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丙酮酸乙酯在老龄小鼠围手术期神经认知障碍中的作用

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[摘　要]　**目的：**评价丙酮酸乙酯(ethyl pyruvate，EP)在小鼠围手术期神经认知障碍(perioperative neurocognitive disorders，PND)中的作用。**方法：**纳入54只无特定病原体(specific pathogen free，SPF)老年雄性C57BL/6小鼠，14~16月龄，体重38~52 g。将54只小鼠采用随机数字表法分为3组(n=18)：正常对照组(C组)、肝部分切除手术组(S组)、EP组。EP组在异氟醚麻醉下行肝部分切除术，术毕时腹腔注射EP 100 mg/kg，C组和S组腹腔注射等容量平衡盐溶液。在术前30 min行痕迹条件恐惧实验(trace fear conditioning，TFC)训练，于术后3 d进行TFC检测，记录小鼠僵直时间。行为实验测试后处死小鼠取海马组织，采用ELISA法检测白细胞介素-1β(interleukin-1β，IL-1β)含量，采用蛋白质印迹法检测高迁移率族蛋白1(high mobility group box-1，HMGB1)、晚期糖基化终末产物受体(receptor for advanced glycation end products，RAGE)、激活核因子-κB p65(nuclear factor-κB p65，NF-κBp65)、胶质纤维酸性蛋白(glial fibrillary acidic protein，GFAP)、离子钙结合衔接分子1(ionized calcium binding adapter molecule，Iba1)及β-actin蛋白质表达。**结果：**与C组相比，在术后3 d时，S组TFC僵直时间缩短，海马IL-1β含量升高，海马HMGB1、RAGE、NF-κBp65、GFAP、Iba1蛋白质表达上调。给予EP治疗，缩短了小鼠术后3 d TFC僵直时间，下调了海马HMGB1、RAGE、NF-κBp65、GFAP、Iba1蛋白质表达和IL-1β含量。**结论：**EP可能是通过抑制HMGB1的分泌和HMGB1/RAGE-NF-κB信号通路改善中枢炎症，从而改善PND。

[关键词]　高迁移率族蛋白1；认知障碍；神经炎症

Effect of ethyl pyruvate on perioperative neurocognitive disorders in aged mice

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Abstract　**Objective:** To evaluate the effect of ethyl pyruvate (EP) on perioperative neurocognitive disorders (PND) in mice. **Methods:** Fifty-four specific pathogen free (SPF) aged male C57BL/6 mice, 14–16 months old, 38–52 g in weight,

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were used in the study. Fifty-four mice were divided into 3 groups (n=18) using the random number table method: a normal control group (C group), a partial hepatectomy group (S group) and an EP group. Partial hepatectomy was performed under isoflurane anesthesia in the EP group with an intraperitoneal injection of EP 100 mg/kg at the end of the operation, and an equal volume of balanced salt solution in the C and S groups. All animals received trace fear conditioning (TFC) training 30 min prior to surgery. The freezing time of mice was recorded by TFC test on the third days after surgery. The mice were then sacrificed and the hippocampus organization was isolated for Western blot to detect the protein expression of high mobility group box-1 (HMGB1), receptor for advanced glycation end products (RAGE), activation of nuclear factor Son-κB p65 (NF-κBp65), glial fibrillary acidic protein (GFAP) and ionized calcium binding adapter molecule 1 (Iba1) and for ELISA to detect interleukin-1β (IL-1β) contents. **Results:** Compared with the C group, the freezing time in the TFC was significantly shortened (P<0.01) and the contents of IL-1β were increased at the third day after surgery. The protein expressions of HMGB1, RAGE, NF-κBp65, GFAP, Iba1 in the hippocampus organization were upregulated at the third day after surgery in S group. Compared with the S group, the freezing time in TFC was significantly prolonged at the third day after surgery in EP group (P<0.05). Compared with the S group, the contents of IL-1β and the protein expressions of HMGB1, RAGE, NF-κBp65, GFAP and Iba1 in the hippocampus organization were reduced at 3 days after surgery in EP group. **Conclusion:** EP showed significant neuroprotection in PND, the mechanism of which might be related to improve neuroinflammation by inhibiting HMGB1 secretion and HMGB1/RAGE-NF-κB signal pathway.

Keywords high mobility group box-1; cognition disorders; neuroinflammation

围手术期神经认知障碍(perioperative neurocognitive disorders，PND)是指发生在术前或术后12个月内，且符合第五版神经障碍手册(Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition，DSM-5)的神经认知功能障碍诊断标准[1]。PND好发于老年患者，主要表现为记忆力、注意力、信息处理能力和执行力的减退。PND的主要发病因素包括高龄、麻醉、手术、受教育程度低下。然而，PND的发病机制尚未完全阐明。

近年来，国内外研究[2-4]认为神经炎症是PND重要发病机制之一。研究[5]已经证实手术创伤可以诱发无菌炎症。然而，高迁移率族蛋白1(high mobility group box-1，HMGB1)是一种广泛存在于哺乳动物细胞的损伤相关模式分子，可以与晚期糖基化终末产物受体(receptor for advanced glycation end products，RAGE)相互作用，激活核因子-κB(nuclear factor-κB，NF-κB)信号通路，引起白细胞介素-1β(interleukin-1β，IL-1β)等炎症因子的释放，从而诱发炎症反应[6]。而且本团队前期研究[7]表明：RAGE信号通路在放大神经炎症起着至关重要的作用。因此，神经炎症是PND防治的一个潜在靶点。而丙酮酸乙酯(ethyl pyruvate，EP)是一种安全、稳定的丙酮酸脂衍生物。越来越多的证据[8-9]证实：EP具有抗炎，抑制HMGB1分泌和NF-κB促炎症信号通路的作用，减少炎症因子肿瘤坏死因子-α(tumor necrosis factor-α，TNF-α)、IL-1β的表达，从而可以促进创伤性脑损伤、蛛网膜下腔出血和神经退行性疾病的神经功能恢复。但EP如何改善老年小鼠PND的研究鲜见报道。本研究采用老年小鼠肝左叶切除术建立PND模型，旨在讨论EP对PND小鼠认知功能的作用及其可能的机制。

1 材料与方法

1.1 实验动物及分组

无特定病原体(specific pathogen free，SPF)级健康老年雄性C57BL/6小鼠54只，14~16月龄，体重38~52g，购自北京华阜康实验动物有限公司。小鼠自由摄食饮水，在室温24~26 ℃，湿度50%~60%，12 h昼夜交替的动物房内适应性饲养2周。采用随机数字表法将小鼠分为3组(n=18)：正常对照组(C组)、手术组(S组)、EP组。本研究取得北京朝阳医院医学研究中心动物福利伦理委员会批准。

1.2 模型制备

S组和EP组小鼠行肝左叶切除术，建立PND模型。将小鼠置于小动物透明麻醉箱(麻醉箱两侧壁分别设有进气口和出气口，进气口连接麻醉机，通入异氟醚和氧气)内，用3.0%异氟醚麻醉诱导，待小鼠翻正反射消失后，将其取出。采用鼻罩，予以1.5%异氟醚维持麻醉。将小鼠仰卧位固定于有保温毯的手术板上，局部备皮、消毒，剑突下纵行切开2 cm手术切口，用撑开器打开腹腔，游离肝脏左叶并结扎切除，用生理盐水冲洗腹腔，逐层缝合腹壁。术前和术后分别给予1.5 mL的生理盐水皮下注射。切口局部给予0.5%罗哌卡因用于术后镇痛。

1.3 痕迹条件恐惧实验

于术前30 min进行痕迹条件恐惧实验(trace fear conditioning，TFC)训练[10]。将小鼠置于检测笼子中适应3 min。开始给予声音的条件刺激(conditioning stimulus，CS)2 700 Hz、85 dB、20 s，间隔20 s后，给予足部电击的非条件刺激(unconditioning stimulus，UCS)0.6 mA、2 s，CS-UCS配对的间隔是30 s。5次配对训练结束后1 min，将小鼠取出放回饲养笼。于术后3 d，小鼠进行场景恐惧记忆实验检测，将小鼠置于术前环境完全相同的检测箱中5 min，不给予任何声电刺激，记录僵直时间。

1.4 蛋白质印迹法检测蛋白质表达

采用蛋白质印迹法检测小鼠海马HMGB1、RAGE、NF-κBp65、胶质纤维酸性蛋白(glial fibrillary acidic protein，GFAP)和离子钙结合衔接分子1(ionized calcium binding adapter molecule，Iba1)蛋白的表达。于术后3 d将小鼠麻醉后断头处死。取出完整脑组织，冰上分离海马，–80 ℃保存。取左侧海马组织，匀浆，使用蛋白质提取试剂盒(美国Thermofisher公司)，BCA(bicinchoninic acid)法检测蛋白浓度，分装，–80 ℃冻存。蛋白加热5 min，变性，分别用10%和12%聚丙烯酰胺凝胶电泳，湿转法将蛋白印迹转至聚偏二氟乙烯(polyvinylidene fluoride，PVDF)膜上，5%脱脂奶粉室温下封闭1 h，分别加入一抗，HMGB1(稀释度1:1 000，英国Abcam公司)，RAGE(稀释度1:500，英国Abcam公司)，Iba1(稀释度1:500，英国Abcam公司)，GFAP（稀释度1:500，英国Abcam公司)，NF-κBp65(稀释度1:1 000，英国Abcam公司)和内参β-actin抗体(稀释度1:1 000，美国Cell Signaling Technology公司)，4 ℃孵育过夜，TBST(Tris buffered saline Tween)洗3次，每次5 min。加入辣根过氧化物标记的二抗(稀释度1:5 000，美国Cell Signaling Technology公司)，室温孵育1 h，TBST洗3次，每次5 min。PVDF膜加入ECL发光液于凝胶成像仪显影。应用Image Lab(Bio-Rad)软件测定蛋白条带灰度值。以目的蛋白条带灰度值与内参β-actin条带灰度值的比值反应目的蛋白的表达水平。

1.5 ELISA 法测定 IL-1β 含量

采用ELISA法测定小鼠海马IL-1β含量。取右侧海马组织，匀浆，4 ℃，12 000转离心15 min。取上清液。按照试剂盒(RayBiotech，美国)说明书操作。

1.6 统计学处理

采用GraphPad Prim 8软件进行分析，正态分布的计量资料以均数±标准差($\bar{x}\pm s$)表示，组间比较采用单因素方差分析，P<0.05为差异有统计学意义。

2 结果

2.1 TFC

与C组[(55±7)%]比较，S组[(38±8)%]小鼠术后3 d的TFC僵直时间缩短(P<0.01)。与S组比较，EP组[(51±5)%]小鼠术后3 d的TFC僵直时间延长(P<0.05)。

2.2 蛋白质印迹法和 ELISA 测定蛋白质表达

与C组比较，在术后3 d，S组小鼠海马HMGB1、RAGE、NF-κBp65蛋白的表达明显升高(P<0.05)；GFAP、Iba1和IL-1β的表达也明显上调(P<0.01)。与S组比较，在术后3 d，EP组下调了小鼠海马HMGB1、RAGE、GFAP、Iba1和IL-1β蛋白的表达(P<0.05)和NF-κBp65蛋白的表达(P<0.01，图1)。

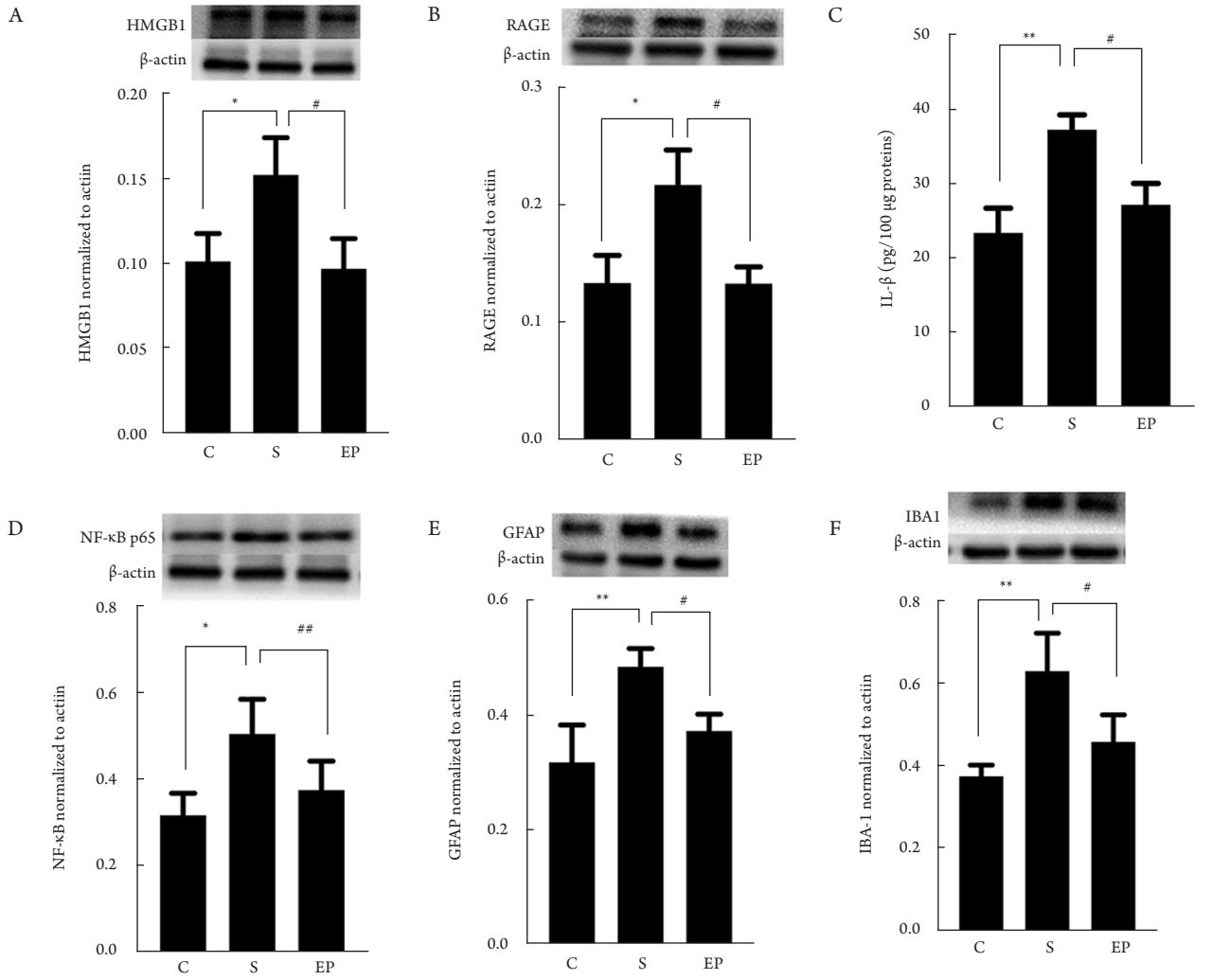

图1 海马(A) HMGB1、(B) RAGE、(C) IL-1β、(D) NF-κBp65、(E) GFAP和(F)Iba1蛋白表达(n=18)

Figure 1 The protein expressions of (A) HMGB1、(B) RAGE、(C) IL-1β、(D) NF-κBp65、(E) GFAP and (F) Iba1 protein in the hippocampus organization (n=18)

与C组比较，*P<0.05，**P<0.01；与S组比较，#P<0.05，##P<0.01。

Compared with the C group, *P<0.05, **P<0.01; compared with the S group, #P<0.05, ##P<0.01.

3 讨论

EP的器官保护作用已经在许多急性炎症模型中得到证实[8-9]。TFC是评价啮齿类动物海马依赖性记忆的经典方法，可通过小鼠僵直程度来判断小鼠对环境和足部电击联合的条件恐惧化的学习情况[11]。本研究结果显示：与C组比较，在术后3 d，S组小鼠的痕迹条件恐惧记忆实验僵直时间缩短，表明小鼠海马依赖性记忆减退，即老年小鼠PND模型制备成功。与S组相比，在术后3 d，EP组小鼠的痕迹条件恐惧记忆实验僵直时间明显延长，表明EP可以改善肝部分切除术后小鼠海马依赖性记忆的降低。

研究[4,12-13]表明：无菌性手术创伤引起的神经炎症伴随着胶质细胞的激活，在调节海马可塑性和记忆过程中起着重要作用。在中枢神经系统，小胶质细胞和星形胶质细胞可由损伤相关分子模式激活，如HMGB1和S100，在术后早期由损伤细胞释放，通过上调促炎性细胞因子，诱导和放大炎症[14]。HMGB1作为全身炎症的驱动因子，HMGB1的升高与术后小鼠和人的认知能力下降密切相关[7,15]。外周注射HMGB1可以导致认知功能障碍，而给予HMGB1抗体可以减轻神经炎症并防治认知能力下降[16]。给予RAGE受体拮抗剂(FPS-ZM1)，可以改善术后3 d老年小鼠的认知功能，可能是通过RAGE/NF-κB信号通路，减少炎症因子

IL-1β的表达和改善海马胶质细胞的激活[17]。本研究结果显示：在术后3 d，小鼠海马的HMGB1，RAGE和NF-κBp65表达升高，IL-1β含量升高，表明手术诱导了海马HMGB1蛋白表达增加，可能与RAGE受体相互作用，通过NF-κB信号通路，促进了IL-1β的表达上调。另外，在术后3 d，海马的GFAP和Iba1蛋白表达上调，表明手术应激激活星形胶质细胞和小胶质细胞，加重神经炎症反应。

EP是一种高亲脂性的内源性能量底物，可以穿透血脑屏障[18]，对多种疾病模型发挥潜在的抗炎和神经保护作用，如阿尔茨海默病、脑出血、蛛网膜下腔出血、创伤性脑损伤[19-20]。Pellegrini等[21]发现：EP作为HMGB1抑制剂，通过抑制HMGB1/RAGE信号通路，可以降低小鼠血清HMGB1水平，并抑制恶性间皮瘤的生长。而且EP通过抑制NF-κB依赖的促炎性信号通路，降低失血性休克模型HMGB1和NF-κB蛋白表达，减轻神经炎症[8]。进一步的研究[22-23]报道：在创伤性脑损伤和慢性脑灌注不足模型中，EP能改善认知功能障碍，抑制小胶质细胞活化，减少促炎细胞因子的表达。本研究结果表明：与手术组相比，EP治疗组小鼠海马的HMGB1、RAGE、NF-κBp65、GFAP、Iba1蛋白表达和IL-1β含量降低，表明EP可以改善老龄小鼠术后海马胶质细胞的激活和炎症反应，推测其一方面可能通过减少HMGB1的表达，改善认知功能；另一方面可能通过HMGB1/RAGE-NF-κB信号通路，降低炎症因子的表达，从而改善患者的认知功能障碍。

综上所述，在术后3 d，EP减轻了老年小鼠海马神经炎症，改善PND，其分子机制可能与抑制HMGB1的分泌和HMGB1/RAGE-NF-κB信号通路有关。因此，EP可能是有效的潜在治疗PND的药物。

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