

纤溶药物出血机制的进展及分析

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摘要: 传统的溶栓药多为纤溶酶原激活剂类药物, 严重依赖于患者本身的纤溶酶原水平, 溶栓效果受到限制。与之不同的是, 纤溶药物具有直接纤维蛋白水解活性, 能够直接溶解血栓, 是一种新型的具有“直接疗效”的溶栓药物, 具有更高的溶栓效率和安全性, 是目前的研究热点之一。虽然能作为纤溶药物的纤溶物质不断被发现研究, 但真正开发成新药、成功运用于临床的却很少, 主要是其出血风险限制了发展。本文综合国内外有关纤溶药物的最新研究进展, 就其出血机制及应对策略进行系统综述, 总结了纤溶药物的5类出血机制, 提出了3类应对策略, 为研发更安全、高效的纤溶药物提供理论依据。

关键词: 血栓; 纤溶; 出血; 金属蛋白酶; 丝氨酸蛋白酶

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Progresses and analysis in fibrinolytic drugs induced hemorrhage

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Abstract: At present, most clinical thrombolytic drugs are plasminogen activators, which are highly dependent on the plasminogen level of the patient. Therefore, the efficacy of those drugs is restricted. Unlike the conventional thrombolytic plasminogen activator drugs, fibrinolytic drugs have direct fibrinolytic activity. Thus, fibrinolytic drugs can directly dissolve the thrombus, and its thrombolysis efficacy is not restricted by the patients' plasminogen. This is a new type of thrombolytic drug with higher thrombolytic efficiency and safety, and has become one of the research hotspots at present. Although more and more agents that can be used as fibrinolytic drugs have been discovered, only a few of them can successfully be applied in clinical practice. The mainly underlying reason is the risk of bleeding. In this paper, based on the latest research progress of fibrinolytic drugs, the bleeding mechanisms and coping strategies of fibrinolytic drugs were systematically reviewed, five types of bleeding mechanisms of fibrinolytic drugs were summarized, and three types of coping strategies were proposed. We hope our work can provide theoretical basis for the development of safer and more efficient fibrinolytic drugs.

Key words: thrombus; fibrinolytic; hemorrhage; metalloproteinase; serine protease

血栓性疾病 (thrombotic disease, TD) 严重威胁人类的生命健康, 其发病率高居各种疾病之首, 且近年来还有剧增之势, 是现代医学研究的重点和热点之一^[1]。作为目前市售临床溶栓特效药物, 具备直接纤维蛋白

溶解能力的纤溶药物 (fibrinolytic drugs) 比传统的通过激活纤溶酶原发挥纤维蛋白溶解作用的纤溶酶原激活剂类 (plasminogen activator, PA) 溶栓药具有更好的疗效和安全性, 但纤溶药物的出血风险仍然不能掉以轻心。本文对纤溶药物的出血机制及对应改进措施进行了综述, 为今后新型纤溶药物的开发提供有益的建言与参考。

1 临床常见溶栓药

目前, 临床上的血栓治疗药物按其作用机制可分

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为抗凝药、抗血小板药和溶栓药三大类^[2]。其中,以肝素、华法林和利伐沙班等为代表的抗凝药^[3],和以阿司匹林、氯吡格雷和替格瑞洛等为代表的抗血小板药^[4]都只能缓解血栓形成,而不能溶解已形成的血栓^[5],只有溶栓药才能有效地溶解业已形成的血栓。溶栓药物根据其作用机制的不同,又可以进一步分为纤溶酶原激活剂类溶栓药物和纤溶药物两类。前者包括尿激酶 (urokinase, UK)、组织型纤溶酶原激活剂 (tissue-type plasminogen activator, tPA)、链激酶 (streptokinase, SK) 等,通过将体内原有的纤溶酶原激活为纤溶酶 (plasmin),从而溶解血栓^[6-11]。后者包括以蛇毒金属蛋白酶 (snake venom metalloproteinases, SVMPs) 为代表的各种金属蛋白纤溶酶 (metalloproteinase)^[12-15]和以纤溶酶为代表的丝氨酸蛋白纤溶酶 (serine proteases)^[16]。纤溶药物能直接降解血栓中的纤维蛋白,实现血栓的快速溶解^[17,13] (表1)。值得注意的是,纤溶酶原激活剂必须依赖患者体内的纤溶酶原才能发挥作用,而患者体内的纤溶酶原大多呈现低表达状态,这严重影响了此类溶栓药的治疗效果^[18];而纤溶药则完全不受限于患者的纤溶酶原状态,是一种新型的具有“直接效应”的溶栓药物,具有更高的溶栓效率和安全性^[19,20]。然

而,越来越多的临床数据表明,纤溶药物虽然具有更强的溶栓效果,但同样也会伴随着用药引起出血并发症,严重限制了其进一步的使用^[21]。

2 纤溶药物出血机制

纤溶药物诱导出血是一个非常复杂的过程,不仅涉及到血管及血液成分的物理学改变,也受流体动力学的影响。根据目前已有研究,可将其归纳成5个主要机制 (图1)。

2.1 降解血管基底膜

由内皮细胞和基底膜构成的内膜层是药物与血管作用的第一道防线,是维持血管正常功能的最内层屏障。而基底膜作为内皮细胞的附着层膜,由层黏连蛋白、IV型胶原、巢蛋白、硫酸类肝素蛋白等多种蛋白质组成。其中,IV型胶原通过巢蛋白的桥接作用,与层黏连蛋白形成了基底膜的骨骼结构^[22]。研究证明,SVMPs可以水解纤连蛋白、层黏连蛋白、IV型胶原和巢蛋白,表明SVMPs可以降解血管基底膜^[23]。进一步体内外实验表明,弱出血性的CcMP-II (P-II类SVMPs)对纤维蛋白原、层黏连蛋白和IV型胶原具有选择特异性,能同时导致基底膜缺失和内皮细胞脱落^[24]。此外,出血性弱的BaP1 (P-I类SVMPs)和出血性强的CsH1 (P-III类SVMPs)两种金属蛋白酶都能

Table 1 Systematic comparison of clinical thrombolytic drugs

Name	Proteinase	Fibrinolytic agent	Immunogenicity	Fibrin specificity	Plasminogen activation	Half-life /min	Ref
Streptokinase	Serine proteinase	Plasminogen activator	Yes	No	Indirect	15-30	[9]
Urokinase	Serine proteinase	Plasminogen activator	No	No	Indirect	15	[10]
Tissue-type plasminogen activator	Serine proteinase	Plasminogen activator	No	No	Direct	4-6	[11]
Batroxobin	Metalloproteinase	Plasmin	No	No	No	360	[14]
Defibrase	Metalloproteinase	Plasmin	No	No	No	180-360	[15]
Fibrinogenase for injection	Metalloproteinase	Plasmin	Yes	No	No	150-250	[13]

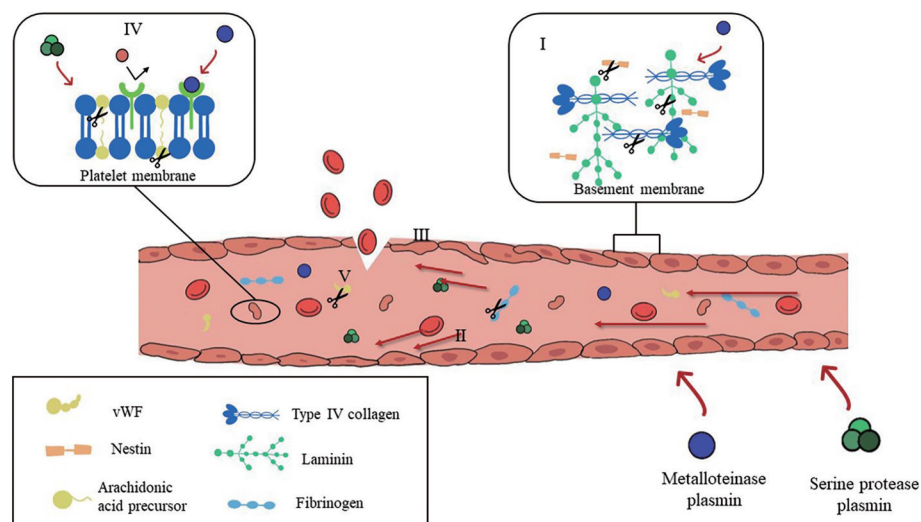


Figure 1 Mechanisms of fibrinolytic drugs induced hemorrhage. I: Degradation of capillary basement membrane; II: Increase of hemodynamic factors; III: Apoptosis of endothelial cells; IV: Inhibition of platelet aggregation; V: Degradation of coagulation factor

显著降低层黏连蛋白、IV型胶原和巢蛋白的水平,但BaP1只是轻微水解IV型胶原,而CsH1则能够强烈降解IV型胶原,这一点与它们各自的出血强度基本一致^[25]。因此,纤溶药的出血强度与它们对基底膜中的IV型胶原特异性降解程度呈正相关^[26]。体外实验中,P-I类SVMPs在3D球体和基质模型中表现出明显的抗血管生成特性,将该球体移植进小鼠体内后,周围的血管被明显破坏。这表明P-I类SVMPs除了可以破坏血管基底膜,也可以抑制血管生成^[27]。不难理解,随着基底膜成分尤其是巢蛋白被降解,内皮细胞中的囊泡逐渐外溢丢失,内皮细胞逐渐变薄并从基底膜的层黏连蛋白上脱落,形成基底膜和内皮细胞层缺口,最终导致血液成分外溢。此外,纤溶药物诱导的局部炎症也会诱发基底膜变薄,促使内皮细胞从基底膜上脱落,从而加剧出血进程^[28]。

2.2 改变血流动力学因素 在研究SVMPs对基底膜的降解过程中,科研人员发现BaP1在不同血流供应状态下对小鼠腓肠肌出血效应差异巨大。当血流阻断时,腓肠肌内无红细胞和炎性细胞,而在不阻断血流的情况下,腓肠肌内有明显的出血和水肿,这种差异表明血流动力学因素在出血过程中起重要作用^[29,30]。血管壁的膨胀性对血管壁张力起着关键作用,这种膨胀性主要是由其基质的机械性能决定的^[31]。因此,基底膜力学稳定性的任何影响都将直接影响血管壁的膨胀性。

针对血流动力学因素与出血的相互作用,Gutiérrez等^[32]首次提出了“两阶段”论的假说。即第一阶段,SVMPs降解基底膜和黏附蛋白,使基底膜变薄,进而减弱内皮细胞与基底膜的相互作用;第二阶段,由于基底膜的削弱,内皮细胞受到的渗透压、剪切力等血流动力学因素增加,随之发生压缩、变形、囊泡外溢,并最终达到破裂临界值时,血液成分外溢。值得注意的是,SVMPs在体内对血管损伤是十分迅速的,而在体外培养时往往需要几个小时以上才会引起损伤。因此可以推测,在体内SVMPs引起的快速损伤并不是由于这些酶对内皮细胞的直接作用的结果,而是由于SVMPs对基底膜组分的作用削弱了血管壁的机械稳定性而产生的间接作用^[28]。因此,单纯改变血流动力学因素虽不能引起出血,但在基底膜降解的动态作用过程中,SVMPs的出血活性将因此得到放大,使出血更快、更剧烈。

2.3 内皮细胞凋亡 在纤溶药物诱发出血时,内皮细胞呈现脱离基底膜、丧失活力的明显变化趋势^[33],暗示其可能发生了失巢性凋亡^[34]。早期多项实验结果表明,SVMPs能调控多条细胞凋亡通路。BaP1对人内皮细胞EA.hy926的凋亡研究显示,虽然BaP1对抗凋亡的Bcl-xL和促凋亡Bax的表达没有明显变化,但能引起

NFκB的抑制剂IκBα的低表达,以及procaspase-8的表达完全消失。这说明BaP1可独立于Bcl-xL和Bax诱导内皮细胞凋亡,并与caspase-8的激活有关^[35]。进一步转录组测序实验表明,经*Crotalus atrox*毒液和Jararhagin两种金属蛋白酶处理后,人脐静脉内皮中有高达33个Fas配体/TNF-α受体凋亡通路的基因上调表达^[36]。与BaP1类似,Jararhagin可以通过激活procaspase-3,改变Bcl-xL/Bax平衡来诱导内皮细胞凋亡^[37]。此外,P-I类SVMPs能阻断G0/G1期细胞,进一步诱导内皮细胞坏死和凋亡^[27]。*C. atrox*毒液能刺激HUVEC中的锌运输,10 μg·mL⁻¹毒液刺激单层HUVEC细胞3 h就能观察到细胞回缩,而100 μg·mL⁻¹毒液浓度则能完全破坏细胞黏附,使细胞脱离^[38]。

除失巢性凋亡途径外,SVMPs家族的整合素血管凋亡诱导蛋白-1(VAP1)可以在不降解细胞外基质或抑制细胞黏附的情况下,特异性地诱导血管内皮细胞凋亡,但诱导效应可被整合蛋白α3、α6、β1和CD9抗体所抑制。这些结果表明,整合蛋白在VAP1诱导的内皮细胞凋亡中发挥着相当重要的作用,而不只是黏附于细胞外的基质而已^[39];SVMPs可引起内皮细胞的非失巢性凋亡。因此,研究者提出整合蛋白介导的内皮细胞凋亡假说,即SVMPs在去整合蛋白/类整合蛋白和富半胱氨酸的结构域,通过与内皮细胞表面的整合蛋白结合,从而诱导内皮细胞凋亡^[40]。

早期研究SVMPs对内皮细胞的作用时,使用的都是单层细胞模型,这一模型的缺点是与人体血管内皮细胞在表型上存在差异。2015年,建立了在细胞外基质支架中对内皮细胞进行二维和三维培养的模式^[41],这一模型更接近人体血管内皮细胞生长条件。实验观察发现,胶原蛋白和人工基底膜基质增强了SVMPs对内皮细胞的损伤,使SVMPs破坏了应力纤维,引起内皮细胞的脱离与凋亡。

综上所述,SVMPs诱导内皮细胞凋亡的途径复杂多样,可发生在细胞脱离前或脱离后。这些凋亡过程往往开始于出血的早期,并影响体内出血的严重程度。

2.4 抑制血小板聚集 众所周知,血栓形成过程涉及血管性血友病因子(von Willebrand factor, vWF, FVII因子的载体)作用于内皮细胞基底膜的胶原,胶原进一步诱导血小板聚集,而血小板聚集过程主要由整合蛋白α2β1(GPIa/IIa)、糖蛋白VI(GPVI)、CD36(GPIV)和GPIIb/IIIa等膜受体介导^[42],vWF在整个聚集过程中起到血小板膜表面GPIIb的“桥梁”作用^[43]。因此,P-II型SVMPs由于存在能与血小板膜表面GPIIb/IIIa结合的RGD序列,在结合血小板的同时阻碍了纤维蛋白原和vWF与活化的血小板结合,抑制血小板聚集,抑制凝

血^[44]。同样, P-III型SVMPs通过其SECD序列与血小板表面的GPVI和GPIa/IIa结合,进而强烈抑制血小板聚集及凝血^[45]。此外,SVMPs的富半胱氨酸结构域可与vWF的vWA结构域结合,增强SVMPs对vWF的特异性水解,并抑制vWF介导的胶原诱导的血小板聚集及凝血^[39]。

与金属蛋白纤溶酶类似的是,作为丝氨酸蛋白纤溶酶的代表,人纤溶酶可以在不同部位剪切凝血酶并激活受体PAR1和PAR4,从而阻断凝血酶诱导的血小板活化过程及凝血^[46]。同时,纤溶酶可以通过特异性抑制血小板膜磷脂池内源性花生四烯酸的动员来抑制血小板聚集及凝血。此外,由于纤溶酶的抑制作用是钙依赖的,因此纤溶酶也可能通过破坏游离钙的膜结合或易位来抑制血小板聚集及凝血^[47]。

因此,金属蛋白纤溶酶与丝氨酸蛋白纤溶酶都能够调控血小板活性,抑制其聚集。不同的是,金属蛋白纤溶酶对血小板的调控作用是由RGD和SECD两个序列引起的,而丝氨酸蛋白纤溶酶则是通过抑制血小板膜磷脂池内源性花生四烯酸的动员,或破坏游离钙的膜结合或易位来达到抑制血小板聚集的目的。

2.5 降解凝血因子 目前,一些P-III类的SVMPs被证明可以激活或降解凝血级联的一些成分,如纤维蛋白原、FII(凝血酶原)、FX、组织因子和vWF^[48]。其中,HF3(P-III类SVMPs)能够体外裂解抗凝血酶III、补体成分C3和C4、FII和纤溶酶原。值得注意的是,HF3还能在体内外诱导出血时裂解血小板衍生生长因子受体(PDGFR)和血小板衍生生长因子(PDGF)^[49]。PDGF能够促进创伤愈合,而其被裂解则会减弱止血功能。

同样的,丝氨酸纤溶酶也可以降解和失活FV、FVIII、FIX、FX等凝血因子^[50]。早期在进行tPA和人纤溶酶的出血风险比较研究中发现,只有当纤溶酶的剂量达到溶栓剂量(1 mg·kg⁻¹)的8倍时,纤溶酶方才开始降解纤维蛋白原和vWF并引起出血,这一安全阈值是tPA的16倍^[51]。此外,丝氨酸蛋白纤溶酶可作为人血管性血友病因子裂解酶(ADAMTS13)的替代药物用于血栓性血小板减少性紫癜(TTP)小鼠模型研究。虽然两者都以剂量依赖的方式切割vWF,但却拥有完全不同的切割位点。ADAMTS13主要剪切vWF的A2结构域的Tyr1605-Met1606肽键,而丝氨酸蛋白纤溶酶则切割vWF的A1-A2连接区域内的K1491-R1492肽键^[52]。

凝血因子作为止血的关键因素,能够调节出血与止血。而过量使用溶栓药,尤其是使用间接型的溶栓药容易突破止血阈值,导致出血。这一机制提示在临床治疗过程中,应密切观察患者的凝血因子水平,并及时调整用药量,减少出血。

3 应对纤溶药物出血的策略

3.1 蛋白修饰及结构优化 出血实验模型显示,N-糖基化后的出血性Jararhagin和ACLH均未引起小鼠皮肤明显出血,而未处理的对照组却呈现明显出血点,表明SVMPs在经过N-糖基化后,其出血活性显著降低。同时,通过基因工程方法在大肠杆菌中无糖基化表达的产品rCDJARA(Jararhagin的重组催化结构域)和rACLH(ACLH的重组催化结构域)同样不具有出血性活性,这意味着单独的催化结构域可能是非出血性的^[53]。研究发现,BaP1的出血活性是通过围绕活性位点的Ω环来实现的,而经过位点突变消除Ω环残基后,出血活性即消失。与未处理的对照组相比,小鼠皮肤未出现明显出血点,且位点突变后的BaP1只有部分蛋白的水解活性降低^[54]。此外,解整合素水解后的MT-d-I(P-II类SVMPs)对血管基底膜蛋白的水解特异性发生显著改变,这说明在调节SVMPs的水解特异性中,解整合素结构域可能发挥了重要作用^[55]。

在人纤溶酶中,Kringle2~5(K2~K5)结构域与纤溶出血相关,而Kringle1(K1)结构域与识别纤维蛋白相关^[56]。因此,通过去除K2~K5结构域并保留K1结构域可制备重组微纤溶酶(ΔK2~5纤溶酶)。结果发现,ΔK2~5纤溶酶与纤维蛋白和α2-抗纤溶酶的结合特性保持不变,表明重组纤溶酶保留了全长纤溶酶的特性^[57],并且分子量的降低也增加了微纤溶酶的比活性。在随后进行的纤溶启动剂量及最高剂量下产生最大纤溶活性测试发现,ΔK2~5纤溶酶与全长纤溶酶所需要的剂量没有差异。并且在大量ΔK2~5纤溶酶实验后,残留的凝血因子和纤维蛋白原足以完成止血,从而大幅度降低出血风险。据此,在2009年首次进行了微纤溶酶的临床试验,研究了长期静脉血栓患者应用微纤溶酶恢复导管功能的安全性和有效性^[58]。临床试验结果显示,样本中80%以上的患者康复,且无出血并发症或其他不良事件。经结构优化后的微纤溶酶的安全性也得到了充分的证实。

3.2 纳米载药 缓解出血问题的另一种有效方法是用纳米颗粒先阻断纤溶药物的纤溶活性,直至到达血栓凝块再释放^[59]。在2020年,Kline等^[60]发明了一种新型的纳米颗粒。经这种纳米颗粒包装后,纤溶酶的水解活性将被暂时性封闭,但保留了其与血栓凝块结合的特异性,一旦结合血栓凝块,纳米结构发生变化,并恢复纤溶酶的水解活性。随后的体外溶栓实验也没有观察到出血现象,表明这是一种理想的包裹纤溶酶的纳米颗粒。另外,由红细胞膜和纳米粒子组装起来的纳米颗粒载体同时具备了天然红细胞膜与纳米材料的优点,是近期兴起的新型纳米载体^[61]。这一新型纳米

载体同样也被证明可以递送纤溶富勒烯醇 (Fols), 使活性 Fols 在体内具有更为高效安全的溶栓效果^[62]。

纳米颗粒不仅可以增强纤溶药物对血栓纤维蛋白的结合活性, 抵抗敏感的水解酶和抑制剂, 如 $\alpha 2$ -抗纤溶酶, 而且还可以实现纤溶药物在特定的血栓凝块中释放, 从而改善出血风险。此外, 纳米颗粒的材料、释放方法、包被药物等方面的创新层出不穷, 为未来纤溶药物的开发提供了更为广阔的理论依据, 赋予了安全型纤溶药物更多的可能性。

3.3 增强特异性 尽管已经提出了一些创新的应对措施, 但纤溶药物引起出血的根本问题——非特异性仍然没有解决。对此, 有学者提出了新的策略方向——纤维蛋白特异性靶向元件插入。通过设计并构建可选择性识别纤维蛋白 α 链 N 端肽的抗体噬菌体库, 最终分离出一种适合的抗体, 命名为 AP2, 该抗体能够高亲和力识别纤维蛋白的 5 个 N 端氨基酸 ($K_d = 44 \text{ nmol} \cdot \text{L}^{-1}$)^[63]。另一项研究显示, 中性粒细胞上的 CD11c/CD18 可以识别纤维蛋白原 α 链 N 端结构域的 Gly-Pro-Arg 序列^[64]。类似的, 一种来自化脓链球菌的蛋白 F, 具有能与纤维连接蛋白结合的化脓性黏附素, 包含了重复结构域 (RD2) 和额外结构域 (UR) 两个结构域, 这两个结构域都能最大限度结合纤维连接蛋白。且 UR 的结合力更高于 RD2, 并能识别出包含纤维蛋白和胶原结合域的大片段^[65]。此外, 一种名为高温需求 a 蛋白 (HtrA) 的新型非纤溶酶依赖性的酶被成功筛选, 其 PDZ 结构域可以特异性结合血栓中错误折叠的纤维蛋白。在血栓被识别后, HtrA 的丝氨酸结构域就能够水解被识别的纤维蛋白^[66]。

纤维蛋白特异性抗体和结构域的鉴定可促进纤维蛋白特异性治疗剂的开发。因此, 添加纤维蛋白靶向基段 (如 AP2、RD2、UR 和 PDZ 等) 或其他能够加强纤溶药物靶向特异性的方法, 是未来更安全的纤溶药物开发的可靠策略。

4 总结与展望

血栓是全球性高发生率的疾病, 它会导致多种心血管病、神经系统疾病及其他严重的并发症。金属蛋白纤溶酶和丝氨酸蛋白纤溶酶作为两种纤溶药物, 已在临床实践或试验中作为溶栓药使用。然而, 治疗过程中出血并发症的频繁发生, 使得治疗风险上升。因此, 在治疗过程中, 应该更加重视治疗的安全性, 并采取有效的措施来防止出血的发生。

目前, 金属蛋白纤溶酶比丝氨酸蛋白纤溶酶应用更广泛, 尽管丝氨酸蛋白纤溶酶显示具有更高的安全性^[67]。在本文综合总结出的 5 种出血机制中, 各个机制并不是孤立的, 而是相互作用、相互影响的, 其中降解血管基底膜是最基础的, 它可以加速第二种机制, 即

血流动力学因素对血管基底膜的影响。另一方面, 内皮细胞的凋亡可以进一步促进血管基底膜的降解。最后, 抑制血小板聚集的机制会进一步扰乱止血, 增强纤溶酶的出血性活性, 而降解凝血因子的机制会突破止血阈值, 导致出血。同时, 文中提出的解决方案的实用性和有效性还需要更多的临床研究来评估, 以期达到真正的临床实用性。

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