

瓜氨酸对辐射致小鼠肠道损伤的防护作用 及机制研究

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摘要 电离辐射会导致动物肠道的损伤,瓜氨酸在小肠上皮细胞内合成,同时被证明对肠道具有一定的保护作用。通过构建小鼠急性辐射致肠道损伤模型,设置正常对照组、单纯辐射组以及辐射加瓜氨酸组,研究瓜氨酸的辐射防护作用及机制。实验结果表明:在辐射后连续一周腹腔注射 $1\text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ 的瓜氨酸能够显著延长受照小鼠中位生存期,增加小鼠体重,显著降低小鼠血浆内毒素含量,提高黏着斑激酶(Focal Adhesion Kinase, FAK)和肠道紧密连接(Occludin)蛋白的表达水平,同时降低一氧化氮(Nitric Oxide, NO)和诱导型一氧化氮合酶(inducible Nitric Oxide Synthase, iNOS)的表达量。本实验证明:瓜氨酸可以通过改善受照小鼠肠道屏障完整性,改善肠道屏障功能,缓解亚硝化应激,对电离辐射小鼠肠道损伤具有保护作用。

关键词 电离辐射, 肠道损伤, 瓜氨酸, 一氧化氮

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Protective effect and mechanism of citrulline on intestinal injury induced by radiation in mice

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Abstract [Background] Ionizing radiation can cause damage to animal's intestinal tissue. Citrulline is produced in the intestinal epithelial cell and has been proven to possess a protective effect on the gastrointestinal tract. [Purpose] This study aims to investigate the protective effects and the underlying mechanisms of citrulline in the context of radiation-induced intestinal injuries. [Methods] Firstly, a mouse model of an acute radiation-induced intestinal injury was established, incorporating a normal control, a simple irradiation, and an irradiation plus citrulline group. Then, these groups were employed to scrutinize the protective effects and mechanisms associated with citrulline. Subsequently, hematoxylin-eosin staining was used to examine the morphology of the mice's intestinal tissue, and the Elisa kit was employed to quantify endotoxin levels in plasma, as well as nitric oxide and inducible nitric oxide synthase in the intestinal tissue. Finally, focal adhesion kinase and Occludin levels in the intestinal tissue were assessed using western blotting. [Results] The experimental results demonstrate that intraperitoneal injection of $1\text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ citrulline for one week following irradiation significantly extend the median survival time of irradiated

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mice and increase their body weight. Moreover, it markedly reduces plasma endotoxin levels, elevate the expression levels of focal adhesion kinase (FAK) and intestinal tight junction protein (Occludin), and decreases the expression levels of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) in the intestinal tissue. **[Conclusions]** Citrulline enhances the integrity of the intestinal barrier in irradiated mice, improves barrier function, mitigates nitrosative stress, and demonstrates a protective impact on radiation-induced intestinal damage in mice.

Key words Ionizing radiation, Intestinal injury, Citrulline, Nitric oxide

现如今, 电离辐射在多个领域展现出良好的发展与应用前景, 尤其是医疗领域^[1]。伴随着电离辐射的发展, 研究发现其对多个领域的从业人员都可能存在潜在危害^[2-3], 电离辐射对人体的不同组织都会产生损伤, 包括生殖器官损伤^[4]、脾脏损伤^[5]、胃肠道损伤等。由于胃肠道对电离辐射的敏感性, 其导致了复杂的病理过程, 如吸收不良、细菌性肠炎、腹泻等, 阻碍治疗进展, 甚至导致死亡^[6], 因此, 辐射防护尤为重要。在我们早期研究中发现, 电离辐射损伤引起的敏感代谢物有瓜氨酸(Citrulline, Cit)、肌酸、脯氨酸和尿酸。瓜氨酸为 α 氨基酸, 主要产生于肠道。由于瓜氨酸与肠道有较为紧密的相关性, 血浆瓜氨酸水平与X射线诱导小肠上皮细胞损伤和小肠形态具有密切联系, 其可能作为评估电离辐射致小肠损伤最有前途的生物标志物之一, 具有较好的应用前景^[7-8]。瓜氨酸对于急性肠损伤状态下的小鼠肠黏膜上皮细胞形态及功能有促进作用^[9], 补充外源性瓜氨酸对于溃疡性结肠炎的肠道炎症损伤具有保护作用^[10], 也可以抑制脓毒症早期促炎因子的释放, 减轻心、肝、肾等机体重要脏器的炎症反应程度^[11-12], 减轻肠黏膜组织炎症反应, 降低受损肠黏膜的通透性, 同时口服瓜氨酸可以保护肠道黏膜的破坏和肠道屏障功能^[13]。因此, 瓜氨酸可能是一种很有潜力针对辐射致肠道损伤的防护药物。

瓜氨酸是精氨酸(Arginine, ARG)的代谢产物^[14]。其作为一种有机补充剂, 在恢复败血症危重患者精氨酸代谢方面具有治疗潜力^[15-16], 补充L-瓜氨酸可通过促进肠道形态发育和氨基酸转运而产生节约精氨酸的效果^[17], 并且口服瓜氨酸不会为胃肠道带来更多负担^[18]。此外, 长期给用瓜氨酸治疗尿素循环缺陷患者没有任何副作用^[19]。口服瓜氨酸在改善非酒精性脂肪肝、肠道疾病、心血管疾病等方面都有一定的作用^[20-21]。一氧化氮(Nitric Oxide, NO)和诱导型一氧化氮合酶(inducible Nitric Oxide Synthase, iNOS)在瓜氨酸代谢循环中起到了重要作用, 黏着斑激酶(Focal Adhesion Kinase, FAK)为肠细胞迁移的信号分子, 可以促进肠道黏膜修复, Occludin是一种肠道紧密连接蛋白, 可以维持肠道屏障功能, 我们通过上述指标来论述瓜氨酸对肠道

的保护作用。

本文拟利用 γ 射线对小鼠进行全身照射, 构建急性辐射致肠道损伤动物模型, 并通过补充外源性瓜氨酸, 研究瓜氨酸对辐射致肠道损伤的影响, 探讨瓜氨酸对辐射致肠道损伤治疗的机制。

1 材料与方法

1.1 实验材料与仪器

实验动物: ICR小鼠(Institute of Cancer Research Mouse)起源于美国的癌症研究机构, 本研究中ICR小鼠(SPF级)购自南京市江宁区青龙山动物养殖场(江苏南京)。许可证号为: SCXK(豫)2020-0001。体重约为20 g, 周龄在8~10周, 动物室笼中饲养1周, 动物室的条件为: 温度23℃左右、湿度55%左右、明暗周期12 h。实验符合伦理要求, 在保证实验能够正常进行的条件下, 尽量减少实验小鼠的使用数量以及实验过程中尽量减轻其痛苦。

材料与试剂: L-瓜氨酸(纯度>99%)购买于上海Macklin公司; NO测定试剂盒、iNOS分型试剂盒均购自南京建成公司; β -actin一抗、山羊抗兔二抗、兔抗小鼠Occludin多克隆抗体、兔抗小鼠FAK多克隆抗体均购自英国Abcam公司。

主要仪器与设备: γ 射线采用南京航空航天大学辐照中心⁶⁰Co源, 辐射源活度为 6.1124×10^{15} Bq, 剂量场采用重铬酸银剂量计(量程0.5~5 kGy)测量, 剂量率为 $1.06 \text{ Gy} \cdot \text{min}^{-1}$ 。

1.2 实验方法

1.2.1 实验动物与分组

设置空白对照组、单纯辐照组(全身照射, Total Body Irradiation, TBI)、辐照+瓜氨酸组, 每组15只。瓜氨酸腹腔注射0.15 mL, 注射剂量为 $1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ^[22], 辐照后每天注射一次, 持续一周。对照组给予生理盐水, 腹腔注射0.15 mL, 每天一次, 其他环境均相同。辐射剂量为8 Gy、10 Gy和12 Gy, 全身照射。记录各组小鼠体重、毛发、粪便、生存状态和生存期, 于辐照后第8天取材, 检测小鼠肠道组织形态学、血

浆内毒素、肠道 NO 与 iNOS、肠道组织黏着斑激酶和肠道紧密连接蛋白水平开展机制研究。

1.2.2 苏木精-伊红染色 (Hematoxylin-Eosin staining, HE) 观察肠道组织

在全身辐照后的第 8 天对小鼠取材,将取得的小肠组织浸泡至磷酸缓冲盐溶液(Phosphate Buffer Saline, PBS)溶液中。为保存小肠组织的形态,将小鼠小肠固定在 10% 的中性福尔马林溶液中,随后在浓度为 10% 的乙二胺三乙酸微波脱钙,并进行石蜡包埋、连续切片展片的操作。最后将石蜡切片染色晾干,镜下观察。

1.2.3 血浆中内毒素(Lipopolysaccharide, LPS)检测

眼球取血,迅速于 $3\,000\text{ r}\cdot\text{min}^{-1}$ 的离心机中离心 2 min,吸出富含血小板的血浆上清液,置于 $2\sim 8\text{ }^{\circ}\text{C}$ 备用。将血浆和样本处理液按照 1:9 混匀,制成 10 倍稀释液备用。将 10 倍稀释液于 $70\text{ }^{\circ}\text{C}$ 水浴锅中加热 10 min,然后用冰水冷却 3 min。血浆内毒素试剂盒具体步骤如下:配置细菌内毒素标准溶液,溶解试剂盒中的试剂,将上述稀释液和空白对照组(内毒素检查用水)按照试剂盒中的步骤添加混匀和温育,最后静置 5 min,在酶标仪 545 nm 处读取吸光度。

1.2.4 肠道组织 NO、iNOS 检测

小鼠麻醉后颈椎脱臼法致死,剪开腹部取小鼠肠道组织一段,用冷生理盐水冲洗掉肠道内容物和表面血液,按照重量:体积=1:9 加入预冷生理盐水后低温研磨,制成 10% 的肠道组织匀浆,离心,按南京建成生物工程研究所提供的试剂盒说明书操作。

1.2.5 免疫印迹法检测 FAK 和 Occludin

用 PBS 溶液冲洗肠道组织,裂解,提取蛋白。使用聚羧基丙烯酸正丁酯(Bicinchoninic Acid, BCA)蛋白试剂盒测定溶液蛋白浓度。蛋白质在 200 V 下分离,在 100 V 下电泳后转移到聚偏二氟乙烯膜(Polyvinylidene Fluoride, PVDF)上。用含 0.4% Tween20 的 Tris-HCl 缓冲盐溶液(Tris Buffered Saline, TBST)和 5% 脱脂牛奶在室温下封闭膜,分别加入 FAK 和 Occludin 的一抗工作液,后加入用 TBST 按照 1:5 000 的比例进行稀释的二抗。最后用化学发光底物(Enhanced Chemiluminescence, ECL)试剂盒对整张膜进行显影定型。用 ImageJ 程序对实验结果进行数据量化。

1.3 数据统计与分析

各组数据采用平均数 $\pm$ 标准差(Mean $\pm$ SD)表示,使用 GraphPad Prism 9.0 软件(GraphPad, San Diego, CA, United States)。采用单因素方差分析

(One-way variance, ANOVA)和 Tukey 的多重比较检验比较两组间的差异。 $p<0.05$ 视为统计学上有显著性差异。

2 结果与分析

2.1 小鼠体重和生存时间变化

对照组小鼠行为敏捷,反应迅速,饮食正常,排便正常。受照射小鼠出现精神萎靡,毛发凌乱,排便较稀。10 Gy 辐照生存时间如图 1 所示(蓝色箭头表示 10 Gy 组中位生存期,红色箭头表示 10 Gy+Cit 组的中位生存期),瓜氨酸给药组的中位生存期为 13 d,与单纯辐照组相比,中位生存期延长 3 d($p<0.05$)(图 1)。8 Gy 与 12 Gy 辐照小鼠体重如图 2 所示(*表示 $p<0.05$, **表示 $p<0.001$),辐照后小鼠体重显著降低,与单纯辐照组相比,腹腔注射瓜氨酸能够显著增加辐照小鼠的体重。

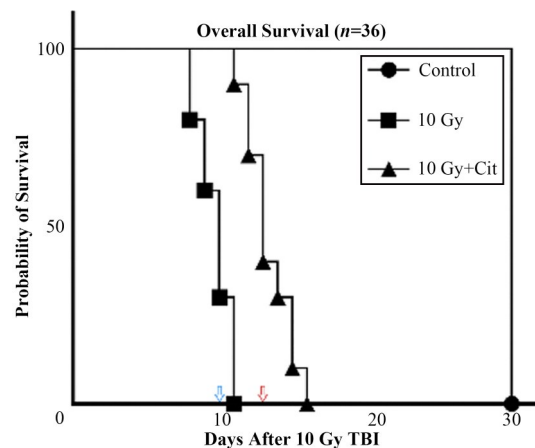


图 1 瓜氨酸对辐照小鼠生存时间的影响(彩图见网络版)
Fig.1 The effect of citrulline on the survival time of irradiated mice (color online)

2.2 肠道组织形态学变化与血浆内毒素含量

对照组肠道光滑无黏连、肠管红润有光泽,腹腔中无积液,肉眼可见肠壁完整无出血点;单纯辐照组小鼠的肠道颜色偏深紫色,出现肠黏连,腹腔中有积液,肉眼可见部分肠管狭窄,肠壁出现明显的出血点,且肠道内容物呈水样便;而瓜氨酸给药组的肠道颜色较辐照组紫色较浅,肠壁出血点明显减少。HE 染色的结果如图 3 所示,8 Gy 辐照后肠黏膜上皮细胞出现变性,无明显坏死,无溃疡形成,12 Gy 辐照后损伤程度较 8 Gy 有较为明显的加重,出现炎细胞。给予瓜氨酸 8 d 后的 HE 染色结果发现,不同剂量辐照组均出现一定程度的损伤修复。

血浆中内毒素的含量可以用来表征肠道屏障的通透性,血浆中内毒素的含量检测结果如图 4 所示

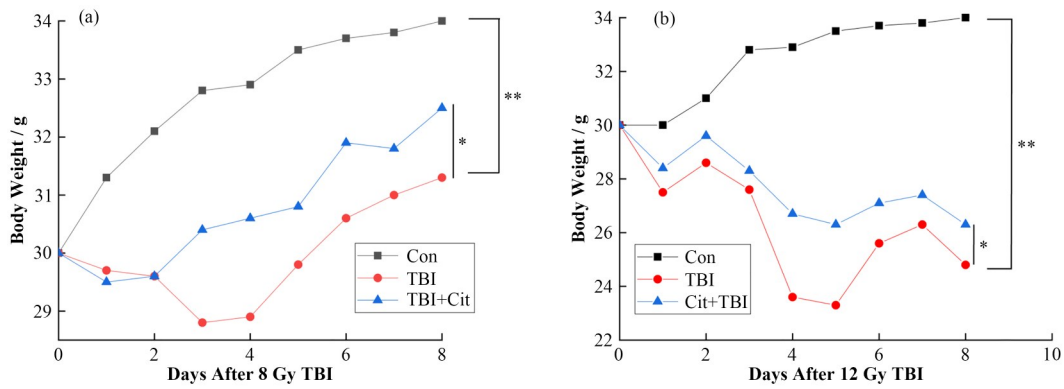


图2 瓜氨酸对辐照小鼠体重的影响
Fig.2 The effect of citrulline on the body weight of irradiated mice

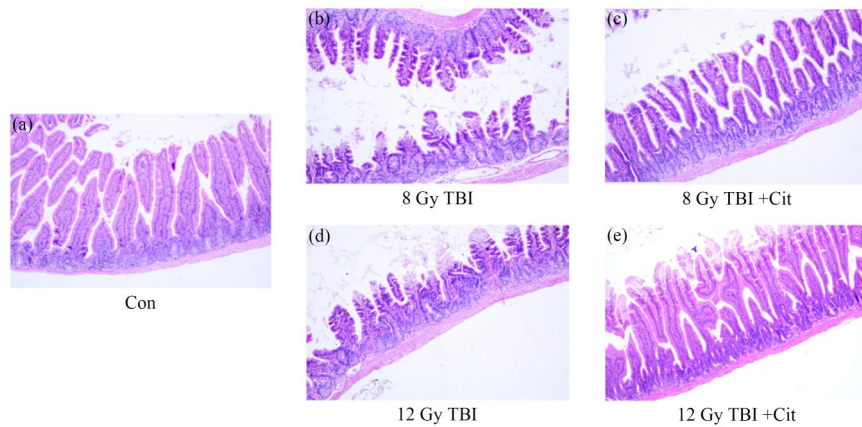


图3 辐照组小鼠和瓜氨酸给药组小鼠的肠道组织HE染色($\times 200$)
Fig.3 HE staining of the intestinal tissue from irradiated mice and citrulline-treated mice ($\times 200$)

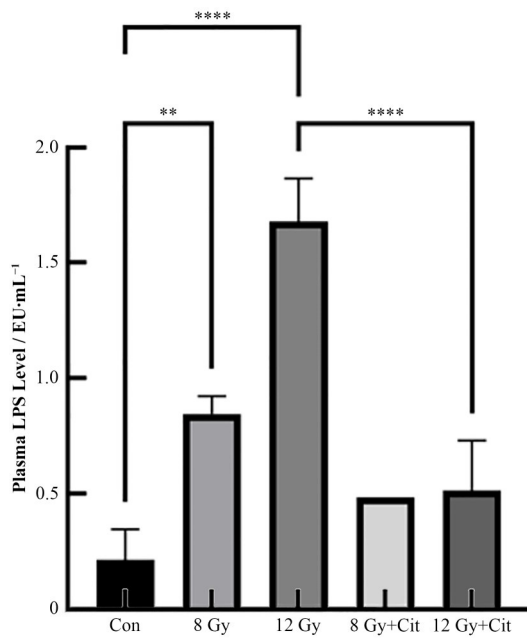


图4 辐照小鼠和腹腔注射瓜氨酸给药组小鼠血浆中内毒素含量
Fig.4 Endotoxin content in the plasma of irradiated mice and the mice treated with intraperitoneal injection of citrulline

(**表示 $p < 0.01$, ****表示 $p < 0.0001$), 结果表明辐照组内毒素含量与对照组相比显著升高($p < 0.05$), 腹腔注射瓜氨酸后能显著降低血浆中内毒素的含量($p < 0.05$), 达到与对照组相比无显著性差异($p > 0.05$), 这一结果提示, 瓜氨酸能够调节辐照小鼠肠道屏障的通透性。

2.3 肠道组织NO、iNOS、FAK、Occludin蛋白含量检测

检测了小鼠肠道中NO和iNOS的表达情况。结果如图5所示(**表示 $p < 0.001$, ****表示 $p < 0.0001$), 辐照后小鼠肠道的NO和iNOS的表达上升, 而瓜氨酸腹腔注射后显著降低了NO和iNOS的表达量, 提示补充瓜氨酸使代谢循环中iNOS依赖的NO合成正常化。

通过免疫印迹检测了FAK和Occludin在小鼠肠道组织的表达情况。结果如图6所示(**表示 $p < 0.01$, ***表示 $p < 0.001$, ****表示 $p < 0.0001$), 辐照后FAK表达量出现显著下降, 下降趋势有剂量依赖性, 而腹腔注射瓜氨酸后, 表达量出现显著的上升; 辐照

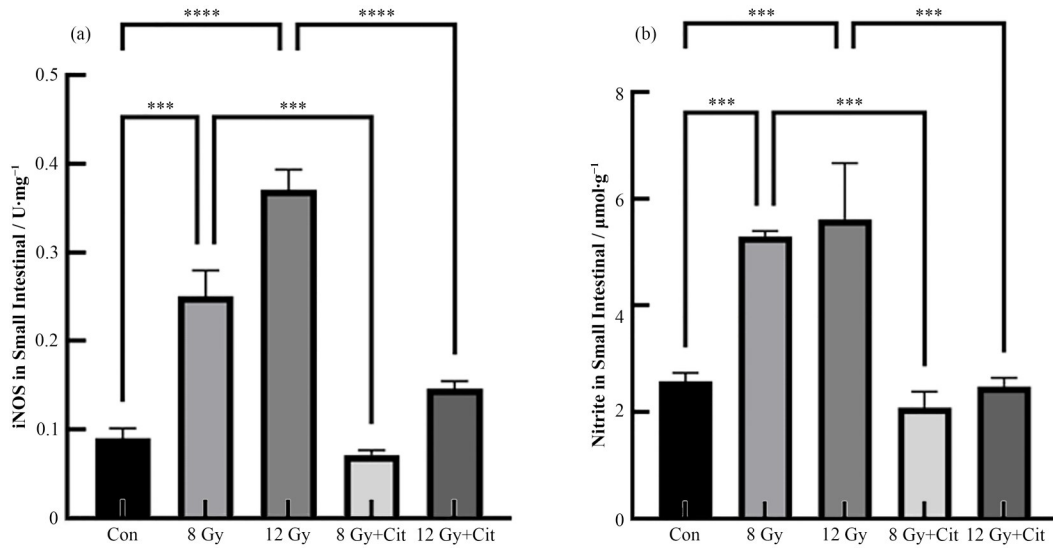


图5 辐照小鼠肠道组织中NO和iNOS的含量
Fig.5 The content of NO and iNOS in the intestinal tissue of irradiated mice

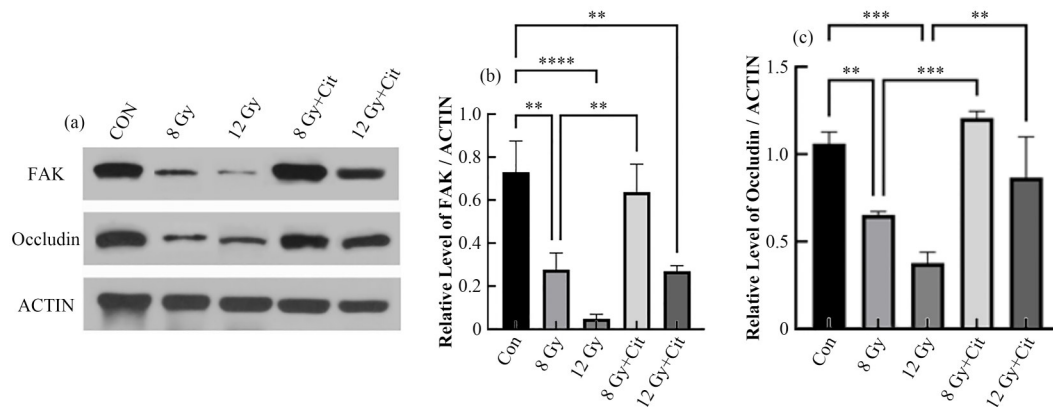


图6 辐照小鼠肠道组织FAK和Occludin表达量的变化
Fig.6 The changes of FAK and Occludin expression in the intestinal tissue of irradiated mice

后 Occludin 蛋白表达量显著下降,下降趋势随着辐照剂量增大而降低,而瓜氨酸注射后,表达量出现显著上升。实验结果提示,瓜氨酸对辐射损伤后肠黏膜屏障有修复和保护的作用。

3 讨论

本研究采用 γ 射线对小鼠进行全身照射,构建急性辐射致肠道损伤动物模型,补充外源性瓜氨酸,探讨瓜氨酸对辐射致肠道损伤的修复作用。结果显示,辐射加瓜氨酸组与对照组和单纯辐照组相比,瓜氨酸可以显著提高急性辐射损伤小鼠的存活时间,并且能够有效减缓辐照损伤带来的体重下降。Villareal等^[23]的一项研究表明,口服瓜氨酸15 d可以显著提高小鼠骨骼肌的重量,增强小鼠的运动能力。瓜氨酸导致体重增长的原因可能与瓜氨酸促进生长相关激素分泌以及瓜氨酸的生理代谢过程有关,瓜

氨酸能够促进肌肉蛋白质合成^[23-24],饲料中添加不同剂量的瓜氨酸能够显著增加肉鸡、黄颡鱼幼鱼、小鼠、湖羊母羊及肉兔的体重^[25-26]。此外,在结肠炎小鼠模型中,给予瓜氨酸能够显著提高小鼠的存活率^[27],这与我们的研究结果相近。

肠道损伤会导致肠道黏膜通透性升高,屏障功能障碍,最终使得肠腔内的革兰氏阴性菌及其产物(内毒素)易位入血^[28]。本实验发现,辐照后小鼠肠道受损,血浆中内毒素含量增高。瓜氨酸给药一周后,辐射致肠道损伤得到缓解,血浆内毒素含量下降。Sellmann等^[29]也报道了类似的结果,他们采用非酒精性脂肪肝模型,发现口服瓜氨酸可以保护肠道黏膜和肠道屏障功能。Batista等^[30]在细菌移位小鼠模型中也证明了瓜氨酸能够降低小鼠肠道黏膜损伤,且保存了更多的完整绒毛结构。Zoghroban等^[31]在兰氏贾第虫感染的小鼠模型中加入瓜氨酸发现肠

道刷状缘恢复,炎症浸润减少。5-氟尿嘧啶致肠黏膜炎模型的小鼠中,瓜氨酸保护小肠黏膜结构完整性,降低肠道通透^[32]。FAK为肠细胞迁移的信号分子,可以促进肠道黏膜修复;Occludin是一种肠道紧密连接蛋白,可以维持肠道屏障功能。在Ho等^[33]的研究中,富含L-瓜氨酸的发酵乳与瑞士乳杆菌可恢复右旋糖酐硫酸诱导的Occludin在内的肠道紧密连接蛋白水平下降。本实验结果显示,辐照后FAK、Occludin表达量显著下降,腹腔注射瓜氨酸后,表达量出现显著的上升,进一步提示瓜氨酸对辐射损伤后肠黏膜屏障具有修复作用。

一方面瓜氨酸参与精氨酸合成,另一方面精氨酸可以在iNOS的诱导下,生成瓜氨酸,同时伴有大量NO的生成。电离辐射损伤可通过自由基介导,此外有研究表明,电离辐射可以增强体内精氨酸参与的iNOS对NO的生物合成^[34-35]。NO能与氧自由基快速反应形成过氧亚硝酸根离子,这是一种强细胞毒性氧化剂,可以攻击DNA、脂质和蛋白质等成分,导致细胞功能受损同时增强炎症反应^[36]。已有研究提出,ROS是启动子,NO是响应电离辐射激活细胞信号转导途径的效应因子之一^[37]。基于瓜氨酸的代谢循环通路以及辐射损伤的特点,我们研究了瓜氨酸对iNOS和NO的表达量的影响。结果显示,电离辐射引起小鼠iNOS和NO表达大量升高,瓜氨酸能够显著降低iNOS和NO的表达,从而减弱过量NO介导的细胞毒性。有研究证实,瓜氨酸可以促进肝脏、大脑等器官iNOS和NO合成正常化^[38], Rajcic等^[39]证明给予非酒精性脂肪肝小鼠瓜氨酸,同样也能够使小鼠小肠中亚硝酸盐和iNOS的含量降低,这与我们的发现一致。提示瓜氨酸有望起到辐射防护的作用。

综上所述,在本实验的研究条件下,辐照后持续一周腹腔注射 $1\text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ 的瓜氨酸,能显著增加小鼠体重、存活率,降低血浆中内毒素、肠道中NO和iNOS的含量,提高FAK和Occludin的表达,提示我们瓜氨酸可能通过增强肠道屏障功能、代谢循环正常化和缓解亚硝化应激起到辐射损伤的防护作用,有望作为一种有效的有潜力针对辐射肠道损伤的治疗药物。

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