Screening and Testing of Endocrine Disruptors in Avian Species Present status and future direction

Masaru Wada

Tokyo Medical and Dental University

Thanks for the introduction. My name is Masaru Wada of Tokyo Medical and Dental University. After the general talk by the first speaker, Mr. Herman, I would like to talk about a more specific topic, the present status of screening and testing of endocrine distruptors in birds, and about our activities in Japan to this issues. The original English title is thus changed to the one above.

I have been interested in avian reproductive endocrinology. If you will permit me to start with some personal information, my work with endocrine disrupters began when I participated in the first meeting of the avian reproduction expert group held in Leipzig in May 1999. This is a photograph which showed the conference center.

One of the objectives of the conference at that time, as our first speaker, Mr. Herman said, was to revise the test guideline 206. Test animals of the guidelines 206 are Japanese quail, bobwhite quail and mallard. A revised guideline would use Japanese quail and bobwhite quail as a test animal omitting mallards, but we were not sure we should select Japanese quail or bobwhite quail and which animal is more suitable for the test animal. Those were the beginning of our discussion.

We discussed about endpoints we should select, experimental design we should conduct, and statistics we should employ. We did not reach any decision, and we began to talk about whether or not it was necessary to conduct a validation test.

At the end of the conference, we felt that guidelines for endocrine disrupters might be necessary beside 206, and decided to continue to discuss the topic.

The second meeting was held in Nashville in November 2000. This is a photograph I took in one of the rooms at the convention center in Nashville. At the second conference, the somewhat different term “endocrine disrupters” came up, and our talk turned to what should be done about guidelines for endocrine disrupters.

By the time of the meeting, it was already decided to consider the two-generation test and to use Japanese quail as a test bird. A draft was prepared and distributed beforehand. We had a lengthy discussion about the 2-generation test. You can see the flow chart hanging on the wall that was a result of the discussion in brainstorming fashion.

The objective at that time was to revise the draft distributed in December 1999, and we reviewed it, and tried to create a new guideline. We also discussed what sort of problems there are with the two-generation test and what kind of test designs is suitable. Finally our conclusion was that we should conduct some actual research and then we make a proposal.

A problem was a relationship between the existing 206 guideline which is a one-generation test and the two-generation test which we are going to propose. Then we talked about the pre-validation test. There was however not much progress. We have currently decided to take a of part of the validation test, but have not reach the final conclusion how and when the pre-validation test will be done.

Concerning the actual revision to test guideline 206, we talked about the need to conduct a comparison test to decide which species are better for the test animal, bobwhite quail or Japanese quail. We still have not specifically decided what to do about this.

Having thus participated in the Leipzig meeting, I returned to Japan with a thought that something must be done about this problem in Japan. Research has been conducted using fish and amphibians, but birds must be included in the research to see the effect of endocrine disruptors because they are ideally

situated in the ecosystem as species that live in aquatic and terrestrial environments. This slide shows about my point as an avian endocrinologist; birds are extremely important.

The main reason is that birds are easy to handle and they lay cleidoic egg; eggs are much easier to handle than mammalian embryos. This is a quail egg. The egg is completely closed when laid. A chick will hatch from the egg, and its nourishment and necessary materials are all contained in the egg.

The egg yolk contains the proteins phosvitin and lipovitellin as nourishment. As the audience has already listen to the topic about vitellogenin in the talk about fish, vitellogenin is also produced by the liver as a precursor protein and is sent to the ovarian follicle in birds.

Triglyceride is also extremely important as a nutrient and source of energy. Lots of triglyceride accumulates inside the yolk. Just as with vitellogenin, the liver produces triglyceride droplets that are covered with lipoproteins by the action of estrogen. Triglycerides are in particular enveloped in very low density lipoprotein (VLDL) with apoprotein B as a covering protein.

This slide shows the gonads and gonoducts of a mature male and female birds. These are the male and these are the female organs, and as you can see, they are completely different. In the case of the female, gonadotropin induces estrogen release which in turn acts on the liver to synthesize vitellogenin and VLDL. They are transported from the liver in the blood and accumulated in the ovaries as you see here. Gonadotropin is similarly produced and secreted in males and stimulates androgen release, but it never causes the liver to produce vitellogenin. VLDL is produced but the quantity is much smaller in the males. Thus only an extremely small quantity of VLDL is found in the blood of the males.

With these backgrounds, we decided to set up a project to study endocrine disruption using birds. Fortunately the Ministry of the Environment supported us and we could form a project. At the beginning the project has two aspects, one was the basic research to study mechanisms of avian endocrine disruption and the other was to establish a method of screening in birds. The research part is currently conducted by Professor Kiyoshi Shimada of Nagoya University and Professor Yukinori Yoshimura of Hiroshima University.

I am now going to talk on the screening part. As was previously mentioned, vitellogenin is an extremely sensitive marker for exposure to estrogenic substances in birds as well as in fish. We therefore set up to develop a measurement method capable of assaying vitellogenin. In the case of birds, vitellogenin has three different fractions according to molecular mass (I – III). Vitellogenin III is just a trace amount and major component is vitellogenin II. In this slide, these are molecules of I and II. As was previously mentioned, the molecules contain lipovitellin I, phosvitin and lipovitellin II. Vitellogenin was taken up by a growing ovum and splitted into these components. Here is major vitellogenin II. The structures of vittellogenin I and II are basically the same, but II has slightly smaller molecular mass than I.

We could have used vitellogenin as an antigen from quail plasma, but lipovitellin in the yolk is more suitable for the antigen because it can be obtained in large quantities and is easy to handle. We separated the lipovitellin I from the yolk of quail eggs and then purified by gel filtration. These slides show the results using the SDS-PGE. In this slide this column is from male serum and this is from female serum. As indicated by an arrow, this band is vitellogenin. And this is lipovitellin separated from vitellogenin. Here we have a fraction of lipovitellin that had a molecular weight of about 120. Using this antigen, we raised antibodies in rabbits, and separated the IgG fraction. We further purified the antibody by removing non-specific antibodies through an affinity column coated with male plasma proteins. This slide shows a dilution test using this final antibody fraction using male and female serum. The female serum shows a clear dilution curve according to antibody concentrations but the male serum did not contain any substance that reacts with the antibody.

This slide shows further characterization of the antibodies as viewed by SDS-PAGE. This is vitellogenin and so is the stuff over here. This is western blotting. Here we have vitellogenin stained by

the antibodies we obtained this time. This is the case in female serum. Here we have a vitellogenin standard sample, vitellogenin in the serum. As this area stained, the antibodies are recognized the protein. Here we used antibodies from a Black-tailed Gull provided by Professor Akihiko Hara of Hokkaido University. Similarly, they are recognized the protein too. So we now very sure that the antibody raised can be used to detect specifically lipovitellin and vitellogenin.

This is the results of ELISA established using the antibody mentioned above. This is a standard curve, from 2000 ng/ml to this concentration here. The standard can be correctly drawn. This graph shows parallelism using female serum. The female serum is definitely parallel. The ELISA system has an enough sensitivity to measure vitellogenin in blood serum.

VLDL on the other hand is measured using HPLC. This shows a diagram of our system for measurement of VLDL. Actually the system has been developed to estimate serum lipoproteins. Simply speaking, the substance is sorted into fractions according to particle diameter using HPLC. Then the fractions are divided into two halves, and the triglyceride and cholesterol contained in each fraction are estimated by being reacted with enzymes and detected by a detector. Data is obtained and processed by a personal computer. The fraction of the largest particles that contain triglyceride can be regarded as VLDL.

As a result of a preliminary study, we have the measurement results in four male and four female Asian Blue Quails. HDL in the blood is shown in green and VLDL is shown in white columns. As you see here, VLDL but not HDL was high only in laying females. This tells us that VLDL increases to carry triglyceride when laying eggs.

This slide shows that a shift of photoperiod from short days where the gonads do not develop to stimulatory long days induced VLDL increases in females but not in males in the Asian Blue Quails.

When males were administered 1 mg of estrogen, a relatively high dose, circulating VLDL definitely increased.

By using these two tools, sexually mature Japanese quail kept on long days were administered test substances for seven days. All of the doses were administered by intraperitoneal injection. We conducted the test using several substances, but this time I will show you only the results of estradiol and diethylstilbestrol.

The vertical axis indicates concentration of VLDL and vitellogenin and the horizontal axis indicates doses. Since it is the first experiment, we started from a somewhat high concentration in order to obtain reliable results. I think you can see a dose response here.

This slide shows a correlation between circulating VLDL and vitellogenin after injected of estradiol. Two endpoints shows a good correlation, although VLDL reaction was somewhat poor in the lower concentrations.

This is a result of diethylstilbestrol. In the case of diethylstilbestrol, the response was saturated in the higher concentrations and the dose-response curve leveled off.

This is a correlation between VLDL and vitellogenin. Just as was mentioned in the results in estradiol, the appearance of VLDL in the lower concentrations is somewhat poor, and the correlation curve is standing. However the correlation is statistically significant.

These are the results we obtained so far. We think that sensitivity of vitellogenin ELISA should be improved. Currently we use polyclonal antibodies for the vitellogenoin ELISA and we are now attempting to improve sensitivity by switching the antibody from polyclonal to monoclonal antibody and by reconsidering the various parts of the ELISA procedures. We are also attempting to improve sensitivity in VLDL assay system.

Using these methods, we are engaged in screening several substances listed by SPEED ’98. These substances include nonylphenol, octylphenol and tributyltin. We are also currently engaged in screening several other substances.

As to the research aspect I talked about in the first part, which are the base to construct the two-generation test, the researchers at Hiroshima University are studying the effect on the offspring when the parents were injected with the substances. With the results from the basic aspects and the screening results, or a combination of the two, we Japanese researchers must consider and contribute to build up the avian two-generation test the OECD is aiming as mentioned by Dr. Herman for the next step.

We are now observing the estrogenic effect, but it is necessary to develop new methods of observing the effect of androgenic effects as well, so we are working on that too.

Concerning establishment of OECD guidelines for endocrine disrupters in birds, I give a talk about the present status and Japanese contribution, specifically what is being done. The development in screening methods in birds presented here were a results of joint research currently being conducted by Tokyo Medical and Dental University and Transgenic Inc.

Thank you for your attention.

Q&A

Koëter: Thank you very much for that nice overview work in birds. Do we have questions on that? Yes, please.

Q: Thank you very much. I have a question about ranking. An assay like the one you have conducted is incorporated in 206 of the OECD as the end point. Is my understanding that you have conducted such work and ranking correct?

Wada: I do not think that vitellogenin is always incorporated into 206. In other words, because we are dealing with reproductive toxicology in 206, eggshell thickness and hatchability and so on are the first priority. I think vitellogenin will be included in the second-generation test. We are currently discussing for this direction.

Q: In the beginning, you said that validation of birds is being conducted at the OECD using the second-generation test. Is it true that there is currently no movement in Japan to participate in validation of the second-generation test?

Wada: As Dr. Herman said a little while ago, the avian expert group includes a large number of ecotoxicologists, and there is much debate and discussion about plans and procedures but not actual studies and experiments. As I think what we need now is the actual experiments to be done, I would like to try to go to the direction.

Q: Thank you very much.

Koëter: Any other questions? If not then, thank you very much for your presentation.