Testing System for Hazard Assessment of Endocrine Disruptors in Fish

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I am Hirofumi Yokota of the Chemicals Evaluation and Research Institute. One of the initiatives of the Ministry of the Environment is to test chemical substances suspected of being endocrine disrupters in order to assess the harmful effect they may have on fish. Today I would therefore like to briefly describe the series of tests we are conducting under the title "Testing System for Hazard Assessment of Endocrine Disrupters in Fish."

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As was previously explained, the Ministry of the Environment has given priority to certain substances among those listed in SPEED '98 for assessment. We are now conducting tests to assess the potential hazard of substances with the highest priority ranking. We are currently assessing the effect of those substances on fish and the health of human beings.

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Here we have the testing system for assessing effect of certain substances on fish. The international system of assessing endocrine disrupters is being studied primarily by the OECD. This system was basically borrowed from the OECD's assessment system. The testing system is roughly divided into "screening" and "testing". "Screening" is a swift method of determining whether or not candidate substances have an endocrine disrupting effect on fish. Screening of substances suspected of having an endocrine disrupting effect is then followed by "testing". Here specimens are exposed to the concerned substance for an extended period of time to clarify at what concentration the endocrine disrupting effect appears. A binding assay using hormone receptors is also conducted to identify the mode of action of test substances and to complement the results of tests using fish.

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All of the tests were conducted using medaka (O*ryzias latipides*). Medaka have been widely used in ecotoxicity tests in Japan and are also given as suitable tests species in the test guidelines for endocrine disrupters being developed by the OECD. Significant advantages of using medaka include the fact that sex reversal and intersex do not occur naturally. They also have a short life cycle, maturing in 6 to 8 weeks of hatching. If a sex differentiation abnormality such as intersex is observed, these features enable one to determine that it was caused by exposure to the concerned chemical substance. It also enables the testing to be completed in a relatively short period of 6 months even if the species are exposed over their entire lifetimes.

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I shall now describe each of the tests. First is the vitellogenin inducition assay.

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As you are aware, vitellogenin is a precursor protein to serve as the vitellus that is normally produced by the female only. Only a minute quantity exists in the male under ordinary circumstances.

Estrogens bind to estrogen receptors in hepatocytes just like a key and keyhole, activating transcription of genes and producing vitellogenin as a protein. The vitellogenin produced is released into the blood and carried to the ovaries. Because these receptors also exist in male individuals, when exposed to hormone-like substances that can bind with the receptors, vitellogenin is likewise produced at high concentrations.

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With this assay, mature medaka are placed in a tank with an exposure device that constantly introduces water containing the dissolved chemical substance. The period of exposure is to a maximum of 21 days. After exposure, the liver is extracted and the vitellogenin concentration is measured.

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Here we have the results of exposing male medaka to ethynylestradiol, a synthetic estrogen. The horizontal axis indicates the concentration of ethynylestradiol and the vertical axis indicates the concentration of vitellogenin in the liver. As you can see, the higher the exposure concentration is, the higher the concentration of vitellogenin is. This assay can detect the estrogen effect on medaka. We are currently conducting assays of a large number of candidate substances.

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Here we have the partial life-cycle test.

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In this test, the test fish are exposed to the concerned substance from the time when they are fertilized eggs to maturity, covering the period of gonadal differentiation and development. When exposure is complete, the males and females are differentiated by the shape of their fins. The tissue of the gonads is also analyzed under a microscope. The primary objective of the test is to evaluate the endocrine disrupting effect on sex differentiation.

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Here we have the results of a test using nonylphenol, one of the priority substances. You are looking at a photograph of gonad tissue. The photograph on the upper left shows normal male testicles. On the outside we have testicular germ cells with numerous spermatozoa in a compacted mass in the center. The photo on the left shows the ovaries of a normal female. Each of the circular cells is an oocyte that is to become an egg. The photograph at the bottom shows testicles of a male exposed to nonylphenol. Cells that may develop into eggs are observed along with concentrations of sperm, or testis-ova. Thus nonylphenol affects differentiation of the gonads in medaka and forms intersex gonads.

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Here we have the full life-cycle test.

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With the full life-cycle test, two generations are exposed to the concerned substance, and various items are studied at each of the stages of growth. In more specific terms, exposure begins with the fertilized eggs and the effect of the concerned substance on the embryo and hatching is observed. Along with daily monitoring of mortality and symptoms after hatching, effect on growth and sex differentiation is observed at the point of about 60 days of hatching. The number of eggs produced per day and fertility are furthermore monitored from about 70 to 100 days of hatching to evaluate effect on reproduction. The

fertilized eggs obtained from exposed parent medaka continue to be exposed and are observed in the same manner as the first generation. In this manner endocrine disrupting effect of the concerned substance over the entire life span of the fish is evaluated to determine the "lowest observed effect concentration" of the candidate substance.

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Finally I would now like to talk about the hormone receptor binding assay.

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All of the assay and test methods described so far use living medaka. The hormone receptor binding assay however uses medaka hormone receptors created by *Escherichia coli* in the form of protein. The slide shows the principle by which this is accomplished. The medaka hormone receptors created by *E. coli* bind with hormones in the same way a key fits a keyhole. When mixed with chemical substances that bind with receptors in the same manner as hormones, some of the receptors bind with the chemical rather than hormones. This assay is used to investigate whether or not the candidate substance has the affinity to bind with hormone receptors.

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This chart gives the tendency for alkylphenol to bind with the estrogen receptor of medaka and receptors of human beings. In the case where binding affinity for estradiol is 100%, the binding affinity of medaka receptors concerning nonylphenol and octylphenol was 8.1% and 16% respectively. The binding affinity for human receptors on the other hand was only 0.061% and 0.032%. This suggests that the estrogen receptors of medaka are much more likely to bind with alkylphenols than those of human beings.

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Thus we are using various types of assay and test methods to evaluate hazard of the candidate substances to fish. We are currently testing ten types of substances.

This completes my presentation. Thank you for your attention.