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基础研究 BASIC RESEARCH

# 氧应激对大鼠肝星状细胞增殖的影响及还原型谷胱甘肽的抗氧化作用

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■背景资料
肝星状细胞(HSC)在肝纤维化的形成过程中起重要作用, "二次打击"学说认为, 氧应激和脂质过氧化是脂肪肝和肝纤维化发病的重要机制之一, 可通过不同途径激活HSC. 还原型谷胱甘肽是一种生理活性物质, 可通过巯基与体内的自由基相结合, 起到清除自由基的作用.

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## Effects of oxidative stress on proliferation of rat hepatic stellate cells and antioxidation of reduced glutathione

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## Abstract

**AIM:** To explore the effects of oxidative stress on the proliferation of rat hepatic stellate cells and the antioxidation of reduced glutathione.

**METHODS:** Rat hepatic stellate cells were incubated with different concentrations of ferric nitrilotriacetic acid (Fe-NTa). With 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphennylterazolium bromide (MTT) colorimetric assay, the effects of Fe-NTa on the proliferation of hepatic stellate cells at 6, 12, 24 and 48 h was detected, and malondialdehyde (MDA) contents and superoxide dismutase (SOD) activity were also detected. At the same time, hepatic stellate cells were incubated with different concentrations of reduced glutathione (0.5, 2.5, 10 mmol/L), and MTT assay was used to SOD activity again.

**RESULTS:** In comparison with that in the blank control group at 12 h, the proliferation of hepatic stellate cells was significantly increased when the ferric nitrilotriacetic acid concentrations were 500 and 1000 μmol/L, respectively (*A* value: 0.369 ± 0.124, 0.485 ± 0.101 *vs* 0.285 ± 0.044, both $P < 0.01$); the proliferation of cells incubated with different concentrations of Fe-NTa was also markedly increased at 24 and 48 h ($P < 0.01$). The proliferation of hepatic stellate cells without Fe-NTa interference at 12, 24 and 48 h was also increased as compared with that at 6 h (*A* value: 0.285 ± 0.044, 0.253 ± 0.033, 0.278 ± 0.037 *vs* 0.111 ± 0.005, all $P < 0.01$), while with the elevation of Fe-NTa concentration, the proliferation of hepatic stellate cells at 12, 24 and 48 h was markedly increased as compared with that at 6 h ($P < 0.01$). In comparison with those in the control group, SOD activity significantly reduced (156.95 ± 21.17, 100.92 ± 10.02 μkat/L *vs* 197.74 ± 17.59 μkat/L, all $P < 0.01$) and MDA contents significantly increased (1123 ± 217, 1549 ± 182 mmol/L *vs* 580 ± 332 mmol/L, all $P < 0.01$) when the concentrations of Fe-NTa were 200 and 500 μmol/L. As compared with the model group (200 μmol/L Fe-NTa), the SOD activities in the cells preincubated with reduced glutathione were significantly increased (5.42 ± 0.58, 6.67 ± 0.18, 8.75 ± 0.58 μkat/L *vs* 2.25 ± 0.35 μkat/L, all $P < 0.01$).

**CONCLUSION:** Oxidative stress can significantly promote the proliferation of rat hepatic stellate cells in time- and concentration-dependent manners. Oxidative stress can also lead to lipid peroxidation, while reduced glutathione may play an anti-oxidative role.

Key Words: Oxidatie stress; Hepatic stellate cell; Proliferation; Reduced glutathione; Rats

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## 摘要

**目的:** 探讨氧应激对肝星状细胞增殖的影响及还原型谷胱甘肽的抗氧化作用.

**方法:** 分别用不同浓度的次氮基三乙酸铁(Fe-NTa)培养大鼠肝星状细胞, 在6, 12, 24和48 h用四甲基偶氮唑盐法(MTT法)检测氧应激对肝星状细胞增殖的影响, 检测丙二醛(MDA)含量和超氧化物歧化酶(SOD)活性; 并用不同浓度的还原型谷胱甘肽与Fe-NTa共同培养细胞, 再检测SOD活性.

**结果:** Fe-NTa作用12 h与同一时间点的空白对照组比较, 500和1000 μmol/L组细胞增殖明显增多(*A*: 0.369±0.124, 0.485±0.101 *vs* 0.285±0.044, *P*<0.01), 作用24和48 h各浓度组与同一时间点的空白对照组比较差异显著(*P*<0.01). 无Fe-NTa干预的空白对照组12, 24和48 h与同样处理的6 h组比较, 细胞增殖明显增多(*A*: 0.285±0.044, 0.253±0.033, 0.278±0.037 *vs* 0.111±0.005, *P*<0.01), 随着Fe-NTa剂量的增加, 作用12, 24和48 h与同样处理的6 h组比较, 细胞增殖亦明显增多(*P*<0.01). 200和500 μmol/L Fe-NTa组与对照组比较, SOD活力明显降低(156.95±21.17, 100.92±10.02 μkat/L *vs* 197.74±17.59 μkat/L, *P*<0.01); MDA含量明显升高(1123±217, 1549±182 mmol/L *vs* 580±332 mmol/L, *P*<0.01). 预先加入GSH的各组与模型组(200 μmol/L Fe-NTa)比较, SOD活力明显升高(5.42±0.58, 6.67±0.18, 8.75±0.58 μkat/L *vs* 2.25±0.35 μkat/L, *P*<0.01).

**结论:** 氧应激促进HSC增殖有剂量和时间依赖性; 且可导致脂质过氧化损伤, 还原型谷胱甘肽有抗氧化作用, 可对抗脂质过氧化损伤.

**关键词: 氧应激; 肝星状细胞; 增殖; 还原型谷胱甘肽; 大鼠**

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## 0 引言

大量研究表明, 肝星状细胞(HSC)在肝纤维化的形成过程中起重要作用. 在正常肝脏HSC是机体贮存维生素A的主要场所. 肝损伤时HSC被激活, 细胞内的维生素A含量逐渐减少, 细胞增殖逐渐明显, 其表型转化为肌成纤维细胞, 产生大量的细胞外基质(ECM)[1-3]. 脂肪肝发病机制的“二次打击”学说认为氧应激和脂质过氧化是脂肪肝及肝纤维化发生的基础机制[4-6]. 氧应激尤其是脂质过氧化产物例如丙二醛(MDA)等在HSC激活早期具有重要意义[7-9]. MDA可促进Ⅰ型胶原mRNA的表达, 提示氧应激与肝纤维化之间存在可能的联系[10]. 但是氧应激和脂质过氧化的强度及作用时间对HSC增殖的影响鲜见报道. 还原型谷胱甘肽(GSH)是一种广泛存在于正常细胞中的生理活性物质, 含有巯基, 可通过巯基与体内的自由基相结合. 我们以肝星状细胞株cFSC为研究对象, 研究氧应激不同强度及作用时间对HSC增殖的影响及GSH的抗氧化作用, 进一步完善脂肪肝发病机制的“二次打击”学说, 并为GSH在脂肪肝和肝纤维化中的应用提供理论和实验依据.

## 1 材料和方法

1.1 *材料* 大鼠肝星状细胞株cFSC由上海长征医院实验诊断科惠赠, 其表型为活化的HSC; 高糖DMEM培养基为美国Gibco公司产品; 胎牛血清为北京鼎国生物技术有限公司产品; 胰蛋白酶、MTT和二甲基亚砜(DMSO)为美国Sigma公司产品; 细胞上清液SOD, MDA检测试剂盒购自南京建成生物工程研究所; 细胞裂解液SOD检测试剂盒-WST购自日本株式会社同仁化学研究所; 次氮基三乙酸铁(Fe-NTa)由0.1 mol/L的$FeNO_3$ (Sigma公司)和$Na_2NAC$ (Fluka公司)两种母液混合配制而成, $NaHCO_3$调节pH至7.4, 0.22 μmol/L的微孔滤器过滤, 现配现用; GSH粉剂(商品名为阿莫拓兰)由重庆药友制药有限责任公司提供, 用含2 mL/L胎牛血清的DMEM培养液溶解, 配制成浓度为10 mmol/L的母液, 0.22 μmol/L微孔滤器过滤后, 加药前用培养液稀释为各所需浓度, 现用现配, 配好的药物保存不超过6 h.

1.2 *方法*

1.2.1 MTT*增殖实验* 取对数生长期的cFSC, 2.5 g/L胰酶消化后, 用含100 mL/L胎牛血清的DMEM配成细胞悬液, 接种于96孔培养板, 密度为$5\times10^7$/L, 每孔加入DMEM培养液100 μL, 置于37℃, 50 mL/L $CO_2$培养箱中培养过夜. 24 h

■*研发前沿*
氧应激和脂质过氧化激活肝星状细胞的途径和机制是肝纤维化研究领域中的热点.

**■应用要点**
本研究证明还原型谷胱甘肽可对抗氧应激导致的自由基损伤, 有抗氧化作用, 为还原型谷胱甘肽在临床上应用于脂肪肝和肝纤维化的治疗提供了理论和实验依据, 也为继续研究其抗纤维化作用打下了理论基础.

表 1 不同浓度和不同时间Fe–NTa对HSC增殖的影响 (mean ± SD)

| Fe–NTa浓度 (μmol/L) | 6 h | 12 h | 24 h | 48 h |
|---|---|---|---|---|
| 0 | 0.111 ± 0.005 | 0.285 ± 0.044[d] | 0.253 ± 0.033[d] | 0.278 ± 0.037[d] |
| 50 | 0.119 ± 0.006 | 0.298 ± 0.097[d] | 0.567 ± 0.133[bd] | 0.743 ± 0.066[bd] |
| 100 | 0.119 ± 0.006 | 0.297 ± 0.089[d] | 0.549 ± 0.187[bd] | 0.734 ± 0.103[bd] |
| 200 | 0.125 ± 0.008 | 0.331 ± 0.126[d] | 0.469 ± 0.167[bd] | 0.873 ± 0.197[bd] |
| 500 | 0.112 ± 0.007 | 0.369 ± 0.124[bd] | 0.452 ± 0.084[bd] | 1.049 ± 0.222[bd] |
| 1000 | 0.106 ± 0.012 | 0.485 ± 0.101[bd] | 0.526 ± 0.150[bd] | 1.546 ± 0.238[bd] |

[b]$P$<0.01 *vs* 空白对照组; [d]$P$<0.01 *vs* 6 h.

后细胞贴壁良好, 换用含2 mL/L胎牛血清的DMEM培养, 使其生长同步化. 再培养24 h后加入不同浓度的Fe-NTa产生氧应激, 设50, 100, 200, 500, 1000 μmol/L 5个浓度梯度, 每个浓度设10复孔, 同时设空白对照组. 分别于继续培养6, 12, 24, 48 h后每孔加入MTT 20 mL, 置37℃, 50 mL/L $CO_2$培养箱培养4 h后, 弃培养上清液, 每孔加入DMSO 100 μL, 振荡器振荡10 min酶联免疫检测仪上570 nm测$A$值, 实验重复2次.

1.2.2 检测细胞上清液中SOD活性和MDA含量 取对数生长期的cFSC, 2.5 g/L的胰酶消化后, 用含100 mL/L胎牛血清的DMEM配成细胞悬液, 以5×10$^7$/L接种于24孔培养板, 每孔培养液为1 mL. 置于37℃, 50 mL/L $CO_2$培养箱中培养24 h后更换培养液并加入不同浓度的Fe-NTa (0, 100, 200和500 μmol/L), 每个浓度设6复孔. 24 h后检测细胞上清液中的SOD活性和MDA含量, 步骤按试剂盒要求进行, 本实验重复2次.

1.2.3 检测细胞裂解液中SOD活性 取对数生长期的cFSC以5×10$^8$/L接种于25 cm$^2$的细胞培养瓶, 每瓶5 mL培养液. 待细胞贴壁良好并同步化后, 更换培养液并加入不同浓度的GSH, 培养2 h后加入不同浓度的Fe-NTa. 分别设空白对照组、模型组和药物干预组, 空白对照组不加任何药物, 模型组分别加入100, 200, 500 μmol/L的Fe-NTa, 药物干预组分为200 μmol/L Fe-NTa+0.5 mmol/L GSH, 200 μmol/L Fe-NTa+2.5 mmol/L GSH和200 μmol/L Fe-NTa+10 mmol/L GSH. 培养24 h后用刮刀刮下细胞, 4℃, 2000 $g$离心10 min, 弃上清. 1 mL冰PBS溶液洗净细胞, 随后4℃, 1000 $g$离心10 min, 弃上清. 重复该步骤1次. 在-20℃下放置20 min, 再在37℃温浴10 min. 重复该步骤2遍, 破碎细胞膜. 加入新的PBS 1 mL, 在4℃, 10 000 $g$下离心15 min. 取上清液用PBS稀释, 制成样品溶液. 用试剂盒检测细胞裂解液中的SOD活性, 步骤按试剂盒说明书进行, 本实验重复2次.

**统计学处理** 计量资料以mean±SD表示, 用SAS 6.12统计软件进行单因素变量的方差分析.

## 2 结果

2.1 MTT增殖实验 Fe-NTa作用6 h对HSC的增殖无明显影响, 在12 h其促进HSC增殖作用逐渐明显, 500和1000 μmol/L组与空白对照组比较有显著差异($P$<0.01). 随着时间的延长, Fe-NTa作用24 h和48 h各浓度组与空白对照组比较均有显著差异($P$<0.01). 无Fe-NTa干预的空白对照组12, 24和48 h之间无明显差异, 但与6 h组比较均有显著差异($P$<0.01). 随着药物剂量的增加, 其他各浓度组的差异越来越显著(表1).

2.2 细胞上清液中SOD活性和MDA含量 不同浓度Fe-NTa对HSC培养上清液中的SOD活性的影响各不相同. 100 μmol/L组与对照组比较无明显差异. 200和500 μmol/L组有明显的降低SOD活性的作用, 与对照组或100 μmol/L组比较均有明显差异($P$<0.05或$P$<0.01). 200与500 μmol/L比较也有显著差异($P$<0.01). 各浓度组均有升高HSC培养上清液中MDA的作用, 与对照组比较有显著差异($P$<0.01). 而且500 μmol/L组与较低剂量组(100和200 μmol/L)比较有显著差异($P$<0.01, 表2).

2.3 细胞裂解液中SOD活性及GSH对SOD的影响 不同浓度Fe-NTa均可明显的降低HSC裂解液中SOD活性($P$<0.01, 表2). 而预先加入GSH的各剂量组均可升高SOD的活力, 与模型组比较有显著差异(200 μmol/L Fe-NTa+0.5 mmol/L GSH时, SOD活力为5.42±0.58 μkat/L; 200 μmol/L Fe-NTa+2.5 mmol/L GSH时, SOD活力为6.67±0.18 μkat/L; 200 μmol/L Fe-NTa+10 mmol/L GSH时, SOD活力为8.75±0.58 μkat/L. $P$<0.01).

## 3 讨论

大量基础和临床研究都显示反应性氧(ROS)和细胞膜的脂质过氧化与肝纤维化发生有关, 这表明氧应激是不同病因慢性肝病共同的发病因素[10-13]. 有研究从基因水平证实脂质过氧化可刺激HSC合成Ⅰ型胶原[14], 而且细胞色素P450 2E1来源的氧化物可促进HSC的增殖和胶原的合成[15-16]. 最近有研究表明, 黄嘌呤/黄嘌呤氧化酶系统产生的ROS能促进HSC的增殖, 此促进作用与基质金属蛋白酶-2 (MMP-2)的激活和表达有关[17-18], 而且在大鼠肺和皮肤成纤维细胞以及心肌成纤维细胞中也发现氧应激可调节MMP-2的表达[19-21]. 由此认为, 氧应激和脂质过氧化可通过不同的机制促进HSC的增殖, 胶原合成增加是HSC增殖的结果. 我们采用Fe-NTa与HSC在体外共同培养, 引起铁超载, 是较为理想的细胞氧应激模型[22]. 用不同浓度的Fe-NTa处理细胞, 在不同的时间点用MTT法检测细胞增殖活力, 结果发现, 随着药物浓度和时间的增加, 细胞增殖越来越明显. 这表明Fe-NTa导致的氧应激有促进HSC增殖的作用, 且随药物剂量的增加和作用时间的延长, 其促进增殖作用越来越明显, 呈现出一定的剂量和时间依从关系, 从铁超载这个不同的侧面再次验证了氧应激和脂质过氧化有促进HSC增殖的作用.

MDA是脂质过氧化的重要终产物之一, 反映了机体细胞受自由基攻击的严重程度, 可促进Ⅰ型胶原mRNA的表达, 与HSC增殖和胶原合成密切相关[23-25]; SOD是机体抗氧化损伤防御体系中最重要的抗氧化酶之一, 反映了机体清除氧自由基的能力[26-28], 所以MDA和SOD是一对相互影响的因素. 我们发现, 氧应激可明显升高MDA含量, 而细胞上清液和细胞裂解液中SOD活力都明显降低, 且呈现出一定的剂量依从关系. 这表明铁超载可产生大量的自由基和脂质过氧化物, 消耗了大量的SOD, 导致机体抗氧化损伤的防御机制受损, 不能清除过多的自由基和MDA等脂质过氧化产物[29]. 而HSC内MDA累积, 通过不同的途径激活HSC, 促进其增殖和合成胶原, 导致肝纤维化的发生. 于洪波 *et al*用高脂饮食喂饲大鼠也得出了类似的结果[30]. GSH含有巯基, 是体内最重要的抗氧化物之一, 是由谷氨酸、半胱氨酸和甘氨酸组成的一种三肽. 他在酶的催化下能与过氧化物和自由基相结合, 对抗氧化物的破坏作用. 我们用Fe-NTa造成氧应激模型, 然后用不同浓度的GSH共同培养细胞, 检测细胞裂解液中SOD的活力, 结果发现GSH可使SOD活力明显升高, 且有一定的剂量依从性. 这表明GSH可对抗氧应激, 保护细胞免受自由基损伤, 有抗氧化作用, 这为GSH在临床上用于脂肪肝和肝纤维化的治疗提供了理论和实验依据, 也为继续研究其抗纤维化作用打下了理论基础.

表 2 Fe-NTa对HSC培养上清液中SOD和MDA及HSC裂解液中SOD的影响 (mean ± SD)

| Fe-NTa浓度(μmol/L) | 培养上清液 | | HSC裂解液 |
|---|---|---|---|
| | SOD活力 (μkat/L) | MDA (mmol/L) | SOD活力 (μkat/L) |
| 0 | 197.74 ± 17.59 | 580 ± 332 | 15.84 ± 1.17 |
| 100 | 186.20 ± 20.44 | 1111 ± 227[b] | 3.17 ± 0.47[b] |
| 200 | 156.95 ± 21.17[bc] | 1123 ± 217[b] | 2.25 ± 0.35[b] |
| 500 | 100.92 ± 10.02[bdf] | 1549 ± 182[bdf] | 1.50 ± 0.23[b] |

[b]$P$<0.01 *vs* 对照组; [c]$P$<0.05, [d]$P$<0.01 *vs* 100 μmol/L; [f]$P$<0.01 *vs* 200 μmol/L.

■名词解释

“二次打击”学说: 初次打击使脂肪肝发生, 各种不同病因通过增加氧应激作为发病的基础机制, 促使来自分子氧的游离基或反应性氧化物(ROS)形成增多和脂肪酸氧化障碍, 导致肝细胞脂肪贮积; 第二次打击经脂质过氧化这一生化和结构破坏性反应的打击导致脂肪肝发生炎症、坏死和纤维化.

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**■同行评价**

本文分别用不同浓度的Fe-NTa培养大鼠肝星状细胞, 在不同时间点检测氧应激对肝星状细胞增殖的影响, 检测MDA和SOD活性, 分析氧应激对大鼠肝星状细胞增殖的影响及还原型谷胱甘肽抗氧化作用, 研究方法可行, 结论可靠, 有临床应用价值.


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