An Approach for Evaluating Low-dose Effects of Suspected Endocrine Disruptors

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Thank you for the introduction. My name is Hiroaki Aoyama, Institute of Environmental Toxicology.

Today, I would like to talk briefly about how to evaluate low-dose effects of suspected endocrine disrupters.

First, most of all, I would like to talk a little bit about the concept of low-dose effects. I have drawn a simple type diagram. Please think of this as a single graph.

When we construe the horizontal axis to be administered dose of the substance and the vertical axis to be reaction of animals to the dose, for example, if animals were administered a certain type of toxic substance and their body weight is measured at certain intervals for certain amount of time, the usual reaction observed is that there would be no significant change up to a certain dose, but once a certain dose is exceeded, the body weight would begin to drop. Or perhaps if animals are administered a pesticide such as DDT and the weight of the liver and so on is observed, there would be no significant reaction up to a certain dose, but once that dose is exceeded, the weight of the liver would be observed to incrementally increase. When such a reaction is observed, it is usually referred to as "No Observed Adverse Effect Level" or NOAEL. There is an area between the point where NOAEL is exceeded and reaction occurs. This is called the "threshold." When the threshold is exceeded, reaction occurs. Concerning this reaction, it is generally understood that "the larger the dose, the stronger the reaction is."

Several years ago, however, when we looked at the weight of prostate gland of males born to mothers administered estrogenic substances such as diethylstilbestrol and bisphenol A during a certain stage of pregnancy, there were numerous reports of a slightly different dose-reaction relationship. In other words, the weight of prostate glands of untreated animals increased slightly at an extremely low dose. An area where there was almost no reaction to the next dose appeared. When an even higher dose was administered, the prostate gland inversely became smaller. The curve for "higher weight at low dose and lower weight at higher dose" appears like an inverted letter U, and is therefore referred to as the "inverted U phenomenon."

From the results of recent research, it has been pointed out that there might be cases of such nonlinear dose-reaction relationship. This is slightly problematic considering risk assessment up to the present.

In other words, as Professor Mori said in his presentation a little while ago, our food contains quite a bit of phytoestrogen. According to Professor Mori's data, phytoestrogen ingested by the mother, albeit a small amount, is transferred to the fetus. Although the concentration is extremely low, chemical substances have also been detected in our drinking water. It had been thought that, if this is so, because we are ingesting the amounts below the NOAEL of toxicity tests, we are all right, but there is now concern that perhaps our environment is not all that safe.

If you however consider the opposite circumstance, you will notice that because the untreated animals, in other words the controls used for comparison, in the experiment mentioned a little while ago eat only ordinary food, they are already exposed to phytoestrogens. Even though they are untreated animals, if they are allowed to drink ordinary tap water, they are ingesting chemical substances, albeit a small amount. If so, we have no idea in what cases this reaction (inverted U phenomenon) will appear.

In order to arrange this a little better, there is now a worldwide movement to obtain more accurate data. Some make-up tests were conducted, but in some cases the effects could not be confirmed at the previously mentioned low doses. This sort of thing has been reported in several papers. There is also data whereby low-dose effects have been confirmed, but the mechanism by which the inverted U phenomenon occurs is still unknown. Due to concern that the conventional test methods used up to now may not be sufficient for studying this, we began our study by considering test methods.

This is an illustration of how newborn babies are tested with the conventional reproductive toxicity test following the most standard protocol. The one with the necktie is the father. This one is the mother. Mice produce a large number of offspring, up to about twenty at one time. In this case thirteen were born at one time. All of the tests given in the table were conducted on the day the babies were born. If there are too many babies, runts begin to appear around the fourth day of nursing, so the animals were culled to a certain number.

Four males and four females are usually culled out and the rest of the animals are eliminated. They are of course disposed of when the test is over. The mice are subsequently weaned at about the twenty-first day of nursing. At this time, one male and one female are kept and the rest are disposed of. By doing so one male and one female representative of the large litter are obtained to be tested for reproductive function, etc.

Even if for example abnormalities occur in one or two babies enclosed in red, they are eliminated during the course of the experiment, and only some of the animals are studied after becoming adults, you could by chance wind up studying only babies not affected. On the other hand, oppositely, there are genetic deformities in some of the individual specimens circled in red here, for example, there could be animals in which genetic formation of sperm did not go very well. In this case, the individual got that way not because it was administered a chemical substance, but because of its physical makeup. If this sort of animals is selected for study by coincidence, even though it was not affected by the chemical substance, abnormal formation of sperm and so on is oppositely observed, inviting a misunderstanding that the abnormality was caused by administration of the chemical substance. We must consider experiments where this never occurs.

We call the test method shown in the figure the "trans-generation assay." The test concept is as follows. The animals are mated, and the mother is administered the chemical substance throughout the pregnancy and nursing periods. The babies are not culled at all; all babies are weaned. Approximately half of the babies are dissected at the point where they are weaned, and certain tests are conducted. All of the other half of the babies are raised until they become adults, during which time sperm and process of sexual maturation are studied. We also attempted to get as accurate reading as possible on the low-dose effect by using animals with a genetically uniform background in order to avoid genetic problems and using food that does not contain phytoestrogens or produces as little incidental effect as possible. As a reference

substance, we tried subcutaneously administering low doses of ethnylestradiol, which is a synthetic estrogen.

Here I have written the test items concerning reproduction capacity. We observe the mother's pregnancy rate, number of implantations, and number of offspring. We carefully observed each individual, one at a time for anogenital distance (AGD), weight of genital organs and accessory reproductive organs, gene expression, sexual maturation, sexual cycle and sperm capacity.

These are measurements of AGD. This photograph shows the measurement of a rat fetus prior to birth, rather than a baby that has been born. This is a male and this one is a female. Here we have the reproductive projection. Here is the anus. The distance for males is twice that of females. You can usually tell males and females apart by observing this distance. At that time we measured the distance and attempted to accurately quantify the variation.

This shows a brief summary of the results. First let's look at the effect of ethnylestradiol on the mother. The dose administrated is given here. From all the way up to the top, we have an extremely low dose of 1 (g/kg. Ethnylestradiol is one of the ingredients of oral contraceptives for human beings. The dose per kilogram of body weight is about 0.3 (g/kg. When used as a morning after contraceptive, I hear the dose is 1 (g/kg, triple that amount. When rats were administered a dose of about 0.3 (g/kg, they achieved pregnancy, but the number of offspring per litter was extremely small for the group administered the contraceptive. Whereas a normal litter consists of about 10 individuals, the average size of litters born to the group administered the contraceptive was about 2.5 individuals.

When the dose is tripled to 1 (g/kg, pregnancy was no longer achieved, and no offspring was born in this dose group. This means the mother rats reacted to the substances about the same as human beings. Low doses below 0.1 (g/kg of course had no effect on ability to reproduce.

Now let's take a look at the offspring. As was shown in the slide a little while ago, almost no offspring are born at doses in excess of 0.3 (g/kg. Because the litters were less than normal, in some cases the mothers did not provide the proper care, resulting in a lack of data. In other words, we only investigated offspring at doses of 0.01, 0.03 and 0.1 (g/kg. This table basically gives the results of when the babies were weaned. Only AGD was examined when the babies were born. Concerning AGD, therefore, a statistically significant extension was observed for all dosage groups starting from the minimum dose of 0.01 (g/kg. This can be interpreted as transition of the female baby expression model to male. As for the weight of the prostate gland and uterus, however, it has been predicted from previous reports that the weight of the prostate gland would increase, but there was in fact no change whatsoever. There was also no change in the weight of any of the other organs.

If you look at manifestation of genes for the prostate gland, the beta (beta type of estrogen receptor) of several of the estrogen receptors studied, you can see that manifestation clearly dropped as the lowest dose. If you look at the androgen receptors (male hormone receptors), you will see that manifestation dropped for the prostate gland. IGF-1 (gene that reacts with estrogen) on the other hand clearly increased for the uterus. When we conducted our study according to the standard for gene manifestation, we found that animals react to the lowest dose.

This is a study conducted after the babies grew into adults. If we look at the results for a similar dose, we find that the weight of the adrenal gland tended to increase slightly, and reacted beginning with the lowest dose. No statistical difference was observed for some of the medium dose groups. All dose groups tended to react similarly. This variation was evident for males beginning with the lowest dose, but first became clear for females at the highest dose of about 0.1 (g/kg. There was still no change in weight of the prostate gland and uterus.

If you look at manifestation of genes -- If we looked at manifestation of genes when the babies were weaned, beta of the estrogen receptors dropped significantly for the prostate gland. The androgen receptors also dropped significantly for the prostate gland. When this was studied in adults, we oppositely found that gene manifestation increased significantly for the prostate gland. Alpha of the estrogen receptors also increased. Direction of increase/decrease in the uterus also varied according to genes. In some cases, genes differ, but the fact that reaction is observed beginning from the lowest dose at the gene manifestation level was confirmed here as well. If you consider the entire picture, however, it seems that the direction of reaction is opposite, compared when the babies are weaned with when they become adults in many cases.

In a nutshell, the experiments we conducted this time enable us to at least determine the influence of estrogen is observed in the mothers at sufficient sensitivity. From manifestation of genes at the lowest dose and changes in the observation item such as AGD in weanlings we were also able to confirm that animals react to extremely low doses of estrogen.

The reaction was such that the inverse U phenomenon described in the beginning of this paper was not observed, there was a linear dose reaction relationship for all cases, and only the same level reaction occurred once a reaction occurred at a low dose and then the dose was gradually raised. The change in gene level was a plastic change, rather than being one whereby once it began to increase, it continued to increase, or once it began to decrease, it continued to decrease. Even in organs where change could be observed at such a gene level, no change was observed in weight. Although I can't provide data here, no abnormality was observed in the index involved with reproduction concerning sexual maturity and ability to form sperm observed in adults.

These results suggest that the type of tests used this time is ideally suited to assessing the effect of low doses of chemical substances with an estrogenic effect. In the future, we hope to continue to work on methods of testing substances in the environment having an estrogenic effect, including the nonylphenol and octylphenol previously mentioned.

Another impression I have is that it has become evident that weanlings react to low dose of exposed estrogen if their mothers are exposed to too low doses of estrogen to affect reproduction function during pregnancy. Because the reaction has plasticity, at the present, it may be proper to interpret the reaction as an adaptive biological phenomenon.

Finally, because this research requires a great deal of work, it was achieved not only by our laboratory, the reproduction and toxicity research laboratory, but also the cooperation of many people at the animal management laboratory, pathology and the biochemistry laboratory. I would like to thank Prof. Suzuki of the Nippon Veterinary and Animal Science University for his various suggestions concerning methodology. I am also indebted to Prof. Kawashima at Nihon NUS for analysis of chemical substances

and handling of compounds, and to Mr. Sumi of the Ministry of the Environment for assistance with the project in general. I would also like to take this opportunity to thank everybody else who helped us with the project. Thank you for your attention.