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Evaluation of the testicular toxicity caused by 2-bromopropane in rats

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Abstract : It has been recently reported that 2-bromopropane (2-BP) induces male reproductive toxicity in both human and experimental animals. However, delayed effects of 2-BP on male reproductive system have not been investigated in detail. The present study was conducted to investigate the testicular toxicity of 2-BP and to determine the recovery of normal spermatogenesis in Sprague-Dawley rats. Male rats aged 5 weeks were administered 1,000mg/kg 2-BP by gavage daily for 4 weeks and sacrificed sequentially at 1, 2, 3, 4 and 12 weeks after initiation of 2-BP treatment. Testicular toxicity was evaluated qualitatively by histopathological examinations and quantitatively by reproductive organ weights, spermatid head count, and repopulation index. In the 2-BP treated rats, the body weights was significantly suppressed and the weights of testes and epididymides were also decreased in a time-dependent manner. On histopathological examination, spermatogonia in stages I-VI and preleptotene and leptotene spermatocytes in stages VII-IX were strongly depleted at 1 week of dosing. Spermatogonia were depleted extensively in all spermatogenic stages at 2 weeks. Continuing with the evolution of spermatogenic cycle, zygotene spermatocytes, pachytene spermatocytes, and round spermatids were sequentially depleted at 2, 3, and 4 weeks of dosing due to the depletion of their precursor cells. Vacuolization of Sertoli cells and spermatid retention were also observed at all time points, suggesting that 2-BP induced Sertoli cell dysfunction. At 12 weeks, after 8 weeks recovery, most of the tubules appeared severely atrophic and were lined by Sertoli cells only. Leydig cell hyperplasia in the interstitial tissue was also found. In addition, dramatic reductions in the number of spermatid heads and repopulation index were observed, indicating that 2-BP-induced testicular injury is irreversible. These results indicate that 4 weeks repeated-dose of 1,000mg/kg 2-BP results in a progressive germ cell loss due to the depletion of spermatogonia followed by long-term testicular atrophy in SD rats.

Key words : testis, atrophy, spermatogonia, Sertoli cell, Leydig cell, spermatogenesis, recovery.

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Introduction

2-bromopropane (2-BP), a halogenated propane analogue, is a substitute for chlorofluorocarbones (CFCs) which have a great potential to destroy the ozone layer and to warm the earth's environment. Because this chemical is nonflammable and volatile and is easily broken down in the environment and is less destructive to the ozone layer than CFCs, the amount used in industry has been increased. Therefore, there has been great concern about its toxic potential in human.

In 1995 a cluster of patients with amenorrhea, oligozoospermia, and anemia were discovered in Korean workers exposed to solvent containing 2-BP[1]. An epidemiological survey suggested that 2-BP might be the causative agent of these health disorders[2,3]. The toxicity of 2-BP has been studied in some aspects[4-11]. However, several aspects remain obscure. Concerning male reproductive toxicity, 2-BP induced reductions in male reproductive organ weight, epididymal sperm count, and motile sperm percentage, an increase in abnormal sperm shape, and a depletion of germ cells in a 9 weeks repeated-dose toxicity study in rats at dose levels of 300ppm or more[12] and produced depletion of germ cells and testicular atrophy in a 4 weeks repeated-dose toxicity study in rats at 1,000mg/kg[13]. Omura *et al*[14] recently reported that 2 weeks subcutaneous injection of 1, 355mg/kg 2-BP to rats produced decreased number of spermatogonia and spermatocytes and increased number of degenerative pachytene spermatocytes, suggesting that spermatogonia are the primary targets for testicular toxicity induced by 2-BP. More recently, Omura and his colleague[15] confirmed that spermatogonia are the target cells of 2-BP in rat testes. In spite of the reports describing the adverse effects of 2-BP on spermatogenesis, the recovery of spermatogenesis has not been investigated in detail.

The present study was conducted to further evaluate the testicular toxicity induced by 4 weeks repeated-dose of 1, 000mg/kg 2-BP and to determine the recovery of normal spermatogenesis in male Sprague-Dawley rats. We evaluated reproductive organ weights and histopathological examinations at 1, 2, 3, 4 and 12 after initiation of 2-BP treatment, and measured spermatid head count and repopulation index at 12 weeks after 8 weeks recovery.

Materials and Methods

Animals : Male Sprague-Dawley rats aged 4 weeks were obtained from the Toxicology Research Center Breeding Facility, KRICT, Daejeon, Korea and used after one week of quarantine and acclimatizaton. Because immature animals are more sensitive for testicular toxicants than mature animals, 5 weeks old rats were chosen in this study. Two animals per cage were housed in an air-conditioned barrier-system animal room with ambient temperature of 23±3℃, relative humidity of 50±10%, and a 12-hr light/dark cycle. They were free access to sterilized tap water and commercial rodent chow (Jeil Feed Co., Daejeon, Korea) before, during, and after treatment.

Test substance : The test agent 2-BP (CAS No. 75-26-3) was purchased from Aldrich (Aldrich Chemical Co., Milwaukee, WI, USA) and was dissolved in corn oil vehicle (Sigma Chemical Co., St. Louis, MO, USA). Its purity was >99% and no significant contaminants were detected. It has the chemical structure of $CH_3CHBrCH_3$ and has a molecular weight of 122.99.

Experimental design : One hundred male rats were randomly assigned to ten groups with ten rats in each group and administered corn oil or 1,000mg/kg 2-BP by gavage daily for 4 weeks. Although human exposure to 2-BP would most likely occur via the respiratory tract or the skin, we rejected these routes because they provide limited accuracy in estimating the amount of 2-BP taken into the organism. The dose level used in the present study were selected by the result of previous study[13]. The dosage volume was 10ml/kg and calculated according to the body weight.

Clinical observation and body weights : All males were observed at least once a day for any mortalilty and signs of intoxication to the treatment, and body weights were measured weekly.

Necropsy and organ weights : Male rats were killed by carbon dioxide asphyxiation 1, 2, 3, 4, and 12 weeks fol-

lowing the initiation of 2-BP exposure, and testes and epididymides were weighed and their weight relative to body weight calculated. Control animals were also sacrificed at all time points.

Tissue preparation : To examine the morphological appearance of seminiferous tubules following 2-BP exposure, the right testis was fixed with Bouin's solution for approximately 24h, stored in 70% ethyl alcohol for several days, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H & E) for histologic examination. Transversal testicular sections were evaluated considering testicular characteristics and spermatogenic stages.

Histopathological examination : Equatorial transverse testicular sections were examined for the presence and integrity of different cell types and cell association, utilizing the description of the rat spermatogenesis by Russell *et al* [16]. Seminiferous tubules were considered to be 'atrophic' if they lacked germ cells more mature than spermatogonia. The spermatogonia in seminiferous tubules were further categorized as either type A spermatogonia or intermediate plus type B spermatogonia. Type A spermatogonia were defined by their nuclear morphology, presence of a visible and limited cytoplasm, and basal location in the seminiferous tubules. Intermediate and type B spermatogonia were identified by their more condensed and basophilic nuclear morphology. To estimate the Sertoli cell damage by 2-BP exposure, we also evaluate the morphology of Sertoli cell nuclei and cytoplasm, degeneration or exfoliation of germ cells, and spermatid retention[17].

Spermatid head count : To count the number of spermatid heads per testis at 12 weeks after 8 weeks recovery, decapsulated left testis was weighed, homogenized, and sonicated with 12ml distilled water according to the method described by Meistrich[18]. The homogenates were counted in a Neubauer improved hemocytometer using the red-cell field and standard hemocytometer technique.

Repopulation index : To evaluate the recovery of spermatogenesis at 12 weeks after 8 weeks recovery, a repopulation index was obtained by all seminiferous tubules in an intact paraffin section of the right testis. A tubule was scored as repopulating if it contained three or more germ cells that had clearly reached the type B spermatogonial stage or later [19]. The repopulation index is the percentage of tubules showing repopulation.

Statistical analysis : All values are expressed as mean and standard deviation. The differences between the treatment and the control group were analyzed using Student's *t*-test. Differences were considered significant at p values of less than 0.05.

Results

Clinical signs : None of the control and 2-BP treated rats showed any changes in general health condition.

Body weight changes : Body weight changes of male rats are shown in Fig 1. The body weights of 2-BP treated rats from 3 to 8 weeks after initiation of treatment were significantly lower than those of control animals.

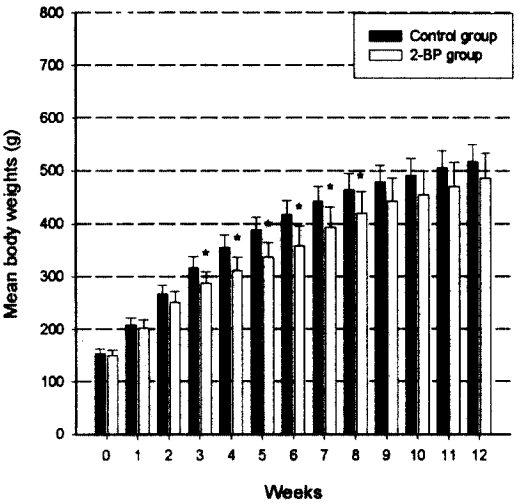

Fig 1. Changes in body weight of male rats treated with 2-BP. *p < 0.05.

Macroscopic findings : Decreases in the size of testes at 1, 2, 3, 4 and 12 weeks and that of epididymides at 12 weeks were found in the treatment group. Except for this reflection of organ atrophy, no abnormalities were observed.

Organ weights : The absolute and relative weights of reproductive organs measured at necropsy are shown in Fig 2 and Fig 3. The absolute weights of testes at 2, 3, 4 and 12

weeks and those of epididymides at 12 weeks in the treatment group were significantly lower than those of the control group. The relative weights of testes at 1, 3, 4 and 12 weeks and those of epididymides at 12 weeks were also significantly decreased in a time-dependent manner.

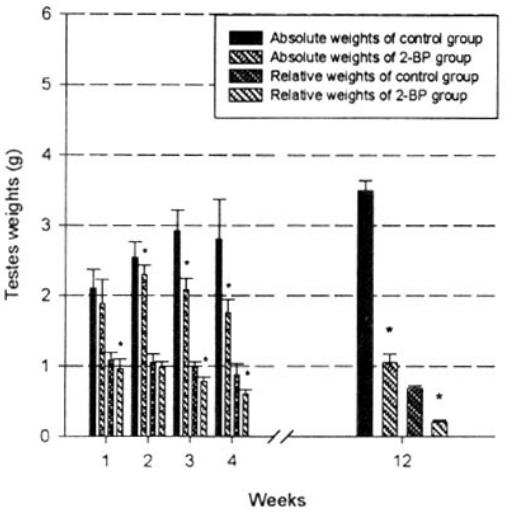

Fig 2. Testes weights of male rats treated with 2-BP. *p < 0.05.

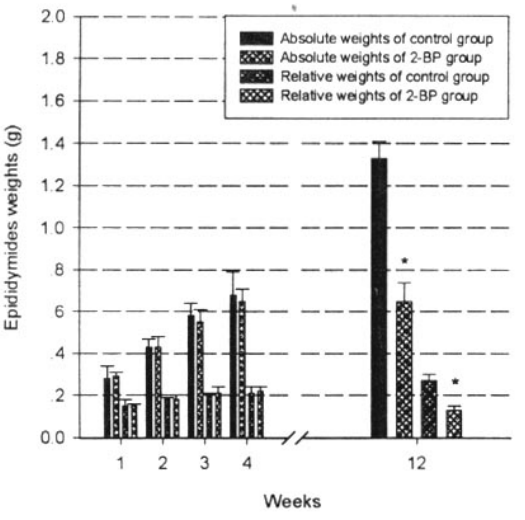

Fig 3. Epididymides weights of male rats treated with 2-BP. *p < 0.05.

Histopathological examination : Control rats showed normal spermatogenesis at all time points (Fig 4A). Rats exposed to 2-BP for one week exhibited minor histologic injury (Fig 4B). The characteristic findings at this time were depletion of intermediate and type B spermatogonia in stages I-VI and that of preleptotene and leptotene spermatocytes in stages VII-IX, some spermatid retention, small vacuoles, and a few degenerative germ cells in the basal region of the seminiferous tubules. Testicular damage was severer at two weeks of 2-BP treatment (Fig 4C). The depleted cell types included not only spermatogonia in all spermatogenic stages and preleptotene and leptotene spermatocytes, but also zygotene spermatocytes in stages XII-XIII and early pachytene spermatocytes in stages I-IV were strongly missing. Small vacuoles, a mass degeneration of pachytene spermatocytes, and spermatid retention were also observed in these animals. At three weeks of 2-BP exposure, the lesion had pregressed severely (Fig 4D). This included degeneration and depletion of pachytene and diplotene spermatocytes, in addition to the germ cells described in the previous time points. At this time, a few pachytene and diplotene spermatocytes were obeserved in later stages, but most of these cells exhibited degenerative changes characterized by pyknotic and/or karyorrhectic nuclei. At four weeks of 2-BP dosing, besides the effects described in the previous time points, complete depletion of pachytene and diplotene spermatocytes, degeneration and depletion of step 1 to 6 spermatids, and large vacuoles were observed. The retention of step 19 spermatids were also observed near the lumen of Stage IX to XI tubules in all rats (Fig 4E). At 12 weeks, 8 weeks after last dosing, most of the tubules were atrophic and lined by Sertoli cells only or few stem spermatogonia. The nonrepopulating tubules were shrunken to about 50% of the diameter of normal tubules in controls (Fig 4F). The basement membrane was more prominent and appeared to thicken slightly. Sertoli cells in seminiferous tubules completely devoid of differentiating germ cell exhibited an altered morphology. The Sertoli cell nuclei were irregularly shaped and were occasionally located towards the tubular lumen away from basement membrane. The Sertoli cell cytoplasm was present as only a thin rim of cytoplasm surrounding as empty seminiferous tubular lumen. Leydig cell hyperplasia in the interstitial tissue was also found.

Spermatid head count : The number of spermatid heads

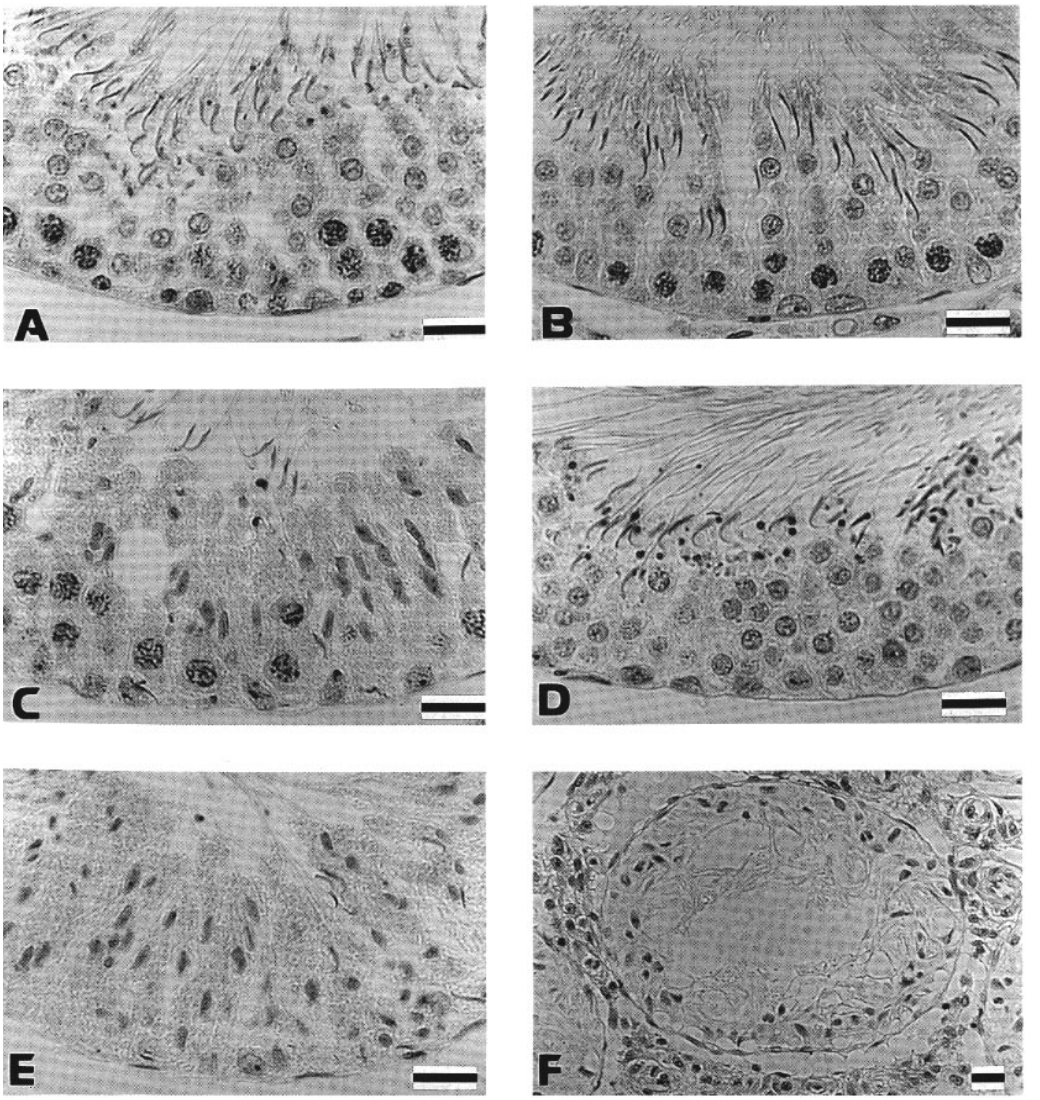

Fig 4. Representative photographs of testis cross sections from control and 2-BP treated rats stained with hematoxylin and eosin. (A) Stage VII seminiferous tubule of a control rat, showing normal spermatogenesis. (B) Stage V seminiferous tubule of a 2-BP treated rat at 1 week, showing depletion of spermatogonia. (C) Stage X seminiferous tubule of a 2-BP treated rat at 2 weeks, showing depletion of spermatogonia and leptotene spermatocytes and spermatid retention. (D) Stage VII seminiferous tubule of a 2-BP treated rat at 3 weeks, showing depletion of spermatogonia and preleptotene and pachytene spermatocytes. (E) Stage X seminiferous tubule of a 2-BP treated rat at 4 weeks, showing depletion of spermatogonia, leptotene and pachytene spermatocytes, and spermatid retention. (F) Seminiferous tubules of a 2-BP treated rat at 12 weeks, showing severe atrophy of tubules, complete loss of germ cells, vacuolization of Sertoli cells, and Leydig cell hyperplasia. Scale bar is 20μm.

at 12 weeks in the treatment group was dramatically decreased when compared with the control value (Fig 5).

Repopulation index : Data for repopulation index is shown in Fig 5. An average of 583±49 tubules (range : 488 to

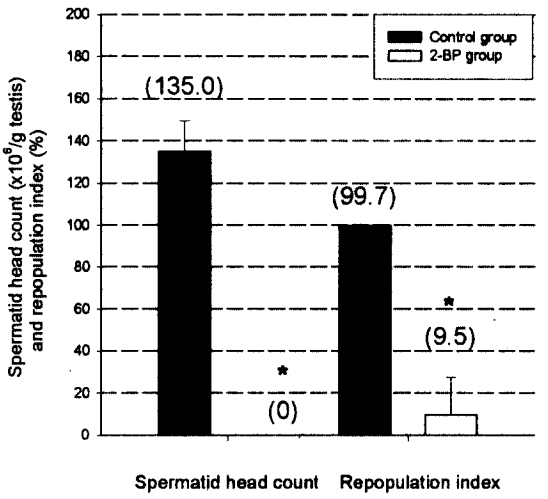

Fig 5. Spermatid head count and repopulation index of male rats treated with 2-BP at 12 weeks. *p < 0.05. ( )Numerical data.

671) were examined per testis to determine the repopulation index. The repopulation index at 12 weeks in the treatment group was remarkably lower than that of the control group. Two out of 10 rats treated with 2-BP had a few repopulating tubules at the rate of 1-2% and other two rats also exhibited some recovery in about 45% of seminiferous tubules. However, the remaining six rats showed no recovery of spermatogenesis in all seminiferous tubules.

Discussion

We have demonstrated that adminstration of 2-BP to male rats produces a progressive germ cell loss in a time-dependent manner followed by testicular atrophy and that 2-BP-induced testicular atrophy is largely irreversible. Because the defects are present after a treatment-free period approximately equal to a cycle of the spermatogenic epithelium and, therefore, reflect effects upon the spermatogonial stem cell, 2-BP may be considered to induce chronic damages of the testis rather than temporary.

This study was undertaken in an attempt to evaluate the effects of 2-BP on testicular function of male rats at 56 days after treatment. The design of the present study allowed for an estimation of the severity of stem cell destruction in rats. Fifty-six days should provide sufficient time for the surviving stem cells to progress through the differentiation pathway and to produce the final stage of spermatid[17]. This time span is also short enough to ensure that only a minimal regeneration of stem cell number occur. Since the period of spermatogenesis is shorter than 8 weeks in rats, it is considered that the testicular sperm head count method can detect testicular lesions even if the testicular toxicant attacks an early phase of spermatogenesis.

A time-dependent reduction in the weights of testes and epididymides may have been a consequence of the progressive germ cell loss induced by 2-BP treatment. A reduction in these organ weights has been previously reported in the toxicity studies of 2-BP in rats[9,12,13].

It has already been described that the chemical structure and toxicologic properties of 2-BP are very similar to those of an alkylating agent 1,2-dibromo-3-chloropropane (DBCP, CAS No. 96-12-8, $C_3H_5Br_2Cl$) which has been reported to cause severe reproductive functional damage in both experimental animals and humans[20,21]. According to Lag *et al* [22], renal and testicular necrotic effects of halogenated propane analogues in rats correlated with their ability to induce renal and testicular DNA damage *in vivo* and also testicular DNA damage *in vitro*. Maeng *et al*[8] reported that 2-BP showed a very similar mutagenic activity in *Salmonella typhimurium* TA100 and TA1535 when compared with that of DBCP reported by McKee *et al*[23]. Although the mechanism of the male reproductive toxicity of 2-BP has not been elucidated, we speculate that 2-BP as an alkylating agent might inhibit rapidly proliferating cells such as germ cells of testis because of the similarities of these chemicals.

Testicular histopathologic evaluation showed that the primary target cells of 2-BP are intermediate and type B spermatogonia, which were strongly depleted at stages I to VI tubules at one week of dosing. These findings are in agreement with the results of Omura *et al*[15] who observed decreased number of spermatogonia at early stages after treatment with 1,355mg/kg 2-BP for 1-5 days in rats. Since spermatogonia are the major proliferative cells of the testis that undergo mitotic division[16], these cells are the primary target

for alkylating agents such as cyclophosphamide[24] and mitomycin C[25]. In the rats, there are three types of spermatogonia in the seminiferous tubules, type A, intermediate, and type B spermatogonia[26]. The depletion of spermatogonia at stages I to VI indicates that differentiating spermatogonia such as intermediate and type B spermatogonia are more vulnerable to 2-BP treatment than type A spermatogonia in rats. Recently, a similar effect was reported by Son *et al*[27] who observed degeneration or depletion of spermatogonia in stages I-IV after 3 consecutive oral doses of 3,500mg/kg 2-BP in rats.

According to Nolte *et al*[28], spermatocytes, spermatids, and spermatozoa past the DNA-synthesizing stages are generally resistant to DNA damaging agents. The decreased number of preleptotene and leptotene spermatocytes in stages VII-IX at one week of dosing are considered to result from the depletion of their precursor cells. Considering the duration of spermatogenic cycle, the results suggest that 2-BP affects differentiating spermatogonia immediately after the exposure and that the depletion of differentiating spermatogonia results in the progressive germ cell loss in a time-dependent manner followed by testicular atrophy. This pattern of germ cell destruction is very similar to the effects of treatment with anticancer agents such as adriamycin[29] and cyclophosphamide[30].

Stem spermatogonia, a subclass of type A spermatogonia, were also strongly affected by two weeks or more repeated-dose of 2-BP, suggesting that the testicular lesions induced by 2-BP were irreversible in rats. This result is also consistent with the data of Omura *et al*[14] who found a reduction in the number of spermatogonia at stages I, V, VII, and XII after treatment with 1,355mg/kg 2-BP for 2 weeks. Stem cell kinetics and recovery of spermatogenesis have been examined in numerous germ cell toxicants[30-33]. The number of stem cells remaining after cytotoxic treatment is directly related to the duration of sterility and the degree of spermatogenic recovery[34]. If there are no stem spermatogonia remaining after cytotoxic treatment, the testicular injury will be irreversible obviously. On the contrary, after irradiation[33] or 2,5-hexanedione exposure[35], no recovery of spermatogenesis was seen despite the presence of stem spermatogonia. Of the nonrepopulating tubules in the current study, some tubules contained a few stem spermatogonia that had become unable to produce differentiated germ cells to enter the spermatogenic pathway. However, the majority of these tubules contained only Sertoli cells. Accordingly, the failure of spermatogenic recovery observed in this study is considered to result from the depletion of stem spermatogonia that was also observed at 12 weeks after 8 weeks recovery. Long term effects of DBCP on reproductive system were also reported by Sod-Moriah *et al*[21]. It was shown that in rats injected once a week for three weeks with 20mg DBCP/kg, the weight of testes at 50 weeks post injection was about one-third of controls with 100% of the seminifeous tubules damaged.

The dramatic reduction in spermatid head count and repopulation index confirmed the subjective estimate of failure of spermatogenic recovery. On the other hand, 4 weeks repeat oral doses of 330mg/kg 2-BP and 3 consecutive oral doses of 3,500mg/kg 2-BP also induced germ cell depletion, but most of the affected tubules were repopulated after 56 or 70 days recovery[13,27]. This apparant discrepancy between the published data and our findings may be explained by the differences in dose level and exposure time. Previous studies indicated that the recovery of testicular dysfunction induced by DBCP was dependent on dose level, exposure time, and recovery time[21,36,37].

Sertoli cells are commonly accepted as performing a critical role in support of spermatogenesis. They provide the appropriate structural and nutritional environment for germ cell development, create the blood-testis barrier, and remodel the seminiferous epithelium as germ cells mature[38]. According to the report of Creasy[17], Sertoli cell damage is frequently recognized by inter- or intracellular vacuoles or by swelling of the basal Sertoli cell cytoplasm, and disturbances in Sertoli cell function result in germ cell degeneration or exfoliation into the tubular lumen and spermatid retention. The vacuolization of Sertoli cell cytoplasm and the spermatid retention observed in this study may be suggestive of Sertoli cell damage. This findings also suggest that the failure of spermatogenic recovery after 2-BP exposure is associated with Sertoli cells dysfunction. Although spermatid retention

is a relatively subtle change, this finding suggests a disturbance of Sertoli cell junctions because release of mature spermatids is a function of the Sertoli cells mediated by specialized cell-to-cell junctions between these cells and the spermatids[39]. Sertoli cell toxicants such as boric acid[40], cyclohexylamine[41], and 1,3-dinitrobenzene[42] have shown to cause this effect, accompanied by other germ cell effects. Whether the effect on the Sertoli cell is due to a direct toxic action of 2-BP or to an indirect effect caused by depletion of germ cells producing an impaired modulation of Sertoli cell function could not be determined in the current study, because the vacuolization of Sertoli cells and the depletion of germ cells which have also been reported by Yu *et al*[9] and Ichihara *et al*[12] were observed simultaneously. Further investigations should be carried out to clarify this point. On the contrary, the results observed in this study are in contrast to the data reported by Omura *et al*[14,15] showing that 2-BP rarely induces Sertoli cell damage in rats. This discrepancy may be due to the differences in administration route, dose level, or exposure time between these studies. Among these factors, the difference in exposure time is considered to be the most plausible.

Spermatocytes and spermatids have been shown to be important modulators of Sertoli cell function[39], raising the possibility that the atrophy persists because of the absence of these cells. However, after irradiation[43] or vinylcyclohexene diepoxide treatment[32], spermatocytes and spermatids are completely eliminated from the seminiferous epithelium, but full recovery can occur[43]. Chemotherpeutic agents such as anthracycline derivatives affect the DNA synthesizing activity of spermatogonia, but high doses of these agents also have damaging effect on Sertoli cell function[44,45]. It is considered that the formation of multinucleated giant cell at 12 weeks is an indicator of continuing degeneration of the spermatogenic epithelium.

It is well known that hyperplasia of Leydig cell is generally observed as a compensatory reaction to chemically or experimentally induced testicular atrophy. In the present study, the Leydig cell hyperplasia at 12 weeks, accompanied by tubules substantially devoid of germinal epithelium, may be due to interstitial shrinkage from testicular atrophy and not a toxicologic effect. Recent studies also showed 2-BP-induced interstitial hyperplasia in rats[9,12,27]. Son *et al*[27] confirmed this finding using immunohistochemical staining with proliferating cell nuclear antigen (PCNA).

The present study concluded that 4 weeks repeated-dose of 1,000mg/kg 2-BP results in a progressive germ cell loss in a time-dependent manner due to the depletion of spermatogonia followed by long-term testicular atrophy in SD rats.

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랫드에 있어서 2-bromopropane에 의해 유발된 정소독성의 평가

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국문초록 : 최근 2-bromopropane(2-BP)이 사람과 실험동물에서 정소독성을 유발한다고 보고된 바 있다. 그러나 수컷 생식기계에 있어서 2-BP의 지연효과에 대해서는 세부적으로 조사된 바가 없다. 본 연구는 Sprague-Dawley 랫드에서 2-BP의 정소독성과 정자발생의 회복을 조사하기 위하여 수행하였다. 5주령의 수컷 랫드에게 2-BP를 1,000mg/kg 용량으로 4주간 반복투여하였고, 투여시작후 1, 2, 3, 4 및 12주째에 부검하였다. 정소독성의 평가는 병리조직학적인 질적평가와 생식기관 중량, 정자두부수 및 재생지수 등의 양적평가로 수행하였다. 시험결과 2-BP를 투여한 랫드에서는 체중과 정소 및 정소상체 중량이 대조군에 비해 시간의존적인 방식으로 억제 또는 감소하였다. 병리조직검사에서는 투여 1주째에 stage Ⅰ~Ⅳ에서 정조세포와 stage Ⅶ~Ⅸ에서 세사전기 및 세사기의 정모세포가 현저하게 소실되었다. 정조세포는 투여 2주째에 모든 stage에서 광범위하게 소실되었으며, 정자발생주기가 진행됨에 따라 2, 3 및 4주째에는 접합기 정모세포, 비후기 정모세포 및 원형 정자세포가 전구세포의 결손에 의해 점진적으로 소실되었다. 지지세포의 기능적 이상을 암시하는 지지세포의 공포화와 정자세포 저류는 상기한 모든 시기에서 관찰되었다. 8주 회복후인 12주째에는 대부분의 곡세정관이 심하게 위축되어 지지세포만 관찰되었으며, 간질조직에서는 간질세포의 과형성이 인정되었다. 또한 2-BP에 의해 유발된 정소의 손상이 비가역적임을 암시해주는 정자두부수와 재생지수의 현저한 감소가 관찰되었다.

상기 결과는 랫드에 2-BP를 1,000mg/kg의 용량으로 4주간 반복투여하면 정조세포의 결손에 의해 점진적으로 생식세포가 감소하고 이로 인하여 장기적인 정소위축이 유발된다는 것을 암시해준다.

Key words : testis, atrophy, spermatogonia, Sertoli cell, Leydig cell, spermatogenesis, recovery.