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Localization and function of tumor suppressor Wwox in postnatal testicular development and spermatogenesis

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WW domain-containing oxidoreductase (*Wwox*) is a well-known putative tumor suppressor, highly expressed in hormonally regulated tissues and considered essentials for normal development of gonads. A Wwox deficient rat model named lethal dwarfism and epilepsy (*lde/lde*) has low gonadotropin and testosterone levels, increased apoptosis of germ cells, decreased number of Sertoli cells (SCs), and retarded growth of Leydig cells (LCs). In the present study, in order to reveal the function of Wwox in testicular development and spermatogenesis, cellular and subcellular localization of Wwox, type of germ cells that cause apoptosis, mechanism of the apoptosis, SCs and LCs differentiation, and steroidogenesis were examined by mainly *in vivo* experiment using normal (+/+) and *lde/lde* rats. Wwox was expressed in almost all testicular cells except spermatids steps 18 to 19, mature sperm and peritubular myoid cells. Wwox localized diffusely in the cytoplasm with focal intense signals (FISWs) which were gradually condensed and changed morphology in germ cells with their differentiation. Along with, Wwox was colocalized with cis-Golgi (GA) marker and resided in isolated GA enriched fractions. These subcellular localization of Wwox was also confirmed in single-cell suspension.

Delayed differentiation of spermatocytes (SPs), increased apoptosis of pachytene spermatocytes (P-SPs), and absence of post-meiotic spermatids indicated the interruption of FRS in *lde/lde* testes. Interestingly, the GA associated protein golgin-160 expression was reduced and formed cytoplasmic abnormal bright condensed signals (ABCSs) outside of GA in P-SPs of *lde/lde* testes. Similarly altered expression of golgin-160 and increased apoptosis were found in GnRH antagonist (Cetrorelix) treated testes, surgically induced cryptorchidism testes, and serum starved embryonic fibroblast cells (REFs) of *lde/lde* rats. These results indicated that Wwox deficiency caused golgin-160 alterations, increased P-SPs apoptosis and disrupted spermatogenesis in late meiosis under depletion of gonadotropins and testosterone in *lde/lde* testes. Significantly increased number of nestin positive cells, increased expression of anti-mullerian hormone and reduced expression of androgen receptor in SCs during FRS indicated that SCs of *lde/lde* were functionally immature. The retarded differentiation of SCs was likely involved with significantly reduction in proliferation and differentiation of LCs lineages in *lde/lde* testes during FRS. Taken together, these results indicated that Wwox is essential for normal SCs and LCs development, spermatogenesis and steroidogenesis in rats during FRS.

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Ventrolateral temporal lobectomy in normal dogs as a counterpart to human anterior temporal lobectomy: a preliminary study on the surgical procedure and complications

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Anterior temporal lobectomy (ATL) is a surgical procedure for drug-resistant mesial temporal lobe epilepsy that is commonly performed in human medicine. The purpose of this study was to determine whether ATL-like surgery, i.e., removal of the amygdala and hippocampal head, is possible in dogs, and to investigate its safety and postoperative complications. Eight healthy beagles underwent ATL-like surgery and were observed for 3 months postoperatively. Samples from the surgically resected tissues and postmortem brain were evaluated pathologically. The surgical survival rate was 62.5%. The major postoperative complications were visual impairment, temporal muscle atrophy on the operative side, and a postoperative acute symptomatic seizure.

Due to the anatomical differences between dogs and humans, the surgically resected area to approach the medial temporal structures in dogs was the ventrolateral part of the temporal lobe. Therefore, the ATL-like surgery described in this study was named "ventrolateral temporal lobectomy" (VTL). This study is the first report of temporal lobectomy including amygdalohippocampectomy in veterinary medicine and demonstrates its feasibility. Although it requires some degree of skill, VTL could be a treatment option for canine drug-resistant epilepsy and lesions in the mesial temporal lobe.

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Decision of Diagnostic Criteria of Feline Obesity Disease

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Obesity is one of the most widespread social problems facing our society health problem. The incidence of obesity in dogs and cats has increased. Prevention of obesity is very important for the health maintenance of dogs and cats. In this study, we settled the diagnostic criteria for obesity disease for cats based on visceral fat accumulation investigated by computed tomography (CT) images and biochemical markers.

In this study, we developed the criteria for determining feline obesity based on computed tomography (CT) images and biochemical markers, and discussed the veterinary medical system to promote the prevention of feline obesity based on the criteria.

We investigated body condition scores (BCS) of clinically healthy dogs and cats, which went to the hospital for health examination at two animal hospitals in Tokyo metropolitan for the past three years. Over 40% of cats were diagnosed as overweight or obese, and its proportion was higher than that of dogs. In obese individuals, excessively accumulated visceral fat secretes free fatty acids (FFA) and some inflammatory cytokines and induces chronic systemic inflammatory. These changes are called lipotoxicity. Similar changes seem to be induced in obese cats, and visceral fat accumulation should be studied.

We divided examinee cats into three groups with different BCS values, BCS 5, BCS 6~7, and BCS 8~9, and measured their plasma metabolites and hormone concentrations, and mass and distribution of subcutaneous and visceral fat (visceral fat /subcutaneous fat ratio, VS ratio) by computed tomography (CT). Plasma triglyceride and FFA concentrations increased accompanying the increase of BCS, adiponectin concentrations were changed among three groups. Plasma SAA concentrations increased with visceral fat accumulation, but BCS did not always reflect changes in the mass of visceral fat.

Simple obesity is not always classified as a disease. JASSO defined pathological obesity which needed medical treatment as 'obesity disease'. Feline obesity should be classified into simple and pathological obesity referring to the criteria for human medicine. Pathological obesity is further divided into with and without visceral fat accumulation. And obesity disease may be diagnosed if overweight cats with BCS >7/9 show two or more of the following, low adiponectin, hypertriglyceridemia, and high SAA values. Obesity disease cats according to these criteria showed significantly higher plasma triglyceride and SAA concentrations and lower adiponectin concentrations than the control (ideal weight) cats. These criteria may be useful for the detection of the early stage of inflammation and prevention of the progress of obesity disease.

Obesity is categorized as chronic systemic inflammation induced by aberrant secretion of inflammatory cytokines from increased visceral fat

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accumulation. Settlement of appropriate diagnostic markers at each stage of obesity is needed to suppress obesity disease. The new criteria for feline obesity disease proposed by our present research may be a useful tool for progressing preventive medicine for dogs and cats.

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Study on the effects of migration and lead pollution on host immunity, and infection of influenza A virus in Black-Headed Gull (*Chroicocephalus ridibundus*)

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Migratory birds are highly susceptible to diseases during their migration period because of the energy trade-off between immune and migration, which results in immunosuppression. This study was conducted on Black-headed gulls (*Chroicocephalus ridibundus*) with high infection rate to influenza A virus, and the basic information about their immunity and body condition, such as body mass, muscle mass, and fat mass, were unknown. The purpose of the study was to determine the effect of lead (Pb) pollution, which results in immunosuppression and changes in body condition during the migration period on the gulls' immunity, and to check if they cause increased infection rate during the migratory period.

First, a study was performed to evaluate the effect of body condition and Pb pollution on the immunity. The gulls were captured from the two areas, Tokyo-bay and Mikawa-bay, and classified based on the three seasons of migration in winter, namely autumn migration, wintering, and spring migration. Pb level in peripheral blood was significantly different in all three seasons and was highest in the wintering season, followed by spring and autumn migrations. Among immune parameters, the proportion of heterophils and lymphocytes had a significant relationship with the blood Pb level, and it was found that an increase in blood Pb level confers immunosuppression. In addition, it was clarified that a blood Pb level of 4.0 μg/dL causes immune suppression. For analyzing the effect of body condition, body condition index (BCI) was set using principal component analysis. On analyzing its relationship with immune components tested, BCI had a positive correlation with the proportion of lymphocytes and the number of white blood cells. It could thus be considered that BCI plays a role in maintaining immune homeostasis.

We found that the proportion of heterophils and lymphocytes in the spring migration was different from that other period. These significant results indicate that there is an energy trade-off between migration and immunity during the spring migration and even during the wintering, suggesting that BCI and Pb contamination have a significant effect on immunity in this period.

Next, the infection status of influenza A was evaluated by enzyme-linked immunosorbent assay (ELISA). The qualitative test results obtained via ELISA were defined as the history of infection and the absorbance, which were measured based on the amounts of antibody. The test results showed that both the parameters increased significantly only during the spring migration. There was a significant difference between BCI and blood Pb

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levels depending on history of infection, and the group with low BCI and high blood Pb levels had a higher history of infection than the group with high BCI and low blood Pb levels. Finally, it was analyzed whether the BCI and blood Pb levels were related to the history of infection and the amounts of antibodies for each period. No significant relationship was found during the autumn migration, and high blood Pb levels were seen along with an increased infection history of influenza A infection during wintering. In the spring migration, the history of infection was high whereas the blood Pb levels increased and BCI decreased, and the amounts of antibodies increased as BCI decreased. These results demonstrate the effects of both BCI and blood Pb levels on the history of infection during the spring migration. In addition, gulls' the existing findings indicate that the amounts of antibodies during the spring migration and the history of infection significantly increased in the Black-headed gulls, suggesting that the characteristics are also related.

This study is expected to contribute to conservation biology related to species diversity by providing an insight into the role of migratory birds in the infection cycle of infectious pathogens and the effects of domestic environmental pollutants through migratory birds.

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Studies on the clinical significance of N-terminal pro-atrial natriuretic peptide in dogs

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Studies have reported that the concentration of plasma N-terminal pro-atrial natriuretic peptide (NT-proANP) increases with a progression of cardiac disease in dogs. However, insufficient studies exist on plasma NT-proANP concentration. Also, influencing factors affecting this concentration remain unclear. Additionally, the clinical usefulness of plasma NT-proANP concentration has not been compared with other cardiac biomarkers (CBs) in dogs. Therefore, this study investigated causes of variation in plasma NT-proANP concentration and their clinical significance in dogs.

First, we evaluated the effect of diet and diurnal variations on plasma NT-proANP concentration in clinically healthy dogs. Autonomic nerve system functions were then investigated to determine whether autonomic function was involved in diurnal variations and if it affected plasma NT-proANP concentration. Results showed that the diet had no effect on plasma NT-proANP concentration. Also, although no statistically significant difference in plasma NT-proANP concentration at different time points was observed, it was proposed that plasma NT-proANP concentration increased when the sympathetic nerve activity increased. Subsequently, plasma NT-proANP concentration was assessed to discover the effect of dehydration on its concentration. As observed, plasma NT-proANP concentration underestimated the severity of cardiac diseases in dehydrated dogs.

Second, the glomerular filtration rate (GFR) was evaluated to investigate the effect of plasma NT-proANP concentration in dogs that were subjected to plasma iohexol clearance test. Results showed that the plasma NT-proANP concentration was affected only when GFR was severely reduced.

Third, we evaluated characteristics and discriminatory abilities of using plasma NT-proANP, NT-proB-type natriuretic peptide (NT-proBNP), ANP, and cardiac troponin I (cTnI) concentrations to discriminate between cardiac dilatation and congestive heart failure (CHF) in dogs with myxomatous mitral valve disease (MMVD). Results showed that plasma NT-proANP, NT-proBNP, and ANP concentrations increased with left atrial enlargement, regardless of the presence or absence of clinical signs. Both plasma NT-proANP and NT-proBNP concentrations were also associated with left atrial enlargements, and their discriminatory abilities against cardiac dilatation and CHF were comparable.

Finally, we investigated the usefulness of plasma NT-proANP and NT-proBNP concentrations in differentiating the cause of coughing in dogs diagnosed with MMVD without signs of CHF and in dogs diagnosed with respiratory diseases.

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Results showed that the ability of these two CBs to discriminate the cause of cough was comparable.

Conclusively, plasma NT-proANP concentration is proposed to increase with increasing sympathetic nerve activity and is influenced by dehydration and GFR. Therefore, the clinical usefulness of plasma NT-proANP concentration for diagnosing MMVD was considered comparable to that of plasma NT-proBNP concentration, which is currently the most widely used in clinical practice. Moreover, these CBs differentiated between causes of coughing in dogs with MMVD without signs of CHF and those with respiratory diseases.

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Studies on transmission cycles of *Mycobacterium marinum* in closed-rearing environment of aquaria in Japan

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Mycobacterium marinum is a nontuberculous mycobacterium (NTM), and causes infectious disease in mammals including humans, fish, amphibians, reptiles, and arthropods. *M. marinum* is widely distributed in nature, especially in the aquatic environment. Additionally, this pathogen has been also isolated from aquatic life such as mollusks, echinoderms, and amoebae. Therefore, there is a hypothesis that transmission of this pathogen includes a variety of animals and environmental factors. However, that has not been proven sufficiently yet. The present study aims to confirm the hypothesis about transmission of *M. marinum* in closed-rearing environment using molecular epidemiological methods (core single nucleotide polymorphism (SNP) analysis). In addition, to evaluate usefulness of variable number of tandem repeats (VNTR) analysis as a rapid method, a comparison of VNTR and core SNP analyses was conducted. We diagnosed *M. marinum* infection and performed isolation of this pathogen from animals and environmental components in two aquaria (aquarium A and B) in Japan. In aquarium A, *M. marinum* infection was diagnosed in a tank rearing flyingfish and a tank exhibiting eelgrasses (*Zostera marina*) bed, and the causative strains were also isolated from fish and filter sand in the both tanks. In aquarium B, it was revealed that the infection occurred in some fish reared in a tank exhibiting eelgrasses (*Zostera marina*) bed, and *M. marinum* strains were isolated from fish, invertebrates, and environmental components in the tank. Core SNP analysis classified isolates from the aquaria into four clusters. In aquarium A, isolates from flyingfish and filter sand in the tanks rearing flyingfish formed a cluster. In addition, isolates from wrasse and bottom sand in the tank exhibiting eelgrasses bed formed another cluster. For aquarium B, isolates from fish, invertebrates and environmental components in the tank exhibiting eelgrasses bed formed another cluster. Hence, it was suggested that fish and environmental components could be involved in the transmissions of *M. marinum* in aquarium A, and fish, invertebrates and environmental components could be involved in the transmission of it in aquarium B. By using VNTR analysis, the genotypes of most isolates were generally classified into three groups, however this method couldn't discriminate some strains obtained at aquarium A and aquarium B, which were geographically isolated. While we conducted the second VNTR analysis with additional VNTR loci which have been previously reported and were newly generated to improve the resolving ability, the analysis still couldn't discriminate those isolates. In this study, to confirm transmission cycles of *M. marinum*, we conducted the diagnosis of *M. marinum* infection, isolation of this pathogen from various factors, and the

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phylogenetic analysis in obtained isolates. The comparison of two genotyping methods was also performed. This molecular-based study epidemiologically confirmed that various factors including fish, invertebrates and environmental components can be involved in transmission cycles of *M. marinum*. Furthermore, it was conducted that further investigation of VNTR locus sets is needed to utilize VNTR analysis.

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Coccidia of raptors: morphological and molecular phylogenetic studies of genus *Eumonospora*

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In this study, parasitological fecal exanimation of raptors imported into Japan was performed. Coccidian oocysts which had characters of single sporocyst with eight sporozoites inside and absence of Stieda body were collected from five genus of Strigiformes and one genus of Falconiformes and these oocysts were all morphologically identified as *Avispora henryae*. However, we resurrected and redescribed genus *Eumonospora* since we found that genus *Avispora* was junior synonym of genus *Eumonospora* during literature review. Genus *Eumonospora* is known as veterinary important parasite for causing diarrhea, blood in feces, and sudden death in raptors, which these clinical symptoms were also observed in those infected individuals and some were recovered after administrated with antiprotozoal, toltrazuril. Although the host specificity of genus *Eumonospora* is regarded as genus-specific, detection of *Eumonospora henryae* from different genera and orders indicates that *E. henryae* might be euryxenous (more than one family of hosts) and the divergent host spectrum between these species. Moreover, the sequence of nuclear 18S rRNA (18S), nuclear 28S rRNA, and mitochondrial cytochrome *c* oxidase subunit 1 amplified from *E. henryae* were used for molecular phylogenetic analyses for resolving the taxonomic position of this genus.

Genus *Eumonospora* clustered in family Sarcocystidae nor than family Eimeriidae with the analyzation of 18S data set of Eimeriorina. Analyzation of data sets including concatenated sequences revealed that genus *Eumonospora* and subfamily Toxoplasmatinae were sister groups with highly supported taxonomic position. On the other hand, phylogenetic topology of genus *Eumonospora* largely corresponded with avian host phylogram might indicated occurrence of cospeciation between parasite and host, while molecular identification of *E. henryae* from different avian orders indicated the possibility of host switching occurred in this species. In the end, the morphological character, single oocyst with eight sporozoites inside, is distinguishable from other subfamilies in family Sarcocystidae. Hence, a new subfamily, Eumonosporinae, was proposed. Furthermore, defining the family Sarcocystidae based on criteria having oocysts with disporocystic and tetrasporozoic, should be modified.

*Supervisor : Prof. KAZUNORI IKE

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Disease ecology of ticks and wildlife: One Health and wildlife management perspectives

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Recently, the association of wildlife with tick-borne diseases has been suspected. For tick-borne diseases prevention and vector control by ecosystem management, it is necessary to understand the distribution of ticks, the influence of urban wildlife, and the relationship between wildlife and ticks on pathogens.

In this study, we surveyed the tick fauna in the western Kanto region (90 x 180 km) using the flagging method, and predicted the distribution of nine major tick species throughout the study area using the MaxEnt model with background information on land use, climate conditions, and wildlife distributions at tick present sites. Forest continuity contributed 18.6-51.1% to seven tick species in the land use factors, and raccoons contributed 7.3-19.1% to six tick species in wildlife distributions.

We investigated ticks dispersed by raccoons, and raccoon dogs, and masked palm civets (Here after, civets), the wildlife which ecological niches overlapped with the raccoons. The evaluated tick dispersing ability based on infestation intensity, removal ability, and resource selection index of ticks. A study in Kanagawa Prefecture comparing raccoons and civets indicated that civets are Ecological Traps with high tick removal ability, while raccoons are Ecological Boosters that can spread ticks. A host selectivity study in Gunma Prefecture comparing raccoons, raccoon dogs, and civets indicated that more tick species selected raccoons and raccoon dogs as the hosts. In addition, although raccoons and raccoon dogs have overlapping habitats, raccoons, which use urban areas more frequently, were thought to be more likely to spread ticks into human dwellings.

By investigating changes in the tick fauna in areas where deer and wild boars, which are considered to be factors of tick distributions, were introduced. *Haemaphysalis megaspinosa* was introduced together with deer and became the dominant species in Niijima, Izu Islands. In Kanagawa Prefecture, where wild boars were introduced, *Amblyomma testudinarium* and *H. hystricis* were introduced together with wild boars, and we found that these two ticks infested the raccoons within 4-7 years of the tick introduction.

An ELISA test for Kabuto mountain uukuvirus-like virus (KAMV) indicated that antibody prevalence in raccoons was affected by habitat suitability of *H. flava* as calculated by the MaxEnt model. The geographic distribution of the probability of KAMV antibody positive raccoons was projected on a map using logistic regression curves.

This study suggested that raccoons are the tick-spreading urban wildlife and that priority measures are needed. The impact of wildlife on

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the tick fauna should be surveyed at least every three years, and that widespread and uniform monitoring strategy is necessary.

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Studies on the clinical significance of serum fibroblast growth factor-23 concentration in dogs and cats with chronic kidney disease

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Fibroblast growth factor (FGF)-23 is a phosphaturic hormone used as an early marker of mineral metabolic disorders in chronic kidney disease (CKD) in humans. In veterinary medicine, some studies on FGF-23 have been recently reported. However, studies evaluating the clinical importance of FGF-23 in dogs and cats are limited. The present study investigated serum FGF-23 concentrations in dogs with CKD and assessed the clinical significance of increased serum FGF-23. Furthermore, this study evaluated serum FGF-23 concentrations in young and adult cats and the association between FGF-23 and hypercalcemia in cats with CKD and upper urolithiasis.

Firstly, the association between serum FGF-23 concentrations and CKD stage in dogs was investigated and compared with other phosphate metabolic markers. The results showed that serum FGF-23 concentrations in dogs with CKD increased in an earlier CKD stage compared with the serum intact parathyroid hormone and phosphorus concentration. Therefore, FGF-23 is a potential earlier marker of mineral metabolic disorders in canine CKD.

Secondly, the clinical significance of increased serum FGF-23 concentration in dogs with CKD without hyperphosphatemia was investigated. Increased serum FGF-23 concentrations were found to be significantly associated with the subsequent development of hyperphosphatemia and CKD progression. The results indicate that reducing serum FGF-23 concentrations can prevent hyperphosphatemia and CKD progression.

Thirdly, this study evaluated serum FGF-23 concentrations in young and adult cats with CKD and found that serum FGF-23 concentration increased with elevated CKD stages. Furthermore, increased serum FGF-23 concentrations were observed in an earlier stage than serum phosphorus concentrations. Therefore, FGF-23 is also a potential early marker of mineral metabolic disorders in CKD in young and adult cats.

Finally, this study investigated whether blood calcium concentrations were related to serum FGF-23 concentrations in cats with CKD and upper urolithiasis.

Increased serum FGF-23 concentrations were significantly associated with hypercalcemia independently of serum creatinine and phosphorus concentrations. Therefore, hypercalcemia is a potential cause of increased serum FGF-23 in cats.

This study demonstrated that increased serum FGF-23 concentrations in dogs with CKD occurred earlier than secondary hyperthyroidism and hyperphosphatemia and presented a risk for the subsequent development of hyperphosphatemia

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and CKD progression. In young and adult cats, FGF-23 was identified as an early marker of mineral metabolic disorders in CKD. Additionally, hypercalcemia was associated with increased serum FGF-23 concentrations in cats with CKD and upper urolithiasis.

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Study on intravenous fluid therapy using a clinical scoring system as an index for the treatment of suckling calves

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A fluid therapy is considered to be the most effective treatment for diarrhea in calf, and it is necessary to evaluate the effectiveness of infusion therapy focusing on sodium (Na) concentration in the blood as well as the pathogenesis of dehydration and acid-base imbalance. In this study, we developed a clinical scoring system that can be applied in clinical practice to treat calf diarrhea and proved the usefulness of fluid therapy strategies using various infusion fluid with different sodium concentrations based on pathological analysis.

In Chapter 1, Kasari's depression score correlated significantly with the concentrations of Hematocrit, total protein, potassium (K), and inorganic phosphorus, blood biochemical parameters that are indicators of dehydration, in Japanese Black cattle and their crossbred diarrheal calves. However, it became clear that when the score generally exceeded 10, it deviated from the reference values of these measurement items. Therefore, to correct dehydration in calf diarrhea, if the depression score is higher than 10, intravenous infusion is actively performed, and if the score is higher than 6.5, sodium bicarbonate is used to correct metabolic acidosis.

In Chapter 2, a model of mild acidemia was created by inducing mild metabolic acidosis due to malabsorption of milk replacer, and isotonic electrolyte infusions (physiological saline [ISS], The correction effects of acid-base imbalance of DL-type lactated Ringer's solution [DLR], L-type Lactated Ringer's solution [LR] and Acetated Ringer's solution [AR]) were compared. Since acetate ion does not rely on liver metabolism alone for BE concentration than lactate ion, it was suggested that AR is superior to DLR and LR in the treatment of mild metabolic acidosis in calves in this study.

On the other hand, resuscitation therapy by intravenous administration of hypertonic saline solution (HSS) is an economical and efficient treatment method in the bovine clinical medicine. However, it is not clear whether rapid fluid movement and increased blood circulation are safe enough for calves which undeveloped cardiovascular and renal urinary systems. Chapter 3 described the usefulness of 2.16% HSS for patients with hyponatremia as an application to HSS diarrhea calves. As a result, we clarified 2.16% hypertonic saline solution is extremely effective in improving dehydration and hyponatremia of diarrhea calves with hyponatremia, although caution is required for plasma K concentration.

To summarize this study, it is the most important and fastest cure method to accurately grasp the condition of diarrhea in suckling calves with the change of time in the field and to select an infusion solution suitable for that condition and treat it early. In the clinical practice of food animals, the scoring system used this time are

comprehensively diagnosed by obtaining a lot of information and an appropriate infusion solution.

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Analysis of the effect of structural change on the function of DNA homologous recombination repair enzyme RAD51

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(Conferred on 10 March 2022, VNT-80)

The eukaryotic RAD51 gene is a homologue of the RecA gene of Escherichia coli. RAD51 forms a complex with the "breast cancer susceptibility gene 2" (BRCA2) and plays a central role in the homologous recombination (HR) repair of a variety of DNA lesions such as the DNA double-strand breaks (DSB). HR is catalysed by the RAD51 recombinase, which forms a nucleoprotein filament on resected single-stranded DNA (ssDNA) at the damage site, and mediates pairing of the homologous DNA sequences and strand invasion. In addition to several reports on cancer-associated mutations in BRCA2, there is also a BRCA2 mutation database (https://brcaexchange.org/). However, there are relatively few reports on RAD51 mutations.

In canine studies, although several mutations have been detected in BRCA2, canine RAD51 (cRAD51) mutations have not been reported. However, a recent report identified the presence of multiple cRAD51 mutations in canine mammary tumours, and predicted that A209S and T225S mutations in cRAD51 might influence its interactions with the "partner and localizer of BRCA2" (PALB2). PALB2 has emerged as an important and versatile player in the maintenance of genome integrity. Biallelic mutations in PALB2 cause Fanconi anaemia, whereas monoallelic mutations predispose an individual to breast, and pancreatic familial cancers. However, canine PALB2 (cPALB2) has not been cloned and investigated, as well as interaction analyses with cRAD51 wild-type and mutants have not been performed.

In this study, we generated the A209S and T225S mutants of cRAD51 in order to understand these interactions in depth. We found that the functional regions of the recombinant cPALB2 shares close homology with its homologs from other species. These highly conserved regions mainly span the N-terminal region, including the coiled-coil and RAD51 interacting domains, as well as the C-terminal domain of cPALB2 protein, including the PALB2_WD domain, which also interacts with RAD51. Based on these observations, we predicted that the interactions of cPALB2 protein with its binding partners might be similar to that reported in other species including humans, where it was shown to interact with the N-terminal of BRCA2. We confirmed the interaction between full-length cPALB2 and the N-terminal region of cBRCA2 using an MTH assay. As canine BRCA2 and PALB2 interact, these molecules were expected to be involved in HR. The side-chain structural changes of A209 and T225 in cRAD51 to serine were not estimated, nor were the major functional changes determined through in-silico simulation because small van der Waals volumes

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are common in these three amino acids. The A209 residue has three hydrogen bonds with the F86 residue of another RAD51 chain, and this structure does not change after the A209S substitution. Only T225S substitution showed the formation of new hydrogen bonds against the intra-strand N267 and Q268 residues. A study done by Ozmen *et al*. showed that while the A209S substitution did not have any negative effects, the T225S substitution was deleterious for the function of RAD51 using protein structure analysis.

The A209 and T225 residues of cRAD51 are a part of the putative interacting domains of PALB2, and therefore, the amino acid substitutions of these residues might affect the binding. To test this hypothesis, we performed MTH and PD assays and investigated the effects of cRAD51 mutation on its interaction with PALB2. First, we examined the effect of A209S and T225S substitutions on the oligomerization of RAD51. The RAD51 proteins with both these substitutions could not oligomerize with WT RAD51, as shown using the MTH and PD assays. Hence, it is possible that in the cells heterozygous for RAD51 mutations, the altered interactions between WT-WT, WT-mutant and mutant-mutant RAD51 may reduce the efficiency of RAD51 oligomerization. Mutant-mutant interactions in MTH assay showed this reduction clearly. RAD51 forms a homo-oligomer in the uninduced, resting state. In response to DNA damage, it converts into monomer and binds to the BRCA2 complex along with PALB2. Following that, this complex is recruited to the site of DNA lesion to perform HR-based DNA repair. Since the BRC repeats of BRCA2 are able to interfere with the oligomeric state of RAD51 by mimicking the self-association mechanism of RAD51, the reduction in the oligomerization of RAD51 may affect its binding with the interacting factors, including BRCA2.

Finally, we examined the influence of A209S and T225S substitutions of cRAD51 on the interaction with cPALB2. Both A209S and T225S showed the significant attenuation of interaction with full length of cPALB2 in the MTH assay. However, in PD assays, the bands of A209S and T225S pulled-down by Halo-tag fused N-terminal domain of cPALB2 were weaker as compared to WT band. On the other hand, only T225S mutant band was weaker when pulled-down by Holo-tag fused cPALB2 C-terminal domain. Even though the results from the MTH assay are not perfectly mirrored by those from the PD assay, we concluded that the A209S and T225S mutations of cRAD51 may adversely impact its interaction with cPALB2. These results show that both T225S and A209S mutations of RAD51 may provide new leads in canine mammary tumours mutation analysis.

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Histological classification and molecular phenotypes of canine mammary tumors

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Mammary tumors in dogs are the most common tumors in females, with 40-60% malignant in epidemiological studies. However, unlike human medical field, which has a large amount of basic data, accurate epidemiological investigation of mammary tumors in dogs is difficult, and the incidence of tumors varies from study to study. The 1999 edition of the World Health Organization (WHO) classification of canine mammary tumors is now widely used for the diagnosis. In 2019, a new classification was proposed, but the association between histological type and malignancy in the new classification is not completely clear. As an example, recent studies have shown that there are different type in what was previously classified as "True SSC (Simple solid carcinoma)". As described above, since canine mammary gland tumors have a complicated tissue morphology, it is difficult to evaluate the malignancy, and simpler and more widely used malignant indicator markers are desired. Therefore, we focused on fibroblast growth factor receptors (FGFR), which have been attracting attention in humans in recent years. FGFR is a receptor for fibroblast growth factor (FGFR), which is a type of growth factor involved in development, wound healing, and so on. Its expression has been reported to be involved in stem cell differentiation, proliferation / infiltration, anti-apoptosis, drug resistance, and is expected to be one of the promising targets in human cancer treatment. However, the analysis of the expression in canine tumors has not progressed and little knowledge is available. In this study, we investigated the occurrence of mammary gland tumors in dogs in Japan, compared the old and new WHO classifications, and analyzed the details of the new classifications. We also analyzed cell characteristics in tumors showing solid growth according to the old classification. In addition, its usefulness as a malignant index marker for FGFRs was examined.

First, we compared the 1999 and 2019 international histological classifications and searched for the incidence of canine mammary tumors at our university based on histological diagnosis. The incidence of malignant tumors was 42.3% in the 1999 edition, but 46.5% in the 2019 edition. The rate of malignancy was not different from that reported in previous overseas studies. The increase in the proportion of malignant tumors in 2019 was due to the addition of classification. It is based on the fact that tumors from myoepithelial cells were found to have malignant phenotype, and the addtion that detail subtypes were classified such as "carcinoma in complex adenoma / benign mixed tumor". As the proportion of malignant tumors of canine mammary gland tumors has increased, it may affect the prognosis and

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treatment policy. In particular, it is hoped that more information will be accumulated in the future, such as biological behavior of tumors in new classifications.

Secondary, we performed morphological and immunohistochemical analysis of canine mammary tumors showing solid growth. Solid cancers could be classified into the following three tumor types. That is, True SSC composed only of glandular epithelial-like tumor cells, MM (malignant myoepithelioma) mainly composed of myoepithelial-like tumor cells, and BC (biphasic carcinoma) characterized by the proliferation of two types of glandular epithelium and myoepithelial-like tumor cells. SSC showed high infiltration to the surroundings and lymphovascular invasion, whereas MM showed expansive growth, suggesting that there is a large difference in prognosis. In addition, the cell morphology of MM was further classified into three types. It has been considered that SSC and MM tumor cells cannot be morphologically distinguished by HE staining, but among MM, clear cell type and basal cell type have some morphological differences even with HE staining alone. However, since some epithelioid types are difficult to distinguish from SSC, it was considered that immunohistochemical staining was still necessary for accurate diagnosis. Additionally, immunohistochemical analysis revealed that all cases of MM contained a small amount of glandular epithelial cells, and that glandular epithelial cell markers were also expressed in myoepithelial cells. From this, it was considered that MM tumor cells may have the property of progenitor cells that differentiate into both glandular epithelium and myoepithelial epithelium.

Finally, the relationship between FGFR expression and malignancy in canine mammary tumors was analyzed by histopathological, immunohistochemical and molecular biological searches. FGFR expression analysis of Real-time PCR confirmed the expression of FGFR in the breast tumor tissue and the breast tumor cell line, and the value was higher in the breast tumor tissue than in the cell line. This was thought to be due to the effects of stroma and myoepithelial cells. Immunohistochemical staining did not show a clear difference in staining between normal tissue and breast tumors. In the results of Western blotting, the expression level of the protein in each tumor cell line did not correspond to the RNA expression, and especially in FGFR3, no specific expression was confirmed . From these, it is necessary to further investigate the cross-reactivity and specificity of the antibody used this experiment, and it is considered that the role of FGFR in tumors can be clarified by identifying the expression cells on the tissue.

This study analyzed canine mammary tumors from various pathological aspects. From the comparison of old and new WHO classifications, the proportion of malignant tumors was expected to increase in the future. In addition, although some tumors are classified in more detail in the new classification, it seems that further classification such as solid tumors needs to be examined. The relationship between FGFR and tumor malignancies was unclear. However, it is possible that the expression on tumor tissue can be analyzed by devising the antibody used, and further analysis is considered necessary.

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Properties of PIK3CA Mutant Isolated from Canine Hemangiosarcoma Cases

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Angiosarcomas in humans are rare, highly aggressive, malignant, endothelial cell tumors of vascular or lymphatic origin. Treatment is challenging in a number of cases, and the prognosis is poor. Canine hemangiosarcoma (HSA) is also an aggressive malignant neoplasm with a poor prognosis. Surgery and chemotherapy have had limited success in prolonging survival and increasing the quality of life of canines with HSA. HSA tissues overexpress vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and their receptors. These growth factors generally induce tyrosine kinase activation of receptors and activate downstream signaling pathways, including the MAPK/ERK and phosphatidyl-inositol-3 kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathways, which are involved in tumor progression. MAPK/ERK pathways in endothelial cells are activated in tumors. The PI3K/Akt/mTOR pathway also participates in the pathogenesis of endothelial cells. Phosphatase and tensin homolog (PTEN) mutation, which is an antagonist of PI3K, has been detected in human and canine HSA cases, and the hyperactivation of the Akt/mTOR pathway has been reported in sporadic human HSA cases. However, the role of the PI3K/Akt/mTOR pathway in canine HSA has not yet been well investigated.

Developments in genome-wide approaches have enabled the comprehensive analysis of disease-related gene mutations in humans and animals. Mutated canine genes related to HSA have been searched using exome sequencing, and the 1047th histidine residue (H1047) of p110 α phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) was found to be highly mutated. PIK3CA acts downstream of the EGFR pathway. In human tumor cases, mutations of the 1047th histidine (e.g., H1047R and H1047L) of PIK3CA have been shown to induce the hyperactivation of EGFR signaling. Akt phosphorylation and tumor cell proliferation are enhanced by this mutation. The importance of the PIK3CA mutation in canine HSA is well recognized; however, the open reading frame of canine PIK3CA has not yet been cloned, at least to the best of our knowledge. The present study thus cloned canine PIK3CA and produced mutants by substituting the H1047 residue and investigated functional alterations in EGFR signaling via Akt phosphorylation. The antiproliferative effects of alpelisib, which suppresses the hyperactivation of EGFR signaling by the PIK3CA mutation, on canine HSA cell lines were also investigated.

The present study demonstrates that there are mutations in PIK3CA H1047 in both canine HSA cases and cell lines. Although canine PIK3CA is also mutated in mammary gland tumor and

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hemangiosarcoma cases, the cloning and molecular characterization of canine PIK3CA have not yet been performed, at least to the best of our knowledge. Therefore, the present study cloned and characterized canine PIK3CA. The novel cloned canine PIK3CA exhibited a high homology with human PIK3CA, and functional domains were well conserved. The H1047 residue was conserved in canine PIK3CA in the same position. These structural properties suggest that canine PIK3CA possesses EGFR signaling activities.

In the mutation analysis of 19 cases of canine PIK3CA in HSA FFPE samples, either a H1047R or H1047L heterozygous mutation was detected. Although the 3140A>G (case no. 7: H1047R) mutant sequence waveform was absent, a small waveform of 3140A>T (case no. 9: H1047L) was detected. This mutant could be cloned and confirmed; however, there may be other invisible mutants in other cases. It has been previously reported that the detection limit of rare alleles by Sanger sequencing is ~10%. High-resolution methods (e.g., pyrosequencing and next-generation sequencing) may be able to detect mutations in the 1047th residue of canine PIK3CA, which was isolated from tumor tissues. A definite pathological difference between the presence or absence of PIK3CA 1047th residue mutation was not detected by microscopic observation.

The analysis of Akt phosphorylation with the enforced expression of canine PIK3CA WT or H1047 mutants in HeLa cells revealed that H1047R- and H1047L-transfected cells exhibited the hyperphosphorylation of Akt; in particular, Akt2 but not Akt1 was phosphorylated with or without EGF stimulation. The Akt isoforms Akt1 and Akt2 play differential roles in tumor metastasis. Akt1 has been demonstrated to suppress, while Akt2 promotes breast cancer cell migration and invasion *in vitro*. This result suggests that the canine PIK3CA H1047 mutation induces the upregulation of EGFR signaling and promote tumor growth and invasion via Akt2 phosphorylation in HeLa cells.

Canine cell lines established from HSA tissue were highly mutated in the H1047th residue of PIK3CA. Herein, the overexpression of the canine PIK3CA H1047 mutation induced the phosphorylation of Akt2, but not Akt1 in HeLa cells. Alternatively, EGF stimulation induced the phosphorylation of endogenous canine Akt at Ser473 in the canine HSA cell lines, Re21, JuA1, and JuB4. JuA1 and JuB4, which were heterozygous/homozygous for the PIK3CA H1047L mutation, and induced the phosphorylation of Akt1 upon EGF stimulation, but not in the PIK3CA normal cell line, Re21. Given that only JuB4 exhibited Akt2 phosphorylation, a homozygous H1047L mutation may induce a stronger gain-of-function in PIK3CA. In canine HSA cell lines, the H1047L mutation of PIK3CA induced the phosphorylation of Akt1 and/or Akt2 with EGF stimulation, which may result in a severe pathogenesis.

Alpelisib (BYL719) is a targeted compound against mutated PIK3CA and is ~50-fold stronger than other isoforms. In the present study, alpelisib inhibited cell proliferation by suppressing Akt phosphorylation and inducing apoptosis via the activation of caspase-3/7 pathways, particularly in PIK3CA-mutant canine HSA cell lines. In MTT assays of canine HSA cell lines exposed to alpelisib, the JuA1 cell line, which is heterozygous for the H1047L mutation, was the most sensitive, although the JuB4 cell line, which is homozygous for the H1047L mutation, exhibited moderate sensitivity to alpelisib. JuA1 cells exhibited higher viability than JuB4 cells in a previous study. Thus, alpelisib may exert a potent antitumor effect on PIK3CA mutant tumor cells by suppressing Akt phosphorylation. On the other hand, although alpelisib suppressed Akt phosphorylation in PIK3CA mutant cell lines, which were derived from canine mammary gland tumors, there was no marked difference in the tumor suppressive effect between normal and mutant PIK3CA cells. It has been reported that canine HSA cells promote migration by interacting with CXCR4 and CXCL12, and the overexpression of CXCR4 promotes the invasion and migration of non-small cell lung cancer via EGFR. These

phenomena suggest that the suppression of abnormal EGFR signaling, induced by PIK3CA mutation, by alpelisib may be able to control canine HSA progression. Furthermore, alpelisib induced significant apoptotic cell death specifically in PIK3CA mutant canine HSA cell lines via caspase-3/7 activation; thus, alpelisib can be used as an agent for the treatment of canine HSA. In addition to the tumor-suppressive effect on canine PIK3CA mutant HSA *in vivo*, alpelisib has been confirmed to be safe for use in dogs based on safety examinations during drug development processes. Therefore, the authors aim to investigate its direct clinical effects on canine HSA cases in future studies.

In conclusion, the present study detected a PIK3CA H1047 mutation in canine HSA tissues and cell lines derived from canine HSA cases. The H1047R and H1047L mutations in canine PIK3CA induced EGFR signaling via Akt hyperphosphorylation. Alpelisib suppressed Akt phosphorylation, cell viability and migration, and induced apoptosis in PIK3CA mutated canine HSA cell lines. These data suggest that the H1047 mutation of PIK3CA is a crucial and useful marker of canine HSA, and alpelisib may prove to be an effective agent against PIK3CA-mutant canine HSA.

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Establishment of safe anesthesia method for preventing hypothermia and hyperglycemia induced by medetomidine-midazolam-butorphanol in mice

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A combination of three anesthetics (MMB), namely medetomidine (Me), midazolam (Mi), and butorphanol (Bu), is commonly used as injectable anesthesia in mice. An original dose of MMB (Me/Mi/Bu = 0.3/4.0/5.0 mg/kg) provides a sufficient anesthetic duration of 40-50 min in mice. In addition, atipamezole (Ati) is available for the reversal of MMB anesthesia. However, severe hypothermia has been observed in mice as an adverse effect of MMB anesthesia. Hypothermia during anesthetic events is a common adverse effect of anesthesia in laboratory animals. In particular, small rodents, such as mice, are susceptible to hypothermia during anesthetic events. Therefore, these animals need an additional thermal support from external heating devices during and after anesthesia. In general, the time of recovery from anesthesia is typically longer in the case of injectable anesthesia than that in the case of inhalant anesthesia. However, the duration of thermal support has been almost limited to about 1 h from the time of administration of anesthesia. In addition to hypothermia, hyperglycemia has been observed in mice under MMB anesthesia. Anesthesia is often used for studying glucose metabolism; therefore, the use of MMB anesthesia may influence the blood glucose level (BGL) of mice. Pentobarbital sodium (Pent), which is a short-acting barbiturate widely used as anesthesia in rodents, has a poor analgesic effect and shows few effects on glucose metabolism. Although Pent can be commercially obtained, it is classified as a non-pharmaceutical-grade compound and is not suitable as an anesthetic agent. Secobarbital (Seco), which is a pharmaceutical- grade barbiturate, is a known substitute for MMB or Pent. In the present study, we aimed 1) to compare the levels of hypothermia induced by injectable anesthesia with MMB and inhalant anesthesia with isoflurane (ISO) and investigate the anesthetic component of MMB responsible for causing hypothermia, 2) to find the adequate duration of thermal support in mice after administration of anesthesia and provide the doses of Ati and MMB mixture for preventing hypothermia, and 3) to evaluate the effects of Seco on BGLs and body temperature in mice. All procedures in this study have been approved by the provisions of Nippon Veterinary and Life Science University (Approval Nos. 28S-62, 29K-25, 30K-26, 2019K-14, 2020K-39, and 2021K-49). The results of this study revealed that 1) α2-agonist Me, a component of MMB, is most likely to induce hypothermia. 2) A 5-h thermal support completely prevented hypothermia in the MMB group and a 1-h-support

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prevented hypothermia in the ISO group. The antagonism of Ati within the proper dose range is effective in promoting recovery from MMB-induced hypothermia, and MMB at our recommended dose of 0.2/6.0/10 mg/kg provides anesthetic effects for 40 min and keeps normothermia after thermal support for 2 h. 3) The administration of Seco alone did not induce surgical anesthetic depth in mice, but the anesthetic combination of Seco and Bu (SB) maintained the surgical anesthetic depth for 40 min. In the MMB group, the blood glucose levels significantly increased compared with the Pent, Seco, and SB groups. In addition to the mild effects of SB on BGL, hypothermia was blocked by a thermal support for 2 h in the SB anesthesia group. The present study provides suitable anesthesia methods for preventing hypothermia and hyperglycemia in mice.

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The Analysis of Consumer Attitudes Toward Animal Welfare

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Introduction

Animal welfare (AW), as defined by the World Organization for Animal Health (OIE), refers to "the physical and mental state of an animal in relation to the conditions in which it lives and dies." Because the definition implies maintaining livestock animals in comfortable conditions, it is primarily an issue for producers. However, promoting AW involves not only producers but also processors and distributors. Whether consumers choose AW livestock products is the most important factor.

Previous research has indicated that labeling products with AW considerations increases consumer willingness to pay more for the products. In such studies, labeling was hypothetical and not based on the actual livestock products with labeling or on consumers' awareness, interest, and knowledge of AW. This study validates the issues that must be addressed for understanding the actual purchase status and consumer awareness regarding livestock animals and livestock products. The study is based on the current status of laws and regulations, labeling, and certification systems for AW livestock products in Japan and the distribution status of AW livestock products.

Materials and Methods

A consumer questionnaire was conducted in March 2021, and 490 valid responses were recorded. Awareness and interest in AW, purchase experience with AW livestock products, and purchase intention were analyzed using demographics and compared with results of a similar survey conducted by our laboratory in March 2014. Moreover, a comparison was made with the EU's 2015 survey titled "Special Eurobarometer 442 Attitudes of Europeans toward Animal Welfare." In August 2021, we further surveyed the products and prices managed at seven supermarkets in Kawasaki City and at an online supermarket in Meguro Ward to understand the distribution status of AW livestock products.

Results and Conclusions

The Japan Livestock Technology Association's guidelines for AW assume that layer hens are kept in battery cages. The EU has laws and regulations concerning AW, such as the prohibition on keeping layer hens in battery cages. Moreover, in Japan, labeling regulations and certifications are not categorized in terms of AW; they are separately stipulated in multiple laws and standards, thereby making it difficult for consumers to be aware of AW in their daily purchasing behavior.

In the survey, the number of supermarkets managing AW livestock products was limited. In addition, 5, 0, 3, and 1 store carried AW livestock, milk, dairy products, and meat and processed meat products, respectively. Online supermarkets handled AW-conscious eggs and organic milk. The price of AW-conscious eggs was 2.6-5.3 times

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higher than that of the regular product. For milk, the price was 3.3 times higher.

The consumer questionnaire included the awareness of AW, interest in animal husbandry methods, the importance of animal husbandry methods when purchasing livestock products, and willingness to pay additional amounts for AW livestock products. Among the consumers, 15.7% were aware of AW in 2021, which is a significant increase from 10.7% in 2014. According to the analysis, the age difference was greater than the gender difference. In the descending order of recognition, 21.4% were in their 70s or older and 20.0% were in their 60s. In terms of whether the method used to raise livestock is crucial when purchasing livestock products, 8.6% of the respondents in their 70s and above and 6.2% of the respondents in their 50s and above were aware of the method. In terms of willingness to pay extra, 58.6% of the respondents were in their 70s and above, whereas 55.7% were in their 60s. In general, consumers in their 60s and 70s were more interested in AW than those in their 50s and less. Most of the respondents who were willing to pay extra said that the price difference they could accept was less than 200%, which indicates that they would have difficulty accepting the prices revealed by the price survey. In comparison with the EU, less than half of the respondents under the age of 60 in Japan were willing to accept a price increase, whereas more than 60% of the respondents under the age of 54 in the EU were willing to accept a price increase.

Furthermore, three issues regarding the increasing awareness of AW were identified in this study. First, the awareness of AW among consumers has increased slightly but remains low. In particular, the younger generation is relatively uninterested. Second, a lack of uniform standards for AW labeling and a limited availability of AW products was observed in the stores. Therefore, there is no environment in which consumers are regularly made aware of AW in their daily purchasing behavior. The only exception is poultry eggs. Finally, there is a wide gap in the price difference between AW products and regular products even for consumers who are willing to pay a difference.

AW is an international requirement. Considering laws and policies labeling and subsidy systems for AW is crucial. Other research topics are raising AW awareness among young people-who are particularly interested in AW-and balancing AW considerations with the elimination of price differences.

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Studies on characteristics of nutritional imprinting in mice

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Recently, nutri-epigenetics, which is induced by maternal nutrition and affects metabolic profiles of embryos and neonatal animals was recognized and reported. Maternal nutrition is transmitted to the metabolism of offspring to alter gene expression and epigenetics fix this condition. Recent works indicated that leptin related to this transmission from mothers to offspring. Usually, leptin is secreted from fatty tissues when animals have the high energy and /or high fat and carbohydrate diet. However, it has been reported that blood levels of leptin reduced when pregnant animals had low protein diet ie the high energy and /or high fat and carbohydrate diet, and it contradict to secretion condition of leptin.

On the other hand, it has been also reported that blood levels of leptin increased when lactating animals had similar with above diet, and it agree to secretion condition of leptin. Those two observations indicated that leptin might specific involved in on nutritional information transmitting from mother to offspring, there were few reports about it.

Therefore, two dose response experiments to dietary protein levels were conducted to clarify the mechanisms of leptin related transmission of maternal nutrition in female mice during pregnant and lactation in experiments 1 and 2. In addition, effects of leucine administration which have insulin like action on glucose tolerance and insulin secretion of neonatal mice were investigated as a third experiment in experiment 3.

20 and 18 female mice from 10 to 12 weeks of age were used foe experiments 1 and 2, respectively, and 33 neonatal male mice from each treatment in experiment 2 were selected and used in experiment 3.

Five levels of CP experimental semi-purified diets containing the same levels of energy were prepared using casein as main protein source at 4.3, 13.0, 21.6, 30.2, 38.8% CP offset by starch. In experiment 1, female mice Day 0 of pregnant, the first time of plugs observation, were assigned to each diet group with the same average body weights and fed ad libitum experimental diets for 17 days. At the end of feeding trial, body weights and feed consumptions were recorded. Then, after inhalation anesthesia with isoflurane, cardiac blood was collected and sucrifyied, fetus, placenta, mammary gland fat, and fat were collected and weighed.

In experiment 2, female mice Day 0 of lactation were assigned to each diet group with the same average body weights and fed ad libitum experimental diets for 21 days. At the end of feeding trial, body weights and feed consumptions were recorded. Then, after inhalation anesthesia with isoflurane, cardiac blood was collected and sucrifyied, fetus, placenta, mammary gland fat, and fat were collected and weighed as the same as experiment 1. Leptin levels of blood and extracts of embryo were determined with commercial

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ELISA kit. Leptin and it's receptor mRNA expression were determined with qPCR.

In experiment 3, each male offspring of mother in each dietary treatment were orally administered leucine solution (4 mg/mL) or water on Day 5 of postnatal, and were continuously cared by each mother until weanling, after then feeding commercial diet ad libitum. After fasting for 6 hours, mice were bled from tail, and then orally administered glucose at 2mg /g body weight. And then, they were bled on 15, 30, 60 and 120 minutes after glucose administration. Plasma glucose and insulin concentration were determined in blood samples.

Results obtained were analyzed by 2-way ANOVA using General Linear Models of SAS and means were separated Tukey's multiple range test, or Kruskal-Wallis test.

In experiment 1, significantly lower data were observed in body weight, fat weights, plasma leptin concentration, fat leptin mRNA expression of mice fed lowest protein diet (4.3%; 4.3% group) than those of mice in other feeding groups ($P< 0.05$). However, leptin contents per g embryo was higher in 4.3% group than 21.6% group ($P< 0.05$). Leptin mRNA expression in the placenta increased with increasing dietary protein from 13.0% to 30.2%, while that in 4.3% group was higher than 13.0% group. Leptin mRNA expression decreased with increasing dietary protein from 13.0% to 30.2%, while plasma leptin concentration was increased and then remained constant. These observations indicate the placenta might regulate the leptin secretion among mother and embryo.

In experiment 2, we decided to remove 4.3% group from experiment 2, because almost of offspring died and mother eat it, and 4.3% group was impossible to control the nutritional condition. The fact indicated that extreme deficiency of dietary protein might induce the protective change in mother physiologically and behaviorally. In all index of mother in experiment 2, there were results agreed to current reports and usual leptin secretion condition.

In experiment 3, there were no differences in body weight at 6 and 20 weeks of age. In glucose tolerance test, there was no difference in plasma insulin levels between two groups, while plasma glucose 60 minute after treatment was lower in mice administered leucine (Leu group) than control administered water ($P< 0.05$). Thus, administration of leucine to animals on early stage might elevate glucose adapting function.

These results suggested that nutritional imprinting induced by extreme deficiency of dietary protein might not due to elevating dietary fat and carbohydrate, but down of assimilation signal.

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Investigation of stress response in immune cells using Ddit4 reporter mice

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[Introduction]

Stress occurs when we are psychologically or physically overloaded by various external causes. When we feel psychological stress, it is transmitted through the hypothalamic-pituitary-adrenal axis (HPA axis) and the sympathetic-adrenal medullary system (SAM axis). Stress is also known to affect the immune system, but the details of the mechanism are not fully understood, and the effects also vary depending on the duration and intensity of the stress. It has been reported that short-term stress activates innate and adaptive immunity, while chronic stress suppresses the immune response. Previous studies have revealed that the expression of Ddit4, Gilz, and Mkp-1 genes are increased in immune cells upon stress stimulation. Ddit4, also known as Rtp801, REDD1, and Dig2, is a cytoplasmic protein that was first shown to be upregulated in mouse embryonic stem cells and fibroblasts. In addition to psychological stress, its expression is upregulated by other cellular stresses such as oxidative stress, endoplasmic reticulum stress, hypoxia, and nutrient deprivation. The Ddit4 reporter mouse (Ddit4 mouse) was generated in which the 2A peptide and green fluorescent protein (GFP) were knocked in (KI) in place of the stop codon of the Ddit4 gene. In this mouse, it is expected that GFP is transcribed and translated along with the Ddit4 gene upon stress stimulation, resulting in the GFP protein expression that emits green fluorescence. In this study, utilizing the characteristics of this mouse, I investigated whether GFP is indeed induced after stress stimulation by restraint stress or administration of dexamethasone (Dex), a synthetic glucocorticoid.

[Materials and Methods]

(1) Restraint stress: Ddit4 mice were restrained in a 50 ml tube with air holes for 5 hours. This was repeated for 2-5 days. (2) Dex administration: 0-30 mg/kg (body weight) of Dex was administered intraperitoneally to mice, and samples were prepared the following day. (3) Sample preparation: Peripheral blood from the cheeks of mice was heparinized, and hemolyzed with Tris-NH_4Cl. Spleen and thymus were excised and then ground using glass slides with rough surface. Splenocytes were hemolyzed for erythrocytes with Tris-NH4Cl. Peripheral blood was treated with Turk's solution, and spleen and thymus cells were treated with 0.02% trypan blue for cell counting and prepared for cell suspensions. (4) Flow cytometry (FACS) staining: After preparing the cell suspensions, the cells were stained with various fluorescently labeled antibodies against cell surface markers, fixed in 1% paraformaldehyde (PFA)/PBS, and subjected to FACS analysis. (5) Cryosections and

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immunofluorescence staining: Spleens from wild-type (WT) and Ddit4 mice were harvested, embedded in OCT compound, frozen, and thinly sliced at 7-10 μm in a cryostat. After staining the cell surface with biotin-conjugated anti-CD3 antibody, anti-CD19 antibody and Cy3-conjugated streptavidin, the cells were fixed with 4% PFA/PBS and permeabilized with NP-40. After reaction with anti-GFP antibody using a fluorescence signal booster kit, the cells were observed under a fluorescence microscope.

[Results and Discussion]

1-1) Following Dex administration at 3 mg/kg, there was almost no change in cell number and GFP mean fluorescence intensity (MFI) in the peripheral blood. Administration at 30 mg/kg caused a remarkable decrease in blood cell number that was uncountable because of the small number. 10 and 20 mg/kg caused no significant change in cell number in comparison between before and after administration. The MFI of GFP in mice treated with 20 mg/kg increased about 1.8-fold in total leukocytes, 3.2-fold in T cells, and 2.8-fold in B cells. 1-2) Following restraint stress more than 2 days, GFP expression in T cells tended to increase in peripheral blood. In the case of B cells, MFI tended to increase in some mice depending on the duration of restraint, but it was not consistent. 2-1) Following Dex administration, MFI of GFP in spleen were examined by FACS for whole leukocytes, T cells, B cells, helper T cells, killer T cells, follicular helper T cells, regulatory T cells, natural killer T cells, activated T cells, activated B cells, dendritic cells, and macrophages. After Dex administration, MFI increased in all the cells examined in this experiment. The MFI of T cells was higher than that of B cells and other cells even without treatment. Activated T cells had the highest MFI. 2-2) During 2-5 days of restraint stress, MFI of T cells was higher than that of whole leukocytes and B cells, but there was little difference depending on the duration of restraint stress. MFI of peripheral blood and spleen were almost the same after restraint stress.

3) In the thymus after restraint stress loading, differentiated CD4 single positive (SP) and CD8SP T cells showed strong GFP expression, and immature $CD4^{+}CD8^{+}$ and $CD4^{-}CD8^{-}$ T cells also expressed GFP, although weakly.

4-1) In tissue sections of spleen, there seemed to be no difference between WT and Ddit4 mice when the spleen was observed without staining or fixation. Since GFP expression was detectable by FACS, and I also tried to detect GFP expression by microscopy in single cell state, and then confirmed it. Next, the sections were stained with three types of Alexa Fluor 488-labeled anti-GFP antibodies and observed, but no fluorescence was observed in any of them. 4-2) In order to enhance GFP fluorescence in tissue sections of spleen after Dex administration, the sections were stained and observed using the fluorescence signal booster kit. As a result, the white pulp of the spleen appeared to glow strongly. Therefore, we stained the sections with anti-CD3 or anti-CD19 antibodies and examined the localization of GFP, but there was no difference in the T and B regions. Therefore, further investigation is needed to find out suitable condition for double staining.

These results indicate that strong GFP expression was observed in Ddit4 mice, especially in T cells, and that GFP expression was further enhanced in peripheral blood and spleen by stress stimuli such as restraint stress and Dex administration. Accordingly, this mouse proved to be a useful experimental system to analyze stress responses in immune cells.

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The study of nutritional and physiological significance of neonatal polyamine signal in chick brain and peripheral tissues

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Metabolic programming, which influences the subsequent growth of nutritional status during the neonatal period, has been identified in various animals, including aves. Hence, animal breeders are careful about nutritional management during this period. In addition, from the viewpoint of sustainable animal production, it is important to construct a feeding management method that efficiently absorbs limited nutrients and maximizes the physiological functions of animals. Since it is known that the feeding behavior and metabolic regulation mechanism of the two commercial chickens (broiler and layer) are different, both were used as comparative nutritional and physiological models. Polyamines (putrescine, spermine, spermidine, and cadaverine), which are intermediate metabolites of essential amino acids, have been shown to have different plasma metabolites in both the chicken species. However, the physiological significance of embryogenesis is not yet fully understood. Therefore, this study aimed to investigate the nutritional and physiological significance of polyamine signals during late embryonic development in chickens.

In experiment 1, the tissue and age specificity of the polyamine transporters (*SLC18B1 and SLC22A16*) were investigated using real-time PCR.

In experiment 2, polyamine (spermine, spermidine, and cadaverine) solution was administered *in ovo* (intra-egg) to embryos on the 18th day after the start of incubation. Hatched chicks were weighed and slaughtered after blood sampling. The weight of each organ (the whole brain, heart, superficial pectoralis major muscle, liver, sartorius muscle, pancreas, and residual yolk sac) was measured. The plasma glucose and free fatty acid concentrations were measured.

In experiment 3, the gene expression levels of polyamine transporters (*SLC18B1* and *SLC22A16*) and insulin/insulin-like growth factor signal regulators (*INSR, IGF-1R, IRS1*, and *IRS2*) in the superficial pectoralis major muscle after administration of cadaverine were quantified. The collected plasma was also quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for 3-methylhistidine, which is a marker of skeletal muscle protein degradation.

In experiment 4, the gene expression levels of central melanocortin peptides (*POMC, AGRP*, and *NPY)* and pancreatic insulin and incretin (*INS, GCGA*, and *GCGB*) gene expression levels were relative quantified by the qPCR method.

One-way ANOVA was performed for all statistical analyses, and the Tukey-Kramer method was used for multiple comparison tests.

In experiment 1, the gene expression of polyamine transporters (*SLC18B1* and *SLC22A16*) was confirmed in the diencephalon, liver, heart, superficial pectoralis major, duodenum, kidney, pancreas, sartorius muscle, rectum, bursa of Fabricius, and

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testis. It was also clarified that the expression of both transporters in skeletal muscle increased from the 15th day after the start of incubation to 0 days after hatching and then gradually decreased.

In experiment 2, the hatching rate, body weight, and organ weight of chicks after *in ovo* administration of polyamine did not differ from those of the control group. In contrast, the plasma glucose concentration was high, and the free fatty acid concentration was decreased by the administration of cadaverine.

In experiment 3, the expression levels of *INSR, IGF-1R, IRS1*, and *IRS2* in skeletal muscle tended to increase following *in ovo* administration of cadaverine. In addition, the plasma concentration of 3-methylhistidine was decreased by *in ovo* administration of cadaverine.

In experiment 4, the expression levels of *AgRP* and *NPY* tended to be higher in the chick diencephalon after *in ovo* administration of cadaverine, and the expression levels of *GCGA* and *GCGB* genes in the pancreas tended to be higher than those in the control.

These results suggested that polyamines in neonatal chicks act as bioactive substances. Among them, cadaverine may contribute to the suppression of muscle proteolysis and/or the promotion of protein synthesis after hatching, suggesting that their regulation may be mediated by AgRP/NPY neurons in the hypothalamus and insulin secretory function in the pancreas.

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Efficient synthesis of theaflavins by crude enzyme extracted from tuberous roots of sweet potato (*Ipomoea batatas*)

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Black tea is the second most widely consumed drink in the world after water. Black tea production is characterized by the fermentation of leaves of tea plant (*Camellia sinensis*), through which catechins in fresh tea leaves are oxidized and condensed by polyphenol oxidase (PPO), an endogenous enzyme, to produce red-orange pigments, such as theaflavins (TFs) and thearubigins. The combination of two selective catechins, i.e. one is a catechol-type ((−)-epicatechin or its gallate (EC, ECg)) and the other is a pyrogallol-type ((−)-epigallocatechin or its gallate (EGC, EGCg)), results in the production of four kinds of TFs (TF1 without galloyl group, TF2A and TF2B of monogallate, TF3 of digallates). TFs have been reported to possess various biological activities related to health benefits. However, the amounts of TFs in black tea leaves and their exudate are not so high. Therefore, it is difficult to extract and purify TFs from black tea leaves in a good yield which is sufficient for use in studies on the mechanisms of biological action of TFs as well as structure-activity relationships. To overcome this problem, the enzymatic synthesis of TFs has been attempted. The synthetic reaction using catechins and PPO is popular, because catechins are abundant in tea leaves and PPO are widely present in not only tea plant but also many plants. So far, it has been reported that crude enzymes originating from several plants have oxidative activities available for synthesis of TFs, and sweet potato (*Ipomoea batatas*) is one of them.

Tuberous roots of sweet potato are harvested and consumed in Japan as agricultural crops. They are sometimes discarded at the production stage due to poor appearance or out of size specifications. If such discarded sweet potatoes can be effectively used as an enzyme source for the synthesis of TFs, it is expected to add value to the unused resources as well as to reduce food loss. Therefore, in this study we investigated the characteristics of synthetic activities of the crude enzyme extracted from tuberous roots of sweet potato, aiming to use them as an enzyme source for construction of a system to synthesize and supply TFs efficiently. Furthermore, in order to synthesize TFs more easily and inexpensively, we attempted to use catechin-rich green tea infusion as a substrate.

1. Characterization of crude enzyme extracted from tuberous roots of sweet potato for synthesis of TFs.

Two kinds of crude enzymes were prepared from peeled tuberous roots of sweet potato by acetone precipitation or ammonium sulfate precipitation. Each of these crude enzymes was mixed with green tea infusion containing catechins (pH 3.0-7.0), and its specific activity for synthesis of TFs was determined spectrophotometrically by

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measuring the rate of increase in absorbance at 520 nm of the reaction mixture. As a result, the crude enzyme prepared by the ammonium sulfate precipitation method showed higher values for both specific activity and protein recovery than that prepared by acetone precipitation. Thus, we determined to use the former enzyme.

After the crude enzyme was added into the solution containing a mixture of two selected catechins, the reaction mixture was incubated and analyzed by RP-HPLC. Consequently, we found that TFs increased during the incubation and that TF1 was the most abundantly produced, followed by TF2B, TF2A, and TF3. Furthermore, catechin oxidation by the crude enzyme was kinetically analyzed. The crude enzyme was found to be highly reactive to catechol-type catechins, especially EC, but have little effect on pyrogallol-type ones. However, the pyrogallol-type catechins decreased rapidly in the presence of catechol-type ones, in the case of synthesis of TFs. Therefore, it was suggested that the crude enzyme preferentially catalyzes the oxidation of EC or ECg, and then, the resulting product, quinone, oxidizes EGC or EGCg non-enzymatically, and two kinds of quinone are condensed to form TFs.

2. Synthesis of TFs using green tea infusion as substrate solution

To take reproducibility and make the initial catechin concentrations in reaction mixtures, the water extract of instant green tea powder (IGT) was used as a substrate solution for the enzymatic synthesis of TFs. When IGT was mixed with the sweet potato crude enzyme, TFs were produced with the highest amount of TF1, followed by TF2B and TF2A.

Considering that the crude enzyme showed high activities for the synthesis of TF1 from EC and EGC as well as the EC oxidation, we treated IGT with tannase, a degalloylase which converts E(G)Cg to E(G)C, in order to prepare the solution rich in both EC and EGC which was expected to allow more selective and effective synthesis of TF1. Addition of the crude enzyme into the tannase-treated IGT solution caused the TF1 production. However, it was observed that the rates of increase of TF1 and decreases of EC and EGC became very slow despite a large amount of remained EC. Thus, it was suggested that the enzyme activity was inhibited by some impurities present in the tannase-treated IGT solution.

Since this solution contained high concentration of gallic acid (GA), which was released from ECg and EGCg by tannase, we investigated the effects of GA on the synthesis of TF1. By adding GA to a reaction mixture of EC, EGC, and the crude enzyme, GA strongly inhibited the synthesis of TF1 in a dose-dependent manner. This result suggests that the removal of GA from the tannase-treated IGT solution is necessary for its use in the enzymatic synthesis of TF1.

3. Removal of GA from tannase-treated green tea infusion and its evaluation

When the tannase-treated IGT solution was passed through an anion exchange resin (DEAE) packed column, GA could be removed by adsorption on the resin. However, recovery of catechins was extremely low. On the other hand, changing DEAE to a cation exchange resin (CM), we found that separation of GA from catechins was possible depending on the eluent types and concentrations of buffer used after loading the tannase-treated IGT solution onto the column. Finally, the resulting EC- and EGC-rich solution obtained by loading the tannase-treated IGT solution on the CM packed column allowed the enhancement of synthesis of TF1 by the sweet potato crude enzyme.

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Investigation of chlorophyll-degrading enzyme-catalyzed phytol increase during preparation of smoothies made from spinach leaves and green kiwi fruits

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Introduction

Phytol is a diterpene alcohol that constitutes the side chain of chlorophylls, which are photosynthetic pigments. It is known that when chlorophylls are turned over in plants, phytol is released from chlorophyll (Chl) and demetallized Chl (pheophytin: Phein) by chlorophyllase (CLH) and pheophytinase (PPH), respectively. Free form of phytol has been attracting attention because it has been reported that the intake of phytol shows anti-obesity and antidiabetic effects in rodents. It is expected that such biological activities may be caused by dietary intake of phytol in human.

One of the opportunities for human to intake phytol is that chlorophylls from leafy vegetables are dephytylated through the digestive tract. However, previous reports have indicated that this rarely occurs. Other potential sources of phytol are smoothies, purees, and juices made from fruits and vegetable, because during preparation of them enzymatic dephytylation of chlorophyll derivatives can occur. In particular, smoothies, which have recently attracted attention due to their health benefits, are made by grinding the raw fruits and vegetables expected to contain active CLH and PPH. Therefore, it is probable that phytol is produced by the CLH- and PPH-catalyzed reaction with chlorophylls during smoothie processing, and the resulting phytol may play a role in the health benefit of the smoothie. However, there is neither data on the amount of phytol nor the activities of CLH and PPH in raw fruits and vegetables and smoothies made from them.

In this study, we measured those parameters in spinach leaves and green kiwi fruits. Furthermore, based on the enzymatic properties, we made the smoothies from the fruits and vegetables to verify whether phytol could be increased in the smoothies or not.

Materials and Methods

Determination of phytol content in spinach leaves and green kiwi fruits

Spinach leaves or green kiwi fruits were immersed in the double volume of chloroform:methanol (2:1, v/v) and ground in a mortar with a pestle and transferred to a centrifuge tube. The residue in a mortar was washed with the same solvent and combined with the first homogenate, followed by centrifugation. The lower chloroform phase of solvent was collected to a new tube. The residual aqueous phase and pellet were washed with 10 mL of the same solvent, and then centrifuged. The lower phase was combined with the first one.

The collected chloroform phases containing lipophilic components were evaporated to dryness,

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and redissolved with 2 mL of acetone to be used as samples for RP-HPLC analysis to measure the phytol content in 100 g of these foodstuffs.

Measurement of CLH and PPH activities

Crude enzymes used to measure CLH and PPH activities were prepared by the ammonium sulfate precipitation method. After Chl a or Phein a was added into the buffer solution containing crude enzyme, the reaction mixture was incubated. Then, it was mixed with acetone to stop the reaction, followed by extraction of phytol from the supernatant with hexane twice. The collected two hexane phases were combined and evaporated to dryness, redissolved with acetone, and used for RP-HPLC to measure phytol.

Preparation of smoothie made from spinach leaves and green kiwi fruits

Spinach leaves and green kiwi fruits were homogenized using a kitchen blender with cold water to make spinach smoothie (**S**) and kiwi smoothie (**K**), respectively. Before and after incubation of these smoothies for 2 h at 40℃ , an aliquot of each was collected and mixed with an equal volume of acetone to stop enzymatic reaction. After the extraction of phytol with hexane was conducted twice, the collected hexane phases were evaporated to dryness, redissolved in acetone, and used as samples for RP-HPLC analysis to investigate changes in phytol content in these smoothies.

Results and Discussion

It was found that both spinach leaves and green kiwi fruits contain phytol.

As phytol detected in both foods implied the presence of CLH or PPH in them, we incubated Chl a or Phein a with crude enzymes extracted from them and measured the increase of phytol during the incubation. As a result, the crude enzymes showed the CLH and PPH activities. When their pH dependences were examined at pH 4.0-9.0 using Chl a as a substrate, the CLH activity of spinach leaves became higher with increasing pH, whereas that of green kiwi fruits remained high between pH 5.0 and 9.0. For Phein a as a substrate, the crude enzyme from spinach leaves showed the PPH activity with the same pH dependence as CLH, but the PPH activity of that from green kiwi fruits was higher around pH 8.0 compared to CLH. In the temperature dependence study using Chl a as substrate, both crude enzymes showed high CLH activities around 45℃ , whereas in the thermal stability test, the activity decreased to less than half by treatment at 60 ℃ for 30 min. The total activities of CLH and PPH per 100 g of foodstuff, which were estimated based on the protein recovery during crude enzyme extraction and the specific activity exhibited by the crude enzyme at pH 8.0 and 40℃ , were higher for green kiwi fruits than those for spinach leaves.

On the basis of characterization of the CLH and PPH activities in both foods as described above, we investigated whether phytol increases during incubation of the smoothie made from each food at pH 8 and 40℃ . Consequently, phytol increased in **"S"**, pH of which was adjusted to pH 8, during incubation at 40℃ . On the other hand, phytol did not increase in **"K"** under the same condition as **"S"**, even though green kiwi fruits were expected to have higher CLH and PPH activities than spinach leaves. This result was thought to be due to the lack of chlorophylls in **"K"**, because we confirmed the increase of phytol after addition of Chl a or Phein a to **"K"**.

Conclusion

These results suggest the possibility that phytol production in smoothies made from spinach leaves and green kiwi fruits occurs via dephytylation of chlorophyll derivatives by endogenous CLH and PPH during the preparation and processing of smoothies depending on the combination of foodstuffs and conditions for the enzymatic reaction.