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Developmental Toxicity by Exposure to Bisphenol A Diglycidyl Ether during Gestation and Lactation Period in Sprague-dawley Male Rats

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Objectives : Bisphenol A diglycidyl ether (BADGE) is the major component in commercial liquid epoxy resins, which are manufactured by co-reacting bisphenol A with epichlorohydrin. This study was performed to show the developmental effects of prenatal and postnatal exposures to BADGE in male rat offspring.

Methods : Mated female rats were divided into four groups, each containing 12 rats. The dosing solutions were prepared by thoroughly mixing BADGE in corn oil at the 0, 375, 1500 and 3000 mg/kg/day concentrations. Mated females were dosed once daily by oral gavage on gestation day (GD) 6 - 20 and postnatal day (PND) 0 - 21. Pregnant female dams were observed general symptoms and body weight. Also, male pups were observed the general symptoms, body weight, developmental parameters (e.g. anogenital distance, pina detachment, incisor eruption, nipple retention, eye opening, testis descent), organ pathologic changes and hormone levels of plasma.

Results : Pregnant rats treated with BADGE died at a rate of about 70% in the 1500 mg/kg/day group and all rats treated with 3000 mg/kg/day died. Body weight, for male pups treated with doses of 375 mg/kg/day, was significantly lower than in the control group at PND 42, 56, and 63 ($p<0.05$). Evaluation of body characteristics including; separation of auricle, eruption of incisor, separation of eyelid, nipple retention, descent of testis, and separation of the prepuce in the BADGE treated group showed no difference in comparisons with the control group. AGD and adjusted AGD (mm/kg) for general developmental items in BADGE 375 mg/kg/day treated pups tended to be longer than in controls, however, these differences were not statistically significant. Relative weights of adrenal gland, lung ($p<0.05$), brain, epididymis, prostate, and testis ($p<0.01$) were heavier than in control in measures at PND 9 weeks. There were no significant changes in comparisons of histological findings of these organs. Loss of spermatids was observed in the seminiferous tubule at PND 9 weeks, but no weight changes were observed. The plasma estrogen levels were similar in the control and treatment groups at PND 3, 6 and 9 weeks. The plasma testosterone levels in the control group tended to increase with age. However, in the BADGE 375 mg/kg/day treated male pups it did not tend to increase.

Conclusions : These findings suggest that BADGE is a chemical that has developmental effects consistent with it being an endocrine disruptor.

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Key words : BADGE, Developmental toxicity, Sprague-dawley rats, Gestation, Lactation

INTRODUCTION

The compound 2,2-bis(4-(2,3-epoxypropyl) phenyl)propane, commonly known as bisphenol A diglycidyl ether (BADGE, CAS no. 1675-54-3) is the reaction product of one mole of 2,2-bis(4-hydroxyphenyl)propane (bisphenol A, BPA) with two moles of epichlorohydrin [1]. BADGE and its oligomers are major components of epoxy resins [2]. Pure BADGE is a crystalline material with a melting point of 40 - 44 ° C and is the main component found in commercial liquid epoxy resins (>80% of composition); it is present at the lowest molecular weight oligomer in solid epoxy resins (up to 20% depending on molecular weight). Cured epoxy systems (e.g. can coatings) are basically inert and characterized by a unique combination of properties including: excellent adhesion to various substrates, outstanding corrosion and chemical resistance and excellent thermal, mechanical and electrical insulation properties. The major application areas for use of epoxy resins are protective coatings (steel structures, pipes, ships tanks, automotive primers, appliances) and civil engineering (industrial flooring) together accounting for 75% of the total use. Additional applications include composites (windmills, aircraft, automotive), printed circuit boards, adhesives and tooling. A relatively small amount of epoxy resin (<10%) is used for the interior lining of food and drink cans where BADGE is only a minor component [1]. When heating or preserving canned food, hydrolyzed or chlorinated derivatives of BADGE (BADGE · 2O, BADGE · H_2O, BADGE · Cl · 2O, BADGE · Cl, BADGE · HCl) have been found to have migrated into food [3-5].

BPA (starting agent of BADGE), already known as an endocrine disruptor [6], has been associated with abnormalities of the reproductive tract in experimental animals, and may act at very low doses in a range that could have an impact on human and wildlife environmental exposures [7,8]. However, the identification of BADGE as an endocrine

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disruptor [1] is controversial. The potency of the estrogenic activity of BADGE was found to be lower than 1/100 times compared with BPA [6]. Currently, BADGE is manufactured commercially instead of the BPA.

In 1999, the Scientific Committee of Food (SCF) published a new review on the safety of BADGE and concluded that there was no evidence for a systemic tumorigenic effect of topically applied pure or technical grade BADGE [9]. A review of one- and two-generation reproduction studies and developmental investigations found no evidence of reproductive or endocrine toxicity the upper ranges of dosing were determined by maternal toxicity [1]. However, there are many reports on the estrogenic effects found on in vitro tests [10,11]. Reproductive and developmental studies were performed to determine the NOAEL for the risk assessment of BADGE as an endocrine disruptor. However, the administered dose level was variable from 50 to 750 mg/kg/day. The results of this work were not published and the criteria used by NOAEL were not clear.

Therefore, this study was performed to show the developmental effects of prenatal and postnatal exposures to BADGE in male rat offspring.

METHOD

Ⅰ. Developmental and Reproductive Toxicity Study Design

The developmental toxicity study was performed in accordance with the Good Laboratory Practice (GLP) guidelines [12] for Animal Experiments of Chemon Co. Ltd. The dose range was based on a developmental toxicity range-finding study in which minimally maternally toxic and non-maternally toxic dose levels were identified [1]. Specific pathogen-free (SPF) Sprague-dawley rats eight weeks of age (30 male rats and 60 female rats) were purchased from the Charles River breeding laboratory (Wilmington, Mass. U.S.). The rats were identified and singly housed in suspended wire cages. They were housed in an animal room controlled at 23±3 ° C, relative humidity at 55±15% with a light/dark cycle of 12 h (light from 20:00 to 08:00 hours) and given free access to food, Teklad Global 18% Protein Rodent Diet (Harlan Co. Ltd, U.S.). After acclimatization to the light/dark cycle for one week, the experiment was started. Males and females were co-housed (1:1) until mating was confirmed by observation of a copulatory plug or the presence of sperm in a vaginal rinse. The day that mating was confirmed was recorded as gestation day (GD) 0. After confirmation of mating, the rats were returned to their own cages. Mated females were subsequently assigned to dose groups by a computer-generated body weight sorting program using the GD 0 body weights to ensure that mean body weights were similar. This resulted in four groups, each containing 12 rats (including extra two rats). In this study, we used 10 mated rats for all measured items excepts general observation items.

The dosing solutions were prepared by thoroughly mixing BADGE in corn oil at the proper concentrations. Mated females were dosed once daily by oral gavage on GD 6 - 20 and postnatal day (PND) 0-21. Dosing volumes were 10 mg/kg, and doses were based on the most recent individual body weights. Dams were examined on GD 20. The numbers and locations of implantation sites were recorded; early and late resorptions and live and dead fetuses were counted. Each fetus was weighed and the sex was determined by external observation. All pups were individually identified. The litters were culled on PND 4 to three male pups (if possible) per dam. Thirty pups were allocated in control and BADGE treated (375 mg/kg/day) group, respectively. BADGE 1500 mg/kg/day treated dams were almost died before labor. Only one dam was delivered three pups. All allocations were performed by computerized random selection. The remaining offspring were weighed and euthanized. All dams were allowed to deliver naturally and to rear their offspring to weaning on PND 21 then were sacrificed using ether (Figure 1).

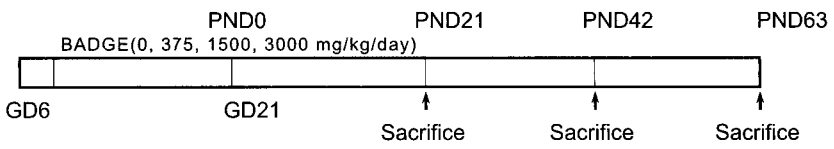

Figure 1. Experimental schedule for the reproduction study (PND: postnatal day, GD: gestational day).

Table 1. The effect of BADGE on the reproduction parameters in dams during the pregnancy and lactation

Items	BADGE (mg/kg/day)		
	0 (n=10)	375 (n=10)	1500 (n=1)
No. of implantations per litter	13.45±2.16	11.82±3.97	14.33±0.58
No. of live fetuses per litter			
Postpartum day 0	12.91±2.12	10.91±3.88	8.80±5.54
Postpartum day 4	12.91±2.12	10.91±3.88	1.80*±3.03
Postpartum day 21	6.00	5.45 ±0.93	1.00*±2.24
No. of dead fetuses per litter	0.09±0.30	0.09 ±0.30	3.20±4.97
% postimplantation loss per litter	3.37	6.92	18.60
Sex ratio of live fetuses(M:F)†	71:72	46:75	33:27
Viability index[a]	99.2	99.1	15
Viability index[b]	100	98.3	22.7

*: p<0.01
†: (M:F) : (Male:Female)
a : (No. of live offspring at day 4 / No. of offspring at birth) × 100
b : (No. of live offspring at day 21 / No. of offspring after litter size control) × 100

Ⅱ. Observed Items

A. General Symptoms and Dead Animals

All animals were observed once daily with detailed evaluations during the study period.

B. Reproduction Parameters in Dams

Dams were sacrificed by ether followed by exsanguination on PND 21, and the uterine contents were removed, examined and weighed. The number and locations of implantation sites were recorded; early and late resorptions and live and dead fetuses were counted. Fetal sex was determined by external observation.

C. General Observations on Development

Each pup was weighed and received a detailed physical examination on PND 1, 4, 7, 14 and 21 and weekly thereafter. Anogenital distance (AGD) was measured on PND 0, 4, 7, 14 and 21. Pina detachment was measured on PND 2, 3 and 4. Incisor eruption was measured on PND 8 to after. Thoracic nipple retention was evaluated for all male pups on PND 11 to 14. Eye opening was measured on PND 12 and thereafter. Testis descent was measured on PND 16 to 22.

D. Body Weight and Organ Weight

Female dams had their body weight measured on GD 0, 6, 9, 12, 15 and 20 as well as on PND 0, 7, 14 and 21. Male pups had their body weight measured on PND 4, 7, 14, 21, 28, 35, 42, 49, 56 and 63. The weight of the main organs including reproductive organs were measured on PND 21, 42, and 63.

E. Histological Observations

For each group necropsy and selective histological examination were conducted on PND 21, 42 and 63. Organs weighed at termination included: brain, thyroid, heart, lung, liver, kidney, testis, epididymis, spleen, prostate and adrenal glands. The testis and epididymis were preserved in Bouin's solution and other organs were preserved in 10% neutral buffered formalin.

F. Plasma Hormone Level

Measurement of plasma estrogen levels was performed using the double antibody method. In the double-antibody precipitation technique, a limited amount of primary antibody was reacted with labeled estrogen; the resulting antigen-antibody complexes were precipitated with a second antibody directed against the primary antibody, leaving the unreacted labeled antigen in solution. Plasma testosterone levels were measured using a radioimmunoassay (RIA) technique.

Table 2. The date (day) of observation of the general development in male pups after birth

Items	BADGE (mg/kg/day)		
	0 (n=10)	375 (n=10)	1500 (n=1)
Separation of auricle	2.33 ± 0.50	2.11 ± 0.33	3
Eruption of incisor	10.56 ± 0.73	10.78 ± 0.97	11
Separation of eyelid	13.56 ± 0.53	13.22 ± 0.67	15
Nipple retention	12.00 ± 0.71	11.89 ± 0.33	13
Descent of testis	17.00 ± 1.00	17.22 ± 0.97	17
Separation of prepuce	43.22 ± 0.44	43.22 ± 0.44	44

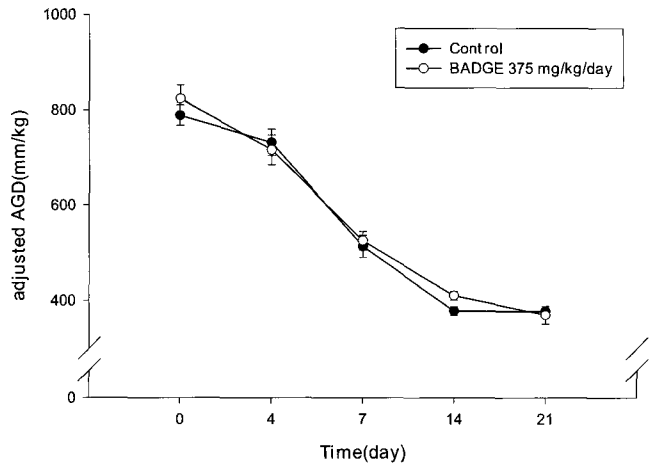

Figure 2. The changes of weight-adjusted anogenital distance (AGD, mm/kg) in SD male pups.

III. Statistical Analysis

Equality of means on adult body weight was tested by appropriate Wilcoxon two-sample test and a test for ordered response in the dose groups. A p-value <0.05 was considered to be significant. Equality of means on organ weight and plasma hormone levels between control and treatment groups was tested using the Wilcoxon two-sample test. Three pups delivered from BADGE 1500 mg/kg/day fed dams were excluded from the statistical analysis because of the small number of observations relative to the control and the 375 mg/kg/day treated group; the results are presented in tables and figures.

RESULTS

I. General Observation of Dams

All of the BADGE 375 mg/kg/day fed 12 dams were healthy from gestation to lactation. Four of the BADGE 1,500 mg/kg/day fed 12 dams were alive before labor and 1 of 4 living dams labored three male pups. All of the three pups delivered from BADGE 1,500 mg/kg/day fed dam was observed to have low body weight and low temperature. All 12 dams fed BADGE 3,000 mg/kg/day died before labor.

II. Reproduction Parameters in Dams

Reproduction parameters were measured in all of the dams in BADGE 375 mg/kg/day fed group and one dam in the BADGE 1,500 mg/kg/day group (Table 1).

III. General Observations on Development in Male Pups

General development items studied (separation of auricle, eruption of incisor, separation of eyelid, nipple retention, descent of testis, and separation of prepuce) in the treatment group showed no difference compared with the control group (Table 2). AGD-adjusted weight measured on PND 0, 4, 7, 14 and 21 compared between control and

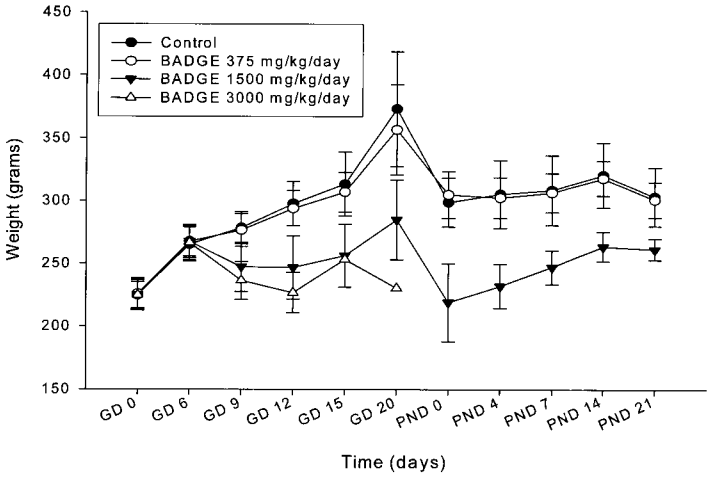

Figure 3. The changes of maternal body weight (grams) in dams exposed to BADGE during pregnancy and lactation (GD: gestational day, PND; postnatal day).

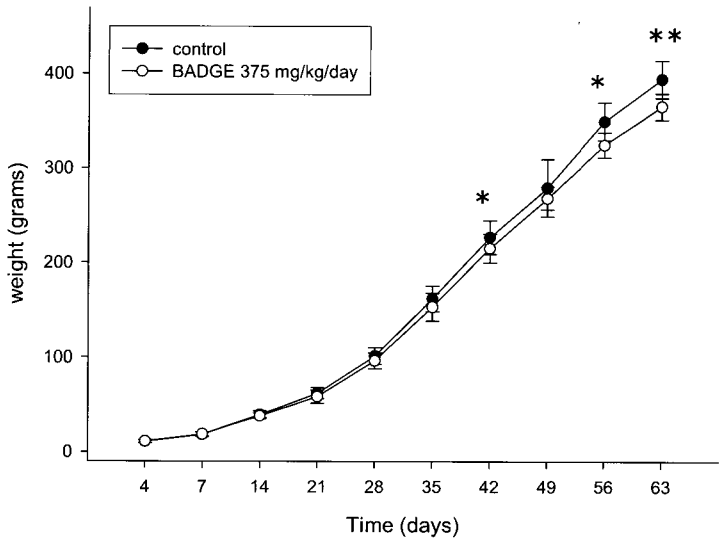

Figure 4. The changes of body weight by age in SD male pups exposed to BADGE (*: p<0.05, **: p<0.01).

BADGE 375 mg/kg/day treated pups did not show a significant difference (Figure 2).

IV. The Changes in Body Weight in Dams and Male Pups and Relative Organ Weight Changes

There was no difference in body weight between the BADGE 375 mg/kg/day treated dams and the control group dams (Figure 3). There was a statistically significant difference between BADGE 1,500 mg/kg/day treated and control group dams. In cases of dams treated with BADGE 1,500 mg/kg/day weight loss on GD 6 to 12 was observed. After those points in time BADGE 1,500 mg/kg/day treated dams tended to increase body weight on GD 20. Until PND 21, however, BADGE 1,500 mg/kg/day treated dams body weight did not recover compared to the control group. Dams treated with BADGE 3,000 mg/kg/day all died before delivery.

In each necropsy day, each organ weight was measured for one of BADGE 1,500 mg/kg/day treated male pups. Therefore, this data was not applied for statistical analysis. In cases of BADGE 375 mg/kg/day treated male pups, there was no statistical difference observed until PND 14 to 35 compared to control group male pups. After then, BADGE 375 mg/kg/day treated male pups body weight gain was observed to be gradually lower than that of the control group ($p>0.05$)(Figure 4).

The relative organ weights for thyroid, heart, liver and kidney were not different compared to the control group (Table 3). The relative weight of the lung in BADGE 375 mg/kg/day treated male pups was much heavier than in the control group on PND 3, 6 and 9 weeks. Especially at PND 6 and 9 weeks, the relative lung and adrenal gland weights were observed to be statistically different. The relative brain, epididymis, prostate and testis weights in BADGE 375 mg/kg/day treated male pups were statistically heavier than in control male pups at PND 9 weeks ($p<0.01$). The relative spleen weights in BADGE 375 mg/kg/day treated male pups were statistically lower than in control group male pups at PND 9 weeks ($p<0.05$).

V. Histopathologic Changes of Pups Organs

Major organs (brain, thyroid, heart, lung, liver, kidney, adrenal) and reproductive organs (testis, epididymis, prostate) were observed by light microscope (Figure 5). All major and reproductive organs, except for the testis, showed no pathological changes in the treatment groups. Decreased numbers of spermatids in the seminiferous tubules of the testis were observed.

VI. Hormone Level Changes in Plasma

The plasma sex hormones (estrogen and testosterone) in control and BADGE 375 mg/kg/day treated rats were studied (Figure 6). The plasma estrogen level decreased after 3 weeks it was similar in the control and treatment groups at PND 3, 6 and 9 weeks. The plasma testosterone level in the control group tended to increase by age in 3, 6 and 9 weeks (2.4, 4.6, and 10.7 ng/ml) for the BADGE 375 mg/kg/day treated male pups it did not tend to increase at 3, 6 and 9 weeks (4.2, 4.5, and 6.8 ng/ml). At PND 3 weeks, the testosterone level in BADGE 375 mg/kg/day treated male pups was statistically higher than in control male pups ($p=0.048$).

DISCUSSION

Pregnant rats treated with BADGE died at a rate of about 70% in the 1,500 mg/kg/day group all rats treated with 3,000 mg/kg/day died. Therefore, BADGE treatment with a dose above 1,500 mg/kg/day was lethal pregnant SD rats. This phenomenon might have resulted not only from the toxicity of BADGE but also from rejection of food after lactation or indigestion due to gastric toxicity [13].

Body weight, for male pups treated with doses of 375 mg/kg/day, was significantly lower than in the control group at PND 42, 56 and 63 ($P<0.05$). This result was similar to the results of Hanley et al. where decreased body weight in male offspring of treated rats, at dose levels of 540 and 750 mg/kg/day, was observed in a two-generation reproduction study [14]. Another study showed that while treatment at all dose levels had no adverse effects on reproductive performance, a slight reduction in mean pup weight in the 540 mg/kg group was observed on day 21 [15]. In other sub-chronic dietary studies, rats receiving the highest dose levels (i.e. 4,500 mg/kg/day) rejected the diet and failed to gain weight [16]. Further studies on the digestive effects of BADGE are needed.

Evaluation of body characteristics including: separation of auricle, eruption of incisor, separation of eyelid, nipple retention, decedent of testis, and separation of the prepuce in the BADGE treated group showed no difference in comparisons with the control group. AGD and adjusted AGD (AGD/BW, mm/kg) for general developmental items in BADGE 375 mg/kg/day treated pups tended to be longer than in controls. However, these differences were not statistically significant, and showed no difference at PND 21 (Figure 1). Other studies have evaluated endocrine disruptors including: BPA, phthalate, and diethylstilbestrol showed a shortening of the AGD [17-23].

Relative organ weights in the BADGE treated group were not different for thyroid, heart, liver and kidney compared to the control group. On PND 6 weeks, relative weights of lung and adrenal glands were heavier than in controls ($p<0.01$ and $p<0.05$). However, there were no changes observed in the histological findings in comparisons of the treated and control groups. Relative weights of lung ($p<0.05$), epididymis, prostate, testis and brain ($p<0.01$) were heavier than in controls and relative spleen weight was lower than in control in measures at PND 9 weeks, but there were no significant changes in histological evaluations. Loss of spermatids was observed in the seminiferous tubule at PND 9 weeks, but no weight changes were observed. These findings may indicate the need for additional electron microscopy and functional studies. Declining spermatids in the seminiferous tubules in adult life (PND 9 weeks) may be associated with adverse effects during the prenatal period [24].

Table 3. The changes of relative organ weight of brain, thyroid, liver, lung, heart, spleen, adrenal gland, kidney, prostate, epididymis and testis by age in SD male pups exposed to BADGE 375 mg/kg/day. Comparison of relative organ weight between control and BADGE 375 mg/kg/day treated group was done by Wilcoxon two-sample test

Items	BADGE (mg/kg/day)		
	0 (n=10)	375 (n=10)	1500 (n=1)
PND21			
Testis	0.423±0.059	0.403±0.040	0.344
Epididymis	0.067±0.016	0.065±0.017	0.068
Prostate	0.050±0.015	0.040±0.008	0.014
Kidney	0.962±0.057	0.992±0.070	1.012
Adrenal gland	0.026±0.005	0.025±0.005	0.038
Liver	4.016±0.212	4.065±0.336	4.013
Spleen	0.470±0.080	0.489±0.126	0.688
Lung	0.799±0.061	0.834±0.060	1.067
Heart	0.491±0.030	0.510±0.039	0.678
Thyroid	1.040±0.420	0.980±0.350	0.680
Brain	2.359±0.179	2.495±0.199	4.054
PND42			
Testis	0.878±0.047	0.923±0.118	0.496
Epididymis	0.117±0.015	0.129±0.022	0.082
Prostate	0.075±0.019	0.089±0.037	0.076
Kidney	0.808±0.047	0.847±0.126	0.552
Adrenal gland	0.015±0.003	0.018±0.003*	0.011
Liver	4.549±0.231	4.669±0.411	3.501
Spleen	0.314±0.042	0.318±0.074	0.317
Lung	0.475±0.044	0.540±0.064†	0.395
Heart	0.394±0.024	0.422±0.035	0.352
Thyroid	0.650±0.190	0.750±0.240	0.440
Brain	0.808±0.075	0.880±0.113	0.703
PND63			
Testis	0.804±0.112	0.860±0.056†	0.682
Epididymis	0.174±0.014	0.186±0.014†	0.248
Prostate	0.100±0.017	0.114±0.039†	0.069
Kidney	0.631±0.170	0.629±0.137	0.301
Adrenal gland	0.014±0.002	0.014±0.001*	0.012
Liver	4.210±0.313	4.005±0.190	4.059
Spleen	0.212±0.029	0.206±0.018*	0.270
Lung	0.365±0.031	0.388±0.022*	0.402
Heart	0.314±0.067	0.287±0.099	0.412
Thyroid	0.540±0.150	0.490±0.110	0.370
Brain	0.521±0.022	0.551±0.020†	0.561

*: $p<0.05$, †: $p<0.01$

The plasma estrogen levels were similar in the control and treatment groups at PND 3, 6 and 9 weeks. The plasma testosterone levels in the control group tended to increase with age. However, in the BADGE 375 mg/kg/day treated male pups it did not tend to increase. This result suggested that exposure to BADGE during the prenatal and postnatal period influenced testosterone production in adult rats.

The secretion of testosterone is regulated by the negative feedback mechanism of the hypothalamus-pituitary-testes axis [13]. Gonadotropin-releasing hormone (GnRH), a peptide hormone, is produced in the medial basal portion of the hypothalamus. GnRH stimulates the pituitary gland to produce and secrete luteinizing hormone (LH) and follicle-

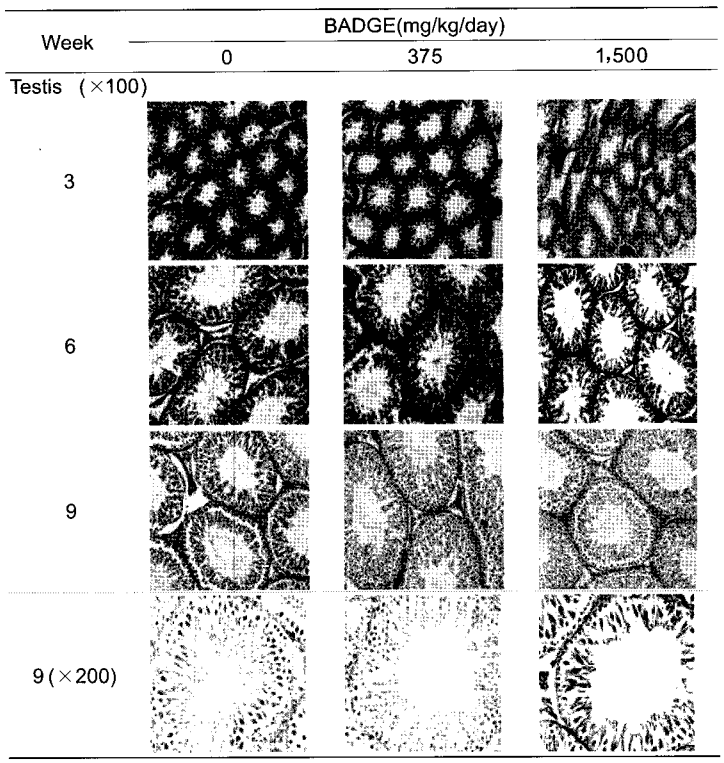

Figure 5. Histological findings in organs. No histological changes were found in any organs except the testis. A decreased number of spermatids were observed in the seminiferous tubules of 9 week male pups.

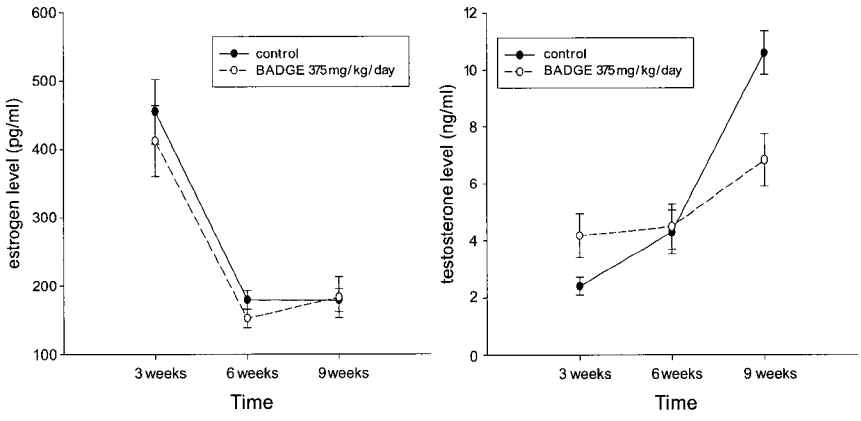

Figure 6. The changes of plasma sex hormone (estrogen and testosterone) levels(mean±s.e.) in SD male pups exposed to BADGE 375 mg/kg/day.

stimulating hormone (FSH). LH is primarily responsible for stimulating testicular Leydig cell secretion of testosterone. FSH acts on the Sertoli cells of the testes to stimulate spermatogenesis and inhibin [25].

Tohei reported that the plasma testosterone concentrations in male rats were decreased and plasma LH levels were increased when the animals were given BPA 1 mg/kg/day over 2 weeks [26]. However, Watanabe reported that the plasma testosterone concentration in male rats at 9 weeks were statistically increased in a dose-dependent manner, when the animals were given BPA 0, 4, and 40 mg/kg/day starting from gestation day 6 through lactation day 20 by gavage [13]. The LH concentration in the BPA groups was at the same level as in the controls. Other investigators have found reduction in the blood testosterone level [26,27]. There discrepancies in study results may be primarily attributable to the doses of BPA, the animal species used in the experiments and the age of the animals when treated [13]. BADGE is prepared by adding epichlorohydrin and BPA; they are regarded as having similar hormonal effects. Therefore, these results suggested that BADGE exposure alters the function and morphology of reproductive organs in the animals directly exposed to it. To confirm the effect of BADGE on LH secretion, a luteinizing hormone-releasing hormone challenge test should be performed.

Testicular toxicity and changes of relative reproductive organ weight appeared in 9 weeks at a dose of 375 mg/kg/day of oral exposure to BADGE during pregnancy and lactation. There have been a few studies to determine the NOAEL of BADGE such as 50 mg/kg/day in males and 540 mg/kg/day in females on slight weight changes [28], 180 mg/kg/day for parental NOEL [15], 750 mg/kg/day for female SD rats [14], 100 mg/kg/day for rabbit dermal erythema [29], and 30 mg/kg/day for rabbit skin effect [30]. However, these results were based on variable doses based on arbitrary decisions, and not data from reproductive studies. Moreover, most of the results are from studies conducted by the chemical company that manufactures the product. This result showed that NOAEL of BADGE for reproductive system of male rat was less than 375 mg/kg/day.

CONCLUSION

Body weight and relative organ weight(lung, adrenal gland, epididymis, prostate, spleen testis and brain) changed in 6 or 9 weeks, testicular toxicity appeared at 375 mg/kg/day of oral exposure in 9 weeks, and plasma testosterone level by age did not change with exposure to BADGE during prenatal and postnatal period. These findings suggest that BADGE is a chemical endocrine disruptor.

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