PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2004;10(3):433-436
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

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

Chao-Pin Li, Li-Fa Xu, Qun-Hong Liu, Chao Zhang, Jian Wang, Yu-Xia Zhu

Chao-Pin Li, Li-Fa Xu, Qun-Hong Liu, Chao Zhang, Jian Wang, Yu-Xia Zhu, Medical College, Anhui University of Science and Technology, Huainan 232001, Anhui Province, China
Supported by the Natural Science Foundation of Anhui Province, No. 2000jl222
Correspondence to: Dr. Chao-Pin Li, Department of Etiology and Immunology, Huainan 232001, Anhui Province, China. cpli@aust.edu.cn
Telephone: +86-554-6658770 **Fax:** +86-554-6662469
Received: 2003-04-10 **Accepted:** 2003-10-07

Abstract

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 cytotoxicity 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 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml and 160 μg/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 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml and 160 μg/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 μg/ml and 80 μg/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 cytotoxicity to liver cells. Further study and application of PPN are warranted.

Li CP, Xu LF, Liu QH, Zhang C, Wang J, Zhu YX. Extraction of protoporphyrin disodium and its inhibitory effects on HBV-DNA. *World J Gastroenterol* 2004; 10(3):433-436
http://www.wjgnet.com/1007-9327/10/433.asp

INTRODUCTION

Protoporphyrin disodium (PPN) is a macrocyclic compound with a conjugated double bond, which is consisted of four pyrrole rings connected by four methylene bonds, and is a derivative of porphine. PPN can be prepared with extracted haemachrome originated from ferrohemoglobin in serum and other chemicals. PPN is a pharmaceutics to improve liver functions and can be used in clinical therapy for recovery of injured liver cells.

PPN could be obtained from fresh anticoagulant animal blood according to reports[1-3]. But the PPN in this study was obtained from fresh unanticoagualted animal blood, and the method has not been reported in the literature.

Hepatitis B is widely occurred in China, and severely affects people' s health and the quality of life. In order to find out the relationship between PPN and the expression and duplication of HBV-DNA, hoping to seek for an effective therapy of Hepatitis B, we designed an experimental *in vitro* study.

MATERIALS AND METHODS

Reagents

Unanticoagulated pig blood (from Huainan area), NaOH, zinc powder, chloroform, skellysolve G, 2.2.15 cell strain (from the Institute of Infectious Disease, Peking Medical University), kit for ELISA (from Huamei Biological Engineering Co.,Ltd), kit for PCR (from Huamei Biological Engineering Co.,Ltd).

Instruments

Tissue homogenate instrument (from Shanghai Biaomo), magnetic stirring apparatus, glassy device of reflux, glassy filter, chromatography column with neutral aluminium oxide (from Shanghai-Jinhua Chromatography Equipment Factory), drying oven by electrothermal blow (form Shanghai-Yuejin Equipment Factory), gamma radio immunoassay counters (from State-Operated Factory 262), CO_2 incubator (from Japan), SLT-Spectra-I enzyme analyzer (from America), DNA amplifier (from Zhuhai Hema Bio-Tech Institute), ultraviolet fluorescent analyzer (from Shanghai-Kanghua Biochemical Instrument Manufactory).

Methods

Extraction of protoheme 1L homogenate of unanticogulated pig bloods was added to a container, and 100 g NaOH was added simultaneously during stirring until they were well mixed. When the mixture changed into dilute solution after placement for 24 h, it was heated and stirred by magnetic stirring apparatus at the 80 ℃ to 90 ℃ for 12 hours. When the mixture was naturally cooled to 60 ℃, 1M HCL aqueous solution was added into the mixture till precipitation of protoheme occurred. The precipitation was taken out, washed 3 times with water and dried. Finally about 1.7g of protoheme was obtained.

Preparation of crude protoporphyrin 1.5 g zinc powder divided into six portions was added into the mixture of 250 ml formic acid aqueous solution (85%) and 5 g protoheme under the condition of heating, stirring and regurgitating respectively. Each addition had an interval of 5 minutes. Then they were heated and regurgitated once more for 20 min and then cooled and filtered. Ammonium acetate aqueous solution (20%) was

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 cystalline 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 cystalline materials was obtained. Then the brown cystalline 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 8 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, $HepG_2$, which had been infected with HBV-DNA, could express all marks of duplications of HBV-DNA effectively[4-17]. Regarding 2.2.15 cell strain as the target cell, the effect of PPN on HBV-DNA was confirmed by detecting the levels of HBeAg in culture supernatants with quantitative methods.

Procedure was as follows: 1×10^5/ml cell suspension was prepared with 2.2.15 cell strain which grew well. One ml cell suspension was added into each of the 24-hole plastic plate respectively and cultivated. After incubation at 37 ℃ for 48 h, 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 ℃ for detection of HBeAg levels and cytotoxicity of PPN to 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 ID_{50} 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 (A_{595}-A_{650})/ values of the control holes (A_{595}-A_{650})×100%. CD_{50} 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 were evaluated with TI ($TI=CD_{50}/ID_{50}$). 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. $HepG_2$ 2.2.15 cells in good cultivation were dispensed to the suspension with a concentration of 1×10^5/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 CASSupper blood genomic DNA isolation kit and further amplified by PCR. The amplification system included 10×buffer 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 ℃ for 5 min, at 94 ℃ for 30 s, at 55 ℃ for 30 s, at 72 ℃ for 30 s, 35 cycles and extension at 72 ℃ 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 characterisics 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 $E_{1cm}^{1\%}$ was 4.81×10^3, 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

CD_{50}=279.4 μg / ml, ID_{50}=37.4 μg / ml, TI=7.47.

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 cellar survival rates were 98.7% and 99.2%, TI=7.47.

It was obvious that the luminance 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 pyrrolase 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 and metallic 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[22-32]. 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 luminance 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 (ASONs) located at pre-C and C gene regions of HBV and also adjust genes ENH I of HBV through ASONs. Then it could finally inhibit the duplication of HBV-DNA and the expression of HBeAg in host cells[33-35]. Therefore, further study and application of PPN in clinical therapy of hepatitis B should be carried out.

ACKNOWLEDGEMENTS

The authors thank the staff of Department of Biochemistry for their help and the instruction of Professor YE Ke-qiang for the instruction.

REFERENCES

1 **Chamberlain MP,** Lock EA, Gaskell BA, Reed CJ. The role of glutathione S-transferase- and cytochrome P450-dependent metabolism in the olfactory toxicity of methyl iodide in the rat. *Arch Toxicol* 1998; **72**: 420-428
2 **Ratra GS,** Cottrell S, Powell CJ. Effects of induction and inhibition of cytochromes P450 on the hepatotoxicity of methapyrilene. *Toxicol Sci* 1998; **46**: 185-196
3 **Ackley KL,** Day JA, Caruso JA. Separation of metalloporphyrins by capillary electrophoresis with UV detection and inductively coupled plasma mass spectrometric detection. *J Chromatogr A* 2000; **888**: 293-298
4 **Zhang S,** Wang S, Wang Y. Inhibition of hepatitis B virus replication *in vitro* by phosphorothioate and tetradecyl phosphorothioate analogs of antisense oligonucleotide directed against precore and core regions. *Zhonghua Neike Zazhi* 1996; **35**: 95-98
5 **Ji W,** St CW. Inhibition of hepatitis B virus by retroviral vectors expressing antisense RNA. *J Viral Hepat* 1997; **4**: 167-173
6 **Lara-Pezzi E,** Majano PL, Gomez-Gonzalo M, Garcia-Monzon C, Moreno-Otero R, Levrero M, Lopez-Cabrera M. The hepatitis B virus X protein up-regulates tumor necrosis factor alpha gene expression in hepatocytes. *Hepatology* 1998; **28**: 1013-1021
7 **Ji W,** Wang Q, Yu M. Transfer and expression of antisense genes of hepatitis B virus (HBV) and their anti-HBV effects. *Zhonghua Yixue Zazhi* 1997; **77:** 425-429
8 **Zhang J,** Chen F, Zhong S, Tang K, Shi X, Wang M, Peng J. Anti-HBV effect of targeted antisense RNA against HBV C gene. *Zhonghua Ganzangbing Zazhi* 2000; **8**: 169-170
9 **Yang Z,** Deng X, Zhang F, Wu W, Lei C, Zhu Y. The effect of attenuated varicella-zoster virus on replication of HBV. *Zhonghua Ganzangbing Zazhi* 2001; **9:** 28-30
10 **Lu X,** Lu Y, Geschwindt R, Dwek RA, Block TM. Hepatitis B virus MHBs antigen is selectively sensitive to glucosidase-mediated processing in the endoplasmic reticulum. *DNA Cell Biol* 2001; **20**: 647-656
11 **Wu C,** Zeng Z, Wang Q. Experimental study of inhibition of hepatitis B by dual-target antisense RNA. *Zhonghua Yixue Zazhi* 2001; **81**: 605-608
12 **Cao H,** Tao P. Anti-hepatitis B virus effects of lamivudine and other five drugs *in vitro. Zhonghua Yixue Zazhi* 2001**; 81**: 1004-1007
13 **Chih HW,** Chiu HF, Tang KS, Chang FR, Wu YC. Bullatacin, a potent antitumor annonaceous acetogenin, inhibits proliferation of human hepatocarcinoma cell line 2.2.15 by apoptosis induction. *Life Sci* 2001; **69**: 1321-1331
14 **Zhong S,** Wen S, Zhang D. Inhibition of HBV gene expression by antisense oligonucleotides using galactosylated poly (L-lysine) as a hepatotropic carrier. *Zhonghua Shiyan He Linchuang Bingduxue Zazhi* 2001; **15**: 150-153
15 **Sun D,** Hu D, Wu G, Hu X, Li J, Fan G. Construction and expression of recombinant retrovirus vector carrying HBV vector. *Zhonghua Shiyan He Linchuang Bingduxue Zazhi* 2002; **16**: 162-165
16 **Dandri M,** Burda MR, Burkle A, Zuckerman DM, Will H, Rogler CE, Greten H, Petersen J. Increase in de novo HBV DNA integrations in response to oxidative DNA damage or inhibition of poly (ADP-ribosyl) ation. *Hepatology* 2002; **35**: 217-223
17 **Tan TM,** Zhou L, Houssais S, Seet BL, Jaenicke S, Peter F, Lim SG. Intracellular inhibition of hepatitis B virus S gene expression by chimeric DNA-RNA phosphorothioate minimized ribozyme. *Antisense Nucleic Acid Drug Dev* 2002; **12**: 257-264
18 **Oh SH,** Yeh BI, Kim SH. Inhibition of HBV replication by antisense oligodeoxyribonucleotides in HepG2 cells transfected with a cloned HBV DNA. *Yonsei Med J* 1995; **36**: 527-533
19 **Zhong S,** Zheng SJ, Chen F, Wen SM, Wang SQ, Zhang JJ, Deng CL. *In vivo* inhibition of hepatitis B virus replication and gene expression by targeted phosphorothioate modified antisense oligodeoxynucleotides. *Zhonghua Ganzangbing Zazhi* 2002; **10**: 283-286
20 **Zekri AR,** Awlia AA, El Mahalawi H, Ismail EF, Mabrouk GM. Evaluation of blood units with isolated anti HBC for the presence of HBVDNA. *Dis Markers* 2002; **18**: 107-110
21 **Jacob Blackmon B,** Dailey TA, Lianchun X, Dailey HA. Characterization of a human and mouse tetrapyrrole-binding protein. *Arch Biochem Biophys* 2002; **407**: 196-201
22 **Fiala ES,** Sohn OS, Li H, El-Bayoumy K, Sodum RS. Inhibition of 2-nitropropane-induced rat liver DNA and RNA damage by benzyl selenocyanate. *Carcinogenesis* 1997; **18**: 1809-1815
23 **Cable EE,** Gildemeister OS, Pepe JA, Lambrecht RW, Bonkovsky HL. Mechanism of induction of heme oxygenase by

metalloporphyrins in primary chick embryo liver cells: evidence against a stress-mediated response. *Mol Cell Biochem* 1997; **169**: 13-20

24 **Iyer S,** Woo J, Cornejo MC, Gao L, McCoubrey W, Maines M, Buelow R.Characterization and biological significance of immunosuppressive peptide D2702.75-84(E–>V) binding protein. Isolation of heme oxygenase-1. *J Biol Chem* 1998; **273**: 2692-2697

25 **Woo J,** Iyer S, Cornejo MC, Mori N, Gao L, Sipos I, Maines M, Buelow R. Stress protein-induced immunosuppression: inhibition of cellular immune effector functions following overexpression of haem oxygenase (HSP 32). *Transpl Immunol* 1998; **6**: 84-93

26 **Chamberlain MP,** Lock EA, Gaskell BA, Reed CJ. The role of glutathione S-transferase- and cytochrome P450-dependent metabolism in the olfactory toxicity of methyl iodide in the rat. *Arch Toxicol* 1998; **72**: 420-428

27 **Ratra GS,** Cottrell S, Powell CJ. Effects of induction and inhibition of cytochromes P450 on the hepatotoxicity of methapyrilene. *Toxicol Sci* 1998; **46**: 185-196

28 **Amersi F,** Buelow R, Kato H, Ke B, Coito AJ, Shen XD, Zhao D, Zaky J, Melinek J, Lassman CR, Kolls JK, Alam J, Ritter T, Volk HD, Farmer DG, Ghobrial RM, Busuttil RW, Kupiec-Weglinski JW. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia / reperfusion injury. *J Clin Invest* 1999; **104**: 1631-1639

29 **Shan Y,** Pepe J, Lu TH, Elbirt KK, Lambrecht RW, Bonkovsky HL. Induction of the heme oxygenase-1 gene by metalloporphyrins. *Arch Biochem Biophys* 2000; **380**: 219-227

30 **Kato H,** Amersi F, Buelow R, Melinek J, Coito AJ, Ke B, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 overexpression protects rat livers from ischemia / reperfusion injury with extended cold preservation. *Am J Transplant* 2001; **1**: 121-128

31 **Redaelli CA,** Tian YH, Schaffner T, Ledermann M, Baer HU, Dufour JF. Extended preservation of rat liver graft by induction of heme oxygenase-1. *Hepatology* 2002; **35**: 1082-1092

32 **Shan Y,** Pepe J, Lambrecht RW, Bonkovsky HL. Mapping of the chick heme oxygenase-1 proximal promoter for responsiveness to metalloporphyrins. *Arch Biochem Biophys* 2002; **399**: 159-166

33 **Zhoug S,** Wen SM, Zhang DF, Wang QL, Wang SQ, Ren H. Sequencing of PCR amplified HBV DNA pre-c and c regions in the 2.2.15 cells and antiviral action by targeted antisense oligonucleotide directed against sequence. *World J Gastroenterol* 1998; **4**: 434-436

34 **Zhong S,** Wen S, Zhang D. Inhibition of HBV gene expression by antisense oligonucleotides using galactosylated poly (L-lysine) as a hepatotropic carrier. *Zhonghua Shiyan He Linchuang Bingduxue Zazhi* 2001; **15**: 150-153

35 **Schulte-Frohlinde E,** Seidler B, Burkard I, Freilinger T, Lersch C, Erfle V, Foster GR, Classen M. Different activities of type I interferons on hepatitis B virus core promoter regulated transcription. *Cytokine* 2002; **17**: 214-220

Edited by Xu JY and Wang XL