MORPHOLOGICAL EVALUATION OF CYCLOPHOSPHAMIDE TESTICULAR TOXICITY IN RATS USING QUANTITATIVE MORPHOMETRY OF SPERMATOGENIC CYCLE STAGES

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(Received May 8, 1995 ; Accepted July 14, 1995)

ABSTRACT—The testicular toxicity of cyclophosphamide (Cp) in rats was evaluated by quantitative morphometry of spermatogenic cycle stages. Nine-week-old male Sprague-Dawley rats in Group 1 were given a single oral administration of 100 mg/kg of Cp, and were sacrificed at 1, 7, 14 and 21 days thereafter. Rats in Group 2 were orally given 100 mg/kg/day of Cp for 2 days, followed by 50 mg/kg/day for the next 3 days, and were sacrificed at 1 and 4 days after the last administration. The numbers of seminiferous epithelia were counted in the seminiferous tubules of stages II, V, VII and XII of the spermatogenic cycle. The data were expressed as numbers of spermatogenic cells per Sertoli cells per seminiferous tubule cross section. Animals in Group 1 showed decreased preleptotene spermatocytes at Day 7, decreased zygotene spermatocytes at Day 14, and decreased pachytene spermatocytes at Day 21. In group 2, testicular toxicity could also be clearly detected by this morphometric approach. The present morphometric study thus indicates that testicular toxicity can be detected from Day 7 even after a single administration of Cp.

KEY WORDS : Testicular toxicity, Quantitative morphometry, Cyclophosphamide.

INTRODUCTION

Cyclophosphamide (Cp) is an antineoplastic agent whose active metabolites are alkylating agents which cross-link DNA. In the clinical field, Cp has been used effectively for the control of proteinuria (Penso *et al.*, 1974) and Behcet's disease, a systemic disorder with ocular effects (Hijikata and Masuda, 1978). Some previous studies revealed the possibility that Cp produces serious side effects, particularly on the male re-

productive system (Fairly *et al.*, 1972). It was reported that adult male patients treated with this chemical showed diminished sperm counts and the absence of spermatogenic cells in their testicular tissue. In addition, from studies using laboratory animals, it was reported that Cp induced sperm abnormalities in mice (Pamerantsevs and Ramaya, 1980 ; Wyrobek and Bruce, 1975), with spermatogonia being the most sensitive to Cp treatment (Lu and Meistrich, 1979). Cp may be also mutagenic for mammalian germinal cells, since it has shown positive in some mutagenicity studies (Sotomayor *et al.*, 1978). In light of the above, it is considered that Cp causes testicular

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toxicity by inhibiting cellular division of spermatogonia. However, several reports have indicated that this chemical exerts no effects on the testis (Penn, 1979) and thus the testicular toxicity of Cp appears to depend on differences in experimental conditions or observation parameters. Such discrepancies constitute one of the major problems in evaluation of testicular toxicity.

It is sometimes difficult to detect toxic changes in the testis by traditional histopathological examination methods, unless marked degenerative morphological lesions are observed in spermatogenic cells, Leydig cells or Sertoli cells. In previous Cp studies where no morphological effects were documented for the rat testis, the suspicion is that early changes such as loss of spermatogonia induced by Cp were too minimal to be detected by routine/traditional histopathological examination or by toxicologic pathologists who do not take into account the spermatogenic cycle stages. In the present study, we evaluated Cp-induced testicular toxicity morphometrically by counting the numbers of spermatogenic cells at four selected stages of the spermatogenic cycle, using a simplified quantitative morphometric approach.

MATERIALS AND METHODS

Nine-week-old male Sprague-Dawley rats supplied by Charles River Japan Inc. (Shiga, Japan) were used. They were maintained in an air-conditioned barrier-system animal room controlled with an ambient temperature of $23 \pm 2^\circ\text{C}$, relative humidity of $55 \pm 5\%$ and 12-hr on/off light cycle, and food and water were provided *ad libitum*. Animals were divided into two groups to receive oral doses of Cp. Since it was thought that the toxic changes in the testis induced by Cp are slight in severity, animals were treated with a dose of Cp as high as possible. In group 1, twelve rats were given a single administration of 100 mg/kg of Cp which was equal to the oral LD_{50} , and 3 animals each were sacrificed at 1, 7, 14 and 21 days after the treatment. In group 2, six animals were given 100 mg/kg/day of Cp for 2 days, followed by 50 mg/kg/day of Cp for the next 3 days, because rats showed deterioration of general condition and hematuria after the second treatment of 100 mg/kg/day of Cp. On days 1

and 4 after completion of this dosing, 3 rats each were sacrificed. Control animals received a single dose of physiological saline, and were sacrificed at 1, 7 and 21 days thereafter. Each animal was weighed, and the testes were removed and the absolute and relative weights were determined.

All testes were preserved in Bouin's solution, embedded in paraffin and stained with HE and PAS for light microscopy. For examination of seminiferous tubules, quantitation of spermatogenic cells using a simplified morphometrical method was performed for stages II-III, V, VII and XII of the spermatogenic cycle. A total of 5 seminiferous tubules per animal were randomly chosen for each stage of the spermatogenic cycle, and the numbers of seminiferous epithelia were counted. The rat spermatogenic cycle can be subdivided into 14 stages and each stage is characterized by a unique and fixed association of spermatogenic cells (Clermont, 1972). The stages evaluated in the present study include major steps of germ cell development. The germ cells are type A spermatogonia, intermediate type spermatogonia, type B spermatogonia, preleptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes, round spermatids and elongate spermatids. In addition, the duration of stages II-III, V, VII and XII occupies the major parts of the spermatogenic cycle. For this reason, stages II-III, V, VII and XII were chosen as being representative. The following spermatogenic cell types were distinguished in the present study in accordance with the criteria of Leblond and Clermont, 1952: spermatogonia, preleptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes and round spermatids. The data were expressed as the numbers of spermatogenic cells per Sertoli cells per seminiferous tubule cross section, and differences between treatment groups and control group were analyzed using Student's *t*-test. The level of significance was set at $P < 0.05$.

RESULTS

Body weights and testes weights (Table 1): In group 1, the body weights of the Cp treated animals were lower than those of the control animals at 1, 7 and 21 days after the Cp treat-

Table 1. Body and testes weights in male rats treated with cyclophosphamide.

		Number of animals examined	Body weight (g)	Testes weight	
				Absolute (g)	Relative (g%)
Single administration					
Day 1	Cont.	3	336.4±16.6 ^{a)}	3.12±0.40	0.93±0.14
	CP	3	311.6± 5.0	3.02±0.24	0.90±0.07
Day 7	Cont.	3	392.6±12.4	3.40±0.16	0.87±0.04
	CP	3	359.9±16.6	3.26±0.21	0.91±0.06
Day 14	Cont.	NE			
	CP	3	379.7±38.1	3.21±0.60	0.84±0.09
Day 21	Cont.	3	437.8±11.0	3.30±0.04	0.76±0.01
	CP	3	392.9±46.1	3.05±0.38	0.78±0.12
Repeated administration					
Day 1	CP	3	306.5±17.3	3.02±0.28	0.98±0.06
Day 4	CP	3	276.9±19.1	3.12±0.14	1.13±0.04

^{a)} : Data are mean ± S.D. values

CP : Cyclophosphamide

NE : Not examined

ment. Absolute weights of the testes also showed a tendency to decrease in comparison with the control group values. However, remarkable changes were not observed in relative weights of the testes. In group 2, the body weights of Cp treated animals was decreased and absolute weights of the testes were lower than in the control animals at day 7.

Quantitative morphometry of spermatogenic cells (Figs. 1, 2) : In group 1, none of the types of spermatogenic cell at stages II-III, V, VII and XII showed any change at 1 day after the Cp treatment. At day 7, significant decreases of spermatogonia in stage V and preleptotene spermatocytes in stage VII were seen, the numbers of preleptotene spermatocytes in stage VII and zygotene spermatocytes in stage XII being decreased at 14 days after the treatment. At day 21, the decrease of preleptotene spermatocytes in stage VII was still present, with numbers of pachytene spermatocytes in stage V and VII

being significantly lower than in the control animals.

In group 2, decreases of spermatogonia in stages II-III, V, VII and XII were clearly detected at 1 day after the final Cp treatment, with numbers of preleptotene spermatocytes in stage VII also being decreased. At 4 days after the treatment, decreases of zygotene spermatocytes in stage XII were observed in addition to the changes seen at day 1.

Although decreased numbers of spermatogenic cells were detected in groups 1 and 2, conspicuous morphological changes, such as inflammatory cell infiltration or circulatory disturbance, were not observed in any animal treated with Cp. However, necrosis of spermatogonia at the base of seminiferous tubules could be detected by careful observation. The degree was very slight, but the frequency of necrotic spermatogonia was increased as compared to that in the control (Photos. 1, 2).

Fig. 1. Numbers of spermatogenic cells in seminiferous tubules of rats that had received a single administration of cyclophosphamide. *, **: Significantly different from the control values at $P < 0.05$ and $P < 0.01$, respectively.

Fig. 2. Numbers of spermatogenic cells in seminiferous tubules of rats that had received five administrations of cyclophosphamide. *, **, Significantly different from the control values at $P < 0.05$ and $P < 0.01$, respectively.

Photo. 1. Normal seminiferous tubule. Control group. HE staining, $\times 133$.

Photo. 2. Seminiferous tubule showing necrosis of spermatogonia ($\uparrow$). One day after the five repeated cyclophosphamide treatments. HE staining, $\times 133$.

DISCUSSION

Thorough studies of the testicular toxicity of Cp using analysis based on the spermatogenic cycle have not been performed previously. In our previous report, we proposed a convenient method for screening testicular toxicity dividing spermatogenic stages into four groups (I-VI, VII-VIII, IX-XI and XII-XIV) (Takahashi and Matsui, 1993). First of all, we adopted this convenient method in the present study to clarify whether toxic changes induced by Cp could be detected, although the data are not shown. However, we could not obtain the evidence that Cp attacked spermatogonia in this method. Based on this attempt, we have concluded that it

may be difficult to identify the target cell type only by this convenient method, while this is considered to be very useful for screening testicular damages. Therefore, we used the simplified quantitative morphometric method at four selected stages of spermatogenic cycle in the present study, to examine the onset of minimal toxic changes and identify the target cells. Application of this simplified morphometric approach allowed testicular damage to be detected from day 7 even after a single administration of Cp. Thus at this time point, the numbers of spermatogonia at stage V and preleptotene spermatocytes at stage VII began to be decreased. Thereafter, each type of spermatocyte was sequentially decreased. Although the morphological changes induced by Cp were not conspicuous, our simplified morphometric method was successful for their definition. Russell and Russell (1991) evaluated changes in the testes of mice, 15 days after two injections of 65 mg/kg of Cp, and reported consistent decreases of leptotene, zygotene and pachytene spermatocytes. Our results at day 14 after the single administration of Cp are similar but in addition give information on stage dependence. Furthermore, testicular toxicity could be detected more clearly after repeated Cp administration with decreases of spermatogonia being clearly evident in different stages only 1 day after the final administration. These changes were more marked after 4 days, as expected from the doses applied.

For the analysis of target cell type, the chart proposed by Ettlin *et al.* is very helpful (Ettlin *et al.*, 1984), allowing us to extrapolate back to the cell type initially affected by an agent from time sequence findings (Fig. 3). When the point of intersection of the vertical line extending up from the cell type affected and the horizontal line indicating the sacrifice interval is moved to the base line along the preexisting line drawn at a slant in Fig. 3, we can predict which cells were affected initially from the intersection of the line at the base. From analysis of our group 1 results using this chart, we can conclude that type A spermatogonia are the target cells most affected initially by Cp (Fig. 3). This conclusion is in agreement with the suggestions of other investigators (Russell and Russell, 1991). On the other hand, the results of group 2 indicated type A, B

Fig. 3. Development chart to aid extrapolation back to target cells in the rat testis from the findings observed in the present study, using the procedure proposed by Ettlin *et al.* (1984).

and intermediate spermatogonia to be target cells from analysis using this chart (Fig. 3). Although it is generally difficult to predict the target cells by repeated dosing, the greater range of target cell types might have been the result of enhanced and cumulated toxic effects with the repeated administration of Cp. Lu and Meistrich performed a morphological evaluation at 11 days after a single dose of 200 mg/kg Cp and determined that types A through B spermatogonia were most sensitive to Cp in mice (Lu and Meistrich, 1979). They also suggested that preleptotene spermatocytes were partially destroyed by Cp. Thus, there may be some variation in the target cells depending on the severity of toxic injuries. The range detected in our study, type A through B spermatogonia, was different from

that of Lu and Meistrich, but this might be attributable to differences in doses of Cp, time point after the treatment or species applied.

In conclusion, the method performed in the present study, a simplified morphometric approach based on the spermatogenic cycle, was found to be particularly useful for detection of the testicular toxicity of an agent which damages spermatogonia, with distinct advantages as compared to routine/traditional histopathological examination.

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