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Effects of cyclophosphamide on protein expression of rat embryo at end of pre-gastrulation stage *in vivo*[1]

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KEY WORDS fetal development; drug-induced abnormalities; blastocyst; mutagens; gene expression regulation

ABSTRACT

AIM: To seek a sensitive time point for pre-gastrulation embryos exposed to developmental toxic agents, and to establish a molecular biomarker to evaluate the mechanism of cyclophosphamide-induced embryonic abnormalities *in vivo*. **METHODS**: Pregnant rats on d 3 of gestation were given ip cyclophosphamide (Cyc) 10, 20, 40 mg·kg^{-1}. SDS-PAGE was performed to qualitatively observe the target proteins in d 8 rat embryos. **RESULTS**: The expression of the protein with a molecular weight (M_r) of approximately 70 kDa distinctively increased and that of the blastocyst-specific protein (M_r 14.4 kDa) disappeared in Cyc 40 mg·kg^{-1} group. **CONCLUSION**: Day 8 of rat gestation could be an optimum time point for understanding developmental toxicity of mammalian embryo during pre-gastrulation, and the expression of the proteins with M_r 70 kDa and 14.4 kDa at this point could be employed as a molecular biomarker to demonstrate embryoteratology objectively and sensitively.

INTRODUCTION

The pre-gastrulation stage in mammalian embryogenesis is a fertile area for studying the origin of birth defects. This period involves stages from embryo cleavage and extends through gastrula stages to the beginning of organogenesis. However, the exposures at different time points in pre-gastrulation development result in both shifting of sensitivity and wide variation in endpoints based on the agents used[1]. So it is important to seek an optimum time point during pre-gastrulation for developmental toxicity studies.

Recently, research in reproductive toxicology has been emphasized on molecular mechanisms of developmental anomalies[2,3]. Therefore, it is necessary to establish a molecular biomarker to explore the mechanisms of embryonic anomalies after *in vivo* treatment. Since Cyc has significant genotoxicity and can induce early resorptions in rats[4], it was taken as a tool drug to develop a more precise method for observing reproductive toxicology. The present work was carried out to seek a time point to verify embryonic toxicity during pre-gastrulation stages, and to determine the changes in protein expression at this point after *in vivo* exposure to cyclophosphamide (Cyc).

MATERIALS AND METHODS

Drug Cyc was purchased from (Sigma St. Lois, MO).

Animal treatment Sprague-Dawley rats, 10 wk. (♀, weighing 229 ± *s* 8 g; ♂, weighing 251 ± *s* 12 g), were obtained from the Experimental Animal Center of Zhejiang University (Grade Ⅱ, certificate № 9601018). After being raised under 12 h light/12 h dark for 2 wk, rats (4♀ : 1♂) were housed together at 18:00. The next day when sperm was found in the vaginal smear was defined as d 0 of gestation. The mated females were divided randomly into experimental groups given ip Cyc 10, 20, 40 mg·kg^{-1} dissolved in 0.85 % normal saline (NS) on d 3 at 9:00. Control group was given ip 0.85 % NS 10 mL·kg^{-1}.

Isolation of embryos on d 8 On d 8 of gestation uteri were removed from the rats and placed in a 50-mm petri dish containing PB1 (a modified Dulbecco's phosphate-buffered saline) /10 % FCS at 0 ℃. With watchmakers' forceps, the uterine muscle was torn along

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the antimesometrial aspect of each implantation site to expose the decidua, then the embryos were gently pushed away from the decidual tissue by taking hold of the ectoplacental cone.

Measurement of d 8 embryonicprotein contents The isolated d 8 embryos were sonicated in a buffer containing Tris·HCl 10 mmol·L^{-1} pH 7.4, edetic acid 1.5 mmol·L^{-1}, DTT (dithiothreitol) 0.5 mmol·L^{-1}, and PMSF (phenylmethylsulfonyl fluoride) 0.1 mmol·L^{-1}. Homogenates were centrifuged for at 4 ℃ 60 min at 36 300 × g and the supernatant was collected. Total protein contents in each sample was determined by the Lowry assay[5].

Seperation of embryonic proteins by SDS-PAGE The soluble supernatant of the homogenate from each embryo was mixed with 2 × sample buffer, and proteins were separated by 13.5 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (n = 3)[6]. After 4–5 h, the modified silver-staining technique was used to visualize the proteins as follows: the gel was soaked in ethanol:HAc:H_2O (3:1:6) for at least 3 h; the solution A (30 % 2-propanol, 2 % glutaraldehyde, 0.2 % $Na_2S_2O_3$·5 H_2O and 6.8 % CH_3COONa·3 H_2O) was prepared and the gel was fixed for 2 h; the gel was washed in distilled water thrice; solution B (143 μL formaldehyde solution, 0.5 g $AgNO_3$) was prepared and the gel for 1 h with constant gentle agitation; washed the gel in deionized water and prepared solution C (143 μL formaldehyde solution, 0.25 % Na_2CO_3) was prepared; the silver stain was developed by soaking the gel in solution C until bands appeared (bands usually appeared in less than 10 min); the staining was stopped with 1.86 % edetic acid.

The protein contents of d 8 embryos were compared using t test.

RESULTS

Effects of Cyc on the protein expression in rat embryos could be observed both quantitatively and qualitatively. The protein content in d 8 embryos was markedly decreased ($P < 0.01$) after the pregnant rats were treated with Cyc on d 3 of gestation (Tab 1). The expression of 70 kDa protein was up-regulated in a dose dependent manner, most distinctly with Cyc 40 mg·kg^{-1}. Meanwhile, Cyc obviously down-regulated the expression of blastocyst-specific protein (M_r 14.4 kDa), which disappeared with Cyc 40 mg·kg^{-1} treatment (Fig 1).

Tab 1. Effects of Cyc on the protein content in d 8 embryos. n = 6 – 7 embryos from 3 rats. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Groups	Dose/mg·kg^{-1}	Protein contents/μg
NS	10 mL·kg^{-1}	1804 ± 137
Cyc	10	1410 ± 112^c
	20	872 ± 84^c
	40	817 ± 64^c

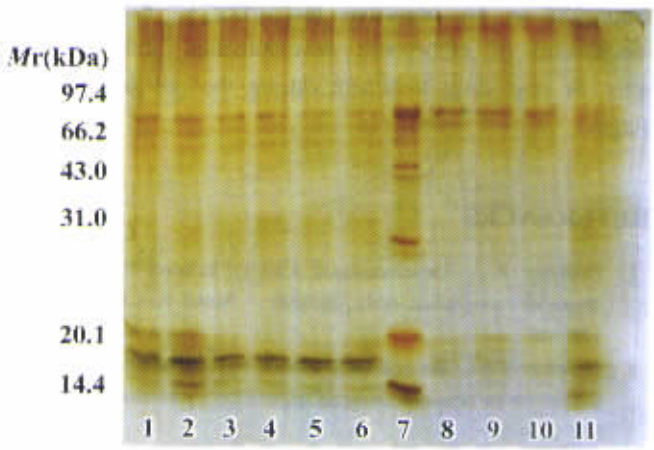

Fig 1. SDS-PAGE patterns of d 8 embryonic proteins from Cyc-treated rats on d 3 of gestation. 1, 2, 11: Control group(NS); 3,4: Cyc 20 mg·kg^{-1}; 5,6: Cyc 10 mg·kg^{-1}; 7: Marker; 8,9,10: Cyc 40 mg·kg^{-1}

DISCUSSION

Pre-gastrulation stages start from gamotogenesis and extend upto the beginning of organogenesis. A common feature among xenobiotic agents which are toxic at pre-gastrulation stages is the induction of embryonic lethality that occurs around or shortly after implantation[1]. Considering rat developmental phases, d 8 of gestation is the most suitable time point for evaluating embryonic toxicity.

The electrophoretic patterns have revealed in the present study two types of protein alterations in d 8 embryos exposed to Cyc on d 3 of gestation. The up-regulation of the 70 kDa protein and the down-regulation of the blastocyst-specific protein (M_r 14.4 kDa) were observed in a dose-dependent manner.

Since M_r at 70 kDa could be one of the definite biomarkers for heat shock protein (HSP)[7], the proteins at 70 kDa observed in this study may be postulated as HSP. Studies have shown that[8] the heat shock reaction

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acts directly on embryos and causes the subsequent apoptosis of inner cell mass (ICM). The blastocyst-specific proteins with M_r 14.4 kDa are expressed only after blastulation[6]. It is postulated that the disappearance of the proteins (M_r 14.4 kDa) with Cyc 40 mg · kg^{-1} treatment may be due to the disturbed expression of some key elements of ICM. Considering that ICM predominantly constitutes the embryonic tissues of conceptus, the changes of protein expression with (M_r 70 kDa and 14.4 kDa) on d 8 of gestation can be employed as an objective biomarker to explore the mechanism of embryonic developmental abnormalities after *in vivo* drug treatment during the pre-gastrulation stages.

REFERENCES

1 Rutledge JC. Developmental toxicity induced during early stages of mammalian embryogenesis. Mutat Res 1997; 396: 113 – 27.
2 Gelineau VWJ, Bennett GD, Finnell RH. Phenytoin-induced alterations in craniofacial gene expression. Teratology 1999; 59: 23 – 34.
3 Ivnitsky I, Torchinsky A, Gorivodsky M, Zemliak I, Orenstein H, Savion S, *et al*. TNF-alpha expression in embryos exposed to a teratogen. Am J Reprod Immunol 1998; 40: 431 – 40.
4 Spielmann H, Eibs HG. Recent progress in teratology. A survey of methods for the study of drug actions during the preimplantation period. Drug Res 1978; 28: 1733 – 42.
5 Lowry OH, Rosenbrough NH, Farr AL, Randall RJ. Protein measurement with the Folin reagent. J Biol Chem 1951; 193: 265 – 75.
6 Howlett SK. Qualitative analysis of protein changes in early mouse development. In: Monk M, editor. Mammalian development – a practical approach. Oxford: IRL press; 1987. p163 – 181.
7 Mirkes PE. Molecular/cellular biology of the heat stress response and its role in agent-induced teratogenesis. Mutat Res 1997; 396: 163 – 73.
8 Edwards MJ, Walsh DA, Li Z. Hyperthermia, teratogenesis and the heat shock response in mammalian embryos in culture. Int J Dev Biol 1997; 41: 345 – 58.

环磷酰胺对大鼠原肠胚前期末胚胎蛋白表达的影响[1]

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关键词 胎儿发育；药物性畸形；胚泡；致突变剂；基因表达调控

目的：确定原肠胚前期胚胎对发育毒性药物敏感的时间点，并建立评价 Cyc 诱导胚胎发育异常机制的分子生物学指标. **方法**：大鼠孕 d 3 *ip* Cyc 10，20，40 mg·kg^{-1}，孕 d 8 SDS-聚丙烯酰胺凝胶电泳定位大鼠胚胎蛋白. **结果**：Cyc 40 mg·kg^{-1}组 70 kDa 蛋白表达显著上调，14.4 kDa 胚泡化特征性蛋白表达消失. **结论**：大鼠妊娠 d 8 为研究原肠胚前期胚胎发育毒性的较适时间点，孕 d 8 70 kDa 蛋白表达上调伴随 14.4 kDa 胚泡化特征性蛋白表达下调可作为该发育阶段胚胎毒性分子生物学评价指标.

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