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Menadione reduced doxorubicin resistance in Ehrlich ascites carcinoma cells *in vitro*[1]

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KEY WORDS vitamin K; doxorubicin; Ehrlich tumor carcinoma; neoplasm drug resistance; glutathione; glutathione transferases; membrane fluidity

AIM: To study the effect of menadione (Men) reducing doxorubicin (Dox) resistance in Ehrlich ascites carcinoma (EAC) cells resistant to Dox (EAC/Dox cells). **METHODS:** Glutathione (GSH) content and membrane fluidity were measured by fluorometric assay and fluorescence depolarization assay, respectively. Glutathione *S*-transferase (GST) activity was measured with 1-chloro-2, 4-dinitrobenzene as the substrate. Cell viability was determined by 3-(4, 5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide assay. **RESULTS:** GSH content, GST activity, and membrane fluidity in EAC/Dox cells were higher than those in EAC cells ($P < 0.01$). The IC_{50} (95 % confidence limits) for Dox on EAC/Dox cell was 22.3 (15.8 - 28.8) mg·L^{-1}. Relative resistance of Dox in EAC/Dox cells was 42-fold. Pretreatment of EAC/Dox cells with Men 5 or 10 mg·L^{-1} decreased intracellular GSH content ($P < 0.01$). Men 1 mg·L^{-1} had no obvious effect on GSH content in EAC/Dox cells ($P > 0.05$), but decreased the elevated membrane fluidity efficiently ($P < 0.05$). Men had no obvious effect on GST activity in EAC/Dox cells ($P > 0.05$). IC_{50} of Dox was reduced to 9.6 (7.8 - 11.3), 6.0 (2.8 - 9.2), or 5.3 (3.9 - 6.7) mg·L^{-1} in EAC/Dox cells pretreated with Men 1, 5, or 10 mg·L^{-1}. **CONCLUSION:** Men reduced Dox resistance effectively due in part to its depletion of GSH content in EAC/Dox cells.

Development of resistance to chemotherapeutic agents is a major impediment in the treatment of cancer. Drug resistance is often associated with a decreased intracellular drug accumulation[1]. The elevation of glutathione (GSH) content in drug-resistant tumor cells has also been observed, and the depletion of GSH can partially reduce the drug-resistant phenotype[2]. Menadione (Men) was observed to deplete GSH and increase the formation of O_2^- in L 1210 cells[3]. These findings prompted us to examine whether GSH depletion might result from Men, and doxorubicin (Dox) resistance could be reduced in EAC/Dox cells.

MATERIALS AND METHODS

Reagents RPMI-1640 medium was obtained from Gibco; Dox was got from Farmitalia Carlo Erba Ltd; 3-(4, 5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide (MTT) and phthaldiadehyde were purchased from Fluka; 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) and 1-chloro-2, 4-dinitrobenzene were bought from Sigma; Men was the product of Shanghai 1st Pharmaceutic Factory. MTT, Dox, and Men were dissolved in phosphate buffer solution (PBS) 0.01 mol·L^{-1}, pH 7.2.

Cell culture EAC and EAC/Dox cells in log phase were maintained in RPMI-1640 medium supplemented with 15 % fetal calf serum and antibiotics (benzylpenicillin 100 kU·L^{-1} and streptomycin 100 mg·L^{-1}) in a humidified atmosphere containing 5 % CO_2 at 37 ℃.

Cytotoxicity assay Expontentially growing cells were plated in flat-bottomed 96-well plates at 4×10^4 cells/well for EAC and EAC/Dox cells. Drug was added in serial dilution to give a final volume of 200 μL/well. Control wells contained medium without drug. Plates were incubated for 2 d, and cell viability was assayed. MTT (5 g·L^{-1}) 10 μL was added to each well, and plates were incubated at 37 ℃ for 4 h. The medium was aspirated and formazan crystals were sobulised in 100 μL Me_2SO. The absorbance at

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570 nm was read on ELISA multikan reader. IC_{50} (50 % inhibitory concentration) for a particular drug was defined as the concentration causing 50 % decrease in cell growth. Relative resistance was defined as: IC_{50} of resistant cell/ IC_{50} of sensitive parent cell. Results was calculated[4].

Assay for GSH GSH contents in EAC and EAC/Dox cells were determined with Hitachi 850 spectrofluorometer[5]. EAC and EAC/Dox cells were inclubated with Men 1, 5, 10, or 20 $mg \cdot L^{-1}$ for 24 h (control was treated with PBS), washed by centrifugation, and resuspended in buffers, and GSH contents were determined.

Assay for glutathione *S*-transferase (GST) activity GST activities of EAC and EAC/Dox cells were determined with Shimadzu UV-365 spectrometer[6]. EAC and EAC/Dox cells were incubated with Men 1, 5, 10, or 20 $mg \cdot L^{-1}$ for 24 h (control was treated with PBS), washed by centrifugation, and resuspended, and GST activities were determined.

Procin determination Protein was determined[7].

Fluoroscence deporlarization measurement Steady-state fluoroscence deporlarization measurements of whole cells (expressed as F) were carried out[8,9]. In brief, 5×10^6 washed cells were incubated with DPH 2 $\mu mol \cdot L^{-1}$ at 25 ℃ for 30 min. F values were measured immediately with Hitachi 850 spectrofluorometer (λ_{ex} = 362 nm, λ_{em} = 432 nm). Value was calculated with the equation[9].

$$F = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$$

$$G = I_{HV}/I_{HH}$$

in which I_{VV}, I_{VH}, I_{HV}, I_{HH} represent the fluoresecence intensities which were parellel or perpendicular to the excitation and emission beam, respectively. A decrease in F value indicates an increase in plasma membrane fluidity.

Statistics Data were expressed as $\bar{x} \pm s$ and analyzed by t test.

RESULTS

Effect of Men on GSH content GSH contents in EAC and EAC/Dox cells were 4.6 ± 1.0 $\mu mol/g$ protein and 18 ± 4 $\mu mol/g$ protein, respectively. Men 5, 10, and 20 $mg \cdot L^{-1}$ decreased GSH contents in EAC/Dox cells (Tab 1), and there was obvious linear correlation between GSH content ($\hat{Y}$) and concentration of Men (X): $\hat{Y} = 1.252 - 0.0598X$ ($r = -0.9524$, $n = 4$, $P < 0.05$).

Effect of Men on GST activity GST activities in EAC and EAC/Dox cells were 1.55 ± 0.25 $mmol \cdot g^{-1} \cdot min^{-1}$ and 2.8 ± 0.4 $mmol \cdot g^{-1} \cdot min^{-1}$, respectively. Men had no obvious effect on GST activities in EAC and EAC/Dox cells (Tab 1).

Effect of Men on membrane fluidity F values in EAC and EAC/Dox cells were 0.193 ± 0.008 and 0.150 ± 0.008, respectively. Men 1, 5, 10, or 20 $mg \cdot L^{-1}$ increased F values in EAC/Dox cells, decreasing membrane fluidity significantly (Tab 1).

Effect of Men on Dox cytotoxicity The IC_{50} (95 % confidence limits) of Dox for EAC and EAC/Dox cells were 0.53 (0.40 – 0.66) $mg \cdot L^{-1}$ and 22.3 (15.8 – 28.8) $mg \cdot L^{-1}$, respectively. The relative resistance of Dox is 42-fold. The IC_{50} of Men for EAC and EAC/Dox cells were 10.6 (7.9 – 13.3) $mg \cdot L^{-1}$ and 19.4 (14.3 – 24.5) $mg \cdot L^{-1}$, respectively. The ability of Dox to inhibit EAC/Dox cell growth

Tab 1. GSH contents (μmol/g protein), GST activities ($nmol \cdot g^{-1} \cdot min^{-1}$), and membrane fluidity (F) in EAC and EAC/Dox cells incubated with menadione (Men) for 24 h. $n = 4$ samples, 5×10^6 cells/ sample. $\bar{x} \pm s$.
$^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control EAC cells. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* control EAC/Dox cells.

Men/ $mg \cdot L^{-1}$	EAC cells			EAC/Dox cells		
	GSH	GST	F	GSH	GST	F
0	4.6 ± 1.0	1.55 ± 0.25	0.193 ± 0.008	17.8 ± 4.4	2.8 ± 0.4	0.150 ± 0.008
1	5.8 ± 2.7^a	1.7 ± 0.4^a	0.192 ± 0.015^a	16.7 ± 1.7^d	2.8 ± 0.5^d	0.164 ± 0.009^e
5	9.4 ± 1.4^c	1.3 ± 0.4^a	0.199 ± 0.016^a	12.1 ± 2.1^f	2.9 ± 0.3^d	0.168 ± 0.012^e
10	2.3 ± 1.2^b	1.40 ± 0.25^a	0.208 ± 0.012^b	3.3 ± 0.8^f	2.6 ± 0.7^d	0.171 ± 0.015^e
20	1.3 ± 1.4^b	1.2 ± 0.4^a	0.205 ± 0.014^b	2.5 ± 1.5^f	2.5 ± 0.5^d	0.169 ± 0.011^e

was enhanced by pretreatment with Men 1, 5, or 10 mg·L^{-1} ($P < 0.01$) (Tab 2, Fig 1).

Tab 2. IC_{50} (95 % confidence limits) of doxorubicin (Dox) for EAC/Dox cells pretreated with Men. $n = 4$ samples, 3×10^4 cells/sample. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control IC_{50}; $^fP < 0.01$ *vs* control relative resistance.

Men/ mg·L^{-1}	IC_{50} (95 % confidence limits)/mg·L^{-1}	Relative resistance
0	22.3 (15.8 - 28.8)	42.1 ± 7.7
1	9.6 (7.8 - 11.3)^c	18.1 ± 2.0^f
5	6.0 (2.8 - 9.2)^c	11.4 ± 3.7^f
10	5.3 (3.9 - 6.7)^c	9.9 ± 1.6^f

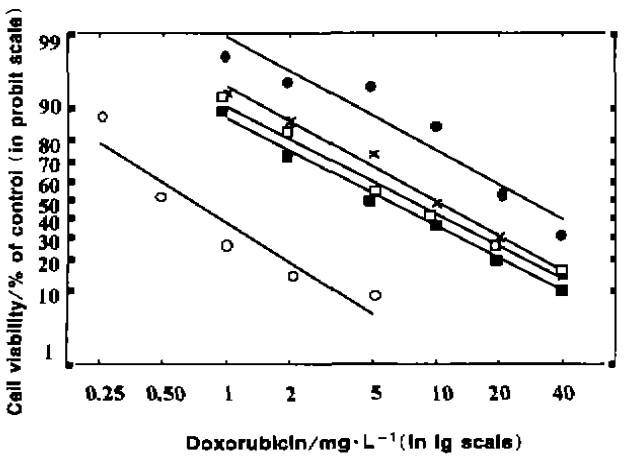

Fig 1. Effect of Men on Dox cytotoxicity in EAC/Dox cells. $n = 4$ samples, $\bar{x} \pm s$. EAC cells without Men (○), EAC/Dox cells: control without Men (●), with Men 1 (×), 5 (□), and 10 mg·L^{-1} (■).

DISCUSSION

We have demonstrated that Men at concentrations not inhibiting tumor cell growth can partially reduce Dox resistance in EAC/Dox cells.

When EAC/Dox cells were incubated without Dox for 36 wk, the sensitivity of Dox was partially recovered and the GSH level in EAC/Dox (R) cells decreased simultaneously[5]. Men 5 or 10 mg·L^{-1} that decreased the intracellular GSH contents in ECA/Dox cells reduced Dox resistance significantly. Because Men had no effect on GST activity in EAC/Dox cells, we concluded that Men might be able to inhibit synthesis or increase degradation of GSH other than increase consumption of GSH.

Membrane stability was very important for the normal functioning of membrane proteins, including receptors and enzymes. Most drug-resistant tumor cells exhibited an increase in membrane fluidity[10]. Men 1 mg·L^{-1} that had no effect on GSH could decrease the membrane fluidity in EAC/Dox cells ($P < 0.05$). While EAC/Dox cells were pretreated with Men 1 mg·L^{-1}, Dox resistance was reduced.

DNA and RNA biosynthesis inhibition was increased in sensitive P 388 and in multidrug-resistant P 388/ADM cells exposed to Dox after pretreatment with Men for 3 h[11]. Men caused a depletion of the intracellular GSH level in P 388 and P 388/ADM cells but at concentrations greater than those that enhanced Dox cytotoxicity.

We concluded from our work that the elevation of GSH was one of the important reasons for Dox resistance in EAC/Dox cells, and Men reduced Dox resistance in EAC/Dox cells efficiently due in part to the depletion of GSH.

REFERENCES

1 McClean S, Hill BT. An overview of membrane, cytosolic and nuclear proteins associated with the expression of resistance to multiple drug *in vitro*. Biochim Biophys Acta 1991; 1114: 107 - 27.
2 Ozols RF, O'Dwyer PJ, Hamilton TC, Young RC. The role of glutathione in drug resistance. Cancer Treat Rev 1990; 17 Suppl A: 45 - 50.
3 Akman SA, Dietrich M, Chlebowski R, Limberg P, Block JB. Modulation of cytotoxicity of menadione sodium bisulfite *versus* leukemia L 1210 by the acid-soluble thiol pool. Cancer Res 1985; 45: 5257 - 62.
4 Marks DC, Belov L, Davey MW, Davey RA, Kidman AD. The MTT cell viability assay for cytotoxicity testing in multidrug- resistant human leukemic cells. Leuk Res 1992; 16: 1165 - 73.
5 Wang QD, Li GD, Liu MJ, Zhang Y, Liu J, Zhang TM. The correlation between multidrug resistance and glutathione (GSH) in EAC, EAC/ADR and EAC/ADR (R) cell lines. Acta Pharm Sin 1994; 29: 20 - 3.
6 Hu YP, Liu J, Wang QD, Yie QX, Zhang TM. Reversal of adriamycin resistance by verapamil and ligustrazini in mouse Ehrlich ascites cancer. Acta Pharm Sin 1993; 28: 75 - 8.
7 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265 - 75.
8 Montaudon D, Vrignaud P, Londos-Gagliardi D, Robert J. Fluorecence anisotropy of cell membranes of doxorubicin-sensitive and -resistant rodent tumoral cells. Cancer Res 1986; 46: 5602 - 5.
9 Lin KC, Nie SQ, Bo HQ, Qiao YL. Research of membrane lipid fluidity in Ehrlich ascites carcinoma with fluorescence probe DPH. Prog Biochem Biophys 1981; (6): 32 - 5.
10 Wiseman H. Tamoxifen: new membrane-mediated mechanisms of action and therapeutic advances. Trends Pharmacol Sci 1994; 15: 83 - 9.
11 Parekh HK, Mansuri-Torshizi H, Srivastava TS, Chitnis MP. Circumvention of adriamycin resistance: effect of 2-methyl-1, 4-naphthoquinone (vitamin K_3) on drug cytotoxicity in sensitive and

MDR P388 leukemia cells. Cancer Lett 1992; 61: 147 - 56.

维生素 K_3 体外降低 Ehrlich 腹水癌细胞对阿霉素的抗药性[1]

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关键词 维生素 K；阿霉素；Ehrlich 瘤癌；瘤抗药性；谷胱甘肽；谷胱甘肽转移酶；膜流动性

目的：研究维生素 K_3（Men）降低 EAC/Dox 细胞对阿霉素（Dox）的抗药性. **方法**：测定谷胱甘肽（GSH），细胞膜流动性及谷胱甘肽 *S*-转移酶（GST）活性. 细胞存活力以甲基四唑蓝法测定. **结果**：EAC/Dox 细胞 GSH，GST 及膜流动性均较 EAC 细胞增加（$P < 0.01$）. Dox 对 EAC/Dox 细胞 IC_{50} 为 22.3（15.8 - 28.8）$mg \cdot L^{-1}$. Men 5 或 10 $mg \cdot L^{-1}$ 可降低 EAC/Dox 细胞 GSH（$P < 0.01$），1 $mg \cdot L^{-1}$ 对 GSH 无影响（$P > 0.05$），但可降低细胞膜流动性（$P < 0.05$）. Men 1，5 或 10 $mg \cdot L^{-1}$ 可使 Dox IC_{50} 降低到 9.6（7.8 - 11.3），6.0（2.8 - 9.2），或 5.3（3.9 - 6.7）$mg \cdot L^{-1}$（$P < 0.01$）. **结论**：Men 在体外降低 EAC/Dox 细胞对 Dox 抗药性与对 GSH 的耗竭有关.

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Negative correlation between cyclophilin mRNA level in leukocytes of renal allograft recipients and ciclosporin concentration in whole blood

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KEY WORDS cyclosporine; cyclophilin; kidney transplantation; polymerase chain reaction; leukocytes

AIM: To study mRNA level of cyclophilin in the white blood cells (WBC) of the renal allograft recipients (RAR) and its correlation with ciclosporin concentration in whole blood. **METHODS:** The cyclophilin mRNA levels and β-actin as controls in the WBC of 47 RAR were measured by quantitative reverse transcription polymerase chain reaction. The blood ciclosporin assay utilized monoclonal antibody fluorescence polarization immunoassay. **RESULTS:** With the increase of ciclosporin concentration in whole blood (from 62 $\mu g \cdot L^{-1}$ to 678 $\mu g \cdot L^{-1}$), relative cyclophilin mRNA level in the WBC of RAR decreased nonlinearly (from 1.1 to 0.03, $r = 0.8195$). **CONCLUSION:** There was a negative correlation between the mRNA level of cyclophilin in the WBC of RAR and the ciclosporin concentration in whole blood.

Ciclosporin (Cic) is an immunosuppressive agent to prevent renal transplant rejection[1]. But often there was a discrepancy between the effects of Cic treatment and its concentration in the whole blood of the renal allograft recipients (RAR). Cyclophilin (CyP) is the receptor of Cic in cytosol, which mediates immunosuppressive action of Cic in the cells. Different expressions of CyP in the cells directly affect the distribution and absorption of Cic *in vivo* as well as the immunosuppressive effect[2]. This paper was to study the mRNA level of CyP in the white blood cells (WBC) of the RAR and its correlation with Cic concentration in whole blood.

MATERIALS AND METHODS

Patients and Cic assay Forty-seven patients (37 M and 10 F) with renal transplantation, aged 17 - 58 ($37 \pm s$ 10) a were given immunosuppressive regimen according to their clinical signs. Blood samples were collected into heparinized tubes at 8:00 before breakfast. The whole blood Cic concentration was measured by the TDX Cic monoclonal antibody whole blood assay (Abbott Laboratory). Serum creatinine

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