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红色诺卡氏菌细胞壁骨架对小鼠肝癌细胞生长的抑制效果*

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摘要 目的:通过构建 H22 荷瘤小鼠模型,观察红色诺卡氏菌细胞壁骨架(Nocardia Rubra Cell Wall Skeleton, N-CWS)对 H22 荷瘤小鼠肝癌细胞生长的抑制效果。**方法:**复苏并培养 H22 细胞,将 H22 细胞以皮下方式接种到小鼠右侧腋部,建立 H22 荷瘤小鼠肝癌模型,随机分为对照组(生理盐水组)、低浓度 N-CWS 组(400 $\mu\text{g/mL}$)与高浓度 N-CWS 组(800 $\mu\text{g/mL}$),分别灌胃 1 次/d,共灌胃 14 d,最长接种肿瘤 15 d 后处死小鼠,观察肝癌细胞的大小和重量。**结果:**治疗组小鼠肿瘤质量明显低于对照组(1.46 ± 0.16)g,且高浓度组小鼠肿瘤质量(0.52 ± 0.13)g 明显低于低浓度组(0.79 ± 0.13)g,组间对比差异有统计学意义($P < 0.05$)。在治疗第 3 d 起直至结束,治疗组小鼠肿瘤体积均明显低于对照组,从第 5 d 起直至结束,高浓度组小鼠肿瘤体积明显低于低浓度组($P < 0.05$)。第 5 d 起至结束,治疗组小鼠肿瘤体积增长的变化明显缓于对照组($P < 0.05$)。高浓度组小鼠肿瘤体积的增长在第 5 d 后明显缓于低浓度组($P < 0.05$)。对照组在第 9 d 时,增长变化最为显著,较第 7 d 增加约 400 mm^3 。低浓度组变化较稳定。高浓度组肿瘤增长明显变缓,第 7 d 后最为明显,后逐渐趋于缓和。**结论:**N-CWS 可以明显抑制肿瘤生长,且高浓度 N-CWS 抑制肿瘤生长效果更佳。

关键词:诺卡氏菌;免疫增强剂;荷瘤小鼠;肿瘤免疫

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The Effect of N-CWS on Tumor Inhibition of H22 BALB/c Mice*

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ABSTRACT Objective: By constructing of H22 tumor-bearing mouse model, observed the inhibiting effect of N-CWS on the tumor growth of H22 tumor-bearing mice. **Methods:** H22 cells were recovered and cultivated, and were subcutaneously inoculated into the right armpit of the mice. The models of H22 tumor BALB/c mice were established and randomly divided into three groups, the control group (normal saline group), the low concentration N-CWS group (400 $\mu\text{g/mL}$) and the high concentration N-CWS group (800 $\mu\text{g/mL}$). The three groups oral administration for 14 d, once a day, and mice were sacrificed after the longest tumor inoculation for 15 days. The size and weight of liver cancer cells were observed. **Results:** The tumor mass of mice in the treatment group was significantly lower than that in the control group (1.46 ± 0.16) g, and the tumor mass of mice in the high concentration group (0.52 ± 0.13) g was significantly lower than that in the low concentration group (0.79 ± 0.13) g. The difference between the groups was statistically significant ($P < 0.05$). From the 3rd day to the end of the treatment, the tumor volume of the mice in the treatment group was significantly lower than that of the control group. From the 5th day to the end, the tumor volume of the mice in the high concentration group was significantly lower than that in the low concentration group ($P < 0.05$). From the 5th day to the end, the change of tumor volume growth in the treatment group was significantly slower than that in the control group ($P < 0.05$). The tumor volume growth of mice in high concentration group was significantly slower than that in low concentration group after 5 days ($P < 0.05$). The control group had the most significant change on the 9th day, with an increase of about 400 mm^3 compared with the 7th day. The change in the low concentration group was more stable. The tumor growth in the high-concentration group was significantly slowed down, the most obvious after 7 days, and then gradually eased. **Conclusion:** N-CWS can significantly inhibit tumor growth, and high concentration of N-CWS has a better effect on tumor growth.

Key words: Nocardia rubrum; Biological Response Modifiers; Tumor BALB/c mice; Tumor immunity

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前言

注射用红色诺卡氏菌细胞壁骨架 (Nocardia Rubra Cell Wall Skeleton for Injection, N-CWS), 又名 "艾克佳", 是一类

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具有广泛生物学活性的生物免疫增强剂 (Biological Response Modifiers, BRMs), 能够通过调动宿主天然防卫机制或外源给予机体某些生物活性物质来取得免疫增强的效应。红色诺卡氏菌细胞壁骨架由枝菌酸、阿拉伯半乳糖和粘肽组成^[1-3]。Azuma 等人首次发现其天然抗肿瘤免疫活性^[4]。国内研究者亦分离提纯得到了红色诺卡氏菌细胞壁骨架, 并通过体外血清试验证实对肿瘤细胞的抑制率可达 18%~30%, 发现具有抑制癌细胞, 防止肿瘤复发, 延长患者存活期^[5]。研究发现 N-CWS 在临床上用于肺癌、膀胱癌、恶性淋巴瘤、晚期胃癌等的辅助治疗^[6,7]。De Boer 等^[8]报道提示 N-CWS 抗肿瘤的机理可能是通过刺激产生肿瘤坏死因子, 使 T 细胞白介素-2 受体和 HLA-DR 表达增加, 从而激活 T 细胞, 产生抗癌作用。但是近几年, 国内外对 N-CWS 与肿瘤的研究较少, 尤其是对抗肿瘤机制研究, 因此本研究观察 N-CWS 对荷瘤小鼠肝癌细胞的抗肿瘤效果, 为肿瘤的治疗和研究机制提供方向和治疗靶点。现报告如下。

1 材料与方法

1.1 一般材料

选择雄性 BALB/c 小鼠 30 只 (北京大学实验动物中心提供), 质量 (27.3±5.2)g, 6~8 周龄。小鼠肝癌 H22 细胞株由清华大学惠赠。倒置显微镜 (OLYMPUS 公司), RPMI-1640、胎牛血清 (美国 Gibco 公司)、注射用红色诺卡氏菌细胞壁骨架 (艾克佳-福建省山河药业有限公司)。

1.2 细胞复苏与培养

取出液氮中保存的 H22 细胞系, 迅速放入 37℃ 水浴锅中, 手动旋转复苏。加入含 10% 胎牛血清 RPMI-1640 培养液进行细胞培养, 待细胞铺满培养皿 80%~90% 时传代。传 2~3 代 (约 20 d), 细胞达到最佳状态, 进行试验。

1.3 动物模型建立

收集对数期的 H22 细胞, 并进行计数, 无血清培养液进行稀释, 调整至研究浓度, 以皮下方式接种到小鼠的右侧腋部, 每只接种的细胞为 $1 \times 10^6/100 \mu\text{L}$ ^[9]。

1.4 分组

接种完的小鼠随机分为对照组 (生理盐水组)、低浓度 N-CWS 组 (400 $\mu\text{g/mL}$) 与高浓度 N-CWS 组 (800 $\mu\text{g/mL}$), 每组

10 只。对照组小鼠生理盐水灌胃 0.2 mL, 低浓度组灌胃 400 $\mu\text{g/mL}$ 规格的 N-CWS 0.2 mL, 高浓度组灌胃 800 $\mu\text{g/mL}$ 的 N-CWS 0.2 mL, 均 1 次/d, 连续 14 d, 当小鼠肿瘤数量出现明显负荷、小鼠快濒死时、接种后 15 d 处死, 观察 3 组肿瘤质量和体积情况。

1.5 观察 N-CWS 对肿瘤生长的作用

(1) 观察小鼠肿瘤质量、体积和生存时间。每隔 2 d, 游标卡尺测量肿瘤的长和宽, 肿瘤体积 (mm^3) = $0.52 \times \text{长} \times \text{宽}^2$; (2) 记录小鼠自发死亡或处死小鼠的时间; (3) 观察 3 组皮毛色泽、食欲及活动变化。

1.6 统计学处理

应用 SPSS 23.0, 计量资料以 ($\bar{x} \pm s$) 示, 多组间比较采用单因素方差分析比较, $P < 0.05$ 有统计学意义。

2 结果

2.1 3 组肿瘤质量比较

治疗组小鼠肿瘤质量明显低于对照组 (1.46 ± 0.16)g, 且高浓度组小鼠肿瘤质量 (0.52 ± 0.13)g 明显低于低浓度组 (0.79 ± 0.13)g, 组间对比差异有统计学意义 ($P < 0.05$), 如表 1。

表 1 N-CWS 对 H22 荷瘤小鼠肿瘤质量的影响

Table 1 The effect of N-CWS on tumor mass of H22 BALB/c mice

Groups	Tumor quality (g)
Control group	1.46 ± 0.16
Low concentration group	$0.79 \pm 0.13^*$
High concentration group	$0.52 \pm 0.13^{**}$

Note: Compared with the control group, $^*P < 0.05$, compared with the low concentration group, $^{**}P < 0.05$.

2.2 N-CWS 对各组小鼠肿瘤体积的影响

治疗第 1 d, 未见明显肿块, 第 3 d 起直至结束, 治疗组小鼠肿瘤体积均明显低于对照组。在第 1~5 d 时, 高浓度组小鼠肿瘤体积与低浓度组小鼠肿瘤体积无明显差异 ($P > 0.05$); 从第 5 d 起直至结束, 高浓度组小鼠肿瘤体积明显低于低浓度处理组 ($P < 0.05$), 见表 2。

表 2 不同时间间隔内 N-CWS 对小鼠肿瘤体积的影响

Table 2 The effect of N-CWS on tumor volume of BALB/c mice in different time

Tumor volume (mm^3)	Control group	Treatment group	
		Low concentration group	High concentration group
d1	-	-	-
d3	222.42 ± 59.12	$172.75 \pm 21.86^*$	$183.75 \pm 15.23^*$
d5	366.57 ± 24.17	$315.64 \pm 40.25^*$	$280.29 \pm 30.11^{**}$
d7	543.32 ± 47.48	$449.85 \pm 37.46^*$	$369.94 \pm 23.27^{**}$
d9	937.46 ± 70.13	$655.72 \pm 48.83^*$	$413.66 \pm 28.62^{**}$
d11	1072.93 ± 121.04	$754.16 \pm 39.69^*$	$458.62 \pm 23.93^{**}$
d13	1273.41 ± 160.36	$877.86 \pm 33.34^*$	$487.51 \pm 17.91^{**}$
d15	1477.87 ± 141.33	$960.19 \pm 47.34^*$	$506.75 \pm 30.77^{**}$

Note: Compared with the control group, $^*P < 0.05$; compared with the low concentration group, $^{**}P < 0.05$.

2.3 各组小鼠肿瘤生长变化比较

在第 1~3 d 内,小鼠肿瘤体积增长的变化无明显差异($P>0.05$)。第 5 d 起至结束,治疗组明显缓于对照组($P<0.05$)。高浓度组小鼠肿瘤体积的增长在第 5 d 后明显缓于低浓度组

($P<0.05$)。对照组在第 9 d 时,增长变化最为显著,较第 7 d 增加约 400 mm³。低浓度组变化较稳定。高浓度组肿瘤增长明显变缓,第 7 d 后最为明显,后逐渐趋于缓和,见表 3。

表 3 N-CWS 对 H22 荷瘤小鼠肿瘤体积增长变化的影响
Table 3 The effect of N-CWS on tumor volume variation of BALA/c mice

Tumor growth change(mm ³)	Control group	Treatment group	
		Low concentration group	High concentration group
d1	-	-	-
d3	222.42± 59.12	172.75± 21.86*	183.75± 15.23*
d5	144.15± 65.92	142.89± 48.22*	96.54± 33.84*#
d7	126.75± 52.98	134.21± 48.90*	89.65± 40.11*#
d9	394.14± 77.66	205.87± 60.44*	43.72± 32.91*#
d11	235.47± 74.73	98.44± 66.48*	44.96± 34.07*#
d13	200.48± 150.41	123.70± 49.21*	28.89± 19.91*#
d15	204.46± 153.70	82.34± 35.27*	19.24± 25.10*#

Note: Compared with the control group, * $P<0.05$, compared with the low concentration group, # $P<0.05$.

3 讨论

N-CWS 首次被 Azuma 等人发现其天然抗肿瘤免疫活性^[2]。其主要成分由枝菌酸、阿拉伯半乳糖和粘肽组成。通过调动宿主天然防卫机制或外源给予机体某些生物活性物质来取得免疫增强的效应,从而发挥宿主抗肿瘤免疫反应^[10]。早有研究发现 N-CWS 具有较强的免疫佐剂活性^[11,12]。后来的研究表明, N-CWS 可以通过活化 CD4⁺T 细胞,促进 CD4⁺T 募集,通过调节 Th1 免疫应答发挥抗肿瘤作用^[13,14]。N-CWS 可以提高 CT26.WT 癌细胞中 PD-1/PD-L1 免疫抑制途径的治疗效果,通过在小鼠皮下注射 N-CWS 后,CT26.WT 癌细胞中 PD-L1 的水平增加,但是 N-CWS 在体外并未诱导 PD-L1 的表达。N-CWS 处理小鼠并同时体内阻断 PD-1/PD-L1 途径时,CT26.WT 癌细胞被显著抑制或消除,说明 N-CWS 可能是 PD-1/PD-L1 免疫疗法的有效佐剂^[15]。同时 Tao Y 等人^[16]研究结果表明,Nr-CWS 可以上调 CD8⁺T 细胞上的 CD69 和 CD25 表达,促进 IFN- γ 和 TNF- α 的分泌,并增强穿孔素和颗粒酶 B 的产生。因此, Nr-CWS 可能通过增加 CD8⁺T 细胞反应而具有增强免疫的治疗活性。

本研究显示,N-CWS 治疗后小鼠肿瘤重量明显低于对照组,且高浓度 N-CWS 组肿瘤重量明显低于低浓度组,较对照组重量分别减少约 46 %和 64 %。在给药过程中,第 1-3 d 治疗组和对照组肿瘤体积无明显差异,3 d 后治疗组肿瘤大小明显低于对照组,5 d 后高浓度 N-CWS 组小鼠肿瘤大小明显小于低浓度组。关于 N-CWS 抑制小鼠肿瘤的研究不多,国内张祝兰等^[17]研究发现,不同浓度 N-CWS 灌胃给药,可抑制小鼠移植性肿瘤生长,其抑瘤率约为 60 %~70 %,与本研究高浓度组结果相当。本研究还发现,治疗 1-3 d 内,小鼠肿瘤体积变化不大,5 d 后治疗组体积变化明显变缓,且高浓度 N-CWS 组增长更慢,治疗中后期基本趋于缓和。N-CWS 是一种针对癌症的免疫治疗剂,已

被证明具有激活免疫反应而不显示毒性的能力。Meng Y^[7]的研究用 TUNEL 染色和膜联蛋白 V / 碘化丙啶测定表明,用 N-CWS 处理后,CIK / NK 细胞可诱导肿瘤细胞中的 DNA 断裂,提示 N-CWS 可以通过 CIK / NK 细胞诱导肝癌细胞凋亡,抑制肿瘤细胞的侵袭和迁移。同时,N-CWS 也可以用于成人急性髓性白血病(AML)的免疫治疗,能够增加患者的缓解期和存活率^[18]。N-CWS 也可以用于伤口的愈合,通过增强巨噬细胞活化和血管生成来加速皮肤伤口愈合,主要是 N-CWS 可以激活巨噬细胞,增加 TGF- β 1 的表达,增强血管生成,从而加速皮肤伤口的愈合^[19]。目前,临床上对于 N-CWS 与肿瘤机制的研究较少,后续需要深入研究。

关于 N-CWS 抗肿瘤机制,有研究认为是由于 N-CWS 募集 CD4⁺T 细胞从而发挥宿主肿瘤免疫反应。CD4⁺T 在免疫调节过程中发挥至关重要作用^[20,21]。活化的 CD4⁺T 细胞可表达 CD69 和 CD25 蛋白^[22,23],且活化后不同的 T 细胞亚群又可分泌不同的细胞因子,Th1 和 Th2 是主要的 CD4⁺T 亚群。Th1 细胞主要分泌 IL-2、TNF- α 和 INF- γ , 这些可活化巨噬细胞和 CD8⁺T 直接对抗肿瘤细胞^[24,26]。而 Th2 细胞通过分泌 IL-4、IL-5 和 IL-10 促进体液免疫,使得 B 淋巴细胞分泌特异性抗体^[27-29]。Wang 等研究通过实验证实 N-CWS 正是通过活化 CD4⁺T 细胞,促进 CD4⁺T 募集,通过调节 Th1 免疫应答发挥抗肿瘤作用^[13,30]。于顺利^[31]等人通过红色诺卡菌细胞壁骨架膀胱灌注预防非肌层浸润性膀胱癌术后复发的疗效和安全性发现,与术后膀胱灌注表柔比星相比,NMIBC 患者 TURBT 术后膀胱灌注 N-CWS 可降低复发风险并延长无复发生存时间,且不良反应少。本研究后续也要在后期进行 N-CWS 在临床中的应用研究。本研究结果提示 N-CWS 具有较好的抑制肿瘤生长的作用。随着免疫治疗方法的蓬勃发展,将有更多深层的机制被阐明,同时,N-CWS 或许能够增加传统治疗方式的疗效,在临床实践过程中使得患者的获益最大化。

综上所述,N-CWS 可以明显抑制肿瘤生长,且高浓度 N-CWS 抑制肿瘤生长效果更佳。

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