

甘草次酸干预对 X 射线照射后小胶质细胞生物学行为的影响

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【摘要】 **目的** 通过甘草次酸(GA)干预 X 射线照射后的小胶质细胞,探究 GA 对小胶质细胞生物学行为的影响及其机制。**方法** 体外培养小胶质细胞 BV2 细胞系,分别给予细胞不同的干预措施:甘草次酸 1 $\mu\text{g}/\text{mL}$ + 射线照射(GA 1 $\mu\text{g}/\text{mL}$ +IR 组)、甘草次酸 10 $\mu\text{g}/\text{mL}$ + 射线照射(GA 10 $\mu\text{g}/\text{mL}$ +IR 组)、空白对照+射线照射(Cont+IR 组)、溶剂对照组+射线照射(DMSO+IR 组)、地塞米松 10 nmol/L+射线照射(Dex 10nmol/L+IR 组)。照射方法:药物预处理 3 h 后,以 6 MV X 射线单次剂量 4 Gy 照射 BV2 细胞,6 h 后检测 BV2 细胞的 ROS 含量、IL-1 β 分泌情况及细胞中 Caspase-1、HO-1 的表达的变化情况。**结果** CCK8 实验结果显示,BV2 细胞经不同浓度 GA 干预后,GA 浓度为 10 $\mu\text{g}/\text{mL}$ 时细胞存活率最高达 73.876%,1 $\mu\text{g}/\text{mL}$ 时次高为 67.226%。流式细胞术结果显示,4 Gy 照射后,GA 组(1 $\mu\text{g}/\text{mL}$)的 ROS 值较 DMSO 组明显上调(14.567% vs 7.167%, $P=0.025$),而与 Dex(10 nM)组相比(14.567% vs 12.900%, $P=0.995$)未见明显改变。ELISA 检测结果表明,GA+IR 组(GA 10 $\mu\text{g}/\text{mL}$ 组)IL-1 β 的水平为照射后 4 组中最低值(0.156),但与 Cont+IR 组(0.156 vs 0.212, $P=0.131$)、DMSO+IR 组(0.156 vs 0.176, $P=0.999$)、Dex(10 nM)组(0.156 vs 0.184, $P=0.961$)相比差异无统计学意义。Western blot 检测结果显示 GA+IR 组的 Caspase-1 相对蛋白水平较 Cont+IR 组(0.147 vs 0.243, $P=0.000$)、DMSO+IR 组(0.147 vs 0.590, $P=0.000$)、Dex+IR 组(0.147 vs 0.565, $P=0.000$)均明显下调,而 GA+IR 组 HO-1 相对蛋白水平(0.537)较其他照射组呈上调趋势($P=0.000$)。**结论** GA 下调小胶质细胞接受 X 射线照射后 Caspase-1 蛋白的表达,上调 HO-1 蛋白水平,表明 GA 能够抑制细胞凋亡,增加脑内小胶质细胞的抗氧化能力。GA 可能在放射性脑损伤过程具有一定的神经保护作用,为临床治疗放射性脑损伤提供可能的治疗机制。

【关键词】 小胶质细胞;甘草次酸;X 射线;放射性脑损伤;生物学行为

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Effect of glycyrrhetic acid intervention on the biological behavior of microglia after X-ray irradiation

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【Abstract】 Objective To investigate the effect of glycyrrhetic acid (GA) on the biological behavior of

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microglia after X-ray irradiation and its mechanism. **Methods** Microglia BV2 cell was cultured in vitro and treated with different intervention groups: Glycyrrhetic acid 1 $\mu\text{g}/\text{mL}$ +irradiation group (GA 1 $\mu\text{g}/\text{mL}$ +IR group), Glycyrrhetic acid 10 $\mu\text{g}/\text{mL}$ +irradiation group (GA 10 $\mu\text{g}/\text{mL}$ +IR group), Blank control+radiation group (cont+IR group), Solvent control group+radiation group (DMSO+IR group), Dexamethasone 10 nmol / L+radiation group (Dex10nmol / L+IR group). Irradiation method: BV2 cells were irradiated with a dose of 4 Gy 6MV X-ray after 3 hours of drug pretreatment, six hours later, we detected the content of ROS, secretion of IL-1 β , expression of caspase-1 and HO-1 in BV2 cells. **Results** After CCK8 results showed that the survival rate of BV2 cells was 73.876% when GA concentration was 10 $\mu\text{g}/\text{mL}$, and 67.226% when GA concentration was 1 $\mu\text{g}/\text{mL}$. Flow cytometry results showed that ROS content in GA group (1 $\mu\text{g}/\text{mL}$) was significantly up-regulated compared with DMSO group (14.567% vs 7.167%, $P=0.025$) after 4 Gy irradiation, but no significant differences was observed compared with Dex group (10 nM) (14.567% vs 12.900%, $P=0.995$). ELISA results showed that the il-1 β level in GA+IR group (GA 10 $\mu\text{g}/\text{mL}$ group) was the lowest (0.156) among the four groups. There was no significant difference compared with Cont+IR group (0.156 vs 0.212, $P=0.131$), DMSO+IR group (0.156 vs 0.176, $P=0.999$) and Dex (10 nM) group (0.156 vs 0.184, $P=0.961$). Western blot analysis showed that the relative protein level of Caspase-1 in GA+IR group was higher than that in Cont+IR group (0.147 vs 0.243, $P=0.000$), DMSO+IR group (0.147 vs 0.590, $P=0.000$), Dex+IR (0.147 vs 0.565, $P=0.000$), while the relative protein level of HO-1 in GA+IR group (0.537) was up-regulated compared with other irradiation groups ($P=0.000$). **Conclusion** GA can reduce the expression of Caspase-1 protein and raise the level of HO-1 protein in microglia after X-ray irradiation, which indicated that GA can inhibit apoptosis and increase the antioxidant capacity of microglia in brain. GA may have a certain neuroprotective effect in the process of radiation-induced brain injury, and provide a possible therapeutic mechanism for clinical treatment of radiation-induced brain injury.

[Key words] Microglia; Glycyrrhetic acid; X-ray; Radiation-induced brain injury; Biological behavior

放射性脑损伤 (radiation-induced brain injury, RBI) 是颅脑、头颈部肿瘤放射治疗后的常见并发症, 对脑肿瘤进行放射治疗时, 周围正常脑组织不可避免地暴露于辐照区, 进而引起放射性脑损伤^[1-2]。随着肿瘤综合治疗的广泛应用及影像诊断技术的日益发展^[3-4], RBI 的发生率呈逐步上升趋势^[5]。值得注意的是, 临床提高放疗剂量有利于改善预后, 但放射性脑坏死等放疗不良反应制约着放疗的应用, 影响患者的治疗效果^[6-7]。放射性脑病一旦出现难以逆转, 在严重的情况下多影响到患者的生活质量和生存期^[8-9]。然而, RBI 的确切机制尚未明确, 临床上尚缺乏有效的防治方法^[5]。

放射性脑损伤过程中伴随多种免疫细胞、免疫因子的相互作用^[10-11]。小胶质细胞是中枢神经系统中主要的免疫细胞, 与脑内炎症密切相关。放疗的过程可以激活大脑内小胶质细胞, 诱导脑组织内的神经炎症反应, 引起脑组织损伤, 进而产生不可逆的神经功能损害, 因此, 小胶质细胞在放射性脑损伤的发生发展过程中占据重要地位^[12-13]。

甘草主要有效成分包括甘草酸、甘草次酸、甘草苷等, 其中甘草酸含量最高, 甘草酸可在肠道进一步水解脱去糖酸键形成的功能性产物甘草次酸 (lycyrrhetic acid, GA) 而进入血液循环, 同时甘草次酸也作为评价甘草质量标准的物质之一, 其具有类固醇样抗炎作用及肾上腺皮质激素样药理作用, 且无严重不良反应^[14], 已有研究证实 GA 有助于放射

性肺炎^[14]等防治, 但 GA 是否有助于减轻放射性脑病, 目前尚未有研究报道。鉴于以上因素, 本研究通过体外培养小胶质细胞 BV2 细胞系, 使用电离辐射刺激细胞, 构建体外 RBI 模型, 并使用 GA 干预细胞, 探索 GA 在小胶质细胞 RBI 过程中的影响作用, 为深入研究 GA 在放射性脑损伤中的作用机制提供可能的理论依据。

1 材料与方法

1.1 细胞与主要试剂 小鼠小胶质细胞 BV2 细胞, 购自武汉普诺赛生命科技有限公司。甘草次酸, 购自购自德国 Sigma 公司。小鼠白细胞介素-1 β (IL-1 β) 购自中国欣博盛公司; ROS 活性氧检测试剂盒购于中国南京凯基公司; Anti-Heme Oxygenase 1 抗体、Anti-Caspase-1 抗体、重组 Anti-NLRP3 抗体均购自英国 Abcam 公司; HO-1 Antibody-Internal、Caspase 1 Antibody-C-terminal、NLRP3 抗体均购自美国 Affinity 公司。

1.2 方法

1.2.1 细胞分组与照射: 将培养基血清浓度调整为 2%, 将 BV2 细胞分为 4 个组别: ①空白对照组; ②二甲基亚砜 (Dimethyl sulfoxide, DMSO) (溶剂) 对照组; ③GA 干预 (1 $\mu\text{g}/\text{mL}$) 组; ④地塞米松干预 (10 nmol/L) 组。照射方法: 药物预处理 3 h 后, 使用 4 Gy 6 MV X 线单次电离辐射各组细胞 (剂量率 500 MU/min), 6 h 后检测各项指标的变化。

1.2.2 CCK8法测定细胞存活率:将BV2细胞铺板至96孔板中(5 000 cells/well),采用高糖DMEM完全培养基梯度稀释甘草次酸至工作浓度为100 μg/mL、50 μg/mL、10 μg/mL、1 μg/mL、0.1 μg/mL、0.01 μg/mL,使用甘草次酸干预48 h后,去除原培养液,PBS冲洗3遍,加入PBS 100 μL,向每孔加入10 μL CCK-8溶液。将培养板放置培养箱内孵育6 h后,应用酶联免疫检测仪,在450 nm双波长处测得相应吸光度。

1.2.3 流式细胞术检测ROS表达水平:将BV2细胞设置为:空白对照组、DMSO(溶剂)对照组、地塞米松干预组(10 nM)、GA干预组(1 μg/mL)、GA干预组(10 μg/mL)、DMSO+IR干预组、地塞米松组(10 nM)+IR干预组、GA(1 μg/mL)+IR干预组、GA(10 μg/mL)+IR干预组;上述药物预处理3 h后采用4 Gy X线照射处理(剂量率500 MU,6 MV X射线),6 h后检测ROS水平。将不同分组的细胞弃去培养基,使用PBS清洗后添加胰酶消化,收集细胞置于EP管中,1 500 r/min离心3 min后,弃去上清,重悬。按体积比1:1将AM酯加入到各组细胞悬液中,37℃孵育60 min。去掉加载液,使用无血清的细胞培养液洗涤细胞2次,1 000 r/min离心5 min,弃上清,使用200 μL PBS使细胞悬浮。采用流式细胞仪484 nm的激发光检测ROS水平。

1.2.4 BCA法检测蛋白浓度:据分组培养细胞,48 h后吸取各组上清液,经1 000 r/min离心15 min后吸取上清于-80℃保存,通过碧云天BCA蛋白浓度测定试剂盒检测蛋白浓度。将标准品按0、1、2、4、8、12、16、20 μL加到96孔板的标准品孔中,加标准品稀释液补足到20 μL(相当于标准品浓度分别为0、0.025、0.05、0.1、0.2、0.3、0.4、0.5 mg/mL稀释),取各组20 μL待测蛋白质样品加入到微孔板中,加入200 μL BCA工作液混匀。37℃反应30 min后用酶标仪于562 nm波长处测定吸光度,重复测定3次。所得标准曲线见图1。

1.2.5 IL-1β的表达水平检测:按上述分组对细胞进行干预,各组细胞上清经BCA蛋白定量法调整各组蛋白量至一致,按照IL-1β ELISA试剂盒说明书进行操作,检测各组细胞上清中IL-1β的表达水平。

1.2.6 Westernblot检测各标记物水平:将BV2细胞分为:空白对照(未照射未给药)、溶剂对照组(DMSO)、甘草次酸干预组(1 μg/mL)、地塞米松干预组(10 nM)。4 Gy 6 MV X线(剂量率500 MU)单次照射照射组BV2细胞。干预72 h后,收集细胞,加入

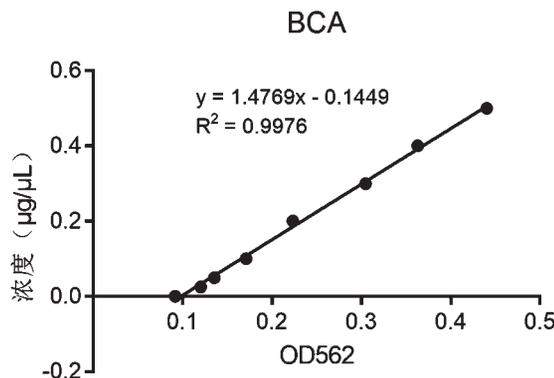


图1 BAC蛋白浓度曲线
Figure 1 BAC protein concentration curve

细胞裂解液裂解细胞,提取细胞蛋白样品并定量。取总蛋白与5×上样缓冲液按照4:1混合,变性后于8% SDS聚丙烯酰胺凝胶上电泳。经转膜,5%脱脂奶粉封闭,分别加一抗(NLRP3/β-Tubulin/HO-1/Caspase-1)4℃孵育过夜。经TBST充分洗涤,采用各抗体的同源二抗孵育,TBST洗涤,用化学发光试剂盒定影显影。

1.3 统计学处理 所有数据采用SPSS 20.0统计软件进行分析,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,然后使用Tukey法(Tukey's multiple comparisons test)进行多重比较。 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 GA干预对BV2细胞存活率的影响 BV2细胞置于不同浓度的GA处理72 h后,通过CCK-8检测细胞存活率,发现BV2细胞经GA干预后细胞存活率降低,但不同浓度GA干预的BV2细胞存活率改变幅度并不显著($P > 0.05$),10 μg/mL时细胞存活率最高为73.876%,1 μg/mL时次高67.226%,100 μg/mL时达最低点55.780%。考虑到过大剂量GA细胞毒性较大可影响细胞正常存活,因此使用1 μg/mL、10 μg/mL作为GA干预剂量(图2)。

2.2 GA干预对GY射线照射后BV2细胞内ROS含量的影响 流式细胞术结果显示,BV2细胞照射0 Gy时,甘草次酸干预组(1 μg/mL)的ROS含量较空白对照组(94.900% vs 41.650%, $P = 0.000$)、溶剂对照组(DMSO)(94.900% vs 21.700%, $P = 0.000$)、GA组(10 μg/mL)(94.900% vs 78.400%, $P = 0.000$)明显上调,但与地塞米松组(10 nM)(94.900% vs 93.533%, $P = 0.999$)相比差异无统计学意义。经4 Gy射线照射后,GA组(1 μg/mL)的ROS含量较溶剂对照组

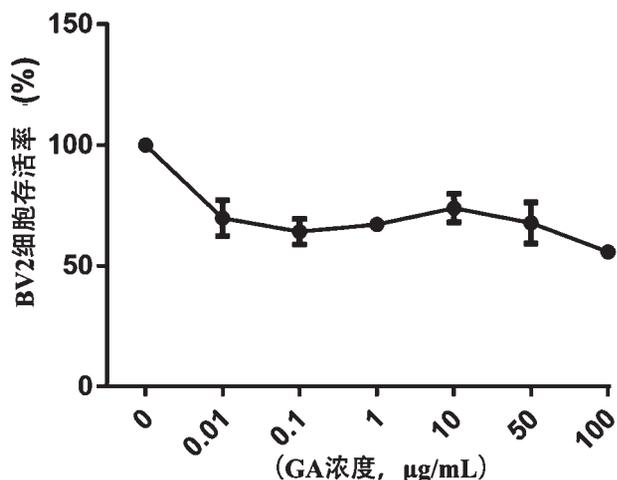


图2 CCK-8检测BV2细胞存活率

Figure 2 Survival rate of BV2 cells detected by CCK8

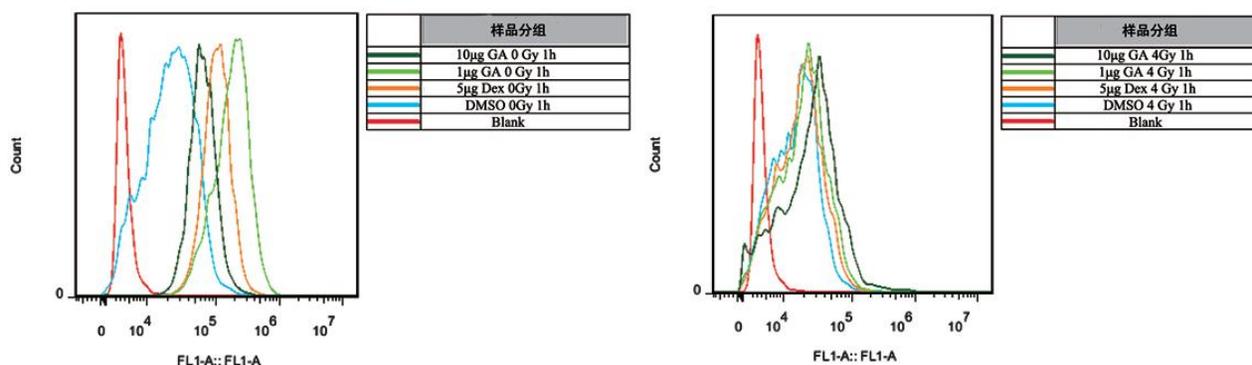


图3 甘草次酸干预后ROS的变化

Figure 3 ROS changes after GA intervention

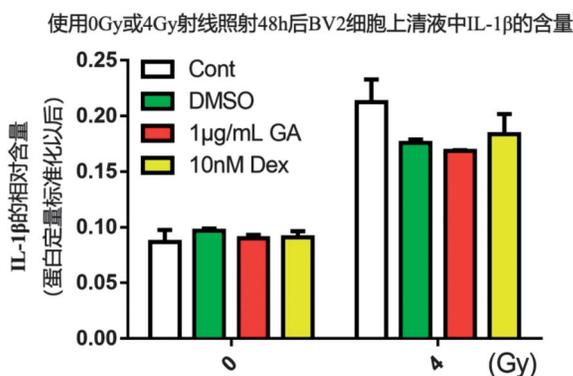


图4 甘草次酸干预后IL-1β水平的变化

Figure 4 Changes of IL-1β levels after GA intervention

2.4 GA 干预对GY 射线照射后BV2 细胞内HO-1、Caspase-1 蛋白水平的影响 Western blot 结果显示, 给予4 Gy 照射后,GA+IR 组的Caspase-1 相对蛋白水平较Cont+IR 组(0.147 vs.0.243, $P=0.000$)、DMSO+IR 组(0.147 vs.0.590, $P=0.000$)、Dex+IR(0.147 vs.0.565, $P=0.000$)组均明显下调,差异均有统计学意义。然而给予4 Gy 照射后各组的HO-1 相对蛋白表达水平

(DMSO)(14.567 % vs.7.167%, $P=0.025$)同样明显上调,而与地塞米松组(10 nM)(14.567 % vs 12.900%, $P=0.995$)相比,ROS 含量差异无统计学意义(图3)。

2.3 GA 干预对GY 射线照射后BV2 细胞内IL-1β 水平的影响 经BCA 定量调整蛋白浓度一致后,ELISA 结果显示:经4 Gy 射线照射后,Cont 对照组(4 Gy)较未照射Cont 对照组(0 Gy)IL-1β 的水平明显升高(0.212 vs 0.087, $P=0.000$)。在甘草次酸(1 μg/mL)的干预下,GA 组(4 Gy)的IL-1β 的水平为4 组中最低含量(0.156)。但与空白对照组(0.156 vs.0.212, $P=0.131$)、溶剂对照组(DMSO)(0.156 vs.0.176, $P=0.999$)、地塞米松干预组(10 nM)(0.156 vs.0.184, $P=0.961$)相比,各组间IL-1β 的含量差异均无统计学意义。见图4。

呈上调趋势,且GA + IR 组与Cont + IR 组(0.537 vs.0.313, $P=0.000$)、DMSO+IR 组(0.537 vs.0.113, $P=0.000$)、Dex+IR 组(0.537 vs.0.047, $P=0.000$)相比,差异均有统计学意义(图5)。

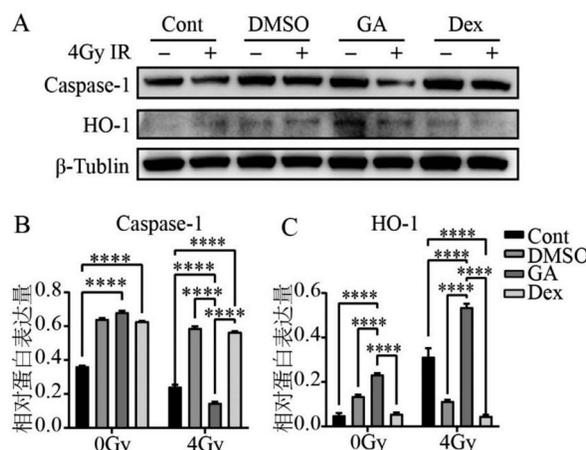


图5 甘草次酸干预后Caspase-1、HO-1 的变化(*** $P<0.001$ vs Cont)

Figure 5 Changes of Caspase-1 and Ho-1 after GA intervention (*** $P<0.001$ vs Cont)

3 讨论

RBI 的发生机制错综复杂,目前尚未明确其发生的确切机制^[15-16]。传统的治疗方法如手术、高压氧^[17]、血管内皮生长因子^[18]、糖皮质激素等只能缓解症状^[19],无法逆转放射性脑损伤,存在一定的局限性^[20-23]。因此,对于 RBI 治疗药物的研究具有重要的实用价值。小胶质细胞是大脑中重要的内源性免疫细胞,在中枢神经系统的损伤过程中^[24-25],包括放射性损伤的病理过程中起着重要作用^[26]。甘草次酸已证实在放射性肺炎等损伤中体现出抗炎、抗氧化等多重作用。本研究中探索了 BV2 小胶质细胞经 X 线照射后,通过使用甘草次酸的干预,检测细胞生物学行为的变化,结果显示 GA 10 μg/mL +IR 组相较于 Cont+IR 组、DMSO+IR 组、IR+DMSO 组、Dex+IR 组明显下调了 Caspase-1 蛋白的含量,增加了 HO-1 蛋白的水平,表明 GA 在脑损伤的过程中具有抗小胶质细胞凋亡,并增加其抗氧化的能力,具有一定的神经保护作用。

本研究采用电离辐射 X 射线照射小胶质细胞,模拟体外脑损伤模型。电离辐射可以激活体内的小胶质细胞,产生脑组织炎症及脑功能损伤,导致认知功能障碍^[27-28]。小胶质细胞是介导神经炎症的关键细胞^[29-30],研究表明 BV2 细胞受到电离辐射时,能刺激炎症反应的产生,包括 ROS^[31]、NLRP3^[32]、IL-1β^[33]等,破坏了体内氧化-抗氧化平衡,引发下游的炎症级联反应,对正常细胞及组织造成损伤并引脑内神经细胞凋亡及神经炎症反应^[34-35]。MISSIRY 等^[36]研究同样发现射线照射后可激活小细胞质胞,引起 IL-1β、TNF-α 增加,ROS 的生成增加。因此,小胶质细胞激活可能是引发放射性脑损伤的关键因素,通过抑制大脑内小胶质细胞的炎症反应尤为重要。本研究以 BV2 细胞为研究对象,接受 4 Gy X 射线照射后,GA+IR 组与 Dex+IR 组相比较,ROS、IL-1β 等指标呈现下调趋势,说明 GA 在 BV2 细胞受到电离辐射刺激时发挥了一定的抗氧化、抗炎的作用,可能具有一定的神经保护作用,但组间比较差异无统计学意义。这可能提示 ROS、IL-1β 可能是下游的指标,GA 可能通过影响其他上游的炎症指标发挥其抗炎、抗氧化的作用。

因此,本文进一步使用 Western blot 检测了其他炎症相关的指标。本研究发现了 GA+IR 组明显下调了 Caspase-1 的含量,Caspase 与真核细胞凋亡密切相关,并参与细胞的生长、分化与凋亡调节^[37, 38],与此同时,还发现 GA 的干预导致 BV2 细胞内 HO-1 蛋白的表达水平上调,HO-1 是一种抗炎、抗氧化和具有

神经保护作用的诱导酶^[39-40],这说明 GA 在减轻脑内胶质细胞凋亡,减轻神经炎症的同时,增加了细胞的抗氧化能力,发挥一定的神经保护作用。同时,本文也存在以下不足之处:(1)本文发现 GA 的神经保护作用可能是通过降低凋亡信号通路发挥作用,但未深入研究其确切的机制,后续应进一步深入研究;(2)本文表明 GA 具有神经保护作用,但是未确定是 GA 中的哪种化合物发挥的作用,后续可通过生物信息学、网络药理学等其他手段进一步分析其确切的组分。

GA 在放射性脑损伤过程发挥了抗凋亡、抗炎、抗氧化的作用,GA 有可能成为临床上治疗放射性脑损伤的潜在药物之一。

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