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弓形虫干扰小鼠肾脏铜代谢途径初探

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摘要 目的 探讨弓形虫感染对小鼠肾脏铜代谢的影响。方法 将80只7~8周龄C57BL/6雌性小鼠适应饲养1周后根据实验方案随机均分为4组:Control组、Cu组、TgCtwh6组和Cu+TgCtwh6组。Control组用普通正常饲料和水喂养;Cu组连续60 d使用含氯化铜1 g/kg加工饲料和0.1%氯化铜的水饲养;TgCtwh6组感染25~30个我国弓形虫Chinese 1优势基因型TgCtwh6虫株包囊,用正常饲料和水喂养;Cu+TgCtwh6组感染25~30个TgCtwh6包囊并且连续60 d使用含氯化铜1 g/kg加工饲料和0.1%氯化铜的水饲养。电感耦合等离子体-质谱法(ICP-MS)测定肾脏组织铜含量变化;采用苏木精-伊红(HE)染色观察小鼠肾脏组织病理变化;采用PI染色法观察凋亡细胞数量;采用Western blot检测谷胱甘肽过氧化物酶4(GPX4)和超氧化物歧化酶(SOD1, SOD2)蛋白表达水平;RT-qPCR法检测铜死亡相关基因mRNA表达。结果 镜下Cu组和TgCtwh6组可见肾间质炎性细胞浸润等病理表现,Cu+TgCtwh6组肾间质炎性浸润细胞减少,肾小球肾小管结构病理表现有所改善;Cu+TgCtwh6组凋亡细胞数(88.36±19)低于Cu组(119.0±20);和Cu组相比,Cu+TgCtwh6组SOD1蛋白表达下调,差异有统计学意义($P<0.05$);RT-qPCR结果显示TgCtwh6感染可逆转铜超载引起的肾脏谷氨酰胺酶(GLS)表达下调和ATP酶铜转运 β (ATP7B)表达上调。结论 弓形虫感染后可干扰小鼠肾脏内铜代谢途径,改善铜超载造成的肾脏损伤,为铜超载疾病的治疗提供新线索。

关键词 弓形虫;肾脏;铜代谢;蛋白质免疫印迹;凋亡;电感耦合等离子体-质谱法

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寄生虫感染可以打破宿主体内微量金属元素的代谢平衡。作为寄生于细胞内的原生动物的弓形虫需要摄取宿主体内的微量金属元素来维持自身生存。课题组前期研究^[1]表明,弓形虫慢性感染会导致小鼠肾脏组织中铜含量降低,说明弓形虫入侵后机体的铜代谢稳态被打破,并对宿主代谢造成一定影响。铜死亡(cuprotoxis)是一种不同于现在已知的所有细胞死亡机制的新机制,通过铜直接与三羧酸循环(tricarboxylic acid cycle, TCA cycle)中的脂酰化组分结合进而导致脂酰化蛋白聚集和随后的铁硫簇蛋白丢失,引发蛋白质毒性应激和线粒体功能障碍,最终导致细胞死亡^[2]。近年来研究^[3]表明机体铜代谢失衡与神经退行性疾病、肝豆状核变

性、多种肾脏疾病以及诸多癌症的发生密切相关。基于此,该研究利用氧化铜构建小鼠铜过载模型,并引入我国流行的弓形虫Chinese 1型TgCtwh6株进行干预,旨在探究弓形虫感染对铜死亡代谢机制的作用,为揭示肾脏铜死亡的致病机制与治疗提供新的方向。

1 材料与方法

1.1 主要试剂和仪器 Annexin V-FITC/PI细胞凋亡检测试剂盒购自无锡耐思生命科技有限公司;二甲苯;浓硝酸(HNO₃, 优级纯)和30%双氧水(30% H₂O₂, 分析纯)、异丙醇、氯仿购自国药集团化学试剂有限公司;Western blot配胶主要试剂购自北京兰杰柯科技有限公司;TRIzol试剂购自南京诺唯赞生物科技股份有限公司;鼠源辣根过氧化物酶(horseradish peroxidase, HRP)标记的甘油醛-3-磷酸脱氢酶(glyceral dehyde-3-phosphate dehydrogenase, GAPDH; 货号:60004-1-Ig; 1:8 000)单抗、兔源超氧化物歧化酶1(superoxide dismutase1, SOD1)(货号:67480-1-Ig; 1:1 000)、SOD2(66474-1-Ig;

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1:1 000)单抗购于武汉三鹰生物技术有限公司;兔源谷胱甘肽过氧化物酶4(glutathione peroxidase 4, GPX4)(货号:T56959;1:1 000)单抗购于艾比玛特医药科技(上海)有限公司。Clin-CP-QMS-I微量元素分析仪(北京毅新博创生物科技有限公司),荧光显微镜(德国 LEICA 公司),微量紫外分光光度计(美国赛默飞公司),隔水式电热恒温培养箱(海跃进医疗器械厂),电泳仪(上海天能生命科学有限公司),化学发光成像仪(美国 Bio-Rad 公司),LightCycler®96实时荧光定量PCR仪(瑞士罗氏公司)。

1.2 实验动物和虫株 7~8周龄雌性C57BL/6小鼠购自杭州子源实验动物科技有限公司(生产许可编号:SCXK2019-0004)。弓形虫Chinese 1优势基因型TgCtwh6株包囊由动物源性传染病安徽省重点实验室传代保种。动物实验均经安徽医科大学动物实验伦理委员会(LLSC:20211187)批准并按照《安徽医科大学实验动物使用和护理指南》的建议严格执行。

1.3 小鼠模型的构建 将80只7~8周龄C57BL/6雌性小鼠适应饲养1周后根据实验方案随机均分为4组:Control组、Cu组、TgCtwh6组和Cu+TgCtwh6组。Control组未感染且用普通正常饲料和水喂养;Cu组连续60 d使用含氯化铜1 g/kg加工饲料和0.1%氯化铜的水饲养;TgCtwh6组第0天感染25~30个TgCtwh6包囊并用正常饲料和水喂养;Cu+TgCtwh6组感染25~30个TgCtwh6包囊^[4]并且连续60 d使用含氯化铜1 g/kg加工饲料和0.1%氯化铜的水饲养。所有小鼠在第60天取材用作后续实验分析。

1.4 电感耦合等离子体-质谱法(inductively coupled plasma-mass spectrometry, ICP-MS)检测小鼠肾脏组织铜元素 各组小鼠肾脏组织用超纯水清洗3次,滤纸吸干后称重,用混合消化液(硝酸:过氧化氢=2:1)在电热消解仪中消解,超纯水定容。用Clin-ICP-QMS-I微量元素分析仪测定并计算铜元素含量,铜元素含量=C×V/m,其中C为样品中铜元素浓度(μg/L),V为样品定容体积(L),m为样品质量(g)。

1.5 苏木精-伊红(HE)染色 取各组小鼠肾脏组织置于4%多聚甲醛通用型组织固定液中固定24 h,用于石蜡包埋切片,经HE染色后显微镜下观察肾脏病理性改变。

1.6 PI染色观察细胞凋亡 按照Annexin V-FITC/PI细胞凋亡检测试剂盒说明书操作,对肾脏组织切

片经脱蜡、复水后滴加5 μL Annexin V-FITC和10 μL PI染色液进行染色,盖玻片封片,置于正置荧光显微镜下观察。

1.7 Western blot 检测 各组小鼠肾脏组织用RIPA裂解液提取蛋白,经BCA定量后加入1/4体积的5×蛋白上样缓冲液,金属浴100 °C煮10 min。制成的蛋白质样品经十二烷基硫酸钠-聚丙烯酰胺凝胶电泳(SDS-PAGE)分离后,用标准程序转移到硝酸纤维素膜上。用5%脱脂牛奶封闭1.5 h,封闭完成后用TBST洗膜3次,每次10 min。在4 °C条件下与GPX4(1:1 000)、SOD1(1:1 000)、SOD2(1:1 000)、GAPDH(1:8 000)一抗孵育过夜后,用TBST洗膜3次,每次10 min。在室温条件下根据抗原反应性分别孵育二抗HRP-conjugated Goat Anti-Mouse IgG(H+L)(1:10 000)和HRP-conjugated Goat Anti-Rabbit IgG(H+L)(1:10 000)1.5 h, TBST洗膜3次,每次10 min,最后凝胶成像仪成像。

1.8 RT-qPCR 采用RT-qPCR技术检测小鼠肾脏组织中金属调节转录因子1(metal regulatory transcription factor 1, MTF1)、细胞周期蛋白依赖性激酶抑制因子2A(cyclin dependent kinase inhibitor 2A, CDKN2A)、谷氨酰胺酶(glutaminase, GLS)、ATP酶铜转运β(ATPase copper transporting beta gene, ATP7B)基因的mRNA表达水平。引物由上海生工合成(表1)。TRIzol法提取各组小鼠肾脏组织RNA,使用微量紫外分光光度计测量RNA的浓度并逆转录成cDNA用于检测。10 μL RT-qPCR反应体系:2×SYBR Green Pro Taq HS Premix 5 μL、cDNA模板1 μL、Premix F 0.3 μL、Premix R 0.3 μL,无酶水补足至10 μL;反应条件:95 °C 10 min预变性,95 °C 10 s, 60 °C 30 s,共40次循环。基于内参基因GAPDH分析PCR结果,并通过 $2^{-\Delta\Delta C_T}$ 方法计算基因相对表达水平。

1.9 统计学处理 所有实验均独立重复3次。使用ImageJ软件对Western blot结果进行分析,使用GraphPad Prism 8.0.2软件进行统计学分析及作图,各组间比较使用单因素方差分析,组间两两比较采用LSD(least-significant difference)-*t*检验,以 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 小鼠肾脏铜含量的ICP-MS检测结果 长期高铜饮食能够引起小鼠肾脏铜蓄积,ICP-MS检测结

表1 RT-qPCR反应引物序列
Tab. 1 Primer sequences used in RT-qPCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>MTF1</i>	ACACCTTCGTCTGTAATCAGGA	CTGCACGTCACACTCAAATGG
<i>CDKN2A</i>	CGCAGGTTCTTGCTCACTGT	TGTTCCAGAAAGCCAGAGCG
<i>GLS</i>	CTACAGGATTGCGAACATCTGAT	ACACCATCTGACGTTGTCTGA
<i>ATP7B</i>	CATCAGTGACGCCATGACAG	TCATCCCGCAGAGCACACC
<i>GAPDH</i>	GGTTGTCTCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC

果显示, Cu组小鼠肾脏铜含量相较Control组增加($P < 0.05$), 提示小鼠铜过载模型构建成功^[5]。TgCtwh6感染组肾脏中的铜元素含量低于Control组, 且差异有统计学意义($P < 0.05$), 与Cu组相比, TgCtwh6+Cu组铜元素含量下降($P < 0.05$)。结果表明, TgCtWh6感染可降低铜过载小鼠肾脏组织的铜水平。见图1。

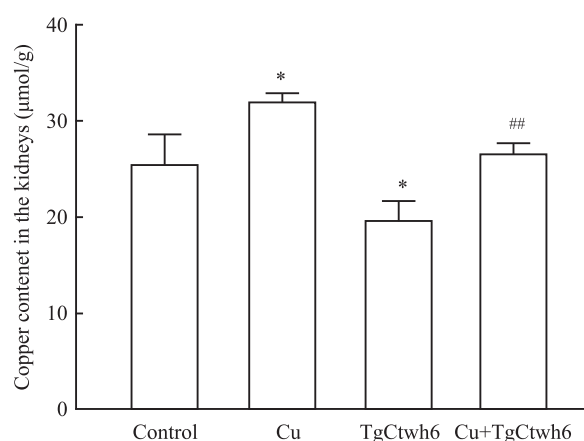


图1 各组小鼠肾脏铜含量的比较

Fig. 1 Comparison of kidney copper content in each group

* $P < 0.05$ vs Control group; ## $P < 0.01$ vs Cu group.

2.2 小鼠肾脏HE染色结果 对各组小鼠肾脏进行组织病理学检查, 经HE染色后, Control组小鼠的肾小管、肾小球形态结构无异, 排列整齐。与Control组相比, Cu组和TgCtwh6组小鼠可见组织局部肾间质炎性浸润, 肾小球变形、肾小管损伤等病理表现; 与Cu组相比, Cu+TgCtwh6组小鼠肾脏炎性细胞明显减少, 病理损伤有所改善。见图2。

2.3 PI染色检测细胞凋亡数 组织中铜过载会通过氧化应激诱导细胞凋亡。对各组小鼠肾脏进行PI染色并在荧光显微镜下观察并计数细胞凋亡情况, 结果显示, 与Control组(53.50 ± 2.6)相比, Cu组凋亡细胞数(119.0 ± 20)和TgCtwh6组(78.57 ± 8.4)增多($P < 0.05$), 而Cu+TgCtwh6组凋亡细胞数(88.36 ± 19)低于Cu组, 差异有统计学意义($P < 0.05$) (见图3、4)。结果表明弓形虫对肾脏过量铜的摄取会改善细胞凋亡情况。

2.4 小鼠肾脏中铜代谢途径中关键蛋白的Western blot结果 对各组小鼠肾脏氧化应激相关蛋白检测分析, 结果显示, 与Control组相比, Cu组、TgCtwh6组肾脏中的SOD1蛋白表达水平上升, 差异有统计学意义($P < 0.05$); 与Cu组相比, Cu+TgCtwh6组

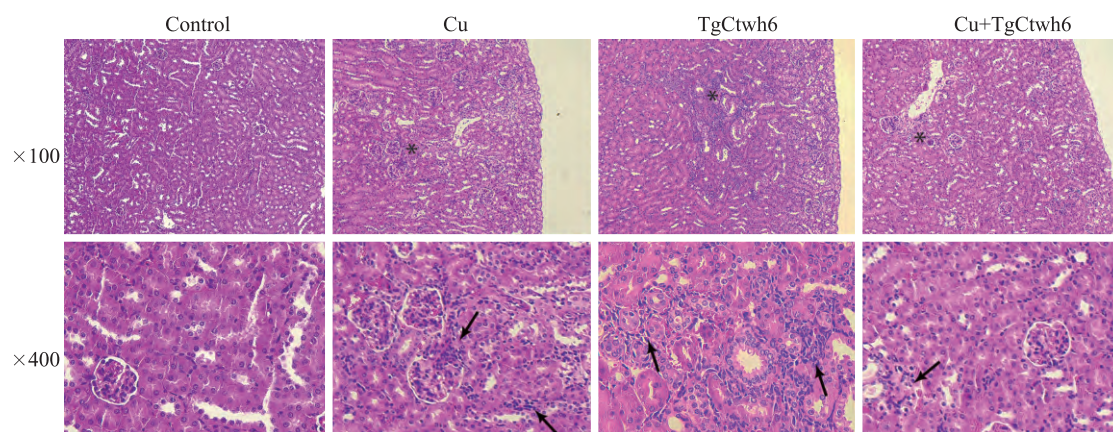


图2 各组小鼠肾脏HE染色结果

Fig. 2 HE staining results of the kidneys of mice in each group

Black asterisk: The occurrence of localized pathological injury in the renal tissue; Black arrows: The occurrence of inflammatory cell infiltration in the renal interstitium.

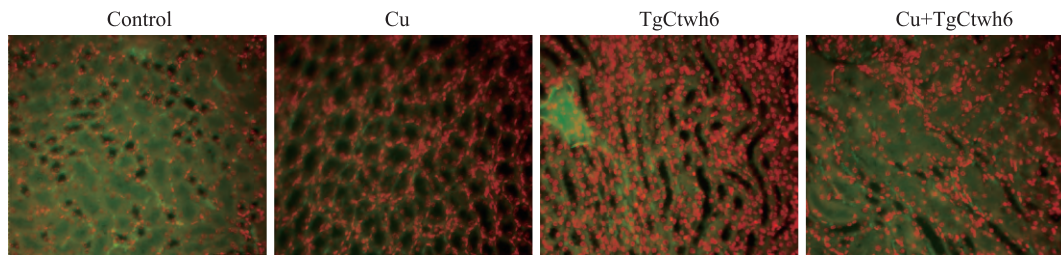


图3 各组小鼠PI染色结果 ×200
Fig. 3 PI staining results in each group ×200

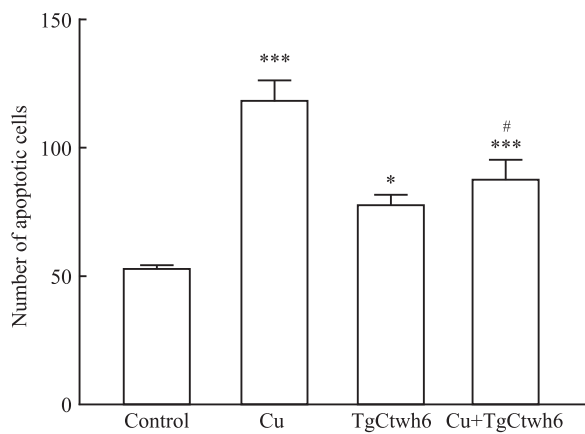


图4 各组小鼠PI染色细胞凋亡数量

Fig. 4 The number of apoptosis stained with PI in each group
* $P < 0.05$, *** $P < 0.001$ vs Control group; # $P < 0.05$ vs Cu group.

SOD1 蛋白表达下调 ($P < 0.05$); 而4组之间 SOD2 表达无明显改变。与 Control 组相比, Cu 组和 TgCtwh6 组肾脏组织中 GPX4 蛋白的表达水平下调 ($P < 0.05$); 而 Cu+TgCtwh6 组 GPX4 表达有所上调, 差异有统计学意义 ($P < 0.05$)。见图5。

2.5 小鼠肾脏铜死亡相关基因的 RT-qPCR 检测结果 通过 RT-qPCR 检测分析, 与 Control 组相比,

Cu 组和 TgCtwh6 组 *MTF1*、*CDKN2A* 基因的 mRNA 表达均显著下调 ($P < 0.05$), 但 Cu + TgCtwh6 组和 Cu 组之间差异无统计学意义。与 Control 组相比, Cu 组的 *GLS* 基因表达显著下调, 但与 Cu 组相比, Cu + TgCtwh6 组 *GLS* 表达上调 ($P < 0.05$)。与 Control 组相比, Cu 组和 TgCtwh6 组 *ATP7B* 的 mRNA 表达显著上调 ($P < 0.05$), 且 Cu + TgCtwh6 组与 Cu 组相比表达减少 ($P < 0.05$) (图6)。结果表明, 弓形虫能够改善铜过载小鼠肾脏组织的 *GLS*、*ATP7B* 基因表达。

3 讨论

弓形虫作为细胞内寄生原虫, 其生存所需要的各种物质都来源于宿主细胞, 而这样的特性势必会打破宿主细胞的代谢平衡。课题组前期研究显示弓形虫感染小鼠后, 宿主体内的多个组织内部金属稳态都发生了改变。金属离子平衡对于宿主-寄生虫相互作用的建立和维持至关重要。铜作为一种必需微量元素, 参与诸多生物活动过程, 其对于呼吸、去除自由基、产生能量、结缔组织发育、铁和氧的代谢、细胞外基质和神经肽的成熟以及神经内分泌信号传导都至关重要^[6]。

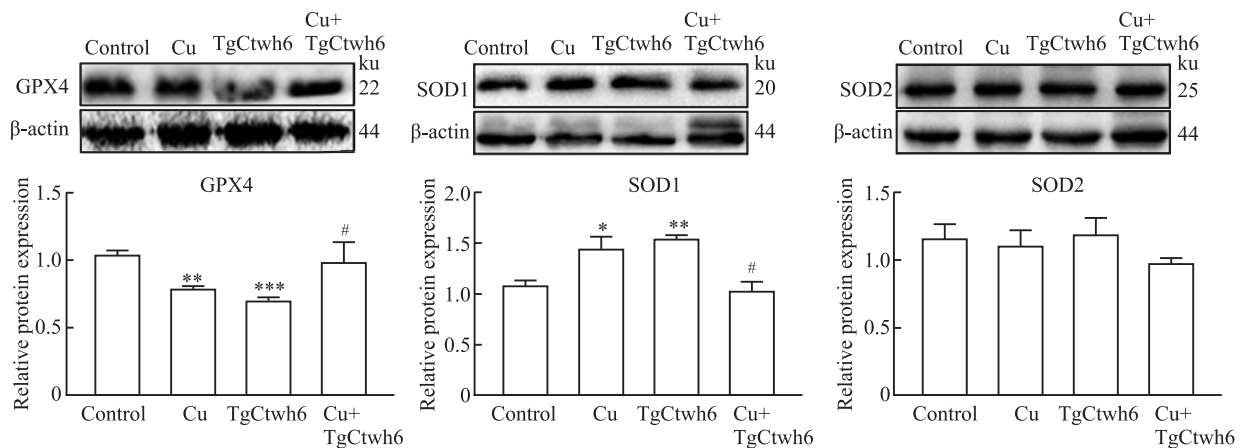


图5 各组小鼠SOD1、SOD2和GPX4蛋白相对表达量的比较

Fig. 5 Comparison of the expression of SOD1, SOD2 and GPX4 proteins in each group
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs Control group; # $P < 0.05$ vs Cu group.

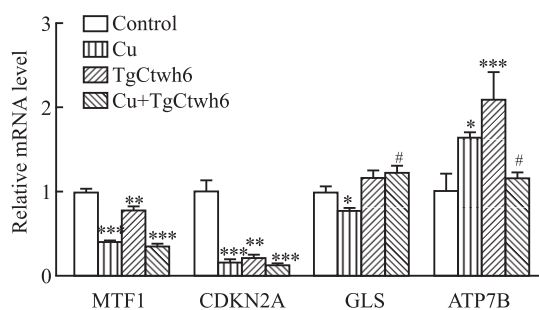


图6 各组小鼠MTF1、CDKN2A、GLS、ATP7B的mRNA表达比较

Fig. 6 Comparison of mRNA expression of MTF1, CDKN2A, GLS, and ATP7B in each group

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs Control group; # $P < 0.05$ vs Cu group.

组织内发生铜超载会诱导细胞死亡,肾是铜超载的主要靶器官之一,高铜浸润在细胞层面会对肾组织造成不可逆的损伤,抑制肾细胞增殖,最终表现为病理组织学上的肾充血水肿、炎性细胞浸润和肾小管的萎缩^[7]。本研究表明,在小鼠铜超载模型构建成功后,TgCtwh6感染能够缓解铜暴露导致的肾脏病理损伤和细胞凋亡。GPX4是一种关键的抗氧化酶,其主要功能是利用谷胱甘肽作为辅助因子来降低脂质过氧化,从而保护细胞膜的完整性^[8]。有研究^[9]表明在铜死亡中铜诱导了GPX4的自噬降解加重细胞死亡。本研究中,铜超载模型鼠GPX4水平显著降低,但介入弓形虫感染后,其表达有所恢复。SOD作为生物体内重要的抗氧化酶,在真核生物中分为SOD1、SOD2,二者区别在于中心的金属离子的不同,SOD1中心为锌和铜离子,SOD2为锰离子^[10]。本研究结果显示,模型鼠的SOD1蛋白表达改变,而SOD2差异无显著性,这可能与SOD和不同的微量元素结合有关。SOD1的表达与铜水平之间存在复杂的相互作用,铜可通过MTF1等调控SOD1的表达^[11]。此外,铜过量可通过氧化应激间接影响SOD1表达。弓形虫感染可激活宿主细胞的抗氧化防御系统,通过上调SOD的表达以应对氧化损伤^[12]。弓形虫和铜联合作用导致SOD1表达降低,是弓形虫与宿主相互作用的复杂结果,可能涉及到铜离子的竞争性摄取、信号通路协同抑制,具体机制有待阐明。谷氨酰胺是一种重要的代谢燃料,通过氨基酸转运蛋白ASCT2/SLC1A5进入细胞,并在线粒体中被GLS催化的脱氨反应转化为谷氨酸。胞质谷氨酸在铜死亡过程中对维持氧化还原反应平衡非常关键,通过产生谷胱甘肽来避免细胞出现氧化应激^[13]。在有关肾透明细胞癌(ccRCC)的研究^[14]中,患者的肿瘤和正常组织之间

的铜代谢相关基因表达存在差异,CDKN2A的表达显著上升,而铁氧还蛋白1(ferredoxin 1,FDX1),二氢脂酰胺S-乙酰转移酶(dihydrolipoamide S-acetyltransferase,DLAT),硫辛酸合成酶(lipoic acid synthetase,LIAS),GLS等则低于正常组织^[14]。铜主要通过铜转运蛋白SLC31A1进入细胞,并与胞质铜伴侣以及可能的其他可溶性蛋白结合。当细胞内铜超过生理水平时,ATP7A和ATP7B从反面高尔基体网络移动到囊泡中,隔离过量的铜并最终将其输出到细胞外^[15]。在本研究中,TgCtwh6感染可恢复铜超载导致的小鼠肾脏GLS表达下调和ATP7B表达上调。初步推测弓形虫感染后可通过对铜死亡相关基因(GLS、ATP7B)的干预调节肾脏内铜代谢,可能与弓形虫对宿主体内过量铜的摄取或者分泌的致密颗粒蛋白(dense granule proteins,GRAs)和棒状体蛋白(rhoptry protein,ROPs)等对相关通路的间接调控有关。其他如FDX1、DLAT、LIAS等本研究未涉及的铜死亡相关基因可能也参与其中,具体机制有待进一步研究。

综上所述,弓形虫感染能够上调肾脏组织GLS基因表达和下调ATP7B基因表达,改变宿主氧化应激状态,调节小鼠肾脏组织中的铜代谢。该研究为利用弓形虫通过干预铜代谢途径治疗如肿瘤等铜超载相关疾病提供了新的线索。

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A preliminary study on *Toxoplasma gondii* interfering with copper metabolism pathways in mouse kidney

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Abstract Objective To investigate the effect of *Toxoplasma gondii* infection on copper metabolism in the kidneys of mice. **Methods** A total of 80 7-8-week-old C57BL/6 female mice were randomly divided into four groups of 20 mice in each group after one week of adaptation, including Control group, Cu group, TgCtwh6 group and Cu+TgCtwh6 group. Mice that were not infected and fed with normal diet and water were used as the Control group; Mice fed with 1 g/kg of copper chloride processing diet and 0.1% copper chloride water for 60 consecutive days were used as Cu group; Mice infected with 25–30 TgCtwh6 cysts (one of the predominant genotype Chinese 1 in China) fed with normal diet and water were used as the TgCtwh6 group; mice infected with 25–30 TgCtwh6 cysts and fed with a processed diet containing 1 g/kg of copper chloride and water with 0.1% copper chloride for 60 consecutive days were used as the Cu+TgCtwh6 group. ICP-MS was used to determine the changes in copper content in kidney tissues. Hematoxylin-eosin (HE) staining was used to observe the pathological changes of mouse kidney tissue. The number of apoptotic cells was observed by PI staining. Western blot was used to detect the protein expression levels of glutathione peroxidase 4 (GPX4) and superoxide dismutase (SOD1, SOD2). RT-qPCR was used to detect the mRNA expression of cuproptosis-related genes. **Results** Pathological manifestations such as inflammatory cell infiltration in the Cu group and TgCtwh6 group were seen under the microscope, and the inflammatory infiltrating cells of the renal interstitial were reduced in the Cu+TgCtwh6 group, and the pathological manifestations (下转第 140 页)

The role of S100A2 in the progression of colorectal cancer

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Abstract Objective To investigate the role of calcium-binding protein S100A2 in colorectal cancer (CRC) progression and its association with fructose metabolism in CRC cells. **Methods** Differential expression of S100A2 between CRC patients and healthy individuals was analyzed using the GEPIA2 tumor database. Western blot and qRT-PCR were performed to compare S100A2 expression levels in CRC cell lines (HCT116, SW480, Caco-2) and normal human colonic epithelial cells (NCM460). Immunohistochemical staining was conducted to assess S100A2 expression in CRC tissues and adjacent non-tumor tissues. S100A2-knockdown stable CRC cell lines and negative control cell lines were established via lentiviral transduction. Functional assays, including CCK-8, wound healing and Transwell experiments were utilized to evaluate the effects of S100A2 downregulation on CRC cell proliferation, migration, and invasion. Western blot and immunofluorescence staining were employed to analyze the impact of S100A2 knockdown on the expression levels of fructose transporter 5 (GLUT5) and ketohexokinase (KHK). Intracellular fructose concentration was measured using a fructose assay kit. A nude mouse CRC xenograft model was established using S100A2-knockdown HCT116 cell lines to investigate the role of S100A2 in tumor proliferation *in vivo*. Tumor tissues from the xenografted mice were analyzed by Western blot and immunofluorescence staining to evaluate the expression levels of GLUT5 and KHK. **Results** S100A2 expression was significantly elevated in CRC patients compared with healthy individuals. All three CRC cell lines exhibited markedly higher S100A2 expression than normal colonic epithelial cells. S100A2 knockdown significantly inhibited CRC cell proliferation, migration, and invasion capacities. Downregulation of S100A2 suppressed the expression of fructose metabolism-related proteins GLUT5 and KHK, accompanied by reduced cellular fructose uptake. *In vivo* experiments demonstrated that S100A2 knockdown effectively inhibited tumor growth and decreased GLUT5/KHK expression in xenograft tissues. **Conclusion** Downregulation of S100A2 inhibits CRC progression by modulating fructose metabolism in CRC cells.

Key words S100A2; colorectal cancer; fructose metabolism; facilitated glucose transporter member 5; ketohexokinase

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(上接第132页)

of glomerular tubular structure were improved. The number of apoptotic cells in the Cu+TgCtwh6 group (88.36±19) was lower than that in the Cu group (119.0±20). Compared with the Cu+TgCtwh6 group, the expression of SOD1 protein was down-regulated, and the difference was statistically significant ($P<0.05$). TgCtwh6 infection could restore the down-regulation of renal glutaminase (GLS) expression and the up-regulation of ATPase copper transporting beta gene (ATP7B) expression caused by copper overload. **Conclusion** *Toxoplasma gondii* infection can interfere with the copper metabolism pathway in the kidney of mice, improve the kidney damage caused by copper overload, and provide new clues for the treatment of copper overload disease.

Key words *Toxoplasma gondii*; kidney; cuproptosis; Western blot; apoptosis; ICP-MS

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