Original Article
Effect of ischemia reperfusion on rabbit VX2 cells in a hepatocellular carcinoma model

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Abstract: Background: We established a rabbit VX2 cell liver carcinoma model to evaluate effects of ischemia reperfusion (IR) on reactive oxygen species (ROS) development and liver cell apoptosis rates. Methods: Thirty-six rabbits were divided into a control (n=6) and a VX2 hepatocellular carcinoma (HCC) model group (n=30), which received VX2 cell suspension injections into their livers. From the 30 HCC rabbits, 6 rabbits served as control without hepatic ischemia and the rest were treated with hepatic artery and portal vein clamps for 60 minutes. At 1 hour, 1 day, 3 days and 7 days of reperfusion, 6 rabbits were sacrificed and changes of catalase (CAT) and super-oxide dismutase (SOD) activities as well as apoptosis rates, measured by TUNEL assays, were compared between tumor tissues, normal tumor surrounding hepatic tissues and controls. Results: All treated animals developed liver tumors. The CAT activity increased in both tissues 1 hour after reperfusion ($P < 0.05$) and dropped to low levels in the hepatocarcinoma cells at day 1 after reperfusion ($P < 0.01$), but increased to higher levels than the control on day 3 ($P < 0.05$). SOD activity decreased significantly in both tissues until day 1 after reperfusion and kept low in the hepatocarcinoma cells until day 7 ($P < 0.05$). The apoptosis rates after IR increased more in cancer than in normal hepatic tissues ($P < 0.01$). Conclusion: Injection of VX2 tumor cell suspension into rabbit liver parenchyma achieved good results for creating a liver tumor model. IR induced apoptosis of tumor tissue and normal hepatic tissues via ROS development.

Keywords: Tumor model, liver cancer, ischemia reperfusion injury, catalase, super-oxide dismutase, reactive oxygen species

Introduction

The annual incidence of hepatocellular carcinoma (HCC) is between 40-150 per 100000 inhabitants worldwide and one million annual cancer related deaths are HCC cases [1]. The VX2 carcinoma cell line, first developed by Shope and Huist is derived from a virus induced rabbit papilloma and characterized by fast growth in rabbit liver, making the cell line suitable for use in HCC rabbit models [2]. Previous studies employed various methods to establish rabbit liver HCCs, such as injection of a VX2 tumor cell suspensions directly into the liver parenchyma [3], hepatic artery and the superior mesenteric vein [4], as well as into the gas-

trointestinal wall [5] and implantation of VX2 tumor fragments into the liver [6]. However, up to now there is no common agreement about an optimum method for VX2 cell HCC induction in rabbits. The liver is prone to damage caused by ischemia reperfusion (IR) injuries [7]. In previous studies, animal models were developed, to study mechanisms of cytotoxic reactive oxygen species (ROS), complement activation, neutrophil adhesion and interactions between complement and neutrophils during liver reperfusion injury [8, 9]. The levels of ROS exposure to a cell is regulated by the activities of super-oxide dismutase (SOD) and catalase (CAT) in order to prevent an increase that eventually could induce cell death [10, 11]. Although ROS have
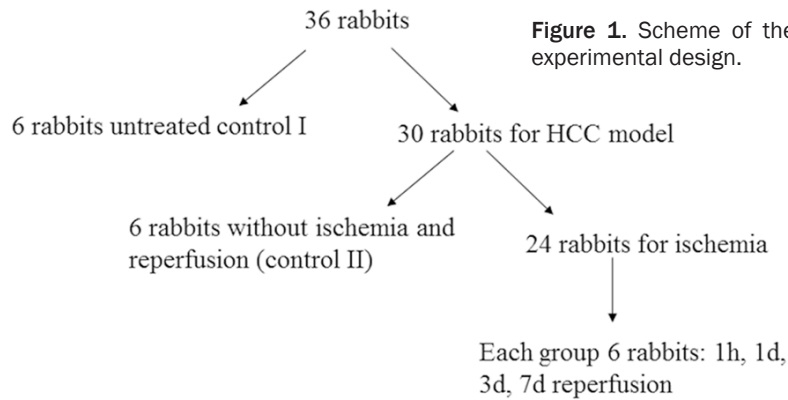
Figure 1. Scheme of the experimental design.

reperfusion for 1 h, 1 day, 3 days, or 7 days (**Figure 1**).

Establishment of the rabbit HCC model

Except for the 6 control animals, the hepatocarcinoma model was established by injecting VX2 tumor cell suspensions into the liver parenchyma of the rabbits. The cell suspension was derived from a previously grown VX2 tumor mass maintained by serial transfer of the tumor homogenate into rabbit thigh muscles. After the tumor grew gradually to a size of 4-5 cm in diameter, it was removed from the hind leg of an animal for preparation of the cell suspension. Tumor masses of about 0.3 × 0.3 × 0.3 cm were dissected from the surrounding tissues and minced to viscous cell suspensions in 1.0 ml physiology buffer saline (PBS) with surgical knives and scissors at room temperature (20°C). After adequate depth of anesthesia with xylazine (Butler Co., Columbus, OH, USA) in a dose of 5 mg/kg applied subcutaneously, ketamine (Parke-Davis, Mooris Plain, NJ, USA) in a dose of 35 mg/kg as well as 0.75 mg/mg acepromazine (Butler Co, Columbus, OH, USA) were intramuscularly injected and a sub-xyphoid laparotomy of 3-4 cm in length was performed to expose the left lobe of the liver for implantation of the VX2 tumor cell suspension. The VX2 tumor cell suspension was slowly injected into the left-middle lobe of the liver under the liver capsule in about 0.3 cm depth, forming an obscure wheal by using a 27-gauge needle with a 1 ml syringe. After injection, the puncture side was gently compressed with sterile cotton for 2 min to prevent leakage of the tumor cell suspension. The wound was the closed and the animals were observed closely and treated for optimal recovery with benzathin penicillin G 5000 IU + 200 mg dihydrostreptomycin (Harbin Pharma Group CO., LTD, Harbin, China) and Temgesic (Buprenorphine 0.03 mg/kg; Schering-Plough, Belgium). Two weeks after VX2 cell suspension injections, control animals were sacrificed via intravenous pentobarbital application and the control liver as well as hepatocarcinoma tissues were collected and dissected for examinations. For the animals of the reperfusion groups, after anaesthesia as

been studied as injurious factors in various models, their role in anticancer effects has received increasing attention in recent years [12-14]. In our present study, we used a VX2 cell induced rabbit HCC model, to compare effects of IR on ROS quenching enzymes and cell apoptosis in normal liver and liver carcinoma tissues.

Material and methods

Animals

Thirty-six adult New Zealand White rabbits (weight 2.0-3.0 kg) were purchased from the experimental center of the 4th military medical University. All animal experiments were approved by the institutional animal care committee and in accordance with the Guide for the Care and Use of Laboratory Animal in our university. The animal underwent an acclimatization period of 7 days after arrival at the facility. All animals were caged individually in stainless steel cages (82 × 66 × 65 cm) and food and water was given ad libitum; the standard rabbit food contained 18.7% protein, 3.1% fat and 14.5% fiber. Environmental conditions in the animal room were temperature 21°C, relative humidity 50-70% and an air exchange rate of 10-room volumes/hour. The light/dark cycle was 12/12 h in addition to the normal day light. The cages, animal bedding and food were sterilized and the entrance to the vivarium was restricted to persons wearing clean coats, surgical caps, masks and overshoes.

The 36 rabbits were divided into 6 groups with 6 animals per group. The groups included untreated rabbits (control I) as well as rabbits with VX2 HCCs and liver ischemia followed by

described above, the hepatic artery and portal vein were exposed by laparotomy and clamped for 60 minutes. After reperfusion for 1 h as well as 1, 3, and 7 days at each time point 6 animals were sacrificed and samples of both normal and hepatocarcinoma tissues were collected and dissected for further analysis. A section of each collected tissue was fixed in phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde for Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays.

Protein quantification of tissue homogenates

Protein contents of all liver homogenates were determined using a Coomassie brilliant blue kit (Bradford) (Thermo scientific, Shanghai, China), according to the manufacturer's manual. Protein levels were calculated with standard curves of BSA dilutions.

Concentration of catalase (CAT)

The CAT concentration was determined according to the instruction of the kit provider (Jiancheng Biotechnology, Nanjing, China). In this method, CAT breaks down hydrogen peroxide into water and oxygen, which is controlled by ammonium molybdate inhibition. The residual hydrogen peroxide reacts with ammonium molybdate to form a complex. Briefly, the control (0.1 ml of distilled water) and examination samples (0.05 ml of 1% tissue homogenate) were mixed with the supplied reagents and absorbance changes at 405 nm OD were determined.

Concentration of super-oxide dismutase (SOD)

The concentration of SOD was determined using a SOD kit according to the manufacturer's instructions (Jiancheng Biotechnology, Nanjing, China). In brief, oxygen produced by xanthine and xanthine oxidase forms a complex with 2-(4-todophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride with maximum absorbance at 532 nm. Superoxide dismutase activity was determined by the degree of reaction inhibition. The samples were 0.3 ml 1% tissues homogenate and the control was 0.3 ml distilled water.

Terminal deoxynucleotidyl transferase- mediated dUTP nick end-labeling (TUNEL) staining

TUNEL staining was performed using the in situ Cell Death Detection Kit according to the manu-

facturer's directions (Roche Diagnostics GmbH, USA). Briefly, tissues were dehydrated using alcohol, embedded in paraffin and sections were cut (3 μm). After deparaffinization, rehydrated sections were treated with proteinase K, quenched with endogenous peroxidase blocker and incubated with TdT labeling buffer. Labeling reaction mix was then added to the samples for 1 h at 37°C. Sections were subsequently washed with PBS and mounted with coverslips and glycerin. Controls included a deoxyribonuclease 1 (DNase1) positive control, an unlabeled experimental control, an experimental negative control, and a counterstaining control as specified in the kit's directions. All samples were observed using fluorescence microscopy and five vision fields were analyzed in each case. Using a 20x objective and white light, the number of positive cells per 100 cells was determined.

Statistical analyses

All statistical analyses were performed using SPSS10.0 software (Chicago, SPSS Inc.). The results of the CAT and SOD analyses are expressed as mean ± SD. Data of apoptosis are expressed as Median (M) and inter-quartile range (Q) and were analyzed using Dunnett t, Kruskal-Wallis and Mann-Whitney tests.

Results

The VX2 tumor cell implantations into the rabbit livers via VX2 cell suspension injection led to 100% tumor development and the tumor sizes in all animals were between 3-5 cm two weeks after injection. Within the 30 rabbits with induced liver tumor growth, leakage and tumor growth in the peritoneal cavity or surrounding organs occurred in six rabbits (20%), whereas lung metastases developed in three rabbits (10%). All tumors were confirmed by histology using H&E staining.

In normal liver tissues, the concentration of CAT increased and restored to normal levels at 3 d after reperfusion, whereas in hepatocarcinoma tissue the CAT activity initially increased at 1 h after reperfusion, reached the lowest level at 1 day after reperfusion and then returned to a normal level after 7 days of reperfusion (**Figure 2**). The SOD concentrations in normal liver tissues and hepotocarcinoma tissues decreased compared with control animals following reperfusion for 1 h and further
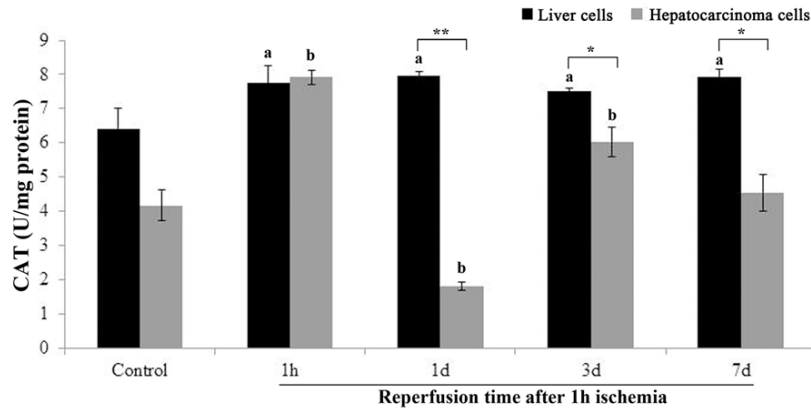
Figure 2. The changes of CAT in normal liver and hepotocarcinoma tissues. The graph represents mean ± SD. Compared with controls: $^aP < 0.05$, $^bP < 0.01$. Compared with the same time point reperfusion values in normal liver tissues: $^{**}P < 0.01$, $^*P < 0.05$.
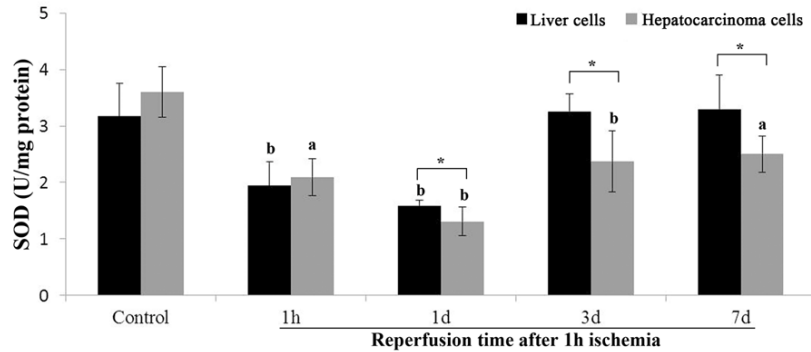

Figure 3. The changes of SOD in normal liver and hepotocarcinoma tissues. The graph represents mean ± SD. Compared with controls: $^aP < 0.05$, $^bP < 0.01$. Compared with the same time point reperfusion values in normal liver tissues: $^{**}P < 0.01$, $^*P < 0.05$.

Table 1. Percent apoptosis rates of normal liver and hepotocarcinoma cells after ischemia reperfusion

	Hepatocarcinoma cells	Liver cells	P value
Control	5.0 (5.5)	1.0 (1.8)	
Reperfusion			
1 h	21.0 (8.0)^b	9.0 (4.3)^b	0.01
1 d	25.5 (13.0)^b	15.5 (9.3)^b	Not significant
3 d	19.5 (2.5)^b	11.5 (5.3)^b	0.01
7 d	8.0 (4.3)	2.5 (1.5)^a	Not significant

Median (M) and inter-quartile range (Q) data are presented. Compared with control: $^aP < 0.05$, $^bP < 0.01$.

decreased in both tissues until 1 d after reperfusion. At day 3 and 7 after reperfusion, the SOD in tumor cells activity still was decreased, whereas in non-tumor liver cells it restored to normal levels from day 3 after reperfusion (**Figure 3**). The activities of both enzymes were lower in tumor than in non-tumor liver cells at day 1 after reperfusion and the SOD activity at day 3 and 7 after reperfusion was lower in tumor cells than in normal liver tissue.

The apoptotic cells in hepotocarcinoma and normal liver tissues increased obviously in all time points following IR. The largest number of positive cells was visible in hepotocarcinoma tissues after reperfusion for 1 d and was markedly different from normal liver tissues. The results demonstrated that apoptosis after IR was more obvious in hepotocarcinoma tissues than normal liver tissues (**Table 1**; **Figure 4**).

Discussion

In this study, we have achieved a high success rate (100%) of VX2 tumor growth using cell suspension injection into the liver. Previous results with 20 rabbits showed, that after VX2 tumor cell suspension containing 1×10^6 cells in a volume of 0.1 ml, 3 rabbits showed no evidence of tumor growth [15]. The reason of our higher success rate might be a larger volume of VX2 suspension cell (viscous fluid in 0.3 ml). Furthermore, it is important to reduce the possibility of implanting non-viable tumor cells into the liver and a tumor mass with solid consistency containing viable cancer cells should be used to prepare the VX2 cell suspension. Our experimental result showed 20% (6/30) leakage of VX2 cell suspensions, which was lower than in a previous study [15] and might be due to their combined injections not only into the parenchyma, but also into blood vessels and bile ducts [3]. In addition, cell leakage from the
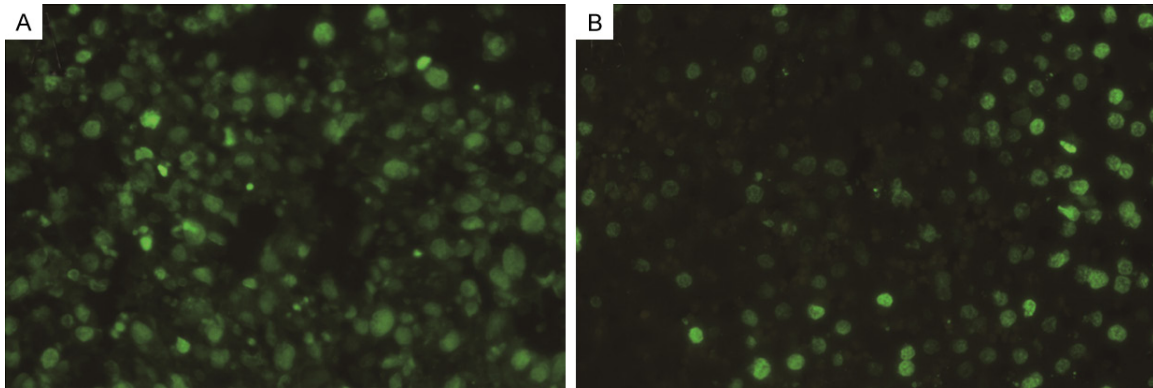
Figure 4. Hepatocarcinoma tissues analyzed by TUNEL staining. Representative images of TUNEL staining for apoptosis detection in hepatocarcinoma tissues subjected to (A) 1 hour ischemia and reperfusion for 24 h; (B) 1 hour ischemia followed by perfusion for 3 days. Both images are 40 × magnified.

puncture site could be avoided by slow injection, compression with cotton gaze and hyperviscosity of the cell suspension. It has been noted that overproduction of ROS in cells after IR induces injury for both normal and hepotocarcinoma tissues [8, 9]. Since ROS development is damaging the cells, quenching mechanisms compensate the effect of free oxygen radicals. In case that the defense mechanisms are overridden by the amount of ROS, cell damages accumulate and eventually lead to apoptosis. CAT activities increased after 1-hour reperfusion in liver and liver cancer tissues. In contrast to normal liver cells, in which the CAT activity kept enhanced until 3 days after reperfusion, in hepatocarcinoma cells it initially decreased after 24 hours and raised again at day 3 to normalize at day 7 after reperfusion (**Figure 2**). SOD activities decreased 1 hour after re-oxygenation, kept lower than the control until 1 day after reperfusion in both tissues and kept decreased in tumor tissue until 7 days after reperfusion (**Figure 3**). Enhanced CAT activities indicated ROS development at 1 hour after reoxygenation in normal liver and hepatocarcinoma cells, but particularly in tumor cells, at day 1 the CAT activities as well as the SOD activities until 7 days of reperfusion were less than the control, which was accompanied by significant higher apoptosis rates of hepatocarcinoma compared to normal liver cells. This is in agreement with previous literature, which noted that enhanced SOD activity inhibits ROS induced apoptosis [16-18]. Cancer cells normally produce more ROS than normal cells [19] and ROS accumulation is an essential therapeutic mechanism associated with radiothera-

py, thermotherapy and photodynamic therapy for the treatment of tumors [20]. One example for induced apoptosis of cancer cells via a ROS developing drug is the traditional Chinese medicine arsenic trioxide (As_2O_3), which inhibits mitochondrial respiration, thereby enhancing ROS occurrence [21-23]. Controlled ischemia reperfusion triggered apoptosis of cancer cells has been reported previously for malignancies especially sited in the pelvic region and extremities [7] and here we showed, that VX2 cell induced rabbit liver tumors are sensitive to ischemia reperfusion triggered apoptosis.

In conclusion, we observed that the apoptosis rates increased from 1 hour to 7 days of reperfusion after 1-hour ischemia with a peak at 1 day particularly in VX2 cell hepatocarcinomas. The results indicated that endogenous ROS induced by IR served as apoptosis trigger of hepotocarcinoma tissues and the VX2 rabbit liver cancer model is suitable for endogenous ROS related drug evaluation.

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Disclosure of conflict of interest

None.

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