·基础研究·

桃红四物汤对大鼠血管吻合模型吻合区 Delta－like4 表达及血管新生的影响

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摘　要　目的:观察桃红四物汤对大鼠血管吻合模型吻合区 Delta－like4 表达及血管新生的影响。**方法**:将 40 只 3 月龄 SPF 级 SD 大鼠随机分为空白对照组、假手术组、生理盐水组、桃红四物汤组,每组 10 只。桃红四物汤组、生理盐水组行尾部动脉血管离断后吻合术;假手术组仅在相应区域作切口,不进行血管离断吻合;空白对照组不做任何处理。造模术后桃红四物汤组以 20 mL·kg^{-1}剂量的桃红四物汤灌胃,生理盐水组给予等量生理盐水灌胃,每日灌胃 2 次;假手术组和空白对照组不给予任何药物干预。分别在药物干预开始后 2 d、5 d 从各组随机抽取 5 只大鼠,采用颈椎脱臼法处死后,桃红四物汤组和生理盐水组切取尾部的吻合处血管,假手术组和空白对照组切取尾部相应部位动脉血管。将所取血管组织制成石蜡切片,分成 2 份,1 份采用免疫组化法检测血管吻合区 Delta－like4 的蛋白表达(光镜下灰度值与 Delta－like4 的蛋白表达量呈反比);1 份经 HE 染色后测量吻合区血管内膜面积。**结果**:①血管吻合区 Delta－like4 的蛋白表达。药物干预开始后 2 d,4 组大鼠血管吻合区 Delta－like4 蛋白表达的灰度值比较,差异无统计学意义(126.27±20.56,111.73±7.99,119.30±16.71,124.53±7.35,F=0.624,P=0.062)。药物干预开始后 5 d,4 组大鼠血管吻合区 Delta－like4 蛋白表达的灰度值比较,差异有统计学意义(85.60±5.58,99.07±3.84,103.13±11.61,111.60±4.49,F=7.041,P=0.012);进一步两两比较,桃红四物汤组血管吻合区 Delta－like4 蛋白表达的灰度值低于生理盐水组(LSD－t=－2.334,P=0.048)、假手术组(LSD－t=－3.035,P=0.016)、空白对照组(LSD－t=－4.450,P=0.002);生理盐水组血管吻合区 Delta－like4 蛋白表达的灰度值与假手术组、空白对照组比较,差异均无统计学意义(LSD－t=－0.703,P=0.502;LSD－t=－2.166,P=0.062);假手术组血管吻合区 Delta－like4 蛋白表达的灰度值与空白对照组比较,差异无统计学意义(LSD－t=－1.464,P=0.181)。②吻合区血管内膜面积。药物干预开始后 2 d,4 组大鼠吻合区血管内膜面积比较,差异无统计学意义[(0.20±0.01)mm^2,(0.18±0.02)mm^2,(0.16±0.01)mm^2,(0.17±0.02)mm^2,F=2.625,P=0.122)。药物干预开始后 5 d,4 组大鼠吻合区血管内膜面积比较,差异有统计学意义[(0.30±0.02)mm^2,(0.20±0.01)mm^2,(0.21±0.01)mm^2,(0.21±0.01)mm^2,F=17.063,P=0.000];进一步两两比较,桃红四物汤组吻合区血管内膜面积大于生理盐水组(LSD－t=－46.275,P=0.020)、假手术组(LSD－t=－68.142,P=0.000)、空白对照组(LSD－t=－66.154,P=0.000);生理盐水组吻合区血管内膜面积与假手术组、空白对照组比较,差异均无统计学意义(LSD－t=－2.187,P=0.060;LSD－t=－1.988,P=0.082);假手术组吻合区血管内膜面积与空白对照组比较,差异无统计学意义(LSD－t=0.199,P=0.847)。**结论**:桃红四物汤能上调血管吻合大鼠模型吻合区 Delta－like4 的表达,促进血管新生。

关键词　桃红四物汤;血管新生;受体,Notch;Delta－like4;大鼠;动物实验

Effect of Taohong Siwu Tang(桃红四物汤) on Delta－like4 expression and angiogenesis in anastomosed area in vascular anastomose rat models

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ABSTRACT　Objective:To observe the effect of Taohong Siwu Tang(桃红四物汤,THSWT) on Delta－like4 expression and angiogenesis in anastomosed area in vascular anastomose rat models. **Methods**:Forty 3－month－old SPF－grade SD rats were randomly divided into blank control group,sham-operated group,normal saline(NS) group and THSWT group,10 rats in each group. The surgery of mutilation－anastomose were performed on the caudal artery of rats in THSWT group and NS group,and sham operation were performed on rats in sham-operated group,while the rats in blank control group were not given any surgical intervention. After the end of the modeling operation,the

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rats in THSWT group were intragastric administrated with THSWT in dosage of 20 ml/kg, and the rats in NS group were intragastric administrated with the same dose of normal saline, twice a day. The rats in sham-operated group and blank control group were not given any drug intervention. At 2 and 5 days after the beginning of drug intervention, 5 rats were randomly selected from each group respectively and were executed by using cervical dislocation method. The caudal anastomotic blood vessels of rats were fetched in THSWT group and NS group, and the caudal arterial vessels of rats in corresponding sites were fetched in sham-operated group and blank control group. The acquired vascular tissues were made into paraffin sections and were divided into 2 parts, one of them was applied to measure the Delta-like 4 protein expression by using immunohistochemical method (the gray values were inversely proportional to the delta-like4 protein expression under light microscope), while the other portion was applied to measure the area of endangium in anastomosed area through HE staining. **Results**: At 2 days after the beginning of the drug intervention, there was no statistical difference in the gray values of Delta-like 4 protein expression in vascular anastomosed area between the 4 groups (126.27 +/- 20.56, 111.73 +/- 7.99, 119.30 +/- 16.71, 124.53 +/- 7.35, F = 0.624, P = 0.062). At 5 days after the beginning of the drug intervention, there was statistical difference in the gray values of Delta-like 4 protein expression in vascular anastomosed area between the 4 groups (85.60 +/- 5.58, 99.07 +/- 3.84, 103.13 +/- 11.61, 111.60 +/- 4.49, F = 7.041, P = 0.012). Further pairwise comparison showed that the gray values of Delta-like 4 protein expression in vascular anastomosed area were lower in THSWT group compared to NS group (LSD-t = -2.334, P = 0.048), sham-operated group (LSD-t = -3.035, P = 0.016) and blank control group (LSD-t = -4.450, P = 0.002); and there was no statistical difference in the gray values of Delta-like 4 protein expression in vascular anastomosed area between NS group and sham-operated group and between NS group and blank control group (LSD-t = -0.703, P = 0.502; LSD-t = -2.166, P = 0.062); and there was no statistical difference in the gray values of Delta-like 4 protein expression in vascular anastomosed area between sham-operated group and blank control group (LSD-t = -1.464, P = 0.181). At 2 days after the beginning of the drug intervention, there was no statistical difference in the area of endangium in anastomosed area between the 4 groups (0.20 +/- 0.01, 0.18 +/- 0.02, 0.16 +/- 0.01, 0.17 +/- 0.02 mm^2, F = 2.625, P = 0.122). At 5 days after the beginning of the drug intervention, there was statistical difference in the area of endangium in anastomosed area between the 4 groups (0.30 +/- 0.02, 0.20 +/- 0.01, 0.21 +/- 0.01, 0.21 +/- 0.01 mm^2, F = 17.063, P = 0.000). Further pairwise comparison showed that the area of endangium in anastomosed area was larger in THSWT group compared to NS group (LSD-t = -46.275, P = 0.020), sham-operated group (LSD-t = -68.142, P = 0.000) and blank control group (LSD-t = -66.154, P = 0.000); and there was no statistical difference in the area of endangium in anastomosed area between NS group and sham-operated group and between NS group and blank control group (LSD-t = -2.187, P = 0.060; LSD-t = -1.988, P = 0.082); and there was no statistical difference in the area of endangium in anastomosed area between sham-operated group and blank control group (LSD-t = 0.199, P = 0.847). **Conclusion**: THSWT can up-regulate Delta-like4 expression and promote angiogenesis in anastomosed area in vascular anastomose rat models.

Key words　Taohong Siwu Tang; angiogenesis; receptors, Notch; Delta-like4; rats; animal experimentation

随着工业化社会的不断发展,断指、断肢的发生率逐年增高,目前已成为临床常见疾病。血管吻合术是断指、断肢再植成功与否的关键所在,而具有活血化瘀药功效的桃红四物汤在血管吻合术后的药物干预中起着重要的作用。我们前期的相关研究[1-2]证实,桃红四物汤能有效促进血管新生。Delta-like4是Notch信号通路中重要的配体,参与血管新生的全过程,与受体及其他配体一起调控血管形成[3-8]。在血管新生过程中,Notch受体和配体在内皮细胞和周围细胞中均有表达,而在血管组织中,主要是Delta配体和Notch受体。本研究以大鼠血管吻合为模型,观察桃红四物汤对血管吻合区Delta-like4表达及血管新生的影响,以期为桃红四物汤在血管吻合术后的应用提供实验依据。

1　材料与仪器

1.1　实验动物　3月龄SPF级SD大鼠40只,体质量200~250 g,雌雄各半,由湖南中医药大学动物实验中心代购,实验动物许可证号:SCXK(湘)2016-0012。实验方案通过医学实验动物伦理委员会批准。

1.2　实验药物　桃红四物汤的方药组成:生地黄20 g、当归20 g、赤芍20 g、川芎10 g、桃仁20 g、红花10 g。上药煎煮后浓缩,制成含生药量为4 g·mL^{-1}的药液冷藏储存备用。

1.3　实验试剂与仪器　免疫组化试剂盒、Notch信号通路Delta样配体4抗体(bioss公司),苏木素-伊红染色(hematoxylin and eosin, HE)试剂盒(碧云天公司)。MF528型光学显微镜(上海医疗器械股份有限公司医用光学仪器厂),显微外科器械、灌胃器(由湖

南中医药大学实验室提供)。

2 方 法

2.1 分组与造模 适应性喂养1周后将40只3月龄SPF级大鼠随机分为空白对照组、假手术组、生理盐水组、桃红四物汤组,每组10只。造模手术前6 h禁食、禁水,用备皮刀对术区进行备皮,以3%戊巴比妥钠(1 mL·kg^{-1})行腹腔麻醉。麻醉成功后,将桃红四物汤组和生理盐水组大鼠仰卧位固定于手术台上;在10倍光学显微镜下切开大鼠尾部中上段皮肤,分离出长约1 cm的动脉血管;将其剪断后,立即用11-0号缝线行断端吻合;吻合通血成功后,分层关闭切口[9-10]。假手术组只切开尾部中上段皮肤即缝合,空白对照组不做任何处理。

2.2 药物干预 术后大鼠给药剂量按人和动物药物等效剂量换算公式[11]计算,桃红四物汤组以20 mL·kg^{-1}剂量的桃红四物汤灌胃,生理盐水组给予等量生理盐水灌胃,每日2次。假手术组和空白对照组不给予任何干预。

2.3 血管吻合区Delta-like4的蛋白表达检测 分别于药物干预开始后2 d、5 d时从各组随机抽取5只大鼠,采用颈椎脱臼法处死后,桃红四物汤组和生理盐水组切取尾部血管吻合处血管,假手术组和空白对照组切取尾部相应部位动脉血管。将所取血管组织制成石蜡切片,分成2份,1份按免疫组化试剂盒上的操作步骤,测定Delta-like4在吻合区的表达。将血管组织切片置于显微镜平台上,首先测量切片中有盖玻片但没有组织区域的灰度作为背景灰度;在400倍光镜下观察切片,阳性标记为黄色或棕色,每张切片随机选取5个视野,将切片中图片输入IMAGE PRO PLUS图像分析软件,得出每个标本平均灰度值,其中灰度值与Delta-like4的蛋白表达量呈反比。

2.4 吻合区血管内膜面积测定 另1份石蜡切片经HE染色后,在400倍显微镜下拍照,图像以TIFF格式存入电脑,在IMAGE PRO PLUS 6.0环境下打开图片,利用软件中的Count and Size功能自动计算出吻合区血管内膜面积。

2.5 数据统计分析 采用SPSS21.0软件对所得数据进行统计学分析,4组大鼠血管吻合区Delta-like4蛋白表达的灰度值及吻合区血管内膜面积的组间比较采用单因素方差分析,组间两两比较采用LSD-t检验,检验水准$\alpha=0.05$。

3 结 果

3.1 血管吻合区Delta-like4的蛋白表达 药物干预开始后2 d,4组大鼠血管吻合区Delta-like4蛋白表达的灰度值比较,差异无统计学意义。药物干预开始后5 d,4组大鼠血管吻合区Delta-like4蛋白表达的灰度值比较,差异有统计学意义;进一步两两比较,桃红四物汤组血管吻合区Delta-like4蛋白表达的灰度值低于生理盐水组(LSD-$t=-2.334$,$P=0.048$)、假手术组(LSD-$t=-3.035$,$P=0.016$)、空白对照组(LSD-$t=-4.450$,$P=0.002$);生理盐水组血管吻合区Delta-like4蛋白表达的灰度值与假手术组、空白对照组比较,差异均无统计学意义(LSD-$t=-0.703$,$P=0.502$;LSD-$t=-2.166$,$P=0.062$);假手术组血管吻合区Delta-like4蛋白表达的灰度值与空白对照组比较,差异无统计学意义(LSD-$t=-1.464$,$P=0.181$)。见表1。

表1 4组大鼠药物干预后血管吻合区Delta-like4蛋白表达量比较

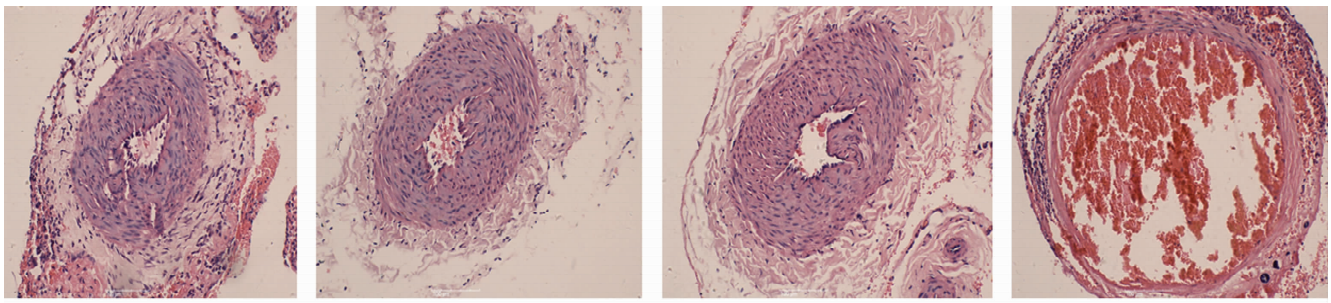
组别	样本量(只)	血管吻合区Delta-like4蛋白表达的灰度值($\bar{x}\pm s$)	
		药物干预开始后2 d	药物干预开始后5 d
空白对照组	5	124.53±7.35	111.60±4.49
假手术组	5	119.30±16.71	103.13±11.61
生理盐水组	5	111.73±7.99	99.07±3.84
桃红四物汤组	5	126.27±20.56	85.60±5.58
F值		0.624	7.041
P值		0.062	0.012

3.2 吻合区血管内膜面积 药物干预开始后2 d,4组大鼠吻合区血管内膜面积比较,差异无统计学意义。药物干预开始后5 d,4组大鼠吻合区血管内膜面积比较,差异有统计学意义;进一步两两比较,桃红四物汤组吻合区血管内膜面积大于生理盐水组(LSD-$t=-46.275$,$P=0.020$)、假手术组(LSD-$t=-68.142$,$P=0.000$)、空白对照组(LSD-$t=-66.154$,$P=0.000$);生理盐水组吻合区血管内膜面

积与假手术组、空白对照组比较，差异均无统计学意义（LSD－t = －2.187，P = 0.060；LSD－t = －1.988，P = 0.082）；假手术组吻合区血管内膜面积与空白对照组比较，差异无统计学意义（LSD－t = 0.199，P = 0.847）。见表 2、图 1、图 2。

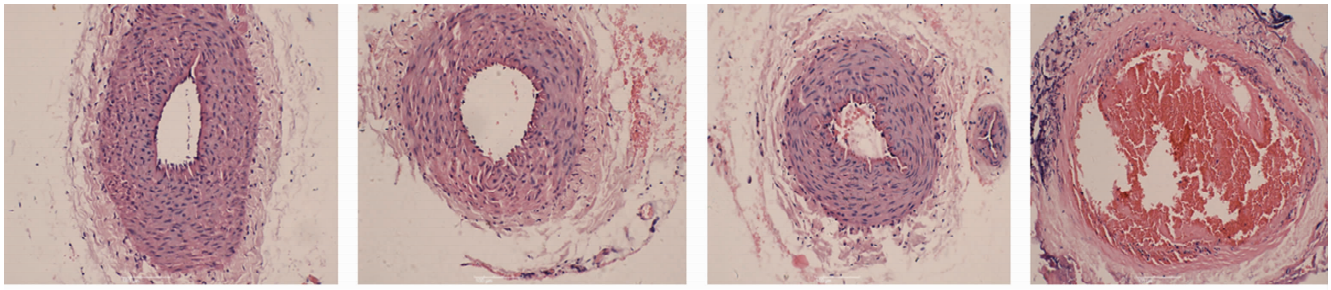
表 2　4 组大鼠药物干预后吻合区血管内膜面积比较

组别	样本量（只）	吻合区血管内膜面积（$\bar{x} \pm s$，mm^2）	
		药物干预开始后 2 d	药物干预开始后 5 d
空白对照组	5	0.17 ± 0.02	0.21 ± 0.01
假手术组	5	0.16 ± 0.01	0.21 ± 0.01
生理盐水组	5	0.18 ± 0.02	0.20 ± 0.01
桃红四物汤组	5	0.20 ± 0.01	0.30 ± 0.02
F 值		2.625	17.063
P 值		0.122	0.000



(1)空白对照组　(2)假手术组　(3)生理盐水组　(4)桃红四物汤组

图 1　4 组大鼠药物干预后 2 d 吻合区血管组织切片（HE 染色　×400）



(1)空白对照组　(2)假手术组　(3)生理盐水组　(4)桃红四物汤组

图 2　4 组大鼠药物干预后 5 d 吻合区血管组织切片（HE 染色　×400）

4　讨　论

Notch 信号通路在血管的增殖与新生中发挥着关键的作用，而 Delta－like4 配体作为 Notch 信号通路调控的重要环节，参与了血管的生成。目前，有关 Delta－like4 对血管生成影响的研究主要集中在肿瘤领域，而 Delta－like4 阻滞剂对肿瘤生成的抑制作用现已成为研究热点。在创伤外科中，利用 Delta－like4 激动剂来促进血管新生和缩短创伤愈合所需时间的研究具有重要的意义。

血管的新生是创伤修复的关键一环，而具有活血化瘀功效的桃红四物汤在创伤疾患的治疗中扮演极其重要的角色。因创伤外科中常会出现“吸收热”，全国名老中医孙达武根据自身经验在临床应用中将桃红四物汤原方中的熟地黄调整为生地黄，发挥其凉血解热的功效；并考虑到现代中药野生较少、药效打折的情况，故在经典方的基础上加大了全方用药剂量。现代药理学研究证实：桃红四物汤具有改善心功能、抑制血小板聚集、改善血液流变学及微循环的作用；在创伤愈合方面，桃红四物汤能促进局部血液循环，扩张血管[12]。桃红四物汤还可以通过干预调节 Notch 通道而促进血管新生；当机体组织受到损伤后，Notch 信号通路激活，Delta－like4 与受体结合后，进入细胞核发挥生物学作用[13]，同时与表皮生长因子形成负反馈[14－15]，影响内皮细胞生长[16－17]。该通路通过增加血管出芽，参与血管修复过程[18－19]。孙绍裘等[1]用大鼠血管吻合模型证实了桃红四物汤能促

进血管内膜增殖,其机制有可能是通过促进血管内膜的血管内皮生长因子的表达而达到修复损伤血管的目的。林念慈等[2]的研究表明,在兔自体皮片移植模型中桃红四物汤可上调 Delta-like4 的表达,增加血管的数目,促进血管的新生。本实验中,药物干预开始后 5 d,桃红四物汤组血管吻合区 Delta-like4 蛋白表达量均高于生理盐水组、假手术组、空白对照组,而且血管内膜面积均大于其他 3 组;这说明桃红四物汤可上调 Delta-like4 的表达,促进血管内膜的增殖。

本研究结果提示,桃红四物汤能上调血管吻合大鼠模型吻合区 Delta-like4 的表达,促进血管新生。但本研究未能明确桃红四物汤的作用时间与其上调 Delta-like4 表达作用之间的关系,以及桃红四物汤中何种物质通过何种途径上调 Delta-like4 的表达。这些问题需在今后的研究中进一步探讨。

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