The influence of joint application of arsenic trioxide and daunorubicin on primary acute promyelocytic leukaemia cells and apoptosis and blood coagulation of cell strain

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Abstract: This test cultivated three groups of acute promyelocytic leukemia (APL) and NB4 cells in liquid in vitro, processed them with arsenic trioxide (ATO), daunorubicin (DNR), ATO+DNR respectively, and then set up blank control group. Apoptosis of cells in each group was observed using flow cytometry, procoagulant activity of APL and NB4 cells in each group was detected with recalcification time, and expressions of tissue factor (TF), thrombomodulin and annexin II of NB4 cells in each group were measured using ELISA method. The results showed that the apoptosis rate increased 4-8 times compared with blank control group after processing APL and NB4 cells with ATO and DNR; procoagulant activity decreased obviously; and expression of TF and annexin II of NB4 cells reduced significantly (P<0.05). We concluded that combination of ATO and DNR could promote APL and NB4 cell apoptosis effectively without aggravating blood coagulation disorders, which might improve coagulation function of APL by inhibiting coagulation and hyperfibrinolysis through reducing expression of TF and annexin II. This drug combination may be a safe and effective method in the treatment of APL of primary high white blood cells type.

Keywords: Acute promyelocytic leukemia; arsenic trioxide; daunorubicin; procoagulant activity; apoptosis.

INTRODUCTION

APL is a kind of special leukemia, characterized by chromosome translocation and formation of promyelocytic leukemia protein-retinoic acid receptor α (PML-RARα) fusion gene (Hongming and Junmin, 2012). Its treatment mainly goes through three stages of combined chemotherapy dominated by anthracyclines, all-transretinoic acid (ATRA) and arsenious acid (As2O3). The experiment verified that ATRA and As2O3 acting on PML- RARα fusion gene might induce the differentiation and apoptosis of it. In recent years, the clinical application of arsenious acid has greatly reduced recurrence rate, and further improves long-term survival rate. However, several APL research teams report that early-stage fatality rate for about 10% still exists in APL patients and patients whose peripheral white blood cells have risen in the first visit usually enjoy a higher rate.

Wu Yanping, et al. (2010) concluded that the application of arsenical could effectively remit APL, improve coagulation function in the early stage effectively, and reduced the possibility of early death through analyzing the effect of As2O3 on APL cardio toxicity clinically (Yanping et al., 2010). Sanz et al. (2011) discovered that As2O3 was an important drug in APL targeted therapy, which could induce the differentiation of promyelocyte, and also the cell apoptosis (Sanz et al., 2011). Through injection treatment of APL, Li Bin, et al. (2012) held that anthracycline-based anticancer drug, a specificity medicine, could inhibit nucleic acid synthesis and mitosis, and kill tumor cells by its own cytotoxicity, thereby inhibiting leukemia clone to the largest extend (Bin et al., 2012). However, the commonly used method is chemotherapy at present, and studies show that application of combined chemotherapy of arsenic trioxide can achieve preferable curative effect (Miguel and Francesco, 2011). Zhao Yanqiu et al. (2012) found that ATO could lower time dependence of procoagulant activity of 8APL-MP, which could explain that ATO induced treatment could not decrease the occurrence of fatal hemorrhage in the early stage though it could effectively improve abnormal coagulation of APL (Yanqiu et al., 2012). DNR is a traditional anthracycline-based antibiotic in the treatment of leukemia (Yueyue et al., 2010).

Therefore, this research explored the influence of joint application of ATO and DNR on cell apoptosis and procoagulant activity of APL and NB4 so as to provide theoretical foundation for the treatment of APL of primary high white blood cells type.

MATERIALS AND METHODS

General materials

Thirty patients with acute promyelocytic leukemia who were diagnosed as primary attack were selected, in which, 18 cases were male, and 12 cases were female, ranging in age from 19 to 61 years (mean: 36.96±2.31 years). 5ml marrow fluid was extracted through posterior superior iliac spine, and mononuclear cell was separated from the liquid by Ficoll-Hypaque. At the same time, NB4 cell of
Platelet-poor plasma (containing sodium citrate) was used for adding 100 µL into coagulometer test cup. Add 100 µL containing 5% CO₂ at 37°C, and cultivate them for 24 h, containing 10% FBS, then incubate them in incubation

APL cell line was cultivated applying RPMI 1640 culture solution (GIBCO Company).

Experiment reagents were: Annexin V-FITC kit (Bioso Technology Co., Ltd., Beijing), DNR (Pharmacia ItaliaS.p.A Company), ATO (Yida Pharmaceutical Co., Ltd., Harbin), thrombomodulin, TF and annexin II ELISA kit (Xin Yue Biotechnology Co., Ltd., Shanghai)

**Methods**

**Inclusion and exclusion standards**

1. All patients' conformed to diagnostic criteria of acute promyelocytic leukemia constituted by World Health Organization (WHO); 2. Patients were detected as positive through testing fusion gene of PML-RARα; 3. Unhappened coagulation situation in blood vessel could be known in patients by test; 4. Patients had good tolerance on the drug used in this study and could stick to treatment; 5. Patients had no mental disorder; 6. Patients and family members actively cooperated with the study, enjoyed the right of information and have signed the informed consent voluntarily.

**Group of experiment**

Cells were divided into four groups when they were inoculated in logarithmic phase into 64-well plates with the density of 2×10⁵/ml: 1. blank control group (without medicine); 2. DNR treatment group (DNR 0.3 µmol/L); 3. ATO treatment group (ATO 1 µmol/L); 4. ATO+DNR treatment group (ATO 1 µmol/L+DNR 0.3 µmol/L). The cultivation of liquid of APL and NB4 cells: cultivate APL and NB4 cells into RPMI 1640 nutrient solution containing 10% FBS, then incubate them in incubation containing 5% CO₂ at 37°C, and cultivate them for 24 h, 48 h, 72 h respectively, after grouping.

**Detection of APL and NB4 apoptosis rate with flow cytometry**

Collect cells in Annexin V-FITC kit, and wash it with PBS; adjust concentration of cell suspension to (0.5~1.0)×10⁶; add 10µL FITC-Annexin V, and incubate it keeping out of sun for 15 min; incubate it in the dark for 5 min after adding propidium iodide (PI). Then add 100 µL binding buffer, and detect it with flow cytometry. Gating was performed by forward scattered light based on logarithm model, with exciting light at the wavelength of 488 nm and emitted light at 530 nm. Then collect 10,000 cells for each sample, and analyze the results using CELL Quest software.

**Detection of plasma recalcification time of APL and NB4 cells**

Test plasma recalcification time with coagulometer in order to detect the procoagulant activity of APL and NB4 cells. Adjust cell concentration to 1×10⁷/ml with PBS, and add 100 µL into coagulometer test cup. Add 100 µL platelet-poor plasma (containing sodium citrate for anticoagulation) from normal people into APL/NB4 cell suspension and mixed it, then incubate it for 3 min at 37°C. Add 100µL 25mmol/L CaCl₂ to activate the reaction. Read experimental results after blood clotting response (data was expressed as second). Then repeat the above procedures for three times and take the average value. Set up saline control group and acellular pure plasma control group.

**Detection of expression of TF, thrombomodulin and annexin II of NB4 cells with ELISA method applying double antibody sandwich method**

Wash NB4 cells twice with PBS, resuspend it to 1×10⁶/ml with PBS, and extract supernatant after multigelation. Add samples (set up blank hole, standard hole, and undertested hole of sample), and incubate it for 60 min at 37°C. Wash it for five times with PBS, then add 50µL color developing agent A and B for each hole successively. Vibrate and blend it slightly. Put the liquid in the dark at 37°C. Fifteen min later, stop the reaction. Then detect absorbance at the wavelength of 450 nm (value A).

**STATISTICAL ANALYSIS**

SPSS 13.0 software was applied to perform statistical analysis on all data. Measurement data was expressed with (mean ± SD) using t test. Enumeration data adopted χ² test. There was statistical significance if P<0.05.

**RESULTS**

**Comparison of apoptosis rate of APL and NB4 cells in each group**

From table 1, we could know that apoptosis rate of APL and NB4 cells after ATO+DNR treatment was the highest, and apoptosis rate of APL and NB4 cells after ATO treatment was lower than DNR group. ATO had shortness for apoptosis; apoptosis rate was higher and higher as time went by. But the apoptosis rate of APL and NB4 cells using ATO or DNR separately was significantly lower than joint group.

**Analysis of procoagulant activity of APL and NB4 cells in each group**

From table 2, we could conclude that the influence of joint application of arsenic trioxide and daunorubicin on primary acute promyelocytic leukemia could be known in patients by test; 4. Patients had good tolerance on the drug used in this study and could stick to treatment; 5. Patients had no mental disorder; 6. Patients and family members actively cooperated with the study, enjoyed the right of information and have signed the informed consent voluntarily.

The influence of joint application of arsenic trioxide and daunorubicin on primary acute promyelocytic leukemia was significantly prolonged (P<0.05), which indicated that ATO could decline coagulation activity of APL; compared with blank control group, the group used DNR alone improved procoagulant activity of APL and NB4 cells significantly (P<0.05). The results showed that ATO+DNR combined treatment could promote APL apoptosis more effectively than ATO monotherapy by adding these two drugs into APL and NB4 cells in the meantime (P<0.05).
From table 3, we could find that, TF antigen expression in ATO treatment group and drug combined treatment group reduced sharply compared with blank control group (P<0.05); TF antigen expression in drug combined treatment group was between ATO treatment group and DNR treatment group (P<0.05 compared with ATO treatment group); expression of thrombomodulin in ATO treatment group had an increasing tendency compared with blank control group, but there was no statistical significance (P>0.05); expression of thrombomodulin decreased after processing NB4 cell with DNR, but there was also no statistical significance (P>0.05); the combined application of ATO and DNR also had no obvious effect on expression of thrombomodulin (P>0.05); expression of annexin II declined dramatically after processing NB4 cell with ATO or DNR (P<0.05); expression of annexin II declined more dramatically after processing NB4 cell with ATO and DNR (P<0.05).

**DISCUSSION**

It is found that, arsenic trioxide can promote mutation of mitochondrial DNA and APL apoptosis to some extent, and induce cell differentiation and degrades PML-RARα fusion gene, so as to achieve the purpose of treating diseases (Li et al., 2011). In 1973, ATO was first applied in the treatment of refractory, recurrent and primary APL patients, and its complete remission rate reached more than 90%. The most outstanding characteristics were: ATO could improve coagulation of APL itself quickly and relevant complications, and decrease fatality rate in the early stage. Previously, the scheme, anthracyclines with standard doses + cytosine arabinoside was considered as preparative regimen in order to reduce the number of increased white blood cell. But 80% APL patients with primary high white blood cells type had obvious blood coagulation disorders when undergoing a definite diagnosis. Traditional chemotherapy would cause or aggravate existing blood coagulation disorders, and increase bleeding and associated fatality rate in the early stage. Single use of chemotherapy drug was bad for APL of primary high white cell type. Preliminary clinical observation found that combined application with DNR not only could improve blood coagulation dysfunction, but also quickly reduced the number of leukemia cells at the same time when ATO therapy alone had ineffective influence on APL of primary high white blood cell type, and the complete remission rate increased significantly. But this method still needs basic theoretical support if applied clinically.

Apoposis rate in ATO+DNR treatment group in this research was higher than in ATO group or DNR group, therefore, joint application could promote apoptosis. In
addition, we discovered that the blood coagulation time in ATO treatment group and DNR treatment group had no statistical significance (P>0.05), and were prolonged obviously compared with control group (P<0.05). It indicated that joint medication of ATO and DNR not only could decline coagulation activity of APL, improve coagulation function significantly, but also quickly reduce the number of leukemia cells, which provided new theoretical foundation for APL of primary high white blood cell type. Joint application of ATO and DNR eased blood coagulation dysfunction of APL through analyzing the influence of coagulant substances secretion and hyperfibrinolysis by further detecting expression of TF, thrombomodulin and annexin II. In normal cells, TF mostly were in inactive state. TF occurred decryption when there was cells apoptosis and it became active, thus promoting clotting cascade reaction. Combination of ATO and DNR could correct blood coagulation dysfunction of APL by reducing expression of TF antigen. It was less efficient than ATO therapy alone. Thrombomodulin is a kind of transmembrane glycoprotein expressed on the surface of endothelial cells, which can unite into compound through thrombin, act as anticoagulation, and inhibit fibrinolysis (Anna et al., 2011; Kitsada and Magarida, 2012). This research explained that DNR promoted coagulation activity not through decreasing thrombomodulin. Joint application of ATO and DNR might not give play to coagulation improving function through influencing expression of thrombomodulin.

In recent years, the study has found that annexin II plays a important role in hyperfibrinolysis. The increase of expression of annexin II on the surface of APL cells speeds up the occurrence of plasmin and generates hyperfibrinolysis, which is one of the important factors for the bleeding complications in APL patients. Expression of annexin II of APL cells reduces and plasma fibrinolytic enzyme decreases after APL patients use ATRA or ATO. Furthermore, ATRA or ATO also decreases mRNA and expression of annexin II of APL cells. ELISA detection results in this research show that ATO and DNR can correct high fibrinolysis state of NB4 cells through lowering expression of annexin II protein of NB4 cells, and joint application enhances the fibrinolytic resistant effect.

REFERENCES


