干细胞及外泌体治疗坏死性小肠结肠炎的临床前研究进展

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摘要 坏死性小肠结肠炎(necrotizing enterocolitis, NEC)是早产儿中最常见的危及生命的胃肠道疾病, 以肠道损伤和坏死为主要特征。该病的确切发病机制尚不清楚, 通常需要手术切除病变肠, 长期预后差, 目前尚无有效的预防和治疗方法。干细胞(stem cells, SCs)广泛的增殖和分化能力以及细胞因子(旁分泌作用)的释放可作为NEC的有效治疗策略。越来越多的临床前研究证实了SCs在实验性NEC中的潜在治疗作用, 然而, 其作用机制和最佳治疗策略仍未解决, 限制了其临床适用性。现对近年来SCs治疗NEC的作用机制及取得的研究进展进行综述, 为下一步SCs治疗NEC临床转化提供思路。

关键词 干细胞; 外泌体; 坏死性小肠结肠炎; 新生儿

Preclinical Progress of Stem Cells and Exosomes in the Treatment of Necrotizing Enterocolitis

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Abstract NEC (necrotizing enterocolitis) is the most common life-threatening gastrointestinal disease in premature infants, characterized by intestinal injury and necrosis. The exact pathogenesis of the disease is unknown, and extensive surgical resection of the diseased intestine is usually required, with poor long-term prognosis and no effective prevention and treatment currently available. The extensive proliferation and differentiation of SCs (stem cells) and the release of cytokines (paracrine action) can be used as an effective treatment strategy for NEC. A growing number of preclinical studies have confirmed the potential therapeutic role of SCs in experimental NEC. However, the mechanisms of action and optimal treatment strategies remain unresolved, limiting their clinical applicability. This paper reviews the mechanism of action and research progress of SCs in the treatment of NEC in recent years, providing ideas for the clinical transformation of SCs in the treatment of NEC in the next step.

Keywords mesenchymal stem cells; exosomes; necrotic enterocolitis; newborn

收稿日期: 2022-02-25 接受日期: 2022-04-17

甘肃省儿科临床医学研究中心(批准号: 18JR2FA004)、甘肃省卫生行业科研计划项目(批准号: GSWSKY-2017-22)、甘肃省青年科技计划(批准号: 21JR7RA013)、甘肃省青年科技计划(批准号: 21JR11RA014)和甘肃自然科学基金(批准号: 20JR5RA602)资助的课题

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Received: February 25, 2022 Accepted: April 17, 2022

This work was supported by Gansu Pediatric Clinical Medical Research Center (Grant No.18JR2FA004), Health Industry Research Program of Gansu Province (Grant No.GSWSKY-2017-22), Gansu Youth Science and Technology Program (Grant No.21JR7RA013), Gansu Youth Science and Technology Program (Grant No.21JR11RA014) and Gansu Natural Science Foundation of China (Grant No.20JR5RA602)

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坏死性小肠结肠炎(necrotizing enterocolitis, NEC)是以肠黏膜损伤、炎症和坏死或肠穿孔为主要特征的危及生命的胃肠道疾病, 严重的情况下会导致心肺衰竭和休克, 常见于早产儿, 尤其是极早产儿[1]。NEC的共同发病机制尚未被阐明, 但一致认为具有多因素病因[2], 主要与早产和胃肠道发育不成熟有关[3]。许多危险因素, 如配方喂养、肠道菌群失调、炎症或肠道灌注不足, 都与NEC的病理机制有关[4]。产前和围产期因素, 如妊娠期高血压、产前使用类固醇、绒毛膜羊膜炎和宫内生长受限也是NEC的高危因素[5]。尽管新生儿危重症护理取得了重大进展, 低出生体重和低胎龄婴儿的生存率有所提高, 但NEC的发病率和死亡率并没有降低, 据统计, 我国NEC的发病率高于大多数发达国家, 近三分之一(27.9%)的患儿需手术治疗[6]。NEC术后幸存者常伴有短肠道综合征和神经发育迟缓, 随后长期依赖肠外营养或需要肠道移植, 总体生活质量差[7]。目前尚无有效的单一预防或治疗NEC的方法, 因此, 临床迫切需要新的治疗策略。干细胞(stem cells, SCs)治疗正在被用于治疗一些早产儿危重疾病, 如支气管肺发育不良(bronchopulmonary dysplasia, BPD)[8]、脑损伤(traumatic brain injurt, TBI)[9]和视网膜病变[10]。越来越多的临床前研究证实了SCs在实验性NEC中的潜在治疗作用[11]。然而, 其作用机制和最佳治疗策略仍未解决, 这限制了其临床适用性。移植到受损组织中的SCs数量极少, 不足以揭示其强大的治疗作用, 以旁分泌方式分泌的生物活性因子可能介导了其保护机制。羊水干细胞(amniotic fluid-derived stem-cells, AFSCs)和间充质干细胞(mesenchymal stem cells, MSCs)是目前用于治疗NEC的两种主要干细胞来源[12-14]。现就目前MSCs、AFSCs及衍生的外泌体(exosomes, Exos)治疗NEC的动物实验研究进展进行总结, 以期为NEC临床治疗带来新希望。

1 SCs和细胞外囊泡(external vesicles, EVs)

1.1 SCs的分类及生物学功能

SCs根据起源可分为胚胎干细胞(embryonic stem cell, ESCs)和成体干细胞(adult stem cell, ASCs), 诱导多能干细胞(induced pluripotent stem cell, iPSCs)近年来作为第三种SCs出现。除了具有基本的再生和分化潜能外, SCs还具有旁分泌作用以及抗炎、抗凋亡、促进增殖、迁移等不同的生物学功能[15]。

1.2 EVs的分类及生物学功能

EVs是一种纳米大小的膜结合囊泡, 是SCs与损伤组织信息交流的主要介质。凋亡小体、微囊泡和外泌体是EVs的三种主要类型, 凋亡小体(0.5~2.0 μm)作为细胞凋亡的产物被释放, 微囊泡(0.1~1.0 μm)是直接从质膜上出芽的囊泡结构, 外泌体(40~120 nm)是由多囊泡体与质膜融合形成的较小的囊泡[16]。已证实EVs可以表达来自其母体细胞的表面标记物[17]。在这三种EVs中, Exos特征最明显, 在各种疾病中研究最多。Exos可由体内许多不同类型的细胞释放, 普遍存在于唾液、尿液和母乳等体液中, 在分泌到细胞外空间后, 其通过与靶细胞膜上的受体相互作用, 修饰靶细胞的细胞外环境, 或与靶细胞膜融合并将其内容物释放到靶细胞细胞质中来影响细胞信号转导, 调节转录和翻译后修饰, 从而改变受体细胞生理状态[18]。Exos包含有microRNA、mRNA和siRNA, 以及生长因子等蛋白质, 具有调节炎症免疫信号、血管生成、纤维化和细胞死亡及修复等生物学功能[19]。Exos不仅比其母体细胞免疫原性更低, 还可被修饰以增强生物利用度和细胞靶向性, 此外, 便于冷冻保存而不会丧失活性, 同时避免了潜在的伦理、法律和科学挑战以及对致瘤性的关注[20]。因此, Exos可能较母体细胞更具优势, 使它们成为早产儿疾病治疗探索的理想候选药物, 但目前仅限于动物实验研究。

2 NEC的发病机制

2.1 肠道发育不成熟导致过度炎症反应

早产儿肠上皮细胞发育不成熟会导致肠蠕动受损、黏液层成分缺陷和肠上皮紧密连接破坏而缺乏防御机制, 随后增加了黏膜通透性使细菌易位进入管腔, 从而导致过度的炎症反应、黏膜损伤、血管损害和缺血坏死。Toll样受体4(Toll-like receptor 4, TLR-4)在这种炎症反应中起着关键作用, 促炎细胞因子(TNF-α、IL-6、IL-8)以及促炎信号NO的释放促进了炎症反应, 从而导致NEC的发生[21]。

2.2 缺氧–缺血性损伤和氧化应激

微循环受损或缺氧引起的灌注不足会导致呼吸衰竭或贫血, 增加了自由基的生成, 而新生儿抗氧化能力不足以抵消自由基的有害影响。新生儿肠道对缺氧的反应包括肠道血管收缩和VEGF/VEGFR2

通路的下调, 受损的肠道VEGF/VEGFR2信号可能通过肠道微血管不足以充分满足代谢需求而增加对NEC的易感性[22]。因此, 新生儿比成人肠道更容易受到肠道缺血/再灌注损伤。

2.3 肠内喂养

肠内喂养与导致促炎基因上调的表观遗传变化有关, 是NEC发生的高危因素。配方喂养缺失母乳中增强免疫功能和有助于维持肠黏膜完整性的重要成分。这造成较高的肠道pH值, 不利于共生菌的生长, 导致厚壁菌门、双歧杆菌和拟杆菌等厌氧菌水平降低, 大肠杆菌、杆菌、变形菌门和梭状芽孢杆菌等病原体比例增加, 致使黏膜通透性增加并刺激促炎细胞因子的产生[23]。此外, 配方喂养可能还和其他危险因素如肠道微生物群和肠道血流相互作用。

2.4 菌群失调

肠道菌群失调是NEC发病的关键特征, 致病性细菌产生的代谢产物诱导了肠黏膜的促炎状态和高通透性。革兰氏阴性菌的定植导致正常肠道菌群的改变和TLR-4信号通路的激活, 促进了肠上皮细胞凋亡、黏膜愈合受损和促炎细胞因子如血小板激活因子(platelet activating factor, PAF)、肿瘤坏死因子(tumor necrosis factor, TNF-α)和表皮生长因子(epidermal growth factor, EGF)的释放, 最终导致NEC的发生[24]。肠道微生物群受分娩方式、配方喂养、长期抗生素治疗和H2受体拮抗剂的影响。

2.5 遗传易感性

单核苷酸多态性与NEC的发病相关, 先天免疫基因、血管张力/生长调节基因、抗氧化反应基因和细胞因子/趋化因子基因参与了炎症、缺氧和氧化应激、趋化、细胞黏附、精氨酸代谢、血管生成、细胞外基质重塑和肌肉收缩的功能途径, 这些通路的失调产生夸大的促炎反应[25]。出现一个或多个功能缺失的NOD2变异更容易患NEC, 而在发生NEC的早产儿中, SIGIRR变异被富集, NOD2和SIGIRR都是TLR信号通路的抑制剂。VEGF和其他血管基因的遗传变异也是NEC易感性的潜在位点。

3 SCs对NEC的治疗应用概况

不同研究表明, MSCs、AFSCs和肠神经干细胞(enteric neural stem cells, E-NSCs)都能影响实验性NEC的疾病进程(表1), 具体如下。

3.1 MSCs

MSCs来源于体内的血管周围细胞, 可以从骨髓(bone marrow, BM)、脐带(umbilical cord, UC)、脐带血(umbilical cord blood, UCB)、羊水(amniotic fluid, AF)、胎盘(placenta, PL)和脂肪组织(adipose tissue, ADSC)等从多种基质组织中分离出来[26]。MSCs的特性是具有体外增殖能力, 基于细胞表面标记物的表达、塑料黏附生长和成骨细胞、脂肪细胞、肌细胞和神经元的分化潜能以及免疫调节作用[27]。其免疫调节作用已在许多关于不同炎症条件的临床前和临床研究中被观察到。虽然已知MSCs的一些独特特征, 但仍缺乏组织特异性的定义。MSCs具有因其缺乏MHC II类受体导致的低免疫原性、易于分离、快速自我更新能力和广泛的体外扩增能力而具有巨大的治疗潜力[28]。最初认为移植和分化导致损伤部位的细胞置换是MSCs作用的关键机制, 然而, 极低的移植率(通常为<1%~5%)[29]以及最近的证据表明, MSC主要通过细胞间的通信和分泌能够调节修复的生物活性分子来发挥其治疗作用, 而不是分化成靶细胞[30]。旁分泌因子是MSCs疗效的关键驱动因素, 因此理论上无细胞制剂与MSCs一样有效[31]。BM和ADSC是目前MSCs最常用的来源, 而UC/UCB-MSCs可以在没有侵入性手术的情况下大量获得, 比BM-MSCs具有更大的免疫调节潜能。ESCs和iPSCs可作为MCSs新的衍生来源, 它们的治疗应用仅限在研究领域中[32]。

3.1.1 BM-MSCs TAYMAN等[33]研究证实了腹腔注射BM-MSCs对NEC大鼠模型体重增加、临床疾病评分和组织病理学的益处, 但对生存率无显著影响。YANG等[34]证实, BM-MSCs不仅对NEC生存有益, 还对组织病理学损伤和肠道通透性有益, 有趣的是, 静脉注射比腹腔注射表现出更强的细胞整合性, 并与肝素结合生长因子(HB-EGF)有协同作用。WEIL等[35]研究发现, BM-MSCs通过分泌IL-6、VEGF和HGF增加了人胎肠上皮细胞缺氧损伤后的增殖和活力。与TAYMAN等[33]研究结果类似, ZANI等[36]观察到静脉注射BM-MSCs对大鼠NEC模型延长生存期方面无效。NIKIFOROU等[37]研究证实, 静脉注射BM-MSCs暴露于脐带闭塞的早产胎羊, 同样没有改善缺血缺氧造成的肠道损伤。其他研究表明, 直接应用BM-MSCs条件培养基缺乏治疗效力, 这可能是由于旁分泌因子释放不足和随后的降解

导致的, 但应用脯氨酸羟化酶2(prolyl hydroxylase 2, *PHD2*)沉默的BM-MSCs条件培养基通过NF-κB依赖的机制增强了MSCs对NEC的旁分泌作用[38]。这些无效的研究结果让研究人员的研究重点转向了其他类型的MSCs。

3.1.2 AF-MSCs GOOD等[39]首次发现, 在小鼠模型中, 将羊水显微注射入胎肠可减轻NEC的炎症反应。AF-MSCs主要是通过表达基质细胞环氧合酶2(cyclooxygenase 2, COX-2)和抑制TLR-4信号通路, 提高NEC的生存率和促进损伤肠的修复的, 而不是直接再生损伤细胞[36]。COX-2是一种诱导酶, 通常在肠道中低表达, 可降低炎症和肠上皮细胞凋亡, 促进上皮细胞增殖。AF-MSCs还可以通过激活Wnt信号促进NEC小鼠的肠上皮再生和ISCs的激活[40]。在猪早产NEC模型中, 在全肠外营养和肠内营养期间均给予AF可增加体重, 提高NEC评分[41]。AF-MSCs还可减少NEC大鼠液体潴留, 降低腹水发生率[42]。

3.1.3 UC-MSCs 与成人组织来源MSCs相比, 脐血中富含的MSCs被认为是更原始的群体且免疫原性更低, 分离效率更高。UC-MSCs已被证明与其他类型的MSCs在实验性NEC中一样有效。研究表明, 在低氧条件下UC-MSCs可以通过旁分泌释放的硫化氢发挥细胞保护、抗氧化和抗炎功能[43]。研究人员还在没有体外扩增的情况下, 给予最低限度处理的UCB, 以快速提供SCs治疗[44]。

3.1.4 其他类型的MSCs 其他类型的MSCs, 如人ESCs或人iPSCs在NEC治疗领域的研究仍然不足。ESCs由于涉及到胚胎有创, 存在伦理限制, iPSCs可以从成人细胞中重新编程来获得。KAGIA等[45]研究结果表明, NEC小鼠模型腹腔分别注射BM-MSCs、UC-MSCs、ESC-MSCs和iPSC-MSCs后, 仅在BM-MSCs和UC-MSCs组可见临床和组织病理学改善, 但iPSC-MSCs延长了生存期。推测相比较于成体组织MSCs, 多能干细胞MSCs可能包含更多不成熟的细胞亚群, 因此可能没有获得在体内对炎症信号的反应能力。

3.2 AFSCs

AFSCs被认为是ESCs和ASCs之间的中间类

表1 干细胞在NEC中的功能和应用

Table 1 Functions and applications of stem cells in NEC

类型 Type	来源 Origin	给药途径 Administration	处理方式 Model	研究结果 Results	参考文献 Reference
BM-MSCs (bone marrow-derived stem cells)					
BM-MSCs	Adult rat	Intraperitoneal injection	*In vivo*	BM-MSCs, AF-MSCs, AF-NSC, and E-NSC treatments all show significant reductions in NEC incidence compared with the control group There is no significant difference in incidence among the four treatment groups	[12]
BM-MSCs	Adult rat	Intraperitoneal injection	*In vivo*	BM-MSCs, AF-MSCs, AF- NSC and E-NSCs treatments all show a significant decrease in intestinal permeability and improved gut barrier function compared with the control group There is no significant difference in intestinal permeability or gut barrier function among the four treatment groups	[13]
BM-MSCs	Human	Intraperitoneal injection	*In vivo*	BM-MSCs administrated by intraperitoneal injection improve pathological changes of the neonatal NEC rat model BM-MSCs injected rats show significant weight gains and clinical sickness score improvement	[33]
BM-MSCs	Mouse homozygotes	Intraperitoneal injection Intravenous injection	*In vitro* *In vivo*	MSCs administrated intravenously have increased engraftment into NEC-injured intestine compared with MSCs administrated intraperitoneally Heparin-binding EGF-like growth factor and MSCs act synergistically to reduce injury and improve survival in experimental NEC	[34]
BM-MSCs	Human	Intravenous injection	*In vivo*	Intravenous MSCs treatment do not ameliorate hypoxia-ischemia induced adverse intestinal events, which are associated with NEC	[37]

续表1

类型 Type	来源 Origin	给药途径 Administration	处理方式 Model	研究结果 Results	参考文献 Reference
BM-MSCs	Adult rat	Intraperitoneal injection (conditioned medium)	*In vitro* *In vivo*	Condition medium of PHD2-silenced BM-MSCs repairs the intestinal damage and improves the survival of NEC rats BM-MSCs' paracrine effect is enhanced by PHD-2 silencing PHD-2 silencing activates NF-κB and promotes IGF-1 as well as TGF-β2 secretion in BM-MSCs	[38]
AF-MSCs (amniotic fluid-derived mesenchymal stem cells)					
AF-MSCs	E14.5 rat	Intraperitoneal injection	*In vivo*	BM-MSCs, AF-MSCs, AF-NSC and E-NSC treatments all show significant reductions in NEC incidence compared with the control group There is no significant difference in incidence among the four treatment groups	[12]
AF-MSCs	E14.5 rat	Intraperitoneal injection	*In vivo*	Pups expose to NEC but treated with BM-MSCs, AF-MSCs, AF-NSC and E-NSC have significantly reduced intestinal permeability and improve gut barrier function There is no significant difference in incidence among the four treatment groups	[13]
AF-MSCs	E14 rat	Intraperitoneal injection	*In vivo*	AF-MSCs improve survival and increase the repair of injured intestine in NEC via a COX-2 dependent mechanism. AF-MSCs decrease bowel inflammation, increase cell proliferation and reduce cell apoptosis AF-MSCs mediated effects do not depend on direct repopulation, but on a paracrine manner	[36]
AF-MSCs	Mouse pup	Intraperitoneal injection	*In vivo* *Ex vivo*	AF-MSCs rescue intestinal injury, restore epithelial regeneration, and increase active ISCs	[40]
AF-MSCs	E14 rat	Intraperitoneal injection	*In vivo*	AF-MSCs decrease fluid retention and lower the incidence of ascites in NEC rats	[90]
UC-MSCs (umbilical cord-derived mesenchymal stem cell)					
UC-MSCs	Human	Intraperitoneal injection	*In vitro* *In vivo*	UC-MSCs exert beneficial effects in NEC via the production of the paracrine mediator H_2S UC-MSCs produce more H_2S under hypoxic conditions.	[43]
NSC (neural stem cell)					
AF-NSCE-NSC	E14.5 rat Rat pup	Intraperitoneal injection	*In vivo*	BM-MSCs, AF-MSCs, AF-NSC, and E-NSC treatments all show significant reductions in NEC incidence compared with the control group There is no significant difference in incidence among the four treatment groups	[12]
AF-NSC E-NSC	E14.5 rat Rat pup	Intraperitoneal injection	*In vivo*	BM-MSCs, AF-MSCs, AF-NSC, and E-NSC treatments all show a significant decrease in intestinal permeability and improved gut barrier function compared with the control group There is no significant difference in intestinal permeability or gut barrier function among the four treatment groups	[38]
NSC	Mouse embryos at 12.5 days post coitum	Intraperitoneal injection	*In vivo*	NSC transplantation improves the enteric nervous system, intestinal integrity, stem cell differentiation,and intestinal transit, as well as decreases the mortality of NEC rats	[53]
NSC	E11.5 mouse	Intraperitoneal injection	*In vitro* *In vivo*	NSC transplantation reduces NEC incidence NSC injection improves gut barrier function and intestinal motility NSC-HB-EGF co-administration or HB-EGF-overexpressed NSC has augmented therapeutic effects on NEC	[54]

型，对MSCs和NSCs的一些表面标记物染色呈阳性，但也表达阶段特异性胚胎抗原-4(stage special embryo antigen 4, SSEA-4)、CD29、CD49e、OCT-4, MHC II类呈弱阳性[46]，因此与AF-MSCs分开进行讨论。EATON等[47]研究表明，AFSCs在培养基中保持稳定及在动物建模中反应良好，比MSCs具有更高的ESCs标记物表达水平，理论上具有更强的增殖能力、多能性、免疫调节活性和更低的致瘤性。AFSCs分泌的蛋白包括丝氨酸/苏氨酸蛋白磷酸酶PP1-γ催化亚基和胰岛素生长因子结合蛋白(insulin growth factor binding protein, IGFBP)家族，PP1-γ亚基参与了广泛的细胞过程，特别是减数分裂和细胞分裂、蛋白质合成、糖原代谢、细胞骨架重组以及膜受体和通道的调控，IGFBP超家族已被证明可以调节许多细胞的存活、迁移和增殖[48]。因此，AFSCs具有强大的刺激肠道SCs增殖和肠道保存的能力。肠内注射羊水本身可以通过激活表皮生长因子受体(epidermal growth factor receptor, EGFR)来减轻实验性NEC的严重程度。事实上，AFSCs比MSCs能分泌更多的生长因子，包括成纤维细胞生长因子、血管内皮生长因子、肝细胞生长因子和IGFBP超家族[49]，这些细胞因子在诱导肠上皮细胞COX-2的表达中发挥了重要作用。AFSCs还能引起隐窝附近COX-2阳性细胞迁移，这与肠道损伤呈负相关，而这些作用可被选择性COX-2抑制剂阻断[50]。在另一项研究中，AFSCs中Wnt的表达水平高于MSCs，这表明AFSCs在调节肠干细胞增殖方面具有更突出的作用[40]。在啮齿动物脓毒症模式下预防性给药AFSCs，AFSCs短暂积累在肝脏、肠系膜和腹膜中，然后释放旁分泌因子诱导巨噬细胞M1向M2极化，巨噬细胞M2的成熟在刺激肠道干细胞维持肠道止血中发挥了重要作用[51]。LI等[40]证实了AFSCs依赖Wnt通路恢复上皮细胞再生和增加$Lgr5^+$肠干细胞来改善NEC诱导的肠道损伤，然而只有极少量的AFSCs被发现可整合在肠壁中，对组织再生可能不是AFSCs发挥有益作用的主要机制。

3.3 E-NSCs

E-NSCs具有独特的自我更新能力，最终分化为神经元和胶质细胞[52]。E-NSCs移植可改善NEC大鼠的肠神经系统、肠道完整性、肠干细胞分化和肠道转运，并降低死亡率[53]。然而，炎症微环境可能对移植E-NSC的存活、增殖和迁移产生负面影响，促炎细胞因子如IL-1和IL-6对神经元分化产生抑制作用。与HB-EGF对BM-MSCs的有益作用相似，HB-EGF在体外能促进NSCs增殖，体内同时给予HB-EGF和E-NSCs增强了治疗效果[54]。

3.4 不同组织来源的SCs比较

在NEC中，研究者一直在试图阐明哪种类型的SCs是最优的，以及如何更好地将它们用于肠道恢复[55]。研究发现，不同类型的SCs对NEC治疗同等有效，AF-MSCs、BM-MSCs、AF-NSCs和E-NSCs治疗NEC均显著降低了NEC发生率，改善了肠道屏障功能，且四个治疗组间差异无统计学意义[13]。NSCs可以从AFSCs中纯化和衍生出来，这些羊水衍生的NSCs可能具有与其他NSCs及MSCs相同的治疗效果。与MSCs相比，AF-NSCs和E-NSCs培养和分离具有挑战性，限制了其临床应用。EGF和FGF的存在有助于确保NSCs处于未分化状态[12]。研究还表明，BM-MSCs和AFSCs都可减少实验性啮齿动物NEC的肠道损伤和炎症反应，但仅AFSCs显著延长了生存期[36,90]。蛋白质组学分析表明，AFSCs主要参与细胞的生长和发育，而MSCs则参与免疫调节，在NEC诱导前给予AFSCs可降低NEC的严重程度和黏膜炎症，肠道增殖和内源性SCs活化增加。然而，给予MSCs则没有显示出有益的作用[56]。DRUCKER等[57]认为，MSCs是NEC的最佳SCs治疗选择，因为它们能够靶向受损组织，具有分化能力并有较低的移植物多样性。AKDUMAN等[58]首次将MSCs成功应用于出生22天新生儿的室上性心动过速(supraventricular tachycardia, SVT)相关性NEC治疗，结果显示MSCs有助于预防短肠综合征，MSCs的应用可能改善了受损但仍存活的肠道，非修复坏死组织。

4 Exos对NEC的治疗应用概况(表2)

4.1 MSCs来源的Exos

研究表明，来源于MSCs的无细胞条件培养基或Exos在NEC中与单独使用MSCs具有同等疗效[59]。Exos主要通过减少炎症并再生肠上皮细胞来促进NEC肠道恢复[46]。NITKIN等[60]研究表明，腹腔注射BM-MSC-Exs可保护大鼠肠道屏障的完整性，并降低大鼠NEC的发生率和严重程度。RAGER等[61]研究表明，BM-MSC-Exs可促进体外肠上皮细胞伤口愈合，耗尽Exos的条件培养基则无伤口愈合能力。Exos还可以作为在NEC治疗中运输工具及传递载

表2 外泌体在NEC中的功能和应用

Table 2 Functions and applications of exosomes in NEC

类型 Type	来源 Origin	给药途径 Administration	处理方式 Model	研究结果 Results	参考文献 Reference
BM-MSC-Exos	Mouse	Intraperitoneal injection	*In vitro* *In vivo*	Wound healing in IEC-6 cells is significantly increased by BM-MSC-derived exosomes BM-MSC-Exos significantly lower intestinal permeability and the incidence of NEC	[31]
AF-MSC-Exos	Rat	Intraperitoneal injection	*In vitro* *In vivo* *Ex viv*o	AF-MSC-Exos increase cellular proliferation reduce inflammation, and regenerate a normal epithelium AF-MSC-Exos attenuate NEC intestinal injury via activating the Wnt signaling pathway	[40]
AF-MSC-Exos BM-MSC-Exos AF-NSC-Exos E-NSC-Exos	Mouse	Intravenous injection	*In vivo*	BM-MSC-Exos, AF-MSC-Exos, AF-NSCExos and E-NSC-Exos demonstrate equivalent reductions in NEC incidence Stem cell-derived exosomes are equivalent to stem cells in NEC therapy	[46]
HM-Exos	Human	Gavage	*Ex vivo* *In vivo*	HM-Exos reduce inflammation and improve mucus production *in vivo* HM-Exos decrease inflammation in hypoxia and LPS treated intestinal organoids Pasteurized HM-Exos are as effective as raw HM-Exos	[63]
BovM-Exos	Cow	Gavage	*In vitro* *In vivo*	BovM-Exos promote goblet cell and endoplasmic reticulum chaperone protein expression both *in vitro* and *in vivo*, which increases mucus production and protect the intestine	[64]
HM-Exos	Human	Gavage	*In vivo*	HM-Exos promote the proliferation and migration of intestinal epithelial cells both *in vitro* and *in vivo* Peptidomic differences between preterm and term milkexosomes are revealed	[65]
HM-Exos	Human	Intraperitoneal injection Gavage	*In vitro* *In vivo*	HM-Exos increase the proliferation and decrease the apoptosis of intestinal epithelial cells HM-Exos administered intraperitoneally or enterally decrease NEC incidence HM-Ex enteral administration has better effects	[66]
HM-Exos	Human	/	*In vivo*	HM-Exos reduce oxidative stressrelated injury on intestinal epithelial cells	[91]
HM-Exos	Human	/	*Ex vivo*	HM-Exos derived from colostrum, transitional or mature human milk prevent inflammatory injury HM-Exos derived from colostrum are most effective in decreasing inflammatory cytokine	[92]
HM-Exos	Human	/	*In vivo*	HM-Exos upregulate Wnt/β-catenin signaling in ISCs and increase cell viability under H_2O_2 exposure compared with the control group	[93]
PM-Exos	Pig	Gavage	*In vitro* *In vivo*	PM-Exos inhibit intestinal epithelial cell apoptosis and decrease TLR4/NF-κB signaling through miRNAs *in vitro* PM-Exos prevent LPS-induced intestinal injury and inflammation *in vivo*	[94]

体, 不仅将重要的蛋白质和细胞产物包裹在脂质层中, 便于运输, 还可以特异性地靶向HB-EGF到NEC损伤部位[62]。有趣的是, 尽管受到消化降解的影响, 但肠内给予MSCs来源的Exos仍具有显著效果。

4.2 人母乳来源的外泌体(HM-Exos)

母乳中天然富含Exos, 而且在初乳中含量最

高。HM-Exs在正常和实验性NEC小鼠中可减少氧化应激和肠上皮细胞死亡, 恢复杯状细胞MUC2的产生和内质网功能[63]。MARTIN等[91]研究表明, HM-Exos可保护肠上皮细胞免受H_2O_2诱导的氧化应激的影响。肠内HM-Exos保留了位于Wnt上游的GRP94, 为母乳的保护作用提供了分子基础[64]。WANG等[65]比较了足月和早产儿母亲的HM-Exos, 结果表明, 早产儿HM-Exos改善肠上皮细胞增殖的能力强于足月HM-Exos。蛋白质组学分析表明, 早产儿HM-Exos中含有参与代谢、发育、免疫系统、生物黏附和细胞增殖的多肽。此外, 通过肠内途径给药与腹腔注射相比, NEC发生率进一步降低, 说明HM-Exos可穿越肠上皮屏障, 肠内给药是更好的选择[66]。HM-Exos降低NEC发病率的机制可能是通过激活Wnt/β-catenin信号通路, 随后增加了ISCs活力并保护细胞免受氧化应激[67]。

4.3 其他SCs来源的外泌体

有研究评估了来自不同类型Exos的治疗效果, 结果表明, BM-MSC-Exs、AF-MSC-Exos、AF-NSC-Exos和E-NSC-Exos在改善大鼠肠道屏障功能和降低NEC发生率方面具有同等作用, 组织学分析显示, Exos效应受剂量, 而不受Exos大小、分布或分离方法的影响[60]。有研究表明, 不同组织来源的Exos对疾病进展有不同影响, 例如从肠上皮细胞或免疫细胞释放的Exos有助于NEC的进展, 而来自MSCs或母乳来源的Exos可改善NEC的严重程度, 并保持体内肠道屏障功能[68]。羊水来源的Exos可减少肠道NEC损伤和炎症, 同时促进ISCs表达和细胞增殖[69]。小肠成纤维细胞来源的Exos携带肠SCs调节所需的生长因子如EGF家族成员, 通过内源性调节Wnt/β-连环蛋白通路来减轻NEC肠道损伤, 并调节肠上皮SCs功能和细胞迁移, 这些因子在维持肠道上皮细胞的完整性和减少黏膜损伤方面发挥重要作用[70]。此外, 在NEC过程中, 来源于人AFSCs的条件培养基同样具有减轻回肠炎症、增加肠上皮细胞增殖和恢复绒毛隐窝内的干细胞生态位以及促进肠血管生成/微血管的作用。目前, 这些不同来源的Exos作用机制尚不清楚, 需要更多的研究来确定被激活的下游靶点。

5 SCs/Exs可能的作用机制

NEC的病理生理学包括细菌感染、炎症和缺血, 因此, NEC发病机制和SCs/Exs作用机制的重叠为SCs和Exs治疗提供了理论可行性(图1)。目前的证据表明, MSCs及其Exos主要通过趋化、抗炎、促血管生成、抗纤维化和抗氧化的机制发挥作用[70], 体现在以下几个方面。

5.1 趋化和异型细胞融合

在肠道中发生NEC时, 炎症介质、组织趋化因子和趋化因子受体的释放增加, 将MSCs向受损和感染的组织趋化[71]。局部植入炎症区域的BM-MSCs不仅分化为结肠间质细胞, 还可以提供多种因子, 如VEGF和TGF-β1, 负责成纤维细胞活化、血管生成和组织修复。但有研究表明, MSCs和AFSCs在NEC模型中的肠绒毛植入部位几天后无法被检测到, 但可能位于偏远的部位, 如脾脏和肝脏[56]。MSCs显示修复作用的另一个机制是异型细胞融合, 即来自不同谱系的两个细胞合并成一个细胞来传输信息和中介。BM-MSCs和心肌细胞之间、浦肯野神经元和肝细胞之间已被证实可以融合[72]。一些研究也证实了BM-MSCs与肠上皮细胞之间的融合, 但也有一些研究指出, 这可能不是这些细胞提供保护作用的机制。

5.2 促进ISCs表达增加

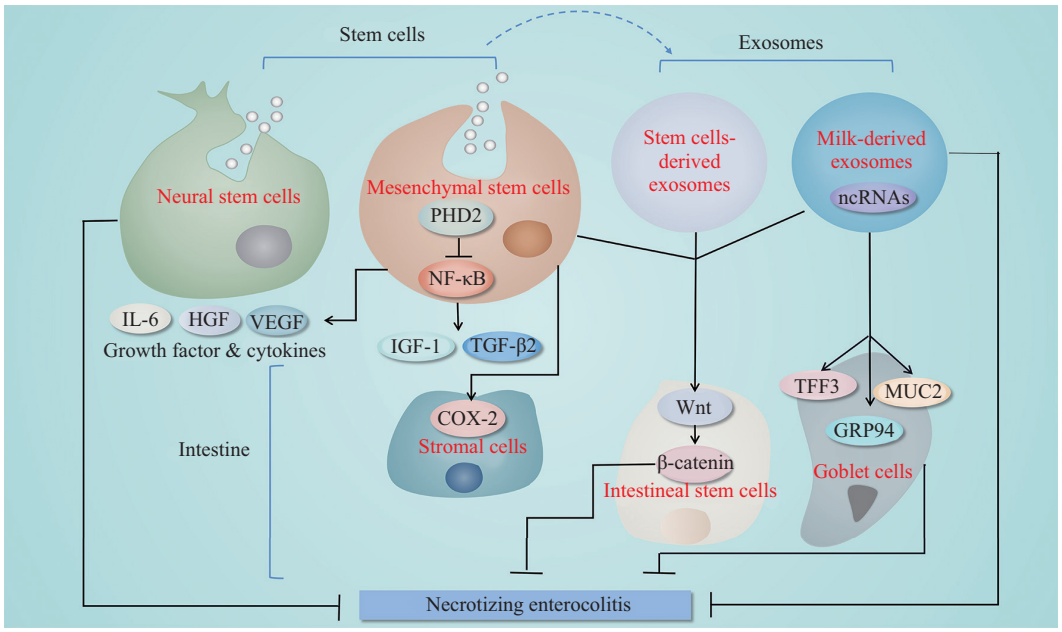
位于隐窝底部的ISCs的增殖和分化是肠黏膜更新的主要细胞学基础。NEC中ISCs群体的消耗与肠道损伤的严重程度成正比。NEC与Wnt/β-连环蛋白通路功能障碍相关, 该通路是ISCs功能和肠道上皮维持所必需的[73-74]。如前所述, AFSCs给药导致Wnt/β-连环蛋白通路基因表达上调。同样, MSCs可以上调生长因子, 促进ISCs表达水平增加来减轻黏膜损伤。

5.3 促进肠上皮细胞的增殖

肠上皮细胞是阻止外源抗原和毒素进入的最重要屏障。已知缺氧可诱导Fas介导的肠上皮细胞促凋亡信号通路, 导致下游促凋亡蛋白如Caspases-3的上调和激活[75]。SCs治疗NEC的益处之一是肠上皮完整性的重建。如前所述, 在大鼠幼鼠NEC模型中, 所有四种类型的SCs都维持了肠上皮的完整性, 并通过旁分泌作用增加了NEC损伤后肠上皮细胞的活力和增殖能力。

5.4 减少炎症细胞因子

炎症是NEC发病机制的重要促进因素, 包括抗炎介质的缺乏和促炎介质表达水平的增加。MSCs



干细胞和外泌体通过各种信号通路对NEC发挥有益的作用: MSCs分泌细胞因子和生长因子, 包括IL-6、VEGF和HGF。PHD2的下调激活了MSCs中的NF-κB信号通路, 从而增加了IGF-1和TGF-β2的旁分泌释放。MSCs通过旁分泌的方式增加基质细胞中COX-2的表达水平。Exos含有有利于NEC损伤的非编码RNA(ncRNAs)。牛奶来源的Exos促进了杯状细胞表达标志物TFF3和MUC2以及GRP94的表达。MSCs和MSC衍生的Exos都能激活Wnt/β-连环蛋白信号通路, 从而提高ISC的活力和肠道再生能力。

Stem cells and exosomes have beneficial effects on NEC through a variety of signaling pathways: MSCs secrete cytokines and growth factors, including IL-6, VEGF, and HGF. Down-regulation of PHD2 activates the NF-κB signaling pathway in MSCs, resulting in increased paracrine release of IGF-1 and TGF-β2. MSCs increased COX-2 expression in stromal cells by paracrine. Exos contains non-coding RNAs (ncRNAs) that are beneficial to NEC damage. Milk-derived Exos increased the expression of goblet cell expression markers TFF3 and MUC2, and GRP94. Both MSCs and MSC-derived Exos activate the Wnt/β-catenin signaling pathway, thereby enhancing ISC activity and intestinal regeneration.

图1 干细胞和外泌体在坏死性小肠结肠炎(NEC)中的作用和机制(根据参考文献[16]修改)

Fig.1 The role and mechanism of stem cells and exosomes in NEC (necrotizing enterocolitis) (modified from reference [16])

在炎症刺激下可产生和分泌多种生物活性分子包括肿瘤坏死因子α刺激基因(tumor necrosis factor-alpha stimulation gene, TSG)、吲哚胺2,3-双加氧酶(indoleamine 2,3-dioxygenase, IDO)、前列腺素E2(prostaglandin E2, PGE2)和TGF-β1。IDO可通过调控色氨酸耗竭和积累代谢产物来抑制免疫细胞过度活化, TGF-β1可通过参与调节性T细胞的分化以及抑制NK细胞来抑制免疫细胞的增殖。MSCs抑制炎症的能力还依赖于许多免疫细胞群之间复杂的相互作用, 在诱导促炎因子如IL-1β、IL-6、TNF-α、IFN-γ、IL-1Rα和PGE2向抗炎细胞因子IL-10和TSG-6的转化的同时[76], 还促进Tregs的Foxp3的上调以及巨噬细胞M1向M2极化的转换, 从而保护肠上皮细胞免受炎症和损伤[77]。虽然MSCs抑制T细胞的能力在体外得到了充分的证明, 但它们在体内的功能知之甚少。最新的研究表明, hUC-MSCs通过与单核细胞和巨噬细胞的相互作用, 间接抑制Th细胞的激活, 这对于抑制促炎适应性免疫反应也至关重要。由此可见, 调节炎症反应可能是MSCs减轻NEC实验动物肠损伤的重要机制[78]。

5.5 促血管生成

MSCs具有促血管生成作用, 主要通过VEGF发挥作用。既往研究表明, 来自胎盘和UCB的MSCs和AFSCs通过分泌VEGF并诱导内源性VEGF分泌, 改善BPD模型中的肺血管密度[79]。在体外, UCB-MSCs条件培养基诱导内皮细胞增殖和小管形成, 与直接应用VEGF诱导的作用类似[80]。

5.6 抗纤维化

纤维细胞参与了炎症和成纤维细胞活化的病理过程, 在急性损伤应激反应中, 纤维细胞可以表达巨噬细胞样炎症基因程序, 该程序编码与抗原呈递和白细胞转运相关的促炎细胞因子和趋化因子受体。肠道纤维化以活化的成纤维细胞产生的过量细胞外基质沉积为特征。研究表明, NEC患者外周血中循环纤维细胞的数量明显高于对照组, 大量纤维细胞浸润NEC肠黏膜[81]。传统的抗炎治疗并不能有

效地阻止肠道纤维化的形成, 对已经形成的纤维化更是无法逆转。SCs/Exos可通过抑制炎性因子表达, 抑制氧化应激反应来抑制上皮间质转分化(epithelial-mesenchymal transition, EMT)的过程来减缓肠道纤维化。其他可能的机制还涉及TGF-β1、基质金属蛋白9(matrix metalloproteinases 9, MMP-9)/基质金属蛋白酶抑制剂-1(tissue inhibitor of metalloproteinase1, TIMP-1)、斯钙素-1(stanniocalcin-1)、肾上腺髓质素、结缔组织生长因子(connective tissue growth factor, CTGF)、弹性蛋白等。杨佳[82]用实验证明, 基因改造过的SCs来源的微囊泡可有效地抑制实验性缓解结肠炎相关肠纤维化。MSCs的抗纤维化作用还不太确定, 但仍然是一种可能的作用机制。

5.7 抗氧化

越来越多的证据支持MSCs在多种疾病动物模型中发挥抗氧化特性, 这可能解释了它们的细胞保护和抗炎特性。氧化应激伴随着细胞损伤、炎症和代谢失调, 因此是多种疾病的关键病理生理机制。在许多疾病模型中, ROS和氧化应激的生物标志物的减少明确地证明了MSCs减轻氧化损伤的潜力[83]。体外模型的证据表明, MSCs直接保护细胞免受氧化刺激[85]。MSCs的抗氧化作用可能依赖于细胞接触, 给予MSCs条件培养可降低体内的氧化应激[84]。目前, MSCs已被提出通过清除自由基, 增强细胞呼吸和线粒体功能, 通过上调其他细胞的抗氧化防御和改变细胞生物能学来间接表现出抗氧化特性[85]。MSCs的免疫抑制特性也可以避免ROS的产生。

5.8 调节肠道菌群失调

最近的研究发现, AD-MSCs和脐带来源的MSCs在早期注射后可维持肠道微生物群的平衡[86]。在盲肠结扎和脓毒症穿刺的大鼠模型中, ADSC-MSCs降低了有害细菌的比例, 增加了有益细菌的比例[87]。

6 临床应用与挑战

越来越多的研究为SCs治疗NEC提供了证据, 但SCs治疗面临着许多挑战。除了存在伦理问题外, 还有排斥反应、潜在生物毒性、未知的免疫效应、致瘤可能以及标本污染等风险。Exos可能是更有前途的选择, 虽然Exos将其内容物(蛋白质和核酸)沉积到受体细胞中的确切机制尚不清楚, 但先前的啮齿动物研究证明了它们能够穿过血脑屏障。Exos不仅以旁分泌的方式对临近细胞产生影响, 还会影响全身偏远部位的细胞。结合Exos在创伤性脑损伤研究中观察到的益处, Exos对NEC诱导的神经损伤可能也具有潜在益处。虽然目前的研究支持Exos治疗NEC的疗效, 但目前尚不清楚Exos中包含的哪些核酸和蛋白质发挥作用。用于治疗这类新生儿疾病的安全有效剂量也尚未确定。Exos的半衰期较短, 其影响可能是短寿命的。研究人员发现反复给药在促进伤口愈合方面比剂量本身更重要, 重复使用剂量不会导致毒性和免疫原性的增加。使用Exos作为治疗的另一个挑战是Exos制剂的纯度, 并被认为与分离技术直接相关。目前用于分离的方法包括差异沉降法(超离心法)、密度梯度法、排阻色谱法和试剂盒分离法。

Exos制剂的安全性对其临床转化也至关重要, 在动物模型中, AFSCs和MSCs都未被证明具有致瘤性。免疫原性是干细胞治疗的另一个潜在的不良结果。普遍认为, 将SCs直接应用于损伤的肠道可能是以最高的局部浓度传递细胞产物并改善损伤肠道恢复的最有效的方法。尽管在动物模型中, 腹腔和静脉给药途径具有相同的疗效, 但在静脉注射后, 许多细胞被困在肺部, 导致可用于治疗目的的细胞数量减少。NEC病程的显著变化使得决定细胞治疗的时间难以确定。理想情况下, SCs治疗将提供给那些确诊NEC但尚未进展到需要手术干预的婴儿; 对超过II A期的非手术NEC的婴儿使用SCs治疗可能被认为是限制疾病进展的最佳方法; 也可以考虑对NEC发展高危的极早产儿预防性使用SCs或用来预防和治疗SBS。

在SCs和Exos治疗中, 可以通过几种策略来优化治疗效果。SCs修饰, 包括基因修饰和预处理修饰, 既能改善SCs的迁移、黏附和存活, 又能减少SCs的过早衰老[88]。将SCs与天然或合成的生物材料支架结合, 可有效提高SCs在体内的生存能力、分化能力和治疗效果[89]。其中, 细胞外基质可以为细胞的黏附和迁移提供平台, 是理想的外泌体载体。纳米工程的研究进展有望使Exos靶向进入损伤的肠道, 优化Exos的治疗效果。Exos上具有相应靶点的锚定肽能够直接加载Exos, 适配体介导的外泌体给药操作简单、疗效高、成本低, 将来可能成为靶向治疗的首选。然而, 对工程Exos的研究还处于起步阶段,

还没有证据表明可将工程化Exos成功应用于NEC的治疗。

7 总结与展望

由于Exos被认为是SCs旁分泌信号的关键介质, 因此逐渐开始向无细胞治疗策略转变, 基于SCs的治疗已经成为新生儿疾病的一种有前途的替代方案, 但目前还没有关于SCs或SCs来源的Exos用于NEC的临床试验。尽管SCs治疗入选临床试验存在显著障碍, 但已被证实的安全性可能有助于将这些临床前研究转化而明显获益。除了难以快速识别那些早期发生NEC的早产儿外, 还存在许多关键待解决的问题, 如SCs及Exos制备过程缺乏统一标准以及SCs治疗的临床指证、治疗时机、合适的给药途径、有效剂量都有待明确。此外, 我们对Exos发挥其作用的机制及存在的生物活性介质知之甚少。一些研究报道了蛋白质介导的Exos效应, 但越来越多的焦点转移到Exos中包含的RNA物种及其对靶细胞的表观遗传调控潜力。最后, 需要进一步的研究来解决SCs或SCs来源的Exos可能的副作用, 并克服与其治疗应用相关的挑战。尽管如此, 我们相信, 基于SCs或SCs来源的Exos的创新疗法正在为新生儿药物作为药物和干细胞的替代品开辟新的途径。

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