的临床相关性。

2. 材料和方法

2.1. 材料

2.1.1. 主要试剂

TBS 缓冲液（伯乐生命医学产品（上海）有限公司，中国）

PBS 磷酸缓冲液（1x，PH 7.2-7.4）（北京索莱宝科技有限公司，中国）

TBST 缓冲液（1x）（北京索莱宝科技有限公司，中国）

考马斯亮蓝溶液（生工生物工程（上海）股份有限公司，中国）：

牛血清白蛋白（BSA，Sigma，USA）

4%多聚甲醛（上海晶都生物技术有限公司，中国）：

甲醇（上海代轩生物科技有限公司，中国）

95%乙醇（上海交通大学医学院附属第九人民医院）

75%乙醇（（上海交通大学医学院附属第九人民医院）

异丙醇(Sigma, USA) （上海代轩生物科技有限公司，中国）

氯仿(Sigma, USA)

兔抗 CD31 多克隆抗体（Abcam，UK）

小鼠抗 Netrin-1 多克隆抗体 (Abcam，Cambridge, UK)

FITC-羊抗小鼠 IgG 多克隆抗体（Abcam，Cambridge, UK）

Fluor 555-羊抗兔 IgG 多克隆抗体（Invitrogen, Carlsbad, CA）

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DAPI（西安赫特生物科技有限公司，中国）

树脂（Epon, Merck, Darmstadt, Germany）

OCT 包埋剂（DAKO，USA）

荧光封片剂（DAKO，USA）

Matrigel 凝胶（Sigma，USA）

ELISA 试剂盒（上海一研生物科技有限公司，中国）

2.1.2. 主要仪器与设备

15 ml 离心管（Corning Inc.，USA）

50 ml 离心管（Corning Inc.，USA）

离心机（Thermo，USA）

去离子水系统（Millipore，USA）

超净工作台（江苏苏净集团，中国）

倒置显微镜（Olympus，Japan）

LumiStation 1800Plus 化学发光酶标仪（上海闪谱生物科技有限公司，中国）

荧光显微镜（Nikon，Japan）

精密天平（Mettler Toledo，Switzerland）

天平（上海澄洋仪器仪表有限公司，中国）

冰箱 （北京天地精仪有限责任公司，中国）

6 孔板（BD Falcon， USA）

培养皿（BD Falcon，USA）

0.22 μm 针头滤器（Millipore，USA）

40 μm 滤网（BD Falcon，USA）

PCR 仪（Thermo Hybaid，USA）

Image J 软件（Rawak Software Inc., Stuttgart, Germany）

解剖显微镜（上海光学仪器一厂，中国）

显微外科手术器械（江苏博美达生命科学有限公司，中国）

真空全封闭型组织脱水机（LABSUN GERMANY，Germany）

KD-2850 低温恒冷切片机（北京世纪科信科学仪器有限公司，中国）

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2.1.3 临床标本

临床标本取自上海交通大学医学院附属第九人民医院血管外科于 2017 到 2019 年接受下肢（大腿及小腿）截肢的患者，其中因糖尿病下肢严重缺血截肢患者 15 人，非糖尿病急性下肢动脉栓塞截肢患者 13 人，患者均签署知情同意书后，医生于术中取患者下肢缺血肌肉组织标本，共 28 例。缺血肌肉标本均取自近患者截肢平面的切除肢体近端。

2.2. 方法

2.2.1 临床样本的获取与处理

2.2.1.1 血液样本处理

本研究纳入的所有因糖尿病下肢严重缺血及急性动脉栓塞截肢患者签署知情同意书后，于术前抽取 10mL 外周静脉血后，保存于凝胶促凝管中，并迅速转运至实验室中。将采血管在 37℃温箱中静置 30 分钟，待血块凝集后，将采血管在离心机上离心，1000g，15 分钟，用移液器分离血清后放入冻存管内，置于-80℃冰箱保存，用于后续 ELISA 检测。

2.2.1.2 临床标本处理

术中获得的标本即时置入冰盒，迅速转移至实验室，在无菌环境中执行处理措施，具体如下：

（1）对于获得下肢缺血肌肉组织标本，应用组织剪将其分离剪至适当大小（＞3×3×3 cm^3）的数块组织块，置于-80℃冰箱保存，用于后续 Western Blot 实验分析；

（2）将其余下肢缺血肌肉组织标本切至约 0.6×0.6×0.6 cm^3 大小后，置入 4%多聚甲醛中浸泡，4℃冰箱内过夜，成功固定。第二日，将固定后的标本包埋入 OCT 包埋剂，应用冷冻切片机将其连续切成厚度为 10 μm 的冰冻切片后，置入-20℃冰箱保存，用于后续免疫组化及免疫荧光检测。

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2.2.2. 标本免疫荧光染色检测 Netrin-1 与 CD31 共定位情况

（1）室温中应用 0.3% Triton-X 进行 10min 破膜，然后用 PBS 进行 3 次漂洗标本，每次持续约 5min；

（2）应用 10%山羊血清于 37℃温度下封闭标本 30min；

（3）按说明书所示，依次加入兔抗 CD31 抗体（1：200 稀释）与小鼠抗 Netrin-1 抗体（1：250 稀释），对照组加入 PBS，均置于 4℃下进行过夜后，用 PBS 漂洗标本 3 次，每次持续约 5min；

（4）依次向标本加入 Fluor 555 羊抗兔 IgG 抗体及 FITC 羊抗小鼠 IgG 抗体（1:500 稀释），注意放置于黑盒中避光，于 37℃下放置约 60min，孵育完成后，用 PBS 漂洗标本 3 次，每次持续约 5min；

（5）用 DAPI（1:1000 稀释）进行细胞核染色，孵育 2min 后用 PBS 漂洗标本，约持续 5min；

（6）用荧光封片剂进行封片，标本荧光片制作完成，然后在荧光显微镜下观察记录。

2.2.3 免疫组化染色检测 Netrin-1 表达及小血管密度

（1）将冰冻切片置入 1mM EDTA 中，于 95℃下加热 10 分钟，于室温下冷却至常温，进行抗原修复；

（2）使用适当的固定液固定细胞或切片，用免疫染色洗涤液洗涤 2 次，每次约持续 5min；

（3）加入免疫染色封闭液，封闭约持续 60min；

（4）按一抗说明书，适当比例稀释一抗。回收封闭液后加入稀释好的一抗，于室温下孵育 1 小时，回收一抗。用 PBS 漂洗标本 3 次，每次持续约 5min；

（5）按二抗说明书，适当比例稀释二抗。回收封闭液后加入稀释好的二抗，于室温下孵育 1 小时，回收二抗。用 PBS 漂洗标本 3 次，每次持续约 5min；

（6）用封片剂封片，标本免疫组化片制作完成，然后光学显微镜下观察记录。

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2.2.4. Western Blot

（1）首先配置裂解液，其次将临床获取的下肢缺血肌肉组织剪碎后与裂解液在 0℃共孵育，获得组织匀浆。超声仪中破碎细胞，离心机中离心后获得上清，即总蛋白提取完成；

（2）总蛋白调整浓度至 2μg/μl 后加入上样缓冲液，放入离心管中，与沸水中加热持续时间 5min，使蛋白变性，4℃冷却后放入-20℃保存；

（3）总蛋白取出后恢复至室温，以 10000rpm 离心 1min 后按说明书配置 SDS-PAGE 凝胶，组装电泳仪器，电泳孔中加入蛋白，空白孔中加入 marker，进行电泳；

（4）用甲醇浸泡 PVDF 滤膜 1min，漂洗后放入转膜缓冲液内，电泳结束获得的凝胶漂洗 10min 后与 PVDF 膜共孵育，缓冲液中进行转膜；

（5）TBS 洗涤 PVDF 滤膜后，孵育于封闭液中，1h 后加入稀释好的一抗共孵育，4℃摇匀过夜；

（6）室温下，PVDF 滤膜进行洗涤后与二抗共孵育 2h；

（7）将 PVDF 滤膜 3 次洗涤后，与 ECL 发光液共孵育后，上机观察与记录。

2.2.5. ELISA 检测血清中炎症因子及 Netrin-1 表达水平

（1）计算并设置标准品孔的梯度浓度与加样量；

（2）向空白孔与待测样品孔中加样，加样后稀释的量和标准品孔保持一致；

（3）封板后于 37℃下孵育，持续时间为 30min；

（4）稀释浓缩洗涤液，备用；

（5）揭开封板膜后甩干，用洗涤液清洗各孔，30s 后倒掉，重复 5 次后甩干；

（6）向除了空白孔外的每个孔中加入相同量的酶标试剂。重复（3）（5）操作；

（7）向各孔中加入显色剂 A 及 B，摇匀后避光，37℃下孵育 15min 显色，然后加入终止液，各孔反应终止；

（8）将空白孔设置为 0，应用酶标仪于 450nm 下测量样品 OD 值。

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2.2.6. 统计学方法

本研究中所有数据的统计及分析均使用 SPSS 18 软件，所有统计学图及表格的绘制均使用 GraphPad Prism 5 软件，所有数据以均值及标准差（SD）表示。采用学生 t 检验和单因素方差分析对定量值进行比较分析，统计学意义定义为*P < 0.05，**p＜0.01；***p＜0.001。每个实验进行了三次以上。

3. 实验结果

3.1. 临床患者基线特征

为了 Netrin-1 蛋白在 DPNV 中的表达及临床相关性情况，我们首先获取了因糖尿病下肢严重缺血截肢患者的下肢肌肉组织及相应血液标本，并以因急性动脉栓塞截肢患者的下肢肌肉组织标本及相应血浆标本作为对照。

经血管外科医师严格临床评估后需要截肢的患者签署知情同意书，术前获得其血液样本，术中获得其截肢肌肉组织。共纳入患者 28 人，其中因糖尿病下肢严重缺血截肢患者 15 人，非糖尿病急性下肢动脉栓塞截肢患者 13 人，所有临床患者的基线特征汇总见表 1-1:

表 1-1 临床患者基线特征一览表

	2 型糖尿病	非糖尿病
男性（人）	9	8
女性（人）	6	5
年龄（岁）	69±9.3	71±8.1
血糖（mmol/L）	10.3±3.1	4.8±1.1***
收缩压（mmHg）	151.4±12.7	157.8±33.2
舒张压（mmHg）	81.9±13.8	90.3±18.7
胆固醇（mmol/L）	3.8±0.5	3.6±0.4
甘油三酯（mmol/L）	1.4±0.2	1.5±0.3
低密度脂蛋白（mmol/L）	2.7±0.4	2.5±0.5
高密度脂蛋白（mmol/L）	0.9±0.4	0.8±0.2
游离脂肪酸（mmol/L）	0.5±0.2	0.6±0.2

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经过对两组患者临床基线特征的对比我们发现，截肢患者中，T2DM 患者 15 人，男性 9 人，女性 6 人，平均年龄 69±9.3 岁。非 DM 患者 13 例，男性 8 人，女性 5 人，平均年龄 71±8.1 岁。年龄及性别组成无统计学差异（P = 0.934）。除了在随机血糖 T2DM 患者为 10.3±3.1mmol/L，远远大于非 DM 患者 4.8±1.1mmol/L，二者具有统计学差异（P＜0.001）。其余在血压、胆固醇、HDL 及 LDL 等方面二者均无统计学差异。因此，可以在两组的临床标本方面进行下一步的蛋白学及组织学检测，观察 T2DM 与非 DM 患者下肢缺血肌肉组织中 Netrin-1 表达的差异。

3.2.Netrin-1 在 T2DM 下肢缺血肌肉组织内及外周血中的表达显著下降

DPNV 最主要的病变在于微血管及大血管的结构破坏与功能丧失。为了探索 Netrin-1 与 DPNV 的临床相关性。首先我们通过免疫荧光技术检测确定了 Netrin-1 在人体内皮细胞上与内皮细胞特异性标志物 CD31 共表达，证明了 Netrin-1 与内皮细胞的共定位（图 1-1）。其次我们应用免疫组织化学技术探明 T2DM 患者与对照组下肢缺血肌肉中 Netrin-1 的表达情况及小血管密度。组织学及统计分析均表明，T2DM 患者下肢肌肉中 Netrin-1 表达水平及小血管密度均显著低于对照组（图 1-2，图 1-3，图 1-4，图 1-5）。同时，下肢缺血肌肉组织的 Western Blot 定量地印证了 T2DM 患者肌肉组织中 Netrin-1 表达水平显著下降（图 1-6）。外周血血清的 ELISA 也表明外周血中 Netrin-1 的表达水平显著下降（图 1-7）。本研究表明 Netrin-1 在受损部位与全身血液循环中的表达水平与 DPNV 的内皮细胞数量及血管破坏程度相关，Netrin-1 表达水平与 DPNV 具有临床相关性。

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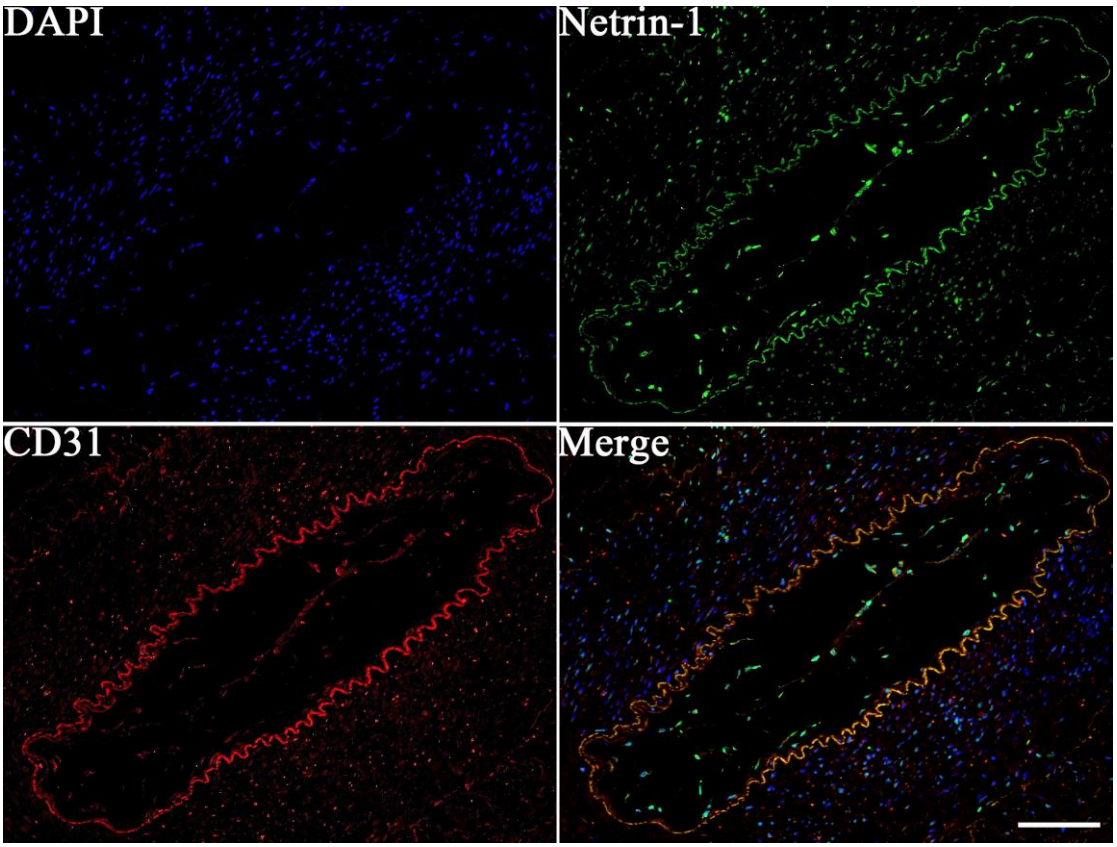

图 1-1 T2DM 患者体内 Netrin-1 与 CD31 共表达，Netrin-1 与内皮细胞共定位

Figure 1-1 Co-expression of Netrin-1 and CD31 in T2DM patients, colocalization of Netrin-1 with endothelial cells

应用 DAPI（蓝色）标明细胞核，Netrin-1 应用绿色荧光抗体标明，CD31 应用红色荧光抗体标明。（比例尺=100μm）

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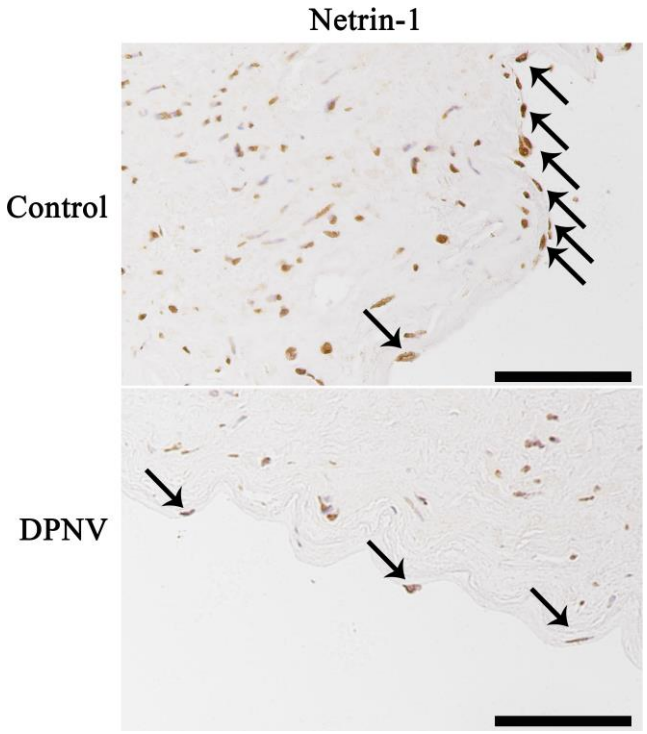

图 1-2 T2DM 患者下肢肌肉内皮细胞 Netrin-1 表达水平明显下降。

Figure 1-2 The expression of Netrin-1 in the lower limb muscle endothelial cells of patients with T2DM was significantly decreased.

箭头所指部位为内皮细胞表达 Netrin-1 情况。（比例尺=100μm）

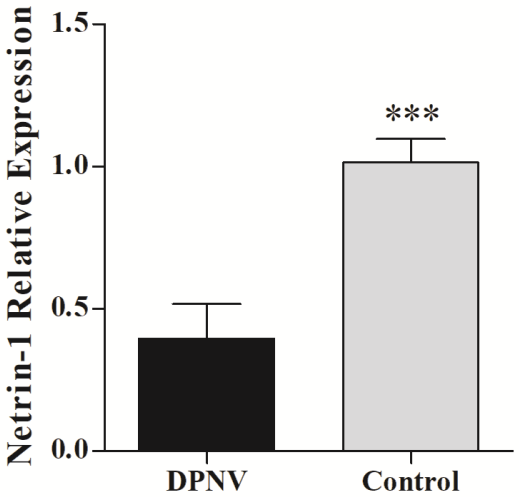

图 1-3 统计学分析表明 T2DM 患者下肢肌肉内皮细胞 Netrin-1 表达水平显著下降 (***，P＜0.001)

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Figure 1-3 Statistical analysis showed that the expression level of Netrin-1 in the endothelial cells of the lower limbs of T2DM patients was significantly decreased(***,P＜0.001).

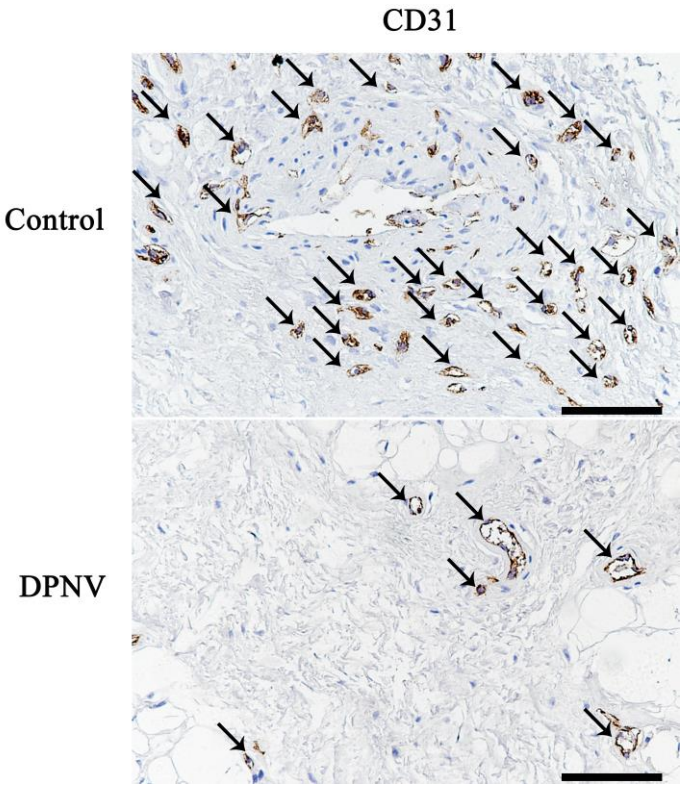

图 1-4 T2DM 患者下肢肌肉小血管密度明显下降。

Figure 1-2 The microvessel densities in the lower limb muscle endothelial cells of patients with T2DM were significantly decreased.

箭头所指部位为小血管内皮细胞表达 CD31 的情况。（比例尺=100μm）

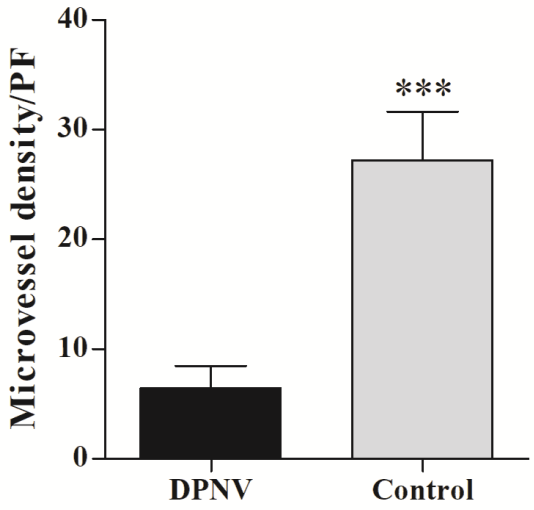

图 1-5 统计学分析表明 T2DM 患者下肢肌肉小血管密度明显下降(***，P＜0.001)。

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Figure 1-5 Statistical analysis showed that the microvessel densities in the lower limb muscle endothelial cells of patients with T2DM were significantly decreased (***,P＜0.001).

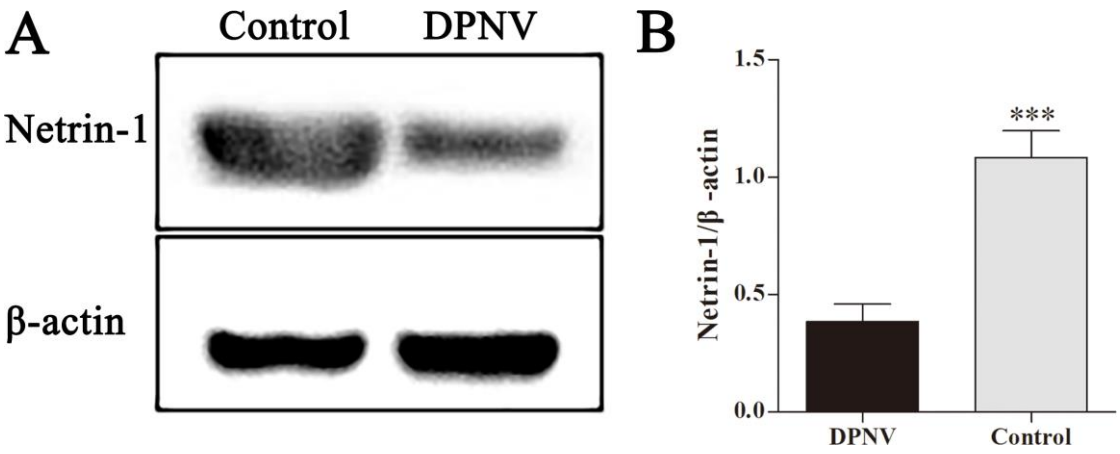

图 1-6 Western Blot 检测(A)及统计学分析(B)表明 T2DM 患者下肢肌肉组织 Netrin-1 表达水平显著下降(***，P＜0.001)

Figure 1-6 Western Blot (A) and statistical analysis (B) showed that the expression level of Netrin-1 of the lower limbs of T2DM patients was significantly decreased(***,P＜0.001).

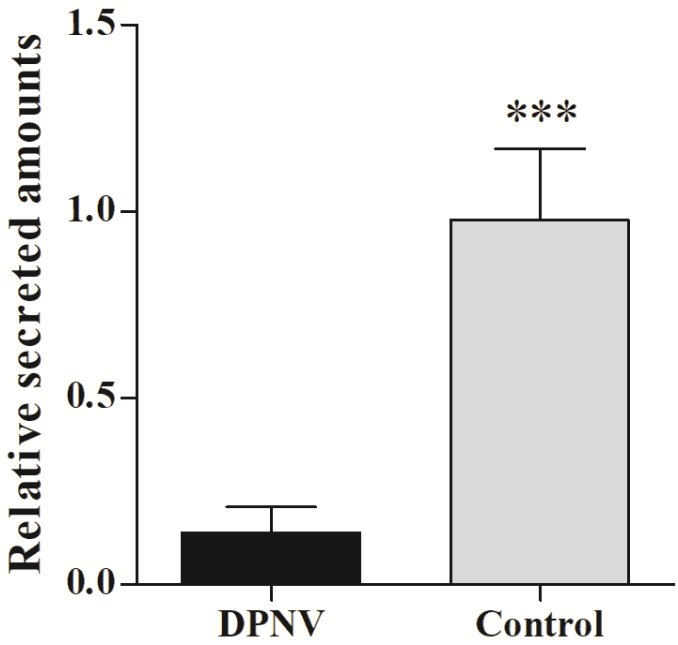

图 1-7 ELISA 检测及统计学分析表明 T2DM 患者外周血中 Netrin-1 表达水平显著下降(***，P＜0.001)

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Figure 1-7 ELISA and statistical analysis demonstrated that the expression level of Netrin-1 in peripheral blood of T2DM patients was significantly decreased(***,P＜0.001).

3.3. 炎症因子在 T2DM 患者外周血中表达水平明显升高

接下来我们应用 ELISA 对 T2DM 和对照组的外周血血清中 IL-6、IL-1β、MCP-1 以及 IL-12 等炎症因子的表达水平进行检测。ELISA 结果及统计学分析表明，与对照组外周血血清相比，T2DM 患者外周血中以上炎症因子的表达水平明显升高（P＜0.05，图 1-8）。本研究结果表明 DPNV 患者体内持续高血糖刺激下，Netrin-1 与炎症因子在外周血中的表达水平具有一定程度的负相关性。

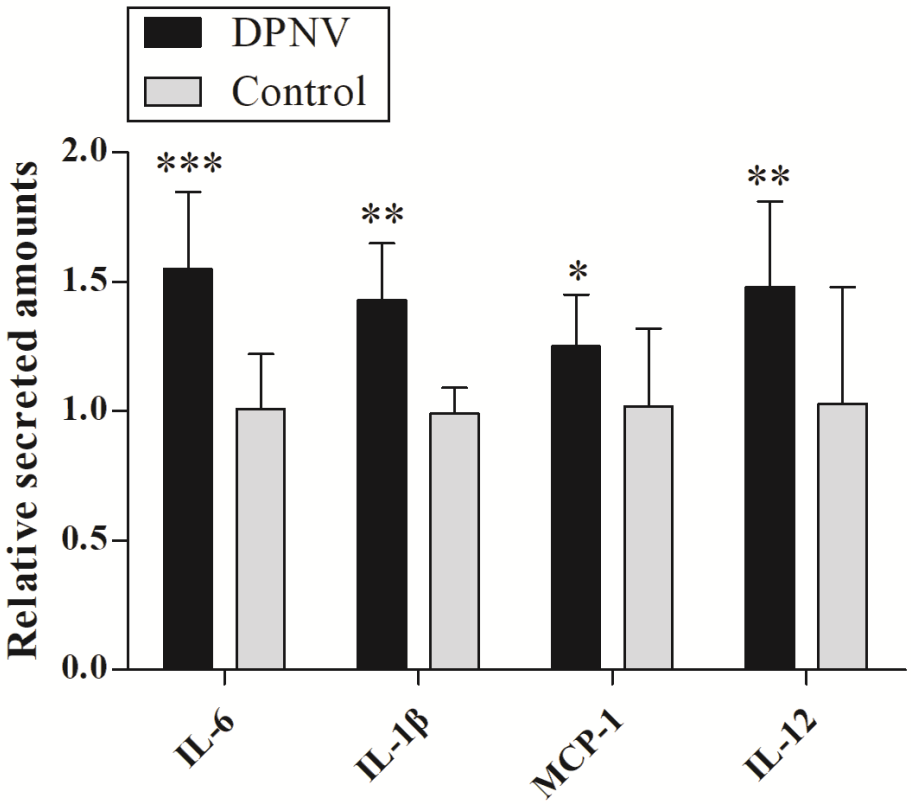

图 1-8 ELISA 检测及统计学分析表明 T2DM 患者外周血中 IL-6、IL-1β、MCP-1 及 IL-12 等炎症因子表达水平显著上升(***，P＜0.001；**，P＜0.01。*，P＜0.05)。

Figure 1-8 ELISA and statistical analysis demonstrated that the expression level of IL-6、IL-1β、MCP-1 and IL-12 in peripheral blood of T2DM patients was significantly increased (***,P＜0.001;**,P＜0.01, *,P＜0.05).

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4. 讨论

DPNV 其实质上是一种由糖尿病人控制不佳的持续升高的血糖所造成的慢性炎症性疾病。Michael Brownlee 等首次提出的“糖尿病并发症发生机制大一统理论”为我们揭示了长期的高血糖状态导致低水平的促炎症微环境，从而通过以氧化应激为主的多种机制破坏血管内皮细胞以及神经细胞的功能[121]。而神经细胞与血管内皮细胞又存在着互相提供营养的相互依存关系，从而导致二者所处环境的进一步恶化，形成瀑布式反应，使得患肢病情迅速恶化，最终发展为糖尿病足，产生无法愈合的溃疡以及坏疽，最终以截肢告终，甚至危及生命[122-129]。临床上现有的诸如最佳药物治疗、血管内球囊扩张以及支架植入、血管旁路血运重建等方式虽能短期控制症状，但长期效果不佳[110-119]。这提示我们更深入的机制仍有待我们去探索，以及如果能在 DPNV 较早的时期从一些生物指标上窥见端倪，或许能收到更好的临床治疗效果。

Netrin-1 作为一个能够抑制炎症的分泌蛋白，广泛存在于血液及组织液中，其与各种炎症性疾病的关系逐渐明了，并被作为许多急慢性炎症性疾病的生物标志物，这可能与机体内 Netrin-1 抑制炎症细胞迁移，抑制炎症细胞因子和趋化因子的产生，通过抑制 NF-κB 活化调控 COX-2 的表达，促进巨噬细胞向抗炎的 M2 样表型分化，以及通过 MAPKs、ERKs、p38 等信号转导途径调节炎症反应等机制相关[136，137]。既往研究表明，Netrin-1 是第一个确定的神经轴突导向因子，并被证明在血管新生以及内皮细胞增殖中发挥重要作用，其在血管与神经领域均十分活跃的“双面性”引起了我们的重视，而 Netrin-1 在 DPNV 这种血管与神经的混合性病变中是否有显著的变化并未见任何报道，所以，本研究着重检测了 Netrin-1 在糖尿病患者的下肢缺血肌肉组织以及外周血血清中的表达水平。

本项研究纳入了 15 例糖尿病下肢慢性缺血截肢患者作为实验组以及 13 例急性下肢动脉栓塞截肢患者作为对照组，统计学分析显示两组除了血糖之外无统计学差异，可以进行对照比较。首先 Netrin-1 与血管内皮细胞的免疫荧光共定位表明 Netrin-1 表达于血管内皮细胞之上，CD31 的免疫组化结果表明糖尿病下肢缺血组织中微血管数量显著减少，这可能是由于下肢动脉栓塞而截肢的患者往往由于房颤或其他栓子脱落进入下肢动脉，往往起病急，进展快，24 小时内无有效

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救肢则只能截肢处理，因此，发病前下肢基本健康，不存在缺血情况；而糖尿病患者的下肢缺血往往是慢性过程，发展到截肢时，持续的高血糖已经将代偿的微血管消耗殆尽，进入失代偿期，因此下肢微血管数量显著减少，呈现出“一片荒漠”状态。而通过 Netrin-1 的免疫组化染色结果和对缺血组织的 Western Blot 检测我们发现，糖尿病患者下肢缺血肌肉内的 Netrin-1 表达水平显著低于因动脉栓塞截肢患者下肢缺血肌肉，并且 ELISA 的结果表明外周血血清中 Netrin-1 蛋白的表达水平也显著降低，而在血液中，IL-6、IL-1β、MCP-1 以及 IL-12 等炎症因子均显著升高，炎症因子的升高印证了 DPNV 是一种慢性炎症性疾病，而 Netrin-1 水平的降低提示我们在 DPNV 病变中，Netrin-1 的表达与炎症因子的表达成负相关性。可能的原因是 Netrin-1 本身是一种对血管和神经抗炎抗凋亡的蛋白，而长期的高血糖状态使得组织细胞一直处于氧化应激的炎症微环境中，在 DPNV 发生的极早期阶段，Netrin-1 或许有反应性的增高，但长期的炎症微环境会逐渐耗竭组织中血管神经细胞内的 Netrin-1，同时血液内的 Netrin-1 也逐渐减少，而这个结果与 Liu C 等的研究结果相一致，他们发现新诊断的 T2DM 患者血液中 Netrin-1 表达水平较非糖尿病患者显著降低[105]。我们的实验研究结果表明，在糖尿病患者血液中，诸如 IL-6、IL-1β、MCP-1 以及 IL-12 等炎症因子均显著升高，印证了 DPNV 是一种慢性炎症性疾病的结论。同时，Netrin-1 作为一种抵抗炎症的分泌蛋白，其组织与血液表达水平与 DPNV 的进展呈负相关。综上我们认为，Netrin-1 在机体抵抗 DPNV 的发生发展中起到重要调节作用，其表达水平的变化可以反映 DPNV 疾病的进展情况，未来可能是一种很有潜力的 DPNV 的生物标志物。然而其具体调控作用与机制需要进一步深入的研究与发现。

5. 小结

本实验研究通过获取临床糖尿病与非糖尿病患者的下肢缺血肌肉组织以及外周血血清，通过免疫组化、免疫荧光、Western Blot、ELISA 等研究方法综合分析了糖尿病患者下肢缺血组织中 Netrin-1 与内皮细胞的共定位情况、Netrin-1 的表达水平、小血管密度以及外周血血清中 Netrin-1 及炎症因子的表达水平。并

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统计分析明确糖尿病高血糖环境下 Netrin-1 的表达与 DPNV 的临床相关性。结果发现 DPNV 患者内皮细胞中表达 Netrin-1 蛋白，但在其外周血血清以及下肢缺血肌肉组织中，Netrin-1 表达水平显著下降，而多项炎症相关因子的表达水平明显上升，微血管的密度较对照组明显下降。本实验研究结果表明，Netrin-1 作为一种抵抗炎症的蛋白，参与了 DPNV 发生发展的病理进程，并发挥重要作用。其组织及血清中的表达水平与 DPNV 疾病的进展呈负相关性。Netrin-1 在未来可能会成为一种有潜力的 DPNV 的生物标志物，其在 DPNV 的发生发展进程中具体的作用原理和分子机制有待于更多的研究进一步探索和发现。

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第二章 Netrin-1 体外调控高糖环境下 ADSCs 功能的研究

1. 引言

DPNV 的发病率高、出现早、起病隐匿、病程长、难以治愈，并且导致截肢甚至生命危险，目前临床尚无有效的长期治疗方案。随着再生医学的高速发展，对于这些临床上尚无令人满意的治疗方法的疾病，科学家逐渐将目光投向了具有多向分化潜能的“种子细胞”们——干细胞。

随着对干细胞研究的深入，目前在实验室及临床应用较多的是间充质干细胞（MSCs），它们是一类未分化的细胞，能够自我更新并分化为多种特定的细胞类型[138]。以往研究得较多的是骨髓间充质干细胞（BMSCs），但在过去几年的研究发现，MSCs 实际上存在于许多器官和结缔组织中。从骨膜、滑膜、骨骼肌、皮肤、外周血和脐带等不同组织来源都成功地分离出与骨髓来源相似的 MSCs[139]。但普遍存在收获细胞产量低和收获组织数量有限的问题。同时，获取 BMSCs 需要进行骨髓抽吸，需要全身麻醉并且相对痛苦与昂贵，而获得的 BMSCs 的细胞产量又很低[140]。最新发现，脂肪组织是获得干细胞的一个很好的替代来源，特别是随着肥胖发病率的增加，使组织丰富且易于获取。在更安全、花费更少的吸脂术过程中，可以大量收集到供体部位的脂肪[141]。美国每年大约进行 40 万次抽脂手术，这些手术产生 100 毫升至 3 升以上的抽脂组织，而这些组织通常被丢弃[142]。此外，脂肪组织中 ADSCs 含量高，无需长期体外培养，降低了染色体异常的风险[141，143，144]。ADSCs 在许多生物学特征方面优于 BMSCs，包括免疫调节作用和多种生长因子和细胞因子的分泌以及抗凋亡和抗炎作用[145-149]。

ADSCs 不仅被基础科学所广泛关注，而且还被广泛用于临床再生医学。ADSCs 可以治疗许多不同疾病，在体内组织修复和调节宿主免疫反应方面表现出巨大的潜力[147]。来自健康供体的 ADSCs 是器官再生的极具吸引力的细胞来源[150]。这些细胞可在体外培养至足够数量，随后用于受损组织再生[151]。ADSCs 在体外诱导时具有广谱的分化潜能，可以分化为从中胚层到外胚层（如肝细胞）和内胚层（如 β 细胞）来源的各种功能细胞[151-154]。ADSCs 可以长期培养，无需传代而不会丧失其分化能力，在低温下亦可存活，而其生存、增殖

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和分化能力均受损较少。ADSCs 最具吸引力的方面是它们的免疫抑制特性，无论宿主和供体之间的人白细胞抗原（HLA）匹配如何，都可以进行 ADSCs 移植[155]。此外，ADSCs 可以表达不同类型的营养因子，能够调节诸如细胞增殖、纤维化、血管生成和免疫抑制等[156-163]。另外，ADSCs 具有明显的抗细胞凋亡，抗氧化，抗炎活性，这也是其有助于组织再生的重要特性[164-168]。迄今为止，在有关 ADSCs 的 160 余项临床试验中，ADSCs 被用于治疗各种疾病，如炎症性疾病，肝功能衰竭，与糖尿病相关的并发症，多发性硬化症，骨科疾病，脱发，生育问题和唾液腺损伤等疾病。不同的临床前和临床试验均证明了 ADSCs 移植促进组织再生的安全性及有效性，并且目前为止并无产生严重副作用的相关报道[169-187]。因此，ADSCs 也有望成为治疗 DPNV 的合适之选。

ADSCs 日益成为应用于基础实验及临床研究中热门的“种子细胞”。其移植后促进组织再生的治疗作用得到了证实，但依然存在诸多局限有待克服。最亟待解决的就是其移植后存活量极低的问题。通过基于导管的经心内膜注射递送的 MSCs 的正电子发射断层扫描（PET）跟踪显示，移植后 10 天，在猪缺血心肌中只存活了大约 6%的 MSCs[188]。Toma 等人报道，在免疫缺陷小鼠心脏移植后第 4 天，存活的 MSCs 不超过 0.44%[189]。同样的，在实验性 MI 的大鼠心脏移植后 24 小时只能检测到大约 1%的 MSCs[190,191]。更多研究发现，移植后糖尿病患者下肢缺血症状及客观指标得到一定程度的改善，但其远期疗效仍然欠佳[36,64-70]。大量研究亦证实：糖尿病周围血管损伤部位存在高级糖基化终产物、产生促炎微环境和诱导氧化应激，使得移植干细胞在靶血管损伤部位及高糖环境下的存活、迁移及向新生血管分化的能力显著减低，是影响干细胞疗效的关键所在[15-17，71-77]。因此，如何促进 ADSCs 在糖尿病周围血管损伤部位的存活、分化及促进失神经支配后糖尿病血管的新生，对防治 DPNV 病变的发生、发展具有重要意义。

研究发现，DPNV 是一种混合性病变，伴有血管，神经和组织损伤[28,78-80]。而 Netrin-1 作为一个最早于神经生长中发现的分泌蛋白，最近被越来越多地发现参与到血管新生、内皮细胞的存活，粘附，迁移，增殖和凋亡，并在多种炎症性疾病包括糖尿病及糖尿病并发症中发挥抑制炎症细胞迁移，抑制炎症细胞因子和趋化因子的产生，通过抑制 NF-κB 活化调控 COX-2 的表达，促进巨噬细胞向抗

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炎的 M2 样表型分化，以及通过 MAPKs、ERKs、p38 等信号转导途径调节炎症反应等抗炎作用[136-137]。因此我们认为通过基因转染 ADSCs 过表达 Netrin-1 可以改善 ADSCs 在高血糖条件下的活力，迁移和向血管内皮细胞分化，促进 DPNV 血管新生。本研究拟获取 C57/BL 小鼠脂肪组织，分离并培养 ADSCs，建立基因转染体系使 ADSCs 过表达绿色荧光蛋白（GFP）及 Netrin-1（N-ADSCs）。通过 CCK-8、Western Blot、流式细胞术、Transwell 等检测 N-ADSCs 与 ADSCs 在高糖环境下的增殖、迁移、粘附、向内皮细胞分化等能力之间的差异，探索体外环境中 Netrin-1 对高糖环境下 ADSCs 的影响。

2. 材料和方法

2.1. 材料

2.1.1. 主要试剂

PBS 磷酸缓冲液（1x，PH 7.2-7.4）（北京索莱宝科技有限公司，中国）

胰酶-EDTA（Trypsin-EDTA，0.25%，北京博奥龙免疫技术有限公司，中国）

DMEM 溶液：

1g/L Dulbecco’s modified eagle medium (DMEM; Gibco, USA)

10% fetal bovine serum (FBS, Hyclone, Australia)

NB4 胶原酶溶液：

0.2%胶原酶 NB4 (Serva, Heidelberg, Germany)

DMEM 溶液配制

TBS 缓冲液（伯乐生命医学产品（上海）有限公司，中国）

PBS 磷酸缓冲液（1x，PH 7.2-7.4）（北京索莱宝科技有限公司，中国）

TBST 缓冲液（1x）（北京索莱宝科技有限公司，中国）

考马斯亮蓝溶液（生工生物工程（上海）股份有限公司，中国）：

牛血清白蛋白（BSA，Sigma，USA）

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4%多聚甲醛（上海晶都生物技术有限公司，中国）：

甲醇（上海代轩生物科技有限公司，中国）

95%乙醇（上海交通大学医学院附属第九人民医院）

75%乙醇（（上海交通大学医学院附属第九人民医院）

异丙醇(Sigma, USA) （上海代轩生物科技有限公司，中国）

氯仿(Sigma, USA)

DAPI（西安赫特生物科技有限公司，中国）

树脂（Epon, Merck, Darmstadt, Germany）

OCT 包埋剂（DAKO，USA）

荧光封片剂（DAKO，USA）

Matrigel 凝胶（Sigma，USA）

胶原 Collagen II (Abcam, Cambridge, MA)

CCK-8 (Dojindo Laboratories, Kumamoto, Japan)

PI 染液（Sigma，USA）

草酸铵结晶紫染色液（结晶紫染色液）(北京索莱宝科技有限公司，中国)

PE-抗小鼠流式抗体 CD90、CD11b、CD31、CD34、CD45、CD133、MHC-II(eBioscience, San Diego, CA)

PE-抗小鼠流式抗体 Sca-1 (eBioscience, San Diego, CA)

FITC-羊抗小鼠 IgG 多克隆抗体（Invitrogen, Carlsbad, CA）

DAPI（Invitrogen, Carlsbad, Canda）

树脂（Epon, Merck, Darmstadt, Germany）

荧光封片剂（DAKO，USA）

Matrigel 凝胶（Sigma，USA）

戊巴比妥钠（上海交通大学医学院附属第九人民医院，中国）

载体 pHBAd-EF1-MCS-GFP（上海汉恒生物科技有限公司，中国）

大肠杆菌菌株 DH5α（Invitrogen，Carlsbad，CA）

限制性内切酶（Thermo Fisher Scientific，USA）

One Step Cloning Kit（南京诺唯赞生物科技有限公司，中国）

质粒 DNA 大量抽提试剂盒（北京康为世纪生物科技有限公司，中国）

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凝胶回收试剂盒（上海捷瑞生物工程有限公司，中国）

琼脂糖，琼脂粉（生工生物工程(上海)股份有限公司，中国）

DNA ladder（Thermo Fisher Scientific，USA）

人胚肾细胞系 HEK293 cell(上海中国科学院,中国)

LipofiterTM 转染试剂（上海汉恒生物科技有限公司，中国）

2.1.2. 主要仪器与设备

恒温 CO_2 培养箱 （Forma Scientific，USA）

离心机（Thermo，USA）

去离子水系统（Millipore，USA）

超净工作台（江苏苏净集团，中国）

倒置显微镜（重庆光电仪器总公司，中国）

荧光显微镜 （Nikon，Japan）

精密天平（Mettler Toledo，Switzerland）

天平（上海舜宇恒平科学仪器有限公司，中国）

冰箱（海尔，中国）

6 孔板（Corning Inc.，USA）

培养皿（Corning Inc.，USA）

50ml 离心管（Corning Inc.，USA）

15ml 离心管（Corning Inc.，USA）

96 孔板（无锡耐思生物科技有限公司）

移液管（无锡耐思生物科技有限公司）

0.22 μm 针头滤器（Millipore，USA）

40 μm 滤网（BD Falcon， USA）

显微手术器械（上海交通大学医学院附属第九人民医院，中国）

流式细胞仪（Beckman Coulter, Fullerton, Canada）

Image-Pro Plus 软件（Media Cybernetics Inc, Rockville, MD）

低温循环水浴 Polystat（Cole-Parmer Instrument Comapny,USA）

电热恒温水浴锅（上海一恒科技有限公司）

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电泳仪（上海天能科技有限公司）

PCR 扩增仪（Applied Biosystems 公司）

电子天平（北京赛多利斯仪器系统有限公司，中国）

蛋白核酸测定仪（Eppendorf，Germany）

MINI 离心机（Tomy Seiko Co., Ltd. Japan）

梯度 PCR 仪（Eppendorf，Germany）

垂直洁净工作台（沪净净化）

冷冻离心机（Eppendorf，Germany）

全温振荡培养箱（上海一恒科技有限公司限公司，中国）

台式多功能高速冷冻离心机（Eppendorf，Germany）

高压灭菌锅（上海申安医疗器械厂，中国）

高速冷冻离心机（Thermo Fisher Scientific，USA）

凝胶成像系统（上海天能科技有限公司，中国）

2.1.3. 实验动物

年龄为 8W 的 C57/BL6 小鼠（上海南方模式生物有限公司，中国）。

2.2. 方法

2.2.1. ADSCs 的获取、培养与扩增

（1）先用戊巴比妥钠麻醉年龄为 8W 的 C57/BL6 小鼠，然后脱臼颈椎处死，放入 75%酒精中浸泡 10min 以充分消毒；

（2）用解剖器械充分暴露双侧腹股沟，剪下脂肪组织，置于少量 DMEM 溶液中；

（3）用显微解剖剪将获取的脂肪组织充分剪碎，直至大小为 $1mm^3$ 左右，放入已用 DMEM 溶液加足量 0.2%NB4 的 50mL 离心管中，放入恒温摇箱，温度设定为 37℃进行 2h 充分消化；

（4）取出后充分吹打 5min，离心机中以 1200rpm 速度转 5min，吸去上清液；加入配置好的 ADSCs 培养液（DMEM 加 10%血清加 5%抗生素）5mL 以重

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悬细胞；

（5）以 10^5 个细胞/cm^2 的密度接种细胞悬液于 10cm 培养皿中，置于细胞培养箱中孵育；

（6）3 天后观察原代细胞，可见细胞融合至 90%左右，即可传代。

（7）将 2ml 0.25%胰酶-EDTA 加入培养皿中，培养箱中放置 3min 后，取出加入 5mL ADSCs 培养液终止消化。充分吹打后转入 50mL 离心管，1500rpm 速度离心 5min；

（8）倒掉上清，加入 ADSCs 培养液 10mL 重悬原代细胞；

（9）以 10^5 个细胞/cm^2 的密度以 1:2 的比例接种细胞悬液于 10cm 培养皿中，置于细胞培养箱中孵育；

（10）48 小时后，重复以上（7）（8）（9）步骤，传代与扩增 ADSCs。

2.2.2. **ADSCs 的流式鉴定**

（1）小心倒掉培养皿的上层清液,在 37℃下用 PBS 冲洗一次，并添加 0.25%的 trypsin-EDTA 2mL。当大多数细胞收缩成圆形时，加入 ADSCs 培养液。用 3mL 液体停止消化;

（2）液体经由 40μm 过滤器过滤入 15mL 离心管，1500rpm 速度离心 5min 后丢弃上层清液;

（3）用 Buffer 重悬使细胞终浓度为 10^6 个/mL，使用 1.5mL EP 管盛装 100μl 液体并编号;

（4）添加 PE 标记的稀释好的 CD90、Sca-1 CD90、CD11b、CD31、CD34、CD45、CD133、MHC-II (1:20)和对照组抗体，4℃冰箱内避光放置 30min;

（5）用缓冲液冲洗一次，1500rpm 速度离心 5min，丢弃上层清液后再冲洗 3 次;

（6）重悬细胞于缓冲液中，冰上保存带至流式检测仪处检测。

2.2.3. GFP-Netrin-1 基因腺病毒载体构建、包装

（1）查阅相关文献，根据文中提到的 Netrin-1 基因在 NCBI 中的 ID 号，打开 NCBI， 在 All Databases 中选择 Nucleotide，将基因 ID 号输入并搜索，获取

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Netrin-1 基因序列。

（2）pHBAd-EF1-MCS-GFP 载体用 EcoRI 酶切，酶切体系如下：

40ul 酶切体系　　37℃，2h 左右

2ul　　载体（400ng/ul）

2ul　　EcoRI

4ul　　10×buffer

32ul　　H_2O

载体酶切完成后将胶回收；

（3）应用 PCR 回收 Netrin-1 片段。

（4）处理好的目的片段与载体连接反应体系（20ul），以上连接液在 37 温浴 30min，然后迅速置于冰上冰浴 1-2min。

（5）转化(感受态细胞: DH5a)。抗性:Amp; 37℃，培养过夜。

（6）转化后的 Netrin-1 平板挑菌，37℃ 250 转/分钟摇菌 14h，用菌液进行 PCR 鉴定，将阳性克隆菌液送测序公司测序。

（7）大量制备重组质粒

（8）重组腺病毒载体的包装，收毒及扩增，具体操作为：铺细胞、转染、换液、收毒（P1）、冻融、扩增、收毒。

（9）P3 代病毒扩增及收毒。

2.2.4. 建立 Netrin-1 体外转染 ADSCs 的体系

（1）取 P3 代生长旺盛的 ADSCs 消化并计数，在 10cm 培养皿中向 6×10^4 个细胞中加入包装好的含 NTN-1 和不含 NTN-1 的 GFP 腺病毒，将感染复数（MOI）设定为三组：250、500 和 1000，持续时间设定为 24 小时和 48 小时。通过直接免疫荧光显微镜下观察，研究转染 ADSCs 的最佳 MOI 和持续时间以获得最大转染效率，建立转染体系；

（2）通过 RT-PCR、Western Blot 等方法比较转染 GFP 和 GFP-Netrin-1 的 ADSCs 的 Netrin-1 表达情况。

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2.2.5.转染及未转染 Netrin-1 的 ADSC 的 CCK8 检测增殖能力

将 P3 代转染及未转染 Netrin-1 的 ADSCs 以每孔 2×10^3 个细胞的密度接种于 7 个 96 孔培养板中，设 4 孔为不加细胞只加培养液的空白对照孔，最后比色时，以空白孔作为调零。培养板放入 CO_2 孵育箱中培养，以后每天相同时间两组分别取 6 孔，每孔加入 CCK-8 溶液（10μl），孵育 2h，吸去上清，选择 450nm 波长，在酶联免疫检测仪上测定各孔光吸收值（OD），记录结果，连测 7 天，标记细胞生长曲线。

2.2.6. 转染及未转染 Netrin-1 的 ADSCs 的流式及 Western Blot 检测凋亡

流式检测：

（1）小心倒掉培养皿的上层清液,在 37℃下用 PBS 冲洗一次，并添加 0.25% 的 trypsin-EDTA 2mL。当大多数 Netrin-1 转染及未转染的 ADSCs 在高糖环境培养下的 P3 代细胞收缩成圆形时，加入 ADSCs 培养液。用 3mL 液体停止消化;

（2）液体经由 40μm 过滤器过滤入 15mL 离心管，1500rpm 速度离心 5min 后丢弃上层清液;

（3）用 Buffer 重悬使细胞终浓度为 10^6 个/mL，使用 1.5mL EP 管盛装 100μl 液体并编号;

（4）添加 PE 标记的稀释好的 Annexin V 及 PI(1:20)和对照组抗体，4℃冰箱内避光放置 30min;

（5）用缓冲液冲洗一次，1500rpm 速度离心 5min，丢弃上层清液后再冲洗 3 次;

（6）重悬细胞于缓冲液中，冰上保存带至流式检测仪处检测。

Western Blot:

（1）首先配置裂解液，其次将临床获取的下肢缺血肌肉组织剪碎后与裂解液在 0℃共孵育，获得组织匀浆。超声仪中破碎细胞，离心机中离心后获得上清，即总蛋白提取完成;

（2）总蛋白调整浓度至 2μg/μl 后加入上样缓冲液，放入离心管中，与沸水中加热持续时间 5min，使蛋白变性，4℃冷却后放入-20℃保存;

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（3）总蛋白取出后恢复至室温，以 10000rpm 离心 1min 后按说明书配置 SDS-PAGE 凝胶，组装电泳仪器，电泳孔中加入蛋白，空白孔中加入 marker，进行电泳；

（4）用甲醇浸泡 PVDF 滤膜 1min，漂洗后放入转膜缓冲液内，电泳结束获得的凝胶漂洗 10min 后与 PVDF 膜共孵育，缓冲液中进行转膜；

（5）TBS 洗涤 PVDF 滤膜后，孵育于封闭液中，1h 后加入稀释好的一抗共孵育，4℃摇匀过夜；

（6）室温下，PVDF 滤膜进行洗涤后与二抗共孵育 2h；

（7）将 PVDF 滤膜 3 次洗涤后，与 ECL 发光液共孵育后，上机观察与记录。

2.2.7. 细胞迁移实验及细胞划痕实验检测 Netrin-1 调控 ADSCs 迁移的能力

2.2.7.1 细胞迁移实验

（1）P3 代 GFP-ADSCs 和 Netrin-1-GFP-ADSCs 分别取 $2x10^3$ 个细胞，悬浮于补充有 0.5％FBS 的 100μL 高葡萄糖 DMEM 中。

（2）使用 Boyden 室和 8-μm 聚对苯二甲酸乙二醇酯（PET）膜，将液体加入 Transwell 腔室上部，在腔室下部加入 600μL ADSCs 培养液。

（3）将 Transwell 板置细胞培养箱中 24h。

（4）除去过滤器，并使用棉花尖端除去上室中的细胞。使用 4％多聚甲醛固定迁移到下室中的 ADSC，并使用 2％乙醇中的 1％结晶紫染色细胞。 实验重复三次。

（5）显微镜下观察拍照。

2.2.7.2 细胞划痕实验

（1）器械准备:所有可灭菌的仪器均应灭菌，并在操作之前对尺、笔照射 30 分钟(超净台内)

（2）先用记号笔在 6 孔板背面，用直尺均匀画一条横过孔的水平线，约 0.5~1cm。每个孔至少通过 5 行线。

（3）加入 10^5 个 P3 代 GFP-ADSCs 和 Netrin-1-GFP-ADSCs。

（4）第二天，用枪尖尽量沿着水平线划出一条直线。枪尖应该垂直而不是倾斜。

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（5）PBS 洗涤细胞三次，取出划掉的细胞，加无血清 DMEM。

（6）置于细胞培养箱中。在 0、6、12、24h 取样并显微镜下观察并拍照。

2.2.8.细胞粘附实验检测 Netrin-1 调控 ADSCs 粘附的能力

（1）取 P3 代 GFP-ADSCs 和 Netrin-1-GFP-ADSCs，trypsin-EDTA 2mL。当大多数 Netrin-1 转染及未转染的 ADSCs 在高糖环境培养下的 P3 代细胞收缩成圆形时，加入 ADSCs 培养液。用 3mL 液体停止消化；

（2）用 PBS 漂洗 3 次细胞，用细胞计数器各取 5×10^4 个细胞，均匀铺板于 6 孔板中；

（3）将 6 孔板放入细胞培养箱中，30min 后取出；

（4）用 PBS 漂洗 6 孔板内 3 次后，用 DAPI（1:1000 稀释）进行细胞核染色，孵育 2min 后用 PBS 漂洗标本，约持续 5min；

（5）用荧光封片剂进行封片，标本荧光片制作完成，然后在荧光显微镜下观察记录拍照。

2.2.9. 细胞成管实验检测 Netrin-1 调控 ADSCs 成管的能力

（1）将 ECM 凝胶化冻，并制备成 1x 的染色缓冲液。

（2）使用消过毒的 96 孔板，分别将 50μL 的液态 ECM 凝胶加入各个孔。

（3）将含有 ECM 凝胶的 96 孔板置于 37℃温箱内，1h 后，ECM 溶液形成固态凝胶状。

（4）取 P3 代 GFP-ADSCs 和 Netrin-1-GFP-ADSCs，trypsin-EDTA 2mL。当大多数 Netrin-1 转染及未转染的 ADSCs 在高糖环境培养下的 P3 代细胞收缩成圆形时，加入 ADSCs 培养液。用 3mL 液体停止消化；

（5）将细胞在含有 10% FBS 的内皮细胞培养基中重悬至 $4x10^5$ 个细胞/mL。

（6）将 150μl 的 ADSCs 悬液移入 ECM 凝胶上，37°C 下孵育 96 孔板 12h。

（7）在高分辨率视野下用显微镜观察 ADSCs 的成管情况。直观观察成管长度和分支点多少来估计成管情况。拍照并应用 ImageJ Pro Plus software (NIH, Bethesda, MD, http://www.imagej.nih.gov)定量分析。

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2.2.10. 统计学方法

本研究中所有数据的统计及分析均使用 SPSS 18 软件，所有统计学图及表格的绘制均使用 GraphPad Prism 5 软件，所有数据以均值及标准差（SD）表示。采用学生 t 检验和单因素方差分析对定量值进行比较分析，统计学意义定义为*P < 0.05，**p＜0.01；***p＜0.001。每个实验进行了三次以上。

3. 结果

3.1. ADSCs 的生物学特性

首先我们成功获取了 C57 BL6 小鼠的腹股沟脂肪组织，然后从中分离出 ADSCs 并进行快速扩增。P3 代 ADSCs 均显示出成纤维细胞样纺锤状的外观（图 2-1A）。接下来，通过流式细胞术进行的表型分析表明，P3 代 ADSCs 对干细胞表面抗原具有强烈的双阳性，如 CD90（99.2 3.73％）和 Sca-1（99.6 3.08％）（图 2-1），而 CD11b，CD31，CD34，CD45，CD133 和 MHC-II 则是阴性的（数据未显示）。然而，P6 代 ADSCs 可能由于培养时间的延长以及体外传代代数的增多，导致其表现出不规则的外形，以及老化或去分化的特点（图 2-1B）。流式细胞术分析显示 ADSCs 干细胞特异性标志物 CD90（65.4±3.42％）和 Sca-1（59.8±3.15％）表面抗原水平（图 2-1C，D）逐渐降低，差异有统计学意义（P <0.05）。因此，接下来我们将采用 P3-P5 代的 ADSCs 细胞进行所有后续的实验。

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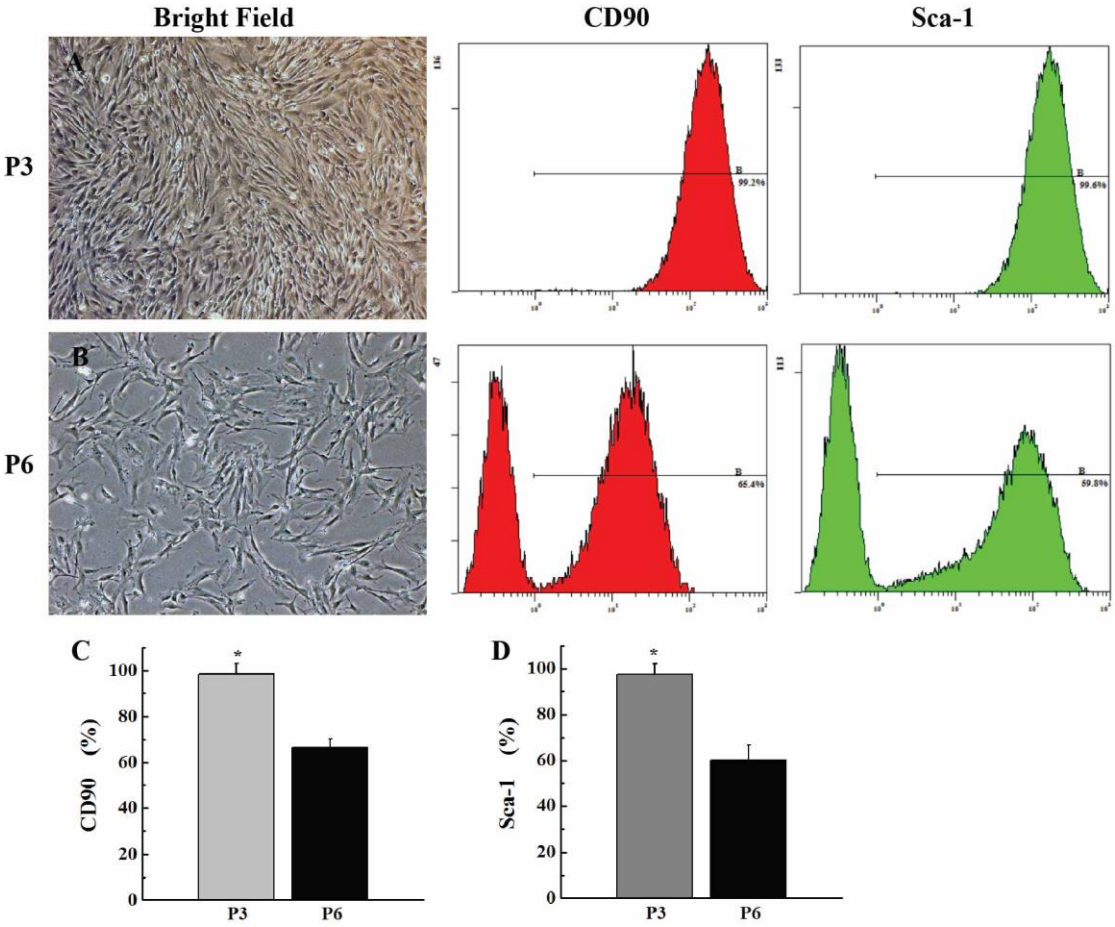

图 2-1 ADSCs 的鉴定及流式细胞术分析。

Figure2-1 Characterization and flow cytometry analysis of ADSCs.

P3 代 ADSCs 为均匀的成纤维细胞样纺锤形群体（A）。P6 代 ADSCs 表现出不规则性增加老化或去分化形状（B）。流式细胞仪检测显示 P3 和 P6 的 ADSCs 的 CD90 和 Sca-1 表面抗原水平逐渐降低，差异有统计学意义（* P <0.05; C，D）比例尺=100μm。 P3，第 3 代; P6，第 6 代; ADSCs，脂肪来源的干细胞。

3.2. 基因转染体系的建立

与其他病毒载体系统相比，腺病毒载体具有广泛的宿主范围并且在人类中具有低致病性。这些载体可以在增殖和非增殖细胞中感染和表达靶基因，而不会整合到染色体中，不具有致突变性，可以同时表达多个基因;此外，它们可以以高滴度生产及包装，可以使转基因表达持续时间长，副作用小[192]。通过腺病毒将 NTN-1 转导入 ADSC 中;通过检测及比较我们发现，最佳感染复数（MOI）为 500，转染 ADSCs 的持续时间为 48 小时，以达到最大转染效率（图 2-2）。经过比较发现，在 ADSCs 细胞中，Netrin-1 和 GFP 之间的转染率没有显著差异（图

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2A，B）。Western Blot、PCR 及统计学分析证实 N-ADSCs 组中 Netrin-1 的表达显著升高，而值得注意的是，在 ADSCs 组的细胞中，Netrin-1 几乎没有表达（P <0.05）（图 2C-E），证明在普通状态的 ADSCs 中本身不表达 Netrin-1 这种分泌蛋白，因此，不需要设置沉默 Netrin-1mRNA 的 si-RNA-NTN-1 组作为空白对照。在此，我们成功建立了一个 NTN-1 基因被高效转染并在 ADSCs 中表达的 Netrin-1 过表达系统。

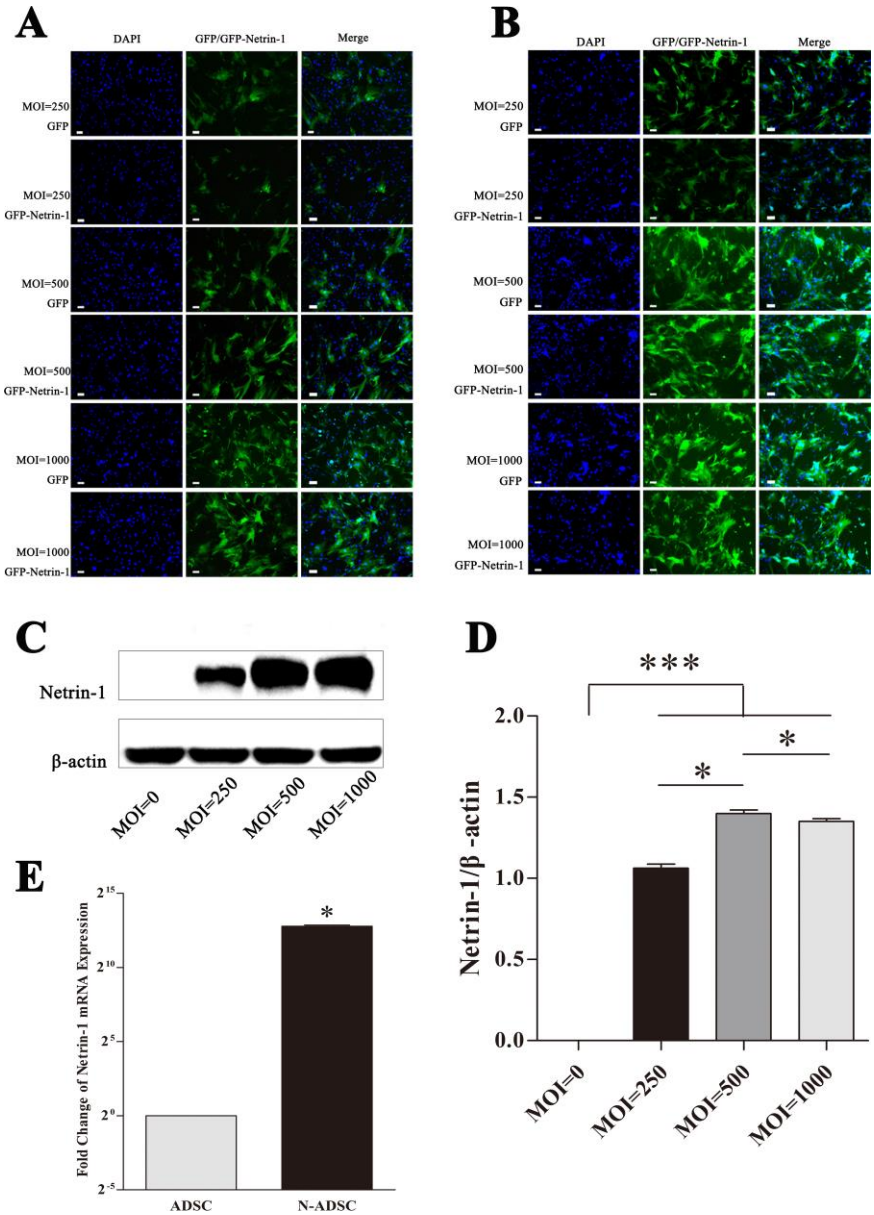

图 2-2：采用基因转染使 ADSCs 过表达 NTN-1。

Fig.2-2 Gene transfection was adopted to overexpress NTN-1 by ADSCs.

在 ADSCs 细胞中观察到 NTN-1 和 GFP 之间的转染率无显著差异（图 A，B）。Western Blot、PCR 和统计学分析证实，与 ADSCs 组中无 Netrin-1 表达相比，N-ADSCs 组中 Netrin-1 的表达显著升高（P <0.05）（图 C-E）。 比例尺=100μm。 * P <0.05。 ADSCs，脂肪来源的干细胞; GFP，绿色荧光蛋白; N-ADSCs，Netrin-1 转染的 ADSCs。

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3.3. Netrin-1 对高糖下 ADSCs 的增殖情况影响

在整个实验研究中，我们采用体外高糖培养基 DMEM(33.3 mmol/L 葡萄糖)配置的 ADSCs 培养液培养 ADSCs，以此模拟 T2DM 体内高血糖情况进行后续实验。从第 1 天到第 7 天，使用 CCK-8 测定法评估 ADSCs 和 N-ADSCs 的增殖情况及生长活力，以探索 Netrin-1 对细胞活力的影响（图 2）。3）。对结果进行统计学分析表明，在前 3 天内，ADSCs 和 N-ADSCs 的生长均相对稳定，差异不大。从第 3 天到第 7 天，两种细胞都表现出了对数生长的模式。与 ADSCs 相比，N-ADSCs 在第 3 天至第 7 天显示出显著更高的生长速率（P <0.05，n = 5）。 本研究证实了 Netrin-1 对 ADSCs 增殖的促进作用。

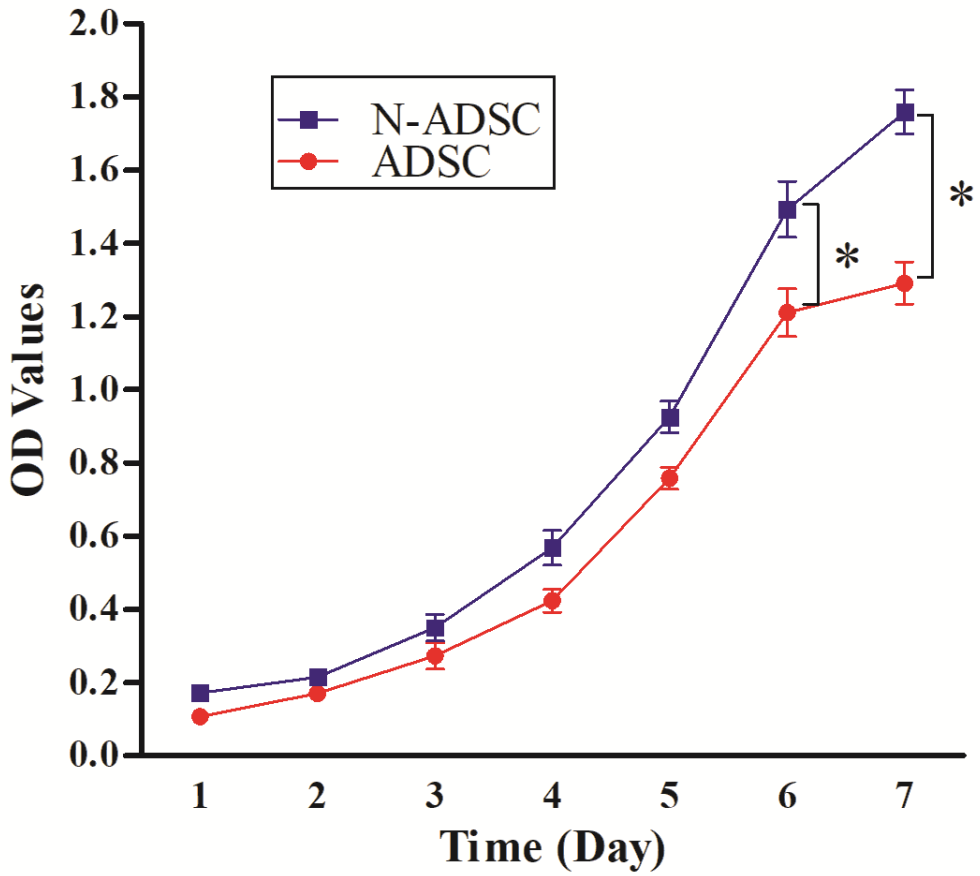

图 2-3：N-ADSCs 及 ADSCs 高糖环境的 CCK-8 生长曲线比较

Figure2-3. Comparison of CCK-8 growth curves of N-ADSCs and ADSCs under high glucose

从第 3 天开始，用 Netrin-1 转染的细胞表现出比未处理的阴性对照 ADSCs 更高的增殖率（P <0.05，n = 5）；* P <0.05。 ADSCs，脂肪来源的干细胞; OD，光密度; N-ADSCs，Netrin-1 转染的 ADSCs。

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3.4. Netrin-1 对高糖下 ADSCs 凋亡的影响

以往的研究发现，高血糖对 T2DM 患者 ADSCs 的增殖和凋亡有负面影响，T2DM 小鼠的 ADSCs 表现出低活力和高凋亡率[15-17，71-77]。为了探讨 Netrin-1 对高糖条件下 ADSCs 凋亡的影响，我们用 Annexin V / PI 染色细胞，并通过流式细胞仪进行分析。N-ADSCs 组的凋亡百分比为 7.7±0.44％，而 ADSCs 组的细胞凋亡率为 10.9±0.32％（图 2-4A，B），二者差异具有统计学意义（P<0.05）（图 2-4C）。 Bcl-2 蛋白代表着抑制凋亡，而 Bax 蛋白代表着促进细胞凋亡。本实验的 Western Blot 和统计分析表明，在高糖条件下，N-ADSCs 的 Bcl-2/Bax 比值显著高于 ADSCs，具有统计学意义（图 2-4D，E），表明 N-ADSCs 的凋亡率受到抑制，从而证明了 Netrin-1 体外对 ADSCs 的抗凋亡作用。综上所述，将 Netrin-1 转染到 ADSCs 中能够促进其在高糖环境下的增殖，同时显著抑制其凋亡。

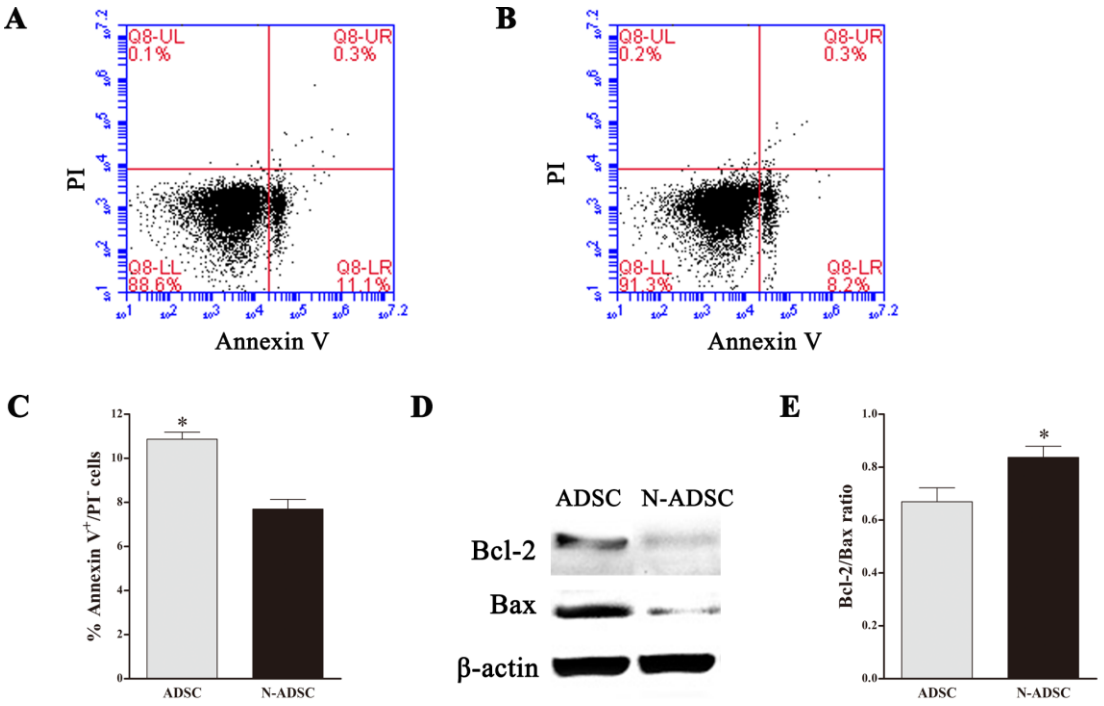

图 2-4：Netrin-1 对高糖条件下 ADSCs 凋亡的影响。

Fig.2-4. Effects of Netrin-1 on the apoptosis of ADSCs under high glucose.

A, B 通过流式细胞学分析在高糖下用或不用 Netrin-1 转染的 GFP-ADSCs 的细胞凋亡图。C 凋亡率统计分析表明 Netrin-1 的过表达显著降低了 ADSCs 的凋亡率，提高了 ADSCs 的活力。D，E Western Blot 和统计学分析显示，在高糖条件下，N-ADSCs 中 Bcl-2 / Bax 的比例显著高于 ADSCs; * P <0.05。ADSCs, 脂肪来源的干细胞; GFP 绿色荧光蛋白; N-ADSCs, Netrin-1 转染的 ADSCs

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3.5. Netrin-1 对高糖下 ADSCs 迁移的影响

研究发现，体内注射移植的 ADSCs 会逐渐迁移到组织受损区域，从而通过分化为特定种类的细胞以及旁分泌大量因子来发挥其修复作用。因此，如何提高 ADSCs 的迁移能力亦成为研究热点而受到广泛关注。本部分实验检测了 Netrin-1 过表达对高糖条件下 ADSCs 迁移能力的影响。划痕实验的结果表明，N-ADSCs 在 12 和 24 小时的迁移速度显著快于 ADSCs（相对间隙区域，P <0.05）（图 2-5A，B）。Transwell 试验（Corning Inc.）的结果表明，N-ADSCs 通过膜的迁移数量显著高于 ADSCs（ADSCs，231.67±9.50 个细胞/视野; N-ADSCs，375.67±6.51 个细胞/视野; P <0.05）（图 2-5A，C）。两个迁移实验更加强有力地证明，Netrin-1 的过表达显著提高了 ADSCs 在高糖条件下的迁移能力。

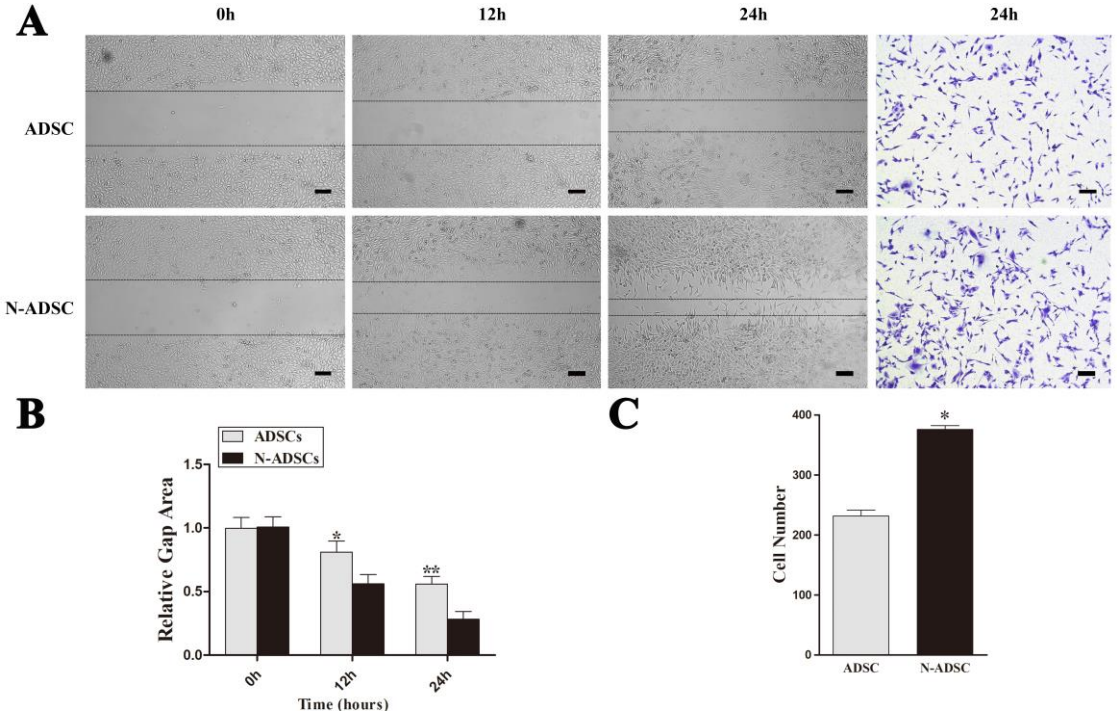

图 2-5：Netrin-1 对 ADSC 迁移的影响。

Fig2-5. Effects of Netrin-1 on the migration of ADSCs.

在高糖条件下培养 24 小时后划痕实验和 Transwell 实验的代表性图像（A）。划痕实验表明，在高糖条件下，N-ADSCs 组的相对间隙面积明显小于 ADSCs 组（B）。Transwell 实验显示，在高糖条件下，N-ADSCs 组中的细胞迁移的数量比 ADSCs 组中的多（C）。 * P <0.05，比例尺= 100mm。 ADSCs，脂肪来源的干细胞; N-ADSCs，Netrin-1 转染的 ADSCs。

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3.6. Netrin-1 对高糖下 ADSCs 粘附以及成管能力的影响

体内移植的 ADSCs 迁移进入受损组织部位后，其粘附能力的高低决定了细胞是否能长期留在受损部位，分化为血管内皮细胞等并旁分泌大量细胞因子，促进血管新生及组织修复。我们对 N-ADSCs 及 ADSCs 进行细胞粘附实验，PBS 清洗后附着于六孔板的细胞表现出圆形的外观，并且在荧光显微镜下可以观察到蓝色荧光和绿色荧光（图 2-6）。统计分析显示，N-ADSCs 组的细胞粘附的数量显著高于 ADSCs 组（ADSCs，20.67±2.08 个/视野; N-ADSCs，60.00±2.65 个/视野，P <0.05）（图 2-6A，B）。目前的结果表明，Netrin-1 的过表达显著提高了 ADSCs 在高糖环境下的粘附能力。而 ADSCs 的成管实验表明，高糖环境下 N-ADSCs 的毛细血管形成比 ADSCs 丰富，而且高糖培养基中 N-ADSCs 的累积成管长度大于 ADSCs（1.21 倍，P <0.05）（图 2-6C，D）。目前的结果表明，在 ADSCs 中 Netrin-1 的过表达显著提高了其高糖环境下促血管生成的能力。

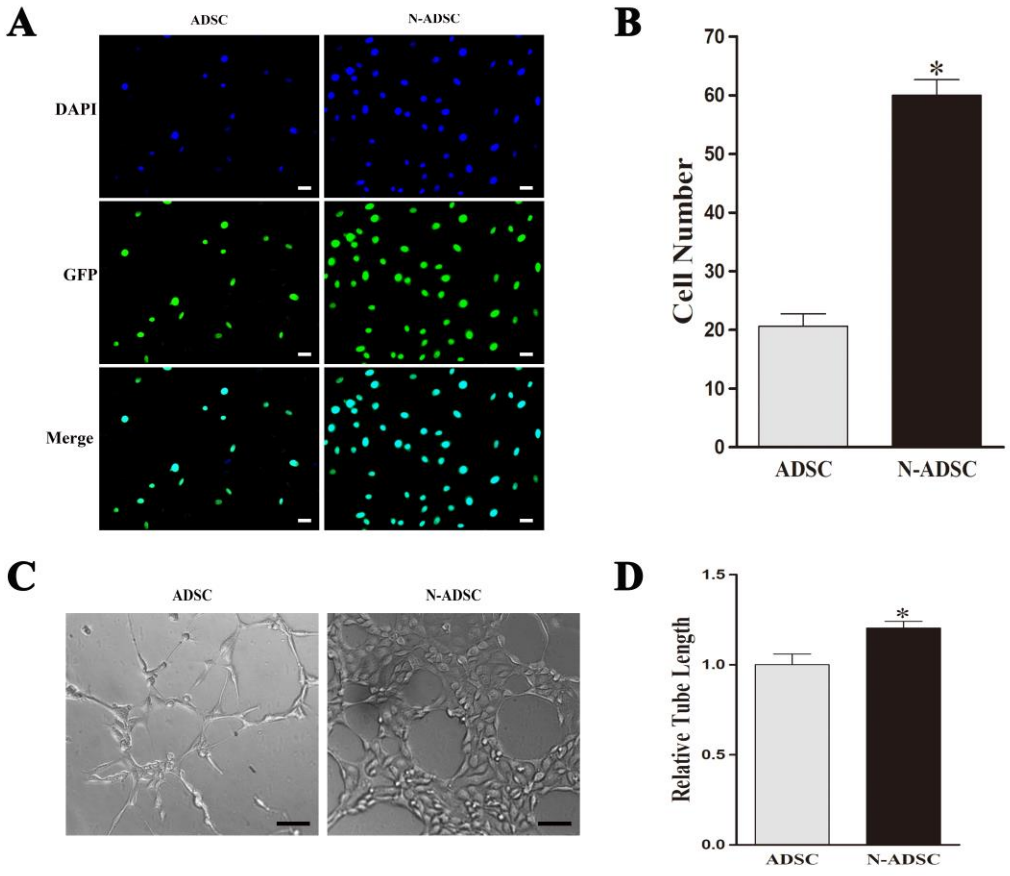

图 2-6：Netrin-1 对高糖条件下 ADSCs 粘附和促血管生成能力的影响。

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Fig.6. Effects of Netrin-1 on adhesion and the proangiogenic ability of ADSCs under high glucose.

与 ADSCs 对照组相比，在 N-ADSC 组中观察到大量附着细胞（A）。定量分析表明，Netrin-1 过表达有效地增强了 ADSCs 的粘附(B)。在高糖下接种到 Matrigel 胶上 12 小时的 N-ADSCs 和 ADSCs 的成管实验的代表性图像(C)。定量分析表明，N-ADSCs 的累积成管长度显著高于 ADSCs (C)。 * P <0.05，比例尺= 100 毫米。 ADSCs，脂肪来源的干细胞; N-ADSCs，Netrin-1 转染的 ADSCs；DAPI，4',6-二脒基-2-苯基吲哚。

4. 讨论

ADSCs 以其易获取、产量高、分化能力强、免疫原性低等优点，日益成为应用于基础实验及临床研究中热门的种子细胞，并有望应用于发病率高、出现早、起病隐匿、病程长、难以治愈的 DPNV 中。其移植后促进组织再生的治疗作用得到了大量基础及临床试验的证实，但一大亟待解决的难题就是其移植后存活量极低。Gyongyosi M 等人通过基于导管的经心内膜注射递送的 MSCs 的正电子发射断层扫描（PET）跟踪显示，移植后 10 天，在猪缺血心肌中只存活了大约 6%的 MSCs[188]。Toma 等人报道，在免疫缺陷小鼠心脏移植后第 4 天，存活的 MSCs 不超过 0.44%[189]。同样的，在实验性 MI 的大鼠心脏移植后 24 小时只能检测到大约 1%的 MSCs[190,191]。更多研究发现，移植后糖尿病患者下肢缺血症状及客观指标得到一定程度的改善，但其远期疗效仍然欠佳[36,64-70]。大量研究亦证实，糖尿病周围血管损伤部位存在高级糖基化终产物、产生促炎微环境和诱导氧化应激，使得移植干细胞在靶血管损伤部位及高糖环境下的存活、迁移及向新生血管分化的能力显著减低，是影响干细胞疗效的关键所在[15-17，71-77]。因此，本身移植后的存活率低的 ADSCs 在糖尿病微环境中存活率进一步降低，使 ADSCs 的治疗作用大打折扣。因此，如何促进 ADSCs 在糖尿病周围血管损伤部位的存活、分化及促进失神经支配后糖尿病血管的新生，对防治 DPNV 病变的发生、发展具有重要意义。

研究发现，DPNV 是一种混合性病变，伴有血管，神经和组织损伤[28,78-80]。而体内血管和神经往往遵循相同的轨迹到达组织器官，这在一定程度上证实了血

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管与神经相互调节的关系。而很多最早发现神经或血管内的蛋白，也逐渐被发现在另一系统中发挥重要的作用。Netrin-1 是第一个确定的神经轴突导向因子，Netrin-1 和 G-netrin 与层粘连蛋白 γ 链具有同源性，该肽由一个氨基末端区域 VI 的近 600 个残基组成，连接着重复的 3 个层粘连型表皮生长因子(V-1、V-2、V-3)和一个羧基末端区域[89]。另一项研究发现，Netrin-1 不仅参与神经系统的功能活动，还参与血管系统的功能活动。Ding 等人证明 Netrin-1 不仅促进中枢神经系统的神经元迁移和分泌，而且还调节非神经组织中内皮细胞和干细胞的存活，粘附，迁移，增殖和分化，并抑制他们的细胞凋亡[90,91]。Wilson 等人系统地研究斑马鱼和哺乳动物，证实 Netrins 可以诱导血管生成。Netrin-1 通过与 UNC5H 受体结合激活 Src/FAK/paxillin 相关信号通路，促进血管内皮细胞粘附，迁移和增殖，形成新的毛细血管网络，该功能可以通过抑制斑马鱼 NTN-1 mRNA 而被阻断[92]。Lu 等人还发现 Netrins 可刺激哺乳动物的血管生成并加速缺血组织的血管生成。该过程依赖于 Netrin-1 受体 DCC 来调节 ERK/eNOS 信号通路[93]。Brunet 等人证明 Netrin-1，Netrin-4 和 VEGF 促进血管生成，但 Netrin-1 在促进内皮细胞分化和神经损伤恢复的双重作用方面具有更大的优势[94]。此外，Netrin-1 不但参与神经生长和血管生成;它还可以增强人体血管和淋巴系统不同阶段内皮细胞的有丝分裂，迁移和粘附[95]。Netrin-1 除了参与到血管新生、内皮细胞的存活，粘附，迁移，增殖和凋亡，近年来，Netrin-1 在多种炎症性疾病中的诊断及治疗价值不断被挖掘出来。研究发现，Netrin-1 能够抑制炎症，保护正常细胞[131-135]。许多急慢性炎症疾病包括糖尿病及糖尿病并发症中，都伴随有 Netrin-1 表达的上升或下降[96-105]。这可能与机体内 Netrin-1 抑制炎症细胞迁移，抑制炎症细胞因子和趋化因子的产生，通过抑制 NF-κB 活化调控 COX-2 的表达，促进巨噬细胞向抗炎的 M2 样表型分化，以及通过 MAPKs、ERKs、p38 等信号转导途径调节炎症反应等机制相关[136-137]。同时，Netrin-1 被报道能够降低心肌缺血再灌注后的炎症反应，抑制神经细胞及血管内皮细胞的氧化应激与炎症，抗细胞凋亡[100-107]。但我们前期实验通过对临床糖尿病与非糖尿病患者的下肢缺血肌肉组织以及外周血血清，通过免疫组化、免疫荧光、Western Blot、ELISA 等分析发现，糖尿病患者下肢缺血组织中 Netrin-1 存在与内皮细胞的共定位情况，而 Netrin-1 表达水平显著降低，炎症因子的表达水平明显上调、小血管

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密度显著下降，数据统计分析表明糖尿病高血糖环境下 Netrin-1 的表达水平与 DPNV 呈负相关。因此，Netrin-1 可能是 DPNV 中重要的生物标志物，提高其表达水平或许能够减缓 DPNV 的病情恶化。

因此，我们认为通过基因转染 ADSCs 过表达 Netrin-1 可以改善 ADSCs 在高血糖条件下的活力、迁移和向血管内皮细胞分化，促进 DPNV 血管新生。本研究成功获取 C57/BL 小鼠脂肪组织，分离、培养并扩增 ADSCs，并首先在体外进行流式细胞学鉴定，发现 P6 代细胞形态变异，老化严重。因此，后续实验采用 P3 至 P5 代 ADSCs。接下来，我们应用腺病毒转染的方式，建立基因转染体系使 ADSCs 过表达绿色荧光蛋白（GFP）及 Netrin-1（N-ADSCs），因为腺病毒所携带的外源基因未插入细胞基因组中，因此其在基础及科研应用中是一种比慢病毒更优越且更安全的方法[193]。通过实验我们发现最佳感染复数（MOI）为 500，转染 ADSCs 的持续时间为 48 小时，为后续此类实验提供了参考依据。值得注意的是，正常的小鼠 ADSCs 本身并不表达 Netrin-1 蛋白，这提示我们不用设置 si-RNA 沉默组同时，也更加凸显了转染外源基因 NTN-1 入 ADSC 的意义。接下来我们通过在高糖环境下的 CCK-8、Western Blot、流式、Transwell、免疫荧光等方法对比 N-ADSCs 与 ADSCs，发现在高糖环境下 N-ADSCs 的增殖、迁移、粘附、成管能力均显著高于 ADSCs，并且 N-ADSCs 的凋亡率显著低于 ADSCs。我们的实验证明了体外过表达 Netrin-1 能够大大提高 ADSCs 的存活、迁移、粘附、成管能力，并且抵抗 ADSCs 细胞在高糖环境下的细胞凋亡。其具体分子机制尚不明确，有待我们进一步探索与发现，同时为后续体内将 ADSCs 移植入糖尿病下肢失神经小鼠模型，探索其体内修复治疗 DPNV 的效果及探讨具体分子机制提供了实验基础。

5. 小结

本研究在前期研究基础上，获取 C57/BL 小鼠脂肪组织，分离并培养 ADSCs，通过腺病毒转染的方法建立基因转染体系使 ADSCs 过表达绿色荧光蛋白（GFP）及 Netrin-1（N-ADSCs）。通过 CCK-8、Western Blot、流式、Transwell、免疫荧光等方法检测 N-ADSCs 与 ADSCs 在高糖环境下的增殖、迁移、粘附、向内皮

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细胞分化等能力之间的差异，证实体外环境中 Netrin-1 能够提高高糖环境下 ADSCs 的增殖、迁移、粘附、向内皮细胞分化的能力，显著降低 ADSCs 的凋亡水平。为后续体内将 ADSCs 移植入糖尿病下肢失神经小鼠模型，探索其体内修复治疗 DPNV 的效果及探讨具体分子机制提供了实验基础。

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第三章 Netrin-1 调控 ADSCs 修复 DPNV 的研究

1. 引言

糖尿病周围神经血管病变（DPNV）是血管与神经以及周围组织损伤的混合性病变，导致起病隐匿，病程长、病情迁延不愈，最终往往以糖尿病足的溃疡及下肢远端缺血坏死告终。据统计，糖尿病患者一生中罹患足部溃疡的风险可能高达 25%。糖尿病患者下肢截肢率是非糖尿病患者的 15 倍，1 型或 2 型糖尿病患者足部溃疡的年发病率为 1.9%至 2.2%[110-112]，发展为糖尿病足溃疡的患者，轻者截趾，重者截肢，更有甚者，由于肢端坏死组织的毒素大量吸收，导致肝肾等全身多器官功能衰竭而死亡[113-115]。临床上虽然有药物治疗、血管重建术、经皮腔内血管成形术（球囊扩张与支架植入）等治疗方法，但存在单纯血糖控制无法抑制 DPNV 进展、重建血管内血栓形成、支架内再闭塞等问题，远期疗效并不理想[116-119]。因此，DPNV 为我国医疗卫生系统带来了巨大的负担。对于此类无有效临床治愈方法的疾病，干细胞移植是一种极有希望的治疗方式，T2DM 的患者身体尤其是腹部富含脂肪组织，通过便宜安全的脂肪抽吸术可以获得大量脂肪组织，通过体外培养而得的 ADSCs 具有多向分化，抵抗炎症，修复组织的优秀特性。但体内移植后存活率低是影响其疗效的关键。尤其是糖尿病患者体内持续高血糖导致的高级糖基化终产物、产生促炎微环境和诱导氧化应激等不良环境进一步降低了 ADSCs 的存活率，使其无法发挥应有的功能。而 Netrin-1 作为一种同时在神经和血管中发挥促进神经细胞和内皮细胞生长、存活、粘附、迁移、增殖和抗凋亡的分泌蛋白，亦可通过 MAPKs、ERKs、p38 等信号转导途径调节炎症反应发挥抗炎作用[136-137]，最近被发现在许多急慢性炎症疾病中，都有其表达水平的显著上升或下降[96-105]。前期我们通过对临床标本的分析发现其在糖尿病高血糖环境下的表达水平与 DPNV 呈负相关，证明了 Netrin-1 与 DPNV 的临床相关性。同时后续体外实验大量获取 ADSCs 后，建立了腺病毒转染 ADSCs 的稳定基因转染体系，随后证实体外环境中 Netrin-1 能够显著提高高糖环境下 ADSCs 的增殖、迁移、粘附、向内皮细胞分化的能力，显著降低 ADSCs 的凋亡水平。为 Netrin-1 提高 ADSCs 体内移植但其体内移植入糖尿病下肢失神经小鼠模型，体内修复治疗 DPNV 的效果以及具体的分子机制尚不明确。

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因此在本研究之中，我们构建 T2DM 小鼠（db/db）下肢失神经模型，体内移植 N-ADSCs 与 ADSCs，并应用激光多普勒观察血流灌注，通过免疫荧光和免疫组化，评估 ADSCs 在体内存活、迁移、分化和促血管新生的效率。应用 Western Blot 探讨 Netrin-1 介导的 ADSCs 增殖，迁移，粘附，分化，促血管生成能力和细胞凋亡的信号通路，应用 ELISA 检测 Netrin-1 介导的 ADSCs 旁分泌多种因子。明确 Netirn-1 调控 ADSCs 增殖、迁移以及治疗糖尿病周围血管神经病变的具体分子机制，为 DPNV 的防治提供新思路和理论依据。

2. 材料和方法

2.1. 材料

2.1.1. 主要试剂

PBS 磷酸缓冲液（1x，PH 7.2-7.4）（北京索莱宝科技有限公司，中国）

胰酶-EDTA（Trypsin-EDTA，0.25%，北京博奥龙免疫技术有限公司，中国）

DMEM 溶液：

1g/L Dulbecco’s modified eagle medium (DMEM; Gibco, USA)

10% fetal bovine serum (FBS, Hyclone, Australia)

NB4 胶原酶溶液：

0.2%胶原酶 NB4 (Serva, Heidelberg, Germany)

DMEM 溶液配制

TBS 缓冲液（伯乐生命医学产品（上海）有限公司，中国）

PBS 磷酸缓冲液（1x，PH 7.2-7.4）（北京索莱宝科技有限公司，中国）

TBST 缓冲液（1x）（北京索莱宝科技有限公司，中国）

考马斯亮蓝溶液（生工生物工程（上海）股份有限公司，中国）：

牛血清白蛋白（BSA，Sigma，USA）

4%多聚甲醛（上海晶都生物技术有限公司，中国）：

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甲醇（上海代轩生物科技有限公司，中国）

95%乙醇（上海交通大学医学院附属第九人民医院）

75%乙醇（（上海交通大学医学院附属第九人民医院）

异丙醇(Sigma, USA) （上海代轩生物科技有限公司，中国）

氯仿(Sigma, USA)

DAPI（西安赫特生物科技有限公司，中国）

树脂（Epon, Merck, Darmstadt, Germany）

OCT 包埋剂（DAKO，USA）

荧光封片剂（DAKO，USA）

Matrigel 凝胶（Sigma，USA）

胶原 Collagen II (Abcam, Cambridge, MA)

草酸铵结晶紫染色液（结晶紫染色液）(1%)(北京索莱宝科技有限公司，中国)

兔抗 CD31 多克隆抗体（Abcam，UK）

Fluor 555-羊抗兔 IgG 多克隆抗体（Invitrogen, Carlsbad, CA）

DAPI（西安赫特生物科技有限公司，中国）

ELISA 试剂盒（上海一研生物科技有限公司，中国）

DAPI（Invitrogen, Carlsbad, Canda）

树脂（Epon, Merck, Darmstadt, Germany）

荧光封片剂（DAKO，USA）

戊巴比妥钠（上海交通大学医学院附属第九人民医院，中国）

2.1.2 主要仪器与设备

恒温 CO_2 培养箱 （Forma Scientific，USA）

离心机（Thermo，USA）

去离子水系统（Millipore，USA）

超净工作台（江苏苏净集团，中国）

倒置显微镜（重庆光电仪器总公司，中国）

荧光显微镜 （Nikon，Japan）

精密天平（Mettler Toledo，Switzerland）

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天平（上海舜宇恒平科学仪器有限公司，中国）

冰箱（海尔，中国）

6 孔板（Corning Inc.，USA）

培养皿（Corning Inc.，USA）

50ml 离心管（Corning Inc.，USA）

15ml 离心管（Corning Inc.，USA）

96 孔板（无锡耐思生物科技有限公司）

移液管（无锡耐思生物科技有限公司）

0.22 μm 针头滤器（Millipore，USA）

40 μm 滤网（BD Falcon， USA）

显微手术器械（上海交通大学医学院附属第九人民医院，中国）

流式细胞仪（Beckman Coulter, Fullerton, Canada）

Image-Pro Plus 软件（Media Cybernetics Inc, Rockville, MD）

低温循环水浴 Polystat（Cole-Parmer Instrument Comapny,USA）

电热恒温水浴锅（上海一恒科技有限公司）

电泳仪（上海天能科技有限公司）

电子天平（北京赛多利斯仪器系统有限公司，中国）

MINI 离心机（Tomy Seiko Co., Ltd. Japan）

垂直洁净工作台（沪净净化）

冷冻离心机（Eppendorf，Germany）

全温振荡培养箱（上海一恒科技有限公司限公司，中国）

台式多功能高速冷冻离心机（Eppendorf，Germany）

高压灭菌锅（上海申安医疗器械厂，中国）

高速冷冻离心机（Thermo Fisher Scientific，USA）

凝胶成像系统（上海天能科技有限公司，中国）

激光多普勒灌注成像仪（moor-FLPI; Moor Instruments，Devon，U.K）

Odyssey 红外成像系统（LI-COR，Lincoln，NE，USA）

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2.1.3. 实验动物

野生型（WT）C57 / BL 小鼠和 2 型糖尿病小鼠（BKS.Cg-m + / + Leprdb）购自中国上海南方模式生物研究中心。所有动物实验均经上海交通大学医学院附属第九人民医院动物伦理委员会批准。糖尿病和高血糖小鼠的血糖水平定义为≥ 16.67 mmol/L，只有这些小鼠用于随后的体内研究。

2.2. 方法

2.2.1. T2DM 小鼠下肢失神经模型的构建与 ADSCs 的移植

所有动物实验均经上海交通大学医学院附属第九人民医院动物伦理委员会批准。将 SPF 级雄性 T2DM 小鼠（BKS.Cg-m +/+ $Lepr^{db}$）（18-20 周龄大小，n = 18;中国上海南方模式生物研究中心）随机分为 3 组。随后，使用戊巴比妥钠（0.5 mg/g）成功麻醉小鼠。将每只小鼠的左后肢全部剃毛。随后在直视下分离坐骨神经，按如前所述的方法，去除一段长 1.0cm 的神经[194,195]。随后用 5-0 尼龙缝合线缝合肌肉，用 3-0 尼龙缝合线缝合皮肤。成功构建 T2DM 小鼠下肢失神经模型。24 小时后，在失神经支配的后肢上的三个不同部位（腓肠肌，股薄肌和股四头肌）注射 100μL 含有 10^5 个细胞的无血清培养基。将小鼠随机分成三组：ADSCs 组（n = 6），N-ADSC 组（n = 6）和接受 PBS 注射的对照组（n = 6）。在移植后第 7,14 和 28 天，应用激光多普勒灌注成像仪（moorFLPI; Moor Instruments，Devon，UK）非侵入性地评估后肢血流灌注情况。

2.2.2.免疫荧光和免疫组织化学染色检测

术后第 7、14、28 天观察各组实验动物的整体情况。在术后第 28 天时，使用戊巴比妥钠（0.5 mg/g）成功麻醉小鼠后，使用 4%多聚甲醛溶液灌注固定小鼠，解剖小鼠，分离并获取双侧后肢肌肉，10%蔗糖脱水 1.5-2h 后组织转入 30%蔗糖浸泡过夜后 OCT 包埋冰冻切片。沿肌肉横轴，以 10μm 厚度连续切片。切片在避光通风处室温下干燥过夜，次日将切片装载入切片盒密封保存于-80℃冰箱内。

2.2.2.1 肌肉组织的免疫荧光检测

（1）室温中应用 0.3% Triton-X 进行 10min 破膜，然后用 PBS 进行 3 次漂

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洗标本，每次持续约 5min；

（2）应用 10%山羊血清于 37℃温度下封闭标本 30min；

（3）按说明书所示，依次加入兔抗 CD31 抗体（1：200 稀释）与小鼠抗 Netrin-1 抗体（1：250 稀释），对照组加入 PBS，均置于 4℃下进行过夜后，用 PBS 漂洗标本 3 次，每次持续约 5min；

（4）依次向标本加入 Fluor 555 羊抗兔 IgG 抗体及 FITC 羊抗小鼠 IgG 抗体（1:500 稀释），注意放置于黑盒中避光，于 37℃下放置约 60min，孵育完成后，用 PBS 漂洗标本 3 次，每次持续约 5min；

（5）用 DAPI（1:1000 稀释）进行细胞核染色，孵育 2min 后用 PBS 漂洗标本，约持续 5min；

（6）用荧光封片剂进行封片，标本荧光片制作完成，然后在荧光显微镜下观察记录。

2.2.2.2 肌肉的免疫组织化学染色

（1）将冰冻切片置入 1mM EDTA 中，于 95℃下加热 10 分钟，于室温下冷却至常温，进行抗原修复；

（2）使用适当的固定液固定细胞或切片，用免疫染色洗涤液洗涤 2 次，每次约持续 5min；

（3）加入免疫染色封闭液，封闭约持续 60min；

（4）按一抗说明书，适当比例稀释一抗。回收封闭液后加入稀释好的一抗，于室温下孵育 1 小时，回收一抗。用 PBS 漂洗标本 3 次，每次持续约 5min；

（5）按二抗说明书，适当比例稀释二抗。回收封闭液后加入稀释好的二抗，于室温下孵育 1 小时，回收二抗。用 PBS 漂洗标本 3 次，每次持续约 5min；

（6）用封片剂封片，标本免疫组化片制作完成，然后光学显微镜下观察记录。

2.2.3. Western Blot 检测 Netrin-1 调控 ADSCs 的信号通路

从在体外高糖条件下培养 48h 的 N-ADSCs 和 ADSCs 中分离获得总蛋白，从分别注射 N-ADSCs、ADSCs 和 PBS 的 T2DM 下肢失神经小鼠的肌肉组织中分离获得总蛋白。对组织样品及细胞裂解物中的总蛋白进行定量，电泳，并转移

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到 PVDF 膜上，用适当的抗体在 4℃下孵育过夜，抗体如下：抗 Akt 和抗 P-AKT 抗体，抗 PI3K 和抗 P-PI3K 抗体，抗 P38 和抗 P-P38 抗体，抗 eNOS 和抗 P-eNOS 抗体，抗 NF-κB 和抗 P-NF-κB 抗体，抗 JNK 抗体，抗 ERK 1/2 抗体和抗 β-actin 抗体（1：500; Abcam）。Odyssey 红外成像系统（LI-COR，Lincoln，NE，USA）用于定量免疫反应条带的相对累积密度。具体实验步骤已在前文部分中详细描述。

2.2.4. ELISA 检测 Netrin-1 调控 ADSCs 的旁分泌因子变化

按照制造商的说明，将 ADSCs 和 N-ADSCs 在高糖培养基中培养 48 小时，离心获取上清液，通过 ELISA 试剂盒检测上清液中血管内皮生长因子（VEGF），碱性成纤维细胞生长因子（b-FGF），肝细胞生长因子（HGF），肿瘤坏死因子-α（TNF-α），血小板衍生生长因子（PDGF），表皮生长因子（EGF）以及胰岛素样生长因子（IGF-1）和 Netrin-1（R＆D Systems Inc）。用高糖 DMEM 培养基用作对照组。每个实验至少在三个孔中重复三次以上。

2.2.5. 统计学方法

本研究中所有数据的统计及分析均使用 SPSS 18 软件，所有统计学图及表格的绘制均使用 GraphPad Prism 5 软件，所有数据以均值及标准差（SD）表示。采用学生 t 检验和单因素方差分析对定量值进行比较分析，统计学意义定义为*P < 0.05，**p＜0.01；***p＜0.001。每个实验至少重复三次以上。

3. 结果

3.1. N-ADSCs 对下肢失神经 T2DM 小鼠后肢血液灌注的影响

先前的研究表明，坐骨神经失神经会导致血管床重塑和毛细血管逐渐丧失，并损害动脉发生和缺血后的恢复，而这加速了 T2DM 小鼠中 DPVN 的进一步恶化[194,195]。在本研究中，我们观察到 Netrin-1 调控 ADSCs 对后肢慢性缺血的坐骨神经去除的 T2DM 小鼠的作用。我们在 ADSCs 与 N-ADSCs 细胞移植入

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T2DM 小鼠下肢失神经模型后，在第 0,7,14 和 28 天通过彩色激光多普勒对后肢血流的状况进行成像检测（图 3-1A）。N-ADSCs 和 ADSCs 组均显示出比 PBS 对照组显著更高的激光多普勒灌注指数。此外，N-ADSCs 组在 28 天时显示出比 ADSCs 组显着更高的激光多普勒灌注指数（ADSCs 组，0.58±0.03; N-ADSCs 组，0.83±0.03; PBS 组，0.26±0.02; P <0.05）。图 3-2）。

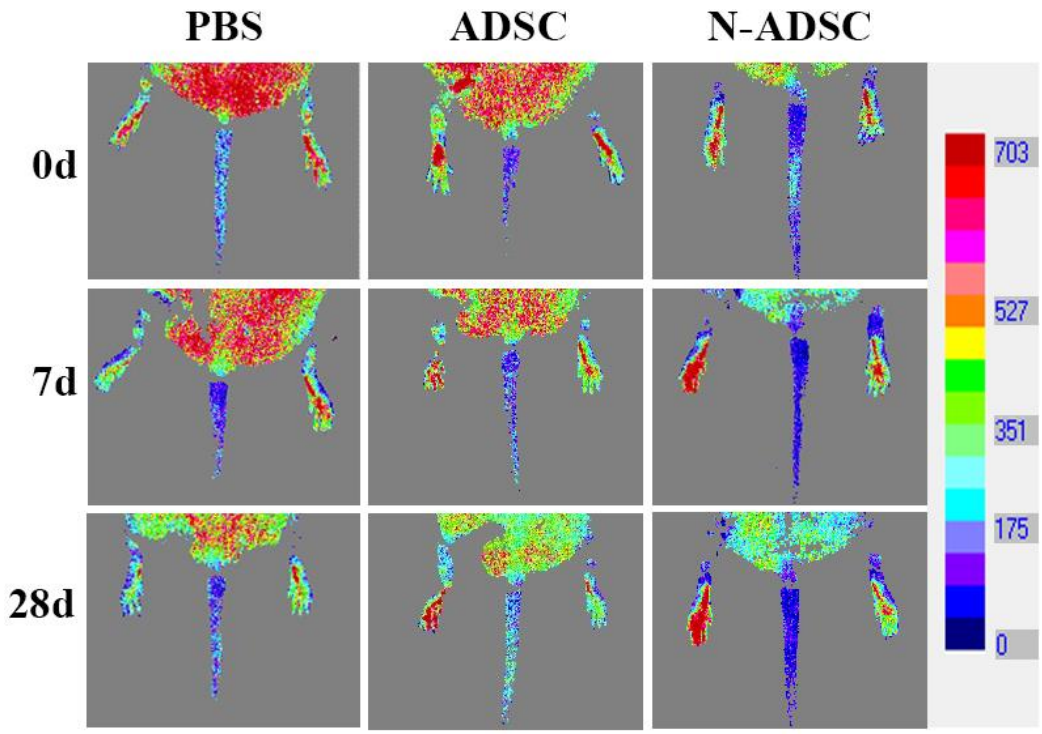

图 3-1 在移植后第 0,7 和 28 天获得代表性的彩色激光多普勒图像，显示失神经后肢的浅表血流。

Figure3-1 Representative color laser Doppler images of the superficial blood flow in the denervated hindlimbs were acquired on days 0, 7, and 28 post-transplantation.

ADSCs，脂肪来源的干细胞; N-ADSCs，Netrin-1 转染的 ADSCs，PBS，磷酸缓冲盐溶液。

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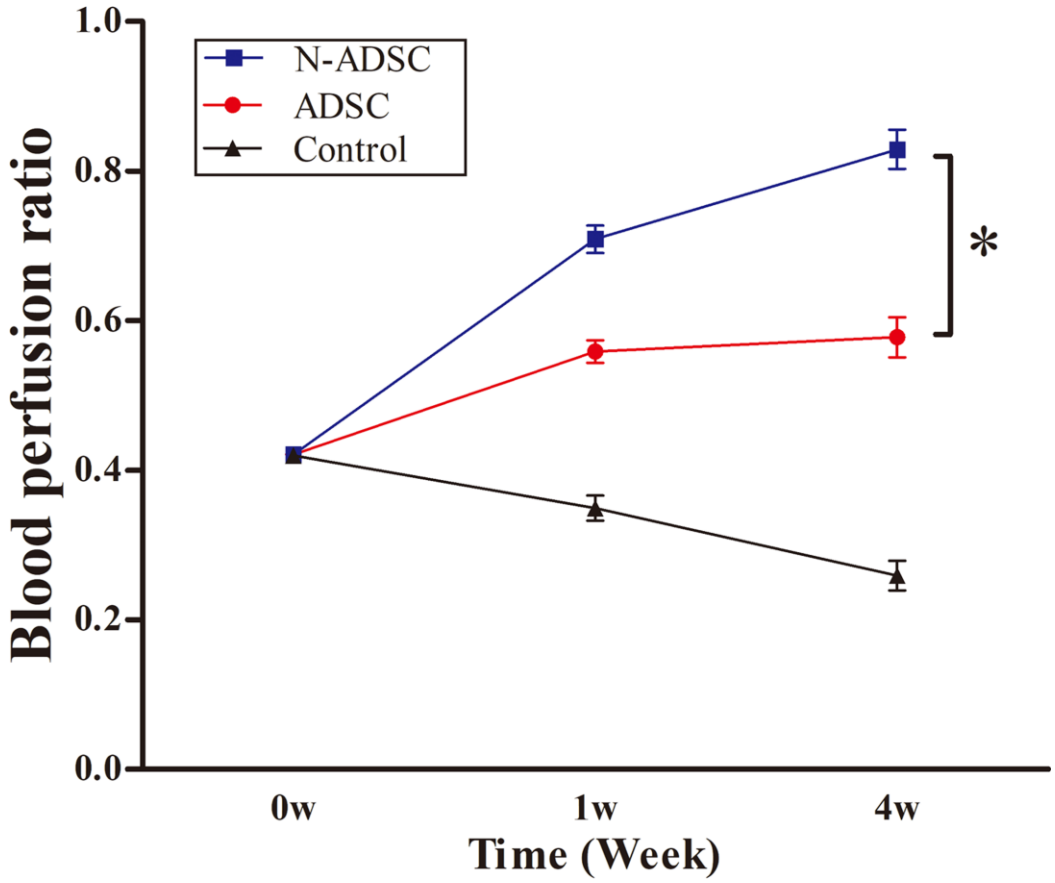

图 3-2 4 周时，与 ADSCs 和 PBS 对照组相比，N-ADSCs 组的血液灌注指数显著升高。

Figure3-2 The blood perfusion index was significantly higher in the N-ADSCs group as compared to that in the ADSCs and PBS control groups over 4 weeks.

ADSCs，脂肪来源的干细胞; N-ADSCs，Netrin-1 转染的 ADSCs，PBS，磷酸缓冲盐溶液。

*P < 0.05。

3.2. N-ADSCs 对下肢失神经 T2DM 小鼠后肢肌肉免疫组化及免疫荧光的影响

本研究发现，在注射 ADSCs 及 N-ADSCs 细胞 28 天后，在失神经支配的缺血肌肉或其他器官中没有形成肿瘤或畸胎瘤。我们通过免疫荧光显微镜在注射 ADSCs 及 N-ADSCs 的小鼠后肢肌肉中检测到事先用 GFP 标记的细胞。CD31（红色），DAPI（蓝色）双染色和转染到细胞中的 GFP（绿色）证实了细胞向慢性缺

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血部位和血管区域受损的迁移，其中一些最终分化成血管内皮细胞（图 3-3）。本研究的结果证明了 N-ADSCs 参与受损血管结构的恢复和血运重建。统计分析表明，N-ADSCs 组存活细胞数明显高于 ADSCs 和 PBS 对照组（ADSCs 组，22.67±4.16 个细胞/视野; N-ADSCs 组，68.33±4.04 个细胞/视野，$P < 0.01$）（图 3-4）。此外，免疫组织化学分析显示，与 ADSCs 和 PBS 组相比，N-ADSCs 显著增加了 T2DM 小鼠失神经后肢的微血管密度（PBS 组，2.50±1.91 个血管/视野; ADSCs 组，4.25±1.71 个血管/视野; N-ADSCs 组，14.25±1.89 个血管/视野，$P < 0.05$）（图 3-5，图 3-6）。证明 Netrin-1 提高了 ADSCs 血管新生的能力。本研究的结果证明了 Netrin-1 在 ADSCs 的体内存活，迁移和分化为内皮细胞中起到了关键的作用。

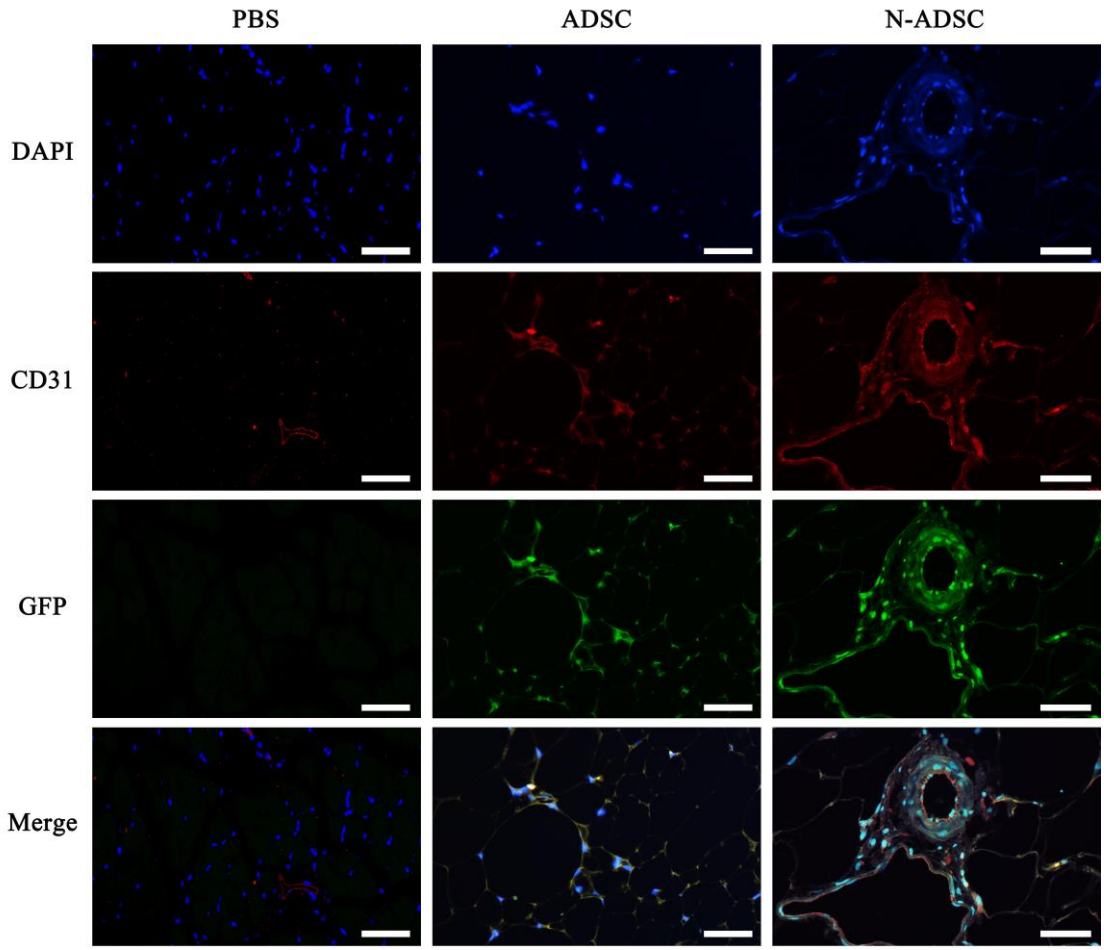

图 3-3 PBS，ADSCs 和 N-ADSCs 组中 CD31（红色），ADSCs（绿色）和具有 DAPI（蓝色）的细胞核的代表性荧光染色。

Figure3-3 Representative fluorescent staining for CD31 (red),ADSCs (green), and nuclei with

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DAPI (blue) in the PBS, ADSC, and N-ADSC groups.

比例尺=100μm。ADSCs，脂肪来源的干细胞；PBS，磷酸盐缓冲溶液；N-ADSCs，Netrin-1 转染的 ADSCs。

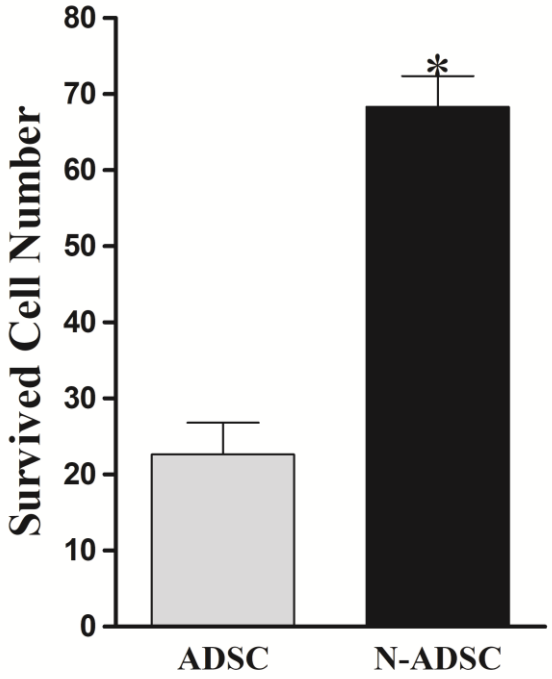

图 3-5 统计分析表明，N-ADSCs 组中存活的 ADSCs 显著高于 ADSCs 组（P <0.05）。

Figure3-5.Statistical analysis indicated that the survived ADSCs were significantly higher in N-ADSCs than in ADSCs (P < 0.05).

* P <0.05。ADSCs，脂肪来源的干细胞; PBS，磷酸盐缓冲溶液; N-ADSCs，Netrin-1 转染的 ADSCs。

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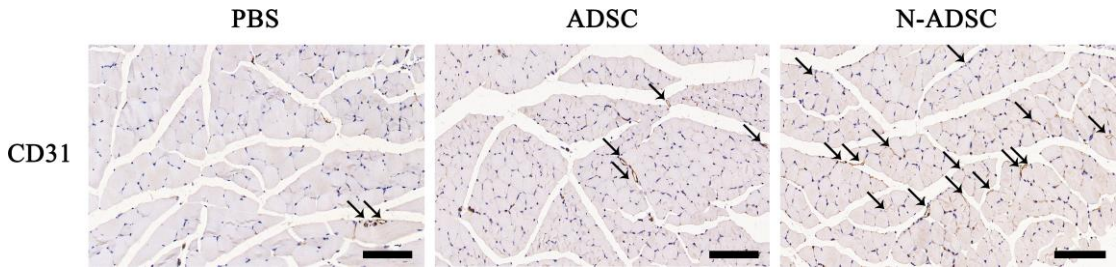

图 3-6 PBS 组，ADSCs 组和 N-ADSCs 组中 CD31 的代表性免疫组织化学染色。

Figure3-6. Representative immunohistochemical staining for CD31 in the PBS, ADSCs, and N-ADSCs groups.

比例尺=100μm。ADSCs，脂肪来源的干细胞；PBS，磷酸盐缓冲溶液；N-ADSCs，Netrin-1 转染的 ADSCs。

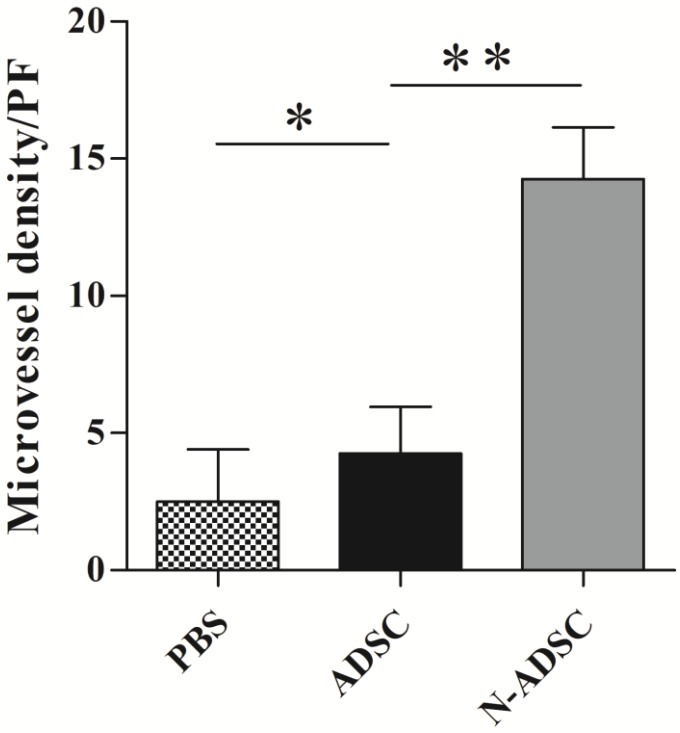

图 3-6 定量分析显示，N-ADSCs 组的微血管密度显著高于 ADSCs 组和 PBS 对照组（P <0.01）

Figure3-6 Quantitative analysis revealed that microvessel densities were significantly higher in the N-ADSCs than in the ADSCs and PBS control groups (P<0.01).

* P <0.05, ** P <0.01。ADSCs，脂肪来源的干细胞；PBS，磷酸盐缓冲溶液；N-ADSCs，Netrin-1 转染的 ADSCs。

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3.3. **Netrin-1 调控 ADSCs 存活，增殖，迁移，粘附和血管生成的分子机制**

Western Blot 证明，无论是在体外还是体内，与 ADSCs 组相比，N-ADSCs 组中的 PI3K / AKT / eNOS / P-38 / NF-κB 信号通路的磷酸化均高度上调（图 3-7A，3-8A）。统计分析表明，在体外和体内，N-ADSCs 中的 PI3K / AKT / eNOS / P-38 / NF-κB 信号传导途径的表达均显著高于 ADSCs 组（P＜0.05，图 3-7B，3-8B）。另一方面，与 ADSCs 组相比，ERK1/2 和 JNK 的表达在 N-ADSCs 中没有上调，由于 ERK1/2、JNK 及 P-38 三者均是 MAPK 重要信号通路的关键上游蛋白，因此我们推测在 Netrin-1 对 ADSCs 的调控中，MAPK 信号通路可能只通过 P-38-MAPK，但是没有 ERK1/2-MAPK 或 JNK-MAPK 信号通路的激活。众所周知，PI3K / AKT / eNOS 的激活对 ADSCs 的增殖，分化和抗凋亡至关重要[196]。此外，文献报道 Netrin-1 可以通过 PI3K / AKT / eNOS 信号通路在高糖状态下恢复血管内皮细胞的细胞损伤和促进血管新生[197]。此外，P38-MAPK 被证实与细胞存活，分化和迁移有关[198]。而 NF-κB 亦具有抗细胞凋亡的作用，并且能够上调 VEGF 的表达，这有利于血管新生。本研究的发现证明了 Netrin-1 在体外和体内调节 ADSCs 的存活，增殖，迁移，粘附和血管生成中的重要功能。

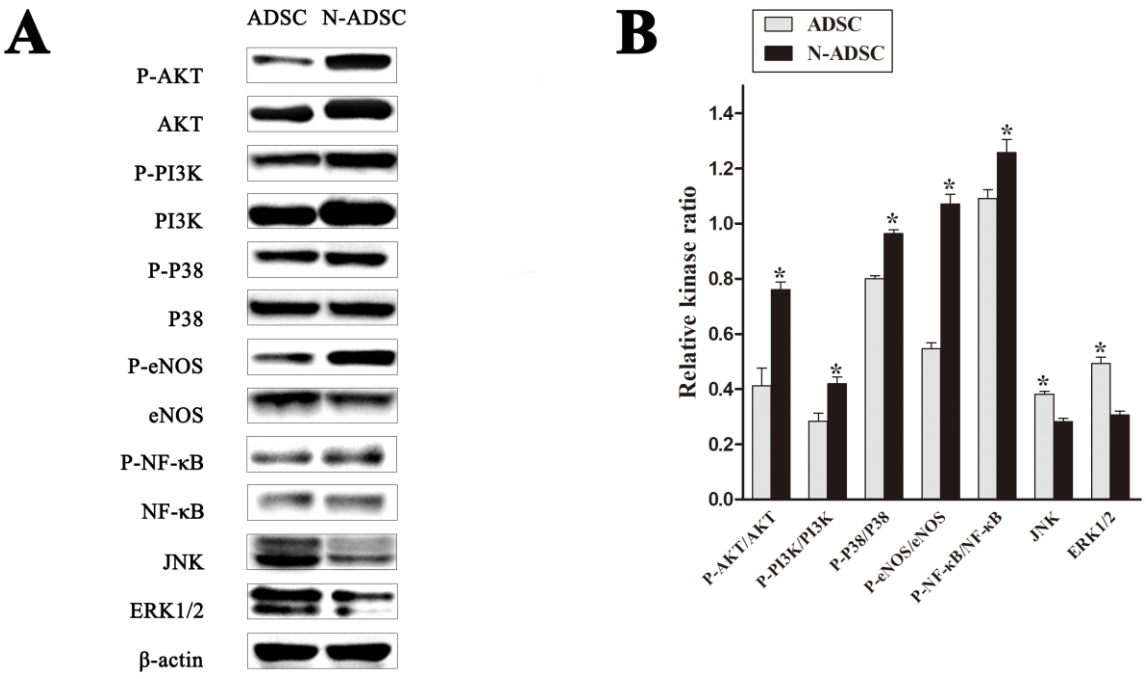

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图 3-7 Netrin-1 在体外调节 ADSCs 的信号通路。

Figure3-7 Signaling pathway of Netrin-1 on ADSCs in vitro.

体外的 ADSCs 与 N-ADSCs 对比的 P-AKT，AKT，P-PI3K，PI3K，P-P38，P38，P-eNOS，eNOS，P-NF-κB，NF-κB，JNK 和 ERK1 / 2 的 Western Blot 结果（A）。统计学分析表明，与 ADSCs 组相比，N-ADSCs 组 PI3K / AKT / eNOS / P-38 / NF-κB 信号通路的磷酸化水平高度显著上调，而 JNK 与 ERK1 / 2 则不然（B）。* P <0.05。ADSCs，脂肪来源的干细胞; N-ADSCs，Netrin-1 转染的 ADSCs。

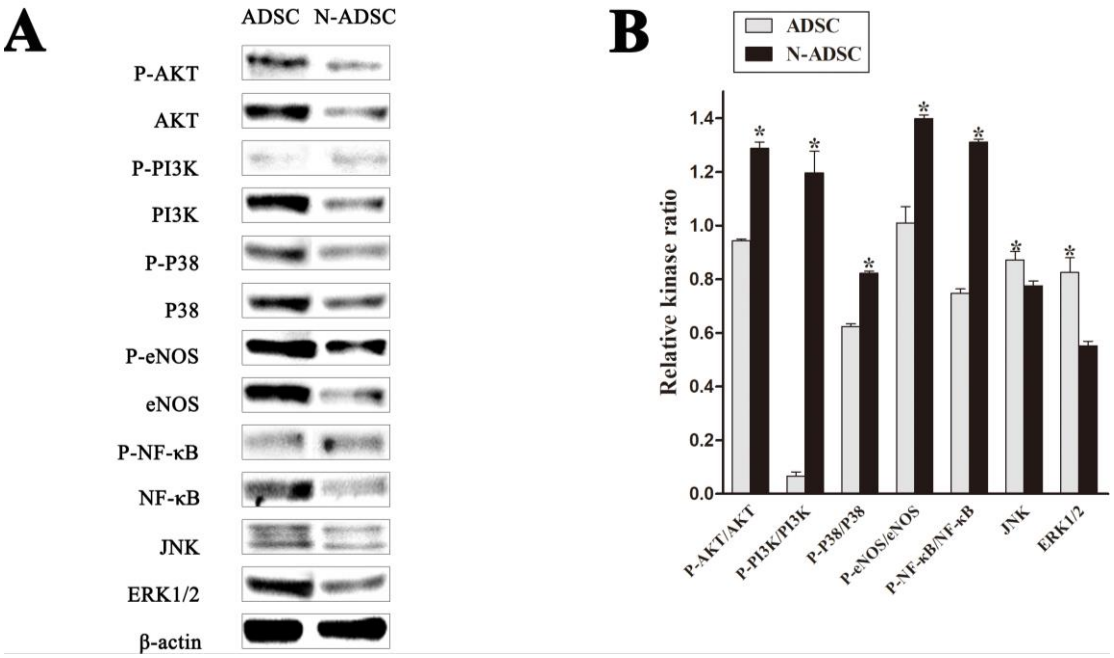

图 3-8：Netrin-1 在后肢失神经 T2DM 小鼠体内调节 ADSCs 的信号通路。

Figure3-8. Signaling pathway of Netrin-1 on ADSCs in vivo in T2DM denerved mice.

体内的 ADSCs 与 N-ADSCs 对比的 P-AKT，AKT，P-PI3K，PI3K，P-P38，P38，P-eNOS，eNOS，P-NF-κB，NF-κB，JNK 和 ERK1/2 的 Western Blot 结果（A）。统计学分析表明，与 ADSCs 组相比，N-ADSCs 组 PI3K / AKT / eNOS / P-38 / NF-κB 信号通路的磷酸化水平高度显著上调，而 JNK 与 ERK1 / 2 则不然（B）。* P <0.05。ADSCs，脂肪来源的干细胞; N-ADSCs，Netrin-1 转染的 ADSCs。

3.4. **Netrin-1 调控 ADSCs 旁分泌细胞因子和生长因子的作用**

将 ADSCs 和 N-ADSCs 在高葡萄糖培养基中培养 48 小时，然后收集上清液并通过 ELISA 进行分析。结果表明，细胞因子和生长因子如 VEGF，b-FGF，HGF，

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TNF-α，PDGF，EGF，IGF-1 和 Netrin-1 的表达在 N-ADSCs 组中显著高于 ADSCs 组（图 8）。越来越多的研究表明干细胞的旁分泌作用在促进组织损伤修复中起到至关重要的作用。而 VEGF，b-FGF，HGF，TNF-α，PDGF，EGF，IGF-1 等为细胞生长、增殖、迁移、粘附和血管新生中必不可少的正向调控因子，ADSCs 旁分泌以上因子可以同时促进 ADSCs 自身以及体内血管内皮的存活与修复，而 Netrin-1 本身具有促进内皮细胞生长和修复的作用，因此，Netrin-1 能够增强 ADSCs 的旁分泌，从而促进体内的血运重建，治疗 DPNV。

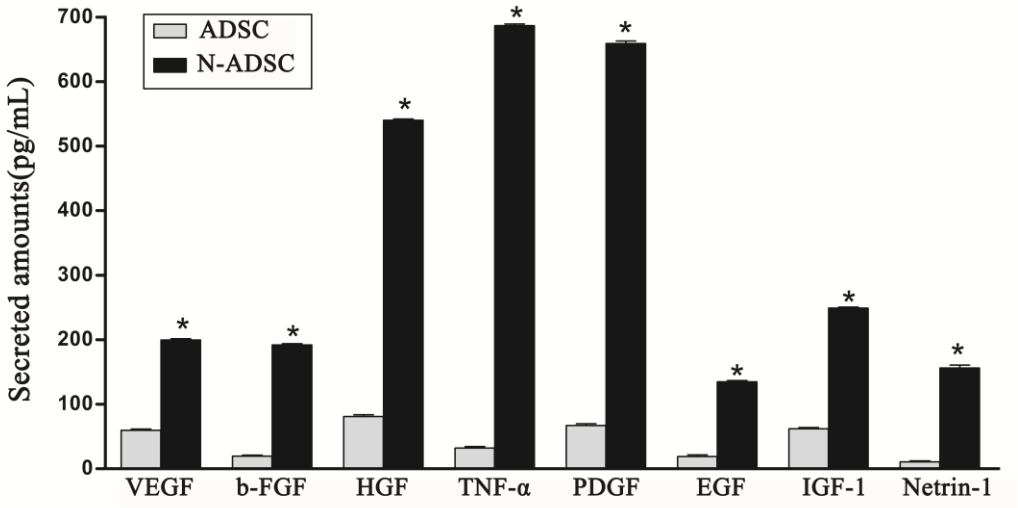

图 3-9 N-ADSCs 中的细胞因子和生长因子。ELISA 显示，与 ADSCs 相比，N-ADSCs 中细胞因子和生长因子（包括 VEGF，b-FGF，HGF，TNF-α，PDGF，EGF，IGF-1 和 Netrin-1）的水平均上调。

Fig3-9 Cytokines and growth factors in N-ADSCs. ELISA showed that the levels of cytokines and growth factors, including VEGF, b-FGF, HGF, TNF- α, PDGF, EGF, IGF-1, and Netrin-1, were upregulated in N-ADSCs as compared to ADSCs.

ADSCs，脂肪来源的干细胞; N-ADSCs Netrin-1 转染的 ADSCs; VEGF 血管内皮生长因子; b-FGF，碱性成纤维细胞生长因子; HGF，肝细胞生长因子; TNF-α，肿瘤坏死因子-α; PDGF，血小板衍生生长因子; EGF，表皮生长因子; IGF-1，胰岛素样生长因子-1，* P <0.05。

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4. 讨论

在前期研究中，我们成功获取了来自小鼠脂肪组织的 ADSCs 并建立了一个高效稳定的腺病毒基因转染系统，获得稳定过表达 Netrin-1 的 N-ADSCs。我们前期体外研究证实 Netrin-1 可以显著改善 ADSCs 的增殖，迁移，粘附和血管生成，并防止高糖诱导的 ADSCs 细胞凋亡，提示我们进一步对体内 N-ADSCs 修复 DPNV 的效果与机制的探讨。本研究中，我们将 N-ADSCs 成功植入坐骨神经失神经支配的 T2DM 的小鼠后肢中。在术后 28 天内的持续观察与监测中，N-ADSCs 组的小鼠后肢血流显示出比 ADSCs 组和 PBS 对照组显著升高的激光多普勒灌注指数。术后 28 天解剖小鼠获得的肌肉标本的免疫荧光检测表明，N-ADSCs 细胞在 T2DM 失神经后慢性缺血的后肢肌肉中存活，并迁移到受损组织及血管附近，一部分分化为内皮细胞并形成小的毛细血管。免疫组织化学结果表明，与 ADSCs 组和 PBS 对照组相比，N-ADSCs 组中后肢肌肉的微血管密度显著升高。对体内外的蛋白进行 Western Blot 检测及统计学分析表明，Netrin-1 改善 ADSCs 体内外存活、增殖、迁移、分化和促进血运重建的机制可能与上调的 PI3K / AKT / eNOS / P-38 / NF-κB 的信号通路有关。

一些研究表明脂肪组织是 ADSCs 的天然丰富储备，ADSCs 具有多向分化，修复组织和器官功能的巨大潜力[199-201]。然而，临床和实验室的研究发现，大量移植的细胞没有带来令人满意的治疗效果。并且如我们的研究所示，培养并传代至 P6 代的 ADSCs 表现出严重衰老的模式，并且干性的减少将进一步导致增殖、分化和迁移能力的减弱。因此，我们只能选择 P3 至 P5 代之间较窄范围的 ADSCs 进行后续研究与实验。此外，T2DM 或高血糖症极大限制了 ADSCs 的血管生成作用，使得有效治疗 DPNV 成为具有挑战性的任务。因此，如何改善移植后干细胞的存活，分化和迁移能力是亟待解决的问题。在本研究中，我们选择了 Netrin-1 蛋白，它是一个同时正向调控神经功能活动和血管系统功能的分泌蛋白。诸多研究表明其在内皮细胞、神经细胞和干细胞的存活、增殖、迁移、粘附和分化以及抑制细胞凋亡中起着至关重要的作用。Ke 等人研究了 Netrin-1 对 BMSCs 在大鼠肢体缺血后血管再生过程中的增殖，迁移，血管形成的积极作用。该研究测量了 VEGF 的血浆和组织水平，并证实 VEGF 的上调是转染 Netrin-1

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的 BMSCs 在大鼠肢体缺血血运重建中的积极作用的主要原因[202]。本研究的体外部分揭示了 Netrin-1 对 ADSCs 的增殖、迁移、粘附和分化的促进作用以及其具体信号通路与分子机制。我们应用的腺病毒由于其上的外源基因并未插入到靶细胞的基因组中，因此在临床使用中它是比慢病毒更优越且更安全的方法[193]。因此，我们通过腺病毒将 NTN-1 基因转染到 ADSCs 中。值得注意的是，Western Blot 的结果证明了自然状态下 Netrin-1 在 ADSCs 中几乎无表达，而转染 NTN-1 的 ADSCs 中 Netrin-1 大量表达，这进一步证实了向 ADSCs 内转染 NTN-1 的必要性。之前有研究表明 Netrin-1 在缺氧条件下通过 DCC / AKT 信号通路抑制间充质干细胞（MSCs）凋亡[203]，增加其存活率。而本研究的 Western Blot 结果显示，N-ADSCs 中 Bcl-2 / Bax 的比例显著高于 ADSCs，因此 Netrin-1 在高糖条件下增强 ADSCs 的增殖并抑制其凋亡。体内免疫荧光和免疫组织化学检测证明，N-ADSCs 在 T2DM 后肢失神经小鼠慢性缺血性肌肉中的存活率显著高于 ADSCs，而一些 ADSCs 也分化成为内皮细胞并形成小毛细血管。这种现象增加了微血管的密度和下肢血流激光多普勒灌注指数，从而极大地增强了后肢的功能。以往的研究表明，分化和旁分泌效应是 ADSCs 移植进入体内后发挥修复受损组织的两种主要机制[62]。本研究的结果证明，Netrin-1 在 ADSCs 中的过表达极大地升高了一系列生长因子和细胞因子（例如 VEGF，HGF，bFGF 和 PDGF）的表达水平，从而为组织再生提供良好的微环境，减少了后肢缺血肌肉的病理学重塑，促进血管再生。与此同时，高表达的分泌蛋白 Netrin-1 本身即是一种强大的血管生成调节因子，可通过 PI3K / AKT-eNOS 信号通路恢复高糖环境下血管内皮细胞的细胞损伤和促进血管新生。此外，我们发现了体内外 PI3K / AKT / eNOS / P-38 / NF-κB 信号传导通路的表达上升，它在 ADSCs 的增殖，分化，抗凋亡，迁移和粘附中发挥着至关重要的作用。但本研究只是对潜在机制的初步探索，后续依旧需要进一步的分子机制相关的实验，明确阐明 Netrin-1 调节 ADSCs 促进 DPNV 血管再生的治疗效果的具体分子机制。尽管本研究有其局限性，但目前的研究结果证明，Netrin-1 可以改善 ADSCs 的在体内外的存活、增殖、迁移、粘附、分化和治疗 DPNV 促进血管新生的效果。总之，本研究的结果为 DPNV 疾病的治疗提供了新的见解。

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5. 小结

本研究通过成功构建 T2DM 小鼠（db/db）后肢失神经模型，体内移植 N-ADSCs 与 ADSCs，并应用激光多普勒观察血流灌注，通过免疫荧光和免疫组化，评估 ADSCs 在体内存活、迁移、分化和促血管新生的效率。应用 Western Blot 探讨 Netrin-1 介导的 ADSCs 增殖，迁移，粘附，分化，促血管生成能力和细胞凋亡的信号通路，应用 ELISA 检测 Netrin-1 介导的 ADSCs 旁分泌多种因子。证实 Netrin-1 可通过 PI3K / AKT / eNOS / P-38 / NF-κB 的信号通路以及促进 VEGF，b-FGF，HGF，TNF-α，PDGF，EGF，IGF-1 和 Netrin-1 等旁分泌因子的表达提高 ADSCs 在体内与体外存活、增殖、迁移、分化、粘附和促血管新生的效率，提高小血管密度以及血流灌注程度，显著改善 T2DM 小鼠失神经后肢的慢性缺血情况与功能，治疗 DPNV。本研究为 DPNV 的防治提供了新思路和理论依据。

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全文总结及创新点

DPNV 的发病率高、出现早、起病隐匿、病程长、难以治愈，并且导致截肢甚至生命危险，目前临床尚无有效治疗方案。干细胞移植是一种极有希望的治疗方式。T2DM 的患者身体尤其是腹部富含脂肪组织，通过便宜安全的脂肪抽吸术可以获得大量脂肪组织，通过体外培养而得的 ADSCs 具有多向分化，抵抗炎症，修复组织的优秀特性。本研究亦证实，实验小鼠腹股沟区可获得大量脂肪组织，成功分离并培养获得大量 ADSCs。因此，ADSCs 是十分具有临床应用前景的治疗 DPNV 的种子细胞。但大量基础及临床研究发现，ADSCs 体内移植后存活率低是影响其疗效的关键。尤其是糖尿病患者体内持续高血糖导致的高级糖基化终产物、产生促炎微环境和诱导氧化应激等不良环境进一步降低了 ADSCs 的存活率，使其无法发挥应有的功能。

Netrin-1 是一种同时在神经和血管中发挥促进神经细胞和内皮细胞生长、存活、粘附、迁移、增殖和抗凋亡、抗炎的分泌蛋白，最近被发现在许多急慢性炎症疾病中，都有其表达水平的显著上升或下降。在本研究中，我们通过对糖尿病患者下肢缺血肌肉临床标本的分析发现 DPNV 患者内皮细胞中表达 Netrin-1 蛋白，但在其外周血血清以及下肢缺血肌肉组织中，Netrin-1 表达水平显著下降，而多项炎症相关因子的表达水平明显上升，微血管的密度较正常组明显下降。证实 Netrin-1 在糖尿病高血糖环境下的表达水平与 DPNV 呈负相关，证明了 Netrin-1 与 DPNV 的临床相关性。因此我们认为通过基因转染 ADSCs 过表达 Netrin-1 可以改善 ADSCs 在高血糖条件下的活力，迁移和向血管内皮细胞分化，促进 DPNV 血管新生。

本研究的体外实验部分成功大量获取 ADSCs 后，构建了腺病毒转染 ADSCs 的稳定基因转染体系，证实体外环境中 Netrin-1 能够显著提高高糖环境下 ADSCs 的增殖、迁移、粘附、向内皮细胞分化的能力，显著降低 ADSCs 的凋亡水平。体内实验部分，本研究成功构建 T2DM 小鼠（db/db）后肢失神经模型，体内移植 N-ADSCs 与 ADSCs，证实 Netrin-1 可通过 PI3K / AKT / eNOS / P-38 / NF-κB 的信号通路以及促进 VEGF，b-FGF，HGF，TNF-α，PDGF，EGF，IGF-1 和 Netrin-1 等旁分泌因子的表达提高 ADSCs 在体内与体外存活、增殖、迁移、分化、粘附

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和促血管新生的效率，提高小血管密度以及血流灌注程度，显著改善 T2DM 小鼠失神经后肢的慢性缺血情况与功能，治疗 DPNV。本研究为 DPNV 的防治提供了新思路和理论依据。

尽管本研究完整地从临床样本分析 Netrin-1 与 DPNV 的临床相关性、通过详实的体内与体外实验证实了 Netrin-1 促进 ADSCs 存活、迁移、分化和修复 DPNV 慢性缺血的重要作用，但依然存在一些不足与局限。首先，本研究由于临床标本珍贵，获得难度大，属于小样本量研究，后续我们将继续扩大样本量，用更大规模的样本量说明 Netrin-1 与 DPNV 的临床相关性，从而获得更有说服力更可信的结论。其次，在体内外 Netrin-1 调控 ADSCs 的具体分子机制的研究中，本研究只是从经典信号通路的蛋白分子方面对其进行了初步的探讨，Netrin-1 如何分别调节 ADSCs 在体内与体外存活、增殖、迁移、分化、粘附和促血管新生的效率的具体的分子机制并未进行探索，在以后的研究中，我们将进一步细化实验步骤，应用信号通路相关激动剂与抑制剂，进一步阐明其具体分子机制，为以后 Netrin-1 修饰的 ADSCs 应用于临床 DPNV 的预防与治疗提供更详实的更有说服力的理论依据。此外，随着越来越多的研究揭示了旁分泌作用在 ADSCs 促进组织损伤修复中至关重要的作用，我们在本研究中检测到的表达水平上升的诸多旁分泌因子在 Netrin-1 调控 ADSCs 治疗 DPNV 中的作用也需要进一步探索，以上不足之处也是后续研究的重点所在，后续研究已经启动并在进行。

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时间如白驹过隙，八年前我还是高考刚刚结束迈入大学生涯的稚嫩学子，曾经以为临床医学八年制的八年如同抗日战争的八年，艰苦而又漫长，但一转眼间，充实的八年学生生活已逝，我即将博士毕业，进入规培，从学生的身份转变为一名医生，迈入人生的全新阶段，用一身本事去报效祖国，做一颗发光发亮的螺丝钉，为祖国的卫生和健康事业奉献自己的一生。

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学术论文及科研成果

论著：

1. Zhang X, Qin J, Wang X, et al. Netrin-1 improves adipose-derived stem cell proliferation, migration, and treatment effect in type 2 diabetic mice with sciatic denervation[J]. Stem cell research & therapy, 2018, 9(1): 285.（**IF = 4.963**）

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参与基金

1. 国家自然科学基金（青年项目），81601621，**Netrin-1 修饰的 ASC 对失神经支配后糖尿病小鼠血管新生的影响及分子机制**，2017.01-2020.01，18 万，在研。

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3. 国家自然科学基金（面上项目），81570432，M2 巨噬细胞通过 TGF-β-RhoA/ROCK 通路介导 BMSCs 定向归巢促进损伤血管再内皮化的机制研究，2016/01-2019/12，58 万，在研。

4. 国家自然科学基金（面上项目），81370423，Periostin 调控 ADSC 存活、迁移及促进损伤血管再内皮化的机制研究，2014/01-2017/12，70 万，已结题。

5. 国家自然科学基金（青年项目），81700432，高糖环境下 GLO1 对 ADSC 促损伤血管再内皮化的作用机制研究，2018.01-2020.12, 20 万，在研。

申请专利：

授权专利：

1. 一种可调速医用光纤自动收放装置，实用新型专利，发明人：**张省**，陆信武等。专利号：CN201820326343.0。

2. 一种实验小鼠尾静脉注射固定器套装，实用新型专利，发明人：陆信武，王新，丁昂昂，秦金保，彭智猷，李博，**张省**，郭信，吴小雨。专利号：CN201621403930.2。

申请中专利：

1. 一种医用可调速光纤自动回撤装置，国家发明专利，发明人：**张省**，陆信武等。申请号：CN201810333667.1，专利已公开，实质审查中，公开号：108420662。

2. 一种新型的医用弹力绷带，国家发明专利，发明人：**张省**，陆信武等。申请号：CN201811125119.6，专利已公开，实质审查中，公开号：109223308。

3. 一种治疗 B 型主动脉夹层的支架，国家发明专利，发明人：陆信武，李维敏，秦金保，**张省**等。申请号：CN201810719924.5，专利已公开，实质审查中，公开号：108553202。

4. 主动脉与双侧颈内动脉颅内转流的装置，国家发明专利，发明人：陆信武，秦金保，李维敏，**张省**等。申请号：201811128258.4，专利已公开，实质审查中，公开号：109125886。

5. 主动脉与左侧颈内动脉颅内转流的装置，国家发明专利，发明人：陆信武，赵振，秦金保，李维敏，**张省**等。申请号：201811128257.X，专利已公开，实质审查中，公开号：109125885。

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获奖：

1. 2016-2017 年度上海交通大学医学院优秀奖学金二等奖

2. 2016-2017 年度罗氏诊断中国医学及生命科学教育研究基金奖学金

3. 2017-2018 年度上海交通大学医学院优秀奖学金一等奖

4. 2018-2019 年度中国红十字基金会院士博爱奖学金

5. 2017-2018 年度上海交通大学“优秀团员”

6. 2017-2018 年度上海交通大学“三好学生”

7. 2012 年全国大学生英语能力竞赛国家级一等奖

8. 2011～2012 年度上海交通大学 A 等奖学金

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八年制学位论文要求

八年制高等医学教育遵循“八年一贯，整体优化，加强基础，注重临床，培养能力，提高素质”的办学原则，培养适应我国经济社会发展需要的、具有崇高的思想品德和社会责任感、高尚的职业道德、良好的敬业精神、伦理行为和法律意识；具有较宽厚的人文社会科学和自然科学知识及扎实的医学科学基础理论、较强的临床实践能力和交流协调能力；具备基本科研能力、终生自主学习能力和创新意识、良好的中、外文沟通能力和信息获取、管理、应用能力；具有较大发展潜能和团队协作精神的高素质临床医学人才，其培养目标定位于临床医学博士专业学位。

根据八年制医学博士学位培养方案总则、应用型学位的特点和上级的有关精神，八年制学生学位论文的要求：

1. 博士学位论文应在导师或指导小组的指导下，由学生本人独立完成，实行导师负责制。

2. 在博士生导师的指导下，在查阅大量文献资料并结合本人工作的基础上确定学位研究课题。选题必须紧密结合临床实际，可以是病例分析报告，或总结临床经验，或改进临床技术方法，也可以是临床和实验研究相结合的研究工作，研究结果对临床工作具有一定的应用价值或应用前景。

3. 表明学生具有运用所学知识解决临床实际问题和从事临床科学研究的能力。

4. 撰写学位论文应恪守学术道德和学术规范,要求概念清楚、立论正确、分析严谨、数据可靠、计算精确、图表清晰、层次分明、文字简练、格式规范。

5. 学位论文内容一般应不少于 2 万字。中文摘要 800 字以内，英文摘要 1500-3000 字符。

6. 学位论文全文要求中英（或法）文对照。

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附：英文版全文

Introduction

Diabetes (DM) is a globally comorbid non-communicable disease. It is essentially a systemic metabolic disorder with elevated blood glucose levels [1, 2]. Diabetes is usually divided into type 1 diabetes (T1DM), type 2 diabetes (T2DM), gestational diabetes (GDM), and other specific types of diabetes. Type 2 diabetes is the most common form. Diabetes has a variety of pathogenesis, and its specific pathogenesis may be impaired insulin secretion or due to insulin resistance of target organ tissues and / or extensive damage of pancreatic beta cells [1,3].

In the past few decades, the incidence and prevalence of diabetes worldwide has shown a significant increase. As more and more people form unhealthy lifestyles and eating habits, the future incidence is expected to continue to rise. According to the International Diabetes Federation and the World Health Organization, the number of people with diabetes worldwide has risen from 153 million in 1980 to 425 million in 2017. It is estimated that by 2020, 629 million people worldwide will have diabetes [4, 5]. Among them, the growth rate of diabetes in developing countries is particularly significant due to the improvement of economic conditions, the improvement of living standards and the acquisition of bad habits from developed countries. As a representative of developing countries, in the past 30 years, Chinese diabetes patients have soared from 1% of the population in 1980 to 10.9% in 2013. At present, more than 121 million people in China have diabetes, and the number of people with diabetes ranks first in the world [6-9]. At the same time, the International Diabetes Federation estimates that as many as 212 million people with diabetes worldwide have not detected diabetes. Therefore, the actual incidence of diabetes is even higher [10,11].

The main hazard of diabetes is the destruction of the target organs and tissues by the continuously elevated blood glucose. Without intervention, it will eventually cause a variety of diabetic complications. Its specific pathogenesis includes certain genetic

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and epigenetic modifications, nutritional factors and sedentary lifestyles [12]. Acute complications of diabetes include, for example, diabetic ketoacidosis (DKA), hyperglycemia, hypertonic state (HHS), lactic acidosis, and hypoglycemia, which are important causes of high morbidity and mortality in diabetes, and greatly increase the difficulty and cost of diabetes nursing[13,14]. Chronic complications are mainly vascular lesions caused by diabetes. Including: (1) diabetic macrovascular complications, mainly cardiovascular disease (CVD), including coronary heart disease, stroke and peripheral arterial disease (PAD), the main reason is a series of atherosclerosis and macroangiopathy caused by diabetes. (2) Diabetic microvascular complications: including end-stage renal disease (ESRD), diabetic retinopathy (DR), diabetic neuropathy (DN), and lower extremity amputation (LEA) caused by diabetes. The pathogenesis of diabetic microvascular complications may include: production of advanced glycation end products (AGEs), creation of a pro-inflammatory microenvironment, induction of oxidative stress, etc. [15-17].

In recent years, as clinicians' awareness of diabetes has deepened, and patients' health awareness and compliance have increased, the application of various hypoglycemic drugs such as insulin, metformin, and antiplatelet and lipid-lowering drugs such as aspirin and statins have become popular. Although the incidence of CVD in diabetic patients is still 2-4 times higher than that in non-diabetic patients, it is gratifying that the prevalence of CVD and CVD-related mortality in diabetic patients have decreased in the past 20 years [18-22]. However, the total amputation caused by diabetic microangiopathy has not only decreased significantly, but reported to be on the rise in some countries [23, 24]. Due to its high incidence, early but insidious onset, long course of disease, and difficulty to cure, a considerable number of patients are not sensitive to pain due to diabetic peripheral neuropathy, and thus cannot be diagnosed early by diabetic peripheral vascular disease. Symptoms of typical arterial stenosis and occlusion, such as intermittent claudication and rest pain caused by lower limb ischemia, are often treated in the event of severe vascular complications [25-28]. The complexity of vascular lesions combined with neuropathy

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often leads to further deterioration of the disease, prolonged unhealed condition, and a considerable number of patients progress to diabetic foot [29-31]. Clinically available treatments, such as conservative drug therapy, vascular reconstruction, and percutaneous transluminal angioplasty, can improve the patient's condition and prognosis to varying degrees, but the long-term efficacy is not ideal, and there exists limitation to clinical applications. Diabetic patients with severe ischemia (CLI) of the lower limbs who have lost the opportunity for surgery and intervention often end in amputation, and some patients are even at risk of life [32-35]. This not only brings great suffering to patients, but also brings a heavy financial burden to medical and health care. Therefore, solving the problem of diabetic peripheral neurovascular disease (DPNV), restoring peripheral nerve vessels, avoiding amputation or even death, has become an urgent problem to be solved.

In recent years, stem cell transplantation technology and regenerative medicine have been developing rapidly. A large number of studies have confirmed that stem cell transplantation can promote the formation of ischemic limb collateral vessels [36-40], improve and restore the blood flow of the affected limb, improve the quality of life of patients, and achieve therapeutic limbs. Stem cell treatment of severe limb ischemia has demonstrated a good clinical application prospect and has become a hot topic in current research [41-45]. Currently, various stem cells have been used in tissue engineering and regenerative medicine. Stem cells are mainly divided into three major categories: embryonic stem cells (ESCs), fetal stem cells (FSCs), and adult stem cells (ASCs). Embryonic-derived stem cells include fetal tissue cells, cord blood cells, placenta and amniocytes, hematopoietic stem cells (HSCs), and mesenchymal stem cells (MSCs). Adult stem cells can be divided into bone marrow stromal cells (BMSCs), muscle satellite cells, neural stem cells (NSCs), hematopoietic stem cells (HSCs), and adipose-derived stem cells (ADSCs) [46, 47]. ESCs are capable of self-renewal and differentiation into any type of cell in the body, but due to ethical and political considerations, it is difficult to apply ESCs to clinical research and practice. Induced pluripotent stem cells (iPSCs) are genetically recombined somatic

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cells that have the characteristics of ESCs. However, the lower induction efficiency of iPSCs and the complexity and cost of acquisition methods make it difficult to obtain and apply in large quantities [48, 49]. Adult stem cells, including BMSCs, HSCs and endothelial progenitor cells (EPCs), have been used clinically and experimentally, and have achieved certain therapeutic effects [50-52], but the risk of collecting peripheral blood or bone marrow stem cells is high, and the patient experience was poor, and most of the patients are elderly, often combined with a variety of underlying diseases, the number of HSCs and EPCs in BMSCs and peripheral blood is not only reduced, but also proliferation, migration, differentiation and angiogenic capacity are significantly reduced, it is difficult to meet a wide range of clinical applications [53]. ADSCs, which have become hot spots in recent years, can be found in any type of white adipose tissue, including subcutaneous fat and omental fat [54]. Humans have abundant subcutaneous fat tissue, especially in patients with diabetes, often with obesity, liposuction can easily acquire large amounts of adipose tissue and was clinically safe without side effects [55, 56]. Studies have found that adipose tissue contains a large number of ADSCs, which can differentiate into adipocytes, osteoblasts, chondrocytes, cardiomyocytes, nerve cells, etc., with strong multi-directional differentiation potential, they can differentiate into endothelial cells and smooth muscle cells under specific induction conditions. And ADSCs can secrete a variety of pro-angiogenic factors, promote autologous blood vessel formation after autologous or allogeneic transplantation, and improve blood flow in the ischemic lower limbs of mice [36, 57]. The study found that compared with autologous bone marrow and peripheral blood stem cells. Adipose tissue acquisition is much cheaper than bone marrow, less invasive, more numerous, and has a higher stem cell proliferation rate than BMSCs, and still retains stem cell phenotype and mesenchymal pluripotency over 25 generations [58-63]. Therefore, adipose tissue is a rich, practical, and attractive source of autologous cell replacement donor tissue. Adipose tissue-derived ADSCs are promising as a suitable treatment for DPNV.

In recent years, domestic and foreign scholars have carried out a lot of research

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work on stem cell transplantation for the treatment of lower limb ischemia in diabetic patients. Some studies have been applied in clinical trials. Although the symptoms of lower limb ischemia and objective indicators of diabetic patients have improved to some extent, its long-term efficacy is still poor [36, 64-70]. At the same time, Kočí Z et al found that ADSCs extracted from patients with diabetic lower extremity ischemia had significantly reduced proliferative capacity and paracrine ability compared with the normal group, and the level of apoptosis increased [71]. Diabetic ADSCs extracted from the body fat of diabetic patients by Ja Hea Gu et al. showed significant damage and deficiency in producing vascular endothelial growth factor (VEGF) and inducing cell proliferation under hypoxic conditions [72]. A large number of animal experiments and studies have also found that the proliferation of ADSCs in diabetic mice is impaired, and the angiogenic ability is impaired or even lost [73-76]. Rennert RC et al. used single-cell analysis and other techniques to discover that the microenvironment of diabetes changes the niche of ADSCs in situ and selectively depletes subpopulations of cells expressing angiogenesis-related genes, thereby making diabetic ADSCs in vitro and in vivo show impaired angiogenic capacity [77]. The above studies show that the persistent high glucose environment in diabetic patients reduces the survival, migration and angiogenic ability of ADSCs transplanted in the target lesions, which makes the treatment effect of ADSCs on DPNV greatly impaired, unable to meet the clinical needs, its mechanism may be associated with advanced glycation end products, production of pro-inflammatory microenvironment and induction of oxidative stress, but the specific mechanism is not fully understood [15-17]. Therefore, how to promote the survival and differentiation of ADSCs in the diabetic vascular injury sites and promote the regeneration of diabetic blood vessels after denervation is of great significance for the prevention and treatment of DPNV lesions.

Studies found that DPNV is a mixed lesion with vascular, neurological and tissue damage [28,78-80]. Previous studies have often focused on the role and significance of a single factor in the treatment of DPNV, without the functional units of nerves and

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blood vessels as a whole, and neglecting the important role of neurological factors in regulating angiogenesis, so the efficacy is limited [26, 81-83]. In addition, nerves and blood vessels exhibit similar complex branches and growth patterns that follow the same migration path to the same target organ or site. The similarities between angiogenesis and axon growth suggest that they may be regulated by some common signaling molecules [84]. Studies by Jones and Li have shown that some signaling molecules can regulate neural and vascular developmental processes [85], especially four pairs of ligands and receptors that were originally thought to affect axon growth-directed regulators, and most studies have confirmed at the same time participate in the formation of blood vessels [86-88]. Netrin-1 is the first identified axon guidance factor. Netrin-1 and G-netrin share homology with the laminin gamma chain. The peptide consists of nearly 600 residues of an amino terminal region VI. Repeated three layers of adhesion-type epidermal growth factor (V-1, V-2, V-3) and one carboxy terminal region [89]. Another study found that Netrin-1 not only participates in the functional activities of the nervous system, but also participates in the functional activities of the vascular system. Ding et al. demonstrated that Netrin-1 not only promotes neuronal migration and secretion in the central nervous system, but also regulates the survival, adhesion, migration, proliferation and differentiation of endothelial cells and stem cells in non-neural tissues, and inhibits their apoptosis [90, 91]. Wilson et al. systematically studied zebrafish and mammals, confirming that Netrins can induce angiogenesis. Netrin-1 activates the Src/FAK/paxillin-related signaling pathway by binding to the UNC5H receptor to promote adhesion, migration and proliferation of vascular endothelial cells, forming a new capillary network that can be inhibited by inhibiting zebrafish NTN-1 mRNA[92]. Lu et al. also found that Netrins stimulates angiogenesis in mammals and accelerates angiogenesis in ischemic tissues. This process relies on the Netrin-1 receptor DCC to regulate the ERK/eNOS signaling pathway [93]. Brunet et al. demonstrated that Netrin-1, Netrin-4 and VEGF promote angiogenesis, but Netrin-1 has a greater advantage in promoting the dual roles of endothelial cell differentiation and recovery of nerve damage [94]. In addition,

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Netrin-1 is involved not only in nerve growth and angiogenesis; it also enhances mitosis, migration and adhesion of endothelial cells at different stages of human blood vessels and lymphatic systems [95]. Therefore, we believe that overexpression of Netrin-1 by gene transfection of ADSCs can improve the viability of ADSCs under hyperglycemia, migration and differentiation into vascular endothelial cells, and promote DPNV angiogenesis.

With regard to the basic research of Netrin-1, which has both the dual effects of promoting nerve growth and angiogenesis, the clinical attention to Netrin-1 is increasing day by day. With the increase and deepening of clinical research, Netrin-1 and the clinical relevance of various inflammatory diseases is also becoming clearer. Mulero P et al.examined 90 patients with multiple sclerosis and 30 blood samples from the control group and found that serum levels of Netrin-1 were significantly lower in patients with multiple sclerosis, especially in relapsed patients.. It is suggested that Netrin-1 may be a biomarker of active inflammation in multiple sclerosis [96]. Reeves WB et al found that the expression of Netrin-1 in the kidney increased in the renal ischemic response, presumably as a homeostatic measure limiting tissue damage. Acute kidney injury (AKI) model induced by renal ischemia in mice showed a decrease in renal tubular Netrin-1 expression and a decrease in Netrin-1 levels in urine [97]. This observation was validated in a prospective clinical trial. Ramesh G et al. confirmed that elevated levels of Netrin-1 in the urine of patients after cardiovascular surgery were significantly associated with AKI, suggesting Netrin-1 can be used as a biomarker for acute kidney injury [98]. In myocardial ischemia-reperfusion, many cell and animal experiments have shown that Netrin-1 plays an important role in protecting myocardium during myocardial ischemia-reperfusion, and may serve as a non-surgical therapeutic target for myocardial ischemia-reperfusion in the future [99-103]. At the same time, many studies have found that Netrin-1 also has potential as a biomarker in diabetes and diabetic complications. A recent clinical study by Jung et al. showed that Netrin-1 may be a new biomarker for early detection of impaired fasting glucose (IFG) or

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T2DM. They found a significant increase in serum Netrin-1 levels in patients with IFG or T2DM compared with controls, serum Netrin-1 versus fasting blood glucose, glycosylated hemoglobin (HbA1c), HOMA insulin resistance index (HOMA-IR), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly positively correlated, and Netrin-1 was significantly negatively correlated with high-density lipoprotein (HDL) cholesterol and glomerular filtration rate (eGFR). In addition, serum Netrin-1 has an independent association with the presence of IFG or T2DM [104]. In contrast, Liu et al. conducted a clinical study of 56 patients, of which 30 new patients with type 2 diabetes were assigned to the treatment group and the rest were assigned to the control group to assess Netrin-1 in diabetic patients. They found that Netrin-1 levels were significantly lower in diabetic patients than in healthy controls. In addition, the degree of Netrin-1 was negatively correlated with the steady-state model evaluation of insulin resistance and blood glucose (fasting and postprandial), fasting insulin, triglyceride (TG), and hemoglobin A1c levels [105]. The findings of the above two clinical studies at the Netrin-1 level and DM clinical relevance are contradictory, so further high quality studies are needed to determine the actual relationship between the two. In terms of diabetic complications, J. Liu et al. included a total of 18 patients with diabetes, including 10 patients with DR and 8 patients without DR. The levels of Netrin-1 and VEGF in the vitreous of patients with DR were significantly higher than those in the control group [106]. K. Miloudi et al found that patients with diabetic macular edema and controls without diabetic macular edema found that intravitreal Netrin-1 was significantly increased 8-fold in patients with diabetic macular edema [107]. Many studies in recent years have shown that Netrin-1 may be a new biomarker and potential therapeutic target in diabetic retinopathy. In terms of diabetic peripheral neuropathy, Netrin-1 has a long-lasting chemical attraction capacity, which can enrich axon extension and is highly expressed in the adult nervous system after injury. Dun and Parkinson's experiments showed that Netrin-1 plays a crucial role in maintaining Schwann cell proliferation, peripheral nerve regeneration and migration. Therefore, in

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order to stimulate the recovery and available recovery of damaged peripheral nerves, targeting the Netrin-1 signaling pathway would be a new therapeutic strategy [108, 109]. These data also suggest that Netrin-1 may be an endogenous trophic factor for Schwann cells in damaged peripheral nerves [109]. Therefore, Netrin-1 may also be a potential biomarker and therapeutic target for diabetic peripheral neuropathy, which is also a diabetic complication.

The relevant basic and clinical research of Netrin-1 is in full swing. However, there are no clinical studies to confirm the clinical correlation between Netrin-1 and DPNV, and whether Netrin-1 has a regulatory effect on the treatment of DPNV in ADSCs. However, combined with previous literature studies and our previous work, we believe that there is a clinical correlation between Netrin-1 and DPNV, perhaps a potential biomarker for DPNV. Furthermore, overexpression of Netrin-1 by ADSCs can improve the ability of ADSCs to proliferate, migrate and differentiate into vascular endothelial cells after transplantation in vivo under conditions of hyperglycemia, thereby promoting angiogenesis of DPNV.

Therefore, this study intends to first obtain the ischemic muscle tissue of the lower limbs and peripheral blood mononuclear cells in clinical diabetic and non-diabetic patients, and analyze the Netrin-1 and inflammation in the pathological tissues of diabetic patients by immunohistochemistry, immunofluorescence, WB, PCR, etc. Factor expression levels, small vessel density, number of macrophages, and colocalization of Netrin-1 and endothelial cells, and statistical analysis of the clinical correlation between the expression of Netrin-1 and DPNV in diabetic hyperglycemia environment. Secondly, in the cell experiment, the adipose tissue of C57/BL mice was obtained, ADSCs were isolated and cultured, and a stable gene transfection system was established to make ADSCs overexpress green fluorescent protein (GFP) and Netrin-1 (N-ADSCs). CCK-8, WB, flow, Transwell, etc. were used to detect the differences in the proliferation, migration, adhesion, and differentiation into endothelial cells of N-ADSCs and ADSCs in a high glucose environment. In vivo, it is proposed to construct a T2DM mouse (db/db) lower limb denervation model,

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transplant N-ADSCs and ADSCs in vivo, and observe the blood perfusion by laser Doppler, and evaluate the ADSCs in vivo by immunofluorescence and immunohistochemistry. Survival, migration, differentiation, and efficiency of angiogenesis. Finally, Western Blot was used to investigate the signaling pathways of proliferation, migration, adhesion, differentiation, proangiogenic ability and apoptosis of Netrin-1 mediated ADSCs. ELISA was used to detect Netrin-1 mediated paracrine secretion of ADSCs to clarify the specific molecular mechanism of Netirn-1 regulating ADSCs proliferation, migration and treatment of diabetic vascular neuropathy.

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Chapter 1 Study on the clinical correlation between Netrin-1 and DPNV

1. Introduction

With the prolongation of life expectancy, the improvement of people's living standards and the popularity of western life and eating habits, diabetes has become a major chronic disease that jeopardizes people's health [1-3]. The danger of diabetes is not only that the persistent hyperglycemia state provides a breeding ground for other diseases such as atherosclerosis, hypertension, cancer and infectious diseases, but also becomes an independent risk factor for many major diseases, and many complications were caused by it. It also constantly threatens the health of the people [12-17]. Among the many complications of diabetes, diabetic peripheral vascular disease (DPNV) is a mixed disease of blood vessels and nerves and surrounding tissue damage, leading to insidious onset, long course of disease, and often ulceration of diabetic foot, ended with the distal avascular ischemia and necrosis. According to statistics, the risk of suffering from foot ulcers in diabetic patients may be as high as 25%. The lower extremity amputation rate of diabetic patients is 15 times that of non-diabetic patients. The annual incidence of foot ulcers in patients with type 1 or type 2 diabetes is 1.9% to 2.2% [110-112], and patients with diabetic foot ulcers develop toe ulcer, in severe cases, amputation, and more, due to the massive absorption of toxins from the extremity of the extremities, leading to death of multiple organ failure such as liver and kidney [113-115]. Although there are medical treatments, revascularization, percutaneous transluminal angioplasty (balloon dilatation and stent implantation), but simply blood glucose control could not control the DPNV progression and intra-stent occlusion, the long-term efficacy is not ideal [116-119]. Therefore, DPNV brings a huge burden to China's health care system.

The main culprit of DPNV is persistent hyperglycemia caused by metabolic disorders in diabetes [120]. More and more studies have found that inflammation

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plays a crucial role in the destruction of peripheral nerve cells and vascular endothelial cells by hyperglycemia. The mechanism of hyperglycemia damage to peripheral nerve cells is mainly due to the increase in intracellular reactive oxygen species (ROS) mitochondria production. ROS causes strand breaks in nuclear DNA, which in turn activates adenosine diphosphate ribose polymerase (PARP). PARP then modified glyceraldehyde phosphate dehydrogenase (GAPDH) to reduce its activity. Finally, reduced GAPDH activity activates the polyol pathway, increases intracellular AGEs formation, activates protein kinase C (PKC) and subsequent nuclear factor-kappa B (NF-κB), and activates the hexosamine pathway. These four mechanisms, in turn, produce more ROS through pro-inflammatory responses such as endoplasmic reticulum stress and activation of p38 mitogen-activated protein kinase (p38 MAPK), thus forming a self-reinforcing disease mechanism [121]. The mechanism of vascular endothelial cell injury caused by hyperglycemia is also related to oxidative stress: high concentration of glucose in cells leads to PKC activation, and then activation is linked by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and p66Shc protein, which produces a lot of ROS. The dramatic increase in oxidative stress causes rapid deactivation of nitric oxide (NO), resulting in the large production of the prooxidant peroxynitrite (ONOO-), which is responsible for protein nitrosylation. At the same time, PKC activates the related enzyme activity, thereby enhancing the uncoupling of endothelial nitric oxide synthase (eNOS) and leading to the further accumulation of free radicals. On the other hand, hyperglycemia reduces eNOS activity and attenuates the activation phosphorylation of Ser1177. At the same time, hyperglycemia-induced PKC activation leads to increased synthesis of endothelin-1 (ET-1), induction of vasoconstriction and platelet aggregation. Superoxide anion accumulation also promotes pro-inflammatory gene monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion by activating NF-κB signaling. Upregulation of molecule-1 (ICAM-1). These events lead to monocyte adhesion, migration and blood cell exudation, forming foam cells in the sub-endothelial layer. Inflammatory factors from

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foam cells cause blood vessels to be in an inflammatory state as well as smooth muscle cell proliferation, thereby accelerating the atherosclerotic process. Endothelial dysfunction in diabetes is also due to increased thromboxane A2 (TXA2) by up-regulating cyclooxygenase-2 (COX-2) and inactivating prostacyclin synthase (PGIS) by increased nitrosation synthesis. In addition, ROS increases the synthesis of the glucose metabolite methylglyoxal, leading to AGE/RAGE signaling and activation of the pro-oxidant hexosamine and polyol pathways [122-129]. Therefore, hyperglycemia mainly causes nerve cells and vascular endothelial cells to enter a series of intensifying inflammatory states, thereby destroying their functions, eventually leading to the outcome of DPNV and diabetic foot ulcer necrosis. At the same time, Papanas N and other studies have found that the low level of pro-inflammatory state produced by persistent hyperglycemia in diabetes makes neuropathy and vascular dysfunction even in pre-diabetes and impaired glucose tolerance [130]. The above research on the mechanism highlights the importance of hyperglycemia to promote inflammation and the need for early intervention.

In recent years, the diagnostic and therapeutic value of Netrin-1 in various inflammatory diseases has been continuously explored. Studies found that Netrin-1 inhibits inflammation and protects normal cells [131-135]. Many acute and chronic inflammatory diseases are accompanied by an increase or decrease in Netrin-1 expression [96-105]. This may inhibit the migration of inflammatory cells, inhibit the production of inflammatory cytokines and chemokines, inhibit the expression of COX-2 by inhibiting NF-κB activation, and promote the differentiation of macrophages to anti-inflammatory M2-like phenotypes. And mechanisms related to the regulation of inflammatory responses through signal transduction pathways such as MAPKs, ERKs, and p38 [136-137]. At the same time, Netrin-1 has been reported to reduce inflammatory response after myocardial ischemia-reperfusion, inhibit oxidative stress and inflammation of nerve cells and vascular endothelial cells, and resist apoptosis [100-107]. However, whether the expression of Netrin-1 is clinically relevant to inflammation-mediated DPNV has not been reported. The specific

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mechanism of action of Netrin-1 in the development of DPNV is not clear. Therefore, this study intends to obtain the lower limb ischemic muscle tissue and peripheral blood serum of clinical diabetic and non-diabetic patients, and analyze the expression levels of Netrin-1 and inflammation factors in diabetic patients pathological tissues and blood, the small vessel density and colocalization of Netrin-1 and endothelial cells by immunohistochemistry, immunofluorescence, Western Blot, ELISA, etc. And make statistical analysis of the clinical correlation between the expression of Netrin-1 and DPNV in diabetic high glucose environment.

2. Materials and methods

2.1. Materials

2.1.1. Primary reagent

TBS Buffer (Bole Life Medical Products (Shanghai) Co., Ltd., China)

PBS phosphate buffer (1x, PH 7.2-7.4) (Beijing Suo Laibao Technology Co., Ltd., China)

TBST Buffer (1x) (Beijing Suo Laibao Technology Co., Ltd., China)

Coomassie Brilliant Blue Solution (Bio-Bio Engineering (Shanghai) Co., Ltd., China):

Bovine serum albumin (BSA, Sigma, USA)

4% paraformaldehyde (Shanghai Jingdu Biotechnology Co., Ltd., China):

Methanol (Shanghai Daixuan Biotechnology Co., Ltd., China)

95% ethanol (Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine)

75% ethanol ((The Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine)

Isopropyl Alcohol (Sigma, USA) (Shanghai Daixuan Biotechnology Co., Ltd., China)

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Chloroform (Sigma, USA)

Rabbit anti-CD31 polyclonal antibody (Abcam, UK)

Mouse anti-Netrin-1 polyclonal antibody (Abcam, Cambridge, UK)

FITC-Sheep Anti-Mouse IgG Polyclonal Antibody (Abcam,Cambridge, UK)

Fluor 555-Sheep Anti-Rabbit IgG Polyclonal Antibody (Invitrogen, Carlsbad, CA)

DAPI (Xi'an Huert Biotechnology Co., Ltd., China)

Resin (Epon, Merck, Darmstadt, Germany)

OCT embedding agent (DAKO, USA)

Fluorescent sealing tablets (DAKO, USA)

Matrigel Gel (Sigma, USA)

ELISA kit (Shanghai Yiyan Biotechnology Co., Ltd., China)

2.1.2. Main instruments and equipment

15 ml centrifuge tube (Corning Inc., USA)

50 ml centrifuge tube (Corning Inc., USA)

Centrifuge (Thermo, USA)

Deionized water system (Millipore, USA)

Ultra-clean workbench (Jiangsu Sujing Group, China)

Inverted microscope (Olympus, Japan)

LumiStation 1800Plus Chemiluminescence Microplate Reader (Shanghai Flash Biotechnology Co., Ltd., China)

Fluorescence microscope (Nikon, Japan)

Precision Balance (Mettler Toledo, Switzerland)

Balance (Shanghai Chengyang Instrument Co., Ltd., China)

Refrigerator (Beijing Tiandi Jingyi Co., Ltd., China)

6-well plate (BD Falcon, USA)

Petri dish (BD Falcon, USA)

0.22 μm needle filter (Millipore, USA)

40 μm filter (BD Falcon, USA)

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PCR instrument (Thermo Hybaid, USA)

Image J software (Rawak Software Inc., Stuttgart, Germany)

Dissecting microscope (Shanghai Optical Instrument Factory, China)

Microsurgical instruments (Jiangsu Bomeida Life Science Co., Ltd., China)

Vacuum fully enclosed tissue dewatering machine (LABSUN GERMANY, Germany)

KD-2850 Low Temperature and Constant Cooling Slicer (Beijing Century Science and Technology Instrument Co., Ltd., China)

2.1.3 Clinical specimens

The clinical specimens were taken from the Department of Vascular Surgery, the Ninth People's Hospital of Shanghai Jiaotong University School of Medicine, who received amputation of the lower extremities (thigh and calf) from 2017 to 2019. Among them, 15 were diabetic patients , 13 patients were non-diabetic with acute lower extremity arterial embolism, the patients signed the informed consent form, the doctor took the patient's lower limb ischemic muscle tissue specimens, a total of 28 cases. The ischemic muscle specimens were taken from the proximal end of the limb of the proximal patient's amputation.

2.2. Methods

2.2.1Acquisition and management of clinical samples

2.2.1. Management of blood sample

All patients with severe ischemic lower limb ischemia and acute arterial embolization were enrolled in the study. After extracting 10 mL of peripheral venous blood, they were stored in a gel-promoting tube and quickly transferred to the laboratory. The blood collection tube was put in a 37 ° C incubator for 30 minutes. After the blood clots were agglutinated, the blood collection tube was centrifuged on a centrifuge, 1000 g, 15 minutes, and the serum was separated by a pipette and placed in a frozen pipe in a -80 ° C refrigerator for subsequent ELISA testing.

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2.2.1.2 Management of tissue sample

The specimens obtained during the operation were immediately placed in the ice box and quickly transferred to the laboratory to perform treatment measures in a sterile environment, as follows:

(1) For obtaining the ischemic muscle tissue specimens of the lower limbs, the tissue was cut into several pieces of tissue pieces of appropriate size (>3×3×3 cm^3) and stored in a refrigerator at
-80 °C for subsequent Western Blot experimental analysis;

(2) Cut the remaining ischemic muscle tissue specimens of lower limbs to a size of about 0.6×0.6×0.6 cm^3, place them in 4% paraformaldehyde, and sterilize them in a refrigerator at 4°C overnight. On the second day, the fixed specimen was embedded in the OCT embedding agent, and it was continuously cut into frozen sections with a thickness of 10 μm using a cryostat, placed in a refrigerator at -20 °C for subsequent immunohistochemistry and immunofluorescence detection.

2.2.2. The co-localization of Netrin-1 and CD31 was detected by immunofluorescence staining

(1) Using a 0.3% Triton-X solution for 10 min at room temperature, and then rinsing the specimen 3 times with PBS for about 5 min each time;

(2) Using 10% goat serum to seal the specimen at 37 ° C for 30 min;

(3) Adding rabbit anti-CD31 antibody (1:200 dilution) and mouse anti-Netrin-1 antibody (diluted 1:250) in turn according to the instructions, and adding PBS to the control group, and place at 4 ° C overnight. Rinsing the specimen 3 times with PBS for about 5 min each time;

(4) Adding Fluor 555 goat anti-rabbit IgG antibody and FITC goat anti-mouse IgG antibody (1:500 dilution) to the specimen in turn, placing it in a black box and protecting it from light, and placing it at 37 ° C for about 60 min. After the incubation, Rinsing the specimen 3 times with PBS for about 5 min each time;

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(5) The cells were stained with DAPI (diluted 1:1000), and after 2 min incubation, the samples were rinsed with PBS for about 5 min;

(6) The film was sealed with a fluorescent sealing tablet, and the fluorescent sheet of the specimen was prepared, and then recorded under a fluorescence microscope.

2.2.3 The expression of Netrin-1 and the density of small blood vessels were detected by immunohistochemical staining

(1) The frozen sections were placed in 1 mM EDTA, heated at 95 ° C for 10 minutes, and cooled to room temperature at room temperature to perform antigen retrieval;

(2) Fixing the cells or sections with an appropriate fixing solution, and washing them twice with the immunostaining washing solution for about 5 minutes each time;

(3) Adding an immunostaining blocking solution, and blocking for about 60 minutes;

(4) According to the primary antibody instructions, the primary antibody was diluted in an appropriate ratio. After the blocking solution was recovered, the diluted primary antibody was added, and the mixture was incubated at room temperature for 1 hour to recover the primary antibody. Rinse the specimen 3 times with PBS for about 5 min each time;

(5) According to the instructions of the second antibody, diluting the secondary antibody in an appropriate ratio. After the blocking solution was recovered, the diluted secondary antibody was added, and the mixture was incubated at room temperature for 1 hour to recover the secondary antibody. Rinsing the specimen 3 times with PBS for about 5 min each time;

(6) The tablet was sealed with a sealing tablet, and the immunohistochemical preparation of the specimen was completed, and then recorded under an optical microscope.

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2.2.4. Western Blot

(1) The lysate was firstly configured, and then the clinically acquired lower limb ischemic muscle tissue was chopped and incubated with the lysate at 0 ° C to obtain a tissue homogenate. The cells were disrupted in the ultrasound system, and the supernatant was obtained by centrifugation in a centrifuge, then, the total protein extraction was completed;

(2) The total protein was adjusted to a concentration of 2 μg/μl, and then the loading buffer was added, placed in a centrifuge tube, and heated in boiling water for 5 min to denature the protein, cooled at 4 ° C, and stored at -20 ° C;

(3) After the total protein was taken out, it was returned to room temperature, centrifuged at 10000 rpm for 1 min, then the SDS-PAGE gel was set according to the instructions, the electrophoresis apparatus was assembled, the protein was added to the electrophoresis well, and the marker was added to the blank well for electrophoresis;

(4) Soaking the PVDF membrane with methanol for 1 min, rinsing it and putting it into the membrane buffer. The gel obtained after electrophoresis was rinsed for 10 min, and then incubated with the PVDF membrane, and the membrane was transferred to the membrane;

(5) After washing the PVDF filter with TBS, incubating it in the blocking solution, and after 1 h, adding the diluted primary antibody and incubate at 4 ° C overnight;

(6) The PVDF filter was washed at room temperature and then incubated with the secondary antibody for 2 h;

(7) The PVDF filter was washed three times, and then incubated with the ECL luminescent solution, and then observed and recorded on the machine.

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2.2.5. Serum levels of inflammatory cytokines and Netrin-1 were detected by ELISA

(1) Calculating and setting the gradient concentration and sample loading of the standard well;

(2) Adding the sample to the blank hole and the sample hole to be tested, and the amount of dilution after the sample was kept consistent with the standard product hole;

(3) Incubating at 37 ° C after sealing, the duration was 30 min;

(4) diluting the concentrated washing solution for use;

(5) After uncovering the sealing film, drying it, washing the holes with liquid, pouring off after 30s, repeating 5 times and then drying;

(6) Adding the same amount of the enzyme labeling reagent to each well except the blank well. Repeating (3) (5) operation;

(7) Adding coloring agents A and B to each well, shaking well, avoiding light, incubating at 37 ° C for 15 min, then adding a stop solution, and the reaction of each well is terminated;

(8) The blank hole was set to 0, and the sample OD value was measured at 450 nm using a microplate reader.

2.2.6. Statistics

All the data in this study were analyzed using SPSS 18 software. All statistical charts and tables were drawn using GraphPad Prism 5 software, and all data were expressed as mean and standard deviation (SD). Quantitative values were compared by Student's t test and one-way ANOVA. The statistical significance was defined as *P < 0.05, **p<0.01; ***p<0.001. Each experiment was performed more than three times.

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3. Results

3.1. Baseline characteristics of clinical patients

In order to express the clinical relevance of Netrin-1 protein in DPNV, we first obtained the lower extremity muscle tissue and corresponding blood specimens of patients with severe ischemic amputation of lower limbs, and the lower limb muscle tissue specimens of patients with acute arterial embolization amputation and the corresponding blood plasma samples were as a control.

Patients who required amputation after a strict clinical evaluation by a vascular surgeon signed an informed consent form, and were obtained a blood sample before surgery, and amputated muscle tissue during surgery. A total of 28 patients were enrolled, including 15 patients with severe ischemic amputation of lower limbs and 13 patients with non-diabetic acute lower extremity arterial embolization. The baseline characteristics of all clinical patients are summarized in Table 1-1.

After comparing the clinical baseline characteristics of the two groups of patients, we found that in the amputation patients, there were 15 T2DM patients, 9 males and 6 females with an average age of 69±9.3 years. There were 13 non-DM patients, 8 males and 5 females with an average age of 71±8.1 years. There was no significant difference in age and gender composition ($P = 0.934$). Except for 10.3±3.1mmol/L in T2DM patients with randomized blood glucose, which was much higher than 4.8±1.1mmol/L in non-DM patients, the difference was statistically significant ($P<0.001$). The rest were not statistically different in terms of blood pressure, cholesterol, HDL and LDL. Therefore, the next step of proteomics and histology could be performed in the clinical specimens of the two groups to observe the difference of Netrin-1 expression in the ischemic muscle tissue of the lower limbs of T2DM and non-DM patients.

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3.2. The expression of Netrin-1 in ischemic muscle tissue and peripheral blood of T2DM was significantly decreased

The most important lesion of DPNV is the structural destruction and loss of function of microvessels and large blood vessels. To explore the clinical relevance of Netrin-1 to DPNV. First, we determined that Netrin-1 was co-expressed with endothelial cell-specific marker CD31 on human endothelial cells by immunofluorescence assay, demonstrating the colocalization of Netrin-1 with endothelial cells (Fig. 1-1). Secondly, we used immunohistochemistry to determine the expression of Netrin-1 and small vessel density in the ischemic muscles of the lower limbs of T2DM patients and the control group. Histological and statistical analysis showed that the expression level of Netrin-1 and small blood vessel density in the lower extremity muscle of T2DM patients were significantly lower than those in the control group (Fig. 1-2, Fig. 1-3, Fig. 1-4, Fig. 1-5). At the same time, Western Blot of ischemic muscle tissue of the lower extremity quantitatively confirmed the significant decrease in Netrin-1 expression in muscle tissue of T2DM patients (Fig. 1-6). ELISA of peripheral blood serum also showed a significant decrease in the expression level of Netrin-1 in peripheral blood (Fig. 1-7). This study showed that the expression level of Netrin-1 in the damaged part and systemic blood circulation was related to the number of endothelial cells and the degree of vascular destruction of DPNV. The expression level of Netrin-1 was clinically correlated with DPNV.

3.3. The expression level of inflammatory cytokines in peripheral blood of patients with T2DM was significantly increased

Next, we used ELISA to detect the expression levels of IL-6, IL-1β, MCP-1 and IL-12 in peripheral blood serum of T2DM and control groups. The results of ELISA and statistical analysis showed that the expression levels of inflammatory factors in peripheral blood of patients with T2DM were significantly higher than those of the control group ($P<0.05$, Figure 1-8). The results of this study indicate that there is a

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certain degree of negative correlation between the expression level of Netrin-1 and inflammatory factors in peripheral blood in patients with DPNV under continuous hyperglycemia.

4. Discussion

DPNV is essentially a chronic inflammatory disease caused by persistently elevated blood sugar that is poorly controlled by diabetics. Michael Brownlee and other first proposed "the theory of the mechanism of diabetes complications" revealed that long-term hyperglycemia leads to low levels of pro-inflammatory microenvironment, thereby destroying vascular endothelial cells through multiple mechanisms based on oxidative stress. And the function of nerve cells [121]. The nerve cells and vascular endothelial cells have mutual interdependence of providing nutrients, which leads to further deterioration of the environment of the two, forming a waterfall reaction, which causes the disease of the affected limb to deteriorate rapidly, and eventually develops into a diabetic foot, which could not be healed. The ulcers and gangrene eventually end in amputation and even endanger life [122-129]. Clinically available methods such as optimal drug therapy, intravascular balloon dilation, stent implantation, and vascular bypass revascularization can control symptoms in a short period of time, but their long-term effects are poor [110-119]. This suggests that deeper mechanism remains to be explored, and that if we can get a glimpse of some biological indicators in the early stages of DPNV, we may receive better clinical treatment results.

As a secreted protein that inhibits inflammation, Netrin-1 is widely found in blood and tissue fluids. Its relationship with various inflammatory diseases is becoming clear, and it is used as a biomarker for many acute and chronic inflammatory diseases, which may be related to the body. Netrin-1 inhibits inflammatory cell migration, inhibits the production of inflammatory cytokines and chemokines, regulates the expression of COX-2 by inhibiting NF-κB activation, promotes the differentiation of macrophages to anti-inflammatory M2-like phenotypes,

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and through MAPKs. Mechanisms such as ERKs, p38 and other signal transduction pathways regulate inflammatory responses [136,137]. Previous studies have shown that Netrin-1 is the first identified axon guidance factor and has been shown to play an important role in angiogenesis and endothelial cell proliferation, with its "double-sided" activity in both the vascular and neurological domains. We paid attention to it, and whether Netrin-1 has significant changes in the mixed lesions of blood vessels and nerves of DPNV has not been reported. Therefore, this study focused on the detection of Netrin-1 in the lower limb tissue and expression levels in peripheral blood serum of diabetic patients.

This study included 15 patients with diabetic lower limbs chronic ischemic amputation as the experimental group and 13 patients with acute lower extremity arterial embolization as the control group. Statistical analysis showed that there was no statistical difference between the two groups except blood glucose, which could be compared. First, the immunofluorescence colocalization of Netrin-1 with vascular endothelial cells indicated that Netrin-1 was expressed on vascular endothelial cells. The immunohistochemical results of CD31 showed that the number of microvessels in the lower limb ischemic tissue was significantly reduced, which may be due to patients with lower extremity arterial embolism often have embolism falling into the lower extremity arteries, they often have acute onset and progress quickly. If there is no effective salvage within 24 hours, they can only be amputated. Therefore, the lower limbs are basically healthy before the onset, and there is no ischemia; while the lower limb ischemia of diabetic patients is often a chronic process, when the amputation develops, the persistent hyperglycemia has exhausted the compensated microvascular depletion and entered the decompensation period, so the number of microvessels in the lower extremities is significantly reduced, showing a picture of desert state. By the immunohistochemical staining results of Netrin-1 and Western Blot detection of ischemic tissue, we found that the expression level of Netrin-1 in the ischemic muscle of the lower limbs of diabetic patients was significantly lower than that of the lower limb ischemia muscles due to arterial embolization. The results of

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ELISA showed that the expression level of Netrin-1 protein in peripheral blood serum was also significantly decreased, while in the blood, inflammatory factors such as IL-6, IL-1β, MCP-1 and IL-12 were significantly increased. Elevation confirms that DPNV is a chronic inflammatory disease, and the decrease in Netrin-1 level suggests that we have a negative correlation between the expression of Netrin-1 and the expression of inflammatory factors in DPNV lesions. The possible reason is that Netrin-1 itself is an anti-inflammatory and anti-apoptotic protein against blood vessels and nerves, and the long-term hyperglycemia state makes tissue cells always in the inflammatory microenvironment of oxidative stress, in the very early stage of DPNV. Netrin-1 may be reactively increased, but the long-term inflammatory microenvironment will gradually deplete Netrin-1 in the vascular nerve cells of the tissue, and Netrin-1 in the blood will gradually decrease, and this result is related to Liu C et al. Consistent with the findings, they found that the level of Netrin-1 expression in the blood of newly diagnosed T2DM patients was significantly lower than that of non-diabetics [105]. Our experimental results show that inflammatory factors such as IL-6, IL-1β, MCP-1 and IL-12 are significantly elevated in the blood of diabetic patients, confirming the conclusion that DPNV is a chronic inflammatory disease. At the same time, Netrin-1 acts as a secreted protein against inflammation, and its tissue and blood expression levels are inversely related to the progression of DPNV. In summary, we believe that Netrin-1 plays an important regulatory role in the development of the body against DPNV. The change in expression level can reflect the progress of DPNV disease, and may be a promising biomarker of DPNV in the future. However, its specific regulatory roles and mechanisms require further research and discovery.

5. Summary

In this study, we obtained the clinical analysis of the lower limb ischemia tissue and the peripheral blood serum of patients with diabetes and non-diabetes. The immunohistochemistry, immunofluorescence, Western Blot, ELISA and other

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methods were used to analyze the Netrin-1 in the ischemic tissue of the lower limbs of diabetic patients. Colocalization with endothelial cells, expression levels of Netrin-1, small vessel density, and expression levels of Netrin-1 and inflammatory factors in peripheral blood serum were also analyzed. And statistical analysis to determine the clinical correlation between the expression of Netrin-1 and DPNV in diabetic hyperglycemia environment was performed. It was found that Netrin-1 protein was expressed in endothelial cells of DPNV patients, but the expression level of Netrin-1 was significantly decreased in peripheral blood serum and ischemic muscle tissues of lower limbs, and the expression levels of multiple inflammation-related factors were significantly increased, and the density of microvessels was significantly lower than the control group. The results of this experiment indicate that Netrin-1, as a protein against inflammation, participates in the pathological process of DPNV development and plays an important role. The expression levels in tissues and serum were negatively correlated with the progression of DPNV disease. In the future, Netrin-1 may become a potential biomarker of DPNV. Its specific mechanism of action and molecular mechanism in the development of DPNV need further research and further exploration.

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Chapter 2 Study on Netrin-1 regulating the function of ADSCs in high-glucose environment in vitro

1. Introduction

The incidence of DPNV is high, with early but insidious onset, long course of disease, difficulty to cure, eventually it leads to amputation and is even life-threatening. There is currently no effective long-term treatment plan in clinical practice. With the rapid development of regenerative medicine, scientists have gradually turned their attention to the "seed cells" with multi-directional differentiation potential--stem cells for these diseases that have no clinically satisfactory treatment methods.

With the deepening of stem cell research, mesenchymal stem cells (MSCs) are currently used in laboratory and clinical applications. They are a class of undifferentiated cells that can self-renew and differentiate into a variety of specific cell types [138]. Bone marrow mesenchymal stem cells (BMSCs) have been studied in the past, but studies in the past few years have found that MSCs actually exist in many organs and connective tissues. MSCs with similar sources of bone marrow were successfully isolated from different tissue sources such as periosteum, synovium, skeletal muscle, skin, peripheral blood and umbilical cord [139]. However, there is a widespread problem of low harvest cell yield and limited harvesting tissue. At the same time, obtaining BMSCs requires bone marrow aspiration, requires general anesthesia and is relatively painful and expensive, and the cell yield of BMSCs obtained is very low [140]. It has recently been discovered that adipose tissue is a good alternative source of stem cells, especially as the incidence of obesity increases, making tissues rich and accessible. In a safer, less expensive liposuction procedure, a large amount of fat can be collected from the donor site [141]. About 400,000 liposuctions are performed each year in the United States, which produce 100 to 3 liters of liposuction, and these tissues are usually discarded [142]. In addition, high

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levels of ADSCs in adipose tissue do not require long-term in vitro culture, reducing the risk of chromosomal abnormalities [141, 143, 144]. ADSCs are superior to BMSCs in many biological characteristics, including immunoregulation and secretion of various growth factors and cytokines as well as anti-apoptotic and anti-inflammatory effects [145-149].

ADSCs are not only widely used by basic sciences, but are also widely used in clinical regenerative medicine. ADSCs can treat many different diseases and show great potential for tissue repair and regulation of host immune responses in vivo [147]. ADSCs from healthy donors are a very attractive source of cells for organ regeneration [150]. These cells can be cultured in vitro to a sufficient number for subsequent regeneration of damaged tissue [151]. ADSCs have broad-spectrum differentiation potential when induced in vitro and can differentiate into various functional cells derived from mesoderm to ectoderm (such as hepatocytes) and endoderm (such as beta cells) [151-154]. ADSCs can be cultured for a long period of time without passaging without losing their ability to differentiate, and can survive at low temperatures, while their ability to survive, proliferate and differentiate is less impaired. The most attractive aspect of ADSCs is their immunosuppressive properties, which allow for the transplantation of ADSCs regardless of the matching of human leukocyte antigen (HLA) between the host and the donor [155]. In addition, ADSCs can express different types of trophic factors, such as cell proliferation, fibrosis, angiogenesis, and immunosuppression [156-163]. In addition, ADSCs have significant anti-apoptotic, anti-oxidative, anti-inflammatory activities, which are important properties that contribute to tissue regeneration [164-168]. To date, in more than 160 clinical trials of ADSCs, ADSCs have been used to treat various diseases such as inflammatory diseases, liver failure, diabetes-related complications, multiple sclerosis, orthopedic diseases, hair loss, and fertility. Problems and diseases such as salivary gland damage. Different preclinical and clinical trials have demonstrated the safety and efficacy of ADSCs transplantation to promote tissue regeneration, and there have been no reports of serious side effects so far [169-187]. Therefore, ADSCs

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are also expected to be a suitable choice for the treatment of DPNV.

ADSCs are increasingly becoming the popular "seed cells" for basic and clinical research. The therapeutic effect of promoting tissue regeneration after transplantation has been confirmed, but there are still many limitations to be overcome. The most urgent problem to be solved is the problem of extremely low survival after transplantation. Positron emission tomography (PET) tracking of MSCs delivered via catheter-based trans-endocardial injection showed that only about 6% of MSCs survived in porcine ischemic myocardium 10 days after transplantation [188]. Toma et al. reported that surviving MSCs did not exceed 0.44% on day 4 after heart transplantation in immune-deficient mice [189]. Similarly, only about 1% of MSCs were detected 24 hours after cardiac transplantation in experimental MI rats [190, 191]. More studies have found that the symptoms of lower limb ischemia and objective indicators of diabetic patients have improved to some extent, but their long-term efficacy is still poor [36, 64-70]. A large number of studies have also confirmed that there are advanced glycation end products, inflammatory microenvironment and oxidative stress in the vascular injury site around diabetes, which makes the survival and migration of transplanted stem cells in the target vascular injury site and high glucose environment. The ability to differentiate significantly is the key to affecting the efficacy of stem cells [15-17, 71-77]. Therefore, how to promote the survival and differentiation of ADSCs in the vascular injury sites in diabetes and promote the regeneration of diabetic blood vessels after denervation is of great significance for the prevention and treatment of DPNV lesions.

Studies found that DPNV is a mixed lesion with vascular, neurological and tissue damage [28, 78-80]. Netrin-1, a secret protein originally discovered in nerve growth, has recently been increasingly found to be involved in angiogenesis, endothelial cell survival, adhesion, migration, proliferation and apoptosis, and in a variety of inflammatory properties. Diseases, including diabetes and diabetic complications, inhibit inflammatory cell migration, inhibit the production of inflammatory cytokines and chemokines, regulate the expression of COX-2 by inhibiting NF-κB activation,

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and promote macrophage to anti-inflammatory M2-like phenotype, as well as anti-inflammatory effects such as inflammatory responses through signal transduction pathways such as MAPKs, ERKs, and p38 [136-137]. Therefore, we believe that overexpression of Netrin-1 by gene transfection of ADSCs can improve the ability of viability, migration and differentiation into vascular endothelial cells of ADSCs under hyperglycemia, and promote DPNV angiogenesis. In this study, we obtained the adipose tissue of C57/BL mice, isolated and cultured ADSCs, and established a gene transfection system to make ADSCs overexpress green fluorescent protein (GFP) and Netrin-1 (N-ADSCs). To explore the difference between the proliferation, migration, adhesion and differentiation of N-ADSCs and ADSCs in high glucose environment by CCK-8, Western Blot, Flow Cytometry, Transwell, etc, and explore the effect of Netrin-1 on ADSCs in high glucose environment in vitro.

2. Materials and methods

2.1. Materials

2.1.1. Primary reagent

PBS phosphate buffer (1x, PH 7.2-7.4) (Beijing Suo Laibao Technology Co., Ltd., China)

Trypsin-EDTA (Trypsin-EDTA, 0.25%, Beijing Boaolong Immunotechnology Co., Ltd., China)

DMEM solution:

1g/L Dulbecco's modified eagle medium (DMEM; Gibco, USA)

10% fetal bovine serum (FBS, Hyclone, Australia)

NB4 collagenase solution:

0.2% collagenase NB4 (Serva, Heidelberg, Germany)

DMEM solution preparation

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TBS Buffer (Bole Life Medical Products (Shanghai) Co., Ltd., China)

PBS phosphate buffer (1x, PH 7.2-7.4) (Beijing Suo Laibao Technology Co., Ltd., China)

TBST Buffer (1x) (Beijing Suo Laibao Technology Co., Ltd., China)

Coomassie Brilliant Blue Solution (Bio-Bio Engineering (Shanghai) Co., Ltd., China):

Bovine serum albumin (BSA, Sigma, USA)

4% paraformaldehyde (Shanghai Jingdu Biotechnology Co., Ltd., China):

Methanol (Shanghai Daixuan Biotechnology Co., Ltd., China)

95% ethanol (Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine)

75% ethanol ((The Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine)

Isopropyl Alcohol (Sigma, USA) (Shanghai Daixuan Biotechnology Co., Ltd., China)

Chloroform (Sigma, USA)

DAPI (Xi'an Huert Biotechnology Co., Ltd., China)

Resin (Epon, Merck, Darmstadt, Germany)

OCT embedding agent (DAKO, USA)

Fluorescent sealing tablets (DAKO, USA)

Matrigel Gel (Sigma, USA)

Collagen Collagen II (Abcam, Cambridge, MA)

CCK-8 (Dojindo Laboratories, Kumamoto, Japan)

PI dye solution (Sigma, USA)

Ammonium oxalate crystal violet staining solution (crystal violet staining solution) (Beijing Suo Laibao Technology Co., Ltd., China)

PE-anti-mouse flow antibody CD90, CD11b, CD31, CD34, CD45, CD133, MHC-II (eBioscience, San Diego, CA)

PE-Anti-Mouse Flow Antibody Sca-1 (eBioscience, San Diego, CA)

FITC-Sheep Anti-Mouse IgG Polyclonal Antibody (Invitrogen, Carlsbad, CA)

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DAPI (Invitrogen, Carlsbad, Canda)

Resin (Epon, Merck, Darmstadt, Germany)

Fluorescent sealing tablets (DAKO, USA)

Matrigel Gel (Sigma, USA)

Sodium pentobarbital (Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, China)

Vector pHBAd-EF1-MCS-GFP (Shanghai Hanheng Biotechnology Co., Ltd., China)

E. coli strain DH5α (Invitrogen, Carlsbad, CA)

Restriction enzyme (Thermo Fisher Scientific, USA)

One Step Cloning Kit (Nanjing Nuoweizan Biotechnology Co., Ltd., China)

Plasmid DNA Large Extraction Kit (Beijing Kangwei Century Biotechnology Co., Ltd., China)

Gel Recovery Kit (Shanghai Jierui Bioengineering Co., Ltd., China)

Agarose, agar powder (Bio-Bio Engineering (Shanghai) Co., Ltd., China)

DNA ladder (Thermo Fisher Scientific, USA)

Human embryonic kidney cell line HEK293 cell (Shanghai Chinese Academy of Sciences, China)

LipofiterTM Transfection Reagent (Shanghai Hanheng Biotechnology Co., Ltd., China)

2.1.2. Main instruments and equipment

Constant temperature CO_2 incubator (Forma Scientific, USA)

Centrifuge (Thermo, USA)

Deionized water system (Millipore, USA)

Ultra-clean workbench (Jiangsu Sujing Group, China)

Inverted microscope (Chongqing Optoelectronic Instrument Corporation, China)

Fluorescence microscope (Nikon, Japan)

Precision Balance (Mettler Toledo, Switzerland)

Tianping (Shanghai Hanyu Hengping Scientific Instrument Co., Ltd., China)

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Refrigerator (Haier, China)

6-well plate (Corning Inc., USA)

Petri dish (Corning Inc., USA)

50ml centrifuge tube (Corning Inc., USA)

15ml centrifuge tube (Corning Inc., USA)

96-well plate (Wuxi Nice Biotechnology Co., Ltd.)

Pipette (Wuxi Nice Biotechnology Co., Ltd.)

0.22 μm needle filter (Millipore, USA)

40 μm filter (BD Falcon, USA)

Microsurgical instruments (Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, China)

Flow Cytometry (Beckman Coulter, Fullerton, Canada)

Image-Pro Plus software (Media Cybernetics Inc, Rockville, MD)

Low temperature circulating water bath Polystat (Cole-Parmer Instrument Comapny, USA)

Electric thermostatic water bath (Shanghai Yiheng Technology Co., Ltd.)

Electrophoresis Instrument (Shanghai Tianneng Technology Co., Ltd.)

PCR Amplifier (Applied Biosystems)

Electronic balance (Beijing Sartorius Instrument System Co., Ltd., China)

Protein Nucleic Acid Analyzer (Eppendorf, Germany)

MINI Centrifuge (Tomy Seiko Co., Ltd. Japan)

Gradient PCR instrument (Eppendorf, Germany)

Vertical clean bench (Shanghai net purification)

Refrigerated centrifuge (Eppendorf, Germany)

Full temperature oscillation incubator (Shanghai Yiheng Technology Co., Ltd., China)

Desktop multi-function high speed refrigerated centrifuge (Eppendorf, Germany)

Autoclave (Shanghai Shen'an Medical Instrument Factory, China)

High speed refrigerated centrifuge (Thermo Fisher Scientific, USA)

Gel Imaging System (Shanghai Tianneng Technology Co., Ltd., China)

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2.1.3. Experimental animal

C57/BL6 mice aged 8W (Shanghai Nan Fang model biology co., LTD., China).

2.2. Methods

2.2.1. Acquisition, culture and amplification of ADSCs

(1) Anesthetized C57/BL6 mice aged 8W with pentobarbital sodium, then sacrificed by dislocation of the cervical vertebrae, and immersed in 75% alcohol for 10 minutes to fully disinfect;

(2) Fully expose the bilateral groin with an anatomical instrument, cut off the adipose tissue, and place it in a small amount of DMEM solution;

(3) Fully cut the adipose tissue obtained by microdissection until the size is about $1mm^3$, put it into a 50mL centrifuge tube which has been filled with 0.2% NB4 in DMEM solution, put it into the constant temperature shaker, and set the temperature. Full digestion for 2h at 37 ° C;

(4) After taking out, thoroughly blow for 5 minutes, centrifuge at 1200 rpm for 5 min, aspirate the supernatant; add 5 mL of the configured ADSCs culture solution (DMEM plus 10% serum plus 5% antibiotic) to resuspend the cells;

(5) Seeding the cell suspension in a 10 cm culture dish at a density of 105 cells/cm 2 and incubating in a cell culture incubator;

(6) After 3 days, the primary cells were observed, and it was found that the cells were fused to about 90%, and they could be passaged.

(7) 2 ml of 0.25% trypsin-EDTA was added to the culture dish, and after being placed in the incubator for 3 minutes, the 5 ml of ADSCs culture solution was taken out and the digestion was terminated. After fully blowing, transfer to a 50mL centrifuge tube and centrifuge at 1500rpm for 5min;

(8) Pour off the supernatant, and resuspend the primary cells by adding 10 mL of ADSCs culture solution;

(9)Seeding the cell suspension in a 10 cm culture dish at a density of 105

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cells/cm 2 at a ratio of 1:2, and incubating in a cell culture incubator;

(10) After 48 hours, the above steps (7), (8) and (9) were repeated, and the ADSCs were passaged and expanded.

2.2.2. Flow cytometry identification of ADSCs

(1) Carefully pour off the supernatant of the Petri dish, rinse once with PBS at 37 ° C, and add 0.25% trypsin-EDTA 2 mL. When most of the cells contracted into a circle, ADSCs medium was added. Stop digestion with 3 mL of liquid;

(2) The liquid was filtered through a 40 μm filter into a 15 mL centrifuge tube, centrifuged at 1500 rpm for 5 min, and the supernatant was discarded;

(3) Resuspend with Buffer to make a final cell concentration of 10^6 cells/mL, and use a 1.5 mL EP tube to hold 100 μl of liquid and number it;

(4) Add PE-labeled diluted CD90, Sca-1 CD90, CD11b, CD31, CD34, CD45, CD133, MHC-II (1:20) and control antibody, and place in the refrigerator at 4 °C for 30 min in the dark;

(5) Rinse once with buffer, centrifuge at 1500 rpm for 5 min, discard the supernatant and rinse 3 times;

(6)Resuspend the cells in buffer and store the strips on the ice to the flow detector for detection.

2.2.3. Construction and packaging of GFP-Netrin-1 gene adenovirus vector

(1) Review the relevant literature, open NCBI according to the ID number of the Netrin-1 gene mentioned in the NCBI, select Nucleotide in All Databases, input and search the gene ID number, and obtain the Netrin-1 gene sequence.

(2) The pHBAd-EF1-MCS-GFP vector was digested with EcoRI, and the enzyme digestion system was as follows:

40ul digestion system 37 ° C, about 2h

2ul carrier (400ng/ul)

2ul EcoRI

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4ul 10×buffer

32ul H2O

After the carrier is cut, the glue is recovered;

(3) The Netrin-1 fragment was recovered by PCR.

(4) The treated target fragment was ligated to the carrier (20 ul), and the above ligated solution was incubated at 37 for 30 min, then rapidly placed on ice for 1-2 min.

(5) Transformation (competent cells: DH5a). Resistance: Amp; cultured overnight at 37 °C.

(6) The transformed Netrin-1 plate was picked up, shaken at 37 °C for 250 rpm for 14 h, and identified by PCR. The positive cloning bacteria were sent to the sequencing company for sequencing.

(7) Preparation of recombinant plasmids in large quantities

(8) Packaging, collection and amplification of recombinant adenovirus vector, the specific operations are: planting cells, transfection, liquid exchange, poisoning (P1), freeze-thaw, amplification, and poison collection.

(9) P3 generation virus amplification and poison collection.

2.2.4. Establishment of a system for transfecting ADSCs in vitro with Netrin-1

(1) The P3 generation vigorously growing ADSCs were digested and counted, and the packaged GFP adenovirus containing NTN-1 and NTN-1 was added to 6×104 cells in a 10 cm culture dish to multiply the infection (MOI).) Set to three groups: 250, 500, and 1000, and the duration is set to 24 hours and 48 hours. The best MOI and duration of transfection of ADSCs were studied by direct immunofluorescence microscopy to obtain the maximum transfection efficiency, and a transfection system was established.

(2) The expression of Netrin-1 in ADSCs transfected with GFP and GFP-Netrin-1 was compared by RT-PCR and Western Blot.

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2.2.5 Proliferation ability of CCK8 in transfected and untransfected ADSCs with Netrin-1

ADSCs transfected with P3 and untransfected with Netrin-1 were seeded in 7 96-well culture plates at a density of 2×103 cells per well, and 4 wells were blank control wells without cells plus culture medium. In the final colorimetric, a blank hole is used as the zero adjustment. The culture plate was cultured in a CO2 incubator, and then 6 wells were taken at the same time every day, and each well was added with CCK-8 solution (10 μl), incubated for 2 h, the supernatant was aspirated, and the wavelength of 450 nm was selected in the enzyme-linked immunosorbent assay. The light absorption value (OD) of each well was measured, and the results were recorded for 7 days, and the cell growth curve was marked.

2.2.6. Flow cytometry and Western Blot detection of apoptosis in transfected and untransfected ADSCs with Netrin-1

Flow cytometry test:

(1) Carefully pour off the supernatant of the Petri dish, rinse once with PBS at 37 ° C, and add 0.25% trypsin-EDTA 2 mL. When most of the Netrin-1 transfected and untransfected ADSCs were contracted into a round shape in a high glucose environment, the ADSCs medium was added. Stop digestion with 3 mL of liquid;

(2) The liquid was filtered through a 40 μm filter into a 15 mL centrifuge tube, centrifuged at 1500 rpm for 5 min, and the supernatant was discarded;

(3) Resuspend with Buffer to make a final cell concentration of 10^6 cells/mL, and use a 1.5 mL EP tube to hold 100 μl of liquid and number it;

(4) Adding PE-labeled diluted Annexin V and PI (1:20) and control antibody, and keeping it in the refrigerator at 4 °C for 30 min;

(5) Rinse once with buffer, centrifuge at 1500 rpm for 5 min, discard the supernatant and rinse 3 times;

(6) Resuspend the cells in buffer and store the strips on the ice to the flow

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detector for detection.

Western Blot:

(1) The lysate was firstly configured, and then the clinically acquired lower limb ischemic muscle tissue was chopped and incubated with the lysate at 0 ° C to obtain a tissue homogenate. The cells are disrupted in the ultrasound system, and the supernatant is obtained by centrifugation in a centrifuge, that is, the total protein extraction is completed;

(2) The total protein is adjusted to a concentration of 2 μg/μl, and then the loading buffer is added, placed in a centrifuge tube, and heated in boiling water for 5 min to denature the protein, cooled at 4 ° C, and stored at -20 ° C;

(3) After the total protein was taken out, it was returned to room temperature, centrifuged at 10000 rpm for 1 min, then the SDS-PAGE gel was set according to the instructions, the electrophoresis apparatus was assembled, the protein was added to the electrophoresis well, and the marker was added to the blank well for electrophoresis;

(4) Soak the PVDF membrane with methanol for 1 min, rinse it and put it into the membrane buffer. The gel obtained after electrophoresis was rinsed for 10 min, and then incubated with the PVDF membrane, and the membrane was transferred to the membrane;

(5) After washing the PVDF filter with TBS, incubated in the blocking solution, and after 1 h, add the diluted primary antibody and incubate at 4 ° C overnight;

(6) The PVDF filter was washed at room temperature and then incubated with the secondary antibody for 2 h;

(7) The PVDF filter was washed three times, and then incubated with the ECL luminescent solution, and then observed and recorded on the machine.

2.2.7. Cell migration assay and cell scratch assay to detect the ability of Netrin-1 to regulate the migration of ADSCs

2.2.7.1 Cell migration experiment

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(1) 2×103 cells of P3 generation GFP-ADSCs and Netrin-1-GFP-ADSCs were respectively suspended in 100 μL of high glucose DMEM supplemented with 0.5% FBS.

(2) Using a Boyden chamber and an 8-μm polyethylene terephthalate (PET) membrane, liquid was added to the upper portion of the Transwell chamber, and 600 μL of ADSCs medium was added to the lower portion of the chamber.

(3) Place the Transwell plate in a cell culture incubator for 24 hours.

(4) Remove the filter and use the cotton tip to remove the cells in the upper chamber. The ADSCs migrated to the lower chamber were fixed using 4% paraformaldehyde, and the cells were stained with 1% crystal violet in 2% ethanol. The experiment was repeated three times.

(5) Take a picture under the microscope.

2.2.7.2 Cell scratch test

(1) Instrument preparation: All sterilizable instruments should be sterilized, and the ruler and pen should be irradiated for 30 minutes before operation (super clean bench)

(2) First use a marker on the back of the 6-well plate, and draw a horizontal line across the hole with a ruler, about 0.5~1cm. Each hole passes at least 5 rows of lines.

(3) 10^5 P3 generation GFP-ADSCs and Netrin-1-GFP-ADSCs were added.

(4) The next day, use the tip of the gun to draw a straight line along the horizontal line. The tip of the gun should be vertical rather than tilted.

(5) Wash the cells three times with PBS, take out the crossed cells, and add serum-free DMEM.

(6) Place in a cell culture incubator. Samples were taken at 0, 6, 12, 24 h and observed under a microscope and photographed.

2.2.8. Cell adhesion assay to detect the ability of Netrin-1 to regulate adhesion of ADSCs

(1) Take P3 generation GFP-ADSCs and Netrin-1-GFP-ADSCs, trypsin-EDTA 2

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mL. When most of the Netrin-1 transfected and untransfected ADSCs were contracted into a round shape in a high glucose environment, the ADSCs medium was added. Stop digestion with 3 mL of liquid;

(2) rinsing the cells three times with PBS, taking 5×104 cells each with a cell counter, and uniformly plating the cells in a 6-well plate;

(3) Put the 6-well plate into the cell culture incubator and take it out after 30 minutes;

(4) After rinsing in a 6-well plate with PBS for 3 times, the cells were stained with DAPI (1:1000 dilution), and after 2 min incubation, the samples were rinsed with PBS for about 5 min;

(5) The slide was sealed with a fluorescent sealer, and the specimen was made into a fluorescent sheet, and then photographed under a fluorescence microscope.

2.2.9. Tube formation assay to detect the ability of Netrin-1 to regulate the formation of ADSCs

(1) The ECM gel was frozen and prepared into a 1x staining buffer.

(2) Using a sterile 96-well plate, 50 μL of liquid ECM gel was added to each well.

(3) The 96-well plate containing the ECM gel was placed in a 37 ° C incubator, and after 1 h, the ECM solution formed a solid gel.

(4) Take P3 generation GFP-ADSCs and Netrin-1-GFP-ADSCs, trypsin-EDTA 2 mL. When most of the Netrin-1 transfected and untransfected ADSCs were contracted into a round shape in a high glucose environment, the ADSCs medium was added. Stop digestion with 3 mL of liquid;

(5) The cells were resuspended in endothelial cell culture medium containing 10% FBS to 4 x 105 cells/mL.

(6) 150 μl of the suspension of ADSCs was transferred to an ECM gel, and the 96-well plate was incubated at 37 ° C for 12 h.

(7) Observing the tube formation of ADSCs with a microscope under high-resolution field of view. Visually observe the length of the tube and the number

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of branch points to estimate the tube condition. Take a photo and apply quantitative analysis using ImageJ Pro Plus software (NIH, Bethesda, MD, http://www.imagej.nih.gov).

2.2.10. Statistics

All the data in this study were analyzed using SPSS 18 software. All statistical charts and tables were drawn using GraphPad Prism 5 software, and all data were expressed as mean and standard deviation (SD). Quantitative values were compared by Student's t test and one-way ANOVA. The statistical significance was defined as *P < 0.05, **p<0.01; ***p<0.001. Each experiment was performed more than three times.

3. Results

3.1. Biological characteristics of ADSCs

First, we successfully obtained the inguinal adipose tissue of C57 BL6 mice, and then isolated ADSCs and rapidly expanded them. P3 generation ADSCs all showed a fibroblast-like spindle-like appearance (Fig. 2-1A). Next, phenotypic analysis by flow cytometry showed that P3 generation ADSCs had strong double positives for stem cell surface antigens, such as CD90 (99.2 3.73%) and Sca-1 (99.6 3.08%) (Figure 2-1). CD11b, CD31, CD34, CD45, CD133 and MHC-II were negative (data not shown). However, P6 generation ADSCs may exhibit irregular shape and aging or dedifferentiation characteristics due to prolonged culture time and increased in vitro passage algebra (Fig. 2-1B). Flow cytometry analysis showed that ADSCs stem cell-specific markers CD90 (65.4±3.42%) and Sca-1 (59.8±3.15%) surface antigen levels (Fig. 2-1C, D) gradually decreased, the difference was statistically significant (P <0.05). Therefore, next we will use P3-P5 generation ADSCs cells for all subsequent experiments.

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3.2. Establishment of gene transfection system

Adenoviral vectors have a broad host range and are less pathogenic in humans than other viral vector systems. These vectors can infect and express target genes in proliferating and non-proliferating cells without integration into chromosomes, they are not mutagenic, and can simultaneously express multiple genes; in addition, they can be produced and packaged at high titers. The expression of the transgene is long and the side effects are small [192]. NTN-1 was transduced into ADSC by adenovirus; by detection and comparison, we found that the optimal multiplicity of infection (MOI) was 500, and the duration of transfection of ADSCs was 48 hours to achieve maximum transfection efficiency (Figure 2-2). After comparison, there was no significant difference in transfection rates between Netrin-1 and GFP in ADSCs cells (Fig. 2A, B). Western Blot, PCR and statistical analysis confirmed that the expression of Netrin-1 was significantly increased in the N-ADSCs group, and it is worth noting that Netrin-1 was almost not expressed in the cells of the ADSCs group ($P < 0.05$) (Fig. 2C-E), it was confirmed that the secreted protein of Netrin-1 was not expressed by itself in the ADSCs in the normal state, and therefore, the si-RNA-NTN-1 group in which Netrin-1 mRNA was silenced was not required to be used as a blank control. Here, we successfully established a Netrin-1 overexpression system in which the NTN-1 gene was efficiently transfected and expressed in ADSCs.

3.3. Effect of Netrin-1 on the proliferation of ADSCs under high glucose

Throughout the experimental study, we used ADSCs cultured in high-glucose medium DMEM (33.3 mmol/L glucose) to culture ADSCs to simulate the hyperglycemia in T2DM for subsequent experiments. From day 1 to day 7, the proliferation and growth viability of ADSCs and N-ADSCs were assessed using the CCK-8 assay to explore the effect of Netrin-1 on cell viability (Figure 2). 3). Statistical analysis of the results showed that the growth of ADSCs and N-ADSCs were relatively stable within the first 3 days, with little difference. From day 3 to day

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7, both cells showed a pattern of logarithmic growth. Compared to ADSCs, N-ADSCs showed significantly higher growth rates from day 3 to day 7 ($P < 0.05$, $n = 5$). This study confirmed the promotion of proliferation of ADSCs by Netrin-1.

3.4. Effect of Netrin-1 on apoptosis of ADSCs under high glucose

Previous studies have found that hyperglycemia has a negative impact on the proliferation and apoptosis of ADSCs in T2DM patients. ADSCs in T2DM mice show low activity and high apoptotic rate [15-17, 71-77]. To investigate the effect of Netrin-1 on apoptosis of ADSCs under high glucose conditions, we stained cells with Annexin V / PI and analyzed by flow cytometry. The percentage of apoptosis in the N-ADSCs group was 7.7±0.44%, while that in the ADSCs group was 10.9±0.32% (Fig. 2-4A, B). The difference was statistically significant ($P<0.05$. Fig.2-4C). Bcl-2 protein represents inhibition of apoptosis, while Bax protein represents the promotion of apoptosis. The Western Blot and statistical analysis of this experiment showed that the Bcl-2/Bax ratio of N-ADSCs was significantly higher than that of ADSCs under high glucose conditions (Fig. 2-4D, E), indicating the apoptosis of N-ADSCs. The mortality rate was inhibited, demonstrating the anti-apoptotic effect of Netrin-1 on ADSCs in vitro. In conclusion, transfection of Netrin-1 into ADSCs promoted its proliferation in a high glucose environment while significantly inhibiting its apoptosis.

3.5. Effect of Netrin-1 on migration of ADSCs under high glucose

The study found that in vivo injection of transplanted ADSCs gradually migrated to the damaged area of the tissue, thereby exerting its repairing effect by differentiating into specific types of cells and by secreting a large number of factors. Therefore, how to improve the migration ability of ADSCs has become a research hotspot and has received extensive attention. This part of the experiment examined the effect of Netrin-1 overexpression on the migration ability of ADSCs under high glucose conditions. The results of the scratch test showed that the migration speed of

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N-ADSCs at 12 and 24 hours was significantly faster than that of ADSCs (relative gap region, $P < 0.05$) (Fig. 2-5A, B). The results of the Transwell test (Corning Inc.) showed that the migration of N-ADSCs through the membrane was significantly higher than that of ADSCs (ADSCs, 231.667±9.50 cells/field; N-ADSCs, 375.67±6.51 cells/field; $P <0.05$) (Figure 2-5A, C). Two migration experiments have more strongly demonstrated that overexpression of Netrin-1 significantly enhances the ability of ADSCs to migrate under high glucose conditions.

3.6. Effect of Netrin-1 on adhesion and tube forming ability of ADSCs under high glucose

After the transplanted ADSCs migrate into the damaged tissue site, the adhesion ability determines whether the cells can remain in the damaged site for a long time, differentiate into vascular endothelial cells, and secrete a large number of cytokines to promote angiogenesis and tissue repair. We performed cell adhesion experiments on N-ADSCs and ADSCs. The cells attached to the six-well plate after PBS washing showed a circular appearance, and blue fluorescence and green fluorescence were observed under a fluorescence microscope (Fig. 2-6). Statistical analysis showed that the number of cell adhesions in the N-ADSCs group was significantly higher than that in the ADSCs group (ADSCs, 20.67±2.08/field; N-ADSCs, 60.00±2.65/field, $P <0.05$) (Fig. 2-6A, B). The current results indicate that overexpression of Netrin-1 significantly improves the adhesion of ADSCs in high glucose environments. The tube formation experiments of ADSCs showed that the capillary formation of N-ADSCs was richer than that of ADSCs in high glucose environment, and the cumulative tube length of N-ADSCs in high glucose medium was larger than that of ADSCs (1.21 times, $P <0.05$) (Fig. 2 -6C, D). The current results indicate that overexpression of Netrin-1 in ADSCs significantly increases the ability of angiogenesis in high glucose environments.

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4. Discussion

ADSCs are increasingly popular seed cells for basic experiments and clinical research because of their advantages of easy access, high yield, strong differentiation ability and low immunogenicity. They are expected to be used in high incidence, early onset, and insidious onset DPNV, which has a long course of disease and is difficult to cure. The therapeutic effect of promoting tissue regeneration after transplantation has been confirmed by a large number of basic and clinical trials, but a major problem to be solved is that the survival rate after transplantation is extremely low. Positron emission tomography (PET) tracking of MSCs delivered by catheter-based transendocardial injection by Gyongyosi M et al. showed that only about 6% of MSCs survived in porcine ischemic myocardium 10 days after transplantation [188]. Toma et al. reported that surviving MSCs did not exceed 0.44% on day 4 after heart transplantation in immunodeficient mice [189]. Similarly, only about 1% of MSCs were detected 24 hours after cardiac transplantation in experimental MI rats [190, 191]. More studies have found that the symptoms of lower limb ischemia and objective indicators of diabetic patients have improved to some extent, but their long-term efficacy is still poor [36, 64-70]. A large number of studies have also confirmed that there are advanced glycation end products, inflammatory microenvironment and oxidative stress in the vascular injury site around diabetes, which makes the survival, migration and migration are low of transplanted stem cells in the target vascular injury site and high glucose environment. The ability to differentiate significantly is the key to affecting the efficacy of stem cells [15-17, 71-77]. Therefore, the survival rate of ADSCs with low survival rate after transplantation is further reduced in the diabetic microenvironment, which greatly reduces the therapeutic effect of ADSCs. Therefore, how to promote the survival and differentiation of ADSCs in the vascular injury sites in diabetes and promote the regeneration of diabetic blood vessels after denervation is of great significance for the prevention and treatment of DPNV lesions.

Studies found that DPNV is a mixed lesion with vascular, neurological and tissue

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damage [28, 78-80]. The blood vessels and nerves in the body often follow the same trajectory to reach the tissues and organs, which confirms the relationship between blood vessels and nerves to some extent. Many of the earliest proteins found in nerves or blood vessels have gradually been found to play an important role in another system. Netrin-1 is the first identified axon guidance factor. Netrin-1 and G-netrin share homology with the laminin gamma chain. The peptide consists of nearly 600 residues of an amino terminal region VI. Repeated three layers of adhesion-type epidermal growth factor (V-1, V-2, V-3) and one carboxy terminal region [89]. Another study found that Netrin-1 not only participates in the functional activities of the nervous system, but also participates in the functional activities of the vascular system. Ding et al. demonstrated that Netrin-1 not only promotes neuronal migration and secretion in the central nervous system, but also regulates the survival, adhesion, migration, proliferation and differentiation of endothelial cells and stem cells in non-neural tissues, and inhibits their apoptosis [90, 91]. Wilson et al. systematically studied zebrafish and mammals, confirming that Netrins can induce angiogenesis. Netrin-1 activates the Src/FAK/paxillin-related signaling pathway by binding to the UNC5H receptor to promote adhesion, migration and proliferation of vascular endothelial cells, forming a new capillary network that can be inhibited by inhibiting zebrafish NTN-1 mRNA[92]. Lu et al. also found that Netrins stimulates angiogenesis in mammals and accelerates angiogenesis in ischemic tissues. This process relies on the Netrin-1 receptor DCC to regulate the ERK/eNOS signaling pathway [93]. Brunet et al. demonstrated that Netrin-1, Netrin-4 and VEGF promote angiogenesis, but Netrin-1 has a greater advantage in promoting the dual roles of endothelial cell differentiation and recovery of nerve damage [94]. In addition, Netrin-1 is involved not only in nerve growth and angiogenesis; it also enhances mitosis, migration and adhesion of endothelial cells at different stages of human blood vessels and lymphatic systems [95]. In addition to participating in angiogenesis, endothelial cell survival, adhesion, migration, proliferation and apoptosis, Netrin-1 has been continuously explored in recent years for the diagnosis and therapeutic value of various

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inflammatory diseases. The study found that Netrin-1 inhibits inflammation and protects normal cells [131-135]. Many acute and chronic inflammatory diseases, including diabetes and diabetic complications, are accompanied by an increase or decrease in Netrin-1 expression [96-105]. This may inhibit the migration of inflammatory cells, inhibit the production of inflammatory cytokines and chemokines, inhibit the expression of COX-2 by inhibiting NF-κB activation, and promote the differentiation of macrophages to anti-inflammatory M2-like phenotypes. And mechanisms related to the regulation of inflammatory responses through signal transduction pathways such as MAPKs, ERKs, and p38 [136-137]. At the same time, Netrin-1 has been reported to reduce inflammatory response after myocardial ischemia-reperfusion, inhibit oxidative stress and inflammation of nerve cells and vascular endothelial cells, and resist apoptosis [100-107]. However, our previous experiments through the analysis of immunohistochemistry, immunofluorescence, Western Blot, ELISA, etc. in the lower limb ischemic muscle tissue and peripheral blood serum of clinical diabetic and non-diabetic patients, the presence of Netrin-1 in the ischemic tissue of the lower limbs of diabetic patients The co-localization of endothelial cells, the expression level of Netrin-1 was significantly decreased, the expression level of inflammatory factors was significantly up-regulated, and the density of small blood vessels was significantly decreased. Statistical analysis showed that the expression level of Netrin-1 was negatively correlated with DPNV in diabetic hyperglycemia environment. Therefore, Netrin-1 may be an important biomarker in DPNV, and increasing its expression level may slow the progression of DPNV.

Therefore, we believe that overexpression of Netrin-1 by gene transfection of ADSCs can improve the viability, migration and differentiation of ADSCs under high blood glucose conditions, and promote DPNV angiogenesis. In this study, the adipose tissue of C57/BL mice was successfully obtained, and ADSCs were isolated, cultured and expanded, and firstly analyzed by flow cytometry in vitro. It was found that the P6 generation cells were morphologically mutated and aging was severe. Therefore, subsequent experiments using P3 to P5 generation ADSCs. Next, we used adenoviral

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transfection to establish a gene transfection system to overexpress green fluorescent protein (GFP) and Netrin-1 (N-ADSCs) in ADSCs, because the foreign gene carried by adenovirus was not inserted into the cell genome. Therefore, it is a superior and safer method than basic lentivirus in basic and scientific applications [193]. Through experiments, we found that the optimal multiplicity of infection (MOI) was 500, and the duration of transfection of ADSCs was 48 hours, which provided a reference for subsequent experiments. It is worth noting that normal mouse ADSCs do not express Netrin-1 protein per se, suggesting that we do not need to set up the si-RNA silencing group, but also highlight the significance of transfecting the foreign gene NTN-1 into ADSC. Next, we compared N-ADSCs and ADSCs by CCK-8, Western Blot, flow cytometry, Transwell, and immunofluorescence in a high glucose environment, and found that the proliferation, migration, adhesion, and formation of N-ADSCs in a high glucose environment. Tube capacity was significantly higher than ADSCs, and the apoptotic rate of N-ADSCs was significantly lower than that of ADSCs. Our experiments demonstrate that overexpression of Netrin-1 in vitro can greatly improve the survival, migration, adhesion, and tube forming ability of ADSCs, and resist the apoptosis of ADSCs cells in high glucose environment. The specific molecular mechanism is still unclear, and we need to further explore and discover it. At the same time, it provides an experimental basis for the subsequent transplantation of ADSCs into the mouse model of diabetic lower limb denervation, exploring the effect of repairing and treating DPNV in vivo and exploring the specific molecular mechanism.

5. Summary

Based on the previous studies, we obtained adipose tissue from C57/BL mice, isolated and cultured ADSCs, and established a stable gene transfection system by adenovirus transfection to overexpress green fluorescent protein (GFP) and Netrin-1 in ADSCs. (N-ADSCs). CCK-8, WB, flow, Transwell, immunofluorescence and other methods to detect the difference in the proliferation, migration, adhesion, differentiation to endothelial cells of N-ADSCs and ADSCs in high glucose

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environment, confirmed in vitro environment Netrin-1 can increase the proliferation, migration, adhesion and differentiation of ADSCs into endothelial cells in high glucose environment, and significantly reduce the apoptosis level of ADSCs. In order to carry out the transplantation of ADSCs into the diabetic lower limb denervation mouse model in vivo, it is necessary to explore the effect of in vivo repair and treatment of DPNV and explore the specific molecular mechanism.

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Chapter 3 Study on the regulation of DPNV by ADSCs regulated by Netrin-1

1. Introduction

Diabetic peripheral neurovascular disease (DPNV) is a mixed disease of blood vessels and nerves and surrounding tissue damage, leading to insidious onset, long course of disease, and eventually end with ulceration of diabetic foot and ischemic necrosis of distal extremities. According to statistics, the risk of suffering from foot ulcers in diabetic patients may be as high as 25%. The lower extremity amputation rate of diabetic patients is 15 times that of non-diabetic patients. The annual incidence of foot ulcers in patients with type 1 or type 2 diabetes is 1.9% to 2.2% [110-112], and patients with diabetic foot ulcers develop toe gangrene, in severe cases, amputation, and more, due to the massive absorption of toxins from the extremities, leading to death of multiple organ failure such as liver and kidney [113-115]. Clinically, although there are medical treatments, revascularization, percutaneous transluminal angioplasty (balloon dilatation and stent implantation) and other treatments, there is no simple blood glucose control to inhibit the progression of DPNV, reconstruction of intravascular thrombosis, and stenting. Problems such as occlusion, long-term efficacy is not ideal [116-119]. Therefore, DPNV brings a huge burden to China's health care system. Stem cell transplantation is a promising treatment for such diseases without effective clinical cure. T2DM patients are rich in adipose tissue, especially in the abdomen. A large amount of adipose tissue can be obtained through cheap and safe liposuction. ADSCs cultured in vitro have multi-directional differentiation, resist inflammation, and repair excellent properties of tissues. However, low survival rate after transplantation in vivo is the key limitation to its efficacy. In particular, adverse conditions such as advanced glycation end products, pro-inflammatory microenvironment and induction of oxidative stress caused by persistent hyperglycemia in diabetic patients further reduce the survival rate of

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ADSCs, making them unable to perform their functions. Netrin-1 acts as a secreted protein that promotes the growth, survival, adhesion, migration, proliferation and anti-apoptosis of nerve cells and endothelial cells in nerves and blood vessels. It can also be transduced by MAPKs, ERKs, p38 and other signals. The pathway regulates the inflammatory response to exert anti-inflammatory effects [136-137], and has recently been found to have a significant increase or decrease in expression levels in many acute and chronic inflammatory diseases [96-105]. In the early stage, we analyzed the clinical specimens and found that its expression level in diabetic hyperglycemia was negatively correlated with DPNV, which proved the clinical correlation between Netrin-1 and DPNV. At the same time, after obtaining a large number of ADSCs in vitro, a stable gene transfection system for adenovirus transfected ADSCs was established. It was confirmed that Netrin-1 can significantly increase the proliferation, migration, adhesion and adhesion to ADSCs in high glucose environment. The ability to differentiate significantly reduces the level of apoptosis in ADSCs. It is not clear if Netrin-1 enhances the transplantation of ADSCs in vivo in a mouse model of diabetic lower limb denervation. The effect of in vivo repair and treatment of DPNV and the specific molecular mechanism are still unclear.

Therefore, in this study, we constructed a T2DM mouse (db/db) lower limb denervation model, transplanted N-ADSCs and ADSCs in vivo, and used laser Doppler to observe blood perfusion, and evaluated by immunofluorescence and immunohistochemistry. The efficiency of ADSCs in survival, migration, differentiation, and angiogenesis in vivo. Using Western Blot to explore Netrin-1-mediated signaling pathways of proliferation, migration, adhesion, differentiation, proangiogenic ability and apoptosis of ADSCs, ELISA was used to detect Netrin-1 mediated paracrine secretion of ADSCs. To clarify the specific molecular mechanism of Netirn-1 regulating ADSCs proliferation, migration and treatment of vascular neuropathy in diabetes, and provide new ideas and theoretical basis for the prevention and treatment of DPNV.

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2. Materials and methods

2.1. Materials

2.1.1. Primary reagent

PBS phosphate buffer (1x, PH 7.2-7.4) (Beijing Suo Laibao Technology Co., Ltd., China)

Trypsin-EDTA (Trypsin-EDTA, 0.25%, Beijing Boaolong Immunotechnology Co., Ltd., China)

DMEM solution:

1g/L Dulbecco's modified eagle medium (DMEM; Gibco, USA)

10% fetal bovine serum (FBS, Hyclone, Australia)

NB4 collagenase solution:

0.2% collagenase NB4 (Serva, Heidelberg, Germany)

DMEM solution preparation

TBS Buffer (Bole Life Medical Products (Shanghai) Co., Ltd., China)

PBS phosphate buffer (1x, PH 7.2-7.4) (Beijing Suo Laibao Technology Co., Ltd., China)

TBST Buffer (1x) (Beijing Suo Laibao Technology Co., Ltd., China)

Coomassie Brilliant Blue Solution (Bio-Bio Engineering (Shanghai) Co., Ltd., China):

Bovine serum albumin (BSA, Sigma, USA)

4% paraformaldehyde (Shanghai Jingdu Biotechnology Co., Ltd., China):

Methanol (Shanghai Daixuan Biotechnology Co., Ltd., China)

95% ethanol (Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine)

75% ethanol ((The Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine)

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Isopropyl Alcohol (Sigma, USA) (Shanghai Daixuan Biotechnology Co., Ltd., China)

Chloroform (Sigma, USA)

DAPI (Xi'an Huert Biotechnology Co., Ltd., China)

Resin (Epon, Merck, Darmstadt, Germany)

OCT embedding agent (DAKO, USA)

Fluorescent sealing tablets (DAKO, USA)

Matrigel Gel (Sigma, USA)

Collagen Collagen II (Abcam, Cambridge, MA)

Ammonium oxalate crystal violet staining solution (crystal violet staining solution) (1%) (Beijing Suo Laibao Technology Co., Ltd., China)

Rabbit anti-CD31 polyclonal antibody (Abcam, UK)

Fluor 555-Sheep Anti-Rabbit IgG Polyclonal Antibody (Invitrogen, Carlsbad, CA)

DAPI (Xi'an Huert Biotechnology Co., Ltd., China)

ELISA kit (Shanghai Yiyan Biotechnology Co., Ltd., China)

DAPI (Invitrogen, Carlsbad, Canda)

Resin (Epon, Merck, Darmstadt, Germany)

Fluorescent sealing tablets (DAKO, USA)

Sodium pentobarbital (Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, China)

2.1.2. Main instruments and equipment

Constant temperature CO_2 incubator (Forma Scientific, USA)

Centrifuge (Thermo, USA)

Deionized water system (Millipore, USA)

Ultra-clean workbench (Jiangsu Sujing Group, China)

Inverted microscope (Chongqing Optoelectronic Instrument Corporation, China)

Fluorescence microscope (Nikon, Japan)

Precision Balance (Mettler Toledo, Switzerland)

Tianping (Shanghai Hanyu Hengping Scientific Instrument Co., Ltd., China)

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Refrigerator (Haier, China)

6-well plate (Corning Inc., USA)

Petri dish (Corning Inc., USA)

50ml centrifuge tube (Corning Inc., USA)

15ml centrifuge tube (Corning Inc., USA)

96-well plate (Wuxi Nice Biotechnology Co., Ltd.)

Pipette (Wuxi Nice Biotechnology Co., Ltd.)

0.22 μm needle filter (Millipore, USA)

40 μm filter (BD Falcon, USA)

Microsurgical instruments (Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, China)

Flow Cytometry (Beckman Coulter, Fullerton, Canada)

Image-Pro Plus software (Media Cybernetics Inc, Rockville, MD)

Low temperature circulating water bath Polystat (Cole-Parmer Instrument Comapny, USA)

Electric thermostatic water bath (Shanghai Yiheng Technology Co., Ltd.)

Electrophoresis Instrument (Shanghai Tianneng Technology Co., Ltd.)

Electronic balance (Beijing Sartorius Instrument System Co., Ltd., China)

MINI Centrifuge (Tomy Seiko Co., Ltd. Japan)

Vertical clean bench (Shanghai net purification)

Refrigerated centrifuge (Eppendorf, Germany)

Full temperature oscillation incubator (Shanghai Yiheng Technology Co., Ltd., China)

Desktop multi-function high speed refrigerated centrifuge (Eppendorf, Germany)

Autoclave (Shanghai Shen'an Medical Instrument Factory, China)

High speed refrigerated centrifuge (Thermo Fisher Scientific, USA)

Gel Imaging System (Shanghai Tianneng Technology Co., Ltd., China)

Laser Doppler perfusion imager (moor-FLPI; Moor Instruments, Devon, U.K)

Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA)

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2.1.3. Experimental animal

Wild type (WT) C57/BL mice and type 2 diabetic mice (BKS.Cg-m + / + Leprdb) were purchased from Shanghai Southern Model Biological Research Center, China. All animal experiments were approved by the Animal Ethics Committee of the Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Blood glucose levels in diabetic and hyperglycemic mice were defined as ≥16.67 mmol/L, and only these mice were used for subsequent in vivo studies.

2.2. Methods

2.2.1. Construction of a model of lower limb denervation in T2DM mice and transplantation of ADSCs

All animal experiments were approved by the Animal Ethics Committee of the Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine. SPF male T2DM mice (BKS.Cg-m +/+ Leprdb) (18-20 weeks old, n = 18; Shanghai Southern Model Biological Research Center, China) were randomly divided into 3 groups. Subsequently, mice were successfully anesthetized with sodium pentobarbital (0.5 mg/g). The left hind limb of each mouse was shaved. The sciatic nerve was then isolated under direct vision and a 1.0 cm long nerve was removed as previously described [194, 195]. The muscles were then sutured with a 5-0 nylon suture and the skin was sutured with a 3-0 nylon suture. Successfully constructed a model of lower limb denervation in T2DM mice. After 24 hours, 100 μL of serum-free medium containing 10^5 cells was injected at three different sites (gastrocnemius, gracilis and quadriceps) on the denervated hind limb. Mice were randomized into three groups: ADSCs (n = 6), N-ADSC (n = 6), and PBS-injected controls (n = 6). On the 7th, 14th and 28th day after transplantation, the blood flow perfusion of the hind limbs was evaluated non-invasively using a laser Doppler perfusion imager (moorFLPI; Moor Instruments, Devon, UK).

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2.2.2. Immunofluorescence and immunohistochemical staining

The overall condition of each group of experimental animals was observed on the 7th, 14th and 28th postoperative day. On the 28th day after surgery, the mice were successfully anesthetized with sodium pentobarbital (0.5 mg/g), and the mice were perfused with 4% paraformaldehyde solution, the mice were dissected, and the bilateral hind limb muscles were isolated and obtained. After the sucrose was dehydrated for 1.5-2 h, the tissue was transferred to 30% sucrose and soaked overnight. A section was continuously sliced at a thickness of 10 μm along the transverse axis of the muscle. The sections were dried overnight at room temperature in a dark place, and the sections were loaded into a slice box the next day and sealed in a -80 ° C refrigerator.

2.2.2.1 Immunofluorescence detection of muscle tissue

(1) using a 0.3% Triton-X solution for 10 min at room temperature, and then rinsing the specimen 3 times with PBS for about 5 min each time;

(2) using 10% goat serum to seal the specimen at 37 ° C for 30 min;

(3) Add rabbit anti-CD31 antibody (1:200 dilution) and mouse anti-Netrin-1 antibody (diluted 1:250) in turn according to the instructions, and add PBS to the control group, and place at 4 ° C overnight. Rinse the specimen 3 times with PBS for about 5 min each time;

(4) Add Fluor 555 goat anti-rabbit IgG antibody and FITC goat anti-mouse IgG antibody (1:500 dilution) to the specimen in turn, place it in a black box and protect it from light, and place it at 37 ° C for about 60 min. After the incubation, Rinse the specimen 3 times with PBS for about 5 min each time;

(5) The cells were stained with DAPI (diluted 1:1000), and after 2 min incubation, the samples were rinsed with PBS for about 5 min;

(6) The film was sealed with a fluorescent sealing tablet, and the fluorescent sheet of the specimen was prepared, and then recorded under a fluorescence microscope.

2.2.2.2 Immunohistochemical staining of muscles

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(1) The frozen sections were placed in 1 mM EDTA, heated at 95 ° C for 10 minutes, and cooled to room temperature at room temperature to perform antigen retrieval;

(2) Fix the cells or sections with an appropriate fixing solution, and wash them twice with the immunostaining washing solution for about 5 minutes each time;

(3) Adding an immunostaining blocking solution, and blocking for about 60 minutes;

(4) According to the primary antibody instructions, the primary antibody is diluted in an appropriate ratio. After the blocking solution was recovered, the diluted primary antibody was added, and the mixture was incubated at room temperature for 1 hour to recover the primary antibody. Rinse the specimen 3 times with PBS for about 5 min each time;

(5) According to the instructions of the second antibody, dilute the secondary antibody in an appropriate ratio. After the blocking solution was recovered, the diluted secondary antibody was added, and the mixture was incubated at room temperature for 1 hour to recover the secondary antibody. Rinse the specimen 3 times with PBS for about 5 min each time;

(6) The tablet was sealed with a sealing tablet, and the immunohistochemical preparation of the specimen was completed, and then recorded under an optical microscope.

2.2.3. Detection of the signaling pathway of Netrin-1 regulating ADSCs with Western Blot

Total protein was isolated from N-ADSCs and ADSCs cultured for 48 h under high glucose conditions in vitro, and total protein was isolated from muscle tissues of T2DM lower limb denervated mice injected with N-ADSCs, ADSCs and PBS, respectively. The total protein in tissue samples and cell lysates was quantified, electrophoresed, and transferred to a PVDF membrane and incubated overnight at 4 °C with appropriate antibodies. The antibodies were as follows: anti-Akt and

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anti-P-AKT antibodies, anti-PI3K and anti-PI3K P-PI3K antibody, anti-P38 and anti-P-P38 antibody, anti-ENOS and anti-P-eNOS antibody, anti-NF-κB and anti-P-NF-κB antibody, anti-JNK antibody, anti-ERK 1/2 antibody and anti-beta Actin antibody (1:500; Abcam). The Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA) was used to quantify the relative cumulative density of immunoreactive bands. The specific experimental steps have been described in detail in the previous section.

2.2.4. Detection of paracrine factors in ADSCs regulated by Netrin-1 by ELISA

ADSCs and N-ADSCs were cultured in high glucose medium for 48 hours according to the manufacturer's instructions. The supernatant was obtained by centrifugation, and the supernatant was assayed for vascular endothelial growth factor (VEGF), basic fibroblasts by ELISA kit. Growth factors (b-FGF), hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin-like growth factor (IGF-1) and Netrin-1 (R&D Systems Inc). High glucose DMEM medium was used as a control group. Each experiment was repeated more than three times in at least three wells.

2.2.5. Statistics

All the data in this study were analyzed using SPSS 18 software. All statistical charts and tables were drawn using GraphPad Prism 5 software, and all data were expressed as mean and standard deviation (SD). Quantitative values were compared by Student's t test and one-way ANOVA. The statistical significance was defined as *P < 0.05, **p<0.01; ***p<0.001. Each experiment was repeated at least three times.

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3. Results

3.1. Effects of N-ADSCs on blood perfusion of hind limbs in lower limb denervated T2DM mice

Previous studies have shown that sciatic nerve denervation leads to vascular bed remodeling and progressive loss of capillaries, and impairs arterial and post-ischemic recovery, which accelerates further deterioration of DPV in T2DM mice [194, 195]. In the present study, we observed the effect of Netrin-1 on the regulation of ADSCs in T2DM mice with sciatic nerve removal from chronic hindlimb ischemia. We performed imaging of the blood flow of the hind limbs by color laser Doppler on days 0, 7, 14 and 28 after transplantation of ADSCs and N-ADSCs into the T2DM mouse lower extremity denervation model (Fig. 3-1A). Both the N-ADSCs and ADSCs groups showed significantly higher laser Doppler perfusion indices than the PBS control group. In addition, the N-ADSCs group showed a significantly higher laser Doppler perfusion index than the ADSCs group at 28 days (ADSCs group, 0.58 ± 0.03; N-ADSCs group, 0.83 ± 0.03; PBS group, 0.26 ± 0.02; $P < 0.05$. Figure 3-2).

3.2. Effects of N-ADSCs on immunohistochemistry and immunofluorescence of hind limb muscles in lower limb denervated T2DM mice

This study found that no tumors or teratomas were formed in denervated ischemic muscles or other organs 28 days after injection of ADSCs and N-ADSCs. We detected GFP-labeled cells in the hind limb muscles of mice injected with ADSCs and N-ADSCs by immunofluorescence microscopy. CD31 (red), DAPI (blue) double staining and GFP (green) transfected into cells confirmed the migration of cells to the chronic ischemic and vascular regions, some of which eventually differentiated into vascular endothelial cells (Figure 3-3). The results of this study demonstrate that N-ADSCs are involved in the recovery and revascularization of damaged vascular structures. Statistical analysis showed that the number of surviving cells in N-ADSCs group was significantly higher than that in ADSCs and PBS control group (ADSCs

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group, 22.67±4.16 cells/field; N-ADSCs group, 68.33±4.04 cells/field, P < 0.01, Figure3-4). In addition, immunohistochemical analysis showed that N-ADSCs significantly increased microvessel density in the denervated hind limbs of T2DM mice compared with ADSCs and PBS groups (PBS, 2.50 ± 1.91 vessels/field of view; ADSCs, 4.25 ± 1.71) vessels/field of view; N-ADSCs group, 14.25 ± 1.89 vessels/field of view, P < 0.05) (Figures 3-5, Figures 3-6). Netrin-1 was shown to increase the ability of angiogenesis in ADSCs. The results of this study demonstrate that Netrin-1 plays a key role in the survival, migration and differentiation of ADSCs into endothelial cells.

3.3. Molecular Mechanism of Netrin-1 Regulating Survival, Proliferation, Migration, Adhesion and Angiogenesis of ADSCs

Western Blot demonstrated that phosphorylation of the PI3K / AKT / eNOS / P-38 / NF-κB signaling pathway was up-regulated in the N-ADSCs group, both in vitro and in vivo, compared to the ADSCs group (Figure 3-7A, 3-8A). Statistical analysis showed that the expression of PI3K / AKT / eNOS / P-38 / NF-κB signaling pathway in N-ADSCs was significantly higher than that in ADSCs group in vitro and in vivo (P<0.05, Figure 3-7B, 3- 8B). On the other hand, the expression of ERK1/2 and JNK was not up-regulated in N-ADSCs compared with the ADSCs group. Since ERK1/2, JNK and P-38 are both key upstream proteins of MAPK important signaling pathways, we It is speculated that in the regulation of ADSCs by Netrin-1, the MAPK signaling pathway may only pass P-38-MAPK, but there is no activation of ERK1/2-MAPK or JNK-MAPK signaling pathway. It is well known that activation of PI3K / AKT / eNOS is critical for proliferation, differentiation and anti-apoptosis of ADSCs [196]. In addition, the literature reports that Netrin-1 can restore vascular endothelial cell damage and promote angiogenesis in the high glucose state through the PI3K / AKT / eNOS signaling pathway [197]. In addition, P38-MAPK has been shown to be involved in cell survival, differentiation and migration [198]. NF-κB also has an anti-apoptotic effect and can up-regulate the expression of VEGF, which is

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beneficial to angiogenesis. The findings of this study demonstrate that Netrin-1 regulates important functions in the survival, proliferation, migration, adhesion, and angiogenesis of ADSCs in vitro and in vivo.

3.4. Effect of Netrin-1 on the regulation of paracrine cytokines and growth factors in ADSCs

ADSCs and N-ADSCs were cultured for 48 hours in high glucose medium, and then the supernatant was collected and analyzed by ELISA. The results showed that the expression of cytokines and growth factors such as VEGF, b-FGF, HGF, TNF-α, PDGF, EGF, IGF-1 and Netrin-1 was significantly higher in the N-ADSCs group than in the ADSCs group (Fig. 8). More and more studies have shown that the paracrine effect of stem cells plays a crucial role in promoting the repair of tissue damage. VEGF, b-FGF, HGF, TNF-α, PDGF, EGF, IGF-1, etc. are essential positive regulators for cell growth, proliferation, migration, adhesion and angiogenesis. The above factors of ADSCs can be paracrine. At the same time, it promotes the survival and repair of ADSCs and vascular endothelium in vivo. Netrin-1 itself promotes the growth and repair of endothelial cells. Therefore, Netrin-1 can enhance the paracrine secretion of ADSCs, thereby promoting revascularization in vivo and treating DPNV.

4. Discussion

In the previous study, we successfully obtained ADSCs from mouse adipose tissue and established a highly efficient and stable adenoviral gene transfection system to obtain N-ADSCs stably overexpressing Netrin-1. Our previous in vitro studies have demonstrated that Netrin-1 can significantly improve the proliferation, migration, adhesion and angiogenesis of ADSCs, and prevent the apoptosis of ADSCs induced by high glucose, suggesting that we further investigate the effect and mechanism of N-ADSCs repairing DPNV in vivo. . In this study, we successfully implanted N-ADSCs into the hind limbs of T2DM mice that are denervated by sciatic nerve. In the continuous observation and monitoring within 28 days after surgery, the

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blood flow in the hind limbs of the N-ADSCs group showed a significantly higher laser Doppler perfusion index than the ADSCs group and the PBS control group. Immunofluorescence measurements of muscle samples obtained from dissected mice 28 days after surgery showed that N-ADSCs survived in the chronic ischemic hind limb muscles after T2DM denervation and migrated to the damaged tissues and blood vessels, and some differentiated into endothelial cells. And small capillaries are formed. Immunohistochemistry results showed that the microvessel density of hind limb muscles was significantly increased in the N-ADSCs group compared with the ADSCs group and the PBS control group. Western Blot and statistical analysis of proteins in vitro and in vivo showed that the mechanism of Netrin-1 improving survival, proliferation, migration, differentiation and promoting revascularization of ADSCs in vitro and in vivo may be related to up-regulation of PI3K / AKT / eNOS / P-38 / The signaling pathway of NF-κB is involved.

Some studies have shown that adipose tissue is a natural abundant reserve of ADSCs, and ADSCs have multipotential differentiation and great potential for repairing tissue and organ function [199-201]. However, clinical and laboratory studies have found that a large number of transplanted cells do not provide a satisfactory therapeutic effect. And as shown in our study, ADSCs cultured and passaged to the P6 generation showed a pattern of severe aging, and a reduction in dryness would further lead to a decrease in proliferation, differentiation, and migration. Therefore, we can only select a narrow range of ADSCs between P3 and P5 for subsequent research and experiments. In addition, T2DM or hyperglycemia greatly limits the angiogenic effects of ADSCs, making effective treatment of DPNV a challenging task. Therefore, how to improve the survival, differentiation and migration ability of stem cells after transplantation is an urgent problem to be solved. In this study, we selected the Netrin-1 protein, a secreted protein that positively regulates both neurological activity and vascular system function. Numerous studies have shown that it plays a vital role in the survival, proliferation, migration, adhesion and differentiation of endothelial cells, nerve cells and stem cells, as well as inhibition

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of apoptosis. Ke et al. studied the positive effects of Netrin-1 on proliferation, migration, and angiogenesis of BMSCs during vascular regeneration following limb ischemia in rats. This study measured plasma and tissue levels of VEGF and demonstrated that up-regulation of VEGF is a major cause of the positive role of BMSCs transfected with Netrin-1 in ischemic revascularization in rats [202]. The in vitro part of this study reveals the role of Netrin-1 in the proliferation, migration, adhesion and differentiation of ADSCs as well as its specific signaling pathways and molecular mechanisms. The adenovirus we use is a superior and safer method than lentivirus in clinical use because the foreign gene on it is not inserted into the genome of the target cell [193]. Therefore, we transfected the NTN-1 gene into ADSCs by adenovirus. It is worth noting that the results of Western Blot demonstrated that Netrin-1 has almost no expression in ADSCs in the natural state, while Netrin-1 is highly expressed in ADSCs transfected with NTN-1, which further confirms the transfection of NTN into ADSCs. The necessity of 1. Previous studies have shown that Netrin-1 inhibits apoptosis of mesenchymal stem cells (MSCs) through DCC/AKT signaling pathway under hypoxic conditions [203], increasing its survival rate. The results of Western Blot in this study showed that the proportion of Bcl-2 / Bax in N-ADSCs was significantly higher than that in ADSCs, so Netrin-1 enhanced the proliferation of ADSCs and inhibited apoptosis under high glucose conditions. In vivo immunofluorescence and immunohistochemistry showed that the survival rate of N-ADSCs in chronic ischemic muscle of T2DM hind limb denervated mice was significantly higher than that of ADSCs, and some ADSCs also differentiated into endothelial cells and formed small capillaries. This phenomenon increases the density of microvessels and the laser Doppler perfusion index of the lower extremity blood flow, thereby greatly enhancing the function of the hind limbs. Previous studies have shown that differentiation and paracrine effects are the two main mechanisms by which ADSCs migrate to the body to repair damaged tissue [62]. The results of this study demonstrate that overexpression of Netrin-1 in ADSCs greatly increases the expression levels of a range of growth factors and cytokines (such as VEGF, HGF,

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bFGF, and PDGF), providing a good microenvironment for tissue regeneration. It reduces the pathological remodeling of hind limb ischemic muscles and promotes angiogenesis. At the same time, the highly expressed secreted protein, Netrin-1 itself, is a potent angiogenesis regulator that restores cellular damage and promotes angiogenesis in vascular endothelial cells in a high glucose environment via the PI3K / AKT-eNOS signaling pathway. In addition, we found increased expression of the PI3K / AKT / eNOS / P-38 / NF-κB signaling pathway in vitro and in vivo, which plays a crucial role in the proliferation, differentiation, anti-apoptosis, migration and adhesion of ADSCs. effect. However, this study is only a preliminary exploration of the underlying mechanism. Further follow-up of molecular mechanisms related experiments is needed to clarify the specific molecular mechanism by which Netrin-1 regulates the therapeutic effect of ADSCs on promoting DPNV angiogenesis. Despite the limitations of this study, current studies have shown that Netrin-1 can improve the survival, proliferation, migration, adhesion, differentiation and treatment of ADSCs in vitro and in vivo to promote angiogenesis. In summary, the results of this study provide new insights into the treatment of DPNV disease.

5. Summary

In this study, T2DM mice (db/db) hind limb denervation model was successfully constructed, N-ADSCs and ADSCs were transplanted in vivo, and blood perfusion was observed by laser Doppler. The survival of ADSCs in vivo was evaluated by immunofluorescence and immunohistochemistry. , migration, differentiation, and the efficiency of angiogenesis. Western Blot was used to investigate the signaling pathways of proliferation, migration, adhesion, differentiation, angiogenesis and apoptosis of Netrin-1 mediated ADSCs. ELISA was used to detect Netrin-1 mediated paracrine secretion of ADSCs. It was confirmed that Netrin-1 can pass the signaling pathway of PI3K / AKT / eNOS / P-38 / NF-κB and promote the secretion of VEGF, b-FGF, HGF, TNF-α, PDGF, EGF, IGF-1 and Netrin-1. The expression of factors enhances the efficiency of survival, proliferation, migration, differentiation, adhesion

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and angiogenesis of ADSCs in vivo and in vitro, increases the density of small blood vessels and the degree of blood perfusion, and significantly improves the chronic ischemia of the denervated hind limbs in T2DM mice. Function to treat DPNV. This study provides a new idea and theoretical basis for the prevention and treatment of DPNV.

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Summary and innovation of the full text

The incidence of DPNV is high, early onset, insidious onset, long course of disease, difficult to cure, and often leads to amputation and even life-threatening. There is currently no effective treatment plan in clinical practice. Stem cell transplantation is a promising treatment. The body of T2DM patients, especially the abdomen, is rich in adipose tissue. A large amount of adipose tissue can be obtained through cheap and safe liposuction. The ADSCs obtained by in vitro culture have multi-directional differentiation, resist inflammation and repair the excellent characteristics of the tissue. This study also confirmed that a large amount of adipose tissue was obtained in the inguinal region of experimental mice, and a large number of ADSCs were successfully isolated and cultured. Therefore, ADSCs are seed cells for the treatment of DPNV with great clinical application prospects. However, a large number of basic and clinical studies have found that the low survival rate of ADSCs after transplantation is the key to its efficacy. In particular, adverse conditions such as advanced glycation end products, pro-inflammatory microenvironment and induction of oxidative stress caused by persistent hyperglycemia in diabetic patients further reduce the survival rate of ADSCs, making them unable to perform their functions.

Netrin-1 is a secreted protein that promotes the growth, survival, adhesion, migration, proliferation, anti-apoptosis and anti-inflammatory of nerve cells and endothelial cells in both nerves and blood vessels. It has recently been found in many acute and chronic inflammatory diseases. There is a significant increase or decrease in its expression level. In this study, we analyzed the expression of Netrin-1 protein in endothelial cells of patients with DPNV by analyzing clinical specimens of ischemic muscles in lower limbs of diabetic patients, but the expression level of Netrin-1 was significant in peripheral blood serum and ischemic muscle tissues of lower limbs. Decreased, and the expression levels of multiple inflammation-related factors increased significantly, and the density of microvessels decreased significantly compared with the normal group. It was confirmed that the expression level of

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Netrin-1 in diabetic hyperglycemia was negatively correlated with DPNV, which proved the clinical correlation between Netrin-1 and DPNV. Therefore, we believe that overexpression of Netrin-1 by gene transfection of ADSCs can improve the viability of ADSCs under hyperglycemia, migration and differentiation into vascular endothelial cells, and promote DPNV angiogenesis.

In the in vitro experiments of this study, a large number of successful acquisitions of ADSCs led to the establishment of a stable gene transfection system for adenovirus transfected ADSCs. It was confirmed that Netrin-1 can significantly increase the proliferation, migration, adhesion and adhesion of ADSCs in high glucose environment. The ability of endothelial cells to differentiate significantly reduces the level of apoptosis in ADSCs. In the in vivo experiment, this study successfully constructed a hind limb denervation model of T2DM mice (db/db), transplanted N-ADSCs and ADSCs in vivo, and confirmed that Netrin-1 can pass PI3K / AKT / eNOS / P-38 / NF-κB signals. Pathway and promotion of expression of paracrine factors such as VEGF, b-FGF, HGF, TNF-α, PDGF, EGF, IGF-1 and Netrin-1 enhance the survival, proliferation, migration, differentiation, adhesion and promotion of ADSCs in vivo and in vitro. The efficiency of angiogenesis, increased small vessel density and blood perfusion, significantly improved the chronic ischemia and function of the denervated hind limbs of T2DM mice, and treated DPNV. This study provides a new idea and theoretical basis for the prevention and treatment of DPNV.

Although this study completely analyzed the clinical correlation between Netrin-1 and DPNV from clinical samples, and confirmed the important role of Netrin-1 in promoting survival, migration, differentiation and repair of chronic ischemia of DPNV by detailed in vivo and in vitro experiments, There are some shortcomings and limitations. First of all, because the clinical specimens are precious and difficult to obtain, this is a small sample size study. We will continue to expand the sample size and use a larger sample size to illustrate the clinical correlation between Netrin-1 and DPNV, thus obtaining more persuasion. A more credible

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conclusion. Secondly, in the study of the specific molecular mechanism of Netrin-1 regulation of ADSCs in vitro and in vivo, this study only discussed the protein molecules of the classical signaling pathway. How does Netrin-1 regulate the survival of ADSCs in vivo and in vitro, respectively? The specific molecular mechanisms of proliferation, migration, differentiation, adhesion, and the efficiency of angiogenesis have not been explored. In future studies, we will further refine the experimental steps and further clarify the use of signaling pathway-related agonists and inhibitors. Its specific molecular mechanism provides a more detailed and more convincing theoretical basis for the application of Netrin-1 modified ADSCs in the prevention and treatment of clinical DPNV. In addition, as more and more studies reveal that paracrine effects play a crucial role in the promotion of tissue damage repair in ADSCs, many of the paracrine factors that have been detected in this study have been elevated in Netrin-1 to regulate ADSCs. The role of treatment in DPNV also needs further exploration. The above deficiencies are also the focus of follow-up research, and follow-up research has been initiated and is underway.

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Time is like a shackle. Eight years ago, I was still a child who just finished college in the college entrance examination. I thought that eight years of clinical medicine eight years was like the eight years of the War of Resistance Against Japanese Aggression. It was hard and long, but in a blink of an eye, the enrichment of eight years' student life is gone, I am about to graduate from doctoral degree, enter the regulation, change from the identity of the student to a doctor, enter a new stage of life, use the ability to serve the motherland, and make a shiny and shiny screw for the motherland. Dedicated to the whole career of health.

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