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·基础研究·

OECs/VitroGel™ 3D-RGD复合物移植治疗新西兰兔脊髓损伤的实验研究

吴官银[1],蓝顺宝[2],江哲琪[1],丁旭秋[1],崔晓萍[3],陈建梅[1] (1.福建医科大学福总临床医学院/联勤保障部队第九〇〇医院骨科,福建 福州 350000;2.福建中医药大学中西医结合学院,福建 福州 350000;3.福建医科大学福总临床医学院/联勤保障部队第九〇〇医院神经内科,福建 福州 350000)

[摘 要] **目的** 探讨嗅鞘细胞(OECs)/VitroGel™ 3D-RGD水凝胶复合物移植对新西兰兔脊髓损伤修复的影响。**方法** 75只新西兰兔随机分为空白对照组、模型组、单纯水凝胶组、OECs组和复合物组,每组15只。除空白对照组外,模型组、单纯水凝胶组、OECs组和复合物组均建立T_9脊髓损伤模型(重物打击致挫伤),并分别于损伤区注射PBS、水凝胶、OECs悬液或OECs/水凝胶复合物。于术后1、2、4、8、12周采用改良Tarlov评分评估实验兔后肢运动功能;采用苏木精-伊红(HE)染色测量脊髓组织空洞面积百分比;神经丝蛋白-200(NF-200)/胶质纤维酸性蛋白(GFAP)评价轴突再生和胶质瘢痕形成情况;分化簇68(CD68)/白细胞介素-10(IL-10)免疫荧光检测炎症细胞浸润和抗炎因子表达。**结果** 复合物组实验兔术后12周的Tarlov评分高于模型组、单纯水凝胶组和OECs组($P<0.01$),脊髓组织空洞面积百分比低于模型组、单纯水凝胶组和OECs组($P<0.01$)。复合物组脊髓组织NF-200阳性轴突数多于单纯水凝胶组和OECs组($P<0.01$),GFAP阳性瘢痕面积低于单纯水凝胶组和OECs组($P<0.01$)。复合物组脊髓组织CD68阳性细胞密度低于单纯水凝胶组和OECs组($P<0.01$)。复合物组脊髓组织IL-10表达水平高于单纯水凝胶组和OECs组($P<0.01$)。**结论** OECs/VitroGel™ 3D-RGD复合物较单一细胞或材料移植更能促进兔脊髓损伤后结构修复、轴突再生和运动功能恢复,其作用可能与三维支架支持、营养因子分泌及炎症微环境改善有关。

[关键词] 嗅鞘细胞;VitroGel™ 3D-RGD;脊髓损伤;组织工程;神经再生;免疫调节

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Experimental study on the transplantation of OECs/VitroGel™ 3D-RGD composite in the treatment of spinal cord injury in New Zealand rabbits

WU Guan-yin[1], LAN Shun-bao[2], JIANG Zhe-qi[1], DING Xu-qiu[1], CUI Xiao-ping[3], CHEN Jian-mei[1] (1. Department of Orthopedics, Fuzong Clinical Medical College of Fujian Medical University/the 900th Hospital of the Joint Logistics Support Force, Fuzhou Fujian 350000, China; 2.College of Integrated Traditional Chinese and Western Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou Fujian 350000, China; 3.Department of Neurology, Fuzong Clinical Medical College of Fujian Medical University/the 900th Hospital of the Joint Logistics Support Force, Fuzhou Fujian 350000, China)

Abstract: Objective To investigate the effects of transplantation of olfactory ensheathing cells (OECs)/VitroGel™ 3D-RGD hydrogel

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[通信作者] 陈建梅,E-mail:cjmei321@163.com;崔晓萍,E-mail:780802527@qq.com

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composite on repair at 12 weeks after spinal cord injury in New Zealand rabbits. **Methods** A total of 75 New Zealand rabbits were randomly divided into the blank control group, the model group, the hydrogel-only group, the OECs group, and the composite group, with 15 rabbits in each group. Except for the blank control group, rabbits in the model group, the hydrogel-only group, the OECs group, and the composite group all underwent establishment of a T_9 spinal cord injury model by weight-drop contusion and received local injections of PBS, hydrogel, OECs suspension, or OECs/hydrogel composite, respectively. Hindlimb motor function of the rabbits was assessed by the modified Tarlov score at 1, 2, 4, 8, and 12 weeks after surgery. Hematoxylin-eosin staining was used to measure the percentage of spinal cord cavity area. Neurofilament-200 (NF-200)/glial fibrillary acidic protein (GFAP) was used to assess axonal regeneration and glial scar formation, and cluster of differentiation 68 (CD68)/interleukin-10 (IL-10) immunofluorescence was used to detect inflammatory cell infiltration and anti-inflammatory factor expression. **Results** At 12 weeks after surgery, the composite group had higher Tarlov scores and lower percentage of spinal cord cavity area compared with the model group, the hydrogel-only group, and the OECs group ($P<0.01$). The composite group had more NF-200-positive axons and less GFAP-positive area in spinal cord tissue compared with the hydrogel-only group and the OECs group ($P<0.01$). The composite group showed lower CD68-positive cell density and higher mean optical density of IL-10 in spinal cord tissue compared with the hydrogel-only group and the OECs group ($P<0.01$). **Conclusion** OECs/VitroGel™ 3D-RGD composite transplantation is more effective in promoting tissue repair, axonal regeneration, and motor function recovery after spinal cord injury in rabbits than transplantation of cells or hydrogel alone. These effects may be related to three-dimensional scaffold support, neurotrophic factor secretion, and improvement of the inflammatory microenvironment.

Keywords: olfactory ensheathing cells; VitroGel™ 3D-RGD; spinal cord injury; tissue engineering; neural regeneration; immunomodulation

脊髓损伤(spinal cord injury, SCI)可导致长期运动、感觉及自主神经功能障碍。继发性炎症、神经营养不足、组织空洞和胶质瘢痕共同限制轴突再生,是SCI后神经功能恢复困难的重要原因[1]。细胞移植为SCI修复提供了新思路,其中嗅鞘细胞(olfactory ensheathing cells, OECs)兼具星形胶质细胞和施万细胞特性,可分泌脑源性神经营养因子(brain-derived neurotrophic factor, BDNF)、神经生长因子(nerve growth factor, NGF)、胶质细胞源性神经营养因子(glial cell line-derived neurotrophic factor, GDNF)等营养因子,表达细胞黏附分子,并具有随再生轴突迁移的能力[2-6]。

然而,单纯OECs移植仍受损伤区恶劣微环境、细胞滞留不足和空间分布不均等因素限制,治疗效果不佳。组织工程支架可为细胞提供三维支持并桥接组织缺损。VitroGel™ 3D-RGD为无动物源合成水凝胶,含RGD序列,具有可注射、透明、成分相对明确和利于细胞黏附等特点[7-9]。基于此,本研究将OECs与VitroGel™ 3D-RGD构建为复合物,并设置单纯水凝胶和单纯OECs对照,探讨该复合物移植对新西兰兔脊髓损伤的修复效果及其炎症调控作用。

1 材料与方法

1.1 实验动物与分组

健康成年新西兰兔75只,雌雄不限,体质量2.5~3.0 kg,由福建省实验动物中心提供,使用许可证号:SYXK(闽)2018-0006。本实验经联勤保障部队第九〇〇医院实验动物福利伦理委员会批准(批准号:2023-22)。采用随机数字表法将新西兰兔分为空白对照组、模型组、单纯水凝胶组、OECs组和复合物组,每组15只。行为学评分和图像定量分析均采用盲法。排除标准包括造模不成功、术后24 h内死亡、严重感染或切片质量不合格。

1.2 OECs分离培养与鉴定

取出生1 d的新西兰兔嗅球组织,剥离脑膜,经胶原酶-胰蛋白酶联合消化制备单细胞悬液。细胞置于无包被培养瓶进行差速贴壁24 h,再接种于多聚赖氨酸包被板,采用含10%胎牛血清、1%青霉素-链霉素、20 ng/mL碱性成纤维细胞生长因子及2 μmol/L毛喉素的DMEM/F12培养基培养;待细胞融合度约60%时,加入终浓度为1 μmol/L的阿糖胞苷,处理48 h进行OECs纯化。取P3代细胞经p75神经生长因子受体(p75 nerve growth factor receptor, p75NGFR)免疫荧光鉴定,并随机计数5个视野计算纯度。

1.3 水凝胶复合物制备与体外评价

根据说明书将VitroGel™ 3D-RGD与DMEM培养基按4∶1混合。单纯水凝胶组使用预凝胶液;复合物组将P3代OECs重悬于预凝胶液,调整细胞浓度为1×10^6/mL。体外评价设置平面培养组(OECs二维培养)和复合物组(OECs/水凝胶三维培养),不含细胞的单纯水凝胶不用于细胞存活、增殖及分泌指标的组间比较。复合物组细胞在37 ℃、5% CO_2条件下孵育30 min,使预凝胶液凝胶化;平面培养组细胞采用不含水凝胶的培养体系;体内移植时以预凝胶状态缓慢注入损伤区,使其原位凝胶化。

采用Live/Dead染色比较平面培养组和复合物组培养24 h的OECs存活情况;MTT法检测细胞培养1、3、5、7 d的增殖活性;ELISA检测共培养7 d的细胞上

清液BDNF浓度。实验独立重复5次。

1.4 SCI模型与移植

实验兔异氟烷吸入麻醉后俯卧位固定,显露T_9段脊髓。除空白对照组外,其余各组采用20 g重物自5 cm高度自由落下打击实验兔脊髓;出现尾部痉挛摆动、双后肢和躯体回缩且术后改良Tarlov评分为0分判断为造模成功。随后分别在模型组、单纯水凝胶组、OECs组及复合物组实验兔损伤中心头尾交界区4点注射PBS、VitroGel™ 3D-RGD、OECs悬液、OECs/VitroGel™ 3D-RGD,总量25 μL,每点约6.25 μL,速度约1 μL/min。术后常规抗感染并人工排尿至膀胱功能恢复。

1.5 行为学、组织学及免疫荧光检测

术前及术后1、2、4、8、12周采用改良Tarlov评分(0~5分)评估实验兔后肢运动功能。术后12周经心脏灌注4%多聚甲醛,取损伤中心上下各约1 cm脊髓组织进行石蜡包埋。每组随机选取8只实验兔用于组织学和免疫荧光检测,每只动物每项染色至少取3张切片,每张切片取3个非重叠高倍视野。采用苏木精-伊红(hematoxylin and eosin,HE)染色,对损伤区脊髓组织切片的空洞面积进行定量分析,结果以空洞面积百分比表示。神经丝蛋白-200(neurofilament-200,NF-200)/胶质纤维酸性蛋白(glial fibrillary acidic protein,GFAP)免疫荧光双标用于评价轴突再生和胶质瘢痕形成情况;分化簇68(cluster of differentiation 68,CD68)/白细胞介素-10(interleukin-10,IL-10)免疫荧光双标用于评价炎症细胞浸润和抗炎因子表达。图像均以Image-Pro Plus 6.0统一阈值分析;CD68以阳性细胞密度表示,IL-10表达水平以平均光密度表示。

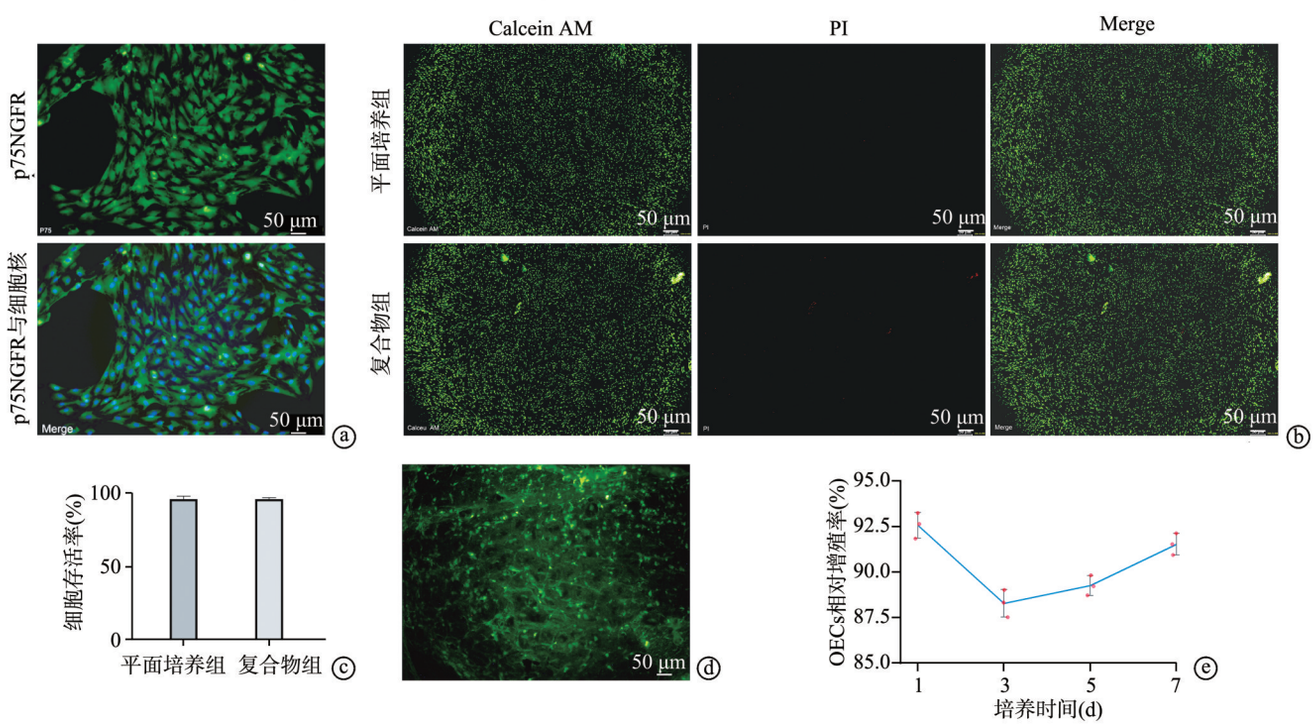
1.6 统计学分析

采用SPSS 26.0统计软件分析数据。计量资料以均数±标准差($\bar{x}\pm s$)表示,多组间比较采用单因素方差分析;方差齐性时组间两两比较采用LSD法,方差不齐时采用Dunnett T3法。行为学评分采用重复测量方差分析。$P<0.05$为差异有统计学意义。

2 结果

2.1 OECs鉴定及复合物体外特性

P3代OECs形态较均一,多呈双极或三极梭形;p75NGFR免疫荧光检测细胞阳性率为(88.5±3.1)%。Live/Dead染色显示,平面培养组和复合物组培养24 h细胞存活率均在95%以上。复合物组细胞可在水凝胶三维网络中伸展突起并相互连接。MTT结果显示,2组细胞培养1、3、5、7 d增殖活性比较差异无统计学意义($P>0.05$),提示材料无明显细胞毒性,见图1。



a:p75NGFR免疫荧光染色及p75NGFR与细胞核共染色;b:平面培养组和复合物组Live/Dead染色;c:2组细胞存活率;d:水凝胶内OECs形态;e:水凝胶内OECs培养1、3、5、7 d的增殖活性

图1 OECs鉴定及OECs/VitroGel™ 3D-RGD复合物体外特性

2.2 实验兔运动功能恢复

空白对照组实验兔改良Tarlov评分始终为5分。除空白对照组外,其余各组实验兔术后1周Tarlov评分均为0分,随后逐渐升高。复合物组实验兔术后2、4、8、12周的Tarlov评分均高于模型组、单纯水凝胶组和OECs组($P<0.001$);OECs组与单纯水凝胶组实验兔术后4、8、12周的Tarlov评分比较差异无统计学意义($P>0.05$),但2组均优于模型组($P<0.001$),见表1。

表1 各组实验兔改良Tarlov评分比较(分)

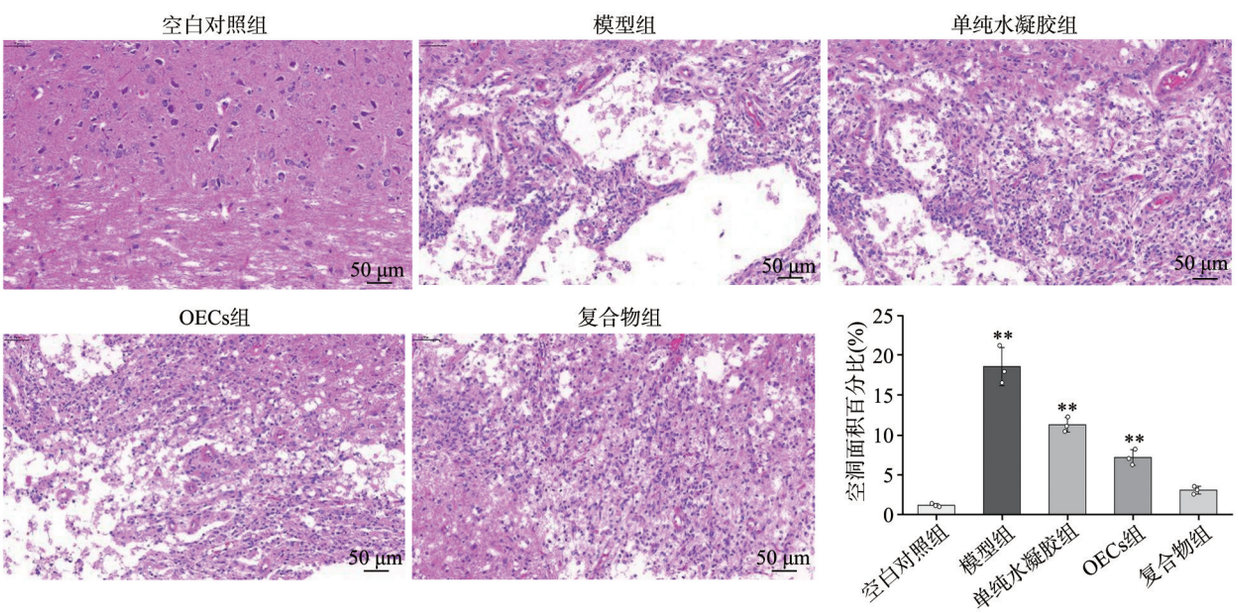
组别	n	术前	术后1周	术后2周	术后4周	术后8周	术后12周
空白对照组	15	5	5	5	5	5	5
模型组	15	5	0	0	0.1±0.1	1.5±0.1	2.0±0.1
单纯水凝胶组	15	5	0	0	1.2±0.1*	2.0±0.1*	2.5±0.2*
OECs组	15	5	0	0	1.9±0.1*	2.5±0.1*	3.0±0.2*
复合物组	15	5	0	1.1±0.1$^{*\#\triangle}$	2.6±0.1$^{*\#\triangle}$	3.5±0.2$^{*\#\triangle}$	4.1±0.2$^{*\#\triangle}$

*:与模型组比较,$P<0.001$;#:与OECs组比较,$P<0.001$;△:与单纯水凝胶组比较,$P<0.001$

2.3 组织病理学和轴突/瘢痕变化

HE染色显示,模型组脊髓组织损伤中心存在较大空洞;单纯水凝胶组和OECs组脊髓组织空洞较模型组减少;复合物组脊髓组织空洞最小,可见新生组织桥接。复合物组脊髓组织空洞面积百分比低于模型组、单纯水凝胶组和OECs组($P<0.01$),见图2。

NF-200/GFAP双标显示,模型组脊髓组织损伤区仅见少量轴突并形成致密GFAP阳性瘢痕;单纯水凝胶组和OECs组脊髓组织轴突数量增加、瘢痕减轻;复合物组脊髓组织NF-200阳性轴突较多,部分成束穿越损伤中心。复合物组脊髓组织NF-200阳性轴突数高于模型组、单纯水凝胶组和OECs组($P<0.01$),GFAP阳性瘢痕面积低于模型组、单纯水凝胶组和OECs组($P<0.01$),见图3。



**:与复合物组比较,$P<0.01$

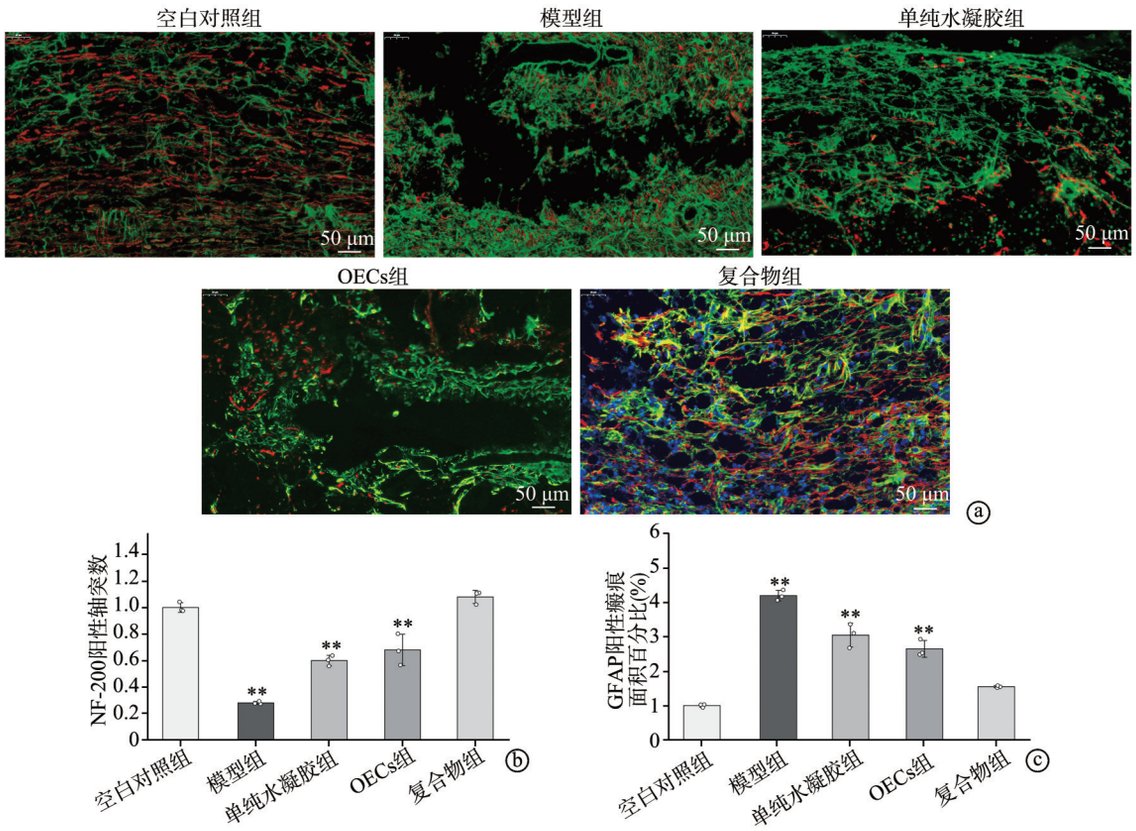
图2 术后12周HE染色评估各组脊髓组织空洞情况

2.4 炎症反应调控

CD68/IL-10双标显示,模型组脊髓组织损伤区仍有大量CD68阳性细胞;单纯水凝胶组和OECs组脊髓组织CD68阳性细胞密度较模型组减少($P<0.05$);复合物组脊髓组织CD68阳性细胞密度低于模型组、单纯水凝胶组和OECs组($P<0.01$)。IL-10免疫荧光染色显示,模型组损伤区荧光信号较弱,单纯水凝胶组和OECs组荧光信号增强,复合物组最强。复合物组脊髓组织IL-10表达水平高于模型组、单纯水凝胶组和OECs组($P<0.01$),见图4。

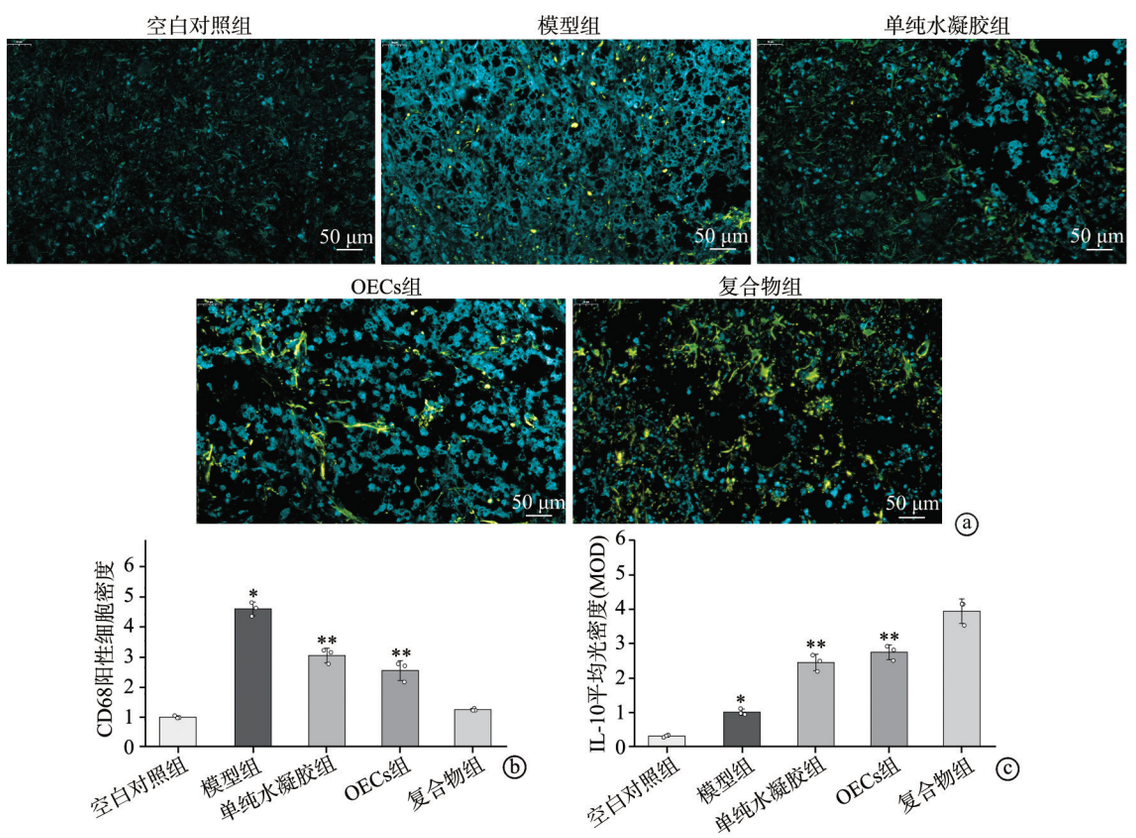
3 讨论

本研究显示,OECs/VitroGel™ 3D-RGD复合物在兔SCI模型中可获得较单纯OECs或单纯水凝胶更优的修复效果,表现为运动功能评分升高、组织空洞减小、NF-200阳性轴突增多、GFAP阳性瘢痕减轻,同时CD68阳性细胞减少、IL-10表达升高。上述结果提示,细胞与支架组合干预,可同时发挥结构支撑、神经营



a:NF-200/GFAP免疫荧光双标染色;b:NF-200阳性轴突数;c:GFAP阳性瘢痕面积百分比 **:与复合物组比较,$P<0.01$

图3 NF-200(红色)/GFAP(绿色)免疫荧光双标染色及定量结果



a:CD68/IL-10免疫荧光双标染色;b:CD68阳性细胞密度;c:IL-10表达水平 *:与空白对照组比较,$P<0.01$;**:与复合物组比较,$P<0.01$

图4 CD68(青色)/IL-10(黄色)免疫荧光双标染色及定量结果

养供给以及调控局部炎症微环境等多重作用,是改善SCI修复的重要原因。

单纯OECs移植能够改善神经营养和轴突生长环境,但细胞在损伤腔隙内易流失,且局部炎症、缺血和瘢痕屏障会削弱其效应。VitroGel™ 3D-RGD提供可注射三维网络和RGD黏附位点,既有助于细胞滞留和分布,又可作为组织桥接材料减少空洞形成。本研究体外结果显示,水凝胶不影响OECs存活和增殖,并促进BDNF分泌;体内结果进一步显示复合物组NF-200阳性轴突数显著增加,说明三维支架可能增强了OECs的营养支持和轴突导向作用。

炎症微环境也是影响SCI修复的重要因素。持续激活的巨噬细胞/小胶质细胞可释放炎性介质并加重继发损伤,而IL-10可抑制过度炎症并参与修复调控[10-14]。本研究中复合物组脊髓组织损伤区CD68阳性细胞密度最低、IL-10表达最高,提示其可促进损伤区由促炎状态向修复状态转变。该变化可能与OECs免疫调节能力、水凝胶空间屏障作用和细胞-支架相互作用共同相关。

然而,本研究仍有局限:未采用细胞示踪技术评价移植OECs的长期存活、迁移和分化;未进行电生理检测;观察期为12周,尚需更长时间随访验证功能维持情况。后续研究应结合细胞示踪、多模态影像、电生理和机制阻断实验,进一步阐明OECs与RGD水凝胶协同修复SCI的关键通路。

综上,OECs/VitroGel™ 3D-RGD复合物移植能促进新西兰兔脊髓损伤后12周运动功能恢复和组织结构修复,效果优于单纯OECs或单纯水凝胶。其优势可能来自水凝胶的三维支撑与桥接、OECs的神经营养作用以及局部炎症微环境改善,为SCI组织工程治疗提供了实验依据。

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