·论 著·

Retrorsine 对小鼠肝损伤后肝细胞增殖的影响

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[摘要] **目的**：比较倒千里光碱（retrorsine）对小鼠与大鼠肝损伤后肝细胞增殖的影响，探讨应用 retrorsine 建立小鼠肝细胞移植模型的可行性。**方法**：雄性小鼠和大鼠均各自分为 retrorsine 处理组和生理盐水注射组（未处理组），每组 30 只。Retrorsine 处理组动物分别接受 2 次（间隔 2 周）retrorsine 注射（小鼠剂量为 70 mg/kg，大鼠剂量为 30 mg/kg）；未处理组用生理盐水代替 retrorsine。末次注射 4 周后，所有的动物均予 CCl_4 注射（0.5 mg/kg），分别在 CCl_4 注射前即刻（记为 0 时）、注射后第 1、2、3、4、6 和 15 天取动物肝组织样本。H-E 染色检查动物肝组织病理学变化，Ki-67 染色检测动物肝细胞增殖情况。**结果**：H-E 染色发现 retrorsine 处理组大鼠肝组织出现明显的巨细胞增多、小胆管增生和小肝细胞增生并形成结节，而未处理组大鼠未出现这些表现，两组间有显著差异；然而，retrorsine 处理组及未处理组小鼠肝组织均表现为肝细胞变性、肝小叶中央静脉周围区坏死，未出现巨细胞增生，两组表现类似。Retrorsine 处理组大鼠 Ki-67 染色阳性肝细胞主要出现在小肝细胞结节中，CCl_4 注射后第 3 天 Ki-67 染色阳性肝细胞数明显少于未处理组（$P<0.05$）；而 retrorsine 处理组小鼠 Ki-67 阳性细胞计数几乎在各时间点均高于未处理组，尤其在 CCl_4 注射后第 4 天（$P<0.001$），两组变化趋势一致。**结论**：应用 retrorsine 可明显抑制大鼠肝损伤后肝细胞增殖，适用于建立大鼠肝细胞移植模型；retrorsine 对小鼠肝损伤后肝细胞增殖无明显抑制作用，不适用于建立小鼠肝细胞移植模型。

[关键词] 倒千里光碱；小鼠；大鼠；肝细胞；细胞增殖
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Influences of retrorsine on mouse hepatocyte proliferation after liver injury

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[**ABSTRACT**] **Objective**: To compare the influences of retrorsine on hepatocytes proliferation in mice and rats after liver injury, so as to investigate the feasibility of using retrorsine for establishment of liver cell transplantation model in mice. **Methods**: Male mice and rats were pretreated with 2 injections of retrorsine (70 mg/kg for mice and 30 mg/kg for rats) (as retrorsine-treated group, $n=30$) at 2 weeks interval or saline (as non-treated group, $n=30$). A single injection of carbon tetrachloride (CCl_4, 0.5 mg/kg) was given to all animals 4 weeks after the final injection of retrorsine or saline. At 0(before administration), 1, 2, 3, 4, 6, 15 d after CCl_4 administration, the animals were sacrificed and their livers were subjected to H-E staining and Ki-67 antibody immunohistochemistry analysis to evaluate the pathological changes and hepatocyte proliferation. **Results**: The liver in rats treated with retrorsine displayed obvious megalocytosis, small bile duct hyperplasia, and small hepatocyte hyperplasia (forming nodule); no such changes were found in non-treated group. However, the liver in mice treated with retrorsine displayed hepatocyte degeneration and necrosis in the perivenous areas and the same was true to the liver in non-treated mice. Ki-67 immunohistochemistry analysis showed that in rats treated with retrorsine, the positive hepatocytes, mainly found in small hepatocyte nodules, were obviously less than those in control group 3 d after CCl_4 administration ($P<0.05$). Ki-67 positive hepatocytes in mice treated with retrorsine were abundant and almost more than those in control group at all time points, especially 4 d after CCl_4 administration ($P<0.01$), with the same changing tendency in both groups. **Conclusion**: Retrorsine can obviously inhibit hepatocyte proliferation after liver injury and is suitable for liver cell transplantation in rats, while it is the contrary in mice.

[**KEY WORDS**] retrorsine; mouse; rat; hepatocytes; cell proliferation
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肝细胞移植可用来治疗肝变性疾病或其他严重的肝损伤[1,2]。在人和其他脊椎动物中观察到，内源性肝细胞的再生能力非常强，胜过外源性细胞[3]。倒千里光碱（retrorsine）是一种吡咯烷类生物碱家族中的化学药物，可以损伤肝细胞的增殖能力，将细胞阻滞在细胞周期中的 G_1/S 期、S 后期和（或）G_2/M 期[4~6]。本研究通过观察 retrorsine 对小鼠肝损伤后肝细胞增殖的影响，探讨其是否适用于小鼠肝

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细胞移植模型。

1　材料和方法

1.1　*实验动物与试剂*　雄性 C57BL/6J 小鼠(6 周龄)和雄性 F344 大鼠(5 周龄)购自中国啮齿类实验动物中心上海分部。动物饲养条件为标准的 12 h 光照/12 h 黑暗节律，所有动物都用标准饲料和饮水饲养。所有动物操作遵循上海第二医科大学实验动物操作条例。retrorsine(Sigma-Aldrich)加入无菌蒸馏水至 10 mg/ml，用 1 mol/L HCl 调节 pH 至 2.5，使药物粉末全部溶解；再用 1 mol/L NaOH 中和，最后加入 0.15 mol/L NaCl；终浓度 retrorsine 为 5 mg/ml，pH 7.0。CCl_4 用无菌矿物油按 1∶10 稀释，保存在带橡皮塞的玻璃瓶中。Ki-67(克隆 SP6)兔单克隆抗体购自 Lab Vision 。

1.2　*动物分组*　动物适应性喂养 1 周后，各自随机分为 2 组：retrorsine 处理组、生理盐水注射组(未处理组)，每组 30 只。Retrorsine 处理组动物腹腔注射 retrorsine(小鼠 70 mg/kg[7]，大鼠 30 mg/kg[4,6])，共注射 2 次，每次间隔 2 周；未处理组动物用生理盐水代替 retrorsine。末次注射 4 周后，所有动物均予 CCl_4 腹腔注射，剂量为 0.5 ml/kg[7,8]。分别在 CCl_4 注射前即刻(记为第 0 天)，注射后第 1、2、3、4、6、15 天各取 3～5 只动物处死，取肝脏组织固定于 40 g/L 甲醛溶液中，进行病理学观察。

1.3　*肝组织病理学观察*

1.3.1　*H-E 染色观察*　肝脏标本经梯度乙醇脱水，石蜡包埋切片，厚度为 5 μm，经常规 H-E 染色分析。

1.3.2　*肝脏组织 Ki-67 染色分析*　将组织切片在 0.01 mol/L 柠檬酸钠缓冲液 (pH 6.0) 中，95℃ 30 min 进行抗原修复；切片与一抗(Ki-67 抗体)4℃孵育过夜，二抗为辣根过氧化物酶标记的羊抗兔 IgG 抗体(Jackson Immunoresearch)，DAB 为显色底物[9]。镜下见棕黑色小点为 Ki-67 染色阳性。每个标本都在 20 倍放大倍数下连续计数 10 个视野中的 Ki-67 阳性肝细胞数，将数据输入 Sigmaplot 软件中进行作图，每个时间点数据由 3～5 只动物的 Ki-67 阳性肝细胞数组成。

1.4　*统计学处理*　计量数据用 $\bar{x}\pm s_{\bar{x}}$ 表示，数据分析和作图使用 Sigmaplot 和 SAS 6.12 软件，参数分析采用 t 检验或 χ^2 分析。

2　结　果

2.1　*H-E 染色结果*　CCl_4 注射前，retrorsine 处理的大鼠和小鼠肝组织形态学观察没有明显改变。注射 CCl_4 后第 1 天，retrorsine 处理组大鼠肝脏可见严重的肝细胞气球样变和中央静脉周围区肝细胞坏死(图 1A)，随后出现巨细胞增生、小胆管增生和小肝细胞增生，常呈结节状(图 1B、1C)。而未处理组中，没有发现类似现象，仅出现肝脏 CCl_4 损伤的病理表现，在中央静脉周围区均可见明显的肝细胞坏死(图 1D～1F)。

CCl_4 注射后第 2 天，retrorsine 处理组(图 2A～2C)、未处理组小鼠(图 2D～2F)仅出现肝脏 CCl_4 损伤的病理表现，在中央静脉周围区均可见明显的肝细胞坏死，此后门静脉周围的肝细胞开始增殖，可见肝细胞分裂相，未发现大鼠肝脏损伤时出现的巨细胞增生等病理改变；15 d 后，两组小鼠的肝实质均恢复正常。

2.2　*Ki-67 免疫组织化学染色结果*　在 CCl_4 注射前，retrorsine 处理的大鼠和小鼠肝组织中均可见少量的 Ki-67 阳性细胞。retrorsine 处理组大鼠 Ki-67 阳性肝细胞数增长缓慢，CCl_4 注射后第 6 天达到高峰，大多数阳性细胞是位于结节中的小肝细胞(图 3A、3B)；而未处理组大鼠 Ki-67 阳性的肝细胞在第 3 天最多(图 3C、3D)。

CCl_4 注射后第 4 天，retrorsine 处理组小鼠 Ki-67 阳性肝细胞达高峰，几乎所有的 Ki-67 阳性细胞均为成熟细胞(图 4A～4C)；而未处理组，Ki-67 阳性肝细胞的高峰出现在第 2 天(图 4D～4F)。

2.3　*Ki-67 阳性细胞数的时间变化曲线*　retrorsine 处理组大鼠 Ki-67 阳性肝细胞数增长缓慢，而未处理组在 CCl_4 处理后第 3 天，Ki-67 阳性肝细胞数明显增多，明显高于 retrorsine 处理组($P<0.05$)，其余各时间点两者无显著差异；而 retrorsine 处理组小鼠 Ki-67 阳性细胞计数几乎在各时间点均高于未处理组，其中以 CCl_4 注射后第 4 天的计数差异和统计学差异最为明显($P<0.001$)，但两者变化趋势一致(图 5)。

3　讨　论

以往研究[4,10]已经成功地应用 retrorsine 来抑制大鼠宿主肝细胞的增殖，并详尽地描述其在大鼠肝细胞抑制过程中的病理变化。我们在应用 retrorsine 抑制小鼠内源性肝细胞增殖后的肝细胞移植中，没有观察到与 Guo 等[7]相似的结果。因此，本研究以大鼠作比较，观察小鼠在接受 retrorsine 和 CCl_4 注射后，肝脏组织的病理改变和肝细胞增殖的动力学变化。

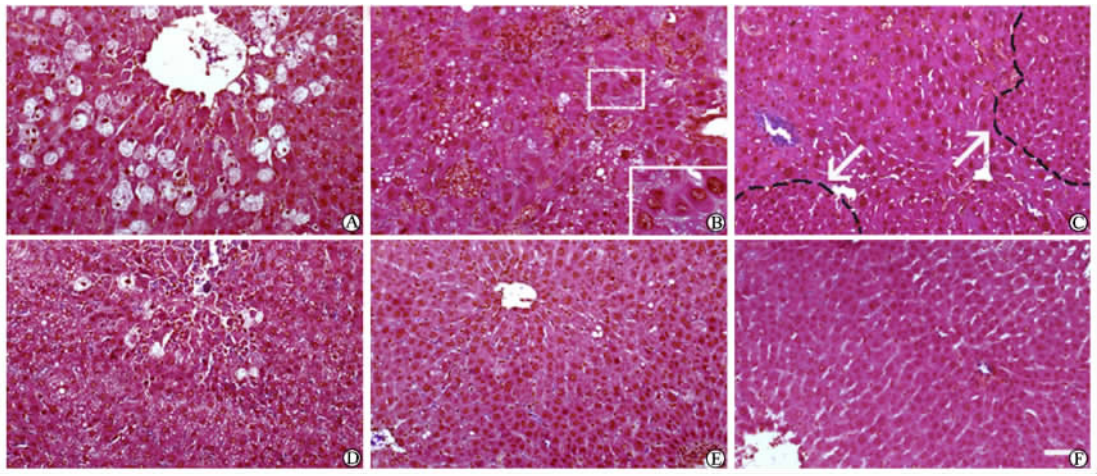

图 1 大鼠肝脏 H-E 染色

Fig 1 H-E staining of rat liver (Scale bar, 100 μm)

A, B and C were the results of retrorsine-treated rat liver on day 1, 6 and 15, respectively; D, E and F were the results of non-treated rat liver on day 1, 6 and 15, respectively. More severe hepatocyte balloon degeneration and necrosis were seen in the perivenous areas in retrorsine-treated rats (A) compared with those in non-treated group (D). (B) Small bile ductular hyperphasia and megalocytosis (the insert showed the area enclosed in the box at high magnification). (C) The proliferation of small hepatocytes (forming nodules) (arrows). (E and F) No obvious pathological changes

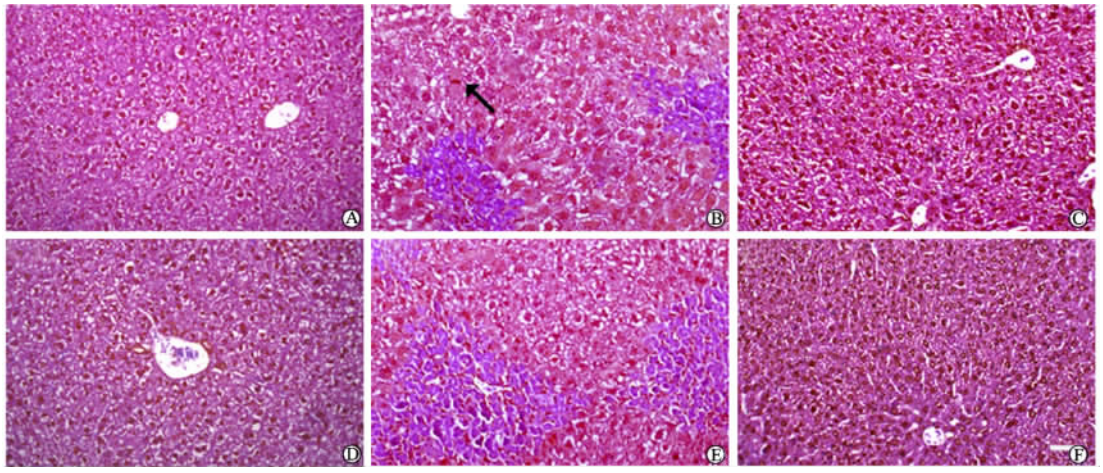

图 2 小鼠肝脏 H-E 染色

Fig 2 H-E staining of mouse liver (Scale bar, 100 μm)

A, B and C were the results of mouse liver in retrorsine-treated group on day 0, 3 and 15, respectively; D, E and F were the results of mouse liver in non-treated group on day 0, 3 and 15, respectively. (A and D) No obvious morphological abnormity. (B and E) Necrosis in perivenous areas; arrow indicates the mitotic figure of a hepatocyte. (C and F) Hepatic parenchyma of both groups became normal

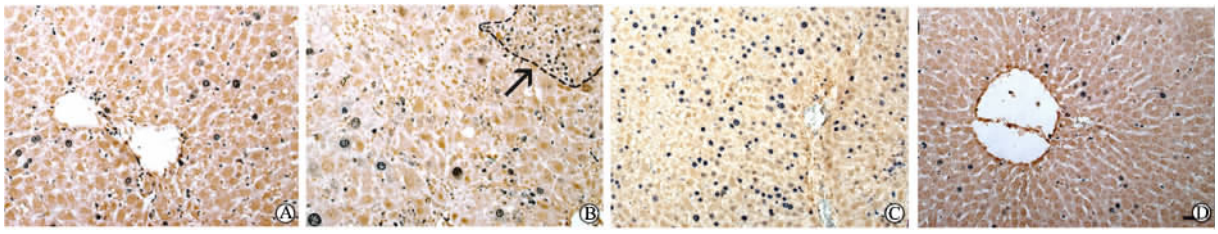

图 3 大鼠肝脏 Ki-67 免疫组织化学染色

Fig 3 Ki-67 immunohistochemical staining of rat liver(Scale bar, 100 μm)

A and B were the results of rat liver in retrorsine-treated group on day 3 and 6, respectively; C and D were the results of rat liver in non-treated group on day 3 and 6, respectively. (A) A few hepatocytes were Ki-67 positive. (B) Ki-67 positive hepatocytes were mainly found in a small hepatocyte nodule as indicated by arrow. (C) Abundant Ki-67 positive cells. (D) Only a few hepatocytes were Ki-67 positive

图 4 小鼠肝脏 Ki-67 免疫组织化学染色

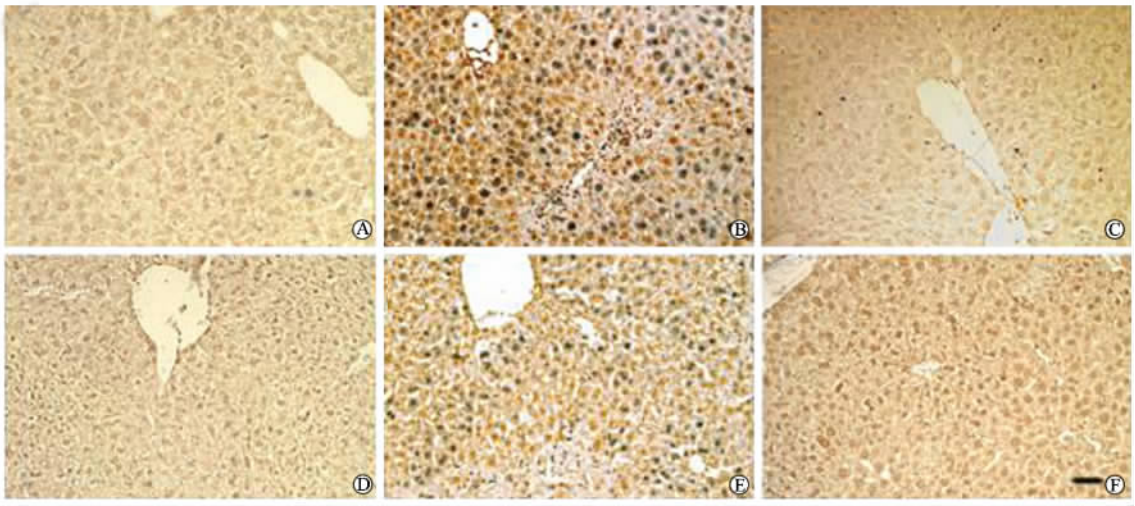

Fig 4　Ki-67 immunohistochemical staining of mouse liver(Scale bar, 100 μm)

A, B and C were the results of retrorsine-treated mouse liver on day 0, 4 and 15, respectively; D, E and F were the results of non-treated mouse liver on day 0, 2, and 15, respectively. Some Ki-67 positive cells appeared in retrorsine-treated mice on day 0 (A) and day 15 (C). The maximum number of Ki-67 positive cells appeared on day 4 in retrorsine-treated mice (B) and on day 2 in non-treated group(E)

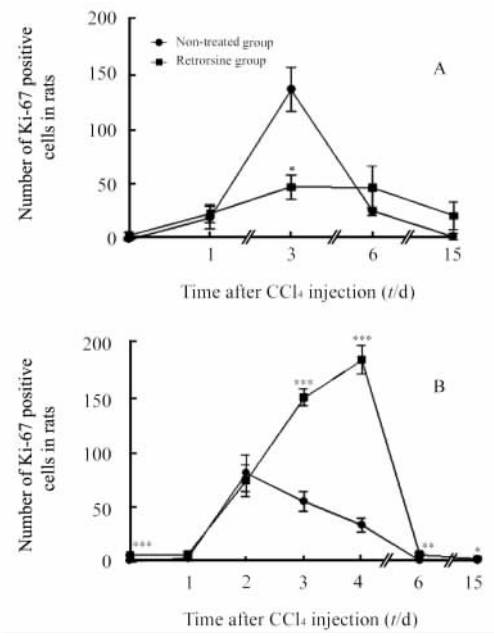

图 5　CCl_4 注射后大鼠(A)和小鼠(B)Ki-67 的动态表达

Fig 5　Kinetic expression of rat(A) and mouse(B) Ki-67 after CCl_4 injection

A: The maximum number of Ki-67 positive hepatocytes in retrorsine-treated rats appeared on day 6 after injection of CCl_4, and on day 3 in the non-treated group; B: The number of Ki-67 positive hepatocytes in retrorsine-treated mice was higher than that in non-treated group, and its peak was delayed. Data expressed as $\bar{x} \pm s_{\bar{x}}$ with $n = 30$-50 fields per group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* non-treated group

Guo 等[7]报道，90%以上的小鼠能耐受 2 次(间隔 2 周)retrorsine(70 mg/kg)处理，如果剂量超过 70 mg/kg，小鼠的死亡率会增加。因此，本研究 retrorsine 的剂量为 70 mg/kg。关于 retrorsine 溶剂的选择，我们应用同一剂量(70 mg/kg)乙醇和水来溶解 retrorsine 分别处理小鼠，两组存活率无显著差异($\chi^2=0.001$, $P=0.980$)，两组小鼠肝脏病理学和 Ki-67 染色也没有明显差别。因此，本研究采用水作 retrorsine 溶剂。

本研究结果显示，retrorsine 处理的大鼠在接受 CCl_4 注射后，肝脏出现巨细胞增生、轻度的胆管增生、小肝细胞增殖并形成结节，而在未处理组大鼠没有出现相应的表现，这与以往的研究[4,6,11]一致。本实验中 Ki-67 免疫组织化学染色分析显示，予 CCl_4 处理后第 3 天，retrorsine 处理组大鼠肝组织 Ki-67 阳性肝细胞数明显少于未处理组，与 Laconi 等[4]的结果相似。这些结果均表明应用 retrorsine 可明显抑制大鼠肝损伤后肝细胞增殖，同时也证明了本研究的实验体系是合适的。而与大鼠相反，本实验结果表明 retrorsine 处理组小鼠肝脏组织的病理学改变与未处理组相似；retrorsine 处理的小鼠肝脏中可见许多 Ki-67 阳性肝细胞，几乎在各时间点其数目均明显高于未处理组，尤其在 CCl_4 注射后第 4 天($P<0.001$)。

Guo 等[7]用增殖细胞核抗原(PCNA)检测肝细胞的增殖情况，显示小鼠单独接受 CCl_4 处理后有 60%以上的肝细胞增殖，这与本研究预实验的结果一致。该研究中却没有显示小鼠接受 retrorsine 和 CCl_4 处理后肝细胞增殖情况。

许多研究[4,6,12]表明在吡咯烷类生物碱处理的大鼠肝脏中常常有巨细胞增生，这一增生被认为是由于将增殖的肝细胞阻滞在 DNA 合成后期和有丝

分裂前期,因此,产生了含有大核的巨细胞。本研究结果显示,巨细胞增生只出现在接受 retrorsine 处理的大鼠肝脏中,而经相同处理的小鼠肝脏中未出现这种现象。小鼠接受 retrorsine 处理后 4 周肝脏 Ki-67 阳性肝细胞数明显高于未处理组($P<0.001$),而两组大鼠间却没有显著差异,与以往研究[13]一致。此外,前期预实验发现,末次 retrorsine 处理后第 2 周接受 CCl_4 注射的大鼠其死亡率高于 retrorsine 处理后第 4 周接受 CCl_4 的大鼠。而对于小鼠,两种处理方式的死亡率却没有明显差异。以上这些结果均提示 retrorsine 对小鼠的作用与对大鼠不同。已有报道[14,15]显示不同的物种对吡咯烷类生物碱有不同的敏感性,主要由于有毒代谢产物的形成和解毒途径不同。虽然大鼠、小鼠同属于啮齿类,但二者的肝脏对吡咯烷类生物碱可能具有不同的代谢和解毒过程,这可能导致小鼠对吡咯烷类生物碱具有抗药性。基于本研究结果,小鼠接受 retrorsine 和 CCl_4 联合处理后,肝脏 Ki-67 阳性肝细胞数目明显高于未处理组,这可能是由于 retrorsine 提高了小鼠肝脏对 CCl_4 损伤的敏感性,因为细胞增殖反应与细胞所受到的损伤程度相关。

总之,应用 retrorsine 可明显抑制大鼠肝损伤后肝细胞增殖,能够建立大鼠肝细胞移植模型;而 retrorsine 对小鼠肝损伤后肝细胞增殖无明显抑制作用,不适用于建立小鼠肝细胞移植模型,相关机制有待进一步探讨。

[参 考 文 献]

[1] Fausto N. Liver regeneration[J]. J Hepatol,2000,32(1 Suppl):19-31.

[2] Ouyang EC, Wu CH, Walton C,et al. Transplantation of human hepatocytes into tolerized genetically immunocompetent rats[J]. World J Gastroenterol,2001,7:324-330.

[3] Overturf K, al-Dhalimy M, Ou CN,et al. Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes[J]. Am J Pathol,1997,151:1273-1280.

[4] Laconi E, Oren R, Mukhopadhyay DK,et al. Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine[J]. Am J Pathol,1998,153:319-329.

[5] Samuel A, Jago MV. Localization in the cell cycle of the antimitotic action of the pyrrolizidine alkaloid, lasiocarpine and of its metabolite, dehydroheliotridine[J]. Chem Biol Interact,1975,10:185-197.

[6] Picard C, Lambotte L, Starkel P, et al. Retrorsine: a kinetic study of its influence on rat liver regeneration in the portal branch ligation model[J]. J Hepatol,2003,39:99-105.

[7] Guo D, Fu T, Nelson JA,et al. Liver repopulation after cell transplantation in mice treated with retrorsine and carbon tetrachloride[J]. Transplantation,2002,73:1818-1824.

[8] Gupta S, Rajvanshi P, Aragona E,et al. Transplanted hepatocytes proliferate differently after CCl_4 treatment and hepatocyte growth factor infusion[J]. Am J Physiol,1999,276(3 Pt 1):G629-G638.

[9] Birner P, Ritzi M, Musahl C,et al. Immunohistochemical detection of cell growth fraction in formalin-fixed and paraffin-embedded murine tissue[J]. Am J Pathol,2001,158:1991-1996.

[10] Laconi S, Curreli F, Diana S,et al. Liver regeneration in response to partial hepatectomy in rats treated with retrorsine: a kinetic study[J]. J Hepatol,1999,31:1069-1074.

[11] Dahlke MH, Popp FC, Bahlmann FH,et al. Liver regeneration in a retrorsine/CCl_4-induced acute liver failure model: do bone marrow-derived cells contribute[J]? J Hepatol,2003,39:365-373.

[12] Laconi S, Pillai S, Porcu PP,et al. Massive liver replacement by transplanted hepatocytes in the absence of exogenous growth stimuli in rats treated with retrorsine[J]. Am J Pathol,2001,158:771-777.

[13] Avril A, Pichard V, Bralet MP, et al. Mature hepatocytes are the source of small hepatocyte-like progenitor cells in the retrorsine model of liver injury[J]. J Hepatol,2004,41:737-743.

[14] Huan JY, Miranda CL, Buhler DR, et al. Species differences in the hepatic microsomal enzyme metabolism of the pyrrolizidine alkaloids[J]. Toxicol Lett,1998,99:127-137.

[15] Chojkier M. Hepatic sinusoidal-obstruction syndrome: toxicity of pyrrolizidine alkaloids[J]. J Hepatol,2003,39:437-446.

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