Extraction of protoporphyrin disodium and its inhibitory effects on HBV-DNA

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AIM: To explore an ideal method for extracting protoporphyrin disodium (PPN) from unanticoagulated animal blood, and to study the inhibitory effects of PPN on HBV-DNA duplication and its cytototoxicity to 2.2.15 cell strain.

METHODS: Protoporphyrin methyl ester and other intermediate products were prepared with protoheme separated from protein hydrolysates of coagulated animal blood, which were finally made into PPN and detected quantitatively with an ultraviolet fluorescent analyzer. Ten \( \mu \text{g/ml} \), 20 \( \mu \text{g/ml} \), 40 \( \mu \text{g/ml} \), 80 \( \mu \text{g/ml} \) and 160 \( \mu \text{g/ml} \) of PPN-aqueous solution were added into culture medium for 2.2.15 cells respectively. Eight days later, the drug concentration in supernatant from the culture medium was detected when inhibition rate of HBeAg, cell survival rate when inhibition rate of HBeAg was 50% (ID50), and when survival cells in experimental group were 50% of those in control group (CD50), and the therapeutic index (TI) was also detected. PPN with different concentration of 10 \( \mu \text{g/ml} \), 20 \( \mu \text{g/ml} \), 40 \( \mu \text{g/ml} \), 80 \( \mu \text{g/ml} \) and 160 \( \mu \text{g/ml} \) was respectively mixed and cultivated with HepG2 2.2.15 cell suspension, and then the inhibition of PPN against HBV-DNA was judged by PCR.

RESULTS: The extract of henna crystal was identified to be PPN. When the concentrations of PPN were 160 \( \mu \text{g/ml} \) and 80 \( \mu \text{g/ml} \), the inhibition rates of HBeAg were 89.8% and 82.4%, and the cell survival rates were 98.7% and 99.2%.

CONCLUSION: It is suggested that PPN can be extracted from unanticoagulated animal blood. PPN can inhibit HBV-DNA expression and duplication in vitro, and has no cytototoxicity to liver cells. Further study and application of PPN are warranted.

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added into the filtrate, then the solution was filtered when crystals occurred after placement for 12 h. After the crystals were dissolved with 600 ml ammonia water (2%), ammonium acetate aqueous solution (30%) was added into the solute and placed for 12 h till crystallization occurred. The crystals were filtered out and washed 3 times with 600 ml ammonium acetate aqueous solution (2%) and 90 ml distilled water, respectively. Then it was dried into crude protoporphyrin, which was a kind of brown crystalline materials of about 2 g.

**Preparation of protoporphyrin methyl ester** 5 g crude protoporphyrin was dissolved in 200 ml hydrochloric methanol aqueous solution (1%), and the reactants were heated, regurgitated for 20 min. Then 1 000 ml ammonium carbonate aqueous solution (1%) was added into the above cooled reactants and placed till crystallization occurred. The crystalline materials were taken, and washed 3 times with distilled water, then dissolved in 200 ml chloroform and the solute was taken to pass through chromatography column with 1 000 g neutral aluminium oxide. After the elution with prepared eluting agent of chloroform and petroleum ether with volumetric proportion at the ratio of 1 to 3 was completed, the eluent of protoporphyrin methyl ester was collected and evaporated, and a kind of brown crystalline materials was obtained. Then the brown crystalline materials were crystallized 2 times with chloroform and methanol, and 2 g protoporphyrin methyl ester was acquired.

**Preparation of pure protoporphyrin** 5 g protoporphyrin methyl ester was dissolved in 150 ml HCL aqueous solution (25%). After placed for 9 h, the reactants were neutralized with NaOH aqueous solution (30%), then crystallization occurred. The crystalline materials were isolated and washed 3 times with distilled water. After being dried, the crystalline materials were re-crystallized 2 times with pyridine, and 2.5 g brown pure protoporphyrin was obtained.

**Preparation of PPN** The mixture of 10 g pure protoporphyrin, 5 g NaOH and 500 ml anhydrous alcohol was heated and regurgitated for 2 h. Crystalline materials occurred after the mixture was cooled. Then the crystalline materials were isolated, washed 3 times with anhydrous alcohol and dried. Finally, 9 g brown crystalline materials of PPN was obtained.

**Inhibitory effects of PPN on HBV-DNA expression in vitro** 2.2.15 cell strain was prepared based on human cancer cell strain of the liver, HepG2, which had been infected with HBV-DNA, could express all marks of duplications of HBV-DNA 

Regarding 2.2.15 cell strain as the target cell, fresh culture media with different concentrations of PPN (10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml, 160 µg/ml) were then replaced every 3 days. Eight days later, supernatants were collected and stored at 20 °C for detection of HBeAg and HBeAg levels and cytotoxicity of PPN in liver cells. ELISA was used for detection of HBeAg. The detailed procedure followed the operating instructions. Inhibition rate (%)=(P/N values of the control holes- P/N values of the study holes)/(P/N values of the control holes -2.1)×100%, and IDso represented the concentration of PPN when the inhibition rate of HBeAg was 50%. The cytotoxicity of PPN to liver cells was detected by MTT to determine the survival rate of liver cells. The survival rate of liver cells (%)=values of the study holes/(A590-As0)/values of the control holes (A590-As0)×100%. CDso represented the concentration of PPN when the number of the survival cells in the detected holes to the survival cells in the study holes was 50%.

Inhibitory effects of PPN on HBV-DNA were evaluated with TI (TI=CDso/IDso). When TI<1, PPN was cytotoxic and poorly effective on liver cells. When 1<TI<2, PPN was cytotoxic and effective. When TI≥2, PPN was mildly cytotoxic and effective. The higher the values of TI, the greater the inhibitory effects of PPN on HBV and the less the cytotoxicity of PPN to liver cells[18,19] (Table 1).

**Inhibitory effects of PPN on HBV-DNA duplication in vitro** PPN was diluted to a series of solutions with different concentrations (including 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml and 160 µg/ml) by aseptic techniques for further use. HepG2 2.2.15 cells in good cultivation were dispensed to the suspension with a concentration of 1×10⁶/2 ml, 6 identical shares of it were subsequently taken out, PPN with the concentration of 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml and 160 µg/ml respectively was added to 5 of 6, the remaining one had no PPN. After a period of cultivation, the cells were collected, then whole genome DNA of them was extracted by using CASsampler blood genomic DNA isolation kit and further amplified by PCR. The amplification system included 10xbuffer 25 ul, dNTP 3 ul, sense 0.5 ul, anti-sense 0.5 ul, TaqE 1.5 ul and template 2 ul. The parameters included at 95 °C for 5 min, at 94 °C for 30 s, at 55 °C for 30 s, at 72 °C for 30 s, 35 cycles and extension at 72 °C for 5 min. The final products with DGL-2000 were further performed with routine electrophoresis. During the process of analysing by using agarose gel electrophoresis containing EB, each hole was added 10 ul of products and placed at 80-100V for 20 min. Lastly, the agar plates were observed by ultraviolet fluorescence analysis.

**RESULTS**

**General characteristics of PPN** The prepared PPN was brown crystalline materials, soluble in water, slightly soluble in methyl alcohol, but not soluble in chloroform, diethyl ether or dimethy ketone. PPN displayed absorption peaks at wavelengths of 600 nm, 556 nm and 408 nm, and the maximal absorption wavelength was 408 nm. When exposed to ultraviolet, strong fluorescence could be observed in red[3].

**Quantitative detection of PPN** According to the fact that when PPN was dissolved in 1.37M HCL and placed at wavelength of 408 nm, the E 1% 1 cm was 4.81×10⁵, the purity of PPN extract was detected to be 97.3%.

**Inhibitory effects of PPN on HBV-DNA in vitro**

| Table 1 | Inhibition of PPN on HBV-DNA expression in vitro |
|---------|-----------------------------------------------|
| PPN (µg/ml) | OD | Inhibition rate of HBeAg (%) | Survival rate of liver cell (%) |
|---------|-----|-----------------------------|-------------------------------|
| 160 | 1.20 | 89.8 | 98.7 |
| 80 | 1.30 | 82.4 | 99.2 |
| 40 | 1.50 | 56.7 | 100 |
| 20 | 1.85 | 32.3 | 100 |
| 10 | 2.30 | 5.5 | 100 |

Table 1 shows that when the concentrations of PPN were 160 µg/ml and 80 µg/ml, the inhibition rates of HBeAg were 89.8% and 82.4% respectively and cellular survival rates were 98.7% and 99.2%, TI=7.47. It was obvious that the luminescence of amplified bands of samples without addition of PPN was the brightest, while that of samples with addition of PPN decreased gradually accompanying increased concentration of PPN.
DISCUSSION

While fresh anticoagulated animal blood was used as raw materials to prepare PPN, the harvest, transportation, and preservation of the raw materials needed a strict condition, which made the source of raw materials limited, working cost increased and the scale of production limited. In this study, unanticoagulated animal blood was used as raw materials to prepare PPN, that is, the form of the raw materials was improved. Using unanticoagulated animal blood as raw materials to prepare PPN, fine PPN could be obtained. The method is an ideal way of preparing PPN at present. Meanwhile, it can solve the problems mentioned. In addition, during the whole process of preparation, the special, expensive reagents are not necessary and also some reagents can be retrieved for reuse. The method is simple and convenient in its process of preparation and its production cost is lower. If some of amino acids produced during the preparation were studied, analyzed and purified, the additional value of the raw materials would be increased, so that large amount of animal blood resource can be fully utilized.

Protoporphyrin is the necessary component of ferrohemoglobin, myohemoglobin, cytochrome, catalase and tryptophan pyrrole in human body. Protoporphyrin showed a circular structure in somatic cells, and could easily combine with metallic ion to form metallic porphyrin[21]. In the case of liver disease, liver functions might be in disorder, porphyrin in bile decrease as well as the activity of catalase in liver would decrease. Therefore, PPN could activate the biosynthesis of porphyrin and metallic porphyrin in cells as demonstrated by in vitro test, increase the quantity of metallic porphyrin in cells, inhibit the decrease of activity of catalase, improve cellular respiration and regeneration, and decrease the necrosis of liver cells with recovery of its function. Based upon the above metabolic properties, PPN could improve blood flow and utilization rate of oxygen by liver and other related organs, accelerate the respiration of histocytes, improve metabolism of proteins and carbohydrates, enhance effects of complement fixation, and enhance immunity, anti-inflammation and anti-anaphylaxis potentials of the organism. Thus, PPN has been constantly studied and applied as an improving agent of liver functions[22-31]. At present, PPN is being studied for its antineoplastic effects in foreign countries. Inhibitory effects of PPN on expression and duplication of HBV-DNA in vitro was studied by us from another point of view. As table 1 shows, PPN could inhibit expression of HBV-DNA in vitro and had no cytotoxic action, and it is not difficult to see that the luminescence of amplified bands decreased gradually with increased concentration of PPN. The mechanism of action in the above results might be that some intermediary metabolites of PPN in liver cells could affect antisense oligonucleotides (ASOs) located at pre-C and C gene regions of HBV and also adjust genes ENH I of HBV through ASOs. Then it could finally inhibit the duplication of HBV-DNA and the expression of HBeAg in host cells[32-33]. Therefore, further study and application of PPN in clinical therapy of hepatitis B should be carried out.

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