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Original Article

Safety evaluation of chicken breast meat extract in chromosomal aberration study, reverse mutation test, and 90-day repeated dose study in rats

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ABSTRACT — To evaluate the safety of chicken breast extract containing plasmalogen, the following three tests were conducted: i) chromosome aberration study with mammalian cells; ii) bacterial reverse mutation test; and iii) 90-day repeated oral dose study in rats. The chromosome aberration study showed that there was no statistically significant increase in the structural aberration rate and the polyploidy aberration rate between the group that was treated with chicken breast extract and the negative-control group. Under the present test conditions, chicken breast extract did not induce chromosomal aberrations. Similarly, in the reverse mutation test, there was no increase in the number of revertant colonies in all of the bacterial strains that more than doubled the negative-control value related to the dose, regardless of the presence or absence of metabolic activation. In other words, chicken breast extract did not exhibit a gene mutation–inducing effect under the present test conditions. No effects were observed in the 90-day repeated oral dose study with the administration of chicken breast extract. Under the present test conditions, the no-observed-adverse-effect level for both male and female rats was 1000 mg/kg/day.

Key words: Chicken breast meat, Plasmalogen, Chromosome aberration study, Reverse mutation test, 90-day repeated oral dose study

INTRODUCTION

Plasmalogen is the generic name of an ether-linked phospholipid with a vinyl-ether bond. In the body, plasmalogen is widely distributed in the brain, heart, and skeletal muscle (Maeba, 2005). Previous studies have reported reduced levels of plasmalogen in the serum of elderly arteriosclerosis patients and in the brain of Alzheimer dementia patients, and it has been suggested that plasmalogen may play an important role in the maintenance of homeostasis in the vascular and cerebral nervous systems (Guanm *et al.*, 1999; Maeba *et al.*, 2007). An increase in the number of dementia patients in recent years in Japan has become a social problem. The Ministry of Health, Labour and Welfare estimates that the number of elderly people with dementia will reach about 7 million by 2025. At present, there is no fundamental therapeutic agent for dementia, and the emphasis has been on medical approaches to prevent disease onset, such as lifestyle and diet modification, as well as physical activity.

Against such a social background, we focused our attention on the breast meat of egg-laying hens, which is rich in plasmalogen, and began to conduct joint research projects on its transformation to food material and association with cognitive function at university-affiliated research institutions. As a result, a manufacturing process to stably extract plasmalogen from chicken breast meat was developed. Plasmalogen's potential for improv-

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ing learning and memory skills in the brain, along with its role in preventing dementia, were also discovered (Kotoura *et al.*, 2017; Kawamura *et al.*, 2019; Hossain *et al.*, 2018). Based on this scientific evidence, we were the first to successfully introduce a chicken breast extract with plasmalogen as a new food material in the Japanese domestic market.

Chicken breast meat has been consumed all over the world since ancient times, and with its rich history of dietary use, it is generally regarded as safe. However, as there are limited data regarding the safety of chicken breast extract containing plasmalogen, in the present study, we conducted the following tests: i) chromosome aberration study using cultured mammalian cells; ii) bacterial reverse mutation test; and iii) a 90-day repeated oral dose study in rats.

The chromosome aberration study with mammalian cultured cells and the bacterial reverse mutation test adhered to the "Ministerial ordinance concerning the standards for the conduct of non-clinical studies on the safety of drugs" (Ministry of Health and Welfare ordinance No. 21, March 26, 1997), and the tests were conducted based on the "Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use" (Pharmaceutical and Food Safety Bureau Notification No. 0920(2), September 20, 2012), "Basic concept related to the appropriate manufacturing of food items in forms such as tablets and soft capsules," and "Guidelines on voluntary inspections for raw material safety of food items such as tablets and soft capsules" (Department of Food Safety Notification No. 0201003, February 1, 2005).

The 90-day repeated oral dose study in rats adhered to the "Ministerial ordinance concerning the standards for the conduct of non-clinical studies on the safety of drugs" (Ministry of Health and Welfare ordinance No. 21, March 26, 1997), and the study was conducted based on the "Partial revision of the guidelines for repeated dose toxicity studies" (Pharmaceutical Affairs Bureau Notification No. 655, April 5, 1999), "Basic concept related to the appropriate manufacturing of food items in forms such as tablets and soft capsules," and "Guidelines on voluntary inspections for raw material safety of food items such as tablets and soft capsules" (Department of Food Safety Notification No. 0201003, February 1, 2005). The tests were conducted according to the study protocol reviewed by the Institutional Animal Care and Use Committee of the testing facility while adhering to the "Act on welfare and management of animals" and "Guidelines for animal testing by Ina Research Inc.". A testing facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (certificate no. 001107) was used.

MATERIALS AND METHODS

Test substance

Chicken breast extract (batch no. 170807) was used in the experiment. The chicken breast extract was prepared by alcohol extraction of freeze-dried chicken breast meat, which was then vacuum concentrated and purified. The extract was a yellowish-brown viscous oil containing plasmalogen, a type of phospholipid (the product specification value of plasmalogen was 12.5% or more, the actual measured value of the batch used in the experiment was 14.47%). In addition, the extract contained approximately 10% ethyl alcohol. The extract can be stored frozen (−18°C or below) for 2 years.

Animal rearing

The rats used in the experiment were reared indoors in a stainless-steel wire cage (29W × 22D × 21H cm) under a 12-hr light-dark cycle (artificial lighting from 0700 to 1900) at a temperature of 21 to 25°C and humidity of 40 to 70%, with a ventilation frequency of 15 to 17 times per hour. The rats were fed a commercial CRF-1 (pelleted feed) (Oriental Yeast Co., Ltd., Chiba, Japan) ad libitum. On the last day of the treatment period, the feed was removed from animals scheduled for necropsy the next day, and the rats were fasted for longer than 16 hr prior to necropsy. The rats were allowed to drink water ad libitum from an automatic water dispenser connected to the municipal water supply. However, during urine collection, a glass water feeder with a built-in touch-drink style tube was used.

Chromosome aberration study using mammalian cells

The chromosome aberration study was carried out according to the 6-hr direct method (−S9 treatment), the metabolic activation method (+S9 treatment), and the 24-hr direct method. The test system for CHL/IU cells (a fibroblast-like cell line derived from the lungs of newborn female Chinese hamsters) was purchased from the Laboratory Products Division of DS Pharma Biomedical Co., Ltd., Japan. Cells were used after reaching the 35th subculture generation in successive cultures. The chicken breast extract was serially diluted with water for injection (Otsuka Pharmaceutical Factory, Inc. Naruto-shi, Tokushima-ken, Japan) before use for each test. In terms of dose selection, the upper limit of the maximum concentration in genotoxicity tests of pharmaceuticals is

recommended as either 1 mM or 0.5 mg/mL, whichever is lowest. However, the test substance in the present study was an extract prepared from a food material. The Organization for Economic Cooperation and Development guidelines no. 473 (OECD, 2016) state that for test substances of unknown composition, the maximum concentration should be increased (5 mg/mL), as well as the concentration of each ingredient. Based on these guidelines, 5 mg/mL (5000 μg/mL) was set as the maximum dose; using a common ratio of two, a total of six doses (156, 313, 625, 1250, 2500, and 5000 μg/mL) were used in the 6-hr treatment direct method and the metabolic activation method. For the 24-hr treatment direct method, a total of seven doses were used (78.1, 156, 313, 625, 1250, 2500, and 5000 μg/mL). Benzo [a] pyrene (B[a]P, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was used as the positive-control substance, and based on the recommended positive-control substance and concentration (Miyake, 2000), B[a]P 30 μg/mL was used for the metabolic activation method. In addition to the above-mentioned treatment group and positive-control group, a group in which water was used for injection was introduced as the negative-control group. The culture medium was prepared by adding penicillin-streptomycin (Life Technologies Corporation, Tokyo, Japan) to liquid Eagle's Minimum Essential Medium (Life Technologies Corporation, Tokyo, Japan) to a final concentration of 100 units/mL and 100 μg/mL, and heat-inactivated fetal bovine serum (Life Technologies Corporation, Tokyo, Japan) was added to a final concentration of 10 vol%. Cell cultures were prepared by seeding CHL/IU cells in tissue culture flasks, 6-well plates, and 60-mm plastic petri dishes. Cells were then maintained in a moist environment under static culture in an incubator at 5% CO_2 and 37°C.

For metabolic activation in +S9 treatment, a mixture of S9 (supernatant fraction of liver homogenate [9000 ×g] of 7-week-old male Sprague-Dawley rats pre-treated with the commonly used drug-metabolizing enzyme-inducing agents phenobarbital and 5,6-benzoflavone) and cofactor-C (a coenzyme for the chromosome aberration study), purchased from Oriental Yeast Co., Ltd., Tokyo, Japan), was used. In the 6-hr direct method, 4×10^4 cells/5 mL were seeded in a 60-mm-diameter plastic petri dish and incubated in a CO_2 incubator (Yamato Scientific Co., Ltd., Tokyo, Japan). The cell culture medium was removed on day 3 after the start of the culture, and a solution containing either a negative-control substance (water for injection) or test substance was added. After incubation in a CO_2 incubator for 6 hr, the cell culture treatment solution was removed, and the cells were washed with phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(−)] (Life Technologies Corporation, Tokyo, Japan). Fresh cell culture medium was then added. After another 18-hr incubation in the CO_2 incubator, a chromosome specimen was prepared. CHL/IU cells were cultured for the metabolic activation method in a manner similar to the 6-hr treatment direct method. The cell culture medium was removed on day 3 after the start of the culture, and the S9 mix was added, followed by either the negative-control substance solution or test-substance solution. After incubation in a CO_2 incubator for 6 hr, the cell culture treatment medium was removed, and the cells were washed with PBS(−). Fresh cell culture medium was then added. After another 18-hr incubation in the CO_2 incubator, a chromosome specimen was prepared. CHL/IU cells were cultured for the 24-hr direct method in a manner similar to the 6-hr treatment direct method. Similar to the 6-hr treatment direct method, either the negative-control substance solution or the test-substance solution was then added. After incubation for 24 hr in the CO_2 incubator, a chromosome specimen was prepared.

Regarding preparation of the chromosome specimens, approximately 2 hr prior to terminating the culture, Colcemid solution (Life Technologies Corporation, Tokyo, Japan) was added to a final concentration of approximately 0.2 μg/mL to accumulate metaphase cells. After the treatment period, the cell culture medium was transferred into a centrifuge tube. After adding 0.25% trypsin solution (Life Technologies Corporation, Tokyo, Japan), the exfoliated cells were transferred into a centrifuge tube, which was then referred to as the cell suspension. After centrifugation, 0.075 mol/L potassium chloride solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added for hypotonic treatment, and the cells were fixed using a cooled fixing solution (methanol:acetic acid = 3:1, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Two slides per petri dish were prepared. The slides were stained for approximately 15 min with Giemsa solution (1 part undiluted Giemsa solution (Merck Ltd., Germany) mixed with 80 parts Sorensen's buffer [1/15 M PBS, pH 6.8] (Wako Pure Chemical Industries, Ltd., Osaka. Japan)), and the chromosome specimens were then examined. The viable cell count was determined, and using the formulas noted below the population doubling (PD) and the relative population doubling (RPD) when the PD of the negative-control group is set to 100% were calculated.

PD = log(mean cell count at termination of treatment with the test substance/mean cell count prior to treatment)/log2; and

RPD% = (PD value of the treated group)/(PD value of the negative-control group) × 100.

As there was no cytotoxicity in the treated group when

the viable cell count was measured, 5000 μg/mL was selected as the maximum dose in both treatment methods, and a total of three doses (1250, 2500, and 5000 μg/mL) were selected and used to observe the chromosome specimens. The presence or absence of structural aberrations was determined from 150 metaphase cells per petri dish (300 cells in each group) observed under a microscope (Olympus BHT, Olympus Corporation, Tokyo, Japan; 600× magnification, 1000× magnification when identification was not clear). The target of observation was a cell with 25 ± 2 chromosomes. Structural aberrations were classified into the following six types: chromatid and chromosome gap (gap; defined as the achromatic section narrower than the chromatid width); chromatid break (ctb), chromatid exchange (cte); chromosome break (csb); chromosome exchange (cse); and other abnormalities (oth). A total of 150 metaphase cells per petri dish (300 cells in each group) were observed under a microscope (400× magnification). Triploids or more (chromosome count of ≥38) were noted as numerical aberrations of polyploid cells (polyploidy, poly). After excluding cell counts that only exhibited a gap, Fisher's direct probability test was used to test for significant differences in the total number of cells with structural aberrations (−gap) and the number of cells with numerical aberrations (poly) between the negative-control group and other groups. The level of significance was set at 5% or 1% (one-sided). SAS software (SAS Institute Inc., USA) was used for statistical analyses.

The test acceptance criteria were: i) the chromosomal aberration rate in the negative-control group (−gap%, poly%) must be within the mean ± 3 standard deviations (S.D.) of the background data; ii) the structural aberration rate in the positive-control group (−gap%) must be significantly higher than that of the negative-control group; and iii) there must be three or more chromosomal analyses of the treated group.

Results were evaluated for both structural aberrations and numerical (polyploidy) aberrations. A test was considered positive when: i) the rate of occurrence of cells with structural and numerical chromosomal aberrations in the treated group was significantly higher than that of the negative-control group, and ii) there was a dose-dependent effect. The structural aberration rate did not include +gap, and only −gap results were considered.

Bacterial reverse mutation test

The test bacterial strains included Salmonella typhimurium TA98, TA100, TA1535, and TA1537, which were purchased from the National Institute of Health Sciences (Division of Genetics and Mutagenesis, Kawasaki-shi, Kanagawa-ken, Japan), and Escherichia coli strain WP2uvrA, which was purchased from the National Institute of Technology and Evaluation (Tokyo, Japan). Chicken breast extract was serially diluted with water for injection before use for each test. The positive-control substances were as follows: sodium azide (NaN_3, Wako Pure Chemical Industries, Ltd., Osaka, Japan); 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2, Wako Pure Chemical Industries, Ltd., Osaka, Japan); 2-aminoanthracene (2AA, Wako Pure Chemical Industries, Ltd.), and 9-aminoacridine hydrochloride (9AA, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The solvents used were as follows: water for injection (for NaN_3) and dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (for AF-2, 2AA, and 9AA). The maximum dose of 5000 μg/plate, as stated in the guidelines, was used as the dose for the test substance, and a total of seven doses were set as the common ratio of two (78.1, 156, 313, 625, 1250, 2500, and 5000 μg/plate). A negative-control group that was administered water for injection was included in addition to the test-substance and positive-control substance groups. Furthermore, nutrient broth no. 2 (2.5 w/v%; Thermo Fisher Scientific Inc., Tokyo, Japan) was used as the preculture medium for the test bacterial strains, and Tesmedia AN (Oriental Yeast Co., Ltd., Tokyo, Japan) was used as the minimal glucose agar plate medium (Oriental Yeast Co., Ltd., Tokyo, Japan). As the top agar (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.6% agar with 0.5 w/v% sodium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used. For the four strains of S. typhimurium, a solution of 0.5 mmol/L histidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.5 mmol/L biotin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added at a 10:1 ratio. Similarly, for E. coli WP2uvrA, a solution of 0.5 mmol/L tryptophan (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added at a 10:1 ratio.

The reverse mutation test was performed according to the pre-incubation method without metabolic –activation (−S9 mix) and with metabolic –activation (+S9 mix). S9 and cofactor-I (a coenzyme for mutagenicity tests that use microorganisms), purchased from Oriental Yeast Co., Ltd., Tokyo, Japan, were mixed and used as metabolic activators in the +S9 mix. In a sterile test tube, 0.1 mL of negative-control substance solution, test-substance solution, or positive-control substance solution was added. For the −S9 mix, 0.5 mL of 0.1 mol/L sodium-phosphate buffered saline (Na-PBS, pH 7.4, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and for the +S9 mix, 0.5 mL of S9 mix was added, and 0.1 mL of the test bacterial solution was added in both methods. Pre-incubation

with shaking at 100 oscillations per minute for 20 min in a shaking incubator (TIETECH Co., Ltd., Nagoya, Japan) at 37°C was performed, and after adding and mixing 2 mL of top agar, the medium was quickly poured onto a minimal glucose agar plate. The plate was incubated at 37°C for longer than 48 hr (approximately 48 to 52 hr) and then observed.

With respect to observations and measurements, an inverted microscope (Olympus Corporation, Tokyo, Japan) was used to observe the presence/absence and degree of bacterial growth inhibition caused by the test substance on the test bacterial strains on each plate. The presence/absence of precipitation of the test substance was observed by visual inspection. Bacterial growth inhibition of the same bacterial strain and under the same treatment conditions (presence/absence of metabolic activation) were compared with the negative (solvent) control. Decreases in viable bacterial count were determined by the change in the growth densities of background colony (background lawn [BG lawn] where minute colonies can be barely confirmed under a microscope over the entire plate of amino acid auxotrophs and proliferation was arrested). The observation was conducted at a 100× magnification. Colonies formed by reverse mutation on each plate were counted using a colony counter. The number of revertant colonies on each plate and the mean number of colonies for each concentration are shown. The presence/absence of precipitation of the test substance at the times of adding the test substance and counting the revertant colonies and the presence/absence of growth inhibition at the time of counting the revertant colonies were noted. Results were considered positive when the number of revertant colonies in the treated groups were twice or more of the negative-control group with dose-relationship was observed. In contrast, results were considered negative when the number of revertant colonies in the treated group was not more than double compared to the negative-control group.

90-day repeated oral dose study in rats

Both male and female rats were used in the experiments, and specific pathogen–free Crl:CD SD rats at 5 weeks of age were purchased from Charles River Laboratories Japan, Inc., Hino-shi, Japan and quarantined for 1 week. During the quarantine period, the general condition was monitored every day, and body weight was measured. Even after the quarantine period, the general condition was monitored once a day until the day before starting the treatment. Using the Provantis system (Instem LSS Limited, Stone, UK), rats that grew favorably in terms of general condition and body weight were assigned to each group based on the body weight measured on the grouping day. Eighteen male and 18 female rats with body weights close to the mean body weight were assigned to each group. The rats were 6 weeks old at the start of treatment.

The dose of the test substance was considered based on the "Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals" (Pharmaceutical and Food Safety Bureau Notification No. 0219 [4], February 19, 2010). Based on the recommended dose limit, 1000 mg/kg/day was set as the high dose and 500 mg/kg/day as the low dose. The test-substance solution for administration was prepared by suspending the test substance in Japanese Pharmacopoeia (JP) water for injection. The high-dose solution was prepared at 100 mg/mL, and the low-dose solution was prepared at 50 mg/mL. An equivalent amount of JP water for injection was used as the control substance in the control group. Each group had six males and six females. The method of administration was gavage using oral gavage tubes.

During the treatment period, general conditions were monitored twice a day, and body weight was measured 1 day prior to the start of treatment. During the treatment, measurements were taken on days 1, 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, 78, 85, and 91. Food consumption was measured by calculating food consumption per day from the difference between food on the weighing day and the residual food consumption on the next weighing day, and using the calculated food consumption as food consumption on each weighing day. Urine tests were conducted during the week 13 of treatment for the following parameters. Urine volume (Vol), color (Col), specific gravity (SG), pH, protein (Pro), glucose (Glu), ketone bodies (Ket), bilirubin (Bil), occult blood (Occ), urobilinogen (Uro), sediment, sodium (Na), potassium (K), and chloride (Cl) were measured using an automated urine analyzer (CLINITEK Advantus, Siemens Healthcare Diagnostics K.K., Tokyo, Japan), a urine specific gravity refractometer (URC-JE, Atago Co., Ltd., Tokyo, Japan), and a 7180 automatic analyzer (Hitachi 7180; Hitachi High-Technologies Corporation, Tokyo, Japan). A hematology test was conducted at the end of the treatment period (during the necropsy) under anesthesia with inhaled JP isoflurane (Mylan Seiyaku Ltd., Tokyo, Japan) by collecting blood samples from both the left and right jugular veins. Blood samples for complete blood cell counts were collected from the jugular vein on one side using a polypropylene injection syringe and needle containing an anticoagulant (1.5% ethylenediaminetetraacetate-2K, (DOJINDO LABORATORIES, Kumamoto-ken, Japan)) solution.

Blood samples for the coagulation test were collected from the jugular vein on the opposite side using a polypropylene injection syringe and needle containing an anticoagulant (3.2 w/v% sodium citrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan)) solution. The following parameters were measured using an ADVIA 120 Hematology System (Siemens Healthcare Diagnostics K.K., Tokyo, Japan): red blood cells (RBCs), hemoglobin concentration (HGB), hematocrit level (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte count (#Retic), reticulocyte ratio (%Retic), platelet count (PLT), white blood cell count (WBC), neutrophil count (#Neut), neutrophil ratio (%Neut), lymphocyte count (#Lymph), lymphocyte ratio (%Lymph), monocyte count (#Mono), monocyte ratio (%Mono), eosinophil count (#Eos), eosinophil ratio (%Eos), basophil count (#Baso), basophil ratio (%Baso), large unstained cells (#LUCs), and large unstained cell ratio (%LUC). Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured using-Automated Blood Coagulation Analyzer CA-510 (Sysmex Corporation, Kobe, Japan). Upon completion of blood sample collection for the hematology tests, the abdominal cavity of the rats was opened under anesthesia with inhaled isoflurane, and blood samples were collected for biochemical tests from the posterior vena cava using a polypropylene injection syringe and needle containing heparin sodium (Wako Pure Chemical Industries, Ltd., Osaka, Japan). A Hitachi 7180 analyzer was used to measure aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LD), creatine kinase (CK), glucose (GLU), total bilirubin (BIL), urea nitrogen (UN), creatinine (CRE), total cholesterol (CHO), triglycerides (TGs), phospholipids (PLs), inorganic phosphorus (IP), calcium (Ca), Na, K, Cl, total protein (TP), albumin (ALB), and albumin-globulin ratio (A/G). The rats were exsanguinated after blood sampling, and the presence or absence of macroscopic abnormalities was determined for each of the organs and tissues of all rats during necropsy. The weight of the following organs was measured: heart, thymus gland, spleen, bronchial tube, lungs, submandibular gland, major sublingual duct, liver, kidneys, pituitary gland, thyroid gland, parathyroid gland, adrenal glands, testis, epididymis, prostate, seminal vesicle, coagulating gland, ovaries, uterus, and brain. In addition to the organs that were weighed, histopathologic examinations were performed on the thoracic aorta, sternum, sternal bone marrow, femur, knee joint, femural bone marrow, submandibular lymph node, mesenteric lymph nodes, trachea, tongue, parotid gland, esophagus, stomach, duodenum, jejunum, ileum (with-, Peyer's patches), cecum, colon, rectum, pancreas, urinary bladder, vagina, spinal cord (thoracic), sciatic nerve, eyes, optic nerve, Harderian gland, skeletal muscle (thigh), skin (abdominal), and mammary glands (females only). The above-mentioned organs and tissues obtained during the necropsy were fixed by immersion in 10 vol% neutral buffered formalin solution (prior to fixing with 10 vol% neutral buffered formalin solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan), the testes were prefixed by immersion in formalin-sucrose-acetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the eyes and optic nerves were prefixed by immersion in phosphate buffer with 1% formaldehyde (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 2.5% glutaraldehyde (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution). The above-mentioned fixed organs and tissues in the control and high-dose groups as well as the organs and tissues of all necropsied animals with abnormalities were examined microscopically after paraffin sections were prepared using a conventional method and stained with hematoxylin-eosin (Sakura Finetek Japan Co., Ltd., Tokyo, Japan).

The mean and standard deviations (SD) of body weight, feed intake, urine, hematology, and blood biochemistry test results as well as organ weights for each group were calculated and analyzed using Bartlett's test for uniformity of variance (significance level at 5%). The Dunnett test was used to compare the mean values of the control group with those of the treated groups when uniformity of variance was confirmed. In contrast, when uniformity of variance was not observed, a natural logarithmic transformation was applied to the data, and using the post-transformation data, the uniformity of variance was once again tested using Bartlett's test (significance level at 5%). When uniformity of variance was confirmed at this stage, differences in mean values between the control group and each of the treated groups were analyzed using the Dunnett test with the post-transformation data. When uniformity of variance could not be confirmed at this point, Steel's test was performed on the data to determine the difference in mean rank between the control group and each of the treated groups. For the weight of organs measured separately between the left and right sides, statistical analysis was performed only on the total value for both the left and right sides. All tests were two-sided, and variability was determined to be significant when there was a difference with the control group at the 5% significance level. The table shows 5% and 1% differences separately. Data were analyzed using the Provantis program.

RESULTS

Chromosome aberration study using mammalian cells

The RPD value for i) 6-hr treatment direct method with a dose range between 156 and 5000 μg/mL was 100.0 to 130.8%; ii) 24-hr treatment direct method with a dose range between 78.1 and 5000 μg/mL was 92.5 to 119.9%; and iii) metabolic activation method with a dose range between 156 and 5000 μg/mL was 86.4 to 109.1%. None of the above results showed dose-dependent inhibition of cell proliferation (Table 1).

Chromosome specimens for three doses in all treatment methods (the highest dose of 5000 μg/mL and two doses [2500 and 1250 μg/mL]) as well as the negative and positive controls for each of the treated groups were observed (Table 2). Precipitation of the test substance was observed at the time of adding the test substance at 1250 to 5000 μg/mL and at the termination of treatment with the test substance at 78.1 to 5000 μg/mL.

The structural aberration rate (−gap%) and polyploidy rate (poly%) in the 6-hr treatment direct method with a dose range of 1250 to 5000 μg/mL were 0.33% and 0.00 to 1.00%, respectively. Compared to the negative-control group (0.33 and 1.00%), there was no statistically significant increase.

The structural aberration rate (−gap%) and the polyploidy rate (poly%) in the 24-hr treatment direct method with a dose range of 1250 to 5000 μg/mL were 0.00 or 0.33% and 0.00 to 1.00%, respectively. Compared to the negative-control group (both 0.00%), there was no statistically significant increase.

The structural aberration rate (−gap%) and the polyploidy rate (poly%) in the metabolic activation method with a dose range of 1250 to 5000 μg/mL were 0.00 or 0.33% and 0.00 or 1.00%, respectively. Compared to the negative-control group (0.67 and 0.00%), there was no statistically significant increase.

According to the metabolic activation method, the structural aberration rate (−gap%) of the positive-control group (B[a]P) was 51.67%, and a statistically significant increase was shown compared to the negative-control group. The structural aberration rate (−gap%) and the polyploidy rate (poly%) in the negative-control group were within the mean ± 3SD of the test facility's background data.

Bacterial reverse mutation test

Regardless of the presence/absence of metabolic activation, none of the tested bacterial strains exhibited a dose-related increases in the number of revertant colonies twice or more of compared with the negative-control group. There was also no growth inhibitory effect against the bacterial strains (Table 3). Precipitation of the test substance was observed in the following dose ranges: in the −S9 mix, 156 to 5000 μg/plate at the time of adding the test substance and 1250 to 5000 μg/plate during plate observation; in the +S9 mix, 313 to 5000 μg/plate at the time of adding the test substance and 1250 to 5000 μg/plate during plate observation.

90-day repeated oral dose study

Throughout the observation period, there were no abnormalities in general conditions or deaths noted in any of the treated groups. Crust formation (behind the neck) was observed in one male rat in the 1000 mg/kg/day group, which started on day 89 and continued until the day of necropsy. However, this change was only observed in one case and not in the other rats of the same group. As this was a change also observed in the testing facility's background values, we assumed that there was no association with the administration of the test substance. The results of body weight and feed intake measurements are shown in Figs. 1 and 2. There were no variations in body weight or feed intake that were attributed to the administration of the test substance. Furthermore, there were no significant variations compared to the control group.

According to the urine test results in Table 4, significantly higher urine volume was observed in male rats in the 500 mg/kg/day group compared to the control group; however, there was no association with dose. Positive urine protein (+++) was detected in one male rat in the 1000 mg/kg/day group; however, the blood biochemistry test, urine test, and histopathologic examination results, which will be described in a subsequent section, exhibited no changes that suggested an effect on the kidneys and urinary tract.

The results of the hematology and blood biochemistry tests are shown in Tables 5 and 6. While PT in the hematology test was significantly higher in male rats in the 1000 mg/kg/day group compared to the control group, there were no variations in APTT or PLT, and no bleeding tendencies were observed. The values were within the range of the test facility's background values. In the blood biochemistry test, there was a significant decrease in BIL in male rats in the 500 and 1000 mg/kg/day groups compared to the control group. However, this may have been a coincidental significant difference due to high levels in the control group, as there was one case in the control group that demonstrated high levels exceeding the test facility's background value. AST levels were also significantly higher among female rats in the 500 and 1000

Table 1-1. Chromosome aberration study of chicken extract using mammalian cultured cells-viable cell counting-Direct method

	6 hr					
Dose (μg/mL)	Viable cell concentration ($\times10^5$cells/mL)	Volume (mL)	Viable cell count ($\times10^5$cells/volume)	Mean viable cell count ($\times10^5$cells)	PD	RPD (%)
Data prior to treatment	1.2	1.0	1.2			
	1.2	1.0	1.2	1.2		
0[a)]	2.7	1.0	2.7			
	2.4	1.0	2.4	2.6	1.1	100.0
156†	2.8	1.0	2.8			
	2.3	1.0	2.3	2.6	1.1	100.0
313†	2.8	1.0	2.8			
	3.0	1.0	3.0	2.9	1.3	114.1
625†	3.4	1.0	3.4			
	3.1	1.0	3.1	3.3	1.5	130.8
1250††	2.8	1.0	2.8			
	3.4	1.0	3.4	3.1	1.4	122.7
2500††	3.1	1.0	3.1			
	2.7	1.0	2.7	2.9	1.3	114.1
5000††	2.6	1.0	2.6			
	2.9	1.0	2.9	2.8	1.2	109.6

Table 1-2. Chromosome aberration study of chicken extract using mammalian cultured cells-viable cell counting-Direct method

	24 hr					
Dose (μg/mL)	Viable cell concentration ($\times10^5$cells/mL)	Volume (mL)	Viable cell count ($\times10^5$cells/volume)	Mean viable cell count ($\times10^5$cells)	PD	RPD (%)
Data prior to treatment	1.2	1.0	1.2			
	1.2	1.0	1.2	1.2		
0[a)]	2.5	1.0	2.5			
	3.4	1.0	3.4	3.0	1.3	100.0
78.1†	3.7	1.0	3.7			
	2.6	1.0	2.6	3.2	1.4	107.0
156†	4.1	1.0	4.1			
	3.1	1.0	3.1	3.6	1.6	119.9
313†	2.9	1.0	2.9			
	2.6	1.0	2.6	2.8	1.2	92.5
625†	3.4	1.0	3.4			
	2.9	1.0	2.9	3.2	1.4	107.0
1250††	2.9	1.0	2.9			
	2.8	1.0	2.8	2.9	1.3	96.3
2500††	3.4	1.0	3.4			
	2.9	1.0	2.9	3.2	1.4	107.0
5000††	3.7	1.0	3.7			
	2.8	1.0	2.8	3.3	1.5	110.4

Table 1-3. Chromosome aberration study of chicken extract using mammalian cultured cells-viable cell counting-
Metabolic activation method

	+S9 treatment					
Dose (μg/mL)	Viable cell concentration ($\times10^5$cells/mL)	Volume (mL)	Viable cell count ($\times10^5$cells/volume)	Mean viable cell count ($\times10^5$cells)	PD	RPD (%)
Data prior to treatment	1.2	1.0	1.2			
	1.2	1.0	1.2	1.2		
0[a)]	3.2	1.0	3.2			
	3.1	1.0	3.1	3.2	1.4	100.0
156††	3.6	1.0	3.6			
	3.3	1.0	3.3	3.5	1.5	109.1
313†	2.8	1.0	2.8			
	2.7	1.0	2.7	2.8	1.2	86.4
625†	3.1	1.0	3.1			
	2.8	1.0	2.8	3.0	1.3	93.4
1250††	2.8	1.0	2.8			
	2.8	1.0	2.8	2.8	1.2	86.4
2500††	3.3	1.0	3.3			
	2.6	1.0	2.6	3.0	1.3	93.4
5000††	3.1	1.0	3.1			
	3.2	1.0	3.2	3.2	1.4	100.0

a): Nagative control, Water for injection
†: Precipitation (at the start of treatment)
††: Pricipitation (at the start and end of treatment)
PD (Population doubling)=log(mean viable cell count at termination of treatment/mean viable cell count pior to treatment)/log2
RPD (Relative population doubling)=PD value in the treated group/PD value in the negative control group×100

Table 2. Chromosome aberration study of chicken extract using mammalian cultured cells-chromosome aberration test-
Direct method

Treatment time[a)](hr)	Dose (μg/mL)	RPD (%)	Number of cells	Number of cells with structural aberrations[b)]								Number of polyploid cells (%)
				gap	ctb	cte	csb	cse	oth	+gap(%)	-gap(%)	
6-18	0[c)]	100.0	300	0	1	0	0	0	0	1(0.33)	1(0.33)	3(1.00)
	1250††	122.7	300	0	1	0	0	0	0	1(0.33)	1(0.33)	2(0.67)
	2500††	114.1	300	2	1	0	0	0	0	3(1.00)	1(0.33)	0(0.00)
	5000††	109.6	300	0	1	0	0	0	0	1(0.33)	1(0.33)	3(1.00)
24-0	0[c)]	100.0	300	0	0	0	0	0	0	0(0.00)	0(0.00)	0(0.00)
	1250††	96.3	300	0	0	0	0	0	0	0(0.00)	0(0.00)	1(0.33)
	2500††	107.0	300	0	1	0	0	0	0	1(0.33)	1(0.33)	0(0.00)
	5000††	110.4	300	2	0	0	0	0	0	2(0.67)	0(0.00)	3(1.00)

Metabolic activation method

Treatment time[a)](hr)	Dose (μg/mL)	RPD (%)	Number of cells	Number of cells with structural aberrations[b)]								Number of polyploid cells (%)
				gap	ctb	cte	csb	cse	oth	+gap(%)	-gap(%)	
6-18	0[c)]	100.0	300	1	1	1	1	0	0	3(1.00)	2(0.67)	0(0.00)
	1250††	86.4	300	0	0	0	1	0	0	1(0.33)	1(0.33)	0(0.00)
	2500††	93.4	300	0	0	0	0	0	0	0(0.00)	0(0.00)	0(0.00)
	5000††	100.0	300	0	0	1	0	0	0	1(0.33)	1(0.33)	3(1.00)
	B[a]P30[d)]	-	300	0	14	144	5	0	0	155(51.67)	155(51.67)**	1(0.33)

a): Treatment time-Recovery time
b): gap; Chromatid and chromosome gap, ctb; Chromatid break, cte; Chromatid exchange, csb; Chromosome break, cse; Chromosome exchange, oth; Multiple aberrant cells, fragmentation, etc.
c): Nagative control, Water for injection
d): Positive control, benzo[a]pyrene
RPD: Relative population doubling
††: Precipitation (at the start and end of treatment)
＊＊：$P < 0.01$, significantly different from the negative control (Fisher's exact test).

Table 3. Reverse mutation test results.

S9 mix		Dose (μg/plate)	Number of revertant colonies/plate (Mean)				
			Base-pair subsitiution type			Frameshift type	
			TA100	TA1535	WP2*uvrA*	TA98	TA1537
-		0[a)]	127	9	27	21	17
		78.1	130	7	20	23	17
		156†	127	10	27	15	17
		313†	123	7	25	19	17
		625†	132	9	19	15	15
		1250††	131	9	24	22	12
		2500††	120	5	28	32	17
		5000††	124	9	24	25	21
+		0[a)]	146	14	31	32	19
		78.1	157	9	27	30	19
		156	149	11	24	28	19
		313†	149	9	25	37	18
		625†	151	11	28	26	18
		1250††	156	10	29	31	19
		2500††	153	15	33	33	19
		5000††	152	13	26	33	18
Positive control	-	Substance	AF-2[b)]	NaN_3[c)]	AF-2	AF-2	9AA[d)]
		Dose (μg/plate)	0.01	0.5	0.01	0.1	80
			865	514	142	403	259
	+	Substance	2AA[e)]	2AA	2AA	2AA	2AA
		Dose (μg/plate)	1	2	10	0.5	2
			1080	231	834	286	78

a): Negative control, Water of injection
b): 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide
c): Sodium azide
d): 9-Aminoacridine hydrochloride
e): 2-Aminoanthracene
†: Precipitation (at the start of treatment)
††: Pricipitation (at the start and end of treatment)

mg/kg/day groups; however, the degree of difference was minimal, and other measurement parameters did not show an effect on the liver. There were also no histologic changes in the liver according to the histopathologic examination.

Tables 7, 8, and 9 show the necropsy, organ weight, and pathologic examination results. After termination of the treatment, crust formation was observed, reflecting the general condition in one male rat in the 1000 mg/kg/day group. One male rat in the control group had a soft testis (unilateral). In the pathologic examination of these specimens, histologically, there was crust formation on the skin and diffuse atrophy of the seminiferous tubule in the testis. Regarding organ weight measurements, while there was a significantly higher organ-to-body weight ratio for the spleen among male rats in the 1000 mg/kg/day group compared to the control group, there was no difference in actual weight. There were no histologic changes suggesting variability in weight, and there was no effect on the hematopoietic system according to the hematology test results described above. Significantly higher actual weight and organ-to-body weight ratio of the thymus gland were observed among the female rats in the 500 mg/kg/day group, however, there were no associations with dose.

DISCUSSION

To evaluate the safety of chicken breast extract containing plasmalogen, the following three tests were conducted: i) chromosome aberration study using mammalian cells; ii) bacterial reverse mutation test; and iii) 90-day repeated oral dose study in rats.

The chromosome aberration study was conducted using CHL/IU cells according to the metabolic activation method (6-hr treatment) and the direct method (6- and 24-hr treatments). The examination of chromosome specimens showed that there was no statistically significant increase in the structural aberration rate or the numerical aberration rate in the treated groups in all treatment methods compared to the respective negative-control groups. Chromosome analyses in all treated groups were conducted at three doses, and there was a statistically significant increase in the structural aberration rate of the positive-control group compared to the negative-control

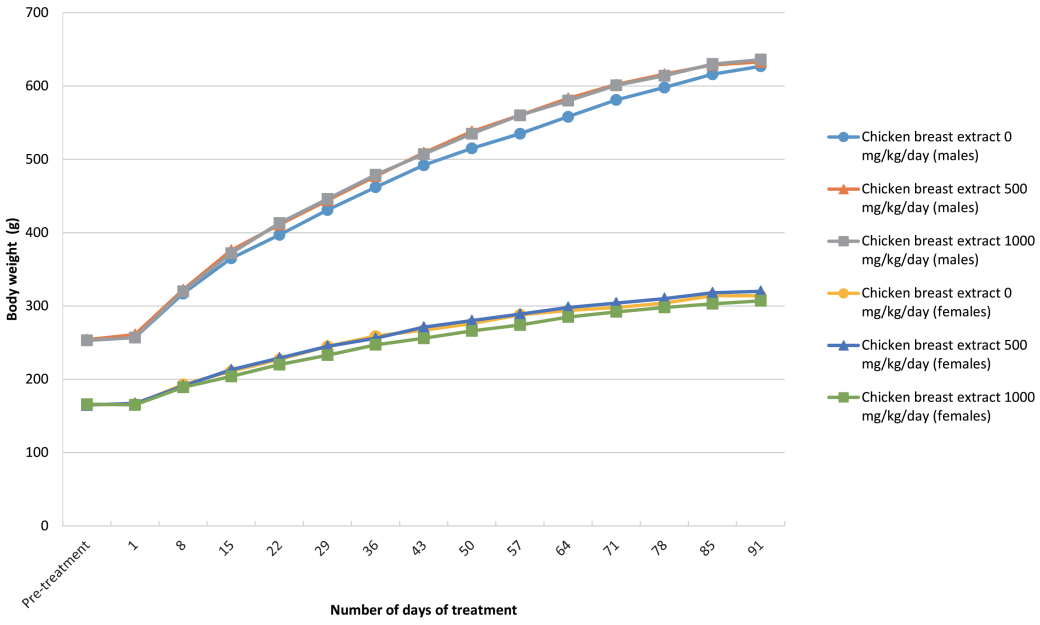

Fig. 1. Change in body weight of rats in the 90-day repeated oral dose study.

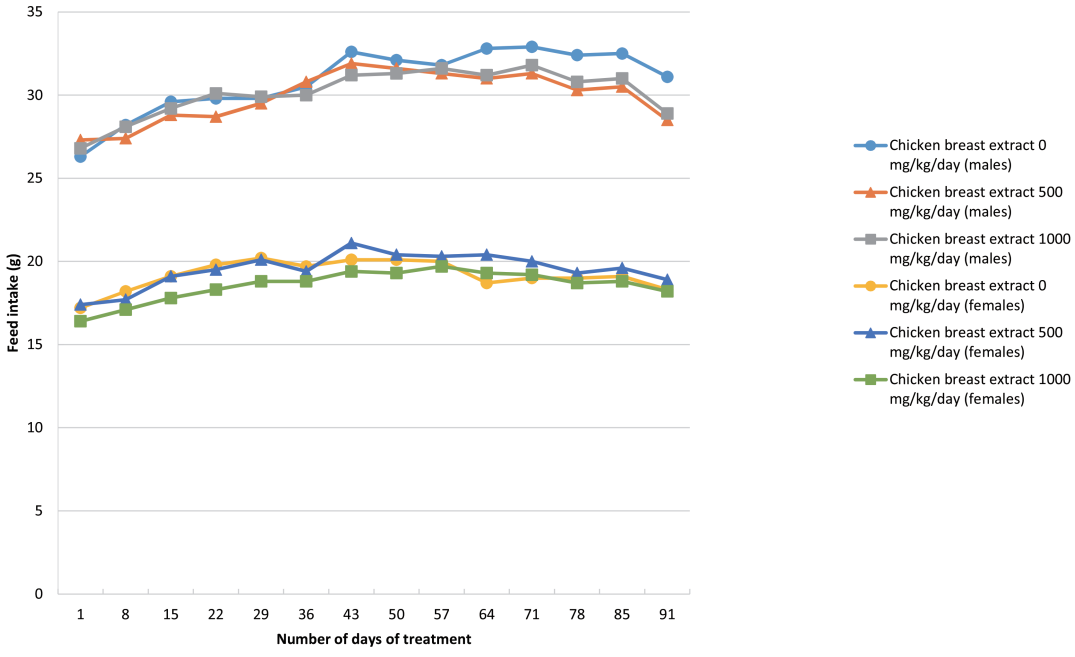

Fig. 2. Change in feed intake of rats in the 90-day repeated oral dose study.

group. Both the structural aberration rate and numerical aberration rate in the negative control group were within the range of the test facility's background data. In other words, chicken breast extract did not induce chromosomal aberrations in CHL/IU cells.

In the bacterial reverse mutation test, there was no dose-related increase in the number of revertant colonies (i.e., twice or more of the negative control) for any of the

Table 4-1. Urine test results of the 90-day repeated oral dose study.

Sex	Test substance	Dose (mg/kg/day)	No. of rats	Vol (mL/day)	S.G	NA (mEq/day)	K (mEq/day)	CL (mEq/day)
Males	Water for injection	0	6	23.2 ± 4.7	1.044 ± 0.012	2.14 ± 0.21	4.49 ± 0.28	2.68 ± 0.34
	Chicken breast extract	500	6	35.4 ± 9.6 *	1.028 ± 0.007	2.02 ± 0.27	4.02 ± 0.70	2.33 ± 0.25
		1000	6	21.4 ± 9.5	1.053 ± 0.015	2.21 ± 0.29	4.70 ± 0.71	2.74 ± 0.23
Females	Water for injection	0	6	16.2 ± 6.7	1.045 ± 0.009	1.75 ± 0.44	3.29 ± 0.73	2.12 ± 0.55
	Chicken breast extract	500	6	17.3 ± 7.2	1.044 ± 0.017	1.71 ± 0.24	3.13 ± 0.38	2.02 ± 0.29
		1000	6	12.9 ± 4.0	1.049 ± 0.015	1.44 ± 0.33	2.80 ± 0.45	1.85 ± 0.43

Significant difference between the control (water for injection) and each measured value: * $p < 0.05$

Table 4-2. Urine test results of the 90-day repeated oral dose study.

Sex	Test substance	Dose (mg/kg/day)		Yellow	L.T.Yellow	pH 8.0	pH 8.5	pH ≧ 9.0	Protein-	Protein+/-	Protein+	Protein++	Protein+++	Glucose-	Ketones-	Ketones+/-	Ketones+	Bilirubin-
Males	Water for injection	0	(N)	5	1	0	5	1	0	4	2	0	0	6	0	4	2	6
	Chicken breast extract	500	(N)	1	5	0	5	1	3	2	1	0	0	6	2	4	0	6
		1000	(N)	5	1	0	4	2	0	4	1	0	1	6	1	2	3	6
Females	Water for injection	0	(N)	5	1	0	5	1	4	1	1	0	0	6	3	3	0	6
	Chicken breast extract	500	(N)	4	2	1	3	2	5	1	0	0	0	6	3	3	0	6
		1000	(N)	6	0	1	4	1	2	2	1	1	0	6	1	2	3	5

Table 4-3. Urine test results of the 90-day repeated oral dose study.

Sex	Test substance	Dose (mg/kg/day)		Bilirubin+	Occ.-	Occ.+/-	Occ.+	Uro.0.1	Uro.1.0	Uro.2.0	RBC-	WBC-	WBC+/-	EC-	Crystal-	Crystal+	Cast-	Other-
Males	Water for injection	0	(N)	0	5	1	0	6	0	0	6	6	0	6	4	2	6	6
	Chicken breast extract	500	(N)	0	4	2	0	6	0	0	6	6	0	6	6	0	6	6
		1000	(N)	0	4	1	1	4	2	0	6	6	0	6	3	3	6	6
Females	Water for injection	0	(N)	0	6	0	0	3	3	0	6	5	1	6	5	1	6	6
	Chicken breast extract	500	(N)	0	6	0	0	3	3	0	6	6	0	6	5	1	6	6
		1000	(N)	1	6	0	0	0	5	1	6	5	1	6	5	1	6	6

Table 5-1. Hematology test results of the 90-day repeated oral dose study.

Sex	Test substance	Dose (mg/kg/day)	No. of rats	RBC (10^6/μL)	HGB (g/dL)	HTC (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Retic (%)	Retic (10^9/μL)	PLT (10^3/μL)	WBC (10^3/μL)	PT (S)	APTT (S)
Males	Water for injection	0	6	9.12 ± 0.30	16.0 ± 0.5	48.7 ± 1.1	53.4 ± 1.4	17.6 ± 0.5	32.9 ± 0.5	1.8 ± 0.2	164.6 ± 14.2	1051 ± 151	10.82 ± 1.51	13.5 ± 2.2	19.7 ± 0.9
	Chicken breast extract	500	6	9.46 ± 0.31	16.2 ± 0.4	49.7 ± 1.5	52.6 ± 1.9	17.2 ± 0.6	32.6 ± 0.3	1.9 ± 0.2	174.4 ± 24.9	1010 ± 49	10.83 ± 2.12	15.0 ± 1.2	20.3 ± 1.1
		1000	6	9.11 ± 0.41	15.9 ± 0.5	48.8 ± 2.2	53.7 ± 1.9	17.5 ± 0.6	32.6 ± 0.6	2.0 ± 0.1	181.3 ± 16.2	1010 ± 102	10.53 ± 2.95	16.0 ± 0.9*	20.7 ± 0.3
Females	Water for injection	0	6	8.41 ± 0.47	15.7 ± 0.5	46.2 ± 1.9	55.0 ± 1.1	18.7 ± 0.6	33.9 ± 0.4	1.7 ± 0.2	137.1 ± 15.3	1099 ± 130	7.05 ± 1.15	9.3 ± 0.0	16.6 ± 0.7
	Chicken breast extract	500	6	8.44 ± 0.26	15.7 ± 0.4	46.3 ± 1.1	54.9 ± 1.3	18.5 ± 0.4	33.8 ± 0.4	1.8 ± 0.3	152.8 ± 22.8	1083 ± 107	7.02 ± 1.52	9.3 ± 0.1	16.6 ± 0.8
		1000	6	8.43 ± 0.25	15.8 ± 0.3	47.0 ± 0.9	55.8 ± 0.8	18.8 ± 0.3	33.6 ± 0.3	1.9 ± 0.5	156.9 ± 41.7	1047 ± 114	7.06 ± 1.39	9.1 ± 0.3	16.6 ± 0.8

Significant difference between the control (water for injection) and each measured value: * $p < 0.05$

Table 5-2. Hematology test results of the 90-day repeated oral dose study

Sex	Test substance	Dose (mg/kg/day)	No. of rats	Neut (%)	Lymph (%)	Mono (%)	Eso (%)	Baso (%)	LUC (%)	Neut (10^3/μL)	Lymph (10^3/μL)	Mono (10^3/μL)	Eso (10^3/μL)	Baso (10^3/μL)	LUC (10^3/μL)
Males	Water for injection	0	6	18.4 ± 3.7	75.0 ± 3.9	3.9 ± 1.3	1.4 ± 0.7	0.3 ± 0.1	1.1 ± 0.4	1.97 ± 0.38	8.12 ± 1.24	0.43 ± 0.17	0.16 ± 0.08	0.03 ± 0.01	0.12 ± 0.05
	Chicken breast extract	500	6	16.6 ± 1.4	75.9 ± 2.4	4.4 ± 1.0	1.7 ± 0.5	0.3 ± 0.1	1.1 ± 0.5	1.80 ± 0.37	8.22 ± 1.58	0.48 ± 0.17	0.18 ± 0.07	0.04 ± 0.02	0.12 ± 0.05
		1000	6	14.5 ± 3.4	78.4 ± 3.3	4.3 ± 0.8	1.7 ± 0.5	0.3 ± 0.1	1.0 ± 0.6	1.52 ± 0.53	8.24 ± 2.39	0.47 ± 0.23	0.17 ± 0.03	0.03 ± 0.02	0.10 ± 0.05
Females	Water for injection	0	6	18.9 ± 12.2	74.0 ± 11.2	4.2 ± 1.1	1.7 ± 0.5	0.2 ± 0.1	1.0 ± 0.5	1.44 ± 1.23	5.12 ± 0.36	0.29 ± 0.07	0.13 ± 0.05	0.02 ± 0.01	0.07 ± 0.04
	Chicken breast extract	500	6	10.7 ± 1.9	82.6 ± 3.1	3.5 ± 1.6	1.8 ± 0.6	0.2 ± 0.1	1.1 ± 0.5	0.74 ± 0.20	5.78 ± 1.20	0.26 ± 0.17	0.13 ± 0.05	0.02 ± 0.01	0.08 ± 0.04
		1000	6	13.1 ± 4.9	80.2 ± 5.0	3.7 ± 0.5	1.9 ± 0.5	0.2 ± 0.1	1.1 ± 0.3	0.92 ± 0.42	5.67 ± 1.25	0.26 ± 0.06	0.13 ± 0.02	0.02 ± 0.01	0.08 ± 0.03

Table 6-1. Blood biochemistry test results of the 90-day repeated oral dose study.

Sex	Test substance	Dose (mg/kg/day)	No. of rats	AST (U/L)	ALT (U/L)	ALP (U/L)	LD (U/L)	CK (U/L)	GLU (mg/dL)	BIL (mg/dL)	UN (mg/dL)	CRE (mg/dL)	CHO (mg/dL)
Males	Water for injection	0	6	62 ± 5	25 ± 3	273 ± 35	116 ± 40	108 ± 12	146 ± 23	0.07 ± 0.02	14.9 ± 1.2	0.37 ± 0.03	55 ± 8
	Chicken breast extract	500	6	69 ± 10	27 ± 4	281 ± 63	127 ± 35	121 ± 20	146 ± 17	0.06 ± 0.01*	13.1 ± 1.5	0.38 ± 0.03	47 ± 12
		1000	6	62 ± 7	24 ± 1	238 ± 37	107 ± 28	102 ± 16	138 ± 18	0.06 ± 0.01*	13.5 ± 1.3	0.36 ± 0.05	43 ± 6
Females	Water for injection	0	6	57 ± 5	20 ± 3	128 ± 26	130 ± 34	104 ± 16	115 ± 9	0.07 ± 0.02	15.4 ± 1.8	0.43 ± 0.04	57 ± 6
	Chicken breast extract	500	6	66 ± 5*	24 ± 5	121 ± 29	132 ± 31	103 ± 25	119 ± 14	0.07 ± 0.01	15.5 ± 1.9	0.41 ± 0.04	56 ± 9
		1000	6	66 ± 7*	25 ± 6	124 ± 27	144 ± 29	104 ± 12	127 ± 16	0.08 ± 0.02	15.1 ± 2.8	0.41 ± 0.05	54 ± 10

Significant difference between the control (water for injection) and each measured value: * $p<0.05$

Table 6-2. Blood biochemistry test results of the 90-day repeated oral dose study.

Sex	Test substance	Dose (mg/kg/day)	No. of rats	TG (mg/dL)	PL (mg/dL)	IP (mg/dL)	CA (mg/dL)	NA (mEq/L)	K (mEq/L)	CL (mEq/L)	TP (g/dL)	ALB (g/dL)	A/G
Males	Water for injection	0	6	67 ± 31	100 ± 14	6.29 ± 0.61	9.93 ± 0.42	146.7 ± 2.0	3.45 ± 0.20	106.1 ± 2.3	5.88 ± 0.25	2.12 ± 0.08	0.56 ± 0.04
	Chicken breast extract	500	6	53 ± 30	86 ± 11	5.99 ± 0.21	10.09 ± 0.18	147.5 ± 1.0	3.43 ± 0.14	106.6 ± 1.1	6.07 ± 0.14	2.18 ± 0.07	0.56 ± 0.04
		1000	6	63 ± 37	82 ± 13	5.87 ± 0.39	10.10 ± 0.17	146.6 ± 1.3	3.53 ± 0.22	105.9 ± 1.8	5.94 ± 0.18	2.11 ± 0.08	0.55 ± 0.04
Females	Water for injection	0	6	10 ± 4	115 ± 6	5.64 ± 0.48	9.99 ± 0.20	146.3 ± 1.2	3.25 ± 0.14	107.7 ± 1.5	6.26 ± 0.27	2.59 ± 0.15	0.71 ± 0.03
	Chicken breast extract	500	6	12 ± 8	110 ± 21	5.24 ± 0.55	9.89 ± 0.38	146.5 ± 1.2	3.32 ± 0.33	109.0 ± 0.9	5.99 ± 0.43	2.43 ± 0.21	0.68 ± 0.04
		1000	6	11 ± 2	107 ± 20	5.69 ± 0.48	9.97 ± 0.41	146.6 ± 0.6	3.38 ± 0.32	108.2 ± 1.4	6.09 ± 0.32	2.44 ± 0.15	0.67 ± 0.02

Table 7. Necropsy results of the 90-day repeated oral dose study.

Organ・organization	Views	Organs/Tissues	Findings	Sex	Males			Females		
				Test substance	Water for injection	Chicken breast extract		Water for injection	Chicken breast extract	
				Dose (mg/kg/day)	0	500	1000	0	500	1000
				No. of rats	6	6	6	6	6	6
Testis	Soft; unilateral	Testis	Soft; unilateral		1	-	-	-	-	-
Skin	Crust fomation	Skin	Crust formation		-	-	1	-	-	-

Necropsies were performed on all rats at the end of the study.

Table 8-1. Organ weight examination results of the 90-day repeated oral dose study.

Sex	Test substance	Dose (mg/kg/day)	No. of rats	Final Bodyweght (g)	Brain (g)	Brain (%)	Pituitary (mg%)	Thyroid (mg)	Thyroid (mg)	Thyroid (mg%)	Submandibular gland (g)	Submandibular gland (%)	Thymus (mg)	Thymus (mg%)	Lung (g)	Lung (%)
Males	Water for injection	0	6	595 ± 55	2.26 ± 0.09	0.382 ± 0.032	12 ± 1	2.1 ± 0.2	27 ± 4	4.5 ± 0.7	0.835 ± 0.101	0.140 ± 0.011	366 ± 160	60.4 ± 22.9	1.71 ± 0.10	0.290 ± 0.034
	Chicken breast extract	500	6	606 ± 33	2.30 ± 0.08	0.380 ± 0.019	13 ± 2	2.1 ± 0.3	32 ± 5	5.3 ± 0.7	0.804 ± 0.089	0.133 ± 0.011	338 ± 91	55.9 ± 15.3	1.74 ± 0.09	0.288 ± 0.021
		1000	6	612 ± 82	2.21 ± 0.05	0.367 ± 0.055	14 ± 1	2.3 ± 0.2	31 ± 2	5.1 ± 0.7	0.867 ± 0.109	0.143 ± 0.017	354 ± 74	57.4 ± 5.6	1.80 ± 0.14	0.297 ± 0.022
Females	Water for injection	0	6	301 ± 34	1.99 ± 0.04	0.666 ± 0.064	15 ± 2	5.0 ± 0.9	18 ± 5	6.1 ± 1.6	0.474 ± 0.060	0.159 ± 0.022	282 ± 30	95.0 ± 15.6	1.15 ± 0.09	0.386 ± 0.038
	Chicken breast extract	500	6	306 ± 37	2.07 ± 0.06	0.684 ± 0.081	16 ± 2	5.2 ± 0.9	22 ± 4	7.2 ± 1.7	0.476 ± 0.039	0.158 ± 0.023	371 ± 75	122.1 ± 26.6*	1.20 ± 0.08	0.398 ± 0.052
		1000	6	295 ± 23	2.07 ± 0.07	0.706 ± 0.072	16 ± 2	5.3 ± 0.6	20 ± 1	6.9 ± 1.0	0.466 ± 0.034	0.158 ± 0.010	247 ± 22	84.6 ± 11.9	1.17 ± 0.08	0.399 ± 0.034

Significant difference between the control (water for injection) and each measured value: * $p < 0.05$

Table 8-2. Organ weight examination results of the 90-day repeated oral dose study.

Sex	Test substance	Dose (mg/kg/day)	No. of rats	Heart (g)	Heart (%)	Liver (g)	Liver (%)	Spleen (g)	Spleen (%)	Adrenal Total (mg)	Adrenal Total (mg%)	Kideny Total (g)	Kideny Total (%)	Testis Total (g)	Testis Total (%)
Males	Water for injection	0	6	1.712 ± 0.195	0.289 ± 0.033	14.9 ± 1.9	2.490 ± 0.102	0.830 ± 0.104	0.139 ± 0.007	66 ± 6	11.1 ± 1.3	3.65 ± 0.40	0.613 ± 0.023	3.45 ± 0.24	0.585 ± 0.072
	Chicken breast extract	500	6	1.743 ± 0.152	0.288 ± 0.020	15.6 ± 0.9	2.585 ± 0.190	0.893 ± 0.112	0.148 ± 0.017	60 ± 7	10.0 ± 1.3	3.65 ± 0.18	0.604 ± 0.039	3.68 ± 0.20	0.609 ± 0.034
		1000	6	1.739 ± 0.151	0.286 ± 0.024	16.4 ± 2.6	2.678 ± 0.181	0.965 ± 0.107	0.158 ± 0.010*	61 ± 10	10.1 ± 2.6	3.86 ± 0.24	0.636 ± 0.059	3.61 ± 0.36	0.596 ± 0.072
Females	Water for injection	0	6	0.927 ± 0.051	0.310 ± 0.022	7.3 ± 0.8	2.414 ± 0.155	0.506 ± 0.075	0.170 ± 0.027	67 ± 11	22.2 ± 2.2	1.91 ± 0.16	0.640 ± 0.074	-	-
	Chicken breast extract	500	6	0.922 ± 0.090	0.302 ± 0.010	7.6 ± 0.9	2.476 ± 0.124	0.568 ± 0.092	0.187 ± 0.029	77 ± 7	25.2 ± 3.1	2.06 ± 0.18	0.674 ± 0.033	-	-
		1000	6	0.874 ± 0.081	0.297 ± 0.019	7.4 ± 0.8	2.527 ± 0.232	0.518 ± 0.109	0.175 ± 0.025	75 ± 9	25.5 ± 2.1	1.94 ± 0.18	0.660 ± 0.049	-	-

Significant difference between the control (water for injection) and each measured value: * $p < 0.05$

Table 8-3. Organ weight examination results of the 90-day repeated oral dose study.

Sex	Test substance	Dose (mg/kg/day)	No. of rats	Epididymis Total (g)	Epididymis Total (%)	Prostate (g)	Prostate (%)	Seminal Vesicle (g)	Seminal Vesicle (%)	Ovary Total (mg)	Ovary Total (mg%)	Uterus (g)	Uterus (%)
Males	Water for injection	0	6	1.43 ± 0.23	0.240 ± 0.029	0.737 ± 0.068	0.125 ± 0.017	2.44 ± 0.13	0.414 ± 0.048	-	-	-	-
	Chicken breast extract	500	6	1.50 ± 0.08	0.248 ± 0.009	0.590 ± 0.175	0.098 ± 0.028	2.40 ± 0.34	0.396 ± 0.055	-	-	-	-
		1000	6	1.54 ± 0.09	0.254 ± 0.030	0.620 ± 0.114	0.103 ± 0.023	2.44 ± 0.26	0.402 ± 0.055	-	-	-	-
Females	Water for injection	0	6	-	-	-	-	-	-	92 ± 18	30.2 ± 3.3	0.56 ± 0.09	0.19 ± 0.04
	Chicken breast extract	500	6	-	-	-	-	-	-	87 ± 14	28.6 ± 4.8	0.71 ± 0.34	0.24 ± 0.12
		1000	6	-	-	-	-	-	-	81 ± 15	27.7 ± 6.2	0.83 ± 0.44	0.28 ± 0.13

Table 9. Histopathologic examination results of the 90-day repeated oral dose study.

Organ・organization	Views		Sex	Males			Females		
			Test substance	Water for injection	Chicken breast extract		Water for injection	Chicken breast extract	
			Dose (mg/kg/day)	0	500	1000	0	500	1000
			No. of rats	6	6	6	6	6	6
Heart	infiltrate,mononuclear cell	±		1	NA	2	0	NA	0
Spleen	extramedullary hematopoiesis	±		3	NA	3	2	NA	1
Lung (Including Bronchus)	alveolar macrophage aggregation	±		1	NA	1	0	NA	0
	metaplasia osseous	P		1	NA	1	0	NA	1
Submandibular Gland	infiltrate, mononuclear cells	±		0	NA	1	0	NA	0
Pancreas	atrophy, aciner cells, focal	±		1	NA	0	0	NA	0
	infiltrate, mononuclear cells	±		0	NA	1	0	NA	0
Liver	microgranuloma	±		5	NA	5	5	NA	4
		+		1	NA	1	0	NA	0
Kidney	cast, hyaline	P		0	NA	0	1	NA	1
	infiltrate, inflammatory cells	±		0	NA	1	0	NA	0
	infiltrate, mononuclear cells	±		1	NA	2	1	NA	0
	cyst	P		0	NA	1	0	NA	0
	basophilia, tubular	±		3	NA	2	0	NA	0
Thyroid	ectopic tissue, thymus	P		0	NA	0	0	NA	1
	ultimobranchial cyst	P		1	NA	2	1	NA	1
Testis	atrophy, tubular, diffuse	+++		1	NA	0	NA	NA	NA
Prostate (Ventral)	infiltrate, mononuclear cells	±		2	NA	5	NA	NA	NA
Uterus	cyst, squamous	P		NA	NA	NA	1	NA	0
Eye	dysplasia, retina	P		0	NA	0	1	NA	1
Harderian Gland	infiltrate, mononuclear cells	±		1	NA	0	0	NA	0
Skin (Abdominal)	infiltrate, mononuclear cells	±		0	NA	0	0	NA	1
Skin	crust fomation	+		NA	NA	1	NA	NA	NA

Test results were: no change (-), very minor change (±), minor change (+), moderate change (++), notable change (+++), appearance (P)

bacterial strains examined, regardless of the presence or absence of metabolic activation. The number of revertant colonies in the negative-control group was within the range of the test facility's background value. There were four or more doses that did not exhibit growth inhibition and five or more levels of analyzable doses in the treated groups, whereas positive outcomes for all of the bacterial strains were observed in the positive-control groups. The above findings demonstrated that under the present test conditions, chicken breast extract did not induce genetic mutations.

In the 90-day repeated oral dose study in rats, no changes attributable to the test substance were observed in general conditions, body weight, feed intake, urine test, hematology, blood biochemistry, necropsy, organ weights, or pathologic examination. Therefore, the NOAEL under the conditions of this study was considered to be 1000 mg per kg/day in both males and females.

These findings indicate that chicken breast extract does not exhibit mutagenicity or sub-chronic toxicity. Chicken breast extract is rich in plasmalogen, a substance that has the potential to contribute to improving brain learning and memory and preventing dementia. We believe that chicken breast extract with guaranteed safety can be expected as a health food material that has the potential to contribute to improving learning memory and preventing dementia.

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

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