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高迁移率族蛋白 1 拮抗剂 BoxA 对细菌性脑膜炎大鼠的治疗作用 *

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摘要 目的:探究高迁移率族蛋白 1(HMGB1)拮抗剂 BoxA 尾静脉注射对细菌性脑膜炎(BM)大鼠模型的临床体征改善和炎症抑制作用。**方法:**除外正常对照的雄性 Sprague-Dawley(SD)大鼠设为对照组(n=20),另取 60 只大鼠行脑室立体定向注射 20 μL 大肠杆菌 *Escherichia coli* (DH5α 1× 10^7 CFU/mL)建立 BM 模型,之后随机分为两组(各组 n=30),一组尾静脉注射 HMGB1 拮抗剂 BoxA,即 BoxA 组;一组麻醉后进行尾静脉注射无菌磷酸盐(PBS),即 Vehicle 组。造模 3 d 后,对各组大鼠的临床指标以及病理生理参数 (颅内压和脑脊液白细胞(WBC)计数)进行评估,使用酶联免疫吸附实验(ELISA)法检测血清中 HMGB1 的相对含量,使用伊文思蓝染色观察血脑屏障(BBB)通透性,使用免疫荧光染色检测大脑皮层炎症因子(IL-1β 和 TNF-α)的表达水平。**结果:**相比 Control 组,Vehicle 组临床指标,颅内压,WBC 计数以及血清 HMGB1 含量明显提升(P<0.05);而 BoxA 组相比 Vehicle 组,以上改变有部分减少(P<0.05)。另外,Vehicle 组较 Control 组 EB 渗漏增加且炎症因子(IL-1β 和 TNF-α)表达水平增高(P<0.05)。与 Vehicle 组相比,BoxA 组的这些变化亦被部分调节(P<0.05)。**结论:**HMGB1 抑制剂 BoxA 尾静脉注射能够下调 HMGB1 表达水平并同时缓解细菌性脑膜炎大鼠的临床症状和炎症反应。

关键词:细菌性脑膜炎;大肠杆菌;BoxA;高迁移率族蛋白 1;炎症

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Effect of High Mobility Group Protein 1 Antagonist BoxA on the Treatment of Bacterial Meningitis in Rats*

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ABSTRACT Objective: To investigate the effect of high mobility group protein 1 (HMGB1) antagonist BoxA via tail vein injection on the improvement of clinical signs and anti-inflammatory effects in a rat model of bacterial meningitis (BM). **Methods:** Male Sprague-Dawley (SD) rats were used and the normal rats were set as the control group (n=20). Another sixty rats were injected with *Escherichia coli* (DH5α 1× 10^7 CFU/mL) to establish BM rat model and then were randomly divided into two groups (n=30): after anesthesia one group received intravenous BoxA (BoxA group), the other one received PBS injection as placebo (Vehicle group). Three days after model establishment, the clinical index of each group and pathophysiological parameters (intracranial pressure and CSF white blood cells (WBC) count) were evaluated, Enzyme-linked immunosorbent assay (ELISA) method were used to detect the relative serum HMGB1 content, Evans blue staining were used to observe the blood-brain barrier (BBB) permeability, immunofluorescence staining were used to detect the expression level of cerebral inflammation cytokines (IL-1β and TNF-α). **Results:** Compared with Control group, clinical indicators, intracranial pressure, WBC count and serum HMGB1 content in Vehicle group has significantly increased (P<0.05). These changes were partially reduced in the BoxA group compared to the Vehicle group (P<0.05). In addition, Vehicle group showed increased EB leakage and increased expression of inflammatory factors (IL-1β and TNF-α) than Control group (P<0.05). These changes were also partially adjusted in the BoxA group compared to the Vehicle group (P<0.05). **Conclusion:** Tail vein injection of HMGB1's inhibitor BoxA can down-regulate HMGB1 expression and alleviate the clinical symptoms and inflammatory responses in BM rats.

Key words: Bacterial meningitis; *E. coli*; BoxA; High mobility group protein 1; Inflammation

Chinese Library Classification(CLC): R-33; R512.3 Document code: A

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前言

细菌性脑膜炎(bacterial meningitis, BM)是欧洲和美国极为常见[1-3],在中国主要儿童为主,如以大肠杆菌型的 BM 等[4]。它仍然是全球成人感染疾病导致死亡的主要原因之一,幸存者经常遭受脑损伤和残余神经功能缺损[5,6]。近年来临床和基础研究均表明,持续性炎症造成的脑损伤是疾病发生发展的重要因素[7-9]。而在 BM 病理生理进程中,危险相关分子模式(damage associated molecular patterns, DAMPs)的释放,如高迁移率族蛋白 1(High mobility group protein 1, HMGB1)等,在炎症的持续中起着重要作用[10-12]。HMGB1 是高迁移率成员,在脊椎动物细胞中广泛表达,最初被描述为非组蛋白 DNA 结合蛋白,可作为核内保护剂。HMGB1 是介导中枢神经系统疾病(如颅脑损伤,缺血性卒中,急性脑出血引起的损伤,脑脊髓炎和癫痫)中的损伤和炎症的重要细胞因子。但目前关于 HMGB1 以及 HMGB1 拮抗剂在细菌性脑膜炎中的潜在作用尚未明晰。

在本研究中,我们评估了 HMGB1 特异性抑制剂 BoxA 作为治疗 BM 模型的可能性。选用 Sprague-Dawley(SD)大鼠进行大肠杆菌脑室注射建立 BM 动物模型之后,对 SD 大鼠临床指标以及病理生理参数进行分析,并进一步探究了血脑屏障(blood-brain barrier, BBB) 通透性和炎症因子 IL-1β 和 TNF-α 的表达水平的测量。

1 材料与方法

1.1 实验动物

共 80 只 4-6 周龄雄性 Sprague-Dawley (SD)大鼠均来自第四军医大学实验动物中心,动物实验方案得到西京医院伦理批准并符合美国国立卫生研究院的指南。所有动物在室内温度为(25± 1) ℃的条件下食物和水源自由供给。

1.2 细菌性脑膜炎模型以及 BoxA 干预

除了 20 只正常对照组(Control 组,脑室内注射无菌磷酸盐(phosphate-buffered saline,PBS,pH 值 7.4), 其他 60 只老鼠均进行了左侧侧脑室(坐标为 AP:1.5 mm; ML:1.0 mm; DV:4.5 mm)立体定向注射 20 μL 大肠杆菌 *Escherichia coli* (DH5α 1× 10^7 CFU/mL)建立细菌性脑膜炎(BM)动物模型[13],紧接着被随机分为 BoxA 组(n=30,尾静脉注射 10 mg/Kg BoxA), Vehicle 组(n=30,相同方法注射 PBS)。实验过程中若有死亡则另行补充。

1.3 临床指标评估和生化检查

经典的细菌性脑膜炎行为学评估即为非常简陋,而本研究采用国际最新的临床行为学评估方式, 内容包括平衡木测试、体位反射测试、是否有竖毛、癫痫发作以及反映迟钝等[14-16]。在健康动物得分为 0 分;13 则为临床表现最差。生化检查方面,用异氟醚短暂麻醉小鼠,固定后将导管置入小脑延髓池取脑脊液标本,测定脑脊液白细胞(WBC)计数,同时测量颅内压。其中白细胞计数等在西京医院传染科进行。另外,血清 HMGB1 相对表达水平使用商业上可用的酶联免疫吸附试验(Enzyme-linked immunosorbent assay,ELISA) 试剂盒(RapidBio Laboratories,美国)测定(其中 Control 数值设置为 100%)。

1.4 伊文思蓝(Evans blue, EB)染色检测血脑屏障通透性

大鼠尾静脉注射 EB(鼎国,中国)染料(1%,2 mL/kg)。允许其在血液循环 2 h 后, 麻醉大鼠进行 200 mL PBS 灌注,2 mL/100 mg 甲酰胺孵育 24 h,最后用分光光度计在 620 nm 吸光度法测定 EB 浓度[17,18]。

1.5 免疫荧光染色分析炎症因子表达

BoxA 组用 10%水合氯醛(3 mL/kg,i.p.)麻醉后 4%多聚甲醛灌注。取脑组织准备成 2 μm 厚度。术后 3 天进行大鼠皮层免疫荧光染色。兔抗 IL-1β (1:100,Abcam,美国),兔抗 TNF-α(1:100,Abcam,美国)添加后,组织切片放于湿盒中进行 4℃过夜。然后用荧光二抗体检测(山羊抗兔 488,Invitrogen,美国)在室温下 4 h。之后,4 0.0001%,6-diamidino-2-phenylindole(DAPI)染色(Beyotime、上海)用于细胞核染色。同时观察 10 个以上视野(200×)。使用共聚焦激光扫描显微镜(FV1000, JPN)获取图像;使用 image pro plus 6.0(放大 200×)统计,阳性细胞的覆盖的区域是按划定的总面积的百分比计算(%of area)。

1.6 统计学分析

采用 SPSS22.0 统计软件, 计量资料以均数± 标准差(Mean± SD)表示,多组间比较采用单因素方差分析,两组间比较采用 SNK 法,$P<0.05$ 提示差异有统计学意义。

2 结果

2.1 BoxA 可改善细菌性脑膜炎大鼠临床评分和生化参数,同时降低 HMGB1 含量

BM 建模 3 d 后对 BoxA 其整体疗效进行评估。临床评分方面,各组的组间差别为 $F(2,14)= 45.91$,$P<0.0001$;颅内压方面,各组组间差别为 $F(2,14)= 46.49$,$P<0.0001$;白细胞计数方面,各组的组间差别为 $F(2,14)= 33.05$,$P<0.0001$。相比 Control 组,Vehicle 组临床指标($P=0.0015$),颅内压($P<0.0001$),WBC 计数($P<0.0001$)以及血清 HMGB1 含量($P<0.0001$)明显提升;而 BoxA 组相比 Vehicle 组, 以上改变有部分减少 (临床评分 $P=0.0003$; 颅内压:$P<0.0001$;WBC 计数:$P=0.0006$; 血清 HMGB1:$P=0.0023$)。

2.2 BoxA 可降低细菌性脑膜炎大鼠血脑屏障 (BBB) 通透性(伊文思蓝检测)并降低炎症因子表达水平

在疗效评估过后紧接着进行 BBB 以及炎症因子水平检测。相比 Control 组,Vehicle 组在 EB 渗漏($P=0.0011$)以及炎症因子表达水平(IL-1β:$P<0.0001$;TNF-α:$P<0.0001$)方面均有明显增高。然而在 BoxA 注射之后相应改变能够被部分抑制:EB 渗漏方面相比 Vehicle 组减少($P=0.0098$);而在炎症因子方面相比 Vehicle 组亦有明显表达水平的降低(IL-1β:$P=0.0122$;TNF-α:$P<0.0001$)

3 讨论

大肠杆菌性脑膜炎是一种潜在的致命性和致残性疾病,脑膜肺炎球菌感染后广泛的中性粒细胞炎症是脑损伤的重要原因[19-21]。现有研究表明,脑膜炎症是在识别病原体相关分子模式(Pathogen-associated molecular patterns,PAMP)时触发的,PAMP 在大肠杆菌自溶过程中通过模式识别受体在宿主免疫细胞上和细胞内表达而释放。结果,中性粒细胞被招募到脑脊液中,并将其所有的抗菌武器(如强氧化剂和蛋白水解酶)释放到细胞外空间。中性粒细胞来源的毒素和细菌毒素(如肺溶解素和过

氧化氢)可对宿主细胞造成应激和损伤[22-24]。本研究中也发现了 这一问题。
在 BM 模型中颅内压和脑脊液 WBC 计数明显增高,也验证了

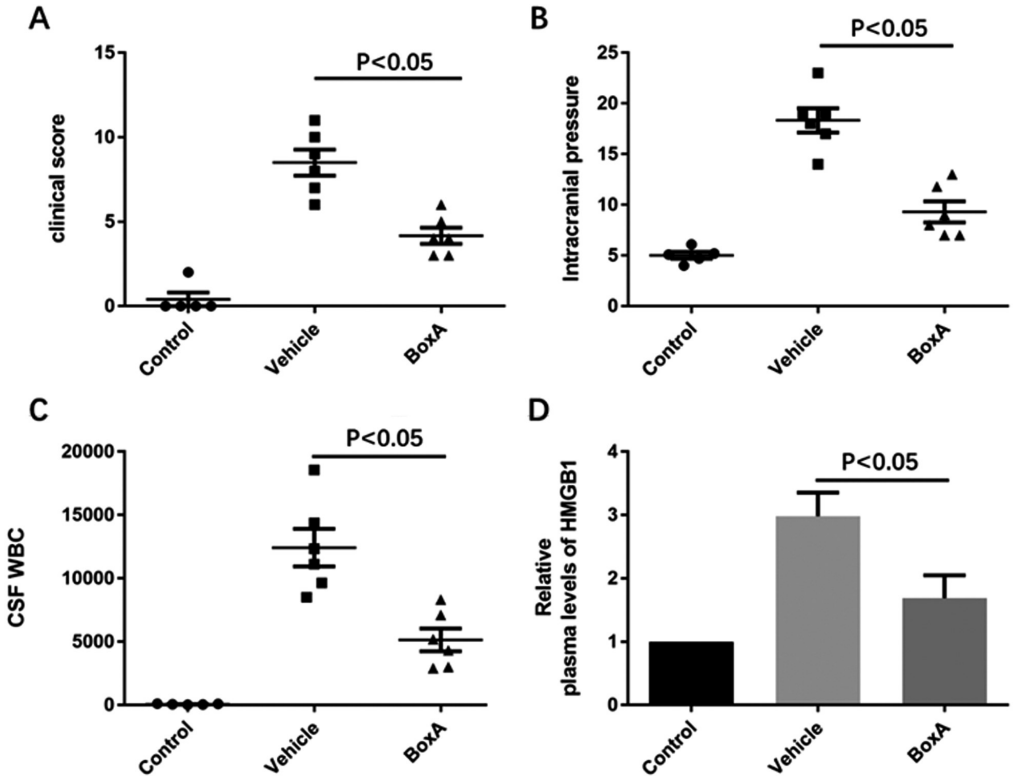

图 1 细菌性脑膜炎大鼠临床以及生化指标

Fig.1 The Clinical and biochemical indexes in rat model of bacterial meningitis. (A)The clinical score; (B) the intracranial pressure; (C) the cerebral-spinal fluid (CSF) white blood cell (WBC) count; (D) the relative plasma HMGB1 expression measured by ELISA and the Control group was set as 100%. Control group (n=5), other groups (n=6). The data are expressed as mean ± SEM. *P*<0.05 vs.Vehicle group.

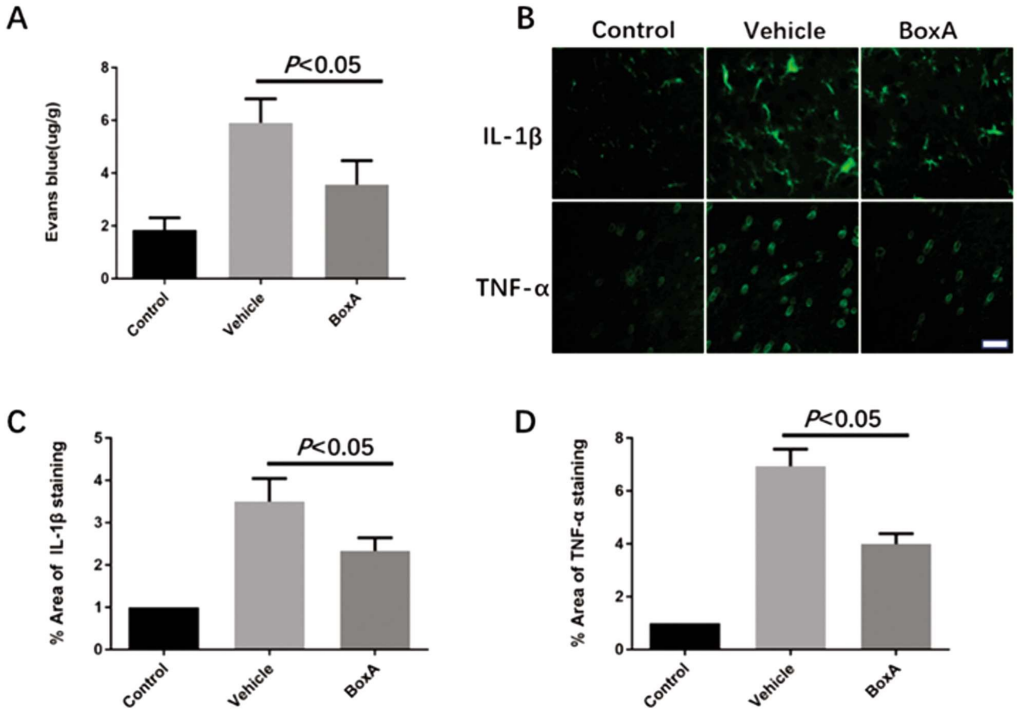

图 2 细菌性脑膜炎大鼠伊文思蓝染色以及免疫荧光分析

Fig.2 The Evans blue dye and immunofluorescence analysis in rat model of bacterial meningitis. (A) The Evans blue leakage (μg/g); (B) the representative images of immunostaining of IL-1β and TNF-α in the brain cortex; the horizontal bar = 50 μm; (C-D): the quantification of the immunofluorescence among the three groups and the control group was set as 100%. Control group (n=5), other groups (n=6). The data are expressed as mean ± SEM. *P*<0.05 vs.Vehicle group.

HMGB1 是与 PAMP 相对应的一种损伤相关分子模式(DAMPs)的非组蛋白 DNA 结合蛋白[25],它一方面可以在颅内感染时主动分泌,一方面也可以通过被动释放等模式参与到例如脑损伤等疾病中来,HMGB1 易位到细胞外空间后起到促炎作用[25,26]。HMGB1 由免疫细胞(如巨噬细胞,单核细胞和树突状细胞)通过无前导的分泌途径分泌。活化的巨噬细胞和单核细胞分泌 HMGB1 作为炎症的细胞因子介质。中和 HMGB1 的抗体可保护其免受关节炎,结肠炎,局部缺血,败血症,内毒素血症和系统性红斑狼疮的损害和组织损伤。激活巨噬细胞细胞因子释放。这将 HMGB1 定位在无菌和感染性炎症反应的交叉点。HMGB1 和 TLR4 之间的相互作用导致 NF-κB 的上调,从而导致细胞因子的产生和释放增加。HMGB1 还能够与嗜中性粒细胞上的 TLR4 相互作用,以刺激 NADPH 氧化酶产生活性氧。现有研究表明,脑缺血后神经元大量释放 HMGB1,导致毗邻神经元的二次损伤[12,27]。此外,重组 HMGB1 可单独诱导急性炎症,而 BoxA 可抑制炎症反应和氧化应激,导致炎症条件下的行为学和生化结果改善 [26]。而我们也发现在 BM 模型中 HMGB1 血清含量明显增高, 说明 HMGB1 可以游离 BBB 出来并发挥促炎作用,下一步可以对其脑内如皮层,海马以及白质纤维区域的 HMGB1 表达情况进行分析。

本研究中所使用的 BoxA 拮抗剂是一种目前已经商业化的,人工合成的短肽,其可以特异性的抑制 HMGB1 下游 Toll 样受体 2/4 的激活,从而阻断 HMGB1 的促进炎症作用[26]。与之前的数据一致,在我们的 BM 模型中,抗 HMGB1 治疗被证明具有临床和病理保护作用。有趣的是,由于抗 HMGB1 的表现略好于其他的 DAMP 分子,我们将研究重点放在前者上。针对给药时间点的问题,有研究观察到在抗生素治疗 3 小时后给予抗 HMGB1 治疗(如中和抗体等)的有益临床效果有相当大的减弱[13]。这说明,尽管 HMGB1 被认为是一种较晚的炎症介质,在疾病晚期 CSF 中含量较高, 但及时抑制其功能是防止免疫反应延长和脑损伤的重要途径。当然拮抗 HMGB1 本身可能将来只是作为一种辅助疗法,与其他抗生素的有效结合才具有最好的效果。在国外 BM 相关报道中,已经有研究表明在动物模型或临床试验中测试的辅助治疗需要与地塞米松、抗生素等同时治疗[6,8,28]。

综上所述,本研究利用大肠杆菌脑室立体定向注射构建的细菌性脑膜炎大鼠模型,发现 HMGB1 特异性拮抗剂 BoxA 可通过降低 HMGB1 的表达,在细菌性脑膜炎大鼠中有明显的治疗作用。一方面能够改善临床症状,降低颅内压和脑脊液 WBC 计数; 另一方面也能够降低 BBB 通透性, 抑制炎症因子的表达。从而较为系统的评估了 HMGB1 特异性抑制剂 BoxA 治疗 BM 模型的可能性,为其进一步应用奠定了基础。

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