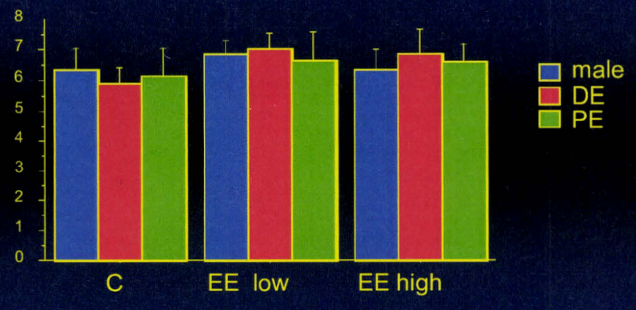
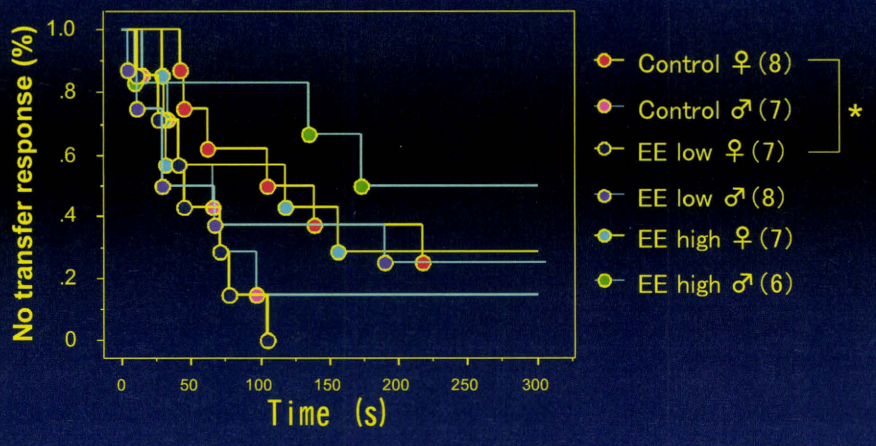
ガラス玉覆い隠し試験においては有意な影響は検出されなかった



もっと性周期がはっきりしている系統を使用する必要があるかもしれない

受動回避学習試験においてメスラットの学習能力が2ug/kgEE投与によって低下した



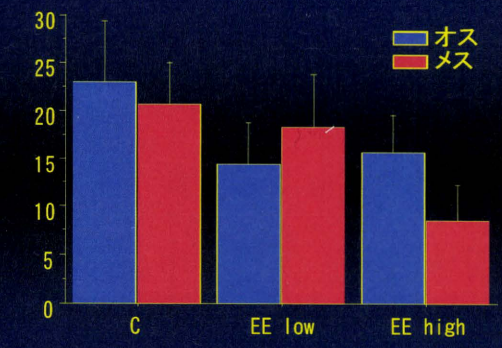
強制水泳時の無動時間

15分間水槽円柱(直径30cm　高さ60cm　水深 約40cm)に入れる

24時間後

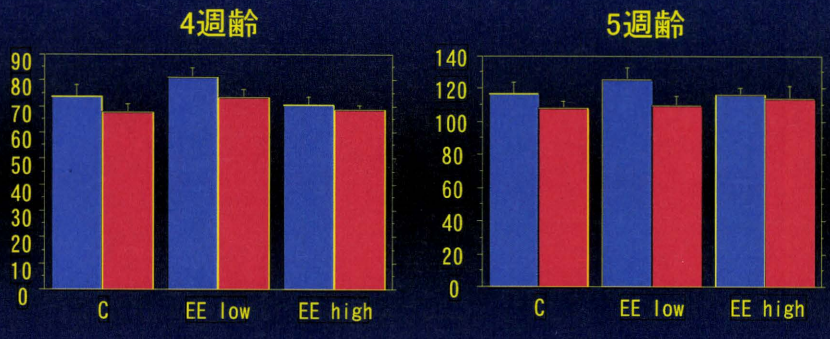
5分間水槽に入れる

総無動時間を測定し、うつ様行動の指標とする



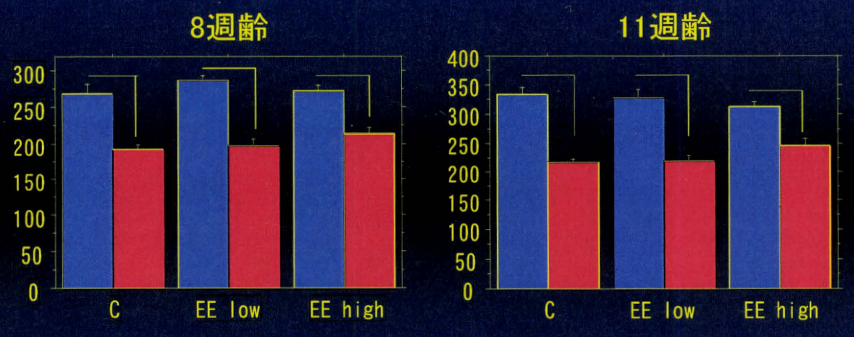
高濃度EEを曝露したメスラットで無動時間が減っている傾向があるが、有意な変化ではない。

性成熟前の体重(g)



高濃度EEを曝露したラットの体重差が減っている傾向があるが、有意な変化ではない。

性成熟後の体重(g)



高濃度EEを曝露したラットの体重差が減っている傾向があるが、有意な変化ではない。

今後の方向性

- 昨年度までに影響が現れた受動回避学習試験は、性成熟前の影響を確認した後、関連する早期指標について検討する

→受動回避学習試験に関連する脳内変化を明らかにする

- ストレスを伴わない学習試験についても検討する

→新規物体認知試験、社会性認知試験を行う

- 昨年度までの結果を鑑み、今年度からは繁殖に関連した項目についても検討する

→性嗜好性試験、性行動試験を行う

- 短期観察で実施できる実験系も別途開始する

→母子分離誘発啼鳴反応実験を行う

- 今年度からはポジティブコントロール群を作成する
- 行動実験と並行して、ホルモン濃度や病理変化など影響が検出される可能性の高い項目について検討をお願いする

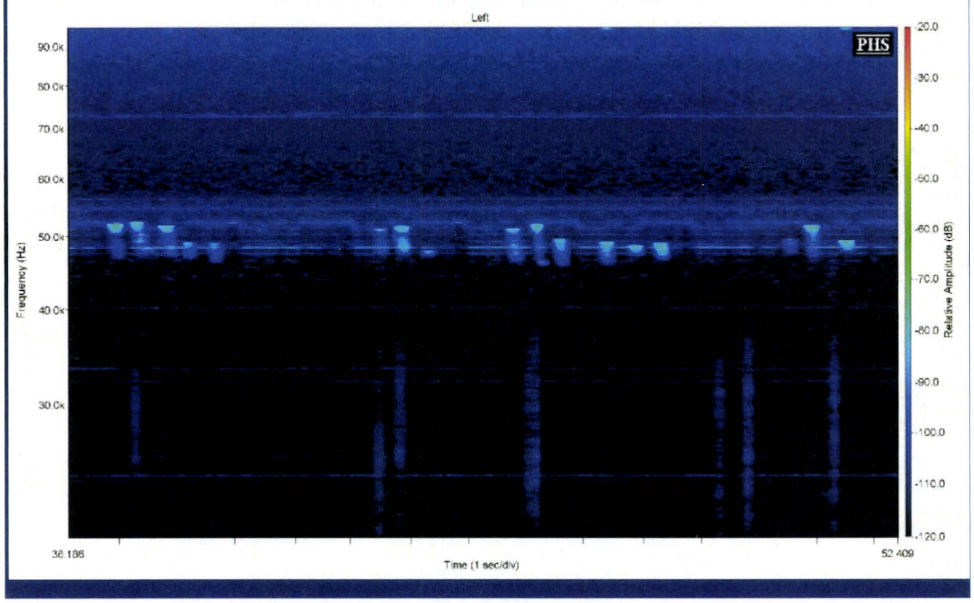
今後の予定
PD1仔ラットにごま油(C)、20ug/kgEE(EE low)、8mg/kgEE(EE high)を投与し以下の試験を行う

- 母子分離誘発啼鳴反応（不安・社会性行動）: 1～3週齢
- 受動回避学習試験（学習行動）: 5週齢
- オープンフィールド試験（一般・不安行動）: 7～8週齢
- 新奇物体認知試験（探索・学習行動）: 7～8週齢
- 社会性認知試験（社会性・学習行動）: 7～8週齢
- 性嗜好性試験: 9～12週齢
- 性行動試験: 9～12週齢

母子分離誘発啼鳴反応とは

- 離乳前の仔ラットは、母親および同腹仔から引き離されると、分離直後から20kHz～60kHzの超音波領域に主成分を持つ啼鳴反応を示す。
- この反応はHPA軸が関与していること、ジアゼパムなどの抗不安薬で抑制されることが報告されている。
- 母子分離誘発啼鳴反応自体には性差がないことが知られている。

母子分離誘発啼鳴反応の例



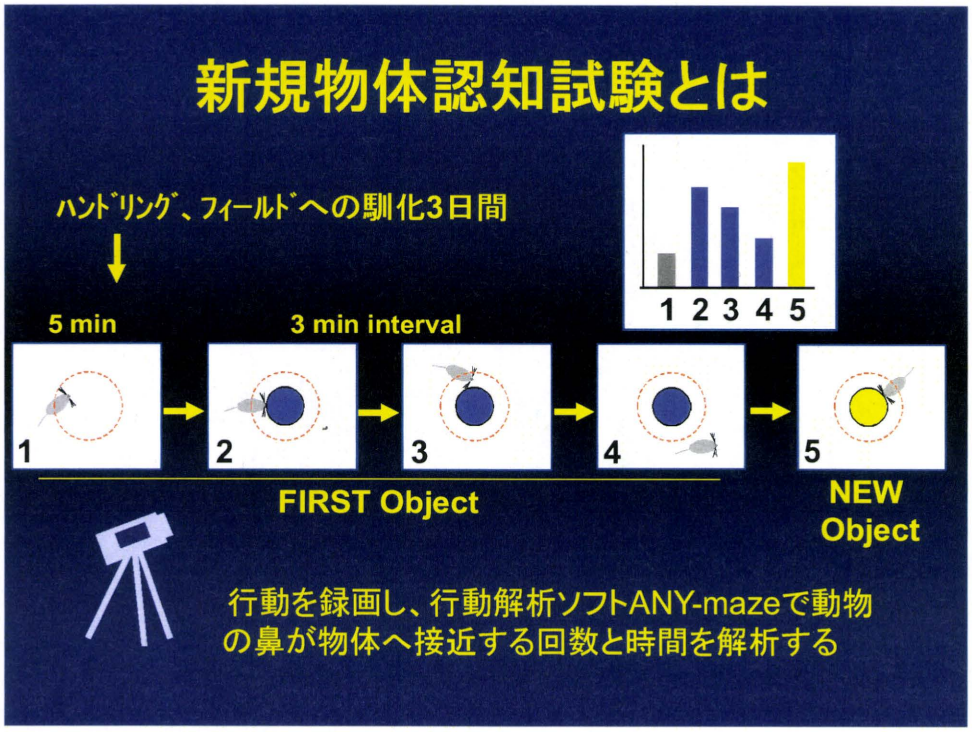
母子分離誘発啼鳴反応試験の目的

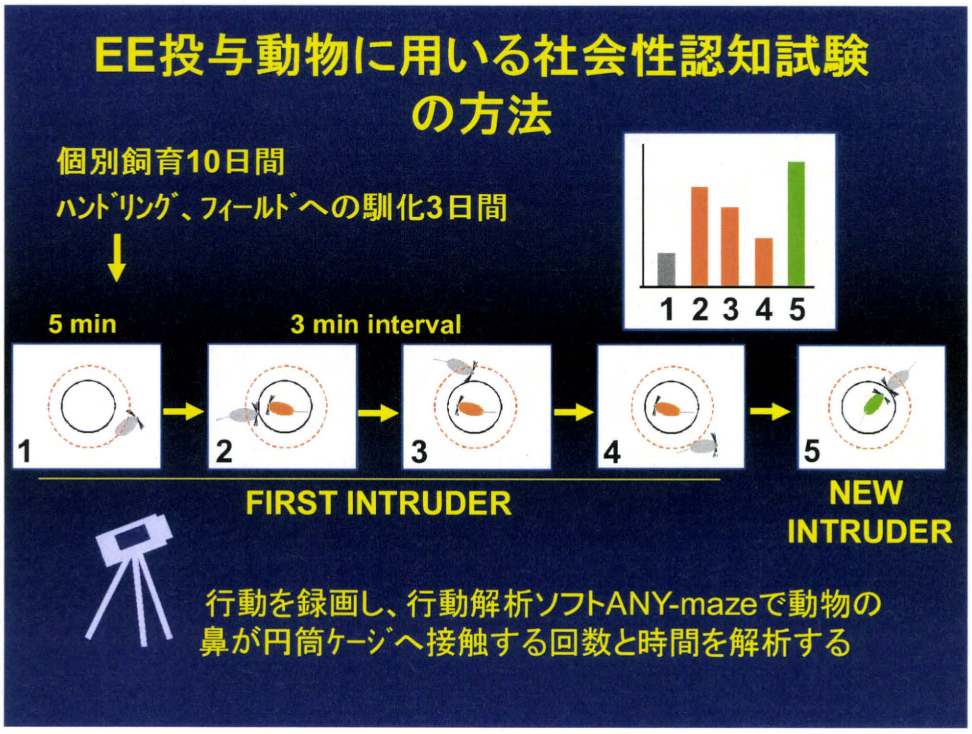
- 母子分離誘発啼鳴反応は社会性行動とストレス応答の二つの側面がある

  →エストロゲン様物質曝露の行動学的な早期指標になり得るか検討する

- ジアゼパムの一部の作用には新生仔期のホルモンに依存する性差が報告されている

  →ジアゼパム投与の母子分離誘発啼鳴反応の性差とEE投与の影響について明らかとする

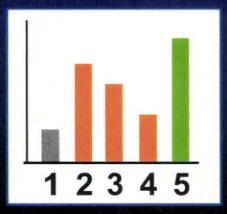

新規物体認知試験とは
ﾊﾝﾄﾞﾘﾝｸﾞ、ﾌｨｰﾙﾄﾞへの馴化3日間
5 min
3 min interval
1 2 3 4 5
1
2
3
4
5
FIRST Object
NEW Object
行動を録画し、行動解析ソフトANY-mazeで動物の鼻が物体へ接近する回数と時間を解析する


EE投与動物に用いる社会性認知試験の方法
個別飼育10日間
ﾊﾝﾄﾞﾘﾝｸﾞ、ﾌｨｰﾙﾄﾞへの馴化3日間
5 min
3 min interval
1 2 3 4 5
1
2
3
4
5
FIRST INTRUDER
NEW INTRUDER
行動を録画し、行動解析ソフトANY-mazeで動物の鼻が円筒ｹｰｼﾞへ接触する回数と時間を解析する

EE投与動物に用いる社会性認知試験の方法

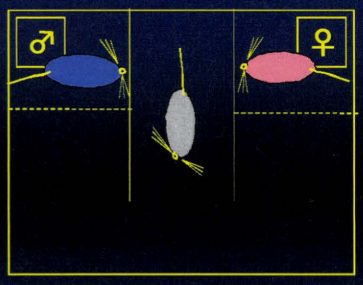
個別飼育10日間

ﾊﾝﾄﾞﾘﾝｸﾞ、ﾌｨｰﾙﾄﾞへの馴化3日間



行動を録画し、行動解析ソフトANY-mazeで動物の鼻が円筒ｹｰｼﾞへ接触する回数と時間を解析する

性嗜好性試験



網で仕切った先に雄ラットと雌ラットを入れ、テストラットがどちらを好むかを、テストラットの滞在領域などを指標として計測する

並行して性行動を検討する

雌ラットはあらかじめ卵巣を摘出し、実験二日前にエストロゲン、当日テスト3時間前にプロゲステロンを皮下投与し、発情期とする

並行して摂食量と体重変化を測定する

Ⅲ. 研究成果の刊行に関する一覧表

雑誌

著者名	タイトル	雑誌名	管・号・ページ	年
Takahashi M, Yoshida M, Inoue K, Morikawa T, Nishikawa A: Age-related susceptibility to induction of osteochondral and vascular lesions by semicarbazide hydrochloride in rats.	Age-related susceptibility to induction of osteochondral and vascular lesions by semicarbazide hydrochloride in rats.	*Tox. Pathol*	38(3):598-605	2010
Kawaguchi, M., Morohoshi, K., Imai H., Morita, M., Kato, N., and Himi, T.	Maternal exposure to isobutyl-paraben impairs social recognition in adult female rats	*Exp. Anim.*,	59(5), 631-5.	2010

Ⅳ. 研究成果の刊行物

Toxicologic Pathology, 38: 598-605, 2010

ISSN: 0192-6233 print / 1533-1601 online
DOI: 10.1177/0192623310368979

Age-related Susceptibility to Induction of Osteochondral and Vascular Lesions by Semicarbazide Hydrochloride in Rats

MIWA TAKAHASHI, MIDORI YOSHIDA, KAORU INOUE, TOMOMI MORIKAWA, AND AKIYOSHI NISHIKAWA

Division of Pathology, National Institute of Health Sciences, Setagaya-ku, Tokyo, Japan

ABSTRACT

To compare the susceptibility to toxicity of semicarbazide hydrochloride (SEM-HCl) between young and adult rats, 3- and 20-week-old female SD rats were given a diet containing SEM-HCl at 0, 500, or 1,000 ppm and 0 or 1,000 ppm, respectively, for 4 weeks. Half of the animals were then maintained on basal diet for a further 2 weeks as recovery groups. Only in young rats was deformation of the knee joints as well as thorax and tail observed at 500 and 1,000 ppm. Histopathologically, severe osteochondral lesions, such as disarrangement and thickening of the epiphyseal cartilage and deformation of articular cartilage, were observed, but the severity of these lesions became reduced during the recovery period. In adult rats, osteochondral lesions were relatively mild. Fissures in the cartilage matrix of the tibia were characteristic of adult rats, and in these, reduction of severity was not obvious in the recovery group. In the thoracic aorta, the appearance of elastic laminae was altered only in young rats in both the 4-week treatment and recovery groups. These results suggest that growing animals are more susceptible to toxicity of SEM-HCl than adults are. Effects and the induced lesions link to the developing stage of the target organs.

Keywords: semicarbazide hydrochloride; osteolathyrism; food contaminant; children.

INTRODUCTION

Semicarbazide (SEM), a metabolite of the banned veterinary antibiotic nitrofurazone, has been proposed as a marker for detection of nitrofurazone abuse (Effkemann and Feldhusen 2004; de la Calle and Anklam 2005). However, SEM has in fact been found in food in glass jars sealed with plastic gaskets manufactured using azodicarbonamide as a blowing agent, such as baby foods, fruit juices, jams, and conserves (European Food Safety Authority 2003; Stadler et al. 2004). Other sources of contamination, such as from food processing by hypochlorite treatment, have further been suggested (Hoenicke et al. 2004; Saari and Peltonen 2004). The possibility of exposure through various foods independent of nitrofurazone usage has thus raised concerns about the health risk of SEM.

SEM is also known to inhibit enzymes, such as lysyl oxidase, semicarbazide-sensitive amine oxidase (SSAO), and glutamic acid decarbocylase (Magyar, Mészáros, and Mátyus 2001; Dawson, Rinaldi, and Pöch 2002; Macedo et al. 2007). It thereby acts as an osteolathyrogen, inducing osteochondral and vascular lesions in young rats due to impaired cross-linking reactions of collagen and elastin through inhibition of lysyl oxidase or SSAO (Ramamurti and Taylor 1959; Langford et al. 1999; Dawson et al. 2002; Mercier et al. 2007). In addition, teratogenic effects such as induction of cleft palate and aortic aneurysms have been reported (Steffek, Verrusio, and Watkins 1972; de la Fuente del Rey 1986; Gong et al. 2006). SEM has weak genotoxicity *in vitro* but not *in vivo* (Parodi et al. 1981; Abramsson-Zetterberg and Svensson 2005; European Food Safety Authority 2005, Food Safety Commission 2007). In mice, SEM hydrochloride (SEM-HCl) at a high dose in the drinking water increased incidences of lung and blood vessel tumors that were commonly observed in untreated mice, suggesting that SEM-HCl might be only a weak carcinogen (Toth, Shumizu, and Erickson 1975). In rats, although no carcinogenic effects were observed after feeding a diet containing SEM-HCl at 500 and 1,000 ppm for 78 or 32 weeks (Weisburger et al. 1981), data for carcinogenicity remain insufficient for evaluation.

So far, the risk of adverse effects of SEM exposure to human beings has been considered low because there is a sufficient margin of exposure (Nestmann et al. 2005). However, intake of SEM for infants is estimated to be much higher than for adults because of high consumption of baby food in glass jars and the infants' small body weight (European Food Safety Authority 2005; Nestmann et al. 2005). Generally, developing bones and cartilage of infants and children have a different susceptibility from that of their adult counterparts (Schwenk et al. 2003), and previous studies of effects of other osteolathyrogens such as beta-aminopropionitrile (BAPN) and aminoacetonitrile hydrochloride indicated young animals to be more susceptible than adults (Morgan and Bellamy 1976; Davies and Schofield 1980). In man, although there has been no report of osteolathyrism caused by SEM, Haque et al. (1997) reported lesions induced by BAPN. Accordingly, because infants or children might be more vulnerable to SEM than adults, evaluation of toxic effects of SEM with the focus on age-related susceptibility is important for risk assessment in human health. In the present study, we therefore compared histopathological

Address correspondence to: Miwa Takahashi, DVM, PhD, Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; e-mail: mtakahashi@nihs.go.jp.

Abbreviations: BAPN, beta-aminopropionitrile; HE, hematoxylin and eosin; SEM, semicarbazide; SEM-HCl, semicarbazide hydrochloride; SSAO, semicarbazide-sensitive amine oxidase.

osteochondral and vascular lesions in young and adult female rats given SEM-HCl for 4 weeks. Reversibility of the induced lesions was also assessed.

Materials and Methods

Chemicals

SEM-HCl (CAS no. 563-41-7) was purchased from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan), as a white powder with a purity of 99.3% and well mixed at concentrations of 0, 500, or 1,000 ppm into a powdered basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan). More than 89% stability of the test compound was confirmed for up to 4 weeks of storage at 4°C and room temperature. Test diets were prepared every 2 weeks and stored at 4°C before use.

Animals and Treatments

Thirteen pregnant Crl:CD (SD) rats were obtained from Charles River Japan Inc. (Kanagawa, Japan) at gestational day 10. They were housed individually in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature 24°C ± 1°C, relative humidity 55% ± 5%, 12-hour light-dark cycle) with powdered basal diet (CRF-1) and tap water available ad libitum. At weaning, 30 female pups (210 or 22-day-old) were allocated to 3 groups, each consisting of 10 animals from different dams, and given diet containing 0, 500, or 1,000 ppm SEM-HCl for 4 weeks (young group). The dietary dose levels were determined as toxic levels in a 2-week dose-finding study conducted on the basis of a previous report (Weisburger et al. 1981). Dams were kept untreated for 5 weeks after weaning, and 12 animals showing no abnormalities at 20 weeks of age were divided into 2 groups and fed diet containing 0 or 1,000 ppm SEM-HCl for 4 weeks (adult group). Observations for mortality and clinical signs, including posture and gait abnormalities and deformation of 4 limbs, thorax, and tail, were conducted daily. Body weight and food consumption were recorded every week. After 4-weeks treatment, half of the animals from each group were euthanized by exsanguination from the abdominal aorta under deep anesthesia with ether. The remaining animals were maintained on basal diet for another 2 weeks as recovery groups and then subjected to necropsy in the same manner. The experimental protocol using animals was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Histopathological Examination

After macroscopic examination, all organs were removed and fixed in 10% neutral buffered formalin. For examination of osteochondral lesions, the nasal cavity, sternum, right femur, right tibia, left knee joint, right and left ankles, spine (cervical, thoracic, lumbar, and caudal vertebrae with corresponding spinal cord) were fixed in 10% neutral buffered formalin and then decalcified in EDTA solution at room temperature for a month. The tissues were then routinely processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin. Victoria blue staining was applied to three transverse sections of the descending thoracic aorta cut at 5-mm intervals to demonstrate elastic fibers. The number of elastic laminae of each section was counted.

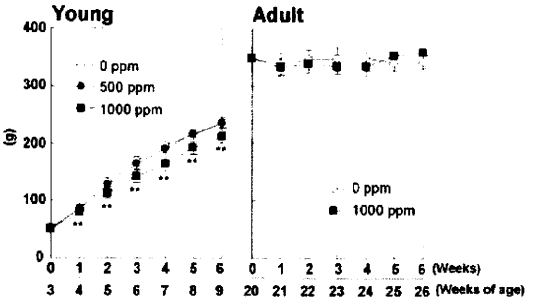


FIGURE 1.—Growth curves for young and adult rats given semicarbazide hydrochloride in diet for 4 weeks and then maintained on basal diet for 2 weeks as a recovery period. **Significantly different from the 0-ppm group at $p < 0.01$.

Statistical Analysis

Variance in data for body weights and food consumption of young rats was checked for homogeneity by Bartlett's procedure. If the variance was homogeneous, the data were assessed by one-way analysis of variance. If not, the Kruskal-Wallis test was applied. When statistically significant differences were detected, the Dunnett's multiple test was employed for comparison between the 0-ppm and treatment groups. In the adult group, the data for body weights and food consumption were analyzed by the Student's or Welch's *t*-test following a test for equal variance.

Results

In-Life Parameters

No deaths occurred during the experiment. Body weights in young groups increased constantly, and body weight gain at 1,000 ppm was significantly suppressed from week 1 (Fig. 1) throughout the study. In contrast, body weights of adult animals in both the 0- and 1,000-ppm groups stayed flat and were comparable between the two groups throughout the study. Data for food consumption and SEM-HCl intake are summarized in Table 1. Although the mean values for food consumption per animal in young and adult rats were similar, the mean value for food consumption per kilogram of body weight in young animals was nearly doubled that in adults. Accordingly, intake of SEM-HCl in young animals was also twice as much as that in adult rats.

Clinical Findings and Necropsy

The external abnormalities observed during the study are summarized in Table 2. Enlargement and deformation of the knee joints was apparent in young rats given 500 and 1,000

TABLE 1.—Food consumption and intake of SEM-HCl by young and adult rats.

Group	Dose (ppm)	No. of animals	Food consumption (g/animal/day)	Food consumption (g/kg BW/day)	Intake of SEM-HCl (mg/kg BW/day)
Young	0	10	14.8 ± 3.1[a]	105.3 ± 15.8	0.0 ± 0.0
	500	10	15.8 ± 2.9	117.0 ± 21.8	58.5 ± 10.9
	1,000	10	11.8 ± 1.5	99.9 ± 19.1	99.9 ± 19.1
Adult	0	6	16.9 ± 1.2	48.9 ± 3.1	0.0 ± 0.0
	1,000	6	15.2 ± 2.6	45.3 ± 7.3	45.3 ± 7.3

Abbreviations: BW, body weight; SEM-HCl, semicarbazide hydrochloride.
[a] Mean ± SD.

TABLE 2.—External clinical observations at necropsy in young and adult rats.

		Young						Adult			
		4-week dosing period			4 weeks + recovery			4-week dosing period		4 weeks + recovery	
Site	Findings	0 ppm	500 ppm	1,000 ppm	0 ppm	500 ppm	1,000 ppm	0 ppm	1,000 ppm	0 ppm	1,000 ppm
No. of animals examined		5	5	5	5	5	5	3	3	3	3
Hind limb	Enlargement and deformation of the knee joint	0	3	5	0	3	5	0	0	0	0
Thorax	Prominence	0	1	5	0	0	3	0	0	0	0
Tail	Stiff flexion	0	0	5	0	0	1	0	0	0	0

ppm from week 1. Tails in these groups exhibited stiff flexion from week 2, and prominence of the thorax was also found at week 4. Such changes remained after the 2-week withdrawal period, although the number of animals with the abnormalities and their severities were somewhat decreased. In adults, no external findings were found in either the 4-week treatment or recovery groups.

Histopathological Examination

Both in young and adult groups, histopathological lesions were mainly observed in the bones, cartilages, and aorta, and no treatment-associated changes were found in any other organs. The findings for histopathological examination of the bones and joints are summarized in Table 3.

In young rats, severe osteochondral lesions were observed in various sites in the body at both 500 and 1,000 ppm. As compared with the 0-ppm group (Fig. 2A and B), the epiphyseal plates at the proximal ends of the tibia were thickened in animals given the two doses at similar severities, and disarrangement of epiphyseal chondrocytes and degeneration of hypertrophic chondrocytes was also observed in the thickened cartilage plates (Fig. 2C and D). Staining intensity of the cartilage matrix in these groups was reduced. After the recovery period, thickening of the epiphyseal plates and the degeneration of hypertrophic chondrocytes had disappeared, and the severity of disarrangement of epiphyseal chondrocytes was reduced (Fig. 2E and F). The staining of the cartilage matrix had completely recovered to that seen in the control animals. The femurs exhibited similar histological lesions to the tibias with the same severity. In the sternum, disarrangement of epiphyseal chondrocytes was observed in young animals at 500 and 1,000 ppm in a dose-dependent manner, and fissures with increased connective tissue and bone deformation were evident at 1,000 ppm. After the recovery period, the severities of these lesions were reduced as in the case in the tibia. In addition, in young animals only, deformation and fissures of articular cartilage were observed in the knee joints, intervertebral joints (from cervical to caudal), and ankles at 500 and 1,000 ppm (Fig. 3). Although some lesions in the articular cartilage were decreased after the recovery period, such reduction was not apparent in the intervertebral joints.

In adult rats, the osteochondral lesions were relatively mild and limited to the femur and tibia. Disarrangement of epiphyseal chondrocytes was found in the femur and tibia but without thickening of the epiphyseal plate and degeneration of hypertrophic chondrocytes. Fissures in the cartilage matrix in the anterior areas of the tibias were characteristic in adult rats of the 1,000-ppm group, accompanied by an increase of connective tissues (Fig. 4C and D), and these persisted in the recovery group. There were no significant lesions in the sternum or articular cartilage in adult rats. In both young and adult groups, no treatment-related changes in cartilage of the nasal cavity, trachea and bronchi, or Achilles tendons were found. Most lesions in young animals at 500 ppm were clearly more severe than those in adult animals at 1,000 ppm.

Histopathological findings for the thoracic aorta are summarized in Table 4. Although the numbers of elastic laminae were unchanged, their edges became roughened only in young rats at 500 and 1,000 ppm, and the interlaminar spaces in

TABLE 3.—Histopathological findings for the bones and joints in young and adult rats.

Site	Findings	Young						Adult			
		4-week dosing period			4 weeks + recovery			4-week dosing period		4 weeks + recovery	
		0 ppm	500 ppm	1,000 ppm	0 ppm	500 ppm	1,000 ppm	0 ppm	1,000 ppm	0 ppm	1,000 ppm
No. of animals examined		5	5	5	5	5	5	3	3	3	3
Femur	Disarrangement of epiphyseal chondrocytes (+/++)[a]	0	5[b] (0/5)[c]	5 (0/5)	0	2 (2/0)	5 (5/0)	0	3 (2/1)	0	3 (3/0)
	Thickening of epiphyseal plate	0	5	5	0	0	0	0	0	0	0
	Degeneration of hypertrophic chondrocytes	0	5	5	0	0	0	0	0	0	0
Tibia	Disarrangement of epiphyseal chondrocytes (+/++)[a]	0	5 (0/5)	5 (0/5)	0	2 (2/0)	5 (5/0)	0	3 (0/3)	0	3 (1/2)
	Thickening of epiphyseal plates	0	5	5	0	0	0	0	0	0	0
	Degeneration of hypertrophic chondrocytes	0	5	5	0	0	0	0	0	0	0
	Fissures and increase of connective tissue in epiphyseal plate	0	0	0	0	0	0	0	3	0	3
Sternum	Disarrangement of epiphyseal chondrocytes (+/++)[a]	0	5 (5/0)	5 (0/5)	0	3 (3/0)	5 (3/2)	0	0	0	0
	Fissures of epiphyseal plates	0	0	5	0	0	2	0	0	0	0
	Increase of connective tissues	0	0	4	0	0	1	0	0	0	0
	Bone deformation	0	0	5	0	0	2	0	0	0	0
Knee joint	Deformation and fissures of articular cartilage	0	3	4	0	0	2	0	0	0	0
Vertebrae	Deformation and fissures of articular cartilage	0	5	5	0	5	5	0	0	0	0
Ankle	Deformation and fissures of articular cartilage	0	0	5	0	0	2	0	0	0	0

[a] Grade of change. +, partly in the epiphyseal plate; ++, mostly or wholly in the epiphyseal plate.
[b] Total number of animals with each finding.
[c] Number of animals with each grade.

treated groups had a rod or globular appearance, in contrast to the fibrillar appearance in the 0-ppm group (Fig. 5). These lesions were similarly found after the recovery period. In adult cases, histology of elastic laminae did not differ between the 0- and 1,000-ppm groups, after both the 4-week treatment and the recovery period.

DISCUSSION

In the present study, toxicologic effects of SEM-HCl were mainly observed in the bones, cartilage, and aorta, and histopathologic changes observed in young groups were basically consistent with the previous reports using rats during the growing period (Ramamurti and Taylor 1959; Langford et al. 1999; Mercier et al. 2007). While the target sites of SEM-HCl were similar in both young and adult groups, the lesions induced were much more diverse and serious in young animals. Young animals generally take more food per body weight during the growing period than their adult counterparts do, and during the present study, the intake of SEM-HCl per kilogram of body weight in young animals was about twice that in adult rats. However, even when comparing young animals at 500 ppm and adults at 1,000 ppm, the lesions in young animals were clearly more severe. Therefore, young rats are more susceptible to SEM-HCl than adults. This is in line with the fact that SEM impairs cross-linking reactions of collagen and elastin, which are essential for maturation of connective tissues, including bones and blood vessels (Ramamurti and Taylor 1959; Langford et al. 1999; Dawson, Rinaldi, and Pöch 2002; Mercier et al. 2007).

After the recovery period, the severities of osteochondral lesions were markedly reduced in young animals, especially in the long bones growing up rapidly, such as the femur and tibia. On the other hand, histological changes of the aorta were similarly observed in both 4-week treatment and recovery groups. In SD rats, the growth of bones is reported to reach equilibrium by approximately 20 weeks of age (Horton et al. 2008). It is known that in the aorta, elastic laminae are formed by 4 weeks of age and mature by 8 weeks of age, and turnover of elastin of the aortic wall is extremely slow in adult animals (Davis 1995; Katsuda et al. 2002). Therefore, the present results suggest that the induced lesions in the bones and cartilages are reversible during the period when they grow vigorously, even if these effects of SEM-HCl are more severe. In contrast, while the aorta is affected by SEM-HCl during the period of formation of elastic laminae, the lesions are irreparable after completing maturation of elastic laminae.

Reduction of staining intensity of the cartilage matrix is considered to reflect the decreased polymerization of cartilage matrix or absence of mineralization (Ramamurti and Taylor 1959; Maranghi et al. 2009), indicating alteration of matrix properties caused by inhibition of cross-linking reactions of collagen. Because fissures specifically occurred in the sites subject to body weight loading such as the anterior area of the

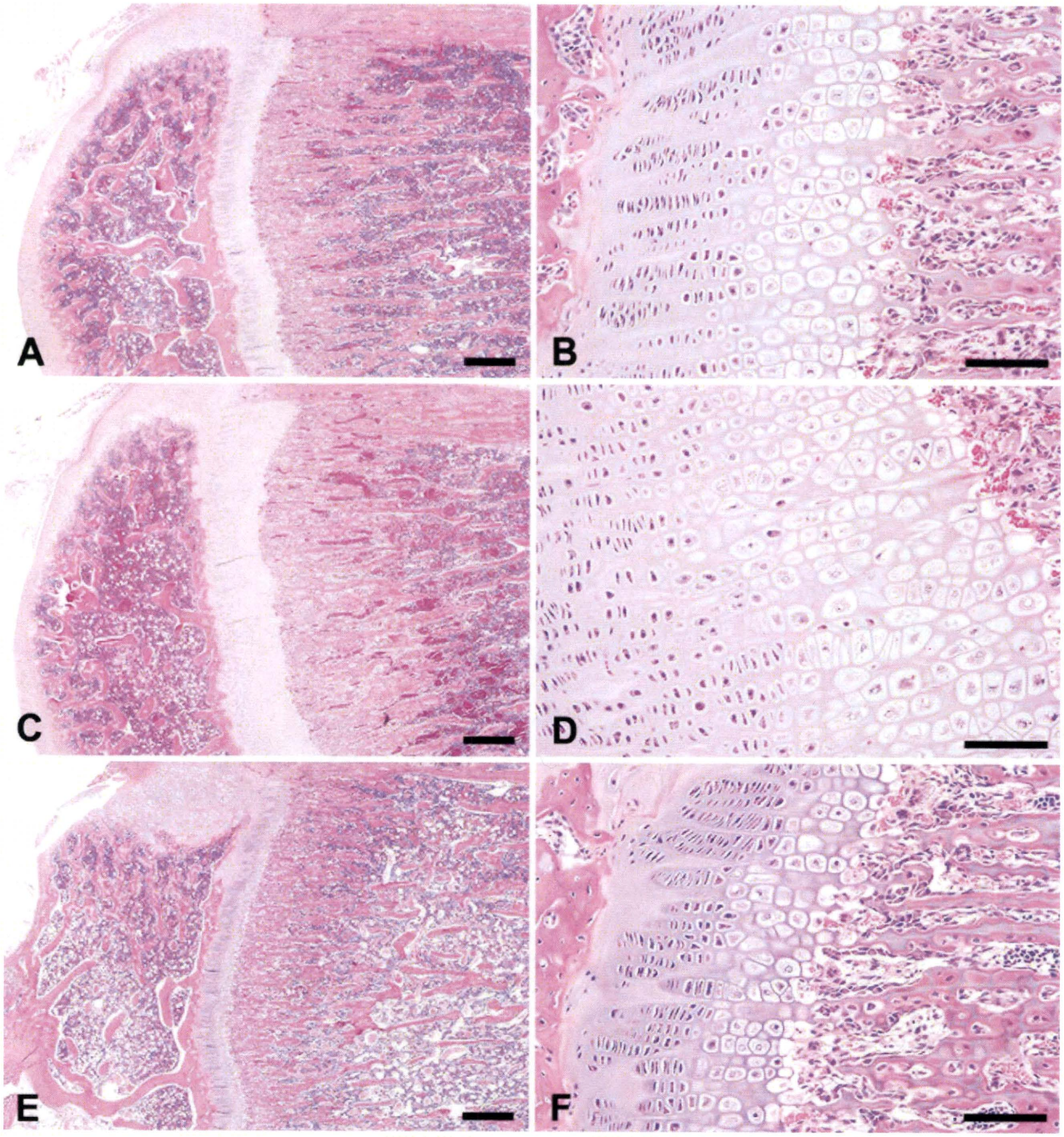


FIGURE 2.—Histopathology of the tibia of young rats. Normal proximal end of a tibia (A) and high magnification of an epiphyseal plate from the 0-ppm group (B). In a rat given 1,000 ppm for 4 weeks, thickening of the epiphyseal plate and reduction of staining intensity of the cartilage matrix are apparent (C). At high power, disarrangement and degeneration of hypertrophic chondrocytes are evident in the thickened cartilage plate (D). After the recovery period, the thickness and staining intensity of the epiphyseal plate are normal (E), and the severity of disarrangement of epiphyseal chondrocytes is reduced (F). Hematoxylin and eosin stain. Bars = 500 μm (A, C, E), 100 μm (B, D, F).

tibia, alteration of matrix properties might result in reduction in the strength of cartilage.

Taking the present results and the literature together, SEM-HCl induces characteristic histopathological lesions of bones, cartilage, and the elastic laminae of arteries. Although the intake of SEM-HCl per kilogram of body weight in young animals was about twice that in adult rats, the lesions in young animals at 500 ppm were clearly of greater intensity than in

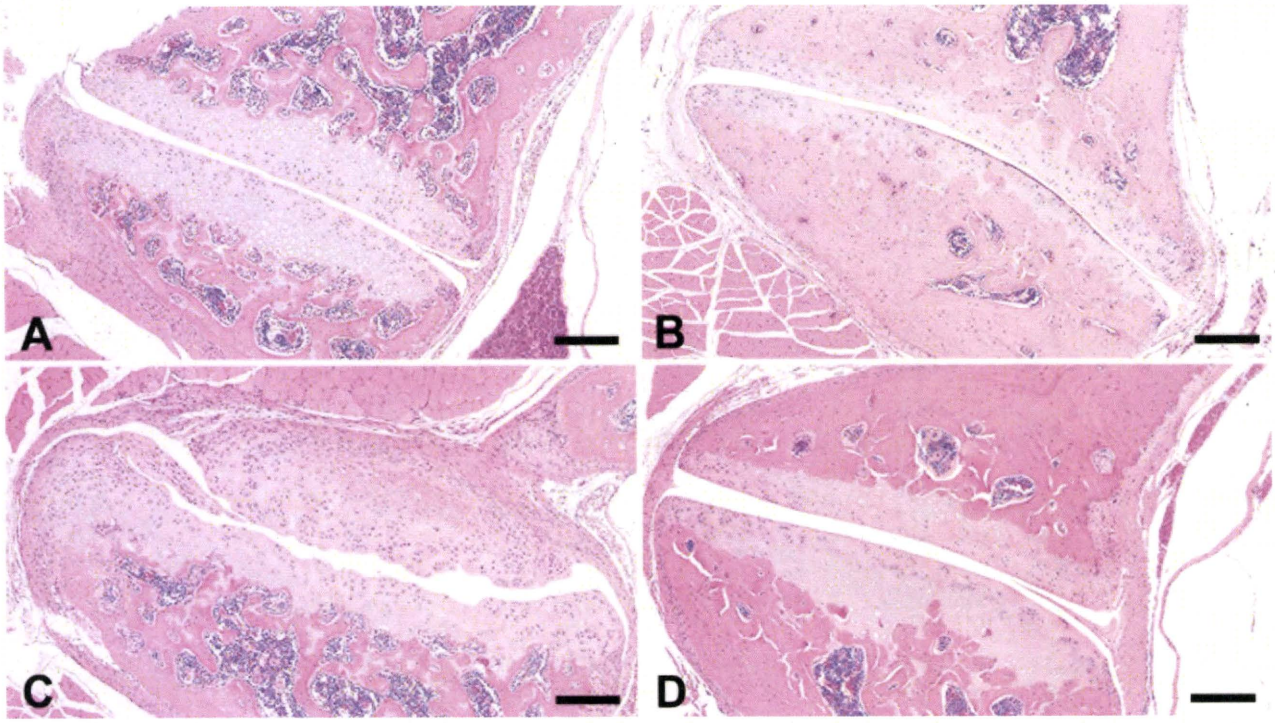


FIGURE 3.—Histopathology of the cervical vertebrae of young and adult rats. Normal intervertebral joints of young (A) and adult (B) rats of the 0-ppm group. In a young animal receiving 1,000 ppm for 4 weeks, deformation and fissures of articular cartilage is apparent (C), while there are no significant lesions in an adult case (D). Hematoxylin and eosin stain. All bars = 200 μm.

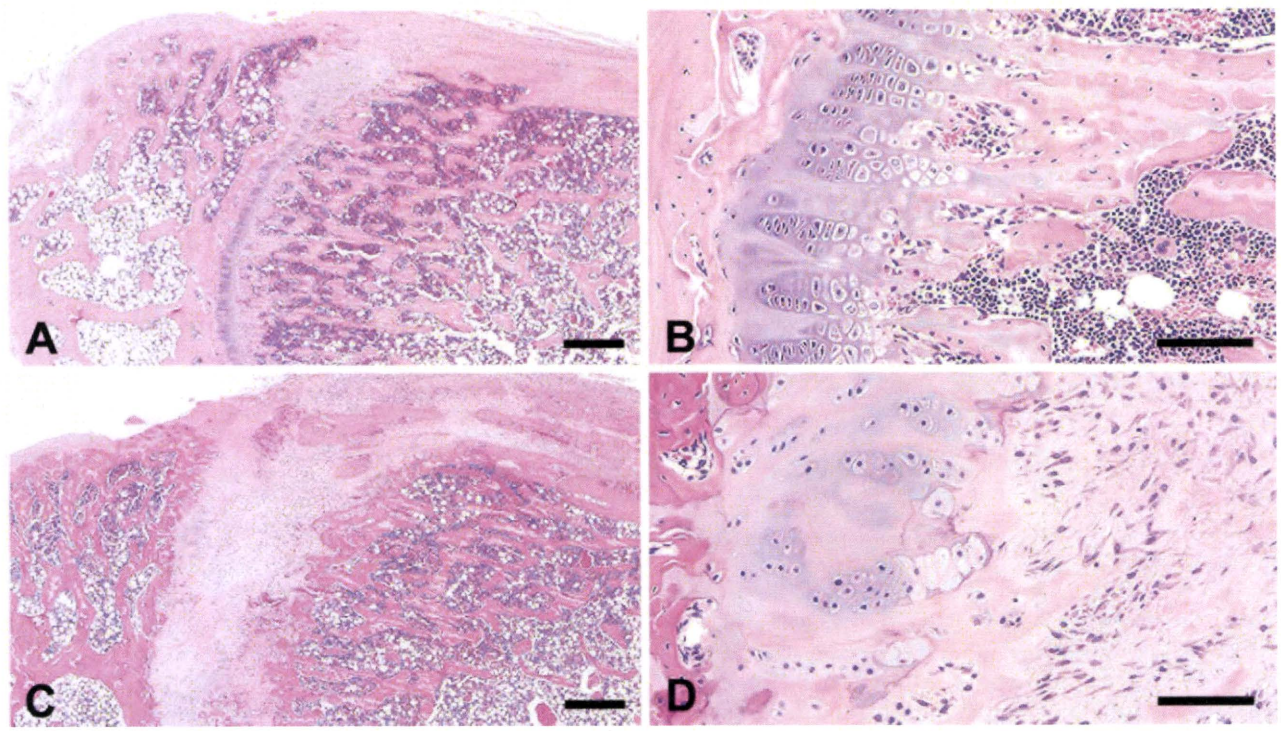


FIGURE 4.—Histopathology of the tibia of adult rats. Normal proximal end of a tibia (A) and high magnification of its epiphyseal plate from the 0-ppm group (B). In the 1,000-ppm group, disarrangement of epiphyseal chondrocytes and the fissures with increased connective tissues are found in the anterior area (C, D). Hematoxylin and eosin stain. Bars = 500 μm (A, C), 100 μm (B, D).

TABLE 4.—Histopathological findings of the thoracic aorta in young and adult rats.

	Young						Adult			
	4-week dosing period			4 weeks + recovery			4-week dosing period		4 weeks + recovery	
Findings	0 ppm	500 ppm	1,000 ppm	0 ppm	500 ppm	1,000 ppm	0 ppm	1,000 ppm	0 ppm	1,000 ppm
No. of animals examined	5	5	5	5	5	5	3	3	3	3
No. of elastic laminae	9.1	9.0	8.5	9.1	9.1	9.5	9.0	9.3	8.7	9.1
Edges of elastic fibers										
Smooth	5	0	0	5	0	0	3	3	3	3
Rough	0	5	5	0	5	5	0	0	0	0
Interlaminar spaces										
Fibrillar	5	0	0	5	0	0	3	3	3	3
Rod to globular	0	5	5	0	5	5	0	0	0	0

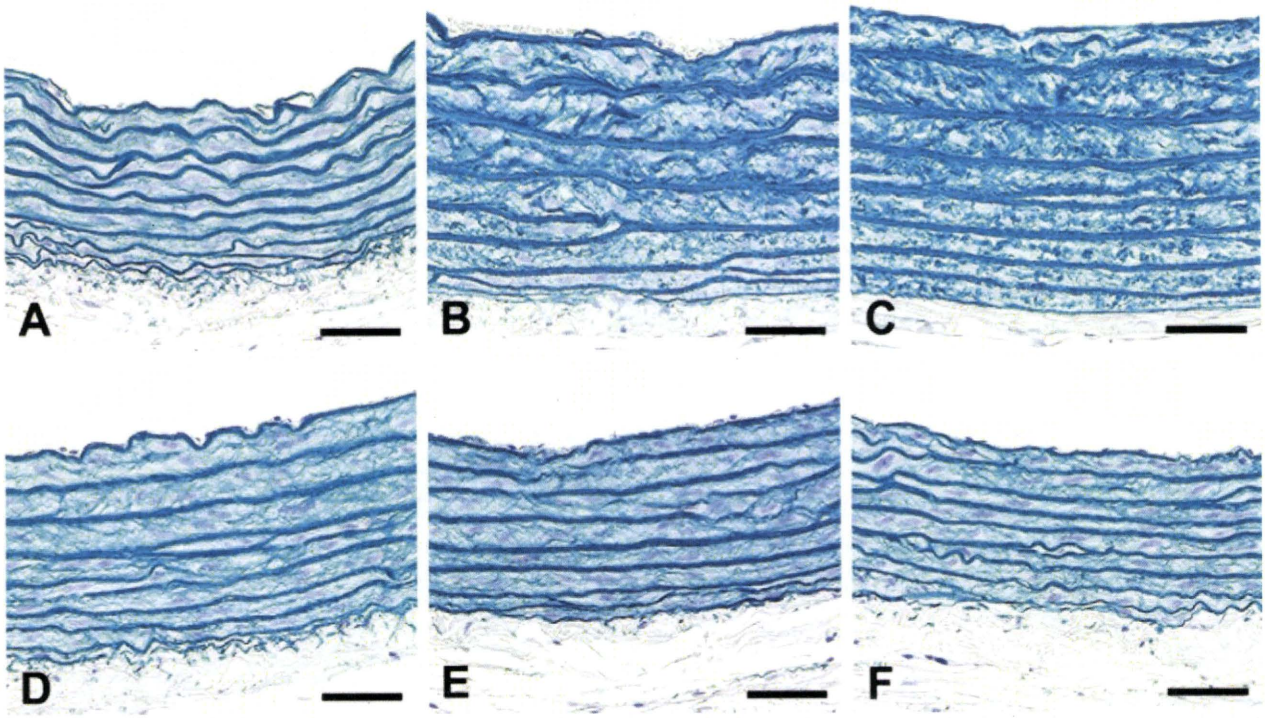


FIGURE 5.—Transverse sections of the thoracic aorta of young and adult rats. As compared with the 0-ppm group (A), elastic laminae in the 1,000-ppm group show rough edges in the young group (B), and the interlaminar spaces in treated groups have a rod or globular appearance, in contrast to the fibrillar appearance in the 0-ppm group. These changes were similarly found after the recovery period (C). In adult cases, the histology of elastic laminae was unchanged between 0 ppm (D) and 1,000 ppm in both the 4-week treatment (E) and recovery groups (F). Victoria blue stain. All bars = 50 μm.

adult animals at 1,000 ppm. Therefore, animals with growing processes are considered to be more susceptible than adults, and the toxicologic effects and induced lesions appear to depend on the growing stage of the target organs. For risk assessment of SEM exposure in humans, it is thus important to take into account the development stage of the bones and blood vessels.

ACKNOWLEDGMENT

We thank Miss Ayako Kaneko for technical assistance in conducting the animal study.

REFERENCES

Abramsson-Zetterberg, L., and Svensson, K. (2005). Semicarbazide is not genotoxic in the flow cytometry-based micronucleus assay in vivo. *Toxicol Lett* **155**, 211–17.

Davies, I., and Schofield, J. D. (1980). Connective tissue ageing: the influence of a lathyrogen (beta-aminopropionitrile) on the life span of female C57BL/Icrfat mice. *Exp Gerontol* **15**, 487–94.

Davis, E. C. (1995). Elastic lamina growth in the developing mouse aorta. *J Histochem Cytochem* **43**, 1115–23.

Dawson, D. A., Rinaldi, A. C., and Pöch, G. (2002). Biochemical and toxicological evaluation of agent-cofactor reactivity as a mechanism of action for osteolathyrism. *Toxicology* **177**, 267–84.

de la Calle, M. B., and Anklam, E. (2005). Semicarbazide: occurrence in food products and state-of-the-art in analytical methods used for its determination. *Anal Bioanal Chem* **382**, 968–77.

de la Fuente del Rey, M. (1986). Teratogenic effect of semicarbazide in Wistar rats. *Biol Neonate* **49**, 150–57.

Effkemann, S., and Feldhusen, F., (2004). Triple-quadrupole LC-MS-MS for quantitative determination of nitrofuran metabolites in complex food matrixes. *Anal Bioanal Chem* **378**, 842–44.

European Food Safety Authority. (2003). Statement of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact With Food Updating the Advice Available on Semicarbazide in Packaged Foods. Adopted October 1, 2003.

European Food Safety Authority. (2005). Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request made from the commission related to semicarbazide in food. *EFSA Journal* **219**, 1–36.

Food Safety Commission. (2007). The 68th expert committee of veterinary medicines [in Japanese]. http://www.fsc.go.jp/senmon/doubutu/d-dai68/doubutu68-siryou2.pdf

Gong, B., Trent, M. B., Srivastava, D., and Boor, P. J. (2006). Chemical-induced, nonlethal, developmental model of dissecting aortic aneurysm. *Birth Defects Res A Clin Mol Teratol* **76**, 29–38.

Haque, A., Hossain, M., Lambein, F., and Bell, E. A. (1997). Evidence of osteolathyrism among patients suffering from neurolathyrism in Bangladesh. *Nat Toxins* **5**, 43–46.

Hoenicke, K., Gatermann, R., Hartig, L., Mandix, M., and Otte, S. (2004). Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurazone abuse? *Food Addit Contam* **21**, 526–37.

Horton, J. A., Bariteau, J. T., Loomis, R. M., Strauss, J. A., and Damron, T. A. (2008). Ontogeny of skeletal maturation in the juvenile rat. *Anat Rec (Hoboken)* **291**, 283–92.

Katsuda, S., Waki, H., Yamasaki, M., Nagayama, T., O-Ishi, H., Katahira, K., Machida, N., Hasegawa, M., and Shimizu, T. (2002). Postnatal changes in the rheological properties of the aorta in Sprague-Dawley rats. *Exp Anim* **51**, 83–93.

Langford, S. D., Trent, M. B., Balakumaran, A., and Boor, P. J. (1999). Developmental vasculotoxicity associated with inhibition of semicarbazide-sensitive amine oxidase. *Toxicol Appl Pharmacol* **155**, 237–44.

Macedo, C. E., Martinez, R. C., Albrechet-Souza, L., Molina, V. A. and Brandão, M. L. (2007). 5-HT2- and D1-mechanisms of the basolateral nucleus of the amygdala enhance conditioned fear and impair unconditioned fear. *Behav Brain Res* **177**, 100–8.

Magyar, K., Mészáros, Z., and Mátyus, P. (2001). Semicarbazide-sensitive amine oxidase: its physiological significance. *Pure Appl Chem* **73**, 1393–400.

Maranghi, F., Tassinari, R., Lagatta, V., Moracci, G., Macrì, C., Eusepi, A., Di Virgilio, A., Scattoni, M. L., and Calamandrei, G. (2009). Effects of the food contaminant semicarbazide following oral administration in juvenile Sprague-Dawley rats. *Food Chem Toxicol* **47**, 472–29.

Mercier, N., El Hadri, K., Osborne-Pellegrin, M., Nehme, J., Perret, C., Labat, C., Regnault, V., Lamazière, J. M., Challande, P., Lacolley, P., and Fève, B. (2007). Modifications of arterial phenotype in response to amine oxidase inhibition by semicarbazide. *Hypertension* **50**, 234–41.

Morgan, A. J., and Bellamy, D. (1976). Effect of a lathyrogen (aminoacetonitrile hydrochloride) on aortic physiosclerosis and skeletal mineralization in growing rats of different age. *Age Ageing* **5**, 155–63.

Nestmann, E. R., Lynch, B. S., Musa-Veloso, K., Goodfellow, G. H., Cheng, E., Haighton, L. A., and Lee-Brotherton, V. M. (2005). Safety assessment and risk-benefit analysis of the use of azodicarbonamide in baby food jar closure technology: putting trace levels of semicarbazide exposure into perspective—a review. *Food Addit Contam* **22**, 875–91.

Parodi, S., De Flora, S., Cavanna, M., Pino, A., Robbiano, L., Bennicelli, C., and Brambilla, G. (1981). DNA-damaging activity in vivo and bacterial mutagenicity of sixteen hydrazine derivatives as related quantitatively to their carcinogenicity. *Cancer Res* **41**, 1469–82.

Ramamurti, P., and Taylor, H. E. (1959). Skeletal lesions produced by semicarbazide and experimental analysis of the action of lathyrogenic compounds. *J Bone Joint Surg Br* **41-B**, 590–99.

Saari, L., and Peltonen, K. (2004). Novel source of semicarbazide: levels of semicarbazide in cooked crayfish samples determined by LC/MS/MS. *Food Addit Contam* **21**, 825–32.

Schwenk, M., Gundert-Remy, U., Heinemeyer, G., Olejniczak, K., Stahlmann, R., Kaufmann, W., Bolt, H. M., Greim, H., von Keutz, E., and Gelbke, H. P. (2003). Children as a sensitive subgroup and their role in regulatory toxicology: DGPT workshop report. *Arch Toxicol* **77**, 2–6.

Stadler, R. H., Mottier, P., Guy, P., Gremaud, E., Varga, N., Lalljie, S., Whitaker, R., Kintscher, J., Dudler, V., Read, W. A., and Castle, L. (2004). Semicarbazide is a minor thermal decomposition product of azodicarbonamide used in the gaskets of certain food jars. *Analyst* **129**, 276–81.

Steffek, A. J., Verrusio, A. C., and Watkins, C. A. (1972). Cleft palate in rodents after maternal treatment with various lathyrogenic agents. *Teratology* **5**, 33–38.

Toth, B., Shumizu, H., and Erickson, J. (1975). Carbamylhydrazine hydrochloride as a lung and blood vessel tumour inducer in Swiss mice. *Eur J Cancer* **11**, 17–22.

Weisburger, E. K., Ulland, B. M., Nam, J., Gart, J. J., and Weisburger, J. H. (1981). Carcinogenicity tests of certain environmental and industrial chemicals. *J Natl Cancer Inst* **67**, 75–88.

Exp. Anim. 59(5), 631–635, 2010

—Note—

Maternal Exposure to Isobutyl-Paraben Impairs Social Recognition in Adult Female Rats

Maiko KAWAGUCHI[1)], Kaori MOROHOSHI[2,8)], Hideki IMAI[3)], Masatoshi MORITA[4,5)], Nobumasa KATO[6,7)], and Toshiyuki HIMI[1)]

[1)]Faculty of Pharmacy and Research Institute of Pharmaceutical Science, Musashino University, 1–1–20 Shinmachi, Nishi-tokyo, Tokyo 202-8585, [2)]Biological Risk Assessment Section, Environmental Health Sciences Division, National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba, Ibaraki 305-8506, [3)]Fuculty of Nursing at Higashigaoka, Tokyo Healthcare University, 2–5–23 Higashigaoka, Meguro, Tokyo 152-8588, [4)]Research Center for Environmental Risk, National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba, Ibaraki 305-8506, [5)]Department of Bioresources, Faculty of Agriculture, Ehime University, 3–5–7 Tarumi, Matsuyama, Ehime 790-8566, [6)]CREST, [7)]Department of Psychiatry, Showa University School of Medicine, 6–11–11 Kitakarasuyama, Setagaya, Tokyo 157-8577, and [8)]Present address: Mitsubishi Chemical Medience Corporation, Medi-Chem Business Segment, Toxicological Science Division, Yokohama Laboratory, 1000 Kamoshida-cho, Aoba, Yokohama, Kanagawa 227-0033, Japan

Abstract: Isobutyl-paraben (IBP), a widely used preservative, exhibits estrogenic activity. We analyzed the effects of exposure to IBP during gestation and lactation *via* dam on social recognition behavior in ovariectomized offspring of Sprague-Dawley rats. Offspring were ovariectomized at 7 weeks of age, and were used in a social recognition test at 16 weeks of age. Each offspring was exposed to a novel ovariectomized rat four times and to a second novel rat in a fifth exposure. We counted the investigations by offspring of intruder rats. The IBP-exposed rats showed impaired social behavior compared with controls. These data imply that early exposure to IBP may have an effect on adult social behavior, which is reported to be an autism spectrum disorders in humans.

Key words: isobutyl-paraben, rat, social recognition

Autism spectrum disorders (ASDs) comprise a range of behavioral phenotypes including impaired social recognition, communication and imagination [1]. The Centers for Disease Control and Prevention indicates a dramatically increased prevalence of identified ASDs among children in the United States [29]. For example, the average prevalence of ASDs among children aged 8 years increased by 57% across 10 locations in the United States from 2002 to 2006. While genetic factors are clearly important, [23, 31] these increasing rates of ASDs suggest that environmental factors are involved in causing this developmental disorder [11, 14, 30, 39].

Exposure to synthetic chemicals through air, water and food is unavoidable in the modern way of life. Synthetic chemicals that mimic or inhibit the action of gonadal steroid hormones are referred to as endocrine-disrupting chemicals [8]. Gonadal steroid hormones are required for development during gestation and the early

(Received 30 April 2010 / Accepted 27 June 2010)

Address corresponding: T. Himi, Faculty of Pharmacy and Research Institute of Pharmaceutical Science, Musashino University, 1–1–20 Shinmachi, Nishi-tokyo, Tokyo 202-8585, Japan

postnatal period, a time when numerous systems including the central nervous system are vulnerable to endocrine-disrupting chemicals. Early exposure to estrogenic chemicals may alter brain development and subsequent social behaviors, since early exposure to estrogenic chemicals alters monogamous behavior in the female pine vole [12], and alters mother-infant interactions in monkeys [26]. To date, however, the effect of early exposure to estrogenic chemicals on adult social recognition of animals of the same sex has not been reported.

Maternal glucocorticoid changes due to environmental factors may be involved in the manifestation of ASDs, because stressful life events during pregnancy correlate with the prevalence rates of ASDs in humans [2, 21, 36]. In addition, maternal glucocorticoids modulate the developing hypothalamic-pituitary-adrenal axis (HPA-axis) and social or maladaptive behavior associated with ASDs-like behaviors in rodents [10, 15, 22, 25, 37, 38] and primates [7, 32, 33].

Parabens are widely-used as preservatives in foods, cosmetics and pharmaceutical products [5, 9, 28], and have been studied to examine their estrogenic activity *in vitro* and *in vivo* [13]. Isobutyl-paraben (IBP) shows a comparatively high potency of estrogenic activity among parabens [13, 24]. In this study, to clarify whether early exposure to estrogenic chemicals predisposes to autistic behaviors, we analyzed the effect of maternal exposure to IBP on social recognition in adult offspring. In addition, we analyzed female offspring, because the major effect of maternal adrenalectomy on maladaptive behavior occurs in female offspring [38], and we previously reported that maternal IBP exposure decreased plasma corticosterone levels in dams [19]. Furthermore, IBP exposure may change estrogen susceptible systems, such as social recognition of female offspring, because we previously elucidated that maternal IBP exposure increased uterine sensitivity to estrogen in adult female offspring [19].

Sprague-Dawley rats were obtained from Charles River Laboratories Japan, Inc. (Ibaraki, Japan), and were maintained under conditions of controlled lighting (lights on, 07.00–19.00), temperature (22.5 ± 0.5°C) and humidity (55 ± 10%). The rats were given *ad libitum* access to food (CE2; CLEA Japan, Inc., Tokyo, Japan) and distilled water from glass bottles. Adult rats and nursing dams with pups were housed in stainless steel cages lined with paper bedding (Paper Clean; Japan SLC, Inc., Shizuoka, Japan). Animals were maintained and used according to the guidelines of the Musashino University Animal Care and Use Committee and the National Institute for Environmental Studies Animal Care and Use Committee.

Thirteen-week-old virgin female rats were paired with 11-week-old male rats from the evening of proestrus to the morning of estrus. The stage of the estrous cycle was determined based on vaginal cytology. Mating was verified by the presence of sperm in vaginal smears (gestational day 0; GD0), and when sperm was found the females were separated from the males. The pups were counted on postnatal day (PD) 3, were culled to four males and four females per litter, and were kept with their respective dams until weaning on PD21 (the day of birth was designated PD0). Three weeks before the mating, under ether anesthesia, female rats were implanted (under the skin of the flank region) with a 20-mm-long Silastic capsule, 2 mm *i.d.*, 3 mm *o.d.* (Kaneka Medix Co., Osaka, Japan) that was either filled with IBP (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) or empty (control). We prevopisly showed that Silastic capsules filled with IBP release about 4.36 mg/l/day IBP into 37°C saline [19]. At 7 weeks of age, female offspring were gonadectomized to remove the effects of inter-animal variability in circulating hormone levels.

Female offspring of treated and untreated dams were subjected to a social recognition test at the age of 16 weeks. Daily, for 3 days before the social recognition test, the rats were individually placed for 10 min in a square open-field apparatus constructed of vinyl chloride walls (50 × 50 × 50 cm) with paved polyethylene paper on the floor, and allowed to acclimate to the environment. On the fourth day, an unfamiliar ovariectomized 16-week-old female intruder was placed in the field for 60 s, and we counted the amount of time and the frequency with which the test animals spent investigating the intruder. Interaction was defined as sniffing of the intruders by the rat being tested. The tested rats were removed and then returned to the field after a 10-min interval. The rats were repeatedly tested for four additional trials (inter-trial interval, 10 min). On the fifth trial, a novel,

ovariectomized, 16-week-old intruder was placed into the field. During the 10-min interval between the trials, the apparatus was cleaned with 70% aqueous ethanol and the paving papers were replaced unless otherwise stated.

All results are expressed as mean ± SEM. Two-way analysis of variance (ANOVA) for repeated measures was applied to examine the effects of IBP exposure on the social recognition behavior (n=6 control litters and n=5 IBP-exposed litters, with one female offspring per litter randomly). If ANOVA showed significant interactions, Fisher's *post-hoc* tests were applied to search for significant differences among the groups. If ANOVA did not show significant interactions, the Tukey-Kramer test, which can be used in the absence of significant ANOVA results was performed. Differences were considered significant when the P value was below 0.05. All analysis were performed using StatView 5.0J software (SAS, Inc., Cary, NC, USA) for Microsoft Windows.

Rats treated with IBP showed impaired social recognition. Two-way ANOVA revealed significant interactions between treatment and test. In the control group, the social response decreased through trials 1–4 (with repeated presentation of the same intruder rat) but increased again during the fifth trial when presented with a novel rat (Fig. 1a). On the other hand, IBP-exposed rats did not show a change in frequency of social interaction toward the repeatedly presented rat and the subsequent novel rat (Fig. 1a). In the fourth trial, the social interest of control rats was significantly lower than that of IBP-exposed rats. The same pattern was observed for the time spent in social interaction: control rats spent progressively less time investigating the repeatedly introduced intruder, then more time interacting with the novel intruder. However, there were no significant differences between IBP-exposed and control rats (Fig. 1b).

The present study demonstrated that female rats given early exposure to IBP had impaired social recognition in adulthood. This provides the first evidence that developmental exposure to estrogenic chemicals *via* the placenta or milk can exert permanent or long-lasting influences on social recognition in offspring.

These results cannot be explained by a generalized difference in total activity or anxiety, because there are

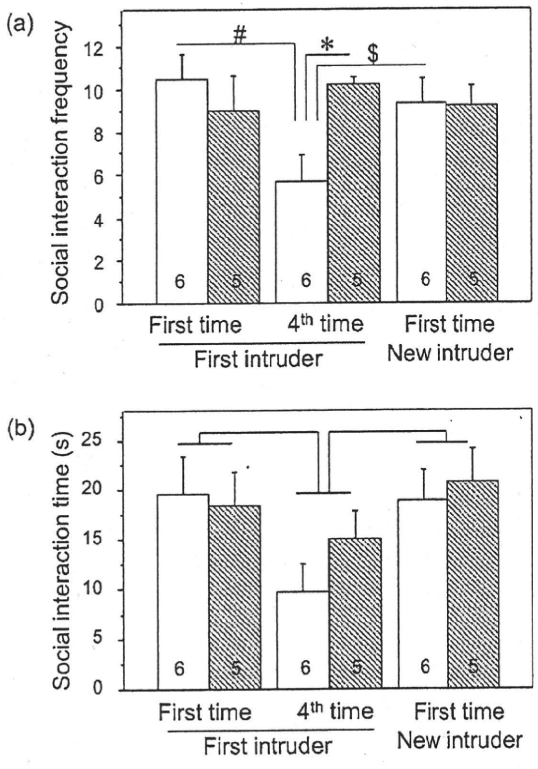


Fig. 1. Effects of developmental exposure to isobutyl-paraben (IBP) on social recognition performance. The following behaviors were quantitated: social interaction frequency (a) and social interaction time (b). The open and hatched columns show the control and IBP-exposed groups, respectively. Values shown are mean ± SEM. Numbers within the bars indicate the number of rats in each group. *: $P<0.05$ for control vs IBP-exposed rats on the fourth trial with the same intruder. #: $P<0.05$ for control rats on the first trial vs the fourth trial with the same intruder. $: $P<0.05$ for control rats on the fourth trial with the same intruder vs the first trial with a new intruder.

no effects of IBP exposure on general, anxiety and learning behaviors in female offspring [18]. In contrast, early exposure to IBP increases anxiety, and disturbs passive avoidance performance in male offspring [18]. Further elucidation is required for an understanding of the effects of IBP on the male offspring.

IBP treatment has been shown to decrease plasma corticosterone levels in dams, suggesting that IBP may inhibit endogenous estrogen activities [19] that stimulate the HPA-axis [4, 17]. Many studies have suggested that changes in maternal glucocorticoids levels may be in-