【DOI】 10.3969 / j. issn. 1671-6450. 2023. 07. 014

论著 · 基础

叶酸通过改善内皮型一氧化氮合酶解偶联抑制腹主动脉瘤发展研究

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基金项目：国家自然科学基金资助项目(81571376)
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【摘　要】 **目的**　探讨叶酸(FA)治疗对血管紧张素Ⅱ(AngⅡ)联合延胡索酸-3-氨基丙腈(BAPN)诱导腹主动脉瘤(AAA)小鼠的预防作用及其可能机制。**方法**　于 2022 年 7—10 月在武汉大学人民医院中心实验室进行实验。将 30 只 C57BL/6 小鼠采用随机数字表法分为正常对照组、腹主动脉瘤组(AAA 组)和 FA 干预组(AAA + FA 组), 每组 10 只。AAA 组：小鼠皮下植入微量渗透泵持续泵入 Ang Ⅱ(1 μg · kg^{-1} · min^{-1}) 28 d, 同时饲喂含 0.25% BAPN 饲料 28 d。AAA + FA 组：同 AAA 组方法造模, 并以 FA(15 mg · kg^{-1} · d^{-1})灌胃 28 d。正常对照组：实验全程用普通饲料喂养小鼠, 自由进水。动脉瘤破裂死亡小鼠立即取材, 第 29 天仍存活小鼠处死取材。取材后, 测量小鼠腹主动脉直径, 冰冻切片进行活性氧(ROS)染色, 石蜡切片进行弹性纤维染色, Western blot 检测基质金属蛋白酶 2(MMP-2)、基质金属蛋白酶 9(MMP-9)、内皮型一氧化氮合酶(eNOS)、二氢叶酸还原酶(DHFR)表达。**结果**　AAA 组发生腹主动脉瘤 8 只(其中 1 只于 21 d 死亡), AAA + FA 组发生 4 只, AAA + FA 组小鼠腹主动脉瘤发生率显著低于 AAA 组(40.0% vs. 80.0%, χ^2/P = 1.667/0.170), AAA + FA 组腹主动脉直径明显小于 AAA 组[(1.44 ± 0.19) mm vs. (2.08 ± 0.50) mm, t/P = 3.598/0.005]。AAA + FA 组腹主动脉弹性纤维结构较 AAA 组完整, 且无明显断裂纤维。AAA + FA 组 ROS 含量及 MMP-2、MMP-9 表达明显低于 AAA 组(t/P = 4.206/0.048、5.267/0.006), eNOS、DHFR 表达高于 AAA 组(t/P = 4.511/0.011、2.914/0.044)。**结论**　FA 可改善 AngⅡ联合 BAPN 诱导的腹主动脉瘤, 可能是通过上调 DHFR 的表达从而恢复 eNOS 偶联状态, 减少 ROS, 并减轻以内侧弹性纤维分解、MMP-2、MMP-9 升高为特征的血管重塑。

【关键词】 腹主动脉瘤；叶酸；内皮型一氧化氮合酶；氧化应激；小鼠
【中图分类号】 R543.1^{+}6　　【文献标识码】 A

Folic acid inhibits the development of abdominal aortic aneurysm by improving endothelial nitric oxide synthase decoupling

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Funding program: National Natural Science Foundation of China Funded Project (81571376)

【**Abstract**】 **Objective**　To explore the preventive effect of folic acid (FA) treatment on abdominal aortic aneurysm (AAA) induced by angiotensin II (Ang II) combined with fumarate 3-aminopropiononitrile (BAPN) in mice and its possible mechanism.**Methods**　The experiment was conducted in the Central Laboratory of Wuhan University People's Hospital from July to October 2022. Thirty C57BL6 mice were randomly divided into a normal control group, an abdominal aortic aneurysm group (AAA group), and a FA treatment group (AAA + FA group) using a random number table method, with 10 mice in each group. AAA group: mice were subcutaneously implanted with a micro osmotic pump to continuously pump Ang II (1 μg · kg^{-1} · min^{-1}) for 28 days, while feeding a feed containing 0.25% BAPN for 28 days. AAA + FA group: The model was made using the same method as the AAA group, and FA (15 mg · kg^{-1} · d^{-1}) was administered orally for 28 days. Normal control group: The mice were fed with regular feed throughout the experiment, with free water intake. Mice who died from ruptured aneurysms were immediately harvested, and the surviving mice were euthanized and harvested on the 29th day. After sampling, the diameter of mouse abdominal aorta was measured, and the frozen sections were stained with reactive oxygen species (ROS), and the paraffin sections were stained with elastic fibers. Western blot was used to detect the expression of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), endothelial nitric oxide syn-

thase (eNOS), and dihydrofolate reductase (DHFR). **Results** Eight abdominal aortic aneurysms occurred in the AAA group (one of which died on 21 days), while four in the AAA + FA group. The incidence of abdominal aortic aneurysms in AAA + FA mice was significantly lower than that in the AAA group (40.0% vs 80.0%, χ^2/P = 1.667/0.110), the diameter of the abdominal aorta in the AAA + FA group was significantly smaller than that in the AAA group [(1.44 ± 0.19) mm vs. (2.08 ± 0.50) mm, t/P = 3.598/0.005]. The elastic fiber structure of the abdominal aorta in the AAA + FA group is more complete than that in the AAA group, and there are no obvious broken fibers. The expression of ROS, MMP-2, and MMP-9 in the AAA + FA group was significantly lower than that in the AAA group (t/P = 4.206/0.048, 5.267/0.006), while the expression of eNOS and DHFR was higher than that in the AAA group (t/P = 4.511/0.011, 2.914/0.044). **Conclusion** FA can improve Ang II combined with BAPN induced abdominal aortic aneurysm, possibly by upregulating the expression of DHFR to restore eNOS coupling state, reduce ROS, and alleviate vascular remodeling characterized by medial elastic fiber breakdown, MMP-2, and MMP-9 elevation.

【**Key words**】 Abdominal aortic aneurysm; Folic acid; Endothelial nitric oxide synthase; Oxidative stress; Mice

当腹主动脉横径超过正常横径 50% 时可被诊断为腹主动脉瘤(abdominal aortic aneurysm, AAA),AAA 的危险系数与动脉横径呈正比,横径越大,破裂风险越高。既往认为 AAA 仅仅是由于动脉中膜的缺陷所导致,动脉细胞外基质降解、平滑肌细胞凋亡、氧化应激和炎性反应导致主动脉壁受损[1]。但近年来研究发现血管内皮也直接或者间接参与了 AAA 病变[2]。本课题组早期研究发现叶酸(folic acid, FA)对内皮细胞功能不全的 AAA 模型小鼠(hph1 小鼠是 GTP 环水解酶 1 缺乏的小鼠,因 BH4 缺乏导致内皮细胞功能不全)有治疗作用[3],但是对其他 AAA 模型小鼠的治疗作用以及机制不明。延胡索酸-3-氨基丙腈(β-aminopropionitrile monofumarate, BAPN)是一种不可逆赖氨酸氧化酶(lysyl oxidase, LOX)抑制剂,可防止赖氨酸衍生醛的形成。LOX 交联弹性蛋白和胶原纤维,在维持弹性层的稳态中起着关键作用。有研究证明通过 NADPH 氧化酶(NADPH oxidase, NOX)的瞬时激活和随之而来的过氧化氢依赖性、内皮特异性四氢生物喋啉(Tetrahydrobiopterin, BH4)补救酶二氢叶酸还原酶(dihydrofolate reductase, DHFR)缺乏来解偶联内皮型一氧化氮合酶(endothelial nitric oxide synthase, eNOS)[4]。故以血管紧张素Ⅱ(angiotensinⅡ, AngⅡ)联合 BAPN 建造另一种 AAA 小鼠模型,探究 FA 对其他模型的 AAA 是否有改善作用,报道如下。

1　材料与方法

1.1　材料　(1)实验动物:8 周龄 C57BL/6 雄性小鼠 30 只,SPF(specific pathogen free)级,由武汉大学人民医院动物实验中心提供。小鼠饲养在 SPF 级屏障环境中,正常昼夜周期,自由饮水,室温控制在(22.0 ± 0.1)℃,湿度(60 ± 10)%。(2)试剂与仪器:Ang Ⅱ(A9290)、叶酸(F8090)、HE 染色试剂盒(G1120)、Weigert 间苯二酚品红染色试剂盒(G0032)购自武汉索莱宝生物科技有限公司,BAPN 购自 sigma 公司,DHE 荧光探针试剂(KFS377)购自北京百奥莱博科技有限公司,eNOS(GB11086)、MMP-2(GB11130)、MMP-9(GB12132)抗体购自武汉市赛维尔生物科技有限公司,DHFR(A9299)抗体购自爱博泰克生物科技有限公司。Altzet 微量渗透泵购自上海玉研科学仪器有限公司,高精度电子游标卡尺购自德国苏测,正置显微镜(型号 BX51,日本奥林巴斯),冰冻切片机(型号 HM550,德国美康),酶标仪(EnSight 美国 Perkin Elmer),化学发光仪(ChemiDocTM Touch 美国 BIO-RAD)。

1.2　实验方法

1.2.1　AAA 造模:于 2022 年 7—10 月在武汉大学人民医院中心实验室进行实验[伦理号:WDRM 动(福)第 20210904 号]。小鼠 30 只称体质量,依照随机数字表法分为 3 组,正常对照组(n = 10):实验全程用普通饲料喂养,自由进水;AAA 组(n = 10):参照孙静媛等[5]造模方法诱导腹主动脉瘤,小鼠皮下植入微量渗透泵持续泵入 Ang Ⅱ(1 μg · kg^{-1} · min^{-1}) 28 d,同时用含 0.25% BAPN 饲料饲喂 28 d;AAA + FA 组(n = 10):小鼠皮下植入微量渗透泵持续泵入 Ang Ⅱ(1 μg · kg^{-1} · min^{-1})28 d,同时用含 0.25% BAPN 饲料饲喂 28 d,叶酸(15 mg · kg^{-1} · d^{-1})灌胃 28 d。

1.2.2　样品采集及处理:于实验第 29 天眼眶取血 0.8 ml,4℃离心留取上清 -80℃保存。取一部分新鲜腹主动脉组织多聚甲醛中固定,行石蜡包埋;另一部分腹主动脉组织液氮冻存,待用。

1.3　观测指标与方法

1.3.1　腹主动脉瘤直径测量:所有小鼠开胸腹并暴露心脏、主动脉及双侧肾脏,用生理盐水冲洗心脏和主动脉,将主动脉与周围组织分离,生理盐水经心脏灌入主动脉中,使主动脉保持正常形状,使用高精度电子游标

卡尺测量主动脉直径，最宽处直径大于正常值的 50% 时，即认定为发生腹主动脉瘤。

1.3.2　腹主动脉壁中弹性纤维变化观测：于实验第 29 天处死小鼠，剖开腹部暴露并分离腹主动脉，剪切腹主动脉用 4% 多聚甲醛保存。（1）腹主动脉弹性纤维染色：主动脉标本从多聚甲醛中取出，行石蜡包埋，切片（横切，片厚 4 μm），切片脱蜡至水，置入 Weigert 间苯二酚品红染色液的染缸，浸染 3 h，冲洗，乙醇去除细胞浆背景着色，冲洗，常规脱水，透明，封片。置于正置显微镜下观察并摄片。（2）腹主动脉 Masson 染色：石蜡切片脱蜡至水，重铬酸钾染液滴染，铁苏木素液 A 与铁苏木素液 B 混合滴染；盐酸酒精分化后丽春红酸性品红染液滴染，磷钼酸分化液分化后苯胺蓝染液滴染；乙醇脱水，二甲苯透明后，中性树胶封片。采用 40 × 放大倍数显微镜镜检，图像采集分析。

1.3.3　ROS 含量检测：采用二氢乙啶（dihydroethidium，DHE）荧光对腹主动脉壁染色。小鼠新鲜腹主动脉组织用冰冻切片包埋剂（OCT）包埋，用冰冻切片机垂直于血管纵轴切片；滴加 DHE 荧光染料，37℃ 避光孵育 30 min，PBS 洗 3 次，抗荧光淬灭剂封片，正置荧光显微镜镜检。

1.3.4　Western blot 检测蛋白表达：取瘤样病变血管组织 46 mg，加入蛋白裂解液和蛋白酶抑制剂冰浴匀浆，离心取上清，BSA 法测定总蛋白浓度。取蛋白样品加 5 × 电泳缓冲液，加热至变性，后凝胶电泳，转膜，BSA 封闭液封闭，一抗采用兔抗 eNOS、MMP-2、MMP-9、DHFR 抗体 4℃ 孵育过夜，HRP 标记羊抗兔二抗孵育，洗脱、发光，曝光显色。采用 Image J 软件进行光密度扫描进行半定量分析。

1.4　统计学方法　利用 Graphpad 8.0 软件对实验数据进行处理分析。正态分布计量资料以 $\bar{x} \pm s$ 表示，组间比较采用单因素方差分析和独立样本 t 检验。$P < 0.05$ 为差异有统计学意义。

2　结　果

2.1　腹主动脉瘤的发生率及腹主动脉直径比较　AAA 组发生腹主动脉瘤 8 只（其中实验第 21 天腹主动脉瘤破裂死亡 1 只），AAA + FA 组发生腹主动脉瘤 4 只，与 AAA 组比较，AAA + FA 组小鼠腹主动脉瘤发生率降低（40.0% vs. 80.0%，$\chi^2/P = 1.667/0.170$）。虽然腹主动脉瘤发生率降低无显著差异，但与正常对照组比较，AAA 组腹主动脉直径明显增大［(0.99 ± 0.07) mm vs. (2.08 ± 0.50) mm，$t/P = 6.417/ < 0.001$］，并且明显大于 AAA + FA 组（1.44 ± 0.19）mm，差异具有统计学意义（$t/P = 3.598/0.005$）。见图 1。

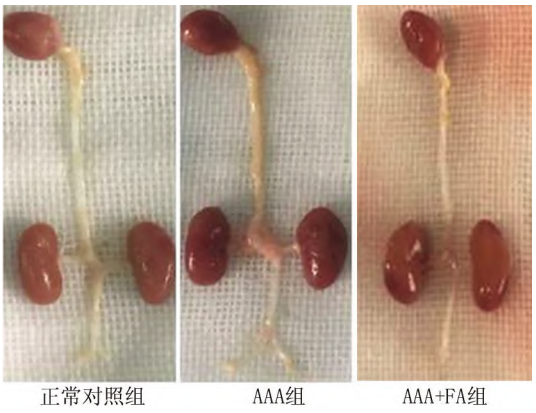

图 1　3 组小鼠腹主动脉大体标本比较

Fig. 1　Comparison of gross specimens of abdominal aorta in three groups of mice

2.2　3 组小鼠腹主动脉壁中弹性纤维变化比较　腹主动脉 Masson、Weigert 间苯二酚碱性品红染色显示，与正常对照组比较，AAA 组小鼠腹主动脉瘤部位主动脉壁弹力纤维排列紊乱，并且有断裂及溶解，失去正常波浪状结构；与 AAA 组比较，AAA + FA 组小鼠腹主动脉壁弹力纤维结构完整平滑，走形规则连续呈波浪状结构，见图 2。

2.3　3 组小鼠腹主动脉壁中 ROS 含量表达比较　腹主动脉 DHE 染色结果显示，与正常对照组比较，AAA 组红色荧光明显增强，表明 AAA 小鼠腹主动脉 ROS 表达增多；与 AAA 组比较，AAA + FA 组小鼠腹主动脉组织中红色荧光强度明显减弱，表明 ROS 含量减少，见图 3。

2.4　3 组小鼠 eNOS、DHFR、MMP-2、MMP-9 蛋白表达差异比较　与正常对照组比较，AAA 组腹主动脉 eNOS、DHFR 蛋白表达明显下降，AAA + FA 组上述 2 种蛋白表达高于 AAA 组（$t/P = 4.511/0.011$，$2.914/0.044$）。MMP 参与 AAA 的细胞外基质（ECM）破坏和主动脉壁重构[6]；通过 Western blot 检测主动脉组织中 MMP 的表达，MMP-2 和 MMP-9 蛋白水平 AAA 组小鼠显著高于正常对照组，而 AAA + FA 组小鼠主动脉 MMP-2 和 MMP-9 蛋白水平显著下降（$t/P = 4.206/0.048$，$5.267/0.011$），见图 4。

3　讨　论

腹主动脉瘤是多因素影响的具有潜在破裂风险的主动脉病理扩张性疾病。目前的治疗方案主要是开放手术和介入治疗，缺乏有效的药物控制 AAA 的发展。本研究采用 Ang Ⅱ 联合 BAPN 诱导小鼠 AAA 模型进行研究，采用口服给药的方式给予叶酸干预。虽然结果显示 AAA 组与 AAA + FA 组的 AAA 发生率无显著

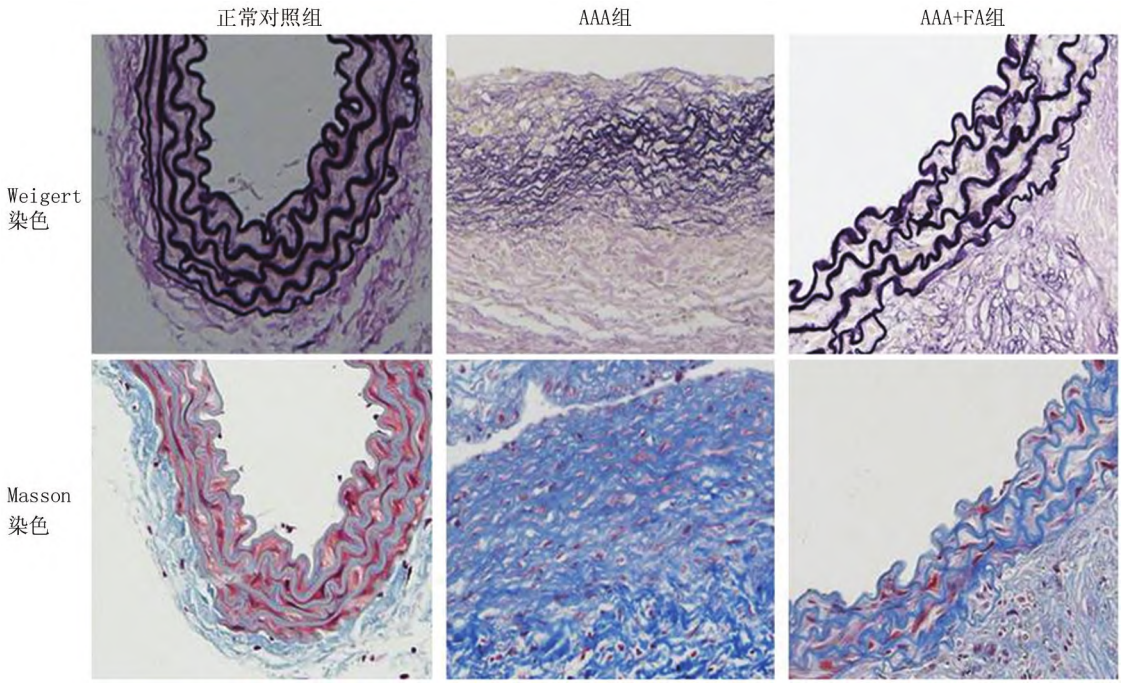

图 2　3 组小鼠腹主动脉壁中弹性纤维变化比较(×40)

Fig. 2　Comparison of changes in elastic fibers in the abdominal aortic wall of three groups of mice (×40)

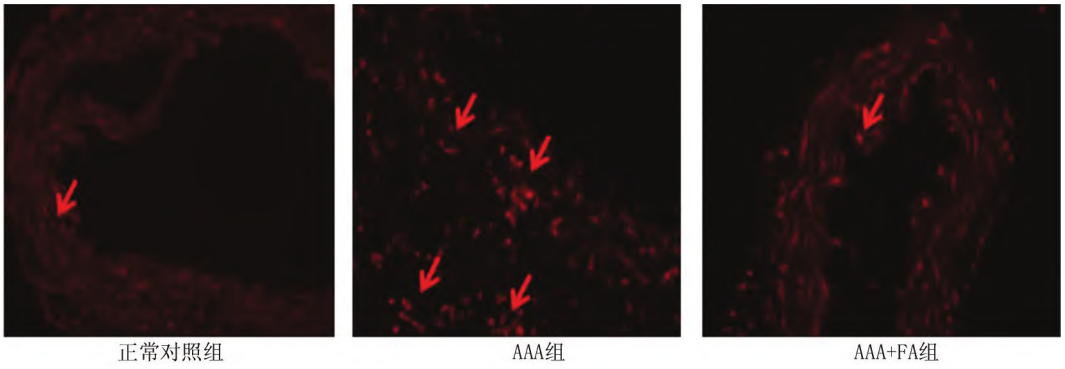

图 3　3 组小鼠腹主动脉活性氧表达比较(二氢乙啶染色, ×40)

Fig. 3　Expression of reactive oxygen species in the abdominal aorta of three groups of mice (stained with dihydroethylidine, ×40)

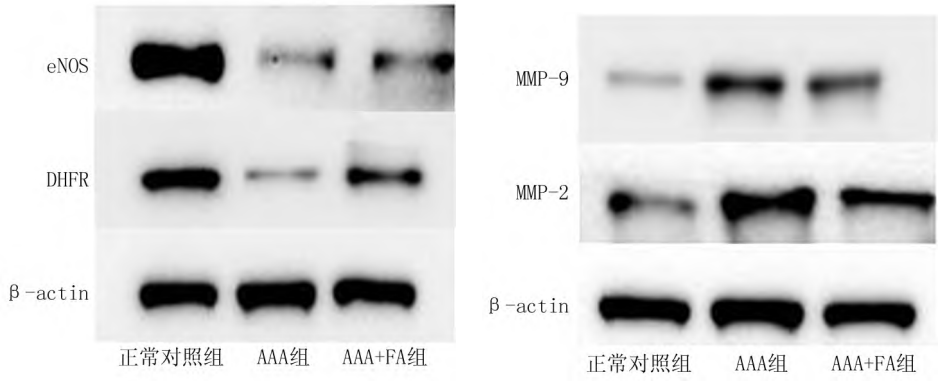

图 4　3 组小鼠腹主动脉各蛋白表达比较

Fig. 4　Comparison of protein expression in abdominal aorta of three groups of mice

差异,这可能是由于样本数量少的原因。但是口服叶酸可以显著抑制 AngⅡ联合 BAPN 诱导的小鼠腹主动脉直径扩张,并且防止主动脉弹性纤维破坏。

AAA 的发病机制是多因素的,包括中性粒细胞、巨噬细胞和肥大细胞对外膜和中膜的浸润;平滑肌细胞的增殖转化和丧失;动脉瘤组织中氧化应激水平的升高;细胞外基质的降解[7]。目前认为,AAA 的发病机制还涉及动脉粥样硬化和血流动力学紊乱,其中内皮细胞发挥了重要作用[8]。有研究已经证明 eNOS 解偶联与 BH4 缺乏的因果作用,以及 eNOS 再偶联在 AAA 形成中的治疗潜力[9]。eNOS 解偶联导致一氧化氮(nitric oxide,NO)生物利用度下降,氮氧化合物累积从而引起内皮氧化应激。BH4 作为 eNOS 偶联的辅助因子,当 BH4 的从头合成及补救合成限速酶 DHFR 缺乏时,同样会导致内皮功能障碍[10-11]。有研究已经证明,叶酸通过恢复 DHFR 以恢复 eNOS 功能来预防 AngⅡ输注 apoE 缺失小鼠 AAA 模型[12]。本研究中使用 AngⅡ联合 BAPN 诱导的 AAA 模型中的结果与之相符。

除此之外,内皮是刺激或抑制底层平滑肌细胞增殖的分子来源。在正常的血管壁中,平滑肌细胞是静止的,但当内皮受损时则会增殖[13]。内皮细胞和平滑肌细胞之间的相互作用是多种心血管病过程的基础,如动脉生成、动脉粥样硬化和动脉重塑[14]。叶酸通过恢复内皮功能进一步维持动脉肌层稳态,抑制 BAPN 联合 AngⅡ诱导的中膜平滑肌细胞紊乱和炎性反应。

MMP 在 AAA 形成过程中起着重要作用[15]。在正常主动脉中,内皮细胞、平滑肌细胞(SMC)和外膜成纤维细胞负责 MMP 的产生。在 AAA 的情况下,炎性细胞充当 MMP 的额外来源[16]。MMP 是与细胞外基质(ECMs)降解机制关联最紧密的一类酶,可降解腹主动脉壁组织中胶原和弹力蛋白成分。AAA 形成过程中起主要作用的种类为 MMP-2、MMP-9。MMP-9 和活化的 MMP-2 在异种移植动脉瘤变性过程中上调,这些酶的进一步增加与破裂有关[17]。MMP-2、MMP-9 由成纤维细胞和 SMC 产生,并在 AAA 形成期间通过浸润外膜巨噬细胞产生[18-19]。据报道,FA 治疗降低了糖尿病大鼠及高同型半胱氨酸(homocysteine,Hcy)血症小鼠诱导的 MMP-2、MMP-9 分泌及活性[20-21]。有研究证明,FA 通过诱导 5-亚甲基四氢叶酸还原酶(5-methylene tetrahydrofolate reductase,5-MTHFR)将 Hcy 重新甲基化为甲硫氨酸来降低同型半胱氨酸水平,并诱导了金属蛋白酶组织抑制剂,共同降低金属蛋白酶[22]。本研究结果也进一步表明,AAA 中 MMP-2、MMP-9 上调明显,叶酸口服治疗后上述 2 种基质金属蛋白酶明显下降。

综上,口服叶酸可能成为预防或治疗 AAA 的一种选择。

利益冲突:所有作者声明无利益冲突

作者贡献声明

金忠调:设计研究方案,实施研究过程,论文撰写;孙静媛、梅瑛娜、夏敏琪:实施研究过程,数据收集;高凌:提出研究方案、研究思路,论文审核

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(收稿日期:2022－12－03)

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(收稿日期:2023－03－20)