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The application of a bioabsorbable scaffold composed of hydroxyapatite and polylactide

Hiroyuki AKAGI*

Laboratory of Veterinary Surgery
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

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Fractures are common in small animal orthopedics, with the rate of incidence being approximately 7%. The incidence rate of nonunion fractures in the dog was about 4%. In some nonunion fracture cases with a large bone defect, restoration of the limb length requires reconstruction. Several methods of bone reconstruction using an autograft, allograft, and a bioabsorbable scaffold have been reported. Bioabsorbable scaffolds have the advantages of being minimally invasive, not requiring any special storage facilities, and ease of intervention; they are therefore needed in veterinary orthopedic surgery.

Bioabsorbable scaffolds are divided into two major groups: bioabsorbable polymer and calcium phosphate. Several bioabsorbable polymers have been used as the bioabsorbable osteosynthetic material, particularly poly-L-lactide (PLLA), because PLLA maintains mechanical strength during bone healing and is absorbed without any inflammatory reaction. Furthermore, PLLA also has a piezoelectric effect and promotes local bone formation. Although the use of bioabsorbable osteosynthetic implants was not popular in veterinary orthopedics, the bioabsorbable scaffold might have a potency to be a usefull implant as an artificial bone for the dog with nonunion. However, it has been pointed out that PLLA caused pain and acute aseptic necrosis five years after implantation in the past report. Then, a bioabsorbable osteosynthetic material composed of a combination of a hydroxyapatite (HA) and PLLA was developed. The biocompatibility and osteoinductive potential of HA are equal to those of beta-tricalcium phosphate (β-TCP), and it maintains mechanical strength longer than β-TCP. It has been reported that a HA/PLLA osteosynthetic implant showed good biocompatibility and direct integration with the host bone. However, PLLA needs a long period for its degradation and absorption more than poly-D-lactide (PDLA), which is an structural isomer of PLLA. Then, HA/PDLLA, which is composed of HA and PDLLA, has recently developed as a new bioabsorbable material. I considered that HA/PDLLA might be suitable for a bioabsorbable scaffold.

In this study, I studied on the application of this bioabsorbable scaffold as an artificial bone. In chapter 2, we discussed the application and issues associated with a frozen cortical allografts (FCAs) for a femoral nonunion patient. In chapter 3, the substitution process and inflammatory reaction of the HA/PLLA composite was compared to the PLLA composite in the cortical bone. In chapter 4, the HA/PDLLA scaffold was studied, and the substitution process and mechanical strength were compared to those of the β-TCP scaffold at the unloading site. The HA/PDLLA composite has a characteristics to be transformed with heat and trimmed with a scalpel. We conducted an experiment to analyze the transforming effect of heat on the substitution process. In chapter 5, the substitution process of HA/PDLLA scaffold was studied histologically and compared to the β-TCP scaffold at the loading site using the canine tibial ostectomy model.

1. The application and issues associated with frozen cortical allografts (FCAs) at a femoral nonunion fracture

I performed reconstructive surgery using a frozen cortical allografts (FCAs) and cancellous autograft for femoral nonunion patients. The remodeling process of the FCAs itself and the interface of proximal and distal portions of the FCAs and host bone were analyzed according to the radiographic score system reported by Weiland et al. The bony union between the proximal or

*Supervisor : Prof. Yasushi HARA

distal host bone and FCAs was observed after 3 months. The remodeling of the FCAs was recognized at six months. The continuousness between the ends of the host bone and FCAs were observed.

Moreover, it was observed that remodeling of the FCAs itself were progressed after 12 months. These results indicated that the FCAs showed good mechanical strength and biocompatibility in the grafted site. However, it has been reported that the implanted allograft needs a long period more than 7 years to be completely substituted by the host bone. These findings indicated that a long-term follow-up would be needed. Moreover, FCAs need special storage to maintain the temperature at −80° and normal dogs to retrieve their bone. Therefore, reconstructive surgery using FCAs was conducted in only a few facilities in Japan in the field of veterinary orthopedics. It was concluded that more convenient bioabsorbavle scaffolds would be needed to treat a bone defect of nonunion fractures.

2. Comparative study of PLLA composite and HA/PLLA composite on the substitution process in canine femur

It has been reported that the HA/PLLA osteosynthetic implants achieved direct union with the host bone and demonstrated a superior biocompatibility to the PLLA osteosynthetic implants. We therefore studied the use of HA and PLLA osteosynthetic implants as a bone scaffold. The basis of this chapter was a study of whether the HA/PLLA osteosynthetic material achieved substitution to the host bone. The aim of this study was to analyze the substitution process of the HA/PLLA osteosynthetic implants to cortical bone histologically and compare it to the PLLA osteosynthetic implants. The HA/PLLA screws and PLLA screws were inserted in the femur and a histological analysis was performed at 12, 36, 60, and 84 months.

It was confirmed that the screw hole were closed radiographically at 60 months in the HA/PLLA group, and histological analysis demonstrated that screw holes were substituted by bony tissue. However, in the PLLA group, screw holes were not closed by the bony tissue at 84 months. Additionally, the PLLA screw showed severe histiocyte cell infiltration at 60 months, whereas the HA/PLLA screw did not show severe infiltration during the follow-up period. This study showed that the HA/PLLA composite showed superior biocompatibility compared to the PLLA screw, direct union with the host bone, and substitution to the host bone. These results indicated that a bioabsorbable composite composed of HA and PLLA might be available for use as a bioabsorbable artificial bone.

3. A comparative study on the substitution process of HA/PDLLA scaffold and β-TCP scaffold, and the heat-transforming difference of HA/PDLLA scaffold at the unloading site

In this chapter, I studied on the application of the HA/PDLLA scaffold compared to the β-TCP scaffold. Experiment 1 was conducted to analyze the area of new bone formation and the residual HA/PDLLA and β-TCP scaffolds. Experiment 2 was conducted to evaluate the transforming influence by heat. Experiment 3 was conducted to analyze the adherence strength of HA/PDLLA scaffold and host bone compared to that of the β-TCP scaffold. Then, the average molecular weight (Mw) of HA/PDLLA scaffold was measured.

The results of experiment 1 showed that bone formation and scaffold absorption of the HA/PDLLA scaffold was delayed compared to the β-TCP scaffold. Although the remodeling process of the HA/PDLLA scaffold was not stopped, numerous Runt-related transcription factor 2 (Runx2)-positive cells and type I collagen (Col-I)-positive tissue were observed in the HA/PDLLA scaffold compared to that in the β-TCP scaffold. The results of experiment 2 showed that the heat-transforming of the HA/PDLLA scaffold did not affect any substitution process. The results of experiment 3 showed that the HA/PDLLA scaffold and β-TCP scaffold had equal bonding strength, and the Mw of HA/PDLLA scaffold decreased significantly with time. These results indicated that the substitution process of the HA/PDLLA scaffold was slightly delayed compared to the β-TCP scaffold at the unloading site.

4. A comparative study on substitution process of the HA/PDLLA scaffold and β-TCP scaffold at the loading site

It has been reported that the degradation rate of PDLLA was promoted at the loading site. In this chapter, I evaluated the substitution process of the HA/PDLLA scaffold at the loading site. The tibial diaphysis was ostectomized (15mm) and the HA/PDLLA scaffold and β-TCP scaffold were inserted on each side, and three experimental groups were prepared (1, 3, and 12 months). After the follow-up period, we conducted a histological analysis. The results of this study showed that the HA/PDLLA scaffold and β-TCP scaffold represented equal bone formation. The HA/PDLLA scaffold showed earlier and better infiltration of the cell

and tissue than the β-TCP scaffold. In the HA/PDLLA scaffold, numerous vessel cavities and osteoclast like-cells as well as Runx2-positive cells were present that were responsible for primary bone formation and scaffold substitution, as well as differentiation to osteoblasts, respectively. These results showed that the HA/PDLLA scaffold had induced numerous cells that were essential for bone remodeling and promoted the substitution process. There were many differences in the substitution process between the HA/PDLLA scaffold and β-TCP scaffold. However, both scaffolds did not show complete substitution at 12 months; a long-term follow-up is therefore needed for observation of complete substitution.

This study showed that the HA/PDLLA scaffold, which was developed as a new artificial bone, showed no inflammatory reaction as well as the the β-TCP scaffold and were substituted to the host bone. The HA/PDLLA scaffold was slightly delayed in the substitution process compared to the β-TCP scaffold at the unloading site, although their bonding strength was equal. Furthermore, the HA/PDLLA scaffold showed equal bone formation compared to the β-TCP scaffold, and superior infiltration of tissue and cell compared to the β-TCP scaffold at the loading site. It was concluded that the HA/PDLLA scaffold was expected to be usefull as a new bioabsorbable artificial bone. However, neither scaffold achieved complete substitution to the host bone, therefore, it was considered that the follow-up study would be needed to confirm the complete substitution.

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Studies on growth inhibitory effects of dasatinib against canine histiocytic sarcoma cell lines

Keita ITO*

Laboratory of Veterinary Clinical Pathology
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

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Introduction

Canine histiocytic sarcoma is an aggressive, often lethal tumor that arises from the histiocytic lineage including macrophages and dendritic cells. There is a highly elevated incidence in several breed, including Bernese Mountain Dog, Flat Coated Retriever, Rottweiler and Golden Retriever, especially, Bernese Mountain Dog and Flat Coated Retriever may share genetic characteristics contributing to tumor initiation and progression.

For the treatment of HS, chemotherapy is often used alone or in combination with surgery and/or radiation therapy. Although overall response rate of HS cases with gross lesions treated by lomustine (CCNU) was around 46%, median survival time of these cases was short with a range of only 3 to 6 months. Therefore, a new therapeutic approach is required for the treatment of HS.

The survival and growth of tumor cells are usually associated with multiple abnormalities, such as aberrantly activated growth signaling pathway, dysregulation of cell cycle, aberrantly arrested apoptosis or angiogenesis and, dysfunctional DNA replication/repair. Since a wide variety of cellular abnormalities, it has been believed to be difficult to elicit tumor cell death by targeting only one aberrant molecular mechanism. However, it has been shown that the growth of certain tumor cells strongly depend on one or a few these abnormalities. Targeted therapy using a compound that blocks the growth of tumor cells by interfering with specific aberrant molecular mechanisms is a potent therapeutic approach in the treatment of these tumors. Many types of compounds are protein kinase inhibitors that show anti-tumor effect by blocking ATP binding site of activated kinases. Recently, targeted therapy has been shown to be effective in the treatment of many types of malignant cancers including human chronic myelogenous leukemia, human non-small lung cancer, human advanced non-small-cell lung cancer, and canine and feline mast cell tumor.

Despite the promising strategy for the treatment of malignancies, no compound for targeted therapy against canine HS has been reported. It has been reported that some aberrant molecular mechanisms, such as abnormal under expression of tumor suppressing genes and abnormalities of transcriptional regulators are related to the growth of HS. However, targeted therapy has not been established because the aberrant mechanism needed for tumor survival and growth has not been detected. Therefore, it is necessary to determine the candidate mechanism in HS to establish a effective targeted therapy against HS.

In this study, molecular mechanisms that play a crucial role in the survival and growth of HS cells were searched by growth inhibition assay using chemical library. The target of chemical compound that suppresses growth of HS cells was then investigated. Lastly, *in vivo* effect of this compound against HS cells was examined using a mouse xenograft model.

1. A comprehensive search to identify crucial molecular mechanisms in the survival and growth of HS cell lines

For the evaluation of the compounds that have growth inhibitory effect on HS cells, two HS cell lines (CHS-1 and MHT-2) and 219 compounds were used. From all of the compounds, only dasatinib clearly inhibited the growth of CHS-1 cells. We then focused on dasatinib and examined its growth inhibitory properties

*Supervisor : Prof. Tsukimi WASHIZU

against six HS cell lines. Dasatinib clearly inhibited the growth of four HS lines (CHS-1, CHS-2, CHS-4 and CHS-7) with calculated IC50 values of 5.4 to 54.5 nM concentrations. There are 15 other compounds that partially overlap with the reported targets of dasatinib. These compounds target Bcr-Abl, Src family kinase, Kit or PDGFR. However, there was no correlation of these compounds with growth inhibition of CHS-1 cells. Therefore, it is apparent that the effect of dasatinib on HS cells does not result from the suppression of these recognized molecular targets, but rather via the inhibition of another target such as EphA2 or other as of yet unknown targets.

These findings suggests that the growth of some subsets of HS cells seems to be critically dependent on a specific kinase targeted by dasatinib and this on-target activity of dasatinib most likely results in marked growth inhibition in these HS cells.

2. Analysis of molecular targets of dasatinib in HS cells

To characterize the molecular mechanism responsible for the responsiveness to dasatinib in HS cells, we analysed expression level, mutation status, and gene amplification level of EphA2, Bcr-Abl, Src family kinase, Kit, and PDGFR genes. Further investigation was performed to evaluate activation level of downstream signaling pathways of reported targets of dasatinib. These analyses were performed by using dasatinib sensitive cell lines (CHS-1, CHS-2, CHS-4, and CHS-7) and dasatinib insensitive cell lines (MHT-2 and CHS-5). Furthermore, we searched for new targeted molecule of dasatinib in CHS-1 cells with comprehensive analysis of phosphorylated protein.

Gene expression and genomic amplification of EphA2 and Bcr were not observed in dasatinib sensitive cell lines. Additionally, mutation of EphA2, Abl, Bcr, Src, and Yes was not detected in CHS-1 cells. Also, phosphorylation of AKT, ERK1/2, and STAT3 was not observed in dasatinib sensitive HS cell lines. These results suggest that the effect of dasatinib on HS cells may not be attributed to the inhibition of reported targets but rather by the inhibition of novel targets.

The result of comprehensive analysis of phosphorylated protein in CHS-1 cells showed that 14-3-3 protein gamma was constitutively phosphotylated. This phosphorylation was clearly suppressed by dasatinib. It is reported that in the DNA damage response phosphorylated 14-3-3 protein gamma promotes cell cycle as per its role in ATR-Chk1-Cdc25A pathway. Therefore, the growth of CHS-1 cells is considered to depend on constitutive phosphorylation of 14-3-3 protein gamma. Since 14-3-3 protein gamma has no kinase activity, it is suggested that the effect of dasatinib could be induced by directly inhibiting some kinases in the upstream pathway of 14-3-3 protein gamma, perhaps in the JNK pathway.

From these findings, the constitutive phosphorylation of 14-3-3 protein gamma is likely to play a crucial role in the growth of CHS-1 cells. Suppression of this phosphorylation by dasatinib could blockade cell cycle progression, resulting in the inhibition of the growth of CHS-1 cells.

3. *In vivo* effect of dasatinib against canine HS cells in a mouse xenograft model

The *in vivo* growth inhibitory activity of dasatinib against CHS-1 cells was evaluated by using a mouse xenograft model. Dasatinib clearly suppressed the growth of CHS-1 xenografted tumors. Tumors from the dasatinib-treated mice showed a significant decrease in mitotic and Ki-67 indices compared to controls. The apoptotic index was significantly increased in tumors excised from the dasatinib-treated mice when compared to controls. These results suggest that the inhibition of tumor growth in CHS-1 cells may be attributed to the inhibition of cell division and the increased cell death triggered by dasatinib.

Dasatinib may inhibit cell cycle progression by suppressing constitutive phosphorylation of 14-3-3 protein gamma in CHS-1 cells *in vitro*. Hence, growth of CHS-1 xenografted tumors may be suppressed by inhibition of phosphorylation of 14-3-3 protein gamma by dasatinib. Unfortunately, the mechanism of apoptosis induction by dasatinib in the CHS-1 xenografted tumor was not clarified.

From these findings, dasatinib had growth inhibitory effect *in vivo* via inhibition of cell proliferation and increased apoptosis-mediated cell death. Therefore, dasatinib could be a novel therapeutic approach in the certain subset of HS cases that present constitutive phosphorylation of 14-3-3 protein gamma in the tumor cells.

Conclusion

Our study suggests that the constitutive phosphorylation of 14-3-3 protein gamma plays a crucial role in the growth of some subsets of HS. Dasatinib showed growth inhibitory effects in HS cells with constitutive phosphorylation of 14-3-3 protein

gamma both *in vitro* and *in vivo*. Therefore, dasatinib could be a novel therapeutic approach in the certain subset of clinical HS cases that present constitutive phosphorylation of 14-3-3 protein gamma in these tumor cells.

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Studies on intervertebral disc degeneration in the chondrodystrophoid dog breed

Munetaka IWATA*

Laboratory of Veterinary Surgery
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

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Introduction

Low back pain resulting from intervertebral disc (IVD) degeneration is a leading cause of incapacity in human and veterinary health. IVD degeneration leads to loss of proteoglycans and water content in the nucleus pulposus (NP), which contains large amounts of aggregating proteoglycans and type II collagen (Col2), typical of compression-resisting tissues. NP cells display a rounded, chondrocyte-like morphology and secrete extracellular matrix (ECM) macromolecules composing the hyaline cartilage.

Cells in the NP originate from the notochord. There is a significant difference in the lifespan of notochordal cells among different species, and their loss correlates with early disc degeneration. In pigs, rabbits, rodents, and non-chondrodystrophoid dogs, the notochordal cell population persists into late adult life. However, in humans, sheep, and chondrodystrophoid breeds (CDBs), such as the Beagle and the Dachshund, those cells disappear with age and are replaced by fibrochondrocyte-like cells. CDBs are affected by a profound degenerative disc disease with early onset, often developing within the first year of life; clinical symptoms derived from abnormal endochondral ossification develop between 3 and 7 years of age, with high incidence and high relative risk of developing disc herniation. Indeed, the relative risk for disc herniation is approximately 10 ～ 12 times higher in the Dachshund than in non-chondrodystrophoid breeds. It is thought that the chondrodystrophoid phenotype of CDB is similar to that of humans.

The mechanism underlying age-related IVD degeneration, however, is poorly understood. Many studies have shown an increase in the expression and activity of matrix metalloproteinases (MMPs) during IVD degeneration, and prominent ECM components of the disc, including Col1 and Col2 and aggrecan, have been shown to be substrates of various MMPs.

It has been reported that, in osteoarthritic articular cartilage, there is increased accumulation of β-catenin and decreased expression of aggrecan and Col2a1. Several research groups have suggested that Wnt/β-catenin signal play an important role in IVD degeneration. Wnt signals typically involve a noncanonical pathway or a canonical pathway, and of these, the canonical Wnt/β-catenin pathway, which activates the transcription factors T-cell factor (TCF) and lymphoid enhancer factor (LEF) through β-catenin activity, is well known. When the Wnt ligand is absent, β-catenin undergoes glycogen synthase kinase 3β (GSK-3β)-mediated phosphorylation and proteasome-mediated degradation. When the Wnt ligand is present, it interacts with its receptor, low-density lipoprotein (LDL) receptor-related protein (LRD) 5/6, which recruits Axin to facilitate its decomposition. In addition, Dishevelled proteins facilitate the dissociation of the adenomatous polyposis coli/Axin/GSK-3β complex, whereas frequently rearranged in advanced T-cell lymphoma/GSK-3 binding protein (FRAT/GBP) directly inhibits GSK-3β phosphorylation activity. As a result, the phosphorylation of β-catenin by GSK-3β is inhibited and the β-catenin is stabilized. The stabilized β-catenin moves into the nucleus and, together with TCF and LEF, controls the formation of the body axis and somites, as well as cellular proliferation and differentiation. The quantitative changes of β-catenin are therefore an extremely important factor. However, the role of Wnt/β-catenin signals in IVD cells is not yet well understood.

*Supervisor : Prof. Yasushi HARA

Another important factor in the process of disc degeneration is Runt-related transcription factor 2 (Runx2) expression. A previous report suggested that Runx2 is also implicated in the progression of intervertebral disk aging and calcification in CDBs. Runx2 is an essential transcription factor for osteoblast differentiation and chondrocyte maturation. Runx2 expression is also induced in the articular cartilage of wild-type mice in early stages of osteoarthritis, and this induction occurs prior to MMP-13 expression, indicating that Runx2 has an important role in the osteoarthritis disease process. In addition, Wnt signaling enhances Runx2 expression through the direct binding of TCF7 or LEF1/β-catenin on the Runx2 promoter and through DNA binding of small mothers against decapentaplegic (SMAD) proteins and TCF7L2/β-catenin to their cognate sequences as well as protein-protein interactions between them.

The objective of this study was to evaluate whether the Wnt/β-catenin signaling enhances NP cell degeneration and calcification. We hypothesized that Wnt/β-catenin signaling would enhance Runx2 expression in intervertebral degeneration and lead IVD calcification.

1. Quantitative evaluation of degeneration of NP tissue

To evaluate its degeneration, we graded the NP tissue based on the MR signal intensity that was measured using Image J software. NP tissue which exhibits signal intensity >86 is classified as grade 1, 45 ～ 85 as grade 2, and <45 as grade 3. The results showed that the average signal intensity (ASI) of grade 1 NP tissue was 112.3, grade 2 was 68.3, and grade 3 was 34.2. Moreover, the percentage of tissue occupied by the grade 1 was 61%, grade 2 was 18%, and grade 3 was 21%. It should be noted that, although all NP tissue was derived from 12-month-old CDBs, grade 3 NP tissues were detected, indicating that CDBs are affected by a profound degenerative disc disease with early onset that often develops within the first year of life.

2. Variations in gene and protein expression in canine chondrodystrophic nucleus pulposus cells following long-term three-dimensional culture

Specifically, we evaluated the potential of a three-dimensional (3D) culture of healthy NP as an in vitro model system to investigate the mechanisms of IVD degeneration. Agarose hydrogels were populated with healthy NP cells from beagles after performing magnetic resonance imaging, and mRNA expression profiles and pericellular extracellular matrix (ECM) protein distribution were determined. After 25 days of 3D culture, there was a tendency for redifferentiation into the native NP phenotype, and mRNA levels of *Col2A1*, *COMP*, and *CK18* were not significantly different from those of freshly isolated cells. Our findings suggest that long-term 3D culture promoted chondrodystrophic NP redifferentiation through reconstruction of the pericellular microenvironment. Further, lipopolysaccharide (LPS) induced expression of *TNF-α*, *MMP3*, *MMP13*, *VEGF*, and *PGES* mRNA in the 3D cultures, creating a molecular milieu that mimics that of degenerated NP. These results suggest that this in vitro model represents a reliable and cost-effective tool for evaluating new therapies for disc degeneration.

3. Wnt/β-catenin signaling enhances intervertebral disc degeneration and calcification through Runx2 signaling

Here, we demonstrate that Wnt/β-catenin signaling would enhance Runx2 expression in intervertebral degeneration and lead to IVD calcification.

NP tissue was obtained from 12-month-old male Beagle dogs after evaluation of the degeneration based on the magnetic resonance (MR) signal intensity. Histological analysis showed that lack of Safranin-O staining, calcified area, and MMP13-positive cells increased with progression of the degeneration. Furthermore, β-catenin- and Runx2-positive cells also increased with the progression of the degeneration. Real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis showed that the MRI signal intensity and mRNA expression levels of β-catenin and Runx2 are correlated in NP tissues.

Moreover, to evaluate the role of the Wnt/β-catenin pathway in the regulation of NP cells degeneration, we studied the effects of LiCl on cultured canine NP cells.

Using western blotting analysis, we found that the levels of β-catenin were consistently upregulated by LiCl. A supplementation of 20 mM LiCl induced β-catenin accumulation and Runx2 expression. In contrast, FH535, an inhibitor of β-catenin/TCF activity, inhibits the upregulation. These results indicate that Wnt/β-catenin signals have a significant role in degeneration and calcification in IVD through Runx2 signal.

In summary, our findings support a pivotal role for culture microenvironment on chondrodystrophic disc cell behavior and further suggest that the length is an

important factor in 3D scaffolds. Because the phenotype of NP cells of CDBs is similar to that of humans, these results also suggest that the same basic mechanism of accelerated degeneration functions in human NP tissue.

In addition, our results suggest that Wnt/β-catenin signals may have a significant role in degeneration and calcification in IVD through Runx2 signal.

Our data support the possibility that Wnt/β-catenin induces Runx2 and MMP expression in disc cells of CDBs.

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Myocardial function assessed by two-dimensional speckle-tracking echocardiography in dogs

Ryohei SUZUKI*

Laboratory of Veterinary Internal Medicine
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

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Echocardiography plays an essential role in the assessment of cardiac disease in veterinary medicine. Two-dimensional speckle-tracking echocardiography (2D-STE) has recently been used to assess myocardial deformations in humans and dogs. This technique has enabled the assessment of myocardial variables, such as strain, strain rate, and torsional measurements, that provide better quantification of regional and global myocardial deformations and might have higher sensitivity than conventional echocardiographic parameters for detecting subtle myocardial function abnormalities. However, the effect of age and heart rate (HR) on 2D-STE variables has not previously been reported.

Myxomatous mitral valve disease (MMVD) is the most common cardiac disease of dogs, and some dogs with MMVD develop myocardial dysfunction due to enlargement and remodeling of the heart. Recently, systolic dysfunction is associated with poor outcomes in dogs with MMVD. However, assessment of systolic function using conventional echocardiographic methods is difficult in mitral regurgitation (MR) owing to altered hemodynamic loading conditions and sympathetic tone. We hypothesized that myocardial deformations assessed by 2D-STE could be useful markers of systolic dysfunction in dogs with MMVD.

This study was designed to assess 1) the effect of HR and age on 2D-STE variables in healthy dogs, 2) multidirectional myocardial deformations derived by 2D-STE in dogs with various stages of MMVD, and 3) myocardial deformations during a dobutamine stress test with 2D-STE in dogs with experimentally induced chronic MR.

1. Influence of heart rate on myocardial function using two-dimensional speckle-tracking echocardiography in healthy dogs (Chapter Ⅱ)

HR is a known important modulator of cardiac function, influencing echocardiographic variables. Therefore, it should always be considered in the evaluation of cardiac function, particularly in the event of cardiac failure. However, influence of HR on myocardial function assessed by 2D-STE has not previously been reported. In this study, thirteen healthy beagles were anesthetized and controlled HR with right atrial pacing. Myocardial function of each was assessed using 2D-STE at the pacing rates of 120, 140, 160, and 180 beats per minute (bpm). All strain and strain rate variables in the longitudinal, circumferential, and radial directions were not significantly different at the range of 120 ～ 180 bpm. The peak early diastolic torsion rate at 180 bpm was significantly increased compared with that at 120 bpm (P=0.003). Torsion rate in early diastole was elevated at a HR of 180 bpm, which may reflect increased myocardial relaxation with increasing HR. Left ventricular torsion and untwisting at higher HR may play an important role in preserving stroke volume in the presence of shortened ejection and filling times.

2. Effect of age on myocardial function assessed by two-dimensional speckle-tracking echocardiography in healthy beagle dogs (Chapter Ⅲ)

Aging might affect cardiac function in dogs. Aging also seems to be related to myocardial torsion, assessed by 2D-STE in healthy humans, which is directly related to the helical orientation of myocardial fibers. As the number and proportion of older dogs in the canine

*Supervisor : Prof. Hidekazu KOYAMA

population will increase, quantitative information on age-associated changes in cardiovascular function in the absence of disease becomes more important. However, the effect of age on myocardial function assessed by 2D-STE in healthy dogs has not been previously reported. Thirty-two healthy beagles were used. Myocardial function was assessed in each dog by using 2D-STE, and the results were compared between young and old dogs. The myocardial deformations in systole, besides the apical rotation rate, were not significantly different between young and old dogs. In contrast, the early diastolic circumferential strain rate, basal rotation rate, and torsion rate were significantly lower in old dogs than in young dogs (P=0.03, P=0.033, and P=0.015, respectively). Late diastolic longitudinal and radial strain rates were significantly higher in old dogs than in young dogs (P=0.002 and P=0.018, respectively). Young and old dogs showed similar systolic myocardial deformations, but significant differences in the values of some diastolic deformation variables were found between young and old dogs, highlighting the need for using age-matched control subjects in studies of diastolic function. Evidences about the effect of age in the heart provide important information for understanding age-related cardiovascular diseases.

3. Clinical assessment of systolic myocardial deformations in dogs with myxomatous mitral valve disease using two-dimensional speckle-tracking echocardiography (Chapter Ⅳ)

This study was designed to quantitatively measure multidirectional myocardial deformations of dogs in various stages of MMVD. Our hypothesis for this study was that myocardial deformations assessed by 2D-STE could be useful markers of systolic dysfunction in dogs with MMVD. Eighty-seven dogs with MMVD were enrolled in the study. Dogs were placed into 1 of 3 classes, based on the International Small Animal Cardiac Health Council classification. In addition, 20 weight- and age-matched healthy dogs were enrolled as controls. The dogs were examined for myocardial deformations using 2D-STE, and the peak systolic strain and strain rate in the longitudinal, circumferential, and radial directions were evaluated. Class II and III dogs had higher circumferential strain than class I dogs (P=0.002 and P=0.001, respectively) and controls (P<0.001 and P<0.001, respectively). Class III dogs had higher radial strain than class I dogs (P=0.001) and controls (P<0.001). Class III dogs had higher radial strain rate than class I dogs (P=0.006) and controls (P=0.001). Other deformations, including longitudinal deformations, were not significantly different between classes of MMVD or between MMVD dogs and controls. In the clinical progression of MMVD in dogs, myocardial deformations, as assessed by 2D-STE, differed according to myocardial contractile direction. Thus, assessments of multidirectional myocardial deformations may be important for better assessment of clinical cardiac function in dogs with MMVD.

4. Noninvasive clinical assessment of systolic torsional motions by two-dimensional speckle-tracking echocardiography in dogs with myxomatous mitral valve disease (Chapter Ⅴ)

This study was designed to quantitatively measure myocardial torsional deformations of dogs in various stages of MMVD. Our hypothesis for this study was that myocardial torsion, which is directly related to helically oriented myocardial fibers, could be useful markers of systolic dysfunction in dogs with MMVD. Sixty-seven client-owned dogs with MMVD classified into 3 classes based on the International Small Animal Cardiac Health Council classification and 16 weight- and age-matched healthy dogs. Dogs were examined for myocardial deformations using 2D-STE and were evaluated for peak systolic rotation and rotation rate at each basal and apical view. Dogs were also evaluated for peak systolic torsion and torsion rate. Peak systolic torsion was higher in class II than in class I (P<0.001) dogs. Peak systolic torsion was lower in class III than in class II (P=0.001) dogs and controls (P=0.003). Torsional deformations assessed by 2D-STE differed among clinical classes of MMVD. The lower torsion in dogs with severe MMVD may contribute to latent systolic dysfunction and seems to be related to severe cardiac clinical signs. Myocardial torsional deformations using 2D-STE may provide more detailed assessment of contractile function in dogs with MMVD.

5. Dobutamine stress echocardiography for assessment of systolic function in dogs with experimentally induced mitral regurgitation (Chapter Ⅵ)

Systolic dysfunction is associated with poor outcomes in dogs with MMVD. However, assessment of systolic variables using conventional echocardiographic methods is difficult in these dogs due to MR. We hypothesized that an inotropic challenge with dobutamine, and assessed by 2D-STE, could reveal early occult cardiac dysfunction not evident at rest. Five anesthetized dogs

with experimentally induced MR were used. Dogs were examined for systolic myocardial deformations using 2D-STE during dobutamine infusion prior to and 3 and 6 months after MR induction. We evaluated peak systolic rotation and the rotation rate in each basal and apical view; peak systolic torsion and torsion rate were also calculated. Invasive peak positive first derivatives of the left ventricular pressure (dp/dt) were significantly decreased in dogs 6 months after induction of MR compared with the pre-MR values. Dogs with 3 and 6 months of MR had diminished peak systolic torsion values and torsion rates in response to dobutamine infusion compared with pre-MR data (3 months, $P<0.001$ and $P=0.006$; 6 months, $P=0.003$ and $P=0.021$). These were significantly correlated with the overall invasive dp/dt ($r=0.644$, $P<0.001$; $r=0.696$, $P<0.001$). Diminished torsion during dobutamine infusion in dogs with advanced MR may reflect latent systolic dysfunction. Contractile reserve, assessed as described, may provide a more detailed assessment of contractile function in dogs with MR.

This study confirmed that the effect of HR and age on myocardial function assessed by 2D-STE in dogs. The present study obtained normal values for 2D-STE variables in various HR and young and old dogs, and these can be used as preliminary data for the establishment of reference intervals of 2D-STE in the dog. This study also indicated that multidirectional myocardial deformations could be assessed using 2D-STE to evaluate myocardial function of non-sedated dogs with adequate repeatability. These multidirectional myocardial deformations might improve the clinical assessment of cardiac function in dogs with MMVD. As the torsion is directly related to helically-oriented myocardial fibers, the lower torsion in dogs with severe MMVD may contribute to latent systolic dysfunction. Finally, this study suggests that contractile reserve assessed by dobutamine stress test with 2D-STE is useful tool for early detection of the systolic dysfunction in dogs with MR.

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The diagnostic significance of plasma N-terminal pro-B type natriuretic peptide concentration in dogs and cats with cardiac diseases

Yoshinori TOMINAGA*

Laboratory of Veterinary Internal Medicine Ⅱ
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

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Cardiac diseases in dogs and cats are one of the most common disorder in clinical settings. Mitral valve insufficiency (MVI) in dogs and hypertrophic cardiomyopathy (HCM) in cats are the most common. These diseases can lead to congestive heart failure (CHF) with poor prognosis.

History-taking, physical examination, electrocardiogram, thoracic radiography and echocardiography are usually performed as diagnostic examinations for dogs and cats with cardiac diseases. N-terminal pro B type natriuretic peptide (NT-proBNP) used as cardiac biomarker in human medicine is also available in dogs and cats, and contradicting results for its diagnostic significance have been reported in these species to date. One cause of such controversy might result from the unknown factors affecting plasma NT-proBNP concentration in dogs and cats. Therefore, the purposes of the present study were to investigate various factors affecting plasma NT-proBNP concentration, and to establish the diagnostic significance of plasma NT-proBNP concentration for evaluating MVI in dogs and HCM in cats.

1. Intra- and inter-assay variations in canine and feline plasma NT-proBNP concentration (Chapter 2)

Intra- and inter-assay variations are significant to determine whether the variation of plasma NT-proBNP concentration resulted from hemodynamics or measurement procedure when there were variations in plasma NT-proBNP concentration in same case. In addition, necessity to add aprotinin to the plasma were also determined.

The plasma obtained from 5 dogs and 5 cats were used. In the evaluation of intra-assay variation, the plasma added to ethylenediamine-tetraacetic acid (EDTA) was divided into 10 tubes, and the plasma NT-proBNP concentrations were simultaneously measured. In the evaluation of inter-assay variation, plasma NT-proBNP concentrations were measured 10 times at interval over 1 day. Mean, standard deviation (SD) and coefficient of variance (CV) were calculated. Then, the plasma NT-proBNP concentration was measured using the plasma added to EDTA and aprotinin, and the concentrations were compared with the concentrations measured using the plasma added EDTA.

The medians of CV indicating intra- and inter-assay variations in plasma NT-proBNP concentrations were 11.4 and 19.9% in dogs, and 10.6 and 16.9% in cats. These variations were larger in those in humans, however comparable to those from dogs and cats by another reports. Inter- and intra- assay variations were similar between sample added to EDTA and sample added to EDTA and aprotinin. There was the tendency that plasma NT-proBNP concentration was less variable in the sample added to EDTA.

Based on these results, it was decided that the canine and feline plasma added to EDTA were used in further research in the present study. Variation of about 20% in plasma NT-proBNP concentration due to measurement procedure was confirmed in this chapter.

*Supervisor : Prof. Naoyuki TAKEMURA

2. Daily and weekly variations of, and effects of dietary intake and walking on plasma NT-proBNP concentration in clinically healthy dogs and cats (Chapter 3)

Existence and absence of daily and weekly variations in plasma NT-proBNP concentration were determined using clinically healthy dogs (n=7) and cats (n=5), and effects of dietary intake and 15 minutes walking were observed only in clinically healthy dogs (n=7).

The plasma were obtained every 3 hours, and significance of daily variation was determined by plasma NT-proBNP concentration at each time. The degree of daily variation was determined by CV. The plasma were obtained every week, and the presence and the degree of weekly variation were analyzed. In the evaluations of effects of diet intake or walking, the plasma was obtained before diet intake or walking, and 5, 15, 30, 60, 90 120 and 180 minutes after diet intake or walking. The dogs were made to walk for 15 minutes at the speed similar to human walking.

Although there were insignificant daily variations in both species, daily or weekly variation of about 20% in dogs and 35% in cats were confirmed. In addition, dietary intake and walking did not affect plasma NT-proBNP concentration.

Based on these results, it was concluded that the time for sampling and dietary intake and walking were not required to consider for the measurement of canine and feline plasma NT-proBNP concentrations. However, it was advised that the intra-individual variation of 20 ～ 35% should be considered when plasma NT-proBNP concentration was interpreted in clinical setting.

3. Effect of glomerular filtration rate on plasma NT-proBNP concentration in dogs and cats (Chapter 4)

Because circulating NT-proBNP is excreted only from the kidney, plasma NT-proBNP concentration may be affect by glomerular filtration rate (GFR). However, association of the concentration and GFR were not investigated in dogs and cats to date. Thus, the effect of GFR on plasma NT-proBNP concentration was investigated using 73 dogs and 34 cats with various GFR and without cardiac disease, which were presented to the cardiology service in the Animal Medical Center, Nippon Veterinary and Animal Science University.

As the results, plasma NT-proBNP concentration significantly and inversely correlated with GFR in dogs but not in cats. Plasma NT-proBNP concentration significantly increased in cats with moderator to severely reduced GFR. In some animal, plasma NT-proBNP concentration exceeded cutoff value for detection of animals with possible cardiac disease.

These results indicated that GFR should be considered when plasma NT-proBNP concentration was used as cardiac biomarker. However, it was impractical that measurement of GFR in all clinical cases, and thus, plasma NT-proBNP concentration should be interpreted with simultaneous plasma creatinine concentration.

4. The diagnostic significance of plasma NT-proBNP concentration in dogs with MVI (Chapter 5)

In this chapter, the diagnostic significance of plasma NT-proBNP concentration in dogs with MVI, dogs with MVI and tricuspid valve insufficiency (TVI), and dogs with MVI, TVI and pulmonary hypertension (PH) secondary to MVI were investigated. In this study, 270 dogs presented to the cardiology service in Animal Medical Center, Nippon Veterinary and Animal Science University were used.

In dogs with MVI, plasma NT-proBNP concentration increased in association with cardiac enlargement due to MVI. However, plasma NT-proBNP concentration in dogs without cardiac enlargement, indicating the concentration was less significance as screening test in such dogs. Since plasma NT-proBNP concentration elevated in association with increases in volume overload of the left atrium and ventricle, the concentration might be useful for continuous monitoring for left-heart volume overload. In addition, plasma NT-proBNP concentration was significantly higher in dogs with MVI, TVI and secondary PH than that in dogs with MVI alone. In dogs classified as International Small Animal Cardiac Health Council stage IIIa, plasma NT-proBNP concentration was found to be sensitive and less specific to detect complication of TVI and PH secondary to MVI. Echocardiography would be essential to confirm the existence of TVI and PH.

5. The diagnostic significance of plasma NT-proBNP concentration in cats with HCM (Chapter 6)

In this chapter, the diagnostic significance of plasma NT-proBNP concentration, and the association with the concentration and several echocardiographic variables were investigated using 95 cats presented to the cardiology service in the Animal Medical Center, Nippon Veterinary and Life Science University. Result in this

chapter indicated that plasma NT-proBNP concentration increased in association with existence of CHF, left ventricular hypertrophy and left atrial dilation. It was also confirmed that plasma NT-proBNP concentration could detect even cats with asymptomatic HCM with reliable sensitivity (83.9%) and specificity (93.9%). It was noteworthy that cats with asymptomatic HCM could be detected using plasma NT-proBNP concentration, because such cats could not be detected by using clinical examination other than echocardiography. Moreover, plasma NT-proBNP concentration might be valuable to follow the degree of ventricular hypertrophy and atrial distention in same cat.

In conclusion, it was considered that the diagnostic value of plasma NT-proBNP concentration was more significant in cat with HCM than in dogs with MVI. Plasma NT-proBNP concentration failed to detect dogs with mild MVI without cardiac enlargement and dogs with complication of TVI and PH. In contrast, clinical value of plasma NT-proBNP concentration was more significant in cats with HCM. Especially, it would be most important finding in the present study that plasma NT-proBNP concentration could detect cats having asymptomatic HCM with acceptable sensitivity and specificity. Cats with HCM suddenly developed CHF and/or arterial thromboembolism, and HCM was found to be inheritance disease in some feline breeds. Including plasma NT-proBNP concentration in feline screening health examination might be helpful in early detection and therapeutic intervention for HCM.

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Study on the control of highly pathogenic avian influenza in South East Asia

Shiro YOSHIMURA*

Laboratory of Veterinary Public Health
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

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After December 2003, worldwide outbreaks of highly pathogenic avian influenza (HPAI) by avian influenza virus (AIV) subtype H5 have been reported, and contentious ones were confirmed particularly in Asia. The total number of chicken in the world that AIV might infect was approx. 20 billion and more than half of them were in Asia. Thus, control of HPAI is an important task for veterinary hygiene and for prevention of livestock epidemic in Asia. Since human infections and deaths by H5-subtype have been confirmed after 2003 and those by H7-subtype have been confirmed in 2013, HPAI has become a major public health problem.

With these as background, this thesis was composed of three chapters that investigated effective HPAI control in Asia, particularly in Southeast Asia (SEA) : Chapter 1, analyses of the situation and factors of the HPAI outbreak in Asia; Chapter 2, investigation of the problems in prevention and control measures (P/C measures) of epidemics at the national level; Chapter 3, evaluation of results from HPAI P/C measures which had been taken by international projects.

Chapter 1. Analyses of situations and factors of HPAI outbreak in Asia

Owing to the emergence of human influenza (H5N1) in Hong Kong in 1997, HPAI began receiving more attention not only in veterinary hygiene but also in public health. In this chapter re-evaluations were made on the situation of HPAI outbreaks, on related literatures and on factors related to the outbreak and expansion of the infection which may disturb the prevention.

The HPAI outbreak spread sequentially from East Asia and SEA after 2004, and outbreaks were reported from 64 countries by 2012. In Japan, HPAI outbreaks were reported from 2004 through 2011, and the involvement of wild birds was suggested in the HPAI outbreaks particularly in 2010 ～ 2011.

As for the outbreak factors of HPAI, investigations were made on poultry farming systems, and on movements of wildlife including birds, people and goods, to evaluate the involvement of birds. As a result, in China and SEA poultry farms with their poultry outside were found to be in lower biosecurity and wild birds could easily come in contact with the poultry, suggesting that this form of poultry farming greatly contributed to the HPAI outbreak. It cannot be denied that wild birds infected with AIV (H5N1) were involved in HPAI outbreaks in Japan, and the epidemiological relationships were suggested between the disease and wildlife, resident birds, movement of persons and vehicle of farms.

On the other hand, it was found that in some cases HPAI broke out in places where live birds are gathered (such as live bird market in SEA etc.), and that the pathogen was carried with poultry meat exported from China. As for veterinary management and social factors related to outbreak and expansion of HPAI, it was estimated that as a result of lengthy vaccination, infection spread and lasted contrary to an initial strategy and expectation because many poultry with subclinical infection were left without being killed. Asian traditional culture such as cock fighting was also found to become an indirect factor in the HPAI outbreak and the expansion. Further, motorcycles were suggested to contribute to the HPAI outbreak because they are used to carry live poultry, in SEA.

Therefore, problems were pointed out in the evaluation of biological, physical and social factors for outbreak, its expansion and control of HPAI in this

*Supervisor : Prof. Fukiko UEDA

chapter.

Chapter 2. Legislation for prevention of epizootics, and analysis of the problems

In this chapter problems that hinder HPAI control were investigated by comparing the law and P/C measures in countries (including Japan) that have succeeded in HPAI control, and those in countries where infection still continued.

Historically, GM Lancisi in Italy and T Bates in England introduced the world first active control measures to kill infected animals, to restrict movement and to incinerate/bury the killed animals, and the measures were established in the 1700s and adopted throughout the world. The measures including "payment of compensation" by Bates is notably said to have become the model of the current stamping-out.

As for Japan, the idea of stamping-out of infected livestock was introduced in the "Rinderpest Control Law, in 1871", and since then the Law and its subsequent laws were subject to revision and abolition. The older version of the "Animal Infectious Disease Control Law" was enacted in 1922 and the current "Animal Infectious Disease Control Law" in 1951. In Japan, foot and mouth disease, HPAI etc. have been brought under control with revised "Animal Infectious Disease Control Law, 1951", "Disease control guidelines" for ensuring rapid coordination and cooperation, and "Law on special measures" enacted as needed. It was demonstrated that preparation of law having legal force and its enforcement has been significantly contributing to the reduction of outbreaks and damage of epizootics.

Although Asian countries also have similar laws, it was found that there were some laws covering only prevention of epizootics and others covering livestock production and veterinary affairs in addition. Although in some countries no signs of disappearance of HPAI is seen despite the national measures such as those in Japan, others showed that the HPAI outbreak was controlled by increasing the number of staff involved in the prevention of the livestock epidemic and by proper surveillance, etc.

Therefore, in this chapter it was found evident that an adequate levels of budget and staff, and an accurate grasp of epidemiological information were essential for HPAI control in addition to preparation of laws.

Chapter 3. HPAI control project internationally implemented in SEA

Human cases of avian influenza (H5N1) due to AIV H5-subtype have become an important problem in public health because of the high mortality of approximately 60%. In this chapter, the effectiveness of the HPAI project in SEA the author participated in was analysed and the result was evaluated. HPAI, firstly reported in Korea and Indonesia in December 2003, and spread widely in East Asia and SEA including Thailand, and international action to HPAI P/C measures was started thereafter. The author joined the measures as an official of the Ministry of Agriculture, Forestry and Fisheries from the beginning.

At the "International Pledging Conference on Avian and Human Pandemic Influenza" in January 2006 held in Beijing China, followed by the "International Conference on Avian and Human Pandemic Influenza" held in Bamako (Republic of Mali), international budgetary assistance was first proposed for avian and human influenza (Beijing Declaration) and Japan pledged to support a budget, capacity-building, provision of equipments and others.

The author was assigned temporarily to the OIE office in Bangkok in 2006, and engaged for one and a half years in the support project (phase 1) that OIE took responsibility targeting Thailand, Cambodia, Laos, Myanmar, Malaysia, Indonesia, Vietnam and the Philippines.

(1) In the analyses of law, although all countries have an animal hygiene-related law and guidance for HPAI prevention, there were differences in their content, and some were sufficiently equivalent to the international standard and others not. Then, even if a law existed, some countries lacked sufficient personnel, equipment and budget needed for effective enforcement of the law.
For countries whose strategies were insufficient, an official proposal was made to improve and strengthen the strategies in close collaboration with the countries.

(2) In information sharing for local early-warning systems reinforcement, some common problems existed among countries. Thus, workshops, development of computer software and infrastructure improvement were performed to strengthen the local early-warning system, and a positive outcome was achieved.

(3) Problems became clear about diagnostic equipment

and techniques. In this respect a positive outcome was also achieved from provision of diagnostic equipments and on-the-job training, and national training for diagnostic techniques reinforcement.

(4) Since a level of knowledge and techniques of veterinarian and para-professional were not sufficiently high, workshops were organized to help all of them gain similar levels of technical expertise for HPAI prevention, and then, a positive outcome was achieved.

In this chapter, it was clarified that the project achieved a positive outcome by "the Japanese support for HPAI P/C measures in poultry" that the author joined.

In conclusion, problems were first highlighted by reevaluation of biological, physical and social factors related to HPAI control based upon the analyses outbreaks. In the next, preparation of laws, an adequate level of budget and staff were essential for HPAI control, and then from the analysis of the HPAI outbreak, it was also demonstrated in SEA that the appropriate enforcement of laws and related regulation were effective for control of the livestock epidemic. The project that the author joined succeeded in HPAI control in several countries. These results should be considered when planning measures for rapid and effective HPAI control, and are considered to be meaningful for those involved in veterinary hygiene and public health.

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Study on the development of a rapid analytical method for inorganic elements in animal blood

Fumihito TAKAHASHI*

Laboratory of Biomolecular Chemistry
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

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The utilization of inductively coupled plasma mass spectrometry (ICP-MS) can be obtained information on multiple elements covering the entire mass range from lithium (Li) to uranium (U) on the condition of low cost, simple operation and short analysis time, which can also be obtained information on the definite amount of inorganic elements even if small samples (<0.1mL). Consequently, these methods have been assumed one of the useful techniques for chemometric analysis requiring a large amount of measurement data. Study on the development of a rapid analytical method for inorganic element was performed using semi-quantitative analysis which is one of the measurement systems of ICP-MS.

In this study, this method was applied to plural biological samples, and the measuring data obtained from this method was analyzed, it was employed to examine whether the type of feed and the type of disease could be classified. As a definitive purpose, we would like to lead the novel development of these techniques for applications in the fields of veterinary medicine and animal husbandry.

1. The analysis of multiple elements for biological samples using semi-quantitative analysis by ICP-MS and the investigation of the applicability for chemometrics analysis

This chapter was placed to the preliminary experiment for this study, it was performed to examine whether the biological samples could be measured by using semi-quantitative analysis by ICP-MS. Also it was performed to investigate whether the type of the feed stuff could be distinguished by the data from multiple elements in plasmas obtained from lambs, or whether the difference of depilation disease could be distinguished by the data from multiple elements in serum obtained from dogs. Multiple elements in plasma obtained from Romney lambs (*Ovis aries*), younger than 1 year old, fed by different feed were simultaneously measured by ICP-MS. It was employed to examine whether the type of feeds could be distinguished using the multivariate data. Twenty elements in plasma obtained from lambs were analyzed by a semi-quantitative method of ICP-MS, obtained data were then analyzed by principal component analysis (PCA). As a result, the lambs were divided into three groups on a score plots depending on the different conditions, it was suggested to be distinguished the fattening conditions. Discriminant analyses of the elements were performed using linear discriminant analysis (LDA) with forward stepwise regression, the following discriminant function was made by Br and Rb. The accuracy of classification of each group, as shown by 10-fold cross-validation, proved the effectiveness of the established discriminant function.

On the other hand, it was employed to examine whether the disease groups could be distinguished using the information of multiple elements in serum obtained from depilation disease dog and normal dog. As a result, the distribution of a score plots based on PCA was not able to classify clearly to two categories depending on disease. Although the discriminant analyses of the elements were performed using LDA, and discriminant function was made by Rb and Sr, some discrimination rate was less than 90%. Because the concentrations of macroelement such as Na, K and Ca were lower than the concentrations of these elements in blood of normal mammals, it was thought to be required for the verification of accuracy and the improvement of the low result of macroelement by using semi-quantitative ICP-MS analysis. Also the differences of classification

*Supervisor : Prof. Hiroyuki TAZAKI

contribution by each sample were seen, it was thought that the quality of data from multivariate analysis was needed to investigate the applicability for PCA and discriminant function. In chapter 2, we were performed the verification of accuracy of multiple elements in biological samples using semi-quantitative ICP-MS analysis.

2. The verification of the accuracy for multiple elements in biological samples using semi-quantitative analysis by ICP-MS

The comparison of measurement data for bovine serum by semi-quantitative analysis with full-quantitative analysis that was fully utilized as traditional analysis, and the verification of analytical results using semi-quantitative ICP-MS were performed. Both semi- and full-quantitative analysis by ICP-MS was performed for bovine serum. The ratio of concentrations (%) was calculated as a comparison of each method against the other. The ratio of the concentrations from a total of thirty-seven elements ranged from 85.0% to 118%, except for K and Ca. While the analytical results obtained for K and Ca using the semi-quantitative analysis was shown low concentrations, each ratio was 65.3% and 44.0% in comparison with the ratio of the concentrations from full-quantitative analysis. The accuracy could be improved by adding both elements to the calibration standard when performing semi-quantitative analysis for K and Ca (the ratio of concentration for full-quantitative analysis, K: 106%, Ca: 95.1%). Additional recovery tests were performed to evaluate accuracy by semi-quantitative analysis for bovine serum. The results of the recovery test for K and Ca were 103 ± 5.8% and 96.6 ± 5.8%, and the results of the recovery test for multiple elements except for K and Ca ranged from 88.6% to 118%. From additional recovery tests and the comparison with full-quantitative analysis, it was shown that the measurement data obtained from bovine serum by using semi-quantitative method were accurate.

3. The classification of the fattening condition and the fattening period using the bovine serum

It was performed to investigate whether the fattening region and the feed stuff (the following; the fattening condition) could be distinguished by the data from multiple elements in serum obtained from cattle (Holsteins, 16.3 ～ 21.3 months of age) using ICP-MS method evaluated accuracy with chapter 2. Twenty-four elements in serum obtained from cattle were analyzed by a semi-quantitative method of ICP-MS. The data were then analyzed by PCA and partial least squares discriminant analysis (PLS-DA). As a result, the cattle were divided into two groups on a score plots depending on the different conditions. Discriminant analyses of the elements were performed using LDA with forward stepwise regression, the following discriminant function was made by Br, Mo and I. This discriminant function classified the samples from each group for the fattening condition and for the fattening period by nearly 100% probability.

As second approach, to investigate whether the types of the fattening period for cattle could be distinguished using the data of elements in the serum, the samples and analytical results using in first approach were reclassified as fattening middle group (N=38) and fattening final group (N=78). PCA and PLS-DA were carried out for data obtained from cattle, the cattle were divided into two groups on a score plots depending on the fattening period though the overlap on figure was seen. Discriminant analyses of the elements were performed using LDA, the following discriminant function was made by P, Ca, Ti and Se. The discriminant function classified the bovine serum from each group by 100% probability.

In this study, same multivariate data was used for the classification of the fattening condition and the fattening period. If the condition which divide each group differed, the behavior of elemental conditions also differed among each group, it was suggested that the utilization of classification responded to each purpose was effective.

4. Selection examination of the variables (elements) in the classification for the fattening condition using a linear discriminant function

Because the development of the selection of the most suitable variables and the combination of variables were taken a large burden whenever a sample changes, it was performed to investigate whether the combination of fixed flexible variables would be possible. When the serum of cattle groups (a total of 27 samples) was newly measured, and the measuring data to a total of three groups of a cattle group and two cattle above-mentioned groups was classified, the discriminat function using Br, Mo, Rb, Sr, I and Ba had the highest distinction accuracy, and the distinction rate was 99.3%. By the classification of biological samples such as bovine serum, it was suggested that the selection of halogen such as Br and I was a very important.

It was performed to investigate whether discriminant function using 6 elements could be used as flexible technique for all animal species, multiple elements in serum from horses (90 samples) were measured. As a result, creating the discriminant function to a total of four groups of a horse group and three cattle above-mentioned groups were classified the bovine serum from each group by 98.3% probability. Discriminat function using Br, Mo, Rb, Sr, I and Ba could be classified according to the high accuracy of cattle three groups and a horse group, it was shown that this technique was possible to flexibility use even if different animals species are classified.

In chapter 2 of this study, for semi-quantitative method that had few performed example and full-quantitative that had lots of performed example, the usefulness of measurement data obtained from semi-quantitative method can be made clear according to evaluate the accuracy in bovine serum. The research of classification and discrimination was performed easily and quickly by using this method, which is thought to be able to promote the establishment of more classification systems. Because the composition of the inorganic elements was different for different types of samples, the elements and the concentrations in the optimal calibration standard needed to change with the types of samples. Preparation of the calibration standards that were able to ensure the accuracy of measuring elements in the different sample types was necessary.

Since macroelement in plasma and serum of animals are high concentration in vivo, and those concentrations should stay constant in metabolism in vivo, it was suggested that macroelement were difficult to become the index of discrimination. On the other hand, it is expected that the variation of trace elements takes place other factor (the fattening condition and the environment condition) rather than metabolism in vivo, trace elements was suggested to be important variable quantities for the classification of plural groups. As the reason the characteristics to be inspected of elements for animals are restricted macroelement, it was thought that the elemental concentration except for macroelement in blood is low, and the measurement sensitivity with generic method is low. The utilization of ICP-MS was taught us the fluctuation of trace elements that did not become measuring objects, and which might lead to the development of robust discriminant technique and the discovery of novel function for trace elements.

In chapter 3, two kinds of group divisions such as the fattening conditions and the fattening period were performed using multivariate data set from same serum samples. There were significant differences ($p<0.05$) in all trace elements on the fattening condition. But on the fattening period, there were no significant differences ($p<0.05$) in trace elements such as Mo and I which were selected as variables of discriminant function in fattening condition. The behavior of multiple elements in serum for the fattening condition and for the fattening period was suggested to differ completely, it was shown that the utilization of classification system responded to each purpose was possible.

In chapter 4, the discriminant function in the variables that consists of elements contained in given quantity in soil could be made clear to be utilizable also for distinction of the fattening conditions of different animal species. The utilization of flexible valiables could be reduced the burden of the development every samples or every animal species, and it was suggested to be rapidly enabled the introduction of inspection technique.

A rapid analytical method for inorganic element using semi-quantitative analysis data can be used in studies of pet animals and domestic animals, and a novel disease diagnosis and a method for distinguishing domestic animals fed special feeds can be developed. As a definitive purpose, we would like to lead the novel development of these techniques for applications in the fields of veterinary medicine and animal husbandry.

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The utility of MRI for abdominal tumors in veterinary medicine

Daiji YASUDA*

Laboratory of Veterinary Radiology
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

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The use of magnetic resonance imaging (MRI) in veterinary medicine in Japan has become increasingly prevalent since its recent introduction. It has been applied to studies of central nervous system such as head and spinal cord, which are the most frequently scanned areas. In human medicine, however, abdominal diseases as well as central nervous system are examined widely by MRI and information obtained from scanned images has been useful for diagnosis and prediction of prognosis after treatment.

Up to now, for MRI systems in veterinary medicine in Japan, low magnetic-fields have often been used, and because it takes long to scan the abdomen, it has rarely been used for abdominal diseases due to the problems such as immobilization under general anesthesia and breath holding during scanning. Most intraperitoneal tumor diseases exhibit non-specific clinical symptoms and there is no specific tumor marker available in veterinary medicine. Consequently, early diagnosis is seldom made and there are often few opportunities for effective treatments when diagnosis is achieved. Therefore, early diagnosis by imaging modalities is markedly important.

Currently, x-ray, ultrasonography, and computed tomography (CT) are the main imaging modalities for diagnosis of intraperitoneal tumors in veterinary medicine. However, considering the scanning range of MRI and contrast resolution of soft tissue, the use of MRI in veterinary medicine is expected to increase diagnostic accuracy. On this background, the utility of high-magnetic-field MRI in veterinary medicine was investigated, especially in the diagnosis of abdominal tumors.

In Chapter 2, as an example of a clinical case, canine peritoneal lymphoma was examined by abdominal MRI and in terms of usefulness MRI was compared with CT carried out at the same time. A hypervascular giant mass was visualized by plain/contrast CT and three-dimensional synthesized images, whereas MRI in T1- and T2-weighted images and contrast MRI in T1-weighted images clearly demonstrated that the mass consisted of a tumor with the adhered gastrointestinal tract. Furthermore, MRI also suggested edema and inflammation in some parts of the gastrointestinal tract from the information of wall thickness and signal intensity of the luminal structure of the gastrointestinal tract. The findings of tumor invasion and the relationship with the surrounding organs, which were confirmed later during open surgery, were comparable to those obtained by MRI, and MRI provided clearer image information than CT scanning.

In Chapter 3, out-of-phase T1-weighted MRI was carried out to evaluate the presence or absence of tumor adhesion to the adjacent normal organs. Firstly, scanning conditions of out-of-phase T1-weighted images for the prostate were explored in two healthy dogs. Exploration of scanning time and image quality revealed that it was possible to clearly evaluate the presence or absence of tumor adhesion to the adjacent normal organs and whole images when echo time (TE) was set around 6.9 ms. Under the optimal scanning conditions, three dogs with prostatic carcinoma characterized by invasion of surrounding tissues and the high incidence of metastases were examined by out-of-phase T1-weighted MRI with and without a contrast agent, and the images were compared with those by CT with and without a contrast agent carried out at the same time. As a result, pre/post-contrast MRI scan had higher contrast resolution than CT in soft tissue, providing clearer image information as to the internal structure.

*Supervisor : Prof. Michio FUJITA

With regard to the presence or absence of adhesion, CT suggested suspicions of adhesion and invasion in all the three dogs. On the other hand, in one of the three dogs, contrast MRI in out-of-phase T1-weighted image clearly showed a black-brimmed line indicating the boundary of water and fat between the prostatic cancer and the rectum and no possible adhesion was diagnosed. These results suggested that MRI provided detailed image information regarding local invasion of malignant tumors that would greatly influence the treatment and the prognosis.

Whether dynamic MRI used for diagnosis of hepatocellular carcinoma (HCC) in humans is applicable in veterinary medicine is discussed in Chapter 4. Scanning conditions of dynamic MRI were adjusted in five healthy beagle dogs based on anatomical sites of the aorta, hepatic artery, portal vein, and normal liver parenchyma and the optimal timing of contrast agent injection in dynamic CT.

Under the adjusted scanning condition, dynamic MRI clearly visualized the hemodynamics in the arterial, portal, and equilibrium phases on aorta, hepatic artery, portal vein, and normal liver parenchyma using the anatomical sites obtained by dynamic CT. In the time-course contrast enhancement curve associated with the influx of the contrast agent, the peak was observed earliest in the aorta and the hepatic artery about 10 sec after starting the injection of contrast agent, followed by the portal vein at about 30 sec, and the normal hepatic parenchyma at about 60 sec, which were normal time-course changes already known through CT studies in dogs. Futhermore, there were clearly different MRI values, indicating signal intensity, between hepatic aorta and normal hepatic parenchyma in the arterial phase. It was suggested that the visualization of tumor hemodynamics and differentiation of feeding vessels between hepatic artery and portal vein could be possible.

On the basis of these results, a mass 1 cm in diameter that developed in the liver in a dog was examined by dynamic MRI and images were compared with those by dynamic CT since it is considered to find the tumor is difficult (Chapter 5). Dynamic CT showed a nodular image suspected of a mass after contrast agent injection, which had been undetected before contrast enhancement. However, the mass showed contrast enhancement in the arterial, portal, and equilibrium phases.

On the other hand, dynamic MRI showed a nodular image consisting of a mixture of iso-intensity and low-intensity signal regions to normal liver parenchyma before contrast agent injection. The iso-intensity signal regions of pre-contrast enhancement started to show high intensity in the arterial phase and then low intensity in the portal and equilibrium phases after contrast agent injection, and the low-intensity signal regions of pre-contrast enhancement started to show high intensity in the portal and equilibrium phases. The mass partially showed contrast enhancement in the arterial phase and washout in the equilibrium phases similar to previously reported contrast studies of HCC in human medicine, and it was highly likely that the mass was diagnosed as HCC fed by the hepatic artery.

After imaging examinations, the mass was extracted by open surgery, and well-differentiated HCC was diagnosed by histopathology. Therefore, dynamic MRI was considered useful for diagnosis of HCC in veterinary clinical practice.

As part of our aim of investigating the potential of MRI in diagnosis of abdominal tumors, another scanning method using a diffusion weighted image (DWI) was examined. DWI has recently been used in human medical care for detection of tumor lesions, differentiation of benign and malignant tumors, diagnosis of progression of tumor lesions, prognosis of diseases, and effect of treatments. DWI has been reported to be more useful than conventional MRI scanning methods such as T1-weighted images, T2-weighted images, and contrast T1-weighted images. In Chapter 6, as a preliminary study on whether DWI would be applicable for veterinary medicine, 13 clinically and hematologically healthy dogs were examined by MRI in DWI, and apparent diffusion coefficient (ADC) that represented the status of water molecule diffusion was calculated from the images.

As a result, it was possible to obtain distinct DWIs and measure ADC only at the spleen, some kidneys, and gallbladder in 13 dogs. Furthermore, with regard to the gallbladder, different DWI signal intensities were obtained at the proximal (upper area) and distal parts (lower area) of the cystic duct. ADC obtained at each site was statistically analyzed by Mann-Whitney's U test, and it was significantly higher at the proximal than at the distal part ($p<0.05$). Similarly, ADC was analyzed between the renal cortex and medulla, and it was significantly higher at the medulla than at the cortex ($p<0.001$).

Although it was necessary to further examine various conditions such as the scanning condition of DWI, as a diagnostic method for abdominal tumors in veterinary

medicine, the results suggested the possibility that DWI in MRI would be a useful diagnostic tool in veterinary medicine as well as it is in human medicine.

This study investigated whether abdominal MRI, which has become increasingly prevalent in human medicine, was a useful diagnostic method especially for abdominal tumors in veterinary medicine when compared with CT generally employed today. As a result, a lesion which was diagnosed by CT as a giant mass with unclear boundary between the tumor and the involved gastrointestinal tract was visualized clearly as a mass consisting of the tumor and the gastrointestinal tract with edema and inflammation. Moreover, compared with CT, out-of-phase T1-weighted MRI could more clearly evaluate the relationship between the tumor and the adjacent normal organs, such as the presence or absence of adhesion. Furthermore, dynamic MRI provided finer images and more detailed information as to the blood vessel feeding the liver tumor in a dog case with HCC compared with conventional dynamic CT under the adjusted scanning conditions that was established for hepatic disease. DWI, as a new scanning method of MRI for abdominal tumors in veterinary medicine, was applied in this preliminary study using healthy dogs. It was found that ADC could be measured, but only some organs could be visualized, unlike human medicine.

Although scanning time was slightly longer than CT and scanning conditions in DWI needs further investigation of such influence on the imaging quality and ADC due to the difference of MPG application direction and b value, MRI had higher contrast resolution than CT as a diagnostic modality for abdominal tumors in veterinary medicine. In summary, the utility of MRI in veterinary medicine was demonstrated in this study by allowing various scanning methods and providing more detailed information than CT.

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Effect of grooming on removal of lice (*Pedicinus* sp.) eggs in japanese macaques (*Macaca fuscata*)

Naomi ISHII*

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

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Background

Grooming behavior is commonly observed in primates. In Japanese macaques (*Macaca fuscata*), a known function of this behavior is the removal of lice eggs (*Pedicinus* sp.). Although the lice-removing effect of grooming has been previously investigated in a few studies, this behavior and its effect has not been investigated with respect to sex- or age-specific differences.

The objective of this study was therefore to analyze the influence of individual sex, age and rank on the lice-removal effect of an individual's grooming of its own hair (autogrooming) and that of another animal's hair (allogrooming). Specifically, this study investigated whether the number of unhatched lice eggs attached to a macaque's hair varies depending on body part, sex, or age class. The study also considered the relationship between number of attached unhatched eggs and hair density, as these factors are known to be correlated.

Materials and methods

Skin samples were collected from wild Japanese macaques captured in Shimokita Peninsula, Aomori Prefecture, and in Fukushima City, Fukushima Prefecture. The samples were grouped based on sex and age class (infant, juvenile, adolescent, or adult), and examined under a stereoscopic microscope to assess the number of lice eggs attached to the hair in a 3 cm^2 area. Skin samples were collected from the wrist and lower back, which are the body parts likely to be subject to autogrooming and allogrooming, respectively. Hair density was calculated as the number of hairs per unit area (0.25 mm^2) as measured by the photographic method.

Results and discussion

Chapter 2 discusses our analysis of the characteristics of lice eggs. Lice eggs attached to the hair on the wrist of the macaques captured in Fukusima were examined. Analysis revealed that unhatched eggs were attached to the root of the hair. This suggests that to remove unhatched eggs which were attached to the hair layer close to the skin is not easy.

Chapter 3 explores the influence of individual sex and age on the effect of autogrooming. This was examined by counting the number of unhatched eggs on the wrist of macaques of different sex and age classes captured in Shimokita. The results showed a significant age-related difference ($p<0.01$), but no sex-related difference ($p>0.05$), in the number of unhatched eggs, with a significantly larger number of eggs found on juveniles than in other age classes. This suggests that the effect of autogrooming is affected by the development of grooming behavior in accordance with the age.

Chapter 4 explores the influence of individual sex, age, and rank on the effect of allogrooming. This was examined by counting the number of unhatched eggs on the lower back of macaques of different sex and age classes captured in Shimokita. The results showed a significant age-related difference ($p<0.01$), but no sex-related difference ($p>0.05$), in the number of unhatched eggs, with a significantly larger number of eggs found on adults than on infants or juveniles. This suggests that the effect of allogrooming is affected by inter-individual relationships among the animals in accordance with the age and rank. Additionally, the fact that the number of eggs on the lower back was significantly lower than that on the wrist, as explained in Chapter 3 ($p<0.01$), suggests a higher efficacy of

*Supervisor : Prof. Hiroshi KAJIGAYA

allogrooming than autogrooming.

Chapter 5 presents verification of the influence of individual age on the lice-removal effect of autogrooming revealed in Chapter 3. Here, we investigated whether the number of unhatched eggs on the wrist was different based on age class or region, using macaques captured in Shimokita and Fukushima. Macaques captured in Fukushima had significantly fewer attached eggs than those captured in Shimokita ($p<0.01$), but juveniles had a significantly higher number of eggs than macaques of other age classes ($p<0.01$), regardless of region. This suggests that the effect of autogrooming is affected by habitat, even among macaques with similar levels of grooming skill.

Chapter 6 describes further analysis of the age- and region-related differences in the number of eggs on the wrist, as presented in Chapters 3 and 5, to determine their relationship with hair density. The results showed different trends of changes in the number of unhatched eggs and hair density. This suggests that factors other than hair density affect the age-related differences in the number of eggs on the wrist. Additionally, the fact that hair density of macaques captured in Fukushima was significantly lower than those captured in Shimokita ($p<0.01$), as with region-related differences of the number of unhatched eggs, suggests region-related differences in the number of eggs on wrist affected by hair density. These suggestions support the findings presented in Chapter 3 and 5.

Conclusion

Results of this study suggest that the number of lice eggs present on the hair of macaques is more impacted by autogrooming than by allogrooming, and effect of autogrooming is also affected by individual factors such as age and habitat. Although the number of lice eggs was not directly correlated with load of lice infestation, it was supposed that lice had many chances to hatch on macaques that had many unhatched eggs due to low effect of autogrooming. This suggests that the load of lice infestation is affected by individual factors.

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Influence of different carbohydrate sources and oral hypoglycemic drugs on glucose metabolism in cats

Kaori UEDA*

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

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Obesity causes insulin resistance in cat, and is a risk factor for Type-2 diabetes mellitus. In their natural habitat, cats would normally consume prey high in protein, with moderate amounts of fat, and a minimal amount of carbohydrate; thus, cats are metabolically adapted for greater metabolism of proteins and lower utilization of carbohydrates, as compared to other omnivores. It has been reported that the quantity of carbohydrate affect postprandial blood glucose and insulin levels. However, there are few reports about whether different carbohydrate sources affect glucose metabolism in a cat. Moreover, high protein foods cannot be fed when the cats have concurrent diseases, such as renal disease. Therefore, application of the specific carbohydrate source which prevents postprandial hyperglycemia might be useful for dietary therapy in a diabetic cat. Also, oral hypoglycemic drugs currently being used with humans suffering from Type 2 diabetes mellitus might be useful for feline diabetic patients with concurrent disease such as renal disease. However, it is uncertain whether oral hypoglycemic drugs affect glucose metabolism in cats.

In the present study, healthy cats were fed different sources of carbohydrate to investigate the effect on postprandial serum glucose, insulin and nonesterified fatty acid (NEFA) concentrations. Next, healthy and obese cats were conducted the same food tolerance tests to compare the effect on glucose metabolism between healthy and obese cats. Lastly, we investigated the effects of oral hypoglycemic drugs on glucose metabolism in healthy and obese cats.

1. Influence of different carbohydrate sources on glucose metabolism in healthy and obese cats

Food tolerance tests were conducted using four types of different carbohydrate sources (Glucose, Maltose, Trehalose, Cornstarch) and Control diet in healthy cats. Postprandial blood glucose, insulin and NEFA concentrations were evaluated.

Postprandial blood glucose and insulin concentrations in Glucose diet and Maltose diet tended to be higher than Control diet. Meanwhile, Cornstarch diet tended to be lower than Control diet. NEFA concentrations in Cornstarch diet tended to be lower than Control diet. Therefore, it suggested that Cornstarch might be useful for food management of diabetic cats. NEFA concentrations in diet with all four carbohydrate sources were lower than that in Control diet. As such, carbohydrate might be useful for healthy cats as an energy source.

Moreover, food tolerance tests were conducted using three types of different carbohydrate sources (Glucose, Maltose, Cornstarch) in healthy and obese cats. Postprandial blood glucose and insulin concentrations in obese cats tended to be higher than those in healthy cats. It was suggested that obese cats had higher insulin resistance than healthy cats.

2. Influence of α-glucosidase inhibitors on glucose metabolism in cats

We investigated the effects of α-glucosidase inhibitors (acarbose, boglibose and miglitol) on postprandial blood glucose and insulin concentration in obese cats with feeding high-carbohydrate diets. Areas under the curve of postprandial blood glucose levels in the acarbose group and the voglibose group were significantly lower than non-medication group. It suggested that α-glucosidase inhibitors also had an effect on inhibition of glucose absorption in obese cats as in humans. Therefore, it was suggested that acarbose was useful

*Supervisor : Prof. Toshinori SAKO

for the treatment of diabetic cats, since the side effect was not observed in acarbose group.

Lastly, we investigated the effects of acarbose on glucose metabolism in healthy cats with feeding maltose diet. Since decreased areas under the curve of glucose levels were observed in acarbose group compared to non-medication group, it suggested that acarbose was also useful for healthy cats with feeding high-energy diet. It was reported that α-glucosidase inhibitors enhanced active glucagon-like peptide-1 (GLP-1) responses and reduced glucose-dependent insulinotropic polypeptide (GIP) responses in humans. However, different reaction was observed in cats. Therefore, the distribution of the feline intestinal cells which secretes GIP and GLP-1 might be different from humans.

In conclusion, different sources of carbohydrate had an effect of glucose metabolism in cats. Also, it suggested that oral hypoglycemic drugs were also useful for cats. We also demonstrate carbohydrate source should be included in the feline diet. Furthermore, oral hypoglycemic drug might be one of the useful treatments for diabetic cats. Since influence of incretin hormone for cats was unknown in the present study, it is necessary to investigate the distribution of the feline intestinal cells which secretes GIP and GLP-1.

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Establishment and characterization of a novel cell line from canine extraskeletal osteosarcoma

Eri ONOZAWA*

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

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Canine osteosarcoma has a poor-prognosis with high metastatic rate that is a median survival of less than one year after multidisciplinary treatment combining surgery, chemotherapy and radiation therapy. As one of the reasons, it was considered that cancer stem cells present at a constant rate to the cancer cell population. Cancer stem cells have not only the ability to self-renew and asymmetric cell division but also have a resistance to radiation therapy and chemotherapy. Therefore it has been considered the reason of recurrence and metastasis that due to remain cancer stem cells after treatment. Although cancer stem cells of various malignant tumors have been identified in human using the tumor-derived cell lines, there are previously-unreported which cancer stem cells were identified in human and canine osteosarcoma. And there are few reports which are established cell lines derived from canine extraskeletal osteosarcoma. In this study, firstly, we established and characterized of a novel cell line derived from canine extraskeletal osteosarcoma, and next, as fundamental study for establishing of treatment strategy targeting cancer stem cells, we investigated the presence of cancer stem cells in established cell line.

A 8-year-old male mongrel dog was admitted to the animal medical center of Nippon Veterinary and Life Science University with a chief complaint of difficulty in urination and hematuria. A calcified cystic mass accounts for a large part of the abdominal cavity to be located in a dorsocephalic of the prostate and a dorsal of the bladder were revealed by clinical examinations such as X-ray and echography. The gastrointestinal tract and the bladder were pressed highly by the mass, and it was removed surgically with the prostate. The parenchyma in the mass which was removed was suspected a malignant tumor by fine-needle aspiration cytology. Therefore a part of the tumor tissue was used for primary culture, and the remaining tissues were fixed in 10% formalin for histopathology. The tumor tissue was minced with scalpel and cultured in 10%FCS-DMEM medium, we succeeded in establishment of the cell line (COS-C) which can repeatedly-subcultured more than 50th times. Logarithmic phase of the cells were 4 ～ 7 days and the mean doubling time of the cells was 31.4 ± 0.28 hr. When plated on the dish, the cells had a spindle-shaped morphology in the growth, but in accordance with confluent, changed to a polygon of osteoblast-like cells. The cells have sometimes concentric circle and palisade arrangement, in addition nuclear abnormalities characterized by large pleomorphic nuclei with conspicuous nucleoli were observed. This pattern of growth and cell morphology were similar to the human osteosarcoma cell lines. The median number of chromosomes was 78 per cell (range: 72 ～ 131) and no chromosome abnormalities such as deletions and translocations were confirmed.

To determine the cell origin of the established cell line, it was investigated that COS-C cells were analyzed expression of intermediate type filament protein (vimentin and cytokeratin), and bone-associated differentiation markers (RUNX2, ALP, BSP, OPN, OCN and Col I). Furthermore measure of ALP activity, von-Kossa reaction and transplantation of the cells into nude mice. Immunohistochemical staining showed that negative for cytokeratin and positive for vimentin. All bone-associated differentiation markers were expressed. Mineralization ability was confirmed in a small number of cells. ALP activity was quite low (0.027μmol/min/mg protein). It was considered that the cell line had the character of an undifferentiated osteoblast. COS-C was transplanted subcutaneously in the left lateral region

*Supervisor : Prof. Toshinori SAKO

of nude mice. 3 months after transplantation, apocrine adenocarcinoma was formed on one mouse. Although it was a result that different from the primary tumor-tissue, COS-C was the cell line established from tumor-tissue which was diagnosed extraskeletal osteosarcoma histopathologically, and it was clear that COS-C had a non-epithelial cell-derived immunohistochemically. Therefore, we considered the possibility of spontaneous tumors in nude mice derived from sweat glands. The others mice were not observed the tumors, and we need the further study of xenograft to use as *in vivo* experimental system.

As basic research to establish treatment strategy targeting cancer stem cells, we next examined to identify cancer stem cells in COS-C by detection of ALDH activity, analysis of CD133 cell surface antigen expression, sphere assay and expression of cancer stem cells-specific marker genes (STAT3, Oct3/4, Nanog and CD133). $ALDH^+$ cells and $CD133^+$ cells constituted 0.67% and 7.21% in COS-C cell line, respectively. It was suggested that COS-C had a small population of cancer stem cells. And as a result of sphere assay, the number of spheres was 90.33 ± 6.83 (mean ± SD)/well in COS-C cells cultured with growth factors (EGF and bFGF). In contrast, spheres were completely absent from was detected in COS-C in the absence of growth factors. This result was suggested presence of cells with sphere-forming ability in COS-C cell line. Furthermore, we evaluated the expression of cancer stem cell-specific marker genes, and all of the specific marker genes were expressed in adherent culture cells and sphere. From the above results, it was revealed that COS-C has a small population of cancer stem cells.

In this study, we succeeded in establishment a novel canine extraskeletal osteosarcoma cell line from a rare clinical case with a histopathologically-diagnosis of extraskeletal osteosarcoma which occurred close to the prostate of the male dog. Base on the characteristics of COS-C, it was considered to be an osteoblast-derived cell line that has the property of undifferentiated osteoblast. Furthermore, it was revealed that established COS-C cell line contained a small number of cancer stem cells. It was a useful cell line to study of treatment strategy targeting cancer stem cells in which case *in vitro* experimental systems.

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Study on viral replication of porcine circovirus in cultured cells

Tsubasa KOBAYASHI*

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

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Porcine circovirus (PCV) is classified as a member of the genus *Circovirus* within the family *Circoviridae*, and is small non-enveloped circular DNA virus with a genome of approximately 1.8 kilo base. There are two genotypes, PCV-1 and PCV-2. Although PCV-1 is nonpathogenic, PCV-2 is identified as a pathogen of post-weaning multisystemic wasting syndrome (PMWS). However, PCV-2 is also thought to be associated with "porcine respiratory disease complex (PRDC)" and "porcine dermatitis and nephropathy syndrome(PDNS)". Therefore, these diseases are usually called as "porcine circovirus associated disease (PCVAD)".

Virological technique using cultured cells is not developed enough for PCV-2, because the replication efficiency of PCV-2 is significantly lower in cultured cells. Therefore, the diagnoses of PCVAD in Japan are usually based on histopathological and epidemiological analyses. Moreover, there are still a lot of unclear points about the mechanism of the pathogenicity and viral replication *in vitro*. In this study, to obtain basic data for developing efficient culture procedures, viral replication pattern of PCV in cultured cells was analyzed.

Establishment of real-time polymerase chain reaction to detect PCV-1 and PCV-2 genomes

Real-time polymerase chain reaction (PCR) which quantitatively and identically detected PCV-1 or PCV-2 genomes was established. Recombinant plasmids pPCV-1 and pPCV-2 were also constructed including the genotype specific region, and were used as the standard DNA for the PCV type-specific real-time PCR, respectively. Real-time PCR was able to specifically detect each viral gene of PCV-1 and PCV-2, respectively. Sensitivity of the real-time PCRs was equal or 10 times higher than conventional PCRs for PCVs. Moreover, detection efficiency of active viruses was equal between the real-time PCRs and the indirect fluorescent antibody (IFA) method using anti-PCV polyclonal antibody. These results suggest that the established real-time PCRs are useful for type-specific and quantitative detection method for PCV-1 and PCV-2, and that the real-time PCRs might be good tools to analyze the replication pattern of PCV in cultured cells.

Analyses of replication pattern of PCV-1 and PCV-2 in cultured cells

When trying viral isolations from a lymph node affected with PMWS, nonpathogenic PCV-1 which is often co-infected with an animal is more efficiently isolated than PCV-2 because PCV-1 can grow well in cultured cells. Namely, it has the possibility to affect the virus isolation or diagnosis of PCV-2. Therefore, to develop more efficient viral cultivation method followed by the diagnosis of PCV-2, viral replication pattern both of PCV-1 and PCV-2 in cultured cells were analyzed.

Pathogenic PCV-2 Yamagata strain was kindly provided from the National Institute of Animal Health (Tsukuba, Japan) and was grown in porcine kidney cell line PPK3F and serum-free porcine kidney cell line CPK-NS. Nonpathogenic PCV-1 was collected from porcine kidney cells CPK which was persistently contaminated with PCV-1. PPK3F and CPK-NS cells were used for this study. Each virus (10^6 copies of viral genome per inoculum) was inoculated to the cells and incubated at 37℃ in a 5% CO_2 incubator, respectively. As for each cell, cell passage was carried out three times in the 4 or 7 days intervals. Each genome of PCV-1 and PCV-2 in intracellular and extracellular fluids was analyzed by the type-specific real-time PCR, respectively. Viral antigen in these cells was also detected by the IFA

*Supervisor : Prof. Toshinori OMI

method using swine antisera against PCV-2. Every passage, the number of copies of viral genome declined in PPK3F cells infected with PCV-1, but increased gently in the cells infected with PCV-2. As a result of IFA, number of cells showing specific fluorescence signals increased every passage, and reached a peak after the 3rd passage. On the other hand, in CPK-NS cells, PCV-1 genome was not detected four days after the first passage, but number of copies of PCV-2 genome increased remarkably. Moreover, cells infected with PCV-2 developed clear cytopathic effect (CPE) 4 days after the first passage, and most of these cells were detached from the cultivation flask. There are several reports that PCV-1 is predominantly isolated from clinical samples of affected animals which PCV-1 and PCV-2 are co-infected with in the field. In this study, however, viral replication of PCV-2 was more efficient than PCV-1 in swine kidney cell lines such as PPK3F and CPK-NS. It suggests that only PCV-2 can be efficiently isolated though the isolation efficiency of PCV-2 depends on character of viral strains, type of cell lines, and culture-conditions. Moreover, these results indicate that it should be necessary to find conditions which PCV2 replicate faster without cell-passages. Interestingly, CPK-NS cells which were infected with PCV-2 showed the clear morphological changes as CPE. Cytopathogenicity of PCV-2 would be useful for the development of a novel diagnostic method for PCV infections.

Cytopathogenicity of PCV-2 on a particular cell line

Generally, PCV-2 is noncytopathogenic virus, and virus can be persistently infected to cultured cells. However, in this study, a particular cell line CPK-NS which are infected with PCV-2 shows clear CPE. Therefore, the cytopathogenicity of PCV-2 on CPK-NS cells was analyzed.

Ten thousand focus forming unit (FFU) of PCV-2 Yamagata strain was inoculated to CPK-NS and PPK3F cells, and these cells were incubated at 37℃ in a 5% CO_2 incubator, respectively. Then, cell passage of these cells was done in the 4 or 7 days intervals. As the result, CPK-NS cells which were infected with PCV-2 indicated the clear CPE four days after 1st passage. The development of these CPEs was inhibited by the neutralization with porcine antiserum against PCV-2. Moreover, appearance time of CPE was delayed when cultured cells were infected with 10-fold diluted viruses. On the other hand, no PPK3F cells which are infected with 10,000 FFU of PCV-2 showed CPE during at least five times passage. These results indicate that the morphological changes of CPK-NS cells would be depend on the infection of PCV-2, and also suggest that PCV-2 would have ability to induce CPE in particular cells. These finding will become useful to develop novel diagnostic methods for PCV-2 infection. However, low titer of PCV-2 could induce persistent infections on CPK-NS cells, and replicate a higher number of copies of PCV-2 genome. It suggests that the infective dose in an inoculum prescribed ability of CPE induction or persistent infection. It is, in the future, necessary to analyze about the cytopathogenicity of PCV-2 with focuses on virus properties and innate immunity of the infected cells.

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Study of distribution of the carbonic anhydrase isoenzymes II, and VI of the stomach in eastern grey kangaroo, *macropus giganteus*

Taro HORIUCHI*

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

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Carbonic anhydrases (CA) catalyze the hydration of CO_2 and the dehydration of H_2CO_3, and were identified in sixteen isoenzymes with characteristic activities, respectively. In the stomach, CAII and IV are known to play important roles for maintenance of acid-base balance in several mammalian species. CAII is the cytosolic type enzyme with high activity and supplies the secretions of bicarbonate through the reaction of "$CO_2+H_2O_2 \rightarrow HCO_3^- + H^+$", while CAVI is the secreted type enzyme and functions as an acid neutralizer through catalyzing the reaction of "$HCO_3^- + H^+ \rightarrow CO_2+H_2O_2$". Almost all single-stomach mammalians, such as human, rat, mouse and guinea pig show the expression of CAII at surface epithelial cells and parietal cells of the proper gastoric region. In the equine, a single-stomach herbivore, CAII is expressed at the surface epithelial cells, parietal cells and chief cells in the proper gastric region, but not at the squamous epithelial cells in the non-glandular region. In cows, however, CAII is expressed in the squamous epithelial cells in the non-glandular forestomach and the parietal cells in the abomasum. CAVI is detected in the squamous epithelial cells in forestomach, surface epithelial cells, parietal cells and chief cells in abomasums in cows, suggesting that CAVI contributes to regulate pH in the stomach with CAII. The single-stomach of simple stomach species and abomasum of ruminants maintain gastric juice pH under 3.5, while the rumen maintains the neutral pH level against the rise of H^+ by microbial fermentation. Therefore, expression patterns of CAII and CAVI are thought to reflect difference in regulatory mechanism of acid-base balance in the stomach cavity among species.

Eastern grey kangaroos (*Macropus giganteus*) have a complex stomach with a large forestomach and a small hindstomach. The forestomach is divided into nonglandular (esophagial) and cardiac gland regions, which serves as the major site of microbial fermentation. The hindstomach contains fundic and pyloric glandular regions covered with proper gastric epithelium and pyloric glandular epilthelium, respectively. The stomach of eastern grey kangaroos is thought to be primitive type of ruminant-like stomach, since the forestomach shows single tubular structure and lack of defined compartmentalization separating from the hindstomach. This characteristic structure may support the free moving of luminal fluid between these two regions. Therefore, it is suggested that eastern grey kangaroos utilize different mechanism to maintain environmental pH in the hindstomach and the forestomach, compared with ruminats. In this study, to clarify the mechanism for pH regulation in the primitive type of herbivore stomach, I examined expression pattern of CAII and CAIV in the stomach of eastern grey kangaroos.

In the present study, stomach samples were obtained from 3 eastern grey kangaroos euthanized to reduce the excess number of the animals at the Queensland Moggill Koala Hospital. The stomachs were fixed in Bouin's solution and embedded in paraffin, and sectioned at 4mm thickness. The sections were stained with haematoxylin and eosin (HE), Alcian blue (AB) and Periodic acid Sciff reaction (PAS). Immunohistochemistry for CAII and CAVI were performed by peroxidase-labeled polymer method. Briefly, after blocking of endogenous peroxidase, sections were incubated in 0.01 M citrate buffer pH6.0 for 60 min at 65 ℃. Then, the

*Supervisor : Prof. Toshinori OMI

sections were treated with 5 % normal goat serum, and incubated with anti-CAII or CAVI rabbit polyclonal antibody. After 6 ～ 10 hours, the sections were incubated with anti-rabbit Ig goat polyclonal antibody conjugated with horseradish peroxidase labeled-polymer. The antigen-antibody reactions were visualized using diaminobenzidine tetrahydrochloride.

In the forestomach, the non-glandular region was covered with the squamous epithelium, while the epithelium of cardiac gland region consisted of surface epithelial cells and cardiac gland cells. In the hindstomach, the epithelium of fundic gland region was composed of surface epithelial cells, mucus neck cells, parietal cells and chief cells. The epithelium of pyloric gland region contained surface epithelial cells and pyloric gland cells.

In AB and PAS staining, both positive reactions were detected at the cytoplasm of surface epithelial cells of the cardiac gland, mucus neck cells of fundic gland and pyloric gland cells.

In non-glandular region of the forestomach, a small number of squamous cells showing CAII-immunoreactivity were scattered in the inermediate and lower regions of the epithelium. In contrast, CAVI-immunoreactivitiy was detected in many squamous cells distributed in the inermediate and lower regions of the epithelium. In the glandular region of the forestomach, both CAII and CAVI-immunoreactivities were detected in surface epithelial cells. In the fundic gland region of the hindstomach, superficial epithelial cells and parietal cells showed both CAII and CAVI-immunoreactivities, although chief cells showed only CAVI-immunoreactivity. The mucus neck cells showed no CAII and CAVI-immunoireactivities. In the poyloric gland region, surface epithelial cells showed both CAII and CAVI-immunoreactivities, whereas pyloric gland cells lacked these immunoreactivities.

In the present study, I found the presence of many CAVI-immunoreactive squamous epithelial cells in the non-glandular region of forestomach in eastern grey kangaroos. However, CAII-immunoreactivity was detected in a small number of squamous cells. In the glandular region of the forestomach, both CAII and CAVI-immunoreactivities were detected in the surface epithelial cells. In the rumen of cattle, squamous epithelial cells express both CAII and CAIV to maintain neutral pH for microbial fermentation. In addition, CAII provides a continuous source of H^+ to lumen and cytoplasm for the transport of volatile fatty acids(VFAs) by the hydration of CO_2. The secretary type CAVI accelerates the neutralization of excess organic acid produced by microbial flora and forms complementary system with CAII. Therefore, the results of the present study indicate that the acid-base balance in the forestomach is regulated by CAII in surface epithelial cells of glandular region and CAVI secreted from both surface epithelial cells of glandular region and squamous epithelial cells of non-glandular region in eastern grey kangaroos. Considering the expression pattern CAII and VI in the rumen of cows, types of CA-supplying cells may change during evolution from primitive forestomach without compartmentalization to highly compartmentalized stomach seen in ruminants. In the fundic gland of cows, CAII is expressed only in parietal cells, while CAVI is expressed in surface epithelial cells, parietal cells and chief cells. In eastern grey kangaroos, however, surface epithelial cells expressed CAII as well as parietal cells in the fundic gland. The stomach of eastern grey kangaroos has a single tubular structure and avoids clear compartmentalization separating the hindstomach and the forestomach. Therefore, the luminal fluid of these two regions seems to easily mix each other. However, Gemmel et al demonstrated that the pH of stomach fluid changed from 7.0 in the forestomach to about 2.0 in the fundic gland region of the hindstomach in tammar wallabys. The expression of CAII in surface epithelial cells may be necessary to change the pH from neutral to acid rapidly in the hindstomach by producing H^+ in surface epithelial cells in eastern grey kangaroos.

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Establishment of quantitative real-time PCR methods for canine periodontal bacterium and its application for prophylaxis in canine periodontal disease

Natsuki MARUYAMA*

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

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Periodontal disease is a significant oral problem of canine and is characterized by gingival inflammation, increased probing depth, and alveolar bone loss with resulting loosening and eventual loss of teeth. Periodontal infections are caused by certain bacteria. A consensus report concerning periodontal diseases and microbial etiology designated *Porphyromonas gulae*, *Tannerella forsythia* and *Campylobacter rectus* as the most important species. In veterinary medicine, accurate quantification of bacterial species in dental plaque is needed for microbiological diagnosis of periodontal disease.

Tooth-brushing is essential for the prevention and treatment of periodontal disease. In addition, "glucoseoxidase-lactoperoxidase anti-bacterial system (GO-LPO)" was shown to exhibit a possible inhibitory effect on bacteria in saliva and oral malodor in human medicine.

In this study, we investigated quantification method of canine periodontal bacterium and the effect of tooth-brushing and GO-LPO gel system for periodontal disease.

1. Establishment of quantitative real-time PCR method for canine periodontal bacterium

We studied 26 healthy canines maintained in our animal facility. Real-time PCR was performed to determine quantity of the bacterial DNA by species-specific sets of primers (*P. gulae*, *T. forsythia* and *C. rectus*). Coefficient of variance (CV) of intra- and inter-assay of the bacterial specific primer was less than 5%, indicating high reproducibility. *P. gulae*, *T. forsythia* and *C. rectus* were detected in all canines in the present study. A DNA copy number of *P. gulae* is significantly higher than other 2 species.

2. The effect of tooth brushing on bacterial DNA copies in canine periodontal disease

Using 26 canines and primer sets of chapter 1, DNA copy number of periodontal bacterium was evaluated to investigate the relationship with gingivitis score (hyperemia, tumentia, and calculus). As a result, but not significant, the DNA copy number tended to be increased correspondence to gingivitis score. In addition, the present experiment was undertaken to determine the effects of tooth-brushing. The mouth of each dog was divided into brushing and non-brushing control. Consequently, in the brushing group, DNA copy number of periodontal bacterium and gingivitis score tend to be lower than non-brushing group.

3. Evaluation of protective benefit for the canine periodontal disease using the GO-LPO anti-bacterial system

GO-LPO anti-bacterial system provides important antimicrobial activities in human saliva and protects oral tissues from oxygen toxicity through oxidation of SCN^- and consumption of H_2O_2. This chapter was undertaken to evaluate effect of GO-LPO gel on periodontal condition for canine. We used 5 healthy canines. Firstly, these dogs were treated by dental scaling under anesthesia. The experiment was performed by applying either GO-LPO gel which containing the enzyme (GO-LPO group), or placebo gel (placebo group) for canine teeth once in a day for 42days. As a result, DNA copy numbers of periodontal bacterium with GO-LPO group

*Supervisor : Prof. Toshinori SAKO

were lower than those of placebo group. In addition, gingivitis score and probing depth were improved in GO-LPO group as compared to placebo group.

Conclusion

The present study established quantification method of canine periodontal bacterium by using real-time PCR method. This study showed that *Porphyromonas gulae*, *Tannerella forsythia* and *Campylobacter rectus* were 3 major species present in canine. In addition, they might be related to gingival inflammation.

The results of the present study suggest that tooth-brushing and GO-LPO anti-bacterial system reduce the amount of periodontal bacterium, and improve gingival inflammation.

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Studies on antioxidant activity of hydrolysates obtained from porcine muscle proteins by protease treatment

Wataru INOUE*

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

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Introduction

Hydrolysates of food-derived proteins (peptides) are known to regulate some biological functions. We have been reported that a hydrolysate of porcine myofibrillar protein (Mf) possess antioxidant activities. However, the antioxidant activity of hydrolysates of other proteins such as sarcoplasma proteins (Sp) and myostroma proteins (Ms) remains unknown.

In this study, we examined the antioxidative effects of various proteins extracted from porcine muscle and the peptides obtained by the enzymatic digestion of these proteins.

Materials and methods

1. Sample Preparation

After preparation of Mf, Sp, and Ms from the porcine *longissimus thoracis* muscle, 1/100 volume (w/w) each of papain was added and the mixtures were incubated at 37 ℃ for 48 h to hydrolyze the proteins. Supernatants of the deproteinized solutions were used as a Mf-derived peptide fraction (MfP), a Sp-derived peptide fraction (SpP), and a Ms-derived peptide fraction (MsP).

2. Inhibitory effect of porcine muscle protein hydrolysates on lipid peroxidation

Potassium phosphate buffer (final concentration 0.1 M, pH7.0) containing TritonX-100 (final concentration 0.5 %), iron chloride II (final concentration 0.05 mM), and an aliquot of each hydrolysate sample (final concentration 0.1 %) were added to linoleic acid, and the resulting mixture was heated at 80 ℃ for 60 min. The amount of lipid peroxide in the mixture was measured immediately before and after the heat treatment to determine lipid peroxide increases caused by the heating.

3. DPPH radical scavenging activity of porcine muscle protein hydrolysates

An aliquot of each sample solution or α-tocopherol (V.E.) was added to a 0.02 % DPPH radical solution, and the amounts of residual DPPH radical were examined by measuring the absorbance (at 517 nm) of the mixture after 60 min.

4. Inhibitory effect of porcine muscle protein hydrolysates on protein degradation induced by various radical

The hypochlorite radical (ClO•) was prepared by diluting sodium hypochlorite with distilled water. The hydroxyl radical (OH•) was prepared by adding 10 parts of H_2O_2 to one part of EDTA, $FeCl_3$, and ascorbic acid (V.C.) solutions (0.1 M). Peroxynitrite radical (ONOO•) was prepared by adding two parts of 1.5-M NaOH to one part of a 0.6-M $NaNO_2$/0.6-M HCl/0.7-M H_2O_2 solution. V.C., V.E., glutathione (GSH), or an aliquot of a hydrolysate/protein sample (final concentration 0.1 %) was added to an egg-white ovalbumin solution (OVA; final concentration 2.0 mg/ml). Then, distilled water or a radical solution (to the 10 mM, final concentration) was added to prepare control or experimental samples, respectively. The mixtures were incubated in a thermostatic bath at 37 ℃ for the following periods: ClO•, for 30 min; OH•, 60 min; and ONOO•, 90 min. The degree of OVA degradation was determined by SDS-PAGE image analysis.

5. Fractionation of antioxidant peptides

SpP was loaded onto a cation exchange column (AG 50W); the non-absorbed neutral/acidic fraction was

*Supervisor : Prof. Toshihide NISHIMURA

eluted with 0.05-M ammonium acetate buffer (pH6.0) and absorbed basic fraction with 0.2-M NH_4OH. The neutral/acidic fraction was loaded onto an anion exchange column (AG 11); the non-absorbed neutral fraction was eluted with 0.05 M of ammonium acetate buffer (pH3.5) and absorbed acidic fraction with 1-N HCl. The antioxidative effect of each fraction against ClO• was measured to obtain the fractions with strong activity.

Results and discussion

1. Inhibitory effect of pig-muscle protein hydrolysates on lipid peroxidation

For all the source proteins examined, enzymatic degradation increased their antioxidant activities. When MfP was used, the degree of lipid peroxidation was 28.0 %, showing a significantly higher inhibitory effect on lipid peroxidation than the control. This value was equivalent to that of GSH.

2. DPPH radical-scavenging activity of porcine muscle protein hydrolysates

Significant DPPH radical scavenging was observed in samples containing MfP, SpP, Ms, and MsP. The following amounts DPPH radicals remained after the addition of each hydrolysate: SpP, 39.7 %; MfP, 54.7 %; Ms, 72.1 %; and MsP, 76.6 %. None of the hydrolysates exceeded the value observed for V.E. (8.6 %). The degradation of Mf and Sp triggered radical scavenging, whereas no increase in scavenging was observed for Ms after its degradation.

3. Inhibitory effect of pig-muscle protein hydrolysates on protein degradation induced by various radicals

In samples incubated with ClO•, 59.2 % of the OVA protein remained in the presence of SpP, suggesting that SpP had a higher inhibitory effect on protein degradation than GSH (46.5 %). For the OH• samples, after the addition of MfP and SpP, 67.4 % and 66.5 % of the protein, respectively, remained undegraded. Thus, MfP and SpP also had a high inhibitory effect, although it was lower than that of V.E. (79.9 %). For ONOO•, after the addition of SpP, 42.3 % of the protein remained undegraded, similar to that obtained for V.C. (44.6 %). The degradation in the presence of ClO• and OH• was completed within 5 min, whereas in samples containing ONOO•, it continued even after 90 min.

4. Fractionation of antioxidative peptides

SpP, which had the highest inhibitory effect on protein degradation by radicals, was subjected to ion exchange chromatography. The inhibitory effects of the acidic, neutral, and basic fractions on the OVA degradation by ClO• were measured. The neutral and basic fractions allowed 66.8 % and 66.2 % of the protein, respectively, to remain undegraded, showing a strong inhibitory activity.

These results showed that the degradation of proteins enhances their antioxidant activity, and the peptides act against different reactive oxygen species depending on their type. This difference might be caused by differences in contents and sequences of amino acids with various degrees of antioxidant activity properties.

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Studies on proteinase from *aspergillus oryzae* which contributes to cheese ripening

Takahiro UCHIDA*

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

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Many fermented foods are made from aspergillus. In the traditional Japanese fermented foods of miso, soy sauce, and sake, the quality is created by various types of enzymes produced and released by mainly aspergillus. They include proteolytic enzymes, lipolytic enzymes, and amylolytic enzymes. In cheese, another fermented food, quality with distinct flavors and textures is formed by the degradation of lactoprotein, milk fat, and lactose by enzymes derived from milk or from starter and non-starter lactobacillus during the heat process in manufacturing.

In this study I investigated the proteases produced by aspergillus, and their features, with the aim of improving the palatability of cheese using aspergillus-derived enzymes, which are rarely used in animal products. I also focused on cultures that produce greater amounts of enzymes from among various cultures, and detected and refined enzymes with consideration of the environment during the heating of cheese.

To culture aspergillus, deionized water was added to medium of wheat bran only and to medium of wheat bran with added skimmed milk, a main component of cheese, as a source of nitrogen. Inocula (0.15 g) of aspergillus (commercial variety aspergillus, *Aspergillus oryzae*, Akita Konno Shoten) were added to each medium and cultured for 4 days at 30 ℃. After culturing, deionized water was added to the media and it was fragmented in a homogenizer. The lysate was then separated into supernatant and precipitate by centrifugation. The supernatant obtained in this centrifugation was taken as the crude enzyme solution. The protease activity was measured by azocasein assay. For the unit of enzyme activity, azo dye released from azocasein during reaction for 45 min was measured at 440 nm, and an absorbance value of 1 was taken to be 1 unit. The activity of the crude enzyme extracted from the medium supplemented with skimmed milk showed a specific activity 2.43 times greater and a total activity 3.05 times greater than that of enzymes extracted from the wheat bran medium.

To investigate the factors in improved enzyme productivity of the skimmed milk-supplemented medium, each type of crude enzyme solution was freeze-dried and then supplied to anion exchange chromatography (Toyopearl DEAE-650M) and eluted on a gradient of 0 ～ 1 M NaCl. For the protease activity of the eluate, activity was confirmed in the non-adsorbed fraction and the adsorbed fraction (near 0.11 M NaCl). The respective peak activities of these two active fractions were supplied to molecular sieve chromatography (Toyopearl DEAE-HW-55F) and both the non-adsorbed fraction and the adsorbed fraction (near 0.11 M NaCl) showed activity in the vicinity of about 40 kDa. Focusing on the active fraction detected in the skimmed milk-supplemented medium, measurements of the optimum temperature and pH of the active fraction revealed an optimum temperature of 40 ℃ and optimum pH near 6 in the non-adsorbed fraction. In the active fractions (near 0.11 M NaCl) detected in both other bran and skimmed milk-supplemented media, the optimum temperature was confirmed to be 30 ℃ and the optimum pH to be ≥9. The above results suggest that the enzymes produced by the aspergillus used in this study include two types, a protease with an optimum pH on the mildly acidic side and a protease with optimum pH on the basic side. Of the two, we focused on the enzyme that is produced significantly on the skimmed milk-supplemented medium and the acidic protease that is suited to the environment during cheese manufacturing. They were supplied to anion exchange chromatography for further isolation.

*Supervisor : Prof. Ryozo AKUZAWA

The non-adsorbed fraction collected was supplied to a TKSgel G20000SWXL column at a flow rate of 1 mL/min, and collected 0.5 mL/tube. Its activity peak was shown to be near 40 kDa. This active fraction was condensed and again supplied to TKSgel G20000SWXL and chromatography was done. Again, an activity peak was shown near 40 kDa. This active fraction was then measured using SDS-PAGE electrophoresis, and a single band was shown at 36 kDa.

This study suggests that the productivity of the specific adsorption fraction enzyme increases markedly with the addition of skimmed milk to the aspergillus inoculate medium as a nitrogen source. This fungus produces two types of enzymes. One was a protease with optimum temperature of 40 ℃ and optimum pH on the mildly acidic side, and the other was protease with an optimum temperature of 30 ℃ and an optimum pH on the basic side. Considering cheese manufacturing, we focused on the acidic protease and attempted to refine it, and obtained results suggesting that it has a molecular weight of about 40 kDa. When integrating this study in the future, we will analyze the primary structure of this enzyme and investigate its enzymological properties. It will be necessary to also describe its contribution to improved flavor of cheese.

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Research on diffusing sex-sorted embryo transfer method in japanese dairy farming

Tomoko OBUCHI*

Laboratory of System Management Administration
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

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Introduction

One of the factors that efficient milk production and the securing cow are important in planning and the durability of the management in dairy farming. Recently sex-sorted technique has developed rapidly. These new techniques have a value for the first time when they are used by dairy farmers in their farms. But there are few reports about use intention and the economy of the dairy farmers for sex-sorted techniques. I think that this situation might disturb the techniques spread to the plane of farming.

This survey was aimed for factor analysis for sex-sorted embryo transfer method that was so-called advanced technique in field of cow reproduction to settle in ground of the farming.

Materials and methods

(1) By the questionnaire survey for 292 dairy farmers, I clarified the use situation and the use intention of sex-sorted embryo transfer method.
(2) According to a method to make five kinds of sex-sorted embryo, I calculated an expense need for the number of the female calf and the production of the female calf to provided by one egg removal.
(3) I performed the lecture about breeding technology of 21 dairy farmers. And by the questionnaire survey that I carried out before and after a lecture, I inspect a spread effect.
(4) I carried out herring investigation for ten dairy farmers of Tokyo and Shizuoka. I made these dairy farmers a type based on the use situation and use intention of the sex-sorted embryo. And I clarified management characteristic and a factor necessary for the technical diffuse.

Results

1. Use intention for sex-sorted embryo method of dairy farmers

As a result questionnaire survey, 292 dairy farmers were comprised of 4% that "have already use" and 35% that "they want to use." This result clarified the existence of the dairy farmer having the active use intention of sex-sorted embryo method. However there was the most intention "not to know whether you wanted to use it." As the reason, the respondent gave that problems of technique itself and the relation between using technique and profit of the management was indistinct.

2. According to a method to make sex-sorted embryo, expense calculation required for the production of one of the female calf

In fine kind of method, SOV-OPU-Sort IVF was the most effective method that 1.35 female calves ware born one egg removal. The technique costs that this method needed one female calf for the production were calculated with approximately 68,000 yen. This result was the cheapest in five kinds of methods.

3. Enforcement of the lecture about sex-sorted embryo and inspection of the spread effect

Before the lecture, the understanding rate of sex-sorted embryo of the dairy farmer of Tokyo was 57%, Shizuoka was 38%. After the lecture, improvement of the understanding was seen in both areas. However, "The negative intention for the use" that not appeared before a lecture occurred after a lecture.

*Supervisor : Prof. Takeyuki OZAWA

4. Hearing investigation and a type by use intention

The characteristic that was common the dairy farmers who used sex-sorted embryo was two points: (1) Enforcement dairy cattle group official approval and lineage registration. (2) Security veterinarian and inseminator who were egg removal and fertilization.

There were dairy farmers who did not use sex-sorted embryo at the time the survey. However some them hoped to use in the future. The background where they did not reach the use included six reasons: (1) lower conception rate, (2) High sex-sorted price, (3) trouble and time require for fertilization, (4) lack of the information about sex-sorted embryo, (5) the acquisition way of sex-sorted embryo was unclear, (6) the lack of veterinarian and inseminator who were fertilization.

Discussion

Generally, it was said that the spread of new techniques process passes through five stages. As a result of survey, it was revealed that recognition and understanding of sex-sorted embryo was low. From this, it was supported that sex-sorted embryo method was located for the first phase "cognitive stage".

In addition, the reporting by the lecture form may contribute to improvement of the recognition and understanding of the new techniques. After the lecture, there was the dairy farmer who changed "did not hope for use of sex-sorted embryo method." Therefore it was guessed that the understanding improvement of the new method was not necessarily tied to formation of the technical use.

Than the questionnaire survey for 292 dairy farmers and investigation into for dairy farmers of Tokyo and Shizuoka made clear that they hoped for of the presentation of concrete profit when dairy farmers introduced new technique in to the place of farming.

However, there few dairy farmers who thought that it became the method that SOV-OPU-Sort IVF was used widely at the farming. Furthermore, the possibility that the dairy farmers did not always grasp an expense for the production of one female calf was suggested.

From the result of this study, I pointed out five conditions necessarily for the spread of sex-sorted embryo method: (1) a breeding technology improve, (2) the research organization including the university approached the dairy farmer about using technology, (3) maintain environment enforce embryo transfer such as upbringing of a engineer performing embryo transfer and cognitive improvement to the new technology of dairy farmer, (4) Exhibit a concrete expense and profitability to occur when dairy farmer introduced it into the place of management, (5) the master of management be aware of the expense that it costs for the securing female cow and succeeding cow. And it was necessary for manager to determine whether it becomes useful to use the method.

So that sex-sorted embryo method became pragmatic technique at a place of farming, I thought that it was necessary to cope in these factors concurrently.

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Studies on insulin sensitive glucose transporter for specific regulation of blood glucose in chicken embryo

Marie KIMIZUKA*

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

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Gluconeogenesis of chicken embryo increases during later period of development. In addition, plasma glucose concentration of chicken embryo is not affected by insulin and metformin which activate the sensitivity to insulin administration while whole glucose content of embryo is elevated by them. Those facts might be meaning that plasma glucose is maintained when that decreases by uptake induced by insulin to tissues. On the other hand, *in ovo* leucine (Leu) administration elevated expression of insulin like growth factor I receptor (IGF-1 R) mRNA which involved in promotion of muscle protein synthesis in broiler breeder chicks at 28 days of age, although that was not resulted in improvement of growth. That might be due to function of Leu that reduces the gluconeogenesis, and this function might oppose to high gluconeogenesis during later period of embryo developing.

However, hexokinase, limiting enzyme of glycolysis activity is so low, and physiological meaning of glucose is not clearly in chicken embryo. To evaluate the glucose utility, it is necessary to clarify the tissues which uptake glucose mainly during incubation, and determination of those tissues requires determination of glucose transporter (GLUT) expression, but not clarify GLUT involved in glucose uptake in chickens.

Therefore, in order to clarify the physiological meaning of glucose when Lue administered, 6 experiments were conducted to study 1) changes in gluconeogenesis after Leu administration (Experiments 1-5), 2) effects of glucose administration with Leu on subsequent growth (Experiment 6), and to search 3) GLUT involved in glucose uptake in chicken embryo (Experiments 7-10).

32, 120, 48, 60, 75, 15, 2, 2, 10, and 40 Chunky strain broiler breeder eggs were purchased from commercial hatchery and utilizing for Experiments 1 to 10, respectively. Eggs were incubated at 37.8 C and over 60 % RH. In Experiment 1, eggs were divided to 4 groups with 8 eggs each. On Day 17 of incubation, 50 μL saline or insulin solution (1 IU/kg egg weight) was injected into sub-eggshell membrane vein, and broke egg to bleed from the yolk vain at 20 or 60 minute after treatment.

In Experiment 2, eggs were divided to 5 groups with 24 eggs each. On Day 17 of incubation, none, 50 μL 0, 0.5, 1.0 or 2.0 U/kg egg weight insulin solution was injected same way as Experiment 1, and 8 egg were selected from each treatment, and broke egg to bleed from the yolk vain at 5, 20 or 60 minute after treatment. In Experiment 3, eggs were divided to 2 groups with 24 eggs each. On Day 17 of incubation, 50 μL saline, or 1.0 U/kg egg weight insulin solution was injected same way as Experiment 1, 8 egg were selected from each treatment, and broke egg to collect the liver and pectoral muscle for determination of the phosphoenolpyruvic acid calboxykinase (PEPCK) activity at 5, 20 or 60 minute after treatment. In Experiment 4, eggs were divided to 4 groups with 15 eggs each. On Day 17 of incubation, 200 μL saline, 7, 14, or 21 mg/mL Leu solution was injected same way as Experiment 1, 5 egg were selected from each treatment, and broke egg to bleed from the yolk vain at 5, 20 or 60 minute after treatment. In Experiment 5, eggs were divided to 5 groups with 15 eggs each. On Day 17 of incubation, 200 μL saline, 1.0 U/kg egg weight insulin solution, 7, 14, or 21 mg/mL Leu solution was injected same way as Experiment 1, 5 egg were selected from each treatment, and broke egg to collect the liver and pectoral muscle for determination of the PEPCK activity and glycogen contents at 5, 20 or 60 minute after treatment.

*Supervisor : Prof. Yoshiyuki OHTA

In Experiment 6, eggs were divided to 3 groups with 5 eggs each. On Day 18 of incubation, 500 μL distilled water, 9 mg/mL Leu solution, or 9 mg Leu and 53 mg glucose/mL solution was *in ovo* injected, and continued incubation. All newly hatched chicks were housed in same litter floor pen and fed commercial feed and water *ad libitum*. At 28 days of age, broilers were weighed, killed by cutting artery and collected left side pectoral muscle to determined IGF-1 R mRNA expression.

In Experiment 7, each one Day 17 broiler embryo and chicks 28 days of age were scarified to collect the pectoral muscle, liver and pancreas for evaluation of GLUT gene expression. In Experiment 8, same age animals were used to collect the pectoral muscle, liver, pancreas, brain hart, spleen kidney, and small intestine for evaluation of tissue specification in GLUT gene expression by agarose gel electrophoresis. In Experiments 9 and 10, eggs were divided to 2 and 8 groups with 5 eggs each, respectively. On Day 17 of incubation, 50 μL saline or insulin solution (1 IU/kg egg weight) was injected like as Experiment 1, and collected the pectoral muscle, liver and pancreas for determination of GLUT 1, 2, 3, 8, and 10 mRNA expression at 5minute in Experiment 9, and 5, 20, and 60 minute after treatment in Experiment 10, respectively.

There was no difference in plasma glucose concentration among treatments in Experiment 1, while that decreased in only embryos administered 1.0 IU/kg egg weight insulin in Experiment 2 ($P<0.05$). There was no change in hexokinase activity, while PEPCK activities decreased at 5minute after insulin administration ($P<0.05$), and then increased in Experiment 3. There were no effects of several dose of Leu administration in plasma glucose concentration in Experiment 4. In Experiment 5, the hepatic glycogen contents was higher in embryos administered Leu than control at 60 minute after treatment ($P<0.05$), and PEPCK activity increased with advancing time after Leu administration like as the result when embryos were administered insulin ($P<0.05$). Chicken embryo might maintain plasma glucose high level, and Leu might have action like as insulin.

In Experiment 6, growth of broilers was increased relatively control birds by glucose *in ovo* administration with Leu on 28 days of age ($P<0.05$). Thus gluconeogenesis during embryonic period might involve in subsequent growth of broilers.

In Experiment 7, the pectoral muscle, liver and pancreas, main glucose metabolize tissues, have expressions of GLUT 1, 2, 3, 8, 10, and 12 mRNA in both age. All of GLUT 1, 2, 3, 8, 10, and 12 mRNA expressions were recognized in all tissues studied in Experiment 8. In Experiment 9 studied the changes of GLUT mRNA expression after 5 minute of insulin administration, both the pancreas GLUT 2 and pectoral muscle GLUT 3 mRNA expressions were decreased in chicken embryos ($P<0.05$). Because glucose affinity of the spleen is low and GLUT 2 mRNA expression involved in insulin secretion decreased by insulin administration, plasma glucose of chicken embryo might be upper regulated. In Experiment 10 studied shorter period after insulin administration, GLUT 3, 8, and 12 mRNA expression tended to decrease after treatment.

These result suggested that embryonic plasma glucose level have important role in subsequent growth, and GLUT 3, 8, and 12 might involve in plasma glucose regulation and respond to insulin in broilers.

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Studies on mechanisms involved in hypotensive activity of protein hydrolysates in spontaneously hypertensive rats (SHR)

Yuki KOKUBUN*

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

(Conferred on 13 March 2014, ALS-56)

Introduction

We have previously shown that oral administration of enzymatic hydrolysates of chicken leg collagen reduces blood pressure in animal models of hypertension and in humans with a high-normal pressure or mild hypertension. However, the effects of hydrolysates derived from other livestock products on the reduction of blood pressure and maintenance of vascular integrity in various pathological animal models (e.g., stroke and obesity) have not yet been studied.

In this study, we assessed the effect of protein hydrolysates from different origins in hypertensive rats, and the effects of chicken collagen hydrolysates (CCH) in hypertensive rats with stroke or obesity.

Materials and methods

1. Sample preparation and experimental animals

Hydrolysates were prepared by treating animal proteins (chicken leg collagen, pig skin collagen, and bovine casein) with yeast-derived enzymes at 50 ℃ for 24 h. Seven-week-old male spontaneously hypertensive rats (SHR) were obtained from the Disease Model Cooperative Research Association and were used as test animals. Each rat received tap water (control group) or hydrolysates (experimental group: 1 g/kg body weight) orally for 7 weeks. In the experiment involving various pathological rats models, 7-week-old male normotensive rats (WKY rats), stroke-prone spontaneously hypertensive rats (SHRSP), and obese hypertensive rats (SHR/NDmcr-cp rats) were used in addition to SHR mentioned above, and each rat received CCH (1 g/kg body weight) orally for 7 weeks. Blood pressure was measured by the tail-cuff method. After the study period, the rats were subjected to laparotomy under ether anesthesia to collect blood samples from the vena cava and other tissues.

2. Assessment of endothelial progenitor cell (EPC) proliferation activity

CD34-positive cells (EPCs) were isolated from cell fractions containing monocytes using magnetic beads. The cells were cultured in a methylcellulose medium containing rat serum, and the resulting colonies were counted. For statistical analysis, we used Student's t-test to compare each experimental group with the control group.

3. Histopathological examination of blood vessels

Blood vessels collected after the study period were fixed with formalin, and tissue sections were prepared. Hematoxylin-eosin Hematoxylin-eosin-stained and Elastic-Van Gieson-stained specimens were examined and pathological features such as fragmentation of elastic fiber were observed.

4. Examination of proteins involved in blood pressure control and blood vessel homeostasis

Real-time PCR was used to quantify the mRNA expression levels of the following proteins: angiotensinogen (AGN), renin, angiotensin-converting enzyme, endothelial nitric oxide synthase (eNOS), and vascular endothelial growth factor (VEGF). The expression level of each protein was also confirmed by western blotting.

*Supervisor : Prof. Toshihide NISHIMURA

Results and discussion

1. Effects on vascular cells (EPCs) by serum samples from SHRs that received protein hydrolysates of different origins

The culture medium of the recovered EPCs was supplemented with serum samples from SHRs that received the protein hydrolysates and as a result, the number of colonies formed in the presence of the serum from the CCH group was significantly increased (1.4-fold) than that in the presence of the control serum. The serum samples from the pig collagen and bovine casein hydrolysate groups also increased the number of colonies by approximately 1.2-fold. In the SHRs that received the hydrolysates, the expression of VEGF mRNA in the kidneys significantly increased. This result suggests that VEGF activates EPC proliferation and contributes to blood vessel homeostasis.

2. Effects of CCH ingestion on various pathological rat models

CCH was administered to the WKY, SHR, SHRSP, and SHR/NDmcr-cp groups, and blood pressure was measured. The elevated blood pressure was significantly reduced in SHRs after 1 ～ 6 weeks of CCH administration. No difference was observed between the control group and either SPSH or SHR/NDmcr-cp groups. Histopathological examinations of blood vessels did not show any difference between the control and CCH groups in any pathological models tested.

Next, we analyzed mRNA levels of the proteins involved in the elevation of blood pressure. Compared with the control group, AGN mRNA levels significantly decreased to approximately 60 % in CCH-treated SHR, SHRSP, and SHR/NDmcr-cp groups. However, there was no difference in protein levels of AGN.

We also examined mRNA levels of eNOS, which is involved in the dilatation and homeostasis of blood vessels. Compared with the control group, eNOS mRNA levels were significantly reduced in the WKY, SHR, and SHRSP groups that received CCH. No differences were observed in eNOS protein levels. Phosphorylation of eNOS leads to the production of nitric oxide, which regulates vasodilatation as well as homeostasis of the vascular endothelial cells. Accordingly, we also quantified the level of phosphorylated eNOS. In SHRs that received CCH, the level of phosphorylated eNOS was significantly higher compared with that of the control group. This finding may be attributed to the reduction of the elevated blood pressure in hypertensive rats after the CCH treatment inhibition of angiotensin-converting enzyme. In this study, we examined the involvement of eNOS phosphorylation in vasodilatation.

According to the above results, we found that CCH did not reduce blood pressure in the rat models of stroke or obesity but significantly reduced blood pressure in SHRs. Our data thus suggest that the mechanism involves eNOS phosphorylation and EPC activation, which repair blood vessels.

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Studies on the effect of low-molecular compounds in "dashi" on its flavor

Shingo GOTO*

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

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Introduction

Free amino acids, sugars, organic acids, and other low-molecular compounds contained in materials are extracted from dashi (Japanese soup stock usually made from bonito flakes and kelp). We found that, when dashi is prepared, the glutamine (Gln) content decreases, and the free amino acid content increases. This effect is because of the conversion of Gln in an aqueous solution to pyroglutamic acid (5-pyrrolidone-2-carboxylic acid, PCA) through heating. However, we discovered that, in addition to PCA, another compound is also produced.

In this research, we examined the change that occurs to the low-molecular compounds in dashi on heating, and analyzed the structure of the unidentified compound produced by heating. We also examined the characteristics of this compound. In addition, we examined the effect of the low-molecular flavor compounds in dashi on the sensory strength of aroma.

Materials and method

1. Changes accompanying the heating of low-molecular compounds in dashi

We used 1 mM aqueous solutions of Gln, (pH6.8) heated to different temperatures, and western-style chicken stock. Low-molecular compounds were measured through high-performance liquid chromatography (HPLC) by using an aqueous solution (pH2.3) of 5 mM hexasulfonic acid and 20 mM phosphoric acid as the mobile phase, a PEGASIL ODS SP100 column with a column temperature of 40℃, and a detection wavelength of 210 nm.

2. Structural analysis of the unidentified compound

A 1 mM aqueous solution of Gln (pH6.8), heated for 5 h at 95℃, was directly injected into a mass spectrometer (MS: Micromass Q-Tof Premier, Waters USA), and the molecular weight of the Gln was measured. An MS/MS analysis of the peaks of the unidentified compound was performed using the fragment ions, and the structure was subsequently estimated. Then, the structure of the purified unidentified compound was analyzed using ^{1}H-NMR (AVANCE 600 NMR spectrometer from Bruker BioSpin Co.).

3. Influence of the unidentified compound on flavor and aroma

The unidentified compound was added to the basic flavor solution or dashi at a concentration of 0.003 %, and the influence of the unidentified compound on flavor and aroma was examined by sensory evaluation. "Flavor reconstituted dashi" was prepared as chicken stock used for sensory evaluation through previous reports by Fujimura et al. and Hofmann et al., and a meat soup stock fragrance composition (provided by Ajinomoto Co., Inc.) was subsequently mixed into the chicken stock to obtain the "reconstituted dashi" used in the evaluation.

4. Influence of the Low-Molecular Flavor Compounds in the Dashi on the Sensory Strength of the Aroma

A sensory evaluation of the chicken stock for aroma similar to "reconstituted dashi" was conducted using trained panelists. More specifically, the evaluation was conducted on a solution obtained by removing the low-molecular flavor compounds from the aforementioned "reconstituted dashi" (omission test: OT) or addition to

*Supervisor : Prof. Toshihide NISHIMURA

it (addition test: AT). In the OT, a solution from which the low-molecular flavor compounds had been removed was evaluated on a scale of 1 ("flavor reconstituted dashi") to 5 points ("reconstituted dashi"). In the AT, a solution to which low-molecular flavor compounds had been added was evaluated on the scale of 1 point ("solution obtained by adding meat soup stock flavoring to a 0.4 % NaCl solution") and 5 points ("reconstituted dashi").

Results and discussion

1. Changes accompanying the heating of low-molecular compounds in dashi

When the Gln aqueous solution was heated, the concentrations of PCA and an unidentified compound increased depend on heating time and temperature. Moreover, with the western-style chicken stock, the quantity of the unidentified compound increased to cooking time and a temperature -dependent manner.

2. Structural analysis of the unidentified compound

The mass spectrum of the heated substance of the Gln aqueous solution contained peaks at m/z 129 and m/z 281, which we presumed to be derived from a compound other than Gln or PCA. MS/MS analysis was conducted on the m/z 281 peak; consequently, a m/z 258 peak indicating decreased Na+ ion content was detected. The fact that these m/z ratios do not match the molecular weight of Gln or PCA suggested the presence of an unidentified compound. A structural analysis of the unidentified compound was then conducted using ^{1}H-NMR. As the results, we presumed that the m/z 129 peak was 5-amino-3,4-dihydro-2H-pyrrole-2(S)-carboxylic acid, which is a heterocyclic compound with a molecular weight of 128. This product was subsequently synthesized and analyzed by ^{1}H-NMR, and the chemical shift of the synthesized compound perfectly matched that of the unidentified compound. When a mass analysis of the m/z 129 peak of the synthesized compound was conducted, ion clustering was detected; we thus concluded that the m/z 258 peak was detected as the mass of two molecules. Although the synthesized product was added to the basic flavor solution or dashi, it did not influence the flavor.

3. Impact of the low-molecular flavor compounds in the dashi on the sensory strength of the aroma

An OT was conducted to clarify the effect of the low-molecular flavor compounds in the chicken stock on the sensory strength of the aroma. Of the 28 types of low-molecular flavor compounds, 14 were observed to affect the sensory strength of the aroma. The effects of glutamic acid (Glu) and inosine monophosphate (IMP) were particularly large. Next, we conducted an AT by adding the 14 components to the chicken stock; consequently, a score as high as 4.55 was reproduced. However, the influence of these components on the aroma was not confirmed by the independent addition of one component at a time. With the addition of 6 components (Glu, IMP, phosphoric acid, potassium, magnesium, and AMP), the aroma sensory strength as that achieved with the addition of all 14 components, was obtained. Based on these results, we concluded that the 6 aforementioned components contribute significantly to the sensory strength of the meat soup stock aroma.

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Influence of types of the solute on flavor release from aqueous solution

Yurie SASAKI*

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

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Flavor is one of the important factors to determine the good taste of food. Food always releases flavor, and humans perceive the difference in the amount of flavor release as a difference in the intensity of flavor. If the flavor intensity changes, the impression of food changes naturally. Therefore, the study of flavor release is important to pursue a good taste. In this study, in order to find the flavor release phenomenon from an aqueous solution including flavor ingredients, we examined the change of the amount of flavor release from an aqueous solution which added the solute as a third ingredient other than water, and the flavor ingredient by types of solute or the difference of the functional group and carbon number. In addition, some things generally included in food (carbon dioxide, salt and sucrose) were used as the solute of the third ingredient.

Materials and methods

1) Carbon dioxide solution

Volatile compounds (n-alkane, 1-alcoh-ol, 1-aldehyde, 2-ketone and acetic ester compounds, and L-menthol, citral, 2-pen-tanone and isoamyl acetate) were added at 10 ～ 100 ppm (1.6×10^{-5} ～ 1.0×10^{-3} M) to a carbon dioxide aqueous solution which was prepared at 0.72 ～ 3.30 gas volume (0.03 ～ 0.15 M). The gas volume was the range from distilled water under atmosphere pressure to the strongest in commercial carbonated drinks. The sample solution (10 mL) was afterwards poured into vials (20 mL), and the amount of flavor release was analyzed by GC/FID connected to headspace automatic sampler (thermal insulation: 40 ℃, injection time: 6 seconds, needle temperature: 100 ℃). Analytical conditions were as follows: Column: DB-WAX (30 mm × 0.25 mm i.d. × 0.25 μm), oven temperature: 40 ℃ (held for 4 min) to 200 ℃ at 8 ℃ /min.

For sensory evaluation, a sample solution to which volatile compounds (L-menthol, citral, 2-pentanone and isoa-myl acetate) having each characteristic flavor were added at 2.5 ～ 10 ppm (7.7×10^{-5} ～ 1.2×10^{-4} M) was prepared. Test panelists swallowed a sample solution and kept it in their mouth for 6 seconds, and then evaluated the perceived flavor intensity on 13-point scale from −3 to +3 at a 0.5 division with a sample of dissolved volatiles in distilled water being 0 as the standard.

2) Sodium chloride solution

Volatile compounds (n-alkane, 1-alcoh-ol, 1-aldehyde, 2-ketone and acetic ester compounds) were added at 100 ～ 500 ppm (6.3×10^{-4} ～ 1.0×10^{-3} M) to a sodium chloride aqueous solution which was prepared at 0 ～ 1 M (0 ～ 58.4 %). The amount of flavor release was analyzed with the same methods as chapter 1.

3) Sucrose solution

Volatile compounds (n-alkane, 1-alcoh-ol, 1-aldehyde, 2-ketone and acetic ester compounds) were added at 100 ～ 500 ppm (6.3×10^{-4} ～ 1.0×10^{-3} M) to a sucrose aqueous solution which was prepared at 0～60 %(w/w) (0～2.25 M). The amount of flavor release was analyzed with the same methods as chapter 1.

For sensory evaluation, the sample solution to which volatile compounds (L-menthol, citral, 2-pentanone and isoa-myl acetate) having each characteristic flavor were added at 2.5 ～ 20 ppm (6.3×10^{-4} ～ 1.0×10^{-3} M) was prepared. Test panelists spit out the sample solution after keeping it in their mouths for 6 seconds, and then evaluated the perceived flavor intensity on 9-point scale from −2 to +2 at a 0.5 division with a sample of dissolved volatiles in distilled water being 0 as the

*Supervisor : Prof. Sachiko ODAKE

standard.

Results and discussion

In the measurement of flavor release by GC/FID on a carbon dioxide solution, although the carbon dioxide concentration increased, the amount of flavor release of volatiles with the lower carbon number in alkane compounds did not statistically change. However, the amount of flavor release of volatiles with the higher carbon number significantly increased with the increase in carbon dioxide concentration. As for alcohol, aldehyde, ketone and ester compounds, the amount of flavor release of volatiles with the lower carbon number significantly increased with the increase in carbon dioxide concentration, but that of volatiles with the higher carbon number did not statistically change or tended to decrease. Moreover, as to the compounds used for sensory evaluation, the amount of flavor release and the perceived flavor intensity of all volatiles significantly increased with the increase in carbon dioxide concentration.

In the measurement of flavor release by GC/FID on sodium chloride solution, the amount of flavor release in alkane compounds did not statistically change although sodium chloride concentration increased. In contrast, as for alcohol, aldehyde, ketone and ester compounds, the release amount of volatiles with the lower carbon number significantly increased with the increase in sodium chloride concentration, and that of volatiles with the higher carbon number did not change.

In the measurement of flavor release by GC/FID on sucrose solution, the amount of flavor release in alkane compounds significantly decreased with the increase in sucrose concentration. However, the release amount of volatiles with the lower carbon number in alcohol, ketone and ester compounds significantly increased with the increase in sucrose concentration. The release amount in aldehyde compounds decreased with the increase in sucrose concentration. As for the compounds used for sensory evaluation, the amount of flavor release of all volatiles significantly increased with the increase in sucrose concentration. The perceived flavor intensity increased when sucrose concentration was low, but tended to decrease when it was 60 %.

These results suggest that the amount of flavor release was influenced by the difference in solute or carbon number of flavor compounds. It is thought that the compounds which increased the amount of flavor release results in a "salting out" effect. It is thought that the compounds which decreased the amount of flavor release interact with the solute and flavor compounds to inhibit the release. Furthermore, the significant perceived flavor intensity decreased in a highly-concentrated sucrose solution can be caused by a masking effect due to the sweetness.

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Studies on the effect of heat-concentrated onion juice on "koku" sensation

Akira NAGAO*

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

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Introduction

Onions are used in many different cuisines, and it is known that the flavor can be enhanced and certain "richness" can be imparted by adding grated or sautéed onions to various recipes. To date, *S*-propenyl cysteine sulfoxide has been reported as the compound that imparts the "richness" of onions. In addition, a phenomenon is known by which sulfides, furans, pyrazines, and other aromatic components are produced and impart "richness" when an onion concentrate is heated. However, details regarding the "richness" effect achieved through the aroma have not been examined.

In this research, we examined the sustainability of aroma present in heated onion concentrate and analyzed the "richness" imparting effect of the aroma. Moreover, we investigated for new "richness" imparting substances.

Materials and methods

1. Preparing heated onion concentrate

After the juice of onions was concentrated to Brix 70 %, it was heated to 160 ℃ for 1 hour. The resulting product was used as the heated onion concentrate (heated concentrate). In addition, onion supernatant concentrate from which the solid content was removed prior to heating was heated in a similar manner and concentrated to obtain a supernatant heated concentrate. The heated concentrate was then separated by centrifugation; the deposited fraction was used as the "solid content," and the supernatant fraction was used as the "supernatant" sample. The sample was added to consommé soup at a ratio of 1.23 g/100 mL.

2. Sensory evaluation of aroma sustainability

The "heated concentrate" and the "supernatant heated concentrate" were added to the consommé soup, and the aroma sustainability of each was compared through a score-based sensory evaluation. In addition, sensory and aroma sustainability evaluations were performed on the "solid content" and "supernatant" fractionated from the "heated concentrate." The evaluations were performed by a group of 10 ～ 12 trained panelists capable of recognizing aroma sustainability.

3. Measuring Aroma Discharge

A "washed solid content" obtained by washing the solid content with hot water and ethanol was used to measure the aroma retention effect due to the onion solid content. Methylpropyl disulfide, furfural, and 2,6-dimethylpyrazine (hereafter, sulfide, furan, and pyrazine) were each added to the washed solid content, and the amount of the aroma components discharged from the mixture at 40 ℃ was measured using headspace -gas chromatography (HS-GC). The same composition was then heated at 90 ℃ for 4.5 hours in an open system, and the amount of aroma components discharged from the mixture was examined using HS-GC. Furthermore, β-sitosterol and stigmasterol were added as aroma components to the "washed solid content" and heated for 4.5 hours at 90 ℃ . Finally, the amounts of discharged aroma components were measured.

4. Structural analysis of the onion solid content

The thermal decomposition of the "washed solid content" was implemented at 610 ℃ , and the decomposition product was analyzed with gas chromatography/ mass spectrometry (GC/MS: GCMS-QP2010 Ultra, SHIMADZU Co.) using a UA-5 column to deduce

*Supervisor : Prof. Toshihide NISHIMURA

the structure. The original samples of the detected components were analyzed in a similar manner, and the components present in the washed solid content were identified.

Results and discussion

1. Impact of heated onion concentrate on aroma sustainability

When heated onion concentrate was added to consommé soup, the aroma sustainability was increased by 1.4 times when compared with the addition of supernatant heated concentrate. The aroma sustainability obtained by adding solid content fractionated from the heated concentrate exhibited values that were 1.5 times or higher than the values obtained by adding the supernatant.

2. Aroma retention effect by onion solid content

The mixture obtained by adding sulfide to the washed onion solid content was heated at 40 ℃, and the amounts of discharged aroma components were measured. The amount of sulfide discharged from the mixture from the second hour of heating and onward was approximately double when compared with the case in which no washed solid content was added. Moreover, when an aqueous solution with added sulfide was heated to 90 ℃, sulfide was not detected. However, when the washed solid content was added to the aqueous solution, sulfide was detected. From these results, it is clear that the onion solid content has an aroma retention effect.

3. Structural analysis of onion solid content

From the results obtained by thermally decomposing the onion solid content and analyzing it with GC/MS, β-sitosterol with a molecular weight of 416.7 and stigmasterol with a molecular weight of 412.7 were detected as decomposition products. The original samples of these products were analyzed in the same manner, and the same mass spectrums were obtained. Therefore, it is clear that these phytosterols are present in the solid content derived from the heated onion concentrate.

4. Physical property of aroma retention by phytosterols

The aroma component retention effect was examined using phytosterols. Sulfide was retained by both β-sitosterol and stigmasterol. In addition, the sulfide retention effect of β-sitosterol exhibited a value that was eight or more times higher than that of stigmasterol.

Similarly, the retention effect was examined when furan or pyrazine was added to phytosterol and heated at 90 ℃, and a difference in the discharge of the aroma components of furan and pyrazine was not recognized between the addition and exclusion of a phytosterol.

From these results, it is clear that the sulfide retention effect from phytosterol (primarily β-sitosterol) contributes to the "richness" imparting effect because of the aroma sustainability of heated onion concentrate.

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Studies on method to estimate amino acid requirement for raptors

Etsuko HASEGAWA*

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

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Many species of the carnivorous birds, namely raptors, are regarded as endangered or near threatened species. Half of wild raptors stored to protect have been dead by the reason why they did not receive the enough nutritional condition to keep them. On the other hand, although it is necessary to keep endangered Japanese endemic species under artificial preservation, those were fed by the methods based on experience of keepers. When the raptors were admitted, because earlier nutritional treatment is essential, easily method to estimate the macro nutritional requirements, like as weighing body weight (BW), is required. While energy requirement for maintenance was able to estimate by the BW using metabolic body size ($BW^{0.75}$), protein metabolism differs among the feeding habits. Therefore, Protein and amino acid requirement have to estimate experimentally for each feeding habit.

However, usually raptors are weak from stress, and stresses affect nutrient requirements, hence the conventional methods to estimate protein and/or amino acid requirements using criterion like as plasma metabolic substance, hepatic enzyme activity, abdominal fat pad and more which collected by bleeding or scarification are not available. In addition, the method for protein and amino acid requirements using the taurine excretion as a criterion is not available too for carnivorous animals

Therefore, the present study was conducted to develop a new method of protein and amino acid requirements for raptors using urinary creatinine which is excreted into as a final product of amino acid regardless of feeding habit.

In Experiments 1 and 2, thirty 8 day-old chunky strain broiler chicks were divided into 3 groups with 10 chicks (each 5 female and male), housed in the electro heated box individually, and fed a three levels of methionine diets (0.25%, 0.50%, and 0.75%) from deficient to excessive level for 7 days. At last 3 days of feeding trial, excreta were collected to analyze creatinine level in Experiment 1. In experiment 2, at the end of feeding trial, chicks were killed by dislocated and the livers were collected to determine arginine glycine amidinotransferase (AGAT) activity.

In Experiments 3 and 4, in order to study dose response to dietary methionine, 3 and 4 adult Eurasian scops owls (*Otus scops*) were allocated to 3 and 4 dietary methionine levels x 3 and 4 periods recommended by Latin square experimental design, respectively. Each period was consisted of the acclimatizing 3 days and the experimental 4 days, and excreta was collected for last 24 hr. Experimental diets used were neonatal Eurasian field voles (*Microtus arvalis*) containing capsule of crystalline amino acids mixture in abdomen. Total dietary methionine levels were 0.22%, 0.47% and 0.72%, and 0.22%, 0.35%, 0.60%, and 0.72% in Experiments 3 and 4, respectively.

Ccreatinine in samples were extracted by conventional method, and creatinine concentrations in excreta were determined by commercial assay kit (Cayman Chemical Company, Michigan, USA). Statistical significance was determined by one-way ANOVA in Experiments 1 and 2, and 3-way ANOVA in Experiments 3 and 4. In Experiment 4, regression exponential equation was employed as follows;

$Y=(A-BX)(1-e^{-1(X+D)})$ ---------- (Toyomizu *et al.*, 1988).

Methionine requirement was estimated at 95% and 100% maximum creatinin excretion.

In Experiment 1, the dose response of creatinine excretion to dietary methionine levels was compared with performance of broiler chicks. Body weight gain

*Supervisor : Prof. Yoshiyuki OHTA

was increased with increasing dietary methionine from 0.25% to 0.50% ($p<0.05$), and then remained constant. Creatinine excretion was the similar response as body weight gain ($p<0.05$).

Experiment 2 was conducted to explain the response of creatinine excretion to dietary methionine levels by the enzymatic method. The hepatic AGAT activity was decreased with increasing dietary methionine levels from 0.25% to 0.50%, and then remained constant. It might suggest that creatinine excreta reflected amino acids metabolism, therefore creatinine excreta would be criterion to estimate amino acid requirement.

In order to make sure the response of creatinine excretion to methionine in raptors, Experiment 3 was done the same experiment as Experiment 1 using scops owls. Change of creatinine excretion of owls showed the similar response of broilers ($P<0.05$). The facts suggest that creatinine excretion will be criterion for estimation of amino acid requirement for carnivorous birds.

At last, methionine requirement was estimated by the method using creatinine excretion as a criterion in experiment 4. Creatinine excretion increased with increasing dietary methionine levels to 0.60%, and then decreased at 0.72% ($p<0.05$). Regression coefficients of fitted curve were A=−51.3, B=−67.3, C=−0.513, and D=−0.192 (R2=0.99), and dietary methionine requirement was estimated as 0.423% ～ 0.582%.

Above facts indicated that amino acid requirements might be able to estimate experimentally in carnivore avian by the method developed in this study using creatinine excretion as a criterion.

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Evaluation of an experimental model of *Corynebacterium ulcerans* in mice

Yu MOCHIZUKI*

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

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Introduction

Corynebacterium ulcerans is a widely distributed species, and toxigenic strains can produce diphtheria-like toxin, which have the same toxic activity as that of the diphtheria toxin. This pathogen is able to induce an infection, which is characterized by inflammation and the formation of a pseudomembrane in the respiratory tract, similar to the diphtheria infection in humans. Moreover, *C. ulcerans* can cause zoonotic infections in a broad range of animals, whereas *C. diphtheriae* causes epidemics in humans. However, little has been documented about the infection route and clearance of this pathogen in animals. There is no definite regulation in domestic law despite this infection becoming increasingly prevalent in Japan. The purpose of this study was to develop an experimental model of the *C. ulcerans* infection using mice and analyze the pathology.

Materials and methods

Animal experiments were approved by the Committee on Experimental Animals, National Institute of Infectious Diseases, Japan (authorization number 113002). *C. ulcerans* 0102 strain, a toxigenic strain, obtained from a clinical isolate from the first human case in Japan was used. Strains of *C. ulcerans* ATCC 51799, *C. pseudotuberculosis* ATCC 19410, *C. glutamicum* ATCC 13032, and *C. diphtheriae* PW8 were used as controls. Each bacterial suspension was prepared from washed bacterial cultures after shaking for 72 h at 37 ℃ under aerobic conditions. Six-week-old female BALB/cCrSlc mice were infected intranasally with 50 µl of each suspension (dose range from 10^6 to 10^8 CFU per mouse). The body weight and body temperature of mice were measured, and samples from mice were sequentially collected up to 14 days after infection. The weight of each organ was measured and histopathology was performed. Quantitative and qualitative microbial colony counts were performed in the lung, liver, kidney, spleen, blood, cecal contents, rectal feces, and swab (nasal, oral, tracheal and eye) samples. Bacterial colonies were tested by polymerase chain reaction assay and confirmed using the API Coryne system (bioMérieux SA). Diphtheria antitoxin titer and cytokine (interleukin-6, interleukin-1β, tumor necrosis factor-α and interferon-γ) concentrations in the serum samples were measured by micro cell culture method using Vero cells and commercially available ELISA kits.

Results

All mice infected with *C. ulcerans* 0102 at a concentration over 10^7 CFU died because of serial weight loss up to 4 days after infection. In contrast, half of the mice infected with the 10^6 CFU concentration died at 14 days after infection. According to the histopathology results, *C. ulcerans* 0102 was extensively distributed throughout the organs and caused the formation of necrotic nodular lesions and hyaline membranes in the lung, swelling in the spleen, and necrosis and abscess formation in the kidneys of the mice *in vivo*. In mice infected with the 10^6 CFU concentration, kinetics of cytokine production, change in weight, and the formation of lesions in the organs were observed in association with the distribution of *C. ulcerans* 0102. However, diphtheria antitoxin titer in the serum samples and *C. ulcerans* 0102 in cecal contents and rectal feces were undetectable.

*Supervisor : Prof. Hiromi AMAO

Discussion

C. ulcerans 0102 exhibited lethal pathogenicity after intranasal infection in mice. Furthermore, infection with the 10^6 concentration CFU is the 50 % lethal dose for mice. This strain extensively distributed itself, multiplied, and caused necrosis particularly in the lungs and kidneys. This provides evidence that *C. ulcerans* is localized to these organs and has the ability to form lesions. Lesion formation and cytokine production in mice showed the same trend as that observed in previous reports of animals and experimental models infected with *C. diphtheriae* and *C. pseudotuberculosis*, which are allied bacteria of *C. ulcerans*. However, *C. ulcerans* did not show distribution within the colon, unlike *C. kutscheri*, which is an allied bacterium of *C. ulcerans* and infects mice.

Conclusion

In this study, we have produced for the first time a lethal nasal infection mouse model using a toxigenic *C. ulcerans* strain obtained from human clinical isolates. Moreover, the distribution of the organism in mice has been characterized. In the future, we expect to contribute to the elucidation of the underlying mechanism of the *C. ulcerans* pathology through the use of this in vivo experimental model.