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研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

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研究テーマ フロン代替品 1-bromopropane と 2-bromopropane による神経、生殖毒性の発生機序

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 有 ・ 無 (学会名・内容)

(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

Yu X, Kamijima M, Ichihara G, Li W, Kitoh J, Xie Z, Shibata E, Hisanaga N, Takeuchi Y.

2-Bromopropane causes ovarian dysfunction by damaging primordial follicles and their oocytes in female rats.

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3．今後の研究計画

今回の研究では 2-Bromopropane (2-BP) 暴露による卵巣毒性は始原卵胞のアポトーシスの増加によることを示唆しました。今後は始原卵胞のアポトーシスに関連する遺伝子の発見を検討するつまりです。また精巣毒性の発生機序も検討する。さらに今　溶剤として多く使われる 1-Bromopropane の生殖神経毒性の発生の分子レベル機序の検討を行います。

4．研究指導者の意見

オゾン層を破壊する溶剤である特定フロンや1,1,1-トリクロロエタンに代って導入されたブロモプロパンがつよい生殖毒性を有することが明らかになった。その生殖毒性の機序を明らかにすることはブロモプロパンの中毒予防に役立つばかりでなく，他の化学物質の生殖毒性を評価する上で重要である。俞君は精力的な研究でブロモプロパンの雌性への毒性機序が始原卵胞のアポトーシスであることを明らかにする成果をあげた。

研究指導者氏名　竹内康浩 ㊞

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

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2－ブロモプロパンによる卵巣障害機序の検討

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要　旨

2-Bromopropane (2-BP) 暴露による卵巣毒性の標的細胞を明らかにするために ラットに 2-BP を吸入暴露して濃度別 9 週間暴露及び 1 回高濃度暴露後の経時的な観察を行った。濃度別実験では始原卵胞、成長卵胞数は量依存的に対象群に比べて著明に減少した。濾胞卵胞数も量依存的に減少した。3000 ppm1 回暴露した 5 日後に始原卵胞の卵子核の不規則、17 日後に始原卵胞の卵子核の縮小、濃縮など著明な組織学変化を認めた。ApopTag を用いて染色で暴露群では暴露後 5 及び 17 日後にアポトーシスと考えられる茶色に染められた始原卵胞の卵子核が観察された。暴露後 17 日に始原卵胞数は対象群に比べで有意に減少した。2-BP による卵巣卵胞の障害は始原卵胞のアポトーシスの増加によることを示唆した。

Key　Words: 2-Bromopropane; ovarian toxicity; histopathology; apoptosis; follicle count; primordial follicle; inhalation exposure.

INTRODUCTION

Reproductive toxicity of alternatives for ozone-depleting solvents has become an important issue since the outbreak of reproductive and hematopoietic disorders in workers exposed to 2-bromopropane in South Korea (2-BP, CAS No. 75-26-3) (Kim et al., 1996; Park et al., 1997; Takeuchi et al., 1997). In the incident, thirty-three workers (25 women, 8 men) were exposed to solvents containing 2-BP as the major ingredient, resulting in amenorrhea in 16 females and azoospermia or oligospermia in 6 males (Kim et al., 1996; Park et al., 1997). No exposure data were available but the geometric mean concentration of 2-BP was estimated to be 12.4 ppm (range 9.2-19.6 ppm) with short-term level of 4,360 ppm, based on a simulation study after the incident. Since the toxicity of 2-BP was unknown then, several efforts have been made to investigate whether such toxicities occur in experimental animals.

A nine-week inhalation study using male rats revealed that exposure to 2-BP at ≥ 300 ppm resulted in a decrease in testicular and epididymal weights, epididymal sperm count, motile sperm percentage and the number of erythrocytes and platelets. All type of germ cells decreased in the 300 ppm group, and germ cells were absent but Sertoli cells were still present in the 1,000 ppm exposure group (Ichihara et al., 1997). Subsequent study revealed that exposure to 1,000 or 3,000 ppm of 2-BP induced a hypoplastic profile with replacement of the bone marrow with fatty spaces and marked reduction in megakaryocytes (Nakajima et al. 1997). In another study using subcutaneous injections of 2-BP, it was demonstrated that spermatogonia, the stem cells of spermatocytes, were the target cells of 2-BP in rat testes (Omura et al., 1997; 1999). As for female reproductive toxicity, a 9-week inhalation study showed that the female rats exposed to 300 or 1000 ppm of 2-BP developed irregular estrous cyclicity. The cycles in the 1,000-ppm group started to lengthen around the second week of exposure, and that those in the 300-ppm group shown a gradual lengthening around the 7th week. Furthermore, luteinizing hormone tended to decrease and follicle-stimulating hormone increased dose-dependently in the 300 and 1,000-ppm groups albeit statistically insignificant. Marked atresia of ovarian follicles accompanied by reduced number of antral and growing follicles were histopathologically observed in the 300 and 1,000 ppm groups (Kamijima et al., 1997). Another study showed that 2-BP induced a significant delay in the estrous cycle, reduced fertility, and tended to decrease the number of pups born in rats treated with daily intraperitoneal injections of 2-BP (900 mg/kg for 14 days) (Lim, et al., 1997). The third study showed that intraperitoneal injection of 2-BP in mice (8 times at intervals of 2 or 3 days at 500, 1,000 and 2,000 mg/kg for 17 days) caused a marked decrease in the number of ovulated ova induced by superovulation treatment in the 1,000 and 2,000 mg/kg groups (Sekiguchi and Honma, 1998). These series of studies indicate that 2-BP exhibits reproductive toxicity in both males and females. However, the primary target site (target cell) of 2-BP in the ovary or the mechanism of target cell toxicity is still unclear. The aims of the present study were to define the primary target site of 2-BP in the ovary and determine the involvement of apoptosis as a mechanism of 2-BP-induced ovotoxicity. We determined the effects of 2-BP on subtle and early changes in ovarian structure by differential follicle counts. As demonstrated previously in male rats, the present results showed that the immature germ cell, the primordial follicle and its oocyte, is primarily vulnerable to 2-BP. We were also able to detect follicle

destruction at 100 ppm, at which significant alteration in estrous cyclicity and gross ovarian histopathology was not observed in the studies of Kamijima et al. (1997). Our results also indicated that differential follicle counts are more sensitive measure of 2-BP-induced ovarian toxicity than estrous cyclicity, as evident in dose-response and time-course experiments.

MATERIALS AND METHODS

Chemicals. 2-BP was obtained form Tosoh. Co. (Japan). Purity was > 99.5% as determined by capillary gas chromatography. In situ terminal deoxynucleotidyl transferase (TdT) commercial kit (ApopTag, No. S7100) was purchased from Oncor (Gaithesburg, MD).

Animals and inhalation exposure. Female Wistar rats (8 weeks old) were purchased from Shizuoka Laboratory Animal Center, Japan. Animals were housed three per stainless steel cages, provided with food and water *ad libitum*. They were maintained in a 12-12 hr light-dark cycle (lights on at 9:00 and off at 21:00) room, with a constant temperature (23.9-25.3°C) and relative humidity (57-60%). Japanese laws concerning the protection and control of animals, standards related to care and management of experimental animals, and the Guidelines on Animal Experimental set out by Nagoya University School of Medicine, were strictly followed throughout the experiments. After 2 weeks of acclimatization, estrous cyclicity was determined by cytology from daily vaginal smear. Cycle days were classified as proestrus, estrus, diestrus I and diestrus II (Cooper et al., 1993). After monitoring the estrous cycle for three weeks (Cooper et al 1993), rats with four consecutive 4- to 5-day cycles were weighed and grouped in a cage according to their estrous stage. Thus, cage mates were at the same stage of cycle and were treated in an identical manner. Vaginal smear was taken daily. We used the inhalation exposure system described previously by Takeuchi et al. (1989) and modified by Ichihara et al. (1997). The vapor concentration in the chamber was measured by gas chromatography and digitally controlled within ±5% of the target concentration using a personal computer.

Experimental design. The general principal of the design of this study was based on the work of Davis (1993) and Davis et al. (1997). In order to determine the dose-response and 2-BP-related pathogenetic changes on cycling female rats, we conducted dose-dependent and time-course experiments. In the dose-dependent experiments, 36 female rats, weighing 230-270 g at 12 weeks of age, were divided into four equal groups

according to their estrous stage. Each group was exposed to 0, 100, 300, or 1,000 ppm 2-BP for 8 hr/day for 9 weeks. The exposure concentrations were modified from a previous study on male rats (Ichihara et al. 1997), where rats were exposed to 0, 300, 1,000, and 3,000 ppm. Rats in the 3,000 ppm group became seriously ill after exposure for 9-10 days and spermatogenesis was impaired at ≥ 300 ppm. During the exposure period, body weight was measured once a week and vaginal smears were obtained every day to monitor ovarian cycle. After the 9-week exposure period, we sacrifice the rats on the day of diestrus I.

In the time-course experiments, 35 female rats were divided into five equal groups according to their estrous stages. Four groups were exposed continuously to 2-BP at 3,000 ppm for 8 hr, and one group to fresh room air only. Exposure commenced at 21:00 on day 0 and was terminated at 5:00 in the morning. The rats were sacrificed 1, 3, 5 or 17 days after exposure. In order to sacrifice animals on the estrous day, exposure in groups of 1, 5 and 17 days commenced on the day of proestrus while for group 3-days, exposure commenced on the day of diestrus I. Most rats were estrous on the day of sacrifice. Two rats in the 3-day group and one in 5-day group were sacrificed on the next day. Two in the 5-day group and one in the 17-day group were sacrificed 2 or 3 days after.

Histopathological examination. The reproductive organs were carefully dissected, removed, weighed immediately and fixed in 10% neutral buffered formalin for light microscopic evaluation. Following standard processing procedures to prepare tissue on slides, tissue blocks were dehydrated with serial concentrations of ethanol and embedded in paraffin. Eight μm serial sections were prepared from the right ovaries, mounted on glass slides, and stained with hematoxylin and eosin. A modified method of Pedersen and Peters (1968) for differential follicle counts was used according to Plowchalk et al.(1993), where types 1-3b, types 4-5b, and type 6-8 were grouped as primordial, growing, and antral follicles, respectively. The primordial follicle group consisted of a range of follicles that contained an oocyte but no surrounding granulosa cells as well as follicles that had one complete ring of granulosa cells surrounding the oocyte. The growing follicle group consisted of follicles that contained an oocyte with multiple layers of surrounding cells. There was no evidence of antrum formation in these follicles. The antral follicle group consisted of follicles that contain a large oocyte, multiple

cell layers surrounding the oocyte, and a fluid-filled antrum. Only follicles that had chromatin-visible oocytes in cross-sections were counted, and no attempt was made to differentiate atretic from normal follicles.

Starting with the first serial section that contained the ovarian tissue, each specific follicle type was counted. Thereafter, every 5th serial section was scored for differential follicle numbers. Once all sections were scored, the number of each type of follicles in all sections were summed to give the total primordial, growing, and antral follicle counts for that ovary. The total number of primordial, growing and antral follicles counted in the control were ranged from 167 to 256 (mean 202.6), 58 to 106 (mean 74.4), and 22 to 44 (mean 37.8), respectively. Light microscopy was used for morphological characterization and detection of cellular damage. The latter included nuclear pyknosis and chromatin redistribution, cytoplasmic condensation, and the shape and arrangement of the oocyte and granulosa cells. Follicle counting and morphological characterization were blindly performed by two authors.

***In situ* terminal deoxynucleotidyl transferase assay.** In the time-course experiment, 5 μm tissue sections of the left ovary were deparaffinized with xylene and washed in serial concentrations of ethanol solutions. Section were then subjected to labeling DNA strand breaks by using a deoxynucleotidyl transferase (TdT) commercial kit according to the instructions provided by the manufacturer. Digoxigenin-conjugated deoxyuridine 5'-triphosphate (d-dUTP) was incorporated into DNA strand breaks by exogenous TdT. The incorporated d-dUTP was then detected with peroxidase-labeled digoxigenin antibodies, followed by the addition of diaminobenzidine. The specimen was then washed with distilled water and counterstained with hematoxylin, dehydrated, mounted, and examined by using a light microscope. Apoptotic cells were identified by their brownish staining. Positive and negative control sections were included in each sample.

Statistical methods. The relative counts of primordial, growing, and antral follicles in 2-BP exposed groups were expressed as percentages of the control. Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. A P value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Dose-dependent follicle damage. Ovaries of the control rats contained follicles of all developing stage, corpora lutea, and interstitial tissue (Fig. 1). Ovaries of the 1,000-ppm group were hypoplastic and characterized by few primordial, growing and antral follicles, few or no corpora lutea (Fig. 2). Follicle counts in this group showed that all types of ovarian follicles were significantly lower than control (Fig 3). Ovaries from rats exposed to 2-BP at 300 ppm for 9 weeks were also hypoplastic and some contained no newly formed corpora lutea (Fig. 4). The numbers of primordial follicles, growing follicles, and antral follicles in this group were significantly lower than control (Fig. 3). Microscopic examination of ovaries of the 100-ppm group showed no significant morphologic changes relative the control (data not shown). Follicle counts in this group, however, significantly reduced numbers of primordial and growing follicles, but the reduction of antral follicle was not statistically significant (Fig. 3). Table 1 compares the detection limit between vaginal smear tests and follicle counts. Estrous cycles were significantly disrupted in 300 and 1,000-ppm exposed groups during the 7 and 9 week of exposure. However, there was no significant change in the estrous cycle of rats exposed to 100 ppm (Table 1). These results showed that follicle count was a more sensitive method for detecting ovarian dysfunction caused by 2-BP than vaginal smears.

Time-course follicle damage. There was no significant change in estrous cyclicity in all groups after a single exposure to 3,000 ppm for 8 hr. However, the single exposure to 2-BP caused apparent histological changes in the primordial follicle complex at days 5 and 17 after exposure (Figs. 5). Under normal conditions, oocytes and their nuclei are round in shape, with the chromatin being dispersed throughout the nucleus in a reticular or granular pattern (Fig. 5a). The symmetry of the oocyte and its nucleus became distorted and irregular at day 5 after exposure (Fig. 5b). Eccentric pyknotic nuclei were present at day 17 after exposure (Fig. 5c), together with shrinkage of nuclei and the appearance of brown granules (Fig. 5c). Granulosa cells appeared unaffected on 5 and 7 days after exposure (Figs. 5b, c). On the other hand, no obvious histopathological changes were detected in growing follicle complex and antral follicle complex after exposure (data not show).

The results of *in situ* end labeling staining for DNA strand breaks are shown in Figure 6. In control rats, apoptotic cells were confined to the granulosa cells of atretic follicles (Fig. 6a), and positive staining was not

detected in primordial and growing follicles. In contrast, in 2-BP-exposed groups, positively-stained cells were detected in several nuclei of oocytes in primordial follicles at days 5 and 17 after exposure, indicating wide-scale apoptosis in these follicles (Fig. 6b and c).

Fig. 7 summarizes the time-course effects of exposure to 2-BP on follicle counts. The percentage of primordial follicles did not change at days 1 and 3 after exposure but their number tended to decrease at day 5 after exposure. There was a significant reduction in the percentage of these follicles at day 17 after exposure (Fig. 7). The percentages of growing and antral follicles were also reduced after exposure, albeit insignificantly. Small effects on these follicles could not be excluded since the errors were so large that an effect must be larger than 25% to be detected. There were no clear-cut changes in the number of atretic growing and antral follicles at any interval after exposure.

DISCUSSION

Morphological evaluation and differential follicle counts in time-course experiment demonstrated that the primordial follicle and its oocyte were the most sensitive components to a single exposure to 2-BP at 3,000 ppm for 8 hr. The effects on growing and antral follicles were minimal. Disruption of the estrous cycle and loss of growing and antral follicles in rats exposed for 9 weeks to 300, 1,000 ppm were caused by a reduction of the primordial follicle pool. Several compounds are known to damage ovarian follicles (Plowchalk and Mattison, 1992; Shiromizu et al., 1984; Smith et al., 1990) and the stage of development at which the follicle is destroyed is important in determining the impact of exposure to the chemical on reproduction function. In this regard, chemicals that selectively damage large growing or antral follicles are known to cause a transient reproductive system dysfunction which is reversible once exposure to these chemicals is terminated (Mattison and Schulman 1980; Generoso et al., 1971). Conversely, chemicals that destroy oocytes in primordial follicles might show a delayed effect on the estrous cycle or prolonged reproductive dysfunction that continues until recruitment of growing and antral follicles can no longer be supported (Mattison and Schulman, 1980; Hooser et al., 1994). Cyclophosphamide (CPA) is known to damage the primordial, growing, and antral follicles in a time-, dose-, strain-, and species-dependent fashions (Shiromizu et al., 1984; Plowchalk and Mattison, 1992). Shiromizu et al. (1984) found that primordial oocytes were the most sensitive to destruction after intraperitoneal treatment with

CPA in Sprague-Dawley rats, C57BL/6N and DBA/2N mice. A delayed decrease in the number of medium sized follicles was noted between 1 and 2 weeks after treatment. In mice treated with CPA at 75, 200 or 500 mg/kg, the primordial follicles showed the highest sensitivity and were affected by all doses, while antral and growing follicles were also impaired (Plowchalk and Mattison, 1992). Busulfan (1,4-butanediol dimethanesulfate), another cytotoxic drug, has also been shown to cause selective degeneration of the primordial follicle (Hishfield, 1994; Reddoch et al., 1986). The present study showed that one-day dose of 3,000 ppm of 2-BP produced no significant change in estrous cycle during a 17-day observation. We have previously demonstrated that the time required for 2-BP to produce obvious estrous cycle disruptions at 300 ppm or 1,000 ppm was 7 or 3 weeks, respectively (Kamijima et al., 1997). As for 100 ppm, we could not observe any estrous cycle disruption.

Several studies have investigated the mechanisms involved in the destruction of ovarian follicles and oocytes by ovotoxicants (Springer et al., 1996a,b; Hoyer and Sipes, 1996). Morphological evaluation of ovaries of 2-BP-exposed rats showed nuclear pyknosis and shrinkage but no evidence of necrosis (e.g., rupture of membrane, cellular swelling, or infiltration by macrophages) (Kerr et al., 1994). Furthermore, *in situ* analysis of DNA fragmentation of the ovaries suggested that oocytes degeneration in adult rats exposed to 2-BP is due to apoptotic cell death. In control rats, apoptotic cells were confined to the granulosa cells of atretic follicles, which was consistent with the results of previous studies demonstrating that atresia of follicles in growing or antral follicles was due to apoptosis (Hsueh et al., 1996; Kaipia et al., 1997). The primordial follicles represent a uniform group with regard to growth pattern; an all or none growth, and very few degenerate (Pedersen, 1970). However, the nuclei of several oocytes in the primordial follicles in the present study were positively stained at days 5 and 17 after exposure to 2-BP. Apoptosis, or programmed cell death, is an important cellular process involved in response to toxicants. Previous studies have shown that destruction of small preantral follicles by 4-vinycyclohexane diepoxide was due to apoptosis, as confirmed by morphological appearance of margination of chromatin along the nuclear membrane in oocytes and disruption of focal contacts between granulosa cells and oocytes (Springer et al., 1996a). Furthermore, overexpression of bax genes in small preantral follicles was thought to represent selective susceptibility of these cells (Springer et al., 1996b).

The ovarian toxicity of 2-BP was first observed in a Korean electronic factory (Kim et al., 1996; Park et al., 1997). Of the 25 female workers exposed to 2-BP, sixteen developed amenorrhea. The latent period between exposure to onset of amenorrhea was 4 to 16 months. The present results suggest that severe ovarian failure in these workers might be irreversible due to failure of ovulation caused by damage of oocyte in the primordial follicles. In a recent report of a 2-year follow-up study, of the 16 intoxicated female workers, most of them except 2 patients failed to show recovery of normal ovarian function. Six patients underwent laparoscopic examination and ovarian biopsy and four showed focal or diffuse fibrosis in the ovarian cortex. Follicles did not show the various stages of development, and the number of primary follicles was markedly decreased. Oocytes and granulosa cells were not seen in the primary follicles (Koh et al., 1998). The histopathological findings in these patients were consistent with severe impairment of ovaries, similar to those shown in the present study.

Vaginal smear test is simple and useful for determining the extent of ovarian dysfunction induced by exposure to toxicants and the test has long been used for screening female reproductive toxicity (Cooper, et al., 1993). We have recently reported that exposure to 1,000 or 300 ppm of 2-BP results in a significant disruption of the estrous cycle, 3 or 7 weeks after exposure, respectively (Kamijima et al., 1997). Clear histopathological changes were observed in 300, and 1,000-ppm 2-BP exposure groups. On the other hand, follicle counts showed that even exposure to 100 ppm of 2-BP reduced the percentages of primordial and growing follicles. These results indicate that differential follicle count is a more sensitive measure of 2BP-induced ovarian toxicity than gross ovarian weight, smear interruption or fertility. Such method provides a quantitative evaluation of ovarian toxicity compared with the conventional descriptive studies of histopathological examination. Several investigators have also shown that follicle count represents a more sensitive measure of toxicity and could be used to assess the normal function state as well as ovarian responses to toxic agents (Takizawa et al., 1984; Plowchalk et al., 1992; Smith et al., 1990; Bolon et al., 1997). Using these parameters, subtle and early changes in ovarian structure and function could be identified before the development of any reduction in the estrous cycle, fertility or fecundity. However, there is still a controversy with regard to the usefulness of the differential follicle count. Bolon et al. (1997) compared differential ovarian counts and reproductive performance for 15 chemicals in mice ovaries from National Toxicology Program Reproductive Assessment by Continuous

Breeding (RACB) bioassays. The RACB protocols demonstrated that six agents altered female reproductive outcome, but only three of six female toxicants [2,2-bis(bromoethyl)-1,3-propanediol, BPD; ethylene glycol monomethyl ether, EGME; methoxyacetic acid, MAA] significantly decreased counts of small and/or growing follicles by 33 to 92% in CD-1 mice and female toxicants di-N-hexyl phthalate, propantheline bromide, and tricresyl phosphate reduced reproductive performance but not follicle numbers. Altered follicle counts without apparent reproductive impairment occurred in CD-1 mice at lower doses of BPD but were not observed for nontoxic chemicals. Combined with the results of the present study, we conclude that the correlation between vaginal smear test and follicle count is dependent upon the target site (cells) or the type of follicle damaged. For example, a number of female toxicants could produce disruption of estrous cycle due to the impairment in neuroendocrine system, oviduct, uterus, cervix, or accessory sex glands by without reducing the follicle number (Lamb, 1989; McLachlan and Newbold, 1989). The type(s) of follicles damaged also have a critical effect on this correlation. For example, if only primordial follicles are destroyed, cyclicity can continue for a long time until no growing or antral follicles remain (Hooser et al. 1994). On the other hand, if the direct site of damage is antral follicle, this loss should be measured at the same time that vaginal cytology is disrupted. Therefore, the follicle differential counting method will be more effective in detecting early damage to primordial and growing follicles, but not necessarily antral follicle damage. The data in Table1 show a nice correlation that low dose exposure disrupts primordial and growing follicle numbers, but not antral folicles. This is well correlated with no disruption of vaginal cytology unless a dose that produces antral follicle damage is used.

In conclusion, the present study demonstrated that ovarian dysfunction induced by 2-BP was caused by the destruction of primordial follicle and its oocyte and that such cell damage was due to apoptotic process. Furthermore, we also demonstrated the usefulness of the follicle differential count as a sensitive test for monitoring female reproductive system dysfunction induced by 2-BP.

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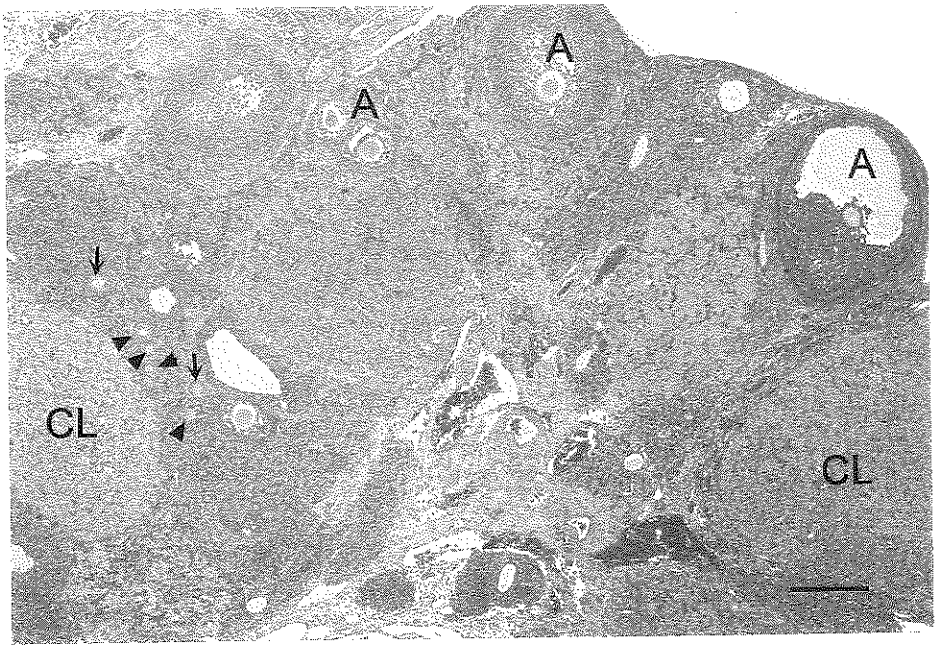

FIG. 1. Photomicrograph of an ovary from a representative control rat. CL, corpora lutea; A, antral follicles. Note the presence of growing follicles (arrows) and primordial follicles (arrowheads). Bar, 250 μm.

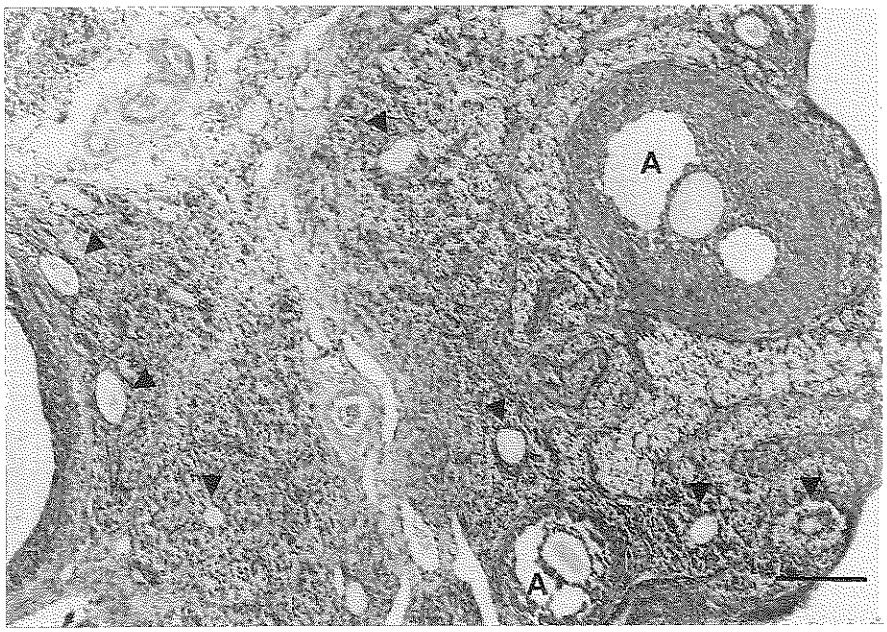

FIG. 2. Photomicrograph of an ovary from a representative rat exposed to 2-BP at 1000 ppm for 8 h/day, 7 days/week for 9 weeks. The ovary contains few primordial, growing, and antral follicles and no or few corpora lutea. A, antral follicles. Note the presence of degenerated follicles (arrowheads). Bar, 100 μm.

TABLE 1
Dose-Dependent Effects of 2-BP on Vaginal Estrous and Ovarian Follicles

		Dose of 2-BP (ppm)			
		Control	100	300	1000
Vaginal smear	Irregular and none	2/7	—(1/7)	↓ ↓ (7/8)	↓ ↓ (9/9)
Follicle differential counts	Primordial follicle	—	↓ ↓	↓ ↓	↓ ↓
	Growing follicle	—	↓ ↓	↓ ↓	↓ ↓
	Antral follicle	—	—	↓ ↓	↓ ↓

Note. Rats were exposed to 2-BP at 100, 300, and 1000 ppm or room air for 8 h/day, 7 days/week for 9 weeks. —, No significant difference from control; ↓ ↓, significantly lower than control at $p<0.01$. Irregular and none, rats showed 0.5–3 cycles/ 3 weeks or 0 cycles/ 3 weeks from Weeks 7 to 9 after commencing exposure.

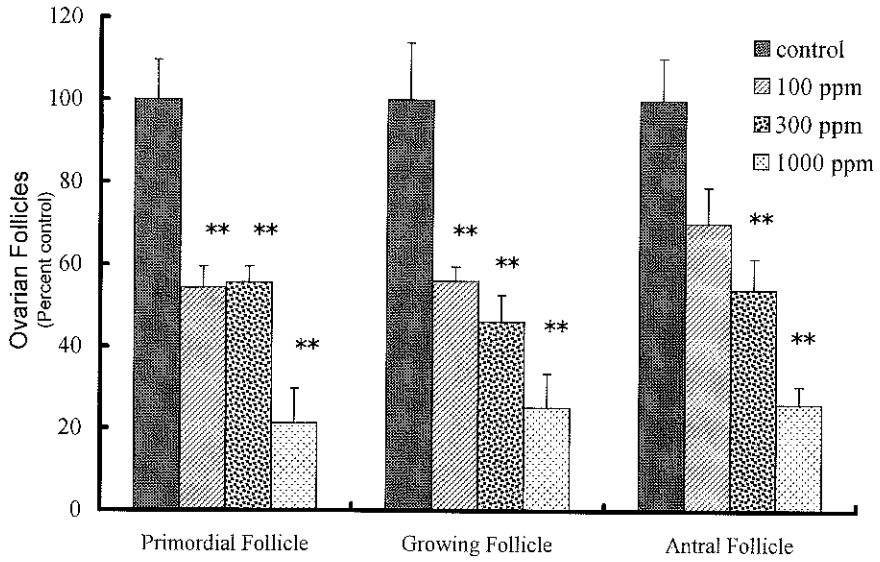

FIG. 3. Dose–effect of 2-BP on ovarian follicles. Rats were exposed to 2-BP at 100, 300, and 1000 ppm or room air for 8 h/day, 7 days/week for 9 weeks. Differential follicle counts were calculated using the modified method of Pedersen and Peters (1968), in which type 1–3b, type 4–5b, and type 6–8 were grouped as primordial, growing, and antral follicles, respectively. The relative counts of primordial, growing, and antral follicles were expressed as percentages of the control. Each bar represents the means ± SEM of six to seven rats. ** Statistically significant at $p < 0.01$.

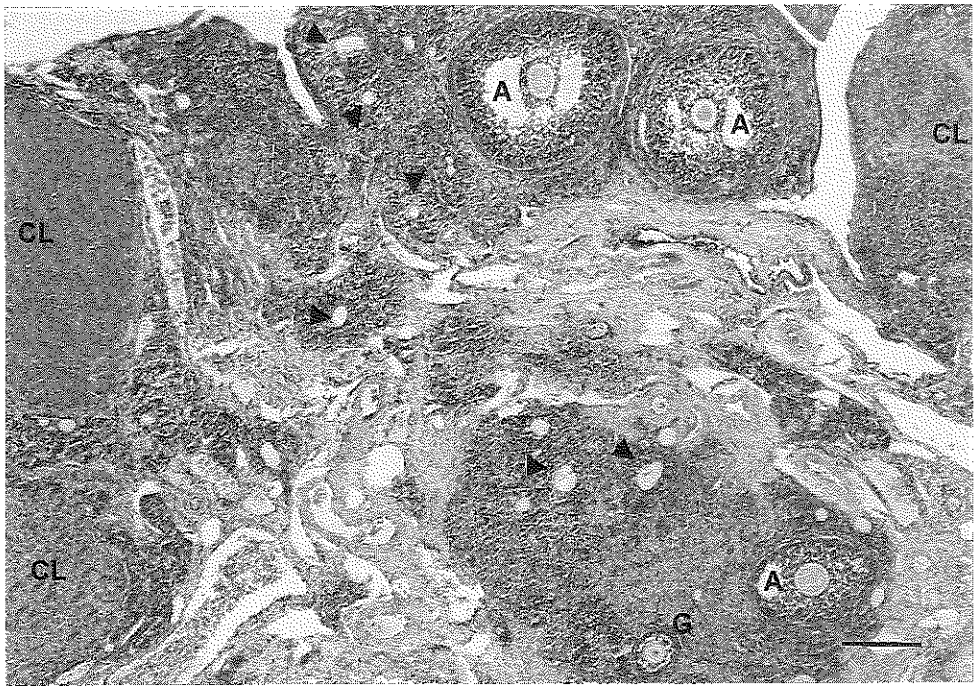

FIG. 4. Photomicrograph of an ovary from a representative rat exposed to 2-BP at 300 ppm for 8 h/day, 7 days/week for 9 weeks. CL, corpora lutea; A, antral follicles. Note the presence of degenerated follicles (arrowheads). The ovary contains few primordial, growing, and antral follicles and only a few newly formed corpora lutea. Bar, 250 μm.

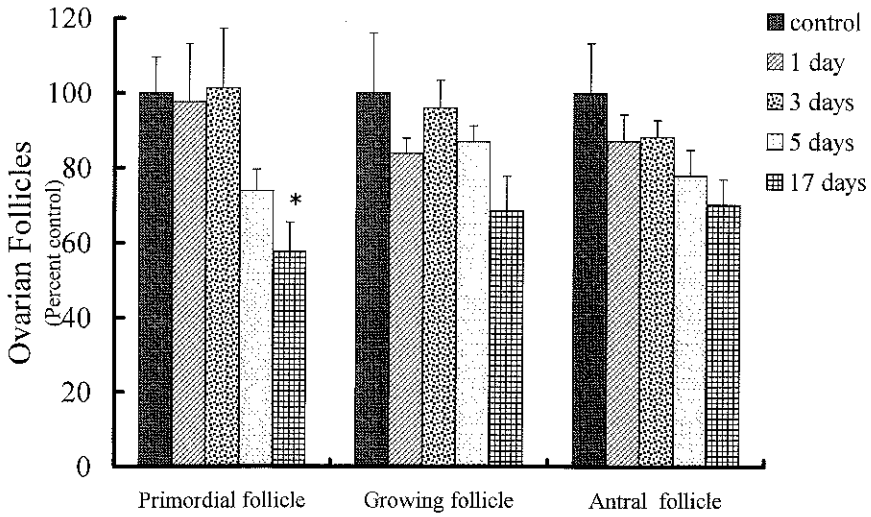

FIG. 7. Effects of a single exposure to 2-BP on ovarian follicles. Rats were exposed to 2-BP at 3000 ppm or room air for 8 h. Rats were euthanized at Days 1, 3, 5, or 17 after exposure. The method used to calculate differential follicle counts was described in Fig. 3. The relative number of primordial, growing, and antral follicles was expressed as percent of the control. Each bar represents the means ± SEM of six to seven rats. *Statistically significant at $p < 0.05$; **statistically significant at $p < 0.01$.

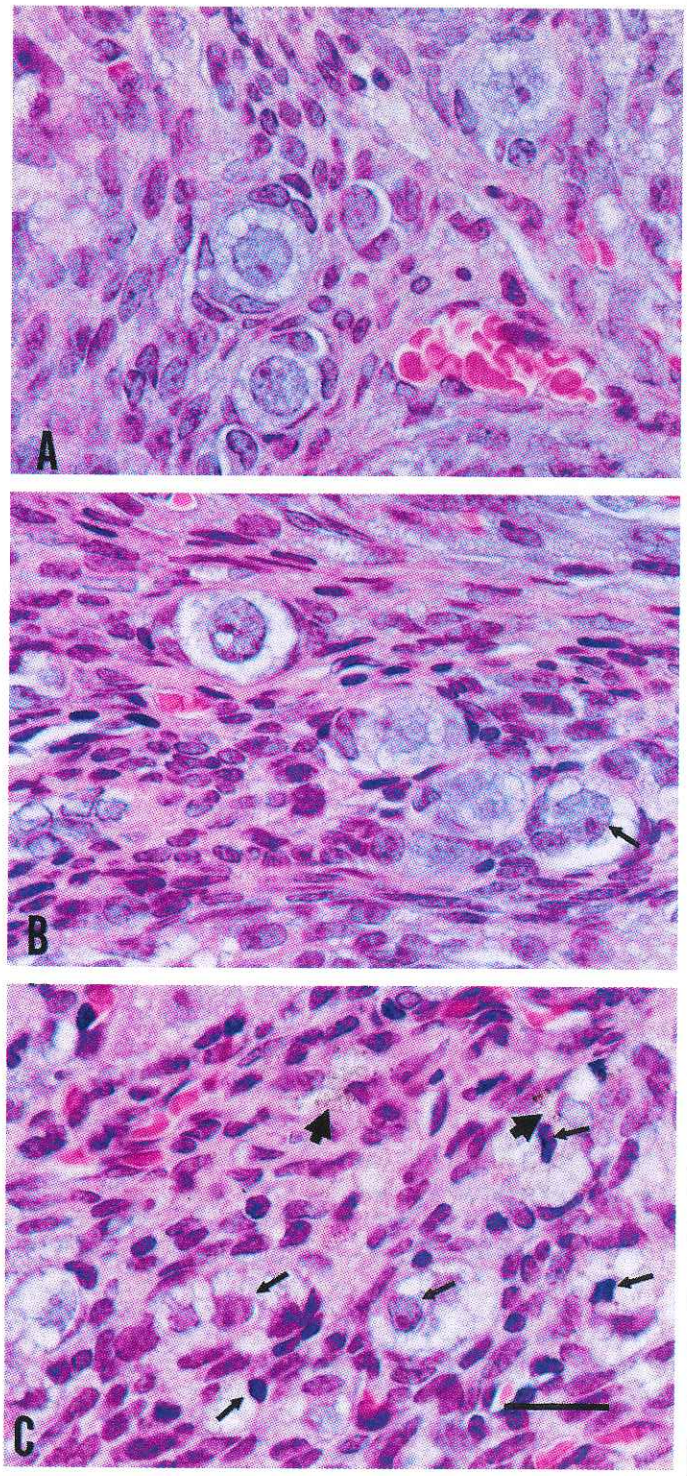

FIG. 5. Photomicrographs of ovarian tissue sections from representative rats exposed to 2-BP. Rats were exposed to 2-BP at 3000 ppm or room air for 8 h. Sections (5-μm thick) were stained with hematoxylin and eosin. (A) A representative example of a primordial follicle from a control rat. Oocytes show a diffuse pattern of nuclear chromatin, a round shape, and each is surrounded by a layer of granulosa cells. (B) A representative example of a primordial follicle from a rat at 5 days after exposure to 2-BP. Note the granular appearance of the nuclear material in oocytes and irregular cell shape (small arrow). (C) A representative example of a primordial follicle from

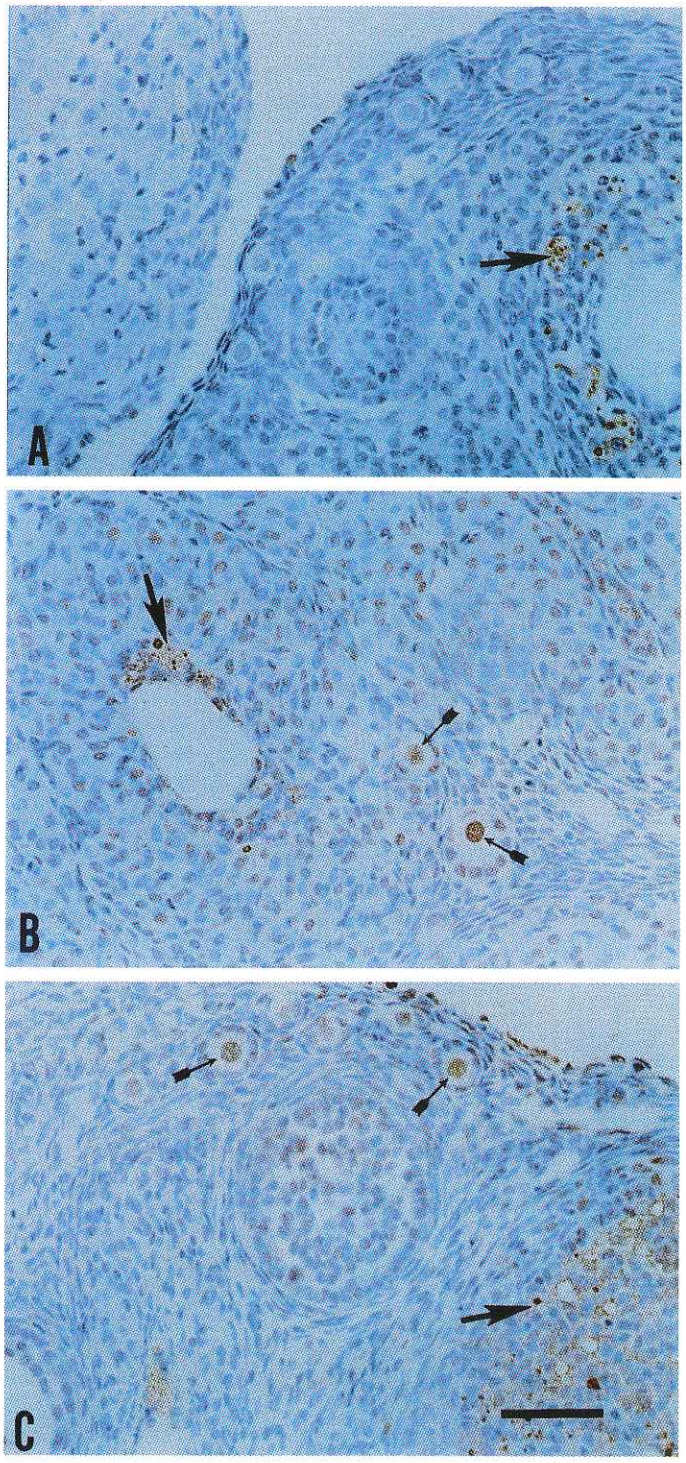

FIG. 6. *In situ* staining of apoptotic cells in ovarian tissues. Rats were exposed to 2-BP at 3000 ppm or room air for 8 h. (A) Positive staining of granulosa cells of the atretic follicle in control rats (large arrow). (B) Five days after exposure. Two oocytes stained brown in type 2 and type 3 primordial follicles (small arrows). Granulosa cells of the atretic follicle are also positively stained (large arrow). (C). Seventeen days after exposure. Apoptotic nuclei with positive brown staining in oocytes of the primordial follicles (small arrows) or granulosa cells of the atretic follicle (large arrow). Bar, 50 μm.