Experimental studies on the inactivation of HBV in blood via riboflavin photochemical treatment

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Abstract. The aim of the study was to examine the influence of riboflavin photochemical method on the inactivation of hepatitis B virus (HBV) and the functions of red blood cells. Twenty patients suffering viral hepatitis B were selected in this study, and venous blood was collected and final concentration of 1,500 μmol/l riboflavin were added, to accept the λ=400-500 nm. The light intensity of 40,000 lux was treated with 2 h. The effect of inactivation was then evaluated and the function of red blood cells was detected. Two hours after treatment of the blood samples with riboflavin (1,500 μmol/l), the numbers of copy of HBV DNA were significantly decreased ($5.1x10^9$±4.2x10 vs. $1.2x10^7$±$1.2x10^6$ after the inactivation, while, 2,3-DPG, Na^+K^+-ATPase, Ca^{2+}-ATPase, Mg^{2+}-ATPase, FHb were unchanged. In conclusion, HBV DNA can be reduced using riboflavin photochemical inactivation method. Inactivate the B virus had no effect on erythrocyte function.

Introduction

With the development of social economy and the improvement of medical level, transfusion therapy has made a significant contribution to saving the lives of patients. However, various pathogenic viruses such as hepatitis B virus (HBV), HCV and HIV contained in blood sample may infected transfected patients and cause serious diseases. As a more severe one, HBV has a very narrow scope of species and is only limited to human and chimpanzee (1). According to the World Health Organization, there are approximately 2 billion individuals all over the world who have been infected by HBV. As well, among which 35 million individuals are chronically-infected individuals (2). Being high incidence area of HBV, China has approximately 20 million HBV infected individuals (3). Moreover, there are approximately 1 million individuals dying from hepatic failure, hepatic cirrhosis and primary hepatocellular carcinoma caused by HBV infection each year (4). However, due to the presence of HBV window phase, some HBV infected blood samples cannot be detected leading to the infection of HBV of receptor (5). Therefore, HBV inactivation may be the key technology to completely eradicate infected transfusion risk in blood products.

Riboflavin photochemical treatment (RPT) is the method that is given close attention in the field of study on the inactivation of pathogenic microorganism in blood component used recently with proven effectiveness and safety (6). Riboflavin is known to be able to combine with nucleic acid and to subsequently induce reaction under photosynthesis that can lead to the breakage of nucleic acid and prevent the replication of nucleic acid. Since this principle is also logically applicable to the inactivation of lymphocytes in blood products, it arouses the interest of relevant investigators. As an essential vitamin for human body, riboflavin has the characteristic of being harmless to human body.

In the present study, RPT was applied by combining visible lights to conduct inactivation treatment for HBV in red cell suspension and its inactivation effects and changes of *in vitro* quality indicators of inactivated red blood cells were observed.

Patients and methods

Specimen source. Twenty cases of patients with viral hepatitis B decompensation were selected randomly in Central Hospital of Xuzhou (Jiangsu, China) from October 2013 to February 2014. Fifteen cases were male and 5 cases female, aged from 20 to 70 years with the median age of 37 years. All the selected patients were without blood, endocrine and autoimmune diseases and all signed informed consent. Fasting venous blood of 20 ml was drawn, 10 ml as specimen before viral inactivation, the rest 10 ml carried out with HBV inactivation through riboflavin photochemical meth.

Methods. HBV DNA in blood sample before and after inactivation was extracted and the copy number of DNA was detected by applying real-time fluorescent quantitative polymerase chain reaction (PCR) method to compare and evaluate the effects of virus inactivation by RPT. Meanwhile, the physiological functions of red cells before and after inactivation were detected to evaluate the effects of RPT on physiological functions of red cells.

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Table I. Comparison of the functions of red cells before and after virus inactivation (mean ± standard deviation).

Time	2,3-DPG (μmol/gHb)	Na^+K^+-ATP enzyme (U/mgprot)	Ca^{2+}-ATP enzyme (U/mgprot)	Mg^{2+}-ATP enzyme (U/mgprot)	Erythrocyte malonaldehyde (nmol/ml)	Plasma-free hemoglobin (mg/l)
Before inactivation	7.8±2.3	6±2	8.8±3.0	6.4±1.1	2.5±0.3	36±4
After inactivation	8.7±2.5	5±2	8.2±1.2	6.0±1.8	9.2±2.0[a]	35±5

[a]Compared with those before inactivation, P<0.05.

Reagents and instruments. DNA extraction kit (Sigma-Aldrich, St. Louis, MO, USA); HBV DNA fluorescence quantitative PCR test kit (Daan Gene Co., Ltd., Guangzhou, China); halogen lamp (100 W; Philips, Amsterdam, Holland); a light illuminometer (ST-80C; Photoelectric Instrument Factory of Beijing Normal University, Beijing, China); Leica inverted microscope (DMIL; Leica, Mannheim, Germany); gene amplification instrument (Eppendorf, Hamburg, Germany); 2,3-diphosphoglyceric acid assay kit, ultra-micro ATP enzyme test kit, malondialdehyde test kit, free hemoglobin test kit and riboflavin (both from Sigma-Aldrich).

Virus inactivation treatment. Experimental group was irradiated by visible lights (λ=400-500 nm) with the illumination of 40,000 lux for 2 h in a low-temperature working cabinet with 4˚C environmental temperature. By contrast, the red cells in the control group were kept in the dark and frozen at 4˚C.

Extraction of HBV DNA and quantitative PCR detection. HBV DNA was extracted from equivalent blood samples before and after inactivation. DNA was extracted according to the extraction steps of Omega and 2 μl DNA sample was taken. The copy number of DNA was detected according to the method specified in the instruction of the DNA fluorescent quantitative PCR kit to evaluate the changes of the carrying capacity of HBV. The PCR cyclic conditions included 93˚C for 45 sec and 55˚C for 60 sec, 10 cycles, and then 93˚C for 30 sec and 55˚C 46 sec, totaled 30 cycles.

Test for quality indicators of red cells

Test for morphological structure of red cells. Morphological scoring of red cells was conducted under inverted microscope. The surface ultra-structure of red cells was observed under scanning electron microscope. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied to analyze erythrocyte membrane skeleton protein.

Test for physiological functions of red cells. The functions of red cells before and after inactivation were detected according to kit instruction so as to evaluate the effects of RPT on it, including 2,3-DPG, concentration of plasma free hemoglobin, 3 types of ultra-micro-ATP enzymes of erythrocyte membrane and concentration of erythrocyte malonaldehyde.

Statistical analysis. Data were expressed by the mean ±standard deviation (SD). SPSS 16.0 software (Chicago, IL, USA) was used for analysis. Various parameters before and after processing were compared by paired t-test. Only P<0.05 meant that the difference was considered statistically significant.

Results

Morphological changes of red cells before and after virus inactivation. The red cells of the experimental group after gradient dilution before and after virus inactivation respectively were added to 96-hole cell culture plate for cultivation. No significant effects were observed under inverted microscope for the forms and membrane structures of red cells before and after HBV inactivation and all red cells took on the shape of normal double-concave disk. Under scanning electron microscope, abnormally protuberant red cells occurred in red cells in occasional cases after inactivation by RPT. Through SDS-PAGE analysis, the difference of erythrocyte membrane skeleton protein band 3 protein and the ratio of other proteins to band 3 protein before and after virus inactivation was not statistically significant.

HBV DNA fluorescence quantification detection before and after inactivation by RPT. The number of copy of HBV DNA before inactivation ($1.2x10^9±1.2x10^8$) was decreased to that after inactivation ($5.1x10^5±4.2x10^4$). This decrease was approximately four orders of magnitudes and the comparative difference had statistical significance (P<0.05). This result suggested that RPT could lower the carrying capacity of HBV DNA.

Effects of RPT on functional indicators of red cells before and after inactivation. Compared with those before inactivation, 2,3-DPG, concentration of plasma-free hemoglobin and 3 types of ultra-micro-ATP enzymes of erythrocyte membrane of venous blood after riboflavin photochemical inactivation had no significant changes, expect for the significantly higher concentration of erythrocyte malonaldehyde (P<0.05) (Table I).

Discussion

As an aromatic compound with polycyclic plane structure naturally existing in mammals, riboflavin can pass through cell membrane, virus lipid film and nuclear membrane to combine with nucleic acid, absorb photon energy, oxidize the guanine

residue on nucleic acid chain to form a covalent compound and mediate the breakage of nucleic acid backbone chain under the activation of ultraviolet rays/visible lights (7). Thus, this prevent the replication, transcription and translation of pathogen nucleic acid to inactivate pathogenic microorganism. Moreover, since riboflavin is electrically neutral, it can diffuse through lipid membrane to effectively kill enveloped viruses, non-enveloped viruses and intracellular pathogen (8). RPT becomes the hot spot of studies on technical field of pathogen inactivation for blood products.

To study the effects of riboflavin photochemistry on inactivation of HBV in red cells, we placed red cell suspension in low-temperature working cabinet at 4˚C to accept irradiation of visible lights with the illumination of 40,000 lux for 20 min. The results showed that riboflavin with the final concentration of 150 μmol/l treated by combining with visible lights (=400-500 nm) for 20 min could lower the number of copy of HBV DNA by 2-fold of magnitudes. This did not indicate that the method could inactivate HBV effectively, but at least, showed at some extent decreased the carrying capacity of HBV in red cells. After illumination, lumiflavin, photopigment and other products were also the normal products generated after the metabolism of riboflavin in human body. It could be deduced that RPT had no side effects on human body.

Red cells are life entities with specific physiological functions and self-support metabolism. The amount of ATP content in red cells not only reflects the capability of energy metabolism of red cells, but is closely related to their form and deformability. Considering ATP and 2,3-DPG as the leading indicator to judge the quality of red cells, has been accepted by a great number of scholars (9). In RPT, Na^+K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase had little change, which showed that it had smaller effect on red cells, but caused abnormally protuberant red cells in occasional cases, and the method values of illumination were worth optimizing further.

In a word, the RPT established in the current experiments could effectively reduce the carrying capacity of HBV in red cells and have no harm to human body. This method may have some clinical application prospects and would generate bring huge economic and social benefits.

However, due to the early stage of our experiments, additional studies are needed to further improved our finding, so as to provide more reliable data.

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