Bull. Nippon Vet. Life Sci. Univ., No.64, 86-88, 2015.

Development of new predictive biomarkers for early diagnosis of obesity in dogs and cats

Gebin LI*

Department of Basic Veterinary Medicine
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 23 May 2014, VA-165)

A number of health problems purported to be associated with or exacerbated by obesity in dogs and cats. Early warning signs of energy metabolism dysregulation in overweight animals could be detected by the presence of insulin and adiponectin signaling genes via PBL qRT-PCR method, and may be valuable in early diagnosis. In addition, better understanding of these mechanisms might assist in the prevention of the development of obesity-related metabolic dysfunction or diabetes in obese animals.

1. Using body fat percentage assessing overweight status in dogs

Currently, 5 point body condition scoring (BCS) is commonly used by veterinarians and clinicians to assess adiposity in dogs in Japan. However, assigning a BCS score is subjective in nature, and most clinicians do not score with half points, instead preferring to round off values, thereby rendering less accurate assessments. Therefore, we sought to determine whether assessing body fat percentage (BF%), using simple morphometric measurements, and supplementing it with 5-point BCS can render increased sensitivity for detecting increasing adiposity in overweight small breed dogs via plasma metabolite validation.

Overall, lean BF % range was determined to be 15-20% for non-neutered male dogs, and 15-25% for female (non-spayed/spayed) and neutered male dogs. BCS categorized overweight animals displayed significantly higher levels of non-esterified fatty acids (NEFA; $p=0.005$) ; whereas significantly higher levels of NEFA ($p=0.006$), Total cholesterol (T-Cho; $p=0.029$), and Triglycerides (TG; $p=0.001$) were observed in BCS+BF % categorized overweight animals as compared to lean animals. The increase in sensitivity, due to BF %, for gauging alterations to plasma metabolite values, may be due to increased correlation strength. BF % positively correlated with plasma insulin ($r=0.627$, $p=0.002$), NEFA ($r=0.674$, $p<0.001$), T-Cho ($r=0.825$, $p<0.0001$), TG ($r=0.5823$, $p<0.005$), Blood Urea Nitrogen ($r=0.429$, $p<0.05$), creatinine ($r=0.490$, $p=0.021$), and Total Protein ($r=0.737$, $p<0.0001$) levels which all tend to increase as a result of increasing adiposity.

In conclusion, BF % supplementation to 5 point BCS, appears to increase the likelihood of validating overweight status in small breed dogs, by detecting alterations in plasma metabolite values, especially lipid metabolites, induced as a result of increasing adiposity.

2. Plasma lipoprotein profiles and malondialdehyde in hyperlipidemia dogs

The aim of this Chapter is to compare metabolic parameters, malondialdehyde (MDA) as a lipid oxidation marker, and lipid profiles between dogs with untreated hyperlipidemia and hyperlipidemia with treatment, in order to examine the usefulness of MDA and lipid profiles as diagnostic parameters at early stages of hyperlipidemia. Dog samples were collected from clinics which were separated into three groups: control, untreated hyperlipidemia based on temporally screening, and hyperlipidemia with current anti-hyperlipidemic (statins and fibrates) treatment. TG levels of untreated hyperlipidemia dogs were significantly higher than those of control dogs. ALT levels of hyperlipidemic dogs with treatment were the highest among three groups. VLDL and LDL of both cholesterol and triglyceride of untreated hyperlipidemia dogs were the highest among three groups. HDL1 levels in triglyceride of hyperlipidemia dogs with treatment were significantly higher than those of control and

*Supervisor : Prof. Toshiro ARAI

untreated hyperlipidemia dog. MDA concentrations of untreated hyperlipidemia dogs were significantly higher than those of control and hyperlipidemic dogs with treatment. The results indicated that dogs with untreated hyperlipidemia clearly showed abnormal lipid status, whereas hyperlipidemic dogs under anti-hyperlipidemia treatment showed more normal lipid status suggesting the effectiveness of the therapy. Anti-hyperlipidemics (statins and fibrates) for dogs are also effective in relieving elevated levels of lipids and lipid oxidation. Plasma lipid (triglyceride and cholesterol) profiles and MDA are useful diagnostic tools for identifying early stages of untreatment hyperlipidemia in dogs.

3. Insulin and adiponectin gene expression of PBL in obese Miniature Dachshunds Peripheral

Blood leukocytes (PBL) continually interact with virtually every organ and tissue in the whole body. A remarkable concordance (80%) of gene expression profiles between peripheral blood mononuclear cells and different tissues has been previously demonstrated in humans. As such, gene expression responses of circulating PBL can therefore potentially provide early warning of any abnormalities they discover. Alteration to weight, when associated with obesity, has been reported to lead to alterations to PBL gene expression, especially those related to insulin and adiponectin signaling genes.

As such, PBL mRNA expression profiles of genes involved in insulin signaling (ADIPOR (-1 and 2) ; IRS (-1 and 2) ; PI3-K) lipogenesis (FAS) and energy homeostasis (G6PDH; MDH) were carried out on lean Miniature Dachshund and compared against similar profiles of breed and age matched overweight Miniature Dachshund in an attempt to identify possible PBL biomarkers for assessing obesity in dogs.

Overweight status resulted in a significant increase in plasma NEFA, T-Cho, triglycerides and ALT, and a significant decrease in plasma adiponectin, as compared to lean Miniature Dachshund. Overweight dogs PBL demonstrated reduced mRNA expression of IRS-1 and -2; PI3-K, ADIPOR1 and FAS genes.

Overall, these findings suggest that dysregulation of energy metabolism, associated with obesity, in overweight dogs may carry over with alterations in PBL gene expression of genes involved in insulin and sterol metabolism. As such, PBL gene expression profiles may aid in early detection of PBL biomarkers for assessing obesity in dogs.

4. High-fat diet cats gene expression in PBL and insulin sensitive tissues

Alterations to gene expression, especially transcriptional changes, occurring in insulin sensitive tissues, may be a good indicator of metabolic changes occurring in the body. The objective of this Chapter is to determine whether PBL can serve as an easily accessible cell type for possibly detecting obesity and subsequent obesity risk in cats.

Regarding insulin signaling activity, high-fat diet cats had a significantly reduced IRS-1 mRNA expression in abdominal fat and peripheral leukocytes, with a significantly increased IRS-1 mRNA expression in liver as compared to control cats. Moreover, in high-fat diet cats, a significant reduction in IRS-2 mRNA expression in subcutaneous and visceral fat, and a significant increase in PI3K p85αmRNA expression in liver and skeletal muscle with a significant reduction in PBL was observed as compared against control cats. With respect to lipid synthesis and adiponectin signaling, high-fat diet fed cats' abdominal adipose demonstrated a significant median increase in ADIPOR1 mRNA expression, with reduced ADIPOR1 mRNA expression in liver and PBL being observed as compared to control cats. In addition, subcutaneous and visceral adipose demonstrated a significant median increase in ADIPOR2 mRNA expression, and FAS mRNA expression was significantly higher in all tissues except PBL as compared to control cats. Lastly, in high-fat diet fed cats, G6PHD mRNA expression was significantly higher in liver and skeletal muscle, but significantly lower in PBL as compared to control cats. In addition, abdominal and subcutaneous adipose demonstrated a significant median increase, while liver and PBL demonstrated a significant reduction in MDH mRNA expression as compared to control cats.

Overall, our results demonstrate that PBL can serve to act as surrogate tissue for various insulin sensitive tissues, depending on 1) the genes of interest, 2) the degree of pathology associated with the insulin sensitive tissue, and 3) the disease condition. Although the expression pattern of the aforementioned genes examined was not completely uniform, there was some correlation between PBL and various tissues. The response to obesity is largely tissue specific with numerous commonly activated pathways suggesting a coordinated attempt by tissues to limit metabolic perturbations occurring in early-stage obesity.

5. Insulin and adiponectin gene expression of PBL in short- and long-term obese cats

Naturally occurring obesity is more representative of the true clinical picture than experimental short-term dietary manipulation in cats. The aim of this preliminary study was to compare plasma metabolite and PBL mRNA transciptome profiles of genes mainly involved with energy homoeostasis, insulin and adiponectin signaling, in short-term high-fat diet induced and long-term naturally occurring obese cats.

Plasma metabolite profiling highlighted the inherent aberrations associated with different types and exposure time of obesity. In addition, PBL transciptome profiles were very consistent regarding the genes used in our study, highlighting the sensitivity of PBL to the effects of obesity regardless of being acute or long term, on a host.

Overall, firstly, present studies have showed that BCS supplementing it with body fat percentage (BF%) provided more accurate assessments for dogs. Secondly, the present investigation indicated that plasma lipid profiles and MDA are most likely useful parameters for identifying early stages of obesity with mild hyperlipidemia in dogs. Thirdly, insulin and adiponectin gene expression responses of circulating PBL can potentially provide early warning of any abnormalities, when associated with obesity in both dogs and cats. Lastly, dogs and cats have been proposed as a valuable animal model for studying human obesity, especially naturally occurring obesity animals.

Bull. Nippon Vet. Life Sci. Univ., No.64, 89-90, 2015.

Study of recently identified porcine parvoviruses in pig herds of Japan and Thailand

Prayuth SAEKHOW*

Department of Preventive Veterinary Medicine
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 26 September 2014, VA-166)

This thesis describes my study on porcine parvoviruses during the PhD course.

There are many parvoviruses infecting a variety of animals including insects and vertebrates. Parvoviruses are non-enveloped, isometric viruses with a linear single stranded DNA of approximately 4-6 kb in size. According to the proposed taxonomy of the family *Parvoviridae* (Cotmore et al., 2014), the family *Parvoviridae* is divided into two subfamilies; *Parvovirinae* infecting vertebrates and *Densovirinae* infecting invertebrates. The subfamily *Parvovirinae* has been subdivided into eight genera. There are several parvoviruses important in the field of veterinary medicine, such as porcine parvovirus (PPV), bovine parvovirus, canine parvovirus, canine minute parvovirus, Aleutian mink disease virus and feline panleukopenia virus, which belong to different genus in the subfamily. Most of parvoviruses are thought to have common characters of the resistance to antiseptic substances and extreme environmental conditions like heat and pH and the requirement of cellular DNA synthesis for its viral replication. These are related to the difficulty to eradicate diseases associated with the viruses and their pathogenesis.

I have studied porcine parvoviruses infecting pigs. The parvovirus initially discovered in pigs is porcine parvoviruses (PPV), which was reported in the 1960s and is now enzootic in most pig-producing countries. PPV mainly causes reproductive failure in naïve dams, and manifestation characterized by stillbirth, mummification, embryonic death and infertility. In contrast, PPV infection of adult pig causes only a subclinical or mild disease.

Besides PPV, several other parvoviruses or their genomes have been recently identified in pigs. The detection of these newly identified porcine parvoviruses was reported in several countries, but their association with diseases is still not known. However, as more multifactorial or idiopathic disorders in pig farms are suspected, these new parvoviruses should be further characterized. I have studied the classical and four newly identified porcine parvoviruses.

In the chapter I, I describe my study of the characterization of porcine parvovirus 2 (PPV2) detected in Japanese pig farms. PPV2 was first detected in specimens from domestic pigs in Myanmar in 2001. The genome was subsequently reported from China, Hungary, the USA and Germany. The prevalence of Japan was 58% in healthy pigs and 100% in sick pigs, which was equivalent to or higher than other countries. When I started to study PPV2, six near full genome sequences of PPV2 have been reported, and there were variations in sizes of ORF1 and ORF2. I therefore molecularly cloned a near complete genome of PPV2, termed JPT68, from the tonsil of a healthy domestic pig. Based on the comparison with other PPV2 sequences, I amplified and sequenced a DNA fragment of a variable region of PPV2 detected from 41 Japanese pigs, and then compared with those of other countries using phylogenetic analyses. The analysis showed that diverged PPV2 strains exist in Japan and clearly distinct strains of PPV2 were detected in 7 of the 10 pig farms. Circulating multiple strains within a farm may be a risk for generating emerging virus as reported in other parvoviruses.

In the chapter II, I describe my study of the five classical and newly identified porcine parvoviruses detected in Thailand. Thailand had not been investigated for the classical PPV or new porcine parvoviruses except for the seroprevalence of PPV (Tummaruk,

*Supervisor : Prof. Hidetoshi IKEDA

et al., 2012). I examined the 80 tonsil samples of pigs collected at a slaughterhouse in the Chiangmai area of Thailand. I investigated the prevalence of the five porcine parvoviruses and characterized the genetic diversity of PPV2 and PPV3. The prevalence was 53% (42/80) for PPV (PPV-Kr or -NADL2 as the new abbreviation), 83% (66/80) for PPV2 (CnP-PARV4), 73% (58/80) for PPV3 (P-PARV4), 44% (41/80) for PPV4 (PPV4) and 23% (18/80) for PBo-likeV (PBoV7). Over 60% of 80 pigs carried more than 3 of the 5 viruses. The phylogenetic analyses for PPV2 and PPV3 indicated the existence of two and one clade(s) of viruses, respectively, suggesting an invasion from a limited source for both viruses.

In the chapter III, I describe the characterization of the infection status of porcine parvoviruses in Japanese pigs and their genetic diversity of PPV3. I previously investigated PPV2 of Japanese pigs, as described in chapter I. With the same specimens, my colleagues and I extended such a screening for 14 other viral genomes including 4 porcine parvoviruses. Only 5 virus genomes were detected; four were the member of the family *Parvoviridae* and another one was PCV2. The prevalence in 120 apparently healthy pigs aged about 6 months was 67% (80/120) for PPV (PPV-Kr or -NADL2 as the new abbreviation), 39% (47/120) for PPV3 (P-PARV4), 33% (32/120) for PPV4 (PPV4), 55% (66/120) for PBo-likeV (PBoV7) and 80% (96/120) for PCV2. PCV2 is a causative agent of porcine circovirus associated disease (PCVAD) and PPV has been considered as one of the cofactors for PCVAD. In the screening, the detection of PCV2 was significantly coincidental with either detection of PPV, PPV2 or PPV3. A coincidental detection of PPV and PPV4 was also observed. Although the exact reason is not known, the concurrent infection with PCV2 and porcine parvoviruses in the subclinically infected pigs may relate to the clinical manifestations of PCVAD. Additionally, I performed a phylogenetic analysis of PPV3 which suggested that Japanese PPV3s showed a slight variation, and possibly, there were farms harboring homogeneous or heterogeneous PPV3s.

Finally, this study shows the infection status of newly identified porcine parvoviruses in pig herds of Japan and Thailand. Since these viruses are not known regarding the association with any disease, our investigation should provide useful information for further studies.

Bull. Nippon Vet. Life Sci. Univ., No.64, 91-93, 2015.

Study of the effect of Excessive Tibial Plateau Angle on degenerative changes of canine cranial cruciate ligament

Tom ICHINOHE*

Department of Veterinary Clinical Medicine
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, VA-167)

1.

Canine cranial cruciate ligament rupture (CrCLR) is a very common orthopaedic disease of the canine stifle. The cranial cruciate ligament (CrCL) prevents cranial tibial displacement relative to the femur, excessive internal tibial rotation, and stifle hyperextension. Cranial tibial thrust (CrTT), which is a force generated during hind limb weight bearing, and an abnormally increased stifle internal rotation may both result from CrCLR. Dogs affected with CrCLR may subsequently develop progressive stifle osteoarthritis and secondary meniscal damage. Although anterior cruciate ligament (ACL) rupture can occur acutely in humans due to trauma, most canine CrCLRs occur secondary to chronic degenerative changes in the CrCL. The degenerative changes in the CrCL are characterized by the degeneration of the extracellular matrix (ECM) in the CrCL, eventually leading to ligament rupture secondary to non-contact injury. Multiple histologic changes, including decreased cell density, disorganization of collagen fibres, and phenotypic changes in ligamentocytes, have been reported in the degenerated CrCL. One key histological characteristic is the alteration in ECM, particularly chondroid metaplasia. Excessive tibial plateau angle (eTPA; TPA $\geqq 35°$), which converts more weight loading to the cranial tibial thrust than the normal tibial plateau angle (TPA) and increases the tensile force in the CrCL, is recognized as one of the risk factors of CrCLR. To date, there are no known studies investigating the relationship between the eTPA and degenerative changes in the CrCL. We hypothesized that degenerative changes in CrCLs, such as chondroid metaplasia, were increased in the stifles with eTPAs.

The objective of this study is to investigate the effect of eTPA on the degenerative changes. In the chapter 2, in order to evaluate the utility of implants for the TPA increasing procedure to generate a model animal for eTPA, we measured the changes of the TPA in cases in which the proximal tibial cylindrical osteotomy was performed with various types of implants. In the chapter 3, we aimed to measure the tensile force in the CrCL, medial and lateral collateral ligament (MCL and LCL) in normal canine stifles and artificial stifle models of eTPA and evaluate the effect of the TPA increasing procedure on the tensile force of these ligaments. In the chapter 4, we aimed to describe the development of chondroid metaplasia, the changes in the expression of ECM components, and the expression of the Sry-type HMG box 9 (SOX9), which is the key factor for the cartilage differentiation and the expression of the cartilage matrix, in CrCLs affected by CrCLR in dogs. In the chapter 5, we aimed to generate an animal model of eTPA and evaluate degenerative changes of the CrCL and the caudal cruciate ligament (CaCL).

2. The effect of plate types on tibial plateau angle and mechanical medial proximal tibial angle after tibial plateau leveling osteotomy

Various types of specialized plates are available for the corrective osteotomy that change the proximal tibial shapes such as tibial plateau leveling osteotomy (TPLO) which changes TPA by the osteotomy in the proximal tibiae. In order to evaluate the utility of these implants for the TPA increasing procedure, we measured TPA and mechanical medial proximal tibial angle (mMPTA) in cases in which TPLO was performed with a Slocum plate (SP), locking compression TPLO plate (LCP), and dynamic compression plate (DCP). The TPA and

*Supervisor : Prof. Yasushi HARA

mMPTA were then compared among different types of plates and after each surgical procedure. The TPA at 1, 2, and 3 months was significantly higher than that immediately after surgery in the SP group. There were no changes in the postoperative TPA over time in the LCP group. The TPA at 2 and 3 months was significantly higher than that immediately after surgery in the DCP group. There were neither changes in the postoperative mMPTA over time in any group nor any significant difference in the mMPTA among the three groups. Compared with SPs or DCPs, LCPs are very useful to maintain the alignment of the proximal tibial fragment, and DCPs are inferior to LCPs specialized for proximal tibial corrective osteotomy in terms of maintenance of the alignment of the proximal tibial fragment. From this result, we decided to apply LCPs to the TPA increasing procedure.

3. The effect of the tibial plateau angle increasing procedure on the tensile force of the cranial cruciate ligament in the canine femorotibial joint

We aimed to measure the tensile force in the CrCL, medial and lateral collateral ligament (MCL and LCL) in normal canine stifles and artificial stifle models of eTPA and evaluate the effect of the TPA increasing procedure on the tensile force of these ligaments. Cadaveric stifles (n=16) were harvested from normal beagles and allocated into (1) the unchanged tibial plateau angle group (normal group; TPA=31.2°) or (2) the excessive TPA group(eTPA group; TPA=41.1°). The eTPA group underwent curvilinear osteotomy at the proximal tibia to increase the TPA. Arobotic system applied a 30 N or 60 N compressive force to the specimens. The craniomedial band (CrMB), caudolateral band (CaLB), MCL, and LCL were sequentially transected and the protocol was repeated. Orthogonal force components were measured and the ligament forces calculated after repeated force measurements as ligament contributions were subtracted by transection. As the compressive force increased, the tensile forces in the CrMB and CaLB also increased, but they remained unchanged in the MCL and LCL. The CrMB tensile force was larger in the eTPA group than in the normal group, and the MCL and LCL tensile force were not larger in the eTPA group than in the normal group. An eTPA may increase the stress on the CrCL, but not the MCL and LCL. The TPA increasing procedure used in this study increases the tensile force in the CrCL without showing a major impact on the MCL and LCL.

4. Degenerative changes of the cranial cruciate ligament harvested from dogs with cranial cruciate ligament rupture

We aimed to describe the development of chondroid metaplasia and the changes in the expression of ECM components in CrCLs affected by CrCLR in dogs. CrCLs from 26 stifle joints with CrCLR (CrCLR group) and normal CrCLs from 12 young beagles (control group) were examined histologically and immunohistochemically for expression of type I (COLI), type II (COLII), type III (COLIII) collagen, and SOX9. Cell density and morphology of CrCLs were quantified using HE staining. In CrCLs, the percentage of round cells was higher in the CrCLR group than in the control group. COLI-positive areas were seen extensively in the connecting fibers, but weakly represented in the cytoplasm of normal CrCLs. In the CrCLR group, there were fewer COLI-positive areas, but COLI-positive cells increased. The percentages of COLII-, COLIII- and SOX9-positive cells were higher in the CrCLR group than in the control group. Deposition of COLI, the main ECM component of ligaments, decreased with increased COLIII expression in degenerated CrCL tissue, which shows that the deposition of the ECM is changed in degenerative CrCL disease. There was no significant correlation between the period from the onset of clinical sign and the expression of COLI, COLI, COLI, and SOX9. On the contrary, expression of SOX9 increased, which may contribute to the synthesis of cartilage matrix. The expression of COLII and SOX9 in ligamentocytes showed that these cells tend to differ to chondrocytes. It is reported that the chondroid metaplasia is s a physiological, not pathological, response, and there is no correlation with the period from the onset of the clinical sign. Therefore it is suggested that these changes occur before the ligaments rupture, although the possibility cannot be denied that ligament rupture have effects on these changes.

5. Degenerative changes of the cranial cruciate ligament harvested from dogs with cranial cruciate ligament rupture

We aimed to generate an animal model of eTPA and evaluate degenerative changes of the CrCL and the caudal cruciate ligament (CaCL). Seven mature female Beagles were included. Cylindrical osteotomy was performed bilaterally in the proximal tibia. The TPA was increased to approximately 40° in the left tibia (eTPA group) and remained unchanged in the right

tibia (control group). The dogs were subjected to exercise stress beginning 3 months postoperatively and were euthanized 12 months postoperatively, and the CrCLs and CaCLs were collected. All specimens were stained with haematoxylin and eosin (HE) to assess the cell morphology and subjected to immunostaining to evaluate COLI, COLII, and COLIII, and the SOX9 expression. Macroscopic CrCL injury was absent in 6 dogs, but was present in one in the eTPA group, which was excluded from the analysis. The cell density decreased and the percentage of round cells increased in the eTPA group. In the eTPA group, there were fewer COLI-positive areas, but COLI-positive cells increased. The percentages of COLII-, COLIII- and SOX9-positive cells were higher in the eTPA group than in the control group. There was no significant difference in the CaCLs between the eTPA group and the control group.

The degenerative changes of the CrCL with eTPA in this study were similar to the degenerative changes in the CrCL from the cases affected by CrCLR. Moreover these changes were not observed in the CaCL with eTPA. It is reported that tensile force in the fibrocytes increase lead to the expression of the SOX9 increase. Therefore the eTPA may increase the tensile force in the CrCL and may increase the expression of the SOX9 and may increase the expression of the cartilage matrix such as COLII. It is also proposed that cartilage-like tissue is more vulnerable to disruption under normal tensile forces, and any fibrocartilaginous transformation may predispose to injury of the CrCL. In the eTPA group, there were fewer COLI-positive areas, but COLI-positive cells increased similar to the degenerative CrCL from the cases affected by CrCLR. It is reported that the turnover of COL I, the principal tensile-resistant fiber increases when the tensile force in the ligamentocyte in the CrCL increases, and that immature collagen crosslinks are increased in ruptured CCLs, which may contribute to a decrease in tensile strength.

Therefore the eTPA may increase the tensile force in the CrCL and may increase the turnover of the COL I and may contribute to a decrease in tensile strength of CrCL. In the eTPA group, COLIII-positive cells increased similar to the degenerative CrCL from the cases affected by CrCLR. Increased expression of COLIII is the first step during injury healing, which is then finally replaced with COLI. Therefore eTPA may increase the tensile force in the CrCL and may increase the turnover of the COL III because of micro injuries increase in the CrCL and may contribute to a decrease in tensile strength of CrCL. Taken together, these observations suggested that eTPA may promote the degenerative changes in the CrCL and may be one risk factor of CrCLR.

Bull. Nippon Vet. Life Sci. Univ., No.64, 94-96, 2015.

Studies on male reproductive characteristics of feral raccoons (*Procyon lotor*) in Kanagawa Prefecture, Japan

Taiki UNO*

Department of Preventive Veterinary Medicine
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, VA-168)

In Japan, the raccoon (*Procyon lotor*), an omnivorous medium-sized member of the Carnivora, was imported from North America as a pet and had naturalized throughout the country. Therefore, feral raccoons are becoming a problematic invasive species by law that was enforced in 2005 and are being commonly captured. An annual damage amount of agricultural crops and number of the capture is increasing year by year. Until now, feral raccoons were captured a lot at Kinki, Hokkaido and Kanto, but in late years the number of the capture is increasing at Kyushu quickly.

In Hokkaido, it has been proposed that feral raccoons could be effectively captured by shifting capture efforts to spring, which is when the animals are caring for their offspring. However, there are regional differences in the parturition periods of feral raccoons. Because in Kamakura, Kanagawa Prefecture, the parturition periods of raccoons is thought to be from February to October and shows a bimodal distribution, capture efforts cannot be concentrated. In North America, the parturition periods of feral raccoons tend to be longer at more southern latitudes. Therefore, the parturition periods of raccoons in Kyushu where the number of the capture is increasing were expected like Kamakura, so management plan like Hokkaido cannot be adopted simply at Kyushu.

As the cause of the bimodal distribution of parturition period of feral raccoons, the factor of the female side was considered until now, but a study is not performed about the factor of the male side. To assess the cause of the bimodal distribution, the present study proposes following two hypotheses: 1) The yearling males reach sexual maturity after spring and produce offspring; 2) Because the number of adult male raccoons being sexually inactive in summer excessively increased, a part of the original peak became dented.

The objective of this study was to study the reproductive characteristics of male raccoons to understand the role of the male for distribution of parturition periods of raccoons and test the above two hypotheses.

First, the parturition periods of raccoons at Kanagawa and Kyoto was estimated in Chapter 2. Then for testing of hypothesis 1, the age when feral raccoons start spermatogenesis and the growth of male sexual organs, such as baculam and prostate, was studied in Chapter 3. Furthermore, it was inspected whether the reproductive characteristics, such as spermatogenesis, and the growth of male sexual organs, such as testis and prostate were different between early litters and late litters in Chapter 4. And for testing of hypothesis 2, the seasonal change of reproductive characteristics was studied in Chapter 5. In addition, the simple evaluation methods to assess reproductive characteristics of male raccoons were developed to make it easy to introduce the study of that for the management plan.

1. Estimation of the parturition period of feral raccoons in Kanagawa and Kyoto (Chapter 2)

2,673 raccoon carcasses were collected by raccoon control programs in Kanagawa Prefecture from November 2005 to October 2014 and 738 raccoon carcasses were collected by raccoon control programs in Kyoto Prefecture from March 2009 to February 2014. To estimate birth month, the crown-rump length of fetuses was measured and age determination was performed by their tooth eruption. In this study, there were significant differences between the parturition period of feral raccoons in Kanagawa and that in Kyoto (χ^2=40.44, d.f.=2, $p<0.01$). That in Kanagawa were

*Supervisor : Prof. Shin-ichi HAYAMA

estimated to range from February to December with two peaks, that big in May and small in August, and the amount decreased in the latter half of that were gradual. In contrast, the parturition period of feral raccoons in Kyoto were estimated to range from January to November with one big peak in May and the amount decreased after May were sudden.

2. Estimation of the age of sexual maturity of male raccoons in Kanagawa and Kyoto (Chapter 3)

1) Estimation of the time of commencing spermatogenesis of feral raccoons in Kanagawa and Kyoto (Chapter 3 section 1)

359 raccoon carcasses were collected by raccoon control programs in Kanagawa from July 2005 to January 2014 and 33 raccoon carcasses were collected by raccoon control programs in Kyoto from December 2007 to December 2013. The ages of the raccoons were determined by the cranial suture obliteration method. Then for estimating the age when feral raccoons start spermatogenesis, the testis and the tail of the epididymis was studied by histologically. The male raccoon in Kanagawa started spermatogenesis at six months of age. In contrast, most male raccoons in Kyoto started spermatogenesis over twenty months of age, expect one that started spermatogenesis at eight months of age. Therefore, it was expected that yearling male breed in Kanagawa at their first mating season and that breed in Kyoto at their second ming season.

2) Growth of the baculum of male raccoons in Kanagawa and Kyoto (Chapter 3 section 2)

189 raccoon carcasses were collected by raccoon control programs in Kanagawa from September 2005 to January 2014 and 31 raccoon carcasses were collected by raccoon control programs in Kyoto from Jury 2011 to December 2013. The ages of the raccoons were determined by the cranial suture obliteration method. After the baculam was removed, boiled and dried, then was measured and weighed. By calculation of using the expression of the growth curve of Gompertz, the growth of the baculum of raccoons at Kanagawa and Kyoto were similar to North America, thus it was expected that the baculam of raccoons at Kanagawa and Kyoto matured at seven months of age.

3) Development of the male reproductive organs of feral raccoons in Kanagawa and Kyoto (Chapter 3 section 3)

359 raccoon carcasses were collected by raccoon control programs in Kanagawa from July 2005 to January 2014 and 33 raccoon carcasses were collected by raccoon control programs in Kyoto from December 2007 to December 2013. The testis, the epididymis and the prostate were analyzed by histologically. Growth rates of various parameters, such as the testicular weight, were calculated using the expression of the growth curve of Gompertz. Because of high growth rate of Kanagawa and high asymptotic value of Kyoto, feral raccoons at Kanagawa were precocious and that at Kyoto were large-sized. Then, the prostate of feral raccoons matured at six months of age in synchrony with spermatogenesis.

3. Difference in reproductive characteristics for birth time (Chapter 4)

To estimate the birth months of feral raccoons at Kanagawa (n=323), specimens of two months of age were analyzed by the cranial suture obliteration methods. Although the parturition season of feral raccoons in Kamakura occurs between February and October, it was considered that feral raccoons born by May represented early litters, whereas those born from June were late litters.

There was no difference about the age that started spermatogenesis and growth rates of various parameters, such as the testicular weight, between early litters and late litters. By analysis according to the capture time, it was expected that the yearling males of the early born group in Kanagawa are a father of early litters, and that of late born group is a father of late litters.

4. Seasonal changes in spermatogenesis of feral raccoons in Kanagawa and Kyoto (Chapter 5)

309 raccoon carcasses were collected by raccoon control programs in Kanagawa from July 2005 to January 2014 and 77 raccoon carcasses were collected by raccoon control programs in Kyoto from December 2007 to December 2013. The testis, the epididymis and the prostate were analyzed histologically. Growth rates of various parameters, such as the testicular weight, were considered about seasonal change. The greatest percentage of adult male raccoons was sexually inactive in summer (spring: 100%; summer: 50%; autumn: 86%;

winter: 100%). In contrast, the ratio of male raccoons being sexually active was constant in 50% throughout the year. Because the ratio of juvenile males being sexually active was increased in summer, it was expected that the juvenile males mated in summer and the late litters was born.

5. Methods of evaluating the spermatogenic ability of male raccoons (Chapter 6)

Generally, spermatogenesis is studied histologically. At present, male spermatogenic ability is not well understood in Japan, unlike female reproductivity, because the histological method to evaluate it is both more difficult and more expensive than macroscopic observation. So, 182 raccoon carcasses were collected by raccoon control programs in Kanagawa from March 2005 to September 2008. In this study, the spermatogenesis of feral male raccoons was evaluated by histologically examining the testis and the tail of the epididymis to establish a simple method of estimating spermatogenesis from external measurements. GSI that is frequently used to monitor breeding activities in fish and body weight were chosen as criteria of spermatogenesis and the judgment rate was 97%, and GSI was chosen as criterion for presence of spermatozoa in the tail of the epididymis and the judgment rate was 98%.

6. Summary and conclusion (Chapter 7)

In Chapter 2, there were significant differences between the parturition period of feral raccoons in Kanagawa and that in Kyoto, and there were more late litters in Kanagawa than Kyoto.

In Chapter 3, the male raccoon in Kanagawa started spermatogenesis at six months of age, and the baculam and the prostate of feral raccoons matured in synchrony with spermatogenesis. From the comparison with other studies, there are regional differences in the sexual maturity of feral male raccoons. Therefore, it was expected that yearling male breed in Kanagawa at their first mating season and that breed in Kyoto and Hokkaido at their second mating season.

In Chapter 4, late litters in Kanagawa were matured at the latter half of their first mating season. Hence, it was expected that the yearling males of the late born group in Kanagawa are a father of late litters.

In Chapter 5, the greatest percentage of male raccoons over twelve months of age were sexually inactive in summer. In contrast, the ratio of male raccoons being sexually active was constant in 50% throughout the year. Because the ratio of juvenile males being sexually active was increased in summer, it was expected that feral raccoons in Kanagawa could mate throughout the year though the raccoon was seasonally breeding mammals.

In Chapter 6, a simple method that was useful for analysis about reproductive characteristics of male raccoons was developed, and it was clarified that GSI that had not been used for mammals very much until now was useful in order to estimate the reproductive characteristics of male raccoons.

First hypothesis was accepted, because male raccoons started spermatogenesis at their first mating season, the ratio of male raccoons being sexually active in Kanagawa was constant in 50% throughout the year, toward that in Kyoto was 20% and the yearling males of the late born group in Kanagawa are a father of late litters. Then, second hypothesis was accepted, because the greatest percentage of adult male raccoons was sexually inactive in summer. However, there was seasonal change in parturition period of feral raccoons in Kanagawa though the ratio of male raccoons being sexually active in Kanagawa was constant in 50% throughout the year. Therefore, it was expected that the factor of female was important for distribution of parturition periods of raccoons. It was thought that new approach such as the histological analysis of the ovary was necessary in future.

As the suggestion of management, I recommend that feral raccoons should be captured at mating season, which is when activity of animal increase to look for a partner, and dispersal season of juveniles. Not usual management of harmful birds and animal that the capture is carried out only at the affected areas, making low density of feral raccoons at establishment areas and prevention of the distributional expansion are important and necessary for management of feral raccoons. So, it is necessary to understand the reproductive characteristics, such as parturition period, thus it is necessary to develop the simple method that was useful for analysis about reproductive characteristics, such as the methods that this study provided. And continuous capture without analysis is not recommended. Local management plans should be formulated with an understanding of local raccoon situation by continuous monitoring. Therefore, it is important to develop the simple method that was useful for analysis about reproductive characteristics for continuous monitoring.

Bull. Nippon Vet. Life Sci. Univ., No.64, 97-99, 2015.

The neuropathogenicity of the Saffold virus in mouse models

Osamu KOTANI*

Department of Veterinary Pathobiology
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, VA-169)

The Saffold virus (SAFV) belongs to the genus *Cardiovirus* in the family *Picornaviridae*. In 2007, SAFV was isolated from a stool sample obtained in 1981 from an infant with a fever of unknown origin. Since its identification, SAFV has been considered to be mainly associated with acute gastroenteritis and acute upper respiratory symptoms in children. Although SAFV is occasionally detected in cases of aseptic meningitis and non-polio acute flaccid paralysis, its role in neurological diseases remains unknown. Hence, this study aimed to elucidate the neuropathogenicity of SAFV in mouse models. Chapter 1 describes an immunohistochemical analysis method we established for identifying the virus in paraffin-embedded tissue samples, using a polyclonal antibody raised against SAFV type 3 (SAFV-3). Chapter 2 describes the determination of the pathogenicity of two clinical isolates of SAFV-3 in mice, along with the isolates' tropism and neurovirulence in the cerebellum. Chapter 3 describes passage of SAFV in the cerebellum of neonatal mice, and the virological, pathological, and immunological characterization of the resultant strain.

Chapter 1. Establishment of a histopathological diagnosis of Saffold virus infection

The detection of pathogen antigens in tissues by immunohistochemistry is necessary for accurate pathological diagnoses and identification of the relationship between a pathogen and an illness. An immunohistochemical analysis on paraffin-embedded tissues using a polyclonal antibody raised against SAFV-3 was established. The antibody against SAFV-3 was tested for specificity to and cross-reactivity with enteroviruses (EV), which are a major cause of aseptic meningitis.

Anti-SAFV polyclonal antibody was produced by hyper-immunizing rabbits with an isolate strain of SAFV-3, the JPN08-404 strain. Three anti-EV polyclonal antibodies were raised against two strains of EV71 and poliovirus type 1 (PV1), with each virus being first denatured with sodium dodecyl sulfate and heat. Other antibodies were also used: one against recombinant viral capsid protein VP1 of Coxsackievirus B3 (CVB3), one against PV1, and one against peptide 2C of PV1.

The specificity and cross-reactivity of the polyclonal antibodies were determined by immunohistochemistry using formalin-fixed, paraffin-embedded cultured cells, and mouse tissues infected with SAFV, EV-A, -B, or EV-C. Degenerated cells within the SAFV-infected cultured cells and mouse tissues tested positive with an anti-SAFV antibody. The SAFV antigen-positive cells were positive for viral RNA, as demonstrated by *in situ* hybridization. Although the specificity of the anti-EV antibodies was low, and they were not able to distinguish between different EV serotypes, they were specific for the EV genus and did not recognize other genera.

In summary, an immunohistochemical method for detecting SAFV-3 viral antigens in paraffin-embedded tissues was established. The anti-SAFV-3 antibody had high specificity for SAFV and was able to distinguish it from the EV agents that cause aseptic meningitis. These antibodies are useful for the diagnosis of not only SAFV infection, but also picornavirus infections.

Chapter 2. Neuropathogenicity of two Saffold virus type 3 isolates in mouse models

SAFV is occasionally detected in children with acute flaccid paralysis, meningitis, and cerebellitis; however, the neuropathogenesis of the virus is still unknown.

*Supervisor : Prof. Fumihiro TAGUCHI

In this chapter, to elucidate the neuropathogenesis of SAFV, two clinical isolates of SAFV-3 were analyzed pathologically, virologically, and immunologically in neonatal ddY mice and 6-week-old BALB/c mice. One clinical isolate was obtained from the cerebrospinal fluid of an aseptic meningitis patient (JPN 08-404, referred to here as the AM strain), and the other was from a throat swab of a patient with upper respiratory tract inflammation (Gunma/176/2008, referred to here as the UR strain) ; both samples were obtained in 2008. Both viruses were inoculated intracerebrally into neonatal ddY mice.

After inoculation, the AM-inoculated mice developed a mild neurological sign suggesting ataxia, but rapidly recovered. During the observational period, the rate of body weight gain was significantly lower for the UR-inoculated mice than that of the MEM-inoculated control mice. However, the difference was not fatal. While both AM- and UR-infected mice had viral antigens in the cerebellum and around the ventricle, as detected by immunohistochemistry, the viral antigen-positive cells were mainly in the Bergmann glia of the cerebellum in AM-inoculated mice and in the neuroepithelial cells surrounding the lateral ventricle in UR-inoculated mice. In addition, the UR-inoculated mice also had viral antigen–positive epithelial cells in the oral mucosa and tooth germ.

In the blood, viral RNA levels peaked at 3 to 5 days post-inoculation (p.i.) in both SAFV-3 inoculated mice, though UR-inoculated mice had higher levels. In addition, viral RNA levels in the brain of both groups increased after day 3 p.i. and were correlated with the level of type I interferon (IFN). The UR-inoculated mice had significantly more viral RNA in the cerebrum and brain stem on days 3 and 5 p.i. than did the AM-inoculated mice, though the UR-inoculated mice had less viral RNA in the cerebellum on day 5 p.i. In addition, the UR-inoculated mice had high expression of type I IFN and inflammatory infiltrations into regions of the brain, including the cerebellum. Next, the neuroinvasiveness of SAFV-3 was evaluated by intraperitoneal inoculation. Histopathological and immunohistochemical examinations revealed that both strains invaded and infected the cerebellum and the region around the ventricle. Inflammatory infiltration was also observed in the brain of the UR-inoculated mice on day 21 p.i.

After examining these phenotypes in neonatal ddY mice, I next examined the neurovirulence of SAFV-3 in 6-week-old BALB/c mice using intracerebral inoculation. Both SAFV-3 strains caused significant body weight loss, compared to MEM, during the first 3 days p.i.; however, the mice exhibited no obvious neurological signs during the observation period. With both strains, the degenerated and necrotic nerve cells around the ventricle were positive for viral antigens on day 3 p.i., whereas only the AM strain had tropism to the cerebellum and triggered expression of type I IFN. Moreover, on day 8 p.i., the AM-inoculated mice exhibited mild inflammation in the cerebellar cortex. The neuroinvasiveness of SAFV-3 in 6-week-old BALB/c mice was also assessed with intraperitoneal, intravenous, intranasal, and per oral inoculation, but there was no evidence for neuroinvasiveness. Notably, oral and intranasal inoculations of the UR strain induced seroconversion in the BALB/c mice.

In summary, two clinical isolates of SAFV-3 had neurotropism and mild neuropathogenesis in neonatal and young mice. In addition, both viral strains were also neuroinvasive in neonatal ddY mice. The two strains had different cell tropism and neurovirulence in mouse models. These results suggest that SAFV-3 is a candidate neurotropic pathogen.

Chapter 3. Infection with Saffold virus passaged in mouse cerebellum affected cerebellar development in neonatal mice

SAFV-3 has tropism to the Bergmann glia of the cerebellum in neonatal mice, and no other picornaviruses have a similar reported tropism. Therefore, I focused on the tropism of SAFV to the cerebellum. The AM strain was passaged in the cerebellum of neonatal mice, and the passaged strain was characterized virologically, pathologically, and immunologically.

The AM strain was serially passaged five times in the cerebellum of neonatal BALB/c mice. Three days after intracerebral inoculation, the cerebellums were collected and homogenized. The supernatant was then serially inoculated five times into neonatal mice. After five passages, the virus was propagated in rhesus monkey kidney epithelial (LLC-MK_2) cells, and is referred to here as the AM-5Cb strain.

During the serial passages, the AM strain obtained two amino-acid replacements at VP2 and VP3, sequentially. According to the prediction of the viral capsid protein structure, the site of the VP2 mutation is adjacent to the virus receptor binding site. Moreover, the AM-5Cb strain has a hydrogen bond between the two mutation sites. Its replication was elevated in LLC-MK_2 cells and human rhabdomyosarcoma (RD) cells,

but reduced in baby hamster kidney (BHK) cells. The murine aneuploid fibrosarcoma cell line L929, the mouse neuroblastoma cell line Neuro2A, and the murine astrocyte cell line KT-5 were not susceptible to either AM or AM-5Cb strains.

The neurovirulence of the AM-5Cb strain in neonatal ddY mice was examined using intracerebral inoculations and the methods of Chapter 2. AM-5Cb-inoculated neonatal mice had noticeable ataxia in the early phase of the infection (days 2-4), and after, some mice developed hydrocephalus. Thus, the virulence of the AM strain increased in neonatal mice after five serial passages. Histopathological and immunohistochemical analyses revealed numerous degenerated/necrotic nerve cells and microglia infiltrations in the cerebrum, brainstem, cerebellum, and spinal cord of the AM-5Cb-inoculated mice. Viral antigen-positive cells were present in the lesions at a high frequency. The number of viral antigen-positive cells in AM-5Cb-inoculated mice was higher than that of AM-inoculated mice, but the cell tropisms were similar. Viral titers and the amount of viral RNA in the cerebrum/brainstem and cerebellum of the AM-5Cb-inoculated mice were significantly higher than those of the AM-inoculated mice on day 3 p.i. This result indicates that the replication of the AM strain in the brains of neonatal mice increased after five serial passages. In addition, high levels of type I IFN and massive inflammatory infiltrations were detected in the AM-5Cb-inoculated mice. Thus, the neuropathogenicity of the AM strain in neonatal mice increased after the passages.

To evaluate the effect of the SAFV infection on cerebellum development in neonatal mice, cerebellum gene expression was investigated. On day 3 p.i., cerebellar expression of the astroglia-specific glutamate transporter *GLAST* (related to the maintenance and differentiation of Bergmann glia) and *Hes5* (related to the induction of astroglia differentiation), was higher in AM-5Cb–inoculated mice than in AM-inoculated mice. In addition, Delta/Notch-like EGF-related receptor (*DNER*) and *Calbindin*, both of which are involved in differentiation and growth of Purkinje cells, were expressed in cerebellum at higher levels in AM-5Cb-inoculated mice than in AM-inoculated mice on day 21 p.i. Histopathologically, the axonal neurites of Purkinje cells seemed to be elongated in both AM- and AM-5Cb-inoculated mice, compared to those of the MEM-inoculated mice. These results suggest that the association of Bergmann glial cells and Purkinje cells during the differentiation and growth of the cerebellar cortex was uncontrolled after the infection.

To confirm the effect on the development of the cerebellar cortex, neonatal mice were intracerebrally inoculated with a high viral load of AM-5Cb. The inoculated mice exhibited cerebellar hypoplasia, with only a rudimentary layer on day 19 p.i. The mice had massive necrosis in the cerebrum and also exhibited cerebellar cortex dysplasia, indicating that AM-5Cb was highly virulent and infective. Because SAFV-3 has the potential to affect cerebellum development in human infants, a mouse model using the cerebellum-passaged strain of SAFV-3 might be useful for studying the neuropathogenesis of SAFV infection.

In conclusion, this is the first study of the neuropathogenesis of SAFV-3 using mouse models and virological, histopathological, and immunological methods. This study revealed that SAFV-3 is a potential neurotropic pathogen. This mouse model using SAFV-3 may be useful for studying the mechanisms controlling the severity of SAFV infection, for identifying antiviral factors, and for developing novel vaccines.

Bull. Nippon Vet. Life Sci. Univ., No.64, 100-102, 2015.

Pathophysiological analysis of the epileptogenic zone in familial spontaneous epileptic cats: electroencephalographic, imaging, and pathological studies

Shunta MIZOGUCHI*

Department of Veterinary Clinical Medicine
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, VA-170)

Epilepsy develops spontaneously in various animal species and is the most common cerebral disease in human and veterinary medicine. In veterinary clinical medicine, the development of idiopathic epilepsy is very rare in cats, and feline genetic epilepsy has not been reported. In 2009, Kuwabara and Hasegawa identified familial spontaneous epileptic cats (FSEC) in which the development of epilepsy was speculated to be inherited in an autosomal recessive manner. FSEC exhibit 2 types of seizure: spontaneous limbic seizures with secondarily generalization that strikingly resemble kindling and kainic acid-induced epilepsy models in cats; and generalized tonic-clonic seizures induced by vestibular stimulation that closely resemble those observed in EL mice. Since the kindling and kainic acid-induced models in cats and EL mice are animal models of human mesial temporal lobe epilepsy (MTLE), FSEC are also considered to be an animal model of human MTLE.

Lüders et al. proposed the presence of the following 6 types of abnormal regions including the epileptogenic zone: 1) structural abnormal zone or epileptogenic lesion, which is a macroscopic lesion causing epileptic seizures by hyperexcitation of the lesion or its adjacent area that is demonstrated by morphological imaging such as high magnetic field magnetic resonance imaging (MRI) ; 2) functional deficit zone, which is a region with abnormal function during an interictal period that is demonstrated by functional imaging such as functional MRI (diffusion-weighted imaging and perfusion-weighted imaging) ; 3) electroencephalographic (EEG) abnormal zone or initiative zone, which is a region in which paroxysmal discharges are observed in an interictal period that is mainly assessed by EEG; 4) ictal-onset zone, which is a seizure-originating region that is mainly assessed by EEG; 5) symptomatogenic zone, which is a region inducing the early symptoms and signs of seizures that is determined symptomatologically in the early stage of seizures; and 6) epileptogenic zone, which is required to induce epileptic seizures and seizures can be suppressed by its resection. These conceptual zones are not necessarily located in an identical position in every case and their interrelation also differs from case to case. In the present study, we identified the epileptogenic zone and performed pathophysiological analysis using electroencephalogical, diagnostic imaging, and histopathological techniques in FSEC based on the concept of these abnormal epileptic zones.

1. Chronic depth EEG analysis and pathological analysis in FSECs (Chapter II)

The most fundamental method for the pathophysiological analysis of epilepsy is the localization of an epileptic focus by EEG recordings. This chapter describes the results of the analysis of ictal EEGs by stereotactic implantation of depth electrodes and subsequent long-term video EEG monitoring in 5 FSECs. These FSECs were also analyzed by conventional pathological methods. In this study, subclinical and clinical spontaneous seizure activity was observed 54 times in total.

Clinically observed focal and secondarily generalized seizures were the typical limbic seizures detected and were synchronized with amygdaloid and/or hippocampal epileptiform activity; therefore, their symptomatic zone and ictal onset zone were suggested to exist in the amygdala and hippocampus.

*Supervisor : Prof. Michio FUJITA

However, the localization of the focus and laterality in subclinical and clinical focal ictal activity differed between individuals. A slight decrease in the number of neuronal cells was observed in the pyramidal cell layer of the hippocampus by histopathological analysis. It is considered, however, that further detailed assessment in intact cats is necessary because the influence of electrode insertion cannot be excluded completely.

2. 3D hippocampal volumetry of FSECs using high magnetic field MRI: time course changes in hippocampal volume (Chapter III)

The measurement of hippocampal volume (HV) by MRI is useful for the detection of hippocampal atrophy and laterality in the epileptogenic lesion of human MTLE patients and has been used commonly for the diagnosis and noninvasive preoperative assessment of hippocampal sclerosis (HS). The purpose of the studies described in this chapter was to detect structural abnormal zones in FSEC by 3D MR hippocampal volumetry. In addition, long-term changes in HV were assessed in 10 FSECs. HV was measured in 18 FSECs and compared with non-age matched control cats, and it was also measured in 14 FSECs and compared with age-matched control cats. In both studies, significant hippocampal asymmetry was found in FSECs, but no significant difference was found in HV between the FSECs and control groups. The age-matched experiment, however, demonstrated that the smaller side of HV in FSECs was significantly reduced compared to mean unilateral HV in controls. In addition, biennial long-term measurements of HV (3 times in total) in 10 FSECs revealed a tendency for a long-term reduction of HV and increase in hippocampal asymmetry, although these differences were not significant. This study demonstrated the presence of a structural abnormal zone in the hippocampus of FSECs; therefore, it is considered that the identification of hippocampal atrophy is useful for the non-invasive diagnosis of an epileptic focus.

3. Functional imaging analysis by diffusion and perfusion imaging using high magnetic field MRI: changes during interictal periods and immediately after seizures (Chapter IV)

Functional imaging analysis can be used as a method for noninvasive focal diagnosis in epilepsy to assess pathology and cerebral function from an aspect that is different from, but complementary to, electrophysiological and morphological assessments. This chapter describes the results of the study of imaging parameters during interictal periods in FSECs as measured by diffusion and perfusion MRI. In some FSECs exhibiting induced seizures, diffusion and perfusion MRI was also performed immediately after seizures, and the changes in imaging parameters between interictal and postictal periods were compared. In FSECs, a significant elevation of diffusibility (ADC value) and reduction of fractional anisotropy (FA value) were found in the hippocampus and amygdala during interictal periods, suggesting that there were microscopic changes in tissue structure such as the expansion of extracellular space in these regions. On the other hand, hypoperfusion was observed in the hippocampus, amygdala, and cerebral cortex, suggesting the presence of a functional deficit zone in these regions. In postictal periods, low diffusion and hyperperfusion areas were observed in and around the hippocampus and amygdala, suggesting the influence of ictal activity and its propagation. These observations were consistent with the origin of seizures in the hippocampus and amygdala on intracranial EEG recordings (Chapter III). In FSECs, the results of diffusion and perfusion MRI indicated a functional deficit zone during interictal periods and possibly reflected epileptogenic foci during postictal periods; therefore, this method is considered to be useful as an approach for the noninvasive diagnosis of epileptogenic foci.

4. Histopathological analysis of the hippocampus and amygdala in FSECs (Chapter V)

This chapter describes the results of the assessment of neuronal cell numbers and gliosis, which are findings for HS in human MTLE patients, in intact homozygous FSECs developing seizures and a small number of heterozygous FSECs that were parents of FSECs. "Intact FSECs" means FSECs not subjected to invasive surgery such as electrode insertion. The results of the examination of granular cell dispersion (GCD) and mossy fiber sprouting (MFS) in the hippocampus are also described. Extensive neuronal cell loss was observed in the pyramidal layer of the hippocampus and amygdala of homozygous and heterozygous FSECs, but this decrease was not associated with gliosis. This finding was not consistent with the typical findings for HS in human MTLE patients. In homozygous FSECs, MFS was not found, but gliosis without neuronal cell loss in the CA4 region of the hippocampus and GCD-like findings characteristic for HS were observed. The hippocampal abnormality in FSEC represented by neuronal cell loss without gliosis was suggested to

be the result of a malformation that is different from typical HS, such as human familial mesial temporal lobe epilepsy (FMTLE).

For the identification of the epileptogenic zone and pathophysiological analysis of FSECs, electrophysiological, diagnostic imaging, and histopathological studies were conducted. Video EEG recordings with depth electrodes demonstrated epileptiform EEG activity (ictal onset zone/symptomatogenic zone) in the hippocampus and amygdala, and the measurement of HV and functional imaging revealed a macroscopic structural abnormality in the hippocampus (structurally abnormal zone) and microscopic structural changes with lowered function (functional deficit zone), respectively. Pathological investigation also demonstrated a decrease in the number of neuronal cells in the hippocampus and amygdala. These results suggested the presence of an epileptogenic zone in the hippocampus and amygdala in FSEC and proved that epilepsy in FSEC was homologous with human MTLE. To prove the epileptogenic zone in FSEC, surgical resection and confirmation of seizure free should be performed in the future study.

The frequency of seizures, their severity, and the location and laterality of the epileptogenic zone differed between individual FSECs, and these heterogeneous phenotypes are also observed in human FMTLE patients. On the basis of these clinical and pathological similarities between FSEC and human FMTLE patients, we have no choice but to consider the possibility that the hippocampal atrophy and decreased number of neuronal cells found in FSEC are also congenital/genetic malformations. Furthermore, similar pathological changes were observed in heterozygous FSECs that had not developed epilepsy. Therefore, it is unlikely that only these pathological changes in the hippocampus are involved in the onset of seizures, and a variety of etiologies are considered to be involved in epileptogenicity in FSEC. Thus, the mode of inheritance in FSEC may not be simple autosomal recessive inheritance as was first supposed upon the discovery of this animal model, and genetic analysis to identify the responsible genes will be required in the future. FSEC are the sole feline model of genetic epilepsy and also represent a unique model in veterinary medicine. FSEC can be a good animal model of MTLE as well as a kindling model, a kainic acid model, and EL mice, and especially it may be a specific model of human FMTLE. Since human FMTLE also has a variety of phenotypes, the responsible genes have not been identified. Therefore, the identification of the responsible genes in FSEC can have an important role in the elucidation of the pathophysiology of human FMTLE and feline epilepsy. It is considered that the elucidation of the pathogenic mechanism for epilepsy in FSEC will play a in the analysis of the pathophysiology of epilepsy that is common between veterinary and human medicine.

Bull. Nippon Vet. Life Sci. Univ., No.64, 103-105, 2015.

Studies on the diagnosis, surgical treatment and outcome of the Thoraco-lumbar Intervertebral Disc Herniation and its associated diseases

Takeshi AIKAWA*

Department of Veterinary Clinical Medicine
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 28 November 2014, VB-319)

Thoracolumbar intervertebral disc herniation (IVDH) is a common disorder of the vertebral column mainly seen in chondrodystrophic dogs. Surgical decompression and removal of the extruded disk material is a widely accepted treatment modality, especially for disk extrusion. Disk protrusion typically occurs in nonchondrodystrophic larger breeds at older ages with chronic onset, but either type of disk herniation can occur in any breed of dog. Although thoracolumbar IVDH in dogs is one of the extensively documented diseases in veterinary neurology, information regarding long-term outcome is still insufficient. The goal of the study reported here was to evaluate a long-term clinical outcome, precise diagnostic evaluation methods and surgical procedures for Thoracolumbar IVDH.

1. Long-term neurologic outcome of hemilaminectomy and disk fenestration for treatment of dogs with thoracolumbar intervertebral disk herniation

Objective: To determine the proportion of dogs with thoracolumbar intervertebral disk herniation (IVDH) that successfully recovered following hemilaminectomy and fenestration, the time to ambulation (TTA) in affected dogs after surgery, and the frequency of urinary and fecal incontinence in recovered dogs and to document long-term complications.

Design: Retrospective case series.

Animals: 831 dogs with thoracolumbar IVDH treated by hemilaminectomy and concomitant disk fenestration by the same surgeon.

Procedures: For all dogs, neurologic deficits before surgery had been assessed with a modified grading system. Dogs were reexamined after surgery over a period of 3 to 6 months, and follow-up evaluation was performed at >12 months. The proportion of dogs that neurologically improved after surgery, TTA, and incidence of fecal or urinary incontinence in recovered dogs were compared among dogs with various grades of neurologic dysfunction before surgery.

Results: Of 831 dogs, 122 had unsuccessful outcomes and 709 had successful outcomes. Of 620 dogs with intact deep nociception before surgery, 606 (97.7%) were ambulatory after surgery. Despite maintaining the ability to walk, 7 dogs were judged to have an unsuccessful outcome because the severity of ataxia did not improve. Of 211 paraplegic dogs with loss of deep nociception, 110 (52.1%) dogs became ambulatory after surgery. Long-term complications included incontinence, permanent neurologic deterioration, and self-mutilation. Dogs with paraplegia before surgery had a higher frequency of urinary or fecal incontinence, compared with dogs that were ambulatory.

Conclusions and clinical relevance: Prognosis for dogs with thoracolumbar IVDH that retain deep nociception in at least 1 of the pelvic limbs or tail before surgery was good.

2. Effectiveness of prophylactic fenestration with hemilaminectomy for thoraco-lumbar intervertebral disc extrusion

Objectives: To determine the incidence and the location of recurrent thoraco-lumber intervertebral disc extrusion (T-L IVDE) in chondrodystrophic dogs after hemilaminectomy with prophylactic fenestration (PF) and to document PF related complications.

*Supervisor : Prof. Yasushi HARA

Study design: Retrospective study.
Sample population: 793 chondrodystrophic dogs.
Methods: Medical records of dogs that recovered from first hemilaminectomy and concomitant PF with >12 months follow-up were reviewed. The rate of recurrent T-L IVDE in dogs that underwent a second surgery and dogs with clinical signs that improved without surgery were evaluated. The rate of second disc extrusion(SDE) within T11-L4 intervertebral discs was compared between the PF discs and non PF discs.
Results: T-L SDE were surgically confirmed in 15 dogs (2.3%), 2-61 months after the first surgery. No dog had recurrence due to further extrusion at the initial extrusion site. Sixty-one dogs had neurological deficits that improved without surgery (mean follow-up: 43.1 months). The SDE occurred at a PF disc (n=1), adjacent to the PF discs (n=8), non-adjacent to the PF discs (n=6). The risk of SDE in non PF discs was 26.2 times higher than that in PF discs (95% CIs 3.4 to 203.4; P<0.001). Major PF related complications included iatrogenic introduction of the disc material into the spinal canal (n=1), and vertebral subluxation or instability (n=3) at 1-88 months postoperatively.
Conclusion and clinical relevance: Performing PF at spaces predisposed to disc extrusion significantly decreases the incidence of T-L IVDE recurrences. PF is a safe and effective treatment to prevent SDE in chondrodystrophic dogs.

3. Diagnostic techniques and surgical treatment for thoracolumbar intervertebral disc associated dynamic compression

Objective: To describe the diagnostic findings, surgical technique and outcome in dogs with thoracolumbar intervertebral disc-associated dynamic compression.
Study design: Retrospective case series.
Animals: Client owned dogs (n=11).
Methods: Medical records (2005-2010) of dogs with a stress myelographic diagnosis of spinal cord injury due to thoracolumbar intervertebral disc-associated dynamic compression with inconclusive compression in the neutral position that had hemilaminectomy and vertebral stabilisation were reviewed. Data on pre- and postoperative neurologic status, diagnostic findings, surgical techniques and outcomes were retrieved. Follow up clinical and radiographic evaluations were performed immediately, and approximately 1, 2, 6 months postoperatively, and at annual follow-up examinations.
Results: The stress myelography demonstrated distinct ventral dynamic compression due to bulging of the disc and additional dorsal compression due to infolding of the ligamentum flavum in some cases. The median percentage of post stress reduction in spinal cord height on lateral view was 18.0% (9.8-27.2%). All dogs recovered after surgery and remained ambulatory at follow up (median: 45 months, range: 7 to 94 months).
Conclusions and clinical relevance: Thoracolumbar intervertebral disc degeneration may result in disc-associated dynamic compression. Stress myelography was an effective means of diagnosing this condition and hemilaminectomy with vertebral stabilisation was an effective treatment resulting in long term neurological improvement in all dogs.

4. Diagnostic techniques and surgical treatment for spinal canal stenosis and vertebral instability caused by congenital thoracic vertebral anomalies

Objective: To describe diagnostic findings, surgical technique and outcome in dogs with thoracic spinal canal stenosis and vertebral instability secondary to congenital vertebral anomalies.
Study design: Retrospective clinical study.
Animals: Dogs (n=9) with thoracic spinal canal stenosis.
Methods: Medical records (1995-1996; 2000-2006) of 9 dogs with a myelographic diagnosis of spinal canal stenosis and/or vertebral instability secondary to congenital vertebral anomaly that were surgically managed by vertebral stabilization with or without laminectomy were reviewed. Data on pre- and postoperative neurologic status, diagnostic findings, surgical techniques and outcomes were retrieved. Follow up evaluations were performed at 1, 2, and 6 months. Long term outcome was assessed by means of clinical examination or owner telephone interviews.
Results: Spinal cord compression was confirmed by myelography and in 2 dogs, dynamic compression by stress myelography. Eight dogs regained the ability to ambulate postoperatively. One dog with a partial recovery regained voluntary movement but did not become ambulatory.
Conclusions: Spinal cord injury secondary to congenital vertebral anomaly may have a good outcome when treated by vertebral stabilization with or without laminectomy. Adequate stabilization of the vertebrae and improved neurologic outcome was achieved in most dogs.
Clinical relevance: Vertebral stabilization using positively

threaded profile pins and polymethylmethacrylate with or without laminectomy is an effective treatment for spinal canal stenosis and vertebral instability secondary to congenital thoracic vertebral anomalies.

5. A comparison of thoracolumbar intervertebral disc extrusion in French Bulldogs and Dachshunds and association with congenital vertebral anomalies

Objectives: To compare data for French Bulldogs and Dachshunds that had hemilaminectomy for thoracolumbar intervertebral disc extrusion (T-L IVDE) by 1 surgeon and to evaluate the association between IVDE and congenital vertebral anomalies.
Design: Retrospective case series.
Animals: French Bulldogs (n=47) and 671 Dachshunds.
Methods: Age, gender, vertebral anomaly, kyphosis/kyphoscoliosis, IVDE site, non-recovery and progressive hemorrhagic myelomalacia development from grade 5 (paraplegia without deep nociception) were compared between the 2 breeds.
Results: French Bulldogs were significantly younger (P=.00001), more likely to be male (P=.023), and more likely to have a congenital vertebral anomaly and kyphosis/kyphoscoliosis (P<.00001) than Dachshunds. The frequencies of French Bulldogs with IVDE within typical sites (T11-L3) were significantly lower (P=.0005) and within caudal sites (L3-L7) significantly higher (P=.0001) compared with Dachshunds. None of the French Bulldogs had IVDE within the kyphotic/kyphoscoliotic segment. The frequency of lumbar IVDE (L1-L5) in French Bulldogs with kyphosis/kyphoscoliosis was significantly higher (P=.003) compared with French Bulldogs without kyphosis/kyphoscoliosis. In grade 5 dogs, the risk of developing progressive hemorrhagic myelomalacia in French Bulldogs was significantly higher (P=.03) than in Dachshunds.
Conclusion: The distribution of IVDE site in French Bulldogs within the thoracolumbar and lumbar spine was different from Dachshunds. IVDE sites were not located at the sites of vertebral anomaly. French Bulldogs appeared to have T-L IVDE at younger ages, with higher male predisposition and higher risk of developing progressive hemorrhagic myelomalacia from grade 5 compared with Dachshunds.

6. A comparison of molecular mechanism of intervertebral disc degeneration between French Bulldogs and Dachshunds

Objectives: Intervertebral disc degeneration (IVDD) has a significant impact on the quality of life. The nucleus pulposus (NP) in chondrodystrophic dog breeds (CDBs) is similar to that in humans, because the cells disappear with age and are replaced with fibrochondrocyte-like cells. IVDD develops within the first year of life in CDBs. We previously reported that French Bulldogs (FB) appeared to have thoracolumbar intervertebral disc extrusion (T-L IVDE) at younger ages with higher male predisposition and higher risk of developing progressive hemorrhagic myelomalacia from grade 5 as compared to Dachshunds. However, the molecular mechanism underlying age-related IVDD is yet to be ascertained. The aim of this study is to investigate the molecular differences in IVDD between FB and Dachshunds.
Methods: Herniated disc material (HNP) was collected from FB and Dachshunds that underwent surgical treatment for intervertebral disk herniation at the Aikawa Veterinary Medical Center, Tokyo, Japan.
Results: Histological analysis showed a safranin O-positive area, and NP cells apoptosis was decreased in the HNP of FB as compare with that of Dachshunds. Real-time reverse-transcription polymerase chain reaction (RT-PCR) showed increased mRNA expression of Type 1 collagen and decreased levels of Type 2 collagen, aggrecan, and matrix metalloprotease (MMPs) in the HNP of FB as compare with that of Dachshunds.
Conclusion: These results suggest that a decrease in the degradation of extracellular matrix and an increase in production of fibrocartilage lead to total NP tissue volume increase in FB as compared with Dachshunds. This was considered to be the cause of early IVDH onset in FB.

In these study, we evaluated a long-term clinical outcome, precise diagnostic evaluation methods and surgical procedures for Thoracolumbar IVDH. Our results may contributes development of clinical management for Thoracolumbar IVDH.

Bull. Nippon Vet. Life Sci. Univ., No.64, 106-107, 2015.

Study of DNA polymorphisms in canine uncoupling protein 2 and 3 genes

Chihiro UDAGAWA*

Department of Basic Science
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, VNT A-3)

Uncoupling proteins (UCPs) in the inner mitochondrial membrane are members of the mitochondrial anion-carrier protein family. Mammals have five UCP homologs, of which UCP1, UCP2, and UCP3 are closely related, while UCP4 and UCP5 are more divergent from the other UCPs. Based on genetic association studies, *UCP2*, *UCP3*, or both are reportedly associated with obesity, insulin resistance, type 2 diabetes mellitus, and metabolic syndrome in humans. For example, a SNP in the 5' untranslated region of the human *UCP3* mRNA, designated the UCP3-55C/T SNP, is a genetic marker associated with elevated high-density lipoprotein cholesterol levels, reduced body mass index (BMI), weight, waist circumference, waist-to-hip ratio, fat mass, low-density lipoprotein (LDL) cholesterol, and total cholesterol (T-Cho). The treatment and prevention of obesity and metabolism-related diseases are also clinically important in dogs. Here, we describe the nucleotide sequences included as part of the 5'-UTRs of *UCP2* and *UCP3* mRNAs. In addition, an mRNA expression study was performed with various tissues and the reverse transcription-polymerase chain reaction (RT-PCR) technique. We also investigated whether canine *UCP2* and *UCP3* are associated with alterations in metabolism.

1. cDNA cloning and expression analysis of the genes encoding canine uncoupling protein 2 and 3

We here present the nucleotide sequences that are included as part of the 5'-UTRs of the *UCP2* and *UCP3* mRNAs as foundational findings for further study. A search of the NCBI database showed that the 5'UTRs of dog *UCP2* and *UCP3* shared 89.7% and 74.6% with respective orthologous sequences in humans. Comparison of the dog genomic and mRNA sequences revealed that canine *UCP2* comprised exons 1 to 8 and that the canine start codon is located in *UCP2* exon 3. Dog *UCP3* comprises exons 1 to 7 and that the start codon is located in exon 2. These results revealed that the numbers of exons and relative locations of the first coding exons in *UCP2* and *UCP3* in dog corresponded with those in the human orthologs. The RT-PCR and RT products obtained from total RNA from each of 30 canine tissues were used to determine the expression patterns of the *UCP2* and *UCP3* mRNA. We found that canine *UCP2* and *UCP3*, like human *UCP2* and *UCP3*, differed from each other with regard to expression profile; specifically, canine *UCP2*, like human *UPC2*, was ubiquitously expressed; in contrast, canine *UCP3*, like human *UPC3*, was highly expressed but in fewer tissues (i.e. skeletal muscle, tongue and diaphragm).

2. Discovery of DNA polymorphisms in canine *UCP2* and *UCP3*

We identified polymorphic DNA sites in coding regions, portions of the 5'- and 3'- flanking sequences, and intron-exon boundaries of canine *UCP2* and *UCP3*. For the *UCP2* analysis, we sequenced six regions; genomic DNA from each of 11 dogs was used to amplify each region as a separate fragment. We identified four SNPs (-3629C/G, -3621T/C, -2931A/T, -2913A/G) and one indel (-2951delTTCA) in intron 1, one SNP (-2613A/C) in exon 2, three SNPs (-916C/T, -748G/A, -636A/G) in intron 2, one SNP (IVS6-108C/T) and one indel (IVS6-133 delTCTCCCC) in intron 6, one SNP (IVS7-106C/T) and one indel (IVS7-187insA) in intron 7. We also identified one indel (IVS7-152delA) in intron 7 of *UCP2* in our analysis of 50 Labrador Retrievers. In all, we identified 10 SNPs and four indels in *UCP2*.

*Supervisor : Prof. Toshiro OMI

For the *UCP3* analysis, we sequenced nine regions; genomic DNA from each of 11 dogs was used to amplify each region as a separate fragment. We identified five SNPs (-4399C/T, -4339T/C, -4010C/T, -930T/C, -803C/T) in intron 1, one SNP (143A/C) in exon 3, three SNPs (IVS3+26T/C, IVS3+69G/A, IVS3+121T/C) in intron 3, two SNPs (IVS5-115G/C, IVS5-100T/C) in intron 5, and one indel (1106delAAG) in exon 7. Additionally, one SNP (838T/C) located in exon7 was identified in an analysis of 30 Shiba individuals, and another SNP (-4160G/A) in intron1 was identified in the analysis of 50 Labrador Retrievers. In all, we identified 13 SNPs and one indel in *UCP3*.

3. Analysis of genetic association between *UCP2* and *UCP3* polymorphisms and metabolic data from dogs

We determined the genotype of 50 Labrador Retrievers for each of the 14 polymorphic sites (13 SNPs and one indel) in *UCP3* and examined whether any of the genotypes were associated with GLU, T-Cho, LDH or TG levels. The average measurements for each metabolic marker were calculated with respect to genotype group. Associations between genotype frequencies and metabolic data were analyzed by one-way analysis of variance (ANOVA). There were no significant differences between genotypes with regard to GLU, LDH, or TG measurements for any polymorphic site. In contrast, T-Cho levels differed significantly between genotype groups for the following four sites: -4399C/T, -4339T/C, -930T/C, -803C/T. We also subjected the 14 polymorphic sites in *UCP2* to this association analysis. None of these DNA polymorphisms was significantly associated with metabolic data. Notably, for the polymorphic sites -3621T/C, -2931A/T, -748G/A, -636A/G, and IVS7-106C/T, the variant allele was not observed within any of the 50 individual Labrador Retrievers analyzed.

The agonists of peroxisome proliferator activated receptors (PPAR) activate *UCP3* expression. Intron 1 of *UCP3* contains the putative binding elements recognized by MyoG/MyoD, PPARγ/RXRα, and SP1/SP3 that enhance the *UCP3* transcription that is mainly regulated by PPARs in hamsters, rats, and mice. Recently, we found nucleotide sequences similar to the PPARγ/RXRα element in intron 1 of dog *UCP3* (Canine Genome Draft, NC_006603.3). These findings indicated that dog *UCP3* intron 1 may be associated with regulation of *UCP3* gene expression. Further studies will be needed to determine whether PPAR ligands bind this intronic region in dogs.

4. Interbreed analysis of Shiba and Shetland sheepdog

Shetland Sheepdogs apparently have a predisposition to primary hyperlipidemia as determined by the levels of cholesterol, triglycerides, and free fatty acids. Therefore, we investigated the distribution of genotypes of the SNPs and indels within *UCP2* and *UCP3* in a population of Shetland Sheepdogs (n=30) ; Shiba (n=30) were also examined for comparison with the Shetland Sheepdogs. The Fisher's exact test was used to determine the statistical significance of differences in allele frequency between the two breeds for each locus.

Statistically significant differences in allele frequency between the two breeds were found for five of the 14 polymorphic sites in *UCP2* (-3629C/G, -2931A/T, -748G/A, -636A/G and IVS6-133delTCTCCCC). Of these 14 polymorphic sites in *UCP3*, four SNPs (-4339T/C, -930T/C, 143A/C and IVS3+121T/C) were significantly different in allele frequency between the two breeds. Despite the differences in genetic background between the dog breeds, the different allele frequencies in the *UCP2* and *UCP3* polymorphic site between the two breeds may result from the susceptibility of Shetland Sheepdogs to hypercholesterolemia in a limited number of individuals.

The T alleles at -4339T/C and -930T/C, which are both located in *UCP3* intron 1, were each associated with higher T-Cho levels, as shown by two different experiments: (i) the association between polymorphisms and metabolic data and (ii) distribution of alleles in the breed that is susceptible to hypercholesterolemia. These results indicated that dog *UCP3* might be associated with T-Cho levels.

The results obtained from a limited number of individuals indicated that *UCP3* in dogs may be associated with total cholesterol levels. Therefore, the *UCP3* gene could be an interesting target, not only for lipid metabolism, but also for the treatment and prevention of obesity and metabolic-related diseases in dogs.

Bull. Nippon Vet. Life Sci. Univ., No.64, 108-109, 2015.

Distribution and dynamics of quasispecies related with bovine viral diarrhea virus infection

Kaoru NISHINE*

Department of Basic Science
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, VNT A-4)

Bovine viral diarrhea virus (BVDV) is an enveloped positive-stranded RNA virus classified into the genus *Pestivirus* within the family *Flaviviridae*. Disease induced by BVDV leads to significant economic losses for cattle producers worldwide. BVDV can be classified into cytopathogenic (CP) and noncytopathogenic (NCP) biotypes; most field-isolated BVDV strains are NCP. Only NCP virus can establish a persistent infection. Persistently infected (PI) calves continuously excrete large amounts of virus particles throughout their lives. Because they show few symptoms, PI calves are a continual source of infection in a herd. Furthermore, PI calves are at risk of developing fatal mucosal disease (MD). A pair of closely related NCP and CP viruses can be isolated from animals that have developed MD.

NCP BVDV can be further divided into two additional biotypes, one of which shows the exaltation of Newcastle disease virus phenomenon (END^+) and the other of which does not (END^-). END^- virus does however interfere with vesicular stomatitis virus. END^+ virus suppresses the induction of type I interferon (IFN) production in cell cultures, whereas END^- virus does not. Previous studies have shown that these viruses coexist in the same strain as quasi-species. However, the distribution of these quasi-species in the field and in infected animals remains unclear, and whether a causal link exists between quasi-species and the clinical condition has not been addressed. The aims of the present study, therefore, were to collect basic data for understanding of the relation between BVDV quasispecies and the various disease state of BVDV infection by examining their distribution and fluctuation among field isolates and within PI cattle.

Chapter 1. Distribution of quasispecies in BVDV field isolates

END^+ and END^- viruses are known to coexist within several laboratory strains of BVDV. In a previous study, I found that END^+ and END^- viruses may be present in varying proportions in BVDV field isolates, but the geographical region examined in that study was limited to Hokkaido Prefecture, Japan. In the present study, 39 field strains of BVDV isolated in another prefecture were analyzed using a peroxidase-linked assay (PLA) with anti-NS3 monoclonal antibody, observation of the cytopathic effect (CPE), the END method, and the reverse plaque formation (RPF) method for quantification of BVDV, CP virus, END^+ virus, and END^- virus, respectively. The isolates were grouped as follows based on the determined virus composition: 32 isolates (82.0%) in which END^- viruses were the major component; 2 isolates (5.1%) containing similar titers of END^+ and END^- viruses; 1 isolate (2.6%) in which END^+ virus was the major component; and 4 isolates (10.3%) containing CP viruses. These results show that END^- viruses are distributed widely in the field and are the major component of many field isolates. Interestingly, these results differed from previous results obtained from Hokkaido Prefecture field isolates, 52% of which contained END^+ viruses as the major component. The data generated in the present study suggest that the distribution of quasi-species in the field varies geographically.

Chapter 2. Changes in quasi-species composition during virus passage in cultured cells

Although BVDV field isolates contain varying proportions of END^+ and END^- viruses, they are usually passaged several times in cultured cells, which calls into

*Supervisor : Prof. Toshiro OMI

question whether virus composition estimates based on titers determined from cultured cells accurately reflect the composition in BVDV-infected animals. In this experiment, changes in virus composition during multiple passages in cultured cells were analyzed. Four field isolates, passaged once in a livestock hygiene center, were passaged 19 additional times at intervals of 4 days in cultured bovine testicular cells. The culture supernatant was sampled at each passage and analyzed using the PLA, END, and RPF methods. The results of these analyses indicated that the virus composition is relatively stable until the 10th passage, suggesting that the composition of field isolates is similar to that within the host. However, after the 10th passage, END^+ viruses began to predominate over END^- viruses. Therefore, the passage history must be taken into consideration in quantitative analyses of virus quasi-species.

Chapter 3. Direct detection of quasi-species from cattle persistently infected with BVDV

In this experiment, END^+ and END^- viruses in PI cattle were examined by direct detection. A total of 50 blood samples were collected from subclinical PI cattle, and active viruses were isolated using the PLA method. Samples from which BVDV was isolated were then tested using the PLA, END, and RPF methods. Active BVDV was isolated from 26 of 50 samples, and END^- virus was detected in 16 of the 26 samples. END^+ virus was detected in 22 of the 26 samples. The 26 samples with active BVDV were divided into 5 groups based on virus composition: 5 samples in which END^+ virus was the predominant component, 1 sample in which END^- viruses were predominant, 1 sample in which the END^+ and END^- virus titers were equal, 16 samples in which the titers determined by PLA were higher than the titers determined using the other methods, and 3 samples in which BVDV was detected only by PLA. These results show that END^+ and END^- viruses, which differentially affect the innate immune response, coexist in PI animals. However, the relationship between these viruses and various aspects of the disease, such as symptoms and duration of persistent infection, has not been elucidated. Further studies are needed to determine whether any association exists between the quasi-species and BVDV infections. Interestingly, three samples did not show neither the END phenomenon nor reverse plaques, suggesting that either these samples contain a new BVDV biotype that cannot be detected by biological methods such as END and RPF or that interfering biologically active factors were present.

Chapter 4. Changes in quasi-species composition in PI cattle

It was hypothesized that the coexistence of quasispecies affects the clinical condition and mode of transmission of BVDV infection because both END^+ and END^- viruses were detected in the blood of PI cattle. In this experiment, changes in virus composition over time were examined in PI cattle. Blood samples were obtained regularly from three breeding PI animals, and the titer of BVDV, END^+ virus, and END^- virus in the serum were determined using the PLA, END, and RPF methods, respectively. Both END^+ and END^- viruses were detected in the serum of one of the animals at all samplings. In another animal, no END^+ virus was detected, although END^- virus was detected at all samplings. This result suggests that a variety of quasispecies compositions may be present even among cows that are born during the same epidemic. In the third PI cow, which survived for an extended period, END^+ virus was detected in all serum samples collected before the age of 23 months but in none of the samples collected after the age of 24 months. END^- virus was detected in all samples until the age of 68 months. Total RNA was extracted from five of the serum samples from the long-surviving PI cow. The BVDV N^{pro} gene region was amplified by reverse transcription polymerase chain reaction and sequenced using next-generation sequencing techniques. The abundance of an interferon-inducible nucleotide sequence within this region was higher in 2 samples that were collected after the age of 61 months, suggesting that the virus composition fluctuates in PI cattle and that END^- viruses eventually predominate in long-surviving PI cows. The results of this study demonstrate that END^- and END^+ viruses are distributed widely in the field and that they coexist in varying proportions as quasispecies in viral strains. The results of this study also show that END^+ and END^- viruses coexist in PI animals and that their composition fluctuates with aging. In addition, the duration of persistent infection appears to be related to the proportion of END^- virus, which tends to increase over time. The results of this study also suggest that the coexistence of END^+ and END^- viruses and fluctuations in their proportions affect the clinical diversity of BVDV infections and the onset of MD. Further research into the distribution of these quasi-species during persistent BVDV infection may lead to elucidation of the mechanisms of both BVDV infection onset and the development of MD.

Bull. Nippon Vet. Life Sci. Univ., No.64, 110-111, 2015.

Studies on cortisol and prolactin concentrations in umbilical cord blood, amniotic fluid, maternal blood, and breast milk related to perinatal factors

Yoko SANO*

Laboratory of Animal Physiology
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, ALS A-4)

Prolactin is known as a hormone that promotes the production of breast milk in mammals. In addition, prolactin shows a variety of physiological functions such as induction of maternal behavior, enhancement of stress tolerance, osmoregulation, and immune regulation. Cortisol is an adrenocortical hormone that regulates energy metabolism. The level of cortisol increases in response to stress. During the delivery, mother undergoes labour pain stress. The fetus is also thought to be undergoing high stress by uterine contractions during the delivery. In this study, based on the stress response of prolactin and cortisol, the correlations between the concentrations of both hormones in umbilical cord blood and amniotic fluids were studied during delivery. In lactation period, prolactin is secreted with high concentration in breast milk as well as maternal blood. It has recently been reported that breast milk prolactin taken at neonatal stage affects on the maternal behavior and stress tolerance at adult stage in rats, suggesting importance of physiological role of the breast milk. In human, however, relationship of prolactin concentrations in mothers' blood and breast milk is not known. In this study, the relationships between the prolactin concentrations in mothers' blood and breast milk were also studied.

This study was reviewed and approved by the ethics committee at Fujinomiya City General Hospital in Shizuoka prefecture. The subjects also signed a consent form after receiving an oral explanation and written documents regarding the study.

1. Correlations of cortisol and prolactin levels in umbilical cord blood with the modes of delivery and duration of delivery

Umbilical cord blood was collected after vaginal delivery and plasma was separated by centrifugation. The concentration of cortisol was determined by Radioimmunoassay using Cortisol kit: FTA (TFB). The concentration of prolactin was measured by Erectro Chemiluminescence Immunoassay using ECLusys Prolactin III kit (Roche-diagnostics). All statistical analyses were performed by Spearman's rank correlation coefficient test using SPSS v15 for Windows.

In human, delivery consists of three stages. The first stage is the period from the beginning of regular uterine contractions until full cervical dilation; the second stage is from full cervical dilation to the birth of a neonate, and the third stage is from the birth to completion of expulsion of the umbilical cord, placenta and foetal membrane. First, relationship between delivery modes and cortisol and prolactin concentrations in the umbilical cord blood were examined. The cortisol concentration, but not prolactin concentration, of the vacuum-assisted delivery group was significantly higher than that of the spontaneous delivery group. Since vacuum-assisted delivery is employed in difficult delivery, neonate may suffer from stronger stress during the delivery. Then, the correlations between cortisol concentrations and delivery duration among the spontaneous delivery groups were analysed. Positive correlations were observed between cortisol concentration and duration of full delivery with stronger extent during the second phase of parturition. These findings suggest that fetuses

*Supervisor : Prof. Minoru TANAKA

suffer from the stress caused by uterine contraction during delivery. On the other hand, the prolactin concentration showed no significant correlation with the duration of delivery. Since stress response of prolactin is temporal, individual prolactin levels in cord blood may fluctuate during delivery.

2. Correlations of cortisol and prolactin concentrations in amniotic fluid with the modes of delivery and duration of delivery

Umbilical fluid was collected at spontaneous or artificial membrane rupture during delivery. Concentrations of cortisol and prolactin were determined by the methods described in Chapter 2. Both the cortisol and prolactin concentrations in amniotic fluid of the vacuum-assisted delivery group were significantly higher than those of the spontaneous delivery group. There was a positive correlation between the cortisol concentration in amniotic fluid and the duration of delivery as observed in the umbilical cord blood, whereas, prolactin concentration showed no correlation with the duration of delivery. The origin of cortisol in the amniotic fluid is considered to be the urine of the fetuses and therefore, the increase of cortisol concentration may reflect the stress response of fetuses during delivery. Prolactin in the amniotic fluid derived from endometrial decidua where stress response of prolactin is unclear.

3. Correlation of prolactin concentrations between maternal plasma and breast milk

Prolactin concentrations in breast milk are markedly increased during the first few days after delivery. Although prolactin is known to be transferred from maternal blood to breast milk, the correlation between prolactin concentration in breast milk and those in maternal blood is not clear. In this study, we investigated the correlation between prolactin concentrations in maternal blood and breast milk collected 4 days after the delivery. First, prolactin concentrations in foremilk collected before breast milk feeding were compared with those in hindmilk collected after breast milk feeding. There was no significant difference in prolactin concentrations between foremilk and hindmilk. Then, prolactin concentrations in maternal plasma were compared with those in hindmilk of primiparas and multiparas. There was a positive correlation between prolactin concentrations in plasma and hindmilk, with a stronger correlation in primiparas than in multiparas. Breastfeeding is known to has many benefits such as enhancement of immune function in neonate, decrease of the risk atopic diseases, and mortality in infants, and decrease breast and ovarian cancer risk in mother. In addition, recent reports have demonstrated the epigenetic effects of breast milk prolactin on the nutritional status and brain function of adult offspring. Therefore, it is important to clarify the maternal factors affecting on prolactin concentration in breast milk.

4. Overall recapitulation

In this study, we first examined relationship between delivery modes and cortisol and prolactin concentrations in the umbilical cord blood. The cortisol concentration was significantly higher in the vacuum-assisted delivery than in the spontaneous delivery and positively correlated with the duration of delivery, with higher extent during the second phase of parturition. These findings suggest that fetuses as well as mothers suffer from the stress caused by uterine contraction during delivery. On the other hand, the prolactin concentration showed neither significant correlation with the duration of full delivery nor the second stage of delivery. Since the stress response of prolactin is temporal, individual prolactin concentrations in cord blood may fluctuate.

Cortisol concentration in amniotic fluid of the vacuum-assisted delivery group was significantly higher than those of the spontaneous delivery group. There was a positive correlation between the concentration of cortisol in amniotic fluid and the duration of parturition as observed in the umbilical cord blood. The origin of cortisol in the amniotic fluid is considered to be the urine of the fetus, and therefore, the increase of cortisol concentration may reflect the stress response of fetus during delivery. Prolactin concentration in amniotic fluid of the vacuum-assisted delivery group was significantly higher than those of the spontaneous delivery group but no correlation was observed between the prolactin concentration and the duration of delivery. Prolactin in the amniotic fluid is derived from endometrial decidua where stress response of prolactin is unclear.

There was a positive correlation between prolactin concentrations in plasma and hindmilk supporting that prolactin in breast milk is mother-blood origin. Recent reports suggest epigenetic effects of breast milk prolactin on the nutritional status and brain function of adult offspring. Therefore, it is important to clarify the maternal factors affecting on prolactin concentrations in breast milk.

Bull. Nippon Vet. Life Sci. Univ., No.64, 112-114, 2015.

Study on the flavor in meat soup stock

Yukiko WASHIO (TAKAKURA)*

Laboratory of Food Function
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 26 September 2014, ALS B-3)

Meat soup stocks of chicken, pork, and beef, which are called "soup stock", "bouillon", "fond" in Western countries, and "shàngtāng", "báitāng", "máotāng" in China, are highly appreciated as a base for savory dishes all over the world due to its desirable meaty flavor profile.

Chicken soup stock is commonly prepared by boiling whole chickens for four hours at low heat though flavoring ingredient is different in individual countries.

On the other hand, there are two types of pork soup stock: the white type (prepared without removing the marrow from the thighbone, followed by boiling at medium to high heat to gradually produce white turbidity), and the clear type (prepared by removing the marrow from the thighbone and boiling at low heat to avoid turbidity). The white type of pork stock is widely used in China and Japan, whereas the clear type of pork stock is widely used in Southeast Asia.

Beef soup stock, which grew on a European rootstock, is appreciated as a base for soup, stew, and sauce. And it became common in the terms of beef extract and is highly appreciated as a base for many commercial food products all over the world. Beef extract is commercially manufactured from beef broth that is produced by boiling meat at approximately 100 ℃, followed by removal of fat from the broth, sterilization at approximately 120 ℃-140 ℃, and concentration processing, which enriches the savory meaty flavor.

Taste compounds of meat soup stock have already been investigated according to the type of meat, and it is known that amino acids, peptides, nucleic acids, organic acids, saccharide, and minerals are the major taste compounds of meat soup stock. However, there has only been limited research on the aroma-active compounds of meat soup stock.

Whereas the aroma compounds from white pork soup stock have been investigated and ranked according to their aroma intensity and possible contribution to the flavor of the stock, there is a lack of research on the aroma-active compounds of clear pork stock, chicken soup stock, and beef extract though they are commonly used as a base for savory dishes all over the world.

Therefore, the aim of this present study was to identify the most aroma-active compounds in chicken soup stock, clear pork stock, and beef extract, and to examine sensory interactions between aroma compounds and taste compounds in meat soup stock.

1. Characterization of the key aroma compounds in chicken soup stock

Aroma extract dilution analysis (AEDA) was performed on an extract prepared from chicken soup stock and 9 aroma-active compounds were selected. On the basis of high flavor dilution (FD) factors in combination with the results of the identification experiments, methylpyrazine, 2-ethyl-4-methylthiazole, 3-(methylthio)propanal, and (*E,E*)-2,4-decadienal were suggested as primary aroma compounds of chicken soup stock.

Recombination and omission experiments of the identified aroma-active compounds in taste-reconstituted chicken soup stock showed that each compound had an individual aroma profile. A comparison of the overall flavor of the recombined mixture and the chicken soup stock revealed a high similarity, suggesting that these four compounds are important contributors to the aroma of chicken soup stock.

Omission experiments which were performed to study sensory profiling of the individual identified compounds showed that methylpyrazine and 2-ethyl-4-methylthiazole contribute to "roast", whereas 2-ethyl-4-methylthiazole contributes to "roast meaty" in addition to "roast" flavors. 3-(Methylthio)propanal and (*E,E*)-2,4-decadienal have similar flavor profiles of "boiled

*Supervisor : Prof. Toshihide NISHIMURA

meaty" but (*E,E*)-2,4-decadienal exhibits "fatty" and "animalic" flavor in addition to "boiled meaty" flavor. Additionally, they are important contributors to each characteristic flavor of chicken soup stock.

2. Characterization of the key aroma compounds in pork soup stock

The aroma extract dilution analysis (AEDA) of an extract prepared from pork stock and subsequent experiments led to the identification of 15 aroma-active compounds in the flavor dilution (FD) factor range of 64-2048. Omission experiments to select the most aroma-active compounds from the 15 odor compounds suggested acetol, octanoic acid, δ-decalactone, and decanoic acid as the main active compounds contributing to the aroma of pork stock. Aroma recombination, addition, and omission experiments of these four aroma compounds in taste-reconstituted pork stock showed that each compound had an individual aroma profile. A comparison of the overall aroma between this recombined mixture and pork stock showed strong similarity, suggesting that the key aroma compounds had been successfully identified.

Addition and omission experiments which were performed to study sensory profiling of the individual identified compounds showed that acetol, octanoic acid, and decanoic acid contributed to the mouthfulness flavor.

Acetol also had a flavor profile of continuity, whereas octanoic acid had roundness flavor. Decanoic acid had a full body flavor profile, in addition to a mouthfulness flavor, whereas δ-decalactone had a specialized flavor profile of roundness flavor. These compounds were therefore important contributors to each characteristic flavor of pork soup stock.

3. Characterization of the key aroma compounds in beef extract

Aroma extract dilution analysis (AEDA) of an ether extract prepared from beef extract (BE) and subsequent identification experiments led to the determination of seven aroma-active compounds in the flavor dilution (FD) factor range of 32-128. Omission experiments to select the most aroma-active compounds from the seven aroma compounds suggested that 2,3,5-trimethyl pyrazine, 1-octen-3-ol, 3-methylbutanoic acid, and 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone were the main active compounds contributing to the aroma of BE. Aroma recombination, addition, and omission experiments of the four aroma compounds in taste-reconstituted BE showed that each compound had an individual aroma profile. A comparison of the overall aroma between this recombination mixture and BE showed a high similarity, suggesting that the key aroma compounds had been identified successfully.

Addition and omission experiments which were performed to study sensory profiling of the individual identified compounds showed that 1-octen-3-ol and 3-methylbutanoic acid contribute to the "boiled meaty flavor." 3-Methylbutanoic acid also contributes to the "sweet meaty flavor," in addition to a "boiled meaty flavor." 2, 3, 5-Trimethyl pyrazine has a specialized "roasted flavor," whereas 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone has a specialized "sweet meaty flavor." Therefore, these compounds are important contributors to each characteristic flavor of BE.

4. Sensory interaction between aroma compounds and taste compounds in meat soup stock

Sensory comparison between a complete mixture of aroma and taste compounds of chicken soup stock (CM28), which was prepared by adding 28 taste compounds to chicken soup stock aroma solution (SA), and chicken soup stock was performed to study interactions of taste compounds with aroma compounds in chicken soup stock. Whereas SA and CM28 have same concentrations of chicken soup stock aroma, sensory retronasal aroma intensities of CM28 (4.3) were found to be strongly increased compared to SA (1.0), and almost reached an intensity of 5.0 which was that for chicken soup stock. This indicated that chicken soup stock aroma was enhanced by taste compounds of chicken soup stock. Moreover, following omission and addition experiment of 28 taste compounds showed that chicken soup stock aroma could significantly be enhanced by the addition of Glu (Glutamic acid) and IMP (Disodium 5'-inosinate).

This is the first study to confirm the key aroma compounds of meat soup stock using aroma recombination and omission experiments and to investigate the roles of each aroma compound in meat soup stock. And we showed that a limited number of volatile compounds actually contribute to the overall aroma of meat soup stock whereas there are hundreds of aroma compounds in meat soup stock. We also found that chicken soup stock aroma could significantly be enhanced by the addition of Glu and IMP. It is already known that Glu has flavor (multiple oral sensation including taste) enhancing effect. However, to our knowledge, no study published in the available literature

focused on aroma enhancement by Glu and IMP. In this study, we could achieve aroma intensity sensory evaluation by trained panelists, and found that soup stock aroma can be enhanced by taste compounds at the first time.

These findings of key aroma compounds and aroma enhancing taste compounds will greatly contribute to the scientific research for “deliciousness” of meat soup stock.

In recent years, there are many scientific studies for the cooking technique of professional chefs. And this study could be the part of the answer to the studies.

Bull. Nippon Vet. Life Sci. Univ., No.64, 115-116, 2015.

Effect on incretin secretion using different dietary carbohydrate and fat sources and amount in healthy dogs

Ran AKIYAMA*

Department of Veterinary Nursing
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, VNT-35)

Gastric inhibitory polypeptide (GIP) and Glucagon-like peptide-1 (GLP-1) are the two major incretin hormones secreted from the intestine. Incretin hormones stimulate insulin secretion from pancreatic beta cells by glucose dependent manner. GIP is secreted from the upper intestine, which enhances postprandial glucagon response and fat deposition in human study. GLP-1 is secreted from the middle section of small intestine, which inhibits glucagon secretion and delays gastric emptying in human study. In human obese and diabetic patients, diets such as inhibiting GIP secretion and promoting GLP-1 secretion are considered to preventing obesity and reducing appetite. In healthy dogs, GIP was secreted from upper small intestine, whereas GLP-1 was secreted from lower small intestine. In addition, incretin is secreted by nutrient ingestion, especially in carbohydrate and fat. However, relationship between incretin hormones and source and amount of dietary carbohydrate and fat has not been evaluated in healthy dogs. Therefore, the aim of this study was to evaluate incretin secretion under different dietary nutrient content in healthy dogs.

1-1. Effect on incretin secretion under different dietary carbohydrate sources in healthy dogs

We used three commercial diets which included three different major carbohydrate sources (rice, corn and wheat). As a result, no significant difference was observed in postprandial blood glucose, insulin and GLP-1 concentrations, between three diets. From the results of insulin secretion, insulin AUC was separated in to two groups ($AUC_{0\text{-}6hrs}$ and $AUC_{6\text{-}12hrs}$). Furthermore, the insulin ratio of $AUC_{0\text{-}6hrs}/AUC_{6\text{-}12hrs}$ was calculated. Result of insulin ratio was higher in rice than corn and wheat diet. GIP AUC was also higher in rice than corn and wheat diet. Insulin ratio shows states of postprandial digestibility, higher value indicates high digestibility. In addition, higher GIP concentration was also considered as high digestibility. Thus, we found that rice diet induced higher postprandial digestibility and promoted GIP secretion as compared to corn and wheat diet.

1-2. Effect on incretin secretion under different amount and sources of dietary fiber in healthy dogs

We used a commercial diet (control diet; fiber: 0.9g/100kcal) and beet pulp (soluble fiber and insoluble fiber) and cellulose (insoluble fiber) as supplemented dietary fiber. The total additive dietary fiber amount were 10% diet (fiber: 3.5g/100kcal) and 20% diet (fiber: 6.1g/100kcal), respectively. When comparing amount of dietary fiber, 20% supplemented diet induced decreased blood insulin concentrations, non-esterified free fatty acid concentration (NEFA) and GIP concentration as compared to control diet. Furthermore, 20% supplemented diet induced increased blood GLP-1 concentration as compared to control diet. Therefore, 20% supplemented diet might help delaying digestion and absorption, thereby preventing obesity and suppressing appetite. However, no significant difference was observed in postprandial blood parameter, between two fiber sources (beet pulp and cellulose). Since beet pulp was mainly composed of insoluble fiber (about 80%), the characteristics of the soluble fiber might be lacked.

*Supervisor : Prof. Toshinori SAKO

2. Effect on incretin secretion under different amount and sources of dietary fat in healthy dogs

We used a commercial diet (control diet; fat: 3.6g/100kcal) and lard (saturated fatty acid) and fish oil (unsaturated fatty acid) as supplemented fat. Each fat was supplemented into control diet as 0.8g/100kcal by reference to previous research. As a result, no significant difference was observed in postprandial blood glucose, insulin and NEFA concentrations, between three diets. GIP AUC was significantly higher in lard diet and fish oil diet as compared to control diet. Therefore, fat supplemented diet promoted GIP secretion. Furthermore, EPA and DHA in fish oil might promote GLP-1 secretion, since GLP-1 AUC of fish oil supplemented diet was significantly higher than control diet.

In conclusion, when comparing sources of dietary carbohydrate, GIP secretion was higher in rice diet than corn and wheat diet. However, no significant difference was observed in postprandial blood GLP-1 concentrations, between three diets. When comparing amount of dietary fiber, high-fiber diet induced decreased blood GIP concentration and increased blood GLP-1 concentration as compared to low-fiber diet. However, when comparing sources of dietary fiber, no significant difference was observed in postprandial blood parameter, between two fiber sources (beet pulp and cellulose). When comparing amount of dietary fat, high-fat diet induced increased blood GIP concentration as compared to low-fat diet. When comparing sources of dietary fat, no significant difference was observed in postprandial blood parameter, between two fat sources (lard and fish oil). Therefore, incretin secretion was affected by different dietary nutrient content as in healthy dogs.

Bull. Nippon Vet. Life Sci. Univ., No.64, 117-118, 2015.

Pathological analysis of epithelial-mesenchymal transition in canine and feline mammary tumors

Tomomi OHE*

Department of Applied Science
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, VNT-36)

Epithelial-mesenchymal transition (EMT) is a phenomenon that neoplastic epithelial cells lose their epithelial characteristics and acquire mesenchymal phenotype during the invasion and metastasis of tumors. Development of EMT in tumors is considered to enhance their malignancy. Moreover, it has been reported that EMT occurs in tumor cells with stemness properties or EMT induces the acquisition of stemness in tumor cells. There have been many reports on EMT in human tumors, but only a few in the animal tumors. This study deals with the mechanism of EMT in the canine and feline mammary tumors.

In this study, EMT was investigated with the immunohistochemistry (IHC) for epithelial markers (CAM5.2 and E-cadherin) and mesenchymal markers (vimentin and N-cadherin) in 36 cases of canine mammary tumors (7 benign and 29 malignant) and malignant 12 cases of feline mammary tumors. Twenty cases (4 benign and 16 malignant) of 36 canine cases and all feline ones were also examined with IHC for EMT-inducing factors (β-catenin and ZEB1) and a stem cell marker (ALDH1).

I. EMT in canine and feline mammary tumors

Double immunofluorescence staining for CAM5.2 and vimentin revealed that the co-expression of these two markers existed in tumor cells of canine malignant tumors (8/29) and feline malignant ones (10/12). This finding suggests that those tumor cells have the ability of EMT. In 7 benign cases of canine mammary tumors, both vimentin and N-cadherin immunoreactivity were not observed, while in canine 29 malignant cases positive immunoreactivity for at least one of them was observed in 25 cases ($P<0.001$). These results revealed that malignant tumor cells showed mesenchymal cell characteristics but benign tumor cells did not show such characteristics. Moreover, in the cases of mammary tumors followed by a recurrence (canine 8 and feline 6 cases), those tumor cells showed positive immunoreactivity for at least either vimentin or N-cadherin in canine 7 and feline 5 cases. In addition, metastatic cells of mammary tumors in the lymph node showed decreased expression of E-cadherin and increased one of vimentin. Double immunofluorescence staining for CAM5.2 and vimentin revealed that the co-expression existed in those metastatic cells. These results suggest that EMT might play an important role in tumor recurrence and metastasis, and could be an indicator of malignancy in canine and feline mammary tumors. Furthermore, it has been suggested that EMT is induced by many signals from the surrounding stroma of the tumor, such as transforming growth factor-β (TGF-β) and integrins. In the present study, the decrease of epithelial markers and the increase of mesenchymal markers were observed in tumor cells on the stromal side of epithelial nests. In canine and feline mammary tumors, therefore, EMT of the tumor cells might occur in close vicinity to the stroma.

II. EMT-inducing factors in canine and feline mammary tumors

The present IHC for β-catenin revealed that both nuclear and cytoplasmic localization was observed in tumor cells of canine malignant tumor (1/16) and feline malignant one (1/12), but not observed in tumor cells of canine benign cases. In those two positive cases, moreover, the reduced expression of epithelial markers was observed, whereas the significant expression of mesenchymal markers was detected on tumor cells observed nuclear and cytoplasmic localization of

*Supervisor : Prof. Sinji KAMIYA

β-catenin. It was considered that β-catenin might be related to EMT in the canine and feline mammary tumors.

The IHC for ZEB1 revealed that the nuclear expression was observed in tumor cells of canine benign (1/4) and malignant (8/16) tumors, and feline malignant ones (9/12). In those ZEB1-positive tumor cells, moreover, E-cadherin expression was reduced. These results indicated that ZEB1 expression might induce depression of E-cadherin. Therefore, ZEB1 may lead to the induction of EMT in the canine and feline mammary tumors.

III. Stem cell marker (ALDH1) in canine and feline mammary tumors

The IHC for ALDH1, a well-known stem cell marker, revealed that the positive immunoreactivity was observed in tumor cells of canine malignant tumors (2/16) and feline malignant ones (9/12), but not observed in tumor cells of canine benign cases. This result indicated that the tumor stem cells existed in the canine and feline malignant mammary tumors and the acquisition of stemness in tumor cells was related to malignancy of feline mammary tumors. In the cases of mammary tumors followed by a recurrence, ALDH1-positive cells were observed in feline mammary tumors (5/6), while those cells were not in canine tumors. From these results, it is considered that the stem cells might play an important role in the recurrence of feline mammary tumors.

In addition, the most of the ALDH1-positive cells (stem cells) were vimentin-positive in feline cases. This result suggests that the tumor stem cells may have the close relationship to EMT expression in feline mammary tumors.

IV. Conclusion

In summary, the present results indicate that EMT acquisition and stem cell formation might play an important role in the malignancy, recurrence and metastasis in canine and feline mammary tumors. Further research is needed to clarify the accurate relationship between EMT and stem cells in tumors.

Bull. Nippon Vet. Life Sci. Univ., No.64, 119-120, 2015.

Estimation of population density and dispersal pattern in Asiatic black bears (*Ursus thibetanus* japonicas) inhabiting around Mt. Asama, Nagano

Masahiro KOMIYA*

Department of Applied Science
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, VNT-38)

Five local populations of the Asiatic black bear (*Ursus thibetanus japonicus*) are designated as threatened in the Red List compiled by the Ministry of the Environment, Japan. While a large number of black bears have recently appeared, most of those that are captured are killed. The number of Asiatic black bears that are killed exceeds 3,000 annually. Since the present population is estimated to be 10,000 to 12,000, there is a concern over the decline in local black bear populations and the possibility of extinction. However, the actual population size remains unverified. Moreover, the Asiatic black bear is wildlife managed under the jurisdiction of prefectural governments. This has caused a delay in understanding the ecology of many Japanese black bears living in prefectural border areas. Accordingly, in Chapter 2, we aimed to estimate the population size and population density of Japanese black bears using a hair-trap method in Karuizawa, Nagano Prefecture, located at the prefectural border, and its surrounding areas.

This study was carried out in the surrounding areas of Mount Asama located at the eastern end of Nagano Prefecture. In approximately 60 km^2 survey area, a grid of 1-km squares was created and at least one trap was set up in each region. A total of 66 traps were set up. Sample collection was conducted for a total of 46 weeks between May 2012 and October 2013. A total of 68 samples were selected from the overall collection and genomic DNA was extracted. Then, individuals were identified using microsatellite analysis based on eight loci. Based on the mark-recapture method proposed by Chapman (1951) and Seber (1992), the population size and population density were estimated.

Of 2,155 hair samples collected over two years, 1,115 samples were identified as belonging to bears. The number of samples collected was highest in the summer for both years and declined during the fall season. Additionally, the mean number of samples collected from the eastern and western parts of the survey area differed, with the former being higher than the latter. The estimated population sizes were 57.75 ± 18.54 and 28.50 ± 2.18 for 2012 and 2013, respectively. The results indicated that the population density in the surrounding areas of Mount Asama was 0.45-0.91 per km^2. Moreover, there was a higher population density (0.91 per km^2) in the eastern area than in the western area (0.67 per km^2). A comparative analysis suggests that the population density of the black bears is higher in the survey area than in other regions. This higher population density could be attributed to the fact that the majority of the survey site is designated as a national wildlife sanctuary. The pressure due to hunting and nuisance killing is low inside the sanctuary, resulting in a relatively high population density of Asiatic black bears.

In Chapter 3, our objective was to elucidate the influence of the differences in population density discussed in the previous chapter on natal dispersal in black bears. Dispersal has a direct impact on the formation and genetic structure of populations; therefore, estimates of dispersal are vital when considering the protection and management of target animals. Studies on the dispersal of bear species have demonstrated sex-biased dispersal in areas with varying population densities and reported that the dispersal of male bears to distant areas plays a role in inbreeding avoidance. However, it has not been directly demonstrated that

*Supervisor : Prof. Hiroshi KAJIGAYA

long-distance dispersal to avoid inbreeding has promoted dispersal in unrelated populations. Therefore, it is important to verify that dispersal occurs for various populations by elucidating the genetic population structure. Accordingly, in Chapter 3, we aimed to demonstrate the genetic structure of Japanese black bears on a local scale and elucidate the dispersal patterns.

Analyses were carried out using a total of 193 hair and blood samples obtained from Japanese black bears captured in the Mount Asama region of Nagano Prefecture between 1999 and 2013. A microsatellite analysis of 14 loci was conducted, and the data were used to estimate the genetic relationships between populations and the genetic population structure. The genetic differentiation coefficient (F_{ST}) was also calculated. The distance and direction of natal dispersal was determined only using data on populations for which mother-offspring relationships could be estimated. The distance and direction of dispersal were calculated by entering the capture point on the geographical information system (GIS).

The genotype was determined for at least 13 loci in all populations. Using these data, an analysis of genetic population structure showed that the female population was divided into three groups (KR, MY, and SP). Estimates of F_{ST} also indicated genetic differentiation among all groups. Moreover, an analysis of the genetic relationships between populations revealed 46 mother-female offspring pairs and 19 mother-male offspring pairs. The average dispersal distance of these populations was 2.69 ± 1.68 km for the mother-female offspring pairs and 10.02 ± 4.57 km for the mother-male offspring pairs. This indicates that male offspring dispersed farther than female offspring, on average. Examining the direction of male offspring dispersal revealed that 12 out of 19 individuals dispersed from the KR group to the MY group, while 3 out of 19 dispersed from the KR group to the SP group. This suggests that males disperse to unrelated populations. Moreover, the population density of the KR group in the eastern region was higher than that of the MY group in the western region based on the results described in Chapter 2. Hence, these results suggest that the dispersal pattern of males is density-dependent.

Bull. Nippon Vet. Life Sci. Univ., No.64, 121, 2015.

Evaluation of postprandial exercise time in diabetic dogs

Saori SHONO*

Department of Veterinary Nursing
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, VNT-39)

Insulin therapy, dietary therapy and exercise therapy are important therapies for patients with diabetes mellitus. In human study, primary effects of exercise therapy are improving blood glucose control, fat combustion and insulin sensitivity. However, exercise therapy also has the risk of hypoglycemia and ketogenesis in diabetic patients. As such, it is necessary to adjust the insulin dose, insulin injection time, site of injection and serving time and composition of meal for diabetic patients. Since diabetic dogs were usually treated with insulin preparations, hypoglycemia was severe side effect under exercise therapy, as well as human patient with diabetes mellitus. However, whether postprandial exercise affected on glucose metabolism was unclear in insulin treated diabetic dogs. Therefore, the objective of this study was to evaluate postprandial exercise affected on glucose metabolism in insulin treated diabetic dogs.

Chapter 1. Effects of exercise therapy with different postprandial time of onset on glucose metabolism in diabetic dogs

In Chapter 1, we examined the risk of hypoglycemia under postprandial exercise therapy at 6 hours (Ex 6hr) and 10 hours (Ex 10hr) in 3 diabetic dogs. As a result, blood glucose concentrations at Ex 6hr was decreased as compared to no exercise state in all 3 diabetic dogs. Since higher insulin levels was observed in all three dogs at Ex 6hr as compared to preprandial value, high insulin concentrations might induced lowering blood glucose concentrations after exercise therapy. However, exercise therapy at Ex 10hr was not observed any changes of blood glucose concentration during exercise therapy in all three diabetic dogs, suggesting postprandial exercise at Ex 10hr did not affect for blood glucose concentrations.

Chapter 2. Effects of dietary fiber amount on postprandial blood glucose concentrations after exercise therapy in diabetic dogs

Section 1, influence of dietary fiber (5.85g/100kcal) on postprandial glucose metabolism after exercise therapy (Ex 6hr or 10hr) was investigated. As a result, lowering of blood glucose concentrations after exercise therapy (at Ex 6hr) with fiber diet was stable as compared to non-fiber diet, suggesting fiber diet delayed glucose absorption from small intestine. However, there was no significant effect on blood glucose concentration during exercise, between non-fiber and high-fiber diet. Furthermore, exercise therapy at Ex 10hr was not observed any changes of blood glucose concentration during exercise therapy both non-fiber and high fiber diet, suggesting fiber diet did not affect for postprandial blood glucose concentrations at Ex 10hr.

Section 2, influence of high dietary fiber amount (10.45g/100kcal) on postprandial glucose metabolism after exercise therapy (Ex 6hr) was investigated. As a result, lowering of blood glucose concentrations with high fiber diet (10.45g/100kcal) at Ex 6hr was higher than that with 5.85g/100kcal fiber diet, suggesting high fiber diet stayed long time period in the stomach by the effect of fiber. However, there was no significant effect on postprandial blood glucose concentration after exercise therapy, between fiber (5.85g/100kcal) diet and high fiber (10.45g/kcal) diet.

In conclusion, adjusting time of onset of exercise therapy can avoid the risk of hypoglycemia. From the results of this study, postprandial exercise should be done at 10 hours postprandially. Furthermore, the amount of dietary fiber (5.85g/100kcal) might maintain patient's glucose concentrations at postprandial 6 hr exercise therapy. These evidences provide useful information for exercise therapy in diabetic dogs.

*Supervisor : Prof. Toshinori SAKO

Bull. Nippon Vet. Life Sci. Univ., No.64, 122-123, 2015.

Studies on splicing species and transcriptional mechanism of Eyes absent 3 mRNA in pars tuberalis of Japanese quail

Ayano OKAZAKI*

Laboratory of Animal Physiology
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, ALS-64)

The change of day length occurs alterations in the physiology of animals living in temperate zone (e.g., seasonal breeding, hibernation, migration). This phenomenon is called photoperiodism. A key factor of the molecular mechanism of photoperiodism on seasonal breeding animals is thyroid stimulating hormone (TSH). The TSH subunit β (TSHβ), released from pars tuberalis (PT) is induced by long-day stimulation. It is essential for photoperiodic-specific light signaling that activates the hypothalamic-pituitary-gonadal axis. In addition, long-day exposure induces tissue-specific and time-dependent expression of Eyes absent 3 (*Eya3*) mRNA as well as *TSHβ* mRNA in the PT. It has been known that the long-day induced expression of *TSHβ* mRNA is controlled by Eya3 in the sheep and mouse. In birds, long-day exposure induces expressions of both *Eya3* and *TSHβ* mRNA in the PT, whereas, it is unclear whether Eya3 is involved in the long-day induced *TSHβ* expression.

In Japanese quail (*Coturnix coturnix*), several *Eya3* (*qEya3*) mRNA variants are generated by alternative transcription and splicing mechanisms. In the quail PT, the *qEya3* gene transcribed from three different transcription start points at exon1A, exon 1B, and exon2. In addition, the transcription variants take different splicing of exons 7, 8, and 9. Among the splicing variants, exon7-containing variant abundantly expresses in the PT, suggesting that exon7-containing variant of *qEya3* mRNA (exon7-*qEya3* mRNA) was involved in PT-specific function. Therefore, the aim of this study is to characterize the alternative transcription and splicing variant of *qEya3* mRNA in the PT.

First, tissue distribution patterns of exon7-*qEya3* mRNA were investigated by RT-PCR-Southern blotting. The expression of exon7-*qEya3* mRNA was detected in all the tissues examined with relatively lower levels in the liver, gallbladder, and pancreas than in other tissues. It has been known that the Eya family protein contains two functional domains, Eya domain 1 (ED1) and Eya domain 2 (ED2). However, *qEya3* exon7 is not the one containing these functional domains. Therefore, physiological significance of the high level expression of exon7-*qEya3* mRNA in the PT remains to be elucidated.

To clarify the mechanisms of transcriptional regulation of *qEya3* mRNA under condition of photoperiodism, expression profiles of *qEya3* mRNA in the PT and cerebrum were investigated by RT-PCR Southern blotting. In the PT, exon7-*qEya3* mRNA transcribed from Exon2 was mainly expressed. In the cerebrum, exon7-*qEya3* mRNA was transcribed from exon1A as a major *qEya3* mRNA. On the other hand, the *qEya3* mRNA lacking exon7 was transcribed from exon1A. These findings indicate that the synthesis of *qEya3* mRNA is regulated by tissue-specific transcription as well as tissue-specific splicing.

Because exon7-*qEya3* mRNA transcribed from Exon2 was mainly produced in the PT, regulatory element(s) for photoperiod-specific transcription of *qEya3* gene may locate within the 5 kb intron between exon1B and exon2. Sequence analysis revealed presence of a TATA box in the 5'-upstream region of exon2. In addition, two E-box motifs were found in the upstream region of the TATA box. In sheep, the photoperiod-specific expression of *Eya3* gene in the PT is controlled by E-box as binding sites for CLOCK/BMAL1, D-box as binding sites for TEF and SIX/EYA3, and Melatonin. These findings suggest that a circadian based photoperiod response of *qEya3* mRNA expression in the quail PT may also be regulated by E-box. Transcriptional mechanism of *qEya3* mRNA is assumed to be independent on melatonin signal

*Supervisor : Prof. Minoru TANAKA

because the light input pathway to the photoperiodic system is different between mammals (melatonin) and birds (light). Further studies are needed to elucidate the regulatory elements located between exon1B and exon2 of *qEya3* gene under condition of photoperiodism.

Bull. Nippon Vet. Life Sci. Univ., No.64, 124-125, 2015.

Regulation of amylin expression by social environment in the mouse medial preoptic area

Misato KANEKO*

Laboratory of Animal Physiology
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, ALS-65)

Introduction

Amylin is identified as a peptide hormone co-secreted with insulin from pancreas. Recently, High level of expression of amylin mRNA has been found in the medial preoptic area (MPOA) of mother rats. Since MPOA is known as the brain center of maternal behavior, amylin may participate in the regulation of maternal behavior. Expression of amylin mRNA has been observed and expression level of lactating mice is higher than that of virgin mice. Group-housing virgin female mice showed high amylin expression but the expression level decreases in single habitation. Therefore, expression of amylin is depend on social environment physiological function of amylin of the MPOA in social activity is not known. In this study, to clarify the environmental factors affected amylin production in MPOA, effects of social environments on expression of amylin mRNA in MPOA.

Animals and methods

C57BL/6J female mice (3 to 5-month-old) were used for the study. Expression levels of amylin mRNA of the mouse MPOA in brain sections were determined by in situ hybridization analysis. The number of positive-staining neurons was determined by Neurolucida (Micro Bright Field).

Results

1. Effect of cohabitation: To investigate the effects of maternal behavior (contact with pups) on amylin mRNA expression in the MPOA, nulliparous mice were cohabitated with pups and their mother or other adult nulliparous mice for 6 days after the 6 days single habitation. The cohabitation showed no effect on the number of amylin mRNA positive cells in the MPOA of nulliparous mice.
2. Effect of immobilized stress: Mice were cohabitated with nulliparous mice for 6 days. Thereafter mice were exposed to immobilized stress for 6 days (2 hours/day). The immobilized stress showed no effect on the number of amylin mRNA positive cells in the MPOA.
3. Effect of odor: To investigate the effect of odor from other mice, each nulliparous mouse was housed alone in a cage for 6 days and thereafter, a part of bedding tips used for other nulliparous female mice were added to the cage for every days. The addition of bedding tips showed no effect on the number of amylin mRNA positive cells in the MPOA. The number of amylin mRNA positive cells in the female mice cohabitated with castrated male mice for 6 days was comparable to that of female mice cohabitated with female mice.
4. Effect of contact stimuli: One of the group-housing mice was moved in an isolated area partitioned with wire mesh where the mouse can contact to other mice. Under the contactable condition, the number of amylin mRNA positive cells in the isolated mice was comparative to that of cohabitated with female mice.

Discussion

In this study, effects of various stimuli from social environment on the expression of amylin mRNA in the MPOA in female mice were investigated. The expression of amylin mRNA was not affected either by immobilized stress or odor from other female mice. Contact stimuli from the cohabited male or female mice are essential for the amylin mRNA expression

*Supervisor : Prof. Minoru TANAKA

in the MPOA in female mice. In the social animals, communication with group members shows social buffering effect to reduce stress-derived reaction. It has been reported that social isolation (single-housing) of female rodents induces depression and delay of wound healing. Together with the findings in this study, it is suggested that amylin produced in the MPOA in female mice by the contact stimuli from the cohabited mice may contributes to maintenance of mental and physical health.

Bull. Nippon Vet. Life Sci. Univ., No.64, 126-127, 2015.

Influence of *Bifidobacterium*-containing fermented soybean milk intake on microbiota of healthy adult

Masahide TSURUTA*

Laboratory of Food Hygiene
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, ALS-70)

Functional foods have recently been attracting attention because the incidence of lifestyle-related diseases is increasing. Soymilk, which originates from soybeans, includes various ingredients such as soybean oligosaccharides, isoflavones, proteins, and saponins; therefore, the nutritive value and functionality of soymilk are very high, and, as such, it has been approved as a special health food in Japan. Previous studies examined the influence of soymilk fermented using lactic acid bacteria such as lactobacilli on the intestinal environment; however, the effects of *Bifidobacterium*-containing fermented soybean milk have not yet been investigated. The efficacy of probiotics is known to vary greatly with the bacterial strains used. Therefore, the influence of *Bifidobacterium*-containing fermented soymilk on the intestinal environment was investigated in the present study. Its prebiotic efficacy by soybean oligosaccharides and probiotic efficacy by viable starter bacteria were expected. An ingredient analysis was performed on the saccharides in *Bifidobacterium*-containing fermented soymilk, and the influence of the intake of *Bifidobacterium*-containing fermented soymilk on fecal microbiota, pH, and metabolic activities was examined in humans. Moreover, the isoflavone metabolic activity of starter bacteria was measured.

Fermented soymilk was made as follows: 1 g of yogurt starter containing *Bifidobacterium* was added to 900ml of soymilk, and these were then incubated at 38℃ for 12h.

The reduction rate of saccharides used in the manufacturing process of fermented soymilk was the highest for glucose, followed by sucrose. Furthermore, the reduction rates of raffinose and stachyose, which are the constituent sugars of soybean oligosaccharides, were significantly lower ($P<0.05$) than those of glucose and sucrose. Among the bacterial strains tested containing starter bacteria, raffinose and stachyose were only utilized by *Bifidobacterim longum* subsp. *longum* ATCC15707^T. This result demonstrated that starter bacteria mainly used glucose and sucrose for growth. These results also revealed that the soybean oligosaccharides in soymilk were not reduced by the fermentation of starter bacteria, suggesting that the probiotic efficacy of the fermented soymilk made in the present study was attributable to starter bacteria containing *Bifidobacterium*, and also to soybean oligosaccharides. Therefore, the fermented soymilk made in this study may be used as a functional synbiotic. We also detected a bacterial strain that had the ability to metabolize isoflavone to isoflavone aglycone.

The influences of the fermented soymilk made in this study and soymilk intake on fecal microbiota, pH, and metabolic activities were investigated in eight healthy adults. The number of fecal *Bifidobacterium* was significantly higher ($P<0.05$) on day 7 and day 14 of the soymilk intake period and on day 7 of the fermented soymilk intake period than before the soymilk intake period. The number of intestinal *Enterobacteriaceae* detected in fecal samples was significantly lower ($P<0.05$) on day 7 and day 14 of the soymilk intake period than before the soymilk intake period. Although no marked changes were observed in the number of *Enterobacteriaceae* during the fermented soymilk intake period, it decreased in six out of the eight subjects examined and its occupancy rate was also slightly lower than that before the soymilk intake period. An investigation of *Bifidobacterium* species revealed that the 16S rDNA copy numbers of *B. longum* subsp. *longum*, *Bifidobacterium adorescentis*, and *Bifidobacterim breve* were significantly higher

*Supervisor : Prof. Tomohiko FUJISAWA

($P<0.05$) during the fermented soymilk intake period, whereas a significant difference was not noted in the 16S rDNA copy number of *Bifidobacterium* species during the soymilk intake period. Fecal pH and the concentrations of ammonia were significantly lower ($P<0.05$) during the fermented soymilk intake period. However, no significant differences were found in these values during the soymilk intake period. These results indicated that decreases in fecal pH and the concentration of ammonia were induced by an increase in the number of intestinal *Bifidobacterium* and decrease in the number of intestinal *Enterobacteriaceae* due to the intake of fermented soymilk. Previous studies reported correlations between ammonia and *p*-cresol concentrations in the feces. Ammonia and *p*-cresol are putrefactive products produced by intestinal bacteria. Therefore, the intake of fermented soymilk may induce not only a decrease in the concentration of ammonia, but also a decrease in the concentration of *p*-cresol. On the other hand, the numbers of *B. longum* subsp. *longum* contained in fermented soymilk, and also *B. adorescentis* and *B. breve* were significantly higher ($P<0.05$) during the fermented soymilk intake period. These results confirmed that the prebiotic efficacy of fermented soymilk was due to soybean oligosaccharides.

The results of the present study suggested that soymilk and fermented soymilk improved the intestinal environment, and fermented soybean milk containing *Bifidobacterium* exhibited probiotic and prebiotic efficacies. Therefore, the soymilk and, in particular, fermented soymilk made in this study are promising functional foods.

Bull. Nippon Vet. Life Sci. Univ., No.64, 128, 2015.

Regulation of prolactin receptor gene expression by prolactin in rat and mouse pituitaries

Masahiro NISHITA*

Laboratory of Animal Physiology
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Conferred on 11 March 2015, ALS-71)

Prolactin (PRL) is a pituitary hormone involved in many physiological functions such as mammary gland development, induction of maternal behavior, osmoregulation, and immune function. These hormonal effects are exerted by binding to its receptor (PRLR). Reflecting these multiple functions, PRLR exists a wide range of tissues. In rodent such as rat and mouse, two types of PRLR isoforms differed from each other in the length and sequence of their cytoplasmic domains are present. These isoforms are generated by alternative splicing of a single primary transcript. Expression of PRL-R gene in rat and mouse is regulated by tissue-specific transcriptional activation of five alternative first exons, E1-1, E1-2, E1-3, E1-4, and E1-5, encoding 5'-untranslated regions.

It has been known that PRL-deficient (PRL-/-) mice show hyperplasia in the pituitary lactotroph at adult stage. It is suggested that the hyperplasia is caused due to the absence of a negative feed back regulation for cell proliferation of lactotroph in PRL-/- mice. However, its mechanism is not fully understood. To clarify the regulatory mechanism of pituitary function by PRL, expression levels of PRLR mRNA in the pituitary during estrous cycle and lactation in rat were examined. In addition, expression levels of PRLR isoform mRNA in PRL-/- mice and PRL+/- mice showing normal PRL secretion were investigated.

First, developmental changes in the expression levels of PRLR mRNA in the pituitary of rat (Wistar Imamich) were examined by real-time PCR of PRLR cDNA synthesized by reverse transcription of pituitary total mRNA. No significant change in the expression level of E1-3 PRLR mRNA was observed during developmental stages at 2, 4, and 8 weeks of either male or female rats. The expression level of the E1-3 PRLR mRNA at diestrus was higher than that at proestrus and 3 days of lactation. These expression patterns were similar to those of total PRLR mRNA. Since plasma PRL concentration was elevated at the proestrus and the lactation, PRL may suppress transcription of E1-3 first exon by a negative feed back system.

The regulatory mechanism of PRLR gene expression in the pituitary by PRL was further investigated using PRL-/- and PRL+/- mice. Expression levels of both E1-3 and total PRLR mRNAs in the pituitary of adult PRL-/- mice were higher than those of PRL+/- mice. Four types of PRLR isoforms, one long form and three short forms, differed sequence and length of cytoplasmic domains are present in mice. The long form mRNA was expressed as a main isofom in the pituitary with higher level in PRL-/- than in PRL+/- mice. Therefore, long form PRLR may be involved in the suppression of PRLR gene expression by PRL in the pituitary.

To confirm the suppressive effect of PRL on the PRLR gene expression, sheep PRL was administrated to adult PRL-/- mice (16-week-old). No effect was observed in the expression levels of total PRLR mRNA of either male or female PRL-/- mice. The pituitary of the adult mice had severe hyperplasia in the pituitary. Therefore, sensitivity of the lactotroph to PRL may be reduced. Further study remains to clarify the suppressive effect of PRL on PRLR gene expression.

*Supervisor : Prof. Minoru TANAKA