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环氧合酶 -2/5- 脂氧合酶抑制剂对酒精相关性口腔癌的抑制作用 *

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摘要 目的:分析和比较选择性环氧合酶 -2 (Cox-2)抑制剂塞来昔布、5- 脂氧合酶(5-Lox)抑制剂齐留通及 Cox/5-Lox 双酶抑制剂利克飞龙对酒精相关性口腔癌的抑制作用。**方法:**选择 66 只 C57BL/6 小鼠,分为阴性对照组、模型组(4NQO 组)、阳性对照组、齐留通干预组、塞来昔布干预组和利克飞龙干预组。阴性对照组不做任何处理,其余各组饮用 50 μg /mL 四硝基喹啉 -1- 氧化物(4NQO)溶液 16 周后,阳性对照组及各干预组以 8%酒精溶液代替饮用水喂养 8 周,同时开始分别用三蒸水和同等药量的齐留通、塞来昔布、利克飞龙(100 mg·kg^{-1}·d^{-1})灌胃 8 周;于 24 周处死动物,取舌行组织病理学观察、BrdU 免疫组化染色、蛋白质印迹法(Western-blot)检测舌组织中 5-Lox、Cox-2 蛋白的表达。**结果:**饮用酒精后,口腔癌发生率从 16.7%增加到 58.3%,5-Lox、Cox-2 蛋白表达显著增加癌组织中 BrdU 阳性率显著升高。齐留通干预后,口腔癌发生率(41.7%)显著降低,5-Lox 表达显著减少,Cox-2 表达显著增加,BrdU 阳性率显著降低;塞来昔布干预后,口腔癌发生率(50.0%)显著降低,Cox-2 表达显著减少,5-Lox 表达显著增加,BrdU 阳性率显著降低;利克飞龙干预后,口腔癌发生率(25%),BrdU 阳性率与阳性对照组、齐留通干预组和塞来昔布干预组相比均显著降低,5-Lox、Cox-2 蛋白表达比阳性对照组显著减少($P<0.05$)。**结论:**酒精促进口腔癌变的过程可能与 5-Lox 和 Cox-2 的表达上调关系密切;齐留通和塞来昔布可以分别抑制 5-Lox 和 Cox-2 活性,使口腔癌的发生率显著降低;利克飞龙对口腔癌的抑制作用优于齐留通和塞来昔布。

关键词:齐留通;塞来昔布;利克飞龙;口腔癌;5- 脂氧合酶;环氧合酶 -2;酒精
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Inhibitory Effect of Cox-2/5-Lox Inhibitors on the Ethanol-related Oral Carcinogenesis in Mice*

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ABSTRACT Objective: To study the inhibitory effect of Zileuton (a specific 5-Lox inhibitor), Celecoxib (a specific Cox-2 inhibitor) and Licofelone (a dual Cox/5-Lox inhibitor) on ethanol-related oral carcinogenesis in mice. **Methods:** 66 C57BL/6 mice were divided into 6 groups randomly. The negative control group (n = 6) was not treated, the remaining mice were divided into 5 groups(12 mice in each group) and treated with 4NQO in their drinking water at a concentration of 50 μg /mL for a period of 16 weeks. Then the mice in 4NQO control group was not treated afterwards, those in the other 4 groups were received by distilled water, Zileuton, Celecoxib and Licofelone respectively. At week 24, all the animals were sacrificed. The tongue was harvested and examined for histopathology, immunohistochemical and western blotting. **Results:** Long-term 8% ethanol consumption significantly increased the oral SCC incidence (from 16.7% to 58.3%), 5-Lox and Cox-2 protein expression, and the BrdU-labeling index The oral SCC incidence and the BrdU-labeling index in Zileuton group, Celecoxib group and Licofelone group were significantly lower than those in positive control group. The oral SCC incidence and the BrdU-labeling index in Licofelone group were significantly lower than those in Zileuton group or Celecoxib group. 5-Lox and Cox-2 protein expression in Licofelone group, 5-Lox protein expression in Zileuton group and Cox-2 protein expression in celecoxib group were significantly lower than those in positive control group. Cox-2 protein expression in Zileuton group and 5-Lox protein expression in Celecoxib group were significantly higher than those in positive control group. **Conclusions:** Ethanol can promot 4NQO-induced oral carcinogenesis through activation of the 5-Lox and Cox-2 pathway of arachidonic acid metabolism. Zileuton and Celecoxib have inhibitory effects against ethanol-related oral carcinogenesis and such inhibition may be related to the suppression of 5-Lox and Cox-2 protein expression. The inhibitory effect of Licofelone is better than that of Zileuton and Celecoxib, and Licofelone may be potentially used for oral cancer prevention in the future.

Key words: Zileuton; Celecoxib; Licofelone; Oral cancer; 5-Lox; Cox-2; Ethanol

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前言

流行病学资料表明长期慢性酒精消耗与口腔癌的发生发展密切相关[1-5]。但到目前为止,酒精导致口腔癌作用的分子机制尚不完全清楚。近年来许多研究表明多种肿瘤的发生与慢性炎症有关,口腔癌的发生也与口腔的长期慢性炎症密切相关[6,7]。在众多炎症介质中,花生四烯酸(Arachidonic Acid,AA)代谢产物与多种肿瘤发生有明确的因果关系。5- 脂氧合酶(Lipoxygenase,Lox)和环氧化物酶(Cyclooxygenase,Cox)-2 是 AA 代谢两个最重要的途径[8,9]。有研究表明长期饮用 8%的酒精,实验小鼠的口腔癌发生率显著升高,5-Lox、Cox-2 的表达增加[10]。本研究采用四硝基喹啉 -1- 氧化物(4-nitroquinoline 1-oxide,4NQO)诱导的小鼠口腔癌模型, 主要对比了 5-Lox 抑制剂齐留通(zileuton)、Cox-2 抑制剂塞来昔布(celecoxib)和(Cox/5-Lox)双重抑制剂利克飞龙(licofelone)对酒精相关性口腔癌的抑制作用,为进一步研究干预、抑制酒精相关性口腔癌奠定基础。

1 材料和方法

1.1 材料

4NQO(Sigma aldrich 公司,美国),无水乙醇及 1,2 丙二醇(国药集团化学试剂有限公司),8 周龄雄性 C57BL/6 小鼠(北京维通利华实验动物技术有限公司),齐留通、塞来昔布(上海翰鸿化工科技有限公司),利克飞龙(默克公司,德国)。

1.2 方法

1.2.1 实验分组及用药 66 只雄性小鼠常规饲养 1 周后开始实验。小鼠随机分为 6 组,阴性对照组 6 只,其余每组 12 只。A 组:阴性对照组;B 组:4NQO 组;C 组:阳性对照组;D 组,齐留通干预组;E 组,塞来昔布干预组;F 组,利克飞龙干预组。阴性对照组不做任何处理。其余 5 组以 50 μg/mL 4NQO 溶液代替饮用水喂养 16 周(4NQO 溶液置于避光瓶中自由饮用,每周更换新鲜溶液)。所有小鼠均单笼饲养。16 周结束后,B 组进行常规饲养,同时每日三蒸水灌胃,持续 8 周。C、D、E、F 四组以 8% 酒精溶液代替饮用水喂养 8 周,同时开始分别用三蒸水和同等药量的齐留通、塞来昔布、利克飞龙(100 $mg\cdot kg^{-1}\cdot d^{-1}$)灌胃 8 周;灌胃三蒸水剂量与用药组剂量相同。每 2 周称重 1 次,每天观察健康状况,肉眼观察小鼠舌粘膜变化情况。实验第 24 周末处死全部动物(处死前 2h 腹腔注射 50 mg/kg 的 BrdU)。

1.2.2 标本处理 取舌,分为两部分,一部分固定在的福尔马林溶液中,用于免疫组织化学染色,另一部分低温冰冻保存后用于 Western-blot 检测。

1.2.3 组织病理学观察 光镜下观察病理改变,并记录其不同病变数目。病理改变分为正常黏膜、轻 - 中度异常增生、重度异常增生和鳞癌,统计各种病变的发生率。

1.2.4 BrdU 细胞增殖指数测定 组织切片行免疫组化 Envision 二步法检测细胞增殖活性。细胞核染成棕黄色者为 BrdU 阳性细胞。每张切片随机选取 3 个以上不同视野 400 倍镜下计数阳性细胞数,除以该视野下总细胞数,计算阳性细胞率。

1.2.5 Western-blot 检测 5-Lox, Cox-2 蛋白表达 取冷冻舌组织称量约 20 mg,每 10 mg 组织加入 200 μL 蛋白裂解液,用玻璃研磨器于冰上匀浆。将匀浆液转移到预冷的 1.5 mL EP 管中,置于冰上 15 min,以充分裂解。然后 4℃ ,12000 rpm 离心 10 min。取上清液,按照试剂盒说明操作,提取各组总蛋白并定量,SDS-PAGE 电泳,PVDF 转膜,蛋白封闭,一抗(按 5-Lox 1:500,Cox-2 1:200)封闭过夜,二抗(1:3000 稀释)反应后,显影曝光分析。将显色后的膜或底片照相,并用 LabWorks 软件对图像进行灰度分析。计算每组标本的灰度值组间的差别。

1.3 统计学分析

应用 SPSS17.0 统计软件进行数据统计分析, 计量资料数据用 $\bar{x}\pm s$ 表示,多组间比较采用单因素方差分析,两两比较采用 LSD-t 检验,计数资料数据用 χ^2 检验,以 $P<0.05$ 为差异有统计学意义。

2 结果

2.1 一般情况

自实验起始至 18 周,小鼠外观健康,体重增长正常。自实验第 18 周,C 组阳性对照组小鼠体重均值开始下降,组内个别小鼠体重下降明显,可能由于恶性肿瘤的发展造成。至第 24 周实验结束时,阳性对照组小鼠体重显著低于其余各实验组($P<0.05$)。其余各实验组间体重无统计学差异(图 1,表 1)。

2.2 组织病理学

光镜下,阴性对照组小鼠舌背黏膜上皮为角化的复层鳞状上皮,细胞排列正常,未发现上皮异常增生或癌变,为正常黏膜上皮。经 4NQO 和 / 或酒精处理后,小鼠舌黏膜上皮出现不同程度的异常增生或癌,固有层和黏膜下层均可见不同程度的炎细胞浸润,部分标本可见炎细胞密集成灶(图 2)。阳性对照组及各干预组的口腔癌发生率显著高于 4NQO 处理组 ($P<0.05$),阳性对照组的口腔癌发生率显著高于各干预组($P<0.05$),齐留通干预组和塞来昔布干预组的口腔癌发生率显著高于利克飞龙干预组($P<0.05$)(表 2)。齐留通干预组和塞来昔布干预组间无显著性差异($P>0.05$)。

2.3 各组小鼠舌组织 BrdU 细胞增殖指数染色结果

BrdU 阳性反应定位于细胞核, 正常小鼠舌粘膜仅在上皮基底层部分个别细胞有表达;异常增生上皮除基底细胞呈阳性表达外,棘层细胞也可见表达;癌组织内,细胞普遍呈阳性表达(图 3)。异常增生上皮中,各实验组间 BrdU 阳性率无统计学差异($P>0.05$)。鳞癌的病变中,阳性对照组及各干预组比 4NQO 处理组的 BrdU 阳性率高,差异有统计学意义($P<0.05$),阳性对照组比各干预组的 BrdU 阳性率高,差异有统计学意义($P<0.05$),齐留通干预组和塞来昔布干预组比利克飞龙干预组的 BrdU 阳性率高,差异有统计学意义($P<0.05$),齐留通干预组和塞来昔布干预组间无显著性差异($P>0.05$)(表 3)。

2.4 各组小鼠舌组织 5-Lox、Cox-2 蛋白表达的比较

阴性对照组小鼠舌组织中可以检测到较低水平的 5-Lox、Cox-2 蛋白表达,经 4NQO 诱导后,舌组织中 5-Lox, Cox-2 蛋白

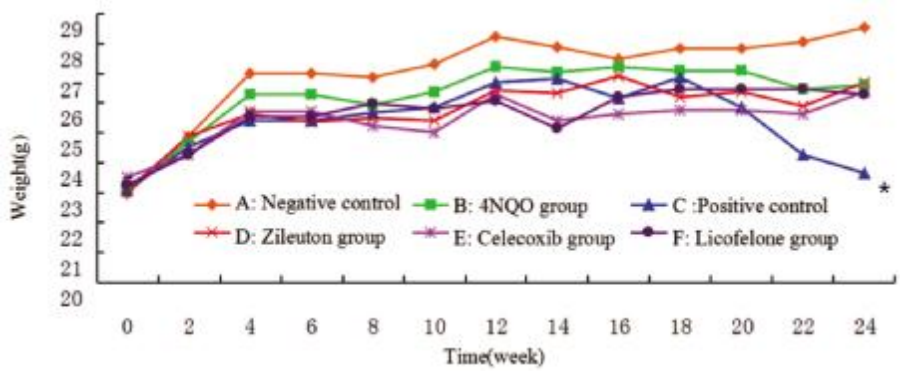

图 1 小鼠体重曲线

Fig.1 Comparison of body weights of different groups

注:与其余各组相比较,*$P<0.05$(ANOVA 检验)。

Note: compared with the other groups after treatment, *$P<0.05$.

表 1 不同组之间小鼠重量的比较

Table 1 Comparison of body weights of different groups

Groups	0 week	24 week
A Negative control	22.94± 0.72	28.52± 0.56
B 4NQO group	23.05± 0.29	25.59± 0.83
C Positive control	23.06± 0.18	23.64± 0,27*
D Zileuton group	23.08± 0.29	26.67± 0.63
E Celecoxib group	23.51± 0.30	26.35± 0.42
F Licofelone group	23.27± 0.46	26.29± 0.39

Note: compared with the other groups after treatment, *$P<0.05$.

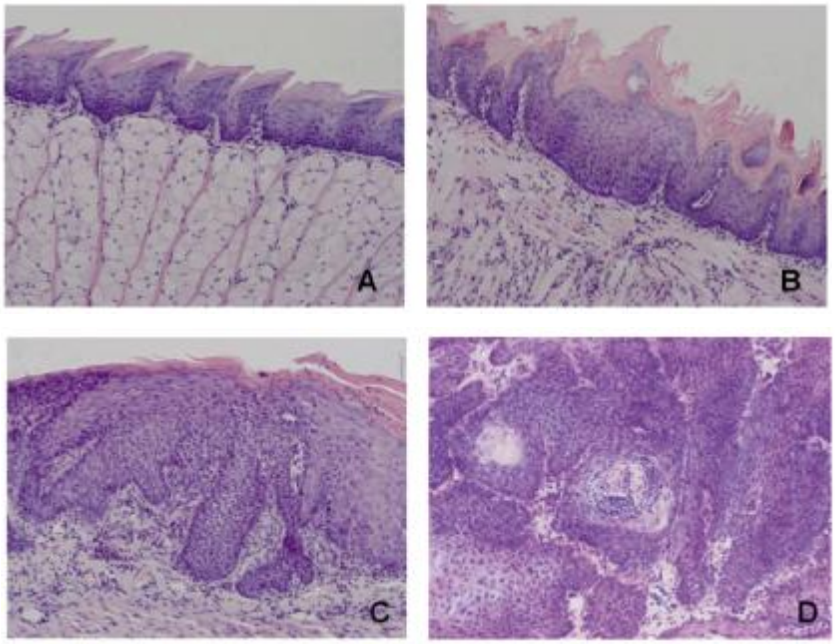

图 2 24 周小鼠舌黏膜病变(HE 染色× 200)

Fig.2 Typical pictures of HE staining in mouse tongue(200×)

注:A:正常黏膜;B:轻 - 中度异常增生;C:重度异常增生;D:鳞癌

Note: A: normal; B: minor dysplasia; C: sever dysplasia; D: SCC

水平较阴性对照组显著增高($P<0.05$)。饮用酒精后舌组织内蛋白含量显著增高,与 4NQO 组间存在显著差异($P<0.01$)。齐留通干预后,与阳性对照组比较 5-Lox 蛋白显著降低($P<0.05$),Cox-2 蛋白显著增加($P<0.05$)。塞来昔布干预后,与阳性对照组比较 5-Lox 蛋白显著增加($P<0.05$),Cox-2 蛋白显著降低($P<0.05$)。利克飞龙干预后,5-Lox 和 Cox-2 蛋白均显著降低,与阳

表 2 24 周小鼠舌 HE 染色病理结果

Table 2 Oral carcinogenesis in mice

Groups	No. of mice	Normal	Mild-moderate dysplasia	Severe dysplasia	SCC
A Negative control	6	6(100%)	0	0	0
B 4NQO group	12		6(50.0%)	4(33.3%)	2(16.7%)
C Positive control	12		3(25%)	2(16.7%)	7(58.3%)*
D Zileuton group	12		4(33.3%)	3(25.0%)	5(41.7%)*▲#
E Celecoxib group	12		3(25.0%)	3(25.0%)	6(50.0%)*▲#
F Licofelone group	12		5(41.7%)	4(33.3%)	3(25%)*▲

注:与 4NQO 处理组比较 *$P<0.05$;与阳性对照组比较▲P<0.05;与利克飞龙干预组比较 # $P<0.05$。

Note: Compared with 4NQO group, *$P<0.05$; Compared with Positive control group, ▲$P<0.05$; Compared with Licofelone group, # $P<0.05$.

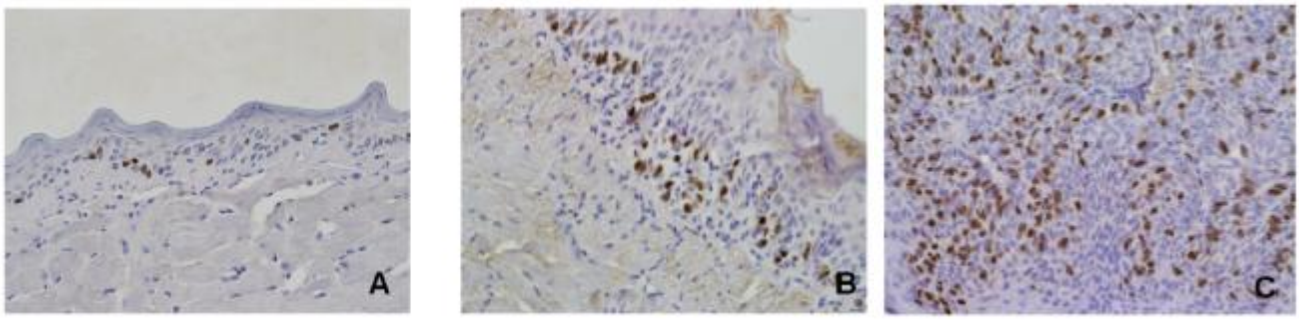

图 3 BrdU 免疫组化染色(× 400)

注:A 正常舌背黏膜;B 上皮异常增生;C 鳞癌

Fig.3 Immunohistochemical results of Brdu expression(× 400)

Note: A: normal; B: dysplasia; C: SCC

表 3 BrdU 增殖指数分析结果:单位($\bar{x}\pm s$,%)

Table 3 BrdU-labelling index($\bar{x}\pm s$,%)

Groups	Normal	Dysplasa	SCC
A Negative control	4.24± 1.88	-	-
B 4NQO group	-	9.95± 3.76	17.93± 3.12
C Positive control	-	10.01± 4.58	27.62± 4.73*
D Zileuton group	-	9.28± 2.49	24.37± 6.56*▲#
E Celecoxib group	-	9.31± 3.13	23.86± 2.83*▲#
F Licofelone group	-	9.05± 2.27	19.61± 4.24*▲

注:与 4NQO 处理组比较 *$P<0.05$;与阳性对照组比较▲$P<0.05$;与利克飞龙干预组比较 # $P<0.05$。

Note: Compared with 4NQO group, *$P<0.05$; Compared with Positive control group, ▲$P<0.05$; Compared with Licofelone group, # $P<0.05$.

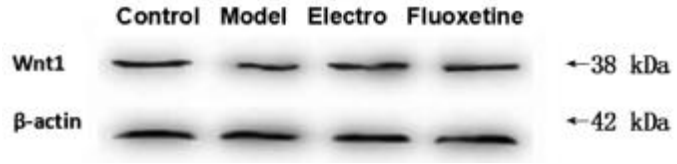

图 4 不同处理组小鼠舌组织中 5-Lox 和 Cox-2 蛋白表达

Fig. 4 Overexpression of 5-Lox and Cox-2 in mouse tongue as determined by Western blotting

性对照组比较有显著性差异($P<0.05$)(图 4)。

3 讨论

AA 代谢与炎症、肿瘤等多种疾病的发病密切相关[11]。以往的研究证实异常 AA 代谢在口腔癌发生发展中也起到了重要作用。单纯增生、异常增生和鳞癌组织中都有 5-Lox 和 Cox-2 的过表达,并且其表达随病变加重而增加。在酒精相关性口腔癌病变动物模型中,也发现黏膜癌变过程中存在 5-Lox 和

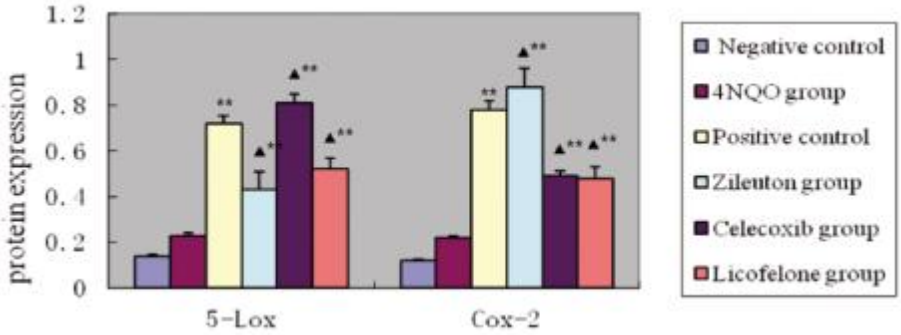

图 5 各组小鼠舌组织中 5-Lox、Cox-2 蛋白表达比较

Fig.5 Comparision of 5-Lox and Cox-2 expression of different groups

注：与 4NQO 组相比，$P<0.01$，与阳性对照组相比 ▲$P<0.05$。**

Note: Compared with 4NQO group, **$P<0.01$; Compared with Positive control group, ▲$P<0.05$.

Cox-2 高水平表达，说明 5-Lox 和 Cox-2 参与了口腔鳞癌的形成[10]。本研究发现小鼠饮用酒精后，口腔癌发生率、BrdU 增殖指数均显著高于只用 4NQO 处理组，表明酒精可以促进实验小鼠口腔癌的发生。Western-blot 检测结果证实，酒精处理后各组舌组织中，5-Lox 和 Cox-2 的蛋白水平较 4NQO 组均有明显升高，说明酒精对 AA 代谢过程中的这两个关键酶都有促进作用。提示我们，酒精促进口腔癌变的过程与 5-Lox 和 Cox-2 通路关系密切。可以推测，对这两条通路的共同激活增加了下游代谢产物前列腺素 E2（Prostaglandin E2，PGE2）和白三烯 B4（Leukotriene B4，LTB4）的生成，加剧了炎症反应，改变了炎症区域内细胞的生物学行为，极大促进了黏膜癌变的发生。

齐留通是一种在体内和体外有效的选择性 5-Lox 抑制剂。其化学防癌作用已在胰腺癌、皮肤癌和结肠癌等动物模型和体外实验中得以证实[12-15]。塞来昔布是第一个用于临床的 Cox-2 的选择性抑制剂。近年来研究发现，塞来昔布具有很好的抗肿瘤活性，可以抑制多种肿瘤细胞的增殖、侵袭及转移[16,17]。与以往研究相一致，在我们的研究中，齐留通和塞来昔布干预后，口腔癌的发生率显著降低。以往的研究证实在 AA 的两条代谢途径之间存在一定的平衡关系，即当 Cox-2 的活性受到抑制时，5-Lox 的活性增强，使更多 AA 进入 5-Lox 代谢途径；同样，当 5-Lox 的活性受到抑制时，则有更多的 AA 进入 Cox-2 代谢途径，二者存在一种此消彼长的关系。用化学药物抑制 Cox-2 活性可以激活 5-Lox 通路；抑制 5-Lox 活性后，炎症细胞产生的 Cox-2 代谢产物增加[18]。本研究 Western-blot 检测结果表明，齐留通干预组 5-Lox 表达降低，而 Cox-2 表达增加。塞来昔布干预组 Cox-2 表达降低，而 5-Lox 表达增加，与以往的研究相一致。

齐留通对肝脏的毒性较大，塞来昔布存在肾毒性，可增加心血管疾病的风险，如果大剂量单用一种药，加大了对某一器官的损害程度。因此，致力于合成双重抑制剂已经成为一种新的方向，利克飞龙具有新的解热、镇痛和抗关节炎作用机制，既是环氧合酶和 5- 脂氧合酶（Cox/5-Lox）双重抑制剂，又在结构上导入硝酸酯，缓解、改善和克服非甾体抗炎药对胃肠道、心血管系统和肾脏等毒性[19-21]。本研究中小鼠给予利克飞龙后，口腔癌发生率显著低于齐留通和塞来昔布干预组，5-Lox 和 Cox-2 表达均明显降低。我们的研究提示，Cox-2 和 5-Lox 单一抑制剂的抑制作用可因代谢分流而减弱，而 Cox-2/5-Lox 双途径抑制可能是一种替代的解决方案。

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