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Immunosurgical studies on cytological and cytogenetic toxicity analysis of rat blastocysts after *in vivo* exposure to cyclophosphamide[1]

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KEY WORDS cyclophosphamide; blastocyst; antibody-dependant cell cytotoxicity; cytogenetics; micronuclei; drug-induced abnormalities

AIM: To establish immunosurgery and indices of cytogenetic assessment for blastocyst and its inner cell mass (ICM), and to evaluate the toxic effects after *in vivo* exposure to cyclophosphamide. **METHODS:** Modified immunosurgery was established by preparation of rabbit-anti-rat spleen antiserum and induction of diluted rat mixed serum as complement. Pregnant rats on d 3 of gestation were injected ip cyclophosphamide (10, 20, and 40 $mg \cdot kg^{-1}$). On d 4, immunosurgery was performed on rat blastocysts. The cell number and the micronuclei of blastocyst and ICM were evaluated respectively. **RESULTS:** In the cyclophosphamide-treated rats, decreases of cell number (35 ± 3, 32 ± 1, 30 ± 1, and 14 ± 2, 11 ± 1, 9 ± 2) and increases of frequency of micronuclei (1.81 %, 2.27 %, 3.14 %, and 2.53 %, 2.98 %, 4.75 %) in blastocysts and ICM were observed in a dose-related manner. The changes of blastocyst were, however, not parallel to those of ICM which were more serious. **CONCLUTION:** Modified immunosurgery, an objective and elegant technique, was used on rat blastocysts. *In vivo* could cyclophosphamide injured ICM more than blastocysts.

Drug-induced mammalian blastocyst abnormality is involved in embryotoxicity and fetal malformations in a dose-dependent manner[1-4]. Development of the blastocyst involves differentiation of the inner cell mass (ICM), which differentiates into primary endoderm and ectoderm and gives rise to the embryo and the extraembryonic membranes of conceptus[5]. To explore the mechanisms of embryonic abnormality after *in vivo* preimplantation treatment, it is important to clarify whether or not drug selectively injuries ICM. The present work was to analyze the selective cytotoxicity and genotoxicity to ICM after *in vivo* exposure to cyclophosphamide (Cyc).

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MATERIALS AND METHODS

Cyc (Sigma). New Zealand white rabbit (♂, 3 months old) and Sprague-Dawley rats (♀, 9 wk, weighing $204 \pm s$ 15 g, ♂, adult, weighing $213 \pm s$ 17 g) were obtained from Experimental Animal Center, Zhejiang Academy of Medical Sciences. Rats were housed under 12-h light/12-h dark, 21 ± 1 ℃, for 2 wk before mating.

Preparation of antiserum and complement Rabbit anti-rat-spleen serum was yielded from rabbits which were bled on d 10 after five iv injections of 2×10^8 ♂ rat spleen cells[6]. Antibodies diluted 1:16 000 were detectable against the rat spleen in sheet glass coagulation test. Serum was heated at 56 ℃ for 30 min before use to inactivate rabbit complement, then stored at −20 ℃ and thawed immediately prior to use. Fresh rat serum was used as the source of complement at a final dilution of 1:5.

Pregnant rats Rats (4 ♀:1 ♂) were housed together at 17:00. The next morning when sperms were found in the vaginal smear was defined as d 0 of gestation. The mated females were divided randomly into experimental groups ($n = 8-10$). On d 3 at 9:00 ip Cyc (dissolved in saline) 10, 20, and 40 $mg \cdot kg^{-1}$. Control group was treated with an equal volume of saline.

Blastocyst immunosurgery During 13:00 − 15:00 on d 4 of gestation the blastocysts were collected in 96-hole plastic culture dishes (NUNU, Nulcon, Denmark), one hole for a blastocyst. The blastocysts were photographed under inverted microscope (×160) before and after immunosurgery[1]. With modifications: omitting the preincubation with pronase, a 15-min incubation of blastocysts with their zonae in heat-inactivated rabbit anti-rat-antiserum (diluted 1:5 vol:vol with M_2) at 37 ℃, washing in M_2 for 3 times, and finally a 60-min incubation in rat complement (diluted 1:5 vol:vol with PBS) at 37 ℃, followed by another washing in M_2. Both incubations were carried out in a humidified 5 % CO_2-in-air.

Sample preparation and cytological and cytogenetic

analyses Immunosurgical blastocysts were incubated in 0.7 % sodium citrate for 2 min, air dried, and stained with Giemsa (pH 7.4) for 2 min. Under microscope (× 400), ICM cells showed the same size, color, and characteristics of blastocysts without undergoing immunosurgery[11], while the TE cells had smaller, dark-stained, and indistinct nucleoli (Fig 1A, Plate 2) The total numbers of two different form nuclei were considered as the cell number per blastocyst. Those which color and focal distance were the same as ICM nuclei (not dark-stained), but the size was only 5 % – 20 % of the ICM nuclei with a smooth margin, were defined as micronuclei from ICM (Fig 1B).

The determinations of the cell number per blastocyst (CNPB), the cell number of ICM (ICMCN), and the frequency of micronuclei (FM) of blastocysts and ICM were employed as the indices to reveal different cytotoxic and genotoxic sensitivities to Cyc in both ICM and TE.

The cell numbers were compared using t test; FM was compared by χ^2 test.

RESULTS

Micromorphology of blastocysts during immunosurgery Preimplantation embryos exposed to antiserum for 15 min appeared unchanged. A few minutes after being transferred to complement, the TE cells appeared lysed, the ICM in blastocysts showed compact cell mass, which were seen within the blastoceles (Fig 2B, Plate 2).

In Cyc 40 mg·kg^{-1} group, blastulation of the embryos were retarded compared to control group. Most of them either delayed at morula stage or became degernating (Fig 2C). No compact cell mass from degerating blastocysts was found but lysed cells.

Cytological and cytogenetic analysis in blastocyst and ICM When pregnant rats were given ip Cyc 10, 20, and 40 mg·kg^{-1} on d 3 of gestation, CNPB was 35 ± 3, 32 ± 1, and 30 ± 1, and ICMCN was 14 ± 2, 11 ± 1, and 9 ± 2, respectively, lower than those of control group. Both of them showed a dose-dependent decrease ($r = -0.993$). The sensitivities of the two kind of cells, however, were not parallel to each other, the decrease of ICMCN was greater than that of CNPB in the same groups. When the % of inhibition was plotted against the logarithm of the Cyc dose, a linear dose-response relationship was obtained for both CNPB and ICMCN ($r = -0.993$). The slopes of two curves were not parallel.

At the same Cyc doses, the decrease of ICMCN was markedly greater than that of CNPB (Tab 1, 2).

Tab 1. Rat blastocysts after *in vivo* exposure to cyclophosphamide (Cyc) on d 3 of gestation and d 4 of immunosurgery. $n = 8-10$, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Cyc/ mg·kg^{-1}	Blastocyst/ recovered	used*	CNPB§	ICMCN‖	Frequency of micronuclei/% blastocyst	ICM
0	44	34	44 ± 2 (1493)	20 ± 2 (691)	0.27	0.29
10	60	53	35 ± 3^c (1878)	14 ± 2^c (792)	1.81^c	2.53^c
20	67	54	32 ± 1^c (1740)	11 ± 1^c (537)	2.27^c	2.98^c
40	78	52	30 ± 1^c (1608)	9 ± 2^c (478)	3.14^c	4.75^c

*Differences between blastocysts recovered and blastocysts used are due to the loss during manipulation or the degeneration of blastulation. §Cell number per blastocyst. ‖ICM cell number.

Tab 2. Cyclophosphamide(Cyc) decrease in cell numbers of per blastocyst (CNPB) and its inner cell mass (ICMCN) isolated by immunosurgery 30 h after treatment of rats on d 3 of pregnancy, $n = 34-54$ (blastocysts), $\bar{x} \pm s$.

Cyc/ mg·kg^{-1}	CNPB	ICMCN	CNPB/as % of control	ICMCN/as % of control
0	44 ± 2	20 ± 2	–	–
10	35 ± 3	14 ± 2	79.5 ± 6.8	70 ± 10
20	32 ± 1	11 ± 1	72.7 ± 2.3	55 ± 5
40	30 ± 1	9 ± 2	68.2 ± 2.3	45 ± 10

FM of blastocyst and ICM were 0.27 % and 0.29 % respectively in control group. In the Cyc groups, FM of blastocyst was 1.81 %, 2.27 %, and 3.14 %. FM of ICM was 2.53 %, 2.98 %, and 4.75 %, respectively, which were obviously different from those of control ($P < 0.01$, Tab 1). At the same dose level, the FM of ICM was particularly higher than that of blastocyst (Tab 1). Both of them showed a dose-dependent increase ($r = 0.999$, $r = 0.990$).

DISCUSSION

Present work employed the immunosurgery

used for isolating ICM from blastocysts, which based on the application of cytotoxic antibody followed by complement to the exterior of the embryo which would lyse the outer cells but not reach the ICM due to the barrier provided by the tight junction permeability seal. The work consisted of two steps and omit preincubation of blastocyst with pronase to remove the zonae[7,8].

Genetic injury of blastocyst has recently provided more important evidence for assessment of drug-induced blastocyst abnormality[1,2,4 11]. No published work on FM of ICM has been found so far, this study takes FM of ICM as a genetoxic index to develop the method for mechanism research.

To conform the reliability of the modified immunosurgery technique and the sensitivity of the evaluating index of FM from ICM, Cyc, a well known cytotoxicity, genotoxicity drug, was selected for present test. Tab 1 showed a dose-dependant decrease in ICMCN and a dose-dependent increase in FM of ICM among Cyc groups. The decrease of cell number and the increase of FM in ICM were markedly greater than those in blastocysts. The results proved that *in vivo* the two groups of cells in blastocysts could, in a dose-related manner, exhibited a different cytological and cytogenetic sensitivities against toxicity induced by Cyc.

In present study the effect of Cyc on CNPB and ICMCN in rats was similar to that in mice[12], but ICM was still more sensitive to cytotoxicity and genotoxicity after Cyc treatment. The differential sensitivity between ICM and TE may in part be due to a greater ability of the TE cells to repair DNA damage[9]. ICM in blastocyst can differentiate into primary endoderm and ectoderm and give rise to the embryo and the extraembryonic membranes of conceptus[5]. The results in this research could be employed to explore the mechanisms of embryonic developmental abnormality after *in vivo* preimplantation treatment.

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免疫外科法评价母体环磷酰胺染毒对大鼠胚泡的细胞学和细胞遗传学毒性[1]

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关键词 环磷酰胺；胚泡；抗体依赖性细胞毒性；细胞遗传毒性；微核；药源性异常

目的：以免疫外科法评价大鼠孕早期环磷酰胺染毒，对胚泡两群不同细胞的选择性影响. 方法：大鼠孕 d 3 ip 环磷酰胺(10, 20, 40 mg·kg^{-1})，孕 d 4 取胚泡行免疫外科术，分离胚泡内细胞团并检测其与胚泡对环磷酰胺损伤敏感性的差异. 结果：环磷酰胺组胚泡及内细胞团平均细胞数减少(35±3, 32±1, 30±1 及 14±2, 11±1, 9±2)，微核率增高(1.81 %, 2.27 %, 3.14 % 及 2.53 %, 2.98 %, 4.75 %)两者改变均呈剂量依赖性. 但胚泡与内细胞团受损随剂量增加不呈平行关系，后者改变更明显. 结论：环磷酰胺在大鼠胚泡着床前给药，对胚泡两群细胞呈剂量依赖性细胞毒性与遗传毒性，其中内细胞团受损尤甚.

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Cyclosporine inhibited calcium-mediated apoptosis of HL-60 cells[1]

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KEY WORDS cyclosporine; calcimycin; harringtonines; camptothecin; thapsigargin; calcium; apoptosis; HL-60 cells; DNA; flow cytometry

AIM: To study the effects of cyclosporine (Cyc) on apoptosis of HL-60 cells. **METHODS**: Apoptotic cells induced by harringtonine (Har), camptothecin (Cam), or calcimycin (Cal), thapsigargin (Tha) were identified with DNA electrophoresis, morphology, and flow cytometry. Relative $[Ca^{2+}]_i$ alteration of apoptotic HL-60 cells were determined with flow cytometry. **RESULTS**: Cal 1 mg·L^{-1} or Tha 0.5 mg·L^{-1} induced apoptosis of HL-60 cells. This effect was inhibited by nontoxic concentration of Cyc 1 mg·L^{-1}. Cyc did not inhibit Har- or Cam-induced apoptosis of HL-60 cells. Both Cal and Tha increased intracellular calcium, whereas Har or Cam did not. **CONCLUSION**: Cyc inhibited apoptosis only induced by calcium increasement in HL-60 cells. The mechanism of apoptosis induced by Cal or Tha was different from that by Har or Cam.

Cyclosporine (Cyc), an immunosuppressive agent, inhibited T lymphocyte activation and activation-driven apoptosis of T-cell lines or T-cell hybridomas[1,2]. Cyc is a good drug of attenuating mutidrug-resistance of cancer cells[3]. Cyc blocks T cell activation pathways associated with an increase of intracellular calcium. Since an early increase of intracellular calcium was found in many types of apoptosis[4], Cyc may block apoptosis in other cell lines. In this study, we investigated whether Cyc prevented apoptosis triggered by drugs that increased intracellular calcium, and compared with apoptosis induced by anticancer drugs.

MATERIALS AND METHODS

Reagents Cyc was purchased from Sino-American Pharmaceutical Factory of East China. Propidium iodide (PI), calcimycin (Cal), thapsigargin (Tha), camptothecin (Cam), and Fluo 3-AM were from Sigma. Hoechst 33342 was from Molecular Probes Inc. Har was from Beijing Union Pharmaceutical Factory.

Cell culture and cell viability assessment HL-60 cells were grown at 37 ℃ in RPMI 1640 medium (Gibco) containing 10 % heat-inactivated fetal bovine serum in an atmosphere containing 5 % CO_2. Exponentially growing HL-60 cells were exposed to drugs. Cells after drug treatment were stained with Hoechst 33342 10 μmol·L^{-1} and PI 50 mg·L^{-1} for 20 min, then washed with PBS and resuspended in PBS. Morphological and quantitative analysis of apoptosis was performed with fluorescence microscopy (Olympus)[5].

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