



·论著·

催乳颗粒通过分泌Frizzled相关蛋白2-Wnt/ β -catenin信号通路改善大鼠产后缺乳*

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【摘要】目的 基于分泌Frizzled相关蛋白2(secreted frizzled-related protein 2, SFRP2)-Wnt/ β -catenin信号通路探讨催乳颗粒(Cuiru Keli, CRKL)治疗产后缺乳的效果及其作用机制。**方法** 通过向分娩后第3天的雌鼠灌胃2 mL 1.6 mg/mL甲磺酸溴隐亭来建立产后缺乳大鼠模型。将产仔时间差小于48 h的雌性大鼠随机分为7组: 正常组(不造模, 不给药); 模型组; CRKL低剂量组(模型组+3 g/kg CRKL); CRKL中剂量组(模型组+6 g/kg CRKL)、CRKL高剂量组(模型组+9 g/kg CRKL); 阳性药组(模型组+3 mg/kg 多潘立酮); NC组(模型组+生理盐水); 每组6只。除正常组和模型组外, 其余5组按分组药物和剂量连续灌胃给药, 每天1次, 共10 d。取7组大鼠, 测量10 d仔鼠总窝质量变化、HE染色观察乳腺病理变化。取除阳性对照组之外的6组大鼠, HE染色观察垂体病理变化, 检测血清中PRL含量(ELISA)、乳腺组织(mammary tissue, MT)中PRLR的表达(免疫组化染色)、MT中乳汁合成相关基因mRNA的表达(RT-qPCR), 然后以网络药理学和分子对接研究CRKL对产后缺乳的治疗作用和机制, 特别是其是否通过SFRP2-Wnt/ β -catenin信号通路治疗产后缺乳; 再通过检测相关通路基因(RT-qPCR)和蛋白(Western blot)进行验证。然后进行细胞实验验证: 取鉴定后的大鼠乳腺上皮细胞(rat mammary epithelial cell, RMEC), 将RMEC分为4组: 正常组(原代培养的RMEC, 不处理)、SFRP2过表达组(原代培养的RMEC+SFRP2过表达载体)、SFRP2过表达+CRKL组(SFRP2过表达组+10%含药血清)、阴性对照组(原代培养的RMEC+空载体)。RT-qPCR检测过表达SFRP2后CRKL对于乳汁合成相关基因FASN、CSN2和GLUT1 mRNA表达的影响。**结果** 与模型组相比, 低、中、高剂量的CRKL均可以提高仔鼠10 d体重质量增加量($P<0.05$ 或 $P<0.01$), 均能有效增加产后缺乳大鼠泌乳量($P<0.01$), 增加乳腺小叶的面积, 增加腺泡腔的大小和填充量, 并促进产后缺乳大鼠催乳素的分泌和表达($P<0.05$ 或 $P<0.01$), 显著促进产后缺乳大鼠乳腺脂肪、乳蛋白和乳糖合成基因的表达($P<0.05$ 或 $P<0.01$)。网络药理学表明, Wnt信号通路可能是CRKL治疗产后缺乳的关键通路。分子对接结果表明CRKL和CCND1、SFRP2具有良好的结合能力。与模型组相比, 低、中、高剂量的CRKL均抑制体内SFRP2基因的表达($P<0.01$), 激活了产后缺乳大鼠乳腺中的Wnt/ β -catenin信号通路中CCND1、c-Myc的基因和蛋白的表达($P<0.05$ 或 $P<0.01$)。细胞实验表明, 过表达SFRP2后, 与正常组相比, 乳汁合成相关基因FASN、CSN2和GLUT1的mRNA水平也降低($P<0.01$)。加入含药血清后, 上述基因的表达上调(与SFRP2过表达组相比, $P<0.01$)。**结论** CRKL通过SFRP2-Wnt/ β -catenin信号通路治疗产后缺乳, SFRP2可能成为产后缺乳诊断和治疗的新靶点。这揭示了CRKL治疗产后缺乳的新机制, 并促进了其临床推广。

【关键词】 催乳颗粒 产后缺乳大鼠模型 网络药理 乳腺上皮细胞 作用机制 中医药

Cuiru Keli Improves Postpartum Hypogalactia in Rats Through Secreted Frizzled-Related Protein 2-Wnt/ β -catenin Signaling Pathway XUE Qiuyun, HUANG Yurong, LI Hui, LI Chen, CHENG Chenglong, WANG Yuting, MIAO Chenggui[△]. School of Integrated Chinese and Western Medicine, Anhui University of Chinese Medicine, Hefei 230012, China

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【Abstract】Objective Based on the secreted frizzled-related protein 2 (SFRP2)-Wnt/ β -catenin signaling pathway, this study explored the effect and mechanism of Cuiru Keli (CRKL) in the treatment of postpartum hypogalactia. **Methods** A rat model of postpartum hypogalactia was established by gavaging 2 mL of 1.6 mg/mL bromocriptine mesylate to female rats on the third day after delivery. Female rats with a delivery time difference of less than 48 hours were selected and randomly assigned to 7 groups, including a normal group (without any modeling or medication), a model group, a CRKL low-dose group of model group model rats receiving CRKL at the dose of 3 g/kg, a CRKL medium-dose group of model rats receiving CRKL at the dose of 6 g/kg, a CRKL high-dose group of model rats receiving CRKL at the dose of 9 g/kg, a positive drug group of model rats receiving domperidone at the dose of 3 mg/kg, and a negative control (NC) group of model rats receiving normal saline. Each group contained 6 rats. Except for the normal and model

* 安徽省科技厅科研项目(No. 2022e07020028, No. 1804a0802218), 安徽省教育厅科研项目(No. KJ2021A0588, No. gxgwx2022019)和安徽中医药大学科研项目(No. 2020rcZD001, No. 2022BHTNXA05)资助

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出版日期: 2024-05-20

groups, the remaining 5 groups were continuously administered with the respective intervention drugs at the specified doses by gavage once a day for 10 days. Changes in the total litter mass of the offspring in the 7 groups within 10 days were measured, and HE staining was performed to identify pathological changes in the mammary tissue (MT). Six groups of rats (excluding the positive control group) were used to observe the pathological changes of eosinophils in pituitary tissue. ELISA was performed to determine the content of prolactin (PRL) in serum, immunohistochemical staining was used to determine the expression of prolactin receptor (PRLR) in MT, and RT-qPCR was used to determine the mRNA expression of genes related to lactation in MT. Network pharmacology and molecular docking were used to study the therapeutic effect and mechanism of CRKL on postpartum hypogalactia, particularly whether it acted through the SFRP2-Wnt/ β -catenin signaling pathway. The mechanism of CRKL treatment was further validated by detecting mRNA (RT-qPCR) and protein expression (Western blot) of related pathway genes. Cell experiments were conducted using primary culture rat mammary epithelial cells (RMEC) from rat MT. RMEC were divided into four groups, including a normal group (primary culture RMEC, untreated), *SFRP2* overexpression group (primary cultured RMEC treated with *SFRP2* overexpression vector), *SFRP2* overexpression+CRKL group (receiving treatment for *SFRP2* overexpression group plus 10% drug-containing serum), and negative control group (primary culture RMEC treated with empty vector). The effect of CRKL on the expression of lactation-related genes *FASN*, *CSN2*, and *GLUT1* mRNA after *SFRP2* overexpression was detected by RT-qPCR. **Results** In this study, CRKL was administered at a dose of 3 g/kg in the CRKL low-dose group, 6 g/kg in the medium-dose group, and 9 g/kg in the high-dose group ($P<0.05$ or $P<0.01$). Compared with the model group, CRKL at all doses significantly increased the total litter weight gain of the offsprings within 10 days ($P<0.05$ or $P<0.01$), and effectively increased lactation ($P<0.01$), the area of mammary lobules, and the size and filling of acinar cavities. CRKL at all doses also increased the number of eosinophils that secreted PRL in the pituitary gland of the postpartum hypogalactia rat model, and increased the content of PRL in the serum ($P<0.05$ or $P<0.01$). CRKL promoted the secretion and expression of PRL in postpartum hypogalactic model rats. In addition, it significantly promoted the expression of genes related to milk fat, milk protein, and lactose synthesis in MT ($P<0.05$ or $P<0.01$). Network pharmacology predicted that the Wnt signaling pathway might be a key pathway for CRKL in treating postpartum hypogalactia. The molecular docking results showed that related chemical components in CRKL had good binding ability with CCND1 and SFRP2. Compared with the model group, CRKL at all doses inhibited the expression of *SFRP2* gene *in vivo* ($P<0.01$) and activated the mRNA and protein expression of CCND1 and c-Myc in the Wnt/ β -catenin signaling pathway in MT ($P<0.05$ or $P<0.01$). Cell experiments showed that, compared to the normal group, *SFRP2* overexpression reduced the mRNA expression of milk synthesis-related genes *FASN*, *CSN2*, and *GLUT1* in RMEC ($P<0.01$). The CCK8 results indicated that 10% of the drug-containing serum was the effective concentration administered to cells ($P<0.01$). After administering drug-containing serum, the expression of the lactation-related genes *FASN*, *CSN2*, and *GLUT1* were up-regulated (compared with the *SFRP2* overexpression group, $P<0.01$). **Conclusion** CRKL alleviates postpartum hypogalactia through the SFRP2-Wnt/ β -catenin signaling pathway. SFRP2 might be a potential new target for the diagnosis and treatment of postpartum hypogalactia. This reveals a new mechanism of CRKL in treating postpartum hypogalactia and promotes its clinical application.

【Key words】 Cuiru Keli Rat model of postpartum hypogalactia Network pharmacology
Mammary epithelial cell Mechanism Traditional Chinese Medicine

产后缺乳是指哺乳期母亲分泌的乳汁少甚至无法喂养婴儿。任何精神刺激,如担忧、恐慌或悲伤,都会减少母乳产量^[1]。乳汁的合成和分泌与内分泌系统密切相关。孕妇分娩后,机体分泌的各类激素经血液运输至乳腺,如催乳素(prolactin, PRL)^[2]。PRL是促进乳汁合成的主要激素^[3]。

催乳颗粒(Cuiru Keli, CRKL)是由黄芪、漏芦、党参、当归、白术、川芎、柴胡、王不留行(炒)和萱草根制成的颗粒剂。取上述九味药材,加水蒸3次,将其与蔗糖、糊精一起煎、过滤、浓缩、制粒,得到CRKL。中医认为,气血虚弱和肝气郁结是产后缺乳的两个主要原因。CRKL具有益气养血,通络下乳的功效,用于产后气虚弱所致的

缺乳少乳^[4]。

分泌Frizzled相关蛋白2(secreted frizzled-related protein 2, SFRP2)是分泌Frizzled相关蛋白家族的一员,与Fz受体结合拮抗Wnts激活Wnt信号通路^[5]。Wnt信号通路激活靶基因在细胞核中的表达,膜受体、Wnt配体蛋白、转录调节和信号转导是该途径的主要组成部分^[6]。研究表明,Wnt/ β -catenin信号通路在乳腺的生长和分化中发挥着非常重要的作用,是脂肪生成基因表达、脂肪生成和脂肪去饱和的重要途径^[7]。小鼠乳腺上皮细胞中经典Wnt信号通路的激活可以促进乳蛋白的产生^[8]。研究Wnt/ β -catenin信号通路的调控机制对产后缺乳的治疗具有重要意义。

本研究发现, *SFRP2*在产后缺乳大鼠模型的乳腺组织(mammary tissue, MT)中异常高表达。因此, CRKL可能通过靶向*SFRP2*来影响Wnt/ β -catenin信号通路, 从而增加产后缺乳大鼠模型的泌乳量。本研究以SD大鼠为实验对象, 建立了合适的产后缺乳大鼠模型。并在体外培养原代大鼠乳腺上皮细胞(rat mammary epithelial cell, RMEC), 研讨CRKL能否通过*SFRP2*-Wnt/ β -catenin信号通路治疗产后缺乳, 进一步阐明CRKL治疗产后缺乳的机制, 为临床推广CRKL提供依据。

1 材料与方法

1.1 实验动物

本实验选用SPF级、2月龄SD大鼠, 体质量为(200±20) g。本研究经安徽中医药大学实验动物伦理委员会批准(动物伦理编号: AHUCM-rats-2022129)。

1.2 主要材料和仪器

CRKL购自蚌埠丰原涂山制药有限公司(中国)。甲磺酸溴隐亭购自DOPPEL Farmaceutici S.r.l.(意大利)。多潘立酮购自西安杨森制药有限公司(中国)。CCK8试剂盒购自上海碧云天生物技术有限公司(中国)。ELISA试剂盒购自武汉基因美有限公司(中国)。DMEM/F12培养基、胎牛血清购自ThermoFisher公司(美国)。 β -catenin、c-Myc、CCND1、 β -actin一抗购自Abcam(美国)。CK18、催乳素受体(prolactin receptor, PRLR)一抗购自北京博奥森生物科技有限公司(中国)。

细胞培养箱购自Thermo公司(美国)。倒置荧光显微镜购自Olympus公司(日本)。罗氏lightCycler 96 荧光定量PCR仪购自Roche公司(瑞士)。Tanon 5200 凝胶成像仪购自上海天能科技有限公司(中国)。Bio-Rad 基础电源电泳仪购自美国伯乐公司(美国)。

1.3 动物实验

1.3.1 产后缺乳大鼠模型的建立

通过向分娩后第3天的雌鼠灌胃2 mL 1.6 mg/mL甲磺酸溴隐亭来建立产后缺乳大鼠模型。

1.3.2 CRKL给药方法及含药血清的制备

经过两天的适应性喂养后, 雄性和雌性配对(1:4)。基于CRKL的临床用药剂量和人大鼠体表面积转换方法, 得到产后缺乳大鼠的日剂量为6 g/kg CRKL。设正常组(不造模, 不给药)、模型组(只造模, 不给药)和模型+不同剂量CRKL(1.5、3、6、9、12 g/kg)组, 每组6只。模型+不同剂量CRKL(1.5、3、6、9、12 g/kg)组取造模大鼠, 以1.5、3、6、9、12 g/kg CRKL灌胃, 每天1次, 连续7 d。通过测量CRKL治疗第7天时产后缺乳大鼠模型单

小时泌乳量, 来确定后继实验选用的CRKL剂量。交配后, 将产仔时间差小于48 h的雌性大鼠随机分为7组: 正常组; 模型组; CRKL低剂量组(模型组+3 g/kg CRKL); CRKL中剂量组(模型组+6 g/kg CRKL)、CRKL高剂量组(模型组+9 g/kg CRKL); 阳性药组(模型组+3 mg/kg 多潘立酮); NC组(模型组+生理盐水); 每组6只。除正常组和模型组外, 其余5组按分组药物和剂量灌胃给药, 每天1次, 连续灌胃给药10 d。取7组大鼠, 测量10 d仔鼠总窝质量变化, HE染色观察乳腺病理变化。取除阳性对照组之外的6组大鼠, HE染色观察垂体病理变化, 检测血清中PRL含量(ELISA)、MT中PRLR的表达(免疫组化染色)、MT中乳汁合成相关基因mRNA的表达(RT-qPCR), 然后以网络药理学和分子对接预测CRKL调节的信号通路, 再通过检测相关通路基因(RT-qPCR)和蛋白(Western blot)进行验证。

另取正常雌鼠6只, 连续灌胃给药CRKL(6 g/kg)5 d, 2次/d, 最后一次给药1 h后, 麻醉雌鼠, 采集血样。离心10 min取血清, 在56 °C水浴下灭活30 min。将CRKL含药血清加入RMEC培养基中, 用于制备不同含量(0、1%、2.5%、5%、10%、15%、20%、25%)的含药血清^[9]。

1.3.3 10 d仔鼠总窝质量变化的测定

称取第一天给药前仔鼠体质量, 记为 W_1 , 给药10 d后, 再次称取仔鼠体质量, 记为 W_2 , $W_2 - W_1$ 记为10 d仔鼠总窝质量的变化。

1.3.4 HE染色观察乳腺和垂体病理变化

随机在各组中取3只雌鼠的MT和垂体组织, 包埋MT和垂体组织, 切片后脱蜡, 常规HE染色后显微镜下观察。

1.3.5 ELISA检测血清中PRL含量

每组取6只雌鼠, 血液离心20 min后收集上清, 按照ELISA试剂盒说明进行实验, 酶标仪测定PRL含量。

1.3.6 免疫组化检测MT中PRLR的表达

每组取3只雌鼠, MT包埋切片后, 置于载玻片上。用二甲苯和不同浓度梯度的乙醇对切片脱蜡。脱蜡冲洗后, 将载玻片置于3% H_2O_2 浸泡, 修复抗原。封闭, 加PRLR一抗(1:200)和二抗(1:400)。加入显色剂后, 进行复染, 脱水, 封片后观察。计算PRLR阳性面积占总视野面积的比例。

1.3.7 RT-qPCR

每组取3只雌鼠, 麻醉雌鼠后取MT, 每80 mg MT加入1 mL Trizol。对于RMEC, 在培养瓶中加入1 mL Trizol试剂, 轻微晃动培养瓶, 使试剂均匀分布, 之后使用玻璃吹打管吹打RMEC 5 min, 使细胞充分裂解脱落。按照

Trizol试剂说明书提取MT和RMEC中RNA,测定RNA的浓度与纯度,以光密度(OD)₂₆₀/OD₂₈₀在1.8~2.0为佳。按试剂盒说明合成cDNA,以13 μL的反应体系进行扩增。按照RT-qPCR试剂盒的说明步骤进行反应,正常对照组为对照样品,β-actin为内参基因,以2^{-ΔΔCt}计算mRNA的相对表达量。引物序列见表1。

表 1 RT-qPCR 引物序列
Table 1 RT-qPCR primer sequences

Gene	Primer sequence
ACACA	F: 5'-TTCCCATCCGCCTCTTCCTGAC-3' R: 5'-TGCTTGCTCTCCATACGCCTGAAAC-3'
β-actin	F: 5'-TGTCACCAACTGGGACGATA-3' R: 5'-GGGGTGTGAAGGTCTCAAA-3'
c-Myc	F: 5'-AGCAGCGACTCTGAAGAAGAACAAG-3' R: 5'-GGATGACCCTGACTCGGACCTC-3'
CSN2	F: 5'-GGTCTTCATCCTTGCTGCCTTG-3' R: 5'-CCTGTCCCATGAGTTTCACCTTCTG-3'
CSN1S1	F: 5'-CTGTGCTCTTGCTCTGCCTAG-3' R: 5'-CCTGTTCTCTACTGCTGCTATTCTC-3'
CCND1	F: 5'-GAGCGGATGAGAACAAGCAGATC-3' R: 5'-GGAGGGTGGGTTGGAAATGAACTTC-3'
FASN	F: 5'-GTGTGGTAGGCTTGGTGAAGTGC-3' R: 5'-GTGAGATGTGCTGCTGAGGTTGG-3'
GLUT1	F: 5'-CATCCACCACACTCACCACAC-3' R: 5'-GCCTGCCAAAGCGATTAAACAAAGAG-3'
SFRP2	F: 5'-CACGCGATCGAGTACCAGAACATG-3' R: 5'-GAGCGSGCACAGGAAGTCTTCT-3'

ACACA: acetyl-CoA carboxylase alpha; c-Myc: V-Myc avian myelocytomatosis viral oncogene homolog; CSN2: casein beta; CSN1S1: casein alpha s1; CCND1: cyclin D1; FASN: fatty acid synthase; GLUT1: glucose transporter type 1; SFRP2: secreted frizzled-related protein 2.

1.3.8 Western blot检测MT中CCND1、c-Myc和β-catenin的蛋白表达量

每组取3只雌鼠,加入RIPA裂解液充分裂解MT后,离心取上清蛋白,按照BCA蛋白浓度测定试剂盒说明书检测蛋白浓度。10% SDS-PAGE电泳90 min后转膜60 min。5%脱脂牛奶室温封闭1 h,加入CCND1(1:10 000)、c-Myc(1:1 000)、β-catenin(1:1 000)、β-actin(1:10 000)4℃一抗孵育过夜,二抗(1:20 000)室温孵育1 h后ECL底物显色。采用Image J分析电泳条带图的灰度值。

1.4 网络药理学分析

采用TCMSP(<http://tcmspw.com/tcmsp.php>)收集CRKL所含九味中草药的化学成分,利用Swisstarget平台对其靶点进行标准化^[10]。通过OMIM(<https://omim.org/>)和GeneCards数据库(<https://www.genecards.org/>)收

集与产后缺乳相关的疾病靶点^[11]。利用STRING平台构建蛋白-蛋白相互作用(protein-protein interaction, PPI)网络^[12]。利用GO富集分析和KEGG富集分析,预测CRKL治疗产后缺乳的关键通路^[13]。

1.5 分子对接

从RCSB PDB数据库获取大分子蛋白靶受体(<https://www.rcsb.org/>),从TCMSP数据库中获得有效成分化合物的2D结构。利用CB-DOCK在线网站(<http://clab.labshare.cn/>)进行了分子对接模拟^[14]。估计值的绝对值表示化合物与蛋白质之间的结合活性,估计值绝对值越大,化合物与蛋白质的结合越稳定。

1.6 细胞实验

通过MT原代培养RMEC。在20%胎牛血清和1%青霉素-链霉素溶液DMEM/F12培养基中培养,实验中使用的细胞是原代培养第三代至第五代的RMEC^[15]。免疫荧光染色后,以CK18阳性鉴定所培养出的细胞是否为RMEC。通过CCK8实验检测不同含量CRKL含药血清对RMEC增殖的影响,挑选合适的CRKL含药血清进行腺病毒过表达细胞实验,然后以RT-qPCR检测过表达SFRP2后CRKL对于乳汁合成相关基因mRNA的表达的影响。

1.6.1 免疫荧光染色鉴定RMEC

用体积分数4%多聚甲醛溶液将RMEC固定在培养板中15 min。用TritonX-100处理细胞10 min。2%BSA密封30 min,加入CK18(1:200)一抗,在4℃下过夜。加入荧光二抗(1:400),每次5 min,并在室温下孵育2 h。加入DAPI染色10 min。每步后使用PBS清洗后再进入下一步。用抗荧光淬灭片(含DAPI)密封片剂。显微镜下观察。CK18阳性则表明所培养出的细胞是RMEC。

1.6.2 CCK8法检测CRKL对RMEC增殖的影响

将RMEC均匀接种到96孔板中。在细胞完全黏附到壁上后,分别用0、1%、2.5%、5%、10%、15%、20%、25%的CRKL含药血清处理24 h。然后按照CCK8试剂盒的说明进行操作,并用酶标仪测量450 nm处的OD值^[16]。OD值正比于细胞增殖率。挑选合适的CRKL含药血清进行腺病毒过表达细胞实验。详见1.15小节。

1.6.3 腺病毒过表达细胞实验

SFRP2腺病毒过表达载体由汉恒生物科技有限公司提供。按照产品说明书对生物安全柜中的腺病毒进行稀释,对原代培养的RMEC进行转染培养^[17]。将RMEC分为4组:正常组(原代培养的RMEC,不处理)、SFRP2过表达组(原代培养的RMEC+SFRP2过表达载体)、SFRP2过表达+CRKL组(SFRP2过表达组+10%含药血清)、阴性对照

组(原代培养的RMEC+空载体)。RT-qPCR检测过表达SFRP2后, CRKL对于乳汁合成相关基因FASN、CSN2和GLUT1 mRNA的表达的影响, 检测方法见1.3.7。

1.7 统计学方法

采用SPSS26.0软件进行统计学分析, 所有数据结果均以 $\bar{x} \pm s$ 表示, 采用单因素方差分析进行多组间分析, 组间两两比较采用LSD法, $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 CRKL显著提高模型大鼠的泌乳量

结果如图1, 与模型组相比, CRKL剂量在3 g/kg以上时, 第7天单小时泌乳量显著增加($P < 0.05$ 或 $P < 0.01$), 表明有治疗效果。因此, 本研究选择3 g/kg的剂量作为CRKL低剂量组, 6 g/kg的剂量作为CRKL中剂量组, 9 g/kg的剂量为CRKL高剂量组。与模型组相比, 低、中、高剂量的CRKL均可以提高仔鼠10 d体质量增加量($P < 0.05$ 或 $P < 0.01$)。此外, CRKL可以增加乳腺小叶的面积, 减少小叶之间的结缔组织, 增加腺泡腔的大小和填充量。

2.2 CRKL促进模型大鼠PRL的分泌和PRLR表达

如图2, 嗜酸性粒细胞在HE染色下细胞质显示桃红色。与模型组相比, 低、中、高剂量的CRKL能够明显增

加垂体中分泌PRL的嗜酸性粒细胞的表达, 增加PRL的分泌。与模型组相比, 低、中、高剂量的CRKL治疗后产后缺乳大鼠模型的血清中PRL的表达增加($P < 0.01$)。低、中、高剂量的CRKL治疗后, MT中PRLR蛋白的褐黄色阳性染色面积均大于模型组($P < 0.01$), 表明CRKL显著促进了PRLR的表达。

2.3 CRKL促进模型大鼠乳脂、乳蛋白和乳糖合成

如图3, CRKL促进MT中乳脂合成基因ACACA和FASN的mRNA表达($P < 0.05$ 或 $P < 0.01$)。此外, CRKL能够上调MT中乳蛋白合成基因CSN2和CSN1S1的mRNA表达($P < 0.05$ 或 $P < 0.01$)。并且CRKL显著增强了MT中GLUT1的mRNA表达($P < 0.01$), 促进乳糖合成。

2.4 基于网络药理学预测CRKL调节的信号通路

本实验根据 $OB \geq 30\%$ 、 $DL \geq 0.18$ 等药代动力学特征, 筛选出CRKL中78种潜在活性成分和919个对应靶点(附图1)。在GeneCards数据库和OMIM数据库中分析和筛选了2 638个产后缺乳疾病靶点。从Venn图工具获得了CRKL和产后缺乳靶基因的459个共有靶点(图4A)。PPI蛋白互作网络分析获得重叠基因相互作用网络(附图2)。选择了前5个高度连接的节点作为核心基因推测通路(图4B)。GO富集分析显示CRKL主要通过生物过程、

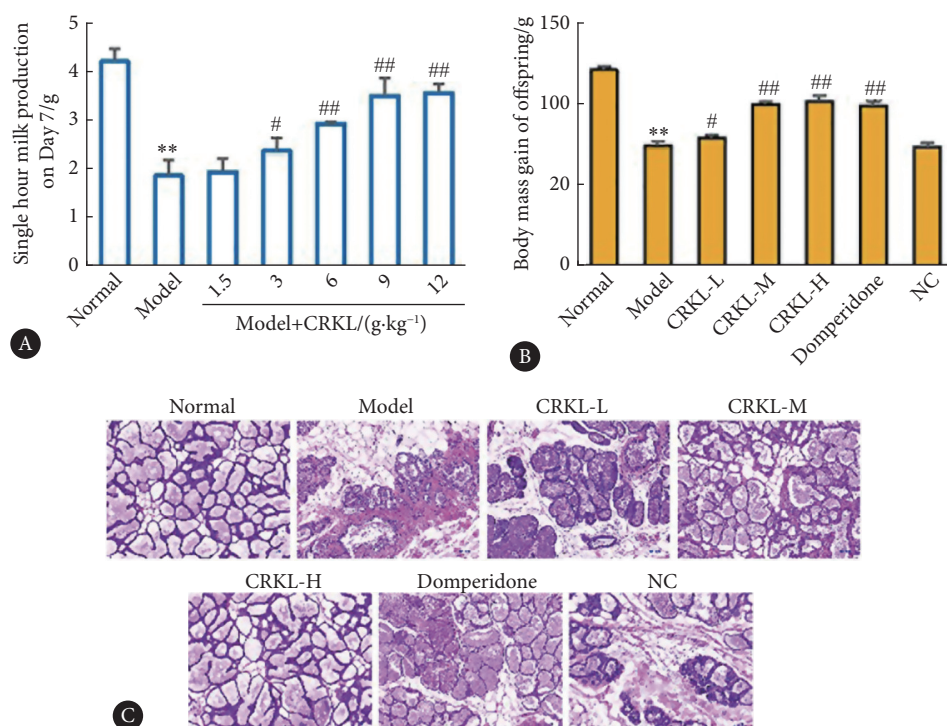


图1 CRKL显著提高模型大鼠的泌乳量

Fig 1 CRKL significantly increases lactation in model rats

A, Lactation volume per hour on the seventh day for female mice given different doses of CRKL ($n=6$). B, Ten day weight gain of offspring mice ($n=6$). C, HE staining of the mammary gland ($\times 100$). CRKL-L: CRKL was given at a low dose of 3 g/kg to model rats; CRKL-M: CRKL was given at a moderate dose of 6 g/kg to model rats; CRKL-H: CRKL was given at a high dose of 9 g/kg to model rats; NC: negative control group. ** $P < 0.01$, vs. normal group; # $P < 0.05$, ## $P < 0.01$, vs. model group.

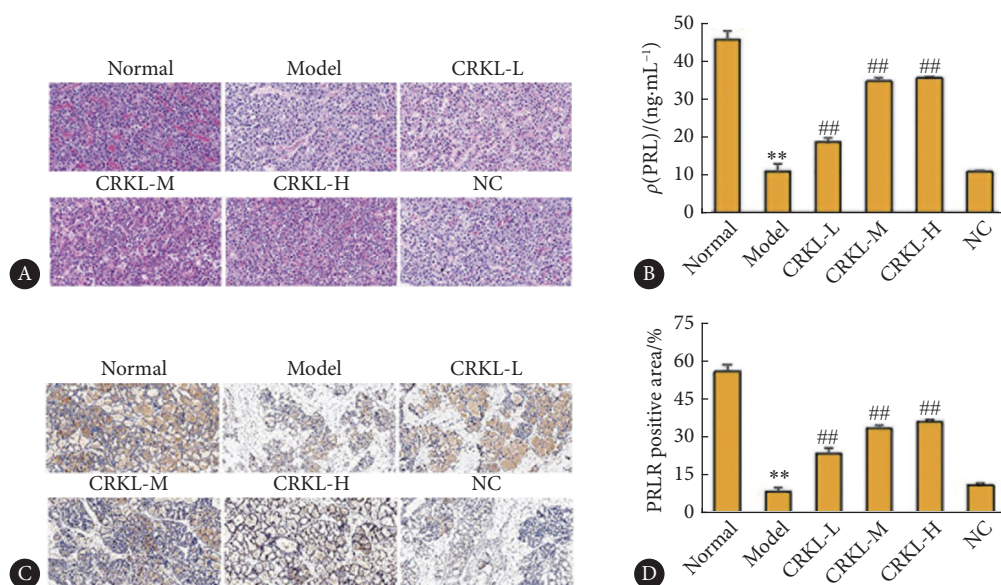


图 2 CRKL促进模型大鼠PRL分泌和PRLR表达

Fig 2 CRKL promotes the PRL secretion and PRLR expression in model rats

A, HE staining of the pituitary tissue (×400). B, Serum PRL levels ($n=6$). C, Immunohistochemical staining of PRLR in MT (×100). D, Quantitative results of PRLR in MT ($n=3$). ** $P<0.01$, vs. normal group; # $P<0.05$, ## $P<0.01$, vs. model group. The other abbreviations are given in the note to Fig 1.

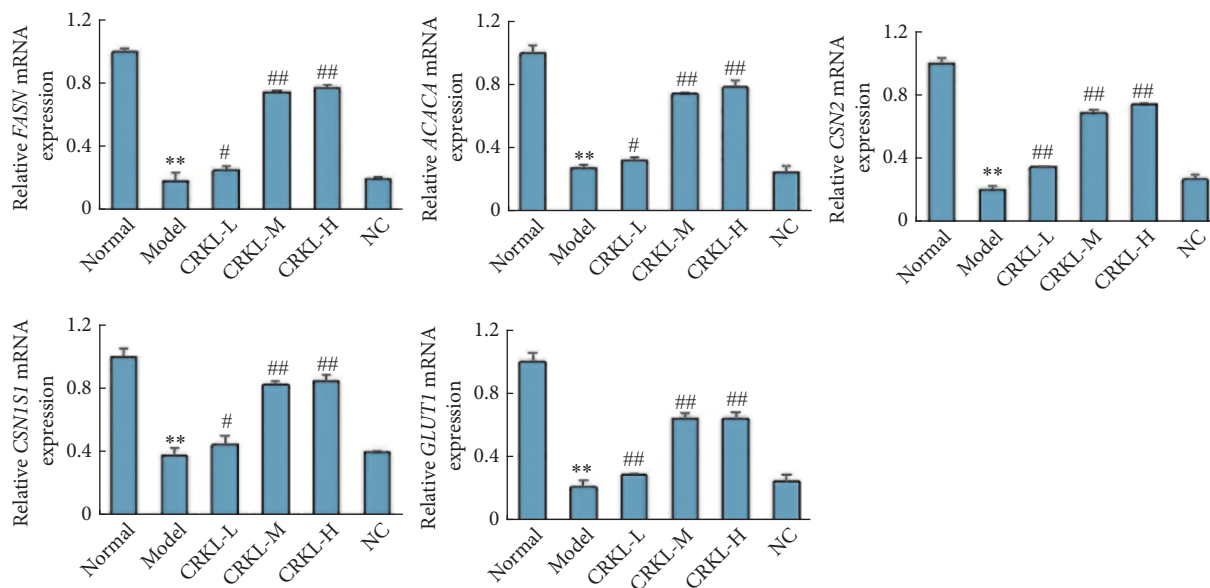


图 3 CRKL促进模型大鼠MT乳脂 (ACACA和FASN)、乳蛋白 (CSN2和CSN1S1) 和乳糖合成基因 (GLUT1) 的表达

Fig 3 CRKL promotes the expression of genes of milk fat (ACACA and FASN), milk protein (CSN2 and CSN1S1), and lactose synthesis in mammary tissue of model rats

$n=3$. ** $P<0.01$, vs. normal group; # $P<0.05$, ## $P<0.01$, vs. model group. The other abbreviations are given in the note to Table 1 and Fig 1.

细胞通信和分子功能发挥作用(附图3)。KEGG结果表明Wnt信号通路是CRKL治疗产后缺乳的关键通路(图4C)。图4D是一个由关键草药、化合物、基因和途径组成的相互作用网络。分子对接结果表明CCND1与CRKL中可能存在的关键化合物具有很强的结合力(图4E)。CCND1分子对接数据见表2。附图均请见本文网络资源附件。

2.5 CRKL通过Wnt/ β -catenin信号通路改善产后缺乳

如图5,与模型组相比,低、中、高剂量的CRKL可以上调产后缺乳大鼠MT中CCND1、c-Myc的mRNA和蛋白表达($P<0.05$ 或 $P<0.01$)。

2.6 CRKL通过SFRP2治疗产后缺乳

根据计算对接的结果,图6A显示了CRKL与SFRP2的相互作用,表现出良好的结合性(表3)。如图6B,CRKL治

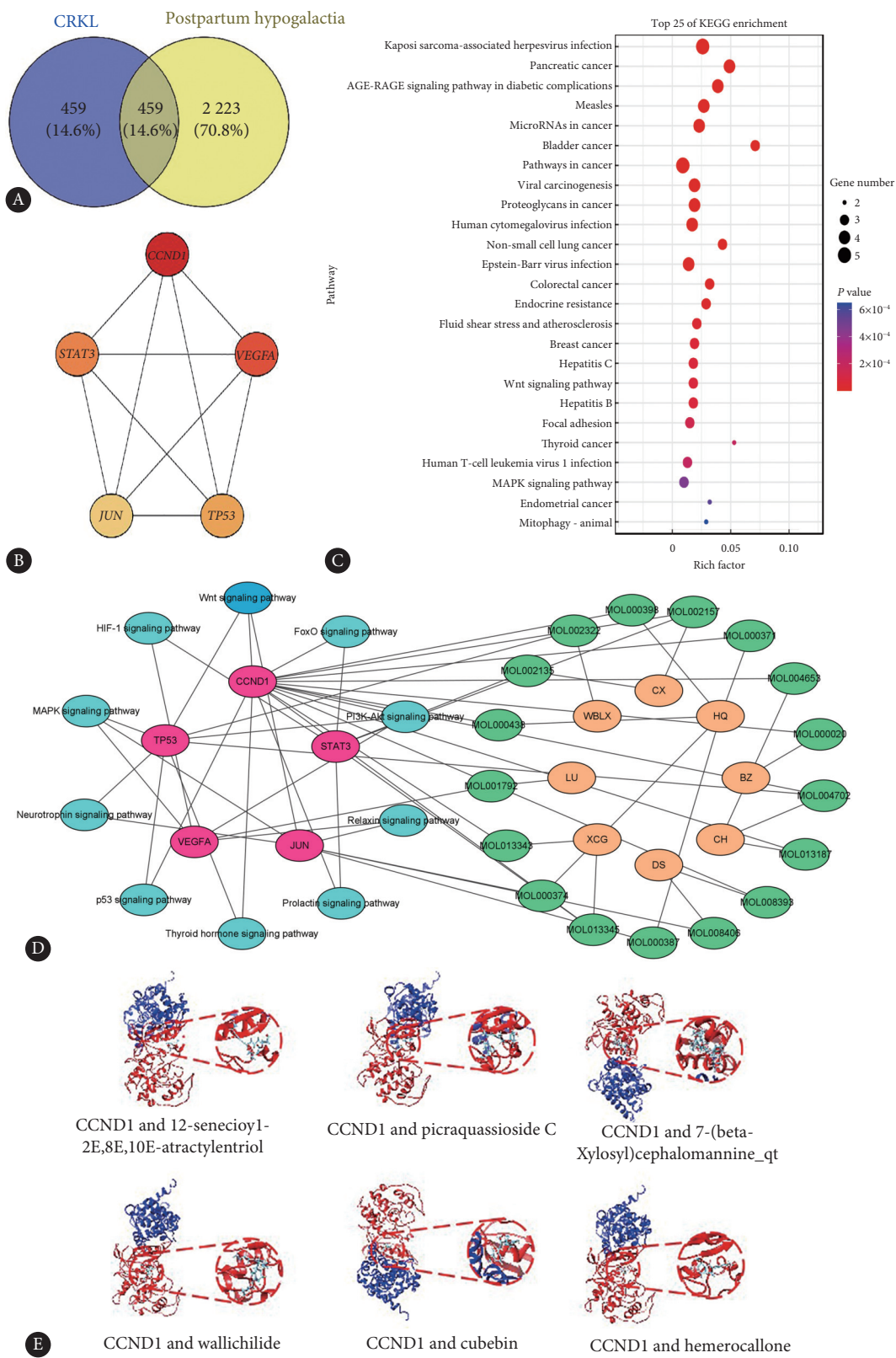


图 4 基于网络药理学预测CRKL调节的信号通路

Fig 4 Prediction of CRKL regulatory signaling pathways based on network pharmacology

A, The Venn diagram. B, The 5 key target genes. C, KEGG enrichment analysis. D, A network of herbal component-targeting pathways. E, Molecular docking.

疗后, *SFRP2*在MT中的mRNA的表达降低, 差异有统计学意义($P<0.01$)。

2.7 CRKL通过SFRP2-Wnt/ β -catenin信号通路治疗产后缺乳

如图7, 利用CK18结果表明所培养出的细胞是

表 2 CCND1分子对接数据
Table 2 CCND1 molecular docking

MOL ID	Molecule name	Herb name	Binding capacity
MOL000020	12-senecieryl-2E,8E,10E-atractylentriol	<i>Atractylodes macrocephala</i> Koidz	-10.01
MOL013345	Picraquassioside C	<i>Hemerocallis Radix</i>	-9.49
MOL008393	7-(beta-Xylosyl)cephalomannine_qt	<i>Codonopsis pilosula</i> (Franch.) Nannf.	-8.98
MOL002157	Wallichilide	<i>Ligusticum chuanxiong</i> Hort.	-8.60
MOL013187	Cubebin	<i>Bupleurum falcatum</i> L.	-8.18
MOL013343	Hemerocallone	<i>Codonopsis pilosula</i> (Franch.) Nannf.	-7.94
MOL004653	(+)-Anomalin	<i>Bupleurum falcatum</i> L.	-7.86
MOL000398	Isoflavanone	<i>Astragalus mongholicus</i> Bunge	-7.82
MOL000371	3,9-di-O-methylnissolin	<i>Astragalus mongholicus</i> Bunge	-7.42
MOL000438	(3R)-3-(2-hydroxy-3,4-dimethoxyphenyl)chroman-7-ol	<i>Astragalus mongholicus</i> Bunge	-7.33

CCND1: Cyclin D1.

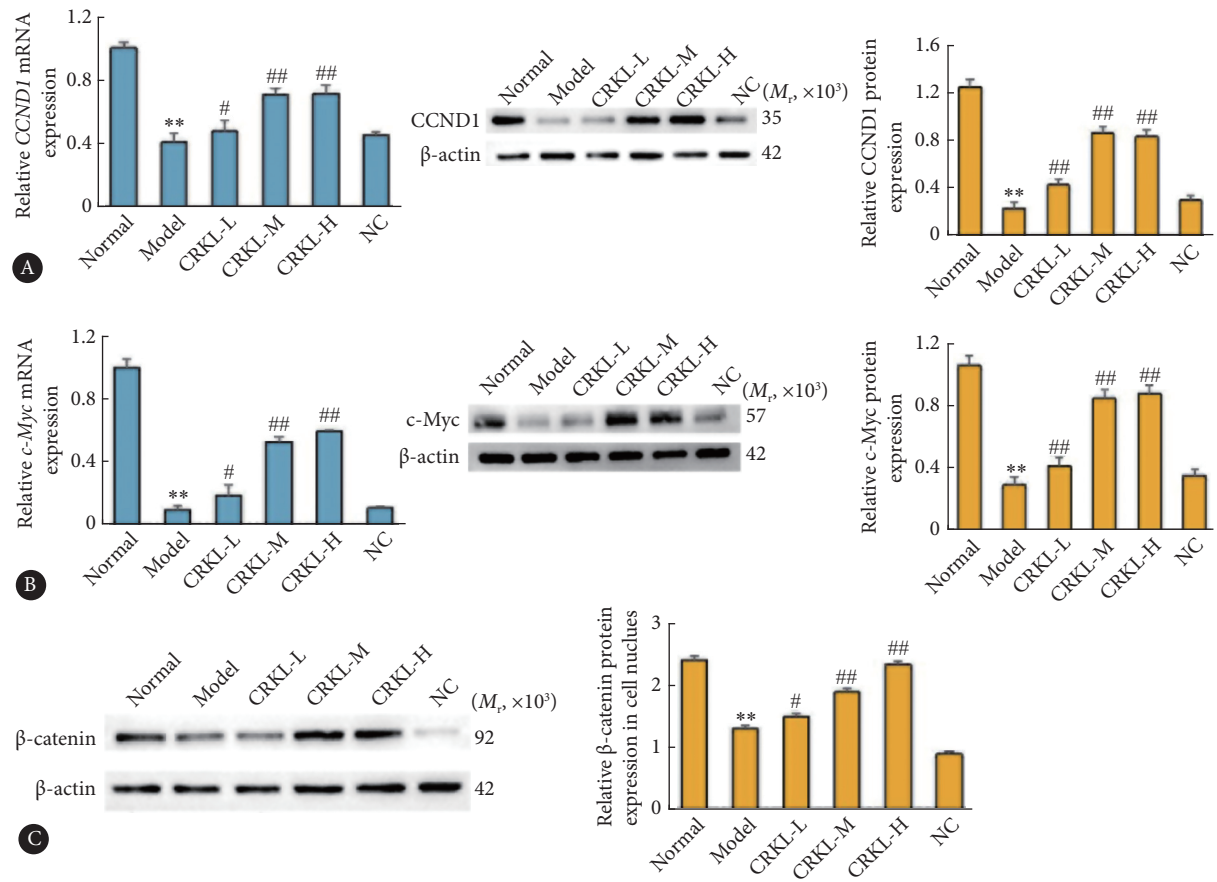


图 5 CRKL通过Wnt/β-catenin信号通路改善产后缺乳

Fig 5 CRKL improves postpartum hypogalactia through the Wnt/β-catenin signaling pathway

A, mRNA and protein expressions of CCND1 in MT. B, mRNA and protein expressions of c-Myc in MT. C, Protein expression of β-catenin in cell nucleus of MT. n=3. ** P<0.01, vs. normal group; # P<0.05, ## P<0.01, vs. model group. The other abbreviations are given in the note to Table 1 and Fig 1.

RMEC。CCK8细胞增殖实验确定CRKL含药血清的含量在10%以后,细胞增殖达到平台期,故选择10%的CRKL含药血清进行后继实验。过表达SFRP2后,与正常组相比,乳汁合成相关基因FASN、CSN2和GLUT1的mRNA水平也降低(P<0.01)。加入含药血清后,上述基因的表达上

调(与SFRP2过表达组相比,P<0.01)。

3 讨论

近年治疗产后缺乳的报道逐渐增多,中医药对于治疗产后缺乳有独特的优势。刘胜春等^[18]收治了66例西医

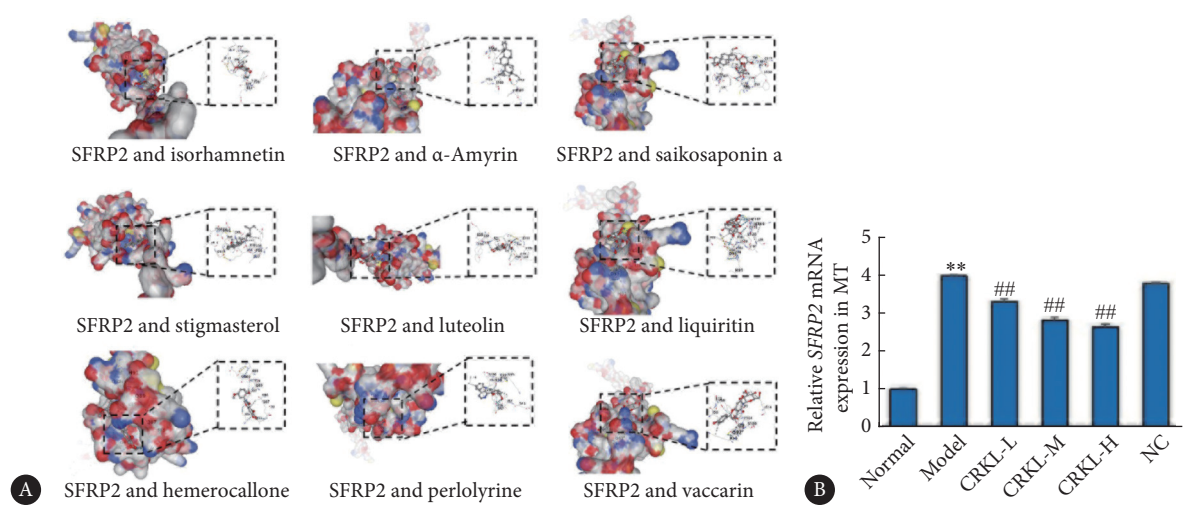


图 6 CRKL通过SFRP2治疗产后缺乳的分子对接结果 (A) 和动物实验结果验证 (B)

Fig 6 Molecular docking results (A) and animal experimental results of CRKL improving postpartum hypogalactia through SFRP2 (B)

n=3. ** $P<0.01$, vs. normal group; ## $P<0.01$, vs. model group.

表 3 SFRP2分子对接数据
Table 3 SFRP2 molecular docking

MOL ID	Molecule name	Herb name	Binding capacity
MOL000354	Isorhamnetin	<i>Astragalus mongholicus</i> Bunge	-6.2
MOL000028	α -Amyrin	<i>Atractylodes Macrocephala</i> Koidz.	-6.6
MOL004635	Saikosaponin a	<i>Radix Bupleuri</i>	-6.7
MOL002140	Perlolyrine	<i>Chuanxiong Rhizoma</i>	-7.2
MOL000449	Stigmasterol	<i>Bupleurum falcatum</i> L.	-6.8
MOL000006	Luteolin	<i>Angelicae Sinensis Radix</i>	-6.8
MOL004903	Liquiritin	<i>Codonopsis pilosula</i> (Franch.) Nannf.	-6.6
MOL008910	Vaccarin	<i>Vaccariae Semen</i>	-7.1
MOL013343	Hemerocallone	<i>Hemerocallis Radix</i>	-5.6

SFRP2: secreted frizzled-related protein 2.

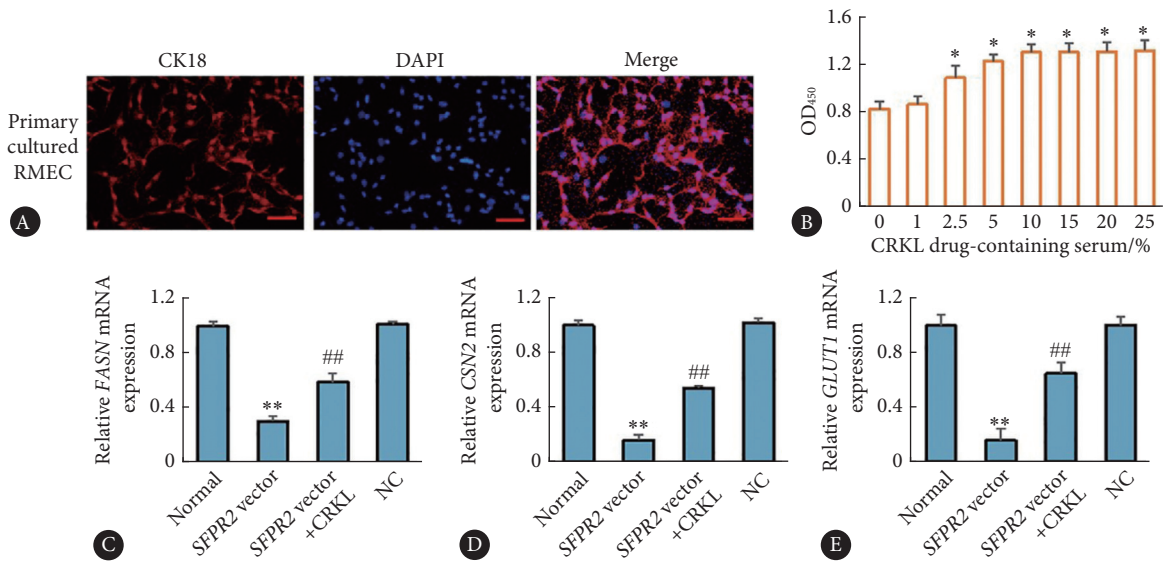


图 7 CRKL通过SFRP2-Wnt/ β -catenin信号通路治疗产后缺乳

Fig 7 CRKL treats postpartum hypogalactia through the SFRP2-Wnt/ β -catenin signaling pathway

A, Relative CK18 fluorescence intensity ($\times 200$). B, The proliferation of RMEC in different CRKL drug-containing serum was detected by CCK8 assay ($n=9$). C-E, The mRNA expression levels of FASN, CSN2 and GLUT1 ($n=3$). * $P<0.01$, vs. 0; ** $P<0.01$, vs. normal group; ## $P<0.01$, vs. SFRP2 vector. NC: negative control group.

诊断为产后缺乳、中医辨证属气血两虚型患者,发现由黄芪、炒白术、当归、川芎和王不留行组成的通乳络方治疗后,患者血PRL升高,雌二醇降低。有研究表明王不留行联合乳房、穴位按摩有利于提高泌乳量。相对于常规的西医疗护理,王不留行联合按摩的治疗后的产妇血清PRL显著升高,并且能有效改善乳房胀痛,提高母乳喂养率^[19]。这些具有临床研究的复方治疗方向与CRKL相似,基本上是调理气血,通络下乳为主。

本研究发现模型组的单小时泌乳量显著低于正常组。CRKL低、中、高剂量组的单小时泌乳量都显著高于模型组,并且CRKL中、高剂量组效果最佳,差异无统计学意义。并且CRKL能够明显增加仔鼠的体重,有利于仔鼠发育。病理HE染色结果显示CRKL可以增加模型组乳腺小叶的面积,减少小叶之间的结缔组织,增加腺泡腔的大小和填充量。此外,CRKL可以促进PRL的分泌,增加MT中PRLR的表达。PRL是一种由垂体前叶产生的多效性激素,参与体内平衡、繁殖和泌乳^[20]。PRLR可被PRL为主的各类激素激活^[21]。CRKL治疗后,产后缺乳模型大鼠脑垂体中分泌PRL的嗜酸性粒细胞数量显著增多,血液中的PRL含量也显著提高,有利于促进泌乳。同时,MT中PRLR表达水平相应升高,能够更好地与PRL结合,增加泌乳量。

ACACA和FASN是新脂肪酸合成的关键限速酶,在母乳中乳脂合成过程中发挥重要作用^[22]。CRKL能显著提高乳脂合成相关基因FASN和ACACA的表达,促进乳脂合成。本研究发现,CRKL可以显著上调产后缺乳大鼠MT中乳蛋白合成相关基因CSN2和CSN1S1的表达,显著改善乳蛋白合成。*GLUT1*在各种组织细胞中广泛表达,以调节葡萄糖摄取^[23]。CRKL能有效提高*GLUT1*的表达水平。综上所述,CRKL不仅能够增加产后缺乳大鼠模型的泌乳量,还能更好的提升乳汁品质,为新生儿提供更好的营养来源。

网络药理学预测出Wnt信号通路是CRKL治疗产后缺乳的关键途径。本研究推测出了CRKL中的78种潜在的活性成分可能是发挥治疗作用的重要成分,但关于其进入外周血,发挥的具体作用仍需进一步研究。本研究发现CRKL可以显著上调Wnt信号通路中关键基因*CCND1*、*c-Myc*和 β -catenin的表达。而SFRP2不仅是一种Wnt结合蛋白,还可以与Wnt蛋白相互拮抗,影响轴突引导。本研究证实了SFRP2不仅在产后缺乳模型中高表达,而且CRKL正是主要影响该基因的表达从而影响Wnt/ β -catenin信号通路,治疗产后缺乳。

综上所述,CRKL可使MT腺泡体积增大,腺泡体积充

盈,有效提高产后缺乳大鼠的泌乳量,对泌乳有一定的促进作用。CRKL可增加PRL和PRLR的含量,促进MT的发育。CRKL还可增加乳蛋白、乳糖和乳脂的含量。总体上看,中、高剂量的CRKL较低剂量的CRKL有更明显的效果,但挑选最合适的剂量并不是本研究的重点,本实验主要是对比CRKL组与模型组的差异,所以未进一步行CRKL剂量组之间的两两比较。本研究证实,CRKL通过SFRP2-Wnt/ β -catenin信号通路治疗产后缺乳,为CRKL治疗产后缺乳的机制提供了新的见解,也为临床推广CRKL提供了理论依据。

* * *

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Author Contribution XUE Qiuyun is responsible for conceptualization and writing--original draft. HUANG Yurong is responsible for formal analysis. LI Hui is responsible for investigation. LI Chen is responsible for methodology. CHENG Chenglong is responsible for software. WANG Yuting is responsible for validation. MIAO Chenggui is responsible for funding acquisition and writing--review and editing. All authors consented to the submission of the article to the Journal. All authors approved the final version to be published and agreed to take responsibility for all aspects of the work.

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Declaration of Conflicting Interests All authors declare no competing interests.

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(2023 – 11 – 14收稿, 2024 – 04 – 29修回)

编辑 吕 熙



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Editorial Office of *Journal of Sichuan University (Medical Science)*