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INDUCTION OF DOMINANT LETHAL MUTATIONS AFTER EXPOSURE OF MALE MICE TO CYCLOPHOSPHAMIDE

INDUKCJA DOMINUJĄCYCH MUTACJI LETALNYCH PO EKSPOZYCJI SAMCÓW MYSZY NA CYKLOFOSFAMID

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The induction of dominant lethal mutations have been studied in the different stages of germ cells of mice exposed to some doses of cyclophosphamide.

INTRODUCTION

Cyclophosphamide (CP) is an alkylating agent commonly used, in man of reproductive age, as an drug which is administered in acute doses for cancer chemotherapy and chronic low doses maintenance chemotherapy and immunosupression. Also, the occupational exposure to cyclophosphamide may occur during industrial processing, in drug manufacture and in hospitals (nursing and pharmacy personnel). Contamination of the work environment was found not only on the working trays of the hoods and on the floors of the different rooms but also in other objects like tables, the sink unit, cleaned urinals and chamber pots, and drug [30].

Cyclophosphamide is indirect-acting mutagen which requires metabolic activation in vitro and in vivo. It is genotoxic after biotransformations. The predominant site of its activity is the liver, and its activation depends on the hepatic microsomal mixed function oxidase system, requiring NADPH and oxygen [8]. It is capable to causing base-pair substitutions and chromosomal aberrations [15, 16, 20]. In vitro and in vivo studies indicate that the major reaction site is the N7- position of guanine [9, 37].

In bacterial and animal test systems cyclophosphamide is mutagenic, carcinogenic and teratogenic [14, 28]. Adult male patients treated with this drugs showed diminished sperm counts and absence of germinal cells in testicular tissue [12]. CP has been also shown to induce sperm abnormalities in mice [24, 38], but not in Syrian hamster [32]. Exposure of male or female rodents to cyclophosphamide can result in dominant lethality and in congenital malformations in offspring [1, 6, 7, 17, 18, 19, 21, 34, 35, 36]. Occupational exposure to cyclophosphamide is significantly associated with fetal loss of nursing personnel in Finland [29] and causes chromosomal aberrations as well

as induces micronuclei in the blood of medical personnel handling antineoplastic drugs [4, 31].

The dominant lethal assay is one of the several tests for mutagenicity currently in use. It is an in vivo mammalian assay considered pertinent to the fundamental question of genetic toxicology, i.e. is a chemical likely to produce genetic change that can be transferred to the offspring. CP has been used as positive control substance in many assay systems. In this paper we would like to compare our own results regarding to dominant lethal mutations to results obtained by another authors.

MATERIALS AND METHODS

The dominant lethal test was performed according to the procedure previously described by *Green et al.* [13].

In the experiment Pzh: Sfis outbread mice, aged 10–12 weeks, were used. Before treatment with CP each male was caged with 2 virgin females. Only males with proven fertility were used. In experiment one male was caged with 3 virgin females for 1 week. Then the females were replaced weekly by 3 new ones. This process was repeated for 7 weeks. Different stages of spermatogenesis may be scored for induced mutation depending upon the interval between treatment and fertilization. The various spermatogenic stages were sampled in the mouse during the following intervals after treatment: spermatozoa (1 week), spermatids (2–3 weeks), spermatocytes (4–5 weeks), spermatogonia (6 week), and stem cells in 7 week [22].

Cyclophosphamide was obtained from Serva Feinbiochemica Company, Heidelberg (Germany). It was dissolved in 0.9% NaCl solution and applied by intraperitoneal injection. The injected volume was 0.1 ml per 10 g body weight. Doses of 25, 50, 100 and 200 mg/kg body weight of CP were used. The control males was injected with an equal volume of saline. Females were killed 18 days after the start of mating. All females that had implants were scored as fertile. The uterine contents of fertilized females were examined and the number of living fetuses, early and late deaths were scored.

The percentage of dominant lethal mutations (DLM) was calculated according to the formula:

$$\% \text{ DML} = \left[1 - \frac{\textit{living embryos / pregnant treated female}}{\textit{living embryos / pregnant control female}}\right]$$

RESULTS

Dominant lethal mutations include the loss of fertilized eggs before and after implantation. A decrease in implantation per female comparing with the control value indicates induced preimplantation death or an excess of unfertilized eggs. The difference between implantation per female and live embryos per female represents postimplantation death.

The results of the CP experiment are summarized in Table I. In control group pregnancy rates varied between 84 and 96 %. The percent of pregnant females was the lowest in week 3, 5 and 6 after exposure to 200 mg/kg bw of cyclophosphamide (between 62 and 76 %). The values are significantly different ($p < 0.05$) from the values for corresponding control groups and represent exposure of early spermatids, early spermatocytes and spermatogonia.

The mean number of live implantations per pregnant female in the control group showed minima and maxima of 10.1 (week 2) and 10.7 (week 5 and 6). After exposure to dose 25 mg/kg bw CP no significant mutagenic effect as determined by comparison

Table I. Results of dominant lethal assay after exposure to cyclophosphamide

Dose	Week	Number of females	% Pregnant females	Implants/pregant female total	live	dead	Dead implants (%)	DML (%)
Control	1	57	93	11,0	10,5	0,5	4,5	–
	2	57	93	10,8	10,1	0,7	6,6	–
	3	57	96	11,4	10,7	0,6	5,7	–
	4	57	84	10,9	10,2	0,7	6,2	–
	5	57	96	11,2	10,7	0,6	4,9	–
	6	57	87	11,2	10,7	0,5	4,8	–
	7	56	91	11,4	10,5	0,9	8,1	–
25 mg/kg	1	45	93	11,7	10,9	0,8	6,5	4,0
	2	45	93	11,9	11,4	0,5	4,4	–13,1
	3	45	91	11,3	10,7	0,6	5,3	–0,1
	4	45	91	12,1	11,4	0,7	5,6	–12,0
	5	42	93	11,2	10,4	0,8	6,9	2,2
	6	45	86	11,6	10,8	0,8	6,5	0,9
	7	45	95	11,2	10,3	0,8	7,3	1,3
50 mg/kg	1	50	86	10,8	9,5*	1,3	11,6	9,5
	2	51	84	10,5	9,4	1,0	9,9	6,9
	3	51	90	11,3	10,5	0,9	7,6	1,9
	4	51	92	10,6	10,0	0,6	5,8	2,0
	5	51	92	10,4	9,9	0,5	4,4	7,5
	6	51	94	11,3	10,9	0,5	10,0	–1,9
	7	51	92	11,1	10,7	0,4	3,9	–1,9
100 mg/kg	1	45	95	9,5	7,4*	2,0	21,6	29,6
	2	45	88	10,0	8,0*	2,0	20,0	20,6
	3	45	88	11,7	10,6	1,1	9,5	1,4
	4	45	93	11,2	10,6	0,5	4,8	4,3
	5	45	84	10,6	10,1	0,6	5,2	4,8
	6	45	88	10,6	10,1	0,5	4,7	5,3
	7	45	93	10,2	9,5*	0,8	7,4	9,6
200 mg/kg	1	30	86	7,3	4,5*	2,8	38,0	57,1
	2	30	86	7,6	4,2*	3,5	45,5	58,4
	3	30	76#	9,4	7,1*	2,3	24,1	33,6
	4	30	80	10,7	9,7	1,0	9,4	4,9
	5	30	63#	9,4	8,7*	0,7	7,3	18,7
	6	30	62#	9,6	9,0	0,6	6,4	15,6
	7	27	92	10,3	9,7*	0,6	6,6	7,6

\# – significantly different ($p < 0.05$) from corresponding control by Chi-square test
* – significantly different ($p < 0.05$) from corresponding control by *Student* t-test

of live embryos in experimental and control groups was detected (*Student* t-test). Sensitivity of germ cells increased with CP doses since 50 to 200 mg/kg bw. A statistically significant dose-dependent reduction of live embryos was observed. The most sensitive stage of spermatogenesis was spermatozoa. After treatment to 200 mg/kg bw the sensitivity of early spermatocytes and early spermatids have been also observed. The average number of live embryos after exposure of spermatozoa and late spermatids was drastically reduced and the percentage of DML was the highest (57–58 %). Values of live implants were significantly different ($p < 0.05$) from the values for corresponding control group by *Student t-test* (Tab. I).

The percent of dead implants increased with dose from 25 mg/kg bw to 200 mg/kg bw of CP (Tab. I). The most sensitive germ cells were those which were treated as late spermatids and mature sperm.

After exposure of spermatozoa and late spermatids to 100 mg/kg bw twice higher percent of dead implants then after exposure to 50 mg/kg bw was observed. Treatment to 200 mg/kg bw CP showed increased incidence of dead embryos in 1, 2 and 3 weeks after exposure. It indicated sensitivity of spermatozoa and spermatids. The highest percent of dead implants was after exposure of late spermatids with this dose (45.5 %).

DISCUSSION

Results obtained from previous and present studies indicate the ability of CP to produce chromosomal damage in male germ cells. In references the lowest effective dose tested in male mice were 25 mg/kg bw by single i.p. injection [27], whereas in the present experiment the same dose no induce dominant lethal mutations. In the present study dominant lethal mutations after exposure of spermatozoa to 50 mg/kg bw was observed. *Dean et al.* [10] reported that i.p. dose of 60 mg/kg bw of CP induced dominant lethals in male mice, and no effects are produced after injection to 48 mg/kg bw. In *Ehling* and *Neuhaser-Klaus* [11] experiment the dose of 40 mg/kg bw clearly induced DLM in spermatozoa and in late spermatids.

CP induced pre- and postimplantation losses resulted in a dose- and time-dependent decrease in the number of live fetuses per litter [34]. Positive responses generally ranged between 15 % and 60% postimplantation loss with single i.p. administration of 100 mg/kg yielding about 25 % and 200 mg/kg yielding about 50%. In all cases the highest increases in post implantation loss in week 1 or week 2 after mating were observed [3]. In our study the dose of 100 mg/kg bw of CP induced DLM (20–30 %) and decreased number of live embryos after exposure of spermatozoa, late spermatids and stem cells. after exposure with 120 mg/kg bw about 40–50 % of DLM in spermatozoa and spermatids were observed by another investigators [11, 23]. After treatment of spermatozoa and late spermatids to 200 mg/kg bw reduction of the total number of implantation was obtained. Similar effect was observed by *Anderson* et al. [2]. The highest single dose tested for dominant lethal effects was 300 mg/kg bw and significant increases in post-implantation loss were seen only after exposure of spermatids [26]. In our study the highest percent of dead implants after exposure of spermatozoa was seen. Less sensitive occurred spermatids. These results are in good agreement with observation described by *Anderson et al.* [2].

In our study the dose-response relationship was calculated using the linear model of regression analyses ($y = C + \alpha D$, where D = dose of CP, y = percent of dead (or the number of live) implants, C = the base-line, α – the linear regression coefficient). The mutagenic effect expressed as percent of dead implants could be described by the equations $y = 2.61 + 18.02$ D for spermatozoa, $y = -2.13 + 23.34$ D for late spermatids and $y = 0.57 + 11.13$ D for early spermatids. The frequencies of dead implants increased with dose and was equal respectively to 0.18; 0.23 and 0.11 per mg/kg of CP.

The linear effects expressed as live implants per female could be described by the equations: $y = 11.48 - 3.44$ D for spermatozoa, $y = 11.52 - 3.76$ D for late spermatids and $y = 11.73 - 2.12$ D for early spermatids. There is a dose-dependent decrease in the survival of implants per female equal respectively to 0.03; 0.04 and 0.02 per mg of CP.

Similar to our results, the authors of previous study have find dose response effects [17, 25–27, 33].

Our study on the induction of dead implants and on decrease of live implants in mice treated to CP shows that the dose-response lines obtained were similar for early and late spermatids as well as for spermatozoa.

It is confirmed that cyclophosphamide is not only a male germ cell mutagen but also its mutagenic effects after exposure of spermatozoa and spermatids increases linearly with dose.

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INDUKCJA DOMINUJĄCYCH MUTACJI LETALNYCH PO EKSPOZYCJI SAMCÓW MYSZY NA CYKLOFOSFAMID

Streszczenie

Zbadano indukcję dominujących mutacji letalnych w komórkach rozrodczych samców myszy szczepu Pzh: Sfis, którym podano różne dawki cyklofosfamidu. Po ekspozycji na 25 mg/kg m.c. nie stwierdzono efektu mutagennego. Potwierdzono indukcję dominujących mutacji letalnych po ekspozycji myszy na wyższe dawki cyklofosfamidu. Wrażliwość męskich komórek rozrodczych wzrasta a wraz ze zwiększaniem dawki cyklofosfamidu od 50 do 200 mg/kg m.c. Liczba żywych płodów zmniejszała się liniowo w zależności od dawki, a odsetek martwych płodów zwiększał się w zależności od dawki. Najbardziej wrażliwymi stadiami spermatogenezy okazały się późne spermatydy i plemniki.

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