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Original Article

Carcinogenicity study of poly-*trans*-[(2-carboxyethyl) germasesquioxane] (Ge-132) in F344 rats

Katsuharu Iwadate[1], Yukari Yamaguchi[1], Mai Sasaki[1], Mikiya Nakatani[1], Yuko Doi[2], Norio Imai[2], Seiko Tamano[2] and Yorifumi Nishihori[1]

[1]Central Institute, New Drug Research Center, Inc., 452-1 Toiso, Eniwa-shi, Hokkaido 061-1405, Japan
[2]DIMS Institute of Medical Science, Inc., 64 Goura, Nishiazai, Azai-cho, Ichinomiya, Aichi 491-0113, Japan

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ABSTRACT — Ge-132 was administered to both sexes of F344 rats at dietary levels of 0, 0.6, 1.3, and 2.5% for 2 years. There were no adverse effects on survival rate, food consumption or hematology data, although diarrhea and body-weight retardation were observed in the male and female 2.5% groups. Significant increases of kidney and adrenal weights were noted in both male and female 2.5% group. Macroscopically, dilatation of the cecum was observed in both male and female 2.5% group, and enlargement of the adrenals was observed in the male of 2.5% group. Significantly higher incidences of benign or malignant pheochromocytoma were observed in the male 1.3% group and both male and female 2.5% groups. Significantly positive trends were noted in the incidences of kidney pelvic and papilla mineralization in both sexes and cortico-medullary junction in females. To investigate possible mechanisms underlying Ge-132-associated development of pheochromocytoma, male F344 rats were administered diets containing 0, 0.6, or 2.5% Ge-132 for 4 or 13 weeks. Although loose stools and increasing water consumption were observed in treated groups, there were no body-weight retardation. Significant elevation of inorganic phosphorus in the serum was found in the 2.5% group at week 13. Dilatation of the cecum and increased cecum weight were evident macroscopically in the 2.5% group. Significant elevation of Ki-67 positive ratio in adrenal medullary cells was also found in the 2.5% group. These data indicated that Ge-132 ingestion induced disturbance in calcium/phosphorus homeostasis, and secondarily induced the development of benign or malignant pheochromocytoma in rats. Such secondary pheochromocytomas are considered to be not relevant for human risk assessment.

Key words: Poly-*trans*-[(2-carboxyethyl)germasesquioxane], Carcinogenicity study, Rat, Pheochromocytoma, Hypercalcemia, Ge-132

INTRODUCTION

Poly-*trans*-[(2-carboxyethyl) germasesquioxane] (Ge-132) is a water-soluble organogermanium compound that was first synthesized at the Asai Germanium Research Institute Co., Ltd.; the chemical structure and physicochemical properties have been reported (Miyao and Tanaka, 1988; Tsutsui *et al*., 1976). Ge-132 has been used as an ingredient in various health foods and supplements available in Japan. Ge-132 is involved in numerous physiologic processes, including anti-tumor activity, induction of interferon-γ production, activation of natural killer cells and macrophages, and regulation of the immune response, and it can help prevent viral infection and relieve rheumatic symptoms (Akiba and Kakimoto, 1994; Aso *et al*., 1985; Aso *et al*., 1989; Arimori *et al*., 1990; Miyao and Tanaka, 1988). Ge-132, an organic germanium compound containing carbon-germanium (C-Ge)

Correspondence: Katsuharu Iwadate (E-mail: k-iwadate@ndrcenter.co.jp)

bonds and has been shown a lack of renal toxicity. In contrast, germanium dioxide (GeO_2), which is an inorganic germanium compound that does not contain C-Ge bonds, has been reported to cause vacuolar degeneration and granular depositions in renal distal tubules and irreversible chronic interstitial fibrosis in rats (Sanai *et al*., 1991a, 1991b). In metabolic studies of Ge-132, it has been reported that Ge-132 does not accumulated in renal tissue and is rapidly excreted from the kidney (Kagoshima *et al*., 1986a, 1986b; Kagoshima and Onishi, 1986; Li *et al*., 2017; Sanai *et al*., 1991b).

Loose stools, diarrhea, increased water consumption, and/or dilatation of the cecum were observed in rats (Sugiya *et al*., 1986a) and mice (Doi *et al.*, 2017) given Ge-132 at very high dietary levels, however, there were no histopathological alteration. It was suggested that these effects were related to increasing osmotic values (unpublished information from Asai Germanium Research Institute Co., Ltd.). Loose stools, diarrhea, and increased water consumption were observed in oral three-month toxicity studies, but not in intraperitoneal 3- and 6-month toxicity studies in rats (Sugiya *et al.*, 1986a, 1986b).

A series of safety studies of Ge-132 performed with Good Laboratory Practice (GLP) status at Asai Germanium Research Institute Co., Ltd. Disclosed no adverse effects with regard to acute, subacute, or chronic toxicity, teratogenicity, or mutagenicity (reverse mutations, chromosome aberrations, and rodent micronuclei) (unpublished data). A Ge-132 carcinogenicity study performed in rasH2 mice, which are sensitive to genotoxic and non-genotoxic human carcinogens, found no evidence of adverse effects associated with ingestion of Ge-132 (Doi *et al*., 2017).

The objective of the present investigation was to assess possible carcinogenicity of Ge-132 given 2 years in the diet to male and female F344 rats under GLP conditions.

MATERIALS AND METHODS

Experiment 1: Carcinogenicity and toxicokinetics study in rats

Regulation and compliance

This carcinogenicity study was performed in accordance with the "Act on Welfare and Management of Animals" (Act No. 105; October 1973) and the "Standards Relating to the Care and Management of Laboratory Animals" (Notice No. 6 of the Prime Minister's Office; March 27, 1980). The study was designed and performed in compliance with GLP principals as detailed in Standard for conducting nonclinical laboratory studies on safety of drugs, Japanese Ministry of Health and Welfare (Ordinance No. 21; March 26, 1997), and in accordance with "Guidelines for Toxicity Studies of Drugs (5) Carcinogenicity Study" (Yakushin 1 No. 24 of the Ministry of Health and Welfare, Japan, September 11, 1989), and "Guidance on Toxicokinetics: the Assessment of Systemic Exposure in Toxicity Studies" (Notification No. 443 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, Japan, July 2, 1996. This study was conducted at the Central Institute, New Drug Research Center, Inc. from July 13, 1998 to June 28, 2002.

Test Material

The Ge-132 had been synthesized from ingots of germanium, which purity was more than 99.9999% (Akiba and Kakimoto, 1994). Poly-*trans*-[(2-carboxyethyl)germasesquioxane] (Ge-132), which is a polymer with the rational formula $[(GeCH_2CH_2COOH)_2O_3]_n$ (Tsutsui *et al*., 1976), is an odorless and acidulous white crystalline powder, and is temperature, humidity and light stable (Miyao and Tanaka, 1988). The Ge-132 (Lot Nos.:0960528A and 0960529A) used in the present study was supplied by Asai Germanium Research Institute Co., Ltd. (Kawasaki, Japan).

Diet preparation and analysis

The basal diet used in the present study was commercial CRF-1 (Oriental Yeast Ltd., Tokyo, Japan). Diet preparation (pelleted) was performed by Oriental Yeast Ltd. (Tokyo, Japan) at monthly intervals: Ge-132 in the prepared diet was stable for 4 months at room temperature (information from the supplier). Content analyses were performed at approximately 3 months intervals during the course of the studies, and the dietary levels were confirmed to be within the acceptable range of the target level. The homogeneity (the first preparation) of the Ge-132-prepared diet was satisfactory.

The dietary levels of 0 (control), 0.6, 1.3, and 2.5% of Ge-132 were selected based on the following criteria. The highest dietary level of 2.5% Ge-132 was judged to be the maximum tolerated dose for the present 2-year carcinogenicity study based on a preliminary 13-week oral toxicity study in F344 rats (unpublished report from Asai Germanium Research Institute Co., Ltd.). The lowest diet level of 0.6%, which was converted to about 300 mg/kg body weight/day, is approximately 6 times the maximum clinical dose of 3,000 mg per person (about 50 mg/kg/day) of Ge-132 (information from the supplier).

Animals and husbandry

5-week-old male and female specific pathogen free

F344/DuCrj (F344) rats were obtained from Charles River Laboratories Japan Inc., Atsugi, Japan and consigned a 1-week acclimation and quarantine period. After confirmation of good health, the 6-week old animals were used for the present studies. Rats were individually housed in stainless steel hanging cages (W255 × D185 × H200 mm) in a dedicated animal facility maintained under standard conditions: room temperature, 22 ± 2°C; relative humidity, 55 ± 15%; ventilation, 13 to 17 air changes per hour; and a 12 hr light/dark cycle, 8:00 to 20:00. Control and test material containing diet, and Eniwa city tap water were available *ad libitum*.

Study design

Using a computerized stratified body weight technique, 506 rats were allocated to one control and 3 treated groups (50 males and 50 females/group) for the carcinogenicity study, and three treated satellite groups (9 males and 9 females/group) for the toxicokinetics study. In the carcinogenicity study, initial mean body weights in control and treated groups were approximately equal, and no significant differences were noted. After animal allocation, the remaining rats (26 males and 26 females) were excluded from the studies. The animals were given diets containing Ge-132 at doses of 0, 0.6, 1.3 and 2.5% for 104 weeks (carcinogenicity study), or at doses of 0.6, 1.3 and 2.5% for up to 30 weeks (toxicokinetics study). The selection of dietary levels of Ge-132 was based on the preliminary 13-week oral toxicity study in F344 rats noted above.

In the carcinogenicity study, the animals were observed daily and palpated weekly. Individual body weights were recorded weekly for the first 13 weeks and once every 4 weeks thereafter. Food consumption was measured over a one-day period at each weighing. Ge-132 intake was calculated from the nominal dietary level, food consumption, and body weight.

After 104 weeks, all surviving rats were euthanized and exsanguinated from the abdominal aorta. Hematologic analysis included erythrocyte count, hemoglobin concentration, hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and leukocyte and platelet count. Blood smears stained with Wright-Giemsa solution were prepared from all animals, either euthanized in a moribund condition or necropsied at termination. However, differential leukocyte count was performed to restrict to animals indicated hematologic disorder from the clinical or macroscopic observation.

Macroscopic examinations were made at necropsy after blood collection. The following organs were weighed and organ-to-body-weight ratios were determined: heart, spleen, lungs (including bronchi), liver, kidneys, brain, pituitary, thymus, adrenals, thyroids (including parathyroids), testes, ovaries, uterus, and salivary glands (submandibular and sublingual). Samples of these organs and of the skin, mammary gland, lymph nodes (submandibular, mesenteric), salivary gland (parotid), bone and bone marrow (sternum, femur), knee joint, larynx, trachea, tongue, esophagus, stomach (glandular, forestomach), small intestine (duodenum, jejunum, ileum), large intestine (cecum, colon, rectum), pancreas, urinary bladder, seminal vesicle, prostate, epididymides, vagina, eyes and optic nerve, spinal cord, thoracic aorta, Harder's glands, skeletal muscle, sciatic nerve, Zymbal's glands, extraorbital lachrymal gland, preputial/clitoral gland, nasal cavity and any other tissues of abnormal appearance were fixed in 10% neutral buffered formalin (excluded eyes and optic nerve, which were preserved in Davidson solution). A full histopathological examination was performed on hematoxylin eosin-stained tissue sections of the organs and the tissues listed above (except Zymbal's glands, extraorbital lacrimal gland, preputial/clitoral gland, nasal cavity which were examined only when a macroscopic abnormality was detected) for all animals. A peer review of the histopathology findings was conducted by a board-certified pathologist, Dr. Hijiri Iwata, LunaPath LLC, Hamamatsu, Japan.

Toxicokinetics (Determinations of Ge-132 in plasma)

Blood was collected from the tail veins of the rats used for toxicokinetics during week 2, week 5, and week 30. Collection was made at 7 time points, every 4 hours from 11:00 to the next 11:00, for the week 2 collection and at 2 time points with a 4-hr interval (11:00 and 15:00) for the week 5 and week 30 collection. Blood (0.1 mL at week 2; 0.2 mL at weeks 5 and 30) was collected from 3 male and 3 female rats per group at each time point at week 2, and from 5 male and 5 female rats per group at each time point at weeks 5 and 30. The remaining one male and one female rats per group were maintained as spare animals. Blood was collected into a heparinized micro blood collection tube, and plasma was obtained by centrifugation at 3,000 rpm for 15 min. Plasma was stored at approximately -20°C until analysis for Ge-132 concentration. The plasma Ge-132 concentrations (μg/mL) in each sample were determined by flameless atomic absorption spectrometry. The maximum plasma concentrations (C_{max}, μg/mL), the area under the plasma concentration-time curve ($AUC_{0\ to\ 24hr}$, μg•hr/mL), and time to maximum plasma concentrations (T_{max}) were determined using blood samples collected during week 2.

Statistical analyses

Statistical comparisons of numerical data from control and Ge-132-treated groups were assessed by the Bartlett method. If homogeneous, the data were analyzed by the one-way ANOVA, and if significant, the data were analyzed by the parametric Dunnett or Scheffé methods. When the data were heterogeneous, they were analyzed by the H test of the Kruskal-Wallis method, and if significant, the data were analyzed by the non-parametric Dunnett or Scheffé methods. Cumulative survival rates were analyzed using the Kaplan and Meier method, and dose-dependency was analyzed using the Cox-Mantel method. Since the cumulative survival rate was not different between the control and the Ge-132-treated groups, statistical analysis of tumor incidence was evaluated by the Fisher's exact probability test (one-tailed). A two-tailed Cochran-Armitage test was used to evaluate the dose-dependency. Steel's test (two-tailed) was employed for comparison of non-neoplastic lesions with different degrees of severity. P values of < 0.05 were considered significant.

Experiment 2: Investigation of mode of action for Ge-132-developed proliferating lesions of the adrenal medullary cell

Animal welfare

This study was approved by the Experimental Animal Committee of the DIMS Institute of Medical Science, Inc., and were performed in accordance with the "Act on Welfare and Management of Animals" (Act No. 105, October 1973), "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notice No. 88 of the Ministry of Environment, April 2006), "Basic policies for the conduct of animal experimentation in the Ministry of Health, Labor and Welfare" (Notice No. 0601005 of the Ministry of Health, Labor and Welfare, June 2006), "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June 2006), and the "Standards for Care and Use of Laboratory Animals of the DIMS Institute of Medical Science, Inc." (May 2, 2014). This study was performed at DIMS Institute of Medical Science, Inc. from September 11, 2014 to April 21, 2015.

Diet preparation

The Ge-132 (Lot No. 012531A) used in present study was supplied by Asai Germanium Research Institute Co., Ltd. (Kanagawa, Japan). Test material was incorporated into MF powdered diet (Oriental Yeast Ltd., Tokyo, Japan) to final levels of 0, 0.6 and 2.5% by weight Ge-132 and mixed with a blender, model HP-50M (Kantou Kongouki Kougyo, Inc., Tokyo, Japan) for 30 min. Treatment diets were shielded from the light and stored at room temperature. Diet preparations were carried out at 5-week intervals: Ge-132 is stable under these conditions for 5 weeks (Doi *et al*., 2017).

Animals and husbandry

5-week-old specific-pathogen free male F344/DuCrl-Crlj (F344) rats were obtained from Charles River Laboratories Japan Inc. (Atsugi, Japan) and consigned a 1-week acclimation and quarantine period. After conformation of good health, the 6-week old animals were used for the present study. Rats were housed in plastic cage (W257 × D426 × H200 mm: 2 rats/cage) on soft chip bedding (Hara Shouten, Inc., Aichi, Japan) in an environment-controlled room: room temperature, 21.4-23.6°C; relative humidity, 51-68%; ventilation, more than 10 times/hr; and a 12 hr light/dark cycle, 7:00 to 19:00. Control and test material containing diets and Ichinomiya city tap water were available *ad libitum*.

Study design

Using a randomized block design, so that the weight distribution was similar and the initial mean body weights were approximately equal, 88 rats were allocated to one control and two treatment groups (26 rats/group). After animal allocation, the remaining 10 rats were excluded from the study. The animals were administered diet containing Ge-132 at doses of 0 (control), 0.6 and 2.5% for 4 and 13weeks. The selection of dietary levels of Ge-132 was based on the carcinogenicity study, experiment 1, described above. The animals were observed daily and individual body weights were measured weekly. Food consumption and water consumption were measured over a 2-day period before each weighing.

At the end of weeks 4 and 13, 10 rats/group were euthanized. Blood samples were collected via the abdominal aorta, and serum electrolytes (calcium, inorganic phosphorus, sodium, potassium, and chlorine) were determined using a Hitachi-Biochemical Automatic Analyzer 7070 (Hitachi Ltd., Tokyo, Japan). Macroscopic examinations were made at necropsy after blood collection. The following organs were weighed and organ-to-body-weight ratios determined: adrenals, cecum (filled and empty), liver, kidneys, pituitary, and thyroids (including parathyroid). Organs were fixed in 10% buffered formalin. A histopathological examination was performed on hematoxylin and eosin-stained tissue sections. In addition, adrenals from all of the rats euthanized at 4 and 13 weeks were examined by Ki-67 immunohistochemistry. At least 2,000 medullary cell nuclei were counted in each adrenal; positive ratios were calculated as the percentages

of cells positive for Ki-67.

The other 6 rats were used for urine analysis. Rats were fasted, but not deprived of drinking water, during the 24-hr urine collecting period at weeks 4 and 13. Urine volumes were measured and levels of urinary electrolytes (calcium, inorganic phosphorus, sodium, potassium and chloride) were determined. After urine collection at week 13, animals were euthanized, but not used for postmortem examination.

Statistical analyses

Statistical comparisons of numerical data from the control and Ge-132-treated groups were assessed by the Bartlett method. If homogeneous, the data were analyzed using the parametric Dunnet method (two-tailed), and if not, they were analyzed using the non-parametric Steel's method (two-tailed). The significance of differences in incidences of findings from gross pathology and histopathological data were analyzed using the Fisher's exact probability test (one-tailed). The Wilcoxon test (two-tailed) was employed for comparison of different degrees of histopathological change. P values of < 0.05 were considered significant.

RESULTS

Experiment 1: Carcinogenicity study

Loose stools and diarrhea were observed in both sexes of the treated groups in a dose-dependent manner during the course of the study (data not shown). The incidence of these clinical signs was higher in males than in females. However, there were no significant differences in mortality between controls and treated animals during the course of the study. At the end of the experiment, the survival rates of rats fed 0, 0.6, 1.3 and 2.5% Ge-132 were 54, 74, 66, and 64%, respectively, for males and 74, 74, 64, and 70%, respectively, for females (Fig. 1).

Significant lower body weight, or a tendency toward lower body weight were observed in the male 2.5% Ge-132 group from week 4 to termination, and in the female 2.5% group from week 66 to termination. No body weight retardation was found in the rats receiving

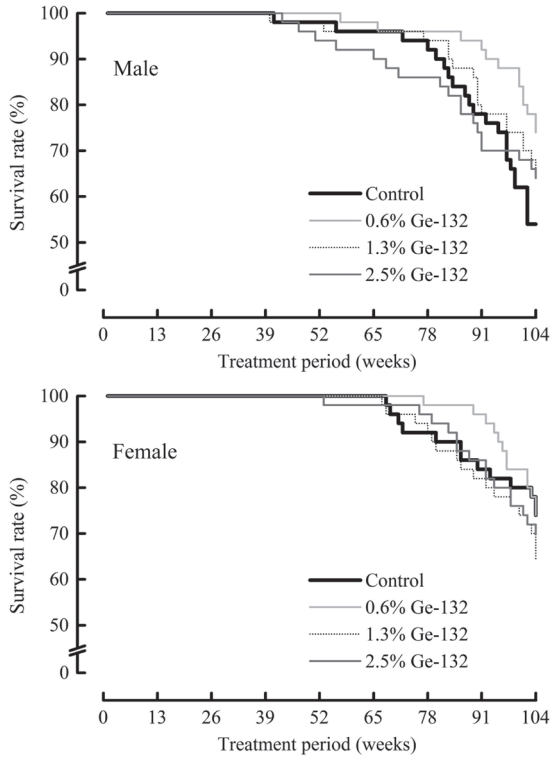

Fig. 1. Cumulative survival curves of male and female rats fed a diet containing Ge-132 for 2 years in the experiment 1.

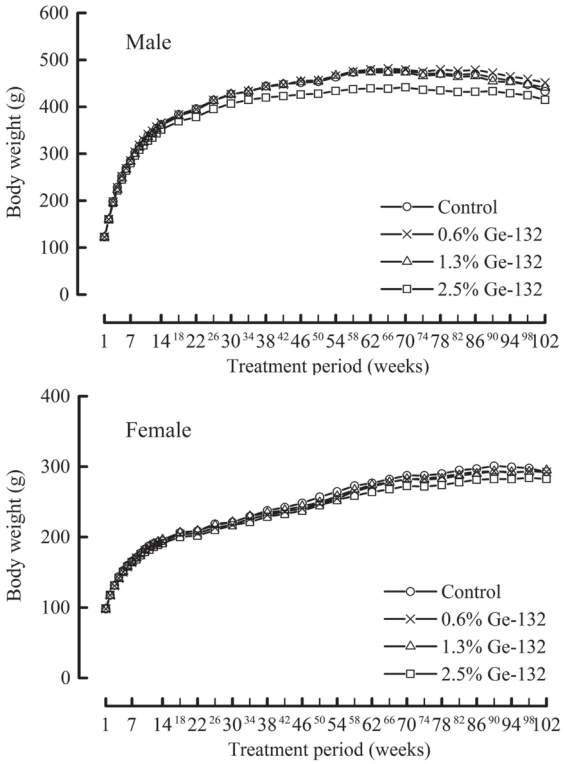

Fig. 2. Body weight changes in male and female rats fed a diet containing Ge-132 for 2 years in the experiment 1.

0.6 and 1.3% Ge-132 (Fig. 2). A tendency toward higher food consumption was observed in all male treated groups and in the female 2.5% group (data not shown). The average Ge-132 intakes for males and females were, 265 and 338 mg/kg body weight/day, respectively, in the 0.6% group, 585 and 746 mg/kg body weight/day, respectively, in the 1.3% group, and 1,195 and 1,509 mg/kg body weight/day, respectively, in the 2.5% group.

No treatment-related adverse effects were apparent in the hematology results (data not shown).

Statistically significant elevations of absolute and relative kidney and adrenal weights were found in both male and female 2.5% group, but not the 0.6 and 1.3% groups. Significant elevations of absolute and relative salivary gland weights were observed in the female 2.5% group, and significant elevation of relative salivary weight was observed in the male 2.5% group (Table 1).

On macroscopic examination (data not shown), dilatations of the cecum were observed in 28 male and 19 female rats given 2.5% Ge-132. Rough surfaces of the kidney were found 6, 8, 16, and 12 males, in the 0%, 0.6%, 1.3%, and 2.5% groups, respectively. Enlargements of the adrenal were noted 2, 3, 2 and 13, males, in the 0%, 0.6%, 1.3%, and 2.5% groups, respectively. These macroscopic findings considered to be related to Ge-132 treatment.

Incidences and severities of non-neoplastic lesions of the kidney, bone (sternum and femur) and parathyroid are shown in Table 2. The incidence and severity of cortico-medullary junction mineralization of the kidney was significantly increased in the female 2.5% group. The incidence and severity of pelvic mineralization of the kidney was significantly increased in all male treated groups and in the female 1.3 and 2.5% groups. The incidence and severity of papilla mineralization of the kidney was also significantly increased in the male 1.3 and 2.5% groups and in the female 2.5% group. Significantly positive trends were noted in the incidences of pelvic and papilla mineralization in both sexes, and cortico-medullary junction mineralization in the female group by the Cochran-Armitage test. The incidence and severity of chronic progressive nephropathy (CPN) of the kidney in treated groups were comparable to the controls. The incidence and severity of increased bone of the sternum and femur was significantly increased in the male 2.5% group. A significantly positive trend was noted in the incidence of increased bone of sternum and femur in male and female rats by the Cochran-Armitage test. The incidences of hyperplasia of the parathyroid in treated groups were comparable to the controls.

Incidences of hyperplastic and neoplastic lesions of the adrenal are shown in Table 3. Significantly higher incidences of focal hyperplasia of adrenal medullary cells were observed in the male and female treated groups and the incidence showed a dose-dependent relationship. The incidences of pheochromocytoma were significantly increased in the male 1.3 and 2.5% groups and the female 2.5% group. The incidence of malignant pheochromocytoma was also significantly elevated in the male 2.5% group. Incidences of benign or malignant pheochromocytoma were significantly increased in the male 1.3 and 2.5% groups and the female 2.5% group. A significant positive trend was noted in the incidences of pheochromocytoma, malignant pheochromocytoma and benign or malignant pheochromocytoma in males, and pheochromocytoma and benign or malignant pheochromocytoma in females by the Cochran-Armitage test.

The incidence of tumors developing in organs other than the adrenal is shown in Table 4. Significant positive trend in the incidence of C-cell adenoma of the thyroid was noted in female rats. However, no significant elevation of incidences of C-cell adenoma was found in any female treated group. Furthermore, a significant positive trend in the incidence of C-cell adenoma or carcinoma of the thyroid was not observed in female rats. A significantly increased incidence of adenoma of the clitoral gland was noted in the female 2.5% group. The incidence of adenoma of the clitoral gland of female rats was also significant by the Cochran-Armitage test. However, the incidence of adenoma or carcinoma of the clitoral gland in the female 2.5% group was comparable to the control value. Overall, the incidences of adenomas, carcinomas, and adenomas or carcinomas of C-cell neoplasia of the thyroid and neoplasia of the clitoral gland suggest that development of C-cell adenoma of the thyroid and adenoma of the clitoral gland were not related to Ge-132 treatment. No significant differences from controls were observed in the incidences of other types of neoplasms.

Toxicokinetics

At week 2 the mean plasma Ge-132 concentrations of the first time point (11:00) for males and females were 3.77 and 7.04 μg/mL, respectively, for the 0.6% group, 15.91 and 16.48 μg/mL, respectively, for the 1.3% group, and 18.00 and 23.59 μg/mL, respectively, for the 2.5% group. Generally, plasma concentrations are high at night when rats actively feeding, and are lower during the day when the rats are less active. However, this pattern was not found in the present study. It might be related to less feeding activity caused by the 7 blood collection one every 4 hours, from same animal. The $AUC_{0\ to\ 24hr}$ (μg•hr/mL) for males and females were 86.4 and 86.0,

Table 1. Final body weight and organ weight in rats fed a diet containing Ge-132 for 2 years in the experiment 1.

Sex	Dose (%)	No. of rats examined	Final B.W.(g)	Salivary glands		Kidneys		Adrenals	
				Absolute (g)	Relative[a]	Absolute (g)	Relative[a]	Absolute (mg)	Relative[b]
Male	0	27	433 ± 37[c]	0.62 ± 0.04	0.14 ± 0.01	2.94 ± 0.27	0.68 ± 0.06	59 ± 10	14 ± 2
	0.6	37	447 ± 42	0.64 ± 0.05	0.14 ± 0.01	3.11 ± 0.29	0.70 ± 0.06	75 ± 43	17 ± 10
	1.3	33	442 ± 40	0.61 ± 0.09	0.14 ± 0.02	3.14 ± 0.32	0.72 ± 0.11	72 ± 20	17 ± 5
	2.5	32	414 ± 26	0.65 ± 0.07	0.16 ± 0.02*	3.24 ± 0.28**	0.79 ± 0.10**	125 ± 86**	31 ± 22**
Female	0	37	299 ± 39	0.47 ± 0.07	0.16 ± 0.03	1.96 ± 0.21	0.67 ± 0.12	73 ± 37	25 ± 14
	0.6	37	294 ± 37	0.45 ± 0.06	0.15 ± 0.02	1.95 ± 0.21	0.67 ± 0.08	80 ± 96	28 ± 35
	1.3	32	299 ± 53	0.47 ± 0.04	0.16 ± 0.02	2.07 ± 0.21	0.71 ± 0.11	72 ± 20	25 ± 8
	2.5	35	281 ± 30	0.49 ± 0.04*[d]	0.18 ± 0.01**[d]	2.22 ± 0.30**	0.80 ± 0.13**	82 ± 36*	30 ± 14**

Data for heart, spleen, lungs, liver, brain, thymus, pituitary, thyroids, testes, ovaries, and uterus were excluded from this Table, since statistically significant changes were not found in Ge-132 treated groups.

[a]: g/100g B.W.

[b]: mg/100g B.W.

[c]: Values are means ± S.D.

[d]: Number of rats examined was 34, since one organ (unilateral) was lost by human error at autopsy.

*, **: Significantly different from control group at $P < 0.05$ and 0.01, respectively.

Table 2. Incidence of non-neoplastic lesions of the kidney, bone (sternum and femur) and parathyroid in rats fed a diet containing Ge-132 for 2 years in the experiment 1.

Organ and tissue	Findings	Sex	Male					Female				
		Dose (%)	0	0.6	1.3	2.5	Trend analysis	0	0.6	1.3	2.5	Trend analysis
		No. of rats examined	50	50	50	50		50	50	50	50	
Kidney	Chronic progressive nephropathy (1)[a]		16	15	21	21		13	15	10	15	
	Chronic progressive nephropathy (2)		23	21	14	16		3	1	5	6	
	Chronic progressive nephropathy (3)		6	11	9	8		0	0	0	3	
	Chronic progressive nephropathy (4)		3	1	3	0		0	1	0	0	
	Mineralization, cortico-medullary junction (1)		0	0	0	0		0	0	0	18 $^{\$\$}$	##
	Mineralization, cortico-medullary junction (2)		0	0	0	0		0	0	0	1	
	Mineralization, pelvic (1)		10	33 $^{\$\$}$	34 $^{\$\$}$	14 $^{\$\$}$	##	24	31	25 $^{\$\$}$	16 $^{\$\$}$	##
	Mineralization, pelvic (2)		1	5	10	24		1	2	13	29	
	Mineralization, pelvic (3)		0	0	2	10		0	1	1	4	
	Mineralization, papilla (1)		1	2	9$^{\$}$	15$^{\$\$}$	##	0	1	2	7$^{\$}$	##
Bone (sternum)	Increased bone (1)		1	0	2	33 $^{\$\$}$	##	5	5	6	20	##
	Increased bone (2)		0	0	0	1		6	8	7	7	
	Increased bone (3)		0	0	0	0		2	0	2	2	
	Increased bone (4)		0	0	0	0		1	0	0	0	
Bone (femur)	Increased bone (1)		1	0	2	21 $^{\$\$}$	##	5	2	10	19	#
	Increased bone (2)		0	0	0	0		4	8	4	2	
	Increased bone (3)		0	0	0	0		5	1	3	1	
Parathyroid	Hyperplasia		6/48[b]	5	4	8		2	4/49	0	2	

[a]: Numbers in parenthesis indicate the grade of lesion: (1) minimal, (2) mild, (3) moderate, (4) marked

[b]: Number of positive/number of examined

$, $$: Significantly different from control group at $P < 0.05$ and 0.01, respectively (Steel's test).

#, ##: Significantly different at $P < 0.05$ and 0.01, respectively (Cochran-Armitage test).

Table 3. Incidence of hyperplastic and neoplastic lesions of the adrenal in rats fed a diet containing Ge-132 for 2 years in the experiment 1.

Findings	Sex	Male					Female				
	Dose (%)	0	0.6	1.3	2.5	Trend analysis	0	0.6	1.3	2.5	Trend analysis
	No. of rats examined	50	50	50	50		50	50	50	50	
Focal hyperplasia of cortical cells		16	13	24	11		19	29*	23	23	
Cortical adenoma		0	0	0	0		0	0	1	0	
Focal hyperplasia of medullary cells		19	35**	41**	44**	##	7	17*	23**	36**	##
Pheochromocytoma		4	8	13*	18**	##	0	2	3	7**	##
Malignant pheochromocytoma		1	3	0	9**	##	2	2	0	3	
Benign or malignant pheochromocytoma		5	11	13*	27**	##	2	4	3	10*	#
Ganglioneuroma		0	0	0	1		0	0	0	0	

*, **: Significantly different from control group at $P < 0.05$ and 0.01, respectively (Fisher's exact probability test).
#, ##: Significantly different at $P < 0.05$ and 0.01, respectively (Cochran-Armitage test).

Table 4. Incidence of neoplastic lesions of organs other than the adrenal in rats fed a diet containing Ge-132 for 2 years in the experiment 1.

Organ and tissue	Findings	Sex	Male				Female				
		Dose (%)	0	0.6	1.3	2.5	0	0.6	1.3	2.5	Trend analysis
		No. of rats examined	50	50	50	50	50	50	50	50	
Hematopoietic system	LGL leukemia[a]		6	7	9	6	5	3	4	4	
Bone marrow (sternum)	Hemangioma		0	0	0	0	0	1	0	0	
Heart	Hemangioma		1	0	0	0	0	0	0	0	
	Schwannoma, intramural		0	1	0	0	0	0	0	0	
Thymus	Thymoma		1/44[b]	0	0/46	0/47	0	0/48	0/49	0/46	
Pituitary	Adenoma, anterior lobe		15/48	14	17	16/48	19/49	21/46	16	13/49	
Thyroid	C-cell adenoma		6	3	11	4	2	1	5	7	#
	C-cell carcinoma		1	1	2	1	1	1	0	0	
	C-cell adenoma or carcinoma		7	4	13	5	3	2	5	7	
	Follicular cell adenoma		1	2	0	0	1	0	1	0	
	Follicular cell carcinoma		0	0	1	0	0	0	0	0	
Parathyroid	Adenoma		0/48	0	0	0	0	1/49	0	0	
Lung	Adenoma, bronchiolo-alveolar		0	3	3	0	0	0	0	0	
	Adenocarcinoma, bronchiolo-alveolar		0	1	1	0	0	0	0	0	
Stomach	Leiomyosarcoma		0	1	0	0	0	0	0	0	
Jejunum	Adenocarcinoma		0	0	1	0	0	0	0	0	
Liver	Cholangioma		0	0	0	0	0	0	1	0	
	Hepatocellular adenoma		0	1	0	1	0	0	0	1	
	Hepatocellular carcinoma		0	1	0	0	0	0	0	0	
	Histiocytic sarcoma		0	1	0	0	0	0	0	0	
Pancreas	Islet cell adenoma		5	5	8	5	2	0	0	1	
Kidney	Lipoma		0	0	0	0	0	0	1	0	
	Nephroblastoma		1	0	0	0	0	0	0	0	
	Transitional cell papilloma		0	1	0	0	0	0	0	1	

Table 4. (Continued).

Organ and tissue	Findings	Sex	Male				Female				
		Dose (%)	0	0.6	1.3	2.5	0	0.6	1.3	2.5	Trend
		No. of rats examined	50	50	50	50	50	50	50	50	analysis
Urinary bladder	Transitional cell papilloma		1	0	0	1	0	0	0	0	
	Transitional cell carcinoma		0	0	1	0	0	0	0	0	
Testis	Interstitial cell tumor		36	36	33	31	-[c]	-	-	-	
Prostate	Adenoma		6	6	4	2/49	-	-	-	-	
Prep./clit. gland	Adenoma		1[2][d]	-	1[2]	1[1]	1[4]	1[1]	2[2]	5[5]*	#
	Carcinoma		0[2]	-	1[2]	0[1]	1[4]	0[1]	0[2]	0[5]	
	Adenoma or carcinoma		1[2]	-	2[2]	1[1]	2[4]	1[1]	2[2]	5[5]	#
Ovary	Granulosa and theca cell tumor		-	-	-	-	1	0	3	0	
	Sertoli cell tumor		-	-	-	-	1	0	0	0	
	Tubular adenocarcinoma		-	-	-	-	1	0	0	0	
Uterus	Endometrial stromal polyp		-	-	-	-	9	5	12	9	
	Endometrial stromal sarcoma		-	-	-	-	0	4	3	0	
	Leiomyosarcoma		-	-	-	-	0	0	0	1	
Mammary gland	Adenoma		0/49	0	0	0	0	1	1	2	
	Fibroadenoma		1/49	0	0	1	15	11	14	12	
	Adenocarcinoma		1/49	0	0	0	2	1	0	1	
Skin/subcutis	Fibroma		5	7	4	3	0	1	0	1	
	Keratoacanthoma		2	0	1	3	1	3	1	0	
	Squamous cell papilloma		1	0	0	0	1	0	0	0	
	Tricholemmoma		0	0	0	1	0	0	0	0	
	Basal cell tumor		0	0	0	0	0	0	1	0	
	Fibrosarcoma		2	1	0	0	0	0	0	0	
	Histiocytic sarcoma		0	1	0	0	0	0	0	0	
	Malignant schwannoma		1	0	0	0	0	0	0	0	
	Sarcoma, NOS		0	0	0	1	0	0	0	0	
	Sebaceous cell carcinoma		0	0	0	0	0	1	0	0	
	Squamous cell carcinoma		1	0	0	0	0	0	0	0	
Brain	Glioma		0	0	0	0	1	0	0	0	
Zymbal's gland	Carcinoma		1[1]	1[1]	-	1[1]	-	-	-	-	
Diaphragm	Rhabdomyosarcoma		-	-	-	-	-	-	-	1[1]	
Bone (limb)	Osteosarcoma		-	-	-	-	-	1[1]	1[1]	-	
Thoracic cavity	Fibrosarcoma		0[1]	0[1]	1[1]	-	-	-	-	-	
Abdominal cavity	Mesothelioma		3[4]	3[6]	0[1]	2[3]	-	-	1[1]	0[2]	

[a] Large granular lymphocytic leukemia
[b] Number of positive/Number of examined
[c] Not applicable
[d] Numbers in square bracket are for animals examined microscopically.
*: Significantly different from control group at $P < 0.05$ (Fisher's exact probability test).
#: Significantly different at $P < 0.05$ (Cochran-Armitage test).

respectively, for the 0.6% group, 173.7 and 143.3, respectively, for the 1.3% group, and 221.7 and 301.1, respectively, for the 2.5% group. Plasma Ge-132 concentrations tended to increase with increasing dietary concentrations of Ge-132.

At week 5, the mean plasma Ge-132 concentrations at the first time point (11:00) for males and females were 7.41 and 3.97 μg/mL, respectively, for the 0.6% group, 19.44 and 9.68 μg/mL, respectively, for the 1.3% group, and 46.47 and 8.89 μg/mL, respectively, for the 2.5% group. At week 30, the mean plasma Ge-132 concentrations at the first time point (11:00) for males and females were 6.56 and 3.35 μg/mL, respectively, for the 0.6% group, 15.72 and 11.46 μg/mL, respectively, for the 1.3% group, and 40.40 and 16.66 μg/mL, respectively, for the 2.5% group. There was a tendency for the concentrations of Ge-132 in the blood at time point 11:00 to be higher than those at time point15:00 except for the female 2.5% group at week 5.

Experiment 2: Investigation of mode of action for Ge-132-developed proliferating lesions of the adrenal medullary cell

The animals' general condition, average body weight, food consumption, and water consumption are summarized in Table 5. Loose stools and significantly increased water consumption were observed in the 0.6 and 2.5% groups. Average body weight and food consumption in the treated groups were comparable to control values, and average intake of Ge-132 in 0.6 and 2.5% groups were 380 and 1,643 mg/kg/day, respectively.

Results for urine and blood biochemistry are summarized in Table 6. Urine volumes decreased in a dose-dependent manner. Urine volumes in the 2.5% group were significantly decreased at week 4 and tended to decrease at week13, and those in the 0.6% group tended to decrease at weeks 4 and 13. Urinary concentration of inorganic phosphorus was significantly elevated in the 2.5% group at weeks 4 and 13. Urinary concentration of calcium was significantly elevated in the 0.6 and 2.5% groups at week 4. On the other hand, total daily urinary excretion of inorganic phosphorus and calcium in Ge-132-treated groups were comparable to control values at weeks 4 and 13. Serum inorganic phosphorus was significantly elevated in the 2.5% group at week 13, but not week 4. A slight but not significantly elevated serum calcium level was observed in the 2.5% group at week 13.

Results for organ weight, macroscopic finding and ki-67 positive cell ratio of adrenal medullary cells are summarized in Table 7. Significant elevations of relative kidney weights were observed in the 0.6 and 2.5% groups at week 13. Significant elevation of relative adrenal weight was noted in the 2.5% group at week 13. Significant elevations of relative cecum (filled and empty) weights were found in the 2.5% group at weeks 4 and 13. Dilatation of the cecum was observed in all rats of the 2.5% group at weeks 4 and 13. A significant increase of Ki-67 positive cell ratio for adrenal medullary cells was observed in the 2.5% group at week 13. However, no treatment-related histopathological findings were observed in any Ge-132-treated rats (data not shown).

DISCUSSION

In the present 2-year carcinogenicity of administration of Ge-132 in the diet using F344 rats, no treatment-related changes in survival rate, food consumption, or hematology results were found; although, body weight retardation was noted in male and female 2.5% groups. Loose stools and diarrhea showed a dose-dependent relationship

Table 5. General condition, average body weight, food consumption and water intake of male rats fed a diet containing Ge-132 for 4 and 13 weeks in the experiment 2.

Items	Administration duration (week)	No. of rats examined	Dose (%)		
			0	0.6	2.5
General condition: Loose stools during 4 weeks	4	26	0/26[a]	2/26	26/26
General condition: Loose stools during 13 weeks	13	16	0/16	8/16	16/16
Average body weight (g) at week 4	4	26	234.5 ± 10.4[b]	236.8 ± 9.0	234.2 ± 9.2
Average body weight (g) at week 13	13	16	323.6 ± 11.2	328.8 ± 11.2	321.8 ± 14.0
Food consumption (g/animal/day) at week 4	4	26	16.4 ± 0.7	16.6 ± 0.6	16.8 ± 0.7
Food consumption (g/animal/day) at week 13	13	16	15.4 ± 0.4	16.1 ± 0.5	16.3 ± 0.7*
Water consumption (g/animal/day) at week 4	4	26	19.4 ± 1.2	21.3 ± 1.3**	27.4 ± 1.1**
Water consumption (g/animal/day) at week 13	13	16	17.0 ± 1.0	18.9 ± 1.0**	25.2 ± 1.2**

[a]: Number of positive/number of examined
[b]: Values are means ± S.D.
*, **: Significantly different from control group at $P < 0.05$ and 0.01, respectively.

Table 6. Urine volume, and urine and blood biochemistry data of male rats fed a diet containing Ge-132 for 4 and 13 weeks in the experiment 2.

Items	Administration duration (week)	No. of rats examined	Dose (%)		
			0	0.6	2.5
Urine volume (mL/day)	4	6	19.98 ± 10.27[a]	7.00 ± 3.73	3.63 ± 1.67*
	13	6	12.08 ± 6.05	9.00 ± 4.14	7.08 ± 3.43
Urine biochemistry					
IP (mg/dL)	4	6	76.83 ± 82.95	147.83 ± 56.61	208.83 ± 54.93**
	13	6	63.83 ± 18.74	112.17 ± 64.70	149.83 ± 55.21*
Ca (mg/dL)	4	6	3.50 ± 1.05	6.33 ± 1.63*	19.83 ± 5.85**
	13	6	8.50 ± 4.28	7.83 ± 2.79	15.50 ± 9.14
IP (mg/24 hr)	4	6	8.83 ± 2.04	9.25 ± 2.90	6.93 ± 1.08
	13	6	7.07 ± 2.39	12.10 ± 13.07	9.37 ± 1.92
Ca (mg/24 hr)	4	6	0.65 ± 0.33	0.40 ± 0.11	0.65 ± 0.18
	13	6	0.85 ± 0.26	0.67 ± 0.29	1.02 ± 0.56
Blood biochemistry					
IP (mg/dL)	4	10	7.46 ± 0.33	7.28 ± 0.33	7.49 ± 0.33
	13	10	5.14 ± 0.33	5.45 ± 0.43	5.78 ± 0.44**
Ca (mg/dL)	4	10	10.77 ± 0.13	10.77 ± 0.09	10.77 ± 0.15
	13	10	10.31 ± 0.10	10.40 ± 0.12	10.45 ± 0.25

[a]: Values are means ± S.D.
*, **: Significantly different from control group at $P < 0.05$ and 0.01, respectively.

Table 7. Final body weight, organ weight, macroscopic findings and Ki-67 positive cell ratio of male rats fed a diet containing Ge-132 for 4 and 13 weeks in the experiment 2.

Items	Administration duration (week)	No. of rats examined	Dose (%)		
			0	0.6	2.5
Final body weight (g)	4	10	238.7 ± 10.9[a]	236.8 ± 6.6	234.5 ± 8.6
	13	10	327.0 ± 12.8	329.9 ± 8.7	327.2 ± 12.8
Organ to body weight ratio (g/100g BW)					
Kidneys	4	10	0.72 ± 0.03	0.72 ± 0.02	0.73 ± 0.03
	13	10	0.59 ± 0.02	0.62 ± 0.02**	0.66 ± 0.02**
Adrenals	4	10	0.013 ± 0.001	0.014 ± 0.001	0.014 ± 0.001
	13	10	0.010 ± 0.001	0.010 ± 0.001	0.011 ± 0.001**
Cecum (filled)	4	10	2.48 ± 0.46	2.89 ± 0.27	4.32 ± 0.59**
	13	10	1.78 ± 0.18	1.98 ± 0.24	3.97 ± 0.52**
Cecum (empty)	4	10	0.36 ± 0.04	0.37 ± 0.04	0.50 ± 0.03**
	13	10	0.26 ± 0.02	0.28 ± 0.03	0.40 ± 0.02**
Macroscopic findings					
Dilatation of the cecum	4	10	0/10[b]	0/10	10/10**
	13	10	0/10	0/10	10/10**
Ki-67 positive cell ratio in adrenal medullary cells (%)	4	10	1.85 ± 0.48	2.21 ± 0.45	2.00 ± 0.42
	13	10	1.07 ± 0.26	1.35 ± 0.42	1.42 ± 0.26*

[a]: Values are means ± S.D.
[b]: Number of positive/number of examined
*, **: Significantly different from control group at $P < 0.05$ and 0.01, respectively.

in both male and female Ge-132 treated rats during the course of the study. Loose stools also showed a dose-dependent relationship in the 13-week study at both 4 and 13 weeks (Experiment 2). Loose stools and diarrhea were also reported in another oral three-month toxicity studies in rats (Sugiya *et al*., 1986a), but not in an intraperitoneal 3- and 6- month toxicity study in rats (Sugiya *et al*., 1986b). Macroscopically, in the 2-year carcinogenicity study, a high incidence of dilatation of the cecum was found in both male and female 2.5% group, but was not found in the 0.6 and 1.3% groups. In the 13-week study, dilatation of the cecum was also found in all examined

rats in the 2.5% group at both weeks 4 and 13. A significant increase in the absolute and relative cecum weights (filled and empty) was also observed in the 2.5% group at both 4 and 13 weeks in this study. A previous report suggested that dilatation of the cecum can be the result of poorly-digested starches increasing the osmotic pressure in the large intestine, and that poorly-digested starches can also cause loose stools and diarrhea (Newberne *et al*., 1988). Therefore, increased osmotic pressure in the large intestine derived from the physicochemical properties of Ge-132 (unpublished information from Asai Germanium Research Institute Co. Ltd.), may have caused the loose stools and diarrhea and cecal dilatation observed in the present study.

Although body-weight retardation was noted in both male and female 2.5% groups, the elevated absolute and relative adrenal weights were associated with enlargement of the adrenal glands in these animals; enlargement of the adrenal gland was especially noticeable in males where it doubled in weight. Significantly higher incidences of pheochromocytoma were identified in the male 1.3% and 2.5% groups and the female 2.5% group, and malignant pheochromocytoma was elevated in the male 2.5% group. These lesions showed evidence of a dose-dependent relationship. No other treatment-related neoplastic lesions were induced by Ge-132 ingestion.

A wide range of chemicals induced hyperplasia and benign or malignant pheochromocytoma in the adrenal medulla of rats. It is well documented that pheochromocytomas occur with relatively high frequency in male rats. Hypoxia, uncoupling of oxidative phosphorylation, disturbance in calcium/phosphorus homeostasis, and disturbance of the hypothalamic endocrine axis increase the incidence of pheochromocytoma (Greim *et al*., 2009). Disturbance in calcium/phosphorus homeostasis appears to be the primary cause of induction of pheochromocytoma in several studies. Roe and Bär, 1985, reported that the development of pheochromocytoma in rats was induced by exposure to polyols (sorbitol, mannitol, xylitol, lactitol) or lactose, and propose that this effect is caused by an increase of calcium absorption from the gastrointestinal tract. Lynch *et al*., 1996, reported dilatation of the cecum, hypercalciuria, and development of adrenal medullary proliferative lesions in rats administered polyols or lactose. Other studies also report that high dietary intake of sorbitol or xylitol causes dilatation of the cecum in rats, increased intestinal absorption and urinary excretion of calcium, and increased development of adrenal medullary hyperplasia and/or tumors associated with calcification in the papilla or the tubules at the cortico-medullary junction of the kidney (Roe, 1984; Lord and Newberne, 1990; Lynch *et al*., 1996). High dose of retinol acetate can also induce pheochromocytoma by promoting an increase in free calcium mediated by enhanced calcium release from bones (Kurokawa *et al*., 1985). These reports suggest that the hypercalcemia caused by these substances is associated with the development of this tumor (Capen *et al*., 2002; Tischler *et al*., 1996).

In the 2-year study, the incidence and severity of pelvic mineralization of the kidney were significantly increased in a dose-dependent manner in Ge-132-treated male and female rats, and the incidences of papilla mineralization of the kidney were significantly increased in the male and female 2.5% groups. It is likely that the increase in kidney weight observed in the male and female 2.5% groups can be related to the pelvic and papilla mineralization that may be induced by Ge-132 treatment. There was also a significant positive trend in the incidence of increased bone of the sternum and femur in male and female rats in the 2-year study. These alterations in the kidney and bone indicate a disturbance of calcium/phosphorus homeostasis.

In the 13-week study, a significantly higher serum inorganic phosphorus level and a slight, but not significant, elevation of serum calcium level was observed in the 2.5% group at week 13. Furthermore, a significant elevation of serum inorganic phosphorus and calcium levels were reported in both sexes of rats administered 4,000 mg/kg Ge-132 for 3 months (unpublished data from Asai Germanium Research Institute Co., Ltd.). These data suggest that calcium/phosphorus homeostasis is impaired by ingestion of Ge-132.

In the 13-week study, the 2.5% group had both loose stools and increased water consumption. Therefore, rat fed Ge-132 exhibited dilatation of the cecum, and decreased urine volume. These effects were most likely caused by increased osmotic pressure in the cecum due to osmotically active Ge-132. These effects were coupled with impaired calcium/phosphorus homeostasis. In addition, the Ki-67 positive cell ratio of adrenal medullary cell was increased, indicating accelerated cellular proliferation. Similar results were also reported for rats administered polyols or lactose in the diet (Lynch *et al*., 1996; Tischler *et al*., 1996). These data are consistent with the argument that Ge-132 ingestion induces impaired calcium/phosphorus homeostasis which in turn stimulated proliferation of chromaffin cells thereby inducing pheochromocytoma (Tischler *et al*., 1996).

The mode of action that applies to the development of adrenal medullary tumors is species-specific. In rats, long-term and excessive intake of poorly absorbable carbohydrates stimulates increased calcium absorption from

the gastrointestinal tract, inducing hypercalcemia which in turn stimulates catecholamine biosynthesis in adrenal medullary cells. The result of this is induction of hyperplasias/neoplasias in the adrenal medulla (Capen *et al*., 2002). In constant, while mice exposed to high level of dietary carbohydrates, such as lactose, exhibit dilatation of the cecum and colon, and enhanced calcium absorption from the gastrointestinal tract, but pheochromocytoma has not been reported (Til *et al*., 1986). Nor was pheochromocyotoma found in a 26-week carcinogenicity study of Ge-132 using rasH2 mice (Doi *et al*., 2017). Additionally, there is rarely spontaneous development of pheochromocyoma in mice and other species (Newberne *et al*., 1988; Maekawa *et al*., 1990). Adrenal medullary pheochromocytoma (chromaffin cell tumor) is very rare in humans and there is no evidence to date to suggest that disturbance in calcium/phosphorus homeostasis induces pheochromocytoma in humans (Greim *et al*., 2009; Roe and Bär, 1985; Newberne, 1988). Consequently, adrenal medullary tumor development in rats as a result of long-term intake of Ge-132 in the diet is considered to be irrelevant to humans.

The upper limit of dietary intake of Ge-132 as a health food supplement in humans is assumed to be 15 mg/kg body weight/day, which is approximately half the 1,500 mg per person that induced no side effects in a previous long-term administration trial to humans (Arimori and Yoshida, 1982; Arimori *et al*., 1990). In the current 2-year feeding carcinogenicity study, the no carcinogenic dose of Ge-132 was 265 mg/kg/day for males (0.6% group) and 746 mg/kg/day for females (1.3% group), which is approximately 18- and 50-fold higher than the maximum daily recommended amount. Importantly, other than pheochromocytomas, up to 2.5% Ge-132 in the diet (1,195 mg/kg/day for males and 1,509 mg/kg/day for females) did not induce the development of either malignant or benign tumors in rats in the present study. These studies do not show any appreciable risk to humans posed by long-term exposure to recommended levels of dietary Ge-132.

In conclusion, dietary supplemented with Ge-132 at 1.3% (585 mg/kg/day) for males and at 2.5% Ge-132 (1,509 mg/kg/day) for females were carcinogenic based on increased incidences of benign or malignant pheochromocytomas of the adrenal. No other neoplastic lesions were induced by up to 2.5% Ge-132 (1,195 mg/kg/day for males and 1,509 mg/kg/day for females) in the diet. In rats, Ge-132 impairs calcium/phosphorus homeostasis which in turn is involved in the development of pheochromocytomas in these animals. However, the mechanism by which Ge-132 induces pheochromocytomas of the adrenal in rats is not relevant to humans. Adrenal medullary pheochromocytoma (chromaffin cell tumor) is very rare in humans and there is no evidence of any relation between a disturbance in calcium/phosphorus homeostasis and increased risk of pheochromocytoma in humans. Consequently, adrenal medullary tumor development as a result of long-term Ge-132 ingestion in rats is considered to be not relevant for human risk assessment.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Akiba, M. and Kakimoto, N. (1994): Synthesis and properties of poly[3,3′-(1,3-dioxo-1,3-digermoxanediyl)bispropanoic acid] (Ge-132) and related compounds as bioactive organogermanium compound. The Chemical Society of Japan, **3**, 286-300. (in Japanese)

Arimori, S. and Yoshida, M. (1982): Effect of Ge-132 on the patients with rheumatoid arthritis. Med. Biol., **104**, 211-213. (in Japanese)

Arimori, S., Yoshida, M. and Ichimura, K. (1990): Improved rheumatoid arthritis case with Ge-132 administration evaluated by clinically and immunologically using two-color flow cytometry. Jpn. J. Clin. Immun., **13**, 80-86. (in Japanese)

Aso, H., Suzuki, F., Yamaguchi, T., Hayashi, Y., Ebina, T. and Ishida, N. (1985): Induction of interferon and activation of NK cells and macrophages in mice by oral administration of Ge-132, an organic germanium compound. Microbiol. Immunol., **29**, 65-74.

Aso, H., Suzuki, F., Ebina, T. and Ishida, N. (1989): Antiviral activity of carboxyethylgermanium sesquioxide (Ge-132) in mice infected with influenza virus. J. Biol. Response Mod., **8**, 180-189.

Capen, C.C., DeLellis, R.A. and Yarrington, J.T. (2002): Endocrine system. In Handbook of Toxicologic Pathology (Second Edition, vol. 2) (Haschek, W.M., Rousseaux, C.G., and Wallig, M.A., eds.), pp.681-783, Academic Press, San Diego.

Doi, Y., Imai, N., Suguro, M., Numano, T. and Furukawa, F. (2017): No carcinogenicity of poly-*trans*-[(2-carboxyethyl)germasesquioxane] (Ge-132): 26-week feeding study using rasH2 mice. Fundam. Toxicol. Sci., **4**, 137-150.

Greim, H., Hartwig, A., Reuter, U., Richter-Reichhelm, H.-B. and Thielmann, H.-W. (2009): Chemically induced pheochromocytomas in rats: mechanisms and relevance for human risk assessment. Crit. Rev. Toxicol., **39**, 695-718.

Kagoshima, M., Onishi, T., Suguro, N. and Tomizawa, S. (1986a): Metabolic fate of 2-carboxyethylgermanium sesquioxide (1)

Oral administration. Oyo Yakuri, **32**, 71-79. (in Japanese)
Kagoshima, M., Suguro, N. and Onishi, T. (1986b): Metabolic fate of 2-carboxyethylgermanium sesquioxide (2) Intravenous administration. Oyo Yakuri, **32**, 81-88. (in Japanese)
Kagoshima, M. and Onishi, T. (1986): Metabolic fate of 2-carboxyethylgermanium sesquioxide (3) Continual administration. Oyo Yakuri, **32**, 89-92. (in Japanese)
Kurokawa, Y., Hayashi, Y., Maekawa, A., Takahashi, M. and Kokubo, T. (1985): High incidences of pheochromocytomas after long-term administration of retinol acetate to F344/DuCrj rats. J. Natl. Cancer Inst., **74**, 715-723.
Li, L., Ruan, T., Lyu, Y. and Wu, B. (2017): Advances in effect of germanium or germanium compounds on animals—A review. J. Biosci. Med., **5**, 56-73.
Lord, G.H. and Newberne, P.M. (1990): Renal mineralization—A ubiquitous lesion in chronic rat studies. Food Chem. Toxicol., **28**, 449-455.
Lynch, B.S., Tischler, A.S., Caoen, C., Munro, I.C., McGirr, L.M. and McClain, R.M. (1996): Low digestible carbohydrates (polyols and lactose): significance of adrenal medullary proliferative lesions in the rat. Regul. Toxicol. Pharmacol., **23**, 256-297.
Maekawa, A., Kurokawa, Y. and Kanno, J. (1990): Neoplasms of the adrenal cortex and medulla. In Atras of Tumor Pathology of the Fischer Rat (Stinson, S.F., Schuller, H.M. and Renzik, G., ed.), pp. 291-313, CRC Press, Florida.
Miyao, K. and Tanaka, N. (1988): Carboxyethylgermanium sesquioxide and related organogermanium compounds – unique synthetic BRM's – a review. Drugs Future, **13**, 441-453.
Newberne, P.M., Conner, M.W. and Estes, P. (1988): The influence of food additives and related materials on lower bowel structure and function. Toxicol. Pathol., **16**, 184-197.
Roe, F.J. (1984): Perspectives in carbohydrate toxicology with special reference to carcinogenicity. Swed. Dent. J., **8**, 99-111.
Roe, F.J. and Bär, A. (1985): Enzootic and epizootic adrenal medullary proliferative disease of rats: influence of dietary factors which affect calcium absorption. Hum. Toxicol., **4**, 27-52.
Sanai, T., Onoyama, K., Osato, S., Motomura, K., Oochi, N., Oh, Y., Okuda, S., Takaichi, S. and Fujishima, M. (1991a): Dose dependency of germanium-dioxide-induced nephrotoxicity in rats. Nephron, **57**, 349-354.
Sanai, T., Okuda, S., Onoyama, K., Oochi, N., Takaichi, S., Mizuhira, V. and Fujishima, M. (1991b): Chronic tubulointerstitial changes induced by germanium dioxide in comparison with carboxyethylgermanium sesquioxide. Kidney Int., **40**, 882-890.
Sugiya, Y., Sakamaki, S., Sugita, T., Abo, Y. and Satoh, H. (1986a): Subacute oral toxicity of carboxyethylgermanium sesquioxide (Ge-132) in rats. Oyo Yakuri, **31**, 1181-1190. (in Japanese)
Sugiya, Y., Sugita, T., Sakamaki, S., Abo, Y. and Satoh, H. (1986b): Subacute and chronic intraperitoneal toxicity of carboxyethylgermanium sesquioxide (Ge-132) in rats. Oyo Yakuri, **32**, 93-111. (in Japanese)
Til, H.P., Feron, V.J., Immel, H.R. and Vogel, W.F. (1986): Chronic (89-week) feeding study with hydroxypropyl distarch phosphate, starch acetate, lactose and sodium alginate in mice. Food Chem. Toxicol., **24**, 825-834.
Tischler, A.S., Powers, J.F., Downing, C.J., Riseberg, J.C., Shahsavari, M., Ziar, J. and McClain, R.M. (1996): Vitamin D_3, lactose, and xylitol stimulate chromaffin cell proliferation in the rat adrenal medulla. Toxicol. Appl. Pharmacol., **140**, 115-123.
Tsutsui, M., Kakimoto, N., Axtell, D.D., Oikawa, H. and Asai, K. (1976): Crystal structure of "carboxyethylgermanium sesquioxide". J. Am. Chem. Soc., **98**, 8287-8289.