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Research on the biofilm formation of *Staphylococcus pseudintermedius* clinical isolates from dogs and cats

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In veterinary medicine, *Staphylococcus pseudintermedius* is isolated as a causative organism of various infections and is also known as biofilm producer. However, since biofilm recognition in the veterinary field is low, sufficient measures against *S. pseudintermedius* infection for biofilm have not been taken. We investigated clinical isolates of *S. pseudintermedius* derived from dogs and cats about the ability of biofilm formation, their source of isolation, antibiotic resistance and the difference of inflammatory responses depending on the potency of biofilm organization.

Firstly, 250 isolates of clinical *S. pseudintermedius* from dogs and cats were intended for isolation and evaluation of the ability of biofilm formation. All isolates were classified as strong (24.8 %), moderate (52.0 %), and weak (23.2 %) biofilm producers. The risk of the biofilm related infections of *S. pseudintermedius* was appeared to be no difference regarding hosts, infection sites, and medical facilities.

The positive correlation was found between minimum inhibitory concentration and biofilm organization potency in 9 antibiotics in the examination about the association between antibiotic resistance and biofilm organization potency. Moreover, in ampicillin-resistant isolates of methicillin-susceptible *S. pseudintermedius* (MSSP), the strong biofilm producers were more resistant than the weak biofilm producers. It was suggested that biofilm formation was associated with antibiotic resistance of the MSSP.

To investigate the pathogenicity of *S. pseudintermedius* biofilm, we compared the expression of inflammatory cytokines in RAW264.7 cells' mouse macrophage cell line, induced by biofilm-conditioned medium (BCM) preparing with filter sterilized culture medium of different of biofilm organization potency. As the results, a significant increase in expression of IL-1 β and TNF-α was observed in RAW264.7 cells cultured with BCM of strong biofilm producer than with BCM of weak biofilm producer ($P < 0.01$). We examined what was the inflammatory attractant and how inflammatory response was occurred in BCM of strong biofilm producer, and revealed that the heat-resistant secreted proteins were induced inflammatory responses via the toll-like receptor (TLR) signaling pathway. Also, the strong biofilm producers had specific banding patterns and peaks when we analyzed by SDS-PAGE and MALDI TOF-MS, respectively. Therefore, it was recognized that a difference in secreted proteins relays on the biofilm organization potency.

*Supervisor : Prof. Kazunori IKE

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Evaluation of the Role of Endoplasmic Reticulum Stress and Novel Neurodegeneration Inducing Factor PRMT8 in the Pathogenesis of Alzheimer's Disease Model Mouse

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Neurodegenerative diseases such as Alzheimer's disease (AD) were refractory disease, thus finding methods for diagnosis and treatment are crucial. It is proposed the 'amyloid cascade hypothesis' that deposition of amyloid-β (Aβ) (amyloid pathology) is the initial pathological event in AD, followed by hyperphosphorylation of tau (tau pathology), and finally neurodegeneration and cell death. However, the mechanistic link(s) between these AD pathological features remains unclear. Familial AD is caused by mutations in *APP, PSEN1* and *PSEN2* genes, which encode amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2), respectively. Several AD model mice containing gene mutations associated with familial AD; these APP and/or PS1-overexpressing Transgenic (Tg) mice have been used widely as AD mouse models. However, there is concern that the elevated ER stress phenotype might be an artifact of overexpression of APP and PS1. Based on these research background, this research have 2 project for the aim of "Re-examining the relationship between AD pathophysiology and endoplasmic reticulum stress", and "Examination of the effect of PRMT 8 on neuropathology".

In project 1, to determine whether the ER stress response is heightened because of Aβ pathology, PS1 mutation, several ER stress markers expression levels were investigated in mouse models of several amyloidosis and tauopathy. In result, no difference in any of the stress markers was observed between wild-type (WT) mouse, *App* knock-in mouse, the model shows Aβ deposition except overexpression of APP, APP-Tg mouse, and Tau-Tg mouse. On the other hand, the mouse of models express genetic modification of PS1 showed upregulation of some ER stress markers. These results indicate that "neither Aβ deposition, APP overexpression, nor tau pathology result in detectable ER stress". It is assumed that the genetic modification of PS1 induces ER stress through a mechanism that is not related to the Aβ pathology.

In project 2, I focused on Protein Arginine Methyltransferase 8 (PRMT 8) that was identified as protein which tau interaction altered by amyloid pathology. To investigate the role of PRMT8 in AD pathogenesis, *PRMT8* gene was deleted or overexpressed in $App^{NL\text{-}G\text{-}F}$ mouse x *MAPT* KI mouse; double KI (dKI) as AD model mouse and pathological features were investigated by biochemical and histological analysis. In result, introducing PRMT8 into dKI mouse via the AAV vector resulted in upregulated phosphorylated tau level, inducted brain inflammation, apoptotic cell-death pathways, and severe vacuole-like structure.

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However, these pathological phenotypes were observed in PRMT8 introduced Tau-KO and WT mouse. Contrary to expectations, this suggests "PRMT8 is a factor that induces neurodegeneration independent of tau pathology".

This research provides new information on the study of neurodegenerative diseases including AD.

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Fundamental Research on Suncus (*Suncus murinus*) as an Animal Model of Lipodystrophy

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Lipodystrophy syndromes characterized by loss of whole or partial body fat are associated with insulin resistance, diabetes and lipid metabolism abnormalities including fatty liver. Leptin is an adipocyte-derived hormone thought to play an important role in the pathophysiology of lipodystrophy. House musk shrew (*Suncus murinus*), a small experimental animal with low body fat, may be a possible model for human lipodystrophy. The objectives of this study were to clarify the structure and distribution of suncus leptin.

To determine the primary structure of suncus leptin, we cloned the suncus *Lep* cDNA using the RACE method. The obtained amino acid residues (aa) sequence was compared with other mammals, and the protein structure was predicted by homology modeling.

The suncus *Lep* cDNA encodes 170 aa of the putative suncus leptin precursor containing a predicted signal peptide of 21 aa, and the mature leptin is consisted of 149 aa. The mature leptin is 75%–82% homologous to that of other mammal species. Insertion of the 3 aa, VPQ, not seen in other mammals was found in the CD-loop. This VPQ insertion is thought to be due to a nucleotide insertion of nine bases by slippage-like microindels. The predicted 3D structure of suncus leptin exhibited a typical four alpha-helix structure, however, the VPQ region protruded compared with human leptin. *Lep* mRNA expression was observed only in white and brown adipose tissues.

This study revealed the structure and distribution of suncus leptin. Because the addition of VPQ, which is not found in other mammals, was observed, suncus leptin attracts attention to its physiological action. It is thought that suncus is useful as a model animal for lipodystrophy in humans, particularly as a model animal without complications.

*Supervisor : Prof. Toshiro ARAI

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Study on function of feline melanocortin 4 receptor and melanocortin 2 receptor accessory protein 2

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Melanocortin 4 receptor (MC4R), which is a member of the G protein-coupled receptor (GPCR) family, mediates regulation of energy homeostasis upon the binding of α-melanocyte-stimulating hormone (α-MSH) in the central nervous system (CNS). Melanocortin 2 receptor accessory protein 2 (MRAP2) is a membrane protein which modulates the function of MC4R. Deletion, mutations or polymorphisms of MC4R and MRAP2 gene are associated with obesity in mice and humans.

To elucidate molecular mechanisms of feline obesity, we performed cDNA cloning of cat MC4R and MRAP2, characterized their amino acid sequences, mRNA expression patterns in cat tissues, protein–protein interactions, functions, and relations between their single nucleotide polymorphisms (SNPs) and body condition score (BCS). We found high sequence homology (>88%) with other mammalian MC4R and MRAP2 encoding 332 and 206 amino acid residues, respectively. Cat MRAP2 contained 2 N-linked glycosylation sites, one each in the N-(N9) and C-(N175) terminus. N175 was not observed in human, mouse, and rat. Moreover, glycosylation in the N- and C-terminus supports the reverse topology hypothesis of MRAP2. Reverse transcription-polymerase chain reaction analysis revealed that cat MC4R and MRAP2 mRNA were expressed highly in the CNS. In CHO-K1 cells transfected with cat MC4R, stimulation with α-MSH increased intracellular cyclic adenosine monophosphate (cAMP) concentration in a dose-dependent manner. Furthermore, the presence of MRAP2 enhanced the cat MC4R-mediated cAMP production. The glycosylation status at N175 is involved in MC4R modulation by MRAP2. Our NanoBiT study showed the dynamics of their interactions in living cells; stimulation with α-MSH slightly affected the interaction between MC4R and MRAP2, and did not affect MC4R homodimerization, suggesting that they interact in the basal state and that structural change of MC4R by activation may affect the interaction between MC4R and MRAP2. In addition, the presence of MRAP2 did not affect MC4R homodimerization. This result denied the hypothesis that dimer or oligomer separation of MC4R by MRAP2 interaction is related to MC4R signaling regulation of MRAP2. The case-control study between SNP and BCS revealed that c.*452C/T in the 3' untranslated region of MC4R and c.*543T/G in the 3' untranslated region of MRAP2 were correlated with BCS. The frequencies for c.*452T>C and c.*543G>T of overweight cats were higher than that of normal cats ($p < 0.05$).

These results suggested that cat MC4R acts as an α-MSH receptor in the CNS; that its function is modulated by MRAP2; and that MC4R and MRAP2 may be involved in weight regulation in cats. Moreover, c.*452T>C and c.*543G>T may be a candidate marker for genetic diagnosis of feline obesity.

*Supervisor : Prof. Toshiro ARAI

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Molecular Pathological Evaluation of Familial Spontaneous Epileptic Cats

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Epilepsy is one of the common neurological disorder which is characterized by recurrent epileptic seizures, and genetic factors are thought to be involved in the disease occurrence in most epileptic patients. Familial spontaneous epileptic cats (FSEC) is the unique colony which is considered as feline genetic epilepsy, and they show two types of seizures, including spontaneous limbic seizures and vestibular stimulation-induced seizures. However, genetic architecture has not yet been elucidated. In this study, comprehensive genetic analysis has been performed that included neuropathological evaluation, candidate gene approach, genome-wide association study (GWAS), genome-wide linkage analysis, and whole genome sequencing (WGS). In the pathological evaluation, the neuronal decrease in the hippocampal CA3 region and amygdaloid central nuclei was found. In addition, gliosis in hippocampal CA4 region without neuronal loss was also found. Mutational analysis for LGI gene family was conducted, which were considered as a candidate gene family of FSEC based on the human and animal model literatures, the causative variant was not detected in this gene family. GWAS was performed to detect the associated loci with epilepsy. Assuming that all the phenotypes were caused by the common variant(s), or each type of seizures (phenotype) was caused by (a) different variant(s), tests were conducted in different patterns. Different loci in each phenotype were detected by GWAS, however, none of them were genome-wide significant. In genome-wide linkage analysis, tests were performed in different patterns as well as GWAS, and multiple loci with suggestive linkage were detected in each phenotype. Furthermore, WGS was performed on four FSECs. After the filtering in different patterns based on the phenotype, unique variants that were not present in feline WGS database were detected in the suggested region by GWAS and linkage analysis. Variants located within genes that were considered to be associated with epilepsy, most of them were not located within the coding region. In this study, the comprehensive genetic analysis was conducted, and it was essential to understand the genetic architecture of epilepsy occurrence in FSEC. This study suggested the complexity of the genetic architecture in FSEC. Variants found in this study are considered not to have large effect, however, these variants may have the contribution to epileptogenesis in FSEC in some way.

*Supervisor : Prof. Michio FUJITA

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Studies on Infectious Disease, Pathology, Clinical Workup and Basic Biology of Zoo Animals

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The author has been working at Zoorasia since the opening preparation period in 1997. This thesis summarized the studies on the diseases and their pathology that the author had experienced in Zoorasia keeping animals so far.

In chapter two, the author described nontuberculous mycobacteriosis in northern carmine bee-eaters. Four norhtern carmine bee-eaters (*Merops nubicus*) kept in the same indoor exhibition died in a row. From sequencing analysis of 16SrRNA gene and *hsp*65 gene using DNA samples extracted from the livers and spleens of dead birds, it was revealed that the obtained base sequences of these house-keeping genes showed 100% homologies with *Mycobacterium genavense* which is a kind of non-tuberculous mycobacteria. As a result, it was concluded that the present serial mortalities were caused by endemic infection of *M. genavense*, non-tuberculous mycobacteria.

In chapter three, the author described the cases of neoplasms observed in the rearing animals of Zoorasia, from the opening period to 2017. Neoplastic conditions in zoo mammals have been recorded in total 45 animals of 24 species from 1999, the opening of Zoorasia, to 2017. The neoplastic conditions were the most frequently recorded in Carnivora (9.2%). In the present survey, squamous cell carcinoma of oral cavity was more common in nonhuman-primates (three cases) and Diprotodonia (two cases).

In chapter four, the author described immune-mediated disorder observed in spectacled bear. A 5-year-old female spectacled bear (Andean bear, *Tremarctos ornatus*) had pruritic eczema all over the body surface. Antihistamines had no effect for the symptoms. The bear was treated with prednisolone, and the symptoms were improved. This skin disease was considered atopic dermatitis because of effect of corticosteroid and disapperarance of clinical signs due to the change of living place. Recent publications regarding a specific dermatological condition in female spectacled bears have been proposing the name of novel immune- mediated skin disorder, Andean bear alopecia syndrome (ABAS). From the clinical features and blood biochemistry, it was suggested that the present case might be diagnosed as ABAS.

In chapter five, the author described cellular susceptibility of proboscis monkey's lymphocytes to Epstein-Barr virus (EBV). Lymphocytes collected from peripheral blood of four proboscis monkeys reared at Zoorasia was infected with EBV in vitro. From expression of EBV related protein and presence of viral genome in the proliferating cells, it was confirmed that proboscis monkey's lymphocytes were susceptible to EBV.

*Supervisor : Prof. Shinpei WADA

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Identification of the genetic attenuation-marker of canine parvovirus vaccine and methodological and epidemiological studies in canine serious infectious diseases

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(Conferred on 28 March 2019, VB-329)

Here, we describe basic studies for development and improvement of vaccines to prevent serious and fatal infectious diseases such as canine parvovirus (CPV) infection, canine distemper, as well as the highly incident canine infectious respiratory disease (CIRD) in owners of multiple dogs. Many challenges remain in the development of more effective and safe vaccines. Regarding live attenuated vaccines for CPV infections, the genetic markers that define the attenuated phenotype have not yet been identified. Evaluation of canine distemper virus (CDV) dynamics using quantitative reverse transcription and polymerase chain reaction (qRT-PCR) did not reveal any relationship to infection outcomes in experimentally infected dogs. In Japan, studies on the surveillance of pathogens that cause CIRD have been limited. The first study identified the minimal determinant for the attenuation of the CPV vaccine strain 9985-46. It showed that attenuation of the 9985-46 strain was defined by at least two mutations in residues 300 and 389 of the VP2 capsid protein. These results are important for the quality control of the 9985-46 live attenuated vaccine strain, and provide insights regarding the rational design of second-generation live attenuated vaccine candidates. The second study evaluated the usefulness of qRT-PCR in investigating the dynamics of CDV in experimentally infected dogs and the relationship between the change and outcome of the infection. The qRT-PCR results showed that CDV replicated irrespective of the degree of clinical manifestation in dogs, as qRT-PCR was more sensitive than virus titration in cell cultures of rectal and nasal sheddings. We also demonstrated that the qRT-PCR results correlated with viral titers in cell culture at the peak of viral RNA. In addition, the peak of viral RNA in symptomatic dogs was consistent with the onset of symptoms. These observations suggested that the peak of viral RNA reflected active CDV replication. This assay will be useful for comparing the multiplication and dissemination among different CDV strains and for determining the protective efficacy of vaccines. The third study conducted an etiological investigation on CIRD in Japan and evaluated the efficacy of its vaccine in the field. The results suggested that *Bordetella bronchiseptica*, canine parainfluenza virus (CPIV), and canine respiratory coronavirus (CRCoV) were the major pathogens that caused CIRD as single or multiple pathogens. In addition, CPIV, canine adenovirus type 2, and CDV were detected less frequently in dogs previously administered with the multivalent live vaccine that included these viruses when compared to unvaccinated dogs, suggesting that the vaccine effectively prevented canine infections. The findings obtained in these studies provide

*Supervisor : Prof. Yoshikazu TANAKA

useful and insightful information regarding the development of improved vaccines against important infectious canine diseases.

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Study on the diagnosis and surgical treatment for atlantoaxial instability in the dog

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Atlantoaxial instability (AAI), commonly affecting young toy-breed dogs (TBDs), results in severe cervical spinal cord injury. In most cases of AAI, ventral fixation of the atlantoaxial joint (AAJ) results in a favorable prognosis. However, in some cases, patient outcome following surgical treatment of AAI was worse than expected. In these cases, pathologic participation of concurrent craniocervical junction abnormality (CJA) that does not include AAI is suspected. The purpose of this study was to clarify the diagnosis and treatment of AAI in dogs with pathologic causation of AAI and CJA.

In chapter 2, we calculated the ratio of the dens length to the axis body length (DALR) and the dens angle (DA), and evaluated morphological characteristics of the dens. Our findings suggested that in AAI-predisposed TBDs with low DALR and great DA, the values of DALR and DA might be important factors for predicting the development of AAI. In chapter 3, we studied incomplete ossification of the dorsal neural arch of the atlas (IODA) in TBDs with AAI. IODA was observed in approximately 70% of AAI-affected TBDs, and the results suggested that IODA was an etiology of AAI, which developed at middle to advanced age. In chapter 4, we evaluated the mechanical strength of implant in three types of ventral fixation techniques for AAJ, using the atlas and axis harvested from healthy Beagle dogs. The currently used multiple metallic implant and polymethylmethacrylate (PMMA) fixation (PMF) was once again proven to be a useful method for AAJ fixation, with the highest fixation strength in the flexural test and with no significant differences in the maximum load between the atlantoaxial plate fixation (APF) group and the PMF group in the torsional test. In addition, APF is considered an alternative fixation method to PMF. In chapter 5, we evaluated how ventral AAJ fixation techniques influenced the fusion of AAJ. The possibility that APF could result in a more ideal AAJ bony fusion in terms of histology than PMF was suggested. In chapter 6, we studied the clinical outcomes after ventral fixation in TBDs with AAI, based on the presence or absence of atlantooccipital overlapping (AOO). The findings suggested that the presence of AOO affects the clinical signs for dogs with AAI but did not directly affect the outcome of the surgical stabilization of AAI. In chapter 7, we studied the cerebral ventricle size, which seemed to be related to AOO, in AAI-affected TBDs with or without AOO. AAI-affected TBDs, with concurrent AOO, exhibited significantly greater dilatation of the lateral and fourth ventricles than those without concurrent AOO.

*Supervisor : Prof. Yasushi HARA

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The study of health of large animals in the activities using animals

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Recently, various types of activities involving animals such as horses and cattle have attracted attention owing to their beneficial effect on the mental, physical, and social health in humans. However, there are inadequate reports about the stress on and welfare of animals, especially large animals such as cattle and horses used in such activities. In the first chapter of the present study, the cortisol (COR) level and several serum biochemical marker levels were assessed in dairy cows before and after the student practical training to investigate stress in these animals. The second chapter describes a study conducted in horses that are widely used in such activities. Differences between breed and serum biochemical marker levels were clarified. Finally, a survey was conducted among the members of an equestrian club regarding their perception of offering treats to horses.

In the first chapter, a significant difference was noted in serum COR level in dairy cattle (n = 29) before and after the training. Thus, contact with many unfamiliar people may induce stress in dairy cattle. However, the increase in serum COR level after the practice was not particularly high when compared to other stress conditions such as transportation and hoof shaving. This was assumed to be because of the usual feeding and milking pattern of the animals during the training. Individual differences were noted among the cattle in this study with one group exhibiting decreased stress with experience and another group being very sensitive to stress.

In the second chapter, serum biochemical marker levels in thoroughbred (n = 50), ponies (n = 49), and miniature horses (n = 14) were investigated. Principal component analysis was conducted using the results obtained, which were divided into three groups based on the breed. The determination rate of thoroughbreds, ponies, and miniature horses by discriminant analysis was 96.0%, 79.59%, and 92.86%, respectively.

The questionnaire survey for the members (n = 43) of an equestrian club was conducted using the information obtained from the previous results in horses. The approval of feeding treats to horses significantly decreased after informing the members regarding triglyceride levels in horses. Proper knowledge of horses resulted in members not excessively feeding treats and becoming aware about feeding management in horses. In the comparison between members owing a horse (owner) and those not owing a horse (general), the approval of feeding treats to horses was significantly lower in owners irrespective of the information provided. It was thought that treats for horses are the significant agita factor for owner. Supporting knowledge acquisition regarding feeding is important for maintain the health of horses.

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Study on early nutritional factors associated with metabolism disorders in modern broilers -Gluconeogenesis specificity and the relation between systemic metabolic control regulation and 25-hydroxychorecalciferol in newly hatched chicks

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[Objective] While modern broilers have improved productivity through breeding improvements, new myodegeneration, such as White striping (WS) and Wooden Breast (WB) has been observed, but its pathogenesis remains unclear. Therefore, in this study, we investigated factors contributing to myodegeneration, examined the glucose (GLU) content of embryos and gluconeogenesis specificity of newly hatched chicks, examined the effect of vitamin D_3 (VD_3) on broiler growth, which has been suggested to be related to metabolic induction of tissue formation, and then examined the effect of 25-hydroxycholecalciferol ($25(OH)D_3$) on the metabolic control system.

[Materials and Methods] In experiment 1, Ross commercial male broilers at 42 days of age were used to select WB probable broiler and non-probable broiler. Gross observations were performed to determine TGF-β, decorin, and vitamin-D receptor (VDR) mRNA expression in pectoral major muscle. In experiment 2-5, Cobb broiler hatching eggs were used to determine the GLU concentration in embryonic tissues. In experiment 6, the dose-response of metformin was confirmed. Then in experiment 7, the GLU concentration in embryonic tissue was measured based on the results of experiment 6. In Experiment 8, Ross broiler hatching eggs were used and distilled water, soybean oil, or VD_3 was in ovo administered to determine the mRNA expression and tibial length of IGF-1 and IGF-1 receptors in the liver and pectoral major muscles at 28 days of age. In Experiment 9, 4 group feeds combined with VD_3 and $25(OH)D_3$ were fed using Ross broiler chicks. VDR mRNA expression levels in pectoral major muscle were measured, and POMC expression levels, AgRP expression, NPY expression, and VDR mRNA expression levels in the diencephalon were measured. Furthermore, sugar tolerance test was performed in each section in experiment 10.

[Results and Discussion] TGF-β and decorin in the pectoral major muscle showed a strong positive correlation, and TGF- β also showed a correlation with VDR, suggesting that VD was related to muscle metabolism. Administration of metformin did not affect the blood GLU concentration, suggesting that strong feedback may be associated with muscle proteolysis. Although the tibial length at 28 days of age was significantly longer in males, the IGF-1 mRNA of the liver was higher than in the control group, but the IGF-1R mRNA of the

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superficial pectoral muscle was different only in females in the VD_3 group, suggesting that the occurrence of WBs was unlikely in females and the relationship between the occurrence of WBs and the soundness of muscle metabolism. The POMC mRNA involved in catabolism correlated with VDR mRNA, and the addition of $25(OH)D_3$ affected the insulin sensitivity of chicks, suggesting that $25(OH)D_3$ should be evaluated instead of VD_3.

These results suggested new guidelines for relevant areas of WB pathogenesis mechanism in broilers and the possibility of WB pathogenesis control in early gluconeogenesis control and catabolism regulation using $25(OH)D_3$.

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Development of new tools for quality evaluation of pasta

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Typical types of cooked spaghetti in Japan were examined for moisture distribution by magnetic resonance imaging (MRI). Moisture content was calculated based on the correlation between spin-spin relaxation time (T_2) and moisture content of standard gel samples. Boiled samples of dried and frozen spaghetti had a moisture gradient from the surface to the core and a distinct low moisture region at the center. On the other hand, the moisture content of long-life spaghetti was almost homogeneous. In mechanical tests of dried and frozen spaghetti, higher force was observed at the region corresponding to the low moisture core, which means that the state known as "al dente" was detected. The luncheon spaghetti and long-life spaghetti exhibited soft and brittle texture caused by higher moisture content at the core.

Moisture distributions and mechanical properties of ordinary dried spaghetti were compared to those of two quick boil spaghetti samples (spaghetti with a V-shaped groove and spaghetti with three windmill-shaped grooves). The quick boil spaghetti samples had lower total moisture content than ordinary dried spaghetti after cooking. The T_2 images of MRI during boiling showed that the region around the tips of their grooves absorbed water and swelled particularly rapidly resulting in closure of the grooves. Moisture distributions of the two quick boil spaghetti samples were not concentric. The force-strain curves of the two quick boil spaghetti samples, therefore, varied depending on the shearing direction. Thin high moisture region at the surface of boiled ordinary dried spaghetti did not have a large effect on the mechanical properties.

As mentioned above, visual evaluation of the textures of pasta using MRI was realized by correlating the data of the moisture distribution to those of the mechanical test.

One hundred twelve commercial dried long pasta collected from all over the world were classified into four groups according to similarities in their sensory characteristics after boiling. A total of fifty samples were selected from each group as representative samples, and they were evaluated sensorially by highly experienced and trained panels to generate expressions. As a result of the consolidation of them by the panel discussion, the final lexicon consisted of 35 terms (5 for appearance, 11 for aroma/flavor, and 19 for texture) were obtained. Twenty-six terms of them were classified as general descriptor, and 9 terms as specific descriptor. In addition, detailed definitions and concrete references were determined for each term. Principal component analysis (PCA) was conducted for results obtained in a trial evaluation of eight representative samples using the general descriptors, and the data was reviewed correlating the physical and chemical analysis data to the sensory characteristics. As a result, all of the general descriptors were found to be appropriate to differentiate the sensory qualities among the samples.

*Supervisor : Prof. Mitsuru YOSHIDA

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Study on Mechanism of Nobiletin-induced Relaxation in Vascular smooth muscle

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(Conferred on 14 March 2019, VNT-62)

Introduction

High blood pressure is one of the risk factors leading to cardiovascular disease, stroke and renal failure. Therefor daily control of blood pressure is extremely important for prevention of these diseases and improvement of prognosis. It has been reported that there was negative correlation between intaking of flavonoids and mortality of cardiovascular disease, suggesting that flavonoids have preventive and therapeutic effects on cardiovascular diseases. Moreover, in small dogs, vasorelaxants are used to lower preload and afterload during congestive heart failure, but drugs with fewer side effects are required. Nobiletin is a flavonoid that it is contained in the peel of citrus fruits, such as *Citrus depressa*, and *Citrus reticulata*. Nobiletin is known to be one of the active ingredients of some herbal medicines. Many studies have demonstrated that nobiletin possesses anti-inflammatory, anti-tumor, anti-oxidative, and anti-Alzheimer's disease effects.

Recent studies have revealed that nobiletin also has cardiovascular protection effects, such as protection against cardiac hypertrophy, anti-platelet aggregation, inhibition of vascular smooth muscle cell proliferation, and the production of nitric oxide (NO) in vascular endothelial cells. However, to our knowledge, there are no reports addressing the direct effects of nobiletin on the contraction of vascular smooth muscle. Therefore, this study was performed to elucidate the precise mechanism underlying nobiletin-induced relaxation of endothelial-denuded rat aortic smooth muscles.

Methods

Male Wistar rats were anesthetized using sodium pentobarbital (50 mg/kg, *i.p.*) and euthanized by exsanguination. The thoracic aorta was isolated and the endothelium was removed by gently rubbing the inner surface of the vessel with forceps. The aortic rings were mounted on stainless steel hooks with a resting tension of 5 mN in a 2 ml organ bath containing PSS. Changes in muscle tension were isometrically recorded using a strain-gage transducer.

cAMP and cGMP levels in the muscle strips were measured using enzyme immunoassays.

Results and Discussion

Nobiletin inhibited phenylephrine (PE, 1 μM)- or KCl (65 mM)-induced contractions in a concentration-dependent manner. However, this relaxation was stronger in PE-induced contractions than in KCl-induced contractions. Nobiletin inhibited receptor agonist-induced contraction more strongly than KCl-induced contraction due to depolarization, suggesting that the mechanism of action may be associated with an increase in cAMP and cGMP.

ODQ (a guanylyl cyclase inhibitor) significantly inhibited the nobiletin-induced relaxation of PE-induced contractions; however, SQ22536 (an

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adenylyl cyclase inhibitor) did not affect the relaxation. These results suggest that nobiletin-induced relaxation may be involved in increases of cGMP levels, but not cAMP levels. In addition, 3-isobutyl-1-methylxanthine IBMX (phosphodiesterase inhibitor) synergistically enhanced the nobiletin-induced relaxation. Nobiletin increased cGMP levels in rat aorta in a concentration-dependent manner. Also, IBMX significantly increased cGMP content in rat aorta, and ODQ significantly reduced cGMP levels. Moreover, these data indicate that nobiletin-induced relaxation is related to increases of cGMP levels via activating guanylyl cyclase (GC). Nobiletin-induced relaxation was significantly inhibited by the iberiotoxin (IbTX) (Ca^{2+}-activated K^+ (BK) channel inhibitor) and the glybenclamide (ATP-sensitive K^+ (K_{ATP}) channel inhibitor). Sodium nitroprusside-induced relaxation was suppressed by IbTX, but not by glybenclamide. These results suggest that nobiletin opens the BK channel via cGMP-dependent signals, but opens the K_{ATP} channel via cGMP-independent signals. However, some researchers reported that the increases in cGMP level is involved in the opening of the K_{ATP} channel, further study is need to clear the relation between K_{ATP} channel and the nobiletin-induced relaxation.

Conclusion

These results suggest that nobiletin inhibits PE-induced contractions of endothelium-denuded rat aorta by increasing cGMP levels via GC activation. Moreover, the present study indicates the possibility that nobiletin opened BK channels by a cGMP-related signal, and K_{ATP} channels by a cGMP-nonrelated signal in rat aorta.

Ischemic heart disease is one of cardiovascular diseases, a condition in which blood is not supplied to the myocardium due to the presence of stenosis or obstruction in the coronary artery. Nitrite (NO donor), calcium antagonist and β-adrenergic antagonist have been used for treatment of ischemic heart disease. Nitrite relaxes coronary artery due to increases cGMP level via activating guanylate cyclase in vascular smooth muscle.

Therefore, it is suggested that the possibility that nobiletin will be used directly or adjunctively for the treatment and prevention of cardiovascular diseases. Furthermore, in small dogs, nitrite is often used in combination with a diuretic when treating pulmonary edema due to congestive heart failure. This study also suggests the possibility that nobiletin can be used directly or adjunctively as a therapeutic agent in severe cases of congestive heart failure in small dogs.

This study shows for the first time that nobiletin directly relaxes vascular smooth muscle and clarifies its mechanism. These findings indicate that nobiletin will be potentially tools to control cardiovascular diseases and blood pressure, which affect not only humans but also small dogs. From the viewpoint of veterinary nurses who are greatly involved in these management, it seems that this study is significance in the field of veterinary nursing and technology, since the management of appropriate blood pressure in daily life is greatly involved in the prognosis of cardiovascular diseases.

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Structural and Functional Analysis of Canine Isocitrate Dehydrogenase 1

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Isocitrate dehydrogenases (IDHs) catalyse the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG), and produce NAD (P) H from NAD (P)$^+$. Eukaryotes have both NAD$^+$- and NADP$^+$-dependent IDHs. IDH3 is an NAD$^+$-dependent IDH localized to the mitochondrial matrix and plays a central role in aerobic energy production in the tricarboxylic acid (TCA) cycle. IDH1 and IDH2 are both NADP$^+$ dependent, but IDH1 localizes to the cytoplasm and peroxisomes of mammalian cells, whereas IDH2 localizes to the mitochondrial matrix. A genome-wide mutation analysis of human cancers revealed predominant somatic mutations in the gene encoding IDH1 in secondary glioblastoma (GBM). Subsequent studies of targeted IDH1 gene sequencing confirmed this finding, identifying IDH1 mutations in over 70% of secondary GBM or low-grade gliomas, but infrequently in primary GBM (approximately 5%). All the IDH1 mutations were identified at the 132nd amino acid (aa) residue (R132). In addition, IDH1 R132 mutations have been identified in acute myeloid leukaemia (AML), but rarely reported in human breast and colorectal cancers. Metabolite profiling analysis showed that IDH1 R132 mutants induced the reduction of α-KG and increased 2-hydroxyglutarate (2-HG) production while converting NADPH to NADP$^+$. 2-HG is known to accumulate in the inherited metabolic disorder 2-hydroxyglutaric aciduria. The elevated level of 2-HG in the brain increases reactive oxygen species, leading to a variety of downstream sequelae.

Although intracranial tumours in dogs such as meningiomas and gliomas are relatively common brain diseases, their pathogenic mechanisms remain unclear. Limited sequencing analysis has been performed around IDH1 R132 but no mutations were identified in canine gliomas. However, a partial canine IDH1 protein sequence (GenBank ABD77219.1) showed a high homology to human IDH1 R132 sequences. Thus, we hypothesised that canine IDH1 is metabolically active in the isocitrate-α-KG conversion process and that mutations involving R132 induce metabolic abnormalities. In a human study, IDH mutations were identified by immunostaining with specific antibodies, but there are no data to support cross-reactivity of human antibodies with canine proteins.

In the present study, we cloned and sequenced the complete open reading frame (ORF) of canine IDH1 (cIDH1) homologue, and induced R132 mutations in cIDH1. We also validated cross-reactivity between human antibodies and cIDH1 mutants. Furthermore, isocitrate dehydrogenase activity between wild type (WT) and mutant cIDH1 in HeLa cells was compared, and HIF-1α expression in these cells was detected.

The ORF of cIDH1 cDNA determined in this study (GenBank LC214936) had 1245 base pairs (bp) and was predicted to code for 414 amino acids.

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The full-length ORF of canine IDH1 has the same sequence length as that of human and murine IDH1 (GenBank NM_005896.3 and NM_001111320.1). A putative isocitrate dehydrogenase sequence was discovered at amino acid residues 11 to 399, suggesting that cIDH1 has isocitrate dehydrogenase activity. The cIDH1 protein shows 96% and 94% homology with the human and murine IDH1 proteins, respectively. Canine IDH1 includes an Arg132 residue identical to that present in human and murine IDH1. The antibodies against R132H and R132S could specifically detect the R132H and R132S transfectants, respectively, in both HeLa and MDCK cells. To further confirm the cross-reactivity of human anti-IDH1-R132H and -R132S, we performed immunocytochemical analyses using canine IDH1-WT, -R132H, or -R132S-transfected HeLa cells. Results showed that anti-IDH1-WT antibody reacted with IDH1-WT-transfected cells. Anti-IDH1-R132H(S) antibody did not react with IDH1-R132S(H)-transfected cells and anti-IDH1-R132H(S) antibody specifically reacted with IDH1-R132H(S)-transfected cells. These findings indicated that both anti-IDH1-R132H and anti-IDH1-R132S are mutation-specific antibodies for canine immunocytochemistry. The mutated IDH1 proteins lose normal catalytic activity for α-KG- and produce less NADPH. Instead, the abnormal enzymatic activity produces 2-HG and consumes NADPH. Therefore, we investigated the formation of NADPH and NADH in multiple types of cIDH1-overexpressing HeLa and MDCK cells by using colorimetric analysis. There were no differences in NADH production between any cells expressing IDH1 WT, R132H, R132S, and the empty vector (negative control). The productivity of NADPH was significantly different between WT, R132H, and R132S in both HeLa and MDCK cells. By western blot analysis, we investigated the induction of HIF-1α protein expression caused by IDH1 mutations. The expression of HIF-1α protein was detected in the WT, R132H, and R132S transfectants, showing increased levels of the protein carrying either the R132H or R132S mutations.

In this study, we cloned, sequenced, and analysed the function of the full-length canine IDH1 ORF, for the first time. Primers for cIDH1 amplification were designed using EST data. Compared to human and murine IDH1 proteins, canine IDH1 showed a highly conserved sequence and primary structure. Isocitrate dehydrogenase domains occupied a large proportion (approximately 80%) of canine IDH1 protein and are well conserved among species (e.g. human and mouse); hence, we predicted that canine IDH1 has isocitrate dehydrogenase activity. The antibodies against human IDH1 R132H and R132S mutations were able to specifically detect cIDH1 mutations in both immunoblotting and immunocytochemistry analysis. We expect that these antibodies can also be used for detection of R132 mutations in canine tissues, and we will perform histopathological analysis against canine gliomas and other tumour tissues in our future studies. The cIDH1 R132H and R132S mutants showed a reduction in NADPH production, whereas NADH production was unchanged. The decreased production of NADPH by the expression of cIDH1 R132 suggests that the R132 mutation in dogs induces 2-HG accumulation and causes tumourigenesis. To validate this prediction, in future experiments we will measure the production of 2-HG in cells expressing cIDH1 mutants. In our study, HIF-1α protein expression was increased in both R132H and R132S transfectants of HeLa and MDCK cells, and the NADPH production ability of the R132H and R132S mutants was lower than that of the WT. These results suggested that both relationships exist. Although NADPH products were reduced in the R132 mutants of canine IDH1 transfectants of both human (HeLa) and canine (MDCK) cells, HIF-1α protein expression was slightly increased.

The mechanisms by which cIDH1 mutations cause canine gliomas and other tumours remain unclear, but the findings reported in this study will make it easier to elucidate the relationship between cIDH1 mutations and tumour formation.

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Analysis of cholesterol lipoprotein separations in dogs with hyperadrenocorticism by anion-exchange high-performance liquid chromatography

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Introduction

As a key component of cell membrane, cholesterol plays a role of precursor of steroid hormone and the likes. Due to its water-insoluble property, lipid including T-Cho and TG exists in a form of lipoprotein in the blood by synthesis with apolipoprotein.

Lipoprotein is classified into chylomicron (CM), very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) and its metabolism is associated with transfer proteins in addition to enzymes. As its abnormality may lead to dyslipidemia to cause arteriosclerosis, hormone abnormalities are associated with the background of the disease.

While conventional classification methods of human lipoprotein include precipitation method, ultracentrifugation method, gel filtration technique, immunochemical assay, electrophoretic method, and anion-exchange high performance liquid chromatography (AEX-HPLC), lipoprotein fraction based on AEX-HPLC method has been developed in recent years.

Characteristics of AEX-HPLC method include a way to classify lipoprotein by number of electric charges on the surface of lipoprotein as well as ability to detect intermediate density lipoprotein (IDL).

IDL causes vascular endothelial disorder with stronger degree than LDL. Vascular endothelial disorder may increase a risk of arteriosclerosis. Measurement of lipoprotein fraction using AEX-HPLC method with a capability of IDL analysis is expected to enhance significance of canine dyslipidemia diagnosis.

Secondary hyperlipidemia in dogs is caused by hypothyroidism, hyperadrenocorticism, diabetes, pancreatitis, hepatic dysfunction, nephrotic syndrome, and iatrogenic disorder. In addition to a report on a case of dog with hyperlipidemia developing hypothyroidism that atherosclerosis was also recognized, there are also reports that hyperadrenocorticism is associated with thrombosis. As of now, however, there are many unclear aspects in canine lipoprotein fraction and only a few reports have been made on it.

Therefore, we have compared lipoprotein fraction between healthy and hyperadrenocorticism dogs as well as determined severity of hyperlipidemia with examination of lipoprotein metabolism based on AEX-HPLC method in the current study in addition to basic consideration and confirmation of reliability of dog lipoprotein fraction.

Chapter 1.
Basic Consideration and Confirmation of

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Reliability of Lipoprotein Analysis by Canine Serum Based on AEX-HPLC Method

Basic consideration was performed on within-run and between-run reproducibility, dilutional linearity, and correlation with gel filtration chromatography which is regarded as a conventional analysis method of canine lipoprotein. Serum of healthy dog was used as a material for the basic consideration.

Preferable result was obtained in within-run and between-run reproducibility with CV values of 10% or less in all items. Dilutional linearity also showed preferable result in all items with r value of 0.97 or larger.

Linearity of correlation with gel filtration chromatography showed a result with r value of 0.93 or larger. However, divergence was observed in a result between part of LDL and VLDL. In case of hypercholesteremia, divergence may have been caused by measurement of LDL, VLDL and CM together in a cluster based on gel filtration chromatography.

Lipoprotein analysis based on AEX-HPLC method is believed to be excellent from a result.

Chapter 2.

Lipoprotein Fraction Comparison Between Healthy and Hyperadrenocorticism Dogs Based on AEX-HPLC Method

Basic range of each fraction based on AEX-HPLC method was set using blood of 40 healthy dogs. Normal distribution was recognized in total cholesterol, HDL and LDL.

Reference value based on average ± standard deviation for T-Cho, HDL, LDL, IDL and VLDL was 202.0 ± 55.3 mg/dL, 162.2 ± 31.6 mg/dL, 30.5 ± 21. 4 mg/dL, 1.9 ± 1.3 mg/dL, and 1.2 ± 0.3 mg/dL, respectively. Healthy dogs resulted in high density in HDL and low density in LDL and VLDL, showing results similar to those of gel filtration chromatography.

Comparison with reference range was performed for 12 dogs suffering from hyperadrenocorticism. VLDL, IDL and LDL increased by 12.6, 4.2 and 3.3 times, respectively, compared with the reference range.

Increased synthesis and insufficient anabolic reaction under insulin resistance were believed to be a cause of lipoprotein increase in VLDL in hyperadrenocorticism dogs.

Similarly to VLDL, lipoprotein increase in IDL was believed to have been caused by reduced catabolic reaction under insulin resistance. In case of LDL, it was believed to have been increased indirectly along with increased VLDL.

In consideration of increased LDL and IDL in hyperadrenocorticism dogs, association with arteriosclerosis and thrombosis is believed to be highly possible.

According to a criterion of Japanese health care, 170 mg/dL or higher non-HDL (LDL + IDL + VLDL) is a judgment value to recommend screening. As a result of applying the judgment value for screening recommendation to those measured in hyperadrenocorticism in the study, 8 out of 12 dogs fell under the judgment value. From those described above, it has been suggested that high cholesterol dog with hyperadrenocorticism may be at risk of possible arteriosclerosis similarly to human.

It is believed that lipoprotein fraction test for dogs based on AEX-HPLC method is useful to evaluate risks caused by LDL and IDL as well as to select treatments for hypercholesteremia and to evaluate the effects.

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Functional Analysis of p53 Mutation in Canine Mammary Tumor-derived Cell Lines

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The tumor suppressor protein p53 plays a central role in the cell cycle and maintenance of genomic integrity. The p53 gene is frequently mutated in human cancers, and germline mutations are the underlying cause of Li-Fraumeni syndrome. The human p53 protein is comprised of an N-terminal transactivation domain, a proline-rich region, a structured DNA-binding domain connected to a tetramerization domain (TD) via a flexible linker, and a C-terminal regulatory domain. The p53 protein is active as a homotetramer which adopts a dimer of dimer topology. The TD in human p53 (amino acid residues 326–356) exhibits a dihedral symmetry of dimers. Two monomers interact with the β-strands (Glu326-Arg333) to form a dimer, and the two dimers then interact as a α-helix bundle (Arg335-Gly356) to form the tetramer. Nine residues in the TD in human p53 (Phe328, Leu330, Ile332, Arg337, Phe338, Met340, Phe341, Leu344 and Leu348) are critical determinants in stabilizing the p53 tetramer. Leu344 mutants lacking the ability to dimerize (L344P), or tetramerize (L344R), have been previously reported, and notably, germline L344P mutation has been found in a family with Li-Fraumeni syndrome. Leu344 is located in the α-helix which forms the hydrophobic core of the tetramer interface.

Although, numerous cell lines containing mutated p53 genes have been established from cancer tissues, there are few cell lines which contain a mutated p53 tetramerization domain. Uyama *et al* established and characterized four pairs of canine mammary tumor cell lines derived from either primary and metastatic origin. One of these cell lines, CTB-m, was passaged 50 times in our laboratory to obtain a line containing a spontaneous L332Q mutation in p53 (corresponding to human L344); we designated this new cell line as CTB-m2. In this study, we assessed the oligomerization abilities of this canine p53 L332Q mutant and performed a tetramerization reporter assay.

Sequencing analysis of genomic p53 from the canine mammary gland tumor cell line CTB-m2 showed the presence of a heterozygous missense mutation L332Q, compared to CTB-m cells, which expressed homozygous L332. However, the sequence of p53 mRNA derived from CTB-m2 cells showed almost exclusively the mutated allele (L332Q). A comparison of a part of the tetramerization domain of the canine p53 protein (GenBank accession: BAJ72203.1) with human, bovine, murine and Xenopus p53 protein (NP_000537.3; NP_776626.1; NP_03570.2; NP_001081567.1) showed that canine Leu332 corresponds to human Leu344, and that this sequence is completely conserved in other species. A cell proliferation analysis was performed using an automatic cell counter every 24 h after cultivation. Seventy-two hours after cultivation, CTB-m2 cell proliferation was found to be

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significantly higher than CTB-m cell proliferation. The p53 tetramer response element, along with the human cytomegalovirus minimal promoter sequence, were cloned upstream of the sequence encoding Nano Luc, and the resulting plasmid was transfected into CTB-m or CTB-m2 cells, or into HeLa cells also expressing exogeneous p53 WT (wild-type) or its L332Q mutant, in order to assess endogenous or exogenous p53 tetramerization. The L332Q heterozygous mutant CTB-m2 had an approximately 50-fold reduction in luciferase activity compared to WT CTB-m cells. Forced expression of the canine p53 L332Q mutant in HeLa cells also showed significantly lower luciferase activity compared to forced expression of WT p53. The oligomerization ability of p53 transfected into HeLa cells was assessed using a glutaraldehyde cross-linking assays. Cell lysates from HeLa cells expressing HA-tagged WT p53 or HA-tagged p53 L332Q were treated with 0.02 and 0.04% glutaraldehyde crosslinker and then analyzed by western blotting. The HA-tagged WT p53 formed both dimers and tetramers following glutaraldehyde treatment. On the other hand, the L332Q mutant formed only a dimer. Thirteen microsatellite loci were compared among the CTB or CIP canine mammary gland tumor cell lines. Two microsatellite loci between the CTB-m and CTB-m2 cell lines were different, while the other 11 were identical. Five microsatellite loci between the CIP-p and CIP-m cell lines were different, while the other 8 were identical.

The functions of the tetramerization domain in the p53 protein and its inhibitory and stabilizing ligands have been well investigated using biochemical and cell biological analysis. Here, we established the cell line CTB-m2 which expresses the p53 L332Q (which corresponds to L344 in human p53) mutant. Microsatellite analysis using 11 markers showed that in CTB-m cells, which are the parental cells for CTB-m2, are nearly identical. Despite being genetically heterozygous at the nucleotides encoding L332, CTB-m2 cells expressed almost only the mutated L332Q p53 mRNA suggesting that in these cells the wild-type p53 allele is inactive. We examined the tetramerization ability of the p53 L332Q mutant using a functional reporter assay. Two p53 response elements and the minimal human cytomegalovirus promoter sequence, were modified and cloned into a vector containing a luciferase reporter. The large difference in luciferase activities observed between CTB-m and CTB-m2 cells, which reflects the endogenous tetramerization abilities of p53 in these two cells. Although there was a significant attenuation in tetramerization for the p53 L332Q mutant, the dimer forming ability of this mutant was retained, as shown by electrophoresis and immunoblotting of p53 cross-linked with glutaraldehyde.

In summary, we established and characterized a new cell line, CTB-m2, which expresses p53 L332Q, a mutant in the p53 tetramerization domain. As a result of this mutation, p53 L332Q lacked tetramerization but not dimerization ability. These CTB-m2 cells can be used to investigate p53 pathogenesis and to evaluate new strategies to restore p53 function.

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Effects of 5-HT_{1A} agonist treatment during the neonatal period on abnormal behaviors in *15q dup* mice with autistic traits

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Autism Spectrum Disorder (ASD) refers to a group of neurodevelopmental disorders characterized by social communication disabilities and restricted, repetitive patterns of behavior. ASD were first reported in 1943 and, despite many decades of research, remains poorly understood. Thus, there is a great societal need for investigations of the pathological mechanisms of ASD and effective treatments. Copy Number Variations (CNVs) are thought to be one potential cause of ASD. Human chromosome 15q11-13 is one of the CNV loci that is frequently observed in ASD. Mice chromosome 7 corresponds to human chromosome 15q11-13. We investigated the effects of an early intervention drug treatment for ASD using a mouse line with duplication of chromosome 7 as a ASD model of 15q11-13 duplication (*15q dup*). *15q dup* mice show abnormal behaviors related to autism and abnormally low brain serotonin (5-HT) levels. Previous reports revealed that treatment with serotonin reuptake inhibitor or 5-HT_{1A} receptor agonists resulted in recovery of social behavior. However, it is still unknown whether the 5-HT_{1A} agonist can affect other behaviors. In this report, we investigated the effects of neonatal 5-HT_{1A} receptor agonist treatment on abnormal behaviors in *15q dup* mice with autistic traits.

Male *15q dup* mice were treated with a 5-HT_{1A} agonist, (R)-(+)-8-hydroxy-2- (di-n-propylamino) tetralin hydrobromide (8OH-DPAT) beginning post natal day (PND) 7 to 21 via S.C. After 9 weeks, the mice were observed during an open field test, rotarod test, acoustic startle response (ASR) test, and fear conditioning test. We used 23 Wildtype(WT)-saline, 24 *15q dup*-saline, 26 WT-DPAT, and 23 *15q dup*-DPAT mice.

We observed a genotype effect during the open field test, in center time. The *15q dup* mice showed anxiety-related behaviors, as previously reported. Clear changes in response to DPAT treatment were not observed. During the rotarod test, a previous report found that *15q dup* mice showed significantly longer time spent on the rod; however, we were unable to replicate these effects. Although the full effects of DPAT treatment were not determined because repetitive behaviors were not observed during this test, DPAT treatment did not appear to affect motor coordination in *15q dup* mice.

During the ASR test, *15q dup* mice displayed a significantly larger duration response to a 110dB tone. This might mean that *15q dup* mice showed autism-like acoustic hyperesthesia. On the other hand, this response was not clearly changed by DPAT treatment in *15q dup* mice. During the 120dB tone ASR test, there were no significant differences between the WT-saline and *15q dup*-saline mice. This might mean that startle responses reached saturation, therefore significant differences were not observed. There was significant difference between the WT-DPAT and

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15q dup-DPAT mice in that the *15q dup*-DPAT mice showed significantly larger response durations, compared to WT-DPAT mice. We are uncertain as to the reason for this. Our results suggest that DPAT treatment during the neonatal period did not prevent acoustic hyperesthesia.

In the Fear conditioning test, we observed no between-group differences in freezing during the contextual test. Considering the hippocampus plays an important role in contextual memory, these data suggest that hippocampal function was not impaired in *15q dup* mice. In addition, there were no changes observed in the DPAT treatment groups. This suggests that DPAT treatment during the neonatal period did not affect contextual memory.

In contrast, during the cued memory test, freezing gradually decreased day-by-day. This suggests a strong retention of cued memory. Moreover, compared with other groups, *15q dup*-saline mice showed higher freezing through the test period. Especially 3 months later, freezing the *15q dup* mice had significantly more freezing than the WT-saline mice. We know the amygdala plays an important role in cued memory, and the amygdala is deeply involved in social behaviors while mediating facial and emotional recognition. Previous reports indicated that postmortem brain neurons were significantly decreased in patients with ASD. Taken together, it is possible that neuronal network mediated by the amygdala is disrupted in *15q dup* mice. On the other hand, *15q dup*-DPAT mice were not significantly different than WT-saline mice. These data indicate that abnormal freezing seen in *15q dup* mice was rescued by DPAT treatment during the neonatal period. Thus, *15q dup* mice showed excessive responses to aversive stimulation over time. Their responses could be rescued by DPAT treatment during the neonatal period.

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Establishment of hair evaluation method based on toy poodle

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Hair is one of the decisive characteristics of mammals. Dog hair-coat plays pivotal role to protect skin from the chemical stimulus, physical stimulus, microorganism. In addition, sensory organ, camouflage and temperature regulation are the function of hair in dogs.

To evaluate human hair, physiological parameters and their measuring methods have been established such as diameter, tensile strength, moisture uptake amount, density, heat resistance, electrical resistance, and etc. These parameters were used for studies on racial characteristics of hair, evaluating damage, growth rate etc. On the other hand, there are few studies on the dog hair, because the human hair measuring method has not been applied.

In the present study, we selected three hair-parameters, the diameter, tensile strength and moisture uptake amount for the basic index evaluating hair properties in dogs. The standardizing dog-hair measuring methods were estimated with applying the human-hair methods with coordinating the dog hair properties. Thereafter, standard values for abdominal regions were calculated. Approval for this study was granted by our institutional animal care and use committee.

Chapter 1. Establishment of Standardization Method for Measurement of Hair Coat in Dogs : the Diameter of a Hair Strand

Dog hair samples were collected from 30 dogs which came to Yamazaki Gakuen University. The breed was limited to the toy poodle. The subjects were 3-9 years-old and 14 males and 16 females. Hair-diameter, one of the hair-parameters, was examined to be applied to evaluate dog-hair properties. In the chapter one, the purpose was to establish a standard method to apply dog-hair.

A digital micrometer was used to measure hair diameter. Hair samples were collected from the seven anatomical regions (crown, left ear, withers, left chest, lower chest, end of left hind limb and tail).

The coarse and fine hair strands were easily recognized. Therefore, observation was done for each coarse hair and fine hair. In fine hair strand, there was no significant difference among regions. On the other hand, the diameters of the coarse hair on the withers and the tail showed significantly larger than one of any strand on the other regions.

The hair of the back, mainly the coarse hair-strand, may develop as a physical barrier, as the coarse hair-strands correspond to long hair length. Fine hair may be secondary hair. The function is thought to regulate the body temperature, as the thick and fine hair strands would work to keep warm air within the coat.

Chapter 2. Establishment of Standardization Method for Measurement of Hair Coat in Dogs : Study on Tensile Strength

Hair samples collected from the same Toy

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Poodle dogs in Chapter 1 was used. The hair tensile strength, which is one of the basic indicators for evaluating the properties of hair in humans, was examined. For measurement of tensile strength, a digital force gauge equipped with a flat chuck was used. After measuring the tensile strength, the hair-diameter of the separated portion was measured under a microscope to calculate the cross-sectional area.

The hair tensile strength was positively correlate to the cross sectional area. Among the anatomical sites, the withers region was the lowest accessed with the tensile strength per unit area. In regard to the ratio of the tensile strength to the cross sectional area, the coarse hair was lower than the fine hair.

The cortex consists of fibers and possesses high tensile strength. The medulla has the hollow structure which is weak in the tensile strength. The present result was considered to be due to the fact that the proportion of hair medulla is small in fine coat and the proportion of hair medulla is large in coarse coat.

The present result is considered to be correlate to the proportion of hair medulla because the coarse hair was smaller than the fine hair.

Chapter 3. Establishment of Standardization Method for Measurement of Hair Coat in Dogs : Moisture Uptake of Hair

Dog hairs were collected from 8 dogs (5 males, 3 females, 3-8 years of age). There were 4 toy poodles and 4 standard poodles. An infrared type moisture meter was used for measuring the moisture content. The measuring condition was heating at 70 ℃. for 10 minutes. In this condition, Standard poodle was significantly higher than toy poodle in the moisture content of hair coat.

We did not demonstrate any factors making the difference in water uptake between the two breeds and will be examined in the future.

Conclusion

Generally, it is thought that toy poodle have single coat. However, even at each anatomical site, there was a significant difference in hair diameter that distinguished main hair from secondary hair. In addition, the fine hair which seemed to be secondary hair had no significant difference in the hair diameter among the anatomical regions. This result was also suggested by histological specimens of the hair follicles. Therefore, toy poodle has double coat like other breeds, unlike the conventional concept.

In this study, a method for measuring physiological indicators to determine the properties of canine hair was established. We showed one breed, toy poodle, was examined on each anatomical site as a standard value. The present study is expected to play an important role in accumulating standard values on hair of other canine breeds and even different animal species.

In human basic nursing assistant skills, personal hygiene is one of the basic activities of daily living requiring nursing assistants. Specifically, bath assistance, partial baths, pubic care, cleansing / shampooing, oral care, cosmetic adjustment, and bedclothes exchange. This study is useful for accumulating physiological data to establish the basic nursing assistant skills. It contributes to the establishment of evidence of bathing assistance, partial bathing, cleansing / shampooing, and conditioning. It is essential for establishing and disseminating basic nursing assistant skills in the field of animal care.

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Role of nestin expression in canine mammary tumor progression

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Introduction

Nestin, a class VI intermediate filament protein, was originally described in 1990 as a neuronal stem cell/progenitor cell marker during central nervous system development. Subsequent study has shown that nestin is also expressed in immature or progenitor cells in non-neuronal cells in normal tissues. Furthermore, nestin is also expressed by various types of human neoplasms, including brain tumors, melanoma, and uterine, cervical, prostate, bladder, head and neck, ovarian, testicular, pancreatic, and breast cancers, and the nestin immunostaining significantly predicts poor survival in some neoplasms. However, expression and the roles of nestin in canine mammary tumors have not been clarified. In this study, I identified the expression of nestin in some canine mammary carcinomas in immunohistochemical and *in situ* hybridization analyses. Next, the correlations between nestin expression and several pathological parameters of mammary carcinomas were assessed. In addition, I examined the relationship between nestin and a mesenchymal marker vimentin expressions in mammary carcinoma tissues and culture cells. Furthermore, I investigated the roles of nestin through knockdown experiments of nestin using an RNA interference (RNAi) technique with a WST-8 cell proliferation assay and Boyden chamber cell migration assay.

Nestin expression in canine mammary carcinomas and the relationship with pathological parameters.

First, I confirmed that the anti-human nestin antibody permits detection of the canine nestin protein by western blot analysis.

By using of the antibody, expression of nestin was examined immunohistochemically in 116 canine mammary samples, including 9 hyperplasia, 30 benign tumors, 63 primary malignant tumors, and 14 metastasis of mammary carcinoma. In the results, all hyperplasias and benign tumors, no expression of nestin was observed in luminal epithelial cells. However, in 16/63 (25%) of the primary malignant tumors and 5/14 (36%) of the metastasis of mammary carcinoma examined, cytoplasmic expression of nestin was detected in luminal epithelial cells.

To provide further proof of the nestin expression in canine mammary carcinomas, I employed an *in situ* hybridization method with a probe specific for mRNA sequence of canine nestin. Dotted signals of nestin mRNA were detected in carcinoma cells that stained immunohistochemically with the antibody to nestin in serial sections.

It was assessed that the relationships between nestin expression and several pathological parameters of mammary carcinomas, including histological types, grades, vascular/lymphatic invasion, lymph node metastasis, and Ki-67 index. The frequency of nestin-positive complex carcinomas (3 of 22, 14%) was comparatively lower than that of simple carcinomas (7 of 23

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tubulopapillary, 30%; 4 of 10 solid, 40%; 1 of 1 anaplastic, 100%). In histological grading, 1 (6%) of 18 grade I, 8 (30%) of 27 grade II, and 7 (39%) of 18 grade III carcinomas were nestin-positive, and there was a significant positive correlation between S100A4 expression and histological grade ($P < 0.01$). Histologically, of the 63 carcinomas, 20 had vascular/lymphatic invasion and 11 (55%) of them expressed nestin, while 5 of the 43 (12%) carcinomas without vascular/lymphatic invasion were nestin positive. Nestin was expressed in 7 of 15 (47%) of the primary carcinomas that showed lymph node metastasis, and in 9 of 48 (19%) of the primary carcinomas that did not have lymph node metastasis. Statistical significant relationships were found between nestin expression and vascular/lymphatic invasion or metastasis ($P < 0.01$). The Ki-67 index was differ significantly ($P < 0.01$) between nestin-positive carcinomas (47.8 ± 26.3) and nestin-negative carcinomas (25.3 ± 15.4).

Fifteen (94%) of 16 nestin-positive carcinomas were also positive for vimentin and 10 (21%) of 47 nestin-negative carcinomas were positive for vimentin. Statistical significant relationship was observed between nestin and vimentin expressions in mammary carcinomas ($P < 0.01$). Double labeling fluorescent immunohistochemistry revealed that some carcinoma cells co-expressed nestin and vimentin.

Nestin expression in canine mammary carcinoma cells and the investigation of role of nestin by RNA interference technique.

By real-time reverse transcription-polymerase chain reaction (RT-PCR), I examined the nestin and vimentin expression levels in nine cell lines from canine mammary carcinoma, including CIPp, CIPm, CTBp, CTBm, CNMp, CNMm, CHMp, CHMm, and NV-CML. Nestin expressed in these nine cell lines in varying degrees and the similarity between nestin and vimentin expression patterns was noted.

Finally, we performed knockdown of nestin using the siRNA technique in CIPp cells that had the highest expression level of nestin and evaluated whether nestin knockdown influenced cell proliferation and migration. SiRNA specifically targeting canine nestin mRNA (siNES) or a siRNA that does not bind to any canine RNA (siNeg) as a negative control were transfected to CIPp cells that had the highest expression level of nestin. Nestin mRNA levels were determined by real-time RT-PCR. The expression level of nestin mRNA in CIPp cells transfected with siNES was decreased to approximately 36% of that with siNeg. By immunocytochemistry, fluorescent intensity for nestin protein in siNES-transfected cells was higher than that in siNeg-transfected cells.

First, cell proliferation after 5 days culture was evaluated by a WST-8 assay. The absorbance measured after treatment with WST-8 reagent was significantly lower in siNES-transfected cells than in siNeg-transfected cells ($P < 0.05$). Next, a cell migration assay was conducted using the Boyden chamber technique. The NES-transfected cells migrated more slowly than the siNeg-transfected cells, and the number of migrating cells in the siNES-transfected cells was significantly fewer by comparison with that in the siNeg-transfected cells ($P < 0.01$). Therefore, nestin at least partially seems to mediate the proliferation and migration of canine mammary carcinoma cells.

Conclusion

Nestin expressed in some canine mammary carcinomas, whereas not in all benign proliferative lesions. Nestin expression in mammary carcinomas was positively correlated with several pathological parameters that indicated high-grade malignancy, including the elevated histological grades, the presence of vascular/lymphatic invasion or metastasis, and high Ki-67 index. Nestin expression was also correlated to vimentin expression in canine mammary carcinomas, suggesting that nestin may relate to epithelial-to-mesenchymal transition of carcinomas.

Cell proliferation and migration were significantly decreased in nestin-downregulated cells compared with control cells. These findings suggest that nestin may be related to progression

of canine mammary carcinomas, via its influence on cell growth and motility; thus, nestin may be a new potential therapeutic target.

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Attempts of cell differentiation and proliferation of mouse eosinophils with cytokines in vitro

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Introduction

Leukocytes mainly include monocytes, lymphocytes, eosinophils, neutrophils and basophils. Among them, eosinophils are described as about 2% of leukocytes in human peripheral blood and about 1% in mice in a textbook, and are extremely small.

The differentiation process of eosinophils is that eosinophil progenitor cells (EoP) derived from bone marrow progenitor cells becomes mature eosinophils via granule-containing eosinophil progenitor cells (preEos). EoP expresses interleukin-5 receptor (IL-5R) on the cell surface and has proliferative capacity. While maintaining the expression of IL-5R, the EoP differentiates to the preEos, and then starts expression of carbohydrate-binding protein Siglec-F on the surface. It is known that preEos becomes mature eosinophil with expression of CCR3 on the cell surface.

It is also known that IL-5, a cytokine, promotes differentiation and proliferation into mature eosinophils in mice and humans, and is also involved in maintaining the survival of eosinophils. As cytokines other than IL-5, IL-3, and granulocyte macrophage colony stimulating factor (GM-CSF) have been shown to induce the formation of colonies containing eosinophils from bone marrow progenitor cells. IL-5R is composed of α chain and β chain, and β chain is common with β chain of IL-3R and GM-CSFR. The β chain is thought to play a role for transmitting necessary signals in eosinophilic increase and tissue invasion. In addition, as eosinophils were still present in IL-5KO or IL-5RαKO mice, it is suggested that eosinophil differentiation may also be performed by molecules other than IL-5.

Eosinophils are important effector (working) cells involved in allergic diseases and parasitic infections. Eosinophils have protein molecules such as major basic protein (MBP) in cytoplasmic granules. Because they have the property of promoting inflammation, tissue degeneration occurs by eosinophil degranulation. It has also been reported that FcεRI expressed on mast cells and basophils is also expressed on eosinophils of human patients with eosinophilia, but it is unknown whether FcεRI can also be expressed in mouse eosinophils.

On the other hand, eosinophils also play an important role during parasitic infections. Larger parasites such as helminths can not be engulfed by phagocytic cells such as macrophages and neutrophils. Instead, eosinophils are known to be responsible for elimination of helminths, but there are still many unclear points in their mechanism. Therefore, in this report, we tried to find out the optimal culture conditions for eosinophils to efficiently differentiate and proliferate, and to find out new molecules other than IL-5. Using eosinophils grown by in vitro culture, we decided to examine their functions as well.

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Materials and Methods

(1) Determination of normal eosinophilc value: peripheral blood cells and peritoneal cells from BALB/c and C57BL/6 mice were collected, and the proportion of each cell population was determined using a flow cytometer (FACS). (2) Production and recovery of cytokines: a retroviral vector pIG expressing a number of cytokines such as IL-5, IL-13, IL-15, IL-33, G-CSF, and GM-CSF was transfected into a virus-packaging cell, Plat-E. Then, the recombinant virus in the culture supernatant was infected into the established cell WEHI-231, and the culture supernatant containing the cytokine was recovered. (3) Culture with cytokine: cells were collected from peripheral blood, peritoneal cavity, bone marrow in mice, and parasite-infected mice, and then the supernatants recovered as above were added 20% in the final concentrations and cultured. Bone marrow cells were also cultured with commercially available rIL-5. (4) FACS analysis: Siglec-F, Gr-1, CCR3, CD3, CD19, FcγR and FcεRI were used as cell surface markers. (5) Degranulation: cells were stimulated with calcium ionophore A23187, and the degranulation was examined using FACS. (6) Binding to nematodes: cultured eosinophils and nematode *Caenorhabditis elegans* were mixed, and anti-*C. elegans* serum was further added to observe the binding between eosinophils and nematodes.

Results and Discussion

(1) First, the normal cell proportion in mice was determined. Eosinophils (SiglecF^{+}) were 0.3-2.7% in peripheral blood and 1.3-7.9% in the peritoneal cavity. (2) IL-5 was used to induce differentiation from peripheral blood cells, peritoneal cavity cells, and bone marrow cells. From peripheral blood cells and peritoneal cells, eosinophils increased to about 4% on day 4-5 in BALB/c mice. From bone marrow cells, eosinophils increased to about 49% on day 11. Thus, the induction of eosinophils with IL-5 was more strongly observed in bone marrow cells than peripheral blood cells and peritoneal cells. Consequently, it seems that the reactivity to IL-5 depends on immaturity of the cells. (3) Next, we examined whether to promote further eosinophilic differentiation from bone marrow cells by combining IL-5 with other cytokines. As a result, it was shown that the cells were remarkably proliferated by mixing IL-5 and IL-13 (IL-5+IL-13) more than IL-5 or IL-13 alone. (4) Therefore, a large-scale cell culture was carried out to investigate cells which increased with IL-5+IL-13. However, the proportion of eosinophils was the rather highest at 32.5% in IL-5 alone. At the same time, in the FS/SS (cell size/internal structure) plot of FACS, the fraction which does not correspond to eosinophils is increased in culture with IL-5+IL-13 compared with IL-5 alone. (5) Furthermore, bone marrow cells were also cultured with commercially available rIL-5, and the properties of eosinophils were investigated. First we attempted degranulation of eosinophils with calcium ionophore. As a result, SS value decreased from 736 to 694, indicating that degranulation can be induced for cultured eosinophils. (6) Expression of FcεRI, FcγR and CCR3 on the cell surface of eosinophils was examined, and expression of FcγR and CCR3 was confirmed, but expression of FcεRI was not observed. That is, the possibility of binding to and attack target via IgG-FcγR was shown. From the above observation, it was suggested that the eosinophils obtained here are mature enough, suggesting that eosinophilic differentiation proceeds roughly or largely even with IL-5 alone, and it seems possible to investigate the function of eosinophils using them.

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Effects of various phosphates on muscle proteins and muscle contraction

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The muscles of livestock animals are hardened after slaughter because of rigor mortis. This is induced by the muscle contraction which consume residual adenosine-5'-triphosphate (ATP). The resolution process of the rigor mortis is called aging, through which the muscle is sufficiently tenderized to be bitten off by human jaw, turning into meat. In the previous studies, it was shown that disintegration of the structure of contracted myofibrils and connective tissues bundling myofibers by endogenous proteases is a major cause of meat tenderization. On the other hand, weakening of actin (A) -myosin (M) interaction was thought to be related to tenderization of meat during aging, but its detail has not been clarified. The purposes of this study are to reveal the effect of various phosphates on the A-M interaction and muscle protein structures and to elucidate the softening mechanism during meat aging.

1. Effects of various phosphates on actomyosin

When actomyosin (AM) was applied to a Sepharose CL-2B column equilibrated with a 0.6 K solution (20 mM Tris-HCl (pH 7.2) / 2 mM NaN_3 / 0.6 M KCl), the M heavy chain and the A monomer were eluted in fraction No.16 together. Therefore, AM was not shown to be dissociated under these conditions. When AM treated with 8 mM pyrophosphate (PYP), triphosphate (TRP) or inosine-5'-monophosphate (IMP) in the presence of 0.6 M KCl was applied to a column equilibrated with 0.6 K solution containing each phosphate, M heavy chain and A monomer detected in fraction No.24 and No.34, respectively, but little protein was detected in fraction No.16. These results indicate that AM is dissociated into A and M by various phosphates. In the presence of 0.2 M KCl, it was known that PYP and IMP are able to dissociate AM into A and M, and TRP solubilizes the whole AM without dissociation (Sawada, 2015), but the present result was different. The presence of 0.6 M KCl was presumed to cause a structural change of AM and to enhance the dissociation of AM induced by TRP. PYP and TRP should similarly act on AM of meat in the presence of 3% (about 0.6 M) NaCl which is necessary for producing hams and sausages, resulting in enhancement of the binding property of meat products. The present study showed that dissociation of AM caused by various phosphates would contribute to the production of meat products from the viewpoint of molecular weight change for the first time. In addition, IMP was suggested to increase the binding ability by dissociating AM to A and M when IMP is used in place of PYP or TRP which are conventionally used for the production of hams and sausages.

2. Effects of various phosphates on myofibrils

Myofibrils incubated in 0.2 K solution (20 mM Tris-HCl (pH 7.2) / 2 mM NaN_3 / 0.2 M KCl) containing 8 mM PYP or TRP were centrifuged and the resulting supernatant was applied to a Sepharose

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CL-2B column. The M heavy chain and the A monomer were detected together in fraction No.16, indicating that PYP or TRP solubilized AM without dissociation. AM is not dissolved in 0.2 M KCl, but actin and myosin is dissolved in 0.2 M KCl. Sawada (2015) conducted an experiment using this difference and assumed that A and M were liberated from myofibrils by PYP and TRP. However, the present study revealed that whole AM was solubilized by PYP and TRP without dissociation. Moreover, PYP and TRP could release AM from particular restraints (probably Z line) in myofibrils, but AM liberated from myofibrils by those phosphates were not dissociated into A and M, suggesting that they cannot degrade another binding like a hoop.

Using a similar method, it was shown that when myofibrils were treated with 8 mM IMP in the presence of 0.2 M KCl, no protein was detected in any fractions eluted from a Sepharose CL-2B column, indicating that nothing was released from myofibrils.

These results showed that PYP and IMP dissociate AM in the presence of 0.6 M or 0.2 M KCl, while TRP dissociates AM only in the presence of 0.6 M KCl.

3. Effect of IMP on contraction of skinned fiber

In skinned fibers (SFs), myofibrils containing AM were incorporated in the structure as bundles and those cell membranes are punctured. Therefore, those intracellular fluid and enzymes are lost. On the other hand, since the structure such as the sarcoplasmic reticulum surrounding the myofibrils are maintained, SFs are contracted / relaxed by addition and removal of Ca^{2+}. Investigation of the change in a tension of SFs enables us to examine how various phosphates influence structures closer to meat than AM and myofibrils. SFs were prepared by the treatment of various muscles (diaphragm, tibialis anterior, soleus and extensor digitorum longus (EDL)) from mice or rats with 30 μM surfactant saponin in PIPES buffer having physiological ionic strength (0.2 M KCl). The tension changes of these SFs in the relaxing solution (4.46 mM ATP / 5.54 mM $MgSO_4$ / 108.6 mM propionic acid / 20 mM NaN_3/20 mM PIPES / 1 mM EGTA (pH 7.0)) or the contracting solution ($CaCl_2$ added to relaxing solution to pCa 4.5) was investigated. As a result, SFs obtained from EDL were most clearly contracted and relaxed. Thus, they were adopted as a new experimental system.

Immersing the contracted SFs in the contracting solution in which ATP was replaced with IMP did not decrease the tension. However, when ATP was partly replaced (2 mM ATP + 4.46 mM IMP) in the solution, the tension decreased. This result suggests that IMP attenuates AM binding only in the presence of ATP in SFs. It is conceivable that some factors included in SFs but not myofibrils limit the action of dissociating AM by IMP. If these factors can be clarified in the future, the SF experimental system might be useful to elucidate the softening mechanism during meat aging. In addition, these results suggest that relaxation by IMP and ATP in the presence of Ca^{2+} like post mortem muscles occurs in a mechanism different from ATP-induced relaxation in the absence of Ca^{2+} in live muscle. The obtained results in the present study support the presumption that IMP is a candidate for factors of rigor resolution during meat aging (Matsuishi et al., 2016).

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Analysis of IgE production mechanisms by administration of the nematode *C. elegans* into mice

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Introduction

The hygiene hypothesis is still being debated as a cause of a significant increase in allergic diseases in the past 30 to 40 years. The hygiene hypothesis is the theory that the degree of exposure to environmental microorganisms in early childhood has some influence on the immune system, which in turn affects sensitivity to allergic diseases. In addition, infectious diseases have decreased due to the development of medical treatment in recent years, and a change of symbiotic microorganisms including intestinal bacteria due to the use of antibiotics might have transformed the natural development of the immune system. These alterations are also considered to contribute to the increase of allergic diseases.

Parasitic infections may also be mentioned as recently decreased infectious diseases. Parasites are classified as unicellular protozoa and multicellular helminths, and helminths are further classified as nematodes, trematodes, and cestodes. Worms in helminth infection, as they migrate tissues in the human body, elicit so-called type 2 immune responses in which IgE, eosinophils, and mast cells increase, thereby promoting worm expulsion. *Caenorhabditis elegans*, a type of helminth, is a non-parasitic nematode that lives mainly in the soil and has a body length of about 1 mm. Since the whole genome sequence has been determined and it is easy to keep it in the laboratory, it is widely used as an experimental material in fields such as embryo engineering. Previous studies revealed that IgE is produced when *C. elegans* is administered to mice, indicating that *C. elegans* can also induce a type 2 immune response with IgE production as parasitic nematode infection dose. Thus, the administration of *C. elegans* which can induce a type 2 immune response can be used as a model of parasitic infection and it should be described that there is a possibility of obtaining a hint for exploring the mechanism of IgE production by parasite infection. However, it is still unknown how IgE production is induced, whether IgE produced recognizes *C. elegans*, and which constituent of the worm induced IgE.

In this study, we aimed to elucidate particular immune cells that recognize and react to *C. elegans*, and a pathway of immune responses leading to IgE production.

Materials and methods

C. elegans were cultured and grown on NGM agar coated with *Escherichia coli*, then treated at 37℃ in the presence of gentamicin, killed and stored frozen and used in the experiment. Mice mainly used were BALB/c strains of 9 to 16 weeks old. First, 5,000 to 50,000 *C. elegans* were administered to mice every 7 days. In addition, blood was collected immediately before the administration and the blood IgE concentration was measured each time, and changes in the IgE concentration

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were compared according to the number of administered worms or the number of times of administrations. Furthermore, splenocytes from mice, into which *C. elegans* were administered, were stimulated with phorbol ester PMA and calcium ionophore A23187, and cytokine production was analyzed.

In addition, 14,000 *C. elegans* were administered intraperitoneally or subcutaneously into mice. After a particular time period, worms, peritoneal exudate cells, and subcutaneously exudate cells were attempted to recover from the mice by washing with PBS. Adherent cells to *C. elegans* that could be recovered were observed by means of fluorescently labeled antibody staining and tried to identify the cells. For exudate cells, antibody staining was performed after removal of *C. elegans* by a filter, and flow cytometry (FACS) analysis was performed.

Results and discussion

Multiple doses of *C. elegans* in mice increased blood IgE levels. Consequently, it was found that the production amount of IgE increases according to the number of times of administrations. On the other hand, it did not vary significantly depending on the number of administered worms. That is, although IgE production was increased by *C. elegans* administration, there was a tendency to depend on the number of times rather than the number of worms administered. The reason for this is that since *C. elegans* administered contains various growth stages, there is a possibility that it is difficult to reflect the increase in the amount of administered protein proportional to the increase in the number of worms. Otherwise, since adult *C. elegans* are about 1 mm in length at the longest, it might be yet only a trace amount for mouse body, thus it is possible that the increase in the number of worms did not affect the amount of IgE production.

Regarding cytokine production, IL-4 production was induced in spleen cells after *C. elegans* administration. On the other hand, no significant change was observed in IFN-γ production regardless of before or after administration. Subsequently, it was suggested that if IL-4 production is started, irrespective of the production of IFN-γ, a type 2 immune response is triggered and the class switch to IgE could be induced.

In order to investigate the initial reaction after administration of *C. elegans* intraperitoneally and subcutaneously into mice, and after a particular period of time passed, the worms, the peritoneal cavity and subcutaneously exudate cells were tried to recover from the mouse body. As a result, in the peritoneal cavity, the number of worms recovered with the experimental time course tended to decrease faster than subcutaneously, although no statistically significant difference.

In FACS analysis of peritoneal exudate cells, Gr-1 positive cells (neutrophils) increased with time after administration, but on the contrary, it was found that the proportion of CD19 positive cells (B cells) and F4/80 positive cells (macrophages) were decreased. When *C. elegans* adherent cells were fluorescently stained, mainly F4/80 and Gr-1 positive cells were observed. From the results of FACS analysis and fluorescent staining, it was considered that tissue-resident macrophages bound to *C. elegans* in advance, followed by the induction of neutrophils.

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Effects of circadian rhythm on dose response of growth and creatinine excretion to dietary protein and methionine levels weaning Rodents

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Introduction

Animals with high genetic diversity need tailor-made nutrition management. This is a method of regulating diet on the premise that nutritional requirement exists for each animal. In Avian already, it is possible to estimate dietary amino acid and crude protein (CP) requirements using urinary creatinine (Cr) excretion as a criterion, and it is possible to estimate nutritional status. On the other hand, mammals that are difficult to collect whole urine have not yet been studied, and studies on herbivorous animals have never been conducted. Therefore, three experiments were conducted to develop the method to evaluat nutritional energy-protein status in mammals using partial urine.

Relationship in diurnal change between Cr which is varies depending on amino acid interaction, and urea nitrogen (BUN) which is common nitrogen excretion were studied in experiment 1. In experiment 2, the responses of urinary excreted Cr and BUN to dietary protein levels were compared in the two species of rodents, mice and field voles, which are omnivorous and herbivorous, respectively. The response of the amount to the amount of dietary CP was examined over time. In addition, a mouse was used to dose-dependently test methionine, one restricted amino acid of Cr substrate, to investigate whether the same tendency as chickens could be obtained.

Materials and methods

In experiment 1, the total amount of urine of 15 weeks old male mice kept on commercial maintenance diet was collected in two periods at 6 to 18 hours and 18 to next day 6 o'clock. In experiment 2, 18 Jcl: ICR mice 16 weeks old and 18 Hungarian Eurasian voles (Microtus arvalis) from 16-25 weeks old were used. Each animal was divided into 3 feed groups and fed for 7 days. Total amount of urine on the final day of the test was collected in 4 times every 6 hours at 0 - 6, 6 - 12, 12 - 18 and 18 - 24 (0), respectively.

After the feeding trial, animals collected liver and kidney after euthanasia by cervical dislocation. For the test feed, referring to the NRC feeding standard (1978, 1995), purified feed using casein as the sole CP source and canceling the CP amount by starch so that the CP amount is in deficient, optimal, excessive three stages was used.

In experiment 3, 18 mice with the same conditions as in experiment 2 were used, experimental feeds were fed for 7 days, the urine for a day on the final day of the test was collected, and after completion of the test the animals were euthanaed by cervical dislocation and the liver and kidney were sampled. Three dietary methionine levels at deficient, adequate and excessive were

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prepared with compensation by glutamic acid to keep dietary ME and CP. Cr and BUN concentrations in urine for each collection unit were measured. In addition, liver and kidney were subjected to analysis of hexokinase (HK: liver) and liver and kidney arginine glycine amidinotransferase (AGAT: liver and kidney) activity.

The results obtained was analyzed by first order regression in experiment 1, two-way analysis of variance was performed in experiment 2, and one-way analysis of variance was performed in experient 3.

Results and discussion

In experiment 1, although the correlation coefficient slightly decreased during the night, a high correlation was found between creatinine and urea nitrogen excretion on the whole day, except for changes in factors such as the amount of exercise such as the amount of exercise under the same diet environment It was suggested that the CP requirement could be obtained at these ratios irrespective of time. In experiment 2, the weight gain increased with increasing dietary CP level and remained constant over the optimal level. The HK activity measured to investigate the change in the amount of carbohydrates in feed and the influence on glycolysis was found in the dose dependent response to the protein content in the feed, showed high values. In addition, mice showed higher values than the voles ($P < 0.05$). Significant effects on creatinine excretion were observed in both species and feed ($P < 0.01$), while there is no interaction. Voles was higher in creatinine /UN level than the mice, and those tended to decrease on adequate CP, and then increased on excessive CP with increasing dietary CP levels. Only the interatomic difference was observed ($P < 0.05$), but the vole showed significantly higher value than the mouse, but the reaction to the diet showed the same tendency as chicken ($P < 0.10$). From the above, it is suggested that there is no interspecific divergence difference in the dose-dependent response depending on the protein content in the feed.

Excretion of creatinine was higher than that of mice, and tended to increase as the amount of dietary CP increased as the amount of dietary CP decreased at the recommended level and became excessive. Only the interatomic difference was observed ($P < 0.05$), but the vole showed significantly higher value than the mouse, but the reaction to the diet showed the same tendency as chicken ($P < 0.10$). From the above, it is suggested that there is no interspecific divergence difference in the dose-dependent response depending on the protein content in the feed.

Even in experiment 3, the weight gain and Cr / UN of mice increased significantly to 0.50% as the content of methionine in the feed increased and became constant thereafter ($P < 0.05$) the liver AGAT activity significantly increased to the methionine content of 0.50% in the feed decreased, and became constant after that ($P < 0.05$). The above tendency is similar to that of chicken, and the amount of creatinine excreted in mammals has been shown to be an indicator of nutritional status between amino acids, protein and energy.

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The methods for preventing from hypothermia induced by three types of mixed anesthetics in mice

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Introduction

Inhalation anesthesia, the most recommended anesthesia for laboratory animals, produces rapid induction and recovery from anesthesia, and it is able to regulate anesthetic depth via an anesthetic vaporizer. Although the anesthetic vaporizers used in animal research are commercially available, it takes measurable costs to purchase and to maintain the devices. Furthermore, handling of the inhalation anesthetic device is comparatively complex and it needs to secure a space of using it. On the other hand, an injectable anesthesia enables a number of the experimental animals to manipulate at once although the regulation of anesthetic depth is not easy in the experimentation. Pentobarbital sodium (PB) had been used as an injectable anesthesia for laboratory animals, however PB anesthesia has severe respiratory depression and poor analgesia, thereby it has been not recommended using by PB anesthesia alone during surgery. Ketamine, which is injected with xylazine usually has been also categorized as a narcotic drug in Japanese law currently. The usage of ketamine is necessary to possess drug handling license. Under these circumstances, injectable three mixed (MMB) anesthesia with medetomidine (Med), midazolam (Mid), and butorphanol (But) has been recommended. As the most advantage of MMB injectable anesthesia, it is able to antagonize by injection of atipamezole (Ati), α_2-antagonist. However, MMB anesthesia has been known several adverse effects during anesthesia and postanesthesia. The hypothermia induced by anesthesia has influence on delayed recovery from anesthesia, bradycardia, disturbed circadian rhythm, and increased infection. It is crucial to support by external thermal units (e.g. hot water-blanket, heating-pad and hot plate) for anesthetized mice, although it has been unknown that its thermal support periods for preventing hypothermia caused by MMB anesthesia. In the present study, we investigated appropriate thermal support periods after injection of MMB anesthesia and the most effective component of MMB anesthesia to induce hypothermia in mice.

Materials and Methods

We used male ICR mice aged 8 weeks in this study, and core body temperature of the mice was measured by the device implanted intraperitoneally for mice at 9 weeks under MMB (Med/Mid/But = 0.3/4.0/5.0 mg/kg) anesthesia. Body temperature of the mice were recorded from 06:00 h on the day before the experiment to 08:00 h on the next day. The mice were allowed recovery periods over 2 weeks after surgery. All of the injectable drugs used in this study were administered by intraperitoneal injection. Isoflurane as inhalational anesthesia were administered at 5 % induction, 1.5-2 % maintenance, and flow rate of 2 L/min. In the recovery from anesthesia, the mice

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under MMB were injected 0.3 mg/kg Ati, and stopped inhalation anesthetic apparatus in ISO at 40 min. The present study was taken in accordance with the Guidelines for Animal Experimental issued by Japanese Association for Laboratory Animal Science and approved by the provisions of Nippon Veterinary and Life Science University.

(1) Thermal support periods preventing hypothermia: To prevent hypothermia, mice were received thermal supports after administration of anesthesia for 1, 2, 3, and 5 h in MMB groups and for 1 h in ISO group.

(2) MMB components caused hypothermia during anesthesia: Each component (0.3 mg/kg Med, 4.0 mg/kg Mid or 5.0 mg/kg But) of MMB was injected alone into adult male mouse.

(3) Dosage of Ati for preventing hypothermia: In addition to the dose of 0.3mg/kg Ati (this dose was reported previously and widely used), 0.6, 1.2 and 2.4 mg/kg Ati were injected. The mice were anesthetized with MMB during 40 min and received thermal supports for 60 min.

Results and Conclusions

In the short periods (1 and 2 h) of thermal supports, mice showed significantly lower (p <0.01) body temperature than the temperature of normal range in MMB anesthesia although its normal range was maintained in ISO anesthesia. In contrast to the short period supports, the mice were maintained normothermia in 3 h and 5 h of thermal supports. In particular, the thermal support periods of 5 h perfectly prevents hypothermia induced by MMB. In each component of MMB, the mice were caused significant hypothermia in Med alone treated group (p <0.01).

In the regard to dose of Ati for preventing hypothermia, the mice were caused hypothermia after treatment of 0.3 and 0.6 mg/kg. However, mice were maintained normothermia in the both dosage of 1.2 and 2.4 mg/kg Ati treatment. Moreover, the time to achieve normothermia and to recovery of the righting reflex were shortened in dose-depend manner (p <0.01).

Our results suggested that 1) 5 h of heating supports after MMB anesthesia perfectly keeps core body temperature in mice, 2) the cause of hypothermia in MMB anesthesia depends on the component of Med, and 3) high dose of Ati has the effects to decrease hypothermia and to promote the recovery of body temperature after injection of MMB anesthesia.

In the conclusions, we recommend that the mice should be received thermal supports over 5 h to prevent hypothermia induced by MMB anesthesia, and this hypothermia was primarily caused by Med.

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Acceptability of once-a-day milking for Japanese dairy farmers

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Background and objective

The number of dairy farming households in Japan is decreasing rapidly as farmers abandon the industry. Reasons for this decline include the aging of proprietors, the lack of successors, as well as the long working hours.

Dairy farmers work longer hours than the average worker in other sectors due to the time-consuming milking process—which takes place at least twice a day—in addition to the processing and transportation of milk. While labor-saving devices are being introduced under the auspices of various public programs, the style of milk production itself has not changed.

In the present study, we investigated "the farming practices" for better clarity of Japanese dairy farmers and their possible interest in introducing once-a-day milking (OAD)—a practice that has become established in New Zealand (NZ) for its ability to reduce labor and operating costs—in order to investigate the percentage of farmers willing to adopt OAD, and assess any associated issues in dairy farming in Japan.

Materials and methods

(1) We surveyed 304 dairy farming households that belong to agricultural cooperatives in Kushiro-Tancho and Hamanaka, which are the major areas of grassland dairy farming in Hokkaido. The farmers were asked about the following: the management strategies of their dairy farms, what their daily work entails, their working hours, and their interest in adopting OAD.

(2) Six dairy farmers who expressed interest in OAD in step (1) were interviewed to understand the unique characteristics of their farms, who want to incorporate OAD into their businesses. In addition, statistical analyses were used to simulate how introducing OAD would impact their revenues.

(3) We surveyed 63 clients of a Japanese company that imports NZ milking cow semen. These data were analyzed together with those from step (1) to profile the dairy farms using the NZ bovine semen and assess their interest in OAD.

(4) Four dairy farmers from NZ, who have introduced OAD were interviewed regarding their business practices, the OAD introduction process, and how their businesses have fared since. We also discuss the possibilities for introducing OAD in Japan.

Results

i. Interest in OAD and working hours

The levels of awareness of, interest in, and desire to introduce OAD were overall low among the surveyed Japanese milk farmers. However, most of those farmers expressed the desire to reduce their working hours. The farmers who were interested in OAD tended to have (1) smaller

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herds and (2) a stronger desire to reduce their working hours compared to the average farmer surveyed. In addition, the farmers who wished to adopt OAD into their businesses tended to (1) obtain lower annual amounts of milk per head, (2) be moving toward shrinking their businesses.

ii. Characteristics of farmers who want to introduce OAD

All six farming households interested in OAD hope it would reduce their working hours, four of whom said they would use these extra hours "not for leisure but to do other farm work." Our simulations showed an expected reduction between 35% and 40% in milk production as a result of implementing OAD, which would have a negative impact on revenue.

iii. Characteristics of and OAD interest among farmers using imported NZ milking cow semen

Compared to the results from point (1) in this section, the Japanese dairy farms that use imported NZ semen tended to have fewer parous cows, produced less milk per parous cow and had more cultivated land per parous cow. In addition, they were significantly more aware of OAD and more interested in its introduction than the farmers surveyed in point (1).

iv. Process of OAD introduction and its impact on profitability

All 4 NZ farming households surveyed have introduced OAD to reduce working hours. Three of them said they did so "to have more time to spend with family"; As a result, daily working hours were reduced by a mean of 3 hours following the introduction of OAD. In addition to improving their life-work balance, the farmers also cited fewer leg injuries and increased conception rates in cows. None of them reported OAD to have negatively impacted their revenues.

Discussion

While dairy farmers' awareness of and interest in OAD remains low, there are some proprietors who wish to introduce OAD into their dairy farms. This interest was found to be stronger among the Japanese farmers who use imported NZ semen, who also tended to have smaller businesses and a stronger desire to reduce their working hours. Additionally, our results suggest that unlike NZ farmers, the Japanese farmers who were interested in introducing OAD did not necessarily want to improve their work-life balance but preferred to re-allocate the time to other farm work.

Going forward, differences in the values of Japanese and NZ farmers regarding dairy farming work should be investigated.

We would like to highlight the following 2 points regarding the introduction of OAD in Japan: First, concrete revenue estimates following OAD introduction need to be performed, and detailed estimates citing various business metrics and data such as milk quality during each lactation period need to be calculated. Second, herds should be improved by importing NZ semen and other methods. Along with this reform of rearing, supervision is needed, such as through grazing management.

It is very possible that introducing OAD would reduce working hours. Going forward, studies that verify the introduction of OAD should be conducted, and how grazing and other rearing supervision methods are carried out needs to be reexamined from a labor-saving perspective.

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Superovulation and In Vitro Fertilization in *Microtus rossiaemeridionalis*

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Introduction

The *Microtus* is 77 species inhabit all over the world, and 5 species are classified beyond an emergency type. As a characteristic, the *Microtus* is monogamous, the number of chromosomes and cell proliferation which differs between species. Thus they are used for various research such as social behavior or brain mechanism. Therefore, maintenance of the *Microtus* is meaningful, and establishment of assisted reproductive technologies for animal supply have been advanced. In recent years, a series of assisted reproductive technologies such as sperm freezing, artificial insemination, superovulation, *in vitro* fertilization, micro-insemination and embryo transfer are established in *M. montebelli*. On the other hand, assisted reproductive technologies mentioned in the others species of *M. montebelli* remain unestablished. In this study, we investigated superovulation and *in vitro* fertilization, which are fundamental technologies for animal reproduction and supply in *M. rossiaemeridionalis*.

Materials and methods

For experiments, 10 to 98 weeks old females and 19 to 62 weeks old males of *M. rossiaemeridionalis*, and/or 16 to 20 weeks old and males aged 23 to 41 weeks old females of *M. montebelli* were used. They have been maintained in our laboratory.

First, superovulation in *M. montebelli* can be applied to the *M. rossiaemeridionalis*. Animals were administered 30 IU equine chorionic gonadotropin (eCG). At 48 hours after administration of eCG, 30 μg/kg of 20% polyvinyl alcohol (PVP) -gonadotropin releasing hormone agonist (GnRH agonist: GnRHa) was subcutaneously administered at the cervix. At 14 hours after administration of GnRHa, oocytes were collected from the fallopian tubes and the nuclear phases were observed. Second, for investigation of the proper eCG concentration of *M. rossiaemeridionalis*. 0, 7.5, 15, or 30 IU of eCG was intraperitoneally administered to female, and then the ovaries and uterus weights isolated were measured 48 hours later. Third, to investigate the effect of different methods for induced ovulation, at 48 hours after administration of 15 IU eCG, 15 IU human chorionic gonadotropin (hCG) or 30 μg/kg of GnRHa was administered. Oocytes were then collected at 14 hours after administration and nuclear phases were observed.

Finally, *in vitro* fertilization with ovulated oocytes in *M. rossiaemeridionalis* was studied. Spermatozoa were collected from the cauda epididymidis, and preincubated for 1.5 hours in human tubal fluid (HTF) supplemented with 1 mM hypotaurine to evaluate motility and viability. The final spermatozoa concentration was 1.0×10^6 cells/ml and insemination was performed for 3 hours in above medium. After the insemination and further culture for 3 hours, the nuclear phase was observed. As this result was not satisfied, in order to improve the result, the following

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experiments were performed; 1) *in vitro* fertilization using *M. montebelli* spermatozoa and *M. rossiaemeridionalis* oocytes to evaluate the fertility of oocytes, 2) the influence of caffeine on sperm motility, and 3) *in vitro* fertilization using caffeine was performed.

All experimental procedures involving animals were reviewed and approved by animal care committees of the each prefectural livestock centers and the Institutional Animal Experiment Committee and the Animal Ethics Committee of Nippon Veterinary and Life Science University (Approval No. 28K-36, 28K-38, 29K-56, 29K-58, 30K-38, 30K-39).

Results and discussion

The number of superovulated oocytes on *M. rossiaemeridionalis* was different among individuals (0 to 7), unlike *M. montebelli*, suggesting that the superovulation of *M. montebelli* is not suitable for *M. rossiaemeridionalis*. Thus, we investigated the eCG concentration. As a result, the ovarian weight was significantly heavier in all treated groups (7.5, 15, 30 IU) than in the 0 IU group (control). Uterine weight also showed significantly heavier in the 7.5 and 15 IU-administrated groups than in the control. As the heaviest weights for both ovarian and uterus weights were shown at 15 IU, the optimum eCG concentration in *M. rossiaemeridionalis* was set to 15 IU. In the induction of ovulation, the number of oocytes tended to be higher in the GnRHa-administrated group. Taken together, it was demonstrated that a combination of 15 IU eCG and 30mμg/kg 20% PVP-GnRHa was suitable for superovulation of *M. rossiaemeridionalis*.

In vitro fertilization of *M. rossiaemeridionalis* in HTF with hypotaurine, sperm penetration was not observed in all oocytes after insemination, and oocytes remained at the second meiotic metaphase. On the other hand, sperm penetration was confirmed by *in vitro* fertilization using *M. montebelli* sperm and *M. rossiaemeridionalis* oocytes. This suggests that *M. rossiaemeridionelis* sperm is a cause of poor sperm penetration. Therefore, we investigated the influence of caffeine, which is known to promote sperm motility and increase penetration rate in other animals, on *M. rossiaemeridionalis* sperm. As a result, there was a tendency that motility was the highest in the pre-incubation and insemination with caffeine. Next, *in vitro* fertilization using HTF with caffeine showed a tendency that sperm penetration and fertilization rates increased in the caffeine group of both pre-incubation and insemination. These results clearly show that caffeine improves sperm motility, sperm penetration rate, and normal fertilization rate in this species.