Bull. Nippon Vet. Life Sci. Univ., No. 60, 147–149, 2011.

Clinical development of BMP-2-based regenerative therapy for the non-union cases

Takamasa ITOI*

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

(Received 11 March 2011, VDK-No. 141)

Fracture is common in small animal orthopedic surgery, and nonunion is one of the most severe complications following fracture. Currently in veterinary medicine, the general treatment for nonunion involves autogenous and allogeneic bone grafts. However, the development of new regeneration therapies as substitutes for bone grafting is desired, because the amount of autologous bone and the route is for obtaining allogeneic bone are limited.

In recent years, studies on bone regeneration have been conducted in various fields. In particular, bone morphogenetic protein (BMP) has been examined as a growth factor that regulates cell growth and differentiation. Recombinant technology has been developed that purifies recombinant human BMP-2 (rhBMP-2) from Chinese hamster ovary (CHO). Various researchers have reported that CHO-derived rhBMP-2 (CHO-BMP-2) enabled bone regeneration in critically sized bone defects in a variety of animal experiments. CHO-BMP-2 has already been utilized clinically ; however, a one of the problem is that the cost of using CHO-BMP-2 is quite high due to the limited yields obtained from mammalian cells. One possible way of solving this problem is by the high-efficiency production of rhBMP-2 in *Escherichia coli* by *ex vivo* biochemical processing. *E. coli*-derived rhBMP-2 (*E*-BMP-2) has been expected to be an inexpensive supply of drugs because it can be produced with high efficiency compared with CHO-BMP-2 production. In the past, it was reported that osteoinduction of *E*-BMP-2 was equivalent to osteoinduction of CHO-BMP-2 in animal experiments using mice and rats. However, no studies have reported the effect of *E*-BMP-2 in dogs.

The purposes of this study were to determine the ideal amount of medication and most appropriate usage method, and to determine the most efficient manner of bone regeneration using simultaneous transplantation of cellular components.

1. Studies on the effect of *E*-BMP-2 on bone differentiation of bone marrow-derived mesenchymal stem cells in the dog

CHO-BMP-2 has been used as a drug for bone regeneration therapy in veterinary medicine. It is reported that *E*-BMP-2 promotes the differentiation of bone in culture experiments using mouse bone marrow-derived mesenchymal stem cells (BMSCs). However, no studies have reported the effect of *E*-BMP-2 in dogs.

The purpose of this study was to examine the effects of *E*-BMP-2 in the dog. This study ex amined cell proliferation and alkaline phosphatase (ALP) activity related to bone differentiation in canine BMSCs that were treated with *E*-BMP-2, dexamethasone, and commercially available bone differentiation medium. The results of this study showed that cell number and ALP activity were significantly increased

* Supervisor : Prof. Masahiro TAGAWA

compared with those of the control group when *E*-BMP-2 was added to the culture medium. In particular, ALP activity in the *E*-BMP-2 group was significantly higher than that in the control, dexamethasone, and commercial osteoinductive medium groups. Thus, these results suggest that *E*-BMP-2 shows greater osteoinductivity compared with dexamethasone and commercially available bone differentiation medium. Additionally, because ALP activity showed a significantly higher dose response, an *E*-BMP-2 dose-dependent effect was observed.

2. Studies on the regeneration of long bone using *E*-BMP-2 in the dog

The osteoinduction of *E*-BMP-2 that has been developed in recent years was reportedly equivalent to osteoinduction of CHO-BMP-2 in animal experiments using mice and rats, and there is great optimism regarding its therapeutic usefulness. However, there have been no reports thus far regarding fractures in dogs.

In this section, we evaluated the healing efficacy of *E*-BMP-2 to determine the necessary amount of medicine in a canine model. A 2.5-cm critically sized segmental ulnar defect was created, and *E*-BMP-2 was implanted in it with 700 mg of artificial bone (*β*-tricalcium phosphate ; *β*-TCP). Based on the amount of *E*-BMP-2 (0, 35, 140, 560, and 2240 *μ*g), 5 experimental groups were created (control, BMP35, BMP140, BMP560, and BMP2240). The high-concentration *E*-BMP-2 groups (BMP2240 and BMP560) developed large calluses around the implanted regions and fusion with host bone (ulna). On the other hand, the low-concentration *E*-BMP-2 groups (BMP140 and BMP35) did not develop a sufficient increase in bone or union with host bone (ulna). Furthermore, it was observed that the number of vacuolar structures (voids) increased depending on the *E*-BMP-2 dose.

The results of this study suggest that *β*-TCP with *E*-BMP-2 at concentrations above 280 *μ*g/cm^3 (BMP560) promotes bone regeneration in animal experiments, especially in dogs, and shows a sufficient bone regeneration effect.

3. Studies on bone regeneration using a *E*-BMP-2/BMSCs/*β*-TCP complex in the dog

CHO-BMP-2 is currently used in veterinary medicine for treating canines with nonunion arthrodesis and mandibular reconstruction. On the other hand, previous studies have suggested that higher doses of CHO-BMP-2 form vacuolar structures (voids) in the regenerated bone tissue. We also observed transient decreases in bone mineral density, which may have been related to the formation of voids in the regeneration process in the higher-dose *E*-BMP-2 group.

In this section, we hypothesized that a combination of BMSCs would improve nonuniformity of internal structures during the bone regeneration process by higher-dose *E*-BMP-2 transplantation, and examined the synergistic effect of the transplantation of a *E*-BMP-2/BMSCs/*β*-TCP complex to 2.5-cm critically sized segmental ulnar defects in the canine. The regeneration bone width was maximum of 1.4 times greater compared with *E*-BMP-2 alone by simultaneous transplantation of *E*-BMP-2 and BMSCs. In addition, the simultaneous transplantation of *E*-BMP-2 and BMSCs inhibited the incidences of void suppression and uniform internal structure.

The results of this study suggest that bone regeneration was further accelerated by transplantation of an *E*-BMP-2/*β*-TCP complex with BMSCs. When performing rhBMP-2 transplantation with cellular components in clinical cases, the formation of high-quality regenerated bone is very important.

4. Studies on the comparison of bone regeneration ability using *E*-BMP-2 and BMSCs (bioartificial bone)

"Bioartificial bone," which involves the attachment of stem cells, including BMSCs, to artificial bone, has been developed and has shown a high bone regenerative effect. However, problems in the clinical application of BMSCs include increased cell culture process and contamination risks. On the other hand, transplantation of biomaterials impregnated with growth factors, including *E*-BMP-2, has been considered to be

clinically useful because there is no risk of cell culture.

In this section, a defect with a 20-mm segmental radius was created, and the effectiveness of a bone regeneration treatment using *E*-BMP-2 and that of a bone regeneration treatment using bioartificial bone were compared. The osteogenic potential and therapeutic efficacy in the BMP group were significantly higher than those in the bioartificial group. Furthermore, the results of this study suggest that the bone regenerative effect of *E*-BMP-2 can be further enhanced by the combined transplantation of bone marrow cells.

Overall, the results of this study suggest that bone regeneration treatment using *E*-BMP-2 shows a higher bone regenerative potential compared with that of bioartificial bone treatment, and that this regenerative potential can be accelerated by transplanting *E*-BMP-2 within bone marrow cells.

5. Clinical application of *E*-BMP-2 for nonunion in the dog and cat

Fractures are common in small animal orthopedic surgery. The number of fracture cases has recently increased in small breed dogs, and in accordance with this, nonunion cases have also tended to increase. There have been numerous radial-ulnar fracture cases in small breed dogs, most of them nonunion cases, at the Nippon Veterinary and Life Science University Veterinary Medical Teaching Hospital.

In this section, we performed bone regeneration treatments using *E*-BMP-2 derived from the results of previous sections for severe nonunion cases in the Nippon Veterinary Medical Teaching Hospital.

Cases 1 and 2 (both severe nonunion cases with bone loss) showed good bone regeneration and no complications or side effects.

Thus, the results suggest that bone regeneration treatment using *E*-BMP-2 is available as a new bone regeneration treatment that substitutes for bone grafting in animal cases.

These results suggest that *E*-BMP-2 demonstrates the ability to promote bone regeneration in large bone defects by differentiating undifferentiated cells into osteoblasts in the dog. Additionally, it inhibits the incidence of void suppression and uniform internal structure, and accelerates bone formation by transplantation with BMSCs. Furthermore, because the simultaneous transplantation of bone marrow cells (without cell culture) also enables further enhancement of the regenerative potential of bone, bone regeneration treatment using *E*-BMP-2 may have a high degree of availability for clinical application compared with bioartificial bone treatment. In addition, the bone regeneration treatment using *E*-BMP-2 was sufficiently effective and showed no clinical complications.

In summary, a bone regeneration treatment as a substitute for bone grafting was developed. Because this bone regeneration treatment using *E*-BMP-2 contributed to an improved therapeutic effect and shortening of the treatment period, it is expected to also contribute to improvements in the quality of life in animal cases.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 150–153, 2011.

Studies on sex-age structure and female reproductive characteristics of feral raccoons (*Procyon lotor*) in Kanagawa Prefecture, Japan

Takuya KATO*

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

(Received 11 March 2011, VDK-No. 142)

The raccoon (*Procyon lotor*) is native to North America, and is considered an invasive alien species in Japan. In recent years, the raccoon has naturalized and expanded its distribution to include numerous prefectures in Japan. This expansion has triggered legitimate concerns that feral raccoons have negative impacts on the surrounding environment, including agricultural damage, invasion of homes, stress on native ecosystems, loss or damage to cultural assets, and disease transmission. As these issues may become more serious with the expanding distribution and growing populations of naturalized raccoons, scientific and practical control programs are necessary to eradicate the feral raccoon population in Japan.

In Kanagawa Prefecture, the first incident of raccoon naturalization was documented in 1988. Subsequent population expansion prompted the Kanagawa local government in 2006 to initiate a raccoon control program, which was based on previous studies in Hokkaido. To date, approximately 10,000 raccoons have been captured in Kanagawa Prefecture, and crop damage has declined over the past few years. Despite these efforts, current control approaches cannot prevent further expansion of feral raccoon distribution in Kanagawa. To explain the cause of the problem, the present study proposes the following 3 hypotheses : 1) Reproductive characteristics of feral raccoons differ between the Kanagawa and Hokkaido populations ; 2) The rate of increase of the expanding Kanagawa population is high ; and 3) Reproductive characteristics fluctuate based on the nutrition and/or density of the population.

The objective of the present study was to analyze the sex-age structure and female reproductive characteristics of feral raccoons in Kanagawa and test the above three hypotheses. In addition, I provided some suggestions for improvement of the feral raccoon control strategies.

Materials and methods of age determination and estimation of reproductive characteristics (Chapter 2)

Based on reports monitoring the distribution of feral raccoons in Kanagawa, the area in which raccoons were captured prior to 2005 was considered as the “establishment area”, while the area in which raccoons were captured from 2006 was considered as the “expanding area”. In this study, 1,648 raccoon carcasses were collected by raccoon control programs in Kamakura, Yokohama, Hayama, Kawasaki, Fujisawa, and Sagamihara in Kanagawa Prefecture from March 2005 to March 2009.

In a previous study, feral raccoons were typically separated into age classes of juveniles,

* Supervisor : Prof. Toshihiko TSUTSUI

yearlings, and adults. However, to examine age-specific reproductive characteristics in greater detail, the age of raccoons was determined by methods based on tooth eruption, root foramina closure of canines, and cranial suture obliteration, as described in Chapter 2-1. Using these approaches, the raccoons were separated into 5 age classes : Class 1 (＜5 months old) ; Class 2 (5–11 months old) ; Class 3 (12–17 months old) ; Class 4 (18–23 months old) ; and Class 5 (＞23 months old).

For annual estimations of parturition season, pregnancy rate, and litter size, it was necessary to examine the reproductive status of all females of reproductive age. From the birth months estimated by fetal growth rate and tooth eruption examination in Class 1 individuals, it was determined when raccoons were born in the region. The pregnancy rate was calculated from the proportion of females observed with fetuses or placental scars in the uterus, while mean litter size was calculated as the average number of fetuses or placental scars among all female (Chapter 2-2).

1. Examination of sex-age structure, parturition season, age-specific pregnancy rate, and litter size in establishment areas in Kanagawa (Chapter 3)

In Kanagawa, raccoon control programs were formulated on the basis of data reported from Hokkaido due to a lack of information regarding parturition season, pregnancy rate, and litter size for raccoon populations in Kanagawa. However, reproductive characteristics may vary between Kanagawa and Hokkaido because climates differ significantly between these areas. The aim of this Chapter was to address the reproductive characteristics of feral raccoons in Kamakura, which is one of the establishment areas in Kanagawa, and to compare and analyze the data with those from Hokkaido.

Comparison of the reproductive characteristics between these two regions revealed that although the parturition periods of feral raccoons in Hokkaido were reported to occur between March and May, those in Kanagawa were estimated to occur from February to October. Clearly, the length of the parturition season of feral raccoons in Kanagawa is longer than that of Hokkaido. In Kanagawa, the pregnancy rate was 50.0% in Class 3 females, which was significantly lower than the 81.5% and 78.0% observed in Class 4 and Class 5, respectively ($p < 0.05$). Also, the pregnancy rate of yearlings (Class 3 and Class 4 combined) was 64.9%, which did not differ from that of adults (Class 5). In Hokkaido, the pregnancy rate was reported to differ between yearlings and adults ; therefore, it appears that the pregnancy rate of yearlings is relatively high in Kanagawa. Although mean litter size tends to be larger in older age classes, no significant differences in mean litter size were observed among the age classes in Kanagawa. In addition, the litter size was not significantly different between female raccoons in Kanagawa and Hokkaido.

2. Examination of sex-age structure, parturition season, age-specific pregnancy rate, and litter size in expanding areas in Kanagawa (Chapter 4)

Due to their adaptability and high rate of population increase, feral raccoons may rapidly increase their distribution range in expanding areas. The purpose of Chapter 4 was to compare the expanding and establishment areas in Kanagawa with respect to the sex-age structure and reproductive characteristics of feral raccoons.

Parturition of feral raccoons in the expanding area was estimated to occur from February to October, identical to the period determined for the establishment area, but distribution of the parturition season was earlier than that of the establishment area population ($p < 0.01$). In addition, the pregnancy rate of yearlings was 63.0%, which differed from that of the adults ($p < 0.05$) ; however, no differences in the age-specific pregnancy rate or litter size were detected between the two areas. In contrast to establishment areas, the proportion of adult females was higher

than that of yearling females in expanding areas, which suggests that a larger number of females with high reproductive rates were present in these areas. In addition, the parturitions of adults tended to be earlier in the season than those of yearlings ($0.05 < p < 0.1$). Notably, because the proportion of adults was higher, parturition tended to be earlier in the season in the expanding areas of feral raccoons. If feral raccoon litters resulting from early parturition have higher growth rates, and if sexual maturity occurs earlier than that of late litters, a greater increase in the rate of population expansion might occur.

3. **Evaluation of a simple index for assessing body condition on the basis of external measurements (Chapter 5)**

In wildlife, although body condition is typically considered to influence reproductive characteristics, no indices were assessed in this study because of the high costs associated with the large sample size required for such calculations. Thus, to evaluate a simple index for the assessment of body condition, the relationship between visible fat index (VFI), which was determined from abdominal subcutaneous and omental fat deposition, and the following external measurements was investigated : body weight, body length, hind foot length, and body mass index (BMI＝(body weight) / (body length $)^2$) (Chapter 5).

In all examined sex-age classes, the VFI in summer was lower than that in winter ($p < 0.01$, * $p < 0.05$ in adult males). In addition, the VFI displayed the highest positive correlation with BMI of all external measurements ($r = 0.65$-0.76, $p < 0.01$). Thus, it was considered that BMI serves as a suitable simple index for the assessment of body condition in feral raccoons.

4. **Influence of body condition on growth rate and primiparity age of early and late litters (Chapter 6)**

The parturition season of feral raccoons occurs over a long period in Kanagawa. Litters born in spring (early litters) were predicted to have higher growth rates and earlier primiparity than litters born from summer to autumn (late litters). Additionally, growth rate and age of primiparity may be affected by differences in body condition between birth months. In Chapter 6, the growth rate, primiparity age, and BMI of early and late litters were analyzed and compared.

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

Pregnant females younger than 16 months of age were not found in late litters, whereas pregnant females were identified from 10 months age in early litters. Furthermore, BMI fluctuations differed between late and early litters, and the growth rate of the late litters was lower than that of the early litters ($p < 0.01$). Taken together, these data suggest that differences in body condition between late and early litters influence the growth rate and primiparity age of feral raccoons.

5. **Study of yearly variation on sex-age structure, pregnancy rate, litter size, and BMI (Chapter 7)**

In Illinois and Missouri, which are regions of native raccoon distribution in the United States, it has been reported that the pregnancy rate of yearlings varied from year to year. This phenomenon must be taken into consideration for the development of long-term raccoon control programs. However, as there were no reports of yearly variation in the pregnancy rate and other parameters among raccoons in Kanagawa, here, the influence of yearly variation on these factors was examined in feral raccoons collected from 2006 to 2009.

Yearly variation in the pregnancy rate was only detected in Class 3 raccoons, with the rate in 2008 found to be lower than that of the other

examined years ($p < 0.05$). No variation between years was found for mean litter size in any of the age classes. In addition, yearly variation was found for BMI, which was lower in 2008 than in other years ($p < 0.01$). Therefore, it was considered that yearly variation of the pregnancy rate was affected by body condition, which was determined by food availability for raccoons during individual years.

6. Examination of the relationship between relative population density and reproductive characteristics (Chapter 8)

The population densities of raccoons in Kanagawa appeared to differ between the establishment and the expanding areas ; however, no differences in the age-specific pregnancy rate or litter size were detected. As population density may influence the reproductive characteristics and body condition of feral raccoons, the purpose of Chapter 8 was to analyze the sex-age structure, pregnancy rate, litter size, and BMI between areas of high and low population densities, using indices of catch per unit effort (CPUE). CPUE was calculated as follows : number of captured raccoons / (number of traps × trapping days). In this study, values less than the median CPUE were considered to represent low population groups, while values over the median CPUE were used to characterize high population groups.

When yearlings and adults were combined in the analysis, the pregnancy rate in the low population group was lower than that in the high population group ($p < 0.05$). Notably, there were no differences in BMI between the two population groups. These findings indicate that the population density of feral raccoons influences the pregnancy rate, regardless of body condition. Therefore, control programs aimed at reducing raccoon population density are expected to lower the pregnancy rate of feral raccoons in Kanagawa.

The primary findings of this thesis are as follows :

1. The parturition season of raccoons in Kanagawa is markedly longer than that of raccoons in Hokkaido. Thus, the reproductive characteristics of feral raccoons vary between these two regions.
2. Between the establishment and expanding areas in Kanagawa, no differences in the age-specific pregnancy rate or litter size were detected. However, as the proportion of adults was higher and parturition tended to be earlier in the season in the expanding area population, the rate of population growth in these areas is higher than that in establishment areas.
3. Body condition of feral raccoons influences growth rate and primiparity as well as contributing to yearly variation in the pregnancy rate. Thus, reproductive characteristics of raccoons fluctuate based on nutrition and population density.
4. Reducing raccoon population density may be an effective method for lowering the pregnancy rate of the feral raccoon population in Kanagawa.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 154–156, 2011.

Biomechanical study of the stabilizer and TPLO in the canine stifle

Nobuo KANNO*

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

(Received 11 March 2011, VDK-No. 143)

Canine cranial cruciate ligament rupture (CrCL-R) is one of the most common diseases in veterinary orthopedics. CrCL-R results in instability of the stifle and secondary osteoarthritis. The pathogenesis and cause of canine CrCL-R are unclear. In recent years, it has been suspected that the occurrence of CrCL in humans is associated with dysfunction of the hamstring, which acts as a dynamic stabilizer. Cauda equina syndrome (CES) is a disease that is known to stimulate a decline in canine hamstring activity. Characteristics of this disease include an uninjured femoral nerve, which innervates the quadriceps, and an injured sciatic nerve, which innervates the hamstring. In human medicine, it has been determined that the hamstring acts as an agonist and the quadriceps acts as an antagonist for the CrCL. Despite dysfunction of the hamstring, the function of the quadriceps is not compromised. Therefore, the CrCL may be exposed to a stronger tensile force. Because CrCL-R patients with concurrent CES visit the Veterinary Medical Teaching Hospital of the Nippon Veterinary Life Science University, the authors have hypothesized that canine CrCL-R is associated with CES. However, this relationship has not been elucidated. Additionally, there is no evidence that supports the function of the hamstring as a dynamic stabilizer.

The tibial plateau leveling osteotomy (TPLO), currently one the most commonly performed surgical procedures for the treatment of CrCL insufficiency, is purported to eliminate cranial tibial thrust by reducing the caudodistally oriented slope of the tibial plateau. However, cranial drawer remains in the stifle after TPLO. If CrCL-R coexists with CES, stifle instability may be increased and the postoperative prognosis will be affected. As previously mentioned, whether the hamstring acts as a dynamic stabilizer in the canine stifle is unclear. Additionally, no studies have been performed on the instability remaining in the stifle after TPLO. Therefore, the purposes of this study were to investigate the functions of the dynamic and static stabilizers in the stifle joint and to evaluate the biomechanical effects following TPLO.

1. Postoperative change in CrCL-R with and without CES after TPLO (Chapter 2)

The purpose of this study was to investigate the effect of CES on the outcome of TPLO for CrCL deficiency in the dog. A total of 38 dogs (54 stifles, body weights＞15 kg) underwent TPLO. Each dog was classified into either the CES-affected group (n＝22, 32 stifles) or the CES-unaffected group (n＝16, 22 stifles) based on clinical signs and diagnostic imaging findings. The peak vertical force (PVF), which was determined by force plate analysis, and the radiographic osteoarthritis score (R-OA) were obtained preoperatively and 1, 2, 3, and 6 months after sur-

* Supervisor : Prof. Masahiro TAGAWA

gery. The patients' PVFs were evaluated and compared with those of 11 normal Labrador retrievers' PVFs (60.8%±4.7% of body weight). The PVFs of the CES-affected group indicated that the functional recovery of the affected limb was delayed compared with that of the CES-unaffected group. The R-OAs of both the CES-affected and -unaffected groups were longitudinally increased after TPLO. However, it is suggested that the progression of the R-OAs was identified earlier in the CES-affected group. These results suggest that the presence of CES will delay the recovery of limb function and accelerate the progression of osteoarthritis in CrCL-R stifles that have undergone TPLO.

2. Comparison of cranial displacement of the stifle between intact and transected CrCL in the dog : an *in vitro* experimental study (Chapter 3)

The purpose of this study was to investigate the effect of cranial stability of the stifle at several joint angles using a biomechanical robotic system.

Experiments were performed at hyperextension and at 135° and 90° stifle joint angles. To evaluate cranial stability, the stifle underwent both the anterior-posterior test (AP test), which mimics the cranial drawer test, and the proximal-distal test (PD test), which mimics the tibial compression test. These experiments were performed on both intact and CrCL-transected stifles.

The AP and PD tests in the CrCL-transected stifles resulted in increased cranial displacement in the intact stifles at all angles. The stifle angle of 135° resulted in the greatest degree of cranial instability. Additionally, the robotic system used in this study made it possible to accurately reproduce cranial tibial thrust, which was the biomechanical character generated in the stifles.

These results suggest that the CrCL is the primary stabilizer for cranial tibial displacement. By using this robotic system, more precise research on stifle biomechanics and the bio mechanical effects of various stifle joint surgeries will be possible.

3. The relationship between the canine hamstring and the CrCL : an *in vitro* experimental study (Chapter 4)

The purpose of this study was to investigate the functions of the quadriceps, gastrocnemius, and hamstring as stabilizers of the canine stifle. We considered the tensile force in each muscle, the cranial tibial displacement, and the tibial internal rotation.

The tensile force of the CrCL-transected hamstring was significantly increased compared with that of the intact stifle ($p=0.01$). The cranial tibial displacement and the tibial internal rotation were approximately 2 mm and 5°, respectively, in the CrCL-transected stifle. However, the tibial cranial displacement and the internal rotation in the CrCL-transected stifle were significantly increased due to the release of the tensile force in the hamstring. When the tensile force was reapplied to the hamstring after releasing the tension, neither the cranial tibial displacement nor the cranial internal rotation returned to the intact position.

These results support the function of the canine hamstring as an agonist of the CrCL. Moreover, the quadriceps and the gastrocnemius muscles may be antagonists of the CrCL. Hamstring dysfunction caused by CES may increase the load on the CrCL.

4. Biomechanical effects of TPLO in a canine CRCL-deficient stifle : an in vitro experimental study (Chapter 5)

The purposes of this study were (1) to determine the optimal tibial plateau angle (optimal-TPA) and (2) to evaluate any changes in craniocaudal or rotational instability following TPLO. In the PD test, the CrCL-transected stifles showed the same cranial stability as that of the intact stifles at a TPA of 6.5°±3.5°. However, in the AP test, cranial displacement and internal instability of the stifles after TPLO were increased compared with those of the CrCL-transected stifles. External rotation exhibited no significant differences among the conditions of

the PD and AP tests.

These results suggest that the CrCL-transected stifles maintained stability under axial loads, but simple craniocaudal displacement and concomitant internal rotation increased following TPLO. These changes may have occurred because TPLO changes the relationship of the femorotibial joint similar to a flexed stifle, creating lax collateral ligaments.

In recent years, some studies have shown that hamstring dysfunction may increase the risk of CrCL-R, while others have suggested that patients with CrCL deficiency may recover the function of the walk without surgical intervention if these patients can control the balance of forces in the hamstring, quadriceps, and gastrocnemius. TPLO does not restore normal biomechanics to the joint, but functionally stabilizes the CrCL-deficient stifle.

Availability of surgical treatment for CrCL-R is often determined by measuring the PVF *in vivo*. Therefore, the effect of surgical treatment is determined by the mechanical recovery of the affected limb. However, we must accomplish not only mechanical recovery, but also motor function recovery in the affected limb.

Recently, kinematic gait analysis has been attempted to evaluate functional recovery following surgical treatment of CrCL-R, and some *in vitro* studies have evaluated the meniscal pressure distribution. These studies demonstrated that surgery maintains the biomechanical characteristics of normal stifles. However, there are few reports on the biomechanical analysis of the stifle in the dog. Therefore, we evaluated the recovery of each surgery based on the load capacity of the affected limb.

We clarified the role of both the static and dynamic stabilizers of the canine stifle. Furthermore, we reproduced the biomechanical changes in the stifle following TPLO.

We hope that these results will not only add to the general knowledge base of CrCL-R, but also help to further develop the surgical procedure for CrCL-R in the dog.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 157–159, 2011.

Fundamental studies on molecular epidemiology of *Listeria monocytogenes* isolates in Japan

Batmunkh OTGONCHIMEG*

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

(Received 11 March 2011, VDK-No. 144)

Listeria monocytogenes is a food-borne pathogen. Listeriosis is a lethal infectious disease among pregnant women, infants, elderly people and immunocompromised people. There is an urgent need to control the infection with *L. monocytogenes*. The aim of this study was to develop a method of genetic classification of *L. monocytogenes*. In order to accomplish this, the author performed genetic classification of strains isolated from foods, human patients and environments in Japan by comparison of nucleotide sequences of the virulence genes *iap* and *actA*, and the housekeeping gene *sigB*. Genetic characteristics of the strains were compared with those isolated in the US to evaluate whether the method developed in the present examination had a high discriminatory ability in classification of this pathogen.

Chapter 1 : Classification of *Listeria monocytogenes* strains isolated in Japan by *iap* region

In this Chapter, the author carried out genetic classification of *L. monocytogenes* strains using nucleotide sequence of 407 bp region of the iap gene. The results obtained showed that 205 strains isolated in Japan and the reference strain EGD-e were classified roughly into three groups (Groups Aiap, Biap, Ciap) and 26 genotypes (Genotypes 0 to 25). Group Aiap was shown to include the strains mainly of serotypes 1/2a and 1/2c, which corresponds to Type (lineage) 2, as proposed by other research groups (Rasmussen, 1995 ; Wiedmann, 1997). Group Biap included the strains mainly of serotypes 1/2b and 4b, which corresponds to Type (lineage) 1. However, all the strains classified to Group Ciap were of serotype 4b, which was not consistent with the serotype assigned to Type 3 as proposed by Rasmussen *et al.* (1995), in which the serotype was 4a. To investigate genetic relationships among the strains classified to Group Ciap and Type 3, a phylogram was constructed using nucleotide sequence for 207 bp region within the *iap* gene. The results showed that, although the strains classified to Group Ciap were located in the same branch as those of Type 3, the genetic distance from Group Ciap to Type 3 was much greater than those from Type 3 to Group Aiap or Biap. In addition, the strains examined were classified genetically by RFLP analysis with genomic DNA, in which three restriction enzymes *Pst* I, *Xba* I, and *Ban* III were used. The results showed that six genotypes (Genotypes 0, 9, 12, 13, 16, 20) were classified to two or three sub-genotypes by RFLP analysis. Eventually, the author concluded that the *L. monocytogenes* strains examined could be classified into 34 genotypes on the basis of a combination of nucleotide sequence for the partial *iap* gene and RFLP analysis. The results showed that Groups Aiap, Biap and Ciap included 17, 13 and four genotypes, respectively.

* Supervisor : Prof. Fukiko UEDA

Chapter 2 : Classification of *Listeria monocytogenes* strains isolated in Japan and US by sigB region

In Chapter 2, in order to aim comparison of genetic characteristics of *L. monocytogenes* strains isolated in Japan and the US, the author investigated nucleotide sequence of the partial *sigB* gene, which was 662 bp in length. In this study, nucleotide sequences of 34 strains isolated in Japan, representative of the genotypes determined by sequence of the *iap* gene and RFLP analysis, were compared with those isolated in the US, for which nucleotide sequence was obtained from the Pathogentracker system developed by the Department of Food Science, Cornell University, New York, USA.

In this Chapter, nucleotide sequence for the partial *sigB* gene of 662 bp was determined and used for genetic classification of isolates that comprised 34 and 172 strains isolated in Japan and the US, respectively. The results showed that the strains could be classified roughly into three groups (Groups Asig, Bsig, Csig), as shown by the classification using sequence of the *iap* gene. However, six subgroups were formed under Groups Bsig (Groups B1sig, B2sig, B3sig) and Csig (Groups C1sig, C2sig, C3sig). The comparison of the phylograms between the *iap* and *sigB* genes showed that Groups Aiap, Biap and Ciap were found to correspond to Groups Asig, B 1sig and C1sig, respectively. The phylogram constructed using the *sigB* gene also showed that Groups Asig and B1sig consisted of strains isolated in both Japan and the US, but that Groups B2sig, B3sig and C2sig consisted of US strains only, whereas Groups C1sig and C3sig consisted of Japanese strains only. The classification using the *sigB* gene showed that the strains investigated could be classified into 38 genotypes and into Groups Asig, B1sig, B2sig, B3sig, C 1sig, C2sig and C3sig that included four, eight, 14, four, three, four genotypes and one genotype, respectively. The strains isolated in Japan were classified into 12 genotypes suggesting that classification using the *sigB* gene does not have a high discriminatory ability for isolates of L. monocytogenes in comparison with the combination of the *iap* gene and RFLP analysis. However, the former classification could divide Genotypes 12 and 13, as determined by sequence of the *iap* gene, into two and three types, respectively, which suggests that nucleotide sequence of the *sigB* gene is also useful for genetic classification in some ways.

Chapter 3 : Classification of *Listeria monocytogenes* strains isolated in Japan and US by *actA* region

Nucleotide sequence of the partial *actA* gene of 561 bp was analyzed in this study. A total of 34 strains isolated in Japan was used for PCR, but four strains (Group Ciap) could not produce detectable amplified products. In addition, nucleotide sequences of three strains (Groups Aiap and Biap) could not be determined. Therefore, nucleotide sequences of 221 isolates, including 27 and 194 strains isolated in Japan and the US, respectively, were used for genetic classification. The results showed that the strains could be classified roughly into four groups (Groups Aact, Bact, Cact, Dact) and that Groups Aact and Bact corresponded to Groups Aiap and Biap, respectively. In addition, the strains examined were classified into 69 genotypes and 24, 17, 21 and seven genotypes were assigned to Groups Aact, Bact, Cact and Dact, respectively. The strains isolated in Japan were classified into 15 genotypes, of which six and nine genotypes were assigned to Groups Aact and Bact, respectively. The number of genotypes in Group Bact was almost same as that in Group Biap suggesting that nucleotide sequence of the *actA* gene has the same classification ability as that of the combination of *iap* gene and RFLP analysis. However, the number of genotypes in Group Aact was less than that in Group A*iap*, which suggests that the classification using the actA gene had a lower ability than that of the combination of the *iap* gene and RFLP analysis to discriminate among the strains of Group Aact and of the corresponding Group Aiap. On the other hand,

the strains isolated in the US were classified to 66 genotypes, which showed that the remaining three genotypes were unique types found in only Japan.

Finally, the author performed genetic classification by multilocus sequence typing (MLST) using two or three regions of nucleotide sequence, partial regions of the actA, *iap* and *sigB* genes. In the first study, the strains isolated in Japan and the US were classified into four groups (Groups A, B, C, D) by MLST using the *sigB* and *actA* genes. Groups C and D were classified into two further subgroups, respectively (Groups C1, C2, D1, D2). This typing method classified the strains examined into 74 genotypes, which shows that this method had a higher discriminatory ability than the genetic method that used sequence of a single gene. In addition, MLST using the *iap*, *sigB* and *actA* genes was employed for classification of the 31 strains isolated in Japan, which were representative of the genotypes determined by sequence of the *iap* gene and RFLP analysis. The results showed that the strains could be classified into 29 genotypes, which suggests that MLST is useful for genetic classification of *L. monocytogenes* isolates ; again, though, genotypes 16-1, 16-2, 20-1 and 20-2 could not be classified by this method. In conclusion, the author suggests that the combination of MLST and RFLP analysis is a powerful tool for elucidation of the epidemiology of *L. monocytogenes* from the source of infection to the human host. The author carried out genetic classification of *L. monocytogenes* strains isolated in Japan for the first time, and elucidated the location in classification by comparison with those isolated in the US. These results are useful for tracking an outbreak of listeriosis from a source to human hosts that could occur in Japan.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 160–161, 2011.

Changes in plasma lipid concentrations in dogs and cats with stress accompanying obesity

Shiro MURANAKA*

Laboratory of Comparative and Behavioral Medicine
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Received 11 March 2011, VDK-No. 145)

1. Changes in environment and nutrition condition, life style with smoking and drinking, and mental fluctuation etc. cause stress in animals. Obesity is considered to be one of inflammation, and hypertrophic adipocytes secrete many kinds of inflammatory cytokines. ER stress and oxidative stress induced by inflammatory cytokines trigger of obesity in animals. I the serious process of lipid metabolism disorders (obesity～insulin resistance～type 2 diabetes), oxidative stress has crucial roles.

2. F2-isoprostanes (F2-IsoP), indicator of tissue damage by lipid peroxidation, concentrations were measured in plasma of control (body condition score, BCS, 3) and obese (BCS 5) dogs. In obese dogs (n＝2), plasma lipid and insulin concentrations increased markedly and insulin resistance was induced. In obese dog plasma, F2 -IsoP concentrations increased (a little under 1% higher than the control value). F2-IsoP is unstable in plasma, and development of other new diagnostic indicator, which substitutes F2-IsoP to determine degree of oxidative stress, is needed for veterinary medicine.

3. Plasma metabolites, enzymes, hormones and lipoprotein profiles were measeured in obese dogs to investigate the effect of obesity on lipid metabolism. In obese dogs with average BSC 4.8, plasma total cholesterol and insulin concentrations and ALT activities increased significantly, whereas plasma adiponectin concentrations decreased significantly compared with control dogs with BSC 3.0. These dogs seemed to be at early state of obesity with moderate insulin resistance. In plasma of obese dogs, HDL2 fraction increased and lipoprotein profiles were changed. Changes in plasma insulin and adiponectin concentrations and lipoprotein profiles may be good indicators to evaluate the degree of obesity also in dogs.

4. Feline obesity generally results in aberrations to plasma metabolite levels, such as lipid concentrations and lipoprotein composition. This study sought to investigate the resultant effect of obesity on cholesterol lipoprotein composition and circulating adiponectin concentrations in cats. Plasma glucose, lipids (triglyceride, cholesterol and free fatty acid), insulin and adiponectin concentrations, and cholesterol lipoprotein composition were measured and compared between BCS determined normal healthy control and obese cats. Although the obese group demonstrated higher levels of plasma cholesterol, glucose, and triglycerides, as compared to healthy controls, the difference was insignificant thus indicating that the BCS determined obese cats may have been overweight and not morbidly obese. Plasma insulin levels were significantly higher (25–30%) versus healthy control animals thereby possibly hinting at the en-

* Supervisor : Prof. Toru R. SAITO

suing emergence of obesity induced insulin resistance. However, the BCS determined obese cat demonstrated a significant reduction ($p < 0.05$) in plasma adiponectin concentration and a significant increase ($p < 0.05$) in LDL-cholesterol % as compared to age matched healthy control animals. This would indicate that changes in plasma adiponectin concentration and cholesterol lipoprotein composition may be good early indicators of obesity in cats.

5. In plasma of rats loaded with binding stress, TG concentrations decreased and FFA concentrations increased. Stress loaded LE rats showed increasing of plasma insulin and adiponectin concentrations, whereas in the plasma of stress loaded Wister rats insulin and adiponectin concentrations decreased. There was obvious difference between lines in biochemical responses against binding stress in rats. Rats with acute stress like binding stress showed marked increase in plasma FFA concentrations differing from animals with chronic stress like obesity accompanying liver injury.

Decreased plasma adionectin concentrations accelerated insulin resistance and induced decrease in AMP-activated protein kinase (AMPK) in obese animal tissues. Decreased AMPK activities caused decreasing in glucose uptake and utilization in skeletal muscle and adipose tissue or accelerated fatty acid synthesis. As a result, hyperglycemia and acceleration of function of pancreatic β cells were continued. Acceleration of β cell function increased production of ROS inducing lipid peroxidation of mitochondrial membrane in β cells with low scanvenging activity of ROS. Above changes spread to wide area of β cells followed by apoptosis of β cells sequent type 1 diabetes mellitus. Prevention of obesity represses onset of diabetes, so increasing in plasma total cholesterol concentrations and LDL-cholesterol ratio as stress response are preventive indicator for lipid disorders such as diabetes.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 162–165, 2011.

Studies on artificial insemination with cryopreserved sperm from feline epididymides stored at low temperature

Mari TOYONAGA*

Laboratory of Veterinary Reproduction,
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Received 9 July 2010, VDO-No. 289)

All 36 species of felidae on the earth today are facing extinction because of reductions and degradation of their habitat. Protecting these threatened species will require continued maintenance of their habitat environment, and it will also be very important to introduce assisted reproductive technologies (ART). Today a variety of studies on ART, including artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic semen injection (ICSI), and cryopreserved embryo transfer are being conducted on domestic cats.

Research on cryopreservation methods is being conducted on the assumption that when a male member of an endangered feline species dies in an unforeseeable accident, etc., the animal's sperm will be recovered from his epididymides, the sites where sperm are stored, and that they will be used for IVF, ICSI, or AI. It is also necessary to take into account the time between the animal's death and the discovery of the carcass, the time required to transport the epididymides to the processing facility, and the transportation conditions, including temperature. Although the effect of cryopreserving epididymides on the condition of epididymal sperm has been investigated in a small number of studies in regard to transportation time and transportation conditions, the qualities of the frozen and thawed sperm and AI with these frozen sperm have still not been investigated.

The author therefore investigated the effect of feline epididymis storage time on epididymal semen qualities by examining their qualities before and after cryopreservation for AI and assessed various modifications of the AI method in an attempt to increase the conception rate when sperm from refrigerated epididymides were used.

1. Qualities of epididymal sperm recovered from feline epididymides stored at low temperature (Chapter 2)

To determine the effect of the interval between the time from death of a male of an endangered feline species and sperm collection on the qualities of the semen, the author investigated the qualities of semen recovered from both epididymides of domestic cats by the mincing method in egg yolk Tris-fructose citric solution (EYT-FC) after the epididymides had been stored at 4℃ for 0 hr, 24 hr, 48 hr, and 72 hr. Each epididymis was divided into the caput and caudal part, and sperm were collected separately from each part. Examination of the qualities of the semen showed no bilateral differences between the qualities of the semen from the two parts of the epididymis, but the mean count of the sperm collected from the caput epididymis (46.2 ± 4.8 [SE] $\times 10^6$) was significantly lower than the mean count of the sperm collected from the caudal epididymis ($99.3 \pm 6.9 \times 10^6$). The motility of the sperm from the caput region was significantly

* Supervisor : Prof. Toshihiko TSUTSUI

lower than the motility of the sperm from the caudal region after all three storage intervals, 0 hr, 24 hr, and 48 hr (all $p<0.01$). There was no significant difference in the motility of the sperm from the caudal part of the epididymides after storage for 0 hr (68.0±3.4%) and 24 hr (60.6±2.8%), but motility was significantly lower in the 48-hr group (42.0±2.6%) and 72-hr group (8.4±1.6%) than in either the 0-hr group or 24-hr group. There were no significant differences in percentages of abnormal sperm among the 0-hr, 24-hr group and 48-hr storage group, but the percentage in the 72-hr storage group was significantly higher than in each of the other groups ($p<0.01$).

In conclusion, the results showed that when semen from epididymides stored at low temperature was used for AI, up to 24 hr was the most useful storage time. However, mean sperm motility in the 48-hr storage group was 42.0%, and significantly lower than in the 0-hr group and the 24-hr storage group. In addition, there was no difference in sperm abnormality between the 0-hr group and 24-hr storage group. Thus, it was necessary to investigate the quality of semen after cryopreservation in the 48-hr storage group.

2. Qualities of cryopreserved epididymal sperm collected from feline epididymides stored at low temperature (Chapter 3)

The qualities of cryopreserved caudal epididymal sperm collected from feline epididymides that had been stored for 0 hr, 24 hr, and 48 hr at low temperature were investigated. The sperm collected was diluted with EYT-FC, and after adjusting the sperm count to 50×10^6/ml with EYT-FC, 0.20 ml of each sample was placed in a 0.25 ml straw, and the straws were frozen by the plunging method. Examination of sperm qualities showed that there were no significant differences in sperm motility, viability, or abnormalities between the 0-hr storage group (38.0±2.5%, 54.8±4.3%, 5.1±1.7%, respectively) and the 24-hr storage group (36.0±5.7%, 44.3±3.8%, 14.5±2.3%), but there were significant differences in these qualities between the 48-hr storage group (16.0±5.4%, 29.3±5.0%, 33.5±4.0%) and all of the other groups (all $p<0.01$). Examination of the qualities of semen that had been allowed to stand at room temperature for 6 hours revealed that at 2 hr after thawing sperm motility and viability were significantly lower in the 48-hr storage group than in the 0-hr storage group ($p<0.01$).

In conclusion, storage of the epididymis for up to 24 hr appears to be useful when using frozen semen collected from the caudal epididymis stored at a low temperature for AI.

3. Qualities of cryopreserved caudal epididymal sperm collected from feline epididymides stored at low temperature after collection with feline seminal plasma (Chapter 4)

A lower conception rate has been reported after intrauterine insemination (IUI) with cryopreserved caudal epididymal sperm than after IUI with cryopreserved ejaculated sperm. The reason of this difference is thought to be that, unlike ejaculated sperm, caudal epididymal sperm have not been sensitized with seminal plasma (SP). Since glycoproteins (GPs) in SP have been found to bind to the head of the sperm and promote fertilization in many animal species, the effect of sensitization with SP or EYT-FC (as a control) during the recovery of caudal epididymal sperm on sperm quality after cryopreservation was investigated by collecting caudal epididymal sperm from epididymides that had been stored at low temperature for 0 hr, 24 hr, and 48 hr. The results showed that the motility of the sperm in the SP group was higher, but there was no significant difference in sperm direction or between the storage groups. After storage for 48 hr, the viability of the sperm that had been collected with SP was significantly lower. None of the differences in percentage of abnormal sperm between the storage groups were significant, but the percentage was higher in the SP group than in the control group after every storage time. There were no significant differences in sperm motility or viability between the SP group and control group, but

sperm motility and viability were both higher in the SP group than in the control group after every storage time. No significant difference was noted in percentages of abnormal sperm.

The above findings show that the qualities of cryopreserved sperm obtained from the caudal epididymis cannot be improved by sensitization with SP during collection.

4. Distribution of glycoproteins on feline testicular sperm, epididymal sperm and ejaculated sperm (Chapter 5)

As described in Chapter 4, it was impossible to improve the qualities of feline sperm after cryopreservation by sensitizing caudal epididymal sperm with SP. Adhesion of GPs, which participate in capacitation, occurs when sperm are sensitized with SP, and that may have protected the acrosome and decreased the damage when canine sperm were frozen. However, it is unknown whether there is a difference between epididymal sperm and ejaculated sperm in cats, because there have been no reports on the adhesion of GPs during the process of sperm maturation from testicular sperm to ejaculated sperm. To determine whether there is a difference, the adhesion of GPs during the process of sperm maturation from testicular sperm to ejaculated sperm was investigated by using the 8 kinds of FITC-lectin that are routinely used in this kind of study.

The results showed that GPs that bound to all of the FITC-lectins used in the experiment were attached to feline sperm from the testis to the epididymis, and no differences in GP binding were found between caudal epididymal sperm and ejaculated sperm.

These results were consistent with the fact that the qualities of the sperm recovered from the epididymis with SP and frozen were not improved in comparison with control group. These findings are consistent with the results reported in Chapter 4.

5. Intrauterine insemination with cryopreserved epididymal sperm from feline epididymides stored at low temperature (Chapter 6)

AI with cryopreserved sperm recovered from epididymides stored at 4℃ was assessed by comparing the fertility of the sperm with that of sperm that had just been collected. Since the sperm number required for conception by felines by intravaginal insemination is 1/10 in IUI, in the experiment reported in this chapter, on day 2-4 of estrus queens were subcutaneously injected with 100 IU of hCG to induce ovulation, and 20 hr later a $40\times10^6/50\mu l$ concentration of semen was surgically introduced into the uterine horn containing the larger number of mature ovarian follicles. The conception rate was low, only 28.6% (2/7), the same as in the group inseminated immediately after sperm collection.

The fertilization capacity of cryopreserved sperm recovered from the epididymides stored at low temperature for 24 hr was the same as that of sperm used for insemination immediately after collection. In order to improve this conception rate, it will be necessary to inseminate both uterine horns or increase the number of sperm used for insemination. However, since 40×10^6 sperm are required to inseminate a single horn by IUI, only one queen can be inseminated with caudal epididymis sperm collected from a single male, and it will be necessary devise an AI method that enables conception with a smaller number of sperm.

6. Intratubal insemination with cryopreserved epididymal sperm from feline epididymides stored at low temperature (Chapter 7)

The results of the experiment had shown that pregnancies can be achieved by IUI with sperm that have been frozen after being recovered from caudal epididymides that had been refrigerated at 4℃ for 24 hr, but since the conception rate was low, the author assessed the feasibility of intratubal insemination (ITI), which could be expected to result in conception with a smaller

number of sperm. In addition, because a catheter is directly inserted into the oviduct to perform ITI, the author also divided the queens used for AI into two groups, a group inseminated before ovulation and a group inseminated after ovulation. Therefore, ITI of a single oviduct was performed surgically 24 hr (before ovulation) or 30 hr (after ovulation) after hCG injection on day 2-4 of estrus to induce ovulation. The sperm count expected to be required for fertilization was $10\times10^6/30\mu$l. The conception rate when inseminated at 24 hr and 30 hr after injection was 80.0% (4/5) and 20.0% (1/5), respectively. The conception rate when IUI was performed before ovulation (24 hr after the hCG injection) was higher, but the difference was not significant.

The above results showed that when ITI was performed with frozen semen containing caudal epididymidal sperm collected from feline epididymides that had been stored at 4℃ for 24 hr, a higher conception rate was achieved with a smaller number of sperm than by IUI.

In conclusion, in order to be able to use sperm for AI when a male of an endangered feline species dies, it is important to store the epididymides at low temperature within 24 hours after the animal has died and to cryopreserve the sperm. In addition, based on the conception rate and the number of sperm used for insemination, the results of this experiment showed that ITI is a more effective fertilization method than IUI. These techniques are considered to be applicable to preservation of caudal epididymal sperm obtained from wild feline species that die of accidents.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 166–169, 2011.

Study on the perioperative changes of blood pressure, heart rate and heart rate variability in dogs

Kiyosi NAKAGAWA*

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

(Received 20 October 2010, VDO-No. 290)

The average lifespan of pet animals has been extended due to continuous development in veterinary medicine and in the quality of emotional and physical care by pet owners in recent years.

The major cause of pet mortality is tumors induced by aging. In fact, 45% of dogs older than 10 yr old died of cancers. The highest morbidity rate in female dogs has been due to to mammary gland tumors (MGT), and the median age of disease induction registers 10–11 yr. Although the first choice of treatment for MGT is complete tumor resection, postoperative algesia/nociception-induced stress has been reported to increase sympathetic nervous activity to eventuate blood pressure (BP) and heart rate (HR) elevations.

Non-steriodal antiinflammatory drugs (NSAID) inhibit cyclooxygenases (COX) and disturb prostaglandin (PG) synthesis to eventually elicit analgesia. Therefore NSAID, which have been approved for perioperative pain treatment in dogs, are extensively employed in Japan. Hitherto, numerous studies have reported postoperative analgesic effects in dogs with pretreatment of selective COX-2 inhibitors, such as carprofen and meloxicam. COX-2 inhibitors have produced efficacious analgesic effects in these studies, and the use of these agents has in fact been extrapolated from subjective evaluation methods in human patients per se. However, reproducibility of such methods in the field of veterinary medicine has been unreliable, and the reported disrepancies have been probably due to interobserver differences in assessment.

In this study, time-related data were extracted from non-anesthetized and unrestrained young (age range : 1–3 yr) and old (age range : 10–11 yr) dogs using the BP telemetry system. The long-term effects of complete unilateral MGT resection-induced invasive surgery on circadian changes of BP, HR and HR variability (HRV) as well as preoperative NSAID tratement were monitored. In addition, circadian variations in BP, HR and HRV induced by aging and feeding in dogs were investigated as the basic reserch.

Ⅰ. Chapter 2 : Effects of aging on BP, HR and HRV in normal dogs

1. BP variation

Circadian BP variations in young dogs portrayed a single-phase pattern with the peak registered in the morning, while those of aged dogs indicated a double-phase pattern with peaks portraying in the morning and afternoon. When meal times were synchronized in the young and aged dogs, effects on the latter were more obvious than the former. Interestingly, the systolic BP (SBP) increased while the diastolic BP (DBP) decreased to apparently induce pulse pressure (PP) in the aged dogs. This could have been attributable to aging-induced compliance decrease in the arterial walls.

2. HR and HR variations

In young dogs, circadian variations in the low-

* Supervisor : Prof. Hisashi HIROSE

frequency (LF)/high-frequency (HF) components of the HR and HRV coincided with peaks of the double-phase pattern duirng meal times in the morning and afternoon. In a similar fashion, those of aged dogs portrayed double-phase peaks under the same experimental conditions. The circadian HR variations of aged dogs indicated a significantly lower value than that of young dogs. This difference, an index for LH/HF of the sympathetic nervous activity, was due to the significantly low variation levels in the old compared with young dogs. In this investigation, as HF, an index for the vagus nerve activity, indicated high variation values in the old compared with the young dogs, vagus nerve regulation was most likely to have been promoted. This, in fact, was due to a decrease in the functional activity of b-adrenergic receptors, thus resulting in the suppression of sympathetic nerve activity.

Ⅱ. Chapter 3 : Effects of the number of meals on BP, HR and HRV in normal dogs

BP, HR and the double-product (DP) increased abruptly before eating and decreased rapidly after eating in dogs. Circardian changes in cardiovascular parameters in dogs with one or two meals portrayed significantly different peak times. The respective values of BP, HR and DP during the 5-min interval immediately before meals indicated marked increases, and established their peak values during eating. The BP of double-meal dogs indicated drastic postprandial decreases in the morning. The highest amplitude increases and decreases respectively observed during the 5-min interval immediately before and the 60-min interval after meals did not indicate any significant differences with respect to the number of meals. Peaks of the LF/HF components coincided well with the feeding time, although the HF component decreased during feeding.

As a result of postprandial secretions of vasodilating digestive tract peptides, such as insulin and neurotensin etc., in humans, blood is pooled in organic veins to induce decreases in cardiac output and subsequent postprandial BP decreases. BP decreases due to elevated postprandial mesenteric bloodflow and intravenous infusion of neurotensin have been documented in dogs as well. Therefore, it is most likely that the postprandial BP decreases observed in the present study were attributed to the same mechanism observed in humans.

In canine cases where aging-related functional decreases of the baroceptor could not adequately compensate for those postprandial BP decreases, aggravated postprandial BP decreases can be induced if a therapeutic for heart failure is administered to the animal by mixing the drug with the animal feed. Symptoms typical of postprandial hypotension encountered in humans may also be found in dogs.

Ⅲ. Chapter 4 : Perioperative variations of BP, HR and HRV in dogs

1. Effects of preoperative single treatment with meroxicam or carprofen on BP, HR and HRV in young dogs

Except for SBP in the meroxicam-treated dogs, short-term postoperative effects in young dogs revealed that the mean values within a 24-hr period of BP, HR and DP of meroxicam- and carprofen-treated dogs did not show any significant differences. When the 24-hr variations were monitored, the respective BP changes were significantly different in the meroxicam-treated dogs. This finding thus advocated the use of this agent with complementary butorphanol administration for acute nociception/algesia for the first postoperative 24 hr after unilateral mammary gland resection, as preoperative single-bolus administration with either meroxicam or carprofen was reckoned to achieve inadequate analgesia/antinociception in such a surgical resection.

With regard to long-term analgesic effects, a single-bolus administration of meloxicam or carprofen was able to buffer 5-day postoperative analgesic aggravation. In our study, NSAID-induced COX-2 inhibition suppressed PGI2 and PGE2 production to eventually nullify peripher-

al hypersalgesia and prevent wind-up at spinal dorsal horn.

Crenitine clearance observed with meloxicam administration in Experiment 2 indicated no changes with or without the use of this agent. This may be due to the highly selective action of meloxicam on COX-2, thus inflicting negligible enzymatic inhibition on the renotrophic COX-1. In addition, differences in the plasma cortisol levels between the 2 groups were not observed. As cortisol levels are influenced by stress and other exogenous factors, the use of this index for assessing analgesia in dogs is though to be unreliable.

2. Age-dependent differences in effects of a single carprofen administration on the BP, HR and HRV

BP, HR and DP of young dogs did not indicate any significant changes 24 hr after operation ; however, these parameters registered significant decreases in the aged dogs treated with carprofen. The liver bloodflow rate decreases with age in humans, and the drug excretion rate from liver is thus attenuated. In dogs, 70% of intravenously administered carprofen is excreted in the feces via bile secretion. As such, delayed carprofen metabolism in aged dogs compared with young dogs could be a factor contributing to differential effects between the 2 age-groups. Furthermore, hypertension has been known to activate the nociception/algesia suppression mechanism via its action on the baroceptor. As such, the higher postoperative BP noted in old than young dogs could also serve as a factor contributing to differences in the antinociceptive/analgesic effect between the 2 age-groups.

With reference to changes during the 14-day postoperative period, BP levels in both young and aged dogs significantly decreased after carprofen administration. This effect may be explained by the same mechanism mentioned above.

Unlike the case of young dogs, there were no significant changes in HR and DP of aged dogs during 14-day postoperative period. In aged dogs, marked HR and DP decreases accompanied carprofen administration immediately before surgery, and these changes stabilized in both age-groups 3 days after operation. Therefore, differences in changes of HR and DP between the 2 age-groups were not noted in the 14-day postoperative period.

3. Evaluation of postoperative analgesia

The effects of surgical invasiveness on BP in both groups persisted for more than 1 week after operation. Although the dogs appeared normal with these postoperative findings, continuous postoperative nociceptive/algesic effects due to surgical invasion were thought to exist, and therefore appropriate subjective assessments have to be performed accordingly.

Although the present study demonstrated novel findings on circadian changes in the cardiovascular system and preemptive analgesic effects of NSAID on surgical invasiveness in both young and aged dogs, further studies are warranted to clarify certain unresolved issues.

The present study also demonstrated circadian changes in the cardiovascuilar parame ters of both young and aged dogs. These circadian cardiovascular variations were found to be associated with eating. Therefore, it is highly possible that the timing of administration of vasodilating agents induced differences in drug efficacy, regardless of whether the treated subject is human or a dog. In cases where drug administration is performed on a single administration per day, the timing of administration to achieve the best efficacy, whether in the morning or afternoon, has yet to be defined. Drug administrations for internal treatment in current small-animal clinical practice often resort to mixing therapeutic agents with feed for convenience. However, our findings revealed that BP decreases were induced after meals, and further studies are warranted to clarify whether the use of vasodilating agents could in fact have aggravated postprandial BP decreases.

In this investigation, dogs with unilateral MGT resection manifested consistently high BP

levels for more than 1 week after surgery. As a single administration of either meloxicam or carprofen immediately before operation could buffer BP elevations, it could clinically be useful to confirm on the required number of postoperative NSAID administrations to establish better quality-of-life for operated dogs.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 170–171, 2011.

Studies on Standardization of Hematology and Serum Biochemistry Values and Its Application in Cetaceans

Fumio TERASAWA*

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

(Received 28 October 2010, VDO-No. 291)

From 1957 through 2003, Enoshima Aquarium Marineland had been carried a total of 257 animals from wilds, representing 18 species (15 *Delphinidae*, 2 *Phocoenidae* and 1 *Physeteridae*). And there were 125 parturitions of 11 species including stillborn and miscarriage. From 1957 to 1967, 21 dolphins of 3 species in total died from the erysipelas epidemic. From 1968 to 1978, the number of dead animal after transported within one month was decreased by the improvement of the transport techniques. From 1979 to 1989, the equipments of an endoscope and ultra sound started to be applied in dolphins. From 1990 to 2003, blood sampling began to be performed by husbandry behavior. The survival rate was improved for these periods. Therefore, the present study summarized over hundreds blood data during routine health examinations was analyzed from several points of view. Hematology and serum chemistry data were compiled from the routine health records of 26 bottlenose dolphins (11 born, 15 wild : 6 males, 20 females) maintained at Enoshima Aquarium Marineland. The Enoshima Standard ranges for hematology and serum chemistry data were established during clinical records for 19-year period (1990-2009).

The present study described how blood values changed before and after feeding with 3 hematological and 18 serum chemistry tests in bottlenose dolphins, *Tursiops truncatus*. *Clinical data* : A total of 286 blood samples were clinically examined from two male and four female adult dolphins from 1990–1997 ; 187 samples were taken between 09 : 00–10 : 00 before feeding, and 99 samples were taken between 13 : 00–14 : 00 after they had been fed 5.0–10.0 kg of mackerel. *Experimental data* : Blood samples from 2 female dolphins were taken at 09 : 00 before feeding and at 13 : 00 after they had been fed on 8.0 kg of mackerel on five separate times. On five other times, blood samples from the same dolphins were also taken at 09 : 00 and 13 : 00 before they had been fed any fish. In both data, it is concluded that TG and BUN will increase, and Cl decrease due to dietary factors. Experimental data, however, suggests that FFA and T-Bil will increase when an animal has not eaten.

To determine how blood values in bottlenose dolphins changed during the year, 504 blood samples were taken from 9 dolphins from 1991 to 1999 and clinical blood examinations were undertaken monthly including 3 hematological and 19 serum chemistry tests. In creatinine, significant seasonal changes were found among three groups of adult males, adult females and juve niles, and the average values in summer were 15–38 % higher than those in winter. In two out of three groups the average total cholesterol values were highest in winter, and the lowest of all groups were in summer. In two other groups the peaks of average FFA value were recorded in

*Supervisor : Prof. Toshiro ARAI

summer, and the lows were in winter.

In the present study values for total cholesterol and triglycerides were measured in 110 blood samples taken from 360 days pre-partum to 90 days post-partum in 10 parturitions of 6 bottlenose dolphins, and in 75 blood samples when the dolphins were not pregnant as a control group. The average total cholesterol values in the second (270 to 181 days pre-partum), third (180 to 91 days pre-partum) and fourth stages (90 days pre-partum to before parturition) and in the puerperium (after parturition to 90 days post-partum) were significantly higher than the average value of the control group by 11.0%, 30.2%, 19.3% and 13.4% respectively. The average triglycerides values for the third and fourth stages and in the puerperium were also significantly higher than those in the control group by 59.7%, 84.3%, and 42.1% respectively.

Plasma fibrinogen concentrations were measured in 136 blood samples from 360 days pre-partum to 90 days post-partum for 12 parturitions of 7 bottlenose dolphins and in 50 blood samples from the dolphins they were not pregnant as a control. The median concentrations increased gradually, and the values during the fourth stage were higher than those during the other stages. The concentrations during the third stage and the puerperium were almost the same. There were significant differences between the plasma fibrinogen concentrations during the third stage and those of the controls and between those of the fourth stages and the controls ($p < 0.01$). The concentrations peaked during the third stage in four cases and during the fourth stage in three cases.

A purulent pneumonia is the most common disease in captive cetaceans, and the most observed pathogen was Staphylococcus aureus. A female false killer whale, Pseudorca crassidens, estimated to be more than 25-years-old, died from chronic purulent pneumonia. A comparison of the total protein, fibrinogen, leukocytes and average monthly temperatures for 8 years revealed an increase in total protein, an increase in subsequent average monthly temperatures, and an increase in both fibrinogen and leukocytes. Three items without total protein were changed similarly. Alpha2-globulin of total protein contains the haptoglobin that is an acute-phase reactant. As a result, α2-globulin would be change at the early stage, and it might be suggested as the reaction of an inflammation.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 172–175, 2011.

Basic plan and background for the Food Safety Basic Act and the role of veterinary medicine and veterinary administrative systems in Japan

Naoto KITAMURA*

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

(Received 28 October 2010, VDO-No. 292)

In September 2001, Japan's first case of bovine spongiform encephalopathy (BSE) was confirmed. As a member of the House of Representatives of Japan as well as a veterinary specialist, the author immediately set up a BSE Countermeasures Headquarters in his political party and became deputy chief of the headquarters, as he then served as a deputy chair of the General Agricultural Policy Research Committee and chaired the Committee on Animal and Plant Quarantine and Food Safety. Through vigorous deliberation and studies on different possible measures, the committee engaged actively in a series of control measures, including a decision made to introduce BSE testing on cattle at slaughterhouses and a total ban on the import, production, shipment and use of meat-and-bone meal and other related feed and fertilizers. On October 18, 2001, a new system for BSE testing at slaughterhouses was thus inaugurated. We also undertook public relations activities and symposiums to provide accurate and scientific information about the disease to customers for the purpose of regaining their trust by communicating the risks. In an effort to identify the route of infection as soon as possible, we introduced a traceability system, dispatched an overseas investigation team, and launched a Special Committee for Food Safety. Working as a chief secretary of the special committee, I took part in the policymaking process for submitting a proposal on food safety.

The Japanese public had some anxiety and distrust about Japan's food safety administration due to problems with residual agricultural chemicals, food and feed additives, and other chemical substances, dioxins and endocrine disrupting chemicals (EDCs). After successive disgraceful incidents, including Escherichia coli O-157 food poisoning, the BSE outbreak, and false food labeling, people feel more victimized than ever before. The most significant duty for politicians under these circumstances is to protect public life and health. The challenge facing the Special Committee for Food Safety was exactly the responsibility facing the politics. Regrettably, it must be acknowledged in view of the current circumstances in Japan that it is very difficult to say that its food safety policies have been based on the standpoint of protecting consumers.

Japan's risk management failed to prevent the BSE outbreak, as the government lacked a sense of urgency when it came to food safety and prioritized producers with little regard for consumer protection.

To comprehensively review Japan's existing system on food safety, the Special Committee

* Supervisor : Prof. Hidetoshi IKEDA

gathered comments from a wide range of experts in different fields for extensive discussions, reporting its findings in the form of a proposal to Prime Minister Junichiro Koizumi and Chief Cabinet Secretary Yasuo Fukuda. Based on the notion that people have the right to seek safety, the report proposed an effective public administrative organization for food safety and called for the development and amendment of relevant laws and institutions, including a longer-term view, urging the government to take the proposal seriously and to put it into action.

This paper reviews the proposal submitted by the Special Committee for Food Safety, in which the author was involved, and examines the construction of legislation on food safety enacted in 2003 for the purpose of ensuring food safety in Japan. It compares food safety measures in Japan and several other countries, and proposes that Japan should adopt a food safety strategy in the near future that leads the world.

1. The Basic Design of Food Safety

Set up as an advisory body to the Minister of Agriculture, Forestry and Fisheries and to the Minister of Health, Labour and Welfare, the BSE Study Committee cited the absence of urgency with respect to food safety in the government sector and the detrimental impacts of the government's stance of prioritizing producers and disregarding consumer protection. These points are not confined to the BSE issues. They are true of overall public administration on food safety. To prevent additional problems like the BSE outbreak that would undermine food safety and to rebuild consumer confidence, it was necessary to radically change the public administrative organization responsible for this area. At the time of redesigning the organization, it was vital to place top priority on protecting consumers' health and to introduce to food safety administration the approaches for risk analysis that it has lacked, which consisted of, first, a risk assessment for a scientific evaluation of the risk ; second, risk management based on a comparison between the risk and the benefit and consideration of the social impact ; and third, risk communication for smooth information exchange and understanding between the government, scientific experts, and the public for encouraging mutual trust. It was also argued that the policies for ensuring food safety should be implemented separately from industrial development.

In November 2001, the Committee for Research and Review on BSE Issues was established. On April 2, 2002, it presented the problems and the points to be redressed and made a decision to institute a provisionally named Food Safety Basic Act in July 11, 2002. In December 2002, an outline of the bill for a Food Safety Basic Act was drawn up and public comments were collected. On February 7, 2003, the bill was approved in the Cabinet meeting and referred to the National Diet on the same day. As a result, the Food Safety Basic Act was finally instituted after confirmation of Japan's first BSE-infected cow and the Food Safety Commission was formed. This move built a platform longed sought by veterinarians, as scientists seeking objectivity and impartiality in policymaking on the basis of scientific findings.

2. Food Safety Measures in Foreign Countries

Food safety can have a significant impact on our lives or health. Unlike the case of an earthquake or a typhoon, any food safety incident must be addressed by taking action to minimize the health hazard to the public, with the appropriate and swift use of risk analysis approaches —risk evaluation, risk management and risk communication—to prevent the spread of the hazard and identify the causes, even if they are presently unknown. Believing that a review of overseas efforts in food safety would help Japan demonstrate global leadership in promoting a food safety strategy in the foreseeable future, the author collected and organized information on overseas food safety initiatives and then compared the results with Japanese measures. Paying particular attention to the fact that Western countries have public bureaus of veterinary

medicine or veterinary affairs, the author believed that Japan should also create a new specialist department or bureau to take positive action on food safety.

3. The Links Between Veterinarians and Legal Development

Traditionally, veterinarians have directly served the public or consumers solely as clinical veterinarians for domestic and livestock animals. Very few members of the general public were aware of the existence of laws, Cabinet orders, and ministerial ordinances governing veterinary practitioners. However, especially after the BSE outbreak, it is increasingly known that veterinarians work in different areas in association with food safety. It is now recognized that their professional activities range from farm to table, or in other words, from production to consumption. As a member of the House of Representatives and as a veterinarian, the author participated in the National Diet debates on legislation amendments in response to BSE issues and food safety concerns. On these occasions, the author stressed the importance of the duties and occupational roles that veterinarians have to fulfill and play. At that time, however, 100% BSE testing was introduced not as measure based on scientific findings but as an emergency step based on a political judgment. It does not guarantee safety from the viewpoint of a scientific veterinary specialist, but the author believed that it was the best possible solution given political realities, and so felt obliged to advise the Minister of Health, Labour and Welfare to implement the 100% BSE testing at the Budget Committee in the House of Representatives.

Given past developments in connection with the BSE outbreak, from legislative initiatives for food safety to the integration of consumer protection administration, future legal improvements should be based on a principle according to which it is the responsibility of the State to guarantee food safety, recognizing that food is essential to life. In fact, however, consumers remain concerned and skeptical about government policies on food safety. Among other concerns, the significance and the role of risk communication in risk analysis are not sufficiently understood. Veterinarians as scientists must represent the views of consumers and public as end beneficiaries from a fair and impartial standpoint on food safety. They have been fulfilling their obligations and playing their occupational roles in their individual domains and positions, and in doing so are making significant contributions, but it is undeniable that their services have not incorporated a consumer-oriented attitude based on the fundamental idea that food supports life. In response to the series of scandals that have occurred, they have been committed to discovery, revelation, investigation into causes, preventive measures, and warnings through veterinary practices to dietary medicine and food-based medical care on the basis of the notion that food is directly linked to life. Their contribution to human interests protected by law is comparable to that made by medical treatment. To enable veterinarians to help provide consumers with the safe food and peace of mind fundamental to dietary medicine and food-based medical care, many different players must unite under the same principle and embody that principal in practice. As a matter of course, compliance with relevant laws and ordinances must be respected. In addition, producers must manage their production farms in consideration of the global environment, animal welfare (which refers to livestock management with special attention to comfort), good agricultural practice (GAP) and hazard analysis critical control points (HACCP), among other aspects. Food processors and distributors are asked to carry out stringent quality control and food labeling. And veterinarians should not only give guidance and advice on them but also pay attention to the proper use of drugs for animals and to the safety of the feed given. They need to accumulate and provide accurate information on dietary medicine and food-based medical care. And especial-

ly as risk communicators and interpreters capable of offering plain information and explanations on scientific findings in risk assessment, they should endeavor to increase their activities of encouraging the public and consumers to carry out risk communications in their communities and facilitating such communications.

As discussed above, the public and consumers will continue to expect veterinarians to provide reassurance about food safety by building more confident relationships. It is vital that veterinarians act with integrity to meet these expectations.

As scientists, veterinarians should engage deeply in food safety and security, in food safety administration, in consumers' rights to seek safety and in animal welfare and take pride in that veterinary medicine or practice includes dietary medicine and food-based medical care. From the perspectives of food safety basics to human lives and human interests protected by law through animals, veterinarians as scientists, the Japan Veterinary Medical Association and institutions relating to education and research in veterinary medicine must be strongly aware of the need to contribute to enhancing public health and food safety in Japan by forming stronger ties and collaboration. Moreover, in consideration of international food safety initiatives, it is essential to enhance, strengthen, and reform veterinary medical education, to create a ministry or agency by means of furthering administrative integration, and to set up a bureau for veterinary medicine or veterinary affairs.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 176–177, 2011.

The study on evaluation and application of virus neutralizing test using an indirect immunoperoxidase technique for rabies

Takashi OGAWA*

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

(Received 2 December 2010, VDO-No. 293)

Rabies is a severe zoonotic viral disease that causes fatal encephalitis. On the other hand, rabies is believed to be one of preventable diseases because of human and animal vaccines which can prevent rabies developing. Especially, dog herd immunization is important public health measure since more than 90% of human deaths due to rabies are caused by rabid dogs.

An animal would not develop rabies if a virus neutralizing titer is more than 0.5 international units (IU) per ml after vaccination. Virus neutralizing tests are therefore useful to confirm the immune status, or vaccine efficacy, and are used as researches for herd immunity of dogs, serological tests for animal quarantine and efficacy tests for rabies vaccines. The international units of virus neutralizing titer are calculated by comparison with a titer of sample and a titer of reference serum which is distributed from World Health Organization (WHO) and World Organization for Animal Health (OIE). At the moment, the rapid fluorescent focus inhibition test (RFFIT) and the fluorescent antibody virus neutralization test (FAVN), which apply fluorescent antibody technique, are used as international virus neutralizing tests. On the other hand, a virus neutralizing test using an indirect immunoperoxidase technique (VNT-IIP) for rabies has been developed as a serological test for dogs and cats in Japan. The advantage is that the VNT-IIP does not need special equipment such as a fluorescent microscope because developed color in the microplate wells show visible results. It is easy to check a large number of microplates since diagnosticians can view the obtained results by the naked eye. The purpose of this study was to demonstrate the availability of the VNT-IIP.

A new method should be validated to show the availability. In this study, we validated the VNT-IIP and compare it with one of the international standard methods, the fluorescent antibody virus neutralization (FAVN) test. The VNT-IIP showed satisfactory repeatability, high analytical specificity, and good accuracy. Regarding the comparison between the VNT-IIP and the FAVN test, the VNT-IIP showed good agreement (91.9%), high diagnostic sensitivity (92.8%) as well as specificity (87.0%), and good correlation ($r=0.92$). As described above, the validation of the VNT-IIP was satisfactory and the performances of the test proved to be equivalent to those of an international standard method.

Stray dogs are a public health risk factor when canine rabies is endemic. The Rabies Prevention Law has introduced measures to control stray dogs, but many dogs are still captured in Japan. In order to estimate the immune status of stray dogs for the purposes of risk management, we conducted a serological survey at the Hyogo Prefecture Animal Well-being Center. Only

* Supervisor : Prof. Hidetoshi IKEDA

27.7% of dogs brought into the Center (n=166) had protective immune status. This result suggests that there is the potential for reintroduction of canine rabies into stray dogs, leading to endemic rabies and its transmission to humans. Continued removal of stray dogs, education on rabies prevention and vaccination of dogs therefore remain important public health issues.

All dogs in international transit should have a veterinary certificate on vaccination and serological test for preventing spread of rabies. To demonstrate the efficacy of a vaccination program for Japanese animal quarantine, we studied antibody titers of 3-month-old dogs immunized with Japanese vaccine (RC-HL strain) at 1-month interval booster inoculation by VNT-IIP. The protective titers (>0.5 IU/ml) were sustained for 15 months. This result indicated that the risk that dogs might develop rabies would be reduced by this vaccination program.

The public health problem is that rabies virus will transmit from wildlife directly to human, or indirectly to human through pets or domestic animals. It has been often pointed out that rabies will be introduced into Japan by illegal dogs from foreign ships, and there is consequently concern that rabies might infect wildlife or stray dogs from rabid dogs. To evaluate an oral vaccine for wildlife, we studied antibody titers of dogs immunized with SAG-2 strain vaccine by VNT-IIP. The protective titers were maintained for 18 months. Because the protective immune period is one of factors to determine the frequency of vaccination campaign, this result would be important data to discuss it. In addition, there are some reports that oral rabies vaccines are useful for dogs, and our result similarly supported the efficacy of SAG2 vaccine for dogs.

In this study, we reported the validation of VNT-IIP and the VNT-IIP application for research of dog herd immunity for rabies, evaluation of rabies vaccination program, and assessment of rabies vaccine efficacy. Taken together, the VNT-IIP was thought to be useful method for a virus neutralizing test. In the future, we will use the VNT-IIP for several investigations and encourage broad use, and consequently make a contribution to improve public health.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 178–180, 2011.

Studies on the spermatogenic function in the cats

Hiromichi OBA*

Laboratory of Veterinary Reproduction,
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Received 2 December 2010, VDO-No. 294)

Female cats are seasonal breeders, and their breeding season (BS) in Japan is January through August. They do not enter estrus under lighting for 8 hours or shorter in captivity, but do so under lighting for 12 hours or longer. Male cats maintain spermatogenesis even during the non-breeding season (NBS) of female cats, showing that they are not seasonal breeders. However, whether spermatogenesis and sex hormones are influenced by the duration of daylight, similarly to female cats, is controversial among researchers. The electroejaculation method (EEM) is generally used to collect semen from male cats, but it requires general anesthesia, which markedly burdens the animal and cannot be frequently performed. Moreover, problems have been pointed out, such as a reduction in the number of sperm, increased seminal plasma, and urinary contamination, compared to semen collected employing the artificial vagina method (AVM). Therefore, to closely observe semen qualities and spermatogenesis in male cats, semen collection employing AVM is essential. However, AVM requires the training of male cats for semen collection, and a female cat in estrus is necessary each time. Because of these difficulties in semen collection employing AVM, spermatogenesis in male cats has not been investigated. In this report, spermatogenesis was investigated in male cats in the following 5 chapters : After Introduction in Chapter 1, the influences of seasons on semen qualities and plasma sex hormones in male cats are investigated in Chapter 2, changes in qualities of semen consecutively collected from male cats in Chapter 3, sperm productivity in male cats in Chapter 4, and the influences of ejaculation interval on semen qualities in elderly cats in Chapter 5.

1. **Seasonal influences on semen qualities and plasma sex hormones in male cats (Chapter 2)**

Male cats are continuous breeders, unlike female cats, but whether or not semen qualities and plasma sex hormones are influenced by BS (January-August) and NBS (September-December) of female cats has not been clarified. Thus, the plasma LH and T levels were measured in male cats in March-April and October-November, and the middle of BS and NBS of female cats, respectively. At the same time, the qualities of semen collected from identical male cats employing AVM were investigated. For the investigation of the plasma sex hormone levels and semen qualities, semen was collected from 5 male cats aged 3.2–3.8 years 10 times at 3-day intervals. On semen quality testing, the semen volume, sperm motility and viability, number of sperm, and incidences of abnormal and immature sperm were measured. The male cats were maintained in individual cages in an animal room controlled at a room temperature of 23±2℃ under natural lighting.

All semen quality items were significantly higher in BS than in NBS ($p < 0.01$). The plasma sex hormone levels were also significantly higher in BS than in NBS ($p < 0.01$).

Supervisor : Prof. Toshihiko TSUTSUI

It was clarified that although male cats are continuous breeders, the sex hormone levels and semen qualities were significantly higher in BS of female cats than in NBS.

2. Changes in semen qualities with consecutive ejaculation in cats (Chapter 3)

Although it is known that several copulations are necessary to induce ovulation and achieve conception in cats, the changes in semen qualities with consecutive ejaculation have not been clarified. Five male cats aged 3.0–5.0 years ejaculated 4 times consecutively using AVM, and changes in the semen qualities were investigated. Semen collection was performed 4 times at 2-week intervals.

The semen volume slightly decreased as ejaculation repeated, but no significant change was noted. The number of sperm decreased with repeated ejaculation. The mean number±SE in the 1st ejaculated semen was $8.3\pm0.4\times10^7$, accounting for 55.0±3.1% of the total number collected from 4 ejaculations on average, and significantly different from those in the 2nd–4th ejaculation ($p<0.01$). Sperm motility decreased with repetition of ejaculation and the level was significantly lower in the 3rd and 4th than in the 1st semen ($p<0.01$), but no significant difference was noted between the 1st and 2nd semen. Sperm viability slightly decreased with repeated ejaculation, and that in the 4th semen was significantly lower than that in the 1st semen ($p<0.05$). The incidences of abnormal and immature sperm slightly increased with repetition, but changes were not significant.

When male cats ejaculated 4 times consecutively, the number of sperm significantly decreased with repetition, and the number in the 1st semen accounted for 55.0±3.1% of the total number. Sperm motility and viability significantly decreased with the repetition of ejaculation, but the incidences of abnormal and immature sperm only slightly increased.

3. Sperm productivity of cats (Chapter 4)

Male cats repeatedly copulate even though sperm is mostly ejaculated the first 2 times, and this may be necessary to raise the ovulation induction rate through copulatory stimulation, rather than to achieve conception. It has been clarified that every other day or a longer interval is necessary for male cats to ejaculate semen with stable qualities. However, spermatogenesis, i.e., sperm productivity per day, in male cats has not been clarified. Thus, sperm levels were exhausted (the number of sperm was smaller than 1×10^7) on consecutive ejaculation in 5 male cats aged 4.5–5.2 years, followed by exhaustion again after 2, 4, 6, 8, and 10 days, and the number of days required to recover the number of sperm before exhaustion was investigated. A 14-day resting period was established after the 2nd exhaustion before the next experiment.

On an exhaustion test after 14-day resting, 5.5±0.3 ejaculations were necessary on average to exhaust the sperm, and no significant difference was noted among the groups which rested for 10–14 days. When the resting period was set to 2 days, sperm was exhausted by 3.8±0.4 ejaculations on average, showing a significant difference compared to that after 14-day resting ($p<0.05$). The mean numbers of ejaculated sperm after various durations of resting were $21.0\pm2.2\times10^7$ in the control group which rested for 14 days, $17.9\pm2.2\times10^7$ in the 10-day resting group, and there were similar numbers in the 8- and 6-day resting groups, showing no significant difference among these 4 groups. In the 4-day resting group, the mean number was $11.6\pm1.1\times10^7$, which was significantly different from those in the control and 8-day resting groups ($p<0.05$). The mean number in the 2-day resting group was $6.4\pm0.5\times10^7$, showing significant differences from those in the 4 groups rested for 6 days or longer ($p<0.01$). It was clarified that male cats required 6 days after exhaustion to recover the number of sperm before exhaustion.

Based on these findings, the mean sperm productivity per day was 3.0×10^7 in male cats.

4. Influence of ejaculation interval on semen qualities in elderly cats (Chapter 5)

It has been clarified that stable semen qualities

can be maintained in male cats when the ejaculation interval is every other day or longer, but spermatogenesis in elderly cats has not been clarified. Elcock and Shoning observed the histology of the testis and epididymis with aging in male cats, in which Sertoli cells, spermatogonia, and spermatocytes were degenerated in cats aged 7 years or older, suggesting a decrease in spermatogenesis. Thus, using 4 elderly cats aged 7.6–9.0 years, we investigated the ejaculation interval at which semen with stable qualities can be collected. Semen collection through 2 consecutive ejaculations using AVM was performed 10 times every day, every other day, and at 3-day intervals.

In semen collected every day, the number of sperm decreased from the following day, and collection was difficult after the 6th day. In semen collected every other day and at 3-day intervals, no changes were noted in sexual behavior or the ejaculatory capacity. The semen volume, sperm motility and viability, and incidence of immature sperm were mostly stable in semen collected every other day and at 3-day intervals, showing no significant differences due to the collection interval or frequency of collection. The number of sperm decreased in semen ejaculated every other day, but it was stable in semen collected at 3-day intervals and significantly higher than that in semen collected every other day ($p < 0.01$). The incidence of abnormal sperm was significantly higher in semen collected every other day than in that collected at 3-day intervals ($p < 0.01$).

Based on the above findings, to maintain stable semen qualities in elderly cats, 3-day ejaculation intervals are necessary.

Although male cats are continuous breeders, the plasma sex hormone levels and semen qualities were significantly higher in BS than in NBS of female cats. Male cats consecutively copulate, but sperm was mostly ejaculated by the 2nd ejaculation. Spermatogenesis was observed by exhausting sperm through consecutive ejaculation and investigating the number of days required to recover the level before exhaustion. The mean sperm productivity per day in male cats was 3.0×10^7. However, spermatogenesis decreased in elderly cats, and 3-day ejaculation intervals were necessary to maintain stable semen qualities.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 181–183, 2011.

Studies on artificial insemination with feline cryopreserved semen

Tatsuji MIZUTANI*

Laboratory of Veterinary Reproduction,
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Received 2 December 2010, VDO-No. 295)

The first reported artificial insemination (AI) with frozen feline semen was performed employing intravaginal insemination (IVI) by Platz *et al.* in 1978, and the conception rate was only 10.7% (6/56). Since then, there have been only 3 and 1 report on AI with ejaculated semen and frozen epididymal sperm employing intrauterine insemination (IUI), respectively. This has been attracting attention as an artificial reproduction technique for not only domestic cats but also wild feline animals on the brink of extinction.

Feline semen is generally collected employing the electroejaculation method (EEM) or artificial vagina method (AVM), but problems with EEM have been pointed out, such as urinary contamination, an increase in seminal plasma, and reduction of the number of sperm. The AVM causes no problem in terms of semen qualities, but it requires the training of male cats for semen sampling and an estrous female cat for mounting.

Frozen feline semen has been investigated with regard to the diluent composition, cryoprotective agent, and packaging, freezing, and thawing methods. For low-freezable swine and canine semen, it has been clarified that sperm acrosome is protected and sperm motility is maintained after thawing when a surfactant, Equex STM paste, is added as a cryoprotective agent in addition to glycerin. However, the usefulness and concentration of these have not been investigated. Moreover, many areas remain to be investigated, such as the equilibration time with glycerin and duration of sensitization with liquid nitrogen vapor.

We investigated the usefulness of the addition of Equex STM paste as a cryoprotective agent, in addition to glycerin, for frozen feline semen to establish an AI method which achieves a higher conception rate.

1. **Usefulness of Equex STM paste as a cryoprotective agent for frozen feline semen (Chapter 2)**

The usefulness of the addition of Equex STM paste as a cryoprotective agent in addition to glycerin for frozen feline semen was investigated. In addition to 7.0% glycerin, Equex STM paste was added at concentrations of 0, 1.0, 2.0, and 4.0% (v/v), and semen qualities after thawing were observed. Frozen semen was prepared using the simple-type quick LNG freezer (LNGF) developed for cattle. Sperm motility after thawing was significantly higher in the 1.0% Equex STM paste group than in the other 3 groups ($p < 0.01$). Sperm viability was not significantly different among the 0, 1.0, and 2.0% Equex STM paste groups, but significantly lower in the 4.0% group than in these 3 groups ($p < 0.01$).

Based on the above findings, the addition of Equex STM paste to feline ejaculated semen was effective to maintain sperm motility after thawing, and 1.0% was the optimum concentration.

* Supervisor : Prof. Toshihiko TSUTSUI

2. Usefulness of sodium lauryl sulfate as a cryoprotective agent for frozen feline semen (Chapter 3)

The addition of 1.0% Equex STM paste to feline semen significantly maintained sperm motility after thawing compared to that without addition. The composition of Equex STM paste is unclear, but the main component has been clarified as sodium lauryl sulfate (SLS). Thus, we investigated the effectiveness of SLS added to feline semen as a cryoprotective agent, in addition to 7.0% glycerin. SLS was added to feline semen at 0, 1.0, 2.0, 3.0, and 4.0 mg/ml, and the semen qualities after thawing were evaluated. A 1.0% Equex STM paste group was established as a control, and the experiment was performed involving 6 groups in total. The protection of sperm acrosome by SLS was also investigated after thawing.

In Experiment 1, the semen qualities after thawing were compared among the 0–4.0 mg/ml SLS and 1.0% Equex STM paste groups (6 groups in total). Sperm motility after thawing was significantly higher in the 3.0 mg/ml than in the 0–2.0 mg/ml SLS groups ($p < 0.01$). On comparison with the 4.0 mg/ml SLS group, sperm motility was higher in the 3.0 mg/ml SLS group, but the difference was not significant. No significant difference was noted between the 1.0% Equex STM paste and 3.0 mg/ml SLS groups.

In Experiment 2, the influence of SLS at the optimum concentration (3.0 mg/ml) identified in Experiment 1 and 1.0% Equex STM paste on sperm acrosome was investigated employing the triple-stain technique (TST). The rate of acrosome-retaining viable sperm after thawing was significantly higher in the Equex STM paste group compared to the non-added group ($p < 0.01$). In the SLS group, the rate was higher than that in the non-added group, but the difference was not significant.

Based on the above findings, the optimum SLS concentration for frozen feline semen was 3.0 mg/ml. However, no significant difference was noted in the acrosome retention rate after thawing, although the value was higher in the added than in the non-added group.

3. Artificial insemination with Equex STM paste- and sodium lauryl sulfate-added frozen feline semen (Chapter 4)

Unilateral intrauterine inseminations (UIUI) with frozen feline semen plus 1.0% Equex STM paste and 3.0 mg/ml SLS were performed, and the conception rate was compared. In UIUI, 100 IU of hCG was administered on day 3 or 4 of estrus to induce ovulation, and 40×10^6 sperm were used to inseminate the uterine horn on the side with a greater number of mature ovarian follicles 20 hours after hCG administration. The conception rate was 70% (7/10) in the Equex STM paste group and 30% (3/10) in the SLS group, showing a higher rate in the Equex STM paste group, but the difference was not significant ($p = 0.1789$).

Based on the above findings, although the semen qualities after thawing were not significantly different between the 2 groups, the conception rate was higher in the Equex STM paste (70%) than in the SLS (30%) group.

4. Preparation of frozen feline semen employing the plunging method (Chapter 5)

We have been employing the LNGF method for freezing feline semen, in which straws are maintained vertically above liquid nitrogen. Since the LNGF is expensive, we investigated the plunging method in which straws are held above but in parallel with the liquid nitrogen surface and then directly plunged into it.

In Experiment 1, 1.0% Equex STM paste was added as a cryoprotective agent in addition to 7.0% glycerin, and semen was used to fill in straws. The straws were sensitized with liquid nitrogen vapor at 7cm above the liquid nitrogen surface for 0, 5, 10, 15, or 20 minutes and then plunged into it to prepare frozen semen. Sperm motility and viability after thawing were significantly decreased in the group sensitized with liquid nitrogen vapor for 0 minutes compared to those in the other 4 groups sensitized for 5–20 minutes ($p < 0.01$), but no significant difference

was noted among these 4 groups. In Experiment 2, the semen qualities after thawing were compared between the group sensitized with liquid nitrogen vapor for 10 minutes, which showed a high sperm motility after thawing in Experiment 1, and frozen semen prepared employing the LNGF method. Sperm motility and viability after thawing were significantly higher in the group prepared employing the plunging method than in the group prepared using the LNGF ($p < 0.01$).

The above findings clarified that the slow freezing of feline semen by keeping straws at 7cm above and in parallel with the liquid nitrogen surface for 10 minutes followed by plunging into it was significantly more effective than the previous rapid freezing technique employing the LNGF method.

5. Intravaginal insemination with frozen feline semen prepared employing the plunging method (Chapter 6)

Non-surgical IVI is anticipated for AI with frozen feline semen, but there has been only one report, and the conception rate in it was low. Thus, we investigated the number of sperm required to achieve conception by IVI using frozen semen prepared employing the plunging method. In IVI, 160×10^6, 240×10^6, and 320×10^6 sperm were inseminated, and the conception rates were 27.3% (3/11), 33.3% (3/9), and 77.8% (7/9), respectively, showing no significant difference among the 3 groups ($p = 0.0549$).

Based on the above results of IVI with frozen feline semen prepared employing the plunging method, a high conception rate (77.8%) was achieved when 320×10^6 sperm were inseminated.

It was clarified that the addition of 1.0% Equex STM paste as a cryoprotective agent, in addition to 7.0% glycerin, is useful to maintain semen qualities after the thawing of frozen feline semen. Regarding the freezing method, semen frozen by keeping straws at 7 cm above and in parallel with the liquid nitrogen surface for 10 minutes followed by plunging them into it showed significantly superior sperm qualities after thawing compared to semen frozen employing the previous LNGF method, and a high conception rate was achieved when this semen was used for IVI at a sperm count of 320×10^6.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 184–185, 2011.

Telomere dynamics and aging of teleost medaka, *Oryzias latipes*

Hitoshi HATAKEYAMA*

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

(Received 7 January 2011, VDO-No. 296)

1. Introduction

The telomeres of vertebrates consist of a simple repeating sequence of six bases, 5'-TTAGGG-3', located at the ends of chromosomes, and are thought to protect them from degeneration and reconstruction. In most vertebrates, including humans, telomeres of somatic cells become progressively shorter with every round of cell division, and this seems to limit the proliferative life span of the cells when serially cultured *in vitro*. When they approach this limit (so-called Hayflick's limit), the cells cease to divide and exhibit a state of replicative senescence. Because rapidly replaced tissues would show a higher number of cell divisions with age, it is considered that the telomeres of such cells would shorten more rapidly with age *in vivo*. In fact, age-related alteration of telomeres has been demonstrated in mammals (mostly humans) and birds.

It is well known that the telomeres of somatic cells shorten with each cell division, and that telomerase for the synthesis of new telomeres *de novo* in stem, progenitor and tumor cells can compensate for this telomere shortening. These findings suggest that telomere shortening in the absence of telomerase acts as a mitotic clock for replicative senescence in normal somatic cells (the so-called telomere hypothesis). On the other hand, considerable levels of telomerase activity have been detected in somatic tissues of normal rodents and birds. Thus, human studies of telomeres and telomerase have been hampered by a lack of suitable fully representative animal model systems. To extend the telomere hypothesis to vertebrates other than humans, further examination of the relationship between telomeres and telomerase is required. To assess the relationships among growth, ageing, telomeres and telomerase, I selected the medaka (*Oryzias latipes*), a small freshwater teleost fish, for the present study because of the relative paucity of reports focusing on the telomere and telomerase biology of fish.

2. The growth, senescence and life span of the medaka

I provided data pertaining to the survival rate, rate of increase in body length and weight, morphological observations over time, and the sequential appearance of age-related characteristics. All the medaka studied died within 5 years. Body length increased significantly with age, confirming that medaka grew continuously throughout life. When the population was divided by age, the growth rate in terms of body length was higher in immature fish than in mature fish. Body weight also increased significantly with age, confirming that growth was continuous. The two sequentially appearing hallmarks of ageing, an abnormal hunchback profile and a pale colour, were apparent in fish 3 years of age or older.

* Supervisor : Prof. Hiroetsu SUZUKI

3. The quantitative analysis of telomere length

I assessed telomere length by Southern blot analysis. The TRF (terminal restriction fragment) length (as telomere length) in samples of whole embryos, the whole body of juvenile medaka aged 1, 3 and 6 months, and the systemic organs of adults showed considerable heterogeneity among age-matched samples, even at the earliest stage examined. This indicated that telomere length diversity was largely attributable to genetic factors. Scatter plot analysis demonstrated that the TRF lengths for both whole-body samples at the developing stage and for five organs during adulthood became significantly reduced with age. Detailed regression analyses for individual life stages showed that young medaka at the developing stage had a higher rate of telomere shortening. On the other hand, the rates of decrease in telomere length for organs of mature individuals were lower. Although TRF lengths were significantly correlated in the various organs of any given individual, there was no significant age-dependent decrease of TRF length in the brain, similar to the situation reported in humans.

4. The quantitative analysis of telomerase activity

I assessed telomerase activity by TRAP (Telomeric Repeat Amplification Protocol) assay using samples from the embryonic stage to senescence. Distinct telomerase activity was detected in all extracts derived from the medaka tissues examined, irrespective of age, with one exception : most samples of intestine. I therefore assayed for the possible presence of a *Taq* DNA polymerase inhibitor in intestine samples using a modified TRAP method, and confirmed that such an inhibitor was present. Therefore, the data suggested that the intestine of medaka does contain telomerase activity, although it is masked. Furthermore, the telomerase activity in most of the samples from this species was about 2-4 times higher than that in a human cancer cell line (SiHa cells) used as a positive control. This suggested that fish constantly require telomerase as a growth-inducing factor regardless of telomere shortening, as fish grow throughout life.

5. Conclusion

High telomerase activity was detected in almost all samples from medaka, suggesting that fish express telomerase activity in all organs throughout their entire life span. Telomere attrition with age was confirmed in medaka, despite the lifelong expression of telomerase. This phenomenon can be explained by continuous cell turnover, during which telomerase levels are insufficient to maintain telomere length.

Precise comparison of the TRF length at particular life stages of medaka revealed that the rate of telomere shortening during the developing stage was distinctly higher than that in adulthood. Therefore, telomere attrition seems to be inversely correlated with body length increase. These findings suggest that this fish does not maintain its telomere length throughout life, even though it possesses continuous telomerase activity, especially during the stages of rapid growth and development.

The present study showed that TRF length in the medaka was approximately 7-15 kbp and varied between individual broods. Thus on an individual basis, this fish shows intrinsic differences in telomere length. Furthermore, in the various organs of any given individual, telomere lengths were significantly correlated. Intriguingly, the regression line for the TRF in the brain was almost flat, and showed almost no correlation with those of other organs within any given individual, suggesting that innate telomere length was maintained in the brain of this fish.

The present data represent the first of their kind for a fish species to date, and should be useful for further studies of telomere and telomerase biology.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 186–188, 2011.

Studies on the pharmacologic effects of butorphanol tartrate and its use in postoperative analgesia in dogs

Kimiyo URANO*

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

(Received 11 March 2011, VDO-No. 297)

Appropriate postoperative pain reduction not only relieves the surgical stress-induced distress of patients, but also prevents a delay in surgical wound healing and complications of the circulatory and respiratory systems, improving the postoperative course. The importance of postoperative pain control has recently been rapidly recognized in small animal clinics, similarly to human medical care, and it has been actively coped with. Currently, various drugs are available as analgesics for veterinary medical care, such as opioids, nonsteroidal anti-inflammatory drugs (NSAIDs), local anesthetics, and N-methyl-D-asparate (NMDA) receptor inhibitors. Since the severity of postoperative pain varies depending on the severity of tissue injury, surgical region, procedure, and the patient age, analgesics should be selected corresponding to the patients. In addition, new concepts of analgesia, such as pre-emptive analgesia and maltimodal analgesia, have recently been proposed, and knowledge of analgesics and analgesia necessary to devise an analgesic plan has become complex.

Opioids play the central role as postoperative analgesics in the veterinary surgery field. Butorphanol is not habit-forming, its handling is simple because it is not subjected to the legal control of narcotics, and it is safe compared to narcotic opioids, such as morphine, suggesting that butorphanol is one of the most applicable analgesics in the veterinary field. However, only a few detailed pharmacology studies of butorphanol in dogs have been reported, and it is used based on experience in veterinary clinical practice in the current situation.

In this study, we paid attention to butorphanol as a perioperative analgesic for dogs. To estab lish an administration method, the pharmacokinetics and pharmacology of the drug were investigated, and safety for clinical use was confirmed. In addition, the effect of combination with NMDA receptor inhibitors and NSAIDs used to control pain was investigated. The results of this study are summarized below.

1. **Pharmacokinetics and pharmacology of butorphanol in dogs and optimum dose setting (Chapter 2)**

The recommended dose of butorphanol for dogs specified in the literature on veterinary anesthesiology is wide (0.1–1.0 mg/kg), information on the dose-dependent potentiation of the analgesic effect is insufficient, and no optimal dose has been established. We administered butorphanol to dogs at 0.05–0.4 mg/kg, and identified the optimal dose by investigating the pharmacokinetics at each dose and evaluating the analgesic effect. Safety of the drug was also assessed by observing the presence or absence of a butorphanol-induced adverse effect. In dogs, the minimum effective dose of butorphanol for postoperative intramuscular injection was 0.1 mg/kg, and no marked potentiation of the anal-

* Supervisor : Prof. Masahiro TAGAWA

gesic effect could be expected even when the dose was increased from the minimum effective dose (0.1 mg/kg). The analgesic effect persisted for about 6 hours, but the effective blood level was maintained for only about 0.5–3 hours, suggesting that administration before the peak of postoperative pain inhibited complex pain and maintained the analgesic effect after the blood level dropped below the effective concentration. The analgesic effect of butorphanol alone was not necessarily strong, and the consideration of maltimodal analgesia or pre-emptive analgesia may be necessary for surgeries expected to cause moderate or severer postoperative pain. Regarding adverse effects, mild bradycardia strongly suspected as having a causal relationship with butorphanol occurred in only 2 animals, showing that the drug is a safe analgesic.

2. Time-course influence of butorphanol on autonomic nerve activity in dogs

Part 1 : Comparison between high- and low-dose butorphanol/influences combinations of butorphanol with droperidol and atropine (Chapter 3)

To use opioids, it is necessary to investigate the influence on autonomic nerve activity to manage circulation and improve the safety of anesthesia because opioids inhibit the cardiovascular function by enhancing vagal activity in many animal species. Reportedly, non-narcotic analgesics including butorphanol inhibit the cardiovascular function only relatively weakly, but butorphanol-associated severe bradycardia may be occasionally encountered in clinical practice. Thus, we assessed the influences of high- and low-dose butorphanol on the autonomic nervous system in beagles employing spectrum analysis using Holter electrocardiography. The influences of combinations with droperidol and atropine were also investigated. It was suggested that butorphanol tended to enhance parasympathetic nerve activity in a dose-dependent manner, but did not influence sympathetic nerve activity in dogs. It was also confirmed that autonomic nerve activity was not affected by butorphanol at the minimum effective dose (0.1 mg/kg). In addition, neither butorphanol alone nor its combination with doroperidol influenced autonomic nerve activity or the heart rate, showing the safety of the combination of these 2 drugs. Furthermore, combination with atropine rapidly inhibited parasympathetic nerve activity and enhanced sympathetic nerve activity, reconfirming that atropine is an effective thera peutic drug for butorphanol-associated bradycardia.

3. Time-course influence of butorphanol on autonomic nerve activity in dogs

Part 2 : Influence of butorphanol pretreatment on tiletamine/zorazepam anesthesia (Chapter 4)

Dissociative anesthetics exhibit an excessive irritant action on the cardiovascular system by enhancing sympathetic nerve activity, to which attention should be paid in patients with heart disease. We showed in our previous studies that butorphanol tends to enhance parasympathetic nerve activity in a dose-dependent manner, suggesting that the combination of butorphanol with a dissociative anesthetic reduces enhanced sympathetic nerve activity-induced excess vascular reactions. Thus, in this study, butorphanol was administered as pretreatment before general anesthesia with a dissociative anesthetic, tiletamine, and a benzodiazepine, zorazepam, and the influence on the autonomic nervous system was investigated employing spectrum analysis using Holter electrocardiography. At the same time, the influence of butorphanol on tiletamine-induced adverse reactions at arousal was also investigated. It was suggested that butorphanol pretreatment reduced the tiletamine-induced enhancement of sympathetic nerve activity and inhibition of parasympathetic nerve activity and increases in the heart rate and cardiovascular irritant reactions accompanied by blood pressure elevation. This effect persisted for a short time (about 30 minutes). Although no effect on adverse reactions developed at arousal from gen-

eral anesthesia with tiletamine/zorazepam was observed, considering the short duration of the actions on the autonomic nerve activity and cardiovascular system, additional treatment at the time of arousal or pre-treatment with more high-dose butorphanol may be effective.

4. **Comparison of the analgesic effect of pre- and postoperative butorphanol administrations in dogs treated with orchiectomy and ovariohysterectomy (Chapter 5)**

The analgesic effect of postoperative butorphanol alone was insufficient for moderate or severer postoperative pain in our previous studies, suggesting that the investigation of pre-emptive analgesia or maltimodal analgesia is necessary to obtain a favorable effect of a butorphanol-centered analgesic plan. In this study, the efficacy and safety of preoperative butorphanol administration (pre-emptive analgesia) and combination with ketamine were investigated in dogs by comparing the effect of pre- and postoperatively administered butorphanol on pain after orchiectomy and ovariohysterectomy. It was suggested that, to reduce postoperative pain, it is most important to administer butorphanol so as to elevate the blood level immediately after arousal, at which time the animal strongly perceives pain. It was con cluded that combined analgesia with butorphanol and ketamine is safe for dogs and markedly effective for postoperative pain in surgeries expected to cause mild postoperative pain, but the analgesic effect is insufficient for those expected to develop moderate or severer postoperative pain, especially visceral pain-dominant surgeries.

5. **Comparison of analgesic effects among butorphanol alone, meloxicam alone, and the combination of these in dogs treated with soft tissue surgery (Chapter 6)**

Butorphanol and meloxicam, classified as NSAIDs, exhibit effects through different analgesic action mechanisms, and the combination of these is expected to exhibit a complementarily potentiated analgesic effect based on the concept of maltimodal analgesia. Thus, the effect of reducing postoperative pain was compared among butorphanol alone, meloxicam alone, and the combination of these in dogs treated with soft tissue surgery. Compared to the effect of butorphanol alone on pain after soft tissue surgery, meloxicam alone or the combination of butorphanol and meloxicam exhibited a more favora ble analgesic effect. The combination of butorphanol and meloxicam and meloxicam alone showed the favorable inhibition of postoperative pain, but no complementary potentiation of the analgesic effect was observed. However, it was suggested that the combination may effectively inhibit stress reactions, such as cortisol production and blood pressure elevation after arousal.

Information on butorphanol collected in this study may facilitate perioperative pain control in dogs, and the results on efficacy and safety demonstrated its usefulness as analgesia for veterinary medical care.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 189–191, 2011.

Diagnostic studies on acute pancreatitis in dogs

Tomoyuki OHMURA*

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

(Received 11 March 2011, VDO-No. 298)

Acute pancreatitis in dogs is a disease that puzzles veterinarians because its clinical mani festations and examination findings vary significantly depending on time since onset and severity. Currently, the diagnosis of acute pancreatitis is made based on clinical manifestations, complete blood cell count (CBC) and other blood chemistry data, and imaging test results. However, changes over time in blood chemistry parameters and imaging data have not been assessed thoroughly. This study examined blood chemistries, imaging data and their changes over time using dogs in which acute pancreatitis was induced and in clinical cases.

1. Inducement of pancreatitis

Despite an increase in the number of acute pancreatitis cases in dogs due to the recent prevalence of canine obesity, it is difficult to secure sufficient clinical cases for a complete study, and, in addition, there are various restrictions on the use of patient dogs. Further, the various breeds, genetic backgrounds and environmental factors make a detailed investigation difficult. Therefore, we induced pancreatitis experimentally in dogs and conducted a range of investigations. This study aims to examine changes in CBC and blood chemistries in dogs with early- to mid-stage acute pancreatitis and to assess their diagnostic values by conducing imaging studies, such as X-ray examination, ultrasonography, and computed tomography (CT). We validated the induced pancreatitis models based on the following criteria : similar characteristics as spontaneous acute necrotizing pancreatitis, original anatomy is not altered, small inter-individual variation, and high reproducibility. Based on a literature review, we used a pancreatitis inducing method in which a solution of trypsin and taurocholate is injected into the pancreatic duct in beagle dogs. The models we created showed conditions extremely similar to those of typical clinical examples. Their clinical manifestations and blood chemistries were very similar to those of clinical cases of acute pancreatitis, and the models were histopathologically confirmed to have acute hemorrhagic necrotizing pancreatitis. Thus, the acute pancreatitis model dogs were used in subsequent experiments.

2. Blood and ascites findings in the induced acute pancreatitis dogs

Pancreatitis was induced in healthy adult beagles using the method described above. Their clinical manifestations were observed, and their CBC and blood chemistries were determined on day 0 (before pancreatitis was induced) and on days 1, 2, 3, 7 and 14 (after treatment), and ascites was examined on day 3. On day 3, conditions consistent with acute abdomen were observed, and CBC and blood chemistries showed neutrophilia with a shift to the left and a significant increase in amylase and lipase. This increase in amylase and lipase was, when considered in combination with clinical manifestations, an important base for the diagnosis of acute pancreatitis. In addition, the amylase and lipase levels in the ascites on day 3 were significantly higher than those in the serum, confirming their very high diagnostic value. The general condi-

* Supervisor : Prof. Hiromitsu ORIMA

tions stabilized over time, and the levels of pancreatic enzymes did not increase after day 14. Pathologically, day 3 was considered to be when the animal models survived acute hemorrhagic necrotizing pancreatitis, and day 14 was considered to be when the models survived acute pancreatitis. Based on these results, the diagnosis of acute pancreatitis by clinical manifestations, CBC and blood chemistries was possible in its early stage. However, the diagnosis by clinical manifestations or blood chemistries alone became difficult as the disease progressed.

3. X-ray findings in the experimental acute pancreatitis dogs

X-ray is the most commonly used diagnostic imaging method, and it is important to determine its usefulness and limitations for acute pancreatitis diagnosis. We examined X-ray imaging findings for acute pancreatitis using the experimental pancreatitis dogs. Plain X-ray examination of the abdomen and an upper GI series were conducted on day 0 (before pancreatitis was induced) and on days 3 and 9 after the treatment. Reduced radiolucency in the front-right abdomen, an unclear mass shadow, the fixation images of the stomach and the duodenum, and an increase in shadow density in the small bowel barium contrast test were characteristic to the X-ray examination of the induced acute pancreatitis dogs, and similar findings were observed in clinical examples. Although these findings were sufficient to suspect pancreatitis, it was difficult to diagnose acute pancreatitis based on these X-ray findings alone. However, since severe acute pancreatitis animals present the above mentioned findings and X-ray findings allow the elimination of other distinguishable causes for acute abdomen, X-ray findings combined with the results of physical examination and blood chemistries narrow the differential diagnosis list. Therefore, X-ray diagnosis is quite useful in the diagnosis of acute pancreatitis.

4. Ultrasonography findings for the experimental acute pancreatitis dogs

After X-ray imaging, ultrasonography is the most commonly used technique in clinical settings. Using the experimental pancreatitis dogs, we conducted abdominal ultrasonography on day 0 (before pancreatitis was induced) and on days 3, 7 and 14 after treatment to examine the usefulness of ultrasonography. Evaluations were made on days 0 and 3 on the visualization capability of ultrasonography in the preabdominal midsagittal, preabdominal transverse and side transverse directions. Scanning in the side transverse direction produced the best results. This scanning method allowed the visualization of the aorta, postcava and portal vein through the liver, and, using them as markers, the pancreas was located to the left of the duodenum. However, regardless of the scanning method used, it was difficult to visualize the entire pancreas, and the edges of the right and left lobes could not be visualized. The healthy pancreas before pancreatitis inducement presented a nearly smooth border, and the interior showed a high echo level equal to or slightly higher than that of the liver. After pancreatitis inducement, the pancreas presented with swelling, border irregularity and uneven echo levels interiorly, which were improved as the days passed.

Based on the above results, and although only being able to visualize a limited portion of the pancreas, ultrasonography allowed a detailed examination of the morphology of the pancreas and can be conducted on un-anesthetized dogs. Therefore, ultrasonography is useful for diagnosing pancreatitis, particularly severe pancreatitis.

5. CT findings for the experimental acute pancreatitis dogs

CT is an increasingly popular diagnostic imaging technique in veterinary practice, and, with its high organ visualization capability, is used mainly for brain, spinal, orthopedic and other regional diseases. However, its usefulness in diagnosing acute pancreatitis has not been determined. Using the experimental dogs with acute pancreatitis, we conducted abdominal plain CT and contrast-enhanced CT on day 0 (before pancreatitis inducement) and on days 3, 7 and 14

after the treatment. We compared the results with a pathological specimen to determine the pathological findings that CT imaging reflects.

The visualization capability of CT for a healthy pancreas was increased compared with other imaging modalities, although it was difficult to distinguish some regions of the pancreas from other organs. The observations became more clear after pancreatitis inducement because of the swelling of the pancreas. Comparison with the findings from the pathological specimen showed that, by both plain and contrast-enhanced CT, peripancreatic panniculitis, inflammation of the stroma and parenchyma of the pancreas, and necrosis of the parenchyma of the pancreas could be distinguished.

Based on these results, despite the requirement of general anesthesia, CT allows a detailed observation of the parenchyma of the pancreas and its surroundings, and was concluded to be highly useful for the diagnosis of acute pancreatitis and estimating its prognosis.

6. Examination using clinical cases

To determine whether the results from the experimental dogs with pancreatitis were similar to clinical cases, we assessed the same examination items and methods as used in the experimental dog on dogs suspected of pancreatitis. Blood lipase, blood amylase, and lipase and amylase in ascites (if the dogs had ascites accumulated) were useful for diagnosing pancreatitis. However, these parameters did not necessarily show increased levels in chronic cases, and, consistent with the results in the experimental dogs, were considered not useful except in dogs with early-stage pancreatitis. While plain X-rays and abdominal contrast-enhancing examination produced some findings that indicated pancreatitis, they were useful mainly for eliminating other diagnosable diseases and identifying complications. Abdominal ultrasonography produced findings that indicated pancreatitis in some cases, but it failed to identify abnormal conditions in some cases. Therefore, ultrasonography was considered useful for diagnosing pancreatitis, but it was difficult to eliminate the possibility of pancreatitis based only on ultrasonic images alone. CT examination visually detected pancreatic abnormalities in all the cases, and, combined with contrast-enhancing techniques, allowed the estimation of the nature of the lesions to some extent. Therefore, CT is considered useful for diagnosing pancreatitis and assessing its prognosis in clinical settings. These results were consistent with those obtained in the experimental pancreatitis dogs.

This study using the experimental pancreatitis dogs and clinical cases allowed an assessment of the usefulness and limitations of CBC and other blood chemistries, ascites examination, and diagnostic imaging in terms of diagnosing acute pancreatitis with consideration of the time elapsed since the occurrence of the disease. Our results showed that blood chemistries and lipase and amylase levels in ascites are useful for diagnosing acute pancreatitis, but are not useful for diagnosing subacute and chronic pancreatitis. X-ray diagnostic imaging is useful for eliminating acute abdomen, but is not useful for diagnosing pancreatitis. Ultrasonography is very useful since it allows the observation of the pancreas in un-anesthetized dogs. However, visualization of a healthy pancreas requires scanning and observation skills, and the edges of the right and left lobes of the pancreas, in particular, can not be visualized. Morphological observation became easier once pancreatitis was induced, but qualitative assessment of the parenchyma of the pancreas still presented difficulties. Although having the disadvantage of requiring general anesthesia, CT easily and clearly visualizes the pancreas and its surroundings in acute and subacute pancreatitis. Furthermore, combined with contrast-enhancing techniques, CT allows the detailed assessment of the lesions of the pancreatic parenchyma. Therefore, CT seems to be the most useful diagnostic imaging technique in the diagnosis of acute pancreatitis in dogs.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 192–194, 2011.

Study of glucose insulin therapy for ketosis in dairy cattle

Yoji TOYODA*

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

(Received 11 March 2011, VDO-No. 299)

Ketosis is caused by an increase in ketone bodies (acetone, acetoacetate, β-hydroxyl butyrate) in body fluid that is induced by carbohydrate and lipid metabolism deficiencies. Ketosis induces clinical symptoms that include anorexia, hypodynamia, weight loss, and the depression of productive capacity. In the clinical field of dairy cows, ketosis is a common disease which decreases its reproductive performance and milk production, and causes considerable economic loss.

Ketosis is generally treated by the intravenous injection of concentrated glucose solution. Glucose is used regularly as the most physiological sugar source in carbohydrate fluid therapy. In addition, xylitol, which can supply carbohydrative energy in insulin independency, is widely utilized as a therapy for cases that are difficult to treat through a single administration of glucose. Several recent reports, however, have demonstrated the recovery of cows with ketosis through the intravenous injection of glucose and xylitol. For these cows, the efficacy of exogenous insulin was acceptable, but the utilization of exogenous insulin was not a safe therapy because there were adverse side effects (hypoglycemia). There has been insufficient investigation into the usage of exogenous insulin.

In this study, I examined the efficacy of xylitol and glucose for the treatment of ketosis by an intravenous bolus infusion, continuous intravenous infusion and mixed intravenous infusion, and examined the curative effect of exogenous insulin. From these results, I designed a new glucose insulin therapy (improved GI therapy), consisting of an intravenous injection of regular insulin and the continuous administration of glucose, as a ketotic therapy for dairy cows, and examined the effects.

In Chapter 1, I examined the effect of an intravenous bolus administration of a 25% solution in 0.1 g/kg BW as an active technique to use the strong insulin secretion-inducing effect of xylitol. I confirmed that this technique induced a peak-shaped strong transient secretion of insulin in healthy cows, but caused a gentle secretion in ketotic cows that continued for a long time. Similarly, a strong secretion of insulin was not observed among ketotic cows in the glucose-treated group. Thus, it was thought that the short-time administration of a concentrated solution of carbohydrate does not have the potential to stimulate a strong insulin secretion in ketotic cows.

In Chapter 2, I examined the effects of a 30-minute continuous administration of 500 ml of 25% xylitol solution, which was expected to induce a continuous secretion of insulin, compared with the peak-shaped strong transient secretion of insulin induced by the bolus infusion described in Chapter 1. However, although it was confirmed that the insulin secretion induced by xylitol administration was stronger than that induced by glucose administration in healthy cows, a strong insulin secretion similar to that reported in Chapter 1 was not confirmed in the

* Supervisor : Prof. Hisashi HIROSE

ketotic cows. Nevertheless, although there were significantly decreases in the serum concentrations of free fatty acids (FFA) and β-hydroxyl butyrate (3HBA) in ketotic cows after administration of either xylitol or glucose, no differences between cows that received xylitol and those which received glucose were observed. Based on these results it was thought that the continuous intravenous administration of xylitol to ketotic cows had an approximately equal anti-ketotic effect as the use of glucose in the same technique in the treatment of ketosis. From these results, it was thought that the anti-ketone effect of xylitol in ketotic cows was not dependent on its strong insulin-releasing action, but its action of the xylitol as an energy source that was independent of insulin.

With Chapter 3, I examined the effectiveness of the mixture of xylitol and glucose solutions that combined the action of insulin secretion induction by the xylitol and the supply of carbohydrate by the glucose. A 1000-ml mixture consisting of 500 ml of a 25% solution of xylitol and 500 ml of a 25% solution of glucose was continually intravenously administered for 60 minutes to healthy and ketotic cows. Similarly to Chapter 1 and Chapter 2, since the strong insulin secretion recognized in healthy cows was not observed in the ketotic cows, it was thought that the administration of the mixture was not useful in stimulating insulin secretion. Moreover, as in Chapter 2, it was suggested that cellular uptake of xylitol in ketotic cows is reduced, and it was thought, from the trends in serum FFA and 3 HBA concentration, that in ketotic cows there is insufficient insulin secretion and that the direct energy production from the cellular uptake of xylitol or glucose is insufficient.

In previous studies, the effectiveness of xylitol as an agent for the treatment of ketotic cows was examined in a variety of administration formats (bolus infusion, continuously intravenous administration, and administration of a mixture of xylitol and glucose) ; however, the results indicated that the efficacy of xylitol administration was not better than the administration of glucose alone. In addition, it was thought that the xylitol was unable to stimulate sufficient insulin secretion in ketotic cows. Therefore, to maintain a high IRI concentration and active cellular glucose uptake in ketotic cows, application of exogenous insulin was studied.

In Chapter 4, I observed the changes of immunoreactive insulin (IRI) and blood glucose concentration after the infusion of insulin. In the healthy cows administrated an intramuscular injection of 0.3 U/kg of NPH insulin, the blood sugar level indicated a significantly low value even after 12 hours following the injection. There were no significant differences between IRI at any time up to 12 hours after the injection. In the healthy cows administrated an intramuscular injection of 0.3 U/kg BW of regular insulin, the minimum blood glucose concentration ap-p eared 90 minutes after injection, but even after 6 hours, the glucose level was significantly lower than before the insulin injection. The IRI value was significantly higher than before the injection until five hours after. Due to the long active duration of the intramuscular injection of 0.3 U/kg of NPH insulin or 0.3 U/kg of regular insulin, it was thought that for safe treatment, the intra venous injection of glucose to prevent hypoglycemia must be administered over a lengthy period. This is thought to be impractical in a clinical practice treating productive animals. The glucose concentration in the blood of cows injected intravenously with 0.1 U/kg BW of regular insulin declined to a minimum at 45 minutes after the injection, and recovered to an almost equivalent concentration as before the injection by 135 minutes. The IRI value recorded a maximum value 15 minutes after the injection and thereafter rapidly decreased. The IRI value was significantly higher than before injection until 45 minutes, and recovered to its level observed before the injection by 75 minutes. Since the active duration of the intravenously injected 0.1 U/kg regular insulin was short, it was thought that a lengthy glucose administration for the

prevention of hypoglycemia is not necessary. Therefore, I thought that the application of intravenously injected 0.1 U/kg BW regular insulin combined with glucose administration was useful for ketosis treatment.

In Chapter 5, based on the result in Chapter 4, I designed a new glucose insulin therapy that was more practical and effective than the previous method for the treatment of ketosis. I call this a new glucose insulin therapy with improved GI therapy, consisting of an intravenous infusion of 0.1 U/kg BW regular insulin followed by an intravenous administration of 500 mL of 25% glucose solution for 45 minutes. When this improved GI therapy was carried out on healthy and on ketotic cows, the blood glucose concentration was lowered by about 5 mg/dL, and it appeared that there was no concern with the development of hypoglycemia unless severe hypoglycemia was noted before administration. Moreover, it appeared that in this improved GI therapy, comparatively high IRI concentration were able to be maintained in ketotic cows to regulate the HSL activity, which suppressed triglyceride decomposition in adipose tissue and reduced the concentration of FFA in serum ; in effect, disrupting the reaction pathway responsible for ketone body generation. This accounts for the efficacy of the therapy in the treatment of ketosis.

A therapeutic trial designed for clinical cases of ketosis was performed to examine the effectiveness of the improved GI therapy. The treatment frequencies of 9 ketotic cows given the improved GI therapy were significantly less than 16 ketotic cows given another therapy. Moreover, hypoglycemia was not admitted in all cases. The intravenously injection of 0.1 U/kg BW regular insulin combined with a gentle intravenous administration of 500 mL of a 25% glucose solution for 45 minutes resulted in high IRI values, and there was little concern for hypoglycemia. It seemed that the improved GI therapy was an effective and safe therapeutic method for the treatment of ketosis.

From these findings, this study concludes as follows : 1) the therapeutic effect of xylitol for ketotic cows is equal to that of glucose ; 2) the improved GI therapy that combined an intravenous injection of regular insulin and gentle administration of a glucose solution is a safe procedure as a technique that utilizes exogenous insulin more positively ; and the effect of the improved GI therapy is higher than that of conventional therapies that only use a carbohydrate solution.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 195–197, 2011.

Important role of the arthroscopic examination of the hip joint for the correct application of triple pelvic osteotomy for hip dysplasia in juvenile dogs

Shinya YAMAGUCHI*

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

(Received 11 March 2011, VDO-No. 300)

Canine hip dysplasia (CHD) develops in middle- to large/giant-sized dogs, and it is recognized as an arthropathy that markedly impairs locomotive function of the pelvic limb and quality of life (QOL) in the affected dogs. CHD is categorized into the following two types : CHD in young dogs, which develop clinical symptoms derived from capsular sprains and sprains of the femoral head ligament associated with abnormal joint laxity during the period of skeletogenesis ; and CHD in mature dogs, which develop clinical symptoms derived from osteoarthritis (OA) at two years of age or older.

Conservative treatment, medication, and surgery are applied in [for] CHD. In cases with severe OA in the femoral head and acetabulum, it is difficult to eliminate clinical symptoms derived from OA by medication and conservative therapy, such as body weight control, restriction of physical activity, and pain management. Instead, although indications are dependent on the body weight of the affected dog, wishes of the owner, and economic reasons, surgical therapy such as femoral head osteotomy and total hip arthroplasty may be applied.

Meanwhile, triple pelvic osteotomy (TPO) and juvenile pubic symphysiodesis have been clinically applied as prophylactic surgery for CHD in young dogs without cartilage injury or deformity in the femoral head and acetabulum. These operations are aimed at preventing the progress of secondary development of OA in CHD by improving the congruity between the femoral head and acetabulum, and it has been suggested that operations soon after the onset are able to improve or maintain the QOL by preserving the load-bearing area of the hip joint in the dog.

TPO is an operation that has been reported for dogs with CHD since the 1990s (Slocum, B. and Devine, T., 1992), and research has been done from a variety of aspects, such as surgical technique and postoperative evaluation, and the operation has been recognized as an effective treatment method with excellent treatment outcomes as long as the indication is appropriate. However, since TPO requires an early diagnosis of hip joint laxity and an operation at as young an age as possible, it is very important to establish a proper evaluation method to determine whether or not the operation is indicated.

In this study, hip joint laxity was evaluated with the objective markers obtained by the traction stress radiography, according to the University of Pennsylvania Hip Improvement Program (Penn HIP) in 115 dogs bred at general homes. The dogs were brought to the clinic for examination of the hip joint to determine whether they

* Supervisor : Prof. Masahiro TAGAWA

had CHD or not. Furthermore, a retrospective survey was carried out regarding OA findings obtained by radiographs of the hip joint according to the criteria by the Orthopedic Foundation for Animals (OFA). Subsequently, hip joints in young dogs with clinical symptoms of CHD were examined by arthroscopy as well as radiography, and the relationship between the findings of radiography and arthroscopy was investigated.

Moreover, joint markers in synovial fluid were measured in some of the cases undergoing arthroscopy, and they were compared with clinical findings of CHD. Lastly, radiographic examinations and arthroscopy were carried out before and after surgery in cases undergoing TPO, and indications for TPO and usefulness of examinations were evaluated by comparing the findings of preoperative and postoperative examinations.

1. Epidemiological study on CHD : study on the severity of OA based on hip joint laxity and joint markers (2nd chapter)

In this chapter, the dog owners who wished to have thorough examinations of their dogs' hip joints were asked about the presence or absence of clinical symptoms in their dogs. OA findings in the hip joints, obtained from a hip-extended ventrodorsal radiographs and several markers for the degree of laxity, were evaluated according to the OFA criteria in order to examine their relationship with clinical signs. In addition, the degree of hip joint laxity was evaluated according to the Penn Hip technique by calculating the distraction index (DI), and the relationship with clinical signs and radiographic markers was examined.

The results of a hip-extended ventrodorsal radiographs were analyzed according to the OFA criteria ; the clinical symptoms had no relationship with OA findings or the evaluated markers. In the canine hip joints diagnosed as CHD, the joint markers measured at the same time revealed an increase in inflammatory cytokine activity involved in cartilage metabolism and protease activity, as well as an increase in concentrations of sulfated glycosaminoglycan, and it was suggested that evaluation of CHD was possible by measurement of joint markers in synovial fluid.

Taken together, our data suggested that OA was already developed at hip joints irrespective of the presence or absence of clinical symptoms. The DI, a marker for hip joint laxity, was significantly higher in cases with clinical symptoms of CHD than in those without them ($p=0.0006$). In cases with clinical symptoms, it was expected that the degree of hip joint laxity was high, but the DI was also high in some cases without clinical symptoms. These results suggested that dogs without clinical symptoms also had some degree of hip joint laxity, and that they might develop clinical symptoms later in life.

2. Arthroscopic findings and hip joint laxity in young dogs suffering from CHD (3rd chapter)

For evaluation of the hip joints in young dogs suffering from CHD, OA findings were analyzed based on a hip-extended ventrodorsal radiographs, according to the OFA criteria. However, this diagnostic method is subjective, and it has been proved that a detailed evaluation of hip joints is difficult (Holsworth, I. G. et al., 2005).

In contrast, since the evaluation of hip joint laxity by the DI calculated according to the Penn HIP method of evaluation is objective in diagnosing CHD, it has been clinically applied as a diagnostic method in CHD at early stages. In Western countries, an indication for TPO is clinically determined based on the results of the Penn HIP radiography technique, however, it has been reported that OA can deteriorate to a severe condition after TPO in some cases that meet the indication criteria, so further examinations are warranted in order to establish the appropriate indication for TPO.

In this chapter, arthroscopy that allowed visual evaluation of the stuructures in the hip joints was carried out, and its relationship with conventional methods to evaluate CHD was examined. As a result, the severity of OA in the hip joints in young dogs that developed clinical sym-

ptoms of CHD was diagnosed at levels ranging from normal to moderate, and the DI was 0.6 or higher in all cases. Arthropathy of the hip joints showed degenerative changes to various degrees, and there was no significant relationship between radiographic and arthroscopic findings.

However, in the cases with a high DI, arthroscopy showed relatively severe degenerative changes in the joint structure in the hip joints. Based on these results, we suggested that arthroscopic findings on hip joints were related to hip joint laxity and that arthroscopy was useful for determining whether TPO is indicated as well as for evaluating the prognosis after TPO.

3. Evaluation of hip joints by radiography and arthroscopy before and after TPO in young dogs suffering from CHD (4th chapter)

In this chapter, TPO was carried out in young dogs with CHD that was diagnosed by radiographs and arthroscopy, and it was investigated how the results of preoperative examinations were related to postoperative outcomes by repeating similar examinations after TPO. As a result, the DI before surgery showed 0.7 or higher in the hip joints in all cases, but it decreased to 0.3 or lower in all cases after surgery.

Arthroscopy showed that the severity was Grade 2 or lower before surgery in all hip joints, and that there was no evident advancement after surgery ; but there was an improvement in the severity in one hip joint. A partial tear of the femoral head ligament recognized before surgery was also observed after surgery, but the swelling that was observed before surgery disappeared. These results suggested that TPO improved hip joint congruity and reduced hip joint laxity. In addition, when preoperative radiographs showed relatively severe OA findings or arthroscopy showed mild injury of joint cartilage in hip joints (Grade 1–2), it was shown that TPO could potentially achieve excellent prognosis.

Taken together, the results of this study showed that hip joint laxity, which has the risk of developing into OA later in life, was also observed in dogs without clinical symptoms associated with CHD, and that the degree of joint laxity was more severe in dogs with clinical symptoms than in those without them. In addition, in considering an indication for TPO for CHD in young dogs, it was revealed that a maximum treatment effect would be achieved by evaluating the hip joint structure based on arthroscopy as well as conventional evaluation methods such as hip joint radiographs. Further more, it was demonstrated that CHD was developing insidiously in animals without clinical signs. These findings indicate that to improve the prognosis after TPO it is necessary to add an objective evaluation of hip joint laxity by the Penn HIP radiography technique and an arthroscopic evaluation of hip joints to the indication criteria that have so far been listed.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 198–200, 2011.

Studies on the mechanism of autumn abortion syndrome in the pig

Kazuo WATANABE*

Laboratory of Veterinary Reproduction,
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Received 11 March 2011, VDO-No. 301)

Non-infectious abortions occurred in August through November in the 1970s in England, and affected 10% of pregnant pigs in serious episodes. These were called swine autumn abortion syndrome (AAS), in which abortion suddenly occurred without clinical symptoms, such as appetite loss, and no lesion was observed in fetuses. Occurrence of AAS has been reported not only in Europe but also many countries including Canada and Australia, and its economic damage was serious. In Japan, incidences of abortion assumed to be AAS have been reported, and the farms suffered serious economic losses. However, the developmental mechanism of AAS has not been fully elucidated, and no preventive measures can be taken in the current situation. In this study, the conditions of swine AAS and changes in sex hormone levels after abortion were investigated to elucidate the developmental mechanism. This report is comprised of introduction in Chapter 1, experimental results in Chapters 2 to 4, and summary in Chapter 5.

1. **Abortion that occurred in an open-type swine farm** (**Chapter 2**)

Maintenance of high reproductive efficiency, i.e., increasing the litter size per dam, is essential for large-scale swine farms, for which many swine farms administer various vaccines to breeder pigs to prevent stillbirth and abortion. However, an outbreak of abortion occurred in a swine farm in Chiba Prefecture.

The farm maintained 110 female SW breeder pigs. Abortion suddenly occurred in 18 pigs without developing a clinical symptom in August-October 1993. It occurred within a very early phase of pregnancy in 14 of the 18 animals (77.8%) : 22–29 days after mating (mean : 24.6± 0.6 days). No abnormality was observed in the aborted fetuses. Recurrence of estrus was observed early, after 4–13 days, in many animals, suggesting that the corpus luteum already regressed at the time of abortion, leading to early recurrence of estrus.

The incidence of abortion in an open-type swine farm was considered to be AAS, and not caused by infection. However, ovarian function could not be objectively evaluated because plasma samples before and after abortion could not be collected, and, thus, a definite diagnosis of AAS could not be made.

2. **Relationship between breeding efficiency and seasons in open-type and windowless swine farms** (**Chapter 3**)

Reduction of reproductive efficiency of pigs in late summer over early fall, such as a delay in recurrence of estrus and reduced conception rate, have been reported mainly in Europe, and these may have been due to that the ancestor of breeder pigs ; European wild pigs, are seasonal breeders breeding in January-March. Since L and W breeds maintained as female breeder pigs in Japan were also derived from European wild

* Supervisor : Prof. Toshihiko TSUTSUI

pigs, reproductive efficiency may decrease in some L and W pigs depending on the season. Thus, we compared reproductive efficiency between pigs maintained in open-type (O group) and windowless (C group) farms, the latter being less likely to be influenced by the season.

No apparent difference was noted in the conception rate between the 2 groups in any season. However, the mean annual conception rates were 91.4±0.7 and 95.2±0.6% in the O and C groups, respectively, being significantly higher in the C group ($P<0.01$). A longer time was required for recurrence of estrus in the O than in the C group in each season, but the difference was not significant. However, the mean times over the year were 8.0±0.8 and 4.6±0.7 days in the O and C groups, respectively, being significantly longer in the O group ($P<0.01$). The number of dams with abortion was 29 of 3,251 pigs (0.89%) and the day of gestation at the time of abortion was 23–101 days (mean : 49.3±3.7 days) in the O group, whereas the abortion rate was 0.50% (19 of 3,775) and the day of gestation was 27–88 days (mean : 50.9±5.2 days) in the C group, being significantly higher in the C group ($P<0.05$).

The above findings revealed that a higher reproductive rate was obtained in a swine farm less affected by the daylight length and external temperature, compared to that in an open-type farm. However, to elucidate the developmental mechanism of AAS, investigation of hormone dynamics in circulation may be necessary.

3. Blood LH and progesterone levels in pregnant and infertile pigs in autumn maintained in an open-type farm (Chapter 4-1)

In AAS, abortion suddenly occurs in early pregnancy without developing clinical symptoms, and no infection is observed in the dam or degeneration in aborted fetuses. Estrus recurred early after abortion, and the conception rate resulted from this estrus was high, suggesting temporary endocrine abnormality as a cause of AAS. Thus, we compared the plasma LH and P_4 levels in pregnant and infertile breeder pigs (LW) maintained in the O swine farm between animals mated in the non-breeding season, August-October (A group), and in the breeding season, January (B group), based on the breeding season of European wild pigs.

The plasma LH level did not markedly change until 35 days of gestation in pregnant pigs in the A group, and the level was 0.7–1.2 ng/ml. In contrast, in the B group, the level was maintained at a significantly higher level (1.0–2.0 ng/ml) than that in the A group ($P<0.01$). Changes in the plasma P_4 level in pregnant pigs were similar in the 2 groups.

In infertile pigs, the plasma LH level was mostly lower than 1 ng/ml throughout the period in the A group. In contrast, the level was higher than 1 ng/ml throughout the period in the B group and it was significantly higher than that in the A group, as observed in pregnant pigs ($P<0.05$). However, changes in the plasma P_4 level were similar in the 2 groups.

The above findings confirmed that female LW breeder pigs resembled European wild pigs : reduction of LH-release from the pituitary in summer through autumn. However, no difference was noted in P_4 secretion from the corpus luteum in pigs maintaining pregnancy even though the plasma LH level was low.

4. Blood LH and progesterone levels before and after abortion in pigs with autumn abortion (Chapter 4-2)

The above findings suggested that endocrine function decreases in summer through autumn and causes AAS in animals that have the genetic profile of European wild pigs. The developmental mechanism of AAS was investigated by measuring the plasma LH and P_4 levels before and after abortion in pigs maintained in the open-type swine farm and suspected as having AAS.

Abortion assumed to be AAS was observed in 22 animals. The incidence was highest in a 2-month period from late August to middle of October, and it occurred in early pregnancy by 35 days of gestation in 14 animals accounting for

63.3%. Estrus recurred early after abortion, and 12 of 19 mated animals (63.2%) were fertilized. Blood was collected before abortion in 2 of the 22 animals with abortion. Both LH and P_4 showed basal levels on the day with abortion. The P_4 level on the abortion day was the basal level in all animals. These findings clarified that the corpus luteum of pregnancy could not be maintained due to reduced pituitary function and resulted in AAS.

It was suggested that pituitary function decreases in the non-breeding season (summer over autumn) in female breeder pigs because they have the genetic profile of European wild pigs, which cannot maintain the corpus luteum of pregnancy, subsequently leading to abortion. Therefore, the most important points to prevent AAS in open-type swine farms may be increasing the insulation of the facility and reduce the influence of shortened daylight length by sufficient lighting. Reviewing such a basic management may be useful to prevent AAS. In addition, breed improvement to prepare pigs insensitive to environmental changes with constantly stable LH pulse is necessary.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 201–202, 2011.

Relationships between Japanese black bear *Ursus thibetanus japonicus* intrusions into Satochi and Satochi environment around the intrusion sites in Numata City, Gunma Prefecture

Kentaro UMEDA*

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

(Received 11 March 2011, NMK-No. 1)

In recent years, many cases of Japanese black bear (*Ursus thibetanus japonicus*) intruding into human living areas have been reported. Several factors are implicated in bear intrusions, and one such factor is the deterioration of the Satochi environment. We analyzed the Satochi environment around bear intrusion sites in Numata City, Gunma Prefecture, to elucidate the relationship between Japanese black bear intrusions into Satochi and the Satochi environment around the intrusion sites.

In Chapter 2, we classified the Satochi environment in the Hocchi area into the following six categories according to land usage : crop fields, paddy fields, orchards, fallow fields, wastelands, and forest edge. Then, the location, planting condition, and management situation of each category were investigated, and macroscopic and microscopic analyses were performed. Data on the Satochi environment were quantified using a geographic information system, and data for the 2004?2008 period associated with bear intrusion sites within the investigated area were analyzed.

Macroscopic analysis revealed a significant relationship between bear intrusion and geographical complexity ($P<0.01$) , and between bear intrusion and the area ratio of the orchards ($P<0.01$). Microscopic analysis showed that bear intrusions were significantly more prevalent in orchards ($P<0.01$) and on sections of the forest edge with heavy tussock growth ($P<0.05$) than in other areas. The results suggest that the factors influencing bear intrusion include geographical complexity and the presence of orchards and tussocks. Based on these factors, we determined High Risk Areas (HRA), which are expected to have a high intrusion rate.

In Chapter 3, we revealed the effectiveness of HRA for future wildlife damage control in the Hocchi area. We also estimated the statistical bias for the frequency of bear intrusions into HRA to reveal whether intrusion sites were biased toward HRA. In addition, we tested whether there were statistically significant differences in the ratio of intrusion sites on HRA for all sites in the Hocchi area in the 2004–2008 and 2009–2010 periods.

The results showed that the bias in the frequency of bear intrusions into HRA was significant ($P<0.05$) and that the ratio of intrusion sites on HRA during the 2004–2008 and 2009–2010 periods was not significant ($P=0.51$). The ratio of intrusion sites on HRA was the average annual about 60% in 2004–2010 periods. We thus concluded that HRA had a universality of the average annual about 60% for bear intrusion in the Hocchi area.

* Supervisor : Prof. Hiroshi KAJIGAYA

In Chapter 4, we tested the relevance of the proposed influential factors (identified in Chapter 2) underlying bear intrusions on Sayama-machi which are adjacent to the Hocchi area. We conducted a survey at Sayama-machi, and extracted the HRA using the same method as that described in Chapter 2. We estimated the statistical bias in the frequency of bear intrusion into HRA in Sayama-machi, and tested whether there were statistically significant differences in the ratio of intrusion sites on HRA of the Hocchi area and Sayama-machi in 2004–2009.

The results showed that the bias in the frequency of bear intrusion into HRA in Sayama-machi was significant ($P<0.01$), and that the ratio of intrusions sites on HRA of the Hocchi area and Sayama-machi was not significant ($P=0.45$). About 80% of intrusion sites were on HRA in Sayama-machi. Consequently, we can determine that geographical complexity and the presence of orchards and tussocks in HRA are factors influencing bear intrusion in Sayama-machi.

In Chapter 5, we studied an apple orchard in Sayama-machi, and revealed the relationship between crop damage by bears in each apple orchard and the relevant damage control per orchard. In addition, we investigated the circumstances of bear intrusion using an infrared sensor camera and organized follow the time series. We then conducted a survey by interview and assessed photographs from the infrared sensor camera.

The results showed that bear damage corresponded with the ripening of fruit in the apple orchards. Furthermore, many apple farmers installed an electric fence in 2010. However, because of the late introduction of these fences, some farmers were unable to prevent crop damage by bears.

The infrared sensor camera revealed that the frequency of bear intrusions was the same in June and July, increased in August, returned to the July level in September, and increased again in October, revealing two peaks. Changes in frequency of bear intrusion according to the photographs appeared to be in line with nut production in autumn. In the future, collecting more information on bear intrusions into Satochi will prove important for developing appropriate preventative measures for bear damage.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 203–204, 2011.

A new technique for Biological Monitoring —A new Index for Evaluation of Lead Pollution

Yuki OKUTOMI*

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

(Received 11 March 2011, NMK-No. 2)

Biological monitoring using wildlife is one of useful method for investigation of the degree of environmental contamination. Thus, there are many reports describing about contamination by various pollutant such as heavy metals, chlorinated organic compound in the studies using wildlife. However, since there is a lack of epidemiological aspects in the studies using wildlife, the researchers cannot make effective use of the obtained data from wildlife. For example, since the amount of Cd in animals increases with ageing, the knowledge of the age of the animals is necessary to understand the degree of Cd contamination in target animals accurately. However, the identification of the age of the wildlife is sometimes difficult. Further, there is a lack of epidemiological aspects such as sex, feeding habits, and the degree of contamination of the habitat. Thus, it was thought that the setup of a normal control group is very difficult for the studies using wildlife.

In mention above, new indexes of Cd, named the Cd standard regression line (CSRL) and the Cd equal probability ellipse, CEPE were established to understanding contamination degree of Cd. Further, a similar index for lead (Pb) was also established. However, the index of Pb was developed using 69 data points, therefore, the index was modified in the present study in first experiment. In the next, to confirm the utility of the established index, the data obtained from experimental animals administered Pb, were compared with the index of Pb. Finally, the data obtained from wild birds captured in Japan were compared with the index.

1. Re-examination for establishment of the index

Pb contents in mammals (19 species of terrestrial mammals, 5 species of marine mammals), birds (5 species of seabirds, 18 species of land birds and waterfowl) and fish (4 species of freshwater fish), 98 data points from 37 reports, in which Pb contents were represented as arithmetic means, were selected in previous publications. The 98 data points cited were plotted on a graph with the Pb content in the liver on the abscissa and the Cd content in the kidney on the ordinate. In conclusion, the regression line obtained after log transformation was $\log (Y) = 0.9256 \log X - 0.0578$ ($R^2 = 0.8087$, $p < 0.01$). After, statistical test using 95% equal probability ellipses, 4 data of 98 points were identified as outliers, although descriptions of Pb contamination of those four points were not described in the references. The remaining 94 data points, excluding these 4 outliers, were tested again using the 95 % equal probability ellipse. As a result, 5 additional points were rejected as outliers.

When the regression line obtained using 69 data points by Mochizuki et al. and the modified regression line obtained using 89 data points, excluding the 9 outliers, were compared, there were no significant differences between the two lines.

* Supervisor : Prof. Hiroshi KAJIGAYA

Further, the data obtained from birds bred under various experimental conditions and wild birds lived in contaminated areas were com pared with the index. The data from the polluted birds were observed outside the index, although the data obtained from the control birds were observed within the index. Those results suggested that the contamination degree with Pb is able to understand using this index. Thus, the following investigations were tried using the obtained index in the present study.

2. The comparison with data obtained from mice injected Pb

Total of 21 male mice (ICR, Clea Japan, Inc, Japan), weighing approx. 20 g, were used in the study. Lead chloride dissolved by distillated water was sterilized using filtration, and then 0.1 ml of the solution was weekly administered intraperitoneally to animals. The applied dose of lead was between 200 and 40000 ppm and the maximum dosing period was 4 weeks. After the experiments, kidney and liver were collected from animals and analyzed using atomic absorption spectrophotometer. The experiments were carried out under ethical consideration of Nippon Veterinary and Animal Science University for experimental animals. The Pb contents of organs were increased depending on the applied dose and dosing period. The obtained data from mice was compared with the index. The data (n=1) obtained from control groups was observed within the index. In the investigation of the higher dose group such as 20000 (n=4) and 40000 (n=3) ppm, all data in each dosing period was divided from the index. In the investigation of group of administered 2000ppm, the data obtained from dosing period of 2 (n=1) and 3 week (n=1) was located within the index, although the data of 4 week (n=1) were divided from the index. Those results suggested that differences of contamination degree in target animals is able to understand using this index.

3. The investigation of contamination degree of Pb in wild birds

In the next, the data obtained from wild birds were compared with the modified Pb-index. The samples from wild birds were collected under other National Investigation conducted by Environmental Agency in Japan. The used wild birds, winter birds in Japan, were as follows ; wigeon (n=11, *Anas penelope*), mallard (n=9, *Anas platyrhynchos*), pintail (n=6, *Anas acuta*), common teal (n=4, *Anas crecca*), gadwall (n=2, *Anas strepera*) and tufted duck (n=1, *Aythya fuligula*). Those birds were collected from Ishikawa (n=7), Ibaraki (n=10), Fukushima (n=7) and Akita (n=5) Prefectures. After analysis using inductively coupled plasma spectrometry, the contents of kidney and liver were investigated. Since one data in kidney obtained from teal was more than 1000 μg/g dry wt., the mean values of kidney of teal were approximately 260 μg/g dry wt. Thus, the higher mean content was observed in Ishikawa where this teal was captured than that of other prefectures. However, after eliminate of this data, there were no significant difference among mean Pb contents. In mention above, the understanding of contamination degree is difficult by the comparison using only mean value. Thus, the data obtained from wild birds were investigated using Pb index. Since the undetectable data was not used for this investigation, total of 30 data was used. As a result, 13 data was observed in the outside of the index. On the other hand, the similar investigation was reported in residential birds such as spot bill duck (n=13, *Anas poecilorhyncha*) and common cormorant (n=9, *Phalacrocorax carbo*). In this investigation, only 2 data was observed in outside of the index. Those result suggested that the degree of Pb contamination is more serious in winter birds.

The utility of the modified index for Pb contamination was investigated in the present study. Further, the contamination degrees of Japanese wild birds were investigated using the modified index. As results, this index is useful to know the contamination degree of Pb in target animals. It was thought that this index will be a solution for understanding of contamination in wildlife.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 205–206, 2011.

Genetic characteristics of isolated small population of Japanese black bear, *Ursus thibetanus japonicus*, inhabiting the Shimokita Peninsula, Aomori prefecture, Japan

Yuri OKUMURA*

Department of Applied Science,
Nippon Veterinary and Life Science University

(Received 11 March 2011, NMK-No. 3)

The Japanese black bears that inhabit the Shimokita Peninsula of Aomori Prefecture live at the northernmost extremity of the animal's range. They are recognized on the Ministry of the Environment's Red List as a local population that is in danger of extinction. It is thought that the population numbers at around 100 bears, and although they are considered to have a long history of isolation, appropriate and practical measures for their protection and management have yet to be implemented in Aomori Prefecture. One reason for this is that without sufficient research about these Asiatic black bears, we have virtually no information about their population size or genetic characteristics. By comparing genetic diversity and evidence of genetic interchange between the populations of Shimokita Peninsula and the Tsugaru region, this research aims to illuminate the genetic characteristics of the Asiatic black bears that inhabit the peninsula.

Materials and Methods

Fur taken from hair traps used as part of Aomori Prefecture's work to protect and manage the Asiatic black bear was used as material. These were comprised of 188 fur samples collected in 2005, 62 in 2006, and 141 in 2007. After extracting DNA from the samples, genotype was determined using nine microsatellite loci (G1A, G 10L, G10M, G10X, Mu23, UamA107, UamD102, UamD2 and Mu64), which was then followed by data analysis.

To evaluate genetic diversity, genetic analysis software was employed to compute each population's allele frequency. Then, in order to ascertain the degree of inbreeding within the population an inbreeding coefficient was calculated. Effective population size was estimated using LDNE software, and the existence of a bottleneck effect was verified with BOTTLENECK1.2.02.

Furthermore, in order to illuminate the degree of gene flow between the populations of Shimokita Peninsula and its neighboring Tsugaru region, assignment tests were conducted to predict which population each individual came from. Also, the coefficient of relatedness was computed using SPAGeDi1.2 software, location data of the hair traps acquired using a GIS (geographical information system), and the relation within the populations between spatial distance and relatedness was examined.

Result

Of the samples in which the genotype of the microsatellite region was determined from six or more of the nine loci, 53 were from the Shimokita population and 77 were from the Tsugaru region population. With regards to the number of alleles for both populations, the expected number of alleles, anomalous number of alleles, and ob-

* Supervisor : Prof. Hiroshi KAJIGAYA

served and expected heterozygosity, in all variables the Shimokita Peninsula population had lower values than the Tsugaru region population. For the former, the inbreeding coefficient was 0.19, and for the latter it was 0.1. Effective group size was 37.8 for the Shimokita Peninsula population (95% confidence interval : 24.9–64.0 individuals), and 101.9 for the Tsugaru region population (65.8–193.8 individuals). The result of the investigation into the occurrence of a bottleneck was that in all mutation models there was no statistically significant deviation and no bottleneck effect was observed in either population. In the assignment tests, 52 individuals (98%) from Shimokita Peninsula and 75 individuals (75%) from the Tsugaru region were identified as belonging to their respective populations, and it was accepted that the populations were genetically differentiated. Furthermore, in the examination into the relation within-group between spatial distance and relatedness a significant negative correlation in both populations was seen.

Discussion

The comparison of observed heterozygosity shows that the Shimokita Peninsula population's genetic diversity is deteriorating, and is now even lower than other local populations in danger of extinction. This indicates that urgent protection and management is needed. One factor in the Shimokita Peninsula population's decreasing genetic diversity could be that it has an effective group size of less than 50 individuals. However, because it has been shown that the population has not experienced a bottleneck in the past, the cause of its present small size and loss of genetic diversity could be due to not only recent deforestation and fragmentation of the habitat, but also the result of sustainment by a limited range over a long period.

Furthermore, examination of the degree of gene flow has shown that with almost no movement occurring in both populations, the Shimokita Peninsula population is becoming progressively isolated. However, as a significant negative correlation in the comparison of relatedness was accepted for both populations, it is shown that the isolation of these Asiatic black bears has not progressed far enough to have interfered with their dispersal behavior and characteristic activity.

The above genetic analysis has shown that the Shimokita Peninsula population inhabits an area that limits their number to a few individuals and restricts gene flow. It is hoped that this information will be used in the immediate implementation of measures for the population's protection and management.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 207, 2011.

Incretin action and effect of the incretin preparation for glucose metabolism in healthy dogs

Hitomi ODA*

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

(Received 11 March 2011, NMK-No. 4)

Incretin was shown to exert their insulinotropic effects through a variety of mechanisms, including by increasing the rates of insulin synthesis, granule docking, and exocytosis. In the presence of matched glucose concentrations, insulin secretion is greater following ingestion of glucose than it is following infusion of glucose. This was referred to as "the incretin effect" and is believed to be modulated at least in part by intestinally secreted hormones such as GLP-1 and GIP. However, the incretin effect in dogs has never been demonstrated. Both GLP-1 and GIP are rapidly inactivated by the enzyme dipeptidyl peptidase-4 (DPP-4). Recently, incretin preparation drugs, DPP-4 resistant GLP-1 analogs and DPP-4 inhibitor, were developed in clinical medicine. Therefore, the aims of this study are twofold. First, we wanted to determine whether canine have the incretin effect. Second, we sought to investigate effect of the incretin preparations (Liraglutide and Sitagliptin) for glucose and insulin metabolism in healthy dogs.

In order to confirm whether dog also have "the incretin effect", we performed the oral glucose tolerance test (OGTT) and intravenous glucose tolerance test (IVGTT) under similar blood glucose variation using artificial pancreas apparatus. No significant difference was observed in temporal serum glucose concentrations between OGTT and IVGTT, since we adjust the intravenous glucose infusion to reproduce similar blood glucose variation as the OGTT. However, insu lin and GLP-1 concentration in OGTT were significantly higher than in IVGTT.

In order to determine the effect of incretin preparations on serum glucose and insulin concentrations, DPP-4 resistant GLP-1 analog, liraglutide and DPP-4 inhibitor, sitagliptin, were administered in 5 healthy dogs. The both of incretin preparations were dispensed medication before starting OGTT. Significant decrease was observed in temporal serum glucose concentrations and glucose $AUC_{0-120min}$ in liraglutide group as compared to control group. Meanwhile, significant increase was observed insulin $AUC_{0-120min}$ in liraglutide group as compared to control group. Also, temporal GLP-1 concentrations and GLP-1 $AUC_{0-120min}$ in liraglutide group were significant higher than that in control group. On the other hand, there was no significant difference in temporal glucose, insulin and GLP-1 concentrations between control and sitagliptin group. Also, no significant difference was observed glucose $AUC_{0-120min}$ and insulin $AUC_{0-120min}$ between both groups. However, significant increase of GLP-1 $AUC_{0-120min}$ in sitagliptin group was observed as compared to control group.

This study demonstrated that healthy dogs have the incretin effect. Moreover, incretin preparations affect glucose and insulin metabolism in healthy dogs.

Key words : healthy dogs ; glucagon-like peptide-1 ; incretin preparation ; the incretin effect

* Supervisor : Prof. Toshinori SAKO

Bull. Nippon Vet. Life Sci. Univ., No. 60, 208, 2011.

Changes in blood biochemical parameter and gene expression of skeletal muscle after exercise test in diabetic dogs

Kaori SAEKI*

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

(Received 11 March 2011, NMK-No. 5)

Therapeutic exercises are beneficial for the treatment and prevention of diabetes mellitus (DM), which ameliorate insulin sensitivity and increase glucose uptake into skeletal muscle. In this study, we investigated whether therapeutic exercise affect for blood biochemical parameter and muscular mRNA expression in diabetic dogs.

The exercises were perfomed below protocol, running for 20 min a day, 5 days/week and during 4 weeks.

Preprandial bood samples were collected from 3 DM dogs before 1week of exercise and under exercise term (1, 2, 3 and 4 week) for evaluating plasma glucose, glycated albumin (GA), non-esterified fatty acid (NEFA) and adiponectin. Skeletal muscle samples were collected before and after 4 week of exercise term. qRT-PCR was perfomed to determine mRNA levels of insulin signaling and glucose metabolism gene : insulin receptor substrate (IRS)-1, IRS-2, phosphatidy linositol3'-kinase (PI3-K), akt kinase2 (AKT2), glucose transporter4 (GLUT4), AMP-activated protein kinase (AMPK), uncoupling protein3 (UCP3) and acetyl-coA carboxylase (ACC).

GA level was significantly decreased after exercises in diabetic dogs. Blood glucose and NEFA concentrations were decreased after exercises. Plasma adiponectin concentrations were increased after exercise. IRS-1, IRS-2, PI3-K, AKT 2, GLUT4, AMPK, and UCP3 mRNA levels in skeletal muscle after exercise were higher than before exercise. ACC mRNA level was decreased after exercises.

In this study, we demonstrate therapeutic exercise does effect for blood biochemical parameter and muscular mRNA expression. Therapeutic exercise in diabetic dogs ameliorate their glycemic control status, which effects might be induced by the alteration of muscular mRNA expression related glucose and insulin metabolism.

Key words : diabetes mellitus, exercise, glucose uptake

* Supervisor : Prof. Toshinori SAKO

Bull. Nippon Vet. Life Sci. Univ., No. 60, 209–210, 2011.

Establishment and characterization of canine rhabdomyosarcoma cell lines

Hana SHIBUTANI*

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

(Received 11 March 2011, NMK-No. 6)

Canine rhabdomyosarcoma (RMS) is a rare tumor that originates from striated muscle cells, striated muscle progenitor cells or primitive mesenchymal cells capable of differentiating into striated muscle cells. It is locally invasive with moderate metastatic potential. Little is known about the biological characteristics of canine RMS cells because of its rare occurrence and lack of study tools. In humans, since several RMS cell lines have been established, many biological properties of human RMS cells, including specific chromosomal translocations, alterations in molecular pathways and sensitivity to various anticancer drugs, are now understood. To understand the biological characteristics of canine RMS, it is necessary to establish a cell line that can be used as a tumor model *in vitro*. To our knowledge, there is no previous report concerning establishment of a cell line originating from canine RMS. This article describes the establishment of novel cell lines from canine RMS and its morphological, immunohistochemical and biological characteristics.

Tumor tissues from 2 dogs with RMS diagnosed by histopathology were used for the establishment of the cell lines. CMS-C cell line was established from tumor tissue raised in the prostate gland of a 14-year-old intact male mixed-breed dog and CMS-J cell line was established from tumor tissue raised in the urinary bladder of a 14-year-old spayed female beagle, respectively. The collected tissue was gently minced by sterile surgical blade and cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, penicillin-streptomycin, and 2 mM L-glutamine at 37°C in humidified 5% CO_2/95% air. The culture medium was changed every 4-5 days. Spontaneous cell growth became evident after 2 months of cultivation, and grew over 50 passages without further additives. Established cell lines displayed the same immunohistochemical characteristics (positive for vimentin and desmin and negative for cytokeratin and smooth muscle actin) as the original tumor cells and express myoD1 and UCP3, known as striated muscle-specific molecules, as shown by RT-PCR assay. The established cell lines were injected to nude mice (BALB/cA Jcl-nu/nu) subcutaneously to confirm the origin of the cells. Ten days after the xenotransplantation, CMS-C cells produced a tumor similar to the original tumor in all of 5 mice injected cell line. By electron microscopy, myofibril-like thin filaments were found in the CMS-C cells. On the other hand, CMS-J cell line did not produced any tumors in 5 mice.

To provide basic data that could potentially be used to help design treatment regimens of canine RMS, we next evaluated the *in vitro* chemosensitivity of CMS-C and CMS-J cells. The anticancer drugs include vincristine, doxorubicin, carboplatin, mitoxantrone, L-asparaginase,

* Supervisor : Prof. Toshinori SAKO

and nimustine were tested. CMS-C and CMS-J cells were sensitive to vincristine, doxorubicin, and mitoxantrone. Vincristine and doxorubicin are the standard regimens of chemotherapy for human rhabdomyosarcoma. Thus, the established canine RMS cell lines could be very important in helping to establish regimens that could be used to canine RMS cases.

From these results, novel canine RMS cell lines were successfully established, which retained morphological and immunocytochemical characteristics of canine RMS tumor cells. A number of established cultured cell lines from human RMS have been characterized in various respects including the morphological features, cellular differentiation, cytogenetics, genes, and effect of anti-cancer drugs or radiation. The development of canine RMS cell lines may enable us to analyze these characteristics about canine RMS, not only for basic studies but also for clinical studies.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 211, 2011.

Genetic variation of Mitochondrial DNA Hypervariable Region 1 Haplotype in dogs

Sho SUGIYAMA*

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

(Received 11 March 2011, NMK-No. 7)

Most of the sequence variation of the mitochondorial DNA (mtDNA) genome among individuals is found in two segments of the control region : hypervariable region 1 (HV1) and hypervariable region 2 (HV2) located between nucleotide position 15458 and 162727. Recently, this variable region is considered an informative DNA maker for dog identification typed from limited or severely degraded DNA. The 660-bp HV1 sequences from 416 dogs were analyzed to characterize the population genetics structure of dog mt DNA, and a 417-bp segment of this sequence compared with a data set reported by Himmelberger et al (2008). Among forty-six haplotypes identified in this study, twenty-one haplotype was corresponded to the data sets but twenty-five haplotype did not to it. Comparison of mt DNA HV1 haplotype in four different pure breeds, highest genetic diversity was shown in toy poodle (0.911). In addition, inter breed investigation showed that genetic diversity rate was 0.904 in 87 breeds (178 animals). These data suggested that dog mtDNA haplotype was useful to established canine mtDNA population data database. Because there were many haplotypes that did not corresponded to the 417-bp data sets, the 660-bp HV1 sequences revealed in this study has been deposited in GenBank (acces sion no. AB622513-AB622568) as fifty-six haplotypes called NVLU001-NVLU056.

* Supervisor : Associate Prof. Toshinori OMI

Bull. Nippon Vet. Life Sci. Univ., No. 60, 212, 2011.

Molecular genetic analysis of *ABO* histo-blood gene in dog

Yong Hwa CHONG*

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

(Received 11 March 2011, NMK-No. 8)

The *ABO* system was discovered by Karl Landsteiner in 1901, that one of the most clinically important blood group systems related to transplantation or transfusion in human. The *ABO* group antigen and its gene in dogs are commonly unknown. Here, we report the isolation and characterization of the cDNA of a dog gene homologous to the human *ABO* histo-blood gene. The cDNA has a 1065-bp open reading frame (ORF) and shares 76.5% nucleotide and 69.7% amino acid identity with the human *ABO* histo-blood gene. Comparison of the dog genomic sequences from data banks of NCBI dog genome resources showed that the cDNA sequences consisted with eight exons, that was identical to that of the human *ABO* histo-blood gene. In addition, we identified the exon-intron boundaries of the cDNA sequences on dog chromosome 9 genomic sequences. The tissue distribution of cDNA has analyzed by the RT-PCR method using total RNAs from thirty tissues. The PCR products of the cDNA were amplified all tissues, that was similar to the ubiquitous expression of human *ABO* histo-blood genes. At present, mRNA sequences similar to *ABO* histo-blood gene are predicted in data banks of NCBI dog genome resources that derived by automated computational analysis using gene prediction method. This predict cDNA sequence consisted with five exons and share 67.5% nucleotide 58.3% amino acid identity with the human *ABO* histo-blood gene. The gene structure of the cDNA isolated in this study is more similar to the human *ABO* histo-blood gene than the predicted cDNA in data bank. Then we conclude the presented cDNA is a dog gene homologous to the human *ABO* histo-blood gene. The sequence of the cDNA has been deposited in GenBank (accession no. AB638847).

* Supervisor : Associate Prof. Toshinori OMI

Bull. Nippon Vet. Life Sci. Univ., No. 60, 213–214, 2011.

Mechanism of Bird Collision on the Glass Surface of the Building

Keiko HATTORI*

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

(Received 11 March 2011, NMK-No. 9)

Objects that wild birds fly into include aircraft, automobiles, windmills, high voltage cables, glass windows and so on. Of all of such man-made causes of avian mortality, it is said that window collisions are one of the most significant. With the appearance of rare species like the Northern Goshawk and the Japanese Sparrowhawk in such reports, from a conservation biology perspective this is an issue that needs resolving. Preventative measures that have been undertaken have included the use of bird-shaped stickers and models of birds of prey. However, actual proof of their effectiveness has not been forthcoming.

Contributing factors to window collision are thought to include migration and time, the proximity of planting grounds, the size of windows, and the presence of birds of prey. In addition, the principal reason is said to be the way that windows reflect the local scenery or their transparency. However, it remains difficult to confirm that this is the true cause. It is still unclear as to what degree the factors considered to present the greatest danger play a part. For this research, the author has undertaken a synthetic analysis that considers anatomical, ecological and even optical aspects with the aim of identifying the true cause of window collision by wild birds.

Materials and Methods

For this research, hazard analyses were conducted from the following three approaches : necropsy, ecology investigation, and video analysis of actual window collisions.

For the necropsies, 58 specimens of wild birds that it was clear from circumstances had been involved in a window collision were examined. At each facility, the specimens were stored in freezers and unfrozen on the day before the dissection. However, in ten cases, dissection took place after the subjects had been in cold storage for a few hours following their collection. The items for inspection were external injuries, deformation or breakage of the beak, muscle injury, bone fractures, bleeding and injury or hemorrhaging of the internal organs. In five of the ten cases that were inspected after cold storage, examinations of brain tissue were conducted. After fixing for over 48 hours with 10% neutral buffered formalin, the brain was cut into paraffin-embedded sections. Apart from HE staining, Kluver-Barrera (KB) staining was also carried out on the paraffin sections.

The ecology investigation was based at Kunitachi City Historical Museum. The investigation was conducted six times in line with the birds' migratory seasons. Items for investigation were time, species of bird, frequency of flight, flight direction and altitude.

For the video analysis, specially developed video analysis software was employed. Color analysis was conducted between the reflected scenery (as well as that seen through the window) and the actual scenery. In each video, corresponding points between the reflected image and the actual scenery were intentionally

* Supervisor : Prof. Hiroshi KAJIGAYA

selected, and within each point the average RGB values, average brightness and average intensity were measured. For the reflected image and that seen through the window, when brightness and intensity were increased or decreased, the change in RGB values was compared to a standard video.

Result and Discussion

Anatomically speaking, of the 58 necropsy specimens, 48 had sustained injuries to the head and neck, and 46 had injuries to the internal organs, many of which had been brought about by a strong external force. It was evident, therefore, that the birds had made no adjustment to their flight velocity when they collided with the window. Furthermore, patterns of injuries to the thorax and internal petechial hemorrhaging in the wings, indicated the possibility that the subjects had approached the glass not face-on but at a somewhat oblique angle.

From an ecology-based perspective, when comparing the seasonal change in population and flight frequency by time period to the actual record of collisions, it appeared unlikely that these factors were greatly involved. Rather, it was considered that it is the conditions for building location, their construction and the avian flight paths they affect which is having a significant impact.

The optical analysis showed that the hues of the reflected images in windows, and the scenery seen through the glass, match that of the actual surroundings. This demonstrates that the transparency and reflection create an "other side" to the window, perhaps a scene in the glass itself that is exactly the same as the surrounding landscape. It is likely that this has a great effect on bird collisions. Also, when the brightness and intensity of the reflected image were reduced, the degree of similarity to the actual scenery dropped, whereas raising the brightness brought about an increase. This demonstrated that, for the reflected image and the actual scenery to be seen as the same, a certain level of brightness is necessary.

Conclusion

Conditions for building location, their construction and the avian flight paths they affect have a strong effect on the occurrence of window collisions by wild birds. Additionally, glass that creates an image that highly resembles the surrounding scenery through reflection or transparency and which lies on a flight path could be bringing about collisions. It is thought that natural scenery seen through or reflected by the window could be a main contributing factor in why wild birds hit the glass. While these two phenomena are based on different optical factors, in terms of their relation to these collisions they are considered to be the same in principle. On the other hand, with respect to the degree of hazard, although the brightness and intensity levels of the reflected image decrease dramatically following their reduction in the image of the natural scenery, the changes for the image seen through the glass are almost in step. It is considered that wild birds would find it difficult to accept the former case, so an image that has passed through transparent glass is thought to present a greater danger than one that has been reflected.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 215, 2011.

Clinical usefulness of serum and urinary 1,5-Anhydroglucitol and myo-inositol in dogs

Yohei MIKI*

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

(Received 11 March 2011, NMK-No. 10)

1, 5-anhydro-D-glucitol (1, 5AG) is a pyranoid polyol compound found in human circulating blood. Myo-inositol (MI) is a stereoisomer of inositol and serves as a precursor of inositol phospholipids. 1, 5AG and MI are filtered by the glomerulus and almost completely reabsorbed through the renal tubules. However, under hyperglycemic conditions, reabsorption through the renal tubules is competitively inhibited because the structures of 1, 5AG and MI resemble that of glucose. In this study, we investigated the kinetics of serum and urine 1, 5AG and MI levels in healthy dogs. We demonstrated that 1, 5AG and MI exist in canine serum and urine by gas chromatography-mass spectrometry. Under continuous hyperglycemic conditions, the serum 1, 5AG concentration in healthy dogs decreased while the serum MI concentration remained unchanged. Urinary excretion of 1, 5AG and MI increased significantly after blood glucose concentrations reached 200 to 220 mg/dl. A significant negative correlation was observed between serum 1, 5AG and glucose concentrations during hyperglycemia. However, no significant correlation was observed between serum MI and glu cose concentrations. In this study, we demonstrated that serum and urine 1, 5AG and MI levels were changed by blood glucose concentrations. The serum 1, 5AG concentration was decreased by continuous hyperglycemia. However, the serum MI concentration does not reflect hyperglycemia.

* Supervisor : Prof. Toshinori SAKO

Bull. Nippon Vet. Life Sci. Univ., No. 60, 216–218, 2011.

Studies on Influence of Bovine Genetic Background of Fatty Acid Synthesis Genes on Fatty Acid Composition in Adipose Tissues

Ju Hwan LEE*

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

(Received 11 2011, LDK-No. 1)

The fatty acid composition of adipose tissue is an important characteristic within the beef cattle industry. A higher proportion of monounsaturated fatty acid (MUFA) and lower melting point (MP) contributes positively to desirable beef flavor and tenderness, as well as to marbling score, which is a standard of carcass. Moreover, it is known that a high MUFA concentration contributes improving fat quality by decreasing the circulating concentration of LDL cholesterol, thereby preventing arteriosclerosis and coronary heart disease in humans. Recently, it has been intensively studied about monounsaturated fatty acid (MUFA) related to taste in beef. Fatty acid composition in bovine adipose tissue can be multifactorially influenced by genetic factors such as lipid synthesis and fatty acid metabolism related genes of cattle, as well as their age and nutrition. In particular, it has been suggested that the polymorphism and expression level of desaturase genes can significantly affect the level of MUFA in beef. This work was to investigate the influence of bovine genetic background on major fatty acid synthesis genes and their effect on the degree of unsaturation in adipose tissues of cattle. Quantitative real-time PCR was performed to profiling mRNA expression level of SCD and other lipogenic genes, and PCR-RFLP methods was utilized to detect nucleotide substitution based on polymorphism. The 1, 2, and 3 studies was explain that bovine genetic background on major fatty acid synthesis genes had any association with the fatty acid composition observed in various adipose tissues.

1. Relationship between adipose maturity and fatty acid composition in various adipose tissues of Japanese Black, Holstein and Crossbred(F1) steers

The amount of monounsaturated fatty acid (MUFA) is intimately related to adipose softness, melting point (MP) and flavor in beef. Stearoyl-CoA desaturase (SCD) is a main gene involved in MUFA synthesis. Mature adipose tends to be highly saturated whereas immature or maturing adipose is highly unsaturated when chronologically based, so the degree of unsaturation can be an index of adipose maturity. In this study, 3 different adipose tissues (coelomic (CL), perirenal (PR), and subcutaneous (SC)) from 3 beef breeds with differing slaughter age (Japanese Black (29.5 months), Holstein (20.1 months), and F1 crossbred (25.6 months)) were examined to 1) determine adipose maturity level as indexed by MUFA % and 2) determine SCD and other lipogenic gene mRNA expression levels in relation to unsaturated fatty acid content. Fatty acid composition was significantly different between adipose tissues ($P < 0.05$). MUFA amount

* Supervisor : Prof. Nobuhiro KIMURA

was high the following order : SC > CL > PR. This pattern corresponded to SCD mRNA expression profile showing higher expression in SC than CL and PR. However, Japanese Black cattle are an exception with CL adipose containing similar UFA % same as SC adipose, yet having the lowest SCD mRNA expression level among all adipose tissues tested. Therefore, SCD mRNA expression and MUFA % appear to be directly related, however differences in SCD mRNA expression among three adipose tissues may reflect differences in the fat development characteristics affected by chronological age of the cattle breeds.

2. Influence of stearoyl-CoA desaturase genotype and mRNA expression level on fatty acid composition of intramuscular adipose tissue in Holstein steers examined by biopsy over time

In this study, we investigated the influence of SCD genotype and mRNA expression on the fatty acid composition of intramuscular adipose tissue in Holstein steers examined by passing time. These cattle were allotted to either SCD AA or VA genotype, respectively (n=3 each) which were raised in the same environment and fed a high-concentrate (74% TDN, 12% CP) and low roughage (rice straw) diet *ad libitum* during the fattening period consisting of 8 months (10 to 18 months of age). Intramuscular adipose tissue was sampled by biopsy of *longissimus dorsi* at three time points (10, 14 and 18 months of age) from the same animals. With regards to SCD genotypes, MUFA% was significantly higher in AA than VA type at 14 months of age ($P<0.05$), but no differences in MUFA composition was noted at 10 and 18 months of age. The mRNA expression of many lipogenic genes, in intramuscular adipose tissue, did not differ between AA and VA type ($P > 0.05$). However, SCD, SREBP-1c, FABP4, FASN, PPARg, C/EBPa and adiponectin mRNA expression was significantly higher at 18 months versus 10 months of age ($P<0.05$). Moreover, irrespective of genotype, SCD mRNA expression positively correlated with MUFA% and C18 : 1 level (r= 0.624 and 0.590, respectively ; $P<0.01$). In addition, SCD mRNA expression and MUFA% appear to be strongly related with body weight of steers (r = 0.720 and 0.862, respectively ; $P<0.001$). There fore, differences in SCD mRNA expression and fatty acid composition of intramuscular adipose tissue in Holstein steers is not be heavily influenced by SCD genotype and may influenced by differences of body weight gain or age.

3. Influence of FASN and SCD polymorphisms on adipose tissue fatty acid composition and fat-melting point of Korean Native cattle (Hanwoo)

Recently, two novel mutations (g.16024A>G and g.16039T>C) were reported in exon 34 of FASN gene. In addition, single nucleotide polymorphisms were observed in the open reading frame of SCD cDNA, and significant effects were determined on MUFA % and the MP of intramuscular fat of Japanese Black cattle. Moreover, two genes have directly influence on the saturated or unsaturated fatty acid levels in final stage of fatty acid synthesis. Whereas, the study related to that has been reported few in Korea, in spite of that it can be used as genetic markers for the improvement of fatty acid composition of beef. Therefore, it was important for us to investigate whether FASN or SCD genotype has any influence on adipose tissue fatty acid composition in Hanwoo steers. The objective of this study was to investigate the influence of FASN and SCD genotypes on fatty acid composition and fat-melting point (MP) observed in various adipose tissues (coelomic (CL), perirenal (PR), intramuscular (IM) and subcutaneous (SC)) of Korean native cattle (Hanwoo) steers (n=44). Specifically, we focused on SNPs occurring at g. 16024A>G in exon 34 of the FASN gene and c.702 A>G in exon 5 of the SCD gene, because these two mutations are known to exert the highest degree of influence on fatty acid composition in Japanese Black cattle. The AA haplotype for FASN and SCD has been reported to be the superior genotype in Japanese Black. In

Hanwoo animals, FASN and SCD gene allele frequencies were slightly biased towards the G and V alleles, respectively. Correspondingly, the frequencies of the AA haplotype for each gene were markedly low, being 0.20 and 0.11 for the FASN and SCD gene, respectively. However, a GG FASN and AA SCD haplotype combination produced the lowest SFA % and highest MUFA % observed in 3 out of 4 adipose tissues, with IM being the exception having an AG FASN and AA SCD haplotype combination. Genotype also significantly influenced MP with lower MPs being associated with a GG FASN haplotype and/or AA SCD haplotype, especially in IM adipose tissue ($P < 0.05$). However, genotypes from both genes did not significantly correlate to fatty acid composition, especially against any individual fatty acid to be exact. Overall, our results suggest that single nucleotide polymorphisms (SNPs) in FASN and SCD genes may be useful markers to improve the beef quality of Hanwoo. Further study is needed to clarify the actual effects of these SNPs in conjunction with other known SNPs, occurring on the same gene, on fatty acid composition in breeding programs by marker-assisted selection.

In summary, biochemical fatty acid analysis demonstrated significant differences between adipose tissues or cattle breeds as well as ages. In addition, MUFA positively correlated against SCD mRNA expression level. However, significant differences in MUFA were not observed between genotypes as reported in other studies with Japanese Black cattle. Moreover, SCD genotype did not impact SCD mRNA expression level in intramuscular adipose tissues, indicating that age of the animal may be more relevant than breed genetics with respect to SCD genotype for Holstein animals. It is important to note that trends observed with mRNA transcripts may not necessarily correspond to similar trends in translated protein contents. These results suggest that differences of lipogenic gene expression may be age sensitive since the degree of adipose maturation depends on localization of the adipose tissue and chronological age in the fattening process. In addition, that may reflect of differences in body weight gain over time. Therefore, further study is needed to clarify the lipogenic gene mRNA expression pattern in nutritional and physiological or hematological aspect.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 219–221, 2011.

Studies on Developing Method the Management of Amino Acid Administration at Early Nutritional Stage of Broilers during Developing Embryo and Posthatch

Naotoshi INOUE*

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

(Received 11 March 2011, LM-No. 24)

Day-old chicks are the end product of the hatchery industry and provide the important starting point for poultry production. However, there was no feeding and managing standard for nutritional management of embryo and neonatal chicks, hence hatchlings fed at farm after 48–72 hours post-hatch. In addition, chicks hatch over 24–48 hour period in commercial hatchery. Difference in the hatching time will result in difference in quality of Day-old chicks. Moreover, fasting around post-hatched period prevent the development of digestive tract which is not only important for digestion and absorption of nutrients but also immune response, and reduce secretion of thyroid hormones are involved in homeostasis in the body temperature, and finally reduced growth of chicks. Recently, pre-starter diets were developed for supplying nutrients to chicks from hatchery to farms. This is able to feed to hatchlings in the hatchery, and packages with chicks in the container for nutritional supply during transportation. However, the feeding behavior of hatchlings is low, feeding works become a load in the hatchery, and nutritional composition is not evaluated experimentally. Thus, pre-starter diets had not developed nutritional status of hatchlings, and the early feeding method for hatchlings was achieved by early transportation to farms. On the other hand, to improve the difference in the quality of hatchlings caused by difference in the hatching time for 24–48 hours, in ovo feeding were developed. In addition, to supply nutrient during treatment in the hatchery, the hypodermic nutrient administration method which was done together with vaccination was developed. Development of those three methods indicates possibility to improve the nutritional condition of hatchlings. However, there are difference in the nutrient administration site among those three methods ; early feeding, *in ovo* feeding, and hypodermic feeding supply nutrient from the intestine, yolk sac, and blood circulation directly, respectively. Hence effects of nutrients supply by these three methods on chicks might be different. Newly-hatched chicks are usually supplied nutrients from 1) feed via the digestive tract, 2) degradation of the hatching muscle at back of neck, and 3) residual yolk sac. Because these nutrient supplying ways are agree to nutrient administration sites of above three methods, chicks may be able to receive nutrients without physiological stress. But many problems are induced by nutrients hugger in hatchlings in many tissues ; the pectoral muscles protein degradation, the prevent of developing immune system in the spleen and intestine, and consumption of Ig-Y in the residual yolk as the energy

* Supervisor : Prof. Dr. Yoshiyuki OHTA

source. It meaning that difference in the nutritional administration site may results in difference in the effectiveness in hatchlings. Therefore, in order to compare and characterize of effects of nutrient administration by above three methods, four experiments were conducted for practical application. Because protein was most important macro nutrient at post-hatch period, AA was administered.

Materials and methods

Chunky strain broiler breeder eggs were used for all studies. In Study 1 In order to evaluate effects of dietary CP levels of pre-starter diet on performance of broiler chicks for first 7 days, newly hatched broiler chicks were fed 3 levels of CP diets from 14 to 23% for 24 hours post-hatch, and evaluated growth performance and humoral immune response to sheep red blood cell until 7 days of age.

In Study 2, in order to compare the skeletal muscle protein degradation between chicks administered AA by the oral and subcutaneous administrations, two experiments were conducted as follows ;

1) hatchlings were subcutaneous administered AA or saline by batch or 4 times fractionated injection, and were bled 1 and 4 hour after first administration, and 2) hatchlings were force injected AA or saline into the crop, and were bled 0, 1, 2, 4 and 8 hour after administration. In both experiments, plasma 3-methyl histidine (3-HM) concentration was analyzed.

In Study 3, in order to clarify the effect of difference in the AA administration site on the gluconeogenesis of hatchlings, hepatic phosphoenolpyruvate carboxykinase activities of Day-old fasted chicks, in ovo AA or saline administered chicks, oral administered chicks, and subcutaneous administered chicks were measured.

In Study 4, in order to clarify the effect of difference in the AA administration site on the immunity of hatchlings, in ovo immunized Day-old fasted chicks, in ovo AA or saline administered chicks, oral administered chicks, and subcutaneous administered chicks were bled to determine the agglutination antibody titer.

In both Studies 3 and 4, in ovo AA or saline administration was done on Day 18 of incubation.

In Study 5, in order to evaluate the possibility of nutritional imprinting by in ovo AA administration, 0.5 mL of total AA solution consisted by whole egg protein composition (whole egg AA), saturated branched chain AA consisted by whole egg protein (BCAA) or saline were in ovo administered to broiler breeder eggs on Day 7 of incubation. Weights of embryos on day 14 of incubation and chicks until 4 weeks of age were recorded, and pectoral muscles were collected to determine the mRNA expression of IGF-1 receptor by Real time PCR. Data obtained in all experiments were analyzed by ANOVA or χ square methods.

Results and discussion

Dietary CP level of pre-starter diet affected plasma AA concentrations, humoral immune response of chicks at 3 day-old, and those were highest in the chicks fed 18% CP diet in Study 1 ($P<0.05$). There were only effect of subcutaneous AA administrations on Plasma 3-HM concentration which was reduced by both batch and fractionated subcutaneous AA administrations to hatchlings ($P<0.05$), while there were no effect of oral AA administration in Study 2. Thus, it was suggested that the difference in the AA administration site affected skeletal muscle protein degradation in hatchlings. In Study 3, hepatic PEPCK of hatchlings were decreased by all AA administration treatments at 2 hours after treatment, but not in other times. In Study 4, frequency of high level the agglutination antibody titer was observed in chicks administered AA by orally and subcutaneously ($P<0.05$). In Study 5, embryonic pectoral muscle weight/embryo weight were heavier in embryos injected BCAA than others ($P<0.05$). Body weight of chicks were lighter in chicks injected BCAA ($P<0.05$), while there was no significant difference in pectral muscle weights. Since expressions of

IGF-1 receptor mRNA of pectoral muscle were higher in chicks injected whole egg AA and BCAA during incubation than other chicks ($P < 0.05$), it was suggested that nutritional imprinting might be induced by in ovo AA administration to broiler breeder embryos.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 222–223, 2011.

Effect of fermented soybean milk intake on fecal microbiota and metabolites in humans

Shunsuke INOGUCHI*

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

(Received 11 March 2011, LMK-No. 25)

Recently, functional foods have attracted attention in Japan because the incidence of life style-related disease is increasing. Functional foods are classified into 3 groups based on their mechanisms of action : prebiotics, probiotics, and biogenics. Prebiotics are defined as non-digestable food components exhibiting beneficial effects on the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already residing in the colon, and, thus, improve the host,s health. Certain oligosaccharides which cannot be digested, except through bacterial activity, are prebiotics. A number of human volunteer trials have demonstrated that oligosaccharides are very efficient at stimulating bifidobacterial growth in the large intestine. Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance. Their beneficial effects on a host,s health, such as the alleviation of lactose intolerance, relief of constipation, imunomodulation, decrease in the activities of harmful fecal enzymes and mutagenicity, hypocholesterolemic effect, antagonistic effect on gastroenteric pathogens, antitumour activities, and reduction of the risk of gastrointestinal disease have been demonstrated in many studies. The probiotic preparations currently on the market are mainly based on lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium*. These genera have been shown to be important components of the gastrointestinal microbiota and are all relatively harmless. The isolates of lactic acid bacteria used in probiotics are mostly intestinal isolates such as *Lactobacillus gasseri*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Bifidobacteium longum*. Recently, it was reported that the ingestion of *Lactobacillus* and *Bifidobacterium*-fermented cow's milk increased the total number of bifidobacteria in humans.

Soybean milk is used as a base in a wide variety of products, including tofu, soy yoghurt, and soy-based cheese. Various nutrients are present in soybean milk, such as saponins, proteins, oligosaccharaides including raffinose and stachyose, and isoflavone. It has been reported that soybeans contain a number of anticarcinogens, and epidemiological studies revealed that a high level of isoflavonoids, particularly in soybean products, was associated with a low risk of colon cancer. Soybean oligosaccharides have also been reported as effective bifidus factors. It has been reported that raffinose and soybean oligosaccharides selectively enhance the growth of *Bifidobacterium* species. The administration of raffinose, and soybean oligosaccharides brings about an increase of bifidobacteria in human feces. The potential health benefits of soy foods milk have been emphasized by many researchers.

In the present study, the possible application

* Supervisor : Prof. Tomohiko FUJISAWA

of commercially available non-fermented soybean milk and soybean milk fermented using starter cultures containing Lactic acid bacteria and *Saccharomyces florentinus* as prebiotics and/or probiotics was investigated. Namely, the utilization of soybean oligosaccharides by fecal bacteria *in vitro*, the saccharide concentrations of non-fermented and fermented soybean milk, and the influences of non-fermented and fermented soybean milk intake on the fecal microbiota, pH, and metabolic activities in human volunteers were studied.

Soybean oligosaccharides, raffinose and stachyose, which are the main components of soybean oligosaccharides, were utilized by bifidobacteria except for *Bifidobacterium bifidum*, but most strains of *Escherichia coli* and *Clostridium perfringens* could not use them. The concentrations of glucose and sucrose in the fermented were significantly ($p<0.05$) lower than those in the non-fermented soybean milk. However, concentrations of raffinose, fructose, and stachyose did not differ in non-fermented and fermented soybean milk. During the dietary administration of non-fermented soybean milk (100 g a day for 2 weeks) to ten healthy adult humans, the numbers of bifidobacteria increased ($p<0.05$) and clostridia in the feces decreased ($p<0.05$). On the other hand, the numbers of bifidobacteria and lactobacilli in the feces increased ($p<0.05$), and *Enterobacteriaceae* and clostridia decreased ($p<0.05$) during fermented soybean milk intake (100 g a day for 2 weeks). Moreover, the concentrations of fecal sulfate were decreased on the intake of non-fermented soybean milk, and fecal acetic acid was increased ($p<0.05$) and fecal sulfide was decreased ($p<0.05$) during fermented soybean milk intake. These results indicate that the consumption of soybean milk, especially fermented soybean milk, is related to improvement of the intestinal environment, and it could be a novel type of probiotic.

In conclusion, it was clarified that both non-fermented and fermented soybean milk are useful as prebiotics, and fermented soybean milk is also useful as a probiotic.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 224, 2011.

The change of EEG (electroencephalogram) by theophylline administration in mice

Akiyo SHIMIZU*

Laboratory of Agriculture Foods
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Received 11 March 2011, LMK-No.26)

Theophylline can induce life-threatening seizures in humans, especially infants, even at serum concentrations below toxic levels. Such drug-induced seizures are a serious problem, but clinical research into them using human subjects tends to run into various ethical obstacles. Theophylline is regularly used as a bronchodilator in the pediatric field, and the relationships between the onset of theophylline-associated seizures (TAS), serum levels of the drug, and/or interictal electroencephalogram(EEG) findings in humans remain unknown. In this study, we used an animal model to investigate the pathophysiology of TAS. The male ddY mice aged 13 weeks were used in this study. Briefly, the mice were anesthetized with ketamine and xylazine, and 4 burr holes were created symmetrically anterior to the coronal and lambdoid sutures. Epidural electrodes, consisting of 1-mm-diameter silver balls coated with epoxy resin. These electrodes were subsequently implanted in the burr holes. A reference electrode was placed on the nasal bone, and a ground electrode was used to connect the electrodes to an EEG device (EEG-8310 ; Nihon Kohden, Japan). EEGs were recorded for at least 1 h once per day for 3-7 days and analyzed with Pc- Wave Form software. The mice were divided into two groups ; control mice (with no theophylline administration) and with theophylline administration. Oral theophylline diluted in drinking water was administered to the mice at a dose of 100 mg/kg/day, and EEGs were recorded as described above. Sera were collected and serum concentrations of theophylline and vitamin B6 were examined. The control sleep EEGs showed, as expected, no spike discharges : ddY mice are generally completely free of seizures. Even in awake state, EEGs also demonstrated no paroxysmal discharges without theophylline administration. However, brief repetitive spike discharges lasting up to 1 s were noted after oral theophylline administration. Theophylline generated spike discharges in each mouse but no actual seizures were observed. Serum theophylline concentrations all mice acquired low serum vitamin B6 concentrations. This result is that very high doses theophylline reduces serum vitamin B6 concentrations in mice. Although no clinical seizures were observed in the present study, I demonstrated that theophylline can play a potent role in subclinical epileptogenicity in ddY mice.

This is the first study to demonstrate that simple oral administration of theophylline can induce subclinical paroxysmal discharges in EEGs of seizure-free animals, even at low serum concentrations.

* Supervisor : Prof. Keiichi Aso

Bull. Nippon Vet. Life Sci. Univ., No. 60, 225–226, 2011.

Development of intestinal microflora and intestinal environment in infant

Chieko TANAKA*

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

(Received 11 March 2011, LMK-No. 27)

A tremendous number of bacteria inhabit in the large intestine and constructs a microflora which is characterized by complex microbial interaction. Bacteria colonize in the gastrointestinal tract of newborn baby through vertical transmission from mothers microflora and horizontal transmission from other environmental source of bacteria, such as hospital. It was well recognized that aerobic bacteria predominantly colonize in the gastrointestinal tract of newborn baby during the first days of life. Thereafter, the predominant bacterial group in the large intestine is gradually transferred from aerobic to facultative and obligate anaerobic bacteria. Moreover, it is well known that the alteration of the dietary components at weaninig period should influence the composition of the large intestinal microflora. This succession of intestinal microflora at infant, particularly at the weaning period closely related with host health. Therefore, analysis of succession in the intestinal microflora and environment is important for the prevention of diseases, and the maintenance and/or the improvement of host health. Although the intestinal microflora has been analyzed with cultivation method, molecular biological techniques are applied to the analysis of the microflora.

In this study, the alteration of intestinal microflora during the first two years of life in the infants was analyzed with molecular biological methods. In addition, organic acids and IgA which are the environmental factors influence the host health were measured to investigate for interaction between the alteration of intestinal microflora and intestinal environment.

(1) Analysis of intestinal microflora in the newborn baby during the first two years of life

The feces were collected from a healthy infant during the first two years of life. The bacterial genomic DNA was extracted from the feces. The fecal microflora was analyzed using the clone library method. Escherichia coli, which is facultative anaerobic bacteria, was predominantly detected during the first two weeks after birth. Thereafter, their detection rate was garadually decreased.At two months after birth, the obligate anaerobic bacteria, such as *Bacteroides fragilis* group and *Veillonella*, were predominantly detected. *Roseburia faecis*, the butyrate-producing bacteria, were also detected at two years old. Furthermore, main human intestinal microbiota were detarmined by real-time PCR. The real-time PCR analysis indicated that *B. fragilis* group and *Veillonella*, which were not detected immediately after birth, were increased with growth of infant. Conversely, *E. coli* was decreased. Thus, the molecular biological methods were available for the analysis of the succession of intestinal microflora as same as cultivation methods. Moreover, the colonization of uncultivable bacteria, such as butyrate-produc-

* Supervisor : Prof. Tomohiko FUJISAWA

ing bacteria, could be detected using the molecular biological methods.

(2) Real-time PCR analysis of intestinal microflora during first one year of infant

The bacterial genomic DNA was extracted from the feces corrected from five healthy in fants during first one year after birth. *Enterobacteriaceae*, *Enterococcus*, *B. fragilis* group, *Clostridium coccoides* group, *Clostridium leptum* subgroup, and *Bifidobacterium*, which were predominant bacteria in the human large intestine, were analyzed by real-time PCR. *Enterobacteriaceae* and *Enterococcus* were stably detected during one year after birth. *B.fragilis* group was stably detected at between three and five months after birth. Although *C. coccoides* group was detected at 10 months after birth, *C. leptum* subgroup was not almost detected during during one year after birth. The timing of colonization of *Bifidobacterium* was individually different. *Bifidobacterium* was stably detected at between one and three month years old, and was most detected at between five and six month years old. *B. infantis* and/or *B. breve* were initially colonized among the *Bifidobacterium*. Thereafter, *B. longum* was colonized at from four to five months after birth. It was suggested that the composition of the Bifidobacterium was altered with the growth of infant. These results indicated that the obligate anaerobic bacteria should not colonized immediately after birth. These bacteria would colonized at three months after birth.

(3) Organic acids and IgA in feces

Succinate, lactate, acetate, propionate, and butyrate were detected in the collected infant feces. Succinate and acetate were detected im mediately after birth. The concentration of succinate decreased at from two to three months after birth. The highest concentration of acetate was detected at from four to five months after birth. Propionate was stably detected at a half year after birth. Although butyrate was not detected before the weaning period, it was detected at from ten to eleven moths after birth. It was suggested that alteration of fecal organic acids profile related with alteration of bacterial composition in the feces. The level of fecal IgA was largely decreased after withdrawal of breast milk. The level of fecal IgA after the suckling period was not recovered to its in the suckling period. Inversely, the kinds of bacterial groups were increased. It suggested that luminal IgA level should influence the colonization of large intestinal bacteria.

The results in this study indicated that the alteration of intestinal environment in infant should relate with the development of intestinal microflora. They would influence the infant health.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 227, 2011.

Studies on the mechanism of cisplatin-induced changes in renal function and food intake in adult male mice

Koichiro CHO*

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

(Received 11 March, 2011, LMK-No. 28)

The typical side effects induced by anticancer agents, including renal dysfunction and suppression of food intake, have a negative effect on successful cancer chemotherapy. Reactive oxygen species (ROS)-mediated side effects have also been reported in addition to the main antineoplastic effects of anticancer agents. In this study, the possible role of the antioxidant enzyme superoxide dismutase (SOD), which converts O_2^- to molecular oxygen and hydrogen peroxide, in the side effects of anticancer agents was investigated. To examine the possibility of alleviating anticancer agent-induced nephrotoxicity and feeding suppression, transgenic (Tg) mice on a FVB/N background overexpressing human CuZn SOD (hSOD-1) were used. Renal function and food intake after administration of cisplatin (CDDP), the most effective chemotherapeutic agent, were examined.

Adult (age : 3-5 months) male hSOD-1-Tg (SOD-Tg) or wild-type FVB/N mice were used in the present study. The control group received a single intraperitoneal (i.p.) injection of saline solution (1 ml/100 g body weight), and the CDDP group received a single i.p. injection of CDDP (5, 10, or 20 mg/kg body weight). Animals were sacrificed at 72 h after CDDP administration, and blood samples were collected to estimate the levels of plasma creatinine (Cre), blood urea nitrogen (BUN), peptide YY (PYY), and glucagon-like peptide-1 (GLP-1). After sacrificing mice, the colon was immediately removed, the colonic mucosa was collected, and the PYY and GLP-1 levels in the plasma and colon were determined by enzyme immunoassay. Cre and BUN levels, which serve as markers of renal function, were determined by the Jaffe method and urease method, respectively (Fuji Dri chem 7000 v).

In FVB/N mice, Cre levels increased significantly in the 20 mg/kg CDDP-treated group compared to the saline-treated group ($p < 0.05$). However, the increase in Cre levels was blocked in SOD-Tg mice treated with 20 mg/kg CDDP. BUN levels increased significantly in both strains treated with 20 mg/kg CDDP compared with the saline-treated group ($p < 0.05$), but the increase was significantly lower in SOD-Tg mice than in FVB/N mice ($p < 0.05$). Plasma PYY and GLP-1 levels significantly increased in the 10 or 20 mg/kg CDDP-treated group compared with the saline-treated group in both FVB/N and SOD-Tg mice ($p < 0.05$). The results were similar for the colonic levels of PYY and GLP-1.

In conclusion, these results suggest that CDDP administration causes ROS-mediated renal dysfunction and PYY- and GLP-1-mediated suppression of food intake in mice. This is the first report to demonstrate that administration of anticancer drugs induces PYY or GLP secretion in mice. In addition, because the increase in PYY and GLP-1 levels induced by CDDP administration could not be blocked in SOD-Tg mice, the secretion of anorexigenic hormones was probably not mediated by ROS.

* Supervisor : Prof. Hiromi AMAO

Bull. Nippon Vet. Life Sci. Univ., No. 60, 228, 2011.

Bisphenol A-induced developmental effects in early chick embryos

Azusa NIIJIMA*

Laboratory of Agricultural Foods
Graduate School of Veterinary Medicine and Life Science
Nippon Veterinary and Life Science University

(Received 11 March 2011, LMK-No. 29)

Environmental exposure to endocrine disrupting compounds may have deleterious effects on human health. One of these compounds, bisphenol A (BPA), binds to estrogen receptors (ERs) and mimics estrogenic effects, and therefore may have adverse effects on human health. The reaction of BPA with ER is similar to that of estrogen with ER. BPA has also been reported to show a connection with the pubertal hormonal activity of children. BPA is used as a raw material in making polycarbonate and epoxy resin. The Japanese Food Safety Commission started investigating BPA after it precipitated in baby bottles in 2008. Many Japanese nursing bottles are made of polycarbonate. Sterilization of these bottles by boiling can cause the leaching of BPA and its mixing into the milk. Humans are also exposed to BPA through the consumption of foods preserved in polycarbonate coated cans. Heating of polycarbonate food containers can generate BPA, which may be mixed into food and absorbed in the body. Concerns have arisen regarding the effect of BPA on child growth. I therefore compared the biological effects of BPA and estrone on chick embryo development.

BPA was diluted to 0.1, 1.0 and 10.0 mM, and E 1 was diluted to 10, 100 nM and 1 μM in PBS. Using a Hamilton syringe, I injected each egg with 5 μl BPA, E1 or DMSO, also diluted in phosphate buffer solution (negative control) through a pin hole made on the shell via the yolk. The holes in the egg shells were sealed with cover silicon gum adhesive with polyethylene film, and the eggs were placed in the incubator and incubated for 48 hr at 37$\pm$0.5℃ and $>$80% relative humidity, with one rotation per hr.

The average number of somites was similar in embryos treated with BPA and control embryos. Malformations were observed in 75.0%, 50.0%, 36.6% of embryos administered 10.0, 1.0, and 0.1 mM BPA, respectively. In the negative control groups, embryos administered 1 : 10, 1 : 100, and 1 : 1000 fold dilutions of DMSO, malformations were observed in 18.8%, 10%, and 18.18%, respec tively. The incidence of malformations was significantly higher in the 10.0 mM BPA than their control group. We have shown here that BPA had deleterious effects on early chick development. Malformations included neural tube and head dysplasia. These embryonic deformities were similar to those induced by E1 administration. These findings indicate that binding of BPA or E1 to the ER activated the latter, resulting in chick embryo deformities. Differences in the incidence of malformation observed with BPA and E1 may be due to their affinity for the ER. The grave abnormality embryo dies of developmental anomaly in the egg shell.

This study is first study to show disturbance that BPA administration can induce developmental on anomaly of chick embryos.

* Supervisor : Prof. Keiichi Aso

Bull. Nippon Vet. Life Sci. Univ., No. 60, 229–230, 2011.

Effect of Canthaxanthin in Egg on Development and Hatchability of Broiler Breeder eggs

Takehiko HAYAKAWA*

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

(Received 11 March 2011, LMK-No. 30)

Introduction

In table egg, Carotenoids have been added in layer feed for improving the egg yolk colour. It has recently acknowledged that Canthaxanthin, one of the Carotenoid, effects for egg yolk pigmentation and productivity in layer industry due to the high pigmenting efficacy from feed to egg yolk.

Mean while, breeding industry is required chicks which are 1. high developing rate, 2. high hatchability, and 3. high chick quality. It is necessary to produce the high quality hatching eggs in breeding farm. Temperature, storage stability of hatching eggs, developing embryo, and hatchability are reported to relate with oxidative stress. And it is also reported that Carotenoids in egg yolk protected embryo from adverse effects or toxic effects of lipid peroxide. These facts can be expected that Canthaxanthin in breeder feed improves the quality of hatching eggs. However, there is evidence to suggest that Carotenoid in hatching eggs improve the storage stability, but there is no evidence to suggest that effect of Carotenoid in developing embryo. Recently, *in ovo* injection enabled nutritional administration into the hatching eggs and it became possible to study the effects of Carotenoid in developing embryo. So, we made it scientifically clear how *in ovo* Canthaxanthin injection influences on developing embryo and conducted to evaluate effects of Canthaxanthin on hatchability of breeder eggs in commercial breeding farm.

Materials and Method

120, 75, and 92 broiler hatching eggs in experiment 1, 2, and 3 respectively were produced from commercial flocks of Cobb strain. After measured egg weight, experimental group was divided 3, 3, and 4 groups with 40, 25, and 23 hatching eggs in Experiments 1, 2, and 3 respectively. Eggs were incubated at 37.8℃ and 60% RH. Experiment 4 was conducted utilizing 7,000 broiler breeders of Chunky (Ross) strain.

Experiment 1 was conducted to evaluate the effects of *in ovo* Canthaxanthin injection on hatchability of broiler breeder eggs. Sterilized water or Canthaxanthin water solution was injected into hatching eggs on Day 18.

Experiment 2 was conducted to evaluate the timing of *in ovo* Canthaxanthin injection on hatchability. Canthaxanthin water solution was injected into hatching eggs on Day 14 and 18 of incubation, respectively.

Experiment 3 was conducted to evaluate the effects of Canthaxanthin on plasma TBARS in broiler chicks due to the evaluation how *in ovo* Canthaxanthin injection influences on the effects of protection for oxidation of embryo. Sterilized water or Canthaxanthin water solution was injected into hatching eggs with drilled 3 holes and without hole on Day 14.

Experiment 4 was conducted to evaluate the

* Supervisor : Yoshiyuki OHTA

effects of Canthaxanthin in feed on hatchability broiler breeder eggs. Control group was fed a commercial breeder feed (CP : 16%, ME : 2,850 Kcal/kg) and Canthaxanthin group was fed a control group diet with 6 ppm Canthaxanthin. Laying rate and hatchability were observed from 43 to 61 weeks of age.

Treatments were analyzed by ANOVA in experiment 1, 2, and 4 using the GLM procedure of SAS software. Experiment 3 was analyzed by 2 -way ANOVA. The comparison of hatchability data was statistically-considered by chi-squared test. Differences were significant at $P < 0.05$.

Result

Experiment 1 : There was no difference in hatchability among treatments but Cantha-xanthin group was 95% compared to 90 % of other groups.

Experiment 2 : There was no difference in BW of hatched chicks. Day 14 group was significant higher hatchability than control group ($P < 0.05$).

Experiment 3 : Egg weights on Day 14 were decreased significantly due to the effects of drilled holes on water loss. Also BW of hatched chicks decreased significantly ($P < 0.05$). Hatchability was significant decreased due to the drilled treatment ($P < 0.05$). On other hand, Canthaxanthin showed an upward tendency on hatchability for all treatments. TBARS value was increased significantly due to the drilled treatment ($P < 0.05$), but Canthaxanthin injection made it decrease significantly ($P < 0.05$).

Experiment 4 : Canthaxanthin group was 85.7% which was significant higher hatchability than control group 82.3%. Laying rate in both groups were 72.3%.

Discussion

This study evaluated the effects of Canthaxanthin on antioxidation property during developing embryo with using *in ovo* feeding and water loss and fatty acid oxidation control method.

In Experiment 1, there was no significant result but observed improvement tendency on hatchability. Experiment 2 was preceded from the result of Experiment 1 and Day 14 group got significant result compared to Day 18 group. It is not clear the relation among size of hatching eggs and hatchability with including mode of action but it is speculated that a presence of Canthaxanthin might be linked with oxidative stress. Oxidative stress was conducted that there is under the metaphase of developing embryo because effects of Canthaxanthin in day 14 were higher than just before hatched chick which is speculated to be susceptible to oxidative stress.

These results indicated that Canthaxanthin effected antioxidation property not only for storage stability of hatching eggs but also for developing embryo. And it is showed to improve hatchability in commercial breeder farm. Thus, Canthaxanthin is indicated to be effective for broiler breeders.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 231, 2011.

Studies on aminotransferase from *Lactococcus lactis* ssp. *cremoris* 317, concerned in cheese ripening

Akihito MORIFUJI*

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

(Received 11 March 2011, LMK-No. 31)

The end products of proteolysis are amino acids, the further breakdown is considered to be an integral part of the overall process of flavor development in cheese. The first step in the degradation of amino acid is transamination, which requires the presence of an alpha-keto acid acceptor for amino group. Specific lactic acid bacteria (LAB) contribute to flavour formation in cheese by amino acid catabolism, but all LABs have not been clarified. The aim of this study was to screening and enzymatic property analysis of aminotrasferase (AT) activities in the catabolism of amino acid phenylalanine using *lactococcus* (Lc). *Lactis* ssp. *Lactis* 303, *cremoris* SK11, *cremoris* AM2, *cremoris* HP, *cremoris* 317, *cremoris* H61, *Lactobacillus* (Lb.) *helveticus* 880, *Lb. acidophilus* IAM 1084, *Lb. delbrueckii* ssp. *bulgaricus* IAM 1120, *Streptcoccus* (St.) *salivarius* ssp. *thermophilus* IAM 10064 strains.

All LABs were cultured with MRS or M17 broth (Becton, Dickinson and Company) and stocked by freeze-dried form. To screening analysis, crude enzyme extracts were obtained from stocked LAB (0.11 g), which were solved in 3ml Tris-HCl buffer (pH7.5) and ultrasonic treatment. Aminotransferase activity was determined by measuring the produced glutamic acid from substrate amino acid with a-ketoglutarate.

Purification of AT from crude extracts was performed with hydrophobic, anion-exchange and gel filtration chromatography column. *Lc.* genus showed relatively high AT activity other than *Lb.* and *St.* genus especially *Lc. Lactis* ssp. *cremoris* 317 was highest activity. Purification of the crude extract of cremoris 317 was showed 72 -fold using above chromatography. Thermal stability of purified active fraction (AT fraction) was indicated over 90% of the initial activity after incubation for at 30–40℃. When temperatures were above 50℃, residual activity gradually decreased and completely deactivation at 70℃ for 1 minuts. The activity of AT fraction was not affected by Na^{+}, Mg^{2+}, K^{+}, but markedly inhibited by other metal ions.

* Supervisor : Prof. Ryozo AKUZAWA

Bull. Nippon Vet. Life Sci. Univ., No. 60, 232, 2011.

Elucidation of the acid phosphatase active molecule from bovine skimmed milk

Shun YAMAGUCHI*

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

(Received 11 March 2011, LMK-No. 32)

Cheese ripening is a very complex biochemical process, it primarily involves glycolysis, lipolysis and proteolysis, together with numerous secondary changes that are responsible for the characteristic taste, flavor and texture of each cheese variety. Proteolysis is probably the most important biochemical event during the ripening of cheese varieties, with a major impact on flavor and texture. However, casein is a phosphoprotein, and phosphorylated protein/peptides are hydrolyzed with difficulty by proteinases/peptidases in cheese. Acid phosphatase (AcP) has been shown to hydrolyze the phosphate ester bonds in casein morecules, and so has been suggested to influence hydrolysis of casein. Thus, the combined action of proteinases/peptidases and AcP on phosphorylated proteins/peptides could be important for cheese quality. Recently, previous our report that newly AcP activity in purified the lactoferrin fraction (LF-rich fraction), although, AcP active molecule tentatively identified as LF, this conclusion is controversial from the viewpoint that purified LF has the possibility of other minor phospatase binding. Therefore, the aim of this study was to determine the principal active molecules with AcP activity in LF-rich fraction. The LF-rich fraction eluted from bobine skimmed milk using cation-exchange resin and column chromatograpy. Consequently, LF-rich fraction applied to gel filtration cheomatography with or without 8 M urea. Enzyme activity was determined by the *p*-nitrophenyl phosphate (*p*-NPP) method. One phosphatase unit was defined as the amount of enzyme that produced 1μmol of *p*-nitrophenol from *p*-NPP per 30 min. Analysis of N-terminal amino acid sequences of AcP active molecule using the automated Edman degradation method. The AcP activity was purified 90,704-fold compared with the raw milk. This fraction was analyzed by SDS-PAGE, which detected only 37 kDa band by silver attaining. The active property of the 37 kDa molecule in purified AcP fraction and commercial LF has an optimal pH at 5.0, optimal temperature at 60℃ and D-value of 95℃, 5min. Thus, these results suggested that principal AcP molecule of purified fraction and commercial LF were corresponding to the 37 kDa molecule. When the 37 kDa molecule was analyzed by a propein sequencer, which amino acid sequence was 95% homologous to bovine uteroferrin (UF). The porcine UF is well documented to belong to the so- called purple acid phospha tase (PAP) family.It has been repoted some AcP prpperty of UF were molecular weight in the range 35–40 kDa, optimal pH range of 5–6, active increase with specific metalion (Fe^{3+} and Zn^{2+}) or limited prpteolysis (trypsin, chymotrypsin and others) and resistance to tartrate acid.These profiles corresponded to the mammalian PAP family (UF, type-5 and tartaric acid resistance acid phosphatase). It seems reasonable to suppose that the active molecule in the LF-rich fraction is an undocumented bovine PAP family.

* Supervisor : Prof. Ryozo AKUZAWA

Bull. Nippon Vet. Life Sci. Univ., No. 60, 233–234, 2011.

The studies on evaluation in lymphocyte subsets and immunoregulation with cytokine or cytokine like substances in lactating cows to treat the bovine mastitis at the early stage

Ryota YAMADA*

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

(Received 11 March 2011, LMK-No. 33)

To decrease bovine mastitis causing economic damage for a dairy farmer, detection and treatment for bovine mastitis at the early stage were necessary. It is possible to detect the bovine mastitis at the early stage. Therefore, treatment the bovine mastitis at the early stage by immunoregulation treatment was investigated.

In experiment 1, effects of lactating cycle on the peripheral blood or milk lymphocyte subset was studied in lactating cows. In experiment 1-1, six Holstein lactating cows which have no mastitis were divided into the7 lactating period. Peripheral blood samples were collected from tail vein into syringe containing heparin, and the peripheral blood lymphocyte subset was determined using the flow cytometry. In experiment 1-2, twenty-three Holstein lactating cows which have no mastitis were divided into the7 lactating period. Milk samples were collected from each quarter , and the peripheral blood lymphocyte subset was determined using the flow cytometry.

In experiment 2, effects of oral administration of HuIFN-α and Tea extract on the white blood cell count (WBC) and on peripheral blood lymphocyte subsets were studied in mice, field voles (*Microtus arvalis*), and lactating cows, respectively. In experiment 2-1, 24 mice were divided into 4 groups with same average body weights. Then, mice of each group were administered water, HuIFN-α, Tea extract solution or HuIFN-α and Tea extract solution into oral-mucosal or stomach for 3 days, respectively. At Days 4 of treatments, after anesthesia, mice were bled from the heart, and the peripheral blood lymphocyte subset was determined using the flow cytometry. In experiment 2-2, 24 voles were divided into 4 groups with same average body weights. Then, voles of each group were administered water, HuIFN-α, Tea extract solution or HuIFN-α and Tea extract solution into oral-mucosal or stomach for 3 days, respectively. At Days 4 of treatments, after anesthesia, voles were bled from the heart, and the peripheral blood white blood cells (WBC) was determined using the flow cytometry. In experiment 2-3, four Holstein lactating cows which have no mastitis were divided into the 2 groups, and none (control) or HuIFN-α and Tea were administered with feeds for 3 days. At before experiment and days 4 after starting experiment, they were bled from jugular vein, and the peripheral blood lymphocyte subset was determined using the flow cytometry.

In experiment 3, effects of oral administration of stevia extract on the antibody titer to sheep

* Supervisor : Prof. Moriyuki SUGAWARA

red blood cell (SRBC) and on peripheral blood lymphocyte subsets was studied in mice, field voles (*Microtus arvalis*), and lactating cows, respectively.

In experiment 3-1, 12 mice were divided into 2 groups with same average body weights. Then, mice of each group were administered water, or Stevia extract solution into stomach for 7 days, respectively. At Days 8 of treatments, after anesthesia, mice were bled from the heart, and the peripheral blood lymphocyte subset was determined using the flow cytometry. In experiment 3-2, 18 voles were divided into 3 groups with same average body weights, and they were intraperitoneal injected sheep red blood cell (SRBC) to immunize. Then, voles of each group were administered none (control), water, or Stevia extract solution into stomach for 7 days, respectively. At Days 8 of treatments, after anesthesia, voles were bled from the heart, and the agglomeration antibody titer to SRBC was evaluated. In experiment 3-3, six Holstein lactating cows which have no mastitis were divided into the 2 groups, and none (control) or Stevia pellet were administered with feeds for 8 days. At Days 3, 8, and 15 after starting experiment, they were bled from jugular vein, and the peripheral blood lymphocyte subset was determined using the flow cytometry.

In experiment 1-1, there were significantly change number of CD4+, CD11a+ and CD25+ CD4+ cells in lactating period. In experiment 1-2, there were significantly change number of CD 8+ cells in lactating period.

In experiment 2-1, the number of lymphocytes were significantly higher in HuIFN-$\dot{\alpha}$ administered mice than Tea extract solution administered mice ($P<0.05$). The number of CD4+ cells were significantly higher in HuIFN-$\dot{\alpha}$ administered mice than Tea extract solution and HuIFN-$\dot{\alpha}$ Tea extract solution administered mice ($P<0.05$).The number of CD8+ cells were significantly higher in HuIFN-$\dot{\alpha}$ administered mice than those of other groups ($P<0.05$).

In experiment 2-2, the number of WBC were significantly higher in HuIFN-$\dot{\alpha}$ Tea extract solution administered vole than those of other groups ($P<0.05$). In experiment 2-3, the number of CD4+ cells tended to be lower in HuIFN-$\dot{\alpha}$ Tea administered cows, but not significantly. The number of CD11a+ cells were significantly higher in HuIFN-$\dot{\alpha}$ Tea administered cows than those of other groups ($P<0.05$).

In experiment 3-1, the number of WBC, lymphocyte tended to be lower in Stevia extract administered mice, but not significantly. In experiment 3-2, the frequency of appearance of the antibody titer to SRBC at the x16 dilution was higher in the voles administered Stevia extract than those of other groups ($P<0.05$). In experiment 3-3, CD11a increased in the control group with advancing experimental period, but not in the Stevia administered cows, significantly ($P<0.05$).

Thus, Variation in immunity index from Holstein dairy cow during lactating period was identify. Therefore, a potential that oral administration of HuIFN-$\dot{\alpha}$ and Tea or Stevia would fluctuate cytokine network of ruminant stomach animal was indicated.

Bull. Nippon Vet. Life Sci. Univ., No. 60, 235–236, 2011.

The Identification and Tissue Specificity of Hexokinase II and Glucokinase Gene for Investigating the Metabolically Characteristics of Field Voles (*Microtus arvalis*)

Emi YOSHIZAWA*

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

(Received 11 March 2011, LMK-No. 34)

Introduction

Field voles, herbivorous rodents, has a specific developing stomach which is separating three parts and existing bacteria, generates volatile fatty acid (VFA) and use fats as energy source mainly. Hence, voles are expected as an laboratory animals of herbivores for feed judgment. However, it was clearly that field voles are a very weak animal to hyperglycemia because they maintain blood glucose at low level and easier develop the diabetes compared to mice. Above fact indicates they have a feature on the metabolism of glucose. Therefore, in order to use field voles as the laboratory animals of a herbivore and a diabetic model, hexokinase II (HK II) and IV (glucokinase ; GK) which are limiting enzyme of glucose metabolism and have insulin susceptibility are focused. Present studies were conducted to clarify the characteristics on the metabolism of glucose in voles, 1) the identification and tissue specificity of vole were investigated in HK II and GK m RNA, 2) an insulin susceptibility was made sure in HKII and GK, and 3) effects of dietary energy levels and energy sources on metabolic pathway of glucose were investigated using HKII and GK mRNA expression, plasma glucose and insulin levels as criterion.

Materials and methods

Experiment 1, four Eurasian field voles were sacrificed for the experiment. They were cervical vertebrae dislocation, and the heart, liver, spleen, ovary, stomach, pancreas, adipose tissue, and skeletal muscle were collected, and immediately frozen by liquid nitrogen. After extracting RNA, cDNA was produced, the target region was amplified by PCR, and the sequence was investigated. Experiment 2, in the insulin susceptibility test, voles fasted for 24h and administered insulin after it fasted for 24h. Primer was designed from HKII and GK mRNA of a vole and was compared by real-time PCR. Experiment 3, the vole which is 16〜19 week age, 12 males and 8 females were used. In the experiment diets and this adjusted to energy 2.91 Mcal/kg, 3.36 Mcal/kg and added a soy oil or glucose, feed 0.88 kcal for 1g, respectively. Voles fed experiment diets individually for 14 days to tame. They were sacrificed and sampled blood, liver and skeletal muscle. The serum insulin and glucose concentration, HKII and GK mRNA expression levels were measured.

Results and discussion

The base sequences and amino acid sequences of HK II and GK of field vole showed and high homology with mouse, rat, human, and mutation was not accepted in the mutation site in the

* Supervisor : Prof. Moriyuki SUGAWARA

diabetes currently confirmed with the mouse. Although Genotype of HK II of vole was close to that of mouse and rat in the phylogenetic trees, while that of hepatic GK was close to humans more than mouse and rat. Moreover, although organ tropisms was the same as that of mouse in many tissues, expression of HK II was observed in the before stomach and a pyloric stomach, which were parts of three location of specific stomach of voles, and expression of HK II mRNA was not observed in the heart conversely. The GK appearance was admitted only in the liver of voles. These genes were identified as mRNA of HKII and GK in voles, because HKII and GK mRNA expression were increased by insulin administration. Finally, effects of dietary energy levels and sources were investigated in voles. Plasma glucose concentration was elevated by increment of dietary energy levels induced by both fat and glucose supplementation, whereas plasma insulin levels was decreased in voles fed glucose added diet compared to voles fed other high energy diets. In addition, expressions of HKII and GK mRNA increased in all high dietary energy groups compared to 2.91 Mcal/kg group, while there was no difference between that in the glucose added group and other high dietary energy groups. Therefore, these results suggested that muscle HKII and hepatic GK might regulated by insulin independence regulation system in field voles.