Bull. Nippon Vet. Life Sci. Univ., No.61, 106–107, 2012.

Research and Development of Molecular Tools for Genetic Manipulation Studies in Dermatophytes

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(Conferred on 12 March 2012, V A-146)

Dermatophytes are a group of fungi that invades the superficial layer of skin causing lesions in the keratinized layer. Identification of pathogenesis-related factors requires a high throughput genetic manipulation system. In dermatophytes, some elements of this system either are missing or need an improvement and, subsequently, they can participate in elucidation of the pathogenicity at molecular level. In this study, the author tried to develop some of required tools to contribute in creating a better research environment for dermatophyte study.

Similar to other filamentous fungi, dermatophytes possess rigid walls, making genomic DNA extraction time consuming and labor-intensive process, particularly when large number of fungal colonies are implemented. Ampdirect DCPCR approach allowed direct colony PCR from a wide range of fungi including yeast and mold after 1 h of rapid fungal lysis. All the 64 tested colonies, including the genus Candida, Trichophyton and Aspergillus, showed expected PCR bands for their target regions: "ITS1/ITS4". Subsequently, tiny portions of fungal colonies of 36 mold strains were used as PCR template to amplify the same region but without any prior treatment. Thirty-five of these strains (97%), including T. mentagrophytes, yielded positive results on Ampdirect PCR, while only 11 of the 36 strains (31%) showed PCR products when standard PCR reagents were used. Ampdirect DCPCR was also applicable for DNA amplification from spores and hyphae. Thus, using Ampdirect DCPCR approach in primary check of genetically transformed T. mentagrophytes strains enables testing of large number of transformants without genomic DNA extraction.

To clarify relationship between various genes, which control many pathways in T. mentagrophytes, multiple mutations are required. However, only two dominant selectable markers, hygromycin B phosphotransferase gene (hph) and neomycin phosphotransferase gene (nptII), have been utilized in dermatophytic transformation studies. Therefore, further markers are needed to support such studies. Applicability of nat1 gene, derived from Streptomyces noursei, to confer resistance to the aminoglycoside antibiotic nourseothricin was demonstrated. NAT-transformants were stable and did not require con-tinuous supplementations of nourseothricin to maintain their phenotype. They remained stable even after 3 rounds of subculture on nourseothricin-free media. However, transformation frequency using nat1-expression cassette, under control of Pact promoter, was lower than using hph-expression cassette, driven by the Pch promoter 1, as reported in a previous study. This can be attributed to incomplete activity of Pact promoter as it is derived from the basidiomycotic yeast Cryptococcus neoformans, while Pch promoter 1 is derived from Cochliobolus heterostrophus, which is classified in the same phylum of T.

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mentagrophytes, Ascomycota. In addition, only 30% of tested transformants showed an integration of a single copy in the genome of T. mentagrophytes, which is anticipated to be increased if homologous fragments to host genome are included in gene-disruption constructs. Accordingly, nat1-expression cassette can poten- tially participate with other selectable markers, i.e., hph and nptII, in genetic manipulation to determine functions and roles of genes in dermatophytes.

Productivity of gene-knockout experiments in T. mentagrophytes is negatively affected by its high affinity to repair broken double strands DNA through non-homologous end joint (NHEJ) pathway, resulting in low homologous integration frequency. A homolog of lig4 encoding key protein in NHEJ pathway in other filamentous fungi was isolated from T. mentagrophytes, designated as TmLIG4, and characterized in detail. It was identified as a single copy of a 3.4 kb ORF interrupted by 6 introns and expected to contain two tandem conserved BRAC1 domains at the C terminus. Growth ability, sporulation rate and sensitivity to DNA damaging agents were almost identical between a knockout mutant of TmLIG4 locus and its parental strain. Homologous integration frequencies were reached as high as 93% when TmLIG4-deficient mutant used as a host strain in knockout experiments of four different loci, while they ranged from 0%–40% in the wild-type strain. These results suggested that studies in strains lacking TmLIG4 would be beneficial to improve our understanding of dermatophytosis by facilitating the genetic manipulation of dermatophytes.

The present study confirms the successful application of several tools for molecular studies of dermatophytes in general and T. mentagrophytes in particular. They can be involved, together or independently, in experiments, which might contribute in better understanding of pathogenesis related factors and, therefore, suggesting suitable drugs with less risks and low costs.

Bull. Nippon Vet. Life Sci. Univ., No.61, 108–110, 2012.

Clinical and basic studies on the diagnosis of epileptogenic foci: Noninvasive localization of epileptogenic foci and development of a novel animal model of epilepsy

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(Conferred on 12 March 2012, V A-147)

Epilepsy is a chronic, pathophysiological phenomenon of the brain that may occur in all mammalians species. The outward manifestation of epileptic seizures is the transient occurrence of signs or symptoms due to abnormal electrical activity in the brain. Clinical signs of epileptic seizures vary according to the location of the abnormal discharge in the cerebral cortex and the pattern of distribution of the discharge. The area of the brain responsible for generating clinical seizures is the epileptogenic zone, which is defined by anatomic-electro-imaging correlations. Therefore, the location and extent of the epileptogenic zone is never definitively known, but can only be deduced from a variety of diagnostic investigations. The purpose of this study was to establish a noninvasive method for localizing epileptogenic foci and to develop a novel animal model of epilepsy. Toward this end, we evaluated seizure semiology, electroencephalograms, and structural/functional MRIs, from a standpoint of veterinary clinical and experimental epilepsy studies.

1. **Imaging diagnosis of epileptic brain using structural/anatomical MRI: MR volumetry of the hippocampus in 58 epileptic dogs. [Chapter 2]**

In humans, the most common intractable epilepsy is temporal lobe epilepsy characterized by focal (complex partial) seizures and pathological lesions, such as hippocampal sclerosis. In dogs, on the other hand, imaging abnormalities are usually not observed in idiopathic epilepsy. In this study, we retrospectively performed clinical hippocampal volumetry in dogs with idiopathic epilepsy to assess the presence of hippocampal atrophy and the usefulness of hippocampal volumetry for localizing and/or lateralizing epileptic foci. In seven of the 58 epileptic dogs (12%), there were visible abnormalities on the MRIs, consisting of unilateral or bilateral hippocampal atrophy and increased T2 W/FLAIR signals. There were strong positive correlations between hippocampal volume and body weight, depending on breed, in control dogs; thus, body weight and breed must be considered when assessing hippocampal volume in epileptic dogs. To determine whether hippocampal atrophy was present, we first calculated the ideal hippocampal volume for each epileptic dog using a regression equation derived from the control dogs, and then determined the atrophic ratios from the ideal and actual values. Based on this calculation, significant hippocampal atrophy was confirmed in the epileptic dogs. In addition, using a cutoff threshold for the hippocampal volume asymmetry ratio of 6%, as indicated in human epileptic

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patients, 28 epileptic dogs (48%) were chara-cterized as having unilateral hippocampus atrophy. However, our ability to use imaging data to localize epileptic foci in a clinical case study was limited; hence, it was necessary to develop a suitable animal model of epilepsy with which to conduct basic/experimental studies.

2. Development and establishment of a novel animal model of epilepsy:

2.1. Studies on seizure semiology of the spontaneous epileptic cat. [Chapter 3.1]

We identified two generations of cats showing spontaneous seizures induced by specific stimulations, such as removing the cat from a cage, in a closed colony of a commercial trader of laboratory animals. In the current study, we investigated the phenotype and seizure semiology of an inbred strain of these spontaneous epileptic cats. The spontaneous seizures exhibited by these epileptic cats were focal (complex partial) seizures with secondary generalized tonic-clonic convul-sions. Most of the spontaneous seizures occurred during sleep and started as arrest and attention behavior. In addition to these completely spontaneous seizures, we also investigated seizures induced by vestibular stimulation, as suggested by the supplier. The vestibular stimulation-induced seizures were generalized tonic-clonic seizures, and nearly all of them occurred suddenly during the stimulation test. In comparison to previous epilepsy models, the spontaneous seizures were similar to those induced by limbic kindling or by the injection of kainic acid, and the stimulation-induced seizures seemed to resemble those of the EL mouse. These similarities suggest that the seizure focus of the spontaneous epileptic cat may be in the mesial temporal lobe, especially in the hippocampus.

2.2. A novel model of idiopathic epilepsy: Establishment of the spontaneous epileptic cat strain. [Chapter 3.2]

We investigated the clinical features (pedigree, inbreeding, clinical examination, and structural MRI) of these epileptic cats to evaluate their value as a model of epilepsy and to establish a colony of the spontaneous epileptic cat strain. Based on its pedigree, the chi-square test showed that this spontaneous epileptic phenotype is inherited as an autosomal recessive trait. We inbred 3 pairs of epileptic males and females, and 8 kittens were born. These kittens exhibited interictal discharges on scalp EEG from early-life, and 2 of these kittens also exhibited clinical seizures. There were no clinical abnormalities present in MRI, CBC, or CSF examinations suggestive of epileptic seizures. Therefore, we considered that this spontaneous epileptic cat strain might be an available and useful model of genetic and/or idiopathic epilepsy.

3. Diagnosis of epileptic focus based on EEG findings in the spontaneous epileptic cats. [Chapter 4]

We performed interictal scalp EEG by using various agents to determine whether the seizures of the epileptic cats are epileptic convulsion (epileptiform discharges). As a part of this examination, we also evaluated the epileptiform discharges, including their waveform, site of origin, pattern, and cerebral dysrhythmia, and performed quantitative EEG (qEEG) analysis. Most epileptiform discharges appeared dominantly in the unilateral and/or bilateral temporal regions under sedation (medetomidine) and were generalized under anesthesia (sevoflurane). The duration of the epileptiform discharges was highest in the temporal region, suggesting that the epileptogenic zone is present in the limbic system. Furthermore, qEEG analysis showed a relative increase in the delta wave activity in the temporal region and was able to localize the EEG abnor- mality. In a study of EEG activation using bemegride, the doses to induce convulsions and/or abnormal EEG in epileptic cats were significantly lower than those in normal cats, suggesting differences in seizure susceptibility of the brain. In summary, we consider the EEG findings obtained in these cats to be the result of epileptic discharges in the temporal lobe. Thus, we consider this epileptic cat strain to be a possible model for studying the pathogenesis and therapy of epilepsy in human and veterinary medicine.

4. Structural imaging diagnosis of the epileptic cat using high magnetic field MRI (3.0 T): Visual and quantitative three-dimensional MR volumetry of the hippocampus. [Chapter 5]

We performed hippocampal volumetry using 3 Tesla MRI to examine its usefulness as a noninvasive method of localizing epileptic foci, and to assess the presence of hippocampal atrophy in the spontaneous epileptic cat (similar to Chapter 2). Visual assessment of the MRIs detected abnormalities in the hippocampus in 11 of 14 epileptic cats (79%). Significant hippocampal atrophy, compared with control cats, was confirmed in the epileptic cats. In addition, a significant asymmetry ratio was also found in the epileptic cats. Consequently, hippocampal volumetry can quantitatively determine the asymmetric ratio, which was useful as an indicator of the degree of hippocampal atrophy in epileptic cats. In contrast, 3 epileptic cats showed no signal changes and no hippocampal atrophy. These 3 epileptic cats are thought to resemble human patients with nonlesional temporal lobe epilepsy.

5. Functional imaging of the epileptic cats using high magnetic field MRI (3.0 T): Studies on the utility of diffusion-based MRI and perfusion MRI as noninvasive methods of localizing epileptic foci [Chapter 6].

The purpose of this study was to evaluate the utility of diffusion weighted imaging (DWI), diffusion tensor imaging (DTI), and perfusion-weighted imaging (PWI) for localizing epileptic foci in epileptic cats. In the interictal epileptic cats, DWI revealed increased apparent diffusion coefficients (ADC) in the hippocampal region, while PWI showed marked relative hypoperfusion of the hippocampal region. These methods reliably demonstrate functional hippocampal changes that are a consequence of structural lesions. In addition, these MR techniques demonstrated their potential as reliable tools for the noninvasive localization of epileptic foci in epileptic cats.

In this study, we performed a series of experiments to localize the epileptic focus in epileptic animals. First, in a clinical case study, we retrospectively measured hippocampal volume in 58 dogs with idiopathic epilepsy, enabling us to reliably assess the presence of hippocampal atrophy and its correlation to the diagnosis of epilepsy. However, our ability to localize epileptic foci in clinical cases using this imaging method was limited and we required a novel animal model of epilepsy to conduct basic/experimental studies. Given these circumstances, we learned of the existence of cats exhibiting spontaneous seizures that appeared to have the potential to be a suitable model of genetic epilepsy to conduct studies to localize epileptic foci. We investigated these spontaneous epileptic cats clinically (seizure semiology and EEG) and genealogically, and confirmed that they would be an excellent model of experimental epilepsy. In an attempt to noninvasively localize the epileptic foci in these epileptic cats, we conducted functional MRI studies with diffusion and perfusion MRI. The results of these studies were consistent with the epileptic focus being in the hippocampal region. Together, these data suggested the possibility that these studies permitted us to determine the epileptogenic region.

In conclusion, we performed a series of basic and clinical studies to establish noninvasive methods to localize epileptic foci in human and animal. The author demonstrated that functional methods of image analysis were suitable for clinical applications and found a novel and valuable epileptic animal model in this research process. The results of this study may lead to the development of new strategies for studying epileptic pathophysiology and treatments for all epileptic mammalian species in the near future.

Bull. Nippon Vet. Life Sci. Univ., No.61, 111–112, 2012.

Neuroendocrinological studies on the maternal-infant bonding by ultrasonic vocalizations in rats

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(Conferred on 12 March 2012, V A-148)

In general, the neonatal period is a phase when an animal's first social connections are formed. Normal interactions of the mother and pups are important for the proper growth and development of mammals. Maternal behavior in rats consists of various integrated elements related to the nutrition and care of the offspring. In rats, mothers spend a lot of times during the first half of lactation period in the nest with their pups, feeding or licking their litter.

It is well known that rodent pups emit ultrasonic vocalization (USV) in situations such as separation from the nest and cold stress. Further, mothers exposed to USV by pups demonstrate retrieving behavior, nest building, and anogenital licking behavior in rats, mice and hamsters. Prolactin levels in mothers increase in response to USV, resulting in promote maternal behavior.]

In the present age, the relationship between parent and child is becoming weak in human being, therefore the basic study of the communication using laboratory animals is significant.

The subject of this study consists of three categories. First, the ultrasonic vocalization (USV) as communications between pups and their mothers was analyzed qualitatively and quantitatively. Second, in sexually na?ve and experienced females during sexual cycle blood prolactin levels induced by rat pups USV were determined by ELISA. Third, the motor activity and c-Fos expression of the neural nucleus of brain in postpartum lactating females with their pups withdrawn and then returned, and in sexually naïve and experienced females by rat pups USV were investigated.

The first study is presented in Chapter 2. Rat pups emit USV in certain stressful situations such as separation from nest and cold. It has been previously reported that rodent pup USV is the essential communication between pups and their mother. In this study the author attempted to verify the ontogenetic changes of USV emitted by infant rats isolated from their mother during pre-weaning period. The number of calls, median frequency and the first peak of frequency of calls were measured on days 1, 3, 5, 7, 10, 12, and 14 of age in Wistar-Imamichi rats. A pup was placed into a cold and the USV was recorded for 5 min. The results showed that the pups'body weights increased significantly with age except days 7 and 10. The number of calls increased to the peak on day 5, and then decreased gradually. The median frequency of calls decreased slowly until day 12, and showed a small increase on day 14. The first peak frequency of calls was highest on day 1, showed decreased gradually by day 12, and then increased slowly on day 14. Changes in the first frequency were similar to the median frequency of calls correlated to the rapid growth and morphological development of the rat pups during the first two weeks after birth. In conclusion, this chapter showed the ontogenetic changes of USV

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emitted by infant rats.

The second study is presented in Chapter 3. The modal peak frequency of 55 kHz by rat pups elicited maternal behavior, and increased plasma prolactin (PRL) levels in lactating rats. PRL is an anterior pituitary hormone, and plays an important role on lactation, parental behavior and homeostasis. PRL was released in response to physiological changes and external stimuli such as light, sound, smell and stress. A previous study had demonstrated that PRL levels in response to pup USV in sexually experienced, multiparous rats were higher than sexually naïve, nulliparous rats. The author investigated PRL levels during sexual cycle in nulliparous and multiparous Wistar-Imamichi rats. The results showed that plasma PRL levels were higher at proestrous than diestrous stages in nulliparous and multiparous rats. In both nulliparous and multiparous rats with diestrous stage PRL levels were significantly increase after pup USV exposure, compared with rats non-exposed to pup USV. The values of PRL in multiparous rats were almost two times as high as nulliparous rats. No changes of PRL levels showed in multiparous rats exposed to mouse and vole pup USV. This result may suggest that mother-pup communication by USV is a species difference.

The third study is presented in chapter 4. The locomotor activity and the active region of brain were investigated in lactating rats with and without their pups by the Laboratory Animal Motor Behavior Analyzer (LAMBA) and immunohistchemistry method, respectively. In addition, the author researched the neuronal activity of brain at proesrous stage in sexually naïve and experienced rats exposed to pups USV. These results obtained were as follows. The motor activity in lactating rats separated from their pups was higher, compared with lactating rats. When returned their pups it showed the same values at the beginning, and then stayed at nest to care of their pups. Though the c-Fos expression in lactating rats with their pups, withdrawn and then returned was observed in the medial preoptic area (MPA), the bed nucleus of the stria terminalis (BST), the caudal portion of posterior hypothalamic nucleus (PH), and the supramamillary nucleus (SUN), the number of c-Fos positive cells significantly increased in lactating rats returned their pups, compared with other groups. In sexually naïve and experienced rats exposed to pups USV the number of c-Fos expression increased significantly in the SUM and the Periaqueductal gray (PAG), and significantly increase in the supraoptic nucleus (SON) in sexually naïve rats.

In conclusion, this study suggested that the communication between mother and her pups by ultrasonic vocalization was very important for the pup survival and maintenance of species in rodent.

Bull. Nippon Vet. Life Sci. Univ., No.61, 113–115, 2012.

Studies on the role of thioredoxin as a biostress marker in dogs

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(Conferred on 12 March 2012, V A-149)

Life is preserved by maintaining homeostasis when exposed to factors that may impair the internal or external environment. Factors that may disrupt the internal environment are called stressors. Stress is defined as an abnormal condition that occurs within an organism experiencing a stressor. The definition and types of stressors are defined by their cause. Oxidative stress is caused by reactive oxygen species (ROS) produced by endogenous or exogenous sources within an organism, and is a major cause of stress to cells or tissues. The body has developed a redox (oxidation and reduction) regulating system, which acts as a defensive system against oxidative stress. The present study focused on basic *in vivo* and *in vitro* studies of canine thioredoxin (Trx), a redox regulating protein known in human medicine.

Thioredoxin-1 (Trx1) is redox-active small protein (12-kDa) consisting of 105 amino acids. Trx was originally identified in *Escherichia coli* as an electron donor for ribonucleotide reductase, an essential enzyme for DNA synthesis. Human Trx was cloned independently as an adult T cell leukemia-derived factor (ADF) in 1989. Trx is present in all cells of the human body and is induced by various stimuli such as ultraviolet light, radiation, ischemia/reperfusion injury, anticancer drug treatment, infection, and inflammation. Trx acts as a defensive protein against these various stimuli. In human medicine, Trx has been shown to have an important role in inflammation during virus infectious diseases such as human immunodeficiency virus (HIV) or hepatitis C virus (HCV) as well as autoimmune disease and cardiovascular disease. The diagnosis of these disorders can be used as a gauge to measure the stress response of a patient, especially for prognosis and therapy evaluation.

Thus, in human medicine Trx is generally recognized as a biostress marker. However, to date there are no studies regarding Trx in veterinary medicine. To date, measurement of Trx levels in companion animals such as dogs and cats have not been investigated. In this study, we focused on Trx, which is useful as oxidative stress marker, and examined the characteristics of canine Trx specifically. The purpose of this study was to measure the levels of Trx in dogs.

1. The study of immunologic cross-reactivity between anti-human Trx monoclonal antibody and canine Trx. (Chapter 2)

Western blotting was performed to evaluate whether cross-reactivity existed between an anti-human Trx monoclonal antibody and canine Trx to determine the possibility of measuring canine Trx levels using a human Trx ELISA kit.

The solid-phase primary and secondary antibodies of the human Trx ELISA kit were ADF21 and ADF11, respectively. Cell lysate of the Madin-Darby canine kidney (MDCK) epithelial cell line was recognized by ADF21 and ADF11. Therefore, the anti-human Trx monoclonal antibody was found to be immunologically highly cross-reactive with canine Trx. From these findings, we suggest

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the human Trx ELISA kit is applicable for the measurement of canine Trx.

2. The distribution of Trx in dogs (Chapter 3)

In humans, Trx is present throughout the body and expression levels of Trx are increased in various disorders. In this study, we examined the body distribution of canine Trx. By comparing Trx concentrations in each tissue, we observed that the liver contained the highest levels of Trx, although Trx levels were also observed in other tissues in descending order of the highest concentration: intestinal tract, lymph node, lung, spleen, renal cortex, renal medulla, and skin.

Both the liver and intestinal tract demonstrated significantly higher Trx levels compared to skin and renal medulla. In addition, Trx levels in the lymph node were significantly higher compared to skin. Therefore, we confirmed that canine Trx protein was produced in organs that have an abundance of reticuloendothelial functions such as the liver and lung but also in other tissues, which are often subjected to oxidative stress such as the liver and intestinal tract. These findings suggested that various organs might produce Trx as a biological defense mechanism against oxidative stress.

3. The *in vitro* study of Trx release from peripheral blood lymphocytes (PBLs) and Madin-Darby canine kidney cells (MDCK) in response to H_2O_2 (Chapter 4)

It has been shown that the level of Trx released is augmented upon the addition of oxidative stressors such as H_2O_2 in Jurkat cells *in vitro* (a human T cell lymphoma cell line). In this study, we examined whether Trx is expressed in PBLs and MDCK upon the addition of an oxidative stressor such as H_2O_2, using Jurkat cells as a positive control. We observed a significant increase in the level of Trx released in Jurkat cells and PBLs upon the addition of H_2O_2 compared with the control group ($P<0.05$). The levels of Trx were markedly elevated at 24 h after the addition of H_2O_2. Cells incubated with 100 ?M H_2O_2 produced significantly higher levels of Trx than the control group which were not incubated with H_2O_2 ($P<0.05$). However, the level of Trx released in H_2O_2-treated MDCK was not significantly different between the 100 ?M H_2O_2 treated group or controls. These results indicated that expression of Trx in PBLs was enhanced by oxidative stress upon the addition of H_2O_2. Furthermore, it was suggested that lymphoid lineage cells have an increased sensitivity to oxidative stress stimuli and therefore are more likely to produce Trx compared to MDCK.

4. Study of the kinetics of Trx in the blood and urine by oxidative stress

stimuli in dogs. (Chapter 5)

The purpose of this study was to examine Trx levels in the blood and urine of dogs under oxidative stress. We administered 21% or 100% oxygen by inhalation to dogs under isoflurane inhalation anesthesia, to examine whether Trx was produced by the inhalation of high oxygen levels. The concentration of Trx was measured compared to 8-hydroxy-2-deoxy guanosine (8-OHdG), a well-known oxidative stress marker. We observed a minimal change in urinary canine 8-OHdG concentrations in the 21% O_2 inhalation group. In contrast, the urinary canine 8-OHdG concentrations increased in the 100% O_2 inhalation group and reached a maximum value 48 h after anesthesia induction. This result indicated that oxidative stress was clearly induced by the inhalation model of pure oxygen in this study. These findings suggest that oxygen is critically involved in the change of canine Trx levels similar to that observed in humans. Therefore, it was suggested that the Trx concentration in the body increases to protect against superoxides generated by oxidative stress following 100% O_2 inhalation.

5. Study of changes in plasma Trx concentration in a canine inflammation model. (Chapter 6)

In this study, we investigated the use of Trx for veterinary clinical applications using an inflammation model induced by injection of turpentine oil into the femoral muscle of dogs. The levels of C-reactive protein (CRP), a canine inflammatory marker, IL-6, an inflammatory cytokine, and

systemic inflammatory response syndrome (SIRS) were measured following turpentine oil injection. After a positive evaluation of the inflammatory reaction in this model, we examined the presence of induced Trx in canine systemic inflammation by measuring the change in Trx serum concentration. We observed no changes in the concentration of serum CRP and IL-6 in the control group. In the treatment group clinical symptoms, i.e, SIRS symptoms were followed by an increase in the concentration of serum CRP and IL-6. Although the plasma Trx concentration showed a minor increase in the control group, high values of Trx were observed in the treatment group during the experimental period despite the presence of unclear peaks. These results indicated that systemic inflammation was induced by intramuscular injection of turpentine oil in dogs, and that Trx was secreted in the blood and production was maintained constantly during systemic inflammation. This suggests that the concentration of plasma Trx was elevated as a defensive mechanism against the systemic inflammation induced by turpentine oil. It has been shown that when Trx is secreted it can traffic between the intracellular and extracellular fluid by forming complexes with lipid rafts.

6. Study of Trx concentration in various canine disorders. (Chapter 7)

In human medicine, it has been reported that the measurement of Trx levels in subjects is a good predictor for oxidative stress in various disorders. In this study, we measured Trx concentrations in dogs kept in standard homes, which had been brought to a veterinarian with various common diseases, and compared them to Trx levels in healthy dogs. We observed that the mean value of canine plasma Trx concentrations was lower compared to normal humans. Furthermore, plasma Trx concentrations were significantly higher in dogs with pyometra compared to healthy dogs. However, dogs with mammary gland tumors, acute pancreatitis, lymphoma, autoimmune disease, or cardiac disease showed no significant difference in plasma Trx concentrations compared to healthy dogs.

As explained previously, the anti-human Trx monoclonal antibody was found to be highly immunologically cross-reactive with canine Trx. It was observed that Trx was induced in the body under conditions of oxidative stress and SIRS from the various results presented in this study. The findings presented here demonstrate that canine Trx has both anti-oxidative and anti-inflammatory effects similar to human Trx, and therefore Trx is useful as biostress marker. Thus, future studies should concentrate on the clinical applications of Trx.

Bull. Nippon Vet. Life Sci. Univ., No.61, 116–118, 2012.

Studies on Stromal Myofibroblasts and Tenascin-C Expression in Canine and Feline Mammary Tumors

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(Conferred on 12 March 2012, V A-150)

Tumor tissue consists of not only tumor cells but also several types of stromal cells and extracellular matrices. Previously, little attention had been paid to the role of such stromal components. However, it has long been suggested that the stromal components influence the tumor development. For instance, scirrhous carcinoma that is carcinoma accompanied by structural hardness due to the development of dense connective tissue in the stroma has a highly invasive potential. Activated fibroblasts in the cancer stroma often express α-smooth muscle actin (α-SMA), which is characteristic of myofibroblasts. Recently, in human medicine, myofibroblasts within the cancerous tissue, which are often referred to as carcinoma-associated fibroblasts (CAFs), have been considered to play a critical role in carcinogenesis. However, study about stromal myofibroblasts in canine and feline mammary tumors has not been carried out so far.

Tenascin-C (Tn-C) is an extracellular matrix protein, which is expressed transiently during organogenesis and is absent or greatly reduced in most adult tissues. Tn-C increases again in pathological conditions, especially cancer stroma, and plays a role in cell migration, proliferation, and angiogenesis. In human breast cancer, a correlation of stromal Tn-C staining with the tumor grades was noted. However, no information is available on the expression of Tn-C in feline mammary tumors.

The aim of this study were to determine whether the appearance of stromal myofibroblasts and the expression of Tn-C correlate with the grade of malignancy in canine and feline mammary tumors and to determine the cellular source of Tn-C in these tumors by immunohistochemical and *in situ* hybridization approaches.

1. Appearance of Stromal Myofibroblasts and Tenascin-C in Normal, Hyperplastic, and Neoplastic Mammary Tissues of Dogs (Second Chapter)

First, the appearance of stromal myofibroblasts in normal, hyperplastic, and neoplastic mammary tissues of dogs was examined. Myofibroblasts were absent in all normal or hyperplastic mammary tissues, whereas they were present in about one-half (55%) of simple adenomas and almost all cases (93%) of simple carcinoma. The appearance of stromal myofibroblasts correlated well with the grade of malignancy, intravasation, and metastasis of carcinomas with statistical significance.

Second, the distribution of Tn-C expression in normal, hyperplastic, and neoplastic mammary tissues of dogs was evaluated. Two different localization patterns of Tn-C protein were recognized: (1) Tn-C protein present in reactive stroma and (2) Tn-C protein present in the basement membrane zone. Stromal Tn-C

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immunoreactivity was not detected in the normal and hyperplastic mammary tissues. On the other hand, about one-half (55%) of simple adenomas and almost all cases (93%) of simple carcinoma exhibited Tn-C immunoreactivity in the stroma. Statistical analysis indicated that stromal Tn-C expression was significantly in positive correlation with the grade of malignancy and intravasation of simple carcinomas. Both immunohistochemistry using serial sections and double immunohistochemistry revealed that the distribution of stromal Tn-C coincided well with the areas where α-SMA-positive myofibroblasts were present. These findings suggest that stromal Tn-C may be excessively produced by stromal myofibroblasts, resulting in cancer progression.

On the other hand, Tn-C immunoreactivity in the basement membrane zone was observed in normal mammary glands, benign mammary lesions, and low-grade simple carcinomas. This type of Tn-C distribution in carcinomas, unlike stromal Tn-C, did not correlate with any of histopathological parameters associated with a poor prognosis. It seems that Tn-C of the basement membrane zone is produced by neighboring myoepithelial cells but not stromal myofibroblasts on its localizing grounds.

2. Appearance of Stromal Myofibroblasts and Tenascin-C in Normal, Hyperplastic, and Neoplastic Mammary Tissues of Cats (Third Chapter)

Feline mammary carcinoma is well known for its highly infiltrative and metastatic potential. The histology of feline mammary carcinoma is more similar to that of human breast cancer than canine and murine counterpart and hence it is considered to serve as a valuable model for human high-grade breast cancer. The aims of this chapter were to detect the appearance and distribution of stromal myofibroblasts and Tn-C expression in feline mammary tissues using the same methodology of the previous chapter, and to compare the results with the data in dogs.

In feline mammary tissues, stromal myofibroblasts were almost absent in normal and hyperplastic glands, but they were present in some cases (38%) of adenoma and all cases (100%) of carcinoma. Stromal Tn-C immunoreactivity was detected in some cases (38%) of adenoma and almost all cases (93%) of carcinoma, whereas it was hardly noted in normal and hyperplastic glands. Its distribution coincided well with that of stromal myofibroblasts. Feline mammary carcinomas showed abundant stromal myofibroblasts and high expression of Tn-C irrespective of the grades of malignancy, intravasation or metastasis. Thus, stromal myofibroblasts and Tn-C appear to be involved in malignant progression of feline mammary tumor.

On the other hand, Tn-C immunoreactivity in the basement membrane zone was occasionally observed in normal mammary gland and benign mammary lesions, but very seldom in carcinomas. This may be caused by a general lack of the resting myoepithelial cells in feline mammary carcinomas, unlike canine counterparts. In this respect, feline mammary carcinoma is similar to human breast cancer.

3. Identification of Tn-C Producing Cells in Canine Mammary Carcinomas by Highly Sensitive *In Situ* Hybridization (Fourth Chapter)

In canine mammary carcinomas, three sites of Tn-C localization were recognized as follows: 1) Reactive stroma in high-grade simple carcinoma; 2) basement membrane zone in low-grade simple carcinoma; and 3) areas of proliferating myoepithelial cells in complex carcinoma. In this chapter, to identify main cellular sources of Tn-C protein in each site of the localization, *in situ* hybridization for dog Tn-C mRNA was carried out.

According to *in situ* hybridization-immunohistochemistry double labeling analysis, in high-grade simple carcinoma, Tn-C mRNA signals were detected in both α-SMA-positive myofibroblasts and α-SMA-negative fibroblasts. In low-grade simple carcinomas, Tn-C mRNA signals were mainly observed in the myoepithelial cells that lay adjacent to the basement membrane. In complex carcinoma, Tn-C mRNA signals were also detected

in proliferating myoepithelial cells.

These results demonstrate that stromal myofibroblasts/fibroblasts are a major cellular source of Tn-C in high-grade simple carcinomas. On the other hand, Tn-C in the basement membrane zone could be produced by myoepithelial cells and functionally differ from stromal Tn-C.

In conclusion, this study suggests that stromal myofibroblasts overproduce Tn-C protein which may be involved in malignant progression of canine and feline mammary tumors. Therefore, the evaluation of stromal myofibroblasts and Tn-C expression could be helpful for assessing the degree of malignancy of the tumors. Furthermore, this study reveals that a large number of canine mammary tumors contain Tn-C protein which is produced by myoepithelial cells and which does not appear to be directly associated with tumor progression.

Bull. Nippon Vet. Life Sci. Univ., No.61, 119–121, 2012.

Studies on changes in glucose and lipid metabolism in obese and diabetic dogs

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(Conferred on 12 March 2012, V A-151)

At the early stage of obesity (slight obesity) in dogs, certain compensative changes in glucose and lipid metabolism are considered to be induced. However, slight obesity is not morbid condition that needs special treatments. The aims of this study are to investigate the biochemical and genetic alterations in glucose and lipid metabolism in obese and diabetic dogs and to apply the new findings to prevention and treatment for metabolic disorders such as obesity and diabetes in veterinary medicine.

1. Investigation of metabolic parameters in blood of control dogs

Large-scale nationwide survey to measure plasma metabolites, hormones and enzymes values was carried out with 888 dogs. Total 12 metabolic parameters, glucose, triglyceride (TG), total cholesterol (TC), total protein (TP), blood nitrogen urea (BUN), creatinine, lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), insulin, adiponectin and free fatty acids (FFA), were measured in plasma of dogs. Overall, a gender gap that female dogs show higher TG and TC concentrations than male dogs was clarified. In the female dogs, the old dogs with over 11 years old (YO) showed significantly higher values of TG, TP, FFA, insulin and ALT than other age group dogs. The dogs with 6 to 10-YO showed high values of TG and insulin, whereas low value of BUN compared to other age groups. The dogs with 3 to 5-YO showed significantly low value of BUN. In the male dogs, the old dogs with over 11-YO showed significantly higher values of TG, TC, TP and insulin and lower values of adiponectin than those in other age groups. The dogs with 6 to 10-YO showed high values of TC and TP and low values of BUN and AST. The dogs with 3 to 5-YO showed higher TC values than those in dogs with 0 to 2-YO as the control. In the case of classification by body condition score (BCS), 26% (237/888) of dogs examined were overweight or obese with BCS 4 to 5. The overweight dogs with BCS4 showed higher values of plasma TC and insulin and lower values of plasma BUN than those in the control dogs with BCS3. There were no metabolic parameters which were influenced by BCS between genders using multivariate statistics analysis. Analysis of parameters influencing BCS using multivariate statistics showed P value of insulin was 0.12 and insulin was suggested as important factor concerning to induction of obesity in dogs. Investigation using castrated dogs revealed that female dogs were more sensitive for castration than male dogs and plasma TG values were significantly varied as parameters for lipid metabolism.

2. Lipoprotein cholesterol profiles in dogs and cats

Use of lipoprotein cholesterol profile for diagnosis in veterinary medicine is not popular as in human medicine because there was fewer data

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about lipid metabolism in dogs than in human. In this study, significance of analysis of cholesterol profile as diagnostic indicator for lipid metabolism disorders were investigated in obese and control dogs. 64 dogs (1 to 7-YO) were divided by ages into 2 groups, 1 to 7-YO and over 8-YO. Dogs with BCS3 were used as control and dogs with BCS4 and 5 were classified as the obese group. HDL cholesterol sub-fraction was changed accompanying with onset of obesity. In the obese dogs with 1 to 7-YO, each fractions of cholesterol concentrations increased parallel to increased TC concentrations, and rate of each cholesterol fractions was not changed. Whereas old obese dogs with over 8-YO showed increased TC concentrations and changes in cholesterol sub-fractions with increased ratio of HDL1/HDL2 and 3 (0.46 vs 0.22 in 1 to 7-YO obese dogs). Because TC concentrations increased and cholesterol sub-fraction changed markedly in obese dogs, cholesterol profile seemed to be a good indicator for diagnosis of obesity.

3. Difference in between dog species in standard values of metabolic parameters

Dogs show various sizes in every species differing from cats, and alterations in energy metabolism in each species have been discussed. For instance it has been reported that Miniature Schnauzer shows congenital hyperlipidemia in USA, however hyperlipidemia in Miniature Schnauzer (MS) has not been reported except in USA. The aims of this study were to compare the plasma lipid, TG and TC, concentrations between Miniature Schnauzer with hyperlipidemia and other dog species, and to discuss the mechanism of hyperlipidemia in Miniature Schnauzer. 922 dogs (441 female and 481 male, 1 to 17-YO) containing Miniature Schnauzer (MS, 12 female and 13 male) and Shetland sheepdog (SS, 14 female and 6 male) were examined in this study. 9 plasma metabolic parameters (glucose, TG, TC, TP, BUN, creatinine, LDH, AST, ALT), insulin and adiponectin values were measured and lipoprotein cholesterol profiles were investigated using polyacrylamide gel electrophoresis (PAGE). Miniature Schnauzer and Shetland sheepdog showed significantly higher concentrations of plasma TG and TC than other dog species. However the cause of hyperlipidemia was different between 2 species (MS and SS), MS showed hyper-TG concentrations and SS showed hyper-TC concentrations. In both species, hyper-TG and hyper-TC became apparent with aging. In Miniature Schnauzer, severe hyperlipidemia was dominant in female compared to in male.

4. Characterization of adiponectin in obese dogs

Adiponectin is a noted cytokine secreted from adipose tissue (adipcytokine), which decreased greatly in plasma of diabetic patients. There are few data about adiponectin in dogs with metabolic disorders compared to in human. In this study, biochemical and genetic characteristics of adiponectin were investigated in obese and control dogs. 21 obese dogs (10 female, 11 male, 3 to 15-YO) and 6 control dogs (5 female, 1 male, 1 to 10-YO) were used. 12 metabolic parameters above mentioned were measured and mRNA expression of adiponectin receptor 1 and 2 (ADIPOR1 and 2) were investigated using real time PCR (RT-PCR) in blood of dogs. Obese dogs showed significantly higher values of FFA, LDH and TC but lower values of adiponectin than control dogs. Obese dogs showed higher insulin concentrations than control dogs but the difference was not significant. Copy numbers of mRNA of ADIPOR1 were more than ADIPOR2 in both obese and control dogs, and copy number of ADIPOR1 and ADIPOR2 were 30 times and 8 times higher in the obese dogs than those in the control dogs, respectively. Increased expression of ADIPOR1 in obese dogs seemed to suggest induction of insulin resistance coinciding increased plasma insulin concentrations. Increased expression of adiponectin receptor was considered to be possible diagnostic marker to indicate onset of obesity at early stage. However negative data about relationship between obesity and adiponectin have been reported recently. Alterations of plasma adiponectin concentrations are controlled by castration, stage of obesity and meals, so these three factors should be discussed for measurement

of adiponectin in dogs.

5. Alterations in adiponectin and lipid metabolism in diabetic dogs

Changes in plasma adiponectin concentrations and lipid metabolism in diabetic dogs were investigated before (pre-) and after (post-) insulin treatments. In this study, plasma biochemical markers concentrations, lipoprotein profile and cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) activities were measured in diabetic and control dogs. Plasma concentrations of TNF-α, regulating factor for adiponectin, were measured coincidentally. In the diabetic dogs at post-insulin treatments, plasma TG concentrations significantly increased, whereas plasma ALT activities and FFA concentrations decreased significantly. Significant decrease in plasma TC, VLDL and LDL concentrations was obvious and HDL1 2 and 3 tended to decrease in the diabetic dogs post-insulin treatments. After insulin treatments, plasma CETP and PLTP activities increased but adiponectin concentrations decreased significantly (17%) in the diabetic dogs. Copy number of mRNA of ADIPOR1 was 4 times higher than that of ADIPOR2 in the diabetic dogs. mRNA expression of ADIPOR1 and ADIPOR2 decreased 905 and 80%, respectively, after insulin treatment in the diabetic dogs. Significant increases in CETP activity and decreases in adiponectin concentrations and mRNA expression of ADIPORs were prominent as changes in lipid metabolism in the diabetic dogs after insulin treatments. These findings suggest that diabetic dogs have the possibility to become atherosclerosis although dogs are HDL dominant animal with lower risk of atherosclerosis compared to LDL dominant animals. Decrease in plasma adiponectin concentrations are considered to be regulated by different signal pathways in obese and diabetic dogs. On the other hand, body fat is popular indicator for obesity in human medicine, and new trial to clarify the possibility of body fat as diagnostic indicator in dogs as in human are proceeded in our laboratory. Development of objective indicators like body fat is necessary for obesity in veterinary medicine as in human medicine. In the near future, use of objective criteria for metabolic disorders in dogs and cats as metabolic syndrome criteria for human is expected.

Bull. Nippon Vet. Life Sci. Univ., No.61, 122–123, 2012.

Study of an avian influenza vaccine prepared from an H5N1 reassortant virus of Eurasian lineage

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(Conferred on 25 November 2011, V B-302)

Influenza A viruses are divided into 16 H subtypes (H1?H16) and 9 N subtypes (N1?N9) on the basis of antigenic specificities of hemagglutinin and neuraminidase glycoproteins. All subtypes have been isolated from migrating waterfowls that are responsible for the global spread of these viruses. Because of repeated passages through chicken populations, these viruses have acquired high transmissibility and pathogenicity in chickens. This has resulted in highly pathogenic avian influenza (HPAI) outbreaks in domestic poultry.

HPAI outbreaks caused by H5 or H7 subtype viruses have occurred in many parts of the world, and such outbreaks have resulted in huge economic losses in the poultry industry. HPAI has two serologically and genetically distinguishable lineages, i.e., Eurasian and North American. Recent Japanese outbreaks were caused by Eurasian H5N1 type viruses that emerged in South Asia and that spread to Eurasia and Africa. In addition, direct transmission of H5N1 viruses from birds to humans caused high mortality.

Culling poultry is the standard measure adopted in most countries for the control of HPAI. Vaccines would be used as an optional tool to decrease virus shedding from infected chickens when culling could not efficiently control the outbreak. Although various commercial vaccines prepared from the North American lineage are available, these may be less effective for outbreaks caused by viruses of the Eurasian lineage. Thus, a vaccine prepared from a Eurasian lineage virus is required in Japan and Eurasian countries.

To develop a new H5N1 vaccine of Eurasian lineage, we isolated an H5N1 reassortant virus, A/duck/Hokkaido/Vac-1/04 (H5N1), that is non-pathogenic for chickens and chicken embryos and exhibits good growth in embryonated chicken eggs. We then characterized test vaccines of this reassortant virus.

The test vaccines comprised water-in-oil emulsions and various concentrations of the inactivated virus. The potencies of these test vaccines were evaluated by analyzing the triangular relationship among the antigen levels of test vaccines, hemagglutination inhibition (HI) antibody response, and protective effect against challenge with an HPAI virus. Neither clinical signs nor virus shedding after the challenge was observed in chickens when immunized with the test vaccines with an antigen level of 160 HA units/dose or higher. In addition, the minimum antigen level of the vaccine was 160 HA units/dose, and the minimum HI antibody titer was 1:16. An antigen dose-dependent HI antibody response was observed, and accordingly, 640 HA units/dose was concluded to be the optimal antigen level.

To elucidate the duration of effectiveness of ne

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vaccine injection, the test vaccine with 640 HA units/dose was intramuscularly injected into five 4-week-old chickens. The chickens were intranasally challenged with an HPAI virus 138 weeks after vaccination. All five chickens survived without clinical signs of HPAI. Limited virus titers were detected in the laryngopharyngeal swabs from only three of the five vaccinated chickens 2 days post-challenge, but not 4 and 14 days post-challenge. Thus, these results indicate that the test vaccine induces long lasting protective immunity in chickens.

Antibody responses in chickens against antigens are known to vary considerably among chicken breeds. We compared antibody responses to the vaccine in laboratory chickens of white leghorn breed and commercial layers of Julia and Boris-Brown breeds. Consequently, no obvious difference was observed among the breeds. This study thus indicated that the vaccine induced good and sufficient antibody response in both laboratory chickens and commercial layers.

In conclusion, the newly generated H5 N1 AI reassortant virus of the Eurasian lineage A/duck/Hokkaido/Vac-1/04 (H5 N1) can be used as a potent vaccine, particularly useful for outbreaks in Japan and other Eurasian countries.

Bull. Nippon Vet. Life Sci. Univ., No.61, 124–125, 2012.

Studies on characteristics and disorders of glucose and lipid metabolism in cats

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(Conferred on 25 November 2011, V B-303)

1. Characteristics in glucose and lipid metabolism in cats

Metabolite, insulin and adiponectin concentrations and LDH, AST and ALT activities were measured in plasma of 142 client-owned cats (1-13 years old. 16 breeds) to set up a new criterion of hypertriglyceridemia (hyper-TG) with increased plasma insulin concentrations for early diagnosis of lipid metabolism abnormality including obesity. Twenty-five cats with over 165 mg/dl of plasma triglyceride (TG) concentrations were decided as hyper-TG with increased plasma insulin concentrations, and prevalence of hyper-TG was 16.7% in young (1-6 years old) and 18.3% in old (>7 years old) cats examined. In the hyper-TG cats, their plasma TG concentrations increased to 6.6-7.4-fold of the values of control cats with 35-50mg/dl of plasma TG and their plasma cholesterol, free fatty acid (FFA) and insulin concentrations and LDH and ALT activities increased significantly, whereas their plasma adiponectin concentrations decreased significantly compared to those in the control cats. Hyper-TG cats with significantly increased body weights and plasma insulin and decreased plasma adiponectin seemed to be in early stage of obesity accompanying increased plasma insulin concentrations. Increased TG, insulin, LDH and ALT and decreased adiponectin values in plasma seemed to be key factors for diagnosis of lipid metabolism abnormality at early stage in cats.

2. Changes in plasma lipid concentrations in cats with aging

Plasma glucose, triglyceride (TG), total cholesterol (TC),free fatty acid (FFA), insulin and adiponectin concentrations and lipoprotein cholesterol profile (HDL,VLDL and LDL-cholesterol) were measured in cats with two different age categories (1～6 years old and >7 years old) to investigate change in plasma lipid concentrations in cats with aging. There were no significant differences in plasma glucose, TG, FFA and insulin concentrations between two age categories. Plasma TC concentrations in >7 years old cats were significantly higher than those in 1～6 years old cats. Plasma adiponectin concentrations tended to reduce in cats with aging. In plasma of 7 years old cats, HDL- cholesterol ratio decreased, whereas LDL-cholesterol ratio increased.

3. Changes in plasma lipid concentrations in obese cats

Feline obesity generally results in aberrations to plasma metabolite levels, such as lipid concentrations and lipoprotein composition. This study sought to investigate the resultant effect of obesity on cholesterol lipoprotein composition and circulating adiponectin concentrations in cats. Plasma glucose, lipids (triglyceride, cholesterol and

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free fatty acid), insulin and adiponectin concentrations, and cholesterol lipoprotein composition were measured and compared between BCS determined normal healthy control and obese cats. Although the obese group demonstrated higher levels of plasma cholesterol, glucose, and triglycerides, as compared to healthy controls, the difference was insignificant thus indicating that the BCS determined obese cats may have been overweight and not morbidly obese. Plasma insulin levels were significantly higher (25-30%) versus healthy control animals thereby possibly hinting at the ensuing emergence of obesity induced insulin resistance. However, the BCS determined obese cat demonstrated a significant reduction ($p<0.05$) in plasma adiponectin concentration and a significant increase ($p<0.05$) in LDL- cholesterol % as compared to age matched healthy control animals. This would indicate that changes in plasma adiponectin concentration and cholesterol lipoprotein composition may be good early indicators of obesity in cats.

4. Criterion of metabolic syndrome in cats

Onset of metabolic disorders which resembles to human metabolic syndrome (MS) based on visceral fat accumulation and insulin resistance has increased markedly in dogs and cats. We made temporary criteria for MS for cats conforming to human MS criterion. Animal with over 3.5 of body condition score (BCS) or 10% heavier than proper body weight plus any two of the following three factors: raised fasting plasma glucose (≥120 mg/dl), raised triglyceride (TG) (≥165 mg/dl) and/or total cholesterol (TC) (≥180 mg/dl) and raised alanine aminotransferase (ALT) level (≥100 IU/l) is diagnosed as metabolic syndrome. Reduced plasma adiponectin level (<3.0 μg/ml) and/or increased plasma insulin level (>3.0 ng/ml) fix the judgment of MS in cats.

5. Prevalence of metabolic syndrome in cats and clinical application of MS criterion in veterinary medicine

In chapter 4, we have made the temporary criteria of metabolic syndrome (MS) for cats. In this study, we investigated changes in plasma glucose (GLU), triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT), insulin and adiponectin as diagnostic factors in 60 volunteered clinically healthy cats in Setagaya District in Tokyo in September in 2010 to verify the usefulness of the criteria. With the criteria, cats with obesity as an essential factor, plus any two of the following three factors; raised plasma glucose concentrations, raised TG and/or TC concentrations and raised ALT activities, is diagnosed as MS. 16.7% (10/60) of the volunteered cats were diagnosed as MS with the above criteria. In the MS cats, plasma GLU, TC, AST, ALT and ALP levels were significantly higher than those in the controls (n=50) without MS. MS was not detected in cats with <3.5 body condition score (BCS), and the appearance rate of MS was highest in aged cats between 5 and 10 years old. With Additional factors, reduced plasma adiponectin concentrations and/or raised insulin concentration, confirm the diagnosis of MS for 6 of 10(60%) MS cats. The criterion could find latent MS animals at the early stage of obesity. For the animals, personal health record (PHR) appears to be effective to monitor each animal health condition, and its application is possibly useful to prevent shifting to the serious metabolic diseases such as diabetes mellitus (DM) and hyperlipemia. In the animals with MS, plasma adiponectin concentrations decreased significantly. Decreased adiponectin level is related to occurrence of various disease including DM and tumor. The criteria are potentially applied to prevent other diseases except MS in dog and cats. There are some hazards in blood sampling and selection of measurement substances to settle the criteria for dogs and cats, however the original criteria is necessary for study of preventive veterinary medicine.

Bull. Nippon Vet. Life Sci. Univ., No.61, 126–129, 2012.

Epidemiology of *α*-herpes virus infection
– Diagnosis and Seroepidemiology of SHBV and HSV –

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(Conferred on 12 March 2012, V B-304)

Herpesviridae are classified roughly into three sub-classes: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. Infections by Herpes simplex virus type 1 (HSV-1, *Human herpesvirus 1*), Herpes simplex virus type 2 (HSV-2, *Human herpesvirus 2*) and B virus (*Macacine herpesvirus 1*, Simian herpes B virus: SHBV), which are assigned to the *Alphaherpesvirinae*, are known to be important problems in humans. In addition, SHBV, which can cause fatal infection in humans, is recognized to be an important zoonotic pathogen in the field of veterinary science in which humans have opportunities to make contact with animals such as macaques, a natural host of SHBV. In general, it is difficult to distinguish SHBV from HSV-1 and HSV-2 using serological tests because the three viruses share many biological characteristics. Although ELISA has been developed as a serological diagnostic test for SHBV infection, cross reaction is observed among the three viruses. In addition, the serological diagnosis is performed currently with different equipment under different diagnostic standards, and thus the standard method using ELISA is not fully developed. This thesis consists of three chapters that summarize the results of a study that aimed to: develop a method of discrimination of the serological (type) of SHBV from HSV-1 and HSV-2 (Chapter 1); establish a diagnostic standard using the method developed in Chapter 1 (Chapter 2); and examine the current prevalence of HSV infection in Japan (Chapter 3).

Chapter 1: Identification of HSV-1,2 and SHBV antibodies in the serum of humans and monkeys.

In this chapter, the optimal conditions for a fluorescence ELISA were examined using recombinant SHBV glycoprotein D (SHBVgD), HSV-1, -2 gG (1, 2-gG), and HSV-1, 2-ELISA (1, 2-ELISA) to establish serological tests for SHBV, HSV-1 and HSV-2.

【Materials and methods】

A fluorescent unit (FU) value, which was estimated by measurement of the production of 4-MUG, was determined using a fluorescence ELISA. SHBVgD, 1, 2-gG, and 1, 2-ELISA were used as antigens, sera from rabbits immunized with HSV-1 or HSV-2 were used as antisera, and biotin-conjugated anti-human IgG, anti-monkey IgG, and anti-rabbit IgG were used as secondary antibodies. The specificity of the assay developed was evaluated using sera derived from SHBV-infected rhesus macaques and healthy individuals (24 samples), and sera from HSV-infected and healthy humans (26 samples).

【Results and discussion】

The optimum reaction was obtained using 3%

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BSA in blocking buffer, and dilutions of 1:100000, 1:40000 (6.25 ng/ml) and 1:40000 (50 ng/ml) of anti-rabbit IgG, anti-human IgG, and anti-monkey IgG, respectively. The greatest reaction was obtained using a dilution of 1:500 of SHBVgD antigen. Macaque and human sera showed the highest reaction with 0.1 and 1 μg/ml of 1, 2-gG antigen and 1, 2-ELISA antigen, respectively. A curve, dependent on the dilution of antiserum, was obtained under the optimal conditions. Low cross reactivity was observed between SHBVgD antigen and HSV-1, -2 immunized rabbit serum, and between 1, 2-gG antigen and SHBV positive serum. The results showed that the method could discriminate among SHBV specific antibody, HSV-1, -2 specific antibodies, and co-infection with HSV-1 and HSV-2. In the fluorescence ELISA using 1, 2-ELISA antigen, cross reaction between 1-ELISA antigen and SHBV positive serum was observed. Co-infection of SHBV with HSV-1 was thought to have occurred in the macaque because antibody to 1-gG was detected in a monkey infected with SHBV.

In this chapter, the author describes the development of a serological diagnostic method that has the ability to discriminate between SHBV and HSV-1, -2.

Chapter 2: Establishment of diagnostic methods for infection with HSV-1, HSV-2 and SHBV using fluorescence ELISA

In this study, the FU values of 1, 2-gG in sera from patients who had been diagnosed with HSV infection and in healthy persons were determined using the fluorescence ELISA and were used to develop diagnostic criteria (standards). The criteria were evaluated with respect to the discriminatory ability of the test to identify sera obtained from patients infected with HSV-1 and/or HSV-2, and the results were compared between the fluorescence ELISA using the serial dilution method (ordinary method) and the complement fixation test (CFT). Furthermore, the criteria (standards) were also developed for SHBV infection.

【Materials and methods】

All sera were diluted at 1:100 to determine the FU values, and some sera were tested in serial dilutions for titration of the antibody. The CFT was carried out by a standard titration method using a microplate. The sera used in this study were collected from 2007 to 2008; 418 and 32 samples were obtained from healthy persons and from patients, respectively. To develop the criteria for SHBV infection, the results obtained from rhesus macaques and humans in Chapter 1 were also used in this study.

【Results and discussion】

The distribution of FU values obtained from control sera (n=450) followed the Poisson distribution. The distribution of values that were obtained by subtraction of control values from sample values was not identical to the normal distribution. Thus, the FU% (fluorescence unit percentage) of antibody against 1-gG and 2-gG, the ratio of sample serum to control, was calculated for the sera diluted at 1:100. The distribution of the FU% values consisted of two normal distributions that crossed between FU% 130 and 150. In the evaluation of the accuracy of determination using HSV positive serum, high sensitivity and specificity were obtained (ST=SP=1.0). The FU% was not affected by the condition and the preservation period of the serum samples. The results obtained from sera diluted at 1:100 showed that FU%<130, 130≤FU%<150 and 150≤FU% were considered to represent negative, false-positive and positive samples, respectively. This estimation on the basis of FU% yielded a greater number of negative samples in comparison with the results obtained by the ordinary method of the fluorescence ELISA. In the CFT, antibody to HSV was detected in 191 of 418 samples, and the correlation coefficient between FU% and CFT was calculated to be R^2=0.9715. The identity of the results between FU% and CFT was 96.4%. On the other hand, 31 of 32 (96.9%) sera from patients infected with HSV were shown to contain antibody to 1, 2-gG, and the FU% was confirmed to be able to identify single infection with HSV-1 or HSV-2 and co-infection

with HSV-1 and HSV-2.

In the diagnosis of SHBV infection, the BFU% was also calculated as the ratio of the FU values obtained by the fluorescence ELISA using SHBVgD antigen to those using normal antigen. The results showed that the distribution of BFU% also consisted of two normal distributions, and that BFU%<150, 150≤BFU%<250, and 250≤BFU% were considered to represent negative, false-positive and positive results, respectively. This indicated that the false-positive and the negative results for macaque sera determined in the study described in Chapter 1 could be changed to positive and false-positive, respectively, and that false-positive results for human sera could be changed to positive.

In this study, HSV and SHBV infections could be detected by the FU% and BFU% recalculated from the sample FU values obtained by the fluorescence ELISA using 1, 2-gG antigen and SHBVgD antigen. High correlations were observed between the results obtained by the ordinary method of fluorescence ELISA and the CFT. Specific antibody against HSV-1 and HSV-2 was detected in 96.9% of serum samples from patients, which suggests that single and multiple infections with HSV-1 and HSV-2 can be discriminated by the criteria established in this chapter. However, additional examination is required to develop standards for the diagnosis of SHBV infection in humans.

Chapter 3 Seroepidemiology of HSV infection in humans in Japan.

In this chapter, infection with HSV in humans in Japan was evaluated by seroepidemiological analysis. A total of 418 healthy people, ranging from 0 to 88 years old, were examined for the presence of specific antibody to HSV.

【Materials and methods】

The FU% values from 450 serum samples were used, as cited in Chapter 2. In the CFT, an antibody titer of 8≤CF was considered to represent a positive result.

【Results and discussion】

Antibodies to 1-gG and 2-gG were detected in 37% and 5% of the individuals investigated, respectively. Detection of antibody to 2-gG in females was about four times higher than that in males, and the prevalence of antibodies to both 1-gG and 2-gG was also higher in females than in males. In the CFT, females showed higher positive rates than males, except for an antibody titer of 256 fold. In a comparison of 10-year age categories with respect to the FU% values, the prevalence of antibody increased with age and reached approximately 90% at more than 60 years old. Similar results were obtained using CFT. In addition, moving averages of FU% and CFT were calculated to investigate HSV infection. These results also indicated to divide the age range into five categories in males and three categories in females on the basis of the prevalence of HSV-1 and/or HSV-2 in Japan. Comparison of the prevalence of anti-1, 2-gG in 2008 and that of neutralizing antibody to HSV-1, 2 in 1970 showed that, although the prevalence of anti-1 gG was lower in all age groups in 2008 than in 1970, an increase in prevalence with age was observed using both tests. Similar phenomena were also observed in the prevalence of anti-2-gG. In a comparison of seroepidemiological results obtained by ELISA in 1973, 1983 and 1993, although the prevalence at the age of 20 in 2008 was higher than that in 1993, the prevalence at all other ages in 2008 was more than 20% lower than in 1993. The prevalence of anti-2-gG in 2008 was four to nine fold higher than that in 1993.

In this chapter, the author has clarified the characteristics of HSV infection by the use of seroepidemiology in healthy persons. It was shown, by analysis of the prevalence of HSV-1 and HSV-2 in Japan, that primary infection and reactivation from latent infection occurred with high frequency in older individuals. In addition, the presence of a serious problem with regard to HSV infection in Japan was identified by comparison of the results obtained from investigations that occurred at different times and in different locations.

Discrimination of HSV from SHBV infection was accomplished using FU% values, which were calculated from the FU values obtained by

fluorescence ELISA. This method could contribute to the spread of type-specific serological diagnosis and seroepidemiological study of HSV infection. In addition, statistical analysis using the moving average is a powerful tool for study of infection with HSV-1 and HSV-2 in Japan; using this technique, the peak FU% value was found to be identical to the highest CF antibody titer. In conclusion, the serological diagnostic methods developed in the present study can be applied to the diagnosis of SHBV and HSV infections in humans and macaques.

Bull. Nippon Vet. Life Sci. Univ., No.61, 130–132, 2012.

Differential colon DNA damage induced by azo food additives between rats and mice

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(Conferred on 12 March 2012, V B-305)

In Japan, many food additives, including colorings, are used. Some synthetic colorings have an azo structure, and they are reportedly genotoxic and carcinogenic. The relationship between synthetic azo dyes and human colon cancer has recently been suggested by an epidemiological study and a structure activity relationship examination. Of these red dyes widely used for confectionery, beverages, red pickled ginger, etc., amaranth, allura red and new coccine are synthetic azo dyes. These azo dyes were negative in a reverse mutation test using *Salmonella typhimurium* with or without S9 mix. Regarding carcinogenesis, the International Agency for Research on Cancer (IARC) classified amaranth as Group 3: Not classifiable regarding its carcinogenicity to humans, with the reasoning that the carcinogenicity test result was inconclusive. However, the acceptable daily intake (ADI) was established in the evaluation by the FAO/WHO Joint Expert Committee on Food Additives (JECFA). No carcinogenicity was detected in allura red or new coccine, and the ADI was set by the JECFA.

Generally, azo compounds undergo complex metabolic pathway in the body. Amaranth, allura red and new coccine are water-soluble azo dyes, and are poorly absorbed from the intestine when orally administered. When the intact dye reaches the colon, it can undergo extensive metabolic reduction by intestinal microflora, and the reductive cleavage products are rapidly absorbed. The degradation products are mostly aromatic amines. Aromatic amines are oxidatively metabolized by p450 in the liver, and metabolites reach the intestine again through the enterohepatic circulation, where they are further metabolized and show genotoxicity. Thus, the complex metabolic pathway may be the reasons for the negative results in Ames tests even with S9 mix.

The *in vivo* comet assay that has the advantage of reflecting the complicated pharmacokinetics of azo compounds would be more effective tool for detecting the organ specific DNA damage of these food additives. In this method, the nuclei are isolated from organs of animals exposed to the test substance and embedded in agarose gel, and the DNA is electrophoresed. Nuclei with an increased frequency of DNA damage display increased migration of DNA toward the anode. This DNA migration pattern looks like "the comet". An index of the DNA damage in this assay is the length of DNA migration which is determined the differences between the whole comet length and the diameter of the head. Amaranth, allura red and new coccine induced colon specific DNA damage in mice when examined using the *in vivo* comet assay. Thus, examining these red dyes in rats,

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which are often used for carcinogenicity tests, in the same manner as in mice and comparing DNA damage induction in target organs between mice and rats would provide useful information in examining species differences in genotoxicity and carcinogenicity. This study employed the *in vivo* comet assay to examine the genotoxicity of amaranth, allura red and new coccine in specific organs to demonstrate the differences in colon DNA damage between mice and rats.

Chapter 1. DNA damage induced by synthetic azo dyes in the *in vivo* comet assayin mice and rats

The organ specific DNA damage induced by amaranth, allura red and new coccine was examined using the *in vivo* comet assay. Mice were sacrificed 3 hr after the treatment of these red dyes (1 and 10 mg/kg), and rats were sacrificed 3, 6, 12 and 24 hr after the the treatment of these red dyes (10, 100 and 1000 mg/kg), and necropsied; brain, lung, liver, kidney, stomach, colon, bladder and bone marrow were removed.

In mice, each of amaranth, allura red and new coccine induced colon specific DNA damage at a dose of 10 mg/kg at 3 hr after the administration. In rats, neither the colon specific DNA damage nor the damages in the other organs tested were observed for any tested dyes with a wide dose range of 10 to 1000 mg/kg at any sampling times from 3 to 24 hr.

Chapter 2. Gastric emptying and intestinal transport in mice and rats

The information about the gastrointestinal transit of compounds, especially azo compounds, is important when their oral toxicity is assessed, in particular to justify the sampling time. In this experiment brilliant blue FCF (BB) were adopted as an indicator, and gastric emptying and intestinal transport in mice and rats were closely investigated. Mice and rats were orally administered with 3.5 mg/head and 18 mg/head of BB, respectively.

Gastric emptying at 5–60 min after the administration in the animals was calculated using a regression analysis. The half times of the gastric emptying were about 70 min for mice and 80 min for rats. The average contents of 57% and 58% were removed from the stomach 60 min after the oral administration to mice and rats, respectively. The intestinal transport demonstrated that in mice, BB reached the colon at 1 hr after the administration and reached almost the end of the intestine 3 hr after its administration to mice. On the other hand, in rats, after the administration, BB reached the colon at 3 hr after the administration and reached the end of the intestine 12 hr after its administration. These results revealed the intestinal transport was different between mice and rats, although the gastric emptying were almost the same.

Chapter 3. Conclusions

In chapter 1, the present results showing that amaranth, allura red and new coccine induced colon specific DNA damage at 10 mg/kg at 3 hr after the administration in mice confirmed the previously reported results. In rat, neither the colon specific DNA damage nor the damages in the other organs tested were observed for any tested dyes with a wide dose range of 10 to 1000 mg/kg at any sampling times from 3 to 24 hr. In chapter 2, the kinetics of orally administered compounds in the gastrointestinal tract of mice and rats were determined using BB as an index. The gastric emptying was almost the same in mice and rats. The intestinal transport demonstrated that in mice, BB reached the colon at 1 hr after the administration and reached almost the end of the intestine 3 hr after its administration. On the other hand, in rats, BB reached the colon at 3 hr after the administration and reached the end of the intestine 12 hr after its administration. These results show that a species difference exists in the intestinal transport between rats and mice. The assumption that the orally administered dyes should move together with the bowel content seems reasonable. Since none of these red dyes or BB influenced the food bolus movement in the gut of mice and rats, it was assumed that amaranth, allura red, new coccine and BB would show similar kinetics in the gastrointestinal tract. Under such assumptions, in mice, these red dyes may reach

the mouse colon 1 hr after the oral administration and stay in the colon until 3 hr after the administration. Based on the findings presented in Chapters 1 and 2, colon DNA damage in mice caused by these red dyes may appear within 2 hr after the arrival at the colon. In rats, after the administration these red dyes may arrive at the rat colon in 3 hr and stay in the colon until 12 hr after the administration. However, in rats, no DNA damage was induced by any of these red dyes at any dose of 10, 100 and 1000 mg/kg or any time-point of 3, 6, 12 and 24 hr, covering the time required for the induction of DNA damage in mice. These results suggest that based on the wide range of dose selection and the sampling times well covering bowel transit time, the absence of induction of DNA damage by amaranth, allura red and new coccine in rats may be intrinsic to the rat.

Based on the report that all the carcinogens inducing the DNA damage in mouse colon in the *in vivo* comet assay caused it also in rat colon, the sensitivity to genotoxins in the rat colon was considered not to be lower than that in mouse. Therefore, the insensitivity in rat colon may be excluded for the negative result in the *in vivo* comet assay in rats. Azo compounds are reduced by intestinal microflora to aromatic amines. The resulting aromatic amines are oxidatively metabolized by p450 in the liver after the absorption from the intestine. Thus, this unique azo-induced differential DNA damage might reflect the difference in complex metabolic pathway of these red dyes between mice and rats. The studies for the metabolic differences of these red dyes between mice and rats would be warranted.

Considering the current *in vivo* genotoxicity studies that do not include the concept of species differences and usually use only one species, the results of this study would provide useful evidence for necessity of concept for species differences in order to extrapolate animal data to humans. The present study also would be a prelude for the mechanistic studies of *in vivo* genotoxicity.

Bull. Nippon Vet. Life Sci. Univ., No.61, 133–134, 2012.

Studies on feeding, leptin and adiponectin levels in cyclic female rats and in neonatal female rats with high-fat diet

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(Conferred on 12 March 2012, V B-306)

A critical amount of energy reserve is necessary for puberty initiation, normal sexual maturation and maintenance of cyclicity and fertility in females of most species. The metabolic role of adipose tissue is as an energy storage compartment. To meet metabolic demands and needs of energy expenditure, adipose tissue secretes several proteins and bioactive peptides or adipokine. Leptin and adiponectin produced by adipose tissue may control reproductive function. Serum letpin levels are correlated with the degree of adiposity, and are regulated by feeding and fasting, whereas adiponectin levels are reduced in obese humans and rodents. It is found that leptin treatment can advance reproductive maturation in both undernourished and well-fed animals

The subject of this study consists of four categories. First, food intake during sexual cycle in female mice and rats were studied. Second, serum leptin and adiponectin levels and leptin mRNA expression in adipose tissue during estrous cycle in female rats were determined by ELISA and quantitative RT-PCR. Third, effects of estrogen on serum leptin level and leptin mRNA expression in adipose tissue in female rats were investigated. Fourth, early onset of reproductive function in female rats treated with high fat diet was studied.

The first study is presented in Chapter 2. The reproductive hormones have multiple effects on food intake, body weight and composition in rats, and voluntary exercise, suggesting that the hormone induces behavior changes which alter body weight and adiposity. The present study was conducted to determine food intake during 4-day estrous cycle in adult female mice and rats. The results obtained in this study showed that the pattern of food intake in the dark phase was greater ($p<0.01$) than in the light phase in female rats. Food intakes in the light and dark phases at proestrous and light phase at estrous stages were significantly lower ($p<0.05$) than other stages. Similarly, food intake in female mice tended to reduce at proestrous stage. In addition, body weights in female mice and rats tended to decrease at estrous stage. The author also confirmed that the decrease in food intake on proestrous stage is due to a direct effect of estrogen on the mechanisms that terminate short term food intake.

The second study is presented in Chapter 3. White adipose tissue (WAT) has been recognized not only as a reservoir of energy-rich molecules but also as an important and highly specialized endocrine and secretes a wide range of biologically

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active molecules. Leptin and adiponectin are among the cytokines produced by adipose tissue. They participate in the control of food intake, glucose and lipid homeostasis. The present study was conducted to determine serum leptin, adiponectin levels and leptin mRNA expression in adipose tissue during sexual cycle in female rats. The results showed that serum leptin level was significantly higher ($p<0.05$) at proestrous stage than other stages. The variation of adiponectin level throughout estrous cycle was not significant. According to the result, leptin mRNA expression in adipose tissue, likewise, serum leptin level varies significantly during estrous cycle, higher at proestrous stage. These findings suggest that increased serum leptin level and leptin mRNA expression induced by estrogen at proestrous stage may play a role in the regulation of appetitive behavior and reproductive function. Finally, the stable adiponectin level throughout estrous cycle indicates that this adipokine does not seem to play a considerable role in female cyclic reproductive function.

The third study is presented in Chapter 4. Estrogen is a potent anorectic agent that reduced both food intake and body weight, that are in the same direction of leptin, it causes a transient reduction in food intake and a moderate reduction in body weight in ovariectomized female mice. The fluctuation of serum leptin level in ovariectomized rats was eliminated by estrogen replacement. The present study was conducted to determine the effect of estrogen and progesterone on serum leptin level and leptin mRNA expression. Food consumption in control group (OVX) was significantly higher than that in Groups 2 (OVX+E2) and 3 (OVX+E2+P). Body weights were not significant differences among 3 groups. Serum leptin concentration and leptin mRNA expression were significantly higher in Groups 2 and 3 than Group 1, but no significant differences between groups 2 and 3. In conclusion, these results indicated that estrogen influenced on food intake as a consequence of positive effect on leptin mRNA expression and serum leptin concentration.

The forth study is presented in Chapter 5. The timing of sexual maturation is associated with body weight and composition. The increasing of nutrition before puberty as well as elevated weight gain and body fat may affect reproductive maturation and trigger the early onset of puberty. This study was to investigate the effects of high fat diet on serum leptin, adiponectin levels, leptin mRNA expression and puberty onset in weaning female rats. The date of vaginal opening in rats with high fat diet (HF) was significantly earlier than with normal diet (NF) ($p<0.001$). When showing vaginal opening, the body weights in weaning rats with HF diet was significantly lower, compared with NF diet ($p<0.001$). In the second experiment, all animals were sacrificed on day 26 of age. The rats with HF had a significantly heavier uterus ($p<0.05$) than with NF, whereas weights of body, ovaries and oviducts, and serum leptin and adiponectin levels and leptin mRNA expression were not significant differences between rats with HF and NF. These results suggest that leptin and adiponectin are not the primary signal that initiates the onset of puberty.

From the above results, the author confirms that the changes of food intake during estrous cycle were dependent on serum leptin and leptin mRNA expression levels, which are induced by estrogen, not progesterone. The consumption of high fat diet during prepubertal period can modify the endocrine response, not related with leptin level. Further investigation on the relationship between dietary fat and brain function, including reproductive function is needed.

These results may suggest that consumption of fat-enriched meal can alter the reproductive function with cognitive impairments in humans and animals. The author speculates that the fat-induced nutritional imbalance in young females may lead to neuroendocrine dysfunction during adolescence.

Bull. Nippon Vet. Life Sci. Univ., No.61, 135–136, 2012.

Studies on genetic diversity of Mongolian native white horse

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(Conferred on 12 March 2012, V B-307)

Many Mongolian native horses which have various coat colors live in the grassland of Inner Mongolia. In these semi-wildly horses, about 100 heads with white coat color are bred as one group in West-Wuzhumuqin.

In general, the coat color of horse has a lot of characteristics. The white coat color in horse is caused by the mutation of several genes regulating the development of melanocyte with the pigment granule. The grey horse is born colored, and most individuals become white by the age of 6-8 years. And the greying with age is an autosomal dominant trait, associated with gradual loss of hair pigmentation and high incidence of melanoma.

The Mongolian native white horse group included a sub-group of grey horse having a coat of white at birth. Moreover, this coat color did not change throughout the life of such horses and the development of melanoma symptoms in the skin of then was not observed. It is important that we research the isolation and expression of white coat color related-genes and the pathogenic mechanism of no melanoma symptom in the Mongolian native white horses having such characteristics.

At first, in this study, three basic coat color genes, MC1R, ASIP and TYRP1 are analyzed for gene frequency and gene polymorphisms in the Mongolian native white horses.

Peripheral blood was collected from 50 native white horses, and DNA was isolated from the blood by the phenol-chloroform extraction method. The three color coat genes (MC1R, ASIP and TYRP1) were analyzed with PCR amplification, and the PCR products were sequenced using the BigDye Terminator cycle sequencing kit. Three color coat genes were genotyped by PCR-RFLP, PCR-AFLP and PCR-direct sequencing methods. Gene frequency was calculated, and the deviation of the Hardy-Weinberg equilibrium were analyzed.

The Mongolian native white horses were classified into 3 colors, white (4%), grey (89%) and spotted (7%). Single nucleotide polymorphism (SNP, C901T) was found in the second domain region of MC1R gene, and the horses were classified to EE, Ee and ee genotypes. The gene frequency of *E* allele was 0.55. The deletion of 11bp was detected in the exon 2 of ASIP gene of the white horse. The gene frequency of *A* allele was 0.88 and the deviation of Hardy-Weinberg equilibrium was found between the observed value and the expected value of ASIP genotypes. The SNP (C189T) was also found in the exon 2 of TYRP1 gene and was classified to BB and Bb genotypes in the native white horse. No bb phenotype was found. The gene frequency of *B* allele was 0.98.

It is suggested that the Mongolian native white horse have the major coat color genes, however, the expression of those genes could be inhibited by the white coat color related-genes.

In the second, we examined the polymorphisms of Syntaxin 17 (STX17), Membrane-Associated Transporter Protein (MATP), Tobiano (To), Sabino

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(SB1) and KIT genes and its effects on greying or whitening the coat color of Mongolian native white horse. Five white coat color related-genes were genotyped by PCR-RFLP, PCR-AFLP and PCR-direct sequencing methods, respectively.

The genotype frequencies of GG and Gg were high in STX17 gene which related to the greying, and the G gene frequency was 0.69. $C^{cr}C^{cr}$ and CC^{cr} mutant-types were detected in MATP locus which is associated with the coat color dilution, and the C^{cr} gene frequency was 0.2. In the Tobiano gene related to the spotting pattern, Toto mutant-type was detected in four horses. However, the genotype variation was not recognized in KIT and Sabino genes. In addition, each one of cremello, polomino and dun was contained in the Mongolian native white horses. The white coat color in these horses was considered to be influenced by MATP gene.

As a result of analysis of genetic variation of white coat color gene in the Mongolian native white horses, it was confirmed that the early greying of coat color occurred by the STX17 gene.

In next, we elucidated the mechanism of early graying phenomenon by the STX17 gene mutation in Mongolian native grey horse. Skin tissues were collected from Mongolian native grey horses as well as Thoroughbred grey horses. Also melanoma lesions were removed from the multiple papules of the tail on the Thoroughbred grey horses. A histological test was then performed to ascertain the distribution of melanin granule in both the skin tissues and the melanoma samples. The distribution and quantity of melanin granule in the skin tissues of the Mongolian native grey horses were compared to the skin and melanoma samples from the Thoroughbred grey horses.

On the one hand, Melanin granule of only scant amounts was found in the melanocyte of hair follicle within the subcutaneous tissue taken from the Mongolian native grey horses. On the other hand, a SNP, close to the TATA box in the promoter region of the STX17 gene, was detected in such tissue.

Consequently, the early greying phenomenon in the Mongolian native grey horses is thought to be by means of a cis-acting abnormal-regulator with 4.6kb duplication in intron 6 of the STX17 gene, while the abnormal-transportation of melanin granule in melanosome is facilitate by means of a transcriptional regulator with SNP in the promoter region of STX17gene.

Furthermore, the nuclear receptor subfamily 4, group A, member 3 (NR4A3) gene, which is the cell cycle-regulatory gene, is closely associated with the STX17 gene in the chromosome 25 of horses. Therefore, the gene expression level of the STX17 and NR4A3 were then analyzed using Real-Time PCR with mRNA from the skin and melanoma samples taken from the Mongolian native grey horses and the Thoroughbred grey horses, respectively.

It was found that the expression of STX17 and NR4A3 were higher in the melanoma samples from the Thoroughbred grey horses than those in the skin tissues from the Mongolian grey horses.

The relation between coat color and temperaments in the Mongolian native horses was investigated. In Mongolian native horse, however, there was no difference of temperament between white coat and color coat. On a sex difference, the stallion has a high score in nervous, independence and excitability. In the mare, friendship and cooperation showed high value. Furthermore, in regard to the age, adaptation to training, memory and cooperation mark were high under 3 years old.

It is known that dopamine D4 receptor (DRD4) is related with temperament of the horse. In the exon 3 region of DRD4 gene, 18bp tandem repeat (VNTR) was found in the Mongolian native white horses. However, there was no polymorphism in this VNTR. In the exon 3 region of DRD4 gene, two SNPs with C147T and A292G were found in the Mongolian native white horses. Only GG genotype of the A292G substitution was detected in the Mongolian native white horses. This *G* allele showed high correlation with mild temperament including such as cooperation, obedience, patience and friendship in the Mongolian native white horses.

Bull. Nippon Vet. Life Sci. Univ., No.61, 137–140, 2012.

Research on the Improvement in Putting Technique of Golf
– Application of Open-field Behavior Analysis Method –

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(Conferred on 12 March 2012, V B-308)

In the game of golf, putting technique is an extremely important element in scoring. The reason for this is that if a golfer fails with a short putt of 1 0r 2 yards, that he should have, naturally, been able to score, he not only suffers a scoring loss, but in all the subsequent holes, he suffers residual heavy psychological damage.

In this manner, putting maintains an important position in both score making and mental playing, but there are still numerous unclear aspects of the fundamental principles and rules, and scientific clarification of these elements is lagging behind. In addition, throughout the broad range of guidance and correction methodologies, effective methods have not yet been clarified.

In this thesis, an attempt has been made to utilize the science of physics to verify those numerous unclear aspects of the fundamental principles and rules of putting, to bring to light the basic elements necessary for enhancing the cup-in ratio, and further, analyze, by means of an open-field behavior test, the golfer's psychogenic reaction, from the viewpoint of ethology, and, based upon the results of these investigations, to create basic links between those results and the guidance and correction methodologies.

1. **In the context of the basic putting stance, analysis was made upon the basis of data and documents of 80 first-class pro golfers, and, these materials were examined to determine the basic putting stance that should be made the index for all golfers from beginners through first-class.**

Figures were drawn out for such basic forms as grip (opposite overlap grip: 52%), stance (25 to 35 cm wide: 84%), ball placement (on a direct line from the left armpit: 95%), forward leaning posture: 35 to 45 degrees: 87%), body weight distribution (left: 45%, right: 55%), elbow position and form (ark-shaped stroke: 92%), position of hands (correspondence of hand fast and lie angle), line of vision (on the target line), and head position (parallel with the backbone).

Particularly, in the case of the line of vision, 85% of all pro golfers repeat 2 or 3 alternating glances at the cup and the ball as part of their routine, so it is assumed that alternating glances at the ball and the cup are important for the purpose of avoiding errors in the angle of the head and the open stance that is induced as a result.

In addition, as a physics approach, concerning the basic principles of putting stroke and cup-in ratio, an experimental position for putting was devised and produced, after which it was utilized for investigations. As a result, with the putter head

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allowed to float 12 to 14 mm above the ground surface and zero loft, it became clear that upon impact, a satisfactory rolling of the ball was obtained with a regular spin.

2. In order to aim toward improvement of putting technique, as a physics approach, concerning the shifts in the heart and emotions during a golfer's putting, based upon the open-field behavior test that is a method used in research on the shifts in animal behavior, observations and analyses were carried out (see Chapter 3). In the realm of analysis of putting behavior, observations were made of the golfer's routine and behavior during initiation of putting, and considerations were made concerning the relationship between behavior patterns and scoring.

1) Analysis of Behavior from arrival at the golf course to start of playing

As a result of questionnaires and practical surveys, it is thought that the golfer is exposed to psychogenic stress from the first stage of his arrival at the golf course. In other words, it is conjectured that such activities as moving to the golf course and action taken after arrival partially correspond to animal emotional behavior and engender a condition of sympathetic nerve predominance. Subsequently, along with the passing of time and routine behavior, within that environment, a stabilization of emotional behavior was observed (parasympathetic nervous system predominance), and it is thought that this is linked to scoring.

2) Analysis of Behavior Immediately Prior to Putting

It is said that the nearer an athlete approaches top class, the more unified his behavior prior to actual playing and his routine become. On the other hand, as a result of the player feeling pressure, insecurity, and impatience, changes manifest themselves in his immediately prior behavior. However, there is no experimental data that has been derived from actual measurement and analysis of the time taken immediately prior to playing (interval). So we observed the final stage of the putting routine and that behavior, and measured and examined the interval of top class golfers.

On the final day of the Masters, one of the 4 major tournaments in the world, analysis was made of the golfers who were competing for top place, and it was found that the behavior immediately prior to putting had a clear pattern and routine among the top class golfers, in accordance with which, a precise time distribution was memorized by their internal body clocks and carried out as physical behavior. In order to verify this, we carried out an additional experiment utilizing the same method.

The results revealed that pro golfers (A & B) executed the same behavior pattern (practice swings: 2, and line confirmation: 2 to 3) each time, and the time utilized during that behavior (interval) was 14.7 ± 1.28 seconds (A). In other words, it was proven that the behavior immediately prior to putting had a clear pattern and routine, and that it demonstrated a stabilized interval in accordance with their precise internal body clocks. In contrast, it was found that the time utilized by amateur golfers (from beginner to intermediate levels) was 21.3 ± 4.60 seconds (beginning golfer B), and 24.6 ± 3.50 seconds (intermediate golfer B), thus showing a triple to quadruple variation when compared with top class pro golfers.

On this point, the striking difference between pro golfers and amateur golfers (beginner and intermediate), can be considered in the following manner from the viewpoint of animal emotional behavior. In other words, it is thought that along with a buildup of experience, golfers, in the same manner as animals, enhance their response abilities to stress to make it possible for them to quickly regain presence of mind (parasympathetic nervous system predominance).

On the other hand, while Rory McIlroy remained in top position until the final day of the Masters, he subsequently missed short putts repeatedly, thus destroying his score to the extent that he dropped to tie in 15th place at the end. At the young age of

21, Mcllroy was under such pressure as tournament leader that, while he did not break down his putting routine and behavior pattern, in the context of his 'interval his stability and unification were reduced to zero.

It is surmised that it was 'psychological disturbance and 'insecurity, and 'hesitation that brought about this result. Mcllroy's intervals immediately prior to putting ranged from the low of 12.03 second, to 14.31 second, 14.62 seconds, 17.12 seconds, 17.21 seconds, 17.4 second, 18.68 seconds, 21.06 seconds, 24.46 second to a high of 36.87 seconds. Thus there was a 24.84 second difference between his lowest to his highest time, with an average deviation of 6.71 seconds. This shows an 'interval variation that is impossible to imagine among top class pro golfers who are in competition for the championship in the final round of the Masters.

But even so, a mere two months later, in the major tournament 2011 All-America Open, Rory Mcllroy once again held top position on the final day. In this short period of time, he had broadly altered his putting routine and interval. And on top of that, his new style kept him in top position from the opening day of the tournament, and allowing no one to outdo him, he ended up winning the championship.

In the Masters, his behavior pattern and routine consisted of 3 practice swings, 1 target confirmation, and, after setting, 2 more target confirmations. His interval was 20.0 ± 6.71 seconds (n=10). In contrast, his behavior pattern and routine at the All-America Open consisted of approaching the ball while looking at the cup, 2 target confirmations, no practice swings, 2 small movements of the right thumb, and a forward press. His interval was 10.4 ± 0.63 seconds (n=19). The major difference from his participation in the Masters, where he carried out practice swings 3 times before taking his final stance, was that he took no practice swings at all in the All-America Open. And accordingly, the time he took for his interval was reduced from 20.0 seconds to 10.4 seconds, meaning that he had reduced the time to approximately half. Also, his 'interval in which variation was seen at the Masters, was an extremely low average time of 0.63 in the All-America Open, meaning that his precise internal body clock brought about the same interval in his every stroke.

3. Concerning the method for correcting his putting stroke, a new guidance method that is not a conventional technique was developed and a correction tool was devised and a prototype made (see Chapter 4).

In order to efficiently master the basics that I mentioned in Chapter 2, it is thought that he made effective utilization of the correction tool and achieved unexpected results. Also, in Chapter 3-2, I gave the example of psychological pressure that caused a top class pro golfer (Rory Mcllroy) to become insecure in terms of his emotional behavior, destroying his score. And it is thought that he succeeded in correcting a delicate disorder in his stroke, that it had not been possible for a human instructor to discover, through utilization of this correction tool, that was effective as a tool for deleting emotional behavior and reviving his mental stability. I acquired the practical new idea No. 3045327 about devising of the correction tool, and when its time limit had expired, in 2010, I had the American pro coach Deen Thompson develop a putting stroke correction tool (see Figure 2), that took over almost all the guide rail guidance basic principles, excluding my basic principle of floating the head above the ground, and put it on sale on the general market.

As a result of the above accomplishments, it became clear that it is possible to search out a golfer's psychological stress using the open-field behavior test, and suggests that it is possible to apply the animal emotional behavior academic analysis method as one means for grasping the psychological condition of the average person.

In addition, it is thought that this knowledge is a significant index for beginning golfers or people who intend to begin playing golf in the future. Also, it is thought that even for people involved in guidance, this is a profitable guideline as one new

guidance method when focusing attention on ‘interval disruption. Further, it is thought that the devising and production of the putting stroke correction tool (practical new idea acquisition) that was based upon the ‘drilling theory can be utilized in practical education in movement science, and also, in the near future, it may also become applicable to experimental education in veterinary medicine.

Bull. Nippon Vet. Life Sci. Univ., No.61, 141–143, 2012.

Studies on the function of the hypothalamus and the pituitary posterior lobe in the hypophysectomized dog

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(Conferred on 12 March 2012, V B-309)

Pituitary-dependent hyperadrenocorticism (PDH) caused by an adrenocorticotrophic hormone (ACTH)-secreting corticotrophic adenoma is a common endocrine disorder in dogs. The diagnosis and medical treatment for PDH were main purposes in the past studies. In recently, the prevalence of advanced imaging modalities for diagnosis, such as computer tomography (CT) and magnetic resonance imaging (MRI), has enabled a detailed visualization of the pituitary tumors. Around the same time, transsphenoidal hypophysectomy was introduced as a treatment for canine PDH, and has been reported that hypophysectomy is associated with a longer survival time than medical treatment. However, hypohysectomy requires specialized techniques, and in dogs it is often difficult to distinguish adenoma from normal pituitary on imaging. Hence, most dogs with PDH are treated clinically to inhibit cortisol excess. Moreover, the major complication after hypophysectomy in dogs is central diabetes insipidus (CDI) because the whole pituitary is resected. The post-operative CDI presents within 24 hr after surgery, and spontaneously resolves within a few days. However, the mechanism for recovery remains to be clarified.

The aim of the present study was to investigate the posterior pituitary on MR images in normal dogs, and evaluate the diagnostic value of the posterior lobe displacement in PDH dog. Moreover, arginine vasopressin (AVP) secretory capacity in the thalamus and compensatory mechanism of the kidney was evaluated in hypohysectomized dogs to clarify the mechanism for spontaneous CDI recovery after hypophysectomy.

1. Relationship between arginine vasopressin and high signal intensity in the pituitary posterior lobe on T1-weighted MR images in dogs.

The posterior lobe of the pituitary gland (PL) normally shows characteristic high signal intensity (SI) on T1-weighted MR images (T1 WI) in humans. In addition, the characteristically high SI is markedly decreased by depletion of arginine vasopressin (AVP) due to disorders such as central diabetes insipidus. Although the posterior lobe of the normal pituitary in dog exhibits hyperintensity in T1 WIs as observed in humans, the cause of the hyperintensity has not been investigated. Therefore, in this study, we examined the relationship between SI in the PL based on T1 WIs and the concentrations of AVP circulating in the blood of normal Beagles after excessive AVP secretion was induced by hypertonic saline (HS) overload.

Under hypertonic saline overload, plasma AVP concentrations gradually increased, while the SI of

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the PL gradually decreased. We concluded that the high SI in the PL in T1 WIs in normal dogs was caused by AVP stored at the site, and examination of the SI in the PL using MRI is useful for diagnosis of abnormal pituitary glands.

2. Magnetic resonance imaging assessment of pituitary posterior lobe displacement in dogs with pituitary-dependent hyperadrenocorticism.

The prevalence of advanced imaging modalities for diagnosis has enabled a detailed visualization of the pituitary tumors. These imaging methods distinguish enlargements of the pituitary tumor by measuring the pituitary height/brain area (P/B) ratio and enable classification of lesions as macroadenoma or microadenoma. However, some cases of pituitary tumors have no apparent tumorigenesis on imaging, especially in the case of microadenoma. In this study, we investigated the relationship between enlargement of the pituitary and the position and SI of the pituitary posterior lobe using MRI examination of the PDH dogs. In addition, we examined the usefulness of these parameters as a diagnostic method for evaluation of pituitary tumors.

Compared with normal dogs, the posterior lobe was displaced dorsally in the pituitary of the PDH dogs. Correlation between the pituitary height/brain area (P/B) ratio and the displacement of the posterior lobe in the PDH dogs suggests that dorsal displacement of the posterior lobe increases in accordance with enlargement of the pituitary. As to the SI of the posterior lobe, the PDH dogs showed significantly lower SI in comparison to the normal dogs. Taken together, these results suggest that assessment of the displacement and SI of the posterior lobe of the pituitary on T1 WIs is useful for the diagnosis of pituitary adenoma.

3. Functional and morphological changes in the hypothalamus-pituitary posterior lobe system after hypophysectomy in the dog.

Hypophysectomy is recognized as a useful surgical treatment for canine PDH. However, in dogs, hypophysectomy induces acute CDI as a post-operative complication, which is believed to be the result of decreased AVP secretion. This sequence of events is a serious problem in humans as well. In the previous study, severe CDI developed within a few hr after hypophysectomy in normal dogs, but spontaneously resolves within a few days. Although, recovery of the AVP secretion has been believed to resolve the post-operative CDI, no report has clarified the mechanism in dogs. Therefore, in the present study, we investigated the disappearance of postoperative CDI. AVP secretion related to increases in Na^+ concentration and serum osmotic pressure was measured, and immunohistochemical analysis in the paraventricular and supraoptic nuclei was performed after hypophysectomy in normal dog.

In the hypertonic saline test, the plasma AVP concentration slightly increased in hypophysectomized dogs, although the increase was markedly smaller than that in normal dogs. An immunohistochemical study of the hypothalamus nucleus revealed that, AVP-positive cells tended to decrease after hypophysectomy. It suggests that excision of the posterior lobe by surgery injured the axon of magnocellular neuron in the hypothalamus. A decrease in the function and the number of AVP-producing and -secreting magnocellular neurons after hypophysectomy, suggests that the clinical improvement of postoperative CDI may not be related to the recovery of AVP secretion.

4. Expression of vasopressin V2 receptor and aquaporin mRNA in hypophysectomized dogs.

Since the previous chapter denied the recovery of the AVP secretion after hypophysectomy, compensatory increase of urinary concentration ability in the kidney has speculated as to resolve the post-operative CDI. In the renal collecting duct, water re-absorption is regulated by the AVP. Binding of AVP to the vasopressin V2 receptor (AVPR2) leads to the insertion of aquaporin (AQP)-2 water channels in the apical membrane, thereby allowing water re-absorption from the pro-urine to the interstitium. In the present study, we examined

the mRNA expressions of AVPR2 and AQP-1 to -4 in the kidney of hypophysectomized dogs to clarify the mechanism of the post-operative CDI recovery.

The mRNA expressions of AVPR2, AQP1, 2 and 3 were significantly greater in hypophysectomized dogs than in normal dogs.

From the results, it has been suggested that AVPR2 was upregulated in response to reductions of hormones in the state of post-hypophysectomy CDI, followed by an increase of AQP2 and AQP3 expression, which contributed to recover the urine concentrating ability. It was also suggested that an increase of AQP1 expression probably caused by elevated osmotic pressure of the blood was playing a role in recovering post-operative CDI.

As stated above, the present study has examined the imaging characteristic of the pituitary cortictroph adenoma on MRI, and the mechanism for recovery of the post-hypophysectomy CDI. In the study examined the relationship between imaging characteristic of the posterior pituitary on T1 WIs and the concentrations of AVP circulating in the blood of normal Beagles, it has been demonstrated that MRI may be useful for evaluating the AVP secretory capacity. The study examined the pituitary posterior lobe displacement suggested that assessment of the displacement and SI on T1 WIs is useful for the diagnosis of pituitary adenoma.

In the study examined the restorative mechanism of the post-operative CDI, the AVP secretion was not recovered sufficiently to resolve CDI even after a lapse of 3 months from the hypohysectomy. Thus, it has been suggested that the clinical improvement of postoperative CDI may not be related to the recovery of AVP secretion. On the other hand, up-regulations of the AVPR2 and AQP water channels has been shown to be involved in the disappearance of post-operative CDI.

From these studies, I suggest not evaluating the pituitary size alone, but assessing the displacement and SI of the posterior lobe of the pituitary on MRI may lead to more accurate diagnosis of pituitary adenoma. Moreover, the mechanism for recovery of the post-operative CDI had partially clarified. However, the majority still remains unclear. Further research is needed to clarify clinical state of the complications after hypophysectomy.

Bull. Nippon Vet. Life Sci. Univ., No.61, 144–146, 2012.

The immunohistochemical study of local expression of natriuretic peptide family (NPs) and their receptors (NPRs) in canine and feline heart muscle

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(Conferred on 12 March 2012, V B-310)

The present study was devised to clarify the immunohistochemical localization of natriuretic peptide hormones (NPs) and their receptors (NPRs) in the atria and ventricles of dogs and cats for the ultimate aim of using BNP and ANP in the clinical diagnosis and/or treatment of heart failure in small animals.

Chapter 1 deals with the characterization and research history of ANP (Atrial natriuretic peptide), BNP (Brain natriuretic peptide) and CNP (type C natriuretic peptide).

They are three types of natriuretic peptide hormone known to have similar molecular structures. ANP and BNP are synthesized by myocardial cells in connection with stretching of the cardiac wall, and released into the circulation. CNP is not present in the heart muscules, and is instead synthesized and secreted by the central nervous system and endothelial cells of blood vesseles.

Isolation of the natriuretic peptide family began with the isolation of atrial natriuretic peptide (ANP) from the human and the rat in 1983, followed by isolation of brain natriuretic peptide (BNP) from the porcine brain in1988. C-type natriuretic peptide (CNP) was subsequently isolated from the porcine brain in 1990.

ANP is synthesized and secreted in the atria, and BNP is firstly reported in the cerebral ventricles and also synthesized in the heart, in response to stretching of the myocardium, and also control of the rennin-angiotensin-aldosterone (RAA) system.

BNP is mainly secreted by the wall of atria and ventricles. The implication is, therefore, that ANP and BNP coexist within granules in the atrial muscle. This finding has also been confirmed by autoradiography. It appears that peptides synthesized in the atria are modified in the Golgi complex and temporarily stored in granules, before being released in response to a stimulus (regulated pathway), while peptides are released from the ventricles without modification whenever they are synthesized (constitutive pathway). It is, therefore, likely that the differences in distributional patterns of ANP which is mainly located at atria, and BNP which is mainly located at ventricles are a reflection of these differences in the process of secretion and release.

CNP is the third NPs. CNP is secreted in the vessel endothelium and the macrophage of the kidney, the lung and the heart, in response to proliferation control of the fibroblast.

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The distribution of receptors against ANP, BNP, and CNP closely resembles the distribution of ligands (ANP and BNP).

Chapter 2 deals with the results of pre-assessment of relationship between NT-proBNP value in blood of 50 dogs and their clinical diagnosis of cardiac conditions.

When NT-proBNP is evaluated with enzyme immune assay (EIA; canine cardio Screen.Guildhay Co., U.K) and regarded it as normal (lower than 210 pmol/L), grey zone (210~300 pmol/L), and pathologic (higher than 300 pmol/L), 13 out of 50 dogs were normal, 8 were grey and 29 were pathological. However , those scales are modified recently to that less than 900 pmol/L is normal, 900~1,800 pmol/L is grey zone and higher than 1,800 pmol/L is pathological. With re-evaluation of our results using of these new standard rule, it was modified as normal 44, grey five, and pathological one. Present evaluation of NT-proBNP level in dog suggested that one old animal might have the cardiac disorder.

However, the source of pathological NT-proBNP level has never be clarified enough.

In following chapters we studied immuno-histochemical distributions of NPs and NPRs in cardiac muscle of normal dog and cat.

Chapter 3 deals with the immunohistochemical localization of NPs (ANP, BNP and CNP) in heart of normal dog and cat.

Five crossbreed felines (two males and three females, all one year old) and three canines (one seven-year-old neutered female, one two-year-old female, and one three-year-old female) were used in this study. For immunohistochemical staining, Anti-human rabbit ANP polyclonal antibody (α -ANP [1-28]; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) , anti-human rabbit BNP polyclonal antibody (BNP [FL-134]; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-human goat CNP polyclonal antibody (CNP[C-19]]; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used, and the avidin-biotin/peroxidase complex (ABC) method was used to detect the immunoreactivity of primary antibodies in this study. Before every antibodies using of the immunohistochemistry, their immunocross-reactivities between ligands of dog or cat and each antibody were confirmed by the western bloting method.

The level of tissue expressions of ANP were higher in the atria than in the ventricles, and slightly higher in the left atrium than the right. This distributional pattern appears to result from the different secretory pathways of ANP and BNP. Through a regulated pathway in the atria, ANP and BNP, the ligands that bind with NPR-A and NPR-C, are synthesized in cardiac myocytes of the atria, subsequently stored in granular form in the normal myocardial cells, and when necessary released by secretion from the cell. In the ventricles, however, ANP and BNP are controlled by a constitutive pathway, in which the ligand peptides are synthesized and secreted as necessary by the ventricular cardiac myocytes. Thus, in normally functioning heart, ligand expression is constantly higher in the atria than the ventricles.

Chapter 4 deals with the Immunohistochemical localization of NPR-A, NPR-B, and NPR-C in heart of normal dog and cat.

Using animals and immuno-histochemical methods were same as NPs examinations, except for the antibodies for this study.

Specific antibodies were used for the receptor molecules that bonded to the various human NPR-A (H-125) rabbit polyclonal IgG, human NPR-B (N-18) goat polyclonal IgG, and human NPR-C (N-20) goat polyclonal IgG. All of them was purchased from Santa Cruz Biotechnologies Co. Ltd. CA, USA.

Before every antibodies using, their immuno-cro ssreactivities between dog and cat ligand and each antibody were confirmed by the western bloting method.

Comparing the dog to cat tissues, while there were some slight individual differences in the staining, expressions of NPR-A and NPR-C were stronger in dog than in cat. In both normal dog and cat, NPR-B, which bonded to CNP, showed

weak or no expression. Staining showed very little age-or sex-related difference in each species.

Expressions of NPR-A and -C was stronger in the atria than ventricles in dog and cat. Besides, their expressions in the canine ventricles were stronger in the right than the left. NPR-B expression was weak in both the atria and the ventricles, with no difference found between left and right atria or between left and right ventricles in dogs and cats. In addition, each NPR was widely distributed within the cardiac muscular cytoplasm rather than strongly localized on the cell membrane in dog and cat. There was no expression of NPRs in the nucleus of myocardial cells.

In canine and feline heart, the ligands ANP and BNP were more strongly expressed in the atria than in the ventricles, and their expressions were stronger in the left atrium than the right.

Chapter 5 deals with the conclusion of this study.

This study firstly revealed the immuno-histochemical distribution of natriuretic peptide hormones NPs;(ANP, BNP and CNP), and NPs receptor NPRs;(NPR-A, NPR-B and NPR-C) molecules in the heart of normal dog and cat. In general NPs reaction in canine heart was stronger than feline. It is unknown that it may reflect the difference between dog and cat.

This result of these morphological data provide useful information to promote the diagnosis and treatment of heart diseases in small animal clinic.

NPR-A and -C were found in the canine right ventricle than the left one, and as the receptors pattern waso reflected the relationship between ligand and receptor expression. At the same time, the weak expression of NPR-B in the cardiac tissue appears to be reflection of the scarcity or noexistence of CNP in the normal heart, only receptor may existed in the heart. These results are a clear reflection that the distribution of NPRs family matches the intramyocardial distribution of ligand molecules.

Bull. Nippon Vet. Life Sci. Univ., No.61, 147, 2012.

Molecular genetic analysis of *Uncoupling Protein 2* and *3* gene in dog

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(Conferred on 12 March 2012, VNT 11)

A mitochondrial protein called uncoupling protein (UCP) plays an important role in generating heat and burning calories by creating a pathway that allows dissipation of the proton electrochemical gradient across the inner mitochondrial membrane, without coupling to any other energy-consuming process.This pathway has been implicated in the regulation of body temperature, body composition, and glucose metabolism. Therefore, the UCP 2 and 3 have been considered as key candidate genes for dog obesity similarly to human. Here we report that molecular genetic analysis of *UCP2* and *UCP3* gene in dog. The dog UCP2 cDNA has a 321 bp 5'untranslated region (5'UTR) and 930 bp open reading frame (ORF). The dog UCP3 cDNA has a 365 bp 5'UTR and 936 bp ORF. We also identified a transcript variant of dog UCP3, which skipped exon 3.
These sequences have been assigned to GenBank as Accession No. AB611704: UCP2, AB611705: UCP3 and AB611706: UCP3 isoform. Comparison with the gene structure in humans, dog genome sequences and the transcripts in this study showed that dog *UCP2* gene consists of exons 1 to 8, and the start codon is located on exon3, and the dog *UCP3* gene consists of exons 1 to 7, and the start codon is located on exon2.The mRNA expression pattern of the *UCP2* and *UCP3* genes was investigated by RT-PCR using RT product obtained from total RNA of 27 canine tissues. UCP2 mRNA was expressed in all canine tissues. UCP3 mRNA was expressed in all canine tissues except for colon, and particularly abundantly in striated muscle. Differences in the distribution of DNA polymorphisms (SNP, INDEL), haplotypes and diplotypes in the each gene were examined in Shiba(n=19) and Shetland sheepdogs(n=19). Gene frequency was significant difference between the two breed. Haplotype, diplotype and LD block were tend to be difference in the two breed. The present data may provide us with information regarding influence of UCP 2 and UCP3 activity on canine energy balance.

*Supervisor : Prof. Toshinori OMI

Bull. Nippon Vet. Life Sci. Univ., No.61, 148, 2012.

Study on DNA identification by Short Tandem Repeat analysis in dog

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(Conferred on 12 March 2012, VNT 12)

Microsatellite DNA marker, i.e. Short Tandem Repeat (STR) marker is a class of nuclear DNA marker consisting of tandemly repeated sequence motifs of two to six base pairs in lengh. Microsatellite DNA marker is being internationally recognized as a genetic tool for individual identification and parentage control in dogs. In 2007, a new panel of 19 microsatellite markers is marketed from Finnzymes Diagnostics. This "Canine Genotypes Panel 1.1 Kit"contains the following 19 loci: AHTk211, CXX279, REN169O18, INU055, REN54P11, INRA21, AHT137, REN169D01, AHTh260, AHTk253, INU005, INU030, FH2848, AHT121, FH2054, REN162C04, AHTh171, REN247M23 and Amelogenin. These markers are included in the'core panel'of loci recommended by the International Society for Animal Genetics (ISAG). The aim of this study was to analyse the polymorphism of 19 microsatellite markers and their usefulness for individual identification and parentage control in 40 Golden Retriever (G.R), 40 Miniature Dachshund (M.Dachs), 4 Cavalier King Charles Spaniel with child-parent relationship, 2 Doberman Pinscher's oral mucosa and hairs in Japan. Amplified PCR products were analysed using 310 Genetic Analyser (ABI). The results of electrophoretic separation were analysed using GeneMapper v.4.0 sofware. The frequency of alleles detected was used to calculate the observed heterozygosity (H*o*), the expected heterozygosity (H*e*), the polymorphic information contents (PIC), the power of discrimination (PD), the accumulated power of discrimination (APD), the paternity exclusion (PE), the accumulated paternity exclusion (APE) and Hardy-Weinburg test for all 18 loci together except Amelogenin. The number of alleles per locus varied from 3 (AHTk253 and INU030) to 10 (AHT137) in G.R and from 3 (REN247M23) to 11 (AHT121) in M.Dachs. H*e* values ranged from 0.4706 (INU005) to 0.8541 (AHT137) in G.R and from 0.4870 (REN54P11) to 0.8516 (FH2054) in M.Dachs. APD was estimated to be $1 - 1.4337 \times 10^{-14}$ (G.R) and to be $1 - 1.61466 \times 10^{-15}$ (M.Dachs). APE was estimated to be 0.999934 (G.R) and to be 0.999975 (M.Dachs). These results showed the determination of genotype in 19 STR loci using the kit could be useful for DNA identification in digs.

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Bull. Nippon Vet. Life Sci. Univ., No.61, 149–150, 2012.

Evaluation of glucose monitoring used Continuous Glucose Monitoring System in healthy and diabetic dogs

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(Conferred on 12 March 2012, VNT 13)

Insulin administration is essential treatment for canine diabetes mellitus. Monitoring blood glucose concentration is essential for stabilizing newly diagnosed diabetic patients and in determining insulin requirements.

Monitoring blood glucose concentrations is essential to stabilize patients with newly diagnosed diabetes and to determine the insulin requirements. Repeated sampling every 1–2 h over a 12–24-h period is necessary to assess the effectiveness of insulin and its duration of action, as well as identify glucose troughs and peaks. However, repeated venipuncture can be stressful and painful for the patient and, there is a risk that a significant blood glucose peak or nadir will fall between two sampling times and will not be recorded.

A continuous glucose monitoring system (CGMS), Medtronic MiniMed CGMS Gold (Medtronic Inc., Tokyo, Japan), was recently approved in Japan to evaluate daily glucose profiles in patients with diabetes. A patient is usually given this device for 3 days. During this time, the patient intermittently checks the blood glucose level using an SMBG device and inputs the SMBG data into the CGMS device. By calibration with the SMBG data, the CGMS device can provide estimated blood glucose values every 5 min for 3 days. However, it has not been used in veterinary medicine in Japan.

The aim of this study was to evaluate the CGMS by using Medtronic MiniMed for its clinical application in healthy and diabetic dogs. We also tested two insulin preparations that are commonly used in diabetic dogs, neutral protamine Hagedorn (NPH) insulin and insulin detemir to investigate whether these drugs have difference effects on daily glucose variations.

Firstly, three days measurement of glucose concentrations by CGMS was evaluated in healthy dogs. No problems were encountered in placing the sensor in any of the dogs. The CGMS device and monitoring were tolerated by the dogs and the devices were worn for up to 3 days by each dog. None of the dogs showed any signs of inflammation or discomfort at the sensor site. We observed no abnormal behaviors, such as rolling, biting at, or rubbing the site of sensor placement, or chewing. Moreover, postprandial hyperglycemia was not observed in the healthy dogs unlike in humans. These results might be derived from the canine characteristics of glucose and insulin metabolism, slower digestion absorption and higher insulin sensitivity than humans.

Then, three days measurement of glucose

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concentrations by CGMS was evaluated in diabetic dogs. NPH insulin and insulin detemir were used to investigate whether these drugs have difference effects on daily glucose variations. There was a clear difference in the glucose profiles between Beagle dogs and Miniature Dachshund and Miniature Schnauzer when using NPH insulin. Also, there was an individual specificity and the characteristics of glucose profiles with each diabetic dog were not observed when using insulin detemir.

Interstitial glucose concentrations measured by CGMS are closely correlated with whole-blood glucose concentrations, and are sensitive to abrupt changes in blood glucose concentrations. In clinical medicine, CGMS is used to generate the maximum amount of information regarding the direction, extent, durability and frequency of glucose fluctuations throughout the day. Subcutaneous insertion of the sensor and wearing of the device were well tolerated by all of the dogs. The system is able to detect rapid changes in glucose concentrations and provides accurate measurements compared with measurement of venous blood glucose concentrations, with little or no discomfort to the dogs during sensor insertion or observation. In this study, the CGMS, worn similar to a Holter ECG monitor, was able to monitor the glucose concentration without apparent difficulties for 3 days. Furthermore, the increased frequency and simplicity of data collection made possible using CGMS have marked benefits for veterinary medicine.

Bull. Nippon Vet. Life Sci. Univ., No.61, 151, 2012.

Genetic structure of Fukushima macaque (*Macaca fuscata*) population

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(Conferred on 12 March 2012, VNT 14)

Mitochondrial DNA (mtDNA) control region, which is inherited maternally and exhibits a rapid evolutionary rate with high variability making it suitable for the quantification of intraspecific diversity, was examined to investigate the diversity and phylogeography of Japanese macaques. It reported a total of 53 unique haplotypes were observed for the 412 bp partial mtDNA control region sequence. While, human short tandem repeat (STR) polymorphisms are the most widely utilized type of genetic marker in population genetics and forensic DNA testing. Recently, human STR loci have applied nonhuman primates studies, for example chimpanzee, gorilla, orangutan, mandrill, bonobo, Japanese macaque, in Y chromosome.

Here, we reported genetic structure of Fukushima macaque (*Macaca fuscata*) population to use mtDNA and Y chromosome STR (Y-STR). 470 samples genotyping of mtDNA control region yielded a total of four haplotypes, JN01, JN03, JN04 and JN06. The frequency of JN01 was 99.2%, JN03 was 0.4%, JN04 and JN06 were 0.2% respectively and genetic diversity was low, 0.016. It was interested that JN03, JN04 and JN06 were yielded first time in Fukushima prefecture. We prepared 82 human Y-STR markers which reported amplification of nonhuman primates, investigated passively applied Japanese macaque. 12 Y-STR makers were amplification of male specific, and 10 of 12 Y-STR makers contained STR region, in addition, 6 of 10 Y-STR makers found polymorphisms. We determined 164 male samples genotype to use the 6 Y-STR makers which found polymorphisms. Later, we determined a total of 28 haplotypes. The frequency was 0.006 to 0.153 and haplotype diversity was 0.925. In this study, to find new markers and detect high haplotype diversity suggests we get new knowledge in ecosystem of Japanese macaque.

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Bull. Nippon Vet. Life Sci. Univ., No.61, 152–153, 2012.

A study on the prevalence of canine Lafora disease in Japan and analysis of its clinical features using the familial occurring pedigree

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(Conferred on 12 March 2012, VNT 15)

Canine Lafora disease (LD) is an autosomal recessive inherited disease characterized by late-onset, progressive myoclonus epilepsy and progressive neurological deterioration. Histologically, periodic acid Schiff-positive intracytoplasmic inclusions (Lafora bodies) caused by abnormal glycogen accumulation have been identified intracranially, particularly in the cerebellum and in the thalamic regions. Lafora bodies may also be present extracranially, for example, in the liver, spleen, lymph nodes, muscles and skin. Although the clinical diagnosis of canine LD is based on the histological finding of Lafora bodies in biopsy specimen, it is rare to detect it in clinically LD suspected dog. Therefore, the definitive diagnosis can only be made by postmortem examination. Additionally, it is absolutely difficult to diagnose in the early stage, because clinical signs are usually seen in dogs over 5 years of age.

A recent genetic study revealed that the expansion of an unstable dodecamer repeats in the *Epm2 b* gene is responsible for canine LD, especially in miniature wire-haired dachshunds (MWHDs). It allows us to diagnose subclinical canine LD cases by genetic test, but there are no reports concerning prevalence of canine LD in Japan. It is also unclear about latencies for the onset of the disease. To prevent spreading canine LD, we thus investigated breed predilections for canine LD in Japan, age of onset and the symptom of the disease, and further analyzed the clinical features.

First, we determined the prevalence of the duplication mutation using 24 dogs which had myoclonus epilepsy clinically. We found mutated EPM2 b gene in 85% (13/15) of MWHDs and in 100% (2/2) of Welsh corgies. Other canine bleeds did not have mutated gene. From these results, MWHDs who were known to the most common breeds in the world had also identified mutation occurred frequently in Japan, and we showed that the most common disease causing myoclonus epilepsy were Lafora disease. They also found a part of MWHDs in affected animals were inter-familial, and the mutation observed in Welsh corgies, we also detected point mutation.

On receiving the result of this study, next, we determined the prevalence of the duplication mutation of *Epm2 b* gene studing with 83 dogs of the familial occurring pedigree. Gene frequency in these MWHDs accounted for 69.3% of the identified mutations, this results shows duplication mutation were quite common in the pedigree. However, we identified mutation occurred frequently in non-affected dog, we have to assess changes over time.

Hence, in order to analyze for clinical features of

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canine Lafora disease, we followed up after two years. As a result, canine Lafora disease occurred myoclonus epilepsy with aging, and its mean age at onset were 7.5 years old. A change in light and noise were better method of examination among external stimuli for induce expression of myoclonus epilepsy. Additionally, we found that Alkaline Phosphatase in blood serum showed a high value in affected animals, and increased with aging.

This study shows that we detected canine Lafora disease in two breeds : MWHDs and Welsh corgis. Mutational analyses of responsible gene was available for 1) preventing the expansion of the disease especially in purebred dogs, 2) detection of familial forms of canine Lafora disease, 3) identification of mutation occurred frequently in non-affected dogs, and 4) detection of individual animals which potentially had duplication mutation of *Epm2 b* gene. Furthermore, we found that alkaline phosphatase activity in blood serum showed a high value in affected animals, and increased with aging, suggesting application to simple screening method for canine Lafora disease.

Bull. Nippon Vet. Life Sci. Univ., No.61, 154–156, 2012.

Analysis of chasing dog effects on moving the home range of troops in Japanese monkeys

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(Conferred on 12 March 2012, VNT 16)

Introduction

The annual cost of crop damage caused by Japanese macaques (*Macaca fuscata*, hereinafter referred to as "monkeys") throughout Japan has remained steady at approximately 1.6 billion yen in recent years, and this crop damage is also a problem in terms of creating a disincentive to engage in agricultural activities. A variety of countermeasures have been tried, but they have not produced satisfactory results for ecological reasons. A new countermeasure involving the use of dogs to chase monkeys has been conducted since 2005, and at least 54 municipalities have adopted this measure as of 2009. Because no long-term studies on this topic could be found in the literature, our aim in this research was to use long-term data to investigate in microscopic terms the effect of chasing distance and the effect of different types of land use on the appearance rate of monkeys, and to investigate in macroscopic terms the annual variations in home ranges and core areas of monkeys. In this manner, we sought to verify the effectiveness of chasing through comprehensive analysis.

Materials and Methods

A nationwide preliminary survey was carried out in order to gain insight into the use of dogs to chase monkeys and to select survey areas in order to verify the effects of the chasing activities. We examined four dogs for three troops of monkeys in Shibata City, Niigata Prefecture, and two dogs for two troops of monkeys in Mutsu City, Aomori Prefecture (one of these two troops of monkeys had a neighboring troop at the location to which they were chased). The chasing was limited to areas around dog owner's houses in Shibata City, whereas the dogs and their handlers moved around according to where the monkeys appeared in Mutsu City. In Shibata City, data about the location of monkeys and their distance from the dog owner's houses were collected, and the appearance rate of monkeys with respect to the distance from the dog owner's houses and data about the appearance rate of monkeys in farmland, forest areas, and border areas between farmland and forest areas within a radius of 1 km from the dog owner's houses were investigated. These results were classified according to the type of environment (farmland or forest) around the dog owner's houses and the degree (high or low) that monkeys occupied the area around the dog owner's houses before chasing, thereby enabling a comparison between the period before chasing started and the years following the start of chasing. Fisher's exact test was used to verify the significance of the appearance rate of monkeys. Because farmland and settlements are concentrated along roads in Mutsu City, the distances from a number of fixed points established on roads to the migration routes of the troops of monkeys were measured, and two-way analysis of variance was used to investigate whether any

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difference was observed in the number of chasing activities around each of the fixed points before and after the chasing started. In addition, as a common means of analysis between the two areas, home ranges and core areas throughout the year were calculated by using the fixed kernel method, and annual variations in these parameters were investigated.

Results

In Shibata City, combinations of dogs and target troops could be divided into three classes according to the type of environment around the dog owner's houses and the degree to which monkeys occupied the environment in question prior to the start of chasing. In cases where the area around a dog owner's house was forest and the degree to which monkeys occupied the area around the dog owner's house was high, it was found that there was a significant difference between the appearance rate of monkeys within 500 m of the dog owner's house between the period before chasing started (11.6%) and the second year onward after the start of chasing (3.8%, $p<0.05$). In the case mentioned above, a significant difference was found in the appearance rate of monkeys between the period before chasing started (11.1%) and the second year onward following the start of chasing (0.0%, $p<0.01$) in border areas between farmland and forest. In cases where the area around a dog owner's house was farmland and the degree to which monkeys occupied the areashii was high, a significant difference was found in the appearance rate of monkeys within 500 m of the dog owner's house between the period before chasing started (one troop: 6.8%, the other: 11.2%) and the second year onward following the start of chasing (one troop: 0.0%, $p<0.01$; the other: 3.7%, $p<0.05$). Furthermore, a significant difference was found according to the type of land use, with the appearance rate of monkeys on farmland being 4.4% before chasing started and 0.0% ($p<0.05$) from the second year onward following the start of chasing. In cases where the area around a dog owner's house was farmland and the degree to which monkeys occupied the area around the dog owner's house was low, no significant difference was observed, but the appearance rate of monkeys within 1 km of the dog owner's house decreased either partially or completely from the first year onward following the start of chasing. With regards to home ranges, in cases where a dog owner's house fell within a core area, the home range expanded into farmland where monkeys did not appear prior to the start of chasing. In cases where a dog owner's house was located on the outer edge of a home range, a part of the home range or core area moved away from the area around the dog owner's house following the start of chasing.

In Mutsu City, the distance to migration pathways increased following the start of chasing for the troop that had no neighboring troop after being chased, regardless of the number of chasing activities ($F=24.15$, $df=1$, $p<0.01$), and a part of their home range or core area moved away from farmland and settlements and expanded toward the mountains following the start of chasing. In the case of the troop that had a neighboring troop after being chased, interactions were seen in the number of chasing activities before and after the start of chasing ($F=12.97$, $df=2$, $p<0.01$), and as the number of chasing activities increased, the distance to migration pathways increased following the start of chasing ($F=415.77$, $df=2$, $p<0.01$).

Discussion

The distance effects seen in Shibata City were associated with the type of environment around the dog owner's houses and the degree to which monkeys occupied the area around the dog owner's house before the start of chasing. The appearance rate of monkeys according to the distance from a dog owner's house and the appearance rate of monkeys in farmland and border areas between farmland and forest decreased from the second year onward following the start of chasing. It is thought that in the first year after chasing starts, monkeys learn that chasing occurs in the area around a dog owner's house, but as a result of monkeys reappearing at the location and chasing occurring repeatedly in the first year, the chasing

provides negative reinforcement, and from the second year onward, the monkeys avoid the area around the dog owner's house. In terms of the effect on home ranges, the dog owner's house, from which chasing activities originate, being inside a core area was associated with a decrease in the appearance rate of monkeys around the dog owner's house and a corresponding increase in the appearance rate in a new location.

In Mutsu City, chasing the troop that had no neighboring troop after being chased produced distance-related effects even where chasing was not carried out. In the case of the troop that had a neighboring troop after being chased, however, because the distance decreased in locations where chasing was not carried out, it was clear that chasing the troop that had no neighboring troop after being chased exhibited better effects related to chasing distance.

Bull. Nippon Vet. Life Sci. Univ., No.61, 157, 2012.

Studies on Bovine Viral Diarrhea Virus Quasispecies in Field Strains

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(Conferred on 12 March 2012, VNT 17)

Bovine viral diarrhea virus (BVDV) is usually classified into a cytopathogenic (cp) and noncytopathogenic (ncp) virus based on the morphological changes of virus-infected cultured cells. Moreover, ncp BVDV is divided into 2 biotypes; one of the biotypes shows the exaltation of Newcastle disease virus (END) phenomenon (END phenomenon-positive; END^+), and the other does not show the END phenomenon (END phenomenon-negative; END^-) but interfere with the growth of vesicular stomatitis virus (VSV). It has been recognized that most of the Japanese BVDV isolates are END^+ viruses. Although END^- viruses is only isolated by long term passage or reverse plaque formation (RPF) method from population of END^+ viruses in laboratory, there is few report about the epidemic situation of END^- virus. The purpose of this study was to detect and quantify BVDV quasispecies such as the END^- virus in endemic BVDV strains, and to analyze a co-relation between quantity of these quasispecies and clinical sign on bovine viral diarrhea virus infections.

Forty five of BVDV isolates in Hokkaido prefecture ware analyzed by the END method, the RPF method based on heterologous interference phenomenon using VSV, and observation of cytopathic effect. As a result, these isolates investigated were divided into five groups. The first group was consisted of 10 isolates from which END^+ virus was only detected. The second included 25 isolates which consisted of END^+ virus as major and END^- virus as minor virus. Third was consisted of 7 isolates in which each virus was detected with same titer. Two isolates as forth group which showed only END^- virus-positive. The last was only 1 isolate which contained cp virus in this study. In the quantitative analysis, it was shown that the ratio of END^- virus against END^+ virus in the strain that contained each virus varied from 2 times to 435 times. These results suggest that BVDV field isolates not only consist of END^+ virus as major virus but also contain several quantity of END^- virus as minor virus, and that END^- virus surprisingly exists frequently in the field. Moreover, some field strains which mainly consisted of END^- virus were interestingly found, which would be a first report. On the other hand, any co-relations between quantity of END^- virus in the field strain and epidemiological dynamism such as animal age, clinical symptoms of BVD-MD, or BVDV genotype, were not found unfortunately. However, several strains which had no detectable END^- viruses were isolated from asymptomatic infected cows. The result suggests that the END^- virus might have the ability to influence the development of clinical symptom in BVD-MD.

*Supervisor : Prof. Akio FUKUSHO

Bull. Nippon Vet. Life Sci. Univ., No.61, 158–159, 2012.

Effects of Glucocorticoids on Insulin Signaling Gene Expression in Canine Leukocytes

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(Conferred on 12 March 2012, VNT 18)

Glucocorticoids reportedly induce insulin resistance to not only rodent and humans but also dogs and can be a risk factor of diabetes mellitus. However, the research investigated about the mechanism with the molecular level is hardly known for the dogs. As one of reasons, the harvest of classical insulin-responsive sensitive tissues (skeletal muscle and abdominal fat) is difficult. On the other hand, the analysis method of insulin signaling gene via peripheral leukocytes in which easy extraction is possible was reported. In this study, the influence that glucocorticoids cause insulin signal was investigated via canine peripheral leukocytes mainly focus on mRNA expression. The target gene measured insulin receptor substrate (IRS)-1, IRS-2, phoshatidylinositol 3-kinase (PI3-K), Akt-kinase (Akt)-2 and protein kinase C (PKC)-lambda, also glucose transporter (GLUT)-1 mRNA expression levels in canine neutrophil/lymphocytes by quantitative RT-PCR.

1. **Acute medication of glucocorticoids influence insulin signaling gene expression kinetics in canine peripheral neutrophil. (Chapter 1)**

In order to demonstrate that glucocorticoids influence insulin signaling gene expression in dogs, the acute medication experiment was conducted *in vivo*. As a result of administering methylprednisolone (2 mg/kg) intravenously for a healthy beagle dogs, significant decrease in IRS-1, PI3-K, PKC-lambda and GLUT1 mRNA level was observed in peripheral neutrophil. While no significant difference IRS-2 and Akt2 mRNA level was observed in peripheral neutrophil. That is to say, glucocorticoids inhibited the insulin signal of the dogs in the transcriptional level.

2. **In canine cultured lymphocytes, glucocorticoids influence insulin signaling gene expression and basal glucose uptake. (Chapter 2)**

Investigation was done about dosage dependence of glucocorticoids using the lymphocyte which has established the cultured *in vitro*. Dexamethsone of various concentrations added into the medium of cultured lymphocytes. 48 hrs later, the insulin signaling gene expression of the lymphocytes after cultured was measured. At the same time, measurement of basal glucose uptake using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose(2-NBDG) by Flow Cytometer. Significant decrease in IRS-1 while, significant increase in IRS-2 was observed. That is to say, IRS-1/2 mRNA expression level was dosage dependence of dexamethsone. Meanwhile no significant difference PI3-K, Akt2, PKC-lambda and GLUT1 was observed. Basal glucose uptake showed the fall tendency to dosage dependence. Difference between GLUT1 mRNA expression level is not change and basal glucose uptake is change might be caused by dexamethsone-stimulation time. That

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is to say, GLUT1 mRNA level might recover quickly than basal glucose uptake.

3. Revelation analysis of the insulin signaling gene in the peripheral neutrophil of the dogs of Cushing's syndrome. -Approach insulin resistance evaluation method- (Chapter 3)

As a clinical case with chronic hyper-glucocorticoids and reportedly insulin resistance, the dogs of Cushing's syndrom (CS) was used in this study. Insulin signaling mRNA expression in peripheral neutrophil was measured. In comparison with control group, IRS-1 mRNA expression was showed fall tendency and IRS-2 mRNA expression was reduced by ~ about 50% in CS group. Long-term hyper-glucocorticoids might actually inhibit not only IRS-1 mRNA expression level but also IRS-2. In comparison with control group, PI3-K mRNA expression of untreated group was significantly decreased and of treated group was showed recovery tendency. Akt2 mRNA expression of both group (untreated and treated group) was significantly decreased but PKC-lambda mRNA expression of both group was not changed. In comparison with control group, GLUT1 mRNA expression of untreated group was showed fall tendency. On the other hand, GLUT1 mRNA expression of treated group was almost comparable as the value of control group. Hence, it is possible that PI3-K and GLUT-1 mRNA expression was related with clinical state of CS.

For the result of this study, it was shown that inhibiting insulin signaling mRNA expression level by glucocorticoids is a possibility of becoming a cause of insulin resistance. In the dogs of CS, some genes (e.g. IRS-2, Akt2, PKC-lambda) expression kinetics were different. Hence, it was speculated that state in a molecular level is different between acute phase and chronic phase. Furthermore, I would like to perform further examination from now on and to expect that insulin signaling mRNA expression level will become useful as one of the clinical monitoring items of a dog by storing data.

Bull. Nippon Vet. Life Sci. Univ., No.61, 160, 2012.

Effect of diabetic prescription diets and oral hypoglycemic drugs on serum glucose and insulin concentration in obese cats

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(Conferred on 12 March 2012, VNT 19)

The prevalence of obesity is increased in cats. Obesity is a significant risk factor for diabetes in cats, since obesity induce insulin resistance.

The intravenous glucose tolerance tests (IVGTT) were given to healthy and obese cats in order to compare changes in serum glucose and insulin concentration (Chapter.1). IVGTT results indicated that glucose intolerance and excess insulin response were observed in obese cats as compared to healthy cats. These results suggested that obese cats had glucose intolerance and insulin resistance.

One of the treatments for obese cats is weight management. Several study used the test meal such like low-carbohydrate, high protein, low fat, and high fiber for the weight management in obese cats. In this study, the three different commercially available DM diets and prescription diet were used for comparing postprandial serum glucose and insulin concentration in obese cats (Chapter.2). The diets used in this study were as follows: A dry (DM diet: High protein), B dry (DM diet: High fiber), C dry (DM diet: High fat), and C/D dry (Control diet: High carbohydrate, low fiber). No significant difference in serum glucose and insulin levels were observed with all DM diets compared to Control diet. However, a significant increase was observed in Glucose-$AUC_{0\text{-}10hour}$ with the Control diet which had 30~50% higher carbohydrates than a dry or C dry. In addition, a significant reduction was observed in Insulin-$AUC_{0\text{-}10hour}$ with the DM diets as compared to Control diet. Thus, DM diets might provide better glycemic control than control diets in obese cat.

α-glycosidase inhibitor(αGI : Acarbose (AC), Voglibose (VO), and Miglitol (M)) are common oral hypoglycemic agents currently being used with humans suffering from Type 2 DM. None of drugs has been tested with obese cats thus far and it is currently unknown whether these drugs exert any effect in obese cats or not. The objective of this study was to determine the effect of AC, VO, and M on serum glucose and insulin responses in obese cats, in order to determine their potential use in diabetic cats (Chapter.3). No significant difference in serum glucose and insulin levels were observed with αGI group compared to Control group. However, significant decrease was observed in Glucose-$AUC_{0\text{-}10hour}$ with AC, VO group compared to Control group. In addition, Insulin-$AUC_{0\text{-}10hour}$ with AC, VO group was slightly decreased as compared to Control group. αGI reduced postprandial glucose elevation in the obese cats. As such, further studies with αGI need to be carried out in diabetes cats to truly assess the potential of these drugs.

*Supervisor : Prof. Toshinori SAKO

Bull. Nippon Vet. Life Sci. Univ., No.61, 161–162, 2012.

Study on effective utilization of wild boar (*Sus scrofa leucomystax*) in Agatsuma-gun, Gunma

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(Conferred on 12 March 2012, VNT 20)

Background

Wild boars are becoming more widespread across Japan, and crop damage attributed to these animals is a serious problem. The wild boar is highly fertile, and the expanding population has led to territorial expansion. Thus, population control by removal is an important strategy in wild boar management. However, the following problems are hampering efforts: the decline in the number of wild boar hunters due to aging, expected increases in the cost of wild boar management accompanying increases in the number of animals captured, and expected financial burden on the government due to increases in subsidy payments.

Utilization of captured wild boars is considered an option to solve these problems. More precisely, establishing a system wherein captured wild boars are sent to commercial facilities that process wild boar meat will improve the problems surrounding wild boar control. The number of such facilities increased from 13 across eight prefectures in fiscal year 2005 to 63 across 23 prefectures in fiscal year 2010. However, the effect of an increase in the number of wild boar processing facilities on the number of captured wild boars remains unclear.

In this study, we investigated whether wild boar utilization has a positive effect on animal capturing, which in turn contributes to the control of the wild boar population.

Materials and Methods

The status of wild boar capturing and processing, and factors influencing the processing rates and meat yield in Agatsuma-gun, Gunma Prefecture, were analyzed. Necessary items of information, albeit not in all cases, were available in Agatsuma-gun. Of the boars captured as pests over a four-year period between fiscal years 2007 and 2010, data of those with necessary information were extracted for analysis. The annual amount of crude meat was estimated based on the number of captured animals (by hunting and as pests) and their body weight. The proportion of processed animals (processing rate by number of boars) and that of processed crude meat (process rating by estimated crude meat volume) in each year were calculated. The season of capture, issues underlying the decision not to process the captured animals, conditions of animals requested by the processing facility (body weight $\geq$ 30 kg; within 1 h of blood draining) were analyzed as factors influencing the processing rate of captured wild boars. Briefly, captured wild boars were grouped into two groups by body weight ($<$ 30 kg and $\geq$30 kg) to obtain the proportion of each group. They were also grouped into three groups by the time required to reach the processing facility from the location of capture ($\leq$ 30 min, 30–60 min, and $\geq$ 61 min) to obtain details of wild boar capturing and the processing rate in each group. The processing rate in each season was also examined. Information

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on issues underlying the decision not to process the captured animals was obtained through interviews. Factors influencing meat yield were gender and body weight of animals, season and method of captivity, reasons for disposal, person who transported the animal to the processing facility, and bacteriological examination findings.

Results and discussion

Status of wild boar capturing

Between fiscal years 2007 and 2010, 4,176, wild boars were captured, including 1,780 by hunting and 2,396 as pests. The number of captured animals with necessary information was 1,448, and 84–99% were captured by a snare. A total of 627 boars were captured in fiscal year 2007, and this number increased to 1,484 in fiscal year 2010. A high proportion of captured animals were adults, suggesting many of the captured wild boars were sexually mature. On the other hand, a continuous increase in the number of captured wild boars suggested that the absolute number of captured animals was insufficient in this period. The estimated amount of crude meat was approximately 68 t in 2010, which was the highest in the four-year period.

Status of wild boar processing

A total of 799 wild boars were processed in the four-year period, including 270 captured by hunting and 529 captured as pests. The processing rate was highest in 2010 (20%), although it remained largely unchanged over the four-year period. The amount of crude meat processed was approximately 20 t in 2010. The processing rate by estimating crude meat was around 29%.

Factors influencing processing

Over the four-year period, 65–74% of the captured wild boars weighed at least 30 kg. This means that approximately 30% did not meet the weight requirements of the processing facility. The processing rate was 25% when the location of wild boar capture was within 30 min of the processing facility and 7% when more than 30 min from the processing facility. The interview survey revealed that 57% of those who captured wild boars decided not to transfer the animals to the processing facility due to issues associated with time and distance and possible inadequacies of killing and blood draining, suggesting that such factors restrict the processing of captured wild boars.

Factors influencing meat yield

The difference in the meat yield among animals was more than 10%. Damage and sweating decreased the yield. In addition, the adequacy of killing and blood draining procedures used by capturers influenced meat yield.

Conclusion

A continuous increase in the total number of wild boars captured in Agatsuma-gun between fiscal years 2007 and 2010 suggests that the number of captured animals was insufficient.

Our findings showed that the processing rate drops when the location of capture is far from the processing facility. Thus, establishing new facilities is important to process more captured wild boars. Furthermore, establishing an encouraging environment for capturing, such as that with incentives, is necessary. Moreover, it is worth considering the introduction of less strict or modified requirements for accepting animals for processing. Conversely, providing instructions for appropriate killing and blood draining procedures to capturers may increase meat yield.

This study revealed that the utilization of captured animals is currently not sufficient to contribute to the control of the wild boar population. It is important to improve the processing rate across Agatsuma-gun and to capture more wild boars.

Bull. Nippon Vet. Life Sci. Univ., No.61, 163-164, 2012.

Studies on the production mechanism of Wagyu beef aroma

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(Conferred on 12 March 2012, ALS 35)

In our recent studies, some lactones and aldehydes were presumed to be responsible for Wagyu (Japanese Black cattle) beef aroma, the preferable sweet and fatty aroma[1]. Those compounds obtained by simultaneous distillation / extraction from Wagyu beef were detected by gas chromatography-mass spectrometry (GC-MS) and GC-sniffing. Moreover, the differences among aroma components extracted from fats of Wagyu beef, crossbred beef (Wagyu × Holstein), dairy cattle beef and imported beef were demonstrated by a solid-phase microextraction (SPME) technique[2].

Oleic acid proportion has been attracted as the palatability indicator of Wagyu beef, because the previous report showed that oleic acid had a positive effect on beef flavor[3]. Furthermore, oleic acid may have been one of the precursors of lactones.

In order to reveal the production mechanism of Wagyu beef aroma, first, the extraction conditions were examined to obtain aroma components directly from minced Wagyu beef. Second, the relationship was investigated between chemical and grading data (relative proportions of oleic acid in fat, crude fat contents and beef marbling standard (BMS) numbers), the sensory evaluation values and contents of volatile compounds in Wagyu beef. Third, the aroma produced by lipase and/or heat treatments of Wagyu fat was examined by sensory evaluation and gas GC-MS.

1. The examination of conditions for extracting aroma components directly from heated ground beef by SPME technique

The extraction conditions of aroma components from ground beef were examined, because ground beef is more similar to ordinary eating stuff than beef fat. As a result, 1 g of ground beef is put into a glass crimp top vial, sealed with Viton(R) septa and heated at 80 ℃ for 50 min. The SPME fiber (50/30?m DVB/Car/PDMS) was then exposed to the headspace vapor.

2. The relationship between chemical and grading data, sensory evaluation values and aroma components in ground Wagyu beef

1) Correlation between chemical analysis and grading data, and the sensory evaluation values

Nineteen Wagyu beef samples belonging to nine areas classified by three relative proportions of oleic acid (under 51%, 51-54%, and over 54%) and marbling grades (3, 4, and 5) were sliced, cooked in 1% NaCl solution at 83℃ for 2 min, and provided to the sensory evaluation. In the scoring test, tenderness, smoothness of fat, strength of Wagyu beef aroma and strength of umami were evaluated using a five point scale. In the paired comparison test, smoothness of fat, strength of Wagyu beef aroma, strength of umami and total palatability instead of tenderness were evaluated.

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As a result of the scoring test, the scores of tenderness, smoothness of fat, and strength of Wagyu beef aroma correlated moderate-positively with BMS numbers and crude fat contents, with correlation coefficient ranging from 0.3 to 0.8, while that of the aroma strength correlated weak-positively with relative proportions of oleic acid, with correlation coefficient ranging from 0.1 to 0.3. In the three areas of marbling grade 3, all scores of evaluation items were zero or minus. In the three areas of marbling grade 4, the scores tended to rise with increasing the proportion of oleic acid. Although the score changes in the areas of marbling grade 5 was similar to those in the areas of marbling grade 4, there were some differences between two grades.

The result of paired comparison test between two beef samples with high and low proportions of oleic acid in the same marbling grades showed that the beef with high proportion of oleic acid tended to have stronger Wagyu beef aroma than that with the low proportion of oleic acid. Therefore, it was suggested that the value of sensory evaluation items such as strength of Wagyu beef aroma is related clearer with BMS numbers and crude fat contents than with the relative proportion of oleic acid.

2) Correlation between chemical and grading data and the amounts of aroma components

Aroma components obtained by heating Wagyu beef used for sensory evaluation were analyzed by GC-MS. The relative proportion of oleic acid was correlated weak-positively with the amounts of lactones contributing to Wagyu beef aroma and correlated negatively with the amounts of aldehydes. The relative proportion of oleic acid was correlated positively with the amounts of most of acids. The crude fat contents and BMS numbers were correlated weak- or moderate-positively with the amounts of lactones, and correlated weak- or moderate-negatively with the amounts of aldehydes.

These results showed that the relation of the proportion of oleic acid with each aroma component was complicated, and the rise of the proportion of oleic acid did not increase necessarily the amounts of volatile compounds contributing to Wagyu beef aroma.

3. Sensory evaluation and aroma components analysis of the aroma produced by lipase and/or heat treatments of Wagyu fat

1) Sensory evaluation of the aroma produced by lipase and/or heat treatment of Wagyu fat

The paired comparison test demonstrated that lipase treatments of fat samples intensified significantly fatty aroma. When fat samples were treated by heating at 80℃ after lipase treatment, fatty aroma was further intensified.

2) Changes of aroma components from Wagyu fat caused by lipase and/or heat treatments

Aroma components were extracted by SPME technique from four fat samples treated with lipase and/or heated, and analyzed by GC-MS. Consequently, some acids were raised by lipase treatment, suggesting contribution of those to the fatty aroma. Although acids were decreased by heat treatment after lipase treatment, some aldehydes tended to increase. These complex changes might explain fatty aroma increase by heat treatment after lipase treatment. In addition, γ-nonalactone contributing to Wagyu beef aroma tended to increase by either of lipase treatments or heat treatments.

These results showed two possibilities that acids and other components contribute to fatty aroma relating with Wagyu beef aroma, and that lactones and other components contributing to Wagyu beef aroma were produced by either of lipase treatments and heat treatments.

1) Masanori MATUSISHI et al., *Nihon Chikusan Gakkaiho,* 75, 409-415(2004)
2) Yuka TAKAHAMA, *Nippon Veterinary and Life Science University Master Thesis,* (2009)
3) Westerling, D.B. and H.B. Hedrick, *J. Anim. Sci.,* **48**, 1343-1348(1979)

Bull. Nippon Vet. Life Sci. Univ., No.61, 165–166, 2012.

Invention of a novel ripening cheese that reuses whey proteins

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(Conferred on 12 March 2012, ALS 36)

In cheese manufacturing, about 90% of the moisture of cow's milk is discharged as cheese whey. On the drained whey, which contains abundant protein nitrogen, exceeds the emission standard established at the Ministry of Environment and therefore cannot be discarded directly. A major cheese manufacturing company that discharges 330,000 tons of cheese whey per year has established a processing system that reuses organic matter, such as protein. However, smaller cheese manufacturing companies that discharge about 1200 tons per year cannot process whey by product as industrial waste, since it is difficult to perform the above-mentioned processing profitably. Therefore, this study examined a method of condensing the whey protein that a smaller scale manufacturer can also carry out.

Then, in order to examine the quality of the new cheese manufactured with reused whey protein (CW) of the obtained concentration, componential analysis of the CW addition in the ripening period and CW-free cheese was performed. The method of proteinic recovery from cheese whey was examined. As result, it was found that the steam injection method adjusted to pH 4.6 is optimal, and this was used for CW. Two kinds of cheese, the Gouda type and the Grana type, were manufactured using 10 L pasteurized (63℃, 30 minutes) cow's milk for examination of the new cheese manufacturing which reuses CW. CW was added in a specified quantity by milk volume. The fat content in cow milk for the Grana type cheese was adjusted to 1.3%. The CW maintenance rate in cheese was high at 60% in the Grana type and 91% in the Gouda type. The effect and maintenance rate of the steam injection method did not fall even if performed continuously, It is suggested that the cost reduction of cheese processing and mitigation of the environmental impact by abandonment of protein nitrogen can be attained by manufacturing using this process for adding CW for milk. No significant difference was observed in the amount of soluble nitrogen and total amount of amino acid in either the Gouda cheese CW addition type and CW-free type. On the other hand, in the Grana type, the amount of soluble nitrogen in the CW addition division increased more markedly than the CW-free division, and attained about 1.5 times that of the CW-free division in the 90th day of ripening, including the total amount of amino acid. The break examination using a rheo-meter (NRM-2010J) showed the influence which it has on the cheese curd by CW addition. Cheeses ripend 90 days were used for the experiment. It was found that the structure that collapses easily due to fracture stress becoming low was evident in the CW addition division rather than the CW-free division. This suggests that it is possible to obtain a texture peculiar to very-hard type cheese, such as the Parmigiano Reggiano cheese, in the early stage of ripening.

Hence, CW can be obtained simply and efficiently by performing pH control and using the steam injection method to obtain cheese whey.

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Moreover, when CW is taken in the curd by adding the CW in the manufacturing process of very-hard type cheeses, it decomposes with the maturing period, and a peculiar texture is obtained. This suggests that the mitigation and cost reduction of the environmental impact by protein nitrogen abandonment can be attained by repeatedly carrying out this manufacturing process.

Bull. Nippon Vet. Life Sci. Univ., No.61, 167–168, 2012.

Studies on the factors related to cholesteryl ester metabolism in the chicken liver around hatching

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(Conferred on 12 March 2012, ALS 37)

In birds, nutrient sources are changed from lipid-rich yolk at pre-hatching to less-lipid external food at post-hatching and therefore, a large amount of cholesteryl ester accumulates in the liver during late embryonic development and rapidly disappears during early post-hatch period. In mammals, enzymes participating the synthesis and hydrolysis of cholesteryl ester are known to be acyl-CoA+ : cholesterol acyltransferase (ACAT) and lysosomal acid lipase (LAL), respectively. In human, defects of LAL gene results in disorder of liver function due to the excess accumulation of cholesteryl ester. In birds, free fatty acid generated by hydrolysis of the accumulated cholesteryl ester is a major energy source around hatching. Cholesterol derived from the cholesteryl ester is utilized for the synthesis of bile acid required for lipid digestion in the intestinal tract. However, in avian species, enzymes participating in the synthesis and hydrolysis of the cholesteryl ester have not been identified.

In mammals, neurotensin (NT), a brain-gut peptide, has been known to be involved in the regulation of sugar and lipid metabolism as well as the regulation of gastrointestinal motility. In chicken, NT has been shown to participate in enterohepatic circulation of bile acid. Previous studies by Numao have shown that NT precursor mRNA is abundantly expressed in the intestinal tissues from late-embryonic period to early post-hatch period. In addition, our preliminary results showed that the expression levels of NT receptor 1 (NTR1) mRNA markedly increase after hatching in the liver. These results imply that NT synthesized in the intestine is involved in the regulation of the cholesteryl ester metabolism in the liver around hatching. To clarify the function of NT on the cholesteryl ester metabolism around hatching, expression patterns of NT precursor, NTR1, ACAT1, and LAL mRNAs in the intestinal tissues and liver were investigated. In addition, expression profile of a bile acid transporter, SLC10A2, which works for the adsorption of bile acid in intestine was examined.

To determine expression levels of mRNAs for NT precursor, NTR1, ACAT1, LAL, and SLC10A2, total RNA was extracted from the liver and intestinal tissues and was reverse-transcribed. The cDNA products were subjected to real-time PCR. The expression levels of NT precursor mRNA in the intestinal tissues increased around hatching with maximum levels at day 1 post-hatch in the duodenum and jejunum, and at day 3 post-hatch in the ileum and colon/rectum. Our previous study has shown that the expression levels of NTR1 mRNA are higher throughout the late-embryonic period than those of the post-hatch period in the duodenum and jejunum. In the ileum, the expression levels remains constant around hatching and the level of the colon/rectum increases after

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the hatch. Since NT has been shown to stimulate growth of mammalian intestine, NT may possibly participate in the growth of the duodenum and jejunum during the embryonic period as well as in the digestive function during post-hatch period in chicken.

The expression levels NTR1 mRNA in the liver of adult hen was 20-fold higher than that of the colon/rectum and that the expression levels markedly increased during day 3 to day7 post-hatch then gradually decreased to the adult level. These results suggest that NT-NTR1 system may play an important role in the cholesteryl ester metabolism in the chicken liver around hatching. To clarify the roles of NT-NTR1 system, chicken ACAT1 and LAL were identified by cDNA cloning for the first time in avian species. First, The ACAT1 and LAL cDNAs were amplified by PCR from a chicken liver cDNA library. The amplified cDNAs were ligated with a plasmid vector and cloned into E.coli. The plasmid DNAs were isolated from E.coli and subjected to sequencing. Chicken ACAT1 encoded in the cloned cDNA consisted of 551 amino acid residues and contained consensus sequences for the catalytic site, substrate binding site, and disulfide-bridge forming site. Chicken LAL consisted of 398 amino acid residues containing two catalytic sites for esterase and two N-linked glycosylation sites as observed in mammalian LAL. Real time PCR showed that expression of ACAT1 mRNA remained at high level from day 14 embryo to day 1 post-hatch, then decreased. On the other hand, the expression of LAL mRNA remained low level and increased from 3 days after hatching, reached maximum at day 10 post-hatch, then decreased. This expression pattern of LAL mRNA is similar to that of NTR1 mRNA, suggesting that LAL gene may possibly be a target of NT-NTR1 signaling system in the liver during the early post-hatch period.

In chicken, NT has been known to stimulate enterohepatic circulation of bile acid. In mammals, multiple bile-acid-transporter proteins have been identified in the liver and intestine. A bile-acid-transporter protein, SLC10A2, has recently been identified in the chicken intestine by cDNA cloning in our laboratory. The mRNA has been shown to be expressed exclusively in the ileum and colon/rectum. In the present study, real time PCR analysis revealed that SLC10A2 mRNA in the ileum and colon/rectum became detectable after hatching, increased until 7 days after hatching, then decreased. These expression patterns are similar to that of NTR1 mRNA in the liver after hatching. Therefore, it is suggested that NT-NTR1 system may participate in the regulation of SLC10A2 gene expression in the intestine during early post-hatch period. Further study is needed to demonstrate the participation of NT-NTR1 system in the regulation of cholesteryl ester metabolism together with bile acid transport around hatching.

Bull. Nippon Vet. Life Sci. Univ., No.61, 169–171, 2012.

Taxonomic investigation of *Turicibacter* sp. isolated from germ-free mice monocontaminated with the organism

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(Conferred on 12 March 2012, ALS 38)

Germ-free animals that are reared in sterile environments have no detectable viruses, bacteria, or parasites in their intestines or any other part of their bodies. Nowadays, we can rear germ-free animals using flexible plastic isolators while maintaining sterile environments. In our laboratory, we perform a sterility test each month to confirm that mice are germ-free.

In 2008, germ-free FVB/N mice generated and maintained by us were found to be contaminated with an unusual bacterium, which was detected in the feces of all mice during sterility testing. This bacterium grew slowly in an anaerobic liquid medium (thioglycollate) containing fecal suspension samples. Sequence analysis of its 16S rDNA revealed a relatively high overall similarity (97%) to *Turicibacter sanguinis* and showed that this bacterial contamination was caused by only one species. In the present study, we considered the possibility that the isolates from the feces of monocontaminated germ-free mice were a novel species of the genus *Turicibacter* and performed a taxonomic investigation of these isolates.

The accidentally monocontaminated mice were maintained under sterile conditions in a flexible plastic isolator. Cages, bedding, and water for the monocontaminated mice were sterilized either in an autoclave or with chlorine dioxide. Commercial diet pellets, sterilized by gamma irradiation at 50 kGy, and sterilized water were provided *ad libitum*. The mice were maintained in the animal room under controlled conditions of temperature (20–25° C), relative humidity (30–70%), and light (12 h, 07.00–19.00 h). After collecting the fecal samples, 10-fold fecal suspensions (w/v) were prepared by adding appropriate volumes of dilution buffer. These suspensions were further diluted 1000-fold, and 50 μl of the resulting fecal suspensions was spread on sheep blood agar plates and incubated for 24 h at 37℃ under anaerobic condition. Two colonies were isolated and the strains were named LA61 and LA62. *Turicibacter sanguinis* DSM 14220^{T} was used as a comparison control for the two isolates.

Biochemical and enzyme profiles were obtained using commercial simplified identification kits used for the study of carbohydrate metabolism and the identification of anaerobes, following the manufacturer's instructions, except for a modification of the suspension turbidity.

After growth for 18 h at 37℃ in Gifu anaerobic medium (GAM) broth, DNA was extracted using phenol-chloroform and RNA was hydrolyzed with RNase A. Partial *groEL* (also known as *hsp60* or *cpn60*) sequences were amplified by PCR using universal primers. The PCR products were cloned into the pTAC-1 vector and transformed into *Escherichia coli* DH5 α competent cells. Ampicillin (100 μg ml^{-1}) and blue-white screening were used to select the transformants. Colony direct PCR

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was performed on randomly selected white colonies using M13F-pUC(–40) (5′-GTT TTC CCA GTC ACG AC-3′) and M13R-pUC(–40) (5′-CAG GAA ACA GCT ATG AC-3′). Sequences of 16S rDNA were determined using the primers 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′), 530f (5′-GTG CCA GCM GCC GCG G-3′), 1114f (5′-CGC AAC GAG CGC AAC CC-3′), 519r (5′-GWA TTA CCG CGG CKG CTG-3′), 1098r (5′-GGG TTG CGC TCG TTG CG-3′), and 1525r (5′-AAG GAG GTG WTC CAR CC-3′) and *groEL* using M13F-pUC(–40) and M13R-pUC(–40), by a fluorescent dye-terminator cycle sequencing method and a capillary sequencer.

After multiple alignment of the data, phylogenetic trees were constructed according to the neighbor-joining and minimum-evolution methods using phylogenetic analysis software. The topologies of the trees were evaluated by bootstrap analysis with 1000 replicates.

Bacterial whole-cell fatty acid methyl esters, extracted and prepared from cultures grown on GAM agar supplemented with 5% sheep blood for 18h at 37℃, were analyzed by gas chromatography using the MIDI system. We carried out DNA–DNA hybridization according to a published method using photobiotin and microplates. To determine the DNA G+C content, the bacterial DNA was digested to nucleotides with nuclease P1. The G+C mol% of DNA was determined by reverse-phase high-performance liquid chromatography (HPLC) equipped with an octadecylsilyl-silica (ODS) column (diameter, 6.0 mm; length, 150 mm), a manual sample injector with a 20-μl sample loop, a UV-visible absorption spectrum detector, and a chart recorder, using 10 mM H_3PO_4/KH_2PO_4 (pH 2.4) with 0.5% CH_3CN eluent at a flow rate of 1.5 ml min^{-1} at 40 ° C. Each nucleotide was detected using 270 nm as the detection wavelength. The analyses were repeated three times and mean values were obtained.

The isolates were strictly anaerobic, non-spore-forming, gram-positive, long, irregular rod-shaped, chain-forming cells (1.3 μm wide with variable length, mostly 5-23 μm). Long filaments with thick bulbs were also observed under a microscope. The strain cultivated in thioglycollate broth resembled wool or a mycelial substance. The colonies formed on sheep blood agar were 2 mm in diameter, grayish white with a convex elevation, and irregu- lar in form with undulating margins. Growth occurred at 27–43℃ and pH 6.2–8.3. Acid was produced from D-ribose, D-tagatose, and 5-keto-gluconate, but not from 46 other carbohydrates including D-maltose. The isolates were positive for enzyme activities of α-galactosidase, β-galactosidase, α-glucosidase, and pyroglutamic acid arylamidase, but negative for enzyme activities of 29 other enzymes including glycine arylamidase and serine arylamidase.

The neighbor-joining phylogenetic tree based on the 16S rDNA sequences showed that strains LA61 and LA62 joined the type strain of the species *T. sanguinis* MOL361^T with a bootstrap value of 1000. The 16S rDNA sequence similarities between strains LA61 and LA62 and the type strain of *T. sanguinis* were 99.8% and 97.2%, respectively. The relationships between the two strains and *T. sanguinis* MOL361^T were also maintained in the trees constructed using the minimum-evolution method. Furthermore, the *groEL* sequence similarities between strains LA61 and LA62 and *T. sanguinis* DSM 14220^T were 99.8% and 84.8 %, respectively. In this study, the major cellular fatty acids of both strains were $C_{16:0}$ (22.6-23.9% of total), $C_{18:0}$ (9.9–10.0%), and $C_{18:1}$ ω 9c (47.4-48.6%). Similarly, those of *T. sanguinis* DSM 14220^T were $C_{16:0}$ (11.7%), $C_{18:0}$ (9.4%), and $C_{18:1}$ ω 9c (57.9%). However, the amount of $C_{17:0}$ in the two strains (2.23–2.32%) was remarkably higher than that in *T. sanguinis* DSM 14220^T (<1 %). Thus, $C_{17:0}$ is a characteristic fatty acid in the isolates. The DNA G+C contents determined by HPLC for LA61, LA62, and *T. sanguinis* DSM 14220^T were 37.8 ± 0.1 mol%, 37.9 ± 0.0 mol%, and 35.5 ± 0.1 mol% (mean ± standard deviation), respectively. These results suggest that the isolates should be classified into the genus *Turicibacter*. Moreover, the low DNA-DNA hybridization value (21%) between the two strains and *T. sanguinis* DSM 14220^T clearly indicates that strains LA61 and LA62 were different from *T. sanguinis*.

Based on the data produced in this investigation, we conclude that strains LA61 and LA62 represent a novel species in the genus *Turicibacter*.

Additionally, we detected *Turicibacter* sp. in the feces of conventional FVB/N mice in our laboratory by PCR using species-specific primers, suggesting that *Turicibacter* sp. that colonized the germ-free mice came from the normal mouse intestinal microbiota. Moreover, We also detected *Turicibacter* sp. in the feces of rats, Mongolian gerbils, Syrian hamsters, and chinchillas. We suggest that *Turicibacter* sp. is a member of the intestinal flora of rodents. In conclusion, we propose the isolates as a novel species in the genus *Turicibacter* in experimental rodents.

Bull. Nippon Vet. Life Sci. Univ., No.61, 172, 2012.

Studies on bovine lactoferrin concerned in the growth of bifidobacteria

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(Conferred on 12 March 2012 ALS 39)

Bovine lactoferrin (bLF) is an iron-binding glycoprotein that belongs to the transferrin family, which has been shown to promote the growth of *Bifidobacterium* spp. However its physical role and mechanism involved in bacterial growth are unclear. The present study was to investigate the effect of bLF on the growth of *Bifidobacterium infantis* M-63, *B. breve* M-16 V and *B. longum* BB 536.

The growth of *B. infantis* M-63 was stimulated (up to 110–120%) by bLF addition at 2–4 mg/mL. Any dose of bLF had little influence on each growth of other strains. A visible change that occurred in the culture with bLF after 25 hours incubation showed consistent turbidity, though the control culture formed a precipitate in the test tube.

This visible change may be caused by excessive capsular polysaccharides (CPS) production, which synthesized by *B. infantis* M-63 that stimulated bLF. And the electrostatic negative charges of CPS on the bacteria surface caused repulsive interaction among bacteria. To investigate this hypothesis, binding ability of lectins for CPS of *B. infantis* M-63 was demonstrated. As a result, lectins significantly bound to CPS of *B. infantis* M-63 that incubated with bLF.

Hence, bLF plays a role as a natural iron distributor, and it is likely that the acquisition of iron from bLF by *B. infantis* M-63 may be one possible mechanism behind the bLF growth stimulatory effects. However, apo (iron free)–type bLF promoted the growth of *B. infantis* M-63. Therefore, the mechanism of bLF in bacterial growth may not only iron requirement.

It has been reported that the particular amino acid sequence of human LF stimulated the growth of bifidobacteria. bLF has high homology amino acid sequence with the particular amino acid sequence of human LF. The peptide (CAVGGGCIVL) that synthesized on the basis of the particular amino acid sequence of bLF stimulated the growth of *B. infantis* M-63.

These results suggest that bLF promotes the growth and the CPS synthesis of *B. infantis* M-63. Moreover, the mechanism of bLF in bacterial growth may be involved with not only supplying *B. infantis* M-63 with iron from bLF, but also particular amino acid sequence of bLF.

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Bull. Nippon Vet. Life Sci. Univ., No.61, 173–174, 2012.

Examination of a Prolactin Releasing Factor in the Pars Tuberalis of the Rat

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(Conferred on 12 March 2012, ALS 40)

Prolactin is known as a lactogenic hormone mainly secreted from lactotrophs in the adenohypophysis. In addition to the lactogenesis in the mammary gland, prolactin is involved in the induction of maternal behavior and stress response by acting on the brain. In mammals, blood concentration of prolactin greatly increases during lactation. It is known that secretion of prolactin from the adenohypophysis is regulated by various hypothalamic hormones such as thyrotropin-releasing hormone (TRH), prolactin-releasing hormone (PrRP), vasoactive intestinal polypeptide (VIP), tachykinin (TAC1), epidermal growth factor (EGF), oxytocin (OT) and fibroblast growth factor (FGF). In mammals, prolactin secretion from the adenohypophysis is greatly enhanced during lactation, whereas factors responsible to the enhancement of prolactin secretion has not been identified. Recently, a protein factor, tuberalin, has been detected in ovine pars tuberalis. Tuberalin is believed to be involved in the photoperiodic stimulation of prolactin secretion in ovine but the protein is not identified yet in any other animals. In this study, I examined the presence of prolactin-releasing factor in the pars tuberalis during the reproductive cycle of rats.

First, I investigated whether the known prolactin-releasing factors are expressed in pars tuberalis at mid-pregnancy, lactation, and weaning stages of rats using in situ hybridization method. The expression of PrRP and VIP mRNAs were observed in the pars tuberalis at all the reproductive stages, suggesting that PrRP and VIP synthesized in the pars tuberalis may participate in some functions including regulation of prolactin secretion during the reproductive cycle. EGF mRNA was detected only at lactation stage. Since EGF is known to stimulate the synthesis and secretion of prolactin, it is suggested that EGF is a candidate involved in the regulation of prolactin secretion during lactation.

To examine whether prolactin secretion in adenohypophysis was regulated by factor(s) synthesized in the pars tuberalis, protein extract was prepared from the pars tuberalis of diestrus, estrus, pregnancy, and lactating rats and the prolactin-releasing activity was examined using a rat pituitary cell line, GH3, which secretes prolactin in response to prolactin-releasing factors. The amount of the secreted prolactin was measured by a biological method using a rat pre-T-cell-derived cell line, Nb2, which proliferates in response to prolactin. Although, the protein extract from the cerebrum stimulated prolactin secretion, the extract from the pars tuberalis showed no prolactin-releasing activity. Since tuberalin is known to be heat resistant protein, the protein extract from pars tuberalis was heat-treated and subjected to the assay of prolactin-releasing activity. The heat-treated proteins showed prolactin-releasing

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activity in GH3 cells, suggesting that heat-stable prolactin-releasing proteins exist in the rat pars tuberalis. EGF is known to be a heat stable protein and exist in the cerebrum. However, heat-treated proteins extracted from the cerebrum showed no prolactin-releasing activity in GH3 cells. This result indicates that EGF is not the heat stable prolactin-releasing factor. Further studies are needed to identify prolactin-releasing factors in the pars tuberalis.

Bull. Nippon Vet. Life Sci. Univ., No.61, 175–177, 2012.

Studies on Effects of Mycotoxin and Feed Additives on Ruminal Metabolism of Ruminants

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(Conferred on 12 March 2012, ALS B-1)

The digestive physiology of cattle and other ruminants is markedly different to that of monogastric animals. The rumen is essentially a fermentation chamber, in which microbial attack helps digest carbohydrates, proteins, and fiber from the diet. Recently, the majority of ruminants are faced with a high risk of mycotoxicoses, through the consumption of mycotoxin contaminated feed. Mycotoxins are naturally occurring metabolites of fungal species, growing on a wide variety of crops, such as grain cereals, wheat, barley and maize. Approximately 25% of crops worldwide are affected by mycotoxins and the economic consequences are profound, with contaminated crops being forced to be destroyed. *Fusarium* is considered to be the most important toxigenic genus since fungi from this genus produce the most prevalent mycotoxins, which include deoxynivalenol (DON) and zearalenone (ZEN). Alternately, *Aspergillus* is responsible for aflatoxins (AF). To reduce the risk of animal mycotoxicoses, the use of mycotoxin adsorbents, as feed additives, is one of the promising approaches.

Although, microorganisms in the rumen degrade nutrients to produce VFA, this fermentation process is inefficient, having energy losses (methane) which limit production performance and contribute to environmental pollution. Antibiotic ionophores, such as monensin (MO), have been successful in reducing these inefficiencies, and enhanced feed efficiency due to its selective antimicrobial activity. However, the use of antibiotics has come under criticism due to the possibility of giving rise to transmissible resistance factors. Consequently, considerable effort has been devoted towards developing alternatives to feed antibiotics. An extensive variety of organic compounds deriving from the secondary metabolism of plants, essential oils (EO), appear to potential for rumen fermentation modulation. EO can interact with microbial cell membranes and inhibit the growth of some bacteria, which has lead to the isolation and extraction of a number of phenolic derivatives such as thymol (TM).

Overall, the central theme and focus of this thesis is rumen fermentation, and how it can be altered naturally, via the action of mycotoxins, and artificially, via the action of plant derived compounds. The thesis consists of four study chapters as summarized below:

1. Effects of the Fusarium mycotoxin deoxynivalenol (DON) on *in vitro* rumen fermentation

The objective of this study was to determine the effect of DON on *in vitro* rumen fermentation and DON degradation under the two different carbon sources (corn starch or cellulose). Results indicated that carbon sources appeared to markedly influence rumen fermentative parameters, otherwise, DON negatively impacts certain aspects

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of rumen fermentative capacity, such as gas and VFA production. Total gas production was significantly reduced under the corn starch and cellulose treatments, whereas concentrations of acetate and propionate were significantly reduced under the corn starch treatment with DON. Lastly, DON degradation under the cellulose treatment was more efficiently as compared with corn starch treatment, indicating that concentrate/forage ratio in the diet, may have an effect on DON degradation.

2. Comparison of adsorbent efficacy on aflatoxin (AFB_1), deoxynivalenol (DON) and zearalenone (ZEN) prevention, and their secondary effects on *in vitro* rumen fermentation

The efficacy of 6 commercial adsorbents was examined with 3 different mycotoxins: DON, ZEN and AFB_1. Out of the adsorbents examined, the majority were effective on DON, reducing its toxicity by leading to positive indicators such as increased VFA and reduced methane production and A/P ratio. Alternately, the majority of adsorbents appeared to be ineffective with AFB_1 and ZEN especially. It appeared that adsorbent treatment with ZEN reduced VFA and increased methane production. The degree of effectiveness of the various adsorbents may be related to their ability or lack thereof to interact with charged or polar as opposed to non-polar mycotoxins. DON has no polar group, whereas AFB_1 is charged, and ZEN carries a slight charge.

3. Determination of dose-response effects of essential oils (EO) or thymol (TM) as compared to monensin (MO) on *in vitro* rumen fermentation

The aim of this study was to assess and compare the dose-response effects of TM, EO and MO on *in vitro* rumen fermentation and determine whether the bacterial or protozoan population is affected thereof. Using MO, as a positive control, TM exhibited certain similarities with reduced total gas, total VFA, protozoa activity, and methane production and differences with the molar proportions of VFAs, whereas EO had no effect on all parameters. Overall though, the effect of TM would generally be viewed as nutritionally unfavorable, with reduced total VFA, in spite of reduced methane production being observed with higher TM concentration. With regards to which microbial population is affected, lower TM concentration resulted in significant decreases on propionate with a bacterial enriched suspension, whereas EO and MO demonstrated no significant effect as compared to CTR conditions. Alternatively, TM resulted in significant decreases on propionate and increases on acetate with a protozoa enriched suspension, whereas EO and MO demonstrated no significant effect compared to CTR conditions. Therefore this indicates that neither EO nor TM supplementation appear to be beneficial for bovine rumen fermentation based on *in vitro* rumen fermentation.

4. Effect of Thymol glucoside (TMG) on blood metabolism of cattle and rumen fermentation *in vitro*

The objective of this study was to investigate the metabolism of TMG, its effect on the blood biochemical and hematological variables in cattle, and the dose-response effects of TMG during *in vitro* rumen fermentation. Excretion of TMG and its subsequent breakdown products were not detected in the feces or urine for up to 48 h post TMG exposure. TMG demonstrated no significant effects on the blood biochemical and hematological variables except for white blood cell (WBC) counts. Approximately 56% of the WBC counts increased as compared to the initial time, suggesting the potency of the TMG's immuno-stimulation. TMG was shown to degrade into TM and an unknown TMG intermediate in the rumen. Contrary to TM, higher TMG treatment tended to increase the propionate proportion and decrease the acetate and methane production.

Therefore, these results indicate that TMG may serve as a better feed additive than TM with regards to positively influencing rumen fermentation, with less side effects than TM.

As a general conclusion from the results of these studies, it was shown that TMG can be used for

modulating rumen fermentation as an alternative to MO. Moreover, research focusing on ways of minimizing the negative effect of DON, from contaminated feed, such as changes in concentrate/forage ratio, in the diet, should be considered. In addition, further research should be carried out on mycotoxin adsorbents and their efficacy on DON, ZEN and AFB_1. Individual difference in the ability to adsorb is related to the specific physicochemical properties essential of each individual compound. Further work needs to be carried out to extrapolate and isolate more individual compounds from EO, for testing their effects on rumen fermentation.