

Effects of Dimethyl Methylphosphonate (DMMP) and Trimethylphosphate (TMP) on Spermatogenesis of Rat Testis

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Both dimethyl methylphosphonate (DMMP) and trimethylphosphate (TMP) are organophosphorous compounds that can evoke sterility in male rodents. The following studies examined the pathology of reproductive organ, especially on the testis, by light microscopy after treatment with both agents. Adult male rats were treated per oral with DMMP, 1750 mg/Kg, for up to 12 weeks and per oral with TMP, 400 mg/Kg for up to 5 weeks. After 5 weeks of treatment with DMMP there were occasional multinucleated giant cells composed of late spermatids in stages X, XI, XII as well as cytoplasmic vacuolation of Sertoli cell. Anachronistic spermiations were seldom, if ever, seen throughout the experiment. After 7 weeks of DMMP those were markedly diminished. The overall changes after treatment with TMP are somewhat similar to those treated with DMMP. The major changes were composed of aggregate of multinucleated giant cells and maturation arrest at spermatid level, which appear immediately after administration of TMP. The peak frequency in the emergence of multinucleated giant cells in treatment with TMP was noted just a week after treatment, but afterwards declined. Maturation arrest was prominent after 3 weeks in the cases treated with TMP.

Key Words: Dimethyl methylphosphonate, trimethylphosphate, multinucleated giant cell, maturation arrest

The seminiferous epithelium of adult male rat is composed of, in addition to Sertoli cell, various generations of germ cells that are not randomly arranged but form cellular associations of fixed compositions (stage of the cycle) in comparison with that of human (Clermont 1972). Fourteen stages are described in the rat (Leblond and Clermont 1952; Clermont and Leblond 1953; Oakberg 1956a; Oakberg 1956b; Clermont and Bustos-Obregon 1968) and 6 stages in human (Clermont 1966). In rats, the constancy in the composition of the cell associations is so remarkable that the existence of a regulatory role, namely such as Sertoli cell (Lacy 1960), and a residual body (Dalust and

Clermont 1955; Lacy 1960) is suggested by several authors. Sterility inducing agents include organophosphates such as DMMP, TMP, triethylenemelamine, parathion and malathion. Although the etiology of toxicity after treatment with DMMP has been suggested to be an impairment to Sertoli cell, but it is not yet to be elucidated (Chapin *et al.* 1984). TMP is a strong alkylating agent which may impair the motility of germ cells. In this study we have tried to characterize the change of germ cell maturation and find any possible difference between the testes treated with DMMP and strong alkylating agent, TMP.

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MATERIALS AND METHODS

Animals

Random-bred albino Sprague-Dawley-descen-

dent rats (210~220 gm) were used. The rats were fed ad libitum with a nutritionally adequate powdered diet.

Grouping and administration of drugs

Each animal was divided into 3 groups. The first group composed of 60 rats was treated with dimethyl methylphosphonate (DMMP). DMMP(97% Aldrich chemical Co, Inc, Milwaukee, Wisconsin, USA) - $CH_3P(O)(OCH_3)_2$ - was administered by gavage, 5 days/week with 1,750 mg/Kg dissolved in tap water. The second group of 100 rats was treated with trimethylphosphate (TMP). TMP (99% Yakuri pure chemicals Co, LTD/Osaka, Japan)-(CH_3O)₃PO was also administered by gavage. Treated animals were gavaged 5 days/week with 1,500 mg/Kg, 1,000 mg/Kg, 750 mg/Kg, 500 mg/Kg and 400 mg/Kg dissolved in distilled water. Each dose was gavaged into 20 rats. The third of 5 rats as the control group was gavaged with distilled water alone.

Experimental design

The first group was treated for 12 weeks.

The intakes of DMMP were calculated twice a week using the analyzed concentration of DMMP and body weight. Five treated rats from each group were sacrificed weekly for 12 weeks. The second group was treated for 5 weeks. Twenty rats were sacrificed weekly until the 5th weeks. Each subgroup included 4 rats. Sacrificed rats were anesthetized with ether. Both testes and adnexae were removed and stored for 24~48 hour in Bowin's fixative.

Light microscopic examination

For light microscopy, sections of testes were processed through graded alcohol to 95%, embedded in 2-hydroxyethyl methacrylate and sectioned on a Shandon microtome at 2~3µm. Staining with periodic acid and Schiff's stain (PAS) and Masson-Trichrome stain allowed detail visualization of the stages of the spermatogenesis, classified according to Clermont and Leblond (1953). Three hundred seminiferous tubules only with an axial ratio of less than 2 in cross section were examined for maturation staging.

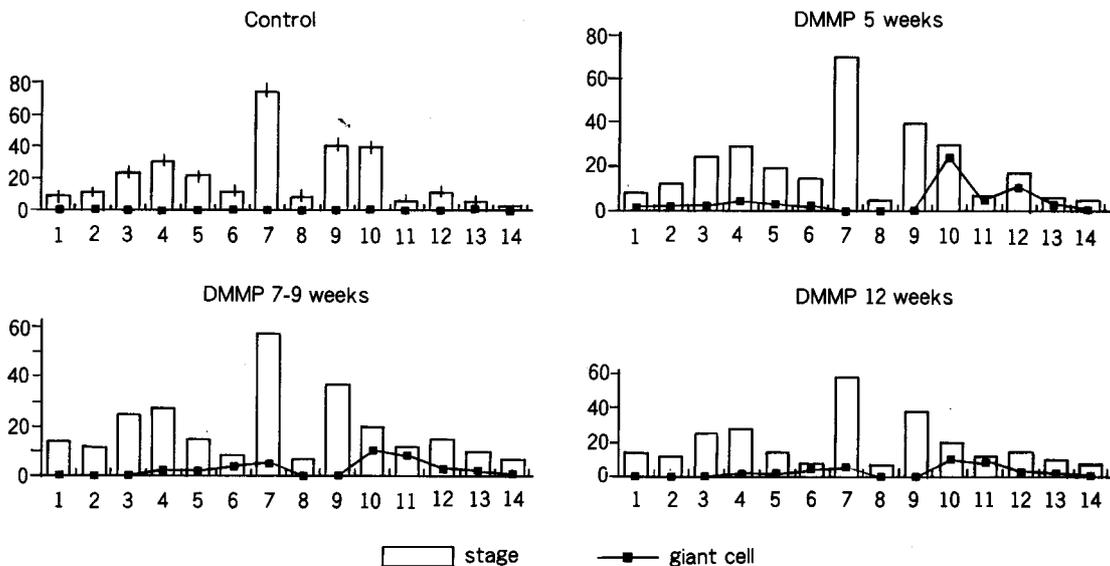


Fig. 1. Distribution of spermatogenic stages (illustrated by bar) in rats (N=5 at each time point) treated with 1750 mg DMMP/kg/day, 5 days/week for 5, 7~9, 12 weeks compared to controls (N=5). Tubules with an axial ratio ≤ 2 were staged according to Leblond and Clermont (1953). Distribution of multinucleated giant cells in rats treated with DMMP was illustrated by linear graph. Data are expressed as means ± SD. (X-axis: stage, Y-axis: No. of tubules)

RESULTS

DMMP treated group

Mortality rate: DMMP induced mortality was much lower (22.6%) than that with TMP.

Distribution of spermatogenic stages: The distribution of spermatogenic stages in the control group showed little variation except for a high peak at stage VII. In the DMMP treated rats there was a notable shift toward the nodal division stages, namely IV, IX. However, the distribution of spermatogenic stages in seminiferous tubules still showed the highest peak at stage VII (Fig. 1).

Histological changes in testes: Several abnormalities were seen in the testes of the DMMP treated groups, the most prominent feature of which was aggregates of multinucleated giant cells (Fig. 2). These cells were characterized by crescentically arranged nuclei of the necrotizing spermatids with central floccular PAS-positive materials. They appeared abruptly after 5 weeks in an average of 64 tubules per testis of all 5 rats. They

were predominantly found in the stages X, XI, XII –namely the early stage of spermatogenesis– and also identified in stages XIII-VII. No aggregates of giant cells were found in the stages VIII and IX, namely ending and beginning point of the spermatogenesis (Fig. 1). These cells were formed by the aggregate of peculiar spermatid levels to their stage. These abnormalities, however, showed abrupt decline after 7 weeks. Other histologic changes in the DMMP treated testes was occurrence of large vacuoles both in cytoplasm of Sertoli cells and their junctions with germinal epithelial cells(Fig. 2*, and 3). These findings appeared closely related with specific stages, being more frequent in stages XII-I. A minute tubulopathy included an anachronistic spermiation which denotes the group of germ cells at the different step of development, namely non-synchronous evolution of spermatogenesis. After 5 weeks there occurred spermatids of step 19 in stage XIII-XIV tubules (Fig. 4).

TMP treated group

Mortality rate: Sixty rats out of 80 rats expired within 5 days after treatment with

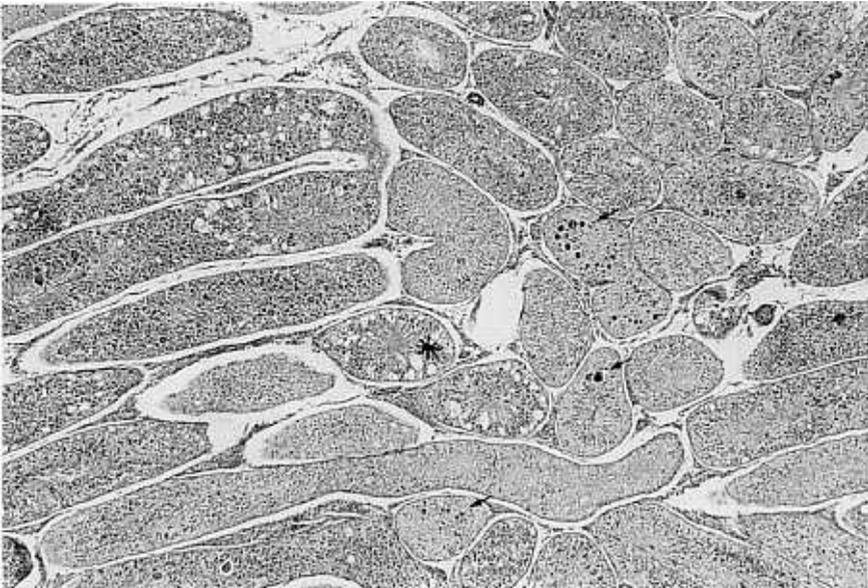


Fig. 2. Variable stages of seminiferous tubules showing features of multinucleated giant cells composed of spermatids(arrow) with cytoplasmic and intercellular vacuolar space () (PAS, ×40).*

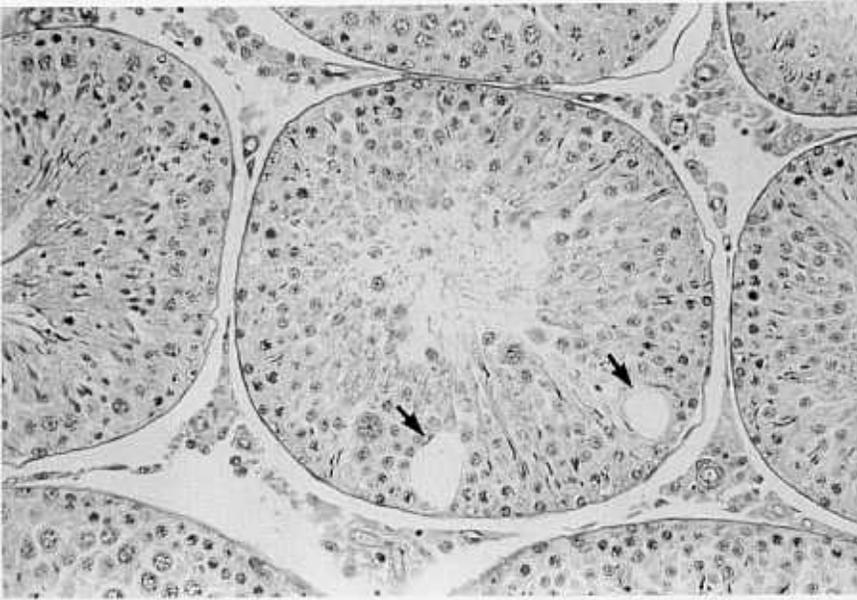


Fig. 3. Cytoplasmic large vacuolation (arrow) at the Sertoli cell and intercellular junction between Sertoli cell and germinal epithelial cells (PAS, $\times 200$).



Fig. 4. Nonsynchronous evolution of step 19 spermatids (arrow) at the stage XIII, so called anachronistic spermiation (PAS, $\times 200$).

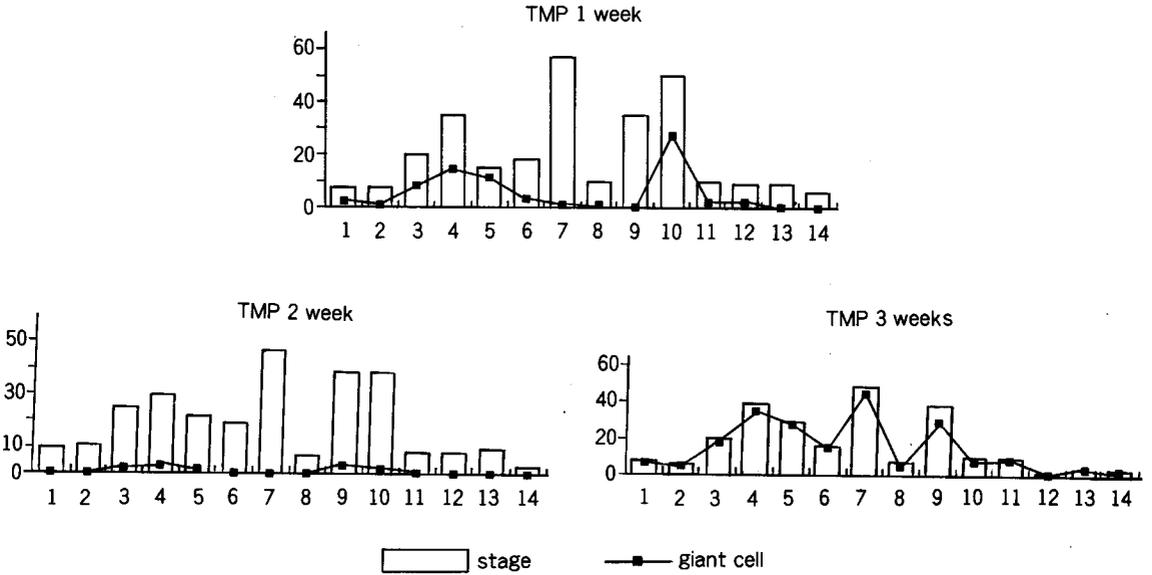


Fig. 5. Distribution of spermatogenic stages (illustrated by bar) in rats (N=5) treated with 400 mg TMP/kg/day, 5 days/week for 1, 2, 3 weeks compared to controls (N=5). Tubules with an axial ratio ≤ 2 were staged. Distribution of giant cells (illustrated by linear graph) in rats treated with TMP. (X-axis: stage, Y-axis: No. of tubules)

TMP (mortality rate 75%). All rats expired within 3 days when TMP was administered with the oral doses in the range from 750 mg/Kg to 1500 mg/Kg. Also, 500 mg/Kg p.o. produced death in 18 of 20 rats within 7 days. Five consecutive oral doses of 400 mg/Kg remained almost all rats alive with only 2 dead. Almost all dead rats had been anuric and hardly taken foods. On autopsy of some dead animals, the urinary bladder was severely distended with urine like a balloon and devoid of obstruction at the distal urinary tract. Microscopic finding revealed multifocal ulceration, loss of urothelial epithelium with marked thinning, and atrophy of the muscle proper. The other organs such as kidney, liver, lung and heart were grossly and microscopically unremarkable. The testes from dead rats were also intact.

Distribution of spermatogenic stage: The distribution of spermatogenic stages showed a similar pattern to that treated with DMMP (Fig. 5). But marked depletion of spermatids and spermatozoa made it impossible to identify stage after 4 weeks.

Testicular lesions and findings: The most prominent change was an appearance of multinucleated giant cells composed of specific spermatids. Nearly all tubules after 1 week showed this abnormality. The amount and degree of this finding were much more extensive than those treated with DMMP (Fig. 6A, and B). After 2 weeks, these findings abruptly disappeared. After 3 weeks, II-V stages were frequent and giant cells reappeared predominantly in stage II-IV, VI-VII, XII. After 4 weeks nearly entire tubules showed aggregate of multinucleated giant cells. The other change was a maturation arrest at the spermatid level. This occurred after 2 weeks and was dose-dependent. After 3 weeks the depletion of spermatids took place in stages II-IV. After 4 weeks almost all tubules became devastated as follows: tubules contained few cells and instead were generally filled with PAS positive cellular debris. Nothing were recognized but nuclei of Sertoli cells, spermatogonia, and rarely those of spermatocytes (Fig. 7). No abnormalities were observed in the inter-tubular connective tissue, interstitial cells, or other

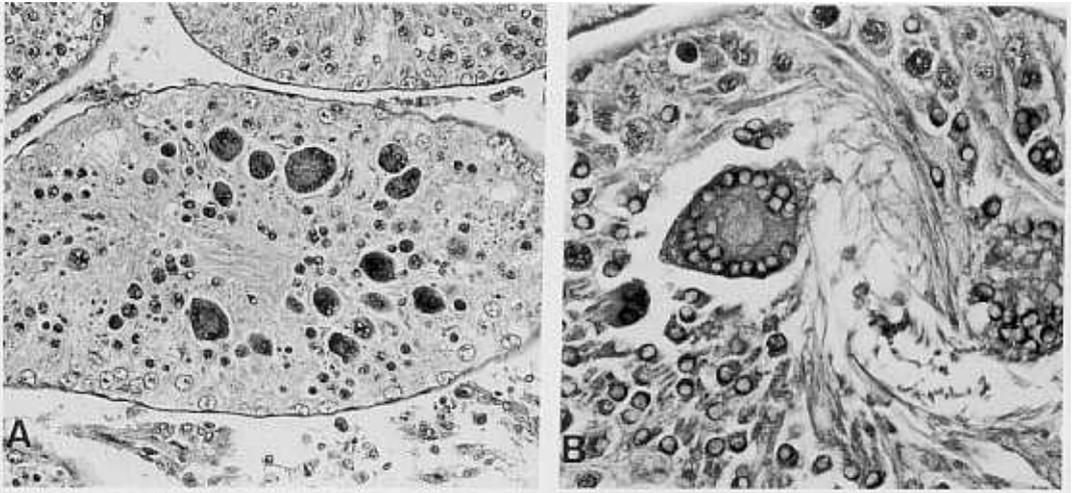


Fig. 6. A,B:Multinucleated giant cells after treatment with TMP are more prominent and exuberant than that with DMMP (A:PAS $\times 200$, B:PAS $\times 400$).

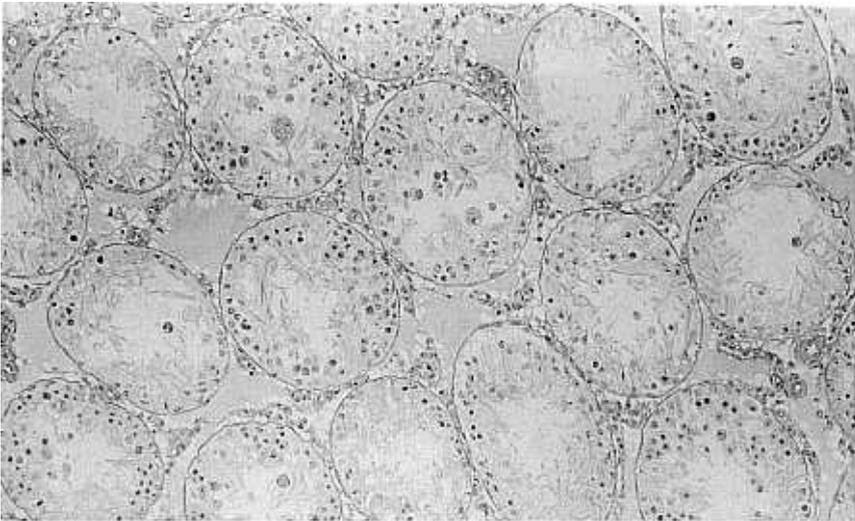


Fig.7. Maturation arrest at the late spermatids level resulting in hollow lumen through nearly entire tubules (PAS, $\times 100$).

structures.

DISCUSSION

Spermatogenesis denotes a continuous, syn-

chronized process which can be divided into 3 distinct phases: mitosis, meiosis, and spermiogenesis. The first phase is referred to as spermatogonial proliferation and renewal; it involves several mitotic divisions of diploid spermatogonia to form spermatocytes and

dedifferentiation to form new spermatogonial stem cells. The second phase concerns that the spermatocytes go through the process of meiotic divisions leading to the formation of haploid cells, spermatids. The third phase involves the spermatids, which go through a complex series of cytological transformations leading to the production of the spermatozoa. This last phase is also nominated as spermiogenesis, metamorphosis of mammalian spermatids, which is composed of 4 distinct sub-phases: Golgi phase, cap, acrosome, and a maturation phase. These detail characteristics had been described by a PAS stained sections as well as electronmicroscopic observation (Leblond and Clermont 1952; Clermont and Leblond 1953; Clermont 1962; Clermont and Bustos-Oblegon 1968). Anatomical steps of spermatogenesis had been already clarified. Widely used terminology includes "stage", "cycle", and "generation". The definition of "generation" is a group of cells which are at about the same step of development. A "cycle" means the regular sequence of events or a complete series of typical cell associations occurring in any given area of tubules. The "stage" of cycle refers to the typical cell association (Oakberg 1956a; Oakberg 1956b; Clermont 1972). Each stage can be identified predominantly according to the nuclear morphology of spermatids and the position of the more mature spermatids within the tubule. These stages are species-specific; for example 14 stages were described in the rat (Table 1), and only 6 stages in human (Clermont and Leblond 1955; Clermont 1962; Clermont 1966). Additional major differences are as follows: groups of germ cells at or approximately at the same step of development may occupy large segments of the tubule and make quite a unique stage in each tubule in a rat, but otherwise in human the number of germ cells seen at the same step of development is relatively small and shows a variable stages in each tubule. These differential points have made the experimentally extensive studies in the rats about the maturation of spermatogenesis possible. The terminating step of spermatogenesis takes place in stage VIII of the cycle when the release of spermatozoa

Table 1. Each step of spermatogenesis in the rat and cellular composition of the stages of the cycle of the seminiferous epithelium (Roman numerals, I- XIV)

Cell types					
I	A	In	P	1	15
II	A	In	P	2	16
III	A	In	P	3	16
IV	A	In _m	P	4	17
V	A	B	P	5	17
VI	A	Bm	P	6	18
VII	A	R	P	7	19
VIII	A	R	P	8	19
IX	A _m	L	P	9	
X	A	L	P	10	
XI	A	L	P	11	
XII	A	L-Z	P	12	
XIII	A	Z	Di	13	
XIV	A	Z	M	14	

Lettering: A, type A spermatogonium; In, intermediate type spermatogonium; B, type B spermatogonium; R, resting primary spermatocyte; L, leptotene spermatocyte; Z, zygotene spermatocyte; P, pachytene spermatocyte; Di, diplotene spermatocyte; M, mitotic division; 1-19 steps of spermiogenesis (Leblond and Clermont 1952)

from the epithelium occurs and the initiation step occurs in stage IX in that time spermatogonia type A undergoes the first of a series of mitosis leading to the production of spermatocytes. The control of these succeeding series of spermatogenesis behaving as a regular cyclic manner has been still unknown, but great emphasis has been put on the role of Sertoli cell as coordinating the evolution of the 5, 6 generations of germ cells (Lacy 1960; Clermont 1972; Kovacs and Asa 1991). Besides the role of Sertoli cells on spermatogenesis, the role of residual body (Lacy 1960), or the interaction of one generation of germ cells on another was once raised, but these theories are no longer accepted.

Several animal experiments inducing sterility with a lot of toxins had been performed in an attempt to reveal the pathology of sterility or regulatory mechanism in spermatogenesis (Kierszenbaum 1970; Carstensen 1971; Harbison *et al.* 1976; Krause 1977; Hanna and Kerr 1981;

Steinberger 1982, Chapin *et al.* 1983; Chapin *et al.* 1984; Creasy *et al.* 1990). In this study, a couple of toxins, DMMP and TMP because their mechanism of action of both agents has been still debated and unsettled. TMP is a strong alkylating agent, whereas DMMP is less strong. The effective dose of DMMP was determined by the previous pilot studies (Chapin *et al.* 1984). To our knowledge, the pathology caused by treatment with TMP has been seldom, if ever, described. The stages of spermatogenesis were rather evenly distributed in control rats apart from high peak in stage VII as in the previous results (Chapin *et al.* 1983; Chapin *et al.* 1984; Creasy *et al.* 1990). Minor peaks were seen in stage I, IV, IX, X. Changes in the distribution of spermatogenic stages after treatment with DMMP or TMP were noted. With increasing doses and time, the frequency of stage VII tended to decline in contrast to stages IV and IX of nodal division. These characteristics may be related with toxin administration and result from the damage to ongoing spermatogenesis. Changes in the distribution of spermatogenic stages had been examined for only few compounds, for example diethyl carbamylmethyl-2,4-dinitroproprrole (Patanelli and Nelson 1964) and 2,5 hexanedione (Chapin *et al.* 1983). They proposed as a mechanism an injury preferentially inflicted to stage VII. We also noted the vulnerability of stage VII to DMMP and TMP and in addition noted an increase in tubules in stage IV, VI, IX. Its mechanism perhaps may be an increased wide-spectrum nodal division as a compensatory proliferation of spermatogenesis.

TMP has proved to be much more lethal than DMMP in this experiment. There are some variations among reports on toxicity. Deichmann and Witherup first reported the LD₅₀ after a single oral dose, in Sprague-Dawley rats to be 1,275 mg/Kg (Connor 1979) and subsequently the amount of lethal dose had been reported to be in the range between 1,000 mg/Kg in minimal dose (Jackson and Jones 1968) and 3,610 mg/Kg (Newell and Jorgenson 1976). Other effect includes carcinogenicity by reaction with host DNA causing endometrial carcinoma (Connor 1979). Jackson

and Jones experimentally produced the sterility in rats with 5 oral doses of 100 mg/Kg~250 mg/Kg between the second and the fifth week of administration (1968). A study by Harbison *et al.* (1976) demonstrated sterility inducing doses as either 100 mg/Kg 5 times per week for 4 weeks, 600 mg/Kg for 5 days or 750 mg/Kg once a week for 12 weeks in rats. But mortality rate of TMP in this study was 75% at the same dose. The cause of death in fatal rats had not been documented. No dead animals had been active with taking foods during life. On autopsy a striking finding was seen in the urinary bladder, of which the contour was saccular and ballooned with paper-thin walls and lumen fulfilled with urine. There was no definite evidence of urinary outflow obstruction in lower urinary tract. The other organs such as lung, liver, kidney and heart were unremarkable.

After treatment with either DMMP or TMP, the most prominent result in this study was the occurrence of multinucleated giant cells. Anton (1979) documented the formation of giant cells after ligation of ductuli efferents which was thought to result from injury to the Sertoli cell. Other reports have documented this finding after direct irradiation (Berliner *et al.* 1964), injection of nitrofurazone (Prior and Ferguson 1950; Uematsu 1966), or administration of 5-thio-D-glucose (Zysk *et al.* 1975). Recent studies also documented similar results after treatment with 2,5 hexanedione (Chapin *et al.* 1983) or cyclohexylamine (Creasy *et al.* 1990). However, multinucleated giant cells after treatment of DMMP or TMP has never been reported before. The mechanism of giant cell formation is still uncertain; some authors have suggested that they may result from nuclear division without cytoplasmic separation (Reddy and Svoboda 1967; Mitranond *et al.* 1979), whereas others postulated that they result from fusion of damaged spermatids (Prior and Ferguson 1950; Maddock *et al.* 1953). This study using DMMP or TMP revealed multinucleated giant cells on intraluminal, inter-spermatids layer, which can be suggested as an aggregate of necrotizing stage-specific spermatids. They occurred at maximal frequency after 5 weeks of DMMP and after 1

week of TMP. In case of DMMP administration the multinucleated giant cells were found predominantly at the stage X (80%), XI (71.4%), XII (62.5%), XIII (50%)-namely early stage of spermatogenesis. Otherwise in case of TMP administration they were seen predominantly at the stage III-V and X in minor peak. Multinucleated giant cells were most frequently noted at the stage V (72.7%). Differentiation into next generation within the same stage, however, has not been disturbed. We are of the same opinion that giant cells are aggregate of necrotizing spermatids, formed by the opening of the syncytium normally connecting adjacent spermatids after meiosis (Uematsu 1966; Anton 1979). Major documents told that the giant cells may be formed by an injury to Sertoli cell (Lacy 1960; Clermont 1972; Kovacs and Asa 1991). We believe that Sertoli cells became injured as evidenced by cytoplasmic vacuolation. It should be noted that these changes occurred most frequently at stage XII-I as did in other reports. Both stages of formation of multinucleated giant cells and vacuolation of Sertoli cell cytoplasm seem to be interrelated. The vacuolation of Sertoli cell cytoplasm was followed by the formation of multinucleated giant cells. It follows that the Sertoli cell may be the primary target of cellular injury, because multinucleated giant cells were preceded largely by Sertoli cell changes. The difference between two experimental groups was that the vulnerable stage after treatment with DMMP was X-XII, early step, whereas that with TMP was III-V, preterminal step. The exact mechanism of these difference was unknown. It is thought, however, that the prevailing stage(III-V) after treatment with TMP may be closely related with the vanishing trend of VII stage. Another notable difference was found in the onset of formation of giant cells. Change of treatment with TMP was far more rapid than that with DMMP. It can hardly be understood why the number of giant cell dropped precipitously after 6 weeks of DMMP and 2 weeks of TMP respectively.

Meiotic prophase and spermiogenesis are dependent on the combined three hormones; pregnant mare serum gonadotropin, human

chorionic gonadotropin (HCG), and testosterone. Unlike spermatogonia which have receptors for both luteinizing hormone (LH) and follicular stimulating hormone (FSH), both spermatocytes and spermatids have testosterone receptors. Thus their metabolism and differentiation depend largely on testosterone. In reference to this, Sertoli cell produce large amounts of androgen binding protein (ABP) in response to FSH stimulation to trap and store testosterone from Leydig cells (Kovacs 1991). Although serum level of testosterone was not investigated, We would like to presume that the negative feedback control of FSH or LH against decreased testosterone may be involved in the great reduction of giant cell formation. In fact some documents have reported that TMP lowered plasma testosterone in rats after 5 weeks of treatment. Further evaluation including measurement of serum hormone level is essentially required. However, reappearance of giant cells after 3 weeks of treatment with TMP did occur when treated with TMP.

Additionally maturation arrest at the late spermatid level was found after 3 weeks of TMP. The immature spermatids were still present in most of affected tubules, sperm being very sparse. After 4 weeks of TMP this change was seen in almost all tubules. TMP was a stronger and more short-acting methylating agent than DMMP with regard to induction of sterility.

The other minor changes after treatment with DMMP or TMP include anachronistic spermiation, occasionally manifested as step 19 spermatids found in stage IX, XIV tubules. These cells are normally found only in stage VII or VIII. Administration of dibutylcyclic AMP (Gravis 1980) or maintenance of subnormal plasma testosterone levels in hypophysectomized rats (Russel 1980) produced a similar defect. The underlying mechanism for this effect on spermiation appears to be the incomplete formation of the tubulobulbar complex, which removes cytoplasm from late stage spermatids for subsequent digestion by the Sertoli cell (Russell and Clermont 1976).

We propose that both DMMP and TMP can produce variable structural defects in testicu-

lar spermatids, and TMP brings about a rapid sterilizing effect as early as 1 week after treatment. Combined hormonal study remains to be further subject of investigation for identification of the effect of feedback control on the temporary disappearance of giant cells.

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