F-3.2.2 **Preservation of genes of endangered wild animals** (Final Report)

Contact Person Susumu Ishii, Department of Biology, School of Education
Waseda University, Nishi-Waseda 1-6-1, Tokyo 169-50
Phone 81-3-5286-1519 Fax 81-3-3207-9694
E-mail susumu@mn.waseda.ac.jp

Total Budget for FY1993-FY1995 21,151,000Yen(FY1995; 5,854,000Yen)

Abstract

Dispersed cells of inner organs of a Japanese male of the Japanese crested ibis, *Nipponia nippon*, and those of a Chinese male of the same species were cryo-preserved. The preserved cells of the former individuals were cultured and revealed to proliferate over several generations. Tissue pieces of the former bird were frozen and preserved in liquid nitrogen. To monitor gonadal developmental conditions, we devised a non-invasive method in which sex steroid hormone concentrations in feces are used as the index. This method may be used for selection of individulas for captive breeding of endangered birds. We also developed a method to induce oocyte growth, ovulation and oviposition by hormone administration. By means of the implantation of primordial germ cells, one of us could produce a cimeric gonads of the domestic fowl and the Jpanese quail, but no chick of the heterologous species has been produced.

Key Words Cryopreservation, Hepatic cells, Kidney cells, Testicular cells, Japanese crested ibis, *Nipponia nippon*, Fecal sex steroid hormone

1. Introduction

When this project has started, we had only two captive individuals the Japanese crested ibis, *Nipponia nippon*, which are the only individuals alive in Japan. It was urgent to organize a scientist group and establish a method to preserve cells and genes of these last individuals in Japan. There are about 60 individuals of this species in wild and captive conditions in China. These are the only individuals of this species alive in the world. Under such a condition, it is also important to establish methods to breed this species artificially under a captive condition. Our group have established these methods and have applied some of the methods to two individuals as described below.

2. Preservation of cells and tissues

For these three years, we have been concentrated mainly in cryo-preserving of cells and tissues of the Japanese crested ibis, *Nipponia nippon*. Until recently, only two

individuals of this species survived in Japan. In China, a wild population of about 40 individuals and about 20 captive individuals have been reported to survive. These are the only individuals alive in the world. One of the Japanese individuals, a male named Midori (or Green), died in April, 1995. We cryo-preserved dispersed cells of his testes, liver, kidneys and lung. A culture test of the preserved cells with an anti-freezing reagent in liquid nitrogen revealed that they could proliferate for several generations. Tissues of most of inner organs of Midori were cut into small pieces and preserved in liquid nitrogen without anti-freezing reagent. We are planning to prepare cDNA and genomic libraries from these tissues and cells. In future, a part of these libraries, preserved cells and tissues will be provided to qualified scientists for scientific research on application. A Chinese male which was borrowed for breeding from Chinese Government died in December, 1994 in Japan. His testicular, hepatic and kidney cells were also cryo-preserved.

3. Non-invasive method to estimate gonadal activities and artificial breeding by administration of hormone

In spite of recent extensive development of biological techniques, little has been incorporated into conservation biology. We have been attempting to apply modern endocrinological methods to conservation biology, especially reproduction of endangered avian species including techniques for captive breeding of them (Ishii, et al., 1995). To select individuals for breeding efficiently from a captive colony of an endangered avian species, it is important to know gametogenic and endocrine conditions of the ovary and testis of birds in the colony. To estimate these conditions, measurement of plasma levels of hormones which are directly related to the gonadal conditions is necessary, since direct observation of the gonad by laparotomy may not be preferred. However, it is also not recommended to catch such precious birds for collection of plasma samples. To solve this problem, we have developed a method to measure sex steroid hormone levels in avian feces which reflect sex steroid hormone levels in blood. We first show some of our data showing that how faithfully fecal sex steroid levels reflect plasma sex steroid levels in Japanese quail which were used as a model. We also present an example of application of the method to an endangered species, the "Toki" or Japanese crested ibis (*Nipponia nippon*).

According to the estimated gonadal condition by the fecal sex steroid analysis method, we may select individuals for mating. If none of the examined individuals shows the active gonadal condition, we have to stimulate the testis or ovary of some individuals in order to breed. For breeding mammals with such a regressed gonad, administration of gonadotropic hormone is used commonly in mammals including the human being. However, the situation had been different in birds until recently. Even in domestic hens, no investigators had been able to induce the ovulation, the final step of the ovarian gametogenic activity, by hormone administration except for a case in domestic hens just after the hypophysectomy and still possessing a developed ovary. Accordingly, we had to develop a method to induce ovarian growth and ovulation in female birds by administration

of hormone (Wakabayashi et al., 1994). Our method for the hormonal induction of a series of the ovarian process, i.e. the oocyte development, ovulation and oviposition, is also described in this paper.

Fecal sex steroid analysis in birds

Extraction methods: We had to employ three different methods to extract sex steroids from fecal samples of birds according to the bird species. The simplest method was extraction using water as the extraction medium followed by radioimmunoassays of the steroids. This method was used in the Japanese quail. In the Japanese crested ibis, we had to use an organic solvent for extraction and LH20 column chromatography for removing substances which disturbed measurement of steroid hormone by radioimmunoassay. A more complicated method was developed to measure sex steroids in feces of the rock ptarmigan. Feces of this bird contained much lipid-like substance which had to be separated from the steroids before radioimmunoassay. For this purpose, we used extraction with an organic solvent followed by a high performance liquid chromato-graphy. *Fecal sex steroid study in the Japanese quail*: To show usefulness of the fecal sex steroid analysis method in estimating the gonadal activities, a part of our results with the Japanese quail is presented.

In an experiment, we used three groups of male Japanese quail, the first group normal intact adults kept under a long-day photoperiodic regimen, the second group normal intact adults kept under a short-day photoperiodic regimen and the third group castrated adults kept under the long-day photoperiodic regimen. Mean concentrations of androgen (testosterone plus dihydrotestosterone) in blood plasma and feces were compared between the three groups and also between plasma and feces. The mean concentrations in feces changed in close association with those in plasma.

In the next experiment using females, feces were collected from two groups of Japanese quail hens, one laying at a certain period of the day and the other non-laying. As well known, progesterone in plasma shows a transient rise around the time of the ovulation in laying hens. Then, we collected fecal samples from laying hens and progesterone in the samples was measured. The mean concentration of progesterone in feces had a clear peak around the time of ovulation that was estimated from the oviposition time in the laying group but no significant peak in the non-laying group. Thus, we could show that the gonadal activity can be estimated by monitoring fecal sex steroid hormone. In the case of birds, both urine and feces are excreted together. This is distinct from mammals. It is well known that sex steroids are metabolized to glucronide or sulfate forms before they are excreted. However, enough amounts of the free form (circulating form) of the steroids were detected in feces in our experiment. We suppose that they moved from the circulation into the content of the digestive duct by diffusion, because they can easily pass through cell membranes.

Fecal sex steroid study in the Japanese crested ibis: We applied the fecal sex steroid analysis method to asses the reproductive condition of a male of the Japanese crested ibis. The mean concentrations of androgen for several days in three summer months in 1994 and a period for several days around the new year's day of 1995. Compared to the summer months, the concentration was significantly higher in the period around the new year's day

when nuptial plumage coloration appeared. It is clear that the fecal androgen concentration reflects well the gonadal function.

Hormonal induction of the oocyte growth, ovulation and oviposition in the Japanese quail.

Repeated injections of gonadotropin preparations could not induce ovulation in hens of female domestic birds (see Wakabayashi et al., 1994). We studied changes in the luteinizing hormone (LH) concentration in blood plasma in Japanese quail. The LH concentration showed a transient increase and dropped to the subnormal level after several hours. Then, we decided to use the osmotic pump (ALZET Co.,) for the chronic treatment of female Japanese quail with gonadotropin. These birds were kept under a short-day regimen for a certain period before the experiment was started and thereafter. Their ovaries were considered to be in the completely regressed condition at the start of the experiment. The pump was lorded with a chicken pituitary glycoprotein fraction which is rich in gonadotropins, and implanted into the abdominal cavity. The flow rate of the glycoprotein was 12.5 μ g /hr. This quantity was equivalent to 1.5 μ g chicken LH/hr. High gonadotropin levels in plasma were maintained over a period of two weeks, when plasma gonadotropin levels were determined by radioimmunoassay. The ovary of the females treated with pituitary glycoprotein for about two weeks by means of the osmotic pump was well developed and contained a number of oocytes containing the yolk. However, no ovulation was induced in these females. To mimic the LH surge which induces ovulation in normal hens, we injected chicken pituitary glycoprotein daily for the last five days of the osmotic pump treatment in female Japanese quail kept under the short-day regimen. Four of the 7 treated females laid 7 eggs during the injection period. One of them laid four eggs and each of the remaining three one egg. All the eggs were incubated artificially and two of them hatched. The chicks were males and one of them was confirmed to be fertile by mating with an adult virgin female. We have been successful in stimulating the testis of male Japanese quail kept under a short-day regimen by the treatment.

Conclusion and Discussion

The fecal sex steroid analysis method is shown to be a useful tool to estimate the reproductive condition of birds non-invasively. We may apply this method to monitor the reproductive condition of birds in feral populations. Furthermore, in the case of captive breeding of birds, we may select individuals for breeding from a captive colony of birds by using this method. However, selection of an appropriate extraction method is important to obtain a satisfactory result. Our hormone administration study was the first successful case of hormonal induction of a series of ovarian events, i.e. oocyte development, ovulation and oviposition, in birds. Combination of the two different means of hormone administration, the osmotic pump and injection, is considered to be the cause of our success. However, for the actual application of this method for captive breeding of endangered birds, problems still remain to be solved. First, the success rate of about 40% may be not high enough. It is also important to clarify a problem on the immunological response to hormones from heterologous species. We are now conducting studies to improve the success rate and also

examining a possibility to use commercial mammalian gonadotropin preparations such as pregnant mare serum gonadotropin and human chorionic gonadotropin. Preliminary data have shown that they are effective.

References

Ishii, S.,M. Wada, S. Wakabayashi, H. Sakai, Y. Kubodera, N. Yamaguchi and M. Kikuchi, 1994. Endocrinological studies for artificial breeding of the Japanese ibis, *Nipponia nippon*, an endangered avian species in Asia. J. Biosci. 19, 491-502.

Wakabayashi, S., M.Kikuchi,M.Wada,H.Sakai and S.Ishii, 1992. Induction of Ovarian Growth and Ovulation by Administration of a Chicken Gonadotrophin Preparation to Japanese Quail kept under a short-day Regimen. British Poultry Sci. 33, 847-858.