HISTOPATHOLOGICAL ANALYSIS OF DUCTULAR REACTION IN RABBIT LIVER AFTER CHRONIC EXPOSURE OF CHROMIUM (VI)

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ABSTRACT

Exposure to hexavalent chromium [Cr(VI)] results in hepatocellular damage. However, no research addresses the response of ductules cells, which harbor hepatic stem/progenitor cells, in Cr(VI) induced liver injury. The main goal of this study is to clarify the toxic effect of Cr(VI) on liver histopathological changes including hepatocellular damage and biliary ductular reaction after 3 months exposure in adult rabbits. Twenty four male and female rabbits were allotted randomly to three groups including control, low and moderate dose of Cr(VI) (fed with 0, 0.35 and 2.09 mg/kg/day Cr(VI), respectively). H&E and immunohistochemistry staining were utilized to analyze changes in the structure of the liver. Histopathological results showed that Cr(VI) exposure resulted in hepatocellular damage in a dose dependent manner. Ductular reaction was observable at the portal area as well as canal of Herings as determined by CK7 and CK19. In addition, low dose of Cr(VI) exposure generated more CK-positive cells compared to control and moderate dose of Cr(VI) exposure. Correspondingly, cholangiofibrosis was accompanied by an increase in the bile duct proliferation. To our knowledge, this is the first report that ductular reaction play an important role in the degree of damage and recovery that the liver undergoes following Cr(VI) intoxication.

Keywords: Cr(VI), Histopathology, Ductular Reaction, Liver, Rabbit.

DOI: 10.19193/0393-6384_2018_5_186

Introduction

Adverse health effects of Cr(VI) exposure include lung cancer, gastrointestinal symptoms, hypotension, and renal failure(1-3). In recent years, risk of exposure to Cr(VI) in non-occupational populations is increasing with the spread of Cr(VI) contamination. For example, due to the leakage of wastewater polluted by Cr(VI), the concentration of Cr(VI) in the groundwater was as high as 63.2 mg/L in some region of China(4). Liver is the primary organ for biotransformation of organic xenobiotics, and alterations in its structure can be significant in the evaluation of the health(5). Cr(VI) has been reported to cause severe liver effects in four of five workers exposed to chromium trioxide in the chrome plating industry(6,7). Based on the findings in animals, liver is an important site of cellular uptake of Cr(VI), and increased levels of chromium with dose were observed in the liver of mice(8). Cr(VI) primarily enters the hepatocytes and undergoes metabolic reduction to Cr(III), resulting in the formation of reactive oxygen species (ROS) together with oxidative tissue damage and a cascade of cellular events, including hepatocyte ultrastructure disruption and apoptosis(9-11). Histopathological analysis revealed Cr(VI) exposed liver resulted in necrosis of hepatocytes, increase of blood sinusoids dilation, and few vacuolated hepatocytes without usual polyhedral shape(12,13).
Small bile ducts at the portal and canals of Hering (CoH) in the parenchyma consist of, or harbor facultative hepatic stem/progenitor cells, which had the capacity to regenerate injured liver when the proliferation of hepatocytes was suppressed\(^{(14)}\). The appearance of ductular structures resembling bile ducts at the portal area was observed following severe liver damage\(^{(15)}\), which is known as the ductular reaction. Therefore, the appearance of ductular reactions often indicates activation and proliferation of hepatic stem/progenitor cells.

Liver tissue regeneration is based on two mechanisms\(^{(16)}\): one led by hepatocytes, and the other by hepatic stem/progenitor cells. These mechanisms are mutually exclusive, the former mechanism being responsible for regeneration processes in acute lesions, while the latter in chronic hepatoocyte lesions. To our knowledge, no research addresses the response of ductules cells in chronic Cr(VI) induced hepatocellular damage.

Laboratory rabbit has some advantages in the study of physiological disorders and toxicology field\(^{(17, 18)}\), especially in the field of hepatic function analysis. Not only can physiological manipulation in the rabbit be more easily carried out than those in mice (because of its larger size), but also it is phylogenetically closer to primates than mice and rat. Therefore, the main goal of this study was to clarify the toxic effect of Cr(VI) on liver histopathological changes including hepatocellular damage and biliary ductular reaction after chronic Cr(VI) exposure in adult rabbits.

**Materials and methods**

**Animals**

New Zealand white rabbits (12 males and 12 females, 2 ± 0.3 Kg in weight) were supplied by Experimental Animal Center of Shaoxing University and placed in the animal house of the Center. They were housed individually in cages under standard laboratory conditions with a period of 12 hours light/dark at 25 to 30 °C and 70 to 80% relative humidity in the animal house. They were allowed to acclimatize for at least 10 days before the start of the experiments. The rabbits were fed with a standard rabbit chow pellet and allowed to drink water ad libitum.

**Experimental Design**

Animals were assigned into three groups which are considered as control, low, and moderate dose of Cr(VI) (4 males and 4 females in each group). Animals in control group were given normal pellet without potassium dichromate (K2Cr2O7) (Haoxin Biotech CO., LTD, Hangzhou, China), while animals in the treatment groups were fed with different doses of potassium dichromate (K2Cr2O7) by syringe with a long thin plastic hose, respectively. Animals in the low and moderate dose group were fed with 0.35 and 2.09 mg/kg/day Cr(VI) for 3 months respectively. We also tried to feed the rabbits with high dose of Cr(VI) (9.05 mg/kg/day), but all rabbits died within 10 days of Cr(VI) exposure.

According to the Shaoxing University research regulation, all research projects dealing with human or animal subjects must be approved in advance by the university research ethics committee. The current project was approved by the committee as complying with the country and university research ethics (approval number: S20110018).

**Histological Examination**

All rabbits were sacrificed after 3 months exposure. Resected liver specimens were fixed in 10% buffered formaldehyde for 24 hours, and embedded into paraffin after 16 hours of alcohol process. Sections of 4-6 \(\mu\)m thick were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Nikon, Japan).

The following criteria were applied in grading the severity of the hepatic necrosis according to Blazka’s classification of liver damage\(^{(19)}\):

- **Grade 0**: Normal histology.
- **Grade 1**: Characterized by minimal congestion and necrosis/degeneration of hepatocytes; many of the lobules are not affected.
- **Grade 2**: Characterized by moderate congestion and necrosis/degeneration of hepatocytes; many of the lobules are affected.
- **Grade 3**: Characterized by serious congestion and necrosis/degeneration of hepatocytes; almost all of the lobules are affected.

For immunohistochemistry analysis of CK7 (Dako, Shanghai, China) and CK19 (Dako, Shanghai, China), antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) at a sub-boiling temperature for 10 minutes. After that, the endogenous peroxidase blocking through hydrogen peroxide incubation was performed. Then, slides were incubated with primary antibody for 60 minutes, and HRP (Dako, Shanghai, China) solution for 30 minutes. Development was performed with...
DAB (Dako, Shanghai, China) solution for 3 to 5 minutes. Nuclei were stained using Mayer’s Hematoxylin.

**Measurement of Cholangiofibrosis**

Photographs were taken at representative areas of liver tissue stained with CK-19. The thickness of myofibroblasts layer of individual bile duct was measured using Zeiss LSM image Examiner software according to the instruction (http://swehsc.pharmacy.arizona.edu/sites/swehsc.pharmacy.arizona.edu/files/docs/ci/lsm_image_browser_help.pdf).

**Statistical Analysis**

All data were expressed as mean ± standard error of the mean (SEM). Student’s t-test was used to determine the significance of differences in multiple comparisons. A test value of P < 0.05 was considered significant.

**Results**

**Histopathology of hepatic parenchymal change**

After 3 months exposure to Cr(VI), all animals in low dose group survived, exhibited normal behavior and gain of body weight, although hair loss was observed in some animals. In moderate dose group, two males and one female rabbits died during the exposure to Cr(VI), other animals showed signs of anorexia, weight loss and hair loss. Liver histopathologic results at various doses of 3 months period are shown in Fig 1.

In the control group, common characteristic lobular organization of the mammalian liver was observed (Grade 0). The hepatic cords were well organized and radiated from a central vein. Neither obvious congestion nor hepatic necrosis/degeneration were observed (Fig. 1A and B). However, in the Cr(VI) exposure groups, arrange of hepatic cord was disorganized in a concentration dependent manner. Blood vessel congestion was obviously visible and the blood sinusoids were dilated between the cords of hepatocytes (Fig. 1C-F). The necrotic and degenerative hepatocellular conditions were characterized by decrease of cell diameter as well as few vacuolated hepatocytes without usual polyhedral shape (Fig. 1C-F). These changes were observed at the low dose of exposure (Grade 1) and became more obvious at the moderate dose of exposure (Grade 2).

After high dose (9.05 mg/kg/day) of Cr (VI) exposure for 5 days, there was serious hepatocellular necrosis, congestion of blood vessel, and increase of blood sinusoids dilation between the cords of hepatocytes (data not shown).

Control liver of rabbit (Grade 0), the central vein was surrounded by cords of hepatocytes. (C, D) After low dose of Cr (VI) exposure (Grade 1), there was minimal hepatocellular necrosis, congestion of blood vessel (star), and increase of blood sinusoids dilation between the cords of hepatocytes (arrow). (E, F) After moderate dose of Cr (VI) exposure (Grade 2), there was mild hepatocellular necrosis, congestion of blood vessel (star), and increase of blood sinusoids dilation between the cords of hepatocytes (arrow).

**Induction of ductular reaction after Cr(VI) exposure**

We then ask whether ductular reaction were occurred in Cr(VI)-induced liver injury via CK7 and CK19 staining. We determined biliary ductal reaction in two distinct zones: the portal and canals of Herring (CoH), by examining the expression of CK7 and CK19. CK7 and CK19 were present in the...
large biliary ducts as well as small branches at the portal area. In the low dose of Cr(VI) exposure group, ductular reaction was most readily observable at the portal area, since the greatest number of CK-positive cells was observed in this area (Fig. 2); while in moderate dose of Cr(VI) exposure group, less number of CK-positive cells were observed compared to the low dose group. The observation of ductular reaction in the moderate group was significantly lower than the low dose group, but slightly higher than the control group, which indicated that cells in portal area exhibited the cytotoxic reaction at moderate dose of Cr(VI) exposure (Fig. 2). We also observed that resident ductules cells were activated and expanded from the perportal to the pericentral zone, where ductular reaction was present in lobules after low dose of Cr(VI) exposure. Liver sections further showed difference in morphology and size with some CK-positive cells in lobules appearing to be larger in cell size and less staining for CKs compared with cells in the portal area (Fig. 2).

![Fig. 2: Ductular reactions were observed after chronic Cr(VI) exposure. Cells (stained by CK7 and CK19) in the portal area expanded from a portal tract into injured target lobe, indicating ductular reactions were appeared. It is noted that more CK-positive cells were observed after low dose of Cr(VI) exposure compared to moderate dose of Cr(VI) exposure. Some CK-positive cells, which retained their original shape and size, were strongly positive to CKs. However, others cells, which exhibited large cell size, were mildly stained for CKs (arrow). Original magnification: × 400.](image)

As the smallest channels containing cholangiocytes, the CoH represent the true hepatocytic-biliary interface of the liver that thus lies within the lobules at the limiting plate or edge of the portal tract. Because CoH form the biliary-hepatocytic interface, it makes biological sense that any stem cells with the potential for biphenotypic differentiation should be located at this interface. CoH are not readily apparent on routine histological staining but are highlighted by the biliary cytokeratins CK7 and CK19. We then tested response of CoH cells in Cr(VI) induced liver. As shown in Fig. 3, immunohistochemical staining with CK7 and CK19 highlights CoH, which are identified as single or two cells and smaller cell diameter with thin, elongated structures, and high nuclear to cytoplasmic ratio. The average cell diameter in CoH is about 1/5 - 1/10 to that of mature hepatocytes. After Cr(VI) exposure for 3 months, the number of CK positive cells in CoH were increased in both Cr(VI) treated groups. Some CK-positive cells retained their original shape and size and were strongly positive to CKs. However, other cells exhibited large cell size and mildly staining for CKs, indicating they were undergoing differentiation to regenerate necrotic hepatocytes.

![Fig. 3: Proliferation of cells in the CoH was observed after chronic Cr(VI) exposure. Cells in the CoH (stained by CK7 and CK19) were expanded in the liver lobules. The region in the yellow box is shown at higher magnification in the red box. Original magnification: × 400.](image)

**Induction of cholangiofibrosis after Cr(VI) exposure**

Ductular reaction normally activates proliferation of portal myofibroblasts which are responsible of periportal fibrogenesis (cholangiofibrosis), we then ask whether cholangiofibrosis were occurred in the Cr(VI)-induced liver with ductular reaction. As expected, after 3 months of Cr(VI) exposure, cholangiofibrosis was observed as an accumulation of fibers and formation of myofibroblasts around the small and moderate bile ducts (Fig 4B and C). In general, cholangiofibrosis was accompanied by an increased proliferation in the bile duct. As shown in Fig. 4 and 5, the thickness of fibers was significantly increased in the low dose of Cr(VI) exposure groups. In addition, cholangiofibrosis was most readily observable in small bile ducts but not in large bile ducts after Cr(VI) exposure (Fig 4A).
Discussion

Cr(VI) exposure has been reported to cause severe liver effects in four of five workers exposed to chromium trioxide in the chrome plating industry, including derangement of the liver cells, necrosis, lymphocytic and histiocytic infiltraton, and increases in Kupffer cells\(^6\). Cases of hepatic effects after oral exposure to Cr(VI) compounds have also been reported after ingestion of 150 mL solution containing 22.5 g potassium dichrome\(^7\). Hepatomegaly and hepatic failure have also been noted in the cases of acute poisoning\(^20\). Consistent with these findings, our results further demonstrated that Cr(VI) exposure also caused liver toxicity in rabbits, with a dose dependent effect.

Low dose of Cr(VI) exposure caused slight damage of liver necrosis, and high dose of Cr(VI) exposure caused acute hepatic failure in rabbits.

Moreover, it seems that the toxic effects of Cr(VI) exposure showed species differences. In our study, two males and one female from 8 rabbits died during the exposure to Cr(VI) in moderate dose group (2.09 mg/kg/day). Meanwhile, other animals showed signs of anorexia, weight loss and hair loss. On the contrary, chronic administration of Cr(VI) in drinking water did not affect survival or produce clinical signs of toxicity in rats or mice, eventhough Cr(VI) intaking is as high as to 7-8 mg/Kg/day\(^21\). Thus, we supposed that rabbits were more sensitive to Cr(VI) than rats and mice. Consistent with our results, Osheroff et al recently observed that, compared to Sprague-Dawley rats and rhesus monkeys, New Zealand White rabbits were the most sensitive species to 1,1’-methyl-enebis[4-[(hydrorxyimino) methyl]-pyridinium] dimethanesulfonate\(^18\).

When mature hepatocyte proliferation is suppressed due to chronic liver damage or prolonged intoxication and viral infection, liver regeneration is carried out by a population of progenitor cells by means of ductular reactions\(^22\). Bile ductules and CoH in liver contain hepatic progenitor cells that can differentiate towards the biliary and hepaticoyctic lineage\(^23\). Ductular reaction refers to an increased number of ductules leading to periportal fibrosis and eventually biliary cirrhosis. It is a phenomenon observed in a variety of liver diseases, such as cholestatic diseases, inflammatory diseases and massive loss of parenchyma. Recently, ductular reaction has gained new interest because of its relationship with putative hepatic progenitor cells\(^24\), which had the capacity to regenerate injured liver. Ductular reaction can be marked by immunostained with CK7 and CK19, which are well-known mark-ers of cholangiocytes and recently were proved to mark their precursor cells\(^25\).

Our results provided the first evidence that ductular and CoH reaction was observed in Cr(VI) induced liver injury in rabbits by immunostained with CK7 and CK19, which are well-known markers of cholangiocytes and recently were proved to mark their precursor cells\(^25\).

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Our results provided the first evidence that ductular and CoH reaction was observed in Cr(VI) induced liver injury in rabbits by immunostained with CK7 and CK19, which suggested that hepatic stem/progenitor cells may be involved to replace the damaged heptocytes through proliferation and differenitatiation. Due to the lack of the rabbit spe-cific antibodies, we failed to provide the double-staining evidence to confirm that hepatic stem/progenitor cells differentiated into heptocytes. But other signs also indicated that proliferative duc-
tules cells might be differentiating into hepatocytes, such as proliferative ductules cells were expanded from the periporal to the pericentral zone, exhibited large cell size, and were mildly stained by CKs.

Ductular reactions can be classified into two types based on their origin and cell morphology. Typical ductular reaction is composed of proliferation of columnar biliary epithelial cells located within well-formed basement membranes. Atypical ductular reactions consist of bile ductules located adjacent to the parenchyma and arranged in anastomosing cords with poorly defined lumina and small cytoplasmic volume of the lining cells\(^{(26)}\). More recently, ductular reactions are classified into primitive, differentiating, and obstructive reactions based on their immunophenotype, such as CD10, CD56, CK7, and EMA. Each type of ductular reaction may imply biological and clinical significance of different liver or bile duct disease\(^{(27)}\). Due to the lack of the rabbit specific antibodies, the detailed immunophenotype of CK positive cells was not further specified. However, cells with different states were observed. Based on the source and morphology of these cells, we speculated that ductular reaction observed in portal area was consistence with typical ductular reaction, while ductular reaction observed in CoH of parenchyma was consistence with atypical ductular reaction.

Hepatocyte damage normally stimulates the proliferation of hepatic stem/progenitor cells, thus resulting in the appearance of ductular reaction. In turn, ductular reaction activates proliferation of portal myofibroblasts which are responsible of periportal fibrogenesis (cholangiofibrosis)\(^{(28)}\). The molecular cross-talk between the ductular reaction and myofibroblasts has been shown both in both experimental animals\(^{(29)}\) and in human\(^{(30)}\). In general, reactive ductules were demonstrated as a source of cell factors such as TGF-β and PDGF which are able to activate myofibroblasts\(^{(30,31)}\).

Consistent with these studies, our results showed that the both ductular reaction and cholangiofibrosis were observed during the process of Cr(VI) induced hepatocyte damage. In addition, cholangiofibrosis was most readily observable in small bile ducts but not in large bile ducts after Cr(VI) exposure. This is not surprise because ductular reaction was most readily observable in small bile ducts at the portal area, but not in large bile ducts, after low dose of Cr(VI) exposure. Cholangiofibrosis is utilized to predict the development of multiple bile duct diseases.

Future experiments will be carried out to study the bile duct diseases induced by Cr(VI) exposure, and its relationship with cholangiofibrosis.

In conclusion, the present work highlights the results of a series of histopathological studies, which demonstrated that Cr(VI) exposure resulted in necrosis of the hepatocytes in the rabbit and ductular reaction were involved in the effect of Cr(VI) on liver damage. To our knowledge, this is the first report that ductular and CoH reaction may play an important role in the degree of damage and recovery that the liver undergoes following Cr(VI) intoxication. This study provides useful information for understanding molecular mechanisms in Cr(VI) toxicity on liver damage.

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Acknowledgements
This work was supported by research grants from Zhejiang Province Science and Technology Project of China [grant number 2013C33189].

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