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Study on relationship between right heart echocardiographic parameters and pulmonary artery pressure, and pharmacokinetics / pharmacodynamics of oral sildenafil in a canine model of chronic embolic pulmonary hypertension

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Pulmonary hypertension (PH) is a progressive disorder characterized by the elevation of pulmonary artery pressure (PAP). Evaluation of pathological conditions via echocardiography related to right heart, and basic information regarding the pharmacokinetics and pharmacodynamics of oral sildenafil in dogs with PH have not been investigated fully. Therefore, the present study aimed to investigate the relatinoship between right heart echocardiographic parameters and pulmonary artery pressure, and examine the pharmacokinetics and pharmacodynamics of oral sildenafil.

Firstly, we investigated the relatinoship between right heart echocardiographic parameters and invasive pulmonary artery pressure using a canine model of chronic embolic pulmonary hypertension (CEPH). As it turned out that the normalized right ventricular internal diameter in diastole, the ratio of the pulmonary artery and aortic diameter in diastole (PA/Ao), the acceleration time to ejection time ratio in pulmonary artery flow profile (AT/ET), and the normalized tricuspid annular plane systolic excursion were correlated with the PAP. In addition, AT/ET and PA/Ao had sufficient sensitivity and specificity for predicting CEPH. Therefore, alterations in these echocardiographic parameters enable us to evaluate pathological condition related to elevated PAP. Secondly,

we aimed to describe the pharmacokinetic properties of oral sildenafil, and determine the effect of feeding and dose proportionality in healthy dogs. As a result, feeding reduced the absorption of sildenafil. For dose proportionality, nonproportional increases in the plasma concentration and absorbed amount of sildenafil were detected by a power model analysis.

Thirdly, we examined the pharmacokinetics of oral sildenafil in a canine model of CEPH. As a consequence, it is likely that the non-proportionality of sildenafil observed in healthy dogs disappeared in dogs with CEPH showing increased PAP and decreased cardiac output (CO). The disappearance of non-proportionality for sildenafil in CEPH models appears attributable to impaired drug absorption due to hypoperfusion of the gastrointestinal tract resulting from reduced CO. In addition, the decrement in the elimination rate was detected when 4 mg/kg sildenafil were administered compared to 1 mg/kg. However, the extent of the decrement in the elimination rate is mild and it is regarded pharmacokinetically and clinically insignificant.

Finally, we evaluated the short-term effects of oral sildenafil on pulmonary and systemic hemodynamics in a canine model of CEPH. As a result, sildenafil decreased PAP and pulmonary vascular resistance in a dose-dependent manner without notable changes in systemic artery pressure and systemic vascular resistance. Therefore, oral sildenafil at high dose is able to enhance the effect of treatment.

In conclusion, alterations in echocardiographic parameters of right-sided heart enable us to evaluate pathological conditions related to elevated PAP. In addition, the effect of feeding and altered pharamcokinetics of oral sildenafil in dogs with PH should be considered for providing maximal therapeutic effects.

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Studies on *KIT* mutations and toceranib susceptibility in canine mast cell tumor

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In the treatment of canine mast cell tumor (MCT), a kinase inhibitor toceranib is frequently used and has been demonstrated anti-tumor activity in certain dogs. Toceranib is a multi-kinase inhibitor that targets receptor tyrosine kinase such as KIT, PDGFR, and VEGFR. One of the mechanisms underlying the action of toceranib is inactivation of KIT that is constitutively activated by a mutation in MCT cells. However, the therapeutic activity of toceranib does not necessarily correlate with the presence of the *KIT* mutation and thus individualized therapy with toceranib have not been established in canine MCTs. To make a foundation of individualized therapy with toceranib in canine MCT, following studies were performed: comprehensive mutation analysis of *KIT* was performed on genomic DNA samples of 164 dog MCTs using next-generation sequencing (NGS) approach. Moreover, recombinant KIT proteins containing mutations identified by NGS and reported but not characterized mutations were prepared and characterized. As a result, there are various types/characteristics of mutations in *KIT* in canine MCTs. Therefore, it is necessary to consider the difference of characteristics among each mutation type for development of individualized therapy with toceranib in canine MCTs. Moreover, low frequency but some mutations conferred toceranib resistance to *KIT*. Thus, it was considered that some MCT cases have minor clones that have a predisposition of toceranib resistance in tumor tissue before starting the toceranib treatment. Therefore, to clarify the developmental process of toceranib-resistant *KIT* mutation, toceranib-resistant cell lines were generated from cloned MCT cell lines and performed NGS analysis of their *KIT* genes. In this analysis, it was considered that the secondary mutation of KIT plays an important role on resistance to toceranib in canine MCTs. Therefore, to develop a strategy to overcome toceranib- resistance in MCT, effects of SHP2 inhibition on the growth of toceranib-resistant MCT cell lines was examined. Although SHP099 alone did not show obvious growth inhibitory effect to toceranib-resistant MCT cell lines, it showed potent growth inhibitory effect when combined with toceranib. In conclusion, it is necessary to consider the difference of characteristics among each mutation type for development of individualized therapy with toceranib in canine MCTs. Particularly, it should be noted that there are tumor cells carrying toceranib-resistant *KIT* mutations that may pre-exist in toceranib-naïve tumor tissue or may occur *de novo* in tumor tissue during toceranib treatment. For canine MCTs carrying toceranib-resistant clones, a combination therapy of toceranib with SHP099 might be a potential therapeutic approach.

*Supervisor : Prof. Makoto BONKOBARA

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Preemptive Veterinary Medicine of Feline Obesity Disease

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Obesity is now a major global health problem. The incidence is rising in recent years not only in developed countries but also in developing countries. Obesity is a non-infectious disease that is a risk factor for serious metabolic diseases such as type 2 diabetes, hypertension, cardiovascular disorders, and cancer, so overcoming it is an urgent issue for human race. In 2000, the Japanese Society of Obesity published the "New Obesity Judgment and Diagnosis Criteria of Obesity disease". And Obesity disease is defined as obesity that is associated with health problems and requires medical weight loss.

Dogs and cats visiting animal hospitals, like humans, tend to increase obesity with age. In our study, overweight to obesity in dogs in 2019 was 38.3%, and cats were 49.1%. Since the subjects were health check-up animals that are relatively interested in health compared with general owners, it is expected that the ratio of actual visiting animals will be higher.

Cats are more likely to be obese than dogs because of their unique glycolipid metabolism characteristics. Based on these metabolic characteristics of cats, we developed a flowchart and criteria for determining obesity disease. It is divided into simple obesity and obesity disease according to the presence or absence of health disorder. Obesity disease in cats was defined as those showing two or more symptoms of overweight, hyperlipidemia, hypoadiponectinemia,and hyper-SAA symptom over BCS7 / 9.

Quercetin derivative Rv-PEM01 is a plant-derived active ingredient phytochemicals having antioxidative and anti-inflammatory effects. The effect of administering this quercetin derivative to healthy cats and obese cats for 4 weeks was examined. Significant increase in liver function-improves lipid metabolism. Quercetin derivatives can be expected to have anti-oxidant and anti-inflammatory effects even when administered to healthy animals. Therefore, quercetin derivatives can be applied to prevent metabolic disorders such as obesity, hyperlipidemia and type 2 diabetes.

Early diagnosis and appropriate early response are effective in suppressing obesity disease. For this purpose, it is also necessary to apply genomics, proteomics and metabolomics analysis data accumulated in the process of developing markers for early diagnosis and digitization as big data. If preemptive medicine is used to detect obesity at an early stage and the occurrence of severe metabolic diseases based on obesity can be suppressed, medical costs will be reduced and healthy life expectancy will be extended.

I think there is no doubt that it will contribute not only to economic aspects but also to the realization of a happy society.

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Study on occurrence of aldosterone breakthrough in dogs

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Aldosterone breakthrough (ABT) is observed during renin-angiotensin-aldosterone system (RAAS) suppression therapy using angiotensin-converting enzyme inhibitor (ACEI) and angiotensin II receptor blocker (ARB). Therefore, in human patients, a mineralocorticoid receptor antagonist (MRA) has been used in combination with an ACEI or ARB to prevent the adverse effects caused by excess aldosterone. However, there are few study that investigate the usefulness of MRA in dogs. Therefore, the objective of the present study was to determine if alacepril or telmisartan has a long duration of suppressive effects on drug-induced RAAS activation in dogs. In addition, this study investigated whether MRA has organ-protecting actions on drug-induced RAAS activation in dogs. Finally, this study investigated whether ABT occurs in dogs with proteinuric kidney disease during telmisartan therapy.

Firstly, the objective of this study was to determine if alacepril has a long duration of action for inhibition of drug-induced RAAS activation in normal dogs. As a result, alacepril temporarily suppressed drug-induced RAAS activation but its clinical application may be limited by its duration of action. In light of this decrease in RAAS inhibitory activity with time, it is possible that ABT occurred.

Secondly, this study investigated whether telmisartan has suppressive effects on drug-induced RAAS activation in dogs. As a result, telmisartan did not fully suppress drug-induced RAAS activation. Therefore, it is necessary to consider the existence of ABT during the ARB therapy in dogs.

Thirdly, the objective of this study was to investigate whether MRA (spironolactone, eplerenone) has organ-protecting actions on drug-induced RAAS activation in dogs. As a result, spironolactone (2 mg/kg, at either q24 h or q12 h, PO) temporarily decreased serum galectin-3 concentration as a biomarker for tissue fibrosis in dogs with drug-induced RAAS activation. Therefore, administration of spironolactone at this dosage could be useful for the organ-protecting actions.

Finally, this study investigated whether ABT occurs in dogs with proteinuric kidney disease during telmisartan therapy. As a result, ABT occurred in dogs with proteinuric kidney disease during RAAS suppression therapy.

In conclusion, this study confirmed that ACEI or ARB dose not fully suppress drug-induced RAAS activation in dogs. Moreover, it becomes clear that ABT is associated with these results. In addition, this study indicated that administration of spironolactone could be useful for the organ-protecting actions in dogs with drug-induced RAAS activation. Finally, the present study revealed that ABT occurred in dogs with proteinuric kidney disease during RAAS suppression therapy.

*Supervisor : Prof. Naoyuki TAKEMURA

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Studies of *PTPN11*/SHP2 mutations in canine histiocytic sarcoma cells

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Some canine cases of histiocytic sarcoma (HS) carry mutations in the src homology 2 domain-containing phosphatase 2 (SHP2) encoded by *PTPN11*. However, the precise mutational positions in SHP2 were not clear because entire coding nucleotide sequence of canine *PTPN11* has not been determined. In addition, functional role of the SHP2 mutations on structure and activity of SHP2 and growth of HS cells is unclear. SHP099 is an allosteric inhibitor of SHP2 that stabilizes SHP2 in a folded, auto-inhibited conformation. The purpose of this study was to make a foundation of SHP2-targeted therapy for canine HS. To investigate expression levels and mutation status of *PTPN11*/SHP2 in HS cell lines, firstly, entire coding nucleotide sequence of canine *PTPN11* was determined using cardiac cDNA isolated from healthy dog

(registered with NCBI: GenBank accession number, MK_372881.1).

Subsequently, expression levels and mutation status of *PTPN11*/SHP2 in HS cell lines were examined. All of six HS cell lines examined with western blot analysis were expressed SHP2 and four out of nine HS cell lines had mutations in *PTPN11*/SHP2 (p.Ala72Gly, CHS-1; p.Glu76Gln, CHS-3; p.Glu76Ala, CHS-6; p.Gly503Val, ROMA).

Moreover, effects of mutations on the structures and the phosphatase activities of canine SHP2 were examined. Recombinant canine SHP2 harboring p.Ala72Gly, p.Glu76Gln and p.Glu76Ala showed constitutive phosphatase activities, while phosphatase activity was not detectable in wild-type SHP2 and SHP2 harboring a p.Gly503Val mutation. The activities SHP2 harboring p.Ala72Gly, p.Glu76Gln and p.Glu76Ala were inhibited by SHP099. *In silico* analysis suggested that mutations p.Glu76Gln and p.Glu76Ala but not p.Ala72Gly and p.Gly503Val promote shifting of the SHP2 conformation from folded to open-active state. Furthermore, the growth inhibitory properties of SHP099 for HS cells were investigated *in vitro* and *in vivo*. Among six HS cell lines, SHP099 potently suppressed the growth of CHS-3 (p.Glu76Gln) and CHS-6 (p.Glu76Ala) cells. In contrast, other cell lines harboring SHP2 p.Ala72Gly, p,Gly503Val or wild-type had lower susceptibilities to SHP099. In HS xenograft mouse model using CHS-6 (p.Glu76Ala), SHP099 exhibited potent anti-tumor activity. In conclusion, p.Glu76Gln and p.Glu76Ala are activating mutations of SHP2 and play a pivotal role for survival/growth of HS cells carrying these mutations. Targeting p.Glu76Gln and p.Glu76Ala SHP2 with SHP099 may be a new therapeutic strategy for canine HS.

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The expression of tumor endothelial marker 8 in mammary gland tumor, and the effects of endotrophin on neoplastic cells

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Tumor endothelial marker 8 (TEM8) is highly expressed in vascular endothelial cells within tumors and involved in tumor angiogenesis. In this study, to clarify the expression and biological significance of TEM8 in canine mammary gland tumors (MGTs), we investigated the histological localization of TEM8 and expression of TEM8-isoforms in canine normal tissues, developmental alterations of TEM8 expression in normal mammary gland (MG) epithelium, and the phenotypical characteristics of TEM8 expressing MGT cells in canine MGT cases and canine MGT cell lines. Furthermore, we also examined the effects of endotrophin (ETP) on the MGT cells. In the mammogenesis, TEM8 expression in MG epithelial cells was increased along with the development of luminal structures and related to the expressions of Notch-1 and c-MET. Previous studies demonstrated that Notch-1 induced the differentiation of luminal cells, and c-MET promoted the luminal structure formation during development of MG epithelial cells, indicating that TEM8 contributes to regulation of the luminal cell differentiation and maturation. In canine MGTs, TEM8 expression was detected in luminal-like (CK19/p63/αSMA; +/-/-) but not in basal-like neoplastic cells (CK19/p63/αSMA; -/+/-). Almost TEM8 (+) MGT cells showed the luminal formation and expressed Notch-1 and c-MET as in the normal MG. In addition, TEM8 (+) MTG cells also showed expression of collagen VI α 3 C5-domain, a source of ETP. Furthermore, ETP-stimulation significantly increased proliferation, cell migration and expressions of *CD44* and *CD49b* mRNA, and significantly decreased expressions of *EpCAM* and *CD133* mRNA in MGT cells. These results indicated that ETP/ TEM8 autocrine signaling might maintain the MGT cells at luminal progenitor stages with high proliferation ability by induction of differentiation from MGT stem cells to the luminal progenitor and suppression of maturation to luminal cells. This study indicated that TEM8 had involved in the expression of the pathological characteristics related to the kinetic of MGT neoplastic cells, and might be an important indicator for estimating the clinical and biological behaviors in canine MGT cases.

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Studies on growth mechanisms and their suppression in canine squamous cell carcinoma cells

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Treatment of unresectable canine squamous cell carcinoma (SCC) remains challenging and new therapeutic strategies is needed. Because previous studies have been demonstrated that survivin and EGFR are overexpressed in canine SCC tissues, these molecules are considered to be closely associated with growth of canine SCC. In this study, to establish new therapeutic strategies for canine SCC, sensitivities of seven SCC cell lines to YM155, afatinib, and osimertinib were examined. Moreover, by focusing to YM155 and afatinib, growth inhibitory mechanisms in canine SCC cell lines were investigated. Furthermore, anti-tumor effect of afatinib against canine SCC cell line *in vivo* was tested. YM155 and afatinib potently and selectively inhibited growth of HAPPY and SQ4 cells and POCO and CSCC-R1 cells, respectively. In contrast, osimertinib did not show such growth inhibitory effects against SCC cell lines. Both YM155-sensitive cell lines HAPPY and SQ4 cells highly expressed survivin, while suppression mechanisms of survivin by YM155 were differed between HAPPY and SQ4 cells, in which YM155 inhibited survivin expression by suppression of *survivin* in HAPPY cells, while it inhibited survivin expression via post-transcriptional mechanism in SQ4 cells. Although YM155 induced autophagy and subsequent PARP-dependent apoptosis in both cell lines, HAPPY cells primarily underwent cell death via PARP-dependent apoptosis, while there were two different cell death mechanisms including PARP-dependent apoptosis and probably autophagic cell death in SQ4 cells. In afatinib-sensitive POCO and CSCC-R1 cell lines, no aberrant of known afatinib target molecules was identified. In a comprehensive analysis of phosphorylated proteins using POCO cells, it was found that afatinib mainly suppressed activation of MAPK pathway. Furthermore, afatinib showed a remarkable anti-tumor effect against POCO cells xenograft mouse. In conclusion, it was suggested that the expression of survivin or phosphorylation of the MAPK pathway plays a crucial role in growth/survival of certain canine SCCs. YM155 and afatinib may be promising as new therapeutic strategies for such canine SCCs.

*Supervisor : Prof. Makoto BONKOBARA

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Expression of vascular endothelial growth factor and its receptors in canine mast cell tumors

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The aim of this thesis is to clarify the involvement of vascular endothelial growth factor (VEGF) -A and its receptors (VEGFRs; Flk-1 and Flt-1) in the differentiation and malignant progression of canine cutaneous mast cell tumors (MCTs). Immunohistochemical examinations revealed that expression of VEGF-A and Flk-1 in MCTs were associated with histological malignancy of the MCTs determined by histological grading systems and c-Kit patterns. In particular, VEGF-A/Flk-1 co-expression was found in highly malignant MCTs, indicating the involvement of their autocrine signaling in the tumor progression. Most of MCT cells expressing Flk-1 and/or VEGF-A also showed poor staining for safranin O (SO) and negative for Gi1 immunohistochemistry. These findings suggest that VEGF-A/Flk-1 signaling maintain the immature state of the tumor cells, which lead to the malignant progression in canine cutaneous MCTs. In normal skin mast cells (MCs) of rats, VEGF-A, Flk-1, and Flt-1 were expressed only in the immature cells during differentiation and maturation. Thus, VEGF-A/Flk-1 and VEGF-A/Flt-1 signaling may regulate the MC differentiation in a coordinated manner. The *in vitro* study using mouse bone-marrow derived MCs (mBMMCs) revealed co-expression of *vegf-A* and *flk-1* at the early, but not late stages of culture. In contrast, *gata2* expression increased at late stages of culture. Inhibition of Flk-1 signaling upregulated *gata2* expression in the mBMMCs. These findings indicate that VEGF-A/Flk-1 signaling suppress MC differentiation and maintain phenotypes of immature MCs by downregulation of GATA2 expression. In conclusion, my thesis suggested that VEGF-A/Flk-1 signaling maintain the immature features by inhibition of GATA2, which lead to tumor progression in canine cutaneous MCTs. Treatment targeted at VEGF-A/Flk-1 signaling would provide a new therapeutic strategy of the highly malignant MCTs.

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Detection of bovine rotavirus C and study on its genetic properties

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In the virus taxonomically, bovine had not generally been included in the natural host of Rotavirus C (RVC). However, the first detection of RVC from cattle in the world is a Shintoku strain isolated from diarrhea of adult cattle in Hokkaido in 1991. Following it, RVC Yamagata strain was detected from diarrhea cases of dairy cattle caused the outbreak in a farm in Yamagata prefecture in April 2002. Since then, the presence of bovine RVC was recognized and, in addition, multiplex RT-PCR to detect major diarrhea-related viruses including bovine RVCs was developed. Therefore, diarrhea cases on bovine RVC were reported one after another. In order to elucidate the occurrence state of bovine RVCs in the field, major diarrhea-related pathogenic microorganisms were detected from the case of diarrhea occurred on farms in Yamagata Prefecture for ten years and the information was organized for the occurrence state and clinical symptoms.

Bovine RVC was suggested to be one of the major pathogenic microorganisms that cause outbreak of diarrhea in dairy cattle from autumn to early spring. High incidence seasons and occurrences of bovine RVC disease showed a common tendency to bovine coronavirus (BCoV) disease and bovine rotavirus B (RVB) disease, and clinical symptoms of bovine RVC disease was similar to bovine RVB disease and slightly different from BCoV disease. The occurrences of bovine RVC in the field have been gradually elucidated, but genetic properties of bovine RVC have not been clarified because of little genetic information. Therefore, all 11 gene segments of RVC 6 strain detected in Chapter 2 were analyzed on genetic diversity, genetic dynamics and ecology of bovine RVC in the field. As a result, it was recognized for the first time that VP4 genes of various bovine RVC strains were resulted in the low homology with nucleotide deletion and insertions. These results indicate that there are various genetic diversities. In addition, it shows that strains belonging to the same genotype acquire genetic diversity by repeating reassortants independently for each segment.

These results lead us to the conclusion that several strains possessing different genetic backgrounds are widely distributed in Japan and are involved in bovine diarrhea. Also, this study revealed at the genetic level for the first time that the same strain or new strain resulted in occurrence repeatedly with bovine RVC disease in neighboring farms and re-occurred in the same farm.

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Studies on the occurrence, pathological conditions, and reduction method for subacute ruminal acidosis (SARA) in dairy cows

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The objectives of this study were to reveal the impacts of subacute ruminal acidosis (SARA) and to establish preventive measures against SARA, which has become a problem associated with the management for lactating cows in recent years. In this study, a field survey of SARA, using a wireless radio transmission pH measurement system (pH sensor), was conducted, and blood levels of various hormones and compounds related to energy metabolism in cows with SARA were evaluated. Additionally, the preventive effect of wood kraft pulp (KP) was investigated in cows with SARA. The first chapter describes the SARA field study in the Mogami region, Yamagata prefecture, using pH sensors. We demonstrated that the prevalence of SARA 45 days before and after the parturition differed among 5 farms, as it ranged from 29.0 to 77.4%. The results of the field survey suggested that ruminal pH values in herds are influenced by the feeding system (total mixed ration or separated), starch concentrations in feed, diet changes of cows during transition, and other factors.

The second chapter describes the investigation of 11 lactating cows, during 2015-2016 at Farm S, presumed to have SARA based on the field survey described in Chapter 1. We continuously monitored ruminal and reticular pH and measured blood concentrations of hormones and metabolites related to energy metabolism. Adiponectin (ADN) concentrations at 4 weeks after parturition correlated with the total amount of time that ruminal or reticulum fluid pH was under the threshold during 1week after parturition. Additionally, there was a strong correlation between the number of days that SARA was detectable based on the criteria in the 30 days after parturition and ADN concentrations at 4 weeks, and average ADN concentrations at 1 and 4 weeks. These results suggest that ADN may serve as an index to assess SARA.

In the third chapter, we describe calculations of Pearson's correlation coefficient for the association of ADN concentration and ruminal characteristics. There was a negative correlation between mole fraction of acetic acid in rumen and ADN concentrations at 4 weeks post-parturition. The result suggests that ADN reflects the ruminal condition, supporting the conclusion described in the second chapter.

In the fourth chapter, we describe the investigation of the effect of KP feed, which is a nutrient-rich feed that is almost pure cellulose fiber and has a slow digestion rate, on reducing SARA and improving lipid metabolism in cows with SARA. The results suggest that supplementation of KP could improve ruminal and reticular pH and lipid metabolism in cows with SARA, although the effects of KP may depend on the constituent concentrations of feeds.

In this study, we conducted a field survey of SARA and demonstrated that the concentration of ADN, which is an adipocytokine involved in the regulation of glucose and fatty acid metabolism, is associated with ruminal fermentation. Additionally, we demonstrated the possibility of decreasing SARA by feeding KP. In the future, further studies measuring inflammatory markers, cytokines, and LPS concentrations in the large intestine may assist in clarifying the relationships among SARA, inflammatory response, and ADN. More investigation about impacts of body weight variation and other inflammatory diseases is also required. This report is expected to lead to future studies to determine the effectiveness of ADN as a biomarker for SARA, and the impacts of SARA as a metabolic disease, as well as effective preventive measures against SARA. This study may also contribute to solving the pathophysiology and prophylaxis of SARA.

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Study on Usefulness of Blood Lactate Measurement and Transrectal Ultrasonography for Diagnosis of Uterine Torsion Severity in Dairy Cows

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In cows, uterine torsion occurs in the uterine body at the base of both uterine horns or at the cervix, unlike in cats where it occurs at a uterine horn. Clockwise torsion, when viewed from the rear of the cow, is also referred to as right torsion, and counterclockwise torsion as left torsion. Known causes of bovine uterine torsion include 1) increased uterine mobility due to extension of the broad ligament of the uterus, which suspends the uterine horns in the abdominal cavity; 2) anatomical factors, such as the uterus compressed by the rumen, a large organ occupying nearly the entire left abdominal cavity; 3) swaying of the uterus when the cow stands up; 4) fall or sliding in a cattle barn; and 5) fetal movement during late pregnancy. Bovine uterine torsion occurs in the second half of pregnancy, during late pregnancy, or during delivery, and is more likely to occur in dairy cows than in beef cows, accounting for about 10% of the causes of bovine dystocia. Prolonged uterine torsion can lead to fetal death or maternal debilitation.

Treatment options for bovine uterine torsion include inserting a hand through the vagina into the uterus to grasp and rotate the fetus in the opposite direction to torsion (fetal version); laying down the cow and then rotating the cow in the same direction as torsion (maternal version); maternal hindlimb lifting; and open surgery. From these options, the optimal reduction/treatment strategy must be selected, taking into account the time from occurrence, severity of torsion, and maternal and fetal conditions. However, with no defined markers or criteria for determining the optimal treatment strategy for uterine torsion, veterinarians have made treatment decisions based on their own personal experience. This has prevented the provision of appropriate treatment for mild, moderate, and sever uterine torsion, resulting in delayed reduction of torsion as well as fetal death and marked maternal debilitation in many cases. The presence and severity of bovine uterine torsion can usually be determined by vaginal or rectal examination, with no other acceptable scientific diagnostic procedures.

Congestion and decreased circulation/hypoxia in organs and tissues are known to cause increased blood lactic acid levels. Studies have suggested the usefulness of blood lactic acid levels for predicting the prognosis of equine colic and bovine abomasum displacement. It has also been reported that destruction of cells in organs and tissues lead to increased blood activities of aspartate aminotransferase (AST) and creatine phosphokinase (CK). Depending on its severity, bovine uterine torsion may also lead to congestive necrosis of the uterine wall. The objectives of this study were to determine whether blood lactic acid levels and blood AST/CK activity levels can be markers for the severity of symptoms in dairy cows with pregnancy-related uterine torsion and can guide the selection of a proper strategy for reducing/treating uterine torsion. We also investigated whether the severity of congestive necrosis of the uterine wall due to uterine torsion as determined by ultrasonography can be a marker for the severity of uterine torsion, treatment selection, and prognosis.

1. Effect of obstetrical procedures after reduction of uterine torsion in dairy cows on maternal/fetal survival and subsequent maternal reproductive performance (Chapter 2)

A total of 112 Holstein cows in which uterine torsion was successfully reduced by non-surgical techniques were divided into 3 groups according to the post-reduction

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obstetrical procedure used to pull/deliver the fetus from the uterus: group A, no treatment or mild pulling (n = 48); group B, moderate or intense pulling (n = 48); and group C, cesarean section due to inability to pull the fetus. These 3 groups were compared for maternal and fetal survival rates and subsequent maternal reproductive performance. The maternal survival rates in groups A, B, and C were 97.9%, 89.6%, and 75.0%, respectively, with the rate in group C significantly lower than that in group A ($p < 0.05$). The post-delivery survival rates of calves in groups A, B, and C were 83.3%, 52.1%, and 18.8%, respectively, with the rates in groups B and C significantly lower than that in group A ($p < 0.05$). The maternal conception rates after artificial insemination during the first year after reduction of uterine torsion in groups A, B, and C were 89.4%, 67.4%, and 66.7%, respectively, with the rates in groups B and C significantly lower than that in group A ($p < 0.05$).

These results demonstrate that excessive pulling of the fetus significantly affects maternal and fetal survival rates. The unfavorable maternal and fetal survival outcomes after cesarean delivery may be due to the tendency for cesarean operations to be performed long after the occurrence of uterine torsion caused by failed attempts of non-surgical reduction. These results suggest the need for diagnostic criteria or markers in favor of earlier cesarean delivery.

2. The usefulness of blood lactic acid levels for diagnosing uterine necrosis due to uterine torsion and prognosis prediction in dairy cows (Chapter 3)

We investigated whether blood lactic acid levels and blood AST/CK activity levels can be used as markers for the severity of uterine torsion and the prognosis of mother cows after reduction/treatment of uterine torsion. Blood samples were collected from 54 Holstein cows with untreated uterine torsion and measured for lactic acid, AST, and CK levels. Cows with congestive necrosis of the uterine wall due to uterine torsion (severe group) showed a mean blood lactic acid level of 15.0 mmol/L, compared with 3.0 mmol/L in cows without necrosis, with a significant difference ($p < 0.01$). Meanwhile, no significant difference was observed in blood AST or CK activity levels. Moreover, the mean blood lactic acid levels in mother cows that died and survived after treatment of uterine torsion were 10.2 and 3.1 mmol/L, respectively, with a significant difference ($p < 0.01$). In contrast, no significant difference was observed in blood AST or CK activity levels between the two groups.

By statistical analysis, the lactic acid cut-off for suspecting uterine necrosis due to uterine torsion was determined as ≥ 5.0 mmol/L, and that for indicating poor prognosis even after reduction of uterine torsion as ≥ 6.5 mmol/L.

These results demonstrate that blood lactic acid levels can be used as a marker for determining the severity of uterine torsion and the prognosis of mother cows. In a clinical setting, cows with uterine torsion should be measured for blood lactic acid levels using a commercially available portable measurement device and lactic acid levels higher than 5.0 mmol/L should prompt early cesarean delivery, rather than attempting non-surgical reduction.

3. Diagnosing the severity of uterine torsion by ultrasonography combined with blood lactic acid measurement (Chapter 4)

A total of 33 Holstein cows with uterine torsion were subjected to measurement of blood lactic acid levels and rectal ultrasonography to determine the thickness of and any damage in the uterine wall. Cows with blood lactic acid levels of ≥ 5.0 mmol/L were found to have an increased uterine wall thickness of 15-25 mm and were more likely to have damage in the uterine wall such as congestive necrosis. The degree of uterine torsion in these cows was considered to be severe. These results demonstrate that the severity of uterine torsion in cows can be accurately diagnosed by blood lactic acid levels and ultrasonography findings of the uterine wall. Cows with reduced uterine blood flow due to uterine torsion tended to have reduced maternal as well as fetal survival rates. Thus, for cows with severe uterine torsion, early cesarean delivery should be considered, rather than wasting time attempting non-surgical reduction, in order to improve fetal survival outcomes, avoid maternal debilitation, and preserve subsequent maternal reproductive performance. In cows with uterine torsion, the combined use of blood lactic acid measurement and ultrasonography of the uterine wall is very effective in determining proper treatment strategies for uterine torsion and for accurate prognosis prediction.

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Study of transmission pattern in sarcoptic mange of raccoon dogs (*Nyctereutes procyonoides*) in Japan

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Sarcoptic mange is a parasitic skin disease caused by the mite *Sarcoptes scabiei*. In Japan, sarcoptic mange outbreaks have been observed in wild raccoon dogs (*Nyctereutes procyonoides*). Previous studies have suggested the cross-transmission of *S. scabiei* between raccoon dogs and other animals, and it is necessary to confirm the transmission pattern of *S. scabiei* in raccoon dogs. The objective of this study was to confirm factors of the epidemic of sarcoptic mange in raccoon dogs and the transmission pattern of *S. scabiei* considering the ecology of raccoon dogs.

In a camera-trapping survey, a sarcoptic mange epizootic occurred after raccoon dog population densities increased. It is suggested that direct contact among raccoon dogs increase with high population densities. Based on genetic analysis of pregnant females and fetuses, there was a possibility of multiple paternities in wild raccoon dogs, which suggests that direct contact of multiple raccoon dogs in the mating season may have occurred. When raccoon dog population densities increase in the mating season, the frequency of transmission of *S. scabiei* may encourage sarcoptic mange spread.

Previous studies were conducted with the suspicion that a factor of the sarcoptic mange epizootic in raccoon dogs was direct contact transmission between parents and offspring. However, in these results, many infested individuals were unrelated. Additionally, the transmission pattern between breeding pairs is not an important factor for the sarcoptic mange epizootic.

Based on genetic structure analysis of raccoon dogs in Gunma Prefecture, wherein genetic groups were distributed and gathered, it was suggested that raccoon dogs had sedentary habits. Considering that the sarcoptic mange epizootic occurred locally, it is suggested that direct contact between closely inhabiting individuals, without blood relationship, is an important factor of the local sarcoptic mange epizootic.

Based on genetic structure analysis of *S. scabiei* in Gunma Prefecture, the distribution of the genetic population structure of *S. scabiei* is not linked to the genetic population of raccoon dogs and mixed population in the same host animals. Additionally, two genetic groups were mixed between raccoon dogs and red foxes, suggesting that cross-transmission of *S. scabiei* among these animals may have occurred. Based on genetic structure analysis of raccoon dogs and *S. scabiei* in Kanagawa Prefecture, the distribution of the genetic population structure of *S. scabiei* is also not linked to the genetic population of raccoon dogs. Thus, it is possible that *S. scabiei* transmission is complex, including raccoon dogs and many other animals. There is a high possibility that many host animals are related to the transmission of *S. scabiei* in raccoon dogs.

*Supervisor : Prof. Toshinori OMI

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Positive and Adverse Effects of Radiotherapy for Canine and Feline Brain Tumor

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Treatment for cancer usually involves surgery, chemotherapy and radiation therapy (RT) in veterinary medicine. The brain tumor has many cases having difficulty in surgery, and RT becomes the adaptation. Therefore, in this study, we examined the therapeutic and adverse effects of RT against brain tumors in dog and cat.

The purpose of chapter 1 was to determine the therapeutic and/or adverse effects of RT against pituitary tumors in dogs with pituitary-dependent hypercortisolism, as monitored by frequent post-RT detailed MRI examinations, clinical signs, and changes in hormone concentrations. In conclusion, RT is effective to reduce pituitary size and the mass effect, but does not appear to affect blood hormone concentrations, necessitating additional medical treatment against hypercortisolism. Periodic MRI imaging post-RT enables early detection of adverse effects of RT.

The purpose of chapter 2 was determine the therapeutic and/or adverse effects of the combination of RT and hydroxyurea against meningioma in dogs, as monitored by frequent post-RT detailed MRI examinations, clinical signs, and tumor size. In conclusion, the combination of RT and hydroxyurea is effective to extend duration of survival. But the neurologic signs were recurred in relation to brain hemorrhage. For the combination of RT and hydroxyurea, survival time was longer than a past report and the serious adverse effect did not happen.

In chapter 3, a 12-year-old, castrated male cat with diabetes mellitus was diagnosed with acromegaly and examined with magnetic resonance imaging (enlarged pituitary gland, 8 mm); serum hormone concentrations were measured. After the first course of radiation therapy (4 Gy, 12 fractions), insulin administration was not required from day 420 after diagnosis. Enlarged pituitary tumor (8 mm) recurred, and insulin dosage amount of the cat was increased on day 1,065. The second course of radiation therapy (6 Gy, 4 fractions) was performed on day 1,201 and insulin administration was again discontinued. However, the cat died from lymphoma on day 1,397. Postmortem examination revealed pituitary adenoma. Most tumor cells were positive for chromogranin A, synaptophysin, and growth hormone immunohistochemistry. The pancreatic islet cells revealed diffuse hyperplasia. We achieved long-term successful management of an acromegalic cat with two courses of RT. However, a protocol for a second course of RT for feline recurrent pituitary tumor should be further discussed.

This study is the preliminarily study to examine the therapeutic and adverse effects of RT against brain tumors in dog and cat. Periodic MRI imaging post-RT enables early detection of adverse effects of RT and neurologic signs. RT against brain tumors in dog and cat is useful method to extend duration of survival time and improve neurologic signs.

*Supervisor : Prof. Toshinori SAKO

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Effects of drinking environment on moisture balance in cats

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Feline lower urinary tract disease (FLUTD) occurs commonly in cats. FLUTD causes problems in the bladder and urethra, with symptoms such as dysuria, hematuria, frequent urination, and inappropriate voiding. Water intake is important for prevention of FLUTD, and it is important for owners to manage water intake on a daily basis in cats. In this study, we focused on the drinking environment on moisture balance in cats. First, we investigated water intake environment of household cats, and estimated risk of FLUTD for each condition. Next, we investigated if the number of water bowls influense drinking behavior, urine volumes, and urine properties of the cats. We used paper sand of toilet, which were proved to be good for urinary examinations based on our examination.

In the first chapter, we investigated water intake environments of household cats by internet questionnaire study. Ultimately, we obtained 406 valid responses, and 325 cases (with clear information of drinking environment) in which were used for analysis. The average number of water bowls was about 2.0 per family and about 1.3 per cat in Japan. The number of water bowls per cat decreased as the number of cats per family increased. The cats with fewer bowls showed a higher risk of FLUTD. Fewer bowls may provide cats less opportunity to access water and restrict their drinking behavior. In addition, stress of group feeding is known as a risk factor of FLUTD, and this may also be affected by competition for water bowls by the cats. In this situation, risk of FLUTD may be increased by urine condensation due to reduced urine volume. Ensuring a sufficient number of water bowls in the rearing environment is important for prevention of FLUTD.

In the second chapter, we examined urine collection conditions in order to collect urine specimens in a home environment. In order to collect urine at home without stressing the cat, we used a two-stage litter box toilet. However, there is not enough data about the effects of litter box sand made of different materials on urine volume and properties. Therefore, we compared the effects of litter box sand made of various materials, such as paper, wood, zeolite, and silica gel to find the optimum material for the examination. In the results, paper sand caused the least effect on urine passage, chemical properties (bilirubin, ketone bodies, protein and glucose), pH, specific gravity, and sediments. We concluded that two-stage litter box toilet with paper sand is optimal to collect urine specimens of cats by spontaneous urination at home.

In the final chapter, we increased the number of water bowls in the cat breeding environment experimentally, and investigated their effects on drinking water volumes, urine output, and urine properties. Five cats kept at home were used with the cooperation of their owners. Urine samples were collected by a spontaneous urination method using a two-stage toilet and non-absorbable paper sand. When we increased the number of bowls, their average water intake volume tended to increase from 72.8 g / day to 94.1 g / day, and the urine volume significantly increased from 36.5 g / day to 52.2 g / day during the two weeks. The increased water bowls may have increased cat access to water and increased drinking and urine output. Our hypothesis that the increment of water bowls number is effective for preventing FLUTD was partially proved. However, to prove this hypothesis, it is necessary to increase the number of cases, extend the measurement schedule, and examine the more numbers of water bowls. It is also necessary to collect data in multiple breeding environments, and we are planning to continue our study with more home-reared cats in the future, using a system litter box that can collect data on an individual basis. According to preventive veterinary medicine, it is important for veterinary nurses to provide appropriate advice to cat owners about the drinking environment.

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Comparison of monosodium glutamate toxicity in two outbred strains of neonatal rat

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Same genetic analysis methods can be used for rats and mice, but more tissues can be collected from a rat than a mouse because rat body size is 10 times bigger than mice. Rats have been widely used in many research fields, including ethology, physiology, and pathophysiology because they are an alternative mammalian model to mice. Particularly, outbred Wistar and Sprague-Dawley (SD) rats are used for pharmacodynamic, toxicity, and safety tests of substances, such as medicines and chemicals.

Cytochrome P450 (CYP), a drug-metabolizing enzyme, is a factor that influences the results of drug efficacy and safety evaluation tests. CYP is most prominently expressed in the liver, with numerous isoforms, many of which show sexual dimorphism. Sexual dimorphism is a condition in which gene expression differs between the two sexes of the same species. Growth hormone (GH) is the only endogenous factor regulating the sexual dimorphism of liver CYP. By blocking GH secretion with neonatal administration of monosodium glutamate (MSG) in SD rats, Shapiro *et al*. revealed that the pituitary GH secretion pattern regulates the hepatic CYP expression. In our previous study, Wistar-Tg (Alb-DsRed2) 34Jmsk rats that express red fluorescent protein (DsRed2) specifically in hepatocytes with albumin promoter were selected for liver regeneration studies. The DsRed2 expression shows sexual dimorphism, is expressed only in adult male rats, and cross-correlates with the GH-dependent sexually dimorphic CYP expression in hepatocytes. However, it is unclear whether GH has a causal relationship with the sexual dimorphism of DsRed2 expression.

Recently, Nishiyama *et al*. revealed that CYP gene expression and activity among rat strains are different. For accurately evaluating the efficacy of a novel drug, it is important to understand the changes in hypothalamic–pituitary GH secretion that dramatically affect CYP expression among rat strains. This study aims to clarify the effects of MSG on strain differences in SD and Wistar-Tg rats. We evaluated the cerebral neurotoxicity and gene expression of GH and CYP by MSG administration in two neonatal rat strains.

SD and Wistar-Tg rats were used in this experiment. Female and male rat pups were injected, with MSG (4 mg/g BW, s.c.) or 0.9 % saline on days 1, 3, 5, 7, and 9. They were euthanized on day 49 of life. In the GH secretion depletion group (positive control of MSG), hypophysectomy was performed on adult male and female Wistar rats. The serum IGF-1 and GH levels, number of neurons in the hypothalamic arcuate nucleus, hippocampal CA1 and cerebral cortex Fr2 region, body weight, various organ weights in each group were measured. Gene expression levels of CYP2C11, CYP2C12, and albumin in the liver were measured with semiquantitative RT-PCR in each group. Statistical analysis was performed using BellCurve for Excel 2016.

The survival rate after MSG administration was 100 % in both male and female SD rats up to 7 weeks of age, whereas the survival rate in Wistar rats decreased sharply by 9 days after administration. At 7 weeks of age, the survival rates for male and female Wistar rats were 33 % and 47 %, respectively, significantly lower than in SD rats ($P<0.01$). In particular, the survival rate was significantly reduced in males. In the GH secretion depletion group, serum IGF-1 concentration was 70.1 ± 40.0 ng/ml in females and 37.0 ± 3.8 ng/ml in males, and the serum GH concentration was below the detection limit. IGF-1 concentrations in the MSG group were 449.6 ± 154.8 ng/ml in female Wistar rats, 302.3 ± 63.5 ng/ml in female SD rats, 294.9 ± 23.0 ng/ml in

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male Wistar rats, and 717.9 ± 150.4 ng/ml in male SD rats. The effect of MSG on suppressing GH secretion was insufficient, especially in the SD MSG group, suggesting that SD rats may have lower neurotoxic sensitivity to MSG than Wistar rats. The number of surviving neurons in the arcuate nucleus of the hypothalamus of the female Wistar MSG group decreased 28.4 % compared with the Wistar saline group (Wistar saline 2546.6 ± 127.7 cells/mm^2, Wistar MSG 1823.2 ± 96.7 cells/mm^2, $P<0.001$), and that in the female SD MSG group also decreased 9.1 % compared with the SD saline group (SD saline 2372.3 ± 539.4 / mm^2, SD MSG 2156.0 ± 189.4 / mm^2). The significant decrease the number of surviving neurons in Wistar rats suggested that the sensitivity of neurotoxicity due to MSG is higher in Wistar rats than SD rats and that they have strain differences.

One of the major adverse effects of MSG may be decreased homeostasis due to blockade of the hypothalamic arcuate secretory hormone. MSG is known to affect the cerebellum, hippocampal CA1 region, and cerebral cortex Fr2 region along with the arcuate nucleus. However, in this experiment, the number of surviving neurons in each region did not differ between the saline and MSG groups. These results conflict with those of the previous reports because more severely impaired rats in the Wistar MSG group died during the experiment, and less impaired rats may have survived. The gene expression level of CYP2C11 mRNA, specifically dominant in males, was 262.7 % ± 299.4 % in the female Wistar MSG group and 106.2 % ± 124.7 % in the SD MSG group, which was higher in the Wistar rather than the SD rats. This result is similar to that obtained when MSG was administered at half the dose adopted in this study, and further enhanced the possibility that MSG has an insufficient inhibitory effect on GHRH and GH secretion. Blocking female GH secretion by hypophysectomy could induce DsRed2 expression, but no DsRed2 expression was observed in the female Wistar MSG group.

In this study, MSG administration reduced the survival rate and neuronal cell count in Wistar rat compared with SD rats. The treatment also influenced IGF-1 concentration, body growth rate, and CYP expression, which is downstream of the hypothalamus–pituitary–liver axis. These results suggested that MSG sensitivity in neonatal rats differs among strains, endogenous GH is not entirely blocked by MSG, and the GH-free phase like male could not be reproduced. The cause of the strain difference is unknown at present, but differences in drug efficacy and toxicity evaluation may occur depending on the strain. Therefore, when using rats to evaluate the safety and efficacy of drugs, it is necessary to comprehensively consider the characteristics of each strain, including the liver, hypothalamus, and pituitary system.

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Establishment and analysis of novel luciferase transgenic mouse line

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In recent years, a bioimaging system for noninvasively visualizing molecular dynamics at biological level has garnered attention. This system is useful from the viewpoint of animal welfare because the same individual can be observed over time. We have conducted basic medical research using green fluorescent protein (*GFP*) and luciferase (*Luc*) transgenic (Tg) animals, particularly rats, and have obtained considerable amount of data. It is easier to collect biological material from rats than from mice; additionally, rats are more suitable for surgical operations such as transplantation. However, when evaluating the efficacy and toxicity of drugs, rats require higher doses, which are expensive, than mice. Therefore, they incur higher costs, which can be a setback during research on these animals. Abundant research outcomes and many Tg strains have been obtained with mice because they have small body size. In a reporter gene-transfected rodent model, the antigenicity of fluorescent proteins such as GFP is a disadvantage in immunological research. Firefly luciferase, which induces luminescence, has no immunogenicity and has excellent tissue permeability. However, at present, there are no reports describing the production of Tg mice with *Luc* singly linked to the ubiquitous expression promoter.

In Chapter 2, to confirm the antigenicity of GFP, skin transplantation was performed using Nippon Veterinary and Life Science University original GFP Tg mice (N14-16, C57BL/6J background), which were created and grown in our laboratory. An immune response analysis was performed. Early rejection of GFP skin grafts into wild-type C57BL/6J mice was confirmed macroscopically and histologically, and the antigenicity of GFP was confirmed for the first time in inbred mice. Because rejection of the wild-type graft did not occur, it was concluded that the GFP Tg mouse was transgenic to the C57BL/6J mouse strain by backcrossing, which has been continued for 9 years since the production.

In Chapter 3, we reported the creation of a novel *Luc*-introduced Tg mouse, which could be an effective tool for medical transplantation research. First, we compared and examined the luciferase activity of expression vectors, in which *Luc* was linked to various ubiquitous promoters. The "hydrodynamic-based transfection: HBT method" was selected as a simple and highly reproducible method for introducing a naked gene from plasmid DNA into living tissue, and luciferase activity in mouse liver was assessed. As a result of comparing the expression of the four *Luc* expression vectors (pAlb-Luc, pRosa-Luc, pCAG-Luc, and pCAcc-Luc$^+$) owned by our laboratory, it was found that pCAcc-Luc$^+$ was stable in terms of expression efficiency. In this regard, it was confirmed that pRosa-Luc was the best expression vector. To the best of our knowledge, this is the first report to reveal the details of the luciferase activity of pCAcc-Luc$^+$ by the same method, and we have produced data that can be used as an index of the expression intensity in tissues after the production of Tg mice.

In Chapter 4, three types of *Luc* gene-transferred Tg mice were produced using the proven microinjection method. For the preparation of Tg mice, linear DNA fragments (4.9, 4.6 and 3.9 kb) were prepared from pRosa-Luc, pCAG-Luc, and pCAcc-Luc$^+$, and each DNA fragment was introduced into the male pronucleus of fertilized eggs by the microinjection (MI) method. The 2-cell stage embryo transplanted into the oviduct of a

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pseudopregnant female mouse. In the Rosa-Luc group, 320 fertilized eggs were injected; the number of offsprings was 41, and the birth rate was 12.8%. In the CAG-Luc group, 86 fertilized eggs were injected; the number of offsprings was 2, and the birth rate was 2.3%. In the CAcc-Luc^{+} group, 88 fertilized eggs were injected; the number of offsprings was 2, and the birth rate was 2.3%. One CAcc-Luc^{+}-introduced neonate who died after birth was *Luc*-positive, but histological findings suggested the possibility of death from respiratory failure due to atelectasis. Finally, Tg mice that grew till the weaning stage were not successful with any of the transgenes. To investigate the cause of the failure, the effects of various Tg fragment insertions during the early stage of embryo development after microinjection were compared and examined. As a result, the incidence was found to be low. To improve the production efficiency, we examined the Rosa-Luc MI group, where an incidence of 30% was observed. The low viability rate in the CAG-Luc and CAcc-Luc^{+} MI groups indicated that the cause of failure was high expression activity of the CAG promoter in early embryonic development and mutations inserted into the genome.

In this study, we demonstrated the GFP antigenicity of GFP in inbred mice in a skin exchange transplantation test for the first time. Comparison of the activities of various luciferase expression vectors by the HBT method revealed the characteristics of each vector and provided valuable data for producing Tg animals. However, in the production of a novel *Luc*-introduced Tg mouse, a mature *Luc*-positive Tg mouse could not be obtained. This study highlights the utility of *Luc*-introduced Tg animals has been proven in the field of transplantation research. In the future, we intend to solve various issues in the production of Tg animals to reduce the invasion of mouse embryos as much as possible.

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Infection dynamics of avian haematozoa in the introduced Melodious Laughing Trush *Garrulax canorus* and native wild birds in suburban area of Tokyo

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Avian haematozoa, including *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. are important pathogens of vector-borne diseases of veterinary medicine. In Japan, the distribution of non-native birds, such as Melodious Laughing Trush, *Garrulax canorus*, has been rapidly expanding in recent years, and there are concerns about the negative impact on native ecosystems. However, the distribution status of the transmission cycle of avian haematozoa and the role of introduced birds in the domestic protozoan infection cycles are not fully understood. The purpose of this study was to estimate the transmission cycle of avian haematozoa in Japan and to examine the effects of introduced birds on native (endemic species in Japan) birds through the blood parasites. We investigated the prevalence of avian haematozoa among native birds, mosquitoes and *G. canorus* that inhabit sympatrically in the field.

From May 2016 to February 2019, blood samples were collected from native birds captured in Hachioji City and Akishima City located in suburban area of Tokyo, Japan. From the obtained blood samples, protozoan bodies were screened by blood smear observation and protozoan DNA was detected by nested polymerase chain reaction (PCR) of the cytochrome b (cyt*b*) gene. Microscopic examination revealed 40.8% (119/292) and PCR showed 64.4% (190/295) positive for haematozoa. The prevalence of *Plasmodium* (avian malaria) was significantly higher in the *Parus minor* and *Sittiparus varius*, and significantly lower in the *Emberiza spodocephala* and *Zosterops japonicus* (χ^2=59.26, df=5, $p<0.01$). The detected avian malaria parasites were classified into 13 lineages, with SGS1 (36.5%), GRW04 (24.0%), and GRW06 (9.6%) respectively. The above results suggested that two species, *Pa. minor* and *S. varius*, were the main hosts in this study area. These lineages of avian malaria such as SGS1 and GRW04 could be transmitted by these bird species. In addition, same lineages were observed for up to 23 months in recaptured individuals, suggesting that these birds could serve as a long-term reservoir of the parasite in the study area.

Prevalence of avian malaria parasites in mosquitoes was examined by microscopic detection and PCR. A total of 1,035 mosquitoes including 5 genera 10 species were collected around the study area in Hachioji City during May to February 2018. Among them, the *Culex pipiens* group (65.4%, 677/1035) and *Aedes albopictus* (22.8%, 236/1035) were the dominant species. Among 216 DNA pools of 986 female adult mosquitoes, amplification of parasite DNAs was observed in 9 pools (minimum infection rate (MIR): 0.9%, 9/986), and only *Cx. pipiens* group was found to be infected with malaria parasites (MIR: 1.3%, 9/670). Oosysts were observed in the midgut of 2 individuals (0.3%) of *Cx. pipiens* group. Sporozoites were found in the salivary gland of 1 individual (0.1%) of these specimens. Positive individuals were obtained almost every month during the study period. Malaria parasite detected from the *Cx. pipens* group were classified into four genetically distinct *Plasmodium* lineages (SGS1, GRW04, CXPIP09, PADOM02). Among them, three lineages (SGS1, GRW04, CXPIP09) had the same genotype as those detected from native wild birds such as *Pa. minor* and *S. varius*. From these results, it was suggested that the transmission cycle of these three

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lineages of avian malaria parasites could be established and transmitted continuously, with the *Parus minor* and *Sittiparus varius* as hosts and the *Cx. pipiens* group as vectors in the study area.

Prevalence of avian haematozoa in the introduced *G. canorus* was examined by microscopic detection and PCR. Microscopic examination revealed 0% (0/108). PCR positive individuals were 7.5% (8/107) for *Plasmodium* and 2.8% (3/107) for *Leucocytozoon* respectively. Malaria parasites detected from the *G. canorus* were classified into only one *Plasmodium* lineage (GRW06). GRW06 was also detected in *Horornis diphone* by PCR and a blood-smear positive individual was found from this species. This is suggesting that *H. diphone* was the host of this lineage, GRW06. Therefore, the transmission cycle of GRW06 was established by *H. diphone* and vector in this site, and *G. canorus*, which shares same habitat with *H. diphone*, was incorporated in the transmission cycle. Since no blood smear-positive individuals were found in this study area, it is unclear whether *G. canorus* has a role of a host for GRW06. Simultaneously, we cannot deny that GRW06 could have been introduced into Japan by *G. canorus* due to reports of transmission in birds of the same family in the country of origin.

The present result suggested that the *Pa. minor* and *S. varius* have important roles as hosts, and the *Cx. pipiens* group plays as vectors in the transmission cycle of avian malaria parasites. Besides, avian malaria parasites such as SGS1, GRW04 and CXPIP09, which are widely distributed in Japan were transmitted in suburban area of Tokyo. Furthermore, it was found that it is currently unlikely that *G. canorus* is involved in that transmission of these three avian malaria parasites.

On the other hand, it was newly discovered that GRW06 might have been transmitted between the introduced bitd, G. canorus, and native bird, *H. diphone*. In the future, it is necessary to identify the mosquito blood-meal in the study area and to investigate the infection status of *G. canorus* in more detail.

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Development of a Draw-a-Horse Scale and Other Evaluation Tools for AAE Programs with Horses

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Introduction

One of the major modern guidelines for Animal Assisted Education was established in 2001 at International Association of Human-Animal Interaction Organizations (IAHAIO) meeting. Animal Assisted Education (AAE) is intended to utilize animals as tools for education of life, and to improve the quality of education and to improve learning motivation. Researches on dogs participating in educational settings are a growing phenomenon (Sandt 2019). Compare to horses, dogs have more opportunities to participate in treatment and education activities, which may be due to the higher cost of raising horses, the special requirements for venues and the difficulty of moving in cities. Consequently, there are few researches on horse assisted education, and very few quantitative studies on the effects.

Even though the opportunities are increasing for children to ride horses in educational or semi-educational settings in Japan, very few studies have been conducted to evaluate the effects of such programs. One of the possible reasons for this is the lack of simple tools.

In this study, student's learning experiences with horse study programs are evaluated by several simple to use evaluation tools. The program is conducted at a public school, and the drawings and other data was provided to the university by a local board of education. Simple methods used in psychology or other AAE programs, such as use of mood scale, indication of anxiety by students with the use of red and white cap for physical education are used.

Since there were no simple method for evaluating drawings by students, a drawing analysis system for horse drawings was developed for this purpose, and was used to evaluate the learning progress during the one-year program. It is based on the Draw A Man test (DAM), which is a developmental psychology test used to evaluate children' s intelligence and or learning experiences.

Method

In 2016 – 2019, an elementary school in Mitaka City, Tokyo Japan, provided 4 time a year AAE with hoses for 3rd graders. In order to evaluate the effects of the program, the local board of education requested Nippon Veterinary and Life Science University for the assistance.

Evaluation of changes in psychological mood

Measurement of student's mood and the riding skill evaluation.

Anxiety measure is taken at the beginning of the program, either the teacher or the handler told students to make sure they express their feelings by using the red and white cap, if anxious, then use the red side and happy, use the white side of the cap. The number of red and white caps were counted by the board of education official at the beginning of the program.

Mood scale; After the riding program and the study session inside, students were asked to fill in the mood scale.

Evaluation of the riding experience

Drawings are used to measure the students' knowledge and cognition about the horses. In order to evaluate the drawings, an original horse-drawing scale, the Draw-a-Horse (DAH) based on the Draw-a-Man (DAM) test was developed. Goodenough developed the DAM test to evaluate children's intelligence in 1926. This was later improved by Harris (1963). A total of

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482 drawings were submitted to the board of education following the riding programs. There were two sets of drawings: 309 first-time and 168 second-time drawings.

The first-time drawings were compared with the second-time ones to evaluate the effects of the program. Drawings were analyzed in two stages: 1) basic data direction of the horse facing and the number of people; and 2) an analysis of the drawing details using DAH.

The DAH analysis was only used paired drawings with the horse facing sideways (126 pairs). The DAH is only applicable to drawings of horses facing sideways.

Result

Evaluation of changes in psychological mood

In anxiety indication using the red-and-white caps, students expressed anxiety the first time. By the fourth time, the number of red caps indicating anxiety decreased. As for the face scale results, students were always happy after each class. The results of the questionnaire in 2018, students rated their riding skill low in the third session. Students were concerned about the condition of the horses in the second and third sessions. Some students felt difficulty when the riding skill level increased, but they seemed to have achieved a sense of accomplishment by the fourth session.

Evaluation of learning by drawing analysis

The direction of the horse, whether it was facing left or right, and the number of people in the drawing were also analyzed. Chi Square analysis showed that more students drew horses from different directions in the 2nd session. More students drew horses from other directions, such as from the front, top or back. Chi Square analysis indicated a significantly greater number of people in the drawing in the second session.

The total results of the DAH for the first and second sessions were compared. The DAH score was higher in the second sessions. It is intuitive to see that the second score increase is session objective.

Further analysis of general body structure, body attachments, and body details were conducted. Some of the details of various parts of the horse body are easier or more difficult to draw, for example, the shape of the eyes, hooves, nose and so on, which appeared in student's drawings, but they were difficult to be accurate.

Discussion

In this study, psychological states at various points of the program were measured, with red and white cap anxiety indicators, body and face scales as a satisfaction indicator, and questionnaires were used to allow students to evaluate their riding experiences. Psychological mood analysis is useful to understand students' overall satisfaction, providing easy-to-handle data for the school and board of education.

HAD was developed in this study to evaluate the educational enhancement of the horse program. It can be used widely to evaluate the effects of similar educational programs. It is easy to administer and also easy to see the changes. It would be a simple to use method for educational horse stables as well.

Since this program was administered four times a year, it was possible to compare the changes in drawings students made in class after riding. Unfortunately, drawings were not always the part of the class, that only some of them have been used here. It is easy to administer and also easy to see the changes. It would be good data for the horse stable, but also for students and families that the accomplishment is measured objectively.

Acknowledgement

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Study on methods to detect bovine viral diarrhea viruses that induce innate immune response within field strains.

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Bovine viral diarrhea virus (BVDV), along with classical swine fever virus and border disease virus, is classified into the *Flaviviridae Pestivirus* genus. BVDV infection is spread worldwide and is severely damaging to the livestock economy. Therefore, BVDV infection is an important infectious disease of cattle and requires hygiene measures. When BVDV infects cattle, a variety of symptom may occur, including subclinical infection, reproductive disorders, respiratory disease, and persistent infection caused following transplacental infection. Cattle that are persistently infected (PI) with BVDV also can develop lethal mucosal disease.

Furthermore, it is known that BVDV can be classified into cytopathogenic and noncytopathopgenic viruses. The latter can be further divided into Exaltation of Newcastle Disease virus (END) -phenomenon positive (END^+) viruses and END-negative (END^-) viruses based on their interference with heterologous viruses. These distinct classes of BVDVs are called quasispecies. Additionally, END^+ viruses have been reported to suppress type I interferon (IFN) production, while END^- viruses strongly induce IFN production. Furthermore, it has been revealed that these BVDVs are present as mixtures at various ratios in field strains recovered from PI cattle in Japan. Based on these reports, we hypothesized that co-infection with both END^+ and END^- viruses may be related to the variety of symptoms associated with BVDV infections. To prove this hypothesis, epidemiological investigation of the distribution of BVDVs in field situations is ongoing. However, the reverse plaque formation method for the detection and quantitation of END^- viruses is complicated and unsuitable for epidemiological investigations that require multiple-sample testing.

In this study, we attempted the establishment of a method to easily detect and quantify END^- viruses among field strains by utilizing the difference in immunological properties between END^+ and END^- viruses.

Chapter 1. Consideration of a method to detect innate immune responses induced by END^- virus infection.

We examined whether the END^- virus could be detected and quantified by measuring the strength of the innate immune responses induced by END^- virus infection. A reporter assay and a quantitative RT-PCR (qRT-PCR) assay were selected as candidates.

First, we tried to establish a reporter assay to measure the innate immune responses. Plasmids carrying a nanoluciferase-encoding gene under the control of the promoter for *bovine interferon-stimulated gene 15 (bISG15)* or for the IFN-beta-encoding gene were constructed, and each plasmid was introduced into 293T cells derived from human fetal kidney. The activity of each promoter activity was significantly induced by IFN addition, indicating that these plasmids were functioning as expected. However, when a similar experiment was performed using bovine-derived cultured cells (the MDBK cell line), IFN-induced promoter activity was not observed. This result was considered to be related to the low gene transfer efficiency of MDBK cells. In an attempt to overcome this problem, we produced a stable cell line harboring reporter and promoter sequences. However, IFN-induced activity was not observed with either promoter in the new cell line. The reason for this shortcoming remains unclear; a detailed examination of conditions will be necessary for use of a reporter assay.

Next, we examined qRT-PCR as another approach for detecting innate immune responses. We observed that the IFN-induced expression of *bISG15* increased

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in a dose-dependent fashion, confirming that the innate immune responses induced in MDBK cells could be detected using by qRT-PCR. Therefore, we propose that the detection of *bISG15* by qRT-PCR could be employed as a method for easily detecting and quantifying the innate immune responses caused by the END$^-$ virus infection.

Chapter 2. Evaluation of qRT-PCR method using artificially synthesized BVDVs.

We evaluated whether qRT-PCR for detection of intracellular *bISG15* can be used as a method for detecting and quantifying END$^-$ virus. First, each of the artificially synthesized END$^-$ and END$^+$ viruses was independently infected into MDBK cells, and expression levels of *bISG15* were measured by qRT-PCR. In a single-infection of END$^+$ virus, the expression of *bISG15* was not induced until 4 days-post infection (dpi). On the other hand, in a single-infection of END$^-$ virus, the expression of *bISG15* was induced from 1 dpi, peaking at 4 dpi. Next, we examined whether qRT-PCR could detect the expression of *bISG15* induced by END$^-$ virus infection when both BVDVs were co-infected. Artificially synthesized BVDVs were co-infected into MDBK cells at various ratios, and the expression levels of *bISG15* were measured by qRT-PCR at 4 dpi. Surprisingly, *bISG15* expression was detected even when the END$^-$ virus was present at only 1/10,000 of the END$^+$ virus amounts. However, no relationship was found between the expression level of *bISG15* and the amount of END$^-$ virus, precluding quantification of END$^-$ virus. The results showed that the qRT-PCR may permit detection of the presence of END$^-$ virus in field strains of BVDV.

Chapter 3. Evaluation of qRT-PCR method using BVDV field strains.

We investigated whether the presence of END$^-$ virus could be determined by measuring the expression of *bISG15* using 12 field strains of BVDV. When the END$^+$ and END$^-$ virus titers were measured in field strains, BVDVs were mixed at various ratios in each field strain. The same amount of each field strain then was inoculated into MDBK cells, and the expression of *bISG15* was measured at 4 dpi. Although the expression of *bISG15* in each strain was induced 10-fold or more compared to mock-infected cells, the level of expression did not correlate with the titer of END$^-$ virus. The experiment also was performed under conditions where the multiplicity of infection was 1.0, but there was no correlation between the titer END$^-$ virus and the degree of *bISG15* expression.

These results indicated that the level of *bISG15* expression in infected cells could serve as an indicator of the presence of END$^-$ virus in field strains, but was insufficient to quantify END$^-$ virus. In order to quantify the END$^-$ virus, it will be necessary to examine the timing of the expression of intracellular *bISG15* and to search for *bISG* genes, other than *bISG15*, that can serve as indicators. In the future, if the quantification of the END$^-$ virus becomes possible, analysis of the association between the quasispecies and various symptoms of BVDV will be further developed.

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Establishment and characterization of canine mammary complex carcinoma cell line

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Canine mammary tumors are one of the most common neoplastic diseases diagnosed among female dogs, and in approximately 30 % of cases, these tumors are malignant. Both the types of tumors, including simple carcinoma, which is composed of only one type of cells, either the luminal epithelial cells or the myoepithelial cells, and complex carcinoma, which is composed of both cell types, occur in the mammary gland of female dogs. However, there is a difference in the malignancy of these tumors. In previous studies, this difference has been attributed to the action of myoepithelial cells, to suppress the progression of tumors, in case of complex tumors. However, to our knowledge, no similar study has been conducted to address the difference in malignancy due to the luminal epithelium themselves, in case of complex tumors. In this study, we established a novel cell line that originated from the luminal epithelium of complex carcinoma, and compared its malignancy grade and gene expression with several other cell lines that originated from the luminal epithelium of simple carcinoma.

At first, we established the cell line originating from the luminal epithelium of complex carcinoma. Tissue samples were obtained from a female dog with canine mammary tumor hosted at the Nippon Veterinary and Life Science University, Tokyo, Japan. Histologically, the tumor was composed of biphasic alveolar structures with luminal epithelial tumor cells located in the center of the structures and myoepithelial tumor cells arranged at the margin of the structures and nests with proliferating myoepithelial tumor cells alone. The luminal epithelial tumor cells were usually cuboidal in shape and, and had abundant and acidophilic cytoplasm. The nuclei showed anisokaryosis, usually oval in shape, and mostly clear. Mitoses were occasionally observed in these cells. The myoepithelial tumor cells were short and spindle-shaped, had clear cytoplasm. The nuclei were generally uniform and circular to elliptical in shape. The tumor was pathologically diagnosed as complex carcinoma. A fragment of the tumor tissue was minced in 0.25 % trypsin-ethylenediaminetetraacetic acid (EDTA) solution, suspended in RPMI-1640 supplemented with penicillin-streptomycin-amphotericin β -suspension and 5 % fetal bovine serum and was incubated at 37 ℃ in a humidified chamber supplied with 5 % CO_2. Thereafter, single cell cloning was carried out using the limit dilution method. After 50 subsequent passages, we established a cell line that was designated as 17-442. To determine the phenotype of the cells, cell blocks were prepared using the cell line and immunohistochemical (IHC) analysis was performed. As a result, the cytokeratin (CK AE1/AE3), which is a pan-epithelial marker and the low molecular cytokeratin (CK8), which is a glandular epithelial marker were positively stained. On the contrary, the α-smooth muscle-actin and p63, which are the myoepithelial cell markers were negatively stained. These results indicate that the 17-442 cell line was specifically derived from the luminal epithelium and not the myoepithelium.

Second, the cell growth ability (using time lapse photography), cell group migration ability (using scratch assay), single cell migration ability (using Boyden chamber assay), and cell proliferation ability (using WST-8 assay) of the newly established 17-442 cell line were compared with those of the five other cell lines (CIPp, CTBp, CNMp, CHMp, NV-CML) that are derived from the luminal epithelium of simple carcinoma. The 17-442 cells were polygonal in shape, adhered to each other, and grew in island structures. In contrast, in the five

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cell lines that were derived from the luminal epithelium of simple carcinoma, individual cells were scattered, and did not adhere to the surrounding cells. The 17-442 cells had the lowest single cell migration ability, but its cell migration distance was the longest after CHMp, and the proliferation ability was the highest when compared with the five cell lines. These results reveal that the 17-442 cells actively proliferate and migrate, but the cell–cell migration ability is extremely low due to the strong adhesion between these cells.

Third, we investigated the difference in the expression level of major genes between the 17-442 cells and the five types of cells derived from the luminal epithelial cells of simple carcinoma. Compared with simple carcinoma cell lines, 17-442 cells exhibited higher expression levels of E-cadherin, which is an epithelial cell adhesion molecule, but exhibited lower expression levels of Snail, Slug, and TWIST2, which suppresses E-cadherin, and N-cadherin, Vimentin, and S100A4, which are mesenchymal markers, and Nestin, which is a stem cell marker. Thus, the 17-442 cells exhibited a low degree of epithelial–mesenchymal transition (EMT) and maintained the properties of the epithelial cells. H19 and HOTAIR, which are the long non-coding RNAs that promote cancer progression, were very high in some simple cancer cell lines, but were relatively low in the 17-442 cells.

As described above, in this study, we established a cell line exhibiting the properties of luminal epithelial cells of complex carcinoma, the malignancy of which is lower than that of luminal epithelial cells of simple carcinoma. It had been considered that the myoepithelial cells in complex carcinoma possess a suppressing effect on cancer progression. However, the results of this study suggest that the luminal epithelial tumor cells themselves derived from complex carcinoma have low malignancy. This result is based on the analysis that was performed using a case of luminal epithelial tumor cells of complex carcinoma. In order to further elucidate the properties of complex carcinoma luminal epithelial cells, we have to establish several new cultured cell lines and confirm their properties in the future.

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Analysis of expression and function of stress-related genes in mouse immune cells

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[Introduction]

When humans and animals feel stress from the external environment, it is transmitted via three types of pathways: ⅰ) hypothalamus-pituitary-adrenal cortex axis (HPA axis), ⅱ) sympathetic nerve-adrenal medulla axis (SAM axis), ⅲ) other hormones.

Activation of the HPA axis pathway increases secretion of glucocorticoid (GC), one of the corticosteroids. GC binds to the intracellular GC receptor (GR) to form a complex, translocates into the nucleus, and regulates the transcription of various genes by binding to the GR responsive site (GRE) on the chromosome. However, the sequence differs depending on the target gene. In addition, the GR gene has the CAG (glutamine) sequence repeat from the position 223 that is 16 times in C57BL/6 (B6) mice and 8 times in BALB/c (BALB) mice, and differs between mouse strains.

Naive helper T cells (Th0) are activated by dendritic cells, one of the antigen presenting cells, and differentiate into Th1 cells or Th2 cells depending on the cytokines which T cells have received. Th1 cells mainly produces IL-2 and IFN-γ, and Th2 cells produces IL-4, IL-5, and IL-13, thereby regulating various immune responses. It has been thought that the bias toward Th2 is more likely to cause allergy, and BALB mice are the predominant Th2 strain. When organisms are exposed to stress, thymus atrophy and aggravation of allergy and inflammation occur, indicating that the immune system is greatly affected. However, the mechanism has not been fully elucidated, and it is not clear how the differences in GR sequences between mouse strains affect the immune response.

In this study, we first examined whether expression of stress-related genes reported in the past was induced by restraint stress. Furthermore, we focused on Gilz among these genes and tried to analyze the promoter of the gene. In addition, we tried gene transduction to investigate how immune cells are affected by Gilz.

[Materials and methods]

(1) Restraint stress: B6 and BALB mice were exposed to restraint stress, and then RNA was extracted from spleen cells, followed by RT-qPCR. Thymocytes were stained with anti-CD4 and anti-CD8 antibodies and analyzed by a flow cytometer (FACS).

(2) GR expression: Thymocytes of B6 mice were stained with anti-CD4 and anti-CD8 antibodies, then stained intracellularly with anti-GR antibodies, and analyzed with FACS. Next, the spleen cells of B6 and BALB mice were stimulated with dexamethasone (Dex), a synthetic glucocorticoid, subjected to cell membrane or nuclear membrane permeabilization treatment, stained with an anti-GR antibody, floowed by FACS analysis. The expression of GR in cell lines was examined.

(3) Promoter analysis: (a) pGL3-Luc vector with the promoter sequence upstream from the translation initiation site of Gilz gene, (b) B6 or BALB GR gene, and (c) pRL-null vector, a relative control of gene transfection efficiency, was transfected into cultured cells, and luciferase activity after Dex stimulation was measured.

(3) Functional analysis: Gilz gene was transfected into spleen T cells using a retrovirus vector under stimulation with anti-CD3 antibody, and the effects on cell proliferation and IL-4 and IFN-γ production were determined by resazurin assay and ELISA, respectively.

[Results and discussion]

(1) In mice subjected to restraint stress, expressions of

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Gilz, Rtp801, and Mkp1 genes were induced in spleen cells, and it was confirmed that they were stress-inducible genes. On the other hand, no change was observed in the expression level of the GR gene.

(2) Next, using the thymus of B6 mice, differences in the expression of GR protein during the maturation process of T cells were examined by intracellular staining. As a result, the expression was higher in the order of $CD4^+CD8^-$ cells, $CD4^+CD8^+$ cells, $CD4^-CD8^-$ cells, and $CD4^-CD8^+$ cells. Next, GR reactivity in spleen cells was examined. When stimulated with Dex, the GR expression in the cytoplasm decreased in both B6 and BALB, indicating the possibility that GR migrated into the nucleus. In addition, in the condition of the nuclear membrane permeabilization, GR expression was higher in the Dex stimulated cells as compared with the unstimulated cells.

(3) Then, we decided to investigate the expression mechanism of the stress-inducible gene by a reporter assay. To that end, we first examined the GR expression of cell lines in our laboratory by RT-PCR in order to find out cell lines suitable for the assay. GR mRNA expression was observed in HEp-2, 293T, Plat-E, and U2OS cells, which are human-derived, and in WEHI-231 cells, which is mouse-derived. Furthermore, GR protein expression was also examined by western blot. In the positive control sample into which the GR gene was introduced, a clear band was observed, but endogenous GR was hardly detected. Therefore, we thought that the effects of endogenous GR could be ruled out, and decided to use Plat-E cells that have the high transfection efficiency for the assay. In the reporter assay, transcriptional activity was first compared in the presence or absence of the TATA box in the Gilz promoter region. As a result, the sequence containing TATA showed a value 100 times higher than the one without TATA. Subsequently, when comparing the effects of B6 or BALB type of GR in the condition of the TATA-containing sequence, B6 type showed a tendency to be slightly enhanced by Dex stimulation, but the transcriptional activity of BALB type was slightly stronger. Thus, it was suggested that the inducibility of Gilz expression may be different among GR polymorphisms. When the promoter sequences of the upstream region containing two or three GRE-like motifs were analyzed, their activities were slightly enhanced by Dex stimulation. When examinined the duration for stimulation and the Dex concentration, it seemed that the culture time should be more than 24 hours and the Dex concentration should be around 10^3 ng/ml.

(4) Lastly, when the Gilz gene was introduced into spleen T cells, there was no effect on cell proliferation and IFN-γ production, but IL-4 production was increased and tended to promote Th2 differentiation. Therefore, it was suggested that Gilz may contribute to the aggravation of allergies due to stress.

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Effect of meat species on retort beef aroma

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Introduction

Corned beef is one of the cured beef products. We feel the unique, continuous and aromatic roast odor in the corned beef (retort beef aroma). Migita et al examined the cause of the odor, and showed that the retort beef aroma was generated by retort heating at 121 ℃ for 20min and that its volatile components, pyrazines, contributed to the odor. On the other hand, it is generally known that heated beef, pork and chicken have different odors. The objective of this study is to reveal the difference of the intensity of retort beef aroma among various kinds of meat heated by retort heating and its cause.

Materials and methods

I. Sensory evaluation and aroma components comparison of various kinds of meat: Commercial beef sirloin, pork loin and chicken leg meat were homogenized with an equal weight of solution of 2% NaCl. The obtained homogenates were placed into plastic bags and vacuum-packed. These samples were placed into larger bags, and were vacuum-packed. The obtained packed materials were heated at 121 ℃ for 20 min (retort-heated). Heated samples were used for sensory evaluation and aroma components comparison. In a scoring test, eight panelists compared strength of retort beef aroma. Moreover, aroma components obtained from samples by simultaneous distillation-extraction were analyzed by the GC-MS. Each component was identified by comparing mass spectrums and retention times with commercial standards. Relative amounts of each component were determined by the internal standard method using 1,2-dichlorobenzene.

II. Comparison of supernatant and precipitate of beef samples: Commercial beef sirloin was homogenized with an equal weight of pure water and the obtained homogenate was centrifuged. The obtained supernatant and precipitate were retort-heated. Strength of retort beef aroma was compared by a pair test. Furthermore, analysis of aroma components obtained from precipitate and supernatant of beef homogenate performed by GC-MS.

III. Quantitation of amino acids and monosaccharides: Amounts of free amino acids and monosaccharides of each kind of meat were analyzed by the ninhydrin method and the anthrone method.

Ⅳ. Quantitation of monosaccharides by HPLC: HPLC analysis of monosaccharides in each kind of meat was performed using precolumn derivatization by 1-phenyl-3-methyl-5-pyrazolone(PMP).

Ⅴ. Quantitation of glucose: Glucose amount in each kind of meat was measured using F-kit (enzyme method).

Ⅵ. Evaluation of retort-heated samples of pork with and without glucose: Commercial pork loin was homogenized with an equal weight of 2% NaCl solution or with an equal weight of 2% NaCl solution containing glucose. The obtained packed materials were heated. Eighteen panelists compared strength of the retort beef aroma by pair test. Moreover, after aroma components were collected from samples and concentrated, analysis of those was performed using GC-MS.

Results and discussion

Ⅰ. As a result of comparing the strength of retort beef aroma among three kinds of meat, the aroma of beef sample was significantly ($P<0.01$) stronger than those of pork and chicken samples. Comparison of pyrazines amounts of these samples demonstrated that the chicken sample had a significantly ($P<0.05$) higher amount of pyrazine than other samples. It was confirmed that the beef sample had a significantly ($P<0.05$) higher amount of some pyrazines such as 2,6-dimetyl-pyrazine, 2,5-dimetyl-pyrazine, trimethyl-pyrazine, 3-ethyl-2,5-

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dimetyl-pyrazine than other samples. From these results, the kind of meat was shown to have similar effects on the pyrazines amount and the strength of retort beef aroma.

Ⅱ. Strength of retort beef aroma of the beef supernatant and precipitate was evaluated by a pair test. As a result, it was found that retort beef aroma of the supernatant was significantly ($P<0.001$) stronger than the precipitate. The result of analysis of odor components of the supernatant and precipitate, the former had significantly ($P<0.05$) higher amounts of some pyrazines such as pyrazine, ethyl-pyrazine, 2,5-dimetyl-pyrazine than the latter. Therefore, it was suggested that retort beef aroma is generated from water-soluble substances that occur in the supernatant fraction of beef.

Ⅲ. Pyrazines arise in Maillard reaction followed by Strecker degradation of sugars with amino acids. Thus, we measured them in meat samples. As a result, there was no significant difference in the amount of free amino acids, while there was significant difference in the amount of sugars between beef and other meats. One of the reasons why strength of retort beef aroma differs among kinds of meat was presumed to be the difference in sugar contents.

Ⅳ. The amounts of glucose, glucose-6-phosphate, ribose and mannose were measured to identify the sugars that contribute to retort beef aroma. Analysis by HPLC showed that the main sugar was glucose in beef, although the main sugar was mannose in pork and chicken, suggesting that not only sugar contents but also sugar types may be responsible for the difference of the strength of retort beef aroma in meat kinds

Ⅴ. The experiment using an enzymatic method that can specifically measure glucose amounts demonstrated that the amounts of glucose in beef, pork and chicken were 0.42g/100g, 0.10g/100g and 0.014g/100g, respectively. This result confirmed that beef contains the highest amount of glucose. Therefore, it was assumed that glucose is responsible for generation of retort beef aroma.

Ⅵ. The results obtained in section Ⅴ let us prepare the pork sample to which glucose was added in the same extent as beef, and analyze the flavor components. Consequently, it was confirmed that the addition of glucose produced significantly larger amounts of 2,6-dimetyl-pyrazine and 3-ethyl-2.5-dimethl-pyrazine. Furthermore, evaluation of the strength of the odor in pork with or without glucose revealed that the addition of glucose tended to increase the roasted odor.

From these results, the difference in glucose contents would cause that the strength of the retort beef aroma differs in various kinds of meat.

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The study on the biochemical properties of the milk-clotting enzymes from *Hericium erinaceum* and the application to the cheese.

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Introduction

The milk-clotting enzyme is essential in cheese production. In general, gastric proteinases from young calves, call calf rennet, have been used for the manufacture of various cheeses. However, low supply of calf rennet and the incidence of BSE are incentives in the search for rennet substitutes from other origins. Microbial rennet and the fermentation-produced chymosin have been developed as the alternatives of calf rennet. Nakamura *et al*. (2018) found that a crude enzyme obtained from the mycelia of the edible mushroom, *Hericium erinaceum* (*H. erinaceum*) had high milk-clotting activity (MCA), and we reported the enzymatic properties of the crude enzyme.

The purpose of the present study was to evaluate the biochemical properties of the milk-clotting enzymes from the mycelia and the fruiting body of *H. erinaceum* and the application to the cheeses.

Materials and Methods

Milk-clotting activity assay and protease activity assay

Milk-clotting activity (MCA) was measured according to the method of Arima *et al*. (1970) with some modifications. The substrate was used defatted commercial milk pasteurized at 66℃ for 30 min with 10 mM $CaCl_2$. The substrate (2.0 mL, pH 6.6) was preincubated for 5 min at 30℃, and 40 μL of the enzyme solution was added. The time needed for the curd fragments to form was recorded as the milk-clotting time.

The protease activity (PA) was assayed using azo-casein according to the method of Sarath *et al*. (1989) with a slight modification.

The protein content was determined using the Pierce™ BCA Protein Assay with bovine serum albumin as the standard.

Effects of pH, temperature on MCA of the crude enzyme from the fruiting body of *H. erinaceum*

The optimum pH and temperature for the activity of the enzyme was determined by assaying MCA. To determine the pH stability, the enzyme solution was mixed in advance with buffers (pH3.0 to 9.0) at 10 ℃ for 24 hr. Thermal stability was analyzed by incubation for 15, 30, 45 and 60 min at different temperature. Residual MCA after each incubation time was measured.

Enzyme purification

The strain of *H. erinaceum* used was obtained from the National Institute of Agrobiological Sciences in Tsukuba, Japan. The cultivation of the mycelia of *H. erinaceum* and the preparation of the crude enzyme were as in our previous report (Sato *et al*., 2018). The crude enzyme solution of mycelium and fruiting body were precipitated using ammonium sulfate. The ammonium sulfate precipitated fractions showing high MCA were further purified with ion-exchange chromatography and gel filtration.

Monitoring the hydrolysis of caseins in the Gouda-type cheeses during ripening

Pasteurized whole milk was placed in a cheese vat and warmed at 30 ℃. After the inoculation of a commercial starter and $CaCl_2$ and incubated at 30 ℃ for 1 hr. This was followed by the addition of the partially purified enzyme from the fruiting body of *H. erinaceum* and

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chymosin, and gentle stirring. The mixture set for 50 min before cutting. To allow syneresis, the curd was incubated at 40 ℃ during gentle stirring. After cooking, the curds were separated from the whey and placed in moulds, pressed for 12 hr, and brined for 8 hr in 20 % salt solution at 10 ℃. The cheeses were vacuum-packaged in plastic film and ripened for 1, 7, 30 and 60 days at 10 ℃. Proteolysis was assessed on the cheeses by the electrophoresis.

Results and Discussion

Biochemical properties of the milk-clotting enzymes of the mycelium and fruiting body from *H. erinaceum*

The milk-clotting enzymes were partially purified from the mycelium and the fruiting body from *H. erinaceum* by ammonium sulfate fractionation, ion-exchange chromatography and gel filtration. The enzymes were purified at approximately 60-fold, 500-fold from the mycelium, the fruiting body from *H. erinaceum*, respectively. The proteolysis of κ-casein occurred with the both purified enzymes from *H. erinaceum*. These results revealed that it might be similar to the degradation observed with chymosin.

Effects of temperature and pH on the crude enzyme of the fruiting body from *H. erinaceum*

The crude enzyme of the fruiting body from *H. erinaceum* was active in the pH range from 3.0 to 8.0 and inactivated completely by heating at 50 ℃ for 30 min.

Effects of the crude enzyme of the fruiting body from *H. erinaceum* on the proteolysis of the Gouda-type cheese during ripening.

In the same manner as chymosin (control cheese), it was possible to prepare the Gouda-type cheese with the crude enzyme of the fruiting body from *H. erinaceum* (trial cheese). During ripening, intensity of the β-casein band of trial cheese was remarkably reduced and several decomposed products appeared, while that of the αs-casein band of control cheese was reduced.

Conclusion

These results indicate that the milk-clotting enzyme of the fruiting body from *H. erinaceum* can be used as effective coagulant on cheese making.

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Analysis of mice with brain-specific deletion of UTX, a histone modifier

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Introduction

Recent studies focus on epigenetics, a regulatory mechanism of gene expression without altering the DNA sequence, since epigenetics is involved not only in physiological processes, such as fertilization and development, but also in pathological processes, such as cancer, lifestyle-related diseases, and neurological disorders.

Epigenetics consists of a number of different mechanisms, such as post-translational modifications of histone tails, including methylation, phosphorylation, acetylation, etc., which regulate gene expression by changing open/closed state of the chromatin and accessibility of transcription factors to DNA. These histone modifications are reported to play essential roles in the proliferation, differentiation and maintenance of various types of somatic cells including stem cells. Among the histone modifications, trimethylation of histone 3 at lysine 4 (H3K4me3) and trimethylation of histone 3 at lysine 27 (H3K27me3) are particularly important, because they contribute to transcriptional activation and transcriptional repression, respectively.

In stem cells, H3K4me3 and H3K27me3 are simultaneously present at silent genes, which is called as a bivalent chromatin state. When stem cells are activated by external stimuli, such as cytokines and chemokines, the bivalent chromatin state is resolved into either H3K4me3 or H3K27me3, which induces transcriptional activation or repression and consequently leads to cell differentiation.

UTX (ubiquitously transcribed tetratricopeptide repeat X chromosome) is an X chromosome-specific histone modifier. UTX functions as a demethylase for H3K27 *per se*, and also contributes to the methylation of H3K4 and acetylation of H3K27 as a component of COMPASS (complex of proteins associated with Set 1)-like complexes. In brain development, histone modifications have been reported to be involved in gene expression regulation of neurons and astrocytes. However, the function of UTX in the development and differentiation of cranial nerves has not been fully elucidated. To address this issue, we generated and analyzed mice with a brain-specific deletion of UTX.

Materials and Methods

Generation of brain-specific UTX-deficient mice

To generate brain-specific UTX-deficient mice, $UTX^{flox/flox}$ mice, in which exons 11 and 12 in the UTX gene were flanked by two loxP sites, were mated to Sox1Cre mice that expressed Cre recombinase specifically in neural stem cells. All the experiments were performed in accordance with regulations in Tokyo Women's Medical University (GE19-066 and AE19-059).

Pathological analysis

Embryos at embryonic days 12.5 (E12.5) and E17.5 and pups on day 0 after birth were collected and macroscopically observed. Brains were fixed in buffered formalin and tissue sections were subjected to pathological analysis.

Immunohistochemical staining

Brains at E12.5, E15.5, E17.5, and E18.5 were fixed in formalin or 4% PFA and tissue sections were subjected to immunohistochemical staining using antibodies for a neural stem cell marker (1:000 for anti-Sox2) and a stem cell differentiation marker (1:1000 for anti-Tbr2)

RNA extraction and quantitative real-time PCR (qPCR)

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Total cellular RNA was extracted using the Trizol Reagent, and qPCR was performed according to the manufacturer's protocol. The primer sequences are;

Utx: 5'-CAGATCCAAATTCTGGCCAGTCC-3' and 5'-CCATGTATCTGCACTTGCTTCTG-3', Aixn2: 5'-GGTTCCGGCTATGTCTTT-3' and 5'-CTCTCTCTGGAGCTGTT-3', Id1: 5'-TACGACATGAACGGCTGCTA-3' and 5'-TCTCCACCTTGCTCACTTT-3', Id4: 5'-CCAACCTTAGAGGCTACATT-3' and 5'-CAACATATCTACAGTGCTCTGGTATATTA-3'.

Western blotting

Tissues were lysed in RIPA buffer (50 mM Tris-HCl Buffer (pH 8.0), 150 mM NaCl, 1% Nonidet P40 Substitute 0.5% Sodium Deoxycholate, and 0.1% SDS). For Western blotting, protein was separated by SDS-PAGE, transferred to a nylon membrane, and incubated with appropriate antibodies (1:1000 for anti-UTX polyclonal antibody, and 1:400 for anti-GAPDH monoclonal antibody). Positive signals were detected with Luminograph.

Scanning electron microscope

E18.5 brains were harvested and fixed with 2.5% glutaraldehyde, postfixed in 2% osmium and dehydrated. The ventricles were photographed with a JSM-6610LA scanning electron microscope.

Results and Discussion

We found that no gross phenotypical differences were observed between $Utx^{flox/flox}$, $Sox1Cre^{+}$ (hereafter referred to as KO) and $Utx^{flox/flox}$, $Sox1Cre^{-}$ (hereafter referred to as Ctrl) mice during embryogenesis, but KO mice died soon after birth.

No apparent differences between Ctrl and KO mice were detected by immunohistochemical staining with antibodies for neural stem cell and differentiation markers. However, pathological analysis revealed that KO mice exhibited significant enlargement of the ventricles, including lateral, third, and fourth ventricles, compared to Ctrl mice at E17.5

Studies have shown that ventricular enlargement is attributed to abnormal development of the choroid plexus and/or defects of motile cilia. Thus, we first compared the expression levels of genes related to the development of choroid plexus by real-time PCR, but could not detect any significant expression differences between Ctrl and KO mice. We then analyzed the development of ventricular cilia by HE staining and scanning electron microscopy. As a result, we found that cilia of KO mice were markedly underdeveloped compared to those of Ctrl mice at E18.5.

These results strongly suggest that brain-specific UTX deficiency impaired normal development of the cilia and induced ventricular enlargement. To clarify the underlying molecular mechanism(s), we are currently performing comprehensive gene expression analysis using neural stem cells and neurons at E12.5 and E18.5 of Ctrl and KO mice, and the results will be subjected to pathway analysis, such as gene set enrichment analysis (GSEA). Our findings will provide novel insights into the roles of histone modifiers in the regulatory mechanisms of gene expression in the neural stem and progenitor cells and also into the development and differentiation of the central nervous system.

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Identification of ion channel important for HCV lifecycle using reporter virus

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[Introduction]

It is reported that about 30,000 people die of liver cancer annually in Japan, approximately 70% of which are due to infection with hepatitis C virus (HCV), and thus HCV elimination would be effective on preventing liver cancer. At present, HCV treatment has been significantly improved in terms of efficacy for infected patients with antiviral medicines targeting HCV proteins. On the other hand, such medicine are very expensive, and the hepatitis often relapses after treatment, and thus, development of new treatment methods is required. HCV mainly infects hepatocytes and has characters that utilize cell membranes, membrane proteins, and membranes of organelles in the process of proliferation. For example, at the time of invasion, it binds to CD81 and Claudin-1 etc. located on the cell membrane and infects cells efficiently. To date, many membrane proteins have been reported in relation to the HCV life cycle, however the reports concerning ion channels, which are major membrane proteins, are very limited. Since HCV also encodes an ion channel-like p7 gene called viroporin in its genome, ion channels could also be involved. Therefore, we focus on ion channels involved in HCV proliferation and sought to identify and elucidate their effects that are clues to new therapeutic methods. To identify the ion channel in my experiment, I used a deficient type of the reporter virus named Jc1-Gluc established by Okubo in 2016 in my laboratory, and analyzed the alteration in virus properties due to the deletion.

[Materials and methods]

1. Characterization of reporter virus: a wild-type or a deficient type of expression plasmid for p7-Gluc gene was introduced into 293 cells, and cleavage between p7 and Gluc was confirmed by Western blot. After expressing the p7 protein in the liver cancer cell line Huh 7.5.1 cells, wild-type RNA for Jc1-Gluc was transfected and evaluated for the complementation effect of the p7 protein.
2. Ion channel screening: siRNA library targeting 338 genes of ion channels was used. This library contains three different sequences per one gene. Huh 7.5.1 cells were transfected with siRNA by lipofection, and then incubated with virus for 2 hours allowing infection. Based on luciferase activity in the supernatant of the following 3 days culture, virus production was evaluated. For the infection experiment, a deficient type of reporter virus Jc1-Gluc was used. The luciferase activity was measured using a renilla luciferase assay kit. Cytotoxicity potentially due to siRNA treatment was evaluated by the XTT assay.
3. Preparation of ion channel knockout (KO) cells and evaluation of virus production: KO cells for particular ion channels were prepared using the CRISPR-Cas9 system. The established KO cells were infected with a deficient type of Jc1-Gluc virus, and then the effect on virus production was evaluated based on the luciferase activity. Luciferase activity was corrected with the amount of protein in KO cells.

[Results and discussion]

1. Characteristic analysis of reporter virus: Gluc expression following transfection of wild-type and deletion-type of p7-Gluc genes was examined, and then revealed cleavage between p7 and Gluc in the deletion type, but not in the wild-type. In addition, by complementing p7 protein to the Jc1-Gluc wild type, virus production increased. In the Jc1-Gluc-deficient

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type, p7-Gluc was normally cleaved, suggesting that the production of functional p7 could help to increase virus production.

2. Ion channel screening: 21 channel genes were selected accoding to the condition as follows: luciferase activity was reduced to 60% or less, but cell activity retains 0.74 or more, as compared with the control. Amonf them, the top four genes with significantly reduced virus production were selected as candidates. Three of the four candidate genes are related to the K^+ channel, suggesting that K^+ channels may be involved in the HCV life cycle.
3. Establishment of ion channel KO cells and evaluation of virus production: KO cells deficient for the particular genes were established using the CRISPR-Cas9 system to evaluate their effect on HCV infection. As a result, virus production was significantly reduced in KCNJ11 and KCNE5 KO cells as compared to control cells. KCNJ11, which has been reported to function in hepatocytes, was analyzed using the HCV tcp system with single infectious virus, capable of monitoring invasion into KO cells, and the subgenome replicon system capable of monitoring the replication process. Both invasion and replication were reduced. KCNJ11 encodes a subunit of the ATP-sensitive inward rectifier K^+ channel, but its function in HCV-infected hepatocytes remains unknown. In the future, I would like to analyze in detail the function of ATP-sensitive inward rectifier K^+ channel during HCV proliferation.

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Mechanisms of new reinfection defense to prevent the mucosal tissue invasion of *Heligmosomoides polygyrus*-infective larvae

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[Introduction]

Heligmosomoides polygyrus (Hp) is an intestinal parasitic nematode isolated from mice in nature. When infective larvae are orally ingested by mice, they once enter the muscular layer through the mucous membrane of the small intestine and develop, and return into the lumen about 8 days after infection. Hp usually persists for more than two months between the villi at the upper part of the small intestine. After expulsion, reinfected Hp is eliminated from the intestinal lumen on the 14th day. Therefore, Hp is studied for protection against reinfection. In the previous experiments, the number of Hp in the muscle layer of small intestine and its distribution on the 6th day of infection were compared between initial and second infection. The results have suggested a protection mechanism that prevents the infective larvae from re-entering the mucosa in the $CD4^+$ T cell-dependent way.

In this study, I investigated the number and distribution of Hp invading tissues within 6 days after infection, and attempted to explore the dynamics of Hp in the early stage of infection, and define the timing and location of protection against reinfection through the mucosal invasion (Chapter 1). Next, I investigated in detail the initial kinetics of Hp infection over time and at sites of invasion and development (Chapter 2).

In addition, the induction site for immunity and effective site for preventing mucosal invasion of infective larvae were examined, and their antigen (parasite species) specificities were examined (Chapter 3). Finally, factors related to this protection for reinfection were selected by gene expression analysis, and some of them were verified (Chapter 4).

[Materials and methods]

1. Hp infection and count

C57BL/6 mice, female, 7 weeks old were used. Two hundred of Hp infective larvae were orally administered, and two weeks later, an anthelmintic was orally administered, and further four weeks later, 200 larvae of Hp were reinfected. Up to day 3 of reinfection, Hp was counted under a microscope by dissolving the stomach and six equal parts of the small intestine with artificial gastric juice. On day 6 post-infection, Hp observable in the muscle layer was directly counted under a stereo microscope. If the infection schedule and administration methods are complicated, it is shown in a chart. For administration via surgical operation, the abdomen was opened under anesthesia, and then Hp was administered to a target site using a 1 mL syringe attached with a 23 G needle.

2. *Nippostrongylus brasiliensis* and *Vampirolepis nana* infections

Nippostrongylus brasiliensis (Nb) is a parasitic nematode that infests small intestine of rats in nature. Infective larvae infest transdermally, pass through the lungs, and colonize into the villi in the upper small intestine. The abdomen of recipient mice was opend under anesthesia, and then adult worms grown in donor mice were administered to the target site of recipient mice using a 1 mL syringe attached with an 18 G needle.

Vampirolepis nana (Vn) is a tapeworm parasitic in the small intestine of rats in nature. Through oral infection of the eggs, the larvae invade into the lamina propria of the upper part of the small intestine, and develop there, and returns to the intestinal lumen around day 5 after the infection. In the intestinal tract, the parasite adheres to the mucous membrane with hooks and suckers. In

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the experiment, 600 eggs were infected orally.

3. Gene analysis

I requested the comprehensive gene expression analysis (RNA-seq) to Hokkaido System Science. RNA extraction and cDNA synthesis in RT-qPCR were performed by a conventional method. The PCR was coducted with the THUNDERBIRD® SYBR® qPCR Mix (TOYOBO), and the settings were based on the recipe of TOYOBO. The amplification process was performed using StepOnePlus (Thermo Fisher Scientific), and its analysis was performed using StepOneSoftware ver. 2.1.

[Results and discussion]

In Chapter 1, two points were revealed. Conventionally, it has been thought that Hp administered orally enters tissue of the small intestine directly. However, after oral administration, Hp enters the stomach, instead of the small intestine, which is the mucous membrane of initial contact for Hp. Then, Hp moves from the stomach to the upper 1/6 part of the small intestine on day 1 to 2 after infection. Furthermore, it is conceivable as follows: once Hp invades the stomach, immune memory is activated, and a mechanism works to prevent the re-invasion into the mucous membrane at the first 1/6 part of the small intestine on day 1 to 2 after infection.

In Chapter 2, it was shown that transmigration from the stomach to the small intestine was via the lumen, re-entering the small intestinal mucosa, and that movement had begun 16 hours after infection. In addition, it was revealed that even when Hp was administered to the ileum with a surgical operation, it migrated to the first 1/6 fraction of the small intestine. These results suggested that the migration of Hp was carried out over time, and that Hp infective larvae as well as adult worms had a preference for the first 1/6 fraction of the small intestine.

In Chapter 3, it was shown that protective immunity against reinfection, which blocks the invasion of infected larvae into mucosal tissues, is triggered throughout the stomach and small intestine, but the effective site itself for protection is located in the stomach and the first 1/6 fraction of small intestine. It also was suggested that this immune response to Hp also affects the colonization of Nb, the same nematode, and the invasion of Vn, a tapeworm.

In Chapter 4, RNA-seq selected 31 genes that were involved in the inhibition of the mucosal invasion in a $CD4^+$ T cell-dependent way. Among them, I focused on the involvement of IL-5 and basophils, and exmined by the method of neutralizing antibody, and then confirmed that they IL-5 and basophils were dispensable for preventing mucosal invasion of Hp infective larvae.

[Summary]

New findings regarding the initial dynamics of Hp and the protective response to reinfection are as follows: 1) Hp infective larvae invade the mucous membrane of the initial contact, and begin to move through the lumen to the first 1/6 fraction of the small intestine from day 1 to 2 post-infection to achieve re-invasion; 2) During this re-invasion, the protection against mucosal invasion is observed in the first 1/6 fraction of the stomach and small intestine; 3) this protection against mucosal invasion is dependent on $CD4^+$ T cells, and also affects the infection of other types of parasites; 4) it was shown that neither IL-5 or basophils were involved.

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Current status and takes of succeeding dairy cattle in Japan -The changing structure of first mated cow production-

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【Background and purpose】

The expansion of raw milk production requires a stable cattle succession plan. In the past, prefectural dairy farms developed a breeding system for "Hitohara sibori," (This is a management style in which the delivered cows that have been purchased are fattened while milking for only one cycle, and are shipped as beef cattle after milking) and the proceeds from the sale of unwanted cattle were used to purchase new calves. However, soaring prices for fattening cattle in recent years have reduced the willingness of dairy farmers to produce dairy calves (successor cattle). In some cases, the price of primiparous cows exceeds 1 million yen, and it is necessary to source high-quality successor cattle. In this study, we analyzed the current status of the succession of cows in Hokkaido and prefectures. We clarified changes in the supply system and trends in measures to secure dairy successor cattle.

【Materials and methods】

(1) We conducted a questionnaire survey of 305 dairy farmers in JA Kushiro Tanchou, Hokkaido, and JA Hamanaka-cho, and analyzed the management situation in the region, the use of sex-sorted technology, and the status of introduction. We also clarified the characteristics of the fertilization status of the first-mated cow to be sold and the successor cow left on the farm.

(2) We conducted a follow-up survey to research conducted by our laboratory in 2011 on all 43 dairy farms in the JA Fuji Kaitaku area, which is a large-scale dairy farming area in Shizuoka prefecture. We compared and analyzed changes in measures to secure successor cattle and the status of the introduction of sex-sorted technology.

(3) We interviewed JA Kushiro Tanchou and JA Higashi Soya on public pasture. Interviews were also conducted with six farmers using public pasture in the JA Kushiro Tanchou area. We investigated the current status and reasons for outsourced rearing cattle.

【Results】

1. Securing dairy cattle in Hokkaido dairy farming and current situation of first-mated cow sales

In the study area, "dairy farmers using sex-sorted semen" tended to have a higher rate of transplantation of Japanese Black fertilized eggs than "dairy farmers not using semen". No differences were found between the insemination of the successor dairy cattle with Japanese Black semen and normal Holstein, or sex-sorted semen. The insemination rate of Japanese Black semen was 60% or more in the first-mated cows sold, regardless of whether gendered semen was used.

2. Changes in JA Fuji Kaitaku

Compared to the 2014 survey, the rate of sex-sorted semen utilization increased significantly from 17% to 75%. Most farmers who did not use sex-sorted semen purchased all first-mated cows externally. The reasons included "no breeding facilities" and "first mated cows can be milked immediately". When we asked about the semen used for the successor first mated cow, most chose sex-sorted semen, while for the cow that was sold, Japanese Black semen was the largest choice. All households answered said they would "buy fertilized Japanese Black semen" when a first-mated cow was introduced as a successor cow from outside their farm.

3. Actual use of public pasture (Hokkaido)

The reasons that dairymen outsourced their work included labor shortages and the lack of land and

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facilities needed for lactation and breeding due to the growing number of delivered cows. In addition, there were several reasons why the externalization of lactation and breeding allowed a specialist focus on the breeding and management of calves, so dairymen could continue to commission breeding cattle in the future. The number of cattle at the current facility had reached its limit, and most of these cattle were affiliated. To maintain public pasture management, it was necessary to increase the occupancy rate of facilities, but it was difficult to raise deposits, so there was a trade-off between facility maintenance and member service.

4. Consideration

It was suggested that dairy farmers who actively used sex-sorted semen in Hokkaido had a higher rate of transplantation of Japanese Black fertilized eggs and no difference in the insemination rates of Japanese Black and Holstein sperm to their own successors. Therefore, the introduction of sex-sorted technology allowed efficient production of the successor cows required for management, and the rearing cattle of that became "surplus" which were bred with profitable semen or fertilized eggs of Japanese Black cattle.

It was confirmed that even in Shizuoka Prefecture, mating to first-mated cows used sex-sorted semen that had a relatively good conception rate, and delivered cows used Japanese Black semen, showing a management response from dairy farmers similar to Hokkaido. Farmers who relied on external introduction to secure their successor cows should purchase primiparous calves using Japanese Black semen because these calves can be sold at high prices. The proceeds from calf sales can be used to fund the introduction of the next generation of successor cattle. However, such management behavior is supported by relatively expensive calf prices for fattening, and the sustainability and stability of the approach cannot be guaranteed.

Furthermore, the externalization of lactation and rearing in Hokkaido is intended to increase gross profits due to the growth in the number of delivered cows, and this is related to favorable milk prices for producers. The Hokkaido public pasture used to accept cattle deposited in the prefecture, but now lactating and breeding cattle occupy this area; therefore, dairy farming in the prefecture has developed its own management to secure successors. Improving lactation and rearing systems will be an urgent issue.

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Studies on Production of the Recombinant Human antibody, Humira, by Genome-edited *Pichia Pastoris* strains.

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【Introduction】

Recombinant monoclonal antibodies (mAbs) are useful for biological research, diagnostics and therapeutic purposes. In most cases, the host cells for production are mammalian cell lines, such as Chinese ovary cells and NS. All approved monoclonal antibodies at present are produced by mammalian cell lines. Since mammalian cell cultures are far more complex and expensive than microbial cell cultures, expression systems with micro-organisms have gained importance. However, bacterial systems, such as *Esherischia coli*, are often not able to modify the product as mammalian cells do.

With their capacity for post-translational modifications and potential to produce large quantities of rather complicated heterologous proteins, yeast production systems are seen as promising substitutes for mammalian cell systems. Among several yeast systems, one using *Pichia pastoris* is increasingly being applied to both research and the production of proteins for diagnostic and therapeutic purposes. Use of its *aox1* -promoter, which controls the expression of the enzyme alcohol oxidase, led to the development of methanol-inducible expression systems. The hyperglycosylation that tends to occur with *Saccharomyces cerevisiae* is much less a problem with *P. pastoris*, and the expressed proteins can either be kept inside the cells or be secreted into the culture medium. For secreted proteins, the purification process is expected to be simpler than that inside cells, since a reasonably high purity could be achieved in the supernatants. The amount of protein produced is usually in the range of 30–80 % of all the secreted proteins. Since the ability to attain very high cell densities (150–400 g wet cell/L can be reached in defined media) by fermentative growth using bioreactors allows for high volumetric productivity, *P. pastoris* has become a robust system for the production of recombinant proteins.

Adalimumab, an antibody directed against the human TNF α produced by CHO, has been clinically used for the treatment of autoimmune diseases. However, the production costs are high, and alternative production systems have been desired.

In this paper, we describe the construction of a *P. pastoris* strain capable of the extra-cellular expression of adalimumab, its production in a flask culture, and results on the comparative characterization of the secretory product with a commercially available adalimumab produced by CHO.

【Materials and methods】

Antibodies, bacterial and yeast strains, restriction enzymes and a plasmid: Humira (adlimumab produced by CHO) was purchased from Roche (USA). Other antibodies used in immunoassays were from R&D Sciences (USA). *JM 109* competent cells used for plasmid construction were from Toyobo Biochemicals (Japan). *Picha pastoris △ och1 strain (△ och1, △ pep4, △ yscB) was* constructed from GS115 following essentially the same methods as previously described. Restriction enzymes were purchased from New England Biolabs (USA).pPICZ A for expression of the target genes were from Life Technology (USA).

Culture media: YPD: 1% yeast extract, 2% peptone, and 2% glucose. YPD plate: YPD + 1.5% agar.YPM: 1% yeast extract, 2% peptone, and 0.5% methanol. YPM plate:YPM + 1.5% agar.YPDSZ;1% yeast extract, 2% peptone, 1 M sorbitol, 1.5% agar, 2% glucose and 50 mg/mL Zeocin (Life Technology). Minimal methanol medium (YNBMM) 6.7% Yeast Nitrogen Base with amino acids (Difco, USA), 0.5% methanol, and 1.5% agar.

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Reagents: Adalimumab was purchased from Abbvie GL (Germany). Human TNF *α* was from Wakko (Japan). Anti-human IgG goat IgG conjugated with HRP was from Rochland (USA). **Genes for adalimumab heavy (H) and light (L) chains**: Genes for adalimumab (Drugbank ID: DB00051) heavy (H) chain and light (L) chain amino acid sequences will be listed elsewhere. Their codons were optimized for expression in *P. pastoris*, were designed, and synthesized by GenScript (USA).

Construction of plasmids: The H and L chain genes synthesized were inserted between the *aox1* promoter and *aox1* transcriptional terminator in pPICZ A to construct an H chain and an L chain expression vector, respectively. The DNA fragment that contained the *aox1* promoter, L chain gene and *aox1* transcription terminator, the L chain expression cassette, was cut out from the L chain expression vector with *Bgl* II and *Bam*H I double digestion. The L chain expression cassette was then inserted into the *Bam*H I site in the H chain expression vector to create an expression cassette vector for adalimumab. The H and L chain expression cassette was then cut off from the vector with double digestion with *Bam*HI and *Bgl* II, and put into a reusable vector pInt3 CRE, which was a derivative of pInt2 CRE. A detail map of a reusable adalimumab expression vector pInt3CRE Hu will be shown elsewhere.

【Conclusion and Discussion】

DNA fragments encoding the light chain and heavy chain genes of an anti-human TNF *α* antibody, adalimumab, fused with an egg-lysozyme signal peptide were synthesized based on the codon bias of *Pichia pastoris*. These fragments were inserted into a site between the AOX 1-promoter and -terminator and put into a reusable vector system. The reusable expression vector was linearized and introduced into *P. pastoris, △ och1 strain*. After checking of several transformants by colony-immunoblot using anti-human IgG to ensure the antibody production, one was selected. Then the selection marker (zeocin resistant) gene integrated in the genome was excised with activation of *cre/loxP* system in the reusable vector. One clone that was zeocin sensitive was chosen to reintroduce the same expression vector. The transformants were checked their increasing levels of the antibody expression with colony-immunoblot. One increased clone was selected to examine its levels of functional antibody production. The levels reached up to 3.7 mg/L in a flask culture with medium containing 1 % methanol. The heavy chain and light chain of the product were assembled to form a hetero tetramer, as detected by SDS-PAGE. N-terminal amino acid sequencing revealed that the signal peptides of both chains were precisely processed. Analysis on the N-glycans of the produced antibody revealed approximately 90% of them were Man_8-$GlcNAc_2$, and the rest were mainly Man_9-$GlcNAc_2$. ELISA studies showed the affinity curve for the human TNF *α* was almost the same as one obtained from the clinically used adalimumab produced by CHO.

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Effects of rearing conditions (group or single rearing) and environmental enrichment on locomotor activity in adult male mice

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Introduction

The 3Rs philosophy is essential to conduct animal experiments properly and ethically. 3Rs is the basic philosophy of animal experiment with three things: "Reduction" refers to reducing the number of animals used, "Replacement" refers to substituting phylogenetically lower animal species or in vitro, "Refinement" refers to reducing pain and improving experimental and breeding methods. In particular, consideration of "Refinement" has been emphasized among them, and improvement of the rearing environment by using environmental enrichment has recently attracted attention as one of the efforts. Environmental enrichment is to enrich the rearing environment in order for animals to show their original behavior and to improve the psychological well-being of them. In the past researches, it has been reported that the environmental enrichment affects the physiological functions and behaviors of animals.

However, there are not many reports examining the effects of environmental enrichment and different rearing conditions (group or single rearing) on locomotor activity. Furthermore, it has been reported that body temperature tends to be lower in single rearing mice than in group rearing mice, and it is thought that such a decreased body temperature may alter locomotor activity.

Based on the above two points, in this study, we examined how the differences in rearing conditions and environmental enrichment affect the locomotor activity of mice, and whether the difference in locomotor activity was related to body temperature.

Materials and Methods

Male ICR mice at 3 weeks of age were reared in four experimental groups, group or single rearing, with or without environmental enrichment. In group rearing, the number of animals per cage was three. In the group using environmental enrichment, one Crawl Ball was installed in the cage as environmental enrichment. At the age of 10 weeks, an implantable activity / temperature measuring device was implanted subcutaneously in the back of mice using a three types of mixed anesthetic (medetomidine / midazolam / butorphanol = 0.3 / 4.0 / 5.0 mg/kg). After implantation, mice were awakened using atipamezole (0.3 mg/kg) and kept warm for more than 5 hours. After a recovery period of about 2 weeks, locomotor activity and body temperature were measured at 13–15 weeks of age. In the data for 36 hours from 13:00 on the measurement day to 1:00 on 2 days later, 19:00 to 7:00 was night-time and 7:00 to 19:00 was day-time. We calculated and compared the ratio of night-time activity to day-time and average body temperature for 24 hours. At 17–18 weeks of age, we collected the striatum of the brain region, which is considered to control locomotor activity and evaluated the dopamine turnover rate (DOPAC/DA) in striatal tissues.

In addition, we also measured the locomotor activity and dopamine turnover rate when the heating treatment was performed on the hot plate in order to examine the relationship between body temperature and locomotor activity. The heating treatment was performed to reduce the difference in the body temperature between each group. Before performing the heating treatment, the locomotor activity was confirmed to be similar

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to the result of the previous experiment under the condition without the heating treatment. Thereafter, at 17 weeks of age, locomotor activity was measured under both conditions with or without heating treatment, and at 18–19 weeks of age, stria-tum was collected under a heating environment to calculate dopamine turnover rate. Locomotor activity and dopamine turnover rate were evaluated by two-way analysis of variance for the effects of rear-ing conditions and environmental enrich-ment.

This study was approved by Nippon Veterinary and Life Science University (30K-26、2019K-14).

Results and Conclusions

In the condition without heating treatment, the ratio of night-time activity to day-time was affected by both rearing conditions and environmental enrichment conditions, and it was suggested that locomotor activity was significantly increased by single rearing and environmental enrichment ($p<0.01$). The dopamine turnover rate in the striatum was affected only by the rearing conditions and showed a significant increase by single rearing ($p<0.05$). Average body temperature for 24 hours was affected by both conditions, and it was suggested that body temperature was significantly increased by group rearing and environmental enrichment ($p<0.01$).

In the condition with heating treatment, the ratio of night-time activity to day-time was affected only by the environmental enrichment conditions, and it was suggested that locomotor activity was significantly increased by environmental enrichment regardless of either rearing condition ($p<0.05$). There was no significant difference in the dopamine turnover rate under either rearing and environmental enrichment condition, and the significant difference observed under the rearing conditions without heating treatment disappeared by heating treatment.

Therefore, it was considered that the behavioral activity of single rearing increased to maintain body temperature. On the other hand, it was suggested that another factor in addition to keeping body temperature may be involved in the increased behavioral activity induced by environmental enrichment. It was also suggested that environmental enrichment may affect the different type of behaviors such as emotional behavior but not pure locomotor activity, because the results of dopamine turnover rate in the striatum and locomotor activity under environmental enrichment conditions did not correlate.

In conclusion, it was suggested that locomotor activity of mice was affected by both the differences in rearing conditions and environmental enrichment, and that locomotor activity caused by these two conditions may be related to different mechanisms such as thermoregulation or emotion.

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Testicular function evaluated by measuring salivary concentration of testosterone in adult male mice

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Introduction

When we performed an animal experiment, it is necessary to comply with the 3Rs principle of Reduction, Replacement, and Refinement. Therefore, our laboratory is focusing on establishing an experimental method that contributes to 3Rs. Saliva samples can be collected with less invasiveness compared to blood samples, and we have reported that salivary corticosterone is available to evaluate stress response in mice.

In this study, we investigated whether testicular function could be evaluated by measuring the concentrations of testosterone in saliva. Saliva samples can be obtained from mice repeatedly and the number of mouse will decrease for testicular toxicity test if the salivary sample is available.

So the purpose of this study is first, we examined the correlation between testosterone concentrations in blood and in saliva samples (Experiment 1), second, we evaluated the response of testosterone secretion against human cholionic gonadotropin (hCG) in saliva samples (Experiment 2), and third, we examined whether testicular dysfunction caused by the environmental estrogen bisphenol A (BPA) could be detected using saliva samples instead of blood samples (Experiment 3).

Materials and Methods

Adult male ICR mice aged 10 weeks were used in this study. Three types of mixed anesthetic agents (medetomidine / midazolam / butrphanol = 0.3 / 4.0 / 5.0 mg/kg) was selected as the anesthesia and atipamezole (0.3 mg/kg) was used for recovery from anesthesia, and saliva secretion was enhanced by pilocarpine hydrochloride (0.5 mg/kg).

This study was approved by Nippon Veterinary and Life Science University (29K-23, 30K-24, 2019K-15).

Experiment 1

Male mice were castrated 1weeks before the implantation of silastic tube filled testosterone (0.1 μg or 0.2 μg) under anesthesia. Saliva and plasma samples were collected at 24 hours after the implantation. The plasma and salivary steroid hormones were extracted using diethyl ether. The concentrations of testosterone in both samples were detected by enzyme immunoassay (EIA).

Experiment 2

To examine the basal levels of testosterone, saliva was collected from adult male ICR mice under anesthesia after administration of saline. One week later, saliva was collected again after administration of hCG (25, 50, 100 IU/kg). The concentration of salivary testosterone was measured by EIA.

Experiment 3

Adult male ICR mice were orally treated with BPA (100, 50, 25, 0 μg/ml) dissolved in 0.01 % ethanol as drinking water for 4weeks. To examine the basal levels of testosterone, saliva was collected from adult male mice under anesthesia after administration of saline. Three days later, saliva collected again after administration of hCG 50 IU/kg. The concentration of salivary testosterone was measured by EIA.

Result

Experiment 1

One week after castration, both plasma and salivary testosterone could not be detected by EIA. The testosterone concentration showed the recovery

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in a dose-dependent manner. In the large dose of testosterone implanting (0.2 μg) group, the concentration of salivary testosterone recovered to same levels to the control group. In addition, it was confirmed that testosterone transferred from blood to saliva was about 10% in the all three groups.

Experiment 2

The salivary testosterone level was significantly increased in all doses of hCG treated group compared with the saline control group (25 IU/kg: $p<0.05$, 50, 100 IU/kg: $p<0.01$). There is not significant difference in the increased level of testosterone between hCG doses.

Experiment 3

The basal level of testosterone in saliva was significantly decreased ($p<0.05$) in the BPA 50 μg/ml and BPA 100 μg/ml treated groups compared with the control group (BPA 0 μg/ml). The exactly same result was obtained in the testosterone responses caused by hCG administration.

Discussion and Conclusions

These results indicate that salivary testosterone concentration reflects approximately 10% of plasma concentration of testosterone, and that the salivary sample is useful as a substitute for blood sample.

In addition, it was suggested that the treatment of hCG 50 IU/kg was effective in evaluating testicular function using saliva samples in mice, and that testicular dysfunction induced by BPA could be detected in saliva samples.

In conclusions, it is suggested that the testicular dysfunction can be detected using saliva samples, and that this method can be applied to testicular toxicity tests and might contribute "Reduction" in the principles of 3Rs.

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A comparative physiological study of differences between dog breeds in maintenance protein requirements

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[Objective]

Food for dogs is made in consideration of acquired factors such as feeding speed and breeding environment. However, dogs vary in genetic distance from their ancestor wolves, but many foods have 0.07 g / kcal of protein per kcal (CP / Energy) obtained from beagle as an experimental animal. It is only devised as a basis. Therefore, the maintenance requirements of protein which were estimated with urine creatinine / urea nitrogen ratio as an index were compared between Shiba-Inu which are 80% homologous to wolves, and Beagle, as a model. Similar comparison was done among Beagle, Toy-Poodle, and Dachshund to determine whether the genetic background affected protein requirements. In addition, we examined nutritional management methods for dogs visiting veterinary clinics during medical examinations. Then, we compared the nucleotide sequence of the Cysteine dioxygenase (CDO) gene, which is likely to be related to eating habits, between dog breeds, and confirmed whether it affected the genome.

[Materials and methods]

In experiment 1, four healthy dogs were selected from Shiba-Inu and Beagle bred at the Japan Pet Food Co., Ltd. Healthy Seafood Research Institute, respectively, and experimental foods (CP 44%, 35%, 28% , 22%) for 7 days, and urine was collected for the last 3 days. In experiment 2, the four Shiba-Inu used in experiment 1 were tested at high protein levels (CP 56%, 44%, 35%, 28%). In experiment 3, four Toy-Poodles and four miniature Dachshunds similarly raised were subjected to the same test as experiment 1. In experiment 4, a total of 66 healthy dogs were fed from 11 healthy dogs visited at the Mizuhodai Animal Hospital in Saitama for a medical checkup, and urine that had been naturally voided at the hospital was used as a sample. The collected urine was measured for creatinine and urea nitrogen by a conventional method, and the protein requirement was estimated. In experiment 5, Exon regions I of the CDO gene sequence were decoded using whole blood samples of 9 species selected from 19 dogs among the individuals used in experiment 4, and comparisons were made between dog breeds.

[Results and discussion]

In experiments 1 and 2, the urinary creatinine / urea nitrogen ratio of both Shiba-Inu and beagle decreased as the amount of protein in the food increased, but the optimal protein requirement (g / g) was 0.07 g / kcal for beagle. kcal), compared to 0.09 g / kcal for Shiba-Inu. The above results indicate that Shiba-Inu requires a higher protein food than Beagle, which is the opposite of general commercial food. In experiment 3, it was inferred that the Toy-Poodle and Miniature Dachshund required the same CP / Energy food as the beagle.

In experiment 4, the change in the urine creatinine urea nitrogen ratio when changing the current food to a higher protein food every other day is used to determine whether the current food is sufficient for protein. It was thought to be useful as an index when performing nutritional management and guidance of individuals in clinical settings. In experiment 5, the homology between the breeds was 100% in the region of Exon I-II that could be decoded. This suggests that differences in food habits between dog breeds at this stage are not likely to be related to the sequence of the exon region of the CDO gene, and that it is necessary to study the regulatory regions such as transcription factors and introns.

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An analysis of Yotsuba grass-fed milk produced by five dairy farmers

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Introduction

In the 1960s, the concept of animal welfare (AW) began to spread in Europe with the review of industrial livestock systems. AW with regard to livestock refers to raising animals in a living state with a high degree of satisfaction of their behavioral demands; it is considered to lead to improvement in productivity and the production of safe livestock products. Although this concept has not been widely used in Japan, five dairy farmers have been grazing their livestock in AW-grazing dairy farms since 2013 in the Makubetsu-cho Churui of Hokkaido. In 2014, sales of "Yotsuba grazing producer-designated non-homo milk" (grass-fed milk) began. In this study, we analyze the current situation of grass-fed milk producers, consumers, and general consumers to clarify the current situation of AW-milk food chains and the issues they face as they spread; we further explore their potential.

Materials and Methods

A survey was conducted with five grass-fed dairy farmers who have obtained grazing dairy farming certification from the Japan Grassland Agriculture & Forage Seed Association in JA-Churui. A questionnaire on the status of milk purchases and awareness of AW was also distributed to 412 grass-fed milk consumers who were members of the joint purchasing group "Yotsubakai (established in 1972)," of which 249 responded.

In addition, 156 general consumers, who were participants of the university's open day, conducted a tasting of grass-fed milk and then completed a questionnaire survey on the status of milk purchases and their awareness of AW.

Results and Conclusion

Among the five dairy farms, the average number of cows is 78.6, the average milk yield is 364.2 tons per year, and the average cultivated land area is 55.6 ha. The grazing period is from May to the end of October. From November to April, grazing is performed in the paddock and breeding is conducted in stanchion stall barns. The AW standards prepared by the Yotsuba Milk Products Co., Ltd., include items such as the cleaning of nursing buckets and the observation of health conditions. As a result of working on AW based on this standard, advantages such as a cleaner cow body, improved hair and gloss, reduced illness, and milder behaviors were mentioned. On the other hand, since stanchion stall barns greatly impede the unrestricted activities of dairy cows, there is room for improvement regarding AW.

Altogether, 91.6% of the Yotsubakai members are female, 79.5% are older than 60, and 69.1% are older than 20. The reasons they joined Yotsubakai were reported as follows: 85.1% "for purchasing safe and secure foods," 63.9% "for family health," and 57.4% "for purchasing delicious foods." A total of 36.5% of members bought grass-fed milk, of which 64.1% was for those who had been members less than 10 years, 40.7% for those who had been members from 10 to 20 years, and 28.5% for those who had been members over 20 years. Short-term members had a higher percentage of grass-fed milk. Regarding the price, 52.7% of members who buy grass-fed milk were satisfied, and 31.9% were slightly satisfied. On the other hand, of those who did not buy grass-fed milk, 35.3% were satisfied and 32.1% were slightly satisfied. Thus, members who buy grass-fed milk were more satisfied with the price. Most people were satisfied with the taste of grass-fed milk, with 84.7% reporting they were "satisfied" and an additional 8.8% reporting

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they were "slightly satisfied." Regarding the reason for buying this milk, "producers and production areas" was 63.1%, "Non-GMO feed" was 39.4%, "grazing" was 28.5%, "price" was 10.8%, and "AW" was 8.0%. Amongst those who reported that AW was their reason for buying this milk, 10.4% of members said they "know it well," and 36.5% said they "would have heard it." Of those who based their preferences on AW, 36.8% said it was "necessary," and 53.8% said it was "slightly necessary."

The general consumers comprised 95 high school students, 51 parents, and 10 students and professors from our university. Altogether, 73.8% reported "price" as being their reason for their milk choices, followed by "producer/production area" at 21.3%, "Non-GMO feed" at 1.6%, and "grazing" and "AW" at 0%. Regarding AW, 17.9% of high school students said they "know it well," and 28.4% said they "would have heard it." In contrast, 4.1% of parents said they "know it well" and 45.1% said they "would have heard it." Of the general consumer participants, 42.5% said that AW was "necessary" and 47.3% said it was "slightly necessary." When comparing prices, 4.9% of respondents said they would buy grass-fed milk even if it its price were "40% higher" than general milk.

Thus, Yotsubakai members prioritized production history and Non-GMO feed while general consumers prioritized price. There was no difference in AW awareness or need. Although more than one-third of Yotsubakai members buy grass-fed milk, it has been found that AW is not well-known and is not used as a standard for purchase. The high degree of satisfaction with the price of grass-fed milk may not have been due to the evaluation of AW but to the evaluation of taste and safety. AW educational activities are necessary, but this is an issue for the future. Despite the fact that the general consumer believes there is a need for AW, grass-fed milk is 40% more expensive than general milk; the issue here is that there is a lack of understanding about this price. In summary, grass-fed milk producers have seen improvements in the health of dairy cows as a result of their AW initiatives, and consumers have gained an understanding of the topic. In addition, consumers of Yotsuba milk are seeking to increase the number of producers working on grazing, increase production of grazing milk, and achieve 100% domestic feed. Therefore, it is necessary to consider interaction between consumers and dairy farmers, hold AW study sessions, and consider policy support.

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Research on support for new entry in prefectural dairy farming

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In Japan, farmers frequently leave dairy farming owing to the absence of a successor, and an average of 740 dairy farmers stop milking each year. The government has taken measures to focus on securing new farmers and fostering leaders, but these responses vary widely from region to region. In Hokkaido, where dairy farming is flourishing, support for new entrants using training ranches is in place. However, the response is delayed in prefectures, which have only isolated cases of support. Most of the previous studies on new entrants have been in agricultural cultivation, such as rice cultivation and upland cultivation, and no studies on dairy management have been conducted. The purpose of this study was to clarify the current situation for dairy farmers who have stopped milking and dairy farmers who were new entrants in prefectural dairy farming. In addition, we explored the success factors for new entrants into dairy farming and presented guidelines for building a support system that can be disseminated.

Chapter 1. Current situation of farming in Tochigi prefecture

We surveyed dairy farmers who had stopped milking in Tochigi Prefecture in 2014–18 and investigated the current state of management resources. Half the dairy farmers in Tochigi prefecture at the time of the survey were over 60 years old. The successor absence rate was 30%. This suggests that a reasonable proportion of dairymen will stop milking in the coming years. In Tochigi Prefecture, 99 dairy farmers had stopped milking in the past five years, of which 72.7% were over 60 years old. The main reasons for stopping milking were "no successor" in 52.5% and "aging of managers" in 43.4% of cases. In addition, some dairymen stopped milking and switched to the beef business. In terms of the cow sheds where dairy farmers had stopped milking, only 28.1% (Sale: 2.8%, Rent: 2.8%, Use by yourself: 22.5%) were effectively used and 70.4% were not used.

Chapter 2. Support system for new entrants

We conducted a survey on support measures for new entrants at the National Dairy Cooperative Association. Responses were received from 11 agricultural cooperatives. The succession rate for 1872 dairymen belonging to 11 agricultural cooperatives was 41%. Ninety-nine dairy farmers said they would stop milking within five years, but only five intended to transfer their management. Five agricultural cooperatives supported new entrants, but there were no cases where agricultural cooperatives took the initiative in providing support. Nine agricultural cooperatives were hoping that related organizations such as livestock cooperatives and governments would take steps to support new entrants. However, no measures were taken. To stop the decline in dairy farming, dairy farmers who stop milking need to be encouraged to non-family-type farm succession. To this end, it should be recognized that support by agricultural cooperatives alone is not sufficient and should be addressed on a "region-wide" basis.

Chapter 3. New entrants in Shimane Prefecture

We surveyed three dairy farmers who were new entrants in Shimane Prefecture. In the survey, we asked about the process up to new entrance, the acquisition method for tangible and intangible assets, and entry support. The three dairy farmers surveyed were in their 30s and 40s, and five to ten years had passed since they entered the dairy industry. Two of the three dairy farms were non-family-type farm succession. One set up a new ranch and started management. They had two

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characteristics. First, there was little involvement with previous managers. Second, there was no understanding from the local residents due to livestock environmental problems. Their success was largely attributed to "information sharing" and "the promotion of understanding of the local residents"' by dairy manufacturers and livestock dealers. From this survey, the following two options were identified as success factors. (1) Build good relationships with local communities, and (2) establish a long-term support system from new entry to stable management with agricultural cooperatives and administrative professionals.

Conclusion

There were several problems with the new entry of prefectures into dairy farming. These were "Stagnation of farmland fluidization due to expensive farmland prices" and "Livestock environmental problems due to the lack of local residents understanding of dairy farming". These factors have closed the door to new entrants. In addition, "the absence of specialized staff who promotes matching in non-family-type farm succession" and "the lack of a support system" were also factors that delayed new entry. However, as in Survey 3, cases where new entrants were dealt with in "the whole region" were confirmed. Until now, the relevant organizations have taken measures individually. However, for successful new entry of prefectures into dairy farming, this needs to change in the future. It is necessary for central organizations to collect information and for the entire region to work together to create a diverse support system.