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RAPID COMMUNICATION

Effects of retrorsine on mouse hepatocyte proliferation after liver injury

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Abstract

AIM: To study the effect of retrorsine on mouse hepatocyte proliferation.

METHODS: Mice and rats were treated respectively with two injections of retrorsine (as retrosine-treated group) or saline (as non-treated group) at 2 wk intervals. They received a single injection of carbon tetrachloride (CCl_4) 4 wk later. On d 0, 1, 2, 3, 4, 6, 15 after CCl_4 administration, the animals were killed and their livers were excised. Hematoxylin and eosin (HE) staining and Ki-67 antibody immunohistochemical analysis of liver samples were used to evaluate the pathological changes and hepatocyte proliferation.

RESULTS: In rats treated with retrorsine and CCl_4, the liver displayed obvious megalocytosis, proliferation of mild bile duct, small hepatocyte-forming nodule, which were not found in liver samples from non-treated group. However, in mice treated with retrorsine combined with CCl_4, the liver displayed hepatocyte degeneration and necrosis in perivenous areas. There was no obvious difference between retrorsine-treated group and non-treated group. Ki-67 immunohistochemical analysis showed that in rats treated with retrorsine, the positive hepatocytes mainly found in small hepatocyte nodules, were obviously less than those in non-treated group. The mice treated with retrorsine showed that the number of Ki-67 positive hepatocytes was very high and more than that in non-treated group.

CONCLUSION: Retrorsine has no effect on mouse hepatocyte proliferation.

Key words: Retrorsine; Mouse; Rat; Hepatocyte proliferation

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INTRODUCTION

Hepatocyte transplantation can not only treat liver degenerative disorders and other serious liver injuries, but also replace liver transplantation[1, 2]. Before clinical application, it is necessary to use an animal model to test whether exogenous liver cells can integrate and grow in the recipient liver. Observations in humans and other vertebrates demonstrated that native liver cells have a very high regenerative potential and outgrow the exogenous cells[3]. Therefore, in animal models for hepatocyte transplantation, it is very important to inhibit the proliferation of native hepatocytes. It has been reported that retrorsine, a member of the pyrrolizidine alkaloid (PAs) family can impair the proliferative capacity of mature hepatocytes. Retrorsine-induced blockade is in G1/S, late "S" and /or "G2/M" phase of cell cycle[4-6]. Laconi *et al*.[4] reported that syngeneic transplantation of hepatocytes in liver of dipeptidyl-peptidase type IV-deficient (DPPIV) rats treated with retrorsine could achieve 95% chimerism and restore its normal function. Although retrorsine has been used in rat model, there are very few reports on its use to create a mouse model for hepatocyte transplantation[7]. In this study, we investigated the effect of retrorsine on mouse mature hepatocytes. The results indicate that retrosine cannot inhibit mouse hepatocyte proliferation after liver injury.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (6 wk) and male F344 rats (5 wk) were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch, China. They were maintained in a 12 h light/dark cycle and fed with standard food and water *ad libitum*. All animals received humane care and study protocols complied with guidelines of Shanghai Second Medical University.

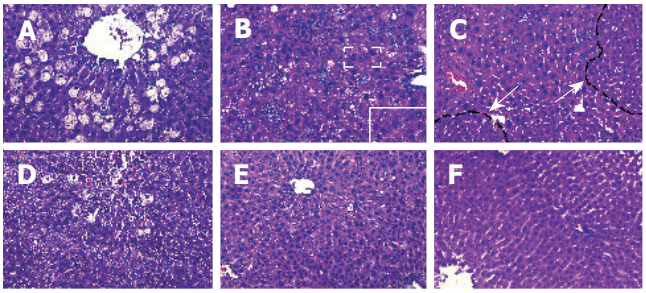

Figure 1 Pathological analysis of rat liver. **A**: Much more severe hepatocyte balloon degeneration and necrosis in perivenous areas of retrorsine-treated group compared with non-treated group (**D**). **B**: Mild bile duct proliferation and megalocytosis (the insert showed the area enclosed in the box at high magnification). **C**: Proliferation of small hepatocytes formed nodules. **E** and **F**: No obvious pathological change was found in non-treated group.

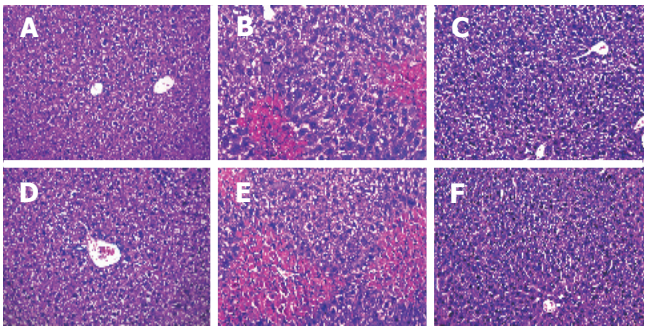

Figure 2 Pathological changes of mouse liver. **A** and **D**: No obvious morphological abnormity. **B** and **E**: Necrosis in perivenous areas, arrow indicates the mitotic figure of hepatocytes. **C** and **F**: Hepatic parenchyma in both groups became normal.

Reagents

Retrorsine (Sigma-Aldrich) was added to distilled water at 10 mg/mL and titrated to pH 2.5 with 1 mol/L HCl to dissolve it completely. The solution was neutralized using 1 mol/L NaOH. Subsequently NaCl was added. The final concentration was 5 mg/mL retrorsine and 0.15 mol/L NaCl, pH 7.0. The working solution was used immediately after preparation.

Carbon tetrachloride (CCl_4) was diluted 1:10 using sterile mineral oil and maintained in a rubber plug-sealed glass tube. Ki-67 (Clone SP6) rabbit monoclonal antibody, a cell proliferation marker[8] was purchased from Lab Vision.

Experimental groups

After one week of acclimatization, the mice and rats were randomly divided into two groups and received two intraperitoneal injections of retrorsine (70 mg/kg for mice[7] and 30 mg/kg for rats[4, 6] (as retrosine-treated group)) or saline (as non-treated group) at 2-week interval. Four weeks after the second injection, diluted CCl_4 was respectively injected into mice and rats, ip 5 mL/kg[7]. Day 0 was set just before CCl_4 injection. On days 1, 2, 3, 4, 6, and 15 after CCl_4 administration, 3-5 animals from each group were killed. The liver of animals was excised and fixed in 40 g/L formaldehyde for the following study.

Pathology and immunohistochemistry

Samples were dehydrated in alcohol and embedded in paraffin. Sections were cut at 5 μm thickness. For pathological analysis, the liver sections were stained with hematoxylin and eosin (HE) according to the standard procedures.

For Ki-67 immunohistochemical staining, antigen retrieval was carried out by incubating slides in antigen retrieval buffer (0.01 mol/L citrated buffer, pH 6.0) at 95 °C for 30 min. The slides were incubated with the primary antibody, Ki-67, at 4°C overnight. The secondary antibody used was anti-rabbit conjugated with horseradish peroxidase (Jackson Immunoresearch). 3,3-diaminobenzidine tetrahydro-chloride containing 0.1g/L hydrogen peroxide was used as a substrate. The proportion of Ki-67 positive hepatocytes was counted from at least 2000 cells from serial fields for each sample under microscope with 20× magnifications.

Statistics analysis

Data were expressed as mean±SE. Sigmaplot 2001 and SAS for windows 6.12 softwares were used for data analysis and plot. The significance of variances was found to be appropriate by Student's *t*- test or χ^2 test.

RESULTS

Pathological analysis

Before CCl_4 administration, liver morphology had no obvious change both in mice and in rats treated with retrorsine. After injecting CCl_4, much more severe hepatocyte balloon degeneration and necrosis in perivenous areas were found on day 1 in retrorsine-treated group than in non-treated group. Some megalocytosis, mild bile duct proliferation and small hepatocyte proliferation-formed nodules occurred in retrosine-treated group but not in non-treated group (Figure 1).

Livers of both retrorsine-treated and non-treated groups showed necrosis in perivenous areas on day 2 after CCl_4 injection. After then hepatocytes in periportal areas began to proliferate, mitotic figures of liver cells could be found. Such a pathological phenomenon might be due to destruction of CCl_4 in liver[9, 10]. There were no megalocytosis and other pathological changes in rats. On the 15th day, hepatic parenchyma in both groups became normal (Figure 2).

Ki-67 immunohistochemical analysis

Before CCl_4 injection, a small number of Ki-67 positive hepatocytes appeared in mice and rats treated with retrorsine. For the rats, the number of positive hepatocytes was increased slowly, reached the peak on day 6 in retrorsine-treated group and most of positive cells were small hepatocytes in nodules. In non-treated group, the maximum number of positive hepatocytes appeared on day 3 (Figures 3 and 5A).

The maximum number of positive hepatocytes was observed on day 4 in retrorsine-treated group of mice and almost all the positive cells were from mature hepatocytes. The maximum number of positive cells appeared on the 2nd day in non-treated group. Both groups showed a similar proliferation pattern (Figures 4 and 5B).

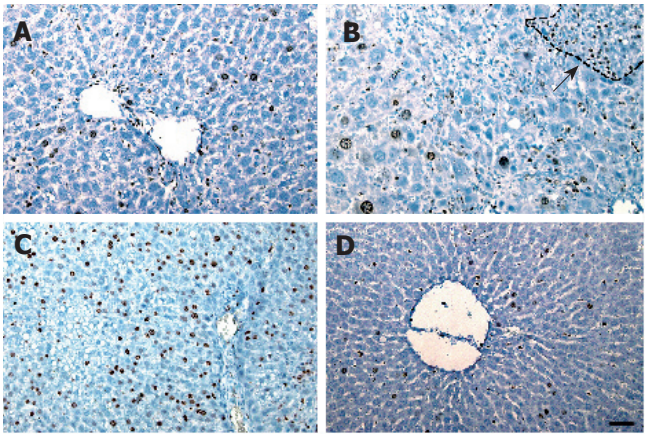

Figure 3 Ki-67 immunohistochemical analysis of rat liver. **A**: A few hepatocytes were Ki-67 positive. **B**: Ki-67 positive hepatocytes were mainly found in small hepatocyte nodules as the arrow indicated. **C**: Abundant Ki-67 positive cells. **D**: Only a few hepatocytes were Ki-67 positive in rat liver.

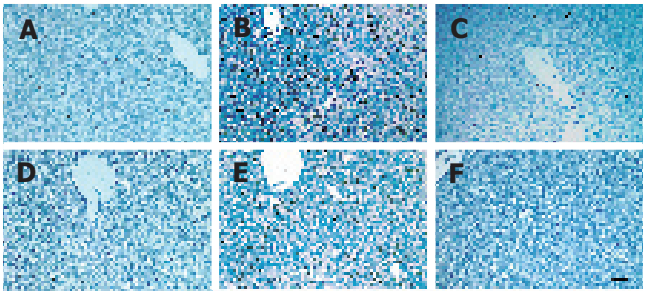

Figure 4 Ki-67 immunohistochemical analysis of mouse liver. **A** and **C**: Some Ki-67 positive cells appeared in retrorsine-treated mice on days 0 and 15. **B** and **D**: The maximum number of Ki-67 positive cells in retrorsine-treated mice on day 4. **E** and **F**: The maximum number of Ki-67 positive cells in non-treated group on day 2.

DISCUSSION

The present study described the comparative pathological changes and kinetic of hepatocyte proliferation in mice and rats treated with retrorsine and CCl_4. Guo *et al* [7] reported that two doses of retrorsine (70 mg/kg at 2 wk interval) could be tolerated by >90% of mice. If the dosage is over 70 mg/kg, the mortality rate of animals would increase. We used this dosage in our experiments and tested ethanol or water as a solvent for retrosine and treated mice with the same dosage (70 mg/kg). The survival rate was 85% (22/26) and 84% (27/32), respectively. No statistically significant difference was displayed between them ($\chi^2=0.001$, $P=0.980$), suggesting that only water can be used as a solvent for retrosine.

The rats treated with retrorsine and CCl_4 showed megalocytosis, mild bile duct proliferation and small hepatocyte proliferation-formed nodules. This phenomenon has been described by many authors[4, 6, 12-15]. But in mice treated with the same protocol, we did not find similar pathological changes. Guo *et al*[7] studied liver repopulation after cell transplantation in mice treated with retrorsine and CCl_4, and found that there are no liver pathological changes.

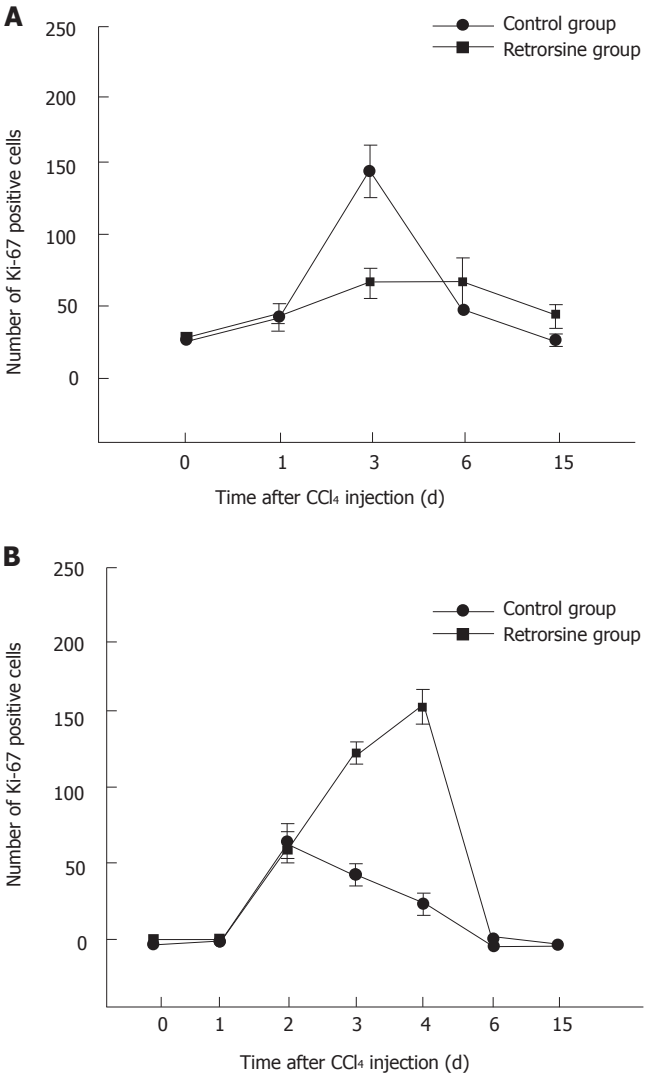

Figure 5 Kinetics of Ki-67 expression in rats (A) and mice (B) after CCl4 injection. **A**: The maximum number of Ki-67 positive hepatocytes on day 6 after injection of CCl4 in retrorsine-treated rats and on day 3 in non-treated group. **B**: The number of Ki-67 positive hepatocytes in retrorsine-treated mice was higher than that in non-treated group delayed. $^aP<0.05$, $^bP<0.01$, $^cP<0.001$ *vs* control group.

Ki-67 immunohistochemical analysis showed that proliferation of hepatocytes in rats treated with retrorsine was blocked. Avril *et al*[16] have reported similar results. Laconi *et al*[6] reported that BrdU labeling index of rats treated with retrorsine combined with partial hepatectomy is also significantly lower than that in non-treated group.

The number of Ki-67 positive hepatocytes in mice treated with the same protocol was higher than that in non-treated group. Guo *et al*[7] reported that the number of proliferating liver cells (detected by proliferating cell nuclear antigen, PCNA) in mice treated with CCl_4 alone is >60%. Our result is consistent with theirs. Since they did not show hepatocyte proliferation after retrorsine treatment combined with CCl_4, we considered that after liver injury mouse hepatocyte proliferation might not inhibited by retrorsine. Since Ki-67 positive hepatocytes in mice treated with retrorsine combined with CCl_4 was higher than that in non-treated group in our study, it is possible that retrorsine might increase the sensitivity of mouse liver to CCl_4 injury because the cell proliferation

response is always dependent on the extent of liver injury.

Retrorsine has long been known for its ability to block hepatocyte division[4-6]. Megalocytosis results from replicating hepatocytes which are blocked after DNA synthesis and prior to mitotic division, thus resulting in a large number of cells with enlarged nuclei (megalocytosis)[4, 6, 17]. Our results demonstrated megalocytosis was only present in livers of rats but not in livers of mice treated with retrorsine, indicating that the effect of retrorsine on mice is different from that on rats. Significant species difference in susceptibility to PAs intoxication has been reported, which is mainly due to the variations in balance between the formation of toxic metabolites and detoxification pathways[18-20]. Although both mice and rats belong to murine, they might have a different process of metabolism or detoxification of PAs in their livers, which may lead to resistance of mice to retrorsine. Moreover, rats receiving CCl_4 2 wk after the second injection of retrorsine displayed higher mortality rate than those receiving CCl_4 4 wk after the second injection of retrorsine (data not shown). There was no obvious difference in the mortality rate and other physiological indices in mice receiving CCl_4 2 wk or 4 wk after the second injection of retrorsine, which proved our hypothesis that retrorsine could seriously injure rat liver but not mouse liver.

As reported by Guo *et al*[7], when hepatocytes are transplanted into mice treated with retrorsine alone, the chimerism rate of exogenous liver cells is less than 1%. It was reported that the chimerism rate of rats treated with retrorsine alone could reach 95%[14]. It is possible that the effect of retrorsine on suppressing proliferation of mouse liver cells is limited in decreasing the chimerism rate. Therefore, retrorsine has no effect on mouse hepatocytes.

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