

## 内质网应激参与蛋氨酸-胆碱缺乏饮食诱导的小鼠 非酒精性脂肪肝的形成

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**摘要:**【目的】探讨内质网应激在蛋氨酸-胆碱缺乏膳食(MCD)诱导的非酒精性脂肪肝炎症以及纤维化形成中的作用。【方法】8周龄C57BL/6雄性小鼠80只,随机分为2个组,8个亚组( $n=10$ ),亚组1~4组给予普通膳食,5~8组给予MCD喂养。并各在第2、4、6、8周处死普通膳食组和MCD组各一组。组织病理染色方法和NAS评分评估小鼠纤维化和炎症程度,内质网应激指标磷酸化蛋白激酶R样内质网R激酶(PERK)、磷酸化真核细胞起始因子2 $\alpha$ (eIF2 $\alpha$ )、CCAAT/增强子结合蛋白同源蛋白(CHOP)采用Western Blot和实时荧光定量PCR(RT-PCR)方法检测。重点分析内质网应激与肝脏炎症以及纤维化形成的关系。【结果】MCD组NAS评分和血清中ALT、AST以及肝组织中TG、TC水平均高于普通膳食组;MCD组p-PERK、p-eIF2 $\alpha$ 、CHOP蛋白表达亦明显高于正常膳食组,而GADD34 mRNA表达显著低于普通膳食组,且随时间推移,MCD组内质网应激逐渐加重;相关分析表明在MCD组中内质网应激相关指标与NAS评分呈显著相关。【结论】在蛋氨酸-胆碱缺乏膳食诱导的小鼠中,小鼠肝脏损伤加重,炎症和纤维化程度加深,而内质网应激是参与了这个过程。

**关键词:**非酒精性脂肪肝;非酒精性脂肪肝炎症活性评分;内质网应激

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## Involvement Endoplasmic Reticulum Stress on Process of Nonalcoholic Fatty Liver Disease Induced by MCD

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**Abstract:** 【Objective】 To investigate the effect of the ER stress on the process of nonalcoholic fatty liver disease which is induced by methionine-choline deficient diet. 【Methods】 C57/BL mice were randomly divided into 8 groups ( $n=10$ ), group 1-4 was given normal diet, 5-8 group was given MCD diet. Then took one group from both normal diet and MCD diet in the 2nd, 4th, 6th, 8th week, recorded each group's body weight and liver weight. The liver fibrosis and inflammation in mice were analyzed by histopathological staining and NAS. The expressions of endoplasmic reticulum stress markers such as p-PERK, p-eIF2 $\alpha$ , CHOP were detected by Western Blot and real time fluorescent quantitative PCR (q-PCR). 【Results】 ①The ALT and AST in serum, TG and TC in liver tissue were both higher in MCD group, the same as NAS; ②The expressions of PERK, eIF2 $\alpha$ , CHOP were significantly higher than the normal diet group, while the GADD34 expression was lower. What's more, as time went by, the difference was more obvious; 3. There was a significant correlation between the ERS index and NAS score in MCD group. 【Conclusion】 The liver damage and inflammatory response was lifted gradually in the mice fed with MCD diet. The ER stress may take an important role in this process.

**Key words:** nonalcoholic fatty liver disease; nonalcoholic steatohepatitis score; endoplasmic reticulum stress

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非酒精性脂肪肝病(nonalcoholic fatty liver disease, NAFLD)是一种与胰岛素抵抗(insulin resistance, IR)和遗传易感密切相关的代谢应激性肝脏损伤,患者无过量饮酒史,疾病谱包括非酒精性单纯性脂肪肝(nonalcoholic simple fatty liver, NAFL)、非酒精性脂肪性肝炎(nonalcoholic steatohepatitis, NASH)及其相关的肝硬化和肝细胞癌<sup>[1-2]</sup>。近20年来,我国的NAFLD的患病率增长迅速并呈低龄化发病趋势,在发达地区比如广州、上海和香港等地成人发病率为15%左右<sup>[3]</sup>。NALFD/NASH的动物模型可以给我们提供关于疾病的许多重要信息,既可以研究疾病的病理过程、寻找相关标志物,也可以研究许多药物对疾病的干预作用<sup>[4]</sup>。蛋氨酸-胆碱缺乏(methionine-choline deficient, MCD)饮食诱发NASH是国际上被广泛应用的研究NASH发病机制的主要动物模型。该膳食主要缺乏了肝细胞 $\beta$ 氧化和合成极低密度脂蛋白(VLDL)所需的蛋氨酸和胆碱<sup>[5]</sup>,导致肝内脂质蓄积并减少VLDL的合成,最终引起包括脂肪变性,小叶炎症和纤维化等形式在内的肝脏损伤<sup>[6]</sup>。内质网应激与脂质代谢密切相关,长期的内质网应激可诱导肝脏脂质代谢异常<sup>[7]</sup>。近年来的研究表明,内质网应激在非酒精性脂肪肝的发病中发挥着重要作用<sup>[8-9]</sup>,但是内质网应激在MCD诱导非酒精性脂肪肝损伤模型中的作用尚不清楚,还需深入阐明。本研究着重于探究MCD饮食诱导脂肪肝模型的过程中小鼠肝损伤的进展情况,探讨非酒精性脂肪性肝病进展过程中病理生理的改变与内质网应激是否存在联系,为进一步了解NAFLD,提出新的理论假说提供线索。

## 1 材料与方法

### 1.1 动物及试剂

SPF级雄性C57/BL小鼠(7~8周龄)80只,购自中山大学实验动物中心。兔抗PERK抗体、兔抗eIF2 $\alpha$ 抗体、兔抗CHOP抗体、兔抗GAPDH抗体、辣根过氧化物酶标记山羊抗兔IgG二抗均购自(Santa Cruz, CA, USA);兔抗p-PERK抗体、兔抗p-eIF2 $\alpha$ 抗体购自(Cell Signaling Technology, Boston, MA, USA)。本实验遵守实验动物伦理原则。

### 1.2 动物分组及模型制备

小鼠于恒温22~26℃,相对湿度60%~80%且

明暗各12 h条件饲养,自由饮食。根据体质量随机分为正常组4组和MCD组,共8组,每组10只。正常组给予普通膳食喂养,MCD组给予蛋氨酸-胆碱缺乏膳食(methionine-choline deficient diet, MCDD)喂养。

### 1.3 实验方法

1.3.1 取材 普通膳食组和MCD组小鼠按批次分别在第2、4、6、8周各取一组,用戊巴比妥麻醉后处死,眼球取血,分离血清用于肝功能检测。肝脏组织摘取后,用磷酸盐缓冲液(PBS)冲洗,取部分固定于40 g/L的多聚甲醛溶液,其余部分于-80℃保存。

1.3.2 肝脏组织HE染色以及天狼猩红染色 取固定后的肝脏组织做石蜡包埋,肝组织切片厚4  $\mu$ m,按照相应步骤进行苏木素-伊红(HE)和天狼猩红染色,光镜下观察。

1.3.3 小鼠血清ALT、AST以及肝脏炎症因子检测 待小鼠血液凝固后,4℃ 3000 r/min离心10 min( $r=10$  cm),取上清液,按照说明书检测血清中ALT、AST以及肝组织中甘油三酯(TG)、胆固醇(TC)含量,相应ELISA试剂盒检测肿瘤坏死因子TNF- $\alpha$ 、白细胞介素IL-6、IL-17A含量。

1.3.4 实时定量聚合酶链反应 称取肝脏100 mg, Trizol法提取总RNA,检测其浓度与纯度,取1  $\mu$ g RNA反转录成c-DNA,反转录体系以及反应条件按照试剂盒说明书,取GAPDH为内参进行校正。

1.3.5 Western blot检测内质网应激通路的表达 取100 mg肝脏组织液氮碾磨,每管加1 mL RIPA和10  $\mu$ L PMSF裂解液裂解,置于冰上反应30 min后,4℃ 12 000 r/min离心15 min,吸取上清液,采用考马斯亮蓝法测定蛋白质的浓度。按照各组浓度,每组加一定量的Loading buffer和双蒸水,调整样本浓度为3~5  $\mu$ g/ $\mu$ L之间,100℃变性10 min,10% SDS-PAGE电泳分离,待蛋白移至PVDF膜上后,用5%脱脂奶粉TBST封闭2 h,敷育一抗,置于4℃冰箱中过夜,室温下敷二抗1 h。每次进行下一步实验前均用Tris缓冲液(TBST)漂洗PVDF膜3次,每次时间10~15 min。暗室X线胶片曝光。以GAPDH进行校正。所得条带用Image-J图像分析软件分析蛋白的灰度值。一抗稀释倍数如下:小鼠抗PERK抗体,1:1000;兔抗p-PERK抗体,1:1000;兔抗CHOP抗体,1:1000;

兔抗 eIF2 $\alpha$  抗体, 1:1000; 兔抗 p-eIF2 $\alpha$  抗体, 1:1000; 小鼠抗 GAPDH 抗体, 1:3000。

1.4 检测指标

1.4.1 Western blot 检测 p-PERK/PERK、p-eIF2 $\alpha$ /eIF2 $\alpha$ 、CHOP 蛋白的表达。

1.4.2 Real-time quantitative PCR 检测 TNF $\alpha$ 、单核细胞趋化蛋白(MCP)-1、IL-6、I型胶原蛋白 A2 (collagen 1A2, Col1a2)、 $\alpha$ -平滑肌激动蛋白( $\alpha$ -SMA)、生长停止和 DNA 损毁诱导性蛋白(GADD)34、CHOP、X-盒结合蛋白(XBP)-1 mRNA 的表达水平。

1.4.3 NAS 评分 NAS 系统基于 NASH 重要组织损伤(即脂肪变性、肝细胞气球样变和小叶、肝门炎症等)对肝脏组织进行病理学评价。将肝脏组织制作成病理切片后, 每张切片选取 5 个视野, 应用 NAS 进行评分, 评分标准: ①脂肪变性, 在方法倍数为 200 倍显微镜观察下随机选取的视野中可见的脂肪空泡所占面积的百分比来评价, 在每个视野中, 脂肪空泡面积 < 5% 评为 0 分, 5% ~ 33%

为 1 分, 34% ~ 66% 为 2 分,  $\geq 67%$  为 3 分; ②小叶炎症, 每个视野中(200 倍)中无炎症病灶为 0 分, < 2 个病灶为 1 分, 2 ~ 4 个病灶为 2 分, > 4 个病灶为 3 分; ③气球样变, 每个视野中(200 倍)中无气球样变为 0 分, 少量但明显的气球样变为 1 分, 大量显著的气球样变为 2 分。

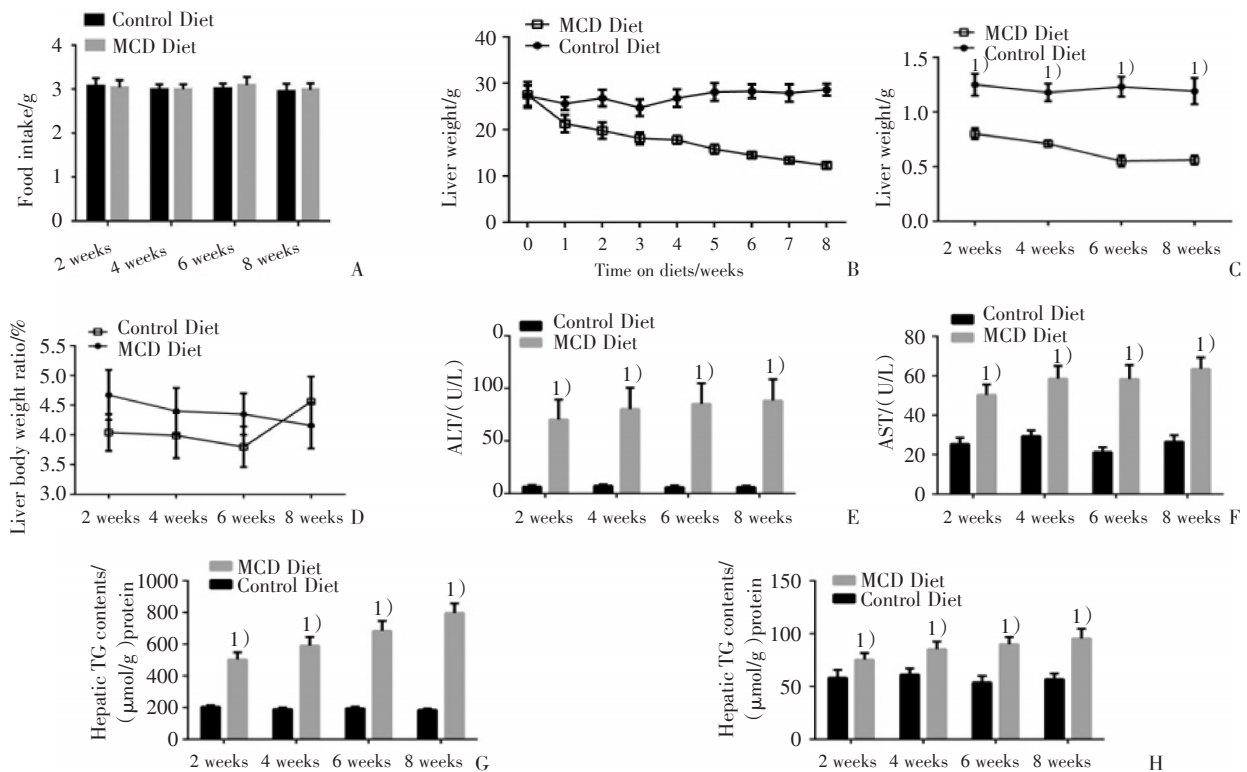
1.5 统计学分析

采用 SPSS20.0 统计软件对数据进行分析, 计量资料结果以表示, 多组间采用单因素方差分析, 检验水准设为 0.05。Western blot 结果用 Image J 分析。Q-PCR 结果采用 GraphPad Prism 5.0 统计软件分析。

2 结果

2.1 小鼠进食量、体质量、肝质量、肝酶以及肝脏脂质含量的时间变化趋势

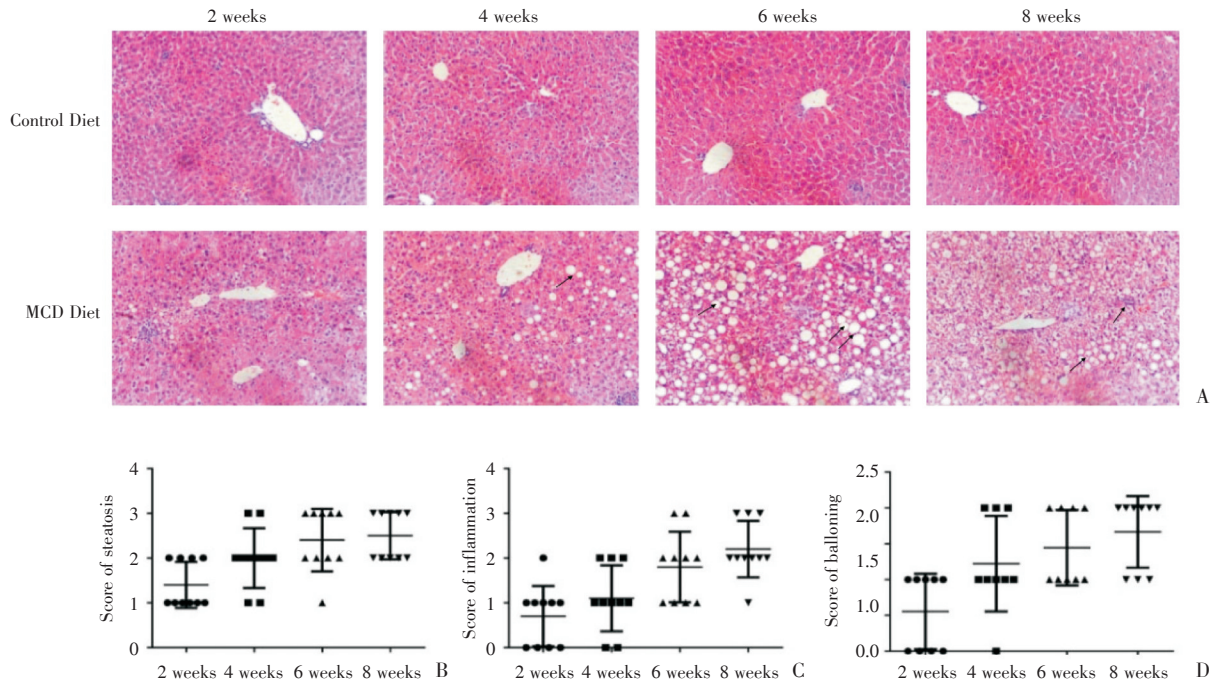
随着不同膳食组饲养时间延长, 小鼠体质量差异越来越明显, 普通膳食组小鼠体质量有所增



The body weight (A) and liver weight (B) of the mice fed a normal diet are much heavier than the MCD diet, while the ratio of liver weight-to-body weight (C) is of no difference. The level of ALT, AST, TG, TC in the normal diet is much lower than the MCD group, which showed that MCD one had a significantly liver damage. 1)  $P < 0.05$  compared with the normal diet mice.

图1 摄食期间各组小鼠进食量、体质量、肝质量以及 ALT、AST、TG、TC 变化趋势

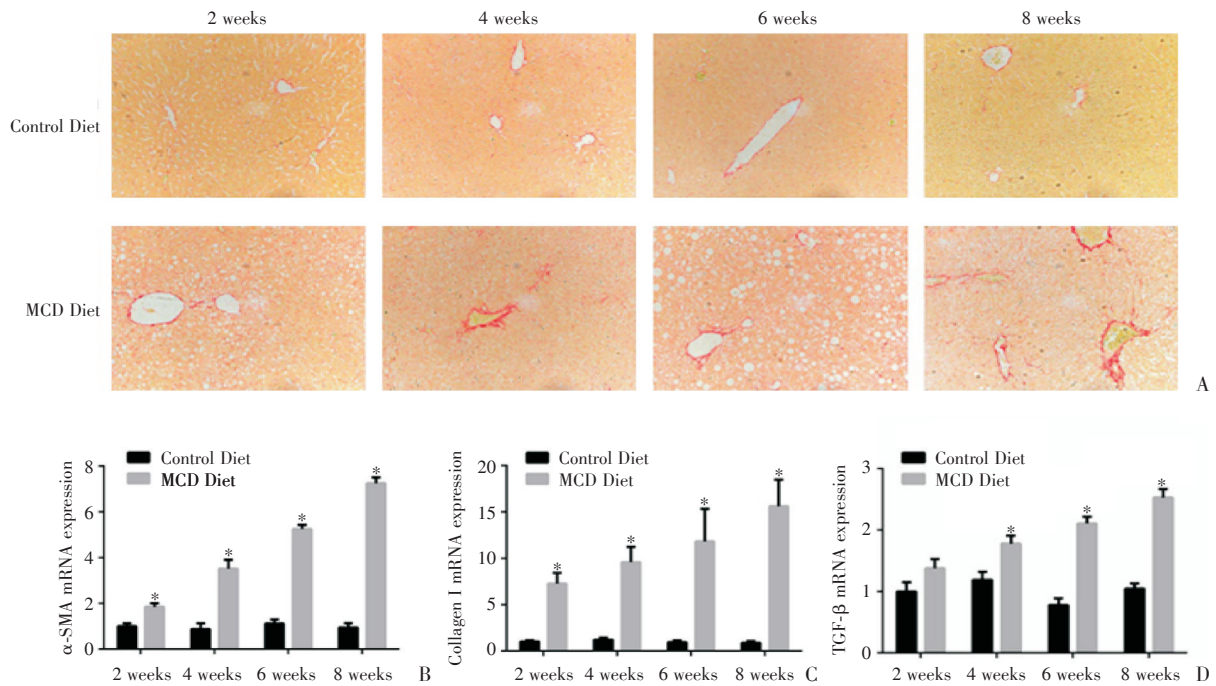
Fig.1 The changes of food intake, body weight, liver weight, ALT, AST, TG, TC in mice during feeding



The score of steatosis (B), inflammation (C) and ballooning (D) is much higher in the MCD diet group mice. Even more, as time went by, it was more serious compared with the normal diet mice. The result of HE staining (A) was shown. Original magnification was 200 ×. 1)  $P < 0.05$  compared with the normal diet mice.

图2 NAS评分

Fig.2 Nonalcoholic steatohepatitis score (NAS)



There is only a little collagen deposition in the normal diet mice while MCD group mice showed a significantly collagen deposition in the liver portal and portal vein (A). Original magnification was 200 ×. Q-PCR showed the mRNA expression of  $\alpha$ -SMA, Col I $\alpha$ 1 and TGF- $\beta$  were much higher in the MCD group. And with longer feeding, the difference was more obvious. 1)  $P < 0.05$  compared with the normal diet mice (B-D).

图3 两组小鼠肝纤维化比较

Fig.3 Fibrosis between the two groups

长,由最初的( $27.3 \pm 2.2$ )g上升至( $28.6 \pm 1.3$ )g,而MCD组体质量下降明显,由最初的( $27.5 \pm 2.8$ )g降低至( $12.3 \pm 0.7$ )g,而两组的进食量没有统计学差异(图1A、B);正常膳食组小鼠肝质量均大于MCD膳食组,而肝质量/体质量则两组之间无差异(图1C、D)。正常膳食组血清中ALT、AST以及肝组织中TG、TC含量均低于MCD组(图1E-H)。

## 2.2 肝脏组织学变化及NAS评分

在第2、4、6、8周,正常膳食组小鼠肝脏脂肪变、水样变以及炎症变化均不明显。MCD组小鼠自第2周开始,肝脏出现少量脂肪变和炎症,随着时间推移,肝脏损伤逐渐加重,脂肪变范围、炎症程度都逐渐加重;且第4周起MCD组出现较为明显的气球样变(图2A)。相应的NAS评分见图2B-D。正常膳食组小鼠肝组织汇管区以及门静脉周围仅有少量胶原沉积,MCD组小鼠肝组织胶原沉积明显(图3A),随着MCD喂养时间的增长其纤维化的程度也逐渐加重,RT-PCR结果亦显示MCD组 $\alpha$ -SMA、Col1A1以及TGF- $\beta$  mRNA表达较正常膳食组明显增加( $P < 0.05$ ;图3B-D)。

## 2.3 血清炎症因子水平及肝脏炎症因子表达变化

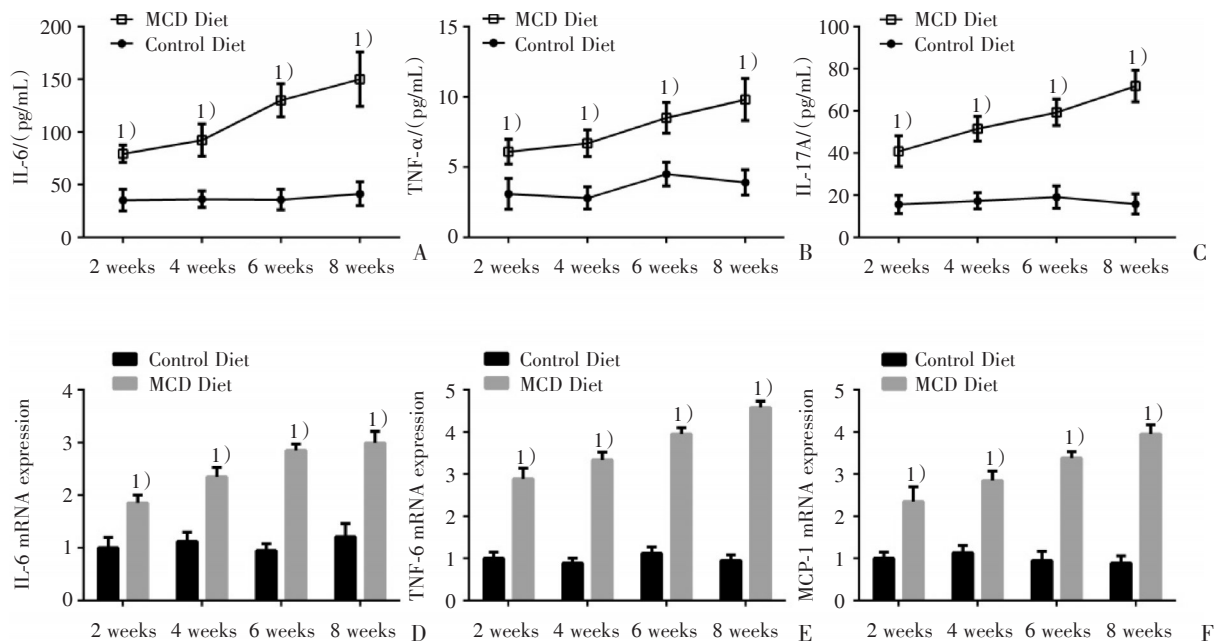
在第2、4、6、8周,MCD组血清中IL-6、TNF- $\alpha$ 、IL-17A炎症因子的表达高于正常膳食组,随着时间逐渐增高。MCD组肝脏组织中IL-6、TNF- $\alpha$ 、MCP-1 mRNA表达水平亦成同样趋势(图4)。

## 2.4 内质网应激相关指标改变

Western blot结果显示MCD组内质网应激标志物p-PERK、p-eIF2 $\alpha$ 和CHOP蛋白较正常膳食组表达增强(图5A);RT-PCR结果显示,MCD组较正常膳食组GADD34 mRNA表达减弱,而CHOP和XBP-1 mRNA表达增强。结果表明随着喂养时间的增长,在MCD组的肝脏组织中,内质网应激逐渐增强(图5B-D)。在MCD组中,相关分析结果显示p-PERK/PERK、p-eIF2 $\alpha$ /eIF2 $\alpha$ 及CHOP/GAPDH与NAS评分呈显著正相关,提示内质网应激与肝脏损伤存在较强关联(图5E-G)。

## 3 讨论

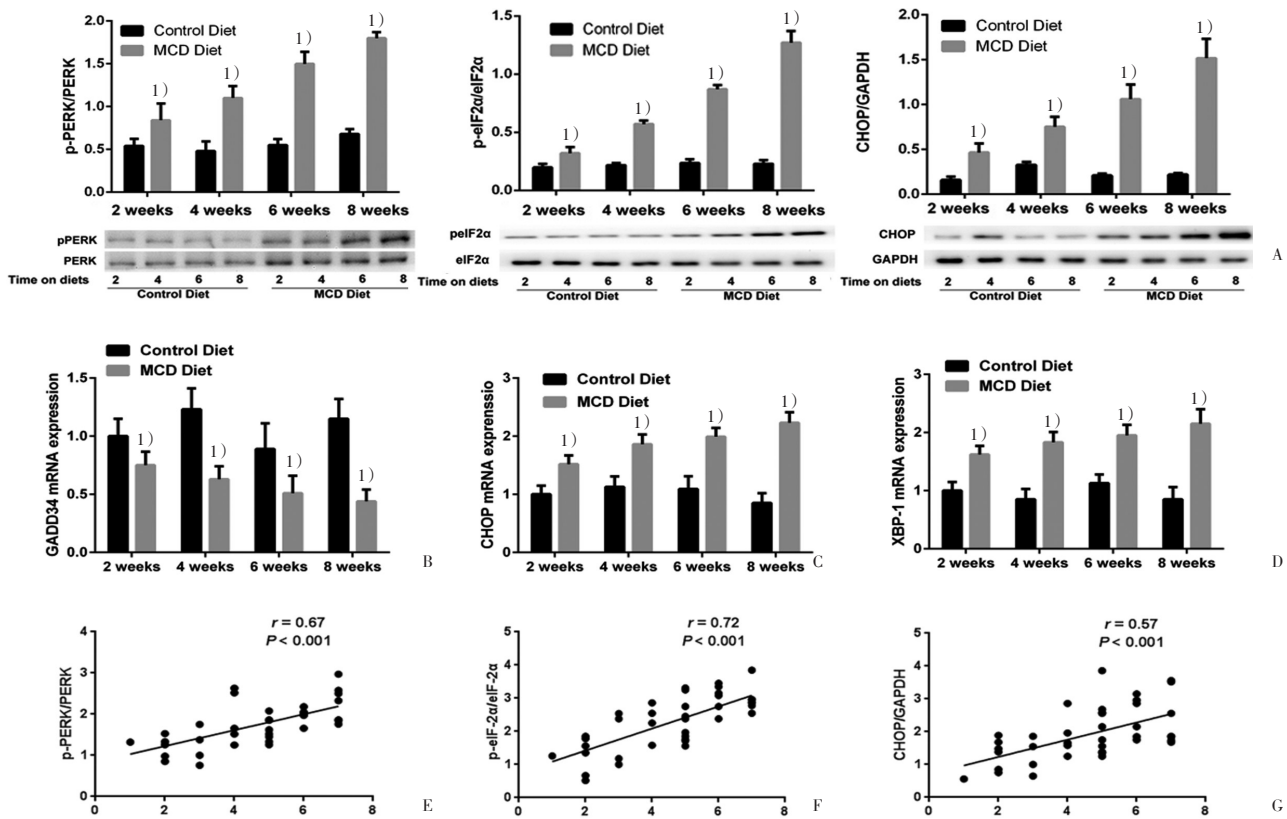
本研究结果显示,在MCD膳食喂养的小鼠



Q-PCR showed that the mRNA expression of IL-6, TNF- $\alpha$  and MCP-1 is much higher in the MCD diet compared with the normal diet (4D-E). Also the level of IL-6, TNF- $\alpha$  and IL-17A in serum showed the same trend (4A-C). 1)  $P < 0.05$  compared with the normal diet.

图4 血浆与肝脏炎症水平

Fig.4 The level of inflammation in serum and liver



Western blot results showed that hepatic protein expression of p-PERK, p-eIF2 $\alpha$  and CHOP in MCD group were significantly enhanced compared with the normal diet (5A). The mRNA expression of CHOP and XBP-1 Q-PCR showed the same trend (5C-D). While the expression of GADD34 was lower in the MCD diet (5B). 1)  $P < 0.05$  compared with the normal diet. p-PERK/PERK, p-eIF2/eIF2 and CHOP/GAPDH were positively correlated with the NAS score which was suggesting that there was a strong correlation between ER stress and liver injury.

图5 内质网应激相关指标

Fig.5 The index of ERS

中,其血清ALT、AST和肝脏TG、TC水平均显著高于普通膳食组,相关的NAS评分、炎症因子IL-6、IL-17A、TNF- $\alpha$ 和MCP-1水平以及纤维化指标 $\alpha$ -SMA、Col1A1的表达亦有同样趋势,提示MCD喂养的小鼠较普通膳食组其肝脏损伤加重,炎症和纤维化程度加深。

内质网(endoplasmic reticulum, ER)是细胞内十分重要的细胞器,它是蛋白质合成、储存、加工的场所,同时也是脂质合成的场所。当未折叠或者错误折叠蛋白质在内质网中累积亦或是内环境中钙稳态失调,都会损伤内质网的正常生理功能,从而引起内质网应激(ER stress, ERS)。发生ERS时,原本与糖调节蛋白78(glucose regulated protein 78, GRP78)结合的三种转录因子PERK、ATF6、IRE-1与之解离,解离后的转录因子活化并启动

相应的信号转导途径。其中IRE-1激活后剪接由ATF6诱导表达的前体mRNA分子内26 bp的内含子,编码产生一个含b-ZIP结构域的转录因子XBP-1<sup>[10]</sup>,XBP-1参与错误折叠蛋白的降解,从而恢复细胞稳态,是内质网应激促生存途径。另一方面,如果损伤过于严重,内质网应激会通过PERK-eIF2 $\alpha$ -CHOP途径、IRE1-JNK-Bcl2途径和caspase 12途径引起细胞凋亡。大量的研究表明CHOP活化在促进细胞凋亡的同时还会导致组织损伤<sup>[11-13]</sup>。本研究结果显示,MCD组小鼠肝脏XBP-1mRNA的表达高于普通膳食组,但是随着喂养时间延长,其表达并无明显增加,提示MCD喂养在最早期即启动了内质网促生存途径,参与了恢复内环境稳态,而由于内质网应激的持续存在,XBP-1的促细胞生存途径被抑制。同时与促

凋亡途径相关的 p-PERK、p-eIF2 $\alpha$  和 CHOP 蛋白在 MCD 膳食小鼠中表达较正常膳食组高,且随着喂养时间的延长,其表达量增加。另一方面,能够扭转内质网应激所致的细胞凋亡,帮助恢复内质网合成功能的 GADD34 因子表达减弱,且随喂养时间呈下降趋势,提示内质网应激诱导的 PERK-eIF2 $\alpha$ -CHOP 促细胞凋亡途径被激活且随着喂养时间的延长,其促凋亡能力持续不断加强。综上所述可以发现,在 MCD 喂养的小鼠中,由内质网应激导致的促细胞生存途径以及促凋亡途径同时被激活。而两者之间的失衡或是 MCD 诱导的肝损伤的重要原因。

非酒精性脂肪肝的发病机制相当复杂,本实验仅仅揭示了内质网应激参与了 MCD 诱导的小鼠非酒精性脂肪肝的进程,阻断内质网应激或许对非酒精性脂肪肝的治疗有着潜在的意义。

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